Functional relationships of FANCC to homologous recombination, translesion synthesis, and BLM

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Some of the restarting events of stalled replication forks lead to sister chromatid exchange (SCE) as a result of homologous recombination (HR) repair with crossing over. The rate of SCE is elevated by the loss of BLM helicase or by a defect in translesion synthesis (TLS). We found that spontaneous SCE levels were elevated ~2-fold in chicken DT40 cells deficient in Fanconi anemia (FA) gene FANCC. To investigate the mechanism of the elevated SCE, we deleted FANCC in cells lacking Rad51 paralog XRCC3, TLS factor RAD18, or BLM. The increased SCE in *fancc* cells required *Xrcc3*, whereas the *fancc/rad18* double mutant exhibited higher SCE than either single mutant. Unexpectedly, SCE in the *fancc/blm* mutant was similar to that in *blm* cells, indicating functional linkage between FANCC and BLM. Furthermore, MMC-induced formation of GFP-BLM nuclear foci was severely compromised in both human and chicken *fancc* or *fancc2* cells. Our cell survival data suggest that the FA proteins serve to facilitate HR, but not global TLS, during crosslink repair.

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Introduction

The integrity of the mammalian genome is essential to suppress oncogenesis, and is maintained by an intricate network of molecules that guard against alterations from DNA damage (Rouse and Jackson, 2002). Fanconi anemia (FA) and Bloom syndrome (BS) are well-known examples of chromosomal instability disorders with an increased incidence of cancer. However, the clinical and cellular features of these diseases are quite distinct. FA is a hereditary disorder characterized by bone marrow failure, skeletal anomalies, and development of leukemia or squamous cell cancer (Grompe and D'Andrea, 2001; Joenje and Patel, 2001; D'Andrea and Grompe, 2003). FA cells are highly sensitive to killing by interstrand crosslinking (ICL) agents such as mitomycin C (MMC) and cisplatin, and display excessive chromosomal aberrations following MMC treatment (Sasaki and Tonomura, 1973; Sasaki, 1975). Complementation analysis indicates that at least 11 causative genes are involved (Levitov *et al*, 2003), and nine of them have been cloned (*FANCA/B/C/D1/D2/E/F/G/L*) (Joenje and Patel, 2001; D'Andrea and Grompe, 2003; Meetei *et al*, 2003a, 2004). Most of the encoded proteins (*FANCA/B/C/E/F/G/L*) form a nuclear core complex, which is responsible for monoubiquitination of FANCD2, a key event in the FA 'pathway' (Garcia-Higuera *et al*, 2001). The exact biochemical function of FANCD2 remains unknown, but recent evidence indicates a role of FANCD2 in promoting homologous recombination (HR) (Yamamoto *et al*, 2005), which is crucial for maintaining genome integrity (Venkitaraman, 2004). Indeed, another FA protein, FANCD1/BRCA2 (Howlett *et al*, 2002), a tumor suppressor for familial breast cancer, is an important regulator of Rad51, the central molecule of HR (Venkitaraman, 2002; West, 2003).

BS is also a hereditary disorder characterized by immunodeficiency, small stature, and development of a variety of cancers (German, 1993). In contrast to FA, BS is caused by a defect in a single gene, BLM helicase (Ellis *et al*, 1995). The hallmark and a diagnostic feature of BS cells is ~10-fold increase in sister chromatid exchange (SCE) levels (Chaganti *et al*, 1974). BLM is a member of the eukaryotic RecQ helicase family, which is thought to play multiple roles in DNA replication, recombination, or repair (Bachrati and Hickson, 2003; Hickson, 2003). For example, BLM is a part of BRCA1-containing mega-protein complex (Wang *et al*, 2000b), and colocalizes in subnuclear foci with Rad51 (Wu *et al*, 2001). The RecQ family contains four more members (RecQ1, WRN, RecQ4, and RecQ5), and a defect in WRN or RecQ4 is known to cause the genome instability disorders, Werner syndrome (Yu *et al*, 1996) or Rothmund-Thomson syndrome (Kitao *et al*, 1999), respectively. Unexpectedly, BLM has been recently detected in the FA core complex (Meetei *et al*, 2003b), suggesting a potential functional link between FA and BLM proteins.

