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Combined experimental and computational analysis of DNA damage signaling reveals context-dependent roles for Erk in apoptosis and G1/S arrest after genotoxic stress

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 September 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. However, they raise several concerns, which should be convincingly addressed in a revision of the study. The recommendations provided by the reviewers are very clear in this regard and refer to the need for some further data analysis and additional clarifications, in particular with regard to the description of the TI-SWR method.

In view of the quantitative nature of this work, it would be also important to include in this submission as much as possible of the quantitative experimental data used in this study. In addition to our capacity to host datasets in our supplementary in formation section, we also provide a functionality on our website, which allows readers to directly download the 'source data' associated with selected figure panels (for example: <http://tinyurl.com/365zpej>), for the purpose of alternative visualization, re-analysis or integration with other data. These files are separate from the traditional supplementary information files and are directly linked to specific figure panels. *In the case of this study, we would strongly encourage you to submit the individual files corresponding to the quantitative signaling, cell cycle and apoptosis measurements displayed in the various figure panels (eg. data showed in figure 2B-D, 3A, 4B, 5A, 7A-C)*. General formatting guidelines for 'source data' are available for download at http://www.nature.com/msb/authors/source-data.pdf.

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If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favourable.

Yours sincerely,

Editor
Molecular Systems Biology

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Referee reports

Reviewer #1 (Remarks to the Author):

This is an elegant and well executed study combining experimental data with computational tools to integrate elements of DNA damage signaling with other signaling pathways, and cell fate decisions. Overall, the work is supported by solid evidence, and there are several innovative conclusions reached by this systems biology approach that allows a broader view on the cellular responses to genotoxic stress, not readily achievable through other methods. As such, this study is important and represents a significant advance in the field, with implications for a broader audience.
I believe the dataset deserves to be published, however a few aspects need attention to provide a more balanced interpretation of the data, as indicated in the critical comments below:

1. The authors should point out that one limitation of this study is the U2OS sarcoma model. While this cell line is very useful and frequently studied (thereby justifying the choice for this multiparameter. systems biology approach), there are multiple genetic defects, and some of these affect the relevant biological responses to DNA damage. including the decisions of cell cycle arrest, surviaval vs. death. In particular, several papers reported a pronounced defect of this cell line in the p53-mediated G1 checkpoint, despite p53 itself is wild-type. This defect almost certainly affects the results that the authors present as surprising, in terms of the relative contribution of G1 vs. other checkpoints in this model, timing of checkpoint duration, relatively rapid entry into S phase despite DNA damage, etc. This does not make this study less valuable, however this needs to be discussed to avoid confusing the readers.

2. The discussion of NFkB pathway is clearly appropriate given the TNFa treatment, and the impartance of NFkB in pro-survival signaling after DNA damage. One important element of the interplay between p53 and NFkB, critical especially for the responses and cell fate decisions under combined doxorubicine and TNFa treatment used in this study, is the status of the NQO1 gene (Fagerholm et al. Nature Genetics 2008). This should be discussed and pointed out, also because the p53-NQO1-NFkB interplay plays an important role in response of human tumors to doxorubicine chemotherapy, affected by the TNFa cytokine present in a significant fraction of e.g. breast carcinomas (Fagerholm et al. 2008). It would be useful to mention also the status of the NQO1 gene in the U2OS cells, I believe that at least one allele is functional in this cell line.

Reviewer #2 (Remarks to the Author):

In this manuscript, multiple components involved in DNA damage signaling are quantitated together with cell-cycle progression and apoptosis in a time-resolved fashion. Signaling measurements are mainly on the cell-population level (immunoblotting); flow cytometry is used to distinguish cell-cycle phases and correlate them with apoptosis markers. The experiments appear to be well conducted and the resulting data are, at present, quite unique in the DNA damage signaling field in

terms of temporal resolution and number of observed variables.

Regression methods are then applied to spot interesting correlations between the signaling time-course data and the cell-cycle behavior and apoptosis. These analyses and subsequent MEK inhibitor experiments show a role of Erk activity for G1/S arrest and apoptosis. The increase of cell death upon TNFa treatment at low dose doxorubicin is also interesting.

