

AD _____

Award Number: DAMD17-02-1-0360

TITLE: Functional Study of the Human BRCA2 Tumor Suppressor

PRINCIPAL INVESTIGATOR: Bing Xia, Ph.D.
David M. Livingston, M.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02115

REPORT DATE: August 2005

TYPE OF REPORT: Annual Summary

20060307 110

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 01-08-2005		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 1 Aug 02 – 31 Jul 05	
4. TITLE AND SUBTITLE Functional Study of the Human BRCA2 Tumor Suppressor				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-02-1-0360	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Bing Xia, Ph.D. David M. Livingston, M.D. E-Mail: bing_xia@dfci.harvard.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dana-Farber Cancer Institute Boston, MA 02115				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT See following page for abstract.					
15. SUBJECT TERMS No subject terms provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 24	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

ABSTRACT

My research is focused on the BRCA2 protein, whose mutations have been implicated in the development of breast, ovarian, male breast, prostate, pancreatic cancers and Fanconi anemia. It is intended to elucidate some of the biological functions of BRCA2 and/or regulation of its in vivo function through generation/utilization of new reagents and identification of new BRCA2 interacting proteins. During the years of grant support, I generated an array of BRCA2 antibodies and identified/cloned a novel protein, PALB2, as a major nuclear partner and localizer of BRCA2. I have established that PALB2 is required for BRCA2 nuclear presence and its functions in homologous recombinational repair of double-strand breaks and intra-S phase damage checkpoint control. Importantly, BRCA2/PALB2 interaction is disrupted by multiple cancer-associated BRCA2 mutations. My results indicate that PALB2 is essential for the tumor suppressing functions of BRCA2 and may be a new tumor suppressor gene in its own right.

A manuscript has been written and is being submitted to Nature.

Table of Contents

Cover	
SF 298	
Table of Contents	
Introduction	4
Body	4
Key Research Accomplishments	11
Reportable Outcomes	11
Conclusions	11
References	12
Appendices	15

INTRODUCTION

Germline mutations in one allele of *BRCA2* predispose carriers to early onset breast and ovarian cancers¹⁻³. The lifetime breast cancer risks among carriers of *BRCA2* mutation range up to 80%^{4, 5}. Mutations in *BRCA2* are also implicated in pancreatic⁶, prostate⁷, male breast⁸ cancers and Fanconi anemia⁹. *BRCA2* tumors exhibit loss of heterozygosity (LOH), indicating that it functions as a typical tumor suppressor.

BRCA2 functions in cell growth, DNA repair, homologous recombination, DNA damage checkpoint control, DNA replication and cell division, as *BRCA2* deficient cells exhibit proliferation arrest^{10, 11}, hypersensitivity to DNA damaging agents^{10, 11}, genomic instability¹¹⁻¹³, defective homology-directed repair (HDR)^{14, 15}, reduced gene targeting efficiency¹⁴, radio-resistant DNA synthesis^{16, 17}, breakdown of stalled DNA replication forks¹⁸ and delayed cytokinesis¹⁹. In addition, *BRCA2* possesses transcriptional activation and coactivation activities^{20, 21}. Targeting the sensitivity of *BRCA2*-deficient tumor cells to therapeutic DNA damaging agents represents a potential avenue to treat such cancers²²⁻²⁴.

Human *BRCA2* consists of 3,418 amino acid residues but has little homology with other proteins and no known enzymatic activities. In addition to its protein partners that include Rad51^{10, 25, 26}, *BRCA1*²⁷, P/CAF^{21, 28}, DSS1²⁹, BRAF³⁵³⁰, androgen receptor (AR)²¹, Polo-like kinase Plk1³¹, FANCD2/G^{32, 33}, BCCIP α/β ³⁴ and the recently identified potential oncoprotein EMSY³⁵, *BRCA2* also binds DNA with its C-terminal OB (oligonucleotide-binding) domains³⁶. As a non-enzyme, *BRCA2* likely functions as a scaffold protein to help organize high-order complex or structure formations in aforementioned cellular processes through highly coordinated interactions with its partner proteins and DNA.

BRCA2 plays a major role in the control of the localization and function of Rad51, a key protein that coats the processed single-stranded DNA overhangs of double strand breaks and promotes the essential homologous-pairing and strand invasion during homologous recombination³⁷. The "BRC" repeats at the center of *BRCA2* directly interact with and structurally mimic Rad51's self-association motif to block its oligomerization and nucleoprotein filament formation^{38, 39}. Nevertheless, the overall control is a positive one, as *BRCA2* is required for the formation of DNA damage-induced Rad51 nuclear foci^{40, 41}, which are likely to be sites of double strand break repair, and for the mobilization of a subpopulation of nuclear Rad51 following DNA damage⁴². Furthermore, a recent biochemical study establishes that a fungi *BRCA2* homologue Brh2 recruits Rad51 to dsDNA-ssDNA junction and nucleates its filament formation⁴³. Importantly, *BRCA2* also interacts with Rad51 with a separate motif located at its C-terminus and this functional interaction is regulated by CDK-dependent phosphorylation⁴⁴.

BODY (Results, Discussion and Methods)

Identification of PALB2 as a major nuclear *BRCA2* partner

In an effort to discover new components of endogenous *BRCA2*-containing protein complexes, whole HeLa cell extract was immunoprecipitated with a *BRCA2* monoclonal antibody. As shown in Fig. 1a, the precipitates consisted of a relatively small number of

major components. Protein bands were excised from the gel and subjected to mass spectrometric analysis. As expected, the most abundant protein with the highest molecular weight and the one migrating slightly above the 39KD marker were confirmed to be BRCA2 and Rad51, respectively. Interestingly, the major band above the 97KD marker was identified as FLJ21816, a hitherto "hypothetical" protein. All three proteins could be eluted from the beads using a specific BRCA2 peptide generated after mapping the exact epitope of this monoclonal antibody (not shown). FLJ21816 was later named "PALB2" for "Partner and Localizer of BRCA2" or "PAL of B2". Other components of the complex were also identified and are currently under verification.

The putative PALB2 open reading frame consists of 1,186 amino-acid residues translating to a protein with a molecular weight of approximately 130KD. Bioinformatic analysis of PALB2 revealed no significant sequence homology with any other proteins and no well conserved motif/domain structures. Apparent homologs of PALB2 were found to exist in other vertebrates but not in lower organisms such as yeast, *C-elegans* and *Drosophila*.

The 3.5KB PALB2 cDNA was cloned into to a FLAG-HA double-tagging recombinant viral vector, transiently expressed and the tagged PALB2 was immunoprecipitated. The precipitate was found to contain large amount of both the predicted 130KD protein and BRCA2 by western analysis (not shown). Multiple rabbit polyclonal antibodies were generated and found to co-immunoprecipitate substantial amount of PALB2 and BRCA2 from lysates of Hela, U2OS and 293 tumor cells as well as non-tumor MCF10A breast epithelial cells and WI38 primary fibroblasts. As an example, an antibody generated against the N-terminal 200 residues (α PALB2N200) was able to effectively pull down not only PALB2 but also BRCA2 from all three cell lysates (Fig. 1b). Reciprocally, another BRCA2 monoclonal antibody (TP15) effectively pulled down PALB2 (Fig. 1b).

PALB2 was found to be a nuclear protein that could only be extracted with high salt (Fig. 1c), indicating that it is stably associated with the chromatin or nuclear matrix. BRCA2 was reproducibly found to be distributed in both cytoplasmic and nuclear fractions, with most nuclear BRCA2 only extractable with high salt. BRCA1 was mainly nuclear with a minor but clear cytoplasmic presence. Next we sequentially extracted U2OS and MCF10A cells with low and high salt lysis buffers and immunodepleted either BRCA2 or PALB2 from the high salt fractions (equivalents of the NS420 fraction in Fig. 1c). As shown in Fig. 1d, BRCA2 antibody not only depleted BRCA2 but also pulled down a significant fraction of PALB2. Strikingly, PALB2 antibody completely depleted both PALB2 and BRCA2, indicating that all BRCA2 stably bound to chromatin or/and nuclear matrix is associated with PALB2. BRCA1 amount was not significantly affected since only a small fraction of cellular BRCA1 is associated with BRCA2. Collectively, above results unambiguously demonstrate that PALB2 is a major physiological nuclear partner of BRCA2.

