Fanconi Anemia Proteins Are Required To Prevent Accumulation of Replication-Associated DNA Double-Strand Breaks†

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Fanconi anemia (FA) is a multigene cancer susceptibility disorder characterized by cellular hypersensitivity to DNA interstrand cross-linking agents such as mitomycin C (MMC). FA proteins are suspected to function at the interface between cell cycle checkpoints, DNA repair, and DNA replication. Using replicating extracts from X. inopex eggs, we developed cell-free assays for FA proteins (xFA). Recruitment of the xFA core complex and xFANCd2 to chromatin is strictly dependent on replication initiation, even in the presence of MMC indicating specific recruitment to DNA lesions encountered by the replication machinery. The increase in xFA chromatin binding following treatment with MMC is part of a caffeine-sensitive S-phase checkpoint that is controlled by xATR. Recruitment of xFANCd2, but not xFANCA, is dependent on the xATR–xATR-interacting protein (xATRIP) complex. Immunodepletion of either xFANCA or xFANCd2 from egg extracts results in accumulation of chromosomal DNA breaks during replicative synthesis. Our results suggest coordinated chromatin recruitment of xFA proteins in response to replication-associated DNA lesions and indicate that xFA proteins function to prevent the accumulation of DNA breaks that arise during unperturbed replication.

The hereditary syndrome Fanconi anemia (FA) belongs to a group of caretaker gene diseases characterized by genomic instability and increased susceptibility to cancer. A hallmark of FA is cellular hypersensitivity to DNA interstrand cross-links (ICLs), suggesting a defect in the DNA damage response (18, 19, 48). Twelve FA complementation groups have been identified, and the majority of the corresponding genes have been cloned (FANCA, FANCB, FANCc, FANCd1, FANCd2, FANCe, FANCf, FANCg, FANCi, FANCl, and FANCm) (22, 23, 25, 43, 55, 60, 66, 83, 93). Although the function of the FA proteins is unknown, identification of BRCA2 (breast cancer-associated gene 2) as FANCd1 and of FANCj as the BRCA1-associated helicase gene Brip1/BACH1, suggests convergence of the FA/BRCA pathway with a larger network of proteins involved in DNA repair (7, 51–53, 95). This is underscored by the discovery that FANCm is related to archaeal Hef, a protein that binds and processes irregular arrangements of DNA in branched structures resembling replication forks (50, 71, 72).

According to current models, the FA pathway consists of an upstream nuclear core complex, including FANCA, FANCB, FANCc, FANCe, FANCf, FANCg, FANCi, and FANCm, required for the activation of its target, FANCd2 (24, 34–36, 59, 60, 66). FANCd2 is monoubiquitinated during S phase and in response to various types of DNA damage, including DNA ICLs, DNA double-strand breaks (DSBs), and replication fork stalling (36, 89). DNA damage-induced monoubiquitination of FANCd2 is also reduced in cells from Seckel syndrome patients with a defect in the ataxia telangiectasia- and RAD3-related gene, ATR (1), suggesting that the FA pathway is under at least partial control of the ATR kinase. Monoubiquitination of FANCd2 is required for its association with chromatin and localization into nuclear foci containing BRCA1, RAD51, MRE11-RAD50-NBS1, replication protein A (RPA), PCNA, and BRCA2 (37, 42, 44, 65, 68, 89). FANCd2 is also phosphorylated in response to different types of DNA damage (68, 78, 90), and it is suspected that FANCd2 phosphorylation is part of two separate pathways that are controlled by one of the two checkpoint kinases, ATR or ATM (ataxia telangiectasia mutated) (68, 78, 90).

