The shieldin complex mediates 53BP1-dependent DNA repair

Sylvie M. Noordermeer1,2,10, Salomé Adam1,10, Dheva Setiaputra1,10, Marco Barazas1, Stephen J. Pettitt4, Alexandra K. Ling5, Michele Olivieri1,6, Alejandro Alvarez–Quilión1, Nathalie Moattt1, Michal Zimmermann1, Stefano Annunziato3, Dragomir B. Krastev1, Feifei Song1, Inger Brandsma4, Jessica Frankum1, Rachel Brough1, Alana Sherker1,6, Sébastien Landry1, Rachel K. Szilard1, Meagan M. Munro1, Andrea Mcewan1, Théo Goullet de Rugy1, Zhen-Yuan Lin1, Traver Hart7, Jason Moffat6,8, Anne–Claude Gingras1,6, Alberto Martin5, Haico van Attikum2, Jos Jonkers3, Christopher J. Lord4, Sven Rottenberg3,9, & Daniel Durocher1,6,*

53BP1 is a chromatin-binding protein that regulates the repair of DNA double-strand breaks by suppressing the nucleolytic resection of DNA termini11. This function of 53BP1 requires interactions with PTIP15 and RIF11,6, the latter of which recruits REV7 (also known as MAD2L2) to break sites10,11. How 53BP1-pathway proteins shield DNA ends is currently unknown, but there are two models that provide the best potential explanation of their action. In one model the 53BP1 complex strengthens the nucleosomal barrier to end-resection nucleases12,13, and in the other 53BP1 recruits effector proteins with end-protection activity. Here we identify a 53BP1 effector complex, shieldin, that includes C20orf196 (also known as SHLD1), FAM35A (SHLD2), CTC-534A2.2 (SHLD3) and REV7. Shieldin localizes to double-strand-break sites in a 53BP1–RIF1-dependent manner, and its SHLD2 subunit binds to single-stranded DNA via OB-fold domains that are analogous to those of RPA1 and POT1. Loss of shieldin impairs non-homologous end-joining, leads to defective immunoglobulin class switching and causes hyper-resection. Mutations in genes that encode shieldin subunits also cause resistance to poly(ADP-ribose) polymerase inhibition in BRCA1-deficient cells and tumours, owing to restoration of homologous recombination. Finally, we show that binding of single-stranded DNA by SHLD2 is critical for shieldin function, consistent with a model in which shieldin protects DNA ends to mediate 53BP1-dependent DNA repair.

To discover proteins acting in the 53BP1 pathway, we searched for genes whose mutation restores homologous recombination in BRCA1-deficient cells and leads to resistance to poly(ADP-ribose) polymerase (PARP) inhibition, which is a hallmark of 53BP1 deficiency14–16. We undertook three independent CRISPR–Cas9 screens that entailed the transduction of BRCA1-deficient cells with lentiviral libraries of single-guide RNAs (sgRNAs) (Extended Data Fig. 1a). The resulting pools of edited cells were exposed to near-lethal doses of two clinically validated PARP inhibitors (PARPi), either olaparib or talazoparib17. We pools of edited cells were exposed to near-lethal doses of two clinically validated PARP inhibitors (PARPi), either olaparib or talazoparib17. We

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data suggest that the complex formed by C20orf196, FAM35A, and REV7 resulted in a phenotype that approached that of 53BP1 loss (Fig. 2c). Furthermore, expressing GFP–SHLD2 in double knockout BRCA1 KO/SHLD2 KO cells resulted in a ~PARPi resistance (~1% and 10 Gy ionizing radiation (6 h recovery). Biologically independent experiments are shown and the bars represent the mean ± s.d. From left to right, the number of replicates was n = 3 (for both conditions in left panel); n = 3, 4, 3, 4, 3 and 3 (for conditions in middle panel, left to right); and n = 4, 6, 6, 6, 6, 6 and 6 (for conditions in right panel, left to right). e, Assessment of gene conversion by traffic light reporter assay. Biologically independent experiments are shown and the bars represent the mean ± s.d. (n = 3 for wild type and 35BP1 KO), n = 4 for SHLD1 KO, SHLD2 KO and REV7 KO). f, Kaplan–Meier curve showing tumour-specific survival of mice transplanted with KB1P4 tumour organoids (WT = 100, KO = 0).

Fig. 2 | Shieldin loss promotes PARPi resistance in cell and tumour models of BRCA1-deficiency. a, CRISPR dropout screen results in RPE1 wild-type cells exposed to ionizing radiation. Gene-level normZ scores < 0 are shown. b, Competitive growth assays using olaparib (16 nM) in RPE1 BRCA1 KO cells. Data are presented as mean ± s.d., normalized to day 0 (n = 3, independent transductions). c, Clonogenic survival in response to 16 nM olaparib. Representative images are shown (left) and quantified (right). Bars represent mean ± s.d. (n = 9: RPE1 wild type and BRCA1 KO/SHLD1 KO; n = 3: BRCA1 KO/SHLD2 KO; n = 4: BRCA1 KO/35BP1 KO; biologically independent experiments). d, Quantification of cells with ≥ 5 RAD51 foci ± 10 Gy ionizing radiation (6 h recovery). Biologically independent experiments are shown and the bars represent the mean ± s.d. For conditions in Supplementary Table 2). e, Kaplan–Meier curve showing tumour-specific survival of mice transplanted with KB1P4 tumour organoids ± olaparib treatment for 80 d (n = 8 per treatment; editing efficiencies found in Supplementary Table 2). P values were calculated using a log-rank test (Mantel–Cox). 1R, ionizing radiation; KO, knock out; WT, wild type.

and CTC-534A2.2 promotes the repair of double-strand breaks (DSBs) by NHEJ. For reasons that will become apparent, we named this complex shieldin and renamed C20orf196, FAM35A and CTC-534A2.2 as SHLD1, SHLD2 and SHLD3, respectively.

Independent sgRNAs targeting SHLD2 or SHLD3 caused sensitivity to the clastogen etoposide in competitive growth assays (Extended Data Fig. 1f) and caused resistance to olaparib in RPE1 BRCA1 KO cells, consistent with SHLD2 and SHLD3 acting with REV7 and SHLD1 (Fig. 2b and Extended Data Fig. 1g). Clonal knockouts of SHLD1 or SHLD2 led to olaparib resistance in BRCA1 KO cells, and SHLD2 KO resulted in a phenotype that approached that of 53BP1 loss (Fig. 2c). Similar results were obtained with 11 independent clonal knockouts of SHLD1 in SUM149PT cells exposed to talazoparib (Extended Data Fig. 1h). Furthermore, expressing GFP–SHLD2 in double knockout BRCA1 KO/SHLD2 KO cells restored olaparib sensitivity (Extended Data Fig. 1i).

Fig. 2 | Shieldin loss promotes PARPi resistance in cell and tumour models of BRCA1-deficiency. a, CRISPR dropout screen results in RPE1 wild-type cells exposed to ionizing radiation. Gene-level normZ scores < 0 are shown. b, Competitive growth assays using olaparib (16 nM) in RPE1 BRCA1 KO cells. Data are presented as mean ± s.d., normalized to day 0 (n = 3, independent transductions). c, Clonogenic survival in response to 16 nM olaparib. Representative images are shown (left) and quantified (right). Bars represent mean ± s.d. (n = 9: RPE1 wild type and BRCA1 KO/SHLD1 KO; n = 3: BRCA1 KO/SHLD2 KO; n = 4: BRCA1 KO/35BP1 KO; biologically independent experiments). d, Quantification of cells with ≥ 5 RAD51 foci ± 10 Gy ionizing radiation (6 h recovery). Biologically independent experiments are shown and the bars represent the mean ± s.d. From left to right, the number of replicates was n = 3 (for both conditions in left panel); n = 3, 4, 3, 4, 3 and 3 (for conditions in middle panel, left to right); and n = 4, 6, 6, 6, 6, 6 and 6 (for conditions in right panel, left to right). e, Assessment of gene conversion by traffic light reporter assay. Biologically independent experiments are shown and the bars represent the mean ± s.d. (n = 3 for wild type and 35BP1 KO), n = 4 for SHLD1 KO, SHLD2 KO and REV7 KO). f, Kaplan–Meier curve showing tumour-specific survival of mice transplanted with KB1P4 tumour organoids ± olaparib treatment for 80 d (n = 8 per treatment; editing efficiencies found in Supplementary Table 2). P values were calculated using a log-rank test (Mantel–Cox). 1R, ionizing radiation; KO, knock out; WT, wild type.
Fig. 3 | Shieldin accumulates at DSB sites downstream of 53BP1–RIF1 and promotes class switch recombination. a,b, Representative micrographs of shieldin subunit accumulation into foci induced by ionizing radiation in U2OS cells (a), or at laser microirradiation sites in RPE1 cells (b) (n = 3 biologically independent experiments). c, Quantification of b. Points represent individual experiments counting ≥100 cells. Data are mean ± s.d. d, Colocalization of GFP-tagged shieldin subunits with mCherry foci in U2OS–FokI cells upon mCherry–LacR–FokI expression. e, Quantification of GFP–SHLD3 or endogenous REV7 focus intensity. Each point represents a cell. Lines represent the mean. Data are the aggregate of two biological replicates with a total of 50 and 120 (GFP–SHLD3 and REV7, respectively), 45 and 122, 48 and 112, 54 and 116, 49 and 111, and 117 (for SHLD3 siRNA, only REV7 foci were quantified) cells counted for control siRNA and siRNAs against RIF1 (Extended Data Fig. 4e, f). 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 (Extended Data Fig. 5a). We also observed that SHLD1 and 53BP1 were epistatic in terms of modulating talazoparib resistance in SUM149PT cells (Extended Data Fig. 5b).

