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A genetic screen identifies BRCA2 and PALB2 as key regulators of G2 checkpoint maintenance

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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 28 March 2011

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, while the referees acknowledge that the study is interesting, they also make a few suggestions of how it could be improved. Both referees 1 and 2 point out that the results from the DNA repair screen should be included in the supplementary information. Referee 1 further indicates that it should be examined whether p53 is required for the BRCA2-dependent maintenance of the G2 checkpoint, and referee 3 feels that the effect of PALB2 silencing on PLK1 phosphorylation needs to be reported. The referees also remark that it should be discussed how a DNA repair factor might regulate the G2/M maintenance checkpoint and why the RSA analysis only was used to determine hits from the siRNA screen.

Given these evaluations and the constructive referee comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your

responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the length of the revised manuscript may not exceed 28,000 characters (including spaces and references) and the maximum of 5 figures. I also would like to add that the supplementary figures should ideally directly relate to one of the main figures in the manuscript and that we aim to restrict the number of supplementary figures to 5 as well. I strongly feel that the current 14 supplementary figures could be reduced in number, given that some of them recapitulate data already shown in the main figures. Figures S1 and S2, for example, may be included in figure 1. Figure S6 seems to report the same as figure 2F and could be deleted. Figure S7 could either be deleted or included in figure S8. Figure S9 could be deleted or combined with main figure 3D. Figure S11 could be deleted or included in figure 3F, figure S12 can be included in figure 3F, and figures S14-17 could be combined into a single, multi-panel supplementary figure, or, as referee 3 suggests figure S14 may be included in figure 4A (and in this case, also figure S15 in figure 4B). Please go through the supplementary figures and delete all redundant or not strictly necessary figures to reduce the total number as much as possible. I also noticed that not all figure legends define the identity of the depicted error bars and not all state the number of experiments performed (n=x). This information must be added. Generally, two repeats of one experiment are not sufficient when calculating standard errors. Either experiments should be performed three times, or the error bars should be removed from the graphs.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

Here Menzel et al discover new regulators of the G2/M checkpoint in human cells. The authors describe two different siRNA screens: the first targeted kinase enzymes, whereas the second targeted DNA repair factors. In both cases, the percentage of cells progressing to mitosis after irradiation was assayed by measuring the extent of Ser10-H3 phosphorylation in the target cells after a nocodazole trap. BRCA2 was identified as a DNA repair factor that regulates the G2/M checkpoint after irradiation. In the siRNA screen, BRCA2 siRNA had an ability several orders of magnitude greater than other DNA repair factors tested to increase the size of the mitotic population after irradiation. By measuring the initial induction and recovery from the G2 checkpoint after irradiation, the authors show that BRCA2 affects maintenance, rather than the intial onset, of the G2 checkpoint. PALB2, which is known to associate with BRCA2, is shown to act in the same pathway as BRCA2 for maintenance of the G2/M checkpoint. The mechanism of the regulatory effect of BRCA2/PALB2 on the G2/M checkpoint is shown to be at the level of inhibition of the Aurora A /Bora/Plk1 signaling axis, which normally acts to promote recovery from the checkpoint.
The Ser10-H3 phosphorylation assay and siRNA screens are robust and the authors provide extensive controls for their experimental system. However, there are some factors which perhaps ought to have been detected in the screens that did not appear in the final lists of regulators. For the kinase screen, it seems surprising, for example, that the DNA damage response kinase ATM was not identified as a regulator of the G2/M checkpoint, considering its well-known role in this process (Xu et al, MCB 2002). Likewise, BRCA1 would be expected to be identified from the screen of DNA repair factors that regulate the G2/M transition (Xu et al, Mol Cell, 1999). Indeed, they show that BRCA1 deficient cells exhibit a defect in the G2 checkpoint maintanence (Fig. 2D), but I don't think they mention this and Brca1 does not appear to come from the screen.. Nonetheless, the

