nature portfolio

Peer Review File

Apoptosis-Resistant Cells Drive Compensatory Proliferation via Cell-Autonomous and Non-Autonomous Functions of the Initiator Caspase Dronc

Corresponding Author: Professor Eli Arama

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript provides some interesting insights into caspase-dependent regulation of tissue repair upon irradiation. Using the fly as a model system, the authors demonstrate that compensatory growth of epithelial cells of the wing imaginal disc is driven by two distinct epithelial cell populations, which appear to communicate with one another: DARE cells (Dronc-activating apoptosis-resistant epithelial cells) and NARE cells (non-Dronc-activating apoptosis-resistant epithelial cells). Upon irradiation, persister DARE cells, which avoid cell death but nevertheless activate Dronc sub-lethally, proliferate extensively 24 hours post-irradiation (hpi) and contribute to tissue regeneration. Upon re-irradiation at 48 hpi, DARE cells appear to remain resistant to irradiation-induced cell death, leading to fewer dying cells. They then go on to study the signalling events in the clones that activate DRONC to sub-lethal levels. First, they assess the role of Dronc throughout the tissue regeneration process and find that Dronc, but not is upstream activating adapter Dark, is required for compensatory proliferation throughout the regeneration process. Effector caspases are not required in this cell population, corroborating the notion that this effect is due to sub-lethal activity of Dronc.

They then investigate the role of Myo1D and Myo7A/Crinkled in this process. Both these proteins were previously associated with regulating Dronc activity, either negatively or positively, respectively. They demonstrate that Myo1D prevents effector caspase activation by sequestering Dronc, ensuring DARE cell survival. In contrast, Myo7A/Crinkled promotes caspase activation, contributing to DARE cell loss. The also find a role for TNF receptors (TNFRs) and JNK signalling in regulating tissue repair (DARE cell proliferation), which in turn influences NARE cell proliferation. All in all, their data demonstrate that the interplay between DARE (caspase signalling) and NARE (no caspase signalling) cells is crucial for maintaining proliferative homeostasis and balanced tissue regeneration.

Taken together, this study suggest that persister cells that activate caspases can trigger a signalling cascade that contributes to tissue repair. As such, this study is an elegant demonstration for a critical role of sublethal caspase activity in coordinating tissue repair/regeneration.

Overall, this study is well conducted, a pleasure to read and will be of significant interest to a wide readership. It integrates previous and new observations in a therapeutically relevant setting (irradiation). Moreover, it leverages Drosophila genetic tools to dissect this mechanism in vivo in a highly informative manner. However, while the role of key cell death-regulatory molecules is addressed, one is left wondering how Dronc regulates compensatory proliferation. Below are some suggestions to might help to further improve the study:

Comments:
1) It would be nice, if the authors could elaborate (or experimentally test) on putative mechanisms, such as eROS/dDUOX-mediated mechanisms, as has previously been suggested by the Bergmann team. Also, are hemocytes required for this process?

2) What is the role of dp53 in compensatory proliferation? Reference 75 demonstrates that dp53 actively regulates compensatory proliferation through Dronc. Given that the authors use a more physiological model involving radiation-induced p53-driven apoptosis, it would be helpful if they could provide data addressing the potential role of dp53 in

compensatory proliferation in this context? Do DARE and/or NARE cells exhibit differential levels of dp53? Do these cells undergo cell cycle arrest?

3) The lack of detailed characterisation of NARE cells and their non-autonomous proliferation represents a missed opportunity (albeit this might be followed up in a separate study). Are the authors aware of any specific markers or phenotypes that distinguish NARE cells beyond their lack of Dronc activation and apoptosis resistance? For instance, could the authors comment on JNK signalling in NARE cells? Additionally, could they provide quantification of NARE volumes in Figure 4?

4) What is the evidence that DRONC activity, rather than its scaffolding function, is required in DARE cells. Given that Dark is dispensable in DARE cells, one is left wondering how Dronc could become activated, independently of Dark? Might it be possible that Dronc mediates its effect as a catalytically inactive scaffold. In this respect it is important to highlight that the initiator caspase-8 can also function as a catalytically inactive scaffold, triggering cytokine production and inter-cellular communication in mammals. Hence, experiment addressing the scaffolding role of Dronc would be highly informative. Alternatively, the authors might wish to tune down their language as no evidence is provided that Dronc activity is required once DARE cells have formed. I assume that Dark is required for the initial creation of DARE cells, an issue that would be important to establish.

5) It would also be informative to evaluate the expression levels of Drice and Dcp1 in DARE/NARE cells following irradiation?

6) What is the source of TNF? Is TNF produced by a specific subpopulation within the wing imaginal disc, or is it delivered to cells in a paracrine manner, perhaps by hemocytes? It will be interesting to stain for Eiger in cross-sections of irradiated or control wing imaginal discs expressing dDBS? Alternatively, an Eiger-lacZ reporter could be used to address this question. While this would be interesting to have, it will not be essential.

7) The statement beginning at line 469 is inaccurate, as the cited studies demonstrate that differential expression of Flower isoforms dictates cell fitness. Specifically, Flower-based fitness recognition depends on the isoform expressed, with fweLoseB marking prospective “loser” cells for elimination. As Rhiner et al. state, “knockdown of all three fwe forms exclusively in loser cells can inhibit/delay competitive interactions.” Did the authors knockdown all Flower isoforms? Could they show isoform-specific effects? Additionally, could they demonstrate that knockout of fwe in the posterior compartment does not autonomously induce cell death or growth effects? I am less convinced about the fwe section, which I think is the weakest part of the ms. I would like to see this section either improved or, alternatively, the authors might decide to remove it.

8) Could the authors provide images of adult wings derived from DARE-ablated wing imaginal discs? Is there any data comparing control wings to those overexpressing Dap (DapOE)? Including such data in Figure 8 would be informative and strengthen the manuscript.

9) The authors state that DARE cells remain resistant to re-irradiation at 48 hpi. What is the proliferation status of epithelial cells at that stage? For irradiation to exhibit an effect, it is essential that cells undergo multiple rounds of the cell cycle. If the tissue at that time slows down its proliferation rate, then such cells will intrinsically be more resistant, because non-proliferative tissue is significantly more resistant to irradiation than proliferative tissue. If this was the case then their observation would have little to do with a ‘resistance memory’. It will be essential that the authors track the proliferative capacity of cells at that stage (at the time point of the 2nd irradiation). Otherwise, their argument is invalid.

10) In the discussion, the authors highlight differences and overlaps between apoptosis-induced proliferation (AiP) and DARE-driven compensatory proliferation. While this is valuable, one could argue that DARE cell proliferation itself represents a non-autonomous effect driven by radiation-induced apoptosis, where dying cells trigger a compensatory proliferation effect. After all, dying cells may not disappear silently, but may also release pro-proliferative factors. Although the authors present data suggesting that DARE cells subsequently induce non-autonomous proliferation of NARE cells, this does not exclude the possibility that the initial trigger for DARE proliferation is AiP (from the dying cells that have not been tracked. To substantiate the claim that DARE proliferation is independent of AiP, the authors would need to demonstrate non-lethal irradiation triggers mild Dronc activation leading to proliferation, and perhaps tissue overgrowth. I understand that the suggested experiment is rather difficult to set up. Therefore, the authors may chose to address/mention this caveat in their discussion.

Minor Comments:
1. Quantification of rDBS and dDBS cells: Could the authors provide quantifications of rDBS and dDBS cells at 4 and 24 hpi? What is the dDBS/rDBS ratio at 48 hpi? An increase in this ratio over time would be expected.
2. Statistical analysis for apoptotic cell numbers: In line 184, a “significant” increase in apoptotic cell numbers is mentioned, but no statistical analysis is provided. While the confocal images are clear, quantifications and statistical analysis would strengthen the findings, particularly in conditions where the fluorescent marker is not diluted.
3. Y-axis labeling: The Y-axis labels throughout the manuscript could be made more informative. For example, in Figure 5E, labelling the axis as “%PARP+/Venus+” would be more immediately informative than the current “%Red/Green.” Similarly, in Figure 4C, “dDBS(G-TRACE)+/Total area (%)” would be clearer than the current label. In Figure 3, the Y-axis should be labelled as “Total fluorescent area” to better reflect the quantification of fluorescence independent of emission spectrum (Green-Yellow-Red). This clarification should also be explicitly stated in the main text.
4. Figure 3C legend: Figure 3C lacks a legend. I recommend moving this panel to a supplementary figure, along with the

corresponding controls for 4 and 24 hpi.
5. Quantification of total fluorescent area: A dedicated graph quantifying the total fluorescent area as a percentage of wing imaginal disc area at all time points (4, 24, and 48 hpi) would facilitate comparisons. This graph should ideally exclude actDrice data, as this construct has not yet been introduced at this point in the manuscript.
6. Endogenous DIAP1 expression: What are the endogenous expression levels of DIAP1 in DARE versus NARE cells?
7. Figure 3 quantification: If quantifications are less variable at later time points, consider placing these in the main figure and moving the 4 hpi data to the supplementary material.
8. Supplementary Figure 2: Could the authors provide quantifications of pHH3 staining at the periphery at different time points post-irradiation? Is there a statistically significant difference in peripheral proliferation between irradiated and unirradiated wing discs with pseudo-clones? Alternatively, quantifications of pHH3 at clonal borders versus clonal centers would suffice.
9. Proliferation Dronc RNAi: Could the authors demonstrate decreased proliferation upon Dronc RNAi using pHH3 or BrdU staining? Similarly, it could be valuable to assess proliferation status (e.g., BrdU/pHH3) in the experimental conditions shown in Figure 5 (Myo1D data).
10. Myo1D localization: Could the authors provide evidence that Myo1D tethers Dronc at the basal membrane following irradiation? Orthogonal confocal views comparing irradiated and non-irradiated wing imaginal discs would be helpful.
11. DapOE effects: Could the authors show the effects of Dap overexpression in the posterior compartment? Does the size of the posterior compartment differ between DapOE and control conditions?
12. Missing references: The statement beginning at line 483 lacks references. Was it not previously established that extracellular matrix (ECM) components sediment in the wing imaginal disc from the hemolymph?

Reviewer #2

(Remarks to the Author)
Braun et al report that X-irradiation causes activation of the initator caspase Dronc, and that some of this is not apoptotic but instead defines a fraction of non-apoptotic cells that survive irradiation to repopulate the wing imaginal disc, both through their own proliferation in response to JNK signaling and thorugh non-autonomous stimulation of nearby cells that never activated Dronc. Additional evidence is provided that non-apoptotic roles of Dronc might be defined by different subcellular localization and interaction with different Myosins. These studies have significant potential to increase understanding of responses to IR and the role of non-apoptotic caspases, which may be important for understanding regulation under stress and have implications for radiation therapy in cancer. At present the data are not sufficient to justify the conclusions, and experimental detail is insufficient for parts of the paper to be assessed.

