The DNA translocase activity of FANCM protects stalled replication forks

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FANCM is the most highly conserved protein within the Fanconi anemia (FA) tumour suppressor pathway. However, although FANCM contains a helicase domain with translocase activity, this is not required for its role in activating the FA pathway. Instead, we show here that FANCM translocase activity is essential for promoting replication fork stability. We demonstrate that cells expressing translocase-defective FANCM show altered global replication dynamics due to increased accumulation of stalled forks that subsequently degenerate into DNA double-strand breaks, leading to ATM activation, CTBP-interacting protein (CTIP)-dependent end resection and homologous recombination repair. Accordingly, abrogation of ATM or CTIP function in FANCM-deficient cells results in decreased cell survival. We also found that FANCM translocase activity protects cells from accumulating 53BP1-OPT domains, which mark lesions resulting from problems arising during replication. Taken together, these data show that FANCM plays an essential role in maintaining chromosomal integrity by promoting the recovery of stalled replication forks and hence preventing tumourigenesis.

INTRODUCTION

The maintenance of replication fork stability is essential for faithful genome duplication. In humans, this is highlighted by the fact that mutations in genes involved in DNA replication and repair lead to diseases with common symptoms, including developmental abnormalities, microcephaly, premature aging and predisposition to cancer (1). One such disorder is Fanconi anemia (FA), a rare genetic condition characterized by bone marrow failure and cellular hypersensitivity to DNA interstrand crosslinking agents, in addition to developmental problems and severe cancer predisposition (2). It is caused by mutations in 1 of 15 genes identified so far, which are designated FANCA-FANCP. Eight of the protein products of these genes form a nuclear core complex with E3 ubiquitin ligase activity that is required for monoubiquitylation of two downstream FA gene products, FANCD2 and FANCI (2). The FA core complex is loaded onto chromatin in S-phase by the FANCM protein (3,4), which is targeted to stalled replication forks by its stable binding partners FAAP24 and the MHF1/2 heterodimer (5–7).

In contrast to most other proteins in the FA pathway, FANCM is an enzyme, with an N-terminal helicase domain that possesses adenosine triphosphate (ATP)-dependent DNA translocase activity (8,9). Full-length FANCM binds specifically to branched DNA molecules that resemble replication intermediates, and can remodel them in vitro (9,10). FANCM translocase activity is not required for the monoubiquitylation of FANCD2 or FANCI, indicating that FANCM has functions outside the FA signalling pathway (11–13). Accordingly, and unlike most other FA cells, FANCM-deficient cells are hypersensitive to a broad range of agents that interfere with DNA replication, such as hydroxyurea (HU) and camptothecin (CPT) (12–15). One role of FANCM outside the FA pathway is to recruit Bloom’s helicase (BLM), the helicase mutated in Bloom’s syndrome, to stalled replication forks (16). Given that it also recruits the FA core complex to

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chromatin, FANCM may act in part as a molecular scaffold to assemble numerous complexes required to promote faithful DNA repair during replication.

Recently, it was shown that FANCM is also required for ATM- and rad3-related (ATR) signalling, independently of its role in the FA pathway (14,15,17). ATR is the master regulator of the cellular response to replication stress (18). It is essential for proper control of replication origin firing and to ensure replisome stability in S-phase, in addition to its functions in checkpoint activation and DNA repair (19). In line with this, FANCM-deficient cells show defective checkpoint activation and excess origin firing (15,17). Thus, FANCM has multiple functions in S-phase, although a specific role for its translocase activity in these processes has not yet been precisely defined.

Here, we investigate the role of the translocase activity of FANCM in relation to its function in the replication stress response. By single-molecule analysis of DNA replication in cells lacking this activity, we find that stalled replication forks are more prone to collapse, generating DNA double-strand breaks (DSBs) that activate ATM and undergo end resection in a CTBP-interacting protein (CTIP)-dependent manner, before being repaired by RAD51-dependent homologous recombination (HR). We also show that FANCM translocase activity protects cells from accumulating increased DNA damage in mitosis that leads to 53BP1-OPT domain formation in G1. Thus, both the structural and enzymatic activities of FANCM are essential to prevent genome instability during DNA replication.

