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Structural basis for recruitment of BRCA2 by PALB2

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The breast cancer 2, early onset protein (BRCA2) is central to the repair of DNA damage by homologous recombination. BRCA2 recruits the recombinase RAD51 to sites of damage, regulates its assembly into nucleoprotein filaments and thereby promotes homologous recombination. Localization of BRCA2 to nuclear foci requires its association with the partner and localizer of BRCA2 (PALB2), mutations in which are associated with cancer predisposition, as well as subtype N of Fanconi anaemia. We have determined the structure of the PALB2 carboxy-terminal β-propeller domain in complex with a BRCA2 peptide. The structure shows the molecular determinants of this important protein–protein interaction and explains the effects of both cancer-associated truncating mutants in PALB2 and missense mutations in the amino-terminal region of BRCA2.

Keywords: PALB2; BRCA2; DNA repair; homologous recombination; cancer

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INTRODUCTION

Repair of DNA double-strand breaks and interstrand crosslinks by homologous recombination in human cells involves the co-ordinated assembly of several multi-protein complexes. Central to these is the scaffold protein BRCA2 (breast cancer 2, early onset), which regulates the assembly of RAD51 recombinase into nucleoprotein filaments (Sharan *et al*, 1997; Yuan *et al*, 1999). Heterozygous germline defects in BRCA2 predispose a person to breast and ovarian cancers, whereas homozygous defects cause Fanconi anaemia (Howlett *et al*, 2002; Gudmundsdottir & Ashworth, 2006). Cells with BRCA2 defects show genetic instability, consistent with abrogation of homologous recombination, and are typified by chromosomal rearrangements and aneuploidy (Patel *et al*, 1998; Tutt *et al*, 1999; Daniels *et al*, 2004).

BRCA2 function depends to a large extent on partner and localizer of BRCA2 (PALB2; also known as FANC-N, for Fanconi anaemia subtype N), for recruitment to nuclear foci and for much of its recombinational activity (Xia *et al*, 2006). As with BRCA2, heterozygous germline mutations in PALB2 are associated with cancer predisposition, and homozygous germline mutations result in a form of Fanconi anaemia (Rahman *et al*, 2007; Reid *et al*, 2007; Xia *et al*, 2007). Recently, PALB2 mutations have also been associated with a susceptibility to pancreatic cancer (Jones *et al*, 2009). PALB2 is a 130-kDa protein with no clear functional domains other than a predicted amino-terminal coiled-coil structure and a carboxy-terminal WD40-repeat motif. Deletion mapping has localized the PALB2-interacting region of BRCA2 to residues 10–40 at the N-terminus of the protein (Xia *et al*, 2006).

We have determined the structure of the C-terminal region of PALB2, showing a seven-bladed WD40-type β-propeller, which provides the binding site for the N-terminus of BRCA2. The structure of the core PALB2–BRCA2 complex shows a new interaction with a pocket on the face of the structure opposite to that seen in most WD40–peptide interactions. Together, these structures explain the effects of cancer-associated truncating mutations in PALB2 and of missense mutations in the N-terminal region of BRCA2. The interaction revealed by our structural studies is found to be both necessary and sufficient for the association of PALB2 and BRCA2 in cells.

RESULTS AND DISCUSSION

Structure of the PALB2 carboxy-terminal domain

Truncating mutations in PALB2 are associated with cancer predisposition (Rahman *et al*, 2007; Reid *et al*, 2007; Tischkowitz *et al*, 2007; Xia *et al*, 2007), implicating the C-terminus in mediating interaction with BRCA2. We defined a C-terminal region in PALB2 that had the characteristics of a seven-bladed β-propeller—a domain commonly involved in protein–protein interactions—and a construct encoding this region (PALB2-C; Fig 1A) was expressed in Sf9 cells, purified and crystallized, and the structure determined by X-ray crystallography (see Methods, supplementary information online and Table 1).