Colocalization of PALB2 and BRCA1 and BRCA2 in Nuclear Foci

BRCA2 and BRCA1 colocalize in nuclear foci in the S-phase of the cell cycle and those induced by DNA damage²⁷. We asked whether PALB2 colocalizes with BRCA1/2 in

these nuclear structures. As PALB2 antibodies able to produce clear nuclear staining remained unavailable, we generated a T98G glioblastoma cell line stably expressing PALB2 from the afore-mentioned viral vector. As shown in Fig. 2a, the N-terminally-tagged PALB2 was found to be exclusively nuclear and concentrate in a number of foci, the majority of which also contained BRCA1. Like BRCA1 foci, PALB2 foci were quickly dispersed following ionizing radiation, indicating that DNA damage initiates a dramatic redistribution of these proteins. DNA damage-induced foci dispersal was also observed for BRCA2 (not shown). Later, both PALB2 and BRCA1 gradually reconcentrated to a same set of presumably new focal locations likely to be sites of DNA repair and/or persistent damage.

Over the course of this study, we generated more than 10 polyclonal antibodies as well as multiple monoclonal antibodies against BRCA2. Among these and all the commercial BRCA2 antibodies we obtained, only one (α BRCA2F8) was able to produce convincing BRCA2 nuclear staining that could be eliminated with BRCA2 specific siRNAs. With α BRCA2F8, we observed colocalization of the tagged PALB2 and BRCA2 in above T98G cells after gamma-irradiation (Fig. 2b). Only a small percentage of these cells contained distinct BRCA2 foci both before and after DNA damage, and colocalization of PALB2 and BRCA2 was mainly observed after damage. Since practically all chromatin-bound BRCA2 is associated with PALB2 and more substantial BRCA1/BRCA2 colocalization occurs in T98G cells without exogenous PALB2 expression (not shown), we infer that significant colocalization between PALB2 and BRCA2 occur in cells. It is possible that proper localization of BRCA2 in above cells is perturbed by the N-terminal tagging or overexpression of PALB2.

Requirement of PALB2 for BRCA2 and Rad51 localizations

To gain insights into the functional relationship between PALB2 and BRCA2, we first knocked down PALB2 and examined the nuclear localization of BRCA2 by staining. U2OS cells treated with control siRNA showed clear nuclear BRCA2 localization and colocalization with BRCA1 (Fig. 3a). Interestingly, cells treated with PALB2 siRNA showed greatly reduced BRCA2 nuclear staining while BRCA1 was not significantly affected 60 hr after transfection (Fig. 3a). Essentially the same observations were made with Rad51 (Fig. 3b), whose focus formation depends on BRCA2⁴⁰. In above experiments, BRCA1 staining also served to identify S-phase cells which should also be BRCA2 and Rad51 staining positive.

To distinguish whether the decreased BRCA2 and Rad51 nuclear staining signals observed after PALB2 loss is caused by a reduction of their amounts in the nucleus or a failure of antibody staining due to their potential conformational changes, we fractionated siRNA treated cells into cytoplasmic and nuclear fractions and examined the protein amounts in both compartments. As shown in Fig. 3c, comparing to cells treated with control siRNA, cells treated with PALB2 siRNA showed a major loss of nuclear BRCA2 without any discernible increase in the cytoplasm; Rad51 followed the same trend while BRCA1 was barely affected. Next, in a parallel experiment we used sequential salt extractions to fractionate above cells into soluble (cytoplasmic and nuclear soluble) and insoluble (chromatin and matrix-bound) fractions. As shown in Fig. 3d, PALB2 siRNA

treatment led to a substantial reduction in the amounts of insoluble BRCA2 but not that of BRCA1. These results are in accordance with the staining data and enable us to conclude that PALB2 is required for the nuclear presence of BRCA2 and to a lesser but significant extent for that of Rad51 as well.

In control siRNA treated cells, a small but significant amount of BRCA1 coimmunoprecipitated with BRCA2 almost exclusively in the high salt fraction (Fig. 3e, lane 2) although there was more total BRCA1 in the low salt fraction (Fig. 3d). PALB2/BRCA2 interaction also occurred primarily in the insoluble fraction (Fig. 3e) where the bulk of PALB2 resided. In contrast, Rad51/BRCA2 association was mainly detected in the soluble fraction, which is however consistent with the fact that the vast majority of Rad51 was freely soluble (Fig. 3d). Similar observations were also made with untransfected Hela and U2OS cells (not shown). In PALB2 knockdown cells, clearly less BRCA2 was present in the high salt fraction and its associated BRCA1 became barely detectable. These results indicate that PALB2/BRCA2 and BRCA1/BRCA2 interactions occurs primarily in chromatin or nuclear matrix while the majority of Rad51/BRCA2 complexes are more mobile and salt sensitive.

PALB2 as an enabler of BRCA2's nuclear functions

The fact that PALB2 is required for BRCA2's nuclear existence suggests that it may be essential for BRCA2 nuclear functions. To test this, we first asked whether loss of PALB2 abrogates the activation of the intra-S phase DNA damage checkpoint, as BRCA2 mutant cells manifest radio-resistant DNA synthesis. U2OS cells treated with control or PALB2 siRNAs were pulse-labeled with BrdU before and after ionizing radiation and the BrdU incorporation was analyzed by flow cytometry. As shown in Figure 4a, no significant difference in DNA synthesis was observed before DNA damage. Notably, 6 hr after radiation, while DNA synthesis of S-phase control cells was strongly inhibited (panel 2, below the line), a significant S-phase population of PALB2 siRNA treated cells still underwent intensive DNA replication, indicating that PALB2 is critical for an efficient S-phase checkpoint.

Subsequently we assayed the major established function of BRCA2 in homology-directed repair (HDR) of double strand breaks. U2OS cells stably integrated with a single copy of DR-GFP repair reporter (DR-U2OS cells) were treated with PALB2 siRNAs and their repair capabilities were tested. As shown in Fig. 4b, PALB2 knockdown substantially reduced the repair efficiency by 2 to 3 fold. The BRCA2 siRNA, as a positive control, produced a more dramatic inhibition, possibly due to its more effective knockdown (not shown). To further validate PALB2's role in double strand break repair, we tested the effect of PALB2 loss on cellular sensitivity to mitomycin C (MMC), which causes interstrand crosslinking eventually leading to double strand breaks. Consistent with the fact that known BRCA2 deficient cells are invariably sensitive to this agent, BRCA2 knockdown markedly sensitized Hela cells to the damage (Fig. 4c). Treatment with two PALB2 siRNAs both significantly sensitized cells to MMC, with the "smartpool" (218S) being as potent as the BRCA2 siRNA. Taken together, above results strongly indicate that PALB2 is an essential enabler of BRCA2's nuclear functions.

Disruption of BRCA2/PALB2 interaction and possible cancer-predisposition

To address the clinical relevance of the BRCA2/PALB2 interaction, we attempted to isolate point mutations of BRCA2 that disrupt the interaction and ask whether such mutated BRCA2 proteins remain functional. First, the exact region of BRCA2 required for PALB2 interaction was determined. As shown in Fig. 5, after six rounds of domain mapping, such a determinant was narrowed down to the extreme N-terminal section of BRCA2 (between residues 10 and 40) encoded by exons 2 and 3 of the gene. Interestingly, this is the very part of BRCA2 that possess transcription activation activity and contains the binding site for the potential oncoprotein EMSY.

