



Published in final edited form as:

*Clin Cancer Res.* 2008 July 15; 14(14): 4672–4680. doi:10.1158/1078-0432.CCR-08-0087.

## A novel breast cancer-associated *BRIP1* (*FANCI/BACH1*) germ line mutation impairs protein stability and function

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### Abstract

**Purpose**—*BRIP1* (*FANCI/BACH1*), which encodes a DNA helicase that interacts with *BRCA1*, has been suggested to be a low penetrance breast cancer predisposing gene. We aimed to assess whether *BRIP1* mutations contribute to breast cancer susceptibility in our population and, if so, to investigate the impact of such mutation(s) on *BRIP1* function.

**Experimental Design**—A series of 49 breast/ovarian cancer families, devoid of a *BRCA1/BRCA2* mutation, were screened for *BRIP1* mutations. Functional analyses, including co-immunoprecipitation and stability assays, were employed to further characterize a previously unreported variant.

**Results**—Five sequence alterations were identified, of which four had been already described. Herein, we report a novel *BRIP1* germ line mutation identified in a woman with early onset breast cancer. The mutation consists of a four-nucleotide deletion (c.2992-2995delAAGA) in *BRIP1* exon 20 that causes a shift in the reading frame, disrupts the *BRCA1*-binding domain of *BRIP1* and creates a premature stop codon. Functional analysis of the recombinant mutant protein in transfected cells showed that the truncation interferes with the stability of the protein and with its ability to interact with *BRCA1*. Loss of the wild type *BRIP1* allele with retention of the mutated one was observed in the patient's breast tumor tissue.

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#### ELECTRONIC DATABASE INFORMATION

The URLs and accession numbers for data presented herein are as follows: GenBank, <http://ncbi.nlm.nih.gov/GenBank/> for *BRIP1* genomic (accession number NT\_010783) and cDNA (accession number NM\_032043) sequences. Primer3 Software, [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

**Conclusions**—These results, by showing that the newly identified *BRIP1* c.2992-2995delAAGA mutation is associated with instability and functional impairment of the encoded protein, provide further evidence of a breast cancer-related role for BRIP1.

### Keywords

BRIP1; breast cancer susceptibility; BRCA1; tumor suppressor genes; Fanconi anaemia

## INTRODUCTION

It is estimated that approximately 5-10% of all breast cancer cases result from an inherited predisposition to the disease (1). Thus far, two major high penetrance susceptibility genes have been identified: *BRCA1* and *BRCA2*. Inheritance of a defective allele of either gene is sufficient to confer breast cancer predisposition with an estimated lifetime risk of ~35-80% ((2-5) and reviewed in (6)). However, mutations in the *BRCA* genes fail to explain occurrence of the neoplasia in all breast cancer prone families (7). Other well-established cancer susceptibility genes, such as *TP53*, *PTEN*, and *LKB1*, contribute only to a small proportion of breast cancer in the general population, as their germ line mutations are very rare. The remainder breast cancer risk is, as yet, unexplained and likely due to cooperative defects in a larger number of low penetrance genes rather than to mutations in highly penetrant ones (7). In keeping with this notion, suitable candidates have been analyzed in search for functionally deficient variants that segregate with the cancer phenotype. Genes whose products are known to interact with *BRCA1* and/or *BRCA2* and to be involved in DNA repair or in the regulation of cell cycle progression are usually prioritized for such investigation (8). As a result, mutations of *ATM*, *CHEK2*, *RAD50*, *Nibrin*, *PALB2* and *BRIP1* have been found to associate with a modest (approximately two-fold) increase of breast cancer risk (9-14).

BRIP1 (BRCA1-interacting protein 1), also known as BACH1 (BRCA1-associated C-terminal helicase 1) was first identified as a 1249 amino acid (aa)-protein that interacts with the *BRCA1* BRCTs *in vivo* (15). BRIP1 is ubiquitously expressed, it co-localizes with *BRCA1* at sites of DNA damage, and contributes to its DNA repair function (15). Specifically, BRIP1-*BRCA1* interaction, which has been shown to depend upon the cell cycle-regulated phosphorylation of BRIP1 at Serine 990 (16-18), is required for timely repair of DNA double strand breaks and for DNA damage-induced checkpoint control during the G2/M phase of the cell cycle (17).

Interestingly, *BRIP1* maps to chromosome 17q22 near the *BRCA1* locus (15). The frequent documentation of breast carcinomas that are wild-type for the *BRCA* genes but exhibit allelic losses encompassing the 17q21-q22 region, suggests that this chromosomal region may harbor an additional breast cancer susceptibility gene (19). Thus, both its functionally relevant interaction with *BRCA1* and its chromosomal location render *BRIP1* a candidate tumor suppressor gene.

In addition, the BRIP1 N-terminus shares substantial sequence homology to the catalytic and nucleotide-binding domains of known members of the DEAH helicase family. Indeed, BRIP1 was shown to be a *bona fide* ATP-dependent, 5'-3' DNA helicase (20). Mutations in helicases (such as *XPB*, *XPD*, *WRN*, *BLM*, *RecQL4*) have been described in association with human genetic disorders (Xeroderma pigmentosum, Werner syndrome, Bloom syndrome, and Rothmund-Thomson syndrome, respectively), which are characterized by genome instability and predisposition to cancer (reviewed in (21-24)).

The demonstration that certain *BRIP1* mutations, detected in women with early onset breast cancer, result in the synthesis of enzymatically defective proteins (20) provides biochemical support for the aforementioned notion that *BRIP1* is a breast cancer gene. However, thus far,

studies have failed to find any highly penetrant *BRIP1* mutations, and most of the newly identified sequence alterations have not been resolved in terms of their biological significance (14,25-28).

More recently, *BRIP1* was shown to be bi-allelically inactivated in patients with Fanconi Anaemia (FA), a genetic disease (which can be autosomal recessive or X-linked) characterized by multiple congenital abnormalities, bone marrow failure, cellular hypersensitivity to interstrand DNA cross-linking agents and susceptibility to cancer (29). Specifically, *BRIP1* mutations were found in patients with FA belonging to the complementation group J (30-33). Similarly, a bi-allelic inactivation of the breast cancer gene *BRCA2* has been previously described in patients with FA complementation group D1 (34). More recently, bi-allelic defects in *PALB2* have been demonstrated to cause FA subtype N (35,36).

The aim of our study was to evaluate the contribution of alterations at the *BRIP1* locus to breast cancer susceptibility in a cohort of 49 breast or breast/ovarian families in which no *BRCA1/BRCA2* mutations were detected.

## MATERIALS and METHODS

### Family recruitment

A total of forty nine breast or breast/ovarian cancer patients belonging to as many families mainly originating from Central-Northern Italy and referred to the University Hospital in Pisa (37), were defined as probands (index individuals) and were analyzed for *BRIP1* germ line mutations. All probands displayed no detectable deleterious mutations upon full screening of *BRCA1* and *BRCA2* genes. In addition, when tested for the *CHEK2* 1100delC disease-linked mutation (11), they all resulted negative. Out of the 49 families, 40 (80%) were associated with breast cancer, 9 (20%) with both breast and ovarian cancer (Supplementary table 1). Most of the families included three or more breast/ovarian cancer cases. Thirty-five pedigrees showed also cases of other types of neoplasia. The average age at diagnosis of breast cancer among patients was 43.8 years (yrs), ranging between 21 and 73 yrs (Supplementary table 1). Family histories were obtained through detailed interviews and pedigrees were traced as far backward and laterally as possible. Families were classified as high-risk or moderate-risk cancer families depending on the criteria they met. Inclusion criteria for the 38 high risk-families were as follows: 1) three or more cases of breast and/or ovarian cancer in first-/second-degree relatives, or 2) two or more cases of breast and/or ovarian cancer in first-/second-degree relatives, of which at least one with bilateral or early onset ( $\leq 40$  yrs) breast cancer or with breast and ovarian cancer. Each of the 4 moderate-risk families displayed two cases of breast/ovarian cancer in first-/second-degree relatives. In addition, 7 families with single cases of bilateral or early onset breast cancer or breast/ovarian cancer along with multiple (two or more) cases of other kinds of tumors in first-/second-degree relatives were included in the study. Written informed consent to collect pedigree data and a blood specimen for a study on cancer susceptibility was obtained from all probands. As controls, fifty individuals, from the same geographical region as the cancer cases, were anonymously recruited. They were all cancer-free at the time of blood donation and their age ranged between 21 and 87 yrs (average 48.5 yrs).

