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Loss of E2F7 confers resistance to poly-ADP-ribose polymerase (PARP) inhibitors in BRCA2-deficient cells

Kristen E. Clements[1], Tanay Thakar[1], Claudia M. Nicolae[1], Xinwen Liang[2], Hong-Gang Wang[2,3] and George-Lucian Moldovan[1,*]

[1]Department of Biochemistry and Molecular Biology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, USA, [2]Department of Pediatrics, The Pennsylvania State University College of Medicine, Hershey, PA 17033, USA and [3]Department of Pharmacology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, USA

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ABSTRACT

BRCA proteins are essential for homologous recombination (HR) DNA repair, and their germline or somatic inactivation is frequently observed in human tumors. Understanding the molecular mechanisms underlying the response of BRCA-deficient tumors to chemotherapy is paramount for developing improved personalized cancer therapies. While PARP inhibitors have been recently approved for treatment of BRCA-mutant breast and ovarian cancers, not all patients respond to this therapy, and resistance to these novel drugs remains a major clinical problem. Several mechanisms of chemoresistance in BRCA2-deficient cells have been identified. Rather than restoring normal recombination, these mechanisms result in stabilization of stalled replication forks, which can be subjected to degradation in BRCA2-mutated cells. Here, we show that the transcriptional repressor E2F7 modulates the chemosensitivity of BRCA2-deficient cells. We found that BRCA2-deficient cells are less sensitive to PARP inhibitor and cisplatin treatment after E2F7 depletion. Moreover, we show that the mechanism underlying this activity involves increased expression of RAD51, a target for E2F7-mediated transcriptional repression, which enhances both HR DNA repair, and replication fork stability in BRCA2-deficient cells. Our work describes a new mechanism of therapy resistance in BRCA2-deficient cells, and identifies E2F7 as a putative biomarker for tumor response to PARP inhibitor therapy.

INTRODUCTION

Improved precision therapy is essential for increasing the survival of cancer patients. PARP1 is a member of the poly-ADP-ribosyltransferase family, catalyzing formation of poly-ADP-ribose chains on target protein substrates (1). PARP1 has diverse substrates and regulates essential cellular processes including DNA replication, DNA repair and transcription. Recently, PARP1 inhibitors have emerged as novel cancer therapeutics, based on groundbreaking work showing that PARP1 is essential for cellular viability in cells with compromised homologous recombination (HR) DNA repair (2–4). Inability to perform PARP1-mediated repair of single stranded DNA breaks leads to replication fork collapse and double strand break formation. In the absence of efficient HR, this results in cell death, underlying the synthetic lethality interactions between PARP1 and HR genes. HR deficiency conferred by germline or somatic mutations in BRCA1, BRCA2, RAD51C, Fanconi Anemia genes and other members of the pathway is observed in a large proportion of adult cancers, including breast, ovarian, pancreatic, prostate and others (5,6). Several PARP inhibitors (PARPi) (olaparib, rucaparib and niraparib) have been approved by the U.S. Food and Drug Administration (FDA) for single agent treatment of BRCA-deficient ovarian and breast cancers.

More recently, it was shown that PARPi also act through a newly described activity known as PARP-trapping which results in crosslinking of the PARP1 protein to DNA (7). These DNA–protein crosslinks can block DNA replication and transcription, making these agents also effective against HR-proficient tumors. Through this mechanism, PARPi act as efficient chemo- and radio-sensitizers (8–10). Thus, use of PARPi is likely to significantly expand in the near future to many different cancers, regardless of HR (BRCA) status. Indeed, there are currently more than 20 active clinical trials

*To whom correspondence should be addressed. Tel: +1 717 531 3610; Fax: +1 717 531 7072; Email: gmoldovan@pennstatehealth.psu.edu

involving PARPi, in tumors ranging from breast to bone to brain, in both children and adults (11).

BRCA2 is an essential HR protein, which catalyzes the loading of RAD51 molecules onto resected DNA at double strand breaks (12). RAD51 loading is required for the subsequent strand invasion and Holliday junction formation steps of the recombination process. BRCA2 was also shown to be required for genomic stability under replication stress conditions (13,14). Upon replication fork stalling at sites of DNA lesions, potentially including trapped PARP1, a set of DNA translocases including ZRANB3, HLTF and SMARCAL1 reverse the fork by annealing the nascent strands of the two newly formed chromatids, forming a structure effectively resembling a one-ended double-stranded DNA break (DSB). This structure needs to be stabilized by BRCA2-mediated loading of RAD51, which protects it against degradation by the MRE11 nuclease (15–17).

While PARPi have excellent anti-tumor activity, they often show only limited efficacy in the clinic. For example, even though olaparib treatment tripled 12-month progression free survival in BRCA2 deficient patients, still only 65% of the olaparib-treated group reached this milestone, indicating that resistance is an important clinical problem (18). Previously described mechanisms of resistance include genetic reversion of BRCA1 and BRCA2 mutations, as well as rewiring of the DNA damage response to restore HR in BRCA1-deficient cells by suppressing recombination-inhibitory proteins such as 53BP1 or RIF1 (19–22). In contrast, in BRCA2-deficient cells, known mechanisms of resistance do not restore HR, but instead act by protecting stalled replication forks against nucleolytic degradation (20,23).

E2F7 is a member of the E2F transcription factor family. Together with E2F8, they are considered atypical E2F family members as they mediate transcription repression rather than activation (24,25). E2F7 levels are induced by DNA damage (26). E2F7 transcriptional repression targets include replication proteins such as CDC6 and MCM2—thereby its induction by DNA damage contributes to G1/S-arrest (27,28). However, among its targets for repression are also HR proteins, including RAD51 and BRCA1 (28). Here, we show that E2F7 promotes sensitivity to PARPi, and its depletion can rescue chemosensitivity of BRCA2-deficient cells by promoting both HR and fork stability.