PALB2-C has the linear topology seen in other WD40-repeat domains and the toroidal structure 'sealed' in the seventh blade by interaction of the C-terminal strand with the incomplete N-terminal blade (Fig 1B). The β-propeller structure of PALB2-C provides an explanation for the cancer-associated mutation (3459C→G; Y1183X) that removes the last four residues (Reid *et al*, 2007). The absence of these residues completely disrupts the

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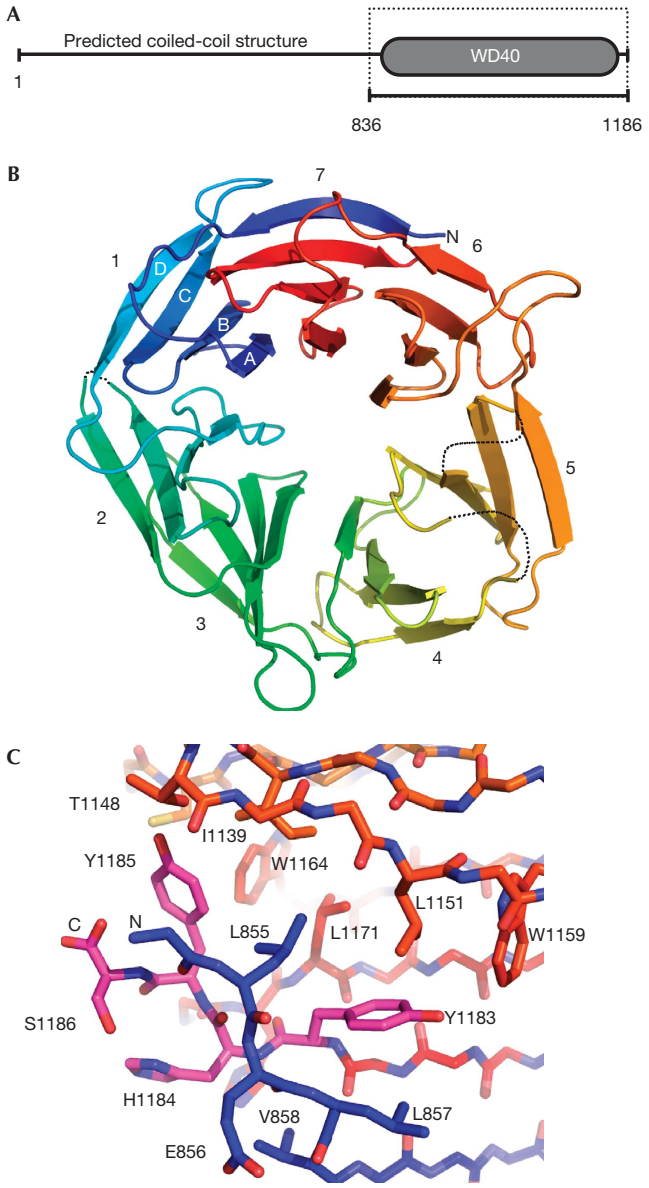

Fig 1 | Structure of the PALB2 carboxy-terminal domain. (**A**) Bioinformatics analysis identifies a WD40-repeat domain forming the C-terminal part of PALB2 whereas the N-terminal and central regions are predicted to contain a coiled-coil structure. The boxed region indicates the amino-acid boundaries of the PALB2-C expression construct used in this study. (**B**) PALB2-C is a seven-bladed β-propeller (rainbow coloured blue→red, N→C terminus). Blade 7 is formed by strands A, B and C from the C-terminus and strand D from the N-terminus, which seal the toriodal structure in a 'molecular Velcro' interaction. (**C**) Details of strand C of blade 7 and its environment. In families with Fanconi anaemia that have Y1183X mutations, the absence of C-terminal residues (magenta) prevents the closure of the WD40 ring and destabilizes the PALB2 protein, which is undetectable in these patients. PALB2, partner and localizer of BRCA2.

