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# Regulated Expression of a FANCL Splicing Variant as a Potential Modifier of DNA Repair Activity

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## Abstract

Fifteen Fanconi Anemia (FA) proteins work altogether to repair DNA interstrand cross-links (ICLs) in response to DNA damage. Monoallelic mutation associated with breast cancer predisposition has been demonstrated for the *FANCD1/BRCA2*, *FANCJ/BRIP1*, *FANCN/PALB2* and *FANCO/RAD51C* genes. The monoubiquitination of FANCD2, which represents the key event of the FANC/BRCA repair pathway is catalyzed by FANCL, which is the only FA protein known to possess an ubiquitin ligase activity. We have identified and studied the expression as well as the impact of a splicing isoform of the *FANCL* gene (*FANCLΔ4*) on DNA repair processes as well as its cellular localization.

A *FANCL* splicing isoform showing the skipping of exon 4 has been isolated in non-BRCA1/2 breast cancer patients and is expressed in several human cell lines. The efficient translation of the *FANCLΔ4* isoform into a functional protein has been shown using the polyribosomal fractions. Although in silico analyses did not reveal any potential implication of any sequence variation, the expression of this isoform has been demonstrated to be under the control of a specific genomic variant localized 11 nucleotides upstream of exon 4. Experiments performed in *FANCL*-deficient cells transfected with the *FANCLΔ4* isoform demonstrated a decreased cell survival, G2/M cell cycle blockade and absence of FANCD2 monoubiquitination. The FANCLΔ4 protein remains localized in the cytoplasm in transfected HEK293T cells following mitomycin C treatment, unlike the FANCL protein that migrates to the nucleus under such conditions.

A splicing isoform of a given FA gene may impact on the severity of the clinical manifestations when occurring in a FA mutation background, such as what is seen with FA and breast cancer patients. Evaluation of the sensitivity of this *FANCLΔ4* isoform to chemotherapeutic agents commonly used in cancer treatment would be of interest in the context of long-term management of affected individuals and their families.

**Keywords:** FANCL; Fanconi Anemia; Splicing; DNA repair

**Abbreviations:** FBS: Fetal Bovine Serum; DNA: deoxyribonucleic acid; cDNA: Complementary Deoxyribonucleic Acid; DAPI: 4', 6-Diamidino-2-Phenylindole; ELF: E2-Like-Fold; HWE: Hardy-Weinberg Equilibrium; INHERIT: Interdisciplinary Health Research International Team on Breast Cancer Susceptibility; kb: kilobase; MMC: Mitomycin C; PBS: Phosphate Buffered Saline; PBS-T: Phosphate Buffered Saline Supplemented With 0,1% Triton; PLB: Polysome Lysis Buffer; RIN: RNA Integrity Number; RING: Really Interesting New Gene; RIPA: radioimmunoprecipitation assay buffer; RNA: ribonucleic acid; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; URD: Ubiquitin-Conjugating-RWD

## Introduction

Breast cancer susceptibility has been demonstrated to be modified by germline mutations in several genes such as *BRCA1*, *BRCA2*, *TP53*, *ATM*, *CHEK2*, *BRIP1*, *PALB2* and *RAD51C*. These breast cancer susceptibility genes represent the major heritability portion of familial breast cancer, and mutations in these genes are found in less than 25% of breast cancer families showing a clear pattern of inheritance [1-3]. Thus, other susceptibility alleles remain to be identified to explain the increased susceptibility in the remnant high-risk families. Of interest, among the genes recently associated with an increased susceptibility to breast cancer, four are Fanconi Anemia (FA) genes: *FANCD1/BRCA2*, *FANCJ/BACH1/BRIP1*, *FANCN/PALB2* and *FANCO/RAD51C* [4-13]. It is noteworthy that most of the genes associated with breast cancer susceptibility, including FA genes, are involved in DNA repair mechanisms.

Until now 15 distinct complementation groups of FA proteins have been described by cell fusion, cell transduction studies or protein complex purification (FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -O, -P) [14-16]. FA is a rare autosomal or X-linked (FANCB) recessive disease associated with congenital abnormalities, progressive bone marrow failure and increased susceptibility to cancer [17-21] such as Acute Myeloid Leukemia, aero-digestive and gynaecological cancers [22-25]. The sensitivity of FA cells to cross-linking agents such as mitomycin C (MMC) is used to determine if two distinct cell lines belong to the same complementation group.

The FANCL protein through its E3 ubiquitin ligase domain, is the only FANC protein to exibit the catalytic function needed for FANCD2 and FANCI monoubiquitination [26,27], and this represents the key event in DNA repair of interstrand cross-link (ICL) damages. Following this monoubiquitination, the activated FANCD2-FANCI proteins recruit or interact with DNA repair proteins such as FAN1, BRCA1/2, FANCN, FANCJ, RAD51C as well as diverse nucleases and

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polymerases, to accurately repair ICL and double-strand DNA breaks (DSBs) by homologous recombination (HR).

In addition, FANCL has been demonstrated to have a key role in foci formation and additional data suggest that FANCL would also own a FANCD2-independant role in ICL repair [28,29]. Cytosolic extracts depleted of FANCL showed a defect in the extent of nucleotide incorporation, (i.e. replication restart) in the presence of MMC and further analysis indicates that the defect is primarily due to the inability of replication forks to elongate past lesions caused by MMC [30]. *FANCL* mutation may also disrupt the core complex by affecting particularly the mobilization of FANCM into sites of cross-link damage [29].

Given the key function of FANCL and the close connection between the FANC and BRCA1/2 proteins in DNA repair, the coding sequence and intron-exon boundaries of the *FANCL* gene were analyzed in a cohort of *BRCA1/2*-negative (BRCAX) breast cancer families and healthy controls from the French Canadian population. We identified a splicing variant that could eventually modulate breast cancer risk and DNA repair efficiency.

## Material and Methods

### Ascertainment of families and genomic DNA extraction

All 96 BRCAX individuals from high-risk French Canadian breast and ovarian cancer families participating in this study were originally part of a larger interdisciplinary program termed INHERIT BRCAs [31,32]. All participants were at least 18 years of age, mentally capable and had to sign an informed consent. Ethics approval was obtained from the participating institutions. The details regarding selection criteria of the breast cancer cases as well as the experimental and clinical procedures have been described previously [33-35]. Lymphocytes from breast cancer individuals were isolated and immortalized as previously described [34,35] and genomic DNA was isolated using the QIAamp DNA Blood kit (Qiagen, Santa Clara, CA). Genomic DNA from peripheral blood of healthy individuals was isolated using Gentra kits (Minneapolis, MN, USA).

### PCR amplification, direct sequencing and variant characterization

PCR amplification of the 14 coding exons of the *FANCL* gene (NM_001114636.1), as well as the flanking intronic regions, was performed on breast cancer cases using a set of 12 primers pairs listed in Supplemental Table 1A. The PCR products were sequenced with an ABI3730XL automated sequencer using the Big Dye 3.1 kit (Applied Biosystems, Foster City, USA) following the manufacturer's instructions. Sequence analyses were performed using Staden preGAP4 and GAP4 programs (http://staden.sourceforge.net/). The frequency of the rs79588315 variant was also determined in healthy individuals. Amplification of the alternative spliced isoform FANCLΔ4 was performed on complementary DNA (cDNA) from BRCAX individuals and human cell lines using primers listed in Supplemental Table 1B.

