

FANCD2 Binds CtIP and Regulates DNA-End Resection during DNA Interstrand Crosslink Repair

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SUMMARY

The Fanconi anemia (FA) pathway is critically involved in the maintenance of hematopoietic stem cells and the suppression of carcinogenesis. A key FA protein, FANCD2, is monoubiquitinated and accumulates in chromatin in response to DNA interstrand crosslinks (ICLs), where it coordinates DNA repair through mechanisms that are still poorly understood. Here, we report that CtIP protein directly interacts with FANCD2. A region spanning amino acids 166 to 273 of CtIP and monoubiquitination of FANCD2 are both essential for the FANCD2-CtIP interaction and mitomycin C (MMC)-induced CtIP foci. Remarkably, both FANCD2 and CtIP are critical for MMC-induced RPA2 hyperphosphorylation, an event that accompanies end resection of double-strand breaks. Collectively, our results reveal a role of monoubiquitinated FANCD2 in end resection that depends on its binding to CtIP during ICL repair.

INTRODUCTION

Fanconi anemia (FA) is a genome-instability disorder characterized by multiple skeletal and deep organ malformations, progressive bone marrow failure, and development of leukemia and solid tumors (Auerbach, 2009). FA can be caused by biallelic germline mutations in any of 16 FA genes that cooperate in a DNA repair pathway often termed the “FA pathway” (Kim and D’Andrea, 2012; Kottmann and Smogorzewska, 2013). The FA pathway is crucial to protect chromosomal integrity during S phase, when DNA replication forks are stalled due to interstrand crosslink (ICL) damage inflicted by agents such as mitomycin C (MMC).

Eight of the FA proteins, together with other non-FA proteins such as FA-associated proteins (FAAPs) and the MHF1/2 complex, interact with each other constitutively, forming the FA core complex (Kim and D’Andrea, 2012; Kottmann and Smogorzewska, 2013). Upon collision of a replication fork with an ICL, the FA core complex is loaded onto damaged chromatin via the activity of its component FANCM/FAAP24 helicase/translocase subcomplex (Kim et al., 2008). The FA core complex then functions as a multisubunit E3 ubiquitin ligase to monoubiquitinate the FANCD2-FANCI (ID) complex (Kim and D’Andrea, 2012; Kottmann and Smogorzewska, 2013). The catalytic subunit in the FA core complex is FANCL, which carries a RING finger domain. The monoubiquitination of the ID complex is a critical regulatory step in the activation of the FA pathway, because the monoubiquitinated ID complex accumulates at damaged chromatin, probably by binding to aberrant DNA structures at the stalled replication fork, where it forms subnuclear foci and orchestrates DNA repair (Kim and D’Andrea, 2012; Kottmann and Smogorzewska, 2013).

The mechanism by which FANCD2 coordinates ICL repair is still poorly understood. Emerging evidence has nonetheless indicated that the monoubiquitinated, chromatin-bound ID complex acts as a scaffold to recruit proteins that contain a ubiquitin-binding domain (UBD) such as FAN1 nuclease or SLX4 (Kim and D’Andrea, 2012; Kottmann and Smogorzewska, 2013). SLX4 is a DNA repair protein that acts as a platform to regulate structure-specific nucleases including XPF/ERCC1, SLX1, and the MUS81/EME1 complex (Kottmann and Smogorzewska, 2013). When an ICL blocks progression of two converging replication forks, DNA is incised at both sides of the ICL, probably by one or more of these nucleases (the so-called unhooking event), leading to the generation of a DNA double-strand break (DSB). The unhooking facilitates DNA synthesis past the lesion in one of the sister chromatids by bypass polymerases (e.g., REV1 and REV3), while the DSB in the other sister chromatid is repaired by homologous recombination (HR) (Kottmann and

