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FANCI phosphorylation functions as a molecular switch to turn on the Fanconi anemia pathway

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In response to DNA damage or replication fork stress, the Fanconi anemia pathway is activated, leading to monoubiquitination of FANCD2 and FANCI and their colocalization in foci. Here we show that, in the chicken DT40 cell system, multiple alanine-substitution mutations in six conserved and clustered Ser/Thr-Gln motifs of FANCI largely abrogate monoubiquitination and focus formation of both FANCI and FANCD2, resulting in loss of DNA repair function. Conversely, FANCI carrying phosphomimic mutations on the same six residues induces constitutive monoubiquitination and focus formation of FANCI and FANCD2, and protects against cell killing and chromosome breakage by DNA interstrand cross-linking agents. We propose that the multiple phosphorylation of FANCI serves as a molecular switch in activation of the Fanconi anemia pathway. Mutational analysis of putative phosphorylation sites in human FANCI indicates that this switch is evolutionarily conserved.

Genome stability is crucial for maintaining integrity of the organism, and therefore all cells have elaborate systems to prevent, repair or tolerate endogenous or exogenous DNA damage. In higher organisms, loss of these functions often leads to cancer predisposition[1] and impaired stem-cell proliferation[2–4]. The rare hereditary disorder Fanconi anemia is a prototype of such conditions. Fanconi anemia is clinically characterized by an increased occurrence of leukemias and solid tumors, progressive bone marrow failure and developmental abnormalities[5,6]. Altogether, 13 genes have been implicated in Fanconi anemia, and their products constitute a common pathway in DNA-damage signaling termed the 'Fanconi anemia pathway'. This pathway responds to stalled replication forks and interstrand cross-links (ICLs) in addition to various types of DNA damage, including double-stranded breaks and UV-induced damage. Upon treatment with ICL inducers such as mitomycin C (MMC) or cisplatin, Fanconi anemia cells show highly increased levels of cell death and chromosome breakages, reflecting a profoundly impaired ability to handle or repair ICLs. Although how the Fanconi anemia pathway participates in ICL repair is currently unknown, it is now presumed that it regulates molecular processes that stabilize and/or resume the arrested fork by affecting homologous recombination and/or translesion DNA synthesis[5,6].

The newest member in the Fanconi anemia pathway, FANCI, was identified through a proteomic screen in an effort to identify ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3–related (ATR) kinase substrates[7], through a search for a FANCD2 homolog in the database[8] and by positional cloning[9]. FANCI physically associates with the key factor FANCD2, resulting in formation of the ID complex[7,8]. Upon DNA damage, FANCD2 and FANCI are monoubiquitinated in a manner dependent on each other[7,8] and on the ATR kinase[10], the E2-conjugating enzyme UBE2T[11] and the Fanconi anemia core complex, a multisubunit E3 ligase formed by eight Fanconi anemia proteins (FANCA/B/C/E/F/G/L/M) and two associated proteins, FAAP24 (ref. 12) and FAAP100 (ref. 13). In turn, FANCD2 and FANCI are both targeted to chromatin and form colocalizing foci together with the homologous recombination proteins breast cancer 1 (BRCA1) and RAD51 (refs. 7,8,14). Monoubiquitin on FANCD2 serves as an attachable chromatin-localization tag[15] and is cleaved off by deubiquitinase ubiquitin-specific peptidase 1 (USP1; ref. 16). Thus, FANCD2 monoubiquitination is crucial for DNA repair via the Fanconi anemia pathway, with downstream or parallel effectors including BRCA2 (also known as FANCD1; ref. 17), partner and localizer of BRCA2 (PALB2, also known as FANCN[18]) and

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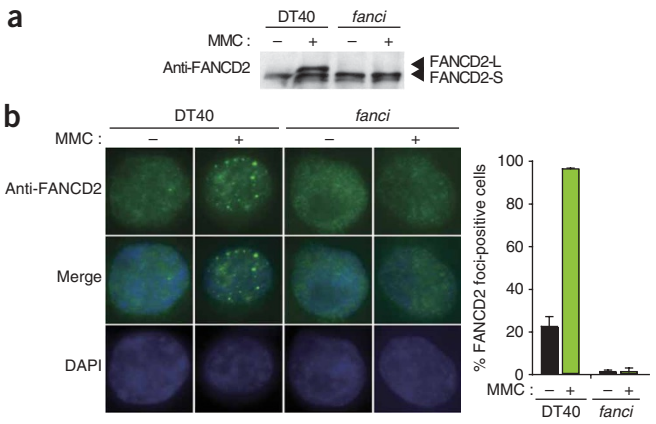

Figure 1 Gene targeting of *FANCI* resulted in loss of FANCD2 monoubiquitination and focus formation. (**a**) Western blot analysis of FANCD2 monoubiquitination induced by MMC treatment. (**b**) FANCD2 localization to nuclear foci by indirect fluorescence using anti-FANCD2 antibodies and DAPI counterstaining. Representative images of wild-type (DT40) or *fanci* cells with or without MMC treatment stained for endogenous FANCD2 are shown. The mean and s.e.m. of the percentage of FANCD2 foci-positive cells in three independent experiments are shown in the bar graph. More than 100 cells were scored in each experiment, and cells containing more than four bright foci were defined as foci-positive.

BRCA1-interacting C-terminal helicase 1 (BRIP1, also known as FANCJ[19]). In addition, the core complex has been suggested to contribute to DNA repair besides having a role as an E3 ligase[15].

As FANCI is an essential cofactor for FANCD2 monoubiquitination, we set out to investigate how FANCI contributes to triggering this key activation event in the Fanconi anemia pathway. We examined the functional role of monoubiquitination and phosphorylation of FANCI in chicken DT40 cells and found that multiple phosphorylation of FANCI but not monoubiquitination is crucial for FANCD2 activation following DNA damage. Thus, we propose that FANCI phosphorylation serves as a molecular switch in the Fanconi anemia pathway.

RESULTS

Generation of FANCI-deficient cells

We disrupted the *FANCI* gene in the chicken DT40 cell line (**Supplementary Fig. 1** online) and observed that *fanci* DT40 cells showed abrogated monoubiquitination and focus formation of FANCD2 protein both before and after MMC treatment (**Fig. 1**), as expected from previous studies using human *FANCI* mutant cell lines[20]. Consistent with a crucial role for FANCD2 monoubiquitination in DNA repair[21], *fanci* cells were extremely cisplatin sensitive (**Fig. 2a**) and showed increased levels of chromosome breakage induced by MMC (**Fig. 2b**). Expression of a GFP-tagged full-length chicken *FANCI* cDNA (GFP–chFANCI-WT) in *fanci* cells fully rescued ICL sensitivity in cell survival (**Fig. 2a**) and chromosome-aberration assays (**Fig. 2b**), as well as monoubiquitination and focus formation of FANCD2 (**Fig. 2c**,**d**). Similarly to what has been observed for human FANCI[7,8], a slower-mobility form of GFP-chFANCI (designated GFP–chFANCI-L, as opposed to the main 'S' form) was detected by anti-GFP western blotting (**Fig. 2c**). This L-form corresponds to monoubiquitinated FANCI, because the GFP–chFANCI-L form accumulated following DNA damage, and when Lys525—equivalent to the monoubiquitination site in human FANCI—was substituted with arginine (K525R), the L form was undetectable.

Genetic requirements for FANCI monoubiquitination

Monoubiquitination of human FANCI is catalyzed by the core complex in a manner strictly dependent on the presence and monoubiquitination of FANCD2 (refs. 7,8). We tested this in DT40 cells by expressing GFP-chFANCI in wild-type and various mutant cell lines followed by western blotting. In wild-type DT40 cells, induction of the GFP–chFANCI-L form was detected following MMC treatment, albeit at low levels, perhaps owing to the presence of endogenous FANCI (**Supplementary Fig. 2a** online). In contrast, the L form was not induced in *fancd2* (ref. 22), *fancd2-K563R-knock in* (in which the monoubiquitination site Lys563 was mutated to arginine[21]), *fancc*[23] or *fancl*[21] cells expressing GFP-chFANCI (**Fig. 3a**, left, and **Supplementary Fig. 2a**). Consistently, we observed MMC-induced GFP-chFANCI focus formation when expressed in wild-type cells but not in *fancd2*, *fancc* or *fancl* cells (**Supplementary Fig. 2b**). These results confirm the data obtained in human cells[7,8] and further support the role of the core complex and FANCD2 monoubiquitiation in inducing FANCI monoubiquitination.

Functional role of FANCI monoubiquitination

To ask whether FANCI monoubiquitination is crucial for FANCD2 monoubiquitination, we introduced GFP-chFANCI carrying the K525R mutation (GFP–chFANCI-K525R) into *fanci* cells. We observed that this construct largely rescued cell survival (**Fig. 2a**) and chromosome breakage (**Fig. 2b**) in response to ICL treatment, although rescue was less than obtained by expression of GFP–chFANCI-WT, indicating that FANCI without monoubiquitination is partially functional in DNA repair. Of note, the experiments in human *fanci* mutant cell lines have also provided similar data regarding MMC sensitivity and chromosome breakage (ref. 7; see figures 6e and 6f, respectively). In support of this, MMC treatment of cells with GFP–chFANCI-K525R induced FANCD2 monoubiquitination, albeit with slightly reduced efficiency (**Figs. 2c** and **3b**), leading to colocalizing focus formation of FANCD2 and FANCI (**Fig. 2d**). Consistently, the FANCD2-L form was targeted to chromatin in these cells in a similar way to in cells expressing GFP–chFANCI-WT (**Fig. 3b**). Furthermore, co-immunoprecipitation experiments showed that, in the chromatin fraction of these cells, FANCD2 interacted with both the GFP–chFANCI-S form (K525R-expressing cells) and L form (wild type–expressing cells), respectively (**Fig. 3b**). Taken together, these data indicate that the role of FANCI monoubiquitination is largely dispensable for FANCD2 monoubiquitination in the Fanconi anemia pathway, and the interaction of the two proteins is monoubiquitination independent.

In a previous study, we showed that cisplatin sensitivity in *fancd2* cells is reversed to near wild-type levels by transfection of FANCD2 fusions with a single ubiquitin moiety or histone H2B (each termed D2KR-Ub or D2KR-H2B, respectively, as their monoubiuqitination site Lys563 is replaced with arginine)[15]. Both of the fusions of FANCD2 are targeted to chromatin despite the absence of the monoubiquitination site; hence, we asked whether, under these conditions, the monoubiquitination of FANCI is supported. To address this question, we transfected the GFP-chFANCI expression vector into these cells and examined monoubiquitination of FANCI. Consistent with the above experiments, we found that GFP-chFANCI was not converted to the L form in these cells (**Fig. 3a**, right), despite the cells having a near normal cisplatin tolerance[15]. Thus, these data further supported the conclusion that monoubiquitination of FANCI is largely dispensable for the DNA repair function of the Fanconi anemia pathway.