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It is well accepted that replication forks often stall at various DNA lesions including endogenous base damage (Cox *et al*, 2000; Cox, 2001). At least two basic processes, translesion synthesis (TLS) and HR, function to ensure that replication can be resumed at stalled or broken forks. When single-strand breaks or blocking lesions are converted to double-strandbreaks or gaps, respectively, during passage of replication forks, these chromatid discontinuities may be repaired by HR occurring between daughter chromatids (Sonoda *et al*, 1999). In classical models, HR involves formation of double Holliday junctions, and their resolution may or may not result in crossing over. However, HR repair events in somatic cells primarily proceed without double Holliday junctions, hence without crossing over, through synthesis-dependent strand annealing pathway (Paques and Haber, 1999; Johnson and Jasin, 2000; Cromie *et al*, 2001). Thus, it is likely that only a fraction of restarting events lead to SCE, which is known to depend on Rad51, Rad54 (Sonoda *et al*, 1999; Dronkert *et al*, 2000), five Rad51 paralogs (Takata *et al*, 2000, 2001), and Nbs1 (Tauchi *et al*, 2002). Since TLS mediates lesion bypass by a group of specialized polymerases, dysfunctional TLS might lead to more SCE if the stalled fork breaks and requires HR to restart. Consistently, at least some of the TLS-deficient cells such as *rad18*, *rev3*, or *dinB* mutants display increased levels of SCE (Okada *et al*, 2002; Yamashita *et al*, 2002; Sonoda *et al*, 2003). In contrast, BLM may directly suppress SCEs by catalyzing the 'dissolution' of the double Holliday junctions (Wu and Hickson, 2003).

In this study, we generated *FANCC*-deficient DT40 cells (hereafter referred to as *fancc*), and found that levels of spontaneous SCEs increased ~2-fold compared to wild-type cells, similar to our *fancc2* cells (Yamamoto *et al*, 2005). To investigate the basis of the elevated SCE, we made double mutants of *FANCC* with mutations in HR (*XRCC3*), TLS (*RAD18*), and BLM helicase. Our genetic analyses indicate functional linkage of *FANCC* with *Xrcc3* or BLM but not with *Rad18*. Furthermore, crosslink damage-induced relocalization of BLM was defective in both human and chicken *fancc* or *fancc2* cells. We propose that BLM, regulated by the FA pathway, functions in restarting stalled replication forks blocked by spontaneous lesions and ICLs.

Results

Disruption of chicken *FANCC* gene in DT40 cells

We obtained a cDNA clone containing full-length chicken *FANCC* by searching the chicken EST database (<http://swallow.gsf.de/DT40/dt40Est.html>). Chicken *FANCC* encodes a putative 559-amino-acid protein (DDBJ accession number AB176529) compared to 557 amino acids of human *FANCC*. The identity and similarity between two proteins are 45 and 59%, respectively. There are no domains or motifs suggestive of biochemical function in either protein.

Based on the sequence of the cDNA, we PCR-amplified a genomic sequence of chicken *FANCC* and designed a targeting vector (Figure 1A). A single transfection with the vector abrogated the band in Southern blot analysis (Figure 1B). This is not surprising, given the localization of *FANCC* on human chromosome 9 and the extensive synteny between human chromosome 9 and chicken Z sex chromosome

Figure 1 Targeted disruption of chicken *FANCC* loci in DT40 cells. (A) Schematic representation of partial chicken *FANCC* locus, the gene disruption construct, and the configuration of targeted allele. S, *SacI*; B, *BamHI*; H, *HindIII*. Only a single exon was deleted by the gene disruption. Arrowheads indicate the positions of primers used in RT-PCR. (B) Southern blot analysis of *SacI*-digested genomic DNA from cells with indicated genotypes using flanking probe as shown in (A). WT, wild type. (C) RT-PCR analysis of total RNA from wild-type and *fancc* cells. Primers were designed from *FANCC* sequences of upstream and downstream exons as shown in (A). As a control, the entire coding region of chicken *Rad51* was amplified from each RT product. (D) Western blot analysis of whole-cell lysate prepared from wild-type and *fancc* cells probed with anti-chicken *FANCC2* serum. Cells were treated with MMC (500 ng/ml) for 1 h, washed, and lysed 6 h later. L or S, long or short form of *FANCC2*, respectively.

(Nanda *et al*, 1999). DT40 was derived from a female chicken, which is hemizygous in terms of the Z chromosome. Another FA gene, *FANCG*, also lies on human chromosome 9, and we were able to disrupt *FANCG* in DT40 by single transfection (Yamamoto *et al*, 2003). RT-PCR analysis confirmed the *FANCC* gene disruption (Figure 1C) that is expected to delete one exon. Nucleotide sequencing revealed that the faint, shorter transcript was owing to anomalous splicing, which is expected to create a truncated protein (residues 1–55 and six additional amino acids) because of a frame shift. We also examined induction of the long, monoubiquitinated form of *FANCC2* protein (*FANCC2*-L) (Gregory *et al*, 2003) by MMC treatment using Western blotting with anti-chicken *FANCC2* antiserum. As shown in Figure 1D, the *FANCC2*-L form existed in untreated wild-type cells, and its proportion was clearly increased after MMC treatment. In contrast, *FANCC2*-L was not detected in *fancc* cells before or after MMC treatment (Figure 1D), similar to human *fancc* cells (Garcia-Higuera *et al*, 2001).