1). The paper is presented as a study of non-apoptotic function of Dronc. It is crucial to establish that the reporters used are actually reporting Dronc activity. Neither this nor the previous paper (ref 81) where these reporters were developed showed that Dronc activates these reporters. Ref 81 even showed that forced Jnk activity could activate the reporters in a Dronc mutant, which implies they can be activated by other mechanisms. It is important to show here that Dronc activates the reporters, and that activity following irradiation is dronc-dependent.
2). Insufficient details are given regarding Drosophila cultures and staging for the results to be properly evaluated. It is not even described at what stage irradiation was carried out. Results are then presented 4h, 24h and 48h post-irradiation as though these represent a time series. For this to be true, all these larvae have to have been irradiated at the identical developmental stage. It is not explained how this was achieved, and this is crucial for interpreting the results. If, for example, eggs were laid over a 24h time window, then larvae dissected 24h after irradiation may very well have been 20h younger at irradiation than the larvae dissected 4h after irradiation. The 24h post-irradiation larvae could have smaller wing discs simply because they were irradiated at an earlier developmental time point, not as a result of apoptosis reducing the tissue size. The results regarding compensatory proliferation of DARE and NARE cells are also undermined by this uncertainty. Sufficient experimental details should be given so that it can be clearly understood how the developmental stage when larvae were irradiated was determined and at what stage or stages larvae were dissected.
3). Area is used as a measure of wing disc size. Area of slide-mounted wing discs depends on the mounting method and amount of mounting medium. Details of how this was standardized should be given.
4). The wing imaginal disc sizes illustrated in Figure 1a are not representative of the graphs shown in Figure 1c. Non-irradiated discs are not shown, the 24h disc shown is much smaller than the 4h disc, and the 48h disc is much the largest. If Figure 1c is correct, then the size changes shown in Figure 1a are not typical. These discrepancies contribute to the concerns raised in points 2,3 above.
5). t-tests are not appropriate for the data in Figures 1b,c, because of the need to correct for multiple testing. These data should be analyzed by one-way ANOVA with an appropriate post hoc correction for multiple testing.
6). Data shown in Figure 1f should be quantified.
7). The main evidence that DARE cells promote proliferation by NARE cells is the distribution of mitotic NARE cells close to DARE cells. This should be quantified, as should the effect on NARE cell proliferation of DARE cell ablation. Otherwise the evidence for a DARE cell-dependent mitotic signal is not compelling, because the wing disc size reduction after DARE cell ablation could be due to DARE cell loss only.

Other points
1) Delay of the delayed activity sensor is substantial. The authors imply that this delay may reflect general inhibition of translation in apoptotic cells. This is puzzling because they are studying non-apoptotic cells. Is there any evidence for altered translation in cells with non-apoptotic caspase activities, or other evidence supporting their model?

2) The authors conclude that Jnk acts as a growth signal during AiP. Ref 81 described Jnk signaling as an apoptotic signal that activates the dronc-reporters. These seemingly contradictory consequences of Jnk signaling should be discussed.
3) Although Braun et al find DARE cells in multiple other imaginal discs besides the wing, they seem surprisingly different. It does not seem expected that DARE cells would be absent from eye imaginal discs. How reproducible are these data?
4) It would be useful to use a Jnk-reporter to map Jnk activity in DARE and NARE cells.

Reviewer #3

(Remarks to the Author)
Report on the manuscript by Braun et al

In this work, Braun et al make full use of the sophisticated genetic technology of Drosophila to explore an important, yet unresolved, developmental problem: the mechanisms behind the restoration of tissue size after generalized cell death, a phenomenon usually referred to as compensatory proliferation (CP). As experimental system they have used the wing imaginal disc, an appropriate choice considering the vast amount of information about its developmental/genetic parameters. Besides, it has been used in several previous studies on the subject. The method to induce damage has been to irradiate (20 Gy) third instar larvae and dissect wing discs for analysis.
This manuscript reports a detailed description of the response to massive cell death caused by the irradiation. Specifically, it is focused on the role of the apical caspase Dronc, a key component pf the apoptosis pathway. Previous work from a number of labs has shown the Dronc not only activates the effector caspases, responsible for the autocrine destruction of the cells, but it is also involved in the secretion of proliferative signals that affect growth of the resident tissue.
The authors have used two sensors of Dronc activity, a rapid response sensor (RDBS) that detects cells in apoptosis, and a delayed reporter sensor (DDBS) that marks surviving Dronc-expressing cells. The logic behind the distinction is that in the DDBS sensor requires the activation of the QF system, which needs two rounds of transcription and translation after the irradiation event. Double staining with an antibody against the effector caspase Dcp1 (Fig 1) shows that RDBS labels cells in apoptosis but the DDBS does not, indicating that the latter marks the Dronc-expressing cells (referred to as DARE cells) that survive the irradiation. This idea is reinforced by additional experiments marking DARE cells with persistent Venus fluorescence label, and especially by the demonstration with the G-Trace method that those cells proliferate and contribute to the repopulation of the irradiated disc (Fig. 3).
Regarding my review of this work, there are two different aspects I consider separately. 1) The role of Dronc in CP, and 2) the response of Dronc to radiation
1) The role of Dronc in compensatory proliferation.
This is the less satisfactory side of the work. The authors have identified two types of cells that survive the irradiation, those that do not activate the apoptosis pathway (NARE), and those that activate Dronc but do not die (DARE) and contribute to the repopulation of the disc. The DARE cells represent another case of non-lethal function of caspases, field to which several of the authors have made significant contributions.
The authors assign the Dronc-expressing DARE cells a leading role in CP, as clearly stated in the title of the manuscript: “The Initiator Caspase Dronc Drives Compensatory Proliferation……..”
It correct, it would be a major advance: a special non-lethal function of Dronc to regenerate a tissue after massive cell death. However, as I detail below, the evidence provided in the manuscript is not convincing.
The problem is the experimental protocol used to assay the role of DARE cells in CP (see the comments below). In a key experiment (illustrated in Fig. 3), the authors eliminate DARE cells (using a constitutively active form of the effector caspase Drice, a nice tool) and check whether there is size compensation in irradiated discs. The results (Fig. 3d.e) appear in principle to support a requirement of DARE cells, as size of 48hpi discs is significantly smaller than non-irradiated controls. However, a drawback of this interpretation is that the authors have not considered the possibility that the growth compensation may take place after 48hpi, mediated by NARE cells; the loss of DARE cells may simply delay the process. In fact, this appears quite likely considering the results of experiments in which the proliferation of DARE cells is impeded by forcing the expression of dacapo or the form of flower that makes the cells unfit (Fig. 7). In absence of contribution from DARE cells there still is growth compensation, indicating the NARE cells are now largely responsible for CP. Along the same lines, the inhibition of JNK signaling in DARE cells was compensated by additional contribution of NARE cells, as to achieve normal disc size (Fig. 6).
Thus, I do not see evidence for a leading role of DARE cells in CP. The cells surviving the irradiation, DARE and NARE, contribute to repopulate the damaged disc. The degree of their contribution may vary depending on the experimental conditions. Nevertheless, it is worth pointing out that these results argue strongly against models proposing that CP is achieved by a specific group of cells located in the hinge region (Verghese and Su, PloS Genet 2018).
2) The response of Dronc to radiation
The demonstration that Dronc-expressing cells play a role in the regeneration of the wing disc is new and reveals a novel non-lethal function of the caspase Dronc. It also illustrates the variability of the response to X-Rays and the diversity of Dronc functions. These viable Dronc-expressing cells proliferate extensively and also secrete proliferative signals that contribute to the regeneration of the disc. They are also able to differentiate adult cuticle. Expectedly, these functions are not dependent on effector caspases, and are also independent of the activity of the adapter dark, involved in the apoptotic functions of Dronc. The interaction of Dronc with the unconventional Myo1D is also of interest as it may indicate a role to prevent the activation of effector caspases in DARE cells. Finally, there is the interesting observation that DARE cells appear to be able to transmit their resistance to radiation to their progeny.

My major concern about this work is that the experimental protocol to assay CP is unsatisfactory. In all the experiments the

authors utilise irradiated third instar discs, and then examine the results at 4, 24, or 48h after the irradiation. Whether or not there is CP is a particular experiment is decided upon the comparison of size of 48 hpi discs with controls. This procedure is inadequate and precludes firm conclusions about growth compensation; it ignores the substantial variability in response to X-rays and that irradiation always generates a developmental delay (see for example Wells and Johnston Dev. Biol 361, 263 (2012). To make a decision on whether an irradiated disc has compensated size would require measuring the size of fully mature discs, when they have achieved final size, regardless of the length of time after the irradiation. In some key experiments, for example, the one in which they kill DARE cells with the actDrice construct, the authors should have allowed the experimental larvae to reach the pre-pupal stage (wandering larvae) and then fix the discs and measure their size. Then the comparison with non-irradiated controls would have been appropriate as to make a firmer conclusion about compensatory growth.

A second concern is the lack of a rigorous measurement of the proportion of disc cells that die after the irradiation, an essential piece of data that would signpost how much compensation is necessary for full recovery. There is a mention in passing in pag. 21: 25% of the total WID area showed dying epithelial cells 4 hours after the irradiation, a very unclear statement, with no indication how the authors have arrived to this figure. The response to IR may be very variable depending of the genetic background, the X-rays dose etc. It should not be too difficult to provide an estimate of how many cells are dying, based, for example, on the comparison of the number of cells expressing Dcp1 and of those expressing GFP after 20Gy irradiation of nub>GFP or sal>GFP discs.

Another (lesser) concern is the title, in which the authors imply a leading role of Dronc in CP, which in my view is not justified. Something like “Cells expressing the initiator caspase Dronc contribute to tissue regeneration after ionizing radiation”, or something like it, would be more appropriate.

In summary, while this manuscript does not report a major advance in the topic of compensatory proliferation, it does provide new insights into the process. It includes a description of the response of the apoptosis machinery to massive generalized cell death that points to a new non-lethal function of Dronc involved in regeneration. It will be of interest to investigate this new Dronc function in other models of regeneration. The manuscript also describes some new properties of viable Dronc-expressing cells; they can proliferate normally and appear to have a molecular memory of their resistance to X-Rays, an observation that may open a new avenue of research of caspase function. The technical quality of the work is very good, including sophisticated high-resolution lineage experiments. It also reports new tools, for example, the constitutively active form of the caspase Drice, which can be very useful in regeneration experiments.
However, as I state above, I have serious concerns about the experiments that assay compensatory growth in different conditions. My recommendation is that the paper is not acceptable in its current form, but that the authors should be given a chance of submitting a much revised version that includes new experiments addressing the criticisms above

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done a great job in addressing my concerns and suggestions. I think the ms is much improved and makes a great read.

Reviewer #2

(Remarks to the Author)
The authors have addressed many of the points raised in the initial review. I thank them for more fully explaining how the larvae were irradiated and staged for these experiments. Although the authors explain how they conclude that all larvae were at a similar developmental stage at the time of dissection, my concern had been more with the time of irradiation. The authors attribute differences seen between wing discs irradiated at different times to processes occurring after the irradiation. It is not certain how they exclude differences caused by the timing of irradiation.

