AWARD NUMBER: W81XWH-13-1-0027

TITLE: Novel Mechanisms of PARP inhibitor resistance in BRCA1-deficient Breast Cancers

PRINCIPAL INVESTIGATOR: Stephanie Yazinski

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114

REPORT DATE: August 2016

TYPE OF REPORT: Final Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2016	Final report	1 Dec 2012 – 31 May 2016

4. TITLE AND SUBTITLE

Novel mechanisms of PARP inhibitor resistance in BRCA1-deficient cancers

5a. CONTRACT NUMBER
W81XWH-13-1-0027

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Stephanie Yazinski

E-Mail: SYazinski@mgh.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
55 Fruit Street
Boston, MA 02114-2621

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Mutations in the BRCA1 gene are associated with a heightened lifetime risk for breast cancer. PARP inhibitors (PARPi) have been tested with promising results for the treatment of BRCA1-associated cancers. BRCA1 is essential for error-free repair of DNA double strand breaks via homologous recombination (HR), while PARPs are thought to primarily function in repair of single stranded DNA breaks especially through activation of base excision repair (BER). A synthetic lethal phenotype occurs when BRCA1- deficiency (HR deficiency) is combined with PARPi (BER defect). However, a majority of BRCA1-deficient tumors do not respond to PARPi, and, of those that do, all tumors recur. I aim to (1) To determine how HR is rescued in BRCA1-deficient cells; (2) to elucidate HR-independent mechanisms of PARPi resistance; (3) to elucidate means of targeting PARPi resistant cancers. I have found the ATRi can synergize with PARPi to resensitize BRCA1-deficient cells to PARPi by preventing Rad51 loading in HR and fork protection. The results of this project may be broadly applicable to BRCA-like and other non-hereditary, spontaneous breast cancers, including triple negative breast cancers. Ultimately, the proposed experiments will provide an understanding of the mechanisms of acquired resistance to PARPi treatment and how resistant tumors can be categorized and targeted for treatment.

15. SUBJECT TERMS

Breast Cancer, BRCA1, PARP inhibitor, homologous recombination, resistance

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	35	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. Introduction……4

2. Keywords…….4

3. Overall Project Summary……..4

4. Key Research Accomplishments……..….13

5. Conclusion……..……14

6. Publications, Abstracts, and Presentations…….…..……..16

7. Inventions, Patents and Licenses…….16

8. Reportable Outcomes..…….16

9. Other Achievements…….16

10. References…….17

11. Appendices……18

Introduction:

Mutations in the BRCA1 gene are associated with a heightened lifetime risk for breast cancer (King, Marks, & Mandell, 2003). PARP inhibitors (PARPi) have been tested with promising results for the treatment of BRCA1-associated cancers (Bryant et al., 2005; Gartner, Burger, & Lorusso, n.d.; Tutt et al., 2010). BRCA1 is essential for error-free repair of DNA double strand breaks via homologous recombination (HR) (Gudmundsdottir & Ashworth, 2006), while PARPs are thought to primarily function in repair of single stranded DNA breaks especially through activation of base excision repair (BER) (Krishnakumar & Kraus, 2010). A synthetic lethal phenotype occurs when BRCA1-deficiency (HR deficiency) is combined with PARPi (BER defect) (Bryant et al., 2005)(Farmer et al., 2005)(Edwards et al., 2008)(Helleday, Bryant, & Schultz, 2005). However, a majority of BRCA1-deficient tumors do not respond to PARPi, and, of those that do, all tumors recur. I hypothesize that in order for BRCA1-deficient cells to overcome PARP inhibition, they must acquire mutations or expression changes that alter their DNA damage response, repair pathways, or checkpoint pathways. I further hypothesize that these changes will result in increased sensitivity to other compounds and can be used to develop biomarkers. To this end, I have derived twelve BRCA1-deficient, PARP inhibitor resistant cell lines from the BRCA1-deficent cancer cell line UWB1.289 by two means (Figure 1A and B). I further characterized the homologous recombination (HR) ability of these cell lines as either "HR-restored" or "HR-deficient" based on the level of Rad51 loading, a marker of HR-repair, following DNA damage compared to BRCA1-deficient or BRCA1-rescue cell lines. The objective of this proposal is to determine the how BRCA1-deficient breast cancers become resistance to PARPi and how resistant tumors can be identified and treated.

Key Words: Breast Cancer, BRCA1, PARP inhibitor, homologous recombination, resistance

Overall Project Summary:

In this study, I predominately used three well-characterized, BRCA1-deficient cell lines, HCC1937 (ATCC), UWB1.289 (ATCC), and BR5-AKT (Orsulic lab, Cedars Sinai) to understand how BRCA1-deficient cancers become resistant to PARPi and how these resistant tumors can be targeted. A PARPi resistant panel of cell lines was derived from UWB1 cells. These cells were confirmed to be resistant to both Olaparib (AZD2281) and ABT-888, a second PARPi (Figure 1A, 1B, 1C and 1D). One PARPi resistant line was derived from BR5-AKT cells (Figure 1E). HCC1937 has *de novo* resistance to PARPi treatment (Figure 1F).

Task 1: Determine how HR is restored in BRCA1-deficient cells (Cells with increased Rad51 foci)

1) Test candidate mechanisms of HR restoration:
   a) Determine HR activity in parental, rescue, and PARP inhibitor resistant lines:
      I showed that the parental line (UWB1) has a defect in HR relative to the rescue line (UWB1+BRCA1) by decreased Rad51 loading after damage; however, this line still has some ability to load Rad51 (Figure 1A, 1B, and 1C). I

showed that HR activity in the PARP inhibitor resistant lines varied; however, all lines showed at least as much HR activity as was seen in the parental, UWB1 line using Rad51 and PALB2 as markers (Figure 2A, 2B, 2C, 2D, and 2E). I measured HR activity after damage by IR, as well as, PARPi treatment itself, both of which result in DNA damage and HR repair. Additionally, I showed in an additional BRCA1-deficient cell line (HCC1937) that HR is partially restored (Figure 2F). This data suggests that a common feature of BRCA1-deficient cell lines is partial HR restoration. This partial restoration does not allow cells to become resistant to PARPi but may be necessary for BRCA1-deficient cells to survive and proliferate.

b) Test BRCA1-restoration:

Because restoration of BRCA2 has been shown to result in cisplatin/PARPi resistance in BRCA2-deficient cancer cell lines and BRCA1 restoration has been shown to occur in response to cisplatin treatment, I tested if BRCA1-restoration may be the cause for resistance in my derived PARPi-resistant lines. BRCA1-deficient, PARP inhibitor resistant cell lines, as well as the parental BRCA1-deficient cell line and the BRCA1-rescue line, which ectopically expresses wild-type BRCA1, were all tested for expression of BRCA1 by Western blot using two different BRCA1-antibodies against different portions of BRCA1. Only the BRCA1-rescue cell line expresses BRCA1 by Western blot (Figure 3A). Furthermore, all cell lines retained the single point mutation that results in a premature stop codon, while only the BRCA1-rescue cell line also expressed a wild type copy of BRCA1. No cell lines had deletion of the region that had been previously described to restore BRCA1 open reading frame, and thus BRCA1 protein expression (Figure 3B). Finally only the BRCA1-rescue line, but not the parental or the PARPi resistant line were affected by siBRCA1, indicating only the rescue line depends on BRCA1 for HR (Figure 3C). Taken together, the results of this sub-aim demonstrate that BRCA1 is not restored in the PARPi resistant lines, and that BRCA1 re-expression does not contribute to PARP inhibitor resistance in these lines.

c) Test 53BP1 and DNA-PK status:

Because candidate approaches have shown that loss of 53BP1 or decreased non-homologous end joining repair, as measured by DNA-PK activity, can rescue the synthetic lethality of combined BRCA1-loss and PARPi treatment, I tested if either of these mechanisms can explain PARPi resistance in the derived cell lines. Expression of 53BP1, Rif1, a protein known to complex with 53BP1, or Ku70 was not altered between cell lines. This data suggests that loss of 53BP1, Rif1, or Ku70 does not contribute to PARP inhibitor resistance in these lines. Additional screens were performed by other labs to identify proteins that could contribute to PARPi resistance and identified that loss of either Rev7 or PTIP could contribute to PARPi resistance through regain of HR or restoration of fork protection, respectively. I then tested if either loss of Rev7 or loss of PTIP contributes to PARPi resistance in my derived lines. Several clones had decreased Rev7 expression or loss of PTIP by Western blot, suggesting increased HR or decreased fork protection may allow for PARPi resistance (Figure 3A).

2) Identify novel mechanisms of HR restoration:
   a) Confirmation of targets with siRNAs:

      Having established that HR activity, as measured by Rad51 loading, is occurring in BRCA1-deficient lines, and is restored to higher level in some PARP inhibitor resistant lines, I next sought to understand which known components of the HR pathway are necessary for Rad51 loading in the absence of BRCA1. To this end, cells were treated with siRNAs directed to known components of the HR pathway, specifically BRCA2 and PALB2, known to function downstream of BRCA1. I also tested knockdown of MRG15, known to function independently of BRCA1. Following 48hr knockdown, cells were treated with PARP inhibitor for 24 hrs, and the level of Rad51 loading was measured by immunofluorescence. Using this approach, I found that HR activity in BRCA1-deficient cells, both the parental and PARP inhibitor resistant lines, is dependent on MRG15, BRCA2, and PALB2, following PARP inhibitor treatment (Figure 4A, 4B, and 4C).

      The residual Rad51 loading in the UWB1 and derived PARPi resistant cell line is not due to restored expression of BRCA1, as knockdown of BRCA1 using siRNA has no effect on Rad51 loading in the BRCA1-deficient cells, as expected. On the other hand, in U2OS cells, which express wild-type BRCA1, knockdown of BRCA1 results in complete loss of Rad51 loading following DNA damage with 10 Gy IR. All cell lines, both BRCA1-expressing cells and BRCA1-deficient lines, require PALB2 to load Rad51. This data demonstrates that BRCA1 deficient cells can undergo PALB2-dependent Rad51 loading in the absence of BRCA1. I further confirmed this dependence on PALB2 for BRCA1-independent Rad51 loading in a second BRCA1 deficient cell line, a human breast cancer line HCC1937 (Figure 5A, 5B, 5C, and 5D). Finally I extended these results to the PARPi resistant cell line, SYr12, and show that all cell lines, BRCA1 rescue, BRCA1-deficient, and PARPi resistant lines, require PALB2 and BRCA2 for Rad51 loading following PARPi treatment (Figure 5E and 5F). Additionally, this Rad51 loading in the PARPi resistant lines is necessary for resistance, as knockdown of either PALB2 or BRCA2 results in restored sensitivity to PARPi treatment (Figure 5G).

   b) Confirmation of targets with inhibitors:

      Because all resistant lines tested were able to load Rad51 to various extents, we can conclude some cell lines were able to partially regain HR function while others were not. Therefore, HR restoration is not required for PARPi resistance. However, all cell lines have some level of HR function, even in the absence of BRCA1, which is PALB2 and BRCA2 dependent. We sought to disrupt the remaining HR in these BRCA1-deficient cells. I have shown that ATR is a critical regulator of homologous recombination repair using an HR reporter assay. An integrated plasmid reporter, DR-GFP, is cut with the rare cutter, I-SceI nuclease, resulting in a double strand break. When this break is repaired via HR, GFP expression occurs, allowing measurement of HR efficiency using flow cytometry. Treatment with increasing doses of either of two ATRi's (VE821 and AZ20) results in significant and dose dependent defects in HR repair, while treatment

with a comparable dose of ATM inhibitor (KU55933) has little effect (Figure 6A, 6B, and 6C). Importantly, treatment with ATRi does not affect cell cycle (Figure 6D and 6E).

To understand where in HR ATR is acting, I systematically stained for markers of Rad51. Treatment of U2OS cells with ATR inhibitor (ATRi) did not significantly decrease BRCA1 loading following 4 Gy IR (Figure 7A). On the other hand, treatment with ATRi prevented BRCA2 loading to damage induced foci in a dose dependent manner, confirming that indeed ATR plays a crucial role in HR (Figure 7B). Since it known that BRCA1, PALB2/BRCA2, and Rad51 load in a sequential manner, this data suggests that ATRi prevents loading of PALB2/BRCA2, which in turn, prevents loading of Rad51, while having no effect on BRCA1 localization after damage. ATRi treatment does not simply delay PALB2 and Rad51 loading, but rather prevents HR repair, as can be seen using the I-SceI reporter assay. Taken together, this data suggests that ATR is a critical regulator of HR that functions downstream of BRCA1, but upstream of PALB2 and BRCA2.

