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## FANCIJ/BRIP1 recruitment and regulation of FANCD2 in DNA damage responses

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### Abstract

FANCIJ/BRIP1 encodes a helicase that has been implicated in the maintenance of genomic stability. Here, to better understand FANCIJ function in DNA damage responses, we have examined the regulation of its cellular localization. FANCIJ nuclear foci assemble spontaneously during S phase and are induced by various stresses. FANCIJ foci colocalize with the replication fork following treatment with hydroxyurea, but not spontaneously. Using FANCIJ mutants, we find that FANCIJ helicase activity and the capacity to bind BRCA1 are both involved in FANCIJ recruitment. Given similarities to the recruitment of another Fanconi anemia protein, FANCD2, we tested for colocalization of FANCIJ and FANCD2. Importantly, these proteins show substantial colocalization, and FANCIJ promotes the assembly of FANCD2 nuclear foci. This process is linked to the proper localization of FANCIJ itself since both FANCIJ and FANCD2 nuclear foci are compromised by FANCIJ mutants that abrogate its helicase activity or interaction with BRCA1. Our results suggest that FANCIJ is recruited in response to replication stress and that FANCIJ/BRIP1 may serve to link FANCD2 to BRCA1.

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## Introduction

Genomic instability is associated with the development of cancer and with a poorer prognosis. Many tumor suppressor proteins function in cellular pathways that maintain a stable genome by responding to DNA damage (Risinger and Groden 2004). FANCI/BRIP1 has been implicated in DNA damage responses through its interaction with the protein encoded by the BRCA1 breast cancer susceptibility gene (Cantor et al. 2001) and by being a Fanconi anemia gene (Levitus et al. 2005; Levrn et al. 2005; Litman et al. 2005).

FANCI was first identified by a physical interaction with BRCA1, which is mediated by phosphorylation of FANCI at S990 (Yu et al. 2003; Xie et al. 2010). Accordingly, this interaction is disrupted in the S990A mutant of FANCI. Like BRCA1, FANCI has been identified as a breast cancer susceptibility protein (Seal et al. 2006). It appears that FANCI functions downstream of BRCA1 in human cells. For example, BRCA1 is required for the assembly of FANCI nuclear foci (Cantor et al. 2001), but it has not been demonstrated that this is mediated through a physical interaction between the proteins. Unlike human FANCI, homologues in chicken and *Caenorhabditis elegans* lack the conserved Ser990-X-X-Phe motif and presumably do not interact with BRCA1 (Bridge et al. 2005; Youds et al. 2008).

Human FANCI has a helicase domain (Cantor et al. 2001). As in other helicases, mutation of a conserved lysine at residue 52 (K52R), which is involved in ATP hydrolysis (Cantor et al. 2001), abrogates the helicase activity of FANCI (Cantor et al. 2004; Gupta et al. 2005). Two mutations of FANCI associated with early onset breast cancer disrupt its helicase activity (Cantor et al. 2004), suggesting the potential importance of this activity to the function of FANCI as a tumor suppressor. FANCI displays preferential binding to a forked duplex substrate and has 5' to 3' helicase activity (Gupta et al. 2005). Both the K52R and S990A mutants of FANCI can alter cellular sensitivity to DNA damage (Peng et al. 2007; Xie et al. 2010), suggesting a role in DNA damage responses for FANCI helicase activity and its capacity to bind BRCA1.

Fanconi anemia (FA) is associated with a predisposition to cancer, including leukemia and various solid tumors, as well as bone marrow failure and assorted congenital anomalies (Mathew 2006; Taniguchi and D'Andrea 2006). Cells from FA patients are characterized by chromosome instability, both spontaneously and in response to DNA interstrand crosslinking agents such as mitomycin C (MMC). There are 14 identified FA genes, and eight of the encoded proteins (FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCD3, FANCD4, and FANCD5) are required for the monoubiquitination of the FA proteins FANCD2 (Garcia-Higuera et al. 2001; Taniguchi and D'Andrea 2006; Wang 2007) and FANCI (Sims et al. 2007; Smogorzewska et al. 2007). Given that a non-ubiquitinable FANCD2 mutant confers no resistance to MMC (Garcia-Higuera et al. 2001; Taniguchi et al. 2002b; Montes de Oca et al. 2005), it appears that monoubiquitination of this protein is critical to the FA pathway.

Among the other FA proteins, FANCI, along with FANCD1/BRCA2, FANCN/PALB2, and FANCO/RAD51C, is not required for FANCD2 monoubiquitination (Howlett et al. 2002; Levitus et al. 2004; Bridge et al., 2005; Litman et al. 2005; Reid et al. 2007; Vaz et al. 2010). For this reason, it has been suggested that FANCI functions downstream of

monoubiquitinated FANCD2 (Bridge et al. 2005; Cantor and Andreassen 2006). It should be noted, in this context, that BRCA1 has not been identified as a FA gene (Cantor and Andreassen 2006). Consistent with a role in DNA damage responses, BRCA1, FANCI, and FANCD2 are all required for cellular resistance to MMC (Garcia-Higuera et al. 2001; Moynahan et al. 2001; Peng et al. 2007) and, to varying degrees, ionizing radiation (IR) (Scully et al. 1999; Taniguchi et al. 2002b; Peng et al. 2006).

Various DNA damage response proteins are recruited in response to DNA damage or replication stress (Andreassen et al. 2006). The mechanism and pattern of recruitment can provide important insights into the function of a particular protein in DNA damage responses. It is known that FANCI foci are inducible in response to IR and that these foci colocalize with DNA damage response proteins such as BRCA1 and RPA (Cantor et al. 2001; Peng et al. 2006; Gupta et al. 2007). The localization of FANCI to DNA damage foci further implicates it in DNA damage responses, but the regulation and role of this localization are not well understood.

