Replication fork stability confers chemoresistance in BRCA−deficient cells

Arnab Ray Chaudhuri1,* Elsa Callen1, Xia Ding2, Ewa Gogola3, Alexandra A. Duarte3, Ji–Eun Lee4, Nancy Wong5, Vanessa Lafarga6, Jennifer A. Calvo6, Nicholas J. Panzarino6, Sam John1, Amanda Day1, Anna Vidal Crespo1, Binghui Shen3, Linda M. Starnes7, Julian R. de Ruiter3, Jeremy A. Daniel4, Panagiotis A. Konstantinopoulos8, David Cortez9, Sharon B. Cantor6, Oscar Fernandez–Capetillo6, Kai Ge5, Jos Jonkers8, Sven Rottenberg10,11, Shyma K. Sharan3,8 & André Nussenzweig1

Cells deficient in the Brca1 and Brca2 genes have reduced capacity to repair DNA double−strand breaks by homologous recombination and consequently are hypersensitive to DNA−damaging agents, including cisplatin and poly(ADP−ribose) polymerase (PARP) inhibitors. Here we show that loss of the ML3/4 complex protein, PTIP, protects Brca1/2−deficient cells from DNA damage and rescues the lethality of Brca2−deficient embryonic stem cells. However, PTIP deficiency does not restore homologous recombination activity at double−strand breaks. Instead, its absence inhibits the recruitment of the MRE11 nuclease to stalled replication forks, which in turn protects nascent DNA strands from extensive degradation. More generally, acquisition of PARP inhibitors and cisplatin resistance is associated with replication fork protection in Brca2−deficient tumour cells that do not develop Brca2 reversal mutations. Disruption of multiple proteins, including PARP1 and CHD4, leads to the same end point of replication fork protection, highlighting the complexities by which tumour cells evade chemotherapeutic interventions and acquire drug resistance.

The role of BRCA1 and BRCA2 in the repair of double-strand breaks (DSBs) is thought to be central to their tumour-suppressor activities, and underlies the hypersensitivity of Brca−deficient cells to DNA−damaging agents. While cisplatin and PARP inhibitors (PARPi) have been shown to be effective chemotherapeutic agents, most Brca−mutant carcinomas acquire resistance1. Besides reduced uptake and increased efflux of drugs, the most well−described mechanism that drives chemotherapeutic resistance in Brca1/2−deficient tumours is through the restoration of homologous recombination (HR)1. Identification of additional mechanisms underlying resistance to DNA damage is crucial for improving therapies and predicting tumour responses in Brca−deficient cancers.

PTIP loss protects RFs in Brca−deficient cells

In addition to their roles in HR, recent studies have uncovered DSB−independent functions for BRCA1 and BRCA2 during replication stress3,4. Since MRE11 has been implicated in mediating replication fork (RF) degradation in cell lines2,4, we tested whether primary cells deficient in BRCA1 or BRCA2 also showed degradation of nascent replication tracts. We therefore conditionally inactivated BRCA1 and BRCA2 in B lymphocytes (Brca1−/−Cd19Cre; Brca2−/−Cd19Cre). B cells were sequentially labelled with CldU−(red) followed by IdU−(green), after which the active RFs were stalled with hydroxyurea (HU) (Fig. 1a). The relative shortening of the IdU tract after HU treatment serves as a measure of RF degradation (Fig. 1a). Upon HU treatment, wild-type (WT) cells showed a mean IdU/CldU tract ratio close to 1 (Fig. 1b). However, Brca1− −− and Brca2−−− B cells exhibited a 30−45% reduction in the IdU tract length (Fig. 1b−e and Extended Data Fig. 1a−c).

Consistent with previous data2,3, RF degradation in B lymphocytes was dependent on MRE11 exonuclease activity (Extended Data Fig. 1a−c). We also tested the role of DNA2 and the Werner syndrome helicase/nuclease (WRN) in degradation of forks in Brca2−deficient B cells. Treatment of Brca2−deficient cells with WRN inhibitor did not result in fork protection, whereas MRE11 and DNA2 were epistatic (Extended Data Fig. 1c).

Since 53BP1, Rif1, and PTIP counteract BRCA1−dependent HR by inhibiting MRE11−dependent DSB resection7−11, we examined whether these factors might also function in RF stability. We thus inactivated BRCA1, BRCA2, Rif1, PTIP and 53BP1 in B lymphocytes (Extended Data Fig. 2a). Short exposure to HU did not promote significant DSB formation (Extended Data Fig. 2b), and fork progression rates were comparable across all genotypes (Extended Data Fig. 2c−f).

Absence of 53BP1 did not protect Brca1−−− B cells from degradation of RFs (Fig. 1b), consistent with the finding that BRCA1 acts in RF stabilization in a manner independent of DSB repair2,3. Nascent strands also shortened considerably in the absence of the 53BP1 effector Rif1 and in Brca1/Rif1 doubly deficient cells (Fig. 1c).

In striking contrast, loss of Ptip protected RFs from HU−induced degradation in both Brca1−−− and Brca2−−−−−− cells (Fig. 1d, e). Moreover, while Brca1−−−, Rif1−−−, and Brca1−−−/Rif1−−− B cells displayed increased genomic instability when treated with HU (Extended Data Fig. 3a), Brca1/Ptip−−−−−− cells challenged with HU (Extended Data Fig. 3b), suggesting that PTIP has functions at
stalled RFs distinct from its DSB-dependent interactions with 53BP1 and Rif1.

We hypothesized that HU-induced degradation would impact RF progression rates. We therefore assayed the ability of WT and mutant cells to incorporate nucleotide analogues in the presence of low concentrations of HU. We observed a significant decrease in IdU tract lengths during HU exposure across all genotypes. However, Brca2-deficient cells had significantly decreased progression rates upon HU treatment, whereas Ptip–/– and Brca2–/–/Ptip–/– cells displayed significantly longer replication tracts (Extended Data Fig. 3c). We also tested the effect of Ptip-deficiency on recovery after replication stalling with high concentrations of HU. We found that although the percentage of restarted RFs did not change among genotypes (Extended Data Fig. 3d), loss of Brca2 resulted in a delayed restart, whereas Brca2/Ptip doubly deficient cells restarted normally (Extended Data Fig. 3e). Thus, loss of Ptip promotes RF progression and timely restart in Brca2-deficient cells, which correlates with decreased RF degradation.

