Title

The p.Ser64Leu and p.Pro104Leu missense variants of PALB2 identified in familial pancreatic cancer patients compromise the DNA damage response

Journal

Human Mutation, 42(2)

ISSN

1059-7794

Authors

Zhang, Yue
Park, Jung-Young
Zhang, Fan
et al.

Publication Date

2021-02-01

DOI

10.1002/humu.24133

Peer reviewed

HHS Public Access

Author manuscript

Hum Mutat. Author manuscript; available in PMC 2022 February 01.

Published in final edited form as:

Hum Mutat. 2021 February ; 42(2): 150–163. doi:10.1002/humu.24133.

The p.Ser64Leu and p.Pro104Leu missense variants of PALB2 identified in familial pancreatic cancer patients compromise the DNA damage response

Yue Zhang[1,2,*], **Jung-Young Park**[1,*], **Fan Zhang**[1], **Sara H. Olson**[3], **Irene Orlow**[3], **Yirong Li**[4], **Robert C. Kurtz**[5], **Marc Ladanyi**[4], **Jie Chen**[2], **Amanda E. Toland**[6,7], **Liying Zhang**[4,8,^], **Paul R. Andreassen**[1,9,^]

[1]Division of Experimental Hematology & Cancer Biology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

[2]Department of Pathology, Peking Union Medical College Hospital, Beijing, P.R. China

[3]Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York, New York

[4]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York

[5]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York

[6]Department of Cancer Biology and Genetics, The Ohio State University Wexner Medical Center, Columbus OH

[7]Department of Internal Medicine, Division of Human Genetics, The Ohio State University Wexner Medical Center, Columbus, OH

[8]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, University of California at Los Angeles (UCLA), Los Angeles, CA

[9]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

Abstract

PALB2 has been identified as a breast and pancreatic cancer susceptibility gene. Utilizing a targeted sequencing approach, we discovered two novel germline missense *PALB2* variants c.191C>T and c.311C>T, encoding p.Ser64Leu and p.Pro104Leu, respectively, in individuals in a pancreatic cancer registry. No missense *PALB2* variants from familial pancreatic cancer patients, and few PALB2 variants overall, have been functionally characterized. Given the known role of

^Correspondence: Liying Zhang: Tel (310) 267-2897, LiyingZhang@mednet.ucla.edu or Paul R. Andreassen: Tel. (513) 636-0499, Paul.Andreassen@cchmc.org.

*Yue Zhang and Jung-Young Park made equal contributions to this study

DATA AVAILABILITY

PALB2 variants identified in probands of the Familial Pancreatic Cancer Family Registry at MSKCC, including p.Ser64Leu, p.Pro104Leu, p.Leu337Ser, p.Gln559Arg, p.Glu672Gln and p.Gly998Glu, have been submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).)

CONFLICTS OF INTEREST

Dr. Liying Zhang declares the following potential conflicts of interest: Honoraria (Future Technology Research LLC, BGI, Illumina); Honoraria and Travel and accommodation expenses (Roche Diagnostics Asia Pacific); Family members holding leadership positions and ownership interests of Shanghai Genome Center. The other authors have no relevant conflicts of interest to declare.

PALB2, we tested the impact of p.Ser64Leu and p.Pro104Leu variants on DNA damage responses. Neither p.Ser64Leu nor p.Pro104Leu had clear effects on interactions with BRCA1 and KEAP1, which are mediated by adjacent motifs in PALB2. However, both variants are associated with defective recruitment of PALB2, and the RAD51 recombinase downstream, to DNA damage foci. Further, p.Ser64Leu and p.Pro104Leu both largely compromise DNA double-strand break-initiated homologous recombination, and confer increased cellular sensitivity to ionizing radiation (IR) and the poly (ADP-ribose) polymerase (PARP) inhibitor Olaparib. Taken together, our results represent the first demonstration of functionally deleterious PALB2 missense variants associated with familial pancreatic cancer and of deleterious variants in the N-terminus outside of the coiled-coil domain. Further, our results suggest the possibility of personalized treatments, using IR or PARP inhibitor, of pancreatic and other cancers that carry a deleterious *PALB2* variant.

Keywords

Pancreatic cancer; Variant of uncertain significance; Missense variants; PALB2; Homologous recombination; Functional studies

INTRODUCTION

PALB2 has been identified as a breast cancer susceptibility gene on the basis of segregation of truncating pathogenic variants with cancer in affected family members (Erkko et al., 2007; Rahman et al., 2007; Tischkowitz et al., 2007). Notably, heterozygosity for *PALB2* pathogenic variants confers a high risk of developing breast cancer similar to pathogenic variants in *BRCA2* (Antoniou, Foulkes, & Tischkowitz, 2014). Given the presence of a WD40 domain at the extreme C-terminus of the PALB2 protein, any truncation resulting from frameshift or nonsense mutations is likely to be pathogenic (Hanenberg & Andreassen, 2018; Tischkowitz et al., 2007). Additionally, biallelic mutation of *PALB2* causes Fanconi anemia (FA), and some of the *PALB2* pathogenic variants found in breast cancer patients are also associated with FA (Rahman et al., 2007; Reid et al., 2007).

More than 200 distinct missense variants of PALB2 in breast cancer patients have been reported in scientific publications, as summarized (Ducy et al., 2019), but the vast majority are variants of uncertain significance (VUS). Further, the ClinVar database alone currently lists more than 1,600 PALB2 missense VUS (https://www.ncbi.nlm.nih.gov/clinvar). Only a small subset of these VUS have been functionally characterized, and even fewer are deleterious. Until now, reported deleterious missense variants have been limited to a small portion of PALB2, occurring within, or directly proximal, to the N-terminal coiled-coil motif (a.a. 9–44) or the C-terminal WD40 domain (a.a. 867–1186). Herein, however, we identify two novel PALB2 missense variants in a region of the N-terminus distinct from the coiled-coil domain.

PALB2 has also been identified as a pancreatic cancer gene by a study that employed whole exome sequencing to find four distinct truncating mutations of *PALB2* in a registry of families with multiple cases of this disease (Jones et al., 2009). Additional studies have also identified pathogenic truncating mutations in pancreatic cancer families (Slater et al., 2010; Zhen et al., 2015) and breast-ovarian-pancreatic cancer families (Blanco et al., 2013). While

some missense variants have been identified in familial pancreatic cancer (Zhen et al., 2015) and in breast-ovarian-pancreatic cancer families (Blanco et al., 2013), all are VUS with unclear clinical effects. None of these missense PALB2 variants have been functionally characterized.

PALB2 is believed to function as a tumor suppressor via a key role in mediating DNA repair by homologous recombination (HR) (Park, Zhang, & Andreassen, 2014). Among its roles in HR, the PALB2 protein directly binds another HR protein, BRCA2, via its C-terminal WD40 domain [amino acids (a.a.) 867–1186], and recruits it to nuclear DNA damage foci (Oliver, Swift, Lord, Ashworth, & Pearl, 2009; Xia et al., 2006; Zhang, Fan, Ren, & Andreassen, 2009). In contrast, a coiled-coil motif at the N-terminus of PALB2 (a.a. 9–44) mediates direct binding to BRCA1 (Sy, Huen, & Chen, 2009; Zhang, Fan, et al., 2009; Zhang, Ma, et al., 2009), and is required for the assembly of PALB2 foci (Zhang, Fan, et al., 2009; Zhang, Ma, et al., 2009). The C-terminal WD40 domain of PALB2 is also required for recruitment of RAD51 to DNA damage foci (Xia et al., 2007; Zhang, Fan, et al., 2009). While there is a KEAP1-binding site in the N-terminus of PALB2 (a.a. 88–94) (Ma et al., 2012), the role of much of the remainder of PALB2 is unclear.

Here, we identify two novel germline missense *PALB2* variants in two different families with a pancreatic cancer history: c.191C>T, which encodes p.Ser64Leu, and c.311C>T, which results in a P to L change at residue 104. Additionally, by expressing p.Ser64Leu and p.Pro104Leu in *PALB2*-deficient cells, we have characterized the effects of these N-terminal variants of PALB2 on the cellular DNA damage response. In particular, we find that p.Ser64Leu and p.Pro104Leu compromise recruitment of both PALB2 and RAD51 to nuclear foci, and impair DNA repair by HR. HR is decreased relative to wild-type (WT) PALB2 and four benign variants, therefore Ser64Leu and Pro104Leu could increase the risk of developing cancer. Further, both p.Ser64Leu and p.Pro104Leu confer increased cellular sensitivity to ionizing radiation (IR) and the poly (ADP-ribose) polymerase (PARP) inhibitor olaparib. This suggests the possibility of personalized treatments of pancreatic cancer patients that harbor deleterious *PALB2* variants, including missense variants, using IR and PARP inhibitors. Indeed, radiation with or without chemotherapy can be utilized to treat pancreatic cancer (Yang et al., 2016), and clinical trials using PARP inhibitors have been initiated on pancreatic and other cancer patients with a genetic deficiency for HR (Kamel, Gray, Walia, & Kumar, 2018; Zhu et al., 2020).

Taken together, by functionally characterizing two novel missense *PALB2* variants identified in familial pancreatic cancer patients, our results yield insight into the impact of PALB2 variants and the role of PALB2 in DNA damage responses. Specifically, we identify the first deleterious missense variants of *PALB2* that are associated with inherited pancreatic cancer, and discover the first deleterious PALB2 missense variants outside of the N-terminal coiled-coil motif and the C-terminal WD40 domain.

MATERIALS AND METHODS

Editorial policies and ethical considerations

Our study based on a Familial Pancreatic Cancer (FPC) Family Registry at Memorial Sloan Kettering Cancer Center (MSKCC) was approved by a MSKCC Institutional Review Board. All participants provided written consent.

Pancreatic cancer patient cohort

Our cohort consisted of 54 probands from a Familial Pancreatic Cancer (FPC) Family Registry at MSKCC (Olson et al., 2010; Saldia et al., 2019). These individuals had familial pancreatic cancer with at least one other affected first degree relative.

Sequencing of *PALB2*

Samples of blood, mouthwash or saliva were used to extract and characterize germline DNA at the Molecular Epidemiology Laboratory at MSKCC. Full gene sequencing analysis of *PALB2* (*PALB2* reference sequence accession number NM_024675.4) was performed on peripheral blood, from probands only, at the Geoffrey Beene Core Facility Laboratory at MSKCC. The newly identified *PALB2* variants, c.191C>T (p.Ser64Leu) and c.311C>T (p.Pro104Leu), were confirmed by Sanger Sequencing using exon-specific primers (exons 2–3, 5'-GTAAAACGACGGCCAGTAGTGCTACTCCCTGCCTCTT-3', 5'-CAGGAAACAGCTATGACTCACACTGTGGGAAAAAGAA-3'; exon 4, 5'-GTAAAACGACGGCCAGTCCTGAATGAAATGTCACTGATT-3', 5'- CAGGAA ACAGCTATGACTCCTTTAGTCTTTTCCCAGACA-3'). The variants were curated and interpreted according to ACMG guidelines (Richards et al., 2015). The mutational status of *BRCA1* and *BRCA2* was, and currently is, unknown for the majority of the probands in this study, including those harboring the *PALB2* Ser64Leu and Pro104Leu variants.

