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Antiparallel Coiled-Coil Interactions Mediate Homodimerization of the DNA Damage Repair Protein, PALB2

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Abstract

Deficits in DNA damage repair pathways are the root cause of several human cancers. In mammalian cells, DNA double-strand break repair is carried out by multiple mechanisms, including homologous recombination (HR). PALB2 (Partner and Localizer of BRCA2), which is an essential factor for HR, binds to the breast cancer susceptibility 1 (BRCA1) protein at DNA double-strand breaks. At the break site, PALB2 also associates with breast cancer susceptibility 2 (BRCA2) protein, to form a multi-protein complex that facilitates HR. The BRCA1-PALB2 interaction is mediated by association of predicted helical coiled-coil regions in both proteins. PALB2 can also homodimerize through the formation of a coiled coil, by self-association of helical elements at the N-terminus of the PALB2 protein, and this homodimerization has been proposed to regulate the efficiency of HR. We have produced a segment of PALB2 (PALB2cc), which forms α -helical structures, and which assembles into stable homodimers or heterodimers with a PALB2-interacting segment of BRCA1 (BRCA1cc). The three-dimensional structure of the homodimer formed by PALB2cc was determined by solution NMR spectroscopy. This PALB2cc homodimer is a classical anti-parallel coiled-coil leucine zipper. NMR chemical shift perturbation studies were used to study dimer formation for both the PALB2cc homodimer and the PALB2cc:BRCA1cc heterodimer. Mutation of residue Leu24 of PALB2cc significantly reduces its homodimer stability, but has a more modest effect on the stability of the heterodimer formed between PALB2cc and BRCA1cc. We show that mutation of Leu24 leads to genomic instability and reduced cell viability after treatment with agents that induce DNA double-strand breaks. These studies may allow the identification of distinct mutations of PALB2 which selectively disrupt homodimeric versus heterodimeric interactions, and reveal the specific role of PALB2 homodimerization in HR.

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Keywords

DNA damage; homologous recombination; PALB2; BRCA1; NMR structure determination; coiled coil

INTRODUCTION

Repair of DNA double-strand breaks in mammalian cells can be achieved by end-joining pathways or by homologous recombination (HR) ^{1, 2}. Whereas end-joining pathways are considered to have mutagenic potential, HR has high fidelity of repair, because it restores the broken DNA using homologous sequence as a template. HR-mediated repair requires pairing of DNA sequences, which is achieved by strand invasion of a resected broken DNA end into a homologous DNA duplex. Strand invasion is catalyzed by RAD51, which is loaded onto DNA around the break site by BRCA2 ³. Recruitment of BRCA2 to DNA double-strand breaks is therefore essential for cellular viability, as it enables efficient HR-mediated repair of breaks that arise during DNA replication and as a result of exposure to exogenous mutagens.

PALB2 (Partner and Localizer of BRCA2) is an essential factor for normal association of BRCA2 with broken DNA ends ⁴. Cells that lack PALB2 show a profound defect in HR and cell cycle checkpoint defects, and individuals who inherit bi-allelic mutations in the *PALB2* gene are affected by a severe form of Fanconi Anemia, equivalent to other genetic mutations that prevent repair of DNA damage ⁵⁻⁷. In mice, PALB2 is essential for normal development, and conditional inactivation of *Palb2* leads to tumorigenesis ^{8, 9}. Mutations affecting the *PALB2* gene have likewise been linked to cancer predisposition in humans ¹⁰⁻¹². Determining the cellular activities of PALB2 is therefore of considerable importance for human health, and for a full understanding of the DNA damage response.

Whereas the structural basis for how PALB2 interacts with BRCA2 has been revealed ¹³, the specific molecular mechanisms by which PALB2 binds other repair factors are not fully characterized. PALB2 is recruited to DNA damage sites partly through direct interaction with DNA, or RPA-coated DNA ^{14,15}, but stable recruitment of PALB2 to break sites has been shown to depend on its interaction with BRCA1 ^{16, 17}. BRCA2, PALB2 and BRCA1 therefore form a multiprotein complex at DNA double-strand break sites that enables normal HR ¹⁸. Several lines of evidence suggest that the BRCA1-PALB2 interaction is mediated by association of predicted helical coiled-coil regions in both proteins ¹⁹. Mutations affecting these predicted coiled-coil motifs in BRCA1 and PALB2 disrupt their ability to interact *in vitro* ^{16, 20}, and mutagenesis of residues in the predicted coiled-coil region of PALB2 causes HR and male fertility defects in gene-targeted mice ²¹.

