Author's response to reviews

Title: Residual gammaH2AX foci as an indication of lethal DNA lesions

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Author's response to reviews: see over
Response to Reviewer #1

1. Was tirapazamine exposure carried out under hypoxic conditions….
This information was inadvertently omitted from the methods and is now included.

2. Fig. 2: The relative gH2AX parameter from the flow cytometry data:
   b. Was this for all cells, or just for the gH2AX positive cells?
   Yes, the signal is for all cells and this is now stated.
   a. Was there some normalization procedure?
   Yes, results were normalized by dividing the signal for the treated cells by the signal for the untreated control. This is now indicated.

3. Page 9, text concerning Fig. 2d and e: The authors should clarify whether they are referring to directly or indirectly induced single-strand breaks or both.
We now indicate that alkali-labile lesions that include both direct single-strand breaks and alkali-labile base damages are included in the measure of single-strand breaks.

4. Fig. 2f: The authors speculate that break rejoining may not have been accurate because of a lack of a concomitant reduction in the H2AX signal. But could base damages have been repaired, affecting alkali comets but not affecting conversion of single-strand breaks to double-strand breaks. In addition it would be useful to know if the H2AX is for all the cells.
A slight twist to this suggestion is that it is known that base damages can lead to H2AX phosphorylation when cells enter S phase. Therefore the lack of a reduction in H2AX may be because base damages cause an increase in H2AX with time after treatment. At the same time, direct single-strand breaks are able to be rejoined so the number of alkali-labile lesions decreases. This idea has been added to the text. Again, H2AX is measured for all of the cells (just like the comet assay).

5. The authors show that cells with RAD51 foci usually have gH2AX foci… Were there less RAD51 foci than gH2AX foci?
It is definitely the case that fewer endogenous and drug-induced RAD51 foci are seen relative to γH2AX foci, but this is not necessarily true for residual foci measured 24h after treatment. This is now stated in the text: Although RAD51 foci may only mark a subset of the double-strand breaks, the number of residual RAD51 foci per cell was not invariably lower than the number of residual γH2AX foci (Fig. 5e).

Reviewer #2

1. All pilot experiments were performed by staining for RAD51. These should be performed for RAD51-GFP as well.

An additional experiment has been added showing the response to different doses of radiation after 24 hours. Early time points are not useful since they are an indication of
the rate of formation/aggregation of the GFP protein itself (sufficient GFP must be present to make foci microscopically visible).

2. To strengthen their interesting observation in Fig. 6E, I would recommend using an additional dose of radiation. Alternatively, a different DNA damaging agent like MNNG… For clarity, the data could be presented in a different way.…

Experiments in Fig. 6E were very labor intensive, and the dose was chosen to give about 50% surviving cells. This is critical for statistical accuracy. A lower or higher surviving fraction would require many more colonies to be scored. For the purposes of this study, radiation experiments were considered adequate since our preliminary results with RAD51 immunostaining used only radiation. Repeating experiment in Fig. 6 with MNNG would require an additional set of antibody experiments as well. We have attempted to improve the clarity of presentation.

3. The aim and setup of experiments in the first part of the manuscript are only briefly described… It is not always clear what assay is being used. Clonogenic surviving fraction should be used throughout.

An effort has been made to explain experiments more clearly and indicate which method was used to measure foci. Axes have been altered to indicate clonogenic surviving fraction.

4. The data of Fig. 1 could be represented better to show the described effect (fewer dots, expand axis).

Unfortunately we are limited by the software available to us in terms of expanding axes, however, the number of dots has been reduced.