MATERIALS AND METHODS

Cell culture and protein techniques

Human HeLa, HCC1395, 293T and U2OS cells were grown in Dulbecco's modified Eagle's medium (DMEM), while SH-SY5Y were grown in DMEM/F12 (1:1). Media was supplemented with 10% fetal calf serum. For *BRCA2* gene knockout, the commercially available BRCA2 CRISPR/Cas9 KO plasmid was used (Santa Cruz Biotechnology sc-400700). Transfected cells were FACS-sorted into 96-well plates using a BD FACSAria II instrument. Resulting colonies were screened by western blot. Cell extracts, chromatin fractionation and western blot experiments were performed as previously described (29–31). Antibodies used for Western blot were: BRCA2 (Bethyl A303-434A), GAPDH (Santa Cruz Biotechnology sc-47724), RAD51 (Santa Cruz Biotechnology sc-8349), Vinculin (Santa Cruz Biotechnology sc-73614), RAD52 (Santa Cruz Biotechnology sc-365341). For gene knockdown, cells were transfected with Stealth siRNA (Life Tech) using Lipofectamine RNAiMAX reagent. For co-depletion experiments, control (non-targeting) siRNA was added to the targeting siRNA in the single knockdown samples to equalize total siRNA levels. The siRNA targeting sequences used were: E2F7 #1: GGACGATGCATTTACAGATTCTCTA; E2F7 #2: GACTATGGGTAACAGGGCATCTATA; E2F7 #3: AAACAAAGGTACGACGCCTCTATGA (used for E2F7 knockdown unless otherwise mentioned); BRCA2: GAGAGGCCTGTAAAGACCTTGAATT; RAD51: CCATACTGTGGAGGCTGTTGCCTAT; RAD52: GGCCAATGAGATGTTTGGTTACAAT.

Immunofluorescence

Immunofluorescence experiments were performed as previously described (31) with small modifications. Briefly, cells were fixed with 4% paraformaldehyde for 10 min, followed by three washes with phosphate-buffered saline (PBS). Cells were then permeabilized with 0.2% Triton X-100 for 10 min. After two washes with PBS, slides were blocked with 3% bovine serum albumin (BSA) in PBS for 10 min, followed by incubation with the primary antibody diluted in 3% BSA in PBS, for 2 h at room temperature. After three washes with PBS, the secondary antibody (Alexa Fluor 488 from Invitrogen) was added for 1 h. Slides were mounted with DAPI-containing Vectashield mounting medium (Vector Labs). Antibodies used for immunofluorescence were: γH2AX (Bethyl A300-081A) and 53BP1 (Bethyl A300-272A).

Functional assays

To measure drug sensitivity, cells were seeded in 96-well plates and incubated with indicated drug concentrations for 3 days. Cellular viability was assayed using the CellTiterGlo reagent (Promega G7572) according to the manufacturer's instructions. Apoptosis Annexin V measurements were performed using the FITC Annexin V kit (Biolegend 640906) according to manufacturer's instructions, using a BD FACSCanto 10 flow cytometer. The neutral comet assay was performed using the CometAssay kit (Trevigen 4250-050). HR and non-homologous end joining (NHEJ) assays were performed as previously described (32).

Quantification of gene expression by real-time quantitative PCR (RT-qPCR)

Total mRNA was purified using TRIzol reagent (Life Tech) according to the manufacturer's instructions. To generate cDNA, 1 μg RNA was subjected to reverse transcription using the RevertAid Reverse Transcriptase Kit (Thermo Fisher Scientific) with oligo dT primers. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with PerfeCTa SYBR Green SuperMix (Quanta), using a CFX Connect Real-Time Cycler (BioRad). The cDNA of GAPDH gene was used for normalization. Primers used were: E2F7

for: GGAAAGGCAACAGCAAACTCT; E2F7 rev: TGGGAGAGCACCAAGAGTAGAAGA; RAD51 for: TGCTTATTGTAGACAGTGCCACC; RAD51 rev: CACCAAACTCATCAGCGAGTC; GAPDH for: AAATCAAGTGGGGCGATGCTG; GAPDH rev: GCAGAGATGATGACCCTTTTG

DNA fiber assay

HeLa cells, pretreated with the indicated siRNA oligonucleotides, were incubated with 100 μM IdU for 30 min. Cells were washed with PBS and incubated with 4 mM hydroxyurea (HU) (with or without 50 μM Mirin as indicated) for 3 h. Following removal of HU media and a PBS wash, fresh media containing 100 μM CldU was added for another 30 min. Next, cells were harvested and DNA fibers were obtained using the FiberPrep kit (Genomic Vision) according to the manufacturer's instructions. DNA fibers were stretched on glass slides using the FiberComb Molecular Combing instrument (Genomic Vision). Slides were incubated with primary antibodies (Abcam 6326 for detecting CIdU; BD 347580 for detecting IdU; Millipore Sigma MAB3034 for detecting DNA), washed with PBS, and incubated with Cy3, Cy5 or BV480-coupled secondary antibodies (Abcam 6946, Abcam 6565, BD Biosciences 564879). Following mounting, slides were imaged using a Leica SP5 confocal microscope. At least 200 tracts were quantified for each sample.

Statistical analyses

With the exception of the DNA fiber data, the statistical analysis performed was the TTEST (two-tailed, unequal variance). For the DNA fiber data, the Mann–Whitney test was performed. Statistical significance is indicated for each graph (ns = not significant, for $P > 0.05$; * for $P < 0.05$; ** for $P < 0.01$; *** for $P < 0.001$; **** for $P < 0.0001$).

