ATR-Dependent Phosphorylation of FANCM at Serine 1045 Is Essential for FANCM Functions

Thiyam Ramsing Singh1,4, Abdullah Mahmood Ali1, Manikandan Paramasivam3, Arun Pradhan1, Kebola Wahengbam1, Michael M. Seidman3, and Amom Ruhikanta Meetei1,2

Abstract

Fanconi anemia (FA) is a genome instability syndrome that has been associated with both cancer predisposition and bone marrow failure. FA proteins are involved in cellular response to replication stress in which they coordinate DNA repair with DNA replication and cell-cycle progression. One regulator of the replication stress response is the ATP-dependent DNA translocase FANCM, which we have shown to be hyperphosphorylated in response to various genotoxic agents. However, the significance of this phosphorylation remained unclear. Here, we show that genotoxic stress–induced FANCM phosphorylation is ATR-dependent and that this modification is highly significant for the cellular response to replication stress. We identified serine (S1045) residue of FANCM that is phosphorylated in response to genotoxic stress and this effect is ATR-dependent. We show that S1045 is required for FANCM functions including its role in FA pathway integrity, recruiting FANCM to the site of interstrand cross links, preventing the cells from entering mitosis prematurely, and efficient activation of the CHK1 and G2–M checkpoints. Overall, our data suggest that an ATR-FANCM feedback loop is present in the FA and replication stress response pathways and that it is required for both efficient ATR/CHK1 checkpoint activation and FANCM function. Cancer Res; 73(14); 1–11.

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Introduction

Fanconi anemia (FA) is a genome instability syndrome characterized by developmental abnormalities, bone marrow failure, chromosome fragility, and a dramatically elevated cancer incidence (1). The FANCM protein is an ATP-dependent DNA translocase and the encoding gene is mutated in a single FA patient belonging to the complementation group FA-M (2, 3). FANCM along with FAAP24 and FANCM-interacting histone fold protein 1 (MHF1) and MHF2 physically and functionally interacts with additional proteins (including FAANCA, FANCB, FANCC, FAACE, FANCF, FANCG, and FANCL, each representing an additional FA complementation group) and their associated factors (including FAAP100, FAAP20, and HES1) to form a larger complex known as the "FA nuclear core complex" (4–7). The FA nuclear core complex acts as an E3 ubiquitin ligase due to the E3 ubiquitin ligase domain in FANCL (8). In response to replication stress, FANCM recruits the core complex to chromatin and this then monoubiquitinates FANCD2 and FANCI to activate the FA pathway (1). Downstream of this event, additional proteins (e.g., FANCD1/BRCA2, FANCF/BACH1, FANCC/PALB2, FANCO/RAD51C, and FANCP/SLX4, each representing an additional FA complementation group) work together with FA-associated proteins (FAN1, RAD18, and RAD51) to affect DNA repair and tolerance reactions. FANCM also plays other significant functions. For example, it provides resistance to ultraviolet (UV) rays and camptothecin (CPT), suppresses spontaneous sister chromatid exchanges (SCE), and activates the synthesis (S)-phase checkpoint (via ATR) in response to replication stress (3, 9, 10). Despite this extensive catalog of FANCM functions and their clear importance for cellular response to replication stress, our understanding of the mechanisms whereby it acts remains unclear.

