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McGill-led team provides new clue to family risk for breast cancer

Discovery could help target drugs to carriers of mutant gene

An international team of researchers led by Dr. William Foulkes of McGill University's Program in Cancer Genetics has linked a particular genetic mutation to increased risk for breast cancer. The discovery, reached after more than a decade of studying two families in Montreal with particularly high rates of breast cancer, is published in this week's Proceedings of the National Academy of Science (PNAS).

Dr. Foulkes and his team, including genetic counselor Nora Wong and Marc Tischkowitz, Assistant Professor and Medical Geneticist at the Jewish General Hospital's Segal Cancer Centre, characterized two mutations in the recently-identified breast cancer susceptibility gene, PALB2, as being associated with a particular breast cancer "signature." The discovery may open new avenues for both identifying PALB2 mutation carriers and for treating women who carry these mutations with drugs that are targeted to components of the novel signature. "The door was already unlocked with the discovery of PALB2," said Dr. Foulkes. "What we've done is rearranged the furniture a bit."

With the help of collaborators at Harvard's Dana Farber Cancer Institute (including Dr. Bing Xia, who first identified PALB2 just over a year ago), Breakthrough Breast Cancer (Institute of Cancer Research, London, UK) and the Netherlands Cancer Institute, Amsterdam, they showed that these two mutations may be associated with a higher-than-expected risk of breast cancer, which could have implications for other PALB2 mutation carriers. In addition, unlike the situation for most other breast cancer susceptibility genes, it now appears that inactivation of the PALB2 gene does not have to be complete for breast cancer to develop.

"This is another "factual brick" in the ongoing construction of our understanding of breast cancer susceptibility," explained Dr. Foulkes. "Approximately 10 genes, including PALB2, have now been associated with a two-fold or greater risk for breast cancer, and carriers of mutations in these genes require special surveillance, including magnetic resonance imaging. Some may opt for preventive surgery."

As those choices are often difficult, Dr. Foulkes pointed out that the Montreal-based Hereditary Breast and Ovarian Cancer foundation (www.hboc.ca), led by McGill physician Harley Eisman, has been established with the specific aim of meeting the needs of families at risk.

The clinical work for this project was carried out by members of the Medical Genetics service at the McGill University Health Centre (MUHC) and the Sir Mortimer B. Davis Institute at the -Jewish General Hospital, a McGill University teaching hospital.

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Biallelic mutations in *PALB2* cause Fanconi anemia subtype FA-N and predispose to childhood cancer

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PALB2 was recently identified as a nuclear binding partner of BRCA2. Biallelic *BRCA2* mutations cause Fanconi anemia subtype FA-D1 and predispose to childhood malignancies. We identified pathogenic mutations in *PALB2* (also known as *FANCN*) in seven families affected with Fanconi anemia and cancer in early childhood, demonstrating that biallelic *PALB2* mutations cause a new subtype of Fanconi anemia, FA-N, and, similar to biallelic *BRCA2* mutations, confer a high risk of childhood cancer.

Fanconi anemia is a rare, recessive, chromosomal instability disorder characterized by growth retardation, congenital malformations, progressive bone marrow failure, cancer predisposition and cellular hypersensitivity to DNA cross-linking agents[1]. The syndrome is genetically heterogeneous with 12 complementation groups currently recognized, 11 of which have been attributed to distinct genes: *FANCA* (FA-A), *FANCB* (FA-B), *FANCC* (FA-C), *BRCA2* (FA-D1), *FANCD2* (FA-D2), *FANCE* (FA-E), *FANCF* (FA-F), *FANCG* (FA-G), *BRIP1* (FA-J), *FANCL* (FA-L) and *FANCM* (FA-M)[2,3].