RESULTS

FANCM translocase activity protects stalled replication forks in vivo

By employing single-molecule analysis of replicated DNA, we showed previously that CPT-treated FANCM-deficient cells complete DNA synthesis by firing dormant origins, rather than by restarting damaged replication forks (15). CPT induces DSBs when forks encounter DNA nicks caused by trapped topoisomerase I complexes. In contrast, HU is a reversible inhibitor of ribonucleotide reductase that interferes with DNA synthesis by depleting the dNTP pool available for replication. Importantly, treatment with HU for short times causes forks to slow or stall rather than collapse (20,21). Therefore, having previously established a role for FANCM in restarting damaged forks, we were interested in determining whether FANCM also plays a role in maintaining the stability of stalled replisomes. Analysis of global replication fork dynamics in FANCM-deficient cells treated with HU rather than CPT allowed us to investigate the requirement for FANCM in promoting fork stability versus that in restarting collapsed forks. Given that FANCM can remodel structures that resemble replication forks in vitro (9,10), we were also interested in analysing the role of FANCM translocase activity in this process.

Therefore, we initially monitored DNA synthesis in wild-type (WT) and FANCM-deficient DT40 cells by single-molecule analysis of newly replicated DNA fibres as described previously (15). A schematic diagram showing the experimental design is depicted in Figure 1A, and a representative image of fibres obtained from these cells is shown in Figure 1B. Analysis of DNA synthesis in populations of untreated, exponentially growing WT and FANCMD203A/2 DT40 cells revealed unaltered global replication fork dynamics, as IdU (red) tract lengths were similar (Fig. 1C). However, transient exposure of FANCMD203A/2 cells to HU caused a significant reduction in average fork velocity in comparison to WT cells, as CldU (green) tract lengths were 2-fold shorter (Fig. 1C). This can also be visualized by a leftward shift of the distribution of CldU/IdU tract length ratios in
FANCM-deficient cells (Fig. 1D). To validate these observations further, we went on to assess fork velocity at various times in response to a range of HU doses in DT40 as well as human cells depleted of FANCM by RNA interference (Supplementary Material, Figs S1 and S2). Consistent with our initial observations, we noticed the same leftward shift of the distribution of fork rates in FANCM-deficient cells regardless of HU dose or incubation time.

Next, we asked whether the translocase activity of FANCM was required to promote efficient DNA synthesis in response to HU treatment. To do this, we used DT40 cells expressing FANCM from the endogenous promoter with a point mutation in the ATP-binding site of the helicase domain (FANCMD203A/2) (12). Strikingly, FANCMD203A/2 cells displayed deficiencies in global replication fork velocity similar to the full knockout (Fig. 1C and D and Supplementary Material, Fig. S1).

To account for the slower fork velocity in FANCM-deficient cells compared with the WT, we could envisage at least two scenarios. First, in FANCM-deficient cells exposed to HU, there is a decreased overall rate of DNA polymerization; or secondly, individual replication forks are more likely to stall when progression is hindered. To distinguish between these two possibilities, we measured the relative number of stalled forks after HU treatment, taking advantage of the double labelling fibre technique employed in the experiments described above. Double labelling allowed us to specifically monitor the fates of two sister forks travelling in opposite directions from the same origin. Forks from the same origin tend to display similar replication rates (22); hence in this experiment, an overall decrease in the rate of DNA polymerization would affect both sister forks. Conversely, if individual forks are more prone to stalling, this would cause greater asymmetry between sister fork tract lengths (Fig. 2A). To examine which of the two scenarios was occurring in FANCM-deficient cells, we analysed DNA fibre spreads for the presence of bidirectional forks emanating from an individual origin. Strikingly, we noticed a significant increase in asymmetric sister forks in both FANCM2/2 and FANCMD203A/2 cells (Fig. 2B and C). This indicates that fork stalling occurs at a higher rate in cells lacking FANCM translocase activity, possibly reflecting an inability to efficiently replicate through certain DNA sequences or chromatin structures. An alternative possibility, not mutually exclusive, is that FANCM maintains the stability of forks once they stall, thus preventing fork collapse and allowing for efficient re-establishment of DNA synthesis.

