# Histone chaperone activity of Fanconi anemia proteins, FANCD2 and FANCI, is required for DNA crosslink repair

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**Running title:** Nucleosome dynamics by FANCD2-FANCI

**Abstract**

Fanconi anemia (FA) is a rare hereditary disorder characterized by genomic instability and cancer susceptibility. A key FA protein, FANCD2, is targeted to chromatin with its partner, FANCI, and plays a critical role in DNA crosslink repair. However, the molecular function of chromatin-bound FANCD2-FANCI is still poorly understood. In the present study, we found that FANCD2 possesses nucleosome-assembly activity *in vitro*. The mobility of histone H3 was reduced in FANCD2-knockdown cells following treatment with an interstrand DNA crosslinker, mitomycin C. Furthermore, cells harboring FANCD2 mutations that were defective in nucleosome assembly displayed impaired survival upon cisplatin treatment. Although FANCI by itself lacked nucleosome assembly activity, it significantly stimulated FANCD2-mediated nucleosome assembly. These observations suggest that FANCD2-FANCI may regulate chromatin dynamics during DNA repair.

**Keywords:** DNA repair/ FANCD2/ FANCI / Fanconi anemia/ histone chaperone

## Introduction

Fanconi anemia (FA) is a rare hereditary disorder characterized by skeletal abnormalities, progressive bone marrow failure, and genomic instability accompanied by cancer susceptibility (Venkitaraman, 2004; Niedernhofer *et al*, 2005; Taniguchi and D'Andrea, 2006; Wang, 2007). FA mutant cells are highly sensitive to interstrand DNA crosslinking reagents, which induce stalled replication forks, suggesting that FA proteins promote the stabilization and restarting of the replisome (Thompson *et al*, 2005; Wang, 2007).

Thirteen genes, *FANCA*, *-B*, *-C*, *-D1* (*BRCA2*), *-D2*, *-E*, *-F*, *-G*, *-I*, *-J* (*BRIP1*), *-L*, *-M*, and *-N* (*PALB2*), corresponding to individual FA complementation groups, have been cloned (Thompson *et al*, 2005; Wang, 2007; Kee and D'Andrea, 2010; Garner and Smogorzewska, 2011; Kitao and Takata, 2011). Additionally, homozygous Rad51C mutations have recently been identified in a family with an FA-like disorder, as the *FANCO* gene (Vaz *et al*, 2010), and *SLX4* has been confirmed as the *FANCP* gene (Crossan *et al*, 2011; Kim *et al*, 2011; Stoepker *et al*, 2011). These FA gene products constitute a common DNA damage response pathway that is often referred to as the "FA pathway". In this pathway, eight proteins, FANCA, -B, -C, -E, -F, -G, -L, and -M, and three FANCA-associated polypeptides (FAAPs) form the FA core E3 ligase complex (Garcia-Higuera *et al*, 2001; Wang, 2007; Ali *et al*, 2012; Kim *et al*, 2012; Leung *et al*, 2012). On the other hand, FANCD2 and FANCI associate with each other to form a different complex, called the ID complex (Sims *et al*, 2007; Smogorzewska *et al*, 2007).

Upon DNA damage during S phase, multiple phosphorylations of FANCI trigger the monoubiquitination of FANCD2 and FANCI by the FA core complex (Ishiai *et al*, 2008). The monoubiquitinated ID complex is then targeted to the chromatin, where it plays a critical role in DNA repair pathways, such as homologous recombination and

translesion synthesis (Matsushita *et al*, 2005; Thompson *et al*, 2005; Yamamoto *et al*, 2005; Wang, 2007; Kee and D'Andrea, 2010; Garner and Smogorzewska, 2011; Kitao and Takata, 2011). Recent studies indicated that monoubiquitinated FANCD2 (and FANCI) recruit the FAN1 nuclease, which possesses endo- and exonuclease activities, providing a partial explanation for their roles in DNA repair (Kratz *et al*, 2010; MacKay *et al*, 2010; Smogorzewska *et al*, 2010; Yoshikiyo *et al*, 2010). Monoubiquitinated FANCD2 also reportedly recruits SLX4 (Garner and Smogorzewska, 2011; Yamamoto *et al*, 2011), which is considered to function as a scaffold that interacts with the other nucleases, SLX1, XPF, and MUS81 (Fekairi *et al*, 2009; Svendsen *et al*, 2009; Yamamoto *et al*, 2011). Furthermore, a recent report found that FANCD2 itself might have exonuclease activity (Pace *et al*, 2010). However, whether chromatin-bound FANCD2 and FANCI have any additional functions remains to be determined.

The nucleosome is the fundamental repeating unit of chromatin (Wolffe, 1998). Four core histones, H2A, H2B, H3, and H4, are the protein components of the nucleosome. H2A forms a specific dimer with H2B (H2A/H2B dimer), and H3 forms a specific dimer with H4 (H3/H4 dimer). During nucleosome assembly, two H3/H4 dimers (H3/H4 tetramer) are first deposited on DNA, forming a tetrasome, in which the DNA is wrapped around the H3/H4 tetramer. Two H2A/H2B dimers are then incorporated into the tetrasome to form the mature nucleosome, in which about 150 base pairs of DNA are wrapped around a histone octamer, containing two each of the H2A/H2B and H3/H4 dimers. In cells, nucleosomes are dynamically assembled and disassembled during the replication, transcription, recombination, and repair processes, and such nucleosome dynamics are accomplished with the aid of histone chaperones and/or ATP-dependent chromatin remodeling factors (Avvakumov *et al*, 2011).

In the present study, we purified the human and chicken FANCD2 proteins, and found that FANCD2 possesses nucleosome-assembly activity *in vitro*. We also purified FANCI, and showed that it significantly stimulated FANCD2-mediated nucleosome assembly, although FANCI itself lacked nucleosome assembly activity. A histone-binding domain was mapped in the chicken FANCD2 C-terminal region (residues 1268-1439). The FANCD2 mutants, in which either the histone-binding domain was deleted or the Arg1336 and Lys1346 residues were replaced by Ala, were significantly defective in nucleosome assembly *in vitro*, and cells bearing these mutants displayed impaired survival upon cisplatin treatment *in vivo*. Furthermore, a disease-related mutation, human FANCD2(R302W) (Timmers *et al*, 2001), compromised histone dynamics, and the corresponding chicken FANCD2(R305W) also showed impaired histone chaperone activity. These data suggest that the histone chaperone activity of FANCD2 is crucial for the histone dynamics and the DNA crosslink repair in cells.

## Results

### *Human FANCD2 promotes nucleosome assembly*

In a proteome analysis to search for proteins in HeLa cell extracts that bind to the histone H3/H4 complex (Supplementary Figure S1), we unexpectedly detected human FANCD2 (hFANCD2) as a candidate interacting protein. Indeed, hFANCD2 was efficiently captured from a HeLa cell extract, using H3/H4 beads (Figure 1A). We purified hFANCD2 as a recombinant protein expressed in insect cells (Supplementary Figure S2A), and confirmed that purified hFANCD2 also bound to H3/H4 (Figure 1B). The

hFANCD2-H3/H4 binding was also detected in the presence of DNaseI (Figure 1B, lane 5), indicating that the interaction is not mediated by DNA contamination. These results indicated that hFANCD2 directly binds to H3/H4, which prompted us to examine its nucleosome assembly activity.

We tested hFANCD2-mediated nucleosome assembly by a topological assay, using relaxed circular DNA in the presence of topoisomerase (Figure 1C). The extent of nucleosome formation was assessed by analyzing the superhelicity of circular DNA fractionated through an agarose gel, because negative supercoils are introduced when nucleosomes are formed. As shown in Figure 1C, the number of superhelical turns in the DNA substrate increased with greater amounts of hFANCD2. The faster migration of the DNA substrate was not due to DNA degradation (Supplementary Figure S2B). Therefore, hFANCD2 actually promoted nucleosome assembly *in vitro*. We next performed the nucleosome assembly assay with a short DNA fragment, to directly detect the nucleosomes by an electrophoretic mobility shift assay. hFANCD2 stimulated the nucleosome assembly in this assay (Figure 1D, lanes 6-9). The nucleosome assembly activity of hFANCD2 was slightly lower than that of human Nap1, which is a prominent nucleosome assembly protein (Figure 1D). These biochemical results suggest that FANCD2 may regulate chromatin reorganization during DNA repair in higher eukaryotes.

Chromatin-bound FANCD2 is known to be monoubiquitinated, and the ubiquitin moiety may function to recruit its associated nucleases (Fekairi *et al*, 2009; Svendsen *et al*, 2009; Kratz *et al*, 2010; MacKay *et al*, 2010; Smogorzewska *et al*, 2010;

Yoshikiyo *et al*, 2010; Yamamoto *et al*, 2011). Therefore, we tested whether FANCD2 monoubiquitination affects the nucleosome assembly activity. For this purpose, we utilized the chicken FANCD2 protein (cFANCD2) (Yamamoto *et al*, 2005), which was bacterially expressed and purified to homogeneity (Supplementary Figure S2C). We then prepared monoubiquitinated cFANCD2, using purified components for the conjugation (i.e., FANCL, UBE2T, E1, and ubiquitin; Supplementary Figure S2D-F). As we previously reported, the cFANCD2 monoubiquitination was robustly enhanced in the presence of DNA (Sato *et al*, 2012), and about 40% of cFANCD2 was monoubiquitinated in this study (Supplementary Figure S2F). This monoubiquitinated cFANCD2 fraction was purified, and was subjected to the topological assay. However, we did not find a clear difference in the nucleosome assembly activities between the fractions containing monoubiquitinated cFANCD2 and the monoubiquitination-deficient cFANCD2(K563R) mutant (Supplementary Figure S2G). Therefore, the monoubiquitination does not affect the activity. However, this could be due to the incomplete monoubiquitination of cFANCD2. Therefore, we prepared the monoubiquitination-mimicking version of FANCD2, by genetically fusing FANCD2(K563R) with ubiquitin to create FANCD2(K563R)-Ub (Supplementary Figure S2H), which is known to complement the DNA-repair defective phenotype in the *FANCD2-/-* DT40 cells (Matsushita *et al*, 2005). We found that purified FANCD2(K563R)-Ub possessed similar histone-binding and nucleosome-assembly activities to those of cFANCD2 (Supplementary Figure S2I and J). These results suggested that the FANCD2 monoubiquitination may not be directly involved in the nucleosome assembly.

