METHODS

Cell lines, cell culture and synchronisations

The human cell lines HEK293T and U2OS cells (kind gift from Dr. Mounira Amor-Gueret) were cultured in DMEM (Eurobio Abcys, Courtaboeuf, France) media containing 25 mM sodium bicarbonate and 2 mM L-Glutamine supplemented with 10% heat inactive FCS (EuroBio Abcys). The BRCA2 deficient colorectal adenocarcinoma cell line DLD1 BRCA2$^{-/-}$ (Hucl, T. et al 2008) (HD 105-007) and the parental cell line DLD1 BRCA2$^{+/+}$ (HD-PAR-008) was purchased from Horizon Discovery (Cambridge, England). The cells were cultured in RPMI media containing 25 mM sodium bicarbonate and 2 mM L-Glutamine (EuroBio Abcys) supplemented with 10% heat inactive FCS (EuroBio Abcys). The DLD1 BRCA2$^{-/-}$ cells were maintained in growth media containing 0.1 mg/ml hygromycin B (Thermo Fisher Scientific). The stable cell lines of DLD1$^{-/-}$ BRCA2 deficient cells expressing BRCA2 WT or variants of interest generated in this study were cultured in growth media containing 0.1 mg/ml hygromycin B and 1 mg/ml G418 (Sigma-Aldrich). All cells were cultured at 37°C with 5% CO_2 in a humidified incubator and all cell lines used in this study have been regularly tested negatively for mycoplasma contamination.

For synchronization of cells in mitosis, nocodazole (100-300 ng/ml, Sigma-Aldrich) was added to the growth media and the cells were cultured for 14h before harvesting. For synchronisation by double thymidine block, the cells were treated with thymidine (2.5 mM, Sigma-Aldrich) for 17h, released for 8h followed by a second thymidine (2.5 mM) treatment for 15h.

Plasmids

2XMBP-, human 2XMBP-BRCA2$_{1-250}$ and EGFP-MBP-BRCA2 subcloning in phCMV1 expression vector were generated as described [1,2]. In the case of 2XMBP and 2XMBP-BRCA2$_{1-250}$, a tandem of 2 nuclear localization signals from RAD51 sequence was added downstream the MBP-tag.

Point mutations (M192T, S193A, S196N, S206C, T200K and T207A) were introduced in the 2xMBP-BRCA2$_{1-250}$, EGFP-MBP-BRCA2 vector using QuikChange II and QuikChange XL site-directed mutagenesis kit (Agilent Technologies), respectively (see Supplementary information, Table S2 for primer sequences).

For expression of BRCA2$_{190-283}$ in bacteria, the human BRCA2$_{190-283}$ was amplified by PCR using full length BRCA2 as template (phCMV1-2xMBP-BRCA2). The PCR product was purified and digested with *BamH1* and *SalI* and cloned into in the pGEX-6P-1 vector (GE Healthcare) to generate GST-BRCA2$_{190-283}$. The point mutations (M192T, T200K and T207A) were introduced in the same way as for 2xMBP-BRCA2$_{1-250}$ and the EGFP-MBP-BRCA2. The introduction of the point mutations was verified by sequencing (see Supplementary information, Table S3 for sequencing primers).

The *PLK1* cDNA (Addgene pTK24) was cloned into the pFast-Bac HT vector using Gibson assembly (NEB) (see Table S5 for primer sequences). To produce PLK1-KD, the point mutation K82R was introduced in the pFast-Bac HT-PLK1 vector using QuikChange XL site-directed mutagenesis kit (Agilent Technologies), see Table S3 for primer sequences.

The Polo-like binding domain (PBD) of PLK1 (amino acid 326 to amino acid 603) was amplified from the pTK24 plasmid (Addgene) and cloned into a pT7-His6-SUMO

expression vector using Gibson assembly (NEB) (see Table S6 for primer sequences). A plasmid containing a smaller PLK1 PBD fragment (amino acid 365 to amino acid 603) with a N-terminal GST tag was a kind gift from Dr. Anne Houdusse (Institute Curie, Paris).

Expression and purification of 2xMBP-BRCA2$_{1-250}$

The 2xMBP-BRCA2$_{1-250}$ was purified as previously described[1]. Briefly, ten 150 mm plates of HEK293T were transient transfected with the 2xMBP-BRCA2$_{1-250}$ using TurboFect (Thermo Fisher Scientific). The cells were harvested 30 h post transfection, lysed in lysis buffer H (50 mM HEPES (pH 7.5), 250 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM DTT, 1 mM PMSF and EDTA-free Protease Inhibitor Cocktail (Roche)) and incubated with amylose resin (NEB) for 3h at 4°C. The 2xMBP-BRCA2$_{1-250}$ was eluted with 10 mM maltose. The eluate was further purified with Bio-Rex 70 cation-exchange resin (Bio-Rad) by NaCl step elution. The size and purity of the final fractions were analysed by SDS-PAGE and western blotting using anti-MBP antibody. The 2xMBP-BRCA2$_{1-250}$ fragments containing the BRCA2 variants (M192T, S193A, S196N, T200K, S206C and T207A) were purified following the same protocol as for WT 2xMBP-BRCA2$_{1-250}$.