FA proteins are involved in cellular response to replication stress in which they coordinate DNA repair with DNA replication and cell-cycle progression. In eukaryotes, DNA replication involves replication initiation, replication fork progression, and replication termination (11, 12). Interruption during replication fork progression results in replication stress (11, 12). Negative influences include the presence of difficult-to-replicate DNA regions (e.g., rDNA, centromeres, telomeres, and other repetitive sequences), DNA damage resulting from...
compounds generated by the cellular metabolism (e.g., reactive oxygen species) and genotoxic agents. The genotoxic agents include compounds that cause interstrand crosslinks (ICL) [e.g., mitomycin C (MMC) and cisplatin], result in failure to remove covalently bound Topo I to DNA (e.g., CPT), and deplete the dNTP pool by inhibiting the enzyme ribonucleotide reductase [e.g., hydroxyurea (HU)]. The consequences of replication fork stalling include the following. First, a long stretch of single-stranded DNA (ssDNA) is generated, and it is recognized as DNA damage by the cell-cycle checkpoint machinery (13). Replication protein A (RPA), a single-strand binding protein, binds ssDNA and recruits the checkpoint kinase ATR (ATM- and Rad3-related) and its binding partner ATRIP (ATR-interacting protein). ATR then phosphorylates a large number of substrates to trigger the checkpoint response (11, 13). One such substrate is CHK1 kinase that phosphorylates CDC25A, resulting in the inactivation of cyclin-dependent kinases (CDK) to promote cell-cycle arrest (11, 13). In addition, ATR/CHK1 signaling is important for activating the FA pathway (12, 14), including the core complex proteins FANCA (S1449), FANCG (S7), and FANC (T346 and S374) and this is necessary for cellular tolerance to MMC. Also, ATR/CHK1-mediated phosphorylation of FANCI and FANCD2 is essential for efficient monoubiquitination of these proteins (12, 14). Recently, FANCM was found to be required for the activation of ATR-mediated checkpoint signaling, and it was suggested to play an "upstream" role in ATR checkpoint activation (9, 15, 16). FANCM and its partner FAAP24 associate with checkpoint protein HCLK2 (9, 17). This activity of FANCM is independent of the FA core complex (9). Silencing of FANCM in mammalian cells confers a phenotype similar to that of cells lacking ATR, HCLK2, or CHK1: increased rates of spontaneous DNA damage, nuclear abnormalities, and supernumerary centrosomes in unperturbed cells and checkpoint defects in cells subjected to replication stress and DNA damage (17). The translocase activity of FANCM is essential for its role in checkpoint signaling (9, 15, 16). Notably, FANCM-deficient cells are sensitive to MMC and CPT. FANCM and FAAP24 possess intrinsic structure–based DNA-binding activity (preference for Y-shaped molecules), and both proteins interact constitutively with chromatin and with HCLK2 (9, 17). On the basis of these findings, it has been proposed that FANCM acts as a sensor of damage and/or mediator of ATR checkpoint signaling (9, 17).

FANCM is phosphorylated during the S and mitosis (M) phases of the cell cycle (2, 3, 18), and it becomes hyperphosphorylated in response to various forms of genotoxic stress (exposure to HU, MMC, or CPT; refs. 2, 3). Several lines of evidence suggest that multiple kinases are involved in FANCM phosphorylation, and the nature and extent of its phosphorylation has been proposed to play important roles in regulating its activity, both as a component of the FA core complex and otherwise (2, 3, 18). Although the majority of FANCM phosphorylation during M phase is mediated by polo-like kinase 1 (PLK1) and results in FANCM degradation, it is not clear which kinases is relevant during S-phase and in the response to genotoxic stress (2, 3, 18). In this study, we have probed the role of ATR in FANCM phosphorylation and found that it is relevant in the context of DNA damage. We also provide evidence for the importance of ATR-dependent phosphorylation of FANCM at serine 1045 (S1045) in the FA pathway and ATR signaling and uncover a feedback loop in the FA pathway. Our report provides the first evidence for a specific posttranslational modification of FANCM in response to genotoxic stress and that this event is required for FANCM function.

Materials and Methods

Cells, cell culture, and chemicals

HeLa, U2OS, and HEK293 cells were maintained using standard procedures (6). Parental HCT116 cells were purchased from American Type Culture Collection, and HCT116 ATRfl/fl– cells were a kind gift from Dr. Stephen J. Elledge. HU (Sigma) was resuspended in water to a stock concentration of 1 mol/L. MMC (Sigma) was dissolved in 70% ethanol (Sigma) to a stock concentration of 500 ng/μL. CPT (Sigma) was dissolved in dimethyl sulfoxide (DMSO; Sigma) to a stock concentration of 10 mmol/L.

Cloning, constructs, and retroviruses

The pMIEG3 bicistronic retroviral vector and pMIEG3-expressing FANCM were described previously (3). The FANCM S1045A mutant was introduced using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies). Retroviral packaging and generation of stable cell lines were as described earlier (3).

Antibodies

Antibodies against actin, ATR, and CDC25A (F-6) were purchased from Santa Cruz Biotechnology. CHK1 and phospho-CHK1 (S317) were purchased from Cell Signaling. Anti-FANCM, -FANCA, and -FANCD2 were described previously (2). Anti-FAAP24 was a kind gift from Dr. Stephen West (London Research Institute, Cancer Research UK). Anti-histone H2A antibody was purchased from Millipore. Custom-made phospho-specific antibody recognizing FANCM S1045 was generated by PhosphoSolutions.