Another remaining concern is how to compare these results with some that have been published previously. A paper by Wells and Johnston Dev. Biol 361, 263 (2012) previously studied wing disc regeneration after irradiation and concluded that Dronc made no contribution. This paper does not seem to be cited, although one of the other reviewers drew attention to it. Given the complexity of the data and interpretations, I think it would be necessary to cite this previous work, and preferably explain why different conclusions are reached, so that readers can be alert to alternative perspectives.

Reviewer #3

(Remarks to the Author)
I have read with care the revised version of the manuscript by Braun et al. They have addressed the criticisms I raised in my previous review of the work, especially what concerned the developmental timing in the experiments eliminating the DARE cells. The new results appear to support their proposal of a specific role of DARE cells in compensatory proliferation. While I may not fully agree with some of the authors interpretations, I think they can publish their work as they see fit.
I also note the authors have gone a long way to address the points made by the other reviewers.

My recommendation is that this work be published in Nature Comm.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript provides some interesting insights into caspase-dependent regulation of tissue repair upon irradiation. Using the fly as a model system, the authors demonstrate that compensatory growth of epithelial cells of the wing imaginal disc is driven by two distinct epithelial cell populations, which appear to communicate with one another: DARE cells (Dronc-activating apoptosis-resistant epithelial cells) and NARE cells (non-Dronc-activating apoptosis-resistant epithelial cells).

Upon irradiation, persister DARE cells, which avoid cell death but nevertheless activate Dronc sub-lethally, proliferate extensively 24 hours post-irradiation (hpi) and contribute to tissue regeneration. Upon re-irradiation at 48 hpi, DARE cells appear to remain resistant to irradiation-induced cell death, leading to fewer dying cells. They then go on to study the signalling events in the clones that activate DRONC to sub-lethal levels. First, they assess the role of Dronc throughout the tissue regeneration process and find that Dronc, but not is upstream activating adapter Dark, is required for compensatory proliferation throughout the regeneration process. Effector caspases are not required in this cell population, corroborating the notion that this effect is due to sub-lethal activity of Dronc.

They then investigate the role of Myo1D and Myo7A/Crinkled in this process. Both these proteins were previously associated with regulating Dronc activity, either negatively or positively, respectively. They demonstrate that Myo1D prevents effector caspase activation by sequestering Dronc, ensuring DARE cell survival. In contrast, Myo7A/Crinkled promotes caspase activation, contributing to DARE cell loss. The also find a role for TNF receptors (TNFRs) and JNK signalling in regulating tissue repair (DARE cell proliferation), which in turn influences NARE cell proliferation. All in all, their data demonstrate that the interplay between DARE (caspase signalling) and NARE (no caspase signalling) cells is crucial for maintaining proliferative homeostasis and balanced tissue regeneration.

Taken together, this study suggest that persister cells that activate caspases can trigger a signalling cascade that contributes to tissue repair. As such, this study is an elegant demonstration for a critical role of sublethal caspase activity in coordinating tissue repair/regeneration. Overall, this study is well conducted, a pleasure to read and will be of significant interest to a wide readership. It integrates previous and new observations in a therapeutically relevant setting (irradiation). Moreover, it leverages Drosophila genetic tools to dissect this mechanism in vivo in a highly informative manner. However, while the role of key cell death-regulatory molecules is addressed, one is left wondering how Dronc regulates compensatory proliferation. Below are some suggestions to might help to further improve the study:

We thank the reviewer for their thoughtful and positive assessment of our work. We are pleased that the reviewer found the study to provide interesting insights into caspase-dependent tissue repair and appreciated our use of *Drosophila* genetics to dissect this mechanism *in vivo*. We are also grateful for the recognition of the clarity, rigor, and broader relevance of our findings, and for the constructive comments.

Comments:
1) It would be nice, if the authors could elaborate (or experimentally test) on putative mechanisms, such as eROS/dDUOX-mediated mechanisms, as has previously been suggested by the Bergmann team. Also, are hemocytes required for this process?

We have now addressed these points through additional experiments.

First, we downregulated the two major ROS-generating enzymes in *Drosophila*, Dual oxidase (Duox) and NADPH oxidase (Nox), specifically in DARE cells. Knockdown of both *duox* and *nox* resulted in a significant reduction in DARE cell numbers and clone size at 48 hpi, with the effect of *duox* knockdown being more pronounced (*Supplementary Fig. 3f-h*). Consistent with the behavior of most signaling molecules involved, no change in WID size was observed at 48 hpi (*Supplementary Fig. 3f, g, i*), and no significant effects were detected at the shorter time points of 4 and 24 hpi (*Supplementary Fig. 1g-j*).

To assess the potential involvement of hemocytes as mediators in compensatory proliferation, we ablated larval hemocytes altogether by overexpressing the *actDrice* construct specifically in hemocytes using Hml^{P2A}*-Gal4*, a strong hemocyte-specific driver (*Stephenson et al., Dev. 2022*). Following irradiation in an otherwise WT background, the already low number of hemocytes in the WID gradually declines from 4 hpi to 24 hpi and further to 48 hpi (*Supplementary Fig. 3j*). Upon *actDrice* expression, hemocyte numbers are dramatically reduced at all time points, with almost no hemocytes detectable in the WIDs (*Supplementary Fig. 3j*). Nevertheless, WID size remained unaffected at 48 hpi, indicating that hemocytes are not required for compensatory proliferation in this context (*Supplementary Fig. 3k*).

Together, these findings suggest that while ROS produced in DARE cells is essential for their proliferation and proper tissue regeneration, hemocytes are dispensable for compensatory proliferation.

2) What is the role of dp53 in compensatory proliferation? Reference 75 demonstrates that dp53 actively regulates compensatory proliferation through Dronc. Given that the authors use a more physiological model involving radiation-induced p53-driven apoptosis, it would be helpful if they could provide data addressing the potential role of dp53 in compensatory proliferation in this context? Do DARE and/or NARE cells exhibit differential levels of dp53? Do these cells undergo cell cycle arrest?

It is important to note that, despite the title of that paper, it describes a role for the *Drosophila* p53 ortholog (Dp53) in promoting Dronc-dependent cell proliferation specifically in the context of AiP, not compensatory proliferation (Wells, Yoshida, & Johnston. *Curr Biol*., 2006). To directly assess its potential involvement in our system, we performed *dp53* knockdown specifically in DARE cells. However, this manipulation had no effect on the number of DARE cells or clones, nor on overall WID size at 48 hpi, suggesting that *dp53* does not play a cell-autonomous role in DARE-mediated compensatory proliferation (*Supplementary Fig. 3d. e*).

However, we cannot rule out an indirect role for Dp53 in the induction of DARE cells by dying cells, as irradiation-induced cell death has been shown to depend on Dp53 (*Brodsky et al., Cell, 2000*). Notably, irradiation-induced cell death occurring after 18 hpi has been reported to be Dp53-independent (*Wichmann, Jaklevic, and Su, PNAS, 2006*), implying that it may be too early to account for the emergence of the majority of DARE cells observed at around 24 hpi onward.

3) The lack of detailed characterisation of NARE cells and their non-autonomous proliferation represents a missed opportunity (albeit this might be followed up in a separate study). Are the authors aware of any specific markers or phenotypes that distinguish NARE cells beyond their lack of Dronc activation and apoptosis resistance? For instance, could the authors comment on JNK signalling in NARE cells? Additionally, could they provide quantification of NARE volumes in Figure 4?

Currently, we do not have a specific marker that can distinguish NARE cells from DARE cells, apart from the absence of Dronc activity in the NARE population. While we agree with the Reviewer that this cell population is of particular interest, future studies involving the isolation of both DARE and NARE cells followed by comprehensive omics analyses may help uncover the molecular characteristics that define these cells.

Regarding JNK signaling, we now assessed JNK pathway activation in DARE and NARE cells at 24 hpi using *TRE-RFP*, an activity-specific reporter of JNK (*Chatterjee & Bohmann. PLoS One. 2012*), and likely also p38 (see details in the main text). Interestingly, and consistent with our genetic data implicating p38/JNK signaling in DARE cell proliferation, TRE-RFP signal was significantly higher in DARE cells compared to NARE cells, suggesting that, unlike DARE cells, NARE cells may not rely on p38/JNK pathway activations for their proliferation. This data is now included in *Fig. 7k, l*.

As for the quantification of NARE cell volume, it is essentially derived as the complementary percentage to that of the DARE cell volume. Specifically, DARE cell volume is calculated by measuring the labeled (colored) area and dividing it by the total WID area. Consequently, the remaining unlabeled area within the WID, particularly at 48 hpi, when most dying cells have already been cleared from the tissue, corresponds primarily to the NARE cell population.

4) What is the evidence that DRONC activity, rather than its scaffolding function, is required in DARE cells. Given that Dark is dispensable in DARE cells, one is left wondering how Dronc could become activated, independently of Dark? Might it be possible that Dronc mediates its effect as a catalytically inactive scaffold. In this respect it is important to highlight that the initiator caspase-8 can also function as a catalytically inactive scaffold, triggering cytokine production and inter-cellular communication in mammals. Hence, experiment addressing the scaffolding role of Dronc would be highly informative. Alternatively, the authors might wish to tune down their language as no evidence is provided that Dronc activity is required once DARE cells have formed. I assume that Dark is required for the initial creation of DARE cells, an issue that would be important to establish.

The Reviewer refers to the fact that the presence of active Dronc in DARE cells, evidenced by its ability to cleave the activity-based *DDBS* reporter, does not necessarily prove that its catalytic activity is required for DARE and NARE cell proliferation. Our study, however, shows that DARE cell overexpression of Diap1, which inhibits both Dronc and effector caspase activity, leads to a strong attenuation of tissue regeneration, closely resembling the effects observed with *dronc* knockdown (*Fig. 4f, g*). In contrast, DARE cell overexpression of P35, which inhibits the activity of the effector caspases but not Dronc, has no such effect (*Fig. 4h, j*). These findings indicate that within DARE cells, the requirement is upstream of effector caspases, specifically pointing to Dronc activity.

5) It would also be informative to evaluate the expression levels of Drice and Dcp1 in DARE/NARE cells following irradiation?

We have now examined protein expression levels of Drice and Dcp-1 in 24 hpi WIDs using the endogenously tagged fly lines, *drice::V5::TurboID* and *dcp-1::V5::TurboID* (*Shinoda et al., PNAS, 2019*). Our analysis reveals that both caspases are expressed at comparable levels in DARE and NARE cells following irradiation. The data have been added to *Supplementary Fig. 2e, f*. This observation is also consistent with previous findings from our lab, which reported similar levels of *drice* and *dcp-1* RNAs in non-irradiated third instar larval WIDs (*Fig. S1 in Florentin & Arama, JCB, 2012*).

6) What is the source of TNF? Is TNF produced by a specific subpopulation within the wing imaginal disc, or is it delivered to cells in a paracrine manner, perhaps by hemocytes? It will be interesting to stain for Eiger in cross-sections of irradiated or control wing imaginal discs expressing dDBS? Alternatively, an Eiger-lacZ reporter could be used to address this question. While this would be interesting to have, it will not be essential.