PTP loss rescues lethality of Brca2-null ESCs

Since elevated levels or stabilized RAD51 filaments could protect RFs from degradation2,3,16, we asked whether PTP deficiency leads to over-expression of RAD51 or enhanced RAD51 activity. RAD51 levels were similar in WT, Ptip−/−, Brca2−/−, and Brca2−/−/Ptip−/− cells (Extended Data Fig. 3f), but the ability of RAD51 to relocate to sites of DNA DSBs was severely impaired in Brca2/Ptip-deficient B cells (Fig. 1g), irradiation-induced RAD51 foci formation was defective in Brca2/Ptip-deficient ESCs (Fig. 2c, d). Moreover, while efficient gene targeting to the Pim-1 locus was observed in WT ESCs using a pro-moterless hygromycin cassette (100% of the hygromycin-resistant WT clones were targeted integrations), we did not observe a single targeted clone in Brca2/Ptip-deficient ESCs (Fig. 2e) indicative of defective HR. Similarly, the synthetic HR reporter substrate DR–GFP revealed impaired HR in Brca2/Ptip-deficient ESCs (Extended Data Fig. 4c). Nevertheless, stalled RFs in PTP-deficient Brca2−/− ESCs displayed RF protection compared with Brca2 hypomorphic mutant ESCs (Y3308X)17 (Fig. 2f). Thus, deficiency in PTP protects RFs from degradation and rescues the lethality of Brca2 knockout ESCs without restoring DSB-induced HR.

BRCA2 is dispensable for HR at RFs

It has been suggested that HR at stalled forks is regulated differently from HR at DSBs18. As a readout for HR at RFs, we assayed for sister chromatid exchanges (SCEs) in WT and Y3308X ESCs. Although Y3308X cells show undetectable levels of irradiation-induced RAD51 formation and loss of targeted integration, indicative of a defect in DSB-induced HR17, the basal frequency of SCE was normal in Y3308X cells (Extended Data Fig. 4d). Moreover, RAD51 was enriched on nascent DNA in Y3308X during normal replication and in presence of HU as measured by isolation of proteins on native DNA (iPOND) analysis (Extended Data Fig. 4e). We also observed similar frequencies of spontaneously generated and DNA damage-induced SCEs in WT, Brca2−/− and Brca2/Ptip-deficient B cells (Extended Data Fig. 4f). Thus, in contrast to RAD51 which is required for DSB- and replication-associated HR19, BRCA2 appears to be dispensable for HR.
PTIP deficiency rescues the lethality of Brca2-null mouse ESCs and confers fork protection. a, Schematic for deletion of Brca2. b, PCR genotyping of ESC clones (M, marker; P, positive control for Brca2+/−; N, no DNA control). c, d, Representative images (c) and quantification (d) of irradiation-induced foci in shPtip and Brca2+/−/shPtip ESCs (n = 110 cells examined). e, Representative Southern blot images (top) and quantification for targeting efficiency (bottom) for 59xDR–GFP36 gene targeting to the Pin-1 locus. f, Ratio of IdU versus CldU (****p < 0.0001, Mann–Whitney test). One hundred and twenty-five replication forks were analysed.

that the nascent sister chromatid to repair DNA lesions during replication.

MRE11 association at RFs depends on PTIP–MLL3/4

Although the recruitment of PTIP to DSBs after irradiation is dependent on 53BP1 (Extended Data Fig. 5a, b),10, we hypothesized that PTIP might be recruited to sites of stalled RFs independently of its interactions with 53BP1. Consistently, we observed PTIP accumulation at sites of replication stalling marked by pan-nuclear γ-H2AX staining. Among cells exhibiting pan-γ-H2AX signal, 71.4% of WT and 78% of 53Bp1−/− mouse embryonic fibroblasts (MEFs) exhibited PTIP foci following HU treatment (Fig. 5a and Extended Data Fig. 5c). Even in the absence of HU treatment, PTIP exhibited extensive co-localization with proliferating cell nuclear antigen (PCNA) during late S phase both in WT and in 53Bp1-deficient cells (Fig. 5b and Extended Data Fig. 5d, e). Collectively, these data suggest that PTIP might function during normal or perturbed replication in a DSB- and 53BP1-independent manner.

Like PTIP, MRE11 also associates with chromatin in a DNA damage-independent but DNA replication-dependent manner (Fig. 3c). Loss of PTIP resulted in a marked decrease of MRE11 association with PCNA foci in unperturbed cycling MEFs (Fig. 3c) and defective MRE11 recruitment to ssDNA regions upon HU treatment (Extended Data Fig. 5f). Re-introduction of WT full-length PTIP into Ptip−/− MEFs restored MRE11 co-localization with PCNA in late S phase (Fig. 3d). Thus, in contrast to irradiation-induced MRE11 foci, localization of MRE11 to sites of DNA replication is PTIP-dependent (Fig. 3c–e and Extended Data Fig. 5g).

To monitor MRE11 and RAD51 association with active and stalled RFs we performed iPOND analysis in WT and Ptip−/− MEFs (Extended Data Fig. 5b)25. WT cells showed an increase in MRE11 and RAD51 association with stalled RFs (Fig. 5f). Ptip-deficient cells also showed an increase in RAD51 association at RFs, but MRE11 association with nascent DNA was reduced upon HU treatment (Fig. 5f), consistent with our immunofluorescence analysis (Fig. 3c and Extended Data Fig. 5f). Thus, MRE11 deposition on newly synthesized DNA is dependent on PTIP, which itself is recruited to stalled forks upon HU treatment (Extended Data Fig. 5i)25.
PTIP is also known to constitutively associate with PA1 and with MLL3/MLL4 histone methyltransferases which catalyse methylation of histone H3 at lysine 4 (refs 23, 24). To identify the region of PTIP that promotes RF degradation in Brca2-deficient cells, we expressed EV (empty vector), FL (full-length PTIP), W165R (disrupting interactions with PA1)25,26, W663R (disrupting interactions with 53BP1 at DSBs)25 or Del-BRCT5-6 (disrupting interaction with MLL3/4 independently of DSBs)23,24,26 in Brca2/PTip doubly deficient cells. We observed that only reconstitution of Brca2/PTip-deficient cells with PTIP-Del-BRCT5-6 maintained fork protection (Extended Data Fig. 6a).

