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The phenotype of FancB-mutant mouse embryonic stem cells

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Abstract

Fanconi anemia (FA) is a rare autosomal recessive disease characterized by bone marrow failure, developmental defects and cancer. There are multiple FA genes that enable the repair of interstrand crosslinks (ICLs) in coordination with a variety of other DNA repair pathways in a way that is poorly understood. Here we present the phenotype of mouse embryonic stem (ES) cells mutated for FancB. We found FancB-mutant cells exhibited reduced cellular proliferation, hypersensitivity to the crosslinking agent mitomycin C (MMC), increased spontaneous and MMC-induced chromosomal abnormalities, reduced spontaneous sister chromatid exchanges (SCEs), reduced gene targeting, reduced MMC-induced Rad51 foci and absent MMC-induced FancD2 foci. Since FancB is on the X chromosome and since ES cells are typically XY, FancB is an excellent target for an epistatic analysis to elucidate FA's role in ICL repair.

Keywords

Fanconi anemia; interstrand crosslink; G2 arrest; Radial; FancD2; Rad51

1. Introduction

There are multiple genes that function in the FA pathway. Their protein products can be categorized into three groups [1]. The first two groups are essential for the initial FA response to DNA damage. Group 1 is composed of 8 FA proteins in the core complex that include FancB along with Fanc(A, C, E–G, L, M). Mutations in genes that code for these proteins are known to cause FA. In addition, there are two proteins in this complex, FAAP24 and FAAP100 that have not been identified with the FA disease. Group 2 is composed of FancI and FancD2; these proteins share sequence homology and interact with each other to form the ID complex [2,3]. This complex is activated after an E3 ubiquitin ligase, FancL monoubiquitinates both proteins in response to DNA damage [4]. The other core complex proteins are also critical for ID monoubiquitination [5]. Group 3 is composed of FancD1 (Brca2), FancN (Palb2, partner and localizer of Brca2)[6,7] and FancJ (Bach1 or Brip1) [8,9]. This group directs downstream repair pathways like homologous recombination (HR), a major DNA double-strand break (DSB) repair pathway. HR repairs DSBs that arise when replication forks collide with ICLs [10]. In addition mutations in two

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Conflicts of interests

The authors declare there are no conflicts of interest.

other genes important for homologous recombination, *RAD51C* [11] and *SLX4* [12] cause a phenotype similar to FA. Group 3 proteins provide an important link to breast cancer since homozygous mutations lead to FA while heterozygous mutations in at least some of these genes cause breast cancer after loss of heterozygosity [13,14]. Unlike the first and second groups, genes mutated in the third group do not impact ID monoubiquitination indicating they function downstream of ID or in a different pathway. Proteins from all three groups are highly conserved in vertebrates with only some proteins present in lower species [15].

Many of the FA genes have been mutated in mouse ES cells and in mice including FancC [16,17], FancA [18–20], FancL (a.k.a. Pog) [21], FancG [22,23] and FancD2 [24]. Most of these mutations delete one or more exons causing a frameshift for downstream sequences [16–20,22–24] while one mutation is a duplication of three exons [21]; all mutations are likely null. Mutating these genes caused a common phenotype: hypersensitivity to crosslinking agents and radial chromosomes. Genetic modifiers influenced their phenotypes since deletion of either FancL or FancD2 caused embryonic lethality in some but not all genetic backgrounds [21,24]. In addition, FancD2-mutant mice displayed more symptoms than these core complex mutations including perinatal lethality and elevated cancer suggesting either a difference in genetic background or function independent of the core complex.

Here we present the phenotype of FancB-mutant ES cells. FancB is X-linked, a member of the core complex and is mutated in about 2% of FA patients [25]. It contains a putative C-terminal nuclear localization signal and a coiled coil domain (intertwined alpha-helices for protein-protein interactions) [26] and is conserved in vertebrates [27]. We found FancB-mutant ES cells exhibited reduced cellular proliferation, increased sensitivity to MMC, increased spontaneous and MMC-induced chromosomal abnormalities, reduced spontaneous SCEs, reduced MMC-induced Rad51 foci and absent MMC-induced FancD2 foci. We believe this X-linked gene is an excellent target for an epistatic analysis since it requires deletion of only one copy in XY ES cells.

