The Fanconi Anemia Protein FANCM Is Controlled by FANCD2 and the ATR/ATM Pathways*

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Genomic stability requires a functional Fanconi anemia (FA) pathway composed of an upstream “core complex” (FA proteins A/B/C/E/F/G/L/M) that mediates monoubiquitination of the downstream targets FANCD2 and FANCI. Unique among FA core complex members, FANCM has processing activities toward replication-associated DNA structures, suggesting a vital role for FANCM during replication. Using Xenopus egg extracts, we analyzed the functions of FANCM in replication and the DNA damage response. xFANCM binds chromatin in a replication-dependent manner and is phosphorylated in response to DNA damage structures. Chromatin binding and DNA damage-induced phosphorylation of xFANCM are mediated in part by the downstream FA pathway protein FANCD2. Moreover, phosphorylation and chromatin recruitment of FANCM is regulated by two major players in the DNA damage response: the cell cycle checkpoint kinases ATR and ATM. Our results indicate that functions of FANCM are controlled by FA- and non-FA pathways in the DNA damage response.

Fanconi anemia is a genetic disease characterized by genomic instability and cancer predisposition. Cells from FA patients show hypersensitivity to DNA interstrand cross-links and have highly elevated chromosomal breakage rates, indicating a role for FA proteins in the cellular DNA damage response. The FA pathway consists of an upstream FA core complex containing at least eight proteins (FANCA, -B, -C, -E, -F, -G, -L, and -M) that is required for the DNA damage-induced monoubiquitination of two downstream proteins, FANCD2 and FANCI. Although the molecular function of the FA pathway is unknown, the identification of additional FA genes FANCD1 (BRCA2), FANCN (PALB2), and the DNA helicase FANCJ (BRIP1) as breast cancer (BRCA) susceptibility genes suggests convergence of the FA/BRCA pathway with a larger network of pathway components (6), suggesting that FANCM is controlled by other, as yet unknown upstream components of the DNA damage response. Here, we used cell-free Xenopus egg extracts to investigate the role of FANCM during replication and in the DNA damage response. We show that Xenopus FANCM (xFANCM) binds chromatin in a replication-dependent manner and is phosphorylated during unperturbed replication as well as in response to various DNA damage structures. Both chromatin recruitment and phosphorylation of xFANCM are partially controlled by xFANCD2, suggesting feedback signaling from xFANCD2 to the upstream xFA core complex via regulation of xFANCM. In addition, chromatin recruitment during unperturbed replication and activation of xFANCM in response to DNA damage are controlled by the xATR and xATM cell cycle kinases.

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1 The abbreviations used are: FA, Fanconi anemia; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.
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**EXPERIMENTAL PROCEDURES**

**Isolation of the Xenopus laevis FANCM Homolog**—Tblastn-based searches were performed at NCBI and JGI as described previously (14). Several short homologous protein fragments were identified using a tblast search with full-length human FANCM in both NCBI and a Xenopus tropicalis data base (JGI). Primers designed from these sequences were used in reverse transcription-PCR to amplify a large fragment of FANCM spanning approximately two-thirds of the FANCM coding region, including the stop codon. The amplified fragment was sequences and primers were designed for 5′-rapid amplification of cDNA ends PCRs. Full-length X. laevis FANCM (xFANCM) was amplified using primers 5′-ATGAGTGGGAACAGAA-AACACTTTTCA-3′ and 5′-TTAAGAGACTCTTGTT-CGCCTAGTGGGTT-3′.

**Plasmid Construction and Protein Purification**—Cloning and sequencing of xFANCD2 was described previously (14). To construct a baculoviral vector expressing full-length glutathione S-transferase-tagged xFANCD2, the xFANCD2 coding region was subcloned from the pDONR201 vector into pDEST20 of the Gateway Cloning System (Invitrogen) according to the manufacturer’s instructions. This vector encodes for a glutathione S-transferase tag, which was used for subsequent purification using glutathione-Sepharose 4B beads (GE Healthcare). Glutathione S-transferase-tagged xFANCD2 protein was expressed in S9F insect cells using the Bac-to-Bac baculovirus expression system (Invitrogen).