Deviation from Hardy-Weinberg equilibrium (HWE) and allelic difference of the rs79588315 variant between both series, were evaluated by two-sided Chi Square test with 1 degree of freedom using a web-based software program located at: http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa2.pl. The prediction of the effect of a given variant on exonic and intronic splicing modulators was assessed using the Human Splicing Finder (HSF) [36] which contains all available matrices from auxiliary sequence prediction softwares such as ESEfinder 3.0 program [37,38] for binding sites of the 9G8, Tra2-β and hnRNP A1 proteins.

### Cell culture maintenance and plasmids

HEK293T cells were grown in DMEM (Wisent Inc, St-Bruno, Qc,CAN) with 10% fetal bovin serum (FBS) and 1% penicillin/streptomycin. EUFA868 (*FANCL*-deficient) cells were obtained from the FA Research Fund and maintained in RPMI-1640 medium supplemented with 15% FBS, 1% L-glutamine and 1% penicillin/streptomycin. All other cell lines are routinely cultured in our laboratory and the conditions have been optimized as recommended by the American Type Culture Collection (ATCC). Concerning MMC treatments, given that the concentrations used was dependent on the experiment, values are indicated in each respective section.

pcDNA3 plasmid (Invitrogen) was modified by inserting a HA tag (YPYDVPDYASL) and stop codon using the Apa1 restriction site. Plasmids pEGFP-N3 and pMSCVneo were obtained from Clontech. FANCL WT or FANCLΔ4 cDNAs were inserted into EcoR1 and Xho1 restriction sites for pcDNA3-HA and pEGFP-N3, and EcoR1 for pMSCVneo. Plasmid transfection was performed using ExGen 500 (Fermentas) according to manufacturer's protocol.

### RNA isolation and qRT-PCR

Total RNA from cell lines was extracted using TRI Reagent® (Molecular Research Center inc, Cincinnati, OH, USA) according to the manufacturer's instructions as previously described [34]. The quality of the RNA samples was verified using a Bioanalyser (Agilent Technologies). Thereafter, reverse transcription of 2.5 µg of standardized RNA samples was performed using 250 ng random hexamers and 200 U of SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the supplier's protocol.

Quantification of mRNA levels was performed using the SyBr Green method as described previously [39]. The reactions were carried out using the amount of cDNA corresponding to 20 ng of initial total RNA. Specific splice-junction oligoprimer pairs (Supplemental Table 1B) that allow the amplification of ~200 bp of indicated specific mRNA were designed by GeneTools software and their specificity were checked by blasting the GenBank database. Data calculation and normalization were performed using the second-derivative and double-correction method [39], and three housekeeping genes. The mRNA levels were expressed as number of copies/µg of total RNA calculated using corresponding standard curves. The spliced/WT mRNAs expression ratio was then determined to compare minigene construct expression.

### Minigene assays

The minigene constructs were assembled into the pcDNA3 (Invitrogen Life Technologies, Burlington, ON) vector. The genomic regions containing the exons 3 to 5, which include large flanking intronic sequences (~6.5 kb) were PCR-amplified from BRCAX genomic DNA. The integrity of the total insert was then confirmed by sequencing. Sequence variations observed in our cohort of French Canadian BRCAX breast cancer families were then introduced into the WT minigene construct by directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Mississauga, ON) following manufacturer's recommendations. Primers used for minigene contructs are listed in Supplemental Table 1C.

For minigene construct transfection, HEK293T cells were plated at 350 000 cells in 6-wells culture plates (BD Biosciences, Mississauga, ON), and 48 hours after plating, cultures were transfected with 3 µg of minigene plasmid. Cells were harvested 24 hours later for RNA extraction. Cells were rinsed with cold phosphate-buffered (PBS) and

| SNP | SNP ID[1] (dbSNP ID) | Location | Amino acid change | Series (N=) | hom | het | rare hom | MAF[2] | p-value[3] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | c.-67-54C/G (rs143259350) | Promoter | - | Cases (96) | 93 | 3 | 0 | 0.016 | 0 |
| 2 | c.112C/T (rs55849827) | Exon 2 | Leu38Phe | Cases (96) | 95 | 1 | 0 | | - |
| 3 | c.216+194T/C (rs56246409) | Intron 3 | - | Cases (96) | 70 | 24 | 2 | 0.146 | - |
| 4 | c.217-141G/A (rs17049422) | Intron 3 | - | Cases (96) | 91 | 5 | 0 | 0.026 | - |
| 5 | c.217-11T/C (rs79588315) | Intron 3 | - | Cases (96) Controls (94) | 69 72 | 25 22 | 2 0 | 0.151 0.115 | 0.33 |
| 6 | c.374+34delT (rs200325071) | Intron 5 | - | Cases (96) | 87 | 9 | 0 | 0.047 | - |
| 7 | c.375-120insATTA (NA) | Intron 5 | - | Cases (96) | 92 | 4 | 0 | 0.021 | - |
| 8 | c.375-85A/G (rs62140045) | Intron 5 | - | Cases (96) | 92 | 4 | 0 | 0.021 | - |
| 9 | c.375-49C/G (rs1404459) | Intron 5 | - | Cases (96) | 80 | 13 | 3 | 0.099 | - |
| 10 | c.706+59delTAA (rs148383677) | Intron 8 | - | Cases (96) | 46 | 43 | 7 | 0.297 | - |
| 11 | c.706+70A/G (rs848288) | Intron 8 | - | Cases (96) | 46 | 43 | 7 | 0.297 | - |
| 12 | c.707-27A/C (rs12624152) | Intron 8 | - | Cases (96) | 82 | 12 | 2 | 0.083 | - |
| 13 | c.790+22C/T (rs10445896) | Intron 9 | - | Cases (96) | 69 | 25 | 2 | 0.151 | - |
| 14 | c.790+31A/G (rs10445895) | Intron 9 | - | Cases (96) | 68 | 26 | 2 | 0.156 | - |
| 15 | c.918+32C/G (rs149412402) | Intron 11 | - | Cases (95) | 93 | 2 | 0 | 0.01 | - |
| 16 | c.996C/T (rs848291) | Exon 12 | Ser332Ser | Cases (96) | 35 | 46 | 15 | 0.396 | - |
| 17 | c.1092T/C (rs11539575) | Exon 13 | Cys364Cys | Cases (96) | 90 | 6 | 0 | 0.031 | - |
| 18 | c.1108-65G/A (rs3732136) | Intron 13 | - | Cases (91) | 43 | 38 | 10 | 0.302 | - |

Exonic variants are displayed in bold characters
1 SNP ID are indicated according to the nomenclature guidelines of the Human Genome Variation Society (RefSeq NM_001114636.1). The first base from the ATG codon is counted as +1. dbSNP ID is indicated according to build 137, NA indicating a SNP not found in the database.
2 MAF: Minor allele frequency.
3 p-value of significance between MAF found in cases and controls

**Table 1:** Sequence variations in *FANCL* gene and genotype frequencies in familial breast cancer cases and controls (rs79588315).

then suspended in Tri-Reagent. RNA extraction and cDNA synthesis were processed as described above. For PCR amplification, specific primers for *FANCLΔ4* and *FANCL* mRNA isoforms in combination with a primer specific to the transcribed region of the minigene vector (5'-ACGACTCACTATAGGGAGACCCA-3') [40,41] were designed with the GeneTools (Biotools Inc., Madrid, Spain) software.