Several findings support the idea that FA proteins function during the S phase of the cell cycle. ICLs, the major genotoxic challenge for FA cells, are processed through generation of
DNA DSB intermediates, which are generated specifically during S phase (5, 21, 28, 82) and repaired by the process of homologous recombination (HR) (83). The hypothesis that FA proteins are likely to function in ICL removal via HR repair during S phase is supported by evidence that FANC D1/BRC A2 is a central component of the HR repair mechanism (47, 79) and interacts with both RAD51 recombina se and FANC D2 (10, 20, 44, 98). Additional evidence supports a role for FANC A, FANCC, FANC G, and FANC D2 in HR (69, 70, 100, 101). New evidence from the DT40 model strongly implicates the FA downstream protein BRIP1/BACH1 helicase in DNA interstrand cross-link repair (7). Furthermore, the FA core complex proteins are part of BRAFT, a larger complex containing the BLM helicase, topoisomerase IIIα, and RPA (61), which supports the hypothesis of a function for FA proteins in replication-associated repair mechanisms.

To elucidate FA protein function(s) in the DNA damage response during replication, we established cell-free assays using Xenopus egg extracts that have been used to understand the role of other DNA repair proteins such as Mre11, Blm, ATR, and ATM during replication (12–17, 38, 49, 54, 56, 85, 102). We have cloned the Xenopus laevis homologs of several of the FA proteins (generally termed xFA) and show that these proteins are recruited to chromatin in response to DNA lesions encountered by the replication machinery. Our findings suggest that the xFA proteins are required to prevent accumulation of DNA breaks that arise not only in response to exogenous DNA damage, but also during unperturbed replication.

**MATERIALS AND METHODS**

Antibodies. Generation of anti-human FANC D2 rabbit polyclonal antiserum was described previously (41). Anti-xFANC A polyclonal rabbit antibodies were raised against an equal mixture of three keyhole limpet hemocyanin-bound xFANC D2 peptides (Global Peptides) corresponding to amino acids 1 to 18, 890 to 908, and 1425 to 1443. The antiserum was affinity purified against the three non-keyhole limpet hemocyanin-bound peptides immobilized on an AminoLink Plus column (Pierce) according to the manufacturer's instructions. Anti-xFANC A and anti-xFANC F polyclonal rabbit antibodies were raised against a chimeric protein containing an N-terminal glutathione S-transferase (GST) tag (GST: xFANC F 1-340) fused to the C-terminal region of xFANC A (amino acids 1205 to 1383) or full-length xFANC F (amino acids 1 to 340) and purified from bacteria as previously described (96). Affinity purification columns were made with chimeric His-xFANC A 1205-1383 and His-xFANC F 1-340 (His/pDEST 17), purified as described in the QIAexpressionist protocol (QIAGEN) and immobilized on an AminoLink Plus column. (For xFA antibody characterization, see Fig. S2 in the supplemental material.) The antibodies for neutralization of xATR and an AminoLink Plus column (Pierce) according to the manufacturer's instructions. Anti-xFANC D2 polyclonal rabbit antibodies were raised against the C-terminal region of xFA proteins, we used extracts prepared from Bacteroides pertussis-conjugated rabbit or mouse secondary antibody (Jackson Laboratories) was used. Protein bands were visualized using an ECL Plus system (Amersham). Antibodies against xTOP IIIα, ORC2, and RPA 70 were a kind gift from W. Dunphy.

**RESULTS**

Conservation of the FA pathway in Xenopus laevis. We identified XENOS homologs of several human FA genes (see Fig. S1 in the supplemental material). A downstream effector of the FA pathway, xFANC D2, has an overall homology of 70% compared with that of human FANC D2; both known modification target sites, i.e., K561 for monoubiquitination (K563 in FANC D2) and K565 for phosphorylation (S224 in Xenopus) are conserved (Fig. 1) (36, 90). Furthermore, interactions between FA core complex members and the DNA damage-induced appearance of FANC D2-2 (36) occur in Xenopus cells and egg extracts (see Fig. S3 in the supplemental material). We infer from these data that the critical components and the activation of the FA pathway are conserved in Xenopus.

xF A proteins associate with chromatin in a replication initiation-dependent manner. To analyze localization and chromatin binding behavior of xFA proteins, we used extracts prepared from Xenopus eggs. These extracts are arrested at the
end of meiosis (M-phase extract) and contain only negligible amounts of DNA. Upon chemical activation, the extracts are released into S phase in tight synchrony. When sperm chromatin is added to S-phase extracts, the DNA decondenses and a nuclear membrane forms (20 min), followed by one round of semiconservative chromosomal replication. Following addition of sperm chromatin, we reisolated nuclei and chromatin fractions at time points before, during, and after replication and assayed for the presence of xFANCA, xFANCF, and xFANCD2. These proteins accumulated in the newly formed

