A rare hereditary disorder Fanconi anemia (FA) is caused by mutations in an array of genes, which interact in a common FA pathway/network. These genes encode components of the FA “core” complex, a key factor FancD2, the familial breast cancer suppressor BRCA2/FancD1, and Brip1/FancJ helicase. Although BRCA2 is known to play a pivotal role in homologous recombination (HR) repair by regulating Rad51 recombinase, the precise functional relationship between BRCA2 and the other FA genes is unclear. Here we show that BRCA2-dependent chromatin loading of Rad51 after MMC treatment was not compromised by disruption of FANCC or FANCD2. Rad51 and FancD2 form co-localizing subnuclear foci in a manner independent of each other. Furthermore, we created a conditional BRCA2 truncating mutation lacking the C-terminal conserved domain (CTD) (brca2ΔCTD), and disrupted the FANCC gene in this background. The fancc/brca2ΔCTD double mutant revealed an epistatic relationship between FANCC and BRCA2 CTD in terms of X-ray sensitivity. In contrast, levels of cisplatin sensitivity and MMC-induced chromosomal aberrations were increased in fancc/brca2ΔCTD cells relative to either single mutant. Taken together, these results indicate that the FA proteins work together with BRCA2/Rad51-mediated HR in DSB repair, while the FA pathway plays a role which is independent of the CTD of BRCA2 in interstrand crosslink repair. These results provide insights into functional interplay between classical FA pathway and BRCA2.

Fanconi anemia (FA) is a rare hereditary disorder characterized by bone marrow failure, genome instability, and an increased susceptibility to cancer (1-3). Cells isolated from FA patients display increased levels of spontaneous and DNA crosslink-induced chromosomal breakage, suggesting that the basic defect in FA could be related to DNA repair (4,5). Indeed, recent studies have indicated that FA cells are defective in two processes, both of which are implicated in restarting stalled replication forks; homologous recombination (HR) repair and translesion synthesis (TLS) (6-9).

To date, twelve genetic FA subgroups have been identified based on the complementation analysis, and eleven of these have been cloned (FancA/B/C/D1/D2/E/F/G/J/L/M) (10,11). Among them, eight gene products interact with each other, forming the FA core complex (FancA/B/C/E/F/G/L/M). This complex is thought to be a multi-subunit ubiquitin E3 ligase (12), which monoubiquitinates FancD2 protein upon DNA damage and during a normal unperturbed S phase (13,14). Monoubiquitination is both necessary and sufficient for FancD2 to be targeted to chromatin following DNA damage, to form...
subnuclear foci, and to participate in DNA repair (15,16).

Recent studies have revealed an increasingly complex picture of the FA pathway/network. It is likely that the core complex functions not only in the monoubiquitination of FancD2, but also in other roles required for the targeting of FancD2 to chromatin, and for DNA repair (16). Indeed, the FA core complex carries subunits required not only for monoubiquitinating FancD2 (E3 ligase FancL), but also for DNA modification (FancM) (17,18). FancM is a vertebrate homolog of the Archeal nuclease/helicase Hef (19), and is therefore likely to play a role in the processing of damaged DNA (20). The core complex also associates with BLM helicase (21) and regulates its subcellular localization (22). Thus the core complex seems to have multiple effector arms, which probably cooperate with FancD2 in DNA repair (16). However, the precise biochemical function of FancD2 is still unknown. Importantly, any patient-derived mutation found in the core complex components appears to disrupt structural integrity of the complex, and may cause complete or partial loss of function in effector molecules. It is currently unclear how the FA pathway couples with DNA repair pathways such as HR or TLS.

Besides the core complex components and FancD2, two additional FA proteins, FancD1 and FancJ, have been identified, and both of them are intimately linked to familial breast cancer. In FANCD1 mutant cells, biallelic hypomorphic mutations of breast cancer susceptibility gene BRCA2 have been identified (23), while FANCJ was found to be the BRCA1-interacting helicase, Brip1 (also known as BACH1) (24-27). Individuals carrying monoallelic BRCA1 or BRCA2 mutation are predisposed to breast and ovarian cancer (28). FANCD1 or FANCJ mutant cells display normal FancD2 monoubiquitination following DNA damage (24), indicating that these genes function independently, or downstream, of the core complex-FancD2 (“classical FA”) pathway. How BRCA2/FancD1 or the Brip1/FancJ helicase functionally interact with rest of the FA proteins is largely unknown. In this study, we focus on the functional relationship between BRCA2 and the classical FA pathway.

