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Precision modeling of breast cancer in the CRISPR era

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The CST Complex Mediates End Protection at Double-Strand Breaks and Promotes PARP Inhibitor Sensitivity in BRCA1-Deficient Cells

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Abstract

Selective elimination of BRCA1-deficient cells by inhibitors of poly(ADP-ribose) polymerase (PARP) is a prime example of the concept of synthetic lethality in cancer therapy. This interaction is counteracted by the restoration of BRCA1-independent homologous recombination through loss of factors such as 53BP1, RIF1, and REV7/MAD2L2, which inhibit end resection of DNA double-strand breaks (DSBs). To identify additional factors involved in this process, we performed CRISPR/SpCas9-based loss-of-function screens and selected for factors that confer PARP inhibitor (PARPi) resistance in BRCA1-deficient cells. Loss of members of the CTC1-STN1-TEN1 (CST) complex were found to cause PARPi resistance in BRCA1-deficient cells *in vitro* and *in vivo*. We show that CTC1 depletion results in the restoration of end resection and that the CST complex may act downstream of 53BP1/RIF1. These data suggest that, in addition to its role in protecting telomeres, the CST complex also contributes to protecting DSBs from end resection.

Introduction

The synthetic lethal interaction between BRCA1 deficiency and poly(ADP-ribose) polymerase (PARP) inhibition is a well-established therapeutic paradigm with encouraging response rates in the clinic (Lord and Ashworth, 2017). This has resulted in the recent regulatory approval of three PARP inhibitors (PARPi) for the treatment of serous ovarian cancers and one PARPi, olaparib, for the treatment of *BRCA*-mutated, HER2-negative breast cancers. Moreover, the BRCA-PARP paradigm might be extended beyond breast and ovarian cancer because recent clinical studies indicate that a subset of prostate cancers harbor a homologous recombination (HR) defect and, hence, might benefit from olaparib treatment (Mateo et al., 2015, Pritchard et al., 2016).

Despite this success, long-lasting clinical response rates in patients with advanced disease are limited by the development of resistance, the mechanisms of which have not been fully elucidated. A major class of resistance mechanisms centers on re-expression of functional BRCA1 or BRCA2 protein, either through promoter demethylation, genetic reversion, or gene fusions (Patch et al., 2015, Swisher et al., 2008, Ter Brugge et al., 2016). However, our previous work also identified the existence of additional BRCA1-independent resistance mechanisms in the *K14cre;Brca1^{F/F};p53^{F/F}* (KB1P) genetically engineered mouse model of hereditary breast cancer (Liu et al., 2007). In this model, re-expression of functional BRCA1 is excluded because of the large, engineered, intragenic *Brca1* deletion, which spans multiple exons. Despite the absence of functional BRCA1 restoration, KB1P tumors acquired resistance to PARPi treatment. In addition to activation of the P-glycoprotein drug efflux transporter (Rottenberg et al., 2008), the BRCA1-independent resistance mechanisms in KB1P tumors predominantly involved the partial restoration of HR activity through re-wiring of the DNA damage response (DDR); for example, by loss of 53BP1 (Bouwman et al., 2010, Bunting et al., 2010, Jaspers et al., 2013). These seminal findings have spurred a number of studies in which additional downstream antagonists of end resection were identified, including RIF1 (Chapman et al., 2013, Di Virgilio et al., 2013, Escribano-Díaz et al., 2013, Zimmermann et al., 2013) and REV7/MAD2L2 (Boersma et al., 2015, Xu et al., 2015). However, the currently known resistance factors cannot explain all PARPi-resistant cases, suggesting that additional proteins functioning in this pathway remain to be identified. Moreover, although the loss of resection antagonists partially restores end resection of DNA double-strand breaks (DSBs), none of these factors have direct functions in DNA metabolism, raising the question of how DNA metabolism at DSBs might be altered to stimulate end resection.

The function of the 53BP1 pathway is not exclusive to canonical DSB repair, but it also acts on telomeres (Panier and Boulton, 2014). Because telomere ends resemble DSBs

located at chromosomal termini, cells have evolved several mechanisms to protect telomeres from DSB end processing and chromosome end-to-end fusions (Sfeir and de Lange, 2012). Mammalian telomeres consist of TTAGG repeats ending with a single-strand G-rich overhang. The single-stranded DNA (ssDNA) overhang is crucial in telomere maintenance because it is required for the formation of the T-loop structure (Makarov et al., 1997, McElligott and Wellinger, 1997). Notably, excessive resection of telomere ends is inhibited by the action of the shelterin complex and by the 53BP1 pathway (Lazzerini-Denchi and Sfeir, 2016, Sfeir and de Lange, 2012).

Besides the mechanisms that have evolved to protect telomeric overhangs from excessive processing, it has recently been shown that the RPA-like CTC1-STN1-TEN1 (CST) complex is able to localize to telomeric ssDNA and mediate a fill-in reaction executed by polymerase-alpha (POLA) to buffer resection activity (Feng et al., 2017, Miyake et al., 2009, Wu et al., 2012). Notably, it was demonstrated that the binding of the CST complex to ssDNA is not particularly sequence-specific, although a partial preference for G-rich regions has been described (Hom and Wuttke, 2017, Miyake et al., 2009). Additionally, CST components do not localize exclusively to telomeres (Miyake et al., 2009). This might argue that the CST complex also has non-telomeric functions.

In this study, three independent forward genetic CRISPR/SpCas9-based loss-of-function screening approaches were employed to identify factors that induce PARPi resistance in BRCA1-deficient cells. Together, these screens identified that defects in *Ctc1*, or its CST complex members *Stn1* or *Ten1*, suppress the synthetic lethal interaction between BRCA1 and PARP inhibition. Inactivation of CTC1 is sufficient to drive PARPi resistance *in vivo*. Depletion of CTC1 increased end resection activity and subsequently restored RAD51 focus formation upon ionizing radiation (IR)-induced DNA damage, providing a mechanistic basis for these observations. Moreover, the CST complex facilitates canonical non-homologous end joining (c-NHEJ)-driven repair. Together, these data demonstrate that the CST complex plays a more global role in DNA repair beyond the protection of telomeres.

Results

Forward Genetic CRISPR/SpCas9 Screens Identify Selective Enrichment for Loss of CTC1 during PARPi Treatment in BRCA1-Deficient Cells

To identify factors that modulate the synthetic lethal interaction between BRCA1 and PARP, we carried out three independent forward genetic loss-of-function CRISPR/SpCas9 screens (Figure 1). All screens were analyzed by harvesting cells before and after PARPi treatment, after which single guide RNA (sgRNA) sequences were amplified from genomic DNA by PCR and analyzed by next-generation sequencing. The screening data were processed by the model-based analysis of genome-wide CRISPR-Cas9 knockout (MAGeCK) or the drugZ algorithm (Li et al., 2014, Wang et al., 2017), and the results were sorted on positive selected gene ranks to allow comparison across screens. Additional experimental details are provided in the Supplemental Experimental Procedures.

The first PARPi resistance screen was performed in SpCas9-expressing KB1P-G3 mouse mammary tumor cells (Jaspers et al., 2013) using a custom-made lentiviral sgRNA library targeting 1,752 DDR-related genes (Table S1) cloned into the doxycycline-inducible pLenti-sgRNA-tetR-T2A-PuroR vector (Prahallad et al., 2015). The screen was performed at 100× coverage, and cells were selected with two different PARPis, olaparib and AZD2461 (Oplustil O'Connor et al., 2016), at the approximate inhibitory concentration 90 (IC90) for 14 days (Figure 1A). Although sgRNAs targeting *Tp53bp1* were deliberately removed from the library to avoid the possibility that this potent PARPi resistance factor might obscure the effects of other genes, its upstream regulatory factor *Rnf8* scored among the top genes (Figure 1B).

The second PARPi resistance screen was performed in SpCas9-expressing *Brca1*^{-/-};*Trp53*^{-/-} mouse embryonic stem cells (mESCs) infected with a genome-wide lentiviral sgRNA library targeting 19,150 genes (Koike-Yusa et al., 2014). The screen was performed at 75× coverage in two independent transductions, and cells were selected with olaparib at a concentration of 15 nM for 10 days. As expected, *Tp53bp1* and *Rnf8* scored among the top genes and ranked #1 and #15, respectively (Figure 1C).

A third PARPi resistance screen was performed in *BRCA1*^{2288delT} mutant SUM149PT human breast cancer cells (Elstrodt et al., 2006). SUM149PT cells expressing doxycycline-inducible SpCas9 were lentivirally infected with a genome-wide sgRNA library targeting 18,010 genes (Tzelepis et al., 2016). This screen was performed at 1,000× coverage, and cells were selected in the presence of doxycycline plus 100 nM talazoparib for 2 weeks. The screen was dominated by sgRNAs targeting *PARP1*, the drug target of talazoparib. Although PARP1 loss is expected to be lethal in BRCA1-deficient cells, the selection for

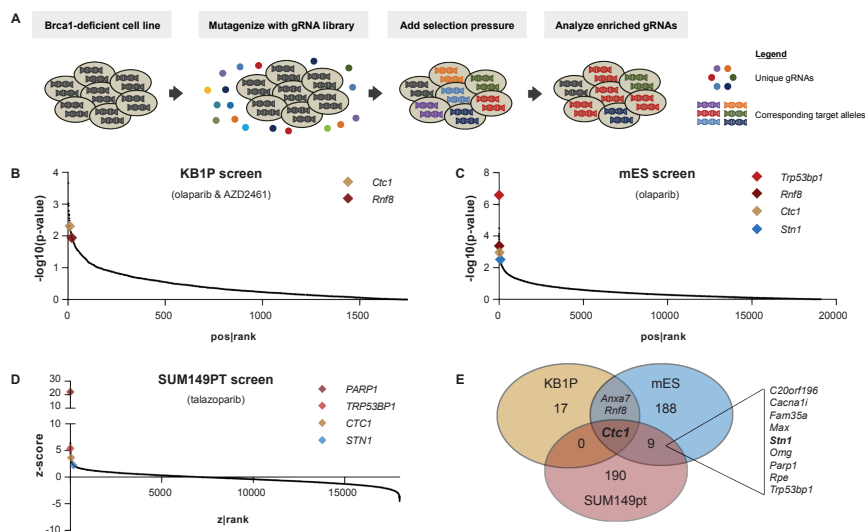


Figure 1 Multiple independent CRISPR/SpCas9 loss-of-function screens identify CTC1 as a driver of PARPi resistance in BRCA1-deficient cells. (A) Schematic overview of the screening approach utilized across the different screens. Each screen was performed on a different cell line and screened with a different library, which is indicated per screen. (B) SpCas9 expressing KB1P-G3 cells were screened with a DNA Damage Response (DDR) focused library, cloned in the doxycycline inducible pLenti-sgRNA-tetR-T2A-PuroR vector, at 100x coverage. Cells were transduced at MOI 0.5 and cultured for 5 days in the presence of puromycin (3 μ g/mL) and doxycycline (3 μ g/mL) to induce sgRNA expression. Cells were next plated for clonogenic growth in the presence of olaparib (75 nM) or AZD2461 (250 nM) for 14 days, after which cells were harvested, sgRNAs were retrieved by PCR and submitted to high-throughput sequencing (HiSeq 2500). The sgRNA abundance in treated populations were compared to the starting population using MAGeCK software. Gene-based p values were log-transformed and plotted based on the positive rank (enrichment). Each dot represents a unique gene. (C) *Brca1*^{-/-};*Trp53*^{-/-} mouse embryonic stem (mES) cells were screened with a genome-wide library in two independent transductions at 75x coverage. After 10 days of culture in the presence of olaparib (15 nM), treated populations were compared to the untreated population cultured for the same period of time using MAGeCK software. Gene based p-values were -log transformed and plotted based on the positive rank (enrichment). Each dot represents a unique gene. (D) A derivative of the *BRCA1* mutant SUM149PT human triple-negative breast tumor cell line carrying a doxycycline-inducible SpCas9 expression construct was lentivirally infected with a genome-wide guide RNA library at >1000x coverage. Cells were cultured in the presence of doxycycline plus 100 nM talazoparib for two weeks. The sgRNA abundance in treated populations were compared to the starting population using drugZ. Gene-based z-scores were log transformed and plotted based on the positive z-rank (enrichment). Each dot represents an individual gene. (E) The top 20 genes in the KB1P-G3 screen and the top 200 genes in the mES and SUM149PT screens were selected and plotted in a Venn diagram to identify consistent outliers.

