Perturbation of PALB2 function by the T413S mutation found in small cell lung cancer [version 2; peer review: 3 approved]

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Abstract

Background: Germline mutations in the PALB2 gene are associated with the genetic disorder Fanconi anaemia and increased predisposition to cancer. Disease-associated variants are mainly protein-truncating mutations, whereas a few missense substitutions are reported to perturb its interaction with breast cancer susceptibility proteins BRCA1 and BRCA2, which play essential roles in homology-directed repair (HDR). More recently, PALB2 was shown to associate with active genes independently of BRCA1, and through this mechanism, safeguards these regions from DNA replicative stresses. However, it is unknown whether PALB2 tumour suppressor function requires its chromatin association.

Methods: Mining the public database of cancer mutations, we identified four potentially deleterious cancer-associated missense mutations within the PALB2 chromatin association motif (ChAM). To assess the impact of these mutations on PALB2 function, we generated cell lines expressing PALB2 variants harbouring corresponding ChAM mutations, and evaluated PALB2 chromatin association properties and the cellular resistance to camptothecin (CPT). Additionally, we examined the accumulation of γH2A.X and the RAD51 recombinase as readouts of DNA damage signalling and HDR, respectively.

Results: We demonstrate that a small-cell lung cancer (SCLC)-associated T413S mutation in PALB2 impairs its chromatin association and confers reduced resistance to CPT, the only FDA-approved drug for relapsed SCLC. Unexpectedly, we found a less efficient γH2A.X nuclear foci formation in PALB2 T413S expressing cells, whereas a near-normal level of RAD51 nuclear foci was visible.

Conclusions: These findings support the importance of PALB2 chromatin association in the suppression of tumours, including SCLC, an unusually aggressive type of cancer with poor prognosis. PALB2 T413S has little impact on RAD51 recruitment, likely due to its intact interaction with BRCA1 and BRCA2. However, this mutant shows inefficient DNA stress signalling. This finding sheds new light on the function of PALB2, playing a role in efficient DNA stress signalling through constitutive chromatin association.

Keywords
Keywords
PALB2, small cell lung cancer, DNA damage, camptothecin

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Author roles: Bleuyard JY: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Butler RM: Investigation, Methodology; Esashi F: Conceptualization, Funding Acquisition, Supervision, Validation, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the Wellcome Trust [101009], Senior Research Fellowship to F.E., and a Wellcome Trust Biomedical Vacation Scholarship to R.M.B.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Bleuyard JY, Butler RM and Esashi F. Perturbation of PALB2 function by the T413S mutation found in small cell lung cancer [version 2; peer review: 3 approved] Wellcome Open Research 2018, 2:110 (https://doi.org/10.12688/wellcomeopenres.13113.2)

First published: 14 Nov 2017, 2:110 (https://doi.org/10.12688/wellcomeopenres.13113.1)
REVISED Amendments from Version 1

We thank the reviewers for their comments and suggestions. Taking these into account, in this version (2), we have made the following amendments:

1. We have included additional text discussing the difficulty in obtaining clonal cell lines expressing the full-length PALB2 ChAM mutants at a similar level as wild-type PALB2, and thus the possibility that ChAM missense substitutions partly destabilise the PALB2 protein, contributing to the observed PALB2 haploinsufficiency for tumour suppression.

2. We explain that, in contrast to the robust impact of ChAM mutations in disrupting the chromatin association of the GFP-ChAM fragment, the partial rescue of chromatin association of full-length PALB2 mutants was somewhat expected, as PALB2 chromatin association is mediated by both ChAM and MRG15 binding.

3. We discuss the notion that the S417Y mutation could be a low-penetrance or neutral variant.

4. We articulate that the role of ChAM in homologous recombination needs to be investigated further.

5. We adjusted our Discussion regarding the pathological impact of the T413S ChAM mutation in the development of SCLC, indicating that this link needs to be further investigated using clinical samples and/or genetic studies.

6. Scale bars have been added to Figure 4A. The statistical data comparing wild-type PALB2 to T413S or S417Y variants, and between the T413S and S417Y variants have been added to Figure 4B. Typos in the Results section have been corrected.

See referee reports

Introduction

BRCA1 and BRCA2 (breast cancer 1 and 2) are two of the best-known cancer susceptibility genes, and mutations in these genes are causally connected to the rare genetic disorder Fanconi anaemia. At the molecular level, BRCA1 and BRCA2 cooperate to promote the homology-directed repair (HDR) of highly genotoxic DNA lesions, such as double-strand breaks (DSBs) or inter-strand crosslinks (ICLs)
.

PALB2 (Partner And Localizer of BRCA2) was more recently identified as a binding partner of BRCA2 and was subsequently shown to bridge BRCA1 and BRCA2 physically
.

Importantly, following studies have demonstrated a link between germline mutations in the PALB2 gene, and Fanconi anaemia and an elevated risk of developing breast, ovarian and pancreatic cancers
, identical to cancer types associated with BRCA1 and BRCA2 germline mutations
.

To date, the majority of disease-associated PALB2 mutations are loss-of-function mutations caused by C-terminal protein truncations of various lengths
. The PALB2 C-terminal WD40-type \( \beta \)-propeller domain is known to promote interaction with BRCA2, and its structure has demonstrated that deleting only the last four residues (the consequence of the PALB2 Y1183X cancer-associated nonsense mutation) is sufficient to disrupt the proper folding of the domain and render the protein unstable
. Another study has revealed that cancer-associated PALB2 C-terminal truncations (such as Q988X or W1038X) can alternatively expose a hidden nuclear export signal (residues 928-945) and lead to the mis-localisation of PALB2 in the cytoplasm
. Since PALB2 forms homo-oligomers through its N-terminal coiled-coil domain
, it is plausible that the Q988X and W1038X truncated proteins can sequester the product of a functional PALB2 allele in the cytoplasm. These variants are hence unable to fulfil PALB2 tumour suppressor function, even in the presence of a wild-type allele.

Recent studies have additionally characterised several missense mutations of PALB2 associated with human disease. For example, an in-frame exclusion of PALB2 exon 6, resulting in the deletion of residues 839-862 within the WD40 repeat domain, has been reported to give rise to a mild form of Fanconi anaemia, without the severe developmental abnormalities usually associated with the disease
. In line with this observation, when exogenously expressed in U2OS cells, this hypomorphic in-frame exclusion variant of PALB2 retained BRCA2 interaction and supported RAD51 accumulation at damage-induced nuclear foci, a crucial step of DSB and ICL repair mediated by the BRCA1-PALB2-BRCA2 complex.

