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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. 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 (*1*). PARP inhibitors (PARPi) have been tested with promising results for the treatment of BRCA1-associated cancers (*2-4*). BRCA1 is essential for error-free repair of DNA double strand breaks via homologous recombination (HR) (*5*), while PARPs are thought to primarily function in repair of single stranded DNA breaks especially through activation of base excision repair (BER) (*6*). A synthetic lethal phenotype occurs when BRCA1-deficiency (HR deficiency) is combined with PARPi (BER defect) (*3, 7-9*). 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). 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.

Body:

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: Homologous recombination ability had previously been measured using Rad51 localization to IR induced foci as a marker for HR (Figure 2A and B). However, HR ability following IR may not reflect the ability of a cell to respond to replication induced DNA damage with HR, such as that which would occur following PARP inhibitor treatment. In order to understand if BRCA1-deficient and PARP inhibitor resistant cells are able to use HR to repair PARP inhibitor induced DNA damage, cells were treated with PARP inhibitor for 24 hours, and stained and for Rad51 as a marker of HR and γH2AX as a marker of DNA damage. As was seen following IR, HR activity was highest in the BRCA1-rescue line and lowest in the BRCA1-deficient, parental cell line. 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 (Figure 3). The level of HR in the resistant lines following IR or PARPi treatment were not exactly the same, and will need to be repeated to confirm which lines have significantly restored HR. HR restoration was further confirmed using PALB2 localization as a marker. PALB2 is known to be upstream of Rad51 loading, but down stream of BRCA1, as a marker. Because staining for PALB2 foci is technically difficult due to high background levels, cells were damaged using a

UV laser to damage DNA in a specific stripe pattern. The laser induced damage stripe was marked using γH2AX antibody. The colocalization of γH2AX and PALB2 was measured for the BRCA1-deficient parental cells, the BRCA1-rescue cells, and the PARP inhibitor resistant lines. As expected, PALB2 is efficiently loaded in the BRCA1-rescue line, while the BRCA1-deficient lines loaded PALB2 less efficiently. One PARP inhibitor resistant line showed an increase in PALB2 loading relative to the parental line (Figure 4A and B), indicating this line may have partially restored HR.

b) Test BRCA1-restoration: Because restoration of BRCA2 has been shown to result in cisplatin/PARPi resistance in BRCA2-deficient cancer cell lines (*10-12*) and BRCA1 restoration has been shown to occur in response to cisplatin treatment (*13*), 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 (Figure 5A) and immunofluorescence (Data not shown). Only the BRCA1-rescue cell line expresses BRCA1 by Western blot using an N-terminally directed antibody which would recognize the fragment of the BRCA1 expression before the premature stop codon (Figure 5A). Furthermore, all cell lines were sequenced to ensure retention of the point mutation (a single base pair deletion resulting in a premature stop codon) in BRCA1 which renders these cells BRCA1-deficient (Figure 5B). Genomic DNA was prepared from the parental line, rescue line, and all resistant lines. Primers were designed against BRCA1 which allowed for sequencing of the point mutation as well as a down stream region of BRCA1, deletion of which had previously been indicated as a potential mechanism to restore the BRCA1 open reading frame. 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 (Figure 5B). No cell lines had deletion of the region that had been previously described to restore BRCA1 open reading frame, and thus BRCA1 protein expression. Finally, cells were treated with an siRNA targeting the C-terminal BRCT domain, which would knockdown any functionally restored BRCA1, even a shorter, partially restored BRCA1 transcript. Only the BRCA1-rescue line, but not the parental or the PARPi resistant line (Figure 5C) were affected by siBRCA, indicating only the rescue line depends on BRCA1 for HR. 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 (*14, 15*) or decreased non-homologous end joining repair, as measured by DNA-PK activity (*16*), 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. Western blot analysis of lysates prepared from the BRCA1-rescue line, the parental BRCA1-deficient line, and all BRCA1-deficient, PARP inhibitor resistant lines was performed before and after DNA damage, including PARP inhibitor treatment.

Expression of 53BP1, Rif1, a protein known to complex with 53bp1 (*17, 18*), or DNA-PK was not altered between cell lines. Additionally, loss phosphorylation of DNA-PK, a known autophosphorylation site, or loss of phosphorylation of RPA at serine 4 and serine 8, a marker of active DNA-PK, was not lost relative to the BRCA1-rescue line (Figure 6). This data suggests that loss of 53BP1 or Rif1 and loss DNA-PK activity does not contribute to PARP inhibitor resistance in these resistant lines. Furthermore, foci formation of 53BP1 after DNA damage (IR) does not change in the resistant lines relative to the rescue line, suggesting that recruitment of 53BP1 is not altered (Data not shown) and cannot account for PARP inhibitor resistance in these cell lines.

