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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

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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 (Figure 2A and B, Figure 3). 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:

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: In my previous two annual reports, 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, BRCA1-deficient line using Rad51 and PALB2 as markers.
    b) Test BRCA1-restoration: In my previous annual reports, I showed 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: In my previous annual reports, I showed that loss of 53BP1, Rif1, or DNA-PK does not contribute to PARP inhibitor resistance in these lines. I have further tested known mechanisms of resistance by candidate approach, including loss of Rev7, which has recently been reported to results in PARPi resistance (Figure 4).
2) Identify novel mechanisms of HR restoration:

a) Confirmation of targets with siRNAs: 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, surprisingly, all cell lines have some level of Rad51 loading following damage, even in the absence of BRCA1 (Figure 2 and 3). This residual Rad51 loading 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 (Figure 5A-C). 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 (Figure 5D-F). All cell lines, both BRCA1-expressing cells and BRCA1-deficient lines, require PALB2 to load Rad51 (Figure 5A-F). 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 5G and H). 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 5I-J). 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 6A and B).
b) Confirmation of targets with inhibitors: I sought to disrupt the remaining HR in these BRCA1-deficient cells using inhibitor treatments. ATR is a critical regulator of homologous recombination repair; however, the precise role of ATR in HR regulation is unclear. In my previous annual report, I demonstrated that ATRi, but not ATMi, can significantly decrease Rad51 loading downstream of BRCA1 through affecting the localization of PALB2-BRCA2. I demonstrated that treatment with ATRi resulted in decreased Rad51 loading and increased PARPi sensitivity in a 53BP1 knockout, BRCA1-deficient cell line, which has been previously shown to be resistant to PARPi treatment. Treatment with ATRi also resulted in decreased PALB2 and BRCA2 loading, and thus Ras51 loading, in the derived BRCA1-deficient PARPi resistant lines as well. Taken together, these data demonstrate that BRCA1-independent Rad51 loading requires PALB2 and BRCA2 localization, and this PALB2-BRCA2 loading can be disrupted by ATRi treatment.

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: In my previous annual report, I showed that PARP inhibitor resistance is not due to efflux pump up-regulation or mutations in PARP that prevent PARP inhibitor from binding to its target.
   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. 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 (Figure 7A). 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 of 1.12 and 1.05, respectively, even in the absence of BRCA1 (Figure 7B and C). This suggests that fork stabilization in the absence of BRCA1 may contribute to PARPi resistance.

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 has a modest effect on resistant cell viability following PARPi treatment, presumably because the stalled forks of these cells are protected, so knockdown of Mre11 has little effect on fork protection. On the other hand, knockdown of Mre11 results in increase resistance to PARPi in the parental UWB1 cells (Figure 7D and E). This data suggests that preventing nascent DNA degradation after fork stalling can result in 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 two resistant cell lines with ATRi results in decreased DNA length, again signifying loss of fork protection. Co-treatment with Mirin, an Mre11 inhibitor, prevents this shortening, demonstrating this degradation following ATRi treatment is Mre11 dependent (Figure 7F).

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. In my previous annual report, I demonstrated that combined treatment with ATRi and PARPi results in synthetic lethality specifically in BRCA1 deficient cell lines. Treatment with a low dose of ATRi results in increased sensitivity to PARPi in BRCA1-deficient cells, both the parental UWB1 cells and the derived resistant cells. Additionally, these BRCA1-deficient cells do not simply fail to proliferate but actually undergo cell death shown by Annexin V staining.

To test if this synthetic lethality of BRCA1-deficient cells in combination with PARPi is unique to ATRi, I performed a targeted drug screen using other DNA damage kinase inhibitors as well as other DNA damaging agents (Figure 8). ATRi combined with PARPi results in increased cell death in all cells, but has a stronger effect in BRCA1-deficient cells, as I found and reported in my previous annual summary. Inhibition of other PI3K-like kinases, ATM or DNA-PK, does not have the same synthetic lethal effect when used at the same concentrations even though they have similar IC50s to their targets. This demonstrates that reliance on ATR pathway for PARPi resistance is pathway specific; there is not an equal dependence on other damage response pathways. Use of another kinase inhibitor, Wee1, which is currently being tested in clinical trials in combination with PARPi, also sensitizes cells to PARPi. However, the effect of Wee1i is not strong in all resistant cell lines, and does not resensitize SYr13. Furthermore, Wee1i alone is quite toxic to the BRCA1 expressing cell line. This suggests that ATRi may be therapeutically better at targeting BRCA1-deficient cells over Wee1i. Finally, all DNA damaging agents (Mitomycin C (MMC), aphidicolin (APH), and Camptothecin (CPT)) seem to kill all cell lines tested similarly, suggesting these drugs may not specifically effect BRCA1-deficient or PARPi resistant cells, although the doses tested may be too high to see a significant difference.