**Antibodies**—Generation of antibodies against xFANCA and xFANCD2 has been described previously (14). Rabbit polyclonal antibodies against xFANCM were generated using a mix of N- and C-terminal peptide regions of xFANCM. The resulting antiserum was used for Western blots at 1:1000 dilutions and for immunodepletions as described later. Antibodies for depletion of xATRIP, neutralization of xATR, and Western blot detection of xRad1 and phosphorylated xChk1 (xChk1 phosphorylated at serine 344) were a kind gift from W. Dunphy. Antibodies to xCdc45 were a kind gift from J. Walter, and antibodies to xRPA70 were a kind gift from W. Dunphy. Commercial antibodies were used against proliferating cell nuclear antigen (sc-56; Santa Cruz), histone H3 (1791; abcam), γ-H2AX (A300-081 A; Bethesda Laboratories), and hChk1 (Cell Signaling Technology).

**Preparation of Xenopus Egg Extracts**—Extracts were prepared from Xenopus eggs according to the method of Murray (15). Tautomycin (3 μM) and recombinant Xenopus geminin (5 μg/μl) were added as indicated. Plasmid DNA was used at 150 ng/μl, as described before (16).

**Preparation of Nuclei and Chromatin Fractions**—The nuclei and chromatin fractions were essentially prepared as described before (14). Briefly, identical aliquots (20 to 50 μl) of egg extracts containing 2000 pronuclei (sperm heads)/μl were each diluted in nuclear isolation buffer (40 mM HEPES, 100 mM KCl, 20 mM MgCl2) or chromatin isolation buffer (40 mM HEPES, 100 mM KCl, 20 mM MgCl2, 0.2% Triton X-100) and purified through a 30% (w/v) sucrose cushion. Nuclear and chromatin pellets were analyzed by SDS-PAGE and immunoblotting.

**DNA Replication Assay**—Replication of sperm chromatin in S phase extracts was monitored as described before (14).

**Preparation of DNA Substrates**—For plasmid DNA, circular plasmid DNA (pBSKS) was prepared from Escherichia coli cultures using a Qiagen Plasmid Maxi kit (Qiagen). For DNA damage structures, the preparation of oligonucleotide-derived DNA structures was done as described previously (16). For bead DNA, streptavidin-conjugated magnetic beads (Dynal, Inc.) coupled to biotinylated DNA structures were used for incubation in egg extracts and analysis of DNA structure-bound proteins as described before (16).

**Immunodepletion**—Immunodepletions were performed essentially as described previously (14). In brief, 200 μl of protein A-Sepharose Fast Flow beads (50% slurry; GE Healthcare) were rotated overnight at 4 °C with 500 μl of phosphate-buffered saline and 100 μl of anti-xFANCA, -xFANCD2, -xFANCM, -xATRIP, or -xATR affinity-purified or raw sera or preimmune sera. The beads were pelleted and washed three times with phosphate-buffered saline and twice with XB buffer. For depletion, 100 μl of extract was added to the beads and rotated for three rounds at 4 °C for 45 min each.

**Immunoblotting**—Protein samples were separated on gradient gels (Invitrogen) and transferred to Immobilon P membranes (Millipore). After being blocked with 5% milk for 1 h, the membranes were incubated with the following primary antibodies: xFANCA (1:1000), xFANCD2 (1:2500), xFANCM (1:1000), xCdc45 (1:2000), xATRIP (1:1000), xATR (1:5000), xChk1-P-Ser344 (1:800), hChk1-P-Ser345 (1:1000), xRAD1 (1:2000), xRPA70 (1:1500), histone H3 (1:2500), or gamma-H2AX (1:3000). Horseradish peroxidase-conjugated rabbit secondary antibody (Jackson Laboratories) was used (1:10,000). The protein bands were visualized using an ECL Plus detection system (GE Healthcare).

