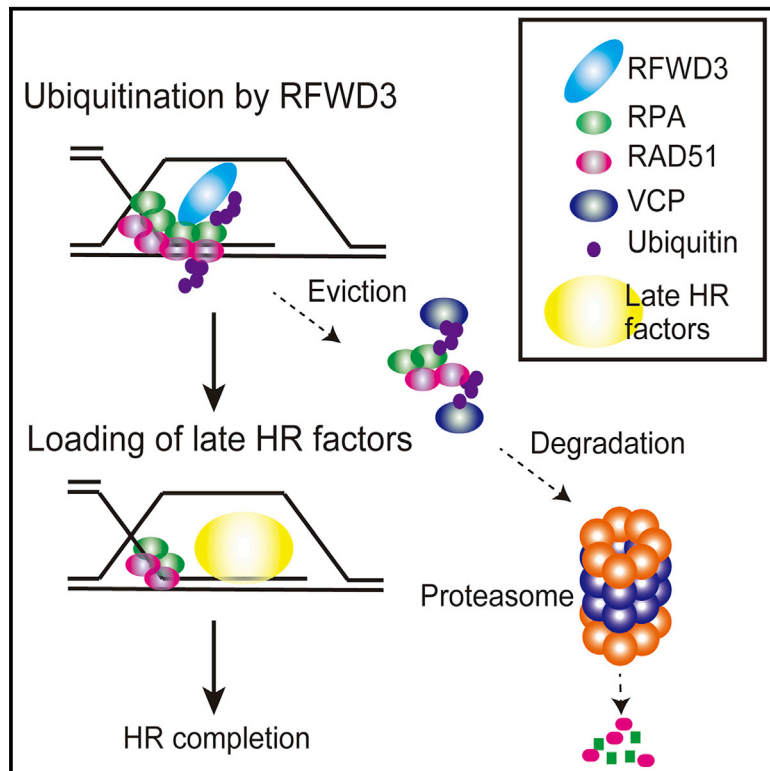


RFWD3-Mediated Ubiquitination Promotes Timely Removal of Both RPA and RAD51 from DNA Damage Sites to Facilitate Homologous Recombination

Graphical Abstract



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In Brief

A novel Fanconi anemia gene FANCW/RFWD3 encodes an E3 ligase that is essential for homologous recombination repair of DNA. Inano et al. show that stepwise repair involves polyubiquitination of RPA and RAD51 and their subsequent removal from chromatin and degradation.

Highlights

- RFWD3 polyubiquitinates both RPA and RAD51 in vitro and in vivo
- Phosphorylation of RFWD3 by ATR and ATM kinases potentiates its E3 ligase activity
- RFWD3 marks MMC-induced RPA and RAD51 foci for clearance by VCP and the proteasome
- Defective clearance impairs HR and loading of MCM8 to chromatin



RFWD3-Mediated Ubiquitination Promotes Timely Removal of Both RPA and RAD51 from DNA Damage Sites to Facilitate Homologous Recombination

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SUMMARY

RFWD3 is a recently identified Fanconi anemia protein FANCW whose E3 ligase activity toward RPA is essential in homologous recombination (HR) repair. However, how RPA ubiquitination promotes HR remained unknown. Here, we identified RAD51, the central HR protein, as another target of RFWD3. We show that RFWD3 polyubiquitinates both RPA and RAD51 in vitro and in vivo. Phosphorylation by ATR and ATM kinases is required for this activity in vivo. RFWD3 inhibits persistent mitomycin C (MMC)-induced RAD51 and RPA foci by promoting VCP/p97-mediated protein dynamics and subsequent degradation. Furthermore, MMC-induced chromatin loading of MCM8 and RAD54 is defective in cells with inactivated *RFWD3* or expressing a ubiquitination-deficient mutant RAD51. Collectively, our data reveal a mechanism that facilitates timely removal of RPA and RAD51 from DNA damage sites, which is crucial for progression to the late-phase HR and suppression of the FA phenotype.

INTRODUCTION

Cells daily incur numerous DNA lesions that threaten genome integrity. To maintain genomic integrity, cells are equipped with a myriad of mechanisms that are each specific for different types of damage. However, DNA interstrand crosslinks (ICLs) require the combined use of various modes of DNA repair, including nucleotide excision repair (NER), translesion synthesis (TLS), and homologous recombination (HR). The Fanconi anemia (FA)

pathway orchestrates these DNA repair activities for ICL repair (Ceccaldi et al., 2016; Kottemann and Smogorzewska, 2013). FA is a hereditary disorder defective in ICL repair and characterized by developmental anomalies, progressive bone marrow failure, leukemia, and solid tumors (Kutler et al., 2003).

An ICL is converted to a double-strand break (DSB) by incision (termed unhooking) through the actions of a structure-specific nuclease SLX4/FANCP-XPF/FANCD2-ERCC1 complex. This is recruited in a manner dependent on the FANCD2-FANCI (D2-I) heterodimeric complex that accumulates in chromatin upon their monoubiquitination by the FA core E3 ligase complex (Ceccaldi et al., 2016; Kottemann and Smogorzewska, 2013). Importantly, a DSB generated at ICL-stalled replication forks is subsequently channeled into the HR repair pathway. Therefore, biallelic mutations in genes of the core HR machinery, such as *BRCA2/FANCD1* or *RAD51C/FANCO*, may also cause FA. More recently, *BRCA1/FANCS* and *RAD51/FANCR*, key molecules in HR, have also been identified as FA genes (Bogliolo and Surralés, 2015; Ceccaldi et al., 2016).

The first step in HR following DSB generation during ICL repair is DNA end resection. The MRE11-RAD50-NBS1 (MRN) complex initiates resection, which is completed by the combined actions of many DNA repair factors that are modulated by FANCD2 itself (Ceccaldi et al., 2016; Symington and Gautier, 2011). The resulting long single-stranded DNA (ssDNA) is rapidly coated with RPA, a trimeric ssDNA binding protein complex that consists of subunits RPA1 (also called RPA70), RPA2 (RPA32), and RPA3 (RPA14). The mediator BRCA2-DSS1 complex plays a major role in loading RAD51 onto RPA-bound ssDNA (Liu et al., 2010; Prakash et al., 2015; Zhao et al., 2015), resulting in RAD51 nucleoprotein filament formation. RAD51 catalyzes the critical activity in HR, i.e., the homology search and strand invasion of homologous duplex DNA, leading to the formation of a displacement loop (D-loop) recombination intermediate. The ssDNA strand

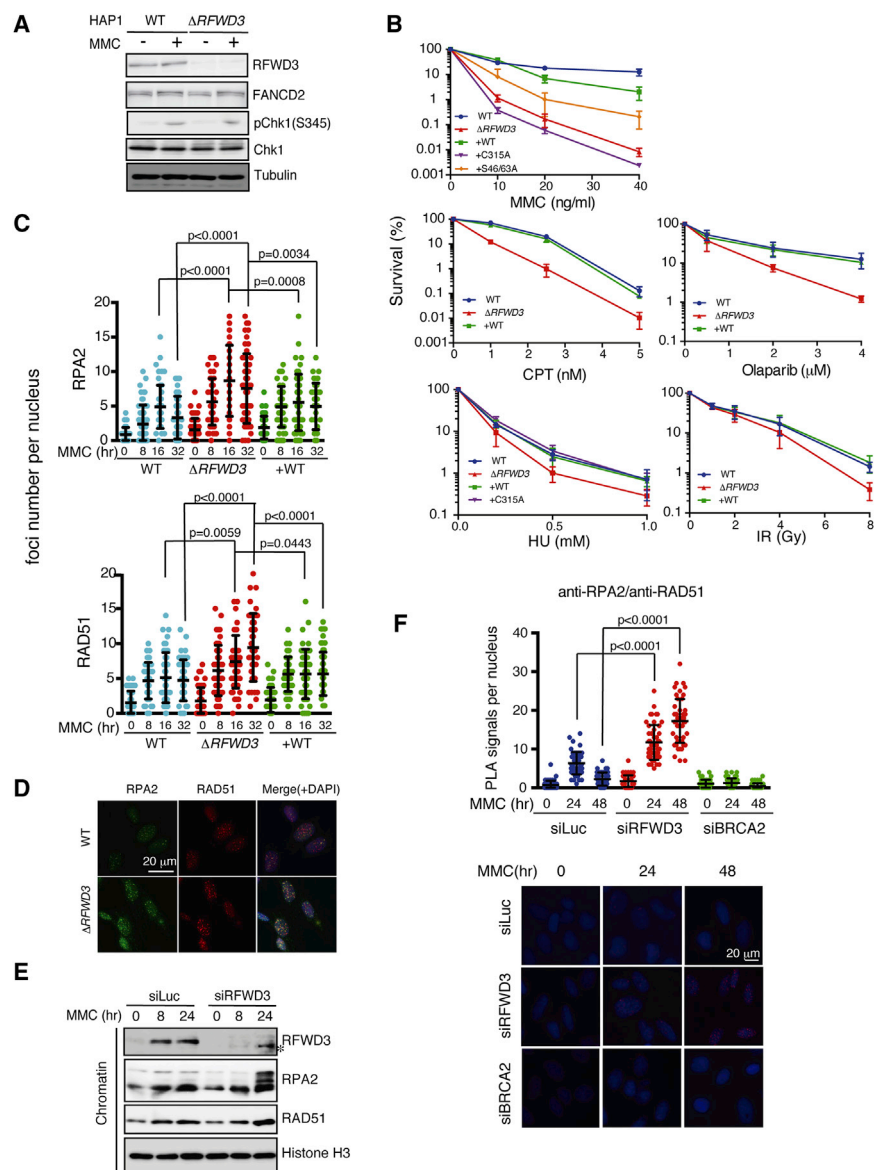


Figure 1. HAP1 Δ RFWD3 Cells Display Increased Persistence of RPA and RAD51 Foci following MMC Damage

(A) Extracts prepared from HAP1 wild-type (WT) and Δ RFWD3 cells treated with MMC for 24 hr were immunoblotted with the indicated antibody. (B) Survival curves of HAP1 WT, Δ RFWD3, or Δ RFWD3 stably transduced with a lentivirus expressing WT or mutant RFWD3. Mean and SD of three independent experiments are shown. (C) Cells were treated with 20 ng/mL MMC for indicated durations up to 16 hr and then released into fresh medium. Cells were fixed and stained with anti-RPA2 or RAD51. Each dot represents a single cell, and the mean and SD are indicated. (D) Representative images of RPA2 and RAD51 foci 16 hr after MMC treatment as analyzed in (C). (E) Levels of RPA2 and RAD51 in chromatin fraction. U2OS cells transfected with the indicated siRNAs were treated with MMC, fractionated, and blotted with the indicated antibodies. An asterisk indicates a non-specific band. (F) In situ PLA between endogenous RPA2 and RAD51 in cells depleted of RFWD3 or BRCA2. U2OS cells transfected with indicated siRNAs were treated with MMC, harvested at 24 and 48 hr. Representative images are shown (below). Each dot represents a single cell, and the mean and SD are shown. See also Figure S1.

displaced from the template DNA during heteroduplex DNA formation also binds RPA. Subsequently, DNA synthesis, from the 3' end of the invading strand as a primer, using a PCNA clamp and DNA polymerases (Heyer, 2015; Sebesta et al., 2013), extends the D-loop, leading to capture and annealing of the other end of the DSB (second end capture). However, it is still poorly understood how these late-phase HR processes are regulated.

RFWD3 is a RING-type E3 ligase that co-localizes with RPA in DNA-damage-induced foci. It binds to RPA2 and functions in replication checkpoints (Gong and Chen, 2011; Liu et al., 2011). It was further reported that RFWD3-mediated ubiquitination of RPA is essential for HR (Elia et al., 2015). However, the mechanisms by which RPA ubiquitination contributes to HR remain unclear. Here, we show that RFWD3 ubiquitinates both RPA and RAD51 in vitro and in vivo and increases their local turn-

over in DNA damage-induced foci. Ubiquitinated RPA2 and RAD51 displayed lowered ssDNA binding, and they eventually underwent proteasomal degradation. In agreement with these observations, we also found higher levels of mitomycin C (MMC)-induced RPA and RAD51 foci that abnormally persisted in the absence of RFWD3. Furthermore, cells expressing ubiquitination-deficient RPA2 and RAD51 mutants displayed HR defects and higher cisplatin sensitivity. Of note, biallelic mutations in the RFWD3 gene have been identified in a patient with FA (K.K., S.I., M.J. Ramírez, M.I., J. Surrallés, M.T., and D.S., unpublished data).

RESULTS

Inactivation of RFWD3 Results in Persistent RPA2 and RAD51 Foci

To gain more insight into the RFWD3 function in HR, Δ RFWD3 cells were constructed in the HAP1 haploid human cell line using CRISPR/Cas9 (Horizon Genomics) and validated by genome sequencing (Figure S1A) and immunoblotting (Figure 1A). Δ RFWD3 cells exhibited hypersensitivity to MMC, Olaparib, and Camptothecin (CPT), with milder sensitivity to gamma-ray irradiation (IR) and hydroxyurea (HU) (Figure 1B). Importantly, reintroduction of wild-type (WT) RFWD3 reversed MMC sensitivity, whereas a RING finger domain mutant (C315A) did not, despite higher

expression levels (Figure S1B). These HAP1 mutant cell lines appeared to maintain a stable DNA ploidy pattern (Figure S1B).

Next, we examined how loss of *RFWD3* affects ICL repair. While Δ *RFWD3* cells were proficient in MMC-induced FANCD2 monoubiquitination (Figure 1A), RPA2 and RAD51 foci numbers in Δ *RFWD3* were drastically elevated at 16–32 hr post-MMC addition (Figures 1C and 1D). A similar tendency was observed after gamma-ray IR in U2OS cells depleted of *RFWD3*, which showed extensive co-localization of RPA2 and RAD51 (Figure S1C). Furthermore, we detected more RPA2 and RAD51 in the chromatin fraction in Δ *RFWD3* than in WT at 24 hr post-MMC stimulation (Figure 1E). Interestingly, the accumulated RPA2 accompanied slower migrating species, which are probably hyperphosphorylated forms of RPA2. We also noted highly increased numbers of proximal ligation assay (PLA) signals between RPA2 and RAD51 proteins in si*RFWD3*-depleted MMC-stimulated U2OS cells (Figure 1F). These PLA signals might reflect the coexistence of RPA and RAD51 in HR intermediates such as D-loops, since *BRCA2* knockdown (Figure S1D) drastically diminished these signals (Figure 1F).

Prior studies have indicated that *RFWD3* is required for checkpoint signaling under replication stress (Elia et al., 2015; Gong and Chen, 2011; Liu et al., 2011). Indeed, we found that Δ *RFWD3* cells exhibited less RPA phosphorylation after HU treatment than WT (Figure S1E). However, MMC-induced phosphorylation was rather enhanced compared with control WT cells (Figure S1E). Unexpectedly, we found that expression of *RFWD3* with the RING domain mutation (C315A) restored RPA2 and Chk1 phosphorylation, as well as HU resistance, in Δ *RFWD3* cells (Figures S1F and 1B). This is in sharp contrast to the situation with the ICL response. We also found that an *RFWD3* construct carrying both C315A and the FA patient-derived mutation I639K, which inhibits chromatin localization of *RFWD3* (K.K., S.I., M.J. Ramirez, M.I., J. Surrallés, M.T., and D.S., unpublished data), only partially reversed these defects (Figure S1F). Since Δ *RFWD3* cells treated with HU for 2 hr displayed a lower percentage of RPA foci-positive cells (Figure S1G), we speculate that *RFWD3* facilitates RPA loading onto ssDNA under HU-induced stress and subsequent ATR-Chk1 activation in a manner independent of its E3 ligase activity.

***RFWD3* Promotes RPA Polyubiquitination following MMC Damage**

Given that *RFWD3* interacts with RPA (Gong and Chen, 2011; Liu et al., 2011), and Δ *RFWD3* cells displayed increased numbers of RPA2 foci formation after MMC damage, we examined whether *RFWD3* polyubiquitinates RPA. In cells transfected with tagged ubiquitin, we found that polyubiquitination of endogenous RPA1 and RPA2 was detectable when treated with MMC in the additional presence of proteasomal inhibitor MG132 (Figure 2A). Polyubiquitination of overexpressed RPA2 (Figure 2B) or RPA3 (Figure S2A) was also observed following combined MMC/MG132 treatment. Importantly, this was markedly suppressed by *RFWD3* depletion (Figures 2A, 2B, and S2A). RPA2 polyubiquitination was further increased when FLAG-*RFWD3* WT was co-expressed (Figure 2B). This effect of *RFWD3* expression was mostly or partially abrogated by the RING domain mutation (C315A) or chromatin localization-defective mutation (I639K) (see the accompanying manuscript) (Figure 2B), respectively.

The latter observation is consistent with the previous report that RPA polyubiquitination occurs in chromatin (Elia et al., 2015). These results are consistent with the possibility that *RFWD3*-dependent RPA polyubiquitination facilitates its proteasomal degradation.

