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BRCA1/2-Mutant Breast Cancers

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14. ABSTRACT Poly(ADP-ribose) polymerase (PARP) inhibition provides a promising therapeutic modality for targeting homologous recombination (HR) deficient tumors such as BRCA1 and BRCA2-mutated triple negative breast cancers (TNBCs). Although PARP inhibitors have shown activity in the BRCA-associated TNBCs, several of these tumors develop de novo as well as acquired PARP inhibitor (PARPi) resistance. Besides attenuation in intracellular uptake of drugs, the only known mechanism that drives chemotherapy resistance of BRCA1/2-deficient cancers is through the restoration of HR. Recent studies from our laboratories (Nussenzweig and D'Andrea) indicate that deregulation of pathways that promote extensive degradation of nascent DNA strands and alternative end-joining (Alt-EJ) can render BRCA1/2-deficient cells resistant to PARPi in a HR-independent manner. The objective of our project is to collaboratively test the hypothesis that complex processes involving Alt-EJ or replication fork stability promote survival and drives resistance to chemotherapy. A detailed assessment of the critical mediators that regulate the balance between HR, Alt-EJ and replication fork degradation should identify novel means to overcome acquired chemoresistance in BRCA1/2-mutated breast cancers. During the first year of the DOD funding, we have made progress in identifying the proteins which contribute to the replication fork stability and we have identified new mechanisms of chemoresistance in BRCA2-deficient tumors.					
15. SUBJECT TERMS Breast cancer, BRCA1, BRCA2, PARP inhibitors, chemotherapy, resistance, HR, replication fork stability, haploinsufficiency, RNF168, 53BP1, PTIP, Shieldin, end resection					
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1. INTRODUCTION:

The inactivation of the tumor suppressor genes *BRCA1* and *BRCA2* by mutations or epigenetic silencing is a critical event in breast and ovarian carcinogenesis. *BRCA1* and *BRCA2* encode proteins that are essential for accurate double strand break (DSB) repair by homologous recombination (HR). *BRCA1* functions early during DSB resection, *BRCA2* functions later in HR by catalyzing RAD51 nucleo-filaments at processed DSBs. Accordingly, HR deficient breast and ovarian tumors are highly sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors, since PARP inhibitors exhibit synthetic lethality in tumors with defective HR DNA repair. PARP inhibitors are currently in development for BRCA- or otherwise HR repair-deficient cancers, with FDA approval of Olaparib, Rucaparib and Niraparib. Of these drugs, Olaparib has been the most widely studied thus far, and it has been approved by the FDA as a monotherapy for treatment of ovarian cancer patients with germline *BRCA1* or *BRCA2* mutations. Nonetheless, *de novo* and acquired PARP inhibitor (PARPi) resistance, is a pressing clinical problem in patients with BRCA-deficient cancers treated with PARP inhibitors. Therefore, identification of the mechanisms underlying PARPi resistance is crucial for improving treatment and predicting tumor responses. Besides attenuation in intracellular uptake and increased efflux of drugs, the other known mechanism of PARPi resistance in BRCA-deficient tumors include restoration of HR due to somatic reversion of *BRCA1/BRCA2* or loss of other genes such as 53BP1, RIF1 or REV7. Recently, it was shown that *BRCA1* and *BRCA2* protect stalled replication forks from Mre11-mediated degradation, independent of their roles in HR. Accordingly, restoration of either HR capacity or replication fork stability is also associated with PARPi resistance in BRCA-deficient tumors. Indeed, a recent study from the Nussenzweig laboratory indicated that loss of *PTIP* protects replication forks from degradation in both *BRCA1*- and *BRCA2*- deficient cells and confers PARPi resistance. The D'Andrea laboratory has recently identified a novel DNA repair pathway, the so-called PARP/POLQ end-joining pathway, which, when upregulated, provides the HR-deficient breast tumor cell with an alternative mechanism of DNA repair. Collectively, recent studies from both laboratories indicate that deregulation of pathways that promote extensive degradation of nascent DNA strands and alternative end-joining (Alt-EJ) can render *BRCA1/2*-deficient cells resistant to PARPi in a HR independent manner. *We had therefore hypothesized that replication fork protection and PARP mediated Alt-EJ are novel and potentially interlinked mechanisms by which BRCA1/2-deficient breast cancers acquire resistance to chemotherapy.* Accordingly, the objective of our project is to provide a more detailed assessment of the factors that contribute to replication fork protection and Alt-EJ. This could lead to therapeutic approaches to overcome acquired resistance by targeting new vulnerabilities in both *BRCA1/2*-mutant and *BRCA1/2*-wildtype breast cancer.

2. KEYWORDS:

Breast cancer, *BRCA1*, *BRCA2*, PARP inhibitors, replication fork stability, haploinsufficiency, RNF168, 53BP1, *PTIP*, Shieldin, end resection, Thap1, REV7, TRIP13, Novobiocin

3. ACCOMPLISHMENTS:

The major goal of the project is to identify the molecular mechanisms of PARPi resistance in BRCA1/BRCA2 mutated breast tumors in order to improve therapeutic options for breast cancer patients.

The following specific aims were proposed:

Specific Aim 1. Understand how PTIP-MLL3/4 and PARP1 confers chemoresistance and replication fork (RF) degradation in BRCA1/2-deficient cells.

Specific Aim 2. Determine the interactions of BRCA2, FANCD2, and POLQ in replication fork (RF) stability and Alt-EJ

Specific Aim 3. Assess mechanisms of PARPi resistance in mouse models and patient derived xenografts

- What was accomplished under these goals?

We have described major activities, specific objectives, significant results or key outcomes, conclusions and other achievements related to each specific aim in the following sections. Both Drs. Nussenzweig and D'Andrea have noted tasks for which they were responsible (**Site 1, Nussenzweig: NCI, NIH; Site 2, D'Andrea: DFCI**).

In our Year 1 annual report (2017), we better defined the relationships between PARylation, Brca2-deficiency and genomic instability that lead to cancer development and additionally identified a novel role of EZH2 (a histone methyltransferase) at replisomes in BRCA2-deficient tumors.

In our Year 2 annual report (2018), we made significant progress in understanding the mechanisms by which proteins such as PTIP and PARP1 contribute to replication fork stability and chemoresistance in BRCA2-deficient tumors. Using a mass spectrometry approach to identify proteins associated with stalled replicating forks, we identified numerous candidate proteins that could contribute to replication fork protection or destabilization. Additionally, we made progress in identifying proteins which contribute to replication fork stability and identified a new mechanism of chemoresistance in BRCA2-deficient tumors. We determined that loss of function of EZH2 in BRCA2-deficient cancer cells promotes PARPi resistance. Importantly, our results identify EZH2 expression as a biomarker of BRCA2-deficient tumor response to chemotherapy.

In our Year 3 annual report (2019), we demonstrated the functional importance of PTIP-53BP1 interactions in regulating homologous recombination (HR), and in doing so identified a new mechanism by which 53BP1 can suppress HR directly by blocking RAD51 loading post-resection. We also determined that restoration of replication fork (RF) protection and HR represent two independent means by which Brca1/2-deficient cells can become chemoresistance. We additionally confirmed a role of the POLQ protein in Alt-EJ and in replication fork stability.

In our final report, Drs. Nussenzweig and D'Andrea have made significant advances for major tasks as they relate to Specific Aims 1 and 2. Using a CRISPR-based genetic screen, Dr. Nussenzweig has identified a novel protein that confers chemosensitivity in Brca1-deficient cells (**Specific Aim 1**) and using an *in vitro* screen, Dr. D'Andrea has discovered a small molecule inhibitor of Polymerase Q which can selectively kill HR-deficient cancer cells (**Specific Aim 2**).

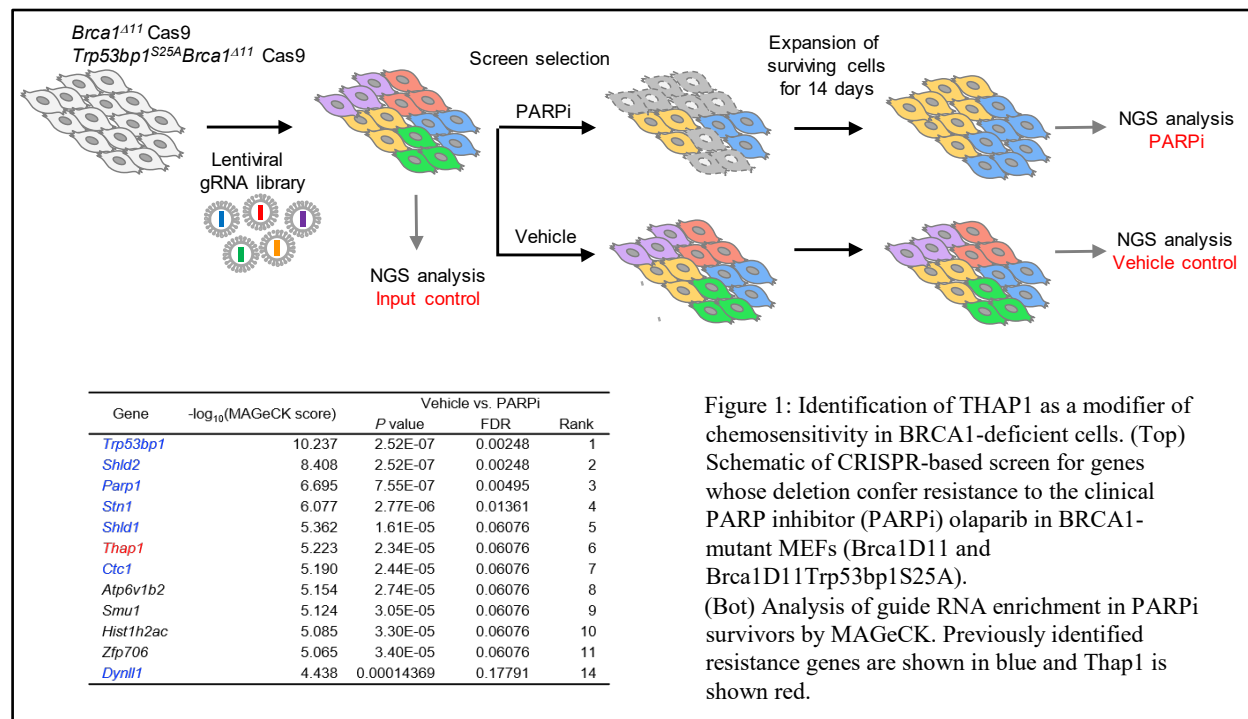
Specific Aim 1:

Major Task 2: Determine the mechanisms by which PARP1 modulates chemosensitivity in BRCA-deficient cells.

To more systematically explore the genetic basis of PARPi resistance, we conducted whole-genome CRISPR-Cas9 synthetic-viability screens in BRCA1-mutant cells. *Brca1^{D11}* and *Brca1^{D11}Trp53bp1^{S25A}* mouse embryonic fibroblasts (MEFs) were transduced with lentiviral libraries of single-guide RNAs (sgRNAs), and the resultant pools of edited cells were exposed to near-lethal doses of the clinically used PARPi olaparib for two weeks (**Figure 1, Top**). Gene enrichment or depletion was calculated for each cell line with positive and negative β -scores indicating positive and negative selection of a given gene deletion event, respectively.

Identification of THAP1 as a modifier of chemosensitivity in BRCA1-deficient cells

We identified multiple genes whose deletion have previously been shown to confer PARPi resistance in BRCA1-deficient cells, including *Trp53bp1*, *Shld1*, *Shld2*, *Ctc1*, *Stn1* and *Dynll1*



(**Figure 1, Bot**), thereby confirming the validity of our approach. In addition, we identified *Thap1* as a candidate gene whose deletion strongly imparts PARPi resistance in both *Brca1^{D11}*

and *Brca1^{D11}Trp53bp1^{S25A}* MEFs (**Figure 1, Bot**). Thap1 encodes the murine homolog of Thanatos-associated protein 1 (THAP1), a transcription factor that is causatively linked to adult-onset torsion dystonia type 6 (DYT6) in humans. To verify the results of the primary screens, we conducted competitive growth assays using sgRNAs targeting either Thap1 or Trp53bp1 and found that each led to the outgrowth of two independently-derived *Brca1^{D11}* MEF cell lines treated with PARPi (data not shown). By contrast, Thap1 deletion did not affect the PARPi sensitivity of BRCA1-proficient cells as measured by cell viability (data not shown).

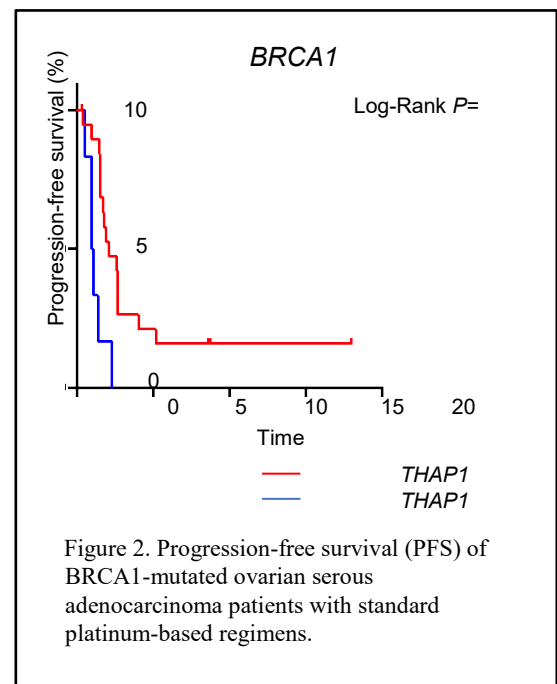
These results support THAP1 as a bona fide modifier of chemosensitivity in mouse and human cell lines with BRCA1 deficiency.

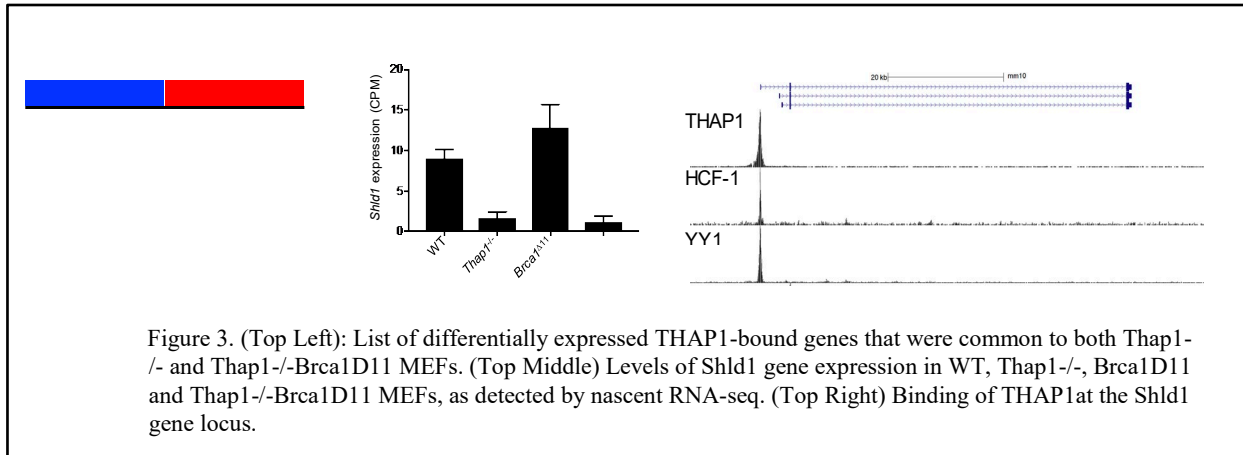
Loss of THAP1 confers chemoresistance in BRCA1-deficient human tumors

To test whether dysregulated THAP1 has an impact on tumorigenesis, we extracted human tumor expression data from The Cancer Genome Atlas (TCGA) database. We found that low expression of THAP1 was significantly correlated with shorter progression-free survival (PFS) of patients with BRCA1-mutated serous ovarian carcinoma (**Figure 2**). Our results suggest that THAP1 loss alleviates PARPi and cisplatin hypersensitivity in BRCA1-deficient cells and tumors, and this effect is not species-, cell lineage- or BRCA1 mutation-specific.

THAP1 is a direct transcriptional regulator of *Shld1* expression

Mutations in THAP1 have been identified as the cause of DYT6 (dystonia), which is associated with abnormal muscle contractions without overt neuropathological lesions. Given that most of the dystonia-causing mutations in THAP1 disrupt its sequence-specific DNA binding activity, we hypothesized that THAP1 may regulate the chemosensitivity of BRCA-deficient cells directly through transcriptional mechanisms. To investigate this possibility, we performed nascent RNA-sequencing (RNA-seq) to systematically probe for genes that are differentially expressed in Thap1^{-/-} and Thap1^{-/-}Brca1D11 MEFs, as compared to wild-type and Brca1D11 parental cells, respectively. 98 genes (57 downregulated and 41 upregulated) were common to both Thap1^{-/-} and Thap1^{-/-}Brca1D11 cells. To determine which of these genes are direct targets of THAP1, we examined published THAP1 ChIP-sequencing (ChIP-seq) dataset in mESCs which identified 2,758 unique THAP1 binding sites and nine differentially expressed genes in Thap1-deficient MEFs were also bound by THAP1 (**Figure 3, Top Left**). Among these genes we identified *Shld1*, the penultimate

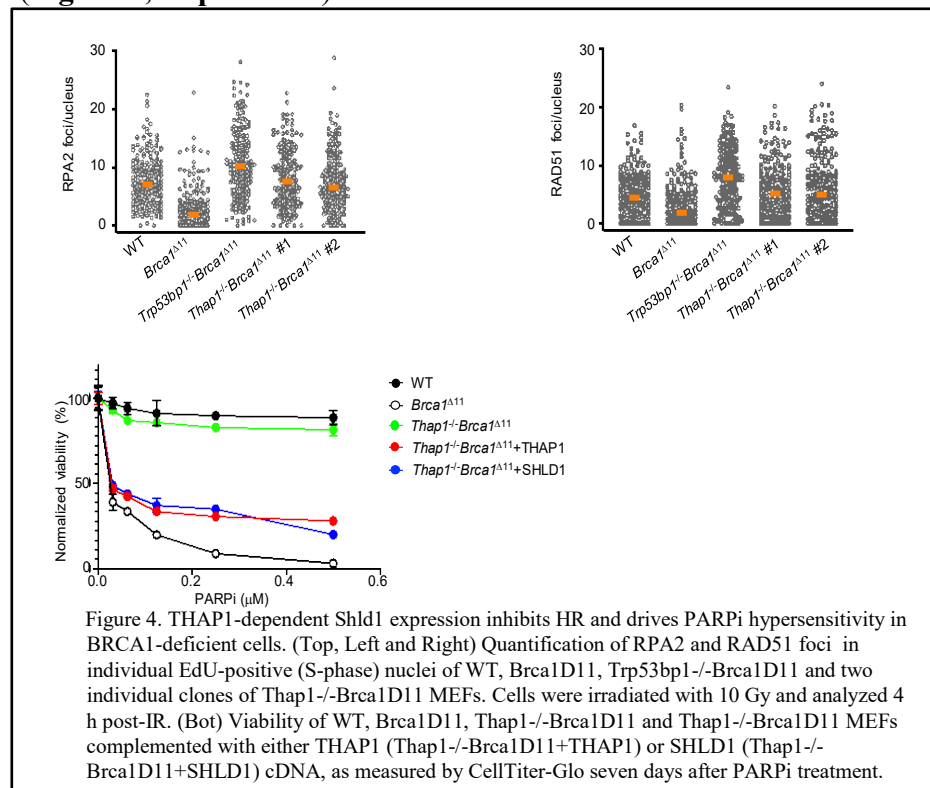




component of the Shieldin complex and a known effector of 53BP1. The Shieldin complex, consisting of SHLD1, SHLD2, SHLD3 and REV7, were identified as critical effectors of 53BP1-mediated end protection downstream of RIF1. In addition, Shieldin has been shown to directly suppress RAD51 nucleofilament formation on ssDNA and notably, depletion of any Shieldin complex component restores HR in BRCA1-deficient cells, and reduced expression of Shieldin is associated with the acquisition of PARPi resistance in PDX models of BRCA1-deficient breast cancers. The *Shld1* promoter contains a consensus THAP1 binding sequence (THABS) motif where a strong THAP1 ChIP-seq signal was detected (**Figure 3, Top Right**) and our RNA-seq datasets confirmed that *Shld1* expression was significantly decreased in *Thap1*^{-/-} and *Thap1*^{-/-}*Brca1D11* MEFs (**Figure 3, Top Middle**).

THAP1 inhibits DNA end resection and RAD51 nucleofilament formation

We found that deletion of THAP1 rescued both irradiation-induced RPA foci and RAD51 nucleofilament formation in *Brca1D11* MEFs to wild-type levels (**Figure 4, Top Left and Right**). Thus, loss of



THAP1 promotes end-resection and HR proficiency in BRCA1-deficient cells, which could explain why doubly deficient cells acquire resistance to PARPi and cisplatin. To provide definitive proof that the observed PARPi resistance in Thap1^{-/-}Brca1D11 MEFs was due to decreased Shld1 expression, we overexpressed SHLD1 in Thap1^{-/-}Brca1D11 MEFs. Overexpression of SHLD1 in Thap1^{-/-}Brca1D11 MEFs restored their hypersensitivity to PARPi. Addback of THAP1 in Thap1^{-/-}Brca1D11 MEFs also restored PARPi hypersensitivity (**Figure 4, Bot**). Moreover, SHLD1 or THAP1 overexpression in Thap1^{-/-}Brca1D11 MEFs increased chromosomal aberrations to levels comparable to or higher than in Brca1D11 parental cells (data not shown). Thus, THAP1-dependent Shld1 transcription is essential for maintaining the functionality of Shieldin.

Specific Aim 2:

Major Task 6: To identify and characterize proteins which cooperate with FANCD2 and POLQ in replication fork stability and Alt-EJ. *In this section, Dr. D'Andrea has cited the six publications from our laboratory which were supported by this grant from the Department of Defense.*

Subtask 1: To identify and characterize proteins which cooperate with FANCD2 and POLQ in replication fork stability and Alt-EJ using iPOND/mass spectrometry (time line:6-18 months) (**Site 2, DFCI, Dr. D'Andrea**).

We have not performed the experiments proposed for this subtask.

Subtask 2: Validation of FANCD2 and POLQ interacting proteins identified by mass spec using coimmunoprecipitation, shRNA knockdown, changes in POLQ nuclear foci and Alt-EJ template assays (proposed timeline: 18-30 months) (**Site 2, DFCI, Dr. D'Andrea**).

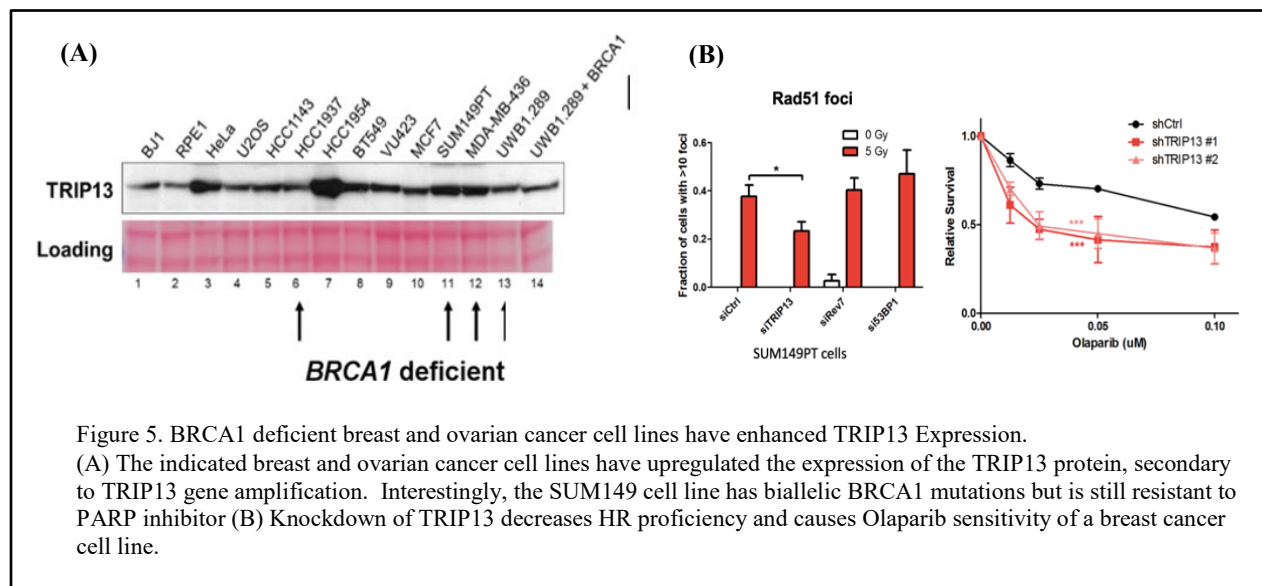
We have not performed the experiments proposed for this subtask.

Rather than focus on Subtask 1 and Subtask 2, we focused on the following highly related projects which turned out to be much more productive. These projects led to novel insights into the proteins **REV7 and TRIP13** and their role in breast cancer. During the last year, we have further evaluated the role of POLQ in alternative end joining (Alt-EJ). We published a review article on Alt-EJ (*Patterson-Fortin J, D'Andrea AD. Exploiting the Microhomology-Mediated End-Joining Pathway in Cancer Therapy. Cancer Res. 2020 Jul 10;canres.1672.2020. doi: 10.1158/0008-5472.CAN-20-1672*). We have demonstrated that inhibition of POLQ blocks Alt-EJ and selectively kills HR-deficient tumor cells. Specifically, we devised an in vitro screen to identify a small molecule inhibitor of Polymerase Q. The POLQ protein has an N-terminal ATPase domain and a C-terminal Polymerase domain. Since the N-terminal ATPase domain has strong ATPase activity in vitro, we were able to perform a high throughput screen. We identified Novobiocin (NVB) as a potent and specific inhibitor of the POLQ-ATPase. We have performed several mouse xenograft and GEMM models to confirm that NVB kills BRCA1-deficient tumor cells, as well as other HR-deficient tumor cells.

As part of Subtask 2, we have identified a new mechanism by which BRCA1-deficient PARP inhibitor sensitive tumor cells became PARP inhibitor resistant. We have shown that BRCA1-deficient tumor cells can amplify the gene TRIP13 as a mechanism for restoring HR repair. Specifically, the TRIP13 gene encodes an ATPase which can inactivate REV7, leading to an opening and inactivation of the SHLD complex. The relevance of the SHLD complex results in increased dsb end resection, increased RAD51 loading, and increased HR, leading to acquired PARPi resistance. This work has recently been published in two papers (*Clairmont CS*, Sarangi P*, Ponnienselvan K, Galli LD, Csete I, Moreau L, Adelmant G, Chowdhury D, Marto JA, D'Andrea AD. TRIP13 Regulates DNA Repair Pathway Choice through REV7 Conformational Change. Nature Cell Biol, 2020.*) (*Sarangi P, Clairmont CS, D'Andrea AD. Disassembly of the Shieldin Complex by TRIP13. Cell Cycle. 2020 Jul;19(13):1565-1575. doi: 10.1080/15384101.2020.1758435. Epub 2020*)

TRIP13 overexpression promotes HDR and PARP inhibitor resistance in BRCA1-deficient breast cancer cells

Since our last progress report, we proceeded to directly assay the effect of TRIP13 in BRCA1-deficient breast cancers using patient-derived cell lines. We assessed TRIP13 protein levels in a panel of normal and patient-derived breast cancer cell lines and observed wide variation (**Figure 5A**). We focused on the SUM149PT cell line, as it is a widely used BRCA1-deficient model

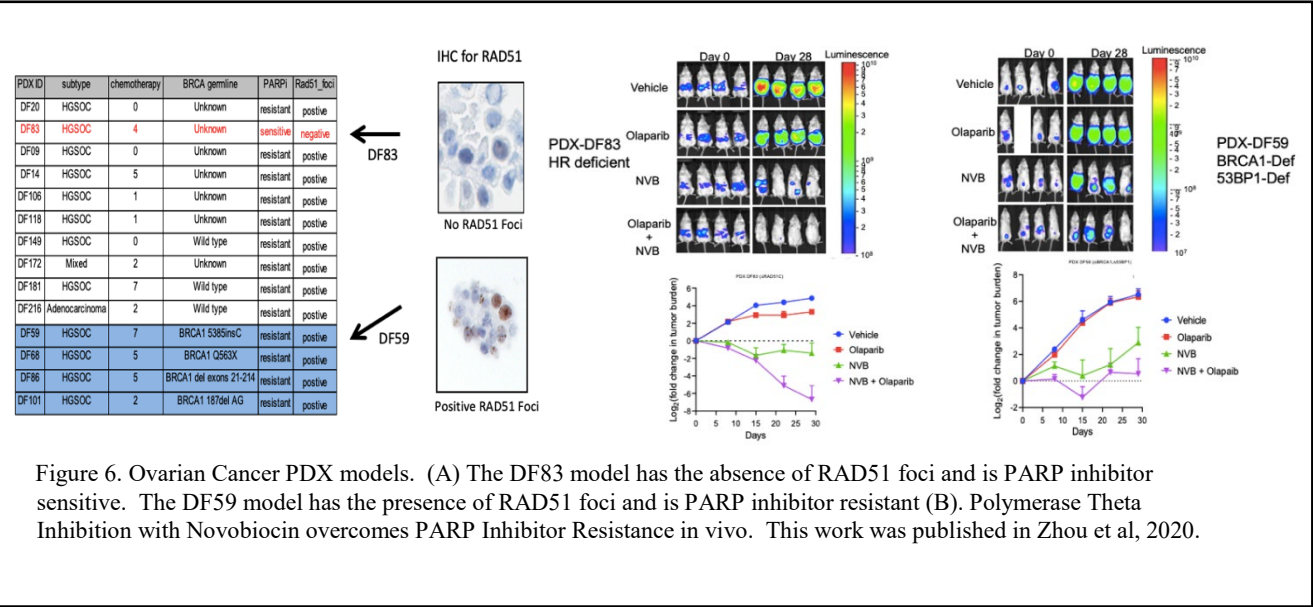


with high TRIP13 expression. Despite their BRCA1 deficiency, SUM149PT cells were partially HDR-proficient as monitored by RAD51 focus formation and Olaparib resistance. We therefore asked whether TRIP13 promotes HDR in these cells. Indeed, stable knockdown of TRIP13 significantly impaired both RAD51 focus formation and Olaparib resistance (**Figure 5B**). Interestingly, knockdown of REV7 or 53BP1 did not appreciably increase RAD51 focus formation in SUM149PT cells, unlike the case in other BRCA1-deficient contexts. This is likely because the 53BP1-REV7-Shieldin axis in these cells is already inactivated by their high TRIP13 expression. This work was also described in our recent review article which was supported by

this DOD grant. (Sarangi P, Clairmont CS, D'Andrea AD. Disassembly of the Shieldin Complex by TRIP13. Cell Cycle. 2020 Jul;19(13):1565-1575. doi: 10.1080/15384101.2020.1758435. Epub 2020). Moreover, using CRISPR screens, we were able to identify novel sgRNAs, and hence gene products, which further regulate DNA repair and

homologous recombination activity in breast cancer cells (1. Li F, Kozono D, Deraska P, Branigan T, Dunn C, Zheng X, Parmar K, Nguyen H, DeCaprio J, Shapiro GI, Chowdhury D, D'Andrea AD. CHK1 Inhibitor Blocks Phosphorylation of FAM122A and Promotes Replication Stress. Mol Cell, 2020; 2. Cai, M., C. Dunn, W. Chen, B. Kochupurakkal, H. Nguyen, L.A. Moreau, G. I. Shapiro, D. Kozono and A. D. D'Andrea. "Cooperation of the ATM and Fanconi Anemia/BRCA Pathways in Double Strand Break End Resection." Cell Rep. 2020 Feb 18;30(7):2402-2415.e5.; 3. Li, N., J. Wang, S. S. Wallace, J. Cheng, J. Zhou and A. D. D'Andrea "Cooperation of the NEIL3 and Fanconi Anemia/BRCA Pathways in Interstrand Crosslink Repair." Nucleic Acids Res. 2020 Jan 25. pii: gkaa038. doi: 10.1093/nar/gkaa038.)
Major Task 8: Evaluate POLQ and PARP inhibitor resistance in murine xenograft and GEMM models (Site 2, DFCI, Dr. D’Andrea)

As described above in Major Task 6, we have identified **Novobiocin (NVB)** as a novel inhibitor of POLθ (reviewed in Patterson-Fortin and D’Andrea, Cancer Research, 2020) and evaluated whether POLθ inhibition could represent a novel therapeutic option for PARPi-resistant tumors, we have tested the efficacy in vivo of NVB in the patient-derived xenograft (PDX) models. The PDX model, DF83, was generated from an HR-deficient (loss of RAD51C expression), human high-grade serous ovarian carcinoma (**Figure 6A**). NSG mice bearing the HR-deficient DF83



ovarian cancer PDX model were treated with olaparib, NVB, or a combination of the two, and tumor growth was monitored by bioluminescence imaging (BLI). In order to determine the combined effect with NVB, olaparib was delivered at a submaximal dose (50 mg/kg daily), and we observed only a small degree of tumor growth inhibition. Treatment of DF83 with NVB

alone (75 mg/kg, twice a day) led to tumor regression, while tumors in vehicle-treated mice showed exponential growth (**Figure 6B**). Strikingly, when the DF83 tumor-bearing mice were given NVB and olaparib in combination, we observed complete tumor regression, and few tumor cells were detectable via BLI on day 28 in this group. These in vivo results suggest NVB may be useful in combination with PARPi to achieve better efficacy and prevent drug resistance in HR-deficient tumors. No significant toxicity to normal mouse tissue was observed from the drug combination.

Next, we evaluated the efficacy of NVB alone and in combination with olaparib in the PARPi-resistant PDX model DF59, which was isolated from a patient with a germline BRCA1 mutation and acquired PARPi resistance. This model harbors biallelic mutations in the TP53BP1 locus, providing a mechanism of the acquired PARPi resistance. In the DF59 PDX model, there was no response to olaparib monotherapy, but NVB monotherapy substantially reduced tumor growth, indicating a lack of cross-resistance between PARP inhibition and NVB. Importantly, the combination of NVB and olaparib further inhibited DF59 tumor growth, with tumor regression in the first two weeks (**Figure 6B**). In contrast to DF83 and DF59 models, the BRCA1 WT, HR proficient PDX model DF149 was resistant to both drugs and to the combination (data not shown). These data demonstrate that NVB can inhibit the growth of a BRCA1-mutated, PARPi-resistant tumors in vivo and that the combination of NVB and a PARPi is particularly effective in treating at least some PARPi-resistant tumors. These new data are posted in our recent Bioarchives paper (Zhou J, Gelot C, Pantelidou C, Li A, Yücel H, Davis RE, Farkkila A, Shapiro GI, Blagg BSJ, Ceccaldi R, D'Andrea AD. *Polymerase Theta Inhibition Overcomes PARP Inhibitor Resistance*. *bioRxiv* 2020. <https://doi.org/10.1101/2020.05.23.111658>). This paper also demonstrates the mechanism of NVB activity-namely, the increase in DSB end resection which leads to cell death.

Summary of the key research accomplishments: Our studies have revealed that the evolutionarily conserved transcription factor THAP1 promotes transcription of the Shld1 gene in mammalian cells. Our observation that loss of THAP1 restores HR and chemoresistance in both Brca1D11 (resection-deficient) and Brca1D11Trp53bp1S25A (RAD51 loading-deficient) MEFs is consistent with a critical function of Shieldin pre- and post-resection. We have also identified Novobiocin (NVB) as a novel small molecule inhibitor of POLθ and determined that POLθ inhibition represents a novel therapeutic option for PARPi-resistant tumors.

What opportunities for training and professional development did the project provide?

Nothing to report

How were the results disseminated to communities of interest?

Results were shared with the scientific community via informal discussions, posters and presentations at scientific meetings and through publications in peer-reviewed journals

What do you plan to do during the next reporting period to accomplish the goals and objectives?

Nothing to report

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have shown that chemosensitivity in *Brcal*/2-deficient cells can result from either loss of RF protection or loss of HR (and perhaps both in some cases). Conversely, restoration of RF stability or HR can independently confer chemoresistance. These findings have a high impact on our ability to identify patient populations who may/may not benefit from chemotherapy and may open new avenues for targeted intervention. We have additionally shown that mutations in different domains of *Brcal* can have distinct effects on HR. This suggested that all *Brcal* mutant tumors may not respond to chemotherapy in the same manner.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

Nothing to Report

Significant changes in use or care of human subjects:

Not applicable

Significant changes in use or care of vertebrate animals:

Nothing to Report

Significant changes in use of biohazards and/or select agents:

Nothing to Report

6. PRODUCTS

Publication: Journal publications.

DOD BCRP Publications October 2019-Present (Nussenzweig) citing DOD support in the acknowledgments

1. Shinoda, K, Zong D, Callen E, Wu W, Dumitrache L.C, Belinky F, Wong N, Ishikawa M, Konstantinopoulos P.A, Ehrlich M, McKinnon P.J, Nussenzweig A. "The Dystonia Gene THAP1 Controls Physiological DNA repair and Chemosensitivity in BRCA-deficient Cells." **Molecular Cell** (in revision)
2. van Wietmarschen N, Sridharan S, Nathan WJ, Tubbs A, Chan EM, Callen E, Wu W, Belinky F, Tripathi V, Wong N, Foster K, Noorbakhsh J, Garimella K, Cruz-Migoni A, Sommers JA, Huang Y, Borah AA, Smith JT, Kalfon J, Kesten N, Fugger K, Walker RL, Dolzhenko E, Eberle MA, Hayward BE, Usdin K, Freudenreich CH, Brosh RM Jr, West SC, McHugh PJ, Meltzer PS, Bass AJ, Nussenzweig A. "Repeat expansions confer WRN dependence in microsatellite-unstable cancers." **Nature**. 2020 Oct;586(7828):292-298. doi: 10.1038/s41586-020-2769-8. Epub 2020 Sep 30
3. Sciascia N, Wu W, Zong D, Sun Y, Wong N, John S, Wangsa D, Ried T, Bunting SF, Pommier Y, Nussenzweig A. "Suppressing proteasome mediated processing of topoisomerase II DNA-protein complexes preserves genome integrity." **Elife**. 2020 Feb 14;9:e53447. doi: 10.7554/eLife.53447.
4. Paiano J, Wu W, Yamada S, Sciascia N, Callen E, Paola Cotrim A, Deshpande RA, Maman Y, Day A, Paull TT, Nussenzweig A. "ATM and PRDM9 regulate SPO11-bound recombination

intermediates during meiosis.” **Nat Commun.** 2020 Feb 12;11(1):857. doi: 10.1038/s41467-020-14654-w.

5. Shinoda K, Maman Y, Canela A, Schatz DG, Livak F, Nussenzweig A. “Intra-V κ Cluster Recombination Shapes the Ig Kappa Locus Repertoire.” **Cell Rep.** 2019 Dec 24;29(13):4471-4481.e6. doi: 10.1016/j.celrep.2019.11.088.

DOD BCRP Publications January 2020-Present (D’Andrea) citing DOD support in the acknowledgments

1. Li N, J. Wang J, Wallace S.S, Cheng J, Zhou J, D’Andrea AD. "Cooperation of the NEIL3 and Fanconi Anemia/BRCA Pathways in Interstrand Crosslink Repair." **Nucleic Acids Res.** 2020 Jan 25. pii: gkaa038. doi: 10.1093/nar/gkaa038.

2. Cai, M, Dunn C, Chen W, Kochupurakkal B, Nguyen H, Moreau L.A, Shapiro G.I, Kozono D, and D’Andrea AD. "Cooperation of the ATM and Fanconi Anemia/BRCA Pathways in Double Strand Break End Resection." **Cell Rep.** 2020 Feb 18;30(7):2402-2415.e5.

3. Zhou J, Gelot C, Pantelidou C, Li A, Yücel H, Davis RE, Farkkila A, Shapiro GI, Blagg BSJ, Ceccaldi R, D’Andrea AD. Polymerase Theta Inhibition Overcomes PARP Inhibitor Resistance. **bioRxiv** 2020. <https://doi.org/10.1101/2020.05.23.111658>.

4. Sarangi P, Clairmont CS, D'Andrea AD. Disassembly of the Shieldin Complex by TRIP13. **Cell Cycle.** 2020 Jul;19(13):1565-1575. doi: 10.1080/15384101.2020.1758435. Epub 2020 May 18.

5. Patterson-Fortin J, D'Andrea AD. Exploiting the Microhomology-Mediated End-Joining Pathway in Cancer Therapy. **Cancer Res.** 2020 Jul 10;canres.1672.2020. doi: 10.1158/0008-5472.CAN-20-1672.

6. Li F, Kozono D, Deraska P, Branigan T, Dunn C, Zheng X, Parmar K, Nguyen H, DeCaprio J, Shapiro GI, Chowdhury D, D’Andrea AD. CHK1 Inhibitor Blocks Phosphorylation of FAM122A and Promotes Replication Stress. **Mol Cell.** 2020 Oct 21:S1097-2765(20)30691-2. doi: 10.1016/j.molcel.2020.10.008.

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers, and presentations.

Dr. Andre Nussenzweig (Site 1, NCI, NIH)

2019

- Invited Speaker, 50th. Annual Meeting of the Environmental Mutagenesis and Genomics Society (EMGS), Washington D.C., September 2019.
- Keynote Speaker, ASBMB-BSC Symposium on the Interplay between Epigenetic Regulation and Genome Integrity, Suzhou, China, October 2019.
- Invited Speaker, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, October 2019.
- Invited Speaker, Department of Cancer Genetics and Epigenetics, Beckman Research Institute, City of Hope, Duarte, CA, October 2019.
- Invited speaker, 9th NCI Symposium on Chromosome Biology, National Institutes of Health, Bethesda, MD, November 2019.
- Invited speaker, National Institutes of Health Wednesday Afternoon Lecture Series (WALS), Bethesda, MD. Postponed.
- Invited speaker, Pruitt Memorial Lecture, Roswell Park Comprehensive Cancer Center, Buffalo, New York. Postponed.
- Invited speaker, Mechanisms of Recombination Conference, Cambridge, UK. Postponed.
- Invited speaker, 85th. Cold Spring Harbor Symposium on Quantitative Biology “Genome Stability and Integrity, Cold Spring Harbor, New York. Postponed.
- Keynote speaker, Nucleotide Excision Repair and Crosslink Repair: Molecules to Mankind, Smolenice, Slovakia. Postponed.
- Invited speaker, Institute of Molecular Genetics of the Czech Academy of Sciences, Prague, Czech Republic. Postponed.
- Keynote speaker, Gordon Research Conference on Genomic Integrity, DNA Repair and Human Diseases, Kowloon, Hong Kong. Postponed.
- Invited Speaker, Instituto de Medicina Molecular (iMM), Lisbon, Portugal, Postponed.

2020

- Invited Speaker, DFHCC Connect: Science Virtual Seminar Series, Boston, MA, July 2020.
- Invited Speaker, Genomic Stability and DNA Repair Keystone Virtual Symposia, Breckenridge, Colorado, September 2020.
- Invited Speaker, Virtual Seminar Department of Cell Biology and Division of Biology, Albert Einstein College of Medicine, New York, September 2020.
- Invited Speaker, Virtual Cancer Center Research Seminars, O’Neal Comprehensive Cancer, University of Alabama at Birmingham (UAB), November 2020.
- Invited speaker, EMBO Virtual Workshop Chromatin Dynamics and Nuclear Organization in Genome Maintenance, Leiden, The Netherlands, December 2020.

Dr. Alan D'Andrea (Site 2, DFCI)

2019

- Keystone Symposium: DNA Replication and Genome Instability: From Mechanism to Disease, Snowbird, Utah
- Stand Up To Cancer Scientific Summit, Santa Monica, CA
- DNA Damage Response Therapeutics Summit, Boston, MA
- Gordon Research Conference on Mammalian DNA Repair, Ventura, CA
- ESMO Targeted Anticancer Therapies Conference, Paris, France
- DF/HCC Breast and Ovarian Cancer Symposium, Boston, MA
- NCI Board of Scientific Counselors Meeting, Bethesda, MD
- Sierra Oncology External Advisory Board Meeting, Atlanta, GA
- AACR Annual Meeting, Atlanta, GA
- Susan F. Smith Center Executive Council Breakfast, Boston, MA
- Mt. Sinai Tisch Cancer Institute External Advisory Board Meeting, New York, NY
- Merck KGA DDR Summit, Boston, MA
- Perspectives in Science Seminar, Fred Hutchinson Cancer Research Center, Seattle, Washington
- Cyteir Scientific Advisory Board Meeting, Lexington, MA
- SU2C Pancreatic Cancer Mini-Summit, Philadelphia, PA
- Aspen Cancer Conference, Aspen, CO
- SU2C Merck-Catalyst Review Meeting, Philadelphia, PA
- AACR Advances in Ovarian Cancer Research Conference, Atlanta, GA
- FARF Annual Scientific Symposium, Chicago, IL
- Ideaya Scientific Advisory Board Meeting, San Francisco, CA
- Invited Seminar, Biomedical Research Institute, National Autonomous University of Mexico (UNAM), Mexico City, Mexico
- BCRF Grantees Meeting, New York, NY
- DFCI Joint Visiting Committee Meeting, Boston, MA
- BRCA Answers from Cancer Interactome Structures (BACIS) Scientific Advisory Board Meeting, MD Anderson Cancer Center, Houston, TX
- Susan F. Smith Center for Women's Cancers Executive Council Beyond Boston Luncheon, Wellesley, MA
- isDDRHD Meeting, Shenzhen, China
- Society for Neuro-Oncology (SNO) Meeting, Phoenix, AZ

2020

- Vanderbilt Cancer Center ESAB Meeting, Nashville, TN
- Wienman Symposium, Honolulu, HI
- SU2C Scientific Summit, Santa Monica, CA
- PARP and DDR Inhibitors Summit, Boston, MA
- FARF SAB Meeting, Orlando, FL
- Gray Foundation Summit, New York, NY

- Keynote Address, 2020 University of Massachusetts Medical School MD/PhD Retreat, Worcester, MA
- AACR Virtual Annual Meeting
- SU2C Pancreatic Cancer Collective Symposium
- Cell Press LabLinks Symposium: Targeting the DNA Damage Response in Cancer
- Ideaya Scientific Advisory Board Meeting
- MD Anderson Cancer Center Ovarian SPORE Virtual EAB Meeting
- Rivkin Ovarian Cancer Research Symposium
- Keynote Address, Keystone Symposium: Genomic Stability and DNA Repair

• **Website(s) or other Internet site(s)**

Nothing to Report

• **Technologies or techniques**

Nothing to Report

• **Inventions, patent applications, and/or licenses**

Nothing to Report

• **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
(Site 1, NCI, NIH)

Name:	Andre Nussenzweig
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Dr. Nussenzweig was responsible for the project management and collaboration with Dr. D'Andrea.
Funding Support:	Dr. Nussenzweig is supported by the NIH Intramural Research Program

Name:	Elsa Callen
Project Role:	Staff Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	Dr. Callen has been instrumental in developing the replication fork protection assay and in assessing fork stability in a variety of genetic contexts
Funding Support:	Dr. Callen is supported by the NIH Intramural Research Program

Name:	Dali Zong
Project Role:	Scientist
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6
Contribution to Project:	Dr. Zong has been a major contributor to the laboratory's research efforts in understanding the mechanisms of DNA repair pathway choice. He has been responsible for conducting fundamental studies on the molecular mechanisms of DNA damage/repair and has developed a number of sophisticated genetic, biological, biochemical and especially imaging assays to further these studies.
Funding Support:	Dr. Zong is supported by the NIH Intramural Research Program

Name:	Kenta Shinoda
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6

Contribution to Project:	Dr. Shinoda has performed work in the area of PARP1 biology and how affecting co-factor (NAD+) levels influences enzymatic activity and genome stability in BRCA-deficient cells
Funding Support:	Dr. Shinoda is supported by the NIH Intramural Research Program

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
(Site 2, DFCI)

Name:	Alan D’Andrea
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-6168-6294
Nearest person month worked:	No change.
Contribution to Project:	No change.
Funding Support:	Dr. D’Andrea is supported by the following grants: DoD <i>BC151331PI</i> , 5R01HL52725-24, BCRF, P50CA168504, SU2C-AACR-DT16-15/Supplement, PCF, 5P01HL048546, Fanconi Anemia Research Fund, Inc, Merck & Co., Inc., Sierra Oncology, Inc., Lilly, SU2C/Lustgarten Foundation, Bridge Project, 1P01CA228696-01A1, GI SPORE, SU2C Phillip A. Sharp Innovation Award, Bassor Initiative, Breast Cancer SPORE, Ovarian Cancer SPORE

Name:	Jessica Filiatrault
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	4 calendar months

Contribution to Project:	She assisted Dr. Zhou in performing cell-based and biochemical assays.
Funding Support:	Ms. Filiatrault is supported by this DoD grant <i>BC151331PI</i> and Ovarian Tumor Living Biobank grant.

Name:	Jia Zhou
Project Role:	Instructor
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	5
Contribution to Project:	Dr. Zhou has performed work in the area on examining the role of EZH2 in replication fork stabilization and PARP inhibitor resistance.
Funding Support:	Dr. Zhou is supported by this DoD Grant and Dr. D'Andrea's BCRF grant.

Name:	Chunyu Yang
Project Role:	Mouse Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	She assisted all the mouse related experiments.
Funding Support:	Ms. Yang is supported by this DoD Grant, Lustgarten grant and AAPR Phil Sharp award.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Nussenzweig has added Dr. Kenta Shinoda back to this project for conducting CRISPR-related screening experiments (described above in Specific Aim 1, Major Task 2).

Dr. D'Andrea has the following changed in his other support:

Ongoing Research Support

1. 5R01HL52725-25 (PI: D'Andrea) 1/1/20-12/31/23

NIH/NHLBI

Molecular Pathogenesis of Fanconi Anemia

This study investigates the molecular pathogenesis of Fanconi Anemia using the recently cloned human Fanconi Anemia Complementation Group C (FACC) cDNA and a newly developed antiserum against the FACC polypeptide.

2. Breast Cancer Research Foundation (D'Andrea) 10/1/11-9/30/21

Extending the use of PARP Inhibitors for Triple Negative Breast Cancer Therapy

Goals/Aims: To determine whether the combination of Velcade plus ABT-888 has enhanced cytotoxic activity for TNBC cell lines in vitro, compared to each agent alone; To generate colonies of mice bearing human TNBC orthotopic implants (derived from TNBC cell lines or from TNBC patient biopsies) and to treat these mice with the combination of Velcade plus ABT-888. As a subaim, we will examine the tumors dissected from these mice with numerous pharmacodynamic markers (i.e., immunohistochemistry for pBRCA1, FANCD2-Ub, and RAD51).

3. 5P01HL048546-23 (PI: Grompe)

Pathophysiology and Treatment of Fanconi/Project 2 and Core 9/1/16-5/31/21

NIH

The major goals of this study are 1) determine the mechanism by which TGF- β inhibitors promote FA cellular growth and regulate DNA repair, 2) to determine whether inhibition of TGF- β pathway rescues hematopoietic defects in FA mouse models, and 3) to determine whether inhibition of TGF- β pathway rescues hematopoietic defects in primary bone marrow cells from FA patients.

Role: Project PI

4. Richard & Susan Smith Family Foundation (PI: D'Andrea) 9/1/17-3/31/21

Ovarian Tumor Living Biobank

Major goals are to establish the Susan F. Smith Center Living Biobank of recurrent and primary ovarian tumors at Dana-Farber, and to disseminate the knowledge gained from the initiative to investigators with specialties that span multiple cancer types.

Role: PI

5. SU2C/Lustgarten Foundation (PI: D'Andrea) 11/1/18-12/31/22

Exploiting DNA Repair Gene Mutations in Pancreatic Cancer

Major goal of this study is to identify the most potent combinatorial strategy, along with predictive biomarkers, that will inform the design of a clinical trial for PDAC patients.

Role: PI

6. Bridge Project (PI: D'Andrea) 3/1/19-2/28/21

Nanoparticle-mediated Drug Delivery to Ovarian Cancer Organoid Cultures

Major goal of this study is to understand; 1) the mechanisms that lead to HGSC sensitivity and resistance to various immune and DNA damage repair therapies and 2) what the best method of delivering specific therapies for these defects is.

Role: PI

7. NIH 1P01CA228696-01A1 (PI: Kantoff) 7/1/19-6/30/24

Project 3: Functional Evaluation and Interpretation of DDR Variants in Prostate Cancer

Major goal of this proposal is to determine whether specific DNA repair gene mutations in prostate cancers are deleterious and vulnerable to targeted therapy.

Role: Project Leader

8. DF/HCC SPORE in Gastrointestinal Cancer 7/1/19-6/30/24

P50CA127003 (Bass/El-Bardeesy) Project 3

Major goal of this proposal is to define optimal strategies for identifying PDAC patients with DDR deficiency and to conduct innovative PARP inhibitor treatment trials to improve care for these patients.

Role: Co-Project Leader

9. SU2C Phillip A. Sharp Innovation in Collaboration Award (D'Andrea/Cubillos-Ruiz) 6/1/19-5/31/2021

Resistance to PARP inhibitor plus anti-PD1 therapy driven by ER stress and bioactive lipids in ovarian cancer

Major goals of this proposal are to evaluate the status of LPA/PERK-controlled signatures in responder vs. non-responder patients, and to determine whether inhibition of LPA-PERK sensitizes OC to Niraparib and Pembrolizumab.

Role: Co-PI

10. Bassett Initiative at Gray Foundation, Team Science Grants (PI: Sung) 7/1/19-6/30/23

Dissection of BRCA-mediated Tumor Suppression Pathways

Major goals of this study are to evaluate the PARPi and platinum-resistant mechanisms, and to determine their impact on patient drug response.

Role: Specific 4 Project Leader

11. P50CA168504-06A1 (Winer) 7/01/19 – 06/30/24

NIH/NCI

Dana-Farber/ Harvard Cancer Center SPORE in Breast Cancer – Developmental Research Program

The overall goal of the Developmental Research Program (DRP) is to advance high quality, novel, early-phase research, to foster new ideas in breast cancer research and to move research projects from a pilot stage to a level where external funding for more mature research is possible. A secondary goal of the DRP is to create opportunities for the career development of junior faculty or senior investigators who are interested in transitioning into breast cancer research. The

DRP will be co-led by Alan D’Andrea, MD and Nikhil Wagle, MD. They will work with a standing committee made up of SPORE and DF/HCC investigators to review applications.
Role: Co-Director, DRP

12. DF/HCC Ovarian SPORE (D’Andrea/Spriggs/Matulonis) 8/5/20-7/31/2025

NIH 1P50CA240243-01A1

DF/HCC Ovarian SPORE

Major goal of this proposal is to make translational progress in understanding resistance mechanisms that will lead to meaningful and improved outcomes of women with newly diagnosed ovarian cancer and those with relapsed ovarian cancer.

Role: Contact PI: D’Andrea

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: We have marked the tasks assigned to us and accordingly we have provided a progress made for each task.

QUAD CHARTS: Not applicable

9. APPENDICES: Publications attached.

View Crossmark data 

REVIEW



Disassembly of the Shieldin Complex by TRIP13

Prabha Sarangi^{a,b}, Connor S. Clairmont^{a,b}, and Alan D. D'Andrea^{a,b}

^aDepartment of Radiation Oncology, Dana-Farber Cancer Institute, Boston, MA, USA; ^bCenter for DNA Damage and Repair, Dana-Farber Cancer Institute, Boston, MA, USA

ABSTRACT

In the past decade, the study of the major DNA double strand break (DSB) repair pathways, homologous recombination (HR) and classical non-homologous end joining (C-NHEJ), has revealed a vast and intricate network of regulation. The choice between HR and C-NHEJ is largely controlled at the step of DNA end-resection. A pro-C-NHEJ cascade commencing with 53BP1 and culminating in the newly discovered REV7-Shieldin complex impedes end resection and therefore HR. Importantly, loss of any component of this pathway confers PARP inhibitor resistance in *BRCA1*-deficient cells; hence, their study is of great clinical importance. The newest entrant on the scene of end resection regulation is the ATPase TRIP13 that disables the pro-C-NHEJ cascade by promoting a novel conformational change of the HORMA protein REV7. Here, we tie these new findings and factors with previous research on the regulation of DSB repair and HORMA proteins, and suggest testable hypotheses for how TRIP13 could specifically inactivate REV7-Shieldin to promote HR. We also discuss these biological questions in the context of clinical therapeutics.