A major cellular function of BRCA2 is to regulate the Rad51 recombinase, which catalyzes the initial strand invasion reaction in HR (28,29). Rad51 forms subnuclear foci upon DNA damage, and this could reflect Rad51 nucleoprotein filaments polymerized onto the single stranded DNA ends generated by nucleolytic processing of DSB (29). This foci formation is severely impaired in BRCA2-deficient cells (28). Consistent with a connection between classical FA pathway and BRCA2, nuclear foci of FancD2 colocalize with both BRCA2 and Rad51 foci, and monoubiquitinated FancD2 coimmunoprecipitates with BRCA2 from chromatin fractions (14,30).

To elucidate the functional hierarchy between FA genes and HR factors, analysis of Rad51 focus formation has been employed. However, this has yielded conflicting results to date. While several groups have reported that Rad51 nuclear foci formation is significantly attenuated in FA cells (30-32), other groups reported that DNA-damage induced Rad51 foci formation is unaffected in FANCD2-deficient PD20 cells and other human or mouse FA cells (33-37). Furthermore, siRNA treatment against BRCA2 does not increase the frequency of MMC-induced quadriradial chromosome formation in FANCA-deficient fibroblasts, while siRNA against BRCA1 does (38). On the other hand, another study found that depletion of BRCA2 or Rad51 by siRNA further sensitizes FANCD2-deficient human cells against MMC (33).

To dissect the functional relationship between the classical FA pathway and BRCA2 more precisely, we analyzed MMC-induced Rad51 chromatin loading and foci formation in cells with or without a functional FA pathway or BRCA2. We observed that BRCA2, but not FANCC or FANCD2, is indispensable for the loading of Rad51 to chromatin. We also found that FancD2 and Rad51 act independently of each other in terms of DNA damage-induced foci formation. To mimic the phenotype of FA-D1 patients (23), we generated a BRCA2 truncating mutation immediately downstream of BRC repeats, which resulted in deletion of the C-terminal domain (CTD) including the OB folds that bind ssDNA (CTD truncation).
Based on this BRCA2 CTD truncation mutant, we created fancc/brea2ACTD double mutant cells. Phenotypic analysis of these set of mutant cells revealed that there is a complex interplay between the BRCA2-Rad51 and FancC-FancD2 pathways depending on the type of DNA damage.

**EXPERIMENTAL PROCEDURES**

**Cells lines and gene targeting** - Wild type and various mutant chicken DT40 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 1% chicken serum, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, penicillin, and streptomycin in a 5% CO2 incubator at 39.5 °C. Generation of fancc (22), fancd2 (7), or brea2ΔBRC (40) cells has been previously described. In the brea2ΔBRC cell line, one allele was designed to produce chicken BRCA2 truncated at a point immediately downstream of the BRC3 repeat, while the second allele was rendered conditionally null by using inducible MerCreMer recombinase (40).

Generation of brea2ACTD and fancc/brea2ACTD cells was schematically described in Figure 3B. We constructed the BRCA2-CTD truncation vector by replacing the upper homology arm of the BRCA2-CTD truncation vector (40) with an ~1 kb genomic PCR fragment. The BRCA2-CTD truncation vector was kindly provided by Drs. Mitsuoyoshi Yamazoe and Shunichi Takeda (Kyoto University). The genomic PCR fragment harbored a sequence change to introduce the truncating mutation (CTD truncation) downstream of the BRC8 repeat (the codon 2151 was changed to stop codon). As with brea2ΔBRC cells, the BRCA2-CTD truncation was achieved in cells also harboring conditionally null allele of BRCA2 (a kind gift from Drs. Mitsuoyoshi Yamazoe and Shunichi Takeda), resulting in BRCA2ACTDcon cells (Figure 3B). Then FANCC gene disruption was achieved using previously described FANCC targeting vector (22). The deletion of BRCA2 conditionally null allele was induced by addition of 4-hydroxy-tamoxifen (TAM) in both BRCA2ACTDcon and fancc/BRCA2ACTDcon and was further verified by subcloning. Genomic Southern blotting and RT-PCR analysis were done as previously described (22,40).

A GFP-FancD2 expression vector was made by subcloning the chicken FANCDD2 cDNA into pEGFP-C1 (Clontech, Palo Alt, CA). A point mutation removing the monoubiquitination site (K563R) was introduced by QuickChange kit (Stratagene, La Jolla, CA). Transfections were done by electroporation as described previously (6).

