Supplemental Information

A histone-fold complex and FANCM form a conserved DNA remodeling complex to maintain genome stability

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Inventory of Supplemental Information

Supplemental Figures and Legends

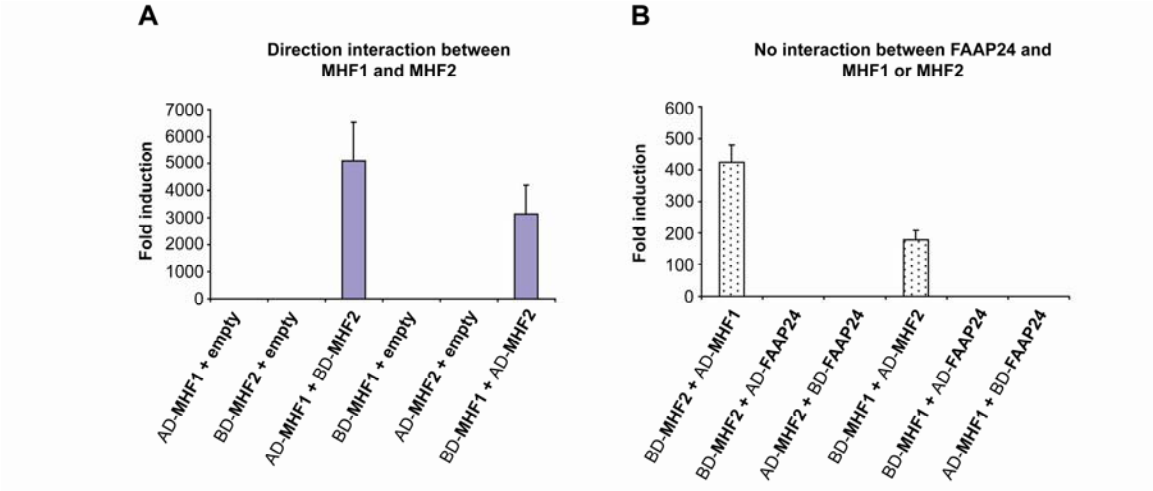


Figure S1 (related to Figure 1). Mammalian two-hybrid assay shows that MHF1 and MHF2 directly interact with each other but not with FAAP24. (A) Mammalian two-hybrid assay showing direct interaction between MHF1 and MHF2. **(B)** The same assay showing no detectable interaction between FAAP24 and MHF1 or MHF2. Indicated proteins are co-expressed in 293 cells as fusion with GAL4 DNA binding domain (BD) or GAL4 activation domain (AD). Fold induction is expressed relative to the luciferase activity obtained with empty vectors (pM and pVP16). Each experimental data set was performed in triplicate.

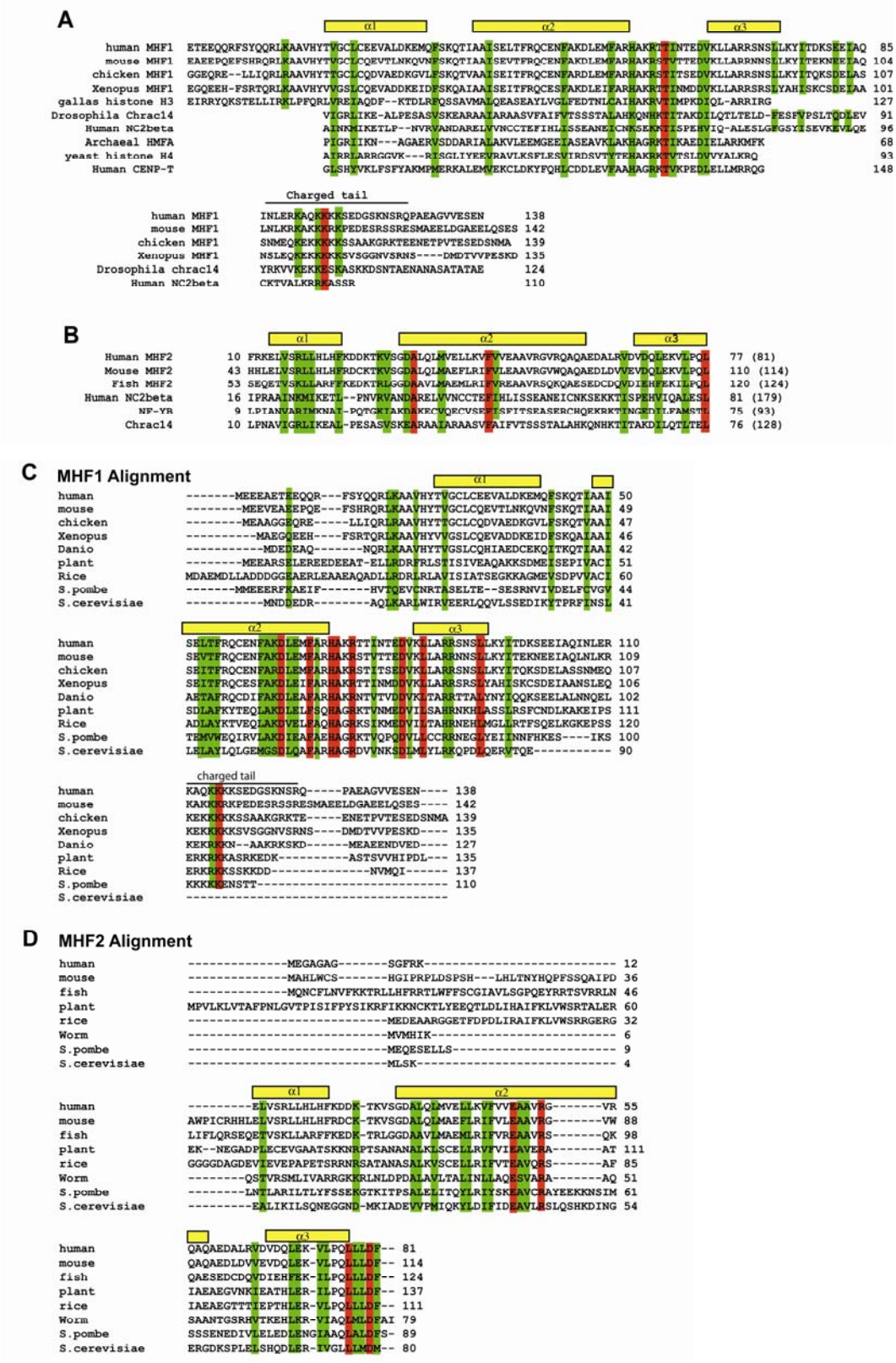


Figure S2 (related to Figure 1). MHF1 and MHF2 are histone-fold proteins conserved from human to yeast. (A) Protein sequence alignment of human MHF1 with