Next, we searched the breast cancer information core (BIC) database for possible BRCA2 mutations and found eight cases of unclassified variants representing six different sequence alterations in this small region (Fig. 6a). To determine whether these variants affect BRCA2's ability to interact with PALB2, we generated these mutations in a construct expressing the N-terminal 60 amino acids of BRCA2 (B2N60) fused to the C-terminus of the Gal4 DNA-binding domain (DBD) which contains an intrinsic nuclear localization signal. PALB2 binding abilities of two variants (W31R and W31C) were found to be completely lost and that of another (G25R) was greatly diminished (Fig. 6b). Two other variants (P9L and Y42C) occurring immediately outside the PALB2 interacting domain were also included in the study and found to have no effect. All above fusion proteins were expressed at similar levels (Fig. 6b, lower panel) and all localized exclusively to the nucleus as examined by immunostaining with an anti-Gal4 monoclonal antibody (not shown). Under the same condition, we failed to detect EMSY-BRCA2 interaction.

Finally, we asked if above BRCA2 variants found in breast cancer patients are functional in homologous recombination and repair. As a pilot experiment showed that the wildtype Gal4-B2N60 was strongly dominant negative when overexpressed in the DR-U2OS cells (Fig. 6c), presumably by titrating PALB2 and thus disrupting the endogenous PALB2/BRCA2 interaction essential in the process, we examined such activities of all variants using this assay system. It was found that, similar to the wildtype, variants still able to interact with PALB2 (P9L, F12V, R18H, I27V and Y42C) invariably remained strongly dominant negative while remarkably, the three sequence alterations (G25R, W31R and W31C) that disrupt BRCA2/PALB2 interaction all led to complete loss of the inhibiting activity (Fig. 6c). The exact correlation between the variants' abilities to bind PALB2 and their activities to inhibit repair in DR-U2OS cells further establishes the essential enabling role of PALB2 for BRCA2 nuclear functions. These results also indicate that G25R, W31R and W31C are cancer-predisposing loss of function BRCA2 mutations.

Discussion

Our results establish that PALB2, as a "fulltime" partner of chromatin-bound BRCA2, plays a crucial role in homologous recombination/double strand break repair and S-phase checkpoint control. Possibly such a role is entirely mediated by its ensuring BRCA2's nuclear presence. Yet it can not be ruled out that PALB2 has a separate function(s) in repair and checkpoint maintenance in addition to holding much of BRCA2 in the nucleus.

Interestingly, while nuclear BRCA2 appears to be dependent of PALB2, the opposite is not true, as PALB2 amount and chromatin association were not significantly affected by knockdown of BRCA2 (not shown).

How PALB2 ensures BRCA2's nuclear presence remains to be answered. We observed a strong reduction of nuclear BRCA2 amount after PALB2 loss without a noticeable increase of cytoplasmic BRCA2. This together with BRCA2's possession of at least two functional nuclear localization signals⁴⁵ suggest that PALB2 may not be required for BRCA2 to enter the nucleus, although such a possibility can not be ruled out. It is more likely that PALB2 plays a role in keeping nuclear BRCA2 stable. To test this possibility we studied the stability of nuclear BRCA2 following PALB2 knockdown. However, presumably due to the fact that the amount of remaining nuclear BRCA2 is small after PALB2 knockdown and that such BRCA2 is still associated with PALB2 (Fig. 3e), our result was unclear. The exact mechanism underlying the loss of nuclear BRCA2 remains a priority of future study.

Importantly, we mapped the BRCA2 domain responsible for PALB2 interaction to a small region in which six different potential cancer-causing mutations were found in breast cancer patients. Three of them were unable to interact with PALB2 and only the same three showed complete loss of function in our dominant negative repair assay, suggesting that BRCA2 tumor-suppressing function relies on its ability to associate with PALB2. Intriguingly, the PALB2-binding domain encoded by exons 2 and 3 overlaps with the transactivation core sequence and the EMSY-binding site^{20, 35}. This region is essential for BRCA2's tumor suppressor function, as an in-frame skipping of exon 3 encoding only 83 residues has been identified as the causative mutation in a breast/ovarian cancer family⁴⁶. It is plausible that cancers in above family are the outcome of the inability of the mutant BRCA2 to interact with PALB2 and therefore its loss of nuclear functions including the transactivating capability. It is also tempting to speculate that if EMSY, when amplified, significantly binds BRCA2, it may function as an oncoprotein by sequestering BRCA2 from interacting with PALB2 and thus abrogating its nuclear functions. Being critical in the repair of double strand breaks and checkpoint maintenance, PALB2 may also be a tumor suppressor in its own right. Efforts to identify and study potential PALB2 mutations in breast cancer patients are currently underway.

Methods

Cells cultures

Hela, U2OS, 293T and T98G cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). WI38 cells were grown in DMEM with 15% FBS. MCF10A cells were grown as described⁴⁷. All cells were grown at 37°C in a humidified incubator with 10% CO₂.

Antibodies

α BRCA2F8 and α BRCA2F9 are rabbit polyclonal antibodies raised against C-terminal fragments of BRCA2 (residues 2,800-3,000 and 3,265-3,418, respectively) as GST fusion proteins. α PALB2N200 was generated against the N-terminal 200 residues of PALB2.

These antibodies were affinity-purified after GST reactive antibodies were removed by multiple passages of the sera through an immobilized GST column. TP15 (BRCA2) was a mouse monoclonal raised against the N-terminal 1,000 residues of BRCA2. Monoclonal Ab-1 (BRCA2) and Ab-4 (BRCA1) were purchased from Oncogene Research Products. SD118 (BRCA1) was described before⁴⁸. Polyclonal anti-BRCA1 (07-434) and anti-Rad51 (FBE) were purchased from Upstate and BD Pharmingen, respectively. Monoclonal anti- α -Tubulin, HA11 and anti-Gal4(RK5C1) were purchased from Sigma, Covance and Santa Cruz, respectively. FITC-conjugated anti-BrdU monoclonal antibody was purchased from DB Biosciences.

Plasmids and siRNAs

The recombinant retroviral vector used to generate T98G cells stably expressing PALB2 was constructed by cloning RT-PCR amplified PALB2 cDNA into pOZ-FH-N (from Dr. Y. Nakatani). For domain mapping, BRCA2 cDNA fragments were PCR-amplified and cloned into a FLAG-HA double tagging vector modified from pCMV-Tag1 (Stratagene). For HDR-inhibition assay, the N-terminal 60 residues of BRCA2 was cloned into pCMX-Gal4(N) (from R. Evans). Site-directed mutagenesis was performed following Stratagene's "QuickChange" protocol. siRNAs were purchased from Dharmacon Inc. or Qiagen Inc.. The sense sequences of NSC (non-specific control), 2182 (PALB2), BRCA1 and BRCA2 siRNAs are UUCGAACGUGUCACGUCAAdTdT, CUUAGAAGAGGACCUUAUUdTdT, CCUAUCGGAAGAAGGCAAGdTdT and GAAGAAUGCAGGUUAAUAdTdT, respectively. 218S (PALB2 custom "smartpool") was synthesized by Dharmacon. Multiple other siRNAs for each of BRCA2, PALB2 and BRCA1 were also used and similar effects were observed.

Cell extracts and fractionations

NETN buffers (20mM Tris (pH7.5 at 25°C), 1mM EDTA, 0.5% NP-40, containing 100, 300 or 420mM NaCl) were used for extract preparations and cell fractionations. Extracts generated by direct lysis/extraction of whole cells with NETN300 or NETN420 were designated as W300 and W420, respectively. For salt fractionation, cells were first lysed with NETN100 and the supernatant was taken as S100. The pellet was then extracted with NETN420 and the supernatant was taken as S4200. The final pellet was resuspended in same volume of NETN420 and saved as P420. To separate cytoplasm and nuclei, cells were first swollen in hypotonic buffer for 10 min on ice and then broken with a tissue homogenizer. Supernatant (cytoplasm) was taken as CYT and the pellet as WN (whole nuclei). If necessary, nuclei were further sequentially extracted with NETN100 and NETN420 to yield NS100, NS420 and NP420 (pellet). All fractionations were carried out in the same volume. When whole cells, nuclei or any pellets were to be analyzed by Western blotting, they were sonicated before SDS sample buffer was added.

