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E3 Ligase RFWD3 Participates in Replication Checkpoint Control*

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Zihua Gong and Junjie Chen[1]

From the Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030

RFWD3 has E3 ligase activity *in vitro*, but its *in vivo* function remains unknown. In this study we identified RFWD3 as a novel replication protein A (RPA)-associated protein. Using purified proteins, we observed a direct interaction between RPA2 and RFWD3. Further analysis showed that RFWD3 is recruited to stalled replication forks and co-localizes with RPA2 in response to replication stress. Moreover, RFWD3 is important for ATR-dependent Chk1 activation in response to replication stress. Upon replication stress, deletion of RPA2 binding region on RFWD3 impairs its localization to stalled replication forks and decreases Chk1 activation. Taken together, our results suggest that RFWD3 and RPA2 functionally interact and participate in replication checkpoint control.

The progression of replication forks is often stalled due to DNA damage or intrinsic DNA secondary structures. The stalled replication forks must be properly resolved to accurately complete DNA replication and prevent genomic instability. At stalled replication forks, single-strand DNA (ssDNA) is generated and extended by helicases. These ssDNA regions are bound by replication protein A, which not only protects these ssDNA regions from nucleolytic digestions but also recruits checkpoint proteins to initiate replication checkpoint signaling (3, 4). One way that RPA[2]-ssDNA complex promotes checkpoint signaling is through its recruitment and interaction with ATRIP/ATR to facilitate ATR-dependent signal transduction (5–7). In addition, RPA-ssDNA complex also recruits RAD51 and other recombination factors to initiate homologous recombination processes (8, 9). Recently, several studies have also revealed a physical and functional interaction between RPA and an annealing helicase SMARCAL1/HARP, which facilitates replication fork stabilization (10–14). Thus, the RPA complex, which consists of three subunits of RPA1, RPA2, and RPA3, acts as a platform to mediate multiple protein-protein interactions at ongoing or stalled replication forks that play an essential role in DNA replication, DNA repair, and recombination (1–3). Here we showed that an E3 ligase RFWD3 interacts directly with RPA2 and is recruited to stalled replication forks in response to replication stress. Moreover, depletion of RFWD3 leads to defective Chk1 activation after replication stress. These results suggest that RFWD3 is a new player involved in DNA replication checkpoint control.

MATERIALS AND METHODS

Constructs—All cDNAs were subcloned into pDONR201 (Invitrogen) as entry clones and were then transferred to destination vectors for the expression of N-terminal-tagged fusion protein. All point or deletion mutants were generated using the site-directed mutagenesis (Stratagene) and verified by sequencing.

Antibodies—Rabbit polyclonal anti-RFWD3 antibodies were raised by immunizing rabbits with maltose-binding protein-fused RFWD3 recombinant proteins containing residues 1–300 and residues 654–954 of RFWD3. The antibodies were affinity-purified using AminoLink plus Immobilization and purification kit (Pierce). The anti-Myc and anti-GST antibodies were obtained from Santa Cruz Biotechnology. Anti-FLAG (M2) were purchased from Sigma. Anti-RPA2 and anti-RPA3 were obtained from Abcam. Anti-RPA1 was obtained from EMD Chemicals.

Cell Culture and Transfection—293T and U2OS cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. All siRNA duplexes were purchased from Dharmacon. The sequences of RFWD3 siRNAs were: #1, GGACCUACUUGCAAACUAUdTdT; #2, GCAGUCAUGUGCAGGAGUUdTdT. The siRNA-resistant wild-type and mutant RFWD3 constructs were generated by changing nucleotides in the RFWD3 siRNA #1 targeting region. The siRNA transfection was performed using Oligofectamine (Invitrogen) following the manufacturer's instructions. Transfection was repeated twice with an interval of 24 h to achieve efficient siRNA-mediated down-regulation of their target genes.

Tandem Affinity Purification of RPA1- or RFWD3-associated Protein Complexes—293T cells stably expressing SFB-RPA1 or SFB-RFWD3 were used for tandem affinity purification. Cells were collected and lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, containing 1 μg/ml each pepstatin A and aprotinin) for 20 min. Crude lysates were collected and incubated with streptavidin-Sepharose beads (Amersham Biosciences) for

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[1] Recipient of an Era of Hope Scholar award from the Department of Defense (W81XWH-05-1-0470). To whom correspondence should be addressed: Dept. of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. E-mail: jchen8@mdanderson.org.