### Mutation screening

A DNA sample of the index individual of each cancer family was analyzed. Genomic DNA was extracted from peripheral blood lymphocytes using a QIAamp DNA Mini kit (Qiagen). Twenty-two fragments of the *BRIP1* gene, including both coding exons and flanking intronic sequences, were amplified by PCR using Taq Gold polymerase (Applied Biosystems). The size of PCR products ranged between 214 and 380 basepairs (bp). Screening for *BRIP1* mutations was carried out by Single Strand Conformation Polymorphism (SSCP). Genomic

DNA from cases showing an altered SSCP migration pattern was amplified and analyzed by bidirectional sequencing using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) on an ABI model 3100 capillary sequencer. Oligonucleotides for SSCP and sequencing were designed using the Primer3 Software based upon the sequence information obtained from the Genbank databases. Primer sequences and PCR conditions are available upon request.

### Loss of heterozygosity analysis

Loss of heterozygosity (LOH) analysis was carried out on tumor tissue excised from the index individual of the Pisa#88 family. Laser-capture microdissection was performed to isolate pure populations of neoplastic and normal breast cells from 5 micron ( $\mu\text{m}$ ) sections of the formalin-fixed paraffin-embedded breast carcinoma tissue block. The DNA was extracted from the dissected tumor and normal cells using a QIAamp DNA Mini Kit (Qiagen). Amplification was carried out by PCR using primers located in exon 20, and the resulting products were analysed by sequencing.

### Plasmids

The full-length BRIP1 cDNA-containing pENTR/D-TOPO (wtBRIP1 pENTR/D-TOPO) vector (15), served as template to generate a 4 nucleotide (nt)-deletion ( $\Delta$ ) at the relevant site using the QuickChange mutagenesis kit (Stratagene). After sequencing to confirm the mutation, the  $\Delta$ BRIP1-pENTR/D-TOPO vector was modified to insert an in frame Flag-encoding C terminal sequence ( $\Delta$ BRIP1Flag-pENTR/D-TOPO). Next, both  $\Delta$ BRIP1 and  $\Delta$ BRIP1 Flag-encoding cDNAs were transferred, by LR recombinase-mediated reaction (Invitrogen), into a pcDNA-DEST47 destination vector (Invitrogen) for expression in mammalian cells. The  $\Delta$ BRIP1-pcDNA-DEST47 vector was further used as template for PCR amplification. The PCR product was NotI-/ApaI-digested and subcloned into a pcDNA3.1 vector (Invitrogen) encoding a C-terminal Myc-6xHys tag ( $\Delta$ BRIP1-pcDNA3.1/MycHis). All cloned cDNA constructs were fully sequenced prior to use. The mammalian expression vector pcDNA3.1 Myc-6xHis containing full length wtBRIP1 cDNA (wtBRIP1-pcDNA3.1/MycHis) was described previously (15). pEGFP-C2 (Clontech) and pRL-TK (Promega) plasmids were used as controls to ensure efficiency of transfection.

### *In vitro* transcription and translation

Expression plasmids, generated as described above, and encoding the wt and the  $\Delta$ BRIP1 protein products, were *in vitro* transcribed and translated using a TNT kit (Promega) according to the manufacturer's specifications.

### Establishment of a lymphoblastoid cell line

A lymphoblastoid cell line (LCL) was established by EBV-infection of Pisa#88 proband's peripheral blood lymphocytes using standard protocols, and served as a source for DNA, RNA and proteins (detailed below).

### DNA extraction and PCR amplification and analysis

Genomic DNA was extracted from the Pisa#88 LCL using a QIAamp DNA Mini Kit (Qiagen), and amplified with appropriate primer pairs depending on the applications. A number of cycles as low as 26 was used to avoid PCR artefacts which might be cause of preferential allelic amplification. Analysis of PCR products by bidirectional sequencing was carried out as described above. For electrophoretic analysis, PCR products were resolved on 4% Low Melting Point (LMP) agarose (Gibco) or 8% polyacrylamide denaturing gels, stained with Ethidium bromide and SYBR Gold (Invitrogen), dilution 1:10,000, respectively. The 4 bp length difference caused by the mutation allowed direct discrimination of the mutant and wt fragments

(amplified with a pair of primers specifically designed to give rise to a small product encompassing the mutation site). Bands were visualized under UV light. The Image J software was utilized for quantitative analysis of the intensity of the bands.

### Cell culture and transfection

HeLa, U2OS and 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gemini) and antibiotics in a humidified incubator at 37°C with an atmosphere containing 10% CO<sub>2</sub>. Grown to 50% confluence, cells were transfected with 5 to 10 micrograms of wtBRIP1 or ΔBRIP1-pcDNA3.1/MycHis constructs using FuGene 6 transfection reagent (Roche Diagnostic).

### RNA extraction, cDNA synthesis and RT-PCR amplification and analysis

RNA was isolated from the Pisa#88 LCL or from GFP-, Myc-wtBRIP1- or Myc-ΔBRIP1-transfected 293T cells using the RNeasy mini kit (Qiagen). cDNA synthesis was carried out utilizing Superscript II RT Rnase H<sup>-</sup> reverse transcriptase (Invitrogen) and random hexamers (Invitrogen) according to the manufacturer's instructions. RT-PCR to analyze the expression levels of endogenous transcripts in the Pisa#88 LCL was carried out with the same primer pair used for DNA, and the amplified fragments were discriminated on agarose and polyacrylamide gels, as previously detailed.

RT-PCR to compare mutant vs wt transcripts in transfected cells was carried out using specific primer pairs that allowed selective amplification of the exogenously expressed constructs. In both cases, mock RT-PCR reactions (from mRNA reverse-transcribed with no addition of reverse transcriptase) served as controls, and confirmed the absence of DNA contamination.

### Immunoprecipitation, immunoblotting, and protein stability analysis

Forty-eight hours after transfection, cells were collected, washed with phosphate buffer saline (PBS) (pH 7.4), and lysed in NETN buffer (0.5% NP-40, 20 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM EDTA) supplemented with 1 tablet/50 ml of Complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation (16000g for 10 min) and incubated on ice for 1 hour with 1-2 μg of the relevant antibody (Ab). Next, 20 μl of slurry protein A or G Sepharose beads (Amersham Biosciences) were added to each lysate, and incubation continued with rotation for 40 min at 4°C. Beads were retrieved by centrifugation and washed three times in NETN buffer. Proteins bound to the beads were eluted by boiling in Laemmli sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred to nitrocellulose membranes (Invitrogen). After blocking in 5% milk, western (W)-blot assays were carried out with the indicated primary Abs for 1 hour at room temperature, followed by incubation with horseradish peroxidase-conjugated secondary Abs for 1 hr at room temperature. Aliquots of lysates were also run and blotted for tubulin as a control for equal protein loading. Equal transfection efficiency among the samples was ensured by western blot detection of the co-transfected pEGFP-C2 plasmid. All blots were developed using ECL-plus western blotting detection system (Amersham Biosciences) and Biomax BLUE XB-1, MR or XAR films (Kodak). Cycloheximide-chase experiments were performed as described (38). The Image J software was used for densitometric analysis of the films.

