Cell Systems

Transparent Peer Review Record

Biphasic JNK–Erk Signaling Separates Induction and Maintenance of Cell Senescence after DNA Damage Induced by Topoisomerase II Inhibition

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Summary

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2 confidential, 0 signed
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Major changes anticipated
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2 original, 0 new
2 confidential, 0 signed
Accepted June 13, 2023

Editorial decision letter with reviewers' comments, first round of review

Dear Dr. Yaffe,

First of all, please accept our sincere apologies for the delay in the review process. I'm enclosing the comments that reviewers made on your paper, which I hope you will find useful and constructive. As you'll see, they express interest in the study, but they also have a number of criticisms and suggestions. Based on these comments, it seems premature to proceed with the paper in its current form; however, if it's possible to address the concerns raised with additional experiments and/or analysis, we'd be interested in considering a revised version of the manuscript.

As a matter of principle, I usually only invite a revision when I'm reasonably certain that the authors' work will align with the reviewers' concerns and produce a publishable manuscript. In the case of this manuscript, the reviewers and I have a couple of make-or-break concerns.

1. Reviewer 1 raises concerns about the generalisability of the conclusions, which we hope can be addressed through producing additional data using more cell lines and DNA damaging agents, or at least additional validation experiments.
2. Both Reviewers raise concerns about the interpretation of the t-PLSR results and some of the overall conclusions, which will need to be resolved.

In addition to the concerns I've detailed above, I've highlighted portions of the reviews that strike me as particularly critical to address. I'd also like to be explicitly clear about an almost philosophical stance that we take at Cell Systems…

We believe that data are of primary importance; text, story, and conclusions are secondary. When forced to make a choice, we would rather have text that hews closely to observations and reflects data precisely than a gloss that obscures alternate interpretations. Please keep this in mind when addressing the inconsistencies between the data and the text pointed out by the Reviewers.

As you address these concerns, it's important that you and I stay on the same page. I'm always happy to talk, either over email or Zoom, if you'd like feedback about whether your efforts are moving the manuscript in a productive direction. Do note that we generally consider papers through only one major round of revision, so the revised manuscript would be either accepted or rejected based on the next round of comments we receive from the reviewers. If you have any questions or concerns, please let me know. More technical information and advice about resubmission can be found below my signature. Please read it carefully, as it can save substantial time and effort later.

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I look forward to seeing your revised manuscript.

All the best,

Bernadett

Bernadett Gaal, DPhil
Editor-in-Chief, Cell Systems

Reviewers' comments:

Reviewer #1: In this manuscript, the authors report a combined experimental and computational analysis of the activation of senescence in U2OS osteosarcoma cells responding to doxorubicin. They identify drug doses for which a 4-hour treatment caused cells to senesce rather than undergo apoptosis. They next made measurements of signaling molecules, response molecules, and overall cellular responses at select time points following doxorubicin treatment. They next used t-PLSR to integrate the measurements to identify cellular components that are most highly correlated to specific cell fates (proliferation, senescence, and apoptosis). They found their first latent variable correlated with proliferation vs senescence and the second latent variable correlated with survival vs apoptosis. One unanticipated result was that levels of phospho-c-Jun correlated with senescence (even though MAPK signaling associated with apoptosis rather than senescence). Treatment with a JNK or MEK inhibitor within the first 12h of doxorubicin treatment attenuated senescence. Signaling through JNK and ERK at later time points was found to contribute to SASP-associated IL-6 and IL-8 secretion. c-Jun knockdown or expression of a dominant-negative form of c-Jun showed c-Jun to be an important regulator of the activation of senescence in response to doxorubicin.

While most of the experimental results and computational analysis are sound overall, there are several major concerns that prevent more enthusiasm for this study. The biggest problems are the limited scope of the study, as described in Major Comments 1 and 2, due to the focus on primarily a single cell line harboring a p16 deficiency and a single DNA damaging agent.

Major comments:
1. The use of (mainly) one cell line limits the scope and generalizability of the results. The fact that the selected cell line, U2OS, that forms the basis of most of the presented work lacks p16 expression, which is an important regulator of senescence in many cells, is a fundamental problem of the study. While an additional cell line, OVCAR-8, was used to validate one set of experiments regarding the effects mediated by the JNK and MEK inhibitors, the effects on senescence in OVCAR-8 were relatively modest.

2. The use of only one DNA damaging compound, doxorubicin, is also severely limiting for this study. Since the focus of the study is presented as an analysis of DNA damage-induced senescence, validation of the findings using several other DNA damaging agents (including ionizing radiation and compounds belonging to other categories of DNA-damaging chemotherapeutics) is warranted.