Figure 2. Loss of FANCM translocase activity leads to increased fork asymmetry. (A) Schematic showing examples of two normally progressing sister forks (symmetric) and two sister forks with one fork stalled (asymmetric). (B) Distribution of CldU tract length ratios between sister forks in WT, FANCM−/− and FANCMD203A−/− DT40 cells (*P ≤ 0.0007; **P ≤ 0.006; two-tailed t-test). Numbers of forks evaluated were for WT, n = 62; FANCM−/−, n = 85; FANCMD203A−/−, n = 81. (C) Frequency of asymmetric forks in WT, FANCM−/− and FANCMD203A−/− DT40 cells. Asymmetric forks are defined as two sister forks with a CldU tract ratio greater than the average plus standard deviation of WT cells (χ^2; P ≤ 0.005). (D) Sensitivity of WT, FANCM−/− and FANCMD203A−/− DT40 cells to HU, measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.
Given these results and the fact that FANCM−/− cells are hypersensitive to HU (15), we speculated that FANCMΔ203A−/− cells would display a similar phenotype. Indeed, when we analysed the survival of WT, FANCM−/−, and FANCMΔ203A−/− cells treated with increasing doses of HU, we found that FANCMΔ203A−/− cells were as sensitive to its killing effects as FANCM−/− cells (Fig. 2D). Taken together, our data indicate that FANCM translocase activity plays an important role in promoting genome integrity at stalled replication forks. However, our data argue against a role for FANCM in promoting global replication stress, possibly because these cells accumulate DSBs at stalled forks.

To test this, we examined CHK2 and H2AX activity was specifically required to limit DNA damage in response to replication stress, possibly because these cells accumulate DSBs at stalled forks. Strikingly, with increasing HU incubation times (and even in untreated cells), FANCM-depleted cells accumulated substantially more F2k foci than control cells (Fig. 3D). These data provide further evidence that FANCM is required to prevent the accumulation of DNA breaks in response to HU, and are in line with a recent report showing that prolonged replication stress leads to DSBs (20).

**FANCM-deficient cells accumulate DNA damage in response to replication stress and rely on ATM for survival**

Genomic integrity is challenged during S-phase by replication over chromosomal regions that are inherently difficult to duplicate (e.g. rDNA sequences, G4 motifs or fragile sites). If cells cannot properly stabilize stalled forks, they accumulate spontaneous DNA damage including DSBs, which can be exacerbated by exogenous agents that inhibit replication or by limiting the number of licensed replication origins (23,24). Having established that FANCM translocase activity plays a key role in limiting stalled fork accumulation in cells exposed to replicative stress, we next wished to examine whether FANCM-deficient cells would also accumulate DNA damage under these conditions. To test this, we first examined H2AX phosphorylation in DT40 cells exposed to HU. Significantly, both FANCM−/− and FANCMΔ203A−/− cells displayed increased levels of γ-H2AX compared with WT cells (Fig. 3A), indicating that FANCM prevents accumulation of DNA damage in response to replication stress.

While H2AX can be phosphorylated after damage by ATM, ATR and DNA-PK, the checkpoint kinase CHK2 is a specific target of ATM and thus serves as a readout of ATM activation (19). In chicken cells, the major ATM phosho-site on CHK2, T68, is not conserved, so we examined this modification in human cells treated with non-targeting or FANCM-specific siRNAs. As in DT40 cells, γ-H2AX levels were elevated in FANCM-depleted HeLa cells in response to HU (Fig. 3B). When we examined CHK2 phosphorylation, we also found it to be elevated in FANCM-depleted cells (Fig. 3B). These results indicate that FANCM-deficient cells show increased ATM signalling in response to replication stress, possibly because these cells accumulate DSBs at stalled forks.

We next wished to know whether FANCM translocase activity was specifically required to limit DNA damage in response to HU. To test this, we examined CHK2 and H2AX phosphorylation in human cells stably expressing siRNA targeting FANCM, complemented with either WT or translocase-deficient (K117R) FANCM (17). CHK2 and H2AX phosphorylation levels were similar in FANCM-depleted cells or in cells expressing FANCM-K117R; however, they were reduced in cells complemented with FANCM-WT (Fig. 3C). Taken together, these results indicate that FANCM translocase activity is required to prevent the formation of replication-associated DSBs and consequently excess DNA damage signalling in response to replication stress.

