The concerted roles of FANCM and Rad52 in the protection of common fragile sites

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Common fragile sites (CFSs) are prone to chromosomal breakage and are hotspots for chromosomal rearrangements in cancer cells. We uncovered a novel function of Fanconi anemia (FA) protein FANCM in the protection of CFSs that is independent of the FA core complex and the FANCI–FANCD2 complex. FANCM, along with its binding partners FAAP24 and MHF1/2, is recruited to CFS-derived structure-prone AT-rich sequences, where it suppresses DNA double-strand break (DSB) formation and mitotic recombination in a manner dependent on FANCM translocase activity. Interestingly, we also identified an indispensable function of Rad52 in the repair of DSBs at CFS-derived AT-rich sequences, despite its non-essential function in general homologous recombination (HR) in mammalian cells. Suppression of Rad52 expression in combination with FANCM knockout drastically reduces cell and tumor growth, suggesting a synthetic lethality interaction between these two genes, which offers a potential targeted treatment strategy for FANCM-deficient tumors with Rad52 inhibition.

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Common fragile sites (CFSs) are large chromosomal regions where gaps and breaks are recurrently generated upon replicative stress. They are preferentially unstable during the early stages of cancer development and often associated with chromosomal rearrangement sites in tumors. Compelling evidence suggests that perturbation of DNA replication at these regions is a major cause for CFS instability. Aberrant oncogene expression promotes CFS breakage (often called CFS expression), likely due to oncogene-induced replication stress. It is believed that CFS instability is one driving force for tumorigenesis.

CFSs are enriched with interrupted AT-dinucleotide repeats (AT-rich), which are predicted to form DNA secondary structures. Such AT-rich sequences derived from FRA16D cause replication fork stalling, and induce double-strand break (DSB) formation and mitotic recombination. DNA combing analysis also demonstrated that fork arrest at the endogenous FRA16C site is preferentially close to the AT-rich sequences. Thus, forming DNA secondary structures at CFSs is an important factor to induce fork stalling and CFS destabilization.

Co-genetic studies have revealed that chromosomal breakpoints in Fanconi anemia (FA) patients often colocalize with CFSs. Consistently, FA proteins play important roles in CFS protection. FA is a genetically heterogeneous disorder characterized by severe genome instability, extreme sensitivity to interstrand crosslinking (ICL) agents, developmental abnormalities, bone marrow failure, and cancer predisposition. Upon DNA damage, the FA core complex (composed of FANCA, FANCB, FANCC, FANC D, FANCF, FANCG, FANCL, and FANCM along with their association proteins) is required for monoubiquitinating the FANCD2 and FANCI heterodimeric complex (ID2), which marks the activation of the FA pathway. Downstream FA proteins including FANC D1/BRCA2, FANCI/ BRIP1, FANCN/PALB2, and FANCO/RAD51 are important for homologous recombination (HR)-mediated DSB repair.

FANCM is a component of the FA core complex and it also forms tight complexes with its binding partners FAAP24 and MHF1/2 (MHF). It contains an N-terminal DEAH helicase domain and exhibits an ATP-dependent DNA-remodeling translocase activity. The localization of the FA core complex to chromatin and monoubiquitination of ID2 complex require FANCM but not its translocase activity. In vitro, FANCM binds specifically to model replication forks and Holliday junctions and promotes fork reversal and migration of junction points in an ATPase-dependent manner. Biochemical studies have demonstrated that both MHF and FAAP24 stimulate DNA binding by FANCM, and MHF promotes the fork remodeling activity of FANCM. In this study, we have identified a new role of FANCM in the maintenance of CFS stability, that is independent of the previously described function of the FA core and ID2 complexes in CFS protection. HR plays an important role in CFS protection, and it has been shown that the AT-rich sequence Flex1 derived from FRA16D induces HR-mediated mitotic recombination. In mammalian cells, Rad51, BRCA1, and BRCA2 are required for HR. However, despite an essential function of Rad52 for HR in yeast, Rad52 is not required for HR in mammalian cells. Knockout (KO) of the Rad52 gene in mice has almost no phenotype in recombination and repair; this is different from Rad51 KO, which shows early embryonic lethality. In this study, we found a novel function of Rad52 for repairing DSBs accumulated at the AT-rich sequences present in CFSs when FANCM is deficient. Combined inactivation of FANCM and Rad52 leads to a strong cell proliferation defect, suggesting a synthetic lethality interaction between these two genes.

Results

FANCM suppresses Flex1-induced mitotic recombination in a manner independent of the FA core complex. We showed that the AT-rich sequence Flex1 derived from FRA16D is genetically unstable, and induces HR-mediated mitotic recombination, as revealed by the EGFP-based HR reporter, HR-Flex (containing Flex1) using HR-Luc reporter (containing a luciferase fragment) as a control. We further showed that the AT-rich sequences 16C-AT1 and 16C-AT3 derived from FRA16C, and 3B-AT derived from FRA3B also induce mitotic recombination in a similar manner as Flex1. Thus, it is a common feature that AT-rich and structure-forming DNA sequences derived from CFSs are unstable and induce mitotic recombination.

To further study the mechanisms of CFS protection, we examined mitotic recombination using the HR-Flex reporter after inactivation of different repair proteins. Depletion of Rad51, Mre11, Nbs1, BrcA1, or BrcA2 by shRNAs leads to a reduction of Flex1-induced mitotic recombination (Supplementary Fig. 2a and 2b). Similar increase of mitotic recombination at Flex1 was observed in other cell lines including Hela, MCF7, and T98G when FANCM expression is suppressed by shRNAs (Fig. 1c and Supplementary Fig. 3a). In addition, mitotic recombination at Flex1 and the AT-rich sequence (3B/AT) derived from FRA3B is substantially higher in HCT116 FANCM KO cells compared to HCT116 wild-type cells.

