Analysis of *PALB2*/*FANCN*-associated breast cancer families

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No more than ≈30% of hereditary breast cancer has been accounted for by mutations in known genes. Most of these genes, such as *BRCA1*, *BRCA2*, *TP53*, *CHEK2*, *ATM*, and *FANCJ/BRIP1*, function in DNA repair, raising the possibility that germ line mutations in other genes that contribute to this process also predispose to breast cancer. Given its close relationship with *BRCA2*, *PALB2* was sequenced in affected probands from 68 *BRCA1/BRCA2*-negative breast cancer families of Ashkenazi Jewish, French Canadian, or mixed ethnic descent. The average BRCAPRO score was 0.58. A truncating mutation (229delT) was identified in one family with a strong history of breast cancer (seven breast cancers in three female mutation carriers). This mutation and its associated breast cancers were characterized with another recently reported but unstudied mutation (2521delA) that is also associated with a strong family history of breast cancer. There was no loss of heterozygosity in tumors with either mutation. Moreover, comparative genomic hybridization analysis showed major similarities to that of *BRCA2* tumors but with some notable differences, especially loss of 18q, a change that was previously unknown in *BRCA2* tumors and less common in sporadic breast cancer. This study supports recent observations that *PALB2* mutations are present, albeit not frequently, in breast cancer families. The apparently high penetrance noted in this study suggests that at least some *PALB2* mutations are associated with a substantially increased risk for the disease.

DNA repair | FANCN | Fanconi anemia | hereditary predisposition

The presence of a family history is the most important predisposing factor for development of breast cancer. Among the genes known to be linked to familial breast cancer, *BRCA1*, *BRCA2*, *CHK2*, *TP53*, and *ATM* all participate in DNA damage responses (1), suggesting that familial breast cancer is, at least partly, a consequence of impaired genome stability control. PALB2 is a recently identified BRCA2-interacting protein, and a high fraction of each protein interacts with the other (2). Their association is essential for BRCA2 anchorage to nuclear structures and for its function in double strand break repair (DSBR) by homologous recombination (HR). Furthermore, introduction of PALB2 siRNAs sensitized cells to mitomycin C like BRCA2 siRNA (2). PALB2-depleted cells, therefore, display a Fanconi anemia (FA)/BRCA2-deficient phenotype (3).

Recent evidence shows that *PALB2* is, in fact, another FA gene (known as *FANCN*), and that *FANCN* disease resembles FA arising from biallelic *BRCA2* mutations in that the affected children are prone to develop embryonal tumors (medulloblastoma, Wilms tumor) and experience early bone marrow failure (4, 5). In other respects, FA-N cases have a typical FA phenotype. Their cells reveal increased chromosome breakage after interstrand cross-linking agent exposure, and these patients reveal growth retardation and various congenital malformations (4, 5). It is unclear why a different cancer predisposition phenotype exists in FA caused by biallelic *BRCA2*/*FANCD1* and *PALB2*/*FANCN* mutations.

In view of the close functional relationship between PALB2/FANCN and BRCA2 and the similar phenotypes associated with biallelic mutations in either of these two genes, it was conceivable that monoallelic *PALB2*/*FANCN* mutations, like those of *BRCA2*, predispose to adult cancer and that *PALB2* mutations account for a proportion of *BRCA1*/*BRCA2*-negative hereditary breast and ovarian cancer families. This has been demonstrated by two very recent studies. Rahman *et al.* (6) identified five different monoallelic *PALB2* truncating mutations in 10 women from a series of 923 individuals with familial breast cancer and estimated that these mutations confer a 2.3-fold increased risk of breast cancer (95% confidence interval 1.4–3.9). At the same time, a founder *PALB2* mutation in Finland has been identified and appears to be associated with a ≈4-fold increased risk (7).

In Montreal, most inhabitants are French Canadian (FC), but there is also a large Ashkenazi Jewish (AJ) population. Both of these groups are affected by founder mutations in the *BRCA1* and *BRCA2* genes. ≈2.5% of individuals of AJ descent harbor one of the three *BRCA1*/*BRCA2* founder mutations which account for 97.5% of all *BRCA1*/*BRCA2* mutations in this ethnic group (8). Five *BRCA1*/*BRCA2* founder mutations have been described in individuals of FC descent. They account for 84% of all *BRCA1*/*BRCA2* mutations in this group (9).