fancc cells have increased levels of spontaneous SCE

The *FANCC*-deficient cells grew more slowly than wild-type cells with an increased proportion of dead cells in the culture (data not shown), and the cell cycle distribution examined by BrdU pulse labelling was not altered (data not shown). As expected, the cells were highly sensitive to the DNA cross-linkers MMC and cisplatin, while sensitivity to X-rays or UV irradiation was only mildly increased (Figure 2A). The cisplatin sensitivity could be complemented to wild-type levels by expression of chicken *FANCC* cDNA, indicating that this defect was indeed caused by *FANCC* disruption (Figure 2B). Although spontaneous chromosomal breaks

Figure 2 Characterization of *fancc* cells. (A) Sensitivity curves of cells to various DNA-damaging agents. The fraction of surviving colonies in methylcellulose plates is shown for each agent. Mean and standard deviation (s.d.) of at least three independent experiments are shown. WT, wild-type cells. (B) Complementation of *fancc* cells with *FANCC* expression. Three *fancc* clones expressing chicken *FANCC* cDNA (+chFANCC#1 to #3) were compared with wild-type and *fancc* cells in colony survival with cisplatin. (C) Chromosome aberrations after MMC treatment in wild-type and *fancc* cells. A total of 200 metaphases were scored.

Table 1 Targeted integration efficiencies in *fancc* cells

Genotypes	Targeting vectors		
	Ku70-His	Xrcc2-puro	Ovalubumin-puro
Wild type	14/34 (41 %)	12/24 (50 %)	10/24 (42 %)
<i>fancc</i>	3/20 (15 %)	1/11 (9 %)	0/35 (0 %)

Data are numbers of targeted clones per number of clones analyzed by Southern blotting. The percentage of targeted integration events is given in parentheses.

were not elevated, MMC-induced aberrations occurred much more frequently in *fancc* cells (Figure 2C).

To test whether *fancc* cells have HR defects, we examined gene targeting at three genomic loci. Wild-type and *fancc* cells were transfected with linearized targeting vectors and selected in media containing appropriate drugs. After expansion, each colony was examined for targeting events by Southern blot analysis of genomic DNA. *Fancc* cells had dramatically reduced gene-targeting efficiency compared to wild-type cells (Table 1). In contrast, we also found that the frequency of spontaneous SCE in *fancc* cells was elevated ~2-fold compared to wild-type cells (Figures 3C and 4B), similar to our *fancc2* cells (Yamamoto *et al*, 2005).

Elevated SCEs in *fancc* cells depend on Rad51 paralog Xrcc3

To investigate the mechanism of SCE elevation in *fancc* cells, and to better define the FA pathway, we performed genetic analysis by disrupting *FANCC* in cells that are deficient in HR (*xrcc3*), TLS (*rad18*), or BLM helicase (*blm*).

We disrupted *FANCC* in conditional *xrcc3* background (Ishiai *et al*, 2004), and exposed the cells, as well as the parental cells, to 4-hydroxy tamoxifen (OH-TAM) (Figure 3A) to excise the *Xrcc3*-EGFP expression cassette by activating the MerCreMer recombinase (Zhang *et al*, 1998). The removal

was ensured by subcloning, and was further verified by the loss of GFP fluorescence and by Southern blotting using human *XRCC3* probe (data not shown). Thus, two independent clones of *fancc/xrcc3* cells were established.

We compared the *fancc/xrcc3* cells with *fancc* cells in terms of cisplatin sensitivity. *Fancc* cells were much more cisplatin sensitive to killing compared to *xrcc3* cells. However, *fancc/xrcc3* cells displayed about the same cisplatin sensitivity as the *fancc* single mutant, indicating functional overlap between *FANCC* and *XRCC3* (Figure 3B). In addition, spontaneous SCE was clearly decreased in *xrcc3*, but elevated in *fancc* cells compared to the parental conditional cells (Figure 3C). Not surprisingly, the SCE frequency in two clones of *fancc/xrcc3* cells was similar to that of *xrcc3* cells (Figure 3C), indicating that spontaneous SCE in *fancc* cells is partially *Xrcc3*-dependent as in wild-type cells (Takata *et al*, 2001).

Genetic analysis of *FANCC* and TLS factor *Rad18*

Since several TLS-deficient vertebrate cells including *Rad18*-deficient DT40 (Yamashita *et al*, 2002) have increased SCE (Okada *et al*, 2002; Sonoda *et al*, 2003), it is quite possible that elevated SCE in *fancc* cells might be related to TLS defects. In yeast *Saccharomyces cerevisiae*, most TLS activity is dependent on *Rad6*, and its heterodimeric partner *Rad18*. To examine the relationship between the FA pathway and TLS, the *FANCC* gene was targeted in *rad18* cells (Yamashita *et al*, 2002). Although both *rad18* and *fancc* cells had high sensitivity to cisplatin or methylmethanesulfonate (MMS), the *fancc/rad18* double mutant displayed even higher sensitivity to both agents, indicating their distinct functions (Figure 4A). Moreover, *fancc/rad18* cells had even greater increased SCE than either *rad18* or *fancc* cells (Figure 4B). Thus, we conclude that spontaneous SCEs in *fancc* cells are elevated by a mechanism separate from *Rad18* function. It is still possible that *FANCC* plays a role in *Rad18*-independent

