The Fanconi anemia group A protein modulates homologous repair of DNA double-strand breaks in mammalian cells

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Fanconi anemia (FA) cells exhibit hypersensitivity to DNA interstrand cross-links (ICLs) and high levels of chromosome instability. FA gene products have been shown to functionally or physically interact with BRCA1, RAD51 and the MRE11/RAD50/NBS1 complex, suggesting that the FA complex may be involved in the repair of DNA double-strand breaks (DSBs). Here, we have investigated specifically the function of the FA group A protein (FANCA) in the repair of DSBs in mammalian cells. We show that the targeted deletion of FANCA exons 37–39 generates a null for FANCA in mice and abolishes ubiquitination of Fancd2, the downstream effector of the FA complex. Cells lacking FANCA exhibit increased chromosomal aberrations and attenuated accumulation of Brca1 and Rad51 foci in response to DNA damage. The absence of FANCA greatly reduces gene-targeting efficiency in mouse embryonic stem (ES) cells and compromises the survival of fibroblast cells in response to ICL agent treatment. FANCA-null cells exhibit compromised homology-directed repair (HDR) of DSBs, particularly affecting the single-strand annealing pathway. These data identify the FANCA protein as an integral component in the early step of HDR of DSBs and thereby minimizing the genomic instability.

Introduction

Fanconi anemia (FA) is a genetically heterogeneous disease characterized by bone marrow failure, congenital abnormalities and an increased incidence of cancer (1,2). Cells of FA patients exhibit hypersensitivity to bifunctional crosslinking agents, such as mitomycin C (MMC), and an elevated frequency of spontaneous chromosome breaks and translocations, which is further increased after exposure to MMC (3,4). The cellular defect responsible for FA is caused by mutation of any of 11 genes defined by the complementation groups, from FA-A to FA-L, established by somatic cell hybridization of patient cells. Nine of the FA genes have been identified: FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCN and FANCL (2,5,6).

MMC induces DNA interstrand cross-links (ICLs) that result in DNA double-strand breaks (DSBs) during DNA replication (7–9). The hypersensitivity of FA cells to ICL agents may be due to the defects in homology-directed repair (HDR) of DSBs. Human FA fibroblasts also exhibit hypersensitivity to restriction enzyme-induced chromosomal DNA DSBs (10). In addition, some FA patients and cells exhibit a mild radio sensitivity, which may reflect the small proportion of radiation-induced DSBs that are repaired by HDR (11). Biochemical studies have indicated that the FA proteins, FANCA, FANCB, FANCN, FANCE, FANCF, FANCN and FANCL form a multisubunit nuclear core complex (6,12). The monoubiquitination of FANC D2 by FANCL (an E3 ubiquitin ligase) is impaired in cells lacking any member of the upstream FA core complex (12,13), thus explaining their common hypersensitivity to DNA cross-linking agents.

FANCD1/BRCA2 has been shown to be involved in the HDR of DSBs presumably through its physical interaction with RAD51 (14–16). Direct interactions between FANCA and BRCA1, FANCN and FANCD1/BRCA2, and between FANCD2 and FANCD1/BRCA2 have been described (17–20). After monoubiquitination, FANC D2 interacts with NBS1 and colocalizes with RAD51 and BRCA1 in nuclear foci as a response to DNA damage (13,21,22), and this is believed to be essential for its function in DNA DSB repair. Therefore, FA proteins are anticipated to repair DSBs generated when the replication machinery encounters an ICL. However, the precise pathway used by FA proteins in DSB repair remains elusive.

Recent studies have shown that the inactivation of the FANCG, FANCC or FANCD2 homologues in chicken B lymphoma-derived DT40 cells results in deficient homologous repair, which strengthens this hypothesis (23–25). However, since human FANCG has only 39% identity to the chicken homologue, and is unable to complement chicken cells lacking FANCG, the relevance of results from this system for the mammalian FA complex remains largely unknown. Finally, FA group A accounts for >60% of total FA patients, and the function of the protein (FANCA) in the DNA repair process has not been studied in mammalian system, nor in chicken cells. Despite great effort, the exact molecular pathways by which FANCA participates in the repair of DSBs in vivo have not been established.