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 9A). This suggests that MEK signaling is required for resistant cells to proliferate. I confirmed this effect using three separate MEK inhibitors (Figure 9B). 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 40 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 9C). 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. But unlikely treatment with ATRi, simultaneous treatment with MEKi and PARPi resulted in increased Rad51 foci in SYr13 cells (Figure 9D). 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 9E). 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:

In my previous annual report, I sought to determine if combining PARPi and ATRi to target BRCA1-deficient cancers could be extending to other BRCA1-deficient cancer types, including breast cancer. In my previous annual report I demonstrated that the combination of PARPi and ATRi could be used to selectively kill BRCA1 deficient human breast cancer cells which were already resistant to PARPi (HCC1937) as well as derived PARPi resistant BRCA1-deficient mouse ovarian cancer cells (BR5-Res1). This suggests that the combination of ATRi and PARPi can be used to target BRCA1-deficient cancer cells, both ovarian and breast cancer.

Key Research Accomplishments:

- 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 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 loading can be prevented after stalled fork by ATRi treatment
- 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.
- All PARPi resistant cells are sensitive to MEKi treatment
- MEKi does not inhibit DNA damage repair, as does ATRi, 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.

Taken together, the results of the experiments contained in this annual 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. 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: Nothing to report

Inventions, Patents, and Licenses: Nothing to report

Reportable outcomes: Nothing to report

Other Achievements: Nothing to report

Training and Professional Development:

- I have been meeting with my mentor bi-weekly and meeting with my co-mentor monthly to discuss results and future experiments.
- I have been presenting at and attending lab meeting, joint journal club, and departmental seminar.
- I have attended MGH Grand Rounds of several talks related to breast cancer.
- I attended the AACR meeting “Advances in Ovarian Cancer” in Orlando, Fl.

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Appendices:

Supporting data:

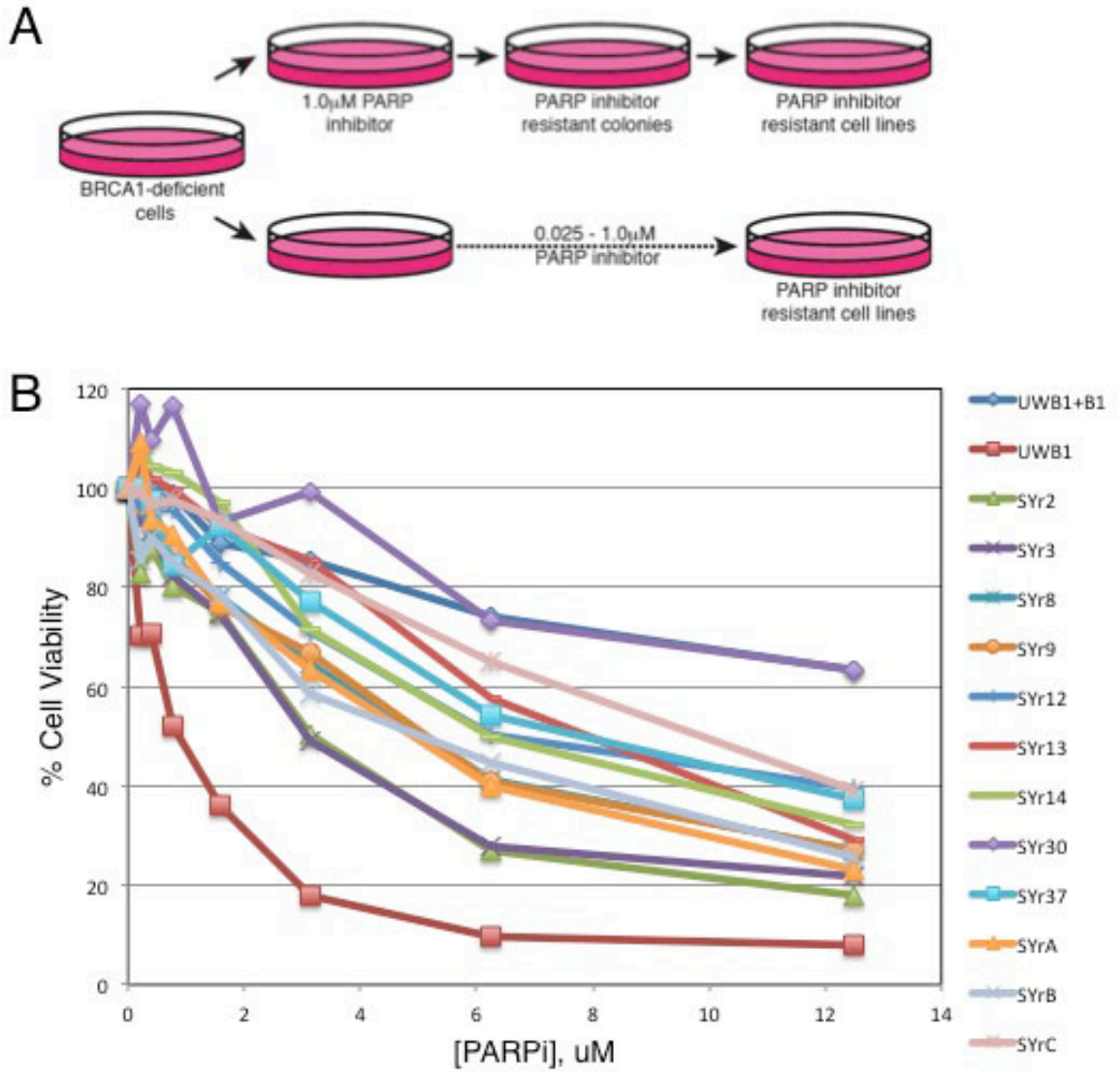

Figure 1: Twelve PARP inhibitor (PARPi) resistant cells lines were derived from a parental, BRCA1-deficient cell line. (A) Schematic of two methods used to derive PARP inhibitor resistant cell lines. In one method (top), parental cells were treated with a high dose of PARPi (1.0uM) such that most cells died. A few surviving cells grew to form resistant colonies after 45 days of treatment. These clones were selected and developed into nine resistant cell lines. In a second method (bottom), parental cells were treated with a sublethal dose of PARPi (0.025uM) and gradually increased after several passages to 1.0uM to allow cells to gradually adapt to the PARPi treatment. (B) Cell viability curve using CellTiter-Glo viability assay with increasing doses of PARPi. The BRCA1-deficient UWB1 cell line is most sensitive to PARPi, while the isogenic BRCA1-rescue line, UWB1+B1, is resistant to PARPi, demonstrating the sensitivity is due to BRCA1 status. The derived BRCA1-deficient, PARPi resistant lines (SYr) are all more resistant to PARPi than the parental line from which they are derived.

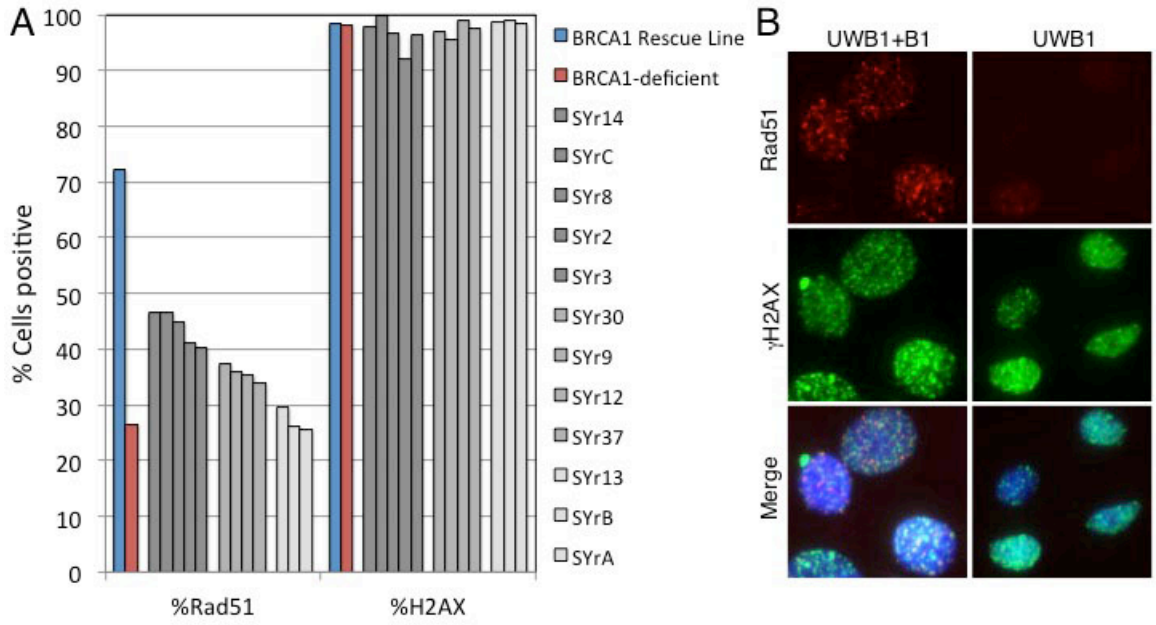

Figure 2: Derived BRCA1-deficient, PARPi resistant cell lines retain some level of homologous recombination (HR) ability, even in the absence of BRCA1. (A) Quantification of Rad51 loading, a marker of HR ability, and γH2AX, a marker of DNA damage, following treatment of cells with 10Gy IR. Positive cells contain greater than eight foci. The BRCA1-rescue line loads Rad51 efficiently, while the BRCA1-deficient line exhibits a defect in Rad51 loading. The derived PARPi resistant lines have varying levels of HR ability, some of which have restored HR ability, while others have levels comparable to the parental cell line. All cells show a similar level of γH2AX, demonstrating cells all received similar levels of damage. (B) Representative images of the BRCA1 rescue cell line (UWB1+B1) or BRCA1-deficient cell line (UWB1) stained by immunofluorescence for γH2AX as a marker for DNA damage and Rad51 as a marker of HR.