2) Identify novel mechanisms of HR restoration:
    a) Confirmation of targets with siRNAs, inhibitors, and overexpression in parental cell line: 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, and 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 7A, B and C).

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 (*19*), 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, except SYrC, after MMS treatment, demonstrating that the PARP inhibitor is retained in the nucleus and that the inhibitor is still able to bind PARP (Figure 8). 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 but one is not due to

mutations in PARP that prevent PARP inhibitor from binding to its target, since the inhibitor has the expected inhibitory effect. Resistance in SYrC may be due to efflux pump up-regulation, PARP mutations, or compensation by other PARP family members that results in high levels of PAR chain formation, despite treatment with PARP inhibitor. To this end, I will test the levels of efflux pump expression using qPCR and western blotting. I will also test if SYrC regains sensitivity to PARP inhibitor when treated with efflux pump inhibitors in combination with PARP inhibitor treatment. Finally, to test if SYrC is resistant due to overexpression of other PARP family members, I will perform Western blotting and knockdown other PARP inhibitor family members using siRNAs.

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 10Gy 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 a mutations in PARP1 or increased activity of redundant PARP family members.

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 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.)

Conclusion:

I have derived BRCA1-deficient, PARP inhibitor resistant lines from a parental PARP inhibitor sensitive line. 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. This HR repair occurs following IR or PARP inhibitor treatment. BRCA1 open reading frame restoration cannot account for this HR, as the parental line and all PARP inhibitor resistant lines do not express BRCA or any BRCA1 fragments by Western blot. Furthermore, sequencing of BRCA1 shows retention of the point mutation that results in loss of BRCA1 protein in the parental and all resistant lines. Finally knockdown of BRCA1 only affected Rad51 loading in the rescue line.

I also ruled out two known mechanisms of HR restoration in the absence of BRCA1, loss of 53BP1 and loss of DNA-PK activity. 53BP1 levels are not altered, and foci formation assays reveal localization has not changed. Similarly, pDNA-PK and pRPA levels are the same or higher than the BRCA1-expressing rescue line, suggesting functional DNA-PK activity. These results show that other novel mechanisms of HR restoration may be responsible for PARP inhibitor resistance.

To begin to identify these novel mechanism of HR restoration in the PARP inhibitor resistant cell lines, I used targeted siRNAs in a candidate approach to selectively knockdown known components of HR pathway. This analysis revealed 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. I have begun to do further experiments using siRNA and specific inhibitors to understand how BRCA2, PALB2, and MRG15 are able to load Rad51 in the absence of BRCA1.

I have further eliminated other trivial explanations for PARP inhibitor resistance that are independent of homologous recombination dependency in all cell lines but one. Because treatment of PARP inhibitor is able to prevent heightened levels of PARylation following DNA damage, several explanations for PARP inhibitor resistance can be excluded, including increased efflux pump activity, increased expression of PARP, compensation by other PARP family members, and mutations in PARP that prevent the inhibitor from binding. I will confirm these results using qPCR and Western blotting to measure efflux pump levels and PARP family member levels.

The results of this years statement of work completion suggest that HR is at least partially functional in many of these BRCA1-deficient cell lines, and that this HR occurs following PARP inhibitor treatment, suggesting HR function may be relevant to PARP inhibitor tolerance and resistance. I was able to rule out other known mechanisms of PARP inhibitor resistance, such as restoration of BRCA1, an increase in efflux pump activity, and loss of 53BP1 or DNA-PK activity. Ultimately, the proposed experiments for year two will provide insight into how HR function occurs in the absence of BRCA1, how BRCA1-mutated breast cancers acquire resistance to PARP inhibitor treatment, and ultimately how recurrent tumors can be treated.

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Supporting Data:

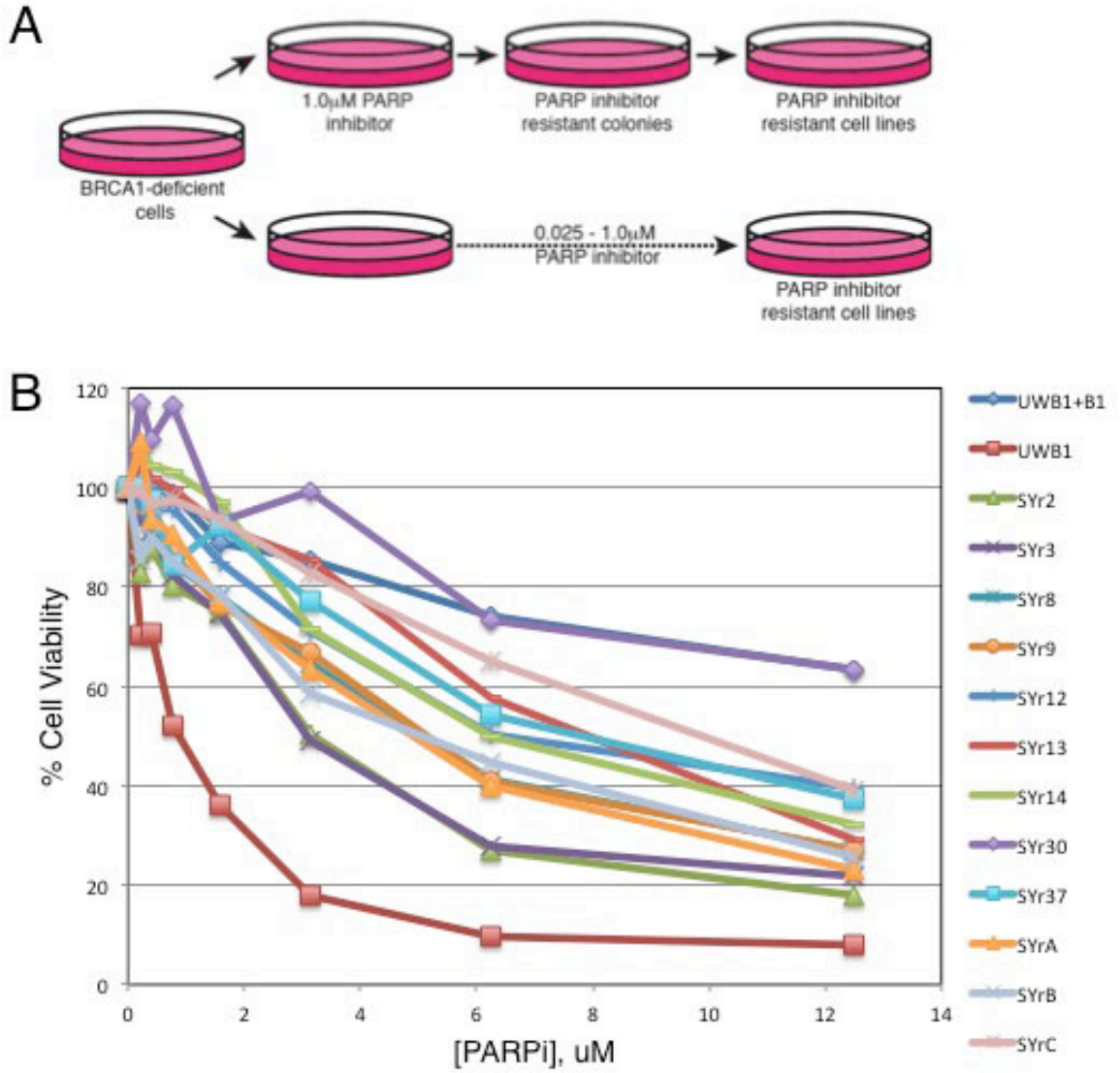

Figure 1: Twelve PARP inhibitor (PARPi) resistant cells lines were derived from a parental, BRCA1-deficient cell line. (A) Schematic of two methods used to derive PARP inhibitor resistant cell lines. In one method (top), parental cells were treated with a high dose of PARPi (1.0uM) such that most cells 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.