***The C-terminal region of FANCD2 is responsible for interacting with histone H3/H4 and for promoting nucleosome assembly***

To gain further insights into the molecular mechanism and the functional relevance of the nucleosome assembly activity of FANCD2, we first searched for the FANCD2 region that interacts with the H3/H4 complex. We subjected cFANCD2 to limited proteolysis, and two fragments, cFANCD2(1-1167) and cFANCD2(1-1389), were identified (Figure 2A). These fragments lacked the acidic region, which is composed of the C-terminal 50 amino acid residues of FANCD2. In addition, FANCD2(1268X), which also lacks the C-terminal region, is present in FA patients (Fanconi Anemia Mutation Database, http://www.rockefeller.edu/fanconi/mutate/), suggesting the functional importance of the FANCD2 C-terminal region. As histone chaperones are generally acidic, we investigated whether this acidic C-terminal region is essential for histone binding. The C-terminal deletion mutants, cFANCD2(1-1167) , cFANCD2(1-1267), and cFANCD2(1-1389), were expressed as GFP-tagged forms in HEK293T cells, and their H3/H4 binding activity was examined by a pull-down assay, using the H3/H4 beads. We found that cFANCD2(1-1167) and cFANCD2(1-1267) displayed diminished H3/H4 binding (Figure 2B, lanes 3 and 4). In contrast, cFANCD2(1-1389) retained residual H3/H4 binding activity (Figure 2B, lane 2). Consistently, cFANCD2(1-1167) and cFANCD2(1-1267) showed significant defects in nucleosome assembly (Figure 2C, lanes 13-17 and 18-22, and D; Supplementary Figure S3A). cFANCD2(1-1389) was completely proficient in the nucleosome assembly activity under low protein concentration conditions (Figure 2C,

lanes 8-12, and D; Supplementary Figure S3B). These observations suggest that the C-terminal region of FANCD2 (amino acids 1268-1389) is important for interacting with H3/H4 and for promoting nucleosome assembly. It should be noted that cFANCD2(1-1389) was defective in nucleosome assembly under high protein concentration conditions (Figure 2C, lanes 10-11, and D; Supplementary Figure S3C). This may reflect the biochemical property of cFANCD2(1-1389), which tends to form large protein-DNA aggregates that are unable to enter an agarose gel during electrophoresis (Supplementary Figure S3D).

Since the cFANCD2 C-terminal deletion may induce the improper folding of the cFANCD2 structure, we next performed the H3/H4 binding and nucleosome assembly experiments with cFANCD2 point mutants. Based on the amino acid conservation among the human, mouse, chicken, frog, fish, and fly FANCD2 proteins, we mutated the conserved amino acid residues, which might be functionally important (Figure 2A). We found that the FANCD2(R1336A/K1346A) mutant, in which the Arg1336 and Lys1346 residues are replaced by Ala, was significantly defective in nucleosome assembly *in vitro*, while another mutant, cFANCD2(D1350A/E1365A/E1382A), in which the Asp1350, Glu1365, and Glu1382 residues are replaced by Ala, did not affect the nucleosome assembly activity (Figure 2A, E, and F; Supplementary Figure S3E). Consistently, the histone-binding activity was substantially reduced in cFANCD2(R1336A/K1346A) (Figure 2B lane 11), but not in cFANCD2(D1350A/E1365A/E1382A) (Figure 2B, lane 10). These results strongly support the conclusion that FANCD2 promotes nucleosome assembly through its

C-terminal histone-binding domain.

Finally, we tested the histone binding of the C-terminal cFANCD2 fragments, cFANCD2(669-1439) and cFANCD2(953-1439), which contain the C-terminal amino acid residues 669-1439 and 953-1439, respectively (Figure 2A). These cFANCD2 fragments were identified by a protease mapping experiment. As expected, the cFANCD2(669-1439) and cFANCD2(953-1439) fragments both efficiently bound to histones (Figure 2B, lanes 5 and 6). Surprisingly, cFANCD2(953-1439), which contained only one-third of cFANCD2, promoted nucleosome assembly (Figure 2C, lanes 23-27, and D). Therefore, we concluded that the histone-binding domain is located in the C-terminal region of FANCD2.

***FANCD2 mediates histone mobilization in living cells in a DNA damage-dependent manner***

To determine whether FANCD2 plays a role in histone dynamics in living cells during DNA repair, we knocked down hFANCD2 in HeLa cells expressing histone H3-GFP (Kimura and Cook, 2001), using small inhibitory RNA (siRNA). Three days after the transfection of the specific siRNA, the level of hFANCD2 had decreased substantially, to <10% of the normal level (Supplementary Figure S4A and B). Using these cells, the mobility of H3 was analyzed by fluorescence recovery after photobleaching (FRAP) (Kimura *et al*, 2006). The recovery kinetics (the curve shapes) of the exchanging fractions were similar in the hFANCD2-knockdown and control cells (Figure 3A), suggesting that hFANCD2 does not play a major role in H3 assembly or exchange under

normal conditions. As FA mutant cells display significant sensitivity to interstrand DNA crosslinking reagents, such as mitomycin C (MMC) (Niedernhofer *et al*, 2005; Thompson *et al*, 2005; Wang, 2007; Kee and D'Andrea, 2010; Garner and Smogorzewska, 2011; Kitao and Takata, 2011), we next tested the effect of MMC on the H3 mobility in the hFANCD2 knockdown HeLa cells. Interestingly, the recovery of H3-GFP in the hFANCD2 knockdown cells was clearly slower in the presence of MMC (Figure 3B). Similar results were obtained with a different FANCD2-specific siRNA (Supplementary Figure S4C). The slower H3-GFP exchange observed in the MMC-treated FANCD2-knockdown cells could be due to a different cell cycle distribution, since the FA deficient cells may be arrested at S and/or G2 due to the deficiency of DNA crosslink repair. We therefore performed FRAP experiments in cells stably expressing both H3-GFP and mCherry-tagged PCNA, which shows characteristic patterns representing replication and repair foci (Leonhardt *et al*, 2000). We first examined the mobility of H3-GFP in different cell cycle stages under the normal growth conditions. The H3-GFP mobility in PCNA foci-positive (S-phase) cells did not differ from that in PCNA foci-negative cells (Figure 3C; Supplementary Figure S4D). In the presence of MMC, the PCNA foci-positive cells were indeed enriched by the hFANCD2 knockdown, but the mobility of H3-GFP was still slower than that in the MMC-treated PCNA foci-positive control cells (Figure 3D; Supplementary Figure S4D). Therefore, the reduced histone H3 mobility in the MMC-treated hFANCD2 knockdown cells does not appear to be attributable to a difference in the cell cycle phase. Furthermore, the slower H3-GFP recovery in the presence of MMC was also detected in the hFANCA knockdown cells, in

which the damage-dependent focus formation of hFANCD2 on chromosomes was significantly inhibited (Supplementary Figure S4E-H). These data suggest that hFANCD2 may mediate nucleosome assembly and/or histone exchange in human cells, in a damage-dependent manner.

***The C terminal histone-binding region of FANCD2 is important for the DNA repair mediated by the FA pathway***

To determine whether the histone assembly activity levels of the FANCD2 mutants *in vitro* correlate with their DNA repair activities *in vivo*, we expressed them in *FANCD2-/-* DT40 cells, and exposed the cells to cisplatin in a colony survival assay. As shown in Figure 4A, full length cFANCD2, cFANCD2(1-1389), and cFANCD2(D1350A/E1365A/E1382A) rescued the cisplatin-sensitive phenotype of the *cFANCD2-/-* cells, in contrast to cFANCD2(1-1167), cFANCD2(1-1267), and cFANCD2(R1336A/K1346A). To ensure the chromatin targeting of these cFANCD2 mutants, we repeated this assay with the cFANCD2 mutants expressed as fusions with histone H2B, since cFANCD2(1-1167), cFANCD2(1-1267), and cFANCD2(R1336A/K1346A) were not monoubiquitinated (Figure 4B), probably due to the weakened interaction with FANCL, which was identified as the catalytic E3 subunit for FANCD2 monoubiquitination (Meetei et al, 2003) (Supplementary Figure S5A). We confirmed that the fusion of H2B to cFANCD2 and cFANCD2(1-1267) did not affect their nucleosome assembly activities (Supplementary Figure S5B-E). Although substantial amounts of cFANCD2(1-1167)-H2B, cFANCD2(1-1267)-H2B, and

cFANCD2(R1336A/K1346A)-H2B were detected at the chromatin (Figure 4D), the mutants still could not complement the cisplatin sensitivity of *cFANCD2-/-* cells, in contrast to the H2B fusion proteins with the full-length cFANCD2 and cFANCD2(K563R), bearing a point mutation at the monoubiquitination site (K563R) (Figure 4C) (Matsushita *et al*, 2005). These results strongly suggest that the nucleosome assembly activity, which depends on the C-terminal region of FANCD2, might be crucial for the DNA repair mediated by the FA pathway. Notably, these cFANCD2 mutants were able to interact with the cFANCI protein in chromatin, as detected by anti-GFP immunoprecipitation followed by western blotting (Figure 4D), and as further supported by the results of *in vitro* binding experiments (Supplementary Figure S7F-H).

***A disease-related FA mutant, human FANCD2(R302W), and its chicken counterpart, cFANCD2(R305W), are monoubiquitinated but do not facilitate histone exchange***

As shown in Figure 4B, the C-terminally deleted or point FANCD2 mutants, which were defective in nucleosome assembly, were also defective in monoubiquitination, probably due to their weakened interactions with FANCL (Supplementary Fig. S5A). This suggested that the histone-binding and FANCL-binding regions may partially overlap. To provide evidence that the FANCD2 histone-chaperone activity functions in DNA repair independently of its monoubiquitination, a FANCD2 mutant, in which the histone-chaperone and monoubiquitination activities are separated, would be useful. We reasoned that such mutations might be found outside the putative histone-binding site. We found that the chicken FANCD2 R305W mutation (Arg305 to Trp), which

corresponds to a disease-related mutation (human FANCD2 R302W) in Fanconi anemia (Timmers *et al*, 2001), is such a separation mutation. cFANCD2(R305W) exhibited reduced histone binding (Figure 2B, lane 8), and was defective in the nucleosome assembly activity (Figure 5A and B). In contrast, cFANCD2(R305W) was proficient in its monoubiquitination (Figure 5C) and chromatin targeting activities (Supplementary Figure S6A). Interestingly, cFANCD2(R305W) was moderately defective in the repair of DNA damage induced by cisplatin (Figure 5D), although its binding to cFANCI was proficient *in vivo* (Supplementary Figure S6A) and *in vitro* (Supplementary Figure S7E). The DNA repair deficiency was also observed when H2B-fused cFANCD2(R305W) was expressed in the cFANCD2-/- cells (Figure 5E). These results indicated that the DNA-repair defect observed in the cFANCD2-/- cells expressing cFANCD2(R305W) may be accounted for by the defective histone chaperone activity of cFANCD2(R305W). Therefore, the histone chaperone activity of FANCD2 may be required in the steps after the FANCD2 monoubiquitination and chromatin targeting, during DNA repair by the FA pathway (Figure 8B).