In silico predictions of whether variants are deleterious

Alamut Visual (Interactive Biosoftware, France) was utilized to predict whether *PALB2* c.191C>T (p.Ser64Leu) and c.311C>T (p.Pro104Leu) affect splicing. Additionally, multiple computational prediction tools (Align GVGD, MutationTaster, SIFT and PolyPhen-2 V2.2.2r398) were utilized to predict whether the Ser64Leu and Pro104Leu variants of *PALB2* are benign or pathogenic.

Cell lines and cell culture

U2OS-DR cells containing a stably integrated reporter to assay DNA double strand break (DSB)-initiated HR (Nakanishi et al., 2005) were grown in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Immortalized EUFA1341 (*PALB2*-deficient cells from a FA patient) (Xia et al., 2007) were cultured in a 1:1 mixture of DMEM and F10 medium containing 15% FBS and 1% penicillin/streptomycin. Stock solutions of olaparib (50 mM in DMSO; Sigma) were kept at −80°C. Irradiation was done with a Mark I-68 Cesium 137 apparatus (J. L. Shepherd and Associates).

Generation of variants and expression in *PALB2*-deficient cells

Cloning of the cDNA for WT *PALB2* into pMMP and pCDNA3.1 containing a N-terminal Flag-HA epitope tag has been described elsewhere (Zhang, Fan, et al., 2009). *PALB2* cDNAs encoding the Ser64Leu and Pro104Leu, as well as the Leu35Pro, Asp134Asn, Leu337Ser, Gln559Arg, Gly998Glu, Arg753Ter, Glu837Ter and Arg1086Ter variants were generated using the QuikChange II Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions.

Following viral packaging in Phoenix cells, EUFA1341 and U2OS-DR were retrovirally transduced (pMMP) with different forms of *PALB2* or the empty vector using filtered (0.45 μm syringe-top filter) viral supernatants along with 8 μg/ml polybrene. Selection of cells transduced using pMMP retroviruses was conducted by selecting with puromycin (0.7 μg/ml, U2OS-DR; 0.5 μg/ml, EUFA1341) at 48 hr after transduction.

Antibodies

Rabbit anti-PALB2 antibodies (Zhang, Fan, et al., 2009) were utilized for immunoblotting and immunofluorescence microscopy. Mouse anti-Flag (M2; Sigma) and anti-HA (HA.11; BioLegend) antibodies were utilized for immunoprecipitation and immunoblotting, respectively. Rabbit anti-RAD51 antibodies (H-92, Santa Cruz) were utilized for immunoblotting and immunofluorescence microscopy, while rabbit anti-BRCA1 (07–434, Millipore), goat anti-KEAP1 (E-20, Santa Cruz), rabbit anti-RAD51AP1 (Ab96666, Abcam), and mouse anti-actin antibodies (C4, Seven Hills Bioreagents) were utilized for immunoblotting.

Secondary antibodies for immunofluorescence microscopy included FITC-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) and Alexa 568-conjugated donkey anti-mouse IgG antibodies (Invitrogen). For immunoblotting, signals from HRP-linked anti-mouse and anti-rabbit IgG secondary antibodies (Amersham), and HRP-linked anti-goat IgG secondary antibodies (Santa Cruz) were detected by chemiluminescence (Amersham).

Immunoblotting

Lysates were prepared as described previously, and equivalent amounts of protein were separated on 4–12% gradient gels (Invitrogen), transferred to nitrocellulose, and incubated with primary and secondary antibodies prior to detection using chemoluminescence, as described previously (Fan, Zhang, Barrett, Ren, & Andreassen, 2009).

Co-immunoprecipitation assays

Cells were lysed in NETN 420 buffer supplemented with protease inhibitors and PMSF as described previously (Zhang, Fan, et al., 2009). Subsequent immunoprecipitations were performed with anti-Flag M2 Affinity Gel (Sigma) as described previously (Zhang, Fan, et al., 2009).

Immunofluorescence microscopy assays

Cells were grown on coverslips coated with poly-D-lysine, fixed with 2% paraformaldehyde for 20 min, and permeabilized with 0.2% Triton X-100 for 3 min. Following washes with

PBS, slides were incubated with primary and secondary antibodies, washed, and mounted with a coverglass in Vectashield (Vector Laboratories) containing DAPI, as described previously (Fan et al., 2009).

Labeled cells were observed with a Nikon Eclipse Ti microscope driven by NIS Elements AR 4.50 Imaging software and images were collected with a Neo/Zyla camera. Images were processed into figures using Photoshop (Adobe). Foci were quantified in two ways: 1) the mean percentage of cells with 3 or more nuclear foci (at least 3 replicate counts of ≥150 cells each) and 2) the mean number of foci/nucleus (3 replicate counts of ≥100 cells each from stored images collected with identical settings).

Homologous recombination assays

U2OS-DR cells with an integrated GFP reporter (Hayakawa et al., 2010), and containing different forms of PALB2 were as described previously (Zhang, Fan, et al., 2009). Briefly, cells were transduced (pMMP) with *PALB2* cDNAs carrying a silent mutation we generated (TCATTTGGATGTCAAGAAA to TCACCTTGACGTGAAAAAG at the beginning of the coding sequence) to make them resistant to siRNA-mediated knockdown of endogenous PALB2 using the following siRNA (UCAUUUGGAUGUCAAGAAAdTdT, Dharmacon). After stable selection of U2OS-DR cells for expression of siRNA-resistant PALB2, cells were transfected with the siRNA above to deplete endogenous PALB2 or with a control siRNA directed against LacZ. At 48 hr after transfection with siRNAs, cells were transfected with pCBASce encoding I-SCEI to induce DNA double-strand breaks (DSBs). Cells were collected 72 hr later by trypsination and placed in fresh medium on ice prior to analysis. The percentage of GFP-positive cells was determined using a FacsCanto (Becton-Dickinson) flow cytometer. Three replicates were performed for each sample and values were calculated relative to the mean value for cells transfected with the control siRNA for each form of PALB2, or the empty vector, and then normalized to the mean value for cells containing WT PALB2. To compare results between individual experiments, U2OS-DR cells reconstituted with empty vector and WT PALB2 were included each time and the normalized values set to 0% and 100% recombination, respectively.

IR and PARP inbibitor sensitivity assays

For measurements of sensitivity to IR, EUFA1341 cells reconstituted with empty vector or different forms of PALB2 were plated into 6 cm dishes in triplicate after irradiating with doses ranging from 0–16 Gy. For measurements of sensitivity to olaparib, cells were plated into 6 cm dishes in triplicate and returned to the incubator overnight, then treated with olaparib with doses ranging from 0 to 0.10 μM the next morning. Cells were incubated for 12 days to form colonies, then washed with PBS, fixed with Wright-Giemsa reagent (EMD), stained with Buffer solution Giordano (Fisher), dried, and manually counted.

Statistical analyses

The statistical significance of different survival curves in colony growth assays was assessed by employing an ANOVA using the Tukey method for multiple comparisons. All other measures of statistical significance were conducted using a Student’s t-test.

RESULTS

Identification of novel *PALB2* variants in a familial pancreatic cancer cohort which do not abrogate interactions with N-terminal partners of PALB2

Following the identification of *PALB2* as a pancreatic cancer gene (Jones et al., 2009),we performed full gene sequencing of *PALB2* from the peripheral blood of 54 pancreatic cancer patients in the Familial Pancreatic Tumor Registry at Memorial Sloan Kettering Cancer Center (MSKCC) (Olson et al., 2010; Saldia et al., 2019). Three of these patients were previously tested for the *BRCA1/2* Ashkenazki Jewish Panel, two patients had *BRCA1/2* full gene sequencing and were tested using the BRCA1 5-site Large Rearrangement Panel, and one patient had *PALB2* and *CDKN2A* testing. All of these tests results were negative.

Among the 54 pancreatic cancer patients in the Familial Pancreatic Tumor Registry at MSKCC sequenced for *PALB2*, two novel missense variants of *PALB2*, which have not been reported previously in the literature, were identified. Neither patient has been tested for *BRCA1/2*. In one family, the father and daughter, deceased at age 76 and 82, respectively, both developed pancreatic cancer (Fig. 1A). The proband (PA000830), who was diagnosed with pancreatic cancer at age 80, carried a germline *PALB2* c.191C>T (p.Ser64Leu) missense variant (Fig. 1B); the mother was unaffected. In another family, the proband (PA000940) was diagnosed with pancreatic cancer at age 78 and was deceased at age 79, while her father and sister were diagnosed with pancreatic cancer and died in their 50's and 60's, respectively. The proband's brother also had pancreatic cancer (age of diagnosis not available), but her mother was unaffected (Fig. 1A). The proband carried a germline c.311C>T (p.Pro104Leu) missense variant of *PALB2* (Fig. 1B). While neither variant has been reported in the literature, both are reported as VUSs in ClinVar (www.ncbi.nlm.nih.gov/clinvar/). There is one submitter for Ser64Leu for a hereditary cancer-predisposing syndrome (HCPS) patient, and two independent submissions for Pro104Leu – one for a HCPS patient and the other for a hereditary breast cancer patient.

Four other missense *PALB2* variants, c.1010T>C (p.Leu337Ser), c.1676A>G (p.Gln559Arg), c.2014G>C (p.Glu672Gln) and c.2993G>A (p.Gly998Glu), all of which are reported as likely benign (LB) or benign (B) in ClinVar, were also detected in the cohort of familial pancreatic cancer patients. Unlike Ser64Leu and Pro104Leu, all of these missense *PALB2* variants have been reported several times in the literature. Leu337Ser, Gln559Arg, Glu672Gln and Gly998Glu have been observed in breast cancer patients (Erkko et al., 2007; Rahman et al., 2007; Thompson et al., 2015; Tischkowitz et al., 2007), Leu337Ser and Gly998Glu in pancreatic cancer patients (Blanco et al., 2013; Hofstatter et al., 2011), and Gln559Arg in Fanconi anemia patients (Xia et al., 2007). Because of these numerous reports and because each is reported as B/LB in ClinVar, we focused our functional studies herein on the p.Ser64Leu and p.Pro104Leu variants of PALB2 rather than p.Leu337Ser, p.Gln559Arg, p.Glu672Gln and p.Gly998Glu.

Based on Alamut Visual, Ser64Leu and Pro104Leu were not predicted to be associated with splicing defects. Further, using four different tools (PolyPhen, SIFT, Mutation Taster, and AlignGVGD), neither p.Ser64Leu nor p.Pro104Leu was predicted as likely to have a negative effect on PALB2 function (Supp. Table S1). Since such *in silico* bioinformatics

tools have not proven highly reliable for predicting the effects of missense variants (Akbari et al., 2011; Plon et al., 2008), we proceeded to functionally characterize the p.Ser64Leu and p.Pro104Leu variants of PALB2.