**Phosphatase Assay**—2 μl of extract were incubated in a total volume of 20 μl containing 0.4 μl of shrimp alkaline phosphatase (Fermentas) and 1× shrimp alkaline phosphatase buffer. Following incubation for 30 min, the reactions were stopped with 7 μl of 4× NuPAGE sample buffer (Invitrogen), and 10 μl/sample were analyzed by SDS-PAGE and immunoblotting.

**RESULTS**

**Cloning of Xenopus FANCM**—For cloning of the X. laevis FANCM gene (xFANCM), we searched for Xenopus DNA sequences homologous to the human FANCM gene via NCBI BLAST (tblastn) search. Partial gene sequences were identified in sequence data bases for X. laevis and Xenopus tropicalis, and the full-length sequence for xFANCM was obtained by a combination of reverse transcription- and rapid amplification of cDNA ends-PCRs (see “Experimental Procedures”). The predicted amino acid sequence of xFANCM is 2166 amino acids long and has an overall homology of 58% compared with its human counterpart. Two regions are highly conserved between human and Xenopus FANCM: the N-terminal DEAH helicase domain (86% homology; supplemental Fig. S1) and the C-terminal ERCC4/XPF-like endonuclease domain (79% homology; supplemental Fig. S2). Similar to the FANCM endonuclease domain in other species, the xFANCM endonuclease domain is degenerate at a conserved residue critical for nuclease function.
sperm chromatin in egg extracts that allow for nuclear assembly and DNA replication under natural cell cycle control. xFANCM associated with chromatin during chromosomal replication in egg extracts (Fig. 1A), and levels of chromatin-bound xFANCM increased in response to DNA-damaging agents mitomycin C and aphidicolin.4 Importantly, chromatin binding of xFANCM was abrogated in the presence of the replication initiation inhibitor geminin (Fig. 1A), indicating that xFANCM is recruited to chromatin in a strictly replication initiation-dependent manner, similar to other xFA proteins (FANCA, -F, -D2) (14). To test whether xFANCM chromatin binding is dependent on the FA core complex during replication, we depleted xFANCA from egg extracts and tested for chromatin binding of xFANCM. As shown in Fig. 1B (upper panel), depletion of xFANCA partially co-depleted xFANCM (residual xFANCM protein levels, 30–40%), indicating that FANCM is only partially associated with the FA core complex in DNA-free egg extracts. Interestingly, the residual xFANCM was able to bind chromatin in xFANCA-depleted extracts (Fig. 1C), indicating that xFANCM can bind chromatin independently of the FA core complex.

Our finding is in agreement with a recent study by Kim et al. (12), showing that human FANCM is required for chromatin recruitment of the FA core complex. Together, these data indicate that FANCM acts upstream and independently of the other FA core complex proteins. We also tested whether chromatin-bound xFANCM is phosphorylated during replication. To trap phosphorylated protein isoforms (17), we added tautomycin, a phosphatase inhibitor, to replicating extracts. Under these conditions, hyperphosphorylated forms of FANCM were detectable in the nuclear fractions, and the more heavily phosphorylated FANCM isoforms were found associated with replicating chromatin (Fig. 1D). In summary, our data support the idea that

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4 A. Sobeck, S. Stone, I. Landais, B. de Graaf, and M. E. Hoatlin, unpublished data.
FANCM binds chromatin in a replication-dependent, but FA core complex-independent manner and is hyperphosphorylated during the unperturbed chromosomal replication process.

