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Fanconi anemia proteins stabilize replication forks

Lily Chien Wang^{1,2}, Stacie Stone³, Maureen Elizabeth Hoatlin³, and Jean Gautier^{1,4*}

¹*Institute for Cancer Genetics, Columbia University, New York, NY 10032 USA*

²*Integrated Program in Cellular, Molecular, Structural and Genetic Studies, Columbia University, New York, NY 10032 USA*

³*Division of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, OR 97239 USA*

⁴*Department of Genetics and Development, Columbia University, New York, NY 10032 USA*

Abstract

Fanconi anemia (FA) is a recessive genetic disorder characterized by hypersensitivity to crosslinking agents that has been attributed to defects in DNA repair and/or replication. FANCD2 and the FA core complex bind to chromatin during DNA replication; however, the role of FA proteins during replication is unknown. Using *Xenopus* cell-free extracts, we show that FANCL depletion results in defective DNA replication restart following treatment with camptothecin, a drug that results in DSBs during DNA replication. This defect is more pronounced following treatment with mitomycin C, presumably because of an additional role of the FA pathway in DNA crosslink repair. Moreover, we show that binding of FA core complex proteins during DNA replication follows origin assembly and origin firing and is dependent on the binding of RPA to ssDNA while FANCD2 additionally requires ATR, consistent with FA proteins acting at replication forks. Together, our data suggest that FA proteins play a role in replication restart at collapsed replication forks.

Keywords

Fanconi anemia; Mitomycin C; Replication forks; Replication restart

1. Introduction

Fanconi anemia (FA) is a rare genetic disorder characterized by chromosomal instability and hypersensitivity to crosslinking agents, such as Mitomycin C (MMC) [1,2], diepoxybutane (DEB) [3], and cisplatin (CDDP) [4]. Thirteen FA genes that participate in the FA pathway have been cloned. A nuclear FA complex (FA core complex), including FANCA, -B, -C, -E, -F, -G, -L, FAAP100, FAAP24 and FANCM, forms the ubiquitin E3 ligase for FANCD2 and FANCI [5–11]. While FANCL is the catalytic component of the E3 ligase [9,12], each component of the FA core complex is required for FANCD2 and FANCI monoubiquitylation and the loss of any component results in the loss of FANCD2 and FANCI monoubiquitylation [6–9,13]. The monoubiquitylation of FANCD2 and FANCI is also co-dependent and the absence of or the mutation of the ubiquitylation site on either protein results in the abrogation

Corresponding Author: Jean Gautier, Tel: 1-212-851-4564, Fax: 1-212-851-5284, jg130@columbia.edu.

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of monoubiquitylation of the other protein [14]. Monoubiquitylated FANCD2 and FANCI assemble into foci with BRCA1, RAD51, FANCD1/BRCA2, FANCI/BRIP1, and FANCN/PALB2 [8,15–20]. Defects in proteins acting downstream of the FANCI-FANCD2 complex (FANCD1, FANCI, and FANCN) result in cells with hypersensitivity to crosslinking agents but show normal FANCD2 and FANCI monoubiquitylation [18,19,21–23].

The FA pathway is activated in response to a variety of DNA damaging agents, including UV light, ionizing irradiation (IR), hydroxyurea treatment and crosslinking agents [24]. Because of the higher sensitivity of FA cells to crosslinking agents, FA proteins are thought to play specific roles in the recognition and/or repair of lesions generated by crosslinking agents, including interstrand crosslinks (ICLs). However, how the FA pathway regulates these processes remains elusive. Treatment of FA cells with crosslinking agents enhances the accumulation of chromosomal rearrangements and triggers arrest in G2/M with 4N DNA content. The latter suggests that FA proteins play a role in the resolution of replication blocks resulting from crosslinking agents [25].