ICL Damage Triggers RPA Polyubiquitination in a Manner Dependent on ATR/ATM Kinase

The DNA damage response (DDR) includes phosphorylation events by three major PIKK family members, ATR, ATM, and DNA-PK. To elucidate how the *RFWD3* E3 ligase is activated during DDR, we tested the effects of inhibitors of ATR (VE-821), ATM (KU-55933), and DNA-PK (NU-7026) on RPA2 polyubiquitination. We found that polyubiquitination was significantly diminished by combined treatment of ATR and ATM inhibitors (Figure S2B). Since it was reported that *RFWD3* Ser46 and Ser63 are phosphorylated by ATM and ATR following DNA damage (Fu et al., 2010), we introduced an *RFWD3* S46/63A mutant construct into HAP1 Δ *RFWD3* cells. We found that the mutant could not fully restore cisplatin tolerance (Figure 1B). Furthermore, overexpressed *RFWD3* S46/63A mutant displayed decreased levels in RPA polyubiquitination (Figure 2C). These results indicated that phosphorylation of *RFWD3* S46/63 mediated by ATR/ATM kinase may be critically involved in activation of *RFWD3*.

Next, we raised a rabbit antibody against an *RFWD3* peptide containing phosphorylated Ser46 (Figures S2C and S5B). This antibody recognized MMC-induced *RFWD3* phosphorylation in *RFWD3* WT, but not the S46/63A mutant, by immunoblotting (Figure S2C). We found that the S46 phosphorylation was sensitive to I639K mutation (Figure S2C) or the combined treatment with ATR and ATM inhibitors (Figure S2D).

Interaction between ssDNA and Ubiquitination-Deficient RPA2 In Vivo

To elucidate the functional role of RPA polyubiquitination, we constructed three types of *RPA2* ubiquitination site mutants. We focused our attention on RPA2 rather than RPA1 or RPA3 since it was shown that multiple RPA1 lysine mutations did not suppress polyubiquitination (Elia et al., 2015), and the contribution of RPA3 ubiquitination to the function of the RPA complex could be limited because of its relatively weak ubiquitination (Figure S2A, and see Figure 4A below). The candidate ubiquitination residues were based on previous proteomics studies and they were replaced with Arg as follows: K37/38R (termed 2KR), K85/127/171R (3KR), and K37/38/85/127/171R (5KR) (Figure S2E). The WT and mutated his-RPA2 constructs were tested for ubiquitination by transient overexpression with *RFWD3*. We found that the 5KR mutation, but not 2KR and 3KR, largely eliminated RPA2 polyubiquitination in cells (compare lanes 2 and 3, Figure 2D).

The increased number of RPA2 foci in cells with inactivated *RFWD3* led us to hypothesize that polyubiquitination of RPA2 reduces the amount of RPA in the chromatin fraction. To test this hypothesis, we transduced the U2OS cell line (harboring a DR-GFP reporter that is repaired to produce functional GFP by HR repair of a site-specific DSB; Prakash et al., 2015) with lentivirus encoding FLAG-tagged RPA2-WT or -5KR under the control of a tetracycline-inducible promoter. Endogenous RPA2 was depleted by a small interfering RNA (siRNA) targeting the

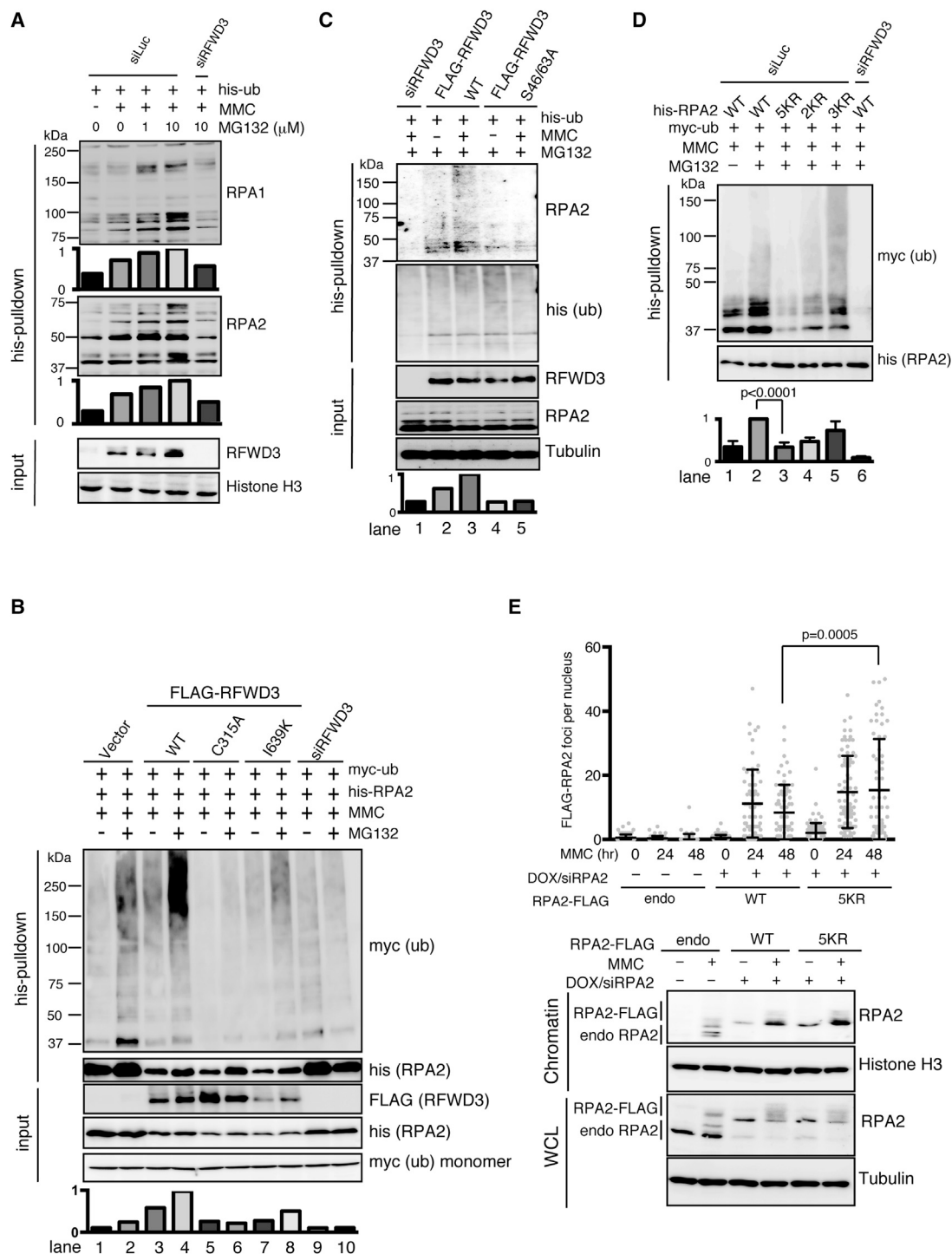


Figure 2. RPA Is Polyubiquitinated by RFWD3 following MMC Treatment

(A) 293T cells simultaneously transfected with his-ubiquitin and indicated siRNA were treated with MMC for 24 hr and with the indicated doses of MG132 (μ M) for 1 hr before harvest. Cells were biochemically fractionated and isolated chromatin was lysed under denaturing conditions. Ubiquitinated proteins were collected by nickel resin and blotted using anti-RPA1 or RPA2 antibody. Signal intensities of the ubiquitinated RPA1 or RPA2 bands normalized to the highest value are shown below each blot.

(B) U2OS cells transfected with myc-ubiquitin, his-RPA2, and FLAG-RFWD3 (and siRFWD3 in lanes 9 and 10) were lysed in denaturing conditions. His-tagged RPA2 was purified and immunoblotted with the indicated antibodies. The ratios of ubiquitinated RPA2 bands (anti-myc) to the total RPA2 (anti-his) were calculated and normalized to the highest value.

(legend continued on next page)

3' UTR, enabling us to replace endogenous RPA2 with the 5KR variant (Figure 2E). In cells expressing the FLAG-RPA2-5KR mutant, we observed modestly increased levels of RPA2 in the chromatin fraction (24 hr post-MMC) and more RPA2 foci formation (48 hr post-MMC) compared to cells expressing RPA2-WT. These results suggest that polyubiquitinated RPA tends to be removed from DNA damage sites (Figure 2E).

RAD51 Is Also Polyubiquitinated by RFWD3 In Vivo

Since *RFWD3* depletion also resulted in augmented numbers of RAD51 foci after ICL damage, we speculated that RAD51 might be another target of *RFWD3* ubiquitination. Indeed, we found that endogenous RAD51 in cells transfected with the his-ubiquitin construct was polyubiquitinated following MMC in a manner dependent on *RFWD3* (Figure 3A). Furthermore, we observed overexpressed his-tagged RAD51 underwent *RFWD3*-dependent ubiquitination when coexpressed with myc-tagged ubiquitin (Figure S3A). Notably, RAD51 polyubiquitination levels were increased by MMC and MG132 treatment and decreased after siRFWD3 depletion (Figure S3A).

Previous studies reported that RAD51 has several potential candidate ubiquitination sites (Chu et al., 2015; Wagner et al., 2011): K58, K64, K107, and K156. In addition, we noticed K57 adjacent to K58 is conserved in vertebrates (Figure S3B). To investigate these putative sites, we generated three RAD51 mutant constructs, 5KR (K57/58/64/107/156R), K58/64R, and K107/156R, and tested them for RAD51 polyubiquitination. Among these mutants, 5KR displayed the most prominent decrease in polyubiquitination levels (Figure 3B).

We then evaluated accumulation of RAD51 on damaged chromatin following MMC. FLAG-tagged siRNA-resistant RAD51-WT or -5KR was lentivirally transduced into the U2OS cell line, and expression was induced by Dox, while endogenous RAD51 was depleted by siRAD51 (Figure 3C). The cells expressing RAD51-5KR showed higher levels of chromatin retention and foci formation than RAD51 at 48 hr post-MMC stimulation. These results suggest that RAD51 polyubiquitination mediated by RFWD3 promotes removal of RAD51 from DNA damage sites.

Purified RFWD3 Protein Polyubiquitinates RPA and RAD51 In Vitro

To examine whether RFWD3 can directly ubiquitinate RPA or RAD51 in vitro, the following recombinant human proteins were purified from *E. coli*: RAD51, the trimeric RPA complex, RFWD3, UBE2T (an E2 enzyme in the FA pathway), and several other E2 proteins that are possible partners of RFWD3 (Markson et al., 2009) (Figures S4A–S4D). Although the RFWD3 preparation contained an ~60 kDa contaminant *E. coli* protein (Fig-

ure S4C), the RPA complex (both RPA1 and RPA2, with weaker ubiquitination on RPA3) and RAD51 were ubiquitinated to various degrees in vitro (Figures 4A and 4B) with UBE2D1 being the most efficient, suggesting that RFWD3 can use several E2 enzymes in a non-selective manner. UBE2T could not support robust polyubiquitination of the RPA subunits, but RAD51 ubiquitination occurred to some extent, with minimal enhancement upon RFWD3 addition (Figure 4B). One of the *RAD51* paralog proteins, DMC1, was modestly ubiquitinated by RFWD3 with UBE2N or UBE2D1 (Figure 4C).

We also examined the interplay among RFWD3, RPA, and ssDNA in vitro. We found that RFWD3 directly interacts with the RPA complex in pull-down assays (Figure S4E). This is consistent with previous reports that show DNA damage-independent RPA-RFWD3 interaction (Gong and Chen, 2011; Liu et al., 2011). We found that the interaction was significantly reduced when RPA was bound to ssDNA (Figure S4Fi), while premixed RPA-RFWD3 could be efficiently pulled down using ssDNA beads (Figure S4Fii). Thus, prior binding of RFWD3 with RPA might be a prerequisite for RFWD3 localization to damaged chromatin. Interestingly, the in vitro RPA ubiquitination efficiency was not affected in the presence of ssDNA (Figure S4G), suggesting that RPA binding with ssDNA itself does not trigger RFWD3 ubiquitination.

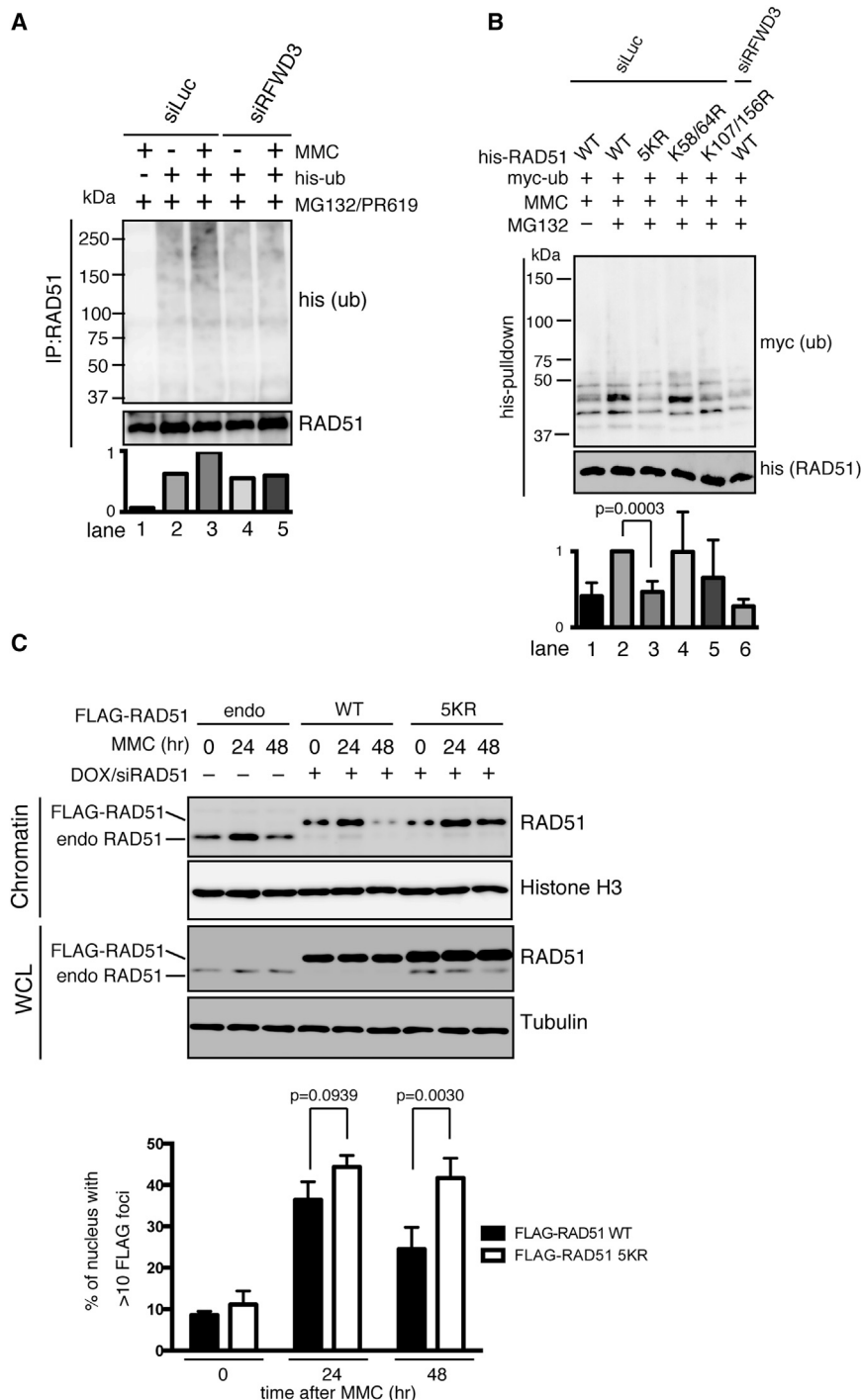
We also carried out ssDNA-bead pull-down experiments with in vitro ubiquitinated RPA or RAD51 and examined the relative amount of ubiquitination in the ssDNA-bound or -unbound fraction. The percentage of ubiquitinated RPA or RAD51 was higher in the unbound than in the bound fraction, suggesting that polyubiquitination significantly reduced the RPA and RAD51 binding with ssDNA (Figures 4D and 4E). This is consistent with the in vivo observation in MMC-treated cells that chromatin retention of RPA2 or RAD51 was higher with the ubiquitination-defective 5KR mutants than the control (Figures 2E and 3C).

RAD51 Directly Interacts with RFWD3

The polyubiquitination of RAD51 by RFWD3 suggests that they may physically interact. We detected an interaction between transfected FLAG-RFWD3 and endogenous RAD51 by co-immunoprecipitation (coIP) (Figure 5A). Importantly, their interaction was modestly enhanced by MMC treatment. We further confirmed the direct interaction between RAD51 and RFWD3 with a pull-down assay using purified proteins (Figure 5B). To fine-map the RAD51-binding site within RFWD3, we performed mammalian two-hybrid assays and found that the N-terminal region (aa 1–278) strongly interacted with RAD51 (Figure 5C). We validated this result using coIP and PLA assays in vivo

(C) 293T cells transfected with his-ubiquitin and siRFWD3 or FLAG-RFWD3 WT or a S46/63A mutant were processed as in (A) and blotted with indicated antibodies. The ratio of ubiquitinated RPA2 bands to total ubiquitinated proteins (anti-his) were quantified, normalized to the highest value, and are shown in the graph.