We therefore tested whether the recruitment of MRE11 at stalled forks was dependent on MLL3/4. We observed that MRE11 association at RFs was dependent on MLL3/4 as monitored by iPOND and immunofluorescence analysis (Extended Data Fig. 6b, c). We also observed an enrichment of H3K4me1 and H3K4me3 at nascent forks upon HU treatment that was PTIP- and MLL3/4-dependent (Fig. 3f and Extended Data Fig. 6b). Thus, deposition of PTIP on nascent chromatin correlates with the establishment of H3K4me1 and H3K4me3 at RFs.

To determine whether MLL4 contributes to degradation of stalled forks in Brca2-deficient cells, we examined RF degradation in Brca1−/−MLL4−/− and Brca2−/−MLL4−/− B cells. Brca1−/−MLL4−/− and Brca2−/−MLL4−/− cells displayed a partial rescue of fork degradation (Extended Data Fig. 6d, e). To test whether MLL4 methyltransferase activity is critical, we targeted the catalytic SET domain of MLL4 in Brca2−/−MLL4−/− SET−/− cells, suggesting that the methyltransferase activity is important for promoting fork degradation (Extended Data Fig. 6f).

**RF protection confers chemoresistance**

RF protection contributes to genome stability in a manner independent of DSB-induced HR2,23,25. Consistently, we observed that Brca2−/−MLL4−/− B cells showed a partial rescue of chromosomal aberrations upon PARPi and cisplatin treatment compared with Brca2−/− cells alone (Extended Data Fig. 6g). However, Rif1-deficient cells, characterized by extensive RF degradation (Fig. 1c) but normal irradiation-induced RAD51 foci formation (Extended Data Fig. 7a), showed increased chromosomal aberrations in response to replication poisons PARPi, HU and cisplatin (Extended Data Figs 3a and 7b, c)27. We therefore speculated that RF stability mediated by loss of PTIP (Fig. 1d, e) might confer genome stability to Brca2-deficient cells exposed to chemotherapeutics that poison DNA replication. Indeed, we found that Ptp deficiency reduced the levels of chromosomal aberrations both in Brca1−/− and in Brca2−/− B cells (Fig. 4a, b).

To test whether differential levels of PTIP expression could be an indicator of patient responses to platinum chemotherapy, we queried clinical information from The Cancer Genome Atlas (TCGA) of patients with Brca1/Brca2-mutated ovarian serous adenocarcinoma treated with platinum chemotherapy (Extended Data Fig. 8a, b). Survival analysis demonstrated that platinum-treated Brca2 mutants with high PTIP expression were correlated with a longer progression-free survival (PFS) (Extended Data Fig. 8a). Lower expression of PTIP also predicted a shorter PFS in Brca2-associated ovarian cancers (Extended Data Fig. 8b). Taken together, these data suggest that PTIP levels could be a biomarker for acquired resistance to platinum-based chemotherapy in Brca1/2-mutated ovarian cancers.

An unbiased shRNA screen recently demonstrated that reduced levels of the nucleosome remodelling factor CHD4 in Brca2 mutant cancers correlated with poor patient response to chemotherapy, and increased tolerance to DNA-damaging agents without restoration of RAD51-dependent HR28. To test whether the resistance mechanism in this case occurs through RF protection, we knocked down CHD4 in the Brca2 mutant ovarian cancer cell line PEO1 (Extended Data Fig. 8c, d). While CHD4 depletion in Brca2 mutant cells did not restore HR28, we observed that it largely conferred protection to nascent replication tracts from degradation upon HU treatment (Fig. 4c).

Moreover, depletion of CHD4 resulted in significantly decreased recruitment of MRE11 upon HU treatment in Brca2 mutant cells (Extended Data Fig. 8e, f). These results suggest that MRE11-mediated degradation contributes to genome instability upon treatment with replication poisons. To test this idea, we pre-incubated Brca2-deficient B cells with the MRE11 nuclease inhibitor mirin before treating with PARPi or cisplatin. Mirin treatment did not alter the frequency of replicating cells monitored by EdU incorporation (Extended Data Fig. 8g). However, incubation with mirin reduced the levels of PARPi-induced chromosomal aberrations approximately twofold. Similarly, Brca2-deficient cells were partly protected from cisplatin-induced DNA damage when MRE11 nuclease activity was inhibited (Extended Data Fig. 8h).

**Figure 4 | Replication fork protection confers genome stability and chemotherapeutic resistance.** **a, b,** Genomic instability measured in metaphase spreads from B cells (n = 50; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, unpaired t-test). Experiments were repeated four times. c, Ratio of IdU versus CldU in Brca2-mutated PEO1 cells either mock (shNSC) infected or infected with shRNA against CHD4 (shCHD4) (*P < 0.05, **P < 0.001, Mann–Whitney test). One hundred and twenty-five replication forks were analysed. d, Ratio of IdU versus CldU in HU-treated B cells (*P < 0.05, **P < 0.001, **P < 0.0001, Mann–Whitney test). One hundred and twenty-five replication forks were analysed. e, Genomic instability in B cells (n = 50; *P < 0.01, unpaired t-test). Experiments repeated four times. f, g, Kaplan–Meier survival curves in mice implanted with either PARPi-naive or -resistant tumours and treated with either topotecan (f) or cisplatin (g) using log-rank (Mantel–Cox) test. h, Ratio of IdU versus CldU in untreated or HU-treated tumours (PARPi naive versus PARPi resistant) (**P < 0.001, Mann–Whitney test). One hundred and twenty-five replication forks were analysed.

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We conclude that MRE11 nuclease promotes genomic instability in Brca2-deficient cells.