**Induction of xFANCM Phosphorylation Can Be Uncoupled from DNA Replication**—The central FA pathway protein, FANCD2, is monoubiquitinated (FANCD2-Ub) during S phase and in response to DNA damage in human cells (18–20). Using Xenopus egg extracts, we recently showed that although xFANCD2-Ub is recruited to chromatin in a replication-dependent manner, we can bypass the need for ongoing replication to trigger xFANCD2 monoubiquitination by adding dsDNA substrates, including circular double-stranded DNA (plasmid DNA), to egg extracts (16). We tested whether xFANCM is also modified in response to plasmid DNA. As shown in Fig. 2A, a slower migrating form of both xFANCM and xFANCD2 was detected in extracts supplemented with plasmid DNA. Importantly, the addition of geminin to plasmid-containing extracts did not affect the xFANCM mobility shift, demonstrating that the plasmid-induced xFANCM modification is not due to plasmid replication in the extracts. Of note, the xFANCM mobility shift occurred at a later time point than the monoubiquitination of xFANCD2 (Fig. 2A, compare lanes 2 and 4) (see results below). To confirm that the slower mobility of plasmid-induced xFANCM isoforms was due to phosphorylation, we treated DNA plasmid-containing egg extracts with tautomycin, suggesting that unlike xFANCD2-Ub, xFANCM is phosphorylated in response to linear and branched ssDNA and dsDNA structures. DNA structures ssDNA70 and dsDNA70 were coupled to beads and incubated in egg extracts (extr.) for 30 min. Extracts were either untreated (lanes 1, 2, 4, 6, and 8) or supplemented with tautomycin (lanes 3, 5, 7, and 9). Following incubation, bead DNA substrates were separated from the extracts and analyzed by SDS-PAGE and immunoblot. C, xFANCM is phosphorylated in response to linear and branched ssDNA and dsDNA structures. DNA structures ssDNA70 and dsDNA70 were coupled to beads and incubated in egg extracts (extr.) for 30 min. Extracts were either untreated (lanes 1, 2, 4, 6, and 8) or supplemented with tautomycin (lanes 3, 5, 7, and 9). Following incubation, bead DNA substrates were separated from the extracts and analyzed by SDS-PAGE and immunoblot. 1 μl of DNA-free extract (lane 1) was used as a negative control.

> FIGURE 2. xFANCM is phosphorylated in response to ssDNA and dsDNA substrates. A, hyperphosphorylation of xFANCM (xFANCM-PPP) is induced in the presence of plasmid DNA, similarly to monoubiquitination of xFANCD2 (xFANCD2-Ub) (16). Egg extracts were incubated with circular plasmid DNA for the indicated time points. Extracts were either untreated (lanes 2, 4, and 6) or treated with geminin (lanes 3, 5, and 7). DNA-free extracts served as a negative control (lane 1). Following incubation, 1 μl of extract was analyzed for xFANCM or xFANCD2 by immunoblotting. B, the DNA plasmid-induced mobility shift of xFANCM is due to phosphorylation. Extracts were incubated for 15 min in the absence (lanes 1 and 2) or presence (lanes 3–6) of plasmid DNA and supplemented with either H2O (lanes 1–4) or tautomycin (lanes 5 and 6). Following incubation, extracts were either untreated (lanes 1, 3, and 5) or treated with shrimp alkaline phosphatase (SAP, lanes 2, 4, and 6). Subsequently, 1 μl of extract was analyzed for xFANCM by SDS-PAGE and immunoblot. C, xFANCM is phosphorylated in response to linear and branched ssDNA and dsDNA structures. DNA structures ssDNA70 and dsDNA70 were coupled to beads and incubated in egg extracts (extr.) for 30 min. Extracts were either untreated (lanes 1, 2, 4, 6, and 8) or supplemented with tautomycin (lanes 3, 5, 7, and 9). Following incubation, bead DNA substrates were separated from the extracts and analyzed by SDS-PAGE and immunoblot. 1 μl of DNA-free extract (lane 1) was used as a negative control.
in response to plasmid DNA (16) (see also Fig. 5A). However, examination of a time course following the addition of plasmid DNA to egg extracts (Fig. 2A) revealed that monoubiquitination of xFANCD2 occurred prior to phosphorylation of xFANCM. Based on this result, we hypothesized that xFANCD2 might also act upstream of FANCM. To test this, we depleted egg extracts of xFANCD2 and tested whether the plasmid DNA-induced xFANCM-PPP formation was affected in the absence of xFANCD2. Interestingly, xFANCM-PPP formation was partially impaired in xFANCD2-depleted extracts compared with mock-depleted extracts (Fig. 3A). To determine whether the effect of xFANCD2 depletion on xFANCM-PPP is directly due to the absence of xFANCD2 and not another protein that might be co-depleted with xFANCD2, we added recombinant wild-type xFANCD2 to extracts depleted of endogenous xFANCD2. As shown in Fig. 3B, recombinant xFANCD2 completely restored the plasmid-triggered FANCM-PPP formation in xFANCD2-depleted extracts, indicating that xFANCM phosphorylation is indeed partly controlled by xFANCD2. The facts that xFANCD2 has partial control over xFANCM phosphorylation and that xFANCM is phosphorylated when bound to replicating chromatin (Fig. 1D) suggested that xFANCD2 might be involved in stabilizing xFANCM on chromatin. To test this idea, we analyzed chromatin binding of xFANCM in xFANCD2-depleted extracts. As shown in Fig. 3C, levels of chromatin-bound xFANCM were reduced in xFANCD2-depleted extracts compared with mock-depleted extracts during replication and on post-replication chromatin. Our results suggest that FANCD2, the proposed downstream target of the FA core complex, is able to signal to the upstream core complex by partially regulating phosphorylation and chromatin association of FANCM.