I next sought to determine if ATRi could disrupt the HR observed in a known mechanism of BRCA1 bypass in BRCA1-deficient cells, since ATR acts downstream of BRCA1. It has been previously shown that the defect in HR that results following loss of BRCA1 can be partially rescued by simultaneous loss of 53BP1, which allows for an increase in resection without BRCA1. U2OS cells were depleted of BRCA1, 53BP1, or both and treated with PARPi to induce damage. Cells were then fixed and stained for Rad51 foci. As expected, following loss of BRCA1, there is a defect in Rad51 loading. This defect is rescued by combined loss of 53BP1, as previously reported. In this BRCA1-deficient context of HR rescue, ATRi significantly inhibits loading of Rad51 (Figure 8A and 8B). Viability assays similarly show loss of BRCA1 alone results in PARPi sensitivity, while combined loss of 53BP1 rescues this sensitivity, but addition of ATRi re-sensitizes the BRCA1 deficient cells to PARPi (Figure 8C and 8D).

I next tested if treatment with ATRi decreased HR in the BRCA1-deficient UWB1 cell line and the derived PARPi resistant cell lines. Cells were treated with PARPi or the combination of PARPi and ATRi. Cells treated with the combination of PARPi and ATRi show a decrease in HR relative to cells treated with PARPi alone, using Rad51 as an indicator of HR (Figure 9A). Furthermore, treatment with ATRi also decreased PALB2 localization to sites of UV laser damage in all BRCA1-deficient cells (Figure 9B). A laser stripe assay must be used because PALB2 foci are not visible in BRCA1-deficient cells. I confirmed this in a second BRCA1-deficient cell line, HCC1937, which also has BRCA1-bypassed Rad51 loading which is inhibited by treatment with ATRi (Figure 9C). Finally, I tested these cells to see if resection was bypassed in a "53BP1-like" state in the UWB1 and derived PARPi resistant cells. To test this, cells were treated with CPT, and resection was measured by staining cells with pRPA S4/S8. All cell lines, including the parental and PARPi resistant lines, were able to undergo resection (Figure 9D). Additionally, PALB2 has been shown to interact with pRPA, suggesting this increased resection and pRPA may be a mechanism of BRCA1-independent PALB2/BRCA2 recruitment. Consistent with this model,

following treatment with ATRi, decreased pRPA signal can be seen in the resistant cell lines SYr12 and SYr13 (Figure 9E). Taken together, these data demonstrate that ATRi treatment decreases BRCA1-independent HR through disruption of PALB2/BRCA2 loading, perhaps through decreased pRPA, thus preventing Rad51 loading.

Task 2: Elucidate HR-independent mechanisms of PARPi resistance (Cells without increased Rad51 Foci)

1) Begin to test candidate HR-independent mechanism of resistance
   a) Test PARP redundancy and efflux pump up-regulation:

      Because mouse models of PARP inhibitor resistance in BRCA1-deficient cancers revealed that up-regulation of efflux pumps is a common occurrence resulting in resistance, I tested if efflux pump up-regulation contributes to PARP inhibitor resistance in the derived lines. The BRCA1-rescue line, the parental BRCA1-deficient line, and all BRCA1-deficient, PARP inhibitor resistant lines were treated with MMS, a DNA damaging agent that is known to induce poly (ADP-ribose) (PAR) chain formation. PAR chain formation was measured by immunofluorescence using an antibody directed to PAR. In all cell lines, an increase in nuclear PAR intensity could be seen after treatment with MMS, demonstrating that PARP is still able to form PAR chains in response to DNA damage. Treatment with PARP inhibitor (AZD) was able to decrease the PAR intensity in all cell lines, after MMS treatment, demonstrating that the PARP inhibitor is retained in the nucleus and that the inhibitor is still able to bind PARP. These results demonstrate that PARP inhibitor resistance in all but one cell line is not due to efflux pump up-regulation, as the inhibitor was retained in the cell and nucleus, and was able to prevent PAR chain formation. Furthermore, these results show that PARP inhibitor resistance in all resistant cell lines is not due to mutations in PARP that prevent PARP inhibitor from binding to its target, since the inhibitor has the expected inhibitory effect (Figure 10).

   b) Test whether replication fork protection is altered in PARPi resistant cell lines:

      It has been shown that BRCA1-deficient cells have a defect in replication fork protection. This results in nascent DNA degradation following fork stalling. This BRCA1-dependent fork protection is Rad51-dependent, but separable from HR. Because several derived resistant cell lines showed decreased PTIP expression, e.g. SYr9 and SYr37. To test whether the derived PARPi resistant cells have altered fork protection, I treated BRCA1-rescue cells, BRCA1-deficient cells, or PARPi resistant cells with a pulse of a thymidine analog CldU followed by a pulse of a second analog, IdU. Cells either were collected at this point, or were treated with HU for 5 hours to stall forks and allow time for degradation, then collected. Cells were then lysed on glass slides and DNA fibers were spread and fixed. The fixed DNA was then denatured and stained for CldU and IdU. The resulting fiber tracts were then measured, and a ratio of CldU to IdU determined. In untreated samples, the IdU/CldU ratio should be 1. If forks undergo degradation following HU treatment and fork stalling, the ratio of IdU/CldU will be <1, while if forks fail to stall the ratio of IdU/CldU will be >1.

The UWB1+B1 rescue cells show no fork regression following HU treatment, as expected, with an IdU/CldU ratio remaining at 1 after HU treatment. On the other hand, the BRCA1-deficient cells show fork degradation after HU treatment, as previously reported, with the IdU/CldU ratio falling to 0.72. Surprisingly, both SYr12 and SYr13 resistant cell lines have stabilized forks following HU treatment with IdU/CldU ratios close to 1, respectively, even in the absence of BRCA1 (Figure 11A and 11B). I further confirmed this observation in three additional resistant cell line (Figure 11C). Thus, increased fork protection is more prevalent in the PARPi resistant cells than restoration of HR. This suggests that fork stabilization in the absence of BRCA1 may contribute to PARPi resistance.

Because we have shown that ATRi can prevent Rad51 loading after DNA damage, resulting in decreased HR, we tested if treatment with ATRi would result in decreased fork protection, another Rad51-dependent process. Treatment with ATRi and HU resulted in resulted in a further loss of fork protection in the parental UWB1 cells, suggesting that these cells, although show a defect in fork protection, may also have some low level of fork protection. This is consistent with a low level of Rad51 loading in the absence of BRCA1 seen when assessing HR after break inducing DNA damage. Importantly, treatment of five resistant cell lines, SYr9, SYr12, SYr13, SYr14, andSYr37 with ATRi results in decreased IdU/CldU ratio, again signifying loss of fork protection (Figure 11B and 11C). Co-treatment with Mirin, an Mre11 inhibitor, prevents this shortening, demonstrating this degradation following ATRi treatment is Mre11 dependent.

To understand if fork protection can contribute to resistance, I knocked down MRE11, the exonuclease which has been shown to degrade nascent DNA in BRCA1 deficient cells following fork stalling, resulting in DNA tract shortening, and treated cells with increasing doses of PARPi treatment. Knockdown of Mre11 results in increased resistance to PARPi in the parental UWB1 cells (Figure 12A and 12B). This data suggests that preventing nascent DNA degradation after fork stalling can result in PARPi resistance. To ensure that fork degradation after ATRi treatment is not due to an increase in DNA damage, I performed pulse field gel electrophoresis. Treatment of both UWB1 and SYr12 with HU and ATRi results in an increase of fragmented DNA, while only SYr12 undergoes fork degradation after ATRi treatment, suggesting the increase in DNA damage alone is not responsible for fork degradation (Figure 13A).

To determine if ATRi treatment is again working through prevention of Rad51 loading, as in inhibition of HR, I performed chromatin fraction experiments to determine if Rad51 is loaded after fork stalling. In UWB1 cells, which are BRCA1-deficient, Rad51 fails to load to chromatin after treatment with HU for 5 hours which stall replication forks, consistent with the observation that these forks are not protected and are vulnerable to MRE11 mediated degradation. However, in the PARPi resistant cells, SYr12, SYr13, SYr14, and SYr9, Rad51 is loaded to chromatin after HU treatment, consistent with the observation that the resistant cells have regained fork protection. However, after treatment with ATRi, the resistant cells have decreased Rad51 loading, consistent with the observation that ATR is essential for fork protection (Figure 13B and 13C). To confirm that Rad51 is loaded to nascent DNA, and not just chromatin localized damage foci,

immunoprecipitation of nascent DNA (iPOND) was performed, in which nascent DNA is pulled down, and associated proteins can be detected. Using iPOND, Rad51 can be seen at nascent DNA in UWB1+BRCA1 after HU treatment, but not in parental BRCA1-deficient UWB1. Rad51 loading is rescued again in the SYr12 PARPi resistant cell line at nascent DNA, consistent with the chromatin fraction data. This rescued Rad51 loading is abolished with ATRi treatment (Figure 13D).

Taken together, these experiments demonstrate that the derived PARPi resistant cell lines have regained fork protection in the absence of BRCA1, and that fork protection can result in PARPi resistance. This aberrant BRCA1-independent fork protection can be overcome by treatment with ATRi, which then allows de-protected forks to undergo Mre11-dependent degradation.

Task 3: Targeting the PARPi resistant breast cancers

1) Complete screen for drugs that selectively kill PIR cells:

The results of Task 1 and Task 2 have shown that derived PARPi resistant cells have some level of HR and some level of fork protection, both have been shown to be Rad51 dependent processes. I have also shown that both BRCA1-independent HR and BRCA1-independent fork protection can be abrogated by treatment with ATRi. To determine the effect of loss of ATR-dependent HR and fork protection on BRCA1-deficient cells, including PARPi resistant lines, colony formation assays were performed using increasing doses of PARPi in the presence or absence of ATRi after 14 days (Figure 14A). PARPi alone reduces the viability of only the BRCA1-deficient parental cell line, UWB1, while the rescue line (UWB1+B1) and the resistant lines (SYr) show no effect. However, the combination of ATRi and PARPi dramatically reduces the viability of all BRCA1-deficient lines, including the PARPi resistant lines. These results were confirmed with a short term 6 day viability assay (Figure 14B). PARPi treatment again only decreased viability of the parental UWB1 cells, while ATRi had little effect on all the cell lines, and the combination of PARPi and ATRi decreased the viability of all BRCA1-deficient cell lines, including the PARPi resistant lines. These results were confirmed using a second ATRi (Figure 14C). A small miniscreen was performed to determine the specificity of this ATRi combination with PARPi. ATMi and DNA-PKi have little effect on viability, even in combination with PARPi. Chk1i combined with PARPi has some specificity to BRCA1-deficient cells, but does not synergize as well as ATRi. Wee1i is currently being tested in combination with PARPi in clinical trials; however, there is more toxicity to all cell lines with Wee1i alone, and there is less specificity to the BRCA1-deficient cell lines (Figure 14D). Taken together, this mini-screen suggests ATRi specifically synergizes with PARPi to overcome PARPi resistance in BRCA1-deficient cells, while other inhibitors do not have this effect. To confirm that this synergy is not simply due to an increase in DNA damage, bliss synergy scores were calculated in UWB1+B1, UWB1, and SYr12 cell lines with PARPi and ATRi or Cisplatin and ATRi. The combination of PARPi and ATRi was only strongly synergistic in SYr12, while Cispltain and PARPi showed synergy most strongly in the BRCA1-expressing UWB1+B1 (Figure 14E and

14F). This data suggests ATRi and PARPi is a more specific combination than Cisplatin and PARPi for targeting BRCA1 deficient cells.

Furthermore, treatment with increasing doses of ATRi results in a shift of the IC50 to PARPi in the BRCA1 deficient cells, suggesting ATRi is re-sensitizing cells to the PARPi treatment (Figure 15A and 15B). The combination has little effect on the rescue cell line exogenously expressing BRCA1. BRCA1-deficient cells, including the PARPi resistant lines SYr12 and SYr13, treated with the combination of ATRi and PARPi do not simply arrest growth, but rather undergo apoptotic cell death, as measured by Annexin V staining, while the rescue cell line expressing BRCA1 is resistant to this combination (Figure 15C). I sought to determine if this synthetic lethality seen with combined PARPi and ATRi could be used to target the parental cells to prevent resistance from immerging. To this end, I treated sparsely plated parental UWB1 or rescue UWB1+B1 cells with PARPi alone, ATRi alone, or the combination for 45 days, changing the media and adding fresh inhibitors every three days. While resistant colonies readily immerged from the ATRi treatment alone and a few resistant colonies immerged from the PARPi treatment alone in the UWB1 cells, no colonies were able to survive the prolonged combined PARPi and ATRi treatment (Figure 15D). Cells were able to survive and proliferate in the rescue UWB1+B1 cells in all three treatments. Importantly, combining PARPi with the same dose of ATMi did not have this same effect, demonstrating that the combined effect is ATRi specific. Taken together, these results suggest combined PARPi and ATRi treatment selectively targets BRCA1-deficient cells to cell death and prevents PARPi resistance from emerging.

