Supplementary Materials: A Functional Analysis of the Unclassified Pro2767Ser *BRCA2* Variant Reveals its Potential Pathogenicity that Acts by Hampering DNA Binding and Homology-Mediated DNA Repair

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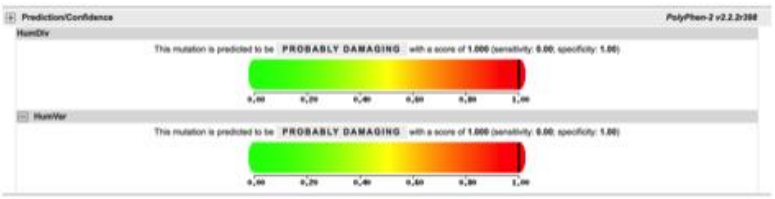
A



B

C

Coordinates	Codons	Transcript ID	Protein ID	Substitution	Region	dbSNP ID	SNP Type	Prediction	SIFT Score	Median Information Content	# Seqs at position
13,32937638,1,C/T	CCT-tCT	ENST00000380152	ENSP00000369497	P2767S	EXON CDS	novel	Nonsynonymous	DAMAGING	0	2.48	71

Figure S1. In silico pathogenicity prediction and multiple sequence alignment variant derived from PolyPhen-2 and SIFT software. (**A**) PolyPhen-2 prediction scores. (**B**) Alignments of the amino acid residue affected by the variant with evidence of high conservation between species. (**C**) SIFT prediction of variant pathogenicity.