Regarding the source of TNF (Eiger), we now show that downregulation of *eiger* in DARE cells does not affect their proliferation or overall tissue regeneration (*Supplementary Fig. 3c-e*). Similarly, as noted in our response to point 1 from this Reviewer, genetic ablation of hemocytes, the source of Eiger in AiP, has no effect on tissue regenerative capacity (*Supplementary Fig. 3j, k*). These results argue against the possibility of autocrine TNF signaling within DARE cells, as well as paracrine signaling originating from hemocytes.

Notably, our findings identify Wengen as the primary TNF receptor on DARE cells that promotes their proliferation. Furthermore, although *eiger* downregulation specifically in DARE cells has no effect on their proliferation (*Supplementary Fig. 3c-e*), its knockdown in the entire posterior region of the WID leads to a modest increase in DARE cell induction, suggesting a negative effect of TNF on DARE cell proliferation (*Fig. 7e, e'*). These results, together with the observed trend of modest negative regulation of DARE cell proliferation by the other TNF receptor, Grindelwald, on DARE cells (*Fig. 6f, h, and Supplementary Fig. 1g, j*), suggest that TNF is secreted either by dying cells or by NARE cells, acting in a paracrine manner to inhibit DARE cell proliferation. Given our evidence that dying cells positively contribute to DARE cell induction (see our response to point 10 from this Reviewer), and the established proliferative balance between DARE and NARE cells, we propose that the source of TNF in this context is the NARE cells.

In contrast to Grindelwald, which is activated by TNF to induce apoptosis, Wengen was recently shown to promote cell survival and tissue regeneration following damage through TNF-independent activation by ROS (*Esteban-Collado et al., EMBO J., 2024*). Consistently, ROS, Wengen, and p38, which was positioned downstream of Wengen in that study, are all required for DARE cell proliferation and tissue regeneration (*Fig. 7d-i*).

Finally, we performed immunostaining on 24 hpi WIDs expressing the *$_{D}$DBS* reporter using anti-Eiger antibodies (a kind gift from Masayuki Miura; *Igaki et al., Dev. Cell, 2009*). However, the staining appeared relatively homogeneous throughout the WID, with no clear pattern or distinction between DARE and NARE cells or the dying cells (*Fig. R1a, b* - for reviewer reference). This

uniform distribution may reflect the modest effect of TNF on DARE cell proliferation observed in our experiments.

7) The statement beginning at line 469 is inaccurate, as the cited studies demonstrate that differential expression of Flower isoforms dictates cell fitness. Specifically, Flower-based fitness recognition depends on the isoform expressed, with fweLoseB marking prospective "loser" cells for elimination. As Rhiner et al. state, "knockdown of all three fwe forms exclusively in loser cells can inhibit/delay competitive interactions." Did the authors knockdown all Flower isoforms? Could they show isoform-specific effects? Additionally, could they demonstrate that knockout of fwe in the posterior compartment does not autonomously induce cell death or growth effects? I am less convinced about the fwe section, which I think is the weakest part of the ms. I would like to see this section either improved or, alternatively, the authors might decide to remove it.

We fully agree with the Reviewer that drawing definitive conclusions about the role of Flower in the proliferative dynamics between DARE and NARE cells would require additional experiments, particularly addressing isoform-specific functions and potential non-autonomous effects. In our current study, we used *flower* knockdown as an additional approach, alongside Dacapo overexpression, to perturb DARE cell fitness and evaluate compensatory proliferation by NARE cells. However, we did not specifically target different Flower isoforms, nor did we dissect isoform-specific contributions, which we acknowledge are critical for interpreting Flower's role in the context of cell competition.

We also recognize that there is ongoing debate in the field regarding the exact mechanisms by which Flower mediates cell competition, and that this topic, while interesting, falls outside the main scope and focus of our manuscript. In light of these considerations and in agreement with the Reviewer's suggestion, we have removed this experiment and related text from the revised version of the manuscript. Consequently, we have substantially expanded our analysis of the effects of Dacapo overexpression (*Fig. 8*).

8) Could the authors provide images of adult wings derived from DARE-ablated wing imaginal discs? Is there any data comparing control wings to those overexpressing Dap (DapOE)? Including such data in Figure 8 would be informative and strengthen the manuscript.

While wild-type larvae irradiated with 20 Gy X-rays still develop into adults and typically form wings that are largely intact, some of which retain residual DARE cell clones shortly after eclosion, prior to their elimination (*Fig. 3e*), flies expressing the *actDrice* transgene specifically in DARE cells die during pupariation. This outcome precludes the analysis of adult wing morphology in this experimental setup. It is important to note that this pupal lethality may result from the ablation of DARE cells throughout the entire animal.

In contrast, irradiated flies with Dacapo-overexpressing DARE cells reach adulthood, but these flies exhibit significantly smaller adult wings compared to irradiated controls (*Fig. 8f, g*). Additionally, these wings display a range of differentiation defects that are more pronounced than those observed in irradiated control wings (*Fig. 8e*). These findings suggest that when NARE cells proliferate to fully compensate for compromised DARE cell proliferation, the resulting organ (wing) becomes highly susceptible to size and differentiation defects. This underscores the importance of balanced proliferation between these two cell populations for proper tissue regeneration.

9) The authors state that DARE cells remain resistant to re-irradiation at 48 hpi. What is the proliferation status of epithelial cells at that stage? For irradiation to exhibit an effect, it is essential that cells undergo multiple rounds of the cell cycle. If the tissue at that time slows down its proliferation rate, then such cells will intrinsically be more resistant, because non-proliferative tissue is significantly more resistant to irradiation than proliferative tissue. If this was the case then their observation would have little to do with a 'resistance memory'. It will be essential that the authors track the proliferative capacity of cells at that stage (at the time point of the 2nd irradiation). Otherwise, their argument is invalid.

In response to the Reviewer's point, we assessed the proliferative index of WIDs at 4, 24, and 48 hpi, the latter being the time point at which the second irradiation is administered. To this end, we performed EdU incorporation assays to track S-phase entry. Our results show that, rather than declining, the proliferative index of the tissue actually increases at 48 hpi compared to both 4 and 24 hpi (*Fig. 9e*). Therefore, the enhanced resistance of DARE cells to re-irradiation at 48 hpi cannot be attributed to reduced proliferation. Instead, it supports the conclusion that DARE cell lineage acquires a form of resistance memory.

10) In the discussion, the authors highlight differences and overlaps between apoptosis-induced proliferation (AiP) and DARE-driven compensatory proliferation. While this is valuable, one could argue that DARE cell proliferation itself represents a non-autonomous effect driven by radiation-induced apoptosis, where dying cells trigger a compensatory proliferation effect. After all, dying cells may not disappear silently, but may also release pro-proliferative factors. Although the authors present data suggesting that DARE cells subsequently induce non-autonomous proliferation of NARE cells, this does not exclude the possibility that the initial trigger for DARE proliferation is AiP (from the dying cells that have not been tracked. To substantiate the claim that DARE proliferation is independent of AiP, the authors would need to demonstrate non-lethal irradiation triggers mild Dronc activation leading to proliferation, and perhaps tissue overgrowth. I understand that the suggested experiment is rather difficult to set up. Therefore, the authors may chose to address/mention this caveat in their discussion.

While we acknowledge the Reviewer's suggestion to test whether non-lethal irradiation might trigger mild Dronc activation and proliferation in the absence of cell death, we agree that, as the Reviewer noted, this experiment is technically challenging. Identifying an irradiation dose that activates Dronc without inducing apoptosis is not straightforward.

As an alternative approach, we now inhibited apoptosis specifically in the posterior region of the WID by either overexpressing P35 or downregulating *dark*, and assessed DARE cell induction relative to the unmanipulated anterior region. P35 is a potent inhibitor of effector caspases, while Dark is the apoptosome adaptor required for Dronc activation during apoptosis. Interestingly, although DARE cell-specific overexpression of P35 or knockdown of *dark* had no effect on DARE cell proliferation and tissue regeneration (*Fig. 4h-k*), applying the same manipulations across the entire posterior compartment of the WID markedly suppressed DARE cell formation (*Fig. 7f, f', g, g'*). These findings suggest that apoptotic cells may release signals that induce DARE cell formation.

It is noteworthy that a distinct form of AiP in the eye region of the eye-antennal imaginal disc was shown to depend on effector caspases rather than Dronc, demonstrating that dying cells can also

induce non-cell-autonomous proliferation downstream of effector caspase activity (Fan & Bergmann, *Dev. Cell*, 2008). Future studies will aim to elucidate the mechanisms and signals released by dying cells that drive the induction of DARE cells.

Minor Comments:

1. Quantification of rDBS and dDBS cells: Could the authors provide quantifications of rDBS and dDBS cells at 4 and 24 hpi? What is the dDBS/rDBS ratio at 48 hpi? An increase in this ratio over time would be expected.

To provide more accurate quantification of $_{R}$*DBS*- and $_{D}$*DBS*-labeled cells, we now used TUNEL staining, which detects fragmented DNA in apoptotic cells, instead of the previously used anti-cDcp-1 antibody. This adjustment was made because Dronc activity, as detected by both the $_{R}$*DBS* and $_{D}$*DBS* reporters, is also localized to the nucleus. We focused our analysis on the 24 hpi time point, when the WID exhibits peak levels of apoptosis.

Importantly, the results are consistent with our earlier findings: $_{R}$*DBS* predominantly marks dying cells, whereas $_{D}$*DBS* mainly labels surviving cells. These updated data and corresponding quantifications are now presented in *Fig. 1f-h*.

2. Statistical analysis for apoptotic cell numbers: In line 184, a "significant" increase in apoptotic cell numbers is mentioned, but no statistical analysis is provided. While the confocal images are clear, quantifications and statistical analysis would strengthen the findings, particularly in conditions where the fluorescent marker is not diluted.

We believe the Reviewer may have inadvertently referred to statistical analysis of apoptotic cells, whereas the section in question (*Fig. 2b*) pertains to DARE cells. Note that the quantification and statistical analysis of apoptotic cells are, in fact, provided in *Fig. 1b*.

In response to the reviewer's comment regarding *Fig. 2b* (line 184 now line 196), we acknowledge that quantifying DARE cells at this setup based on fluorescence intensity may substantially underestimate their numbers, particularly at the latest and most relevant time point (48 hpi), due to progressive dilution of the GFP signal with each round of cell division. To address this technical limitation, we employed the *G-TRACE* system in subsequent experiments. This lineage-tracing approach enables stable fluorescent labeling of DARE cell progeny, allowing for accurate quantification. In light of this limitation, we have revised the manuscript to remove language implying statistical significance in reference to *Fig. 2b*, retaining such language only for the quantified experiments, which represent the vast majority of the data.

3. Y-axis labeling: The Y-axis labels throughout the manuscript could be made more informative. For example, in Figure 5E, labelling the axis as "%PARP+/Venus+" would be more immediately informative than the current "%Red/Green." Similarly, in Figure 4C, "dDBS(G-TRACE)+/Total area (%)" would be clearer than the current label. In Figure 3, the Y-axis should be labelled as "Total fluorescent area" to better reflect the quantification of fluorescence independent of emission spectrum (Green-Yellow-Red). This clarification should also be explicitly stated in the main text.