The results of a second smaller kinase screen (data not shown) revealed that all resistant cells also have increased sensitivity to MEK inhibitor (MEKi) (Figure 16A). This suggests that MEK signaling is required for resistant cells to proliferate. I confirmed this effect using three separate MEK inhibitors (Figure 16B). To understand if MEKi is required for cells to become resistant, I treated BRCA1-deficient parental cells with MEKi, PARPi, or the combination MEKi and PARPi and allowed cells to grow for 45 days, changing the inhibitors every three days. While the cells treated with PARPi alone were able to develop two small PARPi resistant colonies, cells treated with the combination of PARPi and MEKi were not able to develop resistant colonies (Figure 16C). Importantly, MEKi alone did not have the same effect. To test if treatment with MEKi altered Rad51 loading, as does treatment with ATRi, I performed Rad51 immunofluorescence after damaging cells with PARPi. As expected, PARPi treatment induced Rad51 loading in UWB1+B1 cells, while UWB1 and SYr13 were defective for loading (Figure 16D). But unlikely treatment with ATRi, simultaneous treatment with MEKi and PARPi resulted in increased Rad51 foci in SYr13 cells. This suggests that MEKi is not re-sensitizing cells to PARPi by inhibiting Rad51 loading, but is acting through another pathway. Cell cycle analysis further demonstrated that MEKi specifically inhibits the cell cycle progression of the PARPi resistant cell lines (Figure 16E). Taken together, these preliminary data suggest that MEK activity is necessary for cells to develop resistance to PARPi and further necessary for resistant cells to continue to proliferate.

2) Test PARPi resistant targeting drugs on additional breast cancer cell lines:

I next sought to determine if PARPi synergizing with ATRi could be extending to other BRCA1-deficient cancer types, including breast cancer. I tested the effect of combined PARPi and ATRi treatment in viability assay using HCC1937 cells and HCCwt cells. Treatment with PARPi alone has a little effect in HCC1937 cells, despite a well-established HR deficiency in these cells, suggesting this cancer cell line is PARPi resistant. However, combining treatment with the ATRi re-sensitizes these cells to PARPi, with ATRi decreasing the IC50 of PARPi in a dose dependent manner (Figure 17A and 17B). The combination had less effect on the isogenic HCCwt cells in which wtBRCA1 is exogenously expressed, as the IC50 of PARPi remained high. We further extended these studies to another cell line, BR5, a BRCA1-deficient mouse ovarian cancer cell line which is exceptionally sensitive to PARPi treatment (Figure 17C). Though BR5 cells are already sensitive to PARPi treatment, these cells become even more sensitive to PARPi treatment, and undergo increased cell death following addition of ATRi treatment (Figure 17D). Finally, a PARPi resistant line derived from BR5 lines, BR5-R1, shows similar synthetic lethality when PARPi is combined with ATRi, again demonstrating this effect is not cell line specific. These results confirm that a variety of BRCA1-deficient cell lines, which are both resistant and sensitive to PARPi treatment, become more sensitive to PARPi in the presence of ATRi.

3) Test possible biomarkers for PARPi resistance in additional breast cancer lines.

To test the effect of ATRi in a more clinically relevant setting, we analyzed primary tumor cells isolated from a BRCA1-deficient ovarian cancer patient who progressed after Olaparib treatment. DNA fiber analysis revealed that HU-induced fork degradation was modest in these tumor cells. Importantly, consistent with the observations in the derived PARPi-resistant cell lines, VE-821 treatment of the tumor cells increased fork degradation. In contrast, tumor cells isolated from a non-BRCA1/2 ovarian cancer patient did not display fork degradation in HU even in the presence of ATRi (Figure 18A). These results suggest that ATRi preferentially enhances degradation of stalled forks in PARPi-resistant, BRCA1-deficient tumor cells compared to tumor cells without BRCA1/2 mutations. To confirm this, a second set of patient derived cells were used. Circulating tumor cells (CTCs) derived from a breast cancer patient harboring a BRCA2 mutation, but shown to be resistant to PARPi treatment were tested for fork stability. These CTCs did not display fork degradation in HU, but fork degradation was induced by addition of ATRi. On the other hand, a second CTC line derived from a breast cancer patient without any known mutations in HR, but shown to be sensitive to PARPi (AZD7762) demonstrated significant HU-induced fork degradation which was not enhanced by ATRi treatment (Figure 18B). These results confirm that ATRi preferentially enhances degradation of stalled forks in PARPi-resistant, BRCA-deficient tumor cells. Furthermore, these results suggest that DNA fiber analysis is potentially better biomarker of PARPi sensitivity than measurement of

HR by Rad51 foci, which were unable to be seen in the CTC lines (data not shown).

Key Research Accomplishments:

- PARP inhibitor resistant lines have been derived.
- PARP inhibitor resistant lines have been tested for HR restoration as marked by Rad51 loading following 10 Gy IR treatment and 24hr PARP inhibitor treatment.
- PARP inhibitor resistant lines have been tested for HR restoration by PALB2 loading following UV laser stripe induced damage.
- Restoration of BRCA1 was found not to be the cause of PARP inhibitor resistance in the derived resistant lines.
- Loss of DNA-PK or 53BP1 was determined not to result in PARP inhibitor resistance in the derived resistant lines.
- The HR activity in BRCA1-deficieint lines, both the parental lines and all resistant lines tested, is dependent on MRG15, PALB2, and BRCA2.
- PARP inhibitor resistance is not due to an increase in efflux pump expression or activity, in most cell lines.
- PARP inhibitor resistance is not due to mutations in PARP1 or increased activity of redundant PARP family members.
- ATR is a critical regulator of HR
- ATR functions to promote HR down stream of BRCA1 but upstream of PALB2 and BRCA2
- Treatment with ATR inhibitor can disrupt the remaining BRCA1-independent HR remaining in the BRCA1-deficient parental cells as well as the BRCA1-deficient, PARP inhibitor resistant lines.
- Treatment with ATRi re-sensitizes BRCA1-resistant cells to PARPi
- Combination of ATRi with PARPi specifically reduces the viability of BRCA1-deficient cells
- Combination of ATRi with PARPi specifically leads to cell death in the BRCA1-deficient cells
- The combination of ATRi with PARPi specifically reduces the viability of BRCA1-deficient cells in a number of cell lines, including human breast cancer cells, HCC1937, and mouse ovarian cancer cells, BR5-AKT.
- ATRi prevents PARPi resistant colonies from emerging
- I developed new PARPi resistant cells from a mouse BRCA1-deficient ovarian cancer cell line
- Combined ATRi and PARPi can selectively reduce the viability of these PARPi resistant mouse ovarian cells
- Known mechanisms of PARPi resistance, including regaining of BRCA1, loss of Rif1, 53BP1, Ku70/80, or Rev7, or increased efflux of PARPi drug, does not result in PARPi resistance
- BRCA1-deficient cancers have been rewired to allow Rad51 loading to promote HR in the absence of BRCA1
- BRCA1-deficient Rad51 loading requires PALB2 and BRCA2
- PALB2 and BRCA2 are required for PARPi resistance

- BRCA1-deficient PARPi resistant lines have regained fork protection after fork stalling with HU treatment
- Regained fork protection can be reversed by treatment with ATRi
- Rad51 is loaded to chromatin and directly at nascent DNA forks after treatment with HU in PARPi resistant, but not parental, BRCA1-deficient cells
- Regained Rad51 loading at stalled forks is reversed by treatment with ATRi
- ATRi-mediated de-protection results in MRE11-dependent fork degradation
- ATRi was determined to synergize with PARPi specifically in BRCA1-deficient cells in a small DNA damage specific mini-screen.
- Fork protection is re-acquired in PARPi resistant, BRCA-deficient ovarian and breast cancer patient cells
- ATRi treatment prevents fork protection, resulting in fork degradation
- DNA fiber analysis may be a biomarker for PARPi resistance
- All PARPi resistant cells are sensitive to MEKi treatment
- MEKi does not inhibit DNA damage repair, but instead increases damage and stalls cell cycle progression in PARPi resistant cell lines

Conclusion:

In year one of my proposal, I had derived BRCA1-deficient, PARP inhibitor resistant lines from a parental PARP inhibitor sensitive line. I had determined that homologous recombination (HR), as marked by Rad51 loading and PALB2 localization to DNA damage, occurs in all BRCA1-deficient cell lines, including the parental line, and is further restored in several PARP inhibitor resistant lines. I ruled out known mechanisms of PARPi resistance in the absence of BRCA1, including restoration of BRCA1, loss of 53BP1 or Rif1, loss of DNA-PK activity, increased efflux of PARPi from the cell, increased PARP expression, or mutations in PARP that prevent the inhibitor from binding. I used targeted siRNAs in a candidate approach to determine that the restored HR levels, as well as the low level or residual HR seen in the BRCA1-deficient parental cell line depends on BRCA2, PALB2, and MRG15.

In the second year of my proposed experiments, using inhibitors of ATR, I have determined that ATR plays a critical role in HR repair downstream and independently of BRCA1, but upstream of PALB2 and BRCA2. I have determined that ATR plays a role in promoting Rad51 loading even in the absence of BRCA1. Treatment with ATRi prevents HR in the absence of BRCA1, leading to resensitization of these cells to PARPi treatment, resulting in reduced viability specifically in BRCA1-deficient cells, including PARPi resistant lines. The combination of ATRi and PARPi results in cell death specifically in BRCA1-deficient cells. These findings were consistent in a number of cell lines. Finally, using a long-term (42 day) colony assay, I have determined that the combination of PARPi and ATRi in a PARPi sensitive cell line can prevent PARPi resistance from emerging.

In the third year of my proposal, I further characterized the BRCA1-deficient, PARPi resistant lines. First, I determined that resistance is not due to complete loss of Rev7, which was recently shown to confer resistance to PARPi in other cell lines. Secondly, I showed that BRCA1-deficient cancer cell lines is unlike acute BRCA1-depletion, in that BRCA1-deficient cancer cell lines have undergone rewiring to allow inefficient Rad51 loading in the absence of BRCA1. I further showed that this BRCA1-

independent Rad51 loading depends on PALB2 and BRCA2. Additionally, PALB2 and BRCA2 are essential for PARPi resistance in the derived resistant lines.

Additionally, I have completed experiments to understand the mechanism by which ATRi selectively kills BRCA1-deficient cells. To this end, I tested whether fork protection is altered in BRCA1-deficient, PARPi resistant cell lines. I found that in rescue cells, which express BRCA1, forks are protected after stalling with HU, as expected. In the parental BRCA1-deficient cells, forks collapse and undergo fork degradation, as has been previously published. However, in the derived PARPi resistant cells, fork protection was regained. I further demonstrated that this protection is ATR dependent, and that loss of protection results in MRE11-mediated fork degradation. Finally, I showed that preventing fork degradation of unprotected forks by depleting MRE11 can lead to PARPi resistance. In another set of experiments, I performed a DNA damage screen, including DNA damage kinase inhibitors as well as DNA damaging agents, to determine if the resistant cells have an increased dependence on another repair pathway. This screen revealed that ATRi combined with PARPi was specifically lethal in BRCA1-deficient cell lines. Additionally, I found that treatment with other inhibitors (ATMi, Chk1i, Wee1i, or DNA-PKi) or damaging agents (CPT, APH, MMC) did not have this effect.

A second inhibitor screen also revealed resistant cells were particularly sensitive to MEKi, independently of PARPi treatment. MEKi treatment prevents the emergence of PARPi resistant clones. Treatment with MEKi does not result in a decrease in PARPi-induced Rad51 foci, demonstrating that the mechanism of MEKi action is different than that of ATRi. Finally, treatment with MEKi does not result in cell death, but rather a decrease in proliferation, demonstrated by cell cycle analysis.

In the final part of my funding, I extended these results to additional cell lines and patient samples. To this end, I tested the effect of ATRi on fork protection in ovarian cancer cells taken from patients as well as on circulating tumor cell lines derived from patients with BRCA-deficient breast cancer. These experiments showed that ATRi specifically causes fork de-protection in BRCA-deficient cancer cells, suggesting ATRi may resensitize BRCA-deficient cancer cells to PARPi. Furthermore, these studies showed that loss of fork protection may be a better biomarker for PARPi sensitivity than Rad51 loading or BRCA-genotyping.

Taken together, the results of the experiments contained in this final report demonstrate that treatment with ATRi in BRCA1-deficient cancer cells, both ovarian and breast, results in further depletion of Rad51 loading, resulting in loss of HR and loss of fork protection, and ultimately, when combined with PARPi, results in cell death. Fork protection may also serve as biomarker for PARPi sensitivity. Furthmore the combination of ATRi and PARPi prevents PARPi resistant clones from emerging. This suggests that the combination therapy of ATRi and PARPi may be a useful initial therapy as well as a useful treatment for resistant tumors. Additionally, a second inhibitor, MEKi, can also selectively prevent proliferation of resistant cell lines, and prevent resistance from PARPi from emerging. This suggests that MEK activity is required for PARPi resistance to emerge, suggesting co-treatment with MEKi maybe a way to prevent resistance to PARPi from emerging in BRCA1-deficient cancer patients.