Different from FANCM, depletion of the FA core complex protein FANCA and FANCD2 results in a much weaker effect (Fig. 1b and Supplementary Fig. 2c). Furthermore, the FANCM-MM1 mutant, which is defective in FANCM binding to the FA core complex, suppresses Flex1-induced mitotic recombination to a similar level as FANCM-WT (Fig. 1e). Thus, the significant role of FANCM in suppression of Flex1-induced mitotic recombination is largely independent of the FA core and ID2 complexes. We further showed that when a DSB is generated adjacent to Flex1 or Luc by the endonuclease I-SceI, depletion of FANCM, FANCA, or FANCD2 similarly leads to a detectable but mild defect in HR (Fig. 1f and Supplementary Fig. 4), consistent with the role of these proteins in HR. Interestingly, suppression of FANCM expression leads to a substantial increase of spontaneous mitotic recombination at Flex1 in U2OS cells with HR-Luc as a control (Fig. 1b and Supplementary Fig. 2c). Similar increase of mitotic recombination at Flex1 was also observed in other cell lines including Hela, MCF7, and T98G when FANCM expression is suppressed by shRNAs (Fig. 1c and Supplementary Fig. 3a). In this study, we have identified a new role of FANCM in the maintenance of CFS stability, that is independent of the previously described function of the FA core and ID2 complexes in CFS protection. HR plays an important role in CFS protection, and it has been shown that the AT-rich sequence Flex1 derived from FRA16D induces HR-mediated mitotic recombination. In mammalian cells, Rad51, BRCA1, and BRCA2 are required for HR. However, despite an essential function of Rad52 for HR in yeast, Rad52 is not required for HR in mammalian cells. Knockout (KO) of the Rad52 gene in mice has almost no phenotype in recombination and repair; this is different from Rad51 KO, which shows early embryonic lethality. In this study, we found a novel function of Rad52 for repairing DSBs accumulated at the AT-rich sequences present in CFSs when FANCM is deficient. Combined inactivation of FANCM and Rad52 leads to a strong cell proliferation defect, suggesting a synthetic lethality interaction between these two genes.

The translocase activity of FANCM is required for suppressing Flex1-induced mitotic recombination. FANCM exhibits translocase activity, which is important for its fork regression function. We reasoned that upon ssDNA exposure during DNA replication, Flex1 could form secondary structures causing replication stalling and DSB formation. FANCM may promote fork reversal to remove such DNA secondary structures, thus preventing DSB formation at Flex1 (see Discussion). In support of this model, while FANCM-WT suppresses hyper-mitotic recombination at Flex1, FANCM translocase mutant K117R fails to do so (Fig. 1g).

DSBs are accumulated at Flex1 after HU treatment as revealed by ChIP analysis of γH2AX at Flex1 (Supplementary Fig. 5a). Depletion of FANCM by shRNAs or expression of FANCM translocase mutant (FANCM-K117R) with endogenous FANCM silenced by shRNAs leads to substantial increase of γH2AX at
Flex1 upon HU treatment (Fig. 2a, left and middle, and Supplementary Fig. 5b). This is in accordance with the model that the translocase activity of FANCM is important for removing DNA secondary structures at Flex1 to prevent DSB formation.

FAAP24 and MHF1 are important for FANCM to be recruited to Flex1 to suppress Flex1-induced mitotic recombination. As revealed by ChIP analysis, FANCM is recruited to Flex1 in the HR-Flex reporter that is stably integrated into the genome, and
such recruitment is further increased after aphidicolin (APH) treatment (Fig. 2b). FANCM binding partners FAAP24 and MHF1 are also recruited to Flex1 (Supplementary Fig. 5c). Depletion of FAAP24 or MHF1 by shRNAs significantly impairs the recruitment of FANCM to Flex1 (Fig. 2c and Supplementary Fig. 5d), and results in DSB accumulation and hyper-mitotic recombination at Flex1 (Fig. 2a, right, 2d, and Supplementary Fig. 6). While the translocase mutant FANCM-K117R is recruited normally, the C-terminal deletion mutants FANCM-ΔDFA (Δ1846–2048) and FANCM-CA (Δ1362–2048), impaired in both DNA and FAAP24 binding34, are defective in being recruited to Flex1 (Fig. 2e and Supplementary Fig. 5e). The FANCM-ΔDFA and FANCM-CA mutants also fail to suppress mitotic recombination at Flex1 (Fig. 1e). These data suggest that FAAP24 and MHF assist FANCM to be recruited to Flex1, which is important for the suppression of Flex1-induced mitotic recombination.

Further analysis showed that the DNA binding-defective mutant FAAP24-DB (V198A/V199A/G200A)37, but not the FANCM binding mutant FAAP24-MB (V198A/V199A/G200A)38, is impaired in recruitment to Flex1 (Fig. 2f, left and Supplementary Fig. 5f). These data suggest that the binding of FAAP24 to DNA, but not the interaction of FAAP24 with FANCM is important for FAAP24 to be recruited to Flex1. However, both mutants are defective for recruiting FANCM to Flex1 and for suppressing Flex1-induced mitotic recombination (Fig. 2f, right, 2g, and Supplementary Fig. 5g). Thus, the interaction of FAAP24 with FANCM is required for FANCM to be recruited to Flex1 and both DNA binding and FANCM binding activities of FAAP24 are important for suppressing Flex1-induced mitotic recombination.

