Supplementary information

Materials and Methods Tables S1-S9, Figure legends S1-S10

Materials and Methods

Table S1: Number of records in ClinVar and BRCAShare data bases of the variants identified in breast cancer patients altering the amino acids investigated in this work. The specific VUS utilized in this study are highlighted in bold.

VUS	ClinVar	BRCAShare
S196I	4	0
S196N	4	3
S196T	1	0
T200A	2	0
T200I	2	4
T200K	0	1
S206C	1	1
S206Y	1	0
T207A	3	0
T207I	4	0

Table S2: Statistics table for the crystal structure of PBD_pT207, a complex between the Polo-Box Domain of human PLK1 (aa 365 to aa 603) and the 17aa peptide pT207 of BRCA2 (aa 194 to aa 210, threonine 207 being phosphorylated).

	PBD_T207 phosphorylated (PDB 6GY2)
Data collection	
Space group	P_1
Cell dimensions :	
a, *b*, *c* (Å)	50.000 56.040 61.030
α, β, γ (°)	80.79 79.23 65.05
Molecules per a.u	2
Resolution (Å)	59.70 – 3.106 (3.106 – 3.16)

R_{merge}	0.056 (0.346)
R_{meas}	0.076 (0.466)
R_{pim}	0.051 (0.31)
I/σ (I)	10.1 (2.3)
$CC_{1/2}$	0.997 (0.821)
Completeness (spherical, %)	93.7 (96.9)
Redundancy	1.94 (1.977)
B Wilson (Å²)	76.9 (78.86)
Multiplicity	1.9 (2.0)
Refinement	
Resolution (Å)	22.57 - 3.11 (3.11 – 3.15)
No. reflections	9911
R_{work} / R_{free}	0.189/0.215
No. Atoms	
Protein	3788
Heterogen atoms	28
Water	22
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	0.97

*Values in parentheses are for highest-resolution shell.

Table S3: Primers used to introduce point mutations in EGFPMBP-BRCA2, 2xMBP-BRCA2$_{1-250}$ and GST-BRCA2$_{190-283}$ constructs

Mutation	Oligo name	Sequence (5'-3')
S193A	Fw : oAC543	CCC ACC CTT AGT TCT GCT GTG CTC ATA GTC
	Rv : oAC544	GAC TAT GAG CAC AGC AGA ACT AAG GGT GGG
M192T	Fw : oAC283	GTGGATCCTGATACGTCTTGGTCAAGTTC
	Rv : oAC284	GA ACT TGA CCA AGA CGT ATC AGG ATC CAC
S196N	Fw : oAC026	CCTGATATGTCTTGGTCAAATTCTTTAGCTACACCACC
	Rv : oAC027	GGTGGTGTAGCTAAAGAATTTGACCAAGACATATCAGG
S206C	Fw : oAC028	CCACCCACCCTTAGTTGTACTGTGCTCATAGTCAG
	Rv : oAC029	CTGACTATGAGCACAGTACAACTAAGGGTGGGTGG
T200K	Fw : oAC285	CAA GTT CTT TAG CTA AAC CAC CCA CCC TTA G
	Rv : oAC286	CTA AGG GTG GGT GGT TTA GCT AAA GAA CTT G
T207A	Fw : oAC545	GGA TCC TGA TAT GGC TTG GTC AAG TTC TTT AGC
	Rv : oAC546	GCT AAA GAA CTT GAC CAA GCC ATA TCA GGA TCC

Table S4: Sequencing primers

Construct	Oligo name	Binding site	Sequence (5'-3')
GFPMBP-BRCA2, GST-$BRCA2_{190-283}$	Rv : oAC131	aa 273 BRCA2	TTAGTTCGACTTATCCAATGTGGTCTTT
2xMBP-$BRCA2_{1-250}$	Fw : oAC149	aa 1-6 BRCA2	TTATTTGCTAGCCCTATTGGATCCAAAGAG
PLK1	Fw : oAC907	aa 38 PLK1	AAAGAGATCCCGGAGGTCCTAGTG

Table S5: Primers used to subclone $BRCA2_{192-283}$ into the pGEX-6P-1 vector

Construct	Oligo name	Sequence (5'-3')
$BRCA2_{190-283}$	Fw: oAC130	TTAGGATCCATGTCTTGGTCAAGTTCT
	Rv: oAC131	TTAGTTCGACTTATCCAATGTGGTCTTT