We have revised the Y-axes labeling in accordance with the Reviewer's suggestion.

4. Figure 3C legend: Figure 3C lacks a legend. I recommend moving this panel to a supplementary figure, along with the corresponding controls for 4 and 24 hpi.

Previous *Fig. 3c* and legend, along with the corresponding control images for 4 and 24 hpi, has now been moved to *Supplementary Fig. 1b*.

5. Quantification of total fluorescent area: A dedicated graph quantifying the total fluorescent area as a percentage of wing imaginal disc area at all time points (4, 24, and 48 hpi) would facilitate comparisons. This graph should ideally exclude actDrice data, as this construct has not yet been introduced at this point in the manuscript.

As suggested, the graph has now been incorporated into *Fig. 3c*.

6. Endogenous DIAP1 expression: What are the endogenous expression levels of DIAP1 in DARE versus NARE cells?

We performed immunostaining on 24 hpi WIDs expressing the *$_D$DBS*/G-TRACE system using anti-Diap1 BIR domain antibodies ("sk12"; a kind gift from Pascal Meier). However, the staining appeared relatively uniform throughout the WID, without a clear distinction between DARE and NARE cells (*Fig. R1c* - for reviewer reference). Since WIDs at this stage contain numerous dying cells, where Diap1 levels are known to decrease, we conclude that this approach lacks the sensitivity to detect moderate differences in Diap1 expression.

7. Figure 3 quantification: If quantifications are less variable at later time points, consider placing these in the main figure and moving the 4 hpi data to the supplementary material.

While the later time point (48 hpi) shows reduced variability and is consistently included in all relevant figures, an exception was made in *Fig. 3e* (now *Fig. 3d*), where shorter time points (non-irradiated and 4 hpi) are presented. Our rationale is to highlight that although the *$_D$DBS* reporter predominantly labels DARE cells emerging at around 24 hpi, it is still capable of detecting the small number of DARE cells that emerge prior to and shortly after irradiation.

Importantly, the elimination of these early DARE cells upon *actDrice* expression further supports the notion that transcription and translation in DARE cells are already active at these early stages. Therefore, we believe that including these specific early time points is important to demonstrate that transcription and translation inhibition is minimal, if present at all, in DARE cells compared to apoptotic cells. This supports the specificity of the reporter for marking surviving, rather than dying, cells.

8. Supplementary Figure 2: Could the authors provide quantifications of pHH3 staining at the periphery at different time points post-irradiation? Is there a statistically significant difference in peripheral proliferation between irradiated and unirradiated wing discs with pseudo-clones? Alternatively, quantifications of pHH3 at clonal borders versus clonal centers would suffice.

Determining whether DARE cells at clone peripheries divide more frequently than those at the centers is technically challenging, as the borders of many clones are not well-defined due to the close proximity and partial merging of adjacent clones.

However, we now quantified the number of dividing NARE cells located in close proximity to DARE cells versus those positioned further away. Our analysis revealed a significant increase in NARE cell proliferation when these cells were immediately adjacent to DARE cells, within a distance of less than one average cell diameter (*Fig. 8c*). This finding reinforces our genetic analyses showing that DARE cells promote NARE cell proliferation via a non-cell-autonomous mechanism.

We additionally applied an expansion microscopy (ExM) protocol, which enlarged the tissue by approximately fourfold and enabled high-resolution visualization of the spatial relationships between DARE and NARE cells. This revealed that dividing NARE cells located at the DARE-NARE interface are in direct physical contact with adjacent DARE cells (*Fig. 9d*).

9. Proliferation Dronc RNAi: Could the authors demonstrate decreased proliferation upon Dronc RNAi using pHH3 or BrdU staining? Similarly, it could be valuable to assess proliferation status (e.g., BrdU/pHH3) in the experimental conditions shown in Figure 5 (Myo1D data).

We performed the suggested experiments by labeling WIDs with EdU to assess the proliferative index of NARE cells in WIDs where DARE cells were ablated (via *actDrice* expression) or where *dronc* or *myo1D* were knocked down. However, in DARE cell-ablated WIDs at 24 hpi, no significant change in the NARE proliferative index was observed (*Fig. 9f*). Furthermore, downregulation of *dronc* or *myo1D* in DARE cells resulted in an increase, rather than a decrease, in NARE cell proliferation at 24 hpi (*Fig. 9f*). Although these results may appear counterintuitive, they are consistent with our genetic data showing that DARE cells are required for regenerative NARE cell proliferation. When DARE cells are completely absent (ablated), NARE cells are unable to overproliferate to compensate for their loss (*Fig. 10diii*). In contrast, when DARE cell proliferation is reduced, but at least some DARE cells and clones remain, NARE cells respond by overproliferating (*Fig. 10dii*; see also related text in the *Results* section).

10. Myo1D localization: Could the authors provide evidence that Myo1D tethers Dronc at the basal membrane following irradiation? Orthogonal confocal views comparing irradiated and non-irradiated wing imaginal discs would be helpful.

To investigate whether Myo1D tethers Dronc at the basal membrane following irradiation, we analyzed irradiated WIDs carrying the endogenously tagged Dronc line: *dronc::V5::TurboID* (*Shinoda et al., PNAS, 2019*). As a control, we confirmed that anti-V5 antibodies specifically detect Dronc, since *dronc* RNAi expression in the WID pouch (driven by *Sal-Gal4*) markedly reduced Dronc/V5 signal in that region (*Fig. R1d* - for reviewer reference). However, orthogonal confocal analysis of DARE cells at 24 hpi, as suggested, revealed no evidence of Dronc enrichment at the basal membrane (*Fig. R1e* - for reviewer reference).

The absence of sharply localized Dronc signal at the cell surface may reflect a fundamental difference between AiP and compensatory proliferation. In AiP, sequestration of Dronc to the basal membrane has been interpreted as a mechanism to promote extracellular ROS production that attracts hemocytes. By contrast, our data show that DARE cells generate ROS cell-autonomously, and that this ROS is required for their own proliferation. Moreover, hemocytes are dispensable for compensatory proliferation. It is also important to note that AiP has largely been studied in imaginal discs using ‘undead’ cells condition, with conclusions often extrapolated to normal dying cells. In normal dying cells, however, Dronc is distributed throughout the cell, suggesting that the

basal membrane localization of Dronc in AiP may represent a specific feature of the 'undead' context rather than a general property of surviving cells.

11. DapOE effects: Could the authors show the effects of Dap overexpression in the posterior compartment? Does the size of the posterior compartment differ between DapOE and control conditions?

We performed this experiment and observed a general trend toward reduced WID size following Dap overexpression in the posterior region; however, this effect was not statistically significant (*Fig. R1f* - for reviewer reference). Importantly, through several similar genetic manipulations, we realized that while this assay is valuable for monitoring non-cell-autonomous effects on DARE cell induction and proliferation, it is limited for assessing the impact of impaired regeneration on overall WID size. This limitation arises because WID size is strongly confounded by the unmanipulated anterior region of the imaginal disc.

12. Missing references: The statement beginning at line 483 lacks references. Was it not previously established that extracellular matrix (ECM) components sediment in the wing imaginal disc from the hemolymph?

This statement now starts in line 239. It was previously thought that hemocytes in the hemolymph secrete extracellular matrix (ECM) components to form and bond the two cuticle surfaces of the wing (*Kiger, Natzle & Green. PNAS, 2001*). However, subsequent studies, including work from the same laboratory, revealed that these hemocyte-like cells actually originate from the epithelial cells of the wing imaginal disc (*Kiger et al., Dev Biol., 2007*). The sentence has been revised accordingly, and appropriate citations have been added.

Reviewer #2 (Remarks to the Author):

Braun et al report that X-irradiation causes activation of the initator caspase Dronc, and that some of this is not apoptotic but instead defines a fraction of non-apoptotic cells that survive irradiation to repopulate the wing imaginal disc, both through their own proliferation in response to JNK signaling and thorugh non-autonomous stimulation of nearby cells that never activated Dronc. Additional evidence is provided that non-apoptotic roles of Dronc might be defined by different subcellular localization and interaction with different Myosins. These studies have significant potential to increase understanding of responses to IR and the role of non-apoptotic caspases, which may be important for understanding regulation under stress and have implications for radiation therapy in cancer. At present the data are not sufficient to justify the conclusions, and experimental detail is insufficient for parts of the paper to be assessed.

We thank the reviewer for highlighting the potential significance of our findings and its implications for tissue regeneration and radiation therapy, and for the constructive comments.

1). The paper is presented as a study of non-apoptotic function of Dronc. It is crucial to establish that the reporters used are actually reporting Dronc activity. Neither this nor the previous paper (ref 81) where these reporters were developed showed that Dronc activates these reporters. Ref 81 even showed that forced Jnk activity could activate the reporters in a Dronc mutant, which implies

they can be activated by other mechanisms. It is important to show here that Dronc activates the reporters, and that activity following irradiation is dronc-dependent.

We address the reviewer's concern as follows:

I. The reporter used in the current study to identify DARE cells is the *$_{D}$DBS* (originally referred to *as DBS-S-QF* in *Baena-Lopez et al., Development, 2018*; now *Ref. 84*). As shown in Fig. 4C,D of that paper, *dronc* downregulation abolished the reporter signal, indicating that this reporter specifically detects Dronc activity.

II. To further validate this finding, we performed *dronc* knockdown in the posterior region of the WID and observed a significant loss of reporter signal. These new results, consistent with *Ref. 84*, confirm that reporter activation is dependent on Dronc. This data is now included in *Fig. 7h, h'*. Similarly, overexpression of Diap1, which inhibits Dronc activity, in the WID posterior region also led to significant block of the reporter signal (*Fig. 7i, i'*).

III. Moreover, we demonstrate that Dronc activity in DARE cells is functionally important for their proliferation. Specifically, overexpression of Diap1, an endogenous protein inhibitor of both Dronc and effector caspases, within DARE cells significantly reduces their proliferation and impairs tissue regeneration (*Fig. 4f, g*). In contrast, DARE cell-specific overexpression of the baculovirus P35 protein, which inhibits only effector caspases, does not affect DARE cell proliferation and tissue regeneration (*Fig. 4h, j*). These results further support a non-apoptotic role of Dronc in DARE cell proliferation.

IV. Regarding the reviewer's point about reporter activation upon JNK overexpression in the *dronc* mutant background (*Ref. 84*), we first note that this experiment utilized the rapid-response reporter (*$_{R}$DBS*), not the delayed *$_{D}$DBS* reporter employed in our study to detect DARE cells.

There are two possible explanations for the observed residual *$_{R}$DBS* reporter activity in the *dronc* mutant background. First, the *dronc^{I29}* allele used in that experiment may not be a complete null and could retain low levels of Dronc activity. Second, maternally deposited Dronc RNA from heterozygous mothers may persist into the late third instar larval stage. Indeed, a previous study from our lab demonstrated residual but detectable caspase activity in WIDs of *dronc$^{I24/29}$* transheterozygous mutants at this stage, which could be completely abolished by RNAi-mediated knockdown of *dronc* (see Fig. 3A,B in *Florentin & Arama, JCB, 2012*). This study demonstrates that residual Dronc activity persists in the *dronc* mutants during the third instar larval stage, regardless of the exact underlying mechanism, which may account for the residual reporter activity observed upon JNK overexpression.