While p.Ser64Leu and p.Pro104Leu are in the N-terminus of PALB2, they are not present within either the KEAP1-interacting domain or the coiled-coil that mediates interaction with BRCA1 (Ma et al., 2012; Sy et al., 2009; Zhang, Fan, et al., 2009; Zhang, Ma, et al., 2009) (Fig. 1C). Therefore, we began functional characterization of p.Ser64Leu and p.Pro104Leu by testing the effect of these variants on the interaction of PALB2 with other proteins that interact with its N-terminus. For this purpose, different forms of PALB2 were stably expressed in SV40-transformed EUFA1341 fibroblasts derived from a Fanconi anemia patient (N complementation group) (Xia et al., 2007) along with a N-terminal Flag-HA epitope tag. Exogenously expressed WT, p.Ser64Leu and p.Pro104Leu forms of PALB2 were all detected in EUFA1341 cells (Fig. 2) and were immunoprecipitated using anti-Flag antibodies. We tested for effects on interactions with BRCA1, RAD51, KEAP1 and RAD51AP1, since each has been demonstrated to interact *in vitro* and/or in cells with the first 200 amino acids of PALB2, where p.Ser64Leu and p.Pro104Leu reside (Buisson et al., 2010; Dray et al., 2010; Ma et al., 2012; Sy et al., 2009; Zhang, Fan, et al., 2009; Zhang, Ma, et al., 2009). There was no clear effect on the interactions of PALB2-Ser64Leu and PALB2-Pro104Leu with BRCA1, KEAP1, RAD51 or RAD51AP1, as compared to WT PALB2 (Fig. 2), suggesting that if these variants affect PALB2 function it is not via alteration of these interactions.

The p.Ser64Leu and p.Pro104Leu variants compromise recruitment of PALB2 and RAD51 to nuclear foci

To further functionally characterize the missense PALB2 variants we identified in familial pancreatic cancer patients, except for assays of homologous recombination, we similarly utilized *PALB2*-deficient EUFA1341 cells reconstituted with WT PALB2, p.Ser64Leu and p.Pro104Leu, and the empty vector (Fig. 3A). Recruitment of PALB2 to nuclear foci is a sensitive readout for PALB2 function (Park, Zhang, et al., 2014). *PALB2*-deficient EUFA1341 fibroblasts transduced with the empty vector lacked nuclear PALB2 as shown by the example in Fig. 3B. Further, as quantitated for both untreated populations and following exposure to IR, cells reconstituted with the empty vector lacked nuclear foci (Supp. Fig. S1 and 3C-D). In contrast, while also detectable in untreated populations (Supp. Fig. S1), WT PALB2 displayed increased levels of nuclear PALB2 foci in cells treated with IR by two criteria: 1) the percentage of cells with 3 or more nuclear PALB2 foci (Fig. 3C) and 2) the mean number of nuclear foci per cell (Fig. 3D). Strikingly, cells that expressed either the p.Ser64Leu or p.Pro104Leu forms of PALB2 displayed reduced levels of PALB2 foci that were intermediate to those conferred by the empty vector and WT PALB2, both in untreated populations (Supp. Fig. S1) and following exposure to IR (Fig. 3C-D).

To further support the conclusion that PALB2 function is compromised by the p.Ser64Leu and p.Pro104Leu variants, we assayed the assembly of the RAD51 recombinase, which is recruited into nuclear foci in a PALB2-dependent manner (Xia et al., 2007; Zhang, Fan, et al., 2009). The examples shown (Fig. 4A) and quantification (Figs. 4B-C) both demonstrate

that the p.Ser64Leu and p.Pro104Leu variants of PALB2 resulted in decreased recruitment of RAD51 to DNA damage foci following exposure to IR relative to levels in cells reconstituted with WT PALB2. Notably, both the percentage of cells with 3 or more nuclear RAD51 foci (Fig. 4B) and mean number of nuclear RAD51 foci per cell (Fig. 4C) were intermediate to those conferred by WT PALB2 and the empty vector.

The p.Ser64Leu and p.Pro104Leu variants of PALB2 compromise DNA repair by homologous recombination

Next, given the central function of PALB2 in DNA repair via HR (Park, Zhang, et al., 2014; Xia et al., 2006), we sought to assess the effect of the p.Ser64Leu and p.Pro104Leu variants of PALB2 on HR. For this purpose, we utilized U2OS-DR cells with a stably integrated GFP reporter in which DNA double-strand breaks (DSBs) are induced by transfecting with a plasmid that encodes the I-SCEI endonuclease (Nakanishi et al., 2005). These cells were stably transduced with different forms of *PALB2* containing a silent mutation that makes them resistant to a siRNA that depletes endogenous PALB2 (Fig. 5A). In particular, while siPALB2 depleted endogenous PALB2, as seen in cells containing the empty vector, exogenously expressed WT PALB2 was resistant to siPALB2.

HR assays are relevant for assessing the clinical impact of variants in genes such as *BRCA1/2*, as well as *PALB2*, when there is insufficient genetic data (Toland & Andreassen, 2017). Typically, such assays are calibrated based upon known benign and pathogenic variants (Toland & Andreassen, 2017). Although relatively few benign and pathogenic *PALB2* variants have been identified, we tested the effects of the p.Ser64Leu and p.Pro104Leu variants of PALB2 on HR in comparison to 4 benign (B)/likely benign (LB) and 4 pathogenic/complete loss of function (LOF) variants. Three of the B/LB variants, Leu337Ser, Gln559Arg and Gly998Glu, were identified in our cohort of familial pancreatic cancer patients, and in other various types of cancer and/or Fanconi anemia as detailed earlier in this section. The fourth B/LB variant, Asp134Asn, has been observed in breast cancer patients (Thompson et al., 2015; Zheng, Zhang, Niu, Huo, & Olopade, 2012). Given the dearth of known pathogenic (P)/likely pathogenic (LP) or full LOF missense variants of *PALB2*, we tested Leu35Pro, which has been reported as either pathogenic (Foo et al., 2017; Rodrigue et al., 2019) or as a LOF variant (Boonen et al., 2019; Wiltshire et al., 2020), as well as 3 nonsense mutants: Arg753Ter, Glu837Ter and Arg1086Ter. One or more of each of these truncating variants has been associated with familial pancreatic cancer (Jones et al., 2009; Zhen et al., 2015), familial breast cancer (Antoniou et al., 2014; Thompson et al., 2015), germline mutations in unselected breast cancer patients (Sun et al., 2017), familial ovarian cancer (Norquist et al., 2016), and Fanconi anemia (Reid et al., 2007).

The Asp134Asn and Arg1086Ter forms of PALB2, which were resistant to siPALB2, were expressed in U2OS-DR cells at similar levels as WT PALB2 and PALB2-Ser64Leu and PALB2-Pro104Leu (Fig. 5A). Similarly, si-resistant Leu337Ser, Gln559Arg, Gly998Glu, Leu35Pro, Arg753Ter and Glu837Ter were detected in U2OS-DR cells (Supp. Fig. S2).

As an example of the HR assays we performed, WT PALB2 and p.Asp134Asn efficiently promoted HR, based upon levels of GFP-positive cells in dot plots, after induction of DSBs in populations depleted of endogenous PALB2 utilizing siPALB2 (Fig. 5B). In contrast, cells

containing the empty vector or Arg1086Ter were both deficient for HR, as shown by dot plots (Fig. 5B).

Quantification demonstrates that the levels of DSB-initiated HR associated with each of the B/LB missense PALB2 variants, p.Asp134Asn, p.Leu337Ser, p.Gln559Arg and p.Gly998Glu, were indistinguishable from PALB2-WT (Fig. 5C). This is consistent with a recent publication (Boonen et al., 2019). In contrast, each of the P/LP variants, p.Arg753Ter, p.Glu837Ter and p.Arg1086Ter, as well as p.Leu35Pro, displayed a clear loss of function in HR (Fig. 5C). In particular, consistent with recent publications (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2020), the level of HR associated with the empty vector and with the p.Leu35Pro missense variant were indistinguishable. Importantly, both the Ser64Leu and Pro104Leu *PALB2* variants, identified in familial pancreatic cancer patients, strongly compromised HR (Fig. 5C), and were intermediate to the HR activities of WT PALB2 and the 4 B/LB PALB2 variants, and of the empty vector and 3 P/LP variants and the p.Leu35Pro missense variant. This suggests the possibility that Ser64Leu and Pro104Leu could potentially confer an increased or intermediate risk of developing cancer due to compromised HR, which will need to be evaluated in future studies.

The p.Ser64Leu and p.Pro104Leu variants of PALB2 confer increased sensitivity to IR and olaparib, which are DNA damaging agents utilized in cancer therapy

As part of the response to DNA damage, another key function of PALB2 is in mediating cellular resistance to DNA damaging agents (Buisson et al., 2010; Park, Singh, et al., 2014; Xia et al., 2006). Thus, since the p.Ser64Leu and p.Pro104Leu variants of PALB2 both compromised DSB-initiated HR (Fig. 5), we next tested their effects on sensitivity to IR, which induces DSBs. Consistent with a previous report (Park, Singh, et al., 2014), WT PALB2 restores resistance of EUFA1341 cells to IR as measured using clonogenic survival assays (Fig. 6A). Notably, the p.Ser64Leu and p.Pro104Leu variants of PALB2 conferred little resistance to IR when expressed in EUFA1341 cells (Fig. 6A).

Since PARP inhibitors have emerged as an additional therapeutic strategy that is promising for specific targeting of tumor cells with HR deficiencies (Hoppe, Sundar, Tan, & Jeyasekharan, 2018; Zhu et al., 2020), we also tested the effects of the p.Ser64Leu and p.Pro104Leu variants on the resistance of cells to the PARP inhibitor olaparib. In further support of a compromised DNA damage response, both p.Ser64Leu and p.Pro104Leu conferred olaparib sensitivity to EUFA1341 cells which was intermediate to that associated with WT PALB2 and the empty vector, as measured using a clonogenic survival assay (Fig. 6B).

DISCUSSION

We have identified two novel germline missense variants of *PALB2*, Ser64Leu and Pro104Leu, in familial pancreatic cancer patients. These variants have not been reported in publications previously, either in control or cancer populations. Notably, the functional studies we performed here, by reconstituting *PALB2*-deficient cells, demonstrate that both p.Ser64Leu and p.Pro104Leu compromise the normal function of PALB2 in DNA damage responses. This includes decreased recruitment to DNA damage foci, decreased levels of

HR, and increased cellular sensitivity to IR and the PARP inhibitor olaparib. To the best of our knowledge, this is the first demonstration of deleterious missense PALB2 variants outside of a known functional domain as discussed below.

