nature portfolio

Peer Review File

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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this study, Espinar and colleagues identify Inosine monophosphate dehydrogenase 2 (IMPDH2) as a potential regulator of nuclear NAD+ levels that can modulate DNA damage repair. Using triple negative breast cancer as a model system, the authors identified IMPDH2 enrichment on chromatin. Given that triple negative breast cancers often exhibit increased levels of DNA damage, the authors reasoned that IMPDH2 enrichment on chromatin could imply a role in DNA damage repair. Indeed, depletion of IMPDH2 in triple negative breast cancer cells (MDA-MB-231 and Cal51) results in increased levels of DNA damage as measured by gH2AX foci. Mechanistically, IMPDH2 was shown to interact with PARP1 on chromatin, which through its enzymatic activity is postulated to decrease nuclear NAD+, resulting in PARP1 cleavage and apoptosis. The authors therefore conclude that IMPDH2 is a critical regulator of nuclear NAD+ levels and can therefore directly influence DNA damage repair through modulating metabolite availability.

This is an interesting study in an area that is of increasing interest to the DNA repair field. The manuscript is generally well written, and the experimental data is reasonably interpreted. Whilst interesting, I feel that the proposed model needs to be strengthened with additional experimental data. I have outlined the major points to be addressed, below:

Major Comments:

♣ Figure 1B – The way the data is presented is not hugely convincing. The statistical significance between MDA-MB-231 cells and MCF7 and T47D respectively is impressive, however this is not reflected in the way the data is currently presented. Can the authors plot individual data points, to better visualize the data spread, so that it more accurately reflects the statistical analysis?

♣ Extended data 1i, j. The authors state that there is “a trend towards higher IMPDH2 nuclear signal in triple negative breast cancer compared to non-triple negative”. Based on the data presented in this figure, this is not supported. Indeed, the statistical test shows a p-value of 0.3, and the spread of the data for the triple negative cohort is large. The data should be presented better to clarify this claim by the authors, or the claim removed.

♣ Figure 2A+B – Is there a simpler way to represent this data? The data representation in its current form, is not easy to interpret. It would also be helpful to provide

representative images for this analysis.

♣ Based on the analysis using the U2OS Fucci system, the authors claim that there is an enrichment of IMPDH2 in late S and G2 phase cells. The authors should perform an IF co-stain of IMPDH2 with the late S/G2 phase marker mitosin. This should clearly show an enrichment of IMPDH2 in mitosin positive cells.

♣ In Figure 2C-D, depletion of IMPDH2 induces DNA damage. Is this also seen with acute knockdown using siRNA or an inducible shRNA or CRISPR KO system? It would also be useful to assess this DNA damage in the context of cell cycle phase. Assessing gH2AX foci in the context of mitosin positivity should demonstrate more DNA damage in late S/G2 phase cells if the authors' hypothesis is correct.

♣ Using shIMPDH2 cells or the MPA inhibitor, the authors should perform additional DNA repair assays to support their claims as to the importance of IMPDH2 for DNA damage repair. For example, the authors could stain for additional DNA damage markers e.g. 53BP1 in mitosin positive cells. The authors could also perform neutral comet assays to measure DNA double-strand break formation in the absence of IMPDH2 (the authors see a G2/M arrest which is consistent with DNA double-strand breaks). The authors could also perform metaphase spread analysis to measure chromosomal aberrations or immunoblot analysis of key DNA repair signaling pathways (pChk1, pChk2 etc).

♣ In Figure 3B, the authors show an interaction between PARP1 and IMPDH2 in the chromatin fraction. It is not clear however, if this interaction is direct. This IP should be repeated in the presence of benzonase to remove DNA, to rule out that the interaction is seen indirectly via DNA/chromatin binding.

♣ Figures 3C – G are largely correlative. Just because IMPDH2 increases on chromatin after treatment with Etoposide, doesn't necessarily mean that IMPDH2 is involved in the DNA damage response to Etoposide. To better prove this link the authors should:

o Treat IMPDH2 knockdown cells with Etoposide and perform a co-stain analysis for gH2AX and/or 53BP1 foci in Mitosin positive cells. cDNA rescue experiments should also be performed to rescue any phenotypes.

o Clonogenic/cellular survival assays with IMPDH2 knockdown cells treated with Etoposide (with cDNA rescue).

♣ The way the data with Etoposide is described is a little confusing. The authors are

suggesting that the increase of IMPDH2 in response to Etoposide is actually impairing DNA repair. However, this is contradicting earlier data that shows depletion of IMPDH2 impairs DNA repair. Can the authors clarify this?

♣ The authors suggest that IMPDH2 and PARP1 interact, and that IMPDH2 interaction causes PARP1 cleavage into the cytoplasm following DNA damage. If this is correct, then there should be less PARP1 cleavage in IMPDH2 knockout cells following DNA damage, yet the opposite seems to be true in Figure 3H. Can the authors clarify?

♣ The authors' claim that the levels of IMPDH2 nuclear localization is important for DNA repair is further complicated by extended data figure 5A-B. This data shows that reconstitution with wild-type IMPDH2 or a IMPDH2 construct that is localized to the nucleus, has no impact on gH2AX levels in cells. The reasoning that PARP1 activity is required for gH2AX foci accumulation as an explanation for this result is not convincing. Regardless, other markers of DNA damage should still be present (e.g. 53BP1), and this should be tested.

♣ The link between the interaction with PARP1 and IMPDH2 is not particularly convincing (see above regarding concerns about Figure 3B). To strengthen this link, the authors should map the interaction sites for IMPDH2 and PARP1. This will generate mutants to test separation of functon(gH2AX foci, PARP1 cleavage), to further delineate the mechanisms in support of their model.

Minor Comments:

- Line 101 – missing reference.

- Line 174 – missing reference.

- Line 235 – missing reference.

Reviewer #2:
Remarks to the Author:
Espinar, Garcia-Cao, et al. have documented that the enzyme involved in purine synthesis, IMPDH2, translocates to the nucleus and localizes to the chromatin in triple-negative breast cancer cells following DNA damage. The authors assert that both genetic and chemical inhibition of IMPDH2 result in the accumulation of DNA damage. Notably, IMPDH2's chromatin localization onto DNA takes place during a later stage of DNA damage repair. Upon localization to chromatin, IMPDH2 binds to PARP1.

Intriguingly, when IMPDH2 is consistently localized to the nucleus, it causes a depletion of nuclear NAD+, leading to the cleavage of PARP1 and triggering apoptosis.
This work is interesting because it reveals an unanticipated role of IMPDH2 in regulating nuclear NAD+ levels, thereby finely modulating the activation of PARP1 and controlling the DNA damage response.
1) The authors should elucidate the molecular mechanism underlying the basal-level translocation of IMPDH2 to the nucleus in some TN breast cancer cells. If this nuclear translocation is substantial, it is crucial to understand why and how specific breast cancer cell lines exhibit an increase in nuclear IMPDH2 while others do not. Establishing isogenic settings expressing or not expressing HER2 could provide valuable insights.

2) What are the effects of DNA damage on cellular IMPDH2 activity? A metabolite analysis of guanylate levels (IMP, GMP, GDP, or GTP) should be conducted.

3) While increased DNA damage upon IMPDH2 inhibition is anticipated, it is surprising that guanosine or guanine supplementation did not rescue DNA damage accumulation. Do these cells express guanosine and guanine transporters?

4) It remains unclear how the amount of nuclear IMPDH2 increases in response to DNA damage. Is IMPDH2 posttranslationally modified in response to DNA damage regulatory pathways? Is the ATR/ATM pathway involved in this regulation?