3. Interpretations of the t-PLSR results and the PCA analysis are somewhat contradictory and subjective. In particular, in the t-PLSR it seems counter-intuitive that the p-MAPK signals are associated strongly along the "apoptosis" axis, yet a target of the p-MAPKs, p-c-Jun, projects strongly along the "senescence" axis. Furthermore, through the PCA analysis shown in Fig. 5B, the authors highlight that the p-MAPK signals become progressively less PC2 positive and more PC1 positive. However, no quantification in support of this claim is provided, and especially for the p-JNK and p-p38 this appears to be an over-interpretation. Based on similar visual inspection, one could just as readily state that p-c-Jun levels as shown in Fig. 5D are becoming less PC1 positive and more PC2 positive, counter to later analysis highlighting p-c-Jun as a key regulator of senescence.

Minor comment:
1. It would be better to explicitly indicate what the bar colors represent in Fig. 4H, either in the legend or the key inset. Presumably they represent the phenotypes as indicated in Fig. 4F and 4G, but a similar color scheme is used to represent different doxorubicin doses in Fig. 4D.
2. "Reporter" is misspelled in the y-axis label of Fig. 7A.

Reviewer #2: This well-written paper by Yaffe and colleagues is a very interesting exploration of the DNA damage signaling landscape after treatment with various doses of doxorubicin. They measure 27 "signals" (total- and phospho-proteins) and 10 "responses" (measurements of proliferation, senescence, and apoptosis) at 6 timepoints in 4 stimulus conditions - a heroic number of measurements to quantify in single cells. The data also points towards some interesting new findings, including a role for early Jun phosphorylation and AP-1 transcriptional activity in initiating senescence. I am overall enthusiastic and supportive of the work. Nevertheless, I do have some suggestions for improvement, and one suggestion of an experiment that might help to explain the context-dependence of Erk/JNK signaling.

- One difficulty I had in reading the manuscript was related to the inconsistency in how Erk/JNK signaling are treated between the data, text, and figures. When discussing the PLSR results, the authors note that "… p38MAPK, Erk and JNK in the nucleus projected positively along LV2, correlating with apoptosis. These findings further support our biological interpretation of the LV2 axes …". From that point forward Erk and JNK are defined as "apoptotic" stimuli, colored red in all figures and labeled as "apoptotic signals" in Figure 5D - at least until Figure 6, when Erk and JNK are the focus of the remainder of the paper for their roles in senescence.

The reason this is misleading is that even in Figure 3, it is clear that Erk reaches its highest level in the entire dataset under conditions that are exclusively senescence-related: the 96 h timepoint in 0.5 μM doxorubicin. The PCA results in Figure 5D also show that Erk in particular (but all three MAPKs in general) is just as strongly associated with senescence as apoptosis, with early timepoints projecting as apoptosis-related on PC2 and late timepoints projecting as senescence-related on PC1. (This is discussed clearly in the text, despite the corresponding figure still labeling MAPKs as "apoptotic signals").

It would be very helpful to for the authors to clarify this dual role throughout Figures 3-5. It would also be very helpful to comment on how phospho-Erk was exclusively associated with LV2 and apoptosis in the PLSR analysis, despite being strongly correlated with senescence at 96 h in the raw data and PCA.

- The paper's central mechanistic conclusion is that early c-Jun phosphorylation by JNK and Erk plays a crucial role in establishing senescence. But the manuscript left me wondering: why do the high levels of Erk and JNK activity in 10 μM doxorubicin fail to induce c-Jun phosphorylation at those same timepoints? Erk and JNK phosphorylation are highest early in 10 μM doxorubicin (indeed, higher than in 2.5 and 0.5 μM), exactly when the inhibitor experiments suggest they are most important, but phospho-c-Jun remains low.

One may conjecture that this is because total c-Jun might fail to be induced at 10 μM but not 2.5 or 0.5 μM doxorubicin, so that there is nothing to phosphorylate. Is this the case? What about for other AP-1 components (e.g. Fos)? Simply comparing phospho and total Fos/Jun staining at 0.5 and 10 μM doxorubicin would tell us whether phosphorylation of these targets is really context dependent, or whether their expression is, helping to explain how Erk/Jnk can play such distinct roles depending on stimulus conditions.

- My last comment is about the compendium of measurements shown in Figure S2, which has some very strange properties. Some measurements show extremely wide variance between replicates (Cyclin B at 2 μM doxorubicin, IkBa at 0.5 μM dox, p-Hsp27 at 0 μM and 2 μM dox, p-S6 at 0.5 μM and 2 μM dox). In some cases, replicates in a single condition represent both the minimum and maximum values obtained across all timepoints for that quantity! What is even stranger is that both the low and high values are often tightly reproducible across multiple replicates, and then the values "jump" in another set of tightly reproducible replicates. See for example Cyclin B at 2 μM doxorubicin: these are not normally distributed measurement errors on an identical set of underlying data, but what look like two completely different responses (rising Cyclin B nuc + cyto levels in ~half the replicates; falling Cyclin B nuc + cyto levels in the other half) overlaid on top of each other.