The levels of HR associated with p.Ser64Leu and p.Pro104Leu are intermediate to those conferred by 4 benign/likely benign (B/LB) and 3 pathogenic/likely pathogenic (P/LP) PALB2 variants. Thus, Ser64Leu and Pro104Leu may increase the risk of developing cancer by increasing genome instability due to compromised error-free repair, but to a lesser degree than known truncating pathogenic variants of *PALB2*. The Ser64Leu and Pro104Leu variants could, therefore, confer an intermediate risk of cancer compared to truncating variants of *PALB2*.

Our results also yield insight into the function of PALB2 in cellular DNA damage responses. p.Ser64Leu and p.Pro104Leu reside in a largely uncharacterized N-terminal region of PALB2, distinct from the coiled-coil and LDEETGE motifs that interact with BRCA1 and KEAP1, respectively; thus, our results identify residues in a new region of PALB2 required for an optimal response to DNA damage.

Cell-based assays of the effects of the p.Ser64Leu and p.Pro104Leu variants on PALB2 function in DNA damage responses

Relatively few PALB2 missense variants found in cancer patients have been characterized functionally (Boonen et al., 2019; Erkko et al., 2007; Foo et al., 2017; Park, Singh, et al., 2014; Rodrigue et al., 2019; Wiltshire et al., 2020). Thus, an important aspect of our study is conducting tests on p.Ser64Leu and p.Pro104Leu related to the function of PALB2 in DNA damage responses. Previously, only missense variants in the N-terminal coiled-coil and C-terminal WD40 motifs, or directly proximal, primarily identified in breast cancer patients, have been reported as deleterious based at least partly on compromised HR. These deleterious variants include p.Pro8Leu, p.Lys18Arg, p.Leu24Ser, p.Tyr28Cys, p.Lys30Asn, p.Leu35Pro and p.Arg37His in, or directly adjacent, to the coiled-coil motif from a.a. 9–44, and p.Trp912Gly, p.Gly937Arg, p.Leu939Trp, p.Ile944Asn, p.Leu947Phe, p.Leu947Ser, p.Leu961Pro, p.Leu972Gln, p.Thr1030Ile, p.Gly1043Asp, p.Leu1070Pro, p.Trp1140Gly, p.Leu1143Pro, p.Gly1145Arg and p.Leu1172Pro in the WD40 domain from a.a. 867–1186. p.Leu35Pro, which is in the coiled-coil motif, fully disrupts cellular resistance to PARP inhibitor (Foo et al., 2017) and HR [Fig. 5C and (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2020)]. Few missense PALB2 variants have been identified with complete LOF. However, similar to Ser64Leu and Pro104Leu, many of the other deleterious *PALB2* missense variants, mostly identified in breast cancer patients and which are listed above, only partially compromise HR and/or other activities of PALB2 such as cellular resistance to IR and/or PARP inhibitors (Boonen et al., 2019; Erkko et al., 2007; Foo et al., 2017; Park, Singh, et al., 2014; Rodrigue et al., 2019; Wiltshire et al., 2020). It should be noted that many of the variants in the WD40 domain may act by destabilizing PALB2, rather than necessarily having a direct effect on PALB2 function (Boonen et al., 2019; Park, Singh, et al., 2014; Wiltshire et al., 2020). In contrast, while deleterious, neither p.Ser64Leu nor p.Pro104Leu, both in the N-terminus, appears to affect PALB2 stability.

Taken together, here we gain a deeper understanding of PALB2 function by analyzing the effects of p.Ser64Leu and p.Pro104Leu, which are nearby, but outside of, the N-terminal coiled-coil and KEAP-binding motifs (see Fig. 1C). These variants partially compromise the DNA damage response in all assays utilized, including assembly of PALB2 and RAD51 foci, HR-dependent DNA repair, and cellular resistance to IR and the PARP inhibitor olaparib. Thus, p.Ser64Leu and p.Pro104Leu demonstrate the potential importance of an additional functional region of PALB2.

It should be noted that unlike variants of the coiled-coil motif associated with abrogation of binding to BRCA1, which completely perturb PALB2 and RAD51 recruitment to nuclear foci (Foo et al., 2017; Park, Singh, et al., 2014), p.Ser64Leu and p.Pro104Leu only partially impair PALB2 and RAD51 foci. Consistent with apparently normal interactions with BRCA1 (Fig. 2), this suggests that p.Ser64Leu and p.Pro104Leu compromise the DNA damage response independent of the coiled-coil motif.

Implications of the Ser64Leu and Pro104Leu variants for cancer risk and treatment

Since *PALB2* exome sequencing data from the Familial Tumor Registry at the Memorial Sloan Kettering Cancer Center is available only for probands, there is insufficient data to classify the clinical significance of the Ser64Leu and Pro104Leu variants based upon segregation analyses. Functional assays, such as those performed here, are not currently sufficient as a stand-alone basis for classifying these variants on a clinical basis (Toland & Andreassen, 2017). Still, functional assays, such as the ones performed here, can be an important piece of evidence that can be combined with other data, including family history and co-segregation of the variant with cancer in affected families, in multifactorial risk prediction models or ACMG-based rules models to classify variants for clinical use (Chenevix-Trench et al., 2006; Spearman et al., 2008; Tavtigian, Harrison, Boucher, & Biesecker, 2020). For this purpose, functional assays must be calibrated to the functional activities of known B/LB and P/LP variants of the particular gene.

In early studies to classify variants, following the association of a gene with a disease phenotype, the number of available reference variants is typically limited. This was true for the first studies to classify missense variants in *BRCA1* and *BRCA2*, which are HR genes like *PALB2*. Thus, the process of validating functional assays often begins with a small number of truncating and missense variants (Chang, Biswas, Martin, Stauffer, & Sharan, 2009; Wu et al., 2005). We have taken a similar approach for PALB2 variants; the number of defined B/LB and P/LP variants utilized to calibrate our HR assay, which is directly related to the tumor suppressor activity of PALB2 (Park, Zhang, et al., 2014), is within the range of other recent functional studies of PALB2 variants (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2020).

Our study has made an important contribution to setting thresholds, or cutoffs, for B/LB and P/LP *PALB2* variants. In particular, our results (Figure 5C) agree with all other recent PALB2 functional studies that p.Leu35Pro displays full LOF in HR assays, similar to the activity of the empty vector (Boonen et al., 2019; Rodrigue et al., 2019; Wiltshire et al., 2020). In one study, Leu35Pro was classified as pathogenic based upon co-segregation of this variant with cancer in a family with a strong breast cancer predisposition (Foo et al.,

2017). Additionally, we tested the same 4 B/LB missense PALB2 variants as Boonen et al. (2019), p.Asp134Asn, p.Leu337Ser, p.Gln559Arg and p.Gly998Glu, and similarly find that they are fully active in HR. This adds to confidence in future utilization of these B/LB variants to calibrate functional studies. Finally, we tested 3 truncation variants, p.Arg753Ter, p.Glu837Ter and p.Arg1086Ter, which to the best of our knowledge have not been previously tested functionally. These each exhibited equivalent or less HR activity than the p.Leu35Pro missense LOF variant. Thus, this study expands the list of P/LP variants that can be utilized to set functional thresholds in the future. Ultimately, while saturation screens have only been performed for a portion of *BRCA1* (Findlay et al., 2018; Starita et al., 2018), our study and related studies will collectively help subsequent saturation screens for *PALB2* missense variants that will more definitively establish thresholds. At present, we have determined that both p.Ser64Leu and p.Pro104Leu have intermediate HR activities as compared to the least active of the B/LB variants, p.Leu337Ser, and the most active of the P/LP-LOF variants, p.Leu35Pro.

Again, the p.Ser64Leu and p.Pro104Leu variants of PALB2 compromise several aspects of the cellular DNA damage response, including the display of HR activities intermediate to multiple defined B/LB and P/LP variants (Figure 5C). Thus, Ser64Leu and Pro104Leu are strong candidates for further studies to determine whether they are associated with increased cancer risk in individuals. Also, because IR and PARP inhibitors are potentially effective against HR-deficient tumors, our finding that Ser64Leu and Pro104Leu confer increased sensitivity to these agents suggests that germline or somatic detection of these variants may provide a basis for radiotherapy or treatment with PARP inhibitors.

While neither the Ser64Leu nor the Pro104Leu *PALB2* variants has been reported previously in the literature, according to ClinVar both have been identified in hereditary cancer-predisposing syndrome patients. Thus Ser64Leu and Pro104Leu may not be specifically associated with pancreatic cancer. Indeed, various individual *PALB2* variants have been identified in patients from multiple cancer types including pancreatic cancer (Blanco et al., 2013; Jones et al., 2009; Slater et al., 2010; Zhen et al., 2015).

Molecular and cellular mechanisms of the effects of the p.Ser64Leu and p.Pro104Leu variants on PALB2 function in DNA damage responses

While the possibility of a functional domain which contains Ser64 and P104, distinct from the nearby coiled-coil and KEAP1-interacting motifs, is suggested by the very similar phenotypes resulting from the p.Ser64Leu and p.Pro104Leu variants, further work will be required to test this hypothesis. In that context, it is unlikely that any domain containing S64 and P104 is related to RAD51-binding activity, since only P104 is present in the secondary RAD51 binding site from a.a. 101–184 of PALB2 that has been demonstrated using *in vitro* assays (Buisson et al., 2010; Dray et al., 2010). It should be noted that a specific function in the DNA damage response for this secondary RAD51-binding site, which is distinct from the primary RAD51-binding site in the C-terminal WD40 domain of PALB2 (Buisson et al., 2010; Zhang, Fan, et al., 2009), has not been tested in cells. Further, neither variant appears to affect the interaction of RAD51 with PALB2 (Fig. 2).

Neither the p.Ser64Leu nor the p.Pro104Leu variants had an apparent effect on known N-terminal PALB2 protein interactions, such as with BRCA1, which localizes and recruits PALB2 (Zhang, Bick, Park, & Andreassen, 2012; Zhang, Fan, et al., 2009; Zhang, Ma, et al., 2009), KEAP1, which mediates redox homeostasis in conjunction with PALB2 (Ma et al., 2012), or RAD51 and RAD51AP1 (Buisson et al., 2010; Dray et al., 2010) (Fig. 2). Further, the RRKK motif present at a.a. 146–149, distinct from p.Ser64Leu and p.Pro104Leu, is principally responsible for DNA binding by PALB2 (Deveryshetty et al., 2019). It therefore appears unlikely that p.Ser64Leu and p.Pro104Leu mediate their effects via diminished DNA binding by PALB2. One possibility is that p.Ser64Leu and p.Pro104Leu act by affecting an interaction(s) with a protein that is yet to be identified.

Interestingly, it has been reported that PALB2 can be phosphorylated at S64 by a cyclin-dependent kinase, and also at S59 by ATR (Buisson et al., 2017). Phosphorylated S59 and dephosphorylated S64 promote PALB2 recruitment to sites of DNA damage and its function in HR. This was tested utilizing S59E/S64A and S59A/S64E mutants of PALB2, but the effect, if any, of the S64A or S64E variants themselves on these processes was not determined (Buisson et al., 2017). We find that the p.Ser64Leu variant perturbs PALB2 function. Thus p.Ser64Leu could act by disrupting S64 phosphorylation and/or dephosphorylation, perhaps in a cycle, but it is also possible that the change to leucine alters the conformation of PALB2 independently from phosphorylation at this site. In any case, our identification of the Ser64Leu variant in familial pancreatic cancer patients, and demonstration of functional defects in the DNA damage response, suggest the potential importance of S64 phosphorylation.