[2] The abbreviations used are: RPA, replication protein A; SFB, S protein, FLAG, and streptavidin-binding peptide; HU, hydroxyurea.

3 h at 4 °C. The beads were washed 3 times with NETN and then eluted with biotin (Sigma) for 2 h twice at 4 °C. The eluates were combined and then incubated with S-protein-agarose beads (Novagen) for 3 h at 4 °C and then washed 3 times with NETN. The proteins bound to the beads were eluted by boiling with SDS sample buffer, resolved by SDS-PAGE, an visualized by Coomassie Blue staining. The identities of eluted proteins were revealed by mass spectrometry analysis (Taplin Biological Mass Spectrometry Facility, Harvard University).

Co-immunoprecipitation and Western Blotting—For co-immunoprecipitation assays, constructs encoding SFB-tagged and Myc-tagged proteins were transiently co-transfected into 293T cells. Cells were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 20 mM NaF and 1 μg/ml pepstatin A and aprotinin on ice for 30 min. After removal of cell debris by centrifugation, the soluble fractions were collected and incubated with S-protein beads for 3 h at 4 °C. Beads were washed 4 times with NTEN buffer, boiled in 2× SDS loading buffer, and resolved on SDS-PAGE. Membranes were blocked in 5% milk in TBST buffer and then probed with antibodies as indicated.

Immunostaining—Cells cultured on coverslips were treated with 5 mM hydroxyurea (HU) 4 h. Cells were then washed with PBS, permeabilized in CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES, pH6.8) containing 0.5% Triton for 10 min at RT or methanol:acetone (1:1) at −20 °C for 15 min, and then fixed with 3% paraformaldehyde for 20 min. Coverslips were washed with PBS and then incubated with primary antibodies diluted in 5% goat serum for 30 min at room temperature. After washing with PBS twice, coverslips were incubated either with FITC-conjugated or rhodamine-conjugated secondary antibodies for 20 min at 37 °C. Nuclei were counterstained with DAPI and then mounted onto glass slides with anti-Fade solution. Images were taken with a Nikon Eclipse E800 fluorescence microscope.

Cell Survival Assay—Briefly, a total of 1×10^3 cells was seeded onto a 60-mm dish in triplicate. Twenty-four hours after seeding, cells were treated with HU and left for 14 days to allow colonies to form. The resulting colonies were fixed and stained with Coomassie Blue. Numbers of colonies were counted using a GelDoc with Quantity One software (Bio-Rad). Results were the averages of data obtained from three independent experiments.

RESULTS

RFWD3 Is a Novel RPA2-interacting Protein—In an attempt to identify new RPA-associated proteins, we performed tandem affinity purification using 293T cells stably expressing triple epitope (S-peptide, FLAG, and streptavidin-binding peptide)-tagged RPA1 (SFB-RPA1). Mass spectrometry analysis revealed that in addition to RPA2 and RPA3, which normally associate with RPA1 to form a stable heterotrimeric RPA complex, there are several known or potential RPA-associated proteins (Fig. 1*A*). One of them is RFWD3, an E3 ligase that involves in p53 degradation (15). To verify that RFWD3 indeed associates with the RPA complex, we

