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Accumulation of FFA-1, the *Xenopus* Homolog of Werner Helicase, and DNA Polymerase δ on Chromatin in Response to Replication Fork Arrest

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Werner syndrome is a genetic disorder characterized by premature aging and cancer-prone symptoms, and is caused by mutation of the *WRN* gene. WRN is a member of the RecQ helicase family and is thought to function in processes implicated in DNA replication and repair to maintain genome stability; however, its precise function is still unclear. We found that replication fork arrest markedly enhances chromatin binding of focus-forming activity 1 (FFA-1), a *Xenopus* WRN homolog, in *Xenopus* egg extracts. In addition to FFA-1, DNA polymerase δ (Polδ) and replication protein A, but not DNA polymerase ε and proliferating cell nuclear antigen, accumulated increasingly on replication-arrested chromatin. Elevated accumulation of these proteins was dependent on formation of pre-replicative complexes (pre-RCs). Double-strand break (DSB) formation also enhanced chromatin binding of FFA-1, but not Polδ, independently of pre-RC formation. In contrast to FFA-1, chromatin binding of *Xenopus* Bloom syndrome helicase (xBLM) only slightly increased after replication arrest or DSB formation. Thus, WRN-specific, distinct processes can be reproduced in the *in vitro* system in egg extracts, and this system is useful for biochemical analysis of WRN functions during DNA metabolism.

Key words: DNA polymerase δ, FFA-1, replication fork arrest, Werner helicase, *Xenopus* egg extract.

Abbreviations: DSB, double-strand break; FFA-1, focus-forming activity 1; MCM, minichromosome maintenance protein; ORC, origin recognition complex; PCNA, proliferating cell nuclear antigen; Polδ and ε, DNA polymerases δ and ε; pre-RC, pre-replicative complex; RPA, replication protein A.

Werner syndrome (WS) is a rare human genetic disorder characterized by premature aging characteristics, including graying and loss of hair, increased wrinkling and ulceration of the skin, osteoporosis, and atherosclerosis (*1*). WS patients also exhibit an increased frequency of age-related disorders, including cancer, diabetes and cataracts (*2*, *3*). The cells from WS patients have increased genomic instability, and contain deletions, insertions and translocations in the genomic material (*4–6*).

The gene responsible for WS, *WRN*, encodes a 3′ to 5′ helicase that belongs to the RecQ family of DNA helicases (*7*, *8*). The human RecQ family also includes Bloom helicase (BLM), RECQL, RECQL4 and RECQL5 (reviewed in Ref. *9*). In addition to WRN, two other members of the human RecQ family, BLM and RECQ4, are associated with genetic diseases: Bloom syndrome and Rothmund-Thomson syndrome, respectively (*10*, *11*). Notably, increased genomic instability is also a characteristic of these diseases.

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Based on previous analyses of WS cells, WRN has been implicated in various aspects of DNA metabolism, including DNA replication, telomere maintenance, base excision repair and double-strand break (DSB) repair (*12–17*). WRN is predominantly localized in the nucleolus of mammalian cultured cells (*18*, *19*), but treatment of cells with hydroxyurea or DNA damaging agents induces translocation of WRN to the nucleoplasm (*20*, *21*). Biochemical studies have shown that both WRN and BLM helicases can unwind the X-structure DNA substrate (a model substrate for Holliday junction intermediates) more efficiently than a standard substrate such as a tailed duplex DNA (*22*, *23*).

In addition, WRN interacts with a number of proteins involved in DNA metabolism, including replication protein A (RPA) (*24*, *25*), flap endonuclease 1 (FEN1) (*26*), proliferating cell nuclear antigen (PCNA) (*27*), DNA polymerase δ (Polδ) (*19*, *28*), DNA-dependent protein kinase/Ku subunit (*29–32*), DNA topoisomerase I (*27*), BLM (*33*) and WHIP (*34*). These characteristics of WRN have led to the hypothesis that WRN functions at stalled/broken replication forks to prevent aberrant recombination that would cause elevated genomic instability (*9*, *35*). However, the precise role of WRN at stalled replication forks has not been elucidated.

The cell free system using *Xenopus* egg extracts is an excellent model for biochemical analysis of DNA

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metabolism. Focus-forming activity 1 (FFA-1) has been identified as a *Xenopus* WRN homolog that co-localizes with sites of DNA synthesis and RPA in nuclei formed in the egg extracts (*36*, *37*). Although immunodepletion of FFA-1 from egg extracts does not significantly affect DNA synthesis, FFA-1 may have an important role in DNA replication in egg extracts (*38*). More recently, it has been shown that FFA-1 is involved in a specific DSB pathway in egg extracts (*39*).

In this study, the behavior of FFA-1 in response to replication fork arrest in *Xenopus* egg extracts was examined. We found that FFA-1 significantly accumulated on chromatin after replication fork arrest induced by polymerase inhibitors. In addition to FFA-1, Polδ and RPA also specifically accumulated on replication-arrested chromatin. Accumulation of these proteins was dependent on formation of pre-replicative complexes (pre-RCs). Consistent with the possible role of FFA-1 in DSB repair, FFA-1 and RPA, but not Polδ, significantly accumulated on chromatin after induction of DSBs in the egg extracts, and this DSB-induced accumulation occurred independently of pre-RC formation. Thus, distinct processes involving FFA-1 could be reproduced in egg extracts. In contrast to FFA-1, chromatin binding of *Xenopus* BLM helicase (xBLM) (*40*, *41*) increased only slightly after replication fork arrest or DSB formation, implying that FFA-1 may be more strictly associated with processes at stalled forks or DSBs, compared to xBLM.

