Published in final edited form as:
Mol Cell. 2013 March 7; 49(5): 997–1009. doi:10.1016/j.molcel.2012.12.010.

FANCM and FAAP24 Maintain Genome Stability via Cooperative as well as Unique Functions

Yucai Wang[1,2], **Justin W. Leung**[1], **Yingjun Jiang**[1], **Megan G. Lowery**[4], **Do Huong**[1], **Karen M. Vasquez**[4], **Junjie Chen**[1,2], **Weidong Wang**[5], and **Lei Li**[1,2,3,*]

[1]Department of Experimental Radiation Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

[2]The University of Texas Graduate School of Biomedical Sciences at Houston, 6767 Bertner Avenue, Houston, TX 77030, USA

[3]Department of Genetics, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

[4]Department of Molecular Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

[5]Laboratory of Genetics, National Institute of Aging, National Institutes of Health, 251 Bayview Boulevard, Baltimore, MD 21224, USA

SUMMARY

The DNA remodeling enzyme FANCM and its DNA-binding partner, FAAP24, constitute a complex involved in the activation of Fanconi Anemia (FA) DNA damage response mechanism, but neither gene has distinct patient mutants. In this study, we created isogenic models for both *FANCM* and *FAAP24* and investigated their integrated functions in DNA damage response. We found that FANCM and FAAP24 coordinately facilitate FA pathway activation and suppress sister chromatid exchange. Importantly, we show that FANCM and FAAP24 possess non-overlapping functions such that FAAP24 promotes ATR-mediated checkpoint activation particularly in response to DNA crosslinking agents, whereas FANCM participates in recombination-independent interstrand crosslink repair by facilitating recruitment of lesion incision activities which requires its translocase activity. Our data suggest that FANCM and FAAP24 play multiple while not fully epistatic roles in maintaining genomic integrity.

INTRODUCTION

Fanconi anemia (FA) is a cancer prone recessive disorder accompanied by an array of hematological and developmental manifestations (Moldovan and D'Andrea, 2009). The genetic complexity of FA is remarkably high with at least fifteen complementation groups, each corresponding to mutation(s) of a distinct gene. The hallmark of FA patient-derived cells is hypersensitivity to DNA crosslinking agents such as mitomycin C (MMC) (Poll et al., 1985, German et al., 1987), suggesting that FA genes play essential roles in DNA damage response and repair of DNA interstrand crosslinks (ICLs).

*Correspondence: leili@mdanderson.org.

In response to DNA damage, two groups of FA gene products coordinately execute a monoubiquitination reaction. Eight of the FA proteins, FANCA-C, E–G, L and M, together with five FA-associated proteins (FAAP20, 24, and 100, MHF1, MHF2) form the FANC core complex (Ciccia et al., 2007, Ling et al., 2007, Yan et al., 2010, Yan et al., 2012, Leung et al., 2012, Ali et al., 2012, Kim et al., 2012). This complex monoubiquitinates the heterodimeric ID complex consisting of FANCD2 and FANCI upon DNA damage (Meetei et al., 2003a, Smogorzewska et al., 2007, Vandenberg et al., 2003, Wang et al., 2004). The FANCD2- associated nuclease 1 (FAN1) and the FANCP/SLX4 gene product, a scaffolding protein involved in structure-specific nuclease complex, are likely ICL processing components recruited by the ID complex (Kratz et al., 2010, Liu et al., 2010, Smogorzewska et al., 2010, MacKay et al., 2010, Crossan et al., 2011, Stoepker et al., 2011). Four other FA genes, BRCA2/FANCD1, BRIP1/FANCJ, PALB2/FANCN, and Rad51C/FANCO, also known as breast and ovarian cancer susceptibility genes (Howlett et al., 2002, Levitus et al., 2005, Xia et al., 2007, Wang, 2007, Vaz et al., 2010), do not seem to be directly regulated by the FA core complex or involved in the ID complex monoubiquitination (Shen et al., 2009, Ohashi et al., 2005). Given their defined roles in homologous recombination, this group of FA proteins likely functions to reestablish collapsed replication forks as a result of DNA double strand break formation during ICL processing.

Among protein associated with the FA core complex, FANCM is of particular interest because of its DEAH helicase domain and an actual ATP-dependent DNA-remodeling translocase activity (Hoadley et al., 2012a, Meetei et al., 2005, Mosedale et al., 2005). These activities are critical for FANCM to promote repair of ICLs (Rosado et al., 2009, Xue et al., 2008). The ATP-independent DNA binding activity of FANCM is important for FA core complex to monoubiquitinate ID complex (Xue et al., 2008) and may also be important for recruiting FA core complex to chromatin (Kim et al., 2008, Mosedale et al., 2005). FANCM also interacts with RMI1 via its conserved MM2 motif to form the BRAFT super complex. This interaction is thought to recruit the BLM-RMI1-Topo IIIa resolvase complex to ICL-stalled replication forks, thus explaining the SCE phenotype specific to FANCM deficiency (Deans and West, 2009, Meetei et al., 2003b).

FANCM is assisted by two DNA-binding partners FAAP24 and MHF1/MHF2 with distinct DNA binding specificities: the former prefers ssDNA whereas the latter favors dsDNA (Ciccia et al., 2007, Singh et al., 2010, Yan et al., 2010). FANCM and FAAP24 form a complex that exhibits structural resemblance to the XPF/ERCC1 and MUS81/EME1 family of endonucleases, although no nuclease activity has been detected for either protein or the complex. Moreover, FANCM/FAAP24 has been shown to participate in ATR-mediated S phase checkpoint activation (Collis et al., 2008, Schwab et al., 2010, Huang et al., 2010).

While FANCM/FAAP24 has been suggested to play multiple roles in maintaining genomic stability, how these functions integrate together to protect cells from DNA damage remains unclear. Lack of human genetic model systems for FANCM and FAAP24 also precludes epistatic analysis between FANCM and FAAP24. In the present study, we investigated the roles of FANCM and FAAP24 by creating somatic cellular knockout models for each gene. Here we report that FANCM and FAAP24 are not fully epistatic in supporting normal cellular resistance to DNA damage. We identified a novel and specific role for FANCM in promoting repair of interstrand crosslinks during G1/G0, which contributes to cell survival against crosslinking reagent. Conversely, we found that FAAP24 mediates checkpoint activation in a crosslink-specific fashion independent of its interaction with FANCM. We also identified coordinated functions of FANCM and FAAP24 in activating the FA pathway and in suppressing SCE. These findings support a model that FANCM and FAAP24 play both overlapping and distinct functions in maintaining genomic stability.

RESULTS

Inactivation of *FANCM* and *FAAP24* Loci Produces Viable Human Cellular Mutants

To establish genetic models for FANCM and FAAP24, we performed homologous replacement targetings in HCT-116 cells using recombinant adeno-associated virus (rAAV)-based construct delivery (Fig. S1A & B). As shown (Fig. 1A), wild type *FANCM* alleles were inactivated by sequential targeting that resulted in the ablation of exon 2, causing a frame-shift mutation/premature termination in exon 3. Similarly (Fig. 1B), exons 3 and 4 of the FAAP24 gene were deleted from both alleles to generate a null mutant. A $FANCM^{-/-}FAAP24^{-/-}$ double-knockout mutant was constructed by sequential targeting of the *FAAP24* alleles in the $FANCM^{-/-}$ background.

Complete loss of gene products, as demonstrated by western blotting (Fig. 1C), is achieved in $FANCM^{-/-}$ $FAAP24^{-/-}$, and $FANCM^{-/-}FAAP24^{-/-}$ mutant cells. All three mutants are viable with proliferation status comparable to the parental HCT-116 cells. The $FANCM^{--}$ and $FAAP24^{-/-}$ mutants were each complemented with wild type FANCM and FAAP24, respectively. The complemented mutants fully restored MMC-induced FANCD2 monoubiquitination (Fig. 1D & E), indicating that there is minimum clonal or nonspecific effects from the manipulations of homologous targeting. Interestingly, the stability of FANCM was not significantly affected in the absence of FAAP24 and *vice versa*, indicating that there is limited mutual dependency on their stability. This result suggests that FANCM and FAAP24 are not required to stabilize each other as an obligated heterodimer, unlike their structural homologs XPF and ERCC1 (Biggerstaff et al., 1993).

FANCM and FAAP24 Possess Non-overlapping Functions in Cellular Resistance to DNA Interstrand Crosslinking Damage

The isogenic FANCM and FAAP24 mutants allowed direct comparison of hypersensitivities to reveal their genetic interactions. We performed clonogenic survival assays to determine the sensitivities of $FANCM^{-/-}$ and $FAAP24^{-/-}$ mutants to mitomycin C. As shown (Fig. 2A), $FANCM^{-/-}$ and $FAAP24^{-/-}$ single mutants each displayed readily detectable hypersensitivity to MMC, compared to parental HCT-116 cells. However, the degree of MMC sensitivity is markedly lower than an isogenic $FANCB^{-}$ mutant (Fig. S2). In contrast, $FANCM^{-/-}FAAP24^{-/-}$ double-knockout cells exhibited much profound hypersensitivity to MMC than either single knockout cells. Similarly, $FANCM^{-/-}FAAP24^{-/-}$ double mutant exhibited markedly increased sensitivity to cisplatin compared to the single mutants (Fig. 2B). These results indicate that FANCM and FAAP24 may carry out non-overlapping functions in response to DNA interstrand crosslinking damage.

Consistent with this notion, MMC-induced chromosomal breakage occurs with a higher frequency in the $FANCM^{-/-}FAAP24^{-/-}$ double mutant than each single mutant (Fig. 2C & D), indicative of non-redundant functions for FANCM and FAAP24 in preventing the onset of MMC-induced chromosome breaks. On the other hand, tetra-radial chromosomes form at similar frequencies in all three mutants treated with MMC (Fig. 2E). This result suggests that FANCM and FAAP24 carry out epistatic functions in suppressing the formation of tetra-radial structures.