PARP1 loss in SUM149PT cells might be attributed to residual BRCA1 activity, which might enable cell survival in the absence of PARP1 (Pettitt et al., 2017, Wang et al., 2016). Moreover, *TP53BP1* scored among the top enriched genes and ranked #7 (Figure 1D). The results from these three independent screens were collated to identify consistent outliers. The top 20 genes were selected from the DDR-focused library screen in KB1P-G3 cells. Because the genome-wide libraries contain about 10-fold more genes than the DDR-focused library, the top 200 genes were selected from the mESC and SUM149PT screens, and these were plotted in a Venn diagram (Figure 1E). Notably, *Ctc1* was the only gene that consistently scored in all three screens (ranked #10, #39, and #39 in the KB1P-G3, mESC, and SUM149PT screens, respectively). Moreover, *Stn1* (also known as *Obfc1*) scored in two of three screens. These results caught our attention because both CTC1 and STN1 are members of the CST complex. Although the CST complex has known functions in telomere metabolism, these PARPi resistance screens might point toward non-telomeric functions of the CST complex. Because *Ctc1* was a top hit in all three independent screens in both mouse and human cells, we prioritized this gene for further validation.

Depletion of CTC1 Suppresses the Synthetic Lethal Interaction between BRCA1 Deficiency and PARP Inhibition

To validate the effect of CTC1 on PARPi sensitivity in BRCA1-deficient cells, we transfected KB1P-G3 cells with pX330 vectors containing three sgRNAs targeting a putative oligonucleotide-binding (OB) fold domain of *Ctc1* (Figure 2A). The polyclonal targeted populations were efficiently modified for the target site (Figures 2B–2D), as shown by TIDE (tracking of insertions or deletions [indels] by decomposition) analysis (Brinkman et al., 2014). These populations were subsequently treated with olaparib (75 nM) or AZD2461 (250 nM), the same concentrations as used for the screen. As expected, parental KB1P-G3 cells or KB1P-G3 cells targeted by a non-targeting sgRNA (sgNT) showed high sensitivity to PARPi treatment. In contrast, *Ctc1*-targeted cells showed resistance to treatment, indicating that depletion of CTC1 suppresses the synthetic lethal interaction between BRCA1 deficiency and PARP inhibition (Figures 2E and F). This could not be attributed to an effect on cell proliferation because we observed no difference in the doubling time upon depletion of CTC1 (Figure 2G).

We next investigated whether *Ctc1*-mutated cells would be specifically selected out from a mixed population by prolonged PARPi treatment. A competition assay was performed in which the evolution of polyclonal populations was monitored by the TIDE algorithm to quantify changes in allele distributions. sgRNAs were cloned in the pLenti-sgRNA-tetR-T2A-Puro vector and introduced in SpCas9-expressing KB1P-G3 cells by lentiviral transduction. The population was mutagenized by doxycycline-induced expression of

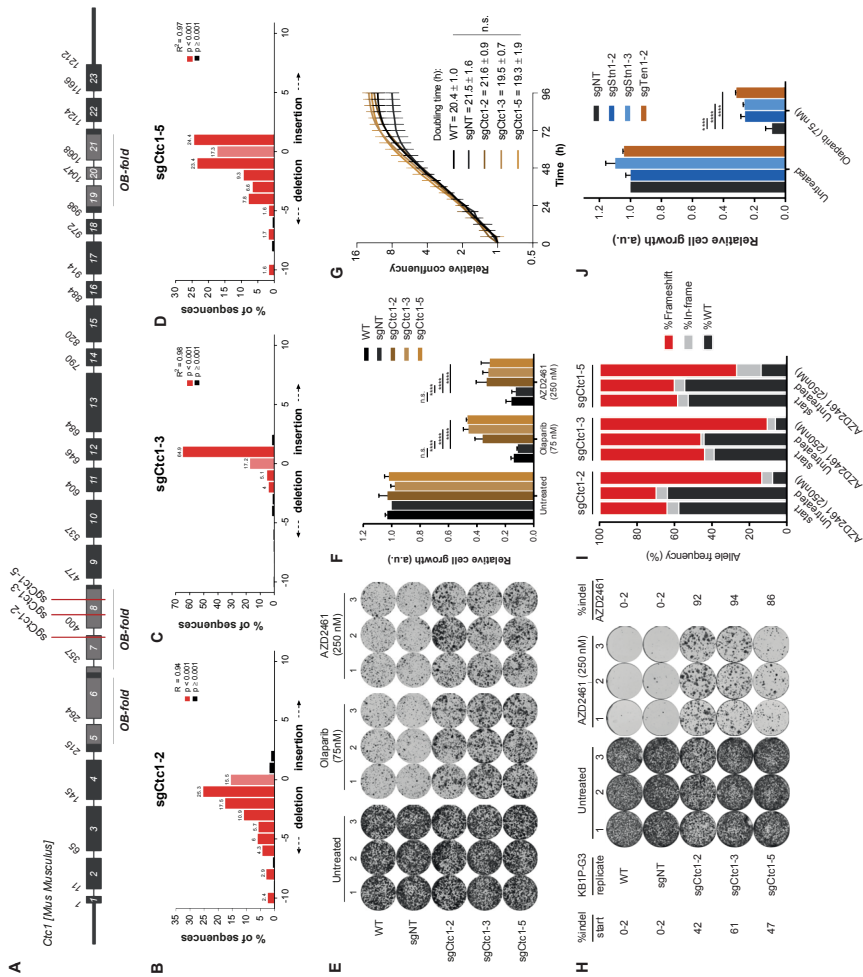


Figure 2

Depletion of CTC1 suppresses the synthetic lethal interaction between BRCA1 deficiency and PARP inhibition. (A) Schematic overview of the *Ctc1* gene in which putative OB-fold domains and sgRNA target locations are indicated (Adapted from Miyake et al., 2009). (B-D) KB1P-G3 cells were transfected with the pX330puro vector in which the indicated sgRNA was cloned. After selection, genomic DNA was isolated and the target region was PCR amplified to verify allele modification using TIDE software. (E) Indicated *Ctc1*-mutated KB1P-G3 cell lines were plated for clonogenic growth and were untreated for 7 days or treated with olaparib (75 nM) or AZD2461 (250 nM) for 10 days. Cells were fixed and stained with crystal violet. Three independent experiments were performed and each condition was plated in triplicate. One representative well per condition is shown for each independent experiment. (F) Quantification of crystal violet staining of (E). Crystal violet was solubilized with 10% acetic acid and measured with the Tecan Infinite 200 PRO plate reader at 562 nM. For each independent experiment the average value for each triplicate measurement was calculated. Data were plotted in GraphPad using relative to the growth of untreated sgNT cells and are presented as mean $\pm$ SD ($n = 3$ independent experiments). Significance was calculated by two-way ANOVA with the Dunnett's multiple comparisons test (* *** = adjusted p -value 0.0001). (G) Relative cell proliferation was determined by IncuCyte Zoom Live – Cell Analysis System measurements. Each data point represents the average of three independent experiments, and in each experiment six replicate wells were measured and averaged. Data represent mean $\pm$ SD ($n = 3$). Doubling times (exponential growth equation) were calculated using GraphPad software, and significance was calculated by two-way ANOVA with the Dunnett's multiple comparisons test. (H-I) SpCas9 expressing KB1P-G3 cells were transduced with doxycycline-inducible pLenti-sgRNA-tetR-T2A-PuroR vectors containing indicated sgRNAs. Cells were selected with puromycin (3 μ g/mL) for 5 days and during the same time period sgRNA expression was induced by doxycycline (3 μ g/mL). Polyclonal populations, containing roughly equal distributions of wildtype and mutated alleles, were plated for clonogenic growth in the presence or absence of AZD2461 (250 nM). Cells were passaged every 10 days for a total of three times. At the end point, wells were fixed and stained by crystal violet and allele distributions were determined from each condition using TIDE software. (J) KB1P-G3 cells were transfected with pX330puro vectors containing sgRNAs targeting *Stn1* and *Ten1*, and cultured in the presence or absence of 75 nM olaparib as in (2E). Data were analyzed as in (2F).

the sgRNA for 5 days, after which cells were plated without doxycycline for clonogenic growth. After 10 days of culture in the presence or absence of AZD2461, the cells were harvested and re-plated at equal amounts every 10 days for an additional two rounds, resulting in a total treatment duration of 30 days. Although non-transduced cells or cells transduced with a non-targeting sgRNA were effectively killed by this prolonged treatment, *Ctc1*-targeted cells survived (Figure 2H). This coincided with an enrichment of *Ctc1* frameshift mutations compared with untreated populations, which were kept in culture for the same duration (Figure 2I).

To study whether this effect is CTC1-specific or a feature of the CST complex, we genetically inactivated the other two CST complex members *Stn1* and *Ten1*, and treated these cells with olaparib under the same conditions as used for *Ctc1*. CRISPR/SpCas9-mediated disruption of *Stn1* or *Ten1* also induced PARPi resistance, recapitulating the effect of *Ctc1* (Figure 2J). This is consistent with the identification of STN1 in the PARPi resistance screens (Figure 1E) and shows that PARPi sensitivity is modulated by all CST complex members rather than CTC1 alone.