Protein knockdown by siRNA/shRNA treatment

All siRNA oligos were purchased from Dharmacon. For ATR knockdown, we used 2 siRNA oligos: ATR#1 targeting the coding region of ATR (CGAGACTTCTGCGGATTCG) and ATR#2 was purchased from Dharmacon (J-003202-19-00). A nonspecific control siRNA (D-001210-01) was used in all experiments. Cells were transfected as described previously (19). Endogenous FANCM was stably knocked down by retroviral-mediated expression of a short hairpin RNA (shRNA) targeting the 3′-untranslated region (UTR) of the FANCM mRNA (AAAGACCTCTCACAATATT). To generate retroviruses encoding shRNAs specific to 3′-UTR region of the FANCM mRNA, we used a pSilencer 1.0-U6 vector (Ambion) containing the RNA PolIII-specific U6 gene promoter. Virus was generated as described previously (5).

Purification and mass spectrometry analyses of FANCM complexes

HeLa cells were treated with 1.5 mmol/L HU for 16 hours, and FANCM protein complex was purified from the nuclear
extract using anti-FANCM antibody. The purified protein complex was resolved on an 8% to 16% SDS-PAGE gel and stained using either the SilverQuest Silver-Staining Kit or Colloidal Blue Staining Kit according to the manufacturer’s instructions (Invitrogen). Phosphorylated FANCM was excised into 3 fragments for 3 protease digestions (trypsin, chymotrypsin, and Glu-C), and phospho-peptides were identified by Ion-trap Mass Spectrometry analysis. All mass spectroscopy work was conducted by the Mass Spectrometry Core at the University of Virginia (Charlottesville, VA).

**Phosphatase treatment**

HeLa cells expressing FLAG-FANCM were treated with MMC for 16 hours. Cells lysates were prepared using lysis buffer, and then FANCM was immunoprecipitated with anti-M2 agarose (Sigma). FLAG immunoprecipitates were mock treated or incubated at 30°C with 400 U of λ-protein phosphatase instructions (New England Biolabs) for 60 minutes according to the manufacturer’s instructions before immunoblot analysis.

**FANC D2 immunofluorescence**

Cells were fixed in 2% paraformaldehyde in PBS for 20 minutes at room temperature (RT) and then deposited on 12-mm diameter glass coverslips coated with poly-o-lysine (centrifugation in a Thermo Shandon Cytospin apparatus at 163 × g for 2 minutes). After being washed with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 3 minutes and washed again with PBS. Then anti-FANC D2 antibody E35 (1:200) in antibody buffer [PBS/3% bovine serum albumin (BSA)/0.05% Tween 20/0.04% sodium azide] was added for 1 hour at 37°C. Cells were washed with PBS and incubated for 30 minutes at 37°C with rhodamine B–conjugated donkey anti-rabbit IgG antibody (1:200; Jackson ImmunoResearch Laboratories). Cells were washed with PBS, mounted in Vectashield with 4',6-diamino-2-phenylindole (DAPI; Vector Laboratories) to stain DNA, and analyzed using a Zeiss Axiovert 200M microscope (Carl Zeiss).

**Drug sensitivity assay**

Drug sensitivity was assessed using the chromogenic Cell Titer 96 Proliferation Assay (Promega Corporation), with optical density recorded at 490 nm. Specifically, 3,000 cells were grown in 96-well plates and treated with the indicated drugs for a week. The number of viable cells was then measured with a Live Cell environmental chamber. After laser treatment, the cells were incubated at 37°C for 10 minutes and fixed immediately in freshly prepared 4% formaldehyde (in PBS) for 10 minutes at room temperature. Fixed cells were permeabilized with a PBS solution containing 0.5% Triton X-100, 1% BSA, 100 mmol/L glycine, and 0.2 mg/mL EDTA on ice for 10 minutes and subsequently digested with RNase A at 37°C. For immunofluorescence staining, cells were incubated at 37°C for 1 hour with anti-γH2AX (Upstate, Millipore) and anti-FLAG (Sigma). Cells were incubated with corresponding secondary antibodies (Alexa Fluor goat anti-mouse or Alexa Fluor goat anti-rabbit; Molecular Probes, Invitrogen). After washing, they were mounted using ProLong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen). The immunostained cells were visualized and imaged using a Hamamatsu EM-CCD digital camera attached to the Nikon Eclipse TE2000 confocal microscope.