Consistent with playing a role in DNA replication, FANCD2 and core complex components FANCA and FANCF bind to chromatin during unperturbed replication in *Xenopus* cell-free extracts [26] and core complex components FANCA, FANCC, and FANCG bind to DNA during normal replication in mammalian cells [27]. Both DNA damage-dependent and -independent FA chromatin binding are inhibited by geminin, which sequesters Cdt1 and prevents pre-RC assembly, and are thus dependent on DNA replication [26,27], underscoring the role of FA protein recruitment to DNA during DNA replication in the activation of the FA pathway. However, FA proteins also bind to small synthetic DNA substrates in replication-incompetent cell-free extracts, pointing to a possible role in DNA repair, independently of DNA replication [28].

Assembly of the pre-replicative complex (pre-RC) at origins of replication starts with the binding of the origin recognition complex (ORC), followed by the Cdc6- and Cdt1-dependent loading of the minichromosome maintenance proteins (MCM2-7) – the replicative helicase. Next, Cdk2 and Cdc7 protein kinases are required for the activation of origins, as seen by the recruitment of Cdc45, GINS, and MCM10 proteins and subsequent origin unwinding. The single-stranded DNA (ssDNA) generated by origin unwinding is then coated by RPA. Finally, DNA polymerases are bound and bi-directional DNA replication takes place [29]. DNA replication forks are the sites of complex DNA transactions and many DNA intermediates form at replication forks. Replication fork progression can slow or stop at sites of secondary DNA structures or protein-DNA complexes or following inhibition of DNA polymerases [30]. Stalled replication forks are normally stabilized by checkpoint kinases [31–34] and failure to properly stabilize and/or restart stalled replication forks can lead to replication fork collapse and the generation of double-strand breaks (DSBs) [32]. Small compounds have been used to inhibit replication fork progression and to distinguish between stalled and collapsed replication forks. Aphidicolin (APH), an inhibitor of DNA polymerases, causes replication fork stalling. Camptothecin (CPT) inhibits DNA topoisomerase I (topoI) by binding to the topoI-DNA intermediate and preventing the religation reaction [35], thus generating DSBs upon collision of the replication fork with the lesion and subsequent replication fork collapse. MMC is a potent DNA crosslinking agent that also causes DSBs and replication fork collapse. In contrast to APH, which does not affect the stability of DNA polymerase ϵ (Pol ϵ) at the replication fork, treatments with CPT and MMC result in the unloading of Pol ϵ from DNA [36].

We examined the role of FA proteins in repair and replication restart after APH, CPT, and MMC treatments in *Xenopus* cell-free extracts. We find that in the absence of a functional FA pathway, restart of replication forks following MMC or CPT treatment was impaired. Notably, CPT treatment does not generate DNA crosslinks. The timing of recruitment of FA proteins

to chromatin during DNA replication coincides with RPA loading and RPA is required for FA proteins loading, thus placing the FA complex at replication forks. Taken together, our results implicate the FA pathway in the restart of collapsed replication forks.

2. Materials and methods

2.1 Preparation of *Xenopus* extracts

Cytosolic interphase *Xenopus* eggs extracts were prepared and tested as described by Shechter D et al. (2004).

2.2 Antibodies and Reagents

Anti-xFANCD2, -xFANCA, -xFANCG, and -xFANCF antibodies were generated as described by Sobeck et al (2006) and Stone et al (2007). Anti-MCM6 antibodies were generated as described by Ying et al. [38]. Anti-RPA p70 antibodies were a gift from P Jackson, anti-Pol α antibodies were a gift from WM Michael and T Wang, and anti-ATR antibodies were a gift from V Costanzo. The xFANCL sequence was described previously [26]. An xFANCL-GST fusion protein was made by inserting the xFANCL sequence into the pDONR201 vector of the Gateway Cloning System (Invitrogen) and recombination reactions to produce the expression vector pDEST GST-xFANCL. Recombinant GST-xFANCL protein was purified with Glutathione-Sepharose A beads (GE) and both bead-bound GST-FANCL and denatured GST-FANCL were used to immunize rabbits.