FIG. 1. Homology between Xenopus and human FANCD2. Full-length Xenopus FANCD2 (xFANCD2) was aligned with full-length human FANCD2 (hFANCD2) with MacVector 7.1.1 software. Amino acid identity is indicated by dark shading; similar amino acids are indicated by light shading. Two known critical residues, S222 and K561 (indicated by arrows), are conserved between human and Xenopus FANCD2.
nuclei and associated with chromatin during replication (Fig. 2A), which typically occurred between 30 to 60 min (Fig. 2B) (67). A slow-mobility form of xFANCD2 was predominant in whole nuclei (Fig. 2A, lanes 1 to 4), as well as in the nuclear chromatin fractions (Fig. 2A, lanes 5 to 7), at the indicated time points. Since the extracts were precisely synchronized throughout S phase and replication of added sperm chromatin started shortly after nuclear membrane formation, it is likely
that xFANCD2-S was converted to its modified form (FANCD2-L) immediately upon nuclear import and then recruited to replicating chromatin. Only the small isoform of xFANCD2 was detected in M-phase and S-phase extracts (Fig. 2C), where only negligible amounts of DNA were present. This observation implies that modification of xFANCD2 during S phase is dependent on the presence of chromatin, consistent with previous evidence that chromatin binding is associated with monoubiquitination of FANCD2 (65, 98). Chromatin-bound FA proteins are labeled with the suffix “-chr” in the text to provide a frame of reference because egg extracts and nuclear chromatin reisolated from egg extracts typically contain only a single isoform of xFANCD2. Egg extracts have a form comparable to FANCD2-S in cellular extracts, while chromatin replicated in egg extracts contained FANCD2-chr, comparable to FANCD2-L on chromatin isolated from cells. The mobility changes for FANCD2-chr and FANCA-chr compared with chromatin-bound counterparts in cell extracts (Fig. 2A, compare lanes 7 and 8) are likely due to large amounts of chromatin and protein in the egg extract samples.

Unlike other DNA replication proteins such as xRPA70 or xPCNA, the xFA proteins remained associated with chromatin once the bulk of replication was completed (Fig. 2D, lane 90). On the other hand, the chromatin binding pattern of the checkpoint signaling kinase xATM, which monitors origin firing during normal replication (85), closely resembled the temporal binding pattern of both xFANCA and xFANCD2. To further investigate the observed chromatin association pattern of xFA proteins, we used egg extracts prepared in the absence of cycloheximide. In classically prepared Xenopus egg extracts, cycloheximide blocks accumulation of cyclin B, which is required for the transition from S to M phase. Thus, in the presence of cycloheximide, extracts were halted in a G2-like state after DNA replication (Fig. 2D), whereas absence of cycloheximide allows the extracts to exit S phase (63, 67). Interestingly, in cycloheximide-free extracts, xFA proteins dissociated from chromatin once replication was over (Fig. 2E). Thus, the release of xFA proteins from chromatin was not triggered by the completion of replicative DNA synthesis alone but occurred only when extracts were allowed to exit S phase (compare Fig. 2D and E).

Accumulating evidence suggests that FA proteins function in the repair of specific DNA lesions that are encountered during transit through S phase (40, 70, 82, 89, 92). To determine if xFA proteins bound to chromatin in a replication-dependent manner, we blocked replication initiation by adding geminin to S-phase egg extracts. Geminin prevents assembly of prereplication complexes and thus inhibits replication initiation but does not affect chromosome decondensation or nuclear membrane formation (88). As shown in Fig. 3A, top left, accumulation of xFA core complex proteins and xFANCD2 on chromatin (compare lanes 1 and 2) was drastically inhibited in the presence of geminin, demonstrating that recruitment of FA proteins to chromatin occurs in a strictly replication initiation-dependent manner.