To test whether the histone chaperone activity of FANCD2 functions in histone dynamics in living cells, we expressed mCherry-tagged hFANCD2, hFANCD2(R302W), or hFANCD2(K561R) in H3-GFP-expressing HeLa cells, in which the endogenous hFANCD2 was knocked down by siRNA (Supplementary Figure S6B). We compared the H3-GFP mobility between mCherry-positive and -negative cells. The mobility of H3-GFP was efficiently restored with the expression of wild-type hFANCD2 (Figure 6A), but not with the monoubiquitination-deficient hFANCD2(K561R) mutant (Figure 6B,

top panel), consistent with its chromatin targeting deficiency (diffusing within the nucleus; Figure 6B, bottom panels). Interestingly, hFANCD2(R302W) also failed to restore the H3-GFP mobility (Figure 6C, top panel), although its chromatin targeting was not defective (forming nuclear foci like the wild-type hFANCD2; Figure 6C, bottom panels). Together with the DNA-repair deficiency of the cFANCD2-/- cells expressing cFANCD2(R305W), these results support the view that the histone-chaperone activity of FANCD2 is important in the DNA repair by the FA pathway.

***FANCI stimulates nucleosome assembly by FANCD2***

Finally, we examined the involvement of FANCI in nucleosome assembly, using purified chicken FANCI (cFANCI) (Figure S7A and B), which could form a stable complex with cFANCD2 (Figure 7A). Although FANCI shares significant homology with FANCD2 (Sims *et al*, 2007; Smogorzewska *et al*, 2007), purified cFANCI alone did not efficiently promote nucleosome assembly (Figure 7B). However, cFANCI clearly stimulated the cFANCD2-mediated nucleosome assembly at low cFANCD2 concentrations, at which cFANCD2 itself did not promote detectable levels of nucleosome assembly (Figure 7C and D). cFANCI did not form a complex with the major histone chaperone Nap1, and did not stimulate its nucleosome assembly (Supplementary Figures 7C and D), suggesting that it may specifically stimulate the cFANCD2-mediated nucleosome assembly. In addition, cFANCI stimulated the nucleosome assembly activity of cFANCD2(1-1389), which retained H3/H4 binding ability, but not that of cFANCD2(1-1267) (Supplementary Figure S7I). Therefore, the ID complex may facilitate nucleosome reorganization during

DNA repair.

**Discussion**

In the present study, we found that the purified FANCD2 protein promotes nucleosome assembly *in vitro*, and that its partner, FANCI, significantly stimulates this activity. We also discovered that the C-terminal region of FANCD2 is critical for the nucleosome assembly activity, and showed that the FANCD2 deletion and point mutants are defective in both nucleosome assembly *in vitro* and tolerance to cisplatin treatment *in vivo*. These findings provide novel insights into the function of the ID complex in chromatin.

The crystal structure of the mouse ID complex revealed that FANCD2 is composed of seven subdomains, solenoid 1 (S1), helical domain 1 (HD1), solenoid 2 (S2), helical domain 2 (HD2), solenoid 3 (S3), solenoid 4 (S4), and the C-terminal acidic region (Joo *et al*. 2011). In the present study, we mapped the histone-binding region within the S4 domain of FANCD2. The FANCD2 S4 domain is separate from the FANCI-binding surface, and is largely accessible (Joo *et al*, 2011). In the S4 domain, the cFANCD2 Arg1336 and Lys1346 residues are perfectly conserved among the human, mouse, chicken, frog, fish, and fly FANCD2 proteins (Figure 2A), and are located on the concave surface (Figure 8A; Joo *et al*, 2011). Our mutational analysis revealed that the cFANCD2 Arg1336 and Lys1346 residues are essential for nucleosome assembly *in vitro* and cisplatin resistance *in vivo*. In the crystal structure, the side chains of the mouse FANCD2 Arg1334 and Lys1344 residues, corresponding to the cFANCD2 Arg1336 and

Lys1346 residues, respectively, are completely exposed to the solvent on the concave surface (Figure 8A). Therefore, the concave surface of the FANCD2 S4 domain may be its histone-binding region.

In cFANCD2, Arg1336 and Lys1346 are positively charged residues, which are considered to be less important for the histone binding of the acidic histone chaperone, Asf1 (English *et al*, 2006; Natsume *et al*, 2007). However, the involvement of positively charged residues in histone binding has been reported in the histone chaperone HJURP, which is the specific chaperone for the histone H3 variant, CENP-A (Dunleavy *et al*, 2009; Foltz *et al*, 2009). In the complex of HJURP with histone H4 and CENP-A, polar interactions exist between the negatively charged Glu96 of CENP-A and the two positively charged Arg32 and Lys39 residues of HJURP, and also between Glu107 of CENP-A and Arg28 of HJURP (Hu *et al*, 2011). Interestingly, the Glu96 and Glu107 residues of CENP-A are both conserved in histone H3 (Tachiwana *et al*, 2011a). Therefore, the histone-binding mechanism of FANCD2 may be similar to that of HJURP.

In the present study, we found that cFANCD2(R305W) was clearly defective in reversing the cisplatin sensitivity of the FANCD2-/- DT40 cells, but was proficient in its monoubiquitination *in vivo*. Interestingly, cFANCD2(R305W) was significantly defective in both the histone binding and nucleosome assembly activities. In addition, the corresponding human FANCD2(R302W) mutant could not rescue the decreased histone H3 mobility in the FANCD2-knockdown cells. These findings indicate that the histone-chaperone activity of FANCD2 is actually involved in the DNA crosslink repair by the FA pathway, and strongly suggest that FANCD2 regulates nucleosome dynamics

after its monoubiquitination. The C-terminal cFANCD2 mutants tested in this study showed more severe cisplatin sensitivity than cFANCD2(R305W), even when localized in the chromatin by the H2B fusion. The residual histone-chaperone activity of cFANCD2(R305W), which was barely detectable in the present nucleosome formation assay, may partially complement the cisplatin sensitivity of the cFANCD2-/- cells. In the crystal structure of mouse FANCD2, the side chain of the corresponding Arg300 directly hydrogen bonds with Asp379 (Joo *et al*, 2011), suggesting that the Arg300-Asp379 interaction may be important in the tertiary structure of the protein. Therefore, the Arg to Trp substitution found in cFANCD2(R305W) or hFANCD2(R302W) may cause a large structural change, which may allosterically impair the histone binding activity of FANCD2.

FANCI-FANCD2 is reportedly required for nucleolytic incisions in a replicating plasmid carrying an interstrand crosslink in a frog egg extract (Knipscheer *et al*, 2009). One can envision that the ID complex-mediated chromatin remodeling may regulate the incision step by promoting the recruitment of nucleases (Figure 8B, I) (e.g. FAN1, Mus81, and XPF) (Nomura *et al*, 2007; Bhagwat *et al*, 2009; Fekairi *et al*, 2009; Svendsen *et al*, 2009; Hicks *et al*, 2010; Kratz *et al*, 2010; MacKay *et al*, 2010; Smogorzewska *et al*, 2010; Yoshikiyo *et al*, 2010; Yamamoto *et al*, 2011). In this step, the proper incision by the repair nucleases may occur on the single-stranded DNA region (Figure 8B, I, arrowhead), while the improper digestion of intact DNA strands may be restricted by the nucleosomes formed by the ID complex (Figure 8B, I, dashed line). Secondly, in the FA pathway, homologous recombination repair may be promoted after FANCD2 assembly

into chromatin. The nucleosome remodeling by the ID complex may facilitate the DNA recombination reaction mediated by RAD51, which is the key protein for homologous recombinational repair (Figure 8B, II). Finally, the histone assembly activity of the ID complex may simply be important for chromatin reformation, following crosslink removal and DNA repair (Figure 8B, III). In addition to the direct interaction of the ID complex with histones, presented here, several chromatin modifiers have been suggested to interact with FA proteins. For example, a histone acetyltransferase, TIP60, reportedly interacts with FANCD2 (Hejna *et al*, 2008). Thus, the function of FANCD2 may be tightly coupled with those of other chromatin remodeling factors in chromatin dynamics, to facilitate DNA crosslink repair.

## Materials and Methods

### *Purifications of FANCD2, FANCI, FANCL, UBE2T, and Nap1*

Experimental procedures are described in Supplementary Materials and Methods. The protein concentration was determined by the Bradford method (Bradford 1976), using bovine serum albumin as the standard protein.

### *Purification of recombinant human H3/H4 and H2A/H2B complexes*

Human histones H2A, H2B, H3, and H4 were overexpressed in *Escherichia coli* cells as $His_6$-tagged proteins, as described (Tanaka *et al*, 2004; Tachiwana *et al*, 2008). The purification and preparation of the histone H3/H4 and H2A/H2B complexes were

performed as previously described (Tachiwana *et al*, 2010; 2011b). The $His_6$-tag was removed during the purification steps.

***Proteome analysis***

The purified recombinant human histone H3/H4 complex was covalently conjugated with Affi-Gel 10 beads (Bio-Rad), according to the manufacturer's protocol. Briefly, the histone H3/H4 beads were incubated with a chromatin extract from HeLa cells at 4°C. The beads were washed with 10 mM PIPES buffer (pH 7.0), containing 1 mM $MgCl_2$, 1 mM EDTA, 0.01% Triton X-100, 300 mM sucrose, and 0.1 M NaCl. The proteins bound to the beads were fractionated by SDS-PAGE. Each lane was cut into nine pieces, and was further treated with trypsin. To identify the peptide fragments, the samples were analyzed by liquid chromatography/tandem mass spectrometry, as previously described (Nozawa *et al*, 2010). The raw data files were analyzed by the Mascot software (Matrix Science).

***Pull-down assay with histone-conjugated beads***

The histone H3/H4 complex was covalently conjugated with Affi-Gel 10 beads (Bio-Rad), according to the manufacturer's protocol. The histone H3/H4 beads were incubated with a HeLa whole cell extract (3 mg of protein), and the beads were washed three times with 100 μL of washing buffer, containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF and Protease Inhibitor Cocktail (Nacalai Tesque). The hFANCD2 that copelleted with the histone H3/H4 beads was fractionated by 8% SDS-PAGE, and was detected by western blotting with the

hFANCD2-specific mouse monoclonal antibody (FI17, Santa Cruz Biotechnology, Inc.).

For the recombinant hFANCD2-binding assay, the histone H3/H4 beads (15 μL) were incubated with purified hFANCD2, with or without 10 U of DNaseI (TOYOBO), at 23°C for 120 min. The beads were then washed three times with 100 μL of 10 mM PIPES-KOH buffer (pH 7.0), containing 0.5 M NaCl, 0.3 M sucrose, 1 mM $MgCl_2$, and 0.01% Triton X-100. The proteins bound to the beads were separated by 15% SDS-PAGE, and were visualized by Coomassie Brilliant Blue staining.

