Shieldin complex promotes DNA end-joining and counters homologous recombination in BRCA1-null cells

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BRCA1 deficiencies cause breast, ovarian, prostate and other cancers, and render tumours hypersensitive to poly(ADP-ribose) polymerase (PARP) inhibitors. To understand the resistance mechanisms, we conducted whole-genome CRISPR–Cas9 synthetic-viability/resistance screens in BRCA1-deficient breast cancer cells treated with PARP inhibitors. We identified two previously uncharacterized proteins, C20orf196 and FAM35A, whose inactivation confers strong PARP-inhibitor resistance. Mechanistically, we show that C20orf196 and FAM35A form a complex, ‘Shieldin’ (SHLD1/2), with FAM35A interacting with single-stranded DNA through its C-terminal oligonucleotide/oligosaccharide-binding fold region. We establish that Shieldin acts as the downstream effector of 53BP1/RIF1/MAD2L2 to promote DNA double-strand break (DSB) end-joining by restricting DSB resection and to counteract homologous recombination by antagonizing BRCA2/RAD51 loading in BRCA1-deficient cells. Notably, Shieldin inactivation further sensitizes BRCA1-deficient cells to cisplatin, suggesting how defining the SHLD1/2 status of BRCA1-deficient tumours might aid patient stratification and yield new treatment opportunities. Highlighting this potential, we document reduced SHLD1/2 expression in human breast cancers displaying intrinsic or acquired PARP-inhibitor resistance.

Double-strand breaks (DSBs) are highly cytotoxic cellular lesions that must be effectively and accurately repaired to maintain genome stability and prevent premature aging, neurodegeneration, immunodeficiency, cancer and other diseases1–3. In response to DSB detection, the apical kinases ATM, ATR and PRKDC (DNA-dependent protein kinase catalytic subunit) become activated and phosphorylate numerous substrates to initiate the cellular DNA damage response (DDR)4. The ensuing cascade of molecular DDR events, which are promoted by various post-translational modifications including protein phosphorylation, ubiquitylation, sumoylation and poly (ADP-ribose)ylation, impacts on a myriad of cellular components, among other things leading to the assembly of DDR factors at DNA-damage sites, arrest or slowing of cell-cycle progression, and activation of DNA repair mechanisms5–13. The two main types of DSB-repair pathway are non-homologous end-joining (NHEJ), which is active throughout the cell cycle, and homologous recombination (HR), which normally requires a sister chromatid as a template and hence only operates in the S and G2 phases of the cell cycle. DSB-repair pathway choice is partly determined by functional antagonism between the HR-promoting factor BRCA1 and NHEJ-promoting proteins such as TP53BP1 (53BP1), RIF1 and MAD2L2 (REV7)6–13.

Inherited or acquired mutations in the BRCA1 or BRCA2 genes that result in protein loss or a mutant BRCA1/2 protein cause breast, ovarian, prostate and other cancers, and render tumours hypersensitive to poly(ADP-ribose) polymerase (PARP)-inhibitor drugs such as olaparib14–17. Unfortunately, intrinsic or acquired PARP-inhibitor resistance frequently leads to a lack of response or to patient relapse and tumour regrowth15,18. In the clinic, the most common PARP-inhibitor resistance mechanisms reported to date are restoration of BRCA1/2 expression or function. Notably, 53BP1 expression is lost in various triple-negative breast cancers15, which may account for certain clinically relevant examples of PARP-inhibitor resistance. Nevertheless, the mechanisms driving PARP-inhibitor resistance in a large proportion of BRCA1/2-deficient tumours remain unexplained15,19.

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To systematically survey for genetic mechanisms of PARP-inhibitor resistance, we conducted whole-genome CRISPR–Cas9 synthetic-viability/resistance screens in human BRCA1-deficient breast cancer cells treated with PARP inhibitors. In addition to identifying known resistance factors such as 53BP1, RIF1 and MAD2L2 loss6–13, we identified two previously uncharacterized proteins, C20orf196 and FAM35A, whose inactivation confers PARP-inhibitor resistance to BRCA1-deficient cells. Our ensuing work led us to define the ‘Shieldin’ (SHLD1C20orf196/SHLD2FAM35A) complex, which promotes NHEJ by serving as the downstream effector of 53BP1, RIF1 and MAD2L2, restricts DSB resection, and counteracts HR in BRCA1-deficient cells by antagonizing replacement of replication protein A (RPA) with BRCA2 and RAD51 on unreplicated single-stranded DNA (ssDNA). Finally, we report that SHLD1C20orf196/SHLD2FAM35A loss confers hypersensitivity to the DNA-crosslinking agent cisplatin, and that reduced SHLD1C20orf196 or SHLD2FAM35A expression is associated with evolution of PARP-inhibitor resistance in a patient-derived BRCA1-deficient breast cancer xenograft model and in BRCA1-mutant cancers displaying intrinsic PARP-inhibitor resistance.

Results

FAM35A or C20orf196 loss suppresses PARP-inhibitor sensitivity of BRCA1-mutant cells. To systematically explore genetic mechanisms imparting PARP-inhibitor resistance, we carried out genome-wide CRISPR–Cas9 gene-inactivation screens with the GeCKO library1 in the BRCA1-mutant breast cancer cell line SUM149PT treated in parallel with the PARP inhibitors olaparib, talazoparib (BMN673) or AZD2461 (Fig. 1a,b and Supplementary Fig. 1d,e). These included DYNLL1, whose inactivation confers hypersensitivity to the DNA-crosslinking agent cisplatin, and that reduced SHLD1C20orf196 or SHLD2FAM35A expression is associated with evolution of PARP-inhibitor resistance in a patient-derived BRCA1-deficient breast cancer xenograft model and in BRCA1-mutant cancers displaying intrinsic PARP-inhibitor resistance.

FAM35A/C20orf196 complex interacts with and acts downstream of 53BP1/RIF1/MAD2L2. Sequence analyses indicated that FAM35A and C20orf196 are well conserved in vertebrates. Moreover, structure prediction modelling (RaptorX; http://raptor.uchicago.edu/) revealed that FAM35A harbours a disordered N terminus and an ordered C-terminal region containing three oligonucleotide/oligosaccharide-binding (OB) folds, with the last C-terminal OB fold/FAM domain containing a CXXC-type zinc finger motif (Fig. 2a). Notably, this organization is highly similar to those of the RPA1 subunit of ssDNA binding protein RPA and the CTC1 subunit of the CST complex that also binds ssDNA14. In this regard, we noted that while the C20orf196 N terminus (residues 1–70) is predicted to be intrinsically disordered, its C-terminal part is more structured and may harbour one- or two-winged helix (WH) domains (Fig. 2a) similar to those in the yeast CST subunit Stn12,24, suggesting that C20orf196 and Stn1 might play analogous or complementary roles.