**UV-induced G2-M arrest analyzed by monitoring the mitotic index**

Cells in PBS were UV-irradiated with 5 J/m² UV and then cultured in complete medium for 16 hours in the presence of 100 ng/mL nocodazole. The cells were then fixed in suspension (10⁶ cells/mL) by the addition of 2 mL of 70% ethanol and incubation at −20°C for 24 h. After fixation, the cells were washed twice with PBS, suspended in 1 mL of PBS containing 0.25% Triton X-100, and incubated on ice for 15 minutes. After centrifugation, the cell pellet was suspended in 100 µL of PBS containing 1% BSA and anti-H3 (S10), a polyclonal antibody that specifically recognizes the phosphorylated form of histone H3 (Cell Signaling), and incubated for 2 hours at room temperature. The cells were then rinsed with PBS containing 1% BSA and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G antibody (Jackson ImmunoResearch Laboratories, Inc.) and diluted 1:200 in PBS containing 1% BSA. After a 1-hour incubation at room temperature in the dark, the cells were washed again, resuspended in PBS containing 25 µg/mL of propidium iodide (PI; Sigma) and 0.1 mg/mL of RNase A (Sigma), and incubated at room temperature for 30 minutes. The percentage of positively staining cells was quantified by fluorescence-activated cell-sorting (FACS) analysis.

**Preparation of chromatin fractions**

Chromatin fractions were prepared as described earlier (3).
Results

Kinetics of FANCM phosphorylation and FANCD2 monoubiquitination in response to genotoxic stress are similar

We previously reported that FANCM is phosphorylated in response to MMC- and HU-induced genotoxic stress (2). FANCD2, a key protein in the FA pathway, becomes mono-ubiquitinated under these conditions. A comparison of the time course of FANCM phosphorylation with that of FANCD2 monoubiquitination in response to both agents (Fig. 1A and Supplementary Fig. S1A) revealed that both phenomena increase over time, reaching a peak at 24 hours posttreatment (Fig. 1A and Supplementary Fig. S1A). The similarity in kinetics is consistent with phosphorylation of FANCM playing a role in the FA pathway.

FANCM phosphorylation in response to genotoxic stress is ATR-dependent

ATR plays an important role in the FA pathway, phosphorylating a number of the relevant proteins (12). We examined the ATR dependence of FANCM phosphorylation in response to genotoxic stress in 2 human cell lines (U2OS and HEK293), using siRNA oligos to knockdown ATR and analyzing FANCM phosphorylation by immunoblotting (Fig. 1B and Supplementary Fig. S1B, respectively). Genotoxic stress–induced phosphorylation (þHU) of FANCM was strongly reduced in ATR knockdown cells of both types, and these effects correlated with a reduction of FANCD2 protein. Similar results were obtained using an siRNA oligo targeting a distinct site in the ATR coding region (Supplementary Fig. S1C). These results are consistent with ATR playing a role in FANCM phosphorylation. We also tested the effects of ATR knockout, infecting cells of the HCT116 ATR<sup>fl<sup>ox</sup>/C</sup> cells, accompanied by loss of FANCM phosphorylation (Fig. 1C). These results confirm that ATR is essential for FANCM phosphorylation in response to genotoxic stress.

Identification of FANCM phosphopeptides and an ATR/ATM/DNA-PK consensus site at FANCM S1045

To purify phosphorylated FANCM and identify any phosphorylation sites, we immunoprecipitated FANCM from HU-treated HeLa cells (Fig. 2A). The band containing immunoprecipitated phospho-FANCM was excised from a colloidal Coomassie-stained gel and digested with 3 different proteases (trypsin, chymotrypsin, and Glu-C), and the resulting peptides were analyzed by liquid chromatography (LC)–mass spectrometry (MS). The peptides analyzed covered about 85% of the FANCM protein (Supplementary Fig. S2), and a total of 18 phosphorylation sites were identified. Given the role of ATR in FANCM phosphorylation, we inspected...
the phosphopeptides for the presence of the PIKK kinases (ATR, ATM, and DNA-PK) consensus site, S/TQ (13), and identified a peptide CTCLLSHSAVNS/C3QQNLELNSLK containing phospho-serine 1045 (pS1045) (Fig. 2B); the relative abundance of this peptide was higher than that of any other peptide in the spectrum (Fig. 2B).