ATM initiates not only a phosphorylation cascade, but also a ubiquitin-dependent sequence of events in response to DNA damage (25). These are mediated by the RNF8 and RNF168 ubiquitin ligases, which produce ubiquitin conjugates at DSB sites that can be readily detected by immunofluorescence and thus serve as a reliable readout for DSB formation. We therefore examined the appearance of ubiquitin foci marked by the monoclonal antibody FK2 in HU-treated control or FANCM-depleted cells. Strikingly, we found that there was a substantial increase in ubiquitin foci formation in cells treated with non-targeting or FANCM-specific siRNAs at various times after HU addition. These results indicate that FANCM-depleted cells rely on the ATM pathway for survival, probably because of increased formation of spontaneous replication-associated DSBs in these cells.

**Collapsed forks in FANCM-deficient cells are repaired by HR after nucleolytic processing**

In S-phase, DSBs may be preferentially repaired by HR if a sister chromatid is present to serve as template (27). The RAD51 recombinase plays a critical role in the strand invasion step of HR repair, and forms foci at sites where stalled forks have collapsed into DSBs (20). As FANCM-deficient cells show signs of increased DSB formation, we monitored RAD51 foci formation in cells treated with non-targeting or FANCM-specific siRNAs at various times after HU addition. Strikingly, we found that there was a substantial increase in the number of RAD51 foci in FANCM-depleted cells, even before treatment (Fig. 4A), indicating that in the absence of FANCM, replication forks are more likely to degenerate into DSBs that are substrates for HR. In addition, we found that the translocase activity of FANCM was required to limit RAD51 foci, as FANCM-depleted cells complemented with the K117R mutant still showed elevated RAD51 foci compared with cells complemented with the WT protein (Fig. 4B).

DSB repair by HR requires processing of DNA ends by CTIP and MRE11 to generate the single-stranded DNA (ssDNA) substrate for RAD51 (28). Previously, we reported that levels of chromatin-bound RPA are elevated in FANCM-
deficient cells (15), but the relevance of this observation was unclear. Interestingly, it has been shown recently that RPA2 hyperphosphorylation is a marker of DNA end resection (29). Therefore, to understand the relevance of increased RPA accumulation on chromatin in the absence of FANCM further, we analysed its phosphorylation status in FANCM-deficient cells. Strikingly, we found that RPA2 phosphorylation was also elevated in FANCM-depleted cells compared with control cells (Fig. 3B). These observations further support our hypothesis that FANCM-depleted cells accumulate significantly higher numbers of DSBs. If this were correct, one would expect that a proportion of these breaks would be processed by CTIP in order to initiate the HR repair process and this might plausibly explain the increased levels of RPA hyperphosphorylation and chromatin accumulation that we observed. To test this hypothesis, we
initially monitored the formation of ssDNA by immunofluorescence of cells incubated in medium containing BrdU under non-denaturing conditions using a method described previously (30). In FANCM-depleted cells and cells complemented with translocase-dead FANCM, more BrdU-ssDNA foci were observed compared with cells complemented with WT FANCM (Fig. 5A), indicating that the enzymatic activity of FANCM prevents the accumulation of ssDNA in response to replication stress. Next, we co-depleted FANCM and CTIP (Fig. 5B), and examined the effect on BrdU foci formation in HU-treated cells. While we could readily observe higher numbers of BrdU-ssDNA foci in FANCM-depleted cells after HU treatment, strikingly co-depletion of CTIP reduced these foci back to normal levels (Fig. 5C). Since CTIP-dependent end resection requires initial processing of the broken DNA ends by MRE11 (31), we also tested the requirement for the latter protein in generating excess ssDNA in FANCM-deficient cells. In line with our initial observations, we obtained similar results when we co-depleted FANCM and MRE11 (Supplementary Material, Fig. S3). Thus, CTIP/MRE11-dependent DNA end-processing, and not excessive DNA unwinding (14), generates increased RPA-ssDNA in FANCM-deficient cells.