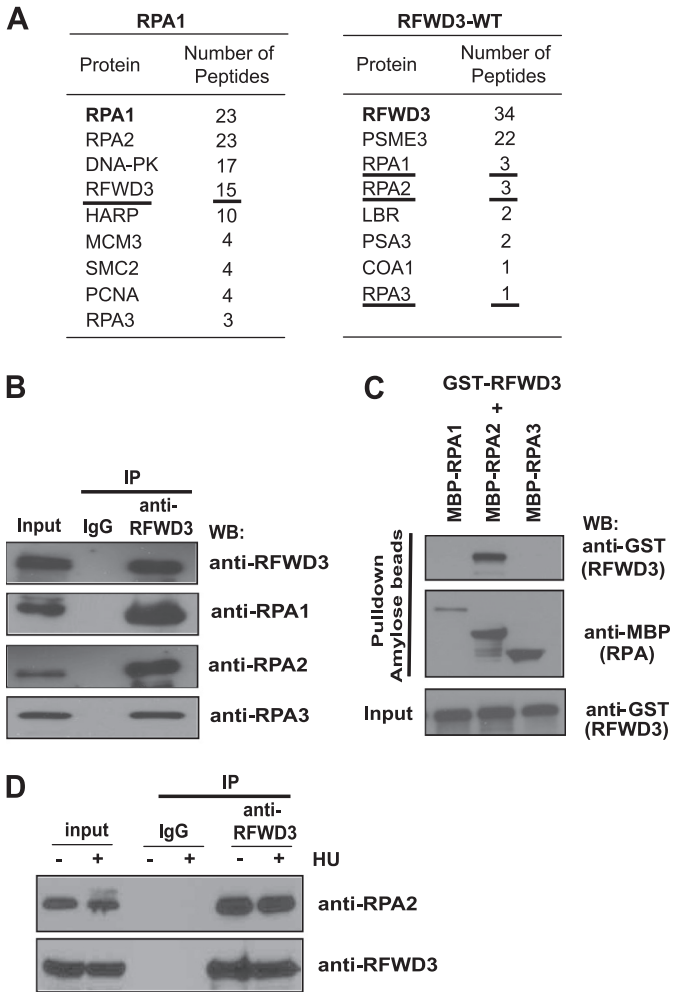

FIGURE 1. **RFWD3 associates with the RPA complex.** *A*, tandem affinity purification was performed using 293T cells stably expressing tagged RPA1 or RFWD3. The data from mass spectrometry analysis are presented in the tables. *B*, immunoprecipitation (*IP*) was carried out using HeLa extract and control IgG or anti-RFWD3 antibody. Western blotting (*WB*) was performed using indicated antibodies. *C*, RFWD3 binds strongly to RPA2 *in vitro*. The *in vitro* binding assay was performed using baculovirally expressed GST-RFWD3 and bacterially expressed MBP-RPA subunits. Pulldown experiments were performed using amylose beads, and Western blotting was carried out using GST antibody. *D*, endogenous interaction between RFWD3 and RPA2 in the absence or presence of HU (after 2 h of treatment with 2 mM HU) is shown. For the control, anti-RFWD3 immunoprecipitates were immunoblotted with indicated antibodies.

repeated tandem affinity purification using 293T cell lines stably expressing SFB-tagged human RFWD3 and identified the RPA complex as RFWD3-associated proteins (Fig. 1*A*). To further confirm this interaction, we generated anti-RFWD3 antibodies and showed that endogenous RFWD3 associated strongly with RPA complex *in vivo* (Fig. 1*B*). Taken together, these data suggest that RFWD3 is a *bona fide* RPA-binding protein.

To determine whether the interaction between RPA and RFWD3 is direct, we expressed and purified MBP-tagged RPA1, RPA2, and RPA3 from *Escherichia coli* and GST-tagged RFWD3 using a baculovirus-insect cell expression system. Pulldown experiments revealed that RFWD3 binds strongly with RPA2 but not with RPA1 or RPA3 (Fig. 1*C*), indicating that RFWD3 interacts with RPA complex through RPA2. Moreover,