Expression and purification of BRCA2$_{190-283}$ for NMR

Recombinant ^{15}N-labelled (WT, T200K, T207A,) and ^{15}N/^{13}C-labelled (WT, T207A) BRCA2$_{190-283}$ were produced by transforming *Escherichia coli* BL21 (DE3) Star cells (Protein Expression and Purification Core Facility, Institut Curie) with the pGEX-6P-1 vector containing human BRCA2$_{190-283}$ (WT and the variants) following standard heat-shock transformation protocols. Cells were grown in a M9 medium containing 0.5 g/l

$^{15}NH_4Cl$ and 2 g/l ^{13}C-glucose when ^{13}C labelling was needed. The bacterial culture was induced with 1 mM IPTG at an OD_{600} of 0.8, and it was further incubated for 3 h at 37°C. Harvested cells were resuspended in buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM DTT, 1 mM EDTA) with 5 % glycerol, 10% Triton X-100, 1 mM PMSF and protease inhibitors cocktail (Roche) and disrupted by sonication. Clarified cell lysate was loaded onto Glutathione (GSH) Sepharose beads (GE Healthcare) equilibrated with buffer A. After 2 h of incubation at room temperature, beads were washed with buffer A and eluted with buffer A containing 20 mM reduced glutathione. The tag was cleaved by the precision protease during an overnight dialysis at 4°C against buffer B (50 mM HEPES pH 7.0, 1 mM EDTA) with 2 mM DTT and 150 mM NaCl. The cleaved GST-tag was removed by heating the sample for 15 min at 95°C and spun it down for 10 min at 16,000 x *g*. Sample concentration was calculated using its estimated molecular extinction coefficient of 10,363 M^{-1} cm^{-1} at 280 nm. The protein sample was characterized for folding using NMR HSQC spectra, before and after the heating at 95°C. $BRCA2_{190-283}$ was dialyzed overnight at 4°C against buffer B with 2 mM DTT.

Expression and purification of PLK1 and PLK1-kinase dead (PLK1-KD)

The recombinant 6xHis-PLK1 and 6xHis-PLK1-K82R mutant (PLK1-KD) were produced in sf9 insect cells by infection for 48h (28°C, 110 rpm shaking) with the recombinant baculovirus (PLK1-pFast-Bac HT vector). Infected cells were collected by centrifugation (1300 rpm, 10 min, 4°C), washed with 1xPBS, resuspended in lysis buffer (1xPBS, 350 mM NaCl, 1% Triton X-100, 10% glycerol, EDTA-free Protease Inhibitor Cocktail (Roche), 30 mM imidazole). After 1h rotation at 4°C the lysate was centrifuged (25000 rpm, 1h, 4°C) and the supernatant was collected, filtered (0.4 µm)

and loaded immediately onto a Ni-NTA column (Macherey Nagel) equilibrated with Buffer A1 (1xPBS with 350 mM NaCl, 10% glycerol and 30 mM imidazole, the column was washed with buffer A2 (1xPBS with 10% glycerol) and the protein was eluted with Buffer B1 (1x PBS with 10% glycerol and 250 mM imidazole). The eluted protein was diluted to 50 mM NaCl with Buffer A before being loaded onto a cationic exchange Capto S column (GE Healthcare) equilibrated with Buffer A1cex (50 mM HEPES (pH 7.4), 50 mM NaCl and 10% glycerol), the column was washed with Buffer A1cex before elution with Buffer B1cex (50 mM HEPES (pH 7.4), 2M NaCl and 10% glycerol). The quality of the purified protein was analysed by SDS-PAGE and the proteins concentration was determined using Bradford protocol with BSA as standard. The purest fractions were pooled and dialyzed against storage buffer (50 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.25 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM PMSF and 25% Glycerol) and stored in -80°C. The purified proteins can be seen in Figure S10a.