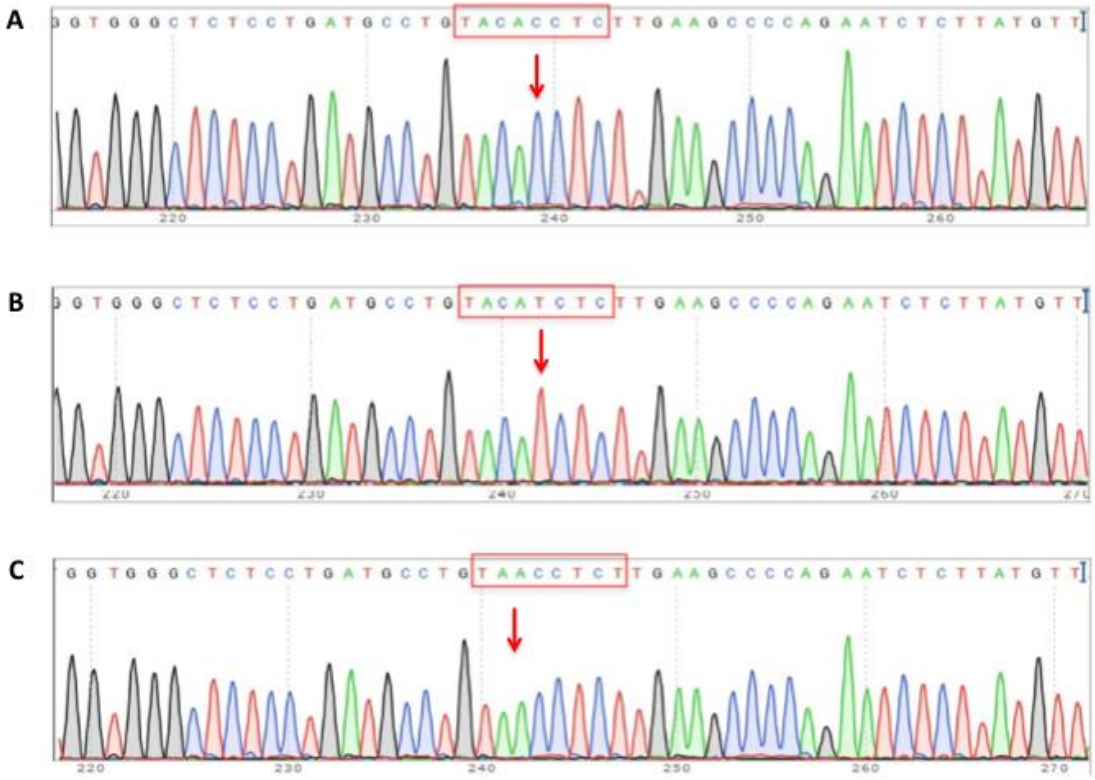


Figure S2. Mutagenesis reactions results evaluated by standard Sanger sequencing. (**A**) Electropherogram showing the wild-type nucleotide C at position 8299 of BRCA2 cDNA. (**B**) Substitution C > T to reproduce the Pro2767Ser variant at c.8299. (**C**) Deletion del C at c.8297 nucleotide position to obtain Thr2766Asnfs.

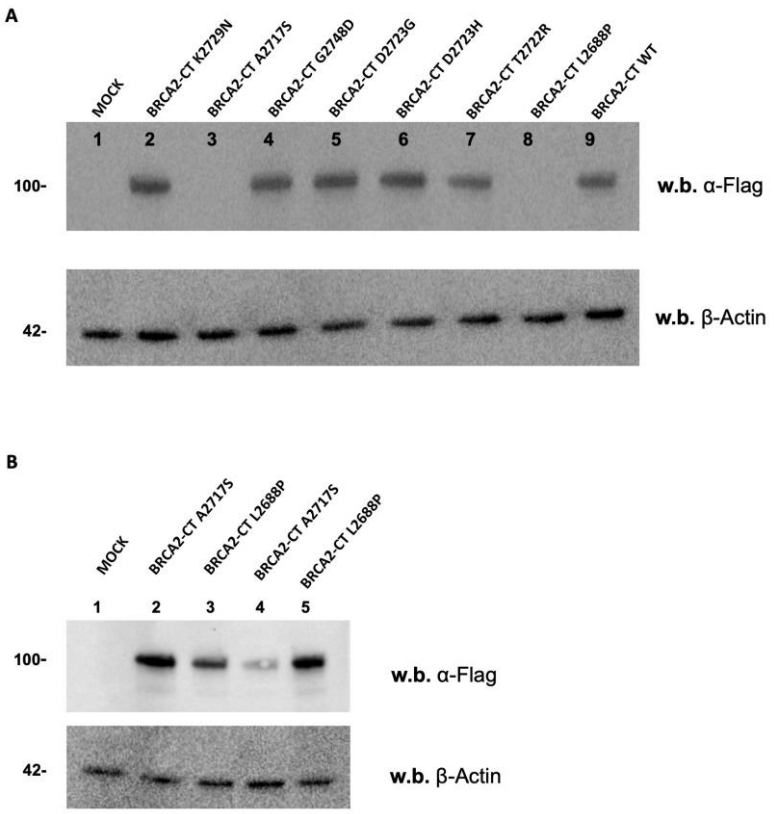


Figure S3. Western Blot analysis of all BRCA2-CT minigenes for validation set analysis. (**A**) All BRCA2-CT CTRs showed proper expression in NIH-3T3 cells. (**B**) Further transfections to assess the expression of the remaining BRCA2-CT A2717S and L2688P controls. A mutant BRCA2 protein of 95kDa was detected in all samples.