(D) U2OS cells transfected with his-RPA2-WT or -KR mutants and FLAG-RFWD3 or siRFWD3 were processed as in (B) and blotted with indicated antibodies. Signal intensities of the ubiquitinated bands were normalized as in (B) and are shown with the mean and SD ($n = 3$). The p value was calculated by Student's t test. (E) Foci formation (upper panel) and chromatin retention (lower panel) of RPA2 WT or 5KR. Expression of FLAG-RPA2-WT or -5KR was induced by Dox in lentivirally transduced U2OS cells, and endogenous RPA2 was depleted by siRNA. Cells were treated with MMC for the indicated time (upper) or 24 hr (lower). Each dot represents a single cell, and the mean and SD are shown. p values were calculated by a one-way ANOVA post hoc test. See also Figure S2.



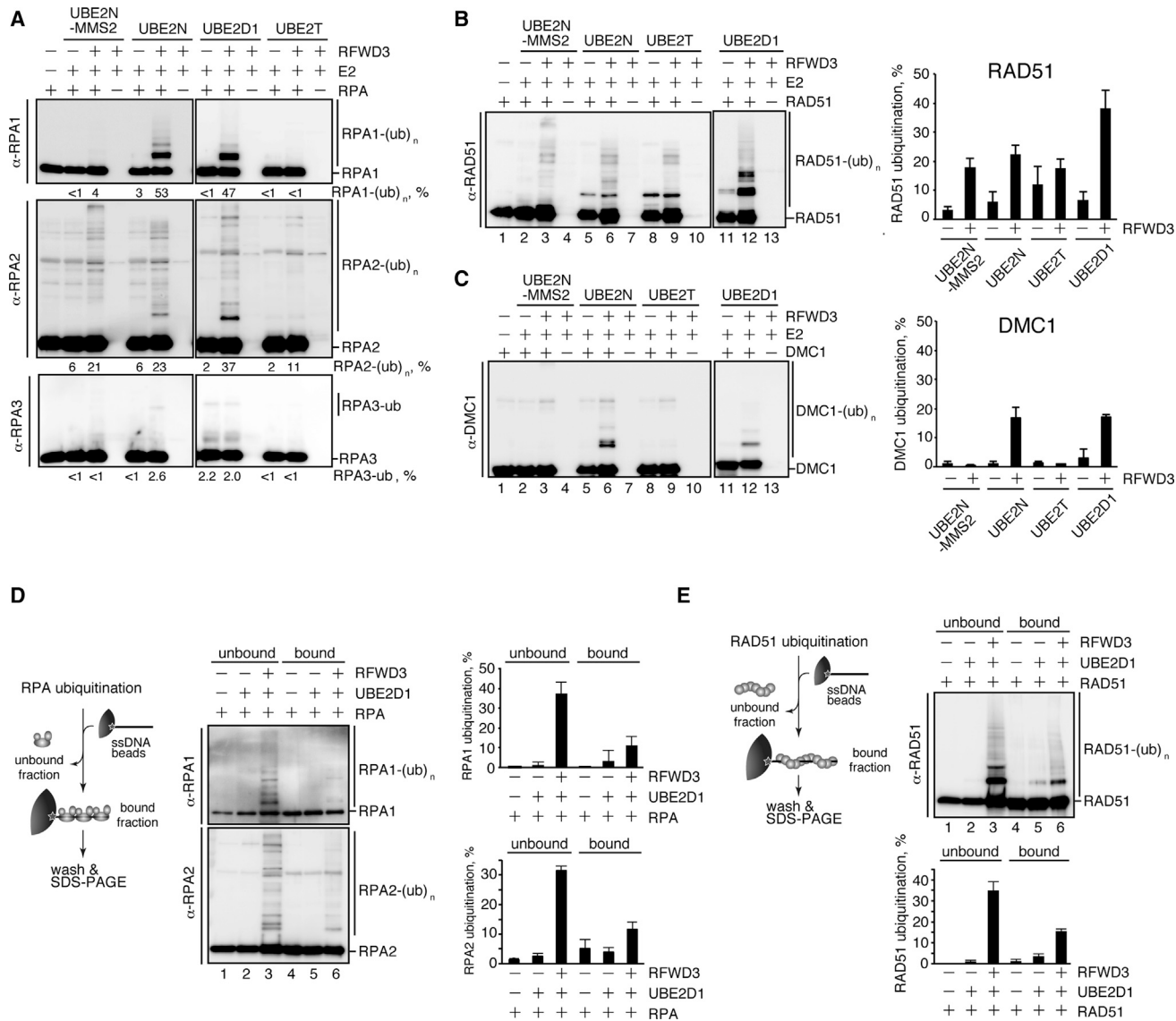


Figure 4. Purified RFWD3 Ubiquitinates RPA and RAD51 In Vitro

(A–C) Ubiquitination assay with RPA (A), RAD51 (B), or DMC1 (C). Ubiquitinated proteins were separated by SDS-PAGE and detected by immunoblotting with indicated antibodies. Percentages of polyubiquitinated proteins (indicated, for example, as RPA1-(ub)_n) in the total amount of separated proteins were quantified and are presented below the lanes in (A) or as bar graphs (mean and SD, n = 3–5) at right in (B) and (C).

(D and E) DNA binding assay with ubiquitinated RPA (D) or RAD51 (E). A schematic diagram of the ssDNA binding assay is shown at left. RPA or RAD51 was ubiquitinated with E1, UBE2D1, RFWD3, and ubiquitin and was incubated with ssDNA beads. Graphs represent the percentage of ubiquitinated RPA1 and RPA2 or RAD51 in the unbound and bound fractions. The mean and SD of three independent experiments are shown.

See also [Figure S4](#).

with this, HAP1 Δ RFWD3 cells expressing the LQP/AAA mutant exhibited higher levels of RAD51 foci (Figures 5G and S1B) and lower cell survival upon cisplatin treatment (Figure S5E) compared to the WT control.

VCP/p97 Interacts with RPA and RAD51 to Promote Their Local Turnover following DNA Damage

Our data suggest that RPA and RAD51 are removed from chromatin in an *RFWD3*-dependent manner. To clarify how this occurs, we examined whether valosin-containing protein (VCP),

also known as p97 or Cdc48, is involved. VCP is a homohexameric molecular chaperone that extracts ubiquitinated proteins from immobile cellular structures in a manner dependent on its ATPase activity and escorts them to the 26S proteasome for their degradation. The role of VCP in eviction of chromatin-bound proteins in DDR has recently been established (Meerang et al., 2011; Vaz et al., 2013).

First, we tested whether VCP interacts with RPA2 or RAD51 in response to MMC damage. We were able to co-immunoprecipitate endogenous VCP with RPA or RAD51 from HAP1 cell

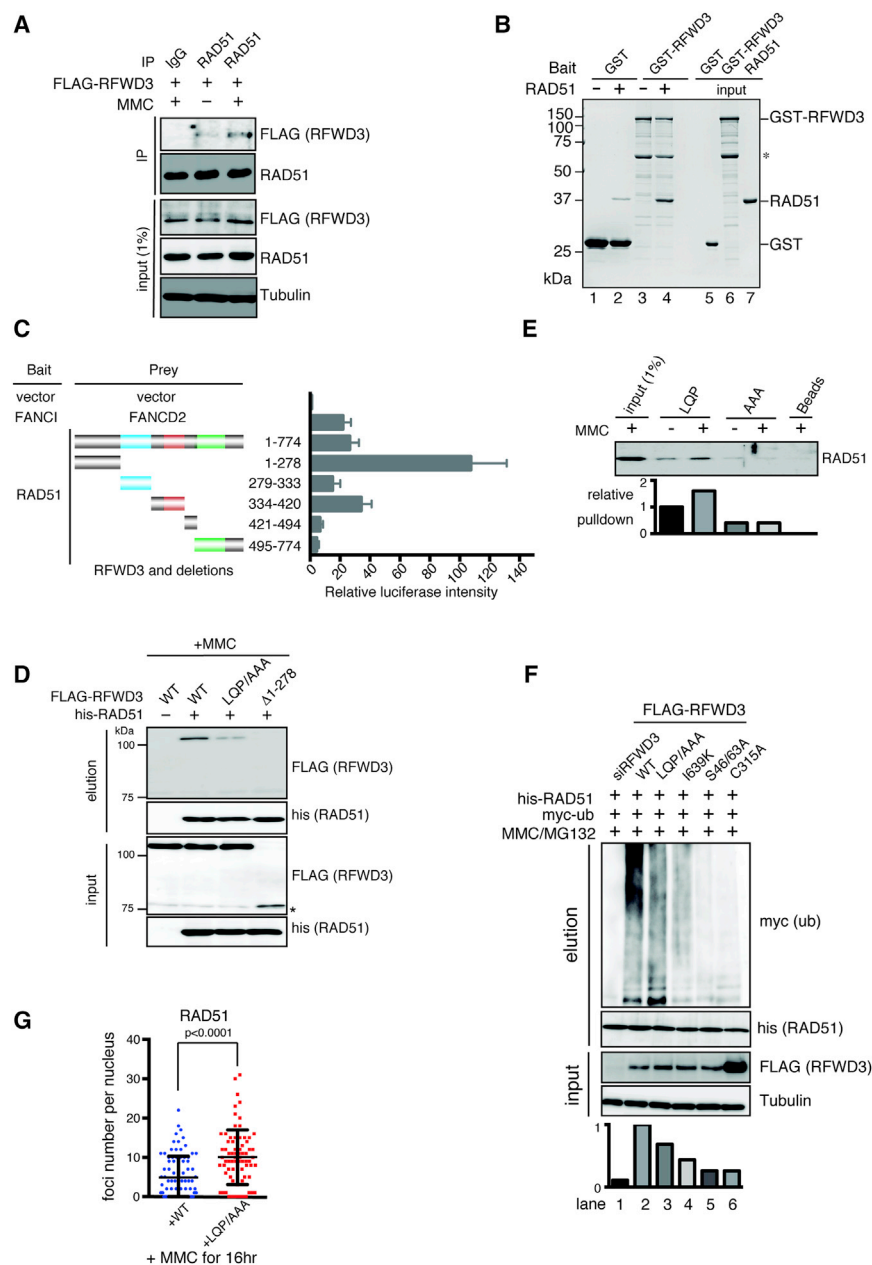


Figure 5. Physical Interaction of RAD51 with the N-Terminal Domain of RFWD3

(A) U2OS cells transfected with FLAG-RFWD3 were treated with or without MMC for 24 hr and subjected to anti-RAD51 immunoprecipitation and immunoblotting.

(B) RAD51 bound to GST-tagged RFWD3 was co-pelleted with glutathione sepharose 4B beads, and the proteins were analyzed by SDS-PAGE with Coomassie brilliant blue (CBB) staining. An asterisk indicates an impurity.

(C) Mammalian two-hybrid interaction assay between RAD51 and RFWD3 subregions. The C315A mutant was used to enhance expression levels by avoiding autoubiquitination. Luminescence signal levels were normalized to the value obtained by co-transfection of empty bait and prey vectors. FANCD2 and FANCI are included as a positive control. The domains of RFWD3 are colored according to the scheme in Figure S5B, and the expressed fragments are indicated by the amino acid number. The mean and SD values in a triplicate experiment are shown.

(D) U2OS cells co-transfected with his-RAD51 and either FLAG-RFWD3 WT or mutants were treated as in (A) and were subjected to pull-down with nickel resin. An asterisk indicates a non-specific band.

(E) Nuclear extracts were prepared from 293T cells treated as in (A) and were subjected to LQP or AAA peptide pull-down. Immunoblot of bound material was probed by anti-RAD51, and the relative efficiency of the pull-down is shown as a graph. Sequences of LQP and AAA peptides are shown in Figure S5B.

(F) 293T cells transfected with indicated plasmids or siRNA were treated with MMC for 24 hr and with 10 μ M MG132 for 1 hr before harvest. Cells were lysed under denaturing conditions and his-RAD51 was captured with Ni-resin and blotted with the indicated antibodies. The ratios of ubiquitinated (anti-myc) to non-ubiquitinated RAD51 (anti-his) bands normalized to the highest value are shown.

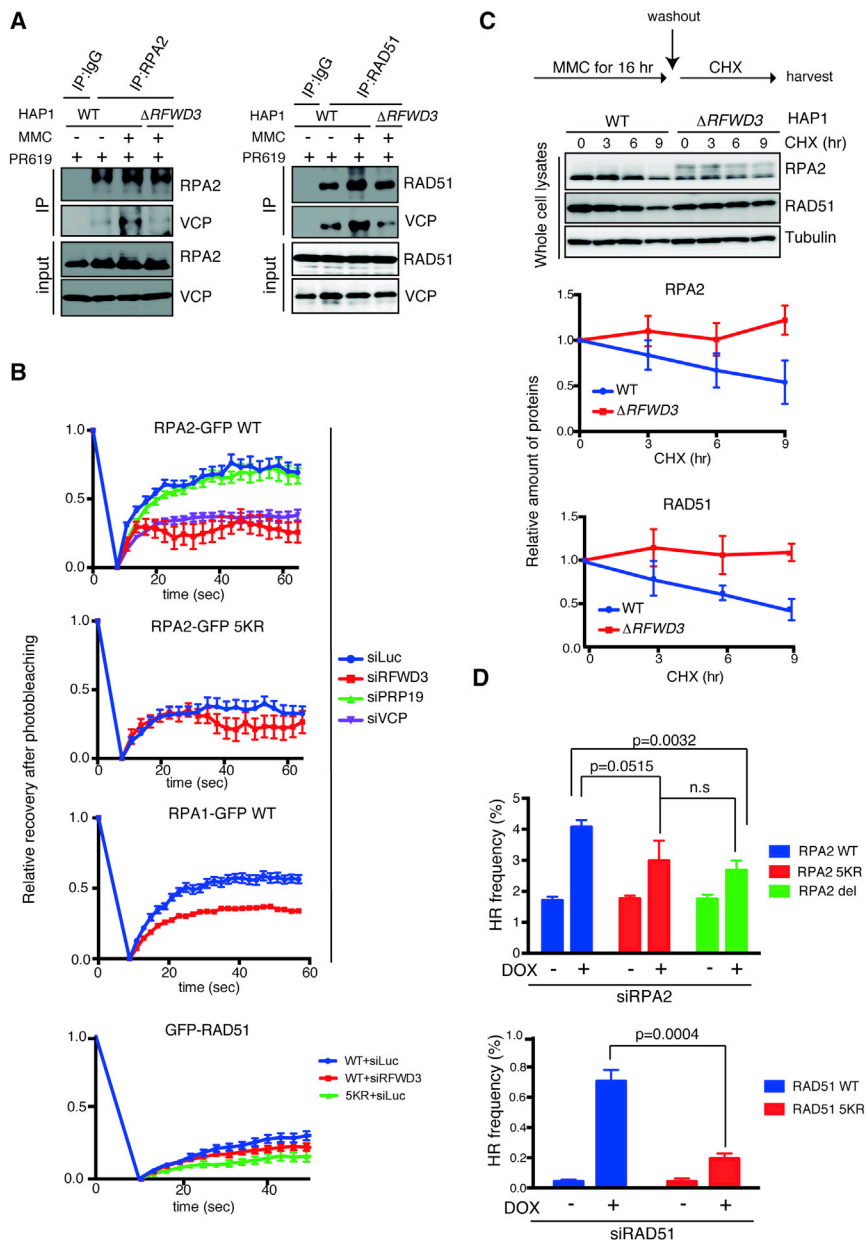
(G) HAP1 Δ RFWD3 cells transduced with RFWD3 WT or LQP/AAA mutant were treated with 20 ng/mL MMC for 16 hr and stained with anti-RAD51. Each dot represents a single cell; the mean and SD are shown.

See also Figure S5.

extracts (Figure 6A). Both interactions were enhanced following MMC treatment and were dependent on RFWD3 (Figure 6A). Furthermore, depletion of VCP enhanced polyubiquitination of RPA1 and RPA2 in chromatin isolated from MMC-treated cells; this was abrogated by siRFWD3 (Figure S6A).

To assess the effects of RFWD3 on protein dynamics of RPA and RAD51 in DNA damage sites, we next performed fluorescence recovery after photobleaching (FRAP) analysis. In each dataset, we photobleached more than 50 individual foci formed after MMC treatment of U2OS cells transiently expressing GFP-tagged RPA1 or RPA2 or expressing inducible GFP-RAD51. Examples of the MMC-induced RPA1, RPA2,

and RAD51 foci are shown in Figure S6B. In line with previous studies (Galanty et al., 2012; Gibb et al., 2014), RPA1 or RPA2 in DNA damage-induced foci was highly mobile, whereas RAD51 was rather static compared to RPA, in keeping with RAD51 in nucleoprotein filaments (Essers et al., 2002; Yu et al., 2003). Strikingly, we found that knockdown of RFWD3 drastically or mildly reduced protein turnover of RPA1 and RPA2, or RAD51, respectively (Figure 6B). Furthermore, VCP depletion reduced protein turnover of RPA2 similarly to siRFWD3 (Figure 6B). We also prepared U2OS cells stably expressing ubiquitination-deficient RPA2-5KR or RAD51-5KR variants tagged with GFP and carried out FRAP analysis. We



found that RPA2-5KR turnover was drastically reduced compared to WT RPA2 and was not affected by siRFWD3 (Figure 6B, middle panel). Knockdown of *PRP19* did not affect RPA2 turnover, which is consistent with a previous report that implicates this gene in RPA polyubiquitination via K63 linkages (Maréchal et al., 2014). Protein dynamics of the GFP-RAD51-5KR mutant were mildly reduced, as expected (Figure 6B).