By combining cellular co-localization and co-immunoprecipitation experiments, we established that FAM35A and C20orf196 directly interact in a manner that is mainly, but not exclusively, mediated by the FAM35A OB3/FAM domain (Fig. 2b,c and Supplementary Fig. 2a,b). Because loss of FAM35A or C20orf196 had similar effects to loss of 53BP1/RIF1/MAD2L2 in BRCA1-deficient cells, we tested for possible interactions between these factors. Thus, via co-immunoprecipitation and mass spectrometry (MS) studies, we found that both C20orf196 and FAM35A interact with MAD2L2, the most distal factor of the 53BP1/RIF1/MAD2L2 axis mediating PARP-inhibitor sensitivity in BRCA1-deficient cells1–15 (Fig. 2d and Supplementary Fig. 2c).

Many DDR proteins accumulate at DSB sites within ionizing radiation (IR)-induced nuclear foci (IRIF). We established that both FAM35A and C20orf196 formed IRIF, and by live-cell imaging studies found that the proteins were also recruited to localized DNA-damage sites induced by laser micro-irradiation (Supplementary Fig. 2d). Furthermore, we determined by confocal and super-resolution microscopy that FAM35A co-localized with the established DSB markers phosphorylated histone H2AFX (yH2AX) and 53BP1 (Supplementary Fig. 2e). Notably, siRNA/shRNA-depletion experiments established that while 53BP1 and MAD2L2 levels and IRIF were not significantly impaired by FAM35A or C20orf196 depletion (Supplementary Fig. 2f–h), IRIF formation by FAM35A and C20orf196 required 53BP1, RIF1 and MAD2L2 but not PTIP (Fig. 2e and Supplementary Fig. 3a–c; note that the total levels of green fluorescent protein (GFP)-tagged FAM35A/C20orf196 were minimally affected by 53BP1/RIF1/MAD2L2 depletion). We also established that C20orf196 IRIF were almost totally abrogated by FAM35A depletion, while C20orf196 depletion reduced but did not abolish FAM35A IRIF (Fig. 2e). In addition, FAM35A formed nuclear foci when cells were treated with the DNA topoisomerase I inhibitor camptothecin (CPT; Supplementary Fig. 3d). Significantly, the FAM35A N terminus was necessary and sufficient for its IRIF formation; these IRIF depended on 53BP1, RIF1, MAD2L2 and C20orf196, and this region could be co-immunoprecipitated with MAD2L2 (Fig. 2f and Supplementary Fig. 3e,f). Collectively, these findings indicated that FAM35A and C20orf196 act as downstream components of 53BP1/RIF1/MAD2L2 molecular assembly at DSB sites.

FAM35A and C20orf196 promote NHEJ. As 53BP1, RIF1 and MAD2L2 promote NHEJ6–13, we tested whether FAM35A and C20orf196 fulfilled a similar role. Indeed, as for depletion of the NHEJ factor XRCC4, siRNA depletion of 53BP1, FAM35A or C20orf196 impaired NHEJ, as measured by random integration of plasmid DNA into chromosomes27 (Fig. 3a). In addition, FAM35A or C20orf196 depletion conferred IR hypersensitivity to both human and mouse cells (Fig. 3b and Supplementary Fig. 4a). 53BP1, RIF1 and MAD2L2 and C20orf196, and this region could be co-immunoprecipitated with MAD2L2 (Fig. 2f and Supplementary Fig. 3e,f). Collectively, these findings indicated that FAM35A and C20orf196 act as downstream components of 53BP1/RIF1/MAD2L2 molecular assembly at DSB sites.

53BP1, RIF1 and MAD2L2 mediate NHEJ by antagonizing redundant mechanisms that result in NHEJ by serving as the downstream effector of 53BP1, RIF1 and MAD2L2, restricts DSB resection, and counteracts HR in BRCA1-deficient cells by antagonizing replacement of replication protein A (RPA) with BRCA2 and RAD51 on unreplicated single-stranded DNA (ssDNA). Finally, we report that SHLD1C20orf196/SHLD2FAM35A loss confers hypersensitivity to the DNA-crosslinking agent cisplatin, and that reduced SHLD1C20orf196 or SHLD2FAM35A expression is associated with evolution of PARP-inhibitor resistance in a patient-derived BRCA1-deficient breast cancer xenograft model and in BRCA1-mutant cancers displaying intrinsic PARP-inhibitor resistance.
**Fig. 1 | CRISPR–Cas9 screens identify suppressors of PARP-inhibitor sensitivity in BRCA1-mutant cells.**

**a,** Schematic of screen procedure. MOI, multiplicity of infection; PARPi, PARP-inhibitor. **b,** MAGeCK analysis of guide enrichments following specified drug treatments; false discovery rate (FDR) of 0.1 indicated by dotted line; \( n = 3 \) technical replicates per drug treatment. **c,** siRNA-mediated verification of hits in clonogenic survival assays; lower panels show area under the curve (AUC); \( n = 3 \) independent experiments. **d,** De novo Cas9-mediated knockout (ko) verification and complementation for FAM35A in clonogenic survival assays (multiple ko clones are shown in AUC); \( n = 4 \) independent experiments except FAM35Ako(#14) (\( n = 2 \)), FAM35Ako(#40) (\( n = 3 \)) and BRCA1ko/FAM35Ako(#34) (\( n = 2 \)) and BRCA1ko/FAM35Ako(#2) + FAM35A (\( n = 3 \)). **e,** As in d but for C20orf196; \( n = 3 \) independent experiments except BRCA1ko/C20orf196ko + C20orf196 (\( n = 2 \)). In c–e, bars represent mean ± s.e.m., one-way analysis of variance (ANOVA); \( * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, \ NS, not significant (\( P \geq 0.05 \)). Individual data points are plotted over bars, and statistical source data including the precise \( P \) values are provided in Supplementary Table 5.
FAM35A and C20orf196 antagonize DNA-end resection. To explore FAM35A and C20orf196 function further, we carried out assays in mouse cells harbouring a temperature-sensitive allele of the telomere-associated factor TRF2 (TRF2ts). TRF2ts inactivation at higher temperatures results in deprotected chromosome ends and causes NHEJ-mediated telomere fusions (Fig. 4a)–(43). Strikingly, through the use of short-hairpin RNA (shRNA) mediated mRNA silencing, we found that such chromosome fusions were diminished by FAM35A or C20orf196 depletion, like that elicited by MAD2L2 depletion (Fig. 4b and Supplementary Fig. 5a,b).

The impacts of 53BP1, RIF1 or MAD2L2 depletion in the TRF2ts system are connected to these factors counteracting DSB resection(42–44). We thus explored whether FAM35A and C20orf196 might also have this function. Indeed, as for 53BP1/RIF1/MAD2L2 inactivation(42–44), loss of FAM35A or C20orf196 in human cells enhanced DSB-resection, as measured by RPA and ssDNA staining intensity in pre-extracted nuclei after treatment with CPT (Fig. 4c–e; RPA1 kinetics at DNA damage sites induced by laser micro-irradiation were not altered by FAM35A or C20orf196 depletion, Supplementary Fig. 5c). In line with this resection being mediated by canonical pathways, it was diminished by depletion of the resection promoting factors RBBP8 (CiTP) or BLM (loom syndrome RecQ-like helicase, Fig. 4f).