Conversely, PALB2 sequencing in non-BRCA1/2 familial breast cancer cases led to the identification of germline missense mutations in the coiled-coil domain (L35P), identified in patients with a truncating mutation in the second allele, and WD40 repeat domain (L939W and L1143P)
. These single-residue changes result in decreased interaction with BRCA1 and BRCA2, respectively, and have been shown to reduce the cellular DSB-repair potential
.

Additionally, breast cancer-associated germline mutations in BRCA2 N-terminus (G25R, W31C and W31R) have also been reported to disrupt the PALB2-BRCA2 interaction
. These findings emphasise the critical role of the BRCA1-PALB2-BRCA2 complex in the maintenance of genome stability and prevention of cancer and provide evidence that heterozygosity for rare missense variants of PALB2 may influence cancer risk.

We previously described the evolutionarily conserved chromatin association motif (ChAM) of PALB2 and showed that it promotes direct interaction with nucleosomes
. Together with the MRG15-binding domain (MBD), which mediates PALB2 interaction with the chromodomain-containing MRG15 protein, ChAM controls PALB2 chromatin association
. In our latest study, we further demonstrated that the ChAM and the MBD act in concert to tether PALB2 to active genes, protecting these loci from replication-associated stress
. Our analysis of missense substitutions within the MBD established that, in the absence of this mechanism, cells accumulate DNA damage at active genes, a process that may ultimately lead to the conversion of pre-cancerous to cancerous cells. Given the emerging evidence that missense substitutions in protein-binding domains of PALB2 are connected with disease predisposition, we aimed to elucidate whether cancer-associated missense mutations may also perturb the function of the PALB2 ChAM.

Methods

Sequence analyses

Sequences of PALB2 orthologues from 40 species were retrieved from the Ensembl and NCBI resources, and aligned with MUSCLE. Dr (Danio rerio, Zebrafish), Tr (Takifugu rubripes, ...
Japanese pufferfish), On (Oreochromis niloticus, Nile tilapia), Ac (Anolis carolinensis, Carolina anole lizard), Xt (Xenopus tropicalis, Western clawed frog), Sh (Sarcophilus harrisii, Tasmanian devil), Md (Monodelphis domestica, Gray short-tailed opossum), Sa (Sorex araneus, Common shrew), MI (Myotis lucifugus, Little brown bat), Cp (Cavia porcellus, Guinea pig), Ss (Sus scrofa, Wild boar), La (Loxodonta africana, African bush elephant), Gg (Cricetulus griseus, Chinese hamster), Mm (Mus musculus, House mouse), Rn (Rattus norvegicus, Brown rat), Ch (Chloephos hoffmanni, Two-toed sloth), Am (Orcinus orca, Common bottlenose dolphin), Oo (Pelodiscus sinensis, Asian pond turtle), Eo (Emydoidea blandingii, Blanding’s turtle), Lf (Ligonia finlaysoni, Finlayson’s leaf-toed elephant shrew), Go (Gorilla gorilla, Western lowland gorilla), Hs (Homo sapiens, human), Cj (Callithrix jacchus, Common marmoset), Pt (Pan troglodytes, Chimpanzee), NI (Nomascus leucogenys, White-cheeked gibbon), Mam (Macaca mulatta, Rhesus macaque), Pa (Pongo abelii, Sumatran orangutan), Paa (Papio anubis, Olive baboon), Clf (Canis lupus familiaris, Dog), Ord (Odobenus rosmarus divergens, Walrus), Bt (Boa, house boa), Oa (Ovis aries, Sheep), Vp (Vicugna pacos, Alpaca), Tt (Tursiops truncates, Common bottlenose dolphin), Oo (Orcinus orca, Killer whale), Tg (Taeniopygia guttata, Zebra finch), Gg (Galus gallus, Red junglefowl), Mg (Meleagris gallopavo, Wild turkey), Ps (Pelodiscus sinensis, Chinese softshell turtle) and Cm (Chelonias mydas, Green sea turtle).

Polyphen-2 prediction of potentially deleterious amino acid substitutions was performed using the J. Craig Venter Institute webserver default parameters. The amino acid substitutions were submitted for batch analysis using human PALB2 Q86YC2 (Uniprot) sequence as reference.

SIFT prediction of potentially deleterious amino acid substitutions was performed using the J. Craig Venter Institute webserver default parameters. The amino acid substitutions were submitted for batch analysis using human PALB2 ENSP00000261584 (Ensembl) sequence as reference.

The secondary structure elements of the human ChAM (PALB2 residues 395 to 450) were predicted using the University of Dundee Jpred427 webserver default parameters.

Cell culture and cell lines
HEK293T and EUFA1341 (PALB2-deficient) cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, D6429, Sigma-Aldrich) supplemented with 10% (v/v) FBS (foetal bovine serum), penicillin (100 U/ml) and streptomycin (0.1 mg/ml). All cells were grown at 37°C in an incubator containing 5% CO2. EUFA1341 cells were transfected with pCEP4-GW/FLAG-PALB2 variants plasmids. Stable cell lines expressing PALB2 cancer-associated variants were first selected and later maintained with 300 µg/ml and 150–200 µg/ml hygromycin, respectively. HEK293T (CRL-3216) were obtained from ATCC and EUFA1341 cells were a kind gift from Dr. H. Joenje (VU University Medical Center, The Netherlands).

Site-Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing. Table 1 provides a list of the mutagenic oligonucleotides used in this study. For GFP-ChAM expression in HEK293T cells, ChAM variants in pENTR1A were transferred to pcdNA-DEST53 (12288015, Invitrogen) using Gateway cloning. For FLAG-PALB2 expression in EUFA1341 cells, PALB2 variants in pENTR3C were transferred to pCEP4-GW/N3xFLAG15 using Gateway cloning.

Transfection
Cells were seeded at a density of 300,000 cells/well in 6 well plates. The next day, the cells were transfected using 0.6136 mg/ml polyethyleneimine (PEI, 408727, Sigma-Aldrich) in MES-HEPES buffered saline (50 mM MES, 50 mM HEPES, 75 mM NaCl, adjusted to pH 7.2 with NaOH), using a ratio of 4 µl PEI for 2 µg DNA. DNA and PEI were diluted separately in 100 µl of plain DMEM (without any supplement), combined and after vortexing for 10 sec incubated at room temperature (RT) for 20 min. 200 µl of transfection mix was added to the cells in 2 ml of plain DMEM. After 6 hours, medium containing transfection mix was exchanged for DMEM supplemented with 10% FBS.