RESULTS

Depletion of E2F7 reverses the PARPi sensitivity of BRCA2-deficient cells

In order to investigate mechanisms of PARPi resistance in BRCA-deficient cells, we first created BRCA2-knockout (labeled BRCA2^{KO}) HeLa cell lines using the CRISPR/Cas9 technology. BRCA2^{KO} cells lack any detectable full-length BRCA2 protein expression by western blot (Figure 1A) and have similar sensitivity to olaparib as parental cells treated with BRCA2-targeting siRNA (Figure 1B). To test its involvement in mediating PARPi-sensitivity in BRCA2-deficient cells, we knocked down E2F7 in BRCA2^{KO} cells and measured olaparib sensitivity. E2F7 knockdown resulted in significant rescue of olaparib sensitivity of these cells (Figure 1C and Supplementary Figure S1). In order to rule out a non-specific effect caused by the CRISPR editing, we also knocked-down E2F7 in parental HeLa cells at the same time with BRCA2 depletion. E2F7 knockdown with three different siRNA oligonucleotides could rescue olaparib sensitivity of BRCA2-knockdown cells (Figure 1D). In contrast, E2F7 knockdown by itself (without BRCA2 co-depletion) did not show a significant effect on olaparib sensitivity (Supplementary Figure S2A and B). All three siRNA oligonucleotides efficiently knock-down E2F7 expression as shown by qPCR-based detection of E2F7 mRNA levels (Figure 1E). Further confirming the specificity of the phenotype, Western blot experiments showed that BRCA2 is equally depleted in cells treated with BRCA2 siRNA alone or in combination with E2F7 siRNA (Figure 1F). Moreover, cellular proliferation and cell-cycle distribution of BRCA2-knockdown cells were not affected by E2F7 co-depletion (Supplementary Figure S2C and D).

BRCA2-deficient cells are sensitive to many genotoxic agents used in cancer therapy, including cisplatin (33). Interestingly, it was recently suggested that PARPi and cisplatin have similar mechanisms of action (34). In order to test the specificity of this novel E2F7-mediated response, we treated E2F7/BRCA2-co-depleted HeLa cells with cisplatin. E2F7 knockdown was able to rescue the cisplatin sensitivity of BRCA2-depleted cells (Figure 1G), indicating that E2F7 has a broad impact on the chemosensitivity of BRCA2-deficient cells. Altogether, these findings show that E2F7 is a novel regulator of DNA damage sensitivity of BRCA2-deficient cells.

We next investigated if the suppression of olaparib sensitivity by loss of E2F7 is restricted to HeLa cells, or is a general phenomenon. BRCA2 depletion in HCC1395 breast cancer cells resulted in olaparib sensitivity, which was rescued by E2F7 knockdown (Figure 2A). Similarly, olaparib sensitivity of BRCA2-depleted SH-SY5Y neuroblastoma cells was rescued by E2F7 depletion (Figure 2B). Olaparib treatment is known to induce apoptosis in BRCA2-deficient cells (35). E2F7 depletion did not only rescue cellular viability of olaparib-treated BRCA2-deficient cells, but also reduced olaparib-induced apoptosis (Figure 2C and Supplementary Figure S2E). Altogether, these results indicate that E2F7 is a novel factor controlling PARPi resistance in BRCA2-deficient cells.

As PARPi are ultimately DNA damaging drugs that act by inducing accumulation of double stranded DNA breaks in BRCA-deficient cells, we next investigated if E2F7 depletion suppresses olaparib-induced genomic instability in these cells. First, we employed the neutral comet assay to detect double stranded DNA breaks in BRCA2-knockout HeLa cells. We found that E2F7 depletion reduces comet tail length, indicating a suppression of DNA damage accumulation (Figure 2D). In line with this, immunofluorescence experiments with these cells showed a reduction in olaparib-induced formation of γH2AX and 53BP1 foci, generally considered markers of double stranded breaks (Supplementary Figure S3). These findings indicate that E2F7 depletion protects against olaparib-induced genomic instability, suggesting that E2F7 suppresses DNA repair in BRCA2-deficient cells.

E2F7 regulates RAD51 levels to promote olaparib resistance of BRCA2-deficient cells

Previously, RAD51 was identified as a possible target for transcriptional repression by E2F7 (28). RAD51 is an essential HR factor, which is loaded by BRCA2 on resected ssDNA and catalyzes the strand invasion step in the re-

