# The Shieldin complex mediates 53BP1-dependent DNA repair

Sylvie M. Noordermeer[1,2]*, Salomé Adam[1]*, Dheva Setiaputra[1]*, Marco Barazas[3], Stephen J. Pettitt[4], Alexanda K. Ling[5], Michele Olivieri[1,6], Alejandro Álvarez-Quilón[1], Nathalie Moatti[1], Michal Zimmermann[1], Stefano Annunziato[3], Dragomir B. Krastev[4], Feifei Song[4], Inger Brandsma[4], Jessica Frankum[4], Rachel Brough[4], Alana Sherker[1,6], Sébastien Landry[1], Rachel K. Szilard[1], Meagan M. Munro[1], Andrea McEwan[1], Théo Goullet de Rugy[1], Zhen-Yuan Lin[1], Traver Hart[7], Jason Moffat[6,8], Anne-Claude Gingras[1,6], Alberto Martin[5], Haico van Attikum[2], Jos Jonkers[3], Christopher J. Lord[4], Sven Rottenberg[3,9] and Daniel Durocher[1,6#]

1 Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, ON, M5G 1X5, Canada

2 Department of Human Genetics, Leiden University Medical Center, Einthovenweg 20, 2333 ZC, Leiden, The Netherlands

3 Division of Molecular Oncology and Molecular Pathology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

4 The CRUK Gene Function Laboratory and Breast Cancer Now Toby Robins Research Centre, The Institute of Cancer Research, London, SW3 6JB, United Kingdom.

5 Department of Immunology, University of Toronto, Medical Sciences Building, Toronto, ON M5S 1A8, Canada

6 Department of Molecular Genetics, University of Toronto, ON, M5S 1A8, Canada

7 Department of Bioinformatics and Computational Biology, University of Texas MD Anderson Cancer Center, Houston, Texas, USA

8 Donnelly Centre, University of Toronto, Toronto, ON, M5S 3E2, Canada.

9 Institute of Animal Pathology, Vetsuisse Faculty, University of Bern, Laenggasstrasse 122, 3012 Bern, Switzerland

*Co-first authors / # corresponding author

**53BP1 regulates DNA double-strand break repair by suppressing the 5'-3' nucleolytic resection of DNA termini. How 53BP1 shields DNA ends is unknown. Here, we describe the identification of a 53BP1 effector complex, Shieldin, which includes C20orf196 (SHLD1), FAM35A (SHLD2), CTC-534A2.2 (SHLD3) and REV7/MAD2L2. Shieldin localizes to break sites in a 53BP1- and RIF1-dependent manner and its SHLD2 subunit binds to ssDNA via predicted OB-fold domains that are analogous to those of RPA1 and POT1. Loss of Shieldin impairs non-homologous end-joining, causes hyper-resection and leads to defective immunoglobulin class switching. Mutations in Shieldin subunit genes also cause resistance to poly(ADP-ribose) polymerase inhibition in BRCA1-deficient cells and tumors due to restoration of homologous recombination. Finally, we show that ssDNA binding by SHLD2 is critical for Shieldin function, consistent with a model where Shieldin protects DNA ends to mediate 53BP1-dependent DNA repair.**

In eukaryotes, the repair systems that protect the genome from DNA double-strand breaks (DSBs) are canonical non-homologous end-joining (cNHEJ), alternative end-joining (A-EJ) and homologous recombination (HR)[1,2]. cNHEJ operates throughout interphase but is inhibited by DNA end-resection, the initiating step of both HR and A-EJ[1]. Conversely, end-protection favors cNHEJ, and thus the processes that modulate DNA end resection control DSB repair pathway choice[3]. End-resection is limited by 53BP1, a nucleosome-binding protein[4,5] that accumulates on the chromatin flanking DSB sites. DSB detection also leads to 53BP1 phosphorylation by the ATM kinase, triggering its interaction with two other proteins (PTIP[6] and RIF1[7-12]) that participate in 53BP1-dependent DNA repair[1]. REV7 (MAD2L2) also acts in the 53BP1 pathway, downstream of RIF1[13,14]. How 53BP1 and its associated proteins limit end-resection is unknown but two models best explain 53BP1 action: in one model, the 53BP1 complex strengthens the nucleosomal barrier to the enzymes that mediate DNA end resection[5,15], whereas in a second model, 53BP1 organizes the recruitment of effector proteins with end-protection activity. However, none of the current 53BP1 pathway proteins harbour biochemical activities that suggest an ability to mediate end-protection, and thus our understanding of the mechanisms governing DSB repair pathway choice remains incomplete.

To discover new proteins acting in the 53BP1 pathway, we searched for genes whose mutation restores HR in BRCA1-deficient cells, leading to resistance to poly(ADP-ribose) polymerase (PARP) inhibition, a phenotype that is a hallmark of 53BP1 deficiency[16-18]. We undertook two sets of CRISPR/Cas9 screens that entailed the transduction of BRCA1-deficient cells expressing Cas9 with lentiviral libraries of single-guide (sg) RNAs (Fig 1a). Following transduction, the resulting pools of edited cells were exposed to a near-lethal concentration of either one of two clinical PARP inhibitors (PARPi), olaparib or talazoparib[19]. We screened both an engineered human RPE1-hTERT *TP53*$^{-/-}$ *BRCA1-KO* cell line (hereafter referred to as RPE1 *BRCA1-KO*) that is highly sensitive to PARPi, and SUM149PT triple negative breast tumour cells that carry a naturally occurring hemizygous *BRCA1* frameshift mutation (c.2288delT)[20]. The gene-based results of the screens are found in Extended Data Table 1.

Visualization of the 20 top-ranking genes from each screen in a Venn diagram indicated that genes coding for 53BP1 and for the uncharacterized protein C20orf196 were top hits in all three screens (Fig 1b and Extended Data Table 1). Among other high-ranking genes in the olaparib-resistance screens, we identified *SCAF1* and *ATMIN*, which encode an SR-family protein and a transcription factor, respectively (Fig 1b and Extended Data Table 1). *PARP1* was a strong hit in the SUM149PT talazoparib-resistance screen, as expected[21], whereas the genes coding for most of the pathway components leading to 53BP1 accumulation at DSB sites (H2AX, MDC1, RNF8 and RNF168) along with RIF1, were hits in the RPE1 *BRCA1-KO* screen (Extended Data Table 1). The presence of 53BP1 and other 53BP1-pathway proteins strongly suggested that these screens could reveal hitherto uncharacterized 53BP1 effectors.

As initial validation of the results, we used a competitive growth assay (Fig 1c). As expected, sgRNAs targeting *53BP1* or *RNF8* led to outgrowth of *BRCA1-KO* cells in the presence of olaparib (Fig 1d; indel frequencies and genotyping information are found in Extended Data Table 2). Similarly, independent sgRNAs targeting *C20orf196* and *ATMIN* led to PARPi resistance, while sgRNAs against *SCAF1* had a more moderate effect (Fig 1d and ED Fig 1a). In another set of experiments, transfection of tracrRNA and crRNAs targeting *C20orf196*, *53BP1* or *PARP1* caused talazoparib resistance in Cas9-expressing SUM149PT cells (Fig 1e and ED Fig 1b). Since C20orf196 was identified as a strong hit in all three screens and validated in independent assays, we sought to determine its role in DNA repair.

C20orf196 is an uncharacterized vertebrate protein of 205 amino acid residues, largely consisting of a domain of unknown function (DUF4521) (Fig 1f). C20orf196 was previously identified as a candidate REV7-interacting protein in a large-scale proteomics study[23], suggesting that it may act downstream of 53BP1-RIF1 by interacting with REV7. To explore this relationship, we carried out immunoprecipitation studies to identify interactors of Flag-tagged C20orf196 or REV7 by mass spectrometry (IP-MS; Fig. 1g and Extended Data Table 3). One protein, FAM35A, was identified as a high-confidence interactor of both C20orf196 and REV7 (Fig 1g and Extended Data Table 3) and was a hit in the talazoparib-resistance screen (Extended Data Table 1). FAM35A was striking due to the presence of three predicted C-terminal OB-fold domains (OBA, OBB and OBC) (Fig 1f) that are organized in a similar fashion to those in the single-stranded (ss) DNA binding protein RPA1[24] and the telomere-binding protein POT1[25]. Subsequent IP-MS experiments with FAM35A recovered another protein, CTC-534A2.2, which was also identified in the REV7 IP-MS (Fig. 1g and Extended Data Table 3). CTC-534A2.2 is a protein of 250 residues (Fig 1f) encoded by an alternative mRNA emanating from the *TRAPPC13* locus (transcript *AC008560.1-201*; ED Fig 1c). The *CTC-534A2.2* gene is not annotated in the consensus CDS project and, as a consequence, guides targeting *CTC-534A2.2* were not present in either of our first-generation sgRNA libraries. IP-MS with CTC-534A2.2 recovered C20orf196, FAM35A and REV7 (Fig 1g), suggesting that these four proteins form a single protein complex, which was confirmed by sequential affinity purification studies using epitope-tagged versions of C20orf196, CTC-534A2.2, FAM35A and REV7 (Fig 1h).

*FAM35A*, *C20orf196* and *CTC-534A2.2* were also identified in a fourth CRISPR/Cas9 screen carried out with a second-generation sgRNA library, TKOv2. This screen was aimed at identifying genes that promote cellular resistance to ionizing radiation (IR) in RPE1 cells (ED Fig 2a). A total of 75 genes scored at a false discovery rate (FDR) <1% and this gene set was highly enriched in genes coding for NHEJ factors, as expected ($p= 1.11 \times 10^{-11}$; Fig 2a and Extended Data Table 4). *RIF1*, *FAM35A*, *C20orf196*, *CTC-534A2.2*, *53BP1* and *REV7* were all hits at FDR <1% (Fig 2a and Extended Data Table 4). These data suggest that the complex formed by C20orf196, FAM35A, REV7 and CTC-534A2.2 promotes DSB repair by cNHEJ. For reasons that will become apparent below, we named this complex Shieldin with C20orf196, FAM35A and CTC-534A2.2 renamed SHLD1, SHLD2 and SHLD3, respectively.

Validating the results of the IR screen, independent sgRNAs targeting *SHLD2* or *SHLD3* caused sensitivity to the DSB-inducing agent etoposide in the competitive growth assay (Fig 2b). The same

sgRNAs caused resistance to olaparib in RPE1 *BRCA1-KO* cells, consistent with SHLD2 and SHLD3 acting with REV7 and SHLD1 (Fig 2c and ED Fig 2b). Next, using RPE1 WT and *BRCA1-KO* cells, we generated clonal knockouts of *SHLD1* and *SHLD2* (*SHLD1-KO* and *SHLD2-KO*, respectively) that were verified by sequencing, due to unavailability of high-quality antibodies against members of the complex (Extended Data Table 2). As expected, loss of SHLD1 or SHLD2 led to olaparib resistance in *BRCA1-KO* cells, with the *SHLD2-KO* resulting in a phenotype that approached that of 53BP1 loss (Fig 2de). Similar results were obtained with 11 independent clonal knockouts of *SHLD1* in SUM149PT cells exposed to talazoparib (ED Fig 2c). Furthermore, olaparib sensitivity was restored in *BRCA1-KO SHLD2-KO* cells by expression of GFP-SHLD2 (ED Fig 2d). We also observed that loss of SHLD2 led to cisplatin resistance in RPE1 *BRCA1-KO* cells (Fig 2de). Resistance to PARPi in *BRCA1-KO* cells was likely due to restoration of HR, as measured both by RAD51 IR-induced focus formation, a proxy for RAD51 nucleofilament formation, and by a reporter for gene conversion, the traffic light reporter assay[26] (Fig 2fg and ED Fig 3).