**Western blotting analysis** - Anti-chicken N-terminal Brca2 and anti-FancD2 antisera were raised as described previously (7,40). Anti-Rad51 polyclonal antibody was kindly provided by Dr. Hitoshi Kurumizaka (Waseda University, Tokyo). Anti-histone H4 antibody was purchased from UBI (Lake Placid, NY). Cell fractionation into the soluble and chromatin fractions (16), and Western blotting (40) were performed as described previously.

**Focus formation assay** - Visualization of subnuclear foci of Rad51 or GFP-FancD2 was done as described previously (7). To detect subnuclear γH2AX foci, cytopsin slides were fixed with 4% paraformaldehyde, and were further treated with 70% ethanol. After blocking, slides were stained with anti-γH2AX monoclonal antibody (UBI, Lake Placid, NY) at 1:1000 dilution followed by Alexa594-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) at 1:250 dilution.

**Cell analysis by flow cytometry** - Cell growth and cell cycle distribution were analysed using FACS Calibur flow cytometer (Becton-Dickinson, Mountain View, CA) as described (6). Anti-BrdU antibody was purchased from BD PharMingen (San Diego, CA). Mitotic index was determined as described previously (41). Briefly, washed cells were fixed in 70% ethanol, stained with anti-phosphorylated histone H3 antibody (UBI, Lake Placid, NY) followed by FITC-conjugated anti-rabbit IgG, and analyzed with FACC Calibur.

**Sensitivity of cells to treatment with DNA damaging agents** - Colony formation was assayed in medium containing 1.4% methylcellulose. Serially diluted cells were irradiated with 4-MV X-rays (Linear Accelerator; Mitsubishi Electric, Inc., Tokyo, Japan) as described previously (6). Alternatively, cells were exposed for 1 hr to MMC (Kyowa-Hakkou, Tokyo, Japan) or...
continuously to cisplatin (Nihon-Kayaku, Tokyo, Japan) and methylmethanesulfonate (MMS) (Sigma, St. Louis, MO).

**Analysis of chromosomal aberrations -** Chromosome analysis was performed as previously described (6). Briefly, colcemid (0.1 µg/ml) was added immediately after the X-ray irradiation and cells were harvested 3 hr later. In some cases, cells were cultured in medium containing 2.5 or 5 ng/ml MMC for 24 hrs and colcemid was added 2 hr before harvesting. Scoring was performed as previously described (6), on coded slides.

**RESULTS**

Disruption of FANCC or FANCD2 does not significantly attenuate MMC-induced chromatin loading of Rad51.

We have shown previously that MMC-induced Rad51 nuclear focus formation is not significantly impaired in fancg (6) or fancd2 (7) cells. We also found that the human fancd2 cell line PD20, as well as PD20 cells complemented with human FancD2, displayed similar levels of Rad51 foci following both X-ray and MMC treatments (supplementary Figure S1). Given a number of contradictory results reported in the literature (30-32), we wished to evaluate the efficiency of Rad51 relocalization after DNA damage in a more quantitative manner. To this end, we separated cell lysates of wild type, fanc, fancd2 and brcA2ABRC chicken DT40 cells into detergent-soluble and insoluble (chromatin) fractions as described previously (16), and quantified the abundance of Rad51 in the chromatin fraction by Western blotting with an anti-Rad51 antibody.

In wild type cells, we observed a significant increase of Rad51 protein in the chromatin fraction in response to MMC treatment, which probably reflects nucleoprotein Rad51 filament formation (Fig. 1). Strikingly, we found similarly increased levels of chromatin bound Rad51 protein in fanc or fancd2 cells (Fig. 1). In contrast, we were not able to detect this induction in brcA2ABRC cells, whose BRCA2 is not functional (Fig. 1). However, chromatin loading of FancD2, which is dependent on its monoubiquitination, was normal in brcA2ABRC but was compromised in fanc cells, which is consistent with previous studies (16) (Fig. 1). These results strongly suggest that, following DNA damage, Rad51 loading onto single-stranded DNA ends is regulated by BRCA2/FancD1 but not by monoubiquitinated FancD2.

**DNA damage-induced FancD2 or Rad51 focus formation proceeds independently of each other**

Next, we examined Rad51 foci formation in relation to FancD2. To conveniently visualize FancD2 distribution in wild type or fanc cells, we expressed a chicken FancD2 protein fused to GFP in fancd2 DT40 cells (designated GFP-FancD2 WT cell). We also then deleted FANCC gene in those cells (GFP-FancD2 fanc cells). We also expressed GFP-FancD2 protein devoid of the monoubiquitination site by mutating Lysine 563 to Arginine in fancd2 cells (GFP-FancD2 KR cells). GFP-FancD2 WT protein was converted to L-form following DNA damage, while in the other two mutant cell line, this conversion was not detectable. Consistently, only GFP-FancD2 WT cells showed cisplatin tolerance comparable to that of wild type DT40 cells.