Taken together, the results of this years progress on proposed work suggest two potential combination therapies, either combined PARPi and ATRi treatment to selectively target BRCA1-deficient cells to cell death, and combined PARPi and MEKi to

prevent PARPi resistance from emerging. Ultimately, the proposed experiments for the next six months will provide insight into how MEK singaling is essential for PARPi resistance to emerge the absence of BRCA1, how BRCA1-mutated breast cancers acquire resistance to PARP inhibitor treatment, and ultimately how recurrent tumors can be treated.

Publications, Abstracts and Presentations:

The BRCA1-interacting protein Abraxas is required for genomic stability and tumor suppression. Castillo A, Paul A, Sun B, Huang TH, Wang Y, Yazinski SA, Tyler J, Li L, You MJ, Zou L, Yao J, Wang B. Cell Reports. 2014 Aug 7;8(3):807-17. PMID: 25066119

Suppression of genome instability in pRB-deficient cells by enhancement of chromosome cohesion. Manning AL, Yazinski SA, Nicolay B, Bryll A, Zou L, Dyson NJ. Molecular Cell. 2014 Mar 20;53(6):993-1004. PMID: 24613344

PRP19 transforms into a sensor of RPA-ssDNA after DNA damage and drives ATR activation via a ubiquitin-mediated circuitry. Maréchal A, Li JM, Ji XY, Wu CS, Yazinski SA, Nguyen HD, Liu S, Jiménez AE, Jin J, Zou L Molecular Cell. 2014 Jan 23;53(2):235-46. PMID: 24332808

Inventions, Patents, and Licences: Nothing to report

Reportable Outcomes:
Meetings attended:

Course: Next Generation Sequencing, Harvard Catalyst

Course: IRB Issues for the Bench and Desk Scientist, MGH

Symposium: Chromatin and DNA repair, Cell Reports, Oct. 2013.

Symposium: Library of Integrated Network-Based Cellular Signatures (LINCS) Consortium, Broad institute, Nov. 2013.

Meeting: "Advances in Ovarian Cancer" in Orlando, Fl., October 2015.

Meeting presentations:

Novel mechanisms of PARP inhibitor resistance in BRCA1-deficient cancers. Stephanie Yazinski, Ron Ho, Hai Dang Nguyen, Leif Ellisen, Cyril Benes, Lee Zou. Massachusetts General Hospital Cancer Center Annual Retreat. Septemeber 2013. (Poster presented. Third place award.)

References:

Bryant, H. E., Schultz, N., Thomas, H. D., Parker, K. M., Flower, D., Lopez, E., … Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*, *434*(7035), 913–7. doi:10.1038/nature03443

Edwards, S. L., Brough, R., Lord, C. J., Natrajan, R., Vatcheva, R., Levine, D. A., … Ashworth, A. (2008). Resistance to therapy caused by intragenic deletion in BRCA2. *Nature*, *451*(7182), 1111–5. doi:10.1038/nature06548

Farmer, H., McCabe, N., Lord, C. J., Tutt, A. N. J., Johnson, D. a, Richardson, T. B., … Ashworth, A. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*, *434*(7035), 917–21. doi:10.1038/nature03445

Gartner, E. M., Burger, A. M., & Lorusso, P. M. (n.d.). Poly(adp-ribose) polymerase inhibitors: a novel drug class with a promising future. *Cancer Journal (Sudbury, Mass.)*, *16*(2), 83–90. doi:10.1097/PPO.0b013e3181d78223

Gudmundsdottir, K., & Ashworth, A. (2006). The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene*, *25*(43), 5864–74. doi:10.1038/sj.onc.1209874

Helleday, T., Bryant, H. E., & Schultz, N. (2005). Poly(ADP-ribose) polymerase (PARP-1) in homologous recombination and as a target for cancer therapy. *Cell Cycle (Georgetown, Tex.)*, *4*(9), 1176–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16123586

King, M.-C., Marks, J. H., & Mandell, J. B. (2003). Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science (New York, N.Y.)*, *302*(5645), 643–6. doi:10.1126/science.1088759

Krishnakumar, R., & Kraus, W. L. (2010). The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets. *Molecular Cell*, *39*(1), 8–24. doi:10.1016/j.molcel.2010.06.017

Pierce, A. J., Johnson, R. D., Thompson, L. H., & Jasin, M. (1999). XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes & Development*, *13*(20), 2633–2638. doi:10.1101/gad.13.20.2633

Tutt, A., Robson, M., Garber, J. E., Domchek, S. M., Audeh, M. W., Weitzel, J. N., … Carmichael, J. (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. *Lancet*, *376*(9737), 235–44. doi:10.1016/S0140-6736(10)60892-6

Appendices:

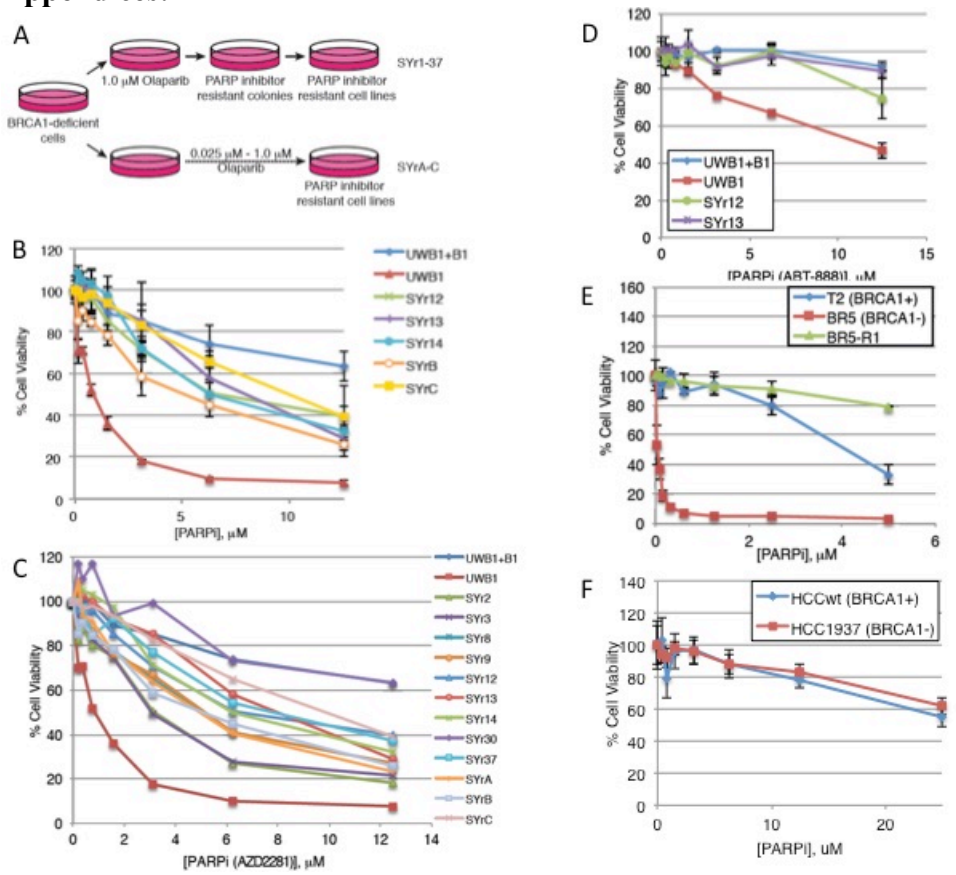

Figure 1: PARPi resistant cells lines were derived from a variety of BRCA1-deficient cell lines. (A) Schematic of derivation of PARPi-resistant cells from the parental BRCA1-deficient UWB1.289 ovarian cancer cell line. (B) Viability assay of UWB1 (parental), UWB+B1 (complemented with wild-type *BRCA1*), and derived PARPi-resistant cell lines after 6 days of increasing doses of PARPi (Olaparib) treatment. n=3 replicates; error bars represent s.d. (C) Viability assay of UWB1 (parental), UWB+B1 (complemented with wild-type *BRCA1*), and all derived PARPi-resistant cell lines after 6 days of increasing doses of PARPi (Olaparib) treatment. (D) Viability assay of UWB1 (parental), UWB+B1 (complemented with wild-type *BRCA1*), and derived PARPi-resistant cell lines after 6 days of increasing doses of PARPi (ABT-888) treatment. (E) Viability assay of BR5 (parental), T2 (wild-type *BRCA1*), and derived PARPi-resistant cell line (BR5-R1) after 6 days of increasing doses of PARPi (Olaparib) treatment. (F) Viability assay of *de novo* resistant HCC1937 (parental) and HCCwt (complemented with wild-type *BRCA1* after 6 days of increasing doses of PARPi (Olaparib) treatment.

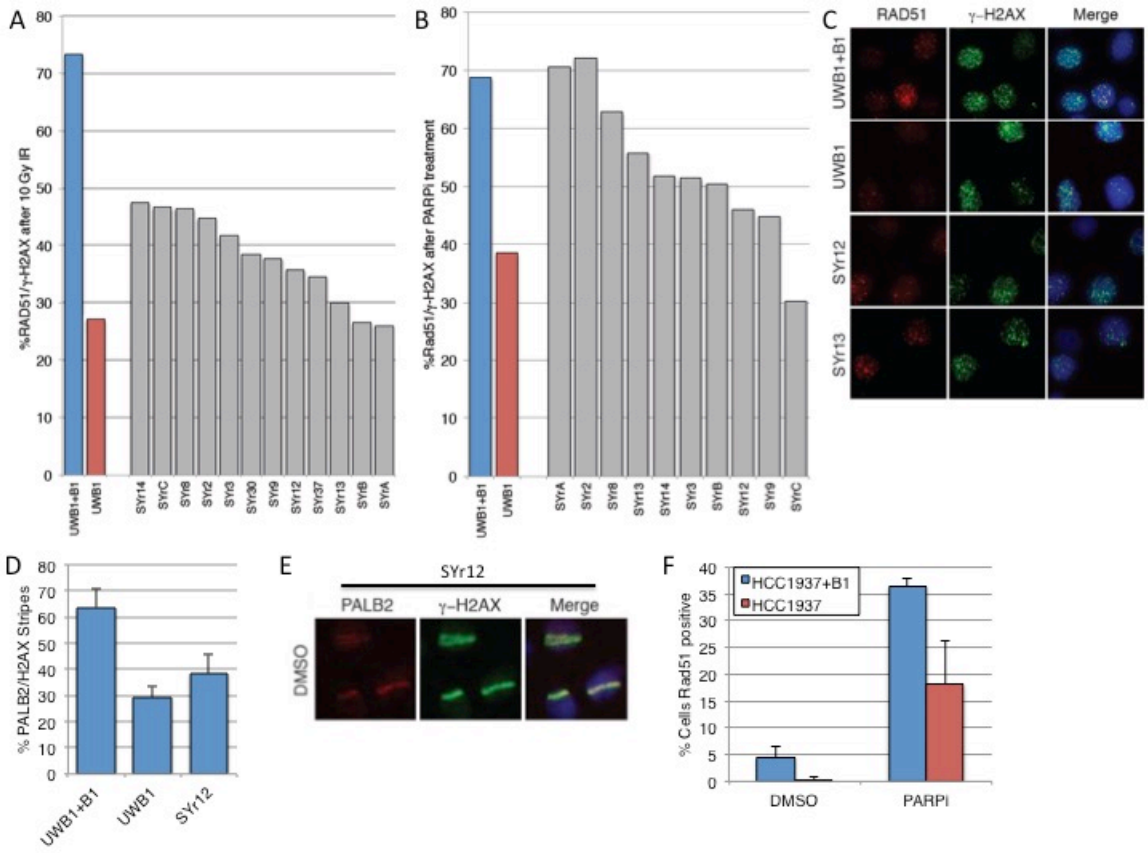

Figure 2: Homologous recombination is restored in BRCA1-deficient parental cells as well as in the derived PARPi resistant cell lines. (A) UWB1, UWB1+B1, and all the PARPi-resistant cell lines were irradiated with IR (10 Gy), and fractions of RAD51 foci positive cells (> 5 foci per cell) were measured 4 hrs later. (B) UWB1, UWB1+B1, and all the PARPi-resistant cell lines were treated with 10μM PARPi, and fractions of RAD51 foci positive cells (> 5 foci per cell) were measured 24 hrs later. (C) Representative images of A are shown. (D) The indicated cell lines were treated with irradiated with ultraviolet (UV) laser, and fractions of cells showing PALB2 staining in γH2AX stripes were measured 1 hr later. n=3 replicates; error bars represent s.d. (E) Representative images of D are shown. (F) HCC1937 and HCC1937+B1 were treated with 10μM PARPi, and fractions of RAD51 foci positive cells (> 5 foci per cell) were measured 24 hrs later.