Recruitment of FA proteins to chromatin during disrupted replication. The hallmark of Fanconi cells is their hypersensitivity to DNA interstrand cross-linking agents such as MMC. As shown in Fig. 3A, top left, chromatin binding of xFANCA, xFANCF, and xFANCD2 increased in the presence of MMC (compare lanes 1 and 3), consistent with fractionation and immunofluorescence data for FA proteins in asynchronously dividing human cells (62, 80). Addition of caffeine, an inhibitor of the major checkpoint kinases, ATM and ATR (84), reversed the MMC-induced recruitment of xFA proteins to chromatin, suggesting that xFA-chromatin binding following MMC treatment was part of a checkpoint controlled by one of the caffeine-sensitive kinases ATM or ATR (Fig. 3A, top left, compare lanes 1 and 5). Importantly, even in the presence of MMC, extracts failed to support chromatin recruitment of xFA proteins when replication initiation was blocked with geminin (Fig. 3A, top left, compare lanes 3 and 4). Replication in these extracts was monitored in matched aliquots by measuring incorporation of radiolabeled nucleotides into the nascent DNA strand. The replication assay shown in Fig. 3A, top right, demonstrated that replication occurred (lane 1), was reduced by exposure to MMC (lane 3), and was rescued by addition of caffeine (lane 2). As expected, no incorporation was detected in the presence of geminin (lane 4). Taken together, these data suggest that chromatin binding of xFA proteins is specifically triggered by DNA lesions that are encountered during DNA replication.

The finding that caffeine restored wild-type-like incorporation of nucleotides during replication in MMC-treated extracts suggests that replication inhibition induced by MMC is due to activation of an S-phase checkpoint, a response that allows cells to repair damage before they proceed to the next cell division, thus preventing the transmission of mutations (4, 9, 73, 75). To further explore the effect of MMC on replication, we determined whether the reduction of replication products occurred in an MMC dose-dependent manner. As shown in Fig. 3A, bottom, DNA replication was significantly blocked at high concentrations of MMC (150 μM) (lane 3) and could not be rescued by caffeine (lane 6). In contrast, at lower MMC concentrations, replication was less inhibited (50 μM, lane 4; 5 μM, lane 5) and could be rescued in the presence of caffeine (lane 7).

To determine if the MMC-induced replication block was under control of the major replication checkpoint kinase, ATR, we added a neutralizing anti-xATR antibody (Fig. 3A, bottom, lanes 8 and 9) to egg extracts to specifically block the xATR kinase function, thereby preventing phosphorylation of the Chk1 protein that is required for activation of the S-phase checkpoint (57). Comparable to the effect observed in the presence of caffeine, inhibition of xATR did not rescue the very strong replication block in high MMC concentrations (lane 7); however, at lower MMC concentrations, reduction of replication products was restored back to wild-type levels when xATR was blocked. Thus, at lower doses the MMC-induced reduction in incorporation of nucleotides during replication is due to activation of an S-phase checkpoint that depends on xATR and results in an increase in xFA-chromatin binding.

To determine the influence of fork stalling during DNA replication on recruitment of FA proteins to chromatin, we added aphidicolin to replicating extracts. Aphidicolin treatment blocks replicative polymerases, while helicases continue to unwind the DNA helix, thereby generating long single-stranded DNA (ssDNA) stretches (74, 85, 97). As shown in Fig. 3B, addition of aphidicolin to replicating extracts resulted in increased chromatin association of xFA proteins, as well as
the ssDNA binding protein xRPA. Interestingly, whereas chromatin binding of xRPA increased significantly when aphidicolin was added before or during ongoing replication, recruitment of xFA proteins to chromatin increased only when aphidicolin was added during ongoing replication at 45 min (midreplication) and 60 min (late replication) (compare lane 1 with lanes 4 and 5). In contrast, when aphidicolin was added to extracts before the onset of replication (0 min or 30 min; compare lane 1 with lanes 2 and 3) or after replication was finished (90 min, compare lane 1 with lane 6), chromatin binding of xFA proteins did not increase. It is also important to note that under our experimental conditions, aphidicolin does not result in detectable DNA DSBs (54). These results suggest that the xFA proteins are recruited when the moving replication fork encounters sites of DNA damage.