Transfections and immunoprecipitations

FuGENE 6 (Roche) was used for DNA and Oligofectamine (Invitrogen) for siRNA transfections. In transient expression/immunoprecipitation experiments, 293T cells were transfected in 6 well plates. 36 to 48 hr after transfection cells were harvested and whole cells extracts prepared in 300 μ l NETN420. FLAG-tagged fragments were precipitated with M2-agarose beads and Gal4-BRCA2N60 fusions were precipitated with RK5C1 bound to protein A beads overnight at 4°C.

Generation of U2OS/DR-GFP reporter cells

U2OS/DR-GFP reporter cells were generated by selection of stable clones following electroporation of the DR-GFP repair substrate⁴⁹ DNA into U2OS cells. Hygromycin-resistant clones were screened by Southern blotting to select those containing a single copy of the substrate. A single clone was used in the study.

Recombination/repair assay

To test the effect of siRNA knockdowns, cells were first transfected with siRNAs using Oligofectamine (Invitrogen) in 6-well plates at approximately 30-40% confluence; 24 hr later the culture medium was refreshed; another 24 hr later, cells were transfected with I-SceI expression plasmid (2 μ g per well); 72 hr after the second transfection, cells were trypsinized and the single cell suspensions were analyzed by flow cytometry on a Becton Dickinson FACScan. In the dominant negative assay, cells in 6-well plates at 50-60% confluence were co-transfected with each of the Gal4-BRCA2N60 expression constructs together with I-SceI expression plasmid (2 μ g each per well) and cells were harvested and analyzed 72 hr later.

Mitomycin C sensitivity assay

Hela cells were treated with siRNAs for 48 hr before seeded into 96 wells plates at a density of 3,000 cells per well. 4 hr after seeding, Mitomycin C (MMC) was added to final concentrations of 0, 5, 10, 15 and 20ng/ml. 96 hr later, cell survivals were measured using a MTT assay kit (Invitrogen).

KEY RESEARCH ACCOMPLISHMENTS:

- Generated an array of BRCA2 antibodies
- Identified and cloned PALB2, a completely novel protein, as a major BRCA2-interacting protein
- Generated several PALB2 polyclonal antibodies
- Established following:
 1. PALB2 is a major physiological nuclear BRCA2 partner
 2. PALB2 is required for the BRCA2 nuclear presence/localization
 3. PALB2 is essential for BRCA2 nuclear functions
 4. BRCA2/PALB2 interactions are disrupted by multiple cancer-associated BRCA2 mutations, indicating that PALB2 is essential for BRCA2 tumor-suppressing function.
- A manuscript has been written and is being submitted to Nature.

REPORTABLE OUTCOMES: N/A

CONCLUSIONS

During the years of grant support, I generated an array of BRCA2 antibodies and identified/cloned a novel protein, PALB2, as a major nuclear partner and localizer of

BRCA2. I have established that PALB2 is required for BRCA2 nuclear presence and its functions in homologous recombinational repair of double-strand breaks and intra-S phase damage checkpoint control. Importantly, BRCA2/PALB2 interaction is disrupted by multiple cancer-associated BRCA2 mutations. My results indicate that PALB2 is essential for the tumor suppressing functions of BRCA2 and may be a new tumor suppressor gene in its own right. A manuscript has been written and is being submitted to Nature.

REFERENCES

1. Welsh, P. L. & King, M. C. BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. *Hum Mol Genet* 10, 705-13 (2001).
2. Venkitaraman, A. R. Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 108, 171-82 (2002).
3. Sowter, H. & Ashworth, A. BRCA1 and BRCA2 as ovarian cancer susceptibility genes. *Carcinogenesis* (2005).
4. Narod, S. A. Modifiers of risk of hereditary breast and ovarian cancer. *Nat Rev Cancer* 2, 113-23 (2002).
5. King, M. C., Marks, J. H. & Mandell, J. B. Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science* 302, 643-6 (2003).
6. Lowenfels, A. B. & Maisonneuve, P. Risk factors for pancreatic cancer. *J Cell Biochem* 95, 649-56 (2005).
7. Simard, J. et al. Prostate cancer susceptibility genes: lessons learned and challenges posed. *Endocr Relat Cancer* 10, 225-59 (2003).
8. Couch, F. J. et al. BRCA2 germline mutations in male breast cancer cases and breast cancer families. *Nat Genet* 13, 123-5 (1996).
9. Howlett, N. G. et al. Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* 297, 606-9 (2002).
10. Sharan, S. K. et al. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature* 386, 804-10 (1997).
11. Patel, K. J. et al. Involvement of Brca2 in DNA repair. *Mol Cell* 1, 347-57 (1998).
12. Yu, V. P. et al. Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. *Genes Dev* 14, 1400-6 (2000).
13. Tutt, A. et al. Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification. *Curr Biol* 9, 1107-10 (1999).
14. Moynahan, M. E., Pierce, A. J. & Jasin, M. BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol Cell* 7, 263-72 (2001).
15. Xia, F. et al. Deficiency of human BRCA2 leads to impaired homologous recombination but maintains normal nonhomologous end joining. *Proc Natl Acad Sci U S A* 98, 8644-9 (2001).
16. Kraakman-van der Zwet, M. et al. Brca2 (XRCC11) deficiency results in radioresistant DNA synthesis and a higher frequency of spontaneous deletions. *Mol Cell Biol* 22, 669-79 (2002).

17. Wang, X., Andreassen, P. R. & D'Andrea, A. D. Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. *Mol Cell Biol* 24, 5850-62 (2004).
18. Lomonosov, M., Anand, S., Sangrithi, M., Davies, R. & Venkitaraman, A. R. Stabilization of stalled DNA replication forks by the BRCA2 breast cancer susceptibility protein. *Genes Dev* 17, 3017-22 (2003).
19. Daniels, M. J., Wang, Y., Lee, M. & Venkitaraman, A. R. Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. *Science* 306, 876-9 (2004).
20. Milner, J., Ponder, B., Hughes-Davies, L., Seltmann, M. & Kouzarides, T. Transcriptional activation functions in BRCA2. *Nature* 386, 772-3 (1997).
21. Shin, S. & Verma, I. M. BRCA2 cooperates with histone acetyltransferases in androgen receptor-mediated transcription. *Proc Natl Acad Sci U S A* 100, 7201-6 (2003).
22. Bryant, H. E. et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434, 913-7 (2005).
23. Farmer, H. et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917-21 (2005).
24. Turner, N., Tutt, A. & Ashworth, A. Targeting the DNA repair defect of BRCA tumours. *Curr Opin Pharmacol* (2005).
25. Wong, A. K., Pero, R., Ormonde, P. A., Tavtigian, S. V. & Bartel, P. L. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene *brca2*. *J Biol Chem* 272, 31941-4 (1997).
26. Chen, P. L. et al. The BRC repeats in BRCA2 are critical for RAD51 binding and resistance to methyl methanesulfonate treatment. *Proc Natl Acad Sci U S A* 95, 5287-92 (1998).
27. Chen, J. et al. Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol Cell* 2, 317-28 (1998).
28. Fuks, F., Milner, J. & Kouzarides, T. BRCA2 associates with acetyltransferase activity when bound to P/CAF. *Oncogene* 17, 2531-4 (1998).
29. Marston, N. J. et al. Interaction between the product of the breast cancer susceptibility gene BRCA2 and DSS1, a protein functionally conserved from yeast to mammals. *Mol Cell Biol* 19, 4633-42 (1999).
30. Marmorstein, L. Y. et al. A human BRCA2 complex containing a structural DNA binding component influences cell cycle progression. *Cell* 104, 247-57 (2001).
31. Lee, M., Daniels, M. J. & Venkitaraman, A. R. Phosphorylation of BRCA2 by the Polo-like kinase Plk1 is regulated by DNA damage and mitotic progression. *Oncogene* 23, 865-72 (2004).
32. Hussain, S. et al. Direct interaction of the Fanconi anaemia protein FANCG with BRCA2/FANCD1. *Hum Mol Genet* 12, 2503-10 (2003).
33. Hussain, S. et al. Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. *Hum Mol Genet* 13, 1241-8 (2004).
34. Lu, H. et al. The BRCA2-interacting protein BCCIP functions in RAD51 and BRCA2 focus formation and homologous recombinational repair. *Mol Cell Biol* 25, 1949-57 (2005).