One interpretation for two tightly clustered responses that vary widely from one another is that something differently was done to the two set of replicates, e.g. leading one sample to have rising Cyclin B in half the samples and falling Cyclin B levels in the other half. It would be helpful for the authors to look over this data once more with a careful eye to ensure that these reproducible but distinct responses are real, and comment on this feature of the data. They may represent a concern about or limitation of the data, given their extreme deviation from typical measurement noise.

The text's discussion of the results also conflicts with the data. For example, the dataset in Figure S2 does not show accumulation of cyclin A and B after 2 μM doxorubicin, as stated on page 9 line 1.

Authors' response to the reviewers' first round comments

Attached.

Editorial decision letter with reviewers' comments, second round of review

Dear Michael,

I'm very pleased to let you know that the reviews of your revised manuscript are back, the peer-review

process is complete, and only a few minor, editorially-guided changes are needed to move forward towards publication.

I've made some suggestions about your manuscript within the "Editorial Notes" section, below. Please consider my editorial suggestions carefully, ask any questions of me that you need, make all warranted changes, and then upload your final files into Editorial Manager.

I'm looking forward to going through these last steps with you. Although we ask that our editorially-guided changes be your primary focus for the moment, you may wish to consult our FAQ (final formatting checks tab) to make the final steps to publication go more smoothly. More technical information can be found below my signature, and please let me know if you have any questions.

All the best,

Bernadett

Bernadett Gaal, DPhil
Editor-in-Chief, Cell Systems

Editorial Notes

Transparent Peer Review: Thank you for electing to make your manuscript's peer review process transparent. As part of our approach to Transparent Peer Review, we ask that you add the following sentence to the end of your abstract: "A record of this paper's Transparent Peer Review process is included in the Supplemental Information." Note that this ***doesn't*** count towards your 150 word total!

Also, if you've deposited your work on a preprint server, that's great! Please drop me a quick email with your preprint's DOI and I'll make sure it's properly credited within your Transparent Peer Review record.

Manuscript Text:

- House style disallows editorializing within the text (e.g. strikingly, surprisingly, importantly, etc.), especially the Results section. These terms are a distraction and they aren't needed—your excellent observations are certainly impactful enough to stand on their own. Please remove these words and others like them. "Notably" is suitably neutral to use once or twice if absolutely necessary.
- We don't allow "priority claims" (e.g. new, novel, etc.). For a discussion of why, read: http://crosstalk.cell.com/blog/getting-priorities-right-with-novelty-claims, http://crosstalk.cell.com/blog/novel-insights-into-priority-claims.
- Please check that you use the word "significantly" in the statistical sense only.

Figures and Legends:

Please look over your figures keeping the following in mind:

- Please ensure that every time you have used a graph, you have defined "n's" specifically and listed statistical tests within your figure legend.
- When figures include micrographs, please ensure that scale bars are included and defined within the legend, montages are made obvious, and any digital adjustments (e.g. brightness) have been applied equally across the entire image in a manner that does not obscure characteristics of the original image (e.g. no "blown out" contrast). ***Note that all accepted papers are screened for image irregularities, and if this advice is not followed, your paper will be flagged.***
- Please ensure that if you include representative images within your figures, a "representative of XXX individual cells"-type statement is made in the legend.
- Please ensure that all figures included in your point-by-point response to the reviewers' comments are present within the final version of the paper, either within the main text or within the Supplemental Information.

STAR Methods: Note that Cell Press has recently changed the way it approaches "availability" statements for the sake of ease and clarity. Please revise the first section of your STAR Methods as follows, noting that the particular examples used might not pertain to your study. Please consult the STAR Methods guidelines for additional information.

> RESOURCE AVAILABILITY
>
> **Lead Contact:** Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jane Doe (janedoe@qwerty.com).
>
> **Materials Availability:** This study did not generate new materials. *-OR-* Plasmids generated in this study have been deposited at [Addgene, name and catalog number]. *-OR-* etc.
>
> **Data and Code Availability:**
>
> - **Source data statement** (described below)
> - **Code statement** (described below)
> - Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Data and Code Availability statements **have three parts and each part must be present. Each part should be listed as a bullet point, as indicated above. I've edited the below to reflect the options for your paper.**

Instructions for section 1: Data. The statements below may be used in any number or combination, but at least one must be present. They can be edited to suit your circumstance. Please ensure that ***all datatypes reported in your paper are represented in section 1***. For more information, please consult this list of standardized datatypes and repositories recommended by Cell Press.

• [Adjective] data have been deposited at [general-purpose repository] and are publicly available as of the date of publication. DOIs are listed in the key resources table.

• [Adjective or all] data reported in this paper will be shared by the lead contact upon request.

Instructions for section 2: Code. The statements below may be used in any number or combination, but at least one must be present. They can be edited to suit your circumstance. ***If you are using GitHub, please follow the instructions here to archive a "version of record" of your GitHub repo at Zenodo, then report the resulting DOI. Additionally, please note that the Cell Systems strongly recommends that you also include an explicit reference to any scripts you may have used throughout your analysis or to generate your figures within section 2.***

• All original code has been deposited at [repository] and is publicly available as of the date of publication. DOIs are listed in the key resources table.