FANCM and FAAP24 Coordinately Support Activation of the Fanconi Anemia Pathway

Compared to $FANCM^{-/-}$ and $FAAP24^{-/-}$ cells, the $FANCM^{-/-}FAAP24^{-/-}$ double-knockout mutant displays drastically increased sensitivity to DNA crosslinkers and more severe phenotypes of chromosomal breakage. One plausible explanation could be that FANCM and FAAP24 act redundantly in activating the FA pathway. As such, disruption of both genes would result in severer disruption of the Fanconi anemia pathway, rendering the

FANCM$^{-/-}$*FAAP24*$^{-/-}$ double mutant much more sensitive to MMC than either single mutant. To test this, we analyzed MMC-induced FANCD2 monoubiquitination in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutants. Consistent with reported studies (Bakker et al., 2009, Ciccia et al., 2007, Rosado et al., 2009), we found that complete loss of FANCM or FAAP24 resulted in significant reductions but not complete loss of FANCD2 monoubiquitination as observed from a FANCB deletion mutant (Fig. 3A & B, Fig. S2). Contrary to our hypothesis, however, *FANCM*$^{-/-}$*FAAP24*$^{-/-}$ double mutant showed no further decrease in FANCD2 monoubiquitination than either single mutant (Fig. 3C). This result argues strongly that FANCM and FAAP24 promote but are not essential for FA pathway activation. It also suggests that the additional hypersensitivity and elevated chromosomal breakage in the *FANCM*$^{-/-}$*FAAP24*$^{-/-}$ double mutant are likely attributed to mechanism(s) independent of the FA pathway.

To further evaluate the roles of FANCM and FAAP24 on FA pathway function, we examined MMC-induced FANCD2 nuclear foci formation (Fig. 3D & F). Although cells lacking FANCM, FAAP24, or both are able to produce a significant amount of monoubiquitinated FANCD2 in response to MMC as shown above, foci formation of FANCD2 in these mutant cells were nearly completely abolished, similar to the *FANCB*$^{-}$ mutant in which no monoubiquitinated FANCD2 is detectable (Fig. 3D), whereas stable re-expression of FANCM and FAAP24 was able to fully restore MMC-induced FANCD2 foci (Fig. 3E & F). These results raise the possibility that the FANCM/FAAP24 complex is important for the chromatin-retention of FANCD2 albeit not essential for its monoubiquitination. We therefore tested chromatin loading of FA core complex components and FANCD2 in response to MMC in FANCM and FAAP24 mutants. As shown in Fig. 3G, chromatin association of FANC core components FANCA, FANCG, and FANCL was severely diminished in *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and the double knockout cells compared to that of the wild type cells, indicating that a coordinated function between FANCM and FAAP24 is essential for chromatin loading of the FA core complex. Chromatin association of monoubiquitinated FANCD2, on the other hand, was less impacted by the loss of FANCM and FAAP24. Together, these data suggest that FANCM and FAAP24 work in concert to promote the FA pathway activation.

FANCM and FAAP24 Cooperatively Suppress Sister Chromatid Exchange

Upon ICL exposure, increased levels of SCEs are found in cells lacking FANCM function (Bakker et al., 2009, Rosado et al., 2009, Deans and West, 2009). Whether FAAP24 is also required for suppressing ICL-induced SCE is unclear. We addressed this question by analyzing SCE in FANCM and FAAP24 knockout cells treated with MMC. As shown in Fig. 4A & B, MMC-induced SCE levels are much higher in *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$ and the *FANCM*$^{-/-}$*FAAP24*$^{-/-}$ double mutants than in wild type and *FANCB*$^{-}$ cells. Noticeably, *FANCM*$^{-/-}$*FAAP24*$^{-/-}$ double mutant did not produce any additional SCE phenotype than each single mutant alone. This result suggests that suppression of SCE is cooperatively achieved by FANCM and FAAP24, most likely through function of the heterodimer formed between the two proteins.

Recent studies suggested that elevated SCE in FANCM mutant cells is likely due to loss of FANCM's interaction with and recruitment of the BLM helicase, since mutation of the BLM-interacting domain on FANCM was shown to abolish BLM foci formation (Deans and West, 2009, Hoadley et al., 2012b). But the role of FAAP24 in this process is unclear. Upon examination of BLM foci formation in both wild type and the knockout mutants (Fig. 4C & D), We found that deletion of FANCM, FAAP24, or both did not yield a detectable impact on MMC-induced BLM nuclear foci formation, suggesting that there exists a FANCM- and FAAP24-independent mechanism for BLM recruitment.

FAAP24 Plays a Primary Role in Promoting ATR-Mediated Checkpoint Activation Induced by Interstrand Crosslinks

FANCM and FAAP24 both harbor DNA-interacting domains and therefore have been variably placed as sensing components upstream of or in parallel to the ATR-mediated checkpoint signaling (Collis et al., 2008, Huang et al., 2010). An unresolved issue is whether FANCM and FAAP24 both contribute to checkpoint signaling. To this end, *FANCM*$^{-/-}$, *FAAP24*$^{-/-}$, and *FANCM*$^{-/-}$*FAAP24*$^{-/-}$ cells were exposed to distinct genotoxic stresses that elicit response from the ATR-dependent checkpoint. Using Chk1 Ser317 and Ser345 as surrogate markers (Fig. 5A & S3), we found that MMC-induced Chk1 phosphorylation showed significant decrease in *FAAP24*$^{-/-}$ and *FANCM*$^{-/-}$*FAAP24*$^{-/-}$ mutant cells, but not in the *FANCM*$^{-/-}$ mutant, which reflects a more important role for FAAP24 in the activation of the ATR-dependent checkpoint.

When cells were exposed to UV and HU (Fig. 5B & C), we found that lack of FANCM and FAAP24 had no visible effect on ATR-dependent checkpoint activation, both in terms of the initial onset and dose/time-dependent accumulation of Chk1 phosphorylation. This result indicates a lesser role for FANCM and an ICL-specific role for FAAP24 in checkpoint signaling, which is consistent with the intrinsic affinity of FAAP24 to ICL DNA and its putative role on RPA recruitment (Huang et al., 2010).

The apparent lack of checkpoint signaling defect in the *FANCM*$^{-/-}$ mutant argues that FAAP24 is able to mediate checkpoint signal activation without forming the heterodimeric complex with FANCM. To test this notion, a FANCM binding-defective mutant was constructed by a three amino acid residue substitution at the C-terminal $(HhH)_2$ domain of FAAP24. The resulting mutant, FAAP24-V198A, failed to interact with FANCM (Fig. 5D). However, when stably expressed in *FAAP24*$^{-/-}$ cells, FAAP24-V198A is fully capable of correcting the Chk1 activation defect caused by FAAP24 loss (Fig. 5E). This result provides further evidence that the checkpoint function of FAAP24 can be segregated from its interaction with FANCM. Consistent with this observation, the FAAP24-V198A mutant partially rescues the MMC sensitivity of *FAAP24*$^{-/-}$ cells, most likely reflecting the deficiency derived from loss of the FANCM-FAAP24 interaction (Fig. 5F). Taken together, these results indicate a complex -independent function of FAAP24 in checkpoint response to ICLs.

FANCM Promotes Recombination-Independent Repair of Interstrand Crosslinks

Lack of checkpoint signaling phenotype from the *FANCM*$^{-/-}$ mutant prompted us to seek checkpoint-independent function for FANCM in response to ICLs. One possibility is that FANCM may be involved in the actual removal of ICLs. To verify this notion, we employed a reporter-based assay to analyze ICL repair activity of FANCM and FAAP24 mutants. This assay relies on reactivation of the luciferase expression to quantify removal of a transcription-blocking site-specific ICL (Fig. 6A). We found that both *FANCM*$^{-/-}$ and *FANCM*$^{-/-}$*FAAP24*$^{-/-}$ mutant cells, but not the *FAAP24*$^{-/-}$ or *FANCB*$^{-}$ mutants, showed reduced ICL repair activity (Fig. 6B), suggesting that loss of FANCM function is specifically linked to compromised recombination-independent ICL repair.

A key step for recombination-independent ICL repair is PCNA monoubiquitination, which enables gap synthesis of the ICL repair intermediate by a lesion bypass polymerase instead of the normal replicative polymerase (Shen et al., 2006, Wang et al., 2001). Thus, we first examined whether disruption of FANCM would affect PCNA monoubiquitination specific to the recombination-independent ICL repair. To eliminate potential interference from PCNA-monoubiquitination from blocked replication forks, cells were grown to confluency to obtain predominantly G1/G0 cells which utilizes the recombination-independent ICL

repair mechanism. As shown (Fig. 6C), FANCM deletion caused a significant reduction of PCNA monoubiquitination in response to ICL, whereas cells lacking FANCB or FAAP24 exhibited wild-type levels of PCNA monoubiquitination. This result argues for a specific role for FANCM, but not FAAP24 or the FANC core complex function in ICL-induced PCNA monoubiquitination.

Impaired PCNA-ubiquitination in FANCM-deficient cells implicates a potential defect in the NER-mediated incision step, which creates a repair intermediate necessary for the onset of PCNA monoubiquitination. We tested this premise by carrying out the eChIP assay (Shen et al., 2009) to determine whether FANCM loss disrupts the ICL-dependent recruitment of XPF/ERCC1, a structure-specific endonuclease responsible for the initial incision during recombination-independent ICL repair. Expectedly (Fig. 6D), ERCC1 was significantly enriched at the defined ICL site in wild type cells. FANCM deletion, however, resulted in a marked decrease of ERCC1 recruitment. In contrast, FAAP24 loss did not affect ICL-induced ERCC1 recruitment. This result further suggests a specific role for FANCM in promoting ICL processing by facilitating localization of incision activities in the absence of DNA replication.

Recombination-independent ICL repair is the predominant means of ICL removal when the lesion is not encountered by DNA replication. Failure in this pathway is expected to impact primarily G1/G0 phase cells and likely causes cell cycle progression attenuation at G1/G0 or the G1/S boundary. To validate this projected outcome, we analyzed cell cycle response of *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutants exposed to elevated doses of MMC. While deletion of *FANCM*, *FAAP24*, or both did not alter cell cycle profile in unperturbed growth conditions (Fig. S4A), exposure to MMC resulted in a prominent increase of G1/G0 phase cells in *FANCM*$^{-/-}$ and *FANCM*$^{-/-}$*FAAP24*$^{-/-}$ mutants, but not in the *FAAP24*$^{-/-}$ or *FANCB*$^{-}$ mutants (Fig. 6E & S4A, C). Consistent with this observation, ERCC1 and XPA mutants, defective in recombination-independent ICL repair (Wang et al., 2001, Zheng et al., 2003), exhibited significant increase in G1/G0 phase cells when identically exposed to MMC (Fig. 6F & S4B). Collectively, these results reflect a novel function of FANCM in ICL repair, independent of FAAP24.