Analyses of the dependencies within the shieldin complex indicate that SHLD3 is the most apical component followed by REV7, SHLD2 and SHLD1 (Fig. 3c–e, Extended Data Figs. 4a–d, 6, 7c–e and Supplementary Note 1 for mapping details). Indeed, SHLD3 interacts with RIF1, which suggests that SHLD3 recruits shieldin to chromatin-bound 53BP1–RIF1 (Fig. 3g and Extended Data Fig. 7f). Further mapping studies suggest that shieldin consists of a DSB-recruitment module composed of SHLD3–REV7 that binds to the N terminus of SHLD2 (residues 1–50; Extended Data Fig. 5b). Analyses of the dependencies within the shieldin complex indicate that SHLD3 is the most apical component followed by REV7, SHLD2 and then SHLD1 (Fig. 3c–e, Extended Data Figs. 4a–d, 6, 7c–e and Supplementary Note 1 for mapping details). Indeed, SHLD3 interacts with RIF1, which suggests that SHLD3 recruits shieldin to chromatin-bound 53BP1–RIF1 (Fig. 3g and Extended Data Fig. 7f). Further mapping studies suggest that shieldin consists of a DSB-recruitment module composed of SHLD3–REV7 that binds to the N terminus of SHLD2 (residues 1–50; Extended Data Figs. 6, 7a–c), and a presumptive DNA-binding module (SHLD2–SHLD1) that features the OB-fold domains at the SHLD2 C terminus (hereafter SHLD2-C, residues 421–904; Extended Data Fig. 7a).

To assess the role of shieldin in NHEJ, we first analysed class switch recombination in CH12F3-2 cells24. Mutation of each of the shieldin subunits compromised class switch recombination, with Shld1-edited cells having a reproducibly milder phenotype (Fig. 3h and Extended Data Fig. 8a–c). Shld2 KO was epistatic with both 53bp1 KO and Shld1 KO mutations, consistent with them acting in the same genetic pathway (Extended Data Fig. 8b, c). The expression of AID—which initiates class switch recombination—was not altered in shieldin mutants, consistent with NHEJ deficiency (Extended Data Fig. 8d). Supporting this possibility, Shld1 and Shld2 mutations impaired random plasmid integration—which occurs largely through NHEJ—to an extent similar to that of 53BP1-deficient cells (Extended Data Fig. 8e, f).
The loss of each shieldin subunit led to ionizing-radiation-induced RPA32 Ser4/Ser8 phosphorylation, which is a surrogate marker of end-resection, suggesting that shieldin protects DNA ends (Fig. 3i and Extended Data Fig. 8g). Supporting this hypothesis, we observed reduced and aberrant ssDNA-binding behaviour (Fig. 4d, lane 2 versus 5). We estimate the binding affinity of SHLD2-C, its presence increased the amount of SHLD2-C purified, and the retarded complex displayed a difference in mobility consistent with the SHLD2-C–SSLD1 complex binding to ssDNA (Fig. 4d, lane 2 versus 5). We estimate the binding affinity of the SHLD2–ssDNA interaction to be about 10 nM (Fig. 4e and Extended Data Fig. 9c). We conclude that SHLD2 possesses ssDNA-binding activity.

To explore whether ssDNA binding is involved in shieldin function, we generated four mutant versions (named m1–m4) of the SHLD2 OB folds by modelling the SHLD2 C terminus on an RPA1 structure (RCSB Protein Data Bank code: 4GNX; Extended Data Fig. 9d). We also employed a splice variant of SHLD2 that disrupts OBb, which we refer to as SHLD2(S). We found that the SHLD2 m1 and SHLD2(S) mutants—either in the context of full-length SHLD2 or SHLD2-C proteins—were unable to suppress RAD51 focus formation in BRCA1KO53BP1KO cells when fused to the RNF8 FHA domain (Fig. 4b and Extended Data Fig. 9a). Expression of full-length SHLD2 m1 and SHLD2(S) in BRCA1KO53BP1KO cells also failed to suppress RAD51 focus formation induced by ionizing radiation, unlike wild-type SHLD2 (Extended Data Fig. 10a, b). Importantly, both mutants localized to DSB sites (Extended Data Fig. 10c, d) and interacted with the other members of the shieldin complex (Extended Data Fig. 10e). Therefore, the SHLD2 m1 and SHLD2(S) mutants are defective in suppressing homologous recombination.

Notably, the SHLD2-C m1 mutant was completely defective in ssDNA binding (Fig. 4d, lane 3) whereas the SHLD2(S)-C mutant displayed reduced and aberrant ssDNA-binding behaviour (Fig. 4d, lane 4). Because the m1 mutation produces a protein that is defective both in ssDNA-binding and suppression of homologous recombination, but which is proficient in both complex assembly and DSB recruitment, we conclude that ssDNA binding by shieldin is critical for 53BP1-dependent DSB repair.

In conclusion, the identification of shieldin forces us to re-evaluate how DNA end stability is regulated in vertebrates. 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. 4f). Our discovery of shieldin also has implications for the management of BRCA1-mutated malignancies, as alterations in shieldin-coding genes may cause clinical resistance to PARPi.

**Online content**

Any Methods, including any statements of data availability and Nature Research reporting summaries, along with any additional references and Source Data files, are available in the online version of the paper at https://doi.org/10.1038/s41586-018-0340-7

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**Fig. 4 | Shieldin is an effector of 53BP1 by binding ssDNA.**

**a.** Schematic for artificially targeting shieldin to DSB sites. **b.** Formation of RAD51 foci by ionizing radiation 3 h after 10 Gy irradiation in BRCA1KO53BP1KO cells expressing the indicated fusion proteins. Data are mean ± s.d. From top to bottom, the number of biologically independent experiments was n = 20, 22, 12, 12, 16, 4, 4, 4, 4, 6, 6 and 3, c, EMSA of the SHLD2–C–SSLD1 complex isolated from 293T cells (see Extended Data Fig. 10c, d). **c, d.** Determination of SHLD2-C–SSLD1 ssDNA binding dissociation constant (Kd). Data are mean ± s.d. (n = 3 independent experiments). Representative EMSA shown in Extended Data Fig. 9c, d. **e, f.** Model of shieldin function. We speculate that the SHLD2 OB-fold domains bind to ssDNA at DSB sites to suppress resection and favour NHEJ.
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METHODS