Previous work has additionally shown that PALB2 forms homo-oligomers through association of predicted coiled coil sequences at the N-terminus of the protein ^{14, 22}. Homo-oligomerization of PALB2 has been proposed to have a regulatory role, potentially by sequestering PALB2 in a form that has a reduced ability to mediate HR. Deletion of the coiled-coil region from PALB2 increases the proportion of monomeric PALB2, which was shown to stimulate RAD51 filament formation *in vitro* ¹⁴. Coimmunoprecipitation

experiments additionally showed that the N-terminal region of PALB2 can disrupt BRCA1-PALB2 heterodimers, by formation of a homo-oligomeric form of PALB2. Size-exclusion chromatography suggested that PALB2 forms a mixture of oligomers, but the stoichiometry of PALB2 homo-oligomers, and their relative stability versus PALB2-BRCA1 heterodimers has not previously been determined. In support of a role for PALB2 homo-oligomers in HR, ionizing radiation-induced DNA damage was reported to increase PALB2 self-association²². On the other hand, Zhang et. al. generated a PALB2 fusion protein which can interact with BRCA1, but which cannot self-associate, to test the importance of homodimerization for DNA repair. Their study indicated that only heterodimerization of PALB2 with BRCA1 was needed for RAD51 foci formation and normal cell growth in the presence of the DNA damaging agent mitomycin C²³. The importance of PALB2 homo-oligomerization is therefore still unclear, and warrants further study.

In order to better understand the nature of oligomeric interactions formed by PALB2, we have tested the ability of polypeptide segments of PALB2 that include the predicted coiled-coil motif to form stable helices and to self-associate. Using circular dichroism (CD) and Nuclear Magnetic Resonance (NMR) spectroscopy, we have characterized fragments of PALB2 that form stable α -helical structures and assemble into stable homodimers. The three-dimensional structure of a homodimer of one of these homodimeric segments of PALB2 was determined by solution-state NMR spectroscopy. The PALB2 homodimer interaction is mediated by an anti-parallel coiled-coil structure, stabilized by key Leu and other hydrophobic residues at the dimer interface. Mutation of residue Leu24 in this segment of PALB2 significantly reduces the PALB2 homodimer affinity, but has a smaller effect on interactions between PALB2cc and the PALB2cc-interaction segment of BRCA1. This work suggests novel approaches for designing PALB2 molecules which disrupt its homodimeric interaction but not its heterodimeric interaction with BRCA1.

EXPERIMENTAL PROCEDURES

Preparation of Protein Samples.

Constructs of the mouse PALB2 coiled-coil domain (residues 1-60, referred to here as PALB2cc), mutant [L24A]-PALB2cc, and the mouse BRCA1 coiled-coil domain (residues 1337-1387, referred to here as BRCA1cc) were cloned, expressed, and purified following the standard protocols of the Northeast Structural Genomics Consortium²⁴. A complete description of the methods used in this work is presented in Supplemental Material. Uniformly ¹³C, ¹⁵N- and 5% ¹³C, U-¹⁵N-enriched PALB2cc and [L24]-PALB2cc samples for NMR studies were prepared using standard protocols²⁴, and concentrated to 1.2-1.5 mM in 95% H₂O and 5% ²H₂O solution containing 20 mM MES buffer at pH 6.5, 200 mM NaCl, 10 mM DTT, and 5 mM CaCl₂ (except where otherwise specified). NMR studies were carried out using 4-mm Shigemi, or 1.7-mm micro, NMR tubes.

NMR Data Collection and Solution Structure Calculations.

All NMR data for resonance assignments and structure determination were collected at 20 °C using Bruker AVANCE II 600 MHz and 800 MHz NMR spectrometers equipped with 5-mm cryoprobes. Complete ¹H, ¹³C, and ¹⁵N resonance assignments for wt-PALB2cc were

determined using conventional triple-resonance NMR methods²⁵ (as summarized in Figures S1 and S2) and deposited, together with raw tripleresonance NMR spectra (fid's), in the Biological Magnetic Resonance Data Bank (BMRB accession number 27534). Resonance assignments were validated using the Assignment Validation Suite (AVS) software package²⁶. The dimer interface in PALB2cc was identified using ¹³C filtered NOESY experiments²⁷ on a 1:2 sample of ¹³C,¹⁵N enriched : unlabeled (natural abundance) protein. Residual dipolar coupling (RDC) data were collected for two bond vectors, ¹⁵N-¹H^N and ¹⁵N-¹³C', in a single alignment medium containing acrylamidopropyltrimethylammonium chloride + 50% acrylamide (see Supplemental Material for details). The PALB2cc homodimer structure was calculated from these NMR data using CYANA Version 3.0²⁸⁻³⁰, and the 20 models out of 100 with the lowest target function were refined by restrained molecular dynamics in explicit water using CNS Version 1.1³¹. All structure calculations included both intra- and interchain NOE data, as well as RDC data. Structural statistics and global structure quality factors, including Verify3D³², ProsaII³³, *PROCHEC*³⁴, and MolProbity³⁵ raw and statistical Z-scores, were computed using the Protein Structure Validation Suite (PSVS Version 1.5) software package³⁶. Values for the global goodness of fit of the final structure ensembles with the NOESY peak list data were determined using the RPF analysis program³⁷. The final refined ensemble of 20 models has been deposited in the Protein Data Bank (code 6e4h), and the corresponding NOESY spectra (fid's) and NOESY peak lists were deposited in the Biological Magnetic Resonance Data Bank (BMRB accession number 27534).

Rotational Correlation Time Measurements and NMR Titration Studies.