In summary, this study identifies the *PALB2* variants Ser64Leu and Pro104Leu in individuals with familial pancreatic cancer, and demonstrates the variants' effects on cellular DNA damage responses. Taken together, our data increase understanding of PALB2 function and suggest a potential contribution of specific PALB2 missense variants to increasing cancer risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We are grateful to Dr. Hans Jonje (Vrije Universiteit Medical Center), and Drs. Maria Jasin and Koji Nakanishi (Memorial Sloan Kettering Cancer Center), for EUFA1341 fibroblasts, and U2OS-DR cells and pCBASce, respectively. Also, we wish to thank Amethyst Saldia, and Pampa Roy, Aliaksandra Samoila and Keimya Sadeghi, for assistance with gathering patient and family histories, and for technical contributions with the handling of biospecimens, derived DNA and related data, respectively (Memorial Sloan Kettering Cancer Center). Additionally, we thank Dr. Adriana Heguy (now at New York University) for her assistance with sequencing and Dr. Adam Lane (Cincinnati Children's Hospital Medical Center) for his assistance with statistical analyses.

Funding information: Epidemiology & Cancer Prevention Fund and The Society of Memorial Sloan Kettering Cancer Center (SHO), Adenocarcinoma Research Fund and The Lucille Castori Seed Grant (RCK), Memorial Sloan Kettering Cancer Center Department of Pathology (LZ); DoD CDMRP (OCRP) W81XWH-18-1-0269 and NIH R01 GM134731 (PRA).

REFERENCES

Akbari MR, Zhang S, Fan I, Royer R, Li S, Risch H, … Narod SA (2011). Clinical impact of unclassified variants of the BRCA1 and BRCA2 genes. J Med Genet, 48(11), 783–786. doi:10.1136/jmedgenet-2011-100305 [PubMed: 21965345]

Antoniou AC, Foulkes WD, & Tischkowitz M. (2014). Breast-cancer risk in families with mutations in PALB2. N Engl J Med, 371(17), 1651–1652. doi:10.1056/NEJMc1410673 [PubMed: 25337756]

Blanco A, de la Hoya M, Osorio A, Diez O, Miramar MD, Infante M, … Vega A. (2013). Analysis of PALB2 gene in BRCA1/BRCA2 negative Spanish hereditary breast/ovarian cancer families with pancreatic cancer cases. PLoS One, 8(7), e67538. doi:10.1371/journal.pone.0067538

Boonen R, Rodrigue A, Stoepker C, Wiegant WW, Vroling B, Sharma M, … van Attikum H. (2019). Functional analysis of genetic variants in the high-risk breast cancer susceptibility gene PALB2. Nat Commun, 10(1), 5296. doi:10.1038/s41467-019-13194-2 [PubMed: 31757951]

Buisson R, Dion-Cote AM, Coulombe Y, Launay H, Cai H, Stasiak AZ, … Masson JY (2010). Cooperation of breast cancer proteins PALB2 and piccolo BRCA2 in stimulating homologous recombination. Nat Struct Mol Biol, 17(10), 1247–1254. doi:10.1038/nsmb.1915 [PubMed: 20871615]

Buisson R, Niraj J, Rodrigue A, Ho CK, Kreuzer J, Foo TK, … Zou L. (2017). Coupling of Homologous Recombination and the Checkpoint by ATR. Mol Cell, 65(2), 336–346. doi:10.1016/j.molcel.2016.12.007 [PubMed: 28089683]

Chang S, Biswas K, Martin BK, Stauffer S, & Sharan SK (2009). Expression of human BRCA1 variants in mouse ES cells allows functional analysis of BRCA1 mutations. J Clin Invest, 119(10), 3160–3171. doi:10.1172/JCI39836 [PubMed: 19770520]

Chenevix-Trench G, Healey S, Lakhani S, Waring P, Cummings M, Brinkworth R, … kConFab I. (2006). Genetic and histopathologic evaluation of BRCA1 and BRCA2 DNA sequence variants of unknown clinical significance. Cancer Res, 66(4), 2019–2027. doi:10.1158/0008-5472.CAN-05-3546 [PubMed: 16489001]

Deveryshetty J, Peterlini T, Ryzhikov M, Brahiti N, Dellaire G, Masson JY, & Korolev S. (2019). Novel RNA and DNA strand exchange activity of the PALB2 DNA binding domain and its critical role for DNA repair in cells. Elife, 8. doi:10.7554/eLife.44063

Dray E, Etchin J, Wiese C, Saro D, Williams GJ, Hammel M, … Sung P. (2010). Enhancement of RAD51 recombinase activity by the tumor suppressor PALB2. Nat Struct Mol Biol, 17(10), 1255–1259. doi:10.1038/nsmb.1916 [PubMed: 20871616]

Ducy M, Sesma-Sanz L, Guitton-Sert L, Lashgari A, Gao Y, Brahiti N, … Masson JY (2019). The Tumor Suppressor PALB2: Inside Out. Trends Biochem Sci, 44(3), 226–240. doi:10.1016/j.tibs.2018.10.008 [PubMed: 30638972]

Erkko H, Xia B, Nikkila J, Schleutker J, Syrjakoski K, Mannermaa A, … Winqvist R. (2007). A recurrent mutation in PALB2 in Finnish cancer families. Nature, 446(7133), 316–319. doi:10.1038/nature05609 [PubMed: 17287723]

Fan Q, Zhang F, Barrett B, Ren K, & Andreassen PR (2009). A role for monoubiquitinated FANCD2 at telomeres in ALT cells. Nucleic Acids Res, 37(6), 1740–1754. doi:10.1093/nar/gkn995 [PubMed: 19129235]

Findlay GM, Daza RM, Martin B, Zhang MD, Leith AP, Gasperini M, … Shendure J. (2018). Accurate classification of BRCA1 variants with saturation genome editing. Nature, 562(7726), 217–222. doi:10.1038/s41586-018-0461-z [PubMed: 30209399]

Foo TK, Tischkowitz M, Simhadri S, Boshari T, Zayed N, Burke KA, … Xia B. (2017). Compromised BRCA1-PALB2 interaction is associated with breast cancer risk. Oncogene, 36(29), 4161–4170. doi:10.1038/onc.2017.46 [PubMed: 28319063]

Hanenberg H, & Andreassen PR (2018). PALB2 (partner and localizer of BRCA2). Atlas Genet Cytogenet Oncol Haematol, 22(12), 484–490. doi:10.4267/2042/69016 [PubMed: 31413733]

Hayakawa T, Zhang F, Hayakawa N, Ohtani Y, Shinmyozu K, Nakayama J, & Andreassen PR (2010). MRG15 binds directly to PALB2 and stimulates homology-directed repair of chromosomal breaks. J Cell Sci, 123(Pt 7), 1124–1130. doi:10.1242/jcs.060178 [PubMed: 20332121]

Hofstatter EW, Domchek SM, Miron A, Garber J, Wang M, Componeschi K, … Tung N. (2011). PALB2 mutations in familial breast and pancreatic cancer. Fam Cancer, 10(2), 225–231. doi:10.1007/s10689-011-9426-1 [PubMed: 21365267]

Hoppe MM, Sundar R, Tan DSP, & Jeyasekharan AD (2018). Biomarkers for Homologous Recombination Deficiency in Cancer. J Natl Cancer Inst, 110(7), 704–713. doi:10.1093/jnci/djy085 [PubMed: 29788099]

Jones S, Hruban RH, Kamiyama M, Borges M, Zhang X, Parsons DW, … Klein AP (2009). Exomic sequencing identifies PALB2 as a pancreatic cancer susceptibility gene. Science, 324(5924), 217. doi:10.1126/science.1171202 [PubMed: 19264984]

Kamel D, Gray C, Walia JS, & Kumar V. (2018). PARP Inhibitor Drugs in the Treatment of Breast, Ovarian, Prostate and Pancreatic Cancers: An Update of Clinical Trials. Curr Drug Targets, 19(1), 21–37. doi:10.2174/1389450118666170711151518 [PubMed: 28699513]

Ma J, Cai H, Wu T, Sobhian B, Huo Y, Alcivar A, … Xia B. (2012). PALB2 interacts with KEAP1 to promote NRF2 nuclear accumulation and function. Mol Cell Biol, 32(8), 1506–1517. doi:10.1128/MCB.06271-11 [PubMed: 22331464]

Nakanishi K, Yang YG, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, … Jasin M. (2005). Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci U S A, 102(4), 1110–1115. doi:10.1073/pnas.0407796102 [PubMed: 15650050]

Norquist BM, Harrell MI, Brady MF, Walsh T, Lee MK, Gulsuner S, … Birrer MJ (2016). Inherited Mutations in Women With Ovarian Carcinoma. JAMA Oncol, 2(4), 482–490. doi:10.1001/jamaoncol.2015.5495 [PubMed: 26720728]

Oliver AW, Swift S, Lord CJ, Ashworth A, & Pearl LH (2009). Structural basis for recruitment of BRCA2 by PALB2. EMBO Rep, 10(9), 990–996. doi:10.1038/embor.2009.126 [PubMed: 19609323]

Olson SH, Chou JF, Ludwig E, O'Reilly E, Allen PJ, Jarnagin WR, … Kurtz RC (2010). Allergies, obesity, other risk factors and survival from pancreatic cancer. Int J Cancer, 127(10), 2412–2419. doi:10.1002/ijc.25240 [PubMed: 20143395]

Park JY, Singh TR, Nassar N, Zhang F, Freund M, Hanenberg H, … Andreassen PR (2014). Breast cancer-associated missense mutants of the PALB2 WD40 domain, which directly binds RAD51C, RAD51 and BRCA2, disrupt DNA repair. Oncogene, 33(40), 4803–4812. doi:10.1038/onc.2013.421 [PubMed: 24141787]

Park JY, Zhang F, & Andreassen PR (2014). PALB2: the hub of a network of tumor suppressors involved in DNA damage responses. Biochim Biophys Acta, 1846(1), 263–275. doi:10.1016/j.bbcan.2014.06.003 [PubMed: 24998779]

Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, … Group, I. U. G. V. W. (2008). Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Hum Mutat, 29(11), 1282–1291. doi:10.1002/humu.20880 [PubMed: 18951446]

Rahman N, Seal S, Thompson D, Kelly P, Renwick A, Elliott A, … Stratton MR (2007). PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet, 39(2), 165–167. doi:10.1038/ng1959 [PubMed: 17200668]