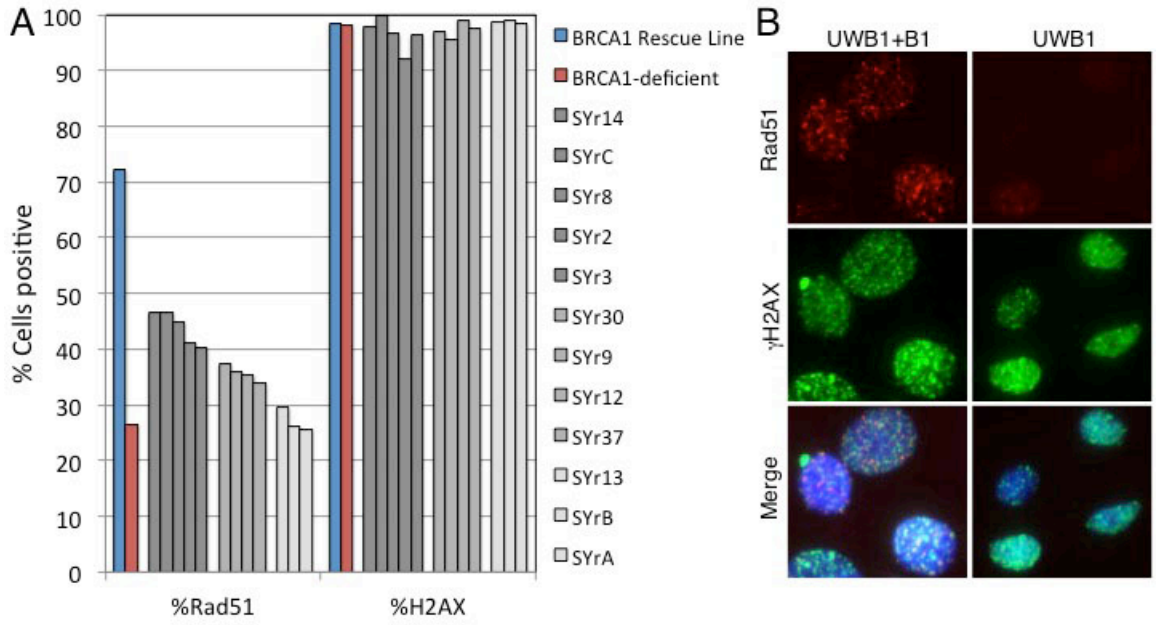

Figure 2: Derived BRCA1-deficient, PARPi resistant cell lines retain some level of homologous recombination (HR) ability, even in the absence of BRCA1. (A) Quantification of Rad51 loading, a marker of HR ability, and γH2AX, a marker of DNA damage, following treatment of cells with 10Gy IR. Positive cells 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.

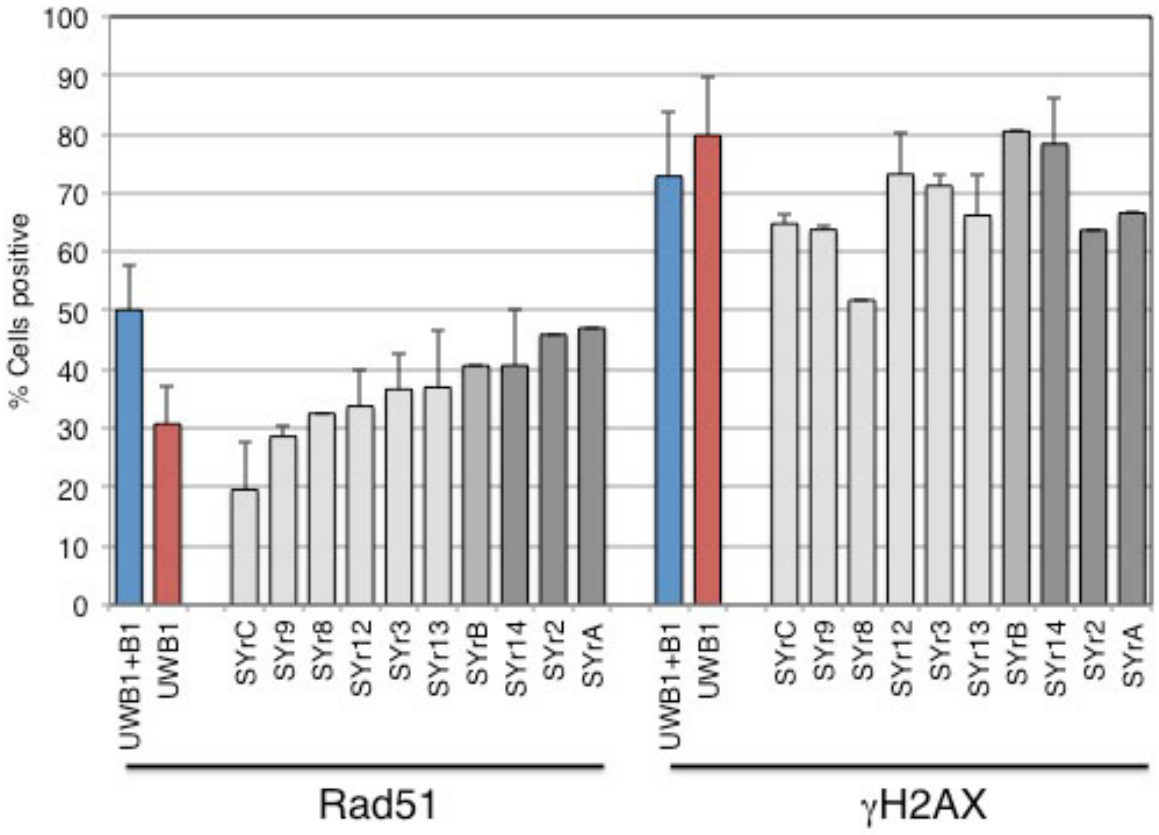

Figure 3: Derived BRCA1-deficient, PARPi resistant cell lines retain some level of homologous recombination (HR) ability, even in the absence of BRCA1. (A) Quantification of Rad51 loading, a marker of HR ability, and γH2AX, a marker of DNA damage, following treatment of cells with 10uM PARPi for 24hr. 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.