Plasmids. DNA corresponding to sgRNAs was cloned into pX330 (Addgene: 42230, Cambridge), LentivGuide-Puro (Addgene: 52963), LentCRISPRv2 (Addgene: 52961), or a modified form in which Cas9 was replaced by NLS-tagged GFP or mCherry using AgeI and BamHI (designated as LentizGuide-NLS–GFP or –mCherry), as described29,30. Sequences of the sgRNAs used in this study are included in Supplementary Table 5. Coding sequences of C20orf196 and the short isoform of FAM35A were obtained from the ORFeome collection (http://orfeome. sfi.harvard.edu/), archived in the Lumenfeld-Tanenbaum Research Institute’s OpenFreezer31. The complete coding sequence of the long isoform of FAM35A was generated by combining a synthesized fragment (GeneArt, Regensburg) corresponding to the long isoform C terminus using an internal KpnI restriction site. The coding sequence of CTC343A2.2 was generated by gene synthesis (GeneArt). The coding sequences were PCR amplified using Ascl and Apal flanking primers and cloned into pcDNA5-FRT/TO-eGFP and pcDNA5-FRT/TO-Flag and N-terminal tagged FAM35A, C20orf196 and CTC343A2.2 pGLUE-HA-Strep-FAM35A was generated by PCR amplification of the long isoform of FAM35A and cloning into pGLUE (Addgene: 15100) using Ascl and NotI. To generate FAM35A fragments and mutants, standard protocols for primer-directed mutagenesis or self-ligation of truncated PCR-products were used. To generate pcDNA5-FRT/TO-V5-CTC343A2.2, eGFP was replaced by a V5 tag in the cloning vector pcDNA5-FRT/TO-eGFP using KpnI and Ascl restriction enzymes after which the coding sequence for CTC343A2.2 was PCR-amplified and inserted into pcDNA5-FRT/TO-V5-MCS using Ascl and Xhol restriction enzymes. To generate RNF8–FHA fusions, the N terminus of RNF8 (amino acids 1–160) was PCR amplified using RNF8-FHA1–160–RNF8-FHA189-RNF8-FHA189 flanking Ascl sites and inserted into pcDNA5-FRT/TO-eGFP-FAM35A, GFP (FHAs) fusions of FAM35A were introduced into pCW7.1 (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 pcDEST-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-LcR 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-sec)(opt)-Ta2-TagBFP: #32627).

Cells and line engineering. 293T and RPE1-hiERT cells line were obtained from ATCC (Manassas), 293 Flp-In cells were obtained from Invitrogen (Carlsbad) and SUM149PT32 cells were obtained from Asterand Bioscience (Detroit). U2OS ER-mCherry-LacFokID-DL cells (U2OS-265, referred to in the text as U2OS–FokI) were a kind gift of R. Greenberg (University of Pennsylvania). All cell lines are routinely authenticated by STR-analysis and tested negative for mycoplasma. 293T and RPE1 cells were cultured in Ham’s F12 medium (Gibco) supplemented with 5% FCS, 10 mM HEPES, 1% peni-streptomycin (Wisent, St-Bruno) at 37 °C, 5% CO2. SUM149PT cells were cultured in DMEM/F-12 medium (Life Technologies, Carlsbad) in the presence of 10% FCS, penicillin–streptomycin (Gibco), 50 ng/ml murine epidermal growth factor (mEGF), 1% l-cysteine (Sigma-Aldrich), and 50 ng/ml murine epidermal growth factor (mEGF) before transfection with EditR crRNA (Dharmacon, Lafayette). Transfection of guides C20orf196_5-1, C20orf196_5-2, PARP1_5-2, PARP1_5-4, C20orf196_5-3 and C20orf196_5-3 and C20orf196_5-3 (see Supplementary Table 5) 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 medium supplemented with 50 nM tazolaparib (without doxycycline) and kept in batch culture or further split to generate single cell colonies. Drug-containing medium was replenished every 3–4 days until PARP inhibitor resistant pools or clones were established. Clones were subsequently picked, expanded and validated by genomic PCR and sequence analysis (for primers used, see Supplementary Table 6, for genomic editing information, see Supplementary Table 5). Four SUM149PT C20orf196F01 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 5-3-C6). To generate the REV7 mouse, double mutant clones, SUM149PT C20orf196F01 clones A and D were infected with a lentivirus expressing an sgRNA targeting TP53BP1 or a non-targeting control sgRNA (for sequences, see Supplementary Table 5) in medium containing 1 µg/ml doxycycline. Forty-eight hours after infection, puromycin (1 µg/ml) was added to the medium. Selection was maintained for 3 d, 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 embryonic stem cells with a selectable conditional Brca1 deletion (Rosa26revErt2bq+bq;Brca1Sc0/Sc1) 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) and 10 μM ESRO LIF (Millipore, Burlington) under normal oxygen conditions (21% O2, 5% CO2, 37 °C). The KB1P-G3 2D cell line was previously established from a Brca1−/−m53−/− mouse mammary tumour and cultured as described34. In brief, cells were cultured in DMEM/F-12 medium (Life Technologies, Carlsbad) in the presence of 10% FCS, penicillin–streptomycin (Gibco), 5 μg/ml insulin (Sigma-Aldrich), 5 ng/ml epidermal growth factor (mEGF) and 0.1% non-essential amino acids (Gibco) (1% O2, 5% CO2, 37 °C). The KB1P-G3 3D tumour organoid line was previously established from a Brca1−/−m53−/− mouse mammary tumour and cultured as described34. Cells were seeded in Basement Membrane Extract Type 2 (BME, Trevigen, Gaithersburg) on 24-well suspension plates (Greiner Bio-One, Kremsmünster) and cultured in AdDMEM/F12 supplemented with 10 μM HEPS (Sigma-Aldrich), GlutaMAX (Invitrogen), penicillin–streptomycin (Gibco), B27 (Gibco), 125 μM N-acetyl-t-cysteine (Sigma-Aldrich), and 50 ng/ml murine epidermal growth factor (Invitrogen).

CH12F2 mutant clones were edited either through transient transfection with pX330 plasmid constructs expressing sgRNAs against Trp53bp1 (sgRNA: Trp53bp1_5e6_834, see Supplementary Table 5), Fam35a, and Ctc534a2.2 or by lentiviral lentizCRISPR v2 transduction for C20orf196. Double knockout cell lines of Fam35a and Trp533p1 or C20orf196 were generated by transient transfection of a pX330 plasmid expressing an sgRNA against Trp53bp1 or by lentiviral transduction with lentizCRISPRv2 with an sgRNA targeting C20orf196.

Antibodies, siRNAs and drugs. An overview of all the antibodies used in this study, including dilution factors, can be found in Supplementary Table 7. The following antibodies were used for western blotting: anti-β-actin (D5B8), clone 6G11, from Santa Cruz Biotechnology, anti-β-tubulin (E6), clone YL1/2, from Sigma-Aldrich, anti-Actin (AC15), clone ACIF, from Sigma-Aldrich, anti-Flag (M2), clone M2, from Sigma-Aldrich, and anti-HA-11A10, from Roche. All antibodies used were diluted at 1:10000. Anti-Flag M2 (32267) and Anti-HA-11A10 (32267) were used to detect Flag and HA-tagged proteins. Cells were analyzed on a Leica TCS SP8 confocal microscope using the LAS AF software. Images were processed with Adobe Photoshop (CS6). The following siRNAs were used in this study: C20orf196 (siRNA1: 5′-GGGAGACAGCUAUGUGAAU-3′, siRNA2: 5′-CAAGUGGACAUUCUGAAAGCUU-3′, siRNA3: 5′-UGGAAACUCUGCUAAAUUCUGUGU-3′), TP53BP1 (siRNA1: 5′-CGAGGAAAACCUAAGAAACCAUAUU-3′), and TRA2 (siRNA1: 5′-GGGAGACAGCUAUGUGAAU-3′, siRNA2: 5′-CAAGUGGACAUUCUGAAAGCUU-3′, siRNA3: 5′-UGGAAACUCUGCUAAAUUCUGUGU-3′). The following drugs were used in the course of this study: olaparib (SelleckChem, Houston, or Astra Zeneca, Cambridge), talazoparib (SelleckChem), cisplatin (Sigma-Aldrich), the ATM inhibitor KU60019 (Sigma-Aldrich), and
Olaparib resistance screens. Viral particles of the TKOv1 sgRNA library were produced as previously described. This library contains 91,320 sgRNA 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 > 100× (SUM149PT) or > 200× (RPE1) (per replicate, if applicable). Twenty-four hours after transduction, transduced cells (~16 nm) or etoposide (~1200 nm) 10 µg/ml puromycin (RPE1) or ~8 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, at which time olaparib (16 nM for RPE1 TP53−/− BRCA1−/−, 2 µM for SUM149PT) was added to the cells. Olaparib-containing medium was refreshed every 4 d. Cells were collected at 3, 9, 18 and 23 d post-infection (RPE1) or at 3, 9, 19 and 26 d post-infection (SUM149PT) for downstream processing as described. In short, total genomic DNA was isolated from 2 × 10^6 (15 sample) or 1 × 10^6 (later time points) cells using the QIAamp DNA Blood Maxi Kit (Qiagen, Germantown). 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) using parameters previously described. Analysis was performed using model-based analysis of genome-wide CRISPRCas9 knockout (MaGeCK) version 0.5.3, 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: magicck run -l/path/to/TKOv1_library/ -n 08-02-2017_141703–sam-ple-label test,CTRL -t 1 -c 0–fastq /path/to/fastq1 /path/to/fastq2.