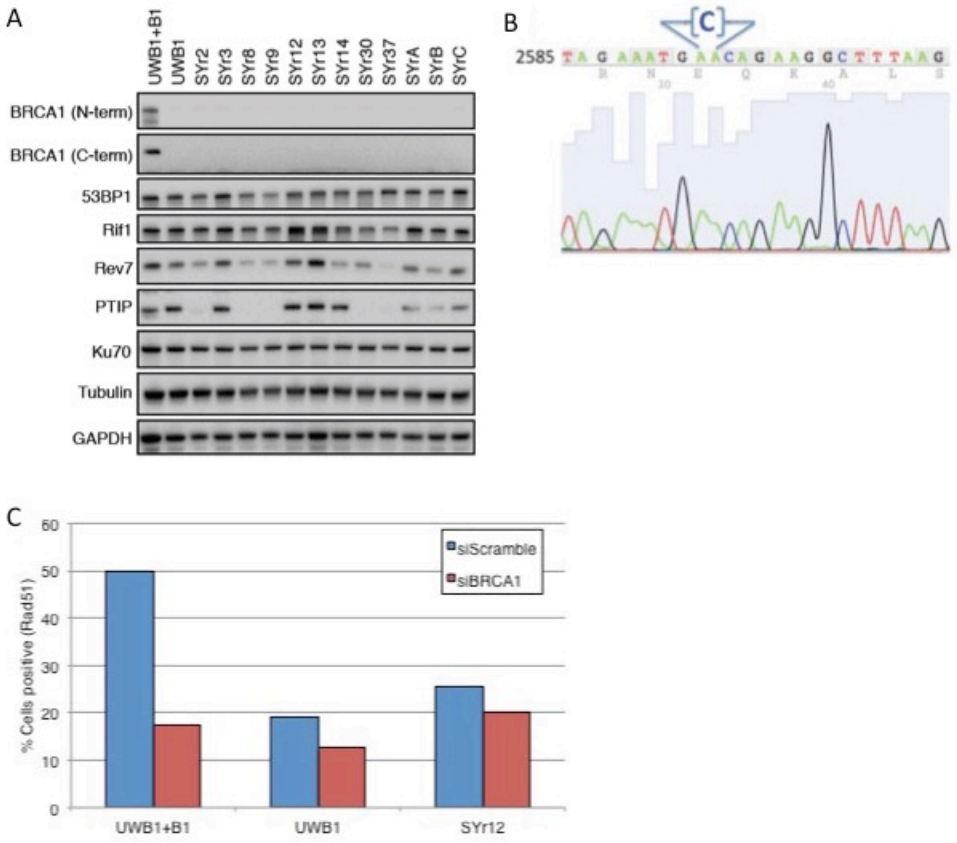

Figure 3: BRCA1 is not restored in the derived PARPi resistant lines. (A) Western blot of UWB1+B1, UWB1, and all derived resistant lines using the indicated antibodies. (B) Representative sequencing trace demonstrating retention of the point mutation resulting in a premature stop codon and loss of BRCA1 in the resistant lines. (C) The indicated cell lines are treated with siScramble or siBRCA1, cell lines were treated with 10μM PARPi, and fractions of RAD51 foci positive cells (> 5 foci per cell) were measured 24 hrs later.

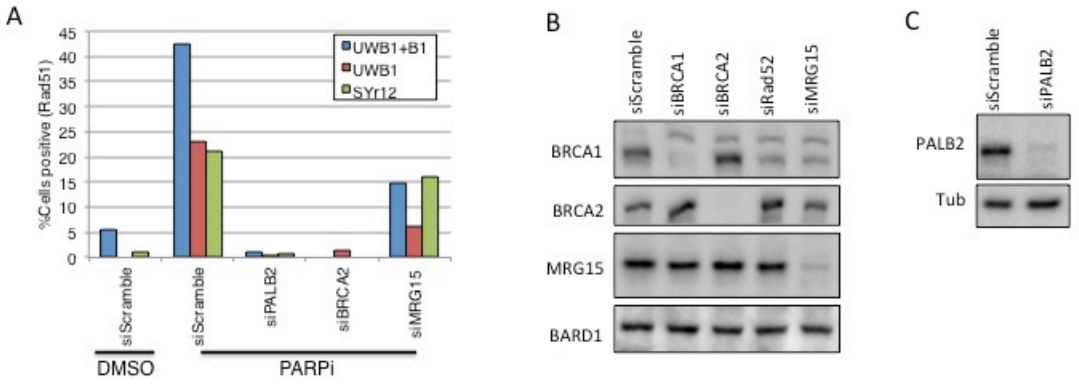

Figure 4: PALB2, BRCA2, and MRG15 contribute to HR in the absence of BRCA1. (A) The indicated cell lines are treated with siScramble, siPALB2, siBRCA2, or siMRG15, cell lines were treated with DMSO or 10μM PARPi, and fractions of RAD51 foci positive cells (> 5 foci per cell) were measured 24 hrs later. (B-C) The indicated cell lines are treated with siScramble, siPALB2, siBRCA2, or siMRG15, and lysate was collected for Western blotting with the indicated antibody.

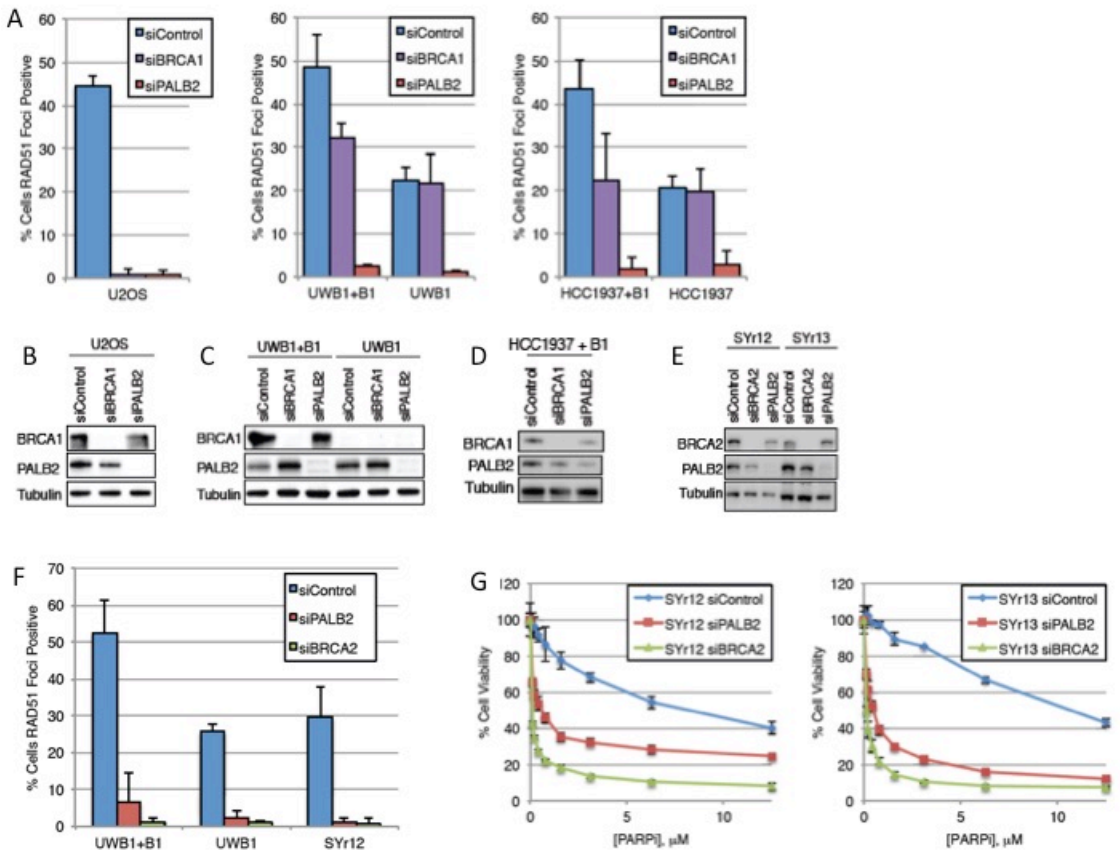

Figure 5: BRCA1 is partially bypassed in BRCA1-deficient cell lines through BRCA2 and PALB2 to load Rad51, contributing to PARPi resistance. (A) The indicated cell lines were treated with siControl, siBRCA1, or siBRCA2, irradiated with IR (10 Gy), and fractions of RAD51 foci positive cells were measured 4 hrs later. n=3 replicates; error bars represent s.d. (B-E) The indicated cell lines were transfected with siControl, siBRCA1, siBRCA2, or siPALB2, and cell lysates were prepared 48 hrs after knockdown. Levels of the indicated proteins were analyzed using specific antibodies. Tubulin serves as a loading control. (F) The indicated cell lines were transfected with siControl, siPALB2, or siBRCA2, treated with PARPi (Olaparib, 10 mM) for 24 hrs, and fractions of RAD51 foci positive cells were determined. n=3 replicates; error bars represent s.d. (G) The PARPi-resistant cell lines were transfected with siControl, siPALB2, or siBRCA2, treated with increasing doses of PARPi (Olaparib) for 6 days, and cell viability was measured. n=3 replicates; error bars represent s.d.

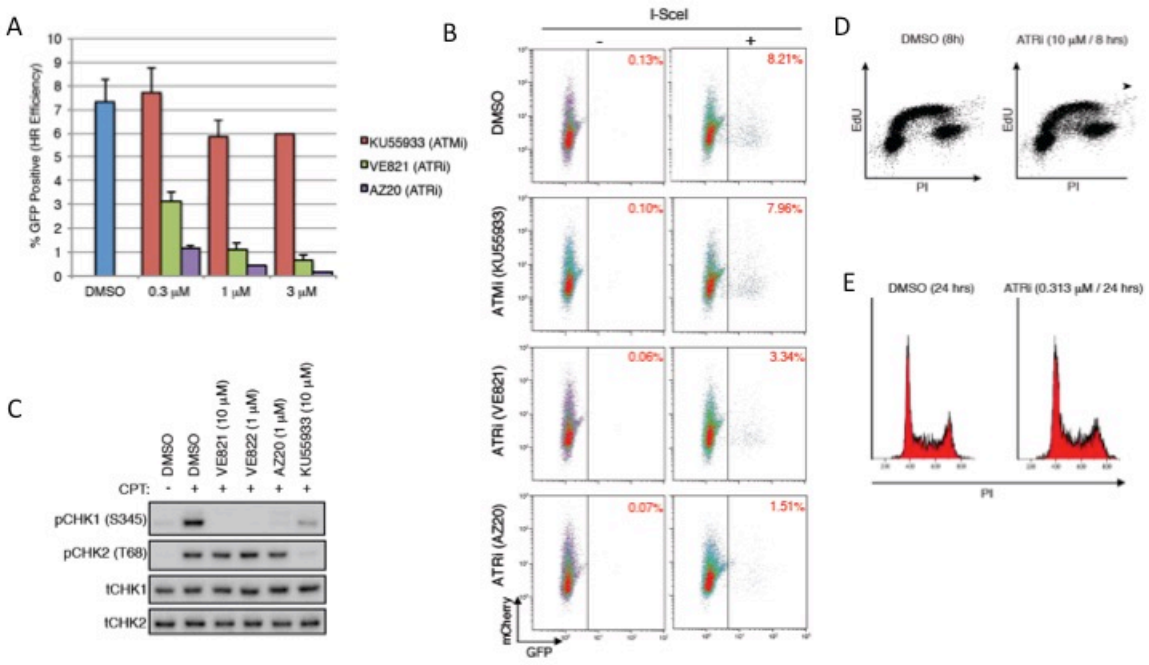

Figure 6: ATR functions in ATR without effecting cell cycle progression. (A) U2OS cells were treated with the indicated doses of ATMi (KU55933) or ATRi (VE821 or AZ20). The efficiency of HR was measured using the DR-GFP reporter 48 hrs after I-SceI transfection. n=3 replicates; error bars represent s.d. (B) Representative FACS data of the U2OS-DR-GFP cell line before or after I-SceI transfection in the absence or presence of ATMi (KU55933, 0.3 μM) or ATRi (VE-821, 0.3 μM; AZ20, 0.3 μM). Cells were co-transfected with a plasmid expressing I-SceI and a second plasmid expressing mCherry, which was used to sort for transfected cells. Fractions of mCherry-positive cells that were also GFP-positive were used to determine the efficiency of HR. (C) U2OS cells were treated with ATRi (VE-821, 10 μM; VE-822, 1 μM; AZ20, 1 μM) or ATMi (KU55933, 10 μM) in combination with Camptothecin (CPT, 1 μM) for 1 hr. Inhibition of ATR or ATM is assessed by loss of pChk1 and pChk2, respectively. (D) EdU incorporation profiles of U2OS cells treated with DMSO or ATRi (VE-821, 10 μM) for 8 hrs. (E) Cell-cycle profiles of UWB1 cells treated with DMSO or ATRi (VE-821, 0.313 μM) for 24 hrs.