Chemical cell fractionation, whole-cell extract and western blot
Cells were collected, washed twice with ice-cold PBS and resuspended in Sucrose buffer (10 mM Tris-HCl pH 7.5, 20 mM KCl, 250 mM Sucrose, 2.5 mM MgCl2, 10 mM Benzamidine Hydrochloride and P2714 protease inhibitor cocktail from Sigma-Aldrich). Triton X-100 was added to a final concentration of 0.2%, and cells were vortexed three times for 10 seconds, followed by centrifugation (500 g, 4°C, 5 min) to separate the cytoplasmic fraction from the nuclei pellet. Nuclei were extracted for 30 min on ice in NETN150 buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10 mM Benzamidine Hydrochloride and P2714 protease inhibitor cocktail from Sigma-Aldrich). The nuclear fraction and chromatin pellet were separated by centrifugation (1,000 g, 4°C, 5 min). The chromatin pellet was finally solubilised for 1 h on ice using NETN150 buffer supplemented with 2 mM MgCl2 and 125 U/ml Benzonase nuclease (71206-3, Novagen). Cytoplasmic, nuclear and chromatin soluble fractions were centrifuged (16,100 g, 4°C, 30 min) to remove cell debris.

For whole-cell extract, the cells were directly lysed in NETN150 buffer supplemented with 2 mM MgCl2 and 125 U/ml for 1 h on ice and centrifuged (16,100 g, 4°C, 30 min) to remove cell debris.

For western blot, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Detection was performed using antibodies against GFP (RRID:AB_439690, G1544, Sigma-Aldrich, 1:2,000 dilution), Tubulin (RRID:AB_1904178, Sigma, 1:1,000 dilution), FLAG (RRID:AB_439702, A8592, Sigma, 1:2,000 dilution) and histone H3 (RRID:AB_2118462, A300-823A, Bethyl, 1:2,000 dilution).

Cell survival assay
EUFA1341 cell lines stably expressing FLAG-PALB2 variants were seeded at a density of 7,500 cells/well in 96-well plates and cultured for 24 h before treatment. Cells were then grown in the
presence of 0–100 nM camptothecin (208925, Calbiochem) for 4 days. Cell proliferation was measured using WST-1 reagent (05015944001, Roche Applied Science), as previously described. 4-day survival is expressed as the percentage of live cells compared to the untreated (no drug) sample. Two technical replicates were performed for each of three experiments. The dose-response curves were fitted to the data pool and the IC50 values calculated using Prism 7 (RRID:SCR_002798, GraphPad Software).

Immunofluorescence staining and automatic nuclear foci quantification
In 12-well plates, approximately 100,000 EUFA1341 cells complemented with FLAG-PALB2 variants were seeded onto glass coverslips pre-coated, 5 min at room temperature, with 1 µg/ml PEI (408727, Sigma-Aldrich) in plain DMEM. The next day, the growth medium was refreshed with medium supplemented with 10 nM CPT in DMSO or the equivalent volume of vehicle. After 17h of incubation (37°C, 5% CO₂), the cells were washed twice with 1x PBS and fixed with 4% formaldehyde (Pierce, 28908) in 1x PBS (15 min, RT). The cells were incubated three times with PBS-T (5 min on an orbital shaker, RT). The coverslips were finally air-dried at RT and mounted on glass slides with ProLong Gold antifade reagent with DAPI (P36935, Invitrogen).

Images acquired on an Olympus Fluoview FV1000 confocal laser-scanning microscope, using fixed parameters, were converted to RGB TIFF format using the Fiji (RRID:SCR_002285)

<table>
<thead>
<tr>
<th>Residue change(s)</th>
<th>Primer sequence (5’ → 3’); F: forward; R: reverse.</th>
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<td>T397S</td>
<td>F: AAGGCCCTCAGGCACTGAGCAAGAATGTTTCTG</td>
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<tr>
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<td>R: CAGAAAACATTTCTGCAGTGGCAGCTAGCCCTT</td>
</tr>
<tr>
<td>F404L</td>
<td>F: CATATAATTTCTGCAAGTAAACAGAAGGCTTCAGG</td>
</tr>
<tr>
<td></td>
<td>R: GCCTGAAGGGCCTTCTGTAACCTGCAAAATATTATG</td>
</tr>
<tr>
<td>P405T</td>
<td>F: GCCTGAAGGGCCTTCTGTTTCTACTGCAAAATATTATG</td>
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<tr>
<td></td>
<td>R: CTCAAAATAATTTCTGCAGTGAACAAAGGAGGCTTCAGG</td>
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<td>R: GTCTGAACATTTATTTCTGCAGCAGCGAGCTTCAGG</td>
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<td>Y408H</td>
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<td>R: GAGACATCTCTCTCAGAAGAAGGAGG</td>
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Table 1. List of oligonucleotides used for mutagenesis.
distribution of ImageJ\textsuperscript{89}. RAD51 and γH2AX nuclear foci were automatically quantified using the FoCo algorithm\textsuperscript{90}. Noteworthy, filters were applied for the minimum radius of nuclei (15 pixels, blue), the minimum radius of foci (γH2AX: 3 pixels, red; RAD51: 2 pixels, green) and the minimum intensity of foci (γH2AX: 0.4, red; RAD51: 0.24, green). The number of foci of the first 180 cells scored was analysed using Prism 7 (RRID:SCR_002798, GraphPad Software).

Statistics and quantitative analysis
For experiments reproduced at least three times in this study, statistical significance was determined using the indicated test (Student’s t test or extra sum-of-squares f test). Data were analysed using Excel 2011 for Mac (Microsoft Software) and Prism 7 (RRID:SCR_002798, GraphPad Software).

For the quantitative analysis of PALB2 level in the chromatin fraction, western blots imaged with film were scanned on a Canon 8800F scanner using backlight illumination. For each protein of interest, an area of fixed size was used to calculate the band intensity in the Fiji (RRID:SCR_002285) distribution of ImageJ\textsuperscript{89}. The intensity of the PALB2 band was normalised against the intensity of the corresponding Lamin A band. The level of PALB2 in the chromatin fraction was then normalised against the protein level in the whole-cell extract and finally expressed as a percentage of the normalised wild-type PALB2 chromatin level.