Recombination-Independent ICL Repair Depends on FANCM Translocase Activity

FANCM displays DNA translocase activity, which contributes to normal resistance to MMC but not FANCD2 monoubiquitination (Xue et al., 2008, Gari et al., 2008b). It is plausible that FANCM acts to recruit NER incision factor(s) via direct interaction, or alternatively, the FAAP24-independent translocase activity acts to create lesion access, allowing the assembly of NER incision factors and subsequent lesion bypass components. To distinguish these premises, we stably complemented *FANCM*$^{-/-}$ cells with a FANCM translocase mutant (K117R) (Fig. S5) and analyzed its function in recombination-independent ICL repair. As shown (Fig. 7A–C), whereas loss of FANCM translocase activity yielded no detectable loss in FANCD2 monoubiquitination and foci formation upon crosslinking damage, recombination-independent ICL repair is rendered deficient in *FANCM*$^{-/-}$ cells complemented with the K117R translocase mutant (Fig. 7D). This result indicates that the translocase activity is important for FANCM's role in ICL repair. On the other hand, FANCM function in recruiting the FANC core complex and subsequent FANCD2 activation was not affected by a disabled translocase function. Consistently, we found that the FANCM-K117R is proficient in interacting with components of the FANC core complex (Fig. 7E).

To further understand the role of FANCM in recombination-independent ICL repair, we examined the K117R translocase mutant for its ability to recruit ERCC1, XPA, and Rev1, which are critical components of the NER-lesion bypass-based ICL repair. Results of the

eChIP experiments (Fig. 7F) showed that, in addition to ERCC1, recruitments of the lesion binding protein XPA, and the lesion bypass factor Rev1 are markedly decreased in cells lacking FANCM translocase activity. Together, these observations suggest that FANCM translocase activity is crucial for recombination-independent ICL removal through promoting the recruitment of multiple repair factors.

DISCUSSION

FANCM and FAAP24 functions have been implicated in the activation of FANC core complex, checkpoint signaling, and suppression of homologous recombination crossovers. However, lack of human genetic model systems restricted in-depth studies of how these functions integrate to protect cells from DNA damage. While no patient mutations have been identified for *FAAP24*, the only FANCM patient also harbors bi-allelic *FANCA* mutations (Singh et al., 2009), rendering the true classification of this patient difficult. In this study, we created isogenic human FANCM- and FAAP24-null mutants, allowing their functional impact on DNA damage response to be precisely determined. Our experiments demonstrated novel functions for FANCM and FAAP24 in response to ICLs, further revealing the complexity of the cooperative and distinct functions of these proteins.

FANCM and FAAP24 Work Cooperatively to Activate the FA Network and to Suppress SCE

The epistatic function between FANCM and FAAP24 are reflected by the comparable phenotypes among FANCM and FAAP24 single and double mutants. We show that FANCD2 monoubiquitination and formation of FANCD2 foci upon MMC exposure are affected to the same extent in FANCM, FAAP24 single and double mutants. Recruitment of the FANC core complex is mediated by the MM1 motif in FANCM (Deans and West, 2009), but the retention of the core complex seems to require both FANCM and FAAP24 as our result shows that FAAP24 deletion impairs damage-induced chromatin association of the core complex, identically to the FANCM mutant (Fig. 3G). We also show that MMC-induced chromosomal tetraradials and SCEs occur with equal frequencies among all three mutants. It is likely that the branch migrating activity of FANCM and FAAP24 observed *in vitro* (Xue et al., 2008, Gari et al., 2008a, Gari et al., 2008b) acts to prevent the onset of tetra-radial structures and the onset of SCE by resolving replication fork stalled by ICLs. Additionally, FANCM-dependent recruitment of the BLM helicase reported previously (Deans and West, 2009) appears minimally affected in the *FANCM*$^{-/-}$ mutant. Possibly, a FANCM-independent mechanism is adapted in the deletion mutant to support the loading of BLM to the sites of ICLs where damage-induced homologous recombination between sister chromatids takes place.

FAAP24 But Not FANCM is Needed for ATR-mediated Checkpoint Activation

Noticeable from the genetic analyses is the finding that deletion of *FANCM* and *FAAP24* each yielded unique phenotypes, which suggests that each gene product exhibits non-overlapping function(s) in dealing with DNA crosslinking damage. This finding is somewhat unexpected, because the prevailing notion that FANCM and FAAP24 function together as a heterodimer is derived from several lines of evidence that include their co-purification, coordinated biochemical activities, and their structural resemblance to the heterodimeric nucleases XPF/ERCC1 and MUS81/EME1. However, immune-depletion showed that the majority of FAAP24 was not co-depleted with FANCM (Ciccia et al., 2007) and that 30% of FANCM was not co-depleted with FAAP24 (Yan et al., 2010), indicating that a significant fractions of these proteins can exist outside the FANCM/FAAP24 complex. Our functional analyses fit well with these data and suggest that free forms of FANCM and FAAP24 may be responsible for the non-overlapping functions of the two proteins

FANCM and FAAP24 have been implicated in checkpoint signaling by separate studies (Collis et al., 2008, Huang et al., 2010). Our result (Fig. 5) showed that defects in MMC-induced Chk1 phosphorylation is manifested specifically by the FAAP24 mutant. It is possible that the knockout cells are able to adapt to loss of FANCM but not FAAP24, whereas previous studies employing siRNA depletion reflect a more acute consequence of FANCM and FAAP24 loss. Our result, on the other hand, is consistent with the finding by Huang *et al* that FAAP24 exhibits affinity to crosslinking lesion in the absence of FANCM (Huang et al., 2010), hinting to a FANCM-independent activity of FAAP24 in checkpoint signaling, presumably via interaction with HCLK2 or directly with the ATR-branch of checkpoint components (Collis et al., 2008). Why FAAP24 is uniquely required for crosslink-specific checkpoint activation is unclear. One unique aspect of interstrand crosslink-mediated replication fork stall is that the MCM replicative helicase is unable to pass the lesion, preventing the uncoupling between the MCM helicase and the replicative polymerase, a step necessary to generate sufficient amount of ssDNA for the initiation of ATR checkpoint (Byun et al., 2005). In this context, it is plausible that the FANCM-independent ICL binding activity of FAAP24 serve as an alternative for an enhanced recruitment or retention of ATR-ATRIP kinase.

The DNA Remodeling Activity of FANCM Promotes Recruitment of Repair Factors in Recombination-independent Repair of ICLs

More importantly, we find that deletion of FANCM led to impaired recombination-independent ICL removal and that MMC-treated FANCM mutant exhibits a significant increase in G1 phase cells, presumably the primary cell cycle manifestation of a defective recombination-independent ICL repair. Our results showed that, while fully capable of FANCD2 activation and damage-induced foci formation, the FANCM translocase mutant exhibits readily detectable deficiency in recombination-independent ICL repair. Upon analyzing loading of ICL repair components, we found that enrichment of multiple repair components (Fig. 7F) were affected by loss of the translocase activity. These results are unlikely explained by recruitment through direct interactions with FANCM since the single K117R substitution is not expected to disrupt multiple protein-protein interactions. Rather it suggests that FANCM translocase activity may function in detecting and creating lesion accessibility for the incision and subsequent steps. The constant association of FANCM with chromatin may reflect a scanning mode for the detection of ICLs similar to that of a glycosylase (Cole et al., 2010). Encountering with an ICL and the impediment of DNA passage could serve as a lesion detection mechanism. On the other hand, NER incision requires a significant span of nucleosome-free duplex DNA (Hara et al., 2000). A feasible role for FANCM is to release DNA flanking the ICL lesion from nucleosome contact via its translocase action, allowing the recruitment of incision activities. Consistently, recent observations by Kelsall *et al* show that FANCM participates in the NER process (Kelsall et al., 2012). Alternatively, the FANCM translocase activity can regress a stalled fork so that the ICL reverts to be flanked by duplex DNA, which enables the NER-based recombination-independent repair. This action would provide an additional means of processing fork-stalling ICLs. Although our data does not distinguish which mechanism is more prevalent, both scenarios are supported by the findings that K117R mutant of FANCM is deficient in recombination-independent ICL repair and in the recruitment of repair factors.

Collectively, our findings lead to a refined understanding that FANCM and FAAP24 maintain cellular resistance to crosslinking lesions via three intercalated mechanisms (Fig. 7G), facilitating FANCD2 monoubiquitination and suppressing SCE, activating DNA damage checkpoint, and promoting recombination-independent ICL repair. When cells are individually devoid of FANCM or FAAP24, the FANC core complex activation is partially disabled and the SCE phenotype manifests, accompanied by the loss of recombination-

independent ICL repair or checkpoint control, respectively, resulting in intermediate sensitivities to crosslinking damage. When FANCM and FAAP24 are both absent, the compounded defects in checkpoint control, recombination-independent ICL repair, and FANC core complex activation is expected to render a heightened sensitivity. The deviated functions of FANCM and FAAP24 from the core FA pathways and their nonessential role in FA pathway activation predict that individuals with FANCM or FAAP24 deficiencies may not exhibit classic manifestations of the FA core components patients. Results from this work revealed novel mechanistic insights into how cells are both coordinately and independently protected by FANCM and FAAP24.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies

HCT-116 and HEK293T cells were obtained from ATCC. Wild type HCT-116, its somatic knockout derivatives, and HEK293T cells were maintained in DMEM plus 10% FBS. *ERCC1*$^{+/+}$ and *ERCC1*$^{-/-}$ CHO mutants were cultured in MEM plus 10% FBS. XPA patient fibroblast XP2OS (*XPA*$^{-/-}$) and its isogenic complement were cultured in MEM plus 15% FBS.