Expression and purification of PLK1$_{PBD}$

The pT7-6His-Sumo-PLK1 PBD (326-603) plasmid was expressed in Tuner pLacI pRare cells (Protein Expression and Purification Core Facility, Institut Curie), 2L of TB medium with Kanamycin and Chloramphenicol antibiotics were inoculated with cells from the pre-culture. The cells were grown at 37°C until an OD_{600} of ~ 0.85. The temperature was decreased to 20°C and the expression was induced by 1mM IPTG overnight. The cells were harvested by 15 min of centrifugation at 4690 *x g*, at 4 °C. The cell pellets were suspended in 80 ml of 1 x PBS, pH 7.4, 150 mM NaCl, 10% glycerol, EDTA-free Protease Inhibitor Cocktail (Roche), 5 mM β-mercapto-ethanol (β-ME). The suspension was treated with benzonase nuclease and $MgCl_2$ at 1 mM

final concentration for 20 min at 4°C. The suspension was lysed by disintegration at 2 kbar (Cell distruptor T75, Cell D) followed by centrifugation at 43000 *x g*, for 45 min, at 4 °C. The supernatant was loaded at 1 ml/min on a His-Trap FF-crude 5 mL column (GE Healthcare) equilibrated with PBS buffer, pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM β-ME (A) and 20 mM imidazole. The proteins were eluted in a linear gradient from 0 to 100 % with the same buffer (A) containing 200 mM imidazole, over 10 column volumes (CV). The purest fractions were pooled and dialyzed (8 kDa cut-off) against 20 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 5 mM β-ME, 10% glycerol at 4 °C. 6xHis-SUMO Protease (Protein Expression and Purification Core Facility, Institut Curie) was added at 1/100 (w/w) and incubated overnight at 4 °C to cleave the 6His-SUMO tag. The cleaved PBD-PLK1 was purified using Ni-NTA agarose resin (Macherey Nagel), washed with the following buffer: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 5mM β-ME and 10 % glycerol. The sample was incubated with the resin for 1h at 4 °C and the flow-through was collected. The sample was concentrated on an Amicon Ultra Centrifugal Filter Unit (10 kDa cut-off) and injected at 0.5 mLl/min on a Hi-Load 16/60 Superdex column (GE healthcare), equilibrated with 20 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 5 mM β-ME. The protein concentration was estimated by spectrophotometric measurement of absorbance at 280 nm. The purified protein is shown in Figure S10a.

The GST-tagged $PLK1_{PBD}$ (365-603) was expressed in *E. coli* BL21 (DE3) STAR cells, induced with 0.5 mM IPTG at an OD_{600} of 0.6, and grown at 37 °C for 3h. The PBD (365-603) was purified by glutathione affinity chromatography. After GST cleavage (using a 6His-TEV protease), the tag and the protease were retained using GST- and NiNTA-agarose affinity chromatography, and the PBD collected in the flow-

through was further purified by gel filtration chromatography. The protein was dialyzed against a buffer containing 50 mM Tris, pH 8, NaCl 150 mM, and 5 mM β-ME.

***In vitro* PLK1 kinase assay**

0.5 µg purified 2xMBP-BRCA2$_{1-250}$ or 25 ng RAD51 protein, was phosphorylated by 0.1 µg recombinant active PLK1 or PLK1-kinase dead (PLK1-KD) (purchased from Abcam or purified from sf9 insect cells as detailed above, see Figure S10b for the comparison of the kinase activity of both PLK1 preparations) in kinase buffer (25 mM HEPES, pH 7.6, 25 mM ß-glycerophosphate, 10 mM $MgCl_2$, 2 mM EDTA, 2mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, 10 µM ATP and 1 µCi [γ^{32}P] ATP (Perkin Elmer)) in a 25 µl total reaction volume. After 30 min incubation at 30°C the reaction was stopped by heating at 95°C for 5 min in SDS-PAGE sample loading buffer. The samples were resolved by 7.5 % SDS-PAGE and [γ^{32}P] ATP labelled bands were analysed with PhosphorImager (Amersham Bioscience) using ImageQuant^{TM} TL software (GE Healthcare Life Science). To control for the amount of substrate in the kinase reaction, before adding [γ^{32}P] ATP, half of the reaction was loaded on a 7.5 % stain free SDS-PAGE gel (BioRad), the protein bands were visualized with ChemiDoc XRS+ System (BioRad) and quantified by Image Lab^{TM} 5.2.1 Software (BioRad). The relative phosphorylation of 2xMBP-BRCA2$_{1-250}$ was quantified as ^{32}P-labelled 2xMBP-BRCA2$_{1-250}$ (ImageQuant^{TM} TL software) divided by the intensity of the 2xMBP-BRCA2$_{1-250}$ band in the SDS-PAGE gel (Image Lab^{TM} 5.2.1 Software). In the control experiment where PLK1 inhibitor was used, 50 nM Bi2536 (Selleckchem) was added to the kinase buffer.