Here, to better understand FANCI function, we have examined its recruitment. We provide quantitative evidence that FANCI foci assemble in response to a variety of stresses and that FANCI is recruited to blocked replication forks in response to treatment with hydroxyurea. These results suggest that FANCI may be recruited to lesions that impede the replication fork. In agreement with this possibility, we find that the ATR checkpoint kinase, which is an important mediator of the replication-stress response (Cimprich and Cortez 2008), influences DNA damage-induced assembly of FANCI foci. Further, we have utilized the FANCI-K52R and FANCI-S990A mutants to determine how FANCI is recruited to nuclear foci. We find that the capacity of FANCI to bind to BRCA1, and its helicase activity, is important for its localization. Interestingly, we also find that both mutants are associated with a defect in the assembly of FANCD2 nuclear foci, both spontaneously and in response to DNA damage induced by MMC. Thus, proper localization of FANCI is associated with efficient recruitment of FANCD2. Together, our results suggest that FANCI may have a role in linking FANCD2 recruitment to BRCA1. This is of interest since each of these proteins is required for the maintenance of genomic stability.

## Materials and methods

### Cell culture

EUFA30-hTERT fibroblasts (Levitus et al. 2005), which were kindly provided by Dr. Hans Joenje (Vrije Universiteit Medical Center), PD20 cells containing the pMMP vector or corrected with wild-type FANCD2, and MCF7 cells were grown in DMEM supplemented as described previously (Garcia-Higuera et al. 2001; Litman et al. 2005; Zhang et al. 2009). Also, PD845 and PD846 fibroblasts (Mankad et al. 2006) immortalized with hTERT were grown as described for EUFA30-hTERT cells (Litman et al. 2005). Cells were kept at 37°C in a humid incubator.

### Retroviral transduction of FA-J cells

K52R and S990A mutations were introduced into the FANCI cDNA in pBluescriptKS (+/-) using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Mutations were verified by sequencing. Following introduction of *Xho*I (5') and *Not*I (3') restriction sites by PCR, these cDNAs were cloned into the pOZ retroviral vector containing a C-terminal Flag-HA tag (Nakatani and Ogryzko 2003). Generation of retroviruses, transduction, and selection of cells using magnetic beads conjugated to IL2 (Upstate) was as previously described (Fan et al. 2009). Cells were analyzed in the first or second week after selection.

### siRNA and transfection

Expression of targeted genes was knocked down by transient transfection with siRNAs directed against FANCI (5'-GTACAGTACCCACCTTAT) (Litman et al. 2005), BRCA1 (5'-GGAACCTGTCTCCACAAAG) (Vandenberg et al. 2003), ATR (5'-CCTCCGTGATGTTGCTTGA), and GFP (5'-CGGCAAGCTGACCCTGAAGTTCAT) (Andreassen et al. 2004). Cells were plated at least 16 h prior to transfection to yield approximately 30% confluency at the time of transfection. siRNA oligos were transfected by Oligofectamine according to the manufacturer's instructions (Invitrogen). To obtain maximal RNA interference, cells were transfected with siRNA again 24 h after the initial siRNA transfection. Cells were analyzed 48 h after the second transfection.

### Immunofluorescence microscopy

Cells were grown on poly-D-lysine coated glass coverslips for a minimum of 16 h prior to experimental manipulation, and unless otherwise noted, cells were fixed with 2% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 3 min, washed, and incubated with primary and secondary antibodies as previously described (Fan et al. 2009). FANCD2, FANCI, BRCA1, and  $\gamma$ -H2AX were detected with E35 rabbit antiserum (Garcia-Higuera et al. 2001), 2G7 mouse monoclonal antibodies (Cantor et al. 2001) (a kind gift of Dr. Sharon Cantor, University of Massachusetts), rabbit polyclonal antibodies (Cell Signaling), and Ser139 rabbit polyclonal antibodies (Upstate), respectively. FANCI-Flag-HA fusion proteins were detected with M2 anti-Flag antibodies (Sigma). Coverslips were mounted over Vectashield containing DAPI and were sealed with fingernail polish.

For double-labeling with anti-PCNA and anti-FANCI antibodies, cells were fixed with 2% paraformaldehyde in PBS for 20 min at RT, washed once with PBS, fixed 10 min with methanol at -20°C, and then incubated with 0.2% Triton X-100 in PBS for 2 min. Cells were then washed with PBS prior to incubation with primary antibodies, as described above. PCNA was detected with a mouse antibody (PC10, Santa Cruz).

For determination of the S phase index, FA-J cells were incubated with 30  $\mu$ M bromodeoxyuridine (BrdU) for 10 min, then fixed with 2% paraformaldehyde, permeabilized, treated with 2 N HCl for antibody access, neutralized, and incubated with anti-BrdU antibody as previously described (Andreassen et al. 2004).

Three counts of 150 or more cells each was made for each treatment condition, unless otherwise noted, and averages and standard deviations were calculated. *P* values were calculated using Student's *t* test. Labeled cells were observed with a Zeiss Axiovert 200M microscope, and images were collected with a Hamamatsu Camera using Openlab software (Improvision). Images were processed into figures using Photoshop (Adobe).

### Immunoblotting

Lysates were electrophoresed by SDS-PAGE (6% poly-acrylamide, bis-acrylamide). Proteins were then transferred to nitrocellulose, and membranes blocked and incubated with primary antibodies, as previously described (Taniguchi et al. 2002a). Antibodies included anti-FANCD2 (E35), E47 rabbit anti-FANCI (a kind gift of Dr. Sharon Cantor), anti-BRCA1 (Upstate), anti-ATR (Sigma), anti-Flag (Sigma), rabbit anti-histone H2A (acidic patch) (Sigma), anti- $\gamma$ -tubulin (Sigma), and C4 anti-actin antibodies (a kind gift of Dr. James Lessard, Cincinnati Children's Research Foundation). Membranes were washed, incubated with HRP-linked secondary antibodies (Amersham), and detected by chemiluminescence (Amersham) as previously described (Taniguchi et al. 2002a).

### Nuclear Fractionation

Tert-immortalized EUFA-30 FA-J fibroblasts that contained the empty pOZ vector alone, or which were corrected by expression of FANCI, were fractionated as described previously (Bogliolo et al. 2007). Briefly, an equivalent number of cells was collected for each cell type, and cells were washed twice with PBS pre-chilled to 4°C. Cells were resuspended in 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and Complete Protease Inhibitor Cocktail (Roche). Cells were incubated for 5 min on ice. Cytoplasmic and soluble nuclear proteins were collected in the supernatant following centrifugation (1,300×*g*, 4 min). Nuclei were then lysed in 3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, and protease inhibitors (Roche) for 30 min on ice. Soluble nuclear proteins were collected by centrifugation (1,700×*g*, 4 min) and were pooled with the previous fraction. Together, this fraction contained soluble proteins. The pellet was washed once in the buffer described above. The pellet, which contained chromatin proteins, was resuspended in SDS sample buffer and was sonicated.