## Ribosomal fractions

T47D cells (approximately $4\times10^7$ cells per fraction) were grown and then collected at 70% confluency. Cycloheximide (CHX 50 µg/ml) was added to cell cultures, incubated for 30 minutes and cells were then rinsed with phosphate-buffered (PBS) - CHX (100 µg/ml) and trypsin. Cells were resuspended in PBS- CHX (100 µg/ml) at a concentration of $10^7$ cells/ml. The total of 12 ml of cells was then split into 3 samples (-EDTA, +EDTA, protein extraction sample). Cells were centrifuged at 1 600 rpm for 5 minutes and then resuspended in lysis buffer and NP40 (0.5%) was then added. After 10 min on ice, cells were centrifuged at 750 g at 4°C and the supernatant was harvested, kept on ice and subsequently used in the sucrose gradient. Sucrose gradients were prepared with gradient Maker (CBS Scientific Compagny inc, model GM-100). Each fraction counts 3 samples, including the one adjusted at 20mM ethylenediaminetetraacetic acid (+EDTA sample). Samples were separated on a 15%-45% sucrose gradient in lysis buffer (without NP40), and centrifuged 2 hours 15 minutes at 36 000 rpm at 4°C. Gradients were fractionated using the Programmable Density Gradient System (Teledyne Isco incorporated, Lincoln, NE) from the top and measured at A260 to determine the polysome profile. Fractions were then selected for RNA extraction. RNA extraction and qRT-PCR were then performed as described above.

## Fluorescence microscopy

HEK293T cells were transfected with FANCL- or FANCLΔ4-EGFP vectors. Cells were then treated (or not) with MMC. Cells ($3.5x10^5$ cells per well) were seeded on microscopic slides coated with L-lysine. HEK293T cells were grown in 15% FBS DMEM medium (Wisent Inc, St-Bruno, Qc, CA) and 16 hours later cells were washed in PBS and then fixed with 4% paraformaldehyde at room temperature (RT). Unspecific binding sites were blocked with 1% BSA and 0,02% sodium azide for 1 hour at RT. Cells were rinsed twice with PBS and then incubated for 15 min with DAPI (4',6-diamidino-2-phenylindole). Cells on cover slides were rinsed again twice with PBS and then mounted on slides with mounting medium (Sigma-Aldrich). Pictures were taken using a Leica DM5000B microscope with the DFC350x camera with the 40x lens, and imported with Photoshop Professional 5 (Adobe Systems Inc., Seattle, WA).

## Retroviral transduction

HEK293T G/P cells ($5\times10^6$) were plated in 10 cm petri dishes and incubated at 37°C with 5% $CO_2$ for 8 hours. The pMSCVneomycin (with or without the cDNA of interest), G/P and VSVG vectors were co-transfected using ExGen500 (Fermentas, Fisher Scientific Limited, Nepean, ON) and then incubated overnight in the same conditions. The day after, the medium was replaced with fresh DMEM and cells were incubated for 48 hours under the same conditions, after what the virus were harvested through filtration, aliquoted and kept at -80°C. For infection, EUFA868 cell line was transduced with pMSCVneo retroviral constructs containing FANCLwt or FANCLΔ4 cDNA and with empty vector as negative control. The transduced cells were selected with neomycin for approximately 2 months. The complemented cell lines were next analyzed for FANCD2 monoubiquitination, cell cycle arrest and cell survival assay upon MMC treatment.

## Western blotting

Proteins were extracted from approximately $40x10^6$ cells using

standard RIPA buffer protocol. Protein concentrations were measured with the Bradford protein assay using an Infinite M1000 microplate reader and the Megellan program. Protein samples were migrated on a 8% acrylamide gel, using the Mini-Protean Tetra Cell kit (BIO-RAD, Life Science Research, Mississauga, ON) and then transferred onto an Amersham Hybond-ECL nitrocellulose membrane (GE HealthCare, Mississauga, ON). After blocking with 5% milk/PBS, the blot was incubated with the FANCD2 antibody (overnight rocking at 4°C) and secondary antibody (1 hour rocking at RT) diluted in 5% milk/PBS. The primary antibodies used were rabbit anti-FANCD2 (Novus Biologicals, 1:10 000) and rabbit anti-Calnexin (Enzo (Life Sciences), 1:1 000) while the secondary antibody was the ECL anti-rabbit IgG horseradish peroxidase-linked antibody (GE HealthCare, 1:10 000). Blotted proteins were detected using Lumiglo reserve chemiluminescent substrate (KPL).

## Flow cytometry

For cell cycle analysis, EUFA868 cells were grown at a concentration of 350 000 cells/ml and either treated (50 ng/ml) or not with MMC for 24 h. Cells were pelleted at 1500 rpm and washed in PBS twice, and then fixed in ice cold 70% ethanol overnight at -20°C. Fixed cells were pelleted, resuspended in 4°C PBS, pelleted again and resuspended in DNA staining solution (1µg/mL DAPI in PBS) for 15 minutes, after which measurements were taken. Stained cells were sorted on a BD SORP LSR II FACS with a 20 mW Coherent® Solid State UV laser 355 nm w/z PMT with a BP 450/50 filter and data were analyzed using the DIVA (BD Biosciences) and FlowJo (Three Star Inc.) programs.

## Survival assays

An average of $3\times10^5$ EUFA868 cells per 10 ml were grown in RPMI medium containing 15% FBS in T-25 flasks. To perform the test, cells were first centrifuged, counted and then diluted to $1.5\times10^4$ cells/100 µl/well. The 100 µl contained the adjusted concentrations of crosslinking agent. The readings were performed after 1, 2, 3, 4, and 5 days. Alamar Blue assays (Invitrogen, Carlsbad CA) were performed according to the manufacturer's instructions. 10 µl of Alamar Blue was added to each well, and after 4 hours the plate was analyzed using an Infinite M1000 (Tecan Systems inc., San Jose, CA) microplate reader with the Megellan program (Tecan). The negative background was subtracted using the measurements obtained from the negative controls (medium and selective agents alone). For the latter, a number of $1.5\times10^4$ EUFA868 cells were plated in a volume of 100 µL in a 96-well plate with corresponding MMC concentrations (0 to 300 nM). Cell survival percentage was measured at day 1 to 5 using Alamar Blue assays according to manufacturer's protocol.

