Author's response to reviews

Title: Functional deficiency of NBN, the Nijmegen Breakage Syndrome protein, in a p.R215W mutant breast cancer cell line

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Reviewer 1: I appreciate that the authors have made revisions in the paper to address some of the previous reviewers comments. However there still are multiple issues as noted below that were not fully addressed. Perhaps the authors misunderstood the prior review comments, but a primary issue is that the cell line also contains a BRCA1 mutation which affects the cellular endpoints under study, namely radiation sensitivity and PARP sensitivity, and not that the BRCA1 mutation has anything to do with the low NBS levels noted.

The study as it is currently set up is flawed in that it is unable to investigate how the R215W missense mutation functionally impacts the breast cancer cells, given the presence of the BRCA1 mutation. Just from a cursory search of the literature it is obvious that the BRCA1 mutation can influence the endpoints under study: “cells without BRCA1 showed decreased TCR and radiosensitivity.” From Abott et al., 1999 JBC. And …

“A major breakthrough in targeted treatment of BRCA1-mutant cancers was heralded by the finding that BRCA1 and BRCA2- mutant cells are exquisitely sensitive to poly(ADP-ribose)poly- merase (PARP) inhibitors” (Farmer et al., 2005; Helleday et al., 2005).

From Popova et al., Ca Res, 2012: “Large-scale state transitions (LST) defined
as chromosomal break between adjacent regions of at least 10 Mb were found to be a robust indicator of BRCA1 status in this setting.”

“…among near-tetraploid cell lines, HCC1395, HCC1937, HCC38, HCC1599 and BC227 carried the highest number of LSTs, which is again consistent with their BRCA1/2-inactivated status.”

Reply from the authors: We thank the reviewer for drawing our attention to some potential of misinterpretations still inherent to our last draft. We principally agree that a BRCA1 mutation can influence radiation and PARP1 inhibitor sensitivity, though not NBN levels, ATM activity and IR-induced focus formation, and these endpoints should be better discriminated. To this end, we have highlighted the presence of the BRCA1 mutation at any point where we think it could be of influence. The three references Abott, Farmer and Hellday have been cited at the respective positions and added to the reference list.

Major Compulsory Revisions:

1) The fact that the cell line contains a BRCA1 mutation should be noted up front in the abstract, and the evidence supporting BRCA1’s influence on these endpoints as noted above should be discussed and cited. The only way to completely ensure that the cellular effects noted are linked to the NBN mutation at R215W, would be to complement the BRCA1 mutation in the HCC1395 line and compare it to the non-transfected cell line for the endpoints measured in this paper. If this is not done than the researchers are currently studying the combined effect of both mutations and thus it should be discussed as such in the very beginning and through out the paper.

Reply: We have now included the information about the BRCA1 mutation already into the abstract as follows: “They also harbour a truncation in BRCA1. Mutations in both genes were already present in the heterozygous state in the patient’s germline.” Furthermore we clarify already in the abstract by adding the BRCA1 mutation that “In line with their deficiency in NBN and BRCA1, HCC1395 cells were particularly sensitive to PARP1 inhibition”. The same has been followed throughout the paper on pp. 11, 15 and 16.

2) The statement in the conclusion “We here report on the first NBN mutant breast cancer cell line which will serve as a useful tool for future molecular studies of NBN function” does not seem to be true, this cell line can not be used for studies of NBN function unless the BRCA1 mutation is complemented as it will always confound the phenotype exhibited.

Reply: We appreciate that this could be misleading and therefore have now modified the conclusion to write “We here report on the first NBN mutant breast
cancer cell line which may serve as a useful tool for future molecular studies of genuine NBN function. The data indicate that NBN*p.R215W is an unstable protein that fails to be recruited into foci after irradiation and also impairs the propagation of gamma-H2AX repair foci.” This should better enable the reader to recognize the main message of our study.

3) The authors state: “Although we cannot formally exclude the possibility that a BRCA1 mutation augments the effect of NBN p.R215W, the functional BRCA1 deficiency does not cause these effects as is also evidenced by the observations of normal NBN levels in other cell lines such as HCC38 or HCC1806 which are functionally deficient in BRCA1.” But what is not mentioned is the radiation sensitivity and PARP sensitivity, these endpoints ARE affected by BRCA1 mutations, this should be CLEARLY stated here, not the fact that it does not change NBS protein levels.

Reply: We agree but we had stated in the sentence before that “This mutation, however, is unlikely to explain the reduced NBN levels or impaired formation of repair foci”, so the functions attributable to NBN had already been restricted appropriately. In the new version, we now modify the sentence under query to state unambiguously that “Although we cannot formally exclude the possibility that a BRCA1 mutation augments the effect of NBN p.R215W on NBN levels and radiation-induced foci formation, the functional BRCA1 deficiency does not cause these effects as is also evidenced by the observations of normal NBN levels and foci formation in other cell lines such as HCC38 or HCC1806 which are functionally deficient in BRCA1 [40,41]. However, the presence of a BRCA1 or BRCA2 mutation has been shown to affect sensitivity towards ionising radiation [42] as well as towards PARP1 inhibition [43,44], so that these endpoints were not specific for the NBN*R215W mutant.”

4) Regarding Fig. 5, it is unclear given HCC1395’s poor growth and cloning efficiency, why it would be plated at the same 5000 cells per well in quadruplicates as the MCF10A line, as noted in methods. Information regarding the percent confluency at the time of Olaparib addition for each line should be noted. An additional experiment using a higher density of HCC1395 cells should be performed if percent confluencies at time of drug addition are not the same between the lines.

Reply: It is true that HCC1395 cells were slowly proliferating but our prior titration experiments revealed the seeding rate of 5,000 cells per well as the optimum for the X-Celligence experiments. It is recommended to seed the cells at a number that reaches an impedance level at cell index 0.8-1.5, therefore the higher number of 7,500 or 10,000 cells per well would have impacted on the quality of the experiment. Furthermore, microscopic inspection of the wells had documented an about 30-40% confluency of cells for both HCC1395 and
MCF10A before the addition of 0.2 µM olaparib, thus there was little room to increase the cell number. At the end of the experiment, MCF10A were confluent whereas HCC1395 cells had rather diminished under the olaparib treatment.

While this experiment is not crucial to the main message of the manuscript, we nevertheless think that it contains valuable information, and the results are sound from their dose-dependent effects and statistical significance. But we understand that the results could be deemphasized due to the presence of mutant BRCA1. To downplay its importance in the revised manuscript, we have moved this figure to the Supplementary Information. It is now provided as Supplementary Figure 5.

5) Others have previously published that NBN and other proteins that affect HR, besides BRCA1/2, may also show some PARP sensitivity (McCabe et.al., Ca Res 2006). This work should also be discussed and cited in the paper.

Reply: We have now included into the discussion on p.16 that “mutations in other proteins that affect HR, besides BRCA1/2, may also confer some PARP1 inhibitor sensitivity” and cited this reference accordingly as Ref.45, as well as a further reference from Jiri Bartek’s group (Ref. 46).