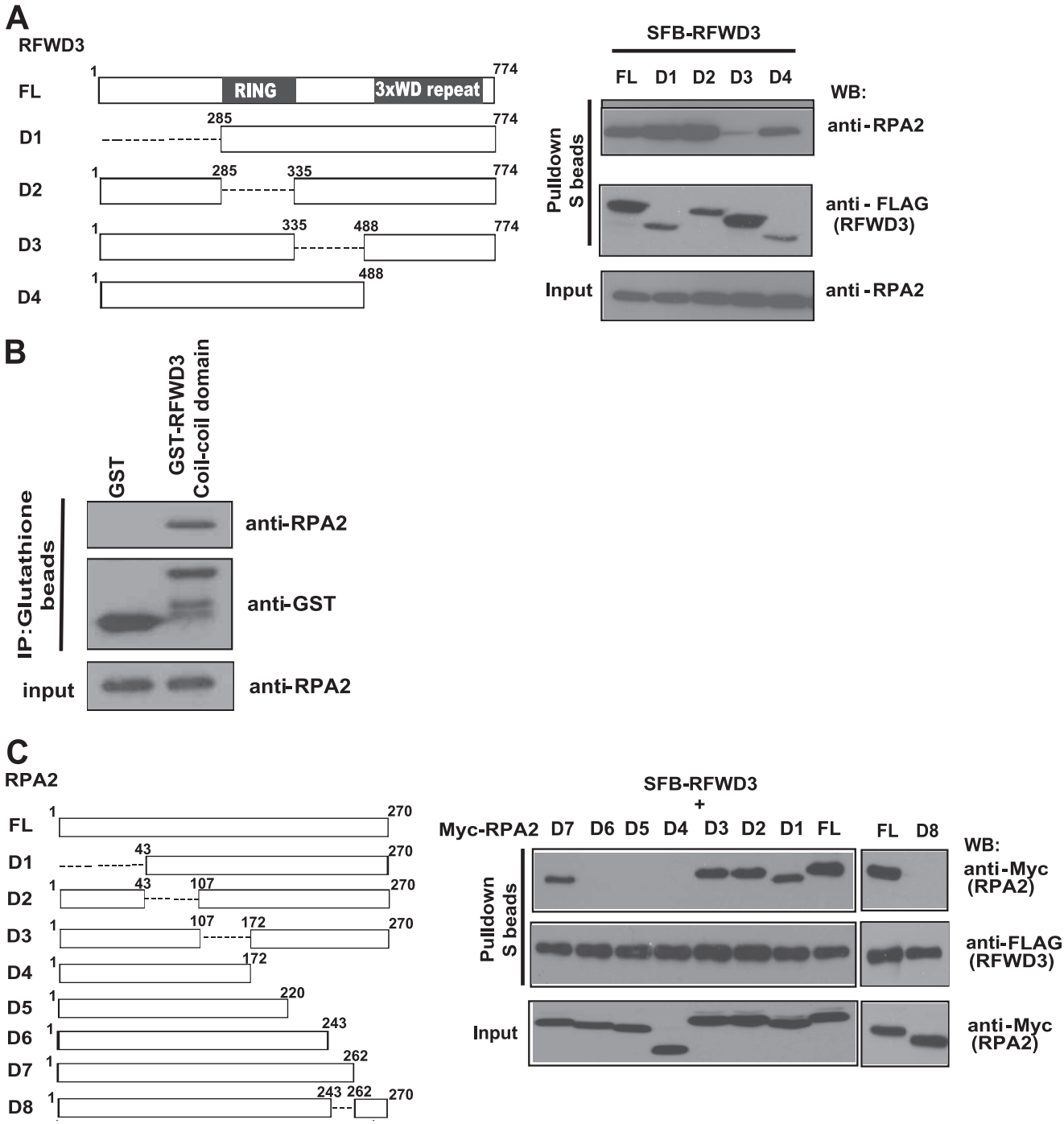

FIGURE 2. **Mapping the regions of RFWD3 and RPA2 that are involved in their interaction.** *A*, shown is a schematic presentation of wild-type and deletion mutants of RFWD3 used in this study (*left*). 293T cells were transfected with plasmids encoding wild-type or deletion mutants of SFB-tagged RFWD3. Pulldown experiments were conducted using S protein beads and then subjected to Western blotting (*WB*) using indicated antibodies (*right*). *FL*, full length. *B*, beads coated with bacterially expressed GST or GST fusion of coiled-coil region of RFWD3 were incubated with HeLa cell lysates. Immunoblotting experiments were carried out using the indicated antibodies. *IP*, immunoprecipitation. *C*, shown is a schematic diagram of wild-type and deletion mutants of RPA2 used in this study (*left*). 293T cells were transfected with plasmids encoding SFB-tagged RFWD3 together with plasmids encoding wild-type or deletion mutants of Myc-tagged RPA2. Pulldown experiments were performed using S-protein beads and then subjected to Western blot analyses using antibodies as indicated (*right*).

the binding of RPA2 to RFWD3 was not changed after cells were exposed to HU (Fig. 1*D*).