Collectively, these data establish that *$_{D}$DBS* reporter activation in our study is Dronc-dependent, and that Dronc activity plays a critical non-apoptotic role in the regulation of DARE cell proliferation.

2). Insufficient details are given regarding Drosophila cultures and staging for the results to be properly evaluated. It is not even described at what stage irradiation was carried out. Results are then presented 4h, 24h and 48h post-irradiation as though these represent a time series. For this to be true, all these larvae have to have been irradiated at the identical developmental stage. It is not explained how this was achieved, and this is crucial for interpreting the results. If, for example, eggs were laid over a 24h time window, then larvae dissected 24h after irradiation may very well have been 20h younger at irradiation than the larvae dissected 4h after irradiation. The 24h post-

irradiation larvae could have smaller wing discs simply because they were irradiated at an earlier developmental time point, not as a result of apoptosis reducing the tissue size. The results regarding compensatory proliferation of DARE and NARE cells are also undermined by this uncertainty. Sufficient experimental details should be given so that it can be clearly understood how the developmental stage when larvae were irradiated was determined and at what stage or stages larvae were dissected.

To clarify our experimental procedure, we have included a schematic of the irradiation and staging protocol in the revised manuscript (*Supplementary Fig. 1a*) and a clarification in the main text that the chosen time points represent distinct phases rather than a continuous time series. We also changed the terminology from "early time points" to "shorter timer points".

Briefly, we irradiated a mixed-stage population of larvae and subsequently collected wandering third instar larvae at 4, 24, and 48 hpi for dissection and analysis. Importantly, this means that while larvae were irradiated at different developmental time points, only those that reached the wandering third instar stage were selected for analysis (spiracle morphology was used as an additional criterion to ensure precise staging). As a result, all dissected larvae were at the same developmental stage, allowing for direct comparison across time points.

This approach ensures that observed differences in wing disc size or cell proliferation are not due to variability in developmental stage at the time of dissection, but rather reflect biological responses to irradiation.

3). Area is used as a measure of wing disc size. Area of slide-mounted wing discs depends on the mounting method and amount of mounting medium. Details of how this was standardized should be given.

To minimize variability that might derive from mounting, all samples were prepared by the same individual (the first author) using a standardized and consistent protocol. Briefly, about 20 WIDs were aligned on a slide in a drop of PBS. The PBS was gently removed using Whatman filter paper, after which 10 µL of mounting medium was added adjacent to the aligned WIDs. A coverslip was then placed at a 45° angle and allowed to lower gradually by gravity, ensuring even distribution of the mounting medium.

Importantly, multiple WIDs from each experimental group were mounted on the same slide to allow direct comparison, and each experiment included at least two biological replicates. These steps helped ensure consistency in mounting conditions across samples. Full details of the procedure have now been included in the *Methods* section.

4). The wing imaginal disc sizes illustrated in Figure 1a are not representative of the graphs shown in Figure 1c. Non-irradiated discs are not shown, the 24h disc shown is much smaller than the 4h disc, and the 48h disc is much the largest. If Figure 1c is correct, then the size changes shown in Figure 1a are not typical. These discrepancies contribute to the concerns raised in points 2,3 above.

We have revised *Fig. 1a* to include a non-irradiated wing imaginal disc and to include more representative images that better reflect the quantitative data shown in *Fig. 1c*. The updated figure now accurately illustrates the typical disc sizes corresponding to each time point post-irradiation.

5). t-tests are not appropriate for the data in Figures 1b,c, because of the need to correct for multiple testing. These data should be analyzed by one-way ANOVA with an appropriate post hoc correction for multiple testing.

We thank the reviewer for this comment. We have replaced the t-tests with one-way ANOVA followed by appropriate post hoc corrections for multiple comparisons in all relevant figures, including *Fig. 1b-c, 3c, 3h, 6g-h, 9e-f and Supplementary Fig. 1g-j, 2d, 3d-e, 3h-i*. This change ensures more robust statistical analysis and accurate interpretation of the data.

6). Data shown in Figure 1f should be quantified.

We now present quantified data in *Fig. 1h*. Please see our reply to minor point 1 from Reviewer #1 for full details about the revised experiments.

7). The main evidence that DARE cells promote proliferation by NARE cells is the distribution of mitotic NARE cells close to DARE cells.

This should be quantified, as should the effect on NARE cell proliferation of DARE cell ablation.

Otherwise the evidence for a DARE cell-dependent mitotic signal is not compelling, because the wing disc size reduction after DARE cell ablation could be due to DARE cell loss only.

We believe the reviewer intended to suggest that "DARE cells promote the proliferation of NARE cells." As the Reviewer suggested, we now quantified the number of dividing NARE cells located in close proximity to DARE cells versus those positioned further away. Our analysis revealed a significant increase in NARE cell proliferation when these cells were immediately adjacent to DARE cells, within a distance of less than one average cell diameter (*Fig. 9c*). Furthermore, we applied an expansion microscopy (ExM) protocol to enlarge the tissue approximately fourfold, enabling clear visualization of the close spatial proximity between DARE cells and dividing NARE cells (*Fig. 9d*). These results reinforce our genetic analyses showing that DARE cells promote NARE cell proliferation via a non-cell-autonomous mechanism.

We also examined NARE cell proliferation index upon DARE cell ablation or downregulation of *dronc* or *myo1D* in DARE cells. In DARE cell-ablated WIDs at 24 hpi, no significant change in the NARE proliferative index was observed (*Fig. 9f*). In contrast, downregulation of *dronc* or *myo1D* in DARE cells resulted in an increase, rather than a decrease, in NARE cell proliferation at 24 hpi (*Fig. 9f*). Although these results may appear counterintuitive, they are consistent with our genetic data showing that DARE cells are required for regenerative NARE cell proliferation. When DARE cells are completely absent (ablated), NARE cells are unable to overproliferate to compensate for their loss (*Fig. 10diii*). In contrast, when DARE cell proliferation is reduced, but at least some DARE cells and clones remain, NARE cells respond by overproliferating (*Fig. 10dii*; see also related text in the *Results* section).

Other points

1) Delay of the delayed activity sensor is substantial. The authors imply that this delay may reflect general inhibition of translation in apoptotic cells. This is puzzling because they are studying non-

apoptotic cells. Is there any evidence for altered translation in cells with non-apoptotic caspase activities, or other evidence supporting their model?

Our intention was to explain that, due to the well-documented inhibition of transcription and translation in apoptotic cells, the delayed reporter preferentially labels non-apoptotic cells such as DARE cells. We agree that this point was not clearly articulated in the original text and have revised the manuscript accordingly to clarify this distinction.

Importantly, there is no evidence of significant transcriptional or translational inhibition in DARE cells, even in non-irradiated WIDs or at 4 hpi when only a few DARE clones have emerged. This is supported by the observation that expression of the *actDrice* transgene in DARE cells, driven by the *$_{D}$DBS* reporter, results in effective cell elimination at 4 hpi (*Fig. 3d*), indicating that both transcriptional and translational machinery are functionally active in these cells. These results indicate a true delay in DARE cell induction during the first ~24 hpi, rather than a delay in reporter detection caused by transcription/translation inhibition in DARE cells.

2) The authors conclude that Jnk acts as a growth signal during AiP. Ref 81 described Jnk signaling as an apoptotic signal that activates the dronc-reporters. These seemingly contradictory consequences of Jnk signaling should be discussed.

Indeed, the JNK signaling pathway has long been recognized for its remarkable versatility (e.g., *Liu & Lin. Cell Res., 2005*; *La Marca & Richardson, Front. Cell Dev. Biol., 2020*). In *Drosophila*, multiple studies, particularly from the Morata and Bergmann groups, have demonstrated that JNK can function as either a pro-apoptotic or pro-proliferative signal, depending on the physiological context (e.g., *Shlevkov & Morata, CDD, 2012; Pinal et al., Open Biol., 2019; Pérez et al., eLife, 2017*). These studies also describe a positive feedback loop between Dronc and JNK signaling, in which each amplifies the activity of the other, rather than reflecting a linear epistatic relationship. Therefore, activation of the reporters by JNK is not inconsistent with its role in promoting proliferation; rather, it highlights the dual capacity of JNK signaling to regulate both apoptosis and regenerative growth in a context-dependent manner (also refer to our response to Major Point 1 from this Reviewer).

It is noteworthy that we now provide genetic evidence showing that p38, another member of the MAPK family, similar to JNK and likewise involved in cellular stress responses, plays a more prominent role than JNK in promoting DARE cell proliferation in a cell-autonomous manner, as well as NARE cell proliferation through a non-cell-autonomous mechanism (*Fig. 6a-d, g-i*). Given that the two TNF receptors appear to play partially opposing roles in regulating DARE cell proliferation, it is possible that their antagonistic effects converge on JNK signaling, thereby dampening its pro-proliferative impact on DARE cells compared to that of p38.

A discussion of this point, in the spirit outlined above, has now been added to the revised *Discussion* section.

3) Although Braun et al find DARE cells in multiple other imaginal discs besides the wing, they seem surprisingly different. It does not seem expected that DARE cells would be absent from eye imaginal discs. How reproducible are these data?

For each imaginal disc type, we analyzed approximately 20 samples, and have now added this *N* number above the images in *Fig. 2c*.

Regarding the limited number of DARE cells in the eye part of the eye-antennal disc, it is important to note that this part of the imaginal disc employs distinct signaling pathways and regulatory mechanisms compared to the adjacent antennal part, both in apoptosis and in AiP (see: *Fan & Bergmann, Dev Cell, 2008*). This difference is likely due to the fact that, at the larval stage examined, the eye field has already initiated photoreceptor specification and differentiation, which may alter its responsiveness to apoptotic or regenerative signaling cues.

We have revised the sentence in the *Results* section that addresses the distinctions between the eye and antennal domains of the eye-antennal imaginal disc as follows: *This observation aligns with the fact that, at this stage, the eye region is already undergoing photoreceptor specification and differentiation (Mollereau and Domingos, 2005), which is also consistent with the distinct mechanisms governing AiP in the eye and antennal domains of the imaginal disc (Fan and Bergmann, 2008).*

4) It would be useful to use a Jnk-reporter to map Jnk activity in DARE and NARE cells.

We have now assessed JNK signaling in both DARE and NARE cells using the JNK (and likely also p38) activity reporter *TRE-RFP*. Our analysis shows that JNK activity is significantly elevated in DARE cells compared to NARE cells. This finding supports our genetic data indicating that JNK and p38 activities are required for DARE cell proliferation (see also our response to Reviewer #1, Point 3). These results are now included in *Fig. 7k, l* of the revised manuscript.