### G2/M Accumulation

EUFA30 fibroblasts containing the empty vector or wild-type FANCI were plated to be subconfluent for the course of the experiment. After 24 h, cells were left untreated or were exposed to 0.35  $\mu$ g/ml melphalan for an additional 48 h. Cells were collected, fixed, and incubated with RNase A and propidium iodide as previously described (Andreassen et al. 2004). For analysis,  $5 \times 10^3$  cells were measured using a FACS Calibur instrument (Becton-Dickinson), aggregates were gated out, and the percentage of cells in G2/M was calculated using ModFit software.

## Results

We conducted an analysis of FANCD1 nuclear foci by immunofluorescence microscopy to gain insight into its potential functions and manner of regulation (Fig. 1). While a previous analysis reported that IR induces FANCD1 foci (Peng et al. 2006), our quantitative analyses demonstrate that FANCD1 foci were also induced by other stresses in MCF7 mammary adenocarcinoma cells. An example of foci assembled in untreated cells and following treatment with MMC is shown in Fig. 1a. Quantification shows that both MMC and hydroxyurea (HU) induced FANCD1 foci, as did exposure to IR (Fig. 1b). Thus, FANCD1 foci are assembled in response to a variety of DNA damages and replication stress.

Importantly, and in agreement with a previous report (Peng et al. 2006), spontaneous FANCD1 foci were detected in a subset of untreated MCF7 mammary adenocarcinoma cells (Fig. 1a, b). We considered the possibility that these spontaneous foci might be cell cycle-linked (Fig. 1c, d). Since certain DNA repair processes, such as HR, are coupled to S phase progression (Rothkamm et al. 2003), we examined whether spontaneous FANCD1 foci assemble strictly at this stage of the cell cycle. For this purpose, we examined cells that were double-labeled with antibodies to FANCD1 and to PCNA. PCNA foci assemble during S phase at the replication fork (Bravo and Macdonald-Bravo 1985). As shown in the example in Fig. 1c, and by quantification in Fig. 1d, nearly all cells that contained spontaneous FANCD1 foci were in S phase and not at other stages of the cell cycle, as determined by the presence or absence of nuclear PCNA.

We then determined whether spontaneous FANCD1 foci colocalize with PCNA foci, which would indicate the association of FANCD1 with the replication fork and a potential function at this site. As shown in the example in Fig. 1c, and by quantification in Fig. 1e, FANCD1 foci showed only a low level of colocalization, if any, with the replication fork in untreated cells ( $1.8 \pm 1.3\%$ ). Similarly, MMC and IR, which unlike HU do not arrest DNA replication, yielded low levels of colocalization of FANCD1 foci with PCNA foci ( $2.8 \pm 0.4\%$  and  $2.0 \pm 0.9\%$  at 7 h after treatment with  $0.5 \mu\text{M}$  MMC or 5 Gy IR, respectively). Interestingly, however, FANCD1 foci strongly colocalized with PCNA foci within 2 h of treatment with HU and also at 7 h of treatment ( $80.2 \pm 2.2\%$  of FANCD1-positive nuclei displayed colocalization at 7 h of treatment with HU). Thus, the recruitment of FANCD1 following exposure to DNA damage may involve replication stress.

To further investigate whether FANCD1 is recruited to DNA damage foci in response to replication stress, we depleted ATR from cells. ATR is a central regulator of the replication-stress response (Cimprich and Cortez 2008). ATR levels were depleted using a siRNA that we have described previously, which targets this protein (Andreassen et al. 2004). Depletion of ATR had no clear effect on FANCD1 protein levels (Supplementary Material, Fig. S1a). However, depletion of ATR resulted in a decrease in the levels of FANCD1 foci following exposure to MMC (Supplementary Material, Fig. S1b), as compared with cells transfected with a control siRNA. There was no effect on FANCD1 foci in untreated cells, consistent with this protein being recruited, in part, by the ATR-mediated replication-stress response.



To better understand the molecular determinants of the assembly of FANCD2 into nuclear foci, we reconstituted EUFA30 FA-J cells, which are deficient for FANCD2 (Levitus et al. 2005), with wild-type FANCD2 or with two different FANCD2 mutants. The K52R mutant is defective for FANCD2 helicase activity (Cantor et al. 2004; Gupta et al. 2006) and the non-phosphorylatable S990A mutant of FANCD2 does not bind to BRCA1 (Yu et al. 2003; Xie et al. 2010). Each form of FANCD2 was expressed as a C-terminal fusion with a Flag-HA epitope tag. Wild-type FANCD2 and each of the mutants were expressed at comparable levels (Fig. 2a) and were detected with antibodies that recognized the Flag epitope tag or FANCD2 itself. Functional correction of the EUFA30 FA-J cell line by expression of wild-type FANCD2-Flag-HA was demonstrated by a clear reduction in G2/M accumulation following treatment with melphalan (Supplementary Material, Fig. S2).

Detection of foci by immunofluorescence microscopy was conducted using anti-Flag antibodies. In the example shown, EUFA30 fibroblasts that contained the empty retroviral vector were devoid of foci detectable with anti-Flag antibodies (Fig. 2b). In contrast, wild-type FANCD2-Flag-HA assembled into distinct nuclear foci in a subset of cells following treatment with 0.5  $\mu$ M MMC for 20 h. A full set of images, including representative examples for FANCD2-K52R and FANCD2-S990A, where only a small fraction of cells assembled FANCD2 foci, is shown in Supplementary Material, Fig. S3. Quantification of the assembly of FANCD2 foci by each mutant, or by wild-type FANCD2, as detected with anti-Flag antibodies, is shown in Fig. 2c. The assembly of FANCD2-S990A or FANCD2-K52R into nuclear foci was compromised, as compared with wild-type FANCD2, both in untreated populations and following exposure to MMC. We did not test these mutants for the assembly of FANCD2 foci following treatment with IR or HU.