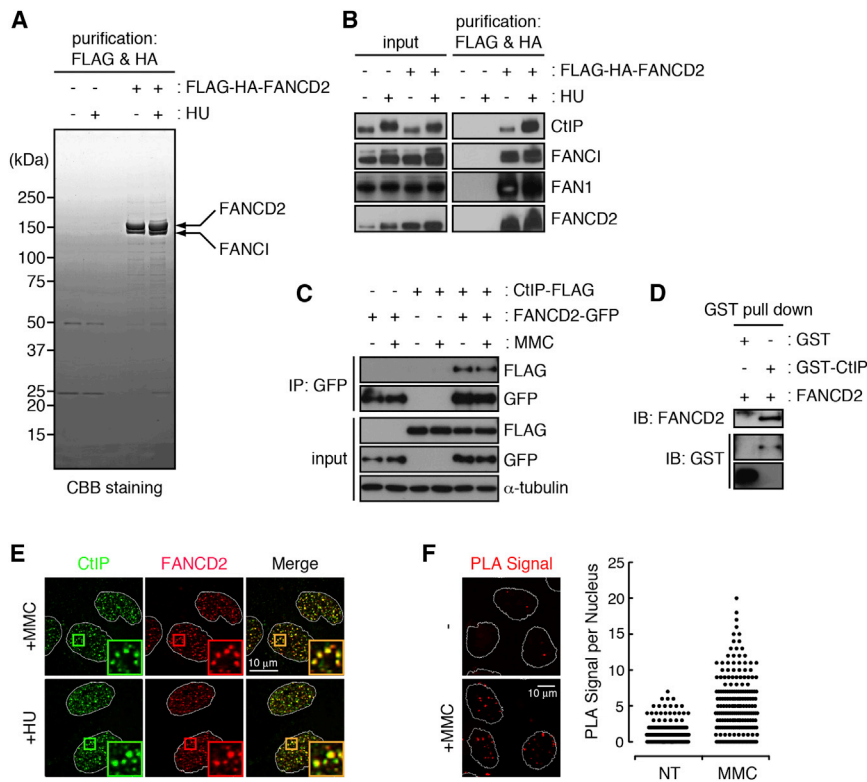


Figure 1. FANCD2 Interacts with CtIP

(A) Purified FANCD2 complex. Two-step purification was carried out from HU-treated (2 mM, 24 hr) HeLa S3 cells expressing FLAG-HA-FANCD2 or transduced with control retrovirus. CBB, Coomassie brilliant blue. (B) Western blotting of the purified FANCD2 complex. (C) CoIP of FANCD2-GFP and CtIP-FLAG in transiently transfected 293T cells. (D) GST pull-down assay using recombinant GST-CtIP and FANCD2. (E) Colocalization of endogenous CtIP and FANCD2 in hTERT RPE-1 cells. (F) The in situ PLA between endogenous CtIP and FANCD2 proteins in U2OS cells. See also Figure S1.

FANCD2 and its monoubiquitination. We propose that FANCD2 is essential for efficient end resection during ICL repair by promoting CtIP localization in chromatin.

RESULTS

Identification of CtIP as an Interactor of FANCD2

To gain insight into FANCD2 function, we sought to identify proteins associated with FANCD2 in response to replication stress. To this end, we expressed human FANCD2 fused with a FLAG-hemagglutinin (HA) epitope tag in HeLa S3 cells (Ikura et al., 2000). The purified FANCD2 complex (Figure 1A) was subjected to mass spectrometric analysis as described in Experimental Procedures. Unexpectedly, we identified CtIP/RBBP8 in the complex (Figure S1A), which was also confirmed by western blotting (Figure 1B). The interaction between FANCD2 and CtIP was verified by coimmunoprecipitation (coIP) of the overexpressed proteins (Figure 1C). The interaction is unlikely to be mediated by contaminating DNA, because the lysates were pretreated with Benzonase before immunoprecipitation. Further, recombinant human FANCD2 protein was pulled down by glutathione S-transferase (GST)-CtIP, but not by GST protein, supporting the notion that the FANCD2-CtIP interaction may be direct (Figure 1D). This interaction was conserved in chicken proteins as shown in Figure 4B.

Both CtIP and FANCD2 proteins form foci following DNA damage and replication stress. As expected, we observed that endogenous FANCD2 and CtIP in hTERT RPE-1 cells (Figure 1E), or endogenous FANCD2 and GFP-CtIP stably expressed in U2OS cells (Figure S1B), colocalized extensively upon exposure to MMC. Next, we employed an in situ proximity ligation assay (PLA) to detect the endogenous FANCD2-CtIP association within cells. The PLA visualizes protein-protein interactions as fluorescent spots by rolling-circle amplification reactions dependent on the close proximity (<40 nm) of the target proteins (Söderberg et al., 2006). We could visualize the interaction as red PLA spots in the nuclei (Figure 1F), and the number of the spots showed a remarkable increase after MMC treatment, while

Smogorzewska, 2013). The incised ICL is then removed by nucleotide excision repair. Among the nucleases associated with SLX4, XPF/ERCC1 seem to be the most important for ICL resistance, while Mus81/EME1 and SLX1 play less prominent roles (Kottemann and Smogorzewska, 2013). SLX4 or XPF have proven to be FA proteins FANCP (Kim et al., 2011; Stoepker et al., 2011) and FANCD2 (Bogliolo et al., 2013), respectively.