Polyclonal antibodies against FAAP24 were raised in rabbits by using MBP-tagged full length FAAP24 protein as the antigen and affinity-purified with GST-FAAP24 fusion protein. Polyclonal rabbit antibodies against FANCM $^{1190–1273}$ were similarly raised and purified. FANCL antibody was described previously (Meetei et al., 2003a). Sources of commercial antibodies: FANCD2 (ab2187, Abcam, sc-20022, Santa Cruz, and NB100-182, Novus); FANCA (A301-980A, Bethyl); FANCG (NB100-2566, Novus); histone H3 (05-928, Millipore); Chk1 (sc-8408, Santa Cruz); phospho-Chk1 Ser317 (2344, Cell Signaling); phospho-Chk1 Ser345 (2348, Cell Signaling); BLM (A300-110A, Bethyl); PCNA (P8825, Sigma-Aldrich); ERCC1 (MS-671-P0, NeoMarkers); REV1 (sc-48806X, Santa Cruz); XPA (MS-650-P1ABX, NeoMarkers).

Somatic Cellular Targeting

FANCM and *FAAP24* targeting vectors were constructed using the USER system (NEB) (Fig. S1). Two approximately 1.5 kb homology arms were PCR-amplified from HCT-116 genomic DNA, and inserted into a modified pAAV-USER vector where they flank a neomycin resistance gene cassette. Packaging of rAAV, infection of HCT-116 cells and PCR screening of targeted clones were described previously (Rago et al., 2007).

Mitotic Spread and SCE Assay

Cells were treated in 40 ng/ml MMC for 18 hr and mitotic spread was prepared as previously described (Wang et al., 2006). Slides were stained with 4% Giemsa and chromosomal abnormalities were quantified by scoring 40–80 metaphases. For SCE assay, cells were cultured in the presence of 100 mM of 5bromodeoxyuridine for approximately 48 hr. For MMC-induced SCE analysis, 20 ng/ml MMC were added 18 hr before harvesting cells.

Immunofluorescence Staining

Cells were cultured on cover slips and treated with desired dosage of MMC for durations specified. Fixed cells were incubated sequentially with desired primary and secondary antibodies for 30 min, and counter stained with DAPI for 2 min. Nuclear foci were counted in at least 100 nuclei at 5 different locations for each sample.

Cell Cycle and Checkpoint Analysis

To determine G1/S transition in response to DNA damage, cells were treated with MMC for 6 or 16 hr, pulse labeled with 10 μM BrdU for 30 min and harvested for staining with Alexa Fluor 488-conjugated anti-BrdU (30 min) and with PI buffer (30 min). Percentage of cells in G1, S, and G2/M phases were determined on an Accuri C6 flow cytometer (BD).

Recombination-Independent ICL Repair Assay

The luciferase reporter reactivation assay was performed as descried previously (Shen et al., 2009). Briefly, 1 ng of reporter substrate with a transcription blocking ICL, or 1 ng of an undamaged control reporter, was transfected into cells together with 50 ng β-galactosidase normalization construct. The relative ICL repair efficiencies were calculated as the percentage of luciferase activity of the crosslinked reporter normalized to that of the undamaged reporter. Each experiment was performed with duplicate transfections.

eChIP Assay

The eChIP assay was carried out according to protocols described earlier (Shen et al., 2009, Wang and Li, 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Dr. Zhenghe Wang for providing reagents and technical advice on rAAV-based targeting and Drs. Lee Zou and Junran Zhang for technical advices on RPA2 immunostaining. This work was supported in part by the Intramural Research Program of the National Institute on Aging (AG000688-07), National Institute of Health (W.W.) and by grants from CPRIT (Multi-Investigator Award; RP110465-P2 to J.C.) and the National Cancer Institute (CA127945 and CA097175 Project 3 to L.L). eChIP substrates preparation and clonogenic survivals were assisted respectively by Core C and Core B (CA097175). J.C. is a recipient of an Era of Hope Scholar award from the Department of Defense (W81XWH-05-1-0470).

References

ALI AM, PRADHAN A, SINGH TR, DU C, LI J, WAHENGBAM K, GRASSMAN E, AUERBACH AD, PANG Q, MEETEI AR. FAAP20: a novel ubiquitin-binding FA nuclear core-complex protein required for functional integrity of the FA-BRCA DNA repair pathway. Blood. 2012; 119:3285–94. [PubMed: 22343915]

BAKKER ST, VAN DE VRUGT HJ, ROOIMANS MA, OOSTRA AB, STELTENPOOL J, DELZENNE-GOETTE E, VAN DER WAL A, VAN DER VALK M, JOENJE H, TERIELE H, DE WINTER JP. Fancm-deficient mice reveal unique features of Fanconi anemia complementation group M. Hum Mol Genet. 2009; 18:3484–95. [PubMed: 19561169]

BIGGERSTAFF M, SZYMKOWSKI DE, WOOD RD. Co-correction of the ERCC1, ERCC4 and xeroderma pigmentosum group F DNA repair defects in vitro. EMBO J. 1993; 12:3685–92. [PubMed: 8253090]

BYUN TS, PACEK M, YEE MC, WALTER JC, CIMPRICH KA. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes Dev. 2005; 19:1040–52. [PubMed: 15833913]

CICCIA A, LING C, COULTHARD R, YAN Z, XUE Y, MEETEI AR, LAGHMANI EL H, JOENJE H, MCDONALD N, DE WINTER JP, WANG W, WEST SC. Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. Mol Cell. 2007; 25:331–43. [PubMed: 17289582]

COLE HA, TABOR-GODWIN JM, HAYES JJ. Uracil DNA glycosylase activity on nucleosomal DNA depends on rotational orientation of targets. The Journal of biological chemistry. 2010; 285:2876–85. [PubMed: 19933279]

COLLIS SJ, CICCIA A, DEANS AJ, HOREJSI Z, MARTIN JS, MASLEN SL, SKEHEL JM, ELLEDGE SJ, WEST SC, BOULTON SJ. FANCM and FAAP24 function in ATR-mediated checkpoint signaling independently of the Fanconi anemia core complex. Mol Cell. 2008; 32:313–24. [PubMed: 18995830]

CROSSAN GP, VAN DER WEYDEN L, ROSADO IV, LANGEVIN F, GAILLARD PH, MCINTYRE RE, GALLAGHER F, KETTUNEN MI, LEWIS DY, BRINDLE K, ARENDS MJ, ADAMS DJ, PATEL KJ. Disruption of mouse Slx4, a regulator of structure-specific nucleases, phenocopies Fanconi anemia. Nat Genet. 2011; 43:147–52. [PubMed: 21240276]

DEANS AJ, WEST SC. FANCM connects the genome instability disorders Bloom's Syndrome and Fanconi Anemia. Mol Cell. 2009; 36:943–53. [PubMed: 20064461]

GARI K, DECAILLET C, DELANNOY M, WU L, CONSTANTINOU A. Remodeling of DNA replication structures by the branch point translocase FANCM. Proc Natl Acad Sci U S A. 2008a; 105:16107–12. [PubMed: 18843105]

GARI K, DECAILLET C, STASIAK AZ, STASIAK A, CONSTANTINOU A. The Fanconi anemia protein FANCM can promote branch migration of Holliday junctions and replication forks. Mol Cell. 2008b; 29:141–8. [PubMed: 18206976]

GERMAN J, SCHONBERG S, CASKIE S, WARBURTON D, FALK C, RAY JH. A test for Fanconi's anemia. Blood. 1987; 69:1637–41. [PubMed: 3107630]

HARA R, MO J, SANCAR A. DNA Damage in the Nucleosome Core Is Refractory to Repair by Human Excision Nuclease. Mol Cell Biol. 2000; 20:9173–9181. [PubMed: 11094069]

HOADLEY KA, XUE Y, LING C, TAKATA M, WANG W, KECK JL. Defining the molecular interface that connects the Fanconi anemia protein FANCM to the Bloom syndrome dissolvasome. Proceedings of the National Academy of Sciences. 2012a

HOADLEY KA, XUE Y, LING C, TAKATA M, WANG W, KECK JL. Defining the molecular interface that connects the Fanconi anemia protein FANCM to the Bloom syndrome dissolvasome. Proc Natl Acad Sci U S A. 2012b; 109:4437–42. [PubMed: 22392978]

HOWLETT NG, TANIGUCHI T, OLSON S, COX B, WAISFISZ Q, DE DIE-SMULDERS C, PERSKY N, GROMPE M, JOENJE H, PALS G, IKEDA H, FOX EA, D'ANDREA AD. Biallelic inactivation of BRCA2 in Fanconi anemia. Science. 2002; 297:606–9. [PubMed: 12065746]

HUANG M, KIM JM, SHIOTANI B, YANG K, ZOU L, D'ANDREA AD. The FANCM/FAAP24 complex is required for the DNA interstrand crosslink-induced checkpoint response. Mol Cell. 2010; 39:259–68. [PubMed: 20670894]

KELSALL IR, LANGENICK J, MACKAY C, PATEL KJ, ALPI AF. The Fanconi anaemia components UBE2T and FANCM are functionally linked to nucleotide excision repair. PLoS One. 2012; 7:e36970. [PubMed: 22615860]

KIM H, YANG K, DEJSUPHONG D, D'ANDREA AD. Regulation of Rev1 by the Fanconi anemia core complex. Nat Struct Mol Biol. 2012; 19:164–70. [PubMed: 22266823]

KIM JM, KEE Y, GURTAN A, D'ANDREA AD. Cell cycle-dependent chromatin loading of the Fanconi anemia core complex by FANCM/FAAP24. Blood. 2008; 111:5215–22. [PubMed: 18174376]

KRATZ K, SCHOPF B, KADEN S, SENDOEL A, EBERHARD R, LADEMANN C, CANNAVO E, SARTORI AA, HENGARTNER MO, JIRICNY J. Deficiency of FANCD2-associated nuclease KIAA1018/FAN1 sensitizes cells to interstrand crosslinking agents. Cell. 2010; 142:77–88. [PubMed: 20603016]

LEUNG JW, WANG Y, FONG KW, HUEN MS, LI L, CHEN J. Fanconi anemia (FA) binding protein FAAP20 stabilizes FA complementation group A (FANCA) and participates in interstrand cross-link repair. Proc Natl Acad Sci U S A. 2012; 109:4491–6. [PubMed: 22396592]

LEVITUS M, WAISFISZ Q, GODTHELP BC, DE VRIES Y, HUSSAIN S, WIEGANT WW, ELGHALBZOURI-MAGHRANI E, STELTENPOOL J, ROOIMANS MA, PALS G, ARWERT F, MATHEW CG, ZDZIENICKA MZ, HIOM K, DE WINTER JP, JOENJE H. The DNA helicase BRIP1 is defective in Fanconi anemia complementation group. J Nat Genet. 2005; 37:934–5.