In vitro protein binding assay

To assess the interaction between recombinant PLK1 and $BRCA2_{1-250}$ after phosphorylation by PLK1, a kinase assay was performed with 0.2 µg recombinant PLK1 or PLK1-kinase dead (PLK1-KD) and 0.5 µg purified 2xMBP-$BRCA2_{1-250}$ (WT or the VUS T207) in kinase buffer supplemented with 250 µM ATP (no [γ^{32}P] ATP) in a total reaction volume of 20 µl. After 30 minutes incubation at 30°C, 15 µl amylose beads was added to the reaction and incubated for 1h at 4°C. The beads were centrifuged at 2000 x g for 2 minutes at 4°C and the unbound fraction was collected before the beads were washed three time in kinase buffer (no ATP) containing 0.5% NP-40 and 0.1% Triton X-100. Bound proteins were eluted from the beads with 10 mM maltose, protein complexes were separated by SDS-PAGE and analysed by western blotting. To control for the amount of proteins in the reaction, 2 µl of the kinase reaction (before adding the amylose beads) was loaded as input.

NMR spectroscopy

NMR experiments were carried out at 283 K on 600 and 700 MHz Bruker spectrometers equipped with a triple resonance cryoprobe. For NMR signal assignments, standard 3D triple resonance NMR experiments were recorded on $BRCA2_{190-283}$ WT and T207A. Analyses of these experiments provided backbone resonance assignment for the non-phosphorylated and phosphorylated forms of these BRCA2 fragments. To follow the PLK1 phosphorylation kinetics, ^{1}H-^{15}N SOFAST-HSQC experiments[3] were recorded at each time point. These experiments were performed using 2048 X 256 time points, 64 scans and an interscan delay of 80 ms. Data processing and analysis were carried out using Topspin and CcpNmr Analysis 2.4.2 softwares.

Analysis of phosphorylation assays followed by NMR

In the HSQC spectra, the intensity of peaks of each phosphorylated residue (pT207, pT226, pT219, pS193) as well as the intensity of peaks corresponding to their non-phosphorylated form was retrieved at each time point of the kinetics. In order to estimate the fraction of phosphorylation for each residue at each point, the function $Intensity_{(phospho)} = f[Intensity_{(non\text{-}phospho)}]$ was drawn for each residue, the trendline was extrapolated to determine the intensity corresponding to the 100% phosphorylated residue and then the percentage of phosphorylation could be calculated at each time point by dividing peak intensities corresponding to the phosphorylated residue by the calculated intensity at 100% phosphorylation. Peaks corresponding to residues closed to a phosphorylated residue (L209 and V211 for pT207; A227, K230, V229 and Y232 for pT226; F221, E218 and A216 for pT219; D191, S197 and S195 for pS193) and thus affected by this phosphorylation were also treated using the same protocol and they were used to obtain a final averaged curve of the evolution of the percentage of phosphorylation at positions 193, 207, 219 and 226 with time.

Isothermal Titration Calorimetry

ITC measurements were performed with the PLK1 PBD protein (amino acid 326 to amino acid 603) and BRCA2 peptides in 50 mM Tris-HCl buffer, pH 8.0 containing 150 mM NaCl and 5 mM β-ME, using a VP-ITC instrument (Malvern), at 293 K. We used automatic injections of 8 or 10 µl. The titration data were analyzed using the program Origin 7.0 (OriginLab) and fitted to a one-site binding model. To evaluate the heat of dilution, control experiments were done with peptide or protein solutions injected into the buffer. The peptides used for the ITC experiments were synthesized by GeneCust (Ellange, LU) or Genscript (Piscataway, NY). The peptides were

acetylated and amidated at the N-terminal and C-terminal ends, respectively (see Table S8 for peptide sequences). Only peptide $BRCA2_{190-283}$ was expressed in bacteria and purified as detailed above (see "Expression and purification of $BRCA2_{190-283}$ for NMR" section).

Crystallization and Structure Determination

The purified PBD protein (amino acid 365 to amino acid 603) was concentrated to 6 mg/ml, and mixed to the ^{194}WSSSLATPPTLSS{pT}VLI^{210} (pT207) BRCA2 peptide at a 3:1 molar ratio. The crystals were obtained by hanging drop vapor diffusion method at room temperature (293 K), by mixing 1µl of complex with 1µl of solution containing 10% PEG 3350, 100 mM BisTris pH 6.5, and 5 mM DTT. Diffraction data were collected at the Proxima 1 beamline (SOLEIL synchrotron, Gif-sur-Yvette, France). The dataset was indexed and integrated using XDS[4] through the autoPROC package[5]. The software performs an anisotropic cut-off (Tickle *et al*., STARANISO (2018) Global Phasing Ltd.) of merged intensity data, a Bayesian estimation of the structure amplitudes, and applies an anisotropic correction to the data. The structure was solved by molecular replacement using PHENIX (Phaser) software[6]. Two molecules of PBD were consecutively positioned. Electron density for the peptide was clearly visible in the position previously reported in other PBD structures in complex with phosphorylated peptides (PDB 4O56 or 3P35). Refinement was performed using BUSTER[57] and PHENIX[8]. The model was built with Coot[9]. A summary of crystallographic statistics is shown in Table S1. The figures were prepared using Pymol v.1.7.4.0 (Schrödinger, LLC).