MATERIALS AND METHODS

Materials—Aphidicolin and AraCTP were obtained from Sigma. *Eco*RI was obtained from Takara Biomedicals.

Recombinant Proteins—A polyhistidine-tagged, nondegradable mutant of *Xenopus* geminin (*42*) was bacterially expressed and purified. The standard proteins used in quantitative immunoblot analyses, polyhistidine-tagged FFA-1 (N.S. and S.W., unpublished), Polδ p66 (*43*) and Polε p60 (*44*), were bacterially expressed and purified under denaturing conditions. The concentration of each full-length polypeptide was determined by SDS–polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining, using bovine serum albumin as a standard.

Analyses of Chromatin Proteins and Replication Products—*Xenopus* egg extracts (low speed supernatant) and demembranated *Xenopus* sperm nuclei were prepared as described previously (*45*, *46*). Demembranated nuclei were mixed with egg extracts (4,000–10,000 per μl of extracts) under the same conditions used in a DNA replication assay described previously (*45*), and aphidicolin was added at 50 μg/ml (stock: 10 mg/ml in DMSO), as described in the figure legends. Analysis of chromatin-bound proteins was performed essentially as described previously (*47*). Briefly, a 25-μl aliquot (equivalent to 24 μl of egg extracts) of the reaction mixture was withdrawn, diluted with 250 μl of 0.25% Triton X-100 and EB buffer (100 mM KCl, 2.5 mM $MgCl_2$, 50 mM HEPES-KOH, pH 7.7), and spun through a cushion (100 μl) of 10% sucrose and EB buffer at 6,000 × *g* for 5 min at 4°C. The chromatin pellet was washed with 2 mM $CaCl_2$ and EB buffer, and then digested with 2 units/ml of micrococcal nuclease (Amersham Biosciences) in EB buffer supplemented with 2 mM $CaCl_2$ at 30°C for 10 min prior to mixing with SDS loading buffer. After SDS polyacrylamide gel electrophoresis, proteins were transferred onto a nitrocellulose membrane (PROTORAN, Schleicher & Schuell) and immunoblot signals were detected with ECL Western blotting detection reagents (Amersham Biosciences). Re-probing with different antibodies was carried out using an ECL detection kit according to the manufacturer's instructions.

For the experiment shown in Fig 4, chromatin isolation was performed with EB buffer containing 0.25% Triton or 10% sucrose and supplemented further with increasing amounts of KCl, as indicated in the figure. Immunoblot signals on film were measured using a gel image documentation system (Dolphins series, Wealtec Corp.) and accompanying image analysis software (Dolphin-1D, Wealtec Corp.).

Antibodies—Rabbit anti–FFA-1 antibodies were raised against bacterially expressed 10X histidine-tagged, C-terminal polypeptide (amino acids 1,330 to 1,418 of FFA-1) or N-terminal polypeptide (amino acids 2 to 95), and rabbit anti-xBLM antibodies were raised against bacterially expressed 10X histidine-tagged, N-terminal polypeptide (amino acids 2 to 98 of xBLM). The rabbit anti-*Xenopus* Polε p60, Polδ p50 and Polα p70 antibodies are described elsewhere (*43*, *44*). Rabbit antibodies against *Xenopus* Polδ p125, RPA, minichromosome maintenance protein 3 (MCM3) and origin recognition complex 2 (ORC2), and a monoclonal antibody against PCNA were generous gifts from Masahiro Akiyama and Hisaji Maki (Nara Institute of Science and Technology, Japan), Johannes Walter (Harvard Medical School, USA), Haruhiko Takisawa (Osaka University, Japan), Yasuo Kawasaki (Osaka University, Japan), and Bruce Stillman (Cold Spring Harbor Laboratory, USA), respectively. Antibodies against phosphorylated histone H2AX (γH2AX) and MCM7 were obtained from Upstate Biotechnology and Sigma, respectively.

Immunodepletion—The anti–FFA-1 antibodies were bound to Dyna beads Protein A (DYNAL) (0.5–1 μl serum/μl bead suspension). Egg extracts (200 μl) were treated twice with 100 μl of beads carrying antibodies against the C-terminal polypeptide of FFA-1, and subsequently once with 100 μl of beads carrying antibodies against the N-terminal polypeptide of FFA-1.

RESULTS

Replication Fork Arrest Markedly Enhances Chromatin Binding of FFA-1—We used the *in vitro* DNA replication system in *Xenopus* egg extracts to investigate the dynamics of chromatin binding of FFA-1 in response to replication fork arrest; this *in vitro* system reproduces faithfully most of the events in cellular DNA replication (*48*). Nuclear formation around DNA or chromatin is a prerequisite for DNA replication in crude egg extracts. The pre-RC is formed on chromatin in parallel with nuclear formation, and DNA synthesis every 10–15 kb begins thereafter. It usually takes about 30 min for DNA synthesis to begin under our conditions, if demembranated sperm nuclei are used as a template.