In order to seek direct evidence for the involvement of the FA proteins in the repair of DSBs in mammalian cells, we engineered mouse cells null for the FANCA protein and analysed the repair pathways of DSBs. FANCA knock-out mice and cells with a defined genetic background, provide a powerful tool for investigation of the authentic functions of FANCA in DSB repair. We show that FANCA is directly involved in the repair of DSBs.
of DNA DSBs by promoting both HDR and, particularly single-strand annealing (SSA), pathways. Failure to activate DSB repair by appropriate pathways is expected to lead to increased use of alternative, error-prone repair processes thus explaining the chromosome instability phenotype of FA patients.

Materials and methods

Gene targeting and cell lines

Targeting vector pTVFlox-Fanca was constructed by cloning PCR-amplified genomic FA fragments into the vector pTVFlox. One lox-P site was inserted into intron 39 and two lox-P sites flanking the neomycin-resistance gene (neo) were introduced into intron 36. A thymidine kinase gene (tk) was used for negative selection. The linearized targeting vector was electroporated into E14.1 embryonic stem (ES) cells and targeted ES clones (Fanca+/Δ) were identified by PCR and Southern blotting. To generate the Fanca deletion allele, Fanca12/12 ES clones were transiently transfected with a Cre-expressing plasmid pMC-Cre, and Fanca1/Δ clones were identified by PCR and Southern-blotting. To delete the remaining wild-type allele of Fanca, two independent Fanca+/Δ ES clones were electroporated with pTVFlox-Fanca and subsequently pMC-Cre. Fanca1/Δ clones were identified by Southern blotting and Western blotting. Dermal fibroblasts were established from ear biopsies of Fanca+/Δ and Fanca1/Δ mice, and immortalized with the transfection of the SV40 large T-antigen-expressing vector, pSVt

Physical analysis of DSBs repair

A DNA fragment located in the random location of the I-Sce I site was amplified by PCR from the genome of mock or I-Sce I-transfected cells as described previously (28). The pathway of DSB repair via either HDR, such as short-track gene conversion (STGC), SSA, long-track gene conversion (LTGC), or nonhomologous end-joining (NHEJ) was measured by the density of DNA bands after staining with ethidium bromide, as described previously (28). The STGC, SSA or LTGC events measured by the I-Sce I-site gains is similar to that obtained by direct sequencing of I-Sce I-resistant fragments as described previously (28). To investigate SSA pathway, we used a combined PCR-Southern blotting method. After I-Sce I expression, genomic DNA was used as PCR template for 2 pairs of PCR primers, either SA-F and SA-R1, or SA-F and SA-R2 (SA-F: GCAACGTGCTGGTTATTGTG; SA-R1 CAAAT- GTGGTATGGCTGATTATG; SA-R2 ATGACCATGATTACGCCAAG). Amplification was for 20 cycles, which was determined to be in the linear range. Southern blots of the PCR products were probed with 32P-labeled probes were used to probe blots: Guinea pig anti-Fanca serum (1:200) and rabbit anti-Fanca serum (1:3000) [both antibodies were raised against amino acids 1-454 of the Fanca protein; kindly provided by Dr Fre Arwert; (30)], and mouse anti-actin serum (1:5000; Santa Cruz, CA, USA). Chemiluminescent detection was performed using the ECL reagent (Amersham Pharmacia Biotech, Freiburg, Germany). For immunoprecipitation, 800 µg of whole cell lysate was incubated with 50 µl of rabbit anti-Fanca serum and antibody-bound proteins were collected by incubation for 16 h with magnetic beads conjugated with sheep anti-rabbit IgG (Dynal Biotech GmbH, Hamburg, Germany).

Detection of monoubiquitinated Fanca2

Cells were treated or untreated with 50 ng/ml of MMC or 5 mM of hydroxyurea (HU). Proteins (80 µg) extracted from cells in lysis buffer (1% TritonX-100, 0.1% SDS, 0.25% DOC, 1 mM EDTA, 50 mM NaF and proteinase inhibitors) were resolved by 6% SDS-PAGE, and electroblotted to nitrocellulose membrane. To detect ubiquitinated Fanca2, an anti-FANCD2 antibody E35 [kindly provided by Dr Alan D.D. Andrea; (13)] was used.