Processing of DNA by CTIP at replication forks in FANCM-depleted cells could either be acting to repair DSBs generated by fork collapse, or as an aberrant event occurring at stalled forks in the absence of FANCM. Such a scenario has been shown recently for BRCA2, which stabilizes stalled forks by restraining nucleases from processing them in a detrimental manner (32). To find out whether a similar situation was occurring in FANCM-deficient cells, we examined whether CTIP co-depletion with FANCM could rescue the genome instability found in these cells by analysing spontaneous micronucleus (MN) formation. In FANCM-deficient cells and in cells expressing translocase-dead FANCM, we found MN to be elevated by ~2-fold (Fig. 5D). CTIP knockdown also resulted in an increase in MN, consistent with its role in HR (Fig. 5E). Strikingly, when we depleted both CTIP and FANCM, the effect was additive (Fig. 5E). To further support these observations, we analysed the survival of cells concomitantly depleted of FANCM and CTIP in response to HU (Fig. 5F).
Interestingly, while FANCM knockdown resulted in hypersensitivity to HU, CTIP depletion by itself also caused significant hypersensitivity to HU, suggesting a previously uncharacterized role in response to replication stress. Crucially, however, FANCM and CTIP co-depletion resulted in an additive effect in terms of HU sensitivity. These results show that in contrast to the situation in BRCA2-deficient cells (where MRE11 pathologically processes stalled forks) (32), CTIP/MRE11-dependent resection is required to repair collapsed forks that accumulate in the absence of FANCM translocase activity. This would be in line with the fact that the HR machinery plays an essential role in cell survival and repair of DSBs formed during replication and in the suppression of tumourigenesis (23,33).

Figure 5.Collapsed forks in FANCM-deficient cells are repaired after processing by CTIP. (A) HEK293 cells expressing siRNA targeting FANCM alone or complemented with either FANCM-WT or FANCM-K117R were incubated in 10 μM BrdU for 24 h before exposure to 2 mM HU. Cells were then fixed and stained with anti-BrdU antibodies to visualize ssDNA under non-denaturing conditions. (B) Western blot showing efficiency of FANCM and CTIP knockdowns. Asterisk denotes a non-specific band detected by the antibody. (C) HeLa cells treated with the indicated siRNAs were incubated in medium containing BrdU for 24 h before incubation in 2 mM HU. Cells were then fixed and stained with anti-BrdU antibodies to visualize ssDNA under non-denaturing conditions. (D) Micronuclei in HEK293 cells expressing siRNA targeting FANCM alone or complemented with either FANCM-WT or FANCM-K117R were scored by immunofluorescence analysis of DAPI-stained cells. (E) Micronuclei in siRNA-treated HeLa cells were scored by immunofluorescence analysis of DAPI-stained cells. (F) Clonogenic survival of HeLa cells treated with non-targeting (siCtrl), FANCM (siFANCM) and/or CTIP-specific siRNAs (siCTIP) after treatment with increasing doses of HU for 24 h. Error bars indicate the standard error of the mean from three independent experiments.
FANCM limits 53BP1-OPT domain formation

In addition to DSB formation, one might expect replication stress to lead to under-replicated chromosomal regions before mitotic entry in cells lacking FANCM translocase activity. Recently, it has been shown that a class of DNA lesions caused by replication stress is transmitted to daughter cells and protected from erosion in nuclear bodies called 53BP1-OPT domains (34,35). These structures persist in G1-phase of the cell cycle but are resolved during the following S-phase, consistent with them being lesions caused by regions of under-replicated DNA rather than classical DSBs. Given the above, we wished to examine whether FANCM-deficient cells display increased levels of these OPT domains by examining 53BP1 focus formation exclusively in unperturbed G1 cells, using Cyclin A as a marker to eliminate S and G2 populations. Strikingly, in FANCM-depleted cells, the number of 53BP1 foci was significantly higher than in control cells (Fig. 6A), indicating that FANCM prevents formation of 53BP1-OPT domains and under-replication of genomic regions that are intrinsically hard to replicate. As the translocase activity of FANCM is required to prevent fork collapse, we also examined 53BP1-OPT domain formation in FANCM-depleted cells alone and in cells complemented with FANCM-WT or FANCM-K117R. Only complementation with FANCM-WT restricted the number of 53BP1-OPT domains, indicating that the translocase activity of FANCM is required to prevent formation of these nuclear bodies (Fig. 6B).