Table S6: Primers used to subclone *PLK1* cDNA into pFastBac HT

Primer name	Sequence (5'-3')
GA_pFBtev_R	GCCCTGAAAATACAGGTTTTCGGTCGTTGGGAT
GA_pFB_UTR_F	TTGTCGAGAAGTACTAGAGGATCATAATCA
GA_hPLK_F	ATCCCAACGACCGAAAACCTGTATTTTCAGGGCATGAGTGCTGCAG TGACTGCA
GA_hPLK_R	TGATTATGATCCTCTAGTACTTCTCGACAATTAGGAGGCCTTGAGAC GGTT

Table S7: Primers used to introduce K82R point mutation in pFastBAC-PLK1 vector to produce PLK1-KD and to subclone $PLK1_{PBD}$ (aa 326-603) into pT7-His6-SUMO

Product	Oligo name	Sequence (5'-3')
K82R-PLK1	Fw : oAC905	GCG GGCAGGATTGTGCCTAAG
	Rv : oAC906	CTTAGGCACAATCCTGCCCGC
$PLK1_{PBD}$	GA_PLKPDB wt_F	ATTGAGGCTCACCGCGAACAGATTGGTGGCTCGATTGC TCCCAGCAGCCT
	GA_PLKPDB wt_R	TTCCTTTCGGGCTTTGTTAGCAGCCGGTCATTAGGAGG CCTTGAGACGGT

Table S8: Synthetic peptide sequences for Isothermal Titration Calorimetry (ITC) and X-ray cristallography

Peptide	Sequence
pS197	DMSWSS{pS}LAT
T207	WSSSLATPPTLSSTVLI
pT207	WSSSLATPPTLSS{pT}VLI
T207A	WSSSLATPPTLSSAVLI
CpT207	WSSSLATPPTLSC{pT}VLI

Table S9: Primers for amplifying BRCA2 (aa 1-267) from genomic DNA

Primer name	Sequence (5'-3')
Fw : OAC035	GGTCGTCAGACTGTCGATGAAGCC
Rv : OAC056	CAAAGAGAAGCTGCAAGTCATGGATTTGAAAAAACATCAGGG

Suplemmentary Figure Legends

Figure S1. Related to Figure 1. **PLK1 phosphorylation kinetics of BRCA2$_{190-283}$ and conservation of PLK1 phosphosites**

(a) Phosphorylation kinetics resulting from a duplicate experiment performed with a different PLK1 kinase aliquot on ^{15}N labelled BRCA2$_{190-283}$ (283K, 600 MHz). The conditions are the same as in Figure 2a. Error bars correspond to SD (n=2). **(b)** Alignment of the region 190-283 of BRCA2 in 30 different species. Amino acids conserved in more than 66 % of the species are highlighted with coloured background. The percentage of identity to human BRCA2 sequence is added after the species name. Arrows show the amino acids identified as phosphorylated by PLK1 in the NMR experiments. **(c)** Bar graph showing the evolutionary conservation of the amino acids phosphorylated by PLK1 detected by NMR.

Figure S2. Related to Figure 2. **PLK1 phosphorylation kinetics of WT vs mutated forms of BRCA2$_{190-283}$**

Phosphorylation kinetics resulting from a duplicate experiment performed with a different PLK1 kinase aliquot on ^{15}N labelled BRCA2$_{190-283}$ (283K, 600 MHz). The conditions are the same as in Figure 2a. Comparisons of the WT kinetics with the phosphorylation kinetics of **(a)** T207A and **(b)** T200K are displayed.

Figure S3. Related to Figure 3. **Isothermal Titration Calorimetry (ITC) thermogram showing binding of PLK1$_{PBD}$ to the fragment BRCA2$_{190-283}$ or a 10 aa BRCA2 peptide containing pS197**

Thermogram showing the binding affinity of PLK1$_{PBD}$ to the **(a)** phosphorylated or **(b)** non-phosphorylated BRCA2$_{190-283}$ fragment, purified from bacteria as explained in the

Methods section and also used in the NMR experiments. **(c)** Thermogram showing the binding affinity of $PLK1_{PBD}$ to a 10 aa BRCA2 synthetic peptide comprising pS197 (see Table S8). Unexpectedly, the affinity of pS197 for PLK1PBD is about 100 fold weaker than that of pT207 for PLK1PBD, even if the two peptides display only 3 mutations within the residues that are highly buried (> 50%) in the crystal structure of pT207 bound to $PLK1_{PBD}$ (P202 to M, L204 to W and L209 to A; see Figure 3j).