2.3 Immunodepletions

Immunodepletions of cytosolic extracts were performed using anti-Pol α , -RPA, -ATR, and -FANCL antibodies coupled to Protein A-Sepharose CL-4B beads (Amersham Biosciences). Two rounds of depletion were performed for anti-Pol α , -RPA, and -ATR and one round of depletion was performed for anti-FANCL by incubation of extracts with bead-bound antibodies at 4°C for 30 min per round. Mock depletion was performed using rabbit IgG (Sigma) coupled to Protein A beads. Immunodepletions were monitored by Western blot analysis.

2.4 Replication Assay

Chromosomal templates for DNA replication were prepared from demembranated *Xenopus* sperm nuclei as described by Murray (1991). Cytosolic extracts were incubated with 1000 sperm nuclei/ μ L at 22°C with 1 μ Ci 32 P- α dCTP for 90 min. The reaction was stopped with replication stop buffer (80 mM Tris-HCL pH 8.8, 8 mM EDTA, 0.5% SDS), followed by incubation with proteinase K at 1mg/mL for 1 h at 60°C, phenol-chloroform extraction, and ethanol precipitation. DNA synthesis was monitored by the incorporation of 32 P- α dCTP following agarose gel electrophoresis. Gels were exposed for autoradiography and quantified using a PhosphorImager. The entire lane (the DNA remaining in the well and the migrated DNA) was quantified.

2.5 Chromatin binding assay

For chromatin binding assays, cytosolic extracts were incubated with 2500 sperm nuclei/ μ L at 22°C. The reactions were incubated for the times indicated and each reaction was diluted with chromatin isolation (CI) buffer (100mM KCl, 2.5 MgCl $_2$, 50 mM Hepes pH 7.8) supplemented with 0.125% Triton X-100. Chromatin was isolated through a 30% sucrose solution in CI buffer at 6000 g for 30 min at 4°C. The samples were run on SDS-PAGE and analyzed by Western blotting.

3. Results

3.1 Chromatin binding of FA proteins requires origin activation and RPA recruitment

We have previously shown that FA proteins loading onto chromatin requires the assembly of the pre-replicative complex [26]. To gain further insight into the function(s) of FA proteins in DNA replication, we determined the requirements for FA protein binding to DNA. We supplemented *Xenopus* cytosolic extracts with geminin, roscovitine, p21^{CIP1}, or APH to identify discrete steps required during DNA replication. Sperm nuclei were assembled and incubated in extracts for 60 min under these experimental conditions, purified from the cytosolic fractions and the association of FA proteins was probed by Western blot of the purified chromatin fraction (Figure 1A and Supplemental figure S1). The long and short forms of FANCD2, corresponding to FANCD2 with and without monoubiquitylation can be detected in the cytosol. In contrast, only the monoubiquitylated form of FANCD2 is present on chromatin, consistent with previous observations (Supplemental figure S2) [26,39]. We first confirmed that geminin reduced chromatin binding of FANCD2 and FA core complex proteins FANCA, FANCG, FANCF, and FANCL (Figure 1A and Sobeck et al. 2006). Roscovitine and p21^{CIP1} are Cdk2 inhibitors that inhibit origin activation. Both reduced the chromatin binding of FANCD2 and FA core complex proteins to the levels observed upon geminin treatment (Figure 1A and Supplemental Figure S1). APH, an inhibitor of replicative DNA polymerases, did not inhibit the chromatin binding of FANCD2 or FA core complex components (Figure 1A). To assess whether polymerase α (Pol α) protein was required for FA chromatin recruitment, we depleted Pol α from extracts using a specific antibody [40]. Depletion of 80% of cytosolic Pol α protein, which resulted in 40% reduction of chromatin-bound Pol α , did not affect the chromatin binding of FA proteins (Figure 1B). From these experiments, we conclude that recruitment of FA proteins to chromatin requires pre-RC assembly and the activation of replication origins, but does not require DNA polymerase activity and complete Pol α recruitment.