In GFP-FancD2 WT cells, MMC or X-ray treatment induced robust Rad51 focus formation (Fig. 2A), and the majority of these foci co-localized with GFP-FancD2 foci (Fig. 2A and data not shown). While GFP foci formation was completely defective in GFP-FancD2 KR or GFP-FancD2 fanc cells (Fig. 2A), Rad51 foci formation occurred with similar kinetics, although there appeared to be some reduction in the fraction of Rad51 foci-positive cells following X-ray exposure (Fig. 2A and Fig. 2B). In these two cell lines, Rad51 foci appeared to be slightly smaller and hence more difficult to visualize which might explain the observed small reduction in percentage of cells with foci. Thus, we conclude that nuclear focus formation of Rad51 remains largely unaffected, irrespective of whether FancD2 protein can form foci or not. Conversely, we observed normal FancD2 focus formation in the absence of Rad51 foci formation caused by brcA2ABRC disruption (Fig. 2C). These data indicated that Rad51 and FancD2 foci formation are independent events, despite the fact that these two proteins accumulate at the same DNA damage sites.

To exclude the possibility that Rad51 focus formation in FA cells in response
to DNA damage is qualitatively different from those in wild type cells (6,7), we examined whether these Rad51 foci are properly localized at sites of DNA damage. Following DSB induction, Histone H2AX is rapidly phosphorylated at its C-terminus in a megabase-sized chromatin region surrounding the DNA damage site, and this leads to the formation of subnuclear foci (42), which serve as a marker for DNA damage sites. We therefore examined the colocalization of Rad51 foci with γH2AX foci in cells lacking FANCC or FANCD2. MMC treatment induced colocalizing nuclear foci of Rad51 and γH2AX in wild type cells, and this colocalization was also preserved in fancc or fancd2 cells (data not shown). Collectively, these results suggest that Rad51 protein is properly recruited to and loaded on to DNA damage sites in chromatin even in cells lacking FANCC or FANCD2.

**fancc/brca2ΔCTD double mutant cells display growth defects similar to brca2ΔCTD cells**

To examine the functional interplay between classical FA pathway and BRCA2/FancD1, attempts at “epistasis-type” analysis have been carried out using BRCA2 siRNA knockdown in FA cells, but this has yielded conflicting results (33,38). To dissect the relationship more precisely, we employed a genetic approach. We first established BRCA2 conditional knockout cells in which one BRCA2 allele expressed a truncated form of the protein that contained all of the BRC repeats, but had lost the C-terminal conserved domain (CTD), while the other allele was conditionally null (referred to as BRCA2ΔCTDcon) (Fig. 3A). In this cell line, both wild type and CTD truncated BRCA2 are expressed (data not shown). Cells harboring the ΔCTD truncation may mimic the defective allele found in FA-D1 patients, since they often have mutation that leads to truncation of the CTD region (23).

Following the above gene manipulations, deletion of the conditional allele expressing wild type BRCA2 protein was achieved by the addition of 4-hydroxy-tamoxifen (TAM), as described previously (40), producing mutant clones that expressed only truncated BRCA2 protein (Fig. 3C) (referred to as brca2ΔCTD). Using BRCA2ΔCTDcon conditional knockout cells, we further disrupted the FANCC gene (referred to as fancc/BRCA2ΔCTDcon), and isolated double mutant clones by TAM treatment (referred to as fancc/brca2ΔCTD) (Fig. 3B). We confirmed each genotype by Southern blotting and RT-PCR analysis (Fig. 3D). FANCC gene disruption was also verified by the loss of FancD2-L form using Western blotting with anti-FancD2 antibody (Fig. 3E). The CTD-truncated BRCA2 protein should contain intact copies of all BRC repeats, which interact with Rad51, and the truncated BRCA2 could be detected by Western blotting using N-terminus-specific anti-BRCA2 antibody (Fig. 3C). However, DNA damage-induced Rad51 foci were abrogated in brca2ΔCTD or fancc/brca2ΔCTD cells (supplementary Fig. S2).