ARTICLE HISTORY

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KEYWORDS

Keywords REV7; Shieldin; TRIP13DNA repair; homologous recombination; PARP inhibitor; HORMA protein; fanconi anemia

Many human cancers, such as ovarian, breast, prostate, and pancreatic cancers, have mutations in homologous recombination (HR) pathway genes. The most commonly altered HR genes are *BRCA1* and *BRCA2*, followed by other genes in the Fanconi Anemia (FA) pathway. Loss of HR causes genomic instability, hyper-dependence on alternative DNA repair mechanisms, and enhanced sensitivity to PARP inhibitors (PARPi) [1,2]. This synthetic lethal interaction with PARPi has been exploited therapeutically, most notably in ovarian cancer, where several PARPis have recently received FDA approval. PARPis are oral drugs and relatively well-tolerated, and they can be combined with other anti-cancer therapies. Unfortunately, PARPi resistance has emerged as a vexing clinical problem for the treatment of *BRCA1/2*-deficient carcinomas.

The most prevalent mechanism of PARPi resistance is secondary events, such as somatic reversion of a germline *BRCA1/2* mutation, leading to restoration of HR repair [3,4]. Another important mechanism of HR restoration in *BRCA1*-deficient cells involves the loss of factors which normally inhibit 5′–3′ DNA end resection at a double

strand break (DSB), an important first step in HR (Figure 1a) [5]. The first gene identified in this pathway was 53BP1, whose loss was found to restore viability in *BRCA1*^{−/−} mice and render *BRCA1*-deficient cancers resistant to DNA damaging treatment, including PARPi [6,7]. Subsequent work showed that upon DNA damage, 53BP1 is phosphorylated by the DNA damage responsive kinase ATM at numerous sites which recruit the downstream effector proteins, RIF1 and PTIP, to promote classical non-homologous end joining (C-NHEJ), a DSB repair pathway that competes with and is more error-prone than HR [8–11]. Loss of RIF1 and PTIP also induces PARPi resistance in *BRCA1/2*-deficient backgrounds, similarly to loss of 53BP1. In a surprising discovery, the small DNA polymerase ζ subunit, REV7, was uncovered as an essential downstream effector of RIF1, independently of any known Pol ζ-related binding partners [12,13].

The Polymerase ζ complex, consisting of REV7 and REV3, has long been known to catalyze mutagenic translesion DNA synthesis (TLS), a pathway that allows cells to replicate DNA across damaged bases [14]. All of the catalytic activity of the complex

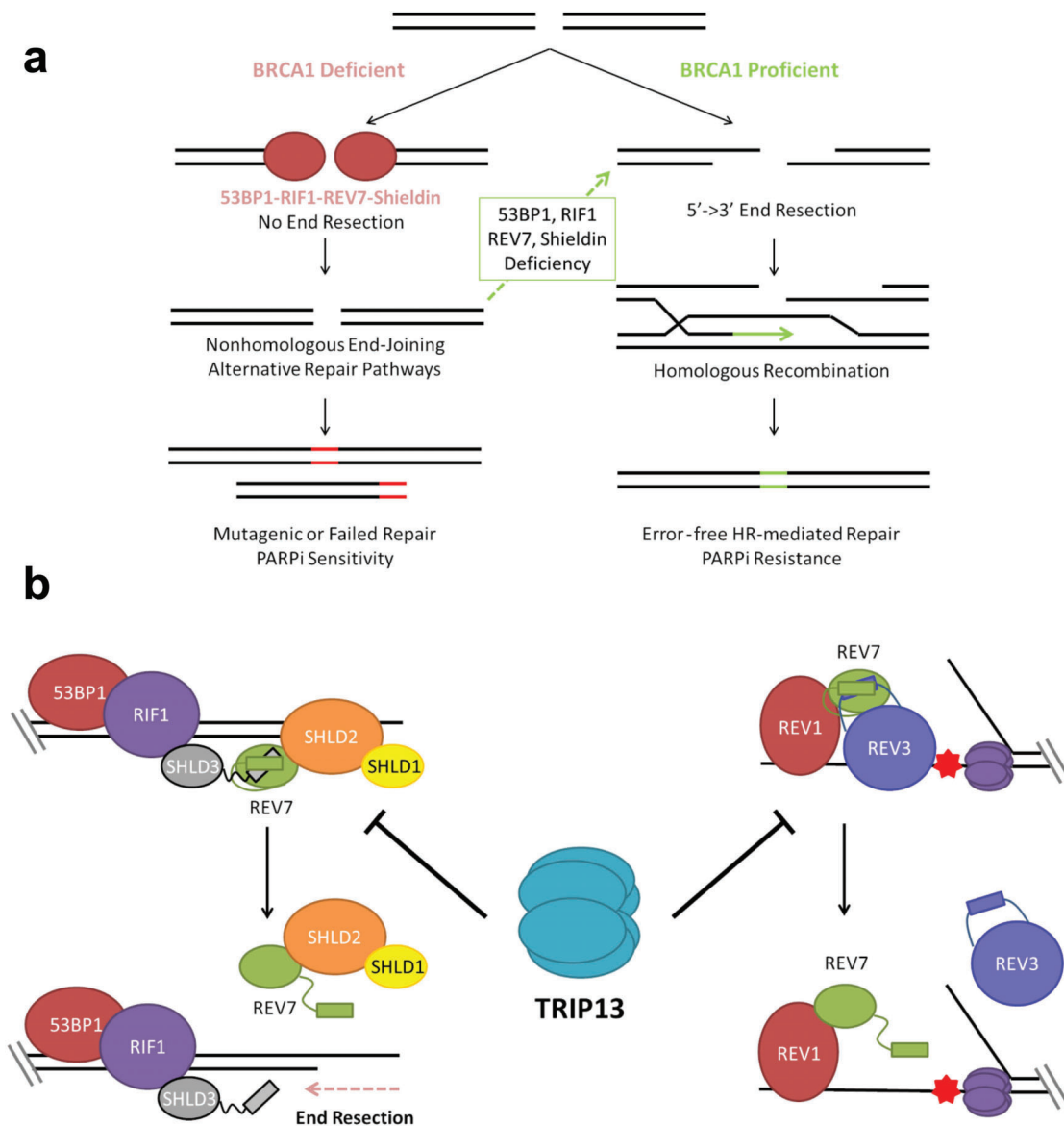


Figure 1. TRIP13 promotes PARP inhibitor sensitivity by disassembling REV7-Shieldin. (a) BRCA1 promotes 5′–3′ DNA end resection, the critical first step in double strand break (DSB) repair through homologous recombination. In the absence of BRCA1; 53BP1, RIF1, REV7 and Shieldin inhibit DNA end resection, preventing repair by HR. End resection and HR can be restored in *BRCA1*-deficient cells by concurrent deficiency of any component of this anti-resection axis. Without HR repair, cells are unable to properly repair all DSBs, particularly those that arise during S phase leading to hypersensitivity to PARP inhibition and other DNA damaging therapies. (b) The Shieldin complex assembles at the break site through sequential recruitment of 53BP1, RIF1, SHLD3, REV7 and SHLD1–SHLD2 (left). REV7 is also recruited to sites of replication blockages, where it interacts with REV1 and REV3 as part of the DNA Polymerase ζ complex (right). TRIP13 catalyzes an inactivating conformational change of REV7, thereby inhibiting both the Shieldin complex and the Polymerase ζ complex.

is contained within the large REV3 subunit, and REV7 has primarily been considered an accessory subunit that may function only to bridge the interaction between Pol ζ and the TLS factor, REV1 [15].

Given the known function of REV7 as an accessory subunit, these studies strongly implied the existence of novel REV7 interaction partners

mediating its newly found role in HR regulation. Indeed, a flurry of studies using complementary techniques of CRISPR screening and physical interactome analysis identified three novel interaction partners – SHLD1, SHLD2 and SHLD3 [16–23]. Together with REV7, these four proteins form a complex known as Shieldin. The entire cascade,

starting with 53BP1 and culminating in the Shieldin complex, inhibits end resection-dependent HR and promotes C-NHEJ, such that loss of any of them in the absence of BRCA1 relieves the inhibition and allows HR to occur. This is clinically relevant because these doubly-deficient cells are resistant to PARPi.

Our laboratory has recently demonstrated that tumor cells can also achieve a state of PARPi resistance by upregulating the expression of the ATPase, TRIP13, and that this mode is linked to REV7 [24]. Importantly, *BRCA1*-deficient tumor cells often have high TRIP13 expression, leading to PARPi resistance. We showed that REV7 exists in active “closed” and inactive “open” conformations (Figure 1b). TRIP13, through ATP hydrolysis, catalyzes an inactivating conformational change in REV7. In doing so, it dissociates the REV7-Shieldin complex, promoting end resection and thereby HR. TRIP13 similarly disassembles the REV7-REV3 TLS complex. Thus, TRIP13 overexpression simultaneously inactivates mutagenic TLS and C-NHEJ and promotes high fidelity HR. Importantly, since TRIP13 is an enzyme, an inhibitor of TRIP13 may be useful in re-sensitizing *BRCA1*-deficient tumor cells to PARPi. Despite some of the more obvious biological and clinical implications of TRIP13 overexpression, such as increased HR and the acquisition of PARPi resistance, there are several unanswered questions regarding its regulation and mechanism of action. This review addresses emerging questions in the field of regulation of end resection, with a brief background on the better known apical players and a detailed look at the newest and most downstream components, REV7 and TRIP13.

Regulation of the 53BP1-RIF1-REV7-Shieldin anti-resection pathway

As end resection is a critical determinant of DSB repair pathway and outcome, it is not surprising that nearly every participating factor is regulated. 53BP1, the apical component of the anti-resection pathway, has ubiquitin binding [25] and methyl-lysine binding domains [26,27], both of which aid in its recruitment. The DNA damage specificity of 53BP1 recruitment is thought to be mediated by its binding to H2AK15 ubiquitylation, catalyzed by the DNA damage responsive ubiquitin ligase,

RNF168 [28]. RNF168 recruitment is in turn regulated by a DNA damage cascade comprising ATM, MDC1 and RNF8 [29–33]. The ATM kinase further phosphorylates 53BP1 itself, which is required for the recruitment of RIF1, REV7 and Shieldin.

The methyl-lysine binding tudor domains bind specifically to methylated histone H4K20, a ubiquitous histone mark [34]. This interaction is normally inhibited by the protein TIRR, which binds directly to the tudor domain, but is released upon DNA damage [35,36]. It has been suggested that H4K20 methylation accumulates slowly following histone deposition on newly replicated DNA, thus favoring 53BP1 binding to unreplicated DNA [37,38]. This is a sensible model, as HR requires the presence of a sister chromatid, and should therefore be repressed in unreplicated DNA. 53BP1 is also inhibited by phosphorylation during M-phase [39–41], when it can cause aberrant DNA repair. It is unclear whether this aberrancy is related to unscheduled resection, which can occur to some degree during M-phase [42].

Only recently have we learned of the downstream REV7-Shieldin components, named so due to their functional and sequence level homology to the telomeric Shelterin complex. Like Shelterin, the Shieldin complex localizes to DNA ends and binds to single stranded DNA (ssDNA), preventing resection, accumulation of the ssDNA binding protein RPA and subsequent ATR signaling. However, unlike Shelterin, which exhibits sequence-specific DNA binding, Shieldin is recruited to DNA damage through 53BP1-RIF1 interaction, with SHLD3 acting as a bridge between RIF1 and REV7, which in turn recruits SHLD1 and SHLD2. Nothing is known about the regulation of these downstream proteins other than the role of TRIP13 and REV7 conformation, the function and regulation of which still present many mysteries.

The HORMA family of proteins

REV7 is a founding member of the HORMA protein family, so-named for HOP1, REV7 and MAD2 [43]. In addition to the founding members, recent studies have identified the autophagy proteins Atg13 and Atg101 as HORMA family

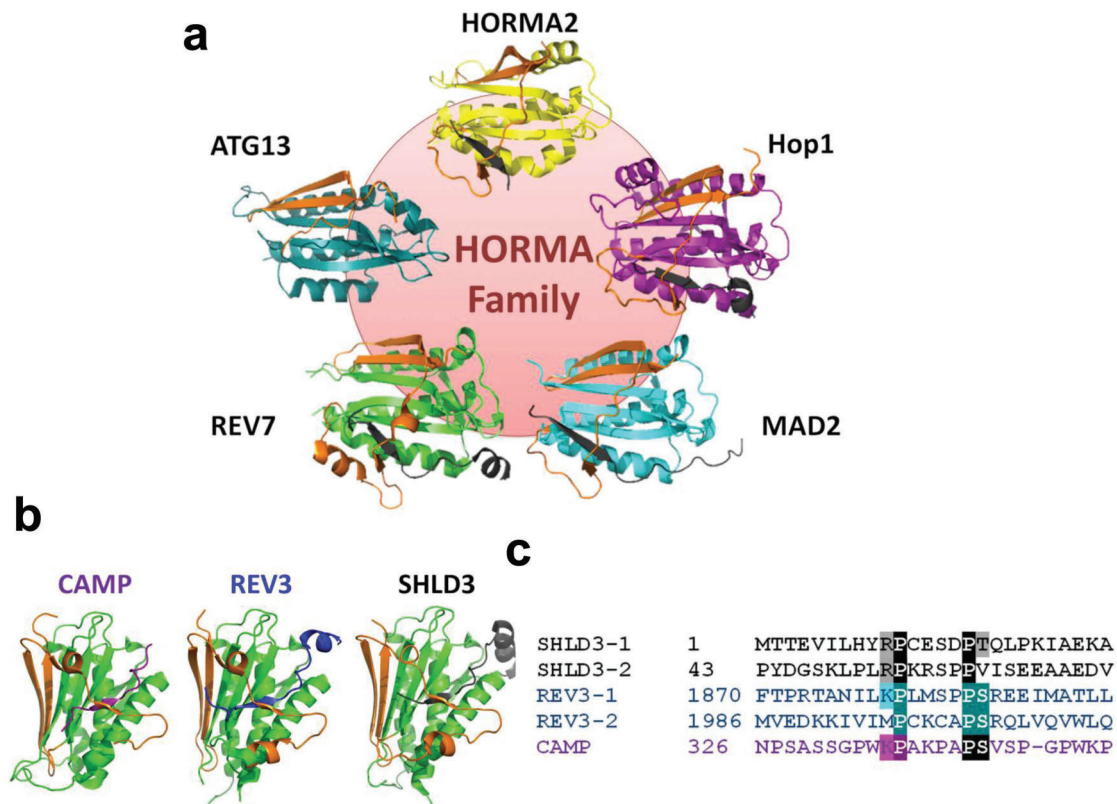


Figure 2. REV7 is a member of the functionally diverse HORMA family and interacts with multiple seatbelt binding partners. (a) The HORMA family is highly structurally conserved across domains of life. Shown are examples of HORMA protein structures in the closed conformation with the seatbelt subdomain highlighted in orange; clockwise from top: HORMA2 – *P. aeruginosa*, PDB: 6P8 U [47]; HIM-3 (Hop1 orthologue) – *C. elegans*, PDB: 4TZJ [60]; MAD2 – *S. pombe*, PDB: 4AEZ [86]; REV7 – *H. sapiens*, PDB: 3VU7 [87], ATG13 – *H. sapiens*, PDB: 5C50 [55]. (b) Structures of closed REV7 bound to seatbelt binding partners: CAMP, REV3 and SHLD3. PDB: 5XPT [62], 3VU7 [87], 6K07 [88] respectively. (c) Primary sequence alignment of REV7 seatbelt binding regions of SHLD3, REV3 and CAMP highlighting conserved amino acids constituting the REV7 seatbelt binding motif (SBM).

members [44–46]. Intriguingly, HORMA proteins and a TRIP13 orthologue have recently been identified in bacteria, suggesting that this family has ancient origins [47]. Proteins of the HORMA family are unified by modest primary sequence homology, but striking homology in tertiary structure (Figure 2a).

Like REV7, Hop1, MAD2 and the bacterial HORMA proteins activity are also regulated by stable conformational changes between open and closed conformations [47–49]. The major structural transition between the open and closed conformation occurs in the C-terminal “seatbelt” subdomain. In the closed conformation, the seatbelt latches tightly to binding partners at so-called seatbelt binding motifs (SBMs), whereas this interaction is not stable in the open conformation. It is this critical binding to SBM-containing partners that is controlled by HORMA conformational

transitions. Intriguingly, Atg13 and Atg101 seem to have non-canonical HORMA function as they appear to be locked in the closed and open states respectively and form a heterodimer [46].

While the HORMA proteins share structural and regulatory features, they function in very distinct capacities. MAD2 plays a clearly defined role in the spindle assembly checkpoint (SAC). During mitosis, pools of inactive, open MAD2 are activated at unattached kinetochores through seatbelt-SBM interactions with MAD1 [50–52]. MAD1 promotes the closing of MAD2 which is propagated in a chain reaction by dimerization of open and closed MAD2 conformers [53,54]. It is noteworthy that the Atg13-Atg101 conformationally-locked heterodimer, resembles the asymmetric closed-open MAD2 dimer [55], suggesting that asymmetric HORMA protein dimers may be a common feature of regulation. The target of

MAD2 is the anaphase promoting complex (APC) activator, Cdc20, another SBM-containing protein [56,57]. Closed MAD2 rapidly binds and sequesters Cdc20, preventing APC activation until each chromatid is attached to the spindle.

Hop1 is a conserved member of the meiosis-specific chromosome axis complex, which organizes homologous chromosomes and promotes crossover formation [58,59]. Unlike MAD2, which forms discrete soluble MAD2:Cdc20 complexes, Hop1 and the related mammalian proteins, HORMAD1 and HORMAD2, appear to form large chromosome-bound chains. Hop1-type proteins have both a HORMA domain and SBMs, allowing for the formation of complex higher-order structures with one Hop1 monomer's seatbelt bound over the adjacent Hop1 monomer's SBM and so on [60].

REV7 is unique among the HORMA proteins in having multiple distinct SBM-containing partners (Figure 2b,c). SHLD3 has two tandem SBMs in its N-terminus, similar to REV3 which has two functionally important SBMs [61]. It is not known if REV7 can bind to both SBMs simultaneously or if this is functionally relevant. In addition to the well-characterized interaction with REV3 and to a lesser extent SHLD3, the zinc-finger protein CAMP is also a confirmed seatbelt interactor [62]. High-throughput interaction profiling of REV7 has revealed numerous additional binding partners, but whether any of these are also seatbelt-mediated remains to be elucidated [17,24]. Why REV7 is involved in these distinct processes is not understood, but is suggestive of some sort of coordinated regulation of REV7-dependent pathways. MAD2 also has two seatbelt-mediated interaction partners, namely MAD1 and Cdc20; however there is a clear distinction, as MAD1 stimulates MAD2 closing, while Cdc20 can only be bound following MAD1 activity, and both these partners are related to the same (and only) function of MAD2. Whether the known REV7 binding partners can play activator functions, analogous to MAD1, or are mainly targets of REV7 activity, like Cdc20, is not yet clear. It is also not known whether REV7 activation is similarly propagated through asymmetric dimerization, like with MAD2. REV7 homodimerization through a similar interface has recently been shown to be functionally important [63], lending support to

this hypothesis; however, more extensive research is still required.

One unifying feature of the HORMA family of proteins is their regulation by the AAA+ ATPase TRIP13. Like REV7, Hop1 and MAD2 are both known to be inactivated by TRIP13 which is thought to remodel closed conformers into the inactive open conformation [64–66]. The involvement of TRIP13 in at least three very distinct cellular processes raises several interesting questions. Is TRIP13 activity somehow directed to specific substrates in the appropriate context, such as through substrate-specific adaptor proteins? Or is the regulation of these seemingly distinct proteins actually coordinated, perhaps at specific cell cycle phases? For instance, TRIP13 may regulate the choice between HR and NHEJ during S phase, and the activity of the SAC in mitosis.

Regulation of TRIP13 activity on HORMA proteins

TRIP13 is a highly conserved AAA+ ATPase that forms single homohexameric rings. It was first linked to the HORMA protein family when studies in yeast revealed a role in meiotic checkpoint and recombination [67–69]. This role is evolutionarily conserved as both yeast Pch2 and mouse Trip13 regulate their respective HORMA substrates, Hop1 and Hormad1 [64,68]. In both organisms, Hop1/Hormad1 monomers bind to DNA in a cooperative manner, and addition of Pch2/Trip13 alters their DNA binding or chromatin association [64,70]. Thus, Pch2/Trip13 has been proposed to remodel Hop1/Hormad1 oligomers already on chromatin, promoting their displacement from DNA and turnover. How Pch2 is targeted to Hop1 is not entirely clear, but it has been shown to interact with the BRCT domains in Xrs2, part of the MRX (Mre11-Rad50-Xrs2) damage sensor in yeast (MRN or MRE11-RAD50-NBS1 in humans), and this interaction is functionally important [68]. Mouse *Hormad1* mutants lacking the N terminal region are refractory to TRIP13 action, indicating that this region is required for the disassembly of HORMAD1 from chromatin.

How TRIP13 is targeted to and inactivates MAD2 is better understood. Like in the case of

Hormad1, the highly conserved N-terminal region of MAD2 is necessary for TRIP13-mediated inactivation [71]. Elegant structural studies have provided insight into why this is so. TRIP13 grasps the N terminus of MAD2, which is fed through a central pore. ATP hydrolysis by TRIP13 causes a limited unfolding of this region of MAD2, which destabilizes the overall closed MAD2 structure by disrupting a conserved hydrogen bond network between residues at the N-terminus and C-terminal seatbelt domain [71]. Interestingly, while TRIP13 can elicit this effect alone, its activity on MAD2 is greatly enhanced by another HORMA-like protein, p31, which directly interacts with both TRIP13 and MAD2, forming a bridge between them [65,72] (Figure 3). p31 binds to MAD2 at the latter's homodimerization interface, and this structural mimicry further attenuates MAD2 signaling by preventing activation through the asymmetric closed-open dimerization pathway [73]. Whether p31 similarly coordinates the interaction of TRIP13 and REV7 is unknown.

Models for TRIP13 activity toward REV7

Given the very recent discovery of TRIP13 action on REV7, we know little about the mechanism and regulation of this interaction; however, using evidence from other HORMA proteins, we can suggest several testable hypotheses. One critical question is whether there is any mechanism for TRIP13 substrate specificity or if all substrates present in the cell are regulated concurrently. A possible mechanism of substrate specificity is through adaptor proteins like p31, which has only been definitively shown to interact with MAD2. If there are alternative p31-like adaptors for REV7 and HORMAD1, this could dictate substrate specificity (Figure 4, Model 1). There is some evidence suggesting that REV7 does in fact interact with p31, contradicting this model; however, more research is required on this topic [63]. The potential universality of the p31 adaptor is also supported by reports that the rice orthologue, CRC1, localizes to the chromosome axis and is implicated in meiotic HR [74],

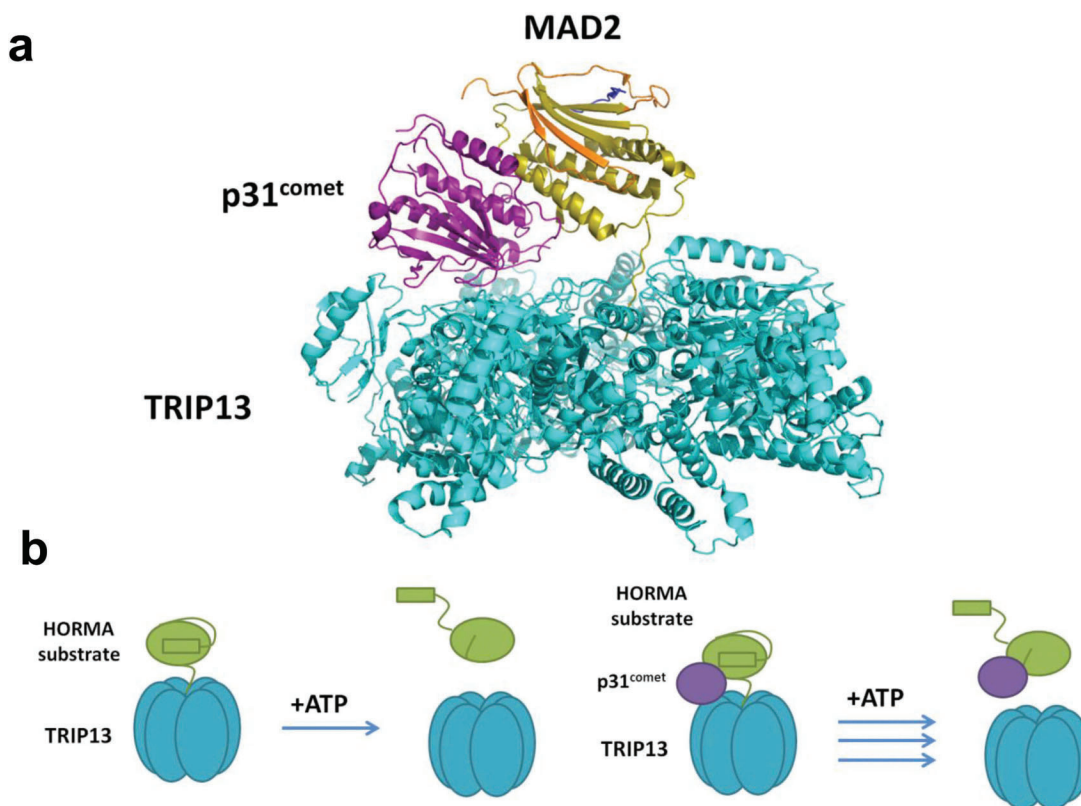


Figure 3. TRIP13 remodels HORMA substrates through N-terminal engagement and is stimulated by an adaptor protein. (a) Cryo-EM structure of TRIP13 acting on C-MAD2, showing bridging by the adaptor protein, p31^{comet}, PDB: 6FOX [89]. (b) Schematic of N-terminal engagement of HORMA substrate by TRIP13 and stimulation by an adaptor, p31^{comet}, which bridges the interaction. Following engagement of the HORMA protein, TRIP13 catalyzes HORMA remodeling coupled to ATP hydrolysis.

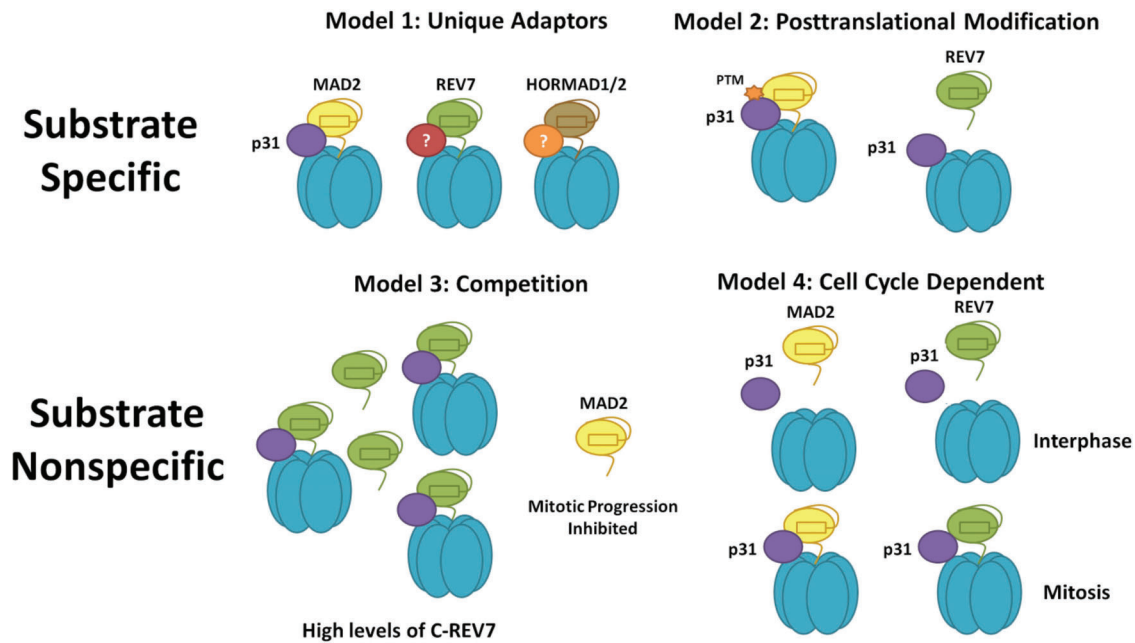


Figure 4. Models for TRIP13 regulation of diverse HORMA proteins. Substrate Specific Models (1): Substrate specificity is enforced by unique adaptors for each HORMA protein; (2): Substrate specificity is enforced by post-translational modification of the appropriate substrate. Substrate Nonspecific Models (3): Each HORMA protein competes for a limited supply of TRIP13-p31. High levels of DNA damage would thereby inhibit anaphase progression through TRIP13-p31 sequestration. C-REV7 indicates closed REV7; (4): TRIP13 acts on all substrates during a specific cell cycle phase or phases. TRIP13 is known to be active in mitosis and may represent another mechanism to suppress DNA repair in mitosis.

suggestive of a HORMAD1 regulatory role. On the other hand, there are some organisms, such as budding yeast, that possess TRIP13 but not p31, and in these cases, TRIP13 appears to have only a meiotic function through Hop1 [75]. If p31 is in fact a universal adaptor, substrate specificity may instead be dictated through posttranslational modifications of TRIP13, p31 or the HORMA substrate (Figure 4, Model 2). Indeed, Plk1-dependent phosphorylation of p31 decreases its affinity for MAD2, thereby dialing down TRIP13-mediated inactivation of MAD2 [76]. Perhaps DNA damage-dependent phosphorylation of p31 or REV7-Shieldin by ATM similarly modulates TRIP13 activity to promote certain types of repair.

It is also possible that TRIP13 does not show substrate specificity; however, this does not mean that TRIP13 activity is not regulated. TRIP13 activity could be controlled by the relative abundance of the closed conformations of each substrate, such that they compete for limited TRIP13-p31 (Figure 4, Model 3). This would have interesting consequences as high levels of DNA damage, and therefore closed REV7,

would effectively sequester TRIP13, preventing inactivation of the SAC. This would represent a novel mechanism of cell cycle arrest in response to DNA damage.

Yet another possibility is that TRIP13 and p31 are sufficiently abundant to prevent any competition. In this case, they would regulate all of their HORMA substrates concurrently. It is noteworthy that p31 protein expression is up-regulated specifically in S and G2/M phases, when REV7 and MAD2 activities need to be regulated. Thus, cells could specifically promote the efficient inactivation of REV7 and MAD2 by limiting p31 expression to these cell cycle phases (Figure 4, Model 4). Apart from these possibilities but perhaps related to them, TRIP13 cellular localization could dictate its activity. Support for this idea comes from studies showing that TRIP13 is normally cytoplasmic, but forms foci at kinetochores during mitosis [77] and on chromosome axes during meiosis [78]. Whether TRIP13 similarly forms foci after DSB induction that co-localize with DNA damage factors would be useful to pinpoint when and where TRIP13 inactivates REV7.

Clinical Implications of the TRIP13-REV7 Interaction

TRIP13 activity is not only biologically interesting, it is also clinically relevant. Previous studies have noted that TRIP13 is overexpressed in various cancers and is a negative prognostic marker [79–81]. One report examining TRIP13 in the context of head and neck cancer similarly found that TRIP13 promotes DSB repair and chemoresistance, but concluded that TRIP13 promotes C-NHEJ, not HR [82]. That study identified a physical interaction between TRIP13 and DNA-PK subunits; however, these are common contaminants that appear in nearly 50% of AP-MS experiments [83]. Additionally, there are no HORMA domains in DNA-PK and no mechanism was proposed for TRIP13 regulation of DNA-PK. It remains possible that TRIP13 affects C-NHEJ in the cancer cell lines used in that study; however, our results demonstrate that the major function of TRIP13 in DSB repair is the promotion of HR through REV7 inactivation. Interestingly, biallelic loss-of-function TRIP13 mutations have been seen in Wilms tumor cells, which not surprisingly have an SAC defect [84]. It would be clinically useful to establish whether they are additionally HR-deficient, and therefore sensitive to PARPis.

Alternative Roles of TRIP13 in Regulating Genomic Stability

TRIP13 overexpression has previously been proposed to engender genomic instability by weakening the SAC, allowing cells to replicate more quickly and become aneuploid, promoting oncogenesis [79]. This idea has been challenged by the finding that TRIP13 expression is highly correlated with that of MAD2, suggesting a homeostatic relationship between the two in maintaining a normal SAC [85]. Given the genomic proximity of TRIP13 to hTERT, a strong oncogene, TRIP13 amplification may often occur as a passenger mutation. Regardless of the mechanism of TRIP13 overexpression in cancer, our studies have shown that the consequences are clear: high TRIP13 levels enhance HR and engender chemoresistance.

While elucidating PARP inhibitor resistance mechanisms through the discovery of 53BP1, RIF1, and the Shieldin complex has been a significant advance, none of those factors are druggable. TRIP13, as the newest entrant to this field and an enzymatic, negative regulator of the Shieldin complex, emerges as a critical player whose inhibition could be used in the clinic to extend the utility of PARP inhibitors.

Disclosure statement

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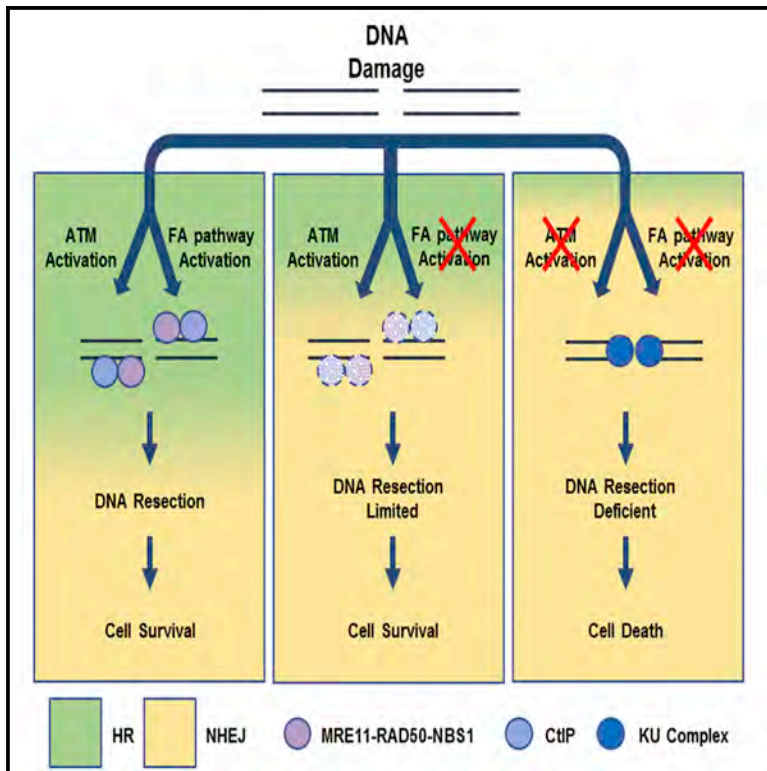
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Cooperation of the ATM and Fanconi Anemia/BRCA Pathways in Double-Strand Break End Resection

Graphical Abstract



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In Brief

ATM inhibitors are currently in clinical development as anti-cancer agents. Using a genome-wide CRISPR screen, Cai et al. demonstrate that cancer cells with Fanconi anemia (FA) pathway deficiency are sensitive to ATM inhibition. The authors also show that synthetic lethality between ATM and the FA pathway is due to reduced DNA resection and increased toxic NHEJ.

Highlights

- Genome-wide CRISPR screen identifies determinants of ATM inhibitor sensitivity
- Loss of Fanconi anemia (FA) pathway and ATM is synthetic lethal
- ATM is required in FA-deficient cells to promote DNA end resection and HR repair
- Loss of FA pathway and ATM results in accumulation of toxic levels of NHEJ



Cooperation of the ATM and Fanconi Anemia/BRCA Pathways in Double-Strand Break End Resection

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SUMMARY

Cells deficient in ataxia telangiectasia mutated (ATM) are hypersensitive to ionizing radiation and other anti-cancer agents that induce double-strand DNA breaks. ATM inhibitors may therefore sensitize cancer cells to these agents. Some cancers may also have underlying genetic defects predisposing them to an ATM inhibitor monotherapy response. We have conducted a genome-wide CRISPR screen to identify genetic vulnerabilities that sensitize lung cancer cells to ATM inhibitors. Knockout of genes in the Fanconi anemia (FA)/BRCA pathway results in hypersensitivity to the ATM inhibitor M3541. Knockdown of either an FA gene or of ATM results in reduced double-strand break end resection, enhanced non-homologous end joining (NHEJ) repair, and decreased homologous recombination repair. Knockout of both the FA/BRCA pathway and ATM strongly inhibits end resection and generates toxic levels of NHEJ, thereby elucidating a mechanism of cellular death by synthetic lethality. ATM inhibitors may therefore be useful for the treatment of tumors with a defective FA/BRCA pathway.

INTRODUCTION

The ataxia telangiectasia mutated (ATM) kinase is a master regulator of the DNA damage response (DDR) (Shiloh and Ziv, 2013), and multiple ATM substrates have been identified. Mutations in the ATM gene result in hypersensitivity to ionizing radiation (IR) and to other DNA damaging agents. ATM coordinates the activity of two DNA double-strand break (DSB) repair pathways, namely, the homologous recombination (HR) pathway and the non-homologous end joining (NHEJ) pathway. Mutations in the ATM gene result in discordant activity of these pathways, persistent DSBs, and genomic instability. Consistent with these cellular phenotypes, humans or mice with biallelic ATM mutations are predisposed to cancers (Swift and Su, 1999).

Since ATM plays such a central role in the DDR, pharmacologic inhibition of ATM is an attractive opportunity for cancer therapy (Batey et al., 2013; Hickson et al., 2004). Nonspecific drugs, such as wortmannin, inhibit ATM and cause radiosensitization; however, this agent also targets other members of the PIKK (phosphatidylinositol-3-kinase-like kinase) family, including DNA-PK (DNA-dependent protein kinase) and ATR (ataxia telangiectasia and Rad3-related protein). KU-55933, another ATM inhibitor (ATMi) (Batey et al., 2013), does not target other PIKKs at effective concentrations; however, early clinical trials have not progressed due to off-target side effects. A recent compound, M3541, is a highly specific and potent inhibitor of ATM (Zimmermann et al., 2018), and a National Cancer Institute (NCI) clinical trial was recently initiated with the drug in combination with radiotherapy (NCT03225105).

Human cancers with an underlying defect in one DNA repair pathway may be hyperdependent on a second pathway. For example, BRCA1/2-deficient tumors with a defect in the HR pathway are hyperdependent on a polyADP ribose polymerase (PARP1)-dependent repair pathway. Accordingly, PARP1 inhibition has proven clinical efficacy in BRCA1/2 mutant ovarian and breast cancer (Bryant et al., 2005; Farmer et al., 2005), and four PARP1 inhibitors have now been granted limited FDA approval. Whether tumors with a deficiency of the ATM pathway also have specific synthetic lethal relationships with other DNA repair pathways remains largely unknown.

In the current study, we performed a genome-wide clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 loss-of-function genetic screen to identify enhancers of cell killing by the ATMi M3541 (Zimmermann et al., 2018). Notably, single guide RNAs (sgRNAs) directed against multiple genes in the Fanconi anemia (FA)/BRCA pathway (Ceccaldi et al., 2016b; Rodríguez and D'Andrea, 2017)—including FANCA, FANCF, and FANCG—and FA-like genes—including FAAP24, FAAP100, and FANCM—were synthetic lethal with ATM. sgRNAs directed against additional DNA repair genes including PTEN, Rad18, and STAG2 also conferred ATMi hypersensitivity. These genes are not FA genes per se, but their knockdown has previously been shown to confer DNA crosslinker sensitivity. Concurrent knockdown of both the FA/BRCA pathway and the ATM pathway resulted in reduced DSB end resection, hyperactivation of NHEJ repair, and enhanced chromosome aberrations, thereby



elucidating a mechanism of the synthetic lethality. Inhibition of NHEJ activity in tumor cells with a dual disruption of the FA/BRCA and ATM pathways restored cell survival. Taken together, our results suggest that the FA/BRCA pathway and the ATM pathway play redundant functions in enhancing DSB resection and suppressing NHEJ. We hypothesize that human tumors with an underlying defect in the FA/BRCA pathway may be hypersensitive to ATMis. Moreover, understanding the potential sensitivity and resistance mechanisms of ATMis may be useful in predicting patient responses, designing ATMi therapy combinations, and implementing mechanisms of re-sensitization.

RESULTS

Identification of sgRNAs That Selectively Enhance or Suppress Cell Killing by ATMi M3541

Initially, we evaluated A549 and NCI-H460 (H460) non-small cell lung cancer cell (NSCLC) lines, which stably express the Cas9 endonuclease. Using a clonogenic assay, we analyzed the cytotoxicity resulting from a combination of M3541—or of another ATMi, KU59333—with IR in these cells (Figures S1A and S1B). In accordance with previous studies, minimal toxicity was observed from ATMi exposure alone, with concentrations ranging up to 1.0 μ M of drug. Enhanced cytotoxicity, however, was observed upon subsequent exposure to 2–6 Gy doses of IR. IR is known to rapidly activate the phosphorylation of ATM at Ser1981 (Bakkenist and Kastan, 2003). Low doses of M3541 or KU59333, in the micromolar range, resulted in a decrease in pS1981 over 2 h post-drug exposure in both the A549 and H460 cell lines (Figure S1C).

We next devised a genome-wide CRISPR screen to identify genes that, when disrupted, enhance or suppress cell killing by the ATMi M3541 (Figure 1). We reasoned that sgRNAs directed against specific genes would either enhance or suppress cell killing by M3541. The screen was conducted in the A549 and H460 cell lines, in the presence or absence of the ATMi M3541. The A549 and H460 cell lines are wild type (WT) for both ATM and the pro-apoptotic gene, p53.

In order to identify sgRNAs that target genes involved in both sensitivity and resistance to M3541, we chose a moderate concentration of the drug that inhibits the phosphorylation of ATM at Ser1981 at a low concentration (1 μ M). Cells were infected with the Brunello v2 lentiviral sgRNA library and grown for 19 days in media treated with DMSO or the ATMi M3541 (Figure 1A). Fresh media, with freshly dissolved drug, were added every 3 days. Cells were collected pre- and post-drug selection, genomic DNA was extracted, and sgRNA sequences were PCR amplified and Illumina sequenced. Top hits among gene knockouts that conferred drug sensitivity or resistance were identified.

To identify highly significant hits, we utilized a STAR score at ≥ 4 when analyzing a single cell line. We included hits with a STAR score at ≥ 2 when analyzing the two cell lines together. The screen identified 62 genes in the A549 cell line and 38 genes in the H460 cell line that modulated cell killing by M3541. Out of these hits, we identified 30 genes that were hits in both cell lines. Many hits corresponded to specific signaling pathways (Figure 1B), and the top hits were DNA repair pathways. Gene Ontology analysis of 70 gene sets revealed strong enrichment for biological processes linked to the FA/BRCA pathway, a

DNA crosslink repair pathway composed of 23 known FA genes (Ceccaldi et al., 2016b; Rodríguez and D'Andrea, 2017). Mapping the 70-gene set on the HumanMine protein-protein interaction database created a highly connected network consisting of multiple DNA damage repair genes, including most of the members of the FA/BRCA pathway (Figure 1C).

Many of the same genes that modulate M3541-mediated cell killing were identified in both lung cancer cell lines (see Figures 1D [scatterplot] and S1D [volcano plots] for the A549 and H460 cell lines). Top hits included FAAP24, FAAP100, FANCF, FANCE, FANCI, FANCA, FANCG, FANCB, FANCC, and FANCD2. Other relevant top hits included RAD18, an E3 ligase known to monoubiquitinate and activate FANCD2 (Williams et al., 2011); the tumor suppressor gene PTEN (McCabe et al., 2015); and the cohesin protein STAG2 (Watrin and Peters, 2009). These genes have also previously been shown to regulate DNA repair processes.

Several genes that regulate DNA DSB end resection were also hits in the screen. Knockdown of CtIP, a known phosphorylation target of ATM (Duquette et al., 2012), and knockdown of EXO1 were synthetic lethal with the ATMi (Figure 1D, lower left quadrant). While CtIP is not an FA gene, per se, it is known to bind to FANCD2 (Murina et al., 2014; Unno et al., 2014; Yeo et al., 2014). Knockdown of either FA/BRCA genes or of nucleases involved in DSB end resection, such as MRE11, conferred cellular hypersensitivity to the ATMi. Accordingly, our results implied that the FA/BRCA pathway and the ATM pathway may directly or indirectly regulate the DSB end resection through interactions with resection nucleases. Moreover, knockdowns of components of the ATM activating MRN complex (Wang et al., 2013), including Rad50 and NBS1, were also hits in the screen.

Interestingly, sgRNAs directed against components of the BRCA1-A complex (including BABAM1, BABAM2, and ABRAXAS1) were shown to increase the growth of both A549 and H460 cells grown in the presence of the ATMi (Figures 1D [upper right quadrant] and S1D). This functional interaction of ATM with subunits of the BRCA1-A complex has been recently demonstrated (Balmus et al., 2019). The BRCA1-A complex is known to suppress HR repair (Balmus et al., 2019; Harris and Khanna, 2011; Sobhian et al., 2007). The loss of this pathway may enhance the HR and increase resistance to ATM inhibition. This finding further supports an underlying role of ATM in enhancing HR repair. Finally, sgRNA directed against ATM itself protected cells treated with M3541. This result further validates the screen and provides further evidence that the ATMi is specific and is acting through its specific target. Loss of a drug target is a common mechanism of acquired drug resistance (Pettitt et al., 2018).

Concomitant Loss of the FA/BRCA Pathway and ATM Function Is Toxic to Cells

We previously demonstrated that a small interfering RNA (siRNA) directed against the FA protein FANCG is synthetically lethal with ATM loss (Kennedy et al., 2007). Whether ATM knockdown is also synthetically lethal with the loss of other genes in the FA/BRCA pathway is unknown. We next validated the CRISPR screen results by CRISPR knockout of individual FA genes and of FA-associated genes FAAP24 (Figures S2A and 2A), FAAP100 (Figures S2A and 2B), and FANCF (Figures S2A and 2C) in A549 and H460 cells. Protein knockdown by CRISPR

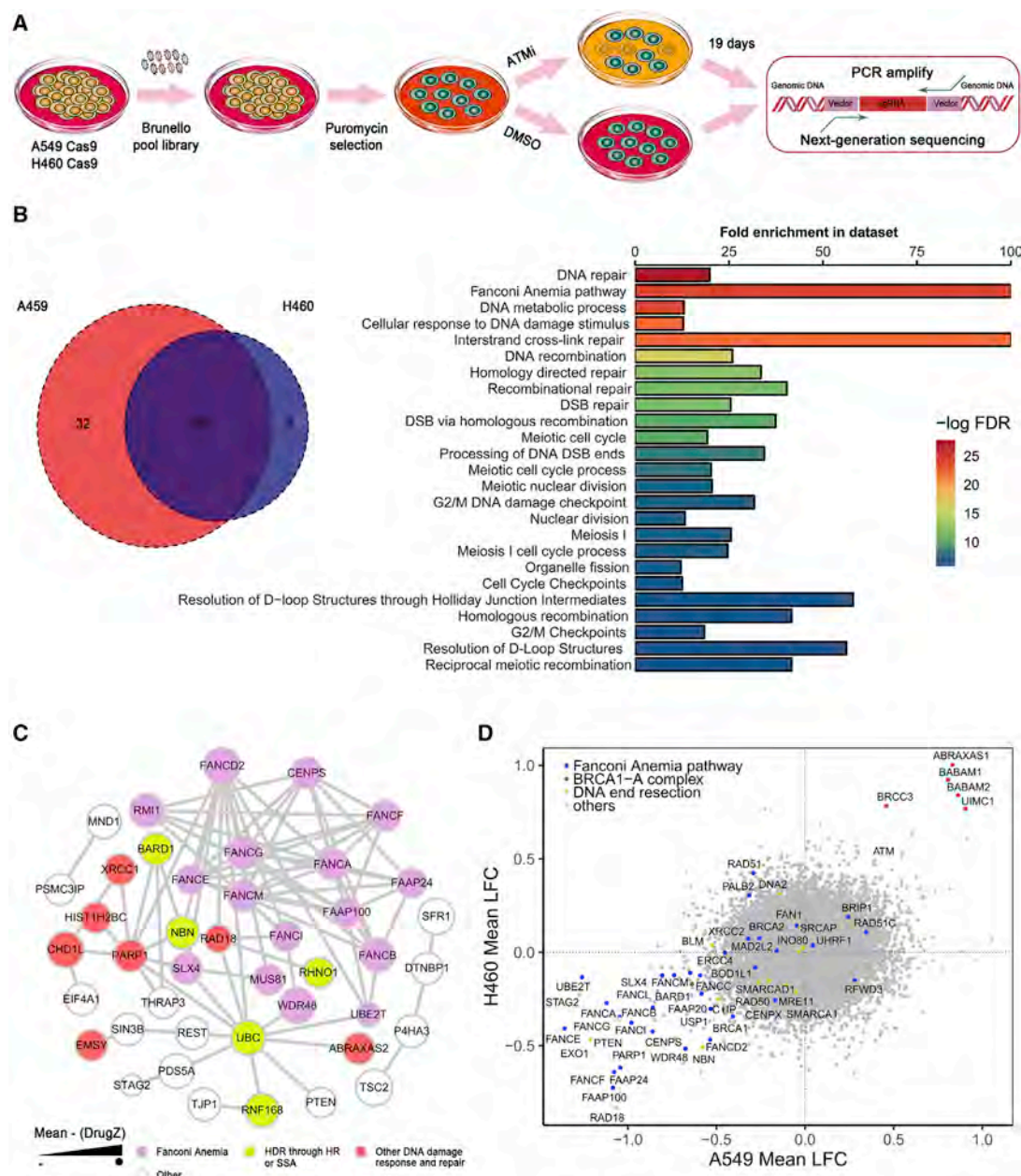


Figure 1. Whole Genome-wide CRISPR Screens Identify Determinants of ATMi Sensitivity

(A) Schematic of the genome-wide CRISPR-Cas9 screens in lung cancer lines.

(B) Venn diagram of all high-confidence hits in the two cell lines (left panel: STAR score at ≥ 4 in one line and STAR score at ≥ 2 in both lines). Gene Ontology (GO) terms significantly enriched in hits common to the two-cell lines (right panel).

(C) easyN network analysis of interactions between hits common to the two cell lines.

(D) Gene-centric visualization of average log₂ fold change (LFC) in ATMi (M3541)- versus DMSO-treated in both cell lines.

See also Figure S1.

knockout was confirmed by immunoblotting with the corresponding antibodies. As expected, knockout of these individual genes resulted in enhanced sensitivity to the ATMi. We next validated synthetic lethality relationships between the ATM and these genes along with RAD18 and PTEN (Figures S2B and S2C). The siRNA-mediated knockdown of FAAP24, FAAP100,

FANCF, RAD18, or PTEN in A549 and NCI-H460 cells resulted in cellular sensitivity to the ATMi, as predicted by the CRISPR screen results. We next determined whether the synthetic lethality relationship of FA genes and ATM could be extended to other cell types. Indeed, knockout of the FA genes FANCA and FANCD2 in HeLa and U2OS cells, respectively, conferred

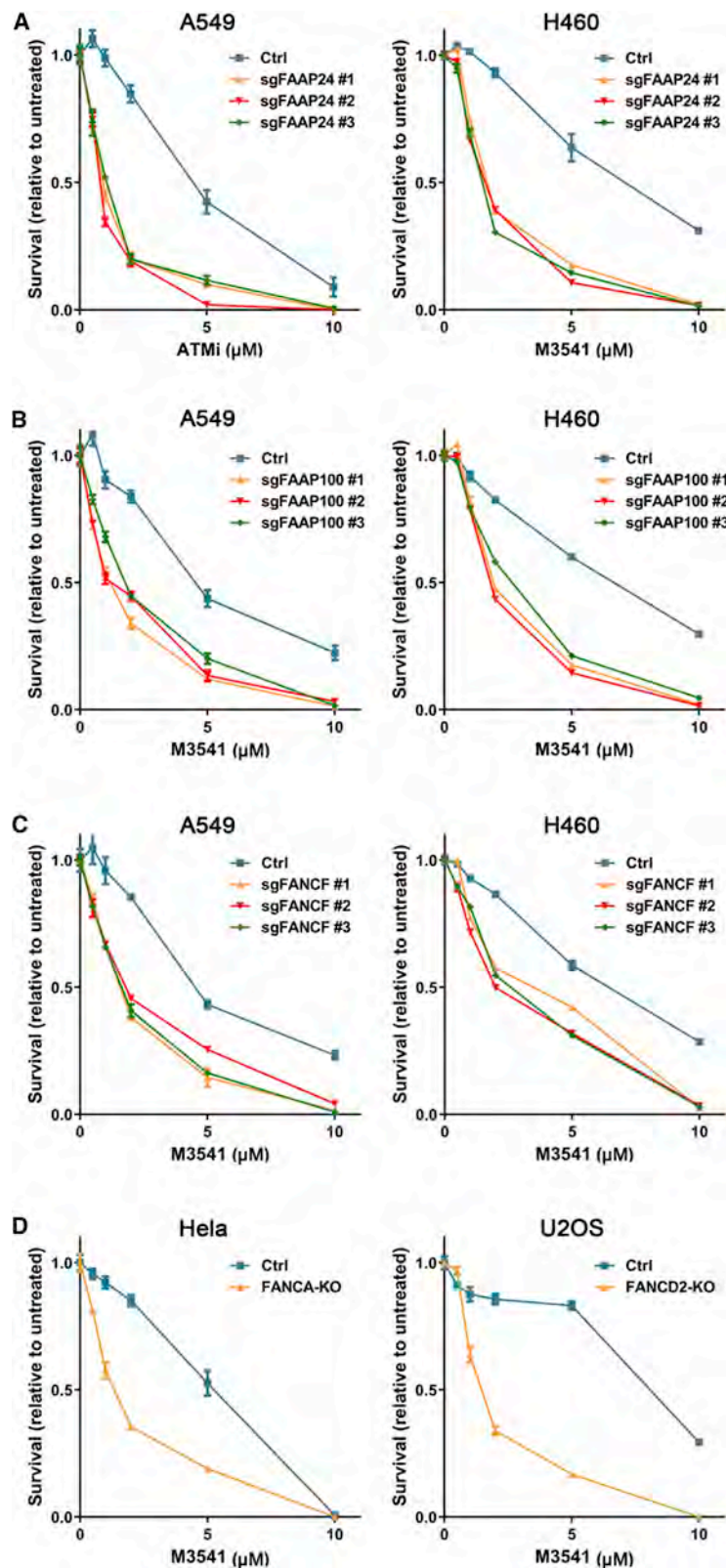


Figure 2. Loss of FA Pathway Confers Sensitivity to ATM Inhibition in Cancer Cells

(A–C) Clonogenic assays of control and knockout (KO) cell pools after treatment with ATM inhibitor (M3541) following transduction with FAAP24- (A), FAAP100- (B), and FANCF-targeting (C) sgRNAs (3 sgRNAs per gene). Survival assays in A549 cells (left panels) and H460 cells (right panels). #1, #2 and #3 indicate three independent sgRNAs for targeting each gene.

(D) Survival assays of HeLa and U2OS cells after treatment with M3541 in stable clones of FANCA and FANCD2 knockout via CRISPR-Cas9. Clonogenic survival assays in HeLa cells (left panel) and U2OS cells (right panel). Data are shown as mean $\pm$ SEM from three independent experiments.

See also [Figure S2](#).