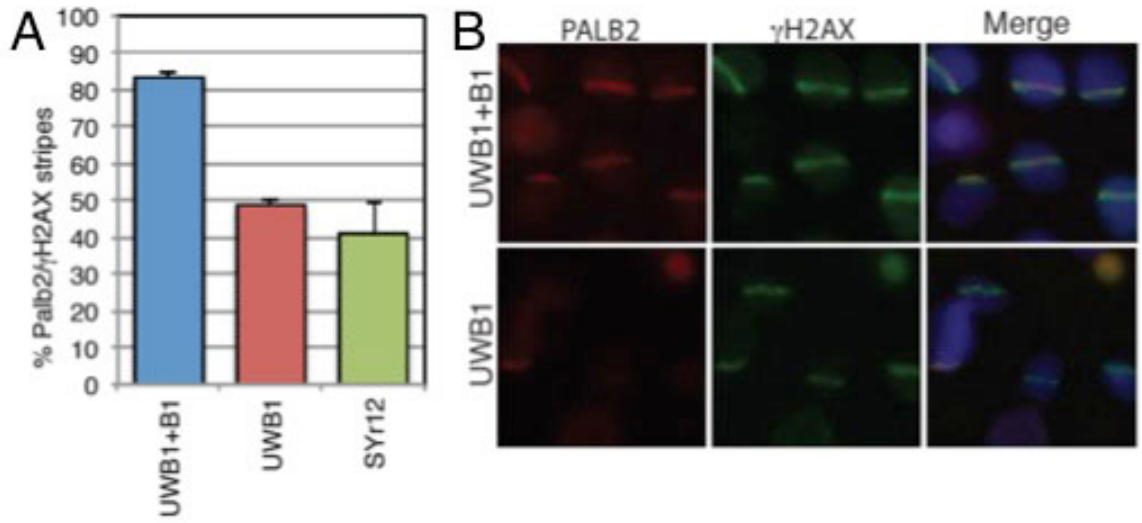

Figure 4: Derived BRCA1-deficient, PARPi resistant cell lines retain some level of homologous recombination (HR) ability, even in the absence of BRCA1, as marked by PALB2 loading. (A) Quantification of PALB2 loading, a marker of HR ability, relative γH2AX, a marker of DNA damage, following treatment of cells with a UV laser stripe after pre-incubation of cells with BrdU for 24hr. The BRCA1-rescue line loads PALB2 efficiently, while the BRCA1-deficient line exhibits a defect in PALB2 loading. The derived PARPi resistant line has a defect in PALB2 loading similar to the parental cell line. γH2AX staining was used to locate and quantify damage stripes. (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 PALB2 as a marker of HR following DNA damaging laser stripes.