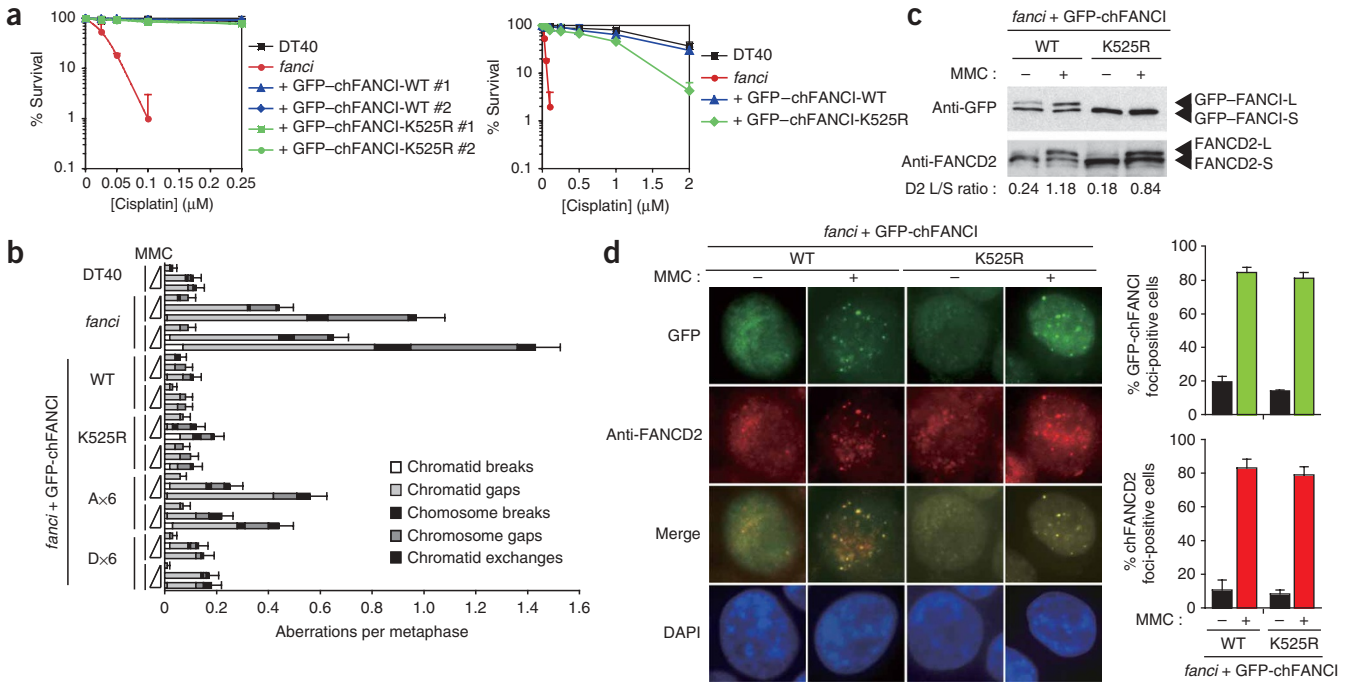

Figure 2 FANCI monoubiquitination has a minor role in inducing Fanconi anemia pathway activation. (**a**) Cisplatin sensitivity of cells with the indicated genotypes were evaluated by colony survival. *fanci* cells were transfected with the GFP–chFANCI-WT or the GFP–chFANCI-K525R expression vector. The experiment was repeated at least three times, and a representative data set with mean and s.d. of the triplicate cultures is shown. (**b**) Chromosome analysis of cells with the indicated genotypes. Along with wild-type cells (DT40) and two clones of *fanci* cells, two independent clones of *fanci* cells expressing GFP–chFANCI-WT or mutant proteins were treated with the increasing concentrations of MMC (0, 20 and 40 ng ml^{-1}) for 24 h. At least 50 metaphases were scored blindly for each preparation. Error bars indicate s.e.m. (**c**) Monoubiquitination of FANCD2 and FANCI. Whole-cell extracts from cells with the indicated genotypes were analyzed using antibodies against GFP or FANCD2. (**d**) GFP-chFANCI or chFANCD2 localization to DNA damage–induced nuclear foci. Representative images of *fanci* cells expressing GFP–chFANCI-WT or the K525R mutant with or without MMC treatment are shown. The graph shows the mean and s.e.m. of the percentage of FANCD2 or GFP-chFANCI foci-positive cells in three independent experiments. Cells with more than four bright foci were defined as foci-positive. More than 100 cells were scored for each data set.

Functional analysis of FANCI phosphorylation

The ATM or ATR kinase generally phosphorylates a Ser/Thr-Gln (S/TQ) motif as a consensus phosphorylation site in substrates. Human FANCI protein harbors numerous S/TQ motifs, and at least five of these sites are phosphorylated in human or mouse FANCI as detected by MS[7,24] (indicated by red letters in **Fig. 4a**; see also **Supplementary Fig. 3** online). Comparison human and chicken FANCI amino acid sequences, we found eight conserved S/TQ sites, and six of these lie in a cluster spanning ~70 amino acids close to the monoubiquitination site Lys523 (human) or Lys525 (chicken) (**Fig. 4a**).

To examine whether phosphorylation of these S/TQ sites has an important role in the Fanconi anemia pathway, we mutated several S/TQ sites in chicken FANCI simultaneously. We isolated chFANCI mutant cDNAs, each carrying seven or nine consecutive alanine substitutions on the S/TQ sites (termed A×7 or A×9), and expressed them in *fanci* cells as GFP-fusion proteins (**Fig. 4a**). To facilitate rapid functional evaluation of the mutants, we developed a cell-viability assay by propidium iodide staining and flow cytometry following 48 h of cisplatin exposure in liquid culture (Methods). Notably, the FANCI-A×7 mutant provided an appreciable rescue of the cell death induced by cisplatin, albeit less efficiently than wild-type FANCI, whereas the A×9 mutant could not (**Fig. 4b**). Colony-survival assays confirmed these results (**Supplementary Fig. 4a** online). These observations suggest that FANCI S/TQ sites might be crucial for DNA repair and also indicate that the two nonoverlapping sites (Ser558 and Ser561) between the A×7 and the A×9 mutants are functionally important. However, another FANCI mutant, A×2, in which these two sites were substituted with alanine, was still capable of reversing cisplatin sensitivity (**Fig. 4a**,**b** and **Supplementary Fig. 4a**), suggesting that, in addition to these two sites, other S/TQ motifs may function in a redundant manner.

To further investigate the importance of potential phosphorylation sites in FANCI, we created A×6 or A×4 mutants in which all or four of the six S/TQ sites in the conserved S/TQ cluster were changed to Ala-Gln, respectively (**Fig. 4a**). *fanci* cells transfected with the A×6 mutant showed levels of cisplatin sensitivity close to those in *fanci* cells, similarly to cells expressing the A×9 protein (**Fig. 4b** and **Supplementary Fig. 4a**). MMC-induced chromosome aberrations in cells with the A×6 mutant were increased compared to cells expressing GFP–chFANCI-WT (**Fig. 2b**). The A×4 mutant showed partial sensitivity to cisplatin damage in the colony-survival assay (**Supplementary Fig. 4a**) and a stronger defect in the liquid culture assay (**Fig. 4b**).

We also looked at these cells expressing GFP-chFANCI mutants by anti-GFP or anti-FANCD2 western blotting. Compared to cells expressing GFP–chFANCI-WT, in cells with the A×4, the A×6 or the A×9 mutants, FANCI or FANCD2 monoubiquitination was virtually abrogated both before and after MMC treatment (**Fig. 4c**). Conversely, we still observed weak levels of monoubiquitination in the A×2 and the A×7 mutants (**Fig. 4c**). These results suggest that

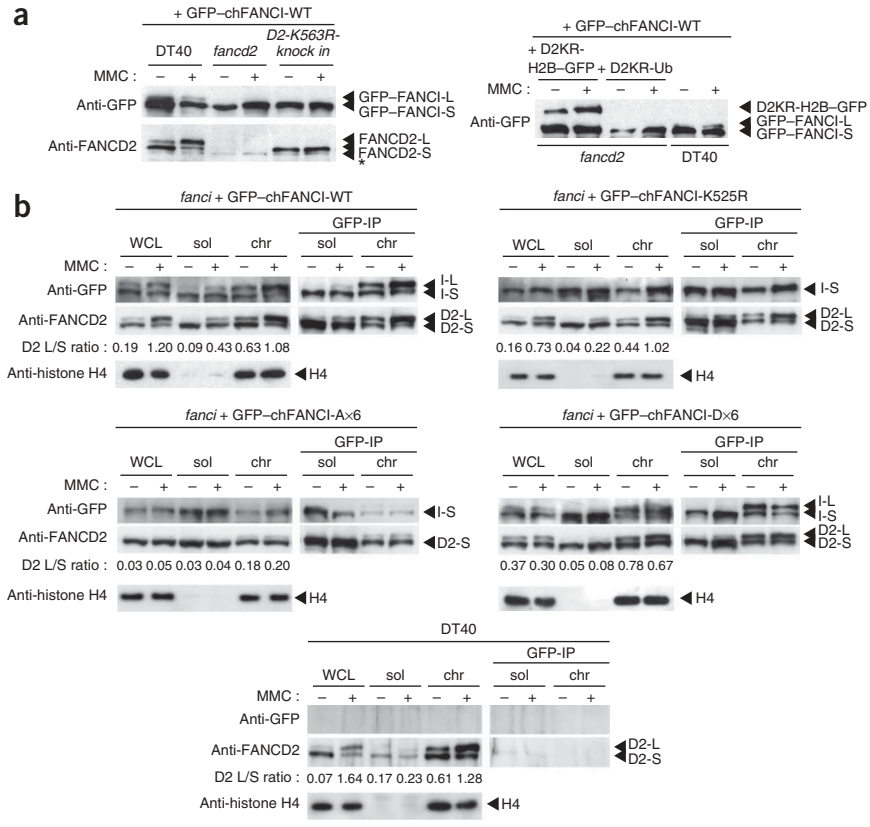

Figure 3 FANCI monoubiquitination depends on FANCD2 monoubiquitination, but their physical interaction is constitutive. (**a**) Genetic requirements for FANCI monoubiquitination. Wild-type DT40 cells, *fancd2* cells, cells with the *FANCD2* monoubiuqitination site K563R knockin mutation (*D2-K563R-knock in*), and *fancd2* cells carrying D2KR-H2B–GFP or D2KR-Ub fusions were stably transfected with GFP–chFANCI-WT, and treated with or without MMC. Whole-cell lysates were blotted with anti-GFP or anti-FANCD2 antibodies. * indicates a nonspecific band. (**b**) Co-immunoprecipitation between FANCD2 and FANCI. Indicated cells were stimulated with MMC and fractionated into soluble (sol) and chromatin (chr) fractions. Immunoprecipitation was carried out using anti-GFP antibody beads. Whole-cell lysates (WCL), fractions (5% of the input) and immunoprecipitates were separated by SDS-PAGE and blotted with antibodies as indicated. As a negative control for anti-GFP immunoprecipitation, lysates from wild-type DT40 cells not expressing GFP-chFANCI were similarly fractionated and analyzed. L or S indicates L form or S form, respectively.

multiple and redundant phosphorylation in the S/TQ cluster domain is a prerequisite for induction of monoubiquitination of FANCD2 and FANCI, and for their DNA repair function.

FANCI is phosphorylated without prior ubiquitination

To support the hypothesis that chicken FANCI protein is indeed phosphorylated following ICL damage, we used *fanci* cells expressing wild-type or mutant FANCI and a Phos-tag reagent that selectively binds to phosphorylated amino acid residues[25]. In polyacrylamide gels containing Phos-tag acrylamide, phosphorylated forms of protein could be detected by slower migration. We treated *fanci* cells expressing GFP-chFANCI-WT, GFP-chFANCI-K525R or the GFP-chFANCI-A×6 with or without MMC, and whole cell lysates of these cells were separated by electrophoresis, with or without the Phos-tag, and blotted with anti-GFP antibodies.

In the presence of Phos-tag, GFP–chFANCI-WT showed two slower-migrating bands in addition to the S and L forms of FANCI, indicating that MMC treatment induced FANCI phosphorylation. Consistent with this, phosphorylated FANCI has been previously detected as two bands using an anti–phospho-SQ antibody[7]. Only the minor fraction of FANCI (~13% of total FANCI, as shown in **Fig. 4d**) showed mobility shift in the Phos-tag gel after MMC treatment. Notably, the GFP–chFANCI-K525R protein from MMC-treated cells showed a single retarded band in a Phos-tag–containing gel (**Fig. 4d**), which was abrogated by λ-phosphatase treatment *in vitro* (**Fig. 4e**). The data suggest that FANCI could be phosphorylated without prior monoubiquitination and that the upper band of phosphorylated FANCI might be monoubiquitinated. The phosphorylation occurs on residues that were mutated in the A×6, because GFP-chFANCI-A×6 did not show a detectable band shift in the presence of Phos-tag (**Fig. 4d**).