The xATRIP/xATR complex controls chromatin binding of xFANC D2 independently of xFANCA. ATR plays a critical role in coordinating the response to DNA damage. Its activation is usually linked to ongoing DNA replication (39, 57, 86, 91). The current model suggests that the ATR complex consisting of the ATR kinase and its binding partner, ATRIP, control S-phase progression in response to DNA damage and replication fork stalling. ATR and ATRIP are mutually dependent partners in the cellular S-phase checkpoint and DNA damage response (2, 3, 6, 11, 30, 45, 46, 94, 103). Generation of RPA-coated ssDNA is the critical signal that triggers recruitment of the tightly associated ATRIP-ATR complex. Our results demonstrated that MMC-induced increase in xFA-chromatin binding was reversed by the ATR-ATM inhibitor caffeine (Fig. 3A, top left, and data not shown). Thus, we asked whether depletion of the xATRIP subunit of the xATR kinase affected recruitment of the xFA proteins to chromatin. As shown in Fig. 3C, recruitment of xFANC D2 to chromatin was negligible in replicating aphidicolin-depleted extracts of xFANCA (Fig. 4D). Our results also showed that xATRIP depletion from replicating xFANCA-depleted extracts (Fig. 3C) did not affect chromatin binding of xFANCA (Fig. 3D). Thus, the xATRIP-xATR complex regulates recruitment of xFANC D2 to chromatin independently of the regulation that xFANCA and other core complex proteins exert in unperturbed replication, as well as in response to replication fork stalling.

Chromatin association of xFANC D2 depends on xFANCA. A functional FA core complex is believed to act upstream of FANC D2 by mediating its monoubiquitination (36, 89). The core complex protein xFANCA was quantitatively depleted from egg extracts to determine its influence on recruitment of xFANC D2 to chromatin (Fig. 4A). Sperm chromatin was added to the xFANCA-depleted extracts, followed by reisolation of replicated chromatin after 90 min. As shown in Fig. 4B, replication-associated binding of xFANC D2 to chromatin was abrogated in the absence of xFANCA. In contrast, immunodepletion of xFANC D2 from egg extracts (Fig. 4C) did not affect chromatin binding of xFANCA (Fig. 4D). Our results indicate that the xFANCA core complex is required for recruitment or stabilization of xFANC D2 at chromatin in response to DNA damage encountered during the replication process.

xFANCA and xFANC D2 are required to prevent accumulation of DNA DSBs during replication. Accumulating evidence points to a role for the FA pathway in repair of DNA DSBs (26, 27, 69, 82, 100, 101). Our results implied that xFA proteins are specifically recruited to chromatin during unperturbed replication and when exogenous DNA damage is encountered during replication. Thus, we first asked whether the absence of
xFANCA (as a representative core complex member) or xFANCD2 (as an effector of the FA core complex) affects the overall kinetics of DNA replication. Following immunodepletion of xFANCA or xFANCD2 from egg extracts, we monitored timing and levels of nucleotide incorporation during chromosomal replication. No gross difference in replication kinetics was observed between mock- and xFANCA-depleted extracts (Fig. 5A and C) or between mock- and xFANCD2-depleted extracts (Fig. 5B and D). Thus, neither xFANCA nor xFANCD2 was required for initiation or elongation in the replication process itself. We then used a TUNEL assay to investigate whether the xFA proteins might be necessary to prevent the accumulation of DNA DSBs that are known to arise during the course of normal replication (15, 54). Chromosomal DNA was added to preimmune serum-, xFANCA-, or xFANCD2-depleted extracts, followed by one round of replication and isolation of replication products. Putative DNA DSBs in the postreplicative chromatin were labeled using terminal transferase and radioactive deoxyribonucleotides. We detected strong signals for incorporation of [32P]dGTP in replication products from extracts depleted of either xFANCA (Fig. 5E) or xFANCD2 (Fig. 5F) compared to mock-depleted extracts. Thus, absence of xFANCA or xFANCD2 from egg extracts results in accumulation of DNA breaks during unperturbed DNA replication.

