

Proper chromosome alignment depends on BRCA2 phosphorylation by PLK1

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Summary

The BRCA2 tumor suppressor protein is involved in the maintenance of genome integrity through its role in homologous recombination. In mitosis, BRCA2 is phosphorylated by Polo-like kinase 1 (PLK1). Here we describe how this phosphorylation contributes to the control of mitosis. We identified two highly conserved phosphorylation sites at S193 and T207 of BRCA2. Phosphorylated-T207 is a *bona fide* docking site for PLK1 as illustrated by the crystal structure of the BRCA2 peptide bound to PLK1 Polo-box domain. We found that BRCA2 bound to PLK1 forms a complex with the phosphatase PP2A and phosphorylated-BUBR1. Reducing BRCA2 binding to PLK1, as observed in *BRCA2* breast cancer variants S206C and T207A, alters the tetrameric complex resulting in misaligned chromosomes, faulty chromosome segregation and aneuploidy. We thus reveal a direct role of BRCA2 in the alignment of chromosomes, distinct from its DNA repair function, with important consequences on chromosome stability. These findings may explain in part the aneuploidy observed in *BRCA2*-mutated tumors.

1
2 **Introduction**
3

4 The BRCA2 tumor suppressor protein plays an important role in DNA repair by homologous
5 recombination (HR)^{1,2} that takes place preferentially during S/G2 phases of the cell cycle³.
6 BRCA2 has also emerging functions in mitosis, for example, at the kinetochore, it forms a
7 complex with BUBR1^{4,5}, an essential factor for the faithful segregation of chromosomes that
8 is required for kinetochore-microtubule attachment and is a component of the spindle
9 assembly checkpoint (SAC)^{6,7}. These two activities of BUBR1 involve different partners and
10 are functionally distinct^{8,9}. BRCA2 has been proposed to contribute to BUBR1 SAC activity
11 through direct binding and promoting its acetylation by the acetyl transferase PCAF^{4,10},
12 although due to confounding results in the BUBR1 interaction site in BRCA2, it is unclear if
13 this interaction is direct^{4,5}. At the end of mitosis, BRCA2 localizes to the midbody and assists
14 cell division by serving as a scaffold protein for the central spindle components¹¹⁻¹³. In
15 mitosis, BRCA2 is a target of phosphorylation by PLK1 both in its N-terminal region^{14 15} and
16 in its central region¹⁵, although the functional role of these phosphorylation events remains
17 unclear.

18 PLK1 is a master regulator of the cell cycle that is upregulated in mitosis^{16,17}. Among other
19 functions, PLK1 directly binds and phosphorylates BUBR1 at several residues including the
20 attachment-sensitive site S670¹⁸ and the two tension-sensitive sites S676¹⁹ and T680²⁰ in
21 prometaphase allowing the formation of stable kinetochore-microtubule attachments. This
22 activity needs to be tightly regulated to ensure proper alignment of the chromosomes at the
23 metaphase plate^{8,9,19}. The kinase activity of Aurora B is an essential to destabilize erroneous
24 kinetochore-microtubule interactions²¹ whereas the phosphatase PP2A protects initial
25 kinetochore-microtubule interactions from excessive destabilization by Aurora B²². This
26 function is achieved through the interaction of PP2A-B56 subunit with BUBR1

27 phosphorylated at the Kinetochores Attachment and Regulatory Domain (KARD) motif
28 (including residues S670, S676 and T680)²⁰. Thus, the interplay between PLK1, BUBR1,
29 Aurora B and PP2A is necessary for the formation of stable kinetochores-microtubule
30 attachments.

31 PLK1 is recruited to specific targets via its Polo-box domain (PBD)²³. PBD interacts with
32 phosphosites characterized by the consensus motif S-[pS/pT]-P/X²⁴. These phosphosites are
33 provided by a priming phosphorylation event, usually mediated by CDK1 or other proline-
34 directed kinases¹⁷; however, there is also evidence that PLK1 itself might create docking sites
35 (“self-priming”) during cytokinesis^{25,26}.

36 Several BRCA2 sites have been suggested as phosphorylated by PLK1 in mitosis, some of
37 which belong to a cluster of serines and threonines located in BRCA2 N-terminus around
38 residue S193¹⁴. We set out to investigate which of these sites are phosphorylated by PLK1,
39 and to reveal whether these phosphorylation events play a role in the regulation of mitotic
40 progression. Here, we demonstrate that S193 and T207 are the only two residues that can be
41 phosphorylated by PLK1 in the conserved region around S193, and we further reveal that
42 phosphorylated BRCA2-T207 is a *bona fide* docking site for PLK1. By investigating the
43 phenotype of BRCA2 missense variants that limit the phosphorylation of BRCA2-T207, we
44 reveal an unexpected role for BRCA2 in the alignment of chromosomes at the metaphase
45 plate. We demonstrate that phosphorylation of BRCA2-T207 by PLK1 facilitates the
46 formation of a complex between BRCA2-PLK1-pBUBR1 and the phosphatase PP2A required
47 to counter excessive Aurora B activity at the kinetochores²⁰. A defect in this function of
48 BRCA2 manifested in chromosome misalignment, chromosome segregation errors, mitotic
49 delay and aneuploidy, leading to chromosome instability.

50

51

52 **Results**

53 ***BRCA2* variants identified in breast cancer reduce the PLK1-dependent** 54 **phosphorylation of *BRCA2* N-terminal region**

55 Several missense variants of uncertain significance (VUS) identified in *BRCA2* in breast
56 cancer patients are located in the N-terminal region predicted to be phosphorylated by PLK1
57 (around S193) (Breast information core (BIC)²⁷ and BRCAShare²⁸), summarized in
58 Supplementary table 1. To find out if any of these variants affected PLK1 phosphorylation in
59 this region, we purified fragments comprising amino acids 1 to 250 of *BRCA2* (hereafter
60 *BRCA2*₁₋₂₅₀) from human embryonic kidney cells (HEK293T) and used an *in vitro* kinase
61 assay to assess the phosphorylation by PLK1 of the fragments containing either the WT
62 sequence, the different *BRCA2* variants M192T, S196N, S206C and T207A, or the mutant
63 S193A, previously reported to reduce the phosphorylation of *BRCA2* by PLK1¹⁴. As
64 expected, S193A reduced the phosphorylation of *BRCA2*₁₋₂₅₀ by PLK1 (Fig. 1a, 1b).
65 Interestingly, variants T207A and S206C also led to a 2-fold decrease in PLK1
66 phosphorylation of *BRCA2*₁₋₂₅₀ (Fig. 1a, b). In contrast, M192T slightly increased the
67 phosphorylation above WT levels whereas S196N did not significantly modify the
68 phosphorylation of *BRCA2*₁₋₂₅₀ by PLK1 (Fig. 1a, b). The phosphorylation observed in the
69 *BRCA2* fragments is specific of the recombinant PLK1 kinase as it is PLK1 concentration
70 dependent (Supplementary Fig. 1a, b) and when replacing the PLK1-WT by a kinase-dead
71 (PLK1-KD) version of the protein (K82R)²⁹, purified using the same protocol, or adding a
72 PLK1 inhibitor (BI2536) to the reaction, the phosphorylation of *BRCA2*₁₋₂₅₀ decreases
73 significantly (Fig. 1c, lanes 4 and 5 compared to lane 3; Fig. 1d).
74 Together, these results show that VUS T207A and S206C identified in breast cancer patients
75 impair phosphorylation of *BRCA2*₁₋₂₅₀ by PLK1 *in vitro*.

76

77 **BRCA2-T207 is a target of phosphorylation by PLK1**

78 The reduction of BRCA2 phosphorylation in BRCA2₁₋₂₅₀ containing T207A and S206C
79 variants suggested that these residues could be targets for PLK1 phosphorylation. We
80 investigated this possibility by following the PLK1 phosphorylation kinetics of two
81 overlapping fragments of BRCA2 N-terminus comprising S206 and T207 (hereafter
82 BRCA2₄₈₋₂₁₈ and BRCA2₁₉₀₋₂₈₄) using Nuclear Magnetic Resonance (NMR) spectroscopy
83 (Fig. 2a). Together, these fragments cover a large N-terminal region of BRCA2 including the
84 cluster of conserved residues around S193 (from amino acid 180 to amino acid 210;
85 Supplementary Fig. 1c). NMR analysis allows residue-specific quantification of ¹⁵N-labelled
86 peptide phosphorylation. Fig. 2b shows superimposed ¹H-¹⁵N HSQC spectra of BRCA2₄₈₋₂₁₈
87 and BRCA2₁₉₀₋₂₈₄ before (black) and after (red) phosphorylation with recombinant PLK1.
88 Analysis of these experiments revealed phosphorylation of S193 and of three other
89 phosphosites, including T207, by PLK1 in the BRCA2 region from amino acid 48 to amino
90 acid 284. Interestingly, while T219 and T226 conservation is poor, T207 and S193 are
91 conserved from mammals to fishes (Supplementary Fig. 1c) suggesting that both residues are
92 important for BRCA2 function.

93

94 **BRCA2 variant T207A alters the phosphorylation kinetics by PLK1**

95 Having identified T207 as a target of phosphorylation of PLK1, we next compared the
96 residue-specific phosphorylation kinetics in the polypeptide WT BRCA2₄₈₋₂₁₈ containing the
97 variant T207A that displayed reduced overall phosphorylation (Fig. 1a, 1b). (The production
98 of a ¹⁵N-labelled recombinant fragment comprising S206C yielded an insoluble protein
99 precluding NMR analysis). Time-resolved NMR experiments revealed that PLK1
100 phosphorylates significantly less BRCA2₄₈₋₂₁₈ containing the variant T207A than the WT
101 peptide (Fig. 2c). The initial phosphorylation rate of S193 was decreased by a factor of 2 (Fig.

102 2d), and T207 was as expected not phosphorylated, being mutated into an alanine. Similar
103 results were obtained using BRCA2₁₉₀₋₂₈₄ (Supplementary Fig. 2). This NMR analysis is in
104 agreement with the *in vitro* kinase assay performed using the BRCA2₁₋₂₅₀ fragment purified
105 from human cells (Fig. 1), in which T207A reduces the phosphorylation of BRCA2₁₋₂₅₀
106 fragment by PLK1.

107

108 **Variants T207A and S206C reduce the interaction of BRCA2 and PLK1**

109 The finding that T207 is efficiently phosphorylated by PLK1 in BRCA2₄₈₋₂₁₈ and BRCA2₁₉₀₋
110 ₂₈₄ (Fig. 2b) together with the observation that T207A mutation causes a global decrease in the
111 phosphorylation of these fragments (Fig. 2c; Supplementary Fig. 2) and the prediction that
112 T207 is a docking site for PLK1_{PBD} binding²⁴ made us hypothesize that T207 might be a
113 “self-priming” phosphorylation event required for the interaction of PLK1 with BRCA2 at
114 this site. If so, the variants that reduce phosphorylation of T207 by PLK1 would be predicted
115 to alter PLK1_{PBD} binding. To test this hypothesis, we examined the interaction of PLK1 with
116 the VUS-containing polypeptides. We overexpressed 2xMBP-BRCA2₁₋₂₅₀ constructs carrying
117 these variants in U2OS cells to detect the endogenous PLK1 that co-immunoprecipitates with
118 2xMBP-BRCA2₁₋₂₅₀ using amylose pull-down. As expected, overexpressed BRCA2₁₋₂₅₀ was
119 able to interact with endogenous PLK1 from mitotic cells but not from asynchronous cells
120 (predominantly in G1/S) where the levels of PLK1 are reduced (Fig. 3a, lane 2 compared to
121 lane 1). Furthermore, the variants T207A and S206C showed a weaker interaction with PLK1
122 than the WT protein (Fig. 3a, pull-down lanes 4, 6 compared to lane 2, Fig. 3b) despite the
123 protein levels of PLK1 remaining unchanged (Fig. 3a, compare PLK1 input lanes 4 and 6 to
124 lane 2). In contrast, the effect of M192T and S196N on the interaction was mild (Fig. 3a,
125 compare pull-down lanes 10 and 12 to lane 8, Fig. 3b). These results are consistent with the
126 idea of a self-priming phosphorylation by PLK1 on T207.