To obtain whole cell lysates (WCEs) from EBV-immortalized lymphocytes, the cells were harvested, washed once in PBS, and lysed in NETN buffer, as previously described. WCEs were separated by SDS-PAGE and subjected to W-blotting as detailed above.



## Antibodies

Two different rabbit polyclonal Abs, I-104 and I-82, were generated against BRIP1 in New Zealand White Rabbits. I-104 was produced against a C-terminal peptide of BRIP1 (N-KTHEIEIKNFKPSKSKNGM-C). I-82 was generated against a recombinant full-length flag-tagged wild-type BRIP1 (rBRIP1) produced in High Five insect cells. I-82 was affinity-purified using rBRIP1 coupled to a solid support. The anti-BRCA1 rabbit antiserum was purchased from Upstate Biotechnology. The anti-BARD1 affinity-purified polyclonal antibody was purchased from Bethyl Laboratories Inc. The following monoclonal antibodies were used: anti-BRCA1 MS110 (39); anti-Myc, clones 4A6 (Upstate Biotechnology) and 9B11 (Cell Signaling Technology); anti- $\alpha$ tubulin, clone DM1A (Sigma); anti-GFP, clone C163 (Zymed Laboratories Inc). Secondary antibodies used for immunodetection were: HRP-conjugated sheep anti-mouse IgG and donkey anti-rabbit Ig (Amersham Biosciences) (dilution 1:5,000).

## RESULTS

Mutational analysis of the *BRIP1* gene performed in our cohort of 49 index individuals led to the identification of four germ line sequence alterations within the *BRIP1* coding region together with one intronic variant (Table 1). Three sequence changes (c.2637G>A, c.2755C>T, c.3411C>T) had already been reported with comparable allelic frequencies (15,25,26,28) and all were classified as neutral polymorphisms. The intronic variant IVS5-31 C>G in the region located between exons 5 and 6 was previously reported, as well (27). Neither of the two heterozygous missense mutations (c.139C>G, c.897G>A) described by Cantor et al. (2001) were found in our set of patients.

Interestingly, we identified a novel heterozygous sequence change in the last exon (exon 20) of *BRIP1*: a four nt-deletion starting at 2992 (c.2992-2995delAAGA) that causes a slippage of the open reading frame, leading to a premature stop at codon 1057 (Figure 1A and B). The sequence from the deletion to the stop bears no homology to wtBRIP1. Importantly, the aa change (aa 998-1057) caused by the newly identified mutation partially encompasses the BRCA1 binding domain (aa 979-1006) of BRIP1 (20) and might be predicted to affect the interaction between BRIP1 and BRCA1.

The c.2992-2995delAAGA mutation was detected in a 49 year-old woman that had been diagnosed with breast cancer at the age of 40 and exhibited a family history of multiple cancers (Figure 1C). No breast cancer cases other than the proband and her mother (who was diagnosed at the age of 76) were known to have occurred in the family. However, the pedigree displayed two cases of colorectal cancer in the proband's paternal aunt and grandmother. Moreover, both brothers of the paternal grandmother had been diagnosed with a neoplastic disease (lymphoma and lung cancer). Unfortunately, since DNA samples from any other suitable family member (s) were not available, co-segregation of the mutated allele with the cancer phenotype through the family could not be tested. Nevertheless, when 50 matched healthy control individuals were analyzed, they all tested negative for the c.2992-2995delAAGA mutation.

*BRIP1* would conform to the classic paradigm for tumor suppressor genes if loss of the wild-type allele could be detected in tumor cells isolated from the affected individual. In order to address this possibility, we searched for LOH at the *BRIP1* locus in breast carcinoma tissue from the patient carrier of the novel mutation. Remarkably, sequence analysis of the DNA extracted from the laser capture-microdissected specimen revealed that loss of the wild-type allele had occurred in the tumor cells, with retention of the mutant allele (Figure 2). This finding underscores the notion that *BRIP1* has tumor-suppressive function and might participate in breast tumorigenesis.

We then sought to elucidate the possible biological consequences of the mutant *BRIP1* allele by investigating whether the deletion had any effect on the function of the corresponding protein. Since the frameshift caused by the mutation was predicted to give rise to a premature translational stop, we decided to characterize the truncated protein product. First, we generated a number of BRIP1 constructs bearing the relevant mutation ( $\Delta$ BRIP1), with or without a C-terminal tag (Flag or Myc). As a control, we utilized the wtBRIP1-pcDNA3.1/MycHis described previously (15). *In vitro* transcription and translation reactions were programmed with the wt and mutant plasmids. The obtained products were resolved by SDS-PAGE and analyzed by western blotting using Abs against BRIP1 or the Myc tag. As predicted, all the  $\Delta$ BRIP1 constructs generated electrophoretically-distinguishable protein products that migrated approximately 20 kDa faster than the wtBRIP1 one, consistent with the expected size for  $\Delta$ BRIP1 being ~200 aa shorter than the wt protein (Figure 3A, lanes 3-5 vs 2). In addition, we ascertained that the mutant BRIP1 was not detected by an antibody generated against the extreme C-terminus of the protein whereas, under the same conditions, the full-length wtBRIP1 was easily recognized (Figure 3B). These results demonstrate that the *BRIP1* c. 2992-2995delAAGA mutation leads to the synthesis of an identifiable truncated protein that lacks the C-terminus.

Next, we aimed to determine the expression of the  $\Delta$ BRIP1 protein *in vivo*. For this purpose, a lymphoblastoid cell line was established, by EBV-infection, from the peripheral blood lymphocytes of the heterozygous patient, and analysis of the endogenous BRIP1 protein was performed by W-blotting. Only a wtBRIP1 product was observed when either a polyclonal antibody (Figure 3C, lane 5) or a pool of monoclonal antibodies (data not shown) were used. This observation indicates that  $\Delta$ BRIP1, if expressed, is significantly less abundant than wtBRIP1 in EBV-immortalized lymphocytes.

Since mRNAs bearing a premature termination codon (PTC) are known to be targeted for degradation via a surveillance mechanism named nonsense-mediated mRNA decay (NMD) (reviewed in (40,41)), we searched for an imbalanced expression of the transcripts (mutant vs wt) that could explain the absence of a detectable mutant protein. As a result, direct sequencing and polyacrylamide gel electrophoresis analysis of RT-PCR products revealed the presence of both mutant and wt alleles in the Pisa#88 LCL (Supplementary figure 1A and B). Furthermore, the expression levels of both transcripts appeared comparable, as confirmed after quantitation of the band intensities (Supplementary figure 1B and C, and data not shown). This finding is consistent with the notion that PTCs residing within the last exon do not trigger NMD (42-44) and argues against preferential allelic degradation as an explanation for the inability to detect the mutant protein.