35. Hughes-Davies, L. et al. EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. *Cell* 115, 523-35 (2003).
36. Yang, H. et al. BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. *Science* 297, 1837-48 (2002).
37. West, S. C. Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol* 4, 435-45 (2003).
38. Davies, A. A. et al. Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol Cell* 7, 273-82 (2001).
39. Pellegrini, L. et al. Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nature* 420, 287-93 (2002).
40. Yuan, S. S. et al. BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. *Cancer Res* 59, 3547-51 (1999).
41. Tarsounas, M., Davies, D. & West, S. C. BRCA2-dependent and independent formation of RAD51 nuclear foci. *Oncogene* 22, 1115-23 (2003).
42. Yu, D. S. et al. Dynamic control of Rad51 recombinase by self-association and interaction with BRCA2. *Mol Cell* 12, 1029-41 (2003).
43. Yang, H., Li, Q., Fan, J., Holloman, W. K. & Pavletich, N. P. The BRCA2 homologue Brh2 nucleates RAD51 filament formation at a dsDNA-ssDNA junction. *Nature* 433, 653-7 (2005).
44. Esashi, F. et al. CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. *Nature* 434, 598-604 (2005).
45. Spain, B. H., Larson, C. J., Shihabuddin, L. S., Gage, F. H. & Verma, I. M. Truncated BRCA2 is cytoplasmic: implications for cancer-linked mutations. *Proc Natl Acad Sci U S A* 96, 13920-5 (1999).
46. Nordling, M. et al. A large deletion disrupts the exon 3 transcription activation domain of the BRCA2 gene in a breast/ovarian cancer family. *Cancer Res* 58, 1372-5 (1998).
47. Debnath, J., Muthuswamy, S. K. & Brugge, J. S. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 30, 256-68 (2003).
48. Scully, R. et al. Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* 88, 265-75 (1997).
49. Nakanishi, K. et al. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. *Proc Natl Acad Sci U S A* 102, 1110-5 (2005).