Mammalian cells utilize two main mechanisms to repair DSBs: HR and nonhomologous end-joining (NHEJ) (Thompson, 2012). End resection of a DSB by nucleolytic degradation of the 5' strand is the first step in initiating DSB repair via the HR pathway (Chapman et al., 2012; Huertas, 2010; Symington and Gautier, 2011). A 3' single-stranded stretch of DNA is exposed by end resection and binds replication protein A (RPA), which is in turn displaced by RAD51 with the aid of RAD51 mediator proteins (Thompson, 2012). CtIP has been shown to be essential for initiation of DSB-end resection (Sartori et al., 2007; You et al., 2009). Its activity and subcellular localization should be tightly regulated by modifications such as phosphorylation by cyclin-dependent kinase during S phase, ATM or ATR kinase upon DNA damage, and by interactions with proteins such as BRCA1 or MRN complex (Huertas, 2010; Raynard et al., 2008; Symington and Gautier, 2011). Recent studies provided evidence for a requirement of CtIP in ICL repair (Duquette et al., 2012; Karanja et al., 2012). However, how CtIP is regulated during ICL repair remains unclear.

To gain more insight into the role of FANCD2, we purified a FANCD2 complex and identified CtIP as a FANCD2-associated protein. Our results indicate that FANCD2 directly interacts with CtIP, which associates with chromatin in a manner dependent on

the spots were mostly absent when either one of the antibodies was omitted (Figure S1C).

MMC-Induced Relocalization of CtIP Depends on FANCD2 and Its Monoubiquitination

To test whether FANCD2 is required for MMC-induced CtIP foci formation, we used *FANCD2*-deficient PD20F cells. In complemented PD20F cells, we observed an increase in MMC-induced CtIP foci that colocalized with GFP-FANCD2 foci (Figure 2A). Importantly, this increase in CtIP foci formation was not observed in PD20F cells expressing the FANCD2 K561R mutant or vector alone (Figure 2A). Consistently, chromatin localization of CtIP protein was induced by MMC treatment in PD20F cells complemented with GFP-FANCD2, but not with vector alone (Figure S2A). Furthermore, the CtIP foci formation as well as chromatin localization was defective in *FANCA*-deficient GM6914 cells (Figures 2B and S2B). Our current results suggest that monoubiquitinated, and therefore chromatin-bound, FANCD2 anchors CtIP via a direct interaction. We also detected MMC-induced CtIP foci formation in previously described patient-derived cell lines lacking either *FAN1* (A1170-22) or *SLX4* (RA3331) following MMC exposure (Kim et al., 2011; Zhou et al., 2012) (Figures S2C and S2D), suggesting that the incision events are not important for CtIP recruitment.

It has been reported that BRCA1 is partially required in mediating accumulation of FANCD2 upon ICL damage (Bunting et al., 2012; Vandenberg et al., 2003). Furthermore, BRCA1 is essential for ionizing radiation (IR)-induced CtIP foci formation (Yu et al., 2006). We found that both MMC-induced FANCD2 and CtIP foci formation were moderately decreased siBRCA1-treated cells (Figures S2E and S2F). We suggest that the role of BRCA1 in the CtIP accumulation is likely to be indirect via mediating FANCD2 foci formation.

It has been reported that CtIP plays an important upstream role in activation of the FA pathway in response to ICLs induced by psoralen plus UV irradiation (Duquette et al., 2012). However, we found that MMC-induced FANCD2 monoubiquitination and foci formation were similar, comparing CtIP-depleted cells to cells treated with control small interfering RNA (siRNA) (Figures 2C and 2D).

Identification of the FANCD2-Interacting Domain in CtIP Protein

To map the FANCD2-interacting domain in CtIP, a series of siRNA-resistant CtIP deletion mutants (Figure 3A) were transiently expressed together with a full-length FANCD2-FLAG in U2OS cells. Because CtIP has been shown to form a dimer (Dubin et al., 2004; Wang et al., 2012), we simultaneously depleted endogenous CtIP using siRNA. We found that the CtIP region spanning amino acids 166–298 might be important for the interaction (Figures 3A and 3B).

We further divided the amino acid 166–298 region into four subsegments and created CtIP deletions (the deleted regions were termed D1 to D4 as shown in Figure 3C). The interaction between FANCD2 (as bait) and CtIP (as prey) was then tested using a mammalian two-hybrid assay. These analyses indicated that three regions (D1, D2, and D3) are each required for the interaction, while the D4 region is dispensable (Figure 3D). We

observed a robust interaction of all of these mutants with CtBP and LMO4 proteins (data not shown), which have been shown to associate with CtIP outside of the regions that we deleted in CtIP (Dubin et al., 2004; Kim et al., 2008; Schaeper et al., 1998). Interestingly, there are stretches of amino acids that are highly conserved from *Xenopus* to humans in segments D1, D2, and D3, but not in D4 (Figure 3C). To identify crucial amino acid residues for the interaction, we introduced alanine substitutions into a pair of conserved amino acids as shown in Figure 3C. The R185A/Y186A mutation in the D1 subregion, but not L224A/V225A (in D2) or V256A/V257A (in D3), reduced the interaction between CtIP and FANCD2 (Figure 3E). We then confirmed the decrease in interaction between FANCD2 and the CtIP mutants by coIP between overexpressed proteins (Figure 3F).