5) Does nuclear IMPDH2 impact transcription and gene expression? Why is IMPDH2 present in the nucleus and not its isoform, IMPDH1? Do other purine enzymes, such as GMPS, ADSL, and ADSS, translocate to the nucleus?

6) The western blots demonstrating increased PARP cleavage in response to NLS-IMPDH2 are not entirely convincing. The authors should consider alternative approaches, such as assessing PARP stabilization in WT or NLS-IMPDH2 cells.

7) If forcing nuclear IMPDH2 localization leads to nuclear NAD+ depletion, then NLS cells should be more sensitive to the NAMPT inhibitor (FK866) than WT cells. The authors could investigate whether chemical NAD+ depletion results in more potent NLS cell death than WT cells.

8) Since the authors claim that nuclear IMPDH2 leads to nuclear NAD+ depletion, increased DNA damage, and cancer cell death, it would be interesting to assess whether nuclear IMPDH2 in breast tumors also leads to nuclear NAD+ depletion and tumor growth reduction.

Reviewer #3:
Remarks to the Author:
Summary
In this manuscript, the authors identified a purine synthesis enzyme, IMPDH2, which is enriched on chromatin in TNBC cell lines. They demonstrate that the IMPDH2 activity is required for proper DNA damage repair and that it interacts with PARP1 on chromatin. Additionally, they report that nuclear IMPDH2 regulates local NAD+ availability and has some potential regulatory role in PARP1 activity.

Review
The work shown here is potentially interesting and adds to the wealth of knowledge regarding the importance of metabolic enzymes in the nucleus. The work also suggests the role of IMPDH2 in modulating DNA damage response pathways in breast cancer cells. Though this work reveals potential novel insights into the role of IMPDH2, it falls short in validating certain key observations and the conclusions are, thus, inflated. Moreover, the mechanistic model put forth in the paper needs further experimental evidence in support.

Strengths: This work identifies a non-canonical role of IMPDH2 in controlling nuclear NAD+ levels to regulate DNA damage response. The authors also showed that the modulation of nuclear IMPDH2 levels impairs DNA repair thus leading to cell death.

Weaknesses: The title of the paper is misleading as it states that nuclear IMPDH2 modulates PARP1 activity. However, the results presented in this manuscript at most are suggestive of the role of nuclear IMPDH2 in PARP1-dependent DNA damage repair or cell death pathways, not strictly related to the regulation of PARP1 catalytic activity. Further validation of key observations and robust support of the model are lacking.
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Major Comments:
1. The authors should be careful using the word “PARP1 activity” (generally taken to mean catalytic activity, not repair activity) and avoid extrapolating the conclusions obtained. Additional experiments that can support their conclusions are listed below.
2. Further evidence is needed to show that IMPDH2 indeed binds to chromatin (e.g., FRAP assay on the nuclear enzyme should be performed).
3. The authors show nuclear translocation of IMPDH2 upon DNA damage, but do not discuss the reasons for this change in localization. Does IMPDH2 have an NLS?, bind to other proteins that mediate this?
4. In Figure 1f, including a western blot detection of γH2AX in MCF7 parental and hormone-insensitive cells might help validate this system and prove the role of nuclear

IMPDH2 in DNA damage repair. Moreover, FDX1 in this panel needs an introduction in the main text.
5. In line 139, there is no introduction about the function of etoposide in its first appearance in this manuscript.
6. The authors briefly introduce the interaction of TOP2A and do not mention/discuss this further. A clear discussion regarding this is warranted especially because the role of TOP2A in DNA damage is also reported. Also, more IP's are needed to validate this interaction, across the other BC cell lines and also to show if IMPDH2 interacts with PARP1 and TOP2A in a mutually exclusive manner or not.
7. In Figure 2, the authors use IMPDH2 inhibition and depletion to conclude that IMPDH2 catalytic activity is required for normal DNA damage repair. Though this may be the case, but it should be tested further by rescue with catalytic dead IMPDH2.
8. In Figure 3c, the data showing the relative abundance changes upon etoposide treatment should be on the same blot. Also, it is imperative to include whole cell lysate as a control for this experiment.
9. In Figure 3h, leveraging the different sizes of cleaved PARP1 the authors comment on apoptosis vs necrosis. This should be supported using other apoptosis and necrosis markers/assays.
10. In line 217, the authors state that the lack of IMPDH2 causes the loss of control of NAD+ cellular levels but never actually show the data. The use of NAD+ sensor is needed like in Figure 5a-d.
11. In line 246, the authors conclude that IMPDH2 accumulation affects PARP1 activity. However, this needs to be tested by monitoring the PARP1 automodification activity.
12. In Figure 4, the authors should include proper controls like WT, IMPDH2 KO alongside KO-WT and KO-NLS cells.
13. In Figure 4a,b, the authors should include the western blot of necrosis-cleaved PARP1 (similar to Figure 3h) to show that IMPDH2 knockout causes necrosis, while IMPDH2 nuclear localization only causes apoptosis. This should be supplemented with other markers as well.
14. In Figure 5a-d, NAD+ needs to be quantified between WT, IMPDH2 KO, KO-WT, and KO-NLS cells to comprehensively show that IMPDH2 nuclear localization affects nuclear NAD+ levels. This assay should also be repeated by supplying the cells with extra NMN to compensate for the possible NAD+ depletion and observe the outcome on overall cell survival.
15. The model in Figure 5d needs a more detailed explanation in the main text.
16. Typos (e.g., PARylation), superscripts (e.g., NAD+) and references need to be corrected in the text.
17. The authors should include details about what type of NLS was added to IMPDH2.

CRG

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Barcelona, 12th of July 2024

Nuclear IMPDH2 controls the DNA damage response by modulating PARP1 activity

Response to reviewers: NCOMMS-23-59999-T

Reviewer #1 - DDR, chromatin factors, HR (Remarks to the Author)
In this study, Espinar and colleagues identify Inosine monophosphate dehydrogenase 2 (IMPDH2) as a potential regulator of nuclear NAD+ levels that can modulate DNA damage repair. Using triple negative breast cancer as a model system, the authors identified IMPDH2 enrichment on chromatin. Given that triple negative breast cancers often exhibit increased levels of DNA damage, the authors reasoned that IMPDH2 enrichment on chromatin could imply a role in DNA damage repair. Indeed, depletion of IMPDH2 in triple negative breast cancer cells (MDA-MB-231 and Cal51) results in increased levels of DNA damage as measured by gH2AX foci. Mechanistically, IMPDH2 was shown to interact with PARP1 on chromatin, which through its enzymatic activity is postulated to decrease nuclear NAD+, resulting in PARP1 cleavage and apoptosis. The authors therefore conclude that IMPDH2 is a critical regulator of nuclear NAD+ levels and can therefore directly influence DNA damage repair through modulating metabolite availability. This is an interesting study in an area that is of increasing interest to the DNA repair field. The manuscript is generally well written, and the experimental data is reasonably interpreted.
Whilst interesting, I feel that the proposed model needs to be strengthened with additional experimental data.

I have outlined the major points to be addressed, below: Major Comments:

We appreciate the reviewers' interest in our manuscript. Their suggestions have helped to further delineate the dynamics of IMPDH2 role during the DDR and strengthen our conclusions, especially for identifying downstream DDR factors and defining the dynamics of γH2AX and IMPDH2 accumulation through the cell cycle.

♣ Figure 1B – The way the data is presented is not hugely convincing. The statistical significance between MDA-MB-231 cells and MCF7 and T47D respectively is impressive, however this is not reflected in the way the data is currently presented. Can the authors plot individual data points, to better visualize the data spread, so that it more accurately reflects the statistical analysis?

We have now plotted the individual data points. Such representation helped improving data visualization.