Generation of stable DLD1 clones

For generation of DLD1 BRCA2$^{-/-}$ cell lines stably expressing human BRCA2 variants of interest, we transfected one 100 mm plate of DLD1 BRCA2$^{-/-}$ cells at 70% of confluence with 10 µg of a plasmid containing human EGFP-MBP-tagged BRCA2 cDNA (corresponding to accession number NM_000059) using TurboFect (Thermo Fisher Scientific), 48h post-transfection the cells were serial diluted and cultured in media containing 1 mg/ml G418 (Sigma-Aldrich) for selection. Single cells were isolated and expanded. To verify and select the clones, cells were resuspended in cold lysis buffer H (50 mM HEPES (pH 7.5), 250 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM DTT, 1 mM PMSF and EDTA-free Protease Inhibitor Cocktail (Roche)), incubated on ice for 30 min, sonicated and centrifuged at 10,000 x *g* for 15 min, 100 µg total protein lysate was run on a 4-15% SDS-PAGE followed by immunoblotting using BRCA2 and GFP antibodies to detect EGFP-MBP-BRCA2. Clones with similar expression levels were selected for functional studies.

The presence of the point mutations in the genome of the clones was confirmed by extraction of genomic DNA using Quick-DNA^{TM} Universal Kit (ZYMO Research) followed by amplification of the N-terminal of BRCA2 (aa 1-267) by PCR using a forward primers that binds to the end of MBP and a reverse primer that binds to amino acid 267 in BRCA2, the presence of the point mutations was confirmed by sequencing of the PCR product (see Supplementary information, Table S8 for primer sequences).

Cell extracts, immunoprecipitation and western blotting

For the interaction between BRCA2$_{1-250}$ and endogenous PLK1, U2OS cells were transfected with 2xMBP-BRCA2$_{1-250}$ construct (WT, M192T, S193A, S196N, T200K, S206C, and T207A) using TurboFect (Thermo Fisher Scientific), 30 h post-

transfection cells were synchronized by nocodazole (300 ng/ml), harvested and lysed in extraction buffer A (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% NP40, 2 mM EGTA, 1.5 mM $MgCl_2$, 50 mM NaF, 10 % glycerol, 1 mM Na_3VO_4, 20 mM ß-glycerophosphate, 1 mM DTT and EDTA-free Protease Inhibitor Cocktail (Roche)). After centrifugation at 18,000 x *g* for 15 min, the supernatant was incubated with amylose resin (NEB) for 1.5h at 4°C. The beads were washed five times in extraction buffer before elution with 10 mM maltose. Bound proteins were separated by SDS-PAGE and analysed by western blotting. Where PLK1 inhibitor was used, the cells were synchronized in mitosis by nocodazole (14h) followed by 2h treatment with either 50-100 nM Bi2536 (Selleckchem) or 50 µM BTO-1 (Sigma-Aldrich) before harvesting. The cells were lysed in extraction buffer, pre-cleared by centrifugation and total protein lysate was separated by SDS-PAGE and analysed by western blotting. For the pull-down experiments after exposure to IR, U2OS cells were transient transfected with 2xMBP-$BRCA2_{1-250}$ WT construct, and exposed to IR (6 Gy) using a ^{137}Cs source after 14h treatment with nocodazole. The cells were immediately harvested and lysed (as described above) after irradiation.

For BUBR1 and pBUBR1 levels in mitosis, nocodazole (100 ng/ml) treated DLD1 $BRCA2^{-/-}$ clones were lysed in extraction buffer A, pre-cleared by centrifugation and total protein lysate was separated by SDS-PAGE and analysed by western blotting. Where PLK1 inhibitor was used, the cells were synchronized in mitosis by nocodazole (100 ng/ml) for 14h followed by 2h treatment with either 50 nM Bi2536 (Selleckchem) or 50 µM BTO-1 (Sigma-Aldrich) before harvesting.

For analysis of the tetrameric-protein complex BRCA2-pBUBR1-PP2A-B56α-PLK1 in mitosis, DLD1 $BRCA2^{-/-}$ stable clones were synchronized with nocodazole, harvested and lysed in extraction buffer A. The lysate were pre-cleared by centrifugation before

incubation with GFP-TRAP beads (Chromotek) for 2h at 4°C to pull-down EGFP-MBP-BRCA2. Around 3 mg total protein lysate was used per pull-down. The beads were washed 5 times in extraction buffer and 2 times in extraction buffer with 500 mM NaCl. Bound proteins were eluted by boiling the samples for 4 min in 3x SDS-PAGE sample loading buffer (SB), eluted proteins were separated by SDS-PAGE and analysed by western blotting using anti-mouse PLK1, anti-rabbit pT680-BUBR1, mouse anti-PP2A C and mouse anti-BRCA2 (OP95) antibodies.