To test the effects of FANCD2 interaction in CtIP foci formation, we introduced GFP-CtIP transgenes into U2OS cells using the Flp-In T-REx system (Figure S3). Strikingly, the focus formation of the mutant CtIP proteins with defective FANCD2 interaction was abrogated in U2OS cells depleted of endogenous CtIP (Figure 3G). Thus, we concluded that CtIP is recruited and tethered to damaged chromatin through its interaction with FANCD2.

Monoubiquitination of FANCD2 Promotes the CtIP-FANCD2 Interaction

The above data collectively may suggest that monoubiquitination of FANCD2 facilitates the interaction between the two proteins. To test this idea, we generated Flp-In T-REx 293 cells that express GFP-FANCD2 with or without the K561R mutation. We observed that coIP of CtIP with GFP-FANCD2 was drastically reduced by the K561R mutation (Figure 4A). This observation is apparently at odds with the finding that the overexpressed proteins can coimmunoprecipitate in similar ratios both before and after MMC exposure. We suggest this could be due to inefficient monoubiquitination of overexpressed FANCD2 (Figure 1C).

Although we could not identify any conserved ubiquitin-binding domains in the CtIP sequence (Hofmann, 2009), it was tempting to speculate that CtIP can recognize ubiquitin. Indeed, we were able to detect ubiquitin-CtIP binding by pull-down assay using ubiquitin-conjugated beads and lysates of U2OS cells transiently transfected with GFP-CtIP (Figure S4A) or with purified GST-CtIP (Figure S4B). However, we could not detect preferential binding of monoubiquitinated over nonubiquitinated chicken FANCD2 with GST-chicken CtIP (Figures 4B and S4C) using ID complex monoubiquitinated in vitro (Figures S4D and S4E) (Sato et al., 2012b). Thus, we concluded that the ubiquitination is unlikely to contribute directly to the FANCD2-CtIP interaction. We propose the role of ubiquitin is to localize FANCD2 to chromatin and thereby expose an interaction surface for CtIP (see Discussion).

MMC-Induced RPA2 Phosphorylation Depends on FANCD2 and CtIP

HR-mediated repair requires DNA-end resection to expose stretches of single-stranded DNA upon which RAD51 polymerizes. Because CtIP is known to be involved in end resection of DSBs (Sartori et al., 2007; You et al., 2009), we tested whether