These data were corroborated in *Brca1*^{-/-};*Trp53*^{-/-} mESCs in which CRISPR/SpCas9-assisted inactivation of *Ctc1* increased survival upon olaparib treatment, which was accompanied by a selection for frameshifting alleles (Figures S1A and S1B). Furthermore, we targeted the CST complex members in R26^{CreERT2};*Brca1*^{Sc^o/Δ} mESCs, which harbor a selectable conditional *Brca1*^{Sc^o} allele that can be inactivated by CreERT2 through the addition of 4-hydroxytamoxifen (4-OHT) (Bouwman et al., 2010). Although 4-OHT-induced inactivation of BRCA1 caused lethality in untransduced R26^{CreERT2};*Brca1*^{Sc^o/Δ} mESCs, clonal outgrowth was observed for cells depleted of CTC1, STN1, or TEN1 (Figure 3A). Complete switching of the conditional *Brca1*^{Sc^o} allele in the surviving population was confirmed by PCR, ruling out that clonal outgrowth was due to a non-recombined *Brca1*^{Sc^o} allele (Figure 3B). Finally, depletion of CTC1 in SUM149PT cells enhanced cell survival in the presence of talazoparib, as did depletion of 53BP1 (Figure 3C), confirming that this effect was not restricted to mouse cells.

In summary, we confirmed that the CST complex promotes PARPi-induced cell lethality in BRCA1-deficient cells. We therefore looked at the role of the CST complex in preventing global DNA damage, focusing on CTC1.

CTC1 Antagonizes End Resection at Non-Telomeric DSBs

During the repair of DSBs, a critical decision is made between initiating repair via NHEJ or via HR, which both require distinct end processing. This decision is tightly balanced by end protection factors, such as 53BP1 or RIF1, which antagonize resection to direct

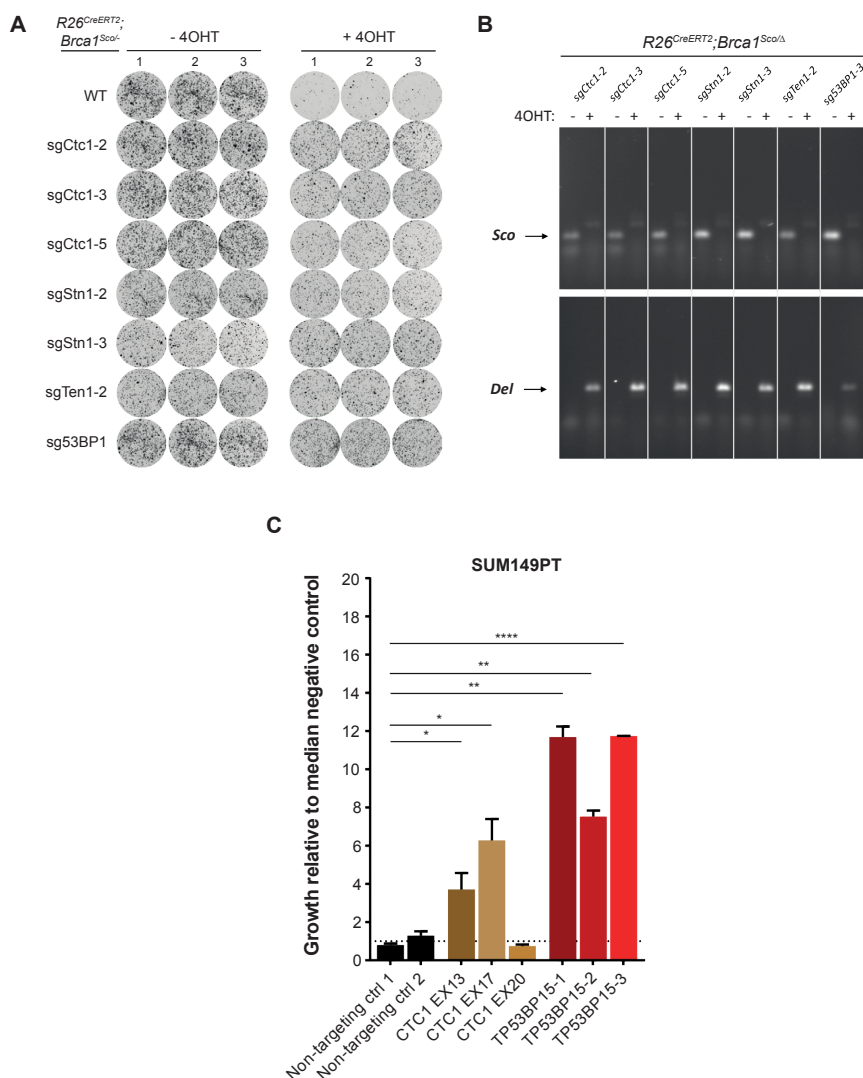


Figure 3 Loss of CST complex members induces PARPi resistance in BRCA1-deficient mouse embryonic stem cells and SUM149PT breast cancer cells. (A) The CST complex members *Ctc1*, *Stn1* and *Ten1* were targeted in *R26^{CreERT2}; Brca1^{Sco^Δ}* mouse embryonic stem (mES) cells using pLentiCRISPRv2 vectors. Following transduction and selection, the *Brca1-Sco* allele was recombined by activation of CreERT2 via addition of 4-OHT, after which cells were plated out for clonogenic growth. Cells were fixed and stained with crystal violet. (B) *Brca1* alleles from surviving populations were PCR amplified using specific primers to detect *Brca1^{Sco}* (*Sco*) and recombined *Brca1^{ΔSco}* (*DelSco*) alleles. (C) *BRCA1*-mutant SUM149PT cells were transfected with the EditR CRISPR system and the indicated crRNA and then continuously cultured in the presence of 50 nM talazoparib over a 14-day period, at which point cell viability was estimated by use of CellTiter-Glo reagent. Median effect from three independent experiments are shown. Error bars represent SEM. **p* = 0.0415 and 0.0201, respectively; ***p* = 0.0013 and 0.0011, respectively; *****p* = <0.0001; Student's *t* test.

repair via NHEJ, and BRCA1, which promotes end resection to direct repair via HR (Chapman et al., 2013, Daley and Sung, 2014, Escribano-Díaz et al., 2013, Feng et al., 2015, Panier and Boulton, 2014). It was previously shown that the end resection defect in BRCA1-deficient cells can be rescued via loss of 53BP1, and this also rescued cell lethality induced by BRCA1 loss (Bouwman et al., 2010, Bunting et al., 2012). Hence, the finding that loss of the CST complex (Figure 3A) rescued BRCA1 lethality points toward a potential inhibitory role in DSB end resection. Moreover, depletion of CTC1 did not induce PARPi resistance in BRCA2-deficient cells (Figures S2A and S2B), which is in line with a possible role of the CST complex upstream of BRCA2.

DSB end resection produces ssDNA overhangs, which are protected from nucleolytic degradation and the formation of secondary structures by the coating of RPA. Therefore, we visualized RPA loading in response to α -particle-induced DNA damage by immunofluorescence as a readout for end resection (Stap et al., 2008). In line with previous studies (Tkáč et al., 2016, Xu et al., 2015), KB1P-G3 tumor cells showed a clear resection defect that was partially restored in *Ctc1*-mutated KB1P-G3 cells but not in sgNT-transfected control cells (Figures 4A and 4B).

We next investigated whether CTC1 loss affects the recruitment of DDR factors to sites of irradiation-induced DNA damage. CRISPR/SpCas9-targeted KB1P-G3 cells were either left untreated or treated with 10 Gy of IR, which potently induced γ H2AX foci (Figures 4C and 4D; Figures S3A and S3B). Although depletion of 53BP1 in KB1P-G3 cells abolished the formation of IR-induced 53BP1 and RIF1 foci, these effects were not observed in CTC1-depleted cells (Figures 4E and 4F and S3C and S3D). Despite the capacity to form 53BP1 and RIF1 foci, KB1P-G3 cells that were depleted of CTC1 restored IR-induced RAD51 focus formation, whereas sgNT-transfected control cells were deficient for this activity (Figures 4G and S3E). Similar conclusions were obtained when DNA damage was induced by treatment with 500 nM olaparib for 24 hr (Figures S3A and S3F–S3M). As expected, PARPi treatment resulted in more heterogeneous DNA damage induction compared with IR because PARP inhibition primarily exerts its cytotoxic effects during replication.

We then tested whether CTC1 loss resulted in productive HR events in conditional BRCA1-deficient R26^{CreERT2}; *Brca1*^{Sc0/Δ}; *Pim1*^{DR-GFP/WT} mESC cells carrying a stably integrated DR-GFP reporter (Bouwman et al., 2013). These cells were transfected to transiently express mCherry and I-SceI, and the percentage of mCherry/GFP double-positive cells was quantified by fluorescence-activated cell sorting (FACS) 24 hr later. Switching of the conditional *Brca1*^{Sc0} allele impaired HR activity, which was partially rescued upon depletion of the CST complex (Figures S3N and S3O).

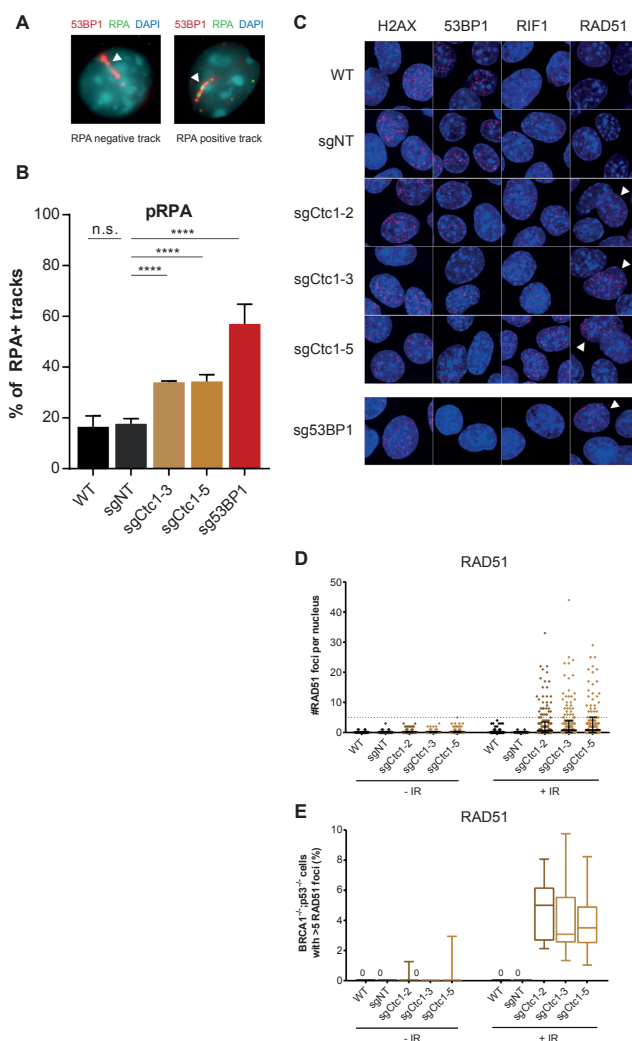


Figure 4 CTC1 functions as a resection antagonist on non-telomeric DSBs. (A-B) CTC1 depletion induces RPA-coated ssDNA overhangs at sites of DNA damage in BRCA1-deficient KB1P cells. (A) Representative images of RPA-negative and RPA-positive 53BP1-labeled alpha tracks in indicated CRISPR/SpCas9-targeted KB1P-G3 cells (highlighted by the white arrowhead). (B) RPA co-localization was quantified 1h after irradiation with a ²⁴¹Am point-source. The experiment was performed three times, and in each independent experiment a minimum of 100 tracks were analyzed. Data is plotted as mean $\pm$ SEM. Significance was calculated by unpaired two-tailed students t-test (** = p-value < 0.01). (C-E) CTC1 depletion restores formation of DNA damage-induced RAD51 foci in BRCA1-deficient cells. (C) Representative confocal images of CRISPR/SpCas9 expressing KB1P-G3 cells targeted with indicated sgRNAs. Cells were stained 3 hours after 10 Gy of ionizing radiation (IR) for indicated proteins. RAD51-positive cells are highlighted by the white arrowhead. (D-E) The experiment was performed three times, and five different confocal fields were imaged per independent experiment (63x magnification). Confocal images were analyzed automatically using an ImageJ macro. The macro detects nuclei based on DAPI intensity and then counts the number of foci within each confocal field. (D) Data plotted as #RAD51 foci per nucleus. (E) Data plotted as percentage of RAD51-positive cells (≥ 5 foci) per field. See also Figure S3.