♣ Extended data 1i, j. The authors state that there is "a trend towards higher IMPDH2 nuclear signal in triple negative breast cancer compared to non-triple negative". Based on the data presented in this figure, this is not supported. Indeed, the statistical test shows a p-value of 0.3, and the spread of the data for the triple negative cohort is large. The data should be presented better to clarify this claim by the authors, or the claim removed.

We thank the reviewer for pointing this out. We realized that there was an error in the analysis of the triple negative vs. the rest of the tumor subtypes comparison. In fact, we had inadvertently included negative values from tissue sections rich in stromal cells (red rectangle in the figure below), which confounded the statistical comparison. We have now revisited the analysis, removed the negative values, and the comparison is statistically significant. We have moved this panel to Fig. 1i,j as it is very compelling with the story of the manuscript.

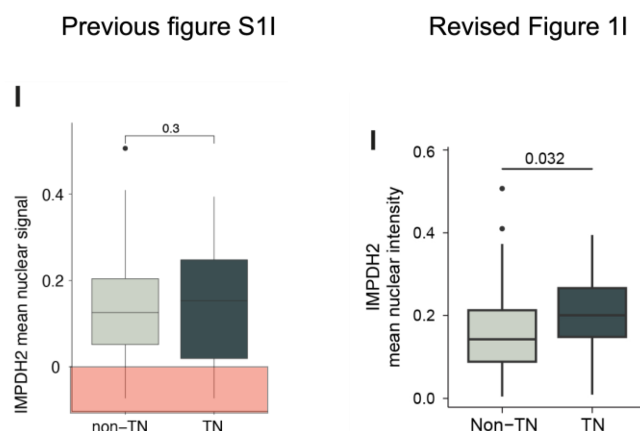

Figure. 1. Comparison of previous and revised analysis for IHC in TNBC patient samples. In the previous analysis, the rectangle in red shows the presence of values below 0, which were removed in the revied analysis. The removal of such values changed the distribution of the population, making the comparison between TNBC and other subtypes significant.

♣ Figure 2A+B – Is there a simpler way to represent this data? The data representation in its current form, is not easy to interpret. It would also be helpful to provide representative images for this analysis.

We have now plotted the bottom and top nuclear/cytoplasmic IMPDH2 cells separately (Fig. 2a and extended data 2a) and plotted the integrated nuclear IMPDH2 intensity in each phase of the cell cycle alongside representative images of cells in different cell cycle phases, which has improved data visualization (Fig. 2b-c).

♣ Based on the analysis using the U2OS Fucci system, the authors claim that there is an enrichment of IMPDH2 in late S and G2 phase cells. The authors should perform an IF co-stain of IMPDH2 with the late S/G2 phase marker mitosin. This should clearly show an enrichment of IMPDH2 in mitosin positive cells.

We agree with the reviewer that Mitosin staining would have allowed the detection of cells in S phase. However, Mitosin is also present in mitosis and this would have complicated the downstream analysis to distinguish cells in S, G2 or M. Following the reviewer's suggestion, we used an alternative approach based on DAPI integrated intensity, which is able to classify cells according to cell cycle in a FACS-like manner (https://www.nature.com/articles/nprot.2015.016). With this approach, we were able to follow γH2AX and IMPDH2 accumulation through the cell cycle, showing an enrichment during the G2M phases (Fig. 2f,h and Extended Data Fig. 3d,e). This strategy allowed us to better delineate the dynamics of γH2AX and IMPDH2 accumulation during DDR. The integration of this approach with the Fucci system strengthens the evidence for an enrichment of IMPDH2 in the nucleus during the S-G2 phase of the cell cycle, concomitant with an increase in γH2AX (Fig. 2a-d).

♣ In Figure 2C-D, depletion of IMPDH2 induces DNA damage. Is this also seen with acute knockdown using siRNA or an inducible shRNA or CRISPR KO system? It would also be useful to assess this DNA damage in the context of cell cycle phase. Assessing gH2AX foci in the context of mitosin positivity should demonstrate more DNA damage in late S/G2 phase cells if the authors' hypothesis is correct.

We have observed DNA damage accumulation upon IMPDH2 inactivation either by inhibition (MPA, Fig. 2g,h), downregulation (knock-down, Fig. 2e,f) or total loss (CRISPR-generated KO, Fig. 2j-k). It is important to point out that our shRNA mediated knock down is performed in acute settings. We infect cell, select them with antibiotics (in this case puromycin) for 2 days and perform the experiment no later than 72 hours post infection. In addition, following the reviewer's suggestion, we have now performed the majority of our analysis in a cell cycle dependent manner.

♣ Using shIMPDH2 cells or the MPA inhibitor, the authors should perform additional DNA repair assays to support their claims as to the importance of IMPDH2 for DNA damage repair. For example, the authors could stain for additional DNA damage markers e.g. 53BP1 in mitosin positive cells. The authors could also perform neutral comet assays

to measure DNA double-strand break formation in the absence of IMPDH2 (the authors see a G2/M arrest which is consistent with DNA double-strand breaks). The authors could also perform metaphase spread analysis to measure chromosomal aberrations or immunoblot analysis of key DNA repair signaling pathways (pChk1, pChk2 etc).
We have now included additional markers such as 53BP1 (Fig. 2i,k and Fig. 5e,f), RPA70 (Extended Data Fig. 2s), pATR (Extended Data Fig. 2i,k), and pChk1 (Extended Data Fig. 2i,k). Results from the analysis of such markers strengthen the evidence for IMPDH2 signalling in DDR.

♣ In Figure 3B, the authors show an interaction between PARP1 and IMPDH2 in the chromatin fraction. It is not clear however, if this interaction is direct. This IP should be repeated in the presence of benzonase to remove DNA, to rule out that the interaction is seen indirectly via DNA/chromatin binding.
We apologize for the lack of this explanation in our previous version of the manuscript. The protocol we used for chromatome purification includes benzonase treatment and sonication (explained in the Methods section). Thus, the interaction between IMPDH2 and either PARP1 or TOP2A is most likely not DNA-mediated.

♣ Figures 3C – G are largely correlative. Just because IMPDH2 increases on chromatin after treatment with Etoposide, doesn't necessarily mean that IMPDH2 is involved in the DNA damage response to Etoposide. To better prove this link the authors should:o Treat IMPDH2 knockdown cells with Etoposide and perform a co-stain analysis for gH2AX and/or 53BP1 foci in Mitosin positive cells. cDNA rescue experiments should also be performed to rescue any phenotypes. Clonogenic/cellular survival assays with IMPDH2 knockdown cells treated with Etoposide (with cDNA rescue).
We thank the reviewer for the suggestion. We have now performed cell cycle analysis of 53BP1, and γH2AX in IMPDH2 KO cells rescued either with IMPDH2 WT or IMPDH2 NLS. These data show a partial phenotype recovery (Fig. 5c-f), similar to what observed for proliferation (Extended Data Fig. 5f,g). Moreover, we performed transcriptome profiling (Fig. 5a,b,g,i and Extended Data Fig. 5a-e) and apoptosis/necrosis studies (Fig. 5h) in IMPDH2 KO and reconstituted cells to delineate the recovery extent in these conditions.