LING C, ISHIAI M, ALI AM, MEDHURST AL, NEVELING K, KALB R, YAN Z, XUE Y, OOSTRA AB, AUERBACH AD, HOATLIN ME, SCHINDLER D, JOENJE H, DE WINTER JP, TAKATA M, MEETEI AR, WANG W. FAAP100 is essential for activation of the Fanconi anemia-associated DNA damage response pathway. Embo J. 2007; 26:2104–14. [PubMed: 17396147]

LIU T, GHOSAL G, YUAN J, CHEN J, HUANG J. FAN1 acts with FANCI-FANCD2 to promote DNA interstrand cross-link repair. Science. 2010; 329:693–6. [PubMed: 20671156]

MACKAY C, DECLAIS AC, LUNDIN C, AGOSTINHO A, DEANS AJ, MACARTNEY TJ, HOFMANN K, GARTNER A, WEST SC, HELLEDAY T, LILLEY DM, ROUSE J. Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. Cell. 2010; 142:65–76. [PubMed: 20603015]

MEETEI AR, DE WINTER JP, MEDHURST AL, WALLISCH M, WAISFISZ Q, VAN DE VRUGT HJ, OOSTRA AB, YAN Z, LING C, BISHOP CE, HOATLIN ME, JOENJE H, WANG W. A novel ubiquitin ligase is deficient in Fanconi anemia. Nat Genet. 2003a; 35:165–70. [PubMed: 12973351]

MEETEI AR, MEDHURST AL, LING C, XUE Y, SINGH TR, BIER P, STELTENPOOL J, STONE S, DOKAL I, MATHEW CG, HOATLIN M, JOENJE H, DE WINTER JP, WANG W. A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. Nat Genet. 2005; 37:958–63. [PubMed: 16116422]

MEETEI AR, SECHI S, WALLISCH M, YANG D, YOUNG MK, JOENJE H, HOATLIN ME, WANG W. A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. Mol Cell Biol. 2003b; 23:3417–26. [PubMed: 12724401]

MOLDOVAN GL, D'ANDREA AD. How the fanconi anemia pathway guards the genome. Annu Rev Genet. 2009; 43:223–49. [PubMed: 19686080]

MOSEDALE G, NIEDZWIEDZ W, ALPI A, PERRINA F, PEREIRA-LEAL JB, JOHNSON M, LANGEVIN F, PACE P, PATEL KJ. The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. Nat Struct Mol Biol. 2005; 12:763–71. [PubMed: 16116434]

OHASHI A, ZDZIENICKA MZ, CHEN J, COUCH FJ. Fanconi anemia complementation group D2 (FANCD2) functions independently of BRCA2- and RAD51-associated homologous recombination in response to DNA damage. J Biol Chem. 2005; 280:14877–83. [PubMed: 15671039]

POLL EH, ARWERT F, JOENJE H, WANAMARTA AH. Differential sensitivity of Fanconi anaemia lymphocytes to the clastogenic action of cis-diamminedichloroplatinum (II) and trans-diamminedichloroplatinum (II). Hum Genet. 1985; 71:206–10. [PubMed: 4065892]

RAGO C, VOGELSTEIN B, BUNZ F. Genetic knockouts and knockins in human somatic cells. Nat Protoc. 2007; 2:2734–46. [PubMed: 18007609]

ROSADO IV, NIEDZWIEDZ W, ALPI AF, PATEL KJ. The Walker B motif in avian FANCM is required to limit sister chromatid exchanges but is dispensable for DNA crosslink repair. Nucleic Acids Res. 2009; 37:4360–70. [PubMed: 19465393]

SCHWAB RA, BLACKFORD AN, NIEDZWIEDZ W. ATR activation and replication fork restart are defective in FANCM-deficient cells. EMBO J. 2010; 29:806–18. [PubMed: 20057355]

SHEN X, DO H, LI Y, CHUNG WH, TOMASZ M, DE WINTER JP, XIA B, ELLEDGE SJ, WANG W, LI L. Recruitment of fanconi anemia and breast cancer proteins to DNA damage sites is differentially governed by replication. Mol Cell. 2009; 35:716–23. [PubMed: 19748364]

SHEN X, JUN S, O'NEAL LE, SONODA E, BEMARK M, SALE JE, LI L. REV3 and REV1 play major roles in recombination-independent repair of DNA interstrand cross-links mediated by monoubiquitinated proliferating cell nuclear antigen (PCNA). J Biol Chem. 2006; 281:13869–72. [PubMed: 16571727]

SINGH TR, BAKKER ST, AGARWAL S, JANSEN M, GRASSMAN E, GODTHELP BC, ALI AM, DU CH, ROOIMANS MA, FAN Q, WAHENGBAM K, STELTENPOOL J, ANDREASSEN PR, WILLIAMS DA, JOENJE H, DE WINTER JP, MEETEI AR. Impaired FANCD2 monoubiquitination and hypersensitivity to camptothecin uniquely characterize Fanconi anemia complementation group M. Blood. 2009; 114:174–80. [PubMed: 19423727]

SINGH TR, SARO D, ALI AM, ZHENG XF, DU CH, KILLEN MW, SACHPATZIDIS A, WAHENGBAM K, PIERCE AJ, XIONG Y, SUNG P, MEETEI AR. MHF1-MHF2, a histone-fold-containing protein complex, participates in the Fanconi anemia pathway via FANCM. Mol Cell. 2010; 37:879–86. [PubMed: 20347429]

SMOGORZEWSKA A, DESETTY R, SAITO TT, SCHLABACH M, LACH FP, SOWA ME, CLARK AB, KUNKEL TA, HARPER JW, COLAIACOVO MP, ELLEDGE SJ. A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. Mol Cell. 2010; 39:36–47. [PubMed: 20603073]

SMOGORZEWSKA A, MATSUOKA S, VINCIGUERRA P, MCDONALD ER 3RD, HUROV KE, LUO J, BALLIF BA, GYGI SP, HOFMANN K, D'ANDREA AD, ELLEDGE SJ. Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. Cell. 2007; 129:289–301. [PubMed: 17412408]

STOEPKER C, HAIN K, SCHUSTER B, HILHORST-HOFSTEE Y, ROOIMANS MA, STELTENPOOL J, OOSTRA AB, EIRICH K, KORTHOF ET, NIEUWINT AW, JASPERS NG, BETTECKEN T, JOENJE H, SCHINDLER D, ROUSE J, DE WINTER JP. SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. Nat Genet. 2011; 43:138–41. [PubMed: 21240277]

VANDENBERG CJ, GERGELY F, ONG CY, PACE P, MALLERY DL, HIOM K, PATEL KJ. BRCA1-independent ubiquitination of FANCD2. Mol Cell. 2003; 12:247–54. [PubMed: 12887909]

VAZ F, HANENBERG H, SCHUSTER B, BARKER K, WIEK C, ERVEN V, NEVELING K, ENDT D, KESTERTON I, AUTORE F, FRATERNALI F, FREUND M, HARTMANN L, GRIMWADE D, ROBERTS RG, SCHAAL H, MOHAMMED S, RAHMAN N, SCHINDLER D, MATHEW CG. Mutation of the RAD51C gene in a Fanconi anemia-like disorder. Nat Genet. 2010; 42:406–9. [PubMed: 20400963]

WANG W. Emergence of a DNA-damage response network consisting of Fanconi anaemia and BRCA proteins. Nat Rev Genet. 2007; 8:735–48. [PubMed: 17768402]

WANG X, ANDREASSEN PR, D'ANDREA AD. Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. Mol Cell Biol. 2004; 24:5850–62. [PubMed: 15199141]

WANG X, PETERSON CA, ZHENG H, NAIRN RS, LEGERSKI RJ, LI L. Involvement of nucleotide excision repair in a recombination-independent and error-prone pathway of DNA interstrand cross-link repair. Mol Cell Biol. 2001; 21:713–20. [PubMed: 11154259]

WANG X, ZOU L, LU T, BAO S, HUROV KE, HITTELMAN WN, ELLEDGE SJ, LI L. Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress. Mol Cell. 2006; 23:331–41. [PubMed: 16885023]

WANG Y, LI L. Detecting recruitment of DNA damage response factors through the eChIP approach. Methods Mol Biol. 2011; 782:245–55. [PubMed: 21870297]

XIA B, DORSMAN JC, AMEZIANE N, DE VRIES Y, ROOIMANS MA, SHENG Q, PALS G, ERRAMI A, GLUCKMAN E, LLERA J, WANG W, LIVINGSTON DM, JOENJE H, DE WINTER JP. Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. Nat Genet. 2007; 39:159–61. [PubMed: 17200672]

XUE Y, LI Y, GUO R, LING C, WANG W. FANCM of the Fanconi anemia core complex is required for both monoubiquitination and DNA repair. Hum Mol Genet. 2008; 17:1641–52. [PubMed: 18285517]

YAN Z, DELANNOY M, LING C, DAEE D, OSMAN F, MUNIANDY PA, SHEN X, OOSTRA AB, DU H, STELTENPOOL J, LIN T, SCHUSTER B, DECAILLET C, STASIAK A, STASIAK AZ, STONE S, HOATLIN ME, SCHINDLER D, WOODCOCK CL, JOENJE H, SEN R, DE WINTER JP, LI L, SEIDMAN MM, WHITBY MC, MYUNG K, CONSTANTINOU A, WANG W. A histone-fold complex and FANCM form a conserved DNA-remodeling complex to maintain genome stability. Mol Cell. 2010; 37:865–78. [PubMed: 20347428]

YAN Z, GUO R, PARAMASIVAM M, SHEN W, LING C, FOX D 3RD, WANG Y, OOSTRA AB, KUEHL J, LEE DY, TAKATA M, HOATLIN ME, SCHINDLER D, JOENJE H, DE WINTER JP, LI L, SEIDMAN MM, WANG W. A ubiquitin-binding protein, FAAP20, links RNF8-

mediated ubiquitination to the Fanconi anemia DNA repair network. Mol Cell. 2012; 47:61–75. [PubMed: 22705371]