'molecular Velcro' hydrogen bonding in the seventh blade (Fig 1C), leaving an incompletely folded protein that is likely to be degraded rapidly. Indeed, PALB2 protein is not detectable in lymphoblastoid cells derived from patients with this mutation (Reid *et al*, 2007).

Interaction of PALB2 with BRCA2

Previous studies localized the PALB2-interacting region of BRCA2 to amino acids 10–40, in which cancer-associated missense mutations have also been observed (Fig 2A). In particular G25R, W31C and W31R result in loss of BRCA2 binding by PALB2 (Xia *et al*, 2006). We found that a peptide containing residues 21–39 of BRCA2 could readily precipitate PALB2-C *in vitro,* whereas a peptide containing the W31C mutation could not (Fig 2B), confirming that the C-terminus of PALB2 contains the BRCA2-binding site. Quantitative analysis of this interaction (Fig 2C) gave a dissociation constant (K_d) of ~0.66 μM. On the basis of this observation, co-crystals of PALB2-C and the wild-type peptide BRCA2(21–39) were obtained, and diffraction data collected and refined against the structure of the ligand-free protein (see Methods). Clear difference electron density for the BRCA2(21–39) peptide was evident and residues 26–36 could be readily fitted and refined (see supplementary Fig S1 online).

In many observations of peptide binding to β-propellers, the interaction centres on the mouth of the axial channel opening onto the face containing the loops connecting consecutive 'blades' (Lodowski *et al*, 2003; Han *et al*, 2006; Jennings *et al*, 2006; Hao *et al*, 2007; Larsen *et al*, 2007). However, this is not universal, and interactions with the outer cylindrical surface are observed in the binding of β-adaptin to the N-terminal WD40 domain of clathrin (ter Haar *et al*, 2000), or in the binding of histone H4 to RbAp46 (Murzina *et al*, 2008).

BRCA2(21–39) binds in a pocket formed by the tips of the fourth and fifth blades, on the face opposite to the common axial site (Fig 3A–C). The core of the interaction is provided by BRCA2 Trp 31, Phe 32 and Leu 35, which project from a short helix into a hydrophobic pocket lined by PALB2 residues Val 1019, Met 1022, Ala 1025, Ile 1037, Leu 1046, Lys 1047, Leu 1070, Pro 1097 and Lys 1098. Hydrophobic interactions N-terminal to the helix involve BRCA2 Ile 27 and Leu 29 and PALB2 Met 1067, Gly 1068 and Leu 1069. The core interface is supported by polar interactions, including a water bridge from the indole nitrogen of BRCA2 Trp 31 to Ser 1065 in the wall of the PALB2 pocket.

The involvement of BRCA2 Trp 31 explains why cancer-associated W31C and W31R mutations disrupt the functional interaction with PALB2. For PALB2 itself, only truncation mutants are described in clinical samples, and these lead to complete loss of the protein. To validate the observed BRCA2-binding site, we generated point mutants in full-length PALB2, designed to disrupt the interaction with BRCA2(21–39), transfected these into human embryonic kidney 293T (HEK-293T) cells, and determined their ability to co-immunoprecipitate endogenous BRCA2 (Fig 3D). Mutation of PALB2 Met 1022, on the side of the hydrophobic pocket, only slightly decreased the BRCA2 co-immunoprecipitation. However, mutation of PALB2 Ala 1025 at the bottom of the pocket, and in contact with BRCA2 Phe 32 in the complex, completely abolished BRCA2 binding, confirming the functional role of the pocket. All the PALB2 mutants were expressed at a level comparable with wild type, and the full-length protein could also be immunoprecipitated at comparable levels, showing that the mutations did not disrupt PALB2 stability. As single missense mutations in