In addition to screening families from nonspecific ethnic backgrounds, we performed sequence analysis of *PALB2* in AJ and FC families in search of possible founder mutations in these popula-

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Abbreviations: aCGH, microarray-based comparative genomic hybridization; AJ, Ashkenazi Jewish; AWS, adaptive weights smoothing; CGH, comparative genomic hybridization; DSBR, double strand break repair; FA, Fanconi anemia; FC, French Canadian; HR, homologous recombination; LOH, loss of heterozygosity.

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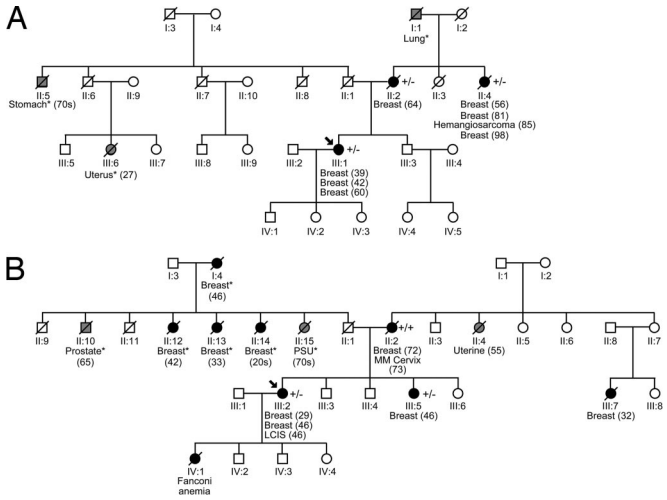

Fig. 1. Pedigrees of the two studied families with *PALB2* mutations. MM, malignant melanoma; PSU, primary site unknown; *, unconfirmed cancer diagnosis. (*A*) Pedigree A. (*B*) Pedigree B. In Family B, the mutation was not found in tumor samples from the proband's mother (BII.2), indicating that the mutation was inherited from the paternal side.

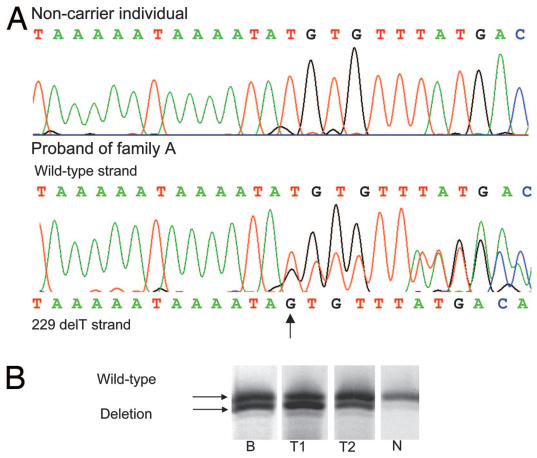

Fig. 2. Sequence chromatogram showing WT exon 4 *PALB2* sequence with the frameshift due to 229delT in the proband of Family A. The WT and mutant strand are indicated. The arrow points to where the T is deleted on the mutant strand. (*A*) LOH studies in *PALB2* 2521delA-related breast cancers. (*B*) A 115-bp region including the mutation *PALB2:*2521delA was amplified by PCR from DNA extracted from patient III:2 (Fig. 1*B*). Genotypes from this patient's blood (*B*) and macrodissected formalin-fixed tumor (T1, right breast tumor, diagnosed age 29; T2, left breast tumor, diagnosed age 46) and from an individual who does not carry the mutation (N) are shown. The larger WT band can be observed in all samples, whereas the fragment amplified from the mutated DNA strand (1 bp smaller) can be observed in the patient's blood and tumors but not in the noncarrier. There is no significant LOH of either the WT or mutant allele in the patient's tumors compared with the blood. A faint ''shadow'' band of the WT allele is present below the WT band in the mutation-negative control, and this band also appears at the same position as the mutant allele in B, T1, and T2. Tumors from AIII:1 and BIII:5 were also analyzed, and no LOH was detected (data not shown).

tions. We also screened *PALB2* in probands affected with prostate cancer, another cancer type associated with *BRCA2* mutations.