Together, these data support a role for CTC1 as a resection antagonist acting on non-telomeric DSBs and as a mediator of the HR defect in BRCA1-deficient cells.

CTC1 Facilitates c-NHEJ-Mediated Repair at Telomeric and Non-Telomeric DSBs

It was previously shown that 53BP1, RIF1, and REV7/MAD2L2 antagonize resection and promote c-NHEJ (Boersma et al., 2015, Bouwman et al., 2010, Bunting et al., 2010, Chapman et al., 2013, Di Virgilio et al., 2013, Escribano-Díaz et al., 2013, Xu et al., 2015, Zimmermann et al., 2013). However, this is not a universal phenotype for resection antagonists because it is not shared by HELB (Tkáč et al., 2016). We therefore sought to determine whether CTC1 affects NHEJ activity. First, we used *Terf2^{-/-};Trp53^{-/-}* mouse embryonic fibroblasts (MEFs) that express a temperature-sensitive TRF2^{lle468Ala} mutant (TRF2ts) (Konishi and de Lange, 2008). TRF2ts is functional and maintains intact TRF2-protected telomeres at 32°C, but it dissociates from telomeres at 37°C–39°C, inducing a DDR response and end-to-end chromosome fusions (Konishi and de Lange, 2008). It was previously demonstrated that these fusions are driven by c-NHEJ and can be rescued by depletion of RNF8, DNA ligase IV, or REV7/MAD2L2 (Boersma et al., 2015, Celli and de Lange, 2005, Peuscher and Jacobs, 2011, Smogorzewska et al., 2002).

We depleted CTC1 in TRF2ts MEFs grown under permissive conditions (Figure 5A), which did not affect cell cycle distribution (Figures S4A and S4B). Cells were then grown at the non-permissive temperature (39°C) for 24 h to uncap telomeres and induce a DDR response prior to harvesting metaphase spreads for telomere fluorescence *in situ* hybridization (FISH). Although chromosome fusions were readily observed in control cells upon temperature-induced TRF2 inactivation, this was significantly reduced in *Ctc1*-mutated cells (Figures 5B and 5C; Figures S4C–S4E). In line with this finding and with NHEJ being inhibited by long ssDNA overhangs, it was previously shown that depletion of CTC1 increased ssG overhang length (Chen et al., 2012, Gu et al., 2012).

We next assessed whether CTC1 depletion in mouse CH12 B cells affects the ability to undergo class switch recombination (CSR) as a measure for non-telomeric c-NHEJ capacity (Muramatsu et al., 2000). CH12 cells were transfected with *Ctc1*-targeting CRISPR/SpCas9 constructs and subcloned to obtain *Ctc1*-mutated CH12 cell clones. Notably, only 2 of 96 tested clones showed heterozygous *Ctc1* allele disruption, and no homozygous knockouts were obtained (Figures S4F and S4G), raising the possibility that complete loss of CTC1 is lethal in CH12 cells. Wild-type and heterozygous *Ctc1* knockout clones were subsequently stimulated with CD40Ab, interleukin-4 (IL-4), and transforming growth factor β -1 (TGF- β -1, CD40Ab, IL-4, and TGF β -1 [CIT]) to induce CSR from immunoglobulin M (IgM) to IgA, which was monitored by flow cytometry. Interestingly, heterozygous knockout of *Ctc1* significantly diminished CSR in both clones (Figures 5D and 5E). We therefore conclude that CTC1 facilitates DSB repair via c-NHEJ at both telomeric and non-telomeric regions.

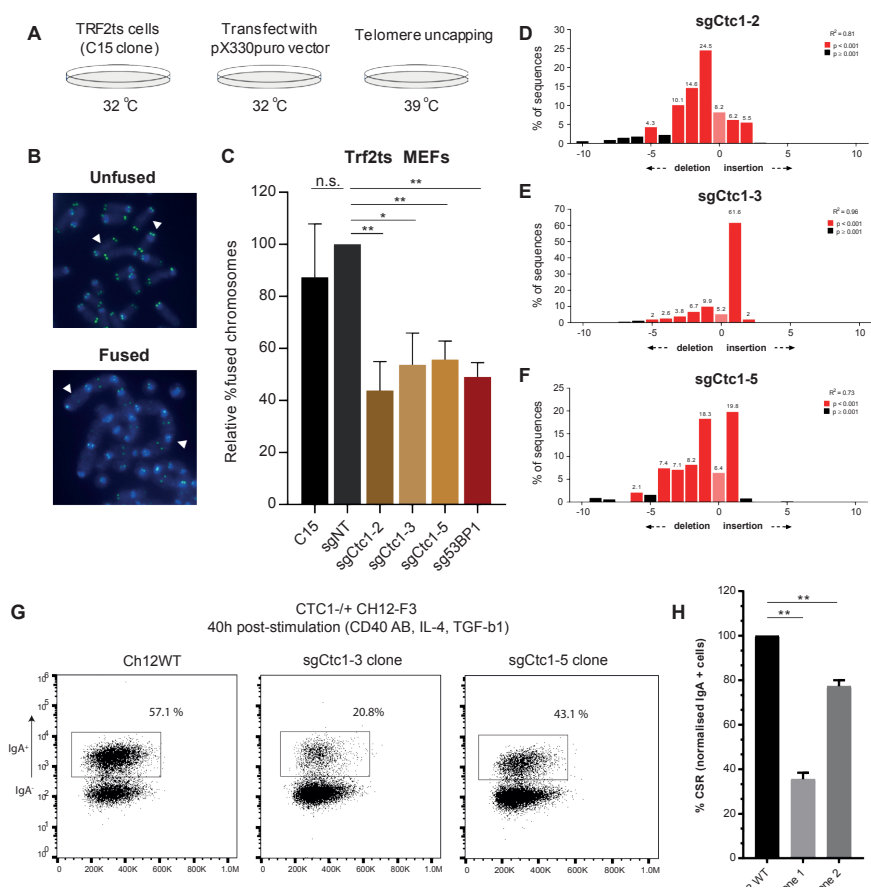


Figure 5 CTC1 facilitates c-NHEJ at telomeric and non-telomeric DSBs. (A-C) CTC1 depletion suppresses end-to-end fusions of uncapped telomeres. (A) Schematic overview of the telomere fusion assay. TRF2ts mouse embryonic fibroblasts (MEFs) of indicated genotypes were cultured at the non-permissive temperature (39°C) for 24h before harvesting. (B) Representative images of metaphase spreads showing chromosomes unfused or fused at their telomeres (examples highlighted by white arrowheads). Chromosomes were stained with DAPI and a telomere specific-FISH probe (green). (C) Metaphases were detected and imaged automatically by Metafer. Three independent experiments were performed, in each independent experiment >2000 chromosomes were counted manually. Genotypes were blinded during counting. Data is plotted as mean $\pm$ SEM (n = 3). Significance was determined by unpaired two-tailed students t-test (* $\leq$ 0.05; ** $\leq$ 0.01). (D-E) Heterozygous inactivation of *Ctc1* impairs IgM to IgA class switch recombination (CSR) in CH12 B cells. (D) FACS analysis of CH12 clones of indicated genotype, 40 h after induction of CSR by incubation with CD40Ab, IL-4 and TGF β -1 (CIT). (E) Quantification of FACS data, representing mean $\pm$ SD of two independent experiments. Significance was calculated by unpaired students t-test (** = p-value $\leq$ 0.01).

Depletion of CTC1 Mediates PARPi Resistance in the KB1P Mouse Model

Last, we explored the *in vivo* effects of CTC1 on the treatment response of BRCA1-deficient tumors to PARP inhibition. We analyzed whether *Ctc1* mRNA expression levels were altered in our previously generated collection of BRCA1- and p53-deficient KB1P and KB1PM mouse mammary tumors with acquired resistance to PARP inhibition (Jaspers et al., 2013). In total, this collection comprises 60 treatment-naïve tumors and 85 matched PARPi-resistant tumors derived from 23 unique donors. To examine the expression levels of *Ctc1* in treatment-naïve and PARPi-resistant tumors, we produced RNA sequencing (RNA-seq) data for all tumors (E.G., unpublished data) and obtained the normalized expression values using edgeR (Robinson et al., 2010). We observed that the expression of *Ctc1* is significantly downregulated in PARPi-resistant tumors compared with naïve tumors ($p = 6.34 \times 10^{-4}$) (Figure 6A). Moreover, in tumors for which copy number variation by sequencing (CNVseq) data were available, CTC1 mRNA downregulation correlated with CNV loss (Figures S5A and S5D). Although a similar correlation was observed for STN1 and TEN1, these factors were not significantly downregulated in resistant tumors (Figures S5B, S5C, S5E, and S5F).

Finally, we used mammary tumor organoid technology (Duarte et al., 2018) to perform an *in vivo* intervention study with the PARPi olaparib in mice carrying tumors derived from isogenic KB1P organoids with or without disruption of *Ctc1*. For this purpose, KB1P4 organoids, derived from a KB1P mammary tumor, were cultured *ex vivo* and co-transduced with lentiviruses produced from pCMV-SpCas9 and pLenti-sg*Ctc1*-tetR-T2A-Puro vectors. Control organoids were generated by co-transduction with pCMV-SpCas9 and pLenti-sgNT-tetR-T2A-Puro lentivirus encoding a non-targeting sgRNA (Figure 6B). The transduced KB1P4 tumor organoids were orthotopically transplanted in mice that were left untreated or treated daily with the PARPi olaparib for 56 consecutive days when tumors reached a size of 50–100 mm³. As expected, the *Ctc1* target site was efficiently disrupted in tumors derived from KB1P4 organoids transduced with pCMV-SpCas9 and pLenti-sg*Ctc1*-tetR-T2A-Puro (Figures 6C and 6D). Although KB1P4 control tumors only relapsed after treatment was stopped, CTC1-depleted tumors relapsed during treatment, resulting in accelerated mammary tumor-related death (median latencies: 39 days for sg*Ctc1*_2 and 42 days for sg*Ctc1*_3 cohorts compared with 73 days for control animals; log rank test, $p = 0.0019$ and $p = 0.0086$, respectively; Figure 6E). These data confirmed that depletion of CTC1 confers PARPi resistance in BRCA1-deficient tumors *in vivo*.