ZHENG HY, WANG X, WARREN AJ, LEGERSKI RJ, NAIRN RS, HAMILTON JW, LI L. Nucleotide excision repair- and polymerase eta-mediated error-prone removal of mitomycin C interstrand cross-links. Molecular & Cellular Biology. 2003; 23:754–761. [PubMed: 12509472]

Article highlights

- FANCM and FAAP24 exhibit non-epistatic functions in cell survival upon DNA damage
- FAAP24 confers unique lesion-specificity in DNA damage cell cycle checkpoint
- FANCM translocase activity is involved in recombination-independent ICL repair

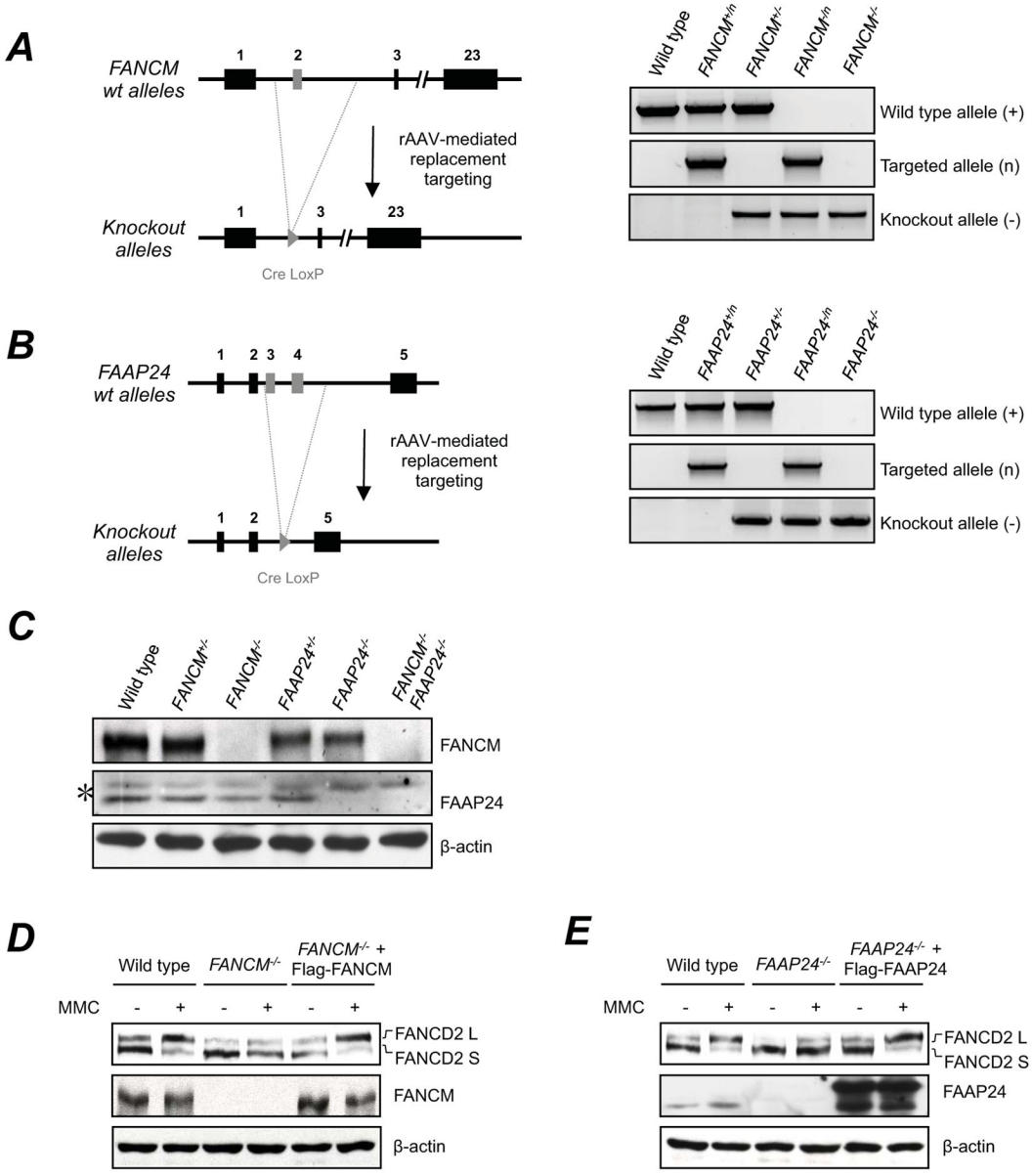

Fig. 1. Homologous replacement targeting of *FANCM* and *FAAP24* loci in HCT-116 cells

(***A***) Left panel, schematics of *FANCM* targeting strategy. Right panel, PCR genotyping of *FANCM*$^{-/-}$ mutant and targeting intermediates. Primers for each PCR amplification are indicated in Fig. S1A.

(***B***) Left panel, schematics of *FAAP24* targeting strategy. Right panel, PCR genotyping of *FAAP24*$^{-/-}$ mutant and targeting intermediates. Primers for each PCR amplification are indicated in Fig. S1B. Numbered boxes indicate exons. Light shaded boxes indicate exon(s) targeted for deletion. Targeted allele (n) depicts the initial targeting event where the targeted exon(s) is replaced by the Neo^{R} cassette. Knockout allele (−) refers to the Cre-treated targeted allele which resulted in the removal of the Neo^{R} cassette.

(***C***) Western blot detecting FANCM and FAAP24 protein loss in somatic knockout cell lines. The asterisk (*) marks a nonspecific band recognized by the FAAP24 antibody.

(***D***) Immunoblot detecting MMC-induced monoubiquitination of FANCD2 in *FANCM*$^{-/-}$ cells complemented with wild-type Flag-FANCM.

(***E***) Immunoblot detecting MMC-induced monoubiquitination of FANCD2 in *FAAP24*$^{-/-}$ cells complemented with wild-type Flag-FAAP24. L and S represent monoubiquitinated and native forms of FANCD2, respectively.

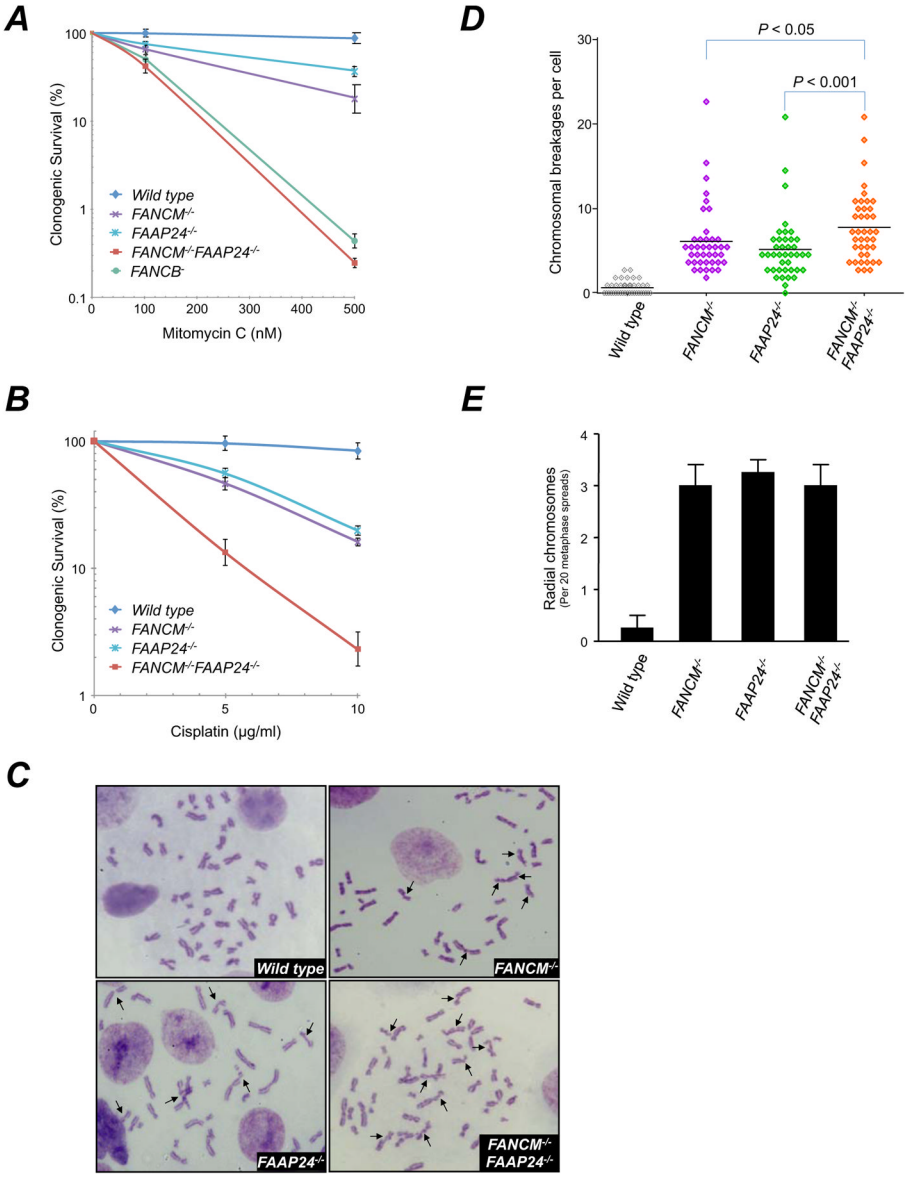

Fig. 2. Differential cellular sensitivities and chromosomal abnormalities in FANCM and FAAP24 single and double mutants

(***A***) Clonogenic survival of *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutants treated with MMC. Error-bars represent standard deviations from six independent experiments with triplicated plates.

(***B***) Clonogenic survival of *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutants treated with cisplatin. Error-bars represent standard deviations from four independent experiments with triplicated plates.

(***C***) Chromosome breakage in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutant cells exposed to MMC (40 ng/ml for 18 hr). Arrows indicate visible chromosome breaks.

(***D***) Quantification of chromosomal abnormality in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutant cells. 40 metaphase spreads were scored for each cell line. Bars represent average chromosomal breakage per spread.

(***E***) MMC-induced radial chromosomes in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutant cells. Quantifications of radial chromosomes in 80 metaphase spreads with each genotype were shown. Error-bars depict standard deviations.