Results
As part of our on-going efforts to characterise the function and regulation of PALB2 chromatin association, we investigate the link between perturbations of PALB2 chromatin association and cancer. To this end, we interrogated the COSMIC (Catalogue Of Somatic Mutations In Cancer) database\textsuperscript{11} to retrieve missense substitutions within the ChAM (human PALB2 residues 395-450). At the time of writing, the COSMIC database references 263 mutations from 30698 tested samples (0.86%), including 160 missense substitutions spanning the whole length of PALB2 (Figure 1). When we initiated this analysis, four distinct missense substitutions were identified in the ChAM: P405T, V410G, T413S, and V425M.

Cancer-associated missense substitutions in the ChAM are predicted to be deleterious
The primary amino acid sequence of the ChAM is highly distinctive and does not present any similarities with other known nucleosome-binding domains. With only predicted secondary structure available (Figure 2A), the amount of information on the structural elements and the overall fold of the domain is also limited. Hence, we first examined the degree of conservation of these residues by aligning the protein sequences of 40 orthologues of PALB2 (Figure 2A). P405, V410 and T413, are identical in all 40 orthologues of PALB2 analysed, while V425 is a conserved residue with some variation. We next used PolyPhen-2\textsuperscript{8,9} and SIFT\textsuperscript{10} prediction tools to obtain an initial assessment of the potentially deleterious effect of these amino acid substitutions (Table 2). Both algorithms predicted P405T, V410G, and T413S substitutions to be deleterious, while the V425M substitution was predicted to be deleterious only by SIFT.

T413S and S417Y missense substitutions hinder PALB2 chromatin association
To directly assess their impact on PALB2 chromatin association, we used site-directed mutagenesis to generate GFP-ChAM variants bearing these missense substitutions and transiently expressed them in HEK293T cells. We additionally tested the effect of three published germline missense substitutions (T397S, Y408H, and S417Y)\textsuperscript{8,9,34} and one variant of unknown significance referenced in the NCBI database of genetic variation (F404L)\textsuperscript{83}. These all affect highly conserved residues and are predicted to be deleterious by the PolyPhen-2 and SIFT algorithms (Figure 2A and Table 2). Surprisingly, most missense substitutions affecting highly conserved residues retained a wild-type level of chromatin association (Figure 2B and Table 2). To further confirm this observation, we tested two additional variants with predicted deleterious mutations at two consecutive conserved residues (F404A/P405A and E407A/Y408A) and again were not able to detect any effect on ChAM-mediated chromatin association (Figure 2A and Table 2). In sharp contrast, we found that T413S and S417Y substitutions dramatically reduced the chromatin association of the GFP-ChAM peptide (Figure 2B), to a comparable extent as our previously

Figure 1. Schematic representation of PALB2 domains and the missense substitutions referenced in the COSMIC database. PALB2 diagram depicting the assigned domains as follows, CC: coiled-coil domain, KBM: KEAP1-binding motif, ChAM: chromatin association motif, MBD: MRG15-binding domain and WD40 repeats 1 to 7. Oblique and vertical striped areas respectively indicate DNA binding regions and nuclear export signal (NES). The histogram indicates the distribution and location of 160 missense substitutions referenced in the COSMIC database. The type of cancer is colour-coded according to the legend and the number of cases indicated in between brackets.
Figure 2. Cancer-associated missense substitutions impair ChAM function. (A) Sequence alignment of the ChAM of 40 species. Red letters highlight the Human PALB2 sequence; green and blue boxes respectively show residues identical in all species analysed and residues with conservative substitutions, across the whole alignment. Asterisks indicate ChAM residues mutated in this study (blue asterisks highlight residues with missense substitutions referenced in the COSMIC database). Secondary structure elements (SSE) of the ChAM as predicted by Jpred4: α: alpha helix. (B) Chromatin association of GFP-ChAM wild-type (WT) and GFP-ChAM bearing the indicated missense substitutions examined by western blot, following transient expression in HEK293T cells. Tubulin, lamin A and histone H3 are markers for extraction of cytoplasmic, nuclear and chromatin proteins, respectively.
Table 2. ChAM missense mutations, deleterious effect prediction and chromatin localisation.
Red and green boxes respectively highlight ChAM missense mutations resulting in impaired chromatin association and those showing no obvious defects, as an arbitrary way to discriminate between potentially harmful and benign mutations.

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<th>Residue change(s)</th>
<th>PolyPhen score</th>
<th>SIFT score</th>
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<td>Impaired</td>
<td>25</td>
<td>–</td>
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</table>

Variants with PolyPhen-2 scores ≥ 0.850 are predicted to be deleterious (highlighted in red).
Variants with SIFT scores ≤ 0.05 are predicted to be deleterious (highlighted in red).
COSMIC: Catalogue Of Somatic Mutations In Cancer database.
dbSNP: NCBI database of genetic variation.

We next set to examine the effect of T413S and S417Y missense substitutions on full-length PALB2 chromatin association. Since PALB2 can form homo-oligomers, we used PALB2-deficient EUFA1341 cells to stably express FLAG-PALB2 variants, while avoiding interference from endogenous PALB2. Interestingly, despite our best efforts to find cell lines with similar level of expression of the FLAG-PALB2 variants analysed, all missense variants displayed a lower level of expression compared to wild type FLAG-PALB2. This observation raises the possibility that ChAM missense substitutions partly destabilise the PALB2 protein and could contribute to the observed PALB2 haploinsufficiency for tumour suppression. Nevertheless, we observed hindered chromatin association of FLAG-PALB2 with T413S or S417Y missense substitutions, when compared to wild-type FLAG-PALB2. Compared to our analysis of GFP-ChAM variants, full-length PALB2 variants, including the ChAM null mutant, only result in a partial loss of chromatin association. This is somewhat expected since PALB2 chromatin association is mediated not only by the ChAM but also through MRG15 binding. However, when we quantified the level of chromatin-associated FLAG-PALB2, we found that only the T413S missense substitution recapitulated the phenotype of our ChAM null mutant, with 35% of the wild-type level of chromatin association. On the
Figure 3. PALB2 with small cell lung cancer T413S mutation behaves as a ChAM null variant. (A) FLAG-PALB2 variants accumulation in the chromatin fraction of stably transfected EUFA1341 cells, as examined by western blot. (B) The level of each FLAG-PALB2 variant in the chromatin fraction was quantified and, following normalisation against their respective level in whole-cell extract and loading control, expressed as % of the wild type (WT). Mean values ±SD (n=4). Asterisks indicate *P values for two-tailed paired Student’s t test with *<0.05, **<0.01 and ***<0.001, ns: non-significant. (C) CPT survival curves fitted from a WST-1 cell proliferation assay comparing EUFA1341 cells complemented with FLAG-PALB2 WT and the indicated variants. Mean values ±SD (n=3). (D) IC50 values for CPT treatment in EUFA1341 complemented cells. n=3, with two technical replicates. Error bars indicate 95% CI. Asterisks indicate the P values for the extra sum-of-squares f test with **<0.01, ***<0.001 and ****<0.0001, ns: non-significant. EV: empty vector.

other hand, the S417Y missense substitution exhibited an intermediate phenotype, with a chromatin association level between that of the wild-type and T413S FLAG-PALB2 variants. These results support that the cancer-associated T413S missense mutation is distinctive in causing an overall reduction of PALB2 chromatin association.