DNA unwinding at origins results in the generation of ssDNA and the binding of RPA to ssDNA and is a prerequisite for DNA polymerase action. To examine if FA recruitment requires RPA recruitment, we compared FA chromatin binding in mock- and RPA-depleted cytosolic extracts. Depletion of RPA resulted in a considerable reduction of FA core complex and FANCD2 recruitment to chromatin (Figure 1C), and FA protein loading was partially rescued by addition of recombinant human RPA protein (Supplemental figure S3), establishing that FA chromatin recruitment requires RPA.

3.2 ATR plays a structural role in the chromatin recruitment of FANCD2

Our data indicate that the chromatin recruitment of FA proteins requires RPA association to ssDNA regions generated at replication forks. ssDNA-RPA intermediates are required for the binding and activation of ATRIP-ATR complexes [2]. ATR is activated following DNA replication stress [41,42] as well as during unperturbed DNA replication [43]. Next, we compared the kinetics of FA core complex proteins, FANCD2, RPA and ATR chromatin binding (Figure 1D and Supplemental figure S4). We observed that the recruitment of FA core complex proteins, FANCA and FANCL, preceded that of FANCD2. Notably, FA core complex binding to chromatin coincided with the recruitment of RPA, whereas, FANCD2 binding to chromatin was concomitant with ATR chromatin recruitment (Figure 1D). These data are consistent with RPA being required for the chromatin binding of FA proteins and further suggest that FANCD2 chromatin recruitment is mechanistically different and could involve ATR.

Next, we compared FA protein loading between mock- and ATR-depleted extracts. ATR-depletion led to a dramatic decrease in FANCD2 chromatin binding, while the chromatin

binding of FA core complex components was not significantly affected (Figure 1E). Consistent with similar observations following ATRIP depletion [26], this result shows that association of an intact FA core complex to chromatin is insufficient for the recruitment of FANCD2 in the absence of ATR-ATRIP. ATR can phosphorylate FA core complex proteins – FANCA, FANCE, FANCG, and FANCM – and FANCD2, FANCI, and FANCN/PALB2 [10,44–48] and ATR-dependent phosphorylations are important for FA-mediated MMC resistance [44]. Next, we determined whether FANCD2 required ATR-dependent phosphorylations. We treated extracts with caffeine, an inhibitor of ATM and ATR kinase activity and found that inhibition of ATR enzymatic activity did not reduce the chromatin binding of FANCD2 or FA core complex proteins (Figure 1E), indicating that ATR plays a structural but not enzymatic role in the chromatin recruitment of FANCD2.

3.3 The FA pathway is required for the repair and replication restart of MMC-induced lesions

Our data suggest that FA core complex and FANCD2 are recruited to DNA at RPA-coated ssDNA regions generated at replication forks during unperturbed replication (Figure 1), further suggesting that FA proteins associate specifically with replication forks and raising the possibility that the FA pathway plays a role in DNA transactions at replication forks. We have previously shown that inactivation of the FA pathway does not inhibit the progression of DNA replication. However, replication in *Xenopus* extracts depleted of FA proteins leads to the accumulation of DSBs, suggesting that FA proteins are required for the processing of DNA lesions generated during DNA replication and/or the restart of replication forks [26].