Since PARP1 (ARTD1) is required for MRE11 localization to stalled replication forks\(^29\) and its loss rescues the embryonic lethality in Brca2-null ESCs\(^30\), we tested the contribution of PARP1 to RF stability and genome integrity by generating Brca1\(^{-/-}\)-Parp1\(^{-/-}\)-B cells (Brca1\(^{-/-}\); Parp1\(^{-/-}\); Cd19Cre). Interestingly, loss of Parp1 protected Brca1-deficient RFs from degradation and resulted in a significant reduction in chromosomal aberrations (Fig. 4d, e). Nevertheless, Parp1 deficiency failed to rescue irradiation-induced RAD51 foci formation in Brca1-deficient cells (Extended Data Fig. 8i). Thus, despite the fact that treatment with PARP inhibitor increases levels of DNA damage in Brca1-deficient cells\(^31,32\), loss of Parp1 before Brca1 loss protects against genome instability.

To determine whether Brca2-deficient tumour cells acquire chemotherapy resistance via RF protection, we induced PARP1-resistance using the KB2P mouse model for Brca2-deficient breast cancer (Extended Data Fig. 9a)\(^33\). A mammary tumour from KB2P mice was transplanted into syngeneic FVB mice and, when the tumour reached a size > 200 mm\(^3\), mice were treated with PARPi (AZD2461) for 28 consecutive days (Extended Data Fig. 9b, c). The tumour was initially responsive to treatment but eventually grew, upon which treatment was repeated until PARPi resistance was achieved (Extended Data Fig. 9c). The stability of acquired resistance was confirmed by re-transplanting matched naive and resistant tumours and treating the animals with vehicle or AZD2461 (Extended Data Fig. 9d). PARPi-resistant tumours also showed cross-resistance to replication poisons topotecan and cisplatin (Fig. 4f, g).

Both naive and resistant Brca2-deficient tumours showed impaired irradiation-induced RAD51 foci formation (Extended Data Fig. 9e, f). We therefore assayed naive and resistant tumours \textit{ex vivo} for RF stability. The mean length of the CldU and the iDu tracts were similar in all samples that were not treated with HU (Fig. 4h and Extended Data Fig. 9g). While naive tumour cells showed degradation of nascent tracts upon HU treatment, resistant tumour cells were protected (Fig. 4h). These data suggest that RF protection rather than restoration of HR may be the main mechanism for acquired resistance in this mammary tumour. However, in this case, acquired resistance was not simply due to loss of PTIP or MRE11 proteins (Extended Data Fig. 9h).

Discussion

Our study and the accompanying paper\(^30\) provide the first examples of genetic alterations that bypass the essential requirement for DSB-induced HR, evidenced by the finding that reduction in PTIP or PARP1 (ARTD1) levels rescues the lethality of Brca2-null ESCs. We speculate that the reason Brca2 nullizygosity is compatible with viability is because RAD51 is able to perform essential HR functions (such as SCE) by loading onto RFs independently of BRCA2 (Extended Data Fig. 10a, b)\(^4\). In Saccharomyces cerevisiae, failure to remove MRE11 from single-stranded DNA can lead to hypersensitivity to a variety of clastogens\(^34,35\). We therefore propose that deficiencies in PTIP, CHD4 and PARP1 could confer drug resistance in Brca2-deficient cells by limiting the access of MRE11 to single-strand DNA at stalled RFs.

In summary, we have shown that protection of nascent DNA from degradation provides a mechanism that can promote synthetic viability and drug resistance in Brca2-deficient cells without restoring HR at DSBs.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 30 July 2015; accepted 15 April 2016.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank A. Bhandoola for discussions; K. Wolcott for flow cytometry; R. Faryabi for help with statistical analysis; T. de Lange for Rif1 mice, J. Tainer for PFM39, R. Brosh for WRNi and J. Petrini for Mre11 antibodies. This work was supported by the Intramural Research Program of the National Institutes of Health (NIH), the National Cancer Institute and the Center for Cancer Research, and by a Department of Defense grant to A.N. (BCRP DOD Idea Expansion Award, grant 11557134), and the Netherlands Organization for Scientific Research, the Dutch Cancer Society and the Swiss National Science Foundation to S.V. A.R.C. was supported by a Prospective Researcher Award from Swiss National Science Foundation (PBZHP3_147302) and Human Frontier Science Program Long-Term Fellowship (LT000393/2013). S.C. was supported by NIH grant R01 CA176166-01A1; B.S. was supported by NIH grant R01CA085344; and J.A.D. was supported by a grant to the Center for Protein Research from the Novo Nordisk Foundation (NNF14CC0001).

Author Contributions A.R.C., E.C., S.S. and A.N. conceived and planned the study, A.R.C., E.C. and X.D. designed, performed experiments and analysed data on B cells, MEFS and ESCs, E.G. and A.A.D. generated and performed experiments on PARPi-resistant tumours, J.J. and S.R. supervised the studies on PARPi-resistant tumours. N.W., A.D. and S.J. helped with experimentation, J.E.L. and K.G. generated Mll4- and Mll4-SET-deficient mice. L.S. and J.D. generated PTIP deletion constructs. B.S. provided reagents for experiments. D.C. provided advice on performing iPOND experiments and supervised iPOND mass spectrometry. J.C., N.P. and S.C. provided shCHD4 PEO1 cells and performed immunofluorescence experiments. P.K. analysed TCGA databases. V.F. and O.F.C. performed high-throughput image analysis of MRE11. S.S. supervised the ESC studies. A.R.C., E.C. and A.N. wrote the manuscript and all authors reviewed it.

Author Information Source tumour measurement data are provided in Supplementary Information. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.N. (andre_nussenzweig@nih.gov).
METHODS