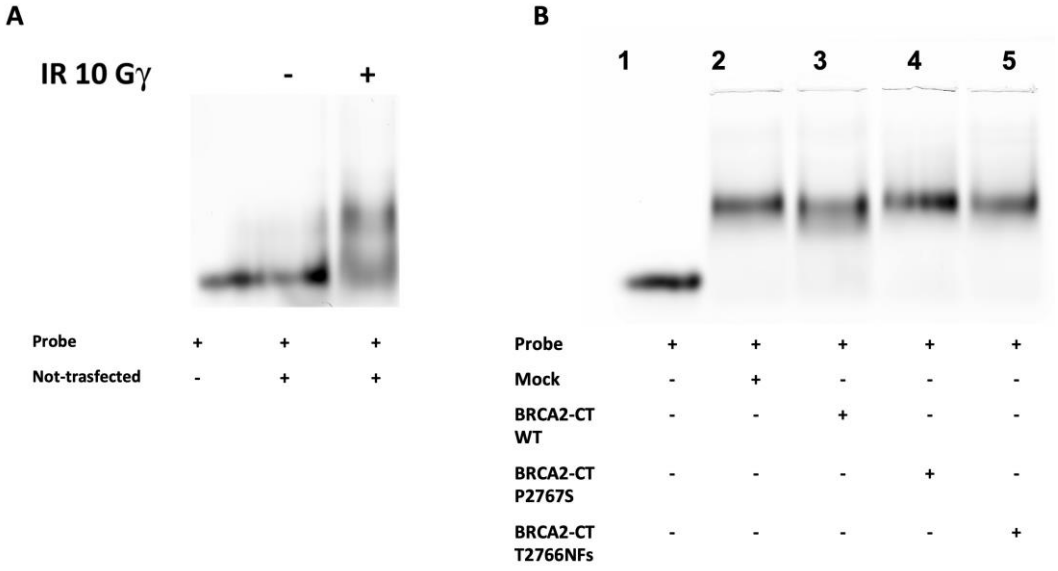


Figure S4. (**A**) In vitro ssDNA binding assay of not-transfected not-treated (NT) and not-transfected irradiated (IR) cells. Migration was not delayed in NT nuclear extracts and in the free probe but there was a slow migrating band in IR nuclear extracts, thereby demonstrating activation of the ssDNA-binding system. (**B**) In vitro ssDNA binding assay of BRCA2-CT WT, P2767S and T2766NFs without IR. All samples show similar band-migration delay, including BRCA2-CT WT-transfected cells that behaved differently respect to BRCA2-CT WT IR sample. This suggests that the damage-sensitive proteins were activated also by transfection procedures, such as by the serum stimulation or by cell replication mechanisms.

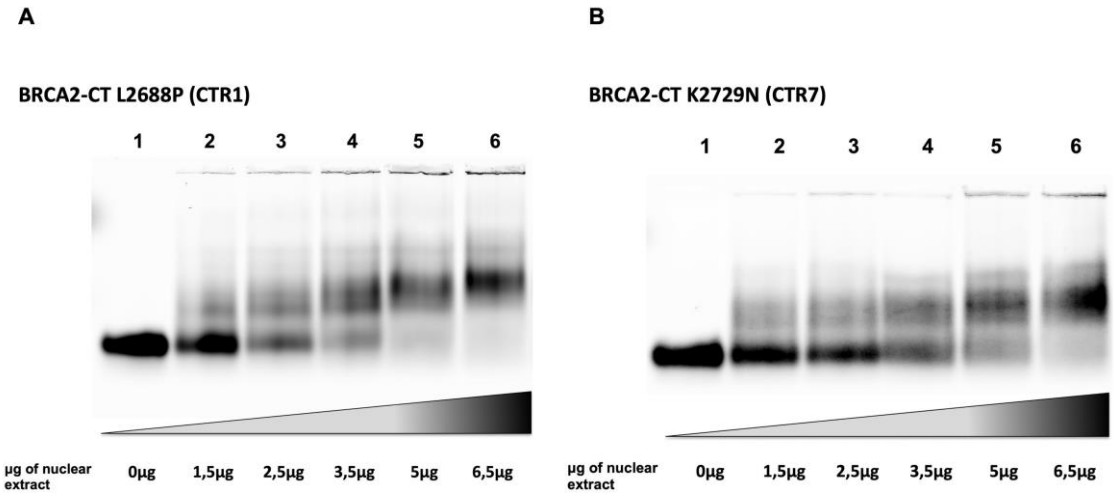


Figure S5. Dose dependent binding assay of the BRCA2-CT L2688P (**A**) and of the BRCA2-CT K2729N (**B**) samples.The ssDNA-binding assay was performed using increasing amount of the nuclear extract. For each sample was loaded: the binding reaction with only free probe, 1.5 µg, 2.5 µg, 3.5 µg, 5 µg, and 6.5 µg of nuclear extract of irradiated cells. The DNA-protein complex formation increases when more protein is present. In particular, differences between BRCA2-CT L2688P and BRCA2-CT K2729N are clearly shown from 5 up to 6.5 µg of nuclear extract (lanes 5–6).