Next, we tested whether loss of Shieldin causes PARPi resistance in the KB1P mouse mammary tumor model deficient in *Brca1* and *Trp53* (p53)[27]. sgRNAs targeting *Shld1* and *Shld2* led to PARPi resistance in clonogenic survival assays in KB1P-G3 cells and in *Brca1*; *Trp53* mutated mouse embryonic stem cells (ED Fig 4a,c). This resistance was also associated with restoration of HR (ED Fig 4b). Furthermore, transduction of *Shld1*- and *Shld2*-targeting sgRNAs suppressed the cell lethality associated with complete *Brca1* loss in p53-proficient cells (ED Fig 4d). We transduced the same sgRNAs into KB1P4 tumor organoids (Extended Data Table 2) and implanted them into the fat pads of 16 mice, which were subsequently separated into control and olaparib-treated cohorts. Olaparib treatment was initiated when tumors reached 50-100 $mm^3$ and was continued for 80 days. While all mice bearing untreated tumors succumbed to excessive tumour burden within 20 days, the control group transduced with control virus (sgEmpty) responded to olaparib for the duration of the treatment, with tumor growth only resuming after termination of olaparib dosing (Fig 2h). Conversely, mice transduced with sgRNAs targeting *Shld1* and *Shld2* exhibited a partial response to olaparib, with tumors resuming rapid growth by day 40 of treatment and mice succumbing by day 60 (Fig 2h). We thus conclude that Shieldin loss causes PARPi resistance in both human and mouse BRCA1-deficient tumour cells by reactivating HR.

**Shieldin acts downstream of 53BP1-RIF1**

As expected of a complex with a direct role in DSB repair, Shieldin accumulates at DSB sites based on the following observations. First, GFP-SHLD2 and -SHLD3 form IR-induced foci in U-2-OS cells (Fig 3a) and SHLD1, 2 and 3 accumulate at DNA damage sites induced by laser microirradiation in RPE1 WT cells (Fig 3bc and ED Fig 5a). We confirmed that Shieldin is recruited to DSB sites using the LacR-FokI assay[28], which we also used to monitor REV7 recruitment (Fig 3d-f and ED Fig 5b). SHLD1, 2 and 3 accumulated at DSB sites in a 53BP1/RIF1-dependent manner (Fig 3a-f and ED Fig 5a-d), as does REV7[14]. However, loss of Shieldin components did not impair formation of 53BP1 or RIF1 IR-induced foci, indicating that Shieldin acts downstream of 53BP1-RIF1 (ED Fig 5ef). Consistent with this possibility, we observed genetic epistasis between *53BP1* and the Shieldin genes using the RAD51 focus formation assay in RPE1 *BRCA1-KO cells* (ED Fig 6a). We also observed that *SHLD1* and *53BP1* were epistatic in terms of modulating talazoparib resistance in SUM149PT cells (ED Fig 6b).

Analyses of the dependencies within the Shieldin complex indicate that SHLD3 is the most apical component followed by REV7 (Fig 3c,f and ED Fig 5a-d). SHLD2 is needed for SHLD1 accumulation on damaged chromatin whereas SHLD1 only partially affected SHLD2 localization (Fig 3c,f and ED Fig 5a-d). We divided SHLD2 into two protein fragments consisting of an N-terminal region (SHLD2N; residues 1-420) containing four segments of high homology among vertebrate orthologs (ED Fig 7a); and a highly conserved C-terminal region (SHLD2C; residues 421-904) consisting of three predicted OB-fold domains (Fig 1h). We found that the SHLD2N fragment, but not SHLD2C, binds SHLD3 and REV7 (ED Fig 8a). REV7 and SHLD3 are essential for SHLD2 recruitment to DSBs and accordingly, SHLD2N but not SHLD2C was recruited to FokI-induced DSBs (Fig 3g). Further mapping experiments using GFP-fusion protein recruitment to a *Lac*O array by a LacR-RIF1 fusion protein (LacR-RIF1$_{1\text{-}967}$; ED Fig 7b-j) and co-immunoprecipitation studies (ED Fig 8a-d) indicated that SHLD3-REV7 binds to the highly conserved first 50 residues of SHLD2 necessary for DSB localization (ED Fig 7k,), with REV7 being itself required for the SHLD3-SHLD2N interaction (Fig 3h and ED Fig 8a-d). Finally, we detected a weak interaction between SHLD3 and RIF1 using two different SHLD3 fusion proteins, suggesting the SHLD3 links Shieldin to 53BP1-RIF1 (Fig 3i and ED Fig 8e). We conclude that Shieldin lies downstream of 53BP1-RIF1 and can be roughly divided in a DSB-recruitment module composed of SHLD3-REV7 that binds to SHLD2N, and a presumptive DNA-binding module (SHLD2C-SHLD1) featuring the SHLD2 OB-fold domains.

**Shieldin promotes class switching and end-protection**

The components of the 53BP1 pathway act during class switch recombination (CSR) by promoting cNHEJ[9-11,13,14,29-33]. We therefore tested the contribution of SHLD1-3 to class switching in the CH12F3-2 murine B cell lymphoma line model[34]. We generated a minimum of two independent clones of *53bp1-*, *Shld1-*, *Shld2-* and *Shld3-KO* in CH12F3-2 cells by genome editing (Extended Data Table 2). As expected, class switching from IgM to IgA in *53bp1-KO* cells was highly reduced compared to controls (Fig 4a and ED Fig 9ab). Similarly, mutation in each of the Shieldin subunits compromised immunoglobulin class switching, with the *Shld2-KO* and *Shld3-KO* cells having switching reduced to near background levels whereas *Shld1*-edited cells had a milder phenotype over multiple independent experiments (Fig 4a and ED Fig 9a). *Shld2-KO* was epistatic with both *53bp1-KO* and *Shld1-KO* mutations, consistent with them acting in the same genetic pathway (ED Fig 9bc). The expression of AID, which initiates CSR, was not altered in the Shieldin mutants, consistent with a DNA repair phenotype (ED Fig 9d). In support of this possibility, analysis of random plasmid integration in RPE1-hTERT cells, a process that occurs largely through cNHEJ[35], showed that *SHLD1* and *SHLD2* mutations impaired NHEJ to an extent similar to that of 53BP1-deficient cells (Fig 4b and ED Fig 9e). Together, these results indicate that Shieldin, like 53BP1 and RIF1, promotes NHEJ.

Since the function of 53BP1 in DNA repair is likely related to its role in limiting end-resection, we tested whether Shieldin loss in CH12F3-2 was associated with hyper-resection by monitoring RPA2 Ser4/Ser8 phosphorylation[24] following induction of DSBs by IR. Loss of each Shieldin subunit led to IR-induced hyperphosphorylation of RPA2, suggesting that Shieldin promotes DNA end protection (Fig 4c). Supporting this hypothesis, both restoration of RAD51 accumulation at DSB sites and olaparib resistance in KB1P-G3 cells expressing *Shld1-* and *Shld2*-targeting sgRNAs were dependent on ATM activity (ED Fig 4ab), which promotes DNA end-resection in the absence of 53BP1[18,36] or REV7[13]. Finally, Mirman *et al*. (co-submitted manuscript) show that *Shld2*-mutated cells have increased end-resection at dysfunctional telomeres. Collectively, these results indicate that Shieldin antagonizes end-resection.

**Shieldin binds ssDNA to regulate DSB repair**

We surmised that if Shieldin is a downstream effector of 53BP1, artificially targeting Shieldin to DSB sites should rescue phenotypes associated with 53BP1 loss. To do this, we fused SHLD2 to the RNF8 FHA domain, which is recruited to damaged chromatin independently of 53BP1 (Fig 5a). We

expressed GFP-tagged FHA-SHLD2 in RPE1 *BRCA1-KO*/*53BP1-KO* cells and found that the FHA-dependent targeting of SHLD2 to DSB sites suppressed RAD51 IR-induced focus formation to levels seen in parental RPE1 *BRCA1-KO* cells (Fig 5b and ED Fig 10a). These results indicated that targeting SHLD2 to DSB sites suppresses HR in *BRCA1-KO* cells in the absence of 53BP1.

We also observed that the FHA-SHLD2C protein, but not FHA-SHLD2N, potently suppressed RAD51 recruitment to DSB sites in the *BRCA1-KO*/*53BP1-KO* cells (Fig 5b and ED Fig 10a). The presence of predicted OB-fold domains in SHLD2C suggests that DNA binding might underpin this effector function of the SHLD2 C-terminus. To test for DNA binding activity, we expressed epitope-tagged SHLD2C in the presence or absence of SHLD1, and affinity-purified SHLD2C from 293T cells (ED Fig 10b). We observed that SHLD2C binds to a radiolabeled ssDNA probe by electrophoretic mobility shift assays (EMSA), and competition with unlabeled oligonucleotides revealed that SHLD2C preferentially binds to ssDNA over dsDNA (Fig 5c). We estimate the binding affinity ($K_D$) of the SHLD2C-ssDNA interaction to be ~10 nM (Fig 5de), which is in the range of human POT1 binding to telomeric ssDNA ($K_D$ ~20 nM)[37]. While SHLD1 is not necessary for SHLD2C DNA-binding, its co-expression with SHLD2C increased the amount of SHLD2C we purified from 293T cells and the retarded complex displayed a difference in mobility consistent with the SHLD2C-SHLD1 complex binding to ssDNA (Fig 5g, lanes 2 vs 5). We conclude that SHLD2, and by extension Shieldin, possesses ssDNA-binding activity.

To explore whether ssDNA-binding is involved in Shieldin function, we generated mutant versions of the SHLD2 OB-folds by modeling the SHLD2 C-terminus on the *Ustilago maydis* RPA1 structure (PDB 4GNX)[38]. The engineered mutations target residues that may be involved in base stacking interactions[38]. These mutations are W489A/F494A/W495A (m1); F527A (m2); W640A, W646A (m3) and F873A (m4), and are located in the OBA (m1, m2), OBB (m3) or OBC (m4) domains (Fig 5f). We also employed a shorter, naturally-occurring splice variant of SHLD2 that disrupts the OBB domain that we call "short" isoform or SHLD2S (Fig 5f). To assess whether any of these mutations disrupt SHLD2 function, we first introduced them in the context of FHA-SHLD2 and expressed the resulting proteins in *53BP1-KO*/*BRCA1-KO* cells. We found that FHA-SHLD2-m1 and FHA-SHLD2S were unable to suppress RAD51 focus formation whereas the other mutants showed varying degrees of defects (Fig 5b and ED Fig 10a). Similar results were obtained when the m1 and SHLD2S mutations were introduced in the more potent FHA-SHLD2C fusion protein (Fig 5b and ED Fig 10a). Expression of full-length SHLD2-m1 and SHLD2S in *BRCA1-KO/SHLD2-KO*

cells failed to suppress RAD51 IR-induced focus formation unlike wild-type SHLD2 (ED Fig 11ab). Importantly, both mutants localized to DSB sites (ED Fig 11cd) and interacted with the other members of the Shieldin complex (ED Fig 11e). Therefore, the SHLD2-m1 and SHLD2S mutants are defective in suppressing HR.