T413S missense substitution impairs PALB2 function in genotoxic drug resistance

The topoisomerase I inhibitor camptothecin (CPT) is a broad-spectrum anticancer drug, and we recently demonstrated that loss of ChAM function sensitises cells to CPT treatment. To further assess the adverse effects of T413S and S417Y missense substitutions, we examined the ability of these PALB2 variants to support cellular resistance to genotoxic stress in response to chronic exposure to CPT. EUFA1341 cells complemented with wild-type and S417Y FLAG-PALB2 displayed comparable levels of survival (Figure 3C and D), with IC50 values of ~30 nM CPT and overlapping growth inhibition curves. In stark contrast, T413S FLAG-PALB2 was unable to fully rescue the CPT sensitivity of EUFA1341 PALB2-deficient cells, which is reminiscent of our previously described ChAM null variant.
We next sought to gain insights into the mechanism underlying the increased CPT-sensitivity of the PALB2 T413S mutant. Since PALB2 is commonly described to play an essential role in HDR by recruiting BRCA2 in complex with RAD51, we scored RAD51 nuclear foci as a proxy to estimate the efficiency of HDR. As expected, CPT-induced RAD51 nuclear foci formation was severely impaired in EUFA1341 cells (Figure 4). To our surprise, however, re-expression of wild-type, T413S, and S417Y FLAG-PALB2 in EUFA1341 cells restored similar levels of RAD51 nuclear foci formation upon CPT treatment (Figure 4B). Although a moderate decrease and increase of the number of RAD51 foci are noticeable in the T413S and S417Y PALB2 variants, respectively, these variations are statistically not significant and are unlikely to explain the differences observed in our CPT survival assay (Figure 3D and Figure 4B). To further verify the efficacy of the DNA stress response in each cell line, we examined the formation of S139-phosphorylated H2A.X (γH2A.X) nuclear foci, a marker of the activation of the DNA damage response (Figure 4). In vehicle-treated cells, the expression of wild-type, T413S, and S417Y FLAG-PALB2 equally suppressed the formation of γH2A.X nuclear foci arising from spontaneous DNA lesions in EUFA1341 cells. Intriguingly, however, CPT treatment induced a notably less efficient γH2A.X nuclear foci formation in EUFA1341 cells supplemented with FLAG-PALB2 T413S (Figure 4B). Collectively, these findings demonstrate T413S as a ChAM defective variant of PALB2, yet point to an unexpected mechanism where DNA

**Figure 4.** PALB2 T413S missense variant supports RAD51 but not γH2A.X nuclear foci formation. (A) Representative pictures, from one experiment, of EUFA1341 cells complemented with FLAG-PALB2 variants and stained for RAD51 (yellow) and γH2A.X (purple) nuclear foci following 17 h treatment with DMSO (left) or 10 nM CPT (right). (B) Automatic quantification of γH2AX (left) and RAD51 (right) nuclear foci using the FoCo algorithm. Cells were treated for 17h with DMSO or 10 nM CPT, as indicated. Dots represent individual cells (n=180), and bars mean values ±SD. *P* values indicate the statistical significance for the two-tailed Mann-Whitney *U* test. EV: empty vector.
stress signalling, rather than RAD51 recruitment to DNA lesions, is altered.

Discussion
Since the discovery of PALB2 in 2006, numerous studies had addressed how the development of various cancers could be linked with PALB2 mutations. For the ~50 protein-truncating mutations classified as cancer risk variants, there is abundant evidence that BRCA2 binding is lost since these variants recapitulate many features of BRCA2 loss of function. Conversely, many missense mutations remain as variants of uncertain significance (VUSs), even though the likely impact of PALB2 missense VUSs hindering BRCA2 or BRCA1 interaction is foreseeable based on the importance of the BRCA1-PALB2-BRCA2 complex in the maintenance of genome stability. There is also an indication that PALB2 is haploinsufficient for tumour suppression, since almost all PALB2 truncating mutations conferring a risk for breast/ovarian cancer are monoallelic. This notion was further supported by the recent identification of a hypomorphic allele of PALB2, with an in-frame deletion of residues 839-862. This variant can support PALB2 function when exogenously expressed in U2OS cells, but not in patient cells where it is expressed at a low level.

In the present study, we provide additional evidence that impaired PALB2 chromatin association may be linked with cancer development. We demonstrate that two cancer-associated missense mutations in the ChAM of PALB2 hinder its chromatin association. The serine to tyrosine substitution at position 417 leads to a partial reduction of ChAM-mediated PALB2 chromatin association, without affecting the cellular resistance to CPT. However, seven independent studies reported PALB2 S417Y as a germline mutation in both cancer patients (mainly breast cancer) and healthy individuals, making this variant the most frequent of the subset we analysed. Although further clinical evidence is required, our observations suggest that PALB2 S417Y may be a low-penetrance genetic variant associated with low cancer risk or a neutral variant.

Surprisingly, the second variant, a conservative threonine to serine substitution at position 413 has a more deleterious effect. To start dissecting the molecular mechanism underlying the CPT-survival defect associated with PALB2 T413S missense variant, we analysed RAD51 and γH2A.X nuclear foci formation in cells challenged with CPT. Remarkably, we found that PALB2 T413S supported a nearly normal level of RAD51 nuclear foci formation, but a reduced level of γH2A.X nuclear foci formation. This observation is somewhat puzzling since most studies report a robust correlation between RAD51 and γH2A.X nuclear foci formation. Nonetheless, the exact nature, meaning and significance of DNA repair proteins nuclear foci remain unclear, and the RAD51 nuclear foci observed in PALB2 T413S expressing cells do not necessarily reflect regular HDR events. It is conceivable that, through its interaction with BRCA1, PALB2 T413S can initiate RAD51 recruitment, but for as-yet-unknown reasons, fail to promote HDR. Notably, BRCA1, in complex with BARD1, can promote the proteasome-mediated degradation of H2A.X, and in this way, supports the attenuation of the γH2A.X-mediated damage signalling following the completion of DNA damage repair. In the case of PALB2 T413S, BRCA1 might be aberrantly trapped at sites of DNA damage due to unproductive HDR events, which then leads to the premature attenuation of the γH2A.X-mediated response. While additional studies will be required to define a potential role of ChAM in HDR, our observations support the notion that PALB2 T413S perturbs DNA stress signalling, resulting in a decreased cellular resistance to CPT treatment. Of note, the T413S PALB2 variant change a residue potentially targeted by protein kinases. Although no direct evidence indicates that kinases phosphorylate this particular residue, it is tempting to speculate that changes in ChAM phosphorylation, rather than its primary amino-acid sequence, could be deleterious, affecting productive DNA stress signalling and repair.