Figure 3 Genetic analysis of *fancc* mutation combined with *xrcc3*. (A) Generation of conditional *xrcc3* cells and disruption of *FANCC* in those cells. OH-TAM treatment activates MerCreMer recombinase that removes the human *Xrcc3* (*hXrcc3*)-IRES-EGFP expression cassette. WT (cond), *xrcc3* cells that express *hXrcc3*, GFP, and MerCreMer. (B) CDDP sensitivity curves of cells with indicated genotypes. *Fancc* cells used were derived from wild-type DT40 cells, not from the conditional *xrcc3* mutant. Mean and s.d. from three independent experiments are shown. (C) Spontaneous SCE levels. Numbers represent mean and s.d. of scores from 50 metaphases. In this analysis, we used *fancc* cells derived from the conditional *xrcc3* mutant, which express *hXrcc3*. Statistical significance was detected between WT (cond) versus *fancc*, WT (cond) versus *xrcc3*, and *fancc* versus *xrcc3* (Bonferroni/Dunn test, $P < 0.0001$ in all comparisons). The difference was insignificant between *xrcc3* versus *fancc/xrcc3* double-mutant clones.

TLS, since not all TLS events require Rad18 in vertebrate cells (Okada *et al*, 2002).

A functional link between FA pathway and BLM in suppressing spontaneous SCE

Next, we deleted *FANCC* in BLM-deficient cells (Wang *et al*, 2000a). In contrast to *fancc/rad18* cells, we found that the *blm* and *fancc/blm* mutants displayed about the same high levels of spontaneous and MMC-induced SCE (Figure 4D and E). Given the functional link suggested by these findings, we measured cell survival of *blm*, *fancc*, and *fancc/blm* cells

following treatment with several mutagens. While MMS-treated *fancc/blm* cells survived more poorly than either single mutant (additive phenotype), survival following cisplatin or MMC treatment was similar between *fancc* and *fancc/blm* cells (Figure 4C). These results suggest that *FANCC* and *BLM* act in a common pathway in ICL repair as well as in controlling SCE levels. However, in repairing DNA lesions created by MMS, they likely have more distinct functions. In addition, *fancc/blm* cells grew more slowly than either *fancc* or *blm* cells (data not shown), indicating that the requirement of both genes extends to cell viability.

To further verify the potential link between FA proteins and BLM, we created double mutant lacking both *FANCD2* and *BLM* by targeting *FANCD2* gene in *blm* cells. *FANCD2* disruption was confirmed by Southern (data not shown) and Western blotting (Supplementary Figure 1A). We found that spontaneous SCE levels and cisplatin sensitivity of the double mutant were essentially the same as those of *blm* mutant or *fancd2* mutant, respectively (Supplementary Figure 1B and C). This result is consistent with the essential role of *FANCC* in activation and/or monoubiquitination of *FANCD2* (Figure 1D).

Defective BLM focus formation in the absence of *FANCC* and *FANCD2*

While *blm* cells have extremely elevated SCEs, the elevation is only ~2-fold both in *fancc* and *fancd2* cells. As BLM helicase was shown to mediate the 'dissolution' of double Holliday junctions without crossing over (Wu and Hickson, 2003), it likely suppresses SCE in a direct manner. We hypothesized that increased SCE in *fancc* and *fancd2* cells is due to mildly compromised BLM function by the absence of the FA 'pathway'. Since BLM resides in a nuclear complex with several FA proteins (Meetei *et al*, 2003b), we examined the distribution of BLM inside *fancc* and *fancd2* cells. GFP-human BLM (GFP-hBLM) has been extensively utilized in a number of studies to examine its subnuclear localization (Hu *et al*, 2001; Suzuki *et al*, 2001; Yankiwski *et al*, 2001; Stavropoulos *et al*, 2002). To visualize chicken BLM localization, we transfected a GFP-chicken BLM (GFP-chBLM) expression construct into wild-type, *fancc*, and *fancd2* cells. Stably expressing transfectants were analyzed by FACSCalibur, and clones with equal expression levels were selected for further analysis. GFP-chBLM was fully functional in suppressing SCE in *blm* DT40 cells (data not shown).

In wild-type cells expressing GFP-chBLM, we observed relatively large foci (5–10 foci per cell) in 2–3% of the untreated cells (Figure 5A and B). These foci might correspond to PML nuclear bodies (Ishov *et al*, 1999), although we could not confirm this because of the lack of suitable reagents. In addition, we detected a number of less intense, smaller foci in untreated cells (Figure 5A and B). MMC treatment produced brighter and more numerous foci (Figure 5A and B). Some cells had a rather faint patch-like GFP accumulation that seems to be BLM localized in the nucleolus (Figure 5B).