Rotational correlation times at 20 °C were determined from ¹⁵N relaxation measurements, as described elsewhere³⁸. Samples were prepared for these NMR measurements in the same buffer at pH 6.5 used for NMR assignments and structure determination. Heterodimer NMR titration studies were carried out using ¹⁵N-enriched PALB2cc, and various amounts of unenriched BRCA1cc, in the same buffer at pH 6.5. Rotational correlation time measurements and NMR titration studies were carried out using a Bruker 600 MHz Avance II NMR System equipped with a 1.7-mm tripleresonance microcryoprobe³⁹, with 30 – 50 µL sample volumes.

Circular dichroism (CD) spectroscopy.

CD measurements were performed in solution using the same buffer at pH 6.5 used for the NMR studies, with an Aviv 62DS CD spectrometer (Aviv Associates). Wavelength scans (200 to 260 nm) were performed on 15 µM protein solutions. Thermal denaturation profiles were obtained by measuring the ellipticity at 222 nm (Θ_{222}) as a function of temperature. Protein solutions (15 µM) were measured at 0.5 degree intervals starting at 0 °C, with an equilibration time of 2 min and an acquisition time of 0.5 min. The apparent melting temperature (T_m) was estimated from the maximum of the first derivative of Θ_{222} with respect to temperature. Thermal denaturation curves used to estimate T_m values were fully reversible.

Analytical Gel Filtration with Multiple Angle Static Light Scattering (AGF-MALS).

Wild-type or mutant PALB2cc were prepared at protein concentration of 3 mg/ml in 20 mM MES at pH 6.5, containing 200 mM NaCl, 5 mM CaCl₂, 10 mM DTT, 1 x protease inhibitors, and 0.02% NaN₃. 30 µl samples were injected onto an analytical gel filtration column (PROTEIN KW-Shodex, Kawasaki, Japan), and the effluent monitored by refractive index (black trace; Optilab rEX) and 90° static light scattering (miniDAWN TREOS, Wyatt Technology) detectors. Data were analyzed to determine shape-independent molecular mass, as described elsewhere ⁴⁰.

Cell culture and transduction.

Mammalian expression plasmids encoding FLAG-tagged coiled-coil domain (amino acid residues 1-60) of mouse PALB2 (WT and L24A) were constructed in the pMX-PIE expression vector. Resting B lymphocytes were isolated from mouse spleens and cultured with LPS (25 µg/ml, Sigma) and IL-4 (5 ng/ml, Sigma) as described ⁴¹. Mouse embryonic fibroblasts (MEFs) and B lymphocytes expressing pMX-PIE vector, WT-PALB2cc and [L24A]-PALB2cc were generated by retroviral transduction. Retrovirus was packaged using BOSC cells and incubated with MEFs or B lymphocytes overnight. Transduced MEFs were selected by 2 µg/ml puromycin while transduced B lymphocytes were not drug selected. Successful transduction of cells was confirmed by western blot using mouse monoclonal anti-FLAG (Sigma, catalog number F1804) antibody.

Preparation of metaphase chromosomes and analysis of chromosome aberrations.

24 hours after transduction of mouse B lymphocytes, cells were mock-treated or treated with 2 µM olaparib overnight. Dividing cells were arrested with 10 µl/ml colcemid for one hour. Metaphase fixation and telomere PNA-FISH was performed as previously described ⁴².

Immunofluorescent detection of RAD51 foci.

Transduced MEFs were grown on sterile coverslips overnight and treated with 10 Gy of ionizing radiation to induce DNA double-strand breaks. After 4 hours recovery at 37°C, cells were fixed using 2% paraformaldehyde followed by treatment with 0.5% Triton X-100. Antibodies used were mouse monoclonal anti-FLAG (Sigma, catalog number F1804) and rabbit polyclonal anti-RAD51 (Santa Cruz, catalog number sc-8349). DAPI was used for nuclei counterstaining. Stained cells were imaged using a Nikon Eclipse E800 epifluorescence microscope.

RESULTS

PALB2cc homodimer formation.

Oligomerization of PALB2 is mediated by a coiled coil motif ⁴³, corresponding to amino acid residues 9 to 44 in the N-terminal region of the protein ^{14, 16, 17, 22, 23, 24, 44, 45}. Several different constructs corresponding to this N-terminal region of PALB2 were cloned and expressed. Some of these constructs provided good expression and solubility, including the PALB2 construct spanning residues 1 to 60. This segment of PALB2 is referred to here as PALB2cc. Backbone amide circular dichroism (CD) analysis (Figure 1A) demonstrates that

PALB2cc is highly helical (55%), and exhibits a highly-cooperative thermal folding/unfolding equilibrium. The oligomerization state of PALB2cc was next characterized, using both estimates of rotational correlations times τ_c from ^{15}N NMR relaxation measurements (Figure 1B) and analytical gel filtration with static light scattering (Figure 1C). These measurements firmly establish that PALB2cc forms a homodimer in solution under the conditions used in this study.

PALB2cc forms a stable antiparallel coiled coil in solution.

