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Impaired Effects of a *FANCC* Splicing Isoform in FANC-BRCA DNA Repair Pathway

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Abstract

The integrity of the *FANC* gene family is essential for the proper reparation of DNA damages. Recently, different *FANCC* splice variants were identified. We characterized sequence variations and studied the impact of the alternative splicing event FANCCΔ7 on DNA repair. The *FANCC*Δ*7* transcript is present in all breast cancer lines analyzed. Genomic and complementary DNA sequencing of non-BRCA1/2 individuals was performed to identify sequence variants and alternative transcripts of the *FANCC* gene. Ribosomal fractions allowed confirming the translation of FANCCΔ7 into a functional protein. The variant protein seems to be secluded in the cytoplasm in transfected HEK293T cells following MMC treatments contrary to the FANCC protein that migrates to the nucleus. Performing localization studies, we observed evidences of colocalization of FANCC and FANCCΔ7 with BRCA1 in centrosomes of cells. We demonstrated that FANCC deficient cells infected with FANCCΔ7 cDNA show a blocking in G2/M in the presence of MMC. Finally, FANCCΔ7 is unable to allow the monoubiquitination of FANCD2. This study unravels the incapability of the FANCCΔ7 splicing event to permit the reparation of interstrand crosslinks induced by MMC. This work unravels a novel layer of complexity to the understanding of the fascinating FANC-BRCA DNA repair pathway.

Keywords: FANCC variants; Alternative splicing; DNA repair; FANC-BRCA

Abbreviations: AB: Alamare Blue; Ab: Antibody; FA: Fanconi Anemia; ASE: Alternative Splicing Event; BRCA1: Breast Cancer Susceptibility Gene 1; BRCA2/FANCD1: Breast Cancer Susceptibility Gene 2/Fanconi Anemia Group D1; BRIP1/FANCJ:BRCA1:Interacting Protein 1 / Fanoni Anemia Group J; CANX: Calnexin; DBS: Double Strand Break; EDTA: Ethylenediaminetetraacetic Acid; FAC FANCC: (Abbreviation Often Used In Literature); FACS: Fluorescence Activated Cell Sorting; FANC: Fanconi Anemia Group; FANCC: Fanconi Anemia Group; FANCE: Fanconi Anemia Group E; FANCD2: Fanconi Anemia Group D2; FAD2: FANCD2; GRP94: Glucose Regulated Protein 94; GSTP1: Glucose S:Transferase P1:1; HEK293T: Human Embryonic Kidney 293T Cells; HSC536: Human Stem Cell 536 (FANCC Deficient Lymphocytes); ICL: Interstrand Crosslink; MAF: Minor Allele Frequency; MMC: Mitomycine C; NMD: Nonsense- Mediated Mrna Decay, Dégradation Des Arnm Non-Sens; OR: Odds Ratio; RT: PCR-Reverse Transcriptase Polymerase Chain Reaction; SNP: Single Nucleotide Polymorphism; WT: Wild Type

Introduction

Pathogenic mutations in *BRCA1*, *BRCA2/FANCD1*, *TP53*, *ATM*, *CHEK2*, *BRIP1/FANCJ*, *PALB2/FANCN* and *RAD51C/FANCO* have been associated with an increased breast cancer risk and, together, are found in less than 25% of breast cancer families showing a clear pattern of inheritance [1-7]. Thus, it is clear that other susceptibility alleles remain to be identified to explain the increased risk in the remnant high-risk families. As the number and characteristics of such alleles are undetermined, a focused candidate gene approach based on genes interacting with the known susceptibility genes (in particular *BRCA1* and *BRCA2*) constituted a study design of choice to identify rare moderate-penetrance susceptibility alleles.

The Fanconi anemia (FA) gene family comprises 15 distinct complementation groups (FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -O, -P) [5,8-23]. A recent study conducted with 944 family members being part of the International Fanconi Anemia Registry revealed an increased risk of breast cancer among grandmothers carriers of *FANCC* mutations [24].

In response to interstrand crosslinking (ICL) damage, this FA/BRCA network activates and coordinates a complex DNA repair mechanism involving homologous recombination (HR), nucleotide excision repair and translesion DNA synthesis [20]. FANCC is a crucial component in the upstream FA nuclear core complex. FANCC-deficient cells show a disable S-phase checkpoint response specific to ICL damage, and also likely have a defect in an ATR-dependent checkpoint, as demonstrated in HSC536N cells [25]. FANCC (localized in both the cytoplasm and nucleus) is implicated in several other cytoplasmic functions such as JAK/STAT and apoptotic signaling [26-29].

Thus, based on the close relation of *FANCC* and known breast cancer susceptibility genes in DNA repair pathways and the possible involvement of *FANCC* alterations in cancer susceptibility, we analyzed the entire coding sequence and intron/exon junctions of the *FANCC* gene by re-sequencing a series of 96 breast cancer cases selected from high-risk families from the French Canadian (FC) population and 96 unrelated healthy controls of the same origin for sequence variations that could possibly modulate breast cancer risk. In addition, we identified a splicing isoform designated FANCCΔ7 which demonstrated deleterious effects on DNA repair efficiency and cellular localization following Mitomycin C (MMC) treatment. Such characteristics could have serious consequences on the cell and therefore deserve in depth investigation.