We next sought to identify the region(s) of RFWD3 that is responsible for its interaction with RPA2. We generated a series of truncation and internal deletion mutants of RFWD3. As shown in Fig. 2*A*, the D3 mutant (deleted of the region containing the coil-coil domain; residues 335–488) of RFWD3 leads to a dramatic decrease in RFWD3/RPA2 interaction, indicating that this region of RFWD3 is important for its binding to RPA2. Furthermore, using bacterially expressed and purified proteins, we found that RPA2 bound to the RFWD3 coiled-coil domain (Fig. 2*B*). Next, we sought

to define the RFWD3 binding region(s) on RPA2. Again, a series of truncation and internal deletion mutants of RPA2 was co-expressed with SFB-tagged RFWD3 in 293T cells. We were able to map the minimal RFWD3 binding region to residues 244–262 of RPA2 (Fig. 2*C*).

RFWD3 Localizes to Stalled Replication Forks via an Interaction with RPA2—The RPA complex is an essential heterotrimeric protein complex composed of three subunits, RPA1, RPA2, and RPA3, that binds and stabilizes ssDNA generated at or near replication forks and/or sites of DNA damage (2, 3). The interaction between RFWD3 and RPA2 suggested that RFWD3 may co-localize with RPA2 at ssDNA regions in the cell. As shown in Fig. 3*A*, endogenous RFWD3 foci is rarely observed in untreated cells. RFWD3 is recruited to nuclear foci in HU-treated cells and colocalizes with RPA2 at these foci (Fig. 3*A*), indicating that RFWD3 localizes to stalled replication forks and may function in cellular response to replication stress.

Next we examined whether the RFWD3 foci formation would depend on its RPA2 binding region. Although distinct nuclear foci of FLAG-tagged RFWD3 were readily detected in HU-treated cells, the D3 mutant (deletion of RPA2 binding region) of RFWD3 fails to form nuclear foci after HU treatment (Fig. 3*B*). Therefore, we conclude that RFWD3 is likely to be recruited to stalled replication forks via its association with RPA2.

RFWD3 Is Required for Chk1 Activation after Replication Stress—Because RFWD3 localizes to stalled replication forks, we decided to explore the role of RFWD3 in replication checkpoint response. As shown in Fig. 4*A*, down-regulation of RFWD3 fails to activate Chk1 phosphorylation after HU treatment. Although the expression of siRNA-resistant wild-type RFWD3 completely rescued Chk1 activation in cells depleted of endogenous RFWD3, reconstitution with RFWD3 with a deletion of its RPA2 binding region failed to do so (Fig. 4*B*). This is consistent with our hypothesis that this region of RFWD3 is required for its association with RPA and its localization to stalled replication forks. Interestingly, RFWD3 mutant deleted of RING domain also failed to rescue HU-induced Chk1 phosphorylation (Fig. 4*B*). Because the RING domain of RFWD3 functions as E3 ubiquitin ligase, we speculate that RFWD3 may regulate certain unknown substrates at stalled replication forks, which are important for proper replication checkpoint control. Indeed, loss of RFWD3 also caused hypersensitivity to hydroxyurea (Fig. 4*C*), further supporting a role of RFWD3 in replication checkpoint control.