**FANCM Phosphorylation and Chromatin Recruitment Are under Partial Control of ATR and ATM**—The two major cell cycle checkpoint kinases, ATR and ATM, are known to regulate many steps in the DNA damage response by phosphorylating downstream proteins involved in DNA repair and cell cycle checkpoint activation. Both ATR and ATM have been linked to the FA pathway; FANCD2 is phosphorylated by ATR and ATM at different sites in response to replication stress and ionizing irradiation, respectively (2–4, 7). Moreover, phosphorylation by ATR, but not ATM, is required for efficient DNA damage-induced monoubiquitination of FANCD2 in human cells (3, 7). We wanted to test whether the observed DNA-stimulated xFANCM-PPP formation in egg extracts is dependent on xATR and/or xATM. As shown in Fig. 4A, xFANCM phosphorylation was reduced in extracts depleted of xATR. To test whether xFANCM-PPP formation was mediated by the xATR kinase activity, we used two strategies: 1) we depleted extracts of xATRIP (ATR-interacting protein), binding partner and “functional subunit” of xATR, required for xATR kinase function (21–25) and 2) we added a xATR neutralizing antibody that inhibits xATR kinase function (26, 27). As a control for efficient suppression of ATR kinase activity, we monitored DNA plasmid-induced xChk1 phosphorylation in the extracts (Fig. 4B, bottom panel). xFANCM-PPP formation was partially reduced in xATRIP-depleted or xATR-neutralized extracts (Fig. 4B, lanes 4 and 5). In addition, in extracts that were depleted of xATRIP and also contained the neutralizing xATR antibody, we observed an additive inhibitory effect on FANCM-PPP formation (Fig. 4B, lane 6). In agreement with our previous observations (16), formation of xFANCD2-Ub was not significantly affected in xATR-depleted or xATR kinase-deficient extracts (Fig. 4, A and B) (16), suggesting that xATR is not required to promote xFANCD2-Ub formation in response to DNA substrates.

![Figure 3](image-url)
In a recent study, we showed that chromatin binding of xFANCD2, but not the FA core complex protein xFANCA, is blocked in xATRIP-depleted extracts (14). Thus, we tested whether xFANCM recruitment to chromatin is affected in the absence of xATR. As shown in Fig. 4C, chromatin binding of xFANCM was blocked in xATR-depleted extracts. Similarly, as expected from our previous results, xFANCD2 chromatin binding was blocked in the absence of xATR. Surprisingly, however, chromatin binding of xFANCA was also fully inhibited in xATR-depleted extracts, whereas xATRIP-depleted extracts show normal chromatin binding of xFANCA (14). This result indicates that in xATRIP-depleted extracts, the residual kinase-deficient xATR protein can still promote chromatin binding of xFANCA. In summary, our data indicate that xATR controls phosphorylation of xFANCM and regulates chromatin binding of the xFA core complex proteins xFANCA and xFANCM as well as the downstream FA pathway protein xFANCD2.