Immunofluorescence staining for foci formation

Cells grown on glass coverslips were treated with 100 ng/ml MMC for 1 h, fixed at indicated time points with ice-cold methanol for 15 min, and permeabilized with ice-cold acetone for 2 min. Cells were stained with rabbit polyclonal anti-Rad51 (1:200, Oncogene, Cambridge, MA, USA), or mouse monoclonal anti-Brc1 (1:10, kindly provided by Dr David MLlvingston) antibodies. Slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized under a Zeiss Axioskop fluorescence microscope equipped with a CCD imaging system (IP Lab Spectrum, NY, USA). For each sample, at least 300 nuclei were scored. A cell with at least five distinct foci in the nucleus was scored as a foci-positive cell.

Results

Generation and characterization of Fanca-null mouse embryonic stem and fibroblast cells

So far, several Fanca mutant mouse strains that carry a homozygous germ-line mutation of Fanca have been reported (31–33). While the deletion of exons 4–7 rendered mice infertile (31), mice carrying exons 1–6 deletion showed various FA symptoms, some of which were dependent on the genetic background (33). In addition, mice with the deletion of exon 37 exhibited some cellular phenotypes mimicking FA, although this strategy generated a ‘leaky’ deletion of Fanca (32). Because intragenic deletion in the region of exons 37–39 are often detected in FA patients, we aimed to investigate the possible effect of mutations in C-terminus of Fanca. To this end, we generated new mouse strains carrying the Fanca gene disrupted in this area. To generate Fanca-null mouse ES and fibroblast cells, we constructed the targeting vector pTVFlox-Fanca by inserting the neomycin gene (neo) cassette flanked by two loxP sites into intron 36 and by placing the third loxP site in intron 39 (Figure 1A). We obtained Fanca+/Δ ES clones (carrying a deletion of exons 37–39) after transiently transfecting a Cre-expressing plasmid terminated into targeted (Fanca+/Δ) ES clones that were generated by gene targeting using the targeting vector pTVFlox-Fanca in E14.1 ES cells (Figure 1B). To disrupt the remaining wild-type allele of Fanca+/Δ cells, we generated FancaΔ/Δ ES clones by electroporating the same targeting vector into Fanca+/Δ cells. After transfection with the Cre-expressing construct into two independent Fanca+/Δ ES clones, five homozygous FancaΔ/Δ ES clones were identified by Southern blotting (Figure 1B). Western blot analysis using antibodies raised against amino acids 1–454 of the Fanca protein detected no protein in these ES clones (Figure 1C), indicating that the deletion of exons 37–39 of Fanca in our knock-out cells and mice is a null mutation.
Fig. 1. Targeted disruption of the mouse *Fanca* gene in ES and fibroblast cells. (A) Structure of the targeting vector and partial restriction map of the *Fanca* locus. *Fanca* locus before and after homologous recombination and Cre-mediated recombination are shown. Exons are indicated by black boxes, and the numbers below indicate the exon number. LoxP sites are represented by empty triangles. The position of the 5' external probe (P) is represented by an empty box. S, SacI. (B) Southern-blot analysis of *Fanca* mutant ES clones after digestion of genomic DNA with SacI, yielding a 3 kb wild-type band, a 4.2 kb targeted allele band, and a 9 kb deleted allele band. Different *Fanca* alleles are indicated as follows: wild-type (+/+ or wt), targeted (t) and deleted (Δ). (C) Western-blot analysis of Fanca protein levels in wild-type (+/+ (W1, W2) and *Fanca*ΔΔ ES clones (H1, C8). Actin is a loading control. (D) Western-blot analysis of Fanca protein levels in *Fanca*ΔΔ (ΔΔ) and wild-type (+/+) fibroblast cells. Left column and right column show total cell lysate and immunoprecipitation products, respectively. The molecular weight is indicated in the middle column. Actin was used to control loading.
To study the role of Fanca in the repair of DSBs, we generated Fanca-deficient mice and cells using two Fanca<sup>-/-</sup> ES clones that were microinjected into blastocysts to generate germline chimeric mice. The Fanca<sup>+/+</sup> mice were obtained at a normal mendelian ratio after intercrossing of Fanca<sup>+/+</sup> mice (M.Digweed and Z.-Q-Wang, unpublished data). To carry out the following studies, we isolated ear fibroblast cells from wild-type and Fanca<sup>−/−</sup> mice. Fanca<sup>−/−</sup> fibroblasts were devoid of the Fanca protein as determined by western blotting and immunoprecipitation (Figure 1D).