Although the data are interesting and important, the broad focus makes this work somewhat vague in a key mechanistic aspect. There are several studies that indicate that the quality of DDR signaling and thus the subsequent cellular response are controlled to a large degree by activation patterns of ATM, p53 and other ATM targets, but this is incompletely understood. Chk kinases play an important role here, but also HiPK2, which has not been considered in the scheme in Figure 1. The measurements of this part of the network are sparse, given its key position in DDR (only one phospho site each in ATM and p53 is monitored, Chk1/2 activity is not measured at all). The authors make interesting observations on ATM and Nbs1 activity that a simple linear pathway model cannot explain but do not attempt to go further here. It might have been more interesting to see how much of the response data can be explained by a reduced set of variables (e.g., the so-called chromatin-integrity module centering around ATM in Fig. 1) rather than by a rather large (PLS) regression model, which suffers the problem of contamination from noise in the present case of a large number of 'independent' variables (as the authors also state).

Regarding the second regression method, named TI-SWR, the description given in the manuscript is unintelligible to me. Hence I cannot evaluate whether it presents an alternative way of looking at the data that successfully copes with potential problems in the PLS regression. The way the text is written, the method appears to have been devised by the authors. There is a toolkit of regression methods for high-dimensional problems available and it would be relevant to know whether TI-SWR is related to an existing method or, otherwise, how its performance has been ascertained. Concise equations in the methods section will help.

A point of interpretation:
- The action of doxorubicin is stated as inducing DSBs by topoisomerase II inhibition. While this is true, doxorubicin also induces other kinds of DNA damage as it forms DNA adducts.
- It is then discussed as "surprising" that apoptosis occurs in G1, as DSBs should be mainly sensed in S phase. DNA integrity is certainly also sensed in G1. It appears rather more surprising that cells arrest in G1, the enter S after some time but arrest again in G2 although doxorubicin has been withdrawn by then. This could indicate defective G1 and/or S phase checkpoints in the cancer cell line used.

Reviewer #3 (Remarks to the Author):

Tentner et al analyzed functional consequences (apoptosis, cell cycle arrest and survival) of crosstalks between multi-signaling pathways that are triggered by doxorubicin, a drug that induces DNA damage, and TNF- , a cytokine commonly found in tumor microenvironments. They exposed U2OS osteosarcoma cells to different doses of doxorubicin and TNF- , and, quantitatively measured molecular signals (such as, pErk, pAkt, pJNK, pp38) at different time points that gave rise to various levels of apoptosis and cell cycle arrest. Using multivariate statistical tools such as, PLSR and "time interval stepwise regression" (a new method developed by the authors), the authors showed that Erk activation which is usually implicated for cell survival and progression into the cell cycle, instead helps in apoptosis and keeping the cells arrested at the G1/S state. This is the main result of their detailed investigation. The result will be of interest to the cell signaling community, as Erk activation is known to influence cell decision in diverse cell types. I think the authors carried out a well planned and thorough study using complementary experiments and computational modeling to arrive at a counter intuitive result, however, I think some of their results need to be re-analyzed to substantiate their main conclusion. I have detailed my comments below.

Major comments:

1. The authors use a MEK inhibitor, PD, to test the role of Erk activation in promoting cell cycle arrest and found that in the presence of PD and doxorubicin the percentage of cells released in the S phase at an earlier time point increases supporting the model prediction. However, it was not clear if

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PD would affect activations of other signaling molecules that may be the main players behind this effect. A direct test of this prediction would be to use the PLSR or T1-SWR model developed in the previous section to predict the results in Fig. 7 using the kinetics of the other molecules as input variables.

2. The authors use principal components to evaluate correlations between activation of different molecular signals and functional outcomes for different treatments (Fig. 5). It was not obvious to me if the model can successfully predict a subset of the training data, and, how many principal components one would need for that. Even though, the top two principal components capture 87% of the variations, the smaller principal components may still play a significant role in affecting predictions. Therefore, including such tests will increase confidence in the model.

Minor comments:
1. pAkt is usually considered a pro-survival signal, however, high doses of doxorubicin that increased apoptosis produced more pAkt, as compared to lower doses of doxorubicin. The authors mentioned this unanticipated result in the paper. Is it possible to get further insight into this by using their models?

2. It will helpful if the error bars are shown in Fig. 4B.

3. On page 4, data from cell proliferation experiments were not shown ("data not shown"). Either it should be noted why the data was not shown or it should be included in the supplementary material.
4. On page 5, it is not clear what is meant by the drug free medium, does it mean a medium with no doxorubicin and TNF- ?