Furthermore, we established that recruitment of BLM to sites of laser micro-irradiation was enhanced by FAM35A or C20orf196 depletion (Fig. 4g). Similarly, as shown for 53BP1 depletion in mouse cells(42), FAM35A or C20orf196 depletion in such cells led to higher levels of the DNA-end resection marker, Ser4/8 phosphorylated RPA2, after IR treatment (Fig. 4d). Together with our other findings, these data established FAM35A and C20orf196 as crucial components of 53BP1/RIF1/MAD2L2-mediated chromosomal NHEJ, and suggested that their pro-NHEJ function is connected to limiting DSB resection.

FAM35A OB fold region interacts with ssDNA and promotes IR survival. Consistent with our prediction of structural similarity between FAM35A and RPA1, the FAM35A C-terminus could be retrieved from cell extracts via interaction with a ssDNA oligonucleotide (Fig. 5a). Sequence alignment to RPA1 and structural modelling of FAM35A identified two Trp (W) residues predicted to be at the protein—ssDNA interface, based on analogous residues critical for RPA binding to ssDNA (Fig. 5b and Supplementary Fig. 5d). In accord with this prediction, we found, via electrophoretic gel-mobility shift assays (EMSAs), that the bacterially expressed, purified FAM35A C-terminal region bound preferentially to ssDNA rather than double-stranded DNA (Fig. 5c and Supplementary Fig. 5e), and ssDNA binding was reduced when the two Trp residues were mutated to Ala (W489/W640A; Fig. 5c). Furthermore, while full-length FAM35A bearing these mutations (FAM35A(W489/W640A)) still interacted with C20orf196 (Supplementary Fig. 5f) and formed IRIF in cells, these IRIF were consistently less pronounced in cells, these IRIF were consistently less pronounced and formed IRIF in cells, these IRIF were consistently less pronounced than those of the wild-type FAM35A protein (Fig. 5d). This suggested that, following IRIF recruitment via its N-terminal region, the FAM35A C-terminal ssDNA binding region may allow further FAM35A recruitment, retention and/or stabilization. In addition, unlike the wild-type protein, FAM35A(W489/W640A) did not confer significant IR resistance when reintroduced into FAM35A null cells (Fig. 5e).

In parallel studies, expression of the FAM35A C-terminus did not complement the IR hypersensitivity of FAM35A null cells. Moreover, expression of the FAM35A N-terminus rendered cells IR-hypersensitive, irrespective of whether they expressed endogenous FAM35A, implying that the N-terminal IRIF-forming domain of FAM35A may have a dominant-negative effect on NHEJ (Fig. 5f; overexpression of these FAM35A derivatives did not affect olaparib sensitivity in a wild-type background, Supplementary Fig. 5g).

FAM35A/C20orf196 loss correlates with PARP-inhibitor resistance in cancers. Having identified SHLD1/C20orf196 and SHLD2/FAM35A as mediating the PARP-inhibitor sensitivity of a BRCA1-deficient breast cancer cell line, we speculated that this might also apply in more physiological settings. Consequently, we employed a patient-derived xenograft (PDx) model of BRCA1-deficient breast cancer propagated in mice in the presence (cohort 2) or absence (cohort 1) of olaparib (Fig. 7a). The resistant tumour was further serially passaged into new hosts that were treated in the presence (cohort 4) or absence (cohort 3) of olaparib to confirm and sustain drug resistance (Fig. 7a; see also Supplementary Fig. 7a). The tumours were then harvested and subjected to whole-genome RNA-sequencing. Notably, our analyses revealed that in contrast to the other cohorts, nearly all resistant tumours after chronic olaparib treatment (cohort 4) correlated with reduced mRNA expression of SHLD1/C20orf196, SHLD2/FAM35A, 53BP1 and/or PARP1 (Fig. 7a; each heatmap column represents one tumour/mouse sample).
Because this tumour model is polyclonal\(^\text{40}\), our data suggested that olaparib resistance mechanisms might arise through parallel evolutionary trajectories converging on loss of Shieldin activity. Furthermore, when we stratified a cohort of BRCA1-deficient PDX tumours by SHLD1/2 expression, ensuing analyses indicated that low SHLD1\(^\text{C20orf196}\) transcript levels correlated with intrinsic olaparib resistance (Fig. 7b). One of the olaparib-resistant models (PDX127) demonstrated concomitant loss of both SHLD1\(^\text{C20orf196}\) and SHLD2\(^\text{FAM35A}\), while two other resistant models exhibiting normal SHLD1/2 transcript levels harboured deleterious 53BP1 mutations. Notably, several of these resistant PDX models also display BRCA1 nuclear foci\(^\text{11}\), suggesting the presence of multiple mechanisms of resistance, due to tumour heterogeneity and/or mechanistic cooperation.
Finally, we found that in contrast to 53BP1 deficiency\textsuperscript{42}, SHLD1\textsuperscript{C20orf196} or SHLD2\textsuperscript{FAM35A} loss increased the sensitivity of BRCA1-proficient and BRCA1-null cells to IR, and even more markedly enhanced their sensitivity to the DNA crosslinking agent cisplatin (Fig. 7c,d and Supplementary Fig. 7b,c). Furthermore, enhanced cisplatin sensitivity following SHLD1\textsuperscript{C20orf196} or SHLD2\textsuperscript{FAM35A} inactivation was associated with increased DNA-damage focus formation by the FANCD2 protein that is involved in the detection and repair of DNA crosslinks (Fig. 7e and Supplementary Fig. 7d). These findings therefore suggested that, if loss/reduced expression of SHLD1/2 occurs in patients, it may provide collateral therapeutic vulnerabilities that could be exploited clinically.

Fig. 3 | FAM35A and C20orf196 promote NHEJ and immunoglobulin CSR. a, Random plasmid integration assay. b, FAM35Ako and C20orf196ko cells were treated with IR and analysed for clonogenic survival. Right, AUC. In a, b, Bars represent mean ± s.e.m., one-way ANOVA; n = 3 independent experiments, except C20orf196ko in b (n = 4), with individual data points plotted over bars; statistical source data are provided in Supplementary Table S. c, Schematic representation of CSR and chromosomal instability in murine IgM\textsuperscript{+} B cells (germline configuration with C\textsubscript{\textgreek{i}} transcription) induced to express AID and undergo CSR to IgA (switch configuration with C\textsubscript{\textgreek{j}} transcription) on addition of anti-CD40, interleukin-4 (IL-4) and transforming growth factor beta (TGF-\beta). CSR levels were measured as percent of IgA-positive cells after 72 h cytokine stimulation, and DNA fluorescence in situ hybridization (FISH) was performed using a chromosome 12-specific paint (grey chromosome) and Ig\textsubscript{\textgreek{h}} locus-specific probes (red and green spots) for the measurement of chromosomal instability at the Ig\textsubscript{\textgreek{h}} locus on induction of CSR. d, CSR levels in Fam35Ako and C20orf196ko CH12-Cas9 cells are reduced compared with wild-type (WT) CH12-Cas9 cells. Bars represent mean ± s.e.m., one-way ANOVA; n = 4 independent experiments of three clones except 53bp1ko-cytokine where n = 3 of two clones, and 53bp1ko-cytokine where n = 2 of two clones; individual data points are plotted over bars. e, Representative images of Ig\textsubscript{\textgreek{h}} translocation and breaks in aberrant metaphases, as quantified in f. f, Quantification of Ig\textsubscript{\textgreek{h}} breaks and translocations in metaphases of the indicated CH12-Cas9 cells. Horizontal bars represent means, Fisher’s exact test; n = 2 independent experiments except Fam35ako and C20orf196ko where n = 3. For a, b, d and f, *P < 0.05, **P < 0.01, ***P < 0.0001; NS, not significant (P ≥ 0.05); statistical source data including precise P values are provided in Supplementary Table S.
Discussion