**FANCM is important for maintaining the genome stability at Flex1 and protecting CFSs.** Flex1 induces plasmid instability in mammalian cells40. We further showed that when FANCM, FAAP24, or MHF1 is depleted by shRNAs, Flex1-containing plasmids become even more unstable (Fig. 3a), likely due to DSB formation at Flex1 when the FANCM pathway is impaired (Fig. 2a). Importantly, FANCM translocase activity is important for preventing Flex1-induced plasmid instability (Fig. 3b and Supplementary Fig. 7).

To examine whether FANCM is important for CFS stability, we spread metaphase chromosomes and found increased chromosomal breakages per cell when FANCM is depleted by shRNAs, which is further elevated following APH treatment under the condition that induces CFS expression (Fig. 3c). To more specifically look at CFSs, we performed fluorescence in situ hybridization (FISH) analysis at FRA16D and FRA3B on metaphase spreads. We showed that FRA16D and FRA3B expression is significantly increased when FANCM is depleted (Fig. 3d). ChiP analysis of γH2AX at endogenous FRA3B locus showed that DSBs are accumulated at the vicinity of AT-rich sequences in FRA3B (Fig. 3e). We further showed that FRA16D and FRA3B expression is also increased when FANCM translocase activity is deficient (Fig. 3f). These data suggest that FANCM and its translocase activity are important for CFS protection to prevent DSB formation.

FRA16D expression is also increased in the FANCM-MM1 mutant defective in binding to the FA core complex, but to a less extent compared to the translocase mutant FANCM-K117R (Supplementary Fig. 8). Since mitotic recombination at Flex1 is not significantly increased in the FANCM-MM1 mutant (Fig. 1e), increased CFS expression in this mutant is not due to a defect of FANCM in replication fork remodeling, but likely in coordination with the FA core complex to protect Flex1. This is consistent with the observation that the FA core complex is important for maintaining CFS stability31,32 but not for the suppression of Flex1-induced mitotic recombination (Fig. 1b). These data also suggest that FANCM protects CFSs mainly through utilizing its translocase activity to preserve stability of AT-rich sequences at CFSs, but the interaction of FANCM with the FA core complex also contributes to CFS protection.

**FANCM protects CFSs upon oncogene expression.** Replication stress is induced upon oncogene expression33,34. Consistently, we observed that H-Ras-V12 overexpression induces ATR activation and DSB formation as revealed by phosphorylation of Chk1 and RPA, and phosphorylation of H2AX, respectively (Supplementary Fig. 9). In accordance with the previous study35, overexpression of H-Ras-V12 induces FRA16D expression (Fig. 4a). We further showed that FANCM and its translocase activity are required for suppression of Ras-induced CFS expression (Fig. 4b, c).

Ras overexpression also induces mitotic recombination at Flex1 (Fig. 4d), likely due to replication stress caused by oncogene expression leading to DSB formation. Indeed, ChiP analysis of γH2AX shows increased DSB formation at Flex1 upon Ras overexpression (Fig. 4e). Loss of FANCM or FANCM translocase activity, as well as suppression of FAAP24 or MHF1 expression, further increases Ras-induced mitotic recombination at Flex1 (Fig. 4f, g). These data suggest that FANCM in complex with FAAP24 and MHF, and the translocase activity of FANCM are important for suppressing oncogene-induced CFS instability, which often occurs at the early stage of cancer development.

**Rad52 is important for HR at Flex1.** Loss of FANCM significantly increases spontaneous mitotic recombination as revealed by the HR-Flex reporter (Fig. 1b), suggesting that HR is used to repair DSBs generated at Flex1 upon loss of the protection mechanism by FANCM. Indeed, inactivation of Rad51 reduces green cell accumulation in FANCM-knockdown HR-Flex reporter cell line (Fig. 5a and Supplementary Fig. 10a). Surprisingly, similar effect was also observed when Rad52 is depleted, although Rad52 is not required for general HR in mammalian cells36,37. Similarly, inactivation of Rad51 or Rad52 both suppresses HU-induced mitotic recombination at Flex1 (Fig. 5b and Supplementary Fig. 10b). ChiP analysis demonstrated that both Rad51 and Rad52 are recruited to Flex1 (Fig. 5c). These data suggest that
Rad52 is involved in HR to repair DSBs generated at Flex1. Indeed, inactivation of Rad52 in FANCM-deficient cells further increases DSB formation at Flex1 as revealed by ChIP analysis of γH2AX (Fig. 5d).

To further illustrate the mechanism underlying the role of Rad52 in mitotic recombination at Flex1, we used I-SceI to create a DSB in the HR reporters with or without Flex1 insertion (Fig. 5e, left and middle). Suppression of Rad52 expression has no
obvious effect when using the general HR reporter without Flex1, but leads to a significant reduction of HR when HR-Flex was used. We reasoned that the presence of Flex1 at the DSB end as a non-homologous sequence to the template, which also forms DNA secondary structure, may block the end and inhibit strand invasion or second-end capture, and consequently Rad52 becomes important for initiating and/or completing HR (Supplementary Fig. 11 and Discussion). When we used the HR-Luc reporter which contains a luciferase sequence (similar size to Flex1) at the side of the I-SceI cleavage site, Rad52 is also required for HR (Fig. 5e, right). These data suggest that when one DSB end is blocked by a non-homologous sequence to the template, Rad52 becomes essential for HR in mammalian cells.