Ionizing radiation dropout screen and TKOv2 library. The TKOv2 lentiviral CRISPR library was used for whole-genome CRISPR knockout screening. To design TKOv2, all possible 20-mer sequences upstream of NGG PAM sites were collected where the SpCas9 double-strand break would occur within a coding exon (defined by hg19/Genode 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 (GATTCG) and BsmBI (GGCTTC). Candidate gRNA + PAM sequences were mapped to hg19 and sequences with predicted offset-target sites in exons or introns, or sequences with more than two predicted offset-target sites (with up to two mismatches) in any location, were discarded. Remaining guides were scored using a ‘sequence score table’ as previously described. Four guides per gene were selected, with a bias towards high sequence scores and maximal coverage across exons (that is, moderate-scoring guides targeting different regions of the same exon) to maximize the diversity of the library.

The final library contains 70,555 gRNA targeting 17,942 protein-coding genes, as well as 142 sequences targeting LacZ, luciferase, and eGFP. Oligonucleotide sequences were ordered from CustomArray (Bothell), PCR-amplified, and cloned into the plCKO vector as previously described.

RPE1-HTERT TP53−/− Cas9-expressing cells were transduced with the lentiviral TKOv2 library (see below) at a low MOI (~0.35) and puromycin-containing medium 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 ionizing radiation 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 ionizing radiation using a Faxitron X-ray cabinet (Faxitron, Tucson) every 3 d after day 6. Cell pellets were frozen at day 18 for DNA isolation. Screens were performed in technical duplicates and library coverage of > 375 cells per sgRNA was maintained at every step. gDNA from cell pellets was isolated using the QIAamp DNA Blood Maxi Kit (Qiagen) and genome-integrated sgRNA sequences were amplified by PCR using the KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington).

Computational Mass Spectrometry at University of California, San Diego (UCSD; mass.wisc.edu). Data are available at MassIVE (http://massive.ucsd.edu/sumo; S3:S00082207). Unique accession numbers are MSV000082207 and PXD0009313, respectively. Data can also be viewed at the proksis website (proksis.w-lunenfeld.ca) under dataset 29: Durocher laboratory. Data in Fig. 1f is represented using Cytoscape, using analyses with an FDR ≤ 0.5. Some 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) and the average probability (AvgP) of the best three out of three (CTC3542A2.2, five out of five (C20orf196 and six (FAM35A, REV7)) biological replicates for each bait was reported as the final SAINT score. P-values with an estimated FDR ≤ 1% were considered true interactors (AvgP ≥ 0.91). 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://www.thegpm.org/crap/index.html; 85,393 entries were searched).

Immunoprecipitation. 293FT cells (1 × 10^6) were transduced with pxDNAs1.1-FRT/ TO-Flag-c20orf196 (10 µg), -GFP-REV (2 µg), -V5-CTC3542A2.2 (14 µg) and pGLUE-HA-Strep-FAM35A (14 µg) or empty vectors using a 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, 100 mM NaF, 10 mM MgCl2, and 10 U/ml Benzonase) in 4 °C for 30 min. Lysates were centrifuged at 15,000g at 4 °C for 30 min, and supernatants were collected and stored at −80 °C. Immunoprecipitation was performed using 10 µl of mAb anti-Diotin (Invitrogen) in lysis buffer for 2 h at 4 °C. When applicable, the eluate was incubated with 20 µl of GFP-Trap_M resin (Chromotek, Planegg-Martinsried) for 1 h at 4°C, washed 5 times with lysis buffer and eluted by boiling.

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in sample buffer. Pull-downs and whole cell extracts were loaded onto SDS-PAGE gels, followed by immunoblotting and probing with indicated antibodies. For GFP–CTC35A2.2 immunoprecipitations, an identical GFP-Trap pulldown procedure as above was used. For V5–CTC35A2.2 immunoprecipitations, lysates from one confluent 10-cm dish of 293T cells transfected with 10 μg pcDNA1.1-FRT/TO-V5-CTC35A2.2 vector was incubated with 10 μg/ml anti-V5 antibody (Invitrogen) for 2 h at 4 °C. Subsequently, 50 μl of protein G Dynabeads (Invitrogen) 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 ≥ DS buffer.

Clonogenic survival assays. RPE1-iTERT TP53−/− cells were seeded in 10-cm dishes (wild type, 250 cells; BRCA1 F53BP1−/−, 500 cells; BRCA1−/− or BRCA1 F53BP1−/− or FAM35A−/−, 1,500 cells; BRCA1 F53BP1−/− or FAM35A−/−, 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 d. After 14 d, 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/Brca1ΔCTC534A2.2 cells, Cre-mediated inactivation of the endogenous mouse Brca1 allele was achieved by overnight incubation of cells with 0.5 μM 4-hydroxytamoxifen (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/Brca1ΔCTC534A2.2 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 PARP1 (olaparib) and ATM (KU55933 treatment were performed as described previously with minor adjustments45. Five thousand KBIP-G3 cells were seeded per well into 6-well plates on day 0, and then PARP1, ATM, both or neither were added. The medium was refreshed every 3 d. On day 6, the ATM alone and untreated groups were stopped and stained with 0.1% crystal violet, the other groups were stopped and stained on day 9. Plates were scanned with a GelCount (Oxford Optronics, Abingdon). 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).

Short-term survival assays. Ten thousand RPE1-iTERT Cas9 TP53−/− parental cells and additional mutants (BRCA1 F53BP1−/− and/or FAM35A−/−) with or without stable integration of integrated eGFP fusions by viral transduction were seeded in 12-well format with or without 200 nM olaparib (and 1 μg/ml doxycycline if applicable). Medium with olaparib (and doxycycline) was replaced after 4 d, and cells were trypsinized and counted after seven days using an automated Z2 Coulter Counter analyser (Beckman Coulter, Indianapolis).

SUM149PT cells were plated at 500 cells per well in 384-well plates and varying amounts of talazoparib in DMSO were added the following day using an Echo 550 (Tecan). After 40 h growth, cell survival was assayed using CellTiter-Glo according to the manufacturer’s protocol (Promega, Madison).

Immunofluorescence. For 53BP1 immunofluorescence, cells were cultured on glass coverslips and treated with 5 or 10 Gy X-ray irradiation and fixed with 2–4% PFA 1–2 h after irradiation. Cells were permeabilized with 0.3% Triton X-100, followed by blocking in 100% goat serum, 1 mM EDTA in PBS (blocking buffer A). Cells were co-stained using 53BP1 and H2AX primary antibodies (see Supplementary Table 7) 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 immunofluorescence, cells were cultured on glass coverslips, treated with 5 or 10 Gy X-ray irradiation and fixed with 2–4% PFA 1–2 h after irradiation. Cells were permeabilized with 0.3% Triton X-100. For REV7 blotting, 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 blotting, primary and secondary antibody incubations (1.5 h at room temperature followed by 4 washes in PBS) were performed in blocking buffer A. Cells were treated with 10 Gy in PBS (blocking buffer) or 10 Gy in PBG buffer (0.2% cold water fish gelatin (Sigma-Aldrich), 0.5% BSA in PBS).

For GFP–shieldin focus and laser stripe analysis, U2OS or RPE1 cells were grown on glass coverslips and either transiently transfected with 1 μg vector expressing GFP–FAM35A or –CTC35A2.2, or virally transduced with GFP–FAM35A-expressing vector. Forty-eight hours after transfection, or 24 h after 0.5 μg/ml doxycycline induction, cells were treated with 5 Gy X-ray irradiation or micro-irradiated, pre-extracted 10 min on ice with NuEx buffer (20 mM HEPES, pH 7.4, 20 mM NaCl, 5 mM MgCl2, 0.5% NP-40, 1 mM DTT and protease inhibitors) followed by 10 min 2% PFA fixation 1 h post-irradiation or micro-irradiation. Antibody staining and blocking were performed as described above except in PBS + 0.1% Tween 20 + 5% BSA using GFP and H2AX antibodies.