Reviewer #3 (Remarks to the Author):

Report on the manuscript by Braun et al

In this work, Braun et al make full use of the sophisticated genetic technology of Drosophila to explore an important, yet unresolved, developmental problem: the mechanisms behind the restoration of tissue size after generalized cell death, a phenomenon usually referred to as compensatory proliferation (CP). As experimental system they have used the wing imaginal disc, an appropriate choice considering the vast amount of information about its developmental/genetic parameters. Besides, it has been used in several previous studies on the subject. The method to induce damage has been to irradiate (20 Gy) third instar larvae and dissect wing discs for analysis. This manuscript reports a detailed description of the response to massive cell death caused by the irradiation. Specifically, it is focused on the role of the apical caspase Dronc, a key component pf the apoptosis pathway. Previous work from a number of labs has shown the Dronc not only activates the effector caspases, responsible for the autocrine destruction of the cells, but it is also involved in the secretion of proliferative signals that affect growth of the resident tissue. The authors have used two sensors of Dronc activity, a rapid response sensor (RDBS) that detects cells in apoptosis, and a delayed reporter sensor (DDBS) that marks surviving Dronc-expressing cells. The logic behind the distinction is that in the DDBS sensor requires the activation of the QF system, which needs two rounds of transcription and translation after the irradiation event. Double staining with an antibody against the effector caspase Dcp1 (Fig 1) shows that RDBS labels cells in apoptosis but the DDBS does not, indicating that the latter marks the Dronc-expressing cells

(referred to as DARE cells) that survive the irradiation. This idea is reinforced by additional experiments marking DARE cells with persistent Venus fluorescence label, and especially by the demonstration with the G-Trace method that those cells proliferate and contribute to the repopulation of the irradiated disc (Fig. 3).

Regarding my review of this work, there are two different aspects I consider separately. 1) The role of Dronc in CP, and 2) the response of Dronc to radiation.

1) The role of Dronc in compensatory proliferation.

This is the less satisfactory side of the work. The authors have identified two types of cells that survive the irradiation, those that do not activate the apoptosis pathway (NARE), and those that activate Dronc but do not die (DARE) and contribute to the repopulation of the disc. The DARE cells represent another case of non-lethal function of caspases, field to which several of the authors have made significant contributions.

The authors assign the Dronc-expressing DARE cells a leading role in CP, as clearly stated in the title of the manuscript: "The Initiator Caspase Dronc Drives Compensatory Proliferation……." It correct, it would be a major advance: a special non-lethal function of Dronc to regenerate a tissue after massive cell death. However, as I detail below, the evidence provided in the manuscript is not convincing.

The problem is the experimental protocol used to assay the role of DARE cells in CP (see the comments below). In a key experiment (illustrated in Fig. 3), the authors eliminate DARE cells (using a constitutively active form of the effector caspase Drice, a nice tool) and check whether there is size compensation in irradiated discs. The results (Fig. 3d.e) appear in principle to support a requirement of DARE cells, as size of 48hpi discs is significantly smaller than non-irradiated controls.

We thank the Reviewer for recognizing the importance of the research question and the technical sophistication, and for the constructive comments.

To assess the extent of compensatory proliferation and specifically the contribution of DARE cells to this process, we used two complementary parameters: (1) the overall WID area, as noted by the reviewer, but also (2) the area occupied by DARE cell clones.

However, a drawback of this interpretation is that the authors have not considered the possibility that the growth compensation may take place after 48hpi, mediated by NARE cells; the loss of DARE cells may simply delay the process. In fact, this appears quite likely considering the results of experiments in which the proliferation of DARE cells is impeded by forcing the expression of dacapo or the form of flower that makes the cells unfit (Fig. 7). In absence of contribution from DARE cells there still is growth compensation, indicating the NARE cells are now largely responsible for CP. Along the same lines, the inhibition of JNK signaling in DARE cells was compensated by additional contribution of NARE cells, as to achieve normal disc size (Fig. 6). Thus, I do not see evidence for a leading role of DARE cells in CP. The cells surviving the irradiation, DARE and NARE, contribute to repopulate the damaged disc. The degree of their contribution may vary depending on the experimental conditions.

We investigated whether a developmental delay induced by irradiation could provide sufficient time for NARE cells to compensate for the absence of DARE cells and restore normal WID size. As a first step, we assessed whether irradiation alone results in a measurable delay in

developmental progression. To this end, wandering third instar larvae from wild-type and DARE cell-ablated backgrounds (achieved via *actDrice* transgene expression) were collected at 48 hpi, transferred to fresh vials, and scored for pupariation at 72 and 96 hpi. While 100% of irradiated wild-type larvae pupariated by 72 hpi, only 60% of DARE cell-ablated larvae had pupariated at this time point, with pupariation increasing to 80% by 96 hpi. These results are now included in *Supplementary Fig. 1f*.

Despite this extended developmental window, WIDs from DARE cell-ablated larvae at both 72 and 96 hpi failed to recover normal size when compared to control WIDs at 48 hpi. This indicates that NARE cell proliferation alone is insufficient to compensate for the loss of DARE cells and restore tissue size. Furthermore, WID size in DARE-ablated larvae significantly decreased by 96 hpi, suggesting that DARE cell loss may impair the survival of NARE cells. These results are now included in *Fig. 3h*.

These findings contradict the assumption that the WID can reach normal size in the absence of DARE cells given sufficient time, reinforcing the idea that DARE cells promote NARE cell proliferation through a non-cell-autonomous mechanism driven by Dronc and p38 activity within DARE cells.

Nevertheless, it is worth pointing out that these results argue strongly against models proposing that CP is achieved by a specific group of cells located in the hinge region (Verghese and Su, PloS Genet 2018).

We agree with the reviewer observation. Specifically, studies mainly by the Su group typically examines a specific region of the WID using a hinge-specific driver. In contrast, the *$_D$DBS* reporter in our study is driven by tubulin regulatory elements, resulting in broad, ubiquitous expression throughout the entire WID (and likely other cells in various relevant tissues). This point is addressed in detail in the *Discussion* section.

2) The response of Dronc to radiation

The demonstration that Dronc-expressing cells play a role in the regeneration of the wing disc is new and reveals a novel non-lethal function of the caspase Dronc. It also illustrates the variability of the response to X-Rays and the diversity of Dronc functions. These viable Dronc-expressing cells proliferate extensively and also secrete proliferative signals that contribute to the regeneration of the disc. They are also able to differentiate adult cuticle. Expectedly, these functions are not dependent on effector caspases, and are also independent of the activity of the adapter dark, involved in the apoptotic functions of Dronc. The interaction of Dronc with the unconventional Myo1D is also of interest as it may indicate a role to prevent the activation of effector caspases in DARE cells. Finally, there is the interesting observation that DARE cells appear to be able to transmit their resistance to radiation to their progeny.

We thank the Reviewer for the thoughtful and encouraging comments.

My major concern about this work is that the experimental protocol to assay CP is unsatisfactory. In all the experiments the authors utilise irradiated third instar discs, and then examine the results at 4, 24, or 48h after the irradiation. Whether or not there is CP is a particular experiment is decided upon the comparison of size of 48 hpi discs with controls. This procedure is inadequate and

precludes firm conclusions about growth compensation; it ignores the substantial variability in response to X-rays and that irradiation always generates a developmental delay (see for example Wells and Johnston Dev. Biol 361, 263 (2012). To make a decision on whether an irradiated disc has compensated size would require measuring the size of fully mature discs, when they have achieved final size, regardless of the length of time after the irradiation. In some key experiments, for example, the one in which they kill DARE cells with the actDrice construct, the authors should have allowed the experimental larvae to reach the pre-pupal stage (wandering larvae) and then fix the discs and measure their size. Then the comparison with non-irradiated controls would have been appropriate as to make a firmer conclusion about compensatory growth.

This concern corresponds to point 1 from this Reviewer. We kindly refer you to our earlier reply for a detailed response.

A second concern is the lack of a rigorous measurement of the proportion of disc cells that die after the irradiation, an essential piece of data that would signpost how much compensation is necessary for full recovery. There is a mention in passing in pag. 21: 25% of the total WID area showed dying epithelial cells 4 hours after the irradiation, a very unclear statement, with no indication how the authors have arrived to this figure. The response to IR may be very variable depending of the genetic background, the X-rays dose etc. It should not be too difficult to provide an estimate of how many cells are dying, based, for example, on the comparison of the number of cells expressing Dcp1 and of those expressing GFP after 20Gy irradiation of nub>GFP or sal>GFP discs.

The 25% figure refers to the percentage of dying cells at 4 hpi (20 Gy) in wild-type WIDs, based on the quantification shown in *Fig. 1b*. This calculation is straightforward, and as noted on (now) page 28 (line 651), the value is derived from that experiment. For clarity, we have now explicitly stated this in the relevant figure legend.

Another (lesser) concern is the title, in which the authors imply a leading role of Dronc in CP, which in my view is not justified. Something like "Cells expressing the initiator caspase Dronc contribute to tissue regeneration after ionizing radiation", or something like it, would be more appropriate.

We have updated the title to: *"Apoptosis-Resistant Cells Drive Compensatory Proliferation via Cell-Autonomous and Non-Autonomous Functions of the Initiator Caspase Dronc."* This revised title more clearly reflects the central findings and mechanistic insights of our study.

In summary, while this manuscript does not report a major advance in the topic of compensatory proliferation, it does provide new insights into the process.

It includes a description of the response of the apoptosis machinery to massive generalized cell death that points to a new non-lethal function of Dronc involved in regeneration. It will be of interest to investigate this new Dronc function in other models of regeneration. The manuscript also describes some new properties of viable Dronc-expressing cells; they can proliferate normally and appear to have a molecular memory of their resistance to X-Rays, an observation that may open a new avenue of research of caspase function.

The technical quality of the work is very good, including sophisticated high-resolution lineage experiments. It also reports new tools, for example, the constitutively active form of the caspase Drice, which can be very useful in regeneration experiments.

However, as I state above, I have serious concerns about the experiments that assay compensatory growth in different conditions. My recommendation is that the paper is not acceptable in its current form, but that the authors should be given a chance of submitting a much revised version that includes new experiments addressing the criticisms above.

We believe that the revised manuscript offers significant new insights that shift the conceptual framework of this process. Most notably, we identify and specifically label apoptosis-resistant cells essential for regeneration by compensatory proliferation and demonstrate that at least two distinct epithelial populations of apoptosis-resistant cells, DARE and NARE cells, coordinate this regenerative response. Importantly, we show that DARE cells exhibit a form of molecular memory of prior irradiation.

While the prevailing AiP model attributes regenerative signaling directly to dying cells, our findings reveal that, in the context of compensatory proliferation, the response is instead driven by surviving cells with sublethal caspase activation, likely triggered by signals from dying cells. The revised manuscript explicitly compares and contrasts the key mechanisms underlying AiP and compensatory proliferation, highlighting shared components, such as Dronc, JNK signaling, and ROS, that are co-opted to drive distinct functional outcomes in each context.

As the reviewer rightly points out, we demonstrate a novel, non-apoptotic function of the initiator caspase Dronc, which acts independently of the apoptosome component Dark and is modulated by the non-conventional myosin Myo1D. This provides mechanistic insight into how DARE cells evade apoptosis and maintain their proliferative capacity, as this study is the first to reveal a role for Myo1D as a guardian against apoptosis (it is worth noting that in the context of 'undead' cells in AiP, Myo1D was previously proposed to sequester Dronc at the basal surface of the cell to promote ROS generation).