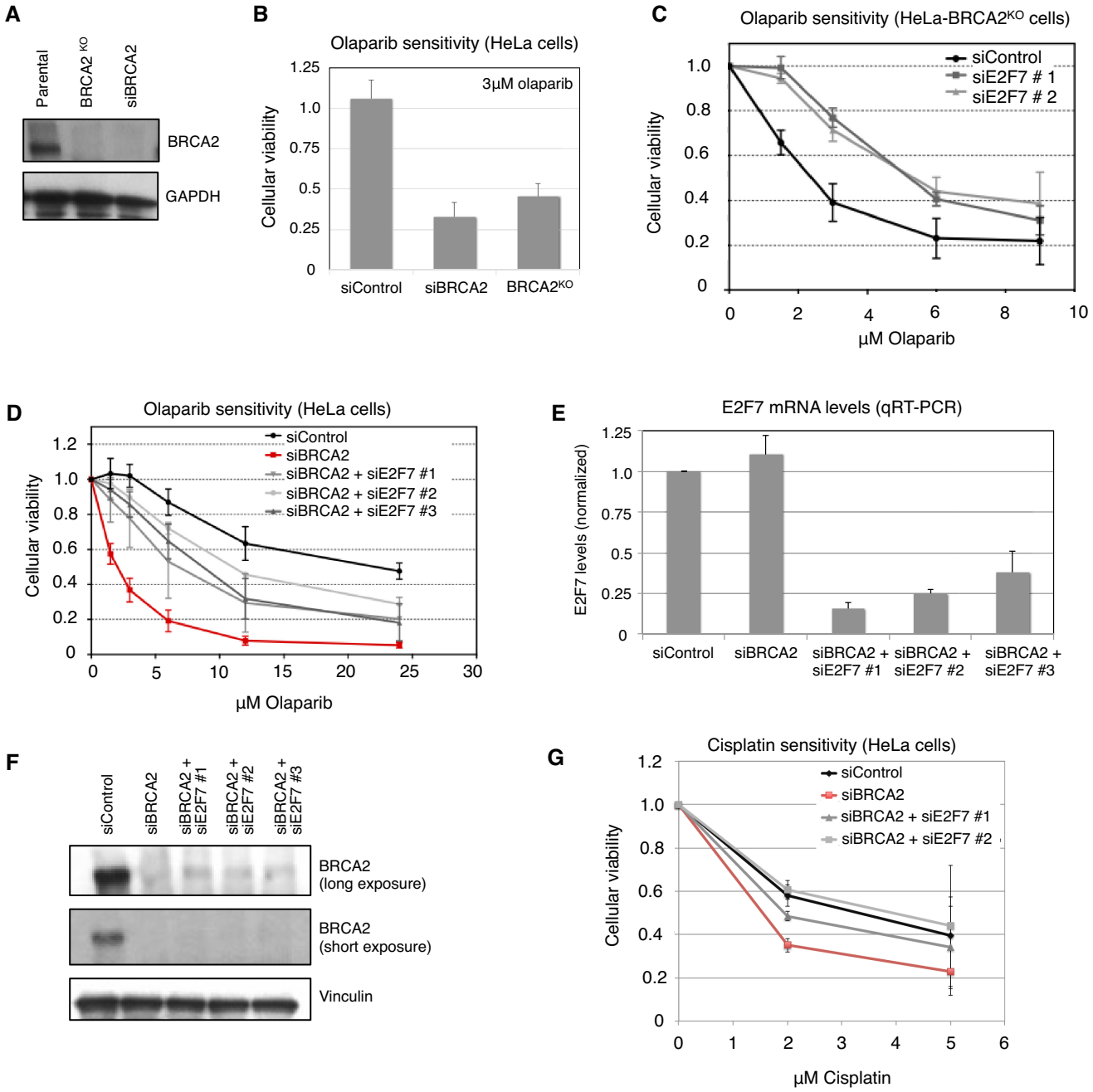

Figure 1. E2F7 depletion in BRCA2-deficient HeLa cells results in olaparib resistance. (**A**) Western blot showing loss of BRCA2 expression in HeLa cells with CRISPR/Cas9-mediated BRCA2 knockout. (**B**) $BRCA2^{KO}$ HeLa cells have similar olaparib sensitivity as HeLa cells treated with BRCA2 siRNA. Results are shown as normalized to control (no drug treatment) for each sample. The average of three experiments, with standard deviations as error bars, is shown. (**C** and **D**) E2F7 knockdown rescues the olaparib sensitivity of BRCA2-knockout (C) and BRCA2-knockdown (D) HeLa cells. The average of three experiments, with standard deviations as error bars, is shown. Rescue of a second BRCA2-knockout clone, independently obtained, is shown in Supplementary Figure S1. (**E**) Quantitative RT-PCR experiment showing efficient E2F7 knockdown by the siRNA oligonucleotides employed. HeLa cells were treated with the indicated siRNA oligonucleotides then incubated with 10 μM olaparib for 24 h before harvesting. The average of three experiments, with standard deviations as error bars, is shown. (**F**) Western blot showing that BRCA2 is efficiently knocked down by the siRNA oligonucleotide employed singly or in combination with E2F7 siRNA oligonucleotides. HeLa cells were treated with the indicated siRNA oligonucleotides then incubated with 10 μM olaparib for 24 h before harvesting. (**G**) E2F7 knockdown rescues the cisplatin sensitivity of BRCA2-knockdown HeLa cells. The average of three experiments, with standard deviations as error bars, is shown.