Over the past two decades, it has become evident that eukaryotic cells have evolved multiple mechanisms of DNA DSB repair that are regulated in complex and sophisticated ways to optimize genome stability. In particular, much attention has focused on how cells strategically employ the two prime modes of DSB repair—NHEJ and HR—which antagonize one another, operate optimally in different contexts and whose relative usage is regulated by factors such as chromatin structure and cell-cycle stage. In addition to being of academic interest, work on such subjects is also of clinical relevance.
particularly in cancer therapy where DSB-inducing chemotherapeutic agents are frequently used, and molecularly targeted drugs such as PARP inhibitors are being increasingly employed in specific settings. Intrinsically or arising PARP-inhibitor resistance in patients with BRCA1/2 mutations is nevertheless an increasing clinical problem. Using whole-genome CRISPR–Cas9 synthetic-viability/...
Fig. 6 | FAM35A or C20orf196 loss restores HR in BRCA1-deficient cells. **a**. Quantification of GFP-FAM35A (left) and GFP-C20orf196 (right) IRIF in U2OS cells after BRCA1 or BRCA2 depletion (5 h after 5 Gy). Bars represent mean ± s.e.m., one-way ANOVA; n = 3 independent experiments, except FAM35A siCTRL (n = 4), FAM35A siBRCA2 (n = 2) and C20orf196 siCTRL (n = 5). Individual data points are plotted over bars. **b**. Quantification of 53BP1 and inducible GFP-FAM35A IRIF in U2OS cells with or without BRCA1 depletion (5 Gy, indicated time points). Bars represent mean ± s.e.m., one-way ANOVA; n = 4 independent experiments, except Groups 4 and 5 (n = 4). Individual data points are plotted over bars. **c**. Representative images (left) and quantification (right) of RAD51 IRIF (5.5 h after 5 Gy) in Cyclin A (CycA) positive RPE1ko cell lines as indicated. Bars represent mean ± s.e.m., one-way ANOVA; n = 4 independent experiments. Individual data points are plotted over bars. **d**. Quantification of 53BP1 and inducible GFP-FAM35A IRIF in U2OS cells with or without BRCA1 depletion (5 Gy, indicated time points). Bars represent mean ± s.e.m., one-way ANOVA; n = 4 independent experiments, except 53BP1, 1.5 h siCTRL (n = 2); 53BP1, 1.5 h siBRCA1 and 53BP1, 16 h siCTRL (n = 3); FAM35A, 1.5 h siCTRL (n = 5). Individual data points are plotted over bars. **e**. Representative images (left) and quantification (right) of RAD51 IRIF per cyclin A positive cell (rel. WT) in U2OS cells treated with indicated siRNAs (for gating strategy see Supplementary Fig. 6f). Individual data points are plotted over bars. **f**. Homologous recombination (HR) assay in U2OS-TLR cells treated with indicated siRNAs (for quantification see Supplementary Fig. 6e). Scale bar, 10 μm. **g**. Olaparib clonogenic survival assay with indicated RPE1ko and complemented cell lines. Bars represent mean ± s.e.m., one-way ANOVA; n = 4 independent experiments, except Groups 4 and 5 (n = 3) and Group 3 (n = 2); AUC is shown in Supplementary Fig. 6g. For a–c, e, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; NS, not significant (P ≥ 0.05); statistical source data including precise P values are provided in Supplementary Table 5.
Fig. 7 | FAM35A or C20orf196 loss correlates with PARP-inhibitor resistance in cancers. a, Top, Schematic of in vivo PDX study. Bottom, Heatmap generated from mRNA sequencing showing scaled expression levels of indicated genes from corresponding PDX samples; n = 6, 5, 7, 8 mice for cohorts 1–4, respectively. b, Expression of C20orf196/FAM35A in breast and ovarian cancer PDXs derived from BRCA1-deficient tumours. y axis: log2 transcript per million (tpm). Lines represent mean ± s.e.m.; n = 12, 4, 15, 1 for SHLD1-high, SHLD1-low, SHLD2-high, SHLD2-low groups, respectively; two-tailed unpaired Student’s t-test; ***P = 0.0003. Statistical source data for PDXs are provided in Supplementary Table 5 and the Methods. c, d, Clonogenic survival assay after IR (c) or cisplatin treatment (d) in the indicated RPE1ko cell lines (AUC shown in Supplementary Fig. 7b,c, respectively). Data shown represent mean ± s.e.m. (n = 3 independent experiments except for Group 7 in c and Group 7 in d where n = 2). e, Loss of FAM35A/C20orf196 leads to increased cisplatin-induced FANCD2 foci. Bars represent mean ± s.e.m., one-way ANOVA; ***P < 0.001, ****P < 0.0001; NS, not significant (P ≥ 0.05); n = 4 independent experiments, with individual data points plotted over bars; statistical source data are provided in Supplementary Table 5. Scale bar, 10 µm. f, Proposed model for the action of SHLD1/2 in DSB repair in the presence or absence of functional BRCA1.
resistance screens, we have uncovered two, previously uncharacterized proteins—SHLD1<sup>Clb0r96</sup> and SHLD2<sup>Fam35a</sup>—whose loss mediates PARP-inhibitor resistance and which we have shown act as the most distal factors of the 53BP1-RIF1/MAD2L2 molecular axis to promote NHEJ and restrict HR in BRCA1-deficient settings. Our screens have also identified additional candidate PARP-inhibitor resistance factors that await validation in future studies.

Mechanistically, we have shown that SHLD1<sup>Clb0r96</sup> and SHLD2<sup>Fam35a</sup> form a complex, termed ‘Shieldin,’ with SHLD1<sup>Clb0r96</sup> recruitment to DNA-damage sites via its interactions with SHLD2<sup>Fam35a</sup> and other factors, and SHLD2<sup>Fam35a</sup> interacting with single-stranded DNA via its C-terminal OB fold region. Moreover, we have established that SHLD1<sup>Clb0r96</sup> and SHLD2<sup>Fam35a</sup> promote NHEJ in a manner that may be mediated via their effects on restricting DNA-end resection, and serve as a barrier to HR by antagonizing the replacement of RPA with BRCA2/RAD51 on resected ssDNA in a manner counteracted by BRCA1. Our work is in line with a recent report<sup>41</sup> that independently identified SHLD1<sup>Clb0r96</sup> and SHLD2<sup>Fam35a</sup> as NHEJ-promoting factors and antagonists of HR in BRCA1-defective cells. This study also identified a third component, RIN1/<s>SHLD3<sup>CtC-Sam4</sup></s>, which is proposed to serve as a molecular bridge from RIF1 to MAD2L2 and SHLD1/2.