2. Materials and Methods

2.1 Cell culture conditions

Mouse ES cells were grown in Minimal Essential Media- α (Invitrogen/Gibco, Carlsbad, California) with 15% fetal bovine serum (Invitrogen/Gibco, Carlsbad, California), 2mM glutamine, 30 μ g penicillin/ml, 50 μ g streptomycin/ml, 10⁻⁴M β -mercaptoethanol and 1 X leukocyte inhibiting factor (Chemicon) and grown on plastic plates coated with gelatin (0.1%) for about one hour or plastic gelatinized plates with a monolayer of murine embryonic fibroblasts (mutant for *HPRT* and positive for puromycin) exposed to 30 Gy to inactivate mitosis.

2.2. Generation of conditional Fancb targeting vector

The conditional targeting vector was made as previously described [28]. Briefly, AB2.2 genomic DNA was used to amplify the left and right arms of homology and the region of exon 2. After amplifying arms, the left arm was digested with *SalI* and *NotI* and cloned into a plasmid backbone (pKO) cut with *SalI* and *NotI*. Then, the right arm was cut with *BamHI* and *NotI* and cloned into the same backbone adjacent to the left arm digested with *BamHI* and *NotI*. The amplified exon 2 was digested with *AscI* and *Rsr2* and then cloned into modified pBluescript. The polymerase was I-proof polymerase (Bio-Rad, Hercules, CA).

Left arm Forward:

5'AAACGCGTCGACTTACTATAAACCACCACATAGCGATGAAGTATACTCCT
ATAG 3'

Left arm Reverse: 5'

CACAGCGCCGCAGGCCACTAAGGCCTTGATCCAGTTCCAAC TGC AAATGG
3'

Conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 1 min -> 64.7°C–70.2°C
for 1 min -> 72°C for 1 min and 30 s; and 1 cycle of 72°C for 10 min

Right arm Forward: 5'

CACAGCGCCGCAGGCCTGCGTGGCCTCTAGTAGTTCTGTTTGTAATCTTGG
G 3'

Right arm Reverse: 5' ACACGGATCCAGAACTTATCCATGAATGTGCAAGC 3'

Conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 1 min -> 64.7°C–70.2°C
for 1 min -> 72°C for 1 min; and 1 cycle of 72°C for 10 min

Exon 2 For: 5' ATATGGCGCGCCATCTAAGATCTATTAGCTAGC 3'

Exon 2 Rev: 5' ATATCGGTCCGAGAGTGATATTAAGCCCCGTACC 3'

Conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 1 min -> 64.7°C–70.2°C
for 1 min -> 72°C for 30 s; and 1 cycle of 72°C for 10 min

Polymorphisms were introduced into *FancB* exon 2 in order to reduce the length of contiguous homology thereby reducing the possibility of a crossover occurring within this area that would exclude the 5' *loxP* in the final targeted clone. *FancB* exon2 was cloned into pBluescriptII and three nucleotide changes were made using the QuickChange® Multi Site-Directed Mutagenesis Kit according to the manufacturer's instruction (Stratagene). These changes did not alter the amino acid. The following primers were used:

SDM1: 5'-GATGAGAACACAAAAATCTTTGACG-3'

SDM2: 5'-TACCCAGAAGCTATCAGAATCAGAC-3'

SDM3 5'-GTTTCAGATTCTTGATGCAGGGAAAAG-3'

This altered *FancB* exon 2 was then cloned into the *AscI* and *Rsr2* sites adjacent to the positive selection cassette, *puAtk*. *puAtk* is a positive/negative selection cassette that fuses puromycin N-acetyltransferase (*pu*) to a truncated version of herpes simplex virus type 1 thymidine kinase (*Atk*) generating resistance to puromycin and sensitivity to FIAU [1-(2-deoxy-2-fluoro-1-β-D-arabino-furanosyl)-5-iodouracil] [29]. This cassette is flanked 5' by an RE mutant *loxP* and 3' by an LE mutant *loxP* [30]. The *AscI* and *Rsr2* sites are in between the RE mutant *loxP* and *puAtk*. The entire cassette (RE mutant *loxP* + *FancB* exon 2 + *puAtk* + LE mutant *loxP*) was cloned into the *SfiI* sites that separate the left and right arms as previously described [28].