Commonly, sister chromatids serve as homologous templates for HR-mediated DSB repair, and non-homologous sequences are not present in the sister chromatid templates. We hypothesize that when DSB ends are blocked by DNA secondary structures even without non-homologous sequences present, Rad52 may also be required for promoting HR from a blocked end (see Discussion). To test this hypothesis, we designed DSB repair substrates (HR-Flex/D-Flex and HR-Luc/D-Luc, Fig. 5f, top) with Flex1 or Luc also inserted in the EGFP donor templates. Due to the presence of Flex1 and Luc in the donor templates, HR would not produce green cells and PCR analysis was used to score the repair events. After I-SceI cleavage, end resection would occur leading to single-stranded DNA (ssDNA) accumulation at DSB ends and Flex1 but not Luc would form secondary structures. Genomic DNA was purified from the reporter cell lines after I-SceI digestion and digested with I-SceI to remove the parental EGFP recipient cassettes (uncut by I-SceI or perfectly religated after I-SceI cleavage in cells), followed by PCR with primers specifically for the parental/repaird EGFP recipient cassettes. The donor templates are marked by BamHI and EcoRI sites, which are absent in the parental cassettes. If HR is used, BamHI and EcoRI sites would be transferred to the HR repair products to replace the I-SceI site, while end joining products would not contain BamHI and EcoRI sites. Thus, the ratio of BamHI and EcoRI cleavable and non-cleavable PCR products (Fig. 5f, right) box would indicate the ratio of the repair products by HR or by imperfect end joining. Interestingly, the percentage of using HR in U2OS (HR-Flex/D-Flex) cells is substantially reduced after Rad52 depletion, but such reduction was not observed in U2OS (HR-Luc/D-Luc) cells (Fig. 5f, bottom). These data suggest that DNA secondary structures formed at Flex1 after end resection would block DSB ends and even when the DSB ends contain perfect homology to the donor templates, Rad52 is required to promote HR-mediated repair from blocked DSB ends.

Loss of Rad52 and FANCN leads to a strong proliferation defect and inactivation of Rad52 suppresses the growth of FANCN-deficient tumors. Neither Rad52 nor FANCN is an essential gene in mice31,40, and inactivation of Rad52 or FANCN does not significantly influence cell growth (Fig. 6a). However, when Rad52 is depleted by shRNAs in FANCN KO cells, a strong proliferation defect is observed, suggesting a synthetic lethality interaction between these two genes. Inhibition of Rad52 in FANCN KO cells expressing FANCN translocase mutant FANCN-K117R also results in a strong cell growth defect, which is in sharp contrast to cells expressing the FANCN wild-type allele (Fig. 6b). These results support the model that Rad52 and FANCN play concerted roles in protecting structure-prone DNA sequences at CSFs, and absence of both would cause cell death.

Inactivation or downregulation of FANCN has been observed in breast cancer and other tumors41-43. To test whether depletion of Rad52 inhibits FANCN-deficient tumor growth, we monitored tumor formation in a mouse xenograft model. While the sizes of tumors derived from FANCN KO cells and Rad52 knockdown cells are initially a bit smaller than the control, they grow at a similar rate as that from wild-type cells at later time points (Fig. 6c). In a stark contrast, depletion of Rad52 in FANCN KO cells completely suppresses tumor growth. Thus, inhibition of Rad52 could offer a new targeted treatment strategy for FANCN-deficient tumors.

Discussion

CSFs are extended over hundreds of kilobases and their instability is induced by multiple mechanisms such as paucity of replication origins, fork stalling at AT-rich DNA sequences, late replication timing, and collision of replication and transcription, which are all associated with perturbation of DNA replication5,44. Our study reveals new mechanisms involving concerted roles of FANCN and Rad52 to maintain stability of structure-prone AT-rich DNA sequences at CSFs, especially upon replication stress and oncogene expression.

We identified a novel function of FANCN in protecting AT-rich sequences at CSFs. Such FANCN function requires its translocase activity and its binding partners FAAP24 and MHF. We propose that AT-rich sequences at CSFs form DNA secondary structures when ssDNA is exposed during replication, and FANCN is recruited by FAAP24 and MHF to these DNA sequences. FANCN promotes fork reversal through its translocase activity to remove secondary structures formed at AT-rich sequences, thereby preventing DSB formation at CSFs (Fig. 7, left, pathway 1), which is supported by accumulation of γH2AX at Flex1 site and at endogenous FRA3B locus in FANCN-deficient cells.
Fig. 3 FANCM translocase activity is important for CFS protection. a, b Plasmid stability assay was performed in cells carrying pCEP4-Flex1 or pCEP4-Luc plasmids after culturing cells without hygromycin for 1 week. U2OS cells expressing indicated shRNAs or control vector (a), and HCT116 WT or FANCM KO cells reconstituted with Flag-FANCM (WT or K117R) (b) were used. c Metaphase spread of HCT116 cells was performed with or without expressing FANCM shRNA or vector before and after APH treatment (0.4 μM, 24 h). Representative images of metaphase spread are shown on the left, and overall chromosome gaps and breaks per cell are shown on the right. d FISH images of HCT116 cells after DAPI staining using probes against FRA3B or FRA16D are shown (left). Frequency of CFS expression at FRA3B or FRA16D in KO cells reconstituted with Flag-FANCM (WT or K117R) (c) was determined. Frequency of CFS expression at FRA3B or FRA16D in U2OS cells expressing FANCM shRNA or control vector (d) was determined (right). FANCM expression is shown by western blots. Bars, 5 μm.

cells. Normal forks can be regenerated and restarted upon fork restoration.

FANCM binds to DNA with a preference for branched structures, such as Holliday junctions, replication forks, and D-loops. FANCM binding partners FAAP24 and MHF stimulate FANCM-DNA binding activity. We showed that the recruitment of FANCM to stalled replication forks at Flex1 requires both FAAP24 and MHF. Biochemically, FANCM exhibits ATP-dependent branch point translocase activity, which promotes replication fork regression, and such fork remodeling activity is stimulated by MHF but is independent of other FA proteins. We showed that the translocase activity of FANCM but not the FA core complex is important for maintaining Flex1 stability, supporting the model that the key role of
FANCM in preserving Flex1 stability is through its translocase activity, which removes DNA secondary structures at stalled replication forks.