While EUFA30 FA-J cells expressing FANCD2-S990A had a slightly elevated rate of passage through S phase, as measured by BrdU incorporation (Andreassen et al. 2004), cells lacking FANCD2 or expressing FANCD2-WT or FANCD2-K52R had comparable rates of S phase progression (Fig. 2d). Thus, effects on the assembly of FANCD2 foci were not due to cell cycle perturbations. We conclude that the assembly of FANCD2 nuclear foci involves both its binding to BRCA1 and its helicase activity.

Colocalization can yield important insight into the function of specific proteins. Given that FANCD2 (Fig. 1) and another FA protein, FANCD1 (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a), form spontaneous S phase foci and are induced by a variety of stresses, we assayed for colocalization of these proteins. Examples of colocalization in untreated MCF7 cells and following exposure to MMC are shown in Fig. 3a. While some dispersed nuclear signal from FANCD2 and FANCD1 was detected, consistent with previous reports (Cantor et al. 2001; Garcia-Higuera et al. 2001), foci were more intense. Quantification in untreated MCF7 cells, or following exposure to MMC, HU, or IR, indicated detectable colocalization of FANCD2 with FANCD1 foci in each case and is shown in Fig. 3b. The colocalization of FANCD2 foci and FANCD1 foci suggests a possible functional interaction between these proteins.

Since FANCD2 has been proposed to function downstream of FANCD1 (Bridge et al. 2005; Cantor and Andreassen 2006), and given the observed colocalization of FANCD2 and

FANCD2 foci (Fig. 3), we sought to determine whether the assembly of FANCD2 foci might be dependent upon FANCD2. Thus, we compared PD20 FA-D2 cells that lacked FANCD2 and their counterparts corrected by expression of FANCD2 but found no difference in FANCD2 foci following treatment with MMC (Supplementary Material, Fig. S4).

Next, to determine whether the assembly of FANCD2 foci is instead dependent upon FANCD2, we examined the assembly of FANCD2 foci in two different FANCD2-deficient cell lines (EUFA30 and IFAR943/1) from FA-J patients (Levitus et al. 2005; Levrin et al. 2005). Both EUFA30 and IFAR943/1 cells displayed undetectable endogenous FANCD2, or low levels relative to PD845 (FA-A) and PD846 (normal control) cells (Fig. 4a). FANCD2-Flag-HA expression in EUFA30 and IFAR943/1 cells was detected with antibodies directed against either FANCD2 or HA (Fig. 4a). FANCD2 was monoubiquitinated in both of these cell lines, and this was induced by treatment with MMC. FANCD2 was not required for FANCD2 monoubiquitination since the levels were similar with or without correction with wild-type FANCD2. In contrast, the PD845 FA-A cell line displayed a low level of FANCD2 monoubiquitination in untreated populations, but no increase was observed following treatment with MMC.

Representative images suggest that the assembly of FANCD2 foci was more efficient in FA-J cells (EUFA30) corrected by the expression of FANCD2 than in uncorrected FA-J cells containing the pOZ empty vector (Fig. 4b). For example, fewer FA-J cells displayed FANCD2 foci than did corrected FA-J cells. Also, within positive cells, FANCD2 foci were generally brighter following reintroduction of FANCD2. A fibroblast line (PD845) from a FA patient with biallelic mutation of FANCA, which unlike FANCD2 is required for FANCD2 monoubiquitination (Garcia-Higuera et al. 2001; Levitus et al. 2004), was also examined for comparison. In contrast to FA-J cells, the PD845 cell line was completely deficient for the assembly of FANCD2 foci (Fig. 4b).

Quantification demonstrates that both EUFA30 and IFAR943/1 FA-J cells displayed some FANCD2 foci. Reconstitution with FANCD2 enhanced the assembly of FANCD2 foci, however, either without treatment or in response to treatment with 0.5  $\mu$ M MMC for 20 h (Fig. 4c). The levels of FANCD2 foci in corrected FA-J cell lines were similar to those observed in PD846 cells from a normal control individual. In contrast, PD845 cells were completely deficient for the assembly of FANCD2 foci, either with or without MMC treatment (Fig. 4c). Thus, FANCD2 promotes the efficient assembly of FANCD2 foci but does so in a manner that is distinguishable from deficient monoubiquitination of FANCD2.

Next, to determine molecular requirements for the role of FANCD2 in promoting the assembly of FANCD2 foci, we utilized EUFA30 FA-J fibroblasts reconstituted with wild-type FANCD2 or with the two different FANCD2 mutants. Quantification demonstrates that unlike wild-type FANCD2, neither the S990A mutant nor the K52R mutant of FANCD2, corrected the defective assembly of FANCD2 foci present in EUFA30 FA-J fibroblasts (Fig. 4d). The percentage of FA-J cells with FANCD2 foci was the same for cells containing the empty vector alone and for those expressing FANCD2-K52R or FANCD2-S990A, either without treatment or following exposure to 0.5  $\mu$ M MMC for 20 h. The assembly of FANCD2 foci following treatment with IR or HU was not measured in FA-J cells reconstituted with FANCD2-S990A or FANCD2-



K52R. Again, as shown by measuring BrdU incorporation (Fig. 2d), these differences were not due to an altered rate of cell cycle progression. Since the efficient assembly of FANCD2 foci also required both FANCD2 helicase activity and FANCD2 binding to BRCA1 (Fig. 2c), normal assembly of FANCD2 foci may be linked to the recruitment of FANCD2.

FANCD2 must be monoubiquitinated to assemble into nuclear foci in chromatin (Montes de Oca et al. 2005). Thus, we hypothesized that FANCD2 may regulate FANCD2 foci by promoting the association of monoubiquitinated FANCD2 with chromatin. To test this possibility, we separated nuclei from FA-J cells, which contained the empty vector or which were corrected with FANCD2, into soluble and chromatin fractions. FANCD2 was detected in the corrected cells and was associated with both the soluble and chromatin-containing fractions from untreated cells (Fig. 5a). Most of the unubiquitinated FANCD2 (-S) was present in soluble fractions (containing both cytoplasmic and nuclear proteins) (lanes 1 and 3), either with or without correction of the FANCD2-deficiency. In contrast, monoubiquitinated FANCD2 (-L) was predominantly detected in chromatin-containing fractions (lanes 2 and 4). Interestingly, monoubiquitinated FANCD2 associated with chromatin less efficiently in uncorrected FA-J cells (FA-J+Vector) than in corrected FA-J cells. This can be seen by the increased percentage of total monoubiquitinated FANCD2, identified in the boxes, which is present in the soluble fractions from uncorrected FA-J cells (for example, compare lane 1 to lane 2, and lane 3 to lane 4).