Compromised DNA damage response and increased chromosome instability in Fanca-null cells

To test the biological consequences of Fanca deletion, we performed colony survival assay using Fanca-null mutant fibroblasts. Fibroblast cells were plated at 250 cells/10 cm culture dish and treated with various doses of MMC. After 10 days of growth, we found that the number of colonies of Fanca<sup>−/−</sup> cells was greatly reduced compared with that of wild-type controls (Figure 2A). Because chromosome instability is a hallmark of FA lymphocytes and fibroblasts, we next investigated, using mouse Fanca-null cells, the instrumental role of Fanca deficiency in chromosome aberrations. We found a very large increase of spontaneous chromatid breaks, translocations and other chromosome aberrations in Fanca<sup>−/−</sup> cells compared with controls. These chromosomal aberrations were significantly elevated in Fanca<sup>−/−</sup> cells after MMC treatment (Figure 2B). This hypersensitivity of Fanca-null cells is consistent with the characteristics of the FA phenotype and indicates a defect in the repair of DNA lesions.

Because FANCA interacts with BRCA1 (17) and the FA complex is important in the DNA damage response (2), we next investigated whether Fanca deletion would affect the accumulation of DSB repair molecules after MMC treatment. While 70% of wild-type cells contained Brca1 foci, only 51% of Fanca<sup>−/−</sup> cells showed foci formation after MMC treatment (Figure 2C). We also found that the population positive for Rad51 foci was always lower in Fanca<sup>−/−</sup> cells than that of wild-type cells (Figure 2D). These results suggest that the recruitment of DNA repair molecules to the DSBs is attenuated in the absence of Fanca.

It has been shown that monoubiquitinated FANCD2 colocalizes with RAD51 and BRCA1 in nuclear foci as a response to DNA damage, and Fanca is in the core complex upstream of the Fancd2 pathway (2), we next tested whether the inactivation of Fanca would affect the modification of Fancd2. As expected, after treatment with MMC and hydroxyurea (HU), the monoubiquitination of Fancd2 was induced in wild-type cells. However, Fancd2 monoubiquitination was not detectable in Fanca-null cells (Figure 2E). These results indicate that the ablation of Fanca affects repair of DSBs by modulating the Fancd2 pathway, and suggest a role for Fanca in the repair of DSBs.

Deficient gene-targeting efficiency in Fanca-null ES cells

The gene-targeting efficiency is a surrogate marker reflecting the relevant homologous recombination activity at a cellular level. To investigate the effect of Fanca depletion on the gene-targeting efficiency, a gene-targeting vector hprt-DRGFP (27) was electroporated into two ES cell clones of wild-type (W1, W2) and Fanca<sup>−/−</sup> (H1, C8) genotype. The gene-targeting efficiency to the hprt locus (i.e. homologous versus total integration) is significantly reduced in Fanca<sup>−/−</sup> ES cells compared with wild-type cells (Table I), suggesting that the disruption of Fanca compromises homologous recombination leading to reduced gene-targeting efficiency.

Compromised HDR in Fanca-null fibroblast cells

To investigate the role of Fanca in DSB repair, we transfected the fluorescence-based reporter substrate (DRGFP) (29) into wild-type and Fanca<sup>−/−</sup> fibroblasts and isolated clones containing a single intact copy of DRGFP analysed by Southern blotting (Figure 3A and B). In order to minimize sources of variability, at least seven independent clones of each genotype were used in the recombination assay. In the presence of I-SceI, a 3-fold reduction of GFP-positive cells (~0.5%) was observed in Fanca<sup>−/−</sup> cells compared with wild-type cells (~1.5% GFP-positive cells) (Figure 3C and D). As expected, we found almost no GFP-positive cells in mock-vector (without the I-SceI gene)-transfected populations for both genotypes (Figure 3C). Moreover, a comparable transfection efficiency with I-SceI expression plasmids was observed for wild-type and Fanca<sup>−/−</sup> fibroblast cells as examined by transfection with a GFP-expressing plasmid (data not shown). The decreased number of the GFP-positive Fanca-null cells suggests that HDR of genomic DSBs is impaired in the absence of Fanca.