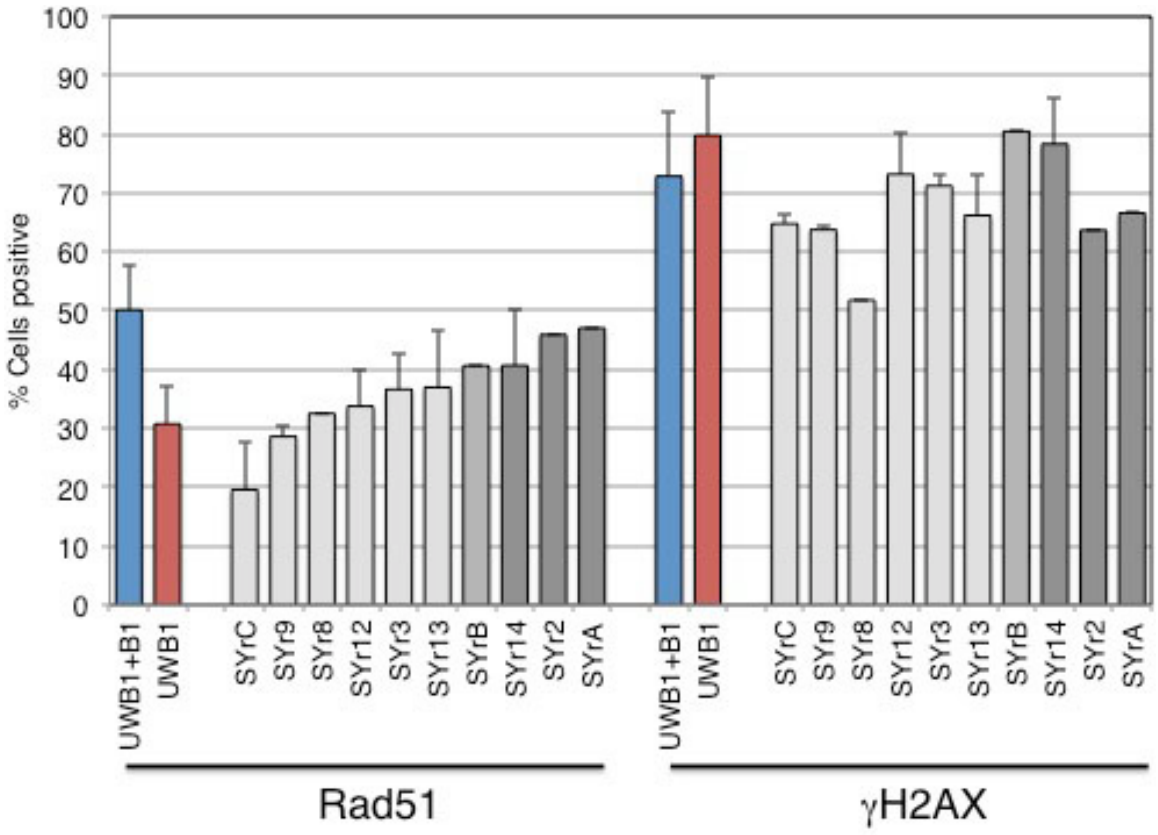

Figure 3: Derived BRCA1-deficient, PARPi resistant cell lines retain some level of homologous recombination (HR) ability, even in the absence of BRCA1. (A) Quantification of Rad51 loading, a marker of HR ability, and γH2AX, a marker of DNA damage, following treatment of cells with 10uM PARPi for 24hr. Positive cells contain greater than eight foci. The BRCA1-rescue line loads Rad51 efficiently, while the BRCA1-deficient line exhibits a defect in Rad51 loading, as when treated with 10Gy IR. The derived PARPi resistant lines have varying levels of HR ability, some of which have restored HR ability, while others have levels lower than the parental cell line. All cells show γH2AX staining, suggesting all were damaged by PARPi treatment. (B) Representative images of the BRCA1 rescue cell line (UWB1+B1) or BRCA1-deficient cell line (UWB1) stained by immunofluorescence for γH2AX as a marker for DNA damage and Rad51 as a marker of HR.