53BP1-OPT domains form in G1 daughter cells as a result of unresolved replication intermediates generated in the parental cell during the previous S-phase (34,35), so these lesions are already visible in the preceding mitosis. γ-H2AX is used as a marker of this mitotic damage (35), as the ubiquitin-dependent signalling required for formation of 53BP1 foci is not present in mitosis (36). Consistent with previous results (35), we found that mitotic γ-H2AX foci appeared to symmetrically mark the condensed chromosomes in anaphase cells and foci numbers on metaphase chromosomes compared with those on a single set of anaphase chromosomes were similar. These observations highlight their origin as a shared lesion on the parental DNA strands that persists in both daughter nuclei after cell division. Strikingly, the numbers of these lesions increased dramatically in FANCM-depleted cells (Fig. 6C), and further experiments showed that the translocase activity of FANCM is required to prevent the appearance of elevated numbers of these mitotic lesions (Fig. 6D).

Taken together, our data indicate that the enzymatic activity of FANCM is required not only to prevent DSB formation at stalled forks, but also to limit other chromosomal aberrations that may escape the G2/M checkpoint, persist through mitosis and manifest as 53BP1-OPT domains in the following G1-phase. Thus, our data firmly establish a critical role for FANCM in the maintenance of genome stability during DNA replication.

DISCUSSION

Cells must maintain stable replisomes in order to faithfully duplicate the genome. However, replication forks frequently stall while progressing through so-called chromosomal ‘replication slow zones’ (37). Prolonged fork stalling can lead to fork collapse (20), and in line with this, replication pause sites are susceptible to chromosomal breaks and genomic rearrangements (38). Importantly, unscheduled recombination between homologous chromosomes can result in loss of heterozygosity that may ultimately lead to cancer. Therefore, understanding mechanisms and pathways required for maintaining fork stability during DNA replication is of great importance. We propose that one such mechanism requires the enzymatic activity of FANCM. This is because in vitro, FANCM is a DNA translocase that can recognize, bind and remodel DNA substrates that resemble replication forks (8–10). However, whether FANCM performs such a function in vivo is still unclear. Here, we have provided experimental evidence to show that FANCM, and specifically its translocase activity, plays a critical role during DNA replication by stabilizing stalled forks to prevent their collapse into DSBs. This conclusion is based on the following observations. First, FANCM translocase activity is required to promote efficient replication fork progression by preventing fork stalling. In support of this, we have shown that cells lacking FANCM translocase activity show greater asymmetry between two sister forks emanating from the same origin (representing the accumulation of stalled forks). Secondly, FANCM-deficient cells treated with the replication inhibitor HU display increased ATM activation. ATM is required to facilitate replication restart when forks degenerate into DSBs, and therefore acts as a backup pathway to allow completion of DNA replication in times of replicative stress (39). Accordingly, we found that FANCM-deficient cells rely on ATM kinase activity for survival. Finally, we examined the fate of broken forks in FANCM-deficient cells, and showed that they undergo extensive CTIP- and MRE11-dependent processing to produce ssDNA filaments required for HR repair. In line with this, ATM or CTIP depletion sensitizes FANCM-deficient cells to HU, suggesting that FANCM and the HR machinery have compensatory roles in promoting genome integrity at stalled forks. This is in contrast to recently published work describing a role for BRCA2 in suppressing pathological resection of DNA by MRE11 within stalled replisomes (32). This suggests that while BRCA2 limits processing by MRE11 of stalled forks that can be restarted, the enzymatic activity of FANCM is required to prevent forks from collapsing into DSBs that actually require resection by MRE11 and CTIP for their faithful repair by HR.

One consequence of increased fork stalling is that DNA replication may be incomplete before cells enter mitosis. Unresolved replication intermediates prevent proper chromosome segregation, resulting in increased anaphase bridging and the formation of 53BP1-OPT domains in G1daughter cells (34,35). In line with this, we have shown that these are upregulated in the absence of FANCM translocase activity. Notably, a recent study showed that cells expressing translocase-defective FANCM also show higher levels of anaphase bridging (40), in agreement with our data reported here.