We, therefore, reasoned that the lack of expression of  $\Delta$ BRIP1 in the patient's immortalized lymphocytes could be due to the inherent instability of the mutant protein. To test this possibility, we set out to characterize an exogenously over-expressed  $\Delta$ BRIP1 protein in cultured cells. HeLa cells were transiently transfected with Myc-wtBRIP1 or Myc- $\Delta$ BRIP1 encoding constructs. Using anti-Myc or anti-BRIP1 antibodies, we were unable to detect a mutant protein product of the predicted size (data not shown). A low expression of the truncated protein was observed after transfection of the relevant constructs in U2OS cells (data not shown). We then exploited the 293T cell line, which expresses SV40 large T antigen, to allow T antigen-dependent amplification of the SV40 origin-containing plasmids. Even in this setting, the expression of the  $\Delta$ BRIP1 protein was lower than the expression of the wt counterpart (Figure 4A). Co-transfection with GFP- or Luciferase-encoding plasmids allowed us to confirm equal efficiency of the transfection for both wt and  $\Delta$ BRIP1 constructs (Figure 4A and data not shown). Furthermore, we ruled out that the mutation could affect the protein by rendering  $\Delta$ BRIP1 insoluble: indeed, the mutated protein was easily extractable in cell lysis buffer from transfected cells with no significant residual amount detected in the pellet (data

not shown). Moreover, a fairly equal level of expression for both Myc-wtBRIP1 and Myc- $\Delta$ BRIP1 transcripts was demonstrated by RT-PCR carried out with primers specifically designed to amplify the exogenous mRNAs (Supplementary figure 2). These observations suggest that the lower level of  $\Delta$ BRIP1 detected after transfection might be due to instability of the truncated protein caused by the mutation.

We sought to address this possibility by utilizing a cycloheximide-chase method (38) to determine the half-lives of both mutant and wt proteins. Transient transfection of 293T cells using equal amounts of the aforementioned Myc-wtBRIP1- and Myc- $\Delta$ BRIP1-encoding constructs, was performed followed by cycloheximide treatment to block new protein synthesis. When cell lysates obtained at different time points were blotted with an anti-Myc specific Ab,  $\Delta$ BRIP1 appeared to be, indeed, significantly less stable than wtBRIP1 (Figure 4B). An estimated half-life of  $\sim$ 1 hour was observed for the mutant protein, whereas wtBRIP1 was shown to be significantly more stable, having a half-life of  $\sim$ 5 hours (Figure 4C), consistent with previous findings (15). These results indicate that the c.2992-2995delAAGA mutation impairs the stability of the corresponding truncated BRIP1 protein.

Although these studies show that the mutant BRIP1 protein is unstable, we were intrigued by the fact that the c.2992-2995delAAGA mutation disrupts the previously described BRCA1-binding domain (15,20) and wondered whether  $\Delta$ BRIP1, even in low concentration, could still interact with BRCA1. To this end, we attempted to assess, by co-immunoprecipitation (Co-IP) analysis, the potential impact of the novel *BRIP1* sequence change on the BRIP1-BRCA1 interaction *in vivo*. 293T cells were transfected with GFP and either Myc-wtBRIP1- or Myc- $\Delta$ BRIP1-encoding constructs, and IP with an anti-Myc monospecific antibody was carried out, followed by W-blotting for BRCA1. Co-immunoprecipitated BRCA1 was always nearly absent in the IP from  $\Delta$ BRIP1-transfected cells, whereas a clearly detectable BRCA1 signal was observed even when serial dilutions of the Myc-IP from wtBRIP1-transfected cells were loaded onto the gel (Figure 5). These results indicate that  $\Delta$ BRIP1, which bears a significantly altered BRCA1-binding domain, is impaired in its ability to interact with BRCA1. In addition, because BRIP1 and BARD1 have been reported to indirectly interact with one another, BRCA1 being the mediator of such interaction (15), we asked whether the defective binding of  $\Delta$ BRIP1 to BRCA1 translated into a defective interaction with BARD1, as well. Indeed, in Myc- $\Delta$ BRIP1 complexes the amount of co-immunoprecipitated BARD1 was significantly decreased compared to what observed in Myc-wtBRIP1 complexes (Figure 5). Furthermore, results from reciprocal IPs (i.e. BRCA1 IP followed by western blotting for Myc) consistently showed a significantly impaired interaction between BRCA1 and the mutant BRIP1 protein (Supplementary figure 3).

Taken together these results indicate that the c.2992-2995delAAGA mutation affects BRIP1 function and impairs its ability to interact with BRCA1.

## DISCUSSION

Our goal was to evaluate whether and to what extent defects in the *BRIP1* gene contribute to familial breast cancer in selected *BRCA1*-/*BRCA2*-negative breast/ovarian cancer families. The mutational screening we carried out led to the identification of five *BRIP1* sequence changes in a series of 49 affected individuals. Four alterations (IVS5-31C>G, c.2637G>A, c.2755C>T, c.3411C>T) had already been reported (15,25-27), of which only one (c.2755C>T) causes a non synonymous aa substitution and introduces a Ser (Pro919Ser) in the BRCA1 binding domain thus generating a potential phosphorylation site. An association between such polymorphism and an increased breast cancer risk by the age of 50 years has been suggested (45) but it is, as yet, not confirmed (28,46).



The fifth sequence variant we characterized (c.2992-2995delAAGA) is a unique mutation that causes a change of 9 out of 28 aa within the BRCA1-binding domain (979-1006) of BRIP1 and introduces a premature stop codon. The mutation was identified in a woman diagnosed with early onset breast cancer who lacked a strong family history for the disease. Importantly, we found LOH at the *BRIP1* locus in breast cancer tissue from the same patient.

To our knowledge, this is the first report of a heterozygous germ line mutation in the *BRIP1* gene, which gives rise to a truncated protein product, predicted to be impaired in its biological role, and is associated with loss of the wt*BRIP1* allele in the corresponding breast tumor tissue. The latter result is consistent with the classic Knudson's two hit hypothesis for loss of tumor suppressor function (47) and supports the speculation that the novel mutation we describe herein might have contributed to the development of breast cancer in the patient. Notably, although most of the *BRIP1* sequence alterations described so far associated with Fanconi Anaemia have a truncating effect (31,32), the c.2992-2995delAAGA mutation was not previously reported in FA-J patients.

Even though analysis of BRIP1 transcripts evidenced a fairly equal expression of both wt and mutant alleles (thus suggesting that the c.2992-2995delAAGA mutation, which falls in *BRIP1* last exon, is not subject to NMD (43)), W-blotting on lysate from Pisa#88 LCL revealed undetectable levels of endogenous mutant protein. Consistent with this observation, the recombinant  $\Delta$ BRIP1 protein was inefficiently expressed in transfected cells because it is unstable. Indeed, instability of mutated BRIP1 proteins has already been reported in studies where cells from the heterozygous carriers could be directly tested (15). Furthermore, truncated BRIP1 species have not been detected, so far, in FA-J cells (31-33).

Given the instability of the truncated protein, the interaction with BRCA1 being preserved or disrupted would likely represent an irrelevant point *in vivo*. However, one cannot rule out the possibility that  $\Delta$ BRIP1 is still expressed at low levels in tissues other than lymphoid. When co-immunoprecipitation experiments were performed in transfected cells, we observed that the described c.2992-2995delAAGA mutation affects BRIP1 interaction with BRCA1. In fact, even though both Ser990 and Phe993 (recently suggested to play a major role in anchoring BRIP1 binding to the first and second BRCT, respectively (16)) are preserved in BRIP1, it is conceivable that the altered aa sequence starting at 998 might have an impact on the interaction.