Although it seems possible that Shieldin loss contributes to HR restoration in BRCA1-deficient cells through effects on both resection and BRCA2/RAD51 loading, the relative importance of these mechanisms needs further investigation. We note that more extensive and possibly faster resection in the absence of Shieldin might enhance BRCA2/RAD51 loading. Alternatively, or in addition, Shieldin might serve as a physical barrier to BRCA2/RAD51 loading at dsDNA/ssDNA junctions in BRCA1-deficient cells—perhaps through it being tethered to DSB-flanking chromatin via the 53BP1-RIF1/MAD2L2 complex allowing interactions between the C termini of distal SHLD2<sup>Fam35a</sup> and ssDNA (see Fig. 7f for a model and Supplementary Fig. 7e for SHLD2<sup>Fam35a</sup> domain function summary). Nevertheless, we found that overexpression of the SHLD2<sup>Fam35a</sup> N terminus but not the C terminus confers olaparib hypersensitivity to BRCA1/FAM35A double knockout cells, suggesting that at least in this context, chromatin binding by SHLD2<sup>Fam35a</sup> plays a dominant role in restricting HR. By contrast, we found that both the SHLD2<sup>Fam35a</sup> N and C termini are important for IR resistance (in BRCA1-proficient cells). As IR sensitivity in Shieldin-deficient cells probably reflects impaired NHEJ, we speculate that Shieldin potentiates NHEJ by restricting DSB resection as well as by assembling with other NHEJ-promoting factors to tether DSB ends together to facilitate their juxtaposition and repair.

Notably, we have found that SHLD1<sup>Clb0r96</sup> or SHLD2<sup>Fam35a</sup> inactivation confers enhanced cisplatin sensitivity to BRCA1-null or BRCA1-proficient cells. Such sensitivity probably does not reflect the roles for Shieldin in promoting NHEJ, restricting DNA-end resection or antagonizing BRCA1-mediated BRCA2/RAD51 loading because, in our hands, 53BP1 loss does not have pronounced effects on cisplatin sensitivity. Intra-strand DNA crosslinks (ICLs) generated by cisplatin and other compounds are detected and repaired by the Fanconi anaemia (FA) pathway, with a key FA protein being FANCd2, which forms foci at sites of these lesions<sup>40</sup>. We have observed that, following cisplatin treatment, FANCd2 foci were more pronounced in cells in which SHLD1<sup>Clb0r96</sup> or SHLD2<sup>Fam35a</sup> was inactivated. It will thus be of interest to determine if Shieldin—like MAD2L2, which (with REV3L) functions as a regulatory subunit of the trans-lesion DNA synthesis (TLS) polymerase Pol zeta, and whose biallelic inactivation has been associated with FA<sup>−/−</sup>—might also promote ICL repair via TLS mechanisms.

Finally, based on our findings, it will be interesting to evaluate SHLD1/2 expression changes in tumour biopsies from patients, establish whether this information can be used in patient stratification for PARP-inhibitor therapies, and determine whether SHLD1/2 expression changes arise in patients whose BRCA1-deficient cancers develop resistance after PARP-inhibitor treatment. In this regard, we note that if downregulation of Shieldin components in BRCA1-deficient cancers does confer clinical resistance, this might allow alternative treatments, such as ones based on platinum compounds.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-018-0140-1.

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Methods
CRISPR–Cas9 screen. CRISPR–Cas9 was performed using genome-scale (GeCKO) v2.0.
SUM149PT cells were transduced at a multiplicity of infection of 1. A further 14 days. IC95–2
used were Olaparib IC95–2 0.3 and 250-fold coverage of the library. Cells were then selected with puromycin
Trf2 mycoplasma free. The U2OS and RPE1 cell lines were recently authenticated using
CRISPR–Cas9 screen. CRISPR–Cas9 was performed using genome-scale
in refs 38,39. The TLR assay and the constructs used herein are described in detail
photobleaching) and association kinetics were performed as described in ref. 50. FRAP and association kinetics.
Whole cell extracts and immunoblotting. Whole cell extracts and immunoblotting were performed as described in ref. 35.
Phosphate-buffered saline (PBS; BioSera), 100 U ml
were generated by transfection of pcDNA5/FRT/TO-neo containing the GFP-
p53 null FRT-derived cells stably expressing inducible GFP-tagged constructs
U2OS Trex or RPE1
Identification of essential genes. Essential genes were identified by combining CRISPR screen and ICL
Mutants deficient in DNA repair were then analysed by colony formation assay on dishes containing 3% agarose and 10 µg ml
Cells were lysed 48 h post-transfection in modified RIPA buffer
and 2 mM
were co-transfected with pMSCV-blas-3xFlag-hMAD2L2 and either pcDNA5.1-
DNA oligos then were resuspended in binding buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP40, 0.5 mM EDTA, 0.5% BSA). Purified GST (glutathione-S-transferase, bacterial expression), GST-FAM5A (Novus Biologicals) and His-C20orf196 (Creative BioMart) were added to the beads at 2 pmol and incubated for 30 min at 4 °C. After washing the beads five times in 10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% NP40, 0.5 mM EDTA and eluted with 100 mM Tris pH 8, 20 mM reduced glutathione, 120 mM NaCl for 15 min rotating at 4 °C. The eluates were boiled for 5 min, loaded on 4–12% Bis-Tris gel (Invitrogen) and subjected to western blotting. The blots were probed with the indicated antibodies.
DNA pulldown experiments. Procedures were described in detail in ref. 37, using oligos with the sequence:
DNA oligos were eluted in a gradient against buffer B (20 mM Tris pH 8.0, 5% glycerol, 150 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole, protease inhibitor (Roche) and 40 µg ml
were dissolved in annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA) to a final concentration of 100 M dithiothreitol, 10 M dithiothreitol.
DNA oligos with the sequence:
Cell cycle profiling. Cell cycle profiling was performed as described in ref. 38, 39.
Clonogenic survival assays. Survival assays were performed as described in refs 27,29.
Whole cell extracts and immunoblotting. Whole cell extracts and immunoblotting were performed as described in ref. 35.
Cell cycle profiling. Cell cycle profiling was performed as described in ref. 38, 39.
Immunoprecipitation. All immunoprecipitation procedures were performed twice as described in ref. 36. For the co-immunoprecipitation shown in Fig. 2d (FAM5A), 293T cells were co-transfected with pMSCV-blas-gEFP-MAD2L2 and either pMSCV-blas-V5-Emetine (EMSA). Equal amount of cells, cells were exposed to 25 Gy IR followed by 3 h recovery. GFP–Trap–MA beads (ChromoTek) were used, and immunoprecipitation was performed according to the manufacturer’s protocol. For C20orf196 (Supplementary Fig. 2c), 293T cells were co-transfected with pMSCV-blas-3XFlag-hMAD2L2 and either pDNAs1.1-GFP for pDNAs1.1-GFP at 200 ng/ml. (ChromoTek) were used, and immunoprecipitation was performed according to the manufacturer’s protocol.
ARTICLES
4°C and protein concentrations were estimated using QuickStart Bradford Protein assay (BioRad). Per SILAC condition, 20 μl of pre-equilibrated GFP-Trap-A beads (ChromoTek) were added to 2 mg of lysate and incubated 1 h at 4°C while rotating, followed by multiple washes with modified RIPA buffer. Bound proteins were eluted in NuPAGE LDS Sample Buffer (Life Technologies) supplemented with 1 mM dithiothreitol, heated at 70°C for 10 min and alkylated with 5.5 mM chloroacetic acid at RT. Samples were loaded onto 4–12% gradient SDS–PAGE gels, and proteins were stained using a Colloidal Blue Staining Kit (Life Technologies) and digested in gel using trypsin. Peptides were extracted from the gel and desalted on reversed-phase C18 Stage Tips.