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Materials and Methods

Ascertainment of families and genomic DNA extraction

All 96 non-*BRCA1/2* (BRCAX) individuals from high-risk French Canadian breast and ovarian cancer families (multiple cases of breast/ovarian cancer are present in close relatives – 3 cases in 1st or 4 cases in 2nd degree relative – or with strong evidence of a familial component) participating in this study were originally part of a larger interdisciplinary program termed INHERIT BRCAs [21,30-34]. All participants were at least 18 years of age, mentally capable and had to sign an informed consent. One individual affected with breast cancer per family was selected for analysis, with a selection preference for the youngest subject available in the family. Ethic 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 [35-38]. In all instances, diagnosis of breast cancer was confirmed by pathology reports. Lymphocytes from breast cancer individuals were isolated and immortalized as previously described [24,37,38] and genomic DNA was isolated using the QIAamp DNA Blood kit (Qiagen, Santa Clara, CA). A cohort of 95 healthy unrelated individuals from the French Canadian population was also included in the study. They were obtained from Dr. Damian Labuda at the Centre de Cancérologie Charles Bruneau, Hôpital Ste-Justine, Montreal, Canada. Genomic DNA from peripheral blood of control individuals was isolated using Gentra kits (Minneapolis, MN, USA).

PCR amplification, direct sequencing and variant characterization

The *FANCC* gene (NM_000136.2) is composed of 15 exons that cover approximately 210 kb of genomic DNA. PCR amplification of the coding sequence (the exon 1 being non-coding was not analyzed), as well as the flanking intronic regions, was performed on breast cancer cases using a set of 14 primers pairs listed in supplemental table 1. 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 presence and frequency of variants identified in the case dataset was also confirmed in the healthy individuals control group. Alternative spliced isoform amplification of FANCCins4A and FANCCΔ7 was performed on complementary DNA (cDNA) from BRCAX individuals and human cell lines using primers listed in Supplemental Table 1.

Deviation from Hardy-Weinberg equilibrium (HWE) and allelic difference between both series, and breast cancer association (Odds Ratio with 95% confidence interval) 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. Nucleotide sequence alignments with other species were performed using data extracted from the National Center for Biotechnology Information (NCBI) and UCSC databases.

Cell culture maintenance

All cells were cultured at 37°C in a humidified atmosphere of 5% carbon dioxide. HEK293T (Human Embryonic Kidney 293 cells stably expressing SV40 large T antigen) cells were grown in DMEM (Wisent Inc, St-Bruno, Qc,CAN) with 10% fetal bovin serum (FBS) and 1% penicillin/streptomycin. The HSC536 (Human Stem Cell 536 *FANCC* deficient lymphocytes) cells were grown in RPMI 1640 (Wisent), with 15% FBS and 1% penicillin/streptomycin. PD331 (Human *FANCC* deficient fibroblasts) cells were grown in MEM (Eagle) Alpha Modification (AMEM) with earl salt (Wisent), with 15% FBS and 1% penicillin/streptomycin. T-47D (Human Breast Cancer ER+ cancer cells) cells were grown in RPMI (Wisent) without phenol red supplemented with 10% FBS, 1% penicillin/streptomycin, and E2. HSC536 and PD331 cell lines were purchased from Coriell Institute. 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 differed between experiments, values are indicated in each respective section.

Variant	SNP ID[1]	dbSNP ID	Series	Number of individuals	Common homozygote No. (expected)[2]	Heterozygote No. (expected)[2]	Rare homozygote No. (expected)[2]	MAF[3]	HWE[4]	p-value	OR[5] (95% CI)
v1	c.-78-73C/T	N/A	Cases	96	95 (95.00)	1 (0.99)	0 (0.00)	0.01	0.96	0.12083	0.192 (0.022-1.656)
			Controls	94	89 (89.07)	5 (4.87)	0 (0.07)	0.03	0.79		
v2	c.-29A/C	rs4647414	Cases	96	95 (95.00)	1 (0.99)	0 (0.00)	0.01	0.96	1.3386	2.953 (0.120-73.948)
			Controls	94	94 (94.00)	0 (0.00)	0 (0.00)	0	1		
v3	c.816G/A	rs34671520	Cases	96	92 (94.04)	4 (3.92)	0 (0.05)	0.02	0.84	0.13638	9.000 (0.481-168.331)
			Controls	94	94 (94.00)	0 (0.00)	0 (0.00)	0	1		
v4	c.896+81 G/A	rs4647512	Cases	96	76 (76.15)	19 (18.70)	1 (1.15)	0.11	0.88	0.02622	2.442 (1.088-5.482)
			Controls	92	85 (85.13)	9 (8.57)	0 (0.22)	0.05	0.7		
v5	c.1155-38 T/C	rs4647534	Cases	96	25 (29.26)	56 (47.48)	15 (19.26)	0.45	0.08	0.73754	1.072 (0.715-1.607)
			Controls	94	30 (30.45)	47 (46.10)	17 (17.45)	0.43	0.85		
v6	c.1677+7C/T	N/A	Cases	96	92 (92.04)	4 (3.92)	0 (0.04)	0.02	0.84	0.13572	8.809 (0.471-164.772)
			Controls	92	92 (92.00)	0 (0.00)	0 (0.00)	0	1		

[1]According to the nomenclature of the Human Genome Variation Society
[2]As expected under Hardy-Weinberg equilibrium
[3]Minor Allele Frequency
[4]p-value for deviation from Hardy-Weinberg equilibrium
[5]Odds Ratio (95% confidence interval)

Table 1: Sequence variations observed in *FANCC* gene and genotype frequencies in familial breast cancer cases and controls.