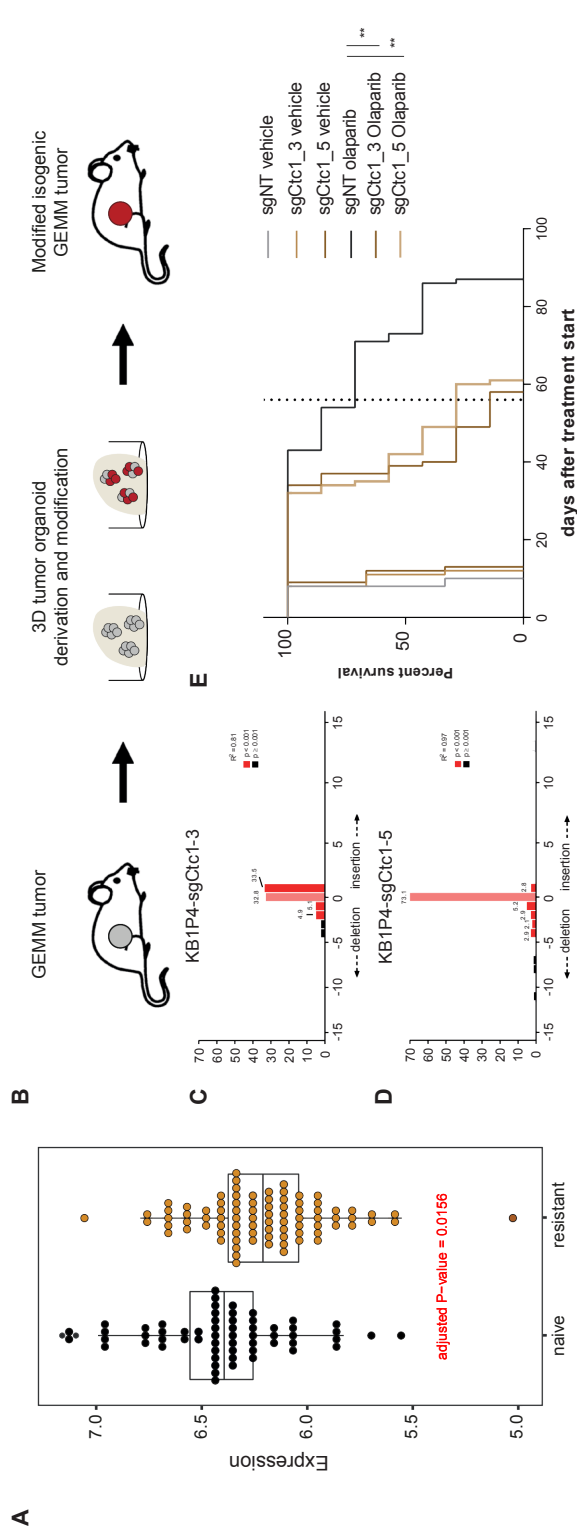


Figure 6 Depletion of CTC1 induces PARP inhibitor resistance *in vivo*. (A) mRNA expression levels of *Ctc1* in matched treatment-naïve and PARPi-resistant BRCA1-deficient mouse mammary tumors. Y-axis indicates the $\log_2$ (Counts per Million) value. (B) Schematic overview of the generation of isogenic *Ctc1*-mutated and control tumors via *ex vivo* manipulation of tumor organoids. (C-D) Example TIDE plots of untreated mammary tumors derived from *Ctc1*-mutated KB1P4 tumor organoids of the indicated genotype. (E) Survival of mice orthotopically transplanted with modified KB1P4 tumor organoids. Mice were stratified into untreated ($n = 3$) or olaparib-treated (100 mg/kg intraperitoneally, daily for 56 consecutive days; $n = 7$) groups when tumors reached a size of 50-100mm³. Significance was calculated by the Log-rank (Mantel-Cox) test (** = $p < 0.01$).

Discussion

In this study, we show that loss of the CST complex members CTC1, STN1, and TEN1 induces PARPi resistance in tumors with irreversible loss of function of BRCA1. Our data highlight the CST complex as a pathway for tumor cells to escape the synthetic lethal effects of PARPi by restoring HR independently of BRCA1. In particular, we demonstrate that the underlying mechanism is a restoration of end resection of DSBs. Together, our findings demonstrate that the CST complex contributes to the regulation of DNA end stability not only at telomeres but also at non-telomeric DSBs.

We and others have recently shown that the 53BP1-RIF1-REV7/MAD2L2 pathway is crucial for blocking end resection of DSBs (Boersma et al., 2015, Bouwman et al., 2010, Bunting et al., 2010, Chapman et al., 2013, Di Virgilio et al., 2013, Escribano-Díaz et al., 2013, Xu et al., 2015, Zimmermann et al., 2013). However, it has remained elusive how DNA end stability is regulated by 53BP1-RIF1-REV7/MAD2L2 because none of these factors have been shown to contain direct DNA binding capacity and do not contain DNA processing activities. Our finding that the CST complex functions as a resection antagonist at DSBs sheds light on this puzzle. The CST complex is an RPA-like complex that can directly bind ssDNA via multiple OB folds (Miyake et al., 2009). In collaboration with the laboratory of Dan Durocher, we recently identified another RPA-like complex, the Shieldin (SHLD) complex, which is composed of SHLD1 (C20ORF196), SHLD2 (FAM35A), SHLD3 (FLJ26957/CTC-534A2.2), and REV7/MAD2L2, as a downstream effector of 53BP1 in DSB repair (unpublished data). Hence, in addition to RPA and the SHLD complex, the CST complex is another trimeric complex that contains direct DNA binding capacity and affects DSB end stability. How these three complexes are recruited to DSBs in time and space remains to be elucidated. Possibly, RPA, SHLD, and CST compete for ssDNA at resected DSBs or collapsed forks to either promote or antagonize HR. Not mutually exclusive with this model, it is conceivable that these complexes might contain specialized functions dependent on the ssDNA substrate since the CST complex has been reported to preferentially bind to and promote melting of G-rich regions and G4-quadruplexes (Bhattacharjee et al., 2017, Lue et al., 2013).

Future work is also required to elucidate whether these complexes form the final step in the regulation of DSB end stability (for instance, through steric hindrance) or whether additional downstream factors are involved. Intriguingly, the CST complex has been described to buffer resection at telomeres via POLA-dependent fill-in DNA synthesis, which is required to prevent excessive telomere erosion (Lazzerini-Denchi and Sfeir, 2016). Our finding that the CST complex antagonizes resection at non-telomeric DSBs raises the question of whether this is dependent on POLA activity. Resection can possibly be antagonized not only by shielding the ends of DSBs from end-processing

activities but also by directly counteracting ongoing resection via fill-in DNA synthesis. This buffering activity might fine-tune the length of ssDNA around the DSB, which is vulnerable for nucleolytic degradation, and it might provide a rescue mechanism in case HR cannot be completed.

The identification of the CST complex as a mediator of PARPi response in BRCA1-deficient tumors might also have clinical implications because loss-of-function mutations in the CST-encoding genes are predicted to cause clinical PARPi resistance. Moreover, we expect that these alterations provide therapeutic vulnerabilities because we recently found that depletion of the 53BP1-dependent DNA repair pathway enhances sensitivity to IR (M.B., unpublished data).

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Author Contributions

Conceptualization, M.B., J.J., and S.R.; Methodology, M.B., S.A., S.J.P., B.E., and J.B.; Software and Formal Analysis, M.B., J.B., J.J., and S.R.; Investigation, M.B., S.A., S.J.P., I.d.K., H.G., S.J.R., C.L., J.F., F.F.S., and R.B.; Resources, B.E. and E.G.; Writing, M.B., J.J., and S.R.; Supervision, M.v.d.V., D.C.v.G., J.J.L.J., R.C., C.J.L., J.J., and S.R.; Funding Acquisition, D.C.v.G., J.J.L.J., R.C., C.J.L., J.J., and S.R.

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Materials and Methods

Cell culture

The KB1P-G3 cell line was previously established from a *K14cre;Brca1^{F/F};Trp53^{F/F}* (KB1P) mouse mammary tumor and cultured as described (Jaspers et al., 2013). The KB2P-3.4 cell line was previously established from a *K14cre;Brca2^{F/F};Trp53^{F/F}* (KB2P) mouse mammary tumor and cultured as described (Evers et al., 2008). Briefly, these cell lines were cultured in DMEM/F-12 medium (Life Technologies) in the presence of 10% FCS, penicillin/streptomycin (Gibco), 5 µg/mL insulin (Sigma), 5 ng/mL epidermal growth factor (Life Technologies) and 5 ng/mL cholera toxin (Gentauro) under low oxygen conditions (3% O₂, 5% CO₂ at 37°C). SUM149PT cells were cultured in Ham's F12 medium (Gibco) supplemented with 5% FCS, 5 µg/ml insulin, 1 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MI, USA). Mouse ES cells with a selectable conditional *Brca1* deletion (*R26^{CreERT2/wt};Brca1^{SCo/Δ}*) (Bouwman et al., 2013) were cultured on gelatin-coated plates in 60% buffalo red liver (BRL) cell conditioned medium supplied with 10% fetal calf serum, 0.1 mM β-mercaptoethanol (Merck) and 10³ U/ml ESGRO LIF (Millipore) under normal oxygen conditions (21% O₂, 5% CO₂, 37°C). CH12F3 cell lines were cultured in RPMI supplemented with 5 % NCTC-109 medium, 10% FCS, 100 U/ml penicillin, 100 ng/ml streptomycin and 2 mM L-glutamine at 37°C with 5% CO₂ under ambient oxygen conditions. The KB1P4 3D tumor organoid line was previously established from a *Brca1^{-/-};p53^{-/-}* mouse mammary tumor and cultured as described (Duarte et al., 2017). Briefly, cells were seeded in Basement Membrane Extract Type 2 (BME, Trevigen) on 24-well suspension plates (Greiner Bio-One) and cultured in AdDMEM/F12 supplemented with 1 M HEPES (Sigma), GlutaMAX (Invitrogen), penicillin/streptomycin (Gibco), B27 (Gibco), 125 µM N-acetyl-L-cysteine (Sigma), 50 ng/mL murine epidermal growth factor (Invitrogen).

PARP inhibitors

Olaparib (CAS 763113-22-0) and AZD2461 (CAS 1174043-16-3) were synthesized by and purchased from Syncom (Groningen, The Netherlands). Talazoparib was purchased from Selleckchem (Catalog No.S7048).

Plasmids, transfection and transduction

Plasmids

pCMV-SpCas9 and pLenti-sgRNA-tetR-T2A-Puro were described previously (Prahallad et al., 2015). Genome-wide mouse lentiviral CRISPR sgRNA library was a gift from Kosuke Yusa (Addgene #50947). Human Improved Genome-wide Knockout CRISPR Library v1

was a gift from Kosuke Yusa (Addgene #67989). pX330-U6-Chimeric_BB-CBh-SpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230). pLentiCRISPRv2 was a gift from Feng Zhang (Addgene plasmid # 52961). The MCherry/I-SceI plasmid has been described previously (Bouwman et al., 2013).