Polyubiquitinated RPA and RAD51 Undergo Proteasomal Degradation after MMC Damage

Polyubiquitinated proteins removed from chromatin by p97/VCP could then be transferred to the 26S proteasome for degradation. To test whether polyubiquitination of RPA or RAD51 results

in proteasomal degradation, we performed a cycloheximide (CHX) chase assay on RPA2 and RAD51 in HAP1 WT and Δ RFWD3 cells. After a 16 hr MMC treatment, cells were cultured in the presence of CHX. During the chase period (up to 9 hr), the levels of both RPA2 and RAD51 in whole-cell lysates steadily decreased in control WT HAP1 cells, but not in Δ RFWD3 cells (Figure 6C). In contrast, the levels were stable, irrespective of the RFWD3 genotype, in cells without prior MMC treatment (Figure S6C).

Ubiquitination-Defective Mutants of RPA2 and RAD51 Display HR Defects

To prove that the critical role of RFWD3 in HR is the polyubiquitination of RPA2 and RAD51, we used U2OS cells in which endogenous RPA2 or RAD51 were replaced with the 5KR mutants as described above (Figures 2E, 3C, and S6D) and tested whether they displayed decreased HR efficiency comparable to Δ RFWD3 cells.

Figure 6. RFWD3 Promotes RPA2 and RAD51 Foci Turnover, Degradation, and HR Repair

(A) HAP1 and HAP1 Δ RFWD3 cells were treated with 20 ng/mL MMC for 16 hr and 10 μ M PR619 for 2 hr before harvest. Cell lysates were immunoprecipitated and blotted with indicated antibodies. (B) FRAP analysis. U2OS cells transiently transfected with the indicated siRNAs and expression constructs were exposed to MMC for 24 hr, and protein dynamics in MMC-induced foci were analyzed by FRAP. For RAD51-GFP foci FRAP, U2OS cells stably transduced with GFP-RAD51-WT or -5KR were transfected with the indicated siRNA and stimulated with Dox (1 ng/mL) and MMC for 24 hr. The mean and SD are shown at each time point. See also Figure S6B for representative images of RPA-GFP or GFP-RAD51 foci. (C) HAP1 and HAP1 Δ RFWD3 cells treated with 20 ng/mL MMC for 16 hr were chased up to 9 hr in the presence of 20 μ g/mL cycloheximide (CHX) and analyzed by immunoblotting. The ratios of RPA2-to-tubulin and RAD51-to-tubulin band intensities were normalized to those at the start of the chase. The experiments were repeated three times; the mean and SD are shown. (D) U2OS DR-GFP reporter cells were stably transduced with indicated RPA2 or RAD51 mutant. Expression was induced by addition of Dox, and endogenous RPA2 or RAD51 was depleted by siRNA (Figure S6D). The next day, the I-SceI expression vector was transfected, and 48 hr later the GFP-positive population was analyzed. The HR frequency was expressed as percentage of GFP positive cells in the viable cell population. The experiments were repeated three times; the mean and SD are shown. RPA2 del, RPA2 deletion mutant lacking residues 244–254. See also Figure S6.

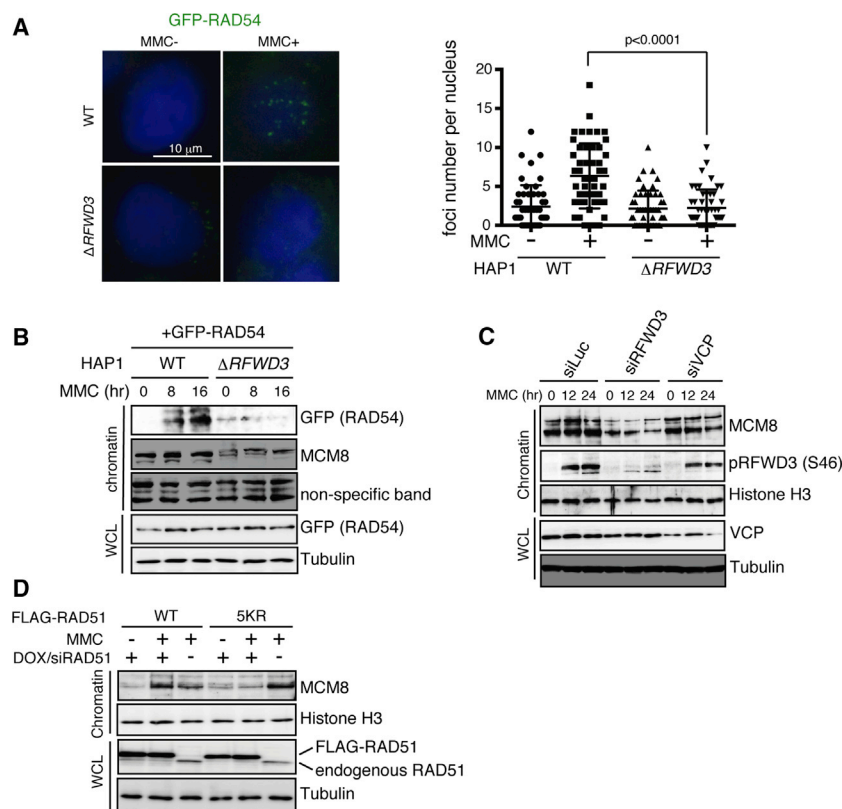


Figure 7. Defective RFWD3-Dependent Ubiquitination Impairs Chromatin Loading of MCM8 and RAD54

(A) HAP1 WT or Δ RFWD3 cells expressing GFP-RAD54 were treated with 20 ng/mL MMC for 16 hr and immunostained with anti-GFP and Alexa-Fluor-488-conjugated antibody. Each dot represents the number of nuclear foci in a single cell; the mean and SD are shown.

(B) Cells in (A) were fractionated and analyzed by immunoblotting. WCL, whole-cell lysates.

(C) U2OS cells transfected with siRNAs were treated with MMC for indicated times and analyzed by immunoblotting after cell fractionation.

(D) U2OS cells expressing Dox-inducible GFP-RAD51-WT or -5KR mutant were transfected with indicated siRNAs and stimulated with Dox and MMC for 24 hr. After biochemical fractionation, samples were analyzed by immunoblotting.

activities in vitro (Figure S4I), excluding the possibility that the 5KR mutation largely compromised RAD51 function.

Inactivation of RFWD3 Inhibits Chromatin Loading of RAD54 and MCM8

To gain insight into how RFWD3 inactivation impairs progression of late phase HR, we examined whether chromatin recruitment of RAD54 and MCM8 occurs normally in the absence of RFWD3. RAD54 belongs to the SWI2/SNF2 family of double-stranded DNA (dsDNA)-dependent ATPase chromatin remodelers and performs important functions including both formation and disruption of RAD51 filaments, branch migration in HR, and RAD51 removal in G2 phase (Heyer et al., 2006; Mason et al., 2015; Spies et al., 2016; Wright and Heyer, 2014). MCM8 is a member of the AAA⁺ superfamily that is thought to work in HR downstream of RAD51 loading (Lutzmann et al., 2012; Nishimura et al., 2012) as well as in DNA end resection (Lee et al., 2015).

To detect RAD54 in cells, HAP1 and HAP1 Δ RFWD3 cells were stably transduced with a lentivirus encoding GFP-RAD54. HAP1 Δ RFWD3 cells displayed greatly reduced numbers of GFP-RAD54 foci (Figure 7A) and chromatin loading (Figure 7B) compared to WT cells following MMC treatment. Similarly, we found decreased chromatin loading of MCM8 in HAP1 Δ RFWD3 (Figure 7B) or in cells treated with siRFWD3 or siVCP, consistent with our previous conclusion that RFWD3 works in a concerted manner with VCP (Figure 7C). Depletion of BRCA2 in U2OS cells abrogated MMC-induced RAD51 chromatin loading as expected and also decreased the amount of MCM8 in chromatin (Figure S7A), as shown before using DT40 cells (Nishimura et al., 2012). These data suggest that stabilized RPA and RAD51 “locked” in recombination intermediates due to defective RFWD3-mediated polyubiquitination compromise progression of HR. Indeed, chromatin loading of MCM8 in response to MMC was eliminated in cells expressing the RAD51-5KR mutant compared to cells with WT RAD51 (Figure 7D).

Cells expressing RPA2-5KR or RAD51-5KR showed mildly and drastically reduced HR efficiencies, respectively, as measured by the DR-GFP reporter compared to the respective WT controls (Figure 6D). The mild reduction in HR by the RPA2-5KR mutant might be due to unperturbed ubiquitination on the RPA1 or RPA3 subunits. Furthermore, cells expressing these variants were cisplatin sensitive compared to control cells (Figure S6E). Collectively, these results suggest that loss of ubiquitination of RPA2 and RAD51 may lead to defective HR in Δ RFWD3 cells.

We also measured the effect of a disrupted RPA2-RFWD3 interaction on HR efficiency. Based on earlier studies (Elia et al., 2015; Gong and Chen, 2011), we prepared an RPA2 deletion mutant lacking residues 244–254, which was sufficient to disrupt the interaction as measured by M2H assay (data not shown). Endogenous RPA2 was replaced with the RPA2 deletion, as in the experiments above (Figure S6D). As expected, HR efficiency was reduced (Figure 6D), consistent with increased levels of cisplatin sensitivity (Figure S6E). Similar defects were previously described by Elia et al. using RPA2 lacking residues 243–262 (Elia et al., 2015).

Of note, we purified the recombinant trimeric RPA complex with the RPA2-5KR subunit (Figure S4A) and RAD51 with 5KR mutation to verify functionality of these mutant proteins (Figure S4B). The RPA complex stoichiometry (Figure S4A) and ssDNA binding (Figure S4H) were indistinguishable from the WT RPA complex. The purified RAD51-5KR protein showed normal levels of ATPase and strand invasion (D-loop formation)

DISCUSSION

A previous study indicated that loss of *RFWD3* function abrogates HR (Elia et al., 2015) and that this results in the FA phenotype in humans (K.K., S.I., M.J. Ramírez, M.I., J. Surrallés, M.T., and D.S., unpublished data). In this study, we identified a role of *RFWD3* in polyubiquitination of RPA and RAD51, their subsequent disassembly from DNA damage sites, and eventual degradation. We propose that their timely removal from recombination intermediates after they finish their prescribed function is essential for HR repair to proceed to later steps (Figure S7B). Thus, our findings indicate a molecular mechanism in the spatiotemporal regulation of the critical HR factors. An analogous process operates in other DNA repair pathways such as NER (Puumalainen et al., 2014) or non-homologous end joining (NHEJ) (Brown et al., 2015; Postow et al., 2008).

In sharp contrast to our results, Elia et al. reported that RPA polyubiquitination by *RFWD3* does not lead to its degradation (Elia et al., 2015). There could be several reasons for this discrepancy. First, they used HU, 4NQO, or UV stimulation, not MMC, with different time frames in their experiments (mostly up to 2 hr post-stimulation), compared to ours (16–48 hr after MMC stimulation). Second, they did not look at RPA foci or RPA protein levels with CHX chase, after DNA damage in the absence of *RFWD3*. Instead, they measured the amount of ubiquitinated RPA peptides following UV by a proteomic approach and found they were not increased by pretreatment with MG132 (described in Figure S3B, the post-UV time point was not specified but probably 2 hr similar to other experiments). They may have missed the RPA degradation because of these different experimental conditions.

Regulation and Activation Mechanism for *RFWD3* during HR Repair

Prior studies have established that the RPA2-*RFWD3* interaction is required for *RFWD3* recruitment to DNA-damage-induced RPA foci (Gong and Chen, 2011; Liu et al., 2011). In keeping with this notion, the FA patient-derived I639K mutation in the *RFWD3* WD40 domain attenuated both MMC-induced *RFWD3* foci (K.K., unpublished data) and the RPA2 interaction. We suggest that *RFWD3* might also promote RPA loading onto chromatin as shown in Figure S1G. Our in vitro binding data suggest that a preformed RPA-*RFWD3* complex is recruited to ssDNA, rather than RPA bound to ssDNA recruits *RFWD3*. In chromatin, *RFWD3* becomes phosphorylated at least on residue S46 by ATM/ATR kinases, which was abrogated by the I639K mutation. We found that the *RFWD3* S46/63A double substitution diminished RPA2 and RAD51 ubiquitination and increased sensitivity to MMC, while there was no effect on the interaction of *RFWD3* with RPA2. *RFWD3* harbors a number of additional SQ motifs that may be involved in the activation upon phosphorylation by ATM/ATR. Thus, our results support a model in which *RFWD3* is recruited and enzymatically activated at the DNA damage sites depending on the interaction with RPA and subsequent multiple phosphorylations by ATM/ATR.

Although it remains unknown exactly at which step of HR *RFWD3* acts to promote progression of ICL repair, it should be in late phase HR; logically, if *RFWD3* were to act at an early

phase, it would function as an anti-recombinase. Given the foci data following MMC or IR, *RFWD3* does not appear to replace RPA with RAD51, which is supposed to be carried out by BRCA2 (Zelensky et al., 2014). Therefore, we speculate that *RFWD3* may be activated after RAD51 is loaded onto ssDNA by BRCA2. The precise mechanism remains unclear and awaits clarification by future studies.

How Persistent RPA2 and RAD51 at DNA Damage Sites Affect HR Repair

Our data suggest that *RFWD3* inactivation should lead to formation of RAD51 filaments scattered with RPA (mixed filaments), which might be reflected by the increased RAD51/RPA2 foci co-localization and the PLA signal between the two proteins. If the mixed filaments were functionally compromised, this may contribute to the loss of HR activities in Δ *RFWD3* cells. Alternatively, *RFWD3* may function at the postsynaptic stage. For example, RAD51 and RPA in a D-loop may need to be removed from the 3' end of the invading ssDNA to initiate efficient DNA repair synthesis. RPA removal may also aid the annealing/capture process with the other DNA end. Further investigation will reveal how *RFWD3* recognizes RPA and RAD51 during HR repair and how the late stages of ICL repair are coordinated. In addition to effects on RPA and RAD51, we found loss of *RFWD3* accompanied decreased chromatin loading of RAD54 and MCM8. Thus *RFWD3* may license *RAD54* or *MCM8* to carry out their tasks. Ubiquitination-defective RAD51 adversely affected chromatin MCM8 loading after MMC damage. We suggest that this could be the direct consequence of persistent accumulation of RAD51.

Conclusions

In this study, we provide evidence that an E3 ligase *RFWD3* functions in timely removal and degradation of RPA and RAD51 to allow HR progression to subsequent steps following MMC damage. These mechanisms set the framework to further elucidate the spatiotemporal regulation of critical components of HR repair, which maintains genome stability to suppress hematopoietic failure in FA and to likely prevent cancer development in humans.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2017.04.022>.