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 [39,40]. Total RNA samples from normal tissues were purchased directly either from Stratagene (breast and ovary) (La Jolla, CA, USA), BioChain Institute Inc. (leukocyte) (Hayward, CA, USA), or Clontech (all other normal tissue samples) (Palo Alto, CA, USA).

RNA samples were then processed as previously described [40,41], and the quality of the RNA samples was verified using a Bioanalyser (Agilent Technologies).

Reverse transcription was performed as previously described [37,42] and for qRT-PCR, 20 ng of cDNA was quantified with the LightCycler 480 (Roche Diagnostics, Laval, Qc, CA). LightCycler 480 SYBR Green I Master was used according to the manufacturer's recommendations. Data calculation and normalization were performed using derivative and double correction method as described previously [43-52].

Minigene assays

The minigene constructs were assembled into the pcDNA3 (Invitrogen Life Technologies, Burlington, ON) vector. The genomic regions containing the exons 6 to 8 as well as large flanking intronic sequences were PCR-amplified from BRCAX genomic DNA using primers containing appropriate restriction sites (BamHI/EcoRI/XhoI/XbaI) into the pcDNA3 vector (Supplemental Table 2).

Sequence variations present in the region of interest were then identified through NCBI and UCSC databases while one variation located in exon 8 was observed in our cohort of FC BRCAX breast cancer families. Directed mutagenesis was then performed using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Mississauga, ON) following manufacturer's recommendations. Primers are described in Supplemental Table 3.

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, rinsed with cold phosphate-buffered (PBS) and suspended in Tri-Reagent. RNA was then extracted and processed as described above and cDNA was prepared.

For PCR amplification, specific primers for *FANCCΔ7* and *FANCC* mRNA isoforms were designed with the GeneTools (Biotools Inc., Madrid, Spain) software (Supplemental Table 2). Worth noticing is that the forward oligo has a portion of the T7 promoter that is transcribed along with the mRNA, making it possible to amplify only what is expressed by the plasmid [53].

Retroviral transduction

HEK293T G/P cells (5×10^6) were plated in 10 cm petri dishes and incubated at 37°C with 5% CO_2 for 8 hours. The pMSCVpuromycin (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 and aliquoted and kept at -80°C. For infection, adherent cells (PD331) were grown to 40% confluency, medium was taken out and retrovirus (1.5 to 2 ml previously aliquoted in the virus production) was added along with culture medium and polybrene (Sigma-Aldrich) at a concentration of 10 µg/ml. The cells (HSC536) were resuspended at 1×10^6 cells per milliliter of virus and polybrene. The next day, culture medium was changed and the antibiotic was added. The selection was operated in the presence of puromycin (0.8 µg/ml) and cells were kept in culture at concentrations of 0.5 µg/ml.

Ribosomal fractions

T47D cells (approximately 4×10^7 cells per fraction) were grown and then collected at 70% confluency. Cycloheximide (50 µg/ml) was added to cell cultures, incubated for 30 minutes and cells were then rinsed with PBS-cycloheximide (CHX 100 µg/ml) and trypsin. Cells were resuspended in PBS-cycloheximide (100 µg/ml) at a concentration of 10^7 cells/ml. The total of 12 ml of cells was then split into 3 samples (-ETDA, +ETDA, protein extraction sample). Cells were centrifuged at 1600 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 20 mM EDTA (+ETDA sample). Samples were separated on a 15-45% sucrose gradient in lysis buffer (without NP40), centrifuged 2 hours 15 minutes at 36000 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 and immunofluorescence

HEK293T cells were transfected with FANCC or FANCCΔ7-EGFP vectors. Cells were then treated (or not) with MMC [50 ng or 100 ng/mL]. Cells (3.5×10^5 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).

For immunofluorescence studies, infected PD331 cells expressing the FANCC-HA and FANCCΔ7-HA protein were treated with MMC [50ng or 100ng/mL], washed, fixed and then permeabilized with PBS-T (0.5% (vol/vol) Triton X-100) for 5 min at RT. Following blocking and washings, slides were incubated with a primary antibody against BRCA1 (monoclonal Ab 17F8,GeneTex, 1: 500) and then with a secondary antibody anti-mouse Alexa Fluor 568 goat, (Invitrogen, 1:600) as well as with another secondary antibody to visualize HA tagged protein (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen, 1:500). Coverslides were mounted on microscopic slides with fluoromount G (Electron Microscopic Sciences, Hatfield, PA).