Cells derived from FA patients are hypersensitive to crosslinking agents such as MMC. Lesions generated by MMC are complex and include mono-adducts, intrastrand and interstrand crosslinks, which can all lead to the formation of nicks and subsequent DSBs during DNA replication. To examine the role of FA proteins in replication fork restart at drug-induced DNA lesions, we adapted a method from Trenz et al. (see scheme in Figure 2A). Cytosolic extract was incubated with sperm nuclei for 45 minutes to allow initiation but not completion of DNA replication. MMC was then added, causing DNA damage and subsequent replication fork collapse. The extract was supplemented with caffeine to inhibit ATM- and ATR-dependent checkpoints and roscovitine to prevent the firing of new origins. Replication fork restart was measured by radioactive nucleotide incorporation as DNA replication resumed at replication forks encountering damage. Depletion of FANCL from extracts inhibited the activation of the FA pathway and prevented the recruitment of FANCD2 to chromatin during DNA replication (Figure 2B), consistent with previous work [37]. Moreover, FA core complex proteins FANCA, FANCG, and FANCF also failed to associate with chromatin in FANCL-depleted extracts (Figure 2B), indicating that an intact FA core complex is required for the recruitment of these proteins to chromatin. Notably, depletion of FANCL resulted in the co-depletion of a significant fraction of FANCA and vice versa, whereas FANCD2 and FANCF protein levels were not reduced (Figure 2C), indicating that FANCL is in a complex with FANCA in *Xenopus* cytosolic extracts.

Cytosolic extracts depleted of FANCL showed a defect in the extent of nucleotide incorporation, i.e. replication restart, in the presence of MMC (Figure 2D–E, 3A). This defect was abrogated when roscovitine was omitted and new origins were allowed to fire (Figure 2D–E, Figure 3A). Since this decrease in nucleotide incorporation in the absence of FANCL is rescued by allowing the firing of new origins, this indicates that the defect is primarily due to the inability of replication forks to elongate past lesions caused by MMC. MMC generates a spectrum of DNA crosslinks and adducts. Therefore, the replication defect may be due to the inability of FANCL-depleted extract to repair lesions and/or the inability to stabilize or restart replication forks that encounter MMC-induced lesions. We attempted to rescue the FANCL-depleted extracts with recombinant FANCL protein. However, the addition of FANCL alone

failed to rescue FANCD2 chromatin recruitment (Supplemental figure S5). This is not surprising since FANCL depletion results in the co-depletion of FANCA, which is itself in a complex with other FA core complex proteins such as FANCG (Stone et al. 2007 and Figure 2C), and it remains to be determined which other components of the FA core complex are also co-depleted with FANCL.

3.4 The FA pathway stabilizes collapsed but not stalled replication forks

To determine whether the defect in resuming DNA replication following MMC treatment was due to a deficiency of FANCL-depleted extracts in crosslink repair or a defect in replication fork stability, we examined replication restart in FANCL-depleted extracts supplemented with APH or CPT, treatments that do not generate DNA crosslinks. APH results in fork stalling due to DNA polymerase inhibition, and the stalled replisome remains chromatin-bound [36]. In contrast, CPT triggers replication-dependent DSBs which lead to replisome disassembly and replication fork collapse [36]. Replication restart of stalled forks induced by APH was not affected by FANCL depletion (Figure 3A). In contrast, FANCL-depleted extracts were deficient in the restart of collapsed replication forks caused by CPT addition (Figure 3A). Similar to MMC treatment, this deficiency was rescued by the firing of new origins when roscovitine was omitted (Figure 3A). These data show that FA proteins do not play a critical role in the restart of stalled replication forks and that FA proteins play a role in restarting collapsed replication forks at DSBs in the absence of DNA crosslinks. FANCL-depleted extracts had a stronger defect in the restart of MMC-induced lesions than that of CPT-induced lesions, suggesting that while FA proteins are facilitating the restart of forks at both types of lesions they could play an additional role in the repair of MMC-induced lesions.