## Results

In an attempt to identify deleterious or truncating mutations of the *FANCL* gene that could be associated with breast cancer susceptibility, we sequenced all 14 exons and adjacent intronic sequences in a cohort of 96 breast cancer cases coming from high-risk families. Although no deleterious mutation was found in the *FANCL* coding region of our French Canadian breast cancer cases, we identified 18 variants in exonic and flanking intronic sequences, including one novel sequence variation (c.375-120insATTA) not reported in the NCBI single nucleotide polymorphism database (dbSNP Build 137) (Table 1). Among the 18 variants, 15 were intronic variants and 3 were located in exonic regions. Two of these coding variations were synonymous changes (c.999C/T and c.1092T/C), while the missense change was a rare variant leading to a Leucine/Phenylalanine amino acid substitution (c.112C/T; Leu38Phe). The Leu38 residue was fully conserved in mammals, therefore suggesting that this amino acid was under strong functional constraint or may have a specific role on protein conformation (data not shown). This amino acid change was predicted to be probably damaging by the Polyphen software with a score of 0.967 (data not shown). This sequence variation was observed in only one breast cancer case (Table 1), and it is located in the E2-like fold protein domain (Figure 1A). All other 15 variants were intronic variations located near intron/exon junctions, including *rs79588315,* which are located 11 base pairs upstream of exon 4. Again the c.217-11T nucleotide is conserved in all mammalian species and given its location, this position could be involved in splicing regulation. This c.217-11T/C variant was therefore further genotyped in a cohort of healthy individuals from the same origin, and no significant difference of MAF between both sample sets was identified (Table 1).

cDNA amplification in BRCAX individuals revealed a significant expression of an alternative spliced isoform of the *FANCL* gene lacking the exon 4 (FANCLΔ4). As illustrated in Figure 1A, this deletion disrupted the E2-like fold and WD-40 protein domains of the FANCL protein. This *FANCLΔ4* isoform is significantly expressed in

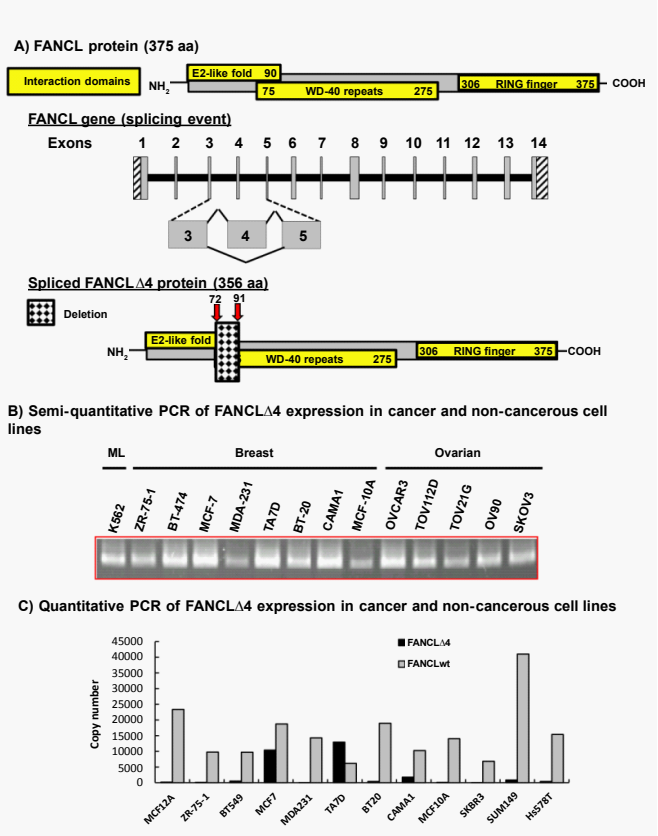


**Figure 1:** FANCL interaction domains, *FANCL* splicing and mRNA expression. (A) Schematic representation of FANCL interaction domains based on the literature. *FANCL* exon structure and schematic representation of the putative protein of the FANCLΔ4 isoform located in the genomic region of exons 3-5 (B) Expression levels of FANCLΔ4 isoforms in cell lines as detected by semi-quantitative PCR experiments (C) Expression levels of FANCLΔ4 and FANCL isoforms in cell lines as measured by quantitative real-time PCR experiments. Relative expression levels of the FANCLΔ4 isoform were calculated as $E^{(\Delta Ct\ \text{wild-type allele} - \Delta Ct\ \text{splice form})}$ in twelve breast cancer and non-cancerous cell lines where E is primer efficiency.

leukemia, breast and ovarian cancer cell lines as demonstrated by semi-quantitative PCR (Figure 1B), whereas accurate qRT-PCR revealed high expression of this isoform in most of the breast cancer cell lines expressing the estrogen receptor (ER+) (Figure 1C). Of interest, this isoform is expressed at low levels in ER- (BT-549, MDA-231, BT-20, SKBR3, SUM149 and Hs578T) and non-cancerous cell lines (MCF10A and 12A), while T47D cells expressed higher levels of the *FANCLΔ4* than of the *FANCL*wt isoform.

Following the identification and expression of the *FANCLΔ4* isoform in BRCAX individuals and cancer cell lines, the putative impact of the relevant *FANCL* periexonic and exonic variants (i.e. located in the vinicity of the exon 4 genomic region) on mRNA splicing was assessed by *in silico* methods and is represented in Table 2. To characterize the putative impact of exonic and intronic variations on splicing, we used the HSF web program, which allows the identification of enhancer and silencer splicing sites as well as branch point sequences. In addition to the two variations identified in our cohort (rs17049422 and rs79588315), we also analyzed variants referenced in dbSNP database which have not been genotyped in our cohort given their deep intronic location. As described in Table 2, most sequence variations could have a significant effect on binding of RNA-binding proteins such as serine/arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNP). Only two variations (rs79588315 and rs115418265) did not seem to exert any potential effect on splicing of exon 4 as predicted by *in silico* analyses. Nevertheless, we analyzed the individual effect of these intronic variants on exon 4 inclusion/exclusion using the minigene assay.

Including a WT version, eight minigene constructs of the *FANCL* gene were transfected into HEK293T cells for 24 hours, and levels of *FANCLΔ4*/*FANCL*wt transcripts encoded by the minigene vector were quantified by qRT-PCR. As described in Figure 2A and 2B, rs79588315 (c.217-11T/C) triggered a 14.5-fold increase of the *FANCLΔ4* mRNA expression and decreased significantly the expression of the *FANCL*wt transcript when compared to other minigene versions. All the other

| Genomic variant | Location | Motif | Reference score/motif (WT) | Variant score/motif | Effect/variation (%) |
|---|---|---|---|---|---|
| rs17049422* (c.217-141G/A) | Intron 3 | Acceptor site[1] | 5.46 | 5.13 | -6.04 |
| | | Branch point site[2] | 73.64 | 66.75 | -9.36 |
| | | SC35[2] | 81.75 | 84.33 | 3.16 |
| | | SRp40[2] | | 78.44 | New site |
| | | Enhancer EIE[3] | present | | Site broken |
| | | Enhancer 9G8[2] | | 65.57 | New site |
| | | Enhancer Tra2-b[2] | | 85.42 | New site |
| rs79588315* (c.217-11T/C) | Intron 3 | Acceptor site[2] | 78.59 | 76.74 | -2.35 |
| | | Acceptor site[1] | 3.01 | 1.83 | -39.2 |
| | | Enhancer EIE[3] | | present | New site |
| | | Silencer IIE[3] | present | | Site broken |
| rs115418265 | Intron 4 | Enhancer EIE[3] | | present | New site |
| | | Silencer Motif 3[4] | 67.6 | | Site broken |
| | | Silencer IIE[3] | | present | New site |
| rs7594702 | Intron 4 | Donor site[2] | 68.02 | 67.78 | -0.35 |
| | | SF2/ASF (IgM-BRCA1)[2] | 74.31 | 75.19 | 1.19 |
| | | SF2/ASF[2] | 82.59 | 75.19 | -8.96 |
| | | Silencer Motif 2[4] | 70.15 | | Site broken |
| | | Silencer Motif 2[4] | 60.23 | | Site broken |
| | | Silencer IIE[3] | present | | Site broken |
| rs73944840 | Intron 4 | SC35[2] | 83.77 | 80.27 | -4.18 |
| | | SRp40[2] | 80.12 | 82.28 | 2.69 |
| | | SF2/ASF (IgM-BRCA1)[2] | 74.08 | 82.28 | 11.07 |
| | | Silencer Motif 3[4] | | 61.51 | New site |
| | | Silencer Motif 3[4] | 62.08 | | Site broken |
| rs28588526 | Intron 4 | Donor site[2] | 50.37 | 77.18 | 53.3 |
| | | SC35[2] | | 75.85 | New site |
| | | SRp40[2] | | 83.71 | New site |
| | | Enhancer EIE[3] | present | | Site broken |
| | | Silencer IIE[3] | | present | New site |
| rs28391566 | Intron 4 | SF2/ASF (IgM-BRCA1)[2] | | 71.15 | New site |
| | | Enhancer EIE[3] | | present | New site |
| | | Silencer hnRNP A1[2] | 74.53 | | Site broken |