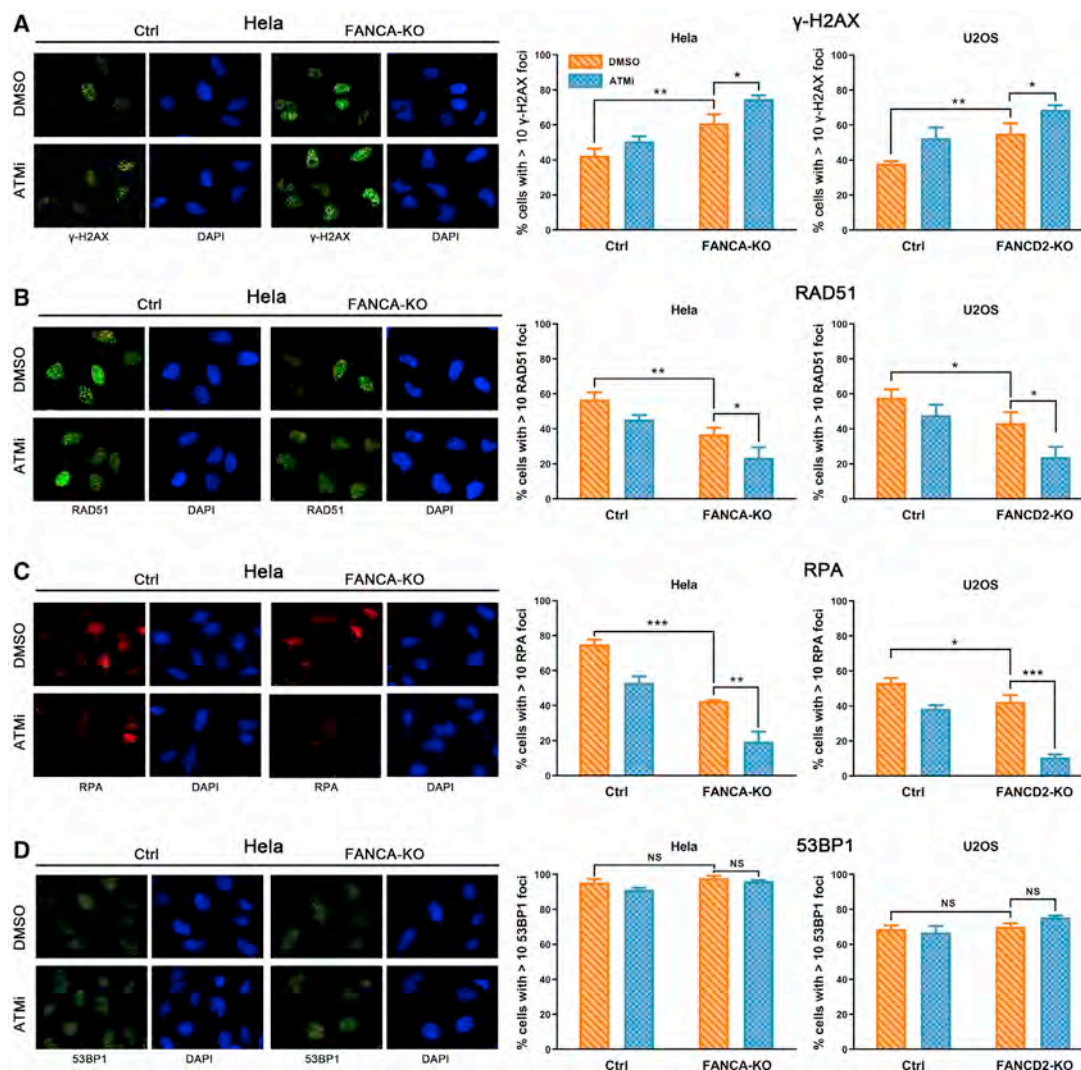


Figure 3. Inhibition of ATM Impairs RAD51 and RPA Focus Formation and Results in DNA Damage in FA-Deficient Cells

Immunofluorescence and quantification of the percentage of cells with more than 10 γ -H2AX (A), RAD51 (B), RPA (C), and 53BP1 (D) foci 3 h after treatment with irradiation (5 Gy) following 24 h exposure to DMSO or M3541 (1 μ M) in control and FANCA-KO cells: representative images (left panels) and graphical quantitation of foci in HeLa cells (middle panels) and U2OS cells (right panels). One hundred nuclei were randomly counted. Data are shown as mean $\pm$ SEM from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 on unpaired two-tailed Student's t test. See also Figure S3.

sensitivity to the ATMi, further validating the CRISPR screen results and extending the data (Figures S2A and 2D).

Mechanism of Synthetic Lethality between the FA Pathway and ATM Pathway

To determine the mechanism of synthetic lethality in cells with a dual knockdown of the FA pathway and ATM, we examined various DNA repair biomarkers (Figure 3). Initially, we examined HeLa cells or U2OS cells that have a CRISPR-engineered knockout of either FANCA or FANCD2, respectively. Knockout of FANCA or FANCD2 in either cell line resulted in increased DNA DSB, as measured by IR-induced H2AX phosphorylation (Figure 3A). Breakage was enhanced by exposure to ATMi, perhaps resulting from slower DNA repair kinetics. In a comet

assay, we also observed longer tails, reflecting increased DNA damage following treatment with the ATMi in the FA-deficient HeLa and U2OS cell lines (Figures S3A and S3B).

We next determined whether the loss of a gene in the FA pathway could confer a reduction in HR repair in these cell lines. Previous studies have indicated that FA gene knockdown can result in a mild reduction in both HR activity and single-strand annealing (SSA) (Nakanishi et al., 2005). Indeed, FANCA or FANCD2 knockout in either HeLa cells or U2OS cells resulted in decreased IR-induced RAD51 foci (Figure 3B). The level of RAD51 focus formation is a measure of BRCA2/RAD51 loading and HR competence. Interestingly, exposure of FANCA-deficient or FANCD2-deficient cells to the ATMi M3541 resulted in a further loss of RAD51 foci (Figure 3B). Taken together, these

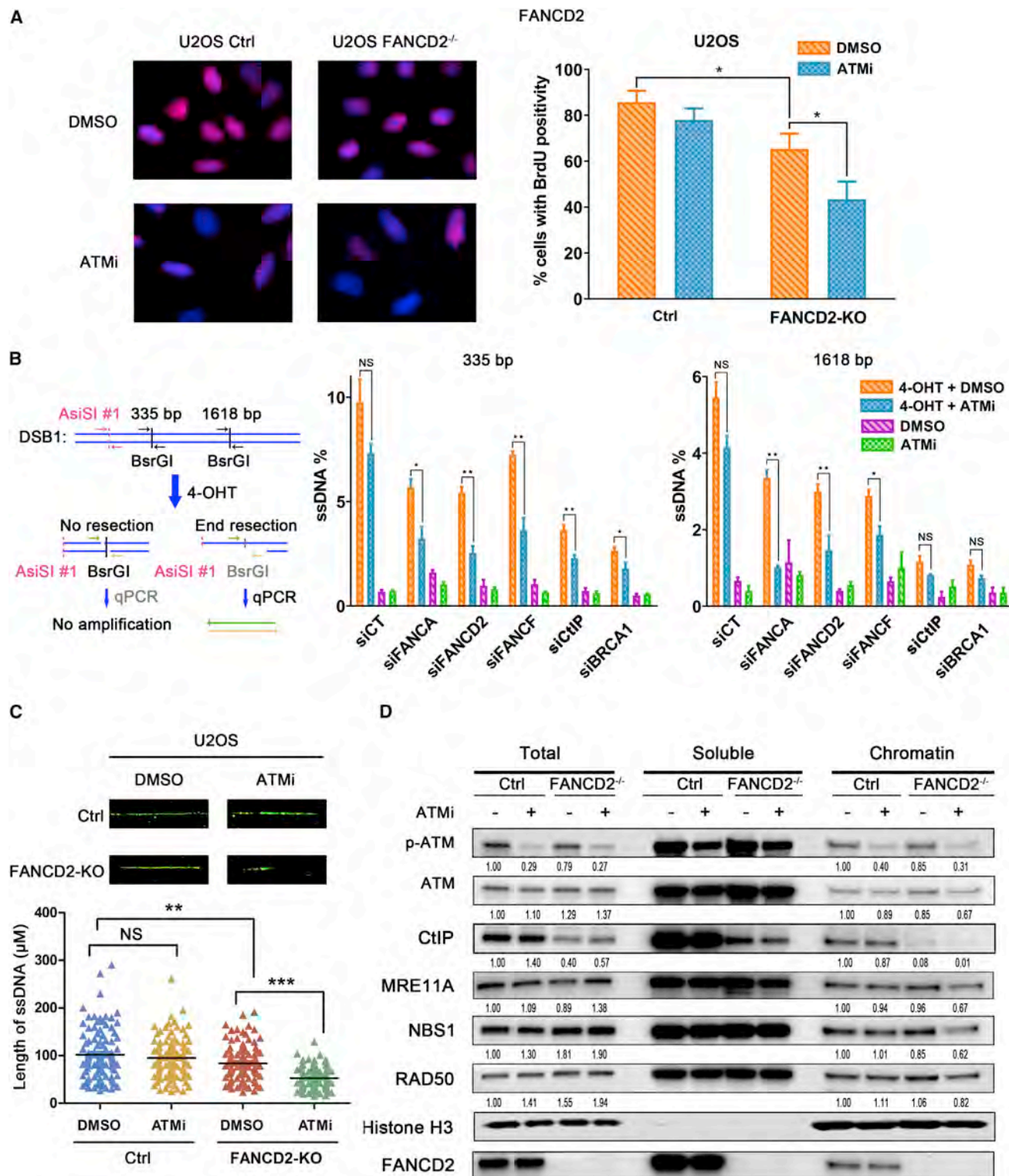


Figure 4. Inhibition of ATM Decreases DNA End Resection Machinery in FA-Pathway-Deficient Cells

(A) Immunofluorescence and quantification of the percentage of cells with BrdU-positive cells 6 h after treatment with irradiation (5 Gy) following 24 h exposure to DMSO or M3541 (1 μM) in control and FANCD2-KO U2OS cells: representative images (left panel) and graphical quantitation of BrdU-positive cells (right panel). One hundred nuclei were randomly counted. *p < 0.05, unpaired two-tailed Student's t test. Data are shown as mean ± SD from three independent experiments.

(legend continued on next page)

results indicate that the FA pathway and the ATM pathway each contribute positively to HR repair. We hypothesized that the loss of both pathways would cause a profound loss of HR repair, further contributing to the lethal phenotypes observed when both pathways are compromised.

Knockdown of FANCA or FANCD2 resulted in decreased DNA DSB resection and decreased single-stranded DNA (ssDNA) generation, as demonstrated by decreased IR-induced replication factor A (RPA) foci (Figure 3C). The decrease in RPA foci following FA gene knockdown suggests that the FA pathway—through its interaction with various resection proteins, such as CtIP and EXO1—can positively regulate the process of end resection and HR repair. In contrast, changes in IR-induced 53BP1 foci were not observed when comparing vehicle control and ATMi treated FANCA^{-/-} or FANCD2^{-/-} cells (Figure 3D).

FANCA-deficient cells exhibited enhanced ATM phosphorylation (Figure S3C), consistent with the synthetic lethality observed when both pathways are disrupted. This increased pATM level suggests that in the absence of the FA/BRCA pathway, cells are hyperdependent on activation of the ATM pathway for survival. Similarly, the loss of ATM resulted in hyperactivation of the FA/BRCA pathway, as detected by elevated FANCD2 monoubiquitination (Figure S3D). Again, these results further support a synthetic lethal relationship of these two pathways. When one pathway is knocked down, the other pathway is upregulated.

Knockout of the FA and ATM Pathways Reduces DSB End Resection, Increases End Joining, and Increases NHEJ

Recent studies demonstrate that the cellular choice between HR repair and NHEJ repair of DSBs is determined by the process of DSB end resection (Ceccaldi et al., 2016a; Symington, 2016). Increased resection favors HR, while the blockage of resection favors NHEJ. We next evaluated the role of the FA and ATM pathways in end resection by using several independent assays.

End resection of a DSB by nucleolytic degradation of the 5' strand is the first step in initiating the HR pathway. The exposed 3' single-strand DNA binds to RPA, which in turn is displaced by the recombinase, RAD51. Previous studies have shown that the ATM kinase can promote DSB end resection by phosphorylating and activating the protein CtIP (Duquette et al., 2012). Other studies have independently shown that monoubiquitinated FANCD2, the downstream regulator in the FA pathway, can recruit CtIP to chromatin and further promote DSB end resection (Murina et al., 2014; Unno et al., 2014; Yeo et al., 2014).

To directly determine the role of FA and ATM in DSB resection, we labeled genomic DNA with 5-bromo-2'-deoxyuridine (BrdU) and used an antibody that detects ssDNA resulting from end resection. Knockout of FANCD2 decreased BrdU-positive cells,

and inhibition of ATM with M3541 further impaired IR-induced BrdU focus formation in FANCD2^{-/-} cells (Figure 4A).

To further quantify the extent of resection, we introduced the AsiSI enzyme fused with a hormone-binding domain in U2OS cells with either FANCD2 or FANCF knockdown. A quantitative PCR (qPCR)-based method was used to examine ssDNA (Figure 4B). A decrease in the amount of ssDNA was found after the depletion of FANCF or of FANCD2. A further decrease in the level of ssDNA was observed following the depletion of both the FA/BRCA pathway and the ATM pathway.

Next, we performed a SMART (single-molecule analysis of resection tracks) assay to further evaluate the effect of FA pathway disruption and/or ATM inhibition on DSB end resection (Figures 4C and S4A). This assay quantifies the amount of ssDNA generated by end resection at a defined DSB (Huertas and Cruz-García, 2018). Disruption of the FANCD2 gene resulted in a minor reduction in DSB end resection, as did exposure to the ATMi. Interestingly, dual disruption of the FA/BRCA and ATM pathways resulted in a further decrease in DSB end resection, consistent with the enhancement of toxic NHEJ activity observed when both pathways are knocked down. Moreover, complementation with FANCD2 in FANCD2-KO cells rescued this phenotype (Figure S4B). Consistent with previous studies, these results indicate that the FA/BRCA pathway normally promotes DSB end resection, leading to a suppression of NHEJ. We next determined whether the FA pathway is required for the recruitment of CtIP to chromatin and whether ATM further promotes this recruitment (Figures 4D and S4C). Interestingly, knockout of FANCD2 (Figure 4D) or of FANCA (Figure S4C) reduced CtIP expression (lanes 3 and 4) and chromatin recruitment of CtIP (lanes 11 and 12), and ATMi further reduced CtIP recruitment. CtIP knockdown resulted in reduced survival of WT U2OS cells. CtIP knockdown resulted in increased toxicity, however, in FANCD2 knockout U2OS cells, suggesting a synthetic lethality between CtIP and FANCD2. Overexpression of CtIP rescued the lethality in both the WT and FANCD2 knockout U2OS cells (Figure S4D). Taken together, these results further demonstrate that the FA and ATM pathways cooperate in enhancing CtIP mediated end resection.

Knockdown of the FA Pathway Results in Increased NHEJ Activity and Decreased HR Activity

Previous studies have suggested that NHEJ is upregulated in cells deficient in the FA pathway (Adamo et al., 2010; Eccles et al., 2018; Pace et al., 2010). Knockdown of components of the NHEJ pathway, such as DNA-PK (Adamo et al., 2010) or DNA ligase 4 (Maruyama et al., 2015), reduces the interstrand crosslinker (ICL) sensitivity of FA cells. Recent studies also indicate that NHEJ is upregulated in ATM-deficient cells (Balmus et al., 2019), suggesting that a principal cellular function of

(B) The schematic of *in vitro* end-resection assay in U2OS cells (left panel). ssDNA was quantified by qPCR at 335 bp or 1,618 bp downstream of the AsiSI-induced break site after indicated siRNA transfection and DSB induction (with 4-hydroxytamoxifen) or no DSB induction (without 4-hydroxytamoxifen) (right panel). Data are shown as mean $\pm$ SEM compiled from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

(C) Change in ssDNA length of control and FANCD2^{-/-} U2OS cells 3 h after treatment with irradiation (5 Gy) following overnight exposure to 1 μ M ATMi. Representative images of SMART assay (top panel) and the quantification (relative to untreated, bottom panel). In total, 100 DNA fibers were assessed per condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired two-tailed Student's *t* test. NS, not significant (bottom panel).

(D) Immunoblots of the indicated proteins in subcellular fractions of control and FANCD2^{-/-} U2OS cells after treatment with 1 μ M ATMi for 6 h. Quantification of the ratio of total and chromatin bound CtIP in control versus FANCD2^{-/-} U2OS cells is shown. See also Figure S4.

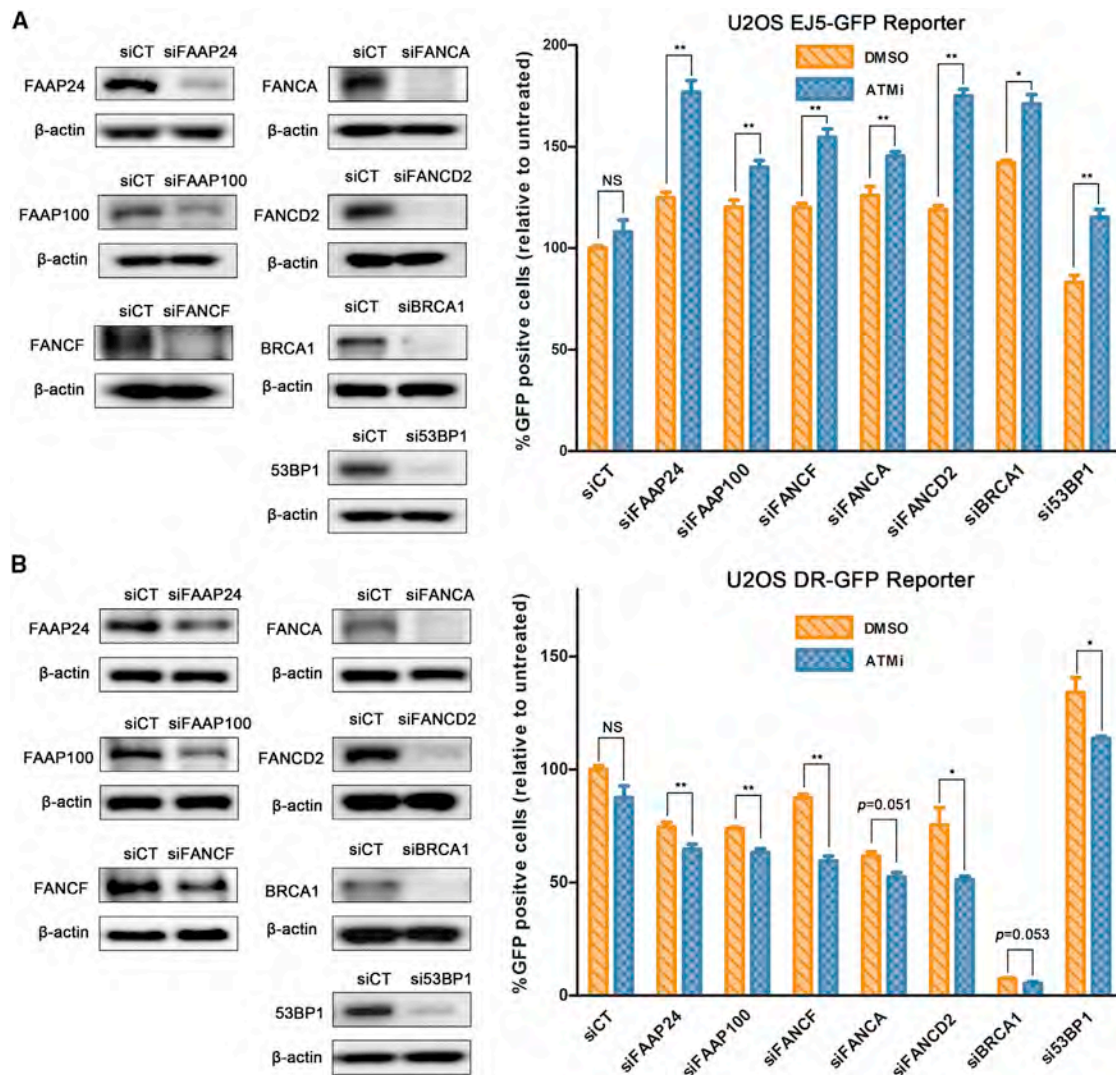


Figure 5. Inhibition of ATM Results in Increased NHEJ and Decreased HR in FA-Pathway-Deficient Cells

U2OS cells stably carrying the EJ5-GFP (A) and DR-GFP (B) reporter were transfected with the indicated siRNAs and were treated with 1 μ M ATMi overnight 36 h after siRNA transfection. Subsequently, the cells were exposed to I-SceI adenovirus for 6 h and then cultured with fresh medium. GFP-positive cells were quantified 72 h later by FACS. siRNA knockdown efficiency was assessed by western blotting assay (left panels). Quantitation for the HR activity and NHEJ activity as % of GFP positive cells is shown (right panels). Data are shown as mean $\pm$ SEM from three independent experiments. * p < 0.05 and ** p < 0.01 on unpaired two-way ANOVA test. See also Figure S5.

ATM is to suppress the NHEJ pathway and to promote DSB end resection and HR. Knockdown of the HR-suppressing BRCA1-A complex can enhance resistance to ATMi (Balmus et al., 2019).

Based on these studies and our evidence of synthetic lethality between FA and ATM, we reasoned that the mechanism of synthetic lethality is a decrease in DSB end resection, an increase in end joining, and an increase in toxic NHEJ following FA and ATM knockdown. Previous studies have indicated that either the FA pathway or ATM is required to remove NHEJ proteins, such as 53BP1, from DSBs that have been generated in the process of DNA crosslink repair or by nuclease processing of reversed forks. We reasoned that loss of both the FA pathway and ATM would further inhibit DSB resection, increase end joining, and further upregulate NHEJ.

To further examine whether the ATMi promotes NHEJ, we used an NHEJ reporter template (Figure 5; Howard et al., 2015). Knockdown of components of the FA/BRCA pathway, including BRCA1, FAAP24, or FANCD2, in U2OS cells increased NHEJ activity. Efficient knockdown of the FA proteins was confirmed by immunoblot. Knockdown of FA protein expression resulted in an enhancement of NHEJ activity. Cellular exposure to the ATMi further increased NHEJ activity in these cells (Figure 5A).

Interestingly, knockdown of the FA pathway and ATM resulted in a decrease in HR, as measured by the DR-GFP assay (Figure 5B). As further evidence to support this mechanism, knockout of FANCA in HeLa cells (Figure S5A), or knockout of FANCD2 in U2OS cells (Figure S5B), resulted in increased phosphorylation of DNA-PK (pDNA-PK), a biomarker of the activated

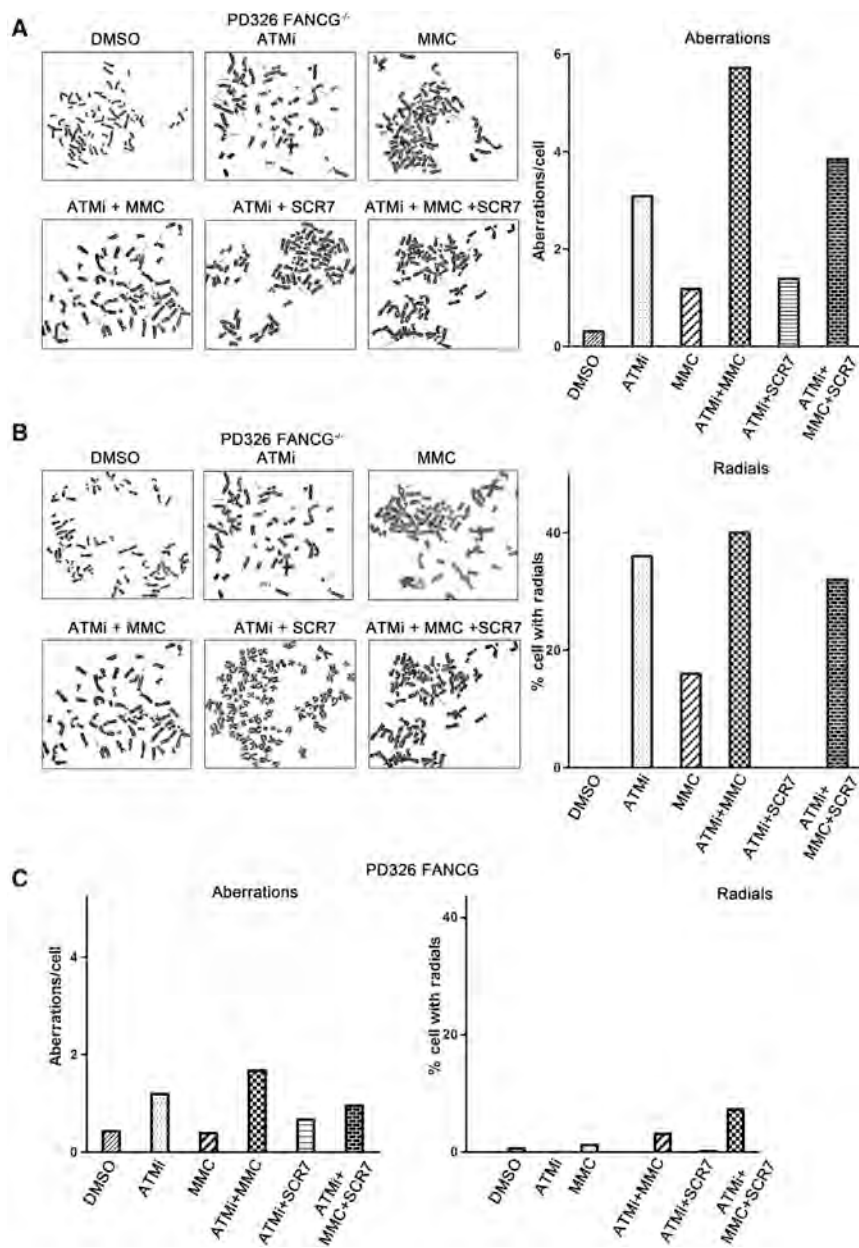


Figure 6. Inhibition of ATM Results in Chromosomal Aberrations and Radials in FA-Pathway-Deficient Cells

(A and B) Representative images (left panels) and quantification (right panels) of chromosomal aberrations (A) and radial chromosomes (B, indicated by arrows) per cell on metaphase spreads of PD326 (FANCG-deficient) cells 48 h after treatment with 5 ng/ml Mitomycin C (MMC) following 24 h exposure to 1 μ M DMSO, 1 μ M ATMi, or 1 μ M ATMi plus 5 μ M Lig4 inhibitor (SCR7). At least 50 cells were counted in each experiment, and the average of 50 cells is shown.

(C) Quantification of chromosomal aberrations (left panel) and radial chromosomes (right panel) per cell as a measure on metaphase spreads 48 h after treatment with 5 ng/ml MMC following 24 h exposure to 1 μ M DMSO, 1 μ M ATMi, or 1 μ M ATMi plus 5 μ M Lig4 inhibitor (SCR7) in PD326 FANCG-corrected cells. See also Figure S6.

DEB (diepoxybutane) or mitomycin C (MMC)-induced increase in chromosome radials. Chromosome radials result from hyperactive non-homologous pairing of chromosomes, indirectly resulting from increased NHEJ activity (Newell et al., 2004). BRCA1-deficient cells exposed to PARP inhibitors exhibit elevated radial chromosomes (Bunting and Nussenzweig, 2010). Knockout of multiple components of the NHEJ pathway, such as the 53BP1 or SHLD complex subunits, in BRCA1-deficient cells results in a reduction of radial chromosomes and in enhanced cell survival (Mirman et al., 2018).

We next determined whether a disruption of ATM activity would further increase NHEJ activity, chromosome aberrations, and radial formation in FA cells (Figure 6). For this purpose, we examined the effect of ATMi exposure on the FANCG-deficient lymphoblast line, PD326. M3541 increased chromosome radials in PD326

NHEJ pathway. The addition of ATMi further increased pDNA-PK and DNA damage, as measured by pH2AX (Figures S5A and S5B). Taken together, these results indicate that the FA pathway and the ATM pathway are non-epistatic, and each suppresses NHEJ activity. Knocking down both pathways resulted in excessive end-joining activity and cellular death, providing additional mechanistic evidence for synthetic lethality.

ATM Is Required for Homologous Recombination Repair in FA/BRCA-Pathway-Deficient Cells

FA-pathway-deficient cells and ATM-deficient cells have a common cellular phenotype, namely, the accumulation of chromosome radial forms. Indeed, the diagnostic test for FA is the

cells, consistent with an enhancement of toxic NHEJ activity. ATMi plus the crosslinker, MMC, further enhanced chromosome radial production. Interestingly, an inhibitor of DNA ligase IV, a downstream enzyme in the NHEJ pathway, reduced chromosome radials, further indicating that the increased radial production resulted from decreased end resection and increased end joining activity (Figures 6A and 6B). As a control, ATMi or MMC exposure did not induce chromosome aberrations or radials in the corrected isogenic cell line, PD326 plus FANCG (Figure 6C). The inhibitor of DNA ligase IV also enhanced clonogenic survival of other FA-pathway-deficient cells, including the FANCA-deficient and FANCD2-deficient cells, when they were exposed to ATMi (Figures S6A and S6B).

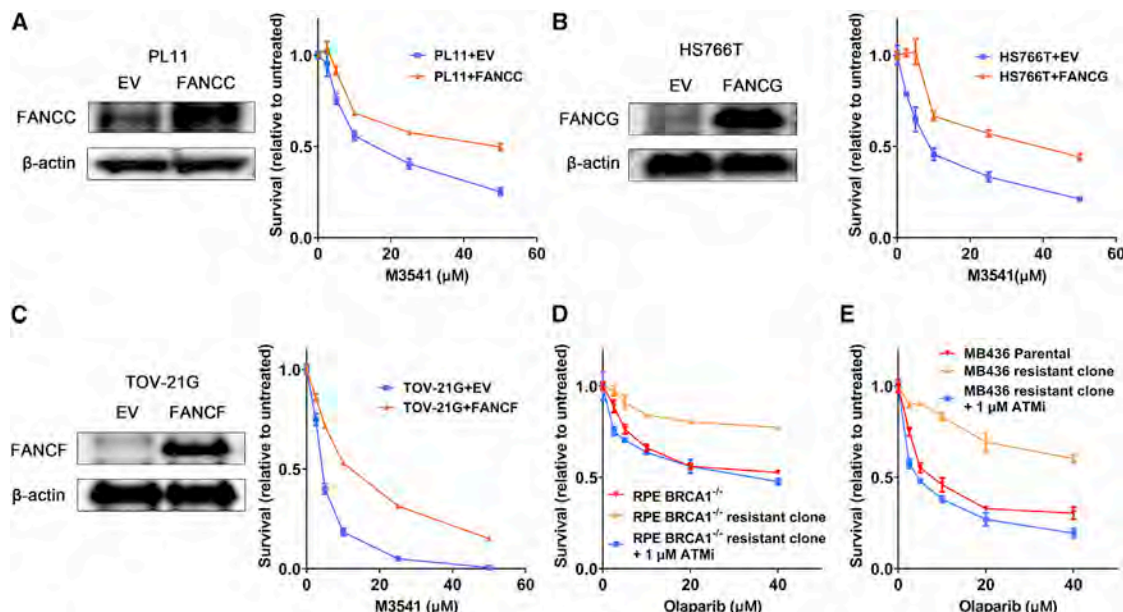


Figure 7. ATM Inhibition Shows Greater Cytotoxicity with FA Pathway Deficiency

(A) Western blot showing FANCC expression (left panel) and quantitation of CellTiter-Glo cell viability assay (right panel) in FANCC mutant PL11 cells and the isogenic FANCC-corrected cells 72 h after M3541 treatment.

(B) Western blot showing FANCG expression (left panel) and quantitation of CellTiter-Glo cell viability assay (right panel) in FANCG mutant HS766T cells and the isogenic FANCG-corrected cells 72 h after M3541 treatment.

(C) Western blot showing FANCF expression (left panel) and quantitation of CellTiter-Glo cell viability assay (right panel) in FANCF mutant TOV-21G cells and the isogenic FANCF-corrected cells 72 h after M3541 treatment.

(D and E) Quantitation of the CellTiter-Glo cell viability assay in BRCA1-KO RPE (D) and MB436 (E) (parental and olaparib-resistant) cells exposed to graded concentrations of the PARP inhibitor olaparib in the presence or absence of the ATMi M3541 (1 μ M). See also Figure S7.

Synthetic Lethality of FA Pathway and ATM in Human Cancer Cell Lines

FA pathway disruption is observed in many human cancers, including pancreatic cancer (van der Heijden et al., 2005) and ovarian cancer (Taniguchi et al., 2003). The FA-pathway-deficient pancreatic cancer cell lines PL11 and HS766T were sensitive to the ATMi. Decreased expression of the FANCC protein in PL11 cells and decreased expression of the FANCG protein in the HS766T cell line were confirmed by immunoblotting with the corresponding antibodies. The cDNA-complemented versions of these cell lines exhibited decreased sensitivity to the ATMi (Figures 7A and 7B). The TOV21G cell line is an ovarian cancer line exhibiting methylation and silencing of the FANCF gene (Taniguchi et al., 2003). An isogenic line TOV21G+FANCF has an intact FA pathway. As predicted, the TOV21G cells were more sensitive to ATMi exposure (Figure 7C) than were the complemented cells. Finally, BRCA1-deficient cells that had acquired resistance to the PARP inhibitor, olaparib, were sensitized by the addition of the ATMi (Figures 7D and 7E).

Validation of Synthetic Lethality of FA and ATM Pathways Using Computational Approaches

Analysis of publicly available tumor and cell line databases was conducted to further validate the synthetic lethal relationship between the FA and ATM pathways. We initially focused on genes that, when lost or mutated, sensitized cells to the

ATMi (M3541) in our CRISPR screen. The gene hits with an average $-\log_{10}(P) < 2.5$ and an average $\log_2$ fold change of at least -0.5 , in both A549 and H460 cells, were RAD18, FAAP24, FAAP100, and FANCF (Figure S1D). Analysis of a cancer-dependency study revealed that cell lines with copy number loss of any one of these four genes, but not cell lines with normal copy number of these genes, are hyperdependent on ATM for survival. (Figure S7A). Moreover, cell lines with mutations in genes encoding proteins in the FA core complex proteins are more dependent on ATM for survival than are the cell lines that are the WT for these genes (Figure S7B).

MMC is a chemotherapeutic agent known to cause DNA crosslinks. Crosslinks are repaired, in part, by the FA pathway. Cells lacking a functional FA pathway are deficient in crosslink repair and are hypersensitive to MMC. As described above, cells lacking the FA pathway are also sensitive to ATMi. We therefore investigated a possible correlation between cellular sensitivity to MMC and ATMi using a drug sensitivity database. Indeed, the cell lines that are sensitive to MMC tend to be more sensitive to the ATMi KU-55933, corroborating the notion that a loss of the FA pathway results in sensitivity to both an ATMi and MMC (Figure S7C).

The synthetic lethal relationship between the FA pathway and ATM described here suggests that cells with defects in both the FA pathway and ATM may not be viable. Tumors with co-occurrence of FA and ATM mutations are therefore hypothesized to

have reduced viability. In order to test this hypothesis, we analyzed clinical data from the PanCancer MSK-IMPACT Clinical Sequencing Cohort. Interestingly, the patients with tumors harboring mutations in both the FA pathway and ATM showed significantly improved survival by a log-rank test, compared to the patients with tumors that are WT for these genes (Figure S7D). Taken together, computational analysis of publicly available databases supports the synthetic lethality between the FA pathway and ATM observed in the CRISPR screen.

DISCUSSION

Cancer cells have multiple DNA repair pathways for coping with intrinsic DNA damage and replication stress. These cells often lose one or more of these pathways through mutation, deletion, or epigenetic silencing, resulting in genomic instability, a mutator phenotype, and a more rapidly evolving malignancy. Cells with one dysfunctional DNA repair pathway may become hyperdependent on an alternative pathway for survival. For instance, tumor cells with a loss of BRCA1 and BRCA2, required for HR repair, are hyperdependent on PARP1-dependent DNA repair mechanisms such as base excision repair (BER) (Lord and Ashworth, 2016) or alternative end joining (Alt-EJ) (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). Accordingly, inhibitors of PARP or other components of the Alt-EJ pathway, such as POLQ (Higgins and Boulton, 2018), may be cytotoxic to HR-deficient tumor cells.

In the current study, we sought to identify genetic vulnerabilities in cancer cell lines that are predisposed to cell death by ATMi monotherapy. We demonstrated that a disruption of the FA/BRCA pathway enhances cell killing by M3541. Further disruption of the terminal components of cNHEJ can reduce the cell killing. Moreover, we demonstrate that one mechanism of the synthetic lethality resulting from a dual disruption of ATM and FA pathway genes is elevated (toxic) cNHEJ activity and decreased HR activity.

Previous studies have suggested that elevated NHEJ activity may account for the cellular features of FA cells. FA cells have elevated ICL sensitivity and an elevation in ICL-induced chromosome radial forms. These radial forms result from enhanced non-homologous pairing of chromosomes, as evident in metaphase chromosome spreads (Newell et al., 2004). Previous studies have also indicated that FA proteins or ATM can function, at least in part, by excluding or removing NHEJ proteins, such as 53BP1, from the sites of DSBs. These breaks may occur during ICL repair or during nuclease processing of reversed replication forks. Loss of both the FA pathway and ATM results in further retention of NHEJ factors at DSBs and persistence of NHEJ activity, extending even into the S phase of the cell cycle. This toxic NHEJ can cause the inappropriate fusion of DNA breaks on different chromosomes. Indeed, the radial chromosomes resulting from fused segments of non-homologous chromosomes resemble the genomic rearrangements found in primary cells from FA patients. Interestingly, this increase in non-homologous pairing correlates with increased NHEJ activity (Adamo et al., 2010; Eccles et al., 2018; Pace et al., 2010). Consistent with this model, a knockdown of NHEJ activity, resulting from cellular exposure to a DNA-PK inhibitor or inhibition of ligase 4, can reverse this phenotype and reduce the ICL sensitivity of FA cells.

ATM is known to promote DSB end resection and suppress NHEJ. The enhancement of DSB end resection results, at least in part, from the ability of ATM to inactivate the 53BP1/RIF1/REV7/SHLD complex, thereby enabling DSB end resection, RAD51 loading, and homologous recombination. Our results indicate that the FA pathway also promotes DSB end resection, although the mechanism of enhanced resection remains unknown. Monoubiquitinated FANCD2, a downstream product of the FA pathway, may bind and stimulate the end resection activity of CtIP. Recent studies also indicate that the FANCA protein can promote the process of SSA (Benitez et al., 2018) and may thereby positively regulate end resection. Accordingly, inactivation of both the ATM and FA pathways appears to further reduce DSB end resection. Inactivation of both pathways simultaneously results in a further decrease in end resection, accounting for the enhancement of NHEJ activity and further contributing to synthetic lethality.

Our results indicate that ATM and the FA pathway cooperate in the regulation of DSB end resection. We previously demonstrated a convergence of this pathway in DNA crosslink repair (Taniguchi et al., 2002). The current study supports a model for the regulation of DSB end resection (Figure S7E). The FA pathway promotes the monoubiquitination and chromatin recruitment of FANCD2. FANCD2-Ub binds to and promotes the chromatin recruitment of CtIP to the site of DSB end resection. ATM further phosphorylates and activates CtIP. Activated CtIP next promotes DSB end resection and suppresses NHEJ. This model provides one of the mechanisms for the synthetic lethal relationship of FA genes and ATM. Loss of both pathways leads to a profound reduction in DSB end resection, leading to toxic NHEJ, as indicated by radial chromosome accumulation.

Our results also suggest that tumor cells may become resistant to an ATMi by finding an alternate mechanism for enhancing DSB end resection, such as by knocking down the BRCA1-A complex or by reducing NHEJ levels. FA cells corresponding to some FA complementation groups also have reduced HR activity and increased PARPi sensitivity. In our study, simultaneous disruption of the FA pathway and ATM resulted in a profound decrease in RAD51 focus formation and cell death, thereby providing a second mechanism of synthetic lethality. Interestingly, the loss of the BRCA1-A complex, a complex known to suppress HR repair, can rescue RAD51 foci, restore HR repair, and rescue cell viability, based on our CRISPR screen results. Accordingly, our CRISPR screen identified components of the BRCA1-A complex, which, when knocked out, enhanced the viability of A549 or H460 cells exposed to the ATMi.

In addition to bona fide members of the FA/BRCA pathway, we identified other genes whose loss is synthetically lethal with ATMi. RAD18 is a ubiquitin E3 ligase known to monoubiquitinate PCNA and to activate TLS (translesion synthesis) repair. Interestingly, RAD18 may also play a role in FA/BRCA pathway function. In one study, RAD18 was shown to promote the FA core complex-dependent monoubiquitination of FANCD2 in a manner that is secondary to its ability to monoubiquitinate PCNA (Song et al., 2010). Moreover, RAD18 was shown to bind to FANCD2 and to promote the monoubiquitination of the FANCD2/FANCI complex (Williams et al., 2011).

The tumor suppressor protein PTEN has also been reported to have a role in the DDR (Gupta et al., 2009; Shen et al., 2007). Loss of PTEN function underlies some cancers, such as prostate cancer and glioblastoma (Li et al., 1997). In addition to its cytoplasmic role in the PI3K pathway, PTEN functions as a nuclear protein and appears to have a direct function in DNA repair. Cells with a deficiency in nuclear targeting of PTEN are defective in HR repair and, like FA cells, are sensitive to DNA crosslinking agents such as cisplatin (Bassi et al., 2013). A recent report further supports a model in which PTEN loss is synthetically lethal with loss of the ATM kinase (McCabe et al., 2015).

STAG2 is a core component of the cohesin complex, which also includes SMC1, SMC2, and Rad21 (Watrin and Peters, 2009). The cohesin complex is required for optimal DNA repair, including DSB repair, in human cells (Terret et al., 2009; Tittel-Eimer et al., 2012). The cohesin complex is known to organize chromatin loops at DNA replication factories, to mediate replication stress tolerance, and to enhance the restart of stalled forks (Wu and Yu, 2012). Somatic mutation of STAG2 in some cancers accounts for crosslinker-dependent chromosome breakage and cisplatin sensitivity, a phenotype similar to cancers with FA/BRCA pathway deficiency (Stoepker et al., 2015). Interestingly, STAG2 also plays a role in replication fork progression and has a synthetic lethal relationship with PARP and ATR loss in DSB repair (Mondal et al., 2019). Taken together, these results are consistent with the synthetic lethal relationship of STAG2 and ATM described herein.

In summary, tumor cells that have an underlying genetic or epigenetic defect in the FA pathway—or in RAD18, PTEN, or STAG2—may be hypersensitive to the ATMi M3541. Accordingly, patients bearing tumors with these defects may be more likely to respond to the ATMi, and these patients may be more likely to benefit from it.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.01.052>.

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AUTHOR CONTRIBUTIONS

M.-Y.C. conceived the study, performed experiments, analyzed data, and wrote the manuscript. C.E.D. performed the experiments and computational analysis, analyzed the data, and helped write the manuscript. W.C. and L.A.M. performed the experiments and analyzed the data. B.S.K. performed the computational analysis, analyzed the data, and helped write the manuscript. H.N. performed the computational analysis and analyzed the data. G.I.S. provided critical advice on the study. K.P. contributed to writing the manuscript. D.K. helped with the design of the CRISPR screen and performed bioinformatics analysis. A.D.D. conceived the study and wrote the manuscript.

DECLARATION OF INTERESTS

A.D.D. is a consultant/advisory board member for Lilly Oncology, Merck-EMD Serono, Intellia Therapeutics, Sierra Oncology, Cytair Therapeutics, Third Rock Ventures, AstraZeneca, Ideaya Inc., and Cedilla Therapeutics Inc.; a stockholder in Ideaya Inc., Cedilla Therapeutics Inc., and Cytair; and reports receiving commercial research grants from Lilly Oncology and Merck-EMD Serono. G.I.S. is a consultant/advisory board member for Lilly, Sierra Oncology, Merck-EMD Serono, Pfizer, Astex, Almac, Roche, Bicycle Therapeutics, Fusion Pharmaceuticals, G1 Therapeutics, Bayer, Ipsen, Cybrexa Therapeutics, Angiex, Daiichi Sankyo, and Seattle Genetics; and reports receiving commercial research grants from Lilly, Sierra Oncology, Merck-EMD Serono, and Merck & Co.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Cas9 (7A9-3A3)	Cell Signaling Technology	Cat# 14697S; RRID:AB_2750916
ATM	Cell Signaling Technology	Cat# 2873S; RRID:AB_2062659
pATM (s1981)	Cell Signaling Technology	Cat# 13050S
β -actin	Cell Signaling Technology	Cat# 4967S; RRID:AB_330288
alpha-Tubulin	Cell Signaling Technology	Cat# 2144S; RRID:AB_2210548
GAPDH	Cell Signaling Technology	Cat# 5174S; RRID:AB_10622025
FAAP24	Fisher Scientific	Cat# PA559464; RRID:AB_2641194
FAAP100	Fisher Scientific	Cat# NBP215635
FANCF	Everest Biotech	Cat# EB10140
RAD18	Cell Signaling Technology	Cat# 9040S; RRID:AB_2756446
PTEN	Cell Signaling Technology	Cat# 9188; RRID:AB_2253290
FANCA	Cell Signaling Technology	Cat# 14657S; RRID:AB_2798558
FANCD2	Novus Biologicals	Cat# NB100-182; RRID:AB_10002867
FANCD2	Santa Cruz Biotechnology	Cat# SC-20022; RRID:AB_2278211
γ -H2AX	Cell Signaling Technology	Cat# 2577S; RRID:AB_2118010
RAD51	Cell Signaling Technology	Cat# 8875S; RRID:AB_2721109
RPA	Cell Signaling Technology	Cat# 2208S; RRID:AB_2238543
53BP1	Cell Signaling Technology	Cat# 4937S; RRID:AB_10694558
FANCG	Fisher Scientific	Cat# NB1002566; RRID:AB_921259
BRCA1	Cell Signaling Technology	Cat# 9010S; RRID:AB_2228244
BrdU	Cell Signaling Technology	Cat# 5292S; RRID:AB_10548898
BrdU	Abcam	Cat# ab6326; RRID:AB_2313786
FANCC	Abcam	Cat# ab54631; RRID:AB_2101904
Histone H3	Cell Signaling Technology	Cat# 9715S; RRID:AB_331563
Ku80	Cell Signaling Technology	Cat# 2180S; RRID:AB_1612531
p-DNAPK	Abcam	Cat# ab18192; RRID:AB_869495
DNAPK	Santa Cruz Biotechnology	Cat# SC-390849
XRCC4	Life Technologies	Cat# MA524383; RRID:AB_2633355
PAXX	Cell Signaling Technology	Cat# 92448; RRID:AB_2800186
XLF	Cell Signaling Technology	Cat# 2854S; RRID:AB_2152954
Ligase IV	Cell Signaling Technology	Cat# 14649S; RRID:AB_2750871
CtIP	Cell Signaling Technology	Cat# 9201S; RRID:AB_10828593
MRE11	Cell Signaling Technology	Cat# 4895S; RRID:AB_2145100
NBS1	Cell Signaling Technology	Cat# 14956S; RRID:AB_2798660
RAD50	Cell Signaling Technology	Cat# 3427S; RRID:AB_2176936
BLM	Cell Signaling Technology	Cat# 2742S; RRID:AB_2064649
EXO1	Sigma-Aldrich	Cat# SAB4503568; RRID:AB_10761635
DNA2	Abcam	Cat# ab220883
Chemicals, Peptides, and Recombinant Proteins		
M3541	EMD Serono/Merck Company	N/A
KU-59333	Selleck Chemicals	Cat# S1092
M3814	Chemgood	Cat# C-1358

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Olaparib (AZD2281)	Selleck Chemicals	Cat# S1060
Mitomycin C	Sigma-Aldrich	Cat# M0503
(Z)-4-Hydroxytamoxifen	Sigma-Aldrich	Cat# H7904
5-Bromo-2'-deoxyuridine	Sigma-Aldrich	Cat# B9285
SCR7	Selleck Chemicals	Cat# S7742
Critical Commercial Assays		
CellTiter-Glo Luminescent Viability Assay	Promega	Cat# G7570
DNeasy Blood & Tissue Kit	QIAGEN	Cat# 69504
Subcellular Protein Fractionation Kit	Life Technologies	Cat# 78840
PowerUp SYBR Green Master Mix	Life Technologies	Cat# A25741
Experimental Models: Cell Lines		
A549-CAS9 Expressing Cell Line	Broad Institute	N/A
NCI-H460-CAS9 Expressing Cell Line	Broad Institute	N/A
HeLa Cell Line	ATCC	N/A
293T Cell Line	ATCC	N/A
U2OS Cell Line	ATCC	N/A
RPE Cell Line	ATCC	N/A
MDA-MB-436 Cell Line	Gift from Dr. Geoffrey Shapiro	N/A
MDA-MB-436-olaparib-resistant Cell Line	Gift from Dr. Geoffrey Shapiro	N/A
MDA-MB-436+BRCA1 Cell Line	Gift from Dr. Geoffrey Shapiro	N/A
PD326 Cell Line	Kennedy et al., 2007	N/A
GM6914 Cell Line	Kennedy et al., 2007	N/A
TOV21G Cell Line	Kennedy et al., 2007	N/A
HS766T Cell Line	Kennedy et al., 2007	N/A
PL-11 Cell Line	Kennedy et al., 2007	N/A
Oligonucleotides		
siRNA targeting sequence: FAAP24: GGGTTAGAAATCCAATAA	Life Technologies	Cat# s40660
siRNA targeting sequence: FAAP100: CCTGCCCGATTATCTGGAG	Life Technologies	Cat# n268651
siRNA targeting sequence: FANCF: GCTATAGAGAGAACCCAAA	Life Technologies	Cat# s5016
siRNA targeting sequence: RAD18: CATATTAGATGAACTGGT	Life Technologies	Cat# s32295
siRNA targeting sequence: PTEN: GCATACGATTTTAAGCGGA	Life Technologies	Cat# s325
siRNA targeting sequence: FANCA: TATTTTCCCTCTTGACCCTT	QIAGEN	N/A
siRNA targeting sequence: FANCD2: CTGGCTCAGGATTCTAATGTA	QIAGEN	N/A
siRNA targeting sequence: BRCA1: GAATGAGCATGATTTTGAA	Life Technologies	Cat# s224682
siRNA targeting sequence: 53BP1: AGAACGAGGAGACGGTAATAG	QIAGEN	N/A
siRNA targeting sequence: negative Control: AATTCTCCGAACGTGTACGCT	QIAGEN	N/A
See Table S3 for remainder of oligonucleotides		

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alan D. D'Andrea (Alan_Dandrea@dfci.harvard.edu). The knockout cell lines generated in this study are available from the Lead Contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

A549 and NCI-H460 cells stably expressing the Cas9 endonuclease were obtained from the Genetic Perturbation Platform at the Broad Institute and were grown at 37°C in a humidified 5% CO₂ incubator in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS, Sigma), 1% penicillin-streptomycin (GIBCO) and 100 µg/mL Normocin (InvivoGen, San Diego, CA). HeLa and 293T cells were purchased from ATCC and grown in DMEM (GIBCO) supplemented with 10% FBS and 1% penicillin-streptomycin (GIBCO). U2OS and RPE cells were purchased from ATCC and grown in DMEM/F12 (GIBCO) supplemented with 10% FBS and 1% penicillin-streptomycin (GIBCO). MDA-MB-436, MDA-MB-436-olaparib-resistant, MDA-MB-436+BRCA1 cells were a kind gift from Dr. Geoffrey Shapiro (Dana-Farber Cancer Institute) and grown in RPMI 1640 (GIBCO) supplemented with 10% FBS and 1% penicillin-streptomycin (GIBCO). Generation and maintenance of the PD326, GM6914, TOV-21G, HS766T and PL-11-derived cell lines has been previously described ([Kennedy et al., 2007](#)).

METHODS DETAILS

Drug selection CRISPR screens

NSCLC cell lines A549 and NCI-H460 stably expressing the Cas9 endonuclease (pXPR_311) were obtained from the Genetic Perturbation Platform at the Broad Institute. The lines were grown at 37°C in a humidified 5% CO₂ incubator in GIBCO RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 100 µg/ml Normocin (InvivoGen, San Diego, CA). The pXPR_311 plasmid contains a blasticidin resistance tag to allow for selection of infected cells. For each line, the selected Cas9 positive cells were infected in four 12-well tissue culture plates with the Brunello whole genome sgRNA pooled lentiviral library consisting of 76,441 sgRNAs targeting 19,114 different genes ([Doench et al., 2016](#)). For adequate representation of each sgRNA, e.g., an average of 500 cells/sgRNA, a total of 135 million cells divided into wells each containing 3 million cells were infected at a multiplicity of infection (MOI) of 0.3 in media containing 8 µg/ml polybrene. Plates were centrifuged at 2000 rpm for 2 hours at 37°C, and then 2 hours after centrifugation, media was replaced with fresh media without polybrene and cells were incubated overnight. Infected cells were transferred to 15 cm tissue culture dishes and selected with 2 µg/mL puromycin for 4 days prior to drug treatment. After selection, 40 million cells (8 plates each containing 5 million cells) were plated for each biological replicate, and an additional 40 million cells per replicate were collected and frozen for subsequent genomic DNA (gDNA) extraction. A549 and NCI-H460 cells were then treated with 1 µM M3541 versus DMSO control, for 19 days. Cells were re-plated every 3-4 days and maintained in drug or DMSO-containing media, each time plating a minimum of 40 million cells per replicate. At the end of the screen, the cells were harvested and frozen for gDNA extraction.

Genomic DNA preparation, sequencing and identification of top hits

gDNA was isolated using QIAGEN QIAamp DNA blood midi and maxi kits, according to manufacturer's protocol. PCR of gDNA was performed to attach sequencing adaptors and barcode samples for sgRNA sequencing, as previously described ([Doench et al., 2016](#)). Samples were sequenced on an Illumina HiSeq2000. Reads per million were log₂-transformed by first adding one to all values, which was necessary to take the log of sgRNAs with zero reads. For analysis, these normalized data were used to determine the log₂-fold change of each sgRNA for the drug treatment group relative to DMSO control group ([Shalem et al., 2014](#)). The STARS gene-ranking algorithm was used to determine the top hits (<https://portals.broadinstitute.org/gpp/public/software/stars>); at least two sgRNAs targeting a gene had to rank within the top 10% of sgRNAs by log₂-fold change in one direction or another ([Doench et al., 2016](#)). The hit genes were scored using the STARS score and the false discovery rate. Additionally, the normalized data were also analyzed with the hypergeometric distribution method (https://github.com/mhegde/volcano_plots). The volcano plots of these results were created using R (<https://www.r-project.org>).

Western blotting

Cells were lysed with lysis buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.4), 1 mM EDTA, 0.5% NP-40], lysates were resolved on denaturing Nu-page (Invitrogen) polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk in TBST, and probed with primary and secondary antibodies respectively, then detected with chemiluminescence (Western Lightning, Perkin Elmer).

CRISPR cas9 Knockout

To generate A549, NCI-H460, HeLa and U2OS cells expressing the indicated sgRNAs, the oligonucleotides described in [Table S1](#) were cloned into the pLentiCRISPR V2 viral vector.

Oligonucleotides used for sgRNA cloning are shown in the supplementary table ([Table S3](#)).

siRNA transfection

For siRNA transfection, cells were seeded at about 30% confluence into 6-well plates and transfected with a specific siRNA duplex using Lipofectamine RNAiMAX Reagent Agent (Life Technologies, #13778150) according to the manufacturer's instructions for 48 hr. The sequences of the siRNAs used in this study are shown in the supplementary table ([Key Resources Table](#) and [Table S3](#)). siRNA resistant CtIP plasmids are described previously ([Chanut et al., 2016](#)).

Clonogenic assay

Cells were seeded at a concentration of 1000–2500 cells per well into 6-well plates. After 24 hr, cells were continuously treated with indicated compounds for about 2 weeks. Colonies were fixed with fixation solution (5 parts methanol + 1 part acetic acid) at room temperature for 20 min and then stained with a solution of 0.5% crystal violet in methanol for 1 hour.

CellTiter-Glo assay

Cells were seeded into 96-well plates at a cell density of 3,000 cells per well, incubated overnight and treated with indicated compounds. The ATP-based, cell viability assay was carried out after incubation for 4 days (CellTiter-Glo, Promega). Survival curves were calculated by best-fit analysis of the log of the drug concentration to fold change of treated cells over vehicle-treated cells. All survival assays included technical triplicates.

Subcellular Fractionation

Subcellular fractions were isolated using a subcellular protein fractionation kit (Thermo Fisher Scientific) according to the manufacturer's instructions, and the resultant fractions were analyzed with western blotting. Antibodies used in this study are listed in the [Key Resources Table](#).

Comet assays

Alkaline comet assays were performed with manufacturer's instruction (Trevigen). Cells were trypsinized and suspended in cold PBS at a concentration of 2.0×10^5 cells/ml. The cells were then mixed with low melting agarose (Trevigen) at a ratio of 1:10 (v/v) and immediately plated onto CometSlide (Trevigen). Alkaline electrophoresis was run at 25 V for 30 minutes in the electrophoresis system. Data were analyzed with a fluorescence microscope (Zeiss).

Reporter assays

NHEJ and HR efficiencies were measured using the EJ5-GFP and DR-GFP reporter assay, respectively ([Ceccaldi et al., 2015](#)). Briefly, 24 h before infection with I-Sce-I adenovirus, U2OS cells with either GFP expression cassette were transfected with the indicated siRNA and/or treated with ATMi (1 μ M). The HR and NHEJ activity was determined by flow cytometric quantification of viable GFP-positive cells 72 h after I-Sce-I adenovirus infection.