To block the progression of replication forks, aphidicolin, an inhibitor of replicative DNA polymerases, was added to a reaction mixture containing demembranated sperm nuclei

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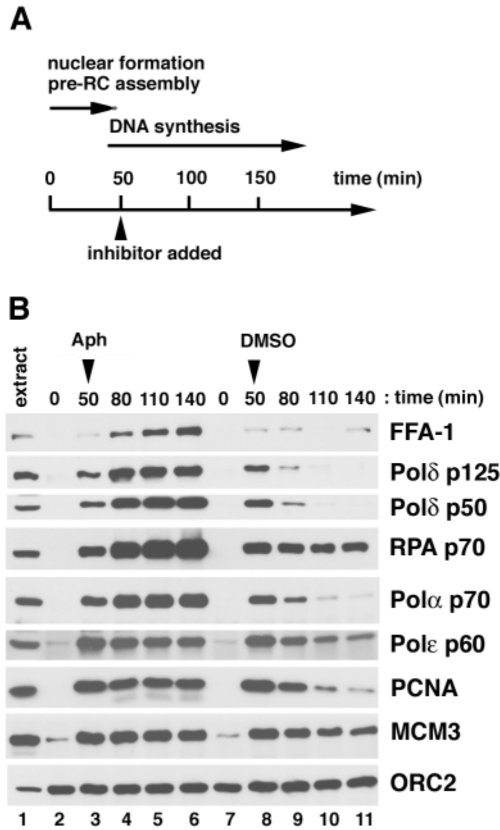

Fig. 1. **Accumulation of FFA-1, Polδ and RPA on chromatin after replication fork arrest.** (A) A scheme for replication fork arrest by DNA polymerase inhibitors in *Xenopus* egg extracts. (B) Demembranated sperm nuclei were mixed with egg extracts at 5,000 nuclei per μl extracts (t = 0 min, lanes 2 and 7) and incubated for 50 min (t = 50 min, lanes 3 and 8). Aphidicolin (50 μg/ml) or an equivalent volume of DMSO was then added, and the incubation was further continued (lanes 4–6 and 9–11). Chromatin was isolated from the reaction mixture at the indicated times, and chromatin-bound proteins were analyzed by immunoblotting using appropriate antibodies. The chromatin sample in each lane is equivalent to 24 μl of egg extracts. Egg extracts (1 μl) were also shown in lane 1.

after the start of DNA synthesis (Fig 1A). Aliquots of the mixture were then withdrawn every 30 min, and proteins bound to chromatin were analyzed by SDS–polyacrylamide gel electrophoresis followed by immunoblotting. DNA synthesis was almost completely inhibited by aphidicolin under the conditions used (data not shown).

As shown in Fig. 1B, addition of aphidicolin markedly enhanced chromatin binding of FFA-1 (Fig. 1B, lanes 2–6), whereas addition of DMSO, the solvent used for aphidicolin, barely enhanced FFA-1 binding (lanes 7–11). Increased chromatin binding of FFA-1 was clearly seen 30 min after addition of aphidicolin (compare lanes 3 and 4, Fig. 1B), and binding was subsequently slightly increased (lanes 4–6, Fig. 1B). Enhanced chromatin binding of FFA-1 was also observed with another DNA polymerase inhibitor, AraCTP (see Fig 3); thus, enhanced chromatin binding of FFA-1 reflects a general response to replication fork arrest. This increased binding of FFA-1 to replication-arrested chromatin highly likely corresponds to the previous observation that the number of FFA-1 foci formed in the reconstituted nuclei was increased when demembranated sperm nuclei were incubated in egg extracts in the presence of aphidicolin (*38*), although in this previous experiment, aphidicolin was added at the beginning of incubation.

DNA Replication Arrest Significantly Enhances Chromatin Binding of Polδ and RPA—We next examined if replication fork arrest would affect replication proteins that function at replication forks. The proteins examined were Polα, Polδ and Polε (all of which are major replicative DNA polymerases), PCNA and replication factor C (RFC) (a clamp and a clamp loader for Polδ and Polε, respectively), RPA (a single-stranded DNA binding protein), and MCM3 and MCM7 (components of the MCM2-7 complex, a putative DNA helicase functioning at the forks) (reviewed in Refs. *49–51*). Without addition of aphidicolin, binding of all these proteins to chromatin gradually decreased, which apparently reflects progression of DNA replication (Fig. 1B, lanes 8–11, and data not shown). As expected, the level of chromatin-bound ORC2 (a component of ORC) remained constant (*51*), regardless of addition of aphidicolin; thus, ORC2 was used as a loading control.

Addition of aphidicolin caused suppression of the decrease in chromatin binding of the replication fork proteins, suggesting that these proteins may stay bound to a stalled replication fork. As shown previously (*52*), chromatin binding of RPA significantly increased after addition of aphidicolin (Fig. 1B, lanes 3–6). Notably, chromatin binding of Polδ was significantly enhanced after addition of aphidicolin (compare lanes 3 and 4 in Fig. 1B); the p125, p66 and p50 subunits of *Xenopus* Polδ all accumulated on chromatin (Fig. 1B, and data not shown), suggesting that the Polδ holoenzyme further accumulates on replication-arrested chromatin. Addition of AraCTP also caused accumulation of Polδ and RPA on chromatin, although the extent of the increase in binding varied among experiments (data not shown, see Fig. 3). In contrast to Polδ, the chromatin-bound levels of Polε, PCNA and RFC did not increase after addition of aphidicolin (compare lanes 3 and 4 in Fig. 1B, and data not shown). Although Fig. 1B shows a slight increase in Polα binding, we also observed that Polα binding did not increase in some experiments, while an increase in chromatin binding of Polδ and RPA was constantly seen (data not shown). Thus, replication fork arrest causes a further increase in chromatin binding of a certain group of replication fork proteins.