BRCA2 is a DNA repair protein with a key role in the repair of DNA double-strand breaks by homologous recombination[4]. *BRCA2* was originally identified through positional cloning of a familial breast cancer predisposition gene, and monoallelic (heterozygous) mutations are associated with high risks of breast and ovarian cancer[5]. Subsequently, biallelic *BRCA2* mutations were found to cause a rare subtype of Fanconi anemia, FA-D1 (ref. 6). The phenotype of biallelic *BRCA2* mutations differs from other Fanconi anemia subtypes, most notably with respect to the high risks of childhood solid tumors, particularly Wilms tumor and medulloblastoma, which occur very rarely in other Fanconi anemia subtypes[6–10].

Although Fanconi anemia and childhood embryonal tumors are attributable to *BRCA2* in many individuals, we identified individuals with this phenotype who lacked *BRCA2* mutations. This raised the possibility that deficiency of other proteins might give rise to this combination of features, and we considered proteins functionally related to BRCA2 the most credible candidates.

PALB2 (for 'partner and localizer of BRCA2') was recently identified as a nuclear partner of BRCA2 (ref. 11). PALB2 colocalizes with BRCA2, promoting its localization and stability in key nuclear structures, which in turn facilitates BRCA2 functions in DNA repair. Furthermore, knockdown of PALB2 sensitizes cells to MMC treatment, which results in interstrand cross-links and double-strand breaks[11]. Sensitivity to MMC is a hallmark of Fanconi anemia, and these data therefore recommended *PALB2* as a candidate Fanconi anemia gene.

We sequenced the 13 exons and intron-exon boundaries of *PALB2* in 82 individuals with Fanconi anemia not due to known genes (**Supplementary Methods** and **Supplementary Table 1** online). We identified pathogenic mutations in seven families (**Fig. 1a** and **Table 1**). In four affected individuals (GESH, IFAR-847, LNEY and IFAR-849), we identified biallelic mutations that resulted in premature protein truncation. Analysis of parental DNA demonstrated that all the mutations had been inherited from different parents, consistent with autosomal recessive inheritance. No sample was available from the affected individuals LOAO, IFAR-007 and ICR-60, but their parents all carried truncating *PALB2* mutations. We also sequenced *PALB2* in 352 control chromosomes (176 normal individuals) and did not identify any truncating mutations, providing further evidence that such mutations are pathogenic in the individuals with Fanconi anemia.

We saw one mutation, 3549C→G (leading to amino acid change Y1183X), in two separate families. We also identified a different mutation in a third family at the same nucleotide, 3549C→A, which also results in Y1183X. 3549C is in the last exon of *PALB2*, and there are only three amino acids after codon 1183 before the protein terminates. Truncating mutations close to the end of a protein are generally expected to escape nonsense-mediated RNA decay[12]. However, there was no detectable PALB2 protein in lymphoblastoid cells from individuals IFAR-847 and IFAR-849, who both carry

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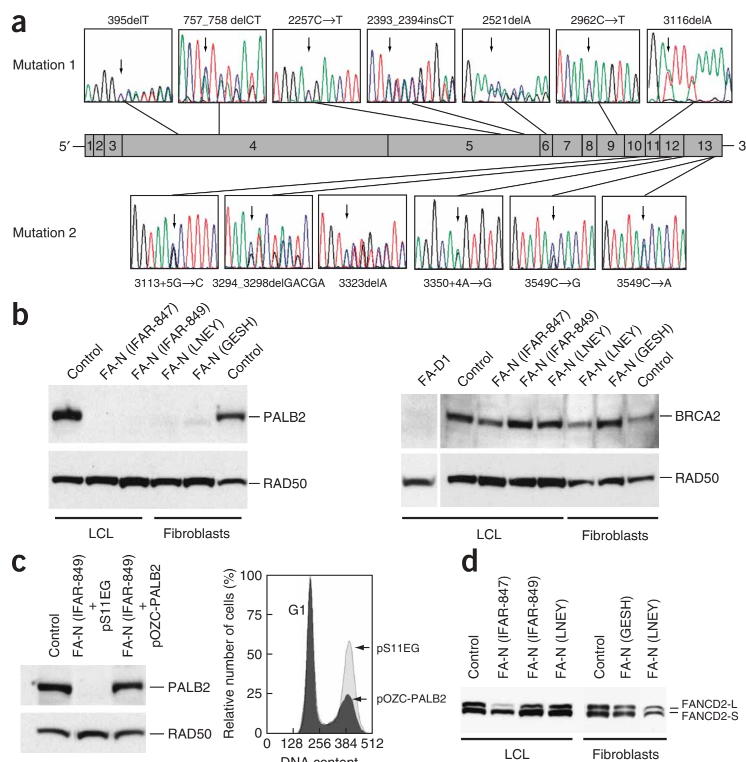