mouse, chicken and xenopus homologues as well as some histones and histone fold proteins as indicated. A charged tail of MHF1 is illustrated by a line. **(B)** Alignment of human MHF2 protein with mouse and fish homologues. Several known histone-fold proteins were included in the alignment. The identical and similar residues are depicted with red and green backgrounds, respectively. The predicted secondary structures comprising three α helices (helix α1, α2, and α3) are indicated by yellow boxes above the sequences. **(C)** Sequence alignment of human MHF1 (NCBI: NP_954988.1) and its orthologs from various species, including mouse (NCBI: EDL14851.1), chicken (NCBI: XP_001234691.1), *Xenopus laevis* (NCBI: NP_001084907.1), *Danio rerio* (NCBI: NP_001122221.1), plant (NCBI: CAN67893.1), rice (NCBI: CAE05152.2), *S. pombe* (NCBI: NP_596235.1) and *S. cerevisiae* (NCBI: EDN63789.1). **(D)** Alignment of human MHF2 (NCBI: A8MT69.1) and its orthologs from several species, including mouse (NCBI: NP_057874.2), fish (NCBI: CAG00206.1), plant (NCBI: CA064206.1), rice (NCBI: NP_001053111.1), worm (NCBI: NP_501398.1), *S. pombe* (NCBI: NP_588439.1) and *S. cerevisiae* (NCBI: NP_878060.1).

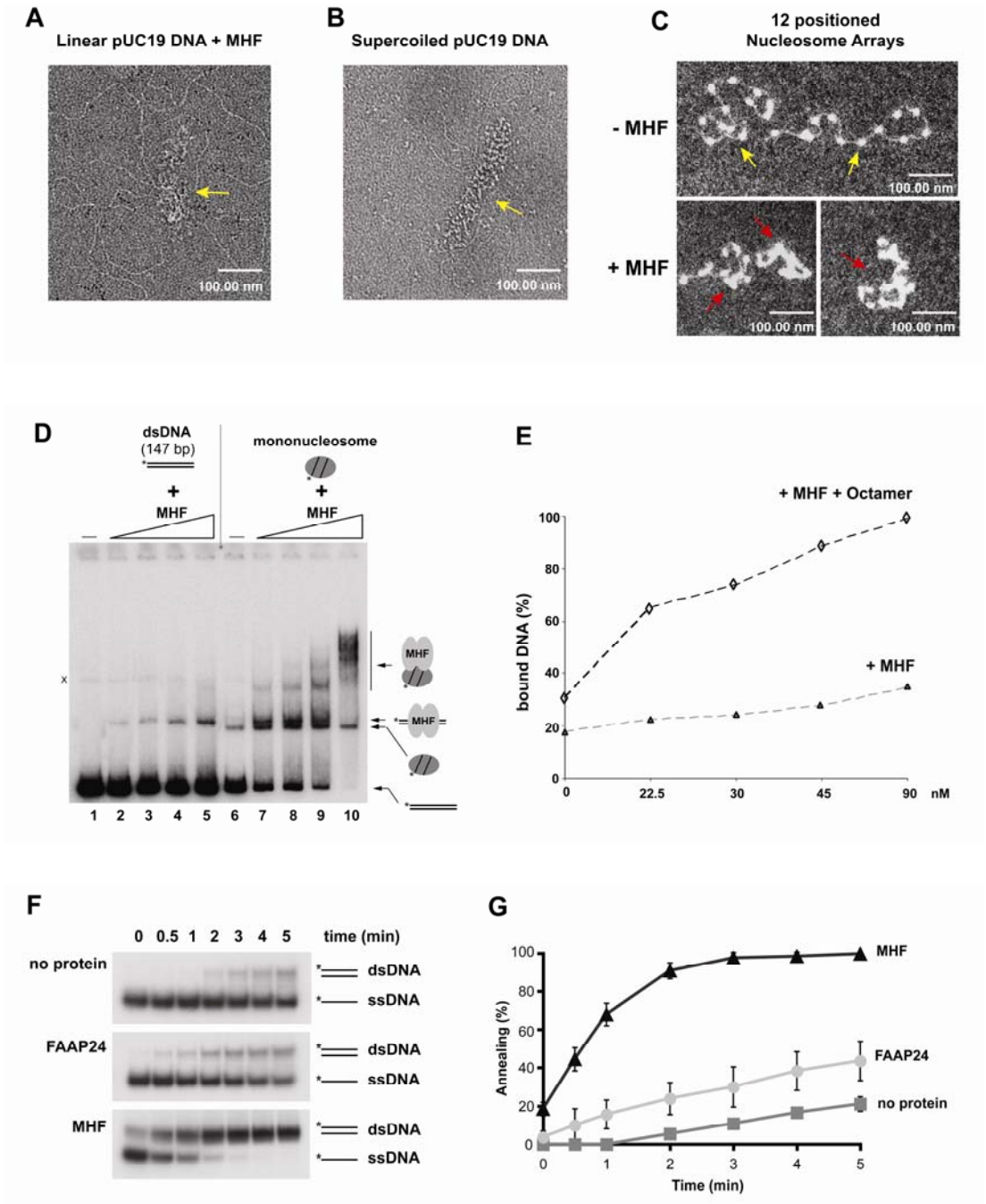


Figure S3 (related to Figure 2). MHF binds dsDNA and chromatin, cooperates with histone octamer to assemble into nucleosome structures, and promotes the annealing of complementary single-stranded DNAs. (A and **B)** Electron microscopy (EM) images of MHF complex bound to long dsDNA structure: linear pUC19 DNA in (A), and supercoiled pUC19 DNA in (B). The yellow arrows indicate MHF clusters on dsDNA. The scale bar represents 100 nm. **(C)** EM images of the chromatin arrays with or without MHF. The upper image shows two in vitro assembled chromatin arrays with 12 positioned nucleosomes as indicated by yellow arrows. The lower two images show MHF

protein-bound chromatin arrays indicated by red arrows. The ratio of protein to DNA is 5 molecules of MHF / 20bp DNA. The scale bar represents 100 nm. In (C), MHF coated the linker DNA, in agreement with the data that MHF binds dsDNA (Figure 3). The presence of MHF increased intra- and inter-array associations, consistent with EM data of naked DNA (A and B) that MHF can self-associate to form higher order structures.

(D) An autoradiograph of electrophoretic mobility shift assay showing that MHF promotes mononucleosome assembly. Increased amounts of MHF complex (22.5nM, lanes 2 and 7; 32nM, lanes 3 and 8; 45nM, lanes 4 and 9; 90nM, lanes 5 and 10) were used. A nonspecific band was marked by "x". **(E)** Quantitative analysis of MHF-bound bands shown in (D).The results were expressed as percentage of bound DNA.