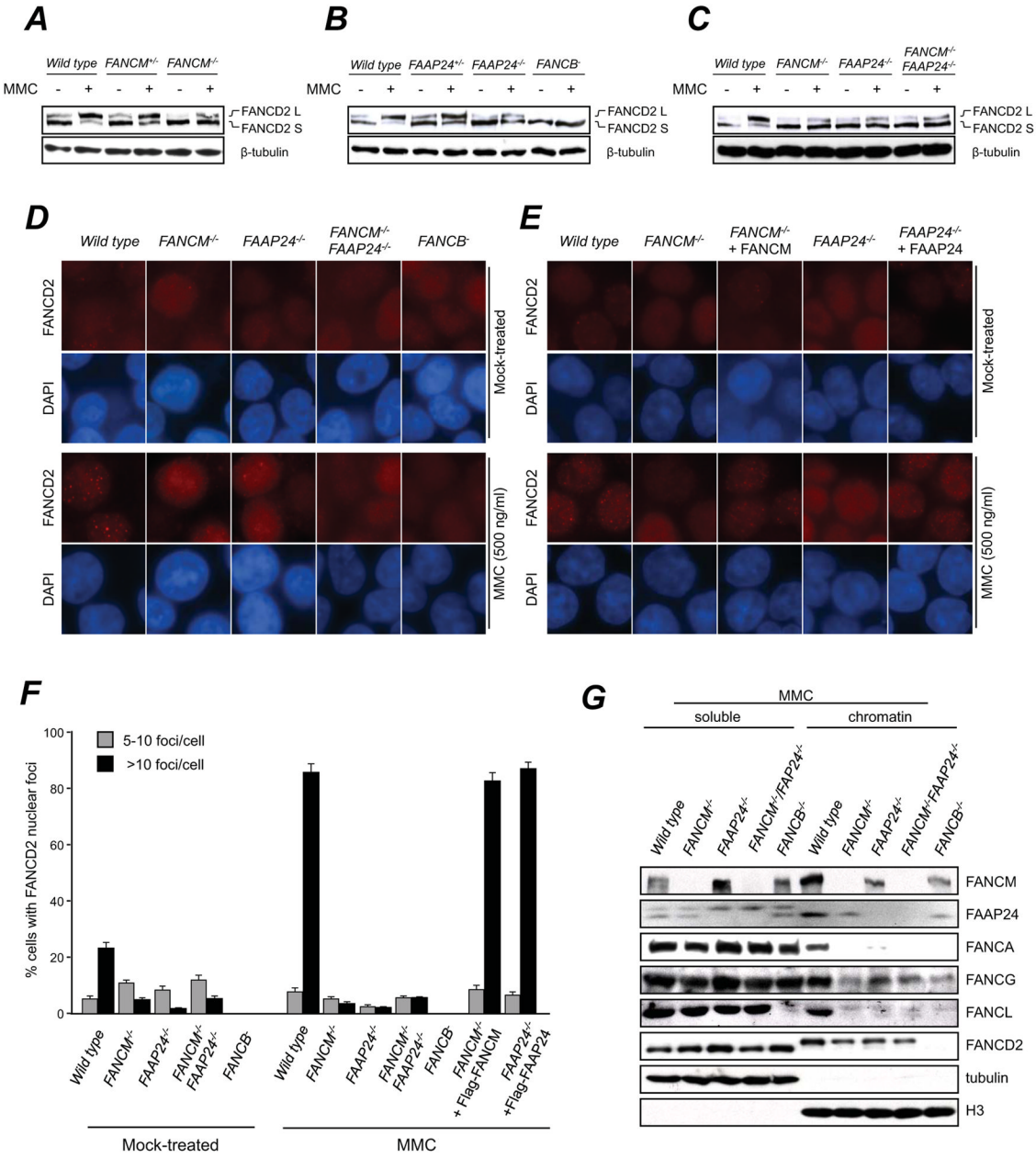

Fig. 3. FANCM and FAAP24 coordinately promote the activation of Fanconi anemia pathway

(***A–C***) Immunoblots detecting FANCD2 monoubiquitination in cells with indicated genotypes, treated or mock-treated with MMC (200 ng/ml, 16 hr).

(***D***) Formation of MMC-induced (500 ng/ml) FANCD2 nuclear foci in FANCM and FAAP24 mutants.

(***E***) Formation of MMC-induced FANCD2 nuclear foci in complemented *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ cells.

(***F***) Quantification of FANCD2 foci in FANCM and FAAP24 mutants and their isogenic complements. Data represent three independent experiments and error bars depict standard deviation derived from 5 data sets.

(***G***) Chromatin association of FANCM, FAAP24, FANCA, FANCG, FANCL and FANCD2 in response to MMC treatment (200 ng/ml, 6 hr).

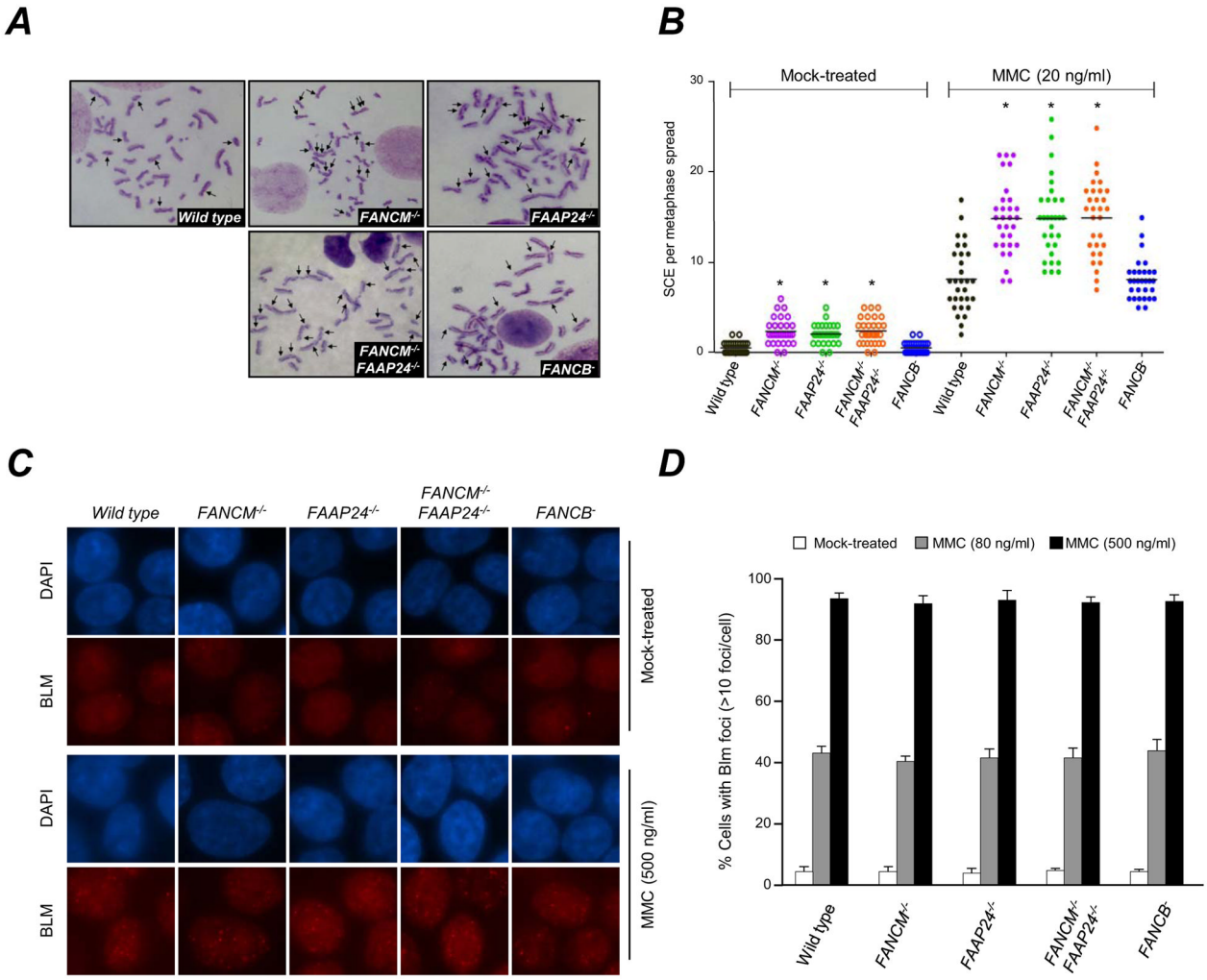

Fig. 4. FANCM and FAAP24 suppress sister chromatid exchanges

(A) Representative metaphase chromosome spreads showing MMC-induced SCEs (arrows) in FANCM and FAAP24 mutants.

(B) Quantification of basal level (mock-treated) and MMC-induced (20 ng/ml for 18 hr) SCEs in FANCM and FAAP24 mutants. 30 metaphases were scored for each sample and bars represent averages. The asterisks (*) denote $P < 0.01$ vs. wild type.

(C) Immunofluorescent staining of BLM foci in FANCM and FAAP24 mutants treated or mock treated with MMC (80 ng/ml or 500 ng/ml for 24 hr).

(D) Quantification of BLM nuclear foci in mock-treated and MMC-treated cells with indicated genotypes. Data are representative of two independent experiments, and error bars represent standard deviation of 5 data points.

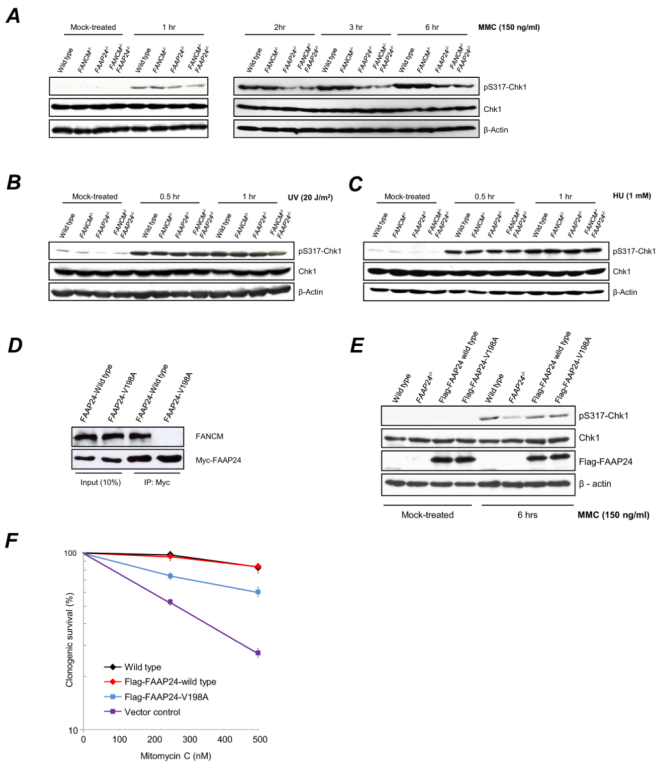

Fig. 5. FAAP24 is required for ATR-mediated checkpoint signaling with distinct damage specificity

(***A***) Ser317 phosphorylation of Chk1 in FANCM and FAAP24 mutant cells exposed to MMC (150 ng/ml) and harvested at indicated time points.