Mice, MEFS, and B cell cultures. The 53BP1Cre-/- (ref. 37), Brca1fl/ffl(Δ111)fl(Δ111) (NCI mouse repository), Brca2fl/ffl(Δ111)fl(Δ111) (NCI mouse repository), Rfp7fl/fl (ref. 27), Ptipfl/fl (ref. 24) and Mll3fl/fl (ref. 38) mice have been described. To generate Mll3-SET-flx/flox mice, exons 50–51 of the Mll3/ΔKmt2d gene were flanked by loxP sites. Cre-mediated deletion of the floxed exons 50–51 causes frame shift and generates a stop codon in exon 52. The resulting protein lacks the carboxy (C)-terminal 276 amino acids including the entire enzymatic SET domain. Resting splenic B cells were isolated from 12-week-old WT or mutant mice with anti-CD43 microbeads (anti-Ly48, Miltenyi Biotech) and were cultured with LPS (25 μg/ml; Sigma), IL-4 (5 ng/ml; Sigma) and RP105 (0.5 μg/ml; BD) as described46. Stimulated B cells were additionally infected with CRE to ensure a high level of gene deletion in these cells. All mice were randomly distributed in experimental groups after genotyping. WT MEFS were immortalized by SV40 retroviral infection, and SV40 immortalized Ptipfl/fl (ref. 39) and Mll3fl/fl MEFS were infected with a CRE retrovirus to delete Ptip and Mll3 respectively. Coding sequences for mouse Ptip–GFP were cloned into the MIGR1 retroviral vector as previously described27. All cell lines used were tested for mycoplasma using a Mycoplasma Detection Kit (Invitrogen) and relevant cell lines were authenticated either by genotyping or western blot analysis. PARP inhibitor (KU55948) was obtained from AstraZeneca. Mirin was obtained from Sigma, PFM39 and WRN inhibitors were gifts from J. Tainer and R. Brosh respectively. DNA2 inhibitor has been described40. Cisplatin and HU were obtained from Sigma. For FISH analysis, metaphases were prepared and imaged as described44. Animal experiments were approved by the Animal Care and Use Committee of NCI-Bethesda and the Animal Ethical Committee of The Netherlands Cancer Institute.

Generation of CHD4-deficient PE01 lines using RNAi. BRCA2-mutated ovarian cancer cell lines PE01 were grown in DMEM with 10% FBS and 1% Glutamax. RNAi for CHD4 was performed as previously described28. ESC lines, shRNA knockdowns and rescue of BRCA2 viability. RNAi for CHD4 was performed as previously described28. For transient knockdown, ESCs were plated under unsaturated DNA signals in the well. Relative DSB levels were obtained by comparing treatment results to the background DSB signals observed for untreated conditions.

Western blotting and immunofluorescence. Primary antibodies were used in the following dilutions: anti-tubulin (1:15,000, Sigma), anti-H3K4me3 (1:5,000, Millipore), anti-H3K4me1 (1:5,000, Millipore), anti-RAD51 (1:50, Santa Cruz), anti-PCNA (1:2,000, Santa Cruz), anti-total H3 (1:1,000 Millipore) and anti-Mre11 (1:10,000, gift from J. Petrin, MSKCC). MEFS were prepared for immunofluorescence by growth on 18 mm × 18 mm glass cover slips. Lymphocytes were dropped onto slides coated with CellTak (BD Biosciences). Cells were fixed with methanol and incubated with primary antibody as indicated.

iPOND and flow cytometry. iPOND was performed essentially as described22. One hundred and fifty million WT, Ptipfl/fl and Mll3fl/fl MEFS were labelled with 10 μM EdU (Life Technologies) and treated with HU as indicated. Two hundred million cells were used for iPOND experiments with ESCs. Cells were cross-linked with 1% formaldehyde for 10 min at room temperature, quenched with 0.125 M glycine and washed with PBS. For the combination of EdU with biotin azide, cells were permeabilized with 0.25% Triton X-100/ PBS, and incubated in click reaction buffer (10 mM sodium-t-acetate, 20 μM biotin azide (Life Technologies), and 2 mM CuSO4) for 2 h at room temperature. Cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, and 1% SDS) supplemented with protease inhibitors (Roche), and chromatin was solubilized by sonication in a Bioruptor (Diagenode-Pico) at 4°C for 2 min. After centrifugation, clarified supernatants were incubated for 1 h with streptavidin-MyOne C1 beads (Life Technologies). Beads were washed and captured proteins were eluted by boiling beads in 2 × NuPAGE LDS Sample Buffer (Life Technologies) containing 200 mM DTT for 40 min at 95°C. Proteins were resolved by electrophoresis using NuPAGE Novex 4–12% Bis-Tris gels and detected by western blotting with the indicated antibodies. For flow cytometric analysis, asynchronous B cells were pulsed with 10 μM EdU for 20 min at 37°C and stained using the Click-IT EdU Alexa Fluor 488 Flow Cytometry Assay Kit according to the manufacturer’s specifications (ThermoFisher C-10425).

Generation of A2D461-resistant KB2P tumours and in situ RAD51 assay. Brca2p53-/- mammary tumours were generated in K14-cre;Brca2fl/fl, p53fl/fl (KB2P) female mice and genotyped as described previously33. Orthotopic transplants into WT FVR/NOL (F1) mice, tumour monitoring, and sampling were conducted as described41. Starting from 2 weeks after transplantation, tumour size was monitored at least three times a week. All treatments were started when tumours reached a size of approximately 200 mm3. A2D461 (100 mg/kg) was given orally for 28 consecutive days. If tumours did not shrink below 100% of the initial volume, treatment was continued for another 28 days. A2D461 was diluted in 0.5% w/v hydroxypropyl methylcellulose in deionized water to a concentration of 10 mg/ml. For testing cross-resistance, mice were given a single treatment regimen of topotecan (2 mg/kg intraperitoneally, days 0–4 and 14–18) or cisplatin (Mayne Pharma, 6 mg/kg intravenously, day 0). Statistical analysis was performed using GraphPad Prism (log-rank Mantel–Cox test). The in situ RAD51 irradiation induced formation assay has been described41.

Statistics. Statistics was performed by two-tailed t-test, Mann–Whitney test or by log-rank Mantel–Cox test unless otherwise specified. Statistical tests were justified appropriate for every figure. The data were normally distributed and the variance between groups being statistically compared was similar. No statistical methods or criteria were used to estimate sample size or to include/exclude samples. The investigators were not blinded to the group allocation during the experiments; however, the samples were coded before analysis unless otherwise specified.
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44. Xu, G. et al. REV7 counteracts DNA double-strand break resection and affects PARP inhibition. *Nature* **521**, 541–544 (2015).
Extended Data Figure 1 | Fork degradation in Brca-deficient B lymphocytes is mediated by MRE11 exonuclease activity. a, b, Ratio of IdU versus CldU upon HU treatment in WT, Brca1<sup>−/−</sup> (a) and WT, Brca2<sup>−/−</sup> (b) B lymphocytes with or without mirin pre-treatment. Numbers in red indicate the mean ± s.d. for each sample (***P ≤ 0.001, ****P ≤ 0.0001, Mann–Whitney test). One hundred and twenty-five replication forks were analysed for each genotype. c, Ratio of IdU versus CldU upon HU treatment in Brca2<sup>−/−</sup> B lymphocytes with or without mirin, PFM39 (MRE11 exonuclease inhibitor), PC5 (DNA2 inhibitor) or WRNi pre-treatment. Numbers in red indicate the mean ± s.d. for each sample (***P ≤ 0.001, ****P ≤ 0.0001, Mann–Whitney test). One hundred and twenty-five replication forks were analysed for each genotype.
Extended Data Figure 2 | Replication fork progression rates and DSBs in B lymphocytes. 