Strikingly, the SHLD2C-m1 mutant was completely defective in ssDNA binding (Fig 5g) whereas the SHLD2C-S mutant displayed reduced and aberrant ssDNA-binding behaviour (Fig 5g). The phenotype of SHLD2-m1 is therefore more informative and since the m1 mutation only alters residues predicted to be involved in base-stacking interactions, produces a protein defective in ssDNA-binding and suppression of HR, we conclude that ssDNA binding by Shieldin is critical for 53BP1-dependent DSB repair.

**Discussion**

The identification of Shieldin forces us to re-evaluate how DNA end stability is regulated in vertebrates. Indeed, our results are consistent with a model in which Shieldin is the ultimate mediator of 53BP1-dependent DNA repair by binding ssDNA and occluding access to resection nucleases (Fig 5h). In this fashion, Shieldin may act similarly to Shelterin where another multi-OB-fold containing protein, POT1, protects telomeric ends from nuclease action, at least in some species[25].

The identification of Shieldin also has implications for the management of *BRCA1*-mutated malignancies, as alterations in Shieldin-coding genes may cause clinical resistance to PARP inhibitors. However, the observation that Shieldin loss-of-function results in sensitivity to DSB-inducing agents also hints that this initial resistance may be accompanied with a new therapeutic vulnerability to ionising radiation and clastogenic chemotherapies. Similarly, the severe class switching phenotype caused by the loss of Shieldin in B cells indicates that loss-of-function mutations in Shieldin genes could conceivably cause immune disorders such as hyper-IgM syndrome.

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**Conflict of interest statement**

The authors declare competing financial interests: details are available in the online version of the paper.

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## Figure legends

**Figure 1. Identification of C20orf196 (SHLD1), FAM35A (SHLD2), CTC-534A2.2 (SHLD3) and REV7 as Shieldin complex subunits. a,** Schematic of the PARPi resistance screens. The indicated Cas9-expressing cell lines were transduced either with the TKOv1 or Yusa lentiviral libraries containing a pool of sgRNAs targeting individual genes. After outgrowth of PARPi-resistant cells, changes in sgRNA representations were quantified by sequencing and gene-based scores were tabulated with MAGeCK. **b,** Venn diagram of the top 20 hits in each of the three

screens. **c**, Schematic of the two-color competitive cell growth assays to validate the screen hits. Cas9-expressing cells are transduced with virus coding for either a control sgRNA or an sgRNA for a gene of interest (GOI) and a fluorescent protein. Cells are mixed at 1:1 ratio and then grown with or without drug while monitoring the green versus red fluorescence ratio over time. **d,** Competitive growth assays testing the capacity of the indicated sgRNAs to cause resistance to olaparib (16 nM) in RPE1 *BRCA1-KO* cells. Data is presented as the mean fraction of GFP-positive cells ± SD, normalized to day 0 (n = 3). Gene editing efficiencies of the sgRNAs can be found in Extended Data Table 2. Note that we have not been able to obtain TIDE data for the *ATMIN*-targeting sgRNAs. **e,** PARPi resistance caused by mutation of *C20orf196* in SUM149PT cells. The indicated crRNAs were transfected into Cas9-expressing SUM149PT cells. Cells were subsequently exposed to 50 nM talazoparib for 14 d at which point cell viability was estimated by CellTiter-Glo. Relative growth was normalized to cells transfected with a non-targeting crRNA (CTRL). Data is presented as the mean ± SD (n=3). **f,** Schematics of the domain architecture of the various Shieldin subunits. SHLD3 is the only member of the complex with a REV7-binding PXXXPP motif[39].
**g,** The protein-protein interaction network surrounding REV7, C20orf196 and FAM35A. Solid arrows represent interactions at an FDR ≤1% with the hatched arrow between FAM35A and C20orf196 indicating an interaction found at FDR ≤5%. The Shieldin complex members are coloured orange. **h,** C20orf196, FAM35A, CTC-534A2.2 and REV7 form the Shieldin complex. Sequential affinity purifications in whole-cell extracts (WCE) of 293T cells transfected with vectors coding for GFP-REV7, Flag-C20orf196 and V5-CTC-534A2.2 along with either an empty Strep/HA vector or the same vector expressing Strep/HA-FAM35A. The first affinity purification was on streptavidin sepharose after which bound proteins were eluted with biotin. The eluate was then bound on GFP-trap beads and the flow-through was kept for analysis. Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

**Figure 2. Loss of Shieldin promotes PARP inhibitor resistance in cell and tumor models of BRCA1 deficiency. a,** Results of a CRISPR dropout screen in RPE1 WT cells aimed at finding genes promoting resistance to IR. Shown is a scatter plot of gene-level normZ scores that are <0. Selected genes are labeled. **b-c,** Competitive growth assays of the indicated sgRNAs in response to etoposide (100 nM) in RPE1 WT cells (b) or olaparib (16 nM) in RPE1 *BRCA1-KO* cells (c). Data is presented as mean ± SD, normalized to day 0 (n = 3). **d-e**, Clonogenic survival assays of the indicated RPE1 *BRCA1-KO* cell derivatives in response to 16 nM olaparib or 800 nM cisplatin.

Representative images of the plates are shown in (d) and in (e) is the quantitation of the experiments. The bar represents the mean ± SD (n=9 for RPE1 WT and *BRCA1-KO*/*SHLD1-KO*; n=3 for *BRCA1-KO*/*SHLD2-KO* and n=4 for *BRCA1-KO*/*53BP1-KO*). **f,** Quantitation of cells with >5 RAD51 foci following treatment (+) or not (-) with a 10 Gy dose of X-rays and recovered for 6 h prior to nuclear extraction and fixation. Individual experiments are shown and the bar represents the mean ± SD (n≥3 for all conditions) with >100 cells counted per experiment. **g,** Left, schematic of the TLR assay to assess HR by gene conversion. Right is the quantitation of gene conversion, as measured by the percentage of GFP-positive cells in RPE1 *BRCA1-KO* cells of the indicated genotypes. Individual experiments are shown and the bar represents the mean ± SD (n=3 for WT, *53BP1-KO*, and *SHLD1-KO*; n=4 for *SHLD2-KO* and *REV7-KO*). **h,** Kaplan-Meier curve showing the tumor-specific survival of mice transplanted with KB1P4 tumor organoids, untreated or treated with olaparib (100 mg/kg intraperitoneally) for 80 consecutive days (n = 8 per treatment). End of treatment is indicated by the dotted line (gene editing efficiencies of the sgRNAs can be found in Extended Data Table 2).

**Figure 3. Shieldin accumulates at DSB sites downstream of 53BP1-RIF1. a,** Representative micrographs of U2OS cells expressing GFP-tagged SHLD1 or SHLD2, 1 h post-IR (5 Gy). Scale bar = 10 μm. **b,** Representative micrographs of the cells displaying GFP-SHLD1 (left), -SHLD2 (middle) and –SHLD3 (right) accumulation at laser-microirradiation sites in RPE1 WT cells. **c,** Quantitation of the cells displaying GFP-SHLD1 (left) and -SHLD2 (right) accumulation at laser-microirradiation sites in RPE1 WT cells. Each point represents an individual experiment with a minimum of 100 cells counted and the bar represents the mean ± SD (n=3 for SHLD1, n=2 for SHLD2). **d,** Schematic of DSB induction at a LacO array by mCherry-LacR-FokI. **e,** Representative micrographs of U2OS-FokI cells expressing GFP-SHLD3 after mCherry-LacR-FokI induction. **f,** Quantitation of focus intensity of either GFP-SHLD3 or endogenous REV7 colocalizing with the mCherry focus, normalized to nuclear background. Each datapoint represents a cell imaged with the line at the mean. The data is the aggregate of two biological replicates with a minimum of 20 cells counted. **g,** Representative micrographs of U2OS-FokI cells expressing the GFP-SHLD2 N- or C-terminal fusions after mCherry-LacR-FokI induction. The mean normalized focus intensity is shown with a minimum of 20 cells counted (n=2). Scale bar = 10 μm. **h,** Affinity purification of Shieldin complex components using N-terminally truncated SHLD2. 293T cells were transfected with vectors encoding the indicated Shieldin subunits and either full length or truncated (Δ1-50)

STREP/HA-SHLD2. Whole cell extracts (WCE) were incubated with streptavidin resin and eluted with biotin. Proteins were resolved by SDS-PAGE and immunoblotted using the indicated antibodies. **i**, Analysis of proteins co-immunoprecipitating with SHLD3. WCE of 293T cells transfected with V5-SHLD3 were incubated with anti-V5 antibody and protein G resin. Cell extracts and bound proteins were resolved by SDS-PAGE and immunoblotted using the indicated antibodies.

**Figure 4. Shieldin promotes class switch recombination (CSR) and suppresses end resection. a,** CSR analysis in the indicated clones of CH12F3-2 cells following stimulation. Each data point represents a biological replicate; the line represents the mean ± SD (n=3). **b,** Random plasmid integration of linearized peGFP-c1 following transfection in the indicated RPE1 derivatives. G418-resistant colonies were quantitated after 14 d. Each data point is a biological replicate; the bar represents the mean of integration, normalized to wild type cells (set at 100) ± SD (n≥3). **c,** The indicated CH12F3-2 clones were irradiated with a 25 Gy dose (+), or left untreated. 3 h post-irradiation, cells were processed and whole cell protein extracts were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

**Figure 5. Shieldin is an effector of 53BP1 by binding ssDNA. a,** Schematic depicting the rationale for artificially targeting Shieldin to DSB sites in *BRCA1-KO 53BP1-KO* cells. **b**, Quantitation of RAD51 IRIF upon 10 Gy irradiation and 3 hrs recovery in *BRCA1-KO 53BP1-KO* cells transduced with virus encoding the indicated fusion proteins. Protein expression was induced with 1 μg/mL doxycycline for 24 hrs prior to IR. Each data point is a biological replicate; the bar represents the mean ± SD (n≥3). **c,** Electrophoretic mobility shift assay of the SHLD2 C-terminus predicted to contain the triple OB-fold (residues 421-904) and SHLD1 complex. SHLD2C-SHLD1 complexes were isolated from 293T cells expressing Strep/HA-SHLD2(421-904) and Flag-SHLD1 by streptavidin affinity purification (see ED Figure 10b). These complexes were assayed for DNA binding through incubation with 20 nM 5'-$^{32}$P-labeled 59nt DNA oligonucleotides and resolved in a native acrylamide gel. Radiolabeled DNA was visualized by phosphorimaging. DNA binding specificity was determined by including either 1x or 5x molar equivalents of unlabelled ssDNA or dsDNA oligonucleotides. **d,** Saturation binding experiments of the SHLD2C-SHLD1 complex. 20 nM of the [$^{32}$P]-labeled ssDNA probe was titrated with increasing amounts of SHLD2C-SHLD1 protein. **e,** Determination of SHLD2C-SHLD1 ssDNA binding dissociation constant ($K_D$). The fraction of bound ssDNA probe was plotted against the calculated concentration of unbound SHLD2C-SHLD1 complex. The $K_D$ value was calculated via nonlinear regression analysis assuming

one site binding. Error bars correspond to the standard deviation between 3 independent replicates. **f,** Structural model of the SHLD2 OB fold domains along with the set of mutations that were engineered (red spheres). **g,** EMSA of SHLD2C constructs. SHLD2C-m1 and SHLD2C-S refer to OB fold mutant 1 and a deletion mutation (Δ655-723) corresponding to the naturally occurring "Short" splice variant. **h,** Model of Shieldin function. We speculate that the SHLD2 OB fold domains bind to DNA structures produced at DSB sites to suppress resection and favour cNHEJ.