As another measure of the influence of FANCD2 on the association of monoubiquitinated FANCD2 with chromatin, we also examined cells following the induction of DNA damage by MMC (Fig. 5b). Higher levels of monoubiquitinated FANCD2 were observed, either with or without correction for FANCD2 (there was more FANCD2-L in lanes 2 and 4 versus FANCD2-S in lanes 1 and 3 when comparing MMC-treated and untreated cells). Under such conditions, FANCD2 again promoted the association of monoubiquitinated FANCD2 with chromatin (compare lane 1 with lane 2 and lane 3 to lane 4). A role for FANCD2 in promoting the association of monoubiquitinated FANCD2 with chromatin was consistently observed in three experiments. Following treatment with MMC, 29.8±6.1% of monoubiquitinated FANCD2 associated with chromatin in uncorrected cells versus 13.0±3.1% in cells corrected by expression of FANCD2. This difference is statistically significant ( $p<0.02$ ).

As an additional measure of whether FANCD2 regulates the assembly of FANCD2 foci, we also depleted FANCD2 using a siRNA in MCF7 cells. Substantial depletion of FANCD2 is demonstrated in Fig. 6a. Suppression of FANCD2 did not substantially alter FANCD2 monoubiquitination, however. Quantification demonstrates that suppression of FANCD2 resulted in partial inhibition of the assembly of FANCD2 foci, relative to controls transfected with a siRNA against GFP, both in untreated populations or following exposure to 0.5  $\mu$ M MMC for 20 h (Fig. 6b). Depletion of FANCD2 also inhibited FANCD2 foci in MCF7 cells exposed to IR or HU (Supplementary Material, Fig. S5). FANCD2 may therefore be involved in regulating the assembly of FANCD2 foci in response to a variety of DNA damage or replication stresses.

Consistent with our finding that a FANCD2 mutant that is defective for binding to BRCA1 does not support efficient assembly of FANCD2 foci (Fig. 4d), we find that depletion of

BRCA1 suppresses the assembly of FANCD2 foci to a similar degree as depletion of FANCD2 (Fig. 6b). Importantly, as observed previously (Cantor et al. 2001; Gupta et al. 2007), BRCA1 also had a role in the assembly of FANCD2 foci (data not shown). These results, along with defects in FANCD2 foci associated with the FANCD2-S990A mutant (Fig. 4d), support the possibility that BRCA1 and FANCD2 cooperate in regulating the assembly of FANCD2 foci.

As an assay of the requirement for FANCD2 in targeting FANCD2 to sites of DNA damage, we examined MCF7 cells, with or without depletion of FANCD2, for the colocalization of FANCD2 foci with  $\gamma$ -H2AX foci at 7 h following exposure to 5 Gy IR. Foci formed by  $\gamma$ -H2AX are indicative of DNA double-strand breaks in DNA, such as those induced by IR (Rothkamm and Lobrich 2003). FANCD2 and  $\gamma$ -H2AX foci displayed colocalization after treatment with IR in untransfected MCF7 cells (Fig. 6c). The majority of untreated cells did not display FANCD2 foci or colocalization of FANCD2 foci with  $\gamma$ -H2AX foci (data not shown).

Depletion of FANCD2 or BRCA1 resulted in a decrease in the colocalization of FANCD2 foci with  $\gamma$ -H2AX foci following exposure to IR, but had no effect on the assembly of  $\gamma$ -H2AX foci (Fig. 6d, e). Taken together, our results suggest that FANCD2, as well as FANCD2 helicase activity and the capacity of FANCD2 to bind to BRCA1, have a role in regulating the recruitment of FANCD2 to sites of DNA damage.

## Discussion

While it is well established that FANCD2 has structure-specific helicase activity in vitro (Gupta et al. 2005), to better understand its functions in DNA damage responses, we have focused here on the regulation of the cellular localization of FANCD2. We have found that FANCD2 foci are induced by a variety of stresses, including MMC and IR, and that FANCD2 is recruited to replication forks blocked by HU. Thus, FANCD2 recruitment in response to DNA damage may involve replication stress. It is already known that lesions such as ICLs and DSBs activate ATR, which is indicative of a replication-stress response (Andreassen et al. 2004; Lin et al. 2004).

Additionally, we have identified molecular determinants of FANCD2 localization, including a role for FANCD2 helicase activity and for the capacity of FANCD2 to bind BRCA1. Each of these mutants alters cellular sensitivity to DNA damage (Xie et al. 2010), suggesting the potential importance of the localization of this protein. It is interesting, in this context, that FANCD2 and BRCA1, to which FANCD2 binds, are both associated with inherited breast cancer (Seal et al. 2006). Further, several mutants identified in breast cancer patients affect FANCD2 helicase activity in vitro (Cantor et al. 2004; Gupta et al. 2005). Thus, the localization of FANCD2 may be important for its function as a tumor suppressor.

We find a novel function for FANCD2 in promoting the recruitment of another Fanconi anemia protein, FANCD2, to sites of DNA damage. Thus, rather than FANCD2 being dependent on monoubiquitinated FANCD2, these proteins may cooperate in DNA damage responses. Interestingly, we also find that FANCD2 helicase activity and its ability to bind to

BRCA1 are both involved in the recruitment of FANCD2 to nuclear foci. This is important because this suggests that FANCD2 may link the behavior of monoubiquitinated FANCD2 to BRCA1, which has a role in DNA damage signaling and in coordinating DNA damage responses (Foray et al. 2003; Kitagawa et al. 2004; Lin et al. 2004).