5. It will be helpful for the community interested in using the results and the models if the data sets (including replicates) shown in Fig. 4 are provided as Excel files.

6. Typo on page 3, I think "though" means "thought".

1st Revision - authors' response 22 December 2011

Response to Reviewers

We would like to thank all of the reviewers for their helpful comments and suggestions.

Reviewer #1

This is an elegant and well executed study combining experimental data with computational tools to integrate elements of DNA damage signaling with other signaling pathways, and cell fate decisions. Overall, the work is supported by solid evidence, and there are several innovative conclusions reached by this systems biology approach that allows a broader view on the cellular responses to genotoxic stress, not readily achievable through other methods. As such, this study is important and represents a significant advance in the field, with implications for a broader audience.

Thank you!

1- *The authors should point out that one limitation of this study is the U2OS sarcoma model. While this cell line is very useful and frequently studied (thereby justifying the choice for this multiparameter. systems biology approach), there are multiple genetic defects, and some of these affect the relevant biological responses to DNA damage. including the decisions of cell cycle arrest, surviaval vs. death. In particular, several papers reported a pronounced defect of this cell line in the p53-mediated G1 checkpoint, despite p53 itself is wild-type. This defect almost certainly affects the results that the authors present as surprising, in terms of the relative contribution of G1 vs. other checkpoints in this model, timing of checkpoint duration, relatively rapid entry into S phase despite DNA damage, etc. This does not make this study less valuable, however this needs to be discussed to avoid confusing the readers.*

We fully agree with the reviewer. We now discuss this explicitly on page 22 of the Discussion in the revised manuscript. The new text reads:

"It is important to note that these findings were made and validated in the U2OS cell line. While U2OS contain 2 wild-type copies of the p53 gene, this cell line is known to be hypermethlyated at the INK4a/ARF locus, leading to lack of expression of both p16ARF and p14ARF (Park et al, 2002). G1/S cell-cycle arrest in response to DNA damage may be expected to be less robust in the absence of p16/p14 molecules to reinforce this response. It is possible that a role for ERK in the maintenance of the G1/S arrest response, and death from this arrest, represents a 'fail-safe' mechanism, and that a role for ERK in this response may be expected to be less pronounced in cell types that express p16/p14. Furthermore, the absence of a robust G1/S checkpoint in these U2OS cells may partially account for the unexpected doxorubicin-induced death in the G1 state. Importantly, hypermethylation and mutations at the INK4a/ARF locus are found in many human cancers, including a large fraction of melanomas and carcinomas (Cheung et al, 2009; Sharpless & Chin, 2003; Tannapfel et al, 2001). Thus, Erk may fulfill this role in a variety of other tumor contexts."

2- *The discussion of NFkB pathway is clearly appropriate given the TNFa treatment, and the importance of NFkB in pro-survival signaling after DNA damage. One important element of the interplay between p53 and NFkB, critical especially for the responses and cell fate decisions under combined doxorubicine and TNFa treatment used in this study, is the status of the NQO1 gene (Fagerholm et al. Nature Genetics 2008). This should be discussed and pointed out, also because the*

p53-NQO1-NFkB interplay plays an important role in response of human tumors to doxorubicine chemotherapy, affected by the TNFa cytokine present in a significant fraction of e.g. breast carcinomas (Fagerholm et al. 2008). It would be useful to mention also the status of the NQO1 gene in the U2OS cells, I believe that at least one allele is functional in this cell line.

Thank you for bringing this important point to our attention. Indeed, NQO1 is likely to be an important genetic determinant of the response. We now explicitly address this on pages 18-19 of the revised Discussion, which now reads:

*"Interestingly, the U2OS cell line used in this investigation contains 2 wild-type copies of a functional NAD(P)H:quinine oxidoreductase 1 gene (NQO1*1) gene (Fagerholm et al, 2008). Recent findings have implicated NQO1 in regulating cell-death in response to both anthracyclines (i.e. epirubicin and doxorubicin) (Fagerholm et al, 2008; Jamshidi et al, 2011), and to TNF! (Ahn et al, 2006) through effects on NF- !B, p53, and cellular anti-oxidant activity. These findings suggest that NQO1 may be a key integrator of the cellular response to these inputs, potentially playing a role in the observed synergistic response to TNFα and doxorubicin co-treatment."*

Reviewer #2

1- There are several studies that indicate that the quality of DDR signaling and thus the subsequent cellular response are controlled to a large degree by activation patterns of ATM, p53 and other ATM targets, but this is incompletely understood. Chk kinases play an important role here, but also HiPK2, which has not been considered in the scheme in Figure 1. The measurements of this part of the network are sparse, given its key position in DDR (only one phospho site each in ATM and p53 is monitored, Chk1/2 activity is not measured at all).