**ED Figure 1. Supporting data for the identification of Shieldin complex. a,** Competitive growth assays assaying the capacity of the indicated sgRNAs (an additional sgRNA for each gene to the data represented in Fig. 1d) to cause resistance to PARP inhibitors in RPE1 *BRCA1-KO* cells. Data is presented as the mean fraction of GFP-positive cells ± SD, normalized to day 0 (n = 3). Gene editing efficiencies of the sgRNAs can be found in Extended Data Table 2. Note that we have not been able to obtain TIDE data for the *ATMIN*-targeting sgRNAs. **b,** Representative images of SUM149PT-Cas9 cells transfected with indicated crRNAs (see Methods) and exposed to 50 nM talazoparib for 14 d. Purple coloration indicates cells detected by Incucyte live cell imaging. Scale bar represents 100 μm. **c,** Screenshot of the genomic locus surrounding human CTC-534A2.2 taken at ENSEMBL.

**ED Figure 2. Supporting data that Shieldin promotes PARP inhibitor resistance in BRCA1-null cells through activation of HR. a,** Schematic of the screen performed in RPE1-hTERT *TP53*$^{-/-}$ cells stably expressing Cas9 to study genes mediating IR-sensitivity. **b,** Competitive growth assays assaying the capacity of the indicated sgRNAs (an additional sgRNA for each gene represented in Fig 2c) to cause resistance to PARP inhibitors in RPE1 *BRCA1-KO* cells. Data is presented as the mean fraction of GFP-positive cells ± SD, normalized to day 0 (n = 3). Gene editing efficiencies of the sgRNAs can be found in Extended Data Table 2. **c,** Talazoparib sensitivity in 11 *SHLD1-KO* SUM149PT clones obtained after co-transfection of tracrRNA and one of four distinct *SHLD1* crRNAs (5-1, 5-2, 5-3 or 5-5). Each clone was exposed to talazoparib in a 384-well plate format for five days. As a comparison, talazoparib sensitivity in parental SUM149PT cells with WT SHLD1 (WT) is shown, as is talazoparib resistance in a *BRCA1* revertant subclone (*BRCA1-rev)* of SUM149PT. Bars represent the mean ± SD (n=3). ANOVA was performed for each SHLD1 clone vs. WT, $p<0.0001$. Gene editing efficiencies of the sgRNAs can be found in Extended Data Table 2. **d,** *BRCA1-KO SHLD2-KO* were virally transduced with GFP alone of GFP-SHLD2 and sensitivity to olaparib (200 nM) was assayed by a short-term survival assay in the presence of 1 μg/mL

doxycycline. Data is represented as dots for every individual experiment with the bar representing the mean ± SD (n=3).

**ED Figure 3. Supporting data that Shieldin inhibits HR.**

**a,** Representative micrographs of RAD51 focus formation in the indicated RPE1 cell lines (data quantitated in Fig 2f). **b,** Traffic light reporter assay testing RPE1 *BRCA-KO* cells virally transduced with sgRNAs targeting *53BP1* or *SHLD3*. Data is represented as dots for individual experiment with the bar representing the mean ± SD (n=3). Gene editing efficiencies of the sgRNAs can be found in Extended Data Table 2). **c,** Representative flow cytometry plots of cells assayed for the TLR assay (data quantitated in Fig 2g). **d,** Representative flow cytometry plots of cells assayed for the TLR assay (data quantitated in ED Fig 3b).

**ED Figure 4. Supporting data mouse Shieldin promotes resistance to PARP inhibition in *Brca1*-mutated cells and tumours. a,** Clonogenic survival assays of transduced KB1P-G3 cells treated with indicated olaparib doses with and without the ATM inhibitor (ATMi) KU60019 (500 nM). On day 6, the ATMi alone and untreated groups were stopped and stained with 0.1% crystal violet, the other groups were stopped and stained on day 9. **b,** Left, quantitation of Rad51 focus formation in parental KB1P-G3 (*Brca1*$^{-/-}$ *Trp53*$^{-/-}$) cells or KB1P-G3 cells that were transduced with the indicated lentiviral sgRNA vectors. Cells were fixed before or 4 h after irradiation (10 Gy dose). Each data point represents a microscopy field containing a minimum of 50 cells; the bar represents the mean ± SD (n=15). Right, representative micrographs of Rad51-negative and -positive cells (the latter is indicated by an arrowhead). DNA was stained with DAPI. **c**, Clonogenic survival assay (right) of *Rosa26*$^{CreERT2/wt}$*; BRCA1*$^{\Delta/\Delta}$*; p53-null* mES-cells treated with or without 15 nM olaparib for 1 week. Gene editing efficiencies of the sgRNAs can be found in Extended Data Table 2. **d,** Clonogenic survival assay (right) of *Rosa26*$^{CreERT2/wt}$*;BRCA1*$^{Sco/\Delta}$ mES-cells treated with or without tamoxifen to induce BRCA1 depletion. Gene editing efficiencies of the sgRNAs can be found in Extended Data Table 2).

**ED Figure 5. Data supporting that Shieldin localizes to DSB sites**

**a,** Representative micrographs of the experiments quantitated in Fig 3c. **b,** Representative micrographs of the experiments quantitated in Fig 3f that are not displayed in Fig 3e. **c,** Reverse transcription and real-time quantitative PCR of mRNAs for *SHLD1*, *SHLD2* and *SHLD3*. RPE1 cells were transfected with siCTRL (non-targeting control siRNA) or siRNA targeting the indicated

Shieldin subunits. 48 h post-transfection, mRNA was purified and reverse transcribed before being quantified using TaqMan probes. Data were normalized to the amount of *GAPDH* mRNA and expressed relative to the corresponding value for cells transfected with siCTRL. Data is presented as the mean ± SD (n=3). **d,** Whole cell extracts from RPE1 WT cells transfected with the indicated siRNAs were processed for immunoblotting with the indicated antibodies. Tubulin is a loading control. **e,** quantitation of 53BP1 and RIF1 recruitment to IR-induced DSBs upon depletion of the indicated SHLD factors. Data is represented as mean ± SD (n=3). **f,** Representative micrographs of the experiments quantitated in ED Fig 5e.

**ED Figure 6. Data supporting epistasis between 53BP1 and Shieldin factors.**
**a,** Quantitation of RAD51 foci formation 3h upon 10 Gy irradiation in RPE1 *BRCA1-KO* (left panel), *BRCA1-KO/53BP1-KO* (middle panel) and *BRCA1-KO/SHLD2-KO* (right panel) cells after viral transduction with indicated sgRNAs. Data is represented as mean ± SD (n≥4 of two (*BRCA1-KO/53BP1-KO*) or three (*BRCA1-KO* and *BRCA1-KO/SHLD2-KO*) independent viral transductions. P-values were calculated using ANOVA with Holm-Sidak's multiple comparison testing (*** p<0.001, ** p<0.01, *p<0.05, n.s.= not significant). **b,** Talazoparib sensitivity of WT or *SHLD1-KO* SUM149PT-dox-Cas9 clones infected with lentiviral sgRNA vectors targeting *53BP1* (*sg53BP1*) or a control non-targeting sgRNA (*sgCTRL*), following induction of Cas9. Data is presented as the mean ± SD (n=3).

**ED Figure 7. Data supporting the co-localization of Shieldin with RIF1 on chromatin.**
**a,** Representation of the deletion mutants of SHLD2N used in ED Fig 7cd. The orange shading indicates blocks of homology. **b,** Schematic of the LacR-RIF1 chromatin recruitment assay. **c,** Quantitation of the experiment shown in ED Fig 7d. Colocalization was considered positive when the average GFP intensity at the mCherry focus was 3-fold over background nuclear intensity. A minimum of 20 cells were imaged per biological replicate (circles); the bar represents the mean ± SD (n=3). **d,** Representative images of the data quantitated in ED Fig 7c. The main focus is shown in inset and the scale bar = 10 μm**. e-h,** Quantitation (e,g) and representative micrographs (f,h) of overexpressed GFP-SHLD2N and mCherry-LacR-RIF1(1-967) cotransfected into uninduced U2OS FokI cells along with siRNA against Shieldin complex subunits after processing for mCherry and GFP (e,f) or mCherry and REV7 (g,h) immunofluorescence. Colocalization was considered positive when the average GFP or REV7 intensity at the mCherry focus was 3-fold over background nuclear

intensity. A minimum of 20 cells were imaged per condition (circles); the bar represents the mean ± SD (n=3). **i,** Representative images of the data quantitated in Fig 3f and ED Fig 7j. The main focus is shown in inset and the scale bar = 10 μm **j,** Quantitation of GFP intensity at the mCherry-LacR-RIF1(1-967), normalized to nuclear background. Each datapoint represents a cell transfected with vectors coding for the indicated GFP fusions. The line is at the median. **k,** Representative micrographs of GFP-SHLD2 full-length or truncated (Δ1-50) transfected in U2OS-FokI cells after mCherry-LacR-FokI induction.

**ED Figure 8. Mapping the architecture of the Shieldin complex. a,** Streptavidin pulldown analysis determining which half of SHLD2 associates with the other Shieldin subunits. Whole cell extracts (WCEs) of 293T cells transfected with Strep/HA-tagged SHLD2, SHLD2N (residues 2-420), SHLD2C (residues 421-904), or empty Strep/HA vector (EV) were incubated with streptavidin resin and bound proteins eluted with biotin. WCEs and elutions were analysed by SDS-PAGE and immunoblotting. **b,** Mapping the SHLD3 and REV7 binding sites on the SHLD2 N-terminus through streptavidin pulldown with different SHLD2 constructs and immunoblotting. **c,** Streptavidin pulldown analysis of SHLD2 association with REV7 and SHLD3. WCEs of 293T cells transfected with Strep/HA-SHLD2, GFP-REV7, V5-SHLD3, and siRNA against REV7 or SHLD3 were incubated with streptavidin resin and bound proteins eluted with biotin. WCEs and elutions were analysed by SDS-PAGE and immunoblotted against the indicated antibodies. **d,** Dependency of V5-SHLD3 co-immunoprecipitation with GFP-REV7 on SHLD2. WCE of 293T cells transfected with V5-SHLD3 or empty V5 vector (EV), GFP-REV7, and siRNA against SHLD2 were incubated with anti-V5 antibody and protein G resin. Bound proteins were boiled in SDS sample buffer and analysed by immunoblotting. **e,** Co-immunoprecipitation experiment of GFP-SHLD3. WCEs of 293T cells transfected with GFP-SHLD3 were incubated with GFPTrap resin. Bound proteins were boiled in SDS sample buffer and analysed by SDS-PAGE and immunoblotting against 53BP1 and RIF1.