### Regulation of FANCD2 recruitment in response to DNA damage

While it was already known that FANCD2 nuclear foci are induced by IR, we provide quantitative evidence that they are induced by multiple stresses, including MMC and HU. This result is consistent with previous studies suggesting diverse functions for FANCD2, including a role in mediating resistance to both IR and MMC (Litman et al. 2005; Peng et al. 2006), and in unwinding G quadruplex DNA that can impede DNA replication and cause genomic instability (Wu et al. 2008).

Several lines of evidence suggest that FANCD2 foci are assembled at sites of damage. For example, it has been demonstrated previously that FANCD2 foci colocalize with  $\gamma$ -H2AX foci at DSBs in response to IR (Peng et al. 2006). Also, FANCD2 colocalizes with BRCA1, a key protein recruited to sites of DNA damage, in response to DNA damage (Cantor et al. 2001).

Here, we have elucidated key molecular determinants of the recruitment of FANCD2 to DNA damage foci. While it was known that BRCA1 is required for the assembly of FANCD2 foci (Cantor et al. 2001; Gupta et al. 2007), it was unknown whether this is through a direct or indirect mechanism. By observing a defect in FANCD2 foci assembly by the S990A mutant, which is deficient for binding to BRCA1, our results suggest that BRCA1 may directly regulate the recruitment of FANCD2 through a physical interaction. Because phosphorylation of FANCD2 at S990 appears to be mediated by a cyclin-dependent kinase (Yu et al. 2003), a dependence upon the interaction with BRCA1 may dictate that FANCD2 foci assemble spontaneously during S phase, as we observe. Further, FANCD2 is required for DNA repair by HR (Litman et al. 2005). Since HR is restricted to S phase and G2 (Rothkamm et al. 2003), regulation by cell cycle-linked phosphorylation may serve to restrict FANCD2 recruitment and function in HR to these phases. Additionally, the assembly of FANCD2 foci is largely abrogated in the K52R helicase-dead mutant. Perhaps binding to BRCA1 might determine the site of FANCD2 localization, while FANCD2 helicase activity might promote enhanced recruitment of FANCD2 by unwinding DNA proximal to the lesion.

We find that FANCD2 does not colocalize with the replication fork spontaneously in untreated cells and therefore may not play a role in normal DNA replication. But, FANCD2 is strongly recruited to sites of blocked replication. We suggest that this may permit recruitment of FANCD2 to any lesion that impedes or slows replication of DNA, presumably for a function in repairing the lesion. Support for such a role for FANCD2 comes from a recent report that deficiency for FANCD2 in human cells results in genomic deletions of potentially difficult to replicate G/C tracts (London et al. 2008).

Consistent with a role for FANCD2 in responding to replication stress, ATR influences the assembly of FANCD2 foci in response to DNA damage induced by MMC. Since depletion of ATR only partially inhibits DNA damage-induced assembly of FANCD2 foci, it appears that other unknown factors may also be involved. It should be noted that no potential sites of

ATR-dependent phosphorylation on FANCI have been reported. Interestingly, it has recently been reported that FANCI may also have a role in signaling ATR-dependent checkpoints (Gong et al. 2010). Further work will be required to determine how FANCI is both regulated by ATR and can also mediate a critical function of ATR.

### **FANCI is involved in the recruitment of FANCD2 to sites of DNA damage**

Our results suggest a novel function for FANCI in recruiting FANCD2 to damaged chromatin in human cells. This is based upon our observation that FANCI is involved in the normal assembly of FANCD2 foci, both in untreated cells and following exposure to exogenous DNA damage. Deficiency for FANCI in two different FA-J cell lines, or following siRNA-mediated depletion of FANCI in MCF7 cells, compromises the assembly of FANCD2 foci (Figs. 4c and 6b; Supplementary Material, Fig. S5). Also, by measuring colocalization of FANCD2 foci with  $\gamma$ -H2AX foci, a marker of DSBs (Rogakou et al. 1999; Rothkamm and Lobrich 2003), in MCF7 cells depleted of FANCI, we demonstrate that FANCI is involved in the recruitment of FANCD2 to DSBs following exposure to IR (Fig. 6e). Further, FANCD2 foci assemble in chromatin (Montes de Oca et al. 2005), and we have discovered that FANCI promotes the association of monoubiquitinated FANCD2 with chromatin, both in untreated cells and following exposure to MMC (Fig. 5). Interestingly, another DNA damage response factor, XPF-ERCC1, also promotes the association of monoubiquitinated FANCD2 with chromatin (Bhagwat et al. 2009).

The FA nuclear core complex and FANCI are required for FANCD2 monoubiquitination (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a; Sims et al. 2007; Smogorzewska et al. 2007). Thus, a deficiency in any of the eight identified FA nuclear core complex proteins or FANCI completely abrogates the assembly of FANCD2 foci (Garcia-Higuera et al. 2001; Taniguchi et al. 2002a; Sims et al. 2007). In contrast, we find that FA-J cells, which lack FANCI, display a compromised, but partial assembly, of FANCD2 foci. This phenotype appears to distinguish the FA-J complementation group from other FA complementation groups. Unlike FANCI-deficient cells, FANCD2 foci are not compromised in FA-D1 cells, which are deficient for BRCA2 (Wang et al. 2004).

Since FANCI is not required for FANCD2 monoubiquitination, it has been suggested that FANCI functions downstream of FANCD2 monoubiquitination. Our results demonstrate that the assembly of FANCI foci is not dependent upon FANCD2 (Suppl. Fig. S4). Instead, FANCI may act as a co-factor that promotes recruitment of monoubiquitinated FANCD2 to chromatin. In this manner, FANCI may not be absolutely required for the assembly of FANCD2 foci, and this may result in the partial assembly of FANCD2 foci that appears to be characteristic of FA-J cells.

FANCI helicase activity is required for the efficient assembly of monoubiquitinated FANCD2 into DNA damage foci. In this light, it should be noted that we (data not shown), and others (Litman et al. 2005), have not found a physical complex between FANCI and FANCD2. We have examined soluble extracts and chromatin-derived extracts, both from untreated cells and following treatment with MMC. FANCI helicase activity could either unwind DNA to unmask a binding site for monoubiquitinated FANCD2 or could generate a FANCD2 binding site by promoting processing of the damaged DNA. Thus, an interaction

between FANCI and FANCD2 may not be required for the recruitment of monoubiquitinated FANCD2 to damaged chromatin.