Instructions for section 3. Section 3 consists of the following statement: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

In addition,

STAR Methods follows a standardized structure. Please reorganize your experimental procedures to include these specific headings in the following order: LEAD CONTACT AND MATERIALS AVAILABILITY (including the three statements detailed above); EXPERIMENTAL MODEL AND SUBJECT DETAILS (when appropriate); METHOD DETAILS (required); QUANTIFICATION AND STATISTICAL ANALYSIS (when appropriate); ADDITIONAL RESOURCES (when appropriate). We're happy to be flexible about how each section is organized and encourage useful subheadings, but the required sections need to be there, with their headings. They should also be in the order listed. Please see the STAR Methods guide for more information or contact me for help.

Currently, you don't have a **Key Resources Table** (KRT). Note that the key resources table is required for manuscripts with an experimental component, and if a purely computational manuscript links to any external datasets (previously published or new), code-containing websites (e.g. a GitHub repo, noting that DOIs are strongly preferred), or uses non-standard software, it needs to include a key resources table that details these aspects of the paper. Purely computational or theoretical papers that don't contain any external links and use standard software don't require a key resources table, although you're welcome to include one if you like. For details, please refer to the Table Template or feel free to ask me for help.

Thank you!

Reviewer comments:

Reviewer #1: The authors have done a great job through additional experiments and analysis to address my concerns with the original manuscript, as well as the concerns raised by the other reviewer. I appreciate the new experiments with two additional cell lines to increase the generalizability of the results, as well as looking at other DNA damaging compounds to better define the cellular stress being analyzed. I also greatly appreciate the clarifications and new analysis that were performed regarding the PCA, as it helped me to better understand the results of the study. I think the clarifications may also help other readers.

I thoroughly enjoyed reading this study, which presents a wealth of data and analysis in a clear manner. I think the results will be of broad interest to the field and have raised many intriguing future directions for the study of chemotherapeutics, MAPK signaling pathways, and the regulation of apoptosis and senescence.

Reviewer #2: I congratulate the authors on a fantastic manuscript & have no further revisions to suggest.

Point-by-point responses

First and foremost, we would like to thank the reviewers for their careful reading of the manuscript, and the thoughtful comments and insights that they offered. We have addressed these comments with extensive additional computational and wet lab experiments, which are now incorporated into the revised version of the manuscript. We believe that these incorporated suggestions have strengthened the conclusions made in this work, and have significantly improved the work overall. A point-by-point response to the reviewers' comments follows below. All figure and page callouts now correspond to the revised version of the manuscript.

Reviewer #1

In this manuscript, the authors report a combined experimental and computational analysis of the activation of senescence in U2OS osteosarcoma cells responding to doxorubicin. They identify drug doses for which a 4-hour treatment caused cells to senesce rather than undergo apoptosis. They next made measurements of signaling molecules, response molecules, and overall cellular responses at select time points following doxorubicin treatment. They next used t-PLSR to integrate the measurements to identify cellular components that are most highly correlated to specific cell fates (proliferation, senescence, and apoptosis). They found their first latent variable correlated with proliferation vs senescence and the second latent variable correlated with survival vs apoptosis. One unanticipated result was that levels of phospho-c-Jun correlated with senescence (even though MAPK signaling associated with apoptosis rather than senescence). Treatment with a JNK or MEK inhibitor within the first 12h of doxorubicin treatment attenuated senescence. Signaling through JNK and ERK at later time points was found to contribute to SASP-associated IL-6 and IL-8 secretion. c-Jun knockdown or expression of a dominant-negative form of c-Jun showed c-Jun to be an important regulator of the activation of senescence in response to doxorubicin.

While most of the experimental results and computational analysis are sound overall, there are several major concerns that prevent more enthusiasm for this study. The biggest problems are the limited scope of the study, as described in Major Comments 1 and 2, due to the focus on primarily a single cell line harboring a p16 deficiency and a single DNA damaging agent.

Thank you for your thorough evaluation of our work! We have addressed each of your comments below.

Major comments:

1. The use of (mainly) one cell line limits the scope and generalizability of the results. The fact that the selected cell line, U2OS, that forms the basis of most of the presented work lacks p16 expression, which is an important regulator of senescence in many cells, is a fundamental problem of the study. While an additional cell line, OVCAR-8, was used to

validate one set of experiments regarding the effects mediated by the JNK and MEK inhibitors, the effects on senescence in OVCAR-8 were relatively modest.