The solution NMR structure of dimeric PALB2cc was determined using ^{15}N - and ^{13}C -edited NOESY, X-filtered NOESY, backbone chemical shift, and both ^{15}N - ^1H and ^{15}N - ^{13}C RDC data (Figure 1D, E). Structural statistics and quality scores for the resulting solution NMR structure are summarized in Table 1.

The structure was determined from 3,142 NOE-based distance restraints, including 303 interchain restraints derived from X-filtered NOESY and iterative NOESY assignment, 51 RDC values, and 164 dihedral restraints derived from backbone chemical shift data⁴⁶ These provided 56.2 restraints per residue, including 5.9 interchain restraints per residue. The average backbone root mean square deviation of ordered residues in the final ensemble is 0.3 Å. Backbone dihedral angle analysis indicated that 100% of the well-ordered residues fall in the most favored regions of the Ramachandran plot³⁵. Overall, the structure quality scores, including RPF scores 37 comparing the structure with the unassigned NOESY peak lists (summarized in Table 1), indicate a high quality solution NMR structure for the dimeric PALB2cc.

The dimerization of the PALB2cc is mediated by hydrophobic interactions. The amino acid residues at the center of each chain show a characteristic heptad repeat pattern with hydrophobic residues located in the *a* and *d* positions and charged residues in the *e* and *g* positions (Figure 1F). The highly conserved hydrophobic residues, including Leu21, Leu24, Thr31 and Leu35 at the *a/d* positions of the two α -helices, form an extensive hydrophobic interface in which their aliphatic side chains interact with each other in a 'knobs-into-holes' manner (Figure S3). Numerous long-distance NOEs are observed between H δ methyl protons in the side chain of residue Leu21 and protons of residues Tyr28, Thr31 and Leu32 (Figure S4). These residues are located more than 10 Å apart within each α -helical chain of PALB2cc, and thus are too far apart to generate intramolecular NOEs. These definitive, long-distance intermolecular NOEs demonstrate unambiguously that the dimer is antiparallel.

Residue Leu24 is critical for PALB2 homodimerization.

Based on the NMR structure of the PALB2cc homodimer, we designed a key mutation expected to disrupt the dimer structure. Residue Leu24 is located in the middle of the dimeric interface (Figure 1D) and is highly conserved in many species (Figure S5). This residue was replaced by alanine. Analytical gel filtration with static light scattering experiments demonstrate that this mutant PALB2cc is predominantly monomeric in solution at room temperature (Figure 2A). Thermal transition temperatures for wt- and [L24A]-PALB2cc demonstrate that the L24A mutation significantly destabilizes PALB2cc, with T_m

for wt- and mutant of higher than 50 °C and ~ 20 °C, respectively (Figure 2B). These thermal transition curves were fully reversible. Interestingly, monomeric PALB2cc retains some helical structure, as CD spectra of [L24A]-PALB2cc exhibit two negative peaks at 209 and 222 nm (Figure 2C). The [¹H-¹⁵N]-HSQC spectrum of [L24A]-PALB2cc further confirms that [L24A]-PALB2cc retains some ordered structure (Figure S6). Taken together, these data confirm that interfacial residue Leu24 is critical for PALB2cc homodimer formation.

NMR titration experiments were next used to determine the homodimeric dissociation constants K_d for wt- and [L24A]-PALB2cc. Qualitative features of complex affinity were assessed based on whether monomers and complexes are in slow-exchange on the NMR time scale, where separate resonance frequencies are measured for atoms in the monomer and in the complex, or in fast-exchange on the NMR timescale, where interconversion between monomer and complex states is sufficiently rapid that NMR resonance frequencies are population-weighted averages of the frequencies in the monomer and complex states. Generally speaking, slow-exchange dynamics are observed for longer-lived complexes, and tighter binding affinities, while fast-exchange dynamics indicate shorter-lived complexes and weaker binding affinities. In the slow-exchange limit, dimer dissociation constants K_d 's were determined from relative intensities of NMR resonances assigned to monomer and dimer states, respectively, measured in titration experiments, while in the fast-exchange limit K_d 's were estimated by fitting titration data to population-weighted chemical shift curves^{47, 48}. Consistent with the solution NMR structure and analytical gel filtration data, these NMR data demonstrate that the L24A mutation disrupts the PALB2cc homodimer. The homodimer dissociation constants estimated from NMR titration data are 100-fold different, with $K_{d\text{homo}}$ being $\sim 80 \pm 10 \mu\text{M}$ and $\sim 7000 \text{ M} \pm 2000 \mu\text{M}$, for the wt and L24A mutant, respectively. Hence, interfacial interactions involving residue Leu24 in the coiled-coil structure (Figure 2D) contribute significantly to stabilizing the PALB2cc homodimer.

The PALB2cc:BRCA1cc heterodimer is more stable than the PALB2cc homodimer.