Immunofluorescence

Cells plated on coverslips were washed once with PBS and fixed with 4% paraformaldehyde for 10 minutes on ice. 0.5% Triton X-100 was added to cells for 30 min for extraction. Coverslips were then blocked with 3% BSA for 30 min and incubated with primary antibody (diluted in 3% BSA) overnight. Following overnight incubation, coverslips were washed three times with PBST, then incubated with anti-rabbit secondary antibody (Alexa Fluor, Life Technologies) for 1 hour. Coverslips were then mounted with DAPI medium and visualized under the fluorescence microscope.

Cytogenetics

Cells were transfected with siRNAs twice for 48 h and incubated for 48 h in the presence or absence of 20 ng/ml MMC. Cells were exposed for 2 hours to colcemid (0.1 μ g/ml) and harvested using a 0.075M KCl hypotonic solution and fixed with 3:1 methanol:acetic acid. Slides were stained with Wright's stain and when possible, 50 metaphase spreads were scored for aberrations. Metaphase spreads were observed using a Zeiss Axio Imager microscope and captured using CytoVision software from Applied Imaging.

SMART assay

Fiber comb assay was performed using the FiberComb machine (Genomic Vision). In brief, cells were treated with bromodeoxyuridine (BrdU, Sigma) for 24 hours. After 24-hour treatment with 1 μ M ATMi, cells were then trypsinized, counted and embedded in low melting point agarose plugs, then treated with proteinase K overnight. Agarose plugs were then washed and digested with agarase. Agarase-treated samples were then poured into FiberComb wells and combed onto silanized coverslips. Coverslips were probed

with rat anti-BrdU antibody (Abcam) and visualized by fluorescence microscopy. Pictures were taken of at least 100 fibers per condition. DNA fibers were measured with ImageJ and graphed. Each experiment was repeated at least three times unless otherwise stated.

***In vitro* DNA end resection**

U2OS cells stably expressing HA-ER-AsiSI were created as described previously (Zhou et al., 2014). ssDNAs were quantitated by the HA-ER-AsiSI system in which AsiSI enzyme is fused to the estrogen receptor. The cells were treated with or without 300 nM 4-OHT for four hours to induce DSBs at specific AsiSI sites. Subsequently, genomic DNA was extracted with the QIAGEN DNeasy Blood & Tissue Kit and ssDNA at the specific AsiSI site in chromosome 1 was measured by qPCR with the primers as describing in Table S3.

Computational Analysis

Data from the Broad Institute Cancer Dependency Map Achilles CRISPR Avana 17Q2 dataset (Meyers et al., 2017), the Cancer Cell Line Encyclopedia (Cancer Cell Line Encyclopedia and Genomics of Drug Sensitivity in Cancer, 2015), the Genomics of Drug Sensitivity in Cancer (GDSC) (<https://www.cancerrxgene.org/>) (Yang et al., 2013), and the PanCancer MSK-IMPACT Clinical Sequencing Cohort (Zehir et al., 2017) were extracted for analysis. The MSK-IMPACT Clinical Sequencing Cohort and Cancer Cell Line Encyclopedia datasets were accessed through the public portal (<http://www.cbioportal.org>). For dependency analysis, copy number loss of genes was indicated by a copy number variation of -2 or -1 relative to diploid. Normal copy number was indicated by 0 variation relative to diploid. Copy number was chosen as a proxy for gene depletion because RAD18, FAAP24, and FAAP100 mutations are not screened for in cell lines. Cell lines that were in the first quartile of IC_{50} to a drug were categorized as sensitive and cell lines that were in the fourth quartile of IC_{50} were categorized as resistant. The cell lines $\pm$ one quartile from the median were not used in our analysis. For analysis of correlation between patient survival and mutations in ATM or FA genes, patient survival data was matched with FANCA, FANCC, and ATM mutation status using the PanCancer MSK-IMPACT Clinical Sequencing Cohort. The study only screened FANCA and FANCC mutations, commonly mutated FA genes. Due to the significant difference in timeline between groups for survival data, all data before 27.25 months (the last patient in the FA and ATM mutated group), was utilized and analyzed.

QUANTITATION AND STATISTICAL ANALYSIS

All quantitative data were analyzed and graphed using Prism 7 GraphPad software. All data are represented as mean $\pm$ SEM calculated using the Prism software, unless indicated otherwise. Significance was tested using the non-parametric Mann-Whitney U Test unless indicated otherwise. Clinical data were analyzed using the Kaplan-Meier method and the log-rank (Mantel-Cox) regression test for significance.

DATA AND CODE AVAILABILITY

The data for $\log_2$ -fold change of each sgRNA for the drug treatment group relative to DMSO control group in A549 and H460 cells in the genome-wide CRISPR screens are available in Table S1. The STARS analysis for each gene is available in Table S2.

Cell Reports, Volume 30

Supplemental Information

Cooperation of the ATM and Fanconi Anemia/BRCA

Pathways in Double-Strand Break End Resection

Mu-Yan Cai, Connor E. Dunn, Wenxu Chen, Bose S. Kochupurakkal, Huy Nguyen, Lisa A. Moreau, Geoffrey I. Shapiro, Kalindi Parmar, David Kozono, and Alan D. D'Andrea

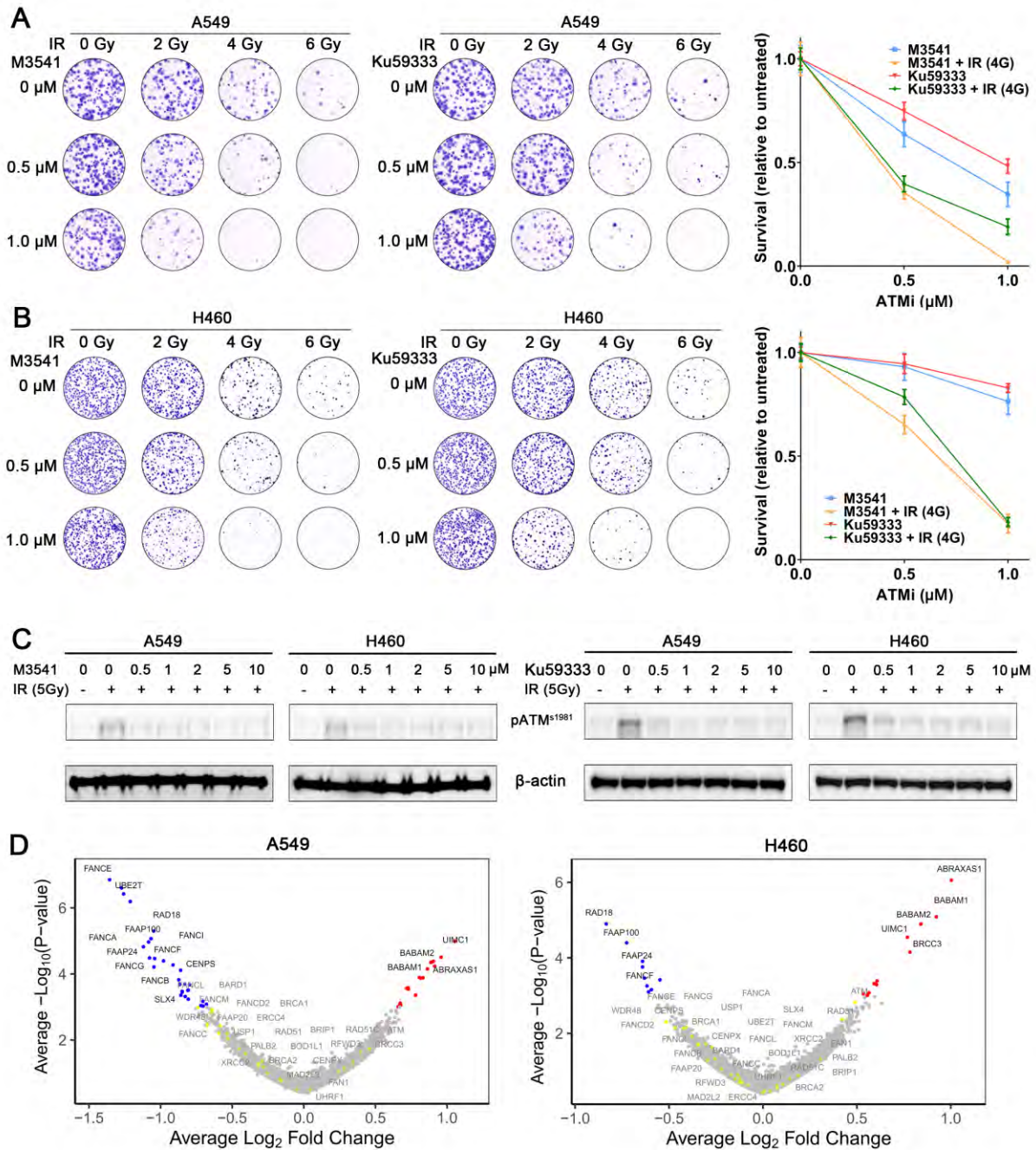


Figure S1. CRISPR screen identifies determinants of M3541 sensitivity. Related to Figure 1. (A) Clonogenic assay of A549 cells treated with 0.5 μ M and 1.0 μ M M3541 or Ku59333, and 2 Gy, 4 Gy, 6 Gy irradiation and DMSO (ctrl). Quantitation of survival assays of the ATMi alone and ATMi in combination with irradiation (right panel). (B) Clonogenic assay of NCI-H460 cells treated with 0.5 μ M and 1.0 μ M M3541 or Ku59333, and 2 Gy, 4 Gy, 6 Gy irradiation and DMSO (ctrl). Quantitation of survival assays of the ATMi alone and ATMi combination with irradiation (right panel). Data are shown as mean $\pm$ SEM. (C) Immunoblot analysis of A549 and NCI-H460 cells treated for 4 hours with the indicated doses of ATMi (M3541 and Ku59333) for detecting phosphorylation of ATM. β -actin was used as a loading control. (D) Volcano plots showing genes targeted by sgRNAs that differentially dropped out (blue) or became enriched (red) in M3541 treated versus DMSO treated control cells in CRISPR screens. Additional Fanconi anemia pathway genes and top hits are also highlighted (yellow).

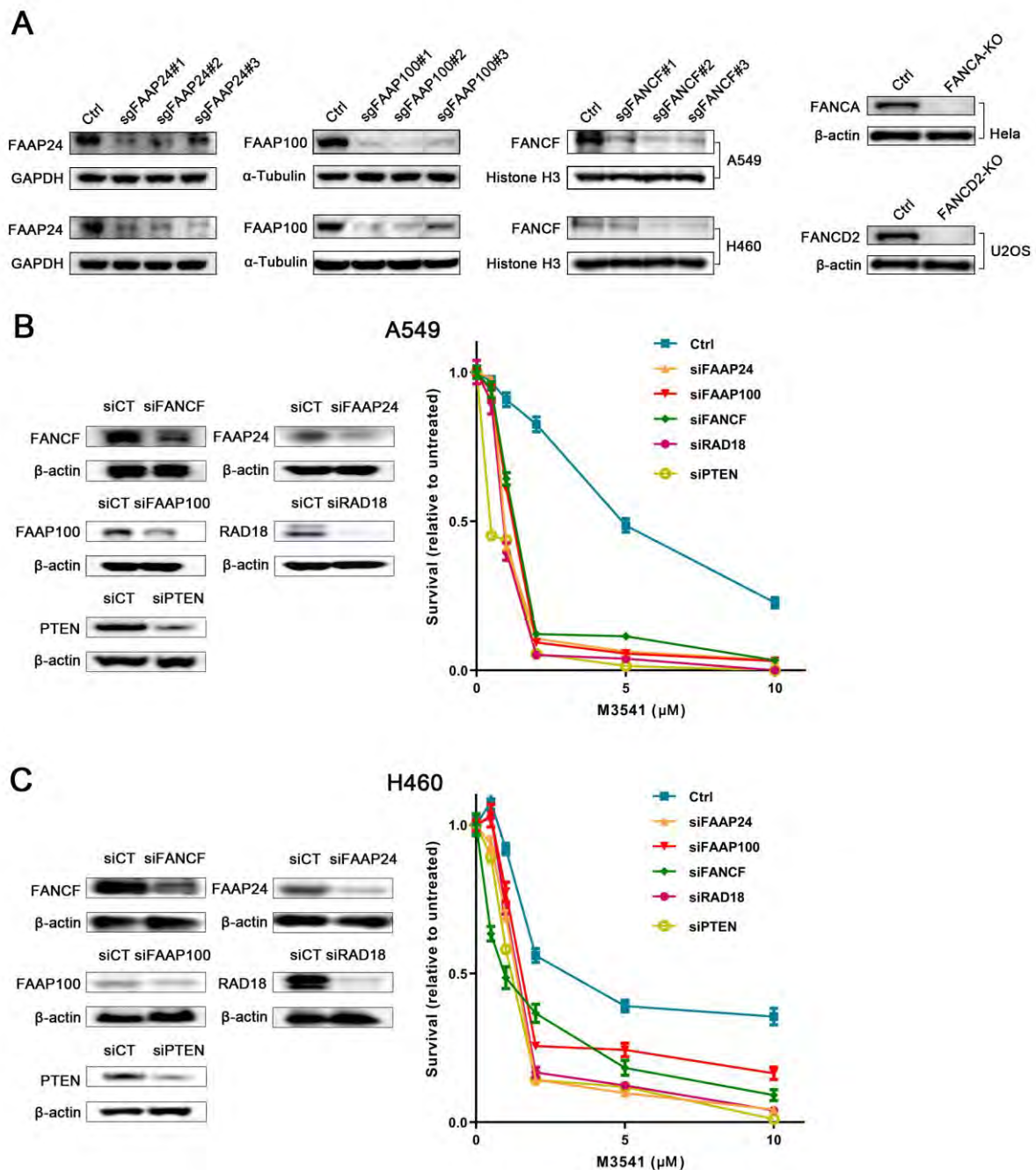


Figure S2. Knockdown of Fanconi anemia pathway confers sensitivity to an ATM inhibitor in cancer cells. Related to Figure 2. (A) Representative western blotting images in A549, H460, HeLa and U2OS cells following transduction with FAAP24-, FAAP100-, FANCF-, FANCA or FANCD2-targeting sgRNAs. #1, #2 and #3 indicate three independent sgRNAs. (B) Clonogenic assays of A549 cells after treatment with ATM inhibitor (M3541) following transfection with siFAAP24, siFAAP100, siFANCF, siRAD18 and siPTEN: representative western blotting images (*left panel*), survival assays (*right panel*). (C) Clonogenic assays of NCI-H460 cells after treatment with ATM inhibitor (M3541) following transfection with siFAAP24, siFAAP100, siFANCF, siRAD18 and siPTEN: representative western blotting images (*left panel*), survival assays (*right panel*).

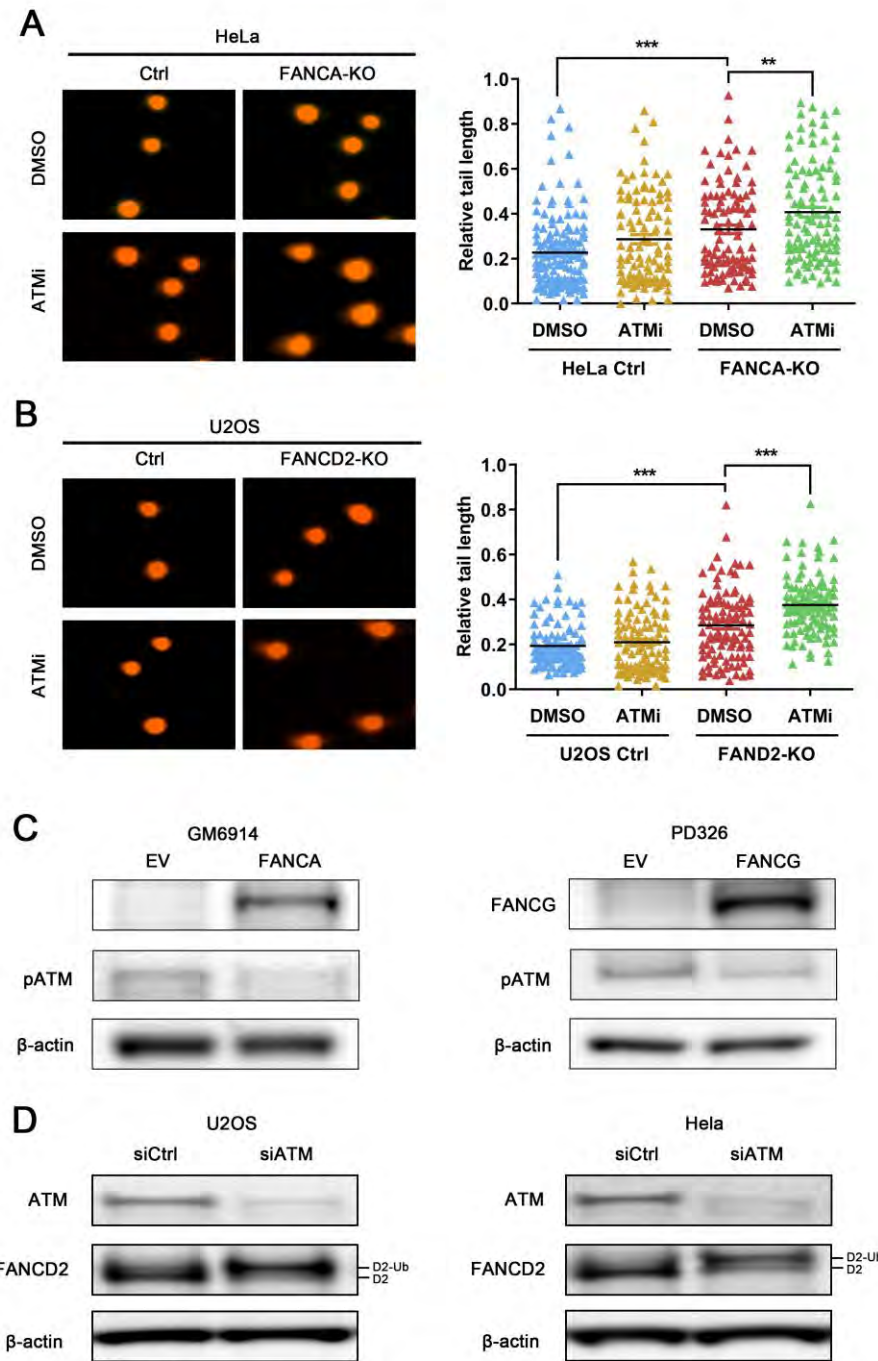


Figure S3. ATM is constitutively activated in FA-deficient cells and inhibition of ATM results in DNA damage. Related to Figure 3. (A) Comet assay images (left panels) and quantification of the tail length relative to nucleus (right panels) 3 h after treatment with irradiation (10 Gy) following 24 h exposure to DMSO or M3541 (1 μ M) in control and FANCA-KO HeLa cells. B. Comet assay images (left) and quantification of the tail length relative to nucleus 3 h after treatment with irradiation (10 Gy) following 24 h exposure to DMSO or M3541 (1 μ M) in control and FANCD2-KO U2OS cells. (C) Immunoblot comparing auto-activation of ATM (phosphorylation at Ser1981) in GM6914 (FANCA-mutated) and isogenic corrected fibroblasts (left panel). Immunoblot comparing auto-activation of ATM (phosphorylation at Ser1981) in PD326 (FANCG-mutated) and isogenic corrected fibroblasts (right panel). (D) Immunoblot comparing FANCD2 monoubiquitination in U2OS cells (left panel) and HeLa cells (right panel) transfected with siControl (siCtrl) or siATM. ** $P < 0.01$, *** $P < 0.001$ on unpaired two-tailed Student's t-test for data shown in panels A and B.

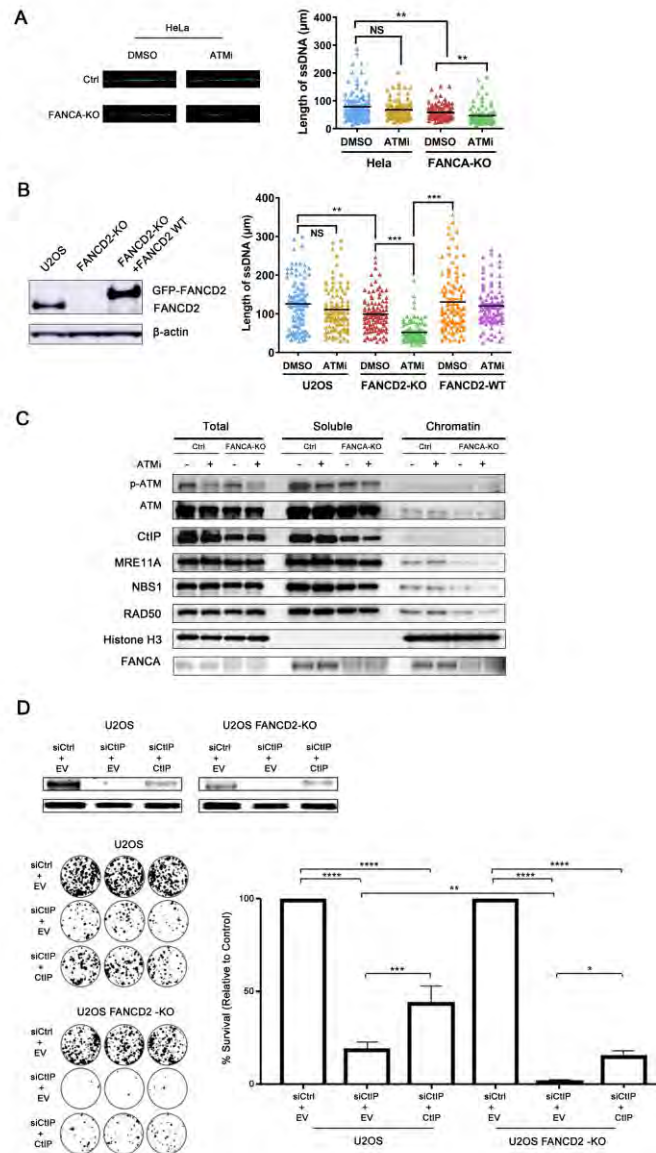


Figure S4. Inhibition of ATM decreases DNA end resection machinery in FA pathway-deficient cells. Related to Figure 4. (A) Change in ssDNA length of control and FANCA^{-/-} HeLa cells 3 h after irradiation (5 Gy) following overnight exposure to 1 μ M ATMi. Representative images of SMART assay (left panel) and the quantification (relative to untreated, right panel). Totally, 100 DNA fibers were assessed per treatment condition. $**P < 0.01$, unpaired two-tailed Student's t-test. NS, not significant. Data are shown as mean $\pm$ SEM from three independent experiments. (B) (right panel) Quantitation of the SMART assay using wild-type (WT) U2OS cells, FANCD2^{-/-} U2OS cells and FANCD2^{-/-} U2OS cells expressing exogenous GFP-FANCD2. The cells were treated with ATMi as described in panel A. (left panel) Immunoblots of the indicated proteins in WT U2OS cells, FANCD2^{-/-} U2OS cells and FANCD2^{-/-} U2OS cells expressing exogenous GFP-FANCD2. (C) Immunoblots of the indicated proteins in subcellular fractions of control and FANCA^{-/-} HeLa cells 6 h after treatment with 1 μ M ATMi. (D) Immunoblots of the indicated proteins (upper panels) and clonogenic survival (lower panels) of WT U2OS cells and FANCD2^{-/-} U2OS cells after transfection with siCtrl or siCtIP with and without siCtIP resistant exogenous CtIP. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ using one-way ANOVA.

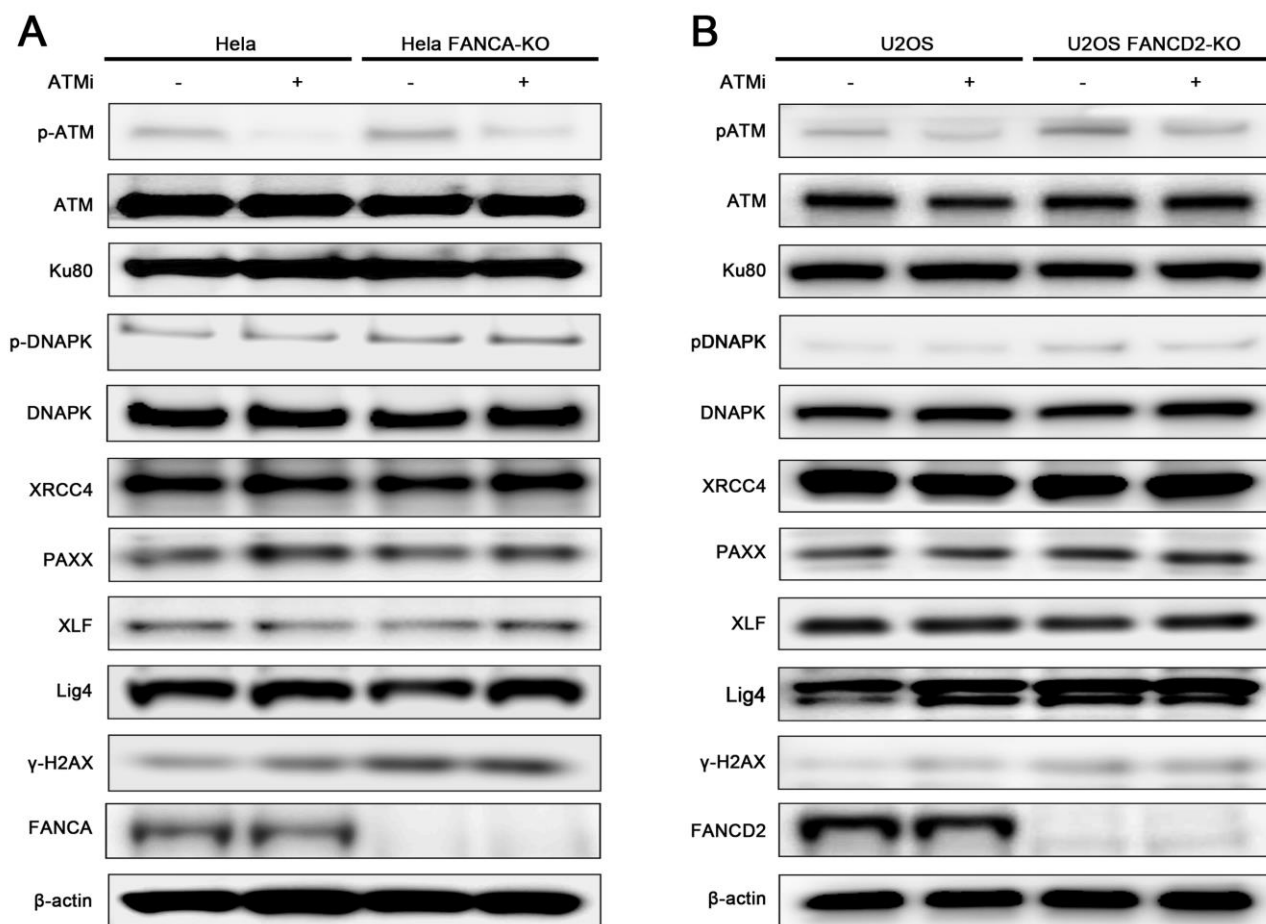


Figure S5. Inhibition of ATM results in increased expression of NHEJ pathway proteins in FA-deficient cells. Related to Figure 5. (A) Representative western blot images depicting activation of ATM (autophosphorylation) proteins in FANCA^{-/-}-HeLa cells as compared to WT cells and inhibition of ATM inducing activation of ATMi-induced increases in DNAPK, Lig4 and γ-H2AX in FANCA^{-/-}-HeLa cells compared to WT. β-actin used as a loading controls. (B) Representative western blot images depicting activation of ATM (autophosphorylation) proteins in FANCD2^{-/-}-U2OS cells as compared to WT and inhibition of ATM inducing activation of ATMi-induced increases in DNAPK, Lig4 and γ-H2AX in FANCD2^{-/-}-U2OS cells. β-actin was used as a loading control.

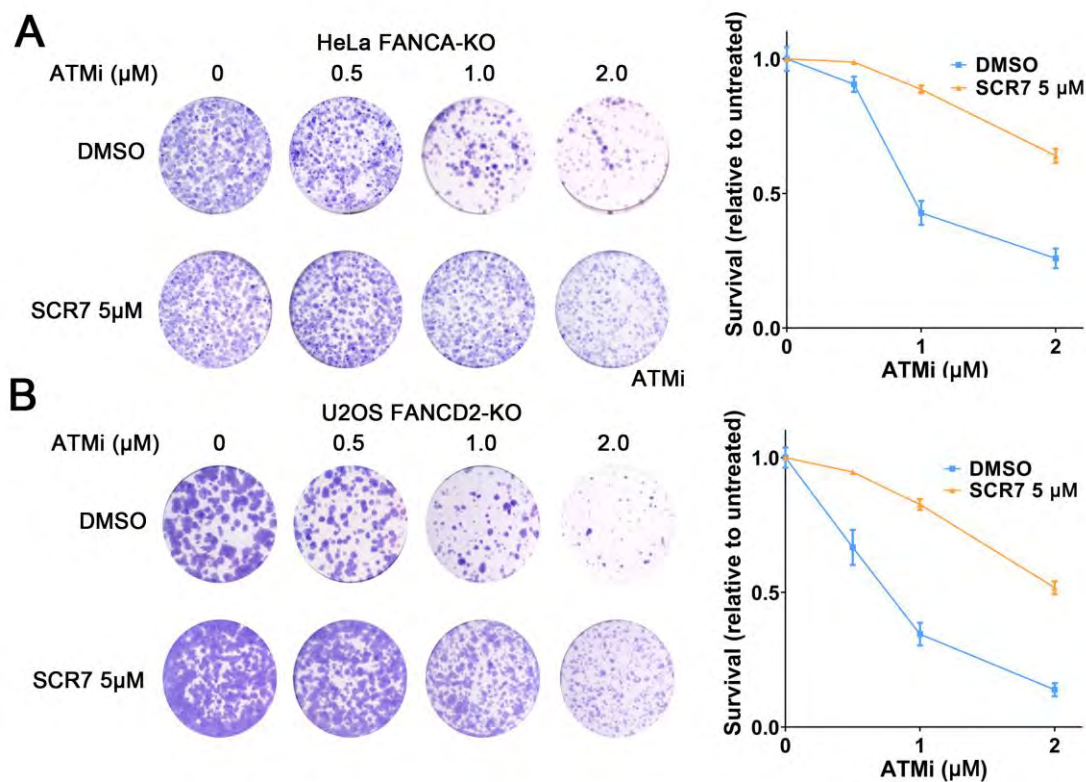


Figure S6. LIG4 inhibitor SCR7 rescues ATMi-induced cytotoxicity in FA-deficient cells. Related to Figure 6. (A) Clonogenic assay of FANCA-KO HeLa cells treated with M3541 $\pm$ 5 μM SCR7 (quantitation in right panel). (B) Clonogenic assay of FANCD2-KO U2OS cells treated with M3541 $\pm$ 5 μM SCR7 (quantitation in right panel).

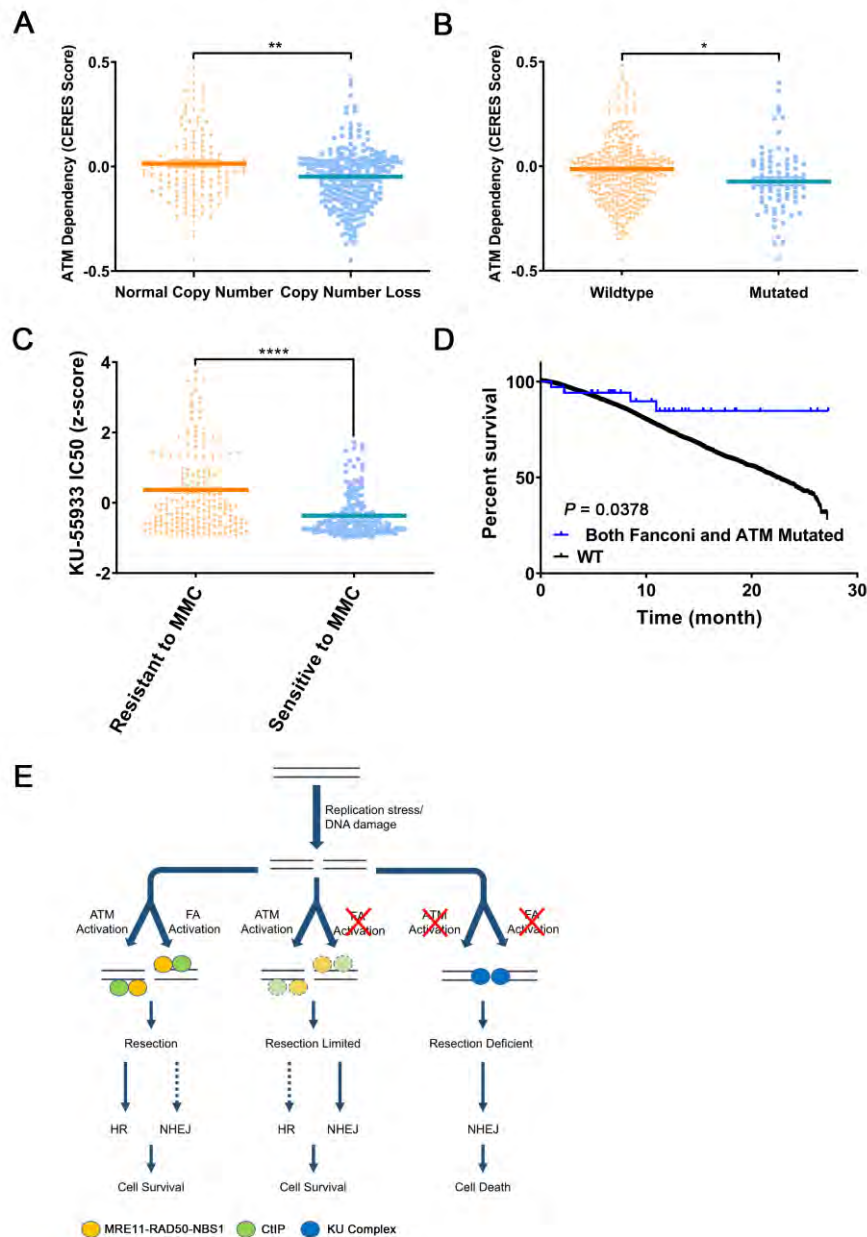
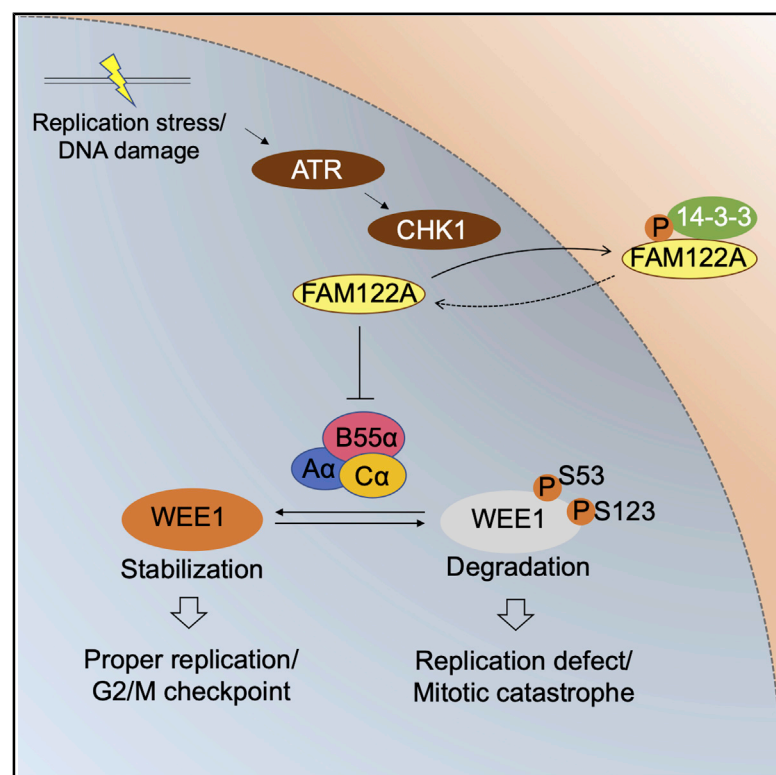


Figure S7. FA-deficient cells depend on ATM for survival. Related to Figure 7. (A) Loss of copy number of RAD18, FANCF, FAAP24, or FAAP100 genes correlates with a higher dependency on ATM (lower CERES score indicates higher dependency) in cell line data from the Cancer Dependency Map. CRISPR gene knockout effects inferred by CERES computational method were compared in cell lines grouped according to gene copy number using data from the BROAD dependency map and Cancer Cell Line Encyclopedia. (B) A mutation in one or more Fanconi anemia genes encoding the Fanconi anemia core complex proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) correlates with a higher dependency on ATM. The data were derived using methods described in panel A. (C) Sensitivity of cancer cell lines to Mitomycin C (MMC) correlates with sensitivity to the ATMi KU-55933. The analysis was performed using the Genomics of Drug Sensitivity in Cancer (GDSC) database. Cell lines that were in the first quartile of MMC IC₅₀ were categorized as sensitive and cell lines which were in the fourth quartile were categorized as resistant. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, Mann-Whitney test for data in panels A,B and C. (D) Kaplan-Meier survival curves of patients with either mutations in both FANCA/FANCC and ATM or wildtype (WT) for these genes from the MSK-IMPACT Clinical Sequencing Cohort. The log rank test was used to assess statistical significance. (E) Model for Fanconi anemia pathway and ATM function in DNA end resection.

CHK1 Inhibitor Blocks Phosphorylation of FAM122A and Promotes Replication Stress

Graphical Abstract



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In Brief

Li and colleagues demonstrate that the cell-cycle checkpoint kinase CHK1 phosphorylates and inactivates FAM122A, an inhibitor of the phosphatase PP2A. Activated PP2A in turn dephosphorylates WEE1, prevents its ubiquitin-mediated proteolysis, increases WEE1 protein levels, and promotes the G2/M checkpoint. Loss of FAM122A expression is a mechanism of acquired CHK1 inhibitor resistance.

Highlights

- CRISPR knockout of FAM122A confers resistance to inhibitors of CHK1 and ATR
- CHK1 phosphorylates FAM122A, thereby activating the phosphatase PP2A
- Activated PP2A dephosphorylates WEE1 and promotes WEE1 stabilization
- Tumor cells acquire drug resistance by deletion or reducing expression of FAM122A

Article

CHK1 Inhibitor Blocks Phosphorylation of FAM122A and Promotes Replication Stress

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SUMMARY

While effective anti-cancer drugs targeting the CHK1 kinase are advancing in the clinic, drug resistance is rapidly emerging. Here, we demonstrate that CRISPR-mediated knockout of the little-known gene FAM122A/PABIR1 confers cellular resistance to CHK1 inhibitors (CHK1i) and cross-resistance to ATR inhibitors. Knockout of FAM122A results in activation of PP2A-B55 α , a phosphatase that dephosphorylates the WEE1 protein and rescues WEE1 from ubiquitin-mediated degradation. The resulting increase in WEE1 protein expression reduces replication stress, activates the G2/M checkpoint, and confers cellular resistance to CHK1i. Interestingly, in tumor cells with oncogene-driven replication stress, CHK1 can directly phosphorylate FAM122A, leading to activation of the PP2A-B55 α phosphatase and increased WEE1 expression. A combination of a CHK1i plus a WEE1 inhibitor can overcome CHK1i resistance of these tumor cells, thereby enhancing anti-cancer activity. The FAM122A expression level in a tumor cell can serve as a useful biomarker for predicting CHK1i sensitivity or resistance.

INTRODUCTION

Oncogene-driven cancer cells exhibit replication stress and are hyperdependent on the ATR-CHK1-WEE1 pathway for survival. In this pathway, the accumulation of single-stranded DNA at stalled replication forks activates the ATR kinase, which in turn phosphorylates and activates the CHK1 kinase (Paulsen and Cimprich, 2007; Zhao and Piwnicka-Worms, 2001). CHK1 subsequently phosphorylates and inactivates the CDC25 phosphatases, preventing dephosphorylation of T14 and Y15 of CDK1, and phosphorylates WEE1, which phosphorylates T14 and Y15 of CDK1 (Patil et al., 2013). Phosphorylated CDK1 has reduced kinase activity, thus resulting in the activation of the G2/M checkpoint. Reduced entry to mitosis allows cells to tolerate replication stress. Whether CHK1 affects WEE1 expression levels or its cellular function in other ways remains unknown.

Among the kinase inhibitors blocking the ATR-CHK1-WEE1 pathway, new CHK1 inhibitors (CHK1i) have demonstrated therapeutic potential in a variety of tumor types (King et al., 2015). Phase 1 clinical trials of the CHK1i prexasertib (LY2606368) have been completed in non-small cell lung cancer (NSCLC) among others (Hong et al., 2016; Hong et al., 2018), and several phase 1 and 2 trials are currently actively recruiting patients. By inhibiting the G2/M- and S-phase checkpoints, CHK1i

cause increased replication stress, DNA damage, and premature entry of cancer cells into mitosis, resulting in mitotic catastrophe and cancer cell death. CHK1i also have enhanced activity when combined with an ATR inhibitor (ATRi) through a mechanism of synthetic lethality (Sanjiv et al., 2016). While these inhibitors provide a therapeutic benefit *in vivo*, acquired resistance can also result. Little is known about how cancer cells become resistant to cell-cycle checkpoint kinase inhibitors. Understanding potential resistance mechanisms may allow the prediction of patient responses, the design of CHK1 therapy combinations, and the implementation of mechanisms for resensitization.

Recent studies indicate that, in addition to WEE1 kinase activity, an increase in WEE1 protein expression level may positively regulate the G2/M checkpoint. WEE1 protein levels are regulated, at least in part, by protein degradation (Watanabe et al., 2004, 2005). The PLK1 kinase and CDC2 kinase phosphorylate WEE1 on two sites (S53 and S123), resulting in Skp1, Cullins, F-box proteins (SCF) E3-ligase-mediated and ubiquitination/protein dependent degradation. Accordingly, downregulation of either WEE1 phosphorylation or of SCF E3 ligase activity can upregulate WEE1 protein expression, thereby enhancing the G2/M checkpoint. Other recent studies suggest that the multisubunit phosphatase PP2A regulates the dephosphorylation of WEE1 and may rescue WEE1 from degradation. Indeed, PP2A

has multiple functions in controlling the G2/M checkpoint (Forester et al., 2007; Wlodarchak and Xing, 2016), such as controlling the phosphorylation state of CDC25A itself. How PP2A activity is regulated during the cell cycle is incompletely known. There are substantially more kinases in the human genome compared to phosphatase genes (Duong-Ly and Peterson, 2013). Target specificity of the phosphatases including PP2A, a heterotrimeric complex consisting of a scaffolding A subunit, a regulatory B subunit, and a catalytic C subunit, is largely conferred by the incorporation of one of a multiple possible B subunits (Sangodkar et al., 2016).

Here, we performed a genome-wide clustered regularly interspersed short palindromic repeat (CRISPR)-Cas9 loss-of-function genetic screen to identify single guide RNAs (sgRNAs) that can either increase or decrease cell killing by the CHK1i prexasertib (King et al., 2015). sgRNAs directed against the protein FAM122A constituted a top hit in the screen, and FAM122A knockout in multiple cell lines resulted in resistance to CHK1i. Since FAM122A was recently shown to be a suppressor of PP2A-B55 α (Fan et al., 2016), we reasoned that downregulation of FAM122A might enhance PP2A-B55 α -mediated stabilization of WEE1 levels. Indeed, FAM122A knockout resulted in increased PP2A activity, increased WEE1 protein levels, reduced replication stress, and restoration of the G2/M checkpoint in cells exposed to the CHK1i. Knockdown of the B55 α regulatory subunit, but not other B subunits, including B56 α or B56 δ , restored prexasertib sensitivity in FAM122A knockout cells, in keeping with the specificity of FAM122A against PP2A-B55 α . Interestingly, CHK1 directly phosphorylated FAM122A on a highly conserved site, S37, leading to its release from the PP2A complex and an upregulation in PP2A activity. Phosphorylated FAM122A was sequestered by 14-3-3 proteins in the cytoplasm and failed to translocate to the nucleus, where it otherwise inhibits PP2A. CHK1i or small interfering RNA (siRNA) knockdown of CHK1 resulted in accumulation of FAM122A in the nucleus. Moreover, FAM122A knockout promoted cellular resistance to not only CHK1i but also ATR inhibitors, which are also currently undergoing clinical development (Qiu et al., 2018; Weber and Ryan, 2015). Taken together, our results indicate a novel process by which CHK1 reduces replication stress and activates the G2/M checkpoint, namely by phosphorylating and inactivating FAM122A and promoting the PP2A-mediated dephosphorylation and stabilization of WEE1 levels and activity. Loss of CHK1-mediated phosphorylation of FAM122A provides a useful pharmacodynamic biomarker for the development of new CHK1i. Moreover, the decreased cellular expression of FAM122A and the increased level of WEE1 protein may serve as useful biomarkers for predicting CHK1i resistance.

RESULTS

Identification of sgRNAs that Selectively Suppress Cell Killing by the CHK1i Prexasertib

Initially, we used the A549 and H460 (NCI-H460) NSCLC lines to conduct a CRISPR screen for sgRNAs that can either increase or decrease cell killing by the CHK1i prexasertib (Figure 1A). These cells were transduced to stably express the Cas9 endonuclease, and they were recently used to conduct a similar screen to iden-

tify cellular enhancers and inhibitors of an ataxia telangiectasia mutated (ATM) inhibitor (Cai et al., 2020). The A549 and H460 cell lines are wild type (WT) for both ATM and the pro-apoptotic gene p53.

In order to identify sgRNAs that target genes involved in resistance to the CHK1i, we chose a moderate concentration of the drug that results in no net cellular proliferation over a 2-week period. A549 and H460 cells exhibit an IC₅₀ for prexasertib in the 10–100 nM range, similar to the drug sensitivity of several other cancer cell lines (Figures S1A and S1B). For the CRISPR screen, we chose a prexasertib concentration of 100 nM for A549 cells and a concentration of 50 nM for H460 cells (Figures S1C and S1D). Cells were infected with the Brunello v2 lentiviral sgRNA library and grown for 19 days in media treated with the CHK1i. Top hits among gene knockouts that conferred drug resistance were identified via the STARS gene-ranking algorithm (Doench et al., 2016).

FAM122A scored as a top hit in both lung cancer cell lines (Figure 1B; Tables S1 and S3). FAM122A is a little-known but highly conserved protein that has recently been shown to inhibit the PP2A multisubunit phosphatase with the B55 α regulatory subunit (PP2A-B55 α) (Fan et al., 2016). sgRNA-mediated knockout of FAM122A (Figure 1C), or siRNA-mediated knockdown of FAM122A, in A549 cells or H460 cells (Figures S1E–S1J) increased resistance to prexasertib, thereby validating the CRISPR screen results. sgRNA knockout of FAM122A also resulted in cellular resistance to another known CHK1i, SRA737 (Figure 1D) and, interestingly, generated cross-resistance to the ATR inhibitor VE822 (Figure 1E). siRNA knockdown of FAM122A also conferred resistance to the ATR inhibitor in another cell line, U2OS (Figure 1F).

Knockout of FAM122A Reduces Replication Stress

CHK1i are known to activate replication stress and disrupt the G2/M checkpoint of cancer cells, and these activities appear to be their major anti-cancer mechanism of action (Parmar et al., 2019). As expected, cellular exposure to CHK1i resulted in a dose-dependent increase in replication stress, as measured by enhanced phosphorylation of KAP1 and CHK1, and enhanced DNA double-strand break (DSB) generation (Figure 2A, lanes 1–4). CHK1i exposure also resulted in an increase in phosphorylation of CHK1, consistent with the loss of a negative feedback loop and the increase in ATR kinase activity toward CHK1. DSB generation was verified by γ H2AX staining (Figure 2B). Interestingly, sgRNA knockout of FAM122A resulted in reduced replication stress, providing a mechanism of increased CHK1 drug resistance (Figure 2A, lanes 5–8). CHK1i also had a strong impact on DNA replication and the cell cycle. Specifically, CHK1i resulted in an increase of unreplicated DNA and accumulation of G2/M cells that was reversed by the sgRNA knockout of FAM122A (Figures 2C and S2A–S2C).

As previously described (Parmar et al., 2019), CHK1i also caused reduced replication fork speed (Figures 2D and 2E); increased DNA damage, as measured by the comet assay (Figures 2F and 2G); and increased chromosome breakage (Figures S2D and S2E). In each case, sgRNA to FAM122A reversed these events, again correlating with the ability of FAM122A knockout to confer cellular resistance to CHK1i.

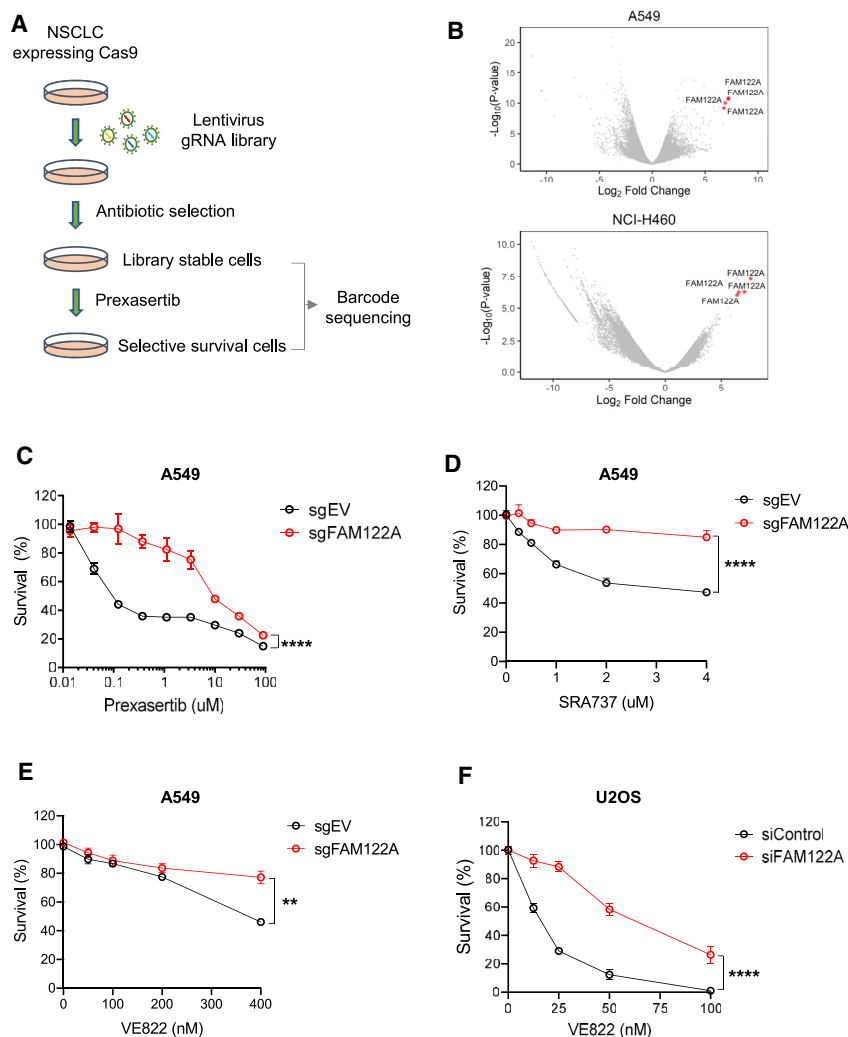


Figure 1. Genome-wide CRISPR Screen Reveals that FAM122A Loss Results in Resistance to CHK1i and ATRi in Lung Cancer Cells

(A) Schematic of CRISPR-based screen for resistance to CHK1i (prexasertib) in lung cancer cells. Cells were infected with the Brunello sgRNA library (containing ~74,000 different sgRNAs) with a multiplicity of infection of 0.3. Following puromycin selection, cells were passaged for three weeks in the presence of prexasertib.

(B) Volcano plots showing genes targeted by sgRNAs that were differentially depleted or enriched following prexasertib selection in A549 and NCI-H460 cells. Note that all four sgRNAs directed to FAM122A were found to be significantly enriched following prexasertib selection in A549 and NCI-H460 cells.

(C) Survival plots of control and FAM122A knockout (FAM122A-KO) A549 cells after exposure to CHK1i (prexasertib) for 5 days. Data are shown as mean $\pm$ SD from three independent experiments. **** p < 0.0001; statistical analysis was performed using two-way ANOVA.

(D) Survival plots of control and FAM122A-KO A549 cells after exposure to CHK1i (SRA737) for 5 days. Data are shown as mean $\pm$ SD from three independent experiments. **** p < 0.0001; statistical analysis was performed using two-way ANOVA.

(E) Survival plots of control and FAM122A-KO A549 cells after exposure to ATRi (VE822) for 5 days. Data are shown as mean $\pm$ SD from three independent experiments. ** p < 0.01; statistical analysis was performed using two-way ANOVA.

(F) Survival plots of siControl and siFAM122A U2OS cells after exposure to ATRi (VE822) for 10 days. Data are shown as mean $\pm$ SD from three independent experiments. **** p < 0.0001; statistical analysis was performed using two-way ANOVA.

Depletion of FAM122A Promotes WEE1 Protein Stability

CHK1 is known to directly phosphorylate WEE1, thereby activating a G2/M checkpoint (Leung-Pineda et al., 2006). Whether CHK1 also directly or indirectly regulates WEE1 expression levels has not been previously demonstrated. We initially observed that CHK1 inhibition in A549 cells resulted in reduced levels of WEE1 protein (Figure 3A, lanes 1 and 2). Interestingly, sgRNA-mediated knockout of FAM122A prevented the CHK1i-activated decline in WEE1 protein level and increased the expression of WEE1 protein (Figure 3A, lanes 3 and 4). Consistent with this, knockout FAM122A also increased resistance to the WEE1 inhibitor AZD1775 (Figure S3B). Again, knockout of FAM122A correlated with reduced γ H2AX levels after cellular exposure to CHK1i (Figure 3A, lanes 3 and 4).

The increase in WEE1 protein expression following FAM122A knockout could either result from increased production or decreased degradation of WEE1. Previous studies have indicated that the WEE1 protein is degraded and that this process is regulated by protein phosphorylation and ubiquitin-mediated and proteasome proteolysis (Watanabe et al., 2004, 2005). Specifically,

WEE1 is phosphorylated on S53 and S123 by the PLK1 and CDC2 kinases, respectively, thereby creating phosphodegrons and resulting in WEE1 degradation of by SCF E3 ligase complex.

To further analyze the role of FAM122A in WEE1 protein degradation, we evaluated the regulation of WEE1 protein degradation in cells with a knockout of FAM122A (Figure 3B). Again, the CHK1i promoted the reduction of WEE1 protein levels (Figure 3B, lanes 2 and 4), and knockout of FAM122A resulted in increased WEE1 protein expression (Figure 3B, lanes 3 and 4). Knockout of FAM122A failed to increase the expression of the WEE1-pS53 or WEE1-pS123 polypeptides. Furthermore, while CHK1i promoted the degradation of WEE1 protein, this degradation was prevented by cellular exposure to the proteasome inhibitor MG132 (Figure S3A). These results are consistent with the known ubiquitin-mediated degradation of the phosphorylated isoforms of WEE1. The stabilization of WEE1 protein, resulting from FAM122A knockout, was confirmed following cellular exposure to a cycloheximide challenge (Figure 3C). Taken together, these results demonstrate that FAM122A knockout regulates

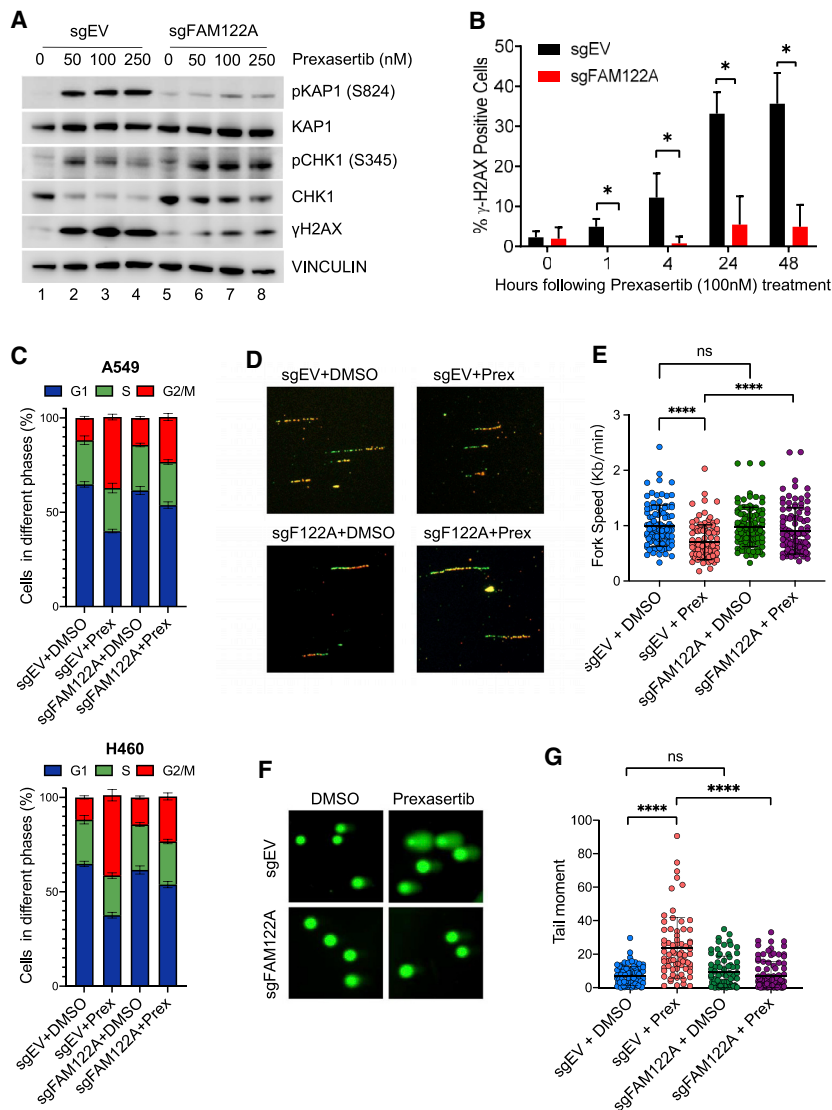


Figure 2. FAM122A Loss Rescues Lung Cancer Cells from Replication Stress, DNA Damage, and G2/M Arrest Caused by CHK1i

(A) Western blots of the lysates from control and FAM122A-KO A549 cells after treatment with prexasertib for 24 h.