We found that MHF did not bind dsDNA within a pre-assembled mononucleosome (data not shown). However, when MHF was incubated with histones and free DNA in the mononucleosome assembly reaction, we observed formation of higher order complexes containing MHF, histones, and DNA (D, compare lanes 7-10 with lanes 2-5). The presence of histones increased the association between MHF and dsDNA (E), implying that MHF may interact with histones so that they can be co-assembled into higher order protein-DNA complexes during post-replication chromatin assembly.

(F) Autoradiographs showing the strand-annealing activity of the MHF complex. Annealing reactions were supplemented with either FAAP24 (100nM) or MHF (100nM), as indicated. The protein FAAP24 has been described before (Gari et al., 2008b). **(G)** Product formation shown in (F) was quantified by PhosphorImaging and expressed as percentage of dsDNA.

MHF promoted the annealing of complementary single-stranded oligonucleotides, which is the activity of several DNA repair proteins (Cheok et al., 2005; Mortensen et al., 1996). FAAP24 had no significant activity in this assay, suggesting that MHF does not simply function as a crowding agent in the annealing reaction. This shows that MHF efficiently overcomes electrostatic repulsions of the negatively charged oligonucleotides, which may play a role in processing of DNA intermediates generated during replication and repair. The annealing activity of MHF was inhibited when the ssDNA was pre-bound by RPA (data not shown). This feature differs from that of RAD52 (Sugiyama et al., 1998), implying that MHF functions differently from RAD52.

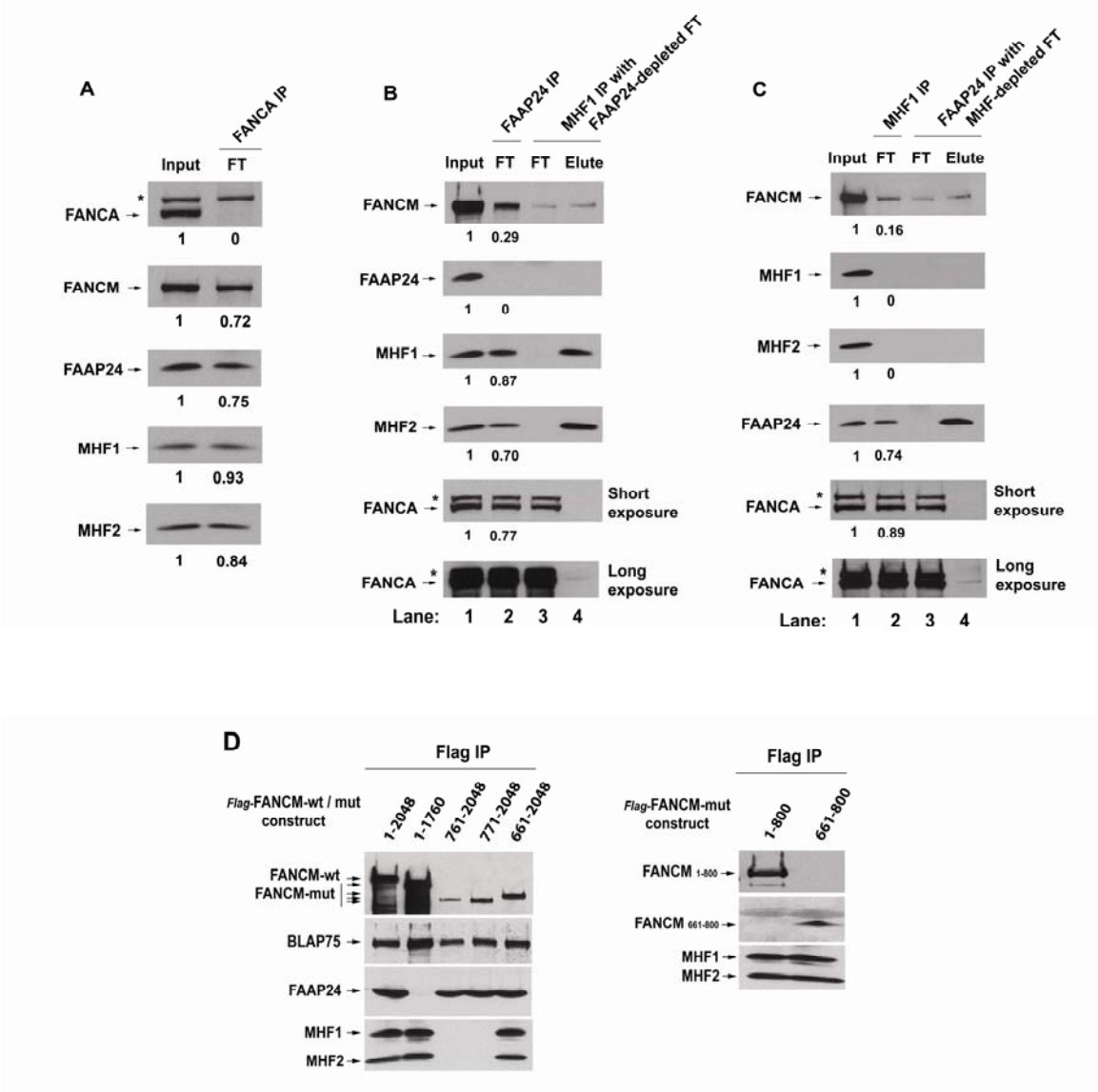


Figure S4 (related to Figure 3). Most of FANCM, MHF and FAAP24 are not associated with the FA core complex; and FANCM can form separate complexes with MHF and FAAP24. MHF interacts with a region near the helicase domain of FANCM. (A) Immunoblotting shows that the majority of FANCM, MHF and FAAP24 retained in the flowthrough (FT) fraction after FA core complex was completely immunodepleted by a FANCA antibody from HeLa nuclear extract. Asterisk indicates a crossreactive polypeptide that can be used as a loading control. The equal amount of nuclear extract was used as an input. The relative levels of indicated proteins on images were obtained by using KODAK Molecular Imaging Software and shown below the blots. The protein level of input was set as "1". **(B)** Immunoblotting shows that a significant fraction of FANCM was co-depleted when FAAP24-associated complexes were immunodepleted. IP-Western (lanes 3 and 4) illustrates co-immunoprecipitation of MHF and FANCM from FAAP24-depleted extract. **(C)** Immunoblotting shows that

majority of FANCM was co-depleted when MHF was depleted by a MHF1 antibody. IP-Western (lanes 3 and 4) shows co-immunoprecipitation of FANCM and FAAP24 in MHF-depleted extract. **(D)** IP-Western to map the MHF-interacting domain in FANCM. Flag-tagged FANCM wildtype (wt) and deletion mutants (mut) were transiently expressed in HEK293 cells, respectively. Immunoprecipitation was carried out using anti-Flag M2 agarose beads. The presence of various FANCM fragments, MHF1, MHF2, FAAP24, and BLAP75 (a control), were detected by Western blotting. A diagram summarizing the FANCM-MHF interaction data is shown in Figure 3B.