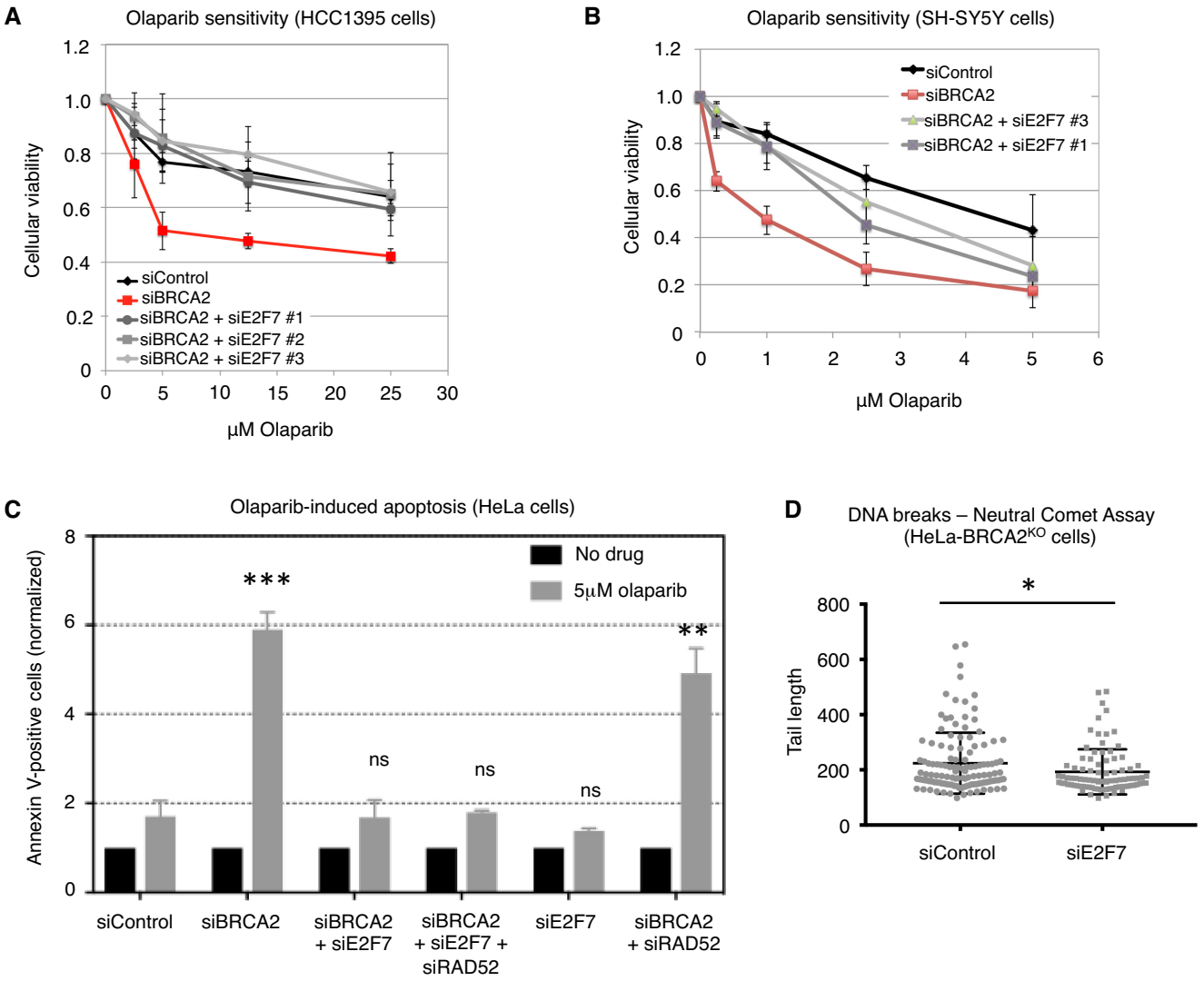

Figure 2. Impact of E2F7 on cellular viability and genomic stability of BRCA2-deficient cells. (**A** and **B**) E2F7 knockdown rescues the olaparib sensitivity of BRCA2-knockdown HCC1395 breast cancer (A) and SH-SY5Y neuroblastoma (B) cells. The average of three experiments, with standard deviations as error bars, is shown. (**C**) Quantification of AnnexinV-positive cells indicating that E2F7 knockdown suppresses olaparib-induced apoptosis of BRCA2-depleted cells. HeLa cells were treated with the indicated siRNA oligonucleotides then incubated with 5 μM olaparib for 3 days. Results are presented as normalized to control (no drug treatment condition) for each knockdown sample. The average of three independent experiments, with standard deviations as error bars, is shown. Asterisks indicate statistical significance compared to siControl olaparib-treated condition. (**D**) Neutral comet assay showing that E2F7 depletion reduces olaparib-induced genomic instability of BRCA2-knockout HeLa cells. Cells were treated with 10 μM olaparib for 24 h. At least 90 comet tails, pooled from three independent experiments, were quantified for each sample. The means with standard deviations are shown. The asterisk indicates statistical significance.

combination process (12). Thus, we decided to investigate the levels of RAD51 in E2F7-depleted HeLa and U2OS cells treated with olaparib. E2F7 knockdown, by itself or in combination with BRCA2 depletion, resulted in increased RAD51 expression at both the mRNA and protein levels (Figure 3A–C). Moreover, E2F7 knockdown resulted in an increase in the levels of chromatin-bound RAD51 upon olaparib treatment (Figure 3D and E). While RAD52 has been previously described to function as a RAD51 loader in BRCA2-deficient cells, its downregulation did not affect the E2F7 depletion-mediated rescue of olaparib sensitivity in BRCA2-knockout cells (Figure 2C and Supplementary Figure S4) suggesting that RAD52 does not play a major role in loading RAD51 upon E2F7 depletion in BRCA2-deficient cells (36). Altogether, these results suggest that E2F7 depletion may promote olaparib resistance in BRCA2-deficient cells by increasing RAD51 levels.

Recently, Chk1 was shown to phosphorylate E2F7 thereby restricting its activity in response to DNA damage (37). This raises the possibility that in BRCA2-deficient cells, Chk1 activity, by inhibiting E2F7, promotes PARPi resistance. Indeed, we found that treatment of BRCA2-knockout HeLa cells with a Chk1 inhibitor significantly sensitized them to olaparib (Figure 3F). This effect was dependent at least in part on E2F7, as its knockdown alleviated this sensitization.