(B) Quantification of γH2AX foci in control and FAM122A-KO A549 cells after treatment with prexasertib for up to 48 h. Error bars indicate standard errors, and p values were calculated using Student's t test (n = 3, *p < 0.001).

(C) Cell-cycle distribution of phosphatidylinositol (PI)-stained control and FAM122A-KO A549 (upper panels) and H460 (lower panels) cells treated with 250 nM prexasertib for 24 h. Data are shown as mean ± SD from three independent experiments.

(D) Representative images of the DNA fibers in control and FAM122A-KO A549 cells after treatment with DMSO or prexasertib (100 nM) for 24 h. Cells were sequentially labeled with IdU and CldU after exposure to prexasertib for 24 h, and the DNA fiber assay was performed to determine the replication fork speed.

(E) Quantitation of the replication fork speed in control and FAM122A-KO A549 cells after treatment with DMSO or prexasertib (100 nM) for 24 h. DNA fibers in (D) were analyzed from >100 ongoing replication forks in each condition. Error bars indicate standard errors, and p values were calculated using Student's t test (****p < 0.0001).

(F) Representative images of alkaline comets in control and FAM122A-KO A549 cells exposed to prexasertib (250 nM) for 24 h.

(G) Quantitation of the comet tail moment in (F) from more than 100 cells in each condition. Error bars indicate standard errors, and p values were calculated using Student's t test (****p < 0.0001).

FAM122A Regulates WEE1 Protein Levels through Inhibition of the PP2A Phosphatase

A recent study indicated that the FAM122A protein binds and inhibits the

the stability of the WEE1 protein, and its effect appears to be mediated via the phosphorylation state of WEE1.

It is well known that inhibitors of ATR, CHK1, or WEE1 can cause replication stress (Matheson et al., 2016; Qiu et al., 2018; Weber and Ryan, 2015), increase phosphorylation of the pKAP1 protein, and increase γH2AX levels. As expected, the knockout of FAM122A reduced the replication stress and DNA damage induced by the CHK1i prexasertib. Interestingly, we observed increased replication stress resulting from the combination of prexasertib plus the WEE1 inhibitor (WEE1i) AZD1775 combination (Figure 3D). Accordingly, while loss of FAM122A conferred a strong level of resistance to prexasertib, these cells were partially resensitized by the combination of prexasertib and WEE1 inhibitor in the media (Figures 3E and S3C). The combination of prexasertib and AZD1775 also increased the replication stress and G2/M accumulation in FAM122A knockout A549 cells (Figures S3D and S3E).

PP2A phosphatase with the B55α regulatory subunit (Fan et al., 2016), although the functional impact on replication stress and cell-cycle transition was not evaluated. PP2A is a ubiquitously expressed, heterotrimeric serine/threonine (S/T) phosphatase consisting of a scaffold (A) subunit, a regulatory (B) subunit, and a catalytic (C) subunit (Janssens and Goris, 2001; Janssens et al., 2008). Based on the assortment and use of these three subunits, there may be as many as eighty distinct trimeric PP2A complexes, some with distinct cell-specific expression and substrate preference. PP2A is known to regulate many cell functions, including several cell-cycle transitions (Harvey et al., 2011; Johnson and Kornbluth, 2012; Wlodarchak and Xing, 2016). Target specificity of PP2A is directed by the variable regulatory (B) subunit. PP2A dephosphorylates several substrates involved in transformation and cancer, including MYC, Wnt, and components of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. By dephosphorylating pCDC25, PP2A also plays

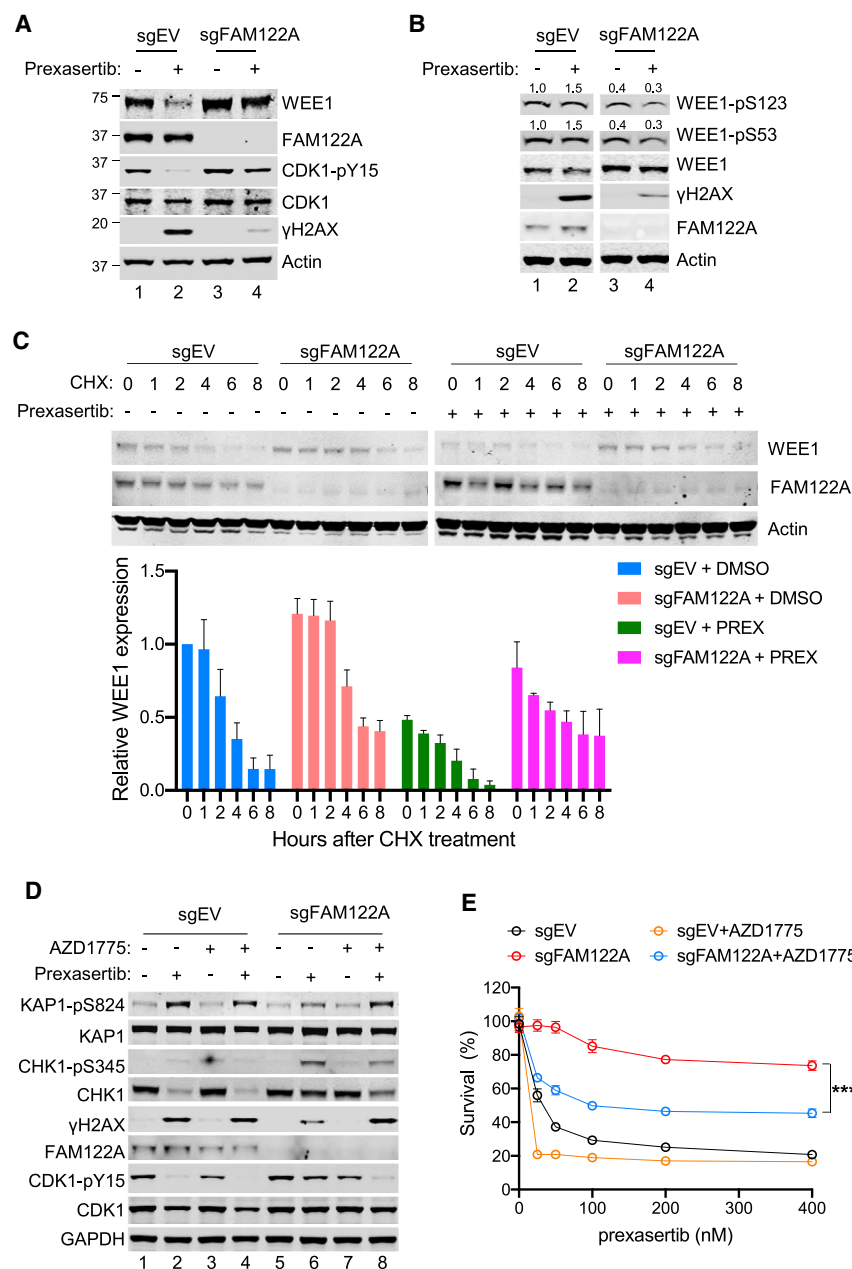


Figure 3. Depletion of FAM122A Promotes WEE1 Stability in A549 Lung Cancer Cells

(A) Western blots of the lysates from control and FAM122A-KO A549 cells after treatment with prexasertib (100 nM) for 24 h.

(B) Western blots of the lysates from control and FAM122A-KO A549 cells treated with/without prexasertib (100 nM) for 16 h. The relative levels of phosphorylated WEE1 compare to WEE1 were quantified as indicated.

(C) Top: Western blots of the lysates from control and FAM122A-KO A549 cells treated with DMSO or prexasertib (100 nM) for 24 h, followed by treatment with cycloheximide (CHX) for the indicated duration hours. Bottom: quantification of WEE1 protein levels. Error bars indicate standard errors (n = 3).

(D) Western blots of the lysates from control and FAM122A-KO A549 cells treated with prexasertib (100 nM) or AZD1775 (50 nM) for 24 h.

(E) Survival plots of control and FAM122A-KO A549 cells treated with graded concentrations of prexasertib along with 50 nM WEE1i (AZD1775). Note that WEE1i sensitizes resistant FAM122A-KO A549 cells to prexasertib. Data are shown as mean $\pm$ SD from three independent experiments. sgFAM122A versus sgFAM122A+AZD1775, ****p < 0.0001; statistical analysis was performed using two-way ANOVA.

PP2A complex and promotes the ubiquitin-dependent degradation of the C α subunit (Fan et al., 2016).

We reasoned that the PP2A complex, and specifically the A α /B55 α /C α trimeric complex, might stabilize WEE1 protein by dephosphorylating WEE1 at the S53 and S123 sites. Furthermore, given its function as an inhibitor of this PP2A-B55 α complex (Fan et al., 2016), the FAM122A protein might play a normal cellular role in inhibiting PP2A-B55 α , blocking WEE1 dephosphorylation, and promoting WEE1 degradation, thereby releasing the G2/M checkpoint. To test this hypothesis, we further analyzed the data collected from our CRISPR screen in the A549 and H460 cells. While sgRNAs

a critical role in the G2/M cell-cycle transition (Forester et al., 2007; Yan et al., 2010).

While loss of function of PP2A is critical for tumor cell growth, mutations in the PP2A subunits are rare, suggesting that other mechanisms can modulate PP2A activity. Indeed, endogenous inhibitors of PP2A, such as SET and CIP2A, have been identified. These inhibitors have abnormal expression patterns in cancer cells and are candidate anti-cancer targets (Janghorban et al., 2014; Junttila et al., 2007; Khanna et al., 2013). FAM122A was recently identified as an endogenous inhibitor of the A α /B55 α /C α trimeric complex of PP2A, and it has direct binding interactions with the B55 α and C α subunits of this complex. FAM122A binding inhibits the catalytic activity of this specific

directed against FAM122A conferred CHK1i resistance, sgRNAs directed against the B55 α regulatory subunit (PPP2R2A) of the PP2A phosphatase complex conferred sensitivity to CHK1is (Figures 4A and 4B; Tables S1, S2, and S3). This observation supported a model in which FAM122A loss leads to PP2A-B55 α dephosphorylation of WEE1, resulting in WEE1 protein stabilization, reduction of replication stress, and an enhanced G2/M checkpoint.

We next further examined the role of specific subunits of the trimeric PP2A complex in the dephosphorylation of WEE1 (Figure 4C). A549 cells were sensitive to the CHK1i prexasertib, and knockout of FAM122A resulted in prexasertib resistance. siRNA knockdown of the B55 α subunit of PP2A resensitized

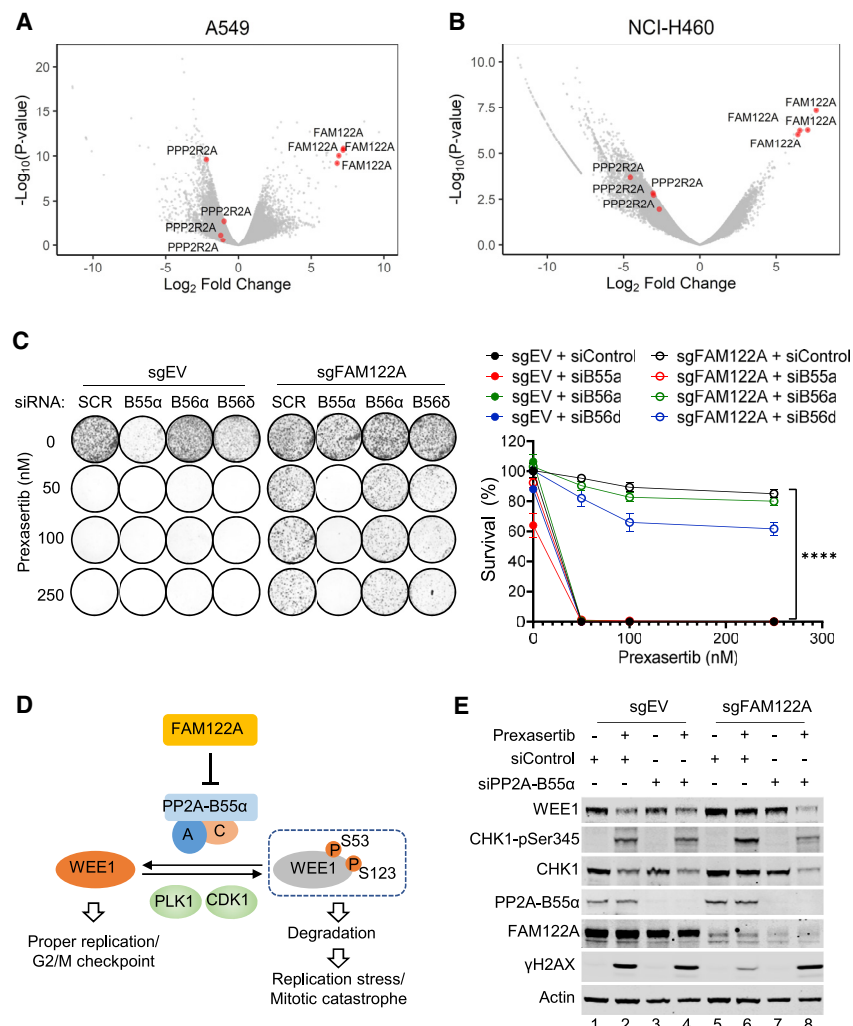


Figure 4. FAM122A Regulates WEE1 Stability by Targeting PP2A-B55α in Lung Cancer Cells

(A) Volcano plots showing genes targeted by sgRNAs that were differentially depleted or enriched following prexasertib selection in A549 cells. Note that all four sgRNAs directed to the PP2A B55α subunit PPP2R2A were found to be decreased following prexasertib selection in A549 cells.

(B) Volcano plots showing genes targeted by sgRNAs that were differentially depleted or enriched following prexasertib selection in H460 (NCI-H460) cells. Note that all four sgRNAs directed to the PP2A B55α subunit PPP2R2A were found to be decreased following prexasertib selection in H460 cells.

(C) Clonogenic assay of control and FAM122A-KO A549 cells treated with indicated siRNAs against PP2A subunits, followed by exposure to prexasertib. Note that knockdown of the B55α subunit of PP2A reverses CHK1i prexasertib resistance in FAM122A-KO A549 cells. In the right panel, siB55α and siB56α are referred as siB55a and siB56a, respectively. siB56δ is referred as siB56d. Data are shown as mean ± SD from three independent experiments. sgFAM122A+siControl versus sgFAM122A+siPP2A-B55α, ****p < 0.0001; statistical analysis was performed using two-way ANOVA.

(D) Schematic of the model for WEE1 regulation by the FAM122A-PP2A pathway.

(E) Western blots of the lysates from control and FAM122A-KO A549 cells treated with prexasertib (100 nM) for 24 h after treatment with siControl or siPP2A-B55α.

FAM122A knockout cells to the drug, demonstrating that B55α is the critical subunit of PP2A required for dephosphorylation of WEE1. Accordingly, loss of B55α resulted in increased WEE1 degradation and increased prexasertib sensitivity. In contrast, siRNA knockdown of other B subunits of PP2A, such as the B56α and B56δ subunits, failed to resensitize the FAM122A knockout cells to prexasertib. Taken together, these results support a model in which PP2A, containing the B55α subunit, is the critical trimeric phosphatase required for FAM122A-regulated dephosphorylation of WEE1 (Figure 4D). To further confirm this model, we determined the effect of PP2A-B55α knockdown on the WEE1 protein expression level (Figure 4E). Again, knockout of FAM122A in A549 cells resulted in an increased WEE1 protein expression level, which was reversed by knockdown of the B55α subunit (Figure 4E, lane 8).

Moreover, knockdown of another PP2A subunit, the Cα catalytic subunit, also reduced the level of WEE1 protein (Figure S4A). Also, a broad-spectrum inhibitor of PP2A activity, okadaic acid (OA), reduced WEE1 protein levels and was able to override the effect of the FAM122A knockout (Figure S4B). The PP2A inhibitor LB-100 was synergistic with prexasertib, and this syner-

gism was increased by sgRNA knockout of FAM122A (Figure S4C). These results suggest that PP2Ai and CHK1i may both contribute to the degradation of WEE1.

Thus, a combination of a PP2A inhibitor plus a CHK1i may be synergistic in an anti-cancer clinical trial. siRNA knockdown of α-endosulfine (ENSA), another endogenous inhibitor of PP2A, but one that targets the B55δ regulatory subunit (Mochida et al., 2010), did not result in a reduction of prexasertib-mediated cell death (Figure S4D), suggesting a specific role of FAM122A in the regulation of CHK1 pathway. Taken together, these data support a model in which a trimeric PP2A complex, composed of the Aα/B55α/Cα subunits, can dephosphorylate WEE1. Consistent with these results, FAM122A is a known inhibitor of this PP2A-B55α complex (Fan et al., 2016). These findings also suggest that a PP2A inhibitor, or a WEE1 inhibitor, may be useful in the treatment of CHK1i-resistant tumor cells.

CHK1 Phosphorylates FAM122A on S37, Resulting in Activation of PP2A

Inspection of the primary amino acid sequence of FAM122A revealed a conserved potential CHK1 phosphorylation site at S37 (Figure S5A). The amino acid consensus sequence for a CHK1 phosphorylation site is RXXS. Moreover, phosphoproteomic databases demonstrated that FAM122A is indeed

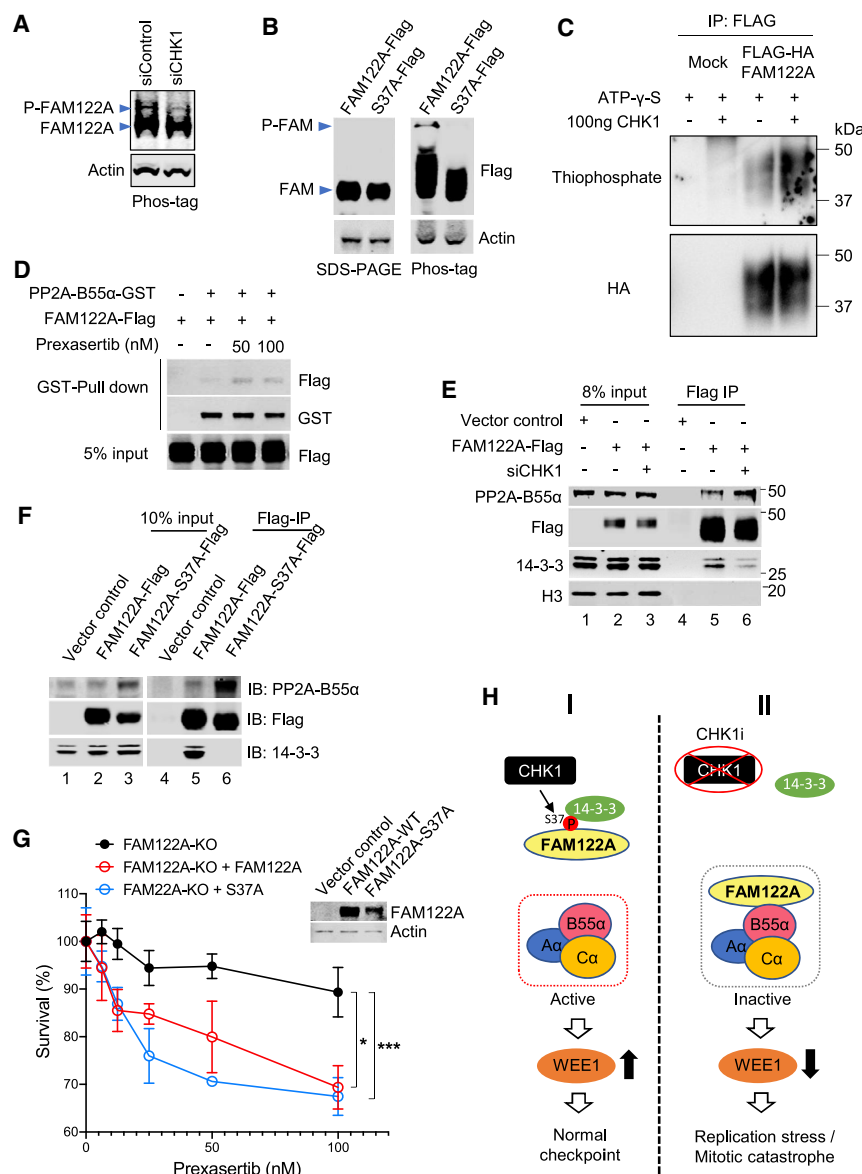


Figure 5. Chk1 Phosphorylates FAM122A at S37

(A) Western blots of the lysates from A549 cells treated with siControl or siCHK1. Samples were separated on a 12% Phos-tag-SDS-PAGE gel. Phosphorylated and unphosphorylated FAM122A are labeled.

(B) Western blots of the lysates from 293T cells expressing FLAG-tagged FAM122A (wild-type [WT] and S37A). Samples were separated on a normal SDS-PAGE gel (left) and a Phos-tag-labeled SDS-PAGE gel (right). Phosphorylated and unphosphorylated FAM122A are labeled.

(C) Kinase assays of 293T cells expressing FLAG-FAM122A. 293T cells were transiently transfected with hemagglutinin (HA)-FLAG-tagged FAM122A, followed by immunoprecipitation with anti-FLAG antibody. HA-FLAG-FAM122A was incubated with ATP-γ-S and with or without purified CHK1. Phosphorylation of FAM122A was detected by thiophosphate.

(D) Anti-glutathione S-transferase (anti-GST) immunoprecipitation assays followed by Western blots of stably expressing FAM122A-FLAG 293T cells. FAM122A-FLAG 293T cells were transiently transfected with PP2A-B55α-GST and then treated with prexasertib at indicated concentrations for 24 h, followed by GST-pull-down assay and Western blots.

(E) Anti-FLAG immunoprecipitation assays followed by Western blots of FAM122A-FLAG 293T cells. Stably expressing FAM122A-FLAG or control 293T cells were treated with siCHK1, followed by FLAG-pull-down assay, and Western blots using anti-FLAG, anti-PP2A-B55α, anti-14-3-3, and anti-H3 antibodies.

(F) Anti-FLAG immunoprecipitation (IP) assays followed by Western blots of FAM122A-FLAG 293T cells. 293T cells expressing FLAG-tagged FAM122A (WT and S37A mutant) were used for FLAG-IP and Western blots.

(G) Survival plots of FAM122A-KO A549 cells transiently transfected with FAM122A-WT and -S37A mutant, followed by treatment with prexasertib for 3 days. Data are shown as mean ± SD from three independent experiments. sgFAM122A versus sgFAM122A+FAM122A, *p < 0.05; sgFAM122A versus sgFAM122A+FAM122A-S37A, ***p < 0.001. Statistical analysis was performed using two-way ANOVA. The expression of the

a phosphoprotein. (<https://www.phosphosite.org/siteGroupAction.action?id=3208890&protOrg=9790&showAllSites=true&showHTPPRefsOnly=true#4>), though the functional importance of its phosphorylation is unknown. We reasoned that the CHK1-mediated phosphorylation of FAM122A might negatively regulate the suppressive activity of FAM122A, resulting in increased PP2A-mediated phosphatase activity directed toward the WEE1 substrate. We therefore tested whether the S37 residue is a bona fide CHK1-mediated phosphorylated site of FAM122A.

Using a phosphotag reagent (Kinoshita et al., 2015), we demonstrated that WT FAM122A is indeed a phosphoprotein (Figures 5A and 5B). siRNA-mediated knockdown of CHK1 reduced FAM122A phosphorylation (Figure 5A). In

contrast, a mutant version of FAM122A, containing the S37A mutation, failed to bind the phosphotag reagent (Figure 5B). Consistent with this result, WT FAM122A was also phosphorylated by CHK1 in an *in vitro* kinase assay (Figure 5C), demonstrating that FAM122A is a direct substrate of CHK1. The CHK1i prexasertib reduced the phosphorylation of WT-FAM122A (Figures S5B and S5C). The effect of siRNA-mediated knockdown of CHK1 (Figure 5A) was comparable to the effect of cellular exposure to the CHK1i (Figures S5B and S5C). Also, hydroxyurea (HU), a known stimulant of CHK1 kinase activity, activated the phosphorylation of FAM122A, and prexasertib inhibited this phosphorylation (Figure S5D).

We next performed a GST-PP2A pull down of cellular FAM122A. GST-PP2A-B55 α could weakly bind to WT FAM122A, but the binding activity was significantly enhanced when prexasertib was used to inhibit the CHK1-mediated phosphorylation of FAM122A (Figure 5D). Taken together, these results further confirm that CHK1 phosphorylates FAM122A on S37 and that phosphorylation dissociates FAM122A from the PP2A complex, resulting in regulated PP2A activation.

Phosphorylated FAM122A Is Released from PP2A Complex and Binds to 14-3-3 Proteins

Previous studies have indicated that 14-3-3 proteins bind to phosphoproteins in the cytoplasm and prevent the nuclear transport of these proteins (Madeira et al., 2015; Margolis et al., 2006). To determine whether pS37-FAM122A binds to 14-3-3 protein, we next pulled down FLAG-FAM122A and immunoblotted for 14-3-3 (Figure 5E). As predicted, siRNA knockdown of CHK1 resulted in reduced coimmunoprecipitation of FAM122A and 14-3-3 (Figure 5E, lane 6), consistent with the reduced level of FAM122A phosphorylation in these cells.

We next examined the cellular localization of 14-3-3 and FAM122A in the absence or presence of CHK1 knockdown or inhibition. 14-3-3 and FAM122A were localized primarily to the cytoplasmic fraction of A549 cells (Figures S5E and S5F). Interestingly, an siRNA knockdown of CHK1 (Figure S5E) or cellular exposure to the CHK1i (Figure S5F) resulted in an increased accumulation of FAM122A in the nucleus. These results are consistent with a model in which FAM122A normally inhibits the PP2A complex in the nucleus. Activation of CHK1 results in the release of FAM122A from PP2A and the sequestration of pFAM122A by 14-3-3 in the cytoplasm. However, knockdown of CHK1 or CHK1 inhibition enhances the binding of unphosphorylated FAM122A to PP2A in the nucleus, thereby blocking PP2A-mediated dephosphorylation of WEE1 and promoting WEE1 degradation.

We next evaluated the ability of FAM122A WT versus FAM122A mutant proteins to differentially bind and coimmunoprecipitate with PP2A and 14-3-3 protein. The WT-FAM122A protein bound constitutively to PP2A or 14-3-3. Interestingly, the S37A-FAM122A mutant protein had enhanced binding to the PP2A complex but no detectable binding to the 14-3-3 protein (Figure 5F). As predicted, WT FAM122A bound to 14-3-3 (Figure 5F, lane 5), but not to PP2A. The converse was observed for the FAM122A-S37A protein (Figure 5F, lane 6). It bound to PP2A, but not to 14-3-3. Taken together, these results confirm that S37 is the critical site of CHK1-mediated phosphorylation on FAM122A and that the unphosphorylated form of FAM122A is a negative regulator of the PP2A-B55 α phosphatase.

Finally, we performed a functional assessment of these two proteins (Figure 5G). A549 cells with a CRISPR knockout of FAM122A, which are resistant to CHK1i, were transiently transfected with either the FAM122A-WT or the FAM122A-S37A mutant. Both proteins were able to partially resensitize the cells to CHK1i. A model describes how CHK1 phosphorylation of FAM122A can upregulate the expression of the WEE1 protein (Figure 5H, panels I and II).

FAM122A Is a Predictive Biomarker for CHK1i Response in Cancer

Since knockout of FAM122A promotes CHK1i as ATRi resistance, we next determined whether mutation or low-level expression of FAM122A is observed in human cancers or cancer cell lines (Figure 6). CBioPortal analysis revealed only a low number of human cancer samples with bona fide FAM122A mutations (data not shown). Cancers did, however, exhibit a low level of FAM122A mRNA expression compared to normal tissue, suggesting a role of FAM122A as a tumor suppressor (Figure 6A).

We next selected ovarian and lung tumor cell lines for CHK1i resistance (Figure S6A). Interestingly, resistant lines exhibited a low level of FAM122A and a high level of WEE1 protein (Figures 6B and S6B–S6D). Consistent with these results, the knockdown of FAM122A in other cell lines, including HEPG2, two pancreatic cancer cell lines, and HeLa cells, also resulted in an increase in WEE1 protein expression levels (Figures S6E–S6H). Correction of one of these cell lines (line ES2), through transfection with WT-FAM122A cDNA, conferred partial restoration of their CHK1i sensitivity (Figures 6C and 6D). We next determined the effect of the WEE1 inhibitor AZD1775 on the CHK1i-sensitive and CHK1i-resistant ES2 ovarian cancer cells (Figures 6E and 6F). For these experiments, we used a low dose of AZD1775 (50 nM), which has little effect on either cell line as a single agent. Indeed, AZD1775 alone did not increase pKAP1 levels in either the ES2 parent cells or the ES2-resistant cells (Figure 6E, lanes 3 and 7). Prexasertib activated replication stress (pKAP1) in ES2 cells, but not in ES2-resistant cells (Figure 6E, lane 2 versus lane 6). Addition of low-dose AZD1775 in combination with a CHK1i (prexasertib) significantly increased replication stress (elevated pKAP1) and DNA damage (increased γ H2AX) in ES2-resistant cells (Figure 6E, lane 8), providing a rationale for this drug combination in the clinic. Similarly, siRNA knockdown of PP2A-B55 α restored replication stress (increased pKAP1) and DNA damage (elevated γ H2AX) in the ES2-resistant cells exposed to prexasertib (Figure S6I, lane 8). We next analyzed the ability of the drug combination of CHK1i plus low-dose (50 nM) AZD1775 on ES2-resistant cells (Figure 6F), and this combination demonstrated significant killing of the resistant cells. Taken together, the cells showing decrease in FAM122A protein expression in response to prexasertib selection have decreased sensitivity to the CHK1i that can be overcome with the addition of a low dose of a WEE1 inhibitor.

FAM122A Is a Novel Tumor Suppressor Gene

Given that the FAM122A level correlates inversely with ATR-CHK1 pathway activity, we reasoned that cancer cells with elevated replication stress and a high dependency on *CHEK1* would exhibit a lower dependency on *FAM122A*. To test this hypothesis, we analyzed more than 500 cancer cell lines covering 20 cancer types (<https://www.broadinstitute.org/ccle> and <https://www.depmap.org>). Indeed, cell lines with high *CHEK1* dependency were less dependent on *FAM122A* for survival (Figures 7A, S7A, and S7B). There was no association between *CHEK2* dependency and *FAM122A* dependency, suggesting that this phenomenon is *CHEK1* specific (Figure 7B).

The rapid growth of tumor cells leads to more replication stress. However, a compensatory low expression of FAM122A

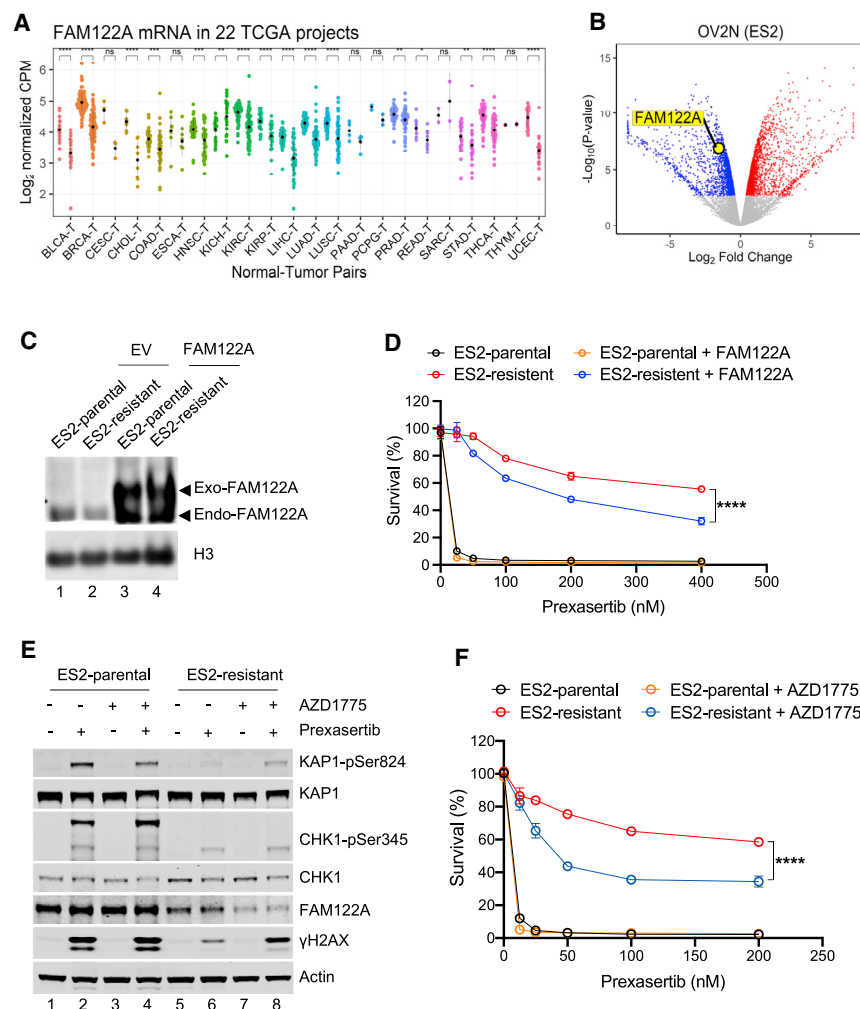


Figure 6. FAM122A Is a Predictive Biomarker for CHK1i Response in Multiple Cancer Types

(A) FAM122A RNA-sequencing (RNA-seq) expression in tumors (right) with matched normal tissues (left). The data were analyzed using 22 The Cancer Genome Atlas (TCGA) projects. Note that FAM122A expression is lower in many cancers compared to matched normal tissues.

(B) Volcano plot showing genes differentially downregulated or upregulated in prexasertib-resistant ES2 ovarian cells (OV2N ES2) compared to parental cells. Note that FAM122A expression is significantly decreased in ES2 cells with acquired resistance to prexasertib.

(C) Western blots of the lysates from stably expressing FAM122A-FLAG or FLAG-control (empty vector [EV]) parental and prexasertib-resistant ES2 cells. Note that anti-H3 was used as a protein loading control.

(D) Survival plots of stably expressing FAM122A-FLAG or FLAG-control parental and prexasertib-resistant ES2 cells after treatment with graded concentrations of prexasertib for 3 days. Data are shown as mean ± SD from three independent experiments. ES2-resistant versus ES2-resistant + FAM122A, ****p < 0.0001; statistical analysis was performed using two-way ANOVA.

(E) Western blots of the lysates from parental and prexasertib-resistant ES2 cells after treatment with prexasertib (50 nM) and AZD1775 (50 nM) for 24 h. (F) Survival plots of parental and prexasertib-resistant ES2 cells after treatment AZD1775 (50 nM) and graded concentrations of prexasertib for 3 days. Data are shown as mean ± SD from three independent experiments. ES2-resistant versus ES2-resistant + AZD1775, ****p < 0.0001; statistical analysis was performed using two-way ANOVA.

in these tumor cells would reduce the replication stress and promote tumor growth. Consistent with this concept, in multiple cancer types, patients whose tumors have low FAM122A expression have significantly worse overall survival than patients whose tumors have high FAM122A expression (Figure 7C).

DISCUSSION

The activation of the ATR-CHK1-WEE1 pathway is a critical upstream event in the G2/M checkpoint. Cancer cells, due to their intrinsic high levels of replication stress, are hyperdependent on this pathway. Accordingly, inhibitors of ATR, CHK1, or WEE1 are under active investigation in anti-cancer clinical trials (Matheson et al., 2016; Qiu et al., 2018; Weber and Ryan, 2015), with early promising results (Fang et al., 2019). Inhibition of the ATR-CHK1-WEE1 pathway disrupts the G2/M checkpoint and promotes rapid exit into mitosis, and cancer cell death.

Role of FAM122A in the Regulation of the G2/M Checkpoint

In the current study, we used a CRISPR screen to uncover a novel mechanism of CHK1i resistance. We identified FAM122A as a

novel physiologic regulator of the ATR-CHK1-WEE1 pathway. In the normal cellular setting, FAM122A binds and inhibits the heterotrimeric PP2A complex containing the B55α subunit, resulting in limited dephosphorylation of WEE1. In this setting, WEE1 levels are maintained at a low level. When CHK1 is activated by replication stress, FAM122A is phosphorylated and released from PP2A. In cancer cells, replication stress can result from any one of several events, including p53 loss, MYC amplification, cyclin E amplification, or loss of a homologous recombination (HR) gene (Zeman and Cimprich, 2014). The activated PP2A now dephosphorylates WEE1, resulting in increased WEE1 expression and contributing to the G2/M checkpoint (Figure 5H, panel I). An inhibitor of CHK1, such as prexasertib, blocks the phosphorylation of FAM122A, resulting in reduced PP2A activity and increased WEE1 degradation (Figure 5H, panel II). Thus, the CHK1i further increases replication stress in the cancer cells, thereby promoting cancer cell death.

Knockout of FAM122A results in increased PP2A-mediated dephosphorylation of WEE1, stabilization and increase levels of WEE1 protein, and restoration of the G2/M checkpoint. Indeed, cancer cells often reduce the expression of FAM122A by genetic or epigenetic means, thereby reducing their replication stress and

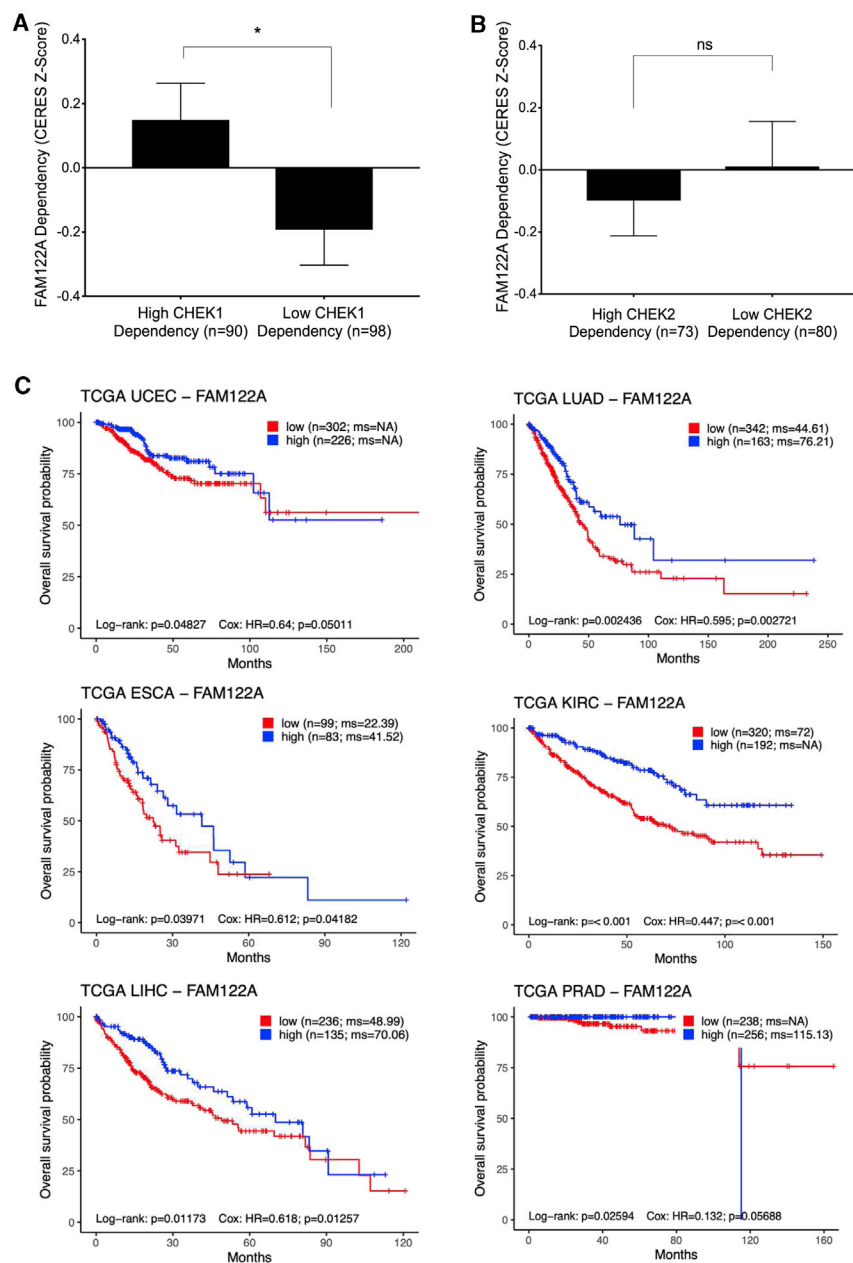


Figure 7. FAM122A Is a Novel Tumor Suppressor Gene

(A) *CHEK1* dependency inversely correlates with *FAM122A* dependency. Cell lines that were 1 SD below or above the mean in terms of *CHEK1* CERES score were grouped into cell lines that have high *CHEK1* dependency and low *CHEK1* dependency, respectively. A lower CERES score indicates a cell line is more dependent on that gene. (B) Absence of an association between *CHEK2* dependency and *FAM122A* dependency. Cell lines that were 1 SD below or above the mean in terms of *CHEK2* CERES score were grouped into cell lines that have high *CHEK2* dependency and low *CHEK2* dependency, respectively. (C) Kaplan-Meier survival curves of patients with indicated cancers stratified by *FAM122A* gene expression level using TCGA data. The log-rank test was used to assess statistical significance.

Role of WEE1 in the Regulation of the G2/M Checkpoint

Our results indicate that CHK1 enhances the WEE1-mediated checkpoint via at least two distinct mechanisms. First, previous studies have shown that CHK1 directly phosphorylates WEE1 (Lee et al., 2001; O’Connell et al., 1997). Activated WEE1 in turn phosphorylates the CDK1/cyclin B complex and promotes the G2/M checkpoint. Second, through its phosphorylation of FAM122A and release of the FAM122A/PP2A-B55 α complex, CHK1 enhances PP2A activity and reduces WEE1 degradation by ubiquitin-mediated degradation. Enhanced WEE1 protein levels further reduce replication stress and augment the G2/M checkpoint. Paradoxically, before mitotic initiation, PP2A-B55 α can also dephosphorylate WEE1 and reduce its kinase activity (Harvey et al., 2011). PP2A-B55 α can also dephosphorylate and subsequently inactivate CDC25 as cells exit mitosis (Forester et al., 2007; Johnson and Kornbluth, 2012). Hence,

allowing them to survive their high replication rate. Adding a PP2A inhibitor, such as OA, in this setting could block WEE1 dephosphorylation and reduce WEE1 protein levels, thereby resensitizing the tumor cells to prexasertib. Of note, the CRISPR screen identified an endogenous inhibitor of PP2A-B55 α as opposed to inhibitors of other heterotrimeric PP2A complexes containing different B subunits such as ENSA. This implies that targets of PP2A-B55 α , including WEE1, are involved in CHK1i resistance conferred by FAM122A loss, as opposed to protein targets of alternate PP2A heterotrimeric complexes. Development of pharmacologic inhibitors specifically of PP2A-B55 α as opposed to PP2A more broadly given its myriad roles would be anticipated to reverse CHK1i resistance with lower toxicity.

PP2A has multiple substrates and may function as both a positive and negative regulator of the G2/M checkpoint.

Effective Anti-cancer Therapy Increases the Replication Stress of Cancer Cells

Our results have important implications for the evolution of cancer and cancer drug development. Cancer cells tend to reduce their expression of FAM122A by either genetic or epigenetic mechanisms. The reduction in FAM122A allows the cells to increase their expression level of WEE1 protein, to enhance their G2/M arrest, and to tolerate the replication stress intrinsic to their oncogene-driven high replication rate. The purpose of inhibiting the ATR-CHK1-WEE1 pathway in cancer therapy is to further increase

replication stress of the cancer cells, to put the cells over the threshold of replication stress, and to kill the cells. A combination of a kinase inhibitor of ATR, CHK1, or WEE1 with a drug that inhibits the PP2A enzyme will further increase replication stress but in many cases also be toxic to normal cells. Since cancer cells have elevated replication stress to begin with, this strategy exploits the underlying therapeutic index of normal versus transformed cells.

Biomarker-Driven Development of New CHK1is

Our study suggests predictive and pharmacodynamic biomarkers for these ongoing trials. Tumors with high baseline levels of replication stress, as measured by high pKAP1 and pRPA, are more likely to respond to these inhibitors. Tumors with low levels of FAM122A are less likely to respond. FAM122A is a critical regulator of PP2A-B55 α phosphorylation activity. Based on its cellular functions, we propose changing the name of FAM122A to PABIR1, standing for PP2A alpha (PPP2R1A) and B55A (PPP2R2A) interacting phosphatase regulator 1. Finally, FAM122A plays a normal function in non-transformed cells. FAM122A likely functions to suppress PP2A-B55 α and promote the phosphorylation and degradation of WEE1 in these cells as well. Under conditions of elevated replication stress, in cancer cells exposed to DNA-damaging agents, CHK1 phosphorylates FAM122A. Phosphorylated FAM122A is sequestered by 14-3-3 proteins in the cytoplasm, thereby freeing active PP2A-B55 α to rescue WEE1 protein levels from degradation. Identification of tumors that are the most likely to respond to CHK1is as monotherapy, or the development of combination therapies that overcome potential mechanisms of CHK1i resistance, will hopefully increase the likelihood that CHK1is will have a durable role in the treatment of cancers.

Limitations of Study

Finally, our study has limitations. First, CHK1 kinase has multiple substrates in addition to FAM122A. Indeed, cellular knockout of FAM122A results in a reduction of replication stress and an increase in the G2/M checkpoint. However, the relative contribution of FAM122A compared to other CHK1 substrates with respect to these cellular activities remains largely unknown. Second, our studies indicate that knockout of FAM122A results in resistance to either a CHK1i or an ATR inhibitor. How FAM122A knockout confers resistance to an ATR inhibitor will require additional experimentation. Third, our results suggest that a combination of a CHK1i plus a WEE1 inhibitor may override the cellular resistance to CHK1i monotherapy. However, this drug combination may prove to be too toxic for use in a clinical setting. Finally, our study focused mostly on cancer cell lines, especially lung cancer cell lines. It will be important to determine whether FAM122A knockdown is also an *in vivo* mechanism of acquired CHK1i resistance of tumors isolated from treated cancer patients.

STAR★METHODS

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Supplemental Information can be found online at <https://doi.org/10.1016/j.molcel.2020.10.008>.

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AUTHOR CONTRIBUTIONS

F.L., D.K., J.D., G.I.S., D.C., and A.D.D. conceived the study, analyzed the data, and wrote the manuscript. P.D., T.B., and X.-F.Z. performed the experiments and computational analysis, analyzed the data, and helped write the manuscript. P.D. and K.P. performed the experiments and analyzed the data. D.K., H.N., and C.D. performed the computational analysis, analyzed the data, and helped write the manuscript. J.D. and C.D. contributed to writing the manuscript. D.K. helped with the design of the CRISPR screen and performed bioinformatics analysis.

DECLARATION OF INTERESTS

D.K. has served as a consultant to Vertex. J.D. has served as a consultant to Merck & Co. and EMD Serono and has received research funding from Constellation Pharmaceuticals. G.I.S. is a consultant/advisory board member for Lilly, Sierra Oncology, Merck-EMD Serono, Pfizer, Astex, Almac, Roche,

Bicycle Therapeutics, Fusion Pharmaceuticals, G1 Therapeutics, Bayer, Ipsen, Cybrexa Therapeutics, Angiex, Daiichi Sankyo, and Seattle Genetics and reports receiving commercial research grants from Lilly, Sierra Oncology, Merck-EMD Serono, and Merck & Co. A.D.D. is a consultant and/or advisory board member for AstraZeneca, Bayer AG, Cedilla Therapeutics, Celgene, Cytair Therapeutics, Epizyme, GalaxoSmithKline, Ideaya, Impact Therapeutics, LAV Global Management Company Limited, L.E.K. Consulting, Lilly Oncology, Merck KGaA, Novartis Bioventures, Novo Ventures, Pfizer, Scholar Rock, and Tango Therapeutics; is a stockholder in Ideaya, Cedilla Therapeutics, and Cytair; and reports receiving commercial research grants from Lilly Oncology and Merck KGaA.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FAM122A	Invitrogen	Cat# MA5-24510; RRID: AB_2633482
β-actin	Cell Signaling Technology	Cat# 4967S; RRID: AB_330288
Vinculin	Cell Signaling Technology	Cat# 13901S; RRID: AB_2728768
KAP1	Abcam	Cat# ab22553; RRID: AB_447151
KAP1-phospho-S824	Abcam	Cat# ab84077; RRID: AB_1861686
CHK1	Cell Signaling Technology	Cat# 2360S; RRID: AB_2080320
CHK1-phospho-S345	Cell Signaling Technology	Cat# 2348S; RRID: AB_2080326
WEE1	Cell Signaling Technology	Cat# 4936S; RRID: AB_2288509
WEE1-phospho-S53	Bioss	Cat# bs-5589R; RRID: AB_11075853
WEE1-phospho-S123	Bioss	Cat# BS-5218R; RRID: AB_11111792
CDK1	Abcam	Cat# ab18; RRID: AB_2074906
CDK1-phospho-Y15	Cell Signaling Technology	Cat# 9111S; RRID: AB_331460
PP2A-B55-alpha	Sigma	Cat# SAB4300573; RRID: AB_10622182
PP2A-B55-alpha	Santa Cruz Biotechnology	Cat# SC-81606; RRID: AB_2252935
HA-Tag	Cell Signaling Technology	Cat# 3724S; RRID: AB_1549585
Flag	Sigma	Cat# F3165; RRID: AB_259529
Flag	Sigma	Cat# F7425; RRID: AB_439687
GST	Cell Signaling Technology	Cat# 2625S; RRID: AB_490796
14-3-3	Cell Signaling Technology	Cat# 8312S; RRID: AB_10860606
GAPDH	Cell Signaling Technology	Cat# 5174S; RRID: AB_10622025
γ-H2AX	Cell Signaling Technology	Cat# 2577S; RRID: AB_2118010
BrdU	Cell Signaling Technology	Cat# 5292S; RRID: AB_10548898
BrdU	Abcam	Cat# ab6326; RRID: AB_305426
Histone H3	Cell Signaling Technology	Cat# 9715S; RRID: AB_331563
Goat anti-Rat IgG (Alexa Fluor 488)	Invitrogen	Cat# A-11006; RRID: AB_2534074
Goat anti-Mouse IgG (Alexa Fluor 488)	Invitrogen	Cat# A32723; RRID: AB_2633275
Chicken anti-mouse IgG (Alexa Fluor 594)	Invitrogen	Cat# A-21201; RRID: AB_141630
Chemicals, Peptides, and Recombinant Proteins		
Prexasertib	Eli Lilly & Co.	Cat# S7178
SRA737	Sierra Oncology	Cat# S8253
VE822	Selleckchem	Cat# S7102
AZD1775	Selleckchem	Cat# S1525
LB-100	Selleckchem	Cat# S7537
5-Chloro-2'-deoxyuridine (CldU)	Sigma Aldrich	Cat# C6891
5-Iodo-2'-deoxyuridine (IdU)	Sigma Aldrich	Cat# I7125
EdU (5-ethynyl-2'-deoxyuridine)	ThermoFisher	Cat# E10415
Cycloheximide	Sigma Aldrich	Cat# 01810
Critical Commercial Assays		
CellTiter-Glo Luminescent Viability Assay	Promega	Cat# G7570
DNeasy Blood & Tissue Kit	QIAGEN	Cat# 69504
Click-iT EdU Imaging Kit with Alexa Fluor 488	ThermoFisher	Cat# C10086
PowerUp SYBR Green Master Mix	Life Technologies	Cat# A25741

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Subcellular Fractionation Kit	Cell Signaling Technology	Cat# 9038
Deposited Data		
Raw imaging data	This paper; Mendeley data	https://data.mendeley.com/datasets/ndvs5mf8hx/1
RNA-seq data	This paper	GEO: GSE158338
Experimental Models: Cell Lines		
NCI-H520	ATCC	N/A
HCC827	ATCC	N/A
NCI-H1563	ATCC	N/A
NCI-H2087	ATCC	N/A
SKLU-1	ATCC	N/A
NCI-H1915	ATCC	N/A
CALU-6	ATCC	N/A
NCI-H1299	ATCC	N/A
NCI-H522	ATCC	N/A
NCI-H23	ATCC	N/A
A549	ATCC	N/A
NCI-H460	ATCC	N/A
U2OS	ATCC	N/A
HeLa	ATCC	N/A
HepG2	ATCC	N/A
MIAPaCa	Gift from Dr. Andrew Aguirre	N/A
PaTu8902	Gift from Dr. Andrew Aguirre	N/A
293T	ATCC	N/A
ES2	ATCC	N/A
A2780	Ceccaldi et al., 2015	N/A
Oligonucleotides		
siFAM122A	Dharmacon	E-015499-00
siPPP2CA	QIAGEN	SI00041853
siPPP2R2A	QIAGEN	SI00041909
siPPP2R5A	QIAGEN	SI02225846
siPPP2R5D	QIAGEN	SI00086240
siCHEK1	QIAGEN	SI02660007
siENSA	QIAGEN	SI00063581
AllStars Neg. Control siRNA	QIAGEN	0001027281
siBRCA1	QIAGEN	SI02654575
siTP53BP1:	QIAGEN	SI02653168
FAM122A-qPCR-Forward Primer: AAGATGGAGCTAGACCTGGAG	eurofins	N/A
FAM122A-qPCR-Reverse Primer: CCGGCGAAGTGCTCACTGAG	eurofins	N/A
gRNA targeting FAM122A #1: GCAGATAAGCCACTCCTGGG	Invitrogen	N/A
gRNA targeting FAM122A #2: CAGTGACACTTCGCCGGTGT	Invitrogen	N/A
Software and Algorithms		
Prism8 GraphPad software	N/A	https://www.graphpad.com/
imageJ software	N/A	https://imagej.nih.gov/ij/

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alan D. D'Andrea (Alan_Dandrea@dfci.harvard.edu).

Materials Availability

The knockout cell lines generated in this study are available from the Lead Contact without restriction.