The PALB2 T413S variant is, however, scarce and reported as a somatic mutation in a single patient with small cell lung cancer (SCLC). It is important to note that SCLC is an exceptionally aggressive type of cancer with poor prognosis, and camptothecin (topotecan) is currently the only drug approved by the US Food and Drug Administration to treat relapsed SCLC. While inactivating (nonsense or essential splice-site) mutations of both TP53 and RB1 is associated with a significant fraction of SCLC cases, whole exome screening of the SCLC patient carrying the PALB2 T413S mutation detected only an inactivating nonsense mutation in TP53. Mutations in PTEN, SOX2 and NOTCH1, which are also frequently associated with SCLC, and BRCA1/2 or other known Fanconi anaemia genes were also not detected. Interestingly, the COSMIC database references three additional SCLC cases with rare missense VUSs within PALB2 WD40 repeat domain (Q921H, V978D and R1086G). The link between SCLC and PALB2 mutations is unidentified to date, and although further investigation will be required to assess the pathological consequences of these mutations (namely T413S, Q921H, V978D and R1086G), our findings may have implications for understanding the development of this disease.

Altogether, we screened 15 variants of PALB2 with missense substitutions at 13 different positions of the ChAM, including 11 highly conserved residues. Given the high level of conservation of the ChAM, it was somewhat surprising to find that most missense substitutions at these residues were well tolerated and did not appear to affect PALB2 chromatin association. Most significantly, our work identifies threonine 413 as a critical residue within the ChAM and implicates its mutation in SCLC. Further investigation of the regulation of PALB2 chromatin association will be essential for
the full understanding of its implication for tumorigenesis and to develop new therapeutic strategies.

**Data availability**

Raw data for this study are available from OSF: https://doi.org/10.17605/OSF.IO/ZVUK8. This includes CPT cell survival data in Microsoft Excel 2011; PALB2 chromatin association quantification data in Microsoft Excel 2011; RAD51 and γH2AX number of foci per nuclei data in Microsoft Excel 2011; TIFF files of uncropped western blots for Figure 2B and Figure 3A.

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

**Competing interests**

No competing interests were disclosed.

**Grant information**

This work was supported by the Wellcome Trust [101009], Senior Research Fellowship to F.E., and a Wellcome Trust Biomedical Vacation Scholarship to R.M.B.

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

**Acknowledgments**

We thank Dr. H. Joenje (VU University Medical Center, The Netherlands) for sharing the EUFA1341/FA-N fibroblasts.

**References**


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Open Peer Review

Current Peer Review Status: ✔ ✔ ✔

Version 1

Reviewer Report 14 December 2017

https://doi.org/10.21956/wellcomeopenres.14225.r27998

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Ashok R. Venkitaraman
MRC Cancer Cell Unit, University of Cambridge, Cambridge, UK

Mutations affecting the PALB2 gene predispose to cancer. Many cancer-associated mutations truncate the encoded PALB2 protein to varying degrees, disrupting its function in the repair of DNA damage by homologous recombination. This interesting paper builds on previous work from Dr. Esashi's laboratory to characterize the functional impact of two mis-sense mutations (T413S and S417Y) altering residues in a PALB2 region (chromatin association motif, or ChAM) previously reported to promote nucleosome interaction.

The authors find that while ChAM fragments bearing the T413S or S417Y mutations exhibit sharply reduced chromatin interaction, full-length PALB2 mutants containing T413S or S417Y still retained a significant proportion of their chromatin-interacting ability, with PALB2 T413S being more severely affected. Consistent with this finding, PALB2 T413S when expressed in PALB2-mutant EUFA1341 cells sensitises them to the topoisomerase inhibitor camptothecin (CPT). In addition, PALB2 T213S (but not S417Y) reduces gamma-H2AX formation after DNA damage, although it has little effect on damage-induced RAD51 foci. The authors suggest that chromatin-bound PALB2 may have functions in the signalling of damage-induced stress distinct from roles in RAD51 recruitment, and that disruption of such functions may account for cancer promotion by mutations affecting the ChAM.

The experiment shown in Figure 3A/B is not easy to interpret. Figure 3A shows that FLAG-tagged PALB2 T413S and S417Y are expressed in significantly lower amounts than the FLAG-PALB2 wild-type protein in whole-cell extracts, and their degree of chromatin association appears proportional to the level of expression (as reflected in Figure 3B). Therefore, is the reduction in mutant PALB2 protein expression – most severe for the functionally compromised T413S mutant – responsible for both the reduced chromatin association and the observed cellular defects?

In this regard, the authors' finding that GFP-tagged T413S or S417Y ChAM fragments exhibit little detectable chromatin association (Figure 2B), whereas full-length PALB2 mutants containing the same mutations retain significant chromatin binding (Figure 3A) is intriguingly unexplained.

The authors make the interesting point that the PALB2 S417Y variant, which has little functional effect in
their studies apart from reduced protein expression, has been found in the germline of cancer patients as well as healthy individuals. While it is possible, as the authors suggest, that this variant represents a low-penetrance cancer gene, it may also represent a neutral variant (particularly if it affects only one of the two PALB2 germline alleles).

Finally, the authors raise the interesting possibility that the PALB2 T413S mutant, identified as a somatic mutation in a single patient with lung cancer, may promote carcinogenesis and resistance to drugs like CPT. However, it was not clear from the accessible sequencing data whether the PALB2 T413S mutation affected one or both PALB2 gene copies in this patient’s sample. Should a functional wild-type copy of PALB2 be retained in this patient’s tumour, experiments showing that the PALB2 T413S mutant can act as a “dominant negative” even when wild-type PALB2 protein is present would strengthen the authors’ conjectures regarding pathogenic impact. This issue should ideally be explained and discussed, in case there are potential limitations to the authors’ conclusions regarding the clinical significance of their findings.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
mutant PALB2 protein expression – most severe for the functionally compromised T413S mutant – responsible for both the reduced chromatin association and the observed cellular defects?