Confocal laser scanning microscopy was performed with a BX-61 microscope equipped with the Fluoview SV500 imaging software 4.3 (Olympus America Inc, Melville, NY), using a 100X Plan-Apochromat

oil-immersion objective (NA 1.35) and a 2–3.5X zoom ratio in the region of interest. The BRCA1-Fluor 568 labeled protein was excited at 603 nm, while the FANCC-HA-Fluor 488 and FANCCΔ7-HA-Fluor 488 labeled proteins were excited at 519 nm using an argon-He laser (Melles Griot Laser Group [Carlsbad, CA]). 0.1 µm confocal z-series were acquired for each observation area and filtered by three-frame Kalman low-speed scans. Acquired z-series images were exported in Imaris Pro Sofware 4.2.0 (Bitplane AG, Zurich, CH). The complete method used is fully described BY Simard et al. [54].

Survival assays

HSC536 cells were selected for approximately 2 months with puromycin. An average of $3x10^5$ 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 1.5×10^4 cells/100 µl/well. The 100 µl contained the adjusted concentrations of crosslinking agent. The readings were performed after 1, 2, 3, 4, 5 and seven days. AlamarBlue assays (Invitrogen, Carlsbad CA) were performed according to the manufacturer's instructions. 10 µl of AlamarBlue 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).

Western blotting

Proteins were extracted from approximately 40×10^6 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. HA-tagged FANCC, FANCCΔ7 or FANCD2 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 appropriate primary (overnight rocking at 4°C) and secondary antibody (1 hour rocking at RT) diluted in 5% milk/PBS. The following antibodies and dilutions were used: rabbit polyclonal IgG anti-HA SC-805 (Santa Cruz, 1:1000), ECL anti-rabbit IgG horseradish peroxidase-linked antibody (GE HealthCare, 1:10 000), rabbit anti-FANCC (FA research Fund, 1:500), rabbit anti-FANCD2 (Novus Biologicals, 1:10 000), anti-FANCE (FA research Fund, 1: 500), rabbit anti-Calnexin (Enzo (Life Sciences), 1:1 000). For confirmation of the viral infection of the PD331 and HSC536 cells, Pierce HA Tag IP/Co-IP kit (Fisher Scientific Limited, Nepean, ON) was used following manufacturer's recommendations.

Flow cytometry

For cell cycle analysis, three different MMC concentrations were used at three time points. HSC536 cells (350000/ml) were infected with the empty virus, the pMSCVpuromycin vector containing FANCC or FANCCΔ7 cDNA and were grown in media containing 0 ng/ml, 100 nM (33 ng/ml), 50 ng/ml or 100 ng/ml of MMC. The cells were harvested after 12, 24, 36 and 48 hours and then washed with RT PBS (1X), centrifuged and washed again in 4°C PBS. Cells were then fixed by adding 4.5 ml of ice cold 70% ethanol and placed at -20°C overnight. Once fixed, cells were washed, centrifuged at 1500 rpm and 400 µl of fresh DNA staining solution was added (PBS 1x, [1 µg/ml] DAPI, [0,2 mg/ml] DNase free RNAse). Samples were incubated 15 minutes in the dark before taking measurements. The 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 (Tree Star Inc., Ashland, OR) programs.

Results

Sequence variations in the FANCC gene

Direct sequencing identified six variants in our cohort of FC breast cancer cases. As represented in table 1 and Supplemental Figure 1, out of the 6 variations, one was located in the coding sequence of exon 8 (c.816G/A), three are found in intronic regions (c.-78-73C/T, c.896+81G/A and c.1155-38T/C) and the two remaining variations are located in untranslated sequences (c.-29A/C; exon 2 and c.1677+7C/T; exon 15). Among the identified variants, two were frequent with a MAF greater than 5%, while the remaining 4 were rare variations. Variants identified in the case dataset were also genotyped in 96 unrelated healthy individuals from the same population. Thus, three nucleotide changes were observed only in the cases series and not in unaffected individuals (Table 1). When genotype frequencies of all variants were compared between both series, the c.896+81G/A intronic variation showed a statistically significant association with breast cancer (OR 2.442, 95% CI: 1.088-5.482; p=0.02). Moreover as displayed in supplemental table 4, this nucleotide is conserved in 23 out of 32 species analyzed, which could suggest that the high conservation of this particular intronic nucleotide could reflect a certain level of functionality.

Identification of FANCC alternative spliced isoforms

Analysis of *FANCC* cDNA highlighted the presence of two distinct alternative splicing events (ASEs) (Figure 1A and 1C). Specific semi-quantitative PCR amplification of both isoforms performed in human cell lines are displayed in Figure 1B (*FANCCins4A* and *FANCCΔ7*). While FANCCΔ7 mRNA seems to be significantly expressed in all leukemia, breast and ovarian cell lines, *FANCCins4A* mRNA analysis displayed an obvious variability of expression particularly in breast cancer cell lines, and this mRNA was not detectable in K562 cell line.

Sequence variants modulate the ratio of FANCCΔ7/FANCC RNA

In order to evaluate the involvement of different genomic variants in the alternative splicing regulation of exon 7, a minigene assay was designed to assess those effects. The variant c.553 C>T was used as a positive control as this variation present in exon 7, leads to a premature stop codon that would severely truncate the FANCC protein and was previously reported as an exon skipping causing mutation [55,56], while the c.816 G>A variant was identified in our high-risk breast cancer families. As illustrated in Figure 1D, two variants namely c.568 C>T and c.553 C>T, increased significantly the exclusion of exon 7 by 74% and 85%, respectively. On the other hand, two variants located at the end of exon 6 (c.530C>T) and exon 8 (c.816G>A) showed the opposite effect by increasing the inclusion of exon 7 by 33% and 39%, respectively.