2.3. Generation and detection of targeted clones

FancB was mutated in two 129 substrains of ES cells. The first strain was AB2.2 and the second strain was IB10. These mouse ES cells (5×10^6 in 800μl DPBS) were electroporated (230V and 500μF, Gene Pulsar apparatus, Bio-Rad) with 5μg of targeting vector previously cut with *PacI*. Then 250μl was seeded onto a 10cm feeder plate. Next day, a final concentration of 1 X puromycin (3μg/ml) was added and puromycin-resistant colonies were picked onto a 96-well feeder plate and maintained in puromycin selection. Cells were expanded and replica plated. One plate was frozen and the other was used to isolate genomic DNA [31]. PCR was used to detect targeted ES cell clones by amplifying a 2.3kb PCR product as follows:

SP1 (in the PGK-reverse orientation) 5'-TCCATTTGTCACGTCCTGCACGACG-3'

SP2 (outside the right arm) 5'-TGTGTTTCAGCTCATGTGTAGGCAGTCATGC-3'.

Conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 1 min -> 70.5°C for 1 min -> 72°C for 1 min and 30 s; and 1 cycle of 72°C for 10 min.

Targeted clones were then tested for the presence of the 5' RE mutant *loxP* using PCR. The RE mutant *loxP* increased the size of the amplified fragment from 828bp to about 900bp.

Forward primer: 5'-TGCTGCTTTGCATATTGCAG-3'

Reverse primer: 5'-TGTGTTCTCATCCAATGCATG-3'

Conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 1 min -> 64°C for 1 min -> 72°C for 30 s; and 1 cycle of 72°C for 10 min.

2.4. Cre-mediated deletion of *Fancb* exon2

Targeted ES cells were expanded in 1 × puromycin media; selection was removed one day before transfection; 5 × 10⁶ cells in 800μl DPBS (Dulbecco's Phosphate Buffered Saline) were electroporated with 10μg of pPGKcreA using a Bio-Rad Gene Pulsar at 230V, 500μF. After transfection, 200μl of electroporation was seeded onto a 10 cm feeder plate without selection for 2days to allow time for removal of *Fancb* exon2-*puA*tk. Then 5 × 10⁴ cells were seeded onto a 10cm feeder plate in 200nM FIAU selection media. FIAU-resistant colonies were picked about 8–10 days later. Cells were expanded in 200nM of FIAU selection media and replica plated. One plate was frozen and the other was used to isolate genomic DNA [31]. Cre-mediated deletion was confirmed by PCR using I-proof polymerase (Bio-Rad Laboratories). For genomic PCR, primers to detect removal of *Fancb* exon2-*puA*tk are as follows:

CreDF1 (in *Fancb* intron 1) 5'-TGCTGCTTTGCATATTGCAG-3'

CreDR1 (in *Fancb* intron, 2): 5'-AGACAGCTACAATGTACTCC-3'.

Conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 1 min -> 64°C for 1 min -> 72°C for 30 s; and 1 cycle of 72°C for 10 min. This amplifies a 1.7kb PCR product in control cells and a 600bp PCR product in *fancb*^{Δex2} cells.

Targeted clones were then expanded and tested for *FancB* transcript using RT-PCR with primers in *FancB* exons 1 and 3 or exons 1 and 2.

FBRTSF1: 5'-TTTGGTGCAACTGGAACG-3',

FBRTSR1: 5'-AGCAAAGTCATTACACCAG-3'

FBRTSR2: 5'-ATATGGATCCTTATGCATCTAGAATCTGAACTG-3'.

Conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 1 min -> 64°C for 1 min -> 72°C for 30 s; and 1 cycle of 72°C for 10 min. The primer pair of FBRTST1 and FBRTSR1 amplifies about 985bp PCR product in control cells while no product is amplified in *fancb*^{Δex2} cells. The primer pair of FBRTST1 and FBRTSR2 amplifies a 1.2kb PCR product in control cells while amplifying 250bp PCR product in *fancb*^{Δex2} cells.

Brca2 exon26–27 transcript was used as an internal control for RT-PCR.