Figure S4. Related to Figures 3-8**. Effect of PLK1 inhibitors on the interaction of BRCA2-PLK1 and BRCA2 protein levels in DLD1 $BRCA2^{-/-}$ stable clones complemented with the cDNA of BRCA2 WT and variants utilized in this study.** **(a)** The effect of PLK1 inhibtors on the interaction between 2xMBP-$BRCA2_{1-250}$ and PLK1. 2xMBP-$BRCA2_{1-250}$ WT or the tag alone (2XMBP) were expressed in U2OS cells by transient transfection for 30h before the cells were treated with nocodazole (300 ng/ml) for 14h. PLK1 inhibitor (Bi2536 (50nM) or BTO (50 μM)) was added to the cells 2h before harvesting. The cells were lysed and immunoprecipitation was performed against the MBP tag using amylose beads. Complexes were resolved on 4-15% SDS-PAGE followed by western blotting using anti-PLK1 and anti-MBP antibodies. **(b)** BRCA2 protein levels in total protein extracts from the DLD1 BRCA2 deficient ($BRCA2^{-/-}$) cells stably expressing GFP-MBP-BRCA2 WT (BRCA2 WT C1) or the variants S206C (clones A7 and A9) and T207A (clones B1 and E4) as detected by western blot using anti-BRCA2 (OP95) and anti-GFP antibodies. **(b)** The effect of PLK1 inhibtors on the interaction between EGFPMBP-BRCA2 (BRCA2 WT) and PLK1. BRCA2 WT cells were treated with nocodazole (100 ng/ml) for 14h, the PLK1 inhibitor (BTO (50 μM) was added to the cells 2h before harvesting. The cells were lysed and EGFPMBP-BRCA2 was immunoprecipitated with GFP-trap beads,

immunocomplexes were resolved on 4-15% SDS-PAGE followed by western blotting, the interaction with PLK1 was revealed by anti-PLK1 and -MBP antibodies. StainFree images of the gels before transfer was used as loading control in (a-c), cropped image is shown.

Figure S5. Related to Figure 5**. Effect of PLK1 inhibitors and phosphatase treatment on pT680-BUBR1 and effect of BRCA2 variants S206C and T207A on CDC25C phosphorylation.**

(a) Protein levels of pT680-BUBR1 in BRCA2 WT stable clone after treatment with PLK1 inhibitors. After 14h culture with media containing nocodazole (100 ng/µl), PLK1 inhibitors (Bi2536 (50 nM) or BTO (50 µM)) were added to the media and the cells were cultured for additional 2h before harvesting and extraction of proteins. The levels of pT680-BUBR1 in total protein lysate was analyzed by western blot. **(b)** Phosphatase (Fast AP phosphatase) treatment of total protein lysate extracted from nocodazole (100 ng/µl) treated DLD1 BRCA2 WT stable clone, levels of pT680-BUBR1 was analyzed by western blot. For (a, b), stain-free images of the 4-15% SDS-PAGE gel before transfer were used as loading control (cropped images are shown).

Figure S6. Related to Figure 5. **Localization of PLK1 and PP2A-B56α to the kinetochores in cells bearing variant S206C**.

(a) Representative images of the localization of PLK1 in metaphase spreads from nocodazole-arrested DLD1 BRCA2$^{-/-}$ stable cell lines expressing BRCA2 WT or BRCA2-S206C, as indicated, showing its co-localization with the centromere marker

CREST. DNA is counterstained with DAPI. **(b)** Quantification of the PLK1 signal co-localizing with CREST signal in (a). **(c)** Representative images of the localization of PP2A-B56α in metaphase spreads from nocodazole-arrested DLD1 BRCA2$^{-/-}$ stable cell lines expressing BRCA2 WT or BRCA2-S206C, as indicated, showing its co-localization with the centromere marker CREST. DNA is counterstained with DAPI. **(d)** Quantification of the PP2A-B56α signal co-localizing with CREST signal in (c).