Quantitation of FFA-1 and Polδ Bound to Replication-Arrested Chromatin—We next quantified the amounts of FFA-1 and Polδ bound to chromatin before and after addition of aphidicolin, using quantitative immunoblotting. As a control, the amount of Polε bound to chromatin was also quantified. Approximately 50 fmol of Polε (in 10^5 sperm nuclei) was bound to chromatin 60 min after incubation without aphidicolin (Table 1); this gives an estimate of approximately one Polε molecule bound per 9.2 kb DNA. Since DNA synthesis was occurring vigorously at the 60-min time point (data not shown), this number (50 fmol) may reflect the number of active replication forks, although the exact number of Polε molecules bound to a single replication fork has not been determined. As shown in Fig. 1, the number of Polε molecules bound to chromatin did not change after

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Table 1. **Quantitation of the proteins bound to chromatin before and after addition of aphidicolin.** After demembranated sperm nuclei (5,000 per μl) were incubated in egg extracts for 60 min, an aliquot was withdrawn from the reaction mixture (aphidicolin –). Aphidicolin was then added and the reaction mixture was further incubated for 60 min (aphidicolin +). Chromatin isolated at each time point was subjected to quantitative immunoblotting along with the standards of the recombinant proteins. The calculated number of the protein molecules (fmol in 10^5 nuclei) is shown. nd: not determined.

	Aphidicolin addition	
	–	+
FFA-1	nd	50
Polδ	55	294
Polε	50	50

replication fork arrest (about 50 fmol in 10^5 nuclei) (Table 1).

Curiously, a similar amount of FFA-1 (about 50 fmol in 10^5 nuclei) accumulated on replication-arrested chromatin, consistent with the possibility that chromatin binding of FFA-1 may be targeted to arrested forks. In contrast, while a similar amount of Polδ (about 55 fmol in 10^5 nuclei) bound to chromatin during ongoing active DNA replication, the amount of Polδ bound to replication-arrested chromatin increased by more than 5-fold (about 300 fmol in 10^5 nuclei) (Table 1). Thus, the amount of Polδ that accumulated on replication-arrested chromatin seemed to be much greater than the amount of Polδ functioning at forks during normal DNA replication.

Levels of FFA-1 and Polδ on Chromatin Are Reduced upon Release of Replication Fork Arrest—As expected, enhanced chromatin binding of FFA-1 was abolished by addition of geminin, which is known to inhibit formation of the pre-RC (Fig 2A, lanes 12–14) (*42*), although the basal level of chromatin binding of FFA-1 persisted even in the presence of geminin (lanes 6–8).

Replication arrest by aphidicolin has been shown to result in activation of ATR [Ataxia-telangiectasia mutated (ATM) and Rad3-related]-dependent replication checkpoint controls (*53*). To examine whether checkpoint activation is involved in enhanced accumulation of FFA-1 and Polδ on replication-arrested chromatin, caffeine, an inhibitor of ATM and ATR, was added to the egg extracts. As shown in Fig. 2B, both FFA-1 and Polδ accumulated on chromatin in the presence of aphidicolin and caffeine (lanes 12–14), indicating that ATM/ATR activation is not required for accumulation of FFA-1 and Polδ on replication-arrested chromatin. It should be noted that chromatin binding of Polδ was significantly increased at 50-min time points in the presence of caffeine (Fig. 2B, lanes 9 and 12). This probably represents accelerated origin firing, resulting from the inactivation of ATM/ATR-dependent signalling pathways as shown previously (*54*).

We next examined whether chromatin binding of FFA-1 would change upon release of replication arrest. To this end, replication was first arrested by AraCTP and then released by adding an excess amount of dCTP (*55*). Chromatin binding of FFA-1 increased 30 min after addition of AraCTP (Fig 3, lanes 4 and 9), and the levels subsequently increased further in the absence of release (lanes 9–11).