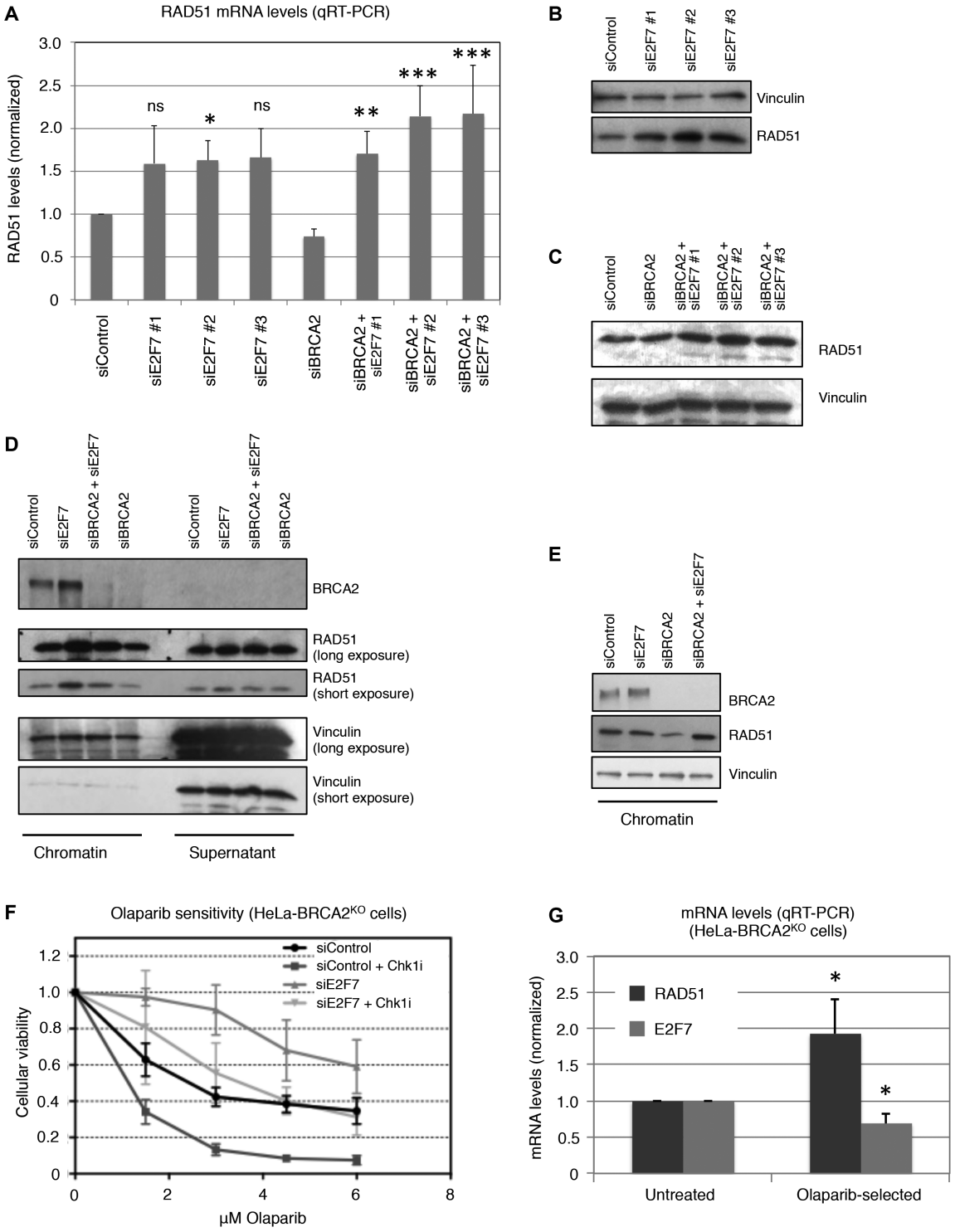

Figure 3. E2F7 regulates RAD51 levels to control olaparib sensitivity. (**A**) Quantitative RT-PCR experiment showing increased RAD51 mRNA levels upon E2F7 depletion in BRCA2-knockdown HeLa cells incubated with 10 μM olaparib for 24 h before harvesting. The average of three experiments, with standard deviations as error bars, is shown. Asterisks denote statistical significance compared to siControl condition (for single E2F7 depletion samples), or siBRCA2 condition (for E2F7/BRCA2 co-depletion samples). (**B** and **C**) Western blots showing that E2F7 knockdown results in increased RAD51 protein levels in U2OS (B) and HeLa (C) cells treated with 10 μM olaparib for 24 h. (**D** and **E**) Chromatin fractionation experiments showing that E2F7 depletion results in increased chromatin-bound RAD51. HeLa (D) or U2OS (E) cells, treated with 10 μM olaparib for 24 h, were used. Vinculin is used as loading control. (**F**) Chk1 inhibition further sensitizes BRCA2-knockout HeLa cells to olaparib treatment, which is suppressed by E2F7 depletion. The average of three independent experiments, with standard deviations as error bars, is shown. Chk1 inhibitor used is Rabusertib (Selleck Chemicals) at a concentration of 300 nM. (**G**) BRCA2-knockout HeLa cells were grown in the presence of 0.25 μM olaparib for 14 days then subjected to qRT-PCR to detect E2F7 and RAD51 mRNA expression. The average of four independent experiments, with standard deviations as error bars, is shown. Asterisks indicate statistical significance compared to control (untreated) samples.

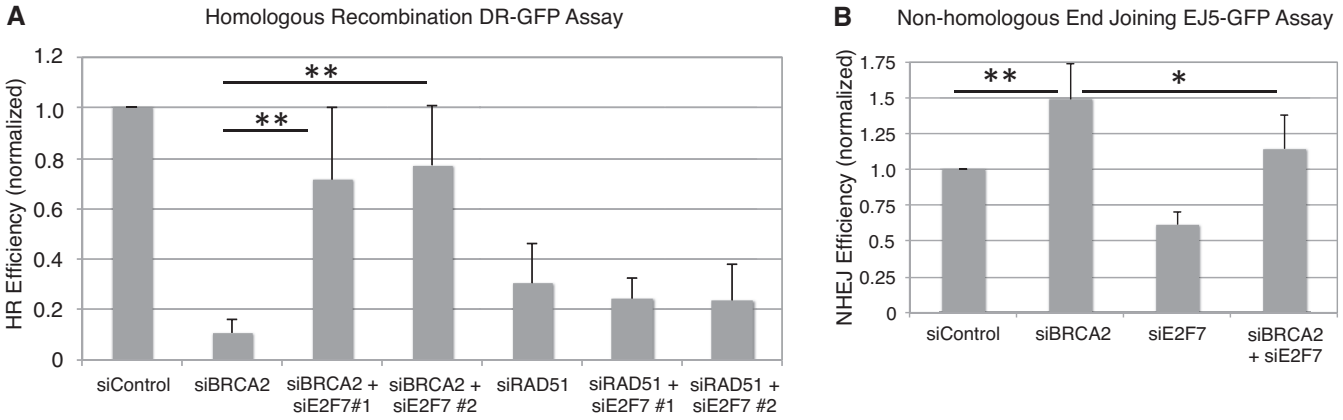

Figure 4. E2F7 depletion restores RAD51-mediated HR in BRCA2-deficient cells. (**A**) DR-GFP assay in U2OS cells showing that E2F7 depletion restores HR levels in BRCA2-knockdown cells, but not in RAD51-knockdown cells. The average of three to five experiments, with standard deviations as error bars, is shown. Asterisks indicate statistical significance. (**B**) EJ5-GFP assay showing the impact of E2F7 co-depletion on the NHEJ levels in BRCA2-depleted cells. The average of four to six experiments, with standard deviations as error bars, is shown. Asterisks indicate statistical significance.