As suggested by the reviewer, we have now examined two additional cell lines, a non-small cell lung cancer line (NCI-H1299) and a non-cancerous primary endothelial cell line (HUVEC), in response to treatment with different doses of doxorubicin. As shown in the new Figures 6 and S6, both of these cell lines demonstrated dose-dependent cell senescence as evidenced by the appearance of cells with enlarged nuclei and cytoplasm (i.e. the classical "fried egg" appearance) that failed to incorporate BrdU into nuclear DNA when serum stimulated at 6 days after doxorubicin treatment (Fig 6F-I, S6B-J) Notably, both cell lines exhibited a higher percentage of BrdU positive (BrdU+) cells in response to the early SP600125, PD98059 and caffeine treatment after doxorubicin treatment, consistent with JNK and Erk-mediated induction of senescence. This data is now described on page 17, lines 14-30 of the revised text. We agree with the reviewer about the modesty of the quantified OVCAR-8 data, and as a result placed the new NCI-H1299 data in figure 6 and moved the OVCAR-8 data to figure S6.

To address the reviewer's second comment about the generalizability of this phenotype to another cell line that expresses p16, we have now performed immunofluorescence for CDK inhibitors p16, p21, and p27 four days after doxorubicin treatment. We found that OVCAR-8 cells had increased levels of nuclear p16 after treatment as shown in Figure S6A. In contrast, we were not able to detect levels of p16 by immunofluorescence in the NCIH1299 or HUVEC cells. Alternatively, the primary HUVEC cells demonstrated increased expression of p21, while the NCI-H1299 NSCLC cells showed elevated levels of both p21 and p27, after doxorubicin treatment as shown in the new Fig S6A). Because OVCAR-8 cells did not detectably upregulate levels of p21 and p27, we surmised the cell cycle arrest in these cells is most likely occurring through p16. OVCAR-8 cells have been previously described to enter long-term cell cycle arrest primarily by induction of p16 (Ha et al. 2000), which is in good agreement with our results.

2. The use of only one DNA damaging compound, doxorubicin, is also severely limiting for this study. Since the focus of the study is presented as an analysis of DNA damage-induced senescence, validation of the findings using several other DNA damaging agents (including ionizing radiation and compounds belonging to other categories of DNA-damaging chemotherapeutics) is warranted.

As requested by the reviewer, we have now conducted additional experiments to address the generalizability of our results to other DNA damaging agents. We describe the results of these experiments in a new subsection of the results (page 21), and show the data in the new Figures 7H-J and S8. Treatment of U2OS with mitoxantrone, a structurally distinct topoisomerase II inhibitor from doxorubicin, also caused the induction of senescence, as evident by appearance of enlarged cells that failed to incorporate nuclear BrdU after mitoxantrone treatment. Strikingly, when the cells treated were co-treated with either the JNK inhibitor SP600125 or the MEK inhibitor PD98059 at early times along with mitoxantrone, there was a large increase in the appearance of BrdU

positive cells, indicating that senescence after mitoxantrone treatment is driven by the JNK and Erk pathways (new Fig 7H).

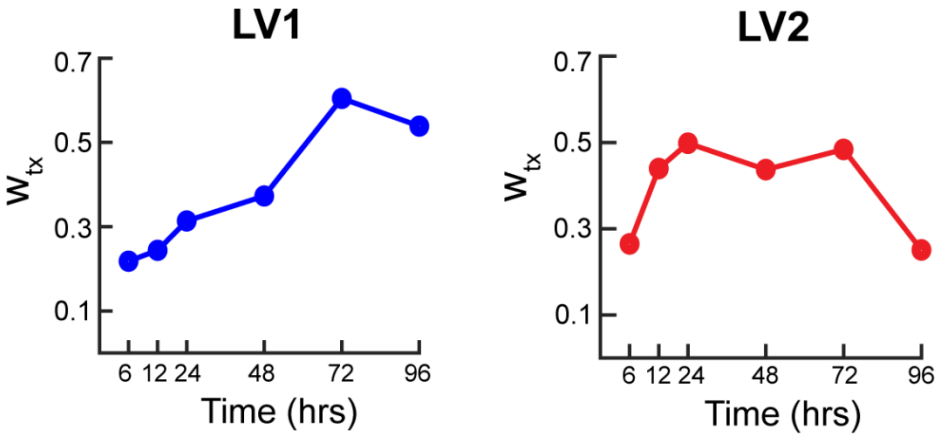
We also treated U2OS cells with with neocarzinostatin (NCS), a radiomimemic double-strand break inducer, or with cisplatin, a platinum-based DNA crosslinker. Both of these drugs, at selected doses, induced cell senescence, as evidenced again by appearance of enlarged flattened cells that were BrdU-negative 6 days after treatment. However, unlike either doxorubicin or mitoxantrone, neither neocarzinostatin or cisplatin-induced cell senescence was reversed by SP600125 and the PD98059 co-treatment (Fig S8A). In seeking to understand the difference in JNK and Erk dependency for senescence induction between these different classes of DNA damaging agents, we noted that topoisomerase inhibitors, in particular, and well known for their ability to readily induce high levels of replication stress (Durr, Wallace, and Citarella 1983; Tewey et al. 1984; Zellweger et al. 2015; Zhao et al. 2012). We therefore next examined whether the extent of induced replication stress distinguished the DNA damaging agents that induced senescence through the JNK and Erk pathways from those that did not. We measured levels of phospho-RPA2 (Ser-8), a widely used marker of replication stress, at early times after the addition of each drug at senescence-inducing doses. Notably, as shown in the the new Figures 7I and J, both doxorubicin and mitoxantrone induced high levels of phospho-RPA2 (S8), while NCS and cisplatin did not (Fig 7I, J). We next measured the levels of phospho- and total JNK, Erk, and c-Jun to see if there was also differential activation of these pathways amongst these distinct DNA damaging agents. Strikingly, only the topoisomerase II inhibitors were able to significantly increase the levels of phospho-JNK, phospho-Erk, and phospho-c-Jun in comparison to vehicle control (Fig 7J, S8B). These results suggest that only DNA damaging agents that strongly induce replication stress at early times after administration activate the JNK and Erk signaling pathways to drive senescence after treatment. These new findings are now described on page 21 of the Results section in the revised text.