For the cFANCD2-binding assay, 293T cells were transiently transfected with GFP-cFANCD2 mutant plasmids, using Lipofectamine2000 (Invitrogen). Cells were disrupted in lysis buffer (Yamamoto *et al*, 2005), and after the lysate was incubated with the histone H3/H4 beads (7.5 μL) at 4°C for 3 hr, the beads were washed four times with lysis buffer. The proteins were separated by 6% SDS-PAGE, and were detected by western blotting with an anti-GFP Ab (Clontech).

***Topological assay for nucleosome formation***

Experimental procedures are described in Supplementary Materials and Methods.

***Gel shift assay for nucleosome formation***

The H2A/H2B complex (8 ng/μL) and the H3/H4 complex (8 ng/μL) were pre-incubated with hFANCD2 or hNap1 at 23°C for 10 min. The nucleosome assembly reaction was initiated by the addition of the 195 bp DNA (8 ng/μL) containing the *Lytechinus variegates* 5S ribosomal RNA gene (Osakabe *et al*, 2010) in a 10 μL reaction mixture,

containing 20 mM Tris-HCl (pH 8.0), 140 mM NaCl, 7% glycerol, and 1 mM dithiothreitol. The reaction was continued at 23°C for 60 min, followed by a further incubation at 42°C for 60 min to eliminate the non-specific DNA binding by free histones. The samples were then separated by 6% PAGE in 0.2x TBE buffer (18 mM Tris base, 18 mM boric acid, and 0.4 mM EDTA). DNA was visualized by ethidium bromide staining.

***Pull-down assay with GST-cFANCL and cFANCD2***

Purified cFANCD2 (6 μg) and GST-cFANCL (5 μg) were incubated at 30°C for 60 min in a 100 μL reaction mixture, containing 20 mM Tris-HCl (pH 8.0), 10% glycerol, 200 mM NaCl, and 5 mM 2-mercaptoethanol. Glutathione Sepharose 4B beads (5 μL) were added to the reaction mixtures, which were gently mixed at 4°C for 60 min. The beads were then washed three times with 1 mL of wash buffer, containing 20 mM Tris-HCl (pH 8.0), 0.4 M NaCl, 10% glycerol, 5 mM 2-mercaptoethanol, and 0.05% Triton X-100. The proteins bound to the beads were separated by 7% SDS-PAGE, and were visualized by Coomassie Brilliant Blue staining.

***In vitro FANCD2 monoubiquitination and purification of monoubiquitinated FANCD2***

Experimental procedures are described in Supplementary Materials and Methods.

***Gel-shift assay***

Circular ϕX174 dsDNA (100 ng) was mixed with cFANCD2 (0.45-1.8 μM) or cFANCD2(1-1389) (0.45-1.8 μM) in 10 μL of reaction buffer, containing 22 mM Tris-HCl (pH 8.0), 70 mM NaCl, 7% glycerol, 2 mM $MgCl_2$, and 2.5 mM dithiothreitol. The samples were incubated at 37°C for 15 min, and were then analyzed by 0.8% agarose gel electrophoresis in TAE buffer. DNA was visualized by ethidium bromide staining.

***siRNA transfection, immunoblotting, and fluorescence recovery after photobleaching (FRAP)***

The FANCD2-specific Stealth RNAs (#1, HSS103525, AAUGAACGCUCUUUAGCAGACAUGG, for Figures 3 and 6, and Supplementary Figure S3; and #2, HSS103527, AAUAGACGACAACUUAUCCAUCACC, for Supplementary Figure S3; Invitrogen), a FANCA-specific Stealth RNA (AAGGGUCAAGAGGGAAAAAUA, for Supplementary Figure S4; Invitrogen), and the control RNA (Negative Control Medium; Invitrogen) were introduced into HeLa cells expressing histone H3-GFP by LipofectAMINE2000 (Invitrogen), as described (Kimura *et al*, 2006). Total cellular proteins were prepared 0-4 days after the RNA introduction, separated by 8% SDS-PAGE, and immunoblotted with a mouse monoclonal antibody directed against either FANCD2 (1:250; FI17, Santa Cruz Biotechnology, Inc.), α-tubulin (1:1000; Sigma) as a loading control, or an antibody against FANCA (1:1000; rabbit polyclonal, Bethyl Laboratories, Inc.). Secondary detection was performed with a sheep anti-mouse IgG, horseradish peroxidase linked species-specific $F(ab')_2$ fragment (GE Healthcare; 1:500 for FANCD2, 1:1000 for α-tubulin) or a sheep anti-rabbit IgG,

horseradish peroxidase linked species-specific $F(ab')_2$ fragment (GE Healthcare; 1:1000 for FANCA). The signals were detected by chemiluminescence (Western Lightning Plus; Perkin Elmer).

To generate HeLa cells expressing both H3-GFP and mCherry-PCNA, cells expressing H3-GFP (blasticidin resistance; Kimura and Cook, 2001) and mCherry-PCNA (puromycin-resistance; generated using pMX-puro-based expression vector carrying mCherry-PCNA; Leonhardt *et al*, 2000; Kitamura *et al*, 2003) were fused using polyethylene glycol (Roche; Kimura and Cook, 2001) and single colonies were selected in the presence of 1 μg/ml blasticidin and 0.5 μg/ml puromycin.

FRAP was performed 3-4 days after siRNA transfection as described (Kimura *et al*, 2006), using a confocal microscope (FV-1000; Olympus) with a 60x UPlanSApo NA = 1.35 lens. Three confocal images of a field containing 4-10 nuclei were collected (800 × 800 pixels, zoom 3, scan speed 2 μs/pixel, pinhole 800 μm, Kalman filtration for 4 scans, LP505 emission filter, and 0.1% transmission of 488-nm Ar laser), one half of each nucleus was bleached using 75% transmission of 488 nm and 100% of 514 nm (2 iterations), and images were obtained using the original setting at 1 min intervals.

For the complementation experiments, RNAi-resistant *hFANCD2* genes were constructed by introducing silent mutations in the FANCD2-specific Stealth RNA (#1) target sequence (5'-TCT GCT AAA GAG-3'), as follows: 5'-TCC GCC AAG GAA-3'. HeLa cells expressing histone H3-GFP were transfected with the FANCD2-specific Stealth RNAs (#1) to knockdown the endogenous hFANCD2. The next day, the cells were transfected with the RNAi-resistant mCherry-*hFANCD2*,

mCherry-*hFANCD2(R302W)*, or mCherry-*hFANCD2(K561R)* gene, using the FuGENE system (Promega), and were further grown for 2 days before FRAP. After FRAP, the glass-bottom dish was processed for immunofluorescence.

The fluorescence intensity of the bleached area was measured using Image J 1.39u (W. Rasband; http://rsb.info.nih.gov/ij/). After subtracting the background, the intensity was normalized to the initial intensity before bleaching.

***Immunofluorescence***

For immunofluorescence, HeLa cells expressing histone H3-GFP were grown on a glass-bottom dish (Mat-tek), transfected with Stealth RNA, and fixed with 4% paraformaldehyde at 3 days after transfection. The fixed cells were permeabilized and stained using mouse anti-FANCD2 (1:250) and goat Cy3-conjugated anti-mouse IgG (1:500; Jackson Immunoresearch). When mCherry-*hFANCD2* was transfected, goat Cy5-conjugated anti-mouse IgG (1:500; Jackson Immunoresearch) was used as the secondary antibody. For double staining with FANCA, rabbit anti-FANCA (1:100; Bethyl Laboratories, Inc.) and goat Cy5-conjugated anti-rabbit IgG (1:500; Jackson Immunoresearch) are also used. DNA was counterstained with DAPI. The fluorescence images were obtained using a confocal microscope (Olympus FV-1000 with a 60x UPlanSApo NA = 1.35 oil-immersion objective lens; or Carl Zeiss LSM510 with a 40x C-Apo NA = 1.2 water-immersion objective lens).

***Generation of the FANCD2-/- DT40 cells producing the FANCD2 mutants***

Plasmids for the cFANCD2 mutant fusions were constructed in the pcDNA3.1-based GFP or histone H2B-GFP expression vector, by inserting FANCD2 fragments using the Gateway system (Invitrogen). To obtain stably expressing clones, these plasmids were transfected into *FANCD2*-deficient DT40 cells (Yamamoto *et al*, 2005), and the clones expressing the fusions were identified by measuring the GFP fluorescence with a FACSCalibur (Becton Dickinson), as described (Ishiai *et al*, 2004; Yamamoto *et al*, 2005).

***Cisplatin sensitivity assay***

Sensitivity to cisplatin (Nihon-Kayaku) was assayed by colony formation, in medium containing 1.4% methylcellulose and the indicated dosage of cisplatin (Ishiai *et al*, 2004; Yamamoto *et al*, 2005).

***Cell fractionation and detection of FANCD2/FANCI proteins***

The indicated cells were either treated with MMC (500 ng/ml for 6 hr) or left untreated, and were fractionated into soluble and chromatin fractions, as described previously (Matsushita *et al*, 2005; Ishiai *et al*, 2008). Pull-down assays of cFANCD2-H2B-GFP proteins were performed using anti-GFP beads (MBL). The proteins were separated by 6% SDS-PAGE, and were detected by western blotting. The anti-chicken FANCD2 and FANCI antibodies were obtained by immunizing rabbits with the bacterially expressed His fusion protein with full-length chicken FANCD2 and the GST fusion protein with chicken FANCI (amino acids 1-251), respectively.

### *Gel filtration analysis*

The purified cFANCD2 and cFANCI proteins were analyzed by Superdex 200 HR 10/30 (GE Healthcare) gel filtration chromatography. The elution buffer contained 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10% glycerol and 1 mM DTT.