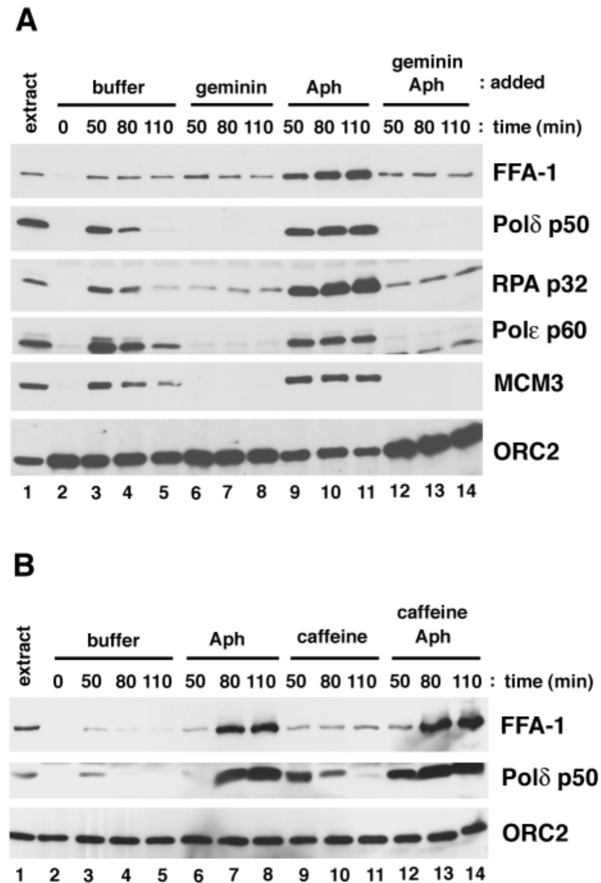

Fig. 2. **Enhanced accumulation of FFA-1 on chromatin requires pre-RC formation but not ATR/ATM activation.** (A) Demembranated sperm nuclei (5,000 per μl extracts) were incubated in egg extracts supplemented with a control buffer (lanes 2–5), 3 μg/ml geminin (lanes 6–8), 50 μg/ml aphidicolin (lanes 9–11) or both (lanes 12–14). (B) Demembranated sperm nuclei (5,000 per μl) were incubated in egg extracts in the absence (lanes 2–8) or presence of 5 mM caffeine (lanes 9–14). After 50-min incubation, aphidicolin (50 μg/ml) (lanes 6–8 and 12–14) or an equivalent volume of DMSO (lanes 3–5 and 9–11) was added (t = 50 min), and the incubation was further continued. Chromatin was isolated at the indicated times and chromatin-bound proteins were analyzed as in Fig. 1. One microliter of egg extract was also loaded on lane 1 in A and B. Note that the bands in lanes 12–14 in the blot with anti-Polε p60 antibodies are non-specific.

In contrast, the bound levels of FFA-1 declined after addition of excess dCTP (lanes 4–6), and a DNA synthesis assay showed that nucleotide incorporation did indeed restart upon addition of excess dCTP to the AraCTP-inhibited reaction (data not shown). These results indicate that FFA-1 transiently associates with chromatin when replication forks are stalled, and then dissociates from chromatin when replication is restarted.

The level of Polδ on chromatin also gradually decreased after release from AraC-arrest (Fig. 3, lanes 3–6), although increased chromatin binding of Polδ was not obvious in this particular experiment. In contrast to FFA-1 and Polδ, the level of RPA on chromatin did not appear to decrease significantly after release from AraC arrest (Fig. 3).

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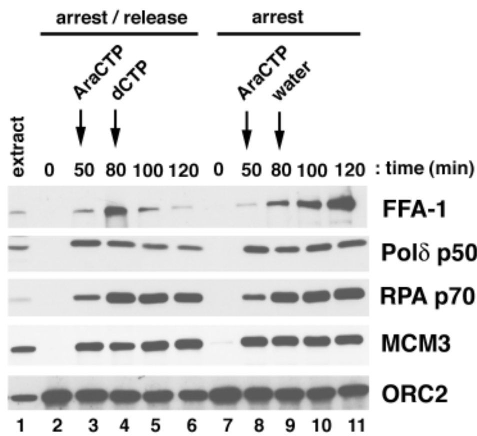

Fig. 3. **Levels of FFA-1 and Polδ on chromatin decline after release of replication fork arrest.** Demembranated sperm nuclei (10,000 per μl) were incubated in egg extracts for 50 min, and then 400 μM AraCTP was added (t = 50 min, lanes 3 and 8). An excess amount (4 mM) of dCTP (lane 4) or an equivalent volume of water (lane 9) was added 30 min after addition of AraCTP (t = 80 min), and the incubation was further continued (lanes 5, 6, 10 and 11). Chromatin was isolated at the indicated times, and chromatin-bound proteins and egg extracts (1 μl) (lane 1) were analyzed as in Fig. 1.

Differential Sensitivity of Chromatin-Bound FFA-1 and Polδ to Salt Extraction—We further investigated how replication arrest would affect chromatin binding of FFA-1 and Polδ by comparing the sensitivity of chromatin-bound FFA-1 and Polδ to salt extraction before and after replication fork arrest. As shown in Fig 4, the majority of FFA-1 molecules bound to replication-arrested chromatin could be extracted with 0.2 M KCl. In contrast, more than 50% of FFA-1 bound to chromatin without addition of aphidicolin remained bound after 0.2 M KCl extraction (Fig. 4 A and C).

In contrast to FFA-1, Polδ bound to replication-arrested chromatin was more resistant to salt extraction, compared to Polδ bound to chromatin with ongoing active DNA replication; more than 50% of Polδ bound to replication-arrested chromatin remained bound after 0.2 M KCl extraction, while a similar extraction resulted in about 80% of Polδ being released from chromatin after initial binding in the absence of aphidicolin (Fig. 4 B and D). These results suggest that FFA-1 and Polδ molecules that additionally accumulate on chromatin upon replication fork arrest may bind to chromatin in a manner distinct from that before induction of replication fork arrest.