We also tested whether phosphorylation of xFANCM is dependent on the xATM kinase. We treated plasmid-containing egg extracts with KU-55933, a specific inhibitor of ATM kinase activity (28). As shown in Fig. 4D, plasmid-induced FANCM-PPP phosphorylation was strongly inhibited in xATM-depleted extracts compared with untreated extracts. In contrast, xFANCD2-Ub formation was not significantly affected (Fig. 4D), consistent with the results of Taniguchi et al. (4) showing that ATM-deficient cells are proficient in FANCD2-Ub formation. In summary, our results indicate that the two major cell cycle checkpoint kinases ATR and ATM mediate FANCM phosphorylation in response to DNA damage.

**FANCM Is Not Required for the ATR-dependent Checkpoint Response to dsDNA Substrates**—Cells with a defective FA pathway are partially deficient in activating a DNA damage-induced checkpoint response (29–34). Several studies have shown that...
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**DISCUSSION**

We cloned the Xenopus FANCM gene and showed that many of its functions are conserved between human and frog: 1) xFANCM is part of the xFA core complex, 2) it is required for xFANCD2 monoubiquitination in response to DNA damage structures, and 3) it binds chromatin during S phase and is hyperphosphorylated during unperturbed DNA replication and in response to DNA-damaging agents. The chromatin binding behavior of xFANCM is similar to that of other xFA proteins, accumulating on chromatin in a strictly replication-dependent manner during S phase, and is in support of the idea that FA pathway functions are linked to ongoing DNA replication (14). Interestingly, approximately 20–30% of all xFANCM in egg extracts are not associated with the FA core complex and bind to replicating chromatin independently, indicating that FANCM may have additional replication-associated functions outside the FA pathway.

Similar to the monoubiquitination of xFANCD2 (16), phosphorylation of xFANCM can be directly triggered in egg extracts in response to small DNA substrates, demonstrating that activation of upstream and downstream FA pathway members can be uncoupled from ongoing replication in the *Xenopus* cell-free system. Consistent with these results, recent studies have demonstrated that human recombinant (nonmodified) FANCM has affinity toward DNA forks and DNA four-way junctions *in vitro*. In egg extracts xFANCM-PPP is inducible by any single or double-stranded DNA structure we tested, indicating that xFANCM activation in this context is not DNA structure-specific, unlike xFANCD2, which is ubiquitinated preferentially in response to branched dsDNA substrates. xFANCM bound to both branched and linear structures with similar affinity; however, because we used whole egg extracts for our studies, association of xFANCM to some of the DNA structures might not be direct but rather mediated by other DNA-binding proteins present in egg extracts.

Interestingly, the presence of circular double-stranded plasmid DNA triggers activation of xFANCD2, xFANCM, and other DNA repair proteins (*e.g.* Mre11 (16)); moreover, it activates an xATR-dependent checkpoint that involves phosphorylation of the checkpoint proteins xChk1 and xRad1. Previous studies did not observe activation of a DNA repair pathway (17) double-stranded or primed single-stranded DNA substrates can trigger an ATR-dependent DNA damage checkpoint response in *Xenopus* egg extracts (35–38). We showed above (Fig. 4, A, B, and D) that the addition of plasmid DNA at sufficiently high DNA concentrations triggers xATR-dependent xFANCM phosphorylation as well as xATR-dependent checkpoint activation, as monitored by phosphorylation of the xATR downstream target, xChk1, at Ser^344^). Thus, we hypothesized that xFANCM might be involved in checkpoint signaling by mediating xATR-dependent phosphorylation of xChk1 and other downstream targets in response to plasmid DNA. As shown in Fig. 5A, depletion of xFANCM from egg extracts did not affect xChk1-P^Ser^344^ formation in response to plasmid DNA. Similarly, we tested plasmid-induced phosphorylation of xRad1, part of the 9-1-1 (Rad9-Hus1-Rad1) complex, as an alternative readout for the xATR-mediated checkpoint response (39). Rad1 phosphorylation was not affected in xFANCM-depleted extracts compared with mock-depleted extracts (Fig. 5A). Finally, we tested the plasmid DNA-induced histone H2AX phosphorylation (γ-H2AX), an indicator of ATR and ATM kinase activity in egg extracts (40, 41). Plasmid DNA-induced γ-H2AX formation was not affected in extracts depleted of xFANCM (Fig. 5A). To further confirm this result, we tested xChk1-P^Ser^344^ formation in response to short dsDNA substrates (dsDNA_{70} bp long (16)) that are known to activate xATR-dependent xChk1 phosphorylation at Ser^344^). As a negative control, we used ssDNA substrates (ssDNA_{70} nt long) that cannot induce xChk1-P formation (reviewed in Ref. 36). As expected, xChk1-P^Ser^344^ was robustly induced by dsDNA_{70} but not by ssDNA_{70} fragments. In agreement with our results above, depletion of xFANCM from egg extracts did not affect this dsDNA_{70}-induced activation of xChk1-P^Ser^344^ (Fig. 5B). Together, our data indicate that xFANCM is not required for the xATR-dependent, dsDNA substrate-induced checkpoint response in S phase extracts.