A previous report has shown that brca2ΔBRC cells grow slowly, which was explained by the high rate of spontaneous cell death (40). We also found that brca2ΔCTD cells displayed slower growth than wild type cells (Fig. 4A) and higher levels (~30%) of spontaneous cell death (Fig. 4B) than did the BRCA2ΔCTDcon cells. In contrast, a milder growth defect was found in fancc/BRCA2ΔCTDcon cells, and this was consistent with the known phenotype of fancc cells (22) (Fig. 4A). Interestingly, the growth rate and levels of spontaneous cell death were almost the same in fancc/brca2ΔCTD cells as compared to brca2ΔCTD cells (Fig. 4A and B).

We next examined cell cycle distribution following BrdU pulse labeling. We observed that both brca2ΔCTD cells and fancc/brca2ΔCTD cells displayed a modest accumulation of cells in the G1 and G2/M phases compared to BRCA2ΔCTDcon or fancc/BRCA2ΔCTDcon cells (Fig. 4C). The apparent increase of cells in the G2/M phases was shown to be caused by G2 accumulation, since the proportion of M phase cells was found to be decreased as determined by flow cytometric analysis using a specific antibody against phosphorylated form of histone H3 (Fig. 4B). These results indicate that both brca2ΔCTD and fancc/brca2ΔCTD cells exhibit higher levels of spontaneous G2 arrest, which might be due to residual unrepaired DNA damage. Furthermore, we observed a
similar (~1-hr) elongation of single cell cycle length in $brc\alpha\Delta$ cells and fancc/$brc\alpha\Delta$ cells when compared to their parental cells (data not shown). We conclude that the growth defect in $brc\alpha\Delta$ cells is caused by spontaneous cell death and aberrant cell cycle progression. In addition, FANCC gene disruption, even though it makes wild type cells grow slower, had no additional effect in $brc\alpha\Delta$ cells on their growth defects, consistent with the possibility that FANCC and BRCA2 cooperate in a common pathway for handling DNA damage that occurs spontaneously.

**Functional interplay between FANCC and BRCA2 in cell survival following DNA damage**

To examine capacity of $BRCA2\Delta$ and $fanche/brc\alpha\Delta$ cells to repair different DNA lesions, we exposed these cell lines to various DNA damaging agents, and measured colony-forming survival. Consistent with our previous report (22), $fanche/brc\alpha\Delta$ cells showed only a moderate level of sensitivity to X-rays or MMS (Fig. 5A and B), while $brc\alpha\Delta$ cells were consistently more sensitive to X-rays or MMS (Fig. 5A and B). Interestingly, levels of sensitivity to X-ray or MMS were comparable in $brc\alpha\Delta$ cells and $fanche/brc\alpha\Delta$ cells (Fig. 5A and B), indicating an epistatic relationship between the classical FA pathway and BRCA2 in repairing chromosomal DSBs. In sharp contrast, $fanche/brc\alpha\Delta$ double mutant cells showed a more severe level of sensitivity to cisplatin and MMC compared to either single mutant (Fig. 5C and D). This additive sensitivity indicates that $BRCA2$ TTD and FANCC have non-overlapping function in repairing ICL lesions on chromosome.

Unrepaired DNA lesions can often be represented in metaphase chromosome spreads as chromosomal aberrations. Hence, we scored several types of chromosomal aberrations in these cells after treatment of MMC or X-rays. X-ray treatment did not induce higher levels of aberrations in $fanche/brc\alpha\Delta$ cells compared to $brc\alpha\Delta$ cells (Fig. 6A), although in both cases this frequency was elevated compared to cells expressing functional BRCA2. In contrast, not only were high levels of chromosomal aberrations induced in $brc\alpha\Delta$ cells using very low concentrations of MMC, but also $fanche/brc\alpha\Delta$ cells displayed a higher level of aberrations than $brc\alpha\Delta$ single mutant cells (Fig. 6B). These results confirmed the conclusions from the epistasis analysis between FANCC and BRCA2 suggested by the colony survival data, and also indicated that the functional interplay between these molecules depends on the particular type of DNA damage involved.

**DISCUSSION**

In response to ionizing radiation, the BRCA2/FancD1 protein is loaded onto chromatin and associates specifically with monoubiquitinated FancD2 (30). BRCA2 also interacts with FancG (43) as well as FancE (30). Given that BRCA2 is one of the FA genes, these data indicate a close functional relationship between the classical FA pathway and BRCA2. It has been hypothesized that $BRCA2/FANCD1$ functions downstream of monoubiquitinated FANC D2, since FancD2 is normally monoubiquitinated and activated in FA-D1 cells. To verify this proposal, the formation of Rad51 foci has been examined as an indication of BRCA2 function in the absence of classical FA proteins. However, the results of such studies have been contradictory, and it has not been possible to draw definite conclusion.