Next, we used NMR spectroscopy to probe the interactions between PALB2cc and BRCA1cc. ¹⁵N-enriched PALB2cc was titrated with unenriched BRCA1cc. These results are illustrated in Fig. 3. In this titration, some ¹⁵N-¹H correlation peaks in the two-dimensional [¹H-¹⁵N]-HSQC spectrum of PALB2cc became weaker or disappeared (Figure 3A). These changes were accompanied by the appearance of the ¹⁵N-¹H resonances of PALB2cc in the complex with BRCA1cc. Estimates of rotational correlation time τ_c based on ¹⁵N-relaxation T_1/T_2 measurements demonstrate that the resonances associated with this complex have a τ_c corresponding to a heterodimer (Figure 3B). NMR titration experiments demonstrate that conformational exchange between homodimeric PALB2cc and the PALB2cc:BRCA1cc complex is slow on the NMR timescale (Figure 3C-E), as resonances of the homodimer decreased in intensity as resonances from the heterodimer increase in intensity. The dissociation constant $K_{d\text{hetero}}$ for this heterodimeric complex, estimated from these NMR data, is $\sim 6 \pm 3 \mu\text{M}$, about 10-fold more stable than the PALB2cc homodimer.

Residue Leu24 contributes less to PALB2 interactions with BRCA1.

Compared to the PALB2 homodimer, the heterodimeric PALB2cc:BRCA1cc complex is less disrupted by the L24A mutation in PALB2. As shown for the wt PALB2cc:BRCA1cc complex, titration of ^{15}N -[L24A]-PALB2cc with unlabeled BRCA1cc can be used to detect complex formation (Figure 3F). In this case, the equilibrium exchange is fast on the NMR chemical shift timescale. NMR titration data provide an estimate of the heterodimer dissociation constant for the [L24A]-PALB2cc:BRCA1cc complex $K_d^{\text{hetero}} \sim 90 \pm 20 \mu\text{M}$, about 10-fold weaker than corresponding $K_d^{\text{hetero}} \sim 6 \pm 3 \mu\text{M}$ for the wt-PALB2cc:BRCA1cc complex. Hence, as illustrated in Figure 4, these NMR titration data demonstrate that (i) the heterodimeric PALB2cc:BRCA1cc complex is significantly more stable than the homodimeric PALB2cc complex, and (ii) the L24A mutation in the PALB2 homodimer interface has a significant (~ 100 -fold) effect on the homodimer stability, but a more modest effect (~ 10 -fold) on the PALB2cc:BRCA1cc heterodimer stability. These results indicate that it may be possible to identify PALB2 mutations that selectively disrupt homodimeric versus heterodimeric interactions.

Mutation of residue Leu24 impairs the DNA damage repair activity of PALB2.

To investigate the effect of the L24A substitution on PALB2 function in cells, peptides containing normal and mutant versions of the PALB2 coiled-coil motif were expressed in mouse B lymphocytes and embryonic fibroblasts (MEFs). Peptides containing the PALB2 coiled-coil motif normally have a dominant-negative effect on PALB2 function when overexpressed, based on their ability to associate with endogenous PALB2 and BRCA1 and prevent them from associating with their normal cellular binding partners¹⁴. As reported previously, overexpression of a FLAG-tagged peptide containing residues 1-60 from PALB2 (i.e. PALB2cc) induced substantial genomic instability in WT B cells (Figure 5A). In contrast, an equivalent peptide carrying the L24A substitution induced a significantly lower level of genomic instability. Replacement of Leu24 with alanine therefore appears to prevent PALB2cc peptides from interfering with normal PALB2 activity, supporting the idea that residue Leu24 is important for PALB2 coiled-coil interactions and for its function in DNA damage repair.

PALB2-mediated localization of BRCA2 to double-strand breaks is essential for normal RAD51 recruitment to damaged DNA⁴. Whereas ionizing radiation induced the formation of RAD51 foci in the nuclei of approximately 35% of control cells, very few cells expressing dominant negative PALB2cc peptide showed RAD51 foci formation (Figure 5B). Cells overexpressing a PALB2cc peptide carrying the L24A mutation showed significantly higher levels of RAD51 foci formation than was seen in cells expressing the wt-PALB2cc construct. This indicates that Leu24 is critical for complex formation by the PALB2 coiled-coil motif. Disruption of this residue alone is sufficient to block much of the dominant negative effect of PALB2cc peptides. Cells showing low levels of FLAG co-staining were excluded from this analysis, so that only cells with high levels of peptide expression were counted. The L24A mutation, which was revealed by CD and NMR spectroscopy to be disruptive for both PALB2cc homodimerization and PALB2cc-BRCA1cc heterodimerization, is therefore also disruptive for normal PALB2 complex formation in cells. Although our results based on biophysical characterizations indicate that the L24A

mutation has a greater effect on PALB2 homodimerization versus heterodimerization, at this stage we cannot distinguish whether the cellular phenotypes associated with expression of [L24A]-PALB2cc arise by disruption of homodimeric or heterodimeric PALB2 complexes.