DAPI (0.8 μg/ml) was included in all experiments to stain nuclear DNA. Coverslips were mounted using Prolong Gold mounting reagent (Invitrogen) or Aqua PolyMount (Polyscience, Warrington). Images were acquired using a Zeiss LSM780 laser-scanning microscope (Oberkochen), a Leica SP8 confocal microscope (Wetzlar) or a Zeiss AxioImager D2 widefield fluorescence microscope. Foci were manually counted.

RAD51 immunofluorescence in KBIP-G3 cells was performed as previously described, with minor modifications45. Cells were grown on 8-well chamber slides (Millipore). Ionizing-irradiation-induced foci were induced by gamma-irradiation (10 Gy) 4 h before sample preparation. Cells were then washed in PBS + (+2% BSA, 0.15% glycerine, 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 room temperature, 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 (Vector Laboratories, Burlingame).

Scale bars indicated in the figure panels represent 10 μm, unless stated otherwise.

LacR–RIF1 N-terminus and FokI-induced focus formation. For monitoring recruitment of GFP-tagged shieldin subunits to mCherry–Lac–Rif1(1–967) foci, 150,000 U2OS–Fok1 cells (known also as U2OS–DSB) were seeded on 6-well plates containing glass coverslips without any induction of Fok1. Twenty-four hours after seeding, cells were transfected using 1 μg of pDEST–mCherry–Lac–Fok1 vector (Invitrogen) using Lipofectamine 3000 transfection reagent (Invitrogen) with P3000D. Twenty-four hours after transfection, the cells were irradiated with 0.5 Gy Shield1, if applicable, and then treated with 0.1 μg/ml shieldin siRNA transfection mix usinguffles 2000 transfection reagent (Invitrogen). For monitoring the localization of the FAM35A 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–Fok1 cells were reverse-transfected with Lipofectamine RNAiMAX–siRNA (10 nM) complex. Twenty-four hours after siRNA transfection, the mCherry–Lac and GFP fusion plasmids were transfected. Cells were fixed with 4% PFA 48 h after DNA transfection. For monitoring recruitment of GFP-tagged shieldin subunits to DSBS at the LacO array, Fok1 stabilization and nuclear translocation was induced by treating cells with 0.1 μM Shield1 (Invitrogen) and 0.05 μM doxycycline, and 24 h later cells were pre-stained with 0.1 μg/ml DAPI (H1500, Vector Laboratories, Burlingame).
gradient gels (Invitrogen), transferred to nitrocellulose and immunoblotted for pRPA32 (S45/S48).

**Mouse mammary tumour models.** All animal experiments were approved by the Animal Ethics Committee of The Netherlands Cancer Institute (Amsterdam) and performed in accordance with the Dutch Act on Animal Experimentation (November 2014). KB1P4 tumour organoids were transduced using spinoculation as previously described. NMRI-mu nude female mice were purchased from Janvier Laboratories and used for transplantation studies at the age of 6–9 weeks. A power analysis was performed to calculate that a minimum of 8 mice per group were needed to achieve a power of 0.8 (two-sided test, alpha = 0.05). Tumour organoids were allografted in mice as previously described with minor adjustments. In brief, tumour organoids were collected, incubated with TrypLE at 37 °C for 5 min, dissociated into single cells, washed and embedded in a 1:1 mixture of tumour organoid culture medium and Basement Membrane Extract (Trevigen) in a cell concentration of 10^6 cells per 40 µL. Subsequently, 10^6 cells were injected in the fourth right mammary fat pad of NMRI nude mice. Mammary tumour size was determined by caliper measurements and tumour volume was calculated (0.5 × length × width^2). Treatment of tumour-bearing mice was initiated when tumours reached a size of 50–100 mm^3. Mice were randomly allocated into the untreated (n = 8) or 80 µCi/kg treatment group (n = 8). Olaparib was administered in a blinded fashion at 100 mg/kg intraperitoneally for 80 consecutive days. Animals were killed with CO₂ when the tumour reached a volume of 1,500 mm^3. The tumour was collected, fixed in formalin for histology and several tumour pieces were collected for DNA analysis.

**Class switch recombination assays.** To induce switching in CH12F3-2 murine B cell lymphoma cells, 2 × 10^6 cells were cultured in CH12 medium supplemented with a mixture of IL4 (10 ng/mL), R&D Systems #7402-ML-050, Minneapolis), TGFβ (1 ng/mL, R&D Systems #7666-MB-005) and anti-CD40 antibody (1 µg/mL, #16-0401-86, eBioscience, Thermo Fisher) for 48 h. Cells were then stained with anti-IgA-PE and fluorescence signal was acquired on an LSR II or Fortessa X-20 flow cytometer (BD Biosciences). To probe AID levels in the seeded cells, immunoblotting was performed on total cell lysates using anti-AID and anti-β-actin antibodies (Supplementary Table 7). Band quantification was analysed by ImageJ.

**Plasmd integration assay.** Two hundred thousand RPE1 cells were seeded into 6-well plates and 24 h later transfected with 2 µg of BamHJ/EcoRI-linearized pEGFP-C1-V5. Seventy-two hours after transfection, cells were sorted 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 days. Forty-two days after seeding, colonies were stained with crystal violet solution and manually counted. NHEJ efficiency was calculated according to the following formula:

\[
\text{Percentage of surviving colonies on selection} = \frac{\text{Number of colonies on selection}}{\text{Number of colonies without selection}} \times 100
\]

The data shown for the different knockout clones in Extended Data Fig. 8e were calculated by measuring GFP-positivity using flow cytometry. Medium with G418 g/ml G418. At this point, transfection efficiency was calculated by measuring 6-well plates and 24 h later transfected with 2 µg of BamHJ/EcoRI-linearized pEGFP-C1-V5. Seventy-two hours after transfection, cells were sorted 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 days. Forty-two days after seeding, colonies were stained with crystal violet solution and manually counted. NHEJ efficiency was calculated according to the following formula:

\[
\text{Percentage of surviving colonies on selection} = \frac{\text{Number of colonies on selection}}{\text{Number of colonies without selection}} \times 100
\]

**DNA binding assays.** Shieldin proteins were isolated using the immunoprecipitation protocol described above with the following modifications. 293T cells were transfected with pGLUE-FAM35A (421-904), the indicated mutants of this construct, or the empty pGLUE Strept/HA-tagging vector and pcDNA5.1-FRT/TO-Flag-C20orf196 in a 2:1 ratio for a 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 SDS–PAGE and Coomassie staining, followed by comparison to a standard curve of known bovine serum albumin (BSA) concentrations measured by fluorescence in the 700-nM channel of the Odyssey imager (LI-COR). A radio-labelled ssDNA probe was prepared by T4 polynucleotide kinase (New England Biolabs, Ipswich) phosphorylation of HPLC-purified 59-nt DNA oligonucleotide (BioBasic, Markham); TACGTTTAGTCGCGCTCTCCCTCCAGAG TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT (using [γ-32P]ATP (3,000 Ci/mmol, 10 mCi/ml; Perkin-Elmer, Woodbridge). Unlabelled competitors were prepared using the same oligonucleotide sequence alone or hybridized to the complementary sequence (AAAAAAAAAAAAAAAAAAAAAAAAAAAAACACATCGAGAAGAACGCATACTACGTA) by heating at 80°C for 10 min and gradual cooling to room temperature overnight.

For electrophoretic mobility shift assays, 20 nM of labelled ssDNA probe was incubated with purified proteins for 20 min in the elution buffer with the addition of 1 mM DTT and 1 mg/10 µL BSA at room temperature. Glycerol was then added to a final concentration of 8.3% and resolved on 6% acrylamide–TAE gels. Gels were adhered onto blotting paper (VWR) and enclosed 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 to be bound probe, as measured in ImageQuant TL (GE Healthcare). The fraction of probe bound is defined as:

\[
\text{Signal of bound probe} \div \text{Signal of free probe}
\]

and the concentration of unbound FABM35A-C-C20orf196 (referred in the text as SHLD2–C–SHLD1) complex is calculated by multiplying the fraction of probe bound by the initial concentration of ssDNA probe, and subtracting this from the initial concentration of SHLD2–C–SHLD1, given the assumption of 1:1 binding.