PCTFF1: 5'-AAAAGAATTCTCCACACCGAACAAGACCC-3'

3226R: 5'-AAAAGCGGCCCGCCTAGCTCCGTGGCGGCTGAAAA-3'

Conditions: 1 cycle of 98°C for 5 min; 35 cycles of 98°C for 1 min -> 70.5°C for 1 min -> 72°C for 15 s; and 1 cycle of 72°C for 10 min. This amplifies a 350bp PCR product.

2.5. Colony forming assay

After deletion of *Fancb* exon2 by Cre-mediated recombination, 8–16 FIAU-resistant colonies were pooled and seeded onto a gelatinized 3.5cm plate. Three days after seeding, cells were counted, and then 2000 cells were seeded onto a gelatinized 3.5cm plate. After incubation for 10 days, the cells were stained with 0.2% methylene blue in 70% ethanol and the image was captured with EPSON Scanner (EPSONPERFECTION, V200 PHOTO). Alternatively, to obtain cell number the colonies were trypsinized, pooled and then counted with a hemacytometer.

2.6. Dose response to MMC

The MMC dose response was performed as described [32]. Briefly, 2000 cells were seeded onto wells of a 24 well plate (day 0). On day 1 the media was changed with MMC at the doses shown in Fig. 3. Cells were counted on day 6 using a hemacytometer. Two clones of *fancb^{mt2}* were compared to the parental ES cells, AB2.2. These experiments were repeated three times.

2.7. Three-color FISH (Fluorescence in situ hybridization)

Cells (70–80% confluent on gelatinized 10cm plate) were treated with 1μg/ml colcemid for 4 hours. Then the media was collected into 15ml conical tube and cells were washed on 10cm plate with 3ml of PBS. The PBS was collected into the same conical tube. Cells were trypsinized on the 10cm plate and then collected into the same conical tube. Slide preparation: the cells were spun down at 800rpm for 7min, and then washed twice in PBS (all PBS washes are pH 7.4 unless otherwise noted.). The pellet was resuspended in 600μl 75mM KCl, dropwise while flicking the tube. Cells were incubated in a 37°C water bath for 15min. Then 600μl fixative (methanol/acetic acid=2:1) was added for pre-fixation, dropwise, flicking tube, and then cells were spun down at 3000rpm for 1min. Fresh fixative (600μl) was added to cells dropwise while flicking tube and then incubated at room temperature for 10min and then spun down 3000rpm for 1min. Fresh fixative was added to cells and then a drop was added onto a glass slide. Hybridization: the slide was aged in 100% methanol over night and then air dried just before hybridization. The slide was incubated in 70% formamide hybridization solution at 72°C for 5–10min, transferred to 30% formamide hybridization solution (pre-warmed) containing major satellite repeat (CY-3 5' TGG AAT ATG GCG AGA AAA CTG AAA ATC ATG GAA AAT GAG A 3') and telomeric [6-FAM 5' (CCCTAA)₇ 3'] probes. The slide was then incubated at 37°C for 10–15 min in the dark. Finally, the slide was washed in PBS, 10 dips, and then coverslipped in Vectasheild (DAPI) mounting media (Vector Labs). Fluorescent microscopy was performed with a Zeiss Axioplan2 microscope.

2.8. Cell cycle analysis

For the cell cycle analysis, 1×10^6 ES cells were seeded onto a gelatinized 10 cm plate and allowed to attach overnight. Cells were treated with or without 20nM MMC for 16h and then cultured for 12, 48, or 48h. At each time point, cells were trypsinized and fixed in 70% ethanol for at least 1hr. Fixed cells were kept in 4°C until use. These cells were washed with PBS twice and resuspended in 1ml PI solution containing 1μl of propidium iodide (PI) from a 10mg/ml stock solution, 81μl of RNase A from a 25mg/ml stock solution and 0.1% Triton X-100. Cells were incubated at 37°C for 20min and analyzed for DNA content using BD Facscliber flow cytometer.

2.9. Sister chromatid exchange (SCE) assay

For the SCE assay, 2×10^6 cells were seeded onto a gelatinized 10 cm plate and allowed to attach overnight. Next day, 10μg/ml BrdU was added to the media. The cells were incubated

in BrdU-containing media for 24hrs, released for 12hrs and then treated with 1 μ g/ml colcemid for 4hrs. These cells were processed in the same way as for FISH analysis on metaphase spreads. Metaphase chromosomes were stained with DAPI (blue), pericentromere probe (red) and fluorescent anti-BrdU antibodies (green).