**FANCM S1045 becomes phosphorylated in response to genotoxic stress**

To detect and study the significance of phosphorylation at FANCM S1045, we raised an antiserum against a 15–amino acid long synthetic peptide that contains pS1045. The antiserum was affinity-purified over a phosphorylated peptide.
column and cross-adsorbed to an unmodified peptide column. The anti-pS1045 thus purified detected FLAGFANCMTWT but not \textit{FLAGFANCMS1045A} by immunoblot analysis in immunoprecipitates from HeLa cells stably expressing \textit{FLAGFANCMTWT} or \textit{FLAGFANCMS1045A} respectively (Supplementary Fig. S3).

Because FANCM phosphorylation was induced in response to genotoxic stress (Fig. 2A), we tested pS1045 immunoreactivity following genotoxic stress. As shown in Fig. 2C and Supplementary Fig. S4, the pS1045 signal increased over time after cells were treated with HU, MMC, or UV. Thus, FANCM phosphorylation at S1045 is upregulated in response to genotoxic agents. Also, treatment of immunoprecipitated \textit{FLAG-FANCMWT} with lambda phosphatase eliminated signal detected with the anti-pS1045 (Fig. 2D), confirming the specificity of anti-pS1045.

\textbf{FANCM S1045 phosphorylation is ATR-dependent}

Because S1045 is part of the ATR consensus motif S/TQ, we assessed whether its phosphorylation depends on ATR. To this end, we knocked out ATR in HCT116 ATR\textsuperscript{lox/lox} cells using Cre recombinase; the resulting reduction of ATR protein was significant (Fig. 2E). FANCM from lysates of ATR-knockout cells were immunoprecipitated using anti-FANCM and immunoblotted with anti-pS1045. As shown in Fig. 2E, phosphorylation at S1045 was significantly diminished in these cells compared with controls, although anti-FANCM detected comparable levels of total FANCM protein in the 2 samples. These findings are consistent with the notion that phosphorylation at S1045 is ATR-dependent (Fig. 2E). Moreover, of cells pretreated with an inhibitor of DNA-PK (NU7026), ATM (KU55933), or ATR (Caffeine), the pS1045 signal was reduced only in the ATR inhibitor-treated group (Fig. 2F). Thus, in human cells subjected to genotoxic stress, ATR specifically regulates FANCM phosphorylation at S1045.

\textbf{Mutant FANCM\textsubscript{S1045A} fails to complement FANCM deficiency}

Cells from patients with FA display a marked hypersensitivity to DNA crosslinking agents such as MMC, and this hypersensitivity can be complemented by ectopically expressing a wild-type cDNA of the defective gene (6). Among the core complex proteins, FANCM-deficient cells are uniquely sensitive to both CPT and MMC (3). If phosphorylation at S1045 is required for FA pathway signaling and CPT resistance, a mutation affecting this residue should cause a phenotype similar to that observed in FANCM-depleted cells. We thus generated a FANCM-encoding construct in which S1045 was substituted with alanine (S1045A). Given that the S1045A form was not expressed in FANCM-deficient EUFA867 lymphoblast cells, which has served as a complementation system in previous analyses of FANCM (3), we established a complementation system using the U2OS cell line, in which endogenous FANCM was stably knocked down by retrovirus-mediated expression of an shRNA directed to the FANCM 3' UTR (shFANCM-UTR; Fig. 3A, lane 2). We then expressed either a wild-type (FANCM\textsubscript{TWT}) or phosphomutant (FANCM\textsubscript{S1045A}) protein in these cells via retroviral gene transfer (Fig. 3A). Immunoprecipitation and immunoblot analyses revealed that FANCM\textsubscript{S1045A} interacted with proteins of the FA core complex in the same way as of FANCM\textsubscript{TWT} (Supplementary Fig. S5A). Nevertheless, the FANCM\textsubscript{S1045A}-expressing cells were not fully functional, as shown by significant reductions in: FANCD2 monoubiquitination (Supplementary Fig. S5B); FANCD2 focus formation (Fig. 3B); reconstitution of MMC and CPT killing (Fig. 3C and D); and MMC-induced chromatin association of the FA-core complex (Supplementary Fig. S6). Thus, phosphorylation at S1045 is essential for the cells to respond appropriately to MMC- and CPT-induced genotoxic stress.