127 To provide further evidence that the PLK1-mediated phosphorylation of BRCA2 favors
128 BRCA2 binding, we performed an *in vitro* kinase assay with recombinant proteins followed
129 by an amylose pull-down and eluted the bound proteins with maltose. PLK1 was found in the
130 maltose elution with BRCA2₁₋₂₅₀-WT demonstrating that PLK1-phosphorylated BRCA2₁₋₂₅₀
131 binds to PLK1 (Fig. 3c lane 4, Fig. 3d). In contrast, the fraction of PLK1 in the eluate of
132 BRCA2₁₋₂₅₀-T207A was substantially reduced (Fig. 3c, lane 8 compared to lane 4, Fig. 3d)
133 indicating that the phosphorylation of T207 is required for efficient binding to PLK1 and
134 confirming our results with cell lysates (Fig. 3a, b). Strikingly, we observed no difference in
135 PLK1 binding between BRCA2₁₋₂₅₀-WT phosphorylated by PLK1 or its kinase dead mutant
136 (PLK1-KD; Fig. 3c, lane 6 compared to lane 4, Fig. 3e) or in the absence of ATP (Fig. 3f,
137 lane 6 compared to lane 4, Fig. 3g). However, pre-incubating BRCA2₁₋₂₅₀-WT with
138 phosphatase before the addition of PLK1 resulted in a 2-fold decrease in the binding to PLK1
139 indicating that the phosphorylation of BRCA2 is required for the interaction with PLK1 (Fig.
140 3f lane 12 compared to 10, Fig. 3g).

141

142 **T207 is a *bona fide* docking site for PLK1**

143 To directly demonstrate the recognition of pT207 by PLK1, we measured the affinity of
144 recombinant PLK1_{PBD} (the target recognition domain of PLK1) for a synthetic 17 aa peptide
145 comprising phosphorylated T207. Using isothermal titration calorimetry (ITC), we found that
146 recombinant PLK1_{PBD} bound to the T207 phosphorylated peptide with an affinity of $K_d = 0.09$
147 ± 0.01 μ M (Fig. 3h), similar to the optimal affinity reported for an interaction between
148 PLK1_{PBD} and its phosphorylated target²⁴. Consistently, PLK1_{PBD} bound to the
149 fragment BRCA2₁₉₀₋₂₈₄ with nanomolar affinity upon phosphorylation by PLK1 ($K_d = 0.14 \pm$
150 0.02 μ M; Supplementary Fig. 3a), whereas it did not bind to the corresponding non-
151 phosphorylated polypeptides (Fig. 3i, Supplementary Fig. 3b). Mutation T207A also

152 abolished the interaction (Fig. 3j), in agreement with the pull-down experiments (Fig. 3a-d).
153 More surprisingly, the phosphomimetic substitution T207D was not sufficient to create a
154 binding site for PLK_{PDB} (Supplementary Fig. 2c). A peptide comprising pT207 and the
155 mutation S206C could not bind to PLK1_{PBD} (Fig. 3k), as predicted from the consensus
156 sequence requirement for PLK1_{PBD} interaction²⁴. Last, a peptide containing phosphorylated
157 S197, which is also a predicted docking site for PLK1, bound with much less affinity to
158 PLK1_{PBD} than pT207 ($K_d = 17 \pm 2 \mu\text{M}$; Supplementary Fig. 2d).
159 To further characterize this molecular interaction, we determined the crystal structure of
160 PLK1_{PBD} bound to the T207 phosphorylated peptide at 3.1 Å resolution (Supplementary table
161 2). Analysis of this 3D structure showed that, as expected, the 17 aa BRCA2 phosphopeptide
162 binds in the cleft formed between the two Polo boxes (Fig. 3l). Twelve residues of the peptide
163 (from A199 to I210) are well-structured upon binding, burying about 694 Å² in the interface
164 with PLK1_{PBD}. BRCA2 residues from P202 to L209 are at least 25% buried in the complex.
165 The interface between BRCA2 and PLK1_{PBD} is stabilized by 12 hydrogen bonds: the
166 backbone of residues T200 to L209 as well as the side chain of S206 are bonded to residues
167 from Polo Box 1, whereas the side chain of phosphorylated T207 is bonded to residues from
168 Polo Box 2 (see the zoom view in Fig. 3l). Specifically, the backbone oxygen of T200 is
169 bonded to the side chain of Y417 of PLK1_{PBD}. Residues L204, S205 and S206 form a β-sheet
170 with residues W414, V415 and D416, characterized by hydrogen bonds between the backbone
171 atoms of BRCA2 L204, S206 and PLK1 D416, W414, respectively. The side chain of S206
172 participates in 2 hydrogen-bonding interactions with the backbone of W414, which explains
173 the strict requirement for this amino acid at this position²⁴. Moreover, the phosphate group of
174 pT207 participates in 3 hydrogen-bonding interactions with the side chains of residues H538,
175 K540 and R557 in Polo Box 2 (see the zoom view in Fig. 3l). This explains the critical
176 dependence on phosphorylation for binding observed by ITC (Fig. 3h, i). The presence of a

177 buried leucine at the pT-3 position ³⁰, as well as the electrostatic interactions of the serine at
178 the pT-1 position with PLK1 W414 ²⁴ and the phosphorylated threonine with PLK1 H538 and
179 K540, have been described as essential for the high affinity and specificity of the interaction
180 in other PLK1_{PBD}-phosphopeptide complexes ^{24,31}.
181 Thus, our biochemical and structural analysis demonstrate that the BRCA2-T207
182 phosphopeptide interacts with PLK1_{PBD} as an optimal and specific PLK1_{PBD} ligand. It
183 supports a mechanism in which phosphorylation of T207 by PLK1 promotes the interaction of
184 PLK1 with BRCA2 through a *bona fide* docking site for PLK1 and favours a cascade of
185 phosphorylation events. In variant T207A, the absence of T207 phosphorylation impairs
186 PLK1 docking explaining the reduction of binding to PLK1 and the global loss of
187 phosphorylation by PLK1. S206C eliminates the serine residue at -1 position required for
188 PLK1_{PBD} interaction resulting as well in a strong reduction of BRCA2 binding.

189

190 **Impairing T207 phosphorylation prolongs mitosis**

191 PLK1 is a master regulator of mitosis ¹⁷. To find out whether the interaction between BRCA2
192 and PLK1 is involved in the control of mitotic progression we examined the functional impact
193 of the variants that reduce PLK1 phosphorylation at T207 (S206C and T207A) in the context
194 of the full-length BRCA2 protein in cells. For this purpose, we generated stable cell lines
195 expressing the BRCA2 cDNA coding for either the GFPMBP-BRCA2 WT or the variants to
196 complement DLD1 BRCA2 deficient human cells (hereafter BRCA2^{-/-}). In this cell line, both
197 alleles of BRCA2 contain a deletion in exon 11 causing a premature stop codon after BRC5
198 and cytoplasmic localization of a truncated form of the protein ³². We selected two stable
199 clones of each variant that show similar protein levels as the BRCA2 WT complemented cells
200 (clone C1, hereafter BRCA2 WT) by western blot (Supplementary Fig. 4a). We then tested
201 the interaction of full-length BRCA2 with PLK1 in these stable clones by GFP pull-down

202 assay. As expected, PLK1 readily co-purified with full-length BRCA2 WT from mitotic cells.
203 Importantly, in cells expressing the variants S206C and T207A, the level of co-purified PLK1
204 was greatly reduced (Fig. 4a, 4b), confirming the results obtained with the overexpressed
205 BRCA2₁₋₂₅₀ fragments (Fig. 3a) now in the context of cells stably expressing the full-length
206 BRCA2 protein. Thus, BRCA2 interaction with endogenous PLK1 is impaired in cells
207 bearing variants S206C and T207A.

208 Also, the binding of BRCA2 to PLK1 in cells stably expressing full-length BRCA2 WT was
209 not reduced by incubating the cells with PLKi (BTO) (Supplementary Fig. 4b), consistently
210 with results obtained *in vitro* (Fig. 3c). Altogether, these data suggest that another binding site
211 primed by a different kinase (presumably T77 phosphorylated by CDK1³³) contributes to
212 BRCA2 binding to PLK1. Consistent with this idea, the binding of overexpressed 2xMBP-
213 BRCA2₁₋₂₅₀ to the endogenous PLK1 in U2OS cells was completely abolished in the presence
214 of the CDK inhibitor (RO3306) (Supplementary Fig. 4c, lane 4 compared to lane 2).

215 Having confirmed that T207 was a docking site for PLK1 in cells, we next examined the
216 impact of BRCA2 variants on mitosis. Therefore, we monitored the time taken for individual
217 cells from mitotic entry (defined as nuclear envelope break down) to mitotic exit using live
218 cell imaging. Cells expressing the endogenous BRCA2 (hereafter BRCA2^{+/+}) and the BRCA2
219 WT cells showed similar kinetics, they completed mitosis, on average, in 47 and 44 min,
220 respectively (Fig. 4c, d) and the majority of the cells (80% for BRCA2^{+/+} and 82% for
221 BRCA2 WT) completed mitosis within 60 min (Fig. 4e). In contrast, cells expressing variants
222 S206C and T207A augmented the time spent in mitosis (average time of 56 and 66 min,
223 respectively, Fig. 4d). This trend was also observed in the frequency of cells dividing within
224 60 min (~49-51%), compared to 82% in BRCA2 WT cells (Fig. 4e). Representative videos of
225 the still images shown in Fig. 4c are included in Supplementary movies 1-3.

226 Taken together, cells expressing variants S206C and T207A display a significant delay in
227 mitotic progression compared to BRCA2 WT expressing cells.

228

229 **Docking of PLK1 at T207 of BRCA2 favours the formation of a complex with PP2A and**
230 **phosphorylated BUBR1 that facilitates chromosome alignment**

231 BRCA2 forms a complex with BUBR1^{4,5}. BUBR1 facilitates kinetochore-microtubule
232 attachments via its interaction with the phosphatase PP2A. Phosphorylation of BUBR1 by
233 PLK1 at the KARD motif comprising the tension sensitive sites S676 and T680 promotes
234 interaction with PP2A²⁰. A defect in the phosphorylation of BUBR1 weakens its interaction
235 with PP2A leading to mitotic delay^{20,34}. The mitotic delay phenotype we observed in BRCA2
236 mutated cell lines led us to ask whether BRCA2 and PLK1 formed a tetrameric complex with
237 pBUBR1 and PP2A. Using a GFP pull-down to capture GFP-MBP-BRCA2 from mitotic
238 BRCA2 WT cells, we observed that PLK1, pT680-BUBR1 and PP2A (detected with an
239 antibody against the catalytic subunit of PP2A, PP2AC) were pull-down together with GFP-
240 MBP-BRCA2, indicating the formation of a tetrameric complex (Fig. 5a). As described for
241 pBUBR1^{19,20}, PLK1³⁴ and PP2A²², we found BRCA2 at the kinetochore in mitotic cells
242 (Supplementary Fig. 5a) as previously reported⁴ supporting the idea that this complex takes
243 place at the kinetochore.

244 Importantly, cells expressing the variants S206C or T207A showed a strong reduction in the
245 interaction of BRCA2 with PLK1, PP2A, BUBR1 and pT680-BUBR1 in the context of the
246 tetrameric complex (Fig. 5a, b). Moreover, the overall levels of BUBR1 and pBUBR1 were
247 also reduced in cells bearing S206C and T207A variants compared to the WT cells, as
248 detected by specific antibodies against BUBR1, pT680-BUBR1 (Fig. 5e-i) and pS676-
249 BUBR1 (Supplementary Fig. 5d), and this was also the case in BRCA2 deficient cells (DLD1
250 BRCA2^{-/-} cells) or U2OS cells depleted of BRCA2 by siRNA (Fig. 5f, g). Furthermore, we

251 observed an overall reduction in the levels of pBUBR1 at the kinetochore (Fig. 5h, i) in cells
252 expressing T207A compared to WT cells. Consistently, when we immunoprecipitated
253 BUBR1 from mitotic cells and detected the levels of co-immunoprecipitated PP2A (PP2AC
254 antibody), we observed that, although PP2A was readily copurified with BUBR1 in the
255 BRCA2 WT cells, expressing BRCA2 variant T207A reduced the levels of PP2A by ~30%
256 (Fig. 5c, 5d) suggesting that BRCA2 facilitates the formation of a complex between BUBR1-
257 and PP2A.