ATR activation is critical for replication fork protection and CFS maintenance. It has been shown that the FANCM/FAAP24 complex is associated with the checkpoint protein HCLK2 and facilitates ATR activation. However, while acute inactivation of FAAP24 or FANCM by siRNAs both lead to an ATR checkpoint defect, only KO of FAAP24 but not FANCM in HCT116 impairs ATR signaling, possibly due to adaptation of cells to the loss of FANCM. In FANCM KO HCT116 cells, mitotic recombination at Flex1 is significantly increased compared to wild-type cells, revealing a defect in Flex1 protection in the presence of normal ATR signaling.

Multiple components of the FA pathway have been linked to CFS protection. Besides FANCM, loss of FA proteins such as FANCA,

**Fig. 4** FANCM is important for suppressing Ras-induced CFS expression and mitotic recombination at Flex1. a–c Frequency of CFS expression at FRA16D in HCT116 cells was determined after expressing H-Ras V12 (Ras) or vector (a), Ras along with FANCM shRNA or control vector (b), or Ras and Flag-FANCM (WT or K117R) with endogenous FANCM silenced by shRNA (c). Expression of Ras and FANCM is shown by western blot analysis. d, f, g Spontaneous recombination was determined in U2OS (HR-Flex) cells with p53 silenced by shRNA (to prevent Ras-induced senescence) 1 week after Ras expression (d), Ras expression following expression of indicated shRNAs or vector (f), or Ras and Flag-FANCM WT or K117R with endogenous FANCM silenced by shRNA (g). Indicated protein expression is shown by western blot analysis. e ChIP analysis of γH2AX at Flex1 was performed in U2OS cells containing pCEP4-Flex1 or pCEP4-Luc plasmids 72 h after Ras overexpression.
FANCB, and FANCD2 and its downstream BRCA1 also has been shown to increase CFS expression. Our designed HR-Flex reporter specifically monitors Flex1-induced mitotic recombination by HR. If DSBs are formed at Flex1 in the reporter and are repaired by HR, the EGFP open reading frame would be restored to produce green cells. We showed that different from loss of FANCM, inactivation of FANCA and FANCD2 does not significantly increase Flex1-induced mitotic recombination, while inactivation of BRCA1 reduces Flex1-induced mitotic recombination. This is consistent with the notion that different FA proteins have distinct roles to...
protect CFSs. BRCA1 is required for HR-mediated DSB repair at Flex1, and its checkpoint function is also needed for CFS protection44. FANCDD2 and FANCI are specifically associated with CFS foci and are involved in preventing fragile site anaphase bridging26. FANCDD2 was also found to facilitate replication through CFSs by suppression of DNA:RNA hybrid formation and by influence of dormant origin firing24. The function of FANCDD to preserve stability of AT-rich sequences at CFSs through its fork remodeling activity is distinct from these previously described roles of FA proteins in CFS protection.

We also showed that the FANCM-MM1 mutant impaired in the interaction with the FA core complex is not defective in suppressing Flex1-induced mitotic recombination, which is consistent with the observation that the FA core complex is not required for maintaining Flex1 stability. However, the FANCM-MM1 mutant still exhibits increased CFS expression. It was shown that the interaction of FANCM and the FA core complex is important for ID2 recruitment and mono-ubiquitination34, and thus the defect of FANCM-MM1 mutant in CFS protection is likely related to modulating the function of the FA core complex and ID2, which is in line with the previous findings that the FA core proteins and ID2 are important for CFS protection14. Collectively, the FA network employs multiple mechanisms to protect CFSs, and our study reveals a novel function of FANCM to remodel forks at the sites containing DNA secondary structures through its translocase activity to maintain CFS stability.

When FANCM is deficient, HR-mediated mitotic recombination is significantly increased to repair DSBs accumulated at Flex1. This suggests that HR is used as a backup mechanism to repair DSBs accumulated at structure-prone DNA sequences at CFSs when FANCM function is impaired (Fig. 5, left, pathway 2). Interestingly, we found that not only Rad51 but also Rad52 is important for suppression of hyper-mitotic recombination induced upon loss of FANCM. In yeast, Rad52 plays a prominent role in all types of HR30,51, but in mammalian cells, Rad52 is not essential for general HR such as gene conversion29. Powell’s group showed that when BRCA2 or several other HR players are inactivated, Rad52 is needed for HR and cell growth39,52,53. Human Rad52 exhibits DNA annealing activity in vitro, but its mediator activity to promote Rad51 filament formation and strand invasion is largely replaced by BRCA234. It was proposed that Rad52 may delineate an alternative backup mediator pathway and function in the absence of BRCA229.

In the HR reporter (HR), both DSB ends share homology to the donor sequence (except 12 bp at the left side and 13 bp at the right side due to the insertion of the I-SceI site), and gene conversion frequency is not obviously altered when Rad52 is depleted (Fig. 5e). However, in the HR-Flex or HR-Luc reporter where a 0.34 kb Flex1 or a similar-sized luciferase sequence non-homologous to the template is present at the DSB, HR is significantly reduced in Rad52-deficient cells. We further showed that even with perfect homology placed in the donor templates, Rad52 is still required when CFS-derived AT-rich sequence Flex1 is present at the DSB ends (Fig. 5f), which is predicted to form DNA secondary structures after end resection. We thus propose that Rad52 is required for HR when DSB ends are blocked by nonhomologous sequences or DNA secondary structures.