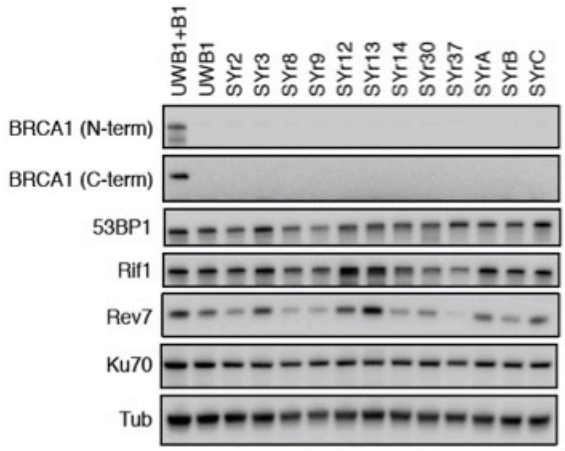

Figure 4: Loss of 53BP1, Rif1, Rev7 or lack of NHEJ are not responsible for PARPi resistance in the derived cell lines. Extracts prepared from BRCA1-rescue line (U+B1), the parental line (U), and the derived resistant lines (SYr) were subjected to Western blotting using antibodies directed to 53BP1, Rif1, Rev7, or Ku70. 53BP1 and Rif1 are known to be in a complex together and loss of either protein is known to result in resistance to PARPi treatment. No derived resistant cell lines showed loss of 53BP1 or Rif1, indicating loss of this complex is not responsible for PARPi resistance in these cell lines. Similarly, loss of Rev7 or Ku70 has also been shown to result in PARPi resistance. Several lines have reduced levels of Rev7 relative to the parental cell from which they are derived. Decreased levels of Rev7 may contribute to PARPi resistance in those lines.