258 The association of phosphorylated BUBR1 with PP2A is required for the formation of stable
259 kinetochore-microtubule attachments^{19,20}, a defect in this interaction resulting in chromosome
260 misalignment. Therefore, we next examined whether cells expressing the BRCA2 variants
261 S206C and T207A altered chromosome alignment. Following thymidine synchronization, the
262 cells were treated with the Eg5 inhibitor Monastrol (100 μ M) for 14h followed by Monastrol
263 washout and release for 1h in normal media supplemented with the proteasome inhibitor
264 MG132 to avoid exit from mitosis¹⁹. Chromosome alignment was then analysed by
265 immunofluorescence. Importantly, the analysis of cells expressing S206C and T207A variants
266 showed high frequency of faulty chromosome congression compared to the BRCA2 WT
267 clone (47% in S206C and 38% in T207 versus 24% in the BRCA2 WT clone), which was
268 exacerbated in BRCA2^{-/-} cells (63%) (Fig. 5j, k), as detected by signals of the centromere
269 marker (CREST) outside the metaphase plate (Fig. 5k).

270 Mutations in the PLK1-targeted sites of BUBR1 lead to chromosome misalignment, a
271 phenotype that can be rescued by the phosphomimic mutant BUBR1-3D (S670D, S676D,
272 T680D)²⁰. To better understand the mechanism behind the phenotype observed in our clones,
273 we overexpressed RFP-BUBR1-3D to test whether this form of BUBR1 could restore the
274 misalignment phenotype observed in cells bearing T207A. Surprisingly, the cells

275 overexpressing RFP-BUBR1-3D presented a similar misalignment phenotype, ~38% of
276 exhibited misaligned chromosomes compared to 33% for cells bearing T207A (Fig. 6a, b).
277 Importantly, BUBR1-3D, which has a weaker affinity for PP2A than BUBR1 phosphorylated
278 by PLK1 *in vitro*³⁵, but is sufficient to restore PP2A binding in BUBR1 deficient cells²⁰,
279 could not rescue the interaction of BUBR1 with PP2A in cells bearing BRCA2 S206C variant
280 (Fig. 6c).

281 Our results strongly suggest that docking of PLK1 onto BRCA2 T207 facilitates the
282 formation of a complex between phosphorylated BUBR1 and PP2A at the kinetochore. This is
283 not due to an increased localization of PLK1 at the kinetochore, as the levels of PLK1 at the
284 kinetochores remained unchanged in BRCA2 WT and variant (Supplementary Fig. 6a, b), nor
285 to an increased interaction of PLK1 with BUBR1 (Supplementary Fig. 6c, d).

286 **The variants that reduce PLK1 phosphorylation of BRCA2 display strong defects in**
287 **chromosome segregation and aneuploidy**

288 Unresolved chromosome misalignment as observed in cells altering BRCA2 phosphorylation
289 by PLK1 is expected to drive chromosome missegregation. To find out if this was the case in
290 cells expressing BRCA2 variants S206C and T207A, we examined chromosome segregation
291 by immunofluorescence in cells synchronized by double-thymidine block and released for
292 15h to enrich the cell population at anaphase/telophase stage. BRCA2^{-/-} cells displayed, as
293 expected, an increased proportion of chromosome bridges (39% vs 16% in cells expressing
294 BRCA2 WT), whereas the fraction of lagging chromosomes was only mildly increased (7%
295 vs 4% in BRCA2 WT). The percentage of chromosome bridges in cells expressing S206C and
296 T207A was moderately increased (23% and 29%, respectively, compared to 16% in BRCA2
297 WT). However, the biggest difference was observed in the percentage of lagging
298 chromosomes increasing between 3- and 5-fold in the cells bearing the variants compared to
299 the BRCA2 WT cells (Fig. 6d, 6e).

300 Erroneous chromosome segregation generates aneuploid cells during cell division³⁶. Given
301 the strong chromosome segregation defects observed in cells expressing S206C and T207A
302 we next analysed the number of chromosomes in these cells. Total chromosome counts
303 carried out on metaphase spreads revealed that 37.1% of BRCA2 WT cells exhibited
304 aneuploidy with chromosome losses or gains. In the case of S206C and T207A, this number
305 was elevated to 52.2% and 61.8% of the cells, respectively (Fig. 7a). An example of the
306 images analyzed can be found in Fig. 7b. As the number of chromosomes was difficult to
307 assess for cells with high content of chromosome gains we arbitrarily discarded cells that
308 contained more than 65 chromosomes. Thus, tetraploid cells were not included in this
309 measurement. Therefore, as a complementary test, we determined the frequency of tetraploid
310 cells by assessing the incorporation of BrdU and measuring the frequency of S-phase cells
311 with >4N DNA content (Fig. 7c). Quantification of these data showed that similar to the
312 BRCA2 WT complemented cells, the frequency of tetraploidy in cells bearing the variants is
313 <1% of the total population (Fig. 7d), while the frequency of BrdU positive cells was
314 equivalent between the BRCA2 WT and the VUS expressing cells (Supplementary Fig. 7).

315 Together, these results indicate that, in addition to the severe chromosome misalignment
316 phenotype, cells expressing S206C and T207A display high frequency of chromosome
317 missegregation, including a strong induction of lagging chromosomes and a mild increase in
318 chromosome bridges. As a consequence, the incidence of aneuploidy, but not tetraploidy, is
319 greatly exacerbated in these cells.

320

321 **The variants altering PLK1 phosphorylation of BRCA2 restore the hypersensitivity of**
322 **BRCA2 deficient cells to DNA damage and PARP inhibition and are HR proficient**

323 Since BRCA2 has a major role in DNA repair by HR, the prolonged mitosis observed in the
324 VUS-expressing stable cell lines (Fig. 4) could result from checkpoint activation through

325 unrepaired DNA. In addition, defects in chromosome segregation have been reported as a
326 cause of DNA damage. Thus, to test the DNA repair efficiency of cells expressing variants
327 S206C and T207A, we performed a clonogenic survival assay in the stable clones after
328 treatment with mitomycin C (MMC), an inter-strand crosslinking agent to which BRCA2
329 deficient cells are highly sensitive³⁷. As expected, BRCA2 deficient cells (BRCA2^{-/-}) showed
330 hypersensitivity to MMC treatment whereas BRCA2 WT cells complemented this phenotype
331 almost to the same survival levels as the cells expressing the endogenous BRCA2
332 (BRCA2^{+/+}). The stable clones expressing variants S206C and T207A also complemented the
333 hypersensitive phenotype of BRCA2^{-/-} cells, although there was a mild reduction in the
334 survival levels compared to the BRCA2 WT cells (Fig. 8a), consistent with the slightly
335 increased levels of chromosome bridges (Fig. 6d). These results suggest that the delay in
336 mitosis is not a consequence of checkpoint activation via unrepaired DNA. Cells expressing
337 VUS S206C and T207A showed a growth defect manifested in a reduced number of colonies
338 observed in unchallenged conditions (Supplementary Fig. 8a), which is consistent with the
339 mitotic phenotype observed (Fig. 4-6). To exclude a possible bias arising from the different
340 ability to form colonies of these cells we also tested the sensitivity to MMC of the cell
341 population by MTT assay. As shown in Fig. 8b, cells expressing S206C and T207A showed
342 similar relative viability compared to BRCA2 WT complemented cells or the cells expressing
343 endogenous BRCA2 (BRCA2^{+/+}), confirming our results. To additionally address the DNA
344 repair capacity of these cells, we tested their viability upon treatment with the poly (ADP-
345 ribose) polymerase (PARP) inhibitor Olaparib. PARP1 is an enzyme required for the sensing
346 of DNA single strand breaks (SSBs) and double strand breaks (DSBs) that becomes essential
347 in the absence of a functional HR pathway³⁸ and therefore is used as a surrogate of HR
348 proficiency. In fact, PARP1 inhibitors, in particular Olaparib, is currently used in the clinic to
349 treat breast and ovarian cancer patients carrying germline mutations in BRCA1/2. In our

350 settings, the relative viability of BRCA2^{-/-} cells was 45% upon 4-day treatment with the
351 highest Olaparib concentration tested (5 μM); in contrast, 74% of BRCA2 WT complemented
352 cells remained viable. Similarly, cells expressing S206C or T207A survived the treatment
353 equally well as the cells expressing BRCA2 WT, the percentage of viable cells at 5 μM
354 treatment ranging from 70 to 83 % depending on the clone (Fig. 8c) indicating that the
355 mutated cells can rescue the phenotype of BRCA2^{-/-} cells.

356 To determine directly the levels of spontaneous DNA damage in these cells and whether or
357 not they can recruit the recombination protein RAD51 to sites of DNA damage, we measured
358 both the number of nuclear foci of both the DSB marker γH2AX and RAD51 protein, in cells
359 unchallenged (-IR) or 2h upon induction of DNA damage by ionizing radiation (6 Gy, (+IR)).
360 Our results show that the number of γH2AX foci in unchallenged conditions, indicative of
361 spontaneous DNA damage, is comparable in all cell lines including in cells transiently
362 depleted of BRCA2 by siRNA (Fig. 8d and Supplementary Fig. 8b); this is probably due to
363 the high genome instability intrinsic to these cancer cells. Similarly, the number of RAD51
364 foci in unchallenged conditions did not change significantly among samples (mean varying
365 between 2-3 foci). In contrast, the number of RAD51 foci upon irradiation increased between
366 5-fold in DLD1 BRCA2^{+/+} cells and BRCA2 WT cells and 3-fold in T207A bearing cells
367 whereas in cells depleted for BRCA2 the number of RAD51 foci decreased to 1 focus per
368 nucleus, on average (Fig. 8e) whereas the number of γH2AX foci upon irradiation among
369 cells remained the same (Fig. 8d). From these results, we conclude that the DNA repair foci
370 are only mildly altered in cells expressing T207A. Representative images of these
371 experiments are shown in Supplementary Fig. 8b.

372 A typical feature of replication stress is the appearance of micronuclei in daughter cells which
373 generally contain DNA fragments. In contrast, the presence of micronuclei with centromeres
374 is an indication of whole chromosome or chromatid, suggesting an event arising from lagging

375 chromosomes. We measured the number of micronuclei with and without centromeres in our
376 cells. BRCA2 deficient cells displayed an overall increased number of micronuclei compared
377 to BRCA2 WT cells. In contrast, cells bearing S206C or T207A variant did not exhibit an
378 increase in micronuclei either with or without centromeres (Supplementary Fig. 8d, e)
379 excluding strong replication stress-induced DNA damage in these cells.

380 Finally, to directly assess the HR proficiency of these cells, we performed a cell-based HR
381 assay by DSB-mediated gene targeting at a specific locus (AAVS1 site) within the
382 endogenous PPP1R12C gene using a site-specific transcription-activator like effector
383 nuclease (TALEN) and a promoter-less mCherry donor flanked by homology sequence to the
384 targeted locus^{39,40}. DSB-mediated gene targeting results in mCherry expression from the
385 endogenous PPP1R12C promoter (Fig. 8f) which can be measured by flow cytometry
386 (Supplementary Fig. 9). Using this system, BRCA2^{+/+} and BRCA2 WT complemented cells
387 show ~ 7% of mCherry positive TALEN-transfected cells (mean of 5.6% for BRCA2^{+/+} and
388 4.9% for WT) whereas the BRCA2 deficiency (BRCA2^{-/-}) led to a reduction to ~2% of
389 mCherry expressing cells, as expected. Importantly, TALEN-transfected cells expressing
390 BRCA2 variants S206C and T207A showed no significant difference with the BRCA2 WT
391 complemented cells indicating an intact HR activity.

392 In summary, these results indicate that the role of BRCA2 in conjunction with PLK1 in
393 mitosis is likely independent of the HR function of BRCA2 as the variants S206C and T207A
394 affecting PLK1 phosphorylation of BRCA2 are only mildly sensitive to DNA damage, do not
395 show an increased number of micronuclei, are able to recruit RAD51 to DNA damage sites
396 (as shown for T207A) and are efficient at DSB-mediated gene targeting.