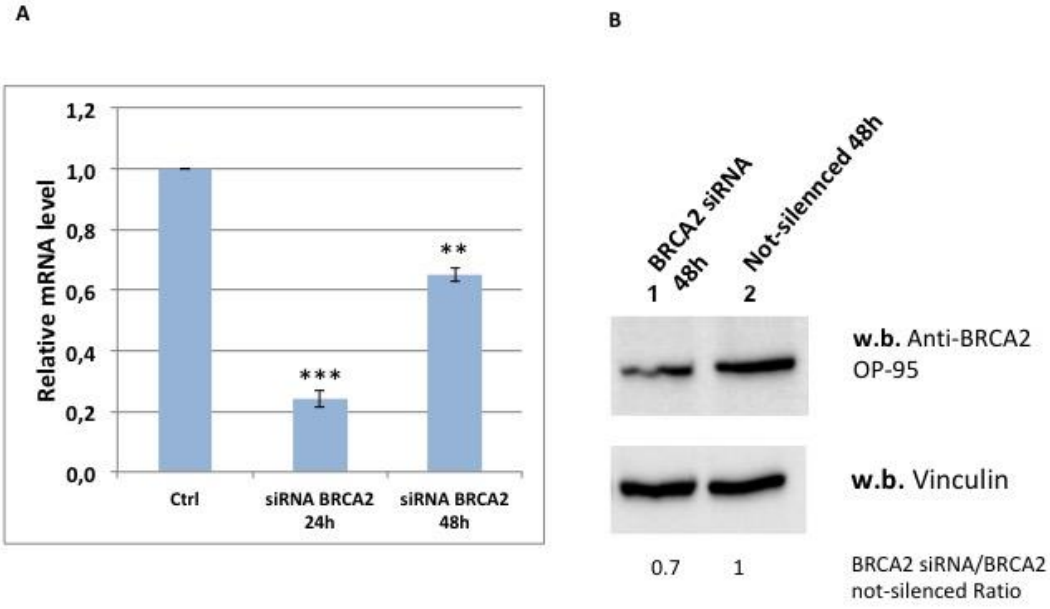


Figure S6. The RNAi results were evaluated by quantitative real-time PCR (qPCR) and Western Blot analysis. (**A**) Real-time qPCR analysis. The mRNA levels of BRCA2 were determined by real-time qPCR and normalized by the Actin mRNA level. Data are expressed as relative to the values obtained on transfection with siRNA negative control. Bars represent means ± sd. *p*-values denoted are reported as follows: ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$). (**B**) Western Blot Analysis of BRCA2 endogenous protein. The ratio of silenced BRCA2 to not-silenced signal is reported at the bottom.