We appreciate this comment. As described in the figure legend, we normalised the signal intensities of PALB2 variants against loading control (Lamin A) in the chromatin fraction, which was then normalised against the respective PALB2/Lamin A ratio in the whole-cell extract; in this way, we ensured that the quantification reflects the proficiency of PALB2 chromatin association. Nonetheless, we agree that the expression levels of PALB2 mutant proteins are lower compared to wild-type PALB2, and now discuss the possible link with PALB2 haploinsufficiency for tumour suppression.

In this regard, the authors’ finding that GFP-tagged T413S or S417Y ChAM fragments exhibit little detectable chromatin association (Figure 2B), whereas full-length PALB2 mutants containing the same mutations retain significant chromatin binding (Figure 3A) is intriguingly unexplained.

We appreciate this comment. The partial chromatin association of full-length PALB2 variants is expected, as wild-type PALB2 chromatin binding is mediated through two mechanisms: direct interaction between ChAM and nucleosomes (Bleuyard et al., 2012 EMBO Rep; this study), and the stable interaction with MRG15, which binds to trimethylated histone H3 at K36 (Bleuyard et al., 2017 PNAS). When both mechanisms are perturbed, full-length PALB2 fails to associate with chromatin. This point is now clarified in the text.

The authors make the interesting point that the PALB2 S417Y variant, which has little functional effect in their studies apart from reduced protein expression, has been found in the germline of cancer patients as well as healthy individuals. While it is possible, as the authors suggest, that this variant represents a low-penetration cancer gene, it may also represent a neutral variant (particularly if it affects only one of the two PALB2 germline alleles).

We agree with this comment and have amended the text accordingly.

Finally, the authors raise the interesting possibility that the PALB2 T413S mutant, identified as a somatic mutation in a single patient with lung cancer, may promote carcinogenesis and resistance to drugs like CPT. However, it was not clear from the accessible sequencing data whether the PALB2 T413S mutation affected one or both PALB2 gene copies in this patient’s sample. Should a functional wild-type copy of PALB2 be retained in this patient’s tumour, experiments showing that the PALB2 T413S mutant can act as a “dominant negative” even when wild-type PALB2 protein is present would strengthen the authors’ conjectures regarding pathogenic impact. This issue should ideally be explained and discussed, in case there are potential limitations to the authors’ conclusions regarding the clinical significance of their findings.

This is a valid point, and it was not possible to obtain the information about the second PALB2 allele in this patient. The PALB2 T413S mutant shows increased sensitivity, rather than resistance, to camptothecin treatment, and to date, we have no evidence that PALB2 T413S acts as a ‘dominant negative’. The pathological consequence of this single point mutation is as yet unknown, and we have amended the text accordingly.

Competing Interests: No competing interests were disclosed.
Kevin Hiom

Division of Cancer Research, Medical Research Institute, Jacqui Wood Cancer Centre, Ninewells Hospital and Medical School, Dundee, UK

This manuscript reports the characterization of cancer-associated mutations in the chromatin associated motif of PALB2 identified through mining of the COSMIC database and analysis of published literature. The authors identified two mutations T413S and S417Y that exhibited defects in chromatin binding when expressed in HEK293T cells with only the former defective in chromatin binding in EUFA13141. Interestingly the T413S mutation has been found in a single case of small cell lung cancer. While expression of the PALB2 T413S mutant in EUFA13141 sensitized these cells to treatment with camptothecin, localization of RAD51 to sites of DNA damage was near normal. Unexpectedly, gH2AX foci in these cells was reduced compared to cells expressing wild type PALB2. The authors conclude that PALB2 that is impaired for chromatin binding is compromised for DNA damage signalling while seemingly proficient in the recruitment of downstream repair proteins. They propose that chromatin binding is required for DNA damage signalling, which might impact on the ability of cancer cells to respond to treatment with the therapeutic agent camptothecin.

This study is a well presented and interesting study describing a functional requirement for the chromatin association motif of PALB2. It demonstrates clearly that mutations in the ChAM that reduce retention of PALB2 on chromatin have an impact on the DNA damage response. What is less clear is why? At the current time the investigation of ChAM mutants on the DDR is limited. It is clear that expression of T413S causes sensitization to camptothecin but this was not reflected in decreased RAD51 recruitment or increased gH2AX foci. However these assays were performed at a single time point and the data does not reflect the kinetics of repair. Is RAD51 recruitment affected over time? Does the decrease in gH2AX foci reflect fewer collapsed replication forks or increased rate of dsb repair (albeit possibly a harmful form of repair leading to decreased survival)? These issues are not addressed and this might explain the difficulty in reconciling a defect in DDR signalling with apparently normal recruitment of downstream repair factors but impaired survival. Given that PALB2 has, through BRCA1 and BRCA2, been linked to homologous recombination repair it might have been useful to quantify levels of HR and investigate sensitivity to Parp inhibitors.

Minor points-
1. The western blot for levels of histone H3 seems irregular
2. Conclusions suggesting the potential importance of ChAM mutations in PALB for the pathology of SCLC are overstated given the single instance of this mutation in the disease.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 12 Jan 2018

Fumiko Esashi, University of Oxford, Oxford, UK

We are grateful for these very insightful comments and suggestions. We totally agree that the molecular and biological functions of the ChAM domain remain unknown, despite the clear phenotypes supporting its role in the DNA damage response. We are currently tackling the question of why the ChAM defect leads to increased sensitivity to camptothecin using various experimental approaches, including those suggested by the referee. We hope to be able to provide clearer answers in our follow-up studies.

To answer your minor points-

1. The western blot for levels of histone H3 seems irregular

This is a blotting artefact due to the bleaching of the histone H3 signal, which was used a marker of the chromatin-associated fraction. As described in the methods, however, we used Lamin A, whose molecular weight is closer to GFP-ChAM, and not histone H3 for normalisation. This does not affect our conclusion.

2. Conclusions suggesting the potential importance of ChAM mutations in PALB for the pathology of SCLC are overstated given the single instance of this mutation in the disease.

We appreciate this comment and have amended the text accordingly.

Competing Interests: No competing interests were disclosed.