Reid S, Schindler D, Hanenberg H, Barker K, Hanks S, Kalb R, … Rahman N. (2007). Biallelic mutations in PALB2 cause Fanconi anemia subtype FA-N and predispose to childhood cancer. Nat Genet, 39(2), 162–164. doi:10.1038/ng1947 [PubMed: 17200671]

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, … Committee, A. L. Q. A. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med, 17(5), 405–424. doi:10.1038/gim.2015.30 [PubMed: 25741868]

Rodrigue A, Margaillan G, Torres Gomes T, Coulombe Y, Montalban G, da Costa ESCS, … Masson JY (2019). A global functional analysis of missense mutations reveals two major hotspots in the PALB2 tumor suppressor. Nucleic Acids Res, 47(20), 10662–10677. doi:10.1093/nar/gkz780 [PubMed: 31586400]

Saldia A, Olson SH, Nunes P, Liang X, Samson ML, Salo-Mullen E, … Kurtz RC (2019). Outcome of Pancreatic Cancer Surveillance Among High-Risk Individuals Tested for Germline Mutations in BRCA1 and BRCA2. Cancer Prev Res (Phila), 12(9), 599–608. doi:10.1158/1940-6207.CAPR-18-0272 [PubMed: 31337648]

Slater EP, Langer P, Niemczyk E, Strauch K, Butler J, Habbe N, … Bartsch DK (2010). PALB2 mutations in European familial pancreatic cancer families. Clin Genet, 78(5), 490–494. doi:10.1111/j.1399-0004.2010.01425.x [PubMed: 20412113]

Spearman AD, Sweet K, Zhou XP, McLennan J, Couch FJ, & Toland AE (2008). Clinically applicable models to characterize BRCA1 and BRCA2 variants of uncertain significance. J Clin Oncol, 26(33), 5393–5400. doi:10.1200/JCO.2008.17.8228 [PubMed: 18824701]

Starita LM, Islam MM, Banerjee T, Adamovich AI, Gullingsrud J, Fields S, … Parvin JD (2018). A Multiplex Homology-Directed DNA Repair Assay Reveals the Impact of More Than 1,000 BRCA1 Missense Substitution Variants on Protein Function. Am J Hum Genet, 103(4), 498–508. doi:10.1016/j.ajhg.2018.07.016 [PubMed: 30219179]

Sun J, Meng H, Yao L, Lv M, Bai J, Zhang J, … Xie Y. (2017). Germline Mutations in Cancer Susceptibility Genes in a Large Series of Unselected Breast Cancer Patients. Clin Cancer Res, 23(20), 6113–6119. doi:10.1158/1078-0432.CCR-16-3227 [PubMed: 28724667]

Sy SM, Huen MS, & Chen J. (2009). PALB2 is an integral component of the BRCA complex required for homologous recombination repair. Proc Natl Acad Sci U S A, 106(17), 7155–7160.doi:0811159106 [pii] 10.1073/pnas.0811159106 [PubMed: 19369211]

Tavtigian SV, Harrison SM, Boucher KM, & Biesecker LG (2020). Fitting a naturally scaled point system to the ACMG/AMP variant classification guidelines. Hum Mutat. doi:10.1002/humu.24088

Thompson ER, Gorringe KL, Rowley SM, Wong-Brown MW, McInerny S, Li N, … Campbell IG (2015). Prevalence of PALB2 mutations in Australian familial breast cancer cases and controls. Breast Cancer Res, 17, 111. doi:10.1186/s13058-015-0627-7 [PubMed: 26283626]

Tischkowitz M, Xia B, Sabbaghian N, Reis-Filho JS, Hamel N, Li G, … Foulkes WD (2007). Analysis of PALB2/FANCN-associated breast cancer families. Proc Natl Acad Sci U S A, 104(16), 6788–6793. doi:10.1073/pnas.0701724104 [PubMed: 17420451]

Toland AE, & Andreassen PR (2017). DNA repair-related functional assays for the classification of BRCA1 and BRCA2 variants: a critical review and needs assessment. J Med Genet, 54(11), 721–731. doi:10.1136/jmedgenet-2017-104707 [PubMed: 28866612]

Wiltshire T, Ducy M, Foo TK, Hu C, Lee KY, Belur Nagaraj A, … Couch FJ (2020). Functional characterization of 84 PALB2 variants of uncertain significance. Genet Med, 22(3), 622–632. doi:10.1038/s41436-019-0682-z [PubMed: 31636395]

Wu K, Hinson SR, Ohashi A, Farrugia D, Wendt P, Tavtigian SV, … Couch FJ (2005). Functional evaluation and cancer risk assessment of BRCA2 unclassified variants. Cancer Res, 65(2), 417–426. [PubMed: 15695382]

Xia B, Dorsman JC, Ameziane N, de Vries Y, Rooimans MA, Sheng Q, … de Winter JP (2007). Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. Nat Genet, 39(2), 159–161. doi:10.1038/ng1942 [PubMed: 17200672]

Xia B, Sheng Q, Nakanishi K, Ohashi A, Wu J, Christ N, … Livingston DM (2006). Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. Mol Cell, 22(6), 719–729. doi:10.1016/j.molcel.2006.05.022 [PubMed: 16793542]

Yang SH, Kuo TC, Wu H, Guo JC, Hsu C, Hsu CH, … Kuo SH (2016). Perspectives on the combination of radiotherapy and targeted therapy with DNA repair inhibitors in the treatment of pancreatic cancer. World J Gastroenterol, 22(32), 7275–7288. doi:10.3748/wjg.v22.i32.7275 [PubMed: 27621574]

Zhang F, Bick G, Park JY, & Andreassen PR (2012). MDC1 and RNF8 function in a pathway that directs BRCA1-dependent localization of PALB2 required for homologous recombination. J Cell Sci, 125(Pt 24), 6049–6057. doi:10.1242/jcs.111872 [PubMed: 23038782]

Zhang F, Fan Q, Ren K, & Andreassen PR (2009). PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2. Mol Cancer Res, 7(7), 1110–1118. doi:1541-7786.MCR-09-0123 [pii] 10.1158/1541-7786.MCR-09-0123 [PubMed: 19584259]

Zhang F, Ma J, Wu J, Ye L, Cai H, Xia B, & Yu X. (2009). PALB2 links BRCA1 and BRCA2 in the DNA-damage response. Curr Biol, 19(6), 524–529. doi:S0960-9822(09)00723-4 [pii] 10.1016/j.cub.2009.02.018 [PubMed: 19268590]

Zhen DB, Rabe KG, Gallinger S, Syngal S, Schwartz AG, Goggins MG, … Petersen GM (2015). BRCA1, BRCA2, PALB2, and CDKN2A mutations in familial pancreatic cancer: a PACGENE study. Genet Med, 17(7), 569–577. doi:10.1038/gim.2014.153 [PubMed: 25356972]

Zheng Y, Zhang J, Niu Q, Huo D, & Olopade OI (2012). Novel germline PALB2 truncating mutations in African American breast cancer patients. Cancer, 118(5), 1362–1370. doi:10.1002/cncr.26388 [PubMed: 21932393]

Zhu H, Wei M, Xu J, Hua J, Liang C, Meng Q, … Shi S. (2020). PARP inhibitors in pancreatic cancer: molecular mechanisms and clinical applications. Mol Cancer, 19(1), 49. doi:10.1186/s12943-020-01167-9 [PubMed: 32122376]

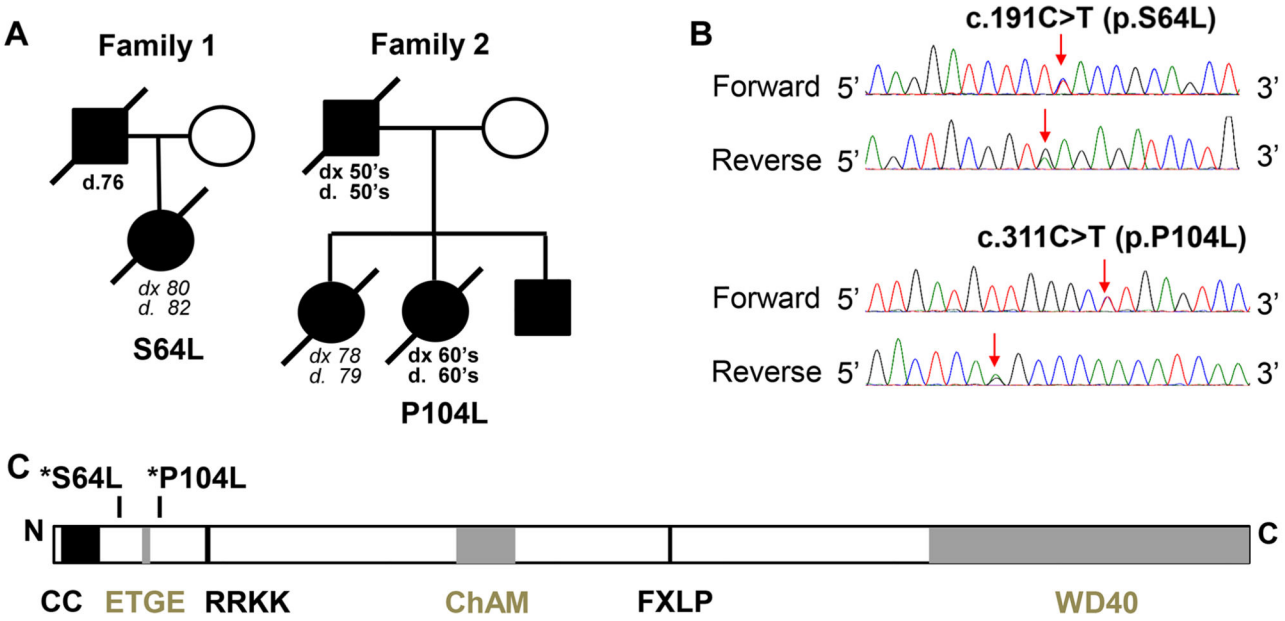

Figure 1. Identification of the Ser64Leu and Pro104Leu variants of *PALB2* in different families with inherited pancreatic cancer.

(A) In Family 1, the father and the proband but not the mother were affected by pancreatic cancer (filled symbols). In Family 2, the father, brother, sister and proband, but not the mother, were affected by pancreatic cancer (filled symbols). A diagonal line indicates that the individual is deceased. Where available, ages at diagnosis with pancreatic cancer (dx) and/or at death (d.) are listed for each individual. Only the probands underwent *PALB2* sequencing, as is indicated by italicization of the diagnosis and/or death information for these individuals. (B) The probands in Family 1 and Family 2 were heterozygous for *PALB2* c.191C>T (p.Ser64Leu, referred to S64L in figures) and *PALB2* c.311C>T (p.Pro104Leu, referred to here as P104L), respectively, detected by full gene sequencing of *PALB2* from peripheral blood. (C) The p.Ser64Leu and p.Pro104Leu missense variants are positioned outside known functional domains in the N-terminus of PALB2, including the coiled-coil motif (a.a. 9–44), the KEAP1-binding motif (a.a. 88–94), and the RRKK DNA binding motif (a.a. 146–149). Other domains elsewhere in PALB2 are the ChAM motif (a.a. 395–446), a MRG15-binding FXLP motif (a.a. 612–615) and a WD40 domain (a.a. 867–1186).