Table 1 | Data collection, phasing and refinement statistics

	PALB2+KAu(CN)$_2$	PALB2 native (2W18)	PALB2+peptide (3EU7)
Data collection			
Space group	C2	C2	C2
Cell dimensions			
a, *b*, *c* (Å)	81.52, 63.15, 77.48	80.78, 64.65, 77.25	82.82, 62.03, 77.98
α, β, γ (°)	90, 109.69, 90	90, 109.65, 90	90, 108.23, 90
Resolution (Å)	40.0–2.1 (2.2–2.1)*	49.3–1.9 (2.0–1.9)	37.5–2.1 (2.32–2.1)
R_{merge}	0.06 (0.41)	0.06 (0.58)	0.07 (0.60)
Mn $I/\sigma I$	17.6 (4.2)	11.2 (2.7)	16.5 (2.9)
Completeness (%)	96.3 (80.6)	94.1 (75.4)	99.9 (99.0)
Redundancy	7.6 (7.2)	4.0 (3.8)	5.8 (5.9)
Refinement			
Resolution (Å)		49.3–1.9	37.5–2.2
Number of reflections		27,818 (3238)	19,026 (2800)
R_{work}/R_{free}		0.21/0.25	0.20/0.25
Number of atoms			
Protein		2,326	2,367
Ligand/ion		30 (glycerol)	12 (glycerol) 108 (peptide)
Water		187	98
B-factors			
Protein		35.9	47.6
Ligand/ion		43.1 (glycerol)	54.9 (glycerol) 55.4 (peptide)
Water		41.4	47.8
r.m.s.d. values			
Bond lengths (Å)		0.012	0.014
Bond angles (°)		1.47	1.63

*Highest resolution shell is shown in parentheses.

either protein can disrupt their interaction, this demonstrates that this interface is both necessary and sufficient for the interaction of BRCA2 and PALB2 in cells.

PALB2 is upstream from BRCA2 in the informational 'flow' from recognition of a DNA double-strand break to assembly of the RAD51 nucleoprotein filament. The interaction we have characterized mediates the recruitment of BRCA2, but unless PALB2 proves to be able to recognize DNA damage directly, it must also interact with other proteins upstream in the DNA-damage information flow. One recently identified possibility is BRCA1, which seems to interact with N-terminal regions of PALB2 (Zhang *et al*, 2009). The surface conservation in PALB2-C (supplementary Figs S2 and S3 online) suggests that, apart from the BRCA2 interaction involving the new 'bottom-face' site, the β-propeller might simultaneously provide interactions through the conventional 'top-face' site at the mouth of the axial channel. The structure-guided PALB2 missense mutants we have developed here, which specifically abrogate BRCA2 interaction without destabilizing the protein, will be invaluable for identifying further PALB2-interacting proteins that mediate the recruitment of the BRCA2 HR repair complex to sites of DNA damage.

Role of the BRCA2 amino-terminus

BRCA2 has a central role in regulating DNA double-strand break and inter-strand crosslink repair through homologous recombination. A significant component of that role is provided by the regulatory and scaffolding interaction of BRCA2 with the RAD51 recombinase (Thorslund & West, 2007), which is mediated by the central BRC repeat and C-terminal regions of BRCA2 (Saeki *et al*, 2006; Davies & Pellegrini, 2007; Esashi *et al*, 2007). Most of the defined pathogenic familial mutations in *BRCA2* cause frameshift or nonsense mutations in the BRCA2 open-reading frame, resulting in the expression of truncated or unstable BRCA2