Interestingly, disease-linked mutations occurring in the BRCT domains are sufficient to abrogate BRCA1-BRIP1 interaction *in vivo* (48). Furthermore, mutations within the helicase domains of BRIP1 were shown to interfere with double-strand break repair in a BRCA1-binding dependent manner (20). One may, therefore, hypothesize that impairment or loss of such interaction, due to *BRIP1* mutations that perturb the stability of the protein and/or its ability to bind to the partner, may be a critical cancer predisposing event. In addition, more recent studies have reported BRCA1-independent functions of BRIP1 (49), and our observations by protein chromatography (R. Drapkin, unpublished) suggest that the majority of BRIP1 exists in a native complex without BRCA1. In such a scenario, one can imagine that in the patient's breast tumor tissue, which, as shown here, has lost the wt allele, the instability of  $\Delta$ BRIP1 combined with possible functional defects of the residual mutant protein might render the tumor cells virtually BRIP1-deficient, thus having a dramatic impact on biological processes in which BRIP1 is involved.

Taken together, our data suggest a causative role for *BRIP1* in breast cancer disease reinforcing the possibility that *BRIP1* mutations may account for a small percentage of inherited breast cancer cases. A recent model of breast cancer susceptibility suggests that a sizeable fraction of the still unaccounted familial aggregation (50) maybe best explained by a large number of low penetrance genes whereby their individual small effects combine multiplicatively (7,51).

Of note, Seal et al. have recently reported that *BRIP1* truncating mutations act as low penetrance breast cancer alleles in the British population (14). Interestingly, *BRIP1* truncating mutations were found to be associated with a high relative risk for carriers aged less than 50, as opposed to *BRIP1* missense variants which were described, instead, to associate with a much lower risk (14).

In conclusion, the screening we carried out revealed a previously unreported constitutive, functionally deleterious *BRIP1* mutation in a woman with early onset breast cancer and, importantly, LOH at the *BRIP1* locus in the corresponding breast tumor tissue. The scarce number of breast cancer cases in the Pisa#88 pedigree is consistent with the low penetrance suggested for *BRIP1* mutations whereby some of the unaffected individuals might be potential carriers. Unfortunately, the inability to check for segregation of the mutation in other individuals of the same family did not allow us to explore the possible link between *BRIP1* mutations and neoplasia in tissues other than breast.

It would be interesting to know whether heterozygous *BRIP1* mutation carriers in FA families have an excess risk of developing breast cancer or other malignancies. So far, unlike *BRCA2*, whose mono-allelic mutations confer high risk of breast cancer, and whose bi-allelic mutations predispose to childhood solid and haematological malignancies (52), *BRIP1* seems to be linked only to a modest risk of breast cancer. Nevertheless, recent studies suggest that *BRIP1* may also be inactivated via an epigenetic mechanism in certain head and neck cancers (53). Future investigation will certainly help to gain insights into the role of *BRIP1* in the FA pathway and to shed light on how that relates to its tumor suppression function.

## ACKNOWLEDGEMENTS

We thank the patients and their family members for cooperation. We are deeply grateful to Prof. D.M. Livingston (Dana-Farber Cancer Institute, Boston, MA) for considerable advice and insights, Dr. V. Joukov (Dana-Farber Cancer Institute, Boston, MA) for helpful discussions and constructive criticism during the preparation of the manuscript, and Prof. S. Cantor (University of Massachusetts Medical School, Worcester, MA) for providing us with reagents and for giving us useful suggestions. We would also like to thank Dr. P. Aretini (MGM Biotecnologie SRL, Pisa, Italy) for invaluable help with artwork, Dr. J. Garber (Dana-Farber Cancer Institute, Boston, MA), Dr. R. Kennedy (Dana-Farber Cancer Institute, Boston, MA), Dr. M. Loda (Dana-Farber Cancer Institute, Boston, MA) and Dr. P. Vermeer (University of Iowa, Iowa City, IA) for reading the manuscript, Dr. C. Colin, Dr. P. O'Donovan, Dr. D. Silver for help with techniques and reagents, and all other members of the Livingston and Drapkin laboratories.

**Funding:** this work was supported by an AIRC (Associazione Italiana per la Ricerca sul Cancro) regional grant to M.A.C. A.D.N. is a recipient of a postdoctoral fellowship by the Susan G. Komen Breast Cancer Foundation. M.T. was funded by the Fondazione Comel at the time of this study. B.S. is a recipient of a doctoral fellowship by the Austrian Academy of Science. R.D. is supported by the NCI (K08 CA108748; Ovarian Cancer SPORE P50CA105009), the Robert and Debra First Fund, the Fannie Rippe Foundation, the Randi and Joel Cutler Ovarian Cancer Research Fund, the Phi Beta Psi Sorority Charity Trust Fund, and the Ovarian Cancer Research Fund (OCRF).

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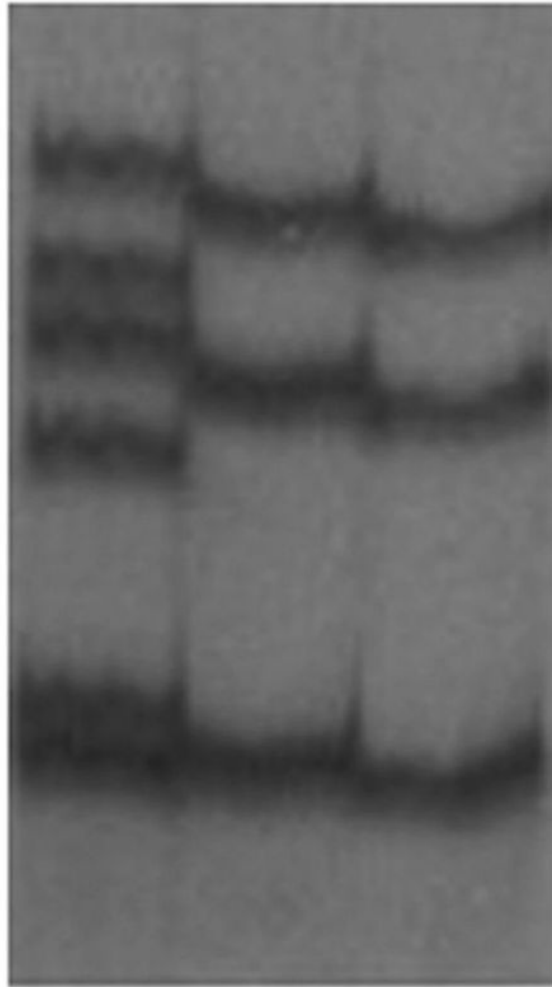
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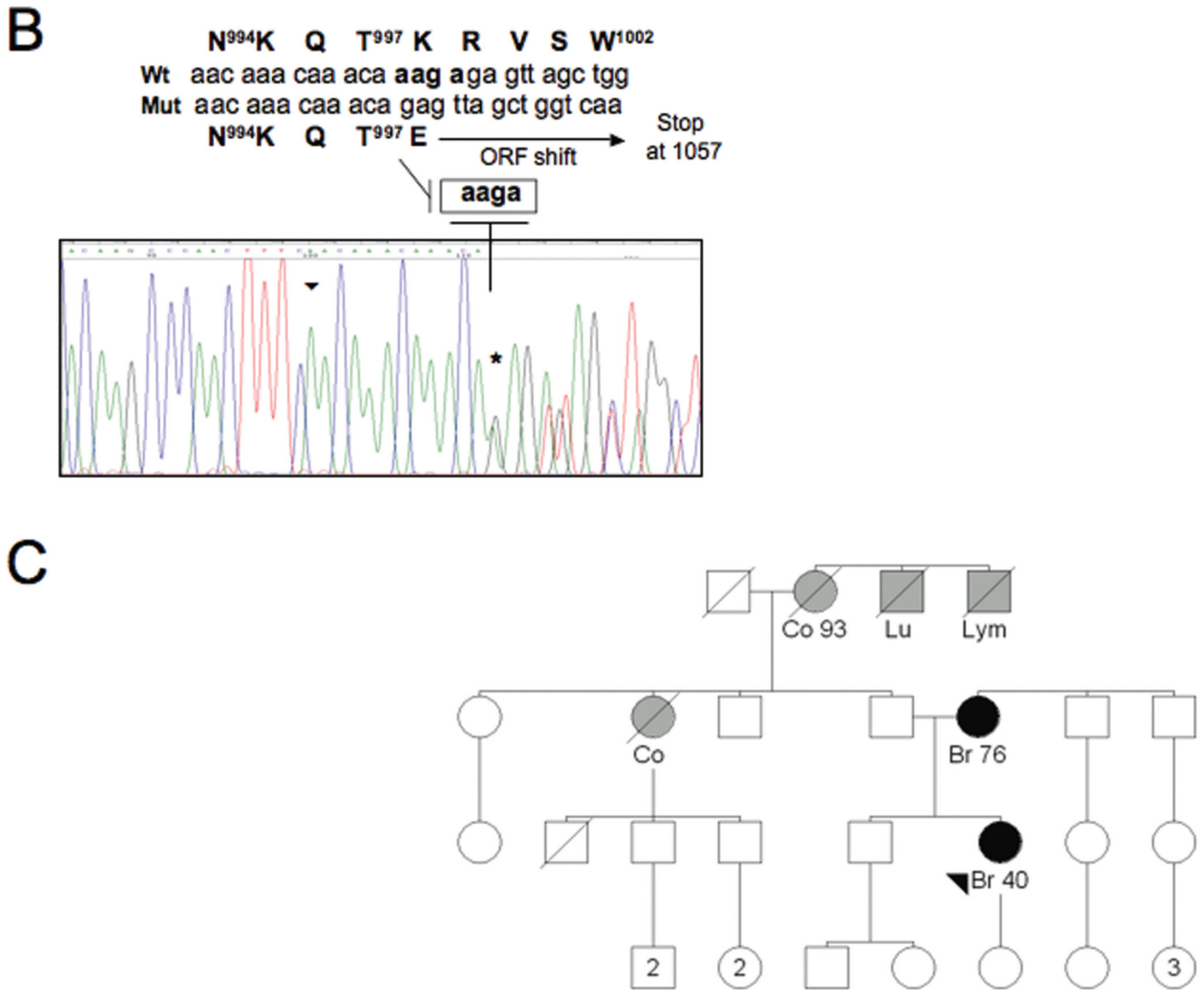
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**A**