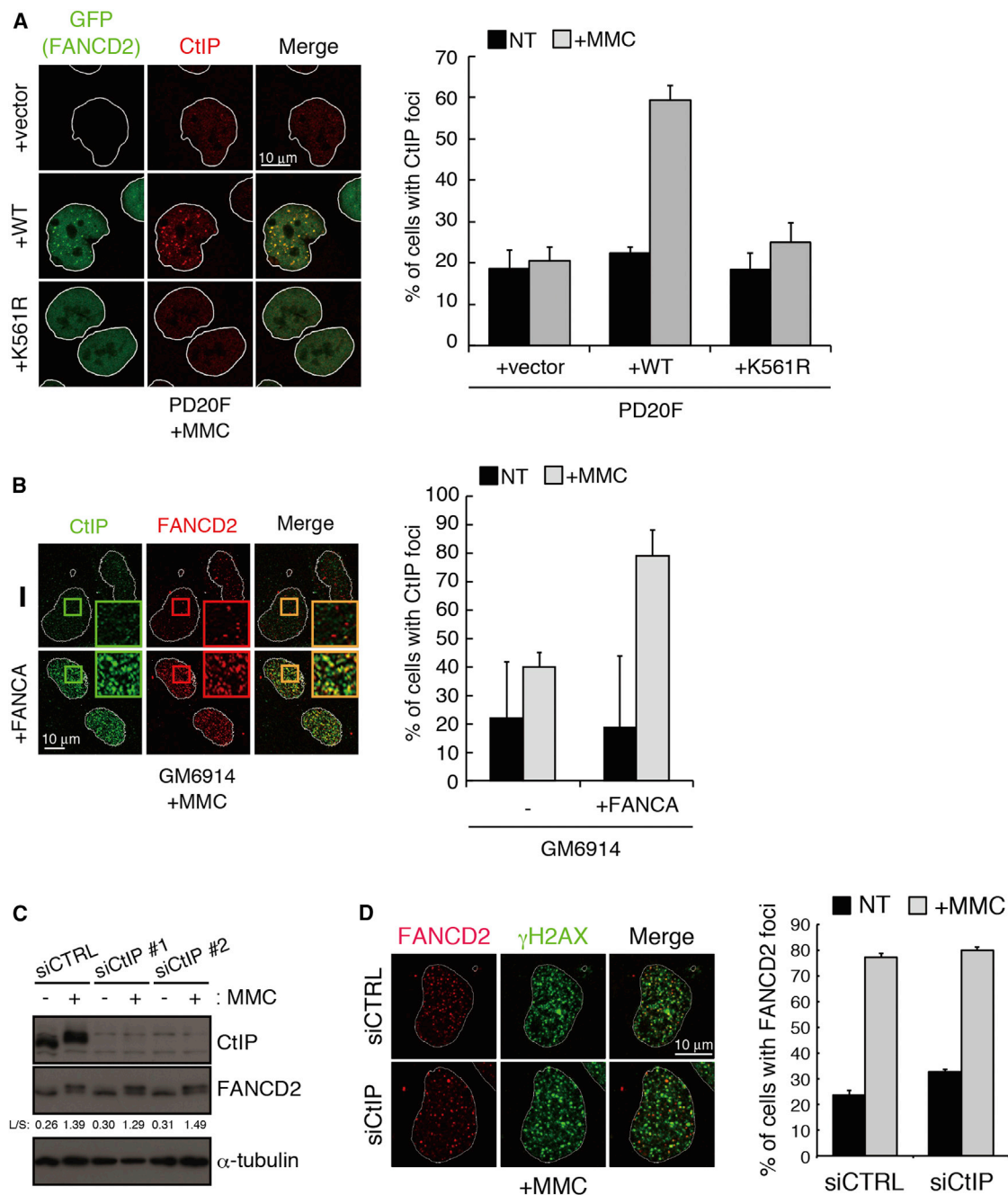


Figure 2. Relocalization of CtIP Is Dependent on FANCD2

(A) MMC-induced foci formation of CtIP. *FANCD2*-deficient PD20F cells transduced with GFP alone, GFP-FANCD2, or GFP-FANCD2 carrying the K561R mutation. The graph shows the mean and SD of the percentage of cells with > 10 CtIP foci.

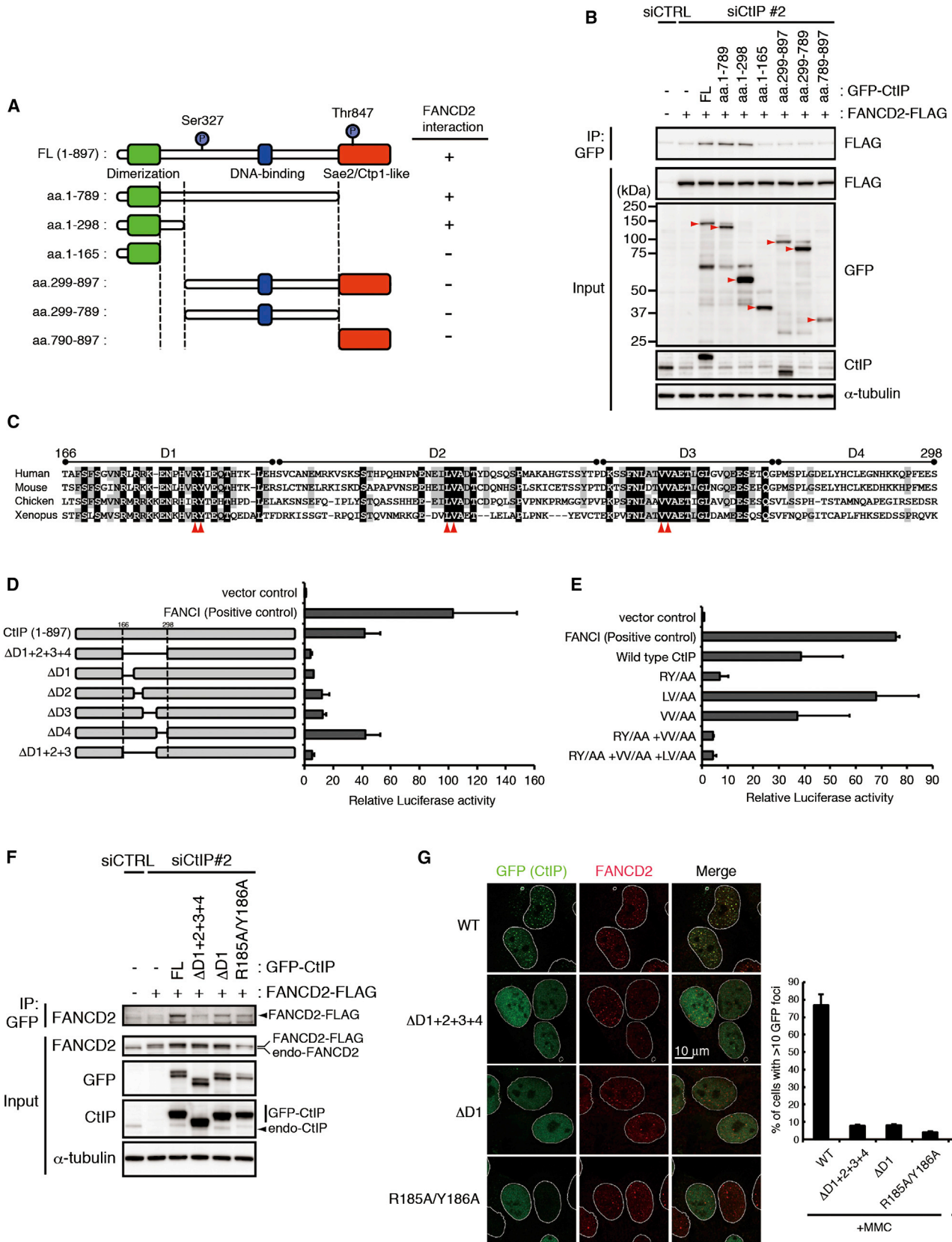
(B) CtIP foci in *FANCA*-deficient GM6914 cells with or without *FANCA* complementation. Cells were treated and examined as in (A).