AUTHOR CONTRIBUTIONS

M.T., D.S., K.K., H.K., and A.T.-K. conceived the study; S.I. carried out experiments with help from Y.K. and M.I.; K.S. carried out in vitro assays with H.K., W.K., and H.T.; K.N., S.N., and H.M. provided unpublished reagents; and M.T., S.I., H.K., and K.S. wrote the paper.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-human RFWD3	Abcam	Cat# ab138030; RRID: AB_10677293
Rabbit polyclonal anti-human phospho-RFWD3 (S46)	this paper	N/A
Rabbit polyclonal anti-human RPA1	Bethyl	Cat# A300-241A; RRID: AB_2180681
Rabbit monoclonal anti-human RPA1	Abcam	Cat# ab199240
Mouse monoclonal anti-human RPA2	Abcam	Cat# ab2175 (9H8); RRID: AB_302873
Rabbit polyclonal anti-human RPA2	Bethyl	Cat# A300-244A; RRID: AB_185548
Rabbit polyclonal anti- human phospho-RPA2 (S4/S8)	Bethyl	Cat# A300-245A; RRID: AB_210547
Rabbit polyclonal anti- human phospho-RPA2 (S33)	Bethyl	Cat# A300-246A; RRID: AB_2180847
Rabbit polyclonal anti- human RPA3	Sigma-Aldrich	Cat# HPA005708; RRID: AB_1856411
Rabbit polyclonal anti- human RAD51	(Kim et al., 2005)	N/A
Mouse monoclonal anti- human Chk1	Santa Cruz	Cat# sc-8408 (G-4); RRID: AB_627257
Rabbit polyclonal anti- human phospho-Chk1 (S345)	Cell Signaling	Cat# 133D3; RRID: AB_331212
Mouse monoclonal anti-DDDDK tag (for cytochemistry)	MBL	Cat# M185-3L; RRID: AB_11123930
Mouse monoclonal anti-FLAG (for immunoblotting)	Sigma-Aldrich	Cat# P2983; RRID: AB_439685
Mouse monoclonal anti-GFP	MBL	Cat# M048-3 (1E4); RRID: AB_591823
Mouse monoclonal anti- α Tubulin	Sigma-Aldrich	Cat# T5168; RRID: AB_477579
Mouse monoclonal anti-Histone H3	Active Motif	Cat# 61647 (1C8B2)
Mouse monoclonal anti-6 x His	Abcam	Cat# ab18184 (HIS.H8); RRID: AB_444306
Mouse monoclonal anti-c-Myc	Santa Cruz	Cat# sc-40 (9E10); RRID: AB_627268
Goat polyclonal anti-human MCM8	Novus	Cat# NB100-325; RRID: AB_2142828
Rabbit polyclonal anti- human VCP	GeneTex	Cat# GTX101089; RRID: AB_1952544
Mouse monoclonal anti- human DMC1	Abcam	Cat# ab11054; RRID: AB_297706
Rabbit polyclonal anti- human FANCD2	Novus	Cat# NB100-502; RRID: AB_10002943
Chemicals, Peptides, and Recombinant Proteins		
MG 132 (proteasome inhibitor)	Millipore	474787
PR 619 (deubiquitinase inhibitor)	Abcam	ab144641
NU-7026 (DNA-PK inhibitor)	Sigma-Aldrich	N1537-5MG
KU-55933 (ATM inhibitor)	Millipore	118500-10MG
VE-821 (ATR inhibitor)	Toronto Research Chemicals	V756700
olaparib (PARP inhibitor)	Selleck Chemicals	S1060
Cycloheximide	Cell Signaling Technology	Cat# 2112
RFWD3 N-terminal peptides	this paper	N/A
Recombinant hRFWD3	this paper	N/A
Recombinant GST-hRFWD3	this paper	N/A
Recombinant hUBE2D1	this paper	N/A
Recombinant hUBE2N	(McKenna et al., 2001)	N/A
Recombinant hMMS2	(McKenna et al., 2001)	N/A
Recombinant hUBE2T	(Sato et al., 2012b)	N/A
Recombinant hRPA complex	(Henricksen et al., 1994)	N/A
Recombinant hRPA complex with the RPA2-5KR subunit	this paper	N/A
Recombinant hRAD51	(Ishida et al., 2008)	N/A
Recombinant hRAD51-5KR	this paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant hDMC1	(Hikiba et al., 2008)	N/A
Recombinant human ubiquitin activating enzyme (E1)	Boston Biochem	Cat# E-305
Recombinant human ubiquitin	Boston Biochem	Cat# U-100H
Critical Commercial Assays		
Duolink In Situ PLA kit	Sigma-Aldrich	Cat# DUO92007-100
Dual-Glo Luciferase Assay System	Promega	Cat# E2940
cOmplete His-Tag Purification Resin	Roche	Cat# 05893682001
AntiINT1-FLAG M2 Affinity Gel	Sigma-Aldrich	Cat# A2220
Anti-FLAG M2 Magnetic Beads	Sigma-Aldrich	Cat# M8823
Dynabeads Protein G Immunoprecipitation Kit	Thermo Fischer	Cat# 10007D
Dynabeads M-280 Streptavidin	Thermo Fischer	Cat# 11206D
Deposited Data		
Raw data	This paper	http://dx.doi.org/10.17632/2nd9gr954b.1
Experimental Models: Cell Lines		
HAP1 control	Horizon Genomics	Cat# C631
HAP1 Δ RFWD3	Horizon Genomics	Cat# HZGHC002532c010
HAP1 Δ RFWD3 FLAG-hRFWD3 WT	this paper	N/A
HAP1 Δ RFWD3 FLAG-hRFWD3 I639K	this paper	N/A
HAP1 Δ RFWD3 FLAG-hRFWD3 S46/63A	this paper	N/A
HAP1 Δ RFWD3 FLAG-hRFWD3 LQP/AAA	this paper	N/A
HAP1 Δ RFWD3 FLAG-hRFWD3 C315A	this paper	N/A
HAP1 Δ RFWD3 FLAG-hRFWD3 C315A/I639K	this paper	N/A
HEK293T	(Unno et al., 2014)	N/A
U2OS	(Unno et al., 2014)	N/A
U2OS DR-GFP	Maria Jasin laboratory	N/A
U2OS DR-GFP tet-inducible RPA2-FLAG WT	this paper	N/A
U2OS DR-GFP tet-inducible RPA2-FLAG 5KR	this paper	N/A
U2OS DR-GFP tet-inducible RPA2-FLAG deletion	this paper	N/A
U2OS DR-GFP tet-inducible FLAG-RAD51 WT	this paper	N/A
U2OS DR-GFP tet-inducible FLAG-RAD51 5KR	this paper	N/A
Oligonucleotides		
siRNA targeting sequence: RFWD3#1; GGACCUAC UUGCAAACUAUtt	(Gong and Chen, 2011)	N/A
siRNA targeting sequence: RFWD3#2; GCAGUCAU GUGCAGGAGUUtt	(Gong and Chen, 2011)	N/A
siRNA targeting sequence: RPA2; AACAGAUUGUGA AACUAGGtt	(Vassin et al., 2004)	N/A
siRNA targeting sequence:: RAD51; AGCUUCUCCAA AUUUCUUCtt	(Biard, 2007)	N/A
siRNA targeting sequence:: VCP; ACAACUGUGAUA CAAUGCGtt	(Meerang et al., 2011)	N/A
siRNA targeting sequence: BRCA2; UAAGGAACGU CAAGAGAUAtt	(Kakaroukas et al., 2013)	N/A
siRNA targeting sequence: PRP19; UGUAAGCAGU GAUCUAGUUUCAUUA	(Maréchal et al., 2014)	N/A
siRNA targeting sequence:Luciferase; UCGAAGUAUU CCGCGUACGtt	(Hira et al., 2015)	N/A
Oligo DNA sequence: 45-mer ssDNA; CCCAGGCCATT ACAGATCAATCCTGAGCATGTTTACCAAGCGCAG	Nihon Gene Research Laboratory	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligo DNA sequence: 49-mer ssDNA; GTCCCAGGCCA TTACAGATCAATCCTGAGCATGTTACCAAGCGCATTG	(Iwasaki et al., 1992)	N/A
Oligo DNA sequence: 70-mer ssDNA; CCGGTATATTCA GCATGGTATGGTCGTAGGCTCTTGCTTGATGAAAGT TAAGCTATTTAAAGGGTCAGGG	(Kobayashi et al., 2016)	N/A
Recombinant DNA		
pcDNA3.1 FLAG-hRFWD3 WT	this paper	N/A
pcDNA3.1 FLAG-hRFWD3 C315A	this paper	N/A
pcDNA3.1 FLAG-hRFWD3 I639K	this paper	N/A
pcDNA3.1 FLAG-hRFWD3 S46/63A	this paper	N/A
pcDNA3.1 FLAG-hRFWD3 aa 1-278	this paper	N/A
pcDNA3.1 FLAG-hRFWD3 LQP/AAA	this paper	N/A
pcDNA4.0 his-ubiquitin	this paper	N/A
pcDNA4.0 myc-ubiquitin	(Nakada et al., 2010)	N/A
pcDNA4.0 his-RPA2 WT	this paper	N/A
pcDNA4.0 his-RPA2 2KR	this paper	N/A
pcDNA4.0 his-RPA2 3KR	this paper	N/A
pcDNA4.0 his-RPA2 5KR	this paper	N/A
pcDNA3.1 FLAG-RPA3	this paper	N/A
pcDNA4.0 his-RAD51 WT	this paper	N/A
pcDNA4.0 his-RAD51 K58/64R	this paper	N/A
pcDNA4.0 his-RAD51 K107/156R	this paper	N/A
pcDNA4.0 his-RAD51 5KR	this paper	N/A
pcDNA3.1 RPA2-GFP WT	this paper	N/A
pcDNA3.1 RPA2-GFP 5KR	this paper	N/A
pcDNA3.1 GFP-RAD51 WT	this paper	N/A
pcDNA3.1 GFP-RAD51 5KR	this paper	N/A
pcDNA3.1 CMV-RPA1-GFP	this paper	N/A
CSIV hRPA2 WT-FLAG	this paper	N/A
CSIV hRPA2 5KR-FLAG	this paper	N/A
CSIV hRPA2 Δ 244-3-25467-FLAG	this paper	N/A
CSIV FLAG-RAD51 WT siResistant	this paper	N/A
CSIV FLAG-RAD51 5KR siResistant	this paper	N/A
CSII GFP-RAD54	this paper	N/A
CSII FLAG-hRFWD3 WT	this paper	N/A
CSII FLAG-hRFWD3 I639K	this paper	N/A
CSII FLAG-hRFWD3 S46/63A	this paper	N/A
CSII FLAG-hRFWD3 C315A	this paper	N/A
CSII FLAG-hRFWD3 C315A/I639K	this paper	N/A
CSII FLAG-hRFWD3 LQP/AAA	this paper	N/A
pM hFANCI	(Unno et al., 2014)	N/A
pM hRAD51	this paper	N/A
pVP FANCD2	(Unno et al., 2014)	N/A
pVP hRFWD3 1-774/C315A	this paper	N/A
pVP hRFWD3 1-278	this paper	N/A
pVP hRFWD3 279-333/C315A	this paper	N/A
pVP hRFWD3 334-420	this paper	N/A
pVP hRFWD3 421-494	this paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
pVP hRFWD3 495-774	this paper	N/A
pENTR hRFWD3	this paper	N/A
pcDNA-FLAG-RPA2	(Unsal-Kaçmaz et al., 2007)	Addgene plasmid #22893
pcDNA3.1 hRAD54	(Tanaka et al., 2000)	N/A
pGEX-6P-1 hRFWD3	this paper	N/A
pGEX-6P-1 hUBE2D1	this paper	N/A
pGEX-6P-1 hUBE2N	this paper	N/A
pGEX-6P-1 hMMS2	this paper	N/A
pGEX-6P-1 hUBE2T	(Sato et al., 2012b)	N/A
p11d-tRPA	(Henricksen et al., 1994)	N/A
p11d-tRPA carrying RPA2-5KR	this paper	N/A
pET-15b hRAD51	(Ishida et al., 2008)	N/A
pET-15b hRAD51-5KR	this paper	N/A
pET-15b hDMC1	(Hikiba et al., 2008)	N/A
Software and Algorithms		
Prism6	GraphPad software	https://www.graphpad.com
ImageQuant TL	GE Healthcare	http://www.gelifesciences.co.jp/catalog/1167.html
BZ analyzer Hybrid Cell Count BZ-H2C	Keyence	N/A
MultiGauge ver. 3.2	Fujifilm	N/A
ImageJ	NCBI	https://imagej.nih.gov/ij/index.html

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Minoru Takata (mtakata@house.rbc.kyoto-u.ac.jp).

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Genetics and culture of cell lines**

HAP1 WT and Δ RFWD3 cells, which were generated by the CRISPR/Cas9 system as shown in Figure S1A, were purchased from Horizon Genomics. HAP1 Δ RFWD3 cells were validated by Sanger Sequencing, and immunoblotting. HAP1 cells and derivatives were cultured in IMDM (Nacalai Tesque) supplemented with 10% fetal calf serum (GIBCO) and Penicillin/Streptomycin (Nacalai Tesque). U2OS, U2OS-DR-GFP (a kind gift from Dr Maria Jasin), and 293T cells were cultured in DMEM (Nacalai Tesque) supplemented with 10% fetal calf serum. Cells were stimulated with MMC at the concentration of 100 ng/ml unless stated otherwise.

METHOD DETAILS**Plasmids**

Human RFWD3 (synthesized), RAD54 (a kind gift from Dr Kiyoshi Miyagawa, University of Tokyo), and RAD51 cDNAs were amplified and subcloned into pENTR entry vector (Invitrogen), and transferred to the expression vectors or lentiviral constructs by LR Clonase II (Invitrogen). Mutations in plasmids were generated by high-fidelity PCR using KOD-plus-neo or KOD-FX polymerases (TOYOBO) and an In-Fusion HD Cloning kit (Takara); all mutations were confirmed by Sanger sequencing. Human RPA1 cDNA derived from U2OS, human RPA2 cDNA in Flag-RPA2 (a kind gift from Dr Aziz Sancar, University of North Carolina, Addgene #22893) and synthesized Ubiquitin cDNA were amplified and subcloned into pcDNA4/HisMax C (Invitrogen) using an In-Fusion HD kit. Myc-ubiquitin constructs were previously described (Nakada et al., 2010). A long linker in the pcDNA4/HisMax C Ubiquitin was removed by PCR-based mutagenesis. Ubiquitin KR mutants were generated by PCR following a standard site-directed mutagenesis protocol using KOD-plus-neo followed by DpnI digestion and transformation into competent cells.

siRNA and plasmid transfections

siRNA duplexes were purchased from Invitrogen and are listed in the key resources table. Transfection was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. For plasmid transfections or co-transfection of plasmids and siRNA, Lipofectamine 3000 (Invitrogen) was used. In all siRFWD3 transfections, siRFWD3#2 was used unless stated otherwise.

Ploidy analysis

Cells at exponential growth were fixed by 70% ethanol, stained with PBS containing propidium iodide (5 μ g/ml) and analyzed using a FACSCalibur flow cytometer (BD).

Colony survival assay

To measure cell survival in the presence of MMC, Cisplatin, Olaparib, CPT, or HU, an appropriate number of HAP1 cells were treated in triplicate with the indicated concentrations of the DNA damaging agents for 24 hr, except for HU (8 hr) (because of extreme toxicity with a 24 hr treatment). Cells were permitted to recover for 24 hr after treatment, and plated with serial dilutions. After 7–10 days incubation, cells were fixed with 100% ethanol, stained with 0.006% crystal violet solution (0.006% crystal violet, 25% methanol), and colonies were counted.

Immunohistochemistry and in situ PLA assay

Cells grown on cover glass were washed with PBS twice and fixed with PBS containing 3% paraformaldehyde, 2% sucrose, 0.5% Triton X-100 on ice for 30 min, and then permeabilized with 0.5% Triton X-100/PBS for 5 min. After blocking with 2% BSA/PBS, staining with antibodies diluted in 2% BSA/PBS was performed for 1 hr at RT. The secondary antibodies used were Alexa Fluor 488-conjugated anti-mouse IgG or Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probes). The experiments were repeated three times, and > 50 cells were analyzed in each experiment. The in situ PLA assay was carried out using a DuoLink in situ PLA kit (Sigma) according to the manufacturer's instructions. Images were captured using a BZ-9000 fluorescence microscope (Keyence) with a Plan Apo λ 40X/NA 0.95 objective lens (Nikon). Counting of the PLA signal dots was done using Hybrid cell count software (Keyence).

Chromatin Isolation

Cells were lysed in Buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M Sucrose, 10% Glycerol, 0.1% Triton X-100, containing 1 mM DTT and 1 mM Na_3VO_4 , and protease inhibitor cocktail (Roche)) on ice for 30 min; samples were then centrifuged at 1700 $\times$ g for 5 min, and remaining pellets were washed once with Buffer A. Pellets were resuspended in Buffer B (3 mM EDTA, 0.3 mM EGTA) containing 1 mM DTT, 1 mM Na_3VO_4 and protease inhibitor cocktail, then incubated on ice for 30 min. After centrifugation at 5000 $\times$ g for 5 min, pellets were solubilized in SDS sample buffer and analyzed as the chromatin fraction.

Immunoprecipitation and blotting

Cells were washed once with PBS, lysed in NETN buffer (150 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl pH8.0, 0.5% NP-40) supplemented with protease inhibitor cocktail, 25 unit/ml Benzonase (Millipore), 2 mM $MgCl_2$, and 1 mM Na_3VO_4 , on ice for 30 min. Then lysates were briefly sonicated and centrifuged at 14000 rpm for 10 min. The pellet was discarded. Dynabeads Protein G (Invitrogen) was incubated with an appropriate primary antibody prior to addition to the supernatant, following the manufacturer's instructions. To capture his-tagged or FLAG-tagged proteins, cOmplete His-Tag Purification Resin (Roche) or anti-FLAG M2 Agarose Affinity Gel (Sigma) was utilized, respectively. Captured proteins were washed five times with NETN buffer and analyzed by immunoblotting. Samples were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). Detection was performed as described previously (Unno et al., 2014).