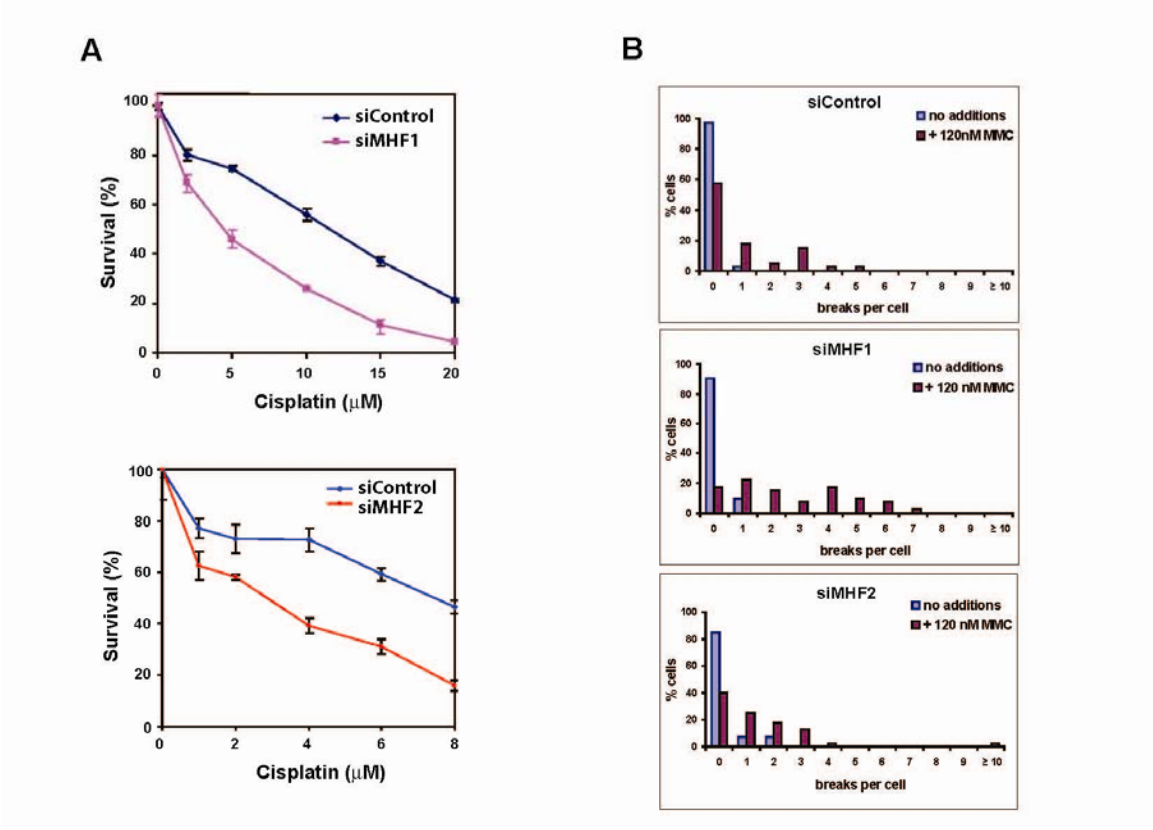
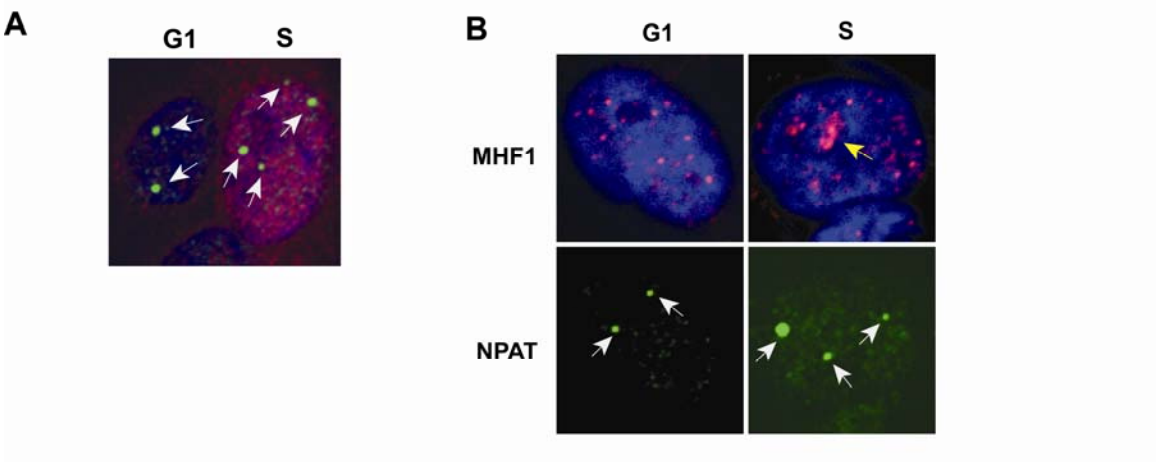


Figure S5 (related to Figure 4). MHF is required for cellular resistance to DNA damage agents and for chromosomal stability. (A) Clonogenic survival assays of HeLa cells depleted of MHF1 (top panel) or MHF2 (bottom panel) by siRNA following the treatment with cisplatin at indicated concentrations. HeLa cells transfected with non-targeting siRNA oligos were used as a control. Three independent experiments were performed, and the results were reproducible. A representative set of data of mean surviving percentage with S.E.M. from triplicate cultures is shown. **(B)** MMC-induced chromosomal aberrations in HeLa cells depleted of MHF1 or MHF2 by siRNA. Non-targeting siRNA oligos were used as a control. At 48hr after siRNA transfection, cells were treated with MMC at indicated concentration for 24 hours or left untreated. Metaphase preparations and chromosomal aberration counting were performed as described previously (Ciccia et al, 2007; Meetei et al, 2003). Percentages of metaphases with and without aberrations after MMC treatment were compared between siControl and siMHF1 or siMHF2 –treated cells using a two-sample Chi2 test. P-values for the differences between siMHF1 and siMHF2 cells were <0.0001 and 0.0587, respectively. When compared to mock transfected cells, p-values were <0.0001 and 0.0068 for siMHF1 and siMHF2 cells, respectively. SiControl and mock transfected cells were not significantly different (p=0.1779).