identification of BRCA2 as a regulator of the G2/M checkpoint is a very novel finding, and is well validated by siRNA in multiple different cell lines. Mechanistically, the evidence presented provides good support for an involvement of BRCA2 in regulating Aurora A autophosphorylation and subsequent Plk1 phosphorylation. BRCA2 and Plk1 have previously been found to interact in vitro (Lee et al, Oncogene, 2003). In that study, BRCA2 was proposed to be a substrate for Plk1, dependent on DNA damage. The evidence presented in Fig. 4 of the current MS instead suggests that Plk1 activation is dependent on BRCA2. In summary the paper demonstrating that DNA repair proteins can regulate cell cycle checkpoints is a very important finding and should be published after attention to the following details.

1. I did not see a list of the DNA repair factors on their siRNA screen. Can the authors please provide this information? Is it possible to list the factors that were not 'hits' in both screens?

2. Do the authors have any idea if the cells that escape G2 make it to G1 phase of the cell cycle? This is likely to be too difficult to address experimentally at this point, but could be relevant (see Lukas C. et al. NCB 13:243-53). In this context the authors should at least discuss what they think is the mechanism by which a DNA repair factor (Brca2/Palb2) might regulate the G2/M maintenance checkpoint. For example, is this a form of adaptation as has been observed in yeast and in mammalian cells where PLK1 controls this process (Sylijuasen R et al. 1:10253-7, Cancer Research 2006).
3. The authors claim that they used a p53 dominant negative system in U20S to disable the G1/S checkpoint and increase the number of cells reaching the G2 checkpoint. However, the cited data (Fig S1) does not justify this to me clearly. What is the significance of the dox in Fig S1? How were the percentages of mitotic cells calculated based on PI alone?
4. Since the authors were using p53 deficient cells, is it possible that p53 deficiency is necessary for the phenomenon (Brca2- dependent escape from the G2 checkpoint) that they are reporting? Or can the same thing be observed in Brca2-deficient, p53 proficient cells?

Referee #2:

Using an unbiased genetic screen, Menzel and colleagues identified BRCA2 as a regulator of the G2 checkpoint. Knockdown of BRCA2 (or its interactor PALB2) allows premature silencing of the checkpoint and premature entry into mitosis. Further knockdown of Plk1 or Aurora A reverts this phenotype by inhibiting recovery.

The paper is a wonderfully clear and concise description of an important result, and the experiments are presented in a straightforward and logical fashion. There are only a few points that should be addressed prior to publication:

1. The list of the 236 DNA damage repair genes should be provided in the Supplements.

2. Fig. 3D: It appears that Cdc25A accumulates five hours after IR despite the activation of Chk1. How do the authors explain this?

3. The authors state: "During checkpoint recovery PLK1 becomes activated by AURORA A/BORA-mediated phosphorylation on T210 (Macurek et al., 2008; Seki et al., 2008; Tsvetkov and Stern, 2005)." However, another important factor in recovery is the re-accumulation of Plk1 (as shown in Fig. 3C) after APC/C-Cdh1-mediated degradation of Plk1 in response to DNA damage (Bassermann et al. 2008). This important regulatory mechanism needs to be mentioned too.

Referee #3:

Menzel et al performed a siRNA screen to determine regulators of the G2 checkpoint in the human osteosarcoma cell line U2OS expressing dominant-negative p53. The authors measured cells over-riding a block in mitosis by the detection phospho-H3 Ser10 positive cells following 2h IR and 8h Nocodazole treatment. The cells were then imaged and quantified on an IN Cell Analyzer and the

screens were analysed by Z score and RSA methods.
The approach of combining siRNA screens with mitotic block over-ride is a similar to those used in previous studies and the hits obtained in both the kinome (screen validation library) and DNA repair library screens are generally unsurprising. Nevertheless, the authors go on to confirm BRCA2 as a hit and then go on to describe the BRCA2 binding partner, PALB2, as also being involved in the G2 checkpoint. However, no mechanistic insight is provided into the mechanism.