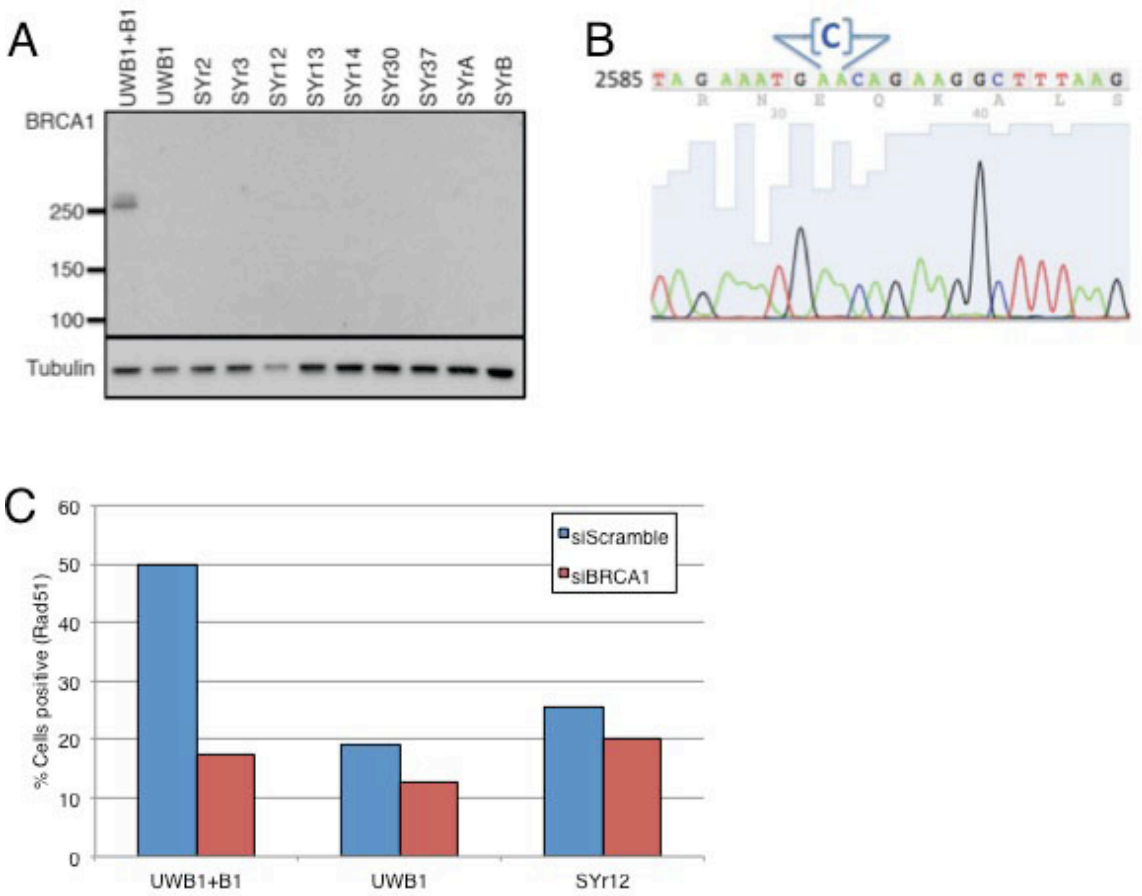

Figure 5: BRCA1 restoration is not the underlying mechanism of PARPi resistance in the derived PARPi resistant cell lines. (A) Western blot analysis of extracts prepared from the BRCA1-rescue line (UWB1+B1), parental line (UWB1), and the derived PARPi resistant lines (SYr) reveals BRCA1 protein is only expressed in the BRCA1-rescue line. An antibody directed to the N-terminal portion of the protein which would be expressed even if the open reading frame was re-established was used. (B) An example of the sequencing data completed for each of the resistant lines. The single base pair deletion that results in a premature stop codon and lack of BRCA1 protein expression is retained in all BRCA1-deficient cell lines, including the PARPi resistant lines. (C) The BRCA1-rescue line, the parental line, and a PARPi resistant line (SYr12) were treated with siBRCA1 targeted to the far C-term BRCT domain which is essential for BRCA1 function, followed by treatment with 10uM PARPi for 24hr to induce DNA damage. Only the rescue line, which expresses exogenous wildtype BRCA1, shows a reduced level of Rad51 loading, indicating an defect in HR after BRCA1 knockdown.

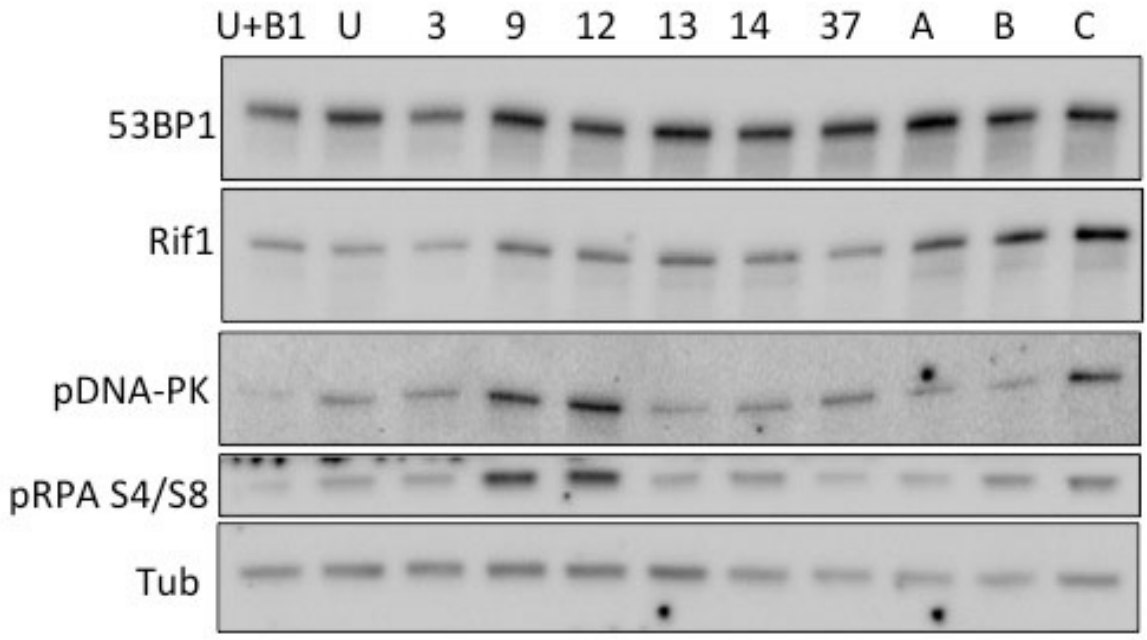

Figure 6: Loss of 53BP1 or lack of NHEJ are not responsible for PARPi resistance in the derived cell lines. Extracts prepared from BRCA1-rescue line (U+B1), the parental line (U), and the derived resistant lines (SYr) were subjected to Western blotting using antibodies directed to 53BP1, Rif1, pDNA-PK, or pRPA S4/S8. 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. Levels pDNA-PK and pRPA are indicative of the activity level of DNA-PK, an essential component of NHEJ. Loss of DNA-PK activity, and thus a decrease in NHEJ, has been shown to reduce the toxicity of PARPi in the absence of BRCA1. While two lines, SYr9 and SYr12, show an increase in pDNA-PK and pRPA, indicating an increase in NHEJ, no cell line showed a loss of DNA-PK activity, or NHEJ ability, relative to the BRCA1-rescue line.