Polδ and RPA Accumulate on Replication-Arrested Chromatin Independently of FFA-1—Since it has been shown that WRN interacts with Polδ (*19*, *28*), the interdependency of FFA-1 and Polδ for accumulation on replication-arrested chromatin was examined, based on analysis of chromatin binding of Polδ in FFA-1–depleted extracts. FFA-1 depletion did not significantly affect DNA replication in egg extracts, as reported previously [(*38*); data not shown]. FFA-1 was undetectable in the FFA-1–depleted extracts and also in chromatin fractions (Fig 5, lanes 8–14), indicating that depletion is nearly complete. However, both Polδ and RPA accumulated on replication-arrested chromatin in the FFA-1–depleted extracts, to a similar level to that seen in mock-depleted extracts (Fig. 5, compare lanes 12–14 with lanes 5–7). These results indicate that accumulation of Polδ and RPA after replication fork arrest occurs independently of FFA-1.

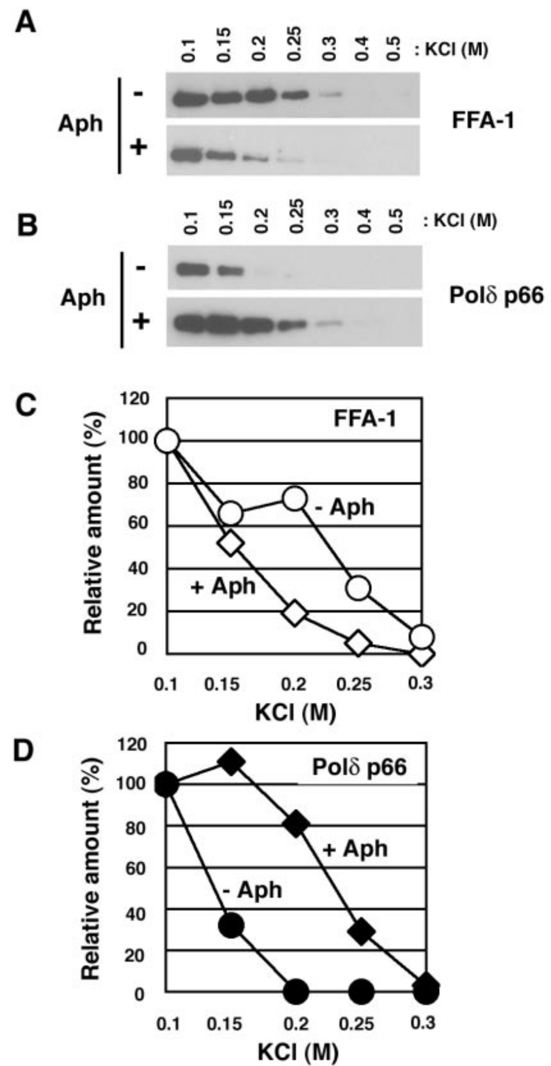

Fig. 4. **Differential sensitivity of chromatin-bound FFA-1 and Polδ to salt extraction before and after replication fork arrest.** (A and B) After demembranated sperm nuclei (5,000 per μl) were incubated in egg extracts for 60 min, aliquots were withdrawn from the reaction mixture (Aph –). Aphidicolin (50 μg/ml) was then added and the reaction mixture was further incubated for 60 min (Aph +). Chromatin was isolated from each reaction mixture with the buffer containing increasing amounts of KCl and analyzed as in Fig. 1 using anti–FFA-1 (A) and anti–Polδ p66 antibodies (B). Note that each loaded sample of Aph (–)-chromatin for immunoblotting with anti–FFA-1 antibodies (top panel in A) was 4 times as much as that of Aph (+)-chromatin and also that the immunoblot signals of Polδ in Aph (–)-chromatin samples (top panel in B) were detected by a longer exposure. (C and D) Intensity of the immunoblot signals shown in A and B was measured and the amounts relative to the signals in chromatin samples after extraction with 0.1 M KCl were plotted.

Double-Strand DNA Breaks Cause Accumulation of FFA-1 on Chromatin, Independently of Pre-RC Formation—Previous studies of WRN in mammalian

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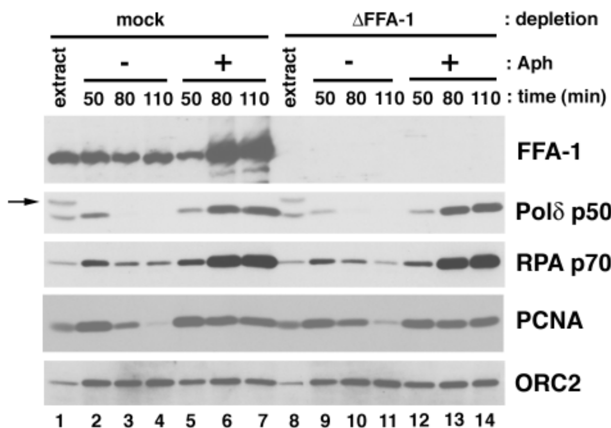

Fig. 5. **Polδ and RPA accumulate on replication-arrested chromatin independently of FFA-1.** Demembranated sperm nuclei (5,000 per μl) were incubated in FFA-1–depleted (lanes 9–14) or mock-depleted extracts (lanes 2–7). Aphidicolin (50 μg/ml) (lanes 5–7 and 12–14) or DMSO (lanes 2–4 and 9–11) was added at t = 50 min and incubation was further continued. Chromatin was isolated at the indicated times, and chromatin-bound proteins and each depleted egg extract (1 μl) (lanes 1 and 8) were analysed as in Fig. 1. Note that the immunoblot with anti–FFA-1 antibodies shown here is a darker image than those shown in other figures, in order to make sure that no FFA-1 bound to chromatin in FFA-1–depleted extracts. An arrow indicates the non-specific bands seen in lanes 1 and 8.

cells and in *Xenopus* egg extracts have shown that WRN is involved in the process of DNA double-strand break (DSB) repair (*14*, *29*, *31*, *32*, *39*). Consistent with this, chromatin binding of FFA-1 markedly increased when sperm chromatin was incubated in egg extracts supplemented with a restriction enzyme, *Eco*RI (Fig 6A, lanes 9–11). The addition of *Eco*RI resulted in phosphorylation of histone H2AX (*56*) and accumulation of RPA, which was apparently hyperphosphorylated (Fig. 6A, lanes 9–11); both these events are indicative of DSBs. We note that addition of aphidicolin did not result in phosphorylation of histone H2AX under these conditions.