**Mass spectrometry analysis.** Peptide fractions were analysed on a quadrupole Orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific) equipped with an EASY-SLC 1000 (Thermo Scientific) source as described in ref. 13. Peptide samples were loaded onto EASY-nLC 1000 (Thermo Scientific) C18 columns and eluted with a linear gradient from 0% to 100% solvent B over a 120 min run with a flow rate of 300 nλ/min. Mass spectra were acquired in data-dependent mode, performing a full mass-scan from m/z 300–1,650 in negative mode, followed by in-source fragmentation of the most abundant peptide ions using HCD (higher-energy C-trap dissociation)54. Fragment spectra were acquired in MS2 acquisition. Survey full-scan MS spectra (m/z 300–1,650) were acquired in the positive ion mode, using a target-decoy approach57. on posterior error probability to arrive at a false discovery rate below 1% estimated using HCD MS2 mode, strict trypsin specificity and allowing up to three miscleavages. Cysteine carbamidomethylation was searched as a fixed modification, whereas protein N-terminal acetylation, methionine oxidation, N-ethylmaleimide modification of cysteines were searched as variable modifications. The data set was filtered based on significant ion intensities and several false discovery rates below 1% estimated using a target-decoy approach.

**Immunofluorescence and microscopy imaging.** Confoal imaging for βH2AX, RAD51, RPA, ssDNA (BrdU), BLM, BRCA2, FANCDC2, Cyclin A and GFP (FAM35A and C20orf196) was performed as described in ref. 13. For RAD51 and Cyclin A, the pre-extraction step was omitted and cells were permeabilised for 15 min in 0.2% Triton X-100 (Sigma) in PBS after fixation. Super-resolution images were acquired using a DeltaVision OMX 3D-SIM System V3 BLAZE (Applied Precision, a GE Healthcare company) equipped with three sCMOS (scientific complementary metal-oxide semiconductor) cameras; 405, 488 and 592.5 nm diode laser illumination, an Olympus Plan Apo N X60.1 1.42 NA oil objective, and standard excitation and emission filter sets. Imaging of each channel was done sequentially using three angles and five phase shifts of the illumination pattern, as described in ref. 14. Sections were acquired at 0.125 μm z-steps. Raw OMX data were reconstructed and channel registered in SoftWoRx software version 6.5.2 (Applied Precision, a GE Healthcare company). Voxelwise nearest-neighbour distances were measured for the GFP-FAM35A signal relative to the 53BP1 signal using a custom script (Butler R) for Fiji (https://github.com/gurdon-institute OMX-Spatial-Analysis). The script maps signal volumes using Kapur’s maximum entropy thresholding method and measures distances using the exact signed 3D Euclidean distance transform with internal distances set to zero for display on the histogram. For all images, scale bars represent 10 μm.

**Multiplex fluorescence in situ hybridization.** Human 24-colour multiplex fluorescence in situ hybridization (M-FISH) probe preparation and slide treatment was carried out as described in ref. 15. For each human cell sample, 10–30 metaphases were karyotyped based on the M-FISH classification and DAPI-bandning pattern. FISH on metaphase spreads using BAC probes was performed as previously described and counted manually. For CSR assays, DNA FISH on metaphase spreads was performed as previously described and counted manually. At least 470 metaphases were evaluated per genotype, using at least two independent clones for each condition. For telomere uncapping, cell harvesting, preparation of metaphase spreads and telomere FISH were performed with an Amaxa Nucleofector Kit V solution (Lonza) and program X-001. Two days later, BFP-positive/pouromycin-resistant CH12-Cas9 cells were selected with 3 μg/ml puromycin for one week. Cells were then single cell diluted into 96-well plates, further cultured and screened by PCR, and Sanger sequenced using PCR primers (listed in Supplementary Table 4). For CSR and cell proliferation assays, CH12 cells were plated at 50,000 cells per ml in complete medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, IL-4 (20 ng/ml), M童年leny) and TGF-β (1 μg/ml). After 3 days, cells were assayed for class-switching by flow cytometry using an Igk-PE antibody (eBiosciences) and a Canto II analyser (BD Biosciences). Viable cells were counted using a Casy cell counter (Roche). CSR and proliferation assays were done on 3× wild-type (WT), 3× FAM35a knockout (FAM35a), 3× C20orf196 knockout (C20orf196), 2× 53BP1 knockout (53BP1) and 3× Mad2l2 knockdown (Mad2l2) cell lines in three independent experiments. For RT–PCR analysis, lgh, a germ-line transcripts (gALT) and Aid mRNA were quantified as previously described. Primers are listed in Supplementary Table 4.

**Patient-derived tumour xenografts.** PDXs were generated and established from breast or ovarian cancer patients’ samples (with consent) as previously described. The research was done with appropriate approval by the National Research Ethics Service, Cambridgeshire 2 REC (REC reference number: 08/ H0300/178), and by the Vall d’Hebron Hospital Clinical Investigation Ethical Committee (PRA1G183/2012). STG201, the PDX model used in this study, is a BRCA1 null model formed by BRCA1 promoter methylation, loss of BRCA1 mRNA and protein expression. We have previously shown its sensitivity in vivo and in PDX-derived cells to PARP inhibitors, including olaparib. STG201 is also linked to deep molecular and drug sensitivity annotation (https://caldaslab.cruk. cam.ac.uk/bcbpe/). All other PDXs were derived from breast or ovarian tumours from BRCA1-mutation carriers or BRCA1 epigenetic silencing due to promoter hypermethylation. PXD127 did not show any co-expression of BRCA1 but it was low in both FAM35A and C20orf196 expression. None of the five PARP-sensitive PDXs exhibited low levels of C20orf196, FAM35A or 53BP1 loss nor BRCA1 hypomorphs. The study was compliant with all relevant ethical regulations regarding research involving animal use and human participants.