The assembly of FANCI foci is largely abrogated by the S990A and K52R mutants of FANCI, which are deficient for binding to BRCA1 and for FANCI helicase activity, respectively (Fig. 2). Importantly, neither of these mutants support the normal assembly of FANCD2 foci either. Thus, the correct localization of FANCI may be important for its function in regulating FANCD2 recruitment. We propose that FANCI has a general role in recruiting FANCD2 to sites of DNA damage, but this function is not required for resistance to MMC. The S990A mutant of FANCI displays normal sensitivity to MMC, unlike deficiency for the entire FANCI protein or other FA proteins (Litman et al. 2005; Peng et al. 2007).

### **FANCI may functionally link BRCA1 to FANCD2**

We find here that BRCA1 and FANCI may function in a pathway involved in regulating the assembly of FANCD2 nuclear foci. This is based upon the results of two different experiments. First, the assembly of FANCD2 foci is compromised in a FA-J cell line expressing the S990A mutant of FANCI (Fig. 4d). Second, siRNA-mediated suppression of FANCI or BRCA1 yields an equivalent decrease in the assembly of FANCD2 foci in MCF7 cells (Fig. 6b). The latter result suggests that FANCI and BRCA1 have equivalent roles in regulating the assembly of FANCD2 foci. Earlier reports, based upon siRNA-mediated suppression of BRCA1 in HeLa cells and the analysis of BRCA1-deficient cancer cell lines (Vandenberg et al. 2003; Burkitt and Ljungman 2007), support the conclusion that BRCA1 is involved in the assembly of FANCD2 foci. Since BRCA1 mediates the assembly of FANCI foci, perhaps BRCA1 functions upstream of FANCI in regulating the assembly of FANCD2 foci in human cells. Together, our results suggest the possibility that FANCI provides a link between BRCA1 and FANCD2. These proteins might cooperate within this network to promote maintenance of genomic stability since each of these proteins is required individually.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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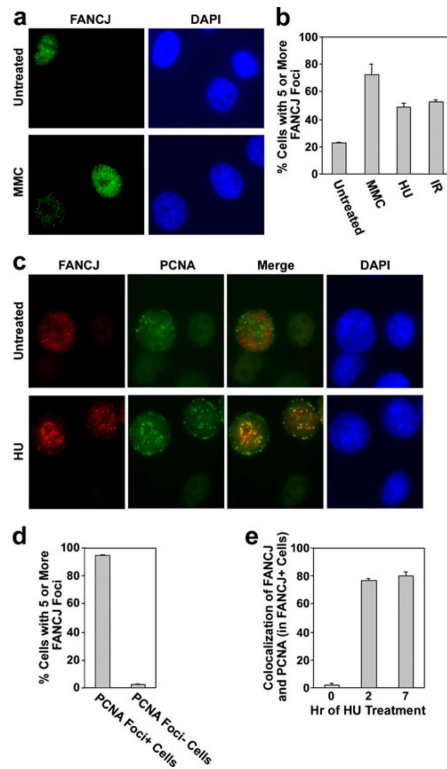
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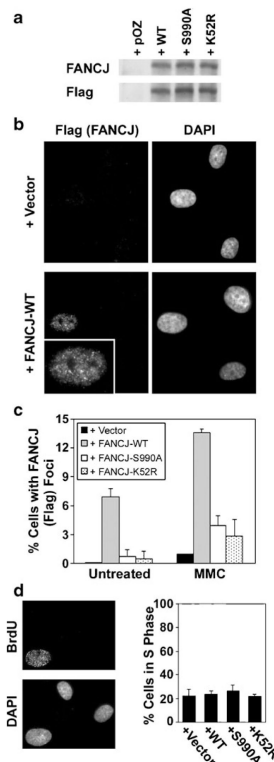


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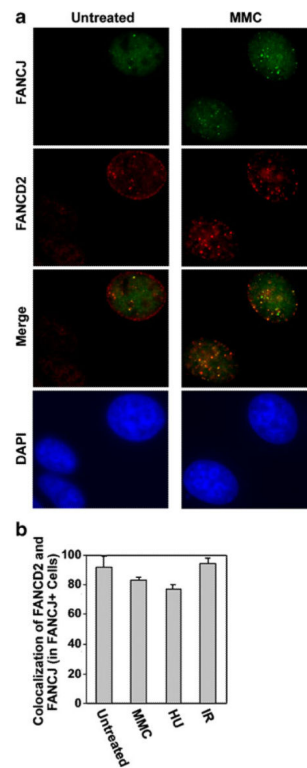
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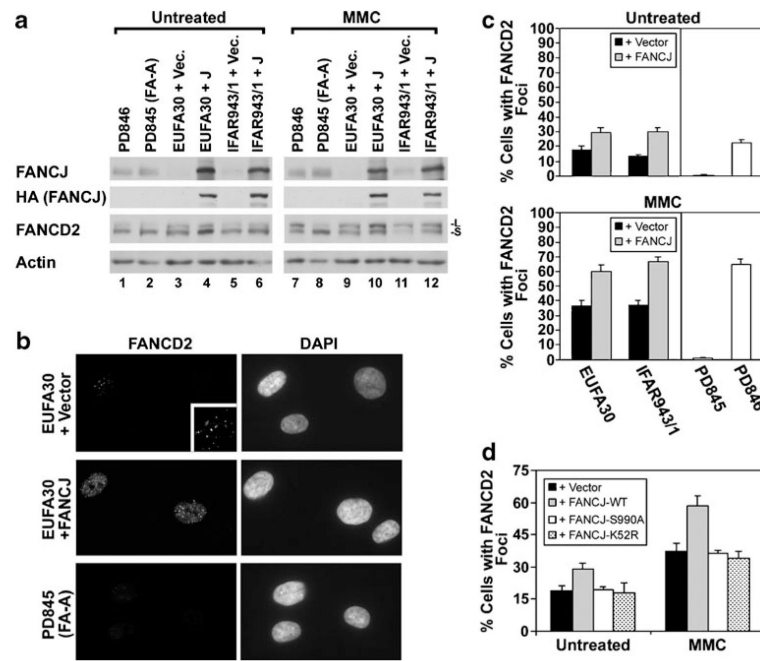
**Fig. 1.** FANCJ nuclear foci assemble spontaneously in S phase cells, are induced by various stresses, and are recruited to blocked replication forks. **a** Examples of FANCJ nuclear foci (*green*) in untreated populations of MCF7 cells or following treatment of cells with 0.5  $\mu$ M MMC for 20 h. Nuclei were stained with DAPI (*blue*) for visualization. **b** Quantification of the percentage of MCF7 cells that displayed five or more FANCJ foci in untreated populations, following exposure to 0.5  $\mu$ M MMC or 2 mM HU for 7 h, or at 7 h following exposure to 5 Gy IR. **c** Examples of FANCJ nuclear foci (*red*) and PCNA foci (*green*) in untreated MCF7 cells or following treatment with 2 mM HU for 20 h. The degree of colocalization is demonstrated in the merged image. **d** The percentage of S phase or non-S phase cells, identified by the presence or absence of nuclear PCNA signal, respectively, that displayed five or more FANCJ foci. **e** The percentage of cells with five or more FANCJ nuclear foci that displayed colocalization with PCNA foci in untreated MCF7 cells, or at 2 or 7 h of treatment with 2 mM HU. PCNA is a marker for the replication fork. Quantitative values represent the average of three counts of 150 or more cells each  $\pm$  standard deviation (**b**, **d–e**). The levels of FANCJ foci, or colocalization of FANCJ and PCNA foci, were significantly increased ( $p < 0.01$ ) following treatment with HU or DNA damage in **b** and **e**, respectively