C

cells / protein	G1		S	
	Recruit	Total	Recruit	Total
MHF1	0	46	8	10
FANCM	0	39	2	6

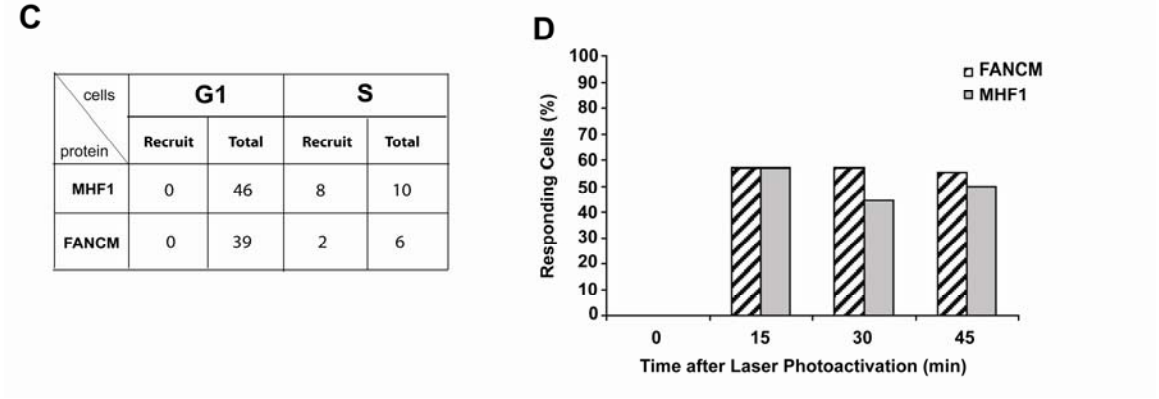


Figure S6 (related to Figure 5). Recruitment of MHF and FANCM to ICLs occurs only in S phase cells. (A) Cell cycle phases were identified by either staining for NPAT (Zhao et al., 2000) (2 spots in G1 or 4 spots in S/G2 cells as indicated by white arrows) or by staining for cyclin A (in red) which is highly expressed in S phase. **(B)** Recruitment of MHF1 was observed in S phase cells, but not in G1 as identified by NPAT staining (green spots as indicated by white arrows. The fourth spot appeared on a different plane). The yellow arrow indicates the laser-targeted region which MHF1 was recruited to. **(C)** In unsynchronized cell population treated randomly with psoralen and laser, recruitment of MHF1 and FANCM were observed only in a fraction of S phase cells and never in G1. **(D)** Bar graph showing the percentage of S phase cells in which MHF or FANCM is recruited to the crosslink at the indicated time points. Cells were synchronized in S phase by double thymidine block and released 2 hr prior to experiments.