Figure 1 Evidence that PALB2 deficiency causes FA-N. (**a**) Mutation analysis. Schematic representation of *PALB2* showing the position of mutations identified in FA-N cases. Further details of the mutations and the individuals in which they were identified are given in **Table 1**. The 3549C→G mutation (leading to amino acid change Y1183X) was identified in two separate families. We obtained informed consent from all families and the research was approved by the London Multicentre Research Ethics Committee (05/MRE02/17). (**b**) PALB2 and BRCA2 immunoblotting. PALB2 protein is absent in cells from FA-N cases IFAR-847 (lymphoblastoid cell line (LCL)), IFAR-849 (LCL), LNEY (fibroblast) and GESH (fibroblast) and is present in control cells (left). BRCA2 is present in FA-N and control cells and is absent in FA-D1 cells (right). RAD50 was used as a loading control. (**c**) Complementation of the cellular phenotype. Transduction of cells from IFAR-849 with the vector pOZC-PALB2 restores PALB2 expression to a similar level as in a normal control, whereas the mock vector pS11EG (expressing GFP) does not (left). Overlay of two cell cycle distributions represented by histograms acquired with flow cytometry after exposure of an LCL from IFAR-849 to 15 ng/ml MMC for 48 h (right). The light gray histogram shows noncomplementation when cells were transduced with a mock vector expressing GFP (pS11EG) (G1 phase, 42%; S, 15%; G2, 43%; G2 phase arrest is typical of FA). The darker histogram demonstrates complementation after transduction of isogenic cells with the PALB2-expressing vector pOZC-PALB2 (G1 phase, 57%; S, 17%; G2, 26%; G2 phase is restored). This effect has been reported in complemented FA cell lines from other subtypes[15]. (**d**) FANCD2 monoubiquitination. There is normal FANCD2 monoubiquitination in FA-N cells, similar to control cells.

Y1183X, indicating that the mutation results in a null allele and confirming its pathogenicity (**Fig. 1b**).

We performed protein blot analysis on lymphoblastoid cells or fibroblasts from four individuals with biallelic *PALB2* mutations. Using an antibody that recognizes the N terminus of PALB2, we found absence of PALB2 protein in each case (**Fig. 1b**). Transduction of cells from IFAR-849 with an expression construct carrying wild-type PALB2 restored PALB2 expression and reversed MMC-induced G2 phase arrest (**Fig. 1c** and **Supplementary Methods**). The combined genetic, protein and complementation data provide strong evidence that *PALB2* mutations underlie a new Fanconi anemia complementation group that we have designated subtype FA-N.

Eight of the known Fanconi anemia proteins (A, B, C, E, F, G, L and M) form a nuclear core complex that mediates monoubiquitination of FANCD2. Activated FANCD2 (FANCD2-L) is translocated to DNA repair foci, where it colocalizes with various proteins involved in the DNA damage response, including BRCA2 (refs. 2,3). Given the close functional relationship between BRCA2 and PALB2, one would predict that PALB2 acts downstream of FANCD2. We confirmed this in different cell types from individuals IFAR-847, IFAR-849, LNEY and GESH, which show normal monoubiquitination of FANCD2 (**Fig. 1d**).