a, Quantitative PCR analysis for Brca1, Brca2, Ptip and Rif1 gene deletions in WT, Brca1\(^{-/-}\), Brca1\(^{-/-}\) Rif1\(^{-/-}\), Brca1\(^{-/-}\)Ptip\(^{-/-}\), Brca2\(^{-/-}\), and Brca2\(^{-/-}\)Ptip\(^{-/-}\) primary B lymphocytes after infection with CRE. b, PFGE analysis for detection of DSBs in WT, Brca2\(^{-/-}\), and Brca2\(^{-/-}\)Ptip\(^{-/-}\) B lymphocytes treated with or without 4 mM HU for 3 h. Positive control for DSBs includes treatment of 15 Gy irradiation and HU + ATRi treatment for 3 h. Quantification of fold change in DSBs across genotypes relative to non-treated WT is plotted on the right. c–f, Replication fork progression rates, measured by tract lengths in micrometres, of CldU (red) and IdU (green) in WT, Brca1\(^{-/-}\), Brca2\(^{-/-}\) 53Bp1\(^{-/-}\), Rif1\(^{-/-}\), Brca1\(^{-/-}\) Rif1\(^{-/-}\), Ptip\(^{-/-}\), Brca2\(^{-/-}\) and Brca2\(^{-/-}\)Ptip\(^{-/-}\) primary B lymphocytes. Samples were not treated with HU. Numbers in red indicate the mean ± s.d. for each sample. One hundred and twenty-five replication forks were analysed for each genotype.

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Extended Data Figure 3 | See next page for caption.
**Extended Data Figure 3 | Loss of PTIP rescues fork progression and restart defects in Brca2-deficient B lymphocytes but does not affect RAD51 IRIF.**

**a,** Genomic instability measured in metaphase spreads from B lymphocytes derived from WT, *Brca1*^−/−*, Rif1*^−/−*, *Brca1*^−/−/*Rif1*^−/−* mice treated for 6 h with 10 mM HU (*P* ≤ 0.05, unpaired *t*-test). Fifty metaphases were analysed per condition. Experiments were repeated three times.

**b,** Genomic instability measured in metaphase spreads from B lymphocytes derived from WT, *Brca2*^−/−*, *Ptip*^−/−*, *Brca2*^−/−/*Ptip*^−/−* mice treated for 6 h with 10 mM HU (*P* ≤ 0.05, unpaired *t*-test). Fifty metaphases were analysed per condition. Experiments were repeated three times.

**c,** Fork progression in B lymphocytes derived from WT, *Brca2*^−/−*, *Ptip*^−/−*, *Brca2*^−/−/*Ptip*^−/−* mice treated for 1 h with 0.2 mM HU. The *y* axis represents the tract lengths in micrometres. Numbers in red indicate the mean ± s.d. for each sample (*****P* ≤ 0.0001, Mann–Whitney test). One hundred and fifty replication forks were analysed for each genotype.

**d,** Percentage of restarted replication forks in WT, *Brca2*^−/−*, *Ptip*^−/−*, *Brca2*^−/−/*Ptip*^−/−* B cells treated for 1 h with 1 mM HU followed by 90 min recovery. Two hundred replication forks were analysed for each genotype.

**e,** Tract lengths of restarted replication forks in WT, *Brca2*^−/−*, *Ptip*^−/−*, *Brca2*^−/−/*Ptip*^−/−* B cells treated for 1 h with 1 mM HU followed by 90 min recovery. The *y* axis represents the tract lengths in micrometres. Numbers in red indicate the mean ± s.d. for each sample (NS, not significant, *****P* < 0.0001, Mann–Whitney test). One hundred and fifty replication forks were analysed for each genotype.

**f,** Western blot analysis for RAD51 levels in WT, *Ptip*^−/−*, *Brca2*^−/−* and *Brca2*^−/−/*Ptip*^−/−* B cell extracts. Tubulin was used as loading control.