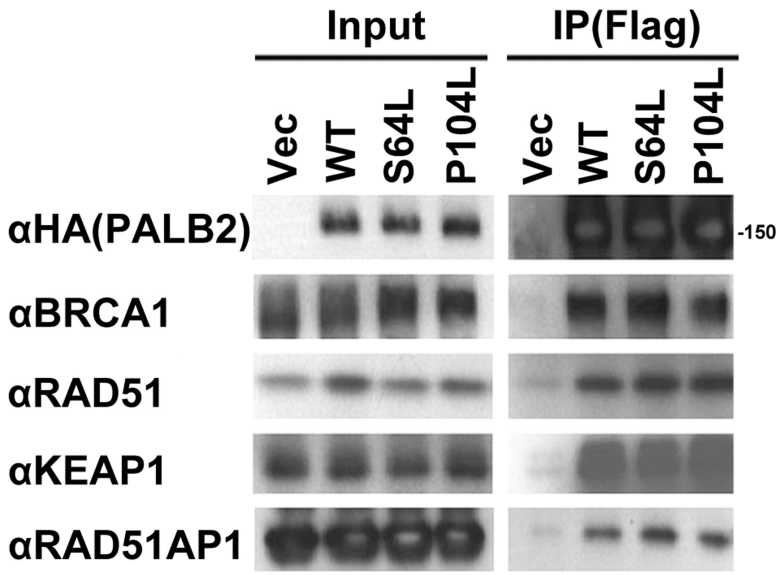

Figure 2. The p.Ser64Leu and p.Pro104Leu PALB2 variants have no clear effect on N-terminal PALB2 interactions.

WT PALB2, or the p.Ser64Leu (S64L) and p.Pro104Leu (P104L) variant forms of PALB2, were stably expressed in untreated *PALB2*-deficient EUFA1341 cells from a Fanconi anemia patient along with a Flag-HA epitope tag. Immunoblots of extracts utilized in immunoprecipitation assays (Input, left) display the levels of PALB2 and interacting proteins, while interactions were detected by immunoprecipitation of PALB2 using Flag antibody and immunoblotting with the indicated antibodies (IP, right). The molecular masses of RAD51, RAD51AP1, KEAP1 and BRCA1 are approximately 37 kD, 38 kD, 70 kD and 210 kD, respectively.

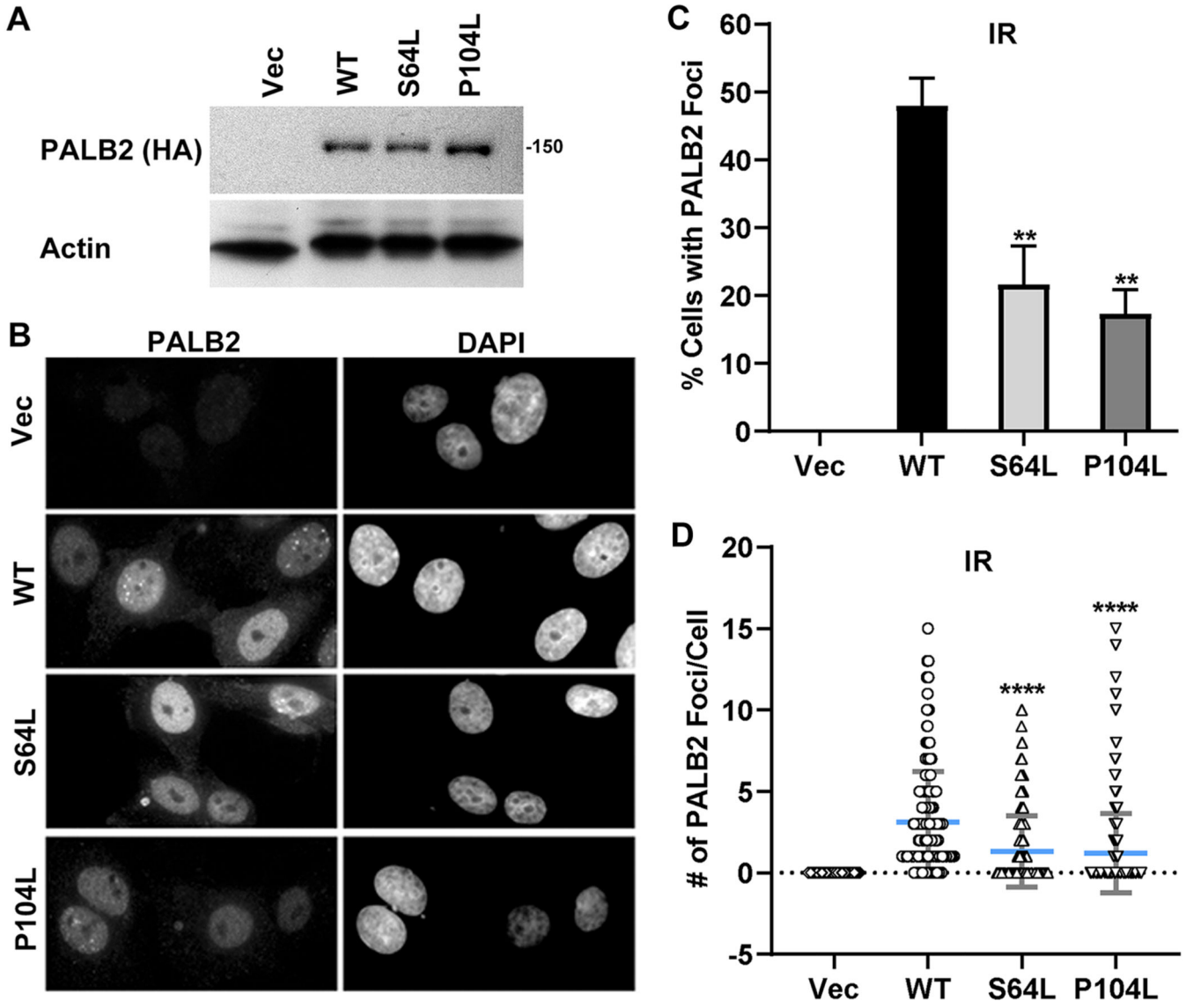

Figure 3. Both the p.Ser64Leu and p.Pro104Leu variants of PALB2 display deficient recruitment to nuclear foci.

(A) An immunoblot demonstrating levels of the WT, p.Ser64Leu (S64L) and p.Pro104Leu (P104L) forms of PALB2 expressed along with a N-terminal Flag-HA tag in *PALB2*-deficient EUFA1341 cells from a FA patient. Actin (42 kDa) is shown as a loading control. (B) Representative images of PALB2 localization in EUFA1341 cells reconstituted with different forms of PALB2, along with a Flag-HA epitope tag, 16 hr after treatment with 10 Gy IR. (C-D) Quantification of the percentage of cells with 3 or more nuclear PALB2 foci (C) and the mean number of nuclear PALB2 foci (D) in cells expressing exogenous PALB2, as detected by the presence of HA signal utilizing immunofluorescence microscopy 16 hr after exposure to 10 Gy IR. The mean ± standard deviation of three counts of at least 150 cells each is shown for each value in C; the mean number of foci (blue) ± standard deviation (grey) per cell, based upon counts of 300 cells expressing exogenous PALB2, is shown in D. Levels of PALB2 foci conferred by the p.Ser64Leu and p.Pro104Leu variants of PALB2

were significantly different than those associated with WT PALB2 or the empty vector (Vec) both with or wihout exposure to IR (**p<0.01; ****p<0.0001).

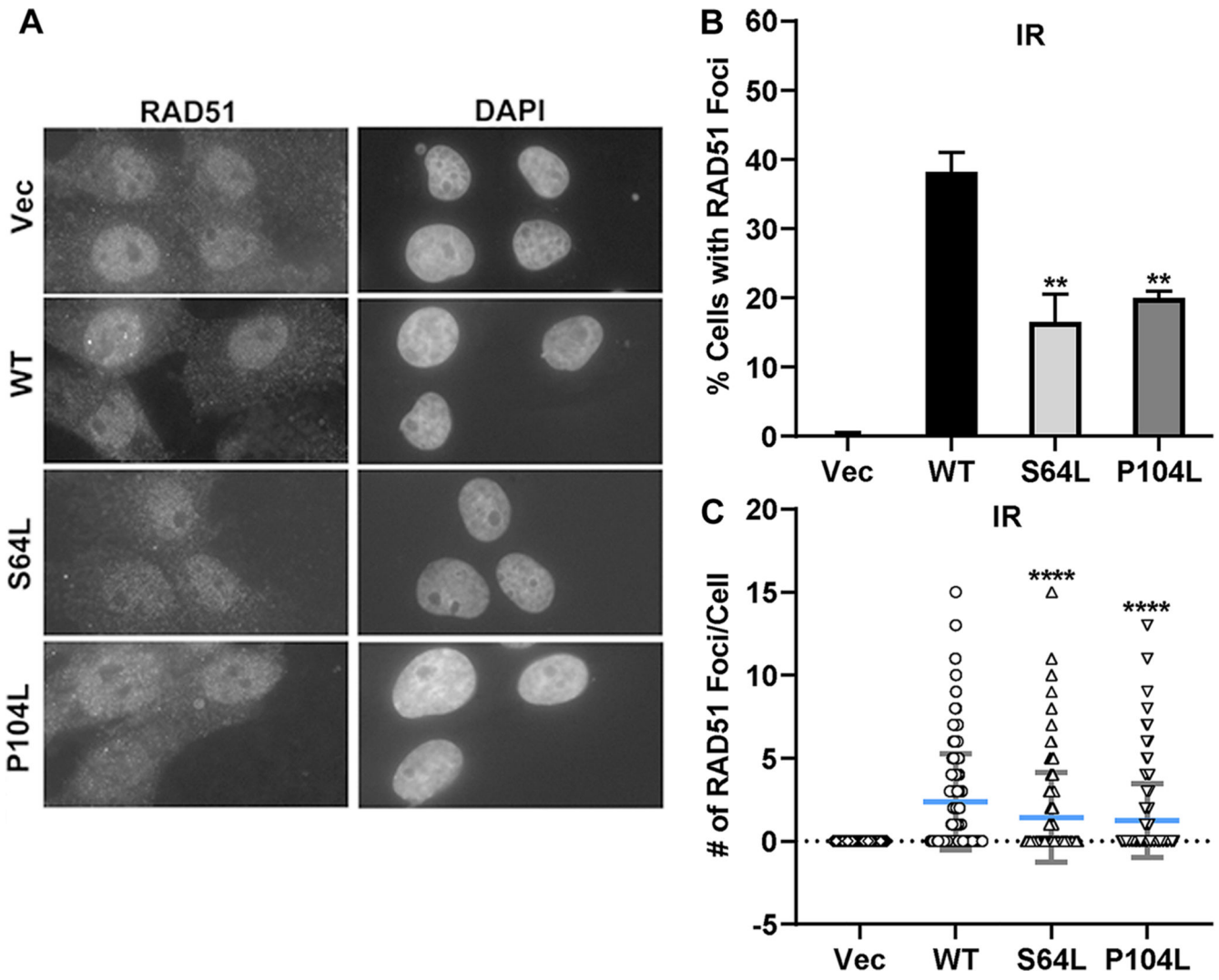

Figure 4. Both the p.Ser64Leu and p.Pro104Leu variants of PALB2 display deficient PALB2-dependent recruitment of RAD51 to nuclear foci.