**ED Figure 9. Controls supporting the role of Shieldin in promoting physiological NHEJ. a,** Representative dot plots of the flow cytometry data obtained to assess class switching in Fig 4a. CSR was determined by substracting the percentage of $IgA^+$ cells after stimulation with the baseline percentage of $IgA^+$ cell in the indicated clones (values in bracket). **b, c,** CSR analysis in *Shld2*-

*double KO* CH12F3-2 cells with *Shld1-KO* (b) or *53bp1-KO* (c) following stimulation. Each data point represents a biological replicate; the line represents the mean ± SD (n=3). Genomic editing efficiencies of the sgRNAs can be found in Extended Data Table 2. **d,** Whole cell extracts of the indicated CH12F2-3 clones were probed for AID and β-actin (loading control) by immunoblotting and were quantitated by densitometry. Each data point represents a biological replicate; the line represents the mean ± SD (n≥3). **e,** Representative images of the plasmid integration assays shown in Fig 4b.

**ED Figure 10. Data supporting the testing of DSB-targeted SHLD2 in the suppression of HR and of the assessment of the SHLD2C-SHLD1 complex binding to ssDNA.**

**a,** Representative micrographs of RPE1 *BRCA1-KO 53BP1-KO* cells transduced with the indicated GFP-fusion proteins, pre-extracted, fixed and stained for RAD51 and GFP 3 h post-IR (10 Gy). Protein expression was induced for 24 hrs before IR using 1 μg/mL doxycycline. Data relates to Fig 5b. **b,** Quantification of Coomassie-stained SDS-PAGE analysis of purified SHLD2C-SHLD1 complex. Strep/HA-SHLD2(421-904)-Flag-SHLD1 complexes were purified from transiently transfected 293T cells. Quantities of purified proteins were estimated by comparison to a standard curve of known BSA concentrations visualized by Coomassie fluorescence at 700 nm. SHLD2C-m1 and SHLD2CS denotes SHLD2C constructs carrying the OB fold m1 mutation and the internal deletion corresponding to the naturally occurring splice variant of SHLD2. * and · mark the bands corresponding to SHLD2C and SHLD1, respectively.

**ED Figure 11. SHLD2 OB-folds are required for suppression of RAD51 IR-induced focus formation.**

**a,** Quantitation of RAD51 foci 3 h upon 10 Gy irradiation in RPE1 *BRCA1-KO/SHLD2-KO* complemented with the indicated eGFP-tagged SHLD2 constructs via viral transduction. Protein expression was induced with 1 μg/mL doxycycline for 24 hrs prior to IR. Each data point is a biological replicate; the bar represents the mean ± SD (n≥3). **b,** Representative micrographs of the data shown in ED Fig 11a. **c,** Representative micrographs of RPE1 *BRCA1-KO/SHLD2-KO* cells virally expressing GFP-tagged SHLD2 WT or mutants, 1 h post 5 Gy IR. Scale bar = 10 μm. **d, e,** SHLD2 mutants did not disrupt Shieldin complex formation. Whole cell extracts (WCE) of 293T cells co-transfected with Strep/HA-SHLD2, its OB-fold m1 mutant (m1), or short splice variant (S) and other Shieldin subunits (Flag-SHLD1, V5-SHLD3, and GFP-REV7) were incubated with

streptavidin resin and bound proteins were eluted with biotin. WCEs and eluted proteins were visualized by SDS-PAGE and immunoblotting against the indicated antibodies.

## Methods

*Plasmids*

DNA corresponding to sgRNAs were cloned into pX330 (Addgene: 42230, Cambridge, MA, USA), LentiGuide-Puro (Addgene: 52963), LentiCRISPRv2 (Addgene: 52961), or a modified form in which Cas9 was replaced by NLS-tagged GFP or mCherry using AgeI and BamHI (designated as LentiGuide-NLS-GFP or –mCherry), as described[40,41]. Sequences of the sgRNAs used in this study are included in Supplemental Table 1. Coding sequences of C20orf196 and the short isoform of FAM35A were obtained from the ORFeome collection (horfdb.dfci.harvard.edu/), archived in the Lunenfeld-Tanenbaum Research Institute's OpenFreezer[42]. The complete coding sequence of the long isoform of FAM35A was generated by combining a synthesized fragment (GeneArt, Regensburg, Germany) corresponding to the long isoform C-terminus using an internal KpnI restriction site. The coding sequence of CTC534A2.2 was generated by gene synthesis (GeneArt). The coding sequences were PCR amplified using AscI and ApaI flanking primers and cloned into pcDNA5-FRT/TO-eGFP and pcDNA5-FRT/TO-Flag to obtain N-terminally tagged FAM35A, C20orf196 and CTC534A2.2. pGLUE-HA-Strep-FAM35A was generated by PCR amplification of the long isoform of FAM35A and cloning into pGLUE (Addgene: 15100) using AscI and NotI. To generate FAM35A fragments and mutants, standard protocols primer-directed mutagenesis or self-ligation of truncated PCR-products were used. To generate pcDNA5-FRT/TO-V5-CTC534A2.2, eGFP was replaced by a V5-tag in the cloning vector pcDNA5-FRT/TO-eGFP using KpnI and AscI restriction enzymes after which the coding sequence for CTC534A2.2 was PCR-amplified and inserted into pcDNA5-FRT/TO-V5-MCS using AscI and XhoI restriction enzymes.

To generate RNF8-FHA fusions, the N-terminus of RNF8 (aa 1-160) was PCR amplified from pcDNA3-RNF8-FHA(1-160)-RNF168 with flanking AscI sites and inserted into pcDNA5-FRT/TO-eGFP-FAM35A. eGFP-(FHA-) fusions of FAM35A were introduced into pCW57.1 (Addgene: 41393) by Gateway cloning using the pDONR221 donor vector. FAM35A amino acid substitution mutations and deletions were introduced by site directed mutagenesis and deletion PCR, respectively.

The REV7 coding sequence was obtained from the ORFeome collection and was cloned into the pDEST-FRT/TO-eGFP vector using Gateway cloning and into the pcDNA5-FRT/TO-Flag vector by PCR amplification. The N-terminal 967 residues of RIF1 were amplified by PCR and cloned into

the pDONR221 vector using Gateway technology. The fragment was then integrated into the pDEST-mCherry-LacR vector by Gateway cloning. Plasmids for the traffic light reporter system were obtained from Addgene (pCVL-TrafficLightReporter-Ef1a-Puro lentivirus: #31482; pCVL-SFFV-d14GFP-Ef1a-HA-NLS-Sce(opt)-T2A-TagBFP: #32627).

*Cell lines and gene editing*

293T and RPE1 hTERT cell lines were obtained from ATCC (Manassas, VA, USA), 293 Flp-In cells were obtained from Invitrogen (Carlsbad, CA, USA) and SUM149PT cells were obtained from Asterand Bioscience (Detroit, MI, USA). U2OS ER-mCherry-LacIFokI-DD cells (U2OS-265, referred to in the text as U2OS-FokI) were a kind gift of R. Greenberg (University of Pennsylvania, Philadelphia, PA, USA). 293T, U2OS and RPE1 cells were cultured in high glucose- and GlutaMAX-supplemented DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) + 1% Penicillin / Streptomycin (Thermo Fisher Scientific) and 10% heat inactivated fetal calf serum (Wisent, St-Bruno, Canada) at 37ºC, 5% $CO_2$. SUM149PT cells were cultured in Ham's F12 medium (Gibco) supplemented with 5% FCS, 10 mM HEPES, 1 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MI, USA), and 5 µg/mL insulin (Sigma-Aldrich) at 37ºC, 5% $CO_2$. Except for RPE1 clonogenic survival assays, which were performed at 3% $O_2$, cells were kept under normoxia conditions. Transient transfections of DNA and siRNA were performed using Lipofectamine 2000, PEI (Sigma-Aldrich), or calcium phosphate and Lipofectamine RNAiMAX, respectively (Thermo Fisher Scientific). siRNA efficiency was analysed by qPCR and immunoblotting. Stable integration of Flag-C20orf196/FAM35A/REV7 with the Flp-In system was achieved by co-transfection of the pcDNA5-FRT/TO plasmid with the recombinase vector pOG44 (Thermo Fisher Scientific) and hygromycin selection for integration. Lentiviral particles were produced in 293T cells by co-transfection of the targeting vector with vectors expressing VSV-G, RRE and REV using calcium phosphate or PEI (Sigma-Aldrich). Viral transductions were performed in the presence of 4-8 µg/µL polybrene (Sigma-Aldrich) at an MOI < 1, unless stated otherwise. Transduced RPE1 cells were selected by culturing in the presence of 15 µg/mL puromycin. For the BRCA1-deficient mouse cell experiments, all experiments were performed using virus produced with the LentiCRISPRv2 backbone (see Supplemental Table 1) and cells were infected using polybrene (8 µg/mL). The medium was refreshed after 12 h and transduced cells were selected with puromycin.

The generation of RPE1 hTERT *TP53*$^{-/-}$ *BRCA1-KO* Cas9 cells has been described elsewhere (Zimmermann *et al*., submitted). *REV7, 53BP1, FAM35A* and *C20orf196* gene knockouts were

generated by electroporation of LentiGuide or LentiCRISPRv2 vectors using a Lonza Amaxa II Nucleofector (Basel, Switzerland) (for sgRNA sequences employed: see Supplemental Table 1; REV7 - sgRNA1, FAM35A - sgRNA2 and C20orf196 - sgRNA1 were used for clonal knockout generation). 24 h following transfection, cells were selected for 24-48 h with 15 μg/mL Puromycin, followed by single clone isolation. Triple knockout cell lines of *TP53*, *BRCA1* and *53BP1* were created by mutating *BRCA1* from the *TP53*$^{-/-}$ *53BP1-KO* double knockout cell line. Triple knockout cell lines of *TP53*, *BRCA1* and *REV7*, *FAM35A* or *C20orf196* were created by mutating *REV7*, *FAM35A* or *C20orf196* in the *TP53*$^{-/-}$ *BRCA1-KO* cells. Loss of protein(s) was verified by immunoblotting when antibodies were available. Gene mutations were further confirmed by PCR amplification and TIDE analysis[22] (for primers used for genomic PCR, see Supplemental Table 2).

To generate SUM149PT *53BP1*, *PARP1* or *C20orf196* knockout populations of cells, SUM149PT-doxCAS9 cells were treated with doxycycline for 24 h at 1 μg/ml prior to transfection with EditR crRNA (Dharmacon, Lafayette, CO, USA). Transfection of guides 53BP1_5_1, 53BP1_5_3, PARP1_5_2, PARP1_5_4, C20orf196_5-1, C20orf196_5-2, C20orf196_5-3 and C20orf196_5-5 (see Supplemental Table 1) was performed at a concentration of 20 nM (crRNA:tracrRNA) in the presence of doxycycline (1 μg/ml) using Lipofectamine RNAiMAX in 48 well plates (35,000 cells per well). The following day cells were split 1:3, fed 24 h later with media supplemented with 50 nM talazoparib (without doxycycline) and kept in batch culture or further split to generate single cell colonies. Drug-containing media was replenished every 3-4 days until PARP inhibitor resistant pools or clones were established. Clones were subsequently picked and expanded and sequence validated by genomic PCR and sequence analysis (for primers used, see Supplemental Table 2). Four SUM149PT *C20orf196-KO* clones with mutations were chosen for further experimentation: clone A (C20orf196 5-1-C1), clone B (C20orf196 5-1-C2), clone C (C20orf196 5-3-C5) and clone D (C20orf196 5-5-C4).