At the onset of the project, we purchased and attempted to validate a large number of antibodies (>100) against many molecular targets involved in the DNA damage response. We also wanted to gain insight into how these core DNA damage response pathways interfaced with other signaling pathways that were not generally considered within the 'core network' (i.e. Erk, JNK, NF!B, AKT, etc). Since doxorubicin was the DNA damaging agent used in these studies, we expected that the majority of DNA damage would be in the form of DNA double strand breaks, and thus the core pathway molecules that were obvious measurement candidates included activated forms of ATM, H2AX, Nbs1, Chk2, and p53. In general we chose antibodies against specific phospho-sites on these molecules to monitor, based on broad evidence in the literature, that phosphorylation at these sites was important either for activation of these molecules (i.e. the pSer-1981 site on ATM (Bakkenist & Kastan, 2003), the pThr-202/pTyr-204 sites on Erk1/2, the pThr-180/pTyr-182 sites on p38, etc) and/or that mutation of these sites to non-phosphorylatable residues conferred a marked phenotype (i.e. the Ser-15 site on p53 (Chao et al, 2000)). The function of other known phosphorylation sites (many of which have only been discovered relatively recently) either is currently unclear, or have minimal, if any, phenotypic consequences upon mutation (i.e. Ser-20 on p53 (Wu et al, 2002)), and were therefore not investigated further. Hopefully, as the function of additional sites on these molecules becomes established, and good antibodies become available, they could be used in future studies.

In order for the resulting data to be useful for systems biology-based computational models, the antibodies that we used must provide a rigorously quantitative and linear response during initial

testing (c.f. Figure 4a,b d, and e), low background reactivity, and minimal day-to-day variability. After testing multiple antibodies from a variety of different vendors we were unable to find any antibodies against Chk2 (particularly the pThr-68 site), or Chk1 (both the pSer-317 and pSer-345 sites) that met these criteria. We explain this in the revised manuscript on pages 9-10 of the revised Results section, which now reads:

"Western blotting and ELISA assays used for this analysis were validated to be both strictly quantitative and highly reproducible, with signaling measurements always performed in the linear regime of the assay, (Janes et al, 2005) (Figure 4A, Methods, Supplementary Table 1). For several signaling molecules of interest, including Chk1 and Chk2, we were unable to develop appropriate assays due to the lack of phospho-site specific antibodies that passed this critical validation step."

HIPK2 is clearly an important molecule in the DNA damage response, however at the inception of our study, most of the published literature suggested that it played a key role in regulating gene transcription (particularly a subset of p53-driven genes), a response that we did not directly measure. In addition, the importance of HIPK2 has not been generally highlighted in traditional reviews of the DNA damage response, although it undoubtedly should be. To address this, we now explicitly point out on page 20 of the revised Discussion:

"Interestingly, a transcriptional modulator, homeodomain interacting protein kinase 2 (HIPK2), has emerged as a player in the core DDR response to DSBs, providing an additional level of regulation between ATM and p53 activities (Puca et al, 2010; Rinaldo et al, 2007; Winter et al, 2008). HIPK2 will be an important molecule to include in future studies investigating the complex regulatory interplay between p53 and ATM and life-death decision following DNA damage."

2- The authors make interesting observations on ATM and Nbs1 activity that a simple linear pathway model cannot explain but do not attempt to go further here. It might have been more interesting to see how much of the response data can be explained by a reduced set of variables (e.g., the so-called chromatin-integrity module centering around ATM in Fig. 1) rather than by a rather large (PLS) regression model, which suffers the problem of contamination from noise in the present case of a large number of 'independent' variables (as the authors also state).