♣ The way the data with Etoposide is described is a little confusing. The authors are suggesting that the increase of IMPDH2 in response to Etoposide is actually impairing DNA repair. However, this is contradicting earlier data that shows depletion of IMPDH2 impairs DNA repair. Can the authors clarify this?
We apologise for the confusion, and we acknowledge the complexity of the mechanism. In the first half of the manuscript, we have shown that IMPDH2 nuclear localization increases during DNA damage, possibly because of a local/nuclear guanosine demand (Fig. 2a-d, Fig. 3 and Extended Data Fig. 3a-e). We then corroborated the role of IMPDH2

in DDR by producing IMPDH2 KO cells. We observed that loss of IMPDH2 leads to high DNA damage accumulation independently of guanosine supplementation (Fig. 2i-l and Extended Data Fig. 2n-r) and beyond replication stress (Extended Data Fig. 2s-t). Loss of IMPDH2 upregulates the activity of PARP1 resulting in reduced nuclear NAD+ availability and cell necrosis (Fig. 4 and Extended Data Fig. 4).
In the second half of the manuscript, we attempted to recover IMPDH2 KO phenotype by rescuing cells with IMPDH2 WT and NLS. However, we observed that forcing IMPDH2 in the nucleus is detrimental because excessive nuclear IMPDH2 reduces NAD+ availability (Fig. 5j and Fig. 6i,j) , shortening PARP1 activation window (Fig. 6g,h) and condemning cells to death even stronger (Fig. 5h and Fig. 6a-f).
This intricated but interesting mechanism of action can in the future be exploited to limit PARP1 activity and scout for synergistic interaction between PARP inhibitors and strategies that would force IMPDH2 nuclear localization. We hope that this explanation, which we tried to convey also in the manuscript, is now more understandable.

♣ The authors suggest that IMPDH2 and PARP1 interact, and that IMPDH2 interaction causes PARP1 cleavage into the cytoplasm following DNA damage. If this is correct, then there should be less PARP1 cleavage in IMPDH2 knockout cells following DNA damage, yet the opposite seems to be true in Figure 3H. Can the authors clarify?
The absence of IMPDH2, on top of causing DNA damage, also causes replication stress (due to guanosine deprivation; Extended Data Fig. 2s), a senescence associated phenotype (SASP; Fig. 5a,b) and deregulated nuclear energetics (Fig. 4f), which altogether induce apoptosis and necrosis (Fig. 4g,h). Moreover, the increased PARP1 cleavage is observed when reconstituting cells with NLS-IMPDH2 which provokes incremented IMPDH2 nuclear levels, much higher than physiological ones, thus provoking nuclear energy deprivation (Fig. 5i,j and Fig. 6g-j) and PARP1 cytoplasmic translocation (Fig. 6a-d). The same is not observed when reconstituting cell with WT-IMPDH2, suggesting that the amount of nuclear IMPDH2 is crucial to fine-tune PARP1 activity and the DDR. We have now tried to convey this message in the manuscript Result and Discussion sections.

♣ The authors' claim that the levels of IMPDH2 nuclear localization is important for DNA repair is further complicated by extended data figure 5A-B. This data shows that reconstitution with wild-type IMPDH2 or a IMPDH2 construct that is localized to the nucleus, has no impact on gH2AX levels in cells. The reasoning that PARP1 activity is required for gH2AX foci accumulation as an explanation for this result is not convincing. Regardless, other markers of DNA damage should still be present (e.g. 53BP1), and this should be tested.
We acknowledge the complexity of this mechanism of action and apologize for the lack of clarity in our initial submission. As suggested by the reviewer, and as explained above, we have now also included the quantification of 53BP1, which shows similar behaviour to γH2AX, thus corroborating our data. Furthermore, the transcriptomic analysis revealed

the complexity of our reconstituted cells, which show alterations in proliferation (Fig. 5a,b), and cell survival (Fig. 5g) and cellular energetics (Fig. 5i,j). It is particularly interesting to observe that only cells reconstituted with nuclear IMPDH2 (NLS) downregulate the sirtuin family, a major consumer of nuclear NAD+, suggesting an important nuclear energy rewiring caused by the supraphysiological presence of IMPDH2 in the nucleus, which limits NAD+ availability in a non-physiological manner. We thank the reviewer for pointing out that the message was unclear and too speculative. We have now removed the speculative arguments from the manuscript and attempted to convey the message as reported here in the hope of providing much clearer information.

♣ The link between the interaction with PARP1 and IMPDH2 is not particularly convincing (see above regarding concerns about Figure 3B). To strengthen this link, the authors should map the interaction sites for IMPDH2 and PARP1. This will generate mutants to test separation of function (gH2AX foci, PARP1 cleavage), to further delineate the mechanisms in support of their model. Minor Comments:- Line 101 – missing reference.- Line 174 – missing reference.- Line 235 – missing reference.

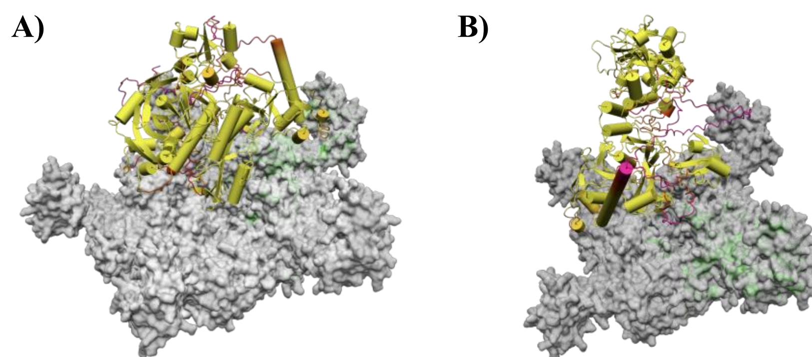

Figure. 2. Prediction models for IMPDH2-PARP1 interaction using AlphaFold3. **A)** Here we modeled with alphafold3 server the interaction between pentameric IMPDH2 observed in nature and monomeric PARP1. The top scoring model shows a docking of PARP1 over the available interface of pentameric IMPDH2 (opposite side of the decamerization). The best AlphaFold3 model shows reliability scores for the global model (pTM: 0.46 with threshold 0.5) slightly over the quality threshold. The figure shows the generated complex, PARP1 residues are coloured following the plddt score computed by AlphaFold3 for each individual amino acid. (yellow high confidence; red low confidence). Pentameric IMPDH2 volume is shown in gray and the secondary structure of one monomer is visible in green. **B)** Here we modeled with alphafold3 server the interaction between tetrameric IMPDH2 and monomeric PARP1. The top scoring model shows a docking of PARP1 over the available interface of tetrameric IMPDH2 (opposite side of the octamerization). The best AlphaFold3 model shows reliability scores for the global model (pTM: 0.55 with threshold 0.5) slightly over the quality threshold. The figure shows the generated complex, PARP1 residues are coloured following the plddt score computed by AlphaFold3 for each individual amino acid. (yellow high confidence; red low confidence). Tetrameric IMPDH2 volume is shown in gray and the secondary structure of one monomer is visible in green.

Although the idea of mapping the residues involved in the interaction is interesting, we believe that this could not be easily solved in the case of IMPDH2-PARP1. The reason

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for this is that these are two large proteins, and in addition IMPDH2 is present in the cell as a tetramer, pentamer or chains of such structures (https://doi.org/10.1074/jbc.m507056200, https://doi.org/10.1021%2Fcr900021w, https://doi.org/10.1186/s13008-018-0038-0, https://doi.org/10.7554/elife.53243). First preliminary results with AlphaFold3 (https://doi.org/10.1038/s41586-024-07487-w) have shown that the pentameric (Figure 2A, this letter) and tetrameric (Figure 2B, this letter) structures of IMPDH2 support the interaction with PARP1 with reliability scores for the global models slightly over the quality threshold. However, at this point we do not feel confident to present these data without additional validation. We plan to decipher the modality of the interaction in the future, as this may lead to a drug discovery project targeting the interaction. finally, as explained above, the co-immunoprecipitation assay was performed on chromatin extracts treated with benzonase and sonicated, suggesting a direct interaction of IMPDH2 and PARP1 that is unlikely to be mediated by DNA or chromatin superstructures.