*Variant genotyped in our breast cancer cohort
EIE: exon-identity elements
IIE: Intron-identity elements
[1]Yeo & Burge, 2004, j Comput Biol 11:377
[2]Based on Human Splicing Finder matrices
[3]Zhang C et al 2008, PNAS 105:5797
[4]Sironi M et al 2004, Nucleic acids research 32:1783

**Table 2:** *In silico* analysis of *FANCL* genomic regions and variants potentially involved in the expression of *FANCLΔ4* spliced transcript.

variants did not alter the *FANCLΔ4*/*FANCL*wt expression ratio (Figure 2A).

To assess the reliability of the experiment and to verify that no other random mutation in the minigene construct could be involved in this effect, a revertant was generated by restoring the WT genotype on the rs79588315 minigene. As illustrated in Figure 2C and 2D, the experiment confirmed that the increase of *FANCLΔ4* expression seems to be almost exclusively dependent on the presence of the rs79588315 variant.

To examine further the effect of this rs79588315 variant on exon 4 exclusion in an *in vivo* context, immortalized lymphoblastoid cell lines (LCLs) RNA of 11 BRCAX WT individuals, 9 heterozygotes and 2 rare homozygote carriers were evaluated regarding the *FANCLΔ4*/*FANCL*wt expression ratio according to their genotype. RNA samples were normalized according the heterozygotes, given that the *FANCLΔ4* isoform is barely expressed in the WT individuals. *FANCLΔ4* mRNA is only expressed at basal levels in WT homozygote individuals, while it represents 25.0 and 40.1% of *FANCL* transcripts in heterozygotes and rare homozygotes, respectively (Figure 3A).

Considering the *FANCLΔ4*/*FANCL*wt expression ratio (Figure 3B), the WT individuals demonstrated an expression ratio of 0.02, while heterozygotes and rare homozygote carriers of the *rs79588315* variant have a relative *FANCLΔ4*/*FANCL*wt expression ratio of 1.0 and 2.01, respectively. These results indicated that the expression of the *FANCLΔ4* isoform is dependent on the number of alleles carrying this c.217-11T/C variation in BRCAX individuals.

In order to confirm the efficient translation of this *FANCLΔ4* mRNA transcript into a functional protein, the action of nonsense-mediated decay (NMD) was first tested by treating (or not) several immortalized LCLs of BRCAX individuals with puromycin, an agent known to inhibit NMD. Given that mRNA extracted from puromycin-treated cells did not show any modulation of the *FANCLΔ4* mRNA expression, this suggested that this isoform was not subject to NMD (data not shown). In addition, the polysome analysis allows determining whether a given mRNA isoform is recruited by the polyribosomes, which drive the translation of mRNAs into functional proteins. Therefore, as illustrated in Figure 4, the ribosomal fractions demonstrated that the *FANCLΔ4* mRNA transcript, being significantly detected in late fractions (fractions 11-15), was associated with multiple ribosomes (polysomes), which confirms its efficient translation. As negative control, each fraction was duplicated and treated with EDTA, which is known to dissociate mRNA from ribosomes.

Thereafter, to further evaluate the effect of the expression of the FANCLΔ4 protein on DNA repair mechanisms, several DNA repair-related assays such as monoubiquitination, survival, cell cycle as well as cell localization analyses have been performed.

FANCD2 monoubiquitination is the key event in the FANC-BRCA DNA repair pathway and this activation is performed by the FANCL protein. As seen in Figure 5, the transduction of FANCL-deficient EUFA868 cells with retroviral vector pMSCV containing the *FANCL* WT or Δ4 cDNAs clearly indicated that following a MMC treatment for 12 hours, only cells complemented with the plasmid pMSCVneomycin-