Surprisingly, in *fancc* or *fancd2* mutant cells, the formation of both large and small GFP-chBLM foci was largely suppressed after MMC treatment. In particular, the number of the small foci per cell and the % positive cells were severely decreased in *fancc* and *fancd2* cells (Figure 5A and B). This reduction was not caused by differences in cell cycle, since

Figure 4 Genetic analysis of *fancc* mutation combined with *rad18* or *blm*. (A) CDDP or MMS survival curves of cells with indicated genotypes. Mean and s.d. from three independent experiments are shown. WT, wild type. (B) Spontaneous SCE levels. Numbers represent mean and s.d. of scores from 50 metaphases. Statistical significance was detected between WT versus *fancc*, WT versus *rad18*, WT versus *fancc/rad18*, *fancc* versus *fancc/rad18*, and *rad18* versus *fancc/rad18* (Bonferroni/Dunn test, $P < 0.0001$ in all comparisons). (C) Survival curves of cells with indicated genotypes. Mean and s.d. from three independent experiments are shown. (D) Spontaneous SCE levels. Numbers represent mean and s.d. of scores from 50 metaphases. Statistical significance was detected between *fancc* versus *blm*, *fancc* versus *fancc/blm#1*, and *fancc* versus *fancc/blm#2* (Bonferroni/Dunn test, $P < 0.0001$ in all comparisons). There was no statistically significant difference between *blm* and *fancc/blm* double mutants. (E) MMC (20 ng/ml)-induced SCE levels. Numbers represent mean and s.d. of scores from 50 metaphases. Statistical significance was detected between *fancc* versus *blm*, *fancc* versus *fancc/blm#1*, and *fancc* versus *fancc/blm#2* (Bonferroni/Dunn test, $P < 0.0001$ in all comparisons). There was no statistically significant difference between *blm* and *fancc/blm* double mutants.

both wild-type and mutant cells showed progressive accumulation at late S to G2 phase in an essentially similar way after MMC treatment (data not shown). In untreated cells, the reduction was not as clear as in MMC-treated cells (Figure 5B). These results indicate that nuclear relocalization of BLM is influenced by the FA proteins.

Next, we looked at whether BLM and FANCD2 can colocalize in DNA damage-induced subnuclear foci. FANCD2 foci were detected by staining with anti-chicken FANCD2. As shown in Figure 5C, GFP-chBLM and FANCD2 foci partially colocalized in MMC-treated cells. Among the 200 cells scored 7 h after MMC treatment, ~40% were positive for FANCD2/GFP-chBLM colocalizing foci, while

~40 or 10% were positive only for FANCD2 foci or GFP-chBLM foci, respectively. The rest of the cells (~10%) had no focus formation. Furthermore, we were able to detect FANCD2-L form in anti-GFP immunoprecipitates from wild-type but not *fancc* cells expressing GFP-chBLM following MMC treatment (Figure 5D). These data suggest that monoubiquitinated FANCD2 physically interacts with BLM either directly or indirectly, resulting in the colocalizing foci. Of note, we could not detect any reproducible mobility change, which may suggest post-translational modifications, in immunoprecipitated GFP-chBLM treated with λ -phosphatase following MMC exposure (data not shown).

Figure 5 Analysis of GFP-chBLM in wild-type, *fancc*, and *fancd2* DT40 cells. (A) Focus formation of GFP-chBLM. Cells expressing GFP-chBLM were observed before and 7 h after MMC treatment (500 ng/ml, 1 h). (B) Fraction of cells having large foci, indicated number of small foci, and patch-like accumulation. Cells were treated as in (A). At least 200 cells were scored in each preparation. (C) Colocalization of GFP-chBLM and FANCD2 in wild-type cells treated with MMC as in (A). (D) Co-immunoprecipitation of GFP-chBLM with FANCD2. Cells with indicated genotypes were treated with MMC (500 ng/ml for 7 h) or left untreated and lysed. A portion of the lysate (2.5%) was saved as an input control, and the rest was subjected to immunoprecipitation with anti-GFP. The lysates and immunoprecipitates were separated and probed with the indicated antibodies. GFP-chBLM (-), wild-type cells that were not transfected with the GFP-chBLM construct serving as negative control. The experiments were repeated three times with similar results.

To test whether defective focus formation in FA cells is specific to BLM or not, we stably expressed GFP-chRad18 into wild-type and mutant (*rad18*, *fancc*, and *fancd2*) DT40 cells. GFP-chRad18 expression was able to restore cisplatin sensitivity of *rad18* mutant to near wild-type levels (data not shown). In all of these transfected cells, GFP-chRad18 protein formed bright and distinct foci in a similar fashion (Supplementary Figure 2), although the percentage of cells with bright foci was quite low even after MMC treatment (2–3% in cells with all genotypes). In addition, colocalization with FANCD2 foci was rather poor (Supplementary Figure 2). These results are consistent with the additive phenotype of *fancc/rad18* cells.