Results

Mutation Analysis. Sequencing of *PALB2* in the proband of a family of Scottish descent identified a frameshift mutation, 229delT, which is predicted to generate a stop 99 codons downstream from the mutation. The pedigree for this family, called Family A, is given in Fig. 1*A*, and the details on cancers that have been confirmed are given in Table 1. The proband, III.1, developed three breast cancers, at ages 39, 42, and 60. Her mother had breast cancer at 64, and her maternal aunt had three breast cancers at 56, 81, and 98 and a sarcoma at 85. DNA from the proband was negative for *BRCA1*/*BRCA2* mutations by full sequencing and deletion analysis (Myriad Genetics, Salt Lake City, UT). Additional screening for *CHEK2:*1100delC (10) was negative. The *PALB2* mutation (shown in Fig. 2*A*) was also found in her aunt (AII.4), and, therefore, the proband's mother (AII.2) was an obligate carrier.

No clearly pathogenic mutations were identified in any of the other 67 strong family-history breast cancer probands sequenced [average score of 0.58, using BRCAPRO (CancerGene 4.3.1, University of Texas Southwestern Medical Center, Dallas, TX)], in the FC moderate family history series or in the familial prostate cancer cases. A number of *PALB2* sequence variants were identified, all of which have been reported in ref. 6. The variant frequencies were similar to those already reported (6), and common variants (frequency >1%), such as Q559R, E672Q, G998E, and T1100T, were present in all three groups tested, with the exception of L337S, which was not seen in the FC population. These data reduce the likelihood that a significant fraction of non-*BRCA1*/*BRCA2* familial breast/ovarian cancer in either the AJ or FC population is due to common founder mutations in *PALB2*.

Table 1. Details of cancers in families A and B

Individual	Tumor, no.	Age, yrs	Cancer Type	Grade	IHC
AIII.1	T1	39	IDC	N/A	ER+,PR+
	T2	42	IDC	3*	ER+,PR+
	T3†	60	IDC	2‡	ER+, PR−, HER2−
AII.2	T1	64	IDC	N/A	N/A
AII.4	T1	56	Type unknown	N/A	N/A
	T2	81	IDC	2*	ER+, PR+
	T3	85	Hemangiosarcoma		
	T4†	98	IDC	3‡	ER+, PR−, HER2+§
BIII.2	T1†	29	IDC	2‡	ER+, PR+
	T2†	46	ILC and LCIS	2‡	ER+, PR+
BIII.5	T1	46	IDC	2‡	ER+, PR+

IHC, immunohistochemistry; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; LCIS, lobular carcinoma *in situ*; N/A, not available.
*Nuclear grade.
†Tumor used in LOH and aCGH studies.
‡Histological grade.
§HER2+IHC score of 2 on a scale of 0–3.