FANCI phosphorylation is caffeine sensitive

Because replication fork arrests generate replication protein A (RPA)-coated single-stranded gaps that activate ATR[26], and FANCI is a putative ATR and ATM substrate[7], it seems likely that ATR kinase phosphorylates FANCI following ICL treatment. As caffeine can inhibit ATM and ATR kinases[27], we examined its effects on FANCI phosphorylation and monoubiquitination of FANCI and FANCD2 by western blotting using gels with or without Phos-tag. Caffeine treatment inhibited the mobility shift of checkpoint kinase CHK1 following MMC treatment (**Fig. 4f**), indicating that ATR activity is suppressed. It also substantially inhibited MMC-induced monoubiquitination of FANCD2 and GFP-chFANCI (**Fig. 4f** and **Supplementary Fig. 5a** online), as well as phosphorylation of GFP–chFANCI-WT or GFP–chFANCI-K525R (**Fig. 4f**). Collectively, these data establish that ICL treatment induces FANCI phosphorylation in a caffeine-sensitive manner.

FANCI phosphomimics activate the Fanconi anemia pathway

To gain more insight into the functional role of phosphorylation in the S/TQ cluster, we replaced all or some of the six alanine residues in the A×6 mutant FANCI with aspartic acid (**Fig. 5a**). Substitution to aspartic acid provides negative charge, thus mimicking phosphorylation. The phosphomimic mutant (termed D×6), in which all of the Ala-Gln sites in the A×6 mutant were changed to Asp-Gln, effectively reversed cisplatin sensitivity (**Fig. 5b** and **Supplementary Fig. 4b**) and MMC-induced chromosome breakage (**Fig. 2b**). In contrast, among mutants in which two adjoining S/TQ sites were converted to Asp-Gln (D×2a, D×2b and D×2c), only the D×2a mutant (Asp558 and Asp561) showed protection against DNA damage with cisplatin. The D×4 mutant, in which the D×2b and D×2c mutations were combined, was as functional as the D×2a mutant (**Fig. 5b**).

In addition, we tried to dissect the D×2a mutation to the two mutants each carrying a single Asp-Gln motif (Asp558 or Asp561) and five Ala-Gln motifs, and found that neither mutant

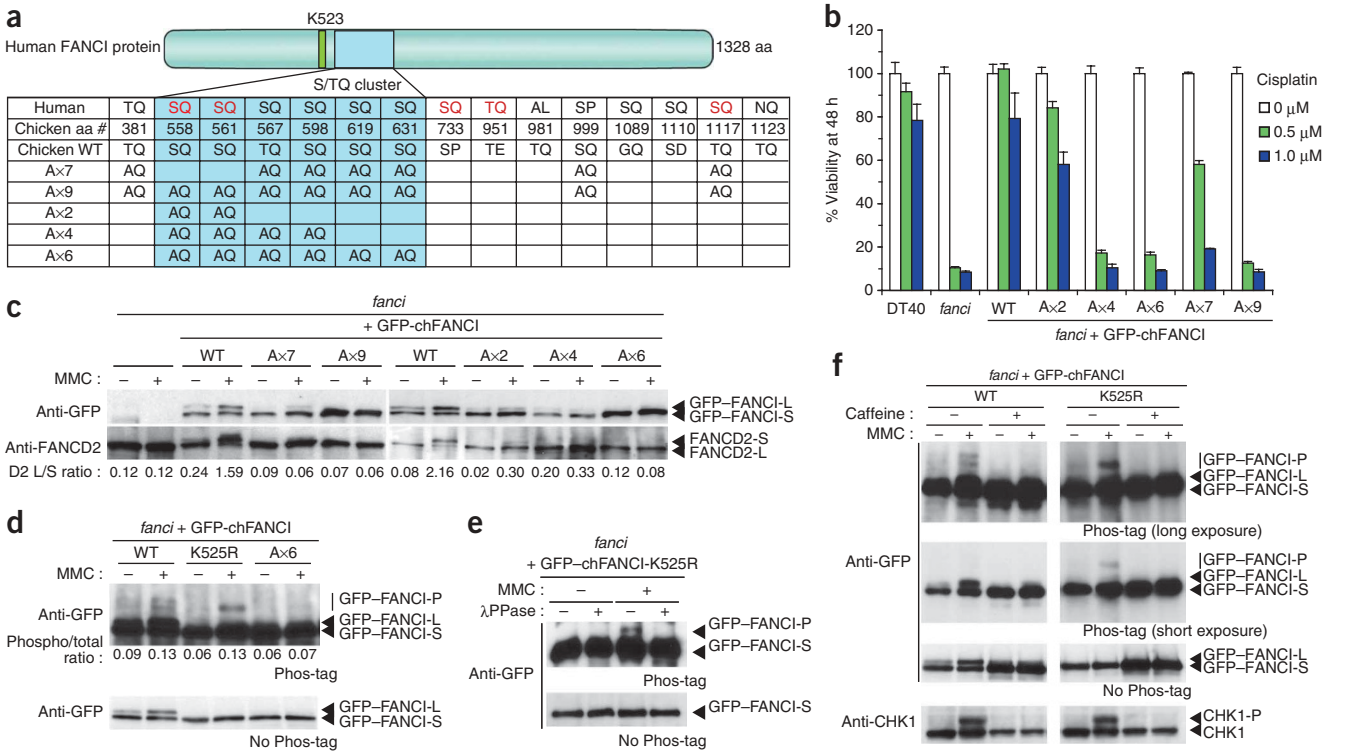

Figure 4 Phosphorylation sites in FANCI are required for Fanconi anemia pathway activation and DNA repair. (**a**) S/TQ sites in human and chicken FANCI proteins were listed with corresponding sites in the other protein. Red lettering indicates sites whose ionizing irradiation–induced phosphorylation was detected by MS[7]. Locations of multiple alanine substitutions in mutant chFANCI proteins are indicated. aa, amino acid. (**b**) Cell survival in cisplatin-containing medium was assayed using propidium iodide staining and FACSCalibur. (**c**) Monoubiquitination of GFP-chFANCI and chFANCD2 in response to MMC. Whole-cell lysates were separated by SDS-PAGE and blotted using antibodies against GFP and chFANCD2. (**d**) Phosphorylation of GFP-chFANCI in response to MMC. Whole-cell lysates were separated by SDS-PAGE using a gel containing Phos-tag acrylamide and blotted using anti-GFP antibodies. Shifted bands are indicated as GFP–chFANCI-P. (**e**) GFP-chFANCI was immunoprecipitated from the indicated cells and treated with λ–phosphatase (λPPase) and detected as in **d**. (**f**) Cells were treated with caffeine (20 mM) and/or MMC (500 ng ml^{-1}) for 6 h or left untreated and analyzed by Phos-tag–containing gel and western blotting as in **d** using antibodies against GFP or CHK1. For Phos-tag western blots, long and short exposures are shown.

supported survival in the liquid culture assay (data not shown). We also found that the D×2a and the D×6 mutants were able to activate monoubiquitination of FANCD2 and FANCI, even without ICL damage (**Fig. 5c**).

The D×6 mutant cell line showed weak cisplatin sensitivity in the liquid culture assay (**Fig. 5b**). This could be due to the fact that, even though there is an increased level of ubiquitinated FANCD2 without DNA damage, the further induction of FANCD2 mono-ubiquitination upon damage seems to be blunted (**Figs. 3b** and **5c,** and **Supplementary Fig. 5a**). There is also a precedent in the literature that, in the setting of USP1 deficiency[28], persistent FANCD2 monoubiquitination results in low levels of DNA-damage sensitivity. Additionally, there is an indication that the FANCI-D×6 protein may have suboptimal function, because cells with combined D×6 and K525R mutations showed higher levels of ICL sensitivity than cells with either single mutant (data not shown). Collectively, results from the mutagenesis of the S/TQ sites suggest that, among the six sites in the S/TQ cluster, phosphorylation on the two most N-terminal sites (Ser558 and Ser561) may have a greater impact on FANCI function compared to the other sites. However, if simultaneously phosphorylated, the other four sites may deliver sufficient signal to activate downstream events such as monoubiquitination of FANCD2.

Constitutive foci formation of the FANCI-D×6 protein

Studying the localization of the phosphomimic mutants of FANCI, we found constitutive foci formation of the GFP–chFANCI-D×6 mutant without ICL treatment. These foci colocalized well with FANCD2 foci (**Fig. 5d**) but only partially localized with γH2AX or RAD51 foci[29] (**Supplementary Fig. 5b**). Upon MMC treatment, the percentage of GFP-labeled foci-positive cells was slightly increased with a modestly enhanced colocalization with γH2AX or RAD51 foci (**Fig. 5d** and **Supplementary Fig. 5b**). Furthermore, cell-fractionation studies revealed that, in cells expressing the D×6 protein, the FANCD2-L form constitutively localized in the chromatin fraction together with L form of the FANCI-D×6, whereas the A×6 mutant induced this relocalization much less efficiently even after MMC treatment (**Fig. 3b**). Collectively, these results suggest that FANCI phosphorylation is sufficient to induce chromatin accumulation and foci formation of FANCD2 and FANCI. We have previously shown that the D2KR-Ub fusion translocates to chromatin without need for additional DNA damage[15]. Our current results are in keeping with this, and they suggest that the machinery for chromatin loading of monoubiquitinated FANCD2 and FANCI is constitutively active and that phosphorylation is the key activating event in the pathway.

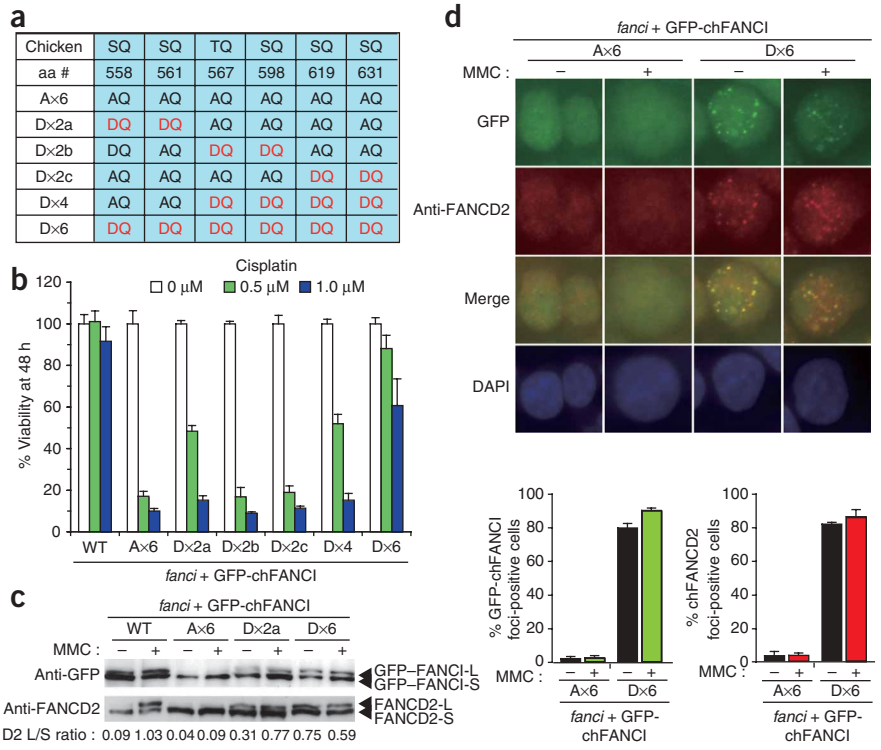

Figure 5 Phosphomimic mutants induce constitutive activation of the Fanconi anemia pathway. (**a**) Phosphomimic mutations in the S/TQ cluster in chFANCI. aa #, amino acid position number. (**b**) Phosphomimic mutations protected *fanci* cells against DNA damage induced by cisplatin. Cell survival was assessed as in **Figure 4b**. (**c**) Constitutive monoubiquitination of GFP-chFANCI and chFANCD2. Whole-cell lysates were separated and blotted using antibodies against GFP or chFANCD2. (**d**) GFP–chFANCI-A×6 or GFP–chFANCI-D×6 localization in cells with or without DNA damage. Fixed cells were stained with anti-FANCD2 antibodies and detected using fluorescence microscopy. The mean and s.e.m. of the percentage of FANCD2 or GFP-chFANCI foci-positive cells in three independent experiments are shown in the bar graph. Cells with more than four bright foci were defined as foci-positive. More than 100 cells were scored for each sample.