**Statistical analysis.** All data are represented as individual replicates and replicate number, mean and error bars are explained in the figure legends. The statistical tests we used (all of which were common tests) and resulting P values are indicated in the figure legends and/or figure panels and have been generated using GraphPad Prism software.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability.** All source data represented in the graphs displayed in this article are available online (Supplementary Data 1–12). Uncropped western blots can be found online as Supplementary Fig. 1. Data of the CRISPR-Cas9 screens are included as Supplementary Table 1 (PARPi positive selection screens) or Supplementary Table 4 (ionizing radiation sensitivity dropout screen). IP-MS data (Supplementary Table 3) are available at MassIVE (http://massive.ucsd.edu/MSV000082207, with unique accession numbers MSV000082207 and PXD009913). IP-MS data can also have been viewed at the prohls website (http://prohls-web.brunelfield.ca) under dataset 29: Durocher laboratory.
Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | The identification of the shieldin complex and its role in the response to genotoxic treatments. a, Schematic of the PARPi resistance screens. b, Competitive growth assays determining the capacity of the indicated sgRNAs to cause resistance to PARP inhibitors in RPE1 BRCA1<sup>KO</sup> cells. Data are presented as the mean fraction of GFP-positive cells ± s.e.m., normalized to day 0 (n = 3, independent viral transductions). Gene-editing efficiencies of the sgRNAs can be found in Supplementary Table 2. Note that we have not been able to obtain TIDE data for the ATMIN-targeting sgRNAs. c, Representative images of SUM149PT–Cas9 cells transfected with indicated crRNAs (see Methods) and exposed to 50 nM talazoparib for 14 d. Purple colour indicates cells detected by Incucyte live-cell imaging. Scale bar, 100 µm. The data are a representative set of images from two biologically independent experiments. d, Screenshot of the genomic locus surrounding human CTC-534A2.2 taken from ENSEMBL. e, Schematic of the screen performed in RPE1-hTERT TP53<sup>−/−</sup> cells stably expressing Cas9 to study genes mediating ionizing radiation-sensitivity. f, g, Competitive growth assays measuring the capacity of the indicated sgRNAs to cause resistance to etoposide (100 nM) in RPE1 wild-type cells (f) or PARPi (16 nM) in RPE1 BRCA1<sup>KO</sup> cells (g). Data are presented as the mean fraction of GFP-positive cells ± s.d., normalized to day 0 (n = 3, independent viral transductions). Gene-editing efficiencies of the sgRNAs can be found in Supplementary Table 2. h, Talazoparib sensitivity in 11 SHLD1<sup>KO</sup> 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 5 d. As a comparison, talazoparib sensitivity in parental SUM149PT cells with wild-type SHLD1 (WT) is shown, as is talazoparib resistance in a BRCA1 revertant subclone (BRCA1-rev) of SUM149PT<sup>WT</sup>. Bars represent the mean ± s.d. (n = 4 biologically independent experiments). ANOVA was performed for each SHLD1<sup>KO</sup> clone versus wild type using Dunnett correction for multiple comparisons, P < 10<sup>−15</sup>. Gene-editing efficiencies can be found in Supplementary Table 2. i, BRCA1<sup>KO</sup> and BRCA1<sup>KO</sup>SHLD2<sup>KO</sup> cells were virally transduced with expression vectors for GFP alone or GFP–SHLD2. Sensitivity to olaparib (200 nM) was determined by a short-term survival assay in the presence of 1 µg ml<sup>−1</sup> doxycycline to induce protein expression. Data are represented as dots for every individual experiment with the bar representing the mean ± s.d. (n = 3).
Extended Data Fig. 2 | Shieldin inhibits homologous recombination.

**a**, Representative micrographs of RAD51 focus formation in the indicated RPE1 cell lines (data quantified in Fig. 2d, n ≥ 3). **b**, Traffic light reporter assay testing RPE1 BRCA1-KO cells virally transduced with sgRNAs targeting 53BP1 or SHLD3. Data are represented as dots for individual experiments with the bar representing the mean ± s.d. (n = 3).

Gene-editing efficiencies of the sgRNAs can be found in Supplementary Table 2. **c**, Representative flow cytometry plots of cells analysed with the traffic light reporter assay (data quantified in Fig. 2e, n ≥ 3). **d**, Representative flow cytometry plots of cells analysed with the traffic light reporter assay (data quantified in b).
Extended Data Fig. 3 | 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 ± 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. Data shown are representative of 3 biologically independent experiments (with 3 technical replicates each). b, Left, quantification 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 without treatment 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 ± s.d. (n = 15). Right, representative micrographs of RAD51-negative and RAD51-positive cells (the latter is indicated by an arrowhead). DNA was stained with DAPI.

c, Clonogenic survival assay of Rosa26CreERT2\(^{wt}\);Brca1\(^{Δ/Δ}\);p53-null mouse embryonic stem cells virally transduced with the indicated sgRNA and treated without or with 15 nM olaparib for 7 d. Gene-editing efficiencies of the sgRNAs can be found in Supplementary Table 2. Data shown are representative of 3 biologically independent experiments (with ≥2 technical replicates each). d, Clonogenic survival assay of Rosa26CreERT2\(^{wt}\);Brca1\(^{IbKO}/Δ\) mouse embryonic stem cells virally transduced with the indicated sgRNA and treated without or with 0.5 µM tamoxifen to induce BRCA1 depletion. Gene-editing efficiencies of the sgRNAs can be found in Supplementary Table 2. Data shown are representative of 2 biologically independent experiments (with 3 technical replicates each).
Extended Data Fig. 4 | Shieldin localizes to DSB sites. a, Representative micrographs of the experiments quantified in Fig. 3c. b, Representative micrographs of the experiments quantified in Fig. 3e. c, Quantification of mRNAs for SHLD1, SHLD2 and SHLD3. RPE1 cells were transfected with siCTRL (non-targeting control siRNA) or siRNA targeting the indicated shieldin subunits. Forty-eight hours after transfection, mRNA was purified and reverse-transcribed before being assayed by quantitative real-time PCR. Data were normalized to the amount of GAPDH mRNA and expressed relative to the corresponding value for cells transfected with siCTRL. Data are presented as the mean ± s.d. (n = 3, independent siRNA transfections). d, Whole cell extracts from RPE1 wild-type cells transfected with the indicated siRNAs were processed for immunoblotting with the indicated antibodies. Tubulin is used as a loading control (n = 1 experiment; siRNA efficiency is also monitored by immunofluorescence). e, Quantification of 53BP1 and RIF1 recruitment to ionizing radiation-induced DSBs (1 h after irradiation with 10 Gy) following depletion of the indicated shieldin components. Data are represented as the mean ± s.d. (n = 3, independent siRNA transfections). f, Representative micrographs of the experiments quantified in e.
Extended Data Fig. 5 | Epistasis between 53BP1 and shieldin factors. 

a, Quantification of RAD51 focus formation 3 h after irradiation (10 Gy) in RPE1 BRCA1KO (left), BRCA1KO53BP1KO (middle) and BRCA1KO\(^{SHLD2}\)KO (right) cells after viral transduction with the indicated sgRNAs (editing efficiency can be found in Supplementary Table 2) or empty vector (EV). Data are represented as the mean ± s.d. (for BRCA1KO53BP1KO, n = 4 biologically independent immunofluorescence experiments; for BRCA1KO and BRCA1KO\(^{SHLD2}\)KO, n = 6 biologically independent immunofluorescence experiments). P values were calculated using a two-tailed unpaired t-test. Left, BRCA1KO EV versus sg53BP1-1 P = 0.0002; EV versus sgSHLD1-1 P = 0.0043; EV versus sgSHLD2-2 P = 0.0348; EV versus sgSHLD3-1 P = 0.0180; EV versus sgREV7-1 P = 0.0012). Middle, right: all comparisons to the EV condition were non-significant (NS). Values for BRCA1KO\(^{53BP1\)}KO EV versus sg53BP1-1 P = 0.2332; EV versus sgSHLD1-1 P = 0.4451; EV versus sgSHLD2-2 P = 0.9632; EV versus sgSHLD3-1 P = 0.1187; EV versus sgREV7-1 P = 0.0568. Values for BRCA1KO\(^{SHLD2\)}KO: EV versus sg53BP1-1 P = 0.0550; EV versus sgSHLD1-1 P = 0.1864; EV versus sgSHLD2-2 P = 0.3568; EV versus sgSHLD3-1 P = 0.4641; EV versus sgREV7-1 P = 0.2888. 