In vivo ubiquitination assay

24 hr after transfection, cells were subjected to 100 ng/ml MMC for an additional 24 hr. 10 μ M MG132 was added as indicated 2 hr before sample collection. Cells were lysed in Guanidine Buffer (6 M Guanidine, 100 mM NaH_2PO_4/Na_2HPO_4 , pH8.0), or in Hot SDS Buffer at 95°C (1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH8.0); in the latter case, samples were then diluted 10-fold with binding buffer (20 mM H_3PO_4 , 300 mM NaCl, pH8.0). After DNA fragmentation by passing through a 27G needle and sonication (Bioruptor, COSMO-BIO), lysates were incubated with Ni-resin at RT for 2 hr. Bound complexes were washed five times with washing buffer (300 mM NaCl, 20 mM H_3PO_4 , pH 8.0, 5mM imidazole), and eluted in SDS sample buffer containing 200 mM imidazole. Eluted samples were analyzed by immunoblotting. For detection of endogenous RAD51 ubiquitination, 10 μ M PR619, a deubiquitinase inhibitor (Abcam), was added 2 hr before harvest. The band intensities of the respective ubiquitinated proteins were quantified with LAS-4000 ImageQuant TL software (GE Healthcare).

Mammalian two-hybrid analysis

Bait and prey vectors (pM and pVP16, respectively, Clontech) with indicated inserts were transfected into 293T cells in triplicate with an expression vector for Renilla luciferase (Unno et al., 2014). Cells were lysed after $\sim$ 48 hr and assayed for luciferase activity using

Dual-Luciferase Reporter Assay System (Promega). Transfection efficiency was normalized by Renilla luciferase activity. Intensity of the interaction was expressed as luciferase activity in relative to the value obtained by empty vectors. pM-FANCI and pVP16-FANCD2 were used as positive control.

Generation of lentivirus and transduction

To complement HAP1 Δ RFWD3 cells, lentiviral particles were prepared by cotransfecting CSII-CMV-MCS-IRES-Bsd vector, in which human RFWD3 WT and mutant cDNAs or GFP-RAD54 were cloned, along with packaging constructs, into 293T cells using Lipofectamine 2000. Following lentivirus infection, Blastcidin S (5 μ g/ml) resistant populations were selected and expanded. To produce U2OS or U2OS-DR-GFP cell lines expressing GFP-RAD51, FLAG-RAD51-WT or -5KR, and FLAG-RPA2-WT, -5KR or the deletion mutant lacking amino acid residue 244-254 under tetracycline-controlled transcriptional activation, cDNAs in the entry vector pENTR were transferred to a puromycin resistant derivative (a kind gift of Drs. Yoshikazu Johmura and Makoto Nakanishi) of CSIV-TRE-RfA-Ubc-KT (Unno et al., 2014) using LR Clonase II (Invitrogen). U2OS cells were infected with the respective lentivirus and selected with puromycin (2 μ g/ml).

Peptide pull-down assay

To prepare nuclear extracts, HAP1 cells collected with a cell scraper were lysed in Buffer A as above, and chilled on ice for 30 min, then cleared by centrifugation at 1700 x g for 5 min. The remaining nuclear pellet was lysed in Buffer C (20 mM HEPES, 25% glycerol, 420 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.1% Triton X-100) containing protease inhibitor cocktail and 1 mM Na₃VO₄. After incubation on ice for 30 min and centrifugation at 14000 rpm for 5 min, the supernatant was obtained as the nuclear extract. After a three-fold dilution with Buffer D (the same as Buffer C, excluding KCl), samples were incubated at 4°C overnight with Dynabeads M-280 (Streptavidin beads, Invitrogen) that had been coated with biotinylated peptides (Scrum). After extensive washing, bound materials were analyzed by immunoblotting.

DR-GFP assay

U2OS-DR-GFP cells were transduced with lentivirus CSIV that expresses FLAG-tagged RPA2 (WT, 5KR, or the deletion lacking residues 244-254) or FLAG-RAD51 (WT or 5KR) under the Doxycycline (Dox) inducible promoter, and were selected with 2 μ g/ml puromycin. The GFP-RAD51 coding sequence was rendered resistant to siRAD51 by silent mutations in the target sequence. Cells were cultured with or without 2 or 10 μ g/ml Dox for 24 hr, then siRNAs targeting endogenous RPA2 or RAD51 were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The following day, the I-SceI expression vector was transfected using Lipofectamine 3000 (Invitrogen) and the cells were further grown for two days with or without Dox. To assess HR frequency, the GFP positive cell population was quantified with a FACSCalibur flow cytometer (Beckton-Dickinson).

FRAP analysis

For RPA1-GFP or RPA2-GFP foci, U2OS cells were co-transfected with an RPA1-GFP or RPA2-GFP (WT or -5KR) expression vector along with indicated siRNAs. 24 hr later, MMC (100 ng/ml) was added and analysis was performed after an additional 24 hr. For GFP-RAD51 foci, U2OS cells expressing GFP-RAD51-WT or -5KR under a Tet-inducible promoter were transfected with indicated siRNA. 16 hr later, GFP-RAD51 expression was induced with Doxycycline (1 ng/ml) along with MMC (100 ng/ml) for 24hr. FRAP was performed using a TCS SP5 II confocal microscope (Leica Microsystems), equipped with an INU incubator system for microscopes (Tokai Hit), at 37°C with 5% CO₂. After two prebleach images were obtained, a rectangular region equivalent to about one third to one fourth of the nucleus was photobleached five times with a 488 nm Argon laser at ~80% transmission, then 20 to 25 images were acquired at two sec intervals. Two or three foci per nucleus were randomly selected and more than 50 foci were analyzed per sample. Measurement of GFP fluorescence level was carried out on ellipsoidal areas with adjustment to foci movement during experiments. The fluorescence intensity in the bleached RPA2-GFP foci was normalized to the pre-bleached signal after subtraction of the background. Data were plotted using Prism 6 software (GraphPad).

Plasmids for recombinant protein expression

The DNA fragments encoding human UBE2D1, UBE2N, and MMS2 were amplified by PCR from human HeLa Marathon-Ready cDNA (Clontech). The amplified DNA fragments encoding UBE2N and MMS2, or UBE2D1, or RFWD3 were ligated into the BamHI-Sall, or EcoRI-XhoI, or BamHI-XhoI sites of the pGEX-6P-1 vector (GE Healthcare), respectively. For the generation of the expression vector for the human RAD51-5KR mutant, the DNA fragment encoding the human RAD51 ORF carrying the 5KR mutation was ligated into the NdeI-BamHI sites of the pET-15b vector (Novagen). For the generation of the expression vector for the human RPA-5KR mutant, the DNA fragment encoding the human RPA2 ORF carrying the 5KR mutation was ligated into the p11d-tRPA vector (Henricksen et al., 1994), using an In-Fusion HD Cloning Kit (Takara).

Purification of recombinant proteins

Human RFWD3 was overexpressed in *Escherichia coli* BL21(DE3) Codon(+)RIL cells (Stratagene) at 16°C. The cells producing GST-tagged RFWD3 were collected by centrifugation, and were resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 10% glycerol, 500 mM NaCl, 30 μ M zinc acetate (ZnOAc), 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 5 mM 2-mercaptoethanol). The

cells were then disrupted by sonication, and the supernatant was separated from the cell debris by centrifugation at 27,700 $\times$ g for 30 min. The supernatant was mixed gently with Glutathione Sepharose 4B resin (3 ml; GE Healthcare) at 4°C for 1 h. The Glutathione Sepharose 4B resin was packed into an Econo-column (Bio-Rad), and was washed with 150 mL of lysis buffer, containing 1 M NaCl. The GST-tagged RFWD3 was eluted with 50 mL of elution buffer (100 mM Tris-HCl (pH 8.8), 10% glycerol, 500 mM NaCl, 30 μ M ZnOAc, 20 mM reduced glutathione, and 5 mM 2-mercaptoethanol). The GST tag was removed from RFWD3 by digestion with PreScission protease (15 unit/mg protein; GE Healthcare) during dialysis against 4 l of dialysis buffer (20 mM Tris-HCl (pH 8.0), 10% glycerol, 150 mM NaCl, and 5 mM 2-mercaptoethanol). The protein sample was concentrated with an Amicon Ultra Centrifugal Filter Device (Millipore), and was then applied to a Superdex 200 gel filtration column (HiLoad 16/60 preparation grade; GE Healthcare) equilibrated with dialysis buffer containing 500 mM NaCl. The purified RFWD3 was concentrated, and aliquots were frozen in liquid nitrogen. Human RPA, human RPA-5KR, human RAD51, human RAD51-5KR, human DMC1, human UBE2T, and the human UBE2N-MMS2 complex were purified as described previously (Sato et al., 2012a, 2012b). Human UBE2D1 was prepared by the same method as that for human UBE2T.

DNA substrates

For the D-loop formation assay, a 70-mer ssDNA, 5'-CCGGT ATATT CAGCA TGGTA TGGTC GTAGG CTCTT GCTTG ATGAA AGTTA AGCTA TTAA AGGGT CAGGG-3', was used. For the ubiquitination and electrophoresis mobility shift assays, a 45-mer ssDNA, 5'-CCCAG GCCAT TACAG ATCAA TCCTG AGCAT GTTTA CCAAG CGCAG-3', and a 49-mer ssDNA, 5'-GTCCC AGGCC ATTAC AGATC AATCC TGAGC ATGTT TACCA AGCGC ATTG-3', were used. All oligonucleotides were purchased from Nihon Gene Research Laboratory, as high-pressure liquid chromatography-purified grade. DNA concentrations are expressed in moles of nucleotides.

In vitro ubiquitination assay

RPA, RAD51, or DMC1 (1 μ M) was mixed with human recombinant E1 (75 nM; Boston Biochem), an E2-conjugating protein (either UBE2D1, UBE2N, the UBE2N-MMS2 complex, or UBE2T; 4 μ M), RFWD3 (1.5 μ M), and ubiquitin (10 μ M; Boston Biochem), in the presence or absence of the 45-mer ssDNA (5 μ M), in 10 μ l of reaction buffer, containing 50 mM Tris-HCl (pH 7.5), 3% glycerol, 45 mM NaCl, 2 mM MgCl₂, 2 mM ATP, 30 μ M ZnOAc, and 0.5 mM dithiothreitol. The reaction mixtures were incubated at 37°C for 90 min, and the reactions were then stopped by the addition of 2% SDS. The reaction products were separated by 15%, 12% or 10% SDS-PAGE, and were transferred to a polyvinylidene fluoride membrane (GE Healthcare). The ubiquitinated RPA1, RPA2, RPA3, RAD51, and DMC1 proteins were detected with an anti-RPA70 antibody (1:1000; ab199240, abcam), an anti-RPA32 antibody (1:1000; A300-244A, Bethyl Laboratories, Inc.), an anti-RPA3 antibody (1:1000; HPA005708, Sigma-Aldrich), an anti-RAD51 antibody (1:1000) (Kim et al., 2005), and an anti-DMC1 antibody (1:1000; ab11054, abcam), respectively. The band intensities of the respective ubiquitinated proteins were quantified with ImageJ (for Figure 4A), or MultiGauge ver. 3.2 software (for Figures 4B–4E and S4G; Fujifilm).

Pull-down assays using purified proteins and ssDNA beads

RPA or RAD51 (4 μ g) was incubated with either GST (18 μ g) or GST-tagged RFWD3 (18 μ g) at 37°C for 60 min in 200 μ L of pull-down buffer, containing 20 mM Tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl, 1 mM ZnOAc, 0.02% NP-40, and 5 mM 2-mercaptoethanol. Glutathione Sepharose 4B beads (3 μ l) were added to the reaction mixtures, and were gently mixed at 23°C for 60 min. The beads were then washed twice with 1 mL pull-down buffer. The RAD51 bound to the beads were analyzed by 12% SDS-PAGE with Coomassie Brilliant Blue staining. The RPA1, RPA2, and RPA3 bound to the beads were analyzed by 15% SDS-PAGE, and detected with an anti-RPA1 antibody (1:1000; ab199240), an anti-RPA2 antibody (1:2000; A300-244A), and an anti-RPA3 antibody (1:1000; HPA005708), respectively. For the pull-down assay with RPA-coated ssDNA beads, RPA (3.6 μ g) was incubated with a 5'-biotinylated 80-mer poly dT ssDNA (27 μ M) conjugated to Dynabeads M-280 Streptavidin (Invitrogen) at 37°C for 15 min, in 5 μ L of reaction buffer, containing 20 mM Tris-HCl (pH 7.5), 8% glycerol, 130 mM NaCl, 0.012% NP-40, 0.6 mM ZnOAc, and 5 mM 2-mercaptoethanol. During the incubation period, the reaction mixtures were gently mixed by tapping at 3 min intervals, and RFWD3 (5 μ g) was preincubated in 50 μ L pull-down buffer at 37°C for 15 min. After the incubation, the beads were washed once with 20 μ L pull-down buffer, and were gently mixed with preincubated RFWD3 at 23°C for 30 min. For the pull-down assay with the ssDNA beads, RPA (3.6 μ g) was pre-incubated with RFWD3 (5 μ g) at 37°C for 15 min in 50 μ L pull-down buffer. A 5'-biotinylated 80-mer poly dT ssDNA conjugated to Dynabeads M-280 Streptavidin (1 μ l, 134 μ M) was added to the reaction mixtures, which were gently mixed at 23°C for 30 min. The beads were then washed twice with 200 μ L pull-down buffer, and the proteins bound to the beads were detected by immunoblotting with an anti-RFWD3 antibody (1:2000; ab138030, abcam), an anti-RPA70 antibody (1:1000; ab199240), an anti-RPA32 antibody (1:2000; A300-244A), and an anti-RPA3 (1:1000; HPA005708).

Electrophoresis mobility shift assay

The 49-mer ssDNA was mixed with 0.2, 0.4, and 0.8 μ M of wild-type RPA or the RPA-5KR mutant in 10 μ L of reaction buffer, containing 23 mM Tris-HCl (pH 8.0), 64 mM NaCl, 5 mM KCl, 4.5% glycerol, 5 mM dithiothreitol and 5 μ g/ml bovine serum albumin, at 37°C for 15 min. The samples were then analyzed by 3.5% PAGE in 0.2 $\times$ TBE (18 mM Tris-borate and 0.4 mM EDTA) buffer. DNAs were visualized by SYBR Gold (Invitrogen) staining.

DNA binding assay with ubiquitinated proteins

RPA or RAD51 (1 μ M) was ubiquitinated with human recombinant E1 (75 nM), UBE2D1 (4 μ M), RFWD3 (1.5 μ M), and ubiquitin (10 μ M) at 37°C for 90 min, in 20 μ L of reaction buffer, containing 50 mM Tris-HCl (pH 7.5), 3% glycerol, 45 mM NaCl, 2 mM MgCl₂, 2 mM ATP, 30 μ M ZnOAc, and 0.5 mM dithiothreitol. A 5'-biotinylated 80-mer poly dT ssDNA conjugated to Dynabeads M-280 Streptavidin (1.2 μ L, 170 μ M) was then added to the reaction mixtures, which were incubated at 37°C for 15 min. During the incubation period, the reaction mixtures were gently mixed by tapping at 3 min intervals. After the incubation, the supernatant was transferred to a new tube (unbound fraction), and the beads were then washed twice with 20 μ L of wash buffer, containing 35 mM Tris-HCl (pH 7.5), 1 mM ATP, 2.5 mM MgCl₂, 0.1% NP-40, and 1 mM dithiothreitol. The proteins bound to the beads were eluted with 10 μ L SDS-sample buffer, containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.7 M 2-mercaptoethanol (bound fraction). The unbound and bound fractions were separated by 12% or 15% SDS-PAGE, and the proteins were detected with anti-RPA70, anti-RPA32, or anti-RAD51 antibodies.

RAD51 ATPase assay

RAD51 or RAD51-5KR (1.5 μ M) was incubated with 5 nCi [γ -³²P]ATP in 10 μ L of reaction buffer containing 26 mM HEPES-NaOH (pH 7.5), 1 mM Tris-HCl (pH 7.5), 45 mM NaCl, 0.01 mM EDTA, 1 mM dithiothreitol, 0.6 mM 2-mercaptoethanol, 3% glycerol, 1 mM MgCl₂, 5 μ M ATP, single-strand ϕ X174 viral (+) strand DNA (20 μ M; New England Biolabs), and 100 μ g/ml bovine serum albumin, at 37°C for 60 min. The reaction was quenched by the addition of 5 μ L of 0.5 M EDTA, and the samples were separated by thin layer chromatography on polyethyleneimine-cellulose, in a solution containing 0.5 M LiCl and 1 M formic acid. The plate was exposed to an imaging plate (Fujifilm), and the products were visualized using an FLA-7000 imaging analyzer (Fujifilm). The signal intensities of the products were quantified using MultiGauge ver. 3.2 software.