Human FANCI serves an evolutionarily conserved function

To examine the role of FANCI phosphorylation in human cells, we studied mutations in ten S/TQ motifs in the human FANCI protein. These included three sites known to be phosphorylated in the human protein (Ser730, Thr952 and Ser1121)[7], three sites consisting of the analogous human sites to known mouse phosphorylation sites (Ser556 and Ser559), a conserved site six residues downstream (Ser565) identified from alignments of vertebrate FancI proteins (**Supplementary Fig. 3**) and a cluster of four sites just downstream of these (Ser596, Ser617, Ser629 and Thr653) (summarized in **Supplementary Fig. 6** online). Proteins carrying various combinations of mutations were expressed in U2OS cells, where we previously showed that the constructs out-compete endogenous *FANCI* and exert a dominant effect[7], and assayed for their effects on FANCD2 foci formation and monoubiquitination in response to MMC treatment.

Simultaneous mutation of S730A, T952A and S1121A did not affect FANCD2 foci formation or ubiquitination (**Fig. 6**). However, the triple mutant of S556A S559A S565A resulted in greatly diminished FANCD2 foci formation, although faint foci were still visible after pre-extraction as well as a 50% reduction in FANCD2 monoubiquitination (**Fig. 6a**,**b**). Of the Ser596, Ser617, Ser629 and Thr653 sites mutated singly or simultaneously, only the S617A mutation resulted in a slight decrease in the intensity of DNA damage–induced FANCD2 foci and monoubiquitination (**Fig. 6** and **Supplementary Fig. 6**). The greatest effect on FANCD2 foci formation was seen in the FANCI–S556A S559A S565A S617A mutant (**Fig. 6a**). Additional FANCI mutations did not visibly affect FANCD2 localization (**Supplementary Fig. 6**). Expression of FANCI–S556A S559A S565A S596A S617A resulted in the lowest levels of FANCD2 monoubiquitination, with or without DNA damage. The fact that the basal level of FANCD2 monoubiquitination was diminished when this mutant was expressed indicates that phosphorylation is important during normal S phase and not only after DNA damage. These data indicated that FANCI phosphorylation exerts an evolutionarily conserved function in inducing FANCD2 monoubiqutination in human cells as well.

DISCUSSION

In this study, we have shown that multiple phosphorylation sites in the S/TQ cluster of FANCI are functionally important for inducing monoubiquitination of FANCD2, a crucial event in the Fanconi anemia pathway and ICL repair. These sites are evolutionarily and functionally conserved even in human cells, and some of them overlap with the sites that were detectably phosphorylated following ionizing irradiation damage[7,24]. Using Phos-tag technology and the mutant GFP-chFANCI proteins, we have shown that MMC-induced phosphorylation occurs at these sites in a caffeine-sensitive manner. Furthermore, introduction of phosphomimic mutations into merely six sites in chFANCI (that is, the D×6 mutant) can activate constitutive monoubiquitination and focus formation of FANCD2 and FANCI.

On the basis of the data presented here, we propose that FANCI phosphorylation functions as a molecular switch to turn on the Fanconi anemia pathway in response to DNA damage or replication fork stress. How is FANCI phosphorylation triggered following DNA damage? Several lines of evidence support the notion that the ATR kinase is involved. First, we found that FANCI phosphorylation is inhibited by caffeine treatment at a concentration that also inhibited CHK1 mobility shift (20 mM, as shown in **Fig. 4f**). Second, FANCI has been identified as a putative ATM and ATR substrate[7], and we found that multiple phosphorylations on the S/TQ cluster domain, the signature ATM and ATR motif, is important for FANCI function. Third, ATR has been previously implicated in inducing FANCD2 monoubiquitination through phosphorylation of FANCD2 (refs. 10,30). However, we found that FANCD2 carrying multiple alanine substitutions on the potential ATR sites (that is, ten S/TQ motifs including six conserved residues that correspond to Ser222, Ser319, Thr691, Ser705, Ser1079 and Ser1401 in the human FANCD2 protein) in chicken FANCD2 still efficiently reversed cisplatin hypersensitivity of *fancd2* cells (data not shown). Our data argue that the phosphorylation of FANCI, not FANCD2, has the most crucial role for induction of FANCD2 monoubiquitination. Of note, there remains a possibility that phosphorylation of FANCD2 or the

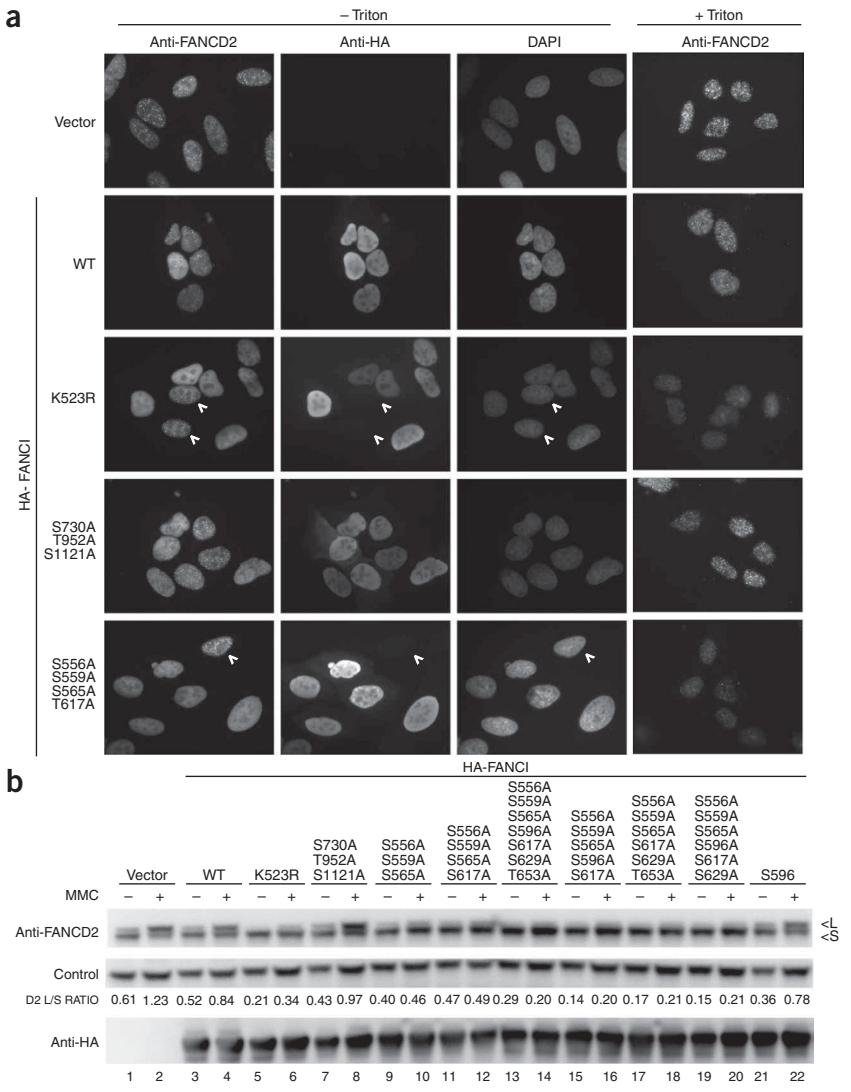

Figure 6 Analysis of phosphorylation mutants in human FANCI. (**a**) Localization of FANCD2 in U2OS cells expressing the indicated hemagglutinin (HA)-tagged FANCI alleles. Cells were treated with 1 μM MMC, and 24 h later were co-stained with antibodies against FANCD2 and the HA-tag. The white arrowheads indicate cells that show no expression of HA-tagged FANCI serving as an internal control for FANCD2 staining. Triton treatment removes most of the nucleoplasmic FANCD2, allowing for better visualization of FANCD2 foci. (**b**) Western analysis of FANCD2 in U2OS cells expressing HA-tagged FANCI alleles. Cells expressing the indicated alleles of FANCI were treated with 1 μM MMC and collected 24 h later. L indicates the long (monoubiquitinated) and S the short form of FANCD2.

core complex components, such as FANCE[31] or FANCM[32], has a minor role in the activation of the Fanconi anemia pathway. Indeed, caffeine treatment resulted in a mild decrease in FANCI monoubiquitination in cells expessing the D×6 mutant (**Supplementary Fig. 5a**). This could be due to a contribution by phosphorylation of FANCD2 or the core complex components.

It is unclear how phosphorylated FANCI promotes monoubiquitination of FANCD2 and itself, a process that must be mediated by the ubiquitin E3 ligase activity of the core complex. In other systems, such as in the Skp1–Cul1–F-box (SCF) E3 ligase complex, the E3 and the substrate often interact upon phosphorylation of the substrate, either by direct binding of the phosphoepitope with the E3 in a manner analogous to the phosphoprotein–SH2 domain interaction, or by exposing cryptic binding sites on the substrate via conformational change[33,34].

Because FANCI and FANCD2 interact with each other (forming the ID complex)[7,8], we tested whether stability of the ID complex is affected by FANCI phosphorylation using co-immunoprecipitation or tandem affinity purification (TAP) pull-down. We could not detect any significant alteration in the interaction between endogenous FANCD2 and GFP-chFANCI (wild type or mutant) in *FANCI*-deficient DT40 cells, both with or without MMC treatment (**Fig. 3b**), or between Flag- or TAP-tagged FANCD2 and GFP-chFANCI (wild type or mutant) transiently expressed in 293T cells (**Supplementary Fig. 7a,b** online). It is conceivable that phosphorylated FANCI somehow recruits FANCD2 to the core complex by interacting with a molecule that associates with the core complex or with a member of the complex itself. So far, we have not detected interaction of FANCI-D×6 with any of the known core complex components or the E2 enzyme UBE2T using the mammalian two-hybrid assay (data not shown). Notably, our current observation is perhaps the first indication that phosphorylation on one protein is necessary for the *in trans* monoubiquitination of its partner. It is important to elucidate how phosphorylated FANCI is involved in the precise molecular mechanism of the E3 ligase activation.

Although the monoubiquitination site on FANCI is exquisitely conserved through evolution, FANCI monoubiquitination seems to be largely dispensable for the function of the Fanconi anemia pathway in DT40 cells. The chFANCI-K525R mutant can support monoubiquitination, as well as focus formation and chromatin loading of FANCD2. We have confirmed this by making DT40 cells carrying the K525R knock in mutation (M.I. and M.T., unpublished results). The differences in the degree to which human and chicken cells require monoubiquitination of FANCI may have to do with the absolute amount of monoubiquitinated FANCD2 needed in their respective cells. The human FANCI-K523R mutant produces less FANCD2-L in response to MMC than the chFANCI-K525R mutant.

In conclusion, we propose that phosphorylation of FANCI on multiple sites may serve as a molecular switch to turn on the Fanconi anemia pathway upon DNA damage. Our data provide a basis for further elucidation of the mechanistic detail of the Fanconi anemia pathway, which should aid development of more rational therapeutics for Fanconi anemia and related conditions.

METHODS

Cells and plasmids. Wild-type and various mutant chicken DT40 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FCS, 1% (v/v)

chicken serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, penicillin and streptomycin in a 5% (v/v) CO_2 incubator at 39.5 °C. Generation of *fancd2*, *fancc*, *fancl* and *fancd2-K563R-knock in* cell lines has been described previously[21–23]. Targeting vectors for *FANCI* gene disruption were constructed by subcloning PCR-amplified genomic fragments on both sides of the resistance gene cassettes. Transfections and selection of the clones were done as previously described[35]. Full-length chicken *FANCD2* or *FANCI* cDNA was cloned into FLAG- or TAP-tag expression vectors or pEGFP-C1 (Clontech). The plasmid for TAP-tag, which consists of calmodulin binding protein and a part of Protein A, was provided by T. Natsume (National Institute of Advanced Industrial Science and Technology, Tokyo). Mutations were introduced using site-directed mutagenesis kits (Stratagene). 293T cells were maintained in DMEM supplemented with 10% (v/v) FCS as described[36] and transfected using Lipofectamine2000 (Invitrogen).