b, Talazoparib sensitivity of wild type or two independent \(SHLD1\)KO SUM149PT-dox-Cas9 clones (A and D) virally transduced with an sgRNA targeting 53BP1 (sg53BP1) or a control non-targeting sgRNA (sgCtrl), following induction of Cas9. Data are presented as the mean ± s.d. (n = 3 biologically independent experiments).
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | The co-localization of shieldin with RIF1 on chromatin. a, Representation of the deletion mutants of SHLD2-N used in c, d. The orange shading indicates blocks of homology. b, Schematic of the LacR–RIF1 chromatin recruitment assay. c, Quantification of the experiment shown in d. Colocalization was considered positive when the average GFP intensity at the mCherry focus was threefold over background nuclear intensity. A minimum of 20 cells were imaged per biological replicate (circles); the bar represents the mean ± s.d. (n = 3). d, Representative images of the data quantified in c. The main focus is shown in inset; scale bar, 10 μm. e–h, Quantification (e, g) and representative micrographs (f, h) of overexpressed GFP–SHLD2-N and mCherry–LacR–RIF1(1–967) co-transfected 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 threefold over background nuclear intensity. A minimum of 20 cells were imaged per condition (circles); the bar represents the mean ± s.d. (n = 3 biologically independent experiments). i, Representative images of the data quantified in j. The main focus is shown in inset; scale bar, 10 μm. j, Quantification of GFP intensity at the mCherry–LacR–RIF1(1–967) focus, normalized to nuclear background. Each data point represents a cell transfected with the vector coding for the indicated GFP fusion. The line is at the median. The data are an aggregate of three independent experiments with a minimum of 20 cells counted (total cells counted: 62, 60 and 61 for GFP, GFP–SHLD2-C and GFP–SHLD3, respectively). k, mCherry–LacR–FokI colocalization with full-length or N-terminally truncated (Δ1–50) GFP–SHLD2. Mean normalized focus intensity is shown from a total of 59 (full-length SHLD2) or 56 (SHLD2 Δ1–50) cells counted (n = 2 biologically independent experiments). © 2018 Springer Nature Limited. All rights reserved.
Extended Data Fig. 7 | Mapping the architecture of the shieldin complex. 

**a**, Streptavidin pulldown analysis determining which region of SHLD2 associates with the other shieldin subunits. WCEs of 293T cells transfected with an expression vectors for Flag–SHLD1, V5–SHLD3, GFP–REV7 and Strept/HA-tagged SHLD2, SHLD2-N (residues 2–420), SHLD2-C (residues 421–904) or empty Strept/HA vector (EV) were incubated with streptavidin resin and bound proteins were eluted with biotin. WCEs and elutions were analysed by SDS–PAGE and immunoblotting with the indicated antibodies. Tubulin was used as a loading control. Results are representative set of immunoblots from two independent experiments. Asterisk denotes a non-specific band.

**b**, Mapping the SHLD3 and REV7 binding sites on the SHLD2 N terminus through streptavidin pulldowns with different SHLD2 constructs (detailed in Extended Data Fig. 6a) and immunoblotting. Results are a representative of a set of immunoblots from three independent experiments.

**c**, Affinity purification of shieldin complex components using N-terminally truncated SHLD2 (Δ1–50) analysed by immunoblotting (representative of three independent experiments).

**d**, Streptavidin pulldown analysis of SHLD2 association with REV7 and SHLD3. 293T cells were transfected with siRNAs and expression vectors for epitope-tagged shieldin components as indicated (EV, empty Strept/HA vector). WCEs were incubated with streptavidin resin and bound proteins were eluted with biotin. WCEs and elutions were analysed by SDS–PAGE and immunoblotted with the indicated antibodies. Short and long exposures are shown for GFP and V5 immunoblots (n = 1).

**e**, Dependency of V5–SHLD3 co-immunoprecipitation with GFP–REV7. 293T cells were transfected with siRNAs and expression vectors for epitope-tagged REV7 and SHLD3 as indicated (EV, empty V5 vector). WCEs were incubated with anti-V5 antibody and protein G resin. Bound proteins were boiled in SDS sample buffer and analysed by immunoblotting with GFP and V5 antibodies (n = 1).

**f**, Association between SHLD3 and RIF1. WCEs of 293T cells transfected with an expression vector for unfused GFP (−) or GFP–SHLD3 (SHLD3) were incubated with GFP-Trap resin. Bound proteins were boiled in SDS sample buffer and analysed by SDS–PAGE and immunoblotting against 53BP1 and RIF1. Results are representative of 2 SHLD3 immunoprecipitations, using SHLD3 fused to GFP (shown here) and V5 (shown in Fig. 3g) affinity tags.
Extended Data Fig. 8 | Controls supporting the role of shieldin in promoting physiological NHEJ. 
a. Representative dot plots of the flow cytometry data obtained (of n = 3 biologically independent experiments) to assess class switching in Fig. 3h. Class switch recombination was determined as the percentage of IgA^+ cells following stimulation after subtracting the baseline percentage of IgA^+ cells in the indicated clones (values in parentheses). b, c, Epistasis analysis of shieldin and 53BP1 in class switch recombination. The percentage of class switching in CH12F3-2 wild type, single knockout or double knockout cells (as indicated) following stimulation is shown. Each data point represents a biological replicate; the line represents the mean ± s.d. (n = 3). Genomic editing efficiencies of the sgRNAs can be found in Supplementary Table 2. d, WCEs of the indicated CH12F2-3 clones were probed for AID and β-actin (loading control) by immunoblotting and were quantified by densitometry. Each data point represents a biological replicate; the line represents the mean ± s.d. (n = 9 for wild type, n = 3 for other samples). e, Random plasmid integration of linearized pcGFP-c1 conferring G418 resistance. Resistant colonies were quantified after 14 d. Bar represents the mean ± s.d. with wild-type cells set at 100% (left, n = 5; right, n = 4 except SHLD2KO (2.7) n = 3 biologically independent experiments). f, Representative images of the plasmid integration assays quantified in e. g, Un-irradiated CH12F3-2 clones were immunoblotted for RPA32 (also known as RPA2) phosphorylation (a representative set from n = 3 biological replicates; data relates to Fig. 3i).
Extended Data Fig. 9 | The role of DSB-targeted SHLD2 in the suppression of homologous recombination and the mapping of the SHLD2-C–SHLD1 complex binding to ssDNA. a, Representative micrographs of RPE1 BRCA1 KO/BRCA1 KO/53BP1 KO/53BP1 KO cells transduced with the indicated GFP-fusion proteins, pre-extracted, fixed and stained for RAD51 and GFP 3 h after ionizing radiation (10 Gy). Protein expression was induced for 24 h before exposure to ionizing radiation using 1 µg ml⁻¹ doxycycline. Data relates to Fig. 4b. Note that owing to the pre-extraction required for visualization of RAD51 foci, the visualization of non-FHA-tagged SHLD2 is lost. b, SDS–PAGE analysis of purified SHLD2-C–SHLD1 complexes. Strep/HA–SHLD2(421–904)–Flag-SHLD1 complexes were purified from transiently transfected 293T cells. Concentrations of purified proteins were estimated by Coomassie staining and comparison to a standard curve of known BSA concentrations visualized by fluorescence at 700 nm. SHLD2-C m1 and SHLD2(S)-C denote SHLD2-C constructs carrying the OB-fold m1 mutation and the internal deletion (Δ655–723) corresponding to the naturally occurring splice variant of SHLD2, respectively. Open and filled arrowheads mark the bands corresponding to SHLD2-C and SHLD1, respectively. EV refers to empty Strep/HA vector. A representative stained gel from two independent experiments is shown. c, Representative image of the ³²P-labelled ssDNA EMSA with SHLD2-C–SHLD1 for Kᵅ determination shown in Fig. 4e. d, Model of the SHLD2 OB-fold domains and the engineered mutations (red spheres, point mutations; red ribbons, splice variant deletion). Model relates to Fig. 4b, d.
Extended Data Fig. 10 | SHLD2 OB-folds are required for suppression of RAD51 focus formation induced by ionizing radiation.
a, Quantification of RAD51 foci 3 h after 10 Gy irradiation in RPE1 BRCA1 KOSHLD2KO cells complemented with the indicated GFP-tagged SHLD2 constructs via viral transduction. Protein expression was induced with 1 µg ml⁻¹ doxycycline for 24 h before exposure to ionizing radiation. Each data point is a biological replicate; the bar represents the mean ± s.d. (n = 6 for BRCA1 untransduced cells, BRCA1 KOSHLD2KO untransduced and GFP-SHLD2 cells, n = 3 for remaining cell lines, biologically independent experiments). b, Representative micrographs of the data shown in a. Note that owing to the pre-extraction required for visualization of RAD51 foci, the visualization of non-FHA tagged SHLD2 foci is lost. c, Representative micrographs of RPE1 BRCA1-KO SHLD2-KO cells virally transduced with vectors expressing GFP-tagged SHLD2 wild type or its OB-fold m1 mutant (m1), or short splice variant (S), 1 h after 5 Gy ionizing radiation. Scale bar, 10 µm. d, Quantification of the data shown in c. Each data point represents an independent biological replicate counting ≥100 cells. Data are represented as mean ± s.d. (n = 3). e, WCEs of 293T cells co-transfected with Strept/HA–SHLD2 wild type, Strept/HA–SHLD2 m1 or Strept/HA–SHLD2(S) mutants, 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 with the indicated antibodies. Results shown are a representative set from two independent experiments. Source Data
Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| Item                                                                 | Confirmed |
|----------------------------------------------------------------------|-----------|
| □ The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement | x         |
| □ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly | x         |
| □ The statistical test(s) used AND whether they are one- or two-sided | x         |
| □ Only common tests should be described solely by name; describe more complex techniques in the Methods section. |           |
| □ A description of all covariates tested                            |           |
| □ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |           |
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| □ Give \( P \) values as exact values whenever suitable.             |           |
| □ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | x         |
| □ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes | x         |
| □ Estimates of effect sizes (e.g. Cohen's \( d \), Pearson's \( r \)), indicating how they were calculated |           |
| □ Clearly defined error bars                                         |           |
| □ State explicitly what error bars represent (e.g. SD, SE, CI)       |           |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