We are very grateful to the reviewer for pointing out this unexplored area in our previous version of the manuscript, and we believe the addition of our findings with other DNA damaging agents further highlights the similarities between the role of replication stress in oncogene-induced senescence and replication stress in DNA damage-induced senescence. We now comment on these parallels in the Discussion section, on lines 9-14 on page 25 of the revised Discussion section. In addition, we have now changed the title of the manuscript to “Biphasic JNK-Erk Signaling Separates Induction and Maintenance of Cell Senescence after DNA Damage Induced by Topoisomerase II Inhibitors” in light of these results.

3. Interpretations of the t-PLSR results and the PCA analysis are somewhat contradictory and subjective. In particular, in the t-PLSR it seems counter-intuitive that the p-MAPK signals are associated strongly along the "apoptosis" axis, yet a target of the p-MAPKs, p-c-Jun, projects strongly along the "senescence" axis. Furthermore, through the PCA analysis shown in Fig. 5B, the authors highlight that the p-MAPK signals become

progressively less PC2 positive and more PC1 positive. However, no quantification in support of this claim is provided, and especially for the p-JNK and p-p38 this appears to be an over-interpretation. Based on similar visual inspection, one could just as readily state that p-c-Jun levels as shown in Fig. 5D are becoming less PC1 positive and more PC2 positive, counter to later analysis highlighting p-c-Jun as a key regulator of senescence.

We agree with the reviewer that it is initially puzzling that MAP kinases that act upstream of c-Jun to be significantly correlated with apoptosis, but not senescence, in tensor PLSR (t-PLSR). One approach to better understand of why certain signals are weighed highly on a particular LV is to study the signal time weights (w_{tx}), which provides information on which timepoints in the signaling data were weighed most heavily in the construction of that particular LV (Fig 4A). When we plotted the w_{tx} from our model for LVs 1 and 2, we found that the w_{tx} for LV1 became more positive over time with the largest value at the 72 hour timepoint, while the w_{tx} for LV2 are largest at intermediate timepoints (Reviewer Figure 1, below).



Reviewer figure 1: Signal time weights from t-PLSR model.

Signal time weights (w_{tx}) from the calibration t-PLSR model plotted over time. The blue curve is the LV1 w_{tx} values, while the red curve is the LV2 w_{tx} values.

This indicates that LV1 (senescence) weighed signal values at later timepoints the most, while LV2 (apoptosis) weighed signal values in the 12-72 hour range the most. When looking at the intensity values in the immunofluorescence data during this intermediate timeframe, the p-JNK and p-Erk are, for the most part, highest at the 10 μM doxorubicin dose in comparison to the other doses. This 24-72 hour window excludes the 6 hour timepoint when p-JNK and p-Erk at the senescence-inducing dose (0.5 μM) are as elevated as the 10 μM dose, and the 96 hour timepoint when p-Erk reaches its maximum value across all measured doses (Figs 3C, S2). Thus, the w_{tx} for LV2 resulted in the intermediate, apoptosis-correlated p-JNK and p-Erk values being weighed more heavily than the early and late senescence-correlating signals when constructing the model. As we noted on page 13 (lines 19-23), one of the pitfalls of t-PLSR is the fact that the aggregate across all signals contribute to these time weights, which makes it not

possible to resolve the t-PLSR contributions of any signal to a measured response at a very specific timepoint. This necessitated the use of PCA to resolve these correlations with cell-fate across time, particularly in the signals that met the significance threshold for correlation with cell-fate in t-PLSR.

As suggested by the reviewer, to quantify the contribution of the PCA loadings to cell fate in principal component space, we have now used the PCA treatment scores to define a senescence axis (using the 0.5 μM dose) and an apoptosis axis (using the 10 μM dose). We then quantified the projection of each PCA loading onto the senescence and apoptosis axes by taking the cosine of the angle between the respective fate axis and the loading vector, as explained in the revised text (pages 13, lines 29-31, and 14, lines 1-5, Fig 5B). When we calculated this for c-Jun, the projections along the senescence axis were highly positive at all timepoints, while the apoptosis projections were negative (Fig 5C). When we looked at these projections for p-JNK and p-Erk, the senescence projections were highly positive at the 6 hour timepoint, negative for the 12-48 hour timepoints, and positive again for the 72 and 96 hour timepoints (Fig 5F).