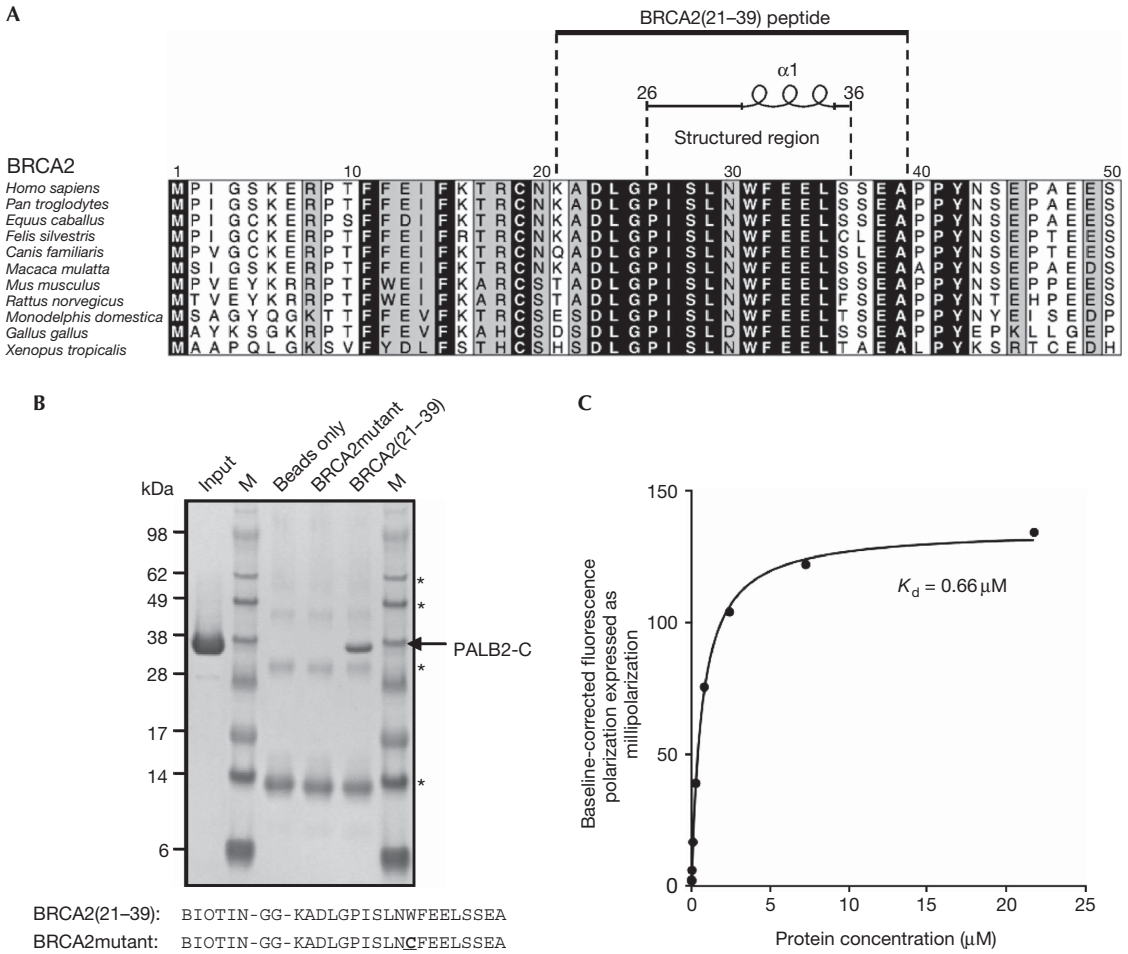

Fig 2 | A minimal PALB2-binding motif in the BRCA2 amino terminus. (**A**) The previously localized PALB2-binding site in residues 10–40 of BRCA2 (Xia *et al*, 2006) was mapped to residues 21–39, encapsulating a minimal ten-residue motif, which is structured in the PALB2-C–BRCA2(21–39) complex. (**B**) Biotinylated wild-type BRCA2(21–39) peptide precipitates the PALB2-C construct, confirming it as the BRCA2-binding site. A peptide containing a breast-cancer-associated W31C mutation failed to co-precipitate PALB2-C. Bands marked with an asterisk arise from the NeutrAvidin resin. (**C**) Fluorescence polarization binding isotherm for interaction of PALB2-C with fluorescein-labelled BRCA2(21–39) peptide (see Methods). K_d as determined by nonlinear fitting of the data with a one-site-specific binding model (GraphPad Prism 5.0). BRCA2, breast cancer 2, early onset; PALB2, partner and localizer of BRCA2.

proteins, which are impaired in RAD51 interactions (Wooster *et al*, 1995; Tavtigian *et al*, 1996). The critical involvement of the C-terminal RAD51-binding domain, in particular, is highlighted by the ability of reverted mutant BRCA2 proteins that contain substantial internal deletions to restore homologous recombination in BRCA2-defective cancer cell lines (Edwards *et al*, 2008; Sakai *et al*, 2008).