DISCUSSION

The homodimer formed by coiled-coil regions of PALB2 has a classic leucine zipper structure (Figure 1), with antiparallel orientation. In this solution NMR structure, the flanking regions of the peptide construct studied are largely disordered, perhaps adopting short-lived transient structures (Figure 1D). Key residues at the dimer interface include Leu17, Leu21, Leu24, Tyr28, Thr31, and Leu35. This homodimer is disrupted by the Leu24 to Ala single-site mutant. Significantly, our CD data also demonstrate that the resulting monomeric form of the PALB2cc peptide retains significant helical structure (Figure 2C). This PALB2cc region also forms a heterodimeric complex with the BRCA1cc region; however, the structure and relative domain orientations of this heterodimer are not yet determined.

Homodimeric PALB2cc has a dissociation constant (K_d) of $\sim 80 \mu\text{M}$, which is weaker than that of the corresponding PALB2cc:BRCA1cc heterodimer ($\sim 6 \mu\text{M}$). Hence, at equilibrium, these polypeptide fragments energetically prefer the heterodimeric form. However, this preference corresponds to only a few kilocalories in free energy, and in the context of full-length proteins and the cellular environment, this equilibrium could be significantly different. Both homodimer and heterodimer formation are impaired by the L24A mutation; however, the homodimer dissociation constant is significantly more sensitive to this mutation than that of the heterodimer. This result suggests it may be possible to design mutants of PALB2 or BRCA1 that preferentially disrupt the PALB2 homodimer relative to the PALB2:BRCA1 heterodimer.

The six amino acid residues (Leu17, Leu21, Leu24, Tyr28, Thr31 and Leu35) that occupy particularly prominent sites at the PALB2 coiled-coil homodimer interface (Figure 1D-F) all show 100% conservation in the PALB2 sequences from human, mouse, cow and horse ²¹. Leu17, Leu21, Tyr28 and Thr31 are additionally conserved in *Xenopus laevis* (Figure S5). Several of these residues have been the subject of earlier studies aimed at understanding the importance of the PALB2 coiled-coil region. Zhang *et al.* generated point mutant versions of PALB2 in which either Leu21 or Leu24 was replaced with proline (PALB2-L21P and PALB2-L24P) ⁴⁵. Neither of these mutant PALB2 mutant proteins was able to interact with BRCA1, nor were they able to rescue HR in cells in which endogenous PALB2 was knocked down. These results do not, however, provide specific insight into the contributions of residues Leu21 and Leu24 to PALB2 activity, as insertion of proline residues into an α -helical region will likely cause disruption of the entire helix structure ⁴⁹. Indeed, based on our structural studies it is likely that these proline mutants would disrupt both the homodimeric and heterodimeric complexes. Foo *et al.* also reported a PALB2 missense mutation in which proline replaces Leu35 ⁵⁰. The L35P variant is associated with breast cancer, and prevents stable association of PALB2 with BRCA1. L35 is a key interface residue in our PALB2 homodimer structure, and its mutation to a proline residue would also be expected to cause a broader disruption of the entire helical region. Accordingly, based on

our experimental structure of the PALB2cc coiled-coil homodimer, none of these previous studies distinguish the roles of residues Leu21, Leu24, or Leu35 in specific dimeric interactions, as replacing any of these Leu residues with Pro is expected to disrupt the overall structures of both the homodimeric PALB2 and heterodimeric PALB2:BRCA1 coiled-coil complexes.

Several other reports have suggested that genetic changes affecting the PALB2 coiled-coil region have the potential to cause disease. Four different splice or frameshift mutations within the region of the *PALB2* gene encoding the coiled-coil motif were identified in a patient study of pathogenic *PALB2* variants⁵¹. Sequencing of 1,250 individuals from the USA further revealed that a *PALB2* variant in which arginine replaces Lys18 is present in 0.89% of the population⁵². Based on *in silico* analysis, this relatively-common *PALB2* variant is predicted to have the potential to be pathogenic by altering the function of the protein. According to our solution NMR structure, the K18R mutation is not predicted to directly alter interface contacts in the PALB2 homodimer, and immunoprecipitation experiments have demonstrated that this mutation has little effect on PALB2 homodimerization or association with BRCA1⁵⁰.

Scanning alanine mutagenesis of triplets of amino acid residues revealed sequence requirements within the PALB2 coiled-coil region that are essential to support homologous recombination²¹. PALB2 protein in which each of residues 24 - 26 were replaced by alanine was unable to support homologous recombination. Knock-in mice expressing PALB2 with this triplealanine mutation (*Palb2*^{CC6/CC6}) were viable, but showed a male fertility defect²¹. Cells from these mice were hypersensitive to mitomycin C (MMC), indicating a defect in DNA repair. These results were interpreted as evidence for an essential role for residue segment 24 – 26 in stabilizing heterodimers of PALB2 with BRCA1. Notably, one of these residues (Leu24) was identified in the present study as a key interface residue for homodimer formation by coiled-coil regions from PALB2. These results are therefore also consistent with the possibility that PALB2 homodimerization is essential for normal repair of DNA damage.