\textbf{Phosphorylation of S1045 is essential for efficient recruitment of FANCM to ICL sites}

FANCM is rapidly recruited at ICL sites generated by laser-activated psoralen conjugation (22). To investigate whether FANCM recruitment to ICL sites is dependent on phosphorylation at S1045, we transfected U2OS cells with \textit{FLAG-FANCMWT} and \textit{FLAG-FANCM\textsubscript{S1045A}} and visualized protein recruitment at ICL sites by indirect immunofluorescence (anti-FLAG) as previously described (23). Analysis of eGFP staining revealed that more than 90% of the cells were transfected. As shown in Fig. 3E and F, recruitment of \textit{FLAG-FANCM\textsubscript{S1045A}} to ICL sites was greatly diminished relative to that of \textit{FLAG-FANCMWT} (occurred in only 11% vs. 35% of cells).

These data suggest that the phosphorylation of FANCM at S1045 is essential for its efficient recruitment to ICL sites. Furthermore, we also observed a defective recruitment of endogenous FANCM to ICL sites in HeLa cells depleted of ATR (Supplementary Fig. S7), thus providing strong evidence for a direct involvement of ATR in FANCM functions.

\textbf{Phosphorylation of FANCM S1045 is required for efficient blockage of mitosis in response to genotoxic stress}

Previous studies suggested that FANCM is required to efficiently block the entry of cell into mitosis following genotoxic stress and failure to block the cells entry into mitosis result in cells with abnormal nuclear morphology (9). This is consistent with FANCM-depleted cells frequently exhibiting abnormalities in nuclear morphology (Fig. 4A and B; ref. 9). We assessed mitotic entry after ectopically expressing FANCM\textsubscript{TWT} and FANCM\textsubscript{S1045A} in FANCM-depleted U2OS cells. Consistent with earlier observations, the incidence of aberrant nuclear morphology increased 4- to 5-fold in the FANCM-depleted cells (Fig. 4A). Ectopic expression of FANCM\textsubscript{TWT}, but not FANCM\textsubscript{S1045A}, corrected this phenotype (Fig. 4A), suggesting that phosphorylation at FANCM S1045 is required to prevent entry into mitosis in response to genotoxic stress.

The above-described block to mitosis is known to be mediated, in part, by the ATR/CHK1 signaling pathway, to ultimately invoke the G2–M checkpoint and to be caused by the phosphorylation and degradation of CDC25A (9). Notably, CDC25A was rapidly degraded in HU-treated cells transduced with a control shRNA and, consistent with the earlier reports (9), this effect was not as pronounced in cells depleted of...
Although the effect was rescued by the expression of FANCMT WT, this was not the case for expression of FANCMS1045A (Fig. 4C). These data suggest that phosphorylation at FANCM S1045 is required for efficient ATR/CHK1 signaling in response to replication stress.

To confirm that phosphorylation at FANCM S1045 is essential for efficient entry into mitosis following genotoxic stress, we assessed the mitotic index of the cells following exposure to UV irradiation. ATR is activated following such treatment, and an important consequence is arrest at the G2–M checkpoint, as measured by a decrease in the percentage of mitotic (histone H3-p-S10–positive) cells in UV-treated versus untreated cells 24 hours posttreatment. As expected, the percentage of cells in mitosis was decreased among those expressing the control shRNA but not among those expressing shFANCM-UTR (Fig. 4D). Ectopic expression of FANCMT WT led to a reduction in the mitotic index similar to that observed in the control cells, whereas that of FANCMS1045A or an ATPase-dead mutant (FANCMK117R) did not (Fig. 4D). Although it is possible that the defect observed in FANCMS1045A-expressing cells resulted from changes in distribution of the cells across the cell cycle, an analysis of the FANCMT WT-, FANCMS1045A-, and FANCMK117R-expressing cells did not reveal differences in this regard (Supplementary Fig. S8). These data indicate that the phosphorylation of FANCM on S1045 is required to prevent inappropriate entry into mitosis in the context of genotoxic stress.