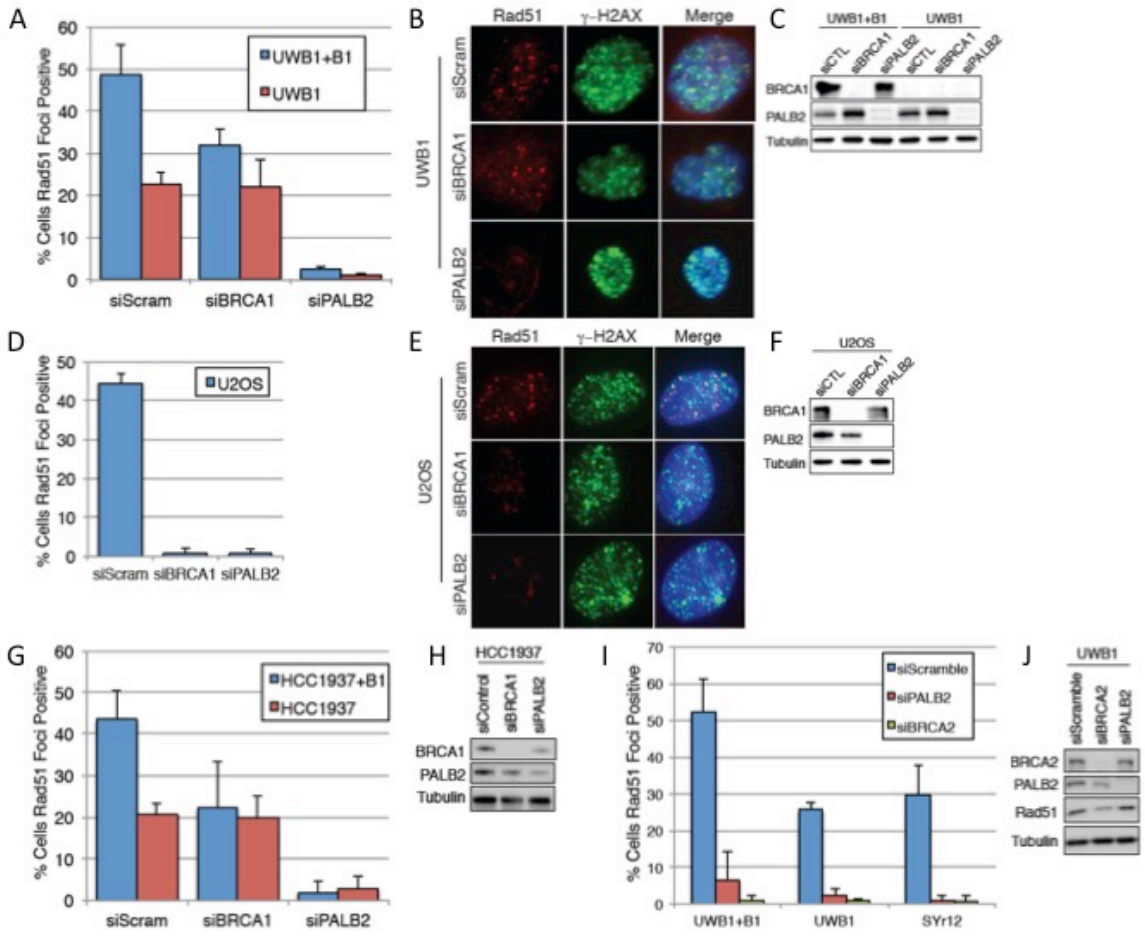

Figure 5: BRCA1 is partially bypassed in BRCA1-deficient cancer cell lines to allow PALB2/BRCA2-dependent Rad51 loading. (A and B) (A) Quantification of Rad51 positive staining and (B) representative images of staining of parental UWB1 cells or rescue UWB1+B1 cells after BRCA1 or PALB2 were knocked down for 72 hours, then treated with 10Gy IR, and stained for Rad51 or γ–H2AX after 4 hours. (C) Western blot showing knockdown of BRCA1 and PALB2 in UWB1+B1 and UWB1 cells. (D and E) (D) Quantification of Rad51 positive staining and (E) representative images of staining of U2OS cells after BRCA1 or PALB2 were knocked down for 72 hours, then treated with 10Gy IR, and stained for Rad51 or γ–H2AX after 4 hours. (F) Western blot showing knockdown of BRCA1 and PALB2 in U2OS cells. (G) Quantification of Rad51 positive staining of human breast cancer HCC1937+B1 or HCC1937 cells after BRCA1 or PALB2 were knocked down for 72 hours, then treated with 10Gy IR, and stained for Rad51 or γ–H2AX after 4 hours. (H) Western blot showing knockdown of BRCA1 and PALB2 in HCC1937 cells. (I) Quantification of Rad51 positive staining of parental UWB1 cells or rescue UWB1+B1 cells after BRCA2 or PALB2 were knocked down for 72 hours, then treated with 10μM PARPi, and stained for Rad51 or γ–H2AX after 24 hours. (J) Western blot showing knockdown of BRCA2 and PALB2 in UWB1+B1 and UWB1 cells.

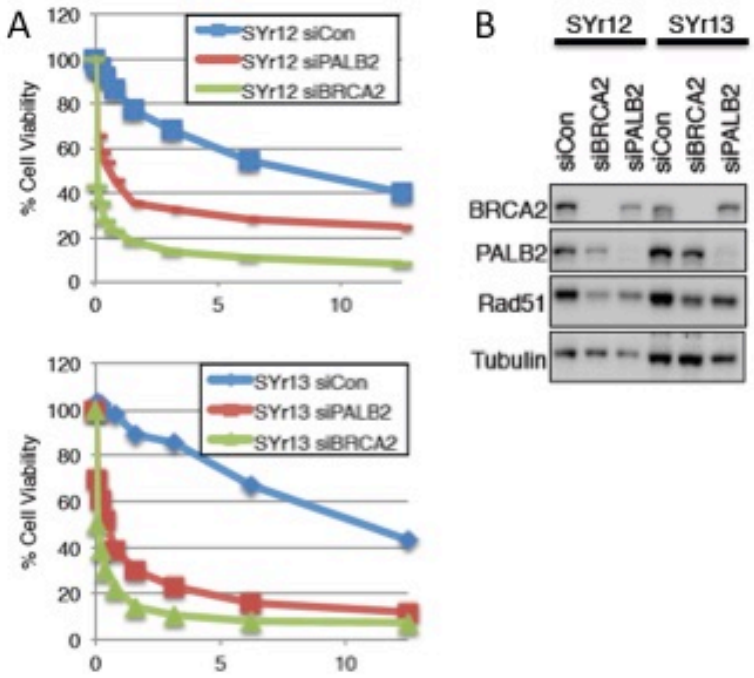

Figure 6: PALB2 and BRCA2 are essential for PARPi resistance in SYr12 and SYr13, two derived PARPi resistant lines. (A) Cells were treated with siRNA against a scramble sequence (siCon), PALB2 (siPALB2), or BRCA2 (siBRCA2). 24 hours later, cells were plated into 96 well plate, and treated with increasing concentrations of PARPi. 6 days later, cell viability was read using Cell Titer Glo. Cell viability is plotted relative to vehicle (DMSO) alone. (B) Western blot showing efficient knockdown of BRCA2 and PALB2 in SYr12 or SYr13 cell lines.