Analysis of cell sensitivity toward ICL treatments. Cell viability in liquid culture containing cisplatin (Nippon Kayaku) was assessed after 48 h using FACSCalibur (BD) and propidium iodide staining. Percentage of viable cells was calculated on the basis of forward-scatter profile and exclusion of propidium iodide fluorescence among the acquired 10,000 events. Colony-formation assays were carried out in medium containing 1.4% (w/v) methylcellulose and the indicated dosages of cisplatin. Chromosomal analysis following MMC (Kyowa Hakkou) treatment was done as described[35].

Subnuclear focus-formation assay. After MMC exposure (500 ng ml^{-1} for 6 h), cytospin slides were fixed and stained with antibodies against chicken FANCD2 (provided by K. Komatsu, Kyoto University) or human RAD51 (provided by H. Kurumizaka, Waseda University). To detect subnuclear γH2AX foci, fixed cells were further treated with 70% (v/v) ethanol and stained with anti-γH2AX monoclonal antibody (UBI) as described[37]. Then cells were stained with fluorescein isothiocyanate (FITC)- or Alexa Fluor 594–conjugated secondary antibody (Invitrogen) with DAPI counterstaining. Images were captured by fluorescent microscopy (Axioplan2 equipped with AxioCam MRm and Axiovision, Zeiss) or confocal laser-scanning microscopy (LSM510META, Zeiss).

Fractionation of cells, immunoprecipication and western blotting. Cells were treated with MMC (500 ng ml^{-1} for 6 h) or left untreated, unless stated otherwise. Samples were separated by PAGE, transferred to a nitrocellulose membrane, and detected with antibodies against GFP (MBL), chicken FANCD2 or CHK1 (SantaCruz), and ECL Plus reagents (GE Healthcare). For immunoprecipitation, cells were lysed in lysis buffer[23] containing phosphatase inhibitor cocktail (Nacalai Tesque) or were fractionated into soluble and chromatin fractions as described previously[15], and precipitated using anti-GFP (MBL) or anti-Flag (Sigma) beads. TAP-tagged chFANCD2 was pulled down using rabbit IgG beads (Sigma). After extensive washing, the immunoprecipitates were treated with λ-phosphatase (NEB) and analyzed by western blotting. Phos-tag–containing polyacrylamide gels were made with Phos-tag acrylamide (3.5 μM; Phos-tag Consortium) and 7 μM $MnCl_2$ to the separation gel[38]. Densitometric analysis of the band intensity was carried out using ImageJ software (http://rsbweb.nih.gov/ij/).

Methods for human U2OS cell experiments. U2OS cells were grown in DMEM supplemented with 100 U ml^{-1}penicillin, 0.1 mg ml^{-1}streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids and 10% (v/v) FBS (Invitrogen). The QuikChange multisite-directed mutagenesis kit (Stratagene) was used to generate mutations in human FANCI. The FANCI mutant cDNAs were introduced into U2OS cells using the lentiviral vector pHAGE_CMV_IP_HAFLAG (J. Lin and J.W. Harper, Harvard Medical School). Antibodies were rabbit anit-FANCD2 (Novus) and anti-hemagluttinin (Covance). For immunofluorescence, cells grown on autoclaved cover slips were rinsed in PBS and fixed in 3.7% (w/v) formaldehyde (Sigma) diluted in PBS for 10 min at room temperature (20–25 °C). Cells were washed once with PBS, permeabilized in 0.5% (v/v) Nonidet P-40 in PBS for 10 min, washed again in PBS and blocked with PBG (0.2% (w/v) cold fish gelatin, 0.5% (w/v) BSA in PBS) for 20 min. Cover slips were incubated for 2 h at room temperature or at 4 °C overnight in a humidified chamber with a primary antibody, and after washing three times for 5 min in PBG, were incubated with the appropriate secondary antibody. After three additional washes in PBG, the coverslips were embedded in Vectashield (Vector Laboratories) supplemented with DAPI. Triton X-100 pre-extraction was performed by incubating cells for 5 min at room temperature with 0.5% (v/v) Triton X-100 in PBS. Cells were fixed and processed as above. Images were captured with Axioplan2 Zeiss microscope with a AxioCam MRm Zeiss digital camera supported by Axiovision 4.6 software.

Accession codes. DNA Data Bank of Japan: Data for chicken *FANCI* cDNA has been deosited in the DDBJ database with accession code AB378696.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

M.T., A. Smogorzewska and S.J.E. conceived and designed the experiments; M.I., H.K., A. Saberi and E.U. performed most of the DT40 cell experiments; M.I., A.K. and S.T. performed microscopic analysis; E.K., E.K.-K., T.K. and J.T. designed and performed Phos-tag experiments; A. Smogorzewska performed human cell experiments; M.I. and H.K. analyzed the DT40 cell data; M.T., A. Smogorzewska and S.J.E. wrote the paper.

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1. Hoeijmakers, J.H. Genome maintenance mechanisms for preventing cancer. *Nature* **411**, 366–374 (2001).
2. Nijnik, A. *et al.* DNA repair is limiting for haematopoietic stem cells during ageing. *Nature* **447**, 686–690 (2007).
3. Ito, K. *et al.* Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* **431**, 997–1002 (2004).
4. Rossi, D.J. *et al.* Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* **447**, 725–729 (2007).
5. D'Andrea, A.D. & Grompe, M. The Fanconi anaemia/BRCA pathway. *Nat. Rev. Cancer* **3**, 23–34 (2003).
6. Wang, W. Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. *Nat. Rev. Genet.* **8**, 735–748 (2007).
7. Smogorzewska, A. *et al.* Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell* **129**, 289–301 (2007).
8. Sims, A.E. *et al.* FANCI is a second monoubiquitinated member of the Fanconi anemia pathway. *Nat. Struct. Mol. Biol.* **14**, 564–567 (2007).
9. Dorsman, J.C. *et al.* Identification of the Fanconi anemia complementation group I gene, FANCI. *Cell. Oncol.* **29**, 211–218 (2007).
10. Andreassen, P.R., D'Andrea, A.D. & Taniguchi, T. ATR couples FANCD2 monoubiquitination to the DNA-damage response. *Genes Dev.* **18**, 1958–1963 (2004).
11. Machida, Y.J. *et al.* UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. *Mol. Cell* **23**, 589–596 (2006).
12. Ciccia, A. *et al.* Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. *Mol. Cell* **25**, 331–343 (2007).
13. Ling, C. *et al.* FAAP100 is essential for activation of the Fanconi anemia-associated DNA damage response pathway. *EMBO J.* **26**, 2104–2114 (2007).
14. Taniguchi, T. *et al.* S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. *Blood* **100**, 2414–2420 (2002).
15. Matsushita, N. *et al.* A FancD2-monoubiquitin fusion reveals hidden functions of Fanconi anemia core complex in DNA repair. *Mol. Cell* **19**, 841–847 (2005).
16. Nijman, S.M. *et al.* The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. *Mol. Cell* **17**, 331–339 (2005).
17. Howlett, N.G. *et al.* Biallelic inactivation of *BRCA2* in Fanconi anemia. *Science* **297**, 606–609 (2002).

18. Xia, B. *et al.* Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol. Cell* **22**, 719–729 (2006).
19. Cantor, S.B. *et al.* BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell* **105**, 149–160 (2001).
20. Levitus, M. *et al.* Heterogeneity in Fanconi anemia: evidence for two new genetic subtypes. *Blood* **103**, 2498–2503 (2004).
21. Seki, S. *et al.* A requirement of FancL and FancD2 monoubiquitination in DNA repair. *Genes Cells* **12**, 299–310 (2007).
22. Yamamoto, K. *et al.* Fanconi anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. *Mol. Cell. Biol.* **25**, 34–43 (2005).
23. Hirano, S. *et al.* Functional relationships of FANCC to homologous recombination, translesion synthesis, and BLM. *EMBO J.* **24**, 418–427 (2005).
24. Matsuoka, S. *et al.* ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**, 1160–1166 (2007).
25. Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K. & Koike, T. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol. Cell. Proteomics* **5**, 749–757 (2006).
26. Zou, L. & Elledge, S.J. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* **300**, 1542–1548 (2003).
27. Sarkaria, J.N. *et al.* Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res.* **59**, 4375–4382 (1999).
28. Oestergaard, V.H. *et al.* Deubiquitination of FANCD2 is required for DNA crosslink repair. *Mol. Cell* **28**, 798–809 (2007).
29. West, S.C. Molecular views of recombination proteins and their control. *Nat. Rev. Mol. Cell Biol.* **4**, 435–445 (2003).
30. Ho, G.P., Margossian, S., Taniguchi, T. & D'Andrea, A.D. Phosphorylation of FANCD2 on two novel sites is required for mitomycin C resistance. *Mol. Cell. Biol.* **26**, 7005–7015 (2006).
31. Wang, X. *et al.* Chk1-mediated phosphorylation of FANCE is required for the Fanconi anemia/BRCA pathway. *Mol. Cell. Biol.* **27**, 3098–3108 (2007).
32. Meetei, A.R. *et al.* A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. *Nat. Genet.* **37**, 958–963 (2005).
33. Hunter, T. The age of crosstalk: phosphorylation, ubiquitination, and beyond. *Mol. Cell* **28**, 730–738 (2007).
34. Harper, J.W. & Elledge, S.J. The DNA damage response: ten years after. *Mol. Cell* **28**, 739–745 (2007).
35. Yamamoto, K. *et al.* Fanconi anemia FANCG protein in mitigating radiation- and enzyme-induced DNA double-strand breaks by homologous recombination in vertebrate cells. *Mol. Cell. Biol.* **23**, 5421–5430 (2003).
36. Ishiai, M. *et al.* DNA cross-link repair protein SNM1A interacts with PIAS1 in nuclear focus formation. *Mol. Cell. Biol.* **24**, 10733–10741 (2004).
37. Kitao, H. *et al.* Functional interplay between BRCA2/FANCD1 and FANCC in DNA repair. *J. Biol. Chem.* **281**, 21312–21320 (2006).
38. Tomida, J., Kitao, H., Kinoshita, E. & Takata, M. Detection of phosphorylation on large proteins by western blotting using Phos-tag containing gel. *Nat. Protocols* doi:10.1038/nprot.2008.232 (in the press).