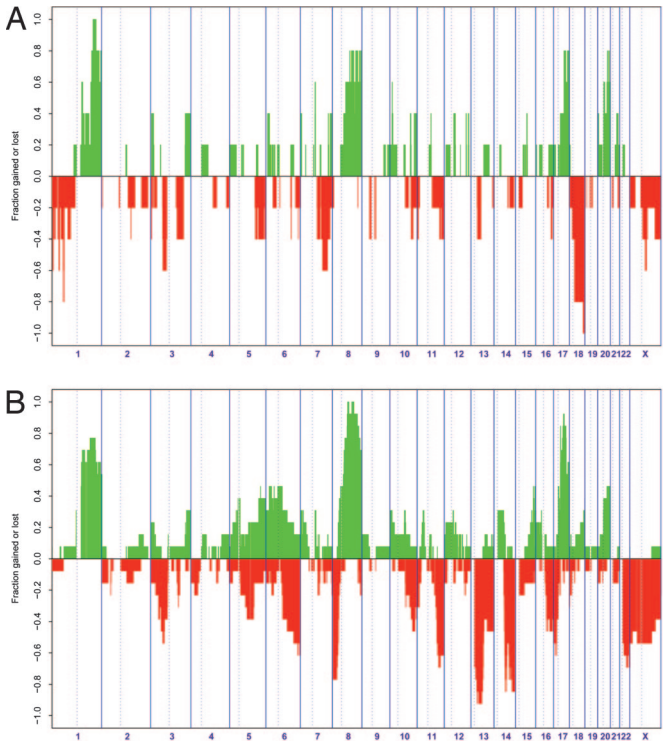

Fig. 3. Frequency of DNA copy number changes in tumors arising in *PALB2* and *BRCA2* germline mutation carriers. (*A*) PALB2 cases [invasive breast cancer ($n = 4$) and lobular carcinoma *in situ* ($n = 1$)]. (*B*) BRCA2 tumors ($n = 13$). Individual BAC clones are plotted according to genomic location along the *x* axis. The proportion of tumors in which each clone is gained (green bars) or lost (red bars) is plotted along the *y* axis. Vertical dotted lines represent chromosome centromeres.

Tumor Characteristics. Tissue was available from four breast cancers and one coexisting area of lobular carcinoma *in situ* from three women with germline *PALB2* mutations. Two tumors originated from the family identified here, and two tumors (one with coexisting lobular carcinoma *in situ*) came from a member of a second family, herewith called Family B (see Fig. 1*B* for pedigree) in which a truncating *PALB2* mutation (2521delA) was identified through ascertainment of FA cases (4). This individual developed breast cancer at the ages of 29 and 46, and this patient's DNA was negative for *BRCA1*/*BRCA2* mutations by full sequencing (Myriad Genetics, Salt Lake City, UT), deletion analysis by quantitative PCR, promoter sequencing, and RNA analysis (to identify mutations that might affect splicing). In addition, genes encoding the *BRCA2*-interacting proteins, RAD51 and DSS1, were sequenced in this family. No pathogenic variants were identified in any of these four genes, and screening for *CHEK2:*1100delC was also negative.

We also investigated certain characteristics of *PALB2*-related breast cancers. The clinical phenotype resembled *BRCA2*-tumors in that they were predominantly ER+, PR+. Moreover, no loss of heterozygosity (LOH) of *PALB2* was seen in any of the four tumors studied (Fig. 2*B* and data not shown). In addition, none of the tumors showed definite evidence of chromosome gains or losses by aCGH [microarray-based comparative genomic hybridization (CGH)] at the *PALB2* locus on 16p12.1 [supporting information (SI) Tables 3 and 4]. All of the breast cancers including the lobular carcinoma *in situ* shared common deletions at 18q. Based on the analysis of the four invasive tumors, only 1q gain, 20q gain, and 18q loss are consistently observed across all of these samples (SI Tables 3 and 4). Of these consistent changes, only the 18q loss was not observed in the analysis of *BRCA2*-related tumors (Fig. 3). Chromosome 1p, 7q, and Xq deletions were also seen in some *PALB2* tumors, and of these aberrations, 7q loss is infrequent in *BRCA1*/*BRCA2* or sporadic cancers. It is also notable that we did not observe loss of 8p, a chromosomal arm that is commonly deleted in *BRCA1*/*BRCA2* and sporadic tumors, or loss of the 17q or 13q loci that contain *BRCA1* and *BRCA2*, respectively. In other respects, such as gain of 1q, 8q, 17q, and 20q, the CGH profile of these tumors resembled that of *BRCA2*-related breast cancers.

Functional Characterization of the Two PALB2 Mutant Proteins. The 229delT mutation generates a fusion protein (C77fs) that retains only 76 residues of native PALB2 sequence but has an unusually long tail of 99 residues. Yet the predicted coiled-coil motif, which mediates protein-protein interactions for some other polypeptides, is still retained (Fig. 4*A*), implying that this dramatically shortened protein may still interact with certain PALB2 partners. The 2521delA mutation results in a much longer protein (T841fs), but all four of the predicted WD40 repeats are deleted (Fig. 4*A*). Because WD40 repeats are also common protein–protein interacting motifs, T841fs has presumably lost the binding site for at least some PALB2 partners.