397

398 **Discussion**

399 Our results demonstrate that residues S193 and T207 of BRCA2 can be efficiently
400 phosphorylated by PLK1 (Fig. 2), thus extending the consensus sequence for phosphorylation
401 by this kinase: position 205 is a serine and not a negatively charged residue, as generally
402 observed at PLK1 phosphorylation sites²⁴. Moreover, we reveal that pT207 constitutes a *bona*
403 *fide* docking site for PLK1_{PBD} (Fig. 3h-l). Accordingly, *BRCA2* missense variants of unknown
404 clinical significance reducing the phosphorylation status of T207 (T207A, S206C) result in a
405 decreased in BRCA2-PLK1 interaction (Fig. 3a-k, 4a). The phenotype of cells expressing
406 these two breast cancer variants in a BRCA2 deficient background was characterized, in order
407 to investigate in detail the possible role of BRCA2 phosphorylation at T207 by PLK1 in the
408 control of mitosis. Unexpectedly, we found that the cells expressing S206C and T207A
409 display defective chromosome congression to the metaphase plate (Fig. 5j, k), causing a
410 substantial delay in mitosis progression (Fig. 4c-e).

411 Proper kinetochore-microtubule attachments require the interaction of BUBR1 with the
412 phosphatase PP2A-B56 α to balance Aurora B kinase activity^{20,22}. This interaction is
413 mediated through the phosphorylation of the KARD motif of BUBR1 by PLK1. *BRCA2*
414 does not alter PLK1 interaction with BUBR1 (Supplementary Fig. 6c, d). However, we found
415 that *BRCA2* forms a tetrameric complex with PLK1-pBUBR1-PP2A, and that this complex is
416 strongly reduced in cells bearing *BRCA2* variants S206C and T207A (Figure 5a, 5b).
417 Furthermore, cells bearing *BRCA2* variants S206C and T207A show reduced overall levels of
418 BUBR1 including pBUBR1(pT680) at the kinetochore (Fig. 5i). Importantly, the fact that in
419 the *BRCA2* mutated cells the interaction of PP2A with total BUBR1 is reduced (Fig. 5c, d)
420 and that neither this interaction (Fig. 6c) nor the chromosome alignment defect can be rescued
421 by BUBR1-3D overexpression (Fig. 6a, b) strongly suggests that PP2A needs to be in
422 complex with PLK1-bound *BRCA2* to bind BUBR1 and facilitate chromosome alignment.

423 Cells bearing T207A and S206C variants display chromosome segregation errors including
424 lagging chromosomes and chromosome bridges (Fig. 6d, e). Importantly, these accumulated
425 errors ultimately lead to a broad spectrum of chromosome gains and losses (aneuploidy)
426 compared to the wild type counterpart (Fig. 7a), but not to tetraploid cells (Fig. 7c),
427 suggesting that cytokinesis *per se*, in which BRCA2 is also involved¹¹⁻¹³, is not affected.

428 Finally, the function of BRCA2-PLK1 interaction in mitosis seems to be independent of the
429 HR function of BRCA2 as cells expressing these variants display mild sensitivity to DNA
430 damage (MMC) and PARP inhibitors (Fig. 8a-c), normal recruitment of RAD51 to DNA
431 damage sites (Fig. 8e, Supplementary Fig. 8b), absence of micronuclei (Supplementary Fig.
432 8d, e), rescue the chromosome bridges phenotype of BRCA2 deficient cells (Figure 6d) and
433 their HR activity, as measured by DSB-mediated gene targeting, is intact (Fig. 8f,
434 Supplementary Fig. 9). Nevertheless, we cannot rule out that the mild phenotype observed in
435 some of these experiments in cells bearing S206C and T207A may arise from a distorted
436 interaction with an unknown DNA repair factor that would bind to the region of BRCA2
437 where these variants localize.

438 Putting our results together we reveal an unexpected chromosome stability control mechanism
439 that depends on the phosphorylation of BRCA2 by PLK1 at T207. We show that BRCA2
440 pT207 is a docking platform for PLK1 that ensures the efficient interaction of BUBR1 with
441 PP2A phosphatase required for chromosome alignment.

442 We propose the following working model-(Fig. 8g): in cells expressing BRCA2 WT, PLK1
443 phosphorylates BRCA2 on T207 leading to the docking of PLK1 at this site. This step
444 promotes the formation of a complex between BRCA2-PLK1-pBUBR1-PP2A in
445 prometaphase at the kinetochore, leading to an enrichment of phosphorylated BUBR1 and the
446 phosphatase PP2A to balance Aurora B activity (Fig. 8g, panel 1). Why is BRCA2 required

447 for PP2A interaction with pBUBR1 and, is this regulated by PLK1? It has been reported that
448 BRCA2 fragment from aa 1001 to aa 1255 (BRCA2₁₀₀₁₋₁₂₅₅) comprising a PP2A-B56 binding
449 motif, binds to the B56 subunit of PP2A⁴¹; in addition, the phosphorylation of positions 2 and
450 8 of this motif enhances the binding to B56⁴². In BRCA2, these positions are occupied by
451 serines that are targets for PLK1. Therefore, it is likely that the recruitment of PLK1 by
452 BRCA2 T207 would favor phosphorylation of BRCA2₁₀₀₁₋₁₂₅₅ and binding of the complex
453 between BRCA2 and PLK1 to PP2A-B56. Non-exclusively, BUBR1 has been reported to
454 bind directly BRCA2 although there are inconsistencies regarding the site of interaction^{4,5}.
455 Thus, either the direct interaction of BRCA2 with PP2A or with BUBR1 or both could be
456 favoring the formation of a complex between PP2A and pBUBR1.

457 Once the kinetochore-microtubule attachments are established and proper tension is achieved,
458 BUBR1 is no longer phosphorylated by PLK1¹⁹. This leads to a full alignment of
459 chromosomes at the metaphase plate, SAC inactivation (Fig. 8g, panel 2) and the subsequent
460 faithful chromosome segregation in anaphase (Fig. 8g, panel 3).

461 In cells expressing the variants that impair T207 phosphorylation (S206C, T207A), PLK1
462 cannot be recruited to pT207-BRCA2, impairing the formation of the complex with PLK1,
463 PP2A and BUBR1 (Fig. 8g, panel 1'), which in turn reduces the amount of pBUBR1 and its
464 binding to PP2A for proper kinetochore-microtubule interactions. This leads to chromosome
465 misalignment defects that prolong mitosis (Fig. 8g, panel 2'); as a consequence, these cells
466 exhibit increased chromosome segregation errors (Fig. 8g, panel 3') and aneuploidy.

467 Although the individual BRCA2 variants analysed here are rare (Supplementary table 1), the
468 majority of pathogenic mutations recorded to date lead to a truncated protein either not
469 expressed or mislocalized⁴³ which would be predicted to affect this function. Consistent with
470 this idea, the BRCA2 deficient cells or cells transiently depleted of BRCA2 used in this study

471 exhibit low levels of phosphorylated BUBR1 (Fig. 5f, g). Thus, the chromosome alignment
472 function described here could be responsible, at least in part, for the numerical chromosomal
473 aberrations observed in *BRCA2*-associated tumors⁴⁴.

474 Finally, the lack of sensitivity to the PARP inhibitor Olaparib observed in our cell lines (Fig.
475 8b) has important clinical implications as breast cancer patients carrying these variants are not
476 predicted to respond to PARP inhibitor treatment (unlike *BRCA2*-mutated tumors that are HR-
477 deficient).

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Author Contributions

A.E. purified WT and mutated BRCA2₁₋₂₅₀, established the stable DLD1^{-/-} cell lines, performed kinase assays, pull-down assays, Western blots, time-lapse microscopy experiments, mitotic index measurements by FACS, clonogenic survival and MTT assays as well as the statistical analysis for all the experiments. C.M. performed IF and image acquisition of metaphase plate alignment, chromosome segregation and karyotype analysis. M.J. performed the NMR experiments assisted by S. M., F.T. and S.Z.J. M.J. and S.M. purified PLK1_{PBD} and performed the ITC experiments. S.M. solved the X-ray structure assisted by V.R. R.B. and G.S. performed IF, image acquisition and quantification of DNA repair foci. V.B. assisted establishing stable clones and performing clonogenic survival assays. P.D. purified PLK1_{PBD}. A.M. cloned and produced PLK1 and PLK1-KD from insect cells. A.C., A.E. and S.Z.J. designed the experiments. A.C. and S.Z.J. supervised the work. A.C. wrote the paper with important contributions from all authors.

The authors declare no conflict of interest.

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Figure legends

Figure 1. BRCA2 VUS alter PLK1 phosphorylation of BRCA2₁₋₂₅₀

(a) PLK1 *in vitro* kinase assay with BRCA2₁₋₂₅₀. Top: The polypeptides encompassing 2x-MBP-BRCA2₁₋₂₅₀ WT or S193A, M192T, S196N, T200K, S206C, T207A mutations or the 2XMBP tag were incubated with recombinant PLK1 in the presence of $\gamma^{32}\text{P}$ -ATP. The samples were resolved on 7.5% SDS-PAGE and the ^{32}P -labeled products were detected by autoradiography. Bottom: 7.5% SDS-PAGE showing the input of purified 2xMBP-BRCA2₁₋₂₅₀ WT and mutated proteins (0.5 μg) used in the reaction as indicated. **(b)** Quantification of the relative phosphorylation in (A). Data in (B) are represented as mean \pm SD from at least four independent experiments (WT (n=9), S193A (n=4), M192T (n=4), S196N (n=4), S206C (n=4), T200K (n=5), T207A (n=4)). **(c)** PLK1 *in vitro* kinase assay performed as in (A) with recombinant PLK1 or the PLK1 kinase dead K82R mutant (PLK1-KD) together with BRCA2₁₋₂₅₀ WT as substrate, in the presence or absence of the PLK1 inhibitor BI2536 (50 nM) in the kinase reaction buffer. **(d)** Quantification of the relative phosphorylation in (c). Data in (d) are represented as mean \pm SD from three independent experiments. (b and c) One-way ANOVA test with Dunnett's multiple comparisons test was used to calculate statistical significance of differences (the p-values show differences compared to WT (b) or PLK1 (d); ns (non-significant)).

Figure 2. PLK1 phosphorylates T207 in BRCA2₄₈₋₂₁₈ and BRCA2₁₉₀₋₂₈₄

Phosphorylation of BRCA2₄₈₋₂₁₈ and BRCA2₁₉₀₋₂₈₄ by PLK1 as observed by NMR. **(a)** Schematic view of the 2 overlapping BRCA2 fragments analysed by NMR. Residues identified as phosphorylated in (b) are indicated. **(b)** Superposition of the ^1H - ^{15}N HSQC spectra recorded before (black) and after (24h; red) incubation with PLK1. Each spectrum contains one peak per backbone NH group. Most peaks are located in the grey region of the spectrum, indicating that they correspond to disordered residues. Assignment of each peak to a BRCA2 residue was performed using a classical heteronuclear NMR strategy. Peaks

corresponding to phosphorylated residues are indicated. **(c)** Comparison of the phosphorylation kinetics recorded for BRCA2₄₈₋₂₁₈ WT and T207A. ¹H-¹⁵N HSQC spectra of BRCA2₄₈₋₂₁₈WT (red) and T207A (green) recorded 5 h after addition of PLK1 are superimposed to highlight the overall decrease of phosphorylation observed for the mutant compared to the WT. **(d)** Fraction of phosphorylated protein deduced from the intensities of the peaks corresponding to the non-phosphorylated and phosphorylated residues plotted as a function of time. WT S193 and T207 time points are represented by red circles and squares, respectively, while T207A S193 timepoints are represented by green circles. The graph represents results of three independent experiments; error bars (SD).