FANCI phosphorylation functions as a molecular switch to turn on the Fanconi anemia pathway

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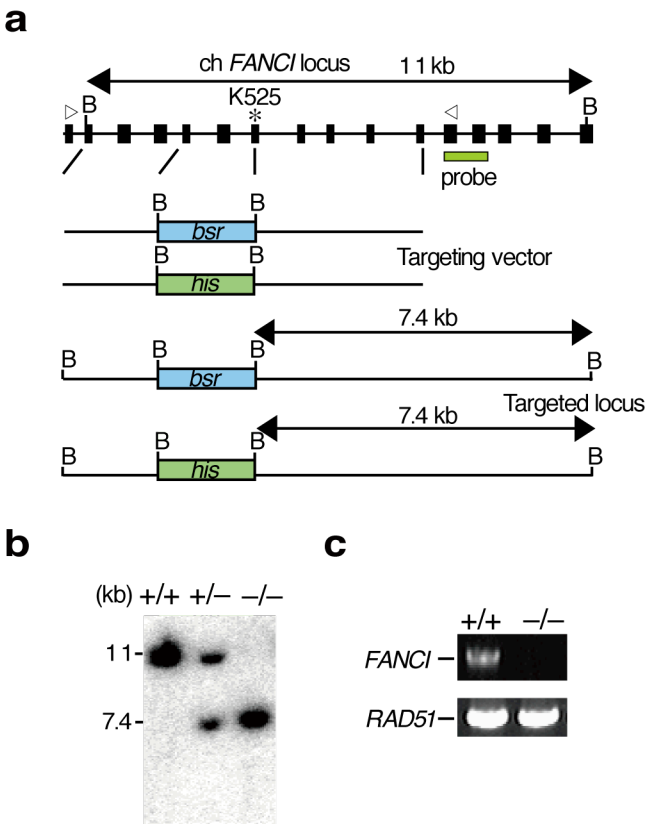
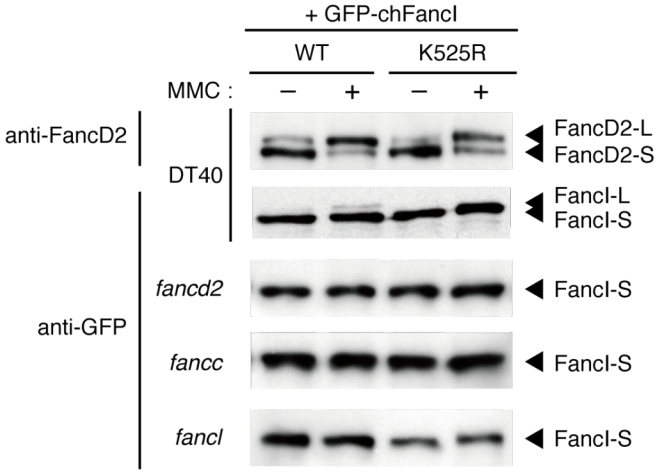


Supplementary Figure 1. Gene targeting of chicken *FANCI* locus. (**a**) A schematic of the *FANCI* gene targeting strategy. Black boxes indicate position of the exons, with an asterisk on the putative monoubiquitination site Lys525. Open triangles indicate positions of the primers used in **c**. B, *Bam*HI. (**b**) Southern blot analysis of *Bam*HI-digested genomic DNA from cells with the indicated genotypes. (**c**) RT-PCR analysis of *FANCI* mRNA expression. *RAD51* was used as control.

a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WT
K525R
MMC :
–
+
–
+
anti-FancD2
DT40
FancD2-L
FancD2-S
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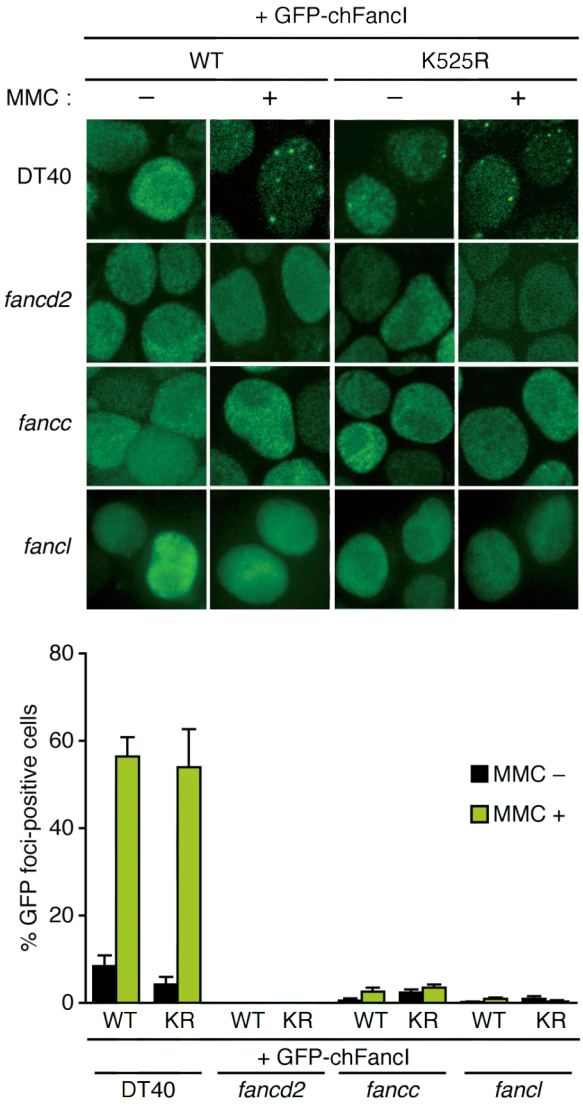
fancl
FancI-S

b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WT
K525R
MMC :
–
+
–
+
DT40
fancd2
fancc
fancl
% GFP foci-positive cells
80
60
40
20
0
MMC –
MMC +
WT
KR
WT
KR
WT
KR
WT
KR
+ GFP-chFancI
DT40
fancd2
fancc
fancl