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## Author contributions

K.S., K.T., S.F., Y.T., A.O., H.T., and W.K. purified FANCD2, FANCI, and histones, and K.S. and K.T. performed biochemical analyses. M.I., H.Kitao, and M.T. performed genetic and cell biological analyses. K.S. and H.Kimura performed FRAP analyses. K.S.

and N.D. performed domain analysis of FANCD2 by mass spectroscopy. A.O., H.T., and C.O. performed proteome analysis of histone binding proteins. H. Kurumizaka and M.T. conceived, designed, and supervised all of the work, and K.S. and H. Kurumizaka wrote the paper. All of the authors discussed the results and commented on the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Figure Legends**

**Figure 1** hFANCD2 promotes nucleosome assembly. (**A**) The H3/H4-conjugated beads were incubated with a HeLa whole cell extract (WCE), and the endogenous FANCD2 bound to the beads was detected by western blotting with an anti-FANCD2 monoclonal antibody (α-FANCD2). N and H indicate the control Affi-Gel 10 beads and the H3/H4-conjugated beads, respectively. Input WCE (30 μg of protein) and hFANCD2 (125 ng) were applied in lanes 4 and 5, respectively. (**B**) The H3/H4 beads were incubated with purified hFANCD2 in the absence or presence of DNaseI, washed with buffer, and mixed with 2-fold SDS sample buffer. The proteins bound to the beads were analyzed by 15% SDS-PAGE. (**C**) Topological assay. A schematic diagram of topological assay is shown in the left panel. Nucleosomes were reconstituted on the relaxed plasmid DNA by hFANCD2 (0.2, 0.5, and 0.9 μM), in the presence of wheat germ topoisomerase I. After deproteinization, the topoisomers were separated by agarose gel electrophoresis. Highly supercoiled and relaxed DNAs are denoted as “sc” and “relaxed”, respectively. (D) Nucleosome assembly assay. A schematic diagram of the nucleosome assembly assay is shown in the left panel. Nucleosomes were reconstituted on the linear 195 base-pair DNA by hNap1 (0.4, 0.8, and 1.6 μM) or hFANCD2 (0.2, 0.4, and 0.8 μM). Nucleosomes positioned at the edge and center of the 195 bp DNA are indicated by cartoons on the right side of the panel.

**Figure 2** The C-terminal region of FANCD2 is responsible for histone binding and nucleosome assembly. (**A**) Schematic representations of full-length cFANCD2, and the

cFANCD2(1-1389), cFANCD2(1-1267), cFANCD2(1-1167), cFANCD2(669-1439), and cFANCD2(953-1439) deletion mutants. The cFANCD2 domains, solenoid 1, helical domain 1, solenoid 2, helical domain 2, solenoid 3, and solenoid 4, are denoted as S1, HD1, S2, HD2, S3, and S4, respectively (Joo *et al*. 2011). The FANCD2 C-terminal acidic region is colored red. The amino acid sequences of the C-terminal regions of the *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus laevis*, *Danio rerio*, and *Drosophila melanogaster* FANCD2 proteins are aligned. The highly conserved residues are colored red. The mutated residues in cFANCD2(R1336A/K1346A) and cFANCD2(D1350A/E1365A/E1382A) are indicated by orange and purple arrowheads, respectively. (**B**) The H3/H4 beads were incubated with extracts of HEK293T cells, producing either GFP-tagged cFANCD2, cFANCD2(1-1389), cFANCD2(1-1267), cFANCD2(1-1167), cFANCD2(669-1439), cFANCD2(953-1439), cFANCD2(R305W), cFANCD2(K563R), cFANCD2(D1350A/E1365A/E1382A), or cFANCD2(R1336A/K1346A). Proteins bound to the beads were detected by western blotting with an anti-chFANCD2 (polyclonal) antibody. The bottom panel indicates negative control experiments with beads lacking histones. (**C**) Nucleosomes were reconstituted on the relaxed plasmid DNA by cFANCD2 (lanes 3-7), cFANCD2(1-1389) (lanes 8-12), cFANCD2(1-1267) (lanes 13-17), cFANCD2(1-1167) (lanes 18-22), and cFANCD2(953-1439) (lanes 23-27) in the presence of wheat germ topoisomerase I. After deproteinization, the topoisomers were separated by agarose gel electrophoresis with ethidium bromide staining. The cFANCD2 concentrations were 0, 0.45, 0.90, and 1.8 μM. Highly supercoiled and relaxed DNAs are denoted as “sc” and “relaxed”, respectively.

(**D**) Graphic representation of nucleosome assembly activities of the cFANCD2 mutants shown in panel C. Representative images are shown in panel C. The supercoiled DNA fractions were generated by nucleosome assembly in the presence of cFANCD2, and the intensities of the bands indicated by the arrows in panel C were quantitated by an LAS-4000 Image Analyzer (GE Healthcare). Means of three independent experiments are shown with standard deviations. (**E**) Nucleosomes were reconstituted on the relaxed plasmid DNA by cFANCD2 (lanes 3-7), cFANCD2(R1336A/K1346A) (lanes 8-12), and cFANCD2(D1350A/E1365A/E1382A) (lanes 13-17) in the presence of wheat germ topoisomerase I. The cFANCD2 concentrations were 0, 0.45, 0.90, and 1.8 μM. Highly supercoiled and relaxed DNAs are denoted as "sc" and "relaxed", respectively. (**F**) Graphic representation of the nucleosome assembly activities of the cFANCD2 mutants shown in panel E. Representative images are shown in panel E. The supercoiled DNA fractions were generated by nucleosome assembly in the presence of cFANCD2, and the intensities of the bands indicated by the arrows in panel E were quantitated by an LAS-4000 Image Analyzer (GE Healthcare). Means of three independent experiments are shown with standard deviations.

**Figure 3** Histone H3 mobility is decreased in FANCD2-knockdown cells in the presence of a DNA crosslinking reagent. (**A, B**) FRAP with HeLa cells. Three days after the transfection of hFANCD2-siRNA or control RNA, the mobility of histone H3-GFP was analyzed by bleaching one-half of a nucleus in the absence (A**)** or presence (B) of 50 ng/mL MMC for 12-18 h. The mean of the relative fluorescence intensity with the

standard deviation (n = 10-11) and examples are shown. (C) FRAP with HeLa cells stably expressing mCherry-PCNA. Three days after the transfection of control RNA, the mobility of histone H3-GFP was analyzed in the absence of MMC. The PCNA foci-positive (S-phase) cells were identified by the characteristic mCherry-PCNA distribution. The mean of the relative fluorescence intensity with the standard deviation (n = 10-14) and examples are shown. (D) FRAP with MMC-treated HeLa cells expressing H3-GFP and mCherry-PCNA in S-phase. Three days after the transfection of hFANCD2-siRNA or control RNA, the mobility of histone H3-GFP in the PCNA foci-positive (S-phase) cells was analyzed in the presence of 50 ng/mL MMC. The mean of the relative fluorescence intensity with the standard deviation (n = 10-18) and examples are shown. Bars: 10 μm.

**Figure 4** DNA repair defects in the cFANCD2 C-terminal mutants. (**A**) Colony survival assay of the cFANCD2-/- DT40 cells expressing GFP fusions with the wild type (WT) and indicated cFANCD2 mutants in the presence of cisplatin. The mean and standard deviation of measurements performed in triplicate are shown. (**B**) FANCD2/FANCI monoubiquitination in cFANCD2-/- cells expressing the indicated cFANCD2 mutants. Cells were treated with or without MMC, and the whole cell extracts were subjected to western blotting. The bands detected just above the original bands (S-forms) correspond to the monoubiquitinated forms (L-forms) of cFANCD2 and cFANCI (lanes 2, 6, 8 and 14). The L-form and S-form bands were quantitated with the Image J software, and the L/S ratios are indicated just above each panel. An asterisk indicates the non-specific

bands. ND: not detectable. (**C**) Colony survival assay of the cFANCD2-/- cells expressing H2B-GFP fusions with the WT and indicated cFANCD2 mutants in the presence of cisplatin. The mean and standard deviation of measurements performed in triplicate are shown. (**D**) Chromatin targeting of cFANCD2 (WT)-H2B, cFANCD2(1-1167)-H2B, cFANCD2(1-1267)-H2B, and cFANCD2(R1336A/K1346A)-H2B. Negative control experiments in the absence of exogenously expressed cFANCD2 are shown in the top panel. The cFANCD2-/- cells expressing the indicated proteins were treated with MMC (500 ng/ml, 6 hr) or left untreated and then fractionated. Each fraction was separated by SDS-PAGE, and western blotting was performed using anti-cFANCD2 and anti-cFANCI antibodies. An asterisk indicates the non-specific band.

**Figure 5** An FA-related mutant, cFANCD2(R305W), is defective in nucleosome assembly activity and DNA repair. (**A**) Nucleosomes were reconstituted on the relaxed plasmid DNA by cFANCD2 (lanes 3-7) and cFANCD2(R305W) (lanes 8-12), in the presence of wheat germ topoisomerase I. After deproteinization, the topoisomers were separated by agarose gel electrophoresis. The cFANCD2 concentrations were 0, 0.45, 0.90, and 1.8 μM. Highly supercoiled and relaxed DNAs are denoted as "sc" and "relaxed", respectively. (**B**) Graphic representation of the nucleosome assembly activity of the cFANCD2(R305W) mutants. Representative images are shown in panel A. The supercoiled DNA fractions were generated by nucleosome assembly in the presence of cFANCD2 (indicated by arrows in panel A), and the intensities of the bands indicated by

the arrows in panel A were quantitated by an LAS-4000 Image Analyzer (GE Healthcare). Means of three independent experiments are shown with standard deviations. (**C**) FANCD2/FANCI monoubiquitination in cFANCD2-/- cells expressing the wild type (WT) FANCD2 or cFANCD2(R305W). Cells were treated with or without MMC, and the whole cell extracts were subjected to western blotting. The bands detected just above the original bands (S-forms) correspond to the monoubiquitinated forms (L-forms) of cFANCD2 and cFANCI (lanes 2 and 4). The L-form and S-form bands were quantitated with the Image J software, and the L/S ratios are indicated just above each panel. An asterisk indicates the non-specific band. (**D**) Colony survival assay of the cFANCD DT40 cells expressing GFP fusions with the wild type (WT) and cFANCD2(R305W), in the presence of cisplatin. Four independent DT40 cells expressing cFANCD2(R305W) were tested and plotted. (**E**) Colony survival assay of the cFANCD-/- DT40 cells expressing H2B-GFP fusions with the wild type (WT) and cFANCD2(R305W), in the presence of cisplatin. The mean and standard deviation of measurements performed in triplicate are shown.