**Generation of acquired drug resistance in vivo.** AZD2281 (Olaparib/Lynparza) as a PARP inhibitor was administered to immunocompromised tumour-bearing mice following randomization, as previously described (50 mg/kg, 3 days per week). To classify the response of the spontaneous and the xenografted murine mammary gland tumours, a modified RECIST (response evaluation criteria in solid tumours) criteria to be based on the percent tumour volume change following continuous olaparib treatment: complete response (CR); best response, ≤−5%; partial response (PR), −5%≤ best response ≤−30%; stable disease (SD), −30% < best response ≤−20%; progressive disease (PD) ≤−30% tumour volume change at day 21, ≤−20% tumour volume change at day 3, ≤−50% tumour volume change at day 15 + 20% PARP-resistant PDXs exhibited PD while PARP-sensitive models exhibited SD, PR or CR. For STG201, time-matched vehicle- and olaparib-treated samples were collected 25 days after treatment (PARPi naïve PDX) and processed for RNA extractions and sequencing. A couple of mice in the study were left with continued exposure to olaparib until tumour regrowth. One of these resistant tumours was serially passed 126 days after treatment into new host mice (PARPi-resistant PDX) and treated with further vehicle or olaparib. At 80 days after treatment, the resistance phenotype was confirmed and samples were collected and processed for RNA-sequencing. Growth curves show average and standard deviation of at least five independent tumour volumes per trial arm. All experimental procedures were approved by the University Animal Welfare Committee and the Vall d’Hebron Hospital Clinical Investigation Ethical Committee and Animal Use Committee. For RNA-sequencing, RNA was extracted from all samples using the Qiagen miRNeasy or RNeasy Mini kit (cat. ID 217004 or 74104) according to the manufacturer’s instructions. Libraries for Illumina sequencing were prepared using a TruSeq Stranded mRNA HT kit or Total RNA Library Prep kit with Ribo-Zero Gold (cat. ID: RS-122-2103 or RS-122-2301, Illumina). 500 ng of total RNA with RNA integrity numbers (RINs) above 8 was used for library preparation. Samples were processed following the manufacturer’s HS (High-Sample) instructions (part no. 15030148 Rev. E, Illumina) with 12 or 15 cycles of PCR used at the Enrichment of DNA Fragments step. All libraries were barcoded using KAPA mRNA Quantitative Illumina Ribo-Zero Normalized (Cat ID, KK4873, KAPA Biosystems) and normalized. Libraries were pooled in equal volumes and pools were used for clustering on a HiSeq4000 sequencing flow cell following the manufacturer’s instructions. Sequencing was performed using 150 bp or 100 bp pair-end runs for dual-indexed libraries. Before alignment, the sequencing quality of the reads was enforced using Trim Galore!
(v0.4.2; http://www.bioinformatics.babraham.ac.uk/projects/trim galore/). Then, as described in refs 48–50, reads were aligned to a combined human (hg19) and mouse (mm10) reference genome using STAR (v2.5.2b)51. Counts were assigned to genome features using featureCounts (v1.5.2), whereby the alignment score is used to discern accurately between reads sourced from human and mouse52. Counts from multiple sequencing runs were merged and then normalized using the edgeR package53.

Statistics and reproducibility. Unless stated otherwise, Prism v7.0b (GraphPad Software) was used to generate graphs, perform statistical tests and calculate P values. Error bars, statistical tests and number of independent repeats (n) are indicated in the figure legends, and statistical source data including precise P values are provided in Supplementary Table 5. Statistical tests included two-tailed Student’s t-tests, Fisher’s exact test, and one-way analysis of variance (ANOVA), the latter all being corrected as recommended for multiple comparisons. Microscopy image analyses were performed using ImageJ/FIJI or Velocity 6.3 (Perkin-Elmer). CRISPR screens were performed with three clones per drug treatment. Mass spectrometry of GFP-FAM35A and GFP-C20orf196 was performed in two independent experiments. RNA-sequencing was performed as three replicates for each trial arm, due to sequencing across multiple lanes (which were merged before any further analysis). This was performed for the following number of independent biological samples: six PDXs in cohort 1, five PDXs in cohort 2, seven PDXs in cohort 3 and eight PDXs in cohort 4. For the SHLD1 high and low expression cohorts, 12 and 4 independent PDXs were evaluated, respectively. All immunofluorescence assay quantification data were evaluated, respectively. All immunofluorescence assay quantification data were evaluated, respectively. All immunofluorescence assay quantification data were evaluated, respectively. All immunofluorescence assay quantification data were evaluated, respectively.

Data availability. The raw data files for the whole-genome CRISPR–Cas9 screen in SUM149 cells are available from the NIH Sequence Read Archive (SRA) via accession no. PRJNA473982. Raw data files for the PDX RNA sequencing are available from the NIH SRA via accession no. PRJNA473981. Raw data files for mass spectrometry are available via the ProteomeXchange Consortium on the PRIDE partner repository with data set identifier PXD009830. Source data for figures are provided in Supplementary Table 5. All other data supporting the findings of this study are available from the corresponding authors on reasonable request.

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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).

- [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- [ ] An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [ ] The statistical test(s) used AND whether they are one- or two-sided
- [ ] A description of all covariates tested
- [ ] A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- [ ] A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- [ ] For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- [ ] For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- [ ] For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- [ ] Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- [ ] Clearly defined error bars

Software and code

Policy information about availability of computer code

Data collection

Data collection using Microsoft Excel for Mac (Microsoft Inc, USA). Adobe Photoshop and Illustrator were used to process data for publication (Adobe Systems Inc, USA).

Data analysis

Above statistical parameters are indicated in the methods section. Graph display and statistical analysis was performed using Prism v7 for MacOs (GraphPad Software Inc., USA). ImageJ was used to quantify immunoblot bands (ImageJ 2.0.0). Flow cytometry analysis using FlowJo (BD Inc, USA). For RNA sequencing studies, prior to alignment, sequencing quality of the reads was enforced using Trim Galore! (v0.4.2). Reads were aligned to reference genomes using STAR (v2.5.2b). Counts were assigned to genome features using featureCounts(v1.5.2). Counts from multiple sequencing runs were merged/normalised using edgeR package. MaxQuant v1.5.2.8 was used for Mass Spectrometry data analysis. Raw OMX data was reconstructed and channel registered in SoftWoRx software version 6.5.2 (Applied Precision, a GE Healthcare company). FIJIv2.0.0 was used for OMX data analysis.

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### Life sciences

#### Study design

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

**Sample size**
- Animal experiments were performed with the available BRCA1 PDX models generated. All experiments were conducted with cell lines with multiple available biological replicates and based on previous experience with specific experimental setup; no statistical method was used to determine sample size.

**Data exclusions**
- No tumors/in-vitro cell line samples were excluded from analysis.

**Replication**
- All experiments were reliably reproduced as stated in the text, and detailed methods provided to aid in their replication by others. Where further methods/data are sought corresponding authors will oblige reasonable requests.