Deletion of the coiled-coil motif from *BRCA1*, which prevents BRCA1 from forming a heterodimer with PALB2, results in severely reduced HR efficiency and hypersensitivity to chemotherapy agents in human cell lines⁵³. This observation matches earlier observations that the integrity of the BRCA1 coiled-coil motif is necessary for HR activity¹⁶. Residues Leu21, Tyr28 and Leu35 were all predicted to occupy the 'a' position in the heptad repeat of the PALB2 coiled-coil region when it associates with a coiled-coil region from BRCA1¹⁶. PALB2 protein in which any of residues Leu21, Tyr28 or Leu35 is replaced with alanine was additionally shown to be non-functional for sustaining HR in cellular reporter assays, and was not able to complement mitomycin C hypersensitivity of PALB2-deficient cells. This deficiency in HR correlated with a failure of L21A, Y28A or L35A PALB2 to interact with BRCA1 in co-IP assays. The structural analysis presented in this study reveals that these same residues are present at the dimer interface for PALB2 homodimerization. In fact, the predicted model for the BRCA1:PALB2 heterodimer interface¹⁶ turns out to be largely consistent with our homodimer structure. This evidence supports the idea that competition

between formation of PALB2 homodimeric coiled coils versus heterodimeric PALB2:BRCA1 coiled coils may influence the efficiency of HR ¹⁴.

The ability of PALB2 to regulate HR is modulated by DNA damage-induced phosphorylation of several residues. Ionizing radiation induces phosphorylation of residues Ser59, Ser157, and Ser376⁵⁴⁻⁵⁶. Of these, Ser59 is part of the PALB2 constructs that we used in this study. We found that Ser59 does not participate in the coiled-coil interactions, but instead lies on the C-terminal side of the coiled-coil α -helix, within an unstructured region. The importance of Ser59 phosphorylation was underscored by the demonstration that ATR-dependent phosphorylation of Ser59 regulates the interaction of PALB2 with BRCA1, and further contributes to recruitment of PALB2 to DNA damage site ⁴⁴. The interaction of human PALB2 with BRCA1 is also influenced by CRL3-KEAP1-mediated ubiquitylation of PALB2 at Lys20, Lys25 or Lys30⁵⁷. The corresponding residues in mouse PALB2, Lys20, Lys25, and Arg30, are within the coiled-coil region. Although the sidechain of residue Lys-25 forms an intrachain salt bridge with Glu-27, none of these proposed ubiquitylation sites are key interface residues of the PALB2 coiled-coil homodimer interaction. It is possible, however, that ubiquitylations and phosphorylations of sites within and around the PALB2 coiled-coil region selectively affect heterodimerization of PALB2 with BRCA1, while leaving intact the interface for PALB2 homodimerization. The solution NMR structure of the PALB2 homodimer described here provides the basis for further studies of the structure-function relationships of the coiled-coil motifs of PALB2 and BRCA1 in HR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PALB2	Partner and localizer of BRCA2
HR	Homologous recombination
BRCA1	Breast cancer susceptibility 1
BRCA2	Breast cancer susceptibility 2
CD	circular dichroism
NMR	nuclear magnetic resonance

ATR Ataxia telangiectasia related

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Figure 1. Biophysical and NMR measurements reveal an antiparallel coiled-coil structure of PALB2cc.

(A) Amide CD spectra indicate a helical structure of PALB2cc. (B) Rotational correlation time (τ_c) at 20 °C *versus* molecular mass plotted for known monomeric proteins (*blue*) and PALB2cc monomer (*red triangle*) demonstrate a dimeric structure. (C) AGF-MALS data indicate a molecular mass of 18 ± 2 kDa PALB2cc (upper dashed red horizontal line). Compared with the expected molecular mass for the single chain including an affinity tag 7.9 kDa, these data further demonstrate the dimeric structure. (D) Ensemble of the 20 lowest-energy NMR-derived conformers. (E) Expanded view of the dimer interface. Side-chains that mediate important interactions are shown as ball-and-sticks, and are labeled. (F) Coiled-coil helical wheel representation of PALB2cc dimer. Labeled residues at "a" and "d" coiled-coil positions form a predominantly hydrophobic interaction interface.

Figure 2. Mutation of residue Leu24 to Ala disrupts the PALB2cc homodimer.

(A) AGF-MALS traces demonstrate that [L24A]-PALB2cc is monomeric in solution. Comparison of (B) CD melting curves and (C) CD wavelength scans of wt (top) and [L24A]-PALB2cc (bottom) confirm the disruption of the homodimeric structure by this interfacial mutation. (D) Expanded view of the dimer interface. Side-chains that mediate important packing interactions are shown as spheres, and the Leu24 side-chain is colored red.

Figure 3. Formation of PALB2cc:BRCA1cc heterodimer and effect of [L24A]-PALB2cc mutation on heterodimerization.