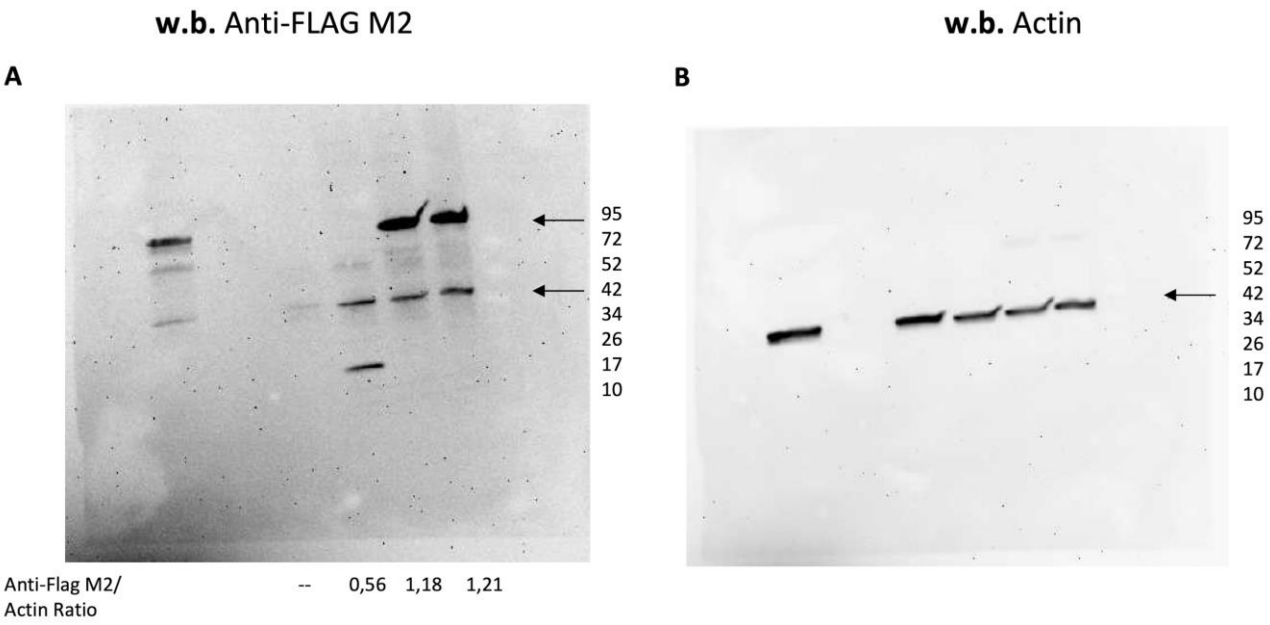


Figure S7. Figure 3C: Western blot analysis of the expression of BRCA2-CT wt, BRCA2-CT P2767S and BRCA2-CT T2766NFs proteins in NIH-3T3 cells. A mutant BRCA2 protein of 95kDa was detected in cells that contained BRCA2-CT wt and BRCA2-CT P2767S proteins; a 15kDa deleted BRCA2-CT T2766NFs protein derived from the Thr2766AsnFs mutation. On the left (A) the entire gel in which are shown the BRCA2-CT flagged bands detected by Anti-Flag M2 (mouse, 1:1000) antibody. On the right (B) the Actin was used as loading control (mouse, 1:1000). The ratio of BRCA2-Flag to the Actin signal is reported at the bottom. The gel was a 4-15% precast. Bands were detected by exposure to the ChemiDoc MP System (Bio-Rad).