We introduced these two mutations into *PALB2*− expressing vectors with FLAG-HA double tags, and asked whether the truncated proteins could bind BRCA2 and function in DNA repair. As shown in Fig. 4*B*, both C77fs and T841fs retained only minimal BRCA2 binding capacity, implying that the C terminus of the protein, which contains the predicted WD-40 domains, is required for PALB2–BRCA2 interaction. The abundance of endogenous BRCA2 was not affected following transient overexpression of either of these mutant proteins (data not shown). In this setting, by comparison with WT, we succeeded in expressing T841fs at a similar level, while the expression of C77fs was clearly lower, suggesting that the fusion protein is less stable than WT or T841fs. Consistent with their failure to bind BRCA2, both proteins were found to be defective in HR/DSBR (Fig. 4*C*) and in the repair of mitomycin C-induced interstrand cross-links (Fig. 4*D*).

Given the strong family histories of breast cancer associated with the two above-noted mutations and the lack of LOH in both cases, it is possible that the truncated and fused proteins perturb normal PALB2–BRCA2 function in HR/DSBR. To address this possibility, we overexpressed C77fs or T841fs in DR-U2OS cells, either transiently or stably, and tested their HR efficiency. No significant defects were observed (data not shown).

Discussion

The data presented here confirm that mutations in *PALB2* are implicated in breast cancer predisposition. Here, we further characterized the *PALB2* breast cancer phenotype in two significant aspects.

First, our results are consistent with the notion that some *PALB2* mutations are associated with a relative risk for breast cancer that is greater than 2.3 (6). The five different monoallelic mutations identified in the familial breast cancer study by Rahman *et al.* (6) were all localized at the 3′ end of the gene, and none of the family histories of cancer were particularly strong. The median age of diagnosis of breast cancer was 46 years, and there was no preponderance of bilateral breast cancer compared with families without mutations. The 229delT sequence variant described here is the most 5′ deleterious mutation observed to date, and the strong breast cancer history associated with this mutation suggests that there may be a genotype-phenotype correlation. In this respect it is of interest that Erkko *et al.* (7) estimate the relative risk of breast cancer associated with Finnish founder mutation (1592delT) to be ≈4-fold increased. Clearly, more data on penetrance in other *PALB2* families are needed before the clinical implications of mutations in this gene are fully apparent. With respect to the importance of *PALB2* mutations to the burden of breast cancer, it is notable that the *PALB2* gene lies on 16p12.1, a region that is not particularly associated with linkage in hereditary breast cancer families (11).