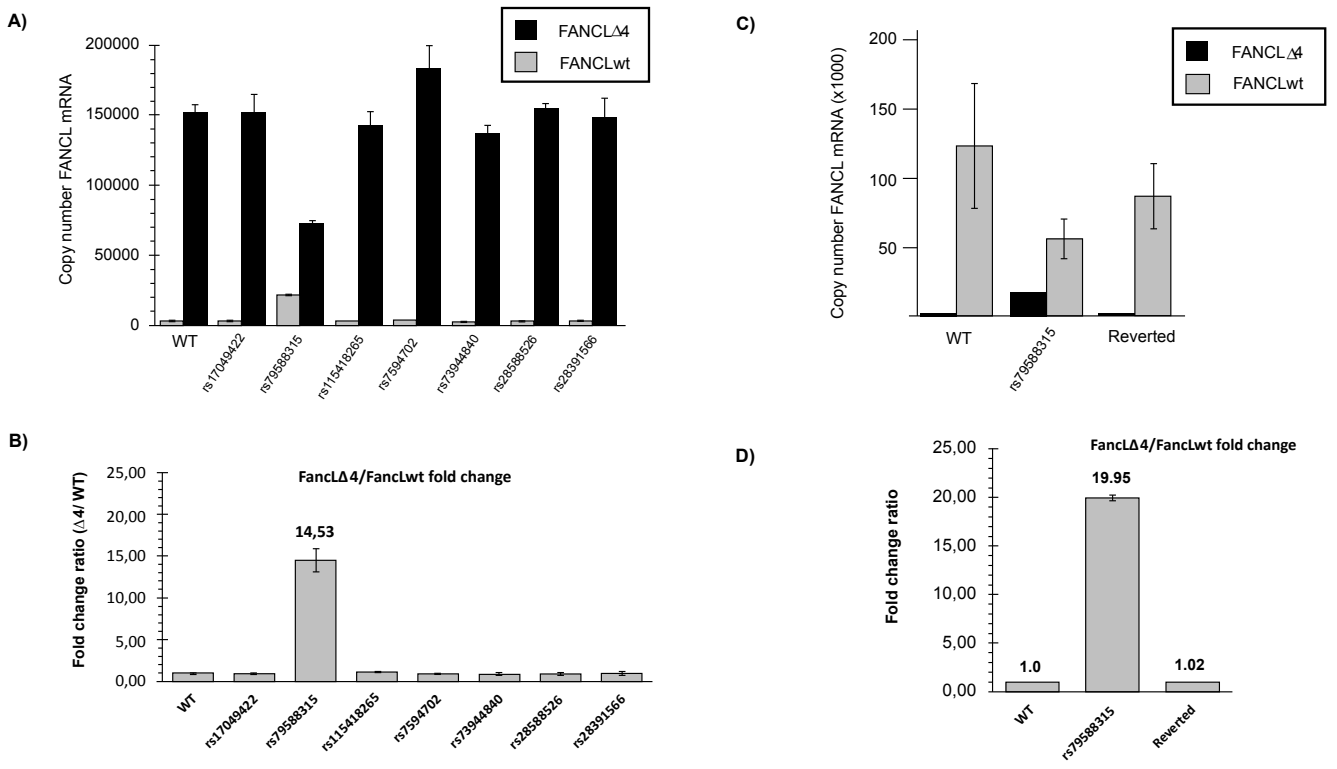


**Figure 2:** Minigene assay. (A) Expression of FANCLΔ4 and FANCL isoforms obtained from 8 different plasmids (constructs) containing specific genomic variants. (B) Based on data observed in (A), FANCLΔ4/FANCL expression ratios are normalized in respect to the wildtype (WT) plasmid. (C) And (D) show the result of the reverse mutagenesis on the c.217-11T/C variation (rs79588315) to reestablish the WT sequence illustrated in copy number and FANCLΔ4/FANCL expression ratios, respectively.

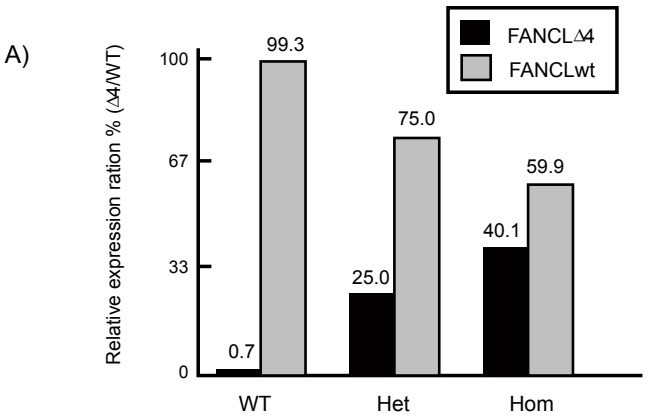


**Figure 3:** Expression of FANCLΔ4 and FANCL isoforms in BRCAX cell lines. mRNA expression levels of FANCLΔ4 and FANCL isoforms in BRCAX cell lines as measured by quantitative real-time PCR experiments. Expression levels are expressed in (A) copy number and (B) FANCLΔ4/FANCL expression ratios. In (A) the numbers at the top of the columns indicated the relative expression of each isoform (%), while in (B) the number indicated the fold change based on the Heterozygotes (Het) normalized at 1.0.

FANCL showed the capacity to monoubiquitinate the FANCD2 protein. Neither in the cells infected with the variant Δ4 nor the empty virus could we observe the long form (monoubiquitinated) of the FANCD2 protein.

In order to assess the ability of the *FANCLΔ4* isoform to support high fidelity DNA repair, cell survival assays were performed. The infected EUFA868 cells were treated with 6 different doses of MMC for five days. As shown in Figure 6A, only the EUFA868 cells infected with the *FANCL* cDNA demonstrated a clear advantage of cell survival when compared with the cells not infected or infected with the empty vector or *FANCLΔ4* cDNA. The whole experiment is shown in Supplemental Figure 1. These data suggest that unlike FANCL, FANCLΔ4 was unable to support cell survival and viability. Being a hallmark of the FA disease, cell cycle analysis is a standard method used to determine whether cells harbor the FA phenotype [42,43]. As displayed in Figure 6B, in absence of treatment with the DNA crosslinking agent MMC, all three EUFA868 cell samples not infected (identified as EUFA) or infected with *FANCL* or *FANCLΔ4* showed a similar cell cycle profile. However, following 24 hours of MMC treatment, a significant G2/M accumulation can be observed in *FANCLΔ4* (27.4%) and EUFA (23.0%) samples, while the one infected with the *FANCL* cDNA, showed a regular transition of the G2/M phase (17.8% of cells accumulated in the G2/M phase). Moreover, based on cell cycle analyses, not only did these results demonstrate that FANCLΔ4 does not complement EUFA868 cells, but also that cells infected with FANCLΔ4 showed a higher cell accumulation in G2/M phase than uncomplemented cells (44,8% in G2/M for FANCLΔ4 cells vs 32,9% for Neo cells) (data not shown). The same trend was observed in survival assays after five days of MMC treatment (Supplemental Figure 1), suggesting a disadvantage caused by the expression of the FANCLΔ4 protein.

The FANCL protein has been reported to be an intracellular protein that localizes in both the cytoplasm and the nucleus [44-46]. Given the alteration of the protein structure of the FANCLΔ4 protein, it was therefore interesting to compare the localization profile of the

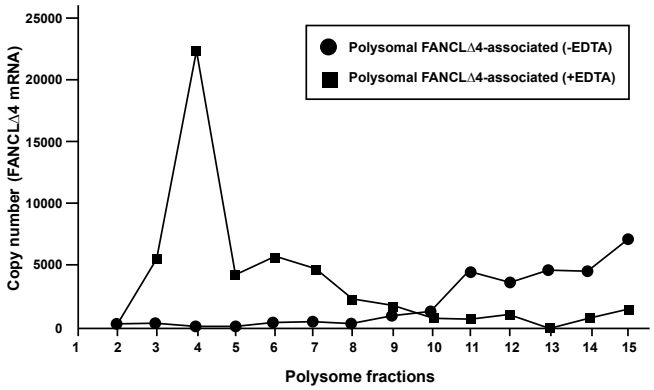


**Figure 4:** Polysome analysis of FANCLΔ4 mRNA. Fractionation of monosomes and polysomes (with or without EDTA) as measured by A260. q-RT PCR of the FANCLΔ4 isoform was performed on T-47D cells and relative expression values for each fraction were calculated by the equation R = (E)(Ctref-Ct), where Ctref is the average cycle threshold of all the fractions for this isoform. The square line is the data acquired from the +EDTA sample whereas the circle line is the data from the -EDTA sample, which illustrates the binding of the FANCLΔ4 transcript with polysomes.

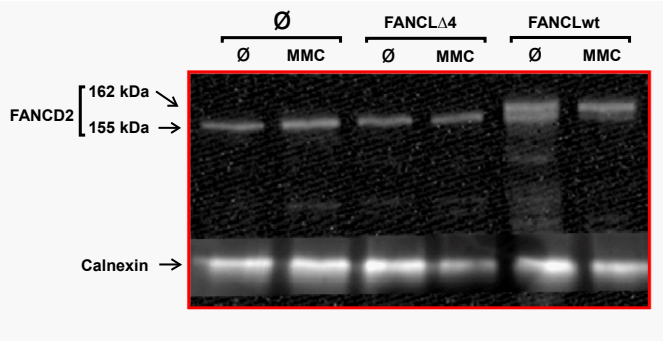


**Figure 5:** Monoubiquitination assays. Western blotting of the FANCD2 protein in whole cell lysate of EUFA868 cells infected with either the *FANCL* or *FANCLΔ4* cDNA, or not infected (ø). Cells were treated or not with 100ng/ml of MMC for 16 hours. FANCD2 associated with an ubiquitin is found at 162 kDa whereas FANCD2 alone weights 155 kDa. Calnexin is used as loading control.