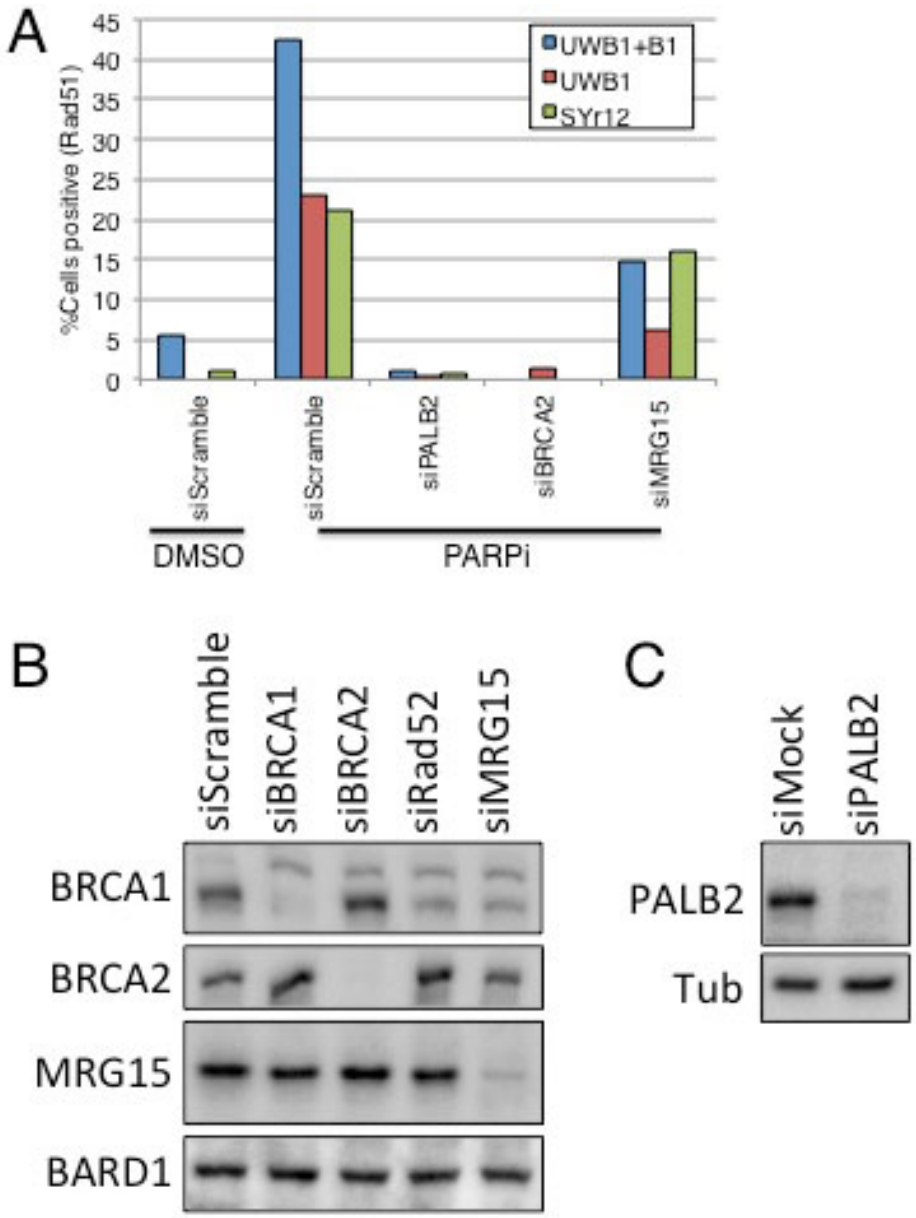

Figure 7: The defective HR that is retained by the parental BRCA1-deficient cell line (UWB1) and a PARPi resistant line (SYr12) depends on BRCA2, PALB2, and MRG15. (A) HR ability, as measure by immunofluorescence staining of Rad51 loading, was quantified for the BRCA1-rescue line (UWB1+B1), the parental line, and a PARPi resistant line after knockdown of components of the HR pathway known to be downstream of BRCA1, namely, PALB2 and BRCA2, or independent of BRCA1, namely MRG15. Knockdown of PALB2, BRCA2, and MRG15 reduced Rad51 loading relative to knockdown with a scramble siRNA sequence in all cell lines indicating these components play a role in HR, even in the absence of BRCA1. (B) Western blotting of UWB1 extracts after treatment with the indicated siRNA shows specific knockdown of BRCA2 and MRG15. BARD1 serves as a loading control. (C) Western blotting of UWB1 extracts after treatment with the indicated siRNA shows specific knockdown of PALB2. Tubulin serves as a loading control.