(C and D) *FANCD2* monoubiquitination and foci formation in siCtIP-treated U2OS cells. siCTRL, control siRNA. The graph shows the mean and SD of the percentage of cells with > 10 *FANCD2* foci.

See also Figure S2.

CtIP and its recruiter *FANCD2* have a role in end resection during ICL repair. We used RPA2 phosphorylation as a surrogate marker for DNA-end resection (Huertas, 2010; Raynard et al., 2008; Symington and Gautier, 2011). In both *FANCD2*- and

CtIP-depleted cells, ratios of phospho/total RPA2 were significantly reduced compared to cells treated with control siRNA (Figure 4C). These results indicate that both *FANCD2* and CtIP have a critical role in DSB-end resection during ICL repair.



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Interplay of FANCD2 and CtIP on Survival of Cells following MMC Treatment

Consistent with the CtIP function in end resection during ICL repair, we found that cells treated with CtIP siRNA showed increased MMC toxicity as evidenced by decreased survival following MMC treatment (Figure 4D). We also found that the cells doubly depleted of both FANCD2 and CtIP were more sensitive to MMC compared to cells transfected with either siRNA alone (Figure 4D). Thus, CtIP appeared to function to some extent independently of the FA pathway. This situation is similar to the case of FAN1 or SLX4, because they are also recruited to the damage sites in a manner dependent on FANCD2, yet their repair activities are not epistatic with the FA pathway (Yamamoto et al., 2011; Yoshikiyo et al., 2010; Zhou et al., 2012). Consistent with this, the CtIP mutants lacking FANCD2 interaction were able to partially restore MMC sensitivity to normal levels in CtIP-depleted cells (Figure 4E). Collectively, these results establish the functional relevance of the CtIP-FANCD2 interaction in ICL repair.

DISCUSSION

In this study, we uncovered a role of FANCD2 in end resection by the identification of CtIP as a FANCD2-interacting protein, expanding its function as a master coordinator of ICL repair. We provided evidence that FANCD2 protein is directly interacting with CtIP and thereby facilitates its chromatin localization. In support of this notion, the CtIP mutants lacking the FANCD2 interaction could not form MMC-induced foci. Furthermore, this interaction is functionally relevant as shown by the clonogenic assay. This is in sharp contrast to the situation in DSB repair. It has been reported that the damage recruitment motif (amino acids 509–557) in the central region of CtIP is necessary and sufficient to be localized to sites of DSB, with additional requirements of ATM kinase and MRN complex (You et al., 2009). BRCA1 has a critical and direct role in CtIP recruitment following IR (Yu et al., 2006), while it has only a partial and likely indirect role through FANCD2 accumulation in MMC-stimulated cells. Thus, in fixing DSBs generated by ICL incision, distinct repair networks are employed than those mobilized during repair of IR-induced DSBs.

Interestingly, our results suggested that CtIP might recognize ubiquitin. However, our data also indicated that ubiquitin-CtIP interaction might not directly contribute to the FANCD2-CtIP interaction. Rather, it seems more plausible that monoubiquiti-

nation and resultant chromatin localization somehow expose a binding surface of FANCD2 for CtIP. For example, DNA binding of FANCD2 likely induces a conformational change, resulting in more efficient monoubiquitination by FANCL in vitro (Sato et al., 2012b). Alternatively, an unknown factor may form a bridge between monoubiquitinated FANCD2 and CtIP.