2.10. FancD2, Rad51 and γ -H2ax foci detection

Immunostaining on mouse ES cell is difficult because they proliferate as multilayer colonies. Some 129 substrains like AB2.2 are extremely difficult while others like TC1 are less difficult. Therefore, we deleted *FancB* in TC1 cells to perform the immunostaining. Two $\times 10^4$ cells were seeded onto a plastic chamber slide (Nalge Nuc International Corp. Nalerville, IL). After 48 hrs, cells were treated with 1.5 μ M MMC for 20 hrs. Following MMC treatment cells were rinsed with PBS and then fixed with 2% formaldehyde (diluted in PBS) by incubating at room temperature for 10min. After three washes with PBS, cells were permeablized with 0.5% Triton in PBS for 10min at room temperature. Following permeablization the cells were washed with PBS three times, and then blocked with blocking buffer (4% non-fat milk in PBS) for 1–2 hrs at room temperature. After wash, FancD2 antibody (Novas Biologicals, 1: 200 dilution) or Rad51 antibody (H92, Santa cruz biotechnology, 1: 200 dilution) or γ -H2ax antibody (Upstate, 1:250 dilution) in 4% non-fat milk in PBS was applied to cells and slides were incubated overnight at 4°C. Upon the completion of antibody incubation, the cells were washed with PBS three times and then incubated with blocking buffer containing a fluorescent-labeled secondary antibody (Alexa Fluor488 @ (AB⁺)₂ fragment of goat anti-rabbit IgG (H+L), Molecular Probe, OR, working dilution-1:1000) for 1 hr at room temperature. After rinsing four to five times with PBS, drops of DAPI-containing mounting medium (VectorShield mounting medium, Vector Laboratory, Inc.) were added to the culture slide, and then a coverslip was placed on top of the mounting medium. Cells were observed under a Zeiss fluorescent microscope (Axioplan2).

3. Results and Discussion

3.1. Generation of a conditional *FancB* mutation

To mutate *FancB*, a conditional targeting vector was designed to delete exon 2 (the 1st coding exon) after Cre-mediated recombination. Mutant *loxPs* surround exon 2 and the selection cassette, *pu Δ tk* (Fig. 1A). This cassette is a fusion of puromycin N-acetyltransferase (*pu*) and a truncated version of herpes simplex virus type 1 thymidine kinase (*tk*) [29]. It is ideal for conditional mutations since it offers positive selection in puromycin and negative selection in FIAU. About 1% of puromycin resistant clones were targeted as detected by PCR (Fig. 1B). Targeted clones are called *fancb^{flex2}*. *FancB* exon 2 was deleted in *fancb^{flex2}* cells by Cre-mediated recombination (Fig. 1C). Exon 2 deletion was confirmed by PCR (Fig. 1C) and RT-PCR. For RT-PCR, primers located in exons 1 and 3 produced a short transcript (sequencing showed splicing from exon 1 to exon 3) but no transcript with primers in exons 1 and 2 (Fig. 1D). These cells are called *fancb^{lex2}*.

3.2. Reduced cellular proliferation

Our goal is to determine if *FancB* exon 2 deletion impairs cellular proliferation/viability by performing a colony forming assay on very early passage cells derived from four *fancb^{flex2}* clones. Cre-mediated recombination was used to delete exon 2 and about 2000 FIAU-resistant colonies grew on gelatinized plastic (no feeders) for all four clones. Colonies were picked and seeded onto gelatinized 96-well plates; but cells failed to proliferate suggesting they were nonviable. In order to measure cell number, 8–16 FIAU-resistant colonies were picked, pooled and seeded onto a 3.5cm gelatinized plate (genomic DNA was extracted to verify exon 2 deletion). Cells seeded at this higher density survived and three days later

were counted. Then cells (1000, 2000 or 5000) were seeded onto a 10cm gelatinized plate and compared to control AB2.2 cells and their parental *fancb^{flex2}* clones before Cre-mediated deletion of exon 2. Colony number was observed 10days later after staining the plates. We found *FancB* exon 2 deletion reduced colony number from all four parental clones at all seeding densities (Fig. 2A, only the 2000 cell density shown). To ensure FIAU did not influence results, AB2.2 cells were observed with and without FIAU exposure; FIAU did not impair colony number. Cell number was measured 10days after seeding by pooling the colonies in trypsin and counting cells with a hemacytometer. We found *FancB* exon 2 deletion reduced cell number from all four *fancb^{flex2}* clones at all seeding densities (Fig. 2B, only the 2000 cell density shown) as compared to AB2.2 cells with and without FIAU exposure ($p=0.0057$ and $p=0.0025$, respectively, student t test with unequal variance) and as compared to their parental *fancb^{flex2}* clones ($p=0.0142$). Thus, *FancB* exon 2 deletion lowered colony and cell number.