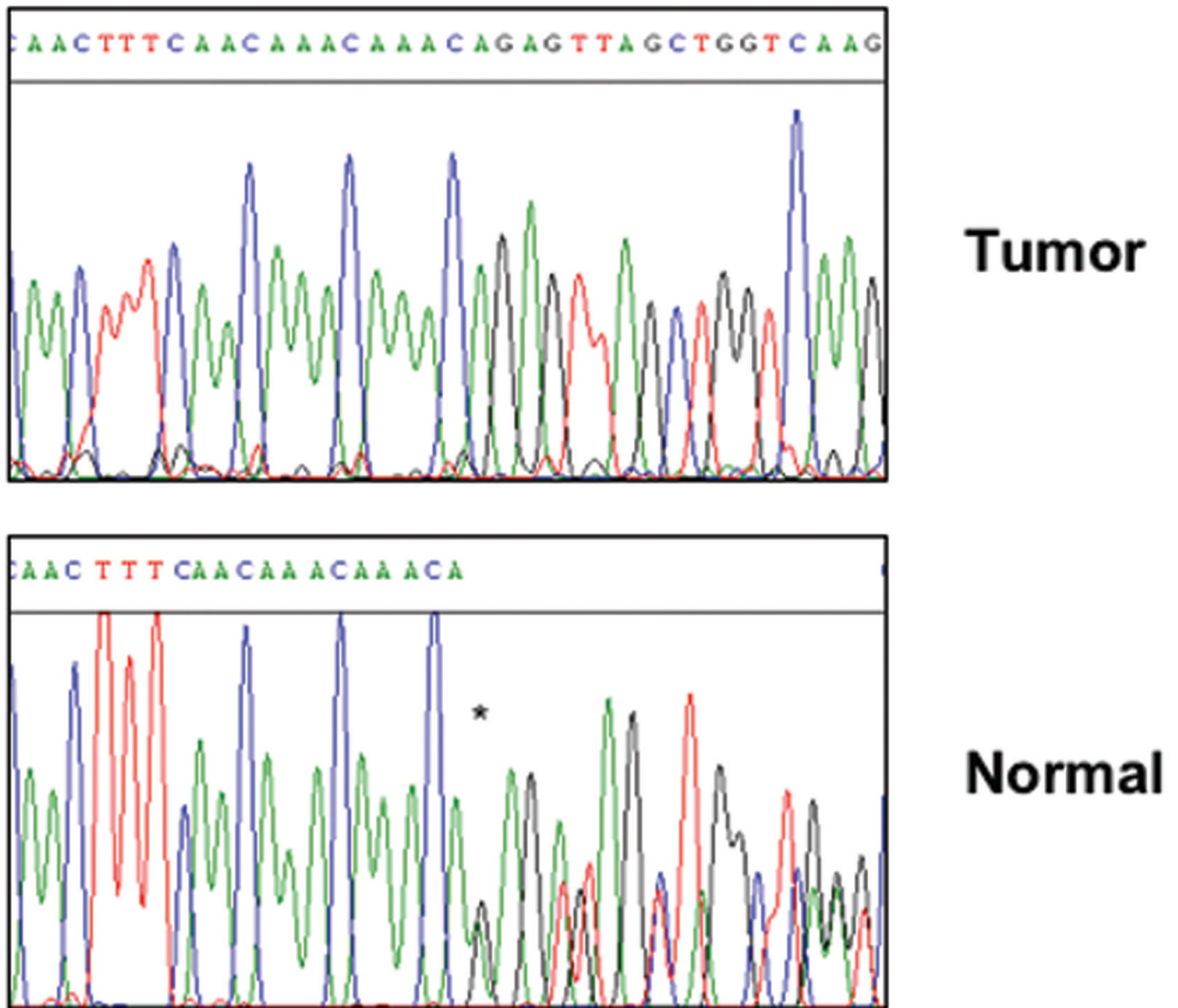
**1 2 3**



**Figure 1. A novel *BRIP1* sequence alteration identified in the family Pisa#88**

(A) Identification of a novel *BRIP1* sequence variant. SSCP analysis after PCR amplification using primers located in *BRIP1* exon 20 reveals an altered electrophoretic pattern in the sample corresponding to the proband of the family Pisa#88 (lane 1). Lanes 2 and 3, corresponding to the families Pisa#61 and Pisa#137, respectively, show a normal migration pattern, as ascertained in healthy control individuals (data not shown). (B) Characterization by sequence analysis of the *BRIP1* gene alteration detected in the index case of the family Pisa#88. The chromatogram displays a 4 nucleotide-deletion, which starts at position 2992 (Genebank database sequence reference NM\_032043, nucleotide numbering starting at codon 1), as indicated by an asterisk, and causes a shift of the open reading frame (ORF). The sequence of the wt and mutated alleles appear overlapped. The wt and mutated cDNA sequences are detailed (starting point marked by an arrowhead in the chromatogram). The corresponding amino acid sequences are reported, as well. The ORF shift (starting at codon 998) caused by the deletion results in a premature stop codon at position 1057. (C) Pedigree of the family Pisa#88, exhibiting the c.2992-2995delAAGA mutation. Black-filled symbols indicate individuals diagnosed with breast cancer (Br= breast cancer). Grey-filled symbols indicate individuals