Figure 1 Xia et al

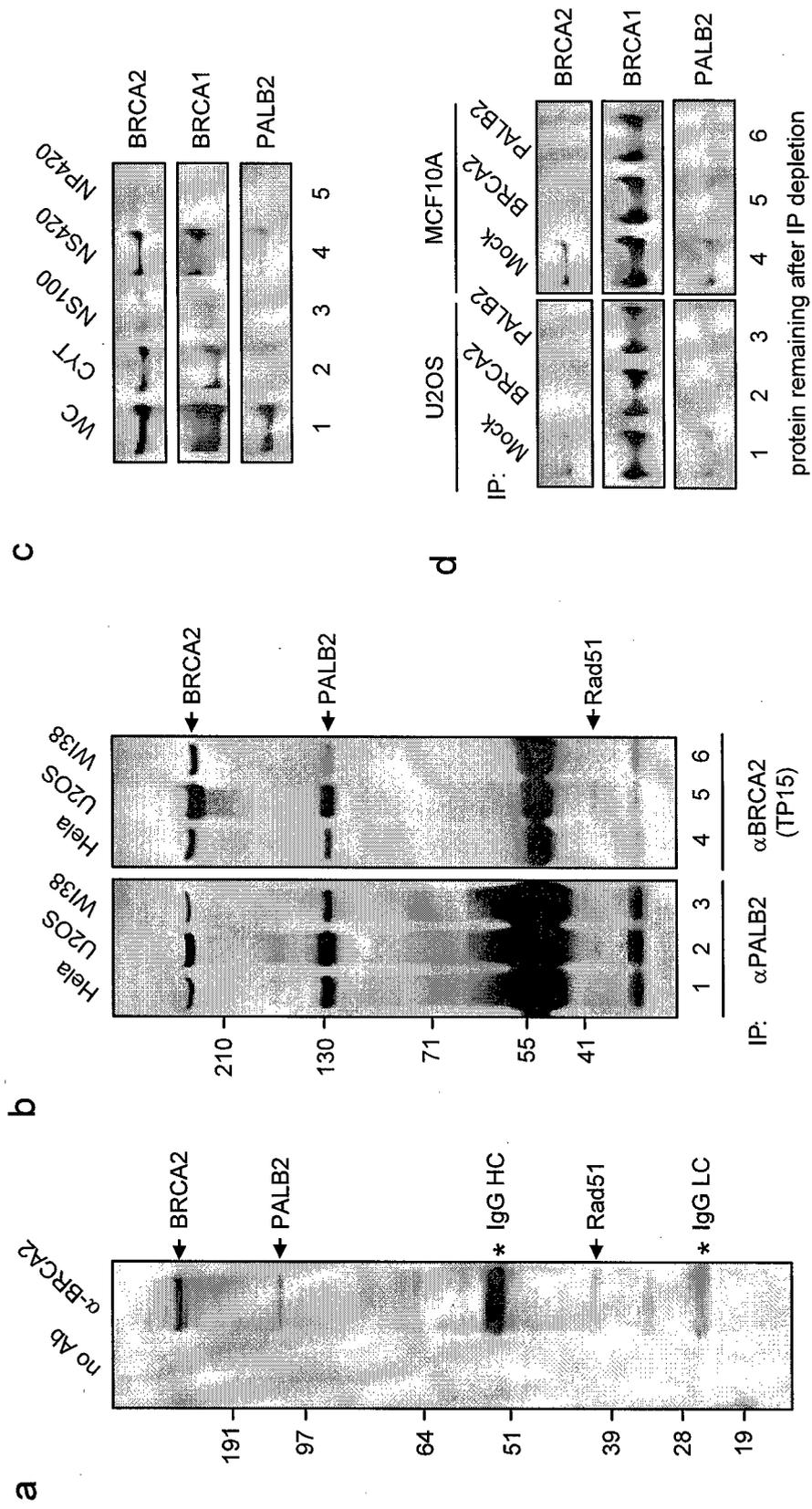


Figure 2 Xia et al

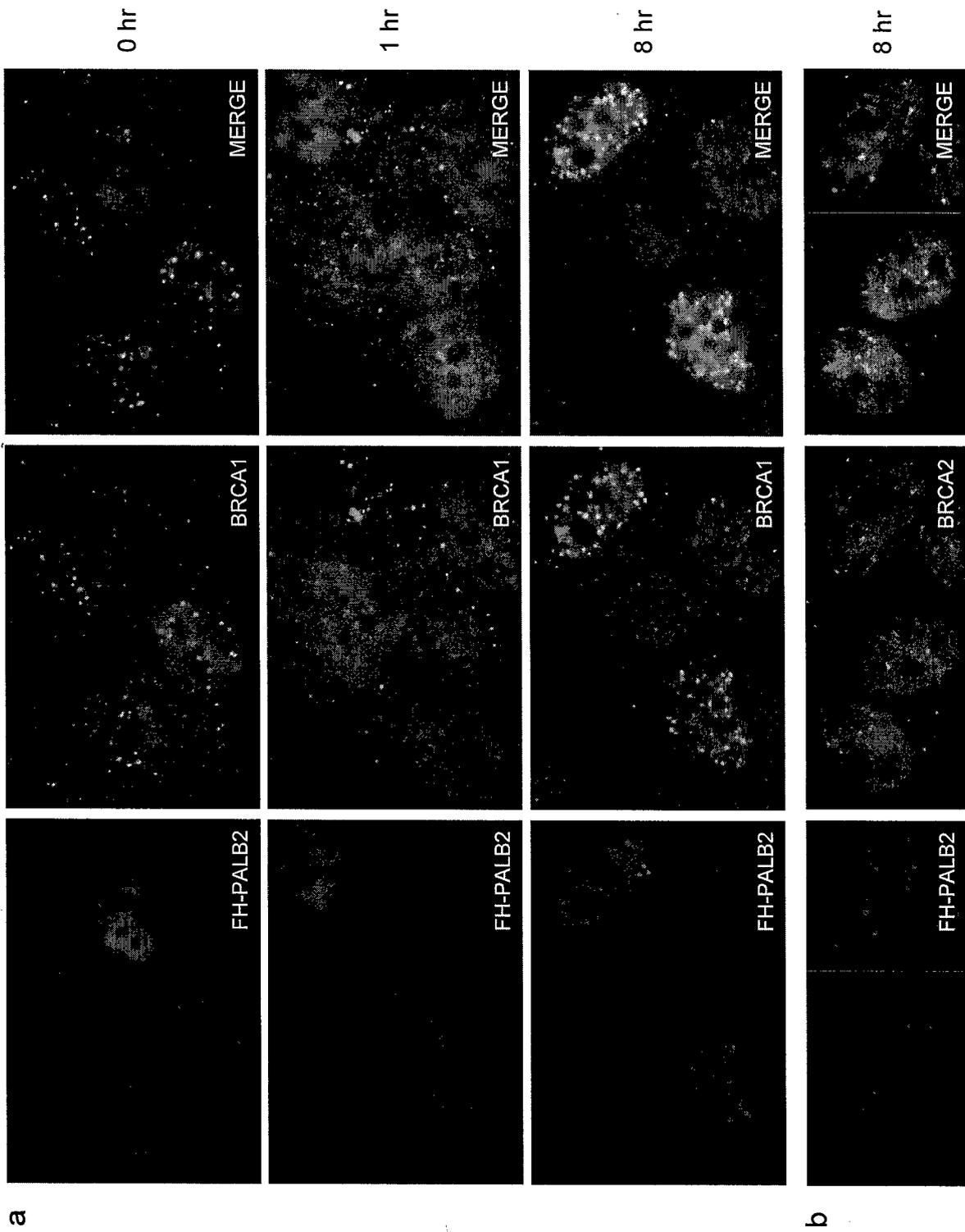


Figure 3 Xia et al