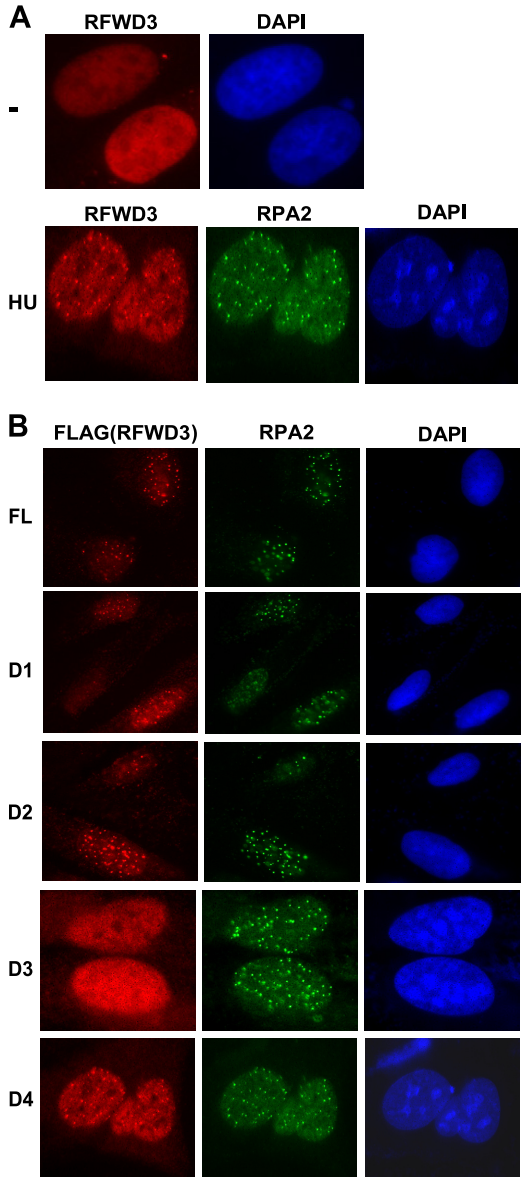

FIGURE 3. **The RPA2 binding region of RFWD3 is required for its foci formation after replication stress.** *A*, RFWD3 localizes at stalled replication forks in response to replication stress. U2OS cells were mock-treated or treated with 5 mM HU for 6 h. Immunostaining experiments were performed using anti-RFWD3 and anti-RPA2 antibodies. *B*, the RPA2 binding region of RFWD3 is important for RFWD3 foci formation after replication stress. U2OS cells were transfected with plasmids encoding SFB-tagged wild-type or deletion mutants of RFWD3. Cells were treated with 5 mM HU. Six hours later cells were fixed and immunostained with antibodies as indicated. *FL*, full length.

DISCUSSION

The RPA complex plays an essential role in DNA replication, DNA repair, and the initiation of DNA damage and replication checkpoints (16–18). It is proposed that these diverse functions of RPA come from its abilities to interact with many binding partners. It has been reported that RPA interacts with RAD9, RAD17, and ATRIP, which are involved in the assembly of the 9-1-1 complex at stalled replication forks, and the activation of ATR signaling during DNA replication checkpoint (6, 7, 19–23). In this study we demonstrated that RFWD3 is a new binding partner of RPA.

RFWD3 was first identified as a substrate of kinase ATM/ATR in a large-scale proteomic analysis of proteins phosphorylated in response to DNA damage (24, 25). RFWD3 contains an SQ-rich region in the N terminus, a RING domain, a coil-coil domain, and a WD40 domain in the C terminus. It has been suggested that RFWD3 displays E3 ubiquitin ligase activity *in vitro* and forms a RFWD3-Mdm2-p53 complex to regulate p53 degradation in response to DNA damage (15). In this study we demonstrated that RFWD3 directly interacts with RPA2, a subunit of the RPA complex. The region containing the coiled-coil motif of RFWD3 is required for its binding to RPA2. Furthermore,