In keeping with a previous report (Meetei *et al*, 2003b), FANCD2 focus formation and FANCD2-L production by MMC treatment were not altered in *blm* cells (Supplementary Figure 3A and B), whereas, as expected (Garcia-Higuera *et al*, 2001), we could not detect any FANCD2 focus formation in *fancc* cells (Supplementary Figure 3A). These data indicate that BLM functions downstream of the FA pathway.

Defective MMC-induced GFP-hBLM relocalization in human FA cells

To extend our observation to human FA cells, we took fibroblast cell lines PD20 (*fancd2*) and PD331 (*fancc*) derived from FA patients and transiently transfected them with GFP-hBLM construct (Suzuki *et al*, 2001). We also included PD20 cells complemented with retrovirus containing human FANCD2 cDNA. As expected, PD20 cells did not express FANCD2, while FANCD2-complemented PD20 or PD331 cells express FANCD2 protein. The FANCD2-L form was not detectable in PD331 cells even after MMC treatment (Figure 6A). Small but bright GFP-hBLM foci were noted in these cells, but the percentage of the focus-positive cells was higher in FANCD2-complemented cells compared to the PD20 or PD331 human mutants. Furthermore, following MMC treatment, the percentage of positive cells with GFP-hBLM foci was increased in the complemented cells but not in PD20 and PD331 cells (Figure 6B and C). We were also able to observe colocalization of FANCD2 and GFP-hBLM foci in the complemented cells following MMC treatment (Figure 6D). Human FA cells displayed accumulation of GFP-hBLM protein in several large nucleolus-like structures, similar to previous reports (Yankiwski *et al*, 2001; Stavropoulos *et al*, 2002). Collectively, these results established that the FA pathway determines MMC-induced GFP-BLM relocalization in both human and chicken cells.

Discussion

In this study, we carried out genetic analyses between the FA pathway and three proteins involved in DNA damage responses. We revealed an overlap between HR factor Xrcc3 and FANCC in cisplatin sensitivity, which indicates that at least some of FANCC's function in repair of cisplatin damage is linked to HR. This result is consistent with our previous findings that there are HR defects in *fancd2* (Yamamoto *et al*, 2005) and *fancc* (Yamamoto *et al*, 2003) DT40 mutant cells. We also found that *fancc* cells had properties similar to those of *fancd2* cells, although the magnitude of the defects was generally more severe in *fancd2* than in *fancc* cells. However, *fancc* cells appeared to be ~2-fold more sensitive to MMC

Figure 6 GFP-hBLM focus formation in human FA cells. (A) Expression and MMC-induced monoubiquitination of FANCD2 in human fibroblasts PD20 (*fancc2*) and PD20 complemented with human FANCD2 cDNA, PD331 (*fancc*). Cells were treated with MMC (40 ng/ml) for 24 h. Western blot analysis was performed using anti-human FANCD2 monoclonal antibody. An asterisk indicates a nonspecific band. (B) Focus formation of GFP-hBLM. Cells transiently transfected with GFP-hBLM expression construct were treated with MMC as in (A). At least ~20% of the populations became GFP-positive 24 h after transfection. (C) Percentage of the focus-positive cells among GFP-hBLM-positive populations before and after MMC treatment. Cells with more than four bright foci were scored as positive. At least 300 cells were scored in each preparation. The experiment was repeated three times with similar results. (D) Colocalization of GFP-hBLM and FANCD2 in FANCD2-complemented cells treated with MMC as in (A). Human FANCD2 foci were detected using anti-human FANCD2 polyclonal antibody.

than *fancc2* cells in colony survival assay (Yamamoto *et al*, 2005). This difference is consistent with the view that FANCC is required for monoubiquitination of FANCD2 but might have an additional role in MMC tolerance (Kruij *et al*, 1998; Cumming *et al*, 2001).

Several lines of evidence suggest linkage between TLS and the FA pathway (reviewed in Thompson *et al*, 2005). First, it has been reported that human FA cells display hypomutability at the *hprt* locus (Papadopoulos *et al*, 1990). Second, we previously showed lower levels of nontemplated mutations in the immunoglobulin light-chain locus in *fancc2* DT40 cells (Yamamoto *et al*, 2005). These data are consistent with the possibility that the FA pathway promotes TLS and base-substitution mutagenesis at spontaneous or induced DNA lesions. Third, a recent report (Niedzwiedz *et al*, 2004) indicated that TLS polymerases Rev1 and Rev3 work in a common pathway with FANCC for ICL repair. Collectively, it seems likely that the FA pathway facilitates both TLS and HR processes in response to blocked or broken replication forks, respectively (Thompson *et al*, 2005).