Supplementary Figure 2. FancI monoubiquitination and focus formation in FA mutant DT40 cells. Cells were stably transfected with GFP-chFancI WT or K525R vector, and examined for (**a**) monoubiqutination of FancD2 or GFP-chFancI, or (**b**) foci formation of GFP-chFancI. The mean and standard error (s.e.m.) of %GFP-chFancI foci-positive cells in three independent experiments are shown in the bar graph. More than 100 cells were scored in each experiment. Foci positive cells were defined as cells having more than four bright foci.

```
HUMAN_I             ------MDQKILSLAAEKTADKLQEFLQTLREGDLTNLLQNQAVKGKVAGALLRAIFKGS 54
MOUSE_I             ------MDLKILSLATDKTTDKLQEFLQTLKDDDLASLLQNQAVKGRAVGTLLRAVLKGS 54
CHICKEN_I           ------MAQRILQLAAEGSPERLQEALQGLTEGELGDMVTRQALRGRETAALLKGIFKGS 54
FISH_I              MSKMKVTVDSILSLSETDDGDELQKHLTLLSDDQLTTMLTNSALKGKDTGILIKAIFKGS 60
FROG_I              ------MDQKILSLAAEEQNDGLQSYLQNLKETELSEIITKHAVKGKDCGALLRGVFKGS 54
                          **.*:     : **. *  * : :*  :: . *::*:  . *:*.::***

HUMAN_I             PCSEEAGTLRRRKIYTCCIQLVESGDLQKEIVSEIIGLLMLEAHHFPGPLLVELANEFIS 114
MOUSE_I             PCSEEDGALRRYKIYSCCIQLVESGDLQQDVASEIIGLLMLEVHHFPGPLLVDLASDFVG 114
CHICKEN_I           PCSQQSGVLRRLQVYKHCVSLVESGDLHVGKVSEIIGLLMLEARQLPGHALAELATLFVE 114
FISH_I              PVSVSHGANRRLLVYKHCIPLCESGDLQTEVASDIIGLLMLDTHSLPGPALATLASLYVD 120
FROG_I              PCSHDVAVRRRLAVYRHCIQLVESGDLQREVASEIMGLLMLEVHHFPGASLIELANLFAD 114
                    * * . .. **  :*  *: * *****:   .*:*:*****:.: :**  *  **. :

HUMAN_I             AVREGSLVNGKSLELLPIILTALATKKENLAYGKGVLSGEECKKQLINTLCSGRWDQQYV 174
MOUSE_I             AVREDRLVNGKSLELLPIILTVLATKKEVLACGKGDLNGEEYKRQLIDTLCSVRWPQRYM 174
CHICKEN_I           VIKRGSLSNGKSLELFSTVLTALSNSKESLAYGKGELNGEEFKKQLINTLCSSKWDPQCV 174
FISH_I              AIKVGEMNSGRSLELFPTILTALS-ATDALAYGKGELTGNEYKKQLINSLCSSRWDPQCV 179
FROG_I              AVKAGTLSNGKSLDLFPTVLTALS-SKESLAYGKGELTGEEFKKQLINSLCSSRWDPQSV 173
                    .:: . : .*:**:*:. :**.*:  .: ** *** *.*:* *:***::*** :*  : :

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MOUSE_I             IQLTSVFKDVCLTPEEMNLVVAKVLTMFSKLNLQEIPPLVYQLLVLSSKGSRRSVLDGII 234
CHICKEN_I           IHLANMFRDIPLSGEELQFVVEKVLRMFSKLDLQEIPPLVYQLLLLSAKGSKKTVLEGII 234
FISH_I              IHLTTMFRDVPLSTEELQFLIEKILRMFLKLDLQEIPPLVYQLLLLSAKGCKKLVLEGII 239
FROG_I              IFLTSMFRDVSLSAEELQFVVEKVLRMLSKLDLQEMPPLVYQLLLLSAKGSKKNIVEGII 233
                    * *:.:*:*: *: **::::: * * *: *::***:********:**:**.:: :::***

HUMAN_I             AFFSALDKQHNEEQSGDE--LLDVVTVPSGELRHVEGTIILHIVFAIKLDYELGRELVKH 292
MOUSE_I             AFFRELDKQHREEQSSDE--LSELITAPADELYHVEGTVILHIVFAIKLDCELGRELLKH 292
CHICKEN_I           SFFNQLDKRQKEEQRVPQSADLEVATVPLDQLRHVEGTVILHIVSAINLDQDIGEELIKH 294
FISH_I              NYFKKQDQLQKEEQRNGECEDVEVQTIPQDQLRHVEGTVILHIVFAIRLDHELGREFFKN 299
FROG_I              TIFNDLDQKQLVQQENSESLDLEDATIPQDQLHHVEGTIILHIVFAIKLDQELGRELLKY 293
                      *   *: :  :*   :    :  * * .:* *****:***** **.** ::*.*:.*

HUMAN_I             LKVGQQGDSNNNLSPFSIALLLSVTRIQRFQDQVLDLLKTSVVKSFKDLQLLQGSKFLQN 352
MOUSE_I             LKAGQQGDPSKCLCPFSIALLLSLTRIQRFEEQVFDLLKTSVVKSFKDLQLLQGSKFLQT 352
CHICKEN_I           LKTEQQKDPGKALCPFSVSLLLSTAVKHRLQEQIFDFLKTSITRSCKDLQILQASKFLQD 354
FISH_I              LKVAQ----SDPLCPFSIALLLSVARIQRYEEQVFEFLKGAITKNFKDDQIQNSSKFLQD 355
FROG_I              LKAGQQGDSSKILCPFSAALLLSVSRIHRFQEQVFDFLKSTILRDYKDLQFHQSSKFLQD 353
                    **. *    .. *.*** :**** :  :* ::*::::** :: :. ** *: :.*****

HUMAN_I             LVPHRSYVSTMILEVVKNSVHSWDHVTQGLVELGFILMDSYGPKKVLDGKTIETSPSLSR 412
MOUSE_I             LVPQRTCVSTMILEVVRNSVHSWDHVTQGLIEFGFILMDSYGPKKILDGKAVEIGTSLSK 412
CHICKEN_I           LCPQQYDVTAVILEVVKNSAFGWDHVTQGLVDLGFSLMESYEPKKSFGGKAAETNLGLSK 414
FISH_I              LLPQFSSVSDMILDTVKNSVFGWDHVTQGLVQLGFILMDSFGPKPGPFGKVTEGTSSTAK 415
FROG_I              LVPQPVCVSSILLETVKNSVYGWDHVTQGLVELGFILMDSFGPKAAFGNKVVELISVHSR 413
                    * *:   *: ::*:.*:**...********:::** **:*: **    .*. *     ::

HUMAN_I             MPNQHACKLGANILLETFKIHEMIRQEILEQVLNRVVTRASSPISHFLDLLSNIVMYAPL 472
MOUSE_I             MTNQHACKLGANILLETFKIHEMIRQEILEQVLNRVVTRTSSPINHFLDLFSDIIMYAPL 472
CHICKEN_I           MPAQQACKLGASILLETFKVHEPIRSDILEQVLNRVLTKAASPVSHFIDLLSNIVVSAPL 474
FISH_I              TPNQLACRLGGQVLLESFKMHEPIRGEILEQVLNRLVTKTASPVTHFIDLLSSIVLSAPM 475
FROG_I              TPSQQACQLGSRILLETFKVHEPIRSEILEQVLNRVITKAATPVTHFIDLLSDIVVSAPL 473
                     . * **:**. :***:**:** ** :********::*::::*:.**:**:*.*:: **:

HUMAN_I             VLQSCSSKVTEAFDYLSFLPLQTVQRLLKAVQPLLKVSMSMRDCLILVLRKAMFANQLDA 532
MOUSE_I             ILQNCS-KVTETFDYLTFLPLQTVQGLLKAVQPLLKISMSMRDSLILVLRKAMFASQLDA 531
CHICKEN_I           VLQNSSSRVTETFDNLSFLPIDTVQGLLRAVQPLLKVSMSVRDSLILVLQKAIFSRQLDA 534
FISH_I              ILLESSSKVTETFDQLSYLPLSTVQGLLKAVQPLLKVSMSMKDALILVLRKAMFSSHLDG 535
FROG_I              ILQNSSSKVTEAFDHLSFLPLTTVQGLLKAVQPLLKISMSMRDSLILVLRKAMFSSQIDA 533
                    :* ..* :***:** *::**: *** **:*******:***::*.*****:**:*: ::*.
                                        * *

HUMAN_I             RKSAVAGFLLLLKNFKVLGSLSSSQCSQSLSVSQVHVDVHSHYNSVANETFCLEIMDSLR 592
MOUSE_I             RKSAVAGFLLLLKNFKVLGSLPSSQCTQSIGVTQVRVDVHSRYSAVANETFCLEIIDSLK 591
CHICKEN_I           RKAAVAGFLLLLRNFKILGSLTSSQCSQAIGATQVQADVHACYNSAANEAFCLEILGSLR 594
FISH_I              RKSAVAGFLLLLKNFRILGSLASSQASQAITSSQVQADVHSRYNTAANEAFCLEILSSLR 595
FROG_I              RKSAVAGFLLLLRNFKVLGSLSSSQCSQAIGASQIQVDVHMRYNAAANEAFCLEILGSLR 593
                    **:*********:**::****.****.:*::  :*::.***  *.:.***:*****:.**:

HUMAN_I             RCLSQQADVRLMLYEGFYDVLRRNSQLANSVMQTLLSQLKQFYEPKPDLLPPLKLEACIL 652
MOUSE_I             RSLGQQADIRLMLYNGFYDVLRRNSQLASSIMQTLFSQLKQFYEPEPDLLPPLKLGACVL 651
CHICKEN_I           RCLSQQADVRLMLYEGFYDVLRRNSQLASSIMETLLSQIKQYYLPQQDLLPPLKLEGCIM 654
FISH_I              RCLNQQADVRLMLYEGFHDVLRRNSQLASSITQTLLSQLKRYFEPEQDLLPPVKLESCIS 655
FROG_I              RCLSQQADVRLMLYEGFYDVLRRNSQLASSVMQTLLSQLKRYYEPEPDLLPPLKLEGCIT 653
                    *.*.****:*****:**:*******.*: :**:**:*::: *: *****:** .*:
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HUMAN_I             TQGDKISLQEPLDYLLCCIQHCLAWYKNTVIPLQ--QGEEEEEEEEAFYEDLDDILESIT 710
MOUSE_I             TQGSQIFLQEPLDHLLSCIQHCLAWYKSRVVPLQ--QGDEGEEGEEELYSELDDMLESIT 709
CHICKEN_I           AQGDQIFLQEPLAHLLCCIQHCLAWYKSTVHLCKGAEDEEEEEDVG-FEQNFEEMLESVT 713
FISH_I              AQGDQVFLQEPLAHLLCCTVHCLLWNQNVRSGGNVSDDEDDEEDVGGVESELQAMLESIT 715
FROG_I              AQGDHIFLQEPLAHLLCCIHHCLQWYKSSLQQHRNPDEDDDDDDQTGCQQDLNDIMESIT 713
                    :**.:: ***** :**.*  *** * :.     .  : :: ::      .::: ::**:*
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HUMAN_I             NRMIKSELEDFELDKSADFSQSTSIGIKNNISAFLVMGVCEVLIEYNFSISSFSKNRFED 770
MOUSE_I             VRMIKSELEDFELDKSADFSQNTNVGIKNNICACLIMGVCEVLMEYNFSISNFSKSKFEE 769
CHICKEN_I           RRMIKSELEDFELDKSADFSPSSGVGVKNNIYAIQVMGICEVLIEYNFKIGNFSKNKFED 773
FISH_I              KRMIKSELEDFELDKCAEFSTGSSVGVKNSIYAVLVMGLNEVLMEYNFITANYSKNHFED 775
FROG_I              RRMIKCDLEDFELDKSADFSLASGVGVKNNIYAVLVMGICEVLIEYNFIIANFSKSKFED 773
                     ****.:********.*:**  :.:*:**.* *  :**: ***:****  ..:**.:**:

HUMAN_I             ILSLFMCYKKLSDILNEKAGKAKTKMANKTSDSLLSMKFVSSLLTALFRDSIQSHQESLS 830
MOUSE_I             ILSLFTCYKKFSDILSEKAGKGKAKMASKASDSLLSLKFVSDLLTALFRDSIQSHEESLS 829
CHICKEN_I           VLGLFTCYNKLSEILKEKAGKNKSTLGNRIARSFLSMGFVSTLLTALFRDNAQSHEESLA 833
FISH_I              VLELFKRYLKVCDILRERAGKGR-PASSKTPRSLLSLGFVSTLLTALFRDSTQSREESLS 834
FROG_I              ILGLFKCCSKLSDILKEKAAKGRQPGNNKTARSLISMTFVSTLLTALFRDSTRSHEESLS 833
                    :* **    *..:** *:*.* :    .: . *::*: *** ********. :*::***:

HUMAN_I             VLRSSNEFMRYAVNVALQKVQQLKETGHVSGPDGQNPEKIFQNLCDLTRVLLWRYTSIPT 890
MOUSE_I             VLRSSGEFMHYAVNVALQKIQQLIRTGHVSGPDGQNPDKIFQNLCDITRVLLWRYTSIPT 889
CHICKEN_I           VLRSSTEFMRYAVSVALQKVQQLEEMGQTDGPDGQNPEKMFQNLCKITRVLLWRYTSIPT 893
FISH_I              VLRSNGDFLRYSVSVALQKIQQLEETGHTDGPEGQSPDKTFRHLCDITSVLMWRYTNVPS 894
FROG_I              ILRANVDFMRYSVCVALQKIQQLEETGVTDGPDGQNSEKMFRSLCEITRVLMWRYTSIPA 893
                    :**:. :*::*:* *****:*** . * ..**:**..:* *: **.:* **:****.:*:

HUMAN_I             SVEESGKKEKGKSISLLCLEGLQKIFSAVQQFYQPKIQQFLRALDVTDKEGEEREDADVS 950
MOUSE_I             SVEESGKKEKGKSISLLCLEGLQKTFSVVLQFYQPKVQQFLQALDVMGTEEEE---AGVT 946
CHICKEN_I           AVEESGKK-KGKSISLLCLEGLLRIFNTMQQLYAARIPQFLQALDITD-GDAEEAD--IN 949
FISH_I              TVEDAGKKEKGQSVSLLCLEGLLRVFTTVLQRYPTRVSNFLSSLDVSG-EGEGDKS---D 950
FROG_I              AAENPGKKDKGKTISLLCLEGLLRVFNTVQQRYPSKIPQFLTALDALG-DEDEEGSREIN 952
                    :.*:.*** **:::******** : *..: * * .:: :** :**  .
                      *

HUMAN_I             VTQRTAFQIRQFQRSLLNLLSSQEEDFNSKEALLLVTVLTSLSKLLEPSSPQFVQMLSWT 1010
MOUSE_I             VTQRASFQIRQFQRSLLNLLSSEEDDFNSKEALLLIAVLSTLSRLLEPTSPQFVQMLSWT 1006
CHICKEN_I           VTEKAAFQIRQFQRSLVNQLSSAEDDFNSKETQLLITILSTLSKLLDPGSQQFLQFLTWT 1009
FISH_I              LTEQTAFYIRQFQRALMNQLSGPEEEFNSKEAQLLVSILSVLSRQLSPSSQQFLQMITWT 1010
FROG_I              VTEKAAFQIKQFQRSLINQLSGGEDDFNSKEALLLVSILSTLSRLLAPSSQQFVQMLSWT 1012
                    :*::::* *:****:*:* **. *::*****: **:::*: **: * * * **:*:::**

HUMAN_I             SKICKENSREDALFCKSLMNLLFSLHVSYKSPVILLRDLSQDIHGHLGDIDQDVEVEKTN 1070
MOUSE_I             SKICKEYSQEDASFCKSLMNLFFSLHVLYKSPVTLLRDLSQDIHGQLGDIDQDVEIEKTD 1066
CHICKEN_I           VKICKENALEDLSCCKGLLTLLFSLHVLYKSPVSLLRELAQDIHACLGDIDQDVEIESRS 1069
FISH_I              VKICKETNFEDIALTKGLLSLLFSLYVLHKSPVSLLWEMCQDIHSQLGDIDQDVEVEKQS 1070
FROG_I              VKICKETNIEDVQFCKGIMNLLFSLHVQFKSPVSVLRELCQDIHGHLGDIDQDIEVEKQS 1072
                     *****   **    *.::.*:***:* .**** :* ::.****. *******:*:*. .
                                                                          *

HUMAN_I             HFAIVNLRTAAPTVCLLVLSQAEKVLEEVDWLITKLKGQVSQETLS-----EEASSQATL 1125
MOUSE_I             HFAVVNLRTAAPTVCLLVLSQAEKVLEEVDWLIAKIKGSANQETLSDKVTPEDASSQAVP 1126
CHICKEN_I           HFAIVNVKTAAPTVCLLVLGQADKVLEEVDWLIKRLT-ILGSD--TS-----EDSTQASN 1121
FISH_I              HFAIICMKTAAS-TTLQVLSQVGKVLDEVDWLIAKKKAQISSDRTIS-----GNTQQPLG 1124
FROG_I              HFATVSLKTAAPTVTLLVLGQAAKVLEEVDWLIIKLKGLLGSEKLST-----EDLTQTSN 1127
                    *** : ::***. . * **.*. ***:****** : .   ..:            *.

HUMAN_I             PNQPVEKAIIMQLGTLLTFFHELVQTALPSGSCVDTLLKDLCKMYTTLTALVRYYLQVCQ 1185
MOUSE_I             PTLLIEKAIVMQLGTLVTFFHELVQTALPSGSCVDTLLKGLSKIYSTLTAFVKYYLQVCQ 1186
CHICKEN_I           QTQALEKGVILQLGTLLTVFHELVQTALPAGSCVDSLLRSLSKTYAILTSLIKHYIQACR 1181
FISH_I              QQDPIEKAITIQLGTLLTALHELVQTALPTGACTDTLMRELSRTYCILTTLTKYYIQLCA 1184
FROG_I              ARVPIEKAIILQLGTLLTACHELVQTALPAGSCTDTLLKELAKMYTILTSVVKYYLQICS 1187
                        :**.: :*****:*  *********:*:*.*:*:: *.: *  **:. ::*:* *

HUMAN_I             SSGG-IPKNMEKLVKLSGSHLTPLCYSFISYVQN---KSKSLNYTGEKKEKPAVVATAM- 1240
MOUSE_I             SSRG-IPNTVEKLVKLSGSHLTPVCYSFISYVQN---KSSDAPKCSEKEK--AAVNTTM- 1239
CHICKEN_I           STSNTVPGRLEKLVKLSGSHLTPQCYSFITYVQNIHSESLSFAEEKKKKKKEDETAVVST 1241
FISH_I              TQPGQLPARLEKLVKLSGSHLTPQCYCFITYVQSGELTAGGSEKLKKKKKEEEAVAAAS- 1243
FROG_I              SHGGQISARLEKLVKLSGSHLTPQCYAFITYVQNIHSESLSLAAEKKKKKKEEALTVTS- 1246
                    :  . :.  :************** **.**:***.    : .     :*::      ..

HUMAN_I             --ARVLRETKPIPNLIFAIEQYEKFLIHLSKKSKVSLMQHMKLSTSRDFKIKGNILDMVL 1298
MOUSE_I             --AKVLRETKPIPNLVFAIEQYEKFLIQLSKKSKVNLMQHMKLSTSRDFKIKGSVLDMVL 1297
CHICKEN_I           VMAKVLRDTKPIPNLIFAIEQYEKFLIHLSKKSKVNLMQYMKLSTSRDFRINASMLDSVL 1301
FISH_I              --AKVLRETKAIPNLIFNIEQYEKFLILLSKKSKVNLMQYMKLSTSRDFRINAATLEAAL 1301
FROG_I              --AKILRDTKPIPNLIFAIEQYEKFLIHLSKKSKVNLMQYMKLSTSRDFRINAATLDAAL 1304
                      *::**:**.****:* ********* *******.***:*********:*:.  *: .*
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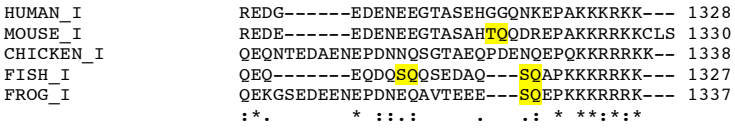
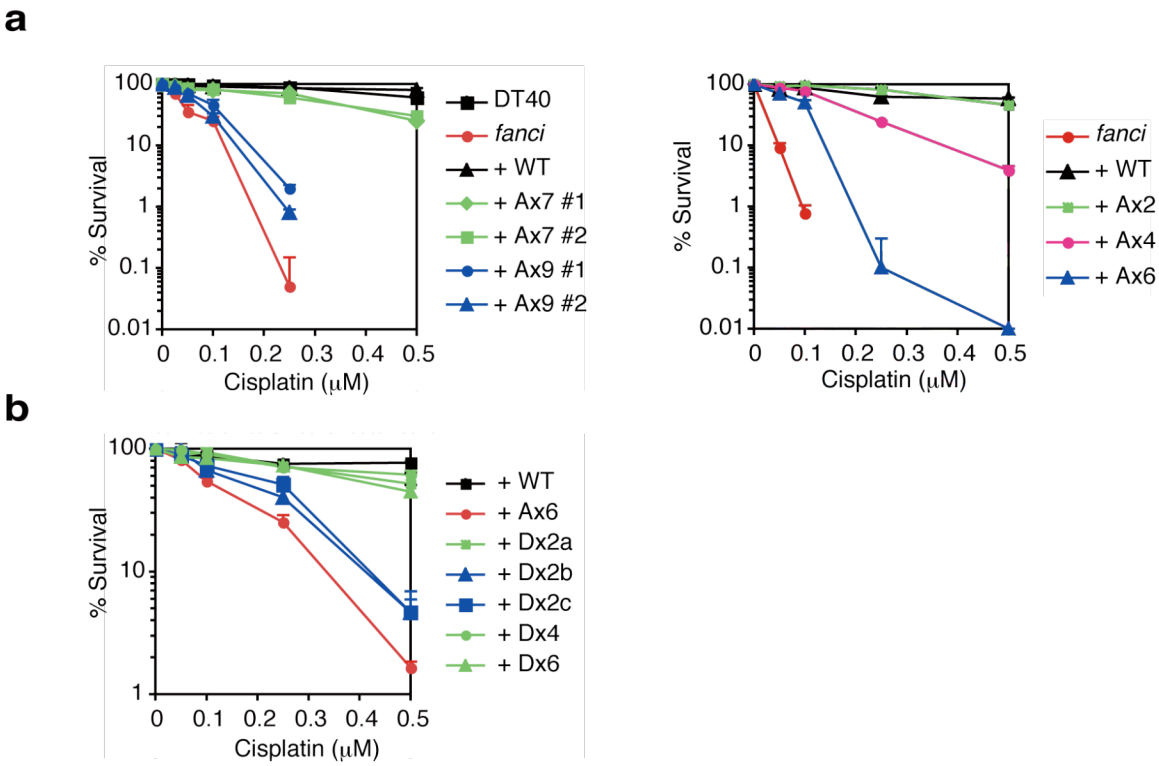
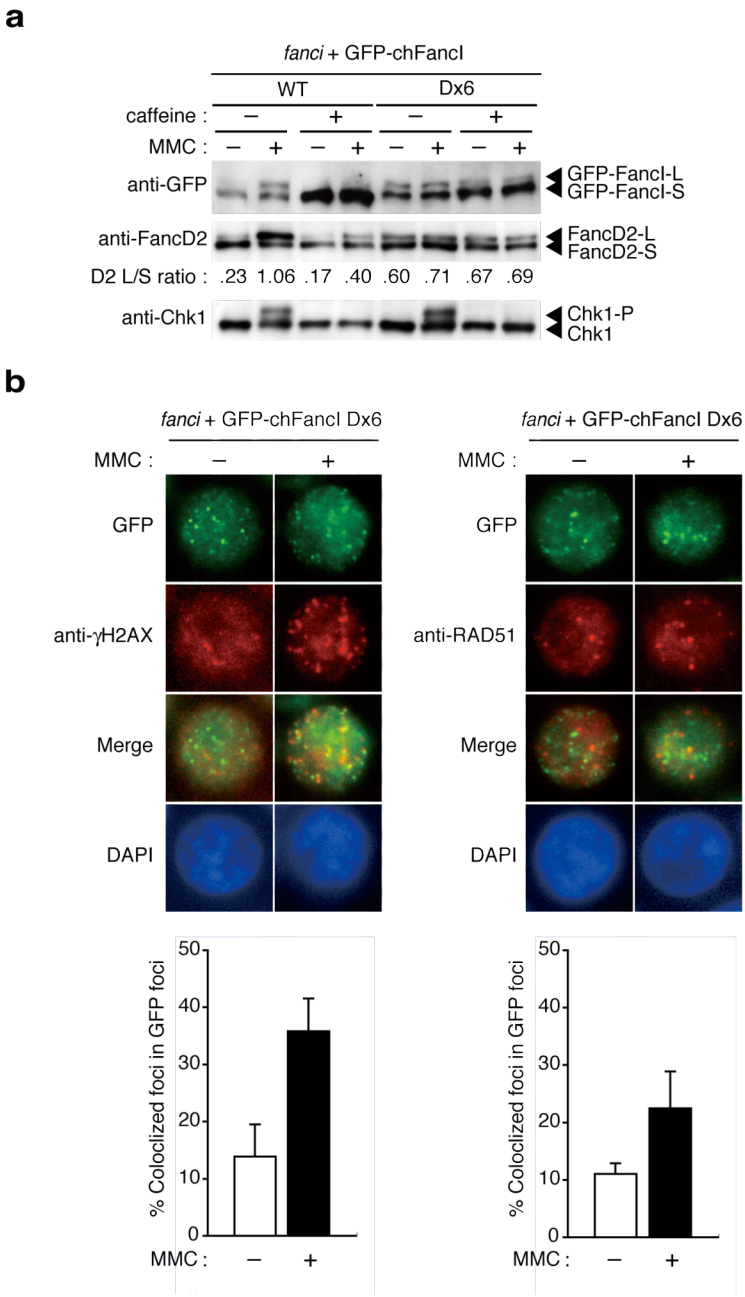


Supplementary Figure 3. Alignment of FANCI amino acid sequences from various vertebrate species. Highlighted in red is the monoubiquitination site. Highlighted in yellow are all the SQ and TQ sites. Red stars indicate phosphorylation sites identified in a human protein after DNA damage[1]. Blue stars indicate phosphorylation sites identified in a mouse protein after DNA damage[1].



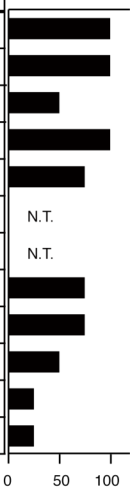
Supplementary Figure 4. Colony survival of cells expressing FancI mutants. (**a** and **b**) Cells were seeded into medium containing methylcellulose and cisplatin, and scored after two weeks. Mean and standard deviation of triplicate cultures are shown.



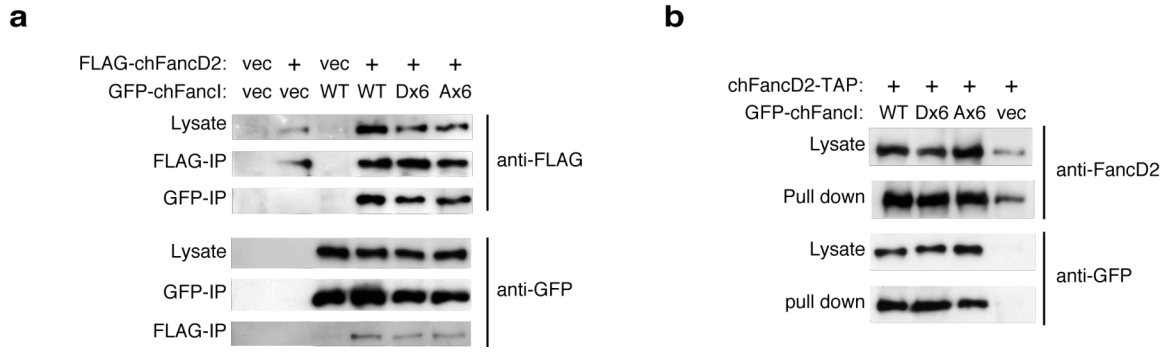
Supplementary Figure 5. Analysis of GFP-chFancI Dx6 protein. (**a**) Effects of GFP-chFancI Dx6 expression and/or caffeine treatment on FancD2 monoubiquitination. Cells were treated with caffeine (20 mM) and/or MMC (500 ng/ml) for 6 h or left untreated, and analyzed as in **Fig. 4f**. (**b**) GFP-chFancI Dx6 and γH2AX or Rad51 localization in

cells with or without DNA damage. Fixed cells were stained with anti-γH2AX or anti-Rad51 antibodies and detected using fluorescence microscopy. The mean and standard error (s.e.m.) of %γH2AX or Rad51 foci colocalized in GFP-chFancI Dx6 foci in three independent experiments are shown in the bar graph. More than 100 bright GFP foci were scored for colocalized γH2AX or Rad51 foci in each experiment.

FANCI mutant	Human amino acid number										FANCD2 foci	FANCD2 ub % of normal
	556	559	565	596	617	629	653	730	952	1121		
FANCI WT	SQ	SQ	SQ	SQ	SQ	SQ	TQ	SQ	TQ	SQ	++++	
S730A_T952A_S1121A								AQ	AQ	AQ	++++	
S556A_S559A_S565A	AQ	AQ	AQ								+	
S596A				AQ							++++	
S617A					AQ						++	
S629A						AQ					++++	N.T.
T653A							AQ				++++	N.T.
S596A_S617A_S629A_T653A				AQ	AQ	AQ	AQ				++	
S596A_S617A_S629A				AQ	AQ	AQ					++	
S556A_S559A_S565A_S617A	AQ	AQ	AQ		AQ						+	
S556A_S559A_S565A_S596A_S617A	AQ	AQ	AQ	AQ	AQ						+	
S556A_S559A_S565A_S596A_S617A_S629A_T653A	AQ	AQ	AQ	AQ	AQ	AQ	AQ				+	



Supplementary Figure 6. Summary of FANCI mutant phenotypes in U2OS cells. Formation of FANCD2 foci was indicated by a semi-quantitative manner based on the microscopic data (**Fig. 6a** and data not shown). In the bar graph showing FANCD2 ubiquitination, " % of normal" indicates the FANCD2 L/S ratio in cells expressing the mutant FANCI relative to the FANCD2 L/S ratio in cells expressing WT FANCI. This number was derived from the D2 L/S ratio shown in **Fig. 6b** and other Westerns (data not shown). Red letters indicate residues that are detectably phosphorylated in either human or mouse FANCI[1]. A blue shaded area indicates homologous residues corresponding to the chicken FancI S/TQ cluster. N.T., not tested.



Supplementary Figure 7. Interaction between overexpressed FancD2 and FancI proteins. Indicated plasmids were transfected into 293T cells using Lipofectamine2000, and cell lysates were prepared. Lysates (10% of the input) and immunoprecipitated or pulled down samples were separated by SDS-PAGE, and blotted with specific antibodies. vec, empty vector control. (**a**) FLAG-or GFP-tagged proteins were immunoprecipitated with anti-FLAG or anti-GFP antibodies. (**b**) chFancD2-TAP was pulled down using rabbit IgG-agarose beads.

References

1. Smogorzewska, A. *et al*. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell* **129**, 289-301 (2007).