To generate *53BP1-KO* double mutant clones, SUM149PT *C20orf196* clones A and D were infected with lentiviral plasmids expressing an sgRNA targeting *TP53BP1* or a non-targeting control sgRNA (for sequences, see Supplemental Table 1) in media containing 1 μg/ml doxycycline. 48 h after infection, puromycin (1 μg/ml) was added to the media. Selection was maintained for 3 days, until the uninfected control cells were killed. Pools of selection-resistant cells were seeded into 384-well plates for short term survival assays (see below) or subcloned to generate clonal lines.

Mouse ES cells with a selectable conditional *Brca1* deletion (*Rosa26*$^{CreERT2/wt}$;*Brca1*$^{SCo/\Delta}$)[43] 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, Kenilworth, NJ, USA) and $10^3$ U/ml ESGRO LIF (Millipore, Burlington, MA, USA) under normal oxygen conditions (21% $O_2$, 5% $CO_2$, 37°C).

The KB1P-G3 2D cell line was previously established from a *Brca1*$^{-/-}$ *p53*$^{-/-}$ mouse mammary tumor and cultured as described[16]. Briefly, cells were cultured in DMEM/F-12 medium (Life Technologies, Carlsbad, CA, USA) in the presence of 10% FCS, penicillin/streptomycin (Gibco), 5 µg/mL insulin (Sigma-Aldrich), 5 ng/mL epidermal growth factor (Life Technologies) and 5 ng/mL cholera toxin (Gentaur, Kampenhout, Belgium) under low oxygen conditions (3% $O_2$, 5% $CO^2$ at 37$^o$C).

The KB1P4 3D tumor organoid line was previously established from a *Brca1*$^{-/-}$ *p53*$^{-/-}$ mouse mammary tumor and cultured as described[44]. Cells were seeded in Basement Membrane Extract Type 2 (BME, Trevigen, Gaithersburg, MD, USA) on 24-well suspension plates (Greiner Bio-One, Kremsmünster, Austria) and cultured in AdDMEM/F12 supplemented with 10 mM HEPES (Sigma-Aldrich), GlutaMAX (Invitrogen), penicillin/streptomycin (Gibco), B27 (Gibco), 125 µM N-acetyl-L-cysteine (Sigma-Aldrich), and 50 ng/mL murine epidermal growth factor (Invitrogen).

CH12F3-2 mutant clones were edited either through transient transfection with pX330 plasmid constructs coding for sgRNAs against *Trp53bp1* (sgRNA: *Trp53bp1*_e6_834, see Supplemental Table 1), *Fam35a*, and *Ctc534a2.2* or by lentiviral lentiCRISPR v2 transduction for *C20orf196*. Double knock out cell lines of *Fam35a* and *Trp53bp1* or *C20orf196* were generated by transient transfection of pX330 plasmid with sgRNAs against *Trp53bp1* or by lentiviral transduction with lentiCRISPRv2 with *C20orf196* sgRNAs.

*Antibodies, siRNAs and drugs*

An overview of all the antibodies used in this study, including dilution factors, can be found in Supplemental Table 3. The following siRNAs from Dharmacon were used in this study: 53BP1: siRNA #2 (D-003548-02-0020); RIF1: siGENOME RIF1 siRNA (D-027983-02-0050); REV7: siGENOME MAD2L2 siRNA (M-003272-03-0010); C20orf196: SMARTpool: siGENOME C20orf196 siRNA (M-018767-00-0005); FAM35A: SMARTpool: siGENOME FAM35A siRNA

(M-013761-01-0005); CTC534A2.2: custom order: siRNA#1: 5'-GGACAAAACUCAAUCAAAU-3', siRNA#2: 5'-CAGUAGAUCUAUUGGAGUU-3', siRNA#3: 5'-CUGGAAGACAUUUGGACAA-3', siRNA#4: 5'-GCAAGAUAGUUUAAAGGCA-3' (used as a pool).

The following drugs were used in the course of this study: olaparib (SelleckChem, Houston, TX, USA, or Astra Zeneca, Cambridge, UK), talazoparib (SelleckChem), cisplatin (Sigma-Aldrich), the ATM-inhibitor KU60019 (Sigma-Aldrich), and etoposide (Sigma-Aldrich). Concentration and duration of treatment are indicated in the corresponding figure legends.

*Olaparib resistance screens*

Viral particles of the TKOv1 sgRNA library were produced as described before[45]. This library contains 91,320 sgRNAs sequences, with a modal number of six sgRNAs per gene. Cas9-expressing cells were infected with an MOI < 0.3 and the coverage of sgRNA representation was maintained at > 100x (SUM149PT) or > 200x (RPE1) (per replicate, if applicable). 24 h following transduction, transduced cells were selected for 120 h with 10 µg/mL puromycin (RPE1) or 48 h with 3 µg/mL puromycin, followed by 72 h with 0.5 µg/mL puromycin (SUM149PT). Three days after transduction, the transduced cells were split into three technical replicates. Cells were passaged once every three days until nine days after infection, upon which olaparib (16 nM for RPE1 *TP53*$^{-/-}$ *BRCA1-KO*, 2 µM for SUM149PT) was added to the cells. Olaparib-containing medium was refreshed every four days. Cells were harvested at 3, 9, 18 and 23 days post-infection (RPE1) or at 3, 9, 19 and 26 d post-infection (SUM149PT) for downstream processing as described[45]. In short, total genomic DNA was isolated from 2 x $10^7$ (t3 sample) or 1 x $10^7$ (later time points) cells using the QIAamp DNA Blood Maxi Kit (Qiagen, Germantown, MD, USA). DNA was precipitated with ethanol and sodium chloride and reconstituted in EB buffer (10 mM Tris-HCl pH 7.5). sgRNA sequences were PCR-amplified using primers harbouring Illumina TruSeq adapters with i5 and i7 barcodes, and the resulting libraries were sequenced on a Illumina NextSeq500 (San Diego, CA, USA) using parameters as described before[45]. Analysis was performed using Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout (MAGeCK) version 0.5.3[46], in conjunction with Python v3.5.1 on a Mac OS X El Capitan operating system. Non-treated samples collected at day 9 after transduction were compared to treated samples collected at day 23 (RPE1) or day 26 (SUM149PT). The positive score for each gene was calculated by using the 'run' function with the following arguments:

```
mageck run -l /path/to/TKOv1_library/ -n 08-02-2017_141703 --sample-label
test,CTRL -t 1 -c 0 --fastq /path/to/fastq1 /path/to/fastq2.
```

*SUM149PT talazoparib resistance screen*

A derivative of SUM149PT with an integrated tetracycline-inducible Cas9 expression allele was lentivirally infected with a genome-wide guide RNA library ("Yusa" library of 90,709 sgRNAs) designed to target 18,010 genes[47], using a multiplicity of infection of 0.3 and infecting >1000 cells per sgRNA. After puromycin selection (3 µg/ml) to remove non-tranduced cells, a sample was removed (t0); 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, gDNA from the remaining cells in the doxycycline-treated sample was recovered. The sgRNA sequences from this gDNA were PCR amplified using barcoded and tailed primers and deep sequenced to identify sgRNAs in the talazoparib-resistant population. sgRNA read data was analysed using a gene-level method (MaGeCK version 0.5.5) as well as using a normalised read frequency method to identify individual sgRNAs associated with resistance, by comparing sgRNA abundances in the resistant and starting populations.

Command used for read count generation:

```
mageck count --output-prefix PREFIX --list-seq
Human_genome_library_guides_for_mageck.csv --fastq T1.fastq T0.fastq --
sample-label T1,T0 --trim-5 0
```

Command used for MLE analysis:

```
mageck mle --norm-method none --output-prefix PREFIX --sgrna-eff-name-
column 3 --sgrna-eff-score-column 4 --sgrna-efficiency
annotation/sgrnas.bed --count-table pptm.counts.txt --design-matrix
designmatrix.txt
```

*IR dropout screen and TKOv2 library*

hTERT RPE1 *TP53*$^{-/-}$ Cas9-expressing cells were transduced with the lentiviral TKOv2 library (see below) at a low MOI (~0.35) and puromycin-containing media was added the next day to select for transductants. Selection was continued until 72 h post transduction, which was considered the initial time point, t0. To identify IR sensitizers, the negative-selection screen was performed by subculturing at days 3 and 6 (t3 and t6), at which point the cultures were split into two populations. One was left untreated while the second was treated with 3 Gy of IR using a Faxitron X-Ray cabinet (Faxitron, Tucson, AZ, USA) every 3 days after day 6. Cell pellets were frozen at day 18 for gDNA

isolation. Screens were performed in technical duplicates and library coverage of ≥375 cells/sgRNA was maintained at every step. gDNA from cell pellets was isolated using the QIAamp Blood Maxi Kit (Qiagen) and genome-integrated sgRNA sequences were amplified by PCR using the KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA). i5 and i7 multiplexing barcodes were added in a second round of PCR and final gel-purified products were sequenced on Illumina NextSeq500 systems to determine sgRNA representation in each sample. DrugZ[48] was used to identify gene knockouts which were depleted from IR-treated t18 populations but not depleted from untreated cells.

The TKOv2 lentiviral CRISPR library was used for whole-genome CRISPR knockout screening. To design TKOv2, all possible 20mer sequences upstream of NGG PAM sites were collected where the SpCas9 double-strand break would occur within a coding exon (defined by hg19/Gencode v19 "appris_principal," "appris_candidate_longest," or "appris_candidate" transcript). Guides with 40-75% GC content were retained and further filtered to exclude homopolymers of length >=4, SNPs (dbSNP138), and relevant restriction sites, including BsmI (GAATCG) and BsmBI (CGTCTC). Candidate gRNA+PAM sequences were mapped to hg19 and sequences with predicted off-target sites in exons or introns, or sequences with more than two predicted off-target sites (with up to two mismatches) in any location, were discarded. Remaining guides were scored using the "sequence score table" described in ref[49]. Four guides per gene were selected, with a bias toward high sequence scores and maximal coverage across exons (i.e. moderate-scoring guides targeting different exons were preferred to high-scoring guides targeting the same exon). The final library contains 70,555 gRNA targeting 17,942 protein-coding genes, as well as 142 sequences targeting LacZ, luciferase, and eGFP. Oligo sequences were ordered from CustomArray (Bothell, WA), PCR amplified, and cloned into the pLCKO vector as described in ref[45].

*Two-color competitive growth assay*

20,000 cells were infected at an MOI of ~ 1.2 to ensure 100% transduction efficiency with either virus particles of NLS-mCherry LacZ-sgRNA or NLS-GFP GOI-sgRNA. 96 h following transduction, mCherry- and GFP-expressing cells were mixed 1:1 (2,500 cells + 2,500 cells) and plated with or without olaparib (16 nM) or etoposide (100 nM) in 12-well format. During the course of the experiment, cells were subcultured when near confluency was reached. Olaparib- or etoposide-containing medium was replaced every three days. Cells were imaged for GFP- and mCherry signal the day of initial plating (t=0) and on days 3, 6, 9, 12, 15 and 18 (olaparib), or, in a

separate set of experiments, on day 5, 10, 15 and 20 (etoposide). Cells were imaged using the automatic InCell Analyzer (GE Healthcare Life Sciences, Marlborough, MA, USA) with a 4x objective. Segmentation and counting of the number of GFP-positive and mCherry-positive cells was performed using an Acapella script (PerkinElmer, Waltham, MA, USA). Efficiency of indel formation was analysed by performing PCR amplification of the region surrounding the sgRNA sequence and TIDE analysis on DNA isolated from GFP-expressing cells 9 d post-transduction.