In contrast to the Rev1/3 study (Niedzwiedz *et al*, 2004), our present data using cells lacking both FANCC and Rad18 revealed independent functions of these two proteins. Upon replication fork stalling, Rad18 promotes lesion bypass together with its partner Rad6. The Rad6/Rad18 complex carries ubiquitin conjugation and ligation activities, and mediates PCNA monoubiquitination, which is required for switching to the specialized polymerase such as *pol eta* (Rad30) (Kannouche *et al*, 2004; Watanabe *et al*, 2004). Although the complex is thought to be a master regulator of TLS in yeast *S. cerevisiae*, not all of the TLS polymerases are Rad18-dependent in vertebrate cells (Okada *et al*, 2002). Therefore, our findings do not exclude the possibility that FA proteins and some TLS polymerases operate together.

Interestingly, both FANCC and FANCD2 are dispensable for spontaneous SCEs despite their roles in certain HR-dependent processes, including gene targeting. In fact, both *fancc* (this paper and Niedzwiedz *et al*, 2004) and *fancc2* (Yamamoto *et al*, 2005) cells have elevated spontaneous SCE. Since SCE is one form of HR, these results suggest that vertebrate cells possess HR processes that are either dependent on or independent of FA proteins. For example, HR repair of cisplatin damage for cell survival requires both Xrcc3 and FANCC, while Xrcc3 but not FANCC is necessary for spontaneous SCE.

The rate of spontaneous SCE per cell might be influenced by (1) the frequency at which replication forks encounter nicks or blocking lesions, (2) HR capacity, (3) overall activity of TLS, and (4) a factor that determines the frequency of crossing over, for example, BLM helicase (Wu and Hickson, 2003). We tested the contributions of the latter three processes to SCE in *fancc* cells by making double mutants and found an overlap between BLM and the FA pathway. We further confirmed this relationship by measuring SCE in *fancc2/blm* double mutants. In addition, both *fancc/blm* and *fancc2/blm* cells also displayed the same levels of sensitivity to killing by ICL reagents as those of *fancc* or *fancc2* cells, respectively. These data suggest that the FA pathway and BLM may function in a common pathway to repair ICLs and suppress SCE.

Several lines of evidence support this conclusion. First, ICL damage-induced focus formation of GFP-BLM was defective in both human and chicken *fancc* and *fancc2* cells. Second, we observed colocalization of FANCD2 and GFP-BLM foci in MMC-treated wild-type DT40 and the complemented human *fancc2* cells. Third, FANCD2-L form was co-immunoprecipitated with GFP-chBLM in wild-type DT40 but not in *fancc* cells following MMC treatment. Fourth, BLM is reported to

co-immunoprecipitate with the FA core complex in human cells (Meetei *et al*, 2003b). Fifth, in contrast to GFP-chBLM, Rad18 focus formation was not defective in *fancc* or *fancd2* DT40 cells, consistent with the additive defects found in *fancc/rad18* cells. Thus, we conclude that the localization of BLM to stalled/broken replication forks caused by ICLs depends on the FA pathway. In suppressing SCE, BLM requires the FA pathway in a partial manner, since elevation of SCE in *fancd2* or *fancc* cells is not as high as in *blm* cells. On the other hand, in repairing potentially lethal ICL damage, BLM functions only inside the pathway that depends on FANCD2 and FANCC.

The components of the FA core complex were found in anti-BLM immunoprecipitates (Meetei *et al*, 2003b). However, FANCD2 appeared not to be tightly bound to the complex (Pace *et al*, 2002). Since components of the core complex have been shown to associate with chromatin/nuclear matrix upon DNA damage (Qiao *et al*, 2001), it is possible that a complex containing FANCC helps mobilize BLM to the sites of stalled/broken replication forks induced by DNA damage. Alternatively, FANCC may participate in BLM redistribution only through FANCD2 activation. Monoubiquitinated FANCD2 localized to stalled/broken replication forks may then mediate the formation of BLM foci through physical interaction. This interaction might be indirect, since BLM is found in the BRCA1-containing megaprotein complex (Wang *et al*, 2000b), and FANCD2-L and BRCA1 co-immunoprecipitate following DNA damage (Garcia-Higuera *et al*, 2001).

In summary, this study provides genetic evidence that defines relationships between the FA pathway and processes required to resolve stalled or broken replication forks. We found that BLM and FANCC act in a common pathway in suppressing spontaneous SCEs and in repairing ICL. The overlap between FA and BS is perhaps unexpected and surprising, given the highly distinct clinical and cellular features of these disorders. We suggest that FANCD2 utilizes BLM as its effector molecule by regulating BLM relocation in the vertebrate nucleus.

Materials and methods

Cell culture and transfection

Wild-type and mutant DT40 cells were cultured at 39.5°C with 5% CO₂ using RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% chicken serum, 2-mercaptoethanol (50 µM), and L-glutamine (2 mM) as described (Yamamoto *et al*, 2003). Transfection by electroporation and subsequent selection were performed as described (Yamamoto *et al*, 2003). *BLM*-deficient (Wang *et al*, 2000a), *RAD18*-deficient (Yamashita *et al*, 2002), and conditional *XRCC3*-deficient (Ishiai *et al*, 2004) DT40 mutant cells were previously described.