The role of the extreme N-terminal region of BRCA2 has been more controversial. Previous studies suggested an involvement in transcriptional activation (Milner *et al*, 1997; Fuks *et al*, 1998), a conjecture reinforced by the discovery of EMSY, a protein able to interact with chromatin-regulatory proteins, as a binding partner for the BRCA2 N-terminus (Hughes-Davies *et al*, 2003). However, the mechanistic implications of possible BRCA2 involvement in transcriptional regulation have not been defined, and the biological significance of this suggestion remains uncertain. The consequences of BRCA2 defects are, however, consistently explained by a primary role in the arrangement and regulation of DNA repair by homologous recombination (Gudmundsdottir & Ashworth, 2006). The discovery of PALB2 as a crucial factor in localizing BRCA2 to sites of DNA damage (Xia *et al*, 2006) provides a strong argument that the main role of the N-terminal region of BRCA2 is to provide a binding site for PALB2. In support of this argument, we found that the BRCA2 residues we identified as being essential for PALB2 binding are totally conserved in organisms (vertebrates) that have a PALB2 orthologue, but not in those that do not. Although the interaction between PALB2 and BRCA2 has now been characterized at the atomic level, the crucial questions about how PALB2 directs BRCA2 to sites of DNA damage, and the identities of other PALB2-interacting proteins that might be involved, remain to be answered.