Assessment of NMD and polysomes analysis

To confirm the *in vivo* translation of the *FANCCΔ7* mRNA transcript, the action of nonsense-mediated decay (NMD) was first tested by treating (or not) several immortalized lymphoblastoid cell lines (LCLs) of BRCAX individuals with puromycin, an agent known to inhibit NMD. mRNA extracted from puromycin-treated cells did not show any modulation of the spliced form relative quantities, suggesting

that both alternative mRNA isoforms were not subject to NMD (data not shown). Moreover, 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 2, the ribosomal fractions demonstrated that the *FANCCΔ7* mRNA transcript, being strongly detected in late fractions (peak at fraction 11), was associated with multiple ribosomes (polysomes), which confirms its efficient translation. As negative control, each fraction was duplicated and treated with 20mM ethylenediaminetetraacetic acid (EDTA), which is known to dissociate mRNA from ribosomes.

The variant FANCCΔ7 does not allow the monoubiquitination to take place

As seen in Figure 3, the transduction of HSC536 (*FANCC*-deficient) cells with retroviral vector pMSCV containing the *FANCC* wild type or Δ7 cDNAs clearly indicated that following a MMC treatment for 12 hours, only cells complemented with the plasmid pMSCVpuromycin-FANCC had the capacity to monoubiquitinate the FANCD2 protein. Neither in the cells infected with the variant Δ7 or the empty virus could we observe the long form (monoubiquitinated) of the protein FANCD2.

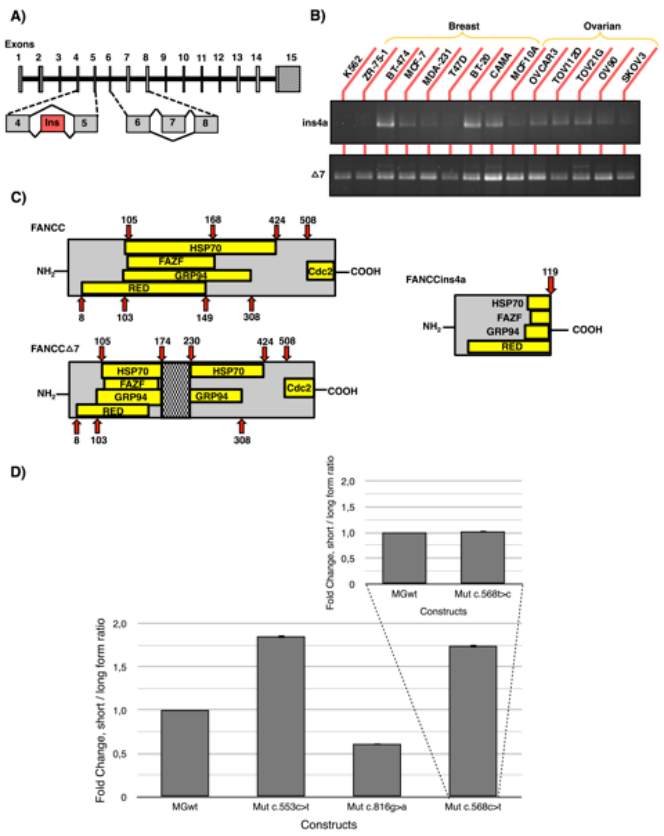

Figure 1: *FANCC* splicing, FANCC interaction domains, and minigene assay. (A) *FANCC* exon structure and the two splicing variants that could be detected via the cDNA analysis: *FANCCins4a* and *FANCCΔ7*. (B) RT-PCR analysis on various cell lines using a set of primers specific for each event. (C) Schematic representation of FANCC, FANCCΔ7 and FANCCins4a interaction domains based on the literature. (D) Minigene assays; ratios of spliced and wildtype isoforms obtained from 3 different plasmids (constructs on which directed mutagenesis was performed). The short form is the *FANCC* mRNA with the exon 7 excluded where the long form includes exon 7. Ratios are normalized with respect to the wildtype plasmid. Upper graph shows the result of the reverse mutagenesis on the c.568c>t mutant to reestablish the wildtype sequence.