Generation of CRISPR/SpCas9 plasmids

Unless otherwise stated, KB1P-G3 experiments were performed using a modified version of the pX330 backbone (Cong et al., 2013) in which a puromycin resistance ORF was cloned under the hPGK promoter (Tim Harmsen, *Nucleic Acids Research* 2017 (in press)). sgRNA sequences were cloned in the pX330puro backbone using custom DNA oligos (IDT) which were melted, annealed and subsequently ligated with quick-ligase (NEB) into BbsI-digested backbone. A similar procedure was used for cloning into the pLenti-sgRNA-tetR-T2A-Puro vector, but using BfuAI-digested backbone. sgRNA sequences are provided in Table S2. All constructs were sequence verified by Sanger sequencing.

Transfection-based genome editing

Transfection in KB1P-G3 cells was performed using TransIT-LT1 (Mirus) reagents following manufacturer's recommendations. In brief, 150,000 cells were plated in 6-well format 1 day before transfection with 1 µg DNA. The medium was refreshed 24 hours after transfection and transfected cells were selected with puromycin for three days. CRISPR/SpCas9 targeted SUM149PT cells were generated with editR crRNA (Dharmacon, Lafayette, CO, USA).

Lentiviral transduction-based genome editing

Cell lines targeted with the pCMV-SpCas9 and pLenti-sgRNA-tetR-T2A-Puro system were generated by lentiviral transduction. Lentivirus was produced in HEK293FT cells as described previously (Follenzi et al., 2000) and mouse KB1P-G3 or *Brca1*^{-/-};*p53*^{-/-} mES cells were infected overnight using polybrene (8 µg/mL). The medium was refreshed after 12 hours and transduced cells were selected with puromycin (3 µg/mL) and blasticidin (500 µg/mL) for five consecutive days. KB1P4 tumor organoids were transduced using spinoculation as described previously (Duarte et al., 2017; Koo et al., 2011). Expression of the sgRNA was induced by incubation with 3 µg/ml doxycycline (Sigma) for at least five days.

Genomic DNA isolation, PCR amplification and TIDE analysis

Allele modification frequencies were quantified from genomic DNA isolated from tumor and cell line samples using Gentra Puregene (Qiagen) according to the manufacturer's protocol. Target loci were amplified by PCR using the following conditions: (1) 98 °C, 30 s, (2) 30 cycles of 98 °C for 10 s, 61 °C for 20 s and 72 °C for 30 s, (3) 72 °C, 5 min.

Reaction mix consisted of 0.75 μ l DMSO, 5 μ l GC Phusion Buffer 5X, 0.5 μ l 2 mM dNTPs, 0.125 μ l 100 μ M Fwd oligo, 0.125 μ l 100 μ M Rev oligo, 0.25 μ l Phusion polymerase in 25 μ l total volume. Amplified products were diluted 50X in dH₂O and 2 μ l of diluted product was submitted for Sanger sequencing to confirm target modification using the TIDE algorithm (Brinkman et al., 2014). Parental cells were used as a reference sequence. PCR primer sequences are provided in Table S3.

CRISPR library screens

The first PARPi resistance screen was performed in the KB1P-G3 tumor cell line, which was previously established from a KB1P tumor (Jaspers et al., 2013). This cell line is BRCA1- and p53-deficient through large intragenic deletions and shows sensitivity to PARPi treatment in the nanomolar range. The DDR sgRNA library was generated based on the gene list from Thanos Halazonetis (University of Geneva) described before (Costantino et al., 2014; Xu et al., 2015) and the NCBI search (terms: “DNA repair”, “DNA damage response”, “DNA replication”, “telomere-associated genes”) . See Table S1 for the full library details. This search resulted in a DDR-related gene list comprising a total of 1,752 genes (Table S1). *Tp53bp1* was specifically removed from this list in anticipation that loss of 53BP1 might dominate the screen results and thereby obscure the effects of other genes. sgRNAs targeting the 1,752 DDR-related genes were synthesized (MYcroarray) and cloned into the pLenti-sgRNA-tetR-T2A-PuroR vector, which allows for doxycycline inducible expression of the sgRNA (Prahallad et al., 2015). KB1P-G3-SpCas9 expressing cells were generated by transduction with the pGSC_SpCas9_Neo vector and transduced cells were selected by 500 μ g/mL G418. The pLenti-sgRNA-tetR-T2A-Puro-DDR library was introduced at 100x coverage. Next, doxycycline was added to the medium for 5 days to mutagenize the population. Cells were subsequently plated in a clonogenic growth format in the presence of the PARP inhibitors olaparib or AZD2461 (Oplustil O’Connor et al., 2016) at the approximate IC₉₀ concentration for 14 days. Cells were harvested before and after PARPi treatment for genomic DNA isolation. Subsequently, sgRNA sequences were amplified from genomic DNA by two rounds of PCR amplification as described previously (Duarte et al., 2017) and sequenced with the HiSeq 2500, using the following barcodes: GTAGCC, TACAAG, CTCTAC, GCGGAC, TTTCAC, GGCCAC. Sequencing reads were aligned to the reference sequences using edgeR software (Robinson et al., 2010). The screening data were processed by the MAGeCK algorithm (Li et al., 2014), and results were sorted on MAGeCK-based positive selected gene ranks to allow comparison across screens.

The second PARPi resistance screen was performed in BRCA1- and p53-deficient, SpCas9-expressing mouse embryonic stem (mES) cells infected with a genome-wide lentiviral sgRNA library targeting 19,150 genes (Koike-Yusa et al., 2014). The screen was

performed at 75x coverage in two independent transductions (MOI 0.5) and cells were first selected with puromycin and subsequently treated with olaparib at a concentration of 15 nM for 10 days. Surviving populations were harvested and processed as described previously (Koike-Yusa et al., 2014), using the following barcodes CGTGAT, ACATCG, GCCTAA, TGGTCA, CACTGT, ATTGGC, GATCTG, TCAAGT, CTGATC, AAGCTA, GTAGCC, TACAAG, TTGACT, GGAAC. The screening data were processed similar to the screen in KB1P-G3 cells.

A third PARPi resistance screen was performed in the SUM149PT human breast cancer cell line. This cell line harbors the *BRCA1*^{2288delT} mutation and LOH (Elstrodt et al., 2006). A derivative of SUM149PT with an integrated tetracycline-inducible SpCas9 was lentivirally infected with a genome-wide sgRNA library designed to target 18,010 genes (Tzelepis et al., 2016), using a multiplicity of infection of 0.3 and infecting >1000 cells per sgRNA. After puromycin selection (3 µg/ml) to remove non-transduced cells, a sample was removed (time or t=0); remaining cells were cultured in the presence or absence of doxycycline plus 100 nM talazoparib, a concentration which normally results in complete inhibition of the cell population. No cells survived in the absence of doxycycline. After two weeks of selection, genomic DNA from the remaining cells in the doxycycline-treated sample was recovered. The sgRNA sequences from this genomic DNA were PCR amplified using barcoded and tailed primers and deep sequenced as previously described (Koike-Yusa et al., 2014) to identify sgRNAs in the talazoparib-resistant population. Read counts were normalized for coverage by converting to parts per ten million (pptm) reads and fold change between starting and resistant population was calculated for each guide. Fold change values were log- and Z-transformed and plotted based on z-rank (Wang et al., 2017).

Clonogenic survival assay

For *R26^{CreERT2/wt};Brca1^{SCo/Δ}* cells, Cre-mediated inactivation of the endogenous mouse *Brca1^{SCo}* allele was achieved by overnight incubation of cells with 0.5 µmol/L 4-OHT (Sigma) (Bouwman et al., 2010). Four days after switching, cells were seeded in triplicate at 10,000 cells per well in 6-well plates for clonogenic survival assay. For experiments with *R26^{CreERT2/wt};Brca1^{SCo/Δ}* p53-null cells, cells were plated without treatment or in the presence of olaparib 2.5 nM. Cells were stained with 0.1% crystal violet one week later, and scanned with the Gelcount (Oxford Optronix). Automated quantification of colony counts was performed using the Gelcount colony counter software.

Clonogenic survival assay with PARPi (olaparib) were performed as described previously with minor modifications (Xu et al., 2015). CRISPR/SpCas9 transfected KB1P-G3 cells were seeded in triplicate at 5 x 10³ cells per well into 6-well plates on day 0, and

then olaparib or AZD2461 was added at the indicated concentrations. On day 6, the untreated group was fixed, the other groups were fixed on day 9 and stained with 0.1% crystal violet. Plates were scanned with the Gelcount (Oxford Optronix). Quantifications were performed by solubilizing crystal violet using 10% acetic acid and the absorbance at 562nm was measured using the Tecan plate reader. The experiment was performed three times.

SUM149PT-SpCas9 cells were transfected with *CTC1* targeting crRNA and tracrRNA using the EditR system (Life Technologies), plated into 48 well plates and then treated with 50 nM talazoparib. Medium was replaced with fresh drug-containing medium as indicated. Images were taken to measure viability of cells in each well every 12 hours using the IncuCyte system. After two weeks, final viability was assessed using CellTiter Glo (Promega).

Competition assays

Competition assays were performed in KB1P-G3 SpCas9 expressing cells, transduced with pLenti-sgRNA-tetR-T2A-Puro vectors containing the indicated sgRNAs. Cells were selected with puromycin (3 µg/mL) for three days and allowed to recover from selection. A sample was harvested for gDNA isolation at $t = 0$, and 5,000 cells were plated in 6-well plates in triplicate per condition, with or without AZD2461 (250 nM). After 10 days of treatment, cells were harvested, counted and re-plated at 5,000 cells per 6-well two times (total treatment time of 30 days). On the last time point, each condition was plated as technical duplicate. At the end point, one technical duplicate well was fixed and stained with crystal violet and the other was used to isolate gDNA. Allele distributions were determined from gDNA samples by PCR followed by Sanger sequencing and TIDE analysis, as described above. Competition assays in KB2P-3.4 cells were performed similarly, except 2,000 cells were seeded per 6-well and cells were treated with olaparib (50 nM).

Growth curves

Growth curves were generated by seeding 1,000 cells per well in 96-well plates, seeding 6 technical replicates per experiment and the well confluency was recorded every 4 hours for 120 hours using an IncuCyte Zoom Live – Cell Analysis System (Essen Bioscience). The images were analyzed using IncuCyte Zoom software. Data were normalized to the confluency at 20h after seeding.