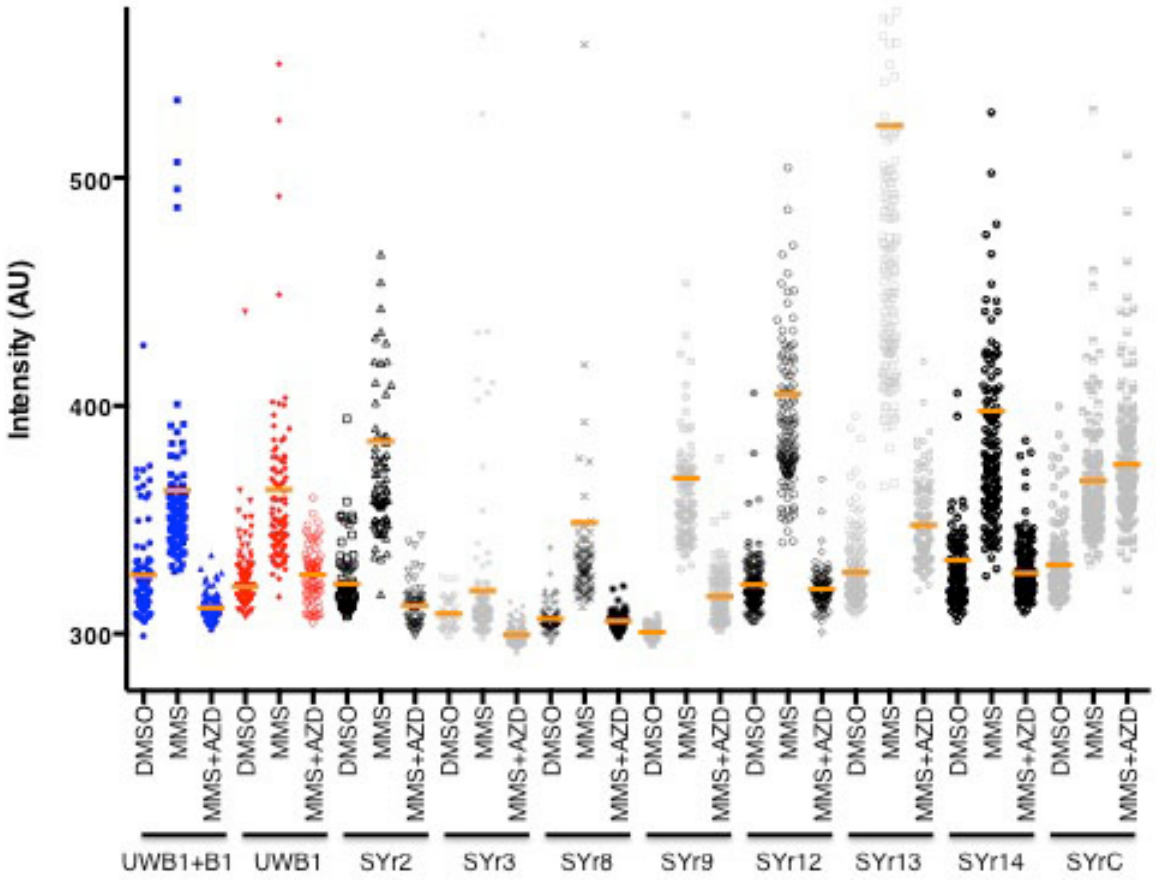

Figure 8: An increase in efflux pump activity, overexpression or compensation by another PARP family member, or mutation in PARP that prevent PARPi from binding its target cannot account for the PARPi resistance of all but one derived cell line. The BRCA1-rescue line (UWB1+B1), the parental line (UWB1), and all derived PARPi resistant lines (SYr) were treated with DMSO, MMS to induce poly (ADP-robose) (PAR) chains, or MMS + PARPi. The level of PAR chain formation in each individual nuclei was quantified using immunofluorescence using an antibody again PAR. The median PAR intensity for each line after each treatment is indicated with an orange bar. Treatment with PARPi was able to suppress the level of PAR chain formation produced following MMS treatment in all cell lines, except SYrC, in which PARPi treatment had no effect.