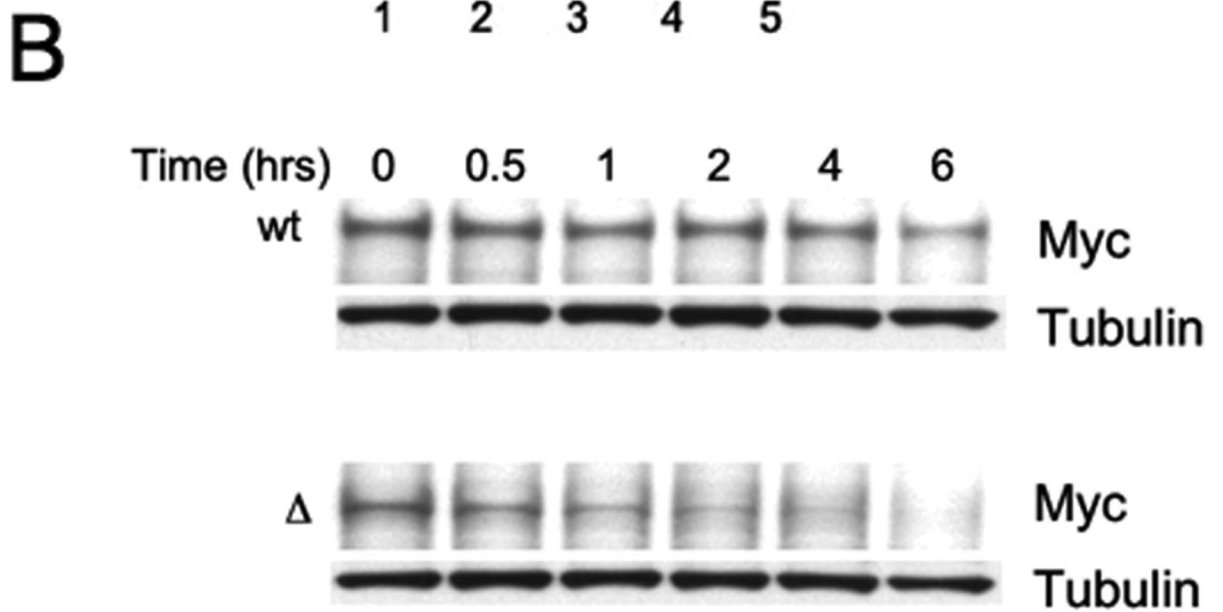
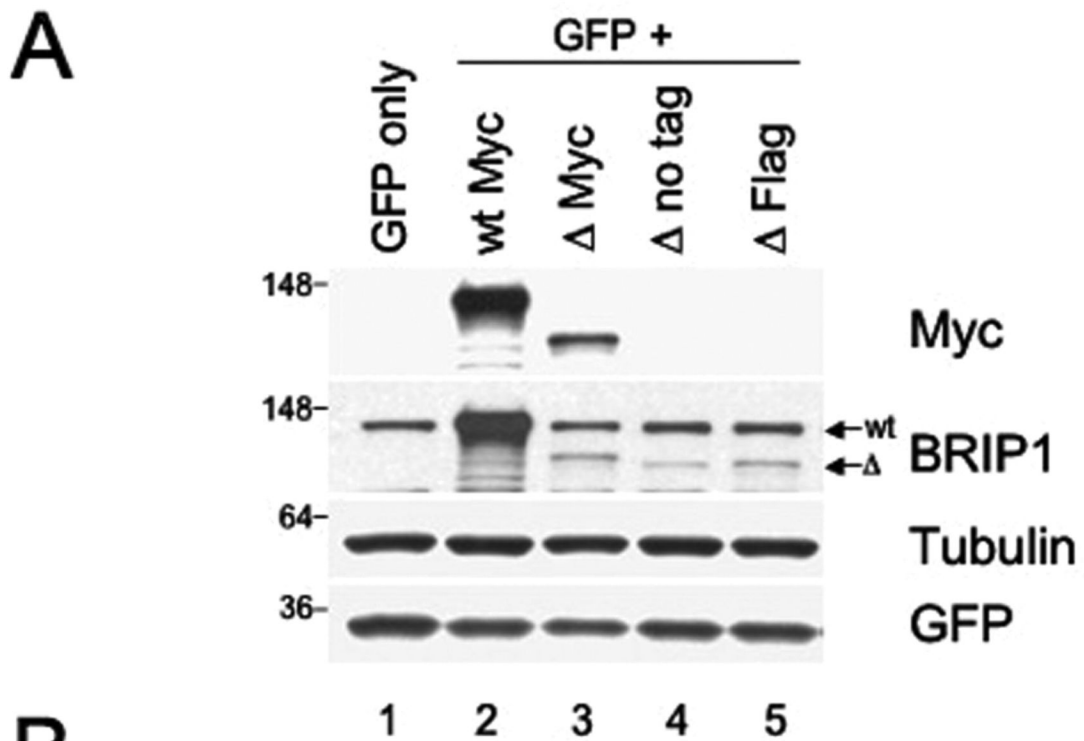
diagnosed with cancer other than breast (Co= colon cancer, Lu= lung cancer, Lym= lymphoma). The arrowhead indicates the index case.



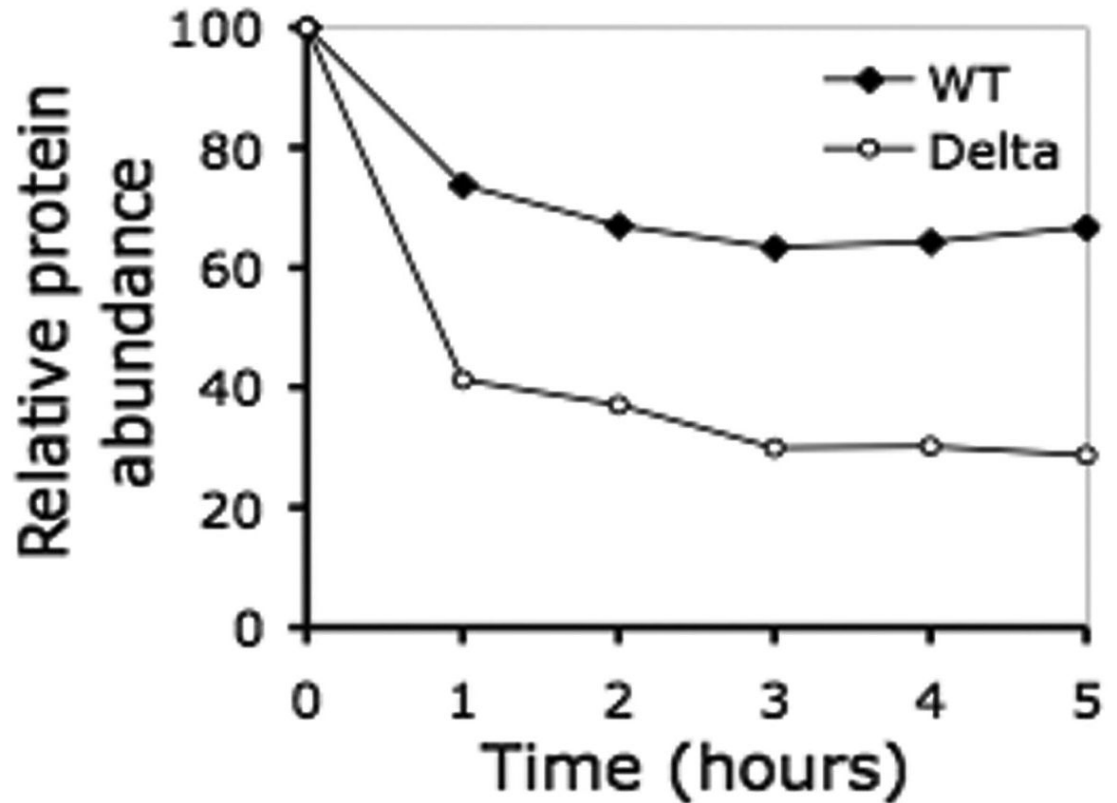
**Figure 2. Breast tumor tissue from Pisa#88 proband displays LOH at the *BRIP1* locus**  
 Chromatograms of tumor and normal breast tissue DNA from Pisa#88 index individual. Loss of the wild type allele with retention of the mutant one is evident in the tumor sequence (upper panel). Normal tissue (lower panel) shows both mutated and wt alleles (an asterisk marks the beginning of the overlapping sequences).