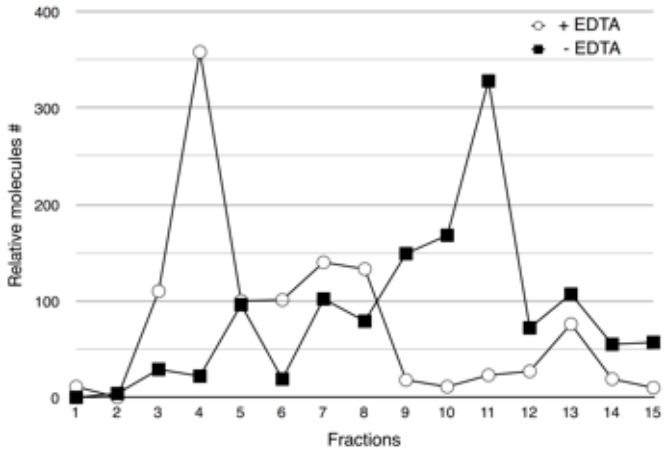

Figure 2: Ribosomal fractions. Polysome analysis of the *FANCCΔ7* alternative transcript in T47D cells. The square line is the data acquired from the -EDTA sample whereas the circle line is the data from the + EDTA sample. Fractions represent the 15-45% sucrose gradient used to separate by weight the mRNA and the ribosomal units associated. Greater the fractions are, heavier the mRNA and ribosomal complexes (polyribosome) meaning more association between ribosomal units and mRNA.

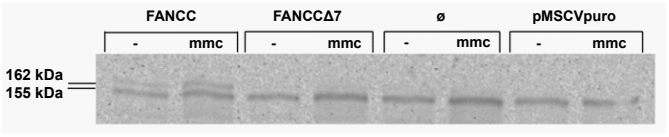

Figure 3: Monoubiquitination assays. Western blotting of the FANCD2 protein in whole cell lysate of HSC536 cells infected with either the *FANCC* cDNA, *FANCCΔ7* cDNA, with the empty viral vector (pMSCVpuro), or not infected (ø). Cells were treated or not with 100ng/ml of MMC for 16 hours. FANCD2 associated with an ubiquitin is found at 162 kDa whereas FANCD2 alone weights 155 kDa.

HSC536 cells complemented with FANCCΔ7 variant harbor deficient cell cycle

Being a hallmark of the FA disease, cell cycle analysis is a standard method used to determine whether cells harbor the FA phenotype [57,58]. As displayed in Figure 4A, in absence of treatment with MMC, all four HSC536 cell samples not infected (identified as HSC536) or infected with FANCC, FANCCΔ7 or empty vector (Supplemental Figure 2) showed the same cell cycle profile. However, following 24 hours of MMC treatment, a significant G2/M accumulation can be observed in all samples except for the one infected with the FANCC cDNA, in which at the highest MMC dose (100 ng/ml) an accumulation in the S stage phase was observed (up to 61.5% in the FANCC sample against 20.3% and 27.3% for the HSC536 and Δ7 samples, respectively). It should be noted that this delayed S phase is not seen in other samples, which rather showed an accumulation of cells in the G2/M phase. As displayed in Supplemental Figure 2, this delayed S phase (24 hours) is progressing further to the G2/M phase at 36 hours. Worth noticing is the fact that only the HSC536/FANCC sample showed a regular progression in its cell cycle throughout this experiment.

FANCCΔ7 fails to rescue the cell survival in FANCC-deficient cells

In order to assess the ability of the FANCCΔ7 isoform to support high fidelity DNA repair, cell survival assays were performed. The infected HSC536 cells were treated with 6 different doses of MMC for seven days. As shown in Figure 4B, only the HSC536 cells infected with the *FANCC* cDNA demonstrated a clear advantage of cell survival when compared with the cells infected with the empty vector or *FANCCΔ7* cDNA. After five days of treatment with 10nM MMC, we barely observe any differences in the FANCC sample unlike the three other samples, where less than 50% of cells survived at this point. These data suggest that unlike FANCC, FANCCΔ7 was unable to support cell survival and viability. The whole experiment is shown in Supplemental Figure 3 where, after only 48 hours with 10nM and 30nM of MMC treatment, a clear advantage was seen in the sample infected with FANCC, which is in accordance with the literature [59].

The FANCCΔ7 and FANCC proteins localized differently following MMC treatment

Given that the protein FANCC is mainly found in the cytoplasm, but also in the nucleus under normal conditions, it was therefore interesting to compare the localization profile of the FANCC and FANCCΔ7 proteins in normal conditions and under MMC treatment. HEK293T cells were therefore transfected with either FANCC-EGPFP or FANCCΔ7-EGFP and then treated (or not) with MMC for a period of 12 hours. In the absence of MMC treatment, both the FANCC and FANCCΔ7 proteins were present mostly in the cytoplasm of cells. However, following 12 hours of MMC treatment, the recruitment of the FANCC-EGFP protein is strongly enhanced in the nucleus of the cell, while the FANCCΔ7-EGFP spliced protein clearly remains in the cytoplasm. This cytoplasmic localization of the spliced protein is similarly observed for both doses of MMC treatments (Figure 5A and Supplemental Figure 4A).

FANCC and FANCCΔ7 proteins seem to localize within centrosomes of cells

Throughout the fluorescence microscopy and immunofluorescence experiments, often did we observe a notable localization of FANCC as well as FANCCΔ7 to a particular place near the nucleus of many cells (HEK293T and PD331). We noted these observations first at a magnitude of 40x (Supplemental Figure 4B). This prompted us to hypothesize that this localization would be most likely the centrosomes of the cells. To confirm our observation, we operated immunofluorescence studies on BRCA1 protein (known to localize in these structures) as well as on FANCC-HA and FANCCΔ7-HA proteins, and we confirmed their co-localization through confocal microscopy (Figure 5B with FANCC-HA).