**DISCUSSION**

We established cell-free assays for functional evaluation of the FA network proteins, which play critical but largely unknown roles in mechanisms that preserve genomic stability. Analysis of the *Xenopus* homologs of FANCD2, FANCA, FANCF, and FANCL revealed regions of strong homology compared to their human counterparts, suggesting that they might contain some as-yet-unrecognized domains. Importantly, the known FA pathway characteristics are conserved in *Xenopus* including (i) conservation of the functional modification target sites of FANCD2, K561 (monoubiquitination), and S222 (phosphorylation); (ii) interactions between core complex members xFANCA and xFANCF (24, 34, 59, 61); and (iii) induction of an xFANCD2-L isoform following DNA damage. Thus, *Xenopus* egg extracts provide a cell-free system for functional analysis of the FA proteins during DNA replication and repair.

The downstream target of the FA pathway, FANCD2, is monoubiquitinated during S phase (FANCD2-L form), and...
this FANCD2-L isoform associates with S-phase chromatin (65, 89). Taking advantage of the fact that proteins can be analyzed in the highly synchronized *Xenopus* egg extracts in M and S phases even in the absence of DNA, we found that entry of the egg extract into S phase alone does not induce the xFANCD2-L form. In contrast, xFANCD2 is very quickly modified upon its import into nuclei that form once sperm chromatin is added to S-phase extracts, suggesting that formation of FANCD2-L is a DNA-dependent process. Following import of xFANCD2, xFANCA, and xFANCF into sperm nuclei, the proteins are recruited to chromatin during unperturbed replication. Chromatin binding of all three xFA proteins increases further in the presence of MMC, consistent with previous work showing that FANCD2-L and FA core complex proteins are chromatin bound during S phase and in response to DNA damage (36, 62, 64, 80, 89). Interestingly, when untreated egg extracts were treated with beads coupled to xFANCA-preimmune serum (lane 1) or beads coupled to anti-xFANCA serum (lane 2). (F) Likewise, extracts were treated with either beads coupled to xFANCD2 preimmune serum (lane 1) or beads coupled to anti-xFANCD2 serum (lane 2). Sperm chromatin was added to treated extracts; after 120 min, TUNEL assays were performed. Samples were subjected to agarose gel electrophoresis and phosphorimaging.
extracts are prohibited from exiting S phase following DNA replication (i.e., they halt in a G2-like state), the xFA proteins stay bound to chromatin, unlike other replication-associated proteins such as xRPA70 or xPCNA. This observation supports a model in which the FA proteins participate in specific DNA repair events that occur during late replication and after the bulk of replication is completed (28, 70, 92, 100). The fact that xFA proteins dissociate from chromatin following replication when extracts are capable of exiting S phase suggests that the release from chromatin requires signaling associated with the G2-M transition of the cell cycle.

Previous reports show S-phase- and DNA damage-dependent chromatin association of several FA proteins (36, 62, 64, 80, 89). However, whether chromatin assembly of the FA proteins is directly associated with the replication process has not been evaluated. We found that in extracts containing the replication initiation inhibitor geminin, xFA proteins did not associate with chromatin even in the presence of MMC. Thus, chromatin association of the xFA proteins is strictly replication dependent. A remaining question is why DNA replication is required for recruitment of FA proteins. Our finding implies that the xFA proteins are selectively recruited to DNA lesions that either arise during normal replication or are recognized in a replication context following exogenous DNA damage.

In agreement with this idea, treatment of extracts with an inhibitor of replicative DNA polymerases, aphidicolin, also resulted in increased recruitment of xFA proteins to chromatin. Aphidicolin treatment leads to the generation of ssDNA regions due to uncoupling of the replicative helicase from the DNA polymerase. These ssDNA regions are generated regardless of whether aphidicolin is added to the egg extract before or during ongoing replication (as monitored by comparing chromatin-bound levels of the ssDNA binding protein xRPA). Interestingly, increased binding of xFA proteins to chromatin is only triggered when aphidicolin is added during the ongoing replication process, suggesting that the generation of ssDNA alone might not be sufficient for recruitment of the xFA proteins. In this regard, Cimprich and coworkers recently showed that functional uncoupling of MCM helicase and DNA polymerase occurs in response to several forms of DNA damage and that the subsequent accumulation of ssDNA is required but not sufficient to trigger the ATR-controlled checkpoint response (8). Further studies will be required to determine if the stalled replication fork itself or subsequently generated DNA intermediates and possibly additional factors trigger recruitment of the xFA proteins.