SV-40 transformed FA-D2 fibroblasts PD20, PD20 complemented with human FANCD2, and FA-C fibroblasts PD331 were kind gifts from Dr Barbara A Cox (Fanconi Anemia Cell Repository, The Oregon Health and Science University). These cells were cultured at 37°C with 5% CO₂ using α -minimal essential medium supplemented with 10% FCS. Transient transfection was performed using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Chicken FANCC cloning and gene targeting vectors

An EST clone containing full-length chicken FANCC cDNA was obtained by searching chicken EST database. A portion of genomic chicken FANCC was PCR-amplified using primers designed on the basis of the cDNA sequence, and subcloned into TOPO-pCR-XL

vector (Invitrogen). FANCC targeting vector was constructed by replacing an ~1.5 kb genomic fragment containing a single exon with a resistance gene cassette. The targeting vector with an appropriate marker cassette was introduced into wild-type, conditional *XRCC3*-deficient, *RAD18*-deficient, and *BLM*-deficient DT40 cells. The targeting event is expected to result in deletion of FANCC amino-acid residues 56–84. By splicing between residual exons, it should create a frame shift with immediate protein truncation. To obtain isogenic sets of *Xrcc3*-deficient and FANCC/*Xrcc3* double-deficient clones, conditional *xrcc3* cells and FANCC-disrupted conditional *xrcc3* cells were cultured for ~48 h in the presence of OH-TAM and subcloned by limiting dilution.

The targeting vectors for FANCD2 deletion were previously described (Yamamoto *et al*, 2005). FANCD2 alleles were disrupted by sequential transfections into *BLM*-deficient cells. The targeting vectors to measure efficiency in gene targeting were previously described (Yamamoto *et al*, 2003).

Expression vectors

Chicken BLM cDNA was kindly provided by Dr Takehisa Matsumoto (RIKEN). Chicken Rad18 (Yamashita *et al*, 2002) was PCR-amplified from DT40 cDNA. To generate expression vectors, chicken FANCC was subcloned into pcDNA4/HisMax (Invitrogen) in-frame. Chicken BLM or Rad18 was inserted into pEGFP-C1 (Clontech, Palo Alto, CA, USA) to create the GFP-chBLM or GFP-chRad18 expression vector, respectively. GFP-hBLM expression vector was previously described (Suzuki *et al*, 2001).

RT-PCR analysis

Total RNA was isolated using TRIzol (Invitrogen), and converted to cDNA by reverse transcriptase (RT) (Invitrogen). A part of chicken FANCC or Rad51 was amplified with Gold Taq polymerase (Applied Biosystems Inc., Foster City, CA, USA).

Sensitivity of cells to irradiations and drugs

Colony formation was assayed in medium containing 1.4% methylcellulose. Cells were irradiated with X-rays (Linear Accelerator, Mitsubishi Electric Inc., Tokyo, Japan) or UV, or exposed to MMC (Kyowa Hakko, Tokyo, Japan) for 1 h and then plated (Yamamoto *et al*, 2003). Sensitivity to cisplatin (Nippon Kayaku, Tokyo, Japan) (Yamamoto *et al*, 2003) or MMS (Sigma-Aldrich, St Louis, MO, USA) was assayed by continuous exposure.

Immunoprecipitation, Western blot analysis, and subnuclear focus assay

For immunoprecipitation, cells were lysed in lysis buffer (0.5% Triton X-100, 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF) supplemented with proteinase inhibitor cocktail (complete EDTA-free tablet) (Roche Diagnostics, Basel, Switzerland). GFP-chBLM was immunoprecipitated using polyclonal rabbit anti-GFP antibody (BD Biosciences, San Jose, CA, USA) and Protein G Plus/Protein A-agarose (EMD Biosciences, Inc., San Diego, CA, USA). Anti-chicken FANCD2 serum was described previously (Yamamoto *et al*, 2005). Anti-human FANCD2 antibodies were purchased from Abcam (Cambridge, UK). Immunoprecipitates or whole-cell lysates were separated with 6% SDS-PAGE, and then transferred to a membrane, and detected by antibodies using ECL plus Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA). To visualize subnuclear focus formation of chicken or human FANCD2, cytospin slides or coverslips were fixed and stained with the antiserum. BLM focus was detected as GFP-BLM. Images were captured using TCS SP2 laser scanning confocal microscopy (Leica microsystems, Bannockburn, IL, USA).

Analysis of chromosomal aberrations and SCE

Chromosome analysis was performed as previously described (Takata *et al*, 1998, 2000; Yamamoto *et al*, 2003). For SCE analysis with or without MMC (20 ng/ml), colcemid (0.1 µg/ml) was added for the last 2 h of 16 h incubation in the presence of BrdU, and scoring was performed on coded slides as previously described (Takata *et al*, 2000). Statistical analysis was carried out by an analysis of variance (ANOVA) with the Bonferroni/Dunn multiple comparison test for intergroup comparison using Statview software (SAS Institute Inc., Cary, NC, USA).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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