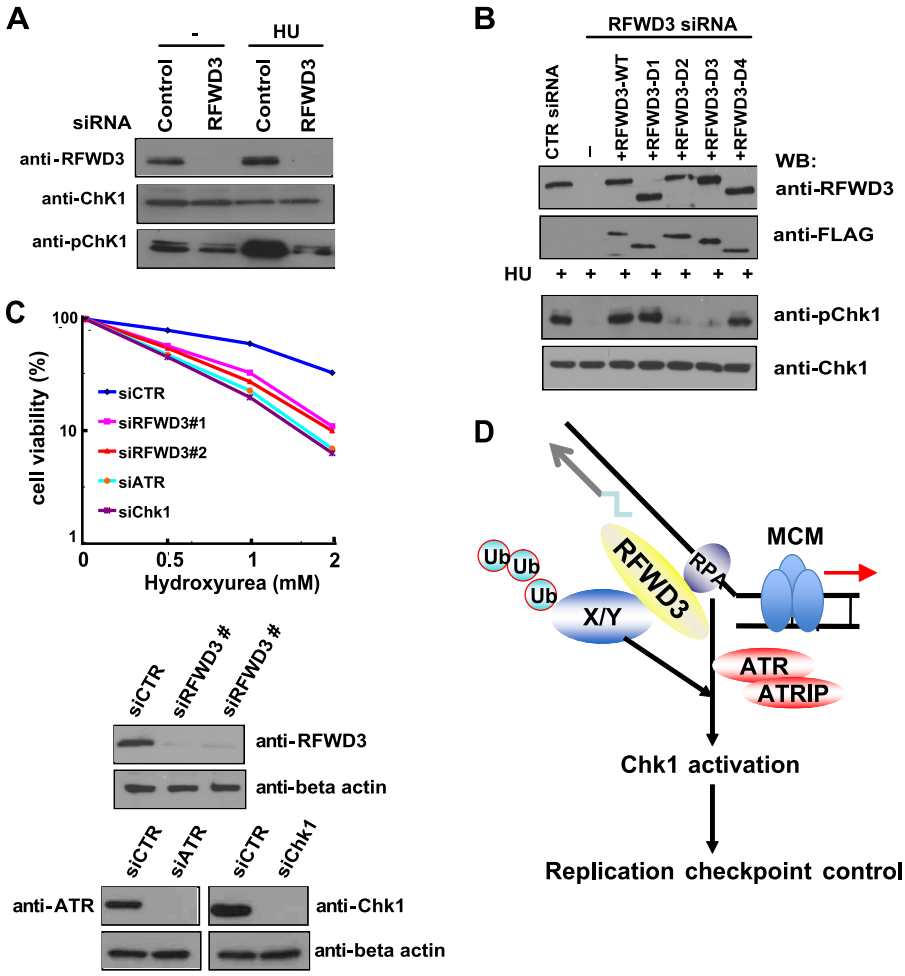

FIGURE 4. **RFWD3 is involved in replication checkpoint control.** *A*, RFWD3 is required for Chk1 activation upon replication stress. U2OS cells transfected with control siRNA or RFWD3 siRNA were mock-treated or treated with HU and harvested 1 h later. Cell lysates were prepared and immunoblotted with antibodies as indicated. *B*, the RING domain and RPA2 binding region of RFWD3 are required for Chk1 activation after replication stress. U2OS cells or U2OS cells stably expressing siRNA-resistant wild-type or deletion mutants of RFWD3 were transfected with RFWD3 siRNA. 72 h after initial siRNA transfection, cells were treated with HU and collected 1 h later. Cell lysates were immunoblotted (*WB*) with indicated antibodies. *CTR*, control. *C*, down-regulation of RFWD3 increases cellular sensitivity to HU treatment. HeLa cells transfected with control, RFWD3 siRNA, ATR siRNA, or Chk1 siRNAs were treated with HU for 24 h. Cell survival after HU treatment was measured by clonogenic assay. *D*, a proposed model of RFWD3 function at replication forks is shown. *MCM*, minichromosome maintenance; *Ub*, ubiquitin. Please refer to the "Discussion" for details.

RFWD3 is recruited to stalled replication forks and co-localizes with RPA2 in response to replication stress. Functional analysis revealed that RFWD3 is important for Chk1 activation after replication stress. In addition, deletion of RPA2 binding region on RFWD3 impairs its localization to stalled replication forks and Chk1 activation upon replication stress. Together, these data strongly support that RFWD3 is a new component involved in replication checkpoint control.

The precise function of RFWD3 at stalled replication forks remains to be elucidated. We showed that deletion of the RING domain of RFWD3 failed to rescue HU-induced Chk1 activation, indicating that this domain and its associated E3 ubiquitin ligase activity are likely to be required for RFWD3 function in replication checkpoint. We propose that in response to replication stress, ssDNA regions generated at stalled replication forks are coated by RPA. Through its interaction with RPA, RFWD3 is recruited to these stalled replication forks and exerts its E3 ligase activity to ubiquitinate yet-to-be-identified substrates, which is required for replication checkpoint control (Fig. 4*D*). The future studies will focus on the identification of these RFWD3 substrates, which will allow us to understand the complex regulation of replication checkpoint pathway.

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