Discussion

The previous identification of *BRCA2/FANCD1*, *PALB2/FANCN*, *BRIP1/FANCJ* and *FANCO/RAD51C* as breast cancer susceptibility genes further strengthens the implication of FA genes in breast cancer susceptibility [21,30,32,34]. As the FC population is considered a founder population, this allows to increase the likelihood of potentially identifying genetic variants associated with breast cancer [35] in non-*BRCA1/2* high-risk families (one individual per family). A number of case-control studies investigated the potential association of *FANCC* germline mutations with breast cancer susceptibility. L554P, c.711+4A/T and c.322delG have been observed at a significantly higher rate than expected among heterozygous *FANCC* mutation carriers' grandmothers [24]. However, Tischkowitz et al. did not find any increase of incidence of the overall cancer risk in FA family members [39]. Although the statistical power of the study conducted by Barroso et al. is limited, they reported an association between rs1045276, located 3' downstream of the *FANCC* gene and poorer survival in breast cancer patients [41]. Our results demonstrated an over-representation of the variant c.896+81G/A in breast cancer cases, which could suggest an association with the disease, however this association remains to be confirmed in larger cohorts.

As clearly demonstrated in Figure 1, a few genomic variants such as c.553 C>T, c.568 C>T and c.816 G>A seem to modulate the *FANCC/Δ7* expression ratio. Changes in the expression ratio of spliced variants of genes can have significant consequences on cell homeostasis [42]. Given that the expression of spliced proteins can exert a dominant negative effect on the function of the wild type protein by several mechanisms [43,45-52], it was therefore of great interest to go deeper into the characterization of the *FANCCΔ7* spliced variant to evaluate its effects on cell physiology.

It is well established in the literature that the FANCC protein localizes to the nucleus following treatments with crosslinking agents (MMC) [1-8,10-12,14,60]. We demonstrated that the FANCCΔ7-EGFP spliced protein was not recruited to the nucleus as opposed to the

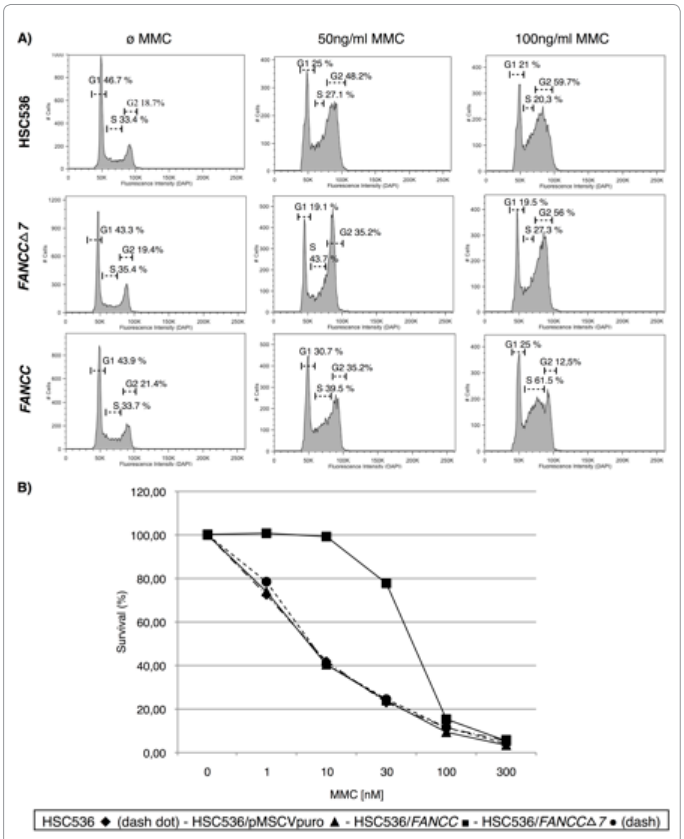

Figure 4: Cell cycle profiles and survival assay. (A) FACs analysis of the *FANCC*- deficient cell line HSC536 infected with *FANCC* cDNA, *FANCCΔ7* cDNA or not infected at all. Cells were treated with indicated concentrations of MMC for 24 hours prior to FACs analysis. (B) MMC-induced cell death. Differential survival of HSC536 (lozenge, dashed and dots), HSC536/pMSCVpuro (triangle, full line), HSC536/*FANCC* (square, full line), HSC536/*FANCCΔ7* (circle, dashed line) cells after 5 days of incubation in escalating concentrations of MMC. The fraction of surviving cells was measured by the AlamarBlue assay.