Taken together, these projection values indicated that p-JNK and p-Erk were correlated with senescence at the 6 hour timepoint, with apoptosis at intermediate timepoints, and increasingly correlated with senescence again at late timepoints. These time-specific correlations with fate are in excellent agreement with the experimentally validated early roles of JNK and Erk in the senescence decision <12 hours after doxorubicin treatment (Fig 6A-E), and the late roles (>72 hours) in secretion of SASP cytokines IL-6 and IL-8 (Fig 6J-N). Additionally, the correlation with apoptosis at intermediate timepoints further confirms why p-JNK and p-Erk were correlated exclusively with apoptosis in t-PLSR, given the LV2 w_{tx}.

Minor comment:

1. It would be better to explicitly indicate what the bar colors represent in Fig. 4H, either in the legend or the key inset. Presumably they represent the phenotypes as indicated in Fig. 4F and 4G, but a similar color scheme is used to represent different doxorubicin doses in Fig. 4D.

Thank you for pointing this out. We have now clarified the meaning of the colors in figure 4H in the legend as suggested.

2. "Reporter" is misspelled in the y-axis label of Fig. 7A.

Thank you for pointing this out. We have now corrected this.

Reviewer #2

Reviewer #2: This well-written paper by Yaffe and colleagues is a very interesting exploration of the DNA damage signaling landscape after treatment with various doses of doxorubicin. They measure 27 "signals" (total- and phospho-proteins) and 10 "responses" (measurements of proliferation, senescence, and apoptosis) at 6 timepoints

in 4 stimulus conditions - a heroic number of measurements to quantify in single cells. The data also points towards some interesting new findings, including a role for early Jun phosphorylation and AP-1 transcriptional activity in initiating senescence. I am overall enthusiastic and supportive of the work. Nevertheless, I do have some suggestions for improvement, and one suggestion of an experiment that might help to explain the context-dependence of Erk/JNK signaling.

Thank you for your enthusiasm about this work! We have addressed all of your suggestions below.

- One difficulty I had in reading the manuscript was related to the inconsistency in how Erk/JNK signaling are treated between the data, text, and figures. When discussing the PLSR results, the authors note that "… p38MAPK, Erk and JNK in the nucleus projected positively along LV2, correlating with apoptosis. These findings further support our biological interpretation of the LV2 axes …". From that point forward Erk and JNK are defined as "apoptotic" stimuli, colored red in all figures and labeled as "apoptotic signals" in Figure 5D - at least until Figure 6, when Erk and JNK are the focus of the remainder of the paper for their roles in senescence.

The reason this is misleading is that even in Figure 3, it is clear that Erk reaches its highest level in the entire dataset under conditions that are exclusively senescence-related: the 96 h timepoint in 0.5 μM doxorubicin. The PCA results in Figure 5D also show that Erk in particular (but all three MAPKs in general) is just as strongly associated with senescence as apoptosis, with early timepoints projecting as apoptosis-related on PC2 and late timepoints projecting as senescence-related on PC1. (This is discussed clearly in the text, despite the corresponding figure still labeling MAPKs as "apoptotic signals").

We apologize for any confusion regarding the labelling of Erk and JNK as apoptotic signals. Additional analysis has now been performed to better clarify the dual senescence and apoptosis roles of JNK and Erk in response to major concern #3 posed by reviewer #1. Please see our response to that comment above for additional details of how we have now addressed this.

In particular, we feel that the additional senescence and apoptosis projection analysis we perform in the PCA analysis (new Fig 5A-F), along with the accompanying text changes, better clarify these time-dependent dual roles. We agree with the reviewer that the label of “apoptotic” in the PCA analysis in the original manuscript was confusing, given the demonstrable time-dependence association with senescence shown in the MAP kinase PCA loadings. To address this, we have now labeled these signals, and others with similar complex fate associations, as “time-dependent signals” in the new Figure 5. We thank the reviewer for pointing out the disconnect between our figures and the text, and hope the implemented figure changes have remedied this.

- The paper's central mechanistic conclusion is that early c-Jun phosphorylation by JNK and Erk plays a crucial role in establishing senescence. But the manuscript left me wondering: why do the high levels of Erk and JNK activity in 10 μM doxorubicin fail to

induce c-Jun phosphorylation at those same timepoints? Erk and JNK phosphorylation are highest early in 10 μM doxorubicin (indeed, higher than in 2.5 and 0.5 μM), exactly when the inhibitor experiments suggest they are most important, but phospho-c-Jun remains low.