**g,** Quantification of RAD51 foci formation in WT, *Brca2*^−/−* and *Brca2*^−/−/*Ptip*^−/−* B cells upon treatment with 5 and 10 Gy irradiation and recovery for 2, 4 and 6 h (*n* = 150 cells analysed).
Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | Depletion of PTIP rescues the lethality of Brca2-deficient ESCs. a, Western blot analysis for PTIP levels in WT and two different clones of Brca2−/− ESCs electroporated with shPtip. b, Southern blot analysis for determination of Brca2 deletion in surviving clones electroporated with shPtip. Probes distinguishing the Brca2-flox allele (4.8 kb) (upper band) and Brca2 KO allele (2.2 kb) (lower band) were used. *Surviving ESC clones with Brca2 deletion and simultaneous downregulation of PTIP (12/96 Brca2-deleted colonies were found with shPtip1 and 3/60 colonies were found with shPtip2). Genotyping was confirmed by PCR (Fig. 2b). c, Top, representative FACS profiles of WT and Brca2−/−/shPtip ESCs electroporated with either pDR–GFP plasmid only (control) or pDRGFP and I-SceI expressing vector for 48 h. Gene conversion of the pDR–GFP construct by HR is determined by the percentage of GFP-positive cells (FL1, green-detection filter; FL2, red-detection filter). Bottom, quantification of the percentage of GFP-positive cells in WT and Brca2−/−/shPtip ESCs across three independent experiments. NS, not significant, *P ≤ 0.05, unpaired t-test. d, Sister chromatid exchange (SCE) analysis in WT and Brca2Y3308X hypomorphic ESCs. Twenty metaphases were analysed per condition; experiments were repeated three times. e, WT and Brca2Y3308X hypomorphic ESCs were preincubated with mirin, EdU-labelled for 15 min and treated with 4 mM HU for 2 h. Proteins associated with replication forks were isolated by iPOND and detected by western blotting with the indicated antibodies. f, SCEs in WT, Brca2−/− and Brca2−/− Ptip−/− B cells either untreated or treated overnight with 1 μM PARPi or with 0.5 μM cisplatin (NS, not significant, *P ≤ 0.05, **P ≤ 0.001, unpaired t-test). Twenty metaphases were analysed per condition; experiments were repeated three times.
Extended Data Figure 5 | See next page for caption.
Extended Data Figure 5 | PTIP localizes to sites of DNA replication independently of DSBs. a, WT and 53Bp1−/− MEFs were retrovirally infected with a GFP-tagged PTIP construct. Cells were then irradiated with 10 Gy and allowed to recover. Co-localization of γ-H2AX (red) and PTIP (green) was assessed. Adjoining graph quantifies the percentage of cells with γ-H2AX foci co-localizing with PTIP upon irradiation (n = 150 cells analysed). Experiments were repeated three times. b, Western Blot analysis for endogenous and overexpressed PTIP levels in WT and 53Bp1−/− MEFs retrovirally infected with a GFP-tagged PTIP construct (GFP–PTIP). c, Quantification of the percentage of pan-nuclear γ-H2AX-positive cells with PTIP foci in WT and 53Bp1−/− MEFs upon treatment with HU (related to Fig. 3a) (n = 150 cells analysed). Experiments were repeated three times. d, Quantification of the percentage of cells with PTIP foci co-localizing with PCNA in WT and 53Bp1−/− MEFs in late S phase (related to Fig. 3b) (n = 150 cells analysed). Experiments were repeated three times. e, Representative immunofluorescence images of PCNA and 53BP1 co-staining in irradiated WT cells (10 Gy) or in late S-phase sites. White arrows indicate that the few 53BP1 foci observed in late S-phase cells do not co-localize with PCNA. (n = 50 cells analysed). Experiments were repeated three times. f, Representative immunofluorescence images of WT and Ptip−/− MEFs treated with 4 mM HU for 2 h and analysed for ssDNA (BrdU) and MRE11 co-localization. Bottom panels shows the quantification of BrdU-positive cells (left) and the percentage of MRE11 co-localization in BrdU-positive cells (right) upon HU treatment in WT and Ptip−/− MEFs. (n = 150 cells analysed). Experiments were repeated three times. g, Quantification of the percentage of cells with MRE11 foci co-localizing with γ-H2AX upon irradiation treatment (10 Gy) in WT and Ptip−/− MEFs (related to Fig. 3e) (n = 150 cells analysed). Experiments were repeated three times. h, Cell cycle profiles in WT and Ptip−/− MEFs as measured by the incorporation of EdU (y axis) vs. DAPI (x axis). i, iPOND coupled to SILAC Mass-Spectrometry analysis for PTIP, H4 and RPA enrichment at stalled forks in 293T cells upon 3 mM HU treatment for 10 min and 4 h (ref. 22). The y axes represent the relative abundance of the indicated proteins on a log2 scale.
Extended Data Figure 6 | See next page for caption.
Extended Data Figure 6 | MLL3/4 promotes replication fork degradation in Brca2−deficient cells. a, Top, schematic of the retroviral PTIP mutant constructs used to identify the domain of PTIP involved in driving replication fork degradation. Different BRCT domains in PTIP are numbered and Q represents the glutamine rich region between the second and the third BRCT domains. Bottom, ratio of IdU versus CldU upon HU treatment of Brca2−/− Ptip−/− B lymphocytes retrovirally infected with either EV, FL, W165R, W663R and Del BRCT 5-6 PTIP-mutant constructs and sorted for GFP or mCherry expression. Numbers in red indicate the mean ± s.d. for each sample (**P ≤ 0.01, ****P ≤ 0.0001, Mann–Whitney test). One hundred and twenty-five replication forks were analysed for each condition. b, WT and Mll3/4−/− MEFs were EdU-labelled for 15 min and treated with 4 mM HU for 4 h. Proteins associated with replication forks were isolated by iPOND and detected by western blotting with the indicated antibodies. c, Quantification of the percentage of cells with MRE11 foci co-localizing with PCNA in late S phase in WT and MLL3/4−/− MEFs (n = 150 cells analysed). Experiments were repeated three times. d–f, Ratio of IdU versus CldU upon HU treatment in WT, Brca1−/−, Brca2−/− Mll4−/−, Mll4−SET−/−, Brca1−/− Mll4−/−, Brca2−/− Mll4−/− and Brca1−/− Mll4−SET−/− B cells. Numbers in red indicate the mean ± s.d. for each sample (NS, not significant, ****P < 0.0001, Mann–Whitney test). At least 125 replication forks were analysed for each genotype. g, Genomic instability measured in metaphase spreads from splenic B cells derived from WT, Brca2−/−, Mll4−/−, Brca2−/− Mll4−/− B cells treated overnight with 1 μM PARPi or with 0.5 μM cisplatin (**P ≤ 0.01, ***P ≤ 0.001, unpaired t-test). Fifty metaphases were analysed per condition. Experiments were repeated three times.
Extended Data Figure 7 | Loss of RIF1 results in chromosomal instability. a. Quantification of RAD51 foci formation in WT, Brca1<sup>−/−</sup> and Rif1<sup>−/−</sup> B cells. Cells were treated with 10 Gy and harvested 4 h after irradiation (NS, not significant, *P ≤ 0.05). At least 100 cells were analysed per condition; experiments were repeated three times. b, c. Genomic instability measured in metaphase spreads from splenic B cells derived from WT, Brca1<sup>−/−</sup> and Rif1<sup>−/−</sup> mice treated overnight with 1 μM PARPi (b) or with 0.5 μM cisplatin (c) (NS, not significant, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, unpaired t-test). Fifty metaphases were analysed per condition; experiments were repeated three times.
Extended Data Figure 8 | See next page for caption.
Extended Data Figure 8 | Multiple mutations can cause resistance to DNA-damaging agents in Brca-deficient cells. a, b, Difference in progression-free survival (PFS) of BRCA2- and BRCA1-mutated ovarian serous adenocarcinoma patients with standard platinum-based regimens. Data were obtained from the TCGA project. Patients were separated into PTIP low- or high-expression on the basis of the 33rd percentile of PTIP expression z-scores. The difference between the PFS of PTIP-low versus PTIP-high was assessed by univariate log-rank P value (P < 0.072 and P < 0.032 in a and b, respectively). Analysis included 38 tumours with BRCA1 mutations and 34 tumours with BRCA2 mutations out of 316 high-grade serous ovarian cancers that underwent whole-exome sequencing. PFS curves for PTIP-low and PTIP-high expressing tumours were generated by the Kaplan–Meier method. All reported P values are two-sided. c, Western blot analysis for CHD4 and PTIP levels in PEO1 cells infected with shNSC and shCHD4. Tubulin was used as loading control. d, Cell cycle profiles in PEO1 cells infected with shNSC and shCHD4 as measured by the incorporation of EdU (y axis) versus DAPI (x axis). e, Immunostaining for MRE11 and PCNA in PEO1 cells infected with shNSC and shCHD4 upon treatment with 4 mM HU. Bottom, quantification for MRE11 recruitment upon HU treatment. At least 100 cells were analysed per condition; experiments were repeated three times. f, Western blot analysis for CHD4 and MRE11 levels in PEO1 cells infected with shNSC and shCHD4. Actin was used as loading control. g, Percentage of EdU-positive cells was analysed 20 h after Brca2−/− B cells were treated with mirin alone, mirin+PARPi or mirin+cisplatin (NS, not significant). EdU was pulsed for 20 min before FACS analysis. Experiments were repeated three times. h, Genomic instability measured in metaphase spreads from B cells derived from Brca2−/− mice pretreated with 25 μM mirin for 2 h followed by overnight treatment with 1 μM PARPi or 0.5 μM cisplatin (NS, not significant, *P ≤ 0.05, **P ≤ 0.001, unpaired t-test). Fifty metaphases were analysed per condition. Experiments were repeated three times. i, Quantification of RAD51 foci formation in WT, Brca1−/−, Parp1−/− and Brca1−/− Parp1−/− B cells treated with 10 Gy irradiation and harvested 4 h after treatment. At least 100 cells were analysed per condition; experiments were repeated three times.
Extended Data Figure 9 | See next page for caption.
Extended Data Figure 9 | Brca2-deficient tumours acquire PARPi resistance without restoration of RAD51 foci formation. a, Schematic depicting the conditional BRCA2 allele of the KB2P (K14CRE;Brca2<sup>fl/fl</sup>;p53<sup>fl/fl</sup>) spontaneous tumour model. b, Outline of the PARPi intervention study. A spontaneous BRCA2-/p53-deficient tumour was generated and re-transplanted into syngeneic WT mice. When the tumours reached 200 mm<sup>3</sup>, they were treated either with vehicle or PARPi AZD2461. c, PARPi response curve of the KB2P tumour (relative tumour volume (rtv) versus days). The treatment for 28 consecutive days was started when the tumour reached 200 mm<sup>3</sup> (rtv = 100%). In response to the treatment, the tumour shrank but eventually grew back. When it reached 100% relative tumour volume, the treatment was repeated (as indicated with red arrows) for another 28 days. This regime was continued until the tumour became resistant to PARPi (black arrow). d, The stability of acquired resistance of the KB2P tumour was confirmed by re-transplanting matched naive and resistant tumours and treating animals either with vehicle or AZD2461 (only one 28-day cycle). Kaplan–Meier survival curve indicates that resistant tumours did not respond to the AZD2461 treatment, while naive tumours exhibited high sensitivity, indicative of a stable genetic mechanism of resistance. e, f, Irradiation-induced RAD51 foci were detected by immunofluorescence in the KB2P donor: RAD51 foci formation was undetectable in naive and resistant tumours, suggesting that HR restoration is not an underlying mechanism of PARPi resistance. Spontaneous tumours from K14 Cre; p53<sup>fl/fl</sup> mice treated with irradiation were used as positive control for RAD51 foci. Unirradiated K14 Cre; p53<sup>fl/fl</sup> cells were used as a negative control. g, Replication fork progression rates measured by tract lengths in micrometres of CldU (red) and IdU (green) in PARPi-naive or PARPi-resistant tumours. Numbers in red indicate the mean ± s.d. for each sample. One hundred and twenty-five replication forks were analysed for each condition. h, Western blot analysis for PTIP and MRE11 levels in PARPi-naive or PARPi-resistant tumours. Tubulin was used as loading control.
Extended Data Figure 10 | Brca2-deficient ESCs have higher levels of chromatin-bound MRE11. 