Reviewer #2

Nucleotide metabolism (Remarks to the Author): Espinar, Garcia-Cao, et al. have documented that the enzyme involved in purine synthesis, IMPDH2, translocates to the nucleus and localizes to the chromatin in triple-negative breast cancer cells following DNA damage. The authors assert that both genetic and chemical inhibition of IMPDH2 result in the accumulation of DNA damage. Notably, IMPDH2's chromatin localization onto DNA takes place during a later stage of DNA damage repair. Upon localization to chromatin, IMPDH2 binds to PARP1. Intriguingly, when IMPDH2 is consistently localized to the nucleus, it causes a depletion of nuclear NAD+, leading to the cleavage of PARP1 and triggering apoptosis.

This work is interesting because it reveals an unanticipated role of IMPDH2 in regulating nuclear NAD+ levels, thereby finely modulating the activation of PARP1 and controlling the DNA damage response.

We appreciate the reviewer's interest and comments, which have allowed us to deepen our knowledge of nuclear IMPDH2 functionality in response to DNA damage. In particular, the reviewer's suggestions have allowed us to reveal that a nucleotide metabolism-related chain of enzymes (including IMPDH2 and other nucleotide-related enzymes such as GART or the NME proteins, Extended Data Fig. 3a) are detected on chromatin upon DNA damage, suggesting an in loco role for nucleotide-related enzymes during DNA damage repair.

1) The authors should elucidate the molecular mechanism underlying the basal-level translocation of IMPDH2 to the nucleus in some TN breast cancer cells. If this nuclear translocation is substantial, it is crucial to understand why and how specific breast cancer cell lines exhibit an increase in nuclear IMPDH2 while others do not. Establishing isogenic settings expressing or not expressing HER2 could provide valuable insights.

We thank the reviewer for this suggestion. We have investigated the drivers of IMPDH2 nuclear translocation in both basal and DNA-damaged conditions. We show that IMPDH2 nuclear translocation is prevented by guanosine supplementation (Fig. 3b), suggesting that IMPDH2 may act as a nucleoside sensor and that nuclear guanosine levels may regulate IMPDH2 nuclear translocation. This idea is further supported by the metabolite analysis study suggested by the reviewer (Extended Data Fig. 3b), which was very informative. In addition, we showed that DNA damage itself, rather than phosphorylation of H2AX, induces IMPDH2 nuclear localization (Extended data Fig. 3g), probably as a consequence of guanosine demand. Finally, we performed a large chromatome analysis (in revision in *Nature Communications* as a separated publication), which confirmed that IMPDH2 localizes to chromatin specifically in TNBC cells, whereas ER+/PrR+ or HER2+ cells do not show high levels of IMPDH2 on chromatin under basal conditions (Figure 3, this letter), supporting the claims presented in Figure 1 of this manuscript.

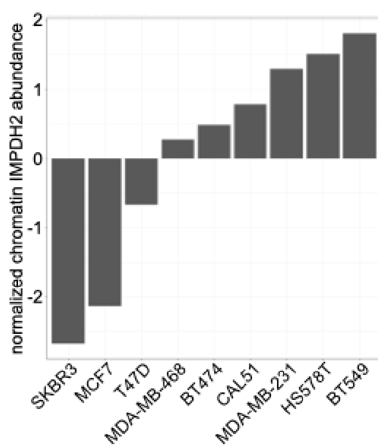

Figure 3. comparison of IMPDH2 chromatin abundance in breast cancer cell lines. Normalized IMPDH2 chromatin abundance in breast cancer cell lines analyzed by MS. Cell lines used: non-TNBC cell lines (SKBR3, MCF7, T47D) and triple negative breast cancer cell lines (MDA-MB-468, BT474, CAL51, MDA-MB-231, HS578, BT549).

2) What are the effects of DNA damage on cellular IMPDH2 activity? A metabolite analysis of guanylate levels (IMP, GMP, GDP, or GTP) should be conducted.

We recently published a dataset of chromatome-MS and metabolomics profiling of U2OS cells treated with etoposide (1µM, 3 hours) to induce DNA damage and released into etoposide-free media to allow DNA damage repair. In the chromatome-MS dataset, IMPDH2 was detected as the most enriched metabolic enzyme on chromatin 24 hours after etoposide release (Extended Data Fig. 3a). In addition, metabolomics profiling analysis revealed a progressive increase in triphosphate nucleosides with a concomitant decrease in monophosphate nucleoside forms with increasing time after etoposide release. Of note, inosine monophosphate (IMP), the substrate of IMPDH2, decreased with time,

while guanosine triphosphate levels increased, suggesting de novo biosynthesis of guanosine nucleotides (Extended Data Fig. 3b).

3) While increased DNA damage upon IMPDH2 inhibition is anticipated, it is surprising that guanosine or guanine supplementation did not rescue DNA damage accumulation. Do these cells express guanosine and guanine transporters?

As part of the review process, we performed transcriptome analysis, which proved to be very helpful not only to address this question, but also to provide mechanistic insights into nuclear IMPDH2 functionality. By analyzing the RNA-seq data, we focused on nucleoside transporters as suggested by the reviewer and found no differences in nucleoside transporter levels between WT and KO cells (Extended Data Fig. 5h). In addition, a guanosine titration assay showed that the cells responded to guanosine supplementation in a dose-dependent manner, demonstrating the ability of these cells to take up guanosine from the media (Fig. 2j-l). However, we did not observe complete recovery of DNA damage or proliferation with any of the concentrations tested, suggesting a role for IMPDH2 in DNA damage signaling beyond guanosine synthesis, which is the focus of this manuscript.

4) It remains unclear how the amount of nuclear IMPDH2 increases in response to DNA damage. Is IMPDH2 posttranslationally modified in response to DNA damage regulatory pathways? Is the ATR/ATM pathway involved in this regulation?

We thank the reviewer for this suggestion, which helped us to better understand the mechanism of IMPDH2 nuclear localization. As suggested, we examined IMPDH2 nuclear localization under basal or etoposide-induced DNA damage conditions in the presence or absence of ATM/ATR inhibitors and found no differences in IMPDH2 nuclear localization, suggesting that other factors may be involved in IMPDH2 recruitment. Nevertheless, the experiment was informative in showing that while ATR inhibition reduces γH2AX accumulation, IMPDH2 nuclear levels still increase in response to DNA damage induction, suggesting that DNA damage, rather than γH2AX itself, mediates IMPDH2 nuclear translocation (Extended Data Fig. 3f,g). In addition, we found that guanosine levels can affect the dynamics of IMPDH2 translocation, perhaps to meet the demands of nucleosides during DNA damage repair (Fig. 3b). Further deciphering how IMPDH2 translocation is regulated in response to DNA damage will certainly be an area of interest for future studies. Regarding the question of post-translational modification, we have recently described a novel workflow for classical immunoprecipitation coupled to mass spectrometry (IP-MS) data that focuses on identifying differential peptidoforms of the bait protein between conditions (https://doi.org/10.1038/s41597-024-03394-x). IMPDH2 could be a good case study to apply this type of analysis in a follow-up study to see if post-translational modifications (not limited to phosphorylation) are involved in cytosol-nucleus shuffling in both basal and DNA damage induced conditions.

5) Does nuclear IMPDH2 impact transcription and gene expression? Why is IMPDH2 present in the nucleus and not its isoform, IMPDH1? Do other purine enzymes, such as GMPS, ADSL, and ADSS, translocate to the nucleus?