Figure 3. BRCA2 variants showing reduced phosphorylation by PLK1 impair PLK1 binding

(a) 2xMBP-BRCA2₁₋₂₅₀ expressing the WT, the variants (M192T, S196N, S206C and T207A) and the 2XMBP-tag were expressed in U2OS cells by transient transfection for 30h before the cells were treated with nocodazole for 14h. Mitotic 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. StainFree images of the gels before transfer was used as loading control (cropped image is shown). **(b)** Quantification of co-immunoprecipitated PLK1 with 2xMBP-BRCA2₁₋₂₅₀ in (a), relative to the input levels of PLK1. Results are presented as the fold change compared to BRCA2₁₋₂₅₀-WT. The data represents the mean \pm SD of 3-4 independent experiments (WT (n=4), M192T (n=4), S196N (n=4), S206C (n=4), T207A (n=3)). Statistical significance of the difference in between WT and the variants was calculated with one-way ANOVA test with Dunnett's multiple comparisons test. **(c)** PLK1 *in vitro* kinase assay followed by *in vitro* pull-down assay using amylose beads. The polypeptides 2x-MBP-

BRCA2₁₋₂₅₀ WT and T207A were incubated with recombinant PLK1-WT or the kinase dead K82R mutant (PLK1-KD, only for the BRCA2₁₋₂₅₀ –WT fragment) in kinase buffer with ATP followed by incubation with amylose beads. The bound complexes were eluted from the beads with maltose and resolved on 10% SDS-PAGE followed by western blotting using anti-PLK1 and anti-MBP antibodies. UB: unbound fraction, E: maltose-eluted fraction. **(d-e)** Quantification of the PLK1 pull-down in (c) relative to the PLK1 levels in the input. Results are presented as the fold change compared to BRCA2₁₋₂₅₀-WT in (d) and PLK1-WT in (e). The data represents the mean \pm SD of three independent experiments. **(f)** PLK1 *in vitro* kinase assay followed by *in vitro* pull-down assay using amylose beads as in (c). *Left panel:* The 2x-MBP-BRCA2₁₋₂₅₀-WT polypeptide was incubated with recombinant PLK1-WT in the absence or presence of ATP followed by incubation with amylose beads. *Right panel:* The 2x-MBP-BRCA2₁₋₂₅₀-WT polypeptide was pre-treated with phosphatase (FastAP Thermosensitive Alkaline Phosphatase) for 1 hour before the incubation with recombinant PLK1-WT followed by the amylose-beads incubation as described in (c). **(g)** Quantification of the PLK1 pull-down in (f) relative to the PLK1 levels in the input. Results are presented as the fold change compared to kinase assay performed with non-phosphatase treated BRCA2₁₋₂₅₀-WT in the presence of ATP. The data represents the mean \pm SD of three-four independent experiments. Statistical significance of the difference in (d-e, g) was calculated with two-tailed t-test (the p-values show significant differences; ns (non-significant)) **(h-k)** Isothermal Titration Calorimetry (ITC) thermograms showing binding of PLK1_{PBD} to a 17 aa BRCA2 peptide containing **(h)** pT207, **(i)** T207, **(j)** A207, **(k)** C206pT207. Residues S206 and pT207 are highlighted in pink (S206) and magenta (pT207) in the peptide sequences. **(l)** 3D cartoon representation of the crystal structure of the interface between PLK1_{PBD} (Polo-box 1 in green and Polo-box 2 in blue) and the BRCA2 peptide containing pT207 (in red except for S206 and pT207 that are highlighted in pink and magenta, respectively, as in (h-i)). The left panel

shows a global view of the structure, whereas the right panel focuses on the interface between PLK1_{PBD} and the BRCA2 peptide (from A199 to I210, marked in italics). In the left panel, S206 and pT207 are represented in sticks, whereas in the right panel, all the BRCA2 residues observed in the crystal structure are displayed in sticks. The hydrogen bonds involving side chain atoms are displayed and depicted as dark grey dots. The amino acids of PLK1_{PBD} involved in these interactions are highlighted in sticks representation.

Figure 4. Cells bearing BRCA2 variants S206C and T207A prolong mitosis

(a) Interaction of full-length BRCA2 and PLK1 in stable clones of BRCA2 deficient DLD1 cells (BRCA2^{-/-}) expressing GFPMBP-BRCA2 WT (BRCA2 WT) or the variants S206C or T207A. EGFPMBP-BRCA2 was immunoprecipitated from whole cell lysates of nocadazole treated cells using GFP-trap beads. Immuno-complexes were resolved on 4-15% SDS-PAGE followed by western blotting using anti-BRCA2 and -PLK1 antibodies. Unsynchronized DLD1 cells with endogenous BRCA2 (BRCA2^{+/+}) were used as control for the immunoprecipitation and StainFree images of the gels before transfer were used as loading control for the input (cropped image is shown). **(b)** Quantification of co-immunoprecipitated PLK1 with EGFP-MBP-BRCA2 in (a) relative to the PLK1 protein levels in the input and the amount of immunoprecipitated (IP) EGFP-MBP-BRCA2 ((PLK1_{IP} /PLK1_{input})/EGFP-MBP-BRCA2_{IP}) Results are presented as the fold change compared to the GFPMBP-BRCA2 WT clone. The data represents the mean ± SD of three independent experiments. Statistical significance of the difference between BRCA2 WT and the variants was calculated one-way ANOVA test with Dunnett's multiple comparisons test. **(c)** Top: Scheme of the double thymidine block procedure used to synchronize DLD1 cells expressing endogenous BRCA2 (BRCA2^{+/+}) or stable clones of DLD1 BRCA2 deficient cells (BRCA2^{-/-}) expressing GFPMBP-BRCA2 WT or VUS as indicated, for live-cell imaging of mitotic progression.

Bottom: Representative still images of the live-cell imaging at different time points. The numbers in each image indicate the time in minutes after nuclear envelope break down (NEBD). Scale bar represents 10 μm . **(d-e)** Quantification of the time the cells spent in mitosis from nuclear envelope break down to mitotic exit monitored by time-lapse microscopy. (d) Scatter dot plot showing the quantification of the live-time imaging in (c). The median time (min) the dividing cells spent in mitosis is indicated by a red line in the plot (median with 95% CI). Each dot represents a single cell, n indicates the total number of cells counted from two to four independent experiments (BRCA2^{+/+} (n=2), WT C1 (n=5), BRCA2^{-/-} (n=4), S206C A7 (n=3), T207A B1 (n=3)), with 45 to 60 cells counted per experiment. For statistical comparison of the differences between the samples we applied a Kruskal-Wallis test followed by Dunn's multiple comparison test, the p-values show significant differences. (e) Frequency distribution of the time spent in mitosis, including cells that fail to divide (DEAD). The error bars represent SD from at least two-four independent experiments (as in d). Statistical significance of the difference in (d and e) was calculated with one- and two-way ANOVA test respectively, with Tukey's multiple comparisons test. The p-values show significant differences.

Figure 5. Cells expressing BRCA2 variants that alter PLK1 phosphorylation display reduced protein levels of phosphorylated BUBR1 and its interaction with PP2A resulting in chromosomes misalignment

(a) Co-immunoprecipitation of the protein complex BRCA2-BUBR1/pBUBR1-PP2A-PLK1 from mitotic cell extracts of BRCA2 WT cells or cells expressing the variant S206C and T207A using GFP-trap beads. The immuno-complexes were resolved on 4-15% SDS-PAGE followed by western blotting, the interactions were revealed by anti-BRCA2, -BUBR1, -pT680-BUBR1, -PLK1 and -PP2AC (PP2A catalytic subunit) antibodies. Mitotic DLD1 cells

with endogenous BRCA2 (BRCA2^{+/+}) were used as control for the immunoprecipitation. **(b)** Quantification of co-immunoprecipitated BUBR1, pBUBR1, PLK1 and PP2A with EGFPMBP-BRCA2 in (a), relative to the input levels of each protein and the amount of immunoprecipitated EGFP-MBP-BRCA2. Results are presented as the fold change compared to the BRCA2 WT clone. The data represents the mean \pm SD of three independent experiments. Statistical significance of the difference was calculated with two-way ANOVA test with Dunnett's multiple comparisons test, the p-values show the significant difference. **(c)** Co-immunoprecipitation of endogenous PP2AC with endogenous BUBR1 from mitotic cell extracts of BRCA2 WT cells or cells expressing the variant T207A using mouse anti-BUBR1 antibody. Mouse IgG was used as control for the BUBR1 immunoprecipitation. The immuno-complexes were resolved on 4-15% SDS-PAGE followed by western blotting, the interactions were revealed by rabbit anti-BUBR1 and anti-mouse PP2AC antibodies. **(d)** Quantification of co-immunoprecipitated PP2A C in (c), relative to the input levels and the amount of immunoprecipitated BUBR1. Results are presented as the fold change compared to the BRCA2 WT clone. The data represents the mean \pm SD of three independent experiments. Statistical significance of the difference was calculated with unpaired two-tailed t-test, the p-values show the significant difference. **(e-g)** Western blots showing the expression levels of endogenous BUBR1 and pT680-BUBR1 in nocodazole treated stable clones of DLD1 BRCA2 deficient cells (BRCA2^{-/-}) expressing GFPMBP-BRCA2 WT (BRCA2 WT) or the variants, as indicated (e) or in nocodazole treated DLD1 BRCA2 deficient cells (BRCA2^{-/-}) or DLD1 cells expressing endogenous BRCA2 (BRCA2^{+/+}) (f). **(g)** Western blot showing the expression levels of endogenous BUBR1 and pT680-BUBR1 in U2OS after down-regulation of endogenous BRCA2 using siRNA. (e-g) The mean BUBR1_{TOT} and pBUBR1 signal relative to the stain free signal is shown for the nocadazole treated samples below the blots, results are presented as percentage compared to BRCA2 WT clone. The data represents the mean \pm SD

of three (e) and two (g and g) independent experiments. The protein levels of PLK1 in (a, e-g) are shown as a G2/M marker. **(h)** Representative images of the localization of pT680-BUBR1 in nocodazole-arrested DLD1 BRCA2^{-/-} cells stably expressing GFP-MBP-BRCA2 WT or the variant T207A as indicated. CREST is used as centromere marker and DNA is counterstained with DAPI. Scale bar represents 1 μ m. **(i)** Quantification of the co-localization of pT680-BUBR1 and CREST in (h). The data represents the intensity ratio (pT680-BUBR1:CREST) relative to the mean ratio of pT680-BUBR1:CREST for the GFP-MBP-BRCA2 WT calculated from a total of 252 pairs of chromosomes analysed from two independent experiments (6 pairs of chromosomes/cell from 21 cells). The red line in the plot indicates the median (95% CI) ratio, each dot represents a pair of chromosomes. For statistical comparison of the differences between the samples we applied a Mann-Whitney test, the p-values show significant differences. **(j)** Top: Scheme of the double thymidine block procedure used to synchronize the DLD1 cells for the analysis of chromosome alignment. Bottom: Quantification of misaligned chromosomes outside the metaphase plate in DLD1 BRCA2 deficient cells (BRCA2^{-/-}) an BRCA2^{-/-} cells stably expressing BRCA2 WT or the S206C and T207A variants. *n* indicates the total number of cells counted for each clone from two (BRCA2^{-/-}, S206C and T207A) and four (BRCA2 WT) independent experiments. Statistical significance of the difference was calculated with unpaired two-way ANOVA test with Tukey's multiple comparisons test, the p-values show the significant difference. **(k)** Representative images of the type of chromosome alignment observed in cells quantified in (j), scale bar represents 10 μ m.

Figure 6. Cells expressing BRCA2 variants S206C and T207A display aberrant chromosome segregation

(a) Top: Scheme of the synchronization procedure for the analysis of chromosome alignment in the T207A cells transient overexpressing the BUBR1-3D mutant (S670D, S676D and T680D). Bottom: Quantification of misaligned chromosomes outside the metaphase plate in DLD1 BRCA2^{-/-} T207A stable clones transient overexpressing the 3xFLAG-BUBR1-3D-RFP mutant (S670D, S676D and T680D). *n* indicates the total number of cells counted for each clone from two independent experiments. Statistical significance of the difference was calculated with unpaired two-tailed t-test, ns (non-significant)). **(b)** Representative images of the type of chromosome alignment observed in cells overexpressing 3xFLAG-BUBR1-3D-RFP quantified in (a); scale bar represents 10 μ m. **(c)** Co-immunoprecipitation of endogenous PP2AC (PP2A catalytic subunit) with transient overexpressed 3xFLAG-BUBR1-3D-RFP mutant (S670D, S676D and T680D) from mitotic cell extracts of BRCA2 WT cells or cells expressing the S206C variant using rabbit anti-RFP antibody. Rabbit IgG was used as control for the BUBR1 immunoprecipitation. The immuno-complexes were resolved on 4-15% SDS-PAGE followed by western blotting, the interactions were revealed by mouse anti-BUBR1 and PP2AC antibodies. The amount of PP2A co-immunoprecipitated with BUBR1-3D-RFP relative to the input levels of PP2A and the amount of immunoprecipitated BUBR1-3D-RFP is presented below the blot as mean \pm SD from two independent experiments. The data is presented relative to the non-treated BRCA2 WT.