**Randomization**
- Mice were randomised into treatment arms.

**Blinding**
- Group allocation and outcome assessment were performed in fully blinded manner.

#### Materials & experimental systems

Policy information about availability of materials

| n/a | Involved in the study |
|-----|-----------------------|
|     | - Antibodies |
|     | - Eukaryotic cell lines |
|     | - Research animals |
|     | - Human research participants |

**Unique materials**
- All established cellular and plasmid reagents are available on reasonable request

**Antibodies**
- **Antibodies used**
  - FAM35A Rabbit Abcam ab105521 WB (1:1000)
  - C20orf196 (E-15) Rabbit Santa-Cruz sc-85394 WB (1:200)
  - 53BP1 Rabbit Novus NB100-304 WB (1:5000)
  - 53BP1 Mouse Millipore MAB3802 IF (1:200)/WB (1:1000)
  - RIF1 Rabbit Bethyl A300-569A WB (1:1000)
  - REV7/MAD2L2 Mouse BD biosciences 612266 IF (1:100)/WB (1:1000)
  - BRCA1 Rabbit Merck G7-434 WB (1:500)
  - FANCd2 Mouse Santa-Cruz sc-20022 (F117) IF(1:100)/WB (1:1000)
  - HA Mouse Santa-Cruz sc7392 WB (1:500)
BLM Rabbit Bethyl A300-110A IF (1:100)
CTIP Mouse Hybridoma supernatant (Richard Baer) WB (1:50)
GFP Mouse Roche WB (1:1000)
Tubulin Rabbit Abcam ab28666 WB (1:1000)
GFP Rabbit Roche IF (1:1000)
PTP Rabbit Abcam ab2614 WB (1:1000)
Abraxas Rabbit Bethyl A302-180A-M WB (1:1000)
Cyclin A Mouse BD Biosciences 611268 IF (1:100)
RAD51 Rabbit Santa Cruz sc-8349 (H-92) IF (1:100)
yH2AX Mouse Millipore 05-636 IF (1:100)
RP42 Mouse Abcam ab2175 IF (1:200)
yH2AX Rabbit Cell Signalling Technology 2577 IF (1:500)
XRCC4 Goat Santa Cruz sc-8285 (C-20) WB (1:1000)
SMC1 Rabbit Bethyl A300-055A WB (1:1000)
Total H2AX Rabbit Abcam ab11175 WB (1:5000)
GAPDH Mouse Abcam ab8245 WB (1:2500)
β-Actin Mouse Abcam ab8266 WB (1:5000)
GFP Rabbit Roche WB (1:1000)
Phospho-RPA32 S4/S8 Rabbit Bethyl A300-245A WB (1:1000)
C20orf196 (E-15) Rabbit Santa Cruz, sc-85394 WB (1:500)
GST Mouse Santa Cruz, sc 138 WB (1:1000)
Alexa Fluor 488 anti mouse/rabbit Goat Molecular Probes A11029/A11034 IF (1:500-1:1000)
Alexa Fluor 594 anti mouse/rabbit Goat Molecular Probes A11005/A11037 IF (1:500-1:1000)
Alexa Fluor 647 anti mouse goat Molecular Probes A21236 IF (1:500)
HRP anti mouse Rabbit Dako Ltd PD260 WB (1:10000)
HRP anti goat Goat Perbio Science 31462 WB (1:20000)
HRP anti goat Rabbit Dako Ltd PD449 WB (1:10000)
S3BP1 Rabbit Bethyl, A300-272A WB (1:1000)
b-Catenin Mouse BD, 610154 WB (1:5000)
MAD2L2 Mouse Santa Cruz sc135977 IF (1:500)
V5 Mouse Invitrogen, R960-25 WB (1:1000)
Flag-M2 Mouse Sigma, F1804 IF (1:200)/WB (1:2000)

Validation
Each experiment had appropriate controls to validate the antibodies. Commercially available antibodies were validated by the supplier and by us using appropriate controls where needed; Supplementary Table 2. Please also refer to the manufacturers’ websites for further details.

Eukaryotic cell lines
Policy information about cell lines
Cell line source(s)
U2OS(TREX), RPE1p53-(FRT), HEK293T, mESC: SP Jackson (Gurdon Institute, Cambridge, UK)
Lenti-X 293T: Clontech Laboratories Inc. (CA, USA)
SUM149PT: C. Lord (Institute of Cancer Research, London, UK)
MEFS(TRF2ts): J. Jacobs (NKI, Netherlands)

Authentication
All cells were originally obtained from the ATCC cell repository, and we have authenticated cell lines used in our study by STR profiling.

Mycoplasma contamination
All cells are routinely tested to be mycoplasma free.

Commonly misidentified lines
(See ICLAC register)
No commonly misidentified cell lines were used.

Research animals
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research
Animals/animal-derived materials
Animals/animal-derived materials PDXTs were generated by implantation of human tumour samples in female highly immunodeficient mice (NOD.Cg-PkdcsidIl2rgtm1Wjl/J or NSGs) at around 4-5 months old. All experimental procedures were approved by the University of Cambridge Animal Welfare and Ethical Review Committee and by the Vall d’Hebron Hospital Clinical Investigation Ethical Committee and Animal Use Committee. Collection to mouse implantation ranges from 30-180 min. Surgically resected tissue samples were embedded in matrigel and then implanted subcutaneously into 2-4 female severe immune compromised NSG mice. Pleural effusion and ascites samples were centrifuged, washed with water twice to eliminate red blood cells, and cell pellets resuspended in 50% matrigel:FBS solution before subcutaneous injection into mice.
Human research participants

Policy information about studies involving human research participants

Population characteristics

Surgically resected primary breast cancer tissue, biopsies from brain, skin, bone, liver, axilla and lymph node metastasis, and pleural effusions or ascites samples were obtained from consenting patients (female, diagnosed with breast or ovarian malignancy). The research was done with the appropriate approval by the National Research Ethics Service, Cambridgeshire 2 REC (REC reference number: 08/H0308/178), and the Vall d’Hebron Hospital Clinical Investigation Ethical Committee (PRIAG183/2012). Comprehensive clinical information on the patients and originating cancer sample implanted to generate PDTXs can be found in Bruna et al. Cell 2016 and Cruz et al. Annals of Oncology 2018.

Method-specific reporting

n/a

Involved in the study
- ChIP-seq
- Flow cytometry
- Magnetic resonance imaging

Flow Cytometry

Plots

- 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

U2OS(TLR) cells (modified from Certo et al. 2011 Nat Methods, Jul 10;8(8):671-6) were treated with the indicated siRNAs and 8 h later cells were transfected with the HR-Donor and I-SceI expression plasmids. After ~72 h, cells were trypsinised and collected in 1% PBS/BSA.

Instrument

Samples were sorted using a BD LSRSorter cell analyser (BD Biosciences).

Software

The data was collected and analysed using FlowJo (BD Inc, USA).

Cell population abundance

Cell sorting was not necessary to evaluate homologous recombination events, and was not performed for this assay.

Gating strategy

FSC/SSC gates define single cell population. For each condition, 10,000 live cells which were successfully expressing donor (BFP) and I-SceI(IFP) were scored for GFP (HR) and mCherry (mutEJ). GFP and mCherry gates were defined using BFP/IFP negative cell population as a negative control. Each siRNA treatment was normalised to a negative control siRNA targeted to firefly luciferase.

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