Following the reviewer's suggestion, we performed RNA-seq in MDA-MB-231 IMPDH2 WT, KO, KO + guanosine, KO reconstituted with IMPDH2 WT, and KO reconstituted with IMPDH2 NLS. This experiment was indeed key to deepening our understanding of the molecular basis of nuclear IMPDH2 functionality. On the one hand, we found that the partial rescue we see in various phenotypic assays is confirmed at the gene expression level, as guanosine supplementation shows an expected partial rescue (Fig. 5b,g). On the other hand, and specifically related to the reviewer's comments, we found that KO cells reconstituted with IMPDH2-NLS deregulate the expression of NAD+-consuming enzymes such as the entire sirtuin family (Fig. 5i) and depict a distinct transcriptional signature in terms of Senescence Associated Secretory Phenotype (SASP; Fig. 5b) or NAD+-ADP-ribosyltransferase activity (Fig. 5i), with relevance for the control of cell fitness, DNA damage response and overall NAD+ utilization.

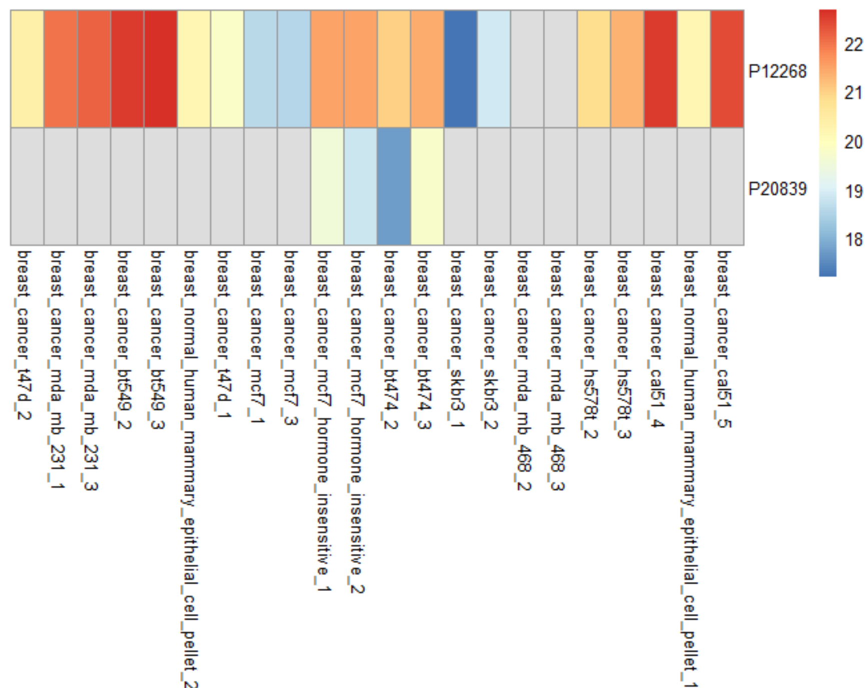

Figure 4. Comparison of IMPDH2 and IMPDH1 chromatin abundance. Pre-normalized abundance of IMPDH2 (P1226; unique peptides only) and IMPDH1 (P20839, unique peptides only) on chromatin in breast cancer cell lines. Gray indicates the absence ofIMPDH2 or IMPDH1 unique peptides in a given sample.

In addition, the reviewer's suggestion to look at other purine enzymes on chromatin has greatly contributed to gain further insight into the mechanistic basis of the nuclear IMPDH2 role in DDR. We detected other purine pathway metabolic enzymes on chromatin, of particular interest the NME proteins - NME1, NME2, NME3 - (Extended Data Fig. 3a), which are nucleoside diphosphate kinases (NDPKs) that catalyse the

transfer of a γ-phosphate group to nucleoside diphosphates, mainly GDP, suggesting a local increase in GTP availability in response to DNA damage. It is tempting to speculate that IMPDH2 uses IMP to produce GMP, which is later converted to GDP and GTP by the NME proteins directly on chromatin. However, follow up studies are required to confirm this hypothesis.
Finally, we do not observe IMPDH1 enrichment on chromatin (Figure 4, this letter), nor its overexpression in IMPDH2 KO cells (given that the used antibody recognises both IMPDHs, Extended Data Fig. 2j) and this explain why IMPDH2 KO cell are guanosine auxotrophic. While it would be interesting to understand how the two proteins are differentially regulated, we believe this is beyond the scope of this study at this time, especially since IMPDH2 expression and activity rather than IMPDH1 has been implicated in cancer progression.

6) The western blots demonstrating increased PARP cleavage in response to NLS-IMPDH2 are not entirely convincing. The authors should consider alternative approaches, such as assessing PARP stabilization in WT or NLS-IMPDH2 cells.
We thank the reviewer for this suggestion. As an alternative approach, we performed quantification of nuclear poly/mono-ADP ribosylation as a readout for PARP activity. We found that NLS-IMPDH2 cells have decreased levels of poly/mono-ADP ribosylation compared to WT MDA-MB-231 cells or IMPDH2 KO cells rescued with IMPDH2 WT (Fig. 6g). These low levels of poly/mono-ADP ribosylation could be rescued by NAD+ supplementation (Fig. 6h), suggesting that nuclear IMPDH2 consumes NAD+, thereby limiting PARP1 activation and provoking PARP1 cleavage due to nuclear NAD+ shortening.

7) If forcing nuclear IMPDH2 localization leads to nuclear NAD+ depletion, then NLS cells should be more sensitive to the NAMPT inhibitor (FK866) than WT cells. The authors could investigate whether chemical NAD+ depletion results in more potent NLS cell death than WT cells.
We performed the suggested experiment and found that KO-NLS are not more sensitive, but rather more resistant than KO-WT (Extended Data Fig. 5i). Although counterintuitive, we believe that this must be one of the strategies that the cell uses to adapt to supraphysiological levels of nuclear IMPDH2. The simplest explanation could be that KO-WT cells express lower levels of NAMPT than KO-NLS cells (as shown by our transcriptomics data, Extended Data Fig. 5j), which could be the reason for their hypersensitivity to NAMPT inhibition compared to KO-NLS cells. In addition, KO-NLS cells are depleted of cytoplasmic IMPDH2, so the consumption of cytoplasmic NAD+ produced by the salvage pathway may be reduced in comparison to KO-WT cells. Finally, NAMPT is known to localize to the nucleus together with GAPDH during DNA damage (https://doi.org/10.1074/jbc.ra119.010571), further complicating experimental interpretation. However, as explaining above and supporting tour claiming, NLS-

CRG

IMPDH2 cells have decreased levels of poly/mono-ADP ribosylation compared to WT MDA-MB-231 cells or IMPDH2 KO cells rescued with IMPDH2 WT (Fig. 6g). These low levels of poly/mono-ADP ribosylation could be rescued by NAD+ supplementation (Fig. 6h), suggesting that nuclear IMPDH2 consumes nuclear NAD+, thereby limiting PARP1 activation and provoking PARP1 cleavage due to nuclear NAD+ shortening.

8) Since the authors claim that nuclear IMPDH2 leads to nuclear NAD+ depletion, increased DNA damage, and cancer cell death, it would be interesting to assess whether nuclear IMPDH2 in breast tumors also leads to nuclear NAD+ depletion and tumor growth reduction.
We thank the reviewer for this suggestion. We think this is of great interest and we will pursue this avenue in the follow-up project we are developing in which we aim to target nuclear IMPDH2 localization to synergize with PARP1 inhibition. For this study, we are using PDX and PDXO models and will assess nuclear IMPDH2 levels along with cytoplasmic PARP1, nuclear NAD+ and DNA damage. We believe that the time required to develop these experiments, together with the amount of data already provided, places the proposed experiments out of scope at this time and we hope that the reviewer will understand these reasons.

Reviewer #3 - PARP signaling (Remarks to the Author):
Summary
In this manuscript, the authors identified a purine synthesis enzyme, IMPDH2, which is enriched on chromatin in TNBC cell lines. They demonstrate that the IMPDH2 activity is required for proper DNA damage repair and that it interacts with PARP1 on chromatin. Additionally, they report that nuclear IMPDH2 regulates local NAD+ availability and has some potential regulatory role in PARP1 activity.