(d) Top: Scheme of the double thymidine block procedure used to synchronize DLD1 BRCA2^{-/-} stable clones for analysis of aberrant chromosome segregation. Bottom: Quantification of cells with aberrant chromosomes segregation in BRCA2^{-/-} cells and in the clones stably expressing BRCA2 WT, S206C and T207A, as indicated. Statistical significance of the difference in (d) was calculated with two-way ANOVA test with Tukey's multiple comparisons test (the p-values show the significant differences compared to WT; ns (non-significant)). *n* in (d) indicates the total number of cells counted for each clone from two

(BRCA2^{-/-}, S206C and T207A) and four (BRCA2 WT) independent experiments. **(e)** Representative images of the type of aberrant chromosome segregation observed in the cells quantified in (d), CREST antibody is used as marker of centromere; nuclei are revealed with DAPI counterstaining. Scale bar represents 10 μ m.

Figure 7. Cells expressing BRCA2 variants S206C and T207A exhibit aneuploidy

(a) Distribution of the number of chromosomes observed in metaphase spreads of stable clones expressing BRCA2 WT, S206C A7 or T207 B1 from two independent experiments (total number of cells counted; BRCA2 WT (n=105), S206C A7 (n=111) and T207A B1 (n=110)); modal number of chromosomes and percentage deviating from the mode are shown at the top; Kruskal-Wallis test followed by Dunn's multiple comparison test was used for statistical comparison of the differences between the samples, the p-values show the significant differences.). The cell passage was between 5 and 9 (BRCA2 WT (p.6 and p.9), S206C A7 (p5 and p9) and T207A B1 (p.6 and p.9)). **(b)** Representative image of metaphase spreads of the DLD1 BRCA2 deficient stable cells bearing the S206C BRCA2 variant stained with CREST and counterstained with DAPI. In this example, the cell contains 45 chromosomes. **(c-d)** Analysis of S-phase tetraploid cells in DLD1 BRCA2 deficient cells expressing BRCA2 WT or the VUS S206C and T207A measured by flow cytometry after 20 minutes of BrdU incorporation. **(c)** Representative flow cytometry plots of cells stained with anti-BrdU-APC antibodies and 7-AAD (DNA). **(d)**. Frequency of S-phase tetraploid cells in stable clones expressing BRCA2 WT or the VUS S206C and T207A. The data represents the mean \pm SD of three independent experiments (cell passage: 6-10). Statistical significance of the difference was calculated with one-way ANOVA test with Tukey's multiple comparisons test (the p-values show the difference compared to WT, ns: non-significant).

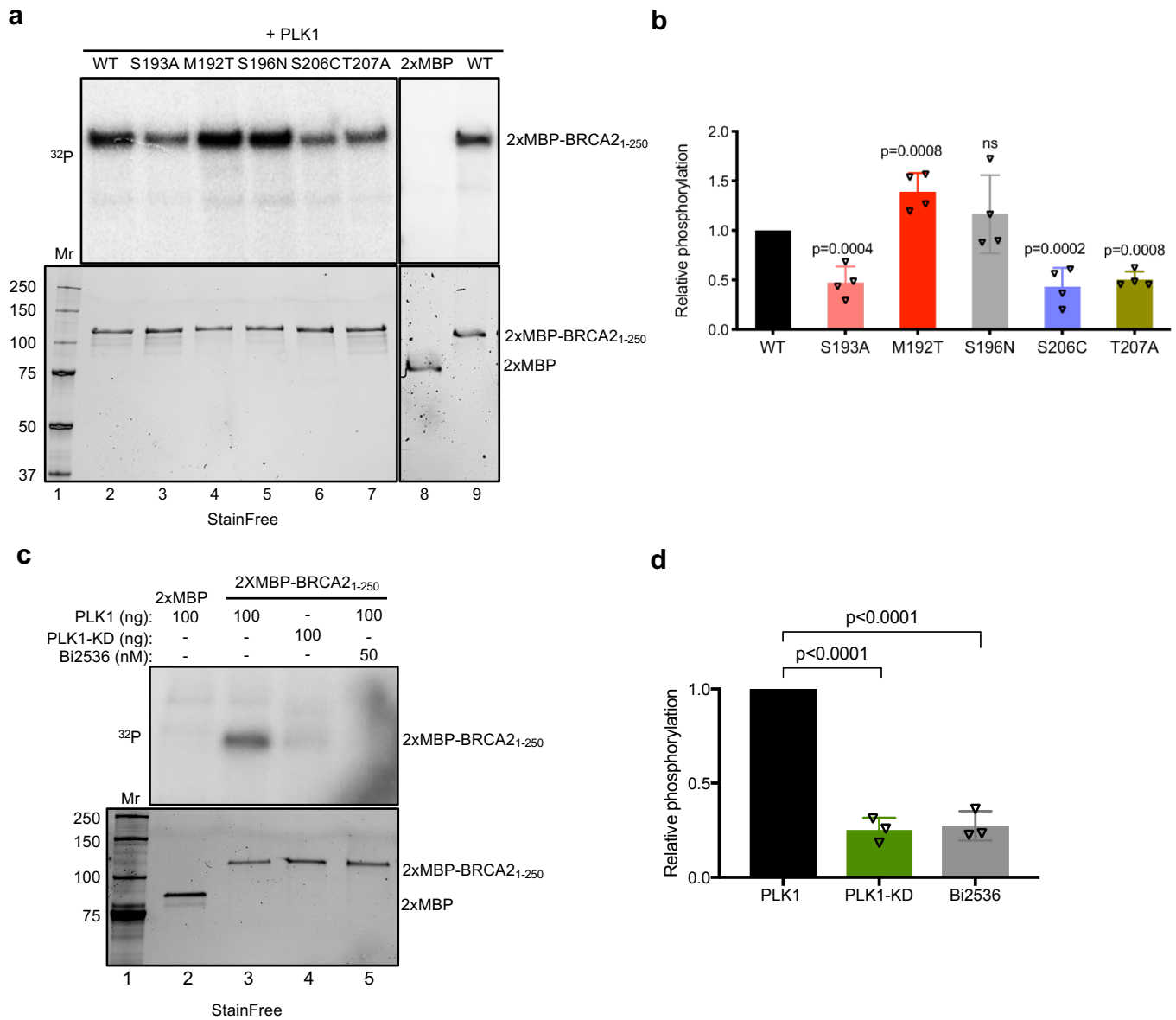
Figure 8. The DNA repair proficiency is not affected in cells bearing BRCA2 variants

S206C and T207A

(a) Quantification of the surviving fraction of DLD1 cells expressing endogenous BRCA2 (BRCA2^{+/+}) or stable clones of BRCA2 deficient DLD1 cells (BRCA2^{-/-}) expressing BRCA2 WT or the variants S206C or T207A assessed by clonogenic survival upon exposure to MMC at concentrations: 0, 0.5, 1.0 and 2.5 μ M. Data are represented as mean \pm SD: BRCA2^{+/+} (red) (n=3), BRCA2^{-/-} (gray) (n=6), WT C1 (black) (n=6), S206C A7 (blue) (n=3), T207A B1 (green) (n=4). **(b-c)** Quantification of the relative cell viability monitored by MTT assay upon treatment with increasing doses of MMC (b) or the PARP inhibitor Olaparib (c), as indicated. The data represents the mean \pm SD of four (b) and three (c) independent experiments. Statistical significance of the difference in (a-c) was calculated with two-way ANOVA test with Tukey's multiple comparisons test (the p-values show the significant differences compared to the BRCA2 WT clone).

(d-e) Quantification of the number of γ H2AX foci (d) or RAD51 foci per nucleus (e) two hours after 6 Gy of γ -irradiation (+IR) versus non-irradiated conditions (-IR), in DLD1 BRCA2^{+/+} cells depleted of BRCA2 (siBRCA2) or control cells (siCTRL) or BRCA2 deficient DLD1 cells (BRCA2^{-/-}) expressing BRCA2 WT or the variant T207A. *n* indicates the total number of cells counted from two independent experiments. For statistical comparison of the differences between the samples we applied a Kruskal-Wallis test followed by Dunn's multiple comparison test, the p-values show significant differences. The red line in the plot indicates the mean \pm SD, each dot represents a single focus. **(f)** Top: Scheme of the DSB-mediated gene targeting HR assay. Bottom: Frequency of mCherry positive cells in cells transfected with the promoter-less donor plasmid (AAVS1-2A-mCherry) without (-TALEN) or with (+TALEN) nucleases. Statistical significance of the difference in (f) was calculated with two-way ANOVA test with Tukey's multiple comparisons test, the p-values show the significant differences. **(g)** Model for the role of PLK1 phosphorylation of BRCA2 T207A by

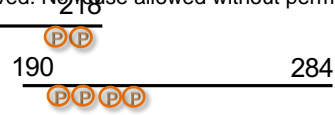
PLK1 in mitosis (see text for details). In panel 1 and 1' the two-sided arrows represent a complex between the two proteins as indicated either favored by BRCA2 WT (green) or impaired in the BRCA2 mutated form (red); in panels 2, 2', 3 and 3' blue blobs represent chromosomes, red circles represent the kinetochores, red cylinders represent the centrioles and orange lines represent the spindle microtubules.



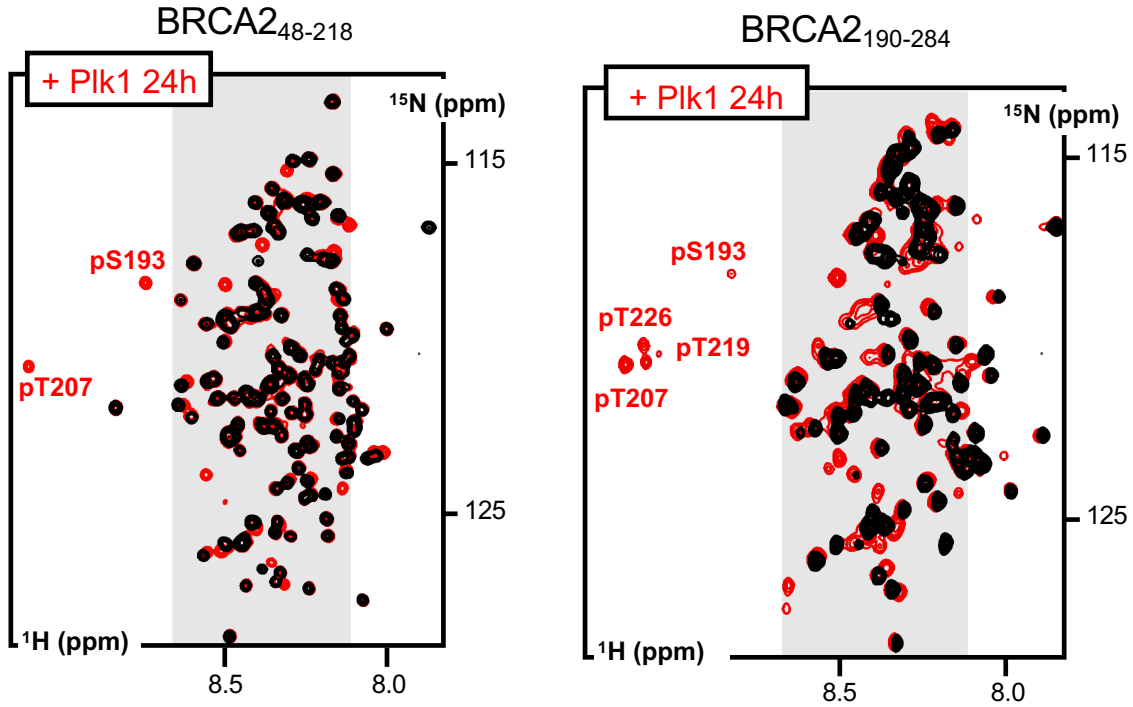
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BRCA2₄₈₋₂₁₈