FANCL and FANCLΔ4 proteins in normal conditions and under MMC treatment. HEK293T cells were therefore transfected with either FANCL-EGPFP or FANCLΔ4-EGFP and then treated (or not) with MMC for a period of 12 hours. As shown in Figure 7, in the absence of MMC treatment, both the FANCL and FANCLΔ4 proteins were present mostly in the cytoplasm of cells. However, following 12 hours of MMC treatment, the recruitment of the FANCL-EGFP protein is strongly enhanced in the nucleus of the cell, while the FANCLΔ4-EGFP spliced protein clearly remains in the cytoplasm.

## Discussion

In order to increase the power to possibly find genetic variants involved in breast cancer susceptibility, we selected individuals from our cohort of FC non-*BRCA1/2* high-risk breast cancer families (one individual per family). Given that this population is considered a founder population, this allowed us to increase the likelihood of potentially identifying deleterious mutations associated with breast cancer [47].

Sequencing of the exonic and neighboring intronic regions led to the identification of 18 variants including one novel variation. None of them has been reported to be causative of a FA diagnostic or any other manifestation. Despite the key role of FANCL in DNA repair and its close connection with other FANC genes already known to be associated with an increased breast cancer susceptibility, to our knowledge only one study investigated the potential involvement of the *FANCL* gene in breast cancer predisposition. Mutation analysis performed in a cohort of 95 BRCA1/2-negative index cases from Spanish high-risk breast cancer families, did not identify any FANCI, FANCL or FANCM deleterious mutation [48]. In that study, among the variants identified in breast cancer cases, three have been found in our cohort including c.-67-54C/G, c.217-11T/C and c.374+34delT, which showed a slight higher MAF than those found in our cohort. Particularly, the c.217-11T/C variant was described to have no significant effect on splicing regulation following *in silico* analyses [48]. In other studies the *FANCL* gene has also been excluded as a major player in esophageal and cervical cancer susceptibility [49,50]. Abnormal expression of the *FANCL* gene has been observed in Calu-6 lung cancer cells resulting in an increased MMC sensitivity [51]. Although no coding mutation was identified, a splicing variant of *FANCL* mRNA lacking exons 10 and 11 was found, resulting in a shorter FANCL protein of 272 amino acids.

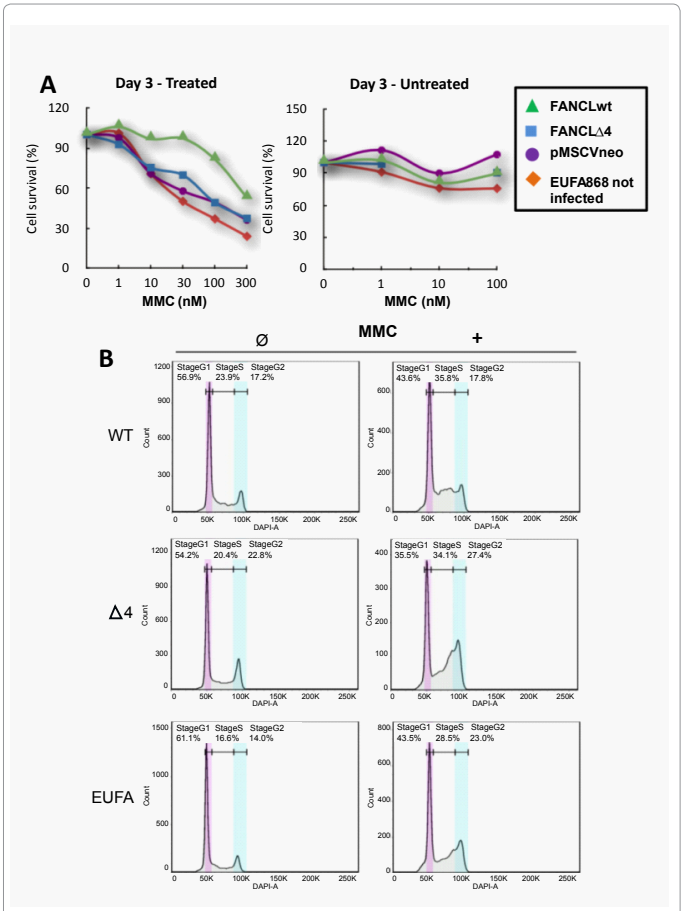


**Figure 6:** Survival assay and cell cycle profiles. (A) MMC-induced cell death. Differential survival of EUFA868 (diamond), EUFA868/pMSCVneo (circle), EUFA868/*FANCL* (triangle) and EUFA868/*FANCCΔ7* (square) cells after 5 days of incubation in escalating concentrations of MMC. The fraction of surviving cells was measured by the Alamar Blue assay. Untreated cells are shown in the right panel. (B) FACs analysis of the EUFA868 FANCL-deficient cell line infected with *FANCL* cDNA, *FANCLΔ4* cDNA or not infected at all. Cells were treated with MMC (50 ng/ml) for 24 hours prior to FACs analysis.

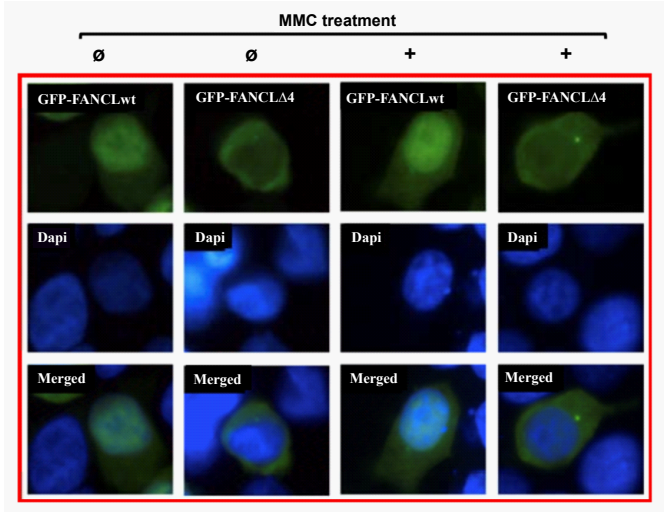


**Figure 7:** Cellular localization. Fluorescence microscopy of FANCL-EGFP and FANCLΔ4-EGFP transiently transfected in HEK293T cells treated or not with 50 ng/ml of MMC for 16 hours. Counterstaining with DAPI is performed to visualize the nucleus.

One of the rare FANCL patients identified so far was carrier of an in-frame 3-bp deletion mutation (p.Ile336_Cys337delinsSer) located in exon 12 and 4-bp duplication (p.Thr367AsnfsX13) in exon 14. These alleles were demonstrated to be unable or correct only partially *FANCL*-deficiency and G2/M arrest in EUFA868 cell line [52]. This EUFA868 cell line has been shown to express FANCL transcripts lacking the exon 11, thus removing the conserved PHD finger and part of the third WD40 repeat [53]. The genomic DNA from this individual showed a homo- or hemizygous insertion of 177 bp into a pyrimidine-rich sequence at the splice junction between intron 10 and exon 11.