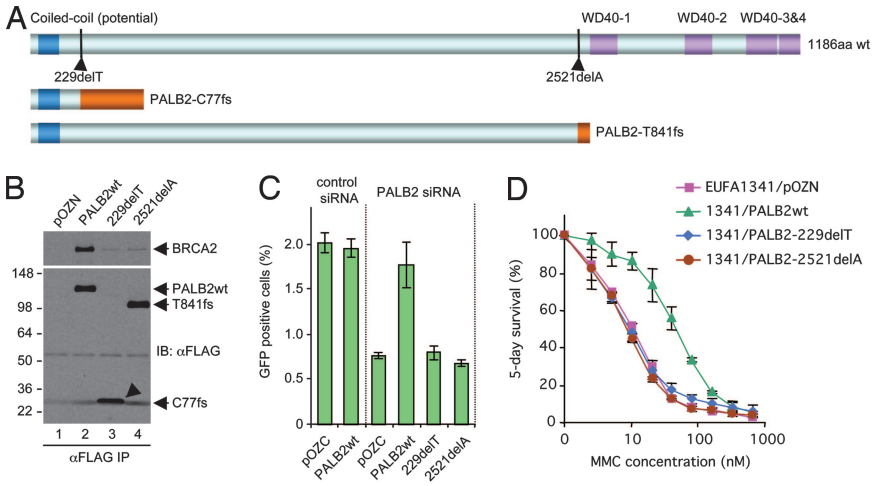

Fig. 4. PALB2 protein structure and assessment of functional consequences of the 229delT and 2521delA mutations. (*A*) Schematic diagram of the protein showing predicted functional domains and the sites of the two truncating mutations studied. The extended reading frames after frameshifts are shown in orange. (*B*) 293T cells were transfected with the indicated plasmids, and N-terminally FLAG-HA-double tagged PALB2 proteins were precipitated with anti-FLAG M2 agarose beads. The abundance of tagged PALB2 proteins and BRCA2 in the precipitates was analyzed by Western blot. (*C*) DR-U2OS HR reporter cells were treated with control or PALB2 siRNAs and then cotransfected with pCBASce together with the pOZC or pOZC-PALB2 vectors, which contain seven silent base changes and are resistant to the siRNA used. GFP-positive cells were counted 72 h later. Data presented is from a representative experiment performed in duplicate. (*D*) Mitomycin C sensitivity of the EUFA1341 (FA-N;PALB2$^{-/-}$) fibroblasts stably expressing the indicated PALB2 species. Results shown are the averages of three independent experiments, each performed in duplicate.

The data presented here indicates that, outside of certain specific ethnic populations, *PALB2* is responsible for a modest proportion of hereditary breast cancer cases (1.5%) and that *PALB2* founder mutations in the AJ or FC populations are unlikely.

Second, analysis of *PALB2*-related breast cancers showed that the tumor characteristics were clearly different from *BRCA1*-related tumors. The tumors revealed some similarities to, but also some differences from, *BRCA2*-related cancers. No consistent LOH of *PALB2* was detected, in keeping with the observations of Erkko *et al.* (7), and CGH showed the presence of alterations such as +8q, +20q, +17q, −13q, and −6q that are all overrepresented in *BRCA2*-related tumors (12, 13). By contrast, all four tumors from the three patients analyzed revealed distal 18q loss, and losses at 1p and 7q were also frequent compared with *BRCA2*-related tumors. The similarities of the clinical, LOH and CGH findings of the *PALB2*-breast cancers studied are all of the more remarkable given the large range of age at diagnosis (29–98 years) in these cases. Even though only a small number of *PALB2*-related tumors were analyzed, the consistent loss of 18q in particular is notable, given that it is rare in *BRCA2*-related breast cancer (12, 13). LOH (14) and CGH studies (15, 16) suggest that 18q losses occur in at most 25–30% of sporadic breast cancers, and that this loss may be associated with a more aggressive phenotype (17, 18). It is possible that the observed phenotype of *PALB2*- related breast cancers reflects a particular carcinogenesis pathway that requires 18q loss. Indeed, if, in a larger series of cases, del18q remains a common characteristic of *PALB2*- but not of *BRCA2*-related breast cancers, then one could argue that, despite the dependence of BRCA2 upon PALB2 function, *PALB2*-related breast carcinomas are not necessarily pure *BRCA2*$^{-/-}$ breast cancer phenocopies.

Because no LOH was detected in the tumors, alternative mechanisms of PALB2 functional inactivation were investigated. C77fs, the product of 229delT, is a remarkable frameshift fusion protein in that it contains an unusually long "alien" tail, leading to the hypothesis that it can act in a dominant negative fashion or is oncogenic. However, it did not act in a dominant negative fashion in the HR assay, whether transiently or stably expressed (data not shown). Despite the lack of LOH, the possibility of a somatic mutation occurring on the retained allele has not been excluded. Another possibility is that *PALB2* is a haploinsufficient tumor suppressor. Conceivably, the close relationship between PALB2 and BRCA2 is particularly sensitive to dosage effects, so reduced amounts of PALB2 affect BRCA2 function sufficiently to cause breast cancer, but only complete absence of PALB2 causes FA. In this respect, it is worth noting that all mutations identified in FA cases, thus far, have been truncating (4, 5).

It remains unclear how heterozygous truncating *PALB2* mutations promote breast cancer development. However, although as yet unproven, it seems unlikely that the mechanism requires a major dominant-negative effect by the truncated, mutant product on DNA cross link repair or HR. The presence of 18q deletions in the absence of LOH at *PALB2* could indicate that a gene on 18q provides a missing functional link that will help in understanding the specific pathogenesis of *PALB2*-related breast cancers. The identification and characterization of additional *PALB2* mutations and the associated tumors, together with some insight into how PALB2 operates biochemically, may help to resolve this mystery.