In this study, we employed biochemical fractionation of DT40 FA cells and examined the MMC-induced chromatin loading of Rad51, which probably reflects relocation of Rad51 to DNA damage sites. We find that Rad51 chromatin loading occurs normally following MMC treatment in $fanche$ or $fancd2$ cells, but not in $brc\alpha$ cells. Using cells expressing GFP-tagged FancD2 protein, we also show that Rad51 and FancD2 form colocalizing foci with similar kinetics in response to DNA damage. However, Rad51 and FancD2 focus formation are independent events, suggesting that the classical FA pathway functions parallel to the BRCA2-Rad51 pathway. Alternatively, it is possible that the FA pathway functions at late stage of HR, downstream of BRCA2-Rad51.
Recent identification of the Brip1 helicase as *FANCJ* (25-27,44) may support this latter possibility, since Brip1 may function downstream of FancD2 (24), and preferentially unwind certain recombination intermediates such as D-loops (45). In agreement with this hypothesis, Rad51 foci form normally in *BRIP1/FANCJ*-deficient cells (26).

We also examined the phenotype of *fancc/brca2ΔCTD* double mutant cells relative to either single mutant. We find that *FANCC* and the CTD of *BRCA2* function together in an epistatic manner in cell proliferation as well as in repairing DSBs induced by X-rays, while they have distinct and non-overlapping functions in repairing ICLs. ICL repair pathway in vertebrates is not very well understood; however, it likely employs a complex network of DNA repair machineries including nucleotide excision repair (NER), HR, TLS, and FA pathways (46,47). In our current understanding, ICL repair proceeds in several steps: (i) incisions mediated by endonuclease XPF/ERCC1 to uncouple the ICL, (ii) DSB formation by a mechanism probably related to replication (iii) translesion DNA synthesis past the unhooked ICL lesion, (iv) a second incision to remove the remaining mono-adduct, and (v) repair of the ICL-associated DSB by HR. Recent studies have indicated that *FANCC* has overlapping roles with both HR and the TLS polymerases Rev1 and Rev3 in repairing ICLs (8,22,48). In support of a role for the FA pathway in TLS, we observed significantly decreased levels of hypermutation in Immunoglobulin (Ig) **V**λ in *fancd2* DT40 mutant cells (7), and this hypermutation is known to depend on the presence of Rev1 (49). In contrast, BRCA2 is known to participate only in the early phases of HR, and DT40 *ΔBRCA* mutant cells display increased Ig hypermutation with loss of gene conversion (40). Thus in the repair of DSBs induced by X-rays, *FANCC* and the *BRCA2* CTD function together in HR. On the other hand, the TLS function of *FANCC* might be required for repairing ICLs, providing an explanation for the distinct roles of *FANCC* and *BRCA2* CTD in this type of DNA repair. *BRCA2* and *FANCC* also appeared to have an epistatic function during normal cell cycle progression, suggesting that they are mainly handling DSBs in the context of the restart of stalled replication forks. These data suggest that relationship between classical FA pathway and function of *BRCA2* is not necessarily a simple, linear pathway depending on types of DNA damage.

*BRCA2* knockout mice display embryonic lethality (50), and only hypomorphic mutant mice can survive. This seems also true for human subjects (23). In contrast, *fancc, fancg, fancd2* mice are able to grow largely unaffected (1,2). Interestingly, *fancd2* mice display overlapping features with *BRCA2* hypomorphic mice, such as epithelial carcinogenesis or germ cell defects (37). However, a number of previous studies have suggested distinct functional defects in FA-D1 patients. First, FA-D1 patients are much more susceptible to cancer, including early-onset leukemia and solid tumor than other subtypes of FA (51,52). Second, a recent study provided evidence that HR defects in *BRCA* mutant human cells are much larger than that in classical FA cells (*fancd2*) (9). Furthermore, these FA genes, but not *BRCA2*, are necessary for one type of HR repair, i.e. single-strand annealing pathway (9). Thus, similarity of the symptoms among FA-D1 patients and other FA patients (e.g. bone marrow failure) could be caused by shared defects in HR repair.