Taken together with previous studies and an accompanying paper in this issue of *Cell Reports* (Murina et al., 2014), we suggest the following model in which FANCD2 bound to chromatin orchestrates ICL repair (Figure 4F): Monoubiquitinated ID complex binds to chromatin in a manner partially dependent on BRCA1. Then, the ID complex recruits nucleases FAN1 and/or SLX4-XPF (via direct binding between ubiquitin and UBD) or CtIP (via binding with FANCD2). Following unhooking by the nucleases, the ID complex promotes end resection by anchoring CtIP in chromatin.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

Cells are maintained in the media specified in Supplemental Experimental Procedures. Plasmid or siRNA transfection was done following the manufacturer's protocol using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen), respectively. Hydroxyurea (HU) (1 mM) or MMC (100 ng/ml) treatment was done for 24 hr unless stated otherwise.

Reagents

All antibodies and siRNAs are listed in Supplemental Experimental Procedures.

Affinity Purification of the FANCD2 Complex and Mass Spectrometric Analysis

FANCD2 complexes were purified as described elsewhere (Ikura et al., 2000). Separated bands were digested and analyzed by liquid chromatography-tandem mass spectrometry as described in Supplemental Experimental Procedures.

Recombinant Proteins and Pull-Down Assays

Human FANCD2 (Sato et al., 2012a) or GST-chicken CtIP was purified from Sf9 or *Escherichia coli*, respectively. GST-human CtIP or ubiquitin-conjugated beads were purchased from Abnova or Boston Biochem, respectively. Monoubiquitinated chicken ID complex was prepared as described elsewhere (Sato et al., 2012b). Pull-down assays were done as described in Supplemental Experimental Procedures.

Immunofluorescence and the In Situ Proximity Ligation Assay

To score foci, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100/PBS for 5 min, and stained with primary antibodies overnight. The experiments were repeated three times, and >100 cells

Figure 3. Mapping of FANCD2-Interacting Region in CtIP

(A) Schematic depicting wild-type CtIP (FL) and CtIP fragments.

(B) Interaction of GFP-CtIP in-frame deletions with FLAG-tagged FANCD2. 293T cells were first transfected with siCtIP#2, and 24 hr later, the constructs encoding siRNA-resistant CtIP deletions and FANCD2-FLAG were cotransfected and further incubated for 48 hr.

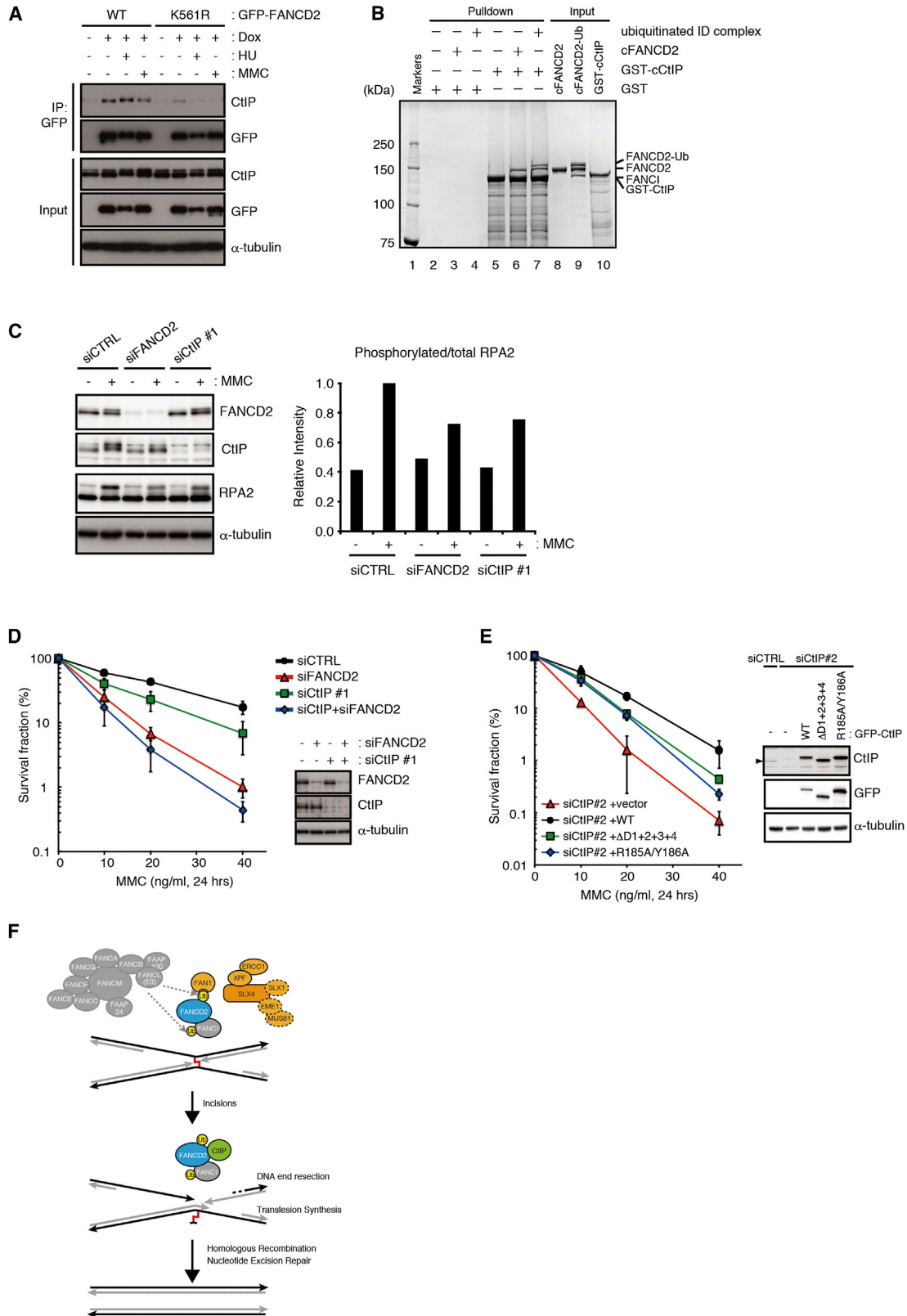
(C) Alignment of human CtIP amino acids 166–298 with CtIP from different species.