After the colony-forming assay, the pools of *fancb^{Aex2}* cells were expanded on gelatinized plastic. Most of these cells exhibited micronuclei and died. Yet out of these populations, some cells formed colonies showing their ability to adapt. Therefore, *FancB* exon 2 deletion induced a lethal response, but deletion was not lethal by itself.

The impact *FancB* exon 2 deletion has on cell proliferation/viability was dependent on tissue culture conditions. Cell density influenced survival since *fancb^{Aex2}* cells were expanded from a pool of 8–16 clones but not a single clone. In addition, pooling ~2000 FIAU-resistant colonies (the entire 10cm plate) improved cell survival to a similar level as controls and there was no obvious micronuclei formation or cell death. There was also no appreciable difference for cells cultured on feeder plates even at low density. Based on these observations we used feeder plates to recover exon 2-deleted clones for future experiments. Thus, *fancb^{Aex2}* cells are sensitive to tissue culture conditions at very early passage.

3.3. Response to MMC

A hallmark of FA cells is extreme sensitivity to crosslinking agents like MMC [33]. When compared to control cells, *fancb^{Aex2}* cells were hypersensitive to MMC as determined by a dose response curve (Fig. 3A). In addition, 48hr after exposure to 20nM MMC, *fancb^{Aex2}* cells exhibited a prolonged G2 arrest and increased sub-G1 population (indicating cell death) (Fig. 3B). Furthermore, as analyzed by three-color fluorescence in situ hybridization (FISH), *fancb^{Aex2}* cells exhibited increased levels of spontaneous chromosomal abnormalities (Fig. 3C) that included chromatid breaks (Table 1, $p<0.001$, Yates corrected Chi-Square), chromosomal breaks (Table 1, $p<0.001$) and radials (Table 1, $p<0.001$). The numbers of these abnormalities increased after exposure to MMC (Table 1). Thus, our *FancB*-mutant cells are hypersensitive to MMC as expected.

It is possible that γ -H2AX is needed to recruit FancD2 to chromatin [34]. Therefore, we tested the formation of γ -H2AX foci after exposure to MMC in *fancb^{Aex2}* cells. The *fancb^{Aex2}* cells appear to exhibit a normal response to DNA DSBs as measured by the formation of γ -H2AX foci after 20hrs exposure to 1.5 μ M MMC (Fig. 3D). This observation is in contrast to immortalized human FA-J cells that showed a 3-fold increase in foci after exposure to MMC [35] and to primary FA fibroblasts that showed delayed γ -H2AX formation after exposure to ionizing radiation [36]. Thus, cell type and perhaps experimental conditions likely influence the formation of γ -H2AX foci after exposure to DNA damaging agents in cells defective for the FA pathway.

3.4. Decreased Homologous Recombination

The *fancb*^{Aex2} cells were tested for spontaneous sister chromatid exchanges (SCEs); a process by which a single chromatid breaks and then recombines with the intact sister chromatid during replication. During this process, regions of parental strands in the duplicated chromosomes are reciprocally exchanged [37]. HR is responsible for at least some spontaneous SCEs since a defect in HR decreased spontaneous SCEs [38,39] while unregulated crossing over caused by a mutation in the RecQ helicase Blm (Bloom's syndrome mutated) increased spontaneous SCEs [40]. We found *fancb*^{Aex2} cells exhibited a reduction in spontaneous SCEs (Fig. 4, $p < 0.0001$) indicating reduced HR. Previously published data in human cells showed FA did not affect SCEs [41] while in chicken cells FA increased SCEs [42–44]. Therefore, the impact FA has on SCEs appears to be species/cell type specific.