Antibodies used for western blotting

mouse anti-MBP (1:5000, R29, Cat. #MA5-14122, Thermo Fisher Scientific), mouse anti-BRCA2 (1:1000, OP95, EMD Millipore), rabbit anti-GFP (1:5000, Protein Expression and Purification Core Facility, Institut Curie), mouse anti-PLK1 (1:5000, clone 35-206, Cat. #05-844, EMD Millipore), mouse anti-BUBR1 (1:1000, Cat. #612502, BD Transduction Laboratories), rabbit anti-BUBR1 (1:2000, Cat. #A300-386A, Bethyl Laboratories), mouse anti-PP2A C subunit (1:1000, clone 1D6, Cat. #05-421, EMD Millipore), rabbit anti-CDC25C (1:1000, 5H9, Cat. #4688, Cell Signaling Technology), rabbit anti-pT680-BUBR1 (1:1000, EPR 19958, Cat. #ab200061, Abcam) and rabbit anti-pS676-BUBR1 (1:1000, R193, kind gift from Dr. Erich A Nigg). Horseradish peroxidase (HRP) conjugated 2nd antibodies used: mouse-IgGκ BP-HRP (IB: 1:10 000, Cat. #sc-516102, Santa Cruz), goat anti-rabbit IgG-HRP (IB: 1:5000, Cat. #sc-2054, Santa Cruz), goat anti-mouse IgG-HRP (1:10 000, Cat.# 115-035-003, Interchim), goat anti-rabbit IgG-HRP (1:10 000, Interchim, Cat.# 111-035-003).

Phosphatase treatment

DLD1 BRCA2$^{-/-}$ cells stably expressing EGFP-MBP-BRCA2 WT were synchronized in mitosis by nocodazole (14h), harvested, lysed in extraction buffer A without phosphatase inhibitors (NaF, Na_3VO_4 and ß-glycerophosphate), and pre-cleared by centrifugation. Increased amount (0-20U) of FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific Cat. #EF0654) was added to 15 µg of total protein lysate in FastAP Buffer in a total reaction volume of 60 µl. After 1h incubation at 37°C the reaction was stopped by heating at 95°C for 5 min in SDS-PAGE sample loading buffer, 30 µl of the reaction was loaded on a 4-15 % SDS-PAGE gel, the gel was transferred onto nitrocellulose membrane and the levels of pT680-BUBR1 and CDC25C were analysed by western blotting.

Cell survival and viability assays

For clonogenic survival assay, DLD1 BRCA2$^{-/-}$ cells stably expressing full-length GFPMBP-BRCA2 and the variants (S206C and T207A) were treated at 70% of confluence with Mitomycin C (Sigma-Aldrich) at concentrations: 0, 0.5, 1.0 and 2.5 µM. After 1 h drug treatment the cells were serial diluted in normal growth media containing penicillin/streptomycin (Eurobio) and seeded in triplicates into 6-well plates. The media was changed every third day, after 10-12 days in culture the plates were stained with crystal violet, colonies were counted and the surviving fraction was determined for each drug concentration.

Cell viability was assessed with 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, #M5655, Sigma Aldrich) after treatment with MMC and the PARP inhibitor Olaparib (AZD2281, Ku-0059436, #S1060, Selleckchem). For MMC, the cells were plated in triplicates in 96-well microplates (3000-5000 cells/well) the day

before treatment. The cells were washed once in PBS before addition of serum-free media containing MMC at the concentrations: 0, 1.0 and 2.5 μM. After 1h treatment the cells were washed once in PBS and incubated for 72h in normal growth media before the viability was measured by MTT assay. For PARP inhibition, the cells were seeded 4h before 4-days treatment in normal growth media with Olaparib at concentrations: 0, 2.5 and 5.0 μM.

Cell cycle analysis and mitotic index

DLD1 BRCA2$^{-/-}$ cells stably expressing full-length GFPMBP-BRCA2 and the variants (S206C and T207A) were nocodazole (100 ng/ml, Sigma-Aldrich) or mock treated for 14h, trypsinized, washed in PBS and fixed in cold 70% ethanol at -20ºC overnight. The cells were washed twice in cold 1 x PBS before staining with rabbit anti-histone3 (phosphor-Ser10) antibody (1:200, Cat. #06-570, EMD Millipore) in staining buffer (1 x PBS, 3% FBS) for 1h in room temperature. The cells were then washed with 1 x PBS before staining for 30 min at room temperature with a chicken anti-rabbit Alexa-Fluor-647-conjugated antibody (1:250, Cat. #A-21443, Life Technologies). After one wash in 1 x PBS the cells were resuspended in 7-AAD (559925, BD Pharmingen) and diluted in staining buffer.