Materials and Methods

Patients. DNA extraction from blood of affected probands from 26 AJ, 22 FC, and 20 mixed ethnicity families was undertaken by using standard methods. All probands apart from one were female, the exception being a male with bilateral breast cancer, and all gave consent to take part in the study. The probands had BRCAPRO scores (19, 20) higher than 0.10. DNA analysis had shown that AJ probands were negative for the three AJ *BRCA1*/*BRCA2* founder mutations (*BRCA1* 187delAG, 5385insC; *BRCA2* 6174delT). DNA from FC and all nonethnic specific probands that were negative for known FC founder mutations underwent full *BRCA1*/*BRCA2* sequencing (Myriad Genetics). Average BRCAPRO scores for each group are given in Table 2. We screened another 16 affected FC women with weaker breast cancer histories (average BRCAPRO score 0.04). Selection criteria and characteristics of the FC families have been described in refs. 9 and 21. We also screened 35 prostate cancer cases (14 AJ and 21 FC) who had a family history of cancer (defined as two or more affected cases) and who were previously screened for the AJ or FC *BRCA1*/*BRCA2* founder mutations. Therefore, we performed *PALB2* mutation analysis on a total of 119 affected probands: 68 cases from FC, AJ and mixed descent families with a strong family history of breast cancer; 16 FC cases

Table 2. Clinical details of families used in the study

Group	Families, no.	BRCAPRO Mean	BRCAPRO Range	Cases with BRCAPRO scores >0.50, no.
Mixed	20	0.68	0.11–0.99	17
AJ	26	0.50	0.11–0.99	16
FC (strong)	22	0.59	0.11–0.99	14
FC (weak)	16	0.04	0.01–0.09	Not applicable

The families in the "mixed" ethnicity group were as follows: British (12), Italian (3), Jamaican (2), Lebanese, Filipino, and Sephardic Jewish.

with a weak family history breast cancer; and 35 prostate cancer cases with a family history of prostate cancer.

PALB2 Sequencing. The *PALB2* genomic sequence was obtained from University of California, Santa Cruz Genome Browser (accession no. NM_024675). Intronic primers were designed by using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA). Because of their large sizes, exons 4 and 5 were amplified in 4 and 2 amplicons, respectively; all primer sequences and annealing temperatures are listed in SI Table 5. A BLAST search did not reveal evidence of *PALB2* pseudogenes. The PCR reactions were carried out in 50-μl volume and consisted of 5 μl of 10× PCR buffer, dNTPs (0.24 mM final concentration; Invitrogen Life Technologies, Burlington, ON, Canada), 0.56 μM final concentration of each primer (Invitrogen Life Technologies), and 1 unit of HotStart TaqPlus (Qiagen, Mississauga, ON, Canada). $MgCl_2$ (1 mM final concentration) was present in analyses of exons 4b, 4d, 5a, 5b, 11, and 13, and 10 μl of Q solution (Qiagen) was added to the exon 1 PCR. The PCR products were purified and then sequenced by using 3730XL DNA Analyzer Systems from Applied Biosystems (Foster City, CA). Sequence data were analyzed by using Multiple Sequence Alignment by Clustalw from Kyoto University Bioinformatics Center (Kyoto, Japan), and the chromatograms were viewed with Chromas 2.31 from Technelysium (Helensvale, Australia).

LOH Analysis. Tumor tissue from affected *PALB2* carriers was both macro- and microdissected (using laser capture microdissection) from formalin-fixed paraffin-embedded tissue, and DNA was extracted from the collected cells using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions for formalin-fixed, paraffin-embedded samples. Primers were designed to produce small PCR products spanning the *PALB2* deletion mutations and were end-labeled with γ-P33, using T4 polynucleotide kinase (Invitrogen Life Technologies) in a forward reaction. Labeled primers were then used to generate PCR products from DNA isolated from blood, normal, and, where relevant, tumor tissue from each designated mutation carrier, using the HotStar TaqPCR system (Qiagen) (primer sequences and annealing temperatures are listed in SI Table 5). Products were separated by electrophoresis in a 6% denaturing acrylamide gel for 2 h at 70 watts and then autoradiographed. The relative intensity of the WT and mutant bands in normal and tumor samples was compared with determine LOH status. As supporting evidence, the mutation from each carrier was also sequenced directly from a PCR product in both blood/normal and tumor tissue, and the relative intensities of the peaks in the normal and mutant traces were visually compared with confirm the LOH results whenever possible.