D-loop formation assay

The supercoiled dsDNA containing tandem repeats of the 5S rDNA sequence was prepared with a method avoiding irreversible denaturation by alkaline treatment of the cells (Kagawa et al., 2001). The 5'-³²P end -labeled 70-mer ssDNA (1 μ M) was incubated with RAD51 or RAD51-5KR (0–0.6 μ M) at 37°C for 10 min, in 9 μ L of reaction buffer containing 24 mM HEPES-NaOH (pH 7.5), 1 mM Tris-HCl (pH 7.5), 30 mM NaCl, 0.01 mM EDTA, 1 mM dithiothreitol, 0.4 mM 2-mercaptoethanol, 2% glycerol, 1 mM MgCl₂, 2 mM CaCl₂, 1 mM ATP, 20 mM creatine phosphate, 75 μ g/ml creatine kinase, and 100 μ g/ml bovine serum albumin. The supercoiled dsDNA (30 μ M, 1 μ L) was then added, and the reaction mixtures were further incubated at 37°C for 10 min. The reactions were stopped by addition of a 2 μ L aliquot of the stop solution, containing 0.2% SDS and 1.4 mg/ml proteinase K (Roche Applied Science). The deproteinized DNA products were separated by 1% agarose gel electrophoresis in 1 $\times$ TAE buffer (40 mM Tris acetate, 1 mM EDTA). The agarose gel was dried, and was exposed to an imaging plate. The gel image was obtained using an FLA-7000 imaging analyzer.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of immunoblotting or protein gel data

For all quantification of band intensities, ImageQuant TL software (GE healthcare), MultiGauge ver. 3.2 software, or ImageJ software (<https://imagej.nih.gov/ij/>) was used.

Quantification in immunocytochemistry and PLA assay

Hybrid Cell Count software (Keyence) was used for scoring the PLA signals and most of the immunocytochemistry images, except for FLAG-RAD51 foci in Figure 3C and GFP-RAD54 foci shown in Figure 7A.

Statistical analysis

P values were calculated by one-way ANOVA post hoc test in multiple comparisons or Student's t test using Prism 6 (Graphpad) software.

DATA AND SOFTWARE AVAILABILITY

The original, unprocessed data have been deposited to Mendeley Data and are available at <http://dx.doi.org/10.17632/2nd9gr954b.1>.

Supplemental Information

**RFWD3-Mediated Ubiquitination Promotes Timely
Removal of Both RPA and RAD51 from DNA Damage
Sites to Facilitate Homologous Recombination**

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Supplemental Figures

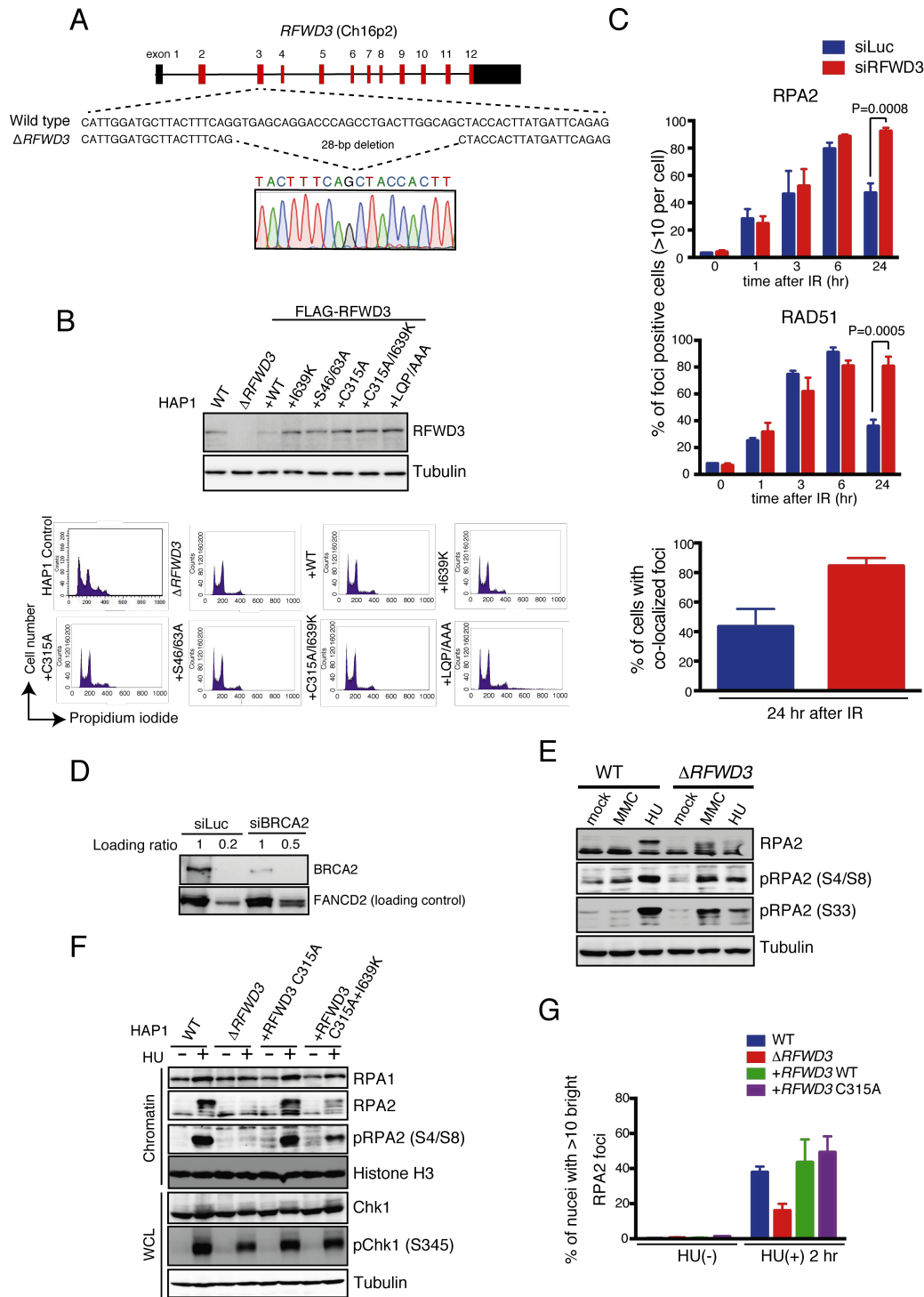


Figure S1

Figure S1. Related to Figure 1. Characterization of HAP1 $\Delta RFW3$ cells in HR repair and replication stress signaling.

- (A) Schematic illustration of the *RFWD3* knockout using the CRISPR/Cas9 system in HAP1 cells. A 28-bp deletion was introduced in exon3, resulting in a frameshift mutation (p.Q138fs46X). The deletion was validated by Sanger sequencing. The black boxes indicate untranslated regions.
- (B) Expression levels of stably transduced *RFWD3* in $\Delta RFW3$ cells were analyzed by immunoblotting (upper). HAP1 cells and derivatives fixed and stained with propidium iodide were analyzed with a FACSCalibur flow cytometer (lower).
- (C) U2OS cells were subjected to 10 Gy of gamma irradiation and permitted to recover for the indicated times before fixation and staining with anti-RAD51 or RPA2 antibodies. Cells with co-localized foci were defined as those in which RAD51 foci were positive (>10 foci per nucleus) and more than half of the RAD51 foci were co-localized with RPA2 foci. The mean and SD of three independent experiments are shown.
- (D) U2OS cells were treated with siLuc or siBRCA2, and analyzed by immunoblotting with different amounts of lysates loaded per lane.
- (E) HAP1 $\Delta RFW3$ cells were treated with 2 mM HU for 2 hr or 20 ng/ml MMC for 16 hr, and whole cell lysates were analyzed by immunoblotting.
- (F) HAP1 WT, $\Delta RFW3$, or $\Delta RFW3$ cells complemented with the indicated *RFWD3* mutants were treated with 2 mM HU for 2 hr, fractionated, and analyzed by immunoblotting. WCL, whole cell lysates.
- (G) Percentage of cells positive for RPA2 foci (>10 bright foci) in HAP1 cells (WT, $\Delta RFW3$, or $\Delta RFW3$ complemented with *RFWD3* WT or the *RFWD3* RING domain (C315A) mutant), comparing untreated cells to cells treated with 2 mM HU for 2 hr. The mean and SD of three independent experiments are shown.

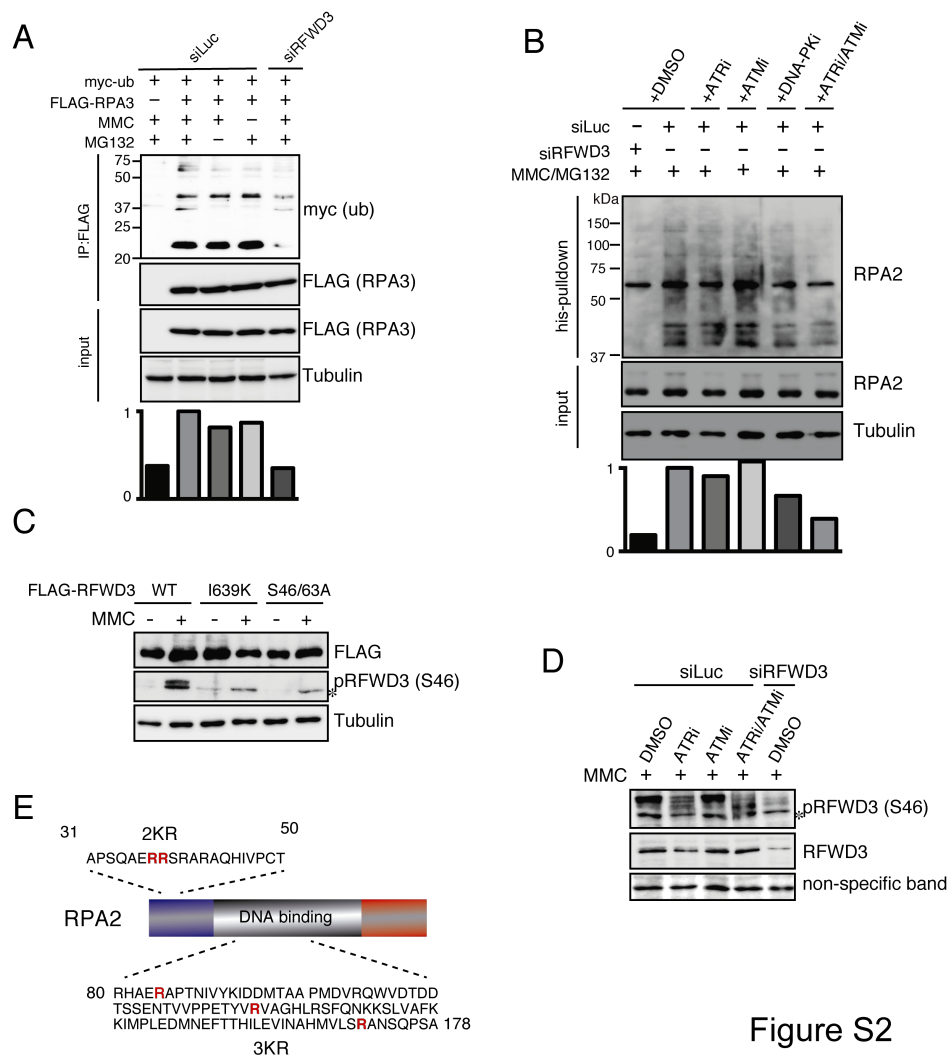
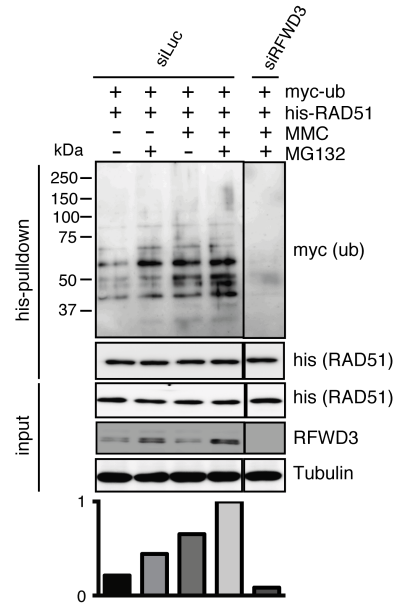


Figure S2

Figure S2. Related to Figure 2. RPA3 ubiquitination and RPA2 phosphorylation in response to MMC damage.

- (A) U2OS cells transfected with myc-ubiquitin and FLAG-RPA3 expression vectors were treated with or without 100 ng/ml MMC for 24 hr and 10 μ M MG132 for 1 hr until harvest. Cell lysates were subjected to anti-FLAG IP and detected by immunoblotting. The ratio of ubiquitinated (anti-myc) to non-ubiquitinated RPA3 (anti-FLAG) was quantified, normalized to the highest value, and is shown beneath the immunoblots.
- (B) U2OS cells transfected with his-ubiquitin were treated as in (A). 10 μ M VE-821 (ATR inhibitor), 10 μ M KU-55933 (ATM inhibitor), or 5 μ M NU-7026 (DNA-PK inhibitor) were added simultaneously with MMC for 24 hr. Cells were lysed in denaturing conditions, and Nickel resin-bound proteins were analyzed by immunoblotting. The ratio of ubiquitinated RPA2 to non-ubiquitinated RPA2 in cell lysates was normalized to the highest value and is shown beneath the immunoblots.
- (C) U2OS cells were transfected with FLAG-RFWD3 WT or indicated mutants and treated with 100 ng/ml MMC for 24hr, and analyzed by immunoblotting. An asterisk indicates a non-specific band.
- (D) U2OS cells treated with the indicated siRNAs were subjected to 100 ng/ml MMC and the indicated inhibitors (10 mM) for 24 hr, and analyzed by immunoblotting.
- (E) Schematic illustration of RPA2 KR mutants (2KR, 3KR, and 5KR). Lysine residues in red are candidate ubiquitination sites as previously suggested, and were replaced by Arginine.

A



B

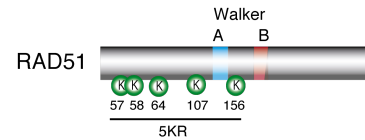


Figure S3

Figure S3. Related to Figure 3. Analysis of RFWD3-dependent polyubiquitination of RAD51.

- (A) U2OS cells transfected with indicated siRNA, his-RAD51, FLAG-RFWD3, and myc-ubiquitin WT or KR mutants were treated with 100 ng/ml MMC for 24 hr and 10 μ M MG132 for 1 hr until harvest. Cells were lysed in denaturing conditions and Nickel resin-bound materials were analyzed by immunoblotting. The ratio of ubiquitinated (anti-myc) to total RAD51 (anti-his) was normalized to the highest value and is shown beneath the immunoblots.
- (B) Schematic illustration of RAD51-KR mutation sites (5KR). Lysine residues in red are candidate ubiquitination sites as suggested by previous proteomics data, and were replaced with Arginine. K58/64R or K107/156R mutations were also generated.

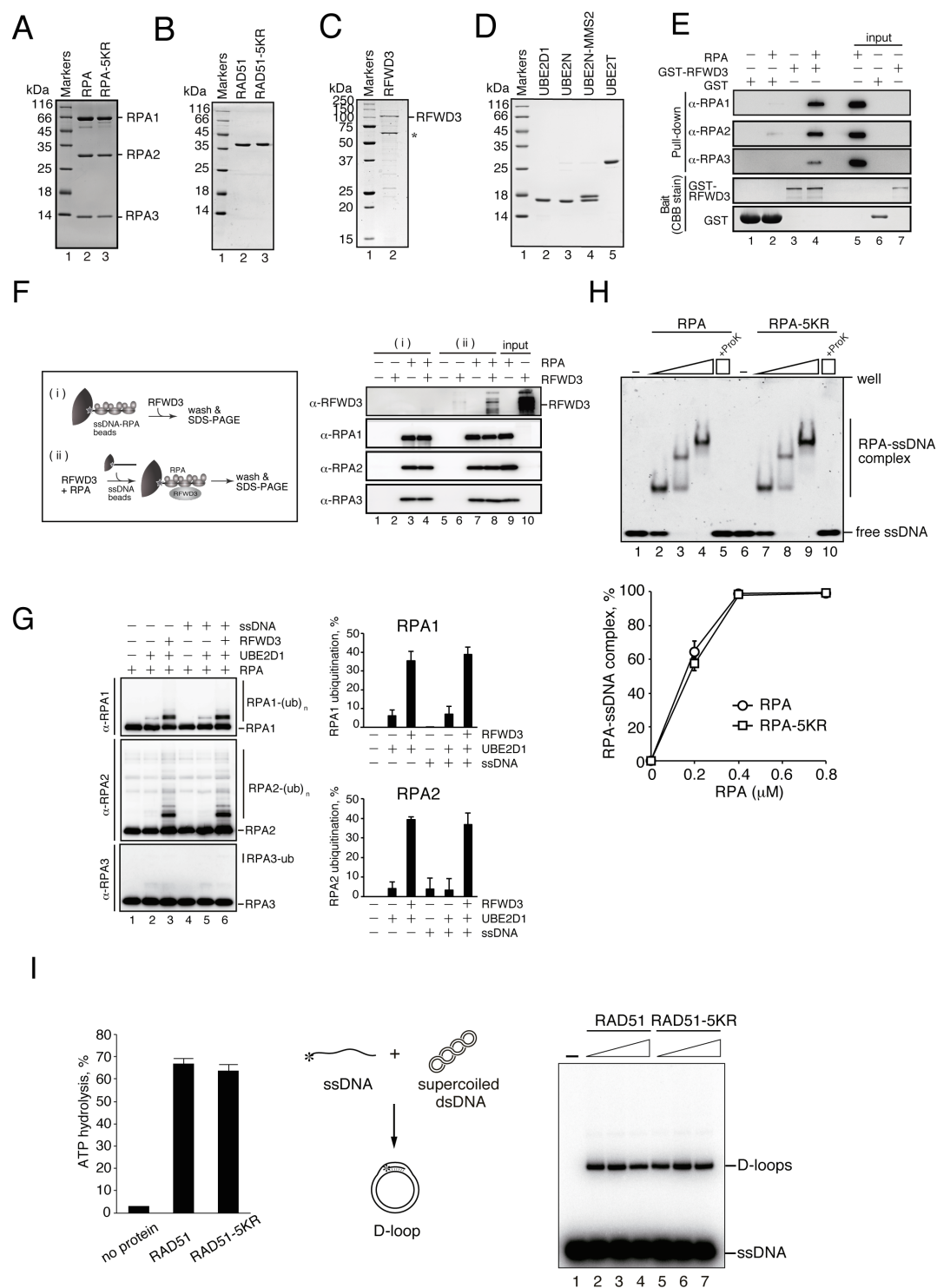


Figure S4

Figure S4. Related to Figure 4. Purification of recombinant proteins and pull-down or functional assays.