One may conjecture that this is because total c-Jun might fail to be induced at 10 μM but not 2.5 or 0.5 μM doxorubicin, so that there is nothing to phosphorylate. Is this the case? What about for other AP-1 components (e.g. Fos)? Simply comparing phospho and total Fos/Jun staining at 0.5 and 10 μM doxorubicin would tell us whether phosphorylation of these targets is really context dependent, or whether their expression is, helping to explain how Erk/Jnk can play such distinct roles depending on stimulus conditions.

This was a brilliant suggestion, and, in fact, the reviewer's conjecture was correct! We are very grateful to the reviewer for this very useful and keen insight. To explore the reviewer's hypothesis, we measured total c-Jun levels at 0, 2, 4, 6, and 8 hours after the addition of 0.5, 2 or 10 μM doxorubicin. As shown in Figure S5, panels H and I, the levels of total c-Jun showed a progressive time-dependent decrease at the 2 and 10 μM dose compared to the 0.5 μM dose, which was evident as early as 6 hours after the addition of doxorubicin, well within the timeframe of when the senescence-proliferation decision is determined by c-Jun (Figs 6 and 7F, G). The decrease in c-Jun also explains why the levels of p-c-Jun in the immunofluorescence data are only moderate at the 2 μM dose and quite low at the 10 μM dose, as seen in Figure 3C, despite the higher p-JNK and p-Erk levels at these same doses. These new results and their interpretation are now discussed on page 20 (lines 18-28) of the revised text, and the reviewer is acknowledged for their suggestion.

- My last comment is about the compendium of measurements shown in Figure S2, which has some very strange properties. Some measurements show extremely wide variance between replicates (Cyclin B at 2 μM doxorubicin, IkBa at 0.5 μM dox, p-Hsp27 at 0 μM and 2 μM dox, p-S6 at 0.5 μM and 2 μM dox). In some cases, replicates in a single condition represent both the minimum and maximum values obtained across all timepoints for that quantity! What is even stranger is that both the low and high values are often tightly reproducible across multiple replicates, and then the values "jump" in another set of tightly reproducible replicates. See for example Cyclin B at 2 μM doxorubicin: these are not normally distributed measurement errors on an identical set of underlying data, but what look like two completely different responses (rising Cyclin B nuc + cyto levels in ~half the replicates; falling Cyclin B nuc + cyto levels in the other half) overlaid on top of each other.

One interpretation for two tightly clustered responses that vary widely from one another is that something differently was done to the two set of replicates, e.g. leading one sample to have rising Cyclin B in half the samples and falling Cyclin B levels in the other half. It would be helpful for the authors to look over this data once more with a careful eye to

ensure that these reproducible but distinct responses are real, and comment on this feature of the data. They may represent a concern about or limitation of the data, given their extreme deviation from typical measurement noise.

We are very grateful to the reviewer for their careful attention to the quantification of the primary immunofluorescence data. We thoroughly examined all of the immunofluorescence data for these signals to determine the source of their outliers, and conducted several additional experiments to further examine their reproducibility, as detailed below.

Cyclin B: The outliers present at the 2 μM dose (the upper two values clustered together) are indeed from the same experiment. Unfortunately, when we opened these images, the original files had been overwritten by the rescaled images. Because the rescaled images from this single experiment seemed to have been saturated, we elected to discard all of the cyclin B values collected at all the doses in all of the prior experiments, and completely re-do the Cyclin B immunofluorescence experiments using three biological replicates for each dose and each timepoint (except for the 72 hour timepoint, where 2 replicates were used because of a technical error in the third replicate). As seen in the new Figures 3C and S2, the new cyclin B values largely recapitulate the trends seen in the original data, with the exception of a slightly larger elevation at the 10 μM dose.

IκBα: We discarded the two outliers present in the data, which were from the same experiment, and replaced them with two new biological replicates from a new experiment that we conducted. As seen in the new Figure S2, the overall trend largely remains the same, but now the variance of the datapoints across experiments has significantly decreased.

p-Hsp27: The source of this outlier was due to a coding error, which inadvertently swapped the NF-κB and the p-Hsp27 wells when processing the CellProfiler outputs in one experiment. The correct NF-κB and the p-Hsp27 are now shown in the new Figures 3C and S2. We apologize for this error, and have double-checked all of the rest of the data processing scripts to ensure that no other coding errors were present.

p-S6: The two outliers again came from one single experiment. To understand if these values were truly outliers, we performed additional experiments using four biological replicates for p-S6 staining. The normalized intensity values from these experiments clustered tightly with the rest of the experimental values, showing strong reproducibility, and confirming that the discarded values from the prior single experiment were indeed outliers.

Using this new experimental data, we then completely rebuilt both the tensor PLSR model and the PCA analysis using the corrected signals. As shown in the revised Figures 4 and 5, these models remained largely unchanged, but the new tensor PLSR model with the improved data actually increased the percent of variance explained by the model from ~75% to ~80%, and decreased the RMSEP in a 3 LV model, indicating that these changes

improved the model! We would like to reiterate our thanks to the reviewer for their meticulous attention to detail, as it greatly improved the quality of the signaling data compendium, as well as the performance of the model.

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