In addition, the main points of concern are the following:

1. The authors use both z score and RSA methods of analysis however only seem to use the RSA scores to determine hits. Since they include the z score analysis in figure 1E, the authors need to include the z scores in the table in figure 1G or explain why they have chosen just the RSA method for hit detection.

2. I find it concerning that the RSA scores of the two replicates shown in figure 1d have a number of siRNAs scoring differently in each screen, with no R2 value stated. The quality control used here is SSMD. The authors state a score of 2 or above represents a reasonable quality screen, however the scores shown in S2 indicate that two of the plates in the kinome screen are below two. The lack of reproducibility in the replicates could mean that hits are missed.

3. The authors show 9 novel candidates in figure 1G, why do the authors not elaborate on these further?

4. As described in 1. same applies to DNA repair screen in figures 2A-C, ie why RSA over z score analysis?

5. The authors need to include the graph in figure S14 in figure 4A. The fold difference referenced in the main text between the mitotic index of PLK1 inhibitor + siBRCA2 with damage (figure 4A) and without damage (figure S14) is difficult to determine when on separate figures.

6. What is the effect of PALB2 silencing on PLK1 and Aurora A phosphorylation as shown for BRCA2 silencing in figure 4C? This needs to be included in figure 4C to make the early phosphorylation of PLK1 more convincing.

1st Revision - authors' response 18 April 2011

Referee #1:

Here Menzel et al discover new regulators of the G2/M checkpoint in human cells. The authors describe two different siRNA screens: the first targeted kinase enzymes, whereas the second targeted DNA repair factors. In both cases, the percentage of cells progressing to mitosis after irradiation was assayed by measuring the extent of Ser10-H3 phosphorylation in the target cells after a nocodazole trap. BRCA2 was identified as a DNA repair factor that regulates the G2/M checkpoint after irradiation. In the siRNA screen, BRCA2 siRNA had an ability several orders of magnitude greater than other DNA repair factors tested to increase the size of the mitotic population after irradiation. By measuring the initial induction and recovery from the G2 checkpoint after irradiation, the authors show that BRCA2 affects maintenance, rather than the intial onset, of the G2 checkpoint. PALB2, which is known to associate with BRCA2, is shown to act in the same pathway as BRCA2 for maintenance of the G2/M checkpoint. The mechanism of the regulatory effect of BRCA2/PALB2 on the G2/M checkpoint is shown to be at the level of inhibition of the Aurora A /Bora/Plk1 signaling axis, which normally acts to promote recovery from the checkpoint. The Ser10-H3 phosphorylation assay and siRNA screens are robust and the authors provide extensive controls for their experimental system. However, there are some factors which perhaps ought to have been detected in the screens that did not appear in the final lists of regulators. For the kinase screen, it seems surprising, for example, that the DNA damage response kinase ATM was not identified as a regulator of the G2/M checkpoint, considering its well-known role in this process (Xu et al, MCB 2002). Likewise, BRCA1 would be expected to be identified from the screen of DNA repair factors that regulate the G2/M transition (Xu et al, Mol Cell, 1999). Indeed, they show that BRCA1

deficient cells exhibit a defect in the G2 checkpoint maintanence (Fig. 2D), but I don't think they mention this and Brca1 does not appear to come from the screen. Nonetheless, the identification of BRCA2 as a regulator of the G2/M checkpoint is a very novel finding, and is well validated by siRNA in multiple different cell lines. Mechanistically, the evidence presented provides good support for an involvement of BRCA2 in regulating Aurora A autophosphorylation and subsequent Plk1 phosphorylation. BRCA2 and Plk1 have previously been found to interact in vitro (Lee et al, Oncogene, 2003). In that study, BRCA2 was proposed to be a substrate for Plk1, dependent on DNA damage. The evidence presented in Fig. 4 of the current MS instead suggests that Plk1 activation is dependent on BRCA2. In summary the paper demonstrating that DNA repair proteins can regulate cell cycle checkpoints is a very important finding and should be published after attention to the following details.