(A) $[^1\text{H}-^{15}\text{N}]$ -HSQC NMR data demonstrate formation of PALB2cc:BRCA1cc heterodimer. Peaks from ^{15}N -enriched PALB2cc (20 μM , blue peaks) appear at new frequencies upon complex formation (red peaks) by addition of 2-fold molar excess of unlabeled BRCA1cc (40 μM). (B) Rotational correlation time (τ_c) at 20 $^\circ\text{C}$ versus molecular mass plotted for known monomeric proteins (blue) and the PALB2cc:BRCA1cc complex (red triangle) demonstrate that the ^{15}N labeled PALB2cc forms a dimeric complex with unlabeled BRCA1cc. (C-E). Titration of 20 μM ^{15}N -PALB2cc with 20, 24, and 36 μM unlabeled BRCA1cc reveals that the equilibrium exchange between ^{15}N -PALB2cc homodimer (blue peaks in each panel) and ^{15}N -PALB2cc:BRCA1cc heterodimer (red peaks) is slow on the NMR chemical shift timescale. The K_d estimated from these data is $\sim 6 \pm 3 \mu\text{M}$. (F) $[^1\text{H}-^{15}\text{N}]$ -HSQC NMR data demonstrate formation of [L24A]-PALB2cc:BRCA1cc heterodimer. Titration of 20 μM ^{15}N -[L24A]-PALB2cc with 40 μM unlabeled BRCA1cc also results in spectral changes indicating complex formation. In this case, the equilibrium between ^{15}N -PALB2cc homodimer (blue peaks) and the ^{15}N -PALB2cc:BRCA1cc heterodimer (red peaks) is fast on the NMR chemical shift timescale, with an estimated K_d

of $90 \pm 20 \mu\text{M}$. All NMR titration and ^{15}N relaxation data were obtained at 600 MHz and 20 °C.

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Figure 4. Schematic representation of homo- and heterodimer formation in wt and L24A PALB2cc.

The homo- and heterodimer dissociation constants determined from NMR are illustrated for the wt- and L24A mutants (denoted with green highlight) of PALB2cc (blue coil) interacting with BRCA1cc (red coil). The heterodimeric complex PALB2cc:BRCA1cc is illustrated hypothetically as a coiled-coil structure, and the monomeric form of BRCA1cc is illustrated as a helix for convenience, when in fact NMR data indicate it is not ordered in the absence of the heterodimeric interaction.

Figure 5. Functional impact of the L24A mutation in PALB2.

(A) Analysis of genomic instability in mouse B lymphocytes overexpressing PALB2 coiled-coil peptides. Left, example of metaphase chromosome spread showing two radial chromosomes (white arrows). Right, average frequencies of radial chromosomes, chromatid breaks (CTD), chromosome breaks (CSB) and all other chromosome aberrations in metaphase chromosome spreads. Error bars show standard deviation of the mean total number of aberrations, based on three experiments. At least 50 metaphases were scored per experiment. (B) Detection of RAD51 foci in irradiated mouse embryonic fibroblasts. Left, immunofluorescence for RAD51 in nuclei exposed to 10 Gy of ionizing radiation. Nuclear DNA is stained with DAPI, and cells overexpressing PALB2 coiled-coil peptides were identified by staining for FLAG. Right, percentage of either all cells (vector control), or cells staining positive for FLAG (others), that showed greater than five RAD51 foci. Error bars show standard deviation of the means from two separate experiments in which at least 50 nuclei were scored. P values (two-tailed) were calculated using Student's t-test.

Table 1.

Summary of NMR data and structure statistics for PALB2cc homodimer.

conformationally-restricting NOE-based distance restraints	
total	3142
intra-residue [$i=j$]	803
sequential [$ i - j = 1$]	1027
medium range [$1 \leq i - j < 5$]	1029
long range [$ i - j \geq 5$] (interchain)	303
hydrogen bond restraints	100
dihedral angle restraints	164
residual dipolar couplings	51
number of conformationally-restricting restraints per restrained residue	56.2
residual restraint violations ^a	
average number of distance restraint violations per structure	
0.1 – 0.2 Å	12.7
0.2 – 0.5 Å	3.65
> 0.5 Å	0
maximum distance restraint violation	0.36 Å
average number of dihedral restraint violations per structure	
1 – 10°	14.25
> 10°	0
maximum dihedral restraint violation	5.10°
root-mean-square deviations of superimposed coordinates ^a	
backbone atoms (for well-ordered residues 11 – 39)	0.3 Å
heavy atoms (for well-ordered residues 11 – 39)	0.6 Å
Ramachandran statistics (for well-ordered residues 11 – 39, Molprobability) ^a	
most favored regions (%)	100
additional allowed regions (%)	0
disallowed regions (%)	0
Global quality scores ^a	
Verify3D	Raw / Z-score
ProsaII	0.14 / -5.14
ProsaII	0.82 / 0.70
Procheck (ϕ - ψ) ^b	0.70 / 3.07
Procheck (all) ^b	0.21 / 1.24
MolProbability Clash	15.6 / -1.16
RPF Scores ^c	
recall, precision, F-measure, DP-Score	0.925, 0.971, 0.948, 0.766

^aValues were calculated using PSVS Version 1.5. Averaged distance violations were calculated using the sum over r^{-6} .^bOrdered residue range: residues 11 – 39.

^cRPF scores reflect the goodness of fit of the final ensemble of structures (including disordered residues) to the NMR data.

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