Review
The work shown here is potentially interesting and adds to the wealth of knowledge regarding the importance of metabolic enzymes in the nucleus. The work also suggests the role of IMPDH2 in modulating DNA damage response pathways in breast cancer cells. Though this work reveals potential novel insights into the role of IMPDH2, it falls short in validating certain key observations and the conclusions are, thus, inflated. Moreover, the mechanistic model put forth in the paper needs further experimental evidence in support.

Strengths: This work identifies a non-canonical role of IMPDH2 in controlling nuclear NAD+ levels to regulate DNA damage response. The authors also showed that the modulation of nuclear IMPDH2 levels impairs DNA repair thus leading to cell death.

Weaknesses: The title of the paper is misleading as it states that nuclear IMPDH2 modulates PARP1 activity. However, the results presented in this manuscript at most are suggestive of the role of nuclear IMPDH2 in PARP1-dependent DNA damage repair or cell death pathways, not strictly related to the regulation of PARP1 catalytic activity. Further validation of key observations and robust support of the model are lacking.

We thank the reviewer for the interest in our study and the insightful comments, which helped us to gain more knowledge about the molecular basis of nuclear IMPDH2, especially regarding the functional relationship between nuclear IMPDH2, PARP and NAD+ levels.

Major Comments:

1. The authors should be careful using the word "PARP1 activity" (generally taken to mean catalytic activity, not repair activity) and avoid extrapolating the conclusions obtained.

We thank the reviewer for pointing this out, in this revised version we have removed speculative arguments and rewritten it to make it more precise and clearer.

Additional experiments that can support their conclusions are listed below.

2. Further evidence is needed to show that IMPDH2 indeed binds to chromatin (e.g., FRAP assay on the nuclear enzyme should be performed).

We agree with the reviewer that in this study we show that IMPDH2 localizes to the chromatin environment, although it does not necessarily bind directly to DNA. In fact, our chromatome protocol includes a benzonase and sonication step that removes DNA (as explained in the methods section). In addition, we performed immunoprecipitation assays with IMPDH2 in the presence of benzonase, suggesting a direct interaction between IMPDH2 and TOP2A or PARP1 proteins without the need for DNA (Fig. 4b,c). Although we cannot exclude the possibility that IMPDH2 binds directly to DNA as shown in Drosophila (https://doi.org/10.1016/j.molcel.2012.04.030), we believe that this is not relevant to the main message of this manuscript. The FRAP experiments suggested by the review are of great value in follow-up studies to investigate the precise dynamics of IMPDH2 binding to DNA under basal as well as DNA damage induced conditions.

3. The authors show nuclear translocation of IMPDH2 upon DNA damage, but do not discuss the reasons for this change in localization. Does IMPDH2 have an NLS?, bind to other proteins that mediate this?

We did not find any nuclear localizing sequence (NLS) in IMPDH2 (predictNLS, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1083765/), suggesting that other proteins, conditions or post-translational modifications may be involved in this regulation. We have tested IMPDH2 nuclear translocation in the presence of ATM or ATR inhibition, two master regulators of the DDR process, and found no alteration in IMPDH2 translocation under these conditions, suggesting the involvement of other factors in this

regulation. One of the factors we found to influence nuclear IMPDH2 levels is guanosine concentration, since nuclear IMPDH2 levels decrease as guanosine concentration increases (Fig. 3b). This suggests that this guanosine concentration-dependent regulation may contribute to a production of nucleotides in the chromatin environment to fulfil an *in loco* demand during the DNA damage repair process. Regarding post-translational modification, we have recently described a novel workflow for classical immunoprecipitation coupled to mass spectrometry (IP-MS) data that focuses on identifying differential peptidoforms of the bait protein between conditions (https://doi.org/10.1038/s41597-024-03394-x). IMPDH2 could be a good case study to apply this type of analysis in a follow-up study to see if post-translational modifications are involved in cytosol-nucleus shuffling under both basal and DNA damage induced conditions. Finally, we do not yet know if importins members interact with IMPDH2, which we could investigate by applying the pipeline of IP-MS mentioned above in a conventional way (to identify interactors). However, IMPDH2 molecular weight (56 kDa) would allow nucleopore shuttling without the need of an active transport.

4. In Figure 1f, including a western blot detection of **γ**H2AX in MCF7 parental and hormone-insensitive cells might help validate this system and prove the role of nuclear IMPDH2 in DNA damage repair. Moreover, FDX1 in this panel needs an introduction in the main text.
We apologize for the lack of information regarding FDX1, which is now appropriately introduced in the text.
We thank the reviewer for the suggestion to perform **γ**H2AX quantification in hormone-independent MCF7 cells. Indeed, we observed a significant increase in **γ**H2AX in these cells (Fig. 1g), which adds value to the characterization and validation of the mechanism of action we have described.

5. In line 139, there is no introduction about the function of etoposide in its first appearance in this manuscript.
We have now appropriately described etoposide functionality in the revised manuscript.

6. The authors briefly introduce the interaction of TOP2A and do not mention/discuss this further. A clear discussion regarding this is warranted especially because the role of TOP2A in DNA damage is also reported. Also, more IP's are needed to validate this interaction, across the other BC cell lines and also to show if IMPDH2 interacts with PARP1 and TOP2A in a mutually exclusive manner or not.
We have now described in more detail the functionality of TOP2A and the consequences of IMPDH2 interaction. In addition, we have validated the interaction of IMPDH2 with TOP2A and with PARP1 in nucleus enriched extract from a different TNBC cell line system (Hs 578T, Fig. 4c). Given the fact that the manuscript focuses on the interaction between IMPDH2 and PARP1, we believe that understanding whether this interaction is

mutually exclusive with the TOP2A interaction, or with the other many interactions IMPDH2 may undertake in the nuclear environment, is out of scope at this time. To perform this experiment, in the future we plan to perform nuclear IMPDH2 immunoprecipitation followed by MS in different conditions (including cell cycle arrest, DNA damage and nucleotide starvation) to get a broad picture of how the nuclear IMPDH2 interactome changes upon perturbations.

7. In Figure 2, the authors use IMPDH2 inhibition and depletion to conclude that IMPDH2 catalytic activity is required for normal DNA damage repair. Though this may be the case, but it should be tested further by rescue with catalytic dead IMPDH2.
We appreciate reviewer suggestions that allowed us to formally assess the involvement of IMPDH2 catalytic activity on DNA damage repair. We generated a catalytic dead mutant (CD) in which the active site residue Cys331 is mutated to Ala (C331A, https://doi.org/10.1016/s0167-4838(01)00277-1) and found that introducing this mutation increase DNA damage (Extended Data Fig. 2m). These data, alongside results with the MPA inhibitor, points to an involvement of the catalytic activity of IMPDH2 in the DDR process.

8. In Figure 3c, the data showing the relative abundance changes upon etoposide treatment should be on the same blot. Also, it is imperative to include whole cell lysate as a control for this experiment.
Indeed, booth the chromatin and the cytosolic fractions, as well as both treatments are part of the same blot, as show in the uncut western blot (Figure 5, this letter and Extended Data Fig. 6). We apologise for the misunderstanding.