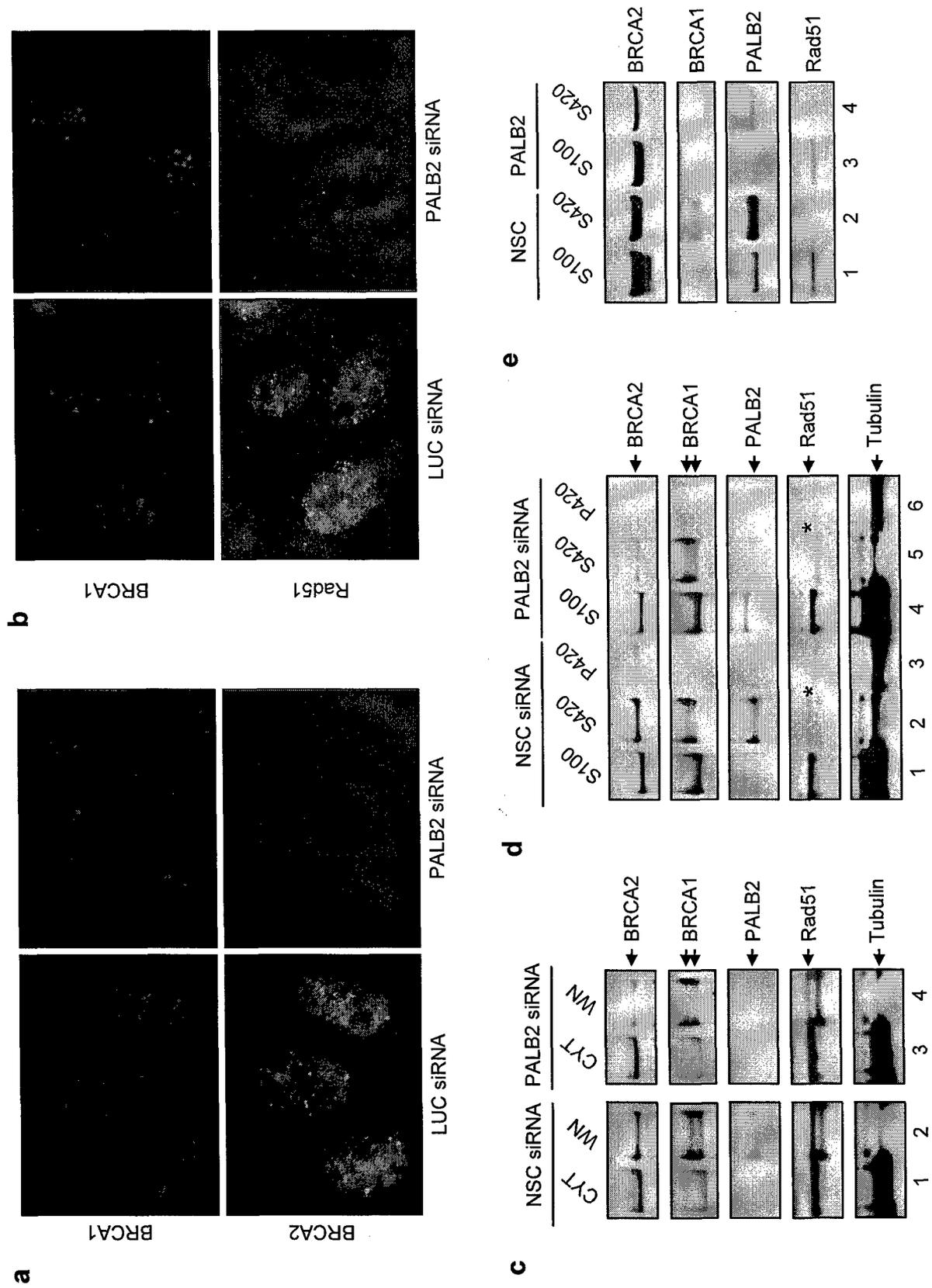


Figure 4 Xia et al

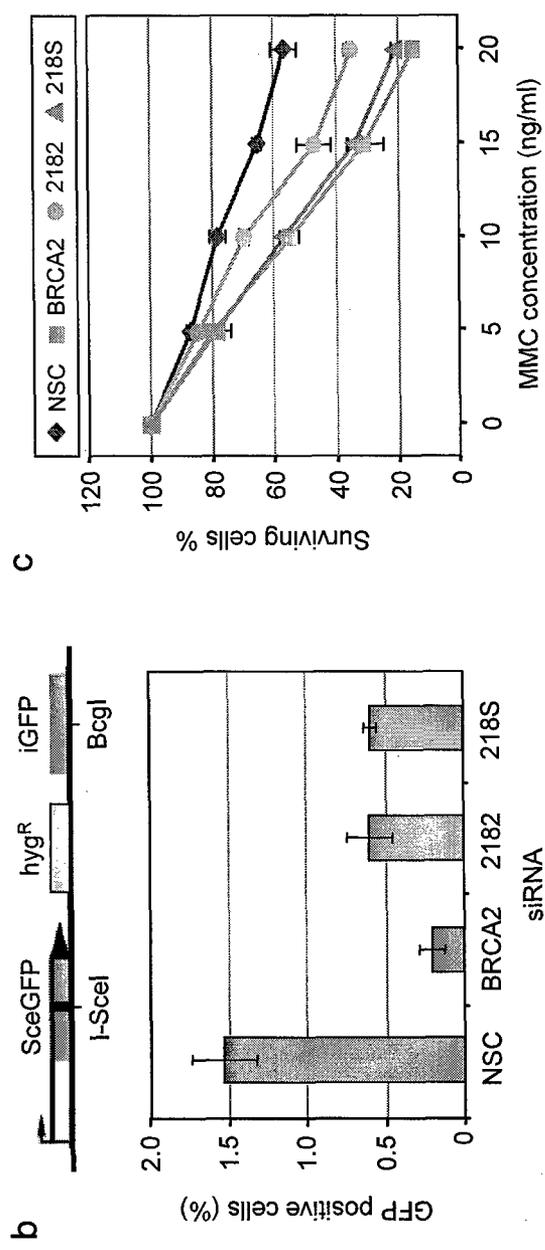
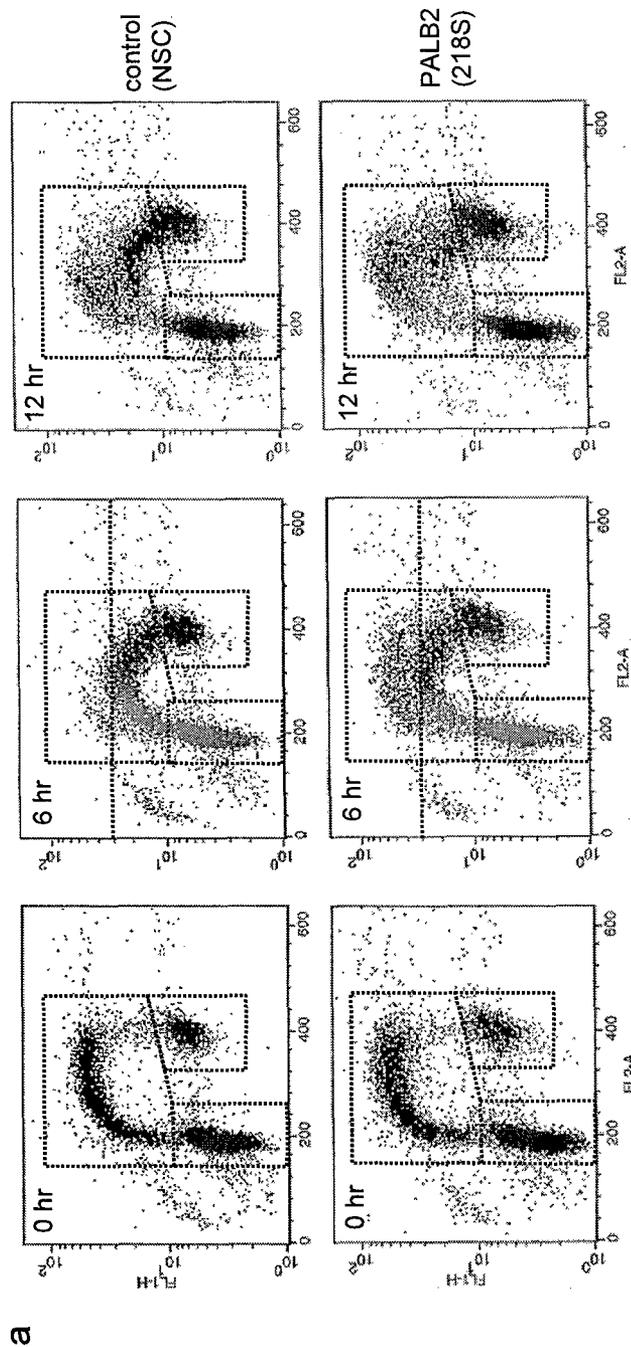