DSBs that form as a result of replication stress are not randomly distributed across the genome, but tend to form within zones that are intrinsically hard to replicate, such as rDNA repeats, G4 sequences and chromosomal fragile sites (37).
53BP1-OPT domains also preferentially form at these regions in the genome (34,35), and significantly such sites are hotspots for increased sister chromatid exchanges (SCEs), which are a hallmark of FANCM-deficient cells (3,16,41). This raises the possibility that the increased SCEs that occur in the absence of FANCM arise due to replication fork collapse and incomplete replication before cell division. In support of this hypothesis, FANCM appears to play no obvious part in the HR repair
process (3), and there is no additional significant increase in SCE frequency in FANCM-deficient mouse embryonic fibroblasts upon treatment with DSB-inducing agents compared with control cells (41).

The enzymatic activity of FANCM has previously been shown to be required for efficient ATR signalling (17), and defects in the ATR/CHK1 pathway give rise to some of the phenotypes we have described here in cells lacking FANCM translocase activity (18,19). It is therefore conceivable that defective ATR signalling accounts for some of the phenotypes of FANCM-deficient cells. However, previous work has demonstrated that inhibition of CHK1 in FANCM-deficient cells exacerbates the replication phenotypes in these cells, and is detrimental to their survival (15 and data not shown). Hence, we believe that the fork stabilization defects found in FANCM-deficient cells are not solely due to defective ATR activation, although as we still do not know the mechanistic basis for the ATR signalling defects in FANCM-deficient cells, it is at present impossible to test this hypothesis.

This work establishes the genetic basis for the role of FANCM in promoting fork stability and preventing formation of replication-associated DSBs, thus revealing a fundamental role for FANCM in response to replicative stress. Both processes are clearly important for tumour suppression, and in line with this FANCM-deficient mice show decreased tumour-free survival (41), and in humans homozygous mutations in FANCM have been found in breast cancer (42) and as a polymorphic risk factor in osteosarcoma (43).

In conclusion, we propose that during replication stress, FANCM acts not only as a molecular platform for the FA core complex and BLM, but also uses its enzymatic activity to coordinate DNA replication with repair at stalled forks (Fig. 7).

**MATERIALS AND METHODS**

**Cells and chemicals**

Human cells were maintained at 37°C in humidified incubators supplied with 5% CO₂. HeLa and U2OS cells were a gift from Dr Fumiko Esashi and were grown in Roswell Park Memorial Institute (medium) and DMEM, respectively, supplemented with 8% FCS and penicillin–streptomycin.

HEK293 cells expressing FANCM-siRNA™ have been described earlier (17). DT40 cells were obtained from Dr K. J. Patel (3,12), and were cultured as described previously (15). ATM inhibitor KU-55933 was from Merck, and all other chemicals used in this study were from Sigma-Aldrich unless stated otherwise.

**Antibodies, SDS–PAGE and western blotting**

Primary antibodies used in this study are described in Supplementary Material, Table S1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were done as described previously (44).

**DNA fibre analysis and cell survival assays**

DNA fibre analyses and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays were performed as described (15). For each DNA fibre experiment, a minimum of 200 individual fibres was counted unless otherwise noted. Colony survival assays were carried out by re-plating cells treated with siRNAs 48 h after transfection at low densities. The following day, cells were treated with HU at the indicated concentrations for 24 h. Cells were then left to grow for 10–12 days, and colonies were counted by staining in 1% methylene blue/50% ethanol solution.

**RNA interference**

Non-targeting siRNAs and siRNAs targeting FANCM, CTIP and MRE11 were ON-TARGET plus SMART pools from Thermo Scientific, and were transfected using Oligofectamine (Invitrogen), as described previously (45).

**Immunofluorescence**

Immunofluorescence analyses were carried out essentially as described previously (44), but with the following modifications: for visualization of γ-H2AX and BrdU foci, cells were pre-extracted for 5 min in piperazine-N,N'-bis(2-ethanesulfonic acid) [PIPES] buffer (20 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100, 10 mM PIPES, pH 6.8) at
4°C before fixation. FK2 and RAD51 foci were visualized by fixing cells in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, followed by permeabilization in 0.5% Triton X-100 in PBS for 5 min, all at RT. For quantification of foci, at least 100 cells were scored in each case unless otherwise noted. Error bars in graphs indicate the standard error of the mean from three independent experiments.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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