aCGH Analysis. ***DNA extraction.*** Tumor samples were microdissected with a sterile needle under a stereomicroscope as described in ref. 22. Microdissected tumor tissue with >80% of neoplastic cells was subjected to phenol:chloroform extraction and ethanol precipitation according to standard protocols (22). Matched normal DNA was obtained from peripheral blood lymphocytes in four cases and from adjacent normal breast tissue (i.e., inflammatory and stromal cells) in one sample. Tumor and reference DNA samples were subjected to a multiplex PCR predictor for aCGH success as described in ref. 23.

aCGH hybridization. The aCGH platform used for this study was constructed in the Breakthrough Breast Cancer Research Centre and comprises ≈16,000 clones, spaced at ≈100 kb throughout the genome and spotted onto Corning GAPSII-coated glass slides (Corning, New York, NY) (24). Labeling, hybridization, and washes were carried out as described in refs. 22 and 24. Arrays were scanned with a GenePix 4000A scanner (Axon Instruments, Union City, CA); fluorescence data were processed with GenePix 4.1 image analysis software (Axon Instruments) (22, 24).

Data analysis. The log2 ratios were normalized for spatial and intensity-dependent biases, using a two-dimensional loess regression. The median of BAC clone replicate spots was calculated after exclusion of excessively flagged clones (flagged in >20% of samples). The median log2 ratio for each clone was averaged across the replicates ("dye-swaps"). This left a final dataset of 11,636 clones with unambiguous mapping information according to the March 2006 build of the human genome (hg17) for five samples. Data were smoothed by using a local polynomial adaptive weights smoothing (AWS) procedure for regression problems with additive errors (25). Thresholds for defining genomic gains and losses were obtained by using data from unamplified female versus female and female versus male genomic DNA, as described in refs. 22 and 24. A categorical analysis was applied to the BACs after classifying them as representing gain (AWS-smoothed $\log_2$ ratios >0.12), high level gains (AWS-smoothed $\log_2$ ratios >0.36), loss (AWS-smoothed log2 ratios <-0.12), or no-change according to their smoothed log2 ratio values. Data preprocessing (normalization, filtering, and rescaling) and analysis were carried out in R software, Version 2.0.1 (www.r-project.org) and BioConductor 1.5 (www.bioconductor.org), making extensive use of modified versions of the packages, in particular aCGH marray and aws (22, 24). CGH analysis for the *BRCA2*-related breast cancers was performed according to methods described in ref. 23. The aCGH platform used for the analysis of *BRCA2*-related breast cancers contains ≈3,500 clones obtained from the Welcome Trust Sanger Institute (Cambridge, U.K.), spaced at ≈1 Mb throughout the genome and spotted in triplicate on CodeLink Activated Slides (Amersham Biosciences, Piscataway, NJ). Arrays were scanned with a G2505B Microarray Scanner (Agilent Technologies, Palo Alto, CA). Average log2 fluorescent ratios were calculated for each triplicate. Thresholds for gain and losses were defined as described above and in refs. 22 and 24.

Functional Analysis. 293T and DR-U2OS (2) cells were cultured in DMEM supplemented with 10% FBS. Cells were cultivated at 37°C in a humidified incubator in an atmosphere containing 5% CO_2. The retroviral PALB2 cDNA vectors, pOZN-PALB2 and pOZC-PALB2, are described in ref. 2. The mutations, 229delT and 2521delA, were introduced into these vectors by site-directed mutagenesis, using the QuikChange method (Stratagene, La Jolla, CA). Whole-cell extracts for protein analysis and immunoprecipitation were generated by using NETN420 (2). Monoclonal anti-FLAG M2 Ab and M2-agarose beads were purchased from Sigma (St. Louis, MO). The HR/DSBR assay was performed as described in ref. 2. The generation of EUFA1341 (FA-N) fibroblasts stably expressing various PALB2 species and subsequent mitomycin C sensitivity assays were performed as described in ref. 5.

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