Figure 3. S1045 phosphorylation is essential for FANCM functions. A, expression of FLAG tagged wild-type (FANCMWT) and S1045A mutant (FANCMS1045A) FANCM in the context of knockdown of the endogenous protein. U2OS cells stably transduced with shRNA targeting the 3'-UTR of FANCM (shFANCM-UTR) were further transduced with retroviral vector alone (vector) or with retrovirus carrying FANCMWT or FANCMK117R. U2OS cells stably expressing a nontargeting shRNA (shControl) served as control. B, requirement of S1045 phosphorylation for assembly of FANCD2 foci. U2OS cells generated as described in A were either left untreated or exposed to 100 ng/mL MMC for 16 hours; the percentage of cells with 5 or more FANCD2 foci was assessed in at least 150 cells. The data represent the average of 3 independent experiments, with SD. C and D, MMC and CPT sensitivity of FANCM-depleted cells. Cells of indicated genotypes were continually exposed to indicated concentrations of MMC or CPT for 10 days, and viable cells were subjected to the Cell Titer 96 Proliferation Assay. The data show the percentage growth compared with that of untreated cells; 1 representative result of 3 independent experiments is shown, with SD. E and F, recruitment of FANCM forms to the ICL site. U2OS cells transfected with FLAG-tagged FANCMWT and FANCMS1045A were tested for recruitment to the ICL sites and were examined by immunofluorescence analysis. Representative images are shown in E, and quantitation is shown in F.
Phosphorylation of FANCM S1045 is essential for efficient ATR/CHK1 signaling in response to genotoxic stress

Previous studies have shown that FANCM is essential for ATR/CHK1 signaling (9, 24), and the data presented above suggest that the phosphorylation of FANCM S1045 is ATR-dependent. We next investigated the role of phosphorylation at S1045 in efficient ATR/CHK1 signaling. We hypothesized that the defect in genotoxic stress–induced CDC25A degradation observed in the context of FANCM S1045A may be due to defective CHK1 activation. We monitored CHK1 activation as it is ATR-mediated phosphorylation at S317. U2OS cells stably expressing shFANCM were transfected with the FANCMWT-, FANCM S1045A-, or FANCM K117R-encoding constructs, treated with 2 μmol/L CPT for 30 minutes, and immunoblotted for phospho-CHK1 (S317). CPT treatment led to strong CHK1 phosphorylation in control cells, and this was diminished in FANCM-knockdown cells (Fig. 5). CHK1 phosphorylation was rescued in FANCMWT-expressing cells but not in FANCM S1045A- or FANCM K117R-expressing counterparts (Fig. 5). Thus, phosphorylation of FANCM at S1045 appears to be essential for ATR/CHK1 signaling in response to genotoxic stress. We also found an increase in spontaneous DNA damage in cells expressing mutant forms of the protein (i.e., an increase in the levels of γH2AX phosphorylated at S139; Fig. 5). Overall, these data suggest that the phosphorylation of FANCM at S1045 is essential for CHK1 activation and that failure of this modification results in an increase in spontaneous DNA damage.
Discussion

Previously, we and others showed that FANCM is hyperphosphorylated in response to genotoxic stress and that cells of a FANCM-deficient line (EUFA867) and others depleted of FANCM by either an siRNA or an shRNA approach are sensitive to MMC as well as CPT (3, 25). Here, we report that the genotoxic stress induced by FANCM phosphorylation is ATR-dependent and identify FANCM residue S1045 as a novel phosphorylation site. Using a phospho-specific antibody, we show for the first time that phosphorylation at this site is induced by genotoxic stress and that ATR knockout or inhibition greatly reduces FANCM phosphorylation at S1045. Notably, phosphorylation at S1045 is essential for FANCM function in both the FA pathway and ATR/CHK1 signaling.