**Fig. 2.**

The assembly of FANCI nuclear foci involves its capacity to bind to BRCA1 and its helicase activity. **a** The levels of FANCI, detected with antibodies to either FANCI or the Flag epitope tag, in EUFA30 FA-J cells in which wild-type FANCI, or the S990A or K52R mutants of FANCI, were expressed. FANCI mutants were expressed as C-terminal Flag-HA fusion proteins. FANCI-Flag-HA was detected with antibodies to either FANCI or the Flag epitope tag. Cells that contained the empty pOZ vector are shown for comparison. **b** Representative images of epitope-tagged FANCI, detected with an antibody against the Flag peptide, for EUFA30 FA-J cells that contained the pOZ vector alone or which were corrected by expression of FANCI-WT. Cells were exposed to 0.5  $\mu$ M MMC for 20 h. An enlargement of the nucleus, which is positive for FANCI foci, is in the *inset*. **c** Quantification of the assembly of FANCI nuclear foci, detected with an anti-Flag antibody, in EUFA30 cells containing empty vector, wild-type FANCI, or its mutants, both in untreated populations or following exposure to 0.5  $\mu$ M MMC for 20 h. **d** Example of BrdU incorporation in untreated EUFA30 cells corrected with wild-type FANCI is shown (left). Determination of the percentage of S phase cells by measuring BrdU incorporation in untreated populations of EUFA30 cells containing empty vector or each form of FANCI described above (right). For each measurement, three counts of 150 or more cells each were made using a microscope and the average  $\pm$  standard deviation is shown. The levels of FANCI foci were statistically different ( $p < 0.01$ ) in cells corrected with wild-type FANCI, as compared with cells expressing no FANCI or other forms of the protein, both in untreated populations and following exposure to MMC (**c**)



**Fig. 3.** FANCJ and FANCD2 foci partially colocalize in untreated MCF7 cells and following exposure to various types of DNA damage. **a** Examples in which FANCJ (*green*) and FANCD2 nuclear foci (*red*) colocalized in untreated populations of MCF7 cells or following treatment of cells with 0.5  $\mu$ M MMC for 20 h. **b** Quantification of the percentage of MCF7 cells with FANCJ foci that displayed three or more colocalized FANCD2 foci in untreated populations, following exposure to 0.5  $\mu$ M MMC or 2 mM HU for 7 h, or at 7 h following exposure to 5 Gy IR. Results represent the average of three counts of 150 or more cells each  $\pm$  standard deviation

**Fig. 4.**

The assembly of FANCD2 foci is compromised in FA-J cells. **a** Levels of endogenous FANCD2 were detected with the mouse monoclonal 2G7 antibody in tert-immortalized fibroblasts from a normal control individual (*PD846*), a FA-A patient (*PD845*), and two different FA-J patients (*EUFA30* and *IFAR943/1*). The FA-J cells contained vector alone or were corrected by the expression of FANCD2. Expression of FANCD2-Flag-FANCD2 in transduced FA-J cell lines was detected with HA.11 mouse anti-HA antibody. Both monoubiquitinated (-L) and unubiquitinated FANCD2 (-S) were detected with E35 antibody. An equivalent amount of protein was loaded from each sample and actin is shown as a loading control. **b** Examples of FANCD2 foci detected by immunofluorescence microscopy in *EUFA30* (FA-J) cells expressing wild-type FANCD2, or containing only the empty pOZ-C vector, or in *PD845* (FA-A) cells. An enlargement of the FANCD2-positive cell from the *EUFA30*+Vector example (*cell at upper left*) is in the inset for better visualization. Cells were treated with 0.5  $\mu$ M MMC for 20 h and nuclei were detected by counterstaining with DAPI. **c** Quantification of the percentage of *EUFA30* or *IFAR943/1* FA-J cells with FANCD2 foci, either with or without genetic correction. Foci were also quantified in untreated *PD845* and *PD846* cells, or those exposed to 0.5  $\mu$ M MMC for 20 h. **d** Quantification of the percentage of *EUFA30* cells containing empty vector alone, wild-type FANCD2, FANCD2-S990A, or FANCD2-K52R, which displayed FANCD2 nuclear foci, both in untreated populations or following treatment with 0.5  $\mu$ M MMC for 20 h. Values represent the average of three blind counts of 150 or more cells each  $\pm$  standard deviation from unidentified slides (**c-d**). For each particular FAJ cell line levels of FANCD2 foci were statistically different ( $p < 0.04$ ) in cells corrected with wild-type FANCD2, as compared with the absence of FANCD2 or expression of other forms of the protein (**c-d**).