Reviewer Report 27 November 2017

https://doi.org/10.21956/wellcomeopenres.14225.r27996
This manuscript describes the identification of cancer-associated missense mutations within the chromatin-association motif of the tumor suppressor gene PALB2. Although the role of PALB2 missense mutations in disrupting its protein interactions with complex members BRCA1 and BRCA2 is well-established, the role of missense mutations in perturbing PALB2 chromatin-binding function and its contribution to tumorigenesis is unknown. These potentially high-impact mutations were identified by mining the public cancer mutation database, COSMIC, and the literature. The fifteen identified missense mutations in the chromatin-association motif were introduced into HEK293T cells, and two mutations (T413S and S417Y) were found to have impaired chromatin binding, one of which is found to be mutated in a single case of SCLC (T413S). These two missense mutations were further introduced into full-length PALB2 and expressed in PALB2-deficient EUFA13141 cells, where only the T413S mutation was found to perturb chromatin binding. PALB2 T413S-expressing cells are more sensitized to camptothecin treatment, and display reduced levels of g-H2AX foci compared to cells expressing wild-type PALB2 and PALB2 S417Y while maintaining comparable levels of Rad51 foci. This suggests failure to efficiently activate DNA damage repair signalling while maintaining downstream effector recruitment. Overall, the authors propose a novel mechanism through which PALB2 missense mutations could promote tumorigenesis by impairing its chromatin-binding ability, albeit found in a very limited number of tumours to date.

Comments:

1. The immunoblot in Figure 2B does not show consistent levels of histone H3 in the chromatin extracts. Although likely an artefact, a better example would strengthen the finding of decreased chromatin binding of GFP-ChAM fragments harbouring T413S and S417Y mutations.

2. It appears that all of the mutants cause a reduction in steady-state PALB2 levels. I appreciate that this could be an artefact of stable cell line selection, but probably worth a comment nonetheless, given the possible relationship between PALB2 haploinsufficiency and disease.

3. In Figure 4B, shouldn't comparisons be made between cells expressing WT PALB2 and the ChAM-mutant PALB2 proteins, in addition to the PALB2-deficient empty vector? The comparison shown, with EV, suggests that T413S is interfering with the action of another effector protein, since it is more defective than cells lacking PALB2. Assuming that the difference between WT and T413S is statistically supported, the data also indicate that T413S is defective relative to WT.

4. In the Discussion, the statement “While the exact role of ChAM in HDR remains to be elucidated” seems premature, given there is no evidence yet of an HDR defect in the mutants studied.

5. “The link between SCLC and PALB2 mutations is unidentified to date, and our findings may have significant implications for understanding the development of this disease.” I would omit ‘significant' given that the mutation seems to be a part of the minority in SCLC and its functional consequences are as of yet undefined.
6. The PALB2 ChAM-null variant response to camptothecin would be a good comparison to include in Figures 3C and 3D.
7. Scale bars should be added to the micrographs in Figure 4A.
8. A couple of typos. Results section two header reads S417S instead of S417Y, and 'cell lines' should be singular in the following sentence: “To further verify the efficacy of the DNA stress response in each cell lines, we examined…”. 

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 12 Jan 2018

**Fumiko Esashi**, University of Oxford, Oxford, UK

We would like to thank you for the time and effort taken to review our manuscript. Please find our point-by-point response (highlighted in bold) to your comments below:

1. The immunoblot in Figure 2B does not show consistent levels of histone H3 in the chromatin extracts. Although likely an artefact, a better example would strengthen the finding of decreased chromatin binding of GFP-ChAM fragments harbouring T413S and S417Y. 

This is indeed a blotting artefact due to the bleaching of the histone H3 signal in the enhanced chemiluminescence (ECL) reaction. Histone H3 was used as a marker of the chromatin-associated fraction, but Lamin A, whose molecular weight is closer to GFP-ChAM, was used for normalisation as described in the methods. The bleached histone H3 signal therefore does not affect our conclusion. Building on the severe phenotype detectable with the GFP-ChAM harbouring T413S and S417Y, we moved on to in-depth analyses of these mutations in the full-length PALB2 context (Figure 3).
2. It appears that all of the mutants cause a reduction in steady-state PALB2 levels. I appreciate that this could be an artefact of stable cell line selection, but probably worth a comment nonetheless, given the possible relationship between PALB2 haploinsufficiency and disease.

We appreciate this comment. Indeed, we generated several stable clones expressing these PALB2 mutants, but were unable to obtain clonal lines expressing equivalent levels. In the revised manuscript, we discuss the level of expression of FLAG-PALB2 variants and the possible link with PALB2 haploinsufficiency for tumour suppression.

3. In Figure 4B, shouldn’t comparisons be made between cells expressing WT PALB2 and the ChAM-mutant PALB2 proteins, in addition to the PALB2-deficient empty vector? The comparison shown, with EV, suggests that T413S is interfering with the action of another effector protein, since it is more defective than cells lacking PALB2. Assuming that the difference between WT and T413S is statistically supported, the data also indicate that T413S is defective relative to WT.

We agree with this comment and have now included the statistical data comparing wild-type PALB2 to T413S or S417Y variants, and the statistical data comparing the T413S and S417Y variants.

4. In the Discussion, the statement “While the exact role of ChAM in HDR remains to be elucidated” seems premature, given there is no evidence yet of an HDR defect in the mutants studied.

We have now amended this statement as the following: “While additional studies will be required to define a potential role of ChAM in HDR”.

5. “The link between SCLC and PALB2 mutations is unidentified to date, and our findings may have significant implications for understanding the development of this disease.” I would omit ‘significant’ given that the mutation seems to be a part of the minority in SCLC and its functional consequences are as of yet undefined.

We appreciate this comment and have amended the text accordingly.

6. The PALB2 ChAM-null variant response to camptothecin would be a good comparison to include in Figures 3C and 3D.

We included the ChAM-null variant (V410P/T413P) in our CPT survival analysis, but unfortunately, had trouble adjusting the seeding for this particular cell line, as reflected by the high WST-1 assay OD reading (50-100% higher than other cell lines) from raw CPT survival data (available on OSF). The elevated cell density for the ChAM-null mutant affected the efficiency of CPT killing, especially at high CPT doses, and, for this reason, we did not include these data in the manuscript.

7. Scale bars should be added to the micrographs in Figure 4A.

Scale bars have now been added to Figure 4A and the legend amended accordingly.

8. A couple of typos. Results section two header reads S417S instead of S417Y, and ‘cell lines’ should be singular in the following sentence: “To further verify the efficacy of the DNA stress
response in each cell lines, we examined….”.

We thank the referee for detecting these typos and have corrected them in the revised manuscript.

Competing Interests: No competing interests were disclosed.