*Mass spectrometry*

Following 24 h of doxycycline-induction of stably integrated 293 FLP-IN cells (expressing Flag, Flag-FAM35A, Flag-REV7, Flag-C20orf196, Flag-CTC-534A2.2), cell pellets from two 150 mm plates were lysed in 50 mM HEPES-KOH (pH8.0), 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 10% glycerol and affinity-purified using Flag-M2 magnetic beads (Sigma-Aldrich). Subsequently, digestion with trypsin (Worthington, Columbus, OH, USA) was performed on-beads. All immunoprecipitations were performed in biological replicates (three for CTC-534A2.2, five for C20orf196 and six for FAM35A and REV7).

For LC-MS/MS analysis, peptides were reconstituted in 5% formic acid and loaded onto a 12-15cm fused silica column with pulled tip packed with C18 reversed-phase material (Reprosil-Pur 120 C18-AQ, 3 µm, Dr. Maisch, Ammerbuch-Entringen, Germany). Peptides were analysed using an LTQ-Orbitrap Velos (Thermo Scientific) or a 6600 Triple TOF (AB SCIEX, Framingham, MA, USA) coupled to an Eksigent NanoLC-Ultra HPLC system and a nano-electrospray ion source (Proxeon Biosystems, Thermo Fisher Scientific). Peptides were eluted from the column using a 90-100 min gradient of acetonitrile in 0.1% formic acid. Tandem MS spectra were acquired in a data-dependent mode for the top 10 most abundant ions using collision-induced dissociation. After each run, the column was washed extensively to prevent carry-over.

Mass spectrometry data extraction and interaction scoring was performed essentially as described previously[50]. In short, raw mass spectrometry files were converted to mzXML and analyzed using the iProphet pipeline[51], implemented within ProHits[52]. The data were searched against the human and adenovirus complements of the Uniprot (forward and reverse) database (Version 2017_09; reviewed Swiss-Prot entries only), to which common epitope tags were added as well as common contaminants (common contaminants are from the Max Planck Institute, http://141.61.102.106:8080/share.cgi?ssid=0f2gfuB, and the Global Proteome Machine,

http://www.thegpm.org/crap/index.html; 85393 entries were searched). Mascot and Comet search engines were used with trypsin specificity (2 missed cleavages allowed) and deamidation (NQ) and methylation (M) as variable modifications. Charges +2, +3 and +4 were allowed with a parental mass tolerance of maximum 12 ppm and a fragment bin tolerance of 0.6 Da selected for Orbitrap instruments, while 35 ppm and 0.15 Da were allowed for the TripleTOF 6600. For subsequent SAINT analysis (see below), only proteins with an iProphet protein probability $\geq 0.95$ were considered, corresponding to an estimated protein false discovery rate (FDR) of ~0.5%.

Interactions were analysed with SAINTexpress (v3.6.1)[53,54]. SAINT probability scores were computed independently for each replicate against eight biological replicate analyses of the negative control (FLAG alone; controls were "compressed" to six virtual controls to increase robustness as described[55]) and the average probability (AvgP) of the best three out of three (CTC534A2.2), five out of five (C20orf196) or six (FAM35A, REV7) biological replicates for each bait was reported as the final SAINT score. Preys with an estimated FDR ≤1% were considered true interactors (AvgP ≥0.93). The entire dataset, including the peptide identification and complete SAINTexpress output was deposited as a complete submission in ProteomeXchange through the partner MassIVE housed at the Center for Computational Mass Spectrometry at University of California, San Diego (UCSD; http://massive.ucsd.edu). Data are available at MassIVE (ftp://MSV000081815@massive.ucsd.edu; currently password-protected: Shieldin). Unique accession numbers are MSV000081815 and PXD008458, respectively. Data in Fig 1g is represented using Cytoscape, using analyses with an FDR ≤ 1 or 5 %.

*Immunoprecipitation*

1 x $10^7$ 293T cells were transfected with pcDNA5.1-FRT/TO -FLAG-c20orf196 (10 µg), -GFP-REV7 (2 µg), -V5-CTC534A2.2 (14 µg) and pGLUE-HA-Strep-FAM35A (14 µg) or empty vectors using standard calcium phosphate or PEI protocol. After 48 h, cells were washed with PBS, scraped, and lysed in 1 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10mM NaF, 10 mM $MgCl_2$ and 10 U/ml Benzonase (Sigma-Aldrich)) on ice for 30 min. Lysates were centrifuged at 15,000g for 5 min at 4 °C, and supernatants were incubated with 100 µl of Streptavidin Sepharose High Performance beads (GE Healthcare) or Dynabeads M-280 Streptavidin magnetic beads (Invitrogen) for 1 h at 4 °C. Beads were washed 5 times with lysis buffer and eluted with 10 mM D-biotin (Invitrogen) in lysis buffer for 2 h at 4 °C. When applicable, the eluate was incubated with 20 µl of GFP-Trap_M (Chromotek, Planegg-Martinsried, Germany) for 1 h at 4 °C,

washed 5 times with lysis buffer and eluted by boiling in sample buffer. Pull down and whole cell extract were loaded onto SDS-PAGE gels, followed by Western blotting and staining with indicated antibodies. For GFP-CTC534A2.2 immunoprecipitations, an identical GFP-Trap_M pulldown procedure as above was used. For V5-CTC534A2.2 immunoprecipitations, lysates from one confluent 10 cm dish of 293T cells transfected with 10 µg pcDNA5.1-FRT/TO-V5-CTC534A2.2 vector was incubated with 10 µg/ml anti-V5 antibody (Invitrogen) for 2 hours at 4 °C. Subsequently 50 µl of protein G Dynabeads (Invitrogen) resin was added to the lysates and incubated for an additional 1 h at 4 °C. Beads were washed 4 times with lysis buffer and boiled in 50 µl 2xSDS buffer.

*Clonogenic survival assays*

RPE1-TERT *TP53*$^{-/-}$ cells were seeded in 10 cm dishes (WT: 250 cells: *BRCA1-KO 53BP1-KO*, 500 cells; *BRCA1-KO* or *BRCA1-KO C20orf196-KO*: 1,500 cells; *BRCA1-KO FAM35A-KO*: 750 cells) in the presence of 800 nM cisplatin or 16 nM olaparib or left untreated. Cisplatin dosing lasted 24 h, after which cells were grown in drug-free medium. Olaparib containing medium was refreshed after 7 days. After 14 days, colonies were stained with crystal violet solution (0.4 % (w/v) crystal violet, 20% methanol) and manually counted. Relative survival was calculated for the drug treatments by setting the number of colonies in non-treated controls at 100%.

For *Rosa26*$^{CreERT2/wt}$ *Brca1*$^{SCo/\Delta}$ cells, Cre-mediated inactivation of the endogenous mouse *Brca1SCo* allele was achieved by overnight incubation of cells with 0.5 µmol/L 4-OHT (Sigma-Aldrich). Four days after switching, cells were seeded in triplicate at 10,000 cells per well in 6-well plates for clonogenic survival assays. For experiments with *Rosa26*$^{CreERT2/wt}$ *Brca1*$^{SCo/\Delta}$ p53-null cells, cells were plated in the presence of 15 nM olaparib. Cells were stained with 0.1% crystal violet one week later. Clonogenic survival assays with PARPi (olaparib) and ATMi (KU60019) combination treatment were performed as described previously with minor adjustments[50]. 5 x $10^3$ KB1P-G3 cells were seeded per well into 6-well plates on day 0, and then PARPi, ATMi or both were added. The medium was refreshed every 3 days. On day 6, the ATMi alone and untreated groups were stopped, the other groups were stopped on day 9 and stained with 0.1% crystal violet. Plates were scanned with a GelCount (Oxford Optronics, Abingdon, UK). Quantifications were performed by solubilizing the retained crystal violet using 10% acetic acid and measuring the absorbance at 562 nm using a Tecan plate reader (Tecan, Männedorf, Switzerland).

*Short term survival assays*

10,000 RPE1-hTERT Cas9 *TP53*$^{-/-}$ parental cells and additional mutants (*BRCA1-KO* and/or *FAM35A-KO*) with or without stable integration of indicated eGFP-fusions by viral transduction were seeded into 12-well format with or without olaparib (and 1 µg/mL doxycycline if applicable). Medium with olaparib (and doxycycline) was replaced after 4 days and cells were trypsinized and counted after seven days using an automated Z2 Coulter Counter analyzer (Beckman Coulter, Indianapolis, IN, USA).

SUM149PT cells were plated at 500 cells per well in 384-well plates and talazoparib in DMSO added the following day using an Echo 550 liquid handler (Labcyte, San Jose, CA, USA). After 5 days growth, cell survival was assayed using CellTiter-Glo according to the manufacturer's protocol (Promega, Madison, WI, USA).

*Immunofluorescence of DNA damage induced foci*

For 53BP1 IF, cells were cultured on coverslips and treated with 5 or 10 Gy irradiation and fixed with 2-4% PFA 1 h after irradiation. Cells were permeabilized with 0.3% Triton X-100, followed by blocking in 10% goat serum, 0.5% saponin, 0.5% NP-40 in PBS (blocking buffer A). Cells were co-stained using 53BP1 and γH2AX primary antibodies (see Supplemental Table 3) in blocking buffer A for 1.5 h at room temperature, followed by 4 washes in PBS, incubation with appropriate secondary antibodies in blocking buffer A plus 0.8 µg/mL DAPI for 1.5 h at room temperature, and finally four washes in PBS. For RAD51 IF, cells with or without stable integration of eGFP-tagged proteins or sgRNAs via viral transduction were grown on glass coverslips and treated with 10 Gy irradiation and recovered for 3 to 6 h (as indicated). Cells were fixed using 1% PFA, 0.5% Triton X-100 in PBS for 20 min at room temperature, followed by a second extraction/fixation using 1% PFA, 0.3% Triton X-100, 0.5% methanol in PBS for 20 min at room temperature. Blocking and primary and secondary antibody incubations (1.5 h at room temperature followed by 4 PBS washes) were performed in BTG buffer (10 mg/mL BSA, 0.5% Triton X-100, 3% goat serum, 1 mM EDTA in PBS) or PBS$^{+}$ (0.5% BSA, 0.15% glycine in PBS). For REV7 and RIF1 IF, cells were grown on glass coverslips and treated with 5 or 10 Gy irradiation and fixed with 2-4% PFA 1-2 h after irradiation. Cells were permeabilized with 0.3% Triton X-100. For REV7, blocking and primary and secondary antibody incubations (1.5 h at room temperature followed by 4 washes in PBS) were performed in blocking buffer A. For RIF1, blocking and primary and secondary antibody incubations (1.5 h at room temperature followed by 4 washes) were performed in PBG buffer (0.2%

cold water fish gelatin (Sigma Aldrich), 0.5% BSA in PBS). 0.8 µg/mL DAPI was included in all experiments to stain nuclear DNA. Coverslips were mounted using Prolong Gold mounting reagent (Invitrogen) or Aqua PolyMount (Polyscience, Warrington, PA, USA). Images were acquired using a Zeiss LSM780 laser-scanning microscope (Oberkochen, Germany), a Leica SP8 confocal microscope (Wetzlar, Germany) or a Zeiss AxioImager D2 widefield fluorescence microscope and foci were manually counted.