The addition of *Eco*RI also resulted in a marked reduction of DNA synthesis in egg extracts, as shown previously [(*57*) and data not shown]. Consistently, chromatin binding of Polδ, Polε and PCNA was significantly suppressed in the presence of *Eco*RI (Fig. 6A, lanes 9–11, also see Fig. 6B), whereas pre-RC formation did not appear to be affected by *Eco*RI (see MCM7 binding in lanes 2–6, Fig. 6B). In contrast to replication arrest, accumulation of FFA-1 on DSB-introduced chromatin was not affected by addition of geminin, indicating that this FFA-1 accumulation is independent of formation and activation of the pre-RC. We also examined the sensitivity to salt extraction of FFA-1 bound to DSB-introduced chromatin. However, there was no significant difference in salt sensitivity of FFA-1 bound to DSB-introduced chromatin and replication-arrested chromatin (Fig. 6 C and D).

Comparison of Chromatin Binding of FFA-1 and xBLM—Recently, it was shown that chromatin binding of xBLM is increased in egg extracts supplemented with aphidicolin (*41*). Thus, we directly compared chromatin binding of FFA-1 and xBLM in the presence of aphidicolin or *Eco*RI. Chromatin binding of xBLM only slightly increased after addition of aphidicolin (compare lanes 6 and 7 in Fig. 6A). Similarly, *Eco*RI also caused only a moderate increase in chromatin binding of xBLM (compare lanes 3–5 and 9–11 in Fig. 6A, also see Fig. 6B). Thus, chromatin binding of FFA-1 seems to change more dramatically upon replication fork arrest or DSB formation, compared to that of xBLM.

DISCUSSION

The cell-free DNA replication system in *Xenopus* egg extracts was used to investigate the dynamics of chromatin binding of Werner helicase and replication fork proteins in response to replication fork arrest. It has been shown that FFA-1 interacts with RPA and this interaction may be important for DNA replication under normal conditions in *Xenopus* egg extracts (*38*). Although a low level of chromatin-bound FFA-1 was observed during normal DNA replication in this study, this level dramatically increased after replication fork arrest. Thus, our study biochemically reproduces the previous observation that the number of FFA-1 foci in the replication-arrested nuclei is increased in *Xenopus* egg extracts (*38*).

Considering previous observations that FFA-1 co-localizes at sites of DNA synthesis (*38*) and that the RecQ helicase in budding yeast, Sgs1, is associated with replication forks (*58*), it seems likely that FFA-1 directly binds to stalled replication forks. This is consistent with our observation that the number of FFA-1 molecules that accumulate on replication-arrested chromatin is almost the same as the number of chromatin-bound Polε or Polδ complexes during active DNA synthesis. Furthermore, FFA-1 can quickly dissociate from chromatin upon restart of fork progression. Thus, FFA-1 may initially recognize a stalled replication fork in the absence of DSBs. Although previous studies suggest that FFA-1 may function at replication forks during normal replication (*38*), the basal level of chromatin binding of FFA-1 does not necessarily reflect the function of FFA-1 at replication forks, because its chromatin binding persisted even in the presence of geminin (Fig. 2A, also see below).

Differential sensitivity of chromatin-bound FFA-1 to salt extraction before and after replication fork arrest implies that FFA-1 binds to chromatin in different ways in these two situations. Based on the DNA binding specificity of mammalian WRN and the many known interactions between WRN/FFA-1 and other proteins, it seems reasonable to speculate that FFA-1 recognizes a specific DNA structure that forms at the stalled replication fork, probably in coordination with other proteins. To understand the mechanism of FFA-1 accumulation on replication-arrested chromatin, it will be necessary to determine the functional domain in FFA-1 that is required for accumulation upon replication fork arrest. On the other hand, the basal level of chromatin binding of FFA-1 without addition of aphidicolin exhibited more resistance to salt extraction and did not depend on pre-RC formation. Thus, FFA-1 binding under normal conditions may reflect another nuclear function of FFA-1, such as base excision repair (*17*).