In conclusion, we have re-examined chromatin loading and focus formation of Rad51 in FA mutant cell lines, and confirmed that these early phase events in HR repair were compromised in *BRCA2 BRCA* or *CTDA* mutants, but not in classical FA mutants. However, genetic analysis indicated that the *BRCA2* CTD and *FANCC* function in the common DSB repair pathway mediated by HR. In contrast, we find that, in dealing with ICLs, the *BRCA2* CTD and *FANCC* have non-overlapping function. How the FA pathway connects with HR or TLS pathway is still unknown. Whether the recently identified FancM or FancJ serve as the link should be clarified in the near future.

**REFERENCES**
FOOTNOTE

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1The abbreviations used are: CTD, C-terminal conserved domain; DSB, double strand break; FA, fanconi anemia; FCS, fetal calf serum; FACS, fluorescence activated cell sorter; HR, homologous recombination; ICL, interstrand crosslinks; Ig, immunoglobulin; MMC, mitomycin C; MMS, methylmethanesulfonate; NER, nucleotide excision repair; PI, propidium iodide; TAM, 4-hydroxy tamoxifen; TLS, translesion DNA synthesis; WCE, whole cell extract.

2Matsushita et al., manuscript in preparation.

FIGURE LEGENDS

Figure 1. MMC-induced chromatin loading of FancD2 and Rad51 protein. Wild type, fancc, fancd2, and brca2ΔBRC cells were untreated or treated with 500 ng/mL MMC for 6 hours, and separated into soluble (Sol) and chromatin fractions (Chr). Western blot using anti-chicken FancD2 (top), anti-Rad51 (middle) and anti-histone H4 (bottom) are shown. An asterisk indicates a non-specific band. WCE, whole cell extract.

Figure 2. DNA damage-induced Rad51 nuclear focus formation is not impaired in the absence of FancD2 foci formation. (A) MMC- or X-ray-induced nuclear foci. GFP-fused chicken FancD2 protein carrying either wild type (WT, Lys563) or mutated (KR, Arg563) monoubiquitination site was expressed in fancd2 background as described in the text. In addition, FANCC gene was deleted in fancd2 cells expressing GFP-FancD2 WT protein. Indicated cell lines were examined following MMC exposure (500 ng/ml for 6 hr) or 6 hr after X-ray irradiation (8 Gy). Rad51 foci were detected by staining with an anti-Rad51 antibody. Merged images are shown in the right. Enlarged images of colocalizing Rad51 and FancD2 foci are shown on the 2nd row. (B) Kinetics of X-ray-induced formation of Rad51 foci (upper panels). At least 300 cells were analyzed at each time point. Cells with more than four bright foci were scored as foci-positive. (C) MMC-induced Rad51 or FancD2 foci in cells with the indicated genotypes. Cells were treated with MMC (500 ng/ml for 6 hr), then harvested.

Figure 3. Generation of fancc/brca2ΔCTD cells. (A) A schematic diagram of wild type and truncated BRCA2 (ΔCTD form). (B) A schematic flowchart illustrating the steps in generation of fancc/brca2ΔCTD cells. (C) Western blot analysis of chicken BRCA2. Lysates from the indicated cell lines were probed with anti-chicken BRCA2 antiserum raised by using N-terminal BRCA2 recombinant protein. (D) RT-PCR analysis for mRNA expression of FANCC (top), BRCA2 (middle) and Rad51 (bottom), and genomic Southern blot analysis of the FANCC locus. Appearance of the shorter ~140 bp FANCC RT-PCR band was due to anomalous splicing.
resulting from gene targeting followed by excision of the resistance marker cassette. This resulted in frame shift, leading to production of short truncated protein (residues 1-55 plus 6 amino acids) as described (22). (E) Western blot analysis of chicken FancD2. Total cell lysates from indicated cell lines were immunoblotted with anti-chicken FancD2 antibody. Cells were either untreated (lanes 1, 3, 5 and 7) or treated with 500 ng/ml MMC for six hours (lanes 2, 4, 6 and 8).

Figure 4. Proliferative characteristics of fancc/BRCA2ΔCTD/con, brca2ΔCTD, and fancc/brca2ΔCTD cells. (A) Proliferation curve of the indicated cell lines. Cell growth was assessed by FACSCalibur flow cytometer using plastic beads as a standard. (B) Spontaneous cell death and percentage of cells in M phase (mitotic index). Cells were fixed with 70 % ethanol, then stained with PI, and analyzed by FACSCalibur. Percentage of cells with less than 2N DNA content were defined as dead cells. To analyze mitotic index, cells were fixed with 70% ethanol, and stained with anti-phosphorylated histone H3. (C) Cell cycle distribution. Cells were pulse-labeled with 10 mM BrdU for 10 min, and were stained with anti-BrdU antibody and propidium iodide (PI). Percentages of cells in G1, S and G2/M phase in the live cell gate are shown.