Data and Code Availability

All the original data for immunofluorescence and western blots have been deposited with Mendeley and can be accessed with <https://data.mendeley.com/datasets/ndvs5mf8hx/1>. The STARS analysis for the CHK1 (Prexasertib) genome-wide CRISPR screens in A549 and H460 cells, positive selection hits, is available in [Table S1](#). The STARS analysis for the CHK1 (Prexasertib) genome-wide CRISPR screens in A549 and H460 cells, negative selection hits, is available in [Table S2](#). edgeR Analysis for the CHK1 (Prexasertib) Genome-wide CRISPR Screens in A549 and H460 cells is available in [Table S3](#). The RNA-seq datasets discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (PMID: 11752295) and are accessible through GEO Series accession number GSE158338.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Lines

A549 and NCI-H460 cells stably expressing the Cas9 endonuclease were obtained from the Genetic Perturbation Platform at the Broad Institute and were cultured at 37°C/5% CO₂ incubator in RPMI 1640 (GIBCO) media supplemented with 10% fetal bovine serum (FBS, Sigma), 1% penicillin-streptomycin (GIBCO). U2OS and HEK293T cells were cultured at 37°C/5% CO₂ in DMEM (high glucose, Hyclone) media supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (GIBCO). Other human NSCLC cell lines A549, Calu-6, HCC827, NCI-H23, NCI-H460, NCI-H520, NCI-H522, NCI-H1299, NCI-H1563, NCI-H1915, NCI-H2087, and SKLU-1 were grown at 37°C in a humidified 5% CO₂ incubator in GIBCO RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1 µg/mL Normocin (GIBCO). Each of these cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and validated by short tandem repeat (STR) testing.

METHOD DETAILS

Generation of Stable Cell Lines

For generation of cells with stable expression of exogenous genes, the constructs were transfected into 293T cells for retroviral packaging and subsequent transduction. For generation of prexasertib-resistant cell lines, cells were cultured with graded concentrations of prexasertib for one week and surviving cells in the drug concentrations of IC₉₀ or IC₈₀ were harvested. These cells were then cultured in the IC₅₀ drug concentrations for 1-2 weeks and were gradually adapted to higher doses of drug for almost 2-3 months. The resistant cells were then maintained in a medium without the drug.

Drug Selection CRISPR Screens

NSCLC cell lines A549 and NCI-H460 stably expressing the Cas9 endonuclease (pXPR_311) were obtained from the Genetic Perturbation Platform at the Broad Institute. The lines were grown at 37°C in a humidified 5% CO₂ incubator in GIBCO RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 100 µg/ml Normocin (InvivoGen, San Diego, CA). The pXPR_311 plasmid contains a blasticidin resistance tag to allow for selection of infected cells. For each line, the selected Cas9 positive cells were infected in four 12-well tissue culture plates with the Brunello whole genome sgRNA pooled lentiviral library consisting of 76,441 sgRNAs targeting 19,114 different genes ([Doench et al., 2016](#)). For adequate representation of each sgRNA, e.g., an average of 500 cells/sgRNA, a total of 135 million cells divided into wells each containing 3 million cells were infected at a multiplicity of infection (MOI) of 0.3 in media containing 8 µg/ml polybrene. Plates were centrifuged at 2000 rpm for 2 h at 37°C, and then 2 h after centrifugation, media was replaced with fresh media without polybrene and cells were incubated overnight. Infected cells were transferred to 15 cm tissue culture dishes and selected with 2 µg/mL puromycin for 4 days prior to drug treatment. After selection, 40 million cells (8 plates each containing 5 million cells) were plated for each biological replicate, and an additional 40 million cells per replicate were collected and frozen for subsequent genomic DNA (gDNA) extraction. A549 and NCI-H460 cells were then treated with 100nM (A549) or 50nM (NCI-H460) prexasertib versus DMSO control, for 19 days. Cells were re-plated every 3-4 days and maintained in drug or DMSO-containing media, each time plating a minimum of 40 million cells per replicate. At the end of the screen, the cells were harvested and frozen for gDNA extraction.

Cells were collected pre- and post-drug selection, genomic DNA was extracted, and sgRNA sequences were PCR amplified and Illumina sequenced. Top hits among the gene knockouts that conferred drug resistance were identified by the STARS gene-ranking

algorithm (Doench et al., 2016). Validation of hits was performed with sgRNAs (Broad Institute) and pooled siRNAs (QIAGEN). LY2606368 IC₅₀s were assessed using colony formation and CellTiter-Glo cell viability assays.

Genomic DNA Preparation, Sequencing, and Identification of Top Hits

gDNA was isolated using QIAGEN QIAamp DNA blood midi and maxi kits, according to manufacturer's protocol. PCR of gDNA was performed to attach sequencing adaptors and barcode samples for sgRNA sequencing, as previously described (Doench et al., 2016). Samples were sequenced on an Illumina HiSeq2000. Reads per million were log₂-transformed by first adding one to all values, which was necessary to take the log of sgRNAs with zero reads. For analysis, these normalized data were used to determine the log₂-fold change of each sgRNA for the drug treatment group relative to DMSO control group (Shalem et al., 2014). The STARS gene-ranking algorithm was used to determine the top hits (Tables S1 and S2) (<https://portals.broadinstitute.org/gpp/public/software/stars>); at least two sgRNAs targeting a gene had to rank within the top 10% of sgRNAs by log₂-fold change in one direction or another (Doench et al., 2016). The hit genes were scored using the STARS score and the false discovery rate. Additionally, edgeR package was used to perform guide-level analysis (Tables S1, S2, and S3). The volcano plots of these results were created using R (<https://www.r-project.org>).

Western Blotting

Cells were lysed with lysis buffer (300 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, 0.5% NP-40), lysates were resolved on denaturing Nu-page (Invitrogen) polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk in TBST, and probed with primary and secondary antibodies respectively, then detected with chemiluminescence (Western Lightning, Perkin Elmer).

CRISPR cas9 Knockout

To generate A549, NCI-H460, HeLa and U2OS cells expressing the indicated sgRNAs, the listed oligonucleotides were cloned into the pLentiCRISPR V2 viral vector.

siRNA Transfection

siRNA negative control and siRNA targeting FAM122A, PP2ACa, ENSA, BRCA1 and 53BP1 were ON-TARGET plus siRNA SMART-pools purchased from Dharmacon. For siRNA transfection, cells were seeded at about 50% confluence into 6-well plates and then transfected with a specific siRNA duplex using Lipofectamine RNAiMAX Reagent Agent (Life Technologies, #13778150) according to the manufacturer's instructions for 48 hr.

Clonogenic Assay

Cells were seeded at a concentration of 500-1000 cells per well into 6-well plates. After 24 hr, cells were continuously treated with indicated compounds for about 2 weeks. Colonies were fixed with fixation solution (methanol:acetic acid = 5:1) at room temperature for 20 min and then stained with a solution of 0.5% crystal violet in methanol for 2 h.

CellTiter-Glo Assay and Synergy Study

Cells were seeded into 96-well plates at a cell density of 1,000-2,000 cells per well overnight and treated with indicated compounds. The ATP-based, cell viability assay was carried out after incubation for 4 days (CellTiter-Glo, Promega). Survival curves were calculated by best-fit analysis of the log of the drug concentration to fold change of treated cells over vehicle-treated cells. All survival assays included technical triplicates.

For the prexasertib/PP2Ai synergy assay, cells were plated in the same fashion as above, and treated with 6 increasing doses of both drugs as a matrix. 72 h after drug treatment, CellTiter-Glo was applied and absorbances were recorded. Each replicate was compared and analyzed using ComBenefit synergy software for BLISS analysis.

Immunofluorescence

Cells plated on coverslips were washed once with PBS and fixed with 4% paraformaldehyde for 10 min on ice. 0.5% Triton X-100 was added to cells for 30 min for extraction. Coverslips were then blocked with 3% BSA for 30 min and incubated with primary antibody (diluted in 3% BSA) overnight. Following overnight incubation, coverslips were washed three times with PBST, then incubated with anti-rabbit secondary antibody (Alexa Fluor, Life Technologies) for 1 h. Coverslips were then mounted with DAPI medium and visualized under the fluorescence microscope.

Measuring Cell Cycle and Rate of DNA Replication

For Cell cycle-FACS analysis, cells were collected and fixed with 70% ethanol. Fixed cells were washed once with PBS and then resuspended with PI/RNase Staining Solution (Invitrogen). After incubation for 20 min at 37°C, the cells were analyzed using a flow cytometer (BD Biosciences). For analysis of DNA replication, cells were pulse-labeled with 10 μM EdU for 30 min, and then detected using the Click-iT EdU Alexa Fluor 488 kit (Invitrogen).

Cytogenetics

Cells were treated with/without 100nM prexasertib before cytogenetic analysis. Cells were exposed for 2 h to colcemid (0.1mg/ml) and harvested using a 0.075M KCl hypotonic solution and fixed with 3:1 methanol: acetic acid. Slides were stained with Wright's stain and when possible, at least 50 metaphase spreads were scored for aberrations. Metaphase spreads were observed using a Zeiss Axio Imager microscope and captured using CytoVision software from Applied Imaging.

Subcellular Fractionation

Subcellular fractionations were isolated using a subcellular fractionation kit (Cell signaling Technology) according to the manufacturer's instruction. The fractionation protein lysates were analyzed with western blotting using indicated antibodies.

Alkaline Single-Cell Electrophoresis (Comet) Assays

Alkaline comet assays were performed as previous described (Cai et al., 2020). Cells were trypsinized and suspended in cold 1 × PBS, then mixed with low melting agarose (Trevigen) at a ratio of 1:10 (v/v) and immediately plated onto Comet slide (Trevigen). Alkaline electrophoresis was run at 25 V for 30 min in the electrophoresis system. Cell comets were imaged with a fluorescence microscope (Zeiss).

DNA Fiber Assay

DNA fiber assays were done as described in Lim et al. (2018). In brief, cells were treated with prexasertib or DMSO for 24 h, then labeled with 25 μ M CldU for 30 min, washed three times with PBS, and labeled with 200 μ M IdU for 30 min. Cells were collected and embedded in low melting point (LMP) agarose plugs, then treated with proteinase K at 42°C overnight. Agarose plugs were then washed and digested with agarase. Agarase-treated samples were then combed onto silanized coverslips using FiberComb (Genomic Vision). Coverslips were blocked with blocking solution (PBS containing 1% BSA) for 30 min, and incubated with Rat anti-BrdU antibody (Abcam) and mouse anti-BrdU antibody (clone B44, BD Biosciences) at 4°C overnight, followed by incubation with Alexa Fluor 594 chicken anti-mouse and Alexa Fluor 488 goat anti-rat antibodies (Invitrogen). DNA fibers were visualized by fluorescence microscopy (Zeiss). Pictures were taken of at least 100 fibers per condition. DNA fibers were measured with ImageJ and graphed.

Kinase Reactions

293T cells were transfected with pHAGE-FAM122A-FLAG-HA and cellular lysates were generated 2 days post transfection with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, and protease and phosphatase inhibitor cocktail sets (Millipore)). Negative control lysates were generated by mock transfecting 293T cells. FLAG-HA-FAM122A was immunoprecipitated from cellular lysates using M2 Magnetic Beads (Sigma-Millipore) at room temperature for 2 h. Immunoprecipitated FLAG-HA-FAM122A protein was added to CHK1 kinase reactions with 100 ng purified CHK1 (Millipore) and 20 μ M ATP-g-S (Abcam). CHK1 was omitted from denoted reactions as a negative control. Kinase Reactions were conducted as previously described (Kim et al., 2020). Briefly, reactions were incubated at 30°C for 30 min and thiophosphate species were alkylated with p-nitrobenzyl mesylate (Abcam) for 2 h at room temperature. 6x SDS buffer was added to the reactions, samples were boiled, and FAM122A phosphorylation was assessed by SDS-PAGE followed by immunoblot.

RNA Sequencing and Analysis

Total RNA was prepared using a QIAGEN kit according to the manufacturer's instructions. Libraries were prepared using Roche Kapa mRNA HyperPrep strand specific sample preparation kits from 200ng of purified total RNA according to the manufacturer's protocol on a Beckman Coulter Biomek i7. The finished dsDNA libraries were quantified by Qubit fluorometer, Agilent TapeStation 2200, and RT-qPCR using the Kapa Biosystems library quantification kit according to the manufacturer's protocols. Uniquely dual-indexed libraries were pooled in equimolar ratios and sequenced on an Illumina NovaSeq 6000 with paired-end 50bp reads by the Dana-Farber Cancer Institute Molecular Biology Core Facilities. Sequenced reads were aligned to the UCSC hg19 reference genome assembly and gene counts were quantified using STAR (v2.7.3a) (Dobin et al., 2013). Differential gene expression testing was performed by DESeq2 (v1.22.1) (Love et al., 2014), and limma (Ritchie et al., 2015). RNAseq analysis was performed using the VIPER snakemake pipeline (Cornwell et al., 2018). The volcano plot of the results was generated using the ggplot2 package in R.

Computational Analysis

The analyses of FAM122A mRNA expression in the Cancer Genome Atlas (TCGA) tumors and matched normal tissues were performed as follows. The RNASeq expression raw HTSeq counts (Anders et al., 2015) of TCGA were downloaded from NCI Genomic Data Commons Data Portal (<https://portal.gdc.cancer.gov/>) using a custom Python script. For each cancer type, lowly expressed genes were filtered out if they did not have counts per million (CPM) of greater than 1 for at least 5% of the samples. The CPM values were normalized by the TMM (weighted trimmed mean of M-values) method using the edgeR package (Robinson et al., 2010). Tumor and matched normal tissue pairs were selected by identifying the sample pairs that were derived from the same patient (same patient barcode), and the tumor sample was a primary solid tumor (sample type code = 1) and the normal sample was a solid tissue normal (sample type code = 11). The ggpubr R package was used to perform the Wilcoxon rank sum test to show the difference in FAM122A

mRNA expression levels ($\log_2$ -transformed normalized CPMs) between the tumor and the matched normal tissue groups for each cancer type. The test results and the CPM data were plotted using the ggplot2 package in R.

The survival analyses of the TCGA patients were performed using the clinical and RNASeq expression data of TCGA Pan-Cancer study for 32 cancer types downloaded from the cBioPortal for Cancer Genomics (cbioportal.org). For each cancer type, samples were grouped into two groups, those with low mRNA expression of FAM122A and those with high mRNA expression of FAM122A. The two groups were determined based on the expression z-scores relative to the diploid samples. Samples with z-scores less than zero belonged to the low group; the remaining samples belonged to the high group. Survival analysis was then performed in R for each cancer type to determine whether there was a difference in the overall survival between the two groups. Kaplan-Meier curves were created, and the log-rank test was used to test for a difference in overall survival using the survival package in R. The p values were calculated from the chi-square distribution. The survminer R package was used to estimate median survivals, and to plot the Kaplan-Meier curves. Additionally, Cox proportional hazards regression was performed to estimate the hazard ratio between the low- and the high-FAM122A groups for each cancer type.

The CRISPR dependency analyses with cancer cell lines were performed using the data from the Broad Institute Cancer Dependency Map (DepMap) (Meyers et al., 2017) (<https://depmap.org/portal/>). The focused dependency analysis with FAM122A and CHEK1 was performed using the Achilles CRISPR Avana 17Q2 dataset. In this analysis, cell lines that were one standard deviation below or above the mean in terms of CHEK1 or CHEK2 CERES score were grouped into cell lines that had high CHEK1 dependency and low CHEK1 dependency, respectively. The CRISPR co-dependency analysis of FAM122A was performed using the Achilles CRISPR DepMap Public 19Q4 dataset. This analysis involved computing association between the dependency of FAM122A and that of every other gene. Only cell lineages with at least 5 cell lines were included in the analysis. The limma R package (Ritchie et al., 2015) was used to compute the associations using the CERES scores from all cell lines, with robust eBayes option and correcting for cell lineage. Volcano plot of the results and the scatterplots of the CERES scores of FAM122A and CHEK1 for the cell lineages were created using the ggplot2 package in R.

QUANTITATION AND STATISTICAL ANALYSIS

All quantitative data were analyzed and graphed using Prism 8 GraphPad software. All data are represented as mean $\pm$ SD calculated using the Prism software, unless indicated otherwise. Significance was tested using 2-tailed Student's t test and two-way ANOVA as indicated. Clinical data were analyzed using the Kaplan-Meier method and the log-rank (Mantel-Cox) regression test for significance.

ADDITIONAL RESOURCES

This study did not generate any additional resources.

Cooperation of the NEIL3 and Fanconi anemia/BRCA pathways in interstrand crosslink repair

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ABSTRACT

The NEIL3 DNA glycosylase is a base excision repair enzyme that excises bulky base lesions from DNA. Although NEIL3 has been shown to unhook interstrand crosslinks (ICL) in *Xenopus* extracts, how NEIL3 participates in ICL repair in human cells and its cooperation with the canonical Fanconi anemia (FA)/BRCA pathway remain unclear. Here we show that the NEIL3 and the FA/BRCA pathways are non-epistatic in psoralen-ICL repair. The NEIL3 pathway is the major pathway for repairing psoralen-ICL, and the FA/BRCA pathway is only activated when NEIL3 is not present. Mechanistically, NEIL3 is recruited to psoralen-ICL in a rapid, PARP-dependent manner. Importantly, the NEIL3 pathway repairs psoralen-ICLs without generating double-strand breaks (DSBs), unlike the FA/BRCA pathway. In addition, we found that the RUVBL1/2 complex physically interact with NEIL3 and function within the NEIL3 pathway in psoralen-ICL repair. Moreover, TRAIP is important for the recruitment of NEIL3 but not FANCD2, and knockdown of TRAIP promotes FA/BRCA pathway activation. Interestingly, TRAIP is non-epistatic with both NEIL3 and FA pathways in psoralen-ICL repair, suggesting that TRAIP may function upstream of the two pathways. Taken together, the NEIL3 pathway is the major pathway to repair psoralen-ICL through a unique DSB-free mechanism in human cells.

INTRODUCTION

DNA interstrand cross-links (ICLs) are toxic lesions that prevent DNA replication and transcription by blocking DNA strand separation, and unrepaired ICLs lead to apoptosis and cell death (1). The Fanconi anemia (FA) pathway is essential for the repair of DNA-ICLs, and defects in the FA pathway result in Fanconi anemia, a chromosomal instability disorder characterized by congenital abnormalities, progressive bone marrow failure, and cancer predisposition (2). The FA proteins function in a multistep pathway required for the repair of endogenous and exogenous ICLs, such as ICLs induced by the therapeutic agent Mitomycin C (MMC). To date, 23 FA genes have been identified, which are grouped into three categories: the FA core complex (an E3 ligase complex), the FANCI/FANCD2 (ID) complex, and the downstream effector proteins, such as structure-specific nuclease and double-strand break (DSB) repair proteins (3,4). When DNA replication is blocked by an ICL, the FA core complex (containing FANCA, B, C, E, F, G, L and M) monoubiquitinates the FANCI–FANCD2 complex (ID2), a pivotal step in the FA pathway (5). The FA core complex is recruited to a stalled replication fork by an ICL via the anchoring complex containing FANCM subunit, along with Fanconi-associated proteins (FAAPs). Nucleolytic processing of the ICLs, which involves nucleases recruited by the SLX4/FANCP scaffold protein (6,7), generates DSBs that can be repaired by multiple downstream repair pathways (8).

Base excision repair (BER) is the major pathway for repairing DNA base damage and single strand breaks. If left unrepaired, these lesions can be mutagenic, blocking repli-

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cation fork (9), or even perturbing epigenetic marks (10,11). The first and most critical step of BER is the searching and excision of damaged bases, a step that is carried out by DNA glycosylases. NEIL3 (DNA Endonuclease VIII-like 3) is a member of the Fpg/Nei glycosylase family (12,13), also including NEIL1 (14) and NEIL2 (15). Like other members of the Fpg/Nei family, NEIL3 contains a DNA glycosylase activity that excises damaged bases and an AP (apurinic/apyrimidinic site) lyase activity that cleaves the DNA backbone at an AP site, thus generating a single-strand break (SSB) (13,16). NEIL3 is distinguished from the other NEILs by its long C-terminal domain (CTD) (13). The glycosylase domain of NEIL3 prefers base lesions in single-stranded DNA (ssDNA) or ssDNA-containing structures (i.e. fork DNA) (13,16). NEIL3 also possesses the unique activity of removing damaged bases from G-quadruplex DNA (17–19).

The biochemical features of NEIL3 have been well characterized in the past decade, although the cellular function of NEIL3 has just begun to be understood. NEIL3 repairs telomere damage and protects telomeres during S phase to ensure accurate segregation of chromosome during mitosis (20). NEIL3 also plays a critical role in preventing autoimmunity, and its glycosylase activity is required for this function (21). NEIL3 appears to be important for cell proliferation, as evidenced by its role in regulating proliferation of cardiac fibroblasts in the heart and neural progenitor cells in the human brain (22,23). Its expression is increased in highly replicative tissues such as bone marrow (12,24) and in cancerous tissues (24). These studies demonstrate that NEIL3 is a versatile DNA glycosylase that functions outside of BER.

A new role for NEIL3 in ICL repair has recently been discovered. NEIL3 directly unhooks psoralen- and AP-ICLs during DNA replication in *Xenopus* egg extracts (25). This function of NEIL3 appears to be the first choice for the repair of a psoralen-ICL or AP-ICL (formed by AP site and an adenosine on opposite strands), and its failure activates the Fanconi anemia pathway. Purified NEIL3 and NEIL1 excises psoralen-induced DNA–DNA cross-links *in vitro* (26,27). Moreover, NEIL3 unhooks ICLs between glycosidic bonds and does not nick the DNA backbone, and accordingly does not generate DSBs (25,26). The ability of NEIL3 to accommodate those bulky lesions is supported by its crystal structure, which features a large lesion binding pocket of its glycosylase domain (28). In addition, mouse embryonic fibroblasts (MEFs) derived from *Neil3*^{-/-} mice are sensitive to ICL-inducing agents such as cisplatin (29). These studies show that NEIL3 plays an important role in ICL repair.

Additional players in ICL repair have been recently discovered. The RING-Type E3 ubiquitin ligase TRAIP (TRAF Interacting Protein) for example is essential for cell proliferation and replication fork progression through DNA damage, and TRAIP mutations cause microcephalic primordial dwarfism (30,31). TRAIP is required for optimal phosphorylation of H2AX and RPA2 during S-phase after UV-C irradiation (31), and knockdown of TRAIP sensitizes cells to MMC (32). More recently, TRAIP was found to operate a switch between the NEIL3 and FA pathways in ICL repair by controlling the length of ubiquitin chain on

CMG complex (33). In addition, the AAA⁺ ATPase family proteins RuvB Like 1 (RUVBL1) and its paralog RUVBL2 are DNA-dependent ATPases/helicases that are involved in diverse cellular activities. The RUVBL1/2 complex facilitates homologous recombination (34) and ICL repair (35,36). The RUVBL1/2 complex also regulates the abundance of FA core complex proteins, and depletion of RUVBL1/2 causes FA phenotypes in human cells, including defective in FA pathway activation, chromosomal instability and sensitivity to MMC (36).

Although NEIL3 was shown to act on ICLs biochemically in the *Xenopus* egg extracts, little is known about how NEIL3 contributes to ICL repair in human cells, and how the NEIL3 and FA pathways interact. Here we show that the NEIL3 pathway is the major pathway that repairs psoralen-induced ICLs (psoralen-ICLs), while the FA pathway is mostly responsible for repairing MMC- and cisplatin- induced ICLs. NEIL3 provides a strong response to psoralen-ICLs, resulting in a rapid, PARP-dependent recruitment to psoralen-ICL sites and protein accumulation/stabilization. Unlike the canonical FA pathway, NEIL3 unhooks psoralen-ICLs without creating DSBs in human cells. TRAIP appears to function upstream of the NEIL3 and the FA pathways, while RUVBL1/2 binds directly to NEIL3 and functions within the NEIL3 pathway. Collectively, we show the NEIL3 pathway is prioritized in psoralen-ICL repair through a unique mechanism in human cells.

MATERIALS AND METHODS

Cell lines and culture conditions

Human FA fibroblasts including PD20 (*FANCD2*^{-/-}), and PD326 (*FANCG*^{-/-}) were derived from FA patients as previously described (37). All human FA fibroblasts, U2OS and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific) in a 5% CO₂ incubator at 37°C. The primary wild-type and *Neil3*^{-/-} mouse embryonic fibroblasts (MEFs) were gifts from Magnar Bjørås. The MEFs were immortalized with SV40 antigen according to the protocol described previously (38). The immortalized MEFs were grown in minimum essential medium supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific), 2 mM L-glutamine and 1% penicillin/streptomycin (Thermo Fisher Scientific) in a 5% CO₂ incubator at 37°C.

Plasmids, siRNA, and transfection

The EGFP-FANCD2 and NEIL3-HA plasmids were described previously (20,39). The RUVBL1-Flag (#OHu26261) and RUVBL2-Flag (#OHu25367) plasmids were purchased from GenScript. Plasmid was transfected using Lipofectamine LTX Reagent (Thermo Fisher Scientific). For siRNA treatment, cells were transfected with specific siRNA (Dharmacon and Qiagen; see Supplementary Table S1) using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific).

Generation of *NEIL3*^{-/-}, *FANCA*^{-/-} and *FANCD2*^{-/-} cells

HeLa-*FANCA*^{-/-} and U2OS-*FANCD2*^{-/-} cell lines were generated using the CRISPR-Cas9 system. To generate HeLa-*NEIL3*^{-/-} and U2OS-*NEIL3*^{-/-} cell lines, HeLa- and U2OS-CAS9 stable cell lines were firstly generated by transfection with the Cas9 plasmid and selected by blasticidin (Thermo Fisher Scientific). Oligonucleotides encoding guide RNAs targeting *NEIL3* (5'-ATTCGCGCGCGGGTGCTCCC-3') was cloned into the lentiGuide-Puro vector, a gift from Feng Zhang (Addgene plasmid # 52963). To produce lentivirus, HEK293T cells were seeded at 60% confluence in 60-mm dishes 24 h before transfection. The gRNA-lentiGuide-Puro construct was transfected together with the packaging plasmids (psPAX2 and PMD2.G) using LipoLTX reagent (Thermo Fisher Scientific). Virus was harvested 48 h after transfection, filtered through a 0.45 µm low-protein-binding membrane (Millipore), and then placed on the HeLa- or U2OS-CAS9 cells with a density of 10%. Mono-clone was collected after 2–3 weeks of selection by puromycin, and knockout clones were identified by western blotting.

PUVA (psoralen plus ultraviolet A) treatment

To induce psoralen-ICLs, the cells were washed using 1× PBS and were treated with the indicated concentrations of psoralen (Sigma-Aldrich #P8399) in 1× Hank's Balanced Salt Solution (HBSS) containing 2% FBS. After incubation for 30 min in the dark, cells were exposed to ultraviolet A (UVA) light (365 nm) at a dose of 0.8 J/cm² (40,41). Following treatment, cells were allowed to recover at 37°C in complete medium.

CellTiter-Glo cell viability assay

The CellTiter-Glo luminescent cell viability assay kit (Promega) was used to measure cell viability after drug treatment. The CellTiter-Glo kit determines the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. Generally, to determine the drug sensitivity of *NEIL3*^{-/-} cells, 2000 cells (HeLa, U2OS or MEF) per well were plated in a 96-well plate. The sources and doses of DNA damaging agents were as following: camptothecin (Selleckchem #S1288: 0, 0.625, 1.25, 2.5, 5, 10 nM), mitomycin C (MMC, Sigma Aldrich #M0503: 0, 1.25, 2.5, 5, 10, 20 ng/ml), cisplatin (Sigma Aldrich #P4394: 0, 0.0625, 0.125, 0.25, 0.5, 1 µM), Hydroxyurea (HU, Sigma Aldrich #H8627: 0, 0.03125, 0.0625, 0.125, 0.25, 0.5 mM), psoralen (Sigma-Aldrich #P8399, 0, 0.03125, 0.0625, 0.125, 0.25, 0.5 µM), X-ray irradiation (IR: 0, 1, 2, 5, 7.5, 10 Gy), PUVA (0, 0.015625, 0.03125, 0.0625, 0.125 or 0.25 µM psoralen incubation for 30 min followed by exposure to 0.8 J/cm² UVA), UVA (0, 0.0625, 0.125, 0.25, 0.5, 1.0 J/cm²), Ultraviolet B (UVB: 0, 50, 100, 200, 400, 800 J/m²), or Ultraviolet C (UVC: 0, 2, 5, 10, 20, 40 J/m²). Cells were cultured in fresh medium for 4–5 days after treatment, by then cell viability was measured. To test knockdown of *NEIL3*, *FANCA*, *FANCD2* and *TRAIP* (Supplementary Table S1), indicated cells were transfected with control or specific siRNA (Final

concentration of 20 nM siRNA) in 6-well plate and the cells were seeded into 96-well plates next day prior to treatment with DNA damage agents, using the condition stated above. To test knockdown of RUVBL1 and RUVBL2 (Supplementary Table S1), cells were transfected with control or specific siRNA (Final concentration of 10 nM siRNA) in 96-well plate (5000 cells/well). 24 h after transfection, the cells were treated with PUVA (0, 0.03125, 0.0625, 0.125, 0.25 or 0.5 µM psoralen incubation for 30 min followed by exposure to 0.8 J/cm² UVA) or MMC (0, 1.25, 2.5, 5, 10, 20 ng/ml) and were subsequently incubated for a further 48 h to measure the cell viability. To test PARP inhibitor, cells were pre-treated with 10 µM olaparib (Selleckchem #AZD8821) for 2 h and then treated with indicated concentrations of psoralen (0, 0.015625, 0.03125, 0.0625, 0.125 or 0.25 µM) in HBSS containing 2% FBS and 10 µM olaparib for 30 min. After exposed with 0.8 J/cm² UVA, cells were cultured in fresh medium with olaparib (0.5 µM) for 4–5 days before measuring cell viability.

Immunofluorescence

Cells were seeded on cover slide in 12-well plate and then were treated with PUVA (50 nM psoralen for 30 min and then irradiation with 0.8 J/cm² UVA) or MMC (5 ng/ml). The γH2AX foci was determined at 0, 1, 6 and 20 h, respectively, after releasing into fresh medium. Briefly, cells were washed by 1× PBS and fixed using 4% paraformaldehyde at room temperature for 20 min. After washing with 1× PBS three times and blocking (blocking buffer, 1× PBS/5% goat serum/0.3% Triton X-100) for 1h, cells were incubated with anti-γH2AX (Ser139) (Cell Signaling Technology #2577) at 4°C overnight. γH2AX foci were generated after incubation with goat anti-rabbit IgG (H+L) secondary antibody (Life Technologies #A32731) for 1 h and detected using fluorescence microscope. For siRNA, 48 h after transfection, cells were then treated with PUVA and followed with above protocols to detect the γH2AX foci formation.

Neutral comet assay

Cells were seeded in six-well plate and exposed to PUVA (10 µM psoralen for 1 h followed by 1.0 J/cm² UVA). After 4 h, the neutral comet assay was performed to detect DSBs, using a commercial kit (Trevigen, # 4250-050-K) according the manufacturer's protocol. In siRNA transfected groups, cells were treated with PUVA (10 µM psoralen for 1 h followed by 1.0 J/cm² UVA) 72 h after siRNA transfection and were subjected to the neutral comet assay 4 h after PUVA. Quantification of the comet length was performed using Image J v.1.8.0.112 software.

Psoralen/UVA laser stripping

To test the recruitment of *NEIL3*, the U2OS cells were seeded in glass bottomed dishes and transfected indicated siRNAs. Forty eight hours after transfection, *NEIL3*-EGFP plasmid was transfection into the cells. Next day the cells were incubated with psoralen (10 µM) for 1 h. A Zeiss PALM microdissection microscope equipped with a 360 nm UV laser at the 34% energy dose was then used to observe the recruitment.

To test the recruitment of FANCD2, we generated the U2OS stable cell line using the EGFP-FANCD2 plasmid by selection with puromycin. Cells were seeded in glass bottomed dishes and treated with indicated siRNAs. Seventy two hours after transfection, cells were incubated with psoralen (10 μ M) in culture medium for 1 h and then used to perform the laser irradiation.

To test the PARPi, NEIL3-EGFP transfection or EFP-FANCD2 stable U2OS cells were pre-treated with DMSO or 10 μ M olaparib for 3 h, and then were used to perform the laser irradiation described as above.

Cell synchronization and Cell cycle analysis

HeLa cells were double blocked with 2 mM thymidine (arrested for 16 h, released for 8 h, and arrested for another 16 h), and then the cells were harvested for cell cycle and NEIL3 expression analysis at indicated time point. Cells were collected by digestion using trypsin and were washed twice with 1 $\times$ cold-PBS. Cells were then fixed in 70% ethanol at -20°C overnight and stained with propidium iodide for 20 min. Cell cycle distribution was analyzed using a FACS Canto machine. The data was analyzed by FlowJo software v10.07 (BD Biosciences) or Modfit LT software v5.0 (Verity Software House).

Mass spectrometry

HEK293T cells were seeded in 15 cm dishes and transfected with NEIL3-EGFP or empty vector. Twenty four hours after transfection, cells were treated with 10 μ M psoralen plus UVA irradiation (0.8 J/cm²) and released to culture for indicated time. Cells were then washed with pre-chilled PBS three times and lysed into the lysis buffer (20 mM HEPES-K⁺, pH 7.6, 0.1 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, 0.01% NP-40, 1 mM DTT, 0.2 mM PMSF, 0.5 mM benzamidine, 1 μ g/ml each of leupeptin, aprotinin and pepstatin A) for 30 min at 4°C . Cell lysates were incubated with GFP-Trap resin (ChromoTek) at 4°C overnight and the resin was washed with cold lysis buffer four times. The immunoprecipitated NEIL3-eGFP and its binding partners were eluted with SDS elution buffer (2% SDS, 50 mM Tris-Cl, pH 6.8). The samples were prepared by trichloroacetic acid (TCA) precipitation as previously described (42), and sent to for mass spec analysis.

Immunoprecipitation (IP)

At 48 h after plasmid transfection in 10 cm dish, HEK293T cells were washed twice with ice-cold phosphate-buffered saline. Cells were scraped from dish using 1 ml cold PBS and then were spun at 500 g for 3 min at 4°C . After removal of the supernatant, the cell pellet were lysed in 600 μ l IP lysis buffer (250 mM NaCl, 50 mM pH 7.5 Tris-HCl, 1 mM EDTA, 10% glycerol, 0.5% NP40) containing EDTA-free protease inhibitor (Roche) at 4°C for 30 min. Centrifuge cell lysate at 20 000 g for 10 min at 4°C and transfer supernatant to a pre-cooled tube. The FLAG[®] M2 magnetic beads (Sigma-Aldrich) or GFP-Trap resin (ChromoTek) were washed three times using the IP lysis buffer

and 25 μ l beads were added to each supernatant tube. After incubating for 4 h at 4°C , the beads were washed three times with the IP lysis buffer and then were boiled with 100 μ l 2 $\times$ SDS-sample buffer for 10 min at 95°C to dissociate immunocomplexes.

Immunoblotting and antibodies

To prepare whole-cell extracts, cells were washed once with ice-cold PBS and lysed with SDS lysis buffer (100 mM pH 6.8 Tris-HCl, 20% glycerol, 1% SDS). Equal amounts of samples were subjected to 3–8% Tris-acetate gel (for FANCD2-ubiquitination detection) or 4–12% Bis-tris gel (Thermo Fisher Scientific). The protein was transferred to a PVDF membrane. Membranes were blocked in 2% BSA in TBS-T for 1 h and then incubated overnight at 4°C with the following antibodies: anti-FLAG M2 antibody (Sigma-Aldrich #F1804), anti-HA-tag (Cell Signaling Technology #3724), anti-GFP (Cell Signaling Technology #2555), anti-beta-actin (Cell Signaling Technology #4967), anti-GAPDH (Cell Signaling Technology #2118), anti-NEIL3 (Proteintech, 11621-1-AP), anti-FANCA (Bethyl Laboratories #A301-980), anti-FANCD2 (Novus Biologicals #NB100-182), anti-FANCI (Santa Cruz Biotechnology #sc-271316), anti- γ H2A.X (Ser139) (Cell Signaling Technology #2577), anti-PARP1 (Cell Signaling Technology #9542), anti-PARP3 (Novus Biologicals #NBP1-31415), anti-MCM7 (Cell Signaling Technology #3735), anti-RUVBL1 (Abcam #ab51500) and anti-RUVBL2 (BD Biosciences #612482). Proteins were detected using a chemiluminescence system with a horseradish peroxidase conjugated secondary antibody.

Quantitative RT-PCR

Total RNA was extracted applying a RNeasy Mini kit (Qiagen), and cDNA was obtained using the SuperScript[™] III First-Strand Synthesis kit (Thermo Fisher Scientific). The following primers were used for qPCR. NEIL3: forward, 5'-GTCCTAATTGTGGTCAGTGCC-3'; reverse, 5'-TCCA GTGCTCTCCGACTTC-3'; TRAP1: forward, 5'-CTAA AAGAGCTCACGGAAGGC-3'; reverse, 5'-TTTCCTGC AGCATCGTTAGC-3'; GAPDH: forward, 5'-CCATCT TCCAGGAGCGAGAT-3'; reverse, 5'-TGCTGATGAT CTTGAGGCTG-3'; NEIL1: forward, 5'-CTCGCCCTAT GTTTCGTGGA -3'; reverse, 5'-TTCAGCCGGTACAG GATCTC-3'.

RESULTS

NEIL3 plays a major role in psoralen-ICL repair independent of the FA/BRCA pathway.

The biochemical role of NEIL3 in psoralen-ICL repair has been demonstrated biochemically *in vitro* (26) and in the *Xenopus* egg extract system (25). Whether NEIL3 plays a role in psoralen-ICL repair or in other types of DNA damage (i.e. double-strand break) repair in human cells remains unclear. We thus generated *NEIL3*^{-/-} human cells and asked whether these cells are sensitive to any DNA damaging agents. *NEIL3*^{-/-} HeLa and U2OS cells were hypersensitive to PUVA (psoralen plus UVA irradiation) (Figure 1A, B

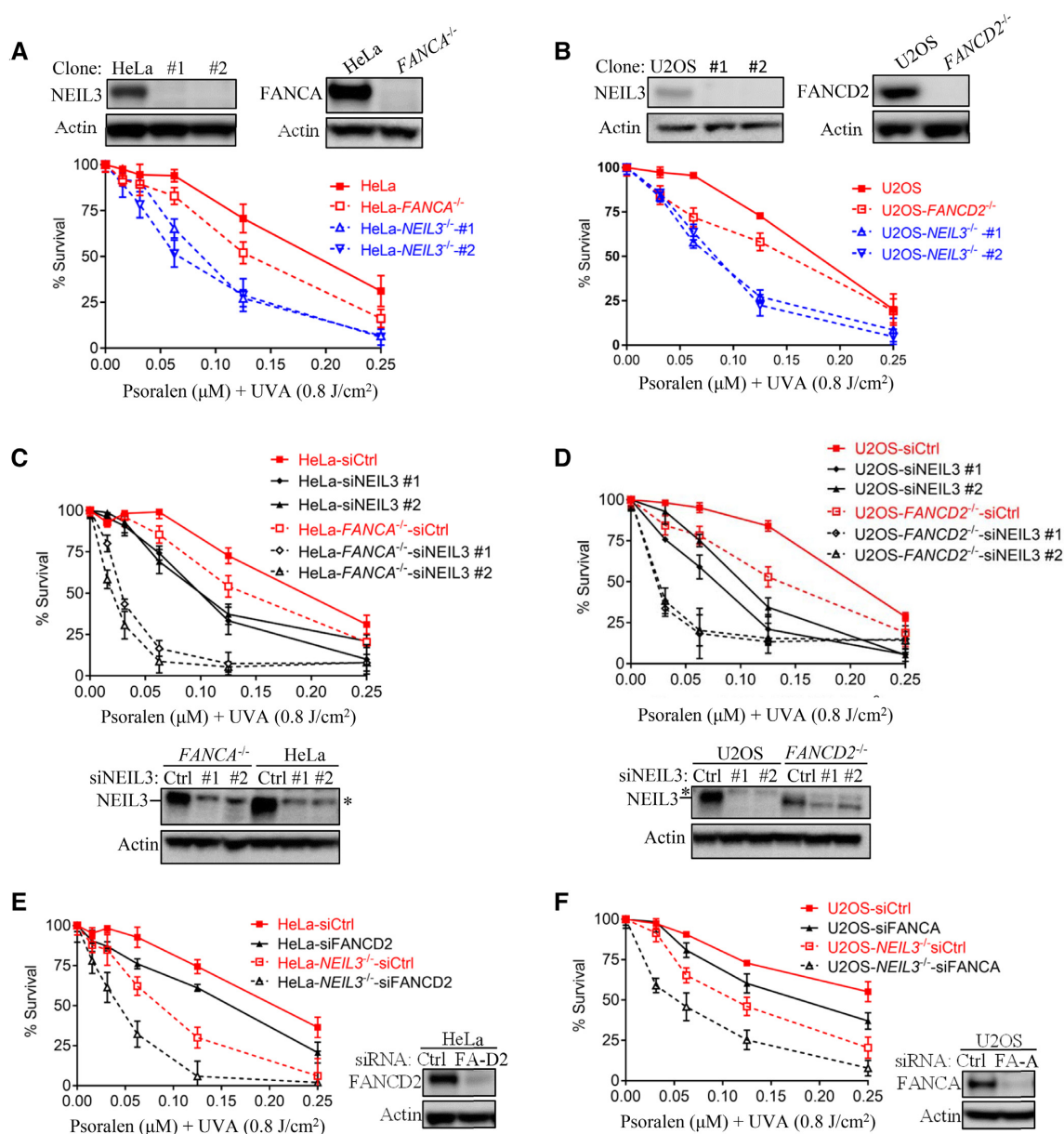


Figure 1. NEIL3 pathway plays a dominant role in psoralen-ICL repair in human cells. (A, B) Single cell HeLa-NEIL3^{-/-}, U2OS-NEIL3^{-/-}, HeLa-FANCA^{-/-} and U2OS-FANCD2^{-/-} clones were validated by Western blot. Cell viability was measured by CellTiter-Glo assays (Promega). PUVA, psoralen plus UVA. knockout cell lines were generated by CRISPR-Cas9. Guide RNA sequences and method used are shown in Materials and Methods. (C, D) Cell viability after PUVA treatment was measured after NEIL3 knockdown in HeLa WT versus HeLa-FANCA^{-/-} and U2OS WT versus U2OS-FANCD2^{-/-} cells. Western blots show that NEIL3 was knocked down. *Asterisk indicates a non-specific band above the NEIL3 band detected by the NEIL3 antibody. (E, F) PUVA sensitivity was determined after knockdown of FANCA or FANCD2 in U2OS-NEIL3^{-/-} and HeLa-NEIL3^{-/-} cells, respectively. Western blots show successful knockdown of FANCD2 and FANCA. Data are mean $\pm$ standard error of the mean (s.e.m.) from three independent experiments.

and Supplementary Figure S1A), while treatment with psoralen or UVA alone had no impact on cell growth (Supplementary Figure S1B). *Neil3*^{-/-} MEFs were also sensitive to PUVA (but not UVA or psoralen) when compared to WT control (Supplementary Figure S1C). *NEIL3*^{-/-} HeLa cells were also sensitive to ICL-inducing agents MMC and cisplatin, but to a lesser extent than PUVA. (Supplementary Figure S1A). *NEIL3*^{-/-} HeLa also showed mild sensitivity to HU and no sensitivity to camptothecin, IR, UVB or UVC (Supplementary Figure S1A). Taken together, the sensitiv-

ity profile results suggest that NEIL3 plays a major role in psoralen-ICL repair.

The FA/BRCA pathway has been extensively studied in MMC- and cisplatin-ICL repair, and it was recently implicated in psoralen-ICL repair (25,33). To study the relative contribution of NEIL3 and FA/BRCA pathways to the repair of those three types of ICLs, the sensitivities of NEIL3-deficient and FA-deficient were compared. Although both *FANCA*^{-/-} and *FANCD2*^{-/-} cells were sensitive to PUVA, *NEIL3*^{-/-} cells showed much more sensitivity to PUVA, sug-

gesting that NEIL3 is the major player in psoralen-ICL repair in HeLa and U2OS cells (Figure 1A and B). Conversely, NEIL3 pathway plays a less important role than the FA/BRCA pathway in MMC- and cisplatin-ICL repair, as demonstrated by the lesser sensitivity of HeLa-*NEIL3*^{-/-} to those two agents (Supplementary Figure S1D).

To determine the relationship between the NEIL3 and FA pathways in psoralen-ICL repair, we knocked down NEIL3 in FA/BRCA-deficient cells and examined their PUVA sensitivity. siRNA knockdown of NEIL3 in FA/BRCA-deficient HeLa and U2OS cells led to additional sensitivity of those cells to PUVA (Figure 1C and D). Similar results were observed in FANCD2-deficient cells (PD20), which are SV40-transformed fibroblast cells derived from a FANCD2-deficient FA patient. We observed additional PUVA sensitivity when NEIL3 was knocked down in PD20 cells (Supplementary Figure S1E). Consistently, depletion of FANCA or FANCD2 by siRNA in *NEIL3*^{-/-} cells also led to an additional sensitivity to PUVA (Figure 1E and F). These PUVA sensitivity data indicates that the NEIL3 pathway and the FA/BRCA pathway are functionally non-epistatic in psoralen-ICL repair. In contrast, HeLa-*NEIL3*^{-/-} cells showed no additional sensitivity to MMC or cisplatin after FANCD2 knockdown (Supplementary Figure S1F), indicating that NEIL3 participates in MMC- and cisplatin-ICLs repair in a FA/BRCA pathway dependent manner. To investigate whether other NEIL DNA glycosylases is involved in psoralen-ICL repair, we knocked down NEIL1 in HeLa cells and tested their sensitivity to PUVA. NEIL1 knockdown resulted in resistance to PUVA in HeLa cells, where NEIL3 was concurrently up-regulated (Supplementary Figure S1G). These results were consistent with a previous study (43) and supported the idea that NEIL3 is the major enzyme to repair psoralen-ICL. Taken together, NEIL3 plays a major role in psoralen-ICL repair in human cells, which is independent of the FA/BRCA pathway. NEIL3 is also involved in MMC- and cisplatin-ICL repair in human cells, which is governed by the FA/BRCA pathway.

NEIL3 knockdown activates the FA/BRCA pathway in psoralen-ICL repair

In *Xenopus* egg extracts, psoralen-ICLs are repaired via the FA/BRCA pathway when the NEIL3 pathway is blocked (25). We asked whether this mechanism is also found in human cells. We first monitored FA/BRCA pathway activation after PUVA by detecting FANCD2 mono-ubiquitination. FANCD2 mono-ubiquitination was not detected in U2OS or HeLa parental cells treated with 50 nM psoralen plus 0.8 J/cm² UVA, when NEIL3 was present. In contrast, mono-ubiquitination of FANCD2 was clearly observed in *NEIL3*^{-/-} HeLa and in *NEIL3*^{-/-} U2OS cells under the same dose of PUVA treatment (Figure 2A). This is consistent with our observation that NEIL3 plays a dominant role in psoralen-ICL repair in human cells (Figure 1), and with the observation in *Xenopus* that the FA pathway is activated in the absence of NEIL3. These results suggest that the NEIL3 pathway is the major and prioritized pathway for repairing psoralen-ICLs in human cells. Only when the psoralen-ICLs exceed the repair capability of NEIL3 does

the FA/BRCA pathway become activated as a backup repair mechanism.

Expression of NEIL3 and FA genes were interdependent at protein level

Since both NEIL3 and FA pathways contribute to the repair of PUVA-ICLs, we expected a compensatory relationship between the NEIL3 and FA pathway proteins. To our surprise, NEIL3 expression was down-regulated at the protein level in U2OS-*FANCD2*^{-/-} and HeLa-*FANCA*^{-/-} cells, compared to their WT counterparts (Figure 2B and Supplementary Figure S2A). Complementation with EGFP-FANCD2 in the U2OS-*FANCD2*^{-/-} cells rescued the expression of NEIL3 (Figure 2B), and similar rescue results could be achieved in FANCD2 deficient (PD20) or FANCG deficient (PD326) FA patient fibroblasts (Supplementary Figure S2B). We excluded the possibility of robust regulation at NEIL3 mRNA level in FA-deficient cells, since NEIL3 mRNA level was not changed in those cells (Supplementary Figure S2C). We also excluded that the decreased level of NEIL3 was a result of cell cycle alteration. Consistent with our previous report (20), NEIL3 expression increases during S phase and reached maximal levels in G2/M (Supplementary Figure S2D). However, there was no significant change in the cell cycle distribution of NEIL3 in U2OS-*FANCD2*^{-/-} and HeLa-*FANCA*^{-/-} cells (Supplementary Figure S2E and F). Next, we studied how NEIL3 deficiency affected FA genes. FANCA and FANCD2 expression levels were decreased in *NEIL3*^{-/-} HeLa and U2OS cells (Figure 2C). Further investigation showed the proteasome inhibitor MG-132 was able to stabilize NEIL3 in *FANCD2* cells and FANCD2 in *NEIL3*^{-/-} cells, suggesting NEIL3 and FANCD2 protein stabilize each other by reducing proteasome degradation (Supplementary Figure S2G). The mutually dependent expression levels led us to speculate that NEIL3 might form a complex with FANCD2 proteins. However, we were not able to detect an interaction between NEIL3 and the FA proteins after many attempts of co-immunoprecipitation experiments, and the FA proteins were not detected in the NEIL3 immunoprecipitant in our IP-MS experiment (Supplementary Figure S2H).

Response of NEIL3 to psoralen-ICLs was enhanced in FA/BRCA-deficient cells

We next studied how NEIL3 responses to PUVA treatment. Upon PUVA treatment, rather than UVA irradiation or psoralen treatment, the NEIL3 expression was up-regulated in both HeLa and U2OS cells, suggesting that NEIL3 plays an active role in the DNA damage response (Figure 2D and E, Supplementary Figure S2A). Interestingly, NEIL3 protein was strongly induced after PUVA exposure in these FA-deficient cells (more than 4-fold), despite a low starting level of NEIL3 protein in these cells (Figure 2D and E). This increase is not likely an effect of cell cycle, since the S and G/M populations combined showed no difference in WT and *FANCA*^{-/-} HeLa cells after PUVA treatment (Supplementary Figure S2I and J). These results demonstrated an active response of NEIL3 to PUVA, and also explained why FA/BRCA-deficient cells were only mildly sensitive to PUVA.

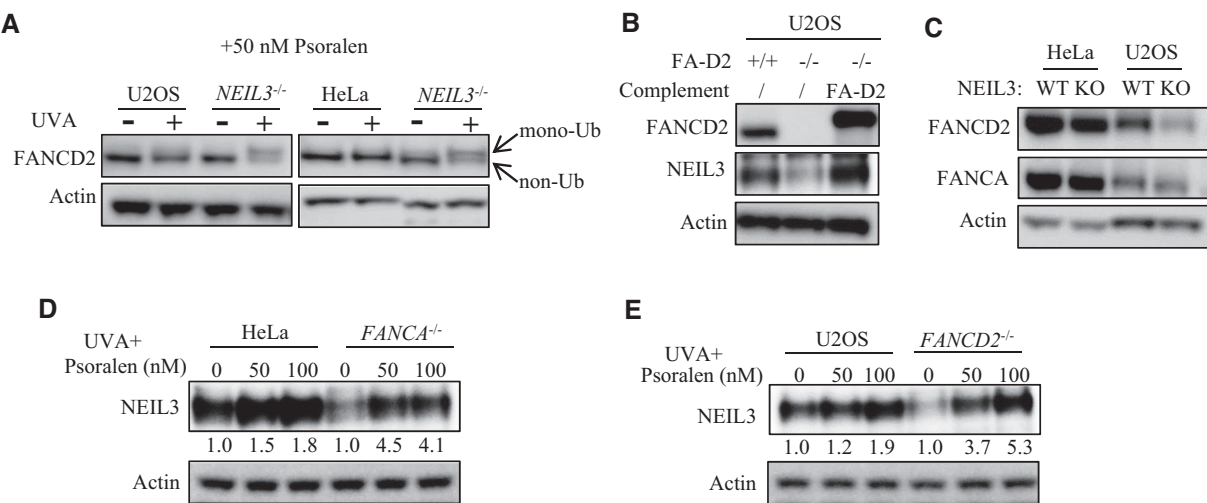


Figure 2. The relationship between the NEIL3 and the FA/BRCA pathways in psoralen-ICL repair. (A) WT and *NEIL3*^{-/-} cells were treated with PUVA (pre-incubated with 50 nM psoralen for 30 min and then irradiation with 0.8 J/cm² UVA). 24 h after PUVA, the level of FANCD2 mono-ubiquitination was detected by WB to determine activation of the FA/BRCA pathway. (B) U2OS-*FANCD2*^{-/-} cells were complemented with EGFP-FANCD2 and NEIL3 expression level was determined by western blot in those cells. (C) The FANCA and FANCD2 expression levels were determined in wild-type and NEIL3 deficiency cells. (D, E) The NEIL3 expression level was determined in wild-type and FANCA gene deficiency cells, 24 h after being treated with indicated dose of psoralen plus UVA (0.8 J/cm²) irradiation. Quantification of the Western blots was performed by ImageJ software and data were normalized to no psoralen.

The NEIL3 pathway repairs psoralen-ICLs without generating double-strand breaks

Previous studies in *Xenopus* egg extracts revealed that NEIL3 unhooks DNA psoralen-ICLs without generating double-strand breaks (DSBs) (25). We next determined whether a similar mechanism was present in human cells. We investigated DSB generation after PUVA treatment in HeLa cells that were defective in either the NEIL3 pathway or the FA/BRCA pathway, using γ H2AX foci as markers. Following PUVA treatment, WT HeLa cells exhibited a slight increase of γ H2AX foci compared to the untreated cells (0 h). *FANCA*^{-/-} cells, which only retained the NEIL3 pathway, had a lower level of γ H2AX foci compared to WT HeLa cells. Conversely, *NEIL3*^{-/-} cells, which only have the FA/BRCA pathway for repair of the psoralen-ICLs, contained an increased number of γ H2AX foci compared to WT HeLa cells (at 6 and 20 h) (Figure 3A and Supplementary Figure S3A). Consistent with the IF results, the γ H2AX level was also increased in the *NEIL3*^{-/-} cells after PUVA treatment as determined by western blot (Figure 3B, lanes 5–8). Moreover, depletion of FANCD2 decreased γ H2AX foci formation in both WT and *NEIL3* HeLa cells (Figure 3C and Supplementary Figure S3B), indicating the FA pathway is responsible for the DSBs generated after PUVA. Since γ H2AX is not a perfect marker for DSBs, we evaluated cellular DSB levels after PUVA treatment using the neutral comet assay, which detects DSBs directly at DNA level. Similar to the γ H2AX foci and Western blot data, we observed that there were more DSBs in *NEIL3*^{-/-} HeLa cells compared to WT counterparts after PUVA, as indicated in the increased tail length of the comets. Consistently, much shorter comet tails were observed in *FANCA*^{-/-} cells (Figure 3D–E). In addition, depletion of FANCD2 decreased the tail length of comets in both HeLa WT and *NEIL3* cells (Figure 3F, G), indicating that DSB genera-

tion after PUVA is dependent on the FA pathway but not the NEIL3 pathway. To note, the elevated γ H2AX foci in *NEIL3*^{-/-} cells were completely rescued by siFANCD2. In addition, we did not observe a difference in γ H2AX foci formation in *FANCA*^{-/-} and *NEIL3*^{-/-} cells following MMC treatment (Supplementary Figure S3C–D), suggesting again that NEIL3 shares the same downstream factors with FA/BRCA pathway in MMC-ICLs repair. Taken together, our data demonstrated that, unlike the FA/BRCA pathway, the unique psoralen-ICLs unhooking mechanism of NEIL3 is not associated with DSB formation in human cells.