1. I did not see a list of the DNA repair factors on their siRNA screen. Can the authors please provide this information? Is it possible to list the factors that were not 'hits' in both screens?

Answer: The screens raw data are now included as supplementary data.

2. Do the authors have any idea if the cells that escape G2 make it to G1 phase of the cell cycle? This is likely to be too difficult to address experimentally at this point, but could be relevant (see Lukas C. et al. NCB 13:243-53). In this context the authors should at least discuss what they think is the mechanism by which a DNA repair factor (Brca2/Palb2) might regulate the G2/M maintenance checkpoint. For example, is this a form of adaptation as has been observed in yeast and in mammalian cells where PLK1 controls this process (Sylijuasen R et al. 1:10253-7, Cancer Research 2006).

Answers: We include time-lapse videos as supplementary data to demonstrate that BRCA2 depleted cells progress through mitosis and divide following checkpoint abrogation. This is in our opinion the best way to precisely document cell cycle progression.
Potential mechanisms how BRCA2 and PALB2 may control AURORA A/BORA /PLK1 and a discussion of our results in light of our previously published findings (SyljuÂsen et.al 2006) are described at the end of the text on page 5-6.

3. The authors claim that they used a p53 dominant negative system in U20S to disable the G1/S checkpoint and increase the number of cells reaching the G2 checkpoint. However, the cited data (Fig S1) does not justify this to me clearly. What is the significance of the dox in Fig S1? How were the percentages of mitotic cells calculated based on PI alone?

Answer: Dnegp53 is expressed in the absence of doxocyclin (-dox), which doubles the amount of mitotic cells observed after siRNA to Chk1. Cells were co-stained with phospho-H3 antibodies to determine the mitotic fraction. Fig S1 was moved to new Fig 1D, and both issues are explained in the new figure legend to Figure 1D.

4. Since the authors were using p53 deficient cells, is it possible that p53 deficiency is necessary for the phenomenon (Brca2- dependent escape from the G2 checkpoint) that they are reporting? Or can the same thing be observed in Brca2-deficient, p53 proficient cells?

Answer: The checkpoint is also abrogated following BRCA2 depletion in p53 wild-type cells, such as TIG-3 fibroblasts (Figure 2F) and parental U2OS cells (Figure S2). The notion that p53 is wild-type in TIG-3 cells is added on page 4.

Referee #2:

Using an unbiased genetic screen, Menzel and colleagues identified BRCA2 as a regulator of the G2 checkpoint. Knockdown of BRCA2 (or its interactor PALB2) allows premature silencing of the checkpoint and premature entry into mitosis. Further knockdown of Plk1 or Aurora A reverts this phenotype by inhibiting recovery.

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1. The list of the 236 DNA damage repair genes should be provided in the Supplements.

Answer: The screens raw data are now included as supplementary data.

2. Fig. 3D: It appears that Cdc25A accumulates five hours after IR despite the activation of Chk1. How do the authors explain this?

Answer: The accumulation of CDC25A at 5 hours is very small in both control and BRCA2 siRNA treated cells (see Fig 3D). Also, CDC25A is not solely regulated by CHK1. Other factors contribute to regulate CDC25A stability such as for instance NEK11 and 14-3-3 proteins.

3. The authors state: "During checkpoint recovery PLK1 becomes activated by AURORA A/BORA-mediated phosphorylation on T210 (Macurek et al., 2008; Seki et al., 2008; Tsvetkov and Stern, 2005)." However, another important factor in recovery is the re-accumulation of Plk1 (as shown in Fig. 3C) after APC/C-Cdh1-mediated degradation of Plk1 in response to DNA damage (Bassermann et al. 2008). This important regulatory mechanism needs to be mentioned too.