(A) Purified human RPA, (B) RAD51 and the RAD51-5KR mutant, (C) RFWD3, and (D) UBE2D1, UBE2N, UBE2N-MMS2 complex, and UBE2T were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining. Asterisk indicates an impurity. (E) Pull-down assay of RPA with GST-tagged RFWD3. RPA bound to GST or GST-tagged RFWD3 was co-pelleted with Glutathione Sepharose 4B beads, and the proteins were detected by immunoblotting. (F) Pull-down assay of RFWD3 with RPA-coated ssDNA beads (i) or RFWD3-RPA with ssDNA beads (ii). A schematic is shown at left. Pulled down proteins were detected by immunoblotting. (G) *In vitro* ubiquitination assay of the RPA complex with or without the addition of ssDNA. The ubiquitinated proteins were detected by immunoblotting. Percentages of polyubiquitinated species (indicated, for example, as RPA1-(ub)_n) in the total amount of RPA1 or RPA2 were determined by densitometric measurements and are presented as bar graphs (mean and SD, n=3) at right. (H) ssDNA binding of the RPA complex with or without the RPA2-5KR mutant was examined using the electrophoretic mobility shift assay. The percentage of shifted bands relative to the total ssDNA signal is shown in the graph beneath the blot (mean and SD, n=3 experiments). (I) The ATP hydrolyzing activities of RAD51 and RAD51-5KR in the presence of ssDNA were analyzed by thin layer chromatography. The average values of three independent experiments are shown, with standard deviations (left). A schematic representation of the D-loop formation assay (middle). The asterisk indicates the ³²P-labeled 5'-end of the ssDNA. A representative D-loop formation assay result (right). The protein concentrations were 0.2 μM (lanes 2 and 5), 0.4 μM (lanes 3 and 6), and 0.6 μM (lanes 4 and 7). Lane 1 indicates a negative control experiment without proteins.

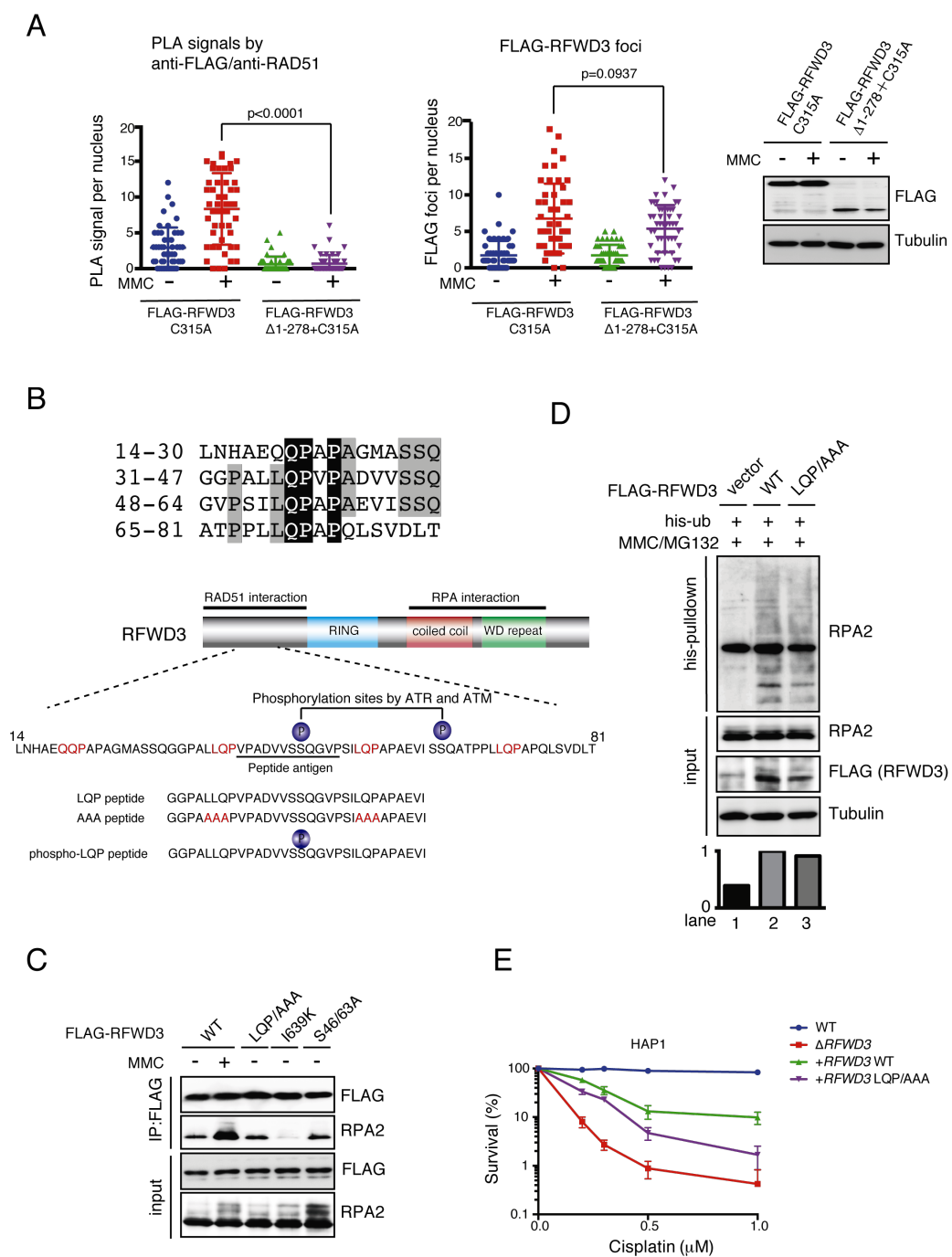


Figure S5

Figure S5. Related to Figure 5. Role of the N-terminal RFWD3 domain and the LQP motif in ubiquitination of RAD51 and RPA.

- (A) PLA signals between RAD51 and FLAG-RFWD3 WT or N-terminal deletion Δ 1-278 (left). Both RFWD3 constructs carried a RING domain mutation C315A to enhance expression levels. U2OS cells transfected with FLAG-RFWD3 C315A or Δ 1-278 + C315A were treated with 100 ng/ml MMC for 24 hr, and analyzed by PLA. Each dot represents the number of PLA signals in a single cell; the mean and SD are shown. Decreased expression levels and foci formation of RFWD3 Δ 1-278 compared to WT are shown at right. U2OS cells transfected with FLAG-RFWD3 C315A or Δ 1-278 + C315A were treated with 100 ng/ml MMC for 24 hr, and foci were analyzed using anti-FLAG antibody (left and middle), and expression was verified by immunoblotting (right).
- (B) Alignment of LQP-SSQ repeats (residue 14 to 81) and a schematic illustration of the RFWD3 domains. A section of the RFWD3 N-terminal amino acid sequence, the synthesized peptides used in Figure 5E, and the phosphorylation sites S46 and S63 are also indicated. The peptide sequence used for raising the anti-phospho-S46 antibody is underlined. The LQP motifs mutated to triple Alanines in RFWD3 LQP/AAA are indicated in red.
- (C) Co-immunoprecipitation of FLAG-RFWD3 mutants (LQP/AAA, I619K, and S46/63A) and RPA2. Lysates from 293T cells transfected with the indicated plasmid constructs were subjected to immunoprecipitation with anti-FLAG antibody, and analyzed by immunoblotting.
- (D) Comparable ubiquitination levels of RPA2 by RFWD3 WT or LQP/AAA mutant. 293T cells transfected with FLAG-RFWD3 WT or the LQP/AAA mutant, or siRNA with his-ubiquitin were treated with 100 ng/ml MMC for 24 hr and with 10 μ M MG132 for 1 hr before the harvest. Cells were lysed under denaturing conditions and ubiquitinated proteins were blotted using anti-RPA2 antibody.
- (E) Survival curves of HAP1 WT, Δ RFWD3, or Δ RFWD3 stably transduced with a lentivirus expressing WT or the RFWD3 LQP/AAA mutant in the presence of Cisplatin. Mean and SD of three independent experiments are shown.

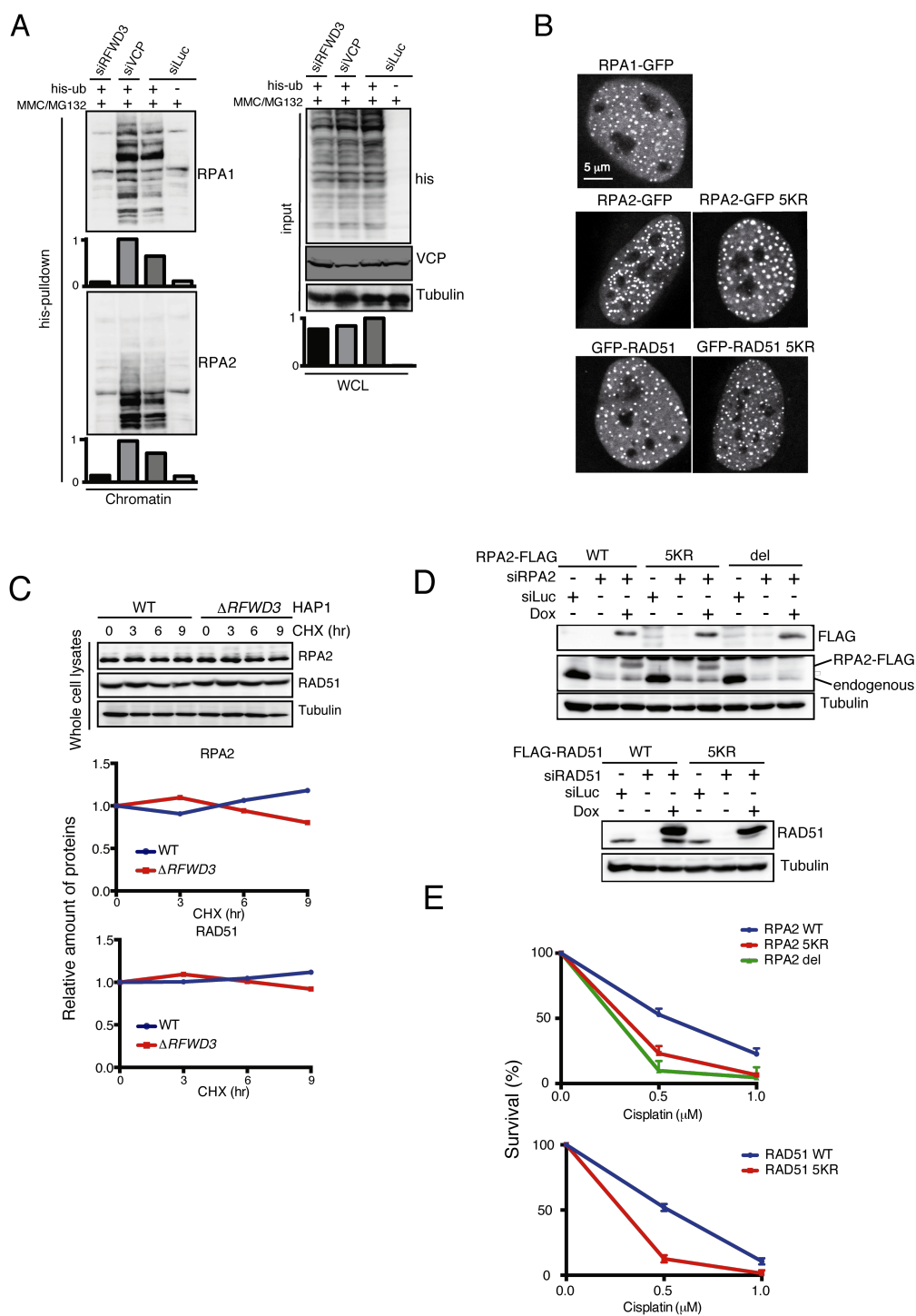
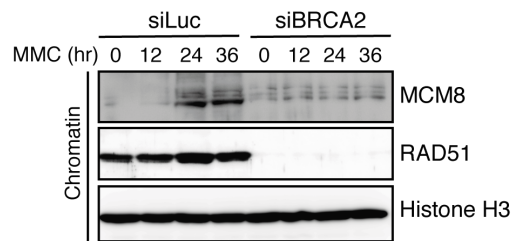


Figure S6

Figure S6. Related to Figure 6. Characterization of cells expressing ubiquitination-deficient RPA2 or RAD51 mutant proteins.

- (A) VCP mediates removal of ubiquitinated RPA2 from the chromatin fraction. 293T cells transfected with his-ubiquitin and indicated siRNAs were harvested after exposure to 100 ng/ml MMC for 24 hr, biochemically fractionated, and analyzed by immunoblotting. WCL, whole cell lysates. Ubiquitinated proteins were quantified, normalized to the highest value, and data are shown below each blot.
- (B) MMC-induced foci formation of RPA1-GFP, RPA2-GFP (WT and 5KR), or GFP-RAD51 (WT and 5KR).
- (C) Stability of RPA and RAD51 proteins in cells without MMC damage. HAP1 WT and *ARFWD3* cells were chased in medium containing 20 μ g/ml cycloheximide for the indicated times, and analyzed by immunoblotting. The band intensity was normalized to those at the start of the chase. See also Figure 6C.
- (D) Dox-induced expression of FLAG-RPA2 (WT, 5KR, or a deletion mutant lacking residue 244-254) or FLAG-RAD51 (WT or 5KR) in U2OS-DR-GFP cells depleted of endogenous RPA2 or RAD51 proteins by siRPA2 (targeting the 3'UTR) or siRAD51. FLAG-RAD51 was rendered insensitive to siRAD51 by synonymous mutations. Note that the RPA2 deletion (del) mutant lacking residues 244-254 was recognized by anti-FLAG but not by the anti-RPA2 that we used.
- (E) Cisplatin sensitivity of U2OS cells in which endogenous RPA2 or RAD51 were replaced with FLAG-tagged RPA2 and RAD51 with the indicated mutations. Cells were exposed to cisplatin for 24 hr at the indicated concentrations. The mean and SD of three independent experiments are shown.

A



B

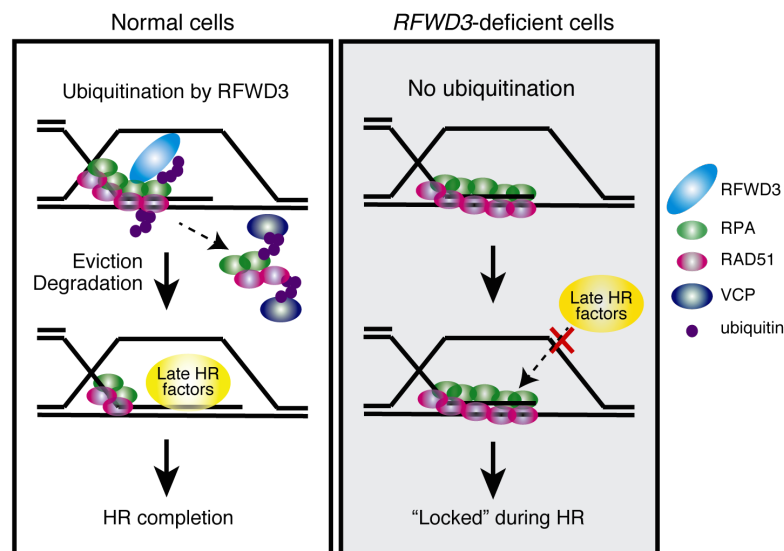


Figure S7

Figure S7. Related to Figure 7. BRCA2 depletion prevents chromatin loading of MCM8.

- (A) BRCA2 depletion inhibits MCM8 chromatin loading upon MMC damage. U2OS cells transfected with the indicated siRNAs were harvested after exposure to 100 ng/ml MMC for the indicated times, biochemically fractionated, and analyzed by immunoblotting.
- (B) Model for RFWD3 mediated RPA and RAD51 ubiquitination that promotes timely removal of the proteins from recombination intermediates and degradation of RPA and RAD51 to allow HR progression to subsequent steps following MMC damage. We propose that the persistent presence of RPA and RAD51 may hinder chromatin recruitment of late-phase HR factors such as RAD54 or MCM8, resulting in a "locked" HR reaction.