The SSA pathway is particularly affected in Fanca-null cells

We next dissected the recombination repair pathways of I-SceI-induced DSBs. In the reporter system used here, either HDR or NHEJ can repair the DSB generated at the I-SceI site (Figure 4A). Pathways of HDR are STGC, LTGC or SSA (Figure 4A). Only the STGC-HDR pathway will restore a functional GFP; SSA and LTGC will cause homologous deletion, resulting in a non-functional GFP; NHEJ may undergo precise or imprecise pathways (Figure 4A). The repair of a DSB via STGC, SSA, LTGC or imprecise NHEJ causes I-SceI site loss and therefore resistance to endonuclease I-SceI. In contrast, SSA and LTGC generate a BcgI site that replaces the I-SceI site in the substrate, rendering sensitivity to BcgI. We amplified by PCR a DNA fragment containing the original location of the I-SceI site from the genome of mock (without the I-SceI gene) or I-SceI-transfected ES cells and digested these PCR products with I-SceI or BcgI. While similar proportions of I-SceI-site loss (~30–35% of PCR products) were detected in both wild-type and Fanca<sup>−/−</sup> cells (Figure 4B), the relative amount of BcgI-site gain, indicative of STGC, SSA or LTGC, were significantly reduced to 12–15% in Fanca<sup>−/−</sup> cells compared with 23–25% in wild-type cells (Figure 4C). These results indicate that the ablation of Fanca affects repair of DSBs by HDR pathways.

We further analysed the status of SSA after the repair of DSBs induced by I-SceI in Fanca<sup>−/−</sup> cells by PCR amplification using the primer set (SAF and SA-R2), and compared these PCR products with PCR products generated through STGC and NHEJ and uncut substrate DRGFP (using primers SAF and SA-R1) (Figure 4A). SSA generated products were significantly reduced in Fanca<sup>−/−</sup> cells compared with wild-type cells (Figure 5A and B). As a control, Brca2/Fancd1-null Chinese hamster ovary cells displayed an elevated SSA activity (Figure 5A and B), consistent with previous observations (36). To test the potential contribution of LTGC, also measured by PCR with primers SAF and SA-R2, to the authentic SSA events, we grew cells in the presence of puromycin after DSBs repair, which would eliminate cells which had
Fig. 2. MMC hypersensitivity and attenuated accumulation of DNA DSB repair molecules in FancaΔ/Δ cells. (A) Colony survival assay after MMC treatment. Curves represent mean colony counts as a percentage of untreated cultures with error bars showing SD. (B) Chromosomal aberration after MMC treatment. Br, chromatid break; Tr, translocation; CBr, isochromatid break, ring or dicentric chromosome. (C and D) Immunofluorescence analysis of foci formation after MMC treatment. Cells were treated with 100 ng/ml MMC for 1 h, and stained for foci formation of Brca1 (C) and Rad51 (D) at 9 h after MMC removal. Quantification of the frequency of foci-positive populations in wild-type and FancaΔ/Δ fibroblast cells. Each bar represents the score of at least 300 nuclei. (E) The lack of DNA damage-mediated monoubiquitination of Fancd2 in the absence of Fanca. Fancd2-S, a low molecular weight isoform; Fancd2-L, a high molecular weight monoubiquitinated isoform. MMC, mitomycin C; HU, hydroxyurea.
Table I. Gene-targeting efficiency in Fanca\(^{+/+}\) and Fanca\(^{\Delta\Delta}\) ES cells