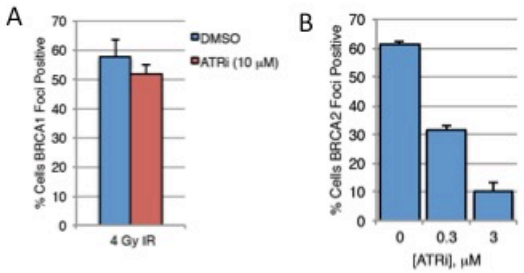

Figure 7: ATR functions downstream of BRCA1 and upstream of BRCA2. (A) U2OS cells were treated with DMSO or ATRi, irradiated with IR (4 Gy), and fractions of BRCA1 foci positive cells were measured 2 hrs later. n=3 replicates; error bars represent s.d. (B) Cells were treated with the indicated doses of ATRi (VE-821), irradiated with IR (10 Gy), and fractions of BRCA2 foci positive cells (> 5 foci per cell) were measured 4 hrs later. n=3 replicates; error bars represent s.d.

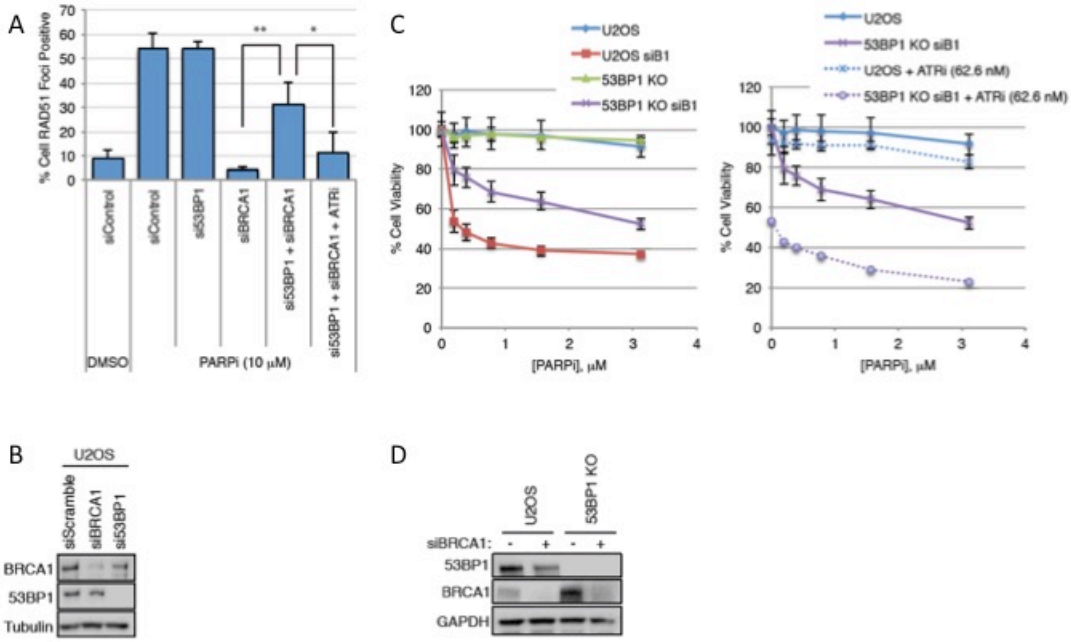

Figure 8: ATR functions in HR and remains indispensable even when BRCA1 is bypassed. (A) U2OS cells were transfected with siControl, si53BP1, siBRCA1, or si53BP1 and siBRCA1. Transfected cells were treated with DMSO, PARPi (Olaparib, 10 μM), or PARPi and ATRi (VE-821, 0.3 μM) for 24 hrs. Fractions of RAD51 foci positive cells were determined. n=3 replicates; error bars represent s.d.; *p<0.05, **p<0.01. (B) Knockdown of BRCA1 and 53BP1 in U2OS cells was confirmed by Western blot. Tubulin or GAPDH serves as loading controls. (C) U2OS cells or U2OS-derived 53BP1 KO cells were transfected with siControl or siBRCA1, treated with increasing doses of PARPi (Olaparib) in the absence or presence of ATRi (VE-821, 62.6 nM) for 6 days. Cell viability was measured. n=3 replicates; error bars represent s.d. (D) Knockout of BRCA1 and 53BP1 in U2OS cells was confirmed by Western blot. Tubulin or GAPDH serves as loading controls.

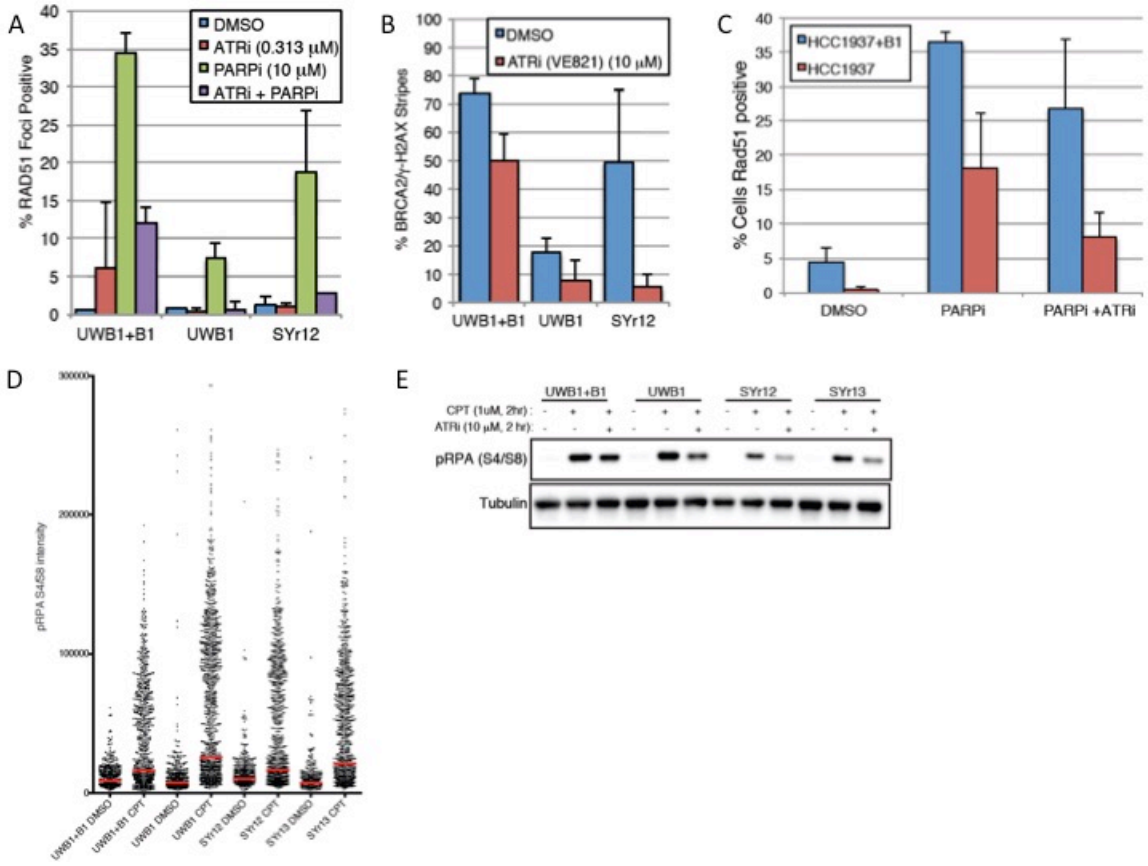

Figure 9: ATR functions in HR downstream of BRCA1 and remains indispensable even when BRCA1 is bypassed. (A) The indicated cell lines were treated with ATRi (VE-821), PARPi (Olaparib), or ATRi and PARPi for 24 hrs. Fractions of RAD51 foci positive cells were measured. n=3 replicates; error bars represent s.d. (B) The indicated cell lines were treated with DMSO or ATRi (VE-821, 10 μM), irradiated with ultraviolet (UV) laser, and fractions of cells showing BRCA2 staining in γH2AX stripes were measured 1 hr later. n=3 replicates; error bars represent s.d. (C) The indicated cell lines were treated with ATRi (VE-821), PARPi (Olaparib), or ATRi and PARPi for 24 hrs. Fractions of RAD51 foci positive cells were measured. n=3 replicates; error bars represent s.d. (D) The indicated cell lines were treated with CPT (1 μM) for 2 hrs. Intensity of pRPA (S4/S8) was measured by immunofluorescence, with each cell plotted individually. n=550 cells for each condition. (E) The indicated cell lines were treated with CPT (1 μM) or CPT and ATRi (10 μM) for 2 hrs. Levels of pRPA (S4/S8) were analyzed by western blot.

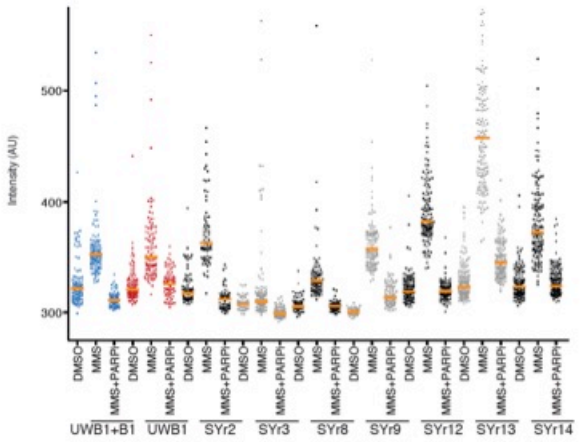

Figure 10: Levels of PAR chain formation in UWB1, UWB1+B1, and the resistant lines as measured by an anti-PAR antibody 30 min after treatments with DMSO, Methyl methanesulfonate (MMS, 1 mM), or MMS and PARPi (Olaparib,10 μM, 5 hr pre-incubation). Bars represent the median PAR intensity.

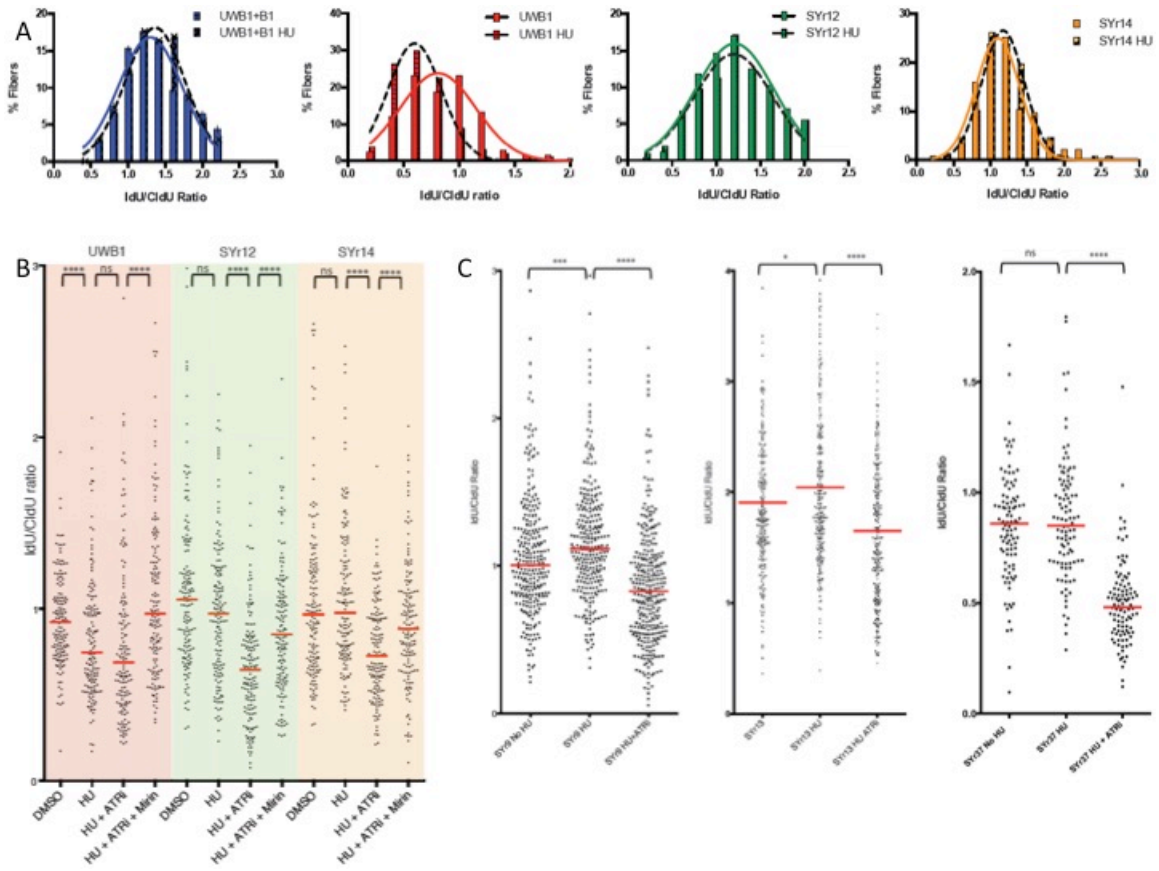

Figure 11: ATRi reactivates degradation of stalled forks in PARPi-resistant cells. (A) DNA fiber analysis of stalled replication forks. Newly synthesized DNA was sequentially labeled with CldU (50 μM, 30 min) and IdU (100 μM, 30 min). Cells were subsequently treated with HU (4 mM) for 5 hrs, and lengths of CIdU and IdU-labeled DNA fibers were measured. IdU/CldU ratio was binned in increments of 0.2 and fit to a Gausian Curve using Prism software. At least n=100 fibers were measured for each condition; experiments were completed in triplicate. (B) DNA fiber analysis of stalled forks as in A, with each fiber plotted individually. The indicated cell lines were untreated or treated with HU (4 mM), HU and ATRi (VE-821, 10 μM), or HU, ATRi and Mirin (50 μM) for 5 hrs. Bars represent the median IdU/CldU ratios. n=150 fibers for each condition; experiments were completed in triplicate. Significance was determined by Mann-Whitney test, **** $p<0.0001$. (C) DNA fiber analysis of stalled forks as in A, with each fiber plotted individually. The indicated cell lines were untreated or treated with HU (4 mM), HU and ATRi (VE-821, 10 μM), or HU, ATRi and Mirin (50 μM) for 5 hrs. Bars represent the median IdU/CldU ratios. Significance was determined by Mann-Whitney test, **** $p<0.0001$.