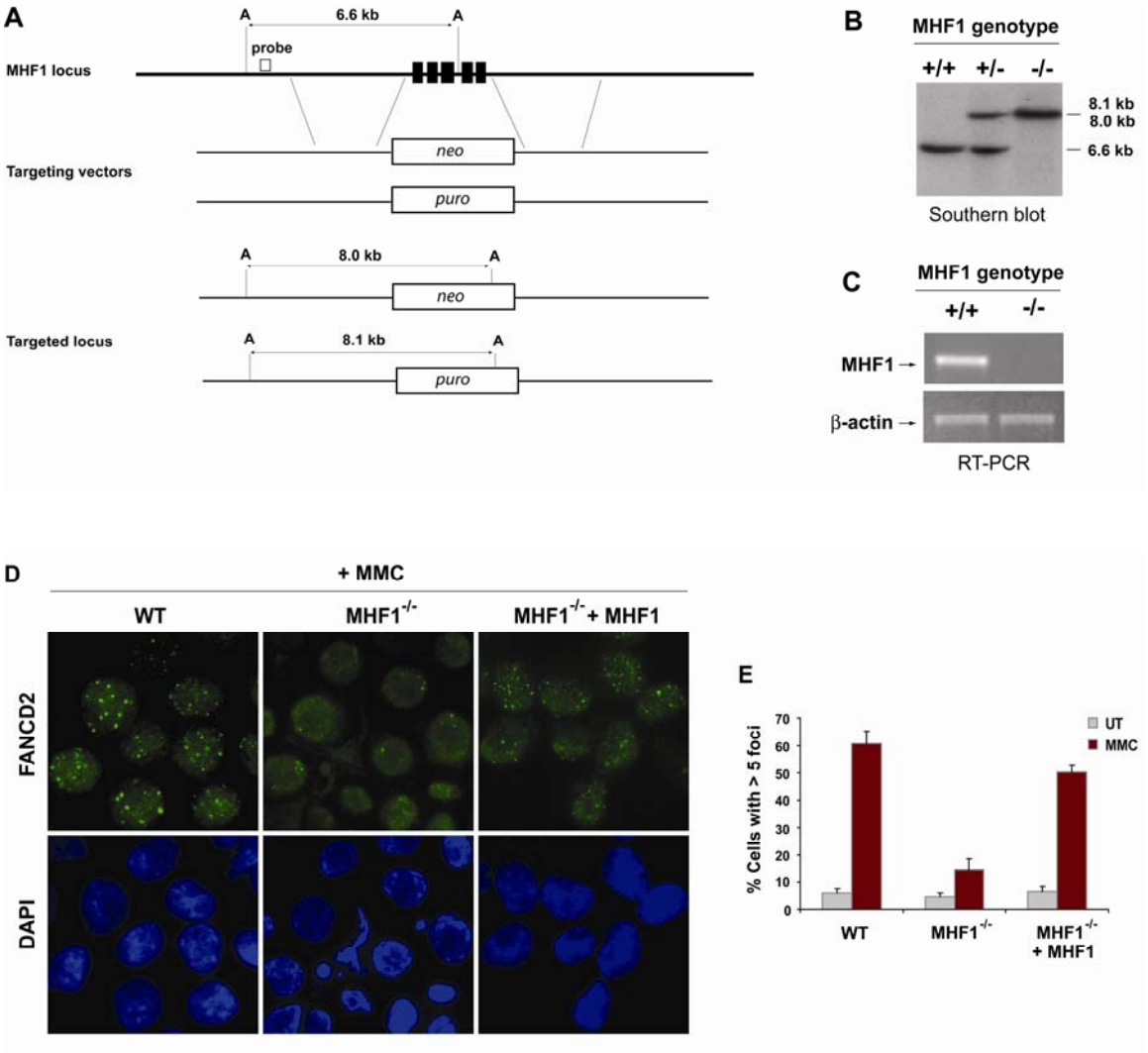


Figure S7 (related to Figure 6). Disruption of MHF1 gene in chicken DT40 cells. MHF is required for formation of FANCD2 foci. (A) A schematic diagram showing chicken MHF1 locus, the gene targeting vectors and targeted alleles. The positions of five exons denoted by black boxes and the probe for Southern blotting are shown. "A" indicates an AflII restriction enzyme site. **(B)** Southern blotting analysis of AflII-digested genomic DNA from the indicated genotypes. **(C)** The expression of MHF1 mRNA in wildtype and MHF1-inactivated DT40 cells was analyzed by RT-PCR. β-actin was used as a control. **(D)** Indirect immunofluorescence shows FANCD2 nuclear foci in DT40 wildtype (WT) cells, *MHF1*$^{-/-}$ cells and *MHF1*$^{-/-}$ cells complemented with human MHF1. Cells were treated with MMC (50 ng/ml) for 18 hr. The images represent immunostaining of FANCD2 in the indicated cells after MMC treatment. **(E)** The bar graphs show the mean values of the percentage of FANCD2-foci-positive cells in untreated (UT) and MMC-treated cells from three independent experiments with standard derivation. A cell containing more than five foci was considered as foci-positive. At least 600 nuclei were counted for each cell line.

Supplemental Experimental Procedures

Cell lines, antibodies and DNA-damaging drugs

HeLa and HEK293 cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS). Wild-type and mutant chicken DT40 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 1% chicken serum, 1% Hepes, in a 5% CO_2 incubator at 39.5^0C. *FANCM*$^{-/-}$ DT40 cell line was kindly provided by Dr. K.J. Patel.

The antibodies against various FANC proteins have been previously described (Ciccia et al., 2007; Ling et al., 2007; Shen et al., 2009; Xue et al., 2008). Anti-MHF1 and MHF2 antibodies were raised against chimeric proteins containing full length MHF1 or MHF2 fused to maltose-binding protein (New England Biolabs). Anti-chicken FANCD2 antibody was kindly provided by Dr. M. Takata. Anti-chicken FANCM antibody was kindly provided by Dr. K.J. Patel. Anti-FANCA antibody for Immunoblotting and anti-actin were purchased from Bethyl laboratories, Inc. Anti-Flag antibody and anti-Flag M2 agarose beads were purchased from Sigma. Anti-histone H3 was purchased from Upstate. Anti-α-tubulin was purchased from Santa Cruz Biotecnology, Inc.

The drugs used for induction of DNA damage, including mitomycin C (MMC), cisplatin, methyl methanesulfonate (MMS), and camptothecin (CPT) were purchased from Sigma.

Plasmid construction, mutagenesis and transfection

Human MHF1 cDNA clone was purchased from InVitrogen. Human MHF2 cDNA was generated by RT-PCR using HeLa mRNA as a source. The expression plasmid pIRES-Flag-MHF2 used for generation of stable cell lines was constructed by inserting MHF2 cDNA with a FLAG tag at its N-terminus into the *EcoRI* and *NotI* sites of pIRES-Neo3 (Clontech). pcDNA3.1-Flag-MHF1 expression plasmid was created by inserting human MHF1 cDNA with a FLAG tag at the N-terminus into the *NheI* and *BamHI* sites of pcDNA3.1-Zeocin (Invitrogen). To generate point mutations in MHF1, site-directed mutagenesis was performed using the QuikChange kit from Stratagene. pcDNA-Flag-FANCM wildtype and deletion mutants were constructed by inserting FANCM full length cDNA or deletion fragment with a FLAG tag at it's N-terminus into

the *NheI* and *BamHI* sites of pcDNA3.1. Lipofectamine 2000 (InVitrogen) was used for transfection in HeLa and HEK293 cells.

Mammalian two-hybrid analysis

Human embryonic kidney HEK293 cells were plated onto six well plates. After 48h, the cells were transiently transfected with MHF1, MHF2 or FAAP24 cDNA fused to either the GAL4 activation domain (pVP16; Clontech) or to the GAL4 DNA-binding domain (pM; Clontech) (1 μg of each), together with a GAL4 driven reporter plasmid (G5E1bLUC, 0.2 μg). The luciferase activity was monitored after 24 h using a Dual-Luciferase Reporter Assay System (Promega) and a single tube luminometer (DLReady, Berthold Detection Systems), according to the manufacturer's instructions. All GAL4 constructs were sequenced to confirm the correct reading frame and each experimental data set was performed in triplicate to overcome the variability inherent to transfections.

siRNA experiments

siRNA pool oligos (ON-TARGETplus SMARTpool L-032895-01 against human MHF1, ON-TARGETplus SMARTpool L-016829-01 against MHF2, ON-TARGETplus SMARTpool L-021955-00 against FANCM and ON-TARGETplus siCONTROL Non-targeting pool D-001810-10) were purchased from Dharmacon. HeLa cells were transfected with these siRNA pool oligos using Lipofectamine RNAi MAX (InVitrogen) according to manufacturer's protocol. Two to three days post-transfection, assays for FANCD2/FANCI monoubiquitination and chromosomal aberrations were performed as described (Ciccia et al., 2007; Meetei et al., 2003). Clonogenic survival assays for MMC, MMS and cisplatin were carried out as described (Meetei et al., 2005). For CPT survival assay, cells were treated with CPT for 48 hours.

Cell extraction and fractionation

The lysis buffer (10 mM Tris-HCl at pH 7.5, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 140mM NaCl, 1 mM EDTA) was used for the preparation of whole cell extract. All buffers were supplemented with complete protease inhibitor cocktail (Roche). For cellular fractionation, the cells were washed with PBS twice and were lysed in low-salt buffer (20mM Tris-HCL, pH 7.5, 100mM NaCl, 1mM EDTA, 0.5% Triton X-100) for 5 min on ice. Following centrifugation, the supernatant was collected for soluble fraction (soluble cytoplasmic and nucleoplasmic proteins). The

pellet was washed once with low salt buffer, once with micrococcal nuclease buffer (20mM HEPES, pH 7.9, 300 mM NaCl, 20mM KCl, 3mM $CaCl_2$, 0.5mM DTT, 0.5mM PMSF) and then digested with micrococcal nuclease (3U/μl) for 20 min at room temperature. Following digestion, 5mM EGTA was added to the extract. After centrifugation, the supernatant was used as chromatin fraction (chromatin-bound proteins).