a, Representative image of a Brca2 hypomorph mouse ESC (denoted Brca2Y3308X) showing MRE11 foci (red) in S phase (identified by PCNA foci (green)). DNA was stained with DAPI (blue).

b, High-throughput microscopy analysis quantifying the overall levels of chromatin-bound MRE11 per individual nucleus in WT and Brca2Y3308X cells treated as indicated (a.u., arbitrary units).
Erratum: Replication fork stability confers chemoresistance in BRCA-deficient cells

Arnab Ray Chaudhuri, Elsa Callen, Xia Ding, Ewa Gogola, Alexandra A. Duarte, Ji-Eun Lee, Nancy Wong, Vanessa Lafarga, Jennifer A. Calvo, Nicholas J. Panzarino, Sam John, Amanda Day, Anna Vidal Crespo, Binghui Shen, Linda M. Starnes, Julian R. de Ruiter, Jeremy A. Daniel, Panagiotis A. Konstantinopoulos, David Cortez, Sharon B. Cantor, Oscar Fernandez-Capetillo, Kai Ge, Jos Jonkers, Sven Rottenberg, Shyam K. Sharan & André Nussenzweig

Nature 535, 382–387 (2016); doi:10.1038/nature18325

It has come to our attention that in this Article, owing to an error during the production process, ‘Brca2’ was mislabelled as ‘Brca1’ in Fig. 4b, and ‘Parp1’ was mislabelled as ‘Ptip’ in Fig. 4e. These errors have been corrected in the online versions of the paper.