**Figure 6** An FA-related mutant, human FANCD2(R302W), is defective in facilitating histone exchange. (**A-C**) FRAP with HeLa cells expressing H3-GFP and mCherry fusions with hFANCD2 (WT) (**A**), hFANCD2(K561R) (**B**), and hFANCD2(R302W) (**C**). After the depletion of endogenous hFANCD2 by siRNA, mCherry-hFANCD2, hFANCD2(K561R), or -hFANCD2(R302W) was expressed. In these cells, the mobility of histone H3-GFP was analyzed in the presence of 50 ng/mL MMC for 12-18 h. The

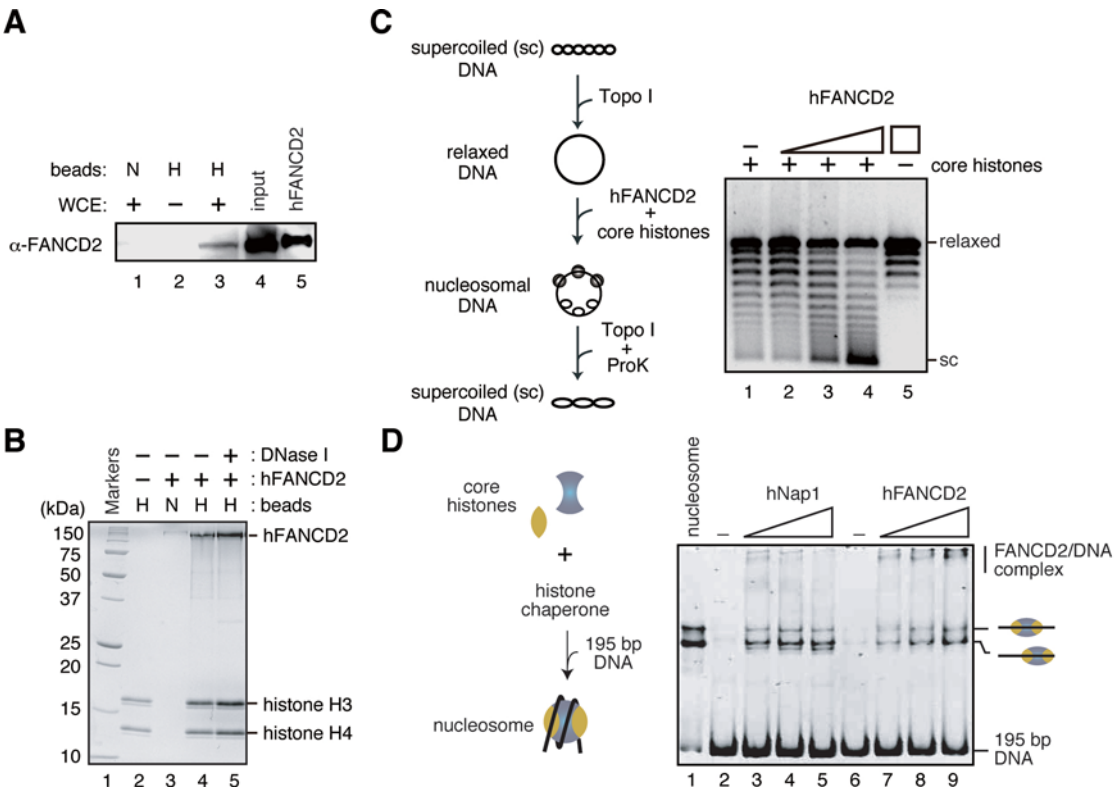
mean of the relative fluorescence intensity with the standard deviation (n = 10-12) and examples are shown. Bars: 10 μm.

**Figure 7** FANCI stimulates the FANCD2-mediated nucleosome assembly. (**A**) Gel filtration analysis of cFANCI-cFANCD2 complex formation. The SDS-PAGE analysis of the cFANCI-cFANCD2 fractions is shown below the gel filtration profiles. (**B, C**) Nucleosomes were reconstituted with relaxed plasmid DNA and cFANCD2 and/or cFANCI in the presence of wheat germ topoisomerase I. After deproteinization, the topoisomers were separated by agarose gel electrophoresis. The protein concentrations used in the assay are indicated at the top of the panels. Highly supercoiled and relaxed DNAs are denoted as "sc" and "relaxed", respectively. (**D**) Graphic representation of the experiments shown in panel C, lanes 3-5. The supercoiled DNA fractions generated by nucleosome assembly in the presence of cFANCD2 and cFANCI (indicated by arrows in C) were quantitated. Means of three independent experiments are shown with standard deviations.

**Figure 8** Models for the FANCD2 function in the FA pathway. (**A**) Locations of the Arg1334 and Lys1344 residues in the mouse FANCD2 structure. Structure of the mouse ID complex (PDB ID: 3S4W; Joo *et al*, 2011). The FANCD2 and FANCI subunits are colored gray and light blue, respectively. The FANCD2 Arg1334 and Lys1344 residues (red), corresponding to the cFANCD2 Arg1336 and Lys1346 residues, respectively, are located on the concave surface of the FANCD2 Solenoid 4 (S4) domain (dark gray). (**B**)

Three possible functions of the histone chaperone activity of the ID complex. (I) After the chromatin binding and monoubiquitination of the ID complex, the nucleosomes assembled by the ID complex on newly synthesized DNA may suppress the improper nucleolytic digestion by the repair nucleases, such as FAN1 and the SLX4 nuclease complex, which are recruited by the monoubiquitinated ID complex. (II) Chromatin remodeling mediated by the ID complex and FANCD2-associated chromatin modifiers may facilitate the RAD51-mediated homologous recombination reaction in chromatin. (III) The nucleosome assembly activity of the ID complex may be required for chromatin reconstitution after DNA repair. RAD51 and RPA are colored red and green, respectively. Histone octamers are shown in brown.

# Figure 1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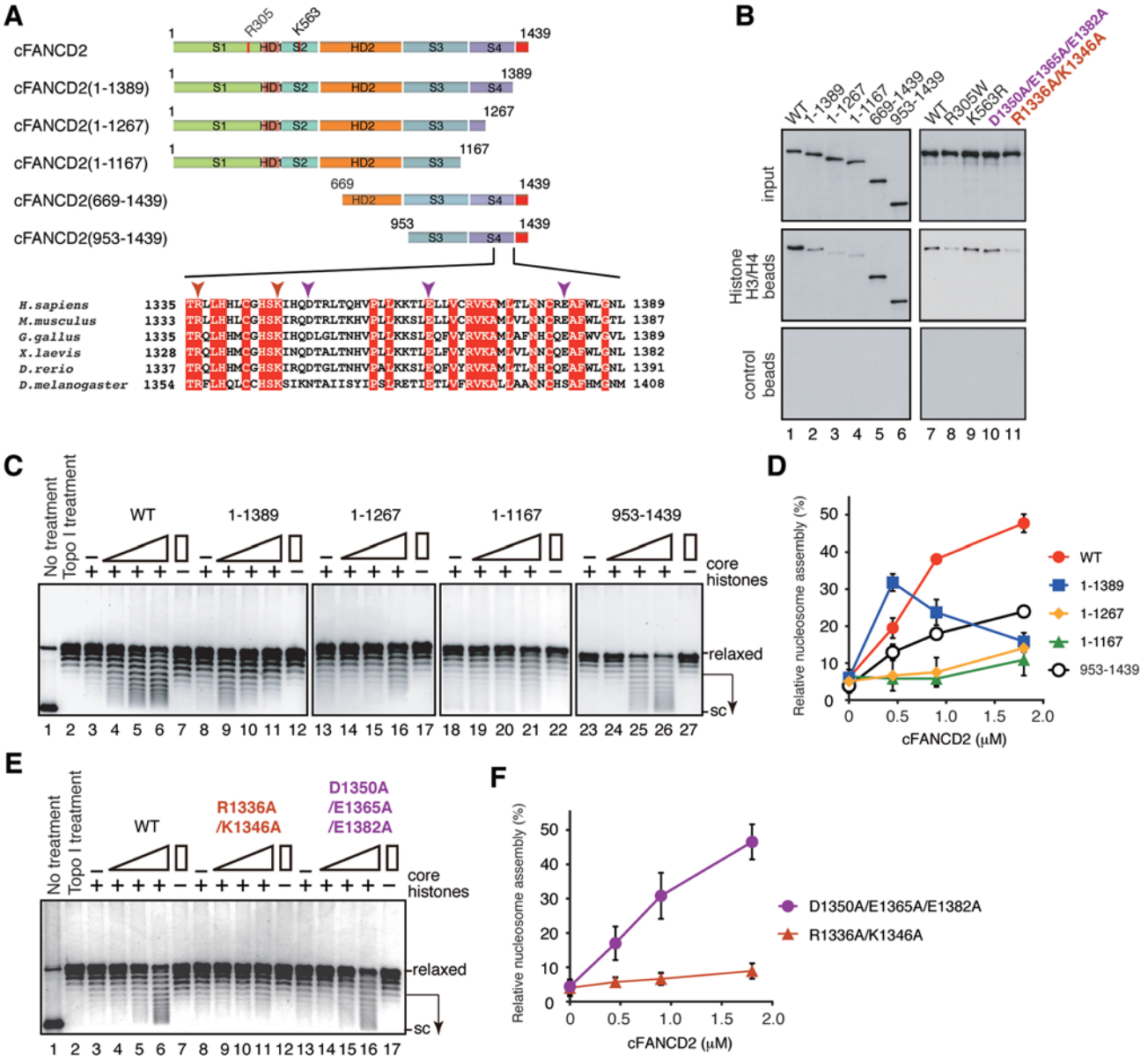
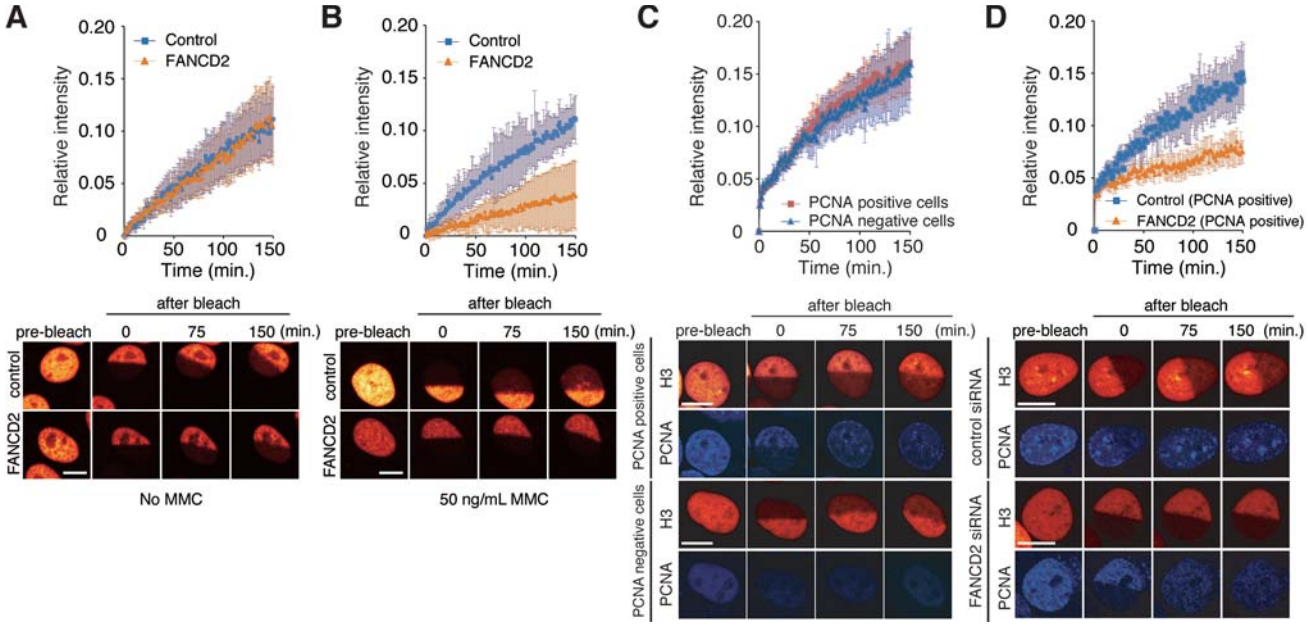
beads: N H H
WCE: + − +
input
hFANCD2
α-FANCD2
1 2 3 4 5
B
Markers
− − − + : DNase I
− + + + : hFANCD2
H N H H : beads
(kDa)
150
75
50
37
25
20
15
10
hFANCD2
histone H3
histone H4
1 2 3 4 5
C
supercoiled (sc) DNA
Topo I
relaxed DNA
hFANCD2 + core histones
nucleosomal DNA
Topo I + ProK
supercoiled (sc) DNA
hFANCD2
core histones
relaxed
sc
1 2 3 4 5
D
core histones
+
histone chaperone
195 bp DNA
nucleosome
nucleosome
hNap1
hFANCD2
FANCD2/DNA complex
195 bp DNA
1 2 3 4 5 6 7 8 9