Kinetics of the genotoxic stress–induced phosphorylation of FANCM correlates with those of FANCD2 monoubiquitination under the same conditions, suggesting that the phosphorylation of FANCM plays an important role in the FA pathway—probably in signaling DNA damage. It is well-established that genotoxic stress leads to the activation of 3 related protein kinases: ATR, ATM, and DNA-PK. While the latter 2 are activated primarily by DNA double-stranded breaks (DSB), ATR responds principally to replication blockage or replication stress (26). ATR plays an important role in the FA pathway and phosphorylates several FA proteins including FANCD2, FANCI, FANCA, FANCG and FANCE (27–29); mutations at the relevant phosphorylation sites impair the monoubiquitination of FANCD2/FANCI. The genotoxic stress–induced phosphorylation of FANCM is ATR-dependent. Activated ATM, ATR, and DNA-PK preferentially phosphorylate serine and threonine residues that are followed by a glutamine residue (S/TQ), and FANCM has 15 such sequences, 9 of which are conserved across FANCM orthologs. However, one of these (S1045) was abundantly represented in MS as being modified, despite the fact that peptides containing all 15 sites were recovered. The other 14 sites may not get phosphorylated at all or get phosphorylated at such a low degree that they were beyond the detection level of MS or may get phosphorylated in some other conditions. The significant increase in phosphorylation at S1045 following DNA damage suggests that this particular site is important for signal transduction. It is notable that although FANCM S1045 is proficient in binding to the FA core complex, nevertheless cells expressing it exhibit impaired FANCD2 monoubiquitination, complete loss of FANCM function with respect to MMC sensitivity, FANCD2 focus formation, loading of the FA core complex onto chromatin in the context of genotoxic stress, and have unique phenotypes of CPT sensitivity and defective ATR/CHK1 signaling.

FANCM is known to stabilize replication forks, and perhaps its phosphorylation is required to perform this response in the context of replication stress—to avoid fork collapse and the formation of DSBs. The fact that we also observed a large increase in spontaneous γ-H2A.X in the FANCM-depleted cells as well as their FANCM S1045A-reconstituted counterparts suggests that FANCM prevents stalled replication forks from developing into DSBs and that FANCM phosphorylation is indispensable.

Although we have shown that FANCM phosphorylation is dependent on ATR, it is formally possible that ATR does not act directly on FANCM. However, the finding that FANCM S1045 is phosphorylated normally after MMC treatment in the presence of a specific inhibitor of ATM or DNA-PK (Fig. 2F) indicates that these kinases are not required. Together with the fact that S1045 is at an ATR/ATM consensus site, this finding highly suggests that the role of ATR is direct. FANCM has been reported to play a role as a sensor of genotoxic stress (9). The findings that phosphorylation at S1045 (i) is induced in the context of genotoxic stress, (ii) is ATR-dependent, and (iii) is...
required for downstream ATR/CHK1 signaling suggest that the activities of 2 genotoxic stress sensors, ATR and FANC, are coordinated via phosphorylation at FANC at S1045.

Previous reports placed FANC upstream of ATR signaling (9, 24). However, our data indicate that following ATR phosphorylation of FANC, the latter signals goes back to ATR. Such mutual control between DNA repair proteins has precedents. For example, studies of Xenopus FANC (xFANC) and FANC D2 (xFANC D2) revealed an interdependence: xFANC is required for the monoubiquitination and recruitment of xFANC D2 to chromatin, but FANC D2 protein signals back to the xFA core complex by regulating FANC phosphorylation (30). Similarly, the Mre11/Rad50/NBS1 complex (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 (31). We show here that FANC and ATR affect one another in the context of genotoxic stress; further studies elaborating on how FANC phosphorylation contributes to ATR function and vice versa will unravel their precise role in DNA damage signaling.

How FANC phosphorylation contributes to DNA repair and the resolution of ICLs or CPT sensitivity is not clear yet. However, our favored model is that ATR phosphorylates FANC at S1045 after detecting a block in replication; that phosphorylated FANC is then translocated along the DNA to the repair site where it stabilizes the replication fork, activates the ATR/CHK1 checkpoint, and recruits the FA core complex and other DNA repair proteins. This model is consistent with the fact that FANC S1045, which cannot be phosphorylated on the relevant serine, is not recruited to ICL sites (Fig. 3E) and that when it is the only form of FANC present, the FA core complex is not recruited to the chromatin following MMC treatment (Supplementary Fig. S6). ATR/CHK1 signaling is reduced, γH2AX levels increase, and the cells are sensitive to MMC and CPT. While definitive proof of the details of the proposed mechanism is not yet available, our results show that phosphorylation of FANC at S1045 is essential for FANC function and show for the first time a link that co-ordinates the FA pathway and ATR checkpoint.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.R. Singh, A.M. Ali, M. Paramasivam, M.M. Seidman, A.R. Meetei
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.R. Singh, A.M. Ali, M. Paramasivam, A. Pradhan, M. Seidman, A.R. Meetei
Writing, review, and/or revision of the manuscript: T.R. Singh, A.M. Ali, M. Paramasivam, M.M. Seidman, A.R. Meetei
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Wahengbam, A.R. Meetei
Study supervision: A.R. Meetei

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