Multiple mechanisms can be proposed for this special requirement of Rad52. First, Rad52 may be required to initiate and/or facilitate Rad51-mediated stranded invasion from a blocked 3′ end (Supplementary Fig. 11, left). We speculate that Rad52 helps Rad51 initiate strand invasion from a blocked end by using its ssDNA annealing function to anneal the 3′ blocked strand to the template when the dsDNA of the template is transiently opened. Alternatively, Rad52 may use its newly identified inverse strand exchange activity55 to initiate the pairing of the invading strand from the blocked end with the template strand. As another possible mechanism, Rad52 may be required for second-end capture of the blocked end if strand invasion occurs from the other homologous end (Supplementary Fig. 11, right). Rad52 ssDNA annealing activity is believed to mediate second-end capture during synthesis-dependent strand annealing (SDSA) in yeast40, but such activity is not required in mammalian cells, since HR is not significantly altered in Rad52-deficient cells. However, when the end is blocked, the annealing activity of Rad52 may become indispensable for second-end capture. The possible roles of Rad52 in promoting strand invasion and in capturing the second-end from a blocked DSB end may not be mutually exclusive, and Rad52 could be involved in both. After Rad52-mediated pairing of the 3′ blocked strand with the template, unpaired DNA tails will be removed by endonuclease cleavage in a manner similar to single strand annealing (SSA) mechanism56, and DNA synthesis will start to complete HR (Supplementary Fig. 11).

When sister chromatids are used as templates for HR-mediated DSB breaks, no non-homologous sequences are present in the sister chromatid templates. However, when replication forks are collapsed at Flex1 or at other structure-prone DNA sequences, single-ended DSBs would be blocked by DNA secondary structures formed after end resection, and under such condition, Rad52 is required for strand invasion to promote replication restart (Fig. 7, middle). If a converging fork arises from the other side, two-ended DSBs with one blocked end would require Rad52 either for strand invasion or for second-end capture (Fig. 7, right). Therefore, while Rad52 is nonessential for general HR, it becomes indispensable for HR when DSBs are generated at special chromosomal regions such as CFSs.

**Fig. 5** Rad52 plays a critical role in repairing DSBs at Flex1. a, b Spontaneous recombination was determined in U2OS (HR-Flex) cells expressing shRNAs for Rad51 or Rad52, or control vector, 6 or 12 days after expression of FANCDD or control vector (a), or 4 days after HU treatment (2 mM, 24 h) or without (b). c Anti-Flag ChIP at Flex1 was performed in U2OS (HR-Flex) cells expressing Flag-Rad51 (left) and Flag-Rad52 (right). The expression of indicated protein is shown by western blots. d ChIP analysis of γH2AX at Flex1 or Luc was performed in U2OS cells containing pCEP4-Flex1 or pCEP4-Luc plasmids and expressing shRNAs for FANCDD, Rad52 or both after treatment of HU (2 mM, 6 h). The ChIP signal of γH2AX is quantified by relative fold to the Ctrl. e Schematic drawing of HR reporters containing the I-SceI site (HR), Flex1 with the I-SceI site (HR-Flex), and Luc with the I-SceI site (HR-Luc) is shown (top). The homology of the I-SceI cleaved EGFP cassettes to the donor template (D-EGFP) is indicated as white boxes. I-SceI-induced HR was assayed in U2OS HR, HR-Flex, and HR-Luc reporter cell lines with expression of Rad52 shRNA or control vector (bottom). Rad52 expression is shown by western blot analysis. f Schematic drawing of HR reporters HR-Flex/D-Flex and HR-Luc/D-Luc, which contain identical Flex1 and Luc sequences in both EGFP recipient cassettes (EGFP-Flex/I-SceI and EGFP-Luc/I-SceI) and the donor templates (D-Flex or D-Luc) is shown on top. The BamHI and EcoRI sites in the donor templates are at the corresponding position of the I-SceI site in the recipient EGFP cassettes. U2OS (HR-Flex/D-Flex) or U2OS (HR-Luc/D-Luc) cells with or without expressing Rad52 shRNAs were infected with I-SceI lentiviruses and 3 days after, genomic DNA was extracted and digested with I-SceI, followed by PCR using indicated primers (shown as arrows). The percentage of BamHI and EcoRI digestible PCR products among total DNA was calculated. Predicted enzyme digestion patterns of parental EGFP recipient cassettes (EGFP-Flex/I-SceI and EGFP-Luc/I-SceI) and the repair products generated by HR or imperfect end joining are shown in the box at right.
Rad52 was shown to be retained at CFSs on mitotic chromatin and is important for mitotic DNA synthesis by recruiting Mus81 and PolD3 to CFSs in early mitosis. Rad52 was also found to be important for promoting BIR upon replication stress. In the context of CFSs, these functions of Rad52 are also expected to be important and may be used cooperatively with the new role that we identified—mediating HR at structure-prone DNA sequences to preserve CFS stability.

Our study reveals the important and concerted roles of FANCM and Rad52 in repairing DSBs at CFSs and preserving CFS stability. In a normal cell, FANCM protects structure-prone DNA sequences by promoting fork reversal using its translocase activity. This is shown in Fig. 7, which presents a proposed model for concerted roles of FANCM and Rad52 in protecting Flex1. Blue-marked (1) and (2) refer to pathway 1 (FANCM-mediated fork reversal to remove DNA secondary structures) and pathway 2 (Rad51/Rad52-dependent HR to repair DSBs accumulated at structure-prone DNA sequences when FANCM is deficient), respectively. See Discussion for more details.
pathway 1), thereby preventing DSBR formation at CFSs, and thus Rad52 is not essential. However, when FANC is deficient, DSBRs are accumulated at structure-prone DNA sequences, which require Rad51/Rad52-dependent HR for repair, and thus Rad52 becomes dispensable (Fig. 7, pathway 2 and Supplementary Fig. 11). This is supported by the observation that inhibition of Rad52 expression in FANC KO cells results in a significant reduction in cell growth, although neither FANC nor Rad52 are essential genes43,45. These studies suggest a synthetic lethality interaction between FANC and Rad52, and Rad52-dependent HR acts as a critical backup pathway for FANC-mediated genome protection function. We also speculate that FANC and Rad52 are important not only for protecting CFSs, but also for maintaining the stability of various DNA sequences that are prone to secondary structure formation throughout the genome. The synthetic lethality interactions between these two genes may reflect their more general roles beyond CFS protection.