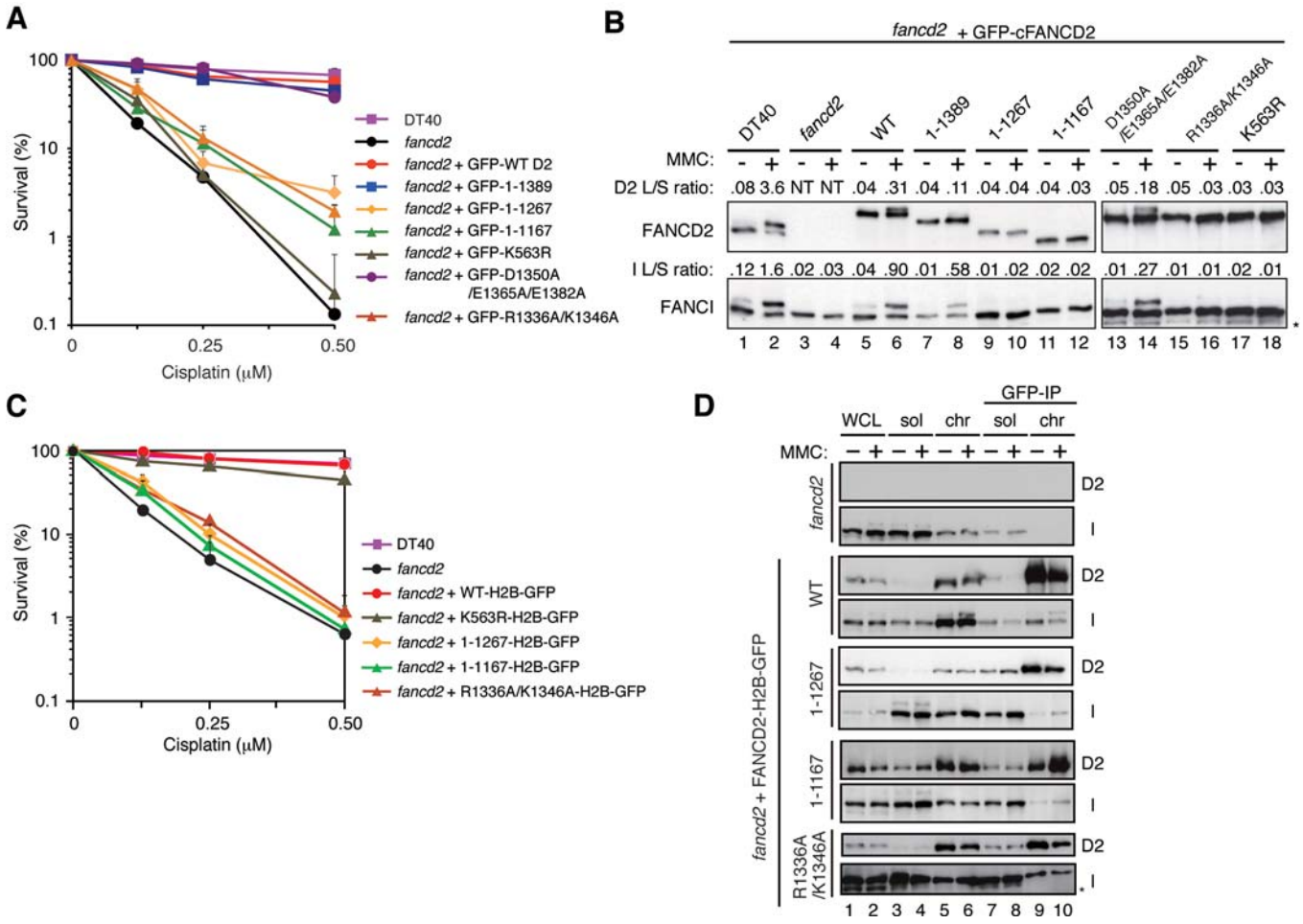
# Figure 2


A
cFANCD2
cFANCD2(1-1389)
cFANCD2(1-1267)
cFANCD2(1-1167)
cFANCD2(669-1439)
cFANCD2(953-1439)
R305
K563
S1
HD1
S2
HD2
S3
S4
H.sapiens
M.musculus
G.gallus
X.laevis
D.rerio
D.melanogaster
B
WT
1-1389
1-1267
1-1167
669-1439
953-1439
R305W
K563R
D1350A/E1365A/E1382A
R1336A/K1346A
input
Histone H3/H4 beads
control beads
C
No treatment
Topo I treatment
core histones
relaxed
sc
D
Relative nucleosome assembly (%)
cFANCD2 (μM)
E
F
D1350A/E1365A/E1382A
R1336A/K1346A

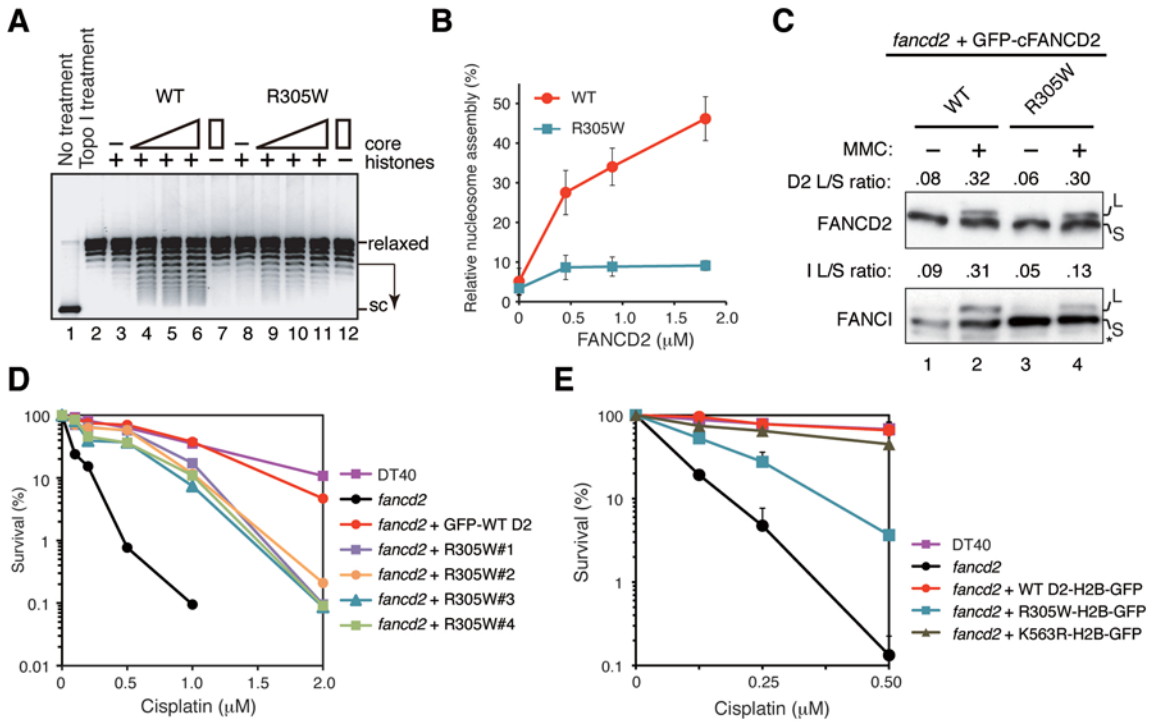
# Figure 3


A
B
C
D
Relative intensity
Time (min.)
Control
FANCD2
PCNA positive cells
PCNA negative cells
Control (PCNA positive)
FANCD2 (PCNA positive)
after bleach
pre-bleach
0
75
150 (min.)
control
FANCD2
No MMC
50 ng/mL MMC
PCNA positive cells
PCNA negative cells
H3
PCNA
control siRNA
FANCD2 siRNA

# Figure 4


A
Survival (%)
100
10
1
0.1
0
0.25
0.50
Cisplatin (μM)
DT40
fancd2
fancd2 + GFP-WT D2
fancd2 + GFP-1-1389
fancd2 + GFP-1-1267
fancd2 + GFP-1-1167
fancd2 + GFP-K563R
fancd2 + GFP-D1350A /E1365A/E1382A
fancd2 + GFP-R1336A/K1346A
B
fancd2 + GFP-cFANCD2
DT40
fancd2
WT
1-1389
1-1267
1-1167
D1350A /E1365A/E1382A
R1336A/K1346A
K563R
MMC: - + - + - + - + - + - + - + - + - +
D2 L/S ratio: .08 3.6 NT NT .04 .31 .04 .11 .04 .04 .04 .03 .05 .18 .05 .03 .03 .03
FANCD2
I L/S ratio: .12 1.6 .02 .03 .04 .90 .01 .58 .01 .02 .02 .02 .01 .27 .01 .01 .02 .01
FANCI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
C
Survival (%)
100
10
1
0.1
0
0.25
0.50
Cisplatin (μM)
DT40
fancd2
fancd2 + WT-H2B-GFP
fancd2 + K563R-H2B-GFP
fancd2 + 1-1267-H2B-GFP
fancd2 + 1-1167-H2B-GFP
fancd2 + R1336A/K1346A-H2B-GFP
D
GFP-IP
WCL sol chr sol chr
MMC: − + − + − + − + − +
fancd2
WT
1-1267
1-1167
R1336A /K1346A
fancd2 + FANCD2-H2B-GFP
D2
I
1 2 3 4 5 6 7 8 9 10