To confirm that our findings are specific to inhibition of the FA pathway, we repeated the replication restart experiments in FANCD2-depleted extracts. We obtained similar results with FANCD2-depletion showing a reduction in replication restart with MMC and CPT but not with APH (Figure 3B and Supplemental figure S6). As with FANCL-depletion, the FANCD2 replication restart defect was rescued when roscovitine was omitted (Figure 3B). Finally, we conducted the replication restart experiments in the presence of curcumin, a small molecule inhibitor of the FA pathway that inhibits CDDP-induced FANCD2 monoubiquitylation in mammalian cells [49], and in *Xenopus* extracts (Landais et al, unpublished data). When 50 μ M curcumin was added to extracts treated with the DNA damaging agent, we observed a reduction in replication restart with MMC and CPT but not APH treatment and this reduction was abrogated when roscovitine was omitted (Figure 3C). The similarity between the phenotypes observed following FANCL and FANCD2 depletion and curcumin treatment strongly argues that the defect seen in replication restart of collapsed forks is specific to the FA pathway.

4. Discussion

FA cells are hypersensitive to crosslinking agents and mildly sensitive to CPT [50]. These agents generate a variety of DNA lesions that can physically block replication fork progression in the case of interstrand crosslinks (ICLs) or be converted into DNA breaks in the case of monoadducts or intrastrand crosslinks. The heterogeneity of the lesions has made it difficult to understand the mechanism(s) of this sensitivity.

By comparing the response to compounds generating different DNA lesions, we show that the absence of FA proteins leads to a defect in replication restart at collapsed replication forks but does not affect the restart of replication at stalled replication forks (Figure 2D–E, Figure 3A–C). This is consistent with studies from several groups supporting the idea that the FA pathway plays a critical role in the stability of replication forks. First, the FA pathway is activated in response to a variety of primary lesions that result in fork stalling and DSB formation [51]. Second, FA proteins are recruited to chromatin in a replication-dependent and damage-

dependent manner [52]. Third, FA deficient cells and FA-depleted *Xenopus* extracts accumulate DNA damage during DNA replication [53]. Finally, the FA pathway interacts with BLM or with proteins associated with homologous recombination [47,54]. While collapsed replication fork restart requires FA proteins, it remains to be determined whether FA proteins participate in the prevention of fork collapse or the reassembly of collapsed forks.

This role of FA proteins at replication forks is supported by the observation that FA proteins assemble onto chromatin during unperturbed DNA replication in mammalian cells and *Xenopus* extract (Mi 2005, Sobeck 2006, this work). While the exact mechanism of FA recruitment to chromatin remains to be fully characterized, we show that FA chromatin recruitment requires pre-RC assembly, origin activation, and RPA binding to ssDNA at replication origins but not polymerase recruitment or activity (Figure 1A–C), suggesting that FA proteins are recruited to replication forks during unperturbed DNA replication. It is tempting to speculate that FA proteins are loaded directly via RPA. First, FA protein loading increases significantly in the presence of aphidicolin (Figure 1A), which increases ssDNA-RPA intermediates. Second, FANCD2 was shown to directly bind to RPA [55]. While RPA at replication forks is sufficient for the recruitment of FA core complex components, it is not sufficient for FANCD2 recruitment to chromatin, which additionally requires ATR (Figure 1E). Our data indicating FANCD2 recruitment requires ATR and previous data showing FANCD2 recruitment requires ATRIP [26] demonstrate that the ATR-ATRIP complex is involved in the chromatin recruitment of FANCD2. Notably, ATR kinase activity is not required for the chromatin recruitment of FANCD2 since caffeine addition does not abolish FANCD2 recruitment (Figure 2D). Together, these data indicate that the ATR-ATRIP complex may act as a scaffold for FANCD2 chromatin binding. The specific requirement of ATR-ATRIP for FANCD2 chromatin association places FANCD2 at replication forks as they stall or slow down during DNA replication.