Expression and purification of *HIS*-tagged recombinant proteins from *E.coli.*

E.coli expression plasmid pET-MHF1 or pET-MHF2 was constructed by cloning human MHF1 or MHF2 cDNA into the *NdeI* and *NotI* sites of pET28a (Novagen). To generate bicistronic MHF1/MHF2 coexpression construct, MHF1 cDNA with a StrepII tag at the N-terminus and an internal ribosomal entry site in the upstream was cloned into the *NotI* and *XhoI* sites of pET-MHF2 plasmid.

E. coli Rosetta (Novagen) cells carrying pET-MHF1, pET-MHF2, pET-MHF2/MHF1 construct or mutant version were grown at 37^0C to OD_{600} of 0.8 and induced with 0.4mM IPTG at 35^0C for 3 h. The cell pellet of 1 liter culture was lysed in 15 ml of 1X BugBuster (Novagen) containing 1mM β-mercaptoethanol, 5 mM imidazole and complete EDTA-free protease inhibitor cocktail (Roche). Following incubation at room temperature for 30 min under constant rotation, the mixture was centrifuged twice (each for 30 min at 15,000rpm in 4^0C). The supernatant was then incubated for 2 hrs with 1ml Talon metal affinity resin (BD Bioscience). The Talon resin was poured into a column, washed with 50 ml of buffer (50mM NaH_2PO_4, 500mM NaCl, 0.1%NP-40, pH 7.8) supplemented with 30 mM imidazole, and eluted (buffer: 50mM NaH_2PO_4, 500mM NaCl , 500mM imidazole, pH 7.8). Peak fractions were pooled and dialyzed against 2 liters of PBS containing 5% glycerol in cold room for 6 hours.

Electrophoretic mobility shift assay

The indicated amounts of proteins were incubated with 0.5nM of 5'-^{32}P-labeled DNA substrates in PBS for 30 min at room temperature. The reactions were loaded on 6% native polyacrylamide gel in TBE and run in 0.5xTBE at 4^0C. Labeled DNA products were visualized by autoradiography. The oligos used for generating DNA substrates have been previously described (Ciccia et al., 2007), unless indicated otherwise.

Cloning of MHF1 and MHF2 cDNAs into baculovirus vectors

The cDNAs were amplified from pIRES-MHF1 and pIRES-MHF2 by PCR with primers containing Gateway attB-sites at their 5'-ends, and subsequently cloned into pDONR221 via gateway BP-reactions according to the manufacturer's instructions (Invitrogen). MHF1 and MHF2 cDNAs were sequence verified and transferred via LR-reactions into the Gateway destination vector pDEST10 (N-terminal 6xHis tag).
Gateway primers:
MHF1 forward:
5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGAGGAGGAGG CGGAGACC3';
MHF1 reverse:
5'GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATTCTCACTTTCCACCACT CC3'.
MHF2 forward:
5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCACCATGGAGGGAGCAG GAGCTGGA3';
MHF2 reverse:
5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCCTAGAAGTCCAGGA GCAGC3'.

Expression and purification of baculoviral recombinant proteins from insect cells

Baculoviruses were prepared according to the manufacturer's instructions (InVitrogen). Insect cells at a concentration of $2x10^6$ cells/ml were infected at an MOI of 1 with recombinant baculoviral particles coding for Flag-FANCM, His-MHF1 and His-MHF2. Cells were collected 48 h post infection and resuspended in 5 PCV of buffer A containing 50 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 150 mM NaCl, 10 % glycerol, 0.1 % NP-40, 0.5 mM EDTA, 2 mM TCEP and protease inhibitors. The suspension was incubated on ice for 30 min, lysed using a Dounce homogeniser (25 strokes with a tight pestle), and centrifuged for 1 h at 100000xg. The supernatant was then incubated with 100µl of anti-Flag M2-agarose bead, and rotated overnight at 4°C. The beads were washed twice for 30 minutes with buffer A supplemented with 100 mM Arginine (pH 7.0), and then once with buffer A. Recombinant Flag-FANCM or Flag-FANCM/His-MHF1/His-MHF2 was eluted with 120 µl of buffer A containing 100 µg/ml 3x-Flag-

peptide (Sigma). The eluate was aliquoted and stored at -80°C. Protein concentration was estimated by silver staining (20 nM).

Annealing assay

Reactions (10 μl) were performed in 25 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 75 mM NaCl, 5 % glycerol, 0.005 % NP-40, 0.25 mM EDTA, 1 mM DTT, 100 μg/ml BSA and contained 3.5 nM 5'-^{32}P labeled oligo 1 (TGGGTGAACCTGCAGGTGGGCAAAGATGTCCTAGCAATCCATTGTCTATGACG), 3.5 nM oligo 2 (CGTCATAGACAATGGATTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCCA) and MHF (100 nM) or FAAP24 (100 nM). At the indicated time, annealing reactions were stopped with 20 mM EDTA, 0.4 % SDS, 2 mg/ml Proteinase K and a 100-fold excess of unlabeled oligo 1. Reaction products were resolved by PAGE through 10% polyacrylamide gels in TBE.

Fork regression assay

The plasmid-based replication fork structure is described in (Gari et al., 2008a; Ralf et al., 2006). Reactions (10μl) were performed in 25 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 75 mM NaCl, 5 % glycerol, 0.005 % NP-40, 0.25 mM EDTA, 1 mM DTT, 100 μg/ml BSA and contained 0.5 nM 5'-^{32}P labeled DNA substrate, 10 nM oligonucleotides (as competitor), 0.5 mM $MgCl_2$, 1 mM ATP, and recombinant proteins at the indicated concentrations. Reactions were carried out for one hour at 37°C, deproteinized for 15 minutes at 37°C with 2mg/ml Proteinase K and 0.4% SDS and resolved on a 0.8% agarose gel containing 0.5 μg/μl ethidium bromide.

Electron microscopy

MHF (1600 nM) was incubated with either linear or supercoiled PUC19 (35 nM) in 25 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 75 mM NaCl, 5 % glycerol, 0.005 % NP-40, 0.25 mM EDTA, 1 mM TCEP for 15 min at 4^0C and then for 15 min at 37^0C . Samples were diluted and washed in 5 mM magnesium acetate, and stained with uranyl acetate, as described (Sogo et al., 1987). Protein-DNA complexes were visualized using a Phillips CM12 electron microscope.