The phenotype of FA-N is in many ways typical of Fanconi anemia and includes growth retardation and variable congenital malformations (**Table 1**). The cellular phenotype of PALB2 deficiency is similar to BRCA2 deficiency and more severe than other Fanconi anemia subtypes, with elevated spontaneous chromosome breakage rates, markedly reduced lymphocyte survival and increased chromosome breakage on exposure to MMC (**Supplementary Fig. 1** online). There was also no formation of nuclear RAD51 foci in PALB2-deficient fibroblasts after ionizing irradiation. Again, this is similar to cells with biallelic *BRCA2* mutations and differs from other Fanconi anemia subtypes[13] (**Supplementary Fig. 1**).

All seven individuals with FA-N developed cancers in early childhood, including three Wilms tumors, five medulloblastomas, two cases of AML and one neuroblastoma (**Table 1**). One individual developed three malignancies within the first year of life, and three individuals had two cancers. Cancer treatment was unsuccessful in six patients, all of whom died before four years of age. LNEY is currently alive at 4.5 years but is in the early stages of treatment for medulloblastoma. The cancer spectrum and early mortality associated with biallelic *PALB2* mutations is thus very similar to that associated with biallelic *BRCA2* mutations[10]. The reasons for the association between childhood solid tumors and deficiency of BRCA2 or PALB2 are unclear but are probably related to functions not shared by other Fanconi anemia proteins.

Monoallelic (heterozygous) *BRCA2* mutations are associated with high risks of breast and ovarian cancer and lesser risks of other cancers such as prostate and pancreatic cancer[5]. Given the intimate functional links between PALB2 and BRCA2 and the similar phenotypes associated with biallelic mutations in the genes that encode them, it is plausible that monoallelic *PALB2* mutations confer susceptibility to adult cancer. Of note in this regard are the cancer histories in the seven FA-N families we have identified. The mother of IFAR-007 had early-onset bilateral breast cancer and has a strong family history of breast cancer affecting her sister, mother and other more distant relatives. *BRCA1* and *BRCA2* mutations have been excluded in this family. No other first-degree relatives of FA-N individuals are known to have developed cancer, although most are still under 50 years of age. However, the maternal grandmother of ICR-60 and a maternal great-grandmother of GESH developed breast cancer at 52 years and 20 years of age, respectively. Mutational analyses of *PALB2* in individuals with adult-onset cancer, particularly familial

Table 1 ***PALB2*** **mutations and clinical features of FA-N**

		Mutation 1		Mutation 2		Clinical features	
ID	Origin	Nucleotide change	Effect	Nucleotide change	Effect	Cancer (age at diagnosis)	Other features
LOAO[a]	Albanian[b], Moroccan[c]	395delT	V132fs	3113+5G→C	r.2835_3113del279/A946fs	Medulloblastoma (3.5 yrs)	Growth retardation, radial ray hypoplasia, absent right kidney
GESH	German	757_758delCT	L253fs	3294_3298 delGACGA	K1098fs	Wilms tumor (0.9 yr), AML (0.9 yr), medulloblastoma (1 yr)	Severe growth retardation, hypoplastic thumbs, left pelvic kidney, anal atresia, microcephaly, congenital cataract, microphthalmia
IFAR-847	Hispanic[b], North American[c]	2257C→T	R753X	3549C→A	Y1183X	Wilms tumor (1 yr)	Severe IUGR, postnatal growth retardation, microcephaly, microphthalmia, skin hyperpigmentation
LNEY	German	2393_2394insCT	T799fs	3350+4A→G	r.3350insGCAG/F1118fs	Medulloblastoma (4 yrs)	Growth retardation, microcephaly, microphthalmia, bifurcated anus
IFAR-007[a,d]	North American[b], African ancestry[c]	2521delA	T841fs	3323delA	Y1108fs	Wilms tumor (1.5 yrs), medulloblastoma (1.5 yrs)	Growth retardation, microcephaly, skin hyper- and hypopigmentation, horseshoe kidney, gonadal dysgenesis
ICR-60[a]	British	2962C→T	Q988X	3549C→G	Y1183X	Medulloblastoma (2.3 yrs)	Growth retardation, microcephaly, hypoplastic thumb
IFAR-849	North American	3116delA	N1039fs	3549C→G	Y1183X	Neuroblastoma (0.7 yrs), AML (2 yrs)	Growth retardation, microcephaly, VSD, ASD, thumb and radial anomalies, skin hyperpigmention