At last, we wanted to investigate if this novel regulatory pathway is involved in acquired resistance to PARP inhibitors. To this end, we treated BRCA2-knockout HeLa cells with a sublethal dose of olaparib (0.25 μM) for 14 days, and measured mRNA expression of E2F7 and RAD51. Compared to controls, cells grown in the presence of olaparib showed a significant reduction in E2F7 levels, with a concomitant increase in RAD51 (Figure 3G). Moreover, these cells were less sensitive to olaparib than control cells (Supplementary Figure S5). These results suggest that prolonged olaparib treatment may result in E2F7 downregulation, as a compensatory mechanism to increase RAD51 levels and promote olaparib resistance.

Depletion of E2F7 restores RAD51-mediated homologous recombination in BRCA2-deficient cells

Next we sought to investigate the mechanism of the observed E2F7 depletion-mediated suppression of olaparib sensitivity in BRCA2-deficient cells. Two activities of BRCA2 have been associated with chemosensitivity: repair of double stranded DNA breaks through HR (by loading of RAD51 to resected DNA ends), and protection of stalled replication forks against nucleolytic degradation by MRE11 (through loading of RAD51 to reversed replication forks) (11,13,38). Thus, we decided to test if the increased RAD51 levels and chromatin loading observed in E2F7-depleted cells may partially alleviate the impact of BRCA2 loss and restore HR proficiency and/or fork protection. To measure HR, we employed the DR-GFP assay that quantifies double strand break-induced recombination between direct repeats in U2OS cells (32). As expected, BRCA2 knockdown significantly reduced HR, while depletion of E2F7 alone did not affect it (Figure 4A and Supplementary Figure S6A). However, co-depletion of E2F7 rescued the HR defect caused by BRCA2 knockdown. In contrast, E2F7 depletion could not rescue the HR defect conferred by loss of RAD51, indicating that the E2F7-mediated rescue observed in BRCA2-deficient cells requires RAD51 expression. Moreover, E2F7 co-depletion did not increase classic or alternative NHEJ rates in BRCA2-knockdown cells (Figure 4B and Supplementary Figure S6B), indicating that its effect is specific for HR and does not occur through other double strand break repair pathways. Altogether, these results indicate that E2F7 depletion increases RAD51 levels, which promotes HR in BRCA2-deficient cells.

E2F7 regulates replication fork stability in BRCA2-deficient cells

Next, we tested the impact of E2F7 on replication fork protection. Recently, degradation of stalled replication forks has emerged as a novel activity underlying the chemosensitivity of BRCA2-deficient cells. In normal cells, BRCA2 protects against fork degradation by loading RAD51 on forks arrested at sites of DNA damage. In BRCA2-deficient cells, RAD51 cannot be loaded, thus rendering stalled forks susceptible to degradation by MRE11 nuclease (13,14,16). In order to test the impact of E2F7 on fork stability in BRCA2-deficient cells, we employed the DNA fiber assay, which allows detection and quantification of nascent DNA strands at the molecular level. We measured the length of replication tracts in BRCA2-knockout HeLa cells upon treatment with HU, an established model of fork degradation in BRCA-deficient cells (15,16,38). In line with previous reports (13,14,16), loss of BRCA2 resulted in HU-induced degradation of nascent DNA (Figure 5 and Supplementary Figure S7). Importantly, E2F7 depletion could rescue the HU-induced fork degradation phenotype of BRCA2-knockout cells (Figure 5B and C; Supplementary Figure S7A). This rescue requires RAD51 loading to stalled forks, as it was abolished by treatment with RAD51 inhibitor B02, previously shown to block RAD51 loading to stalled forks (16). Moreover, addition of the MRE11 inhibitor mirin restored tract length to that of wild-type cells (Figure 5C), confirming that forks are degraded by the canonical MRE11-mediated pathway. These findings indicate that increased RAD51 levels upon E2F7 depletion can protect stalled replication forks against MRE11-mediated degradation in BRCA2-deficient cells.