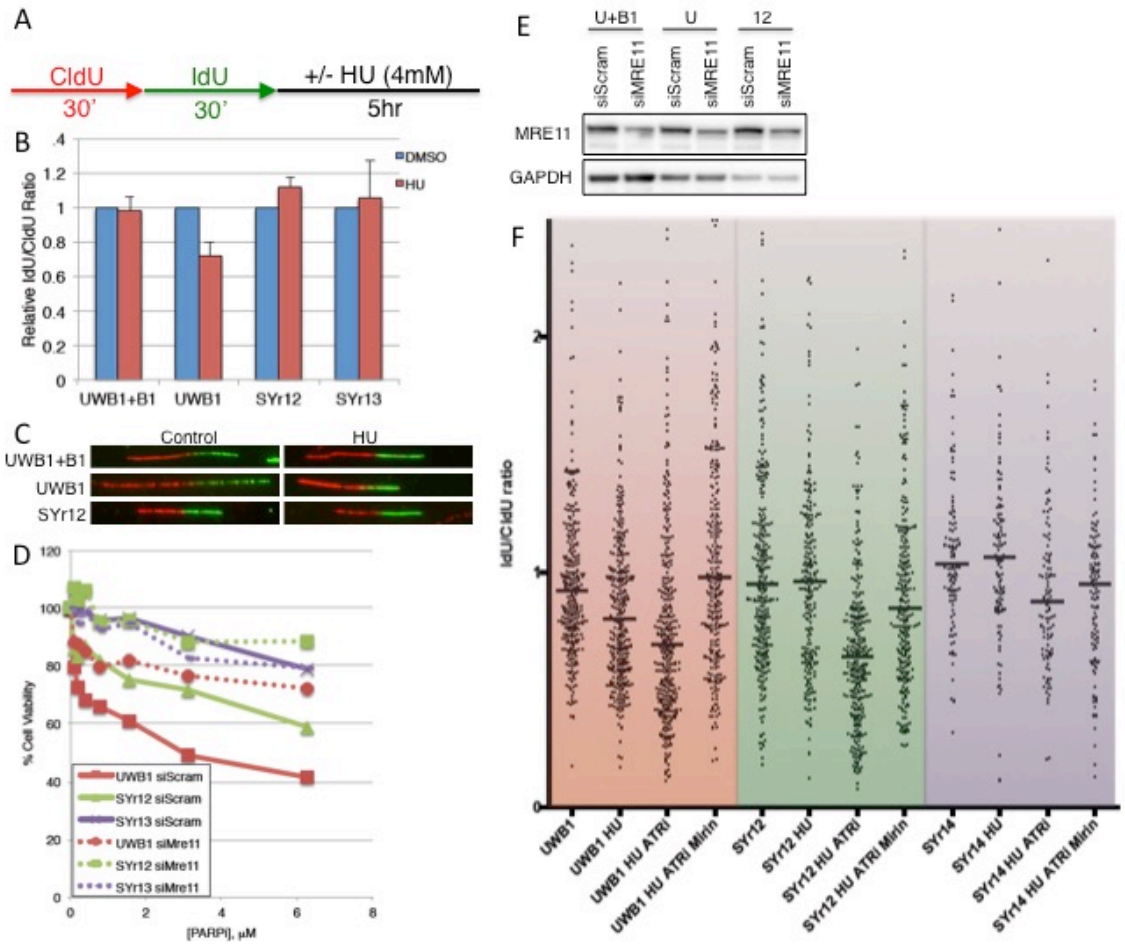

Figure 7: Fork protection, which is necessary for PARPi resistance, is restored in derived PARPi resistant, BRCA1-deficient cell lines. (A) Schematic of DNA fiber assay used to test fork stability. Cells are incubated with a thymidine analog, CldU, for 30min followed by a second analog IdU for 30 min, and the spread or treated with HU for 5hrs to stall forks. A stable fork will not undergo degradation, while an unprotected fork will shorten due to MRE11-mediated exonuclease activity. A stable fork will have an IdU/CldU ratio of 1 while a fork that has undergone shortening will have an IdU/CldU ratio of less than 1. (B) IdU/CldU ratio before and after HU treatment for the rescue UWB1+B1, parental UWB1, and resistant SYr12 and SYr13 cell lines. (C) Representative images of fibers +/- HU in the rescue UWB1+B1, parental UWB1, and resistant SYr12 cell lines. (D) Parental UWB1 cells or resistant SYr12 or SYr13 cells, were treated with siRNA against Mre11. After 24 hours cells were plated in 96 well plates, and treated with increasing doses of PARPi 24 hours later. After 6 days, cell viability was assessed using Cell Titer Glo. (E) Western blot confirming knockdown of Mre11. (F) IdU/CldU ratio of individual forks before and after HU treatment +/- ATRi +/- Mirin, an MRE11 inhibitor, in the parental UWB1, and resistant SYr12 and SYr14 cell lines.