Alpha track assay

Experiments were performed as described previously (Xu et al., 2015) with small modifications. CRISPR/SpCas9 transfected KB1P-G3 cells were seeded on coverslips overnight, washed with PBS and covered with a mylar foil, allowing α -particle irradiation from above, through mylar. Irradiation was done using a ^{241}Am point- source by moving the source over the coverslip for 30s per area, cells were incubated for 1 hour at 37 °C and washed with ice-cold PBS. Subsequently, cells were extracted with cold CSK buffer (10 mM HEPES-KOH, pH 7.9, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl_2 , 1 mM EGTA, 0.5% (v/v) Triton X-100) and cold CSS buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl_2 , 1% (v/v) Tween20, 0.5% (w/v) sodium deoxycholate) for 5 min each before fixation in 4% PFA in PBS for 30 min at room temperature. Fixed cell were washed 2 times for 10 minutes in PBS (0.1% Triton X-100) and washed for 30 minutes in blocking solution (0.5% BSA and 0.15% glycine in PBS). Primary antibodies were diluted in blocking buffer and incubated overnight at 4 °C. Hereafter, cells were washed 2 times for 10 minutes in PBS (0.1% Triton X-100) and 1 time shortly in blocking buffer. Secondary antibodies were diluted in blocking buffer and cells were incubated for at least 1 hour at room temperature in the dark. Finally, cells were washed 2 times in PBS and coverslips were mounted using Vectashield with DAPI. Quantification was done as described previously. Primary antibodies used in this study were as follows: rabbit anti-53BP1 (NB100-304, Novus), 1:1000 dilution; mouse anti-RPA34-20 (Ab-3, CalBiochem), 1:1000 dilution; MRE11 antibody (de Jager et al., 2001), 1:250 dilution. Secondary antibodies used in this study were as follows: Alexa Fluor 594 goat anti-rabbit IgG (A 31631, Invitrogen), Alexa Fluor 488 goat anti-mouse IgG (Thermo Fisher Scientific Cat# A-11001, RRID: AB_2534069), 1:1000 dilution.

Foci formation experiments

RAD51 immunofluorescence in CRISPR/SpCas9 transfected KB1P-G3 cells was performed as described previously, with minor modifications (Xu et al., 2015). Cells were grown on 8-well chamber slides (Millipore). Ionizing-Radiation Induced Foci (IRIF) were induced by γ -irradiation (10 Gy) 4 hours prior to sample preparation. Hereto, cells were washed in PBS++ and fixed with 2% PFA/PBS++ for 20' on ice. Fixed cells were washed with PBS++ and were permeabilized for 20' in 0.2% Triton X-100/PBS++. All subsequent steps were performed in staining buffer (PBS++, BSA (2%), glycine (0.15%), Triton X-100 (0.1%)). Cells were washed 3x and blocked for 30' at RT, incubated with the 1st antibody for 2hrs at RT, washed 3x and incubated with the 2nd antibody for 1hr at RT. Antibodies were diluted in staining buffer. Last, cells were mounted and counterstained using Vectashield mounting medium with DAPI (H1500, Vector Laboratories). Primary antibodies used: rabbit-anti-RAD51; 70-001, BioAcademia, 1:1,000 dilution; rabbit-anti-53BP1; Abcam

Ab21083, 1:2,000 dilution; rabbit-anti-RIF1 was a gift by Ross Chapman, 1:1,000 dilution; mouse anti γ H2AX: Millipore JBW301, 1:1,000 dilution; Alexa fluor 568 F(ab')₂ Fragment goat anti-rabbit; A21069, Invitrogen, 1:400 dilution. Z-stack images were acquired using a confocal microscope (Leica SP5, Leica Microsystems GmbH) and five different confocal fields were imaged (63x objective). Confocal images were analyzed automatically using an ImageJ script (Xu et al., 2015). Briefly, the macro detects nuclei based on DAPI intensity and subsequently counts the number of foci within each nucleus.

DR-GFP

The DR-GFP was performed as described previously (Bouwman et al., 2013).

Assessment of telomere NHEJ

Trf2^{-/-};*Trp53*^{-/-};TRF2ts MEFs (TRF2ts MEFs) were described before (Peuscher and Jacobs, 2011) and maintained at the permissive temperature of 32°C in DMEM with 100 U penicillin, 0.1 mg ml⁻¹ streptomycin, 2 mM l-glutamine and 10% FBS. They were grown for 24 h at the non-permissive temperature of 39°C to inactivate TRF2 and induce NHEJ dependent chromosome end to end fusions as a consequence of telomere uncapping. Cell harvesting, preparation of metaphase spreads and telomere FISH with an Alexa488-labeled C-rich Telomere probe (PN-TC060-005, Panagene/Eurogentec) for metaphase chromosome analysis was done as described before (Boersma et al., 2015).

Digital images of metaphases were captured using the Metafer4/MSearch automated metaphase finder system (MetaSystems, Germany) equipped with an AxioImager Z2 microscope (Carl Zeiss, Germany). After scanning metaphase preparations at 10x magnification, high-resolution images of metaphases were acquired using a 'Plan-Apochromat' 63x/1,40 oil objective.

The cell cycle distribution of TRF2ts MEFs with or without CRISPR/SpCas9 mediated disruption of *Ctc1* or *Tp53bp1* was determined by propidium-iodide staining, acquired on a FACSCalibur (Beckton Dickinson) and analyzed with FlowJo (TreeStar, Ashland, OR) software.

CSR assay

Ctc1-mutated CH12F3 cells were generated by nucleofection (Amaxa Nucleofector 2b, Lonza) with 2 μ g of plasmid and Cell Line Nucleofector Kit R (Lonza), using program D-023. Isogenic cell clones were isolated by limiting dilution and mutated clones were identified by native PAGE resolution of PCR amplicons of the target site, and subsequent confirmation by Sanger sequencing. Immunoglobulin CSR was performed as described previously (Xu et al., 2015). Briefly, CH12 cells were either mock-treated

or stimulated with agonist anti-CD40 antibody (0.5 mg/ml; eBioscience; HM40-3), mouse IL-4 (5 ng/ml; R&D Systems) and TGF- β 1 (1.25 ng/ml; R&D Systems). Cell-surface IgA expression was determined by flow cytometry, immunostaining with biotinylated antimouse IgA antibody (eBioscience; 13-5994), and Alexa488-streptavidin conjugate (Life Technologies).

RNA isolation & RNA-seq analysis

RNA was isolated from fresh-frozen KB1P and KB1PM tumor tissues (Jaspers et al., 2013; Gogola et al, unpublished data). Fresh-frozen tumor tissues were subjected to high-speed shaking in 2 ml microcentrifuge tubes containing 1 ml of TRIsure reagent (Bioline) and stainless steel beads (TissueLyser LT, Qiagen; 10 min, 50 Hz, room temperature). Homogenized lysates were further processed for RNA isolation following TRIsure manufacturer's protocol. Quality and quantity of the total RNA was assessed by the 2100 Bioanalyzer using a Nano chip (Agilent, Santa Clara, CA). Total RNA samples having RIN>8 were subjected to library generation.

Strand-specific libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina Inc., San Diego, RS-122-2101/2) according to the manufacturer's instructions (Illumina, Part # 15031047 Rev. E). Briefly, polyadenylated RNA from intact total RNA was purified using oligo-dT beads. Following purification, the RNA was fragmented, random primed and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, part # 18064-014) with the addition of Actinomycin D. Second strand synthesis was performed using Polymerase I and RNaseH with replacement of dTTP for dUTP. The generated cDNA fragments were 3' end adenylated and ligated to Illumina Paired-end sequencing adapters and subsequently amplified by 12 cycles of PCR. The libraries were analyzed on a 2100 Bioanalyzer using a 7500 chip (Agilent, Santa Clara, CA), diluted and pooled equimolar into a 10 nM sequencing stock solution.

Illumina TruSeq mRNA libraries were sequenced with 50 base single reads on a HiSeq2000 using V3 chemistry (Illumina Inc., San Diego). The resulting reads were trimmed using Cutadapt v.1.12 (Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, 17(1), pp. 10-12) to remove any remaining adapter sequences, filtering reads shorter than 30 bp after trimming to ensure good mappability. The trimmed reads were aligned to the GRCm38 reference genome using STAR v.2.5.2b (Dobin et al., 2013), QC statistics from Fastqc v.0.11.5 (www.bioinformatics.babraham.ac.uk/projects/fastqc) and the above-mentioned tools were collected and summarized using Multiqc (v.0.8; (Ewels et al., 2016)). Gene expression counts were generated by featureCounts (v.1.5.2; (Liao et al., 2014)) using gene definitions from Ensembl GRCm38 version 76. The genes with counts per million (CPM) larger than one at least 20% of total number of samples were taken and used for

further analysis. Then, trimmed mean of M-value (TMM) normalization was performed to obtain normalized expression using edgeR (Robinson et al., 2010) and voom (Law et al., 2014) was used to obtain the DEGs in the comparison of naïve and resistant tumors. The genes with FDR<0.05 were defined as the DEGs.

In vivo studies

All animal experiments were approved by the Animal Ethics Committee of The Netherlands Cancer Institute (Amsterdam, the Netherlands) and performed in accordance with the Dutch Act on Animal Experimentation (November 2014).

Tumor organoids were allografted in mice as described previously (Duarte et al., 2017) with minor adjustments. Briefly, tumor organoids were collected, incubated with TripLE at 37°C for 5', dissociated into single cells, washed in PBS, resuspended in tumor organoid medium and mixed in a 1:1 ratio of tumor organoid suspension and BME in a cell concentration of 10^4 cells per 40 μ l. Subsequently, 10^4 cells were transplanted in the fourth right mammary fat pad of 6-9 week-old NMRI nude mice. Mammary tumor size was determined by caliper measurements and tumor volume was calculated ($0.5 \times \text{length} \times \text{width}^2$). Treatment of tumor bearing mice was initiated when tumors reached a size of 50-100 mm³, at which point mice were stratified into the untreated (n = 3) or olaparib treatment group (n = 7). Olaparib was administered at 100 mg/kg intraperitoneally for 56 consecutive days. Animals were sacrificed with CO₂ when the tumor reached a volume of 1,500mm³. The tumor was collected, fixed in formalin for histology and several tumor pieces were harvested for DNA analysis.

Supplementary Figure Legends

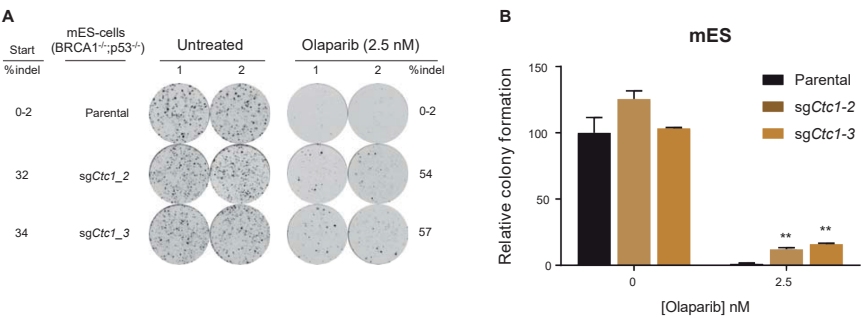


Figure S1
Depletion of CTC1 induces PARPi resistance in *Brca1*^{-/-}; *p53*^{-/-} mouse embryonic stem (mES) cells. (A) Parental and *Ctc1*-mutated *Brca1*^{-/-}; *p53*^{-/-} mES cells were plated for clonogenic growth in the presence or absence of olaparib (2.5 nM) for 7 days before wells were fixed and stained with crystal violet. The experiment was performed in duplicate. *Ctc1* was mutated using the pLenti-sgRNA-tetR-T2A-PuroR vector containing the indicated gRNAs. Allele distributions were determined from the starting population and the surviving population after treatment. (B) Quantification of (A). The number of colonies was determined using GelCount software. Data represent the relative number of colonies compared to the parental untreated mES cells. Data represent mean $\pm$ SD. P-values were determined by unpaired t-test ($P = 0.0074$ and $P = 0.0017$, respectively).