For the analysis of S-phase tetraploid cells in the DLD1 BRCA2$^{-/-}$ stable clones, the cells were incubated with 10 μM BrdU for 20 minutes before they were harvest, fixed and stained for cell cycle analysis using a APC BrdU flow kit (BD Bioscience, Cat. #552598) following the manufacturer's instructions.

Labelled cells were analysed on a BD FACSCanto II (BD Bioscience) using FACSDiva software and data were analysed with FlowJo 10.4.2 software (Tree Star Inc.).

Immunofluorescence

DLD1 BRCA2$^{-/-}$ cells stably expressing full-length EGFP-MBP-BRCA2 and the variants (S206C and T207A) were seeded on coverslips in 6-well tissue culture plates and synchronized in mitosis. For analysis of chromosome alignment, the cells were synchronized by double thymidine block, released for 9h followed by treatment with monastrol (100 µM, Sigma-Aldrich) for 16h before the proteasome inhibitor MG-132 (10 µM, Sigma-Aldrich) was added to the cells for an incubation of 1h. For the monastrol wash-out experiment (Figure S7a), the cells were washed twice in PBS to remove the monastrol before adding the MG-132. For chromosome segregation analysis, the cells were synchronized by double thymidine block and released in normal growth media for 11h. The cells were fixed with 100% methanol for 15 min at -20ºC, rinsed once in PBS before permeabilization with PBS containing 0.1% Triton-X for 15 min at room temperature. Nonspecific epitope binding was blocked with 4% BSA (Sigma-Aldrich) in PBS. The coverslips were rinsed in PBS, incubated with primary antibody diluted in PBS containing 0.1% Tween-20 (PBS-T) and 5% BSA for 1h at room temperature. After incubation, the coverslips were washed three times of 5 min in PBS-T before being incubated for 1h at room temperature with respective Alexa Fluor conjugated secondary antibody diluted in PBS-T with 5% BSA. The coverslips were washed two times of 5 min each in PBS-T followed by one rinse in PBS before being mounted on microscope slides.

For aneuploidy analysis the cells were treated with nocodazole for 14h (0.1 µg/ml) to enable chromosome spread; the cells were rinsed in PBS, incubated for 10 min with KCl (50 mM) at room temperature before they were spread on coverslips at 900 rpm

for 5 min in a Cytospin 4 (Thermo Scientific). The cells were fixed with 3% paraformaldehyde (PFA) in PBS for 20 min followed by 15 min permeabilization in PBS containing 0.1% Triton X-100. The coverslips were rinsed three times in PBS, blocked with 5% BSA in PBS before incubation with primary antibodies diluted in PBS over night at 4°C. After incubation the coverslips were washed three times of 5 min in PBS before 1h incubation at room temperature with respective Alexa Fluor conjugated secondary antibody diluted in PBS. After three washes of 5 min in PBS the coverslips were mounted on microscope slides.

For staining of PLK1 and PP2A-B56 at the kinetochore, the cells were seeded on coverslips and treated with nocodazole (0.25 µg/ml) for 4h, fixed with 4% PFA in PBS containing 0.5% Triton X-100 for 20 minutes at room temperature. The coverslips were rinsed three times in PBS-T and blocked for 30 minutes with 4% BSA in PBS before incubation with primary antibodies diluted in PBS-T with 5% BSA over night at 4°C. After three washes of 5 minutes in PBS-T the coverslips were incubated for 2h incubation at room temperature with respective Alexa Fluor conjugated secondary antibody diluted in PBS-T with 5% BSA. After two washes of 5 min in PBS-T and one rinse in PBS the coverslips were mounted on microscope slides.

For analysis of γH2AX and RAD51 foci, the cells were seeded on coverslips the day before 6 Gy γ–irradiation (GSR D1, Cs-137 irradiator). Two hours after irradiation, the coverslips were washed twice in PBS followed by one wash in CSK Buffer (10 mM PIPES, pH 6.8, 0.1 M NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, EDTA-free Protease Inhibitor Cocktail (Roche)). The cells were permeabilized for 5 minutes at room temperature in CSK buffer containing 0.5% Triton X-100 (CSK-T) followed by one rinse in CSK buffer and one rinse in PBS before fixation for 20 minutes at room

temperature with 2% PFA in PBS. After one rinse in PBS and one in PBS-T, the cells were blocked for 5 minutes at room temperature with 5% BSA in PBS-T before incubation for 2h at room temperature with primary antibodies diluted in PBS-T with 5% BSA. After primary antibody incubation, the coverslips were rinsed in PBS-T followed by two washes of 10 minutes in PBS-T and blocked for 5 minutes at room temperature with 5% BSA in PBS-T before incubation for 1h at room temperature with respective Alexa Fluor conjugated secondary antibody diluted in PBS-T with 5% BSA. After one rinse in PBS-T and two washes of 10 minutes in PBS-T the coverslips were rinsed in PBS before being mounted on microscope slides.
All coverslips were mounted on microscope slides with ProLong Diamond Antifade Mountant with DAPI (Cat. #P36966, Thermo Fisher Scientific).