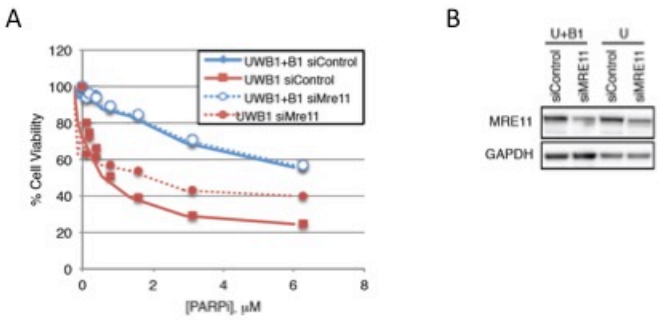

Figure 12: Partial MRE11 knockdown results in decreased sensitivity to PARPi in BRCA1-deficient UWB1 cell lines. (A) UWB1 and UWB1+B1 cells were transfected with siControl or siMRE11, treated with increasing doses of PARPi (Olaparib) for 6 days, and cell viability was measured. (B) Partial knockdown of MRE11 is confirmed by Western blot.

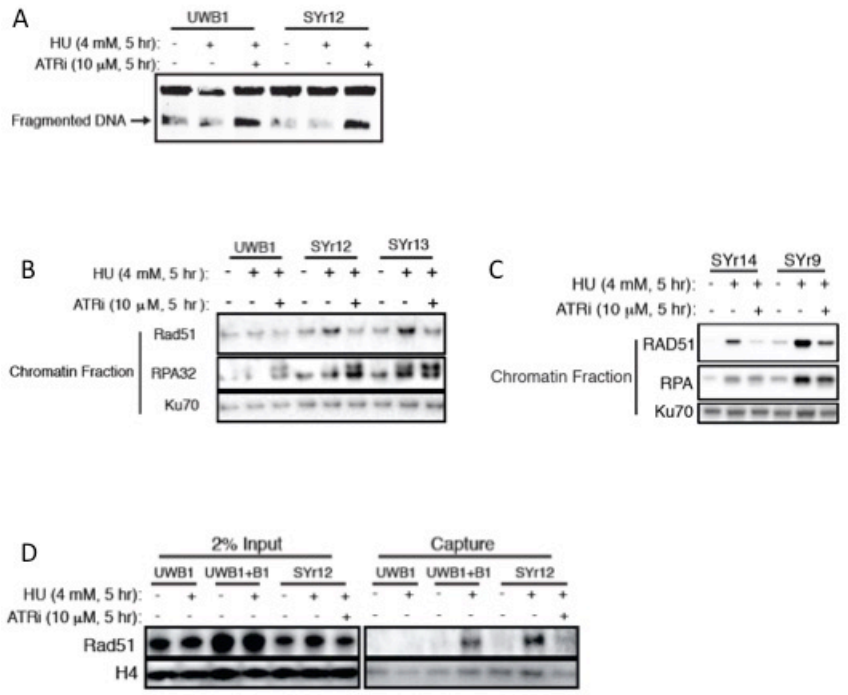

Figure 13: ATRi reactivates degradation through loss of Rad51, but not through increased DNA damage. (A) The indicated cell lines were treated with HU (4 mM) in the absence or presence of ATRi (VE-821, 10 μM) for 5 hrs. The resulting genomic DNA from 5 X 10^5 cells for each condition was subjected to pulse field gel electrophoresis (PFGE) to measure DNA fragmentation. (B-C) The indicated PARPi-resistant cell lines were treated with HU (4 mM) for 5 hrs in the absence or presence of ATRi (VE-821, 10 μM). The levels of chromatin-bound RAD51 and RPA32 were analyzed with KU70 as a loading control. (D) The indicated cell lines were pulsed with EdU for 30 min, then collected or treated with HU (4 mM) in the absence or presence of ATRi (VE-821, 10 μM) for 5 hrs. The association of RAD51 with nascent DNA was analyzed by iPOND with histone H4 as a loading control.

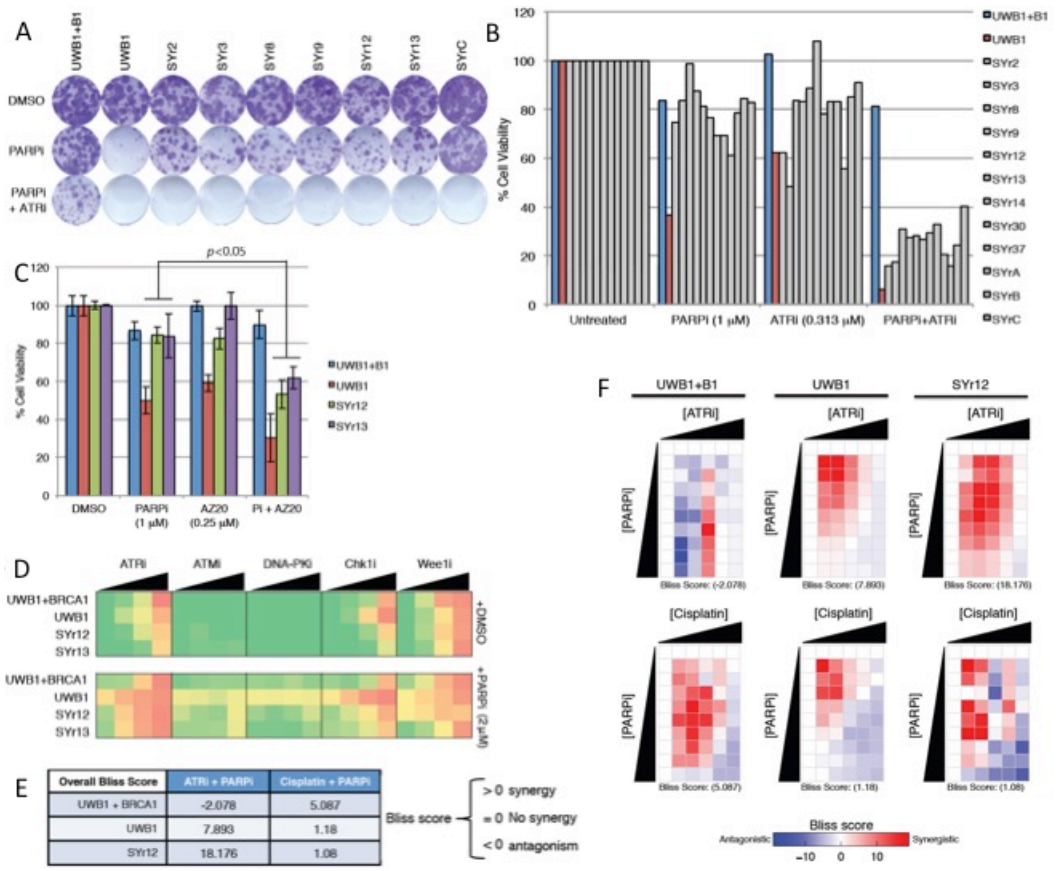

Figure 14: (A) Colony formation assay of the indicated cell lines following 14 days of treatment with DMSO, PARPi (Olaparib, 1 μM), or PARPi and ATRi (VE-821, 0.313 μM). Cells were stained with Crystal Violet. (B) Cell viability following 6 days of treatment across all cell lines using DMSO, PARPi (Olaparib), ATRi (VE-821), or PARPi and ATRi. (C) The indicated cell lines were treated with DMSO or the indicated doses of PARPi (Olaparib), AZ20, or PARPi and AZ20 for 6 days. Cell viability was measured. n=3 replicates; error bars represent s.d.; P<0.05 by Student t-test. (D) Mini drug screen performed on UWB1+B1, UWB1, and resistant lines (SYr12 and SYr13). Cells were treated with increasing doses of ATRi (VE-821, 0-0.625 μM), ATMi (KU55933, 0-0.625 μM), DNA-PKi (NU7441, 0-0.625 μM), Chk1i (MK-8776, 0-0.5 μM), or Wee1i (MK-1775, 0-0.25 μM), in the absence or presence of PARPi (Olaparib, 2.0 mM). Color-coding denotes level of viability, green (100% viability) to red (0% cell viability) relative to DMSO treatment. (E) The indicated cell lines were treated with increasing doses of PARPi (0 - 25 μM) in combination with either increasing doses of ATRi (0 - 2.5 μM) or Cisplatin (0 – 1.25 μM), and an overall Bliss score was calculated. Bliss score < 1, synergistic; Bliss score = 1, additive; Bliss score > 1, antagonistic. (F) The indicated cell lines were treated with increasing doses of PARPi (0 - 25 μM) in combination with either increasing doses of ATRi (0 - 2.5 μM) or Cisplatin (0 - 1.25 μM). Bliss scores were calculated for each drug combination as well as an overall bliss score for each cell line. Bliss score < 1, synergistic (red); Bliss score = 1, additive (white); Bliss score > 1, antagonistic (blue).

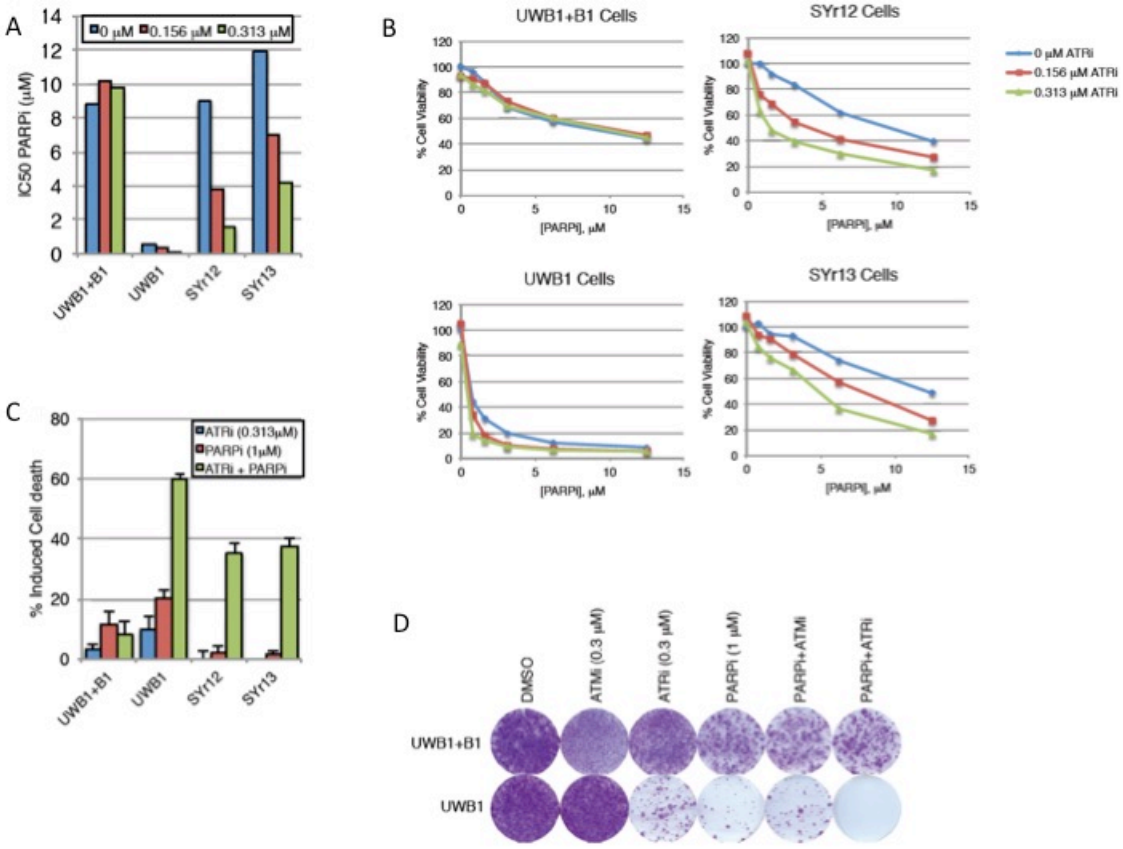

Figure 15: ATRi broadly overcomes the acquired PARPi resistance in BRCA1-deficient cancer cell lines. (A) IC50s of the indicated cell lines to PARPi (Olaparib) were measured after 6 days of Olaparib treatment in the presence of increasing concentrations of ATRi (VE-821). (B) Viability assay of UWB1, UWB1+B1, and the PARPi-resistant cell lines SYr12 and SYr13 after 6 days of treatment with increasing doses of PARPi (Olaparib) in the absence or presence of ATRi (VE-821). Viability curves were used to calculate IC50 to Olaparib for each cell line. (C) The indicated cell lines were treated with ATRi (VE-821), PARPi (Olaparib), or ATRi and PARPi for 7 days. Fractions of cells undergoing cell death (Propidium iodide and Annexin V positive) were measured. n=3 replicates; error bars represent s.d. (D) Colony formation assay of UWB1+B1 and UWB1 cell lines after 45 days of treatment with ATMi, ATRi, PARPi, the ATMi + PARPi combination, or the ATRi + PARPi combination. Cells were stained with Crystal Violet.