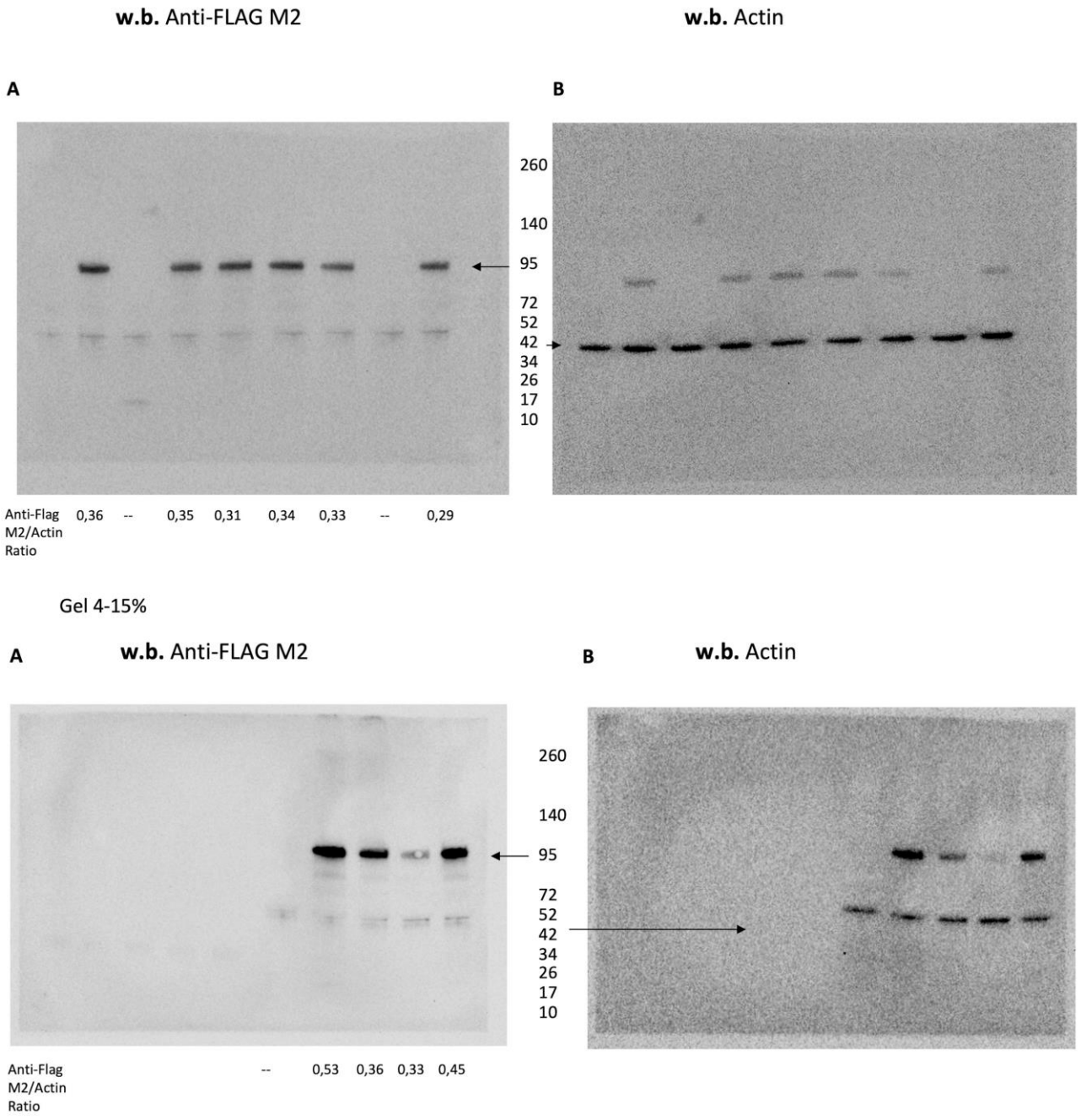


Figure S8. Figure S3 A and B: Western Blot analysis of all BRCA2-CT minigenes for validation set analysis. All BRCA2-CT CTRs showed proper expression in NIH-3T3 cells. A mutant BRCA2 protein of 95kDa was detected in all samples. Respectively, on the left the entire gels in which are shown the BRCA2-CT 95kDa flagged bands detected by Anti-Flag M2 (mouse, 1:1000) antibody. On the right the Actin was used as loading control (mouse, 1:1000). The ratio of BRCA2-Flag to the Actin signal is reported at the bottom. The gel was a 4-15% precast. Bands were detected by exposure to the ChemiDoc MP System (Bio-Rad).