PARP is required for the recruitment of NEIL3 and FANCD2-Ub to DNA damage

We next examined how NEIL3 is recruited to psoralen-ICL sites. Cells were pre-treated with psoralen and irradiated with a UVA laser (357 nm), and the NEIL3 recruitment was monitored by a GFP tag (Supplementary Figure S4A). In the absence of psoralen, UVA laser alone did not trigger the recruitment of NEIL3-EGFP (Figure S4B). The recruitment of NEIL3-EGFP to the PUVA laser stripe was very efficient; 90% of the cells showed positive NEIL3-EGFP recruitment to the laser track. The recruitment was also rapid, which occurs within one minute (Figure 4A, B). To determine whether NEIL3 recruitment to psoralen-ICL is dependent on any known DNA damage response pathways during psoralen-ICLs repair, we tested inhibitors of ATM, ATR, DNA-PK and PARP. We found only the PARP inhibitor (olaparib) strongly blocked NEIL3 recruitment to psoralen-ICLs (Figure 4A, B), while ATM, ATR or DNA-PKcs inhibitors did not affect NEIL3 recruitment (Supplementary Figure S4C). Note that olaparib almost completely blocked the recruitment of NEIL3, indicating this event is highly PARP dependent. In contrast, the recruit-

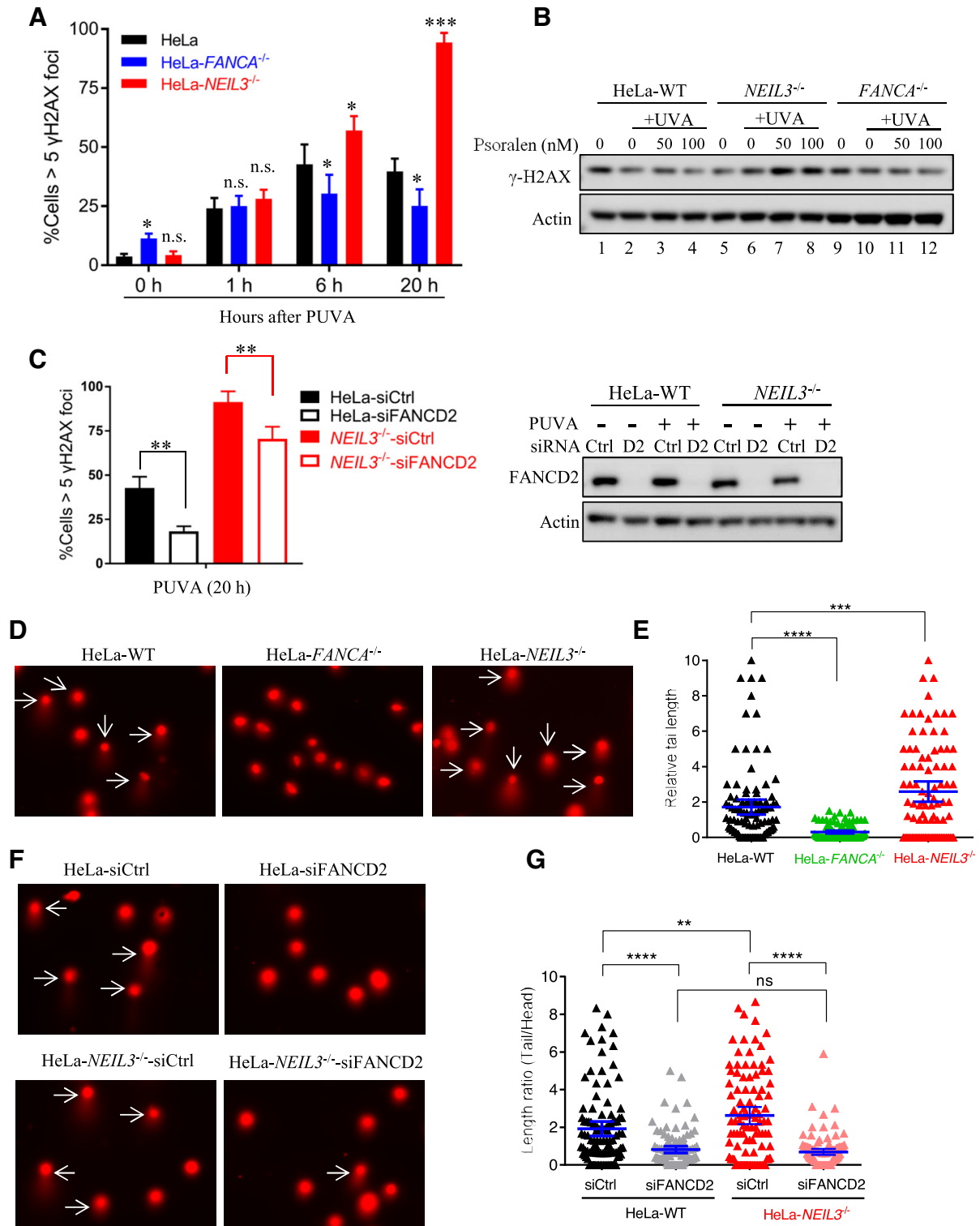


Figure 3. *NEIL3*^{-/-} cells show increased DSBs compared to WT cells after PUVA treatment. (A) Quantification of the γH2AX foci at 0, 1, 6 and 20 h after PUVA. Cells with more than five γH2AX foci were counted and quantified, from IF image shown in Figure S3. Data are mean ± s.e.m. from three independent experiments. (B) γH2AX level was determined in WT, *FANCA*^{-/-} and *NEIL3*^{-/-} HeLa cells after treatment. (C) Quantification and statistical analysis of the γH2AX foci 20 h after PUVA in WT and *NEIL3*^{-/-} HeLa cells after FANCD2 knockdown. Data are mean ± s.e.m. from three independent experiments. Western blot on the right shows the knockdown of FANCD2. PUVA, 50 nM psoralen + 0.8 J/cm² UVA. ***P* < 0.01 (paired *t* test). (D) Representative images of comets, showing tail formation (arrows) 4 h after PUVA in WT, *FANCA*^{-/-} and *NEIL3*^{-/-} HeLa cells. (E) Quantification of the comet length at 4 h after PUVA shown in (D). At least 100 cells were analyzed for each group. (F) Representative images of comets showing tail formation in WT and *NEIL3*^{-/-} HeLa cells after FANCD2 knockdown and PUVA. 72 h after siRNA transfection, cells were treated with PUVA and released for 4 h before comet assays. PUVA, 10 μM psoralen plus 1.0 J/cm² UVA; White arrows, cells with comet tails. (G) Quantification and statistical analysis of comet lengths from experiments shown in (F). ns, not significant, ***P* < 0.01, *****P* < 0.0001 (paired *t* test).

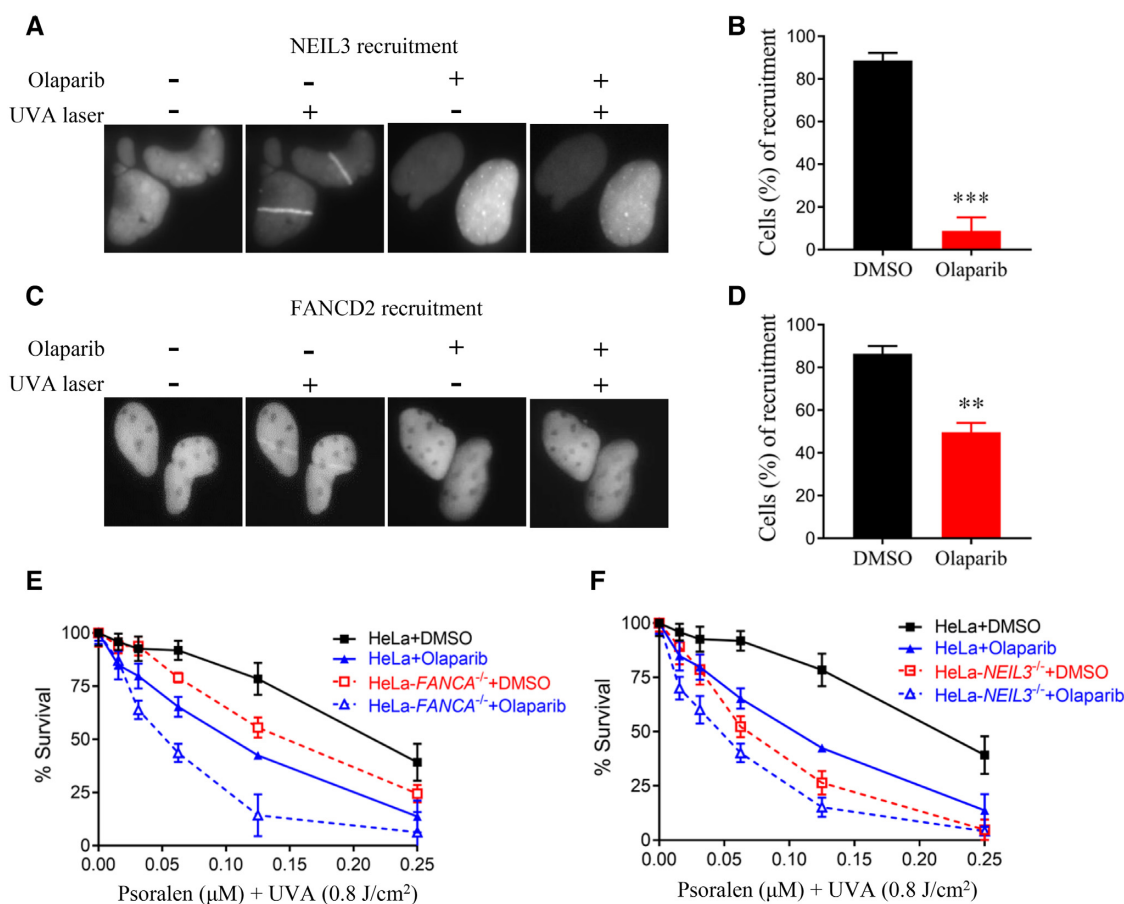


Figure 4. PARP is essential for NEIL3 recruitment to psoralen-ICLs and facilitates FANCD2 recruitment. (A) Recruitment of NEIL3 to PUVA laser tracts. U2OS cells expressing NEIL3-GFP were pre-incubated with $10 \mu\text{M}$ psoralen and the recruitment of NEIL3 was visualized 1 min after exposure to UVA laser. PARP inhibitor (olaparib) was added 1 hour before laser stripping. (B) Quantification of the NEIL3 recruitment to psoralene-ICL laser tracts after treatment with DMSO or olaparib. A total of 100 cells were quantified for each group. (C) U2OS cells stably expressing EGFP-FANCD2 were pre-treated with $10 \mu\text{M}$ psoralen for 1 h before laser stripping. Recruitment efficiency was determined 10 min after exposure to UVA laser. Olaparib or DMSO was added 1 h before laser stripping. (D) Quantification of the recruitment efficiency of EGFP-FANCD2 after treatment with DMSO or olaparib, as shown in (C). A total of 100 cells were counted for each group. (E, F) PUVA sensitivity was determined in WT, *FANCA*^{-/-} and *NEIL3*^{-/-} HeLa cells after DMSO or olaparib treatment. CellTiter-Glo cell viability assays were performed. Mean and s.e.m. of three independent experiments are shown. ** $P < 0.01$; *** $P < 0.001$ (paired t test).

ment of EGFP-FANCD2 was much weaker and less efficient, and the recruitment could only be clearly observed five minutes after PUVA laser irradiation. PARPi olaparib only partially suppressed FANCD2 recruitment (Figure 4C, D and Supplementary Figure S4D). The PARP-dependent recruitment of NEIL3 and FANCD2 (to a lesser extent) was confirmed in PUVA laser irradiation experiments with PARP knockdown (Supplementary Figure S4E–G). Interestingly, the recruitment of NEIL3 and FANCD2 was mediated by different PARP family members. Knockdown of PARP1 blocked NEIL3 recruitment, while depletion of PARP3 blocked FANCD2 recruitment (Supplementary Figure S4E–G). In addition, depletion of NEIL3 had no impact on FANCD2 recruitment, and depletion of FANCD2 did not affect NEIL3 recruitment (Supplementary Figure S4H and I). These results are consistent with the idea that the NEIL3 pathway is promptly activated upon PUVA exposure, while the FA pathway is delayed.

So far, we showed that NEIL3 was critical for psoralen-ICL repair and PARP was indispensable for NEIL3 re-

cruitment to psoralen ICL. We asked whether treating cells with a PARP inhibitor would have same effect as depleting NEIL3 in PUVA sensitivity assays. Consistently, olaparib treatment sensitized HeLa cells to PUVA and caused an additional sensitivity of *FANCA*^{-/-} cells to PUVA (Figure 4E), in agreement with the PUVA laser stripping data (Figure 4A, B) and PUVA sensitivity data with NEIL3 depletion (Figure 1C, D). On the other hand, olaparib caused a mild increase in PUVA sensitivity in *NEIL3*^{-/-} cells (Figure 4F), which may be due to a less efficient inhibition of FANCD2 by olaparib. These results show that NEIL3 repairs PUVA-ICLs in a strictly PARP-dependent manner.

NEIL3 and RUVBL1/2 interact and function in the same pathway

To better understand the function of NEIL3 in psoralen-ICL repair, we performed mass spectrometry to identify novel NEIL3 binding partners upon PUVA treatment. NEIL3-GFP was expressed in HEK293T cells and im-

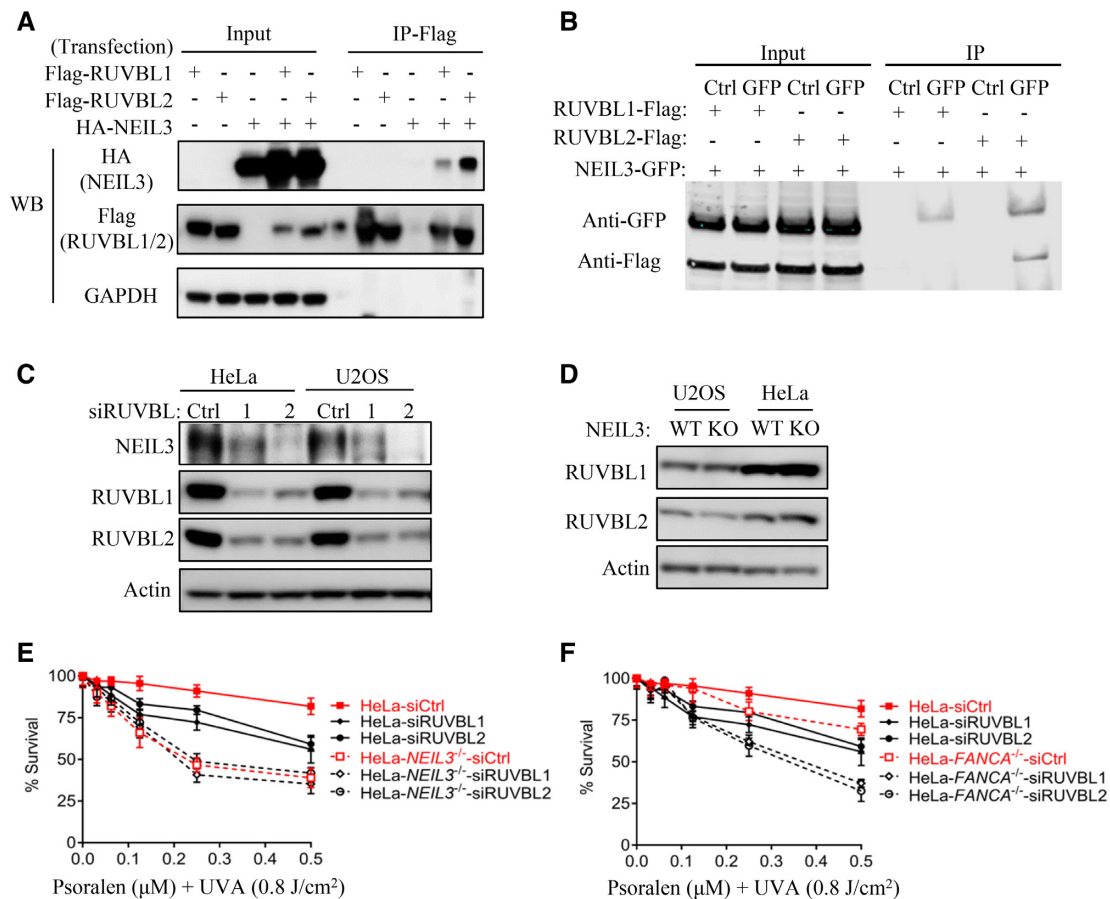


Figure 5. The RUVBL1/RUVBL2 complex is involved in psoralen-ICL repair via the NEIL3 pathway. (A) 293T cells were transiently transfected with indicated plasmids, and co-IP were performed using anti-Flag antibody. WB of the inputs and IPs are shown to detect interactions. (B) 293T cells were transiently transfected with indicated plasmids, and co-IP were performed using normal IgG or anti-GFP antibody. WB of the inputs and IPs are shown to detect interactions. (C) NEIL3 protein levels were analyzed after knockdown of RUVBL1 or RUVBL2 in HeLa or U2OS cells. (D) RUVBL1 and RUVBL2 protein levels were analyzed by WB in WT and *NEIL3*^{-/-} cells. (E, F) Cell viability was measured and compared after knockdown of RUVBL1 or RUVBL2 and PUVA treatment in WT, *NEIL3*^{-/-} (E) and *FANCA*^{-/-} (F) HeLa cells. CellTiter-Glo cell viability assays were performed.

munoprecipitated by the GFP tag. The quality of immunoprecipitation was accessed by silver staining, in which both the bait NEIL3-GFP and the binding partners were evident (Supplementary Figure S5A). A list of the top interactors is shown in Supplementary Figure S5B. Consistent with the PARP-dependent recruitment of NEIL3, PARP1 was detected by the mass spectrometry. To our interest, RUVBL1 and RUVBL2, known to form a heterodimeric complex (44), were among top hits. The RUVBL1/2 complex has been reported to associate with FA/BRCA pathway proteins and its knockdown sensitizes cells to mitomycin C (MMC) (36), a phenotype of FA/BRCA pathway deficient cells. We therefore investigated whether NEIL3 functions in concert with the RUVBL1/RUVBL2 complex. To confirm the interaction between NEIL3 and RUVBL2 and/or RUVBL1, we co-transfected the NEIL3-HA and the RUVBL1-Flag or RUVBL2-Flag plasmids (Supplementary Figure S5C), and co-IP experiments were carried out. RUVBL2, and RUVBL1 to a lesser extent, pulled down NEIL3 (Figure 5A), while NEIL3 pulled down RUVBL2 but not RUVBL1 (Figure 5B). These data suggest NEIL3 primarily binds to RUVBL2 of the RUVBL1/2 complex. Depletion of RUVBL1 or RUVBL2 had no impact on the

recruitment of NEIL3 or FANCD2 to PUVA laser stripes (Supplementary Figure S5D and E). However, a decreased NEIL3 protein level was observed when RUVBL1 or RUVBL2 was knocked down (Figure 5C). A similar reduction of FANCD2 and FANCI levels was observed (Supplementary Figure S5F), which was consistent with previous work (36). Since NEIL3 and RUVBL1/2 form a complex in cells, the down-regulation of NEIL3 might be due to its instability resulting from knockdown of RUVBL1/RUVBL2. On the opposite, RUVBL1 and RUVBL2 levels were not changed significantly in *NEIL3*^{-/-} and *FANCA*^{-/-} cells (Figure 5D and Supplementary Figure S5G). In addition, the cell cycle profiles of RUVBL1-depleted HeLa cells were not altered (Supplementary Figure S5H and (36)). These results show that the RUVBL1/2 complex either stabilize NEIL3 in a complex or regulate NEIL3 through transcription.

We next studied the contribution of the RUVBL1/2 complex to PUVA-ICL repair and its relationship with NEIL3 in this process. Depletion of either RUVBL1 or RUVBL2 led to mild PUVA sensitivity in WT HeLa cells, while *NEIL3*^{-/-} cells were very sensitive to PUVA. We did not observe further PUVA sensitivity after RUVBL1/2 knockdown in *NEIL3*^{-/-} cells, indicating NEIL3 and RUVBL1/2

have an epistatic function in PUVA-ICL repair (Figure 5E). On the other hand, FANCA and RUVBL1/2 showed a non-epistatic relationship in PUVA repair (Figure 5F). Given that the role of RUVBL1/RUVBL2 complex in MMC-ICL repair was dependent on the FA/BRCA pathway (36), which was distinct from psoralen-ICL repair, we next examined MMC sensitivity in *FANCA*^{-/-} and *NEIL3*^{-/-} cells after depletion of RUVBL1/RUVBL2. We did not observe any additional MMC sensitivity after knocking down of RUVBL1 or RUVBL2 in either HeLa-*NEIL3*^{-/-} or HeLa-*FANCA*^{-/-} (Supplementary Figure S5I and J). These data suggest that the RUVBL1/RUVBL2 complex are epistatic with both NEIL3 and FA pathways in MMC-ICL repair. Taken together, these data suggest that RUVBL1/2 and NEIL3 function in the same pathway for psoralen-ICL repair, and this pathway is distinct from the FA/BRCA pathway, which is majorly responsible for MMC-ICL repair.

TRAIP is required for both NEIL3 and FA/BRCA pathways in psoralen-ICLs repair

Recently, TRAIP was identified as a master regulator of psoralen-ICL repair in both the NEIL3 and FA/BRCA pathways in *Xenopus* egg extracts (33). To investigate the role of TRAIP in psoralen-ICLs repair in human cells, we investigated PUVA sensitivity after TRAIP depletion. Lacking a good TRAIP antibody, the efficiency of siRNA-mediated depletion of TRAIP was evaluated by qPCR and Western blotting of exogenously expressed TRAIP-myc (Supplementary Figure S6A and B). Knockdown of TRAIP caused PUVA sensitivity, and more importantly, TRAIP knockdown presented additional PUVA sensitivity in both *FANCA*^{-/-} and *NEIL3*^{-/-} HeLa cells (Figure 6A and B), indicating that TRAIP is non-epistatic with both the FA/BRCA and the NEIL3 pathways in psoralen-ICL repair. In addition, depletion of TRAIP partially impeded NEIL3 recruitment to psoralen-ICLs, but it did not affect FANCD2 recruitment (Figure 6C and Supplementary Figure S6D and E). These results are consistent with a model in which ubiquitylation of the CMG complex by TRAIP is required for NEIL3 recruitment to chromatin in *Xenopus* egg extracts (33). Although MCM7 ubiquitylation has been identified as a key event that is required for NEIL3 recruitment, we only observed a weak impact of MCM7 knockdown in NEIL3 recruitment (Supplementary Figure S6C). Intriguingly, depletion of TRAIP increased the expression of NEIL3 and FANCD2 proteins, and it also increased the mono-ubiquitylation of FANCD2 (Figure 6D). We speculate that this elevated activation of NEIL3 and FA pathways might be a compensatory response to their nonfunctional recruitment in the absence of TRAIP. In conclusion, these data show TRAIP is non-epistatic with both the FA/BRCA and the NEIL3 pathways in psoralen-ICL repair, and suggest that TRAIP may function upstream of both pathways.

DISCUSSION

In this study, we investigated the role of NEIL3 in the repair of psoralen-induced ICLs. We show that NEIL3 plays

a major role in psoralen-ICL repair while the FA pathway is primarily responsible for the repair of MMC- and cisplatin-induced ICLs. The two pathways are non-epistatic in psoralen-ICL repair. Unlike the FA pathway, NEIL3 repair mechanism is PARP dependent and does not introduce DSBs. On the one hand, RUVBL1/2 forms a complex with NEIL3 and is functionally epistatic with NEIL3. On the other hand, TRAIP is functionally non-epistatic with both NEIL3 and FA/BRCA pathways, indicating that TRAIP may function upstream of the two pathways. A model is provided to summarize our findings (Figure 6E).

The hypersensitivity of NEIL3 depleted cells to psoralen-ICLs in multiple human cell lines demonstrates that NEIL3 is the major pathway to repair psoralen-ICLs, and that the NEIL3 pathway is prioritized over the FA pathway in human cells (Figure 1). Based on *in vitro* studies, NEIL3 is also important for repair of AP-ICLs in human cells (25,26). We also tested the sensitivity of *NEIL3*^{-/-} cells to other types of ICLs – namely, cisplatin- and MMC-induced ICLs. Although *NEIL3*^{-/-} cells were sensitive to these ICLs, they were more sensitive to psoralen-ICL, and the repair of cisplatin-ICLs and MMC-ICLs was primarily governed by FA pathway (Supplementary Figure S1). These results are consistent with previous studies indicating that NEIL3 does not process cisplatin-ICL (25,33). It will be interesting to determine how cells choose a pathway upon different ICL exposures. The rapid recruitment kinetics of NEIL3 and relatively slow kinetics of FANCD2 suggest that NEIL3 is the first line of defense. Cells will proceed with the mechanistically simpler NEIL3 pathway when the ICL substrate can be directly unhooked by NEIL3 (i.e. psoralen-ICL) and activate the more complex FA pathway when the ICL cannot be unhooked by NEIL3.

Wu *et al.* showed that both the FA and NEIL3 pathways must to be eliminated in order to generate cellular hypersensitivity to psoralen-ICLs in HAP1 cells (33). Synthetic lethality between FA and NEIL3 pathway was also evident in our study. However, we observed that NEIL3 depletion alone, either by siRNA or CRISPR-Cas9 knock-out, led to hypersensitivity of HeLa and U2OS cells to psoralen-ICLs (PUVA), emphasizing that the NEIL3 pathway is the more efficient pathway in psoralen-ICL repair. This discrepancy may be a result of different cell lines being used. One question was whether the NEIL3 pathway is prioritized in human cells, similar to the observation in *Xenopus* extracts. Our data in human cell lines support this hypothesis. First, NEIL3 is recruited to PUVA-induced DNA damages faster than the FA protein FANCD2 (Figure 4). In our hands, strong NEIL3 recruitment could be observed within one minute, while FANCD2 recruitment was only evident after 5 minutes of PUVA treatment. Secondly, FANCD2 is not mono-ubiquitinated when NEIL3 is present in WT HeLa and U2OS cells, while, in response to PUVA, NEIL3 protein levels are elevated regardless of FA pathway availability (Figure 2). Thirdly, PUVA does not trigger significant phosphorylation of H2AX in WT cells, a sign that NEIL3-mediated repair occurs in the normal setting, while γ H2AX foci levels are higher after PUVA treatment of *NEIL3*^{-/-} cells, indicating that the FA pathway is activated as a backup pathway (Figure 3). In summary, our

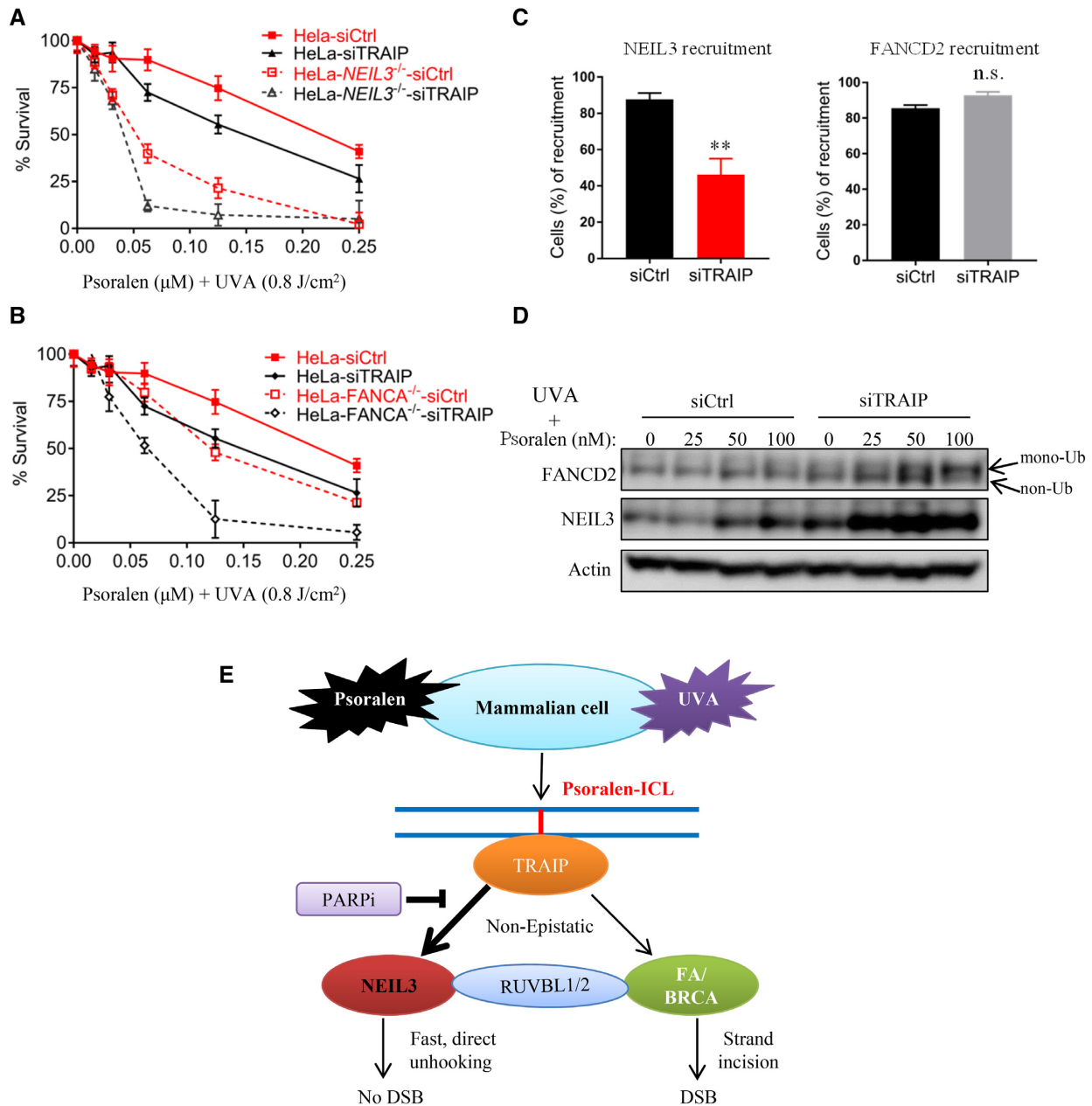


Figure 6. TRAIP shows synthetic lethality with both the NEIL3 pathway and the FA pathway upon PUVA treatment. (A, B) Cell viability was measured after knockdown of TRAIP and PUVA treatment in WT, *FANCA*^{-/-} and *NEIL3*^{-/-} HeLa cells. (C) PUVA laser stripping was used to analyze the recruitment efficiency of NEIL3-EGFP and EGFP-FANCD2 in U2OS cells after siRNA knockdown of TRAIP. A total of 100 cells were counted in each group. ***P* < 0.01, n.s., no significance (paired t test). (D) FANCD2 and NEIL3 were analyzed by Western blotting in cell lysates from HeLa cells, which were transfected with control or TRAIP siRNA and treated with PUVA. (E) A model showing the NEIL3 pathway in psoralen-ICL repair in the context of the FA/BRCA pathway.

data show that NEIL3 pathway is prioritized in PUVA repair in human cells.

The Fanconi Anemia pathway plays an essential role in ICL repair during DNA replication (3). One of the key steps of this pathway is nucleolytic incision of DNA strands flanking the ICL (45), which generates a DSB that need further processing. Both purified NEIL3 and NEIL1 remove psoralen-induced DNA–DNA cross-links *in vitro*, the former does it without nicking DNA backbone (26), which is consistent with results in *Xenopus* extracts (25). Our data in

human cells are consistent with those *in vitro* observations. When NEIL3 was deleted in HeLa cells, a strong accumulation of γ H2AX was observed after PUVA treatment, and this γ H2AX accumulation was attenuated by FANCD2 depletion (Figure 4). Our data demonstrate that the direct unhooking mechanism utilized by NEIL3, which occurs without the generation of DSBs, is conserved in human cells.

Replication fork stalling at a DNA–protein crosslink leads to TRAIP-dependent ubiquitylation and proteasome degradation (46). Wu *et al.* show that CMG ubiquityla-

tion by TRAIP is required to recruit NEIL3 for ICL unhooking. TRAIP associates with the replisome and non-specifically ubiquitylates lysine residues on bulky protein obstacles. The generation of a short ubiquitin chain can recruit NEIL3 (33). Here, we show that NEIL3 is rapidly recruited to PUVA laser tracts in a PARP-dependent manner. The PARP inhibitor olaparib, or knockdown of PARP1, strongly blocks NEIL3 recruitment (Figure 4). TRAIP, on the other hand, only weakly blocks NEIL3 recruitment (Figure 6). Since we looked at an unsynchronized cell population while the *Xenopus* extracts represent S phase, it is possible that the recruitment of NEIL3 relies more on TRAIP during S phase as opposed to other phases of the cell cycle. TRAIP itself is recruited to chromatin following ICL exposure during replication (47). BRCA-related FA proteins (BRCA2, FANCF/BACH1 and FANCD1/PALB2) requires replication for crosslink association, unlike other FA core and I/D2 complexes (48). In agreement with this study, we did not see reduction of FANCD2 recruitment after TRAIP knockdown. Our results suggest that psoralen-ICL repair by NEIL3 is not solely governed by TRAIP, and that it may also function outside of S phase.

Another interesting observation is that the protein levels of NEIL3 and FANCD2 are inter-dependent. We observed FANCD2 and FANCA proteins were decreased in *NEIL3*^{-/-} cells and NEIL3 protein was decreased in the FA cells. Transcription regulation did not appear to be involved, since NEIL3 mRNA levels in *FANCD2*^{-/-} or *FANCA*^{-/-} cells were similar to the WT counterparts. Cell cycle alteration cannot explain the inter-dependency, since cell cycle distribution did not change in those FA cells. A previous study demonstrated that the NEIL1 level is dependent on the presence of an intact FA pathway and NEIL1 undergoes proteolytic degradation in FA cells (49). After treatment with the proteasome inhibitor MG-132, NEIL3 in U2OS-*FANCD2*^{-/-} cells was increased to a similar level as in WT cells. Also, FANCD2 was increased after MG-132 treatment in *NEIL3*^{-/-} HeLa cells, although the increase was not as prominent. These data suggest NEIL3 and FANCD2 stabilize each other by reducing proteasome-mediated protein degradation.

The interdependency between NEIL3 and FANCD2 proteins suggested that they may share a physical interaction. However, we failed to detect a specific protein-protein interaction between NEIL3 and FANCD2 proteins using co-immunoprecipitation. The interaction may be transient or weak in nature and cannot be captured by co-immunoprecipitation. Alternatively, the stabilization between NEIL3 and FANCD2 proteins may not be mediated by a direct interaction; rather, it may be mediated by a shared interaction with additional factors such as the RUVBL1 complex. We have shown that NEIL3 interacts with RUVBL2 and the interaction between FANCD2 protein and RUVBL1/2 complex has been previously reported (36).

A potential role of NEIL1 in ICL repair has also been recently described. Macé-Aimé *et al.* demonstrated that the NEIL1 protein level is dependent on an intact FA pathway, suggesting downregulation of NEIL1 may contribute to hypersensitivity of FA cells to ICLs (49). However, McNeill *et al.* later showed that NEIL1 is recruited to psoralen-ICL, but it interferes and delays psoralen-ICL repair (43). Our

results were consistent with the later study. NEIL1 knock-down cells were mildly resistant to psoralen-ICL. Interestingly, NEIL3 was concurrently upregulated in the NEIL1-depleted cells, providing a mechanism for the resistance of NEIL1-deficient cells to psoralen-ICL. Taken together, these results further suggest that NEIL3, but not the other NEIL glycosylases, is the major enzyme to repair psoralen-ICL.

In summary, our study shows that NEIL3 plays a major role in psoralen-ICL repair, which is non-epistatic with the FA pathway, a pathway which is primarily responsible for MMC- and cisplatin-induced ICLs. The psoralen-ICL repair mechanism of NEIL3 is PARP-dependent and does not introduce DSBs.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (50) with the dataset identifier PXD016256.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Author contributions: N.L., J.Z. and A.D.D. designed the experiments. N.L. and J.Z. carried out the experiments and analyzed the data. N.L., J.Z., S.S.W. and A.D.D. wrote and edited the manuscript. A.D.D., J.Z., J.W. and J.C. supervised this work. J.Z. and A.D.D. conceived the original idea of this study. S.S.W. provided critical reagents. All authors were involved in critical discussions of this project.

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Exploiting the Microhomology-Mediated End-Joining Pathway in Cancer Therapy

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CONFLICT OF INTEREST DISCLOSURE

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ABSTRACT

Repair of DNA double strand breaks (DSB) is performed by two major pathways: homology-dependent repair and classical non-homologous end joining. Recent studies have identified a third pathway: microhomology-mediated end-joining (MMEJ). MMEJ has similarities to homology-dependent repair in that repair is initiated with end resection, leading to single-stranded 3' ends which require microhomology upstream and downstream of the DSB. Importantly, the MMEJ pathway is commonly upregulated in cancers, especially in homologous recombination-deficient cancers which display a distinctive mutational signature. Here we review the molecular process of MMEJ as well as new targets and approaches exploiting the MMEJ pathway in cancer therapy.

INTRODUCTION

DNA Damage and Double-Stranded Breaks

Maintenance of genome integrity is of upmost importance for cellular survival (1). Loss of genomic integrity results in permanent changes to DNA sequence and is the source of many human diseases, notably cancer (2–7). Genome integrity is achieved by DNA repair pathways, collectively known as the DNA damage response (DDR) (8). Double-stranded breaks (DSBs) are the most deleterious form of DNA damage (1). DSBs are generated either exogenously, for example, from exposure to ionizing radiation, or endogenously, for example, from reactive oxygen species secondary to aerobic metabolism or from errors during DNA replication and meiosis (9–11). DSBs were traditionally thought to be repaired by either of two distinct pathways, distinguished by the presence or absence of homology: homologous recombination repair (HR) and classical non-homologous end-joining (NHEJ), respectively (**Figures 1A-B**).

Microhomology-Mediated End-Joining as a Third Pathway for DSB Repair

Evidence for a third pathway that could repair DSBs first emerged following the observation that NHEJ-deficient yeast and hamster cells retained some degree of end-joining activity (12,13). Because this pathway was first reported in NHEJ-deficient cells, it was initially termed alternative end-joining and viewed as a backup mechanism if HR and NHEJ were unavailable. Subsequent work, especially in mammalian cells, has suggested a more substantial role for this pathway, now more commonly referred to as microhomology-mediated end-joining (MMEJ) (14,15).

CURRENT UNDERSTANDING OF DSB REPAIR BY MMEJ

The MMEJ pathway is a stepwise pathway (**Figure 1C**). It is initiated by end-resection near the DSB exposing short regions of complementary sequences ranging from 2 to 20 nucleotides (microhomologies). These microhomologies are used to align the DNA ends with the occurrence of end bridging. Next, the resultant 5' flaps, created following alignment, are processed. A polymerase then fills in any gaps, followed ultimately by ligation (16).

End-Resection

The MMEJ pathway is initiated by end-resection. Following a DSB, poly (ADP-ribose) polymerase-1 (PARP-1) competes with Ku70-Ku80 for binding to DSBs ends, facilitating, though not essential for, recruitment of end-resection factors (17,18). As in HR, the initial end-resection in MMEJ is performed by the MRN complex (MRE11, RAD50, and NBS1) stimulated by C-terminal binding protein interacting protein (CtIP), with the initial creation of a 3' single-strand region (19–21). If the microhomologies are distant, studies have suggested that further resection is performed by Bloom helicase-DNA2 helicase/nuclease and exonuclease 1 (EXO1); however, their roles have not been genetically confirmed. Furthermore, opposing data suggests that long range resection by EXO1 decreases MMEJ (14,19,20,22–27).

Normally, single-stranded DNA (ssDNA) is bound by Replication protein A (RPA) to stabilize the secondary structure. In yeast, RPA functions as a negative regulator of MMEJ. MMEJ is dramatically increased following end resection while HR remains relatively stable when a hypomorphic mutant allele of one of components of yeast RPA is expressed (28). Recently,

Shukla et al. demonstrated that the 5-hydroxymethylcytosine binding, ESC-specific protein (HMCES), previously shown to protect stalled replication forks, can bind ssDNA and promote MMEJ repair (29,30). Furthermore, HMCES binds class switch regions and protects ssDNA overhangs to promote MMEJ, and mice deficient in HMCES have significant defects in class switch recombination, similar to mice deficient in the specialized DNA polymerase of the MMEJ pathway, polymerase theta (POLQ) (30,31). Taken together, these studies suggest that RPA is the critical ssDNA binding protein in HR repair, while HMCES is the critical ssDNA binding protein in MMEJ (**Figures 1B-C**).

End-Bridging and Alignment

End-bridging and alignment of microhomology sequences are next accomplished through the activities of PARP-1, the MRN complex, and POLQ. Recent *in vitro* studies suggest that PARP-1 may compete with Ku70-80 for binding to DSBs and may exhibit end-bridging activity through an unknown mechanism (32). PARP-1 also appears to have a role in the recruitment of POLQ to sites of DNA damage. Regarding the MRN complex, *in vitro* studies have shown that cohesive ends inhibit MRE11-mediated degradation. If a region of microhomology is revealed during resection, MRE11 stalls, thereby stabilizing the junction site (33,34). In addition, scanning force microscopy revealed a complex of MRE11 and RAD50 which can tether linear DNA molecules (35). These findings suggest that the MRN complex, while performing nucleolytic degradation, can facilitate DNA end-bridging and microhomology alignment. POLQ contains a N-terminal helicase domain, a long, unstructured central region, and a C-terminal polymerase domain (36). The crystal structure of the POLQ helicase domain revealed a tetrameric organization,

suggesting a role of microhomology alignment and strand annealing in preparing a substrate for the polymerase domain (37). Indeed, both domains are required for MMEJ, and mutation of a conserved residue in the helicase domain reduces MMEJ (38,39).

Tail and Flap Processing

The processes of DNA end-bridging and alignment result in the generation of nonhomologous 3' tails. These tails must be removed in order to complete the repair process. In some DNA repair processes, such as single-strand annealing, nonhomologous 3' tails are removed by the heterodimeric structure-specific endonuclease ERCC1/XPF (Excision repair cross-complementing group 1 / Xeroderma pigmentosum, complementation group F) (40). ERCC1/XPF is also employed in nucleotide excision repair, in the Fanconi Anemia interstrand crosslink repair, and in base excision repair (41–43). Consequently, it was originally hypothesized that ERCC1/XPF was the endonuclease complex that removes nonhomologous 3' flaps in MMEJ. However, murine ERCC1 ^{-/-} cells demonstrate only a minor MMEJ defect (44). These studies suggest that another functionally-distinct flap endonuclease, such as flap endonuclease 1 (FEN1), might be involved in the removal of nonhomologous 3' tails in the MMEJ pathway.

Gap filling in MMEJ is performed by POLQ, through the process of strand displacement and DNA synthesis, and this process creates displaced 5' ssDNA flaps requiring subsequent removal (45). FEN1 is a structure-specific endonuclease that recognizes and cleaves these 5' ssDNA flaps (46). Using a cell-free system created from rat testes and thymus to study proteins involved in

MMEJ, Sharma et al. demonstrated that siRNA-mediated depletion of FEN1 strongly reduces MMEJ activity (47). More recently, genetic screens to identify genes required for the upregulation of MMEJ and the survival of BRCA2-deficient cells identified FEN1, and specifically the 5' flap endonuclease activity of FEN1 (48). Furthermore, siRNA-mediated depletion of FEN1 resulted in a defect in MMEJ repair in a cell-based MMEJ reporter assay, confirming that FEN1 is an essential enzyme in MMEJ, and suggesting that FEN1 may be the critical endonuclease which cleaves 5' flaps created by POLQ.

Polymerase Theta (POLQ) Gap Filling

Once the DNA microhomology regions are aligned and the 3' nonhomologous tails have been removed, there will be gaps between the duplex DNA. The specialized DNA polymerase involved in MMEJ, POLQ, was first identified from studies in *Drosophila* by using DSBs induced by P-element transposition or sequence-specific endonuclease (38,49). Subsequent studies have shown that POLQ is essential for MMEJ in all mammalian species examined to date (50). POLQ is a 290 kDA A-family DNA polymerase with a unique structure containing a N-terminal helicase domain, a long, unstructured central region, and a C-terminal polymerase domain (36).

The N-terminal domain of POLQ contains a superfamily 2 Hel308-typeS helicase domain. The helicase domain has both ATPase and DNA unwinding activities, which can act independently of each other. The ATPase activity is required for both the suppression of HR by binding RAD51 and inhibiting its assembly along ssDNA, and the survival of HR deficient cells (36,51). Expression of POLQ mutated at ATPase catalytic residues did not reduce RAD51 foci formation,

nor decrease recombination frequency (51). The ATPase activity also facilitates removal of RPA from ssDNA to allow MMEJ and inhibit HR (52). Conceivably, with removal of RPA from ssDNA, HMCES could bind, protecting ssDNA overhangs and promoting MMEJ (30). *In vitro*, the POLQ helicase domain can unwind DNA, thereby facilitating displacement synthesis by the POLQ polymerase domain. Further studies are required to understand the coordination of these domains *in vivo* (53).

The long, unstructured central region of POLQ contains a RAD51 interaction motif. Protein interaction studies of POLQ demonstrated that this site directly interacts with RAD51 via three distinct motifs, though only one region (amino acids 847 to 894) was both necessary and sufficient for RAD51 binding. Interaction of the central domain of POLQ with RAD51 inhibited RAD51-ssDNA nucleoprotein filament assembly. In this way, POLQ acts as an anti-recombinase capable of inhibiting HR and promoting MMEJ (51). This process appears to mirror that of the POLQ helicase domain which can displace RPA, inhibit HR, and promote MMEJ (52). These interactions of the POLQ helicase domain with RPA and the POLQ central domain with RAD51, respectively, demonstrate the competitive nature between MMEJ and HR, occurring at the level of ssDNA binding. In addition, the central domain of POLQ regulates POLQ multimerization (54).

The C-terminal domain of POLQ contains an A-family DNA polymerase domain that performs gap filling. *In vitro* and cellular experiments have elucidated POLQ's gap filling mechanism. POLQ anneals to short regions of ssDNA microhomology of 2 to 6 base-pairs (37,55,56).

Because POLQ can uniquely prime DNA synthesis from non-optimal base-pairing leading to the introduction of insertions at the break sites, with the 3' flaps acting as a template, it is highly error-prone (45,57,58). In addition, the POLQ polymerase is proofreading deficient and quite promiscuous because of its robust terminal transferase activity (59). Biochemically, which domains of POLQ are necessary for DNA synthesis depend on the substrate (54). Purified POLQ polymerase domain alone is active on short ssDNA and short 3' overhangs. Longer ssDNA substrates require both the POLQ N-terminal helicase domain and the C-terminal polymerase domain, and it is the helicase domain's DNA annealing ability that is required rather than its helicase activity. Further, extension of long ssDNA substrates requires multimers of POLQ, and multimerization requires the central region (54).

End Ligation

DSB repair by MMEJ is completed upon ligation of the DNA ends. In mammalian cells, three DNA ligases are encoded: DNA ligase I, DNA ligase III, and DNA ligase IV (60). Ligase IV functions in NHEJ and its deficiency does not impact MMEJ (61). Substantial literature has demonstrated that Ligase III is the major ligase in MMEJ. First, *in vitro* biochemical and plasmid-rejoining studies identified Ligase III (32,62). Second, these studies were confirmed *in vivo*, where mouse cells deficient in Ligase III demonstrated reduced frequency of chromosomal translocations. In this case, the few remaining translocations lacked areas of microhomology commonly seen in MMEJ (63). Third, sequential depletion of Ligase III followed by Ligase I depletion was additive, suggesting the latter is a backup ligase (63). Ligase III forms a stable complex with the scaffold protein, X-ray repair cross-complementing protein 1 (XRCC1), and both proteins in the complex

interact with PARP-1 (64,65). This interaction with PARP-1 is not required for recruitment of Ligase III/XRCC1 to DSBs as there is no reduction in recruitment in the presence of PARP inhibition (15). Rather, it appears that the MRN complex might recruit Ligase III/XRCC1 to DSBs during repair by MMEJ (66).

MMEJ'S ROLE IN HUMAN DISEASE

Because of the mutagenic nature of POLQ, it is likely that POLQ and hence MMEJ are only activated under rare circumstances. Indeed, POLQ protein expression is highly limited in normal tissue (51,67). These rare circumstances might include the following. First, as POLQ expression greatly increases in HR-deficient cancers, the presence of active HR could inhibit expression of POLQ to prevent a more mutagenic DSB repair (51,68). Perhaps the BRCA1 protein, which has some transcriptional functions, reduces the transcription of the POLQ gene (69). Second, embryonic stem cells and somatic cells rely on different DSB repair pathways (70). MMEJ might play a greater role during gametogenesis, embryogenesis, or in hematopoietic stem cells as expression of POLQ is greatest in the testis, human placental tissue, and hematopoietic stem cells (36,71). Third, POLQ appears to have a prominent role in the repair of four-stranded guanine rich structures (G-quadruplex) (72–74). These structures can have both beneficial and deleterious functions. On the one hand, G-quadruplexes can protect telomeres, regulate transcription, and promote immunoglobulin gene recombination. On the other hand, these structures are prone to genomic instability and can inhibit DNA replication through fork stalling (75). Indeed, mice deficient in POLQ have increased genomic instability

(76). Finally, POLQ might play a preferred role in the repair of repetitive DNA, as HR could be a deleterious option given the possibility of recombination between repetitive elements.

MMEJ and Cancer

One of the hallmarks of cancer is genomic instability (77). The repair of DSBs by MMEJ is an intrinsically mutagenic pathway. The creation of templated insertions at DSB junction sites is characteristic of MMEJ (38,51,78,79). Templated insertions are small insertions, typically, 3 to 30 bps, synthesized from DNA flanking the DSB junction into sites of microhomology deletions (38). Templated insertions, are abundant in the ClinVar database, raising the question of MMEJ's contribution to malignancy (80). Microhomology signatures have been observed at sites of oncogenic chromosomal translocation breakpoints in primary human cancer cells, raising the possibility that MMEJ could be the causative mechanism of these translocation (23,26). Further, review of The Cancer Genome Atlas (TCGA) demonstrates that POLQ is overexpressed in breast, lung, bladder, colorectal, gastric, glioblastoma, pancreatic, prostate, melanoma, and uterine cancers, correlating with poor prognosis (67,81). The elevated POLQ expression in these tumors may result, at least in part, from an underlying HR deficiency.

Cancers deficient in HR up-regulate the expression of POLQ as a survival strategy. Aberrant use of MMEJ could create more mutations in cancers, promoting both cancer growth and resistance (82). These cancers become addicted to POLQ expression, routing the repair of DSBs to MMEJ, as evidenced by the accumulation of a distinct pattern of MMEJ-mediated templated insertions (51,68,83). This mutation signature, initially termed mutation signature 3, was identified by

whole genome sequence analysis of HR-deficient tumor and thought to typify tumors that were potentially HR-deficient (84). In actuality, this signature better represents up-regulation and addiction to POLQ rather than a specific signature for HR deficiency (85). Indeed, in a recent revision of mutational signatures in cancers, signature 3 has been further resolved. The MMEJ specific signature, now defined as insertion-deletion signature 6, is characterized predominantly by greater than 5bp deletions with overlapping microhomology at deletion boundaries and is correlated with single-base substitution signature 3 (86).

THERAPEUTIC APPLICATION OF MMEJ FOR CANCER TREATMENT

Loss of DNA repair pathways is a common feature of cancers and likely provides a growth advantage (87). Concomitantly, there is increased dependence on the remaining DNA repair pathways. This dependence renders the cancer vulnerable to inhibition of the remaining DNA repair pathways and is the basis of the synthetic lethality. This concept of synthetic lethality has been successfully exploited by the use of PARP inhibitors in breast and ovarian cancers harboring inactivating mutations in BRCA1 or BRCA2, and could similarly be employed with MMEJ inhibition (88,89). Because PARP-1 is also a component of MMEJ (see section 2), PARP inhibitors also inhibit MMEJ (15). Indeed, inhibition of MMEJ appears to be a major mechanism of synthetic lethality of PARP inhibitors. Loss of expression of POLQ or other MMEJ genes in HR-deficient cells is synthetically lethal (51,68). Some cancers also have mutations in NHEJ genes, and these cancers exhibit synthetic lethality from depletion of POLQ (58,90). Importantly, some HR-deficient tumors acquire PARP inhibitor resistance by downregulating NHEJ. This offers the opportunity for using a POLQ inhibitor, since NHEJ-deficient cells are also dependent

on MMEJ. Taken together these observations provide a strong rationale for targeting MMEJ in the treatment of cancers, including PARP inhibitor resistant cancers (**Figure 2**) (88,89).

Synthetic Lethality: Targeting MMEJ and HR

POLQ has limited protein expression in normal tissue but is overexpressed in wide variety of cancers (51,67,81). This overexpression is increased with concomitant p53 loss (91). These cancers may be addicted to POLQ expression because of the need to repair DSBs (51,68,83). Furthermore, POLQ, via its ATPase activity and RAD51 binding ability, can prevent the accumulation of toxic RAD51 on resected ssDNA, thereby enhancing cancer cell survival. Loss of POLQ in these tumors results in cell death by impairing MMEJ-mediated of DSBs and promoting the buildup of toxic RAD51 (51,68). Thus, POLQ is an attractive target for drug development in HR-deficient cancers (**Figure 2A**) (92).

The POLQ protein has three domains: a N-terminal helicase domain, a long, unstructured central region, and a C-terminal polymerase domain (36). POLQ inhibitors could be designed to target either the N-terminal helicase domain or the C-terminal polymerase domain. Targeting the polymerase domain would inhibit repair of resected DSBs at the gap-filling step in HR-deficient cancers. Targeting the helicase domain, however, might be a better option, as MMEJ repair of DSBs would be inhibited and toxic RAD51 foci would accumulate (51). POLQ inhibitors are currently undergoing active pre-clinical development. Indeed, Zhou et al. have recently identified the antibiotic Novobiocin as a specific POLQ helicase domain inhibitor that selectively kills HR-deficient cancer cells *in vitro* and *in vivo* (93).

In addition to POLQ, FEN1 is an essential enzyme in MMEJ, required at least for removal of the 5' flap and potentially the 3' flap (47,48). Depletion of FEN1 results in a defect in MMEJ repair and is also synthetically lethal with HR deficiency, recapitulating the synthetic lethality observed with POLQ inhibition (48,51,68). Hydroxyurea based FEN1 inhibitors have been developed that induce synthetic lethality and did not induce toxicity as a monotherapy (94). Importantly, FEN1 is not exclusive to the MMEJ pathway. It is also involved in long-patch base excision repair, thereby adding to its value as a target for anti-cancer drug development.

Synthetic Lethality: Targeting More Than HR-Deficiency

Synthetic lethality occurs not only in HR-deficient cells but also in cells deficient in NHEJ, where depletion of POLQ decreases cell survival (58). There are only a few cancers with underlying defects in NHEJ (90). However, DNA-dependent protein kinase (DNA-PK) is a key component of the NHEJ pathway, and DNA-PK inhibitors are currently under development because they block NHEJ and enhance the activity of DNA-damaging agents (95,96). DNA-PK inhibition results in a significant increase in MMEJ-mediated templated insertions and, coupled with genetic downregulation of POLQ, results in significantly reduced viability (91). These results provide evidence for the application of POLQ inhibition beyond HR-deficient cancers and for combination treatment strategies (**Figures 2B-C**).

Cell cycle DNA damage checkpoints allow time for repair by the DDR. The protein kinases, ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia and Rad3-related protein (ATR),

play central roles in these DNA damage checkpoints, creating an opportunity for synthetic lethality (97). ATM is activated by DSBs and initiates a DNA damage response via a signaling cascade of phosphorylation with activation of checkpoint kinase 2 (CHK2) and ultimately p53, as well as activation of the HR pathway. ATR, in contrast, is activated by replication stress. Replication stress is common in oncogene-driven cancer cells, but not in normal tissue (98). Through its kinase activity, ATR activates checkpoint kinase 1 (CHK1) and ultimately Wee1-like protein kinase (WEE1), leading to prolongation of the S/G2 cell cycle phase (99).

Replication stress activates the ATR pathway to prevent replication fork collapse and allow DNA replication to continue (99). By creating single-strand breaks at replication forks, using either a Cas9 nickase or a topoisomerase inhibitor, Wang and colleagues demonstrated synthetic lethality with POLQ deficiency, demonstrating a role for MMEJ in replication stress tolerance (100). Inhibition of the ATR-CHK1-WEE1 signaling cascade is likely to prove useful in combination with POLQ inhibition (**Figure 2B**). Inhibitors of ATR, CHK1, or WEE1 are currently under investigation in clinical trials and have activity through their ability to promote replication stress. Gemcitabine is another agent which can promote replication stress. A randomized phase II clinical trial of the ATR inhibitor M6620 plus gemcitabine demonstrated efficacy through the induction of replication stress in cancer cells (101). Thus, combination treatment strategies involving induction of replication stress with gemcitabine coupled with POLQ inhibition provides a strong rationale for future trials. Finally, a targeted CRISPR screen of 309 murine genes known to be involved in DDR uncovered 140 genes synthetically lethal with POLQ deficiency with the majority of these identified genes being previously unknown. It is likely that

whole genome wide CRISPR screening to identify genetic vulnerabilities that interact with POLQ will reveal new combination therapy targets.