This is an excellent idea. We did exactly what the reviewer suggested, and examined the ability of either a PLSR model using all signals as the independent variable set, or models using a reduced set of independent variables - either only the core DNA damage response proteins, or only the non-core DNA damage response proteins - to predict the extent of apoptosis after doxorubicin administration. As now shown in the new Supplemental Figure 4B, we found that a model built using only the signals from the core DNA damage response proteins was just as good (if not a tiny bit superior) to a model built using all of the signals, as assessed by the predictive capability, Q^2_{cum}. Intriguingly, however, a model built using only the non-core signals (i.e. a model completely lacking ATM, H2AX, Nbs1, p53) had almost as much predictive capability as either the full model or the model built from core DNA damage response molecules, suggesting that these non-core pathways are also information-rich with respect to cell fate decisions. We discuss these findings on pages 12-13 of the revised Results section:

“While signaling from core DDR components is clearly important in controlling cell fate decisions following damage, we were interested in how these signals are integrated with non-core DDR signals, such as the MAP kinases (p38, ERK, JNK), Akt and NF !B, that regularly monitor the cellular environment, and are known to influence life-death decisions following other types of stimuli. We hypothesized that both core and non-core DDR signaling responses would be information rich and have predictive capability with regard to cell fate following DNA damage. To test this, we evaluated the ability of a 2-component PLSR model built with either all of the measured signals, or built with only a reduced set of only core DDR signals or non-core DDR signals as independent variables, to predict cell death between 6 and 12 hr following doxorubicin administration. As shown in Supplementary Figure 4B, a model built with only the core DDR signals as independent variables had a cross-validated predictive capability that was as good or better than either a model built with all of the signals, or a model built with only non-core DDR signals. However, the predictive capability of all three models was quite close (Q^2 = 0.79, 0.78 and 0.70, respectively), and substantially higher than that of a model built using only the total protein levels of signaling molecules as the independent variable set (i.e. neglecting all phosphorylation/activity data; Q^2= 0.36). We interpret these results as evidence that while the core DDR signals are most informative for the prediction of apoptotic response, non-core DDR signals carry a significant amount of complementary predictive capability on their own. We therefore focused further analysis on 2-component PLSR models built with all signals in the independent variable set in order to gain insight into the contributions from both core and non-core signaling pathways.”

Thank you for this suggestion!

3- Regarding the second regression method, named TI-SWR, the description given in the manuscript is unintelligible to me. Hence I cannot evaluate whether it presents an alternative way of looking at the data that successfully copes with potential problems in the PLS regression. The way the text is written, the method appears to have been devised by the authors. There is a toolkit of regression methods for high-dimensional problems available and it would be relevant to know whether TI-SWR is related to an existing method or, otherwise, how its performance has been ascertained. Concise equations in the methods section will help.

We apologize for any confusion or our lack of clarity in describing the TI-SWR method. The method was indeed devised by us, but is essentially a straightforward extension of a well-validated prior method for variable selection known as “stepwise regression with forward selection”. We now explicitly comment on this on page 15 of the revised manuscript, and cite a relevant reference: “We therefore developed time-interval stepwise regression (TI-SWR), a complementary computational method that builds on the established variable selection method, stepwise regression with forward selection (Efroymson, 1960), to identify specific signaling events, or combinations of events, that were highly correlated (and likely causal) for specific phenotypic outcomes.” In addition, we have revised the Methods section to better describe the method (page 27 of the revised manuscript), and also included a step-by-step tutorial (Supplementary Methods) to faciliate the ability of others to use this method in their own research.

4- A point of interpretation:

- The action of doxorubicin is stated as inducing DSBs by topoisomerase II inhibition. While this is

true, doxorubicin also induces other kinds of DNA damage as it forms DNA adducts. - It is then discussed as "surprising" that apoptosis occurs in G1, as DSBs should be mainly sensed in S phase. DNA integrity is certainly also sensed in G1. It appears rather more surprising that cells arrest in G1, the enter S after some time but arrest again in G2 although doxorubicin has been withdrawn by then. This could indicate defective G1 and/or S phase checkpoints in the cancer cell line used.

The reviewer makes an especially vital point. While a major target of doxorubicin is topoisomerase II to cause DSBs, the drug is also capable of causing other types of non-DSB DNA lesions. We therefore thought it was important to experimentally ascertain whether the G1 cells in our study did, or did not, show evidence of DSBs after doxorubicin treatment. We treated cells with low-dose doxorubicin under the exact conditions used in the paper, and then quantitatively examined "H2AX staining as a function of cell cycle stage using flow cytometry. The results are now shown in Supplementary Figure 1, and discussed on page 8 of the revised Results section:

"There is strong evidence that the primary mechanism of doxorubicin action is via Topo II inhibition (Nitiss, 2009). Levels of Topo II in tumors in vivo are correlated with the apoptotic response to doxorubicin, while down-regulation of TopoIIa is associated with acquired resistance of tumors to this drug (Ogiso et al, 2000; Tanner et al, 2006). Importantly, however, doxorubicin is well known to cause additional types of DNA damage through Topo II-independent effects, including, free radical-induced single strand breaks, direct Doxorubicin-DNA adducts, and inter-strand crosslinks ((Swift et al, 2006) and refs therein). To directly address whether doxorubicin-induced double strand breaks were present in the G1 population, we treated cells with 2 μM doxorubicin and used flow cytometry to measure the percentage of cells staining positively for #H2AX as a function of cell cycle stage. As shown in Supplementary Figure 1, elevated levels of #H2AX could be detected in roughly 50% of the G1 cells, a value that while large, was somewhat lower than that observed in S- and G2 cells where the response rate was ~90%. Thus, while other types of doxorubicin induced DNA damage almost certainly exist in G1 cells, our data does indicate the presence of drug-induced DSBs during this phase of the cell cycle. Interestingly, Ju et al have reported topoisomerase-mediated generation of transient DSBs during normal gene transcription (Ju et al, 2006)), providing a possible mechanism for enhanced DSB formation by TopoII inhibition even in the absence of DNA replication."

We completely agree with the reviewer that the escape from G1/S arrest despite the removal of doxorubicin at that time could reflect defective G1 and/or S-phase checkpoints. We now explicitly discuss this on page 22 of the revised Discussion: (see also similar comment 1 from Reviewer 1)

"It is important to note that these findings were made and validated in the U2OS cell line. While U2OS contain 2 wild-type copies of the p53 gene, this cell line is known to be hypermethlyated at the INK4a/ARF locus, leading to lack of expression of both p16ARF and p14ARF (Park et al, 2002). G1/S cell-cycle arrest in response to DNA damage may be expected to be less robust in the absence of p16/p14 molecules to reinforce this response. It is possible that a role for ERK in the maintenance of the G1/S arrest response, and death from this arrest, represents a 'fail-safe' mechanism, and that a role for ERK in this response may be expected to be less pronounced in cell types that express p16/p14. Furthermore, the absence of a robust G1/S checkpoint in these U2OS cells may partially account for the unexpected doxorubicin-induced death in the G1 state. Importantly, hypermethylation and mutations at the INK4a/ARF locus are found in many human cancers, including a large fraction of melanomas and carcinomas (Cheung et al, 2009; Sharpless & Chin, 2003; Tannapfel et al, 2001). Thus, Erk may fulfill this role in a variety of other tumor contexts."

Reviewer #3

1- The authors use a MEK inhibitor, PD, to test the role of Erk activation in promoting cell cycle arrest and found that in the presence of PD and doxorubicin the percentage of cells released in the S phase at an earlier time point increases supporting the model prediction. However, it was not clear if PD would affect activations of other signaling molecules that may be the main players behind this effect. A direct test of this prediction would be to use the PLSR or T1-SWR model developed in the previous section to predict the results in Fig. 7 using the kinetics of the other molecules as input variables.

We agree with the reviewer that this is a very valid issue, and we did exactly as the reviewer suggested to explore this. We first removed all of the Erk signals from the independent variable dataset, and built a series of TI-SWR models from the remaining other molecules to identify which ones correlated with cell cycle progression after DNA damage. Signals at only 4 time points showed statistically significant correlations with S-phase progression: levels of "H2AX + total H2AX at the 2 hr time point, levels of phospho-Nbs1 at the 4 hr time point, and levels of total p53 at the 16 and 24 time points. We then used these molecules as dependent variables, and performed a second round of TI-SWR modeling to examine whether their activities were co-correlated with Erk activity. No such co-correlation between Erk activity and that of any of these other molecules was observed. We now show and discuss these results in Supplemental Table 2 and its associated caption, and summarize the results on page 21 of the revised Discussion section:

"While we cannot rule out the possibility that ERK accomplishes this function indirectly by affecting the activity of other DDR molecules, a computational analysis does not suggest this to be the case (Supplementary Table 2)."

2- The authors use principal components to evaluate correlations between activation of different molecular signals and functional outcomes for different treatments (Fig. 5). It was not obvious to me if the model can successfully predict a subset of the training data, and, how many principal components one would need for that. Even though, the top two principal components capture 87% of the variations, the smaller principal components may still play a significant role in affecting predictions. Therefore, including such tests will increase confidence in the model.