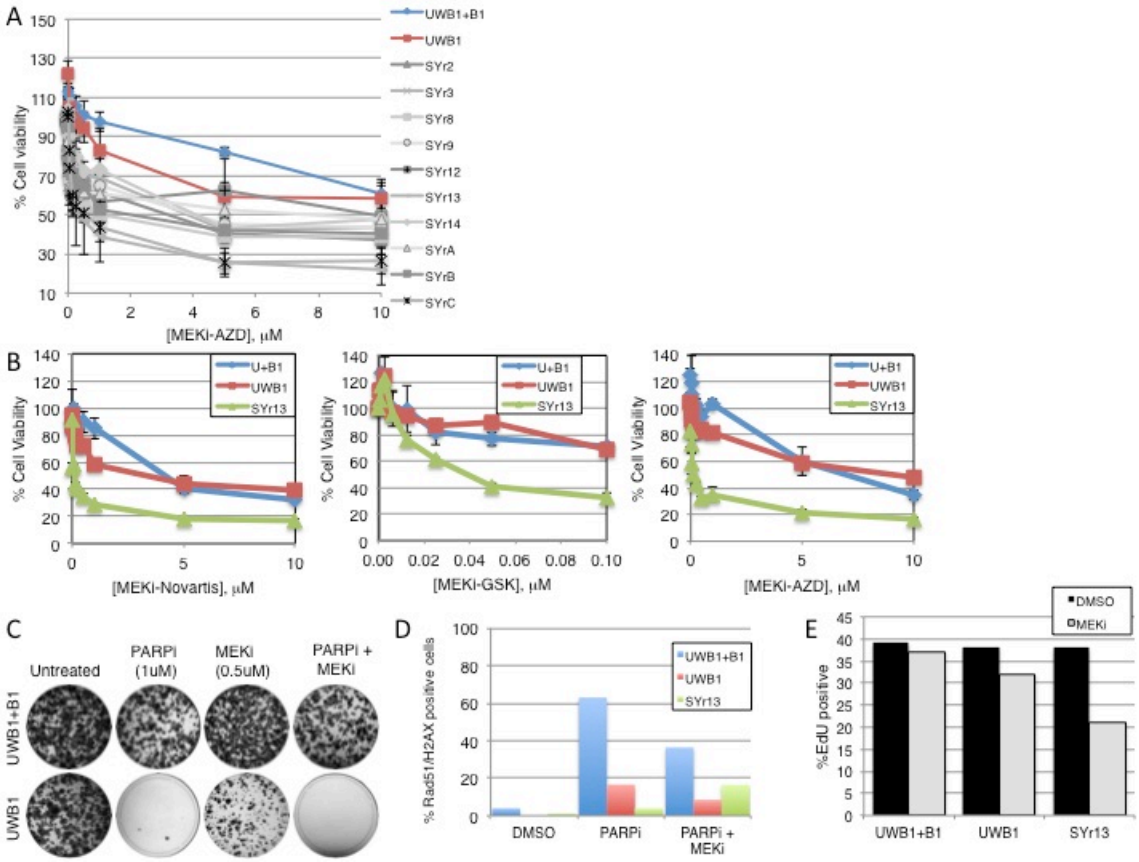

Figure 16: PARPi resistant cells require MEK activity for resistance to arise and to maintain proliferation. (A) Cell viability assay following increasing doses of MEKi demonstrate that all resistant cells have increased sensitivity to MEKi treatment. (B) Treatment with three unique MEKi compounds result in increased sensitivity to PARPi resistant cell line SYr13. (C) Co treatment with PARPi and MEKi for 45 days prevents resistance from emerging in the parental UWB1 cells, while treatment with MEKi alone merely slows growth and treatment with PARPi alone results in a small number of PARPi resistant clones to emerge. (D) Treatment with MEKi does not result in a decrease in Rad51 foci in the SYr13 cells which are sensitive to MEKi treatment. There is a decrease in Rad51 foci in the rescue UWB1+B1 and parental UWB1 cells; however, these cells are not sensitive to MEKi. This suggests that the inhibition of Rad51 loading does not contribute to sensitivity to this MEKi. (E) Decrease in cell cycle progression following MEKi treatment is seen specifically in the SYr13 PARPi resistant cell line.

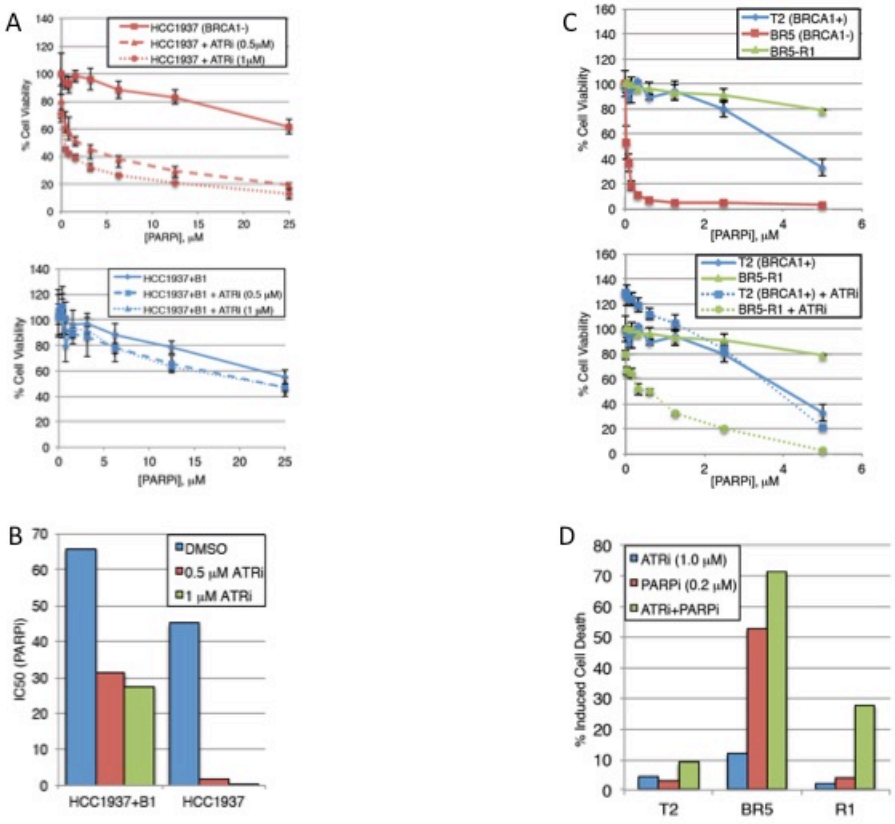

Figure 17: ATRi broadly overcomes the acquired and preexistent PARPi resistance in multiple BRCA1-deficient cancer cell lines of distinct origins. (A) Viability assay of the BRCA1-deficient HCC1937 cell line, which is *de novo* PARPi-resistant, after 6 days of treatment with increasing doses of PARPi (Olaparib) in the absence or presence of increasing doses of ATRi (VE-821). n=3 replicates; error bars represent s.d. (B) IC50s of the indicated cell lines to PARPi (Olaparib) were measured after 6 days of Olaparib treatment in the presence of increasing concentrations of ATRi (VE-821). (C) Viability assay of mouse ovarian cancer cell lines (T2, non-BRCA mutant; BR5, BRCA1-deficient; BR5-R1, BRCA1-deficient and PARPi-resistant) after 6 days of treatment with increasing doses of PARPi (Olaparib) in the absence or presence of ATRi (VE-821, 1.0 μM). n=3 replicates; error bars represent s.d. (D) The indicated cell lines were treated with ATRi (VE-821), PARPi (Olaparib), or ATRi and PARPi for 7 days. Fractions of cells undergoing cell death (Propidium iodide and Annexin V positive) were measured.

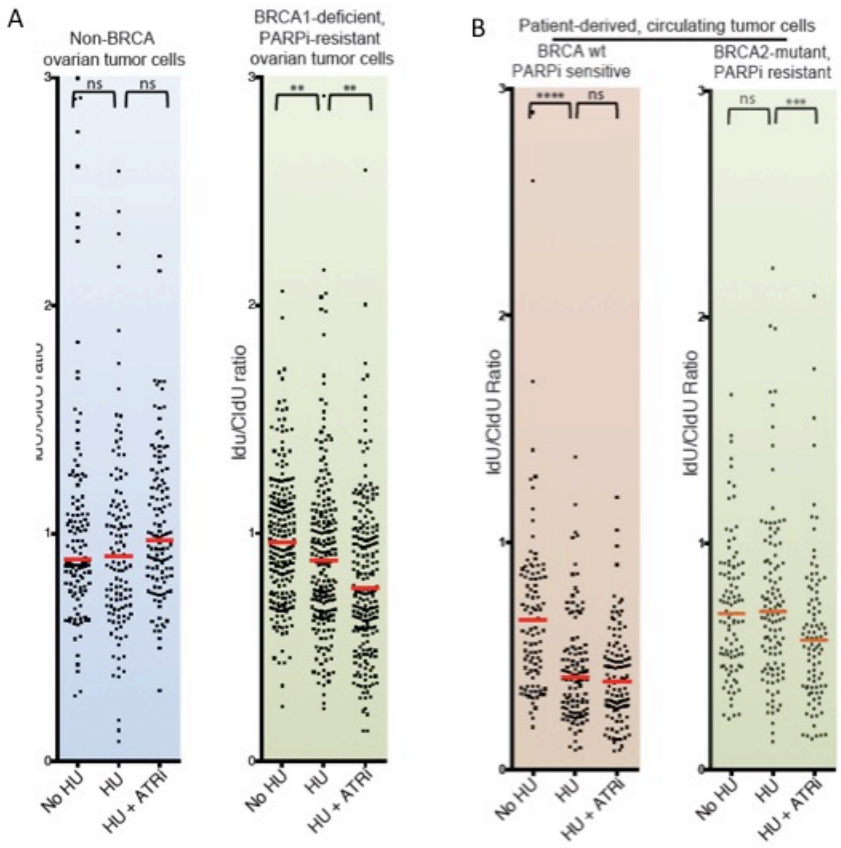

Figure 18: ATRi reactivates fork degradation in tumor cells derived from PARPi-resistant BRCA-deficient patients. (A) DNA fiber analysis of tumor cells from a BRCA1-deficient, PARPi resistant ovarian cancer patient and a non-BRCA ovarian cancer patient, with each fiber plotted individually after no treatment, treatment with HU (4 mM), or treatment with HU and ATRi (VE-821, 10 μM). Red bars represent the median IdU/CldU ratios. n=125 and n=215 for non-BRCA tumor cells and BRCA1-deficient, PARPi-resistant tumor cells, respectively; experiments were performed in duplicate. Significance was determined by Mann-Whitney test, **p<0.01. (B) DNA fiber analysis of circulating tumor cells (CTCs) from a BRCA2-deficient breast cancer patient and a non-BRCA breast cancer patient as in A, with each fiber plotted individually after no treatment, treatment with HU (4 mM), or treatment with HU and ATRi (VE-821, 10 μM). Significance was determined by Mann-Whitney test, **** p<0.0001.