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<th>Cell lines</th>
<th>Experiment 1</th>
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<tr>
<td></td>
<td>(puro^R (\times 10^{-6})^a)</td>
<td>(6-TGR/puro^R (\times 10^{-7})^b)</td>
<td>(puro^R (\times 10^{-6})^a)</td>
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<tr>
<td>Fanca(^{+/+})</td>
<td></td>
<td></td>
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<tr>
<td>W1</td>
<td>325</td>
<td>35.0 (1.08%)</td>
<td>432</td>
</tr>
<tr>
<td>W2</td>
<td>273</td>
<td>24.5 (0.90%)</td>
<td>232</td>
</tr>
<tr>
<td>Fanca(^{\Delta\Delta})</td>
<td></td>
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<td></td>
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<tr>
<td>H1</td>
<td>787</td>
<td>10 (0.12%)</td>
<td>714</td>
</tr>
<tr>
<td>C8</td>
<td>885</td>
<td>10 (0.11%)</td>
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\(^a\)Number of total \((puro^R)\) integrants divided by the number of cells electroporated with the hprt-DRGFP targeting vector.

\(^b\)Number of homologous \((6-TGR/puro^R)\) integrants divided by the number of cells electroporated with the hprt-DRGFP targeting vector.

\(^c\)Gene-targeting efficiency is calculated by dividing the number of homologous \((6-TGR/puro^R)\) integrants with the number of total \((puro^R)\) clones.

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**Fig. 3.** HDR is compromised in Fanca-null cells. (A) The DRGFP recombination substrate integrated at a random chromosomal locus (29). (B) Southern-blot analysis of cells containing the DRGFP reporter. Cells containing the single copy of the substrate (indicated by asterisk) show only two bands with equal density: one is 0.8 kb (the iGFP copy), the other > 3.2 kb band containing the SceGFP sequence. (C) A representative flow cytometric analysis for HDR events (post-DSB) in wild-type (+/+ ) and Fanca\(^{\Delta\Delta}\) (ΔΔ) fibroblastic cells after transfection with I-SceI endonuclease-expressing or mock vectors (without the I-SceI gene). The GFP-positive population (post-DSB) is separated from the GFP-negative population as shifting to greenward. (D) Percentage of GFP-positive cells after transfection with the I-SceI expression vector. Bars represent the mean of at least seven independent clones of each genotype from three independent experiments. See online Supplementary material for a colour version of this figure.
Fig. 4. Analysis of repair pathways of DSBs in Fanca\textsuperscript{+/−} cells. (A) PCR strategy for the detection of DSB repair pathways. The I-SceI site (black segment) and BcgI site (striped segment) located in SceGFP and iGFP, respectively. The binding sites and orientation of PCR primers (DF1, SAF, DR, SA-R1 and SA-R2) are indicated below the corresponding region. Four putative products arising from the original genomic region of the I-SceI site after STGC, SSA, NHEJ (imprecise and precise), or LTGC (coupled to NHEJ), and one product from DRGFP (the uncut substrate) are shown. (B and C) The analysis of homologous repair of DSBs. An ∼723 bp PCR product generated by primers DF1 and DR was digested with I-SceI or BcgI enzymes. (B) I-SceI\textsuperscript{R} fragments result from STGC, SSA, LTGC or imprecise NHEJ, resistant to I-SceI cleavage; I-SceI\textsuperscript{S} fragments represent either cells without generation of DSBs in vivo because of no cleavage by I-SceI, or cells which repaired the DSB by precise NHEJ. (C) BcgI\textsuperscript{R} fragments arise from DSBs, which underwent STGC or SSA or LTGC and are sensitive to BcgI. BcgI\textsuperscript{S} fragments arise from cell populations either without generation of DSBs in vivo or repaired through NHEJ. Mock (without the I-SceI gene)-transfected clones served as controls. Bars (in B and C) represent the mean of three independent experiments.
An intragenic deletion in the region of exons 37–39 has been implicated in recombination repair processes, particularly in the homologous HDR and, particularly, SSA pathways. The present study identifies Fanca for the first time as an integral component in the biological response cascade of DNA DSBs by promoting both HDR and SSA. The absence of Fanca reduces HDR mainly in the SSA pathway, suggesting a putative SSA product shown in Figure 5A. Taken together, the absence of Fanca reduces HDR mainly in the SSA pathway, whereas the deletion of Brca2/Fancd1 seems to enhance SSA.