Our cDNA sequencing analyses revealed the significant expression of the *FANCLΔ4* isoform. The deletion of exon 4 (aa 72-91) located in the N-terminal region resulted in a disruption of the E2-Like-Fold (ELF) domain (aa 1-90) as well as the first of the three WD-40 domains (aa 75-275) of the FANCL protein [54]. Although no specific function or interaction has been reported to date for the ELF domain, this

domain shares 67% similarity with the *Drosophila* FANCL [55] and among the 11 amino acids perfectly conserved in other species 4 are encoded by exon 4, indicating the importance of this coding region in protein function or conformation. The ELF domain (aa 1-90) is always found in combination with WD-40 and RING domains and is suspected to be involved in protein binding [56]. WD-40 proteins are involved in multiple functions such as signal transduction, transcription regulation, cell cycle control and apoptosis while the WD-40 domain is known to be involved in protein interaction [57]. Therefore, the deletion of exon 4 located at the beginning of the first WD-40 domain could affect protein folding and interactions.

Even though *in silico* analyses did not predict any significant effect of the c.217-11C/T variant (rs79588315) described herein (Table 2) and in a previous study [48], our minigene and expression analyses clearly identified a major role for this variant in the deletion of exon 4 of the *FANCL* mRNA. Indeed this expression is almost exclusively dependent on the heterogygous or homozygous state of rs79588315 in the individuals (Figure 3). Splicing mutations reported so far to be involved in several syndromes or cancers are often located in a minimal exon-intron region (5-6 nucleotides), in the polypyrimidine tract or in exons [58], and softwares used for *in silico* analyses could identify significant nucleotide changes for splicing regulation. Given the importance of the c.217-11C/T variant, on exon 4 exclusion, further experiments would be required to identify the splicing factor proteins, for which mRNA binding is impaired. Considering that reduced levels of FANCL can increase cancer susceptibility [51,59], combined with the effect of the c.217-11C/T variant on *FANCLΔ4*/*FANCL* mRNA expression ratio, the frequency of *rs79588315* should be tested in larger cohorts and for other cancer types.

It is well recognized that variation of the spliced/WT expression ratio could have dramatic effects on cell physiology. Candidate genes influencing the susceptibility to complex diseases such as asthma, Alzheimer's and autoimmune diseases have been found to produce alternative spliced isoforms of specific genes, with the expression ratio fluctuating between normal and affected individuals [60]. There is also convincing evidence that in several cancers including breast cancer, the ratio of spliced variants is significantly altered and correlates with disease severity [61-74]. The expression of spliced proteins can exert a dominant negative effect on the function of the WT protein by several mechanisms such as opposite function of the WT and spliced proteins [75,76]; different cellular localization or cytoplasmic solubility [59,77]; heterodimerization between the spliced and the WT protein [59,78-80], or binding of the spliced protein with interactors or DNA response elements which prevents the binding of the WT protein [81,82].

Particularly, a spliced protein encoded by the *FANCL* gene, dubbed FAVL, has been demonstrated to be highly expressed in sporadic bladder cancer as an oncoprotein capable of promoting cancer formation and rendering cells resistant to cisplatin [59,83]. It is therefore suggested that the FA-BRCA-suppressive pathway can be switched to an oncogenic mechanism through alternative splicing of a key component [84].

In contrast to FANCL infection, which leads to normal DNA repair, complementation of EUFA868 *FANCL*-deficient cells with FANCLΔ4 did not allow monoubiquitination of FANCD2 protein, and displayed a cell cycle blockade as well as deficient cell survival rate. It indicates that the intra-FA pathway step of monoubiquitination [53] does not occur in the presence of the protein FANCLΔ4, and therefore that the FANC-BRCA pathway is dysfunctional and may not be able to remove efficiently ICLs present in the DNA. When such lesions are not properly repaired, they can lead to chromosomal breakage, chromosomic instability and ultimately cell death [85].

Ubiquitination is a mechanism of protein modification and is involved in many cellular pathways, including signal transduction and DNA repair [86,87]. In addition to FANCD2, FANCI is also monoubiquitinated by the FANCL protein, which serves as the catalytic ubiquitin E3 ligase [88,89]. FANCI monoubiquitination is critical for activation of the FA pathway and required for the formation and the localization of the FANCI/D2 complex to nuclear foci [28,89-91]. Two E2 proteins namely UBE2T and UBE2W have been shown to interact with FANCL by yeast two hybrid experiments, and the C-terminal RING domain is necessary and sufficient for FANCL binding of UBE2T [26,92]. This protein complex in combination with E1, has been shown to be sufficient for FANCD2 monoubiquitination in an *in vitro* context [92].

A hallmark of the FA phenotype is the disturbance of the cell cycle. The FA alteration in the cell cycle was first described three decades ago where a prolongation of the G2 phase was observed in short-term cultures of FA peripheral blood lymphocytes [93]. Cell cycle blockade and the decrease of surviving cells in EUFA868/FANCLΔ4 cells supported the inefficiency of the FANCLΔ4 protein to promote DNA repair. It is reported in the literature that FA cells defective in the core complex have a S-phase checkpoint defect that appears to be specific for ICL damage [94,95].

Although to our knowledge its nuclear localization was not previously demonstrated, it is well assumed that FANCL must be translocated to the nucleus to exert its function of FANCD2/FANCI monoubiquitination, given that the whole FA protein complex is recruited at the DNA damage sites. Our results demonstrated that the FANCL protein is indeed translocated to the nucleus following MMC treatment in EUFA868 cells, while FANCLΔ4 protein remains in the cytoplasm. This strongly suggests a defect in the recruitment of FANCLΔ4 protein to the nucleus when DNA damage is induced, and points toward the presence of potential nuclear localization signal, post-translation protein modification site or protein binding domain (e.g. FANCB and FAAP100) [96] located in the region of exon 4. FANCC, FANCG and FANCL proteins have been demonstrated to be required for chromatin binding of monoubiquitinated FANCD2 following cisplatin exposure [97]. A missing or dysfunctional protein of the core complex may cause a defect in the mononubiquitination of FANCD2/FANCI [98], and restrict the recruitment of the complex III, thus preventing the assembly of the proteins responsible for DNA repair [99].

## Conclusion

In conclusion, new isoforms of the FANC gene family are identified and new roles are discovered. Studying the splicing isoforms of FANC genes unravels a completely new dimension to the complexity of this gene family. This study confirmed the required integrity of the ELF domain of the FANCL protein for its nuclear translocation and efficient DNA repair. The FA pathway inactivation is considered a predictive biomarker of chemotherapeutic response. Given that a DNA repair defect involves a balance between accumulation of DNA damage and chromosomal instability causing cancer predisposition and, on the other hand, apoptosis reduction resulting in the depletion of hematopoietic stem cells [100], an increased expression of the *FANCLΔ4* isoform could have significant modifier effects on DNA repair efficiency and therefore on action of chemotherapeutic agents used in breast cancer treatment. In the case of alteration of DNA repair

efficiency, the causal *rs79588315* genomic variant of the *FANCLΔ4* isoform expression could thus be genotyped in large cohort of cancer patients as a predictive biomarker of treatment response.

### Conflict of Interest Statemen

The authors declare that they have no competing interests.

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