Figure 5 Xia et al

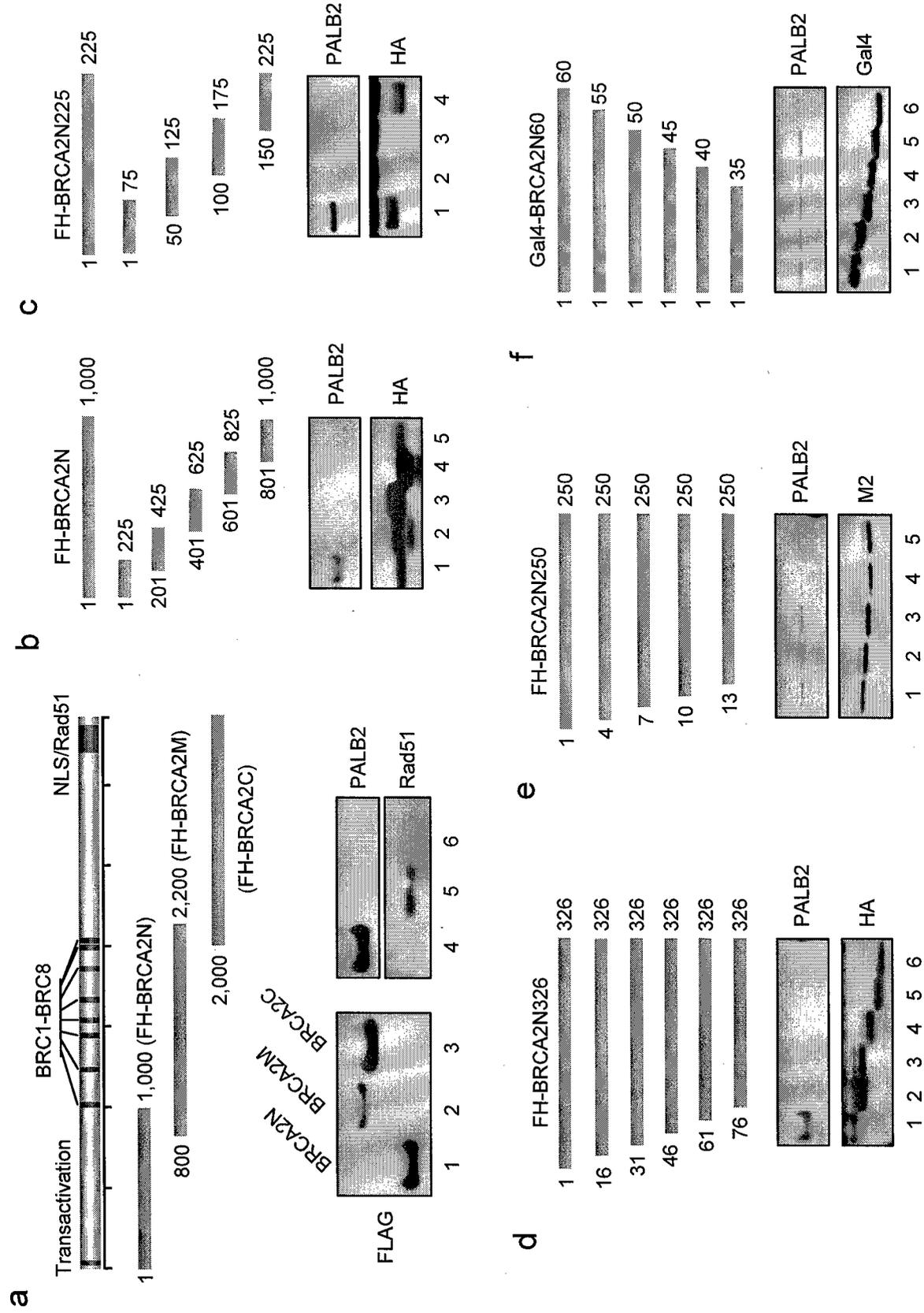


Figure 6 Xia et al

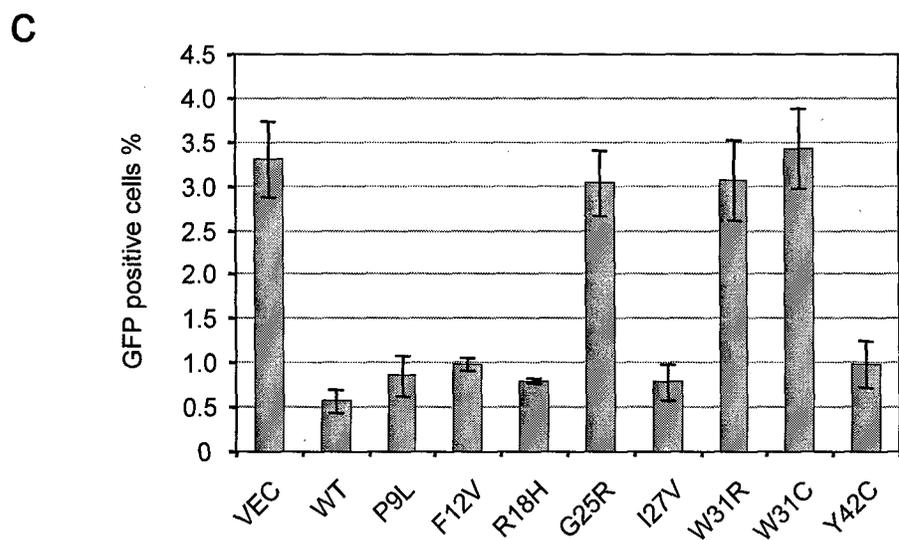
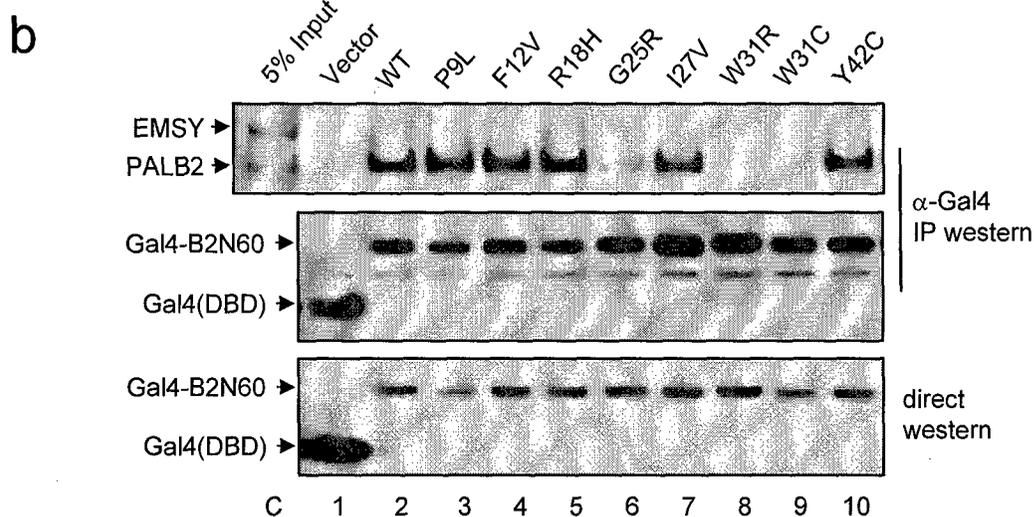
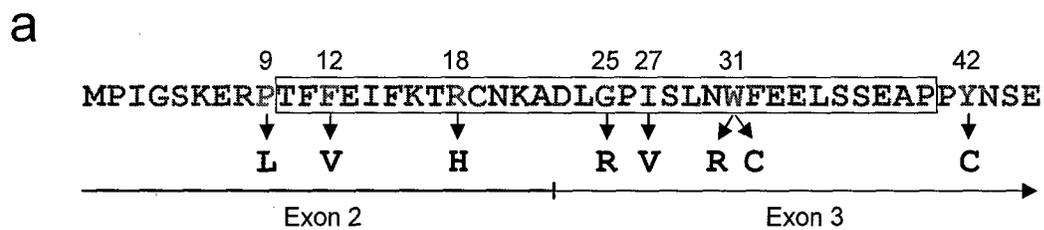


Figure 1. PALB2 is a major physiological BRCA2 partner. **a**, HeLa cells were lysed with NETN300 and the whole cell extract (W300) was immunoprecipitated with protein A beads alone (mock) or a monoclonal BRCA2 antibody (Ab-1). The precipitates were resolved with a 4-12% Bis-Tris gel and stained with silver. BRCA2, Rad51 and PALB2 are marked with arrows. And the asterisks indicate the heavy and light chains of IgG. **b**, W300 extracts of HeLa, U2OS and WI38 cells were immunoprecipitated with either α PALB2N200 or another BRCA2 monoclonal antibody (TP15). The immunoprecipitates were probed with antibodies against BRCA2 (Ab-1), PALB2 (α PALB2N200) and Rad51 (FBE). **c**, U2OS cells were fractionated into cytoplasm (CYT) and nuclei. The nuclei were further fractionated into NS100, NS420 and NP420. WC denotes sonicated whole cell (lane 1). **d**, S420 extracts of U2OS and MCF10A cells were incubated overnight with either protein A beads alone (lane 1) or beads with excess amounts of affinity-purified α BRCA2F9 or α PALB2N200 (lanes 2 and 3, respectively); following immunodepletion the remaining BRCA2 and PALB2 in the supernatant were analyzed by western blotting. BRCA1 serves as an internal control as only a small percentage of it interacts with BRCA2 or PALB2.

Figure 2. PALB2 colocalizes with BRCA1 and BRCA2 in nuclear foci. **a**, T98G cells stably expressing FLAG/HA-double tagged PALB2 were co-stained with antibodies against HA (HA11) or BRCA1 (07-434) at 0, 3 or 8 hr after radiation (15Gy). **b**, Above cells were co-stained with HA11 and α BRCA2F8 antibodies 8 hr after radiation.

Figure 3. PALB2 is required for the nuclear localization of BRCA2 and Rad51. **a-b**, U2OS cells were treated with either control (LUC-luciferase) or PALB2 siRNAs. 60 hr

later, cells were co-stained for BRCA1(SD118)/BRCA2(α BRCA2F8) or BRCA1(SD118)/Rad51(FBE). **c-d**, HeLa cells were treated with control (NSC) or PALB2 (218S) siRNAs for 60 hr and then cytoplasm and nuclei were separated. Total cytoplasm (CYT) and whole nuclei (WN) were analyzed by western blotting to detect BRCA2, PALB2, BRCA1 and Rad51, with α -tubulin as a loading control (**c**). Alternatively, same cells were fractionated into S100, S420 and P420 and then probed for the same set of proteins (**d**). **e**, BRCA2 was immunoprecipitated with α BRCA2F9 from the S100 and S420 fractions in **d** and the amounts of BRCA2, BRCA1, PALB2 and Rad51 in the precipitates were analyzed.

Figure 4. PALB2 plays a crucial role in intra-S phase damage checkpoint control and homologous recombinational repair of double strand breaks. **a**, U2OS cells treated with control (NSC) or PALB2 (218S-“smartpool”) siRNAs for 60 hr were pulse-labeled with BrdU for 15 min at 0, 6 and 12 hr after 15Gy of ionizing radiation and stained with FITC-conjugated anti-BrdU. Incorporation of BrdU (Y axis) and total DNA content (X axis) were analyzed by flow cytometry. Cells in G1, S and G2 phases are indicated by left, upper and right boxes. An arbitrary horizontal line is drawn in 6 hr panels to separate cells that undergo normally reduced (below) or abnormally intensive (above) BrdU incorporation following radiation. **b**, DR-U2OS cells were treated with indicated siRNAs for 48hr and then transfected with I-SceI expression plasmid to induced double strand breaks. GFP positive cells were quantified by flow cytometry 72 hr later. The schematic of the repair substrate integrated in the cells is shown on top. **c**, HeLa cells were treated

with indicated siRNAs for 48 hr and then their mitomycin C (MMC) sensitivities were measured using a MTT assay.

Figure 5. PALB2 interacts with the extreme N-terminus of BRCA2. FLAG-HA-double tagged or Gal4-fusion fragments of BRCA2 were transiently expressed in 293T cells and immunoprecipitated with anti-FLAG M2-agarose beads. The presence of PALB2 in the precipitates was probed by western blotting using either M2 (**a**) or HA11 (**b-f**) antibodies. In panel **a**, co-immunoprecipitation of Rad51 with BRCA2 fragments was also examined.

Figure 6. Loss of PALB2 interaction is correlated with loss of function among unclassified BRCA2 clinical variants. **a**, The N-terminus of BRCA2. The residues targeted by unclassified variations are shown in red, with their positions shown above and changed-to amino acids shown below. The sequence responsible for PALB2 interaction is boxed. **b**, Interactions between BRCA2 clinical variants and PALB2. The Gal4-B2N60 fusions were transiently expressed in 293T cells and then immunoprecipitated with a α Gal4 antibody. The amounts of PALB2 and EMSY (top panel) as well as that of the fusions (middle panel) in the precipitates were analyzed by western blotting. Lane C was loaded with 5% of input extract (from cells transfected with the vector expressing Gal4 DBD alone) and each other lane was loaded with 50% of precipitated material. The expression levels of the fusion proteins were also determined (lower panel). **c**, Inhibition of HDR. Each of above fusion constructs was co-transfected with I-SceI expressing plasmid into DR-U2OS cells. 72 hr later, GFP positive cells were quantified by flow cytometry.