**FIGURE 5.** xFANCM is not required for the dsDNA-induced, xATR-dependent checkpoint response. A, xFANCM depletion does not inhibit plasmid DNA-induced phosphorylation of xChk1, xRad1, or x-γH2AX. Extracts were either mock-depleted (lanes 1, 3, and 5) or depleted of xFANCM (lanes 2, 4, and 6) and incubated with plasmid DNA for the indicated time points. Following incubation, 1 μl of extract was analyzed for the indicated proteins by SDS-PAGE and immunoblot. DNA-free extracts (lanes 1 and 2) were used as a negative control and as a control for protein size and for quantitative depletion of xFANCM. 8, xFANCM depletion does not inhibit dsDNA_{70}-induced phosphorylation of Chk1. Extracts were either mock-depleted (lanes 1, 3, 4, 7, and 8) or depleted of xFANCM (lanes 2, 5, 6, 9, and 10) and incubated with either ssDNA_{70} (lanes 3, 5, 7, and 9) or dsDNA_{70} (lanes 4, 6, 8, and 10) for 30 min. Following incubation, 1 μl of extract was analyzed for the indicated proteins by SDS-PAGE and immunoblot. DNA-free extracts (lanes 1 and 2) were used as a negative control and as a control for protein size and for quantitative depletion of xFANCM. Phosphorylated xChk1 was detected using an anti-xChk1-P^Ser^344^ antibody. (Please note that the low DNA concentration (40 μg/ml) used in this assay triggers a robust xChk1-P response but is not sufficient to induce a robust xFANCM-PPP induction.)
or the \(x\)ATR checkpoint using circular plasmid DNA (36); however, these studies used low DNA plasmid concentrations. We suggest that by using high plasmid DNA concentrations (150 ng of plasmid DNA/µl of extract), we supplement extracts with plasmid subpopulations that mimic DNA damage structures (e.g. gapped, nicked, or broken DNA plasmid molecules) and trigger different DNA damage response pathways.

A surprising finding was the relationship between DNA substrate-induced \(x\)FANCM phosphorylation and \(x\)FANCD2. According to the current FA pathway model the FA core complex, containing FANCM, acts upstream of FANCD2, mediating FANCD2 monoubiquitination and its concurrent chromatin recruitment. However, our data indicate that FANCM and FANCD2 are functionally interdependent and suggest a model where the FANCD2 protein signals back to the \(x\)FA core complex via regulation of FANCM phosphorylation. A precedent exists for the mutual functional control between DNA repair proteins: the interplay between the cell cycle checkpoint kinase ATM and the DNA repair complex, MRN (Mre11-Rad50-NBS1). MRN binds DNA breaks on chromatin independently of ATM but needs to recruit and activate ATM by phosphorylation before it can itself be phosphorylated and activated by ATM. This modification subsequently allows MRN to act as an adapter for downstream signaling to other ATM targets (reviewed in (42, 43). Our data indicate that FANCM and FANCD2 affect each other’s function during replication and the DNA damage response; however, because the molecular function of FANCD2 is currently unknown, further studies are necessary to investigate which function(s) of FANCD2 might be controlled by FANCM and vice versa during DNA damage signaling.