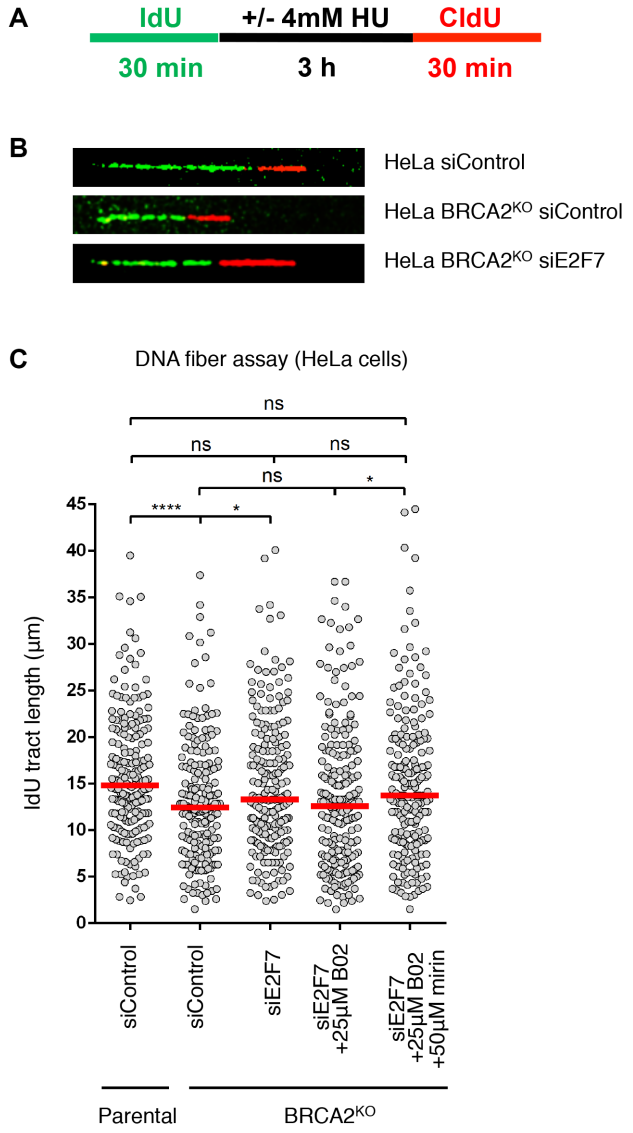

Figure 5. E2F7 controls replication fork stability in control and BRCA2-knockout HeLa cells. (**A**) Schematic representation of the experimental setup for the DNA fiber combing experiment. (**B**) Examples of replication tracts for the indicated genotypes. (**C**) Quantification of the IdU tract length. Loss of BRCA2 reduces the length of the IdU tract upon HU treatment, indicating that the nascent strand is degraded. This degradation can be suppressed by E2F7 depletion. Treatment with RAD51 inhibitor BO2 abolishes this rescue, indicating that it involves RAD51 loading to reversed forks. Incubation with the MRE11 inhibitor mirin can restore tract length, indicating that the fork degradation occurs through the classic MRE11 pathway. At least 200 tracts were analyzed for each sample. The median and statistical significance are indicated. Similar results were obtained with a different E2F7 siRNA oligonucleotide (Supplementary Figure S7A). Sample images showing combing quality are presented in Supplementary Figure S7B. E2F7 knockdown did not impact new origin firing (Supplementary Figure S7C).

DISCUSSION

Identifying clinical biomarkers of PARPi resistance is paramount for more targeted usage of these promising drugs in cancer treatment. Our work identifies E2F7 as a new factor regulating PARPi and cisplatin resistance of BRCA2-deficient cells (Supplementary Figure S8). We show that E2F7 depletion results in increased levels of RAD51, a previously described target for transcriptional repression by E2F7. This, in turn, promotes both HR and fork stability in BRCA2-deficient cells. Thus, our work suggests that E2F7 levels may represent a putative biomarker predicting PARPi responses of human tumors. Future clinical studies are important to validate this prediction. Moreover, upstream regulators of E2F7 may also serve as biomarkers and provide opportunities for therapeutic intervention. Recently, Chk1 was shown to phosphorylate E2F7 thereby restricting its activity in response to DNA damage (37). We show here that Chk1 inhibition further sensitizes BRCA2-deficient cells to olaparib, in a manner at least partially dependent on E2F7. This suggests that Chk1 activity may promote PARPi resistance by inhibiting E2F7. Pharmacological inhibition of this pathway may potentially be employed to restore PARPi sensitivity in tumors with acquired resistance. Our studies also indicate the possibility that E2F7 may be involved in acquired resistance, as prolonged olaparib treatment resulted in a compensatory reduction in E2F7 levels accompanied by increased RAD51 levels and reduced sensitivity to olaparib. Whether such a downregulation occurs in patient tumors upon olaparib treatment is not yet known, but if so, it may represent one of the mechanisms explaining why olaparib treatment only increases progression free survival by 5–15 months in various clinical trials (11).

Previous studies have shown that mechanisms of PARPi resistance differ between BRCA1 and BRCA2-deficient cells. Sensitivity of BRCA1-deficient cells can be suppressed by restoring HR, while in BRCA2-deficient cells resistance occurs through promoting replication fork stability and protection against nucleolytic degradation of stalled replication forks. For example, depletion of ZRANB3, HLTF or SMARCAL1 abolishes formation of the reversed fork structures targeted by the MRE11 nuclease and thus results in chemoresistance of BRCA2-deficient cells (16). Inhibition of MRE11 (13,14), or loss of PTIP (which recruits MRE11 to reversed forks) (38), or of RADX (which inhibits RAD51 accumulation to stalled replication forks) (39) can similarly rescue PARPi sensitivity of BRCA2-deficient cells without restoring HR. Finally, inhibition of a parallel fork degradation pathway governed by the chromatin modifier EZH2 which recruits the nuclease MUS81 to stalled forks (40), or channeling the processing stalled forks toward translesion synthesis-mediated lesion bypass rather than fork reversal (33), can also suppress chemosensitivity of BRCA2-deficient cells. Here, we show that depletion of E2F7, a transcriptional repressor of RAD51, also results in protection against degradation of stalled replication forks by MRE11. Our data suggest that increased RAD51 levels are enough to promote its loading to stalled replication forks even in the absence of BRCA2 activity.

Surprisingly, we found that E2F7 knockdown also restores HR in BRCA2-deficient cells. This effect also involves regulation of RAD51 expression, as E2F7 knockdown could not rescue the HR defect of RAD51-depleted cells, thus ruling out an involvement of other E2F7 targets involved in HR (such as BRCA1). How RAD51 is loaded to DSB ends in BRCA2-depleted cells is not clear. Our data indicates that it is unlikely to involve the activity of RAD52,

which has been previously implicated in RAD51 loading in BRCA2-deficient cells (36).

At this time, the relative contribution of HR rescue and fork protection activities to the E2F7-mediated chemoresistance of BRCA2-depleted cells is unclear. Nevertheless, to our knowledge this is the first time that restoration of HR (independent of a reversion mutation) is identified as a mechanism of PARPi resistance in BRCA2-deficient cells. Recently, E2F7 was reported to bind to double strand break sites and inhibit their repair, potentially through altering chromatin status at these sites (41). While it is not known if this activity requires RAD51, it is nevertheless possible that the rescue of BRCA2 chemosensitivity by E2F7 depletion also reflects this transcription-independent role of E2F7 in repressing DNA double strand break repair.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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