Figure 5. DNA damage sensitivity of fancc/BRCA2ΔCTD/con, brca2ΔCTD, and fancc/brca2ΔCTD cells. Cells with indicated genotypes were exposed to X-ray (A), MMS (B), cisplatin (C), and MMC (D), and colony survival was analyzed. The mean and standard deviation are shown from independent experiments repeated at least three times.

Figure 6. Chromosomal aberrations of fancc/BRCA2ΔCTD/con, brca2ΔCTD, and fancc/brca2ΔCTD cells. (A) X-ray-induced chromosomal aberrations. Cells were irradiated with the indicated dose of X-rays, and were harvested 3hr later. At least 50 metaphases from several spreads were analyzed. (B) MMC-induced chromosomal aberrations. Cells were exposed to indicated dosage of MMC for 24 hrs. At least 40 metaphases from several spreads were analyzed. Error bars represent standard error of total aberrations per metaphase.

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Figure 2

A

GFP-FancD2 WT

\textit{fancd}2

GFP-FancD2 KR

\textit{fancd}2

GFP-FancD2 WT

\textit{fanc}c/\textit{fancd}2

MMC

X-ray

B

\% Rad51 foci positive cells

Time following MMC treatment (hrs)

C

\textit{BRCA2}^\textit{ABRC/con}

\textit{BRCA2}^\textit{ABRC/con}

\textit{BRCA2}^\textit{ABRC/con}

\textit{BRCA2}^\textit{ABRC/con}
Figure 3

A

| BRCA2 WT | BRC repeats | CTD | NLS |
|----------|-------------|-----|-----|
| BRCA2ΔCTD |             |     |     |

3397

1

2150

B

Wild type (BRCA2 \(^{+/+}\)) (WT)

\[ \xrightarrow{BRCA2\text{ conditional null targeting}} \]

BRCA2\(^{+/+}\)\(_{\text{con}}\)

\[ \xrightarrow{BRCA2\Delta\text{CTD targeting}} \]

BRCA2\(^{\Delta\text{CTD/con}}\)

\[ \xrightarrow{\text{FANCC targeting}} \]

fanc/BRCA2\(^{\Delta\text{CTD/con}}\)

\[ \xrightarrow{\text{+TAM}} \]

BRCA2\(^{\Delta\text{CTD/-}}\) (brca2\(^{\Delta\text{CTD}}\)) fanc/BRCA2\(^{\Delta\text{CTD/-}}\) (fanc/brca2\(^{\Delta\text{CTD}}\))

C

WT

brca2\(^{\Delta\text{CTD}}\)

250kDa

BRCA2 WT

BRCA2 \(^{\Delta\text{CTD}}\)

IB: anti-BRCA2 (N terminal)

D

RT-PCR

FANCC

220bp

140bp

BRCA2

700bp

Rad51

1.0kb

FANCC

Genomic Southern

9.0kb

6.5kb

E

| BRCA2\(^{\Delta\text{CTD/con}}\) | fanc/BRCA2\(^{\Delta\text{CTD/con}}\) | brca2\(^{\Delta\text{CTD}}\) | fanc/brca2\(^{\Delta\text{CTD}}\) |
|-------------------------------|-------------------------------|-------------------|-------------------------------|
| MMC: - | - | - | - |
| MMC: + | + | + | + |

IB: anti-FancD2

FancD2-L

FancD2-S
A

![Graph showing cell growth over days](image)

B

| Sample                        | % of dead cells   | % of mitotic cells |
|-------------------------------|-------------------|-------------------|
| BRCA2ΔCTD/con                 | 3.6 ±1.6          | 2.84 ±0.34        |
| fancc/BRCA2ΔCTD/con           | 10.6 ±1.4         | 2.67 ±0.24        |
| brca2ΔCTD #1                  | 31.8 ±3.5         | 1.55 ±0.23        |
| fancc/brca2ΔCTD #1            | 30.6 ±6.5         | 1.42 ±0.14        |

C

![Bar plots showing BrdU uptake](image)
A

X-ray

B

MMC

![Graphs showing aberrations per metaphase](http://www.jbc.org)

- Chromatid breaks
- Chromatid gaps
- Chromosome breaks
- Chromosome gaps
- Exchanges

Figure 6