**Discussion**

Studies using human FA cells and mutant chicken DT40 cells have suggested a role for various FA proteins in the repair of DSBs. The present study, using mouse Fanca-null cells, identifies Fanca as the first time as an integral component in the biological response cascade of DNA DSBs by promoting both HDR and, particularly, SSA, pathways. The present study ties the Fanca protein to an early step in the homologous recombination repair process.

FA patients show a high heterogeneity in genetic background and mutations in the FA protein range from intragenic deletion, protein truncation to null mutation. Because intragenic deletion in the region of exons 37–39 has been detected in FA patients, we generated cells with a corresponding disruption at the C-terminus of the Fanca gene. Western-blot analysis confirmed the complete absence of the Fanca protein in these cells. Using newly engineered Fanca-null mouse cells, the present study shows direct genetic evidence for a role of the FA complex in HDR repair of DSBs in mammalian cells. The homologous repair defects in Fanca mutant cells are most likely to be responsible for chromosomal instability phenotype (chromatid breaks and chromosome fusions) observed in FA cells as well as in our Fanca knockout cells. There are several putative mechanisms by which Fanca may participate in HDR. Fanca may modulate the recruitment of molecules that are operative in DSB repair or signaling to DSB sites. It has been shown that Fanca is required for a stable, functioning core complex and thus for monoubiquitination of FANCD2 and only the monoubiquitated form of FANCD2 is able to co-localize with BRCA1 and RAD51 (13, 22). In this regard, we found that Fanca is required for monoubiquitination of Fancd2 and that the foci formation of Rad51 and Brca1 at DNA breaks was reduced in Fanca-null murine cells. These findings are consistent with previous studies showing attenuated recruitment of BRCA1 and RAD51 to nuclear foci in human FA cells (FANCA/C/G) (37, 38). It is possible that Fanca is involved in homologues repair directly through its interaction with Brca1 (17). Moreover, Fanca may serve as a docking point at DSB sites for the assembly of component of the BRCA1-associated complex, such as BRCA1 and RAD51. Finally, Fanca may be involved in the initial detection of DSBs. In this regard, it is worth noting that FANC2D interacts with NBS1 in DNA damage response (21) and delayed Mre11 foci formation has been found in FANCC/D cells after MMC treatment (37). A recent study also shows that ATR couples FANC2D monoubiquitination to the DNA-damage response (39). Interestingly, we also found that γ-H2Ax foci formation was down-regulated in Fanca-null cells (data not shown).

The gene conversion pathway repairs DSBs by retrieving genetic information from an intact homologue (sister chromatid or homologous chromosome), through strand exchange. As an alternative homology-dependent DSB repair pathway, SSA events may cause deletions of certain sequences (error prone) (40). SSA and gene conversion have some common characteristics, as they both use single-stranded DNA as a common intermediate and share components, such as RAD52 (40). We show that the absence of Fanca abolished ubiquitination of the downstream effector of the FA complex, Fancd2 and that the repair products through the SSA pathway are specifically low in Fanca-null cells, which contributes to the overall HDR deficiency. These data are in agreement with observations in human FA cells showing that the FA complex and FANC2D ubiquitination are involved in SSA and HDR (41). Therefore, although the reduction of the SSA pathway (error prone) would cause less genomic instability, the general defects in HDR are most probably responsible for the chromosomal instability phenotype of FA cells. Taken together, our data place the FA pathway in the early step of HDR of DSBs. These findings also suggest that DNA strand resection is one step that could be regulated by the FA pathway, given the importance of single strands as substrates for both gene conversion and SSA.

While global DSB repair by HDR is predominant in lower eukaryotes, e.g. yeast, higher eukaryotic cells have evolved multiple pathways to repair DNA DSB lesions, namely by...
NHEJ and HDR (42). FA cells, which are defective in SSA, are unable to fine-tune their choice of the HDR pathways and may use NHEJ more extensively, and this may increase the frequency of chromosome aberrations and mutation rates leading to neoplasia.

Supplementary material

Supplementary material is available online at: http://arclin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

References


Role of Fanca in homologous repair in mouse cells

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