Analysis of MMC-treated extracts revealed that the MMC-induced increase in xFA-chromatin binding coincided with a reduction in replicative products. We demonstrated that this is due to activation of an intra-S-phase checkpoint that is controlled by the caffeine-sensitive checkpoint kinase ATR. In extracts containing MMC at concentrations between 5 and 50 µM, caffeine restored replication products back to wild-type levels and blocked the MMC-induced increase of xFA-chromatin association. Similarly, neutralization of the xATR kinase domain with a specific antibody inhibited checkpoint activation and subsequent reduction of replication products. In contrast, at higher MMC concentrations (150 µM), the replication block could not be overcome by caffeine or xATR neutralization. It is possible that at such high MMC concentrations the DNA lesions typically induced by this agent (intra- and interstrand cross-links) reach a level at which each replicon contains a cross-link. This could cause quantitative inhibition of replication by physical impairment of the DNA polymerase-helicase to get through the cross-links, despite the caffeine-xATR-induced block of checkpoint activation. We conclude from these findings that the additional recruitment of xFA proteins to chromatin in response to exogenous DNA damage occurs as part of an intra-S-phase checkpoint controlled by ATR. In agreement with this, Andreassen et al. recently showed that the MMC-induced increase in FANCD2 monoubiquitination, a step required for its targeting to chromatin, is dependent on ATR (1).

Moreover, we could demonstrate that chromatin recruitment of xFANCDe2 is abrogated in extracts that either contained the ATR-neutralizing antibody or were depleted of xATRIP, a functional subunit of the xATR kinase. Strikingly, depletion of xATRIP had no effect on chromatin binding of xFANCA, raising the question of whether xATRIP was positioned between xFANCA (and perhaps the entire core complex) and the downstream target, xFANCDe2, or whether xATRIP controlled chromatin recruitment of xFANCDe2 independently of xFANCA. Testing chromatin binding of xATRIP in xFANCA-depleted extracts demonstrated that it is recruited to chromatin independently of xFANCA. In addition, xATRIP-chromatin binding was unaffected in extracts depleted of xFANCDe2. These data support a model where recruitment of xFANCDe2 to DNA lesions encountered by the replication machinery is under control of two entities: the xFA core complex and the xATR/xATRIP complex. The fact that absence of xATRIP blocked xFANCDe2-chromatin binding even in the absence of exogenous DNA damage suggests that the xATR-xATRIP complex regulates FANCDe2 not only during checkpoint activation but also as part of the DNA repair response that deals with basal levels of DNA damage during normal replication.

The current FA model suggests that modification and chromatin recruitment of FANCDe2 are dependent on a functional FA core complex (1, 24, 36, 59, 62, 98). In support of this model, we found that the replication-associated chromatin binding of xFANCDe2 is completely abrogated in egg extracts depleted of xFANCA. In contrast, depletion of xFANCDe2 from S-phase extracts did not affect association of xFANCA with replicating chromatin. We conclude that the xFA proteins associate with chromatin in a coordinated manner. One possibility is that the xFA core complex binds to chromatin to recruit and activate xFANCDe2 at specific types of DNA lesions encountered by the replication machinery.

Since our results demonstrated that accumulation of xFA proteins on chromatin is dependent on replication origin unwinding, we also investigated a potential requirement of xFA proteins in the replication process itself. As predicted by the absence of gross defects in genomic duplication in cells from FA patients, the rate and timing of replicative DNA synthesis were not affected in the absence of either xFANCA or xFANCDe2. However, we found strikingly increased levels of DNA breaks in replication products from xFANCA- or xFANCDe2-depleted extracts.

These results provide first proof that even in the absence of exogenous DNA damage, the xFA proteins are required to


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