Rad52 is synthetically lethal with BRCA2 deficiency39. However, in contrast to an essential function of BRCA1 and BRCA2 in HR59, FANC is only minimally involved in HR. In this respect, disruption of BRCA1 or BRCA2 in mice leads to early embryonic lethality60 but FANC KO mice develop normally40. In addition, loss of FANC leads to a substantial increase of HR-mediated mitotic recombinational at Flex1, whereas deficiency of either BRCA1 or BRCA2 suppresses Flex1-induced HR (Fig. 1b and Supplementary Fig. 2a and 2b). Thus, the synthetic lethality interaction between FANC and Rad52 is distinct from the genetic interaction of Rad52 with BRCA1/BRCA2.

FANC has been identified as a breast cancer susceptibility gene, and its deficiency is associated with breast cancer, especially triple-negative breast cancer43,61,62. Mutations in FANCM confer a predisposition to high grade serous ovarian cancer41. Down-regulation of FANC was also described in sporadic head and neck squamous cell carcinoma42. Our study identifies an essential function of Rad52 in FANC-deficient cells. Therefore, inhibition of Rad52 can serve as a new targeted therapeutic strategy for treating FANC-deficient tumors, which is expected to have low toxicity to normal cells.

Methods

Cell cultures. U2OS, T98G, MCF7, Hela, HCT116, and 293T cells were from ATCC and cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in the presence of antibiotics. Cell lines tested negative for mycoplasma contamination. All reporter cell lines were verified by Southern blot analysis. FANC knockout HCT116 cells were previously described63.

Plasmid construction and recombinant protein expression. FANC wild-type and indicated mutants were generated by PCR and subcloned into NBLV0051 (Novo Bio) vector containing a 3x N-terminal Flag-tag. Small hairpin RNA (shRNA) target site-resistant mutations were generated by site-directed mutagenesis (QuikChange, Agilent). HA, MCF7, Rad51, and Rad52 were subcloned into pBabe-Puro, with 3x N-terminal Flag-tag. K171A/K173A and V198A/V199A/200A mutations of FAAP24, and shRNA-resistant mutations of FAAP24 and MHF1 were subcloned into pLKO vector containing a 3x N-terminal Flag-tag. Small hairpin RNA and indicated mutants were generated by PCR and subcloned into NBLV0051 (Novo Bio) vector containing a 3x N-terminal Flag-tag. Small hairpin RNA and indicated mutants were generated by PCR and subcloned into NBLV0051 (Novo Bio). FANCM wild-type and indicated mutants were generated by PCR and subcloned into pLKO vectors.

Immunoblotting and antibodies used. Whole cell lysates and immunoblotting were performed as described63,65. Antibodies against FANC and FAAP24, and antibody against FANC were kindly provided by Dr. Weidong Wang65 and Dr. Jun Huang66, respectively. Antibodies against Mre11 and CtIP were used previously66,68. Commercial antibodies used were: anti-FAA2 (Bethyl Laboratories A301-980, 1:1000), anti-MFH1 (Cell Signaling, #2577, 1:500), anti-H2AX (Cell Signaling, #2577, 1:500), anti-H2A.2 (Bethyl Laboratories A301-980, 1:1000), anti-MFH1 (Abcam, ab69385, 1:1000), anti-Rad51 (Santa Cruz Bio-technology, sc-398587, 1:500), anti-H2A.X-131p (Cell Signaling, #2577, 1:500), anti-H2A (Cell signaling, #2578, 1:500), anti-FLAG (Sigma, F1804, 1:2000), anti-Rad52 (Santa Cruz Biotechnology, sc-36341, 1:500), anti-Ras (Santa Cruz Biotechnology, sc-520, 1:1000), anti-β-actin (Sigma, A5441, 1:2000), anti-Chk1 (Santa Cruz Biotechnology, sc-8408, 1:500), anti-Chk1 (Cell Signaling, #2348, 1:500), anti-RPA32 (Bethyl Laboratories A300-244A, 1:1000), BRCAl (Bethyl Laboratories, A300–000A, 1:1000), and FITC-conjugated anti-mouse IgA (BD, 559354).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as described with some modifications101,102. Cells were fixed with 1% formaldehyde for 10 min at room temperature and the reaction was stopped with 12.5 mM glycine solution. After washing twice with cold PBS, cells were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1) supplemented with 12 N- and C-terminal truncated EGFP cassettes carrying Flex1 and Luc sequences respectively, which are identical to the I-SceI-containing recipient cassettes (EGFP::Flex1/I-SceI and EGFP::Luc/I-SceI, Fig. 5f) except that the corresponding I-SceI site is replaced with the BamHI/EcoRI sites.