For analysis of chromosome alignment and segregation, images were acquired in an upright Leica DM6000B wide-field microscope equipped with a Leica Plan Apo 63x NA 1.4 oil immersion objective. The camera used is a Hamamatsu Flash 4.0 sCMOS controlled with MetaMorph2.1 software (Molecular Devices). For Figures 6c and 7b, 7 to 20 Z-stacks were taken at 0.2 µm intervals to generate a maximal intensity projection image using ImageJ. For aneuploidy and kinetochore localization analysis, images were acquired in an inverted confocal Leica SP5 microscope with a plan Apo 63x NA 1.4 oil immersion objective. For Figure 7b, Z-stacks were taken at 0.13 µm intervals to generate a maximal intensity projection image using ImageJ. For the counting of chromosomes in the aneuploidy experiment, the quantification was performed in zoomed areas counting the CREST signal in separated stacks to ensure the counting of all chromosomes. We were able to count up to 65 chromosomes with certainty, thus >65 CREST signals were discarded and not included in the analysis. For the analysis of γH2AX and RAD51 foci, 20 Z-

stacks were taken at 0.2 µm intervals to generate a maximal intensity projection using Image J. γH2AX and RAD51 foci per nucleus were counted by a customized macro using a semi-automated procedure; the nucleus was defined by an auto-threshold on DAPI, a mask was generated and applied onto the Z-projection to only count foci within the nucleus. For the definition of foci we applied the Plug In Find Maxima (ImageJ).

Antibodies used for immunofluorescence

human anti-CREST (1:100, Cat. #15-234-0001, Antibodies Online), mouse anti-PP2A C subunit (1:500, Clone 1D6, Cat. #05-421, EMD Millipore), mouse anti-PLK1 (1;500, clone F-8, Santa Cruz Biotechnology, Cat. #sc-17783), anti-pT680-BUBR1 (1:100, clone EPR 19958, Abcam, Cat. #ab200061), mouse anti-pSer139-γH2AX (1:1000, clone JBW301, EMD-Millipore, Cat. #05-636), rabbit anti-RAD51 (1:100, clone H-92, Santa Cruz Biotechnology, Cat. #sc-8349) and mouse anti-α-tubulin (1:5000, GT114, Cat. #GTX628802, Euromedex). Alexa Fluor conjugated secondary antibodies used: goat anti-human Alexa-488 (1:1000, Cat. # A11013, Life Technologies), donkey anti-mouse Alexa-594 (1:1000, Cat. #A-21203, Thermo Fisher Scientific), donkey anti-rabbit Alexa-488 (1:1000, Cat. #A-21206, Thermo Fisher Scientific), goat anti-human Alexa-555 (1:1000, Cat. #A-21433, Thermo Fisher Scientific), donkey anti-mouse Alexa-488 (1:1000, Cat. #A-21202, Thermo Fisher Scientific).

Time-lapse video microscopy of mitotic cells

For phase-contrast video-microscopy DLD1 BRCA2$^{-/-}$ cells stably expressing full-length GFPMBP-BRCA2 and the variants (S206C and T207A) were seeded in Ibidi 4 well µ-slide with glass bottom (Ibidi, Cat. #80427), synchronized by double thymidine block. At the time of release from the second block, the cells were washed twice in PBS, released and cultured for 7h in phenol red free culture media before the filming was started. To stain the DNA, 50 nM SiR-DNA probe (far red absorption) and 3 µM verapamil (SiR-DNA Kit, Cat. #SC007, Spirochrome) were added to the culture media at the time of release from the second block. The cells were imaged for 14h every 2 min, at 40x dry objective using an inverted spinning-disk microscope (Ti-E, Nikon and Yokogawa CSU-X1 spinning head) equipped with sCMOS Hamamatsu Orca Flash 4.0 (pixel size 6.5 µm) camera controlled by Metamorph software (Molecular Devices). Images were mounted using Image J software (1.51s, NIH).

Statistical analysis

In all graphs error bars represent the standard deviation (SD) from at least three independent experiments unless otherwise stated. Statistical significance of differences was calculated with unpaired two-tailed t-test, one/two-way ANOVA with Tukey's multiple comparisons test or Mann-Whitney two-tailed test as indicated in the figure legends. All analyses were conducted using GraphPad Prism (version Mac OS X 7.0b).

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