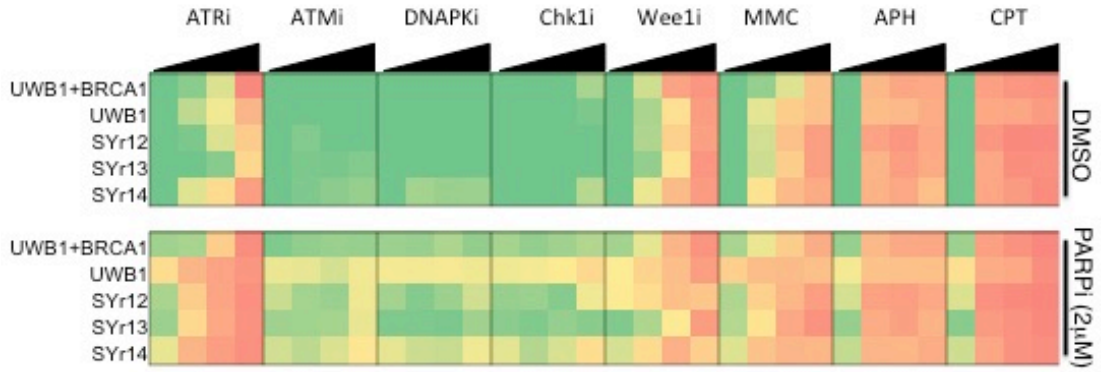

Figure 8: A small scale DNA damage drug screen reveals ATRi can resensitize PARPi resistant cells to low dose PARPi treatment. ATRi also further sensitized BRCA1-deficient UWB1 cells to PARPi treatment., while cells which express BRCA1 (UWB1+BRCA1) remain relatively resistant to PARPi, ATRi, or the combination. Treatment with other kinase inhibitors (ATMi, DNA-PKi, Chk1i, or Wee1i) did not have this same synergistic and specific effect. Combination treatment with damaging agents (MMC, APH, CPT) increased cell death when combined with PARPi, but not specifically in BRCA1-deficient cells.

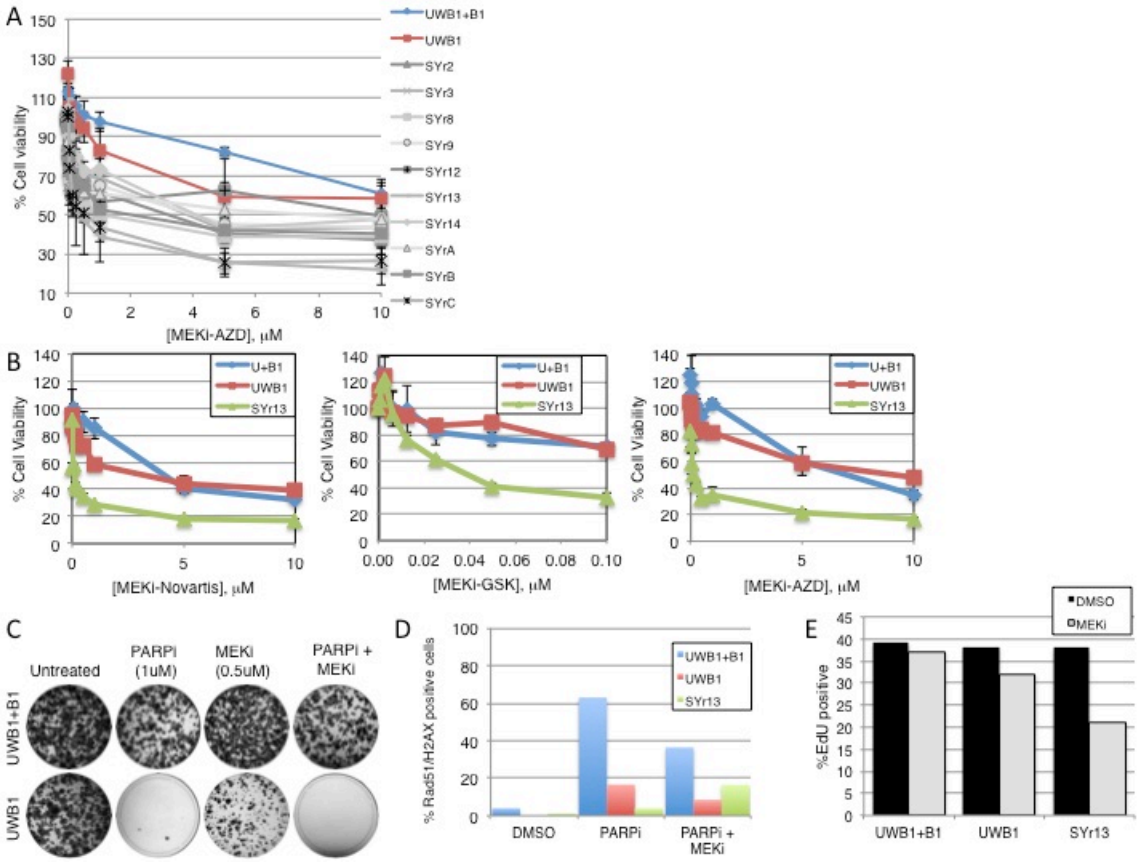

Figure 9: PARPi resistant cells require MEK activity for resistance to arise and to maintain proliferation. (A) Cell viability assay following increasing doses of MEKi demonstrate that all resistant cells have increased sensitivity to MEKi treatment. (B) Treatment with three unique MEKi compounds result 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.