For GFP-Shieldin foci analysis, U2OS cells were grown on glass coverslips and transfected with GFP-SHLD2/3. 48 h post transfection, cells were treated with 5 Gy irradiation, and 1 hr later pre-extracted with NuEx buffer (20mM HEPES, pH 7.4, 20mM NaCl, 5mM MgCl2, 0.5% NP-40, 1mM DTT and protease inhibitors) and PFA-fixed. Immunofluorescence was then performed as described above.

RAD51 immunofluorescence in KB1P-G3 cells was performed as described previously, with minor modifications[13]. Cells were grown on 8-well chamber slides (Millipore). Ionizing-radiation induced foci were induced by γ-irradiation (10 Gy) 4 h prior to sample preparation. Cells were then washed in PBS++ (2% BSA, 0.15% glycine, 0.1% Triton X-100) and fixed with 2% PFA/PBS++ for 20 min on ice. Fixed cells were washed with PBS++ and were permeabilized for 20 min in 0.2% Triton X-100/PBS++. All subsequent steps were performed in PBS++. Cells were washed thrice and blocked for 30 min at room temperature, incubated with the primary antibody for 2 h at RT, washed thrice and incubated with the secondary antibody for 1 h at room temperature. Lastly, cells were mounted and counterstained using Vectashield mounting medium with DAPI (H1500, Vector Laboratories, Burlingame, CA, USA).

*Traffic Light reporter assay*

Cells were infected with pCVL.TrafficLightReporter.Ef1a.Puro lentivirus at a low MOI (0.3-0.5) and selected with puromycin (15 µg/µl). 7 x $10^5$ cells were nucleofected with 5 µg of pCVL.SFFV.d14GFP.Ef1a.HA.NLS.Sce(opt).T2A.TagBFP plasmid DNA in 100 µL of electroporation buffer (25 mM Na2HPO4.7H2O pH 7.75, 2.5 mM KCl, 11 mM MgCl2), using program T23 on a Nucleofector 2b (Lonza). After 72 h, GFP and mCherry fluorescence was assessed in BFP-positive cells using a Fortessa X-20 (BD Biosciences, San Jose, CA, USA) flow cytometer.

*Phospho-RPA immunoblotting*

For phospho-RPA staining, CH12 cells were left untreated, or were treated with 25 Gy of ionizing radiation using a Faxitron x-ray cabinet, then collected by centrifugation 3 h later. Pellets were lysed on ice for 10 min in high salt lysis buffer (50 mM Tris-HCl pH 7.6, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, 1X EDTA-free protease inhibitor cocktail (Roche, Basel, Switerzerland)), cleared by centrifugation at 20, 000 x *g* for 10 min at 4 °C, and quantified by bicinchoninic acid assay (BCA; Pierce, Thermo Fisher Scientific). Equal amounts of whole-cell extracts were separated by SDS-PAGE on 4-12% Bis-Tris gradient gels (Invitrogen) and transferred to nitrocellulose, and then immunoblotted for pRPA32 (S4/S8).

*LacR-RIF1N and FokI-induced focus formation*

For monitoring recruitment of GFP-tagged Shieldin subunits to mCherry-LacR-Rif1(1-967) foci, 150,000 U2OS FokI cells (known also as U2OS-DSB)[28] were seeded on 6-well plates containing glass coverslips without any induction of FokI. 24 h after seeding, cells were transfected using 1µg of pDEST-mCherry-LacR or pDEST-mCherry-LacR-Rif1(1-967) and 0.5-1 µg of GFP fusion expression vectors. Cells were fixed 24-48 h after transfection. For monitoring the localization of the SHLD2 N-terminus to Rif1(1-967) foci with siRNA knockdown of other Shieldin subunits, an essentially identical protocol was used with the following adjustments: 350,000 U2OS FokI cells were reverse transfected with Lipofectamine RNAiMAX-siRNA (10 nM) complex. 24 h after siRNA transfection, the mCherry-LacR and GFP fusion plasmids were transfected. Cells were fixed 48 h after DNA transfection. For monitoring recruitment of GFP-tagged Shieldin subunits to DSBs at the LacO array, FokI stabilization and nuclear translocation was induced by incubating cells with 0.1 µM Shield1 (Clontech, Mountain View, CA, USA) and 10 µg/mL hydroxytamoxifen for 4 h.

ImageJ (https://imagej.nih.gov/ij/) was used to quantify foci in the U2OS FokI system. An mCherry focus and DAPI nuclear signal were used to generate masks. The average GFP or immunofluorescence intensity in the mCherry focus mask was divided by the corresponding average nuclear intensity, and the ratio is reported. Cells displaying a ratio of focus/nuclear average intensity >3 are defined as containing a focus.

*Microirradiation*

For laser microirradiation, virally transduced RPE1 cells expressing the indicated eGFP-tagged proteins were grown on glass coverslips and transfected with siRNAs. 48 h post-transfection, protein

expression was induced using 0.5 µg/mL doxycycline and 24 h later, cells were presensitized with 1 µg/mL Hoechst for 15 min at 37°C. DNA damage was introduced with a 355 nm laser (Coherent, Santa Clara, CA, USA, 40mW) focused through a Plan-Apochromat 40x oil objective to yield a spot size of 0.5-1 mm using a LSM780 confocal microscope (Zeiss) and the following laser settings: 100% power, 1 iteration, frame size 128 x 128, line step 7, pixel dwell: 25.21 µs. Nuclei were pre-extracted using NuEx pre-extraction buffer (20 mM HEPES pH 7.4, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40 and Complete protease inhibitors (GE Healthcare)) for 10 min on ice, followed by fixation in 2% PFA. Antibody staining and blocking were performed in PBS + 0.1% Tween-20 and 5% BSA as described above using GFP and γH2AX antibodies and imaged on a Zeiss LSM780 confocal microscope.

*Mouse mammary tumour models*

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). KB1P4 tumor organoids were transduced using spinoculation as described previously[56]. Tumor organoids were allografted in mice as described previously with minor adjustments[44]. Briefly, tumor organoids were collected, incubated with TripLE at 37$^{o}$C for 5 min, dissociated into single cells, washed and embedded in a 1:1 mixture of tumor organoid culture medium and Basement Membrane Extract (Trevigen) in a cell concentration of $10^4$ cells per 40 µl. Subsequently, $10^4$ cells were injected in the fourth right mammary fat pad of NMRI nude mice. Mammary tumor size was determined by caliper measurements and tumor volume was calculated (0.5 x length x width$^2$). Treatment of tumor bearing mice was initiated when tumors reached a size of 50-100 mm$^3$. Mice were randomly allocated into the untreated (n = 8) or olaparib treatment group (n = 8). Olaparib was administered at 100mg/kg intraperitoneally for 80 consecutive days. Animals were sacrificed with $CO_2$ when the tumor reached a volume of 1,500mm$^3$. The tumor was collected, fixed in formalin for histology and several tumor pieces were harvested for DNA analysis.

*Class switch recombination assays*

To induce switching in CH12 murine B cell lymphoma cells, 200,000 cells were cultured in CH12 media supplemented with a mixture of IL4 (10 ng/mL, R&D Systems #404-ML-050, Minneapolis, MN, USA), TGFβ (1 ng/mL, R&D Systems #7666-MB-005) and anti-CD40 antibody (1 µg/mL, #16-0401-86, eBioscience, Thermo Fisher) for 48h, stained with anti-IgA-PE and fluorescence signal was acquired on an LSR II or Fortessa X-20 flow cytometers (BD Biosciences). To probe

AID levels in the stimulated cells, immunoblotting was performed on total cell lysates and stained for anti-AID and anti-β-actin antibodies (Supplemental Table 3). Band quantification was analysed by ImageJ.

*Plasmid Integration assay*

200,000 RPE1 cells were seeded into 6-well plates and 24 h later transfected with 2 µg peGFP-c1, linearized by BamHI and EcoRI digestion, using PEI. 72 h post-transfection, cells were seeded for colony formation into 10 cm dishes in the presence (50,000 cells per dish) or absence (500 cells per dish) of 600 µg/mL G418. At this point, transfection efficiency was analysed by measuring GFP-positivity using flow cytometry. Medium with G418 was refreshed every 3 d. 14 d after seeding, colonies were stained with crystal violet solution and manually counted. NHEJ efficiency was calculated according to the following formula:

$$\frac{\%\ \text{surviving colonies on selection}}{(\%\ \text{of surviving colonies without selection}) \text{x} (\%\ \text{of transfected cells})}$$

*DNA binding assays*

Shieldin proteins were isolated using the above immunoprecipitation protocol with modifications. 293T cells were transfected with pGLUE-Strep-HA-FAM35A(421-904), the indicated mutants of this vector, or the empty vector and pcDNA5.1-FRT/TO -FLAG-c20orf196 in a 2:1 ratio for total of 10 µg per 10 cm dish. Complexes were immunoprecipitated as described, except using a reduced NP-40 detergent concentration (0.1%) for the last two washes and elution buffer. Eluted proteins were concentrated using Amicon Ultra 0.5 ml 10K centrifugal filter units (Millipore). Concentrations of isolated proteins were estimated by Coomassie-stained SDS-PAGE compared to a standard curve of known BSA concentrations measured by fluorescence in the 700 nM channel of the Odyssey imager (LI-COR). ssDNA probe was prepared by PNK phosphorylation of HPLC-purified 59 nt DNA oligo (BioBasic; TACGTTAGTATGCGTTCTTCCTTCCAGAGGTTTTTTTTTTTTTTTTTTTTTTTTTTT) using [γ-32P]-ATP (3000 Ci/mmol, 10 mCi/ml; Perkin-Elmer). Unlabeled competitors were prepared using the same oligo sequence alone or hybridized with the complementary sequence (AAAAAAAAAAAAAAAAAAAAAAAAAAAACCTCTGGAAGGAAGAACGCATACTAAC GTA) by heating at 80 °C and gradual cooling.

For electrophoretic mobility shift assays, 20 nM of labeled ssDNA probe was incubated with purified proteins for 20 minutes in the elution buffer with the addition of 1mM DTT and 1mg/ml BSA at room temperature. Glycerol was then added to a final concentration of 8.3% and resolved on a 6% acrylamide-TAE gels. Gels were adhered onto blotting paper (VWR) and wrapped in plastic wrap. Gels were exposed to a storage phosphor screen (GE Healthcare) and visualized using a Typhoon FLA 9500 biomolecular imager (GE Healthcare). Dissociation constant (Kd) was determined in GraphPad Prism from nonlinear regression analysis assuming single site specific binding of saturation titration experiments defining all signal above the free probe band as bound probe measured in ImageQuant TL(GE Healthcare).

**Data availability statement**

All data generated or analysed during this study will be included in the published article (and its supplementary information) or available upon request.