Answer: We have included the reference.

Referee #3:

Menzel et al performed a siRNA screen to determine regulators of the G2 checkpoint in the human osteosarcoma cell line U2OS expressing dominant-negative p53. The authors measured cells over-riding a block in mitosis by the detection phospho-H3 Ser10 positive cells following 2h IR and 8h Nocodazole treatment. The cells were then imaged and quantified on an IN Cell Analyzer and the screens were analysed by Z score and RSA methods.
The approach of combining siRNA screens with mitotic block over-ride is a similar to those used in previous studies and the hits obtained in both the kinome (screen validation library) and DNA repair library screens are generally unsurprising. Nevertheless, the authors go on to confirm BRCA2 as a hit and then go on to describe the BRCA2 binding partner, PALB2, as also being involved in the G2 checkpoint. However, no mechanistic insight is provided into the mechanism.

In addition, the main points of concern are the following:

1. The authors use both z score and RSA methods of analysis however only seem to use the RSA scores to determine hits. Since they include the z score analysis in figure 1E, the authors need to include the z scores in the table in figure 1G or explain why they have chosen just the RSA method for hit detection.

Answer: The z-score plots - which have been and are widely used in the field - 1E (and 1D), 2A and S4 were primarily used as visual aids to investigate distribution and behavior of the data (before and after normalization) within screens and between replicate screens e.g. to see how the distribution of positive and negative controls related to the remaining data.
Contrary to most alternative hit detection algorithms (incl. the z-score), RSA uses the combined information from all siRNAs targeting the same gene, thereby increasing the statistical power. As opposed to the z-score, RSA as a rank-based method is also more robust to outliers in the data.

2. I find it concerning that the RSA scores of the two replicates shown in figure 1d have a number of siRNAs scoring differently in each screen, with no R2 value stated. The quality control used here is SSMD. The authors state a score of 2 or above represents a reasonable quality screen, however the scores shown in S2 indicate that two of the plates in the kinome screen are below two. The lack of reproducibility in the replicates could mean that hits are missed.

Answer: The quality of the individual plates vary as judged by the SSMD score (and two of which in figure 1F are borderline) and are not perfect. The two plates of lower quality (figure 1F) are in separate screens and are furthermore not identical (i.e. A2!=B2), so the negative effect on the analysis of these two plates should be relatively limited. We agree with the reviewer that one unfortunate effect of this can be that some hits may be missed.

Correlation values for the plot in 1E:
Pearson correlation: R2=0.7958299; p-value < 2.2e-16
Spearman correlation: rho=0.2454546; p-value < 2.2e-16

3. The authors show 9 novel candidates in figure 1G, why do the authors not elaborate on these further?

Answer: We chose to focus on BRCA2 since we uncovered a completely new role of BRCA2 in G2 checkpoint maintenance. As BRCA2 is an important tumor suppressor we reasoned that validating this hit was more important to the field.

4. As described in 1. same applies to DNA repair screen in figures 2A-C, ie why RSA over z score analysis?

Answer: See answer to point #1 above

5. The authors need to include the graph in figure S14 in figure 4A. The fold difference referenced in the main text between the mitotic index of PLK1 inhibitor + siBRCA2 with damage (figure 4A) and without damage (figure S14) is difficult to determine when on separate figures.

Answer: We have done as the reviewer suggested.

6. What is the effect of PALB2 silencing on PLK1 and Aurora A phosphorylation as shown for BRCA2 silencing in figure 4C? This needs to be included in figure 4C to make the early phosphorylation of PLK1 more convincing.

Answer: We agree that this is an important point. We have done as the reviewer suggested, and the data are figure S6.

2nd Editorial Decision 03 May 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORT:

Referee #1:

The authors have done an excellent job in addressing all of the points from all the referees and the paper should be published.
I would recommend publication as soon as possible as this is very competitive.