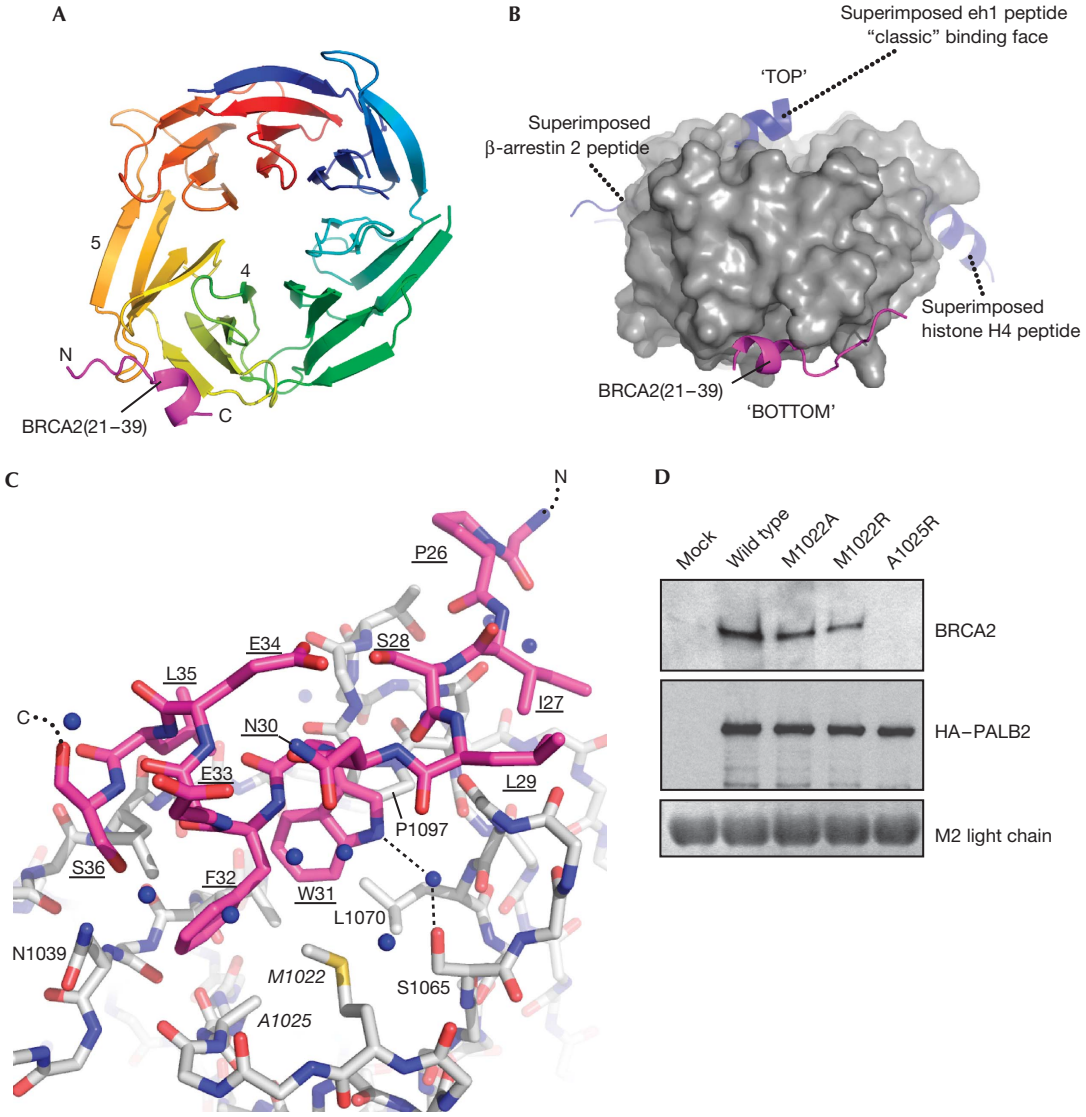

Fig 3 | Structure of PALB2-C–BRCA2(21–39) complex. (**A**) BRCA2(21–39) (magenta) binds across a hydrophobic pocket at the crossover between blades 4 and 5 of the PALB2-C β-propeller, forming a short α-helix. The r.m.s.d. between the apo-bound and peptide-bound PALB2-C structures is 0.329 Å. (**B**) Molecular surface of PALB2-C (grey) highlighting the location of BRCA2 binding (magenta) compared with other β-propeller–peptide complexes. The superimposed eh1 motif from a complex with the WD40 domain of TLE1 (Jennings *et al*, 2006) marks the 'classic' peptide motif-binding site found in most other WD40–peptide complexes. The positions of β-arrestin 2 and histone H4 peptides (blue), from complexes with the WD40 domains of clathrin and RBBP7, respectively (ter Haar *et al*, 2000; Murzina *et al*, 2008), are known examples of non-canonical sites, but are distinct from that observed for the PALB2-C–BRCA2(21–39) complex. (**C**) Details of the PALB2-C–BRCA2(21–39) interface. The core of the interaction is provided by Trp 31, Phe 32 and Leu 35 inserting into a hydrophobic pocket on PALB2-C (see text). The complex buries ~1,100 Å^2 of the molecular surface, typical of a reversible regulatory interaction. (**D**) Immunoprecipitation of FLAG–HA-tagged wild-type PALB2 and PALB2 with missense mutations in residues contributing to the BRCA2-binding site (see Methods). Endogenous BRCA2 is precipitated by wild-type PALB2, and to a lesser degree by the Met 1022 mutants. However, BRCA2 binding is abolished by mutation of Ala 1025, which lies at the bottom of the PALB2-hydrophobic pocket. The light chain of the M2 FLAG antibody provides a loading control. BRCA2, breast cancer 2, early onset; HA, haemagglutinin; PALB2, partner and localizer of BRCA2; TLE1, transducin-like enhancer of split 1.