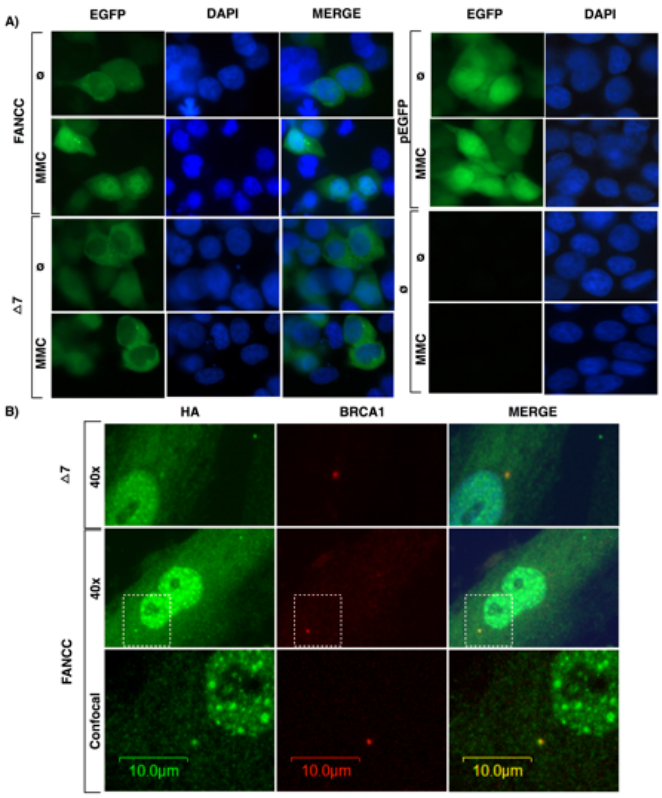

Figure 5: Cellular localization. (A) Fluorescence microscopy of FANCC-EGFP and FANCCΔ7-EGFP transiently transfected in HEK293T cells treated or not with 50 ng/ml of MMC for 16 hours. Counterstaining with DAPI is performed to visualize the nucleus. pEGFP are cells transfected with empty EGFP vector and ø are non transfected cells. (B) Immunofluorescence of FANCC-HA and FANCCΔ7-HA (in green) with BRCA1 (red) in *FANCC*-deficient cells PD331 infected by retrovirus (using viral vector pMSCVpuro and indicated cDNA). Dashed white squares show the region analyzed by confocal microscopy to confirm co-localization. Scale bars: 100 µm.

FANCC protein after a MMC treatment. Because the FA core complex needs all of its 8 FA components (FANCA-B-C-E-F-G-L-M), as well as FA associated protein 24 and 100 (FAAP24 and FAAP100) to be functional, a missing or dysfunctional protein of the core complex may cause a defect in the mononubiquitination of FANCD2/FANCI [5,8-23,61], and restrict the recruitment of the complex III, thus preventing the assembly of the proteins responsible for DNA repair [24,62]. Hence, the absence of recruitment in the nucleus of the Δ7 protein suggests strongly that this spliced isoform is unlikely involved in DNA repair.

The crucial step of monoubiquitination does not occur in the presence of the protein FANCCΔ7 alone, meaning 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 [20,63].

A hallmark of the FA phenotype is the disturbance of the cell cycle. The fluorescence-activated cell sorting (FACs) analysis demonstrated that HSC536 cells infected with the variant Δ7 cDNA showed an evident accumulation of cells in the G2/M stage, which is similar to the empty virus-transfected or not transfected cells. Indeed, when cells are deficient in any of the FA genes, an accumulation of cells can be observed in the G2/M stage following DNA damage [25,57,64,65]. However, it is unknown whether this delay is strictly related to the G2/M phase or could be in fact shared with the late S phase [26-29,65]. In normal cells, such MMC treatment has for effect to slow down the S phase, which represents the stage where most of DNA repair occurs [55,66-68], and this is consistent with what we observed in the HSC536/*FANCC* after 24 hours of MMC treatment (Figure 4A). Twelve hours after this reading, we observe a progression of the FANCC-infected cells into the G2/M phase, while the FANCCΔ7-infected cells seem to fail at showing a progression in their cell cycle. This observation is consistent with the data obtained with the survival assay. 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 [57,58,64,65]. In contrast to normal cells, FA cells cannot stop the S-phase progression due to DNA damages, which supports our results.

During the cell localization experiments, we noticed a localization of both FANCC and FANCCΔ7 proteins near the nucleus in many cells under normal and crosslinking agent (MMC) conditions. The co-localization of FANCC-HA or FANCCΔ7-HA with BRCA1 by immunofluorescence assays suggests their location in the centrosomes of the cells. This localization was seen independently of MMC treatments. Until now, no connections have been proposed between members of the FANC protein family and centrosomes of cells. However, it has been previously demonstrated that FANCA can directly interact with BRCA1 [59,69]. Therefore, such an interaction may pave the way to a possible implication of other FANC proteins of the core complex in the centrosomes of cells. Since FANCC is a multifunctional protein, specific and yet unknown roles of the FANCCΔ7 protein remain to be elucidated.

In conclusion, new isoforms of the FANC family are identified and new roles are discovered. In this study, we characterized a spliced isoform that is unable to participate in the FANC-BRCA pathway to trigger an efficient DNA repair activity. We cannot exclude a significant effect of this splicing variant on the normal activity of the FANCC protein, which remains to be investigated. In addition, this study is the first to describe the localization of the FANCC protein in centrosomes of cells. Having ASE whose functions differ from their respective wildtype gene brings up an additional level of complexity to the already understudied notion of alternative splicing. Because the FANC proteins are known to be implicated in numerous ways for cell homeostasis but are also associated with pathological conditions such as cancers, going further into the characterization of these genes by unraveling their ASE is a most interesting path worth investigating further.

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Conflict of Interes

The authors declare that they have no conflict of interest.

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