In addition to FFA-1, replication fork arrest induces further accumulation of Polδ and RPA. Addition of aphidicolin has been suggested to cause uncoupling between DNA synthesis and DNA helicase activity, leading to

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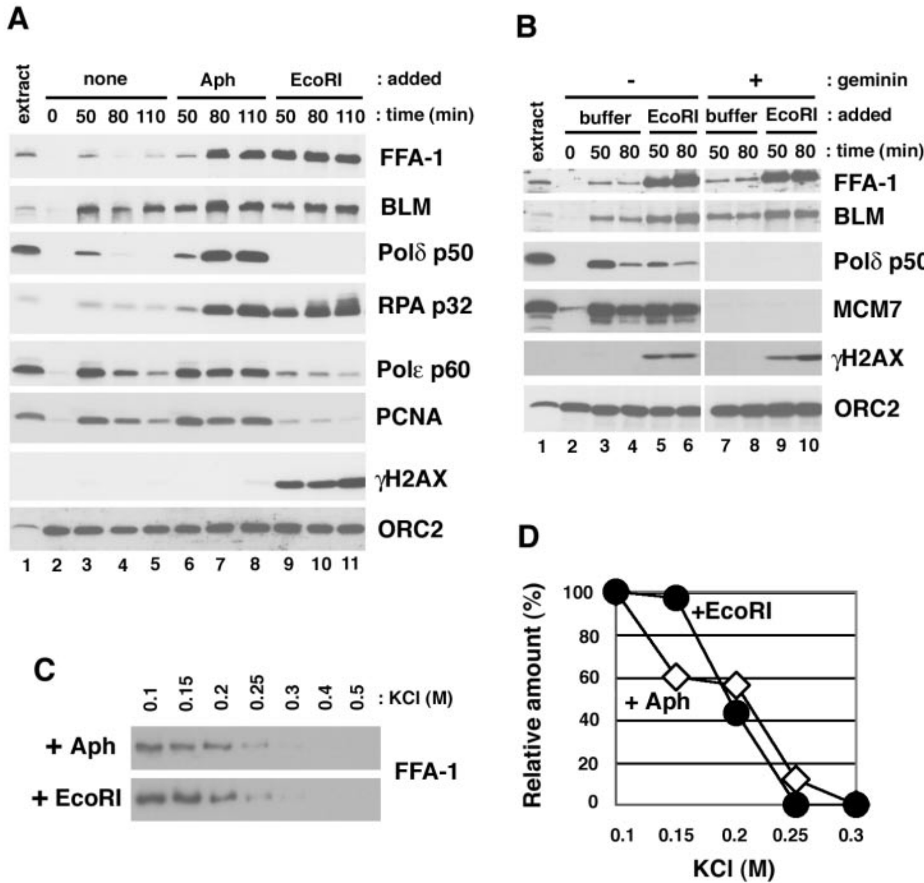

Fig. 6. **Differential accumulation of FFA-1, xBLM, and Polδ on chromatin in response to replication fork arrest and DSB formation.** (A) Demembranated sperm nuclei (5,000 per μl) were mixed with egg extracts supplemented with DMSO (lanes 2–5) or *Eco*RI (0.02 unit/1,000 nuclei) (lanes 9–11) and incubated for the indicated times. Aphidicolin (50 μg/ml) was also added at t = 50 min as in Fig. 1 (lanes 6–8). (B) Demembranated sperm nuclei (5,000 per μl) were incubated in egg extracts supplemented with 3 μg/ml geminin and *Eco*RI for the indicated times. Chromatin was then isolated from the reactions in A and B and analyzed as in Fig. 1. (C and D) Demembranated sperm nuclei (5,000 per μl) were mixed with egg extracts supplemented with *Eco*RI and incubated for 60 min. In a separate reaction, aphidicolin was added at t = 60 min and further incubated for 60 min. Chromatin was then isolated from each reaction mixture with the buffer containing increasing amounts of KCl and analyzed by immunoblotting with anti–FFA-1 antibodies as in Fig. 4.

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formation of extended single-stranded DNA regions and accumulation of RPA on chromatin (*59*); however, this uncoupling mechanism does not explain Polδ accumulation on replication-arrested chromatin. Since the level of chromatin-bound Polε did not change after replication arrest, accumulation is a Polδ-specific event; moreover, this event is not accompanied by accumulation of chromatin-bound PCNA or RFC, both of which are necessary for formation of a processive Polδ complex at the primer terminus (*49*). Therefore, Polδ accumulation on replication-arrested chromatin may reflect an unknown, replication arrest-dependent mechanism that specifically involves Polδ. With respect to this, WRN and WHIP have been shown to stimulate Polδ activity without PCNA (*28*, *60*), and also *Mgs1*, a yeast WHIP homolog, genetically interacts with *Pol3*, which encodes the catalytic subunit of Polδ (*61–63*). Thus, although Polδ accumulation occurs independently of FFA-1, it may still be possible that Polδ has a specific role at a stalled replication fork in coordination with FFA-1, probably to prevent aberrant recombination and/or breakage of the stalled fork.

In conclusion, our results demonstrate that two distinct pathways involving FFA-1 are reproduced in the *Xenopus* egg *in vitro* system: one in response to replication fork arrest and the other in response to DSB formation. Since depletion of FFA-1 from egg extracts does not appear to affect overall DNA synthesis during DNA replication, this system can be used to investigate whether the absence of FFA-1 causes symptoms of genomic instability, which may be induced during normal DNA replication and/or after replication fork arrest, as demonstrated for xBLM depletion (*41*). Furthermore, since we detected a more dramatic increase in chromatin binding of FFA-1 in response to replication fork arrest and DSB formation, compared to xBLM (Fig. 6), FFA-1 might be more strictly associated mechanistically with stalled replication forks and DSB sites. Thus, it will be interesting to see whether double depletion of FFA-1 and xBLM would augment genomic instability induced by xBLM depletion.

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