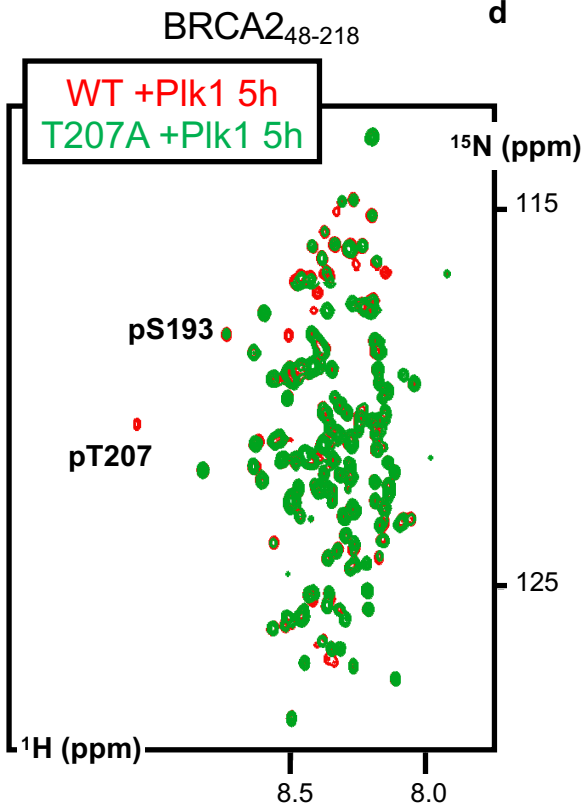
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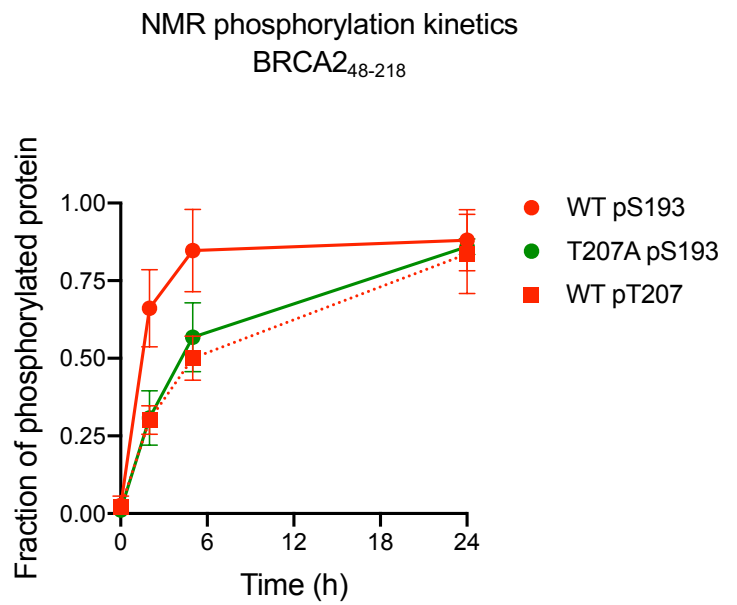
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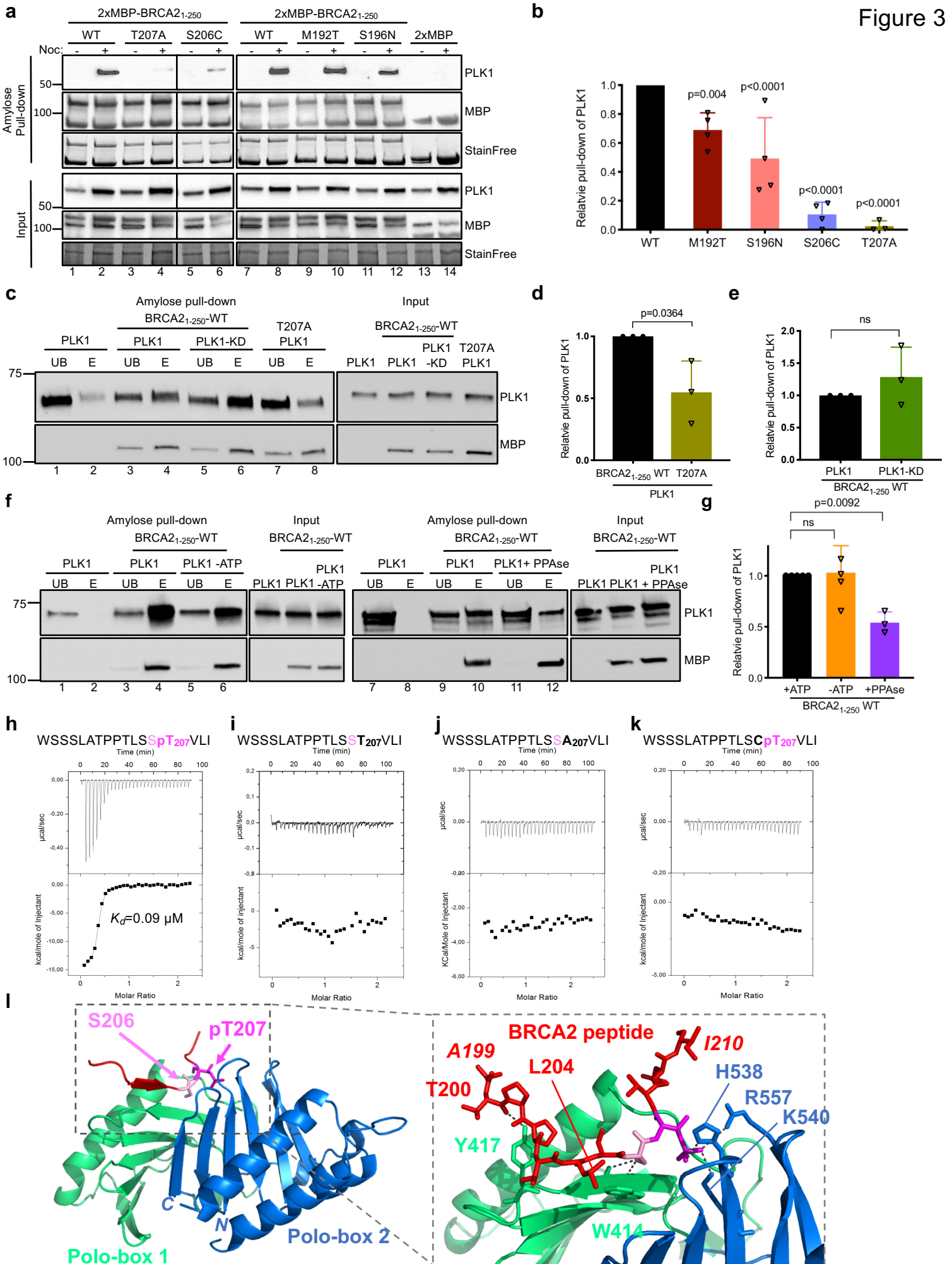