This is an excellent point. To address this, we compared the ability of 1, 2, and 3-component PLSR models to capture the variance in the data (R^2_{cum}), as well as to predict the responses in a subset of the data that was not used for model training (Q^2_{cum}) for both apoptosis and cell cycle progression. The results are now shown explicitly in Supplementary Figures 4A and 5, and discussed in the Supplementary Figure captions and on page 12 of the Results section in the revised manuscript, where we now state:

"We found that 93% of the variance in the cell-death and cell-cycle responses could be captured by three principal components, with the first two principal components capturing 87% of the variance and having clear biological correlates. A two component PLSR model often has the advantage of often being more intuitive and interpretable for analysis. However if retention of a third principal component substantially enhances the ability of the model to predict the dependent variable (i.e. cellular responses in our case), then a three component model may be justified. To address the marginal predictive capability gained by retaining additional principal components we evaluated the ability of 1,

2, and 3-component PLSR models to predict the change in apoptosis between 6 and 12 hrs, or the accumulation of cells in G1 and G2 at 24 hrs, following doxorubicin administration. As shown in Supplementary Figures 4A and 5, the cross-validated cumulative predictive capability Q^2_{cum} of the models for these responses did not substantially increase when moving from two to three components."

Minor comments:

3- pAkt is usually considered a pro-survival signal, however, high doses of doxorubicin that increased apoptosis produced more pAkt, as compared to lower doses of doxorubicin. The authors mentioned this unanticipated result in the paper. Is it possible to get further insight into this by using their models?

The reviewer brings up a good point. We thought it was important to definitively address this experimentally. To do this, we examined whether the late peak in pAKT activity in response to high-dose doxorubicin was pro- or anti-apoptotic by having an AKT inhibitor present only during the first 2 hrs of doxorubicin exposure (i.e. inhibiting early pAKT signaling), or only during late times (from 4 hrs onwards to inhibit the late pAKT signaling), and then measuring apoptotic cell death 12 hrs after doxorubicin administration. The results, shown in Supplementary Figure 6, clearly show that the late pAKT peak is a pro-survival signal. These results are now discussed on page 18 of the revised manuscript, where we state:

"The detailed molecular mechanism underlying this enhancement of doxorubicin-induced cell death by TNFα remains unclear, but since TNFα was found to elicit an increase in early JNK and p53 signaling and a loss of late AKT signaling, interplay between these pathways may be implicated in the observed enhancement of cell death. Consistent with this, we observed that the specific inhibition of robust late AKT activity, but not early AKT activity, following high-dose doxorubicin resulted in a greater than 2.5-fold increase in apoptotic cell death (Supplementary Figure 6), indicating that late Akt signaling is a pro-survival signal under these treatment conditions. The loss of this signal in cells co-treated with TNF! may therefore at least partially explain the dramatically higher levels of apoptotic cell death seen in that treatment condition."

Thank you for this suggestion!

4- It will helpful if the error bars are shown in Fig. 4B.

To maximize the legibility of Figure 4B (particularly if the Figures are reduced in size), we have provided the graphs with error bars as Supplementary Figure 3.

5- On page 4, data from cell proliferation experiments were not shown ("data not shown"). Either it should be noted why the data was not shown or it should be included in the supplementary material.

This particular piece of data dealing with the long-term irreversibility of cell cycle arrest has not been included in the manuscript, as this story is being expanded in much greater detail to address the potential mechanisms involved, including understanding why, and under what conditions, the cells cell-cycle arrest appears to be permanent. This manuscript is in preparation and should be submitted for publication shortly. We now make note of this in the current manuscript, and hope the reviewer is

amenable to allowing us to publish this as a separate story.

6- On page 5, it is not clear what is meant by the drug free medium, does it mean a medium with no doxorubicin and TNFα?

Yes. This has now been clarified in the text to read:

"…transfer to drug free medium without doxorubicin or TNF#."

7- It will be helpful for the community interested in using the results and the models if the data sets (including replicates) shown in Fig. 4 are provided as Excel files.

Absolutely! We now provide all of the source data for Figures 2, 3, 4, 5 and 7.

8- Typo on page 3, I think "though" means "thought".

Thank you for catching this typo. This has now been fixed in the manuscript.

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