(A) Representative images of RAD51 foci in EUFA1341 cells reconstituted with different forms of PALB2, along with a Flag-HA epitope tag, 16 hr after treatment with 10 Gy IR. (B) Quantification of the percentage of cells with 3 or more nuclear RAD51 foci in cells expressing exogenous PALB2, as detected by immunofluorescence microscopy, 16 hr after exposure to 10 Gy IR. The mean ± standard deviation of three or more counts of at least 150 cells each is shown for each value. (C) Quantification of the number of nuclear RAD51 foci in each of 300 cells that exogenously express each form of PALB2, shown as the mean (blue) ± standard deviation (grey). Levels of RAD51 foci in cells reconstituted with the p.Ser64Leu (S64L) and p.Pro104Leu (P104L) PALB2 variants were significantly different than those conferred by WT PALB2 and the empty vector (Vec) (**p<0.01; ****p<0.0001).

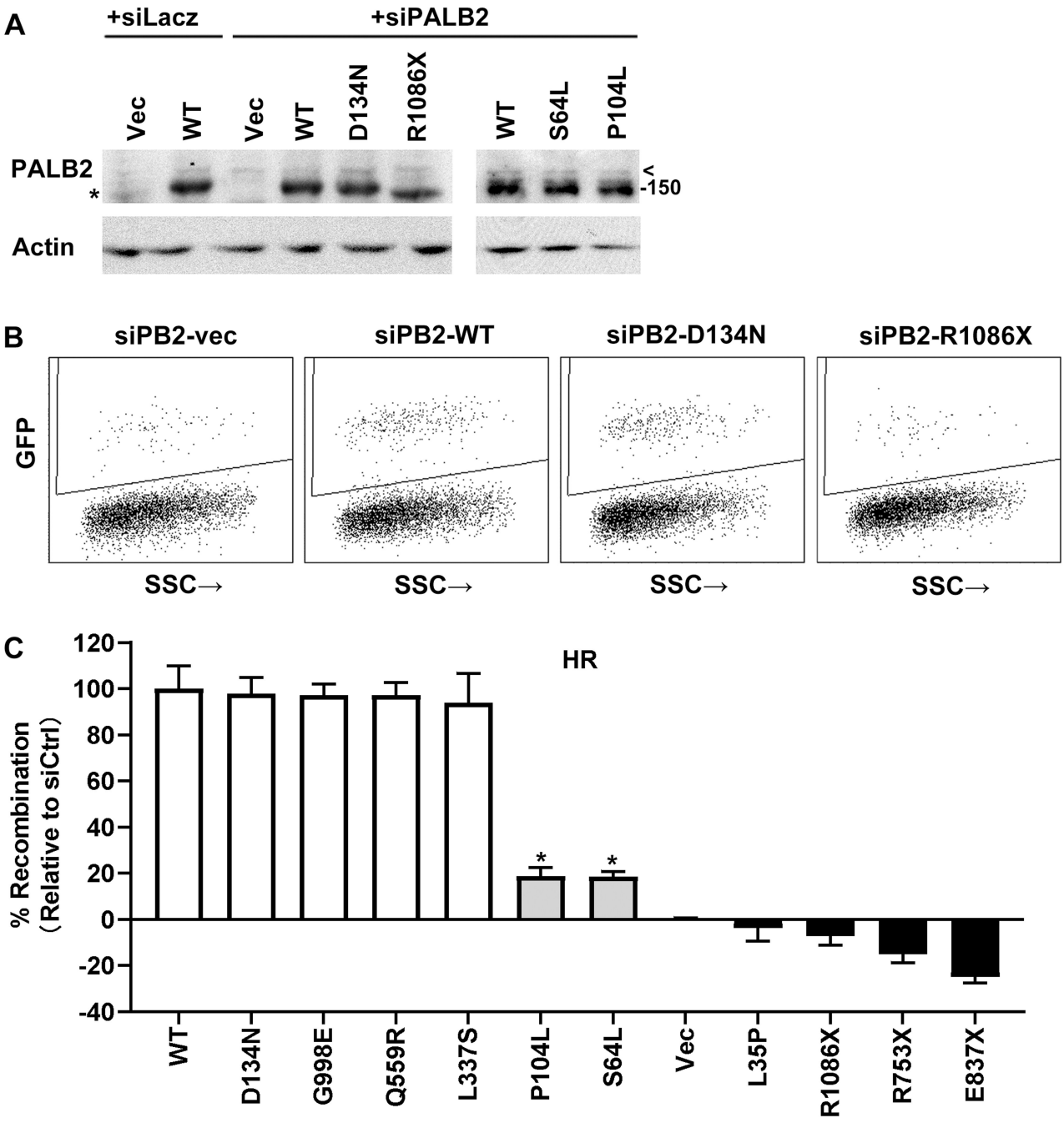

Figure 5. Both the p.Ser64Leu and p.Pro104Leu variants of PALB2 are deficient for homologous recombination.

(A) Immunoblots displaying levels of PALB2 in U2OS-DR cells with knockdown of PALB2 (siPALB2) or transfected with a control siRNA (siLacZ), and expressing different siPALB2-resistant forms of PALB2, including the p.Ser64Leu (S64L) and p.Pro104Leu (P104L) variants, along with a N-terminal Flag-HA epitope tag. Actin is shown as a loading control. A non-specific band present in this overexposed PALB2 blot is indicated (<), and endogenous PALB2 present in U2OS-DR cells containing empty vector and transfected with siLacZ is also indicated (*). (B) Representative dot plots for HR, indicated by increased

levels of GFP, in boxes, plotted versus side-scatter (SSC) for U2OS-DR cells depleted of endogenous PALB2 using siPALB2 and reconstituted with WT PALB2, the p.Asp134Asn and p.Arg1086Ter variants of PALB2, or the empty vector, and transfected with the I-SceI endonuclease to induce DSBs. (C) Quantification of HR for cells depleted of endogenous PALB2 and reconstituted with the p.Ser64Leu and p.Pro104Leu variants identified in familial pancreatic cancer patients (grey bars), WT PALB2 or 4 B/LB missense variants [p.Asp134Asn (D134N), p.Leu337Ser (L337S), p.Gln559Arg (Q559R) and p.Gly998Glu (G998E)] (white bars), or empty vector or 3 pathogenic/likely pathogenic variants [p.Arg753Ter (R753X), p.Glu837Ter (E837X) and p.Arg1086Ter (R1086X)] or a LOF missense variant p.Leu35Pro (L35P) [black bars]. The mean ± standard deviation from three replicates was determined for each form of PALB2 or the empty vector following depletion of endogenous PALB2 in cells transfected with the I-SCEI endonuclease. Values for each form of PALB2 are shown relative to the values for cells reconstituted with that form of PALB2, but not depleted of endogenous PALB2 following transfection with siLacZ, and then normalized to the mean value for WT PALB2. The values for the empty vector and WT PALB2 were set to 0% and 100%, respectively, and all other values are shown relative to this scale. Levels of HR conferred by the p.Ser64Leu and p.Pro104Leu variants were significantly different than those associated with WT PALB2 and benign variants, and from the empty vector and pathogenic-LOF variants (*$p<0.02$).

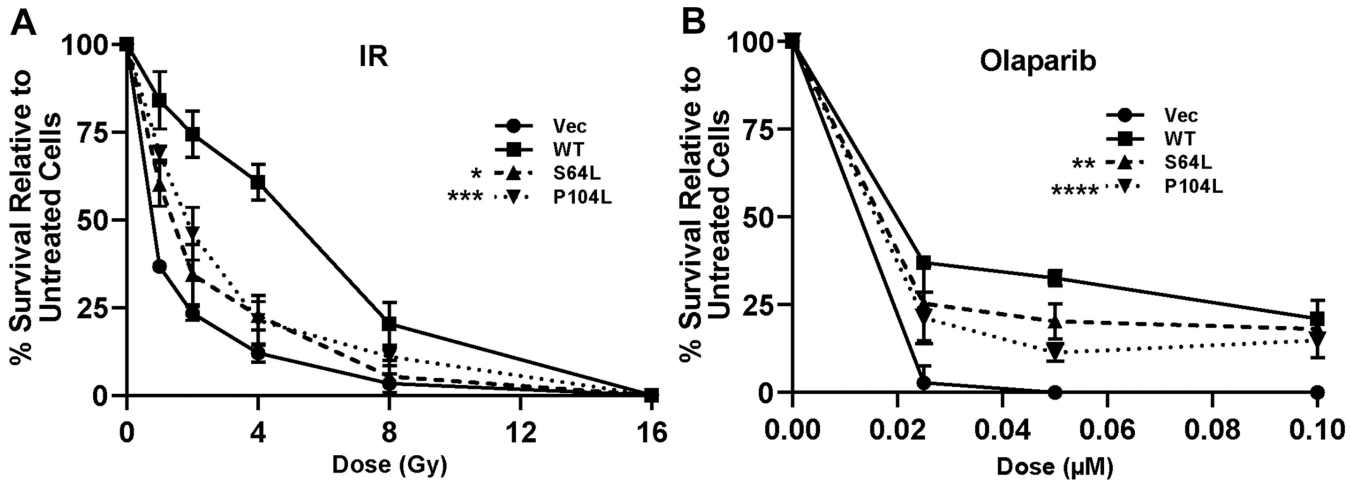

Figure 6. Both the p.Ser64Leu and p.Pro104Leu variants of PALB2 are defective for cellular resistance to ionizing radiation and the PARP inhibitor olaparib.

(A) *PALB2*-deficient EUFA1341 cells reconstituted with WT PALB2, the p.Ser64Leu (S64L) and p.Pro104Leu (P104L) variant forms of PALB2, or the empty vector alone, were tested for cellular resistance to IR using a colony growth assay. (B) *PALB2*-deficient EUFA1341 cells reconstituted with WT PALB2, the p.Ser64Leu and p.Pro104Leu variant forms of PALB2, or the empty vector alone, were tested for cellular resistance to olaparib using a colony growth assay. Each value represents the mean ± standard deviation of three replicates. Survival for p.Ser64Leu and p.Pro104Leu were significantly different than for WT PALB2 and the empty vector following treatment with either IR or olaparib (*$p<0.03$; **$p<0.007$; ***$p<0.0002$; ****$p<0.0001$).