FancA deletion reduced gene targeting efficiency [20]; therefore, *fancb*^{Aex2} cells were tested for gene targeting efficiency using a targeting vector to *Rad51* (to be described elsewhere). Gene targeting efficiency was 16.2% (6/37) for control cells and 0% (0/96) for *fancb*^{Aex2} cells ($p = 0.0007$, Fisher's exact test). Thus, FancB deletion reduced gene targeting suggesting a defect in HR.

Rad51 is required for homology directed repair so *fancb*^{Aex2} cells were tested for Rad51 nuclear foci. Rad51 enables ATP-dependent homologous pairing and strand transfer of a single DNA strand at the end of a DSB to the complementary strand provided by the sister chromatid during DNA replication [45]. The Rad51 recombinase polymerizes onto ssDNA to yield a right-handed nucleoprotein filament, called the presynaptic filament that searches for homology and pairs the DNA molecules to form a DNA joint. Rad51 foci often co-localize with FancD2 foci during S phase [46] and in response to DNA damaging agents including MMC [47]. FA primary human fibroblasts exhibited decreased Rad51 foci in response to ionizing radiation in one study [48] while FA lymphoblasts did not show any change in Rad51 foci after exposure to ionizing radiation or MMC [49]. Similar to the lymphoblast study, FancD2-deleted DT40 chicken B-cells exhibited intact Rad51 foci formation after exposure to either MMC or ionizing radiation [50,51]. These discordances indicate Rad51-mediated homology directed repair genetically interacts with the FA pathway in a cell-type specific manner.

We tested mouse ES cells for Rad51 foci formation after exposure to MMC. We found *fancb*^{Aex2} cells exhibited lower levels of MMC-induced Rad51 foci as compared to control cells (Fig. 5, $p = 0.0017$, Yates Corrected Chi square). Thus, *fancb*^{Aex2} cells are likely defective for homology directed repair of DSBs associated with interstrand crosslinks. This study is similar to one reported for FancA-defective mouse ES cells suggesting this is a common phenotype for disruption of the FA core complex in mouse ES cells [20].

3.5. Decreased FancD2 foci

In response to crosslinking agents, FancD2 forms nuclear foci that are dependent on the FA core complex while a diffuse nuclear staining was observed independent of the core complex [4]. In a cell-free system, FancD2 was required for replication-coupled ICL repair during S phase [52]. Similarly, *fancb*^{Aex2} cells failed to exhibit MMC-induced FancD2 nuclear foci (Fig. 6A–C, $p < 0.0001$). Thus, FancD2 no longer forms foci in response to MMC-induced DNA damage in FancB-mutant cells as expected. This observation demonstrates deleting *FancB* exon 2 disrupts the FA pathway.

3.6. Conclusions

Here we describe the phenotype for deleting FancB exon 2 in mouse ES cells. We found deleting FancB exon 2 caused reduced proliferation and hypersensitivity to MMC. The MMC defects included reduced survival, increased cell death, G2 arrest and chromosomal abnormalities. With the exception of the cell proliferation/viability defect, these phenotypes are typical for an FA phenotype. It is likely mutations in other FA genes also caused reduced cellular proliferation but this phenotype would not be observed in cancer derived cells or even ES cells cultured under certain conditions (high density and with feeders). In addition to these phenotypes we also observed reduced spontaneous SCEs, gene targeting and MMC-induced Rad51 foci. These phenotypes seem to be influenced by cell type and possibly by experimental conditions. We propose FancB is a good target for an epistatic analysis in mouse ES cells due to its location on chromosome X; thus, being hemizygous in XY cells.

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Fig. 1.

Targeting *FancB*. (A) The *puAtk* positive/negative selection cassette is a fusion of puromycin N-acetyltransferase (*pu*) to truncated herpes simplex virus type 1 thymidine kinase (*Δtk*) [29]. The phosphoglycerate kinase 1 (PGK) promoter [53] and bovine growth hormone polyadenylation sequences (*bpA*) [54] are used. (B) Targeting *FancB* using a conditional targeting vector that floxes exon 2 with a RE mutant *loxP* (blue-green arrow) and a LE mutant *loxP* [30] to generate *fancB^{flex2}* cells. Select in puromycin. Targeted clones were detected by PCR (black half arrows, two targeted clones shown). Lanes 1 and 3 are targeted. These confirmed clones were then tested for the presence of the RE mutant *loxP* (green half arrows). (C) Cre-mediated deletion of exon 2 and *puAtk* to generate *fancB^{Δex2}* cells. Selected in FIAU. FIAU-resistant clones were confirmed for exon 2 deletion by PCR (green and blue half arrow. 1. Control, 2–4. *FancB* exon 2 deletion). (D) Confirm mutation by RT-PCR with red half arrows. *Brca2* was used as a loading control (1. Control, 2–4. *FancB* exon 2 deletion, 5. No DNA).