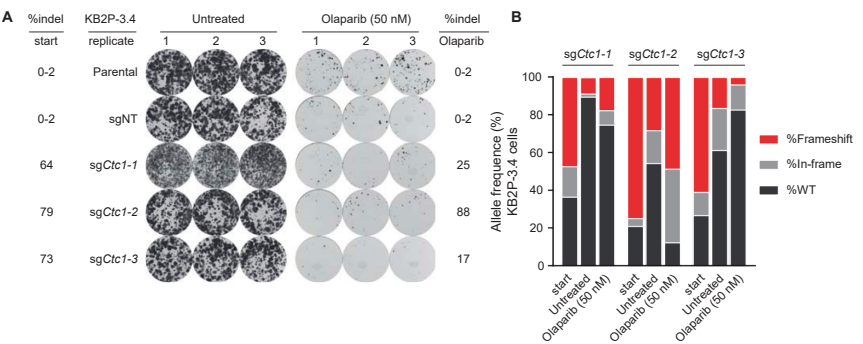
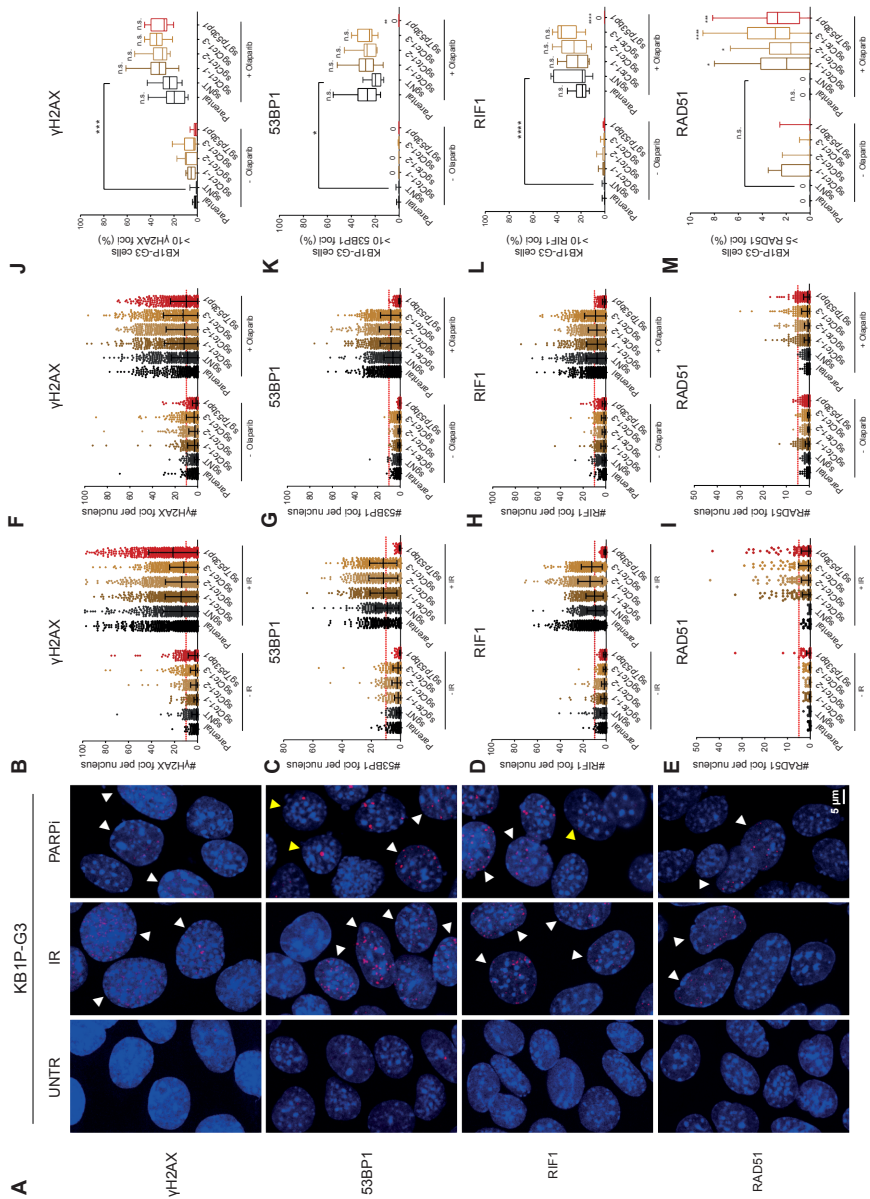


Figure S2
Depletion of CTC1 does not improve PARPi survival in BRCA2-deficient cells. (A-B) Parental, sgNT or *Ctc1*-mutated KB2P-3.4 SpCas9 expressing cells were plated at 2,000 cells per 6-well for clonogenic growth in the presence or absence of olaparib (50 nM) for 10 days. Then, cells were harvested and re-plated at 2,000 cells per 6-well under the same treatment for 10 days, and this was repeated one more time (total treatment duration 30 days). *Ctc1* was mutated using the pLenti-sgRNA-tetR-T2A-PuroR vector containing the indicated gRNAs. Cells were selected with puromycin (3 μ g/mL) and gRNA expression was induced with doxycycline (3 μ g/mL) for 5 days. Allele distributions were determined from the starting, untreated and olaparib-treated populations and the percentage indel is shown and plotted in **b**.



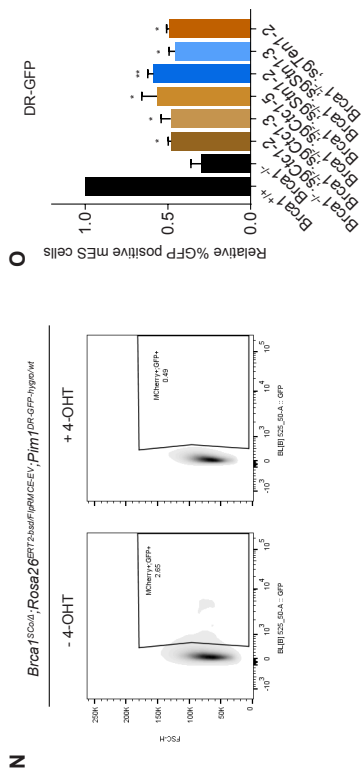


Figure S3

CTC1 functions as a resection antagonist on non-telomeric DSBs. (A-F) Quantifications of confocal images shown in figure 4C. KB1P-G3 SpCas9 expressing cells of indicated genotype were irradiated (10 Gy) and harvested 3 hours later for immunofluorescence of indicated proteins. The experiment was performed three times, and five different confocal fields were imaged per independent experiment (63x magnification, approximately 50 cells per field). Confocal images were analyzed automatically using an ImageJ macro. The macro detects nuclei based on DAPI intensity and then counts the number of foci within each ROI. Data is plotted as #foci per nucleus **a**, **c** and **e** and as percentage of positive cells (≥ 5 foci) per field **b**, **d** and **f**. The following proteins were visualized: γ H2AX **a** and **b**, 53BP1 **c** and **e** and pLentiCRISPR2 vectors containing indicated sgRNAs. Cells were treated with 4-OHT to inactivate the *Brca1*^{Scd} allele and subsequently transfected with mCherry/*I-SceI* constructs. HR activity was determined by flow cytometry and was calculated as the percentage of GFP+ cells in the mCherry+ population relative to BRCA1 proficient parental cells.

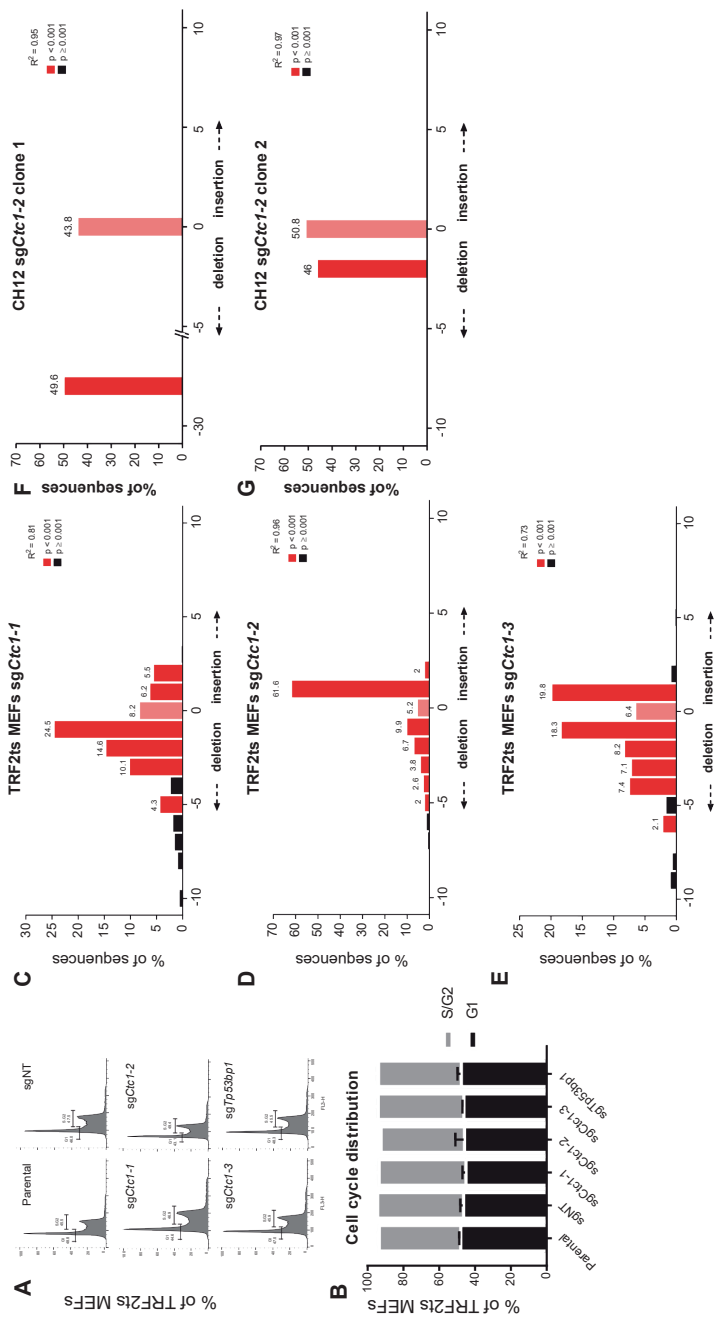


Figure S4 CTC1 facilitates cNHEJ at telomeric and non-telomeric DSBs. (A-B) Cell cycle distribution profiles were determined by flow cytometry from TRF2ts MEFs transfected with pX330puro vectors containing indicated sgRNAs. The experiment was performed two times. (C-D) TIDE plots of TRF2ts MEFs targeted by indicated sgRNAs. (E-F) TIDE plots of the two CH12 clones that were successfully targeted with sgCtcf1-2.