AML, acute myelogenous leukemia; IUGR, intrauterine growth retardation; ASD, atrial septal defect, VSD, ventricular septal defect. North American individuals are of European ancestry.
[a]No samples were available from the affected individuals; therefore, the mutations were identified in parental samples. [b]Origin relates to mutation 1. [c]Origin relates to mutation 2. [d]This individual's clinical details have been published previously[14].

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breast cancer, will clarify the role of monoallelic *PALB2* mutations in cancer susceptibility.

Mutations in *BRCA2* and *PALB2* together account for almost all the individuals we studied having both Fanconi anemia and childhood solid tumors. However, there are rare individuals with Fanconi anemia not due to known genes whose cells show normal monoubiquitination of FANCD2. Thus, it is possible that other genes encoding proteins that physically and/or functionally interact with BRCA2 cause currently unrecognized subtypes of Fanconi anemia.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS
The study was designed by N.R. The mutational and PALB2 protein blot analyses were performed by S.S., K.B., S.H., P.K. and S.S. under the direction of N.R. The cellular, protein blot and complementation analyses and investigation of effects of splicing mutations were performed by D.S., H.H., R.K., K.N., M.F., M.W. and H.N. The phenotypic assessment, sample collection and characterization with respect to Fanconi anemia subgroups was performed by D.S., H.H., S.D.B., F.P.L., S.Y., H.A., M.T., C.G.M. and A.D.A. The manuscript was written by N.R with contributions from the other authors.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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Analysis of PALB2/FANCN-associated breast cancer families

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Notes:

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

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Contributed by David M. Livingston, February 26, 2007 (sent for review February 9, 2007)

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.

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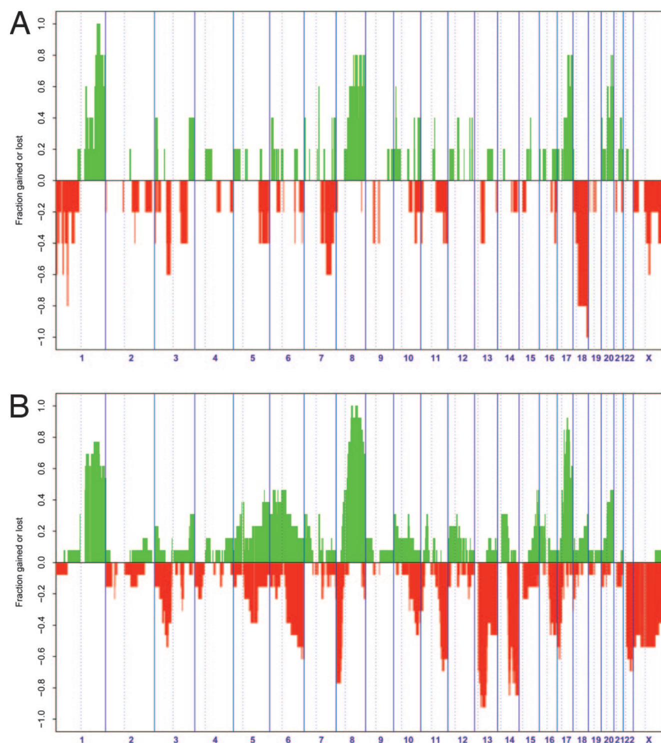

Fig. 3. Frequency of DNA copy number changes in tumors arising in *PALB2* and *BRCA2* germline mutation carriers. (*A*) PALB2 cases [invasive breast cancer (n = 4) and lobular carcinoma *in situ* (n = 1)]. (*B*) BRCA2 tumors (n = 13). Individual BAC clones are plotted according to genomic location along 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		Cases with BRCAPRO scores >0.50, no.
		Mean	Range	
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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