Figure 4

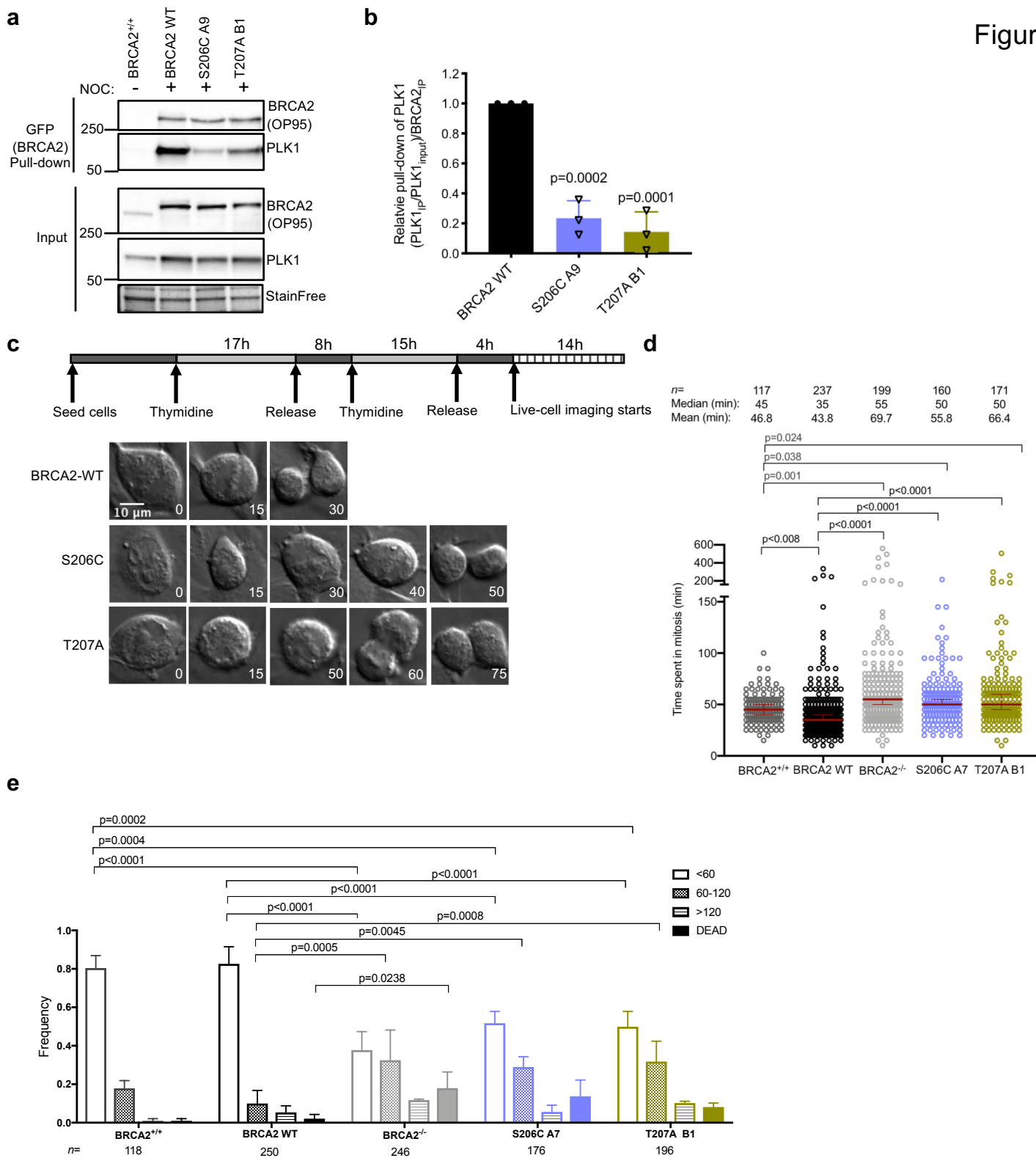


Figure 5

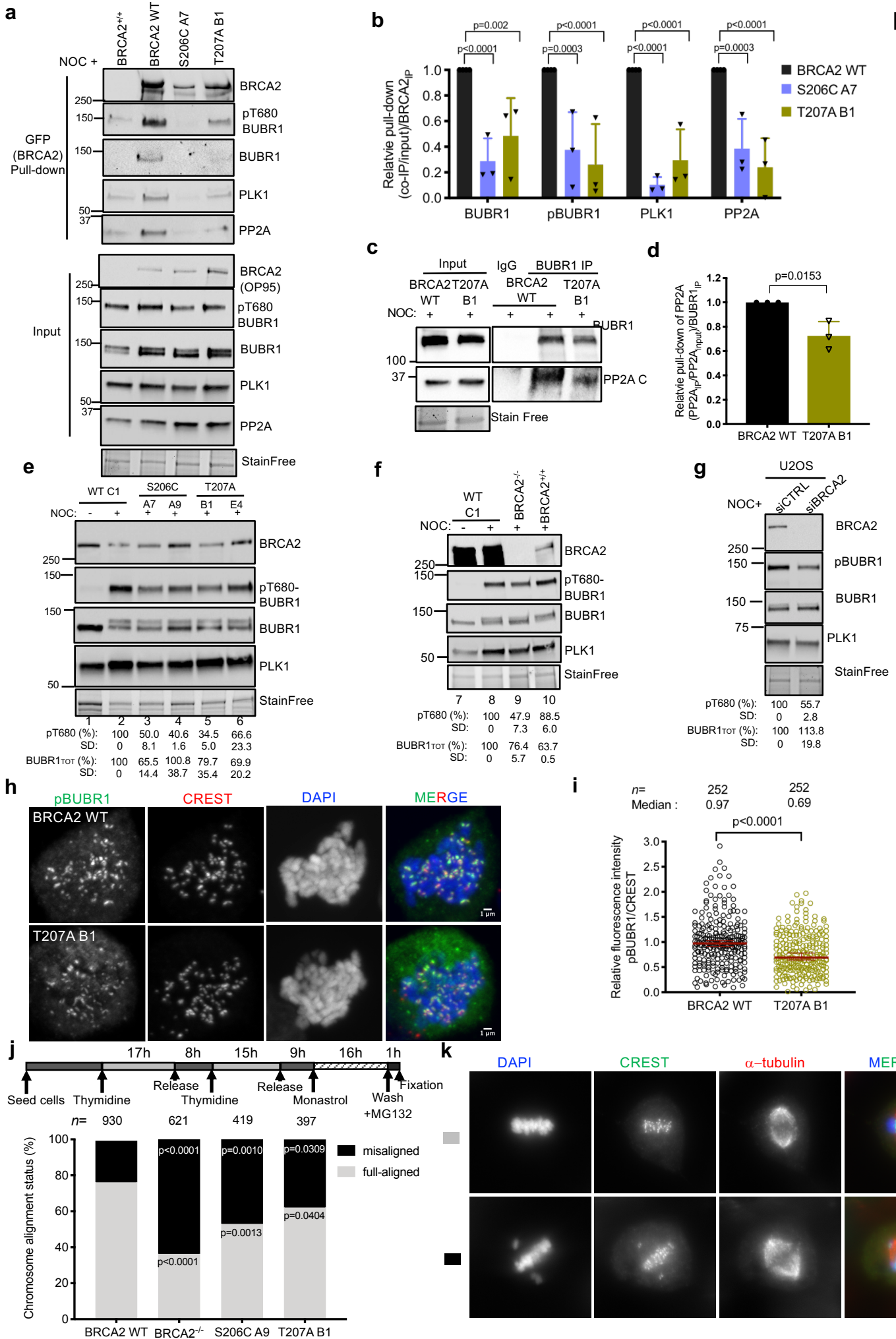


Figure 6

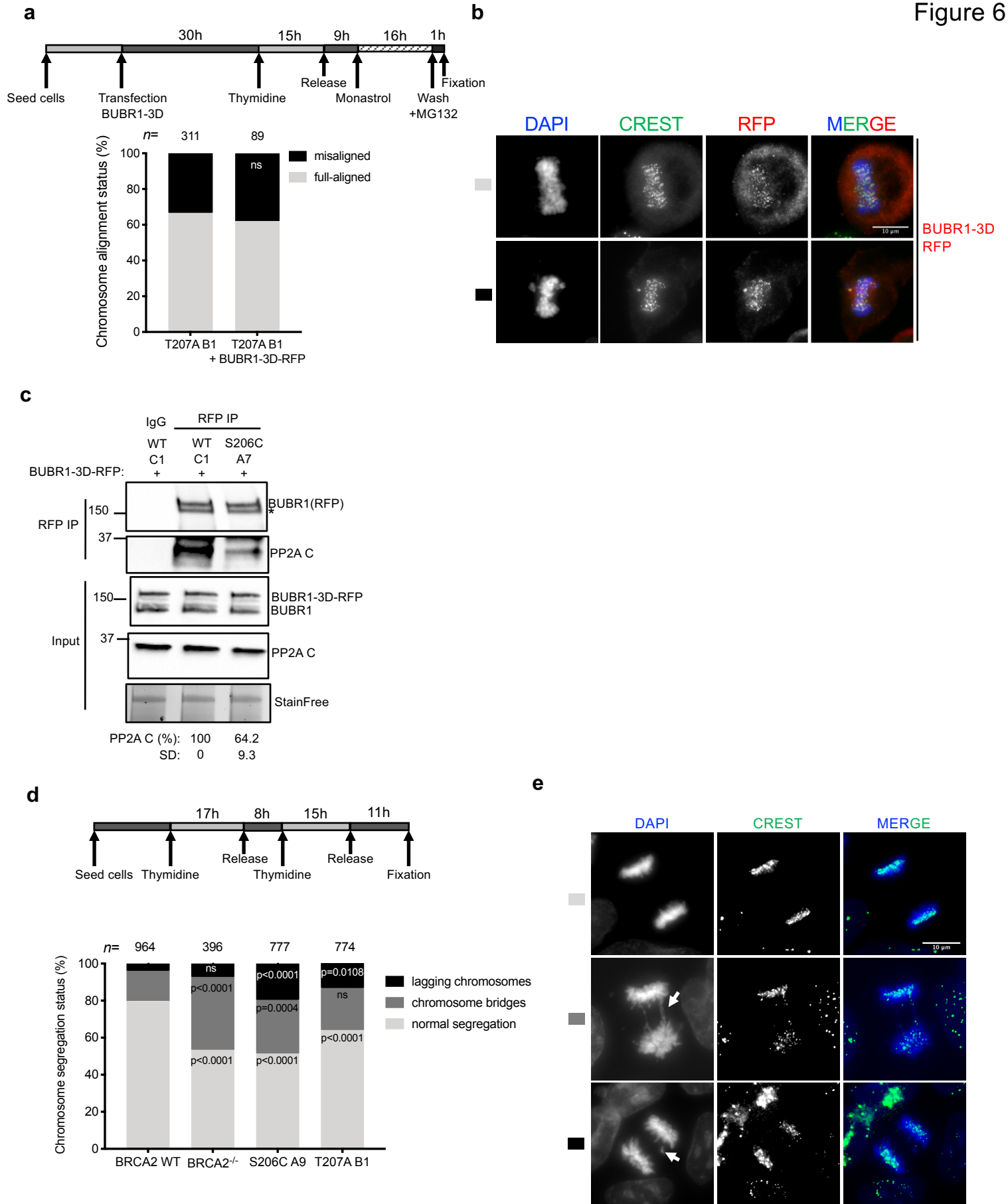
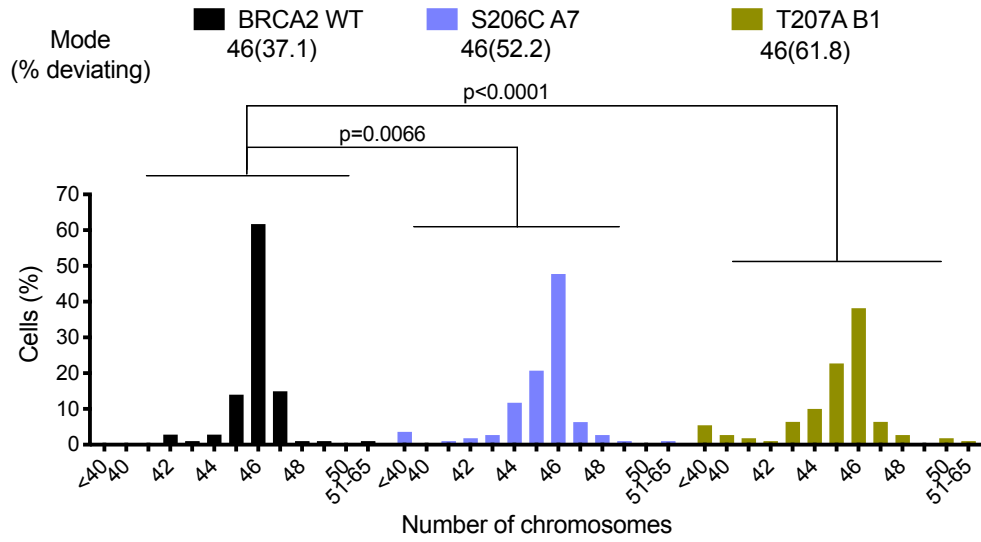
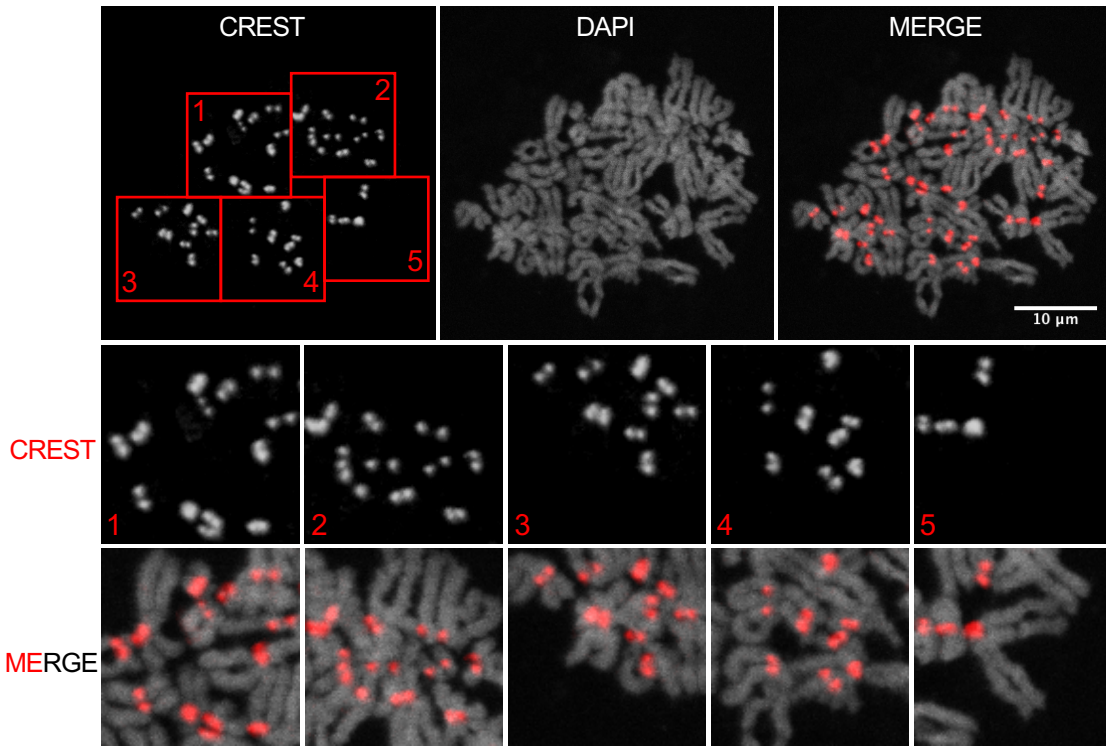


Figure 7

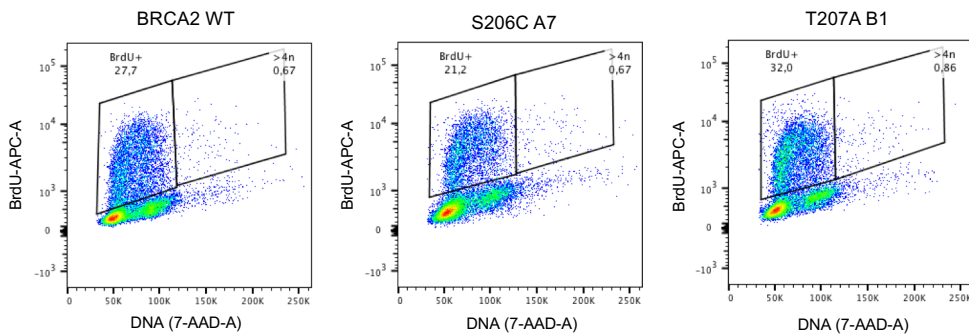
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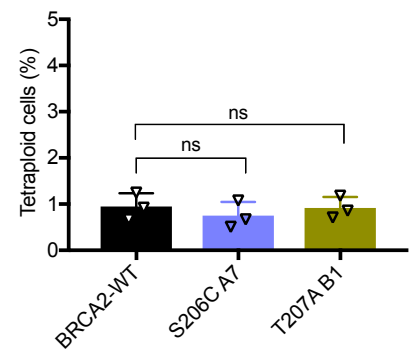


Figure 8