(***B***) Ser317 phosphorylation of Chk1 in FANCM and FAAP24 mutant cells exposed to UV (20 J/m^2) and harvested at indicated time points.

(***C***) Ser317 phosphorylation of Chk1 in FANCM and FAAP24 mutant cells exposed to HU (1 mM) and harvested at indicated time points. Chk1 and β-Actin serve as loading controls.

(***D***) Co-immunoprecipitation of wild-type FAAP24 and the FAAP24-V198A mutant with FANCM.

(***E***) MMC-induced Chk1 activation in *FAAP24*$^{-/-}$ cells complemented with Flag-FAAP24-wild type or Flag-FAAP24-V198A mutant.

(***F***) Clonogenic survival of *FAAP24*$^{-/-}$ cells complemented with Flag-FAAP24-wild type or Flag-FAAP24-V198A. Error-bars represent standard deviations from three independent experiments with triplicated plates.

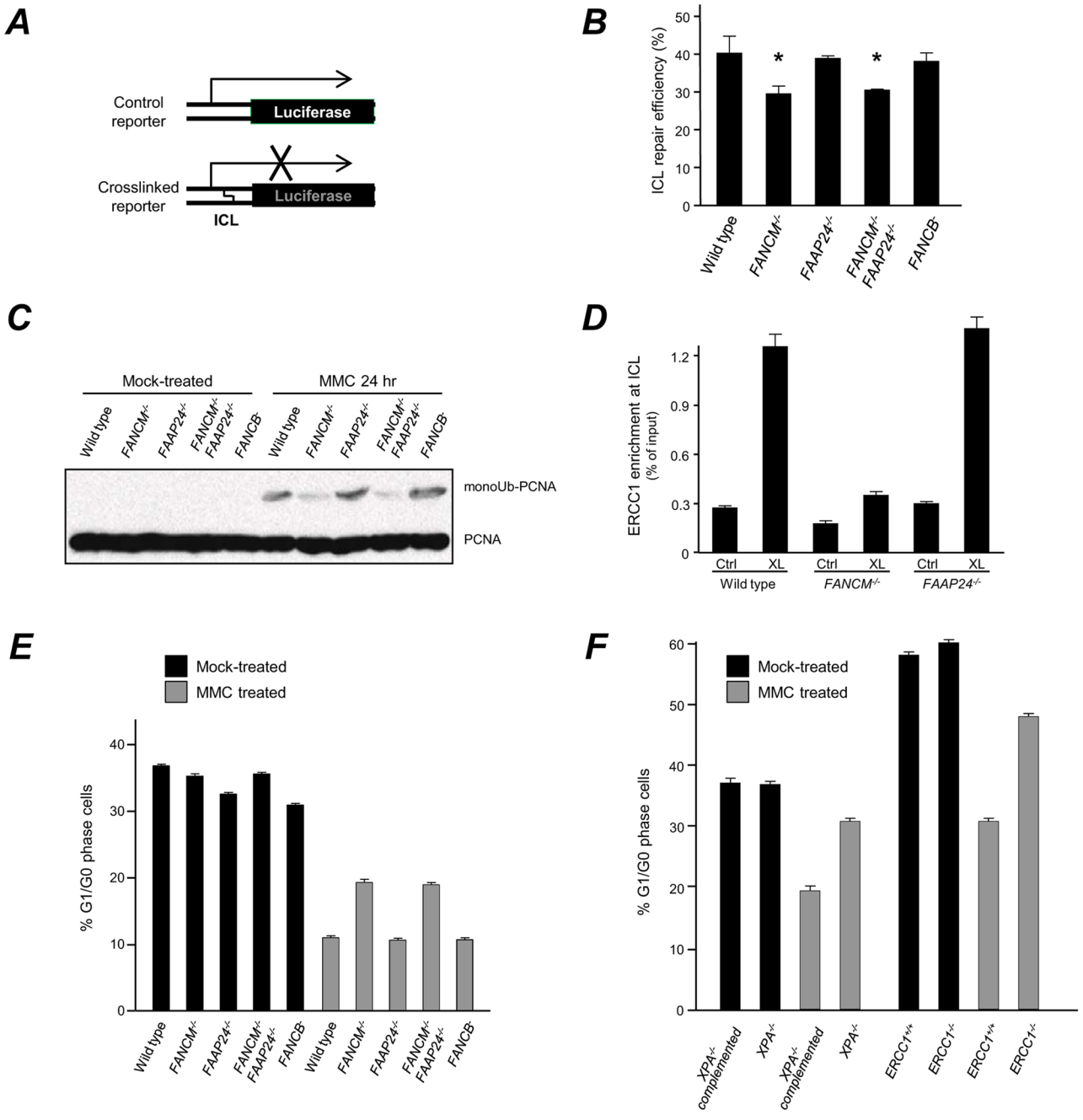

Fig. 6. FANCM deletion leads to defective recombination-independent repair of ICLs

(A) Schematics of the reporter reactivation-based recombination-independent ICL repair assay. Control and crosslinked reporter represent the unmodified and site-specifically crosslinked plasmid substrates, respectively.

(B) Recombination-independent ICL repair efficiencies in FANCM and FAAP24 mutant cell lines. Error bars represent standard deviation of 6 independent experiments (* $P < 0.05$ vs. wild type).

(C) Immunoblot of PCNA monoubiquitination in confluent FANCM and FAAP24 mutants exposed to MMC. Upper and lower bands represent monoubiquitinated and native forms of PCNA respectively.

(D) eChIP assay for ERCC1 recruitment to a site-specific ICL in *FANCM*$^{-/-}$ and *FAAP24*$^{-/-}$ mutant cells. Ctrl: unmodified substrate. XL: crosslinked substrate. Error bars were derived from multiple experiments with duplicated transfections.

(E) G1/G0 cell populations in FANCM and FAAP24 mutant cells exposed to MMC (1000 ng/ml, 16 hr). Cell cycle profile was acquired via BrdU pulse labeling/PI bivariate flow cytometry 16 hours after MMC treatment. Error-bars represent standard deviations from three independent experiments.

(F) G1/G0 populations in XPA and ERCC1 mutant cells treated as in (E). Error-bars represent standard deviations from three independent experiments.

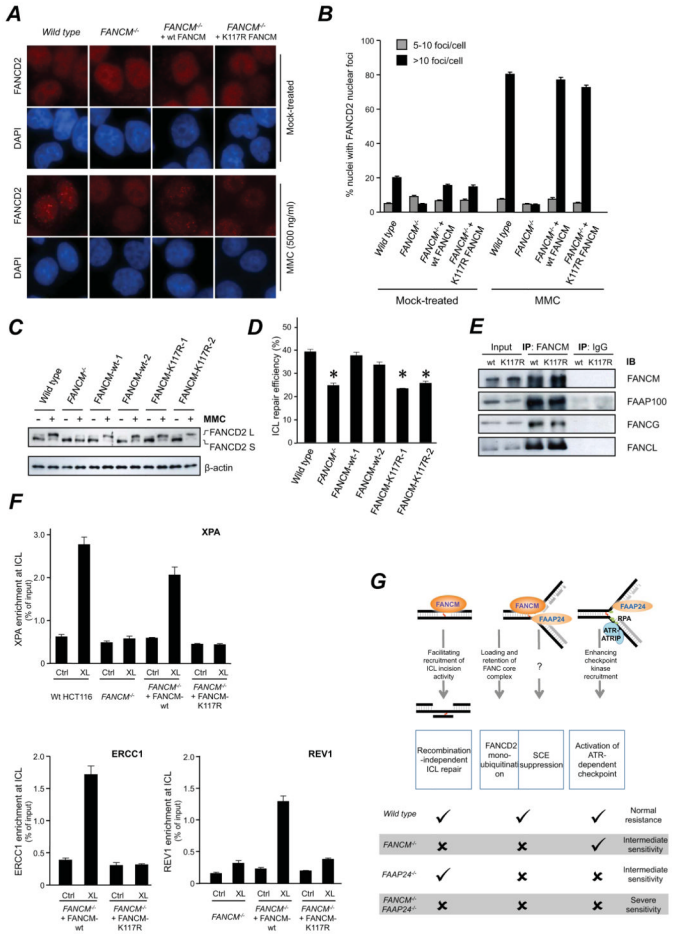

Fig. 7. FANCM translocase activity in recombination-independent ICL repair

(***A***) Formation of MMC-induced FANCD2 nuclear foci in *FANCM*$^{-/-}$ cells stably complemented with wt or K117R FANCM.

(***B***) Quantification of (A). Error bars were derived from three independent experiments.

(***C***) Immunoblot detecting MMC-induced monoubiquitination of FANCD2 in *FANCM*$^{-/-}$ cells complemented with wt or K117R FANCM.

(***D***) Recombination-independent ICL repair efficiencies in *FANCM*$^{-/-}$ cells complemented with wt or K117R FANCM. Error bars represent standard deviation of 6 independent experiments (* $P < 0.05$ vs. wild type).

(***E***) Co-immunoprecipitation detecting interactions between indicated FANC core components and wt or K117R FANCM.

(***F***) eChIP assays detecting recruitments of XPA, ERCC1, and REV1 to a site-specific ICL in *FANCM*$^{-/-}$ cells expressing wild type FANCM or K117R translocase mutant. Ctrl: unmodified substrate. XL: crosslinked substrate. Error-bars represent standard deviations from three independent experiments with duplications.

(***G***) Coordinated and non-epistatic functions of FANCM and FAAP24 in response to DNA interstrand crosslink damage.