shRNA interference. Silencing of indicated endogenous genes was performed by retrovirally or lentivirally infection using pMKO and pLKO vectors, respectively, to express corresponding shRNAs43,65. shRNA target sequences were designed by Dharmatac (http://www.dharmacon.com). U2OS, T98G, MCF7, Hela, HCT116, and 293T cells were from ATCC and cultured at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in the presence of antibiotics. Cell lines tested negative for mycoplasma contamination. All reporter cell lines were verified by Southern blot analysis. FANC knockout HCT116 cells were previously described38.
with protease inhibitor cocktail ("PIC", oComplete, Roche) and subject to sonication to break chromatin into fragments with an average length of 0.2–1 kb. The lysate supernatant was pre-cleared with Protein A/G Sepharose beads (Amersham Biosciences). IP was performed using H2AX-S139p (Cell Signaling #2577) or Anti-FLAG antibody (Sigma F1804) followed by washing with 1 ml TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl) with PIC, TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl) with PIC, buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1) with PIC, and TE with PIC. The protein–DNA complex was eluted from beads by elution buffer (1% SDS, 0.1 M NaHCO3), and cross-linking was reversed by adding in 4 M urea and incubating for 12 h at 4 °C. DNA was purified by QIAquick kit (QIAGEN) according to the manufacturer’s instructions. For ChIP at Flex1 in the HR reporter stably integrated in the genome, DNA binding to Flex1. The primers used for ChIP at Flex1 in HR reporter: P1F 5′-CTCCAATTCGCCCTATAGTGAGTCGTATTA-3′, P1R 5′-CTTGAACTTTTCTCAGGTTACTAGTTGACGGAAGG-3′, P2F 5′-TGTTGGGGCCGTTCTAGTGC-3′, P2R 5′-TGGAGGAGGTTACTAGTTGACGGAAGG-3′ and P3R 5′-CAAGGCTACACAGCTTCAAAAG-3′, and PCR products were resolved by 1.5% agarose DNA gel10.

Metaphase chromosome analysis and fluorescence in situ hybridization

Metaphase chromosome analysis and FISH were performed as described10. Cells were treated with 0.4 μM APH for 18 h, followed by treatment of 0.1 μg/ml colcemid at 37 °C for 45 min. Cells were then collected and resuspended in 75 mM KCl hypotonic solution pre-warmed to 37 °C and incubated at 37 °C for 30 min, followed by several changes of fixative solution (3:1 methanol/acetic acid). Cells were dropped onto slides and incubated for 2 h at 60 °C prior to Giemsa staining or fluorescence in situ hybridization analysis. Breaks and gaps were quantified on Giemsa-stained metaphases. For FISH analysis, breaks and gaps were scored for the number of overall chromosome gaps and breaks. FISH experiments were performed according to standard protocols98. Green 5-Fluorescein dUTP-labeled probes 264L1 (FRA16D) and 641C17 (FRA3B) labeled with a 3:1 methanol/acetic acid. Cells were dropped onto slides and incubated for 2 h at 60 °C prior to Giemsa staining or fluorescence in situ hybridization analysis. Breaks and gaps were quantified on Giemsa-stained metaphases. For FISH analysis, breaks and gaps were scored for the number of overall chromosome gaps and breaks. FISH experiments were performed according to standard protocols98. Green 5-Fluorescein dUTP-labeled probes 264L1 (FRA16D) and 641C17 (FRA3B) labeled with a 3:1 methanol/acetic acid. Cells were dropped onto slides and incubated for 2 h at 60 °C prior to Giemsa staining or fluorescence in situ hybridization analysis. Breaks and gaps were quantified on Giemsa-stained metaphases. For FISH analysis, breaks and gaps were scored for the number of overall chromosome gaps and breaks. FISH experiments were performed according to standard protocols98. Green 5-Fluorescein dUTP-labeled probes 264L1 (FRA16D) and 641C17 (FRA3B) labeled with a 3:1 methanol/acetic acid. Cells were dropped onto slides and incubated for 2 h at 60 °C prior to Giemsa staining or fluorescence in situ hybridization analysis. Breaks and gaps were quantified on Giemsa-stained metaphases. For FISH analysis, breaks and gaps were scored for the number of overall chromosome gaps and breaks. FISH experiments were performed according to standard protocols98. Green 5-Fluorescein dUTP-labeled probes 264L1 (FRA16D) and 641C17 (FRA3B) labeled with a 3:1 methanol/acetic acid.
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Acknowledgements
FANCM wild-type and mutant-containing plasmids were kindly provided by Dr. Stephen C. West (The Francis Crick Institute, UK) and Dr. Weidong Wang (NIH). Plasmids containing wild-type FAAP24, MHF1, Rad52, or Rad52 were provided by Dr. Stephen C. West, Dr. Yong Xiong (Yale University), Dr. Jeremy Stark (City of Hope), and Dr. Patrick Sung (Yale University), respectively. We thank Dr. Weidong Wang and Dr. Jun Huang (Zhejiang University) for providing anti-FANCM and anti-FAAP24 antibodies and BRCA2 antibody, respectively and Dr. Catherine Freudenreich (Tufts University) for sharing the Flex1-containing plasmid. The siRNA vectors pML0.1-puro (#8452), pKLO.1-TRC (#10878), and pKLO.1-blast (#26653) are from Addgene. This work is supported by NIH grants CA187052, CA197995, and GM080677 to X.W.; National Basic Research Program of China (2015CB910602) and National Natural Science Foundation of China (31370841) to H.W.; NIH grants CA179441 and CA193124–Project 3 to L.L.

Author contributions
H.W., S.L., and X.W. designed and performed experiments, and analyzed the data; I.O. performed experiments and assisted in manuscript preparation; J.R. performed experiments and analyzed data; L.L. provided FANCM KO cell lines and gave valuable input to the manuscript; X.W. is responsible for the project’s planning and experimental designing. X.W., H.W., and S.L. wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-05066-y.

Competing interests: The authors declare no competing interests.

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