Figure S7. Related to Figure 5 and 7**. Chromosome alignement status and cell cycle profile of the BRCA2$^{-/-}$ stable clones in nocodazole versus untreated conditions.**

(a) Frequency of misaligned chromosomes outside the metaphase plate in DLD1 BRCA2 deficient cells expressing BRCA2 WT or S206C and T207A variants performed as in Figure 5e but with Monastrol washout before addition of the MG132. Statistical significance of the difference was calculated with two-way ANOVA test with Tukey's multiple comparisons test (the p-values show differences compared to WT). *n* indicates the number of total cells counted in each cell clone from a total of 2 independent experiments. **(b-c)** Representative flow cytometry plots for the analysis of p-histone 3 (S10) expression (quantified in Figure 6a) and cell cycle phase distribution (quantified in Figure 7d, e) in the stable BRCA2 WT clone after DMSO (b) or nocodazole (c) treatment. Viable cells were gated from the Forward Scatter (FSC-A) versus Side Scatter (SSC-A) plot and displayed in a FSC-W versus FSC-A plot to further exclude doublets. The singlet FSC population was displayed in a Alexa Fluor 647 (p-histone 3 (S10)) versus 7-AAD-A (DNA) plot or a histogram against 7-AAD-A (DNA). Gates are showing p-hisone 3 positive cells and, in the histogram, the

popluations in the different cell cycle phases (G1, S and G2/M). 10 000 FSC singlet events were collected for each experiment. **(d-e)** Cell cycle distribution of DLD1 BRCA2$^{-/-}$ stable clones expressing EGFP-MBP-BRCA2 WT or the VUS treated with nocodazole (d) or untreated (e). Statistical significance of differences in (d and e) was calculated with two-way ANOVA test with Tukey's multiple comparisons test (the p-values show differences compared to WT).

Figure S8. Related to Figure 7**. BrdU incorporation measured by flow cytometry of DLD1 BRCA2$^{-/-}$ stable cell lines expressing BRCA2 WT or BRCA2 variants S206C and T207A.**

(a-c) Representative flow cytometry plots for the analysis of S-phase tetraploid cells (quantified in Figure 7c) in the stable DLD1 BRCA2 deficient cells expressing BRCA2 WT (a) or the VUS S206C (b) and T207A (c). Viable cells were gated from the Forward Scatter (FSC-A) versus Side Scatter (SSC-A) plots and displayed in a 7-AAD-W versus 7-AAD-A plot to exclude doublets. The gated singlet population was displayed in a APC-A (BrdU) versus 7-AAD-A (DNA) plot. The S-phase tetraploid population was gated as BrdU^{+} cells with DNA content >4N. 20 000 singlet events were collected for each experiment. **(d)** Frequency of BrdU^{+} cells in the stable clones expressing BRCA2 WT or the VUS as indicated. The data represents the mean ± SD of three independent experiments. Statistical significance of the difference was calculated with one-way ANOVA test with Tukey's multiple comparisons test (the p value show the difference compared to WT, ns:non-significant).

Figure S9. Related to Figure 8**. Plating efficiency of unchallenged DLD1 BRCA2$^{-/-}$ stable clones expressing EGFP-MBP-BRCA2 WT or the VUS and representative images of DNA damage foci in these cells**.

(a) Representative plates showing the number of colonies in unchallenged conditions of the cells assessed for MMC-clonogenic survival assay quantified in Figure 8a (500 cells seeded). **(b)** Representative immunofluorescence images of γH2AX and RAD51 foci in BRCA2 WT and S206 A7 stable cells 2h treated or not with 6 Gy of γ-irradiation (IR), as indicated, as quantified in Figure 8d, e.

Figure S10. SDS-PAGE of the PLK1, PLK1-KD and PLK1-PBD recombinant proteins utilized in this study and comparison of the kinase activity of each batch of PLK1.

(a) SDS-PAGE showing purified PLK1, PLK1-K82R mutant (PLK1-KD) and PLK1$_{PBD}$. Human PLK1 was expressed and purified from sf9 insect cells using Ni-NTA column followed by a second purification step with a cationic exchange Capto S column. Purified PLK1 and PLK1-K82R protein (3 µg) were loaded on a 4-15% SDS-PAGE Stain-Free gel. For purification of PLK1$_{PBD,}$ 6His-Sumo-PLK1$_{PBD}$ was expressed and purified from bacteria using a His-TRAP column, the His-tag was cleaved with 6xHis-SUMO Protease and the cleaved PLK1$_{PBD}$ was further purified using Ni-NTA agarose resin. The purified protein was loaded on a 4-20% SDS-PAGE (1.4 µg) and detected by Coomassie staining. **(c)** *In vitro* kinase assay with the purified PLK1 (0.1 µg) from (a) or PLK1 purchased from Abcam, 0.1 µg PLK1 was used in the kinase reaction with either RAD51 (25 ng) or purified 2xMBP-BRCA2$_{1-250}$ WT (0.5 µg) as substrate in the presence of [γ^{32}P]-ATP. The samples were resolved by 7.5 % SDS-PAGE and ^{32}P-labeled products were detected by autoradiography.