Speculation

The PALB2-binding site on BRCA2 directly overlaps that of EMSY, a protein amplified and overexpressed in many breast and ovarian cancers (Hughes-Davies *et al*, 2003). Tumours with EMSY amplifications and a cell line overexpressing EMSY show chromosomal instability and mitomycin-C sensitivity characteristic of cells defective in BRCA2 or PALB2 (Turner *et al*, 2004; Raouf *et al*, 2005; Xia *et al*, 2006). However, a role for EMSY in BRCA2-mediated homologous recombination or a mechanism by which EMSY overexpression affects the BRCA2 function has not

been described. As the PALB2- and EMSY-binding sites on BRCA2 overlap, and preliminary data (supplementary Fig S3 online) indicate mutually exclusive binding, we speculate that high EMSY protein levels due to amplification and/or overexpression in tumours might outcompete PALB2, disrupting the functionally important PALB2–BRCA2 interaction in homologous recombination and engendering the observed genetic instability.

METHODS

Peptides. All peptides were chemically synthesized and purified at Peptide Protein Research Ltd, Fareham, UK.

Cloning, expression and purification. PALB2-C (residues 835–1186) was expressed in Sf9 insect cells and purified using immobilized metal-affinity and size-exclusion chromatography. Complete details are provided in the supplementary information online.

Crystallization, phasing and data refinement. PALB2-C was crystallized by vapour diffusion in hanging drops, at a protein concentration of 10 mg/ml and a temperature of 20 °C, using a precipitant containing 100 mM 2-[*N*-morpholino]ethanesulphonic acid pH 6.0, 50 mM KH_2PO_4 and PEG 8000 at concentrations between 12 and 20% (wt/vol). Crystals were cryoprotected for data collection by stepwise transfer, to a final concentration of 30% glycerol (vol/vol). A heavy-atom derivative was obtained by soaking native crystals overnight in a stabilizing buffer containing 10 mM $KAu(CN)_2$, before freezing. Co-crystals with the BRCA2(21–39) peptide were grown under similar conditions, with an ~1:4 ratio of protein:peptide.

All diffraction data were collected on station ID14.1 at the European Synchrotron Radiation Facility, Grenoble, France, and the native PALB2-C structure was phased using single-wavelength anomalous dispersion from the $KAu(CN)_2$ derivative crystals. The native structure was built from Fourier maps and refined to 1.9 Å. This model was then used to phase the PALB2-C–BRCA2(21–39) co-crystals, and the structure of the complex was refined to 2.1 Å. Complete experimental details are provided in the supplementary information online, and statistics for the crystallography are provided in Table 1.

Peptide binding and competition assays. Experiments were carried out with biotinylated peptides corresponding to either residues 21–39 of human BRCA2 (biotin–GG–KALDGPISLNW-FEELSSEA) or the same sequence containing a single point mutant W31C (biotin–GG–KALDGPISLNCFEELSSEA). The affinity of BRCA2(21–39) for PALB2-C was determined by fluorescence polarization measurements using an N-terminally fluorescein-labelled wild-type BRCA2(21–39) peptide. Complete details are provided in the supplementary information online.

Mammalian expression and M2-agarose immunoprecipitation. Single point mutants of full-length PALB2, in the mammalian expression vector pOZ-FH-C, were generated using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's recommended protocol. HEK-293T cells were obtained from the American Type Culture Collection (ATCC; LGC Standards, Teddington, UK) and maintained in DMEM containing 10% (vol/vol) fetal calf serum. Cells were transfected in six-well plates using FUGENE6 (Roche, Burgess Hill, UK), according to the manufacturer's instructions. Whole-cell extracts were prepared in 1 ml of NETN420 buffer (20 mM Tris–HCl (pH 7.5), 420 mM NaCl, 1 mM EDTA, 0.5% (vol/vol) IGEPAL CA-630) as described in Xia *et al* (2006), 48 h after transfection. FLAG-tagged PALB2 was precipitated from these extracts with anti-FLAG M2-agarose beads (Sigma-Aldrich, Gillingham, UK), for a period of 20 h at 4 °C.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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