(D and E) Mammalian two-hybrid (M2H) interaction assay between FANCD2 and CtIP deletions (D) or point mutants (E). Luminescence signal levels were normalized to the value obtained by cotransfection of empty bait and prey vectors. The experiments were repeated three times, and the mean and SD values are shown.

(F) CoIP assay between FANCD2 and CtIP mutants. U2OS cells depleted of endogenous CtIP were transfected with the indicated plasmids.

(G) MMC-induced GFP-CtIP foci carrying deletions or the R185A/Y186A mutation. U2OS Flp-In T-REX cells stably integrated with GFP-CtIP-NLS-FLAG wild-type or mutant constructs were treated with siCtIP #2 and stimulated with doxycycline. The graph shows the mean and SD of the percentage of cells with > 10 GFP-CtIP foci.

See also Figure S3.



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were analyzed in each experiment. The in situ PLA was carried out using specific antibodies and a DuoLink in situ PLA kit (Olink Bioscience).

Mammalian Two-Hybrid Analysis

Mammalian two-hybrid assays were carried out as described previously (Pace et al., 2002).

Clonogenic survival assay

Cells were treated with MMC for 24 hr, washed with PBS, and then plated on six-well plates and incubated for 10–14 days.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.04.005>.

AUTHOR CONTRIBUTIONS

A.I. purified the FANCD2 complex with J.T. and T. Ikura; M. Taoka and T. Isobe performed mass spectrometry; K. Sato and H.K. purified proteins and performed in vitro reactions; J.U. performed the rest of the experiments with M.I.; W.S. and K. Sugawara provided unpublished cell lines; and M. Takata wrote the paper with J.U., K. Sato, and H.K.

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Figure 4. Functional Interplay between Monoubiquitinated FANCD2 and CtIP

- (A) CoIP between endogenous CtIP and GFP-FANCD2 WT or lacking the monoubiquitination site in Flp-In T-REx 293 cells.
- (B) Pull-down assay with purified GST-chicken CtIP (cCtIP) and monoubiquitinated chicken FANCD2 (cFANCD2).
- (C) RPA2 phosphorylation in U2OS cells depleted of FANCD2 or CtIP. The ratio of phospho- versus total RPA2 was normalized to the value from the cells treated with control siRNA and MMC. This experiment was repeated twice with similar results.
- (D) Clonogenic cell survival. U2OS cells were transfected with the respective siRNAs, as indicated, and after 48 hr stimulated with MMC for an additional 24 hr. Data shown are the mean and SE of three independent experiments.
- (E) U2OS cells stably expressing wild-type or mutant siRNA-resistant GFP-CtIP were transfected with siCtIP#2 and tested for MMC sensitivity as in (D). Position of the endogenous CtIP was indicated by the arrowhead. See also Figure S4.
- (F) A model of CtIP regulation by the ID complex during ICL repair. See text for details.

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