Chromatin array preparation, binding reaction and electron microscopy

Nucleosomal arrays were reconstituted by salt dialysis onto DNA consisting of 12 tandem units of a 208 bp segment containing the '601' nucleosome positioning unit as described (Nikitina et al., 2007). Arrays (final DNA concentration 10 μg/ml) were mixed with the desired amount of protein in 50 mM NaCl, 10 mM HEPES pH 7.8, 0.2 mM EDTA, held at room temperature for 20 min, then placed on ice and fixed with 1.2% glutaraldehyde for 15 min before EM preparation. EM samples were prepared essentially as described (Nikitina et al., 2007). The fixed arrays were diluted with buffer as needed, applied to thin, glow-discharged carbon films for 5 min, rinsed with 5 mM MgAcetate, stained for 10 sec with 0.01% aqueous uranyl acetate, rinsed extensively with water, and air dried. Grids were examined in a Tecnai 12 TEM (FEI Corp, Hillsboro, OR) operated at 100 KV using tilted darkfield illumination, and images recorded on a 2048x2048 CCD camera (TVIPS, Gauting, Germany).

Mononucleosome assembly and binding assay

147bp substrate was generated by PCR amplification of pGEM-3Z-601 using forward primer (ACAGGATGTATATATCTGAC) and reverse primer (CTGGAGAATCCCGGTGCCGA). The DNA product was then end-labeled using radioactive γ- ^{32}P-ATP. 100,000cpm DNA mixed with and without 30 ng recombinant histone octamer in the presence of 1M NaCl, incubated at 30 ^{0}C for 30 min. The mixture was serially diluted with increasing volumes of Buffer A (50mM HEPES pH7.9, 1mM EDTA, 5mM DTT and 0.5mM PMSF) of 1.8μl, 3.5μl, 4.7μl, 13μl, incubated at 30 ^{0}C for 30 min for each dilution. Increasing amounts of MHF complex were added to the final dilution and incubated for another 30 min at 30 ^{0}C. To stop the reaction, 67μl of Buffer B (10mM Tris.HCl, pH 7.5, 1mM EDTA, 0.1% NP40, 5mM DTT, 0.5mM PMSF, 20% Glycerol, and 100μg/ml BSA) was added. 2μl was loaded on 6% native polyacrylamide gel.

Protein Recruitment to laser-induced localized ICLs

We followed a previous protocol to detect proteins recruited at laser-induced localized ICLs (Thazhathveetil et al., 2007). Cells seeded in a 35mm glass bottom culture dish (MatTek™) were incubated with 5 μM psoralen (which forms crosslinks) or 40 μM angelicin (which can form only monoadducts) at 37^{o}C for 20 minutes prior to laser photoactivation. In some experiments, cells were synchronized in S phase using a double

thymidine block and released 2 hours prior to an experiment. Localized irradiation was performed using the Nikon Eclipse TE2000 confocal microscope equipped with an SRS NL100 nitrogen laser-pumped dye laser (Photonics Instruments, St Charles, IL) that fires 5 ns pulses with a repetition rate of 10 Hz at 365 nm, with a power of 0.7 nW, measured at the back aperture of the 60X objective. The diffraction limited spot size was approximately 300 nm. The laser, controlled by Volocity-5 software (Improvision, Perkin Elmer), was directed to deliver pulses to a specified rectangular region of interest (ROI) within the nucleus of a cell (4x20 pixel, 0.16 micron/pixel) visualized with a Plan Fluor 60X/NA1.25 oil objective. The laser beam fired randomly throughout the ROI until the entire region was exposed, after which the photoactivation of the ROI was repeated. Throughout an experiment, cells were maintained at 37 ^{o}C, 5% CO_2 and 80% humidity using Live Cell™ environmental chamber. At different time intervals, cells from different areas of the dish were treated with the laser to generate a time-course on a single plate. After the final time point, cells were fixed immediately in freshly prepared 4% formaldehyde in PBS for 10 minutes at room temperature. Cells were then permeabilized with 0.5% Triton X-100, 1% BSA, 100 mM Glycine and 0.2 mg/mL EDTA in PBS on ice for 10 minutes. The cells were subsequently digested with RNAse A in PBS-EDTA (5 mM) solution for 30 minutes at 37 ^{o}C and blocked in 10% goat serum in PBS containing 0.01% sodium azide for 1 hour at 37 ^{o}C or overnight at 4 ^{o}C. They were then incubated with appropriate primary antibody (FANCM or MHF1) diluted in blocking solution for 1 hour at 37 ^{o}C. After three 10 minute washes using 0.05% Tween-20 in PBS, cells were incubated with a corresponding fluorescent tagged secondary antibody (Alexa Fluor goat anti-rabbit-488 or 633, Molecular Probes, Invitrogen). After another three 10 minute washes cells were mounted with ProLong Gold antifade reagent with DAPI from Molecular Probes. Stained cells were visualized and imaged using Hamamatsu EM-CCD digital camera attached to the Nikon Eclipse TE2000 confocal microscope.

eChIP DNA substrate construction, preparation and chromatin immunoprecipitation

The eChIP substrate contains two essential components, the EBV OriP and site-specific DNA crosslinks downstream of the replication origin (approximately 500 bp). Preparation and insertion of psoralen-crosslinked oligonucleotide into plasmid vectors

have been described previously (Li et al., 1999; Wang et al., 2001). Crosslinked or uncrosslinked control substrates were electroporated into cells (1.5 x10^7 HEK293 or HEK293-EBNA cells) with an Amaxa device (Amaxa Biosystems, Germany) and conditions recommended by the manufacture. Chromatin immunoprecipitation was carried out as described (Shen et al., 2009).

Generation of MHF1-deficient DT40 cells, SCE analysis and FANCD2 focus formation assay

The chicken MHF1 gene locus was identified by searching chicken genome sequence database with human MHF1 protein sequence. The MHF1 targeting vectors for replacement of all five MHF1 gene exons with selection markers (neomycin and puromycin) were constructed by using MultiSite Gateway System kit (Invitrogen) according to the manufacturer's protocol. The following two pairs of Gateway PCR primers were used for amplifying genomic fragments to generate 5'- and 3'-arms:
5'-arm FW:
GGGGACAACTTTGTATAGAAAAGTTGGAGGACATACGCAAGGTGAGTCATTG
;
5'-arm REV:
GGGGACTGCTTTTTTGTACAAACTTGGATGCAGGCTTCTCTATCTTGATTCTAT
GAG;
3'-arm FW:
GGGGACAGCTTTCTTGTACAAAGTGGGTTAGCCTGCCAGCAATTGCT
3'-arm REV:
GGGGACAACTTTGTATAATAAAGTTGGCTAGCTCAGCTTAGCAATGGCAA.

Electroporation transfection in DT40 cells was carried out using Nucleofector kit (Amaxa) according to the manufacturer's manual. Neomycin (2 mg/ml) or puromycin (0.5 μg/mL) was used for selection of targeted clones. Southern blotting analysis and RT-PCR were performed as described (Yan et al., 2008