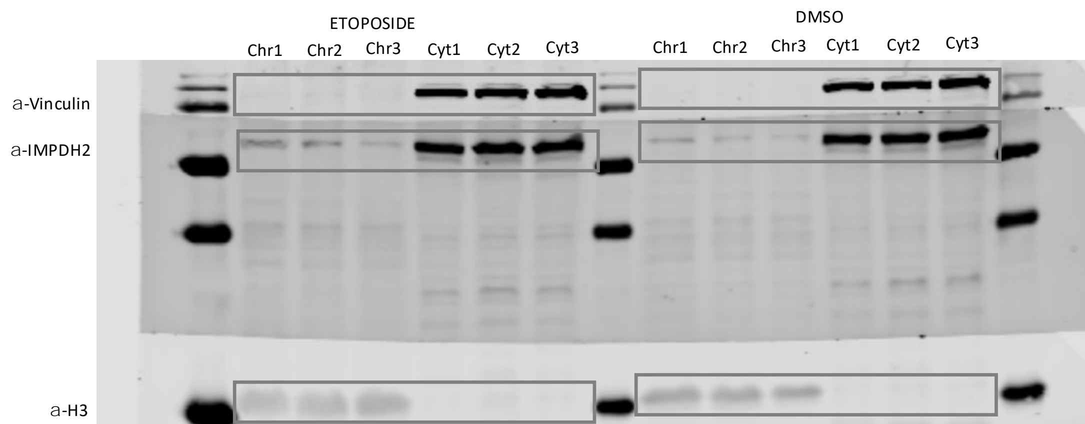

Figure 5. Western blot of MDA-MB231 cells. Cytosolic (cyt) and Chromatin (chr) fractions in the control condition (DMSO) and after 3h of etoposide treatment and 24h of release. Numbers indicate the replicate performed.

9. In Figure 3h, leveraging the different sizes of cleaved PARP1 the authors comment on apoptosis vs necrosis. This should be supported using other apoptosis and necrosis markers/assays.

Following the reviewer's comment, we have used alternative approaches in WT and KO cells (Fig. 4g,h) as well as in reconstituted cells (Fig. 5h) to look at different types of cell death (early apoptosis/late apoptosis/necrosis) such as DAPI-Annexin V staining assays. In addition, we performed RNA sequencing which revealed that each cell death pathway (i.e., apoptosis, necrosis, or necroptosis) is altered in the absence of IMPDH2 and only partially rescued when KO cells are treated with guanosine or reconstituted with either IMPDH2 WT or NLS (Fig.5g). In addition, we observed that specifically the IMPDH2-NLS reconstituted KO cells downregulate the sirtuin family of enzymes (Fig. 5i), which are known NAD+ consumers and compete with PARP1 in the nucleus for NAD+ availability (https://doi.org/10.1016%2Fj.mam.2013.01.004, https://doi.org/10.4161/cc.5.8.2690). This interesting observation led us to quantify NAD+ in the nucleus of reconstituted cells, and we observed that KO NLS cells have lower basal nuclear NAD levels (Fig.5j), which suggest that they may downregulate the sirtuin family to avoid excessive nuclear NAD+ consumption.

10. In line 217, the authors state that the lack of IMPDH2 causes the loss of control of NAD+ cellular levels but never actually show the data. The use of NAD+ sensor is needed like in Figure 5a-d.
We thank the reviewer for the suggestion and agree that this information was missing. We have now used the NAD+ sensor in WT and KO cells and have shown that KO cells have lower nuclear NAD+ levels (Fig. 4f), likely due to their apoptotic/necrotic state even in the absence of DNA damage stimuli (Fig. 4e). In addition, we also analysed nuclear poly/mono-ADP-ribose levels as a measure of PARP1 activity and found that IMPDH2 loss resulted in increased nuclear poly/mono-ADP-ribose levels (Fig. 4k), reflecting increased PARP activity in these cells, consistent with reduced nuclear NAD+ levels.

11. In line 246, the authors conclude that IMPDH2 accumulation affects PARP1 activity. However, this needs to be tested by monitoring the PARP1 automodification activity.
We thank the reviewer for this suggestion. We have now used the quantification of nuclear poly/mono ADP-ribose levels as a readout of PARP1 activity (Fig. 6g).

12. In Figure 4, the authors should include proper controls like WT, IMPDH2 KO alongside KO-WT and KO-NLS cells.
Our transcriptomic analysis showed that IMPDH2 WT cells, IMPDH2 KO cells and the KO reconstituted lines display specific phenotypes with respect to several key cellular pathways such as proliferation, DNA damage, cell death, senescence (Fig. 5a,b,g,i). Therefore, we would be cautious to refer to the WT IMPDH2 cells and the KO IMPDH2 cells as the control for the KO-WT or KO-NLS cells. We would be more inclined to call them independent lines. Nevertheless, we have performed HT-IF of poly/mono ADP-ribose levels (Fig. 6g), NAD+ quantification (Fig. 5j) as well as cell death assay (Fig. 5h)

of each condition in comparison to show their intrinsic differences. We apologize for not having these data in the first submission and thank the reviewer for pointing this out.

13. In Figure 4a,b, the authors should include the western blot of necrosis-cleaved PARP1 (similar to Figure 3h) to show that IMPDH2 knockout causes necrosis, while IMPDH2 nuclear localization only causes apoptosis. This should be supplemented with other markers as well.
We thank the reviewer for this suggestion and included the necrotic band for the western blot panel (Fig.6 a,b). Nevertheless, the revision process led us to a deeper characterization of the phenotypes observed either in the KO cells or in the reconstituted cells and we now know that all reconstitutions show specific cell death profiles, as shown by the transcriptomic analysis, which are different from those observed in the IMPDH2 KO cells (Fig. 5b,g,i). With these data in hand, we performed the above explained experiments (NAD+ quantification, cell death assay, and nuclear poly/mono ADP-ribose quantification) to rule out differences between IMPDH2 WT, KO, and reconstituted cells.

14. In Figure 5a-d, NAD+ needs to be quantified between WT, IMPDH2 KO, KO-WT, and KO-NLS cells to comprehensively show that IMPDH2 nuclear localization affects nuclear NAD+ levels. This assay should also be repeated by supplying the cells with extra NMN to compensate for the possible NAD+ depletion and observe the outcome on overall cell survival.
We sincerely thank the reviewer because we think this suggestion was very helpful to strengthen the functional relationship between nuclear IMPDH2, nuclear NAD levels and PARP. We found a decrease in the nuclear NAD+ pool in KO-NLS cells compared to WT cells (as well as in KO-WT cells; Fig. 5j). Furthermore, using nuclear poly/mono-ADP ribosylation as a measure of PARP activity, we found decreased levels in the nucleus of KO-NLS cells (Fig. 6g). Thus, we supplemented the cells with NAD+, as suggested by the reviewer, and observed a rescue of poly/mono ADP-ribosylation (Fig. 6h), suggesting a causal relationship between nuclear NAD+ levels and PARP function.

15. The model in Figure 5d needs a more detailed explanation in the main text.
Given the evolution of the project during the review process, we feel that the previous scheme was inadequate and have decided to remove it.

16. Typos (e.g., PARylation), superscripts (e.g., NAD+) and references need to be corrected in the text.
We have now corrected these typos.

17. The authors should include details about what type of NLS was added to IMPDH2.
We have included these details in the methods section.
In particular the 3x NLS come from:

C-MYC NLS:

CCTGCTGCTAAGAGAGTGAAACTGGAT

Nucleoplasmin NLS:

AAGCGGCCCGCTGCTACTAAGAAGGCTGGTCAGGCTAAGAAGAAGAAG

SV40 NLS:

GATCCGAAGAAGAAGCGAAAGGTC

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors' have addressed most of my concerns with additional experimental data or with text clarifications, and I believe the manuscript is acceptable for publication. I would like to congratulate the authors on a very nice study, performed to an excellent standard.

Reviewer #2:
Remarks to the Author:
The authors have essentially addressed my comments.

Reviewer #3:
Remarks to the Author:
The authors have done a careful, thoughtful, and thorough job addressing my major concerns. The paper has been improved. The work is sound and will make a good contribution to the literature.