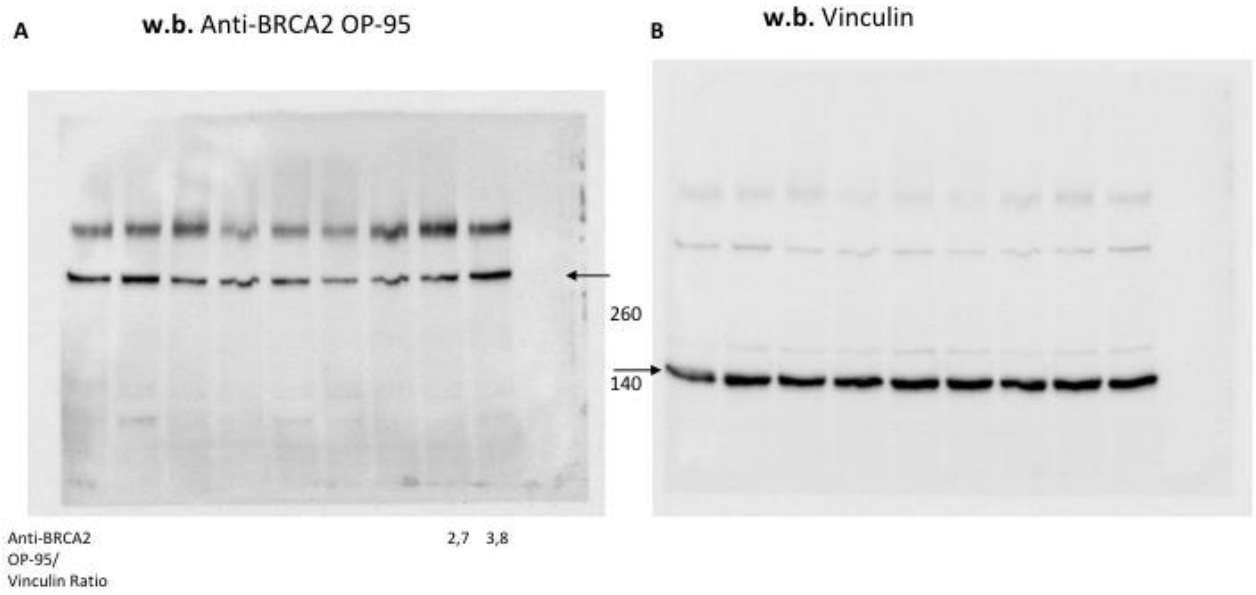


Figure S9. Figure S6 B: The RNAi results evaluated by Western Blot analysis 48 hours after transfection. On the left (A) the entire gel in which are shown the endogenous BRCA2 bands detected by Anti-BRCA2 OP-95 (Ab-1) Mouse mAb 2 (1:1000) antibody. On the right the Vinculin of about 130 kDa (goat, 1:1000) was used as loading control. The ratio of BRCA2 to the Vinculin signal is reported at the bottom. The gel was a 6% polyacrylamide gel. Bands were detected by exposure to the ChemiDoc MP System (Bio-Rad).

Table S1. Sequence of primers used for cloning and in vitro ssDNA binding reactions.

	Primer Names	Primer Sequences
a	**BRCA2_DBD _Fw_Not**	5′-AGTTCAGCGGCCGCAGTCAGAATGGAATGTGCCTTTCCTAAGGAATTTGCTA-3′
b	**BRCA2_DBD _Rev_BamHI**	5′-AGAggatccTTAGATATATTTTTTAGTTGTAATTGTGTCCTGC-3′
c	**Linear φX174 ss-DNA**	5′-GGGCGAATTGGGCCCGACGTCGCATGCTCCTCTAGACTCGAGGAATTCGGTAC CCCGGGTTCGAAATCGATAAGCTTACAGTCTCCATTTAAAGGACAAG-3′

Table S2. Sequence of primers used for qPCR analysis.

	Primer Names	Primer Sequences
a	**BRCA2_mouse_Fw**	5′-CGAGATGCAGCACAGCAGATTTAGGACCG-3′
b	**BRCA2_mouse_Rev_**	5′-CCACAGCTGTTTAAAACACCACAGAGG-3′
c	**Actin_mouse _Fw_**	5′-CTAAGGCCAACCGTGAAAAGAT-3′
d	**Actin_mouse_Rev**	5′-′GCCTGGATGGCTACGTACATG-3′