Fig. 2. *fanchb*^{Δex2} cells exhibited reduced (A) colony number and (B) cell number. Four *fanchb*^{Δex2} clones and their *fanchb*^{flex2} parental clones are shown. The AB2.2 substrain was used.

Fig. 3. *fancb*^{Δex2} cells exhibited increased sensitivity to MMC. (A) Dose response that measures cellular proliferation. This graph shows the average of three experiments. Standard deviation is shown. SF means survival fraction. The AB2.2 substrain was used. (B) G2 arrest and increase cell death (sub-G1 peak) 48hr after exposure to 20nM MMC. The AB2.2 substrain was used. (C) MMC-induced chromosomal abnormalities. Chromatid break (top, arrow) and radial (bottom). A probe was used to detect the major satellite repeats in the pericentromere (red) and the CCCTAA repeats in the telomere (green). DAPI was used to stain the long chromosomal arms blue. The AB2.2 substrain was used. (D) DNA damage response to 20hrs exposure to 1.5μM MMC. Top: control cells (left) and *fancb*^{Δex2} cells (right) stained

for γ -H2AX either exposed (+) or not exposed (–) to 1.5 μ M MMC. Bottom: summary of data. The TC1 substrain was used.

Fig. 4. *fancb*^{Δex2} cells exhibited reduced levels of spontaneous SCEs. Four *fancb*^{Δex2} clones are shown. The AB2.2 substrain was used.

Fig. 5. *fancb^{Δex2}* cells exhibited low levels of MMC-induced Rad51 foci. (A) Control cells, no treatment, NT. (B) Control cells, 20h exposure to 1.5μM MMC. (C) *fancb^{Δex2}* cells, no treatment. (D) *fancb^{Δex2}* cells, 20h exposure to 1.5μM MMC. (E) Summary. Total number of cells observed and total number of cells observed with < 10 or > 10 Rad51 foci. The TC1 substrain was used.

Fig. 6. *fancb Δ ex2* cells failed to exhibit MMC-induced FancD2 foci. After 20hr exposure to 1.5 μ M MMC, (A) control cells but not (B) *fancb Δ ex2* cells exhibited FancD2 foci. (C) Summary. Total number of cells observed and total number of cells observed with FancD2 foci. The TC1 substrain was used.

Table 1
Summary of the number of chromosomal abnormalities observed in a metaphase spread (MPS)

	agent	MPS	chromatid breaks					chromosomal breaks					radials						
			0	1	2	3	4	5>	0	1	2	3	4	5>	0	1	2	3	4>
1. control	-	168	166	2	0	0	0	0	164	4	0	0	0	0	168	0	0	0	0
2. <i>fancB</i> ^{lex2} (#1)	-	212	176	25	8	0	3	0	152	48	11	1	0	0	192	19	1	0	0
3. <i>fancB</i> ^{lex2} (#2)	-	203	175	25	3	0	0	0	156	43	4	0	0	0	182	21	0	0	0
4. <i>fancB</i> ^{lex2} (#3)	-	174	129	36	7	1	0	1	123	43	5	2	1	0	160	12	2	0	0
5. control	MMC	166	160	6	0	0	0	0	144	16	4	0	0	0	166	0	0	0	0
6. <i>fancB</i> ^{lex2} (#1)	MMC	100	24	26	6	4	1	1	35	40	14	4	4	3	24	38	19	11	8
7. <i>fancB</i> ^{lex2} (#2)	MMC	100	29	24	14	4	3	5	32	31	13	16	4	4	29	34	15	14	8
8. <i>fancB</i> ^{lex2} (#3)	MMC	100	20	16	16	7	8	6	23	19	25	21	8	5	20	36	27	11	6