In summary, our study places the FA core complex and FANCD2 at the replication fork where they are poised to respond to both endogenous and exogenous sources of DNA damage. Surveying the progression of DNA replication, the FA core complex and the FANCD2/I complex can help stabilize replication forks that encounter damage and recruit DNA repair proteins, including FA downstream components – FANCD1/BRCA2, FANCD2/BRIP1, FANCD3/PALB2, BRCA1, RAD51, and components of the BRAF complex – including BLM, RPA, BLAP75 and DNA topoisomerase III α [56]. The role of FA proteins as guardians might be particularly important in the case of ICLs because these lesions not only require replication fork stabilization but a combination of repair processes. Indeed, translesion synthesis, nucleotide excision repair, and homologous recombination are likely to be required for the repair of a single ICL. Therefore, maintaining replication fork stability and coordinated activation of these repair pathways is essential. In the absence of FA proteins, replication forks encountering ICLs fail to be stabilized and repaired, leading to the generation of DSBs that can participate in illegitimate recombination and chromosomal rearrangements. The defective FA pathway found in FA patients can result in replication fork collapse and genomic instability that is responsible for predisposition to leukemias and squamous cell carcinomas observed in this population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FA chromatin binding follows origin firing and precedes polymerase loading

(A) Chromatin binding of FA proteins. Cytosolic extracts were incubated with buffer, 50 ng/ μ L Geminin, 500 μ M Roscovitine, 20 ng/ μ L p21^{Cip1}, or 100 ng/ μ L APH, as indicated, for 10 min. Sperm nuclei were incubated with the extracts for 60 min and chromatin-bound proteins were isolated and analyzed by SDS-PAGE and Western blotting with xFANCD2, xFANCA, xFANCG, xFANCF, xFANCL, xMCM6 and RPA p70 antibodies. Bottom panel: The extent of replication in these extracts was assessed by ³²P- α dCTP incorporation into genomic DNA. (B) Sperm nuclei were incubated with mock- and Pol α -depleted extracts for 60 min and chromatin-bound proteins were isolated and analyzed. Bottom panel: Soluble Pol α was monitored by Western blot in mock- and Pol α -depleted extracts. (C) Sperm nuclei were incubated with mock- or RPA-depleted cytosolic extracts for 60 min and chromatin-bound proteins were isolated and analyzed. Bottom panel: Mock- and RPA-depleted extracts were probed with RPA p70 antibody. (D) Sperm nuclei were incubated with cytosolic extracts for the times indicated in the presence of 100 ng/ μ L APH and 5 mM caffeine, and chromatin-bound proteins were isolated and analyzed. (E) Sperm nuclei were incubated in mock- or ATR-depleted cytosolic extracts (lanes 1 and 2) or in cytosolic extract supplemented with buffer or 5 mM caffeine (lanes 3 and 4) for 60 min. Chromatin-bound proteins were isolated and analyzed. Bottom panel: Mock- and ATR-depleted extracts were probed with ATR antibody. Where shown, quantification of bands was performed with ImageJ and normalized to values in mock (B and E left panel) or buffer-treated (E right panel) values.

Figure 3. FANCL promotes the restart of collapsed but not stalled replication forks

(A) Sperm nuclei were incubated in mock- or FANCL-depleted cytosolic extracts. 300 μ M MMC (black bars), 40 μ M APH (white bars), or 56 μ M CPT (gray bars) were used in the replication restart, as indicated, with the addition of 5 mM caffeine, 32 P- α dCTP, and with or without 500 μ M Roscovitine (+ or -) for 90 min. Chromatin was isolated and analyzed by agarose gel and quantified as described above. Values from FANCL-depleted experiments were expressed as a percentage of the corresponding mock-depleted values. Each bar represents the average of at least 3 experiments – mean $\pm$ SEM. * Statistically significant with p-value = 0.0007 ** Statistically significant with p-value = 0.0015 (B) As in 3A, with mock- or FANCD2-depleted cytosolic extracts. * Statistically significant with p-value = 0.0378 **

Statistically significant with p-value = 0.0123 (C) As in 3A, with extracts supplemented with buffer or 50 μ M curcumin. * Statistically significant with p-value = 0.0011 ** Statistically significant with p-value = 0.0126