# Figure 5


A
No treatment
Topo I treatment
WT
R305W
core histones
relaxed
sc
1 2 3 4 5 6 7 8 9 10 11 12
B
Relative nucleosome assembly (%)
WT
R305W
FANCD2 (μM)
C
fancd2 + GFP-cFANCD2
WT
R305W
MMC:
D2 L/S ratio: .08 .32 .06 .30
FANCD2
I L/S ratio: .09 .31 .05 .13
FANCI
1 2 3 4
D
Survival (%)
Cisplatin (μM)
DT40
fancd2
fancd2 + GFP-WT D2
fancd2 + R305W#1
fancd2 + R305W#2
fancd2 + R305W#3
fancd2 + R305W#4
E
Survival (%)
Cisplatin (μM)
DT40
fancd2
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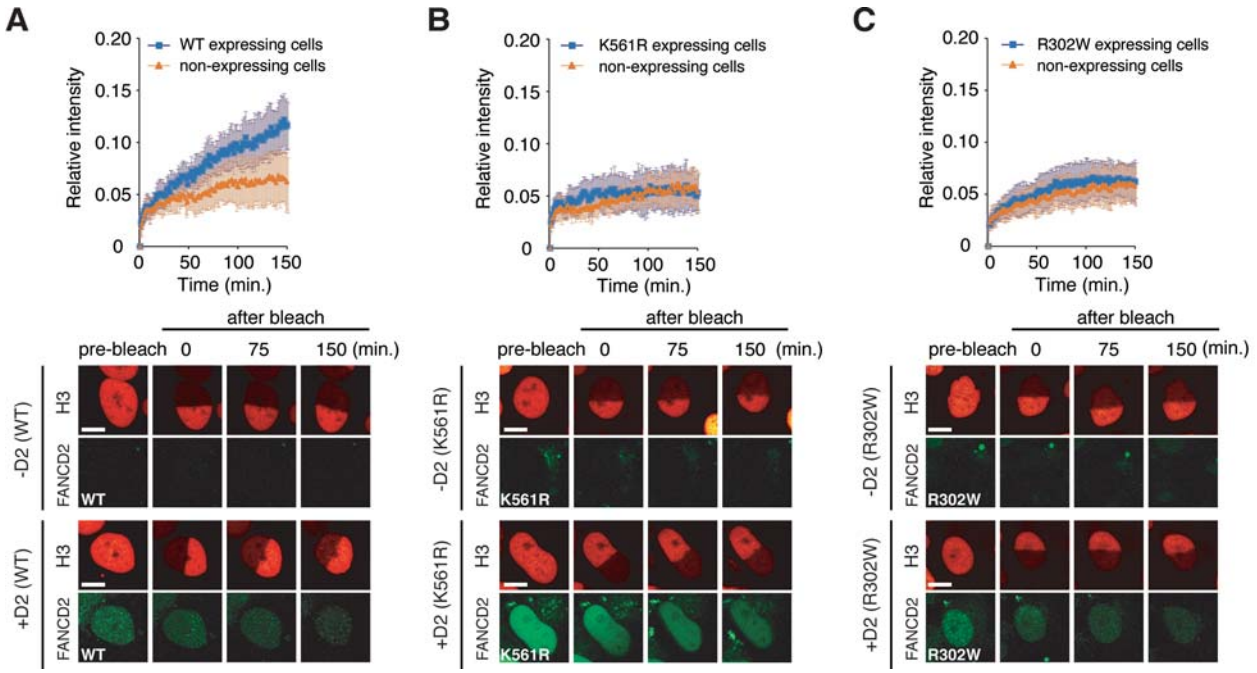
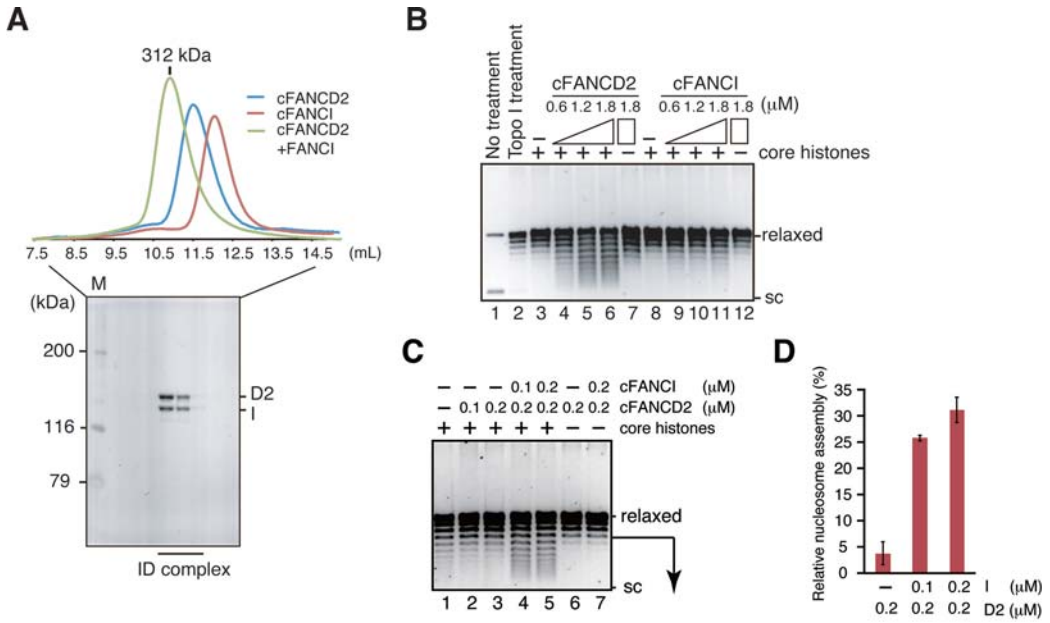
fancd2 + R305W-H2B-GFP
fancd2 + K563R-H2B-GFP

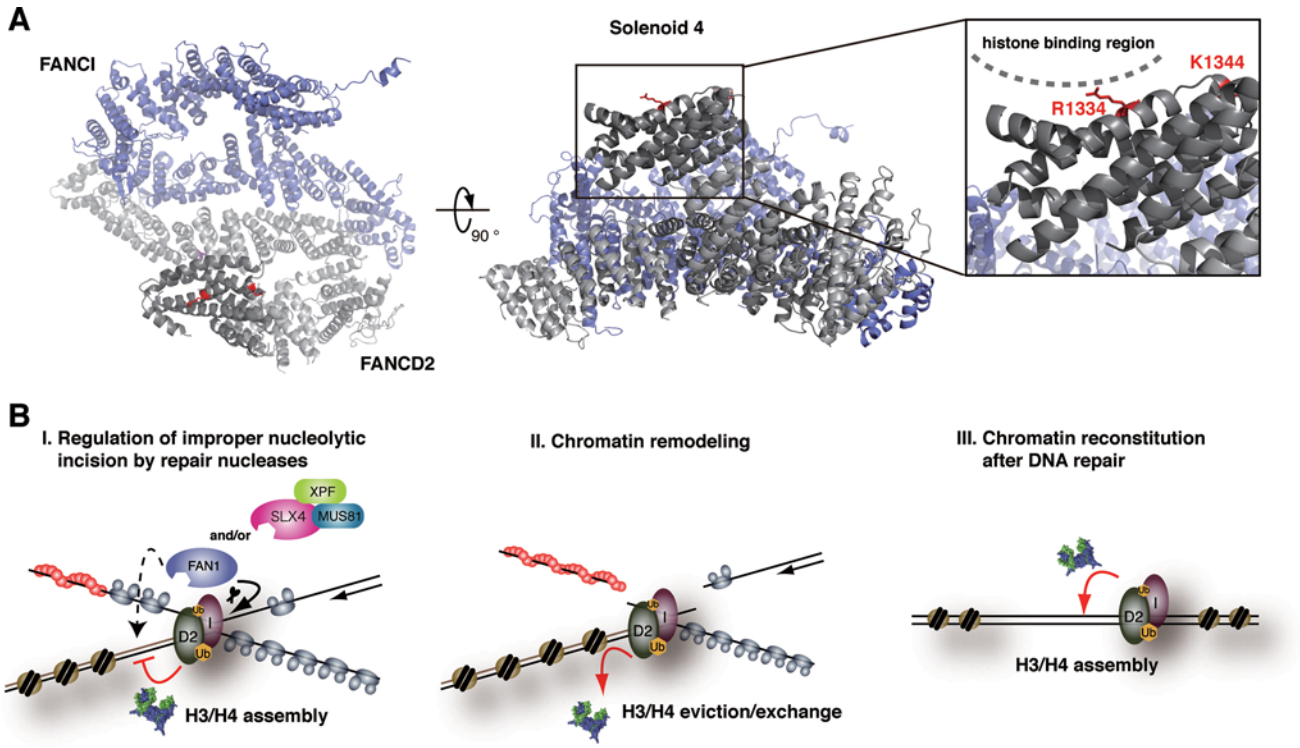
# Figure 6


A
B
C
WT expressing cells
non-expressing cells
K561R expressing cells
non-expressing cells
R302W expressing cells
non-expressing cells
Relative intensity
Time (min.)
after bleach
pre-bleach
0
75
150 (min.)
-D2 (WT)
+D2 (WT)
-D2 (K561R)
+D2 (K561R)
-D2 (R302W)
+D2 (R302W)
H3
FANCD2
WT
K561R
R302W

# Figure 7


A
312 kDa
cFANCD2
cFANCI
cFANCD2
+FANCI
7.5 8.5 9.5 10.5 11.5 12.5 13.5 14.5 (mL)
M
(kDa)
200
116
79
D2
I
ID complex
B
No treatment
Topo I treatment
cFANCD2
0.6 1.2 1.8 1.8
cFANCI
0.6 1.2 1.8 1.8 (μM)
core histones
relaxed
sc
1 2 3 4 5 6 7 8 9 10 11 12
C
cFANCI (μM)
cFANCD2 (μM)
core histones
relaxed
sc
1 2 3 4 5 6 7
D
Relative nucleosome assembly (%)
35
30
25
20
15
10
5
0
I (μM)
D2 (μM)

# Figure 8


A
FANCI
FANCD2
Solenoid 4
90 °
histone binding region
K1344
R1334
B
I. Regulation of improper nucleolytic incision by repair nucleases
XPF
SLX4
MUS81
and/or
FAN1
Ub
I
D2
Ub
H3/H4 assembly
II. Chromatin remodeling
H3/H4 eviction/exchange
III. Chromatin reconstitution after DNA repair
H3/H4 assembly