Targeting MMEJ to Overcome Therapy Resistance

The success of PARP inhibitors (PARPi) in the treatment of breast and ovarian cancers harboring inactivating mutations in BRCA1 or BRCA2 has demonstrated the power of synthetic lethality (88,89). Unfortunately, these cancers ultimately acquire PARPi resistance, with the primary mechanisms of resistance being either restoration of HR repair or replication fork stabilization (102). Reactivation of HR in PARPi resistant cancers would be predicted to alleviate dependence on MMEJ for DSB repair. However, POLQ via its RAD51 binding ability, prevents accumulation of toxic RAD51 secondary to abortive HR. Though HR is often reactivated in PARPi resistant cancers, through somatic reversion of BRCA1/2 mutations or via downregulation of NHEJ, inhibition of POLQ could still result in accumulation of toxic RAD51, resulting in cellular toxicity (51). Furthermore, POLQ and the MMEJ are required for coping with replication stress (100). Inhibition of POLQ in PARPi resistant cancers would still result in increased replication stress and cellular toxicity. Indeed, PARPi resistant tumors are sensitive to the specific POLQ inhibitor, Novobiocin, both *in vitro* and *in vivo* (93). Targeting MMEJ by inhibition of POLQ might help prevent the development of PARPi resistance or re-sensitize PARP inhibitor resistant cancers (**Figure 2C**).

Cancer cells often lose expression of p53 or express a mutant version (103). Loss of p53 function in cancer cells increases resistance to ionizing radiation, a common treatment modality

for cancer (104,105). Given POLQ is overexpressed in p53-deficient cancers, this raises the question of whether POLQ inhibition might re-sensitize p53-deficient cancers to ionizing radiation or if POLQ inhibition could be used as an adjuvant with radiation treatment (91). Indeed, treatment of bone marrow stromal cells derived from POLQ deficient mice demonstrated increased sensitivity to ionizing radiation (106).

Biomarker-Guided Development of MMEJ Inhibitors

The development of predictive biomarkers to guide the application of MMEJ inhibition in cancers is crucial. There are at least four potential MMEJ/POLQ biomarkers. First, since POLQ is overexpressed in HR-deficient cancers or in p53-deficient cancers, dependence on POLQ activity would be predicted by upregulation of POLQ protein expression (51,68,83,91). Assessment of POLQ expression in cancers, via immunohistochemistry or RT-PCR, could serve as a predictive biomarker for response to POLQ inhibition. Second, depletion of POLQ in HR-deficient cancers leads to RAD51 accumulation (51). Assessing RAD51 accumulation could serve as a pharmacodynamic biomarker for POLQ inhibition. Third, the MMEJ pathway is required for alleviation of replication stress and is synthetically lethal with ATR inhibition (100). Replication stress activates the ATR pathway (99). Phosphorylation of RPA is downstream of ATR activation and is an immunohistochemical marker of replication stress (107). Thus, the assessment of phosphorylated-RPA could be a biomarker of the effectiveness of an MMEJ inhibitor. Finally, templated insertions are a biomarker of ongoing (or previous) MMEJ activity in the tumor cells and are a potential indicator of POLQ inhibitor efficacy (84,85). The development of biomarkers will be extremely useful in future clinical trials of POLQ inhibitors.

SUMMARY

DNA is under constant threat from both endogenous and exogenous damage, and the maintenance of genomic integrity is critical for cell survival (1). The most deleterious type of DNA damage is DSBs. Mammalian cells have three main pathways to repair DSBs: NHEJ, HR, and MMEJ. Initially, NHEJ and HR were the best studied DSB repair pathways and were thought to be the dominant pathways, with MMEJ as a weaker alternative pathway. However, evidence has since emerged to support MMEJ as a more substantial third pathway given the functional relevance of the pathway to both DSB repair and human disease. Indeed, the prevalence of a distinctive MMEJ mutational signature of templated insertions in up to 20% of all human cancers provides evidence of the widespread relevance of this pathway. Given the success of targeting DDR dependencies as a therapeutic approach to cancers, for example with PARP inhibitors in HR-deficient ovarian and breast cancers, targeting MMEJ is especially enticing. Furthermore, targeting MMEJ could be helpful in the setting of acquired drug resistance to prior therapies.

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FIGURE LEGENDS

FIGURE 1: DSB Repair Pathways. Repair of DSBs is a stepwise pathway. **(A)** NHEJ. (1) Ku70-Ku80 (Ku) heterodimer recognizes DSB end. (2) Recruitment of DNA-PKcs to Ku70-Ku80 heterodimer-DSB ends. If incompatible ends, further processing is required. (3) Ligation of DNA ends by XRCC4-DNA ligase IV. **(B)** HR. (1) MRN, stimulated by CtIP performs end-resection. (2) ssDNA is bound by RPA. (3) RAD51 replaces RPA, forming a RAD51-ssDNA nucleoprotein filament. (4) RAD51-ssDNA nucleoprotein filament performs strand invasion of the sister chromatid, leading to template-dependent strand extension followed by strand annealing. **(C)** MMEJ. (1) PARP-1 competes with Ku70-80 to bind DSBs ends, recruiting MRN and CtIP. (2) End-resection is

initiated by MRN, stimulated by CtIP, in a stepwise manner first with the creation of a 3' single-strand region endonucleolytically, subsequently followed by 3' to 5' exonucleolytic digestion. (3) Competition for binding of single-stranded DNA by RPA or HMCES with HMCES-ssDNA coating favored by MMEJ. (4) End-bridging and microhomology alignment. End-bridging is mediated by a combination of PARP-1, MRN, and POLQ. (5) Flap processing. Non-homologous 3' tails are removed by ERCC1/XPF, FEN1, and likely as-yet identified endonucleases. Non-homologous 5' tails are removed by FEN1. (6) POLQ gap filling. POLQ can uniquely prime DNA synthesis from non-optimal base-pairing leading distinctive templated insertions. (7) Ligation of ends by LIG3/XRCC1. See text for more details.

FIGURE 2: Therapeutic Application of MMEJ for Cancer Treatment. (A) HR-deficient cancers require MMEJ to repair DSBs. Inhibition of POLQ results in synthetic lethality in HR-deficient cancers. (B) POLQ deficiency is synthetically lethal with ATR inhibition. Inhibition of the ATR-CHK1-WEE1 signaling cascade leading to replication stress in combination with POLQ inhibition would result in synthetic lethality. (C) PARP inhibition results in DSBs which can be repaired by MMEJ. Inhibition of both PARP and POLQ (MMEJ) induces synthetic lethality.

Figure 1:

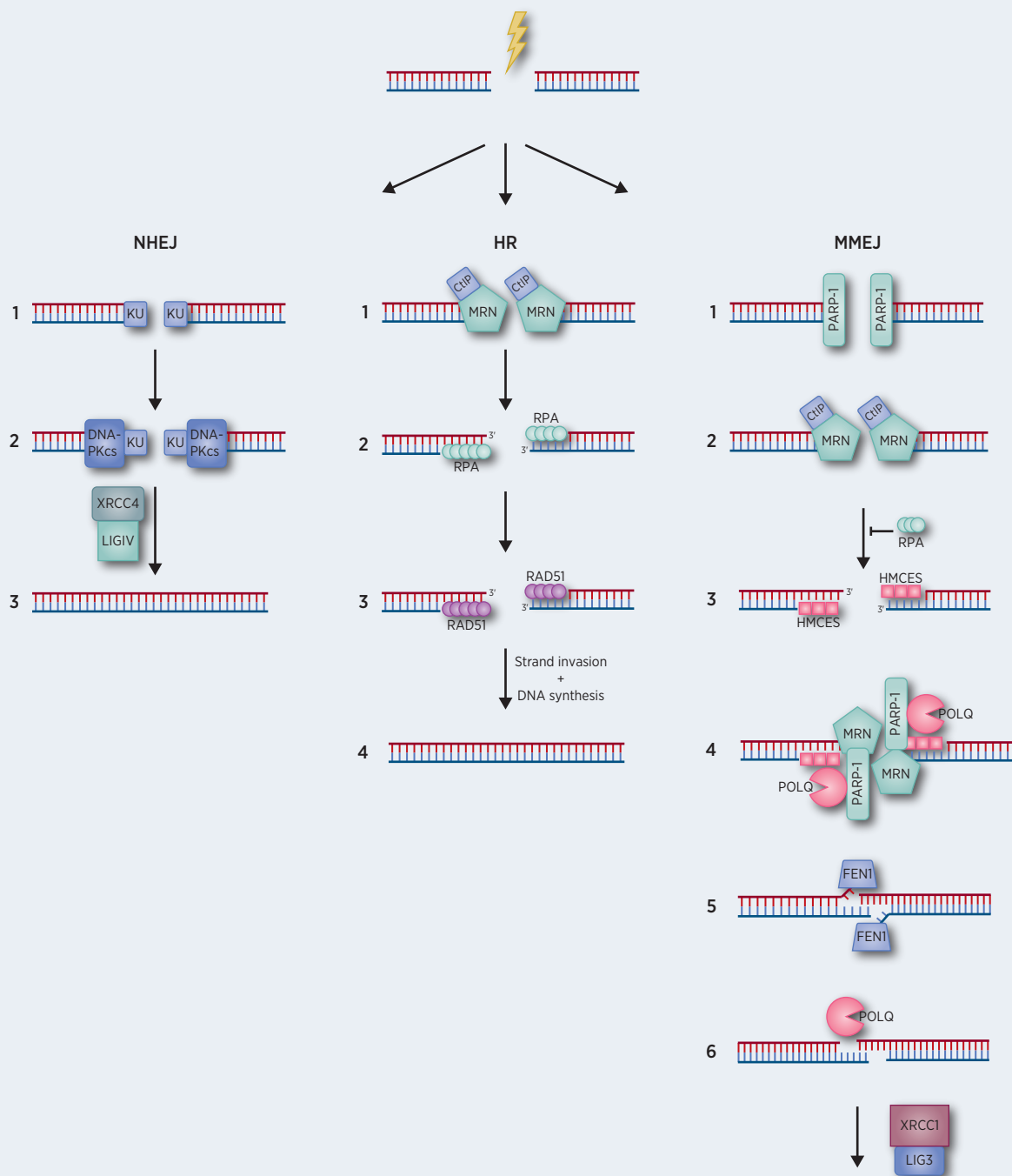
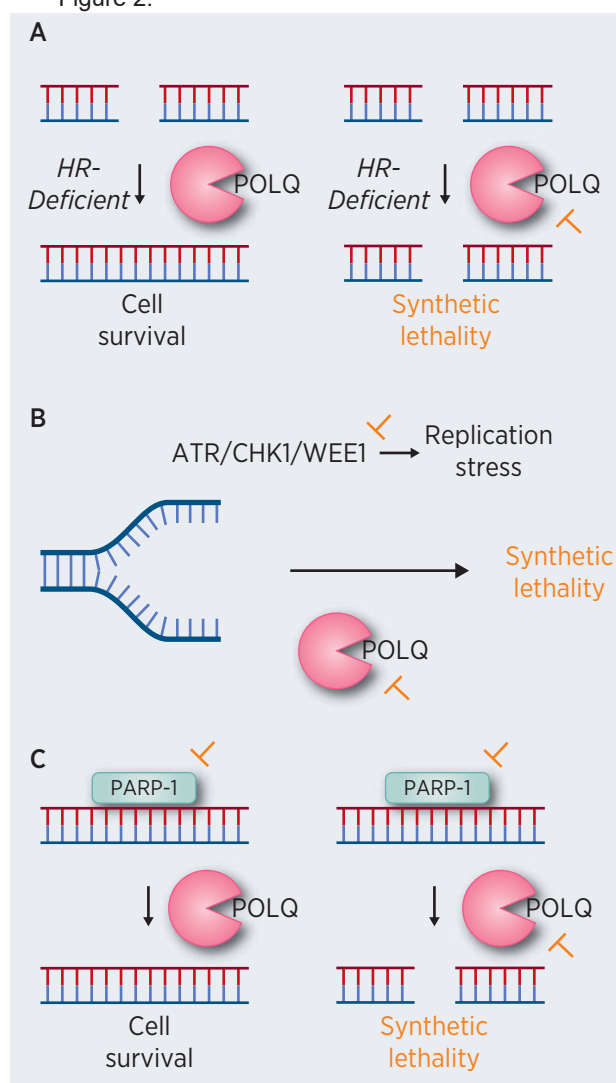


Figure 2:



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Exploiting the Microhomology-Mediated End-Joining Pathway in Cancer Therapy

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Polymerase Theta Inhibition Kills Homologous Recombination Deficient Tumors

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Abstract

PARP inhibitors (PARPi) have become a new line of therapy for Homologous Recombination (HR)-deficient cancers. However, resistance to PARPi has emerged as a major clinical problem. DNA polymerase theta (POLθ) is synthetic lethal with HR and a druggable target in HR-deficient cancers. Here, we identified the antibiotic Novobiocin (NVB) as a specific POLθ inhibitor that selectively kills HR-deficient tumor cells *in vitro* and *in vivo*. NVB directly binds to the POLθ ATPase domain, inhibits its ATPase activity, and phenocopies POLθ depletion. BRCA-deficient tumor cells and those with acquired PARPi resistance are sensitive to NVB *in vitro* and *in vivo*. Increased POLθ expression levels predict NVB sensitivity. The mechanism of NVB-mediated cell death in PARPi resistant cells is the accumulation of toxic RAD51 foci, which also provides a pharmacodynamic biomarker for NVB response. Our results demonstrate that NVB may be useful alone or in combination with PARPi in treating HR-deficient tumors, including those with acquired PARPi resistance.

One Sentence Summary: We identified Novobiocin as a specific POLθ inhibitor that selectively kills naïve and PARPi resistance HR-deficient tumors *in vitro* and *in vivo*.

Since the discovery of the synthetic lethal relationship between PARP inhibition and homologous recombination (HR) repair deficiency (1, 2), PARP inhibitors (PARPi) have become a new line of therapy for HR-deficient cancers (3-7). However, resistance to PARPi has emerged as a major obstacle to their clinical effectiveness (8). While several mechanisms of PARPi resistance are known (9-14), a rational and effective method for overcoming such resistance is still lacking. DNA polymerase theta (POL θ or POLQ), a key enzyme in microhomology-mediated end joining (MMEJ), is synthetic lethal with HR and a druggable target in HR-deficient cancers (15-18). A small-molecule screen identified novobiocin (NVB) as a specific POLQ inhibitor. NVB inhibited POLQ *in vitro* and *in vivo*. Moreover, we demonstrated that NVB can be used alone or in combination with PARPi in treating HR-deficient tumors, even after they have acquired PARPi resistance.

RESULTS

A small-molecule screen identified NVB as a specific POL θ inhibitor

Polymerase theta (POL θ , encoded by the *POLQ* gene) is a critical enzyme for repairing DNA double-strand breaks (DSBs) through a microhomology-mediated end joining (MMEJ) mechanism (19, 20). POL θ expression is elevated in several types of cancers where it correlates with poor prognosis (21, 22). HR-deficient tumors upregulate POL θ -mediated MMEJ as a survival strategy, and loss of POL θ in these tumors results in cell death (15, 17). This synthetic lethality with HR defines the rationale for developing POL θ inhibitors for the treatment of HR-deficient cancers (16).

The ATPase activity of POL θ is required for the survival of HR-deficient cells (17). We took advantage of the strong *in vitro* ATPase activity of purified POL θ (**Fig. S1A**) and designed a large-scale small-molecule screen to identify POL θ inhibitors based on the ADP-Glo luminescent assay (**Fig. 1A, B**). The hits from the primary screen were evaluated in a

secondary screen (**Fig. S1B-D**). The four most potent POL θ ATPase inhibitors were Mitoxantrone (MTX), Suramin (SUR), Novobiocin (NVB) and Aurintricarboxylic acid (AUR), which were individually verified in a ^{32}P -based radiometric ATPase assay (**Fig. S1E**). To test the specificity of the most potent compounds, we purified two other known DNA repair-related ATPases, namely SMARCAL1 and CHD1 (23, 24), and performed ATPase inhibition assays. Only NVB showed high specific inhibition of POL θ with little effect on SMARCAL1 or CHD1 activity, while MTX, SUR and AUR were nonspecific pan-ATPase inhibitors that inhibited all three ATPases (**Fig. 1C and S1F, G**).

To further evaluate the specificity of Novobiocin (NVB) for POL θ , we analyzed its binding capacity. NVB-conjugated beads pulled down the purified ATPase domain of POL θ but not of SMARCAL1, suggesting that NVB directly binds to the POL θ ATPase domain *in vitro* (**Fig. 1D, S1H**). Active NVB-beads also pulled down GFP-tagged full-length POL θ overexpressed in HEK293T cells (**Fig. S1I**). Excess free NVB competed the POL θ binding to the NVB-beads (**Fig. S1J**). Next, we performed protein thermal shift assays, an assay commonly used to demonstrate ligand-protein binding (25, 26). NVB significantly increased the thermal stability of POL θ when compared to DMSO (**Fig. 1E**), while the PARP1 inhibitor olaparib, used as an internal control, effectively stabilized the PARP1 protein (**Fig. S1K**). Finally, we performed molecular docking to find the NVB binding site using the published crystal structure of POLQ ATPase domain (27). The computational docking results revealed that a deep tunnel within the POLQ ATPase domain could accommodate NVB, and that one end of NVB could reach the active site to prevent ATP binding to POLQ and inhibit its ATPase activity (**Fig. 1F**). Taken together, these data demonstrate that NVB binds directly and specifically to the POL θ ATPase domain *in vitro*.

We next assessed whether cellular exposure to NVB has the same consequences as POL θ depletion. Both the ATPase and the polymerase domains contribute to efficient

MMEJ repair activity (28), and the PARP-dependent recruitment of POL θ to DSB sites is a crucial step in this function (15, 17). The inhibition of POL θ by NVB prevented the recruitment of POL θ to laser micro-irradiated DNA damage sites in human cells (**Fig. 1G**). Consequently, NVB inhibited the MMEJ activity in U2OS cells, as demonstrated by the GFP-based EJ2 repair assay (**Fig. 1H**). In contrast, NVB had little effect on HR activity, as measured by the DR-GFP reporter assay (**Fig. S2A**). POL θ also functions as an anti-recombinase that antagonizes HR, and depletion of POL θ increases RAD51 (17) and γ H2AX (29, 30) foci assembly. Again, cellular exposure to NVB recapitulated the RAD51 and γ H2AX phenotypes of a POL θ knockdown. NVB activated more RAD51 foci (**Fig. S2B**) and γ H2AX foci (**Fig. S2C**) in RPE1 cells, regardless of IR exposure. Taken together, these results establish that NVB-mediated POL θ inhibition impairs POL θ DNA repair function and phenocopies POL θ depletion in human cells.

NVB specifically Kills HR-deficient cells *in vivo* and *in vitro*

Depletion of POL θ is synthetically lethal in HR-deficient tumor cells (15, 17). Since NVB has been previously approved for human use, we decided to directly test whether NVB reduces the survival of HR-deficient tumor cells in animal models. First, we evaluated the efficacy of NVB in tumors derived from the K14-Cre-*Brca1*^{fl/fl}; *Trp53*^{fl/fl} genetically-engineered mouse model (GEMM) of triple-negative breast cancer (TNBC), in which spontaneous mammary carcinomas develop after approximately 7 months (31, 32). Tumors from this model were transplanted into immunocompetent FVB/129P2 syngeneic mice that were subsequently treated with NVB. NVB strongly suppressed the growth of these *Brca1*-deficient GEMM-derived tumors (**Fig. 2A** and individual mouse data in **Fig. S3A**). The NVB-treated tumors were significantly smaller compared to the vehicle-treated after 7 days of treatment and beyond. NVB prolonged the overall survival of the tumor-bearing mice by 3-

fold compared to vehicle, with median survival times of 29 and 10 days for NVB- and vehicle-treated mice, respectively (**Fig. 2B**).

Next we tested whether the efficacy of NVB *in vivo* was specific to HR-deficient tumors. We performed a mouse xenograft study with a FANCF-deficient, HR-deficient ovarian cancer cell line [TOV21G + empty vector (EV)] or with the FANCF-complemented, HR-proficient cell line (TOV21G + FANCF cDNA) (**Fig. 2C**). TOV21G + EV cells but not TOV21G + FANCF cDNA cells were sensitive to olaparib and NVB (**Fig. S3B, C**) (33). While vehicle treatment had no impact on the growth of FANCF-deficient or FANCF-complemented tumors, NVB specifically impaired the growth of the FANCF-deficient tumors, with no effect on FANCF-complemented tumors (**Fig. 2C**). We also measured RAD51 foci as a pharmacodynamic biomarker and showed that RAD51 foci were strongly induced in the treated tumors (**Fig. S3D-F**). These results demonstrate that NVB strongly and specifically suppresses the growth of HR-deficient tumors *in vivo*.

To confirm and strengthen the *in vivo* results, we studied isogenic pairs of HR-deficient and HR-proficient cells *in vitro*. We generated *BRCA1* (34) and *BRCA2* knockout RPE1 cells (referred as *BRCA1*^{-/-} and *BRCA2*^{-/-}) in a *TP53*^{-/-} background, which were sensitive to PARPi (**Fig. S4A**). Consistent with our *in vivo* data, clonogenic survival assays showed that NVB significantly reduced the survival of *BRCA1*^{-/-} and *BRCA2*^{-/-} cells, compared to the isogenic WT cells (**Fig. 2D, S4B and Fig. S4C**). We also tested the top 15 initial hits from our screen for their ability to differentially affect the survival of *BRCA1*^{-/-} and WT cells. Among those, NVB had the highest efficacy in selectively killing the *BRCA1*^{-/-} cells (**Fig. S4D**).

To demonstrate the cellular outcome of NVB exposure, we next showed that NVB exposure induces apoptosis in *BRCA1*^{-/-} but not WT cells in a dose dependent manner (**Fig. S5A**). NVB induced chromosomal aberrations and radial chromosomes (a marker of

genomic instability) in *BRCA1*^{-/-} cells, even in the absence of the crosslinking agent mitomycin C (MMC) (**Fig. S5B-D**), confirming that NVB increases DNA damage. These results further demonstrate that NVB phenocopies POLθ depletion, and indicate that NVB-mediated POLθ inhibition is synthetically lethal with HR deficiency and specifically induces cell death in HR-deficient tumor cells.

POLθ is the major target of NVB in human cells

Off target effects are major concerns of biological small inhibitors. To further evaluate whether POLθ is the specific target of NVB and to demonstrate the specificity of NVB in cells, we knocked out the *POLQ* gene in U2OS and RPE1 cells using CRISPR-Cas9 and asked whether loss of POLθ alleviates the cytotoxicity of NVB (**Fig. 2E and S6**). *POLQ* knockout cells were confirmed by genomic sequencing, immunoblotting, increased RAD51 foci, increased sensitivity to irradiation, and increased micronuclei formation (**Fig. S6A-F**). Importantly, *POLQ*^{-/-} cells showed increased tolerance to NVB, both in U2OS and RPE1 cells, as compared to the WT cells (**Fig. 2E and S6G**), indicating that POLθ is the major target of NVB in human cells.

NVB is a known coumarin antibiotic that inhibits DNA gyrase in bacteria (35). NVB has been reported to inhibit the ATPase of HSP90 and of the DNA gyrase homolog topoisomerase II (TOP2) in eukaryotes (36) (37) (38), suggesting that these enzymes may be off-target enzymes of NVB. However, other reports suggest that NVB does not inhibit TOP2 in eukaryotic cells (39). Additionally, the reported IC₅₀ of these targets are very high (approximately 700 μM for HSP90 and 300 μM for TOP2), compared with the IC₅₀ of POLθ. Nevertheless, we next examined whether the cytotoxic effect of NVB in HR-deficient cells resulted from off-target inhibition of HSP90 or TOP2. Unlike the well-characterized HSP90 inhibitor PU-H71, NVB treatment at a concentration that killed HR-deficient cells (i.e. 100

μM) did not induce the degradation of HSP90 clients such as AKT1, BRCA1, or CDK6, or increase the expression of HSP70 (**Fig. S7A-C**). These results suggest that the cytotoxic effect of NVB is not related to HSP90 inhibition. Furthermore, the combination of NVB and the TOP2 inhibitor etoposide exhibited additive cytotoxicity in TOV21G and CAPAN1 cells (**Fig. S7D, E**), suggesting that NVB and etoposide do not share TOP2 as a target and that NVB cytotoxicity is unrelated to TOP2 inhibition.

NVB potentiates the effect of PARPi in HR-deficient tumors

PARPi are currently being evaluated in different combination therapy settings with cytotoxic chemotherapy, radiation, targeted therapies, and immunotherapies (40). We explored whether the combination of NVB and a PARPi may be more effective than PARP inhibition alone in killing HR-deficient tumors. Indeed, NVB further sensitized *BRCA1*^{-/-} RPE1 cells to the PARPi rucaparib, but not BRCA-proficient WT cells in clonogenic assays (**Fig. 2F**). We studied the combination with a second PARPi, olaparib, where synergism of NVB and olaparib was also observed in HR-deficient TOV21G+EV cells, but not in HR-proficient TOV21G+FANCF cells (**Fig. S7F**). Importantly, NVB reduced the IC₅₀ of rucaparib by more than 20-fold in *BRCA1*^{-/-} RPE1 cells and the IC₅₀ of olaparib more than 40-fold in TOV21G cells compared to their respective HR-proficient counterparts (**Fig. 2F and S7F**). One strategy to prevent acquired resistance during treatment is combination therapy. The observed synergy between NVB and PARPi prompted us to evaluate whether NVB could overcome PARPi resistance in HR-deficient tumors.

POLθ inhibition by NVB overcomes acquired PARPi resistance *in vivo* and *in vitro*

Multiple PARP inhibitors (PARPi) including olaparib, niraparib, rucaparib, and talazoparib have received approval for the treatment of ovarian and breast tumors with HR

deficiency (41-43). Despite the remarkable initial tumor response and improved progression-free survival (PFS) in HR-deficient patients (4-6, 44), acquired PARPi resistance is emerging as an unmet medical need (8).

To evaluate whether POLθ inhibition could represent a novel therapeutic option for PARPi-resistant tumors, we first tested the efficacy *in vivo* of NVB in the patient-derived xenograft (PDX) model DF83, generated from an HR-deficient (loss of RAD51C expression), human high-grade serous ovarian carcinoma (45). NSG mice bearing the HR-deficient DF83 ovarian cancer PDX model were treated with olaparib, NVB, or a combination of the two, and tumor growth was monitored by bioluminescence imaging (BLI) (**Fig. 3A**). In order to determine the combined effect with NVB, olaparib was delivered at a submaximal dose (50 mg/kg daily), and we observed only a small degree of tumor growth inhibition. Treatment of DF83 with NVB alone (75 mg/kg, twice a day) led to tumor regression, while tumors in vehicle-treated mice showed exponential growth (**Fig. 3B**). Strikingly, when the DF83 tumor-bearing mice were given NVB and olaparib in combination, we observed complete tumor regression, and few tumor cells were detectable via BLI on day 28 in this group (**Fig. 3A-B**). These *in vivo* results suggest NVB may be useful in combination with PARPi to achieve better efficacy and prevent drug resistance in HR-deficient tumors. No significant toxicity to normal mouse tissue was observed from the drug combination.

Next, we evaluated the efficacy of NVB alone and in combination with olaparib in the PARPi-resistant PDX model DF59, which was isolated from a patient with a germline *BRCA1* mutation and acquired PARPi resistance. This model harbors biallelic mutations in the *TP53BP1* locus, providing a mechanism of the acquired PARPi resistance (45). In the DF59 PDX model, there was no response to olaparib monotherapy, but NVB monotherapy substantially reduced tumor growth, indicating a lack of cross-resistance between PARP inhibition and NVB. Importantly, the combination of NVB and olaparib further inhibited DF59

tumor growth, with tumor regression in the first two weeks (**Fig. 3C-D**). In contrast to DF83 and DF59 models, the BRCA1 WT, HR proficient PDX model DF149 was resistant to both drugs and to the combination (**Fig. 3E-F**). These data demonstrate that NVB can inhibit the growth of a *BRCA1*-mutated, PARPi-resistant tumors *in vivo* and that the combination of NVB and a PARPi is particularly effective in treating at least some PARPi-resistant tumors.

RAD51 focus is a pharmacodynamic biomarker for NVB response *in vivo*

We previously showed that depletion of POL θ in HR-deficient tumors resulted in RAD51 accumulation (17). Similarly, 53BP1 and POL θ are synthetic lethal, and the double knockout cells accumulate large, toxic RAD51 foci (46). We thus reasoned that NVB may induce RAD51 foci in HR-deficient tumors with PARPi resistance *in vivo*, and the RAD51 foci may serve as a pharmacodynamic biomarker for NVB response. To test this hypothesis, we collected tumor cells from the PARPi and/or NVB treated PDX models and evaluated RAD51 foci by immunohistochemistry (**Fig. 3G**). Since DF83 is an HR-deficient and PARPi sensitive model, RAD51 foci were not observed. The HR-restored and PARPi-resistant DF59 model, with biallelic 53BP1 mutations, exhibited a strong increase in RAD51 foci when treated with NVB alone or in combination with olaparib (**Fig. 3G**). Similarly, TOV21G xenograft tumors also exhibited increased RAD51 foci by immunohistochemistry *in vivo* after NVB treatment, which was further enhanced by olaparib (**Fig. S3D-F**). Results from these two *in vivo* studies demonstrate that RAD51 focus can serve as a pharmacodynamic biomarker for the response of tumors to NVB or other POL θ inhibitors. Interestingly, we also observed that two NVB-responsive tumor models had elevated levels of POL θ mRNA expression, compared to the NVB-resistant DF149 model, suggesting that elevated POL θ may be a useful predictive biomarker for POL θ inhibitor response (**Fig. 3H**).

NVB overcomes multiple PARPi resistance mechanisms but not perfect BRCA reversions

To better understand the mechanism by which NVB overcomes PARPi resistance and, we generated several PARPi-resistant clones from *BRCA1*^{-/-} RPE1 cells by gradually exposing them to increasing concentrations of PARPi (**Fig. 4A**). We selected four clones (named R1 to R4), all of which have acquired resistance to olaparib (**Fig. S8A-C**). Multiple PARPi resistance mechanisms were identified in these clones, corresponding to published mechanisms (8). Replication fork stabilization was evident in the R1 and R2 clones (**Fig. S8D**). The R1, R3, and R4 clones have restored RAD51 foci, a marker for restored HR activity (**Fig. S8E**). The restoration of HR repair in these clones may have resulted in part from their downregulation of NHEJ repair. Indeed, the R3 clone had decreased REV7 expression, and the R4 clone had decreased 53BP1 expression (**Fig. S8F-G**). None of the clones had re-expressed BRCA1, thereby excluding somatic reversion of BRCA1 as an underlying PARPi resistance mechanism (**Fig. 4B and S8H**). Strikingly, all four PARPi-resistant clones remained sensitive to NVB to a similar extent as the parental RPE-*BRCA1*^{-/-} cells (**Fig. 4C-D**), suggesting that NVB may overcome multiple mechanisms of acquired resistance to PARPi. To confirm that POLθ inhibition was responsible for cell death in the PARPi-resistant *BRCA1*^{-/-} clones treated with NVB, we knocked out *POLQ* in R1, R2, and the parental cells and measured their cell survival. Upon *POLQ* knockout, the PARPi-resistant clones showed reduced survival similar to the parental *BRCA1*^{-/-} cells, demonstrating that *POLQ* inhibition was the mechanism for NVB cytotoxicity in these cells (**Fig. S8I**).

Next, we examined more clinically relevant PARPi-resistant, HR-deficient models, using tumor cells derived from patients. First, we evaluated the effect of NVB on a PARPi resistant clone (MDA-MB-436-R), previously derived from the BRCA1-mutated breast

cancer cell line MDA-MB-436 (47). These resistant cells restored HR by two mechanisms—namely, stabilization and increased expression of a mutant BRCA1 protein and loss of 53BP1—resulting in increased RAD51 loading. While highly resistant to olaparib, the MDA-MB-436-R cells were NVB sensitive, like the parental cells (**Fig. S9A-B**). In addition, we studied the PARPi-resistant clone UWB1.289-YSR12 (derived from the *BRCA1*-null human ovarian cancer cell line UWB1.289), which acquired resistance to PARPi via stabilization of replication forks (48). Although highly resistant to olaparib, these cells also remained sensitive to NVB, like the parental cells (**Fig. S9C-D**), once again suggesting that acquired resistance to PARPi does not determine cross-resistance to NVB.

Another known mechanism of PARPi resistance in *BRCA1/2*^{-/-} tumors in the clinic is somatic reversion of the *BRCA1/2* genes (18). We next determined whether NVB could overcome PARPi resistance caused by *BRCA2* reversion. Unlike other PARPi resistant cells we analyzed, *BRCA2*-deficient tumor cells with near-perfect somatic reversion or CRISPR-edited reversion of *BRCA2* (PEO4 and CAPAN1-CR) (49, 50) were not only resistant to PARPi but also to NVB, when compared to their HR-deficient parental cell lines (PEO1 and CAPAN1) (**Fig. S10A-D**). These data suggest that NVB can overcome some but not all mechanisms of PARPi resistance.

POLθ expression is a predictive biomarker for NVB sensitivity

POLθ mRNA expression is increased in HR-deficient tumors and in several other cancers associated with poor prognosis (17, 21). We asked whether increased POLθ mRNA and protein expression may serve as a predictive biomarker for NVB sensitivity. As expected, CRISPR-mediated knockout of *BRCA1* in RPE-1 cells resulted in increased POLθ expression as compared to the parental cells (**Fig. 4E**). Interestingly, all PARPi-resistant clones (R1-R4) retain high POLθ expression, revealing a direct correlation between POLθ

expression and NVB sensitivity (**Fig. 4E**). This correlation was also present in the BRCA1-deficient MDA-MB-436 cells. MDA-MB-436-R cells with acquired PARPi-resistance exhibited elevated POLθ mRNA expression and retained NVB sensitivity, while complementation of the parental cells with WT BRCA1 cDNA decreased POLθ expression and NVB sensitivity (**Fig. 4F and S9A-B**). More importantly, the direct correlation of POLθ expression and NVB sensitivity was observed in HR-deficient PDX models, where the elevated POLθ expression in DF83 and DF59 correlated with their high sensitivity to NVB *in vivo* (**Fig. 3H and 3A-F**).

Conversely, low POLθ expression was a predictive biomarker for NVB resistance. In the HR-proficient PDX cells (DF149) with wildtype BRCA1 and in the PARPi-resistant cell lines with BRCA2 reversion mutations (PEO4 and CAPAN1-CR), the cellular resistance to NVB was associated with low POLθ expression levels (**Fig. 3G and S10E**). Also, the loss of POLθ in *POLQ*^{-/-} cells correlated with resistance to NVB (**Fig. 2E**). Taken together, in all cases examined, POLθ expression level correlated with cellular sensitivity to NVB, suggesting that POLθ expression in a tumor biopsy could provide a convenient predictive biomarker for patient enrollment in future clinical trials with NVB or other POLθ inhibitors.

Toxic RAD51 foci is a mechanism of NVB cytotoxicity in PARPi resistant cells

Depletion of POLθ in HR-deficient tumors resulted in RAD51 accumulation (17), which can be toxic if not regulated. Similarly, 53BP1 and POLθ are synthetic lethal, and the double knockout cells accumulate toxic RAD51 foci (46). We reasoned that accumulation of toxic RAD51 foci induced by NVB may be responsible for killing the PARPi resistant tumor cells. This is supported by the RAD51 pharmacodynamic results, where RAD51 foci were greatly increased in tumors after NVB treatment *in vivo* (**Fig. 3G and S3D-F**). We next determined RAD51 foci dynamics in our PARPi resistant cell line models. NVB induced

accumulation RAD51 foci in PARPi-resistant RPE1-BRCA1^{-/-} clones (R1 and R4) more readily than in WT RPE1 cells after irradiation (**Fig. 4G**). Similarly, NVB treatment alone strongly induced RAD51 foci in the PARPi resistant MDA-MB-436-R cells (**Fig. 4H**). In addition, knockdown of RAD51 sensitized RPE1 WT cells to NVB but rescued *BRCA1*^{-/-} R1 clone from NVB cytotoxicity (**Fig. 4I**). Taken together, these results suggest toxic RAD51 foci, induced by NVB, is a mechanism for NVB cytotoxicity in HR-deficient cells with acquired PARPi resistance.

DISCUSSION

In summary, NVB is a specific and potent POLθ inhibitor which phenocopies POLθ depletion and is synthetic lethal in HR-deficient tumors. Many tumors types with PARPi resistance remain sensitive to NVB, depending on their mechanism of resistance. Tumors which acquire PARPi resistance by downregulating NHEJ and partially restoring HR repair remain sensitive to NVB. For these tumors, the persistent high level of POLθ expression is a predictive biomarker for their NVB responsiveness, and the NVB-induced RAD51 accumulation is both a pharmacodynamic biomarker and a mechanism of NVB cytotoxic activity.

Finally, our findings may translate to a potential new therapy for treating HR-deficient tumors in addition to PARP inhibition. Importantly, NVB, which is an oral and well-tolerated drug, has previously been investigated in a Phase 1 trial in which partial responses were documented, even though the enrolled patients were not preselected to have HR-deficient cancers (51). Results in this study provide a strong rationale for future clinical studies of NVB alone or in combination with PARPi to prevent or overcome PARPi resistance.

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COMPETING INTERESTS

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DATA AND MATERIALS AVAILABILITY

All related data have been presented in the main manuscript or in supplementary data.

SUPPLEMENTARY MATERIALS

Figs. S1 to S10

Legends for Figs. S1 to S10

Materials and Methods

Supplementary References

FIGURE LEGENDS

Figure 1. A small-molecule screen identifies Novobiocin (NVB) as a specific POL θ ATPase inhibitor.

A, The domain structures of full length (FL) and ATPase domain (or Δ Pol, a.a. 1-987) of POL θ , a Coomassie-stained gel of the purified POL θ ATPase domain, and a schematic of the small molecule screen for POL θ inhibitors. **B**, Results of the small-molecule screen. Shown is quantification of POL θ ATPase activity measured by the ADP-Glo assay in the presence of small-molecule libraries. We screened a total of 23,883 small-molecules in duplicate across multiple libraries enriched with known bioactive compounds, and we identified 72 compounds (0.3% of total) that significantly reduced POL θ ATPase activity (z-score < -4). Four top hits verified in the secondary screen (Fig. S1B-E) were labeled; Mitoxantrone (MTX), Suramin (SUR), Novobiocin (NVB) and Aurintricarboxylic acid (AUR). Data shown are mean $\pm$ s.e.m, n = 2. **C**, Quantification of POL θ and SMARCAL1 ATPase activity in the presence of indicated small-molecules. Mean $\pm$ s.d., n = 3. **D**, Pulldown experiments *in vitro* with NVB-conjugated beads and purified POL θ and SMARCAL1 ATPase domains (Flag-tag purification from SF9 cells). Input and pulldown proteins were detected by Coomassie staining on SDS gel. **E**, Thermal shift assay with NVB at indicated temperatures. Full length GFP-POL θ was detected by Western blot using the GFP antibody and PARP1 by a PARP1 antibody. Data in **D** and **E** are representative images of three independent experiments. **F**, The NVB binding tunnel in POLQ ATPase domain (shown in surface - PDB 5AGA) was predicted by extra precision glide docking and lowest binding free energy from prime MM-GBSA calculations to have multiple hydrogen bonds and close hydrophobic packing. Top and side views showing NVB docking into the tunnel. Binding modes of NVB (green sticks) and AMP-PNP (magenta sticks) in POLQ helicase domain (PDB 5AGA) with green sphere showing active site Mg²⁺ ion. **G**, Representative images and quantification of POL θ accumulation at sites of laser micro-irradiated DNA damage in cells overexpressing GFP-tagged, full-length POL θ in the presence of indicated inhibitors. Mean $\pm$ s.e.m, n = 3. **H**, MMEJ repair reporter assay in U2OS cells treated with indicated inhibitors. Mean $\pm$ s.d., n = 3. Statistics analysis between groups was performed using *t*-test with Welch's correction. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001; ns, not significant.

Figure 2. NVB kills HR-deficient tumors *in vivo* and *in vitro* and synergizes with PARPi. **A**, Tumor growth of the GEMM model (*Brca1*^{-/-} TNBC) after treatment with vehicle (PBS) or NVB. Tumor chunks from GEMM mice were implanted in syngeneic FVB/129P2 mice, which were treated with PBS or 100 mg/kg NVB via IP injection twice a day for 5 weeks. n = 6 for PBS group and n = 7 for NVB group. Mean ± s.e.m. is shown. Statistical analyses were *t*-test. **B**, Survival plot of the experiment shown in (A). Median survival and *p* value are shown. Statistics analyses were *t*-test. **C**, Tumor growth of the xenograft model with TOV21G (FANCF deficient ovarian cancer) or FANCF-complemented TOV21G cells. The tumor bearing mice were treated with vehicle or 100 mg/kg NVB via IP injection twice a day for 4 weeks. Fold change of tumor was calculated using the formula Fold = T/T₀, where T is the tumor size at given time and T₀ is the initial tumor size. Data are Mean ± s.e.m., n = 10 in each group. Dunn's multiple comparisons test. Representative images are TOV21G xenograft tumors at the end of NVB treatment. **D**, Clonogenic survival of *BRCA1*^{-/-} and WT RPE1 cells under increasing concentrations of the POLθ inhibitor NVB. Survival is normalized to the untreated (Unt) sample. Data shown are mean ± s.e.m., n = 4. **E**, Representative images and quantification of clonogenic survival of *POLQ*^{-/-} and WT U2OS cells under increasing concentrations of NVB. Survival is shown as relative to the untreated (Unt) sample. Data shown are mean ± SD, n = 5. **F**, Clonogenic survival of *BRCA1*^{-/-} and WT RPE1 cells under increasing concentrations of the PARPi Rucaparib alone or in combination with NVB. Quantifications, representative images and calculated IC₅₀ values are shown. Data shown are mean ± s.e.m., n = 3.

Figure 3. NVB overrides PARPi resistance *in vivo* in PDX models and RAD51 is a dynamic pharmacodynamic biomarker. **A-B**, Efficacy of NVB in PDX model DF83 (RAD51C deficient). NSG mice (n = 7 mice/group) bearing luciferized DF83 cells derived from an ovarian cancer patient were treated with 50 mg/kg of olaparib (daily, orally), 75 mg/kg NVB (via IP, b.i.d.), or both for 4 weeks. Tumor growth was monitored weekly by bioluminescence imaging. Representative images of tumor burden on day 0 and day 28 are shown, and quantifications shown are mean $\pm$ s.e.m. $\log_2$ (fold change in tumor size) was calculated using the formula $\log_2(T/T_0)$, where T is the tumor volume at given time and T₀ is the initial tumor volume. **C-D**, Efficacy of NVB in PARPi resistant PDX model DF59 (BRCA1-deficient, 53BP1 loss), n = 6 mice/group. Same conditions and analysis were applied as in **A-B**. **E-F**, Efficacy of NVB in PARPi resistant PDX model DF149 (BRCA1-WT, HR proficient), n = 7 mice/group. Same conditions and analysis were applied as in **A-B**. **G**, Representative IHC images and quantifications of RAD51 foci in DF-59 and DF-83 PDX models. Tumor bearing mice were dosed with indicated drugs and as in efficacy study in Fig 3, and tumor cells were collected from ascites 52 hours after first dose. FFPE sections of the isolated tumor cells were stained with a RAD51 antibody. The percentage of RAD51-foci positive cells (> 4 foci/cell) in 3 random 40X fields was estimated in DF-59. No RAD51 focus was observed in the DF-83 model. Statistical analysis method was one-way ANOVA Bonferroni's multiple comparisons test. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. **H**. POLQ expression at mRNA level in PDX models DF53, DF83 and DF149.

Figure 4. POLQ expression level is a predictive biomarker for NVB sensitivity. **A.** A schematic shows how the PARPi resistant clones R1-R4 were generated. TP53 was knocked out in order to generate *BRCA1* knockout (*BRCA1*^{-/-}) RPE1 cells, which were then used for selection of PARPi resistant clones. **B.** A Western blot of WT and *BRCA1*^{-/-} RPE1 cells and the PARPi resistant clones, using an anti-BRCA1 antibody (Millipore #OP92). No BRCA1 reversion was observed in the clones. **C-D.** NVB sensitivity of WT RPE1, *BRCA1*^{-/-} RPE1, and PARPi-resistant *BRCA1*^{-/-} clones (R1-R4). R1 was selected by olaparib and R2-R4 were selected by Niraparib. R1 and R2 were tested in clonogenic survival assay (**C**). R3 and R4 were tested in CellTiter-Glo cell viability assay (**D**). Data are mean ± s.e.m., n = 3. **E.** POLQ expression at mRNA level and protein level in WT RPE1, *BRCA1*^{-/-} RPE1, and PARPi-resistant *BRCA1*^{-/-} clones (R1-R4). POLQ mRNA was measured by qRT-PCR and normalized to beta-Actin (ACTB). **F.** POLQ expression at mRNA level in parental (P), PARPi resistant (R), and empty vector (+EV) or BRCA1-cDNA (+BR1) complemented MDA-MB-436 cells. **G,** RAD51 foci assay with DMSO or NVB in RPE1-*BRCA1* WT RPE1, *BRCA1*^{-/-} RPE1, and PARPi-resistant *BRCA1*^{-/-} clones (R1-R4) 4 hours after 5 Gy of IR. Cells with more than 10 foci were counted as positive. Data were Mean ± SEM from two independent experiments. **H,** RAD51 foci assay with DMSO or NVB in MDA-MB-436 or the PARPi-resistant MDA-MB-436-R. Cells with more than 5 foci were counted as positive. Data were Mean ± SEM from two independent experiments. **I.** Novobiocin sensitivity of RPE1-WT and the R1 PARPi-resistant clone after being treated with Control or RAD51 siRNA. A Western blot shows the efficiency of RAD51 knockdown in RPE1-WT and the R1 PARPi-resistant clone.

Fig 1

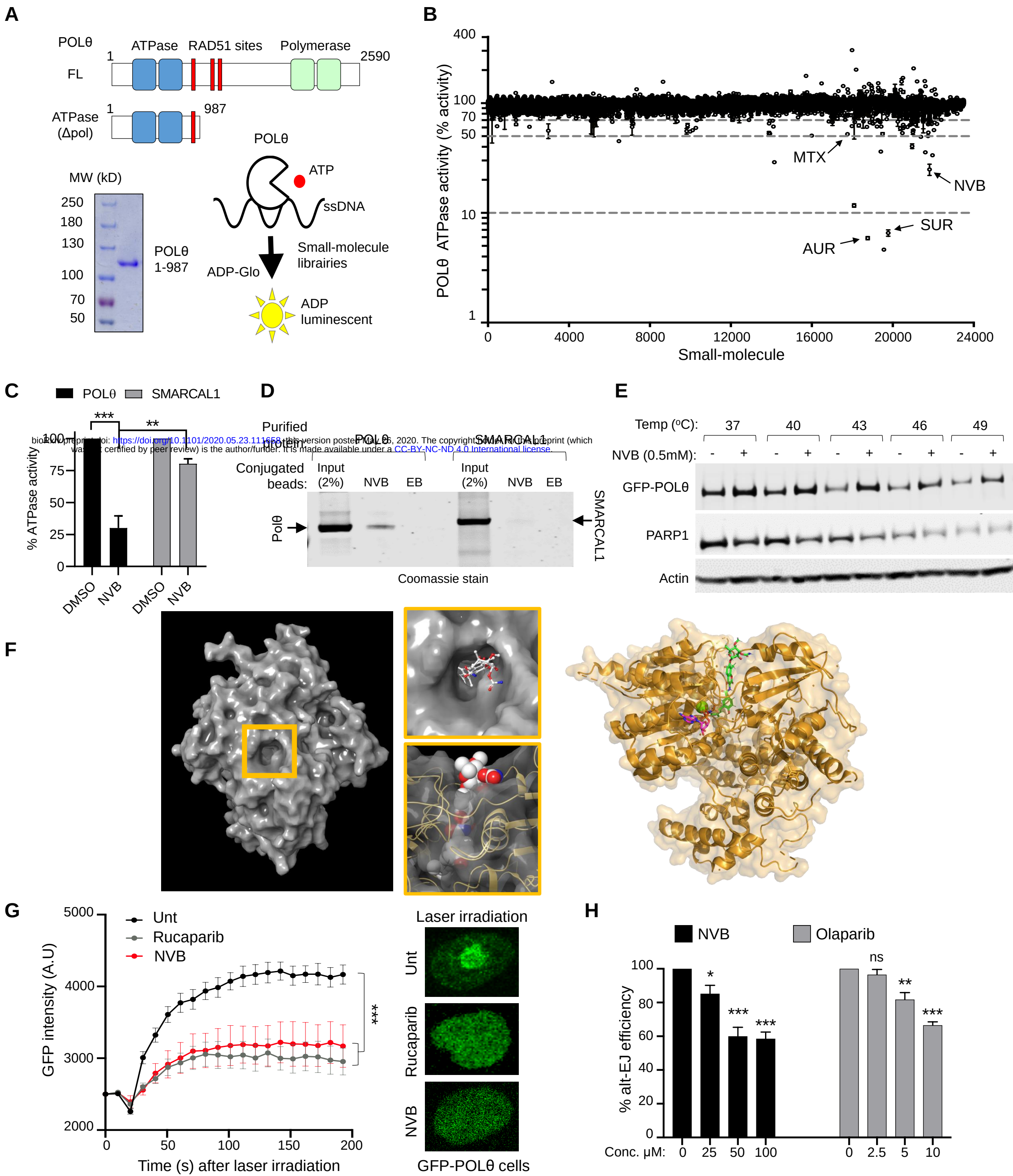


Fig 2

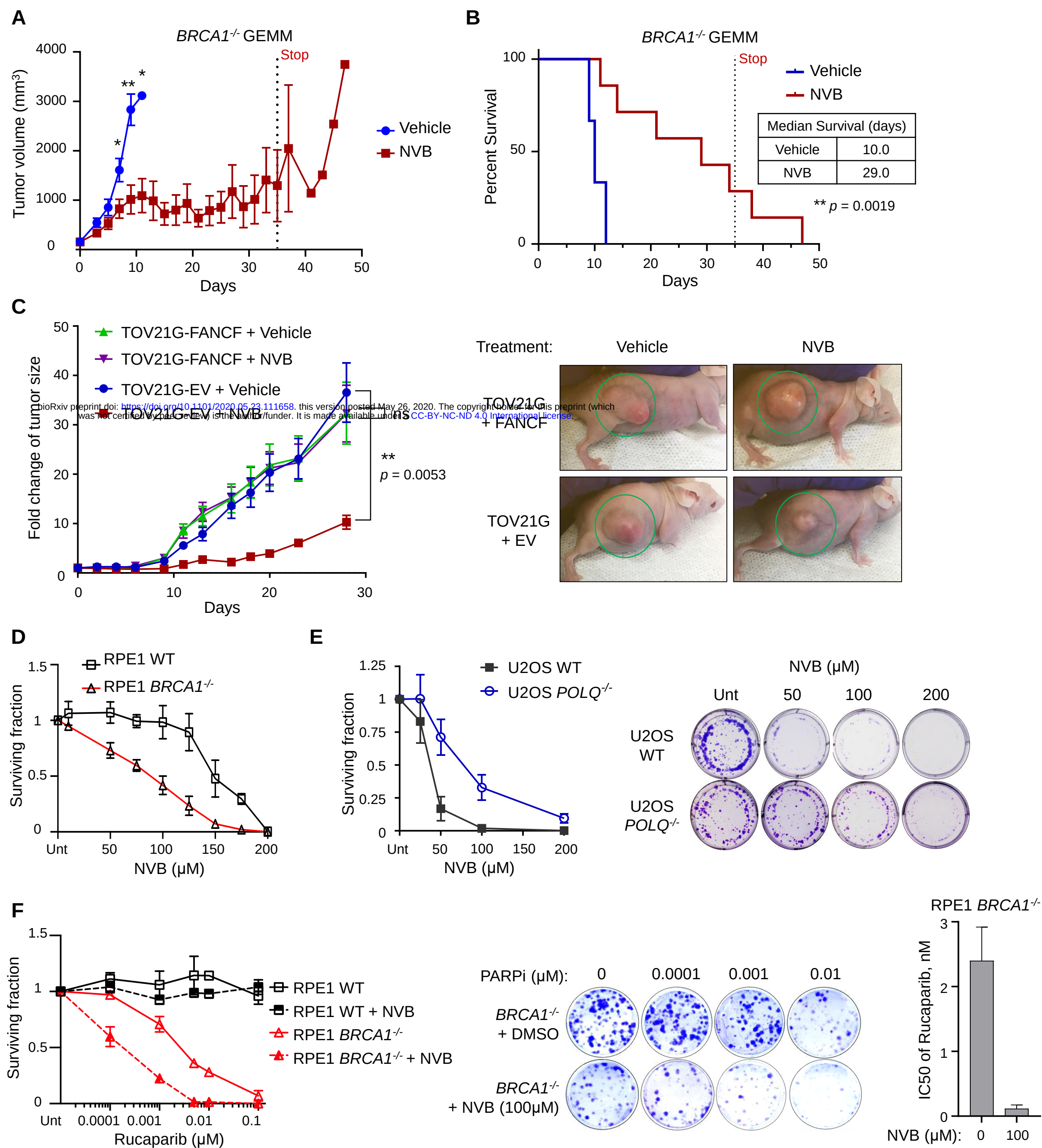


Fig 3

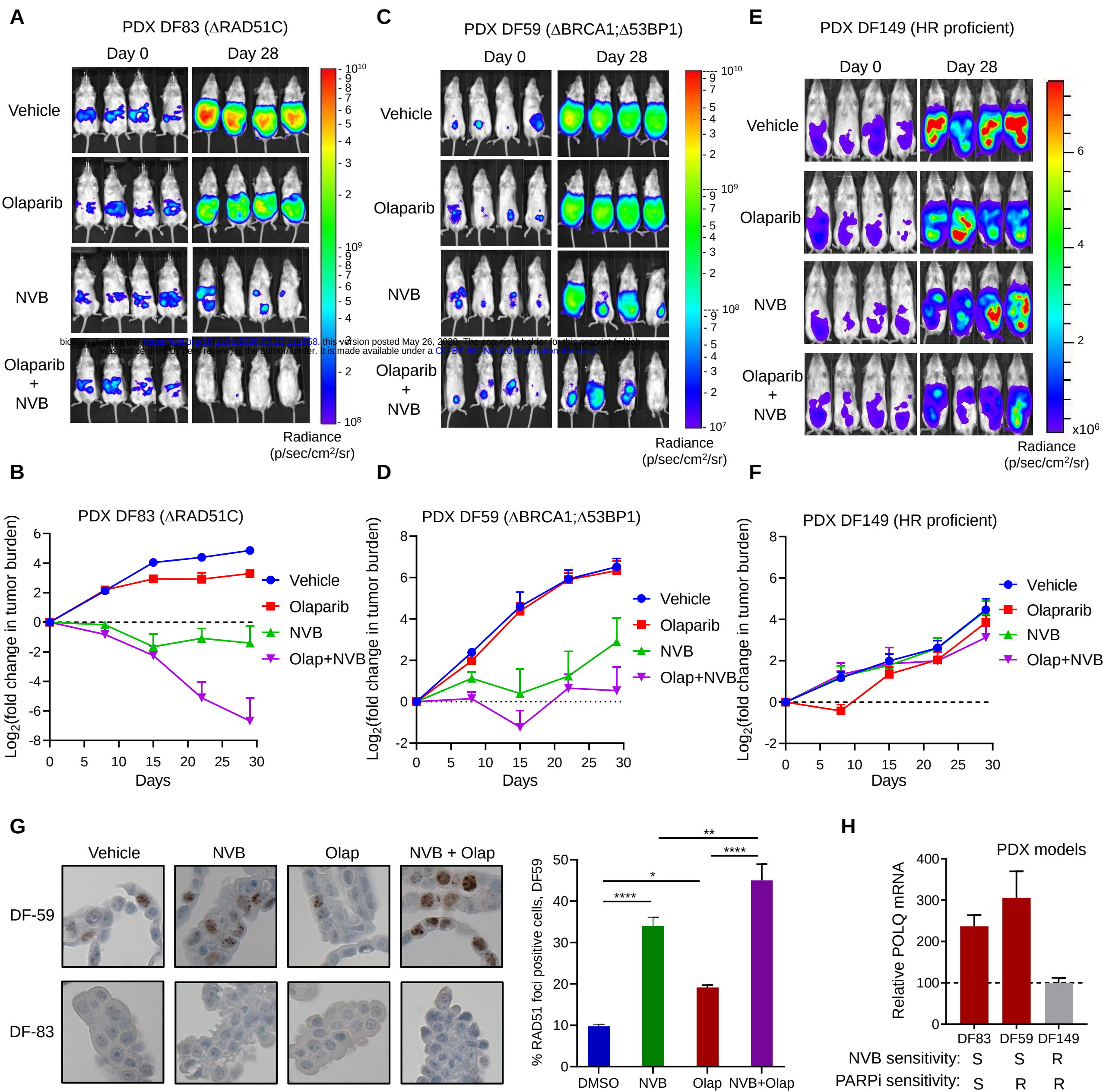


Fig 4