Furthermore, we now present initial evidence that dying cells may contribute to the induction of DARE cells. Specifically, overexpression of P35 or downregulation of *dark* in the posterior region of the WID significantly attenuated DARE cell formation, whereas both manipulations within DARE cells themselves had no effect on DARE cell proliferation or tissue regeneration. This suggests that signals emanating from dying cells downstream of effector caspase activity, are required to induce the formation of DARE cells. This means that, under normal conditions, signals from dying cells promote regeneration indirectly, by inducing the formation of DARE cells, rather than through direct mitogenic signaling, as proposed in the classical AiP model.

This realization also helps reconcile previously conflicting findings regarding mitogenic signals from dying cells. In the AiP model involving 'undead' cells, artificially sustained apoptotic cells are proposed to directly stimulate proliferation in neighboring cells through the secretion of mitogenic factors such as Wingless and Dpp. However, studies from the Morata group showed that compensatory proliferation after irradiation can proceed even without these signals (*Pérez-Garijo, Shlevkov, & Morata. Dev. 2009*). Our findings suggest an alternative model in which dying cells promote regeneration indirectly by inducing DARE cells, which then drive compensatory proliferation through complex interaction apoptosis-resistant NARE cells, which may provide an explanation for the Morata group's observations.

Taken together, we believe that this work represents a substantive advance in the understanding of compensatory proliferation, further strengthened by the substantial revisions made in response to Reviewers' feedback.

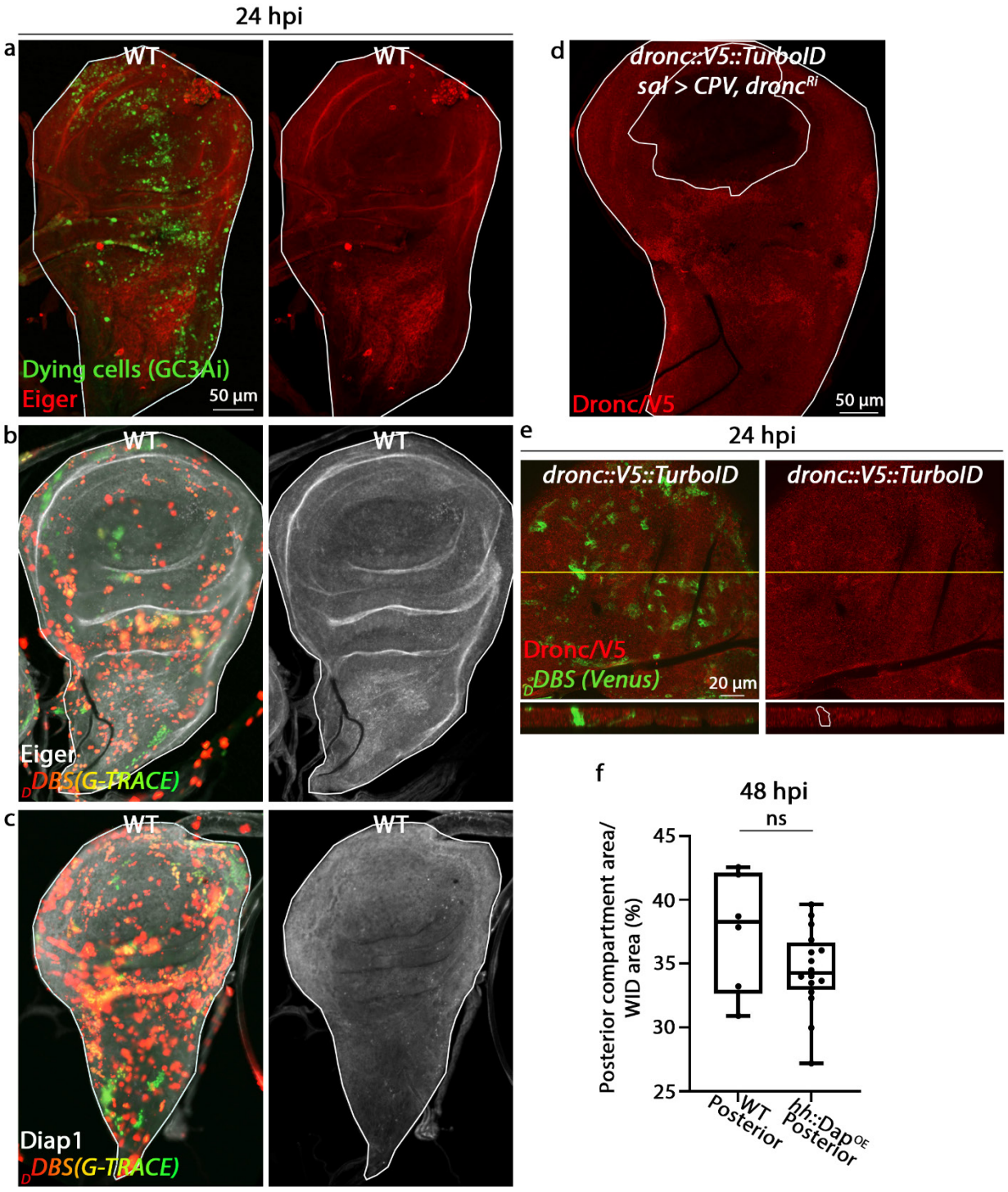

Fig. R1. For reviewer reference. a, b TNF/Eiger expression shows no significant preferential association with DARE, NARE, or dying cells. At 24 hpi, WIDs expressing the GC3Ai reporter of effector caspase activation (dying cells, green) and immunostained with anti-Eiger antibodies (red) reveal no staining bias in dying cells (**a**). Similarly, at 24 hpi, WIDs expressing the G-TRACE system (colored cells) and immunostained for Eiger (white) show no significant difference in

background staining between DARE (colored) and NARE (non-colored) cells (**b**). **c** Diap1 shows no preferential association with DARE or NARE cells. A representative WID at 24 hpi, expressing the *$_{D}$DBS*/G-TRACE system to mark DARE cells (colored) and immunostained with anti-Diap1 antibody (white), illustrates this lack of bias. **d, e** Dronc is not specifically sequestered at the basal membrane in DARE cells. Dronc expression (red) was detected by anti-V5 immunostaining of WIDs carrying an endogenously tagged *dronc* gene (*dronc::V5::TurboID*). Specificity of the signal was confirmed by *Sal-Gal4*-driven *dronc* RNAi, which reduced V5 staining (Dronc) in the pouch area as expected (**d**). At 24 hpi, WIDs carrying *dronc::V5::TurboID* and expressing *$_{D}$DBS*/Venus to mark DARE cells (green) show Dronc expression (anti-V5; red) distributed throughout DARE cells, rather than restricted to the basal membrane. An orthogonal view (bottom) further illustrates a representative DARE cell lacking enrichment of Dronc at the basal membrane (**e**). **f** Overexpression of Dap in the posterior region of 48 hpi WIDs shows a trend toward reduced size, although this decrease was not statistically significant, likely due to confounding signals and physical constraints from the unmanipulated anterior region.

REVIEWER COMMENTS AFTER REVISION

Reviewer #1 (Remarks to the Author):

The authors have done a great job in addressing my concerns and suggestions. I think the ms is much improved and makes a great read.

We thank the reviewer for their positive assessment of our revised manuscript.

Reviewer #2 (Remarks to the Author):

The authors have addressed many of the points raised in the initial review. I thank them for more fully explaining how the larvae were irradiated and staged for these experiments. Although the authors explain how they conclude that all larvae were at a similar developmental stage at the time of dissection, my concern had been more with the time of irradiation. The authors attribute differences seen between wing discs irradiated at different times to processes occurring after the irradiation. It is not certain how they exclude differences caused by the timing of irradiation.

We thank the reviewer for their positive assessment of our revised manuscript. We agree that a limitation of this system is the variation in developmental stages of wing discs at the time of irradiation. For this reason, we restrict our analyses to a maximum of 48 hpi. In our hands, using different assays, later time points (i.e., 72 and 96 hpi) are considerably less reliable because irradiation occurs too early in development. Nevertheless, we now show that developmental timing is extended in larvae in which DARE cells are ablated, allowing us to assess regeneration in animals irradiated at the same developmental stage as 48 hpi wild-type larvae, but examined at 72 and 96 hpi while they remain in the wandering third instar stage due to delayed progression to the pupal stage (*Fig. 3h*). These results demonstrate that DARE cells are essential for tissue regeneration.

Another remaining concern is how to compare these results with some that have been published previously. A paper by Wells and Johnston Dev. Biol 361, 263 (2012) previously studied wing disc regeneration after irradiation and concluded that Dronc made no contribution. This paper does not seem to be cited, although one of the other reviewers drew attention to it. Given the complexity of the data and interpretations, I think it would be necessary to cite this previous work, and preferably explain why different conclusions are reached, so that readers can be alert to alternative perspectives.

We thank the reviewer for drawing our attention to the 2012 study, which we had originally overlooked. However, we note that an earlier work by the same authors, which we cite in our manuscript (DOI: 10.1016/j.cub.2006.07.046), concluded that p53-dependent Dronc activity is required for compensatory proliferation. Furthermore, in the *Discussion* section of the 2012 paper, the authors cautiously state that "*at a minimum, the data indicate that expression of pro-death genes and caspase activation are not sufficient to trigger regeneration.*" leaving open the possibility that Dronc may be required for tissue regeneration but not sufficient on its own. Importantly, our study indicates that p53 has no essential cell-autonomous role in DARE cell compensatory proliferation, even though Dronc activity in these cells is crucial for their proliferation (*Supplementary Fig. 3d, e*). The finding that Dronc activation in DARE cells is

independent of Dark, and thus distinct from its activation during radiation-induced apoptosis, where it requires both Dark and p53, is consistent with our observation that p53 is not required for the proliferation of these cells. Therefore, a possible explanation for the apparent discrepancy is that p53 fulfills at least two roles in promoting compensatory proliferation after irradiation: first, by activating caspases and promoting apoptosis, as dying cells induce DARE cell formation; and second, as a provocative hypothesis, that p53 may function in NARE cells, which by definition do not activate Dronc. These distinct roles of p53 may therefore account for the apparent inconsistency in its reported net effects on compensatory proliferation in imaginal discs. A paragraph along these lines has been incorporated into the *Discussion* section.

Reviewer #3 (Remarks to the Author):

I have read with care the revised version of the manuscript by Braun et al. They have addressed the criticisms I raised in my previous review of the work, especially what concerned the developmental timing in the experiments eliminating the DARE cells. The new results appear to support their proposal of a specific role of DARE cells in compensatory proliferation. While I may not fully agree with some of the authors interpretations, I think they can publish their work as they see fit. I also note the authors have gone a long way to address the points made by the other reviewers. My recommendation is that this work be published in Nature Comm.

We thank the reviewer for their positive assessment of our revised manuscript and hope that future studies using this system will further elucidate additional underlying mechanisms, leading to more definitive interpretations of this important phenomenon.