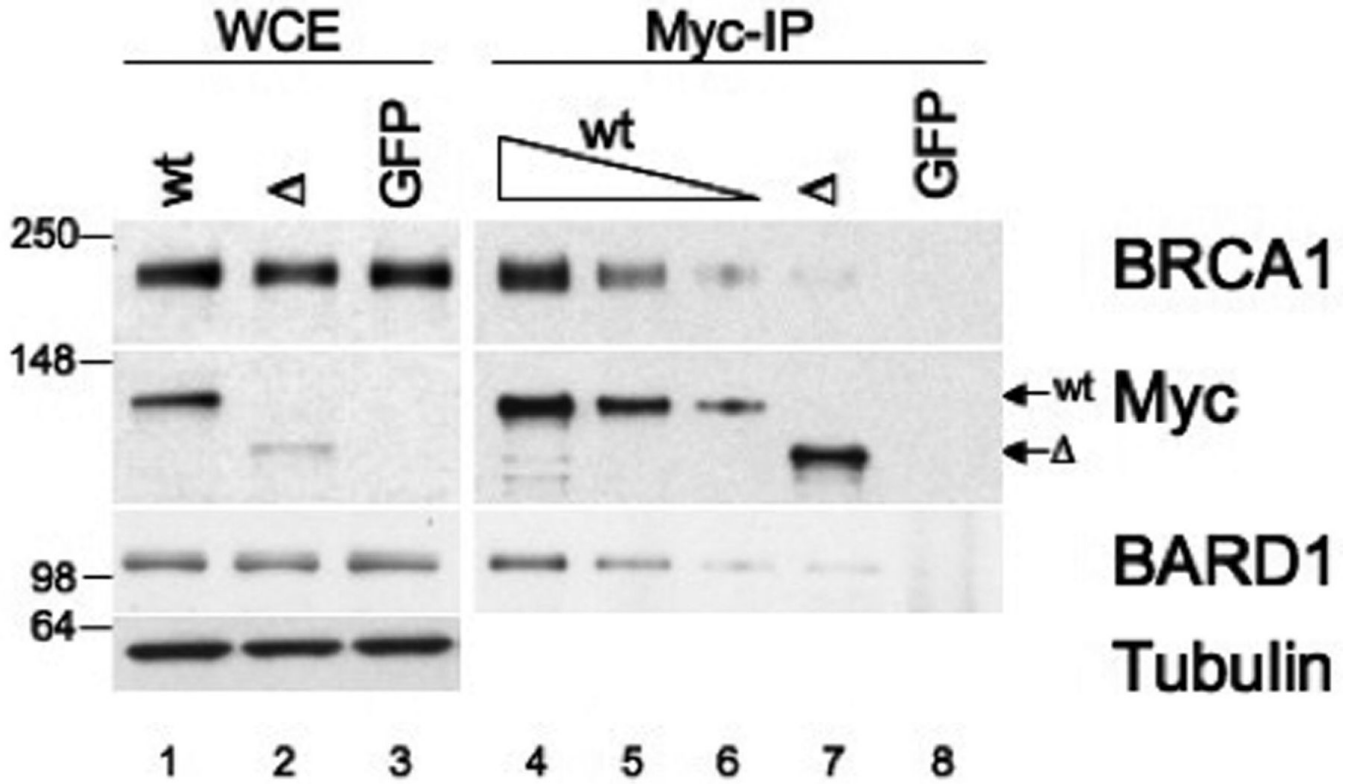


C



**Figure 4. The recombinant  $\Delta$ BRIP1 is unstable when expressed in 293T cells**

(A) Lysates from 293T cells transfected with a GFP-encoding plasmid alone (lane 1) or in combination with either Myc-wtBRIP1- (lane 2) or Myc- $\Delta$ BRIP1-encoding constructs (lanes 3-5) were resolved by SDS-PAGE and probed with the indicated Abs. A significantly reduced abundance of  $\Delta$ BRIP1 compared to wtBRIP1 was evidenced with both anti-Myc (lane 3 vs lane 2) and anti-BRIP1 (lanes 3-5 vs lane 2) Abs. (B) Cycloheximide-chase analysis of 293T cells expressing Myc-tagged wt or  $\Delta$ BRIP1 proteins. Equal amounts of lysates, prepared at the indicated time points, were analyzed by western blot with anti-Myc and anti-tubulin Abs. A significant decrease of the  $\Delta$ BRIP1 signal intensity was detectable already after 1 hour, as opposed to the steady level of wtBRIP1 signal. The experiment was repeated three times with consistent results. (C) Densitometric analysis of a representative cycloheximide chase experiment. Films were scanned and analyzed using the Image J software. The intensity of each band, after normalization to tubulin, which served as loading control, was plotted to determine the half-lives of the wt and mutant BRIP1 proteins.



**Figure 5. ΔBRIP1 exhibits defective ability to interact with BRCA1**

293T cells were transfected with Myc-wtBRIP1-, Myc-ΔBRIP1- or GFP-encoding plasmids. Lysates (lanes 1-3) and corresponding Myc-IPs (lanes 4-8) were analyzed by W-blotting with the indicated antibodies. BRCA1 and BARD1 were detected in Myc-wtBRIP1 complexes even when the IP was diluted up to 10 fold (lanes 4-6), whereas they were both barely detectable in undiluted Myc-ΔBRIP1 complexes (lane 7). IP from GFP-transfected cells (lane 8) served as control.

**Table 1*****BRIP1* sequence alterations**

Exon/Intron	Nucleotide change <sup>a</sup>	Effect on protein	Allele frequency <sup>b</sup> (%)
Intron 5	IVS5-31C>G	unknown	1/98 (1%)
Exon 19	2637G>A	Glu879Glu	32/98 (33%)
Exon 19	2755C>T	Pro919Ser	48/98 (49%)
Exon 20	2992-2995delAAGA	Glu998fsX1057	1/98 (1%)
Exon 20	3411C>T	Tyr1137Tyr	81/98 (83%)

<sup>a</sup>Numbering based on RefSeq NT\_010783.13 (for the intronic variant) and NM\_032043 (for all the variants within the coding region). For exonic variants, numbering starts at codon 1.

<sup>b</sup>Frequency of second allele listed.