- Data collection: Leica Zen 2012 software, Biorad CFX manager.
- Data analysis: Graphpad Prism v7, Flowjo v10, TIDE, ProHits, SAINTEXpress (v3.6.1), cytoscape, ImageJ, ImageQuant TL, MaGeCK, DrugZ, Python v3.5.1, Acapella, Image Studio Lite

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Raw data of CRISPR Cas9 screens (Fig 1b and 2a) is included as supplementary tables. Raw data for IP-MS experiments has been deposited elsewhere (ftp://
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

All experiments represented in this manuscript are performed as biological replicates, with at least three independent replicates, as mentioned in each figure panel (except fig 3f, n=2, with a minimum of 20 cells each replicate). For the mouse transplantation studies, a power analysis was performed to calculate that a minimum of 8 mice per group were needed to achieve a power of 0.8 (two-sided test, alpha=0.05). Otherwise, no power analyses were performed a priori. For those experiments not having power analysis, biological replicates of n=3 or more were considered to have enough power to find biologically significant effects, with for IF studies, a minimum of 20 cells per sample for foci/stripe intensity studies and a minimum of 100 cells for foci number analysis.

Data exclusions

Data from experiments was only excluded in the rare event when control samples didn’t perform as expected. All graphs are represented as dot plots showing variation among individual biological replicates. The type of error bars are explained in the corresponding figure legends.

Replication

In addition to internal biological replicates within experiments, we show data in multiple mouse and human models in this manuscript, all confirming the role of the shieldin complex in NHEJ and limiting end resection. Since the data was acquired by multiple research groups, the data show a strong reproducibility.

Randomization

For the mouse transplantation study, animals were stratified into the different treatment arms by randomly allocating them into the untreated or olaparib treated group. There was no additional randomization in the experiments described in this manuscript.

Blinding

For the mouse transplantation study, treatment of mice with tumours of the different genotypes was performed blind. No other experiments were collected or analyzed in a blinded manner.

Reporting for specific materials, systems and methods

### Materials & experimental systems

| Involved in the study | n/a |
|-----------------------|-----|
| ☒ Unique biological materials |
| ☒ Antibodies |
| ☒ Eukaryotic cell lines |
| ☒ Palaeontology |
| ☒ Animals and other organisms |
| ☒ Human research participants |

### Methods

| Involved in the study | n/a |
|-----------------------|-----|
| ☒ ChIP-seq |
| ☒ Flow cytometry |
| ☒ MRI-based neuroimaging |

### Antibodies

| Antibodies used | For a tabular overview: see Supplementary Table 7. Primary antibodies: Rb anti RAD51 Santa Cruz, sc-8349 1:150; Rb anti RAD51 Bio Academia, #70-001 1:2,000; Rb anti REV7 Abcam, ab180579 1:1,000 1:500; Rb anti 53BP1 Santa Cruz, sc-22760 1:5,000; M anti 53BP1 Becton Dickinson, #612523 1:1,000; M anti RIF1 Santa Cruz, sc-55979 1:1,000 1:200; R anti RIF1 Bethyl A300-569A 1:7,500; M anti γH2AX Millipore, #05-636 1:5,000; Rb anti BRCA1 homemade 1:1,000; Rb anti pRPA32 (S4/S8) Bethyl, A300-245A 1:1,000; Rb anti KAP1 Bethyl, A300-274A 1:2,000. |
### Eukaryotic cell lines

**Policy information about** [cell lines](#)

**Cell line source(s)**  
All human cell lines original from ATCC or are derivatives of ATCC. More detailed information can be found in the Methods section. The mouse cell lines used in this study have been described before (Jaspers et al., 2013; Bouwman et al., 2013; Duarte et al., 2017; Nakamura et al., 1996)

**Authentication**  
We routinely authenticate our cell lines with STR analysis. Genetic knockouts were validated by genomic sequencing and using antibodies, when available.

**Mycoplasma contamination**  
All cell lines were routinely tested negative for mycoplasma contamination.

**Commonly misidentified lines**  
None of the used cell lines are listed in the ICLAC database.

### Animals and other organisms

**Policy information about** [studies involving animals](#), [ARRIVE guidelines](#) recommended for reporting animal research

**Laboratory animals**  
NMRI-nude female mice were purchased from Janvier Laboratories and used for transplantation studies at the age of 6-9 weeks. The derivation of KB1P4 tumor organoids was described previously (Duarte et al. Nat Methods, 2017).

**Wild animals**  
No wild animals were used in this study.

**Field-collected samples**  
No field-collected samples were used in this study.

### Flow Cytometry

**Plots**

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.
## Methodology

### Sample preparation

For Traffic Light Reporter (TLR) assays, cells were collected from tissue culture plates by trypsinization, diluted into a larger volume of PBS with 10% FBS, then sorted immediately.

For class-switch recombination (CSR) assays, live cells were collected +/- CSR induction and stained with PE-conjugated anti-IgA antibody prior to FACS.

### Instrument

BD Biosciences Fortessa and BD Biosciences Fortessa X20-HTS.

### Software

FlowJo version 10

### Cell population abundance

No cell sorting was performed for this project.

### Gating strategy

For all assays, live cells were first gated from the FSC/SSC plots, with events of very low or very high FSC and/or SSC being excluded. The live cell fraction constituted >80% of total events in TLR assays, and >50% of total events in CSR assays.

For the TLR assay, the boundary between BFP-negative and BFP-positive was drawn from the plots of control samples where cells hadn’t been transfected with BFP-Iscel. The BFP-positive population was then used to determine percent of GFP-positive cells. The boundary between GFP-negative and GFP-positive was drawn from the plots of the total live cells from control samples where cells hadn’t been transfected with BFP-Iscel.

For the CSR assay, the boundary between PE-negative and PE-positive cells were applied from the plots of simultaneously stained unstimulated control samples.

For examples of how boundaries between "positive" and "negative" cells were drawn in the TLR assay, please see Extended Data Figure 3c,d.

For examples of how boundaries between "positive" and "negative" cells were drawn in the CSR assay, please see Extended Data Figure 9a.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.