In human cells, ATR and ATM phosphorylate FANCD2 at different sites in response to DNA damage, indicating that important FA pathway functions are controlled by these two cell cycle kinases. Here, we show that \(x\)ATR and \(x\)ATM also control DNA damage-induced phosphorylation of \(x\)FANCM; moreover, \(x\)ATR is required to recruit \(x\)FANCM to undamaged replicating chromatin. We were surprised to find that the absence of \(x\)ATR in egg extracts also blocked chromatin recruitment of \(x\)FANCA, because we observed previously that depletion of \(x\)ATRIP, the binding partner and “functional subunit” of \(x\)ATR (22, 24), does not inhibit chromatin binding of \(x\)FANCA (14) (or \(x\)FANCM). However, although depletion of \(x\)ATRIP inactivates the \(x\)ATR kinase, it only partially co-depletes \(x\)ATR from egg extracts. We conclude that the residual, kinase-inactive \(x\)ATR protein in \(x\)ATRIP-depleted extracts is capable of recruiting the \(x\)FA core complex proteins \(x\)FANCA and \(x\)FANCM to replicating chromatin. In turn, this indicates that the \(x\)ATR kinase domain alone is required but not sufficient to fully support chromatin recruitment of the FA core complex.

\(x\)ATR-dependent phosphorylation of \(x\)FANCM occurred in parallel with activation of an \(x\)ATR-mediated checkpoint response in egg extracts and raised the question of whether \(x\)FANCM is part of the checkpoint signaling cascade, downstream of \(x\)ATR but upstream of other \(x\)ATR phosphorylation targets. Our data indicate that \(x\)FANCM is not required for phosphorylation of \(x\)Chk1 or \(x\)Rad1 in response to dsDNA, both used as readout for \(x\)ATR-dependent checkpoint activation. In agreement with our data, Pichierri and Rosselli (2) demonstrated that in human cells, a DNA cross-link-induced S phase checkpoint is branched downstream of \(x\)ATR, with one branch depending on \(x\)Chk1 and the other depending on the FA proteins, indicating that \(x\)Chk1 and FA pathway act independently of each other. On the other hand, a recent study by Collis et al. (44) showed that HeLa cells lacking FANCM exhibit strongly reduced levels of \(x\)Chk1 phosphorylation at Ser\(^{317}\) in response to replication fork stalling. In human cells, \(x\)Chk1 phosphorylation by \(x\)ATR occurs at two sites, Ser\(^{317}\) and Ser\(^{345}\), in response to replication fork stalling. In contrast, only phosphorylation of the second site (Ser\(^{345}\) in \textit{Xenopus} Chk1) has been reported in \textit{Xenopus} (37). Interestingly, it has been suggested that stalled replication forks and dsDNA\(^{70}\) structures activate ATR through different pathways (36); ATR activation in response to replication fork stalling is RPA-dependent, whereas dsDNA\(^{70}\)-induced ATR activation does not require RPA (35). Conversely, Claspin, another protein required for \(x\)Chk1-P formation, is required for \(x\)Chk1-P\(_{Ser344}\) activation in response to dsDNA\(^{70}\) or double-stranded DNA breaks but not stalled replication forks (46). Together, these data indicate that FANCM is involved in checkpoint signaling following replication fork arrest, but not in response to DNA double strand breaks.

In addition, two recent studies indicate that \(x\)Chk1 and/or \(x\)Rad1 are involved in regulating FA pathway activation in response to DNA damage (45, 47), raising the possibility that FANCM might have functions downstream of \(x\)Chk1 in the \(x\)ATR-dependent checkpoint signaling. In summary, we propose that FANCM has multiple roles during chromosomal replication. It is required for a functional FA pathway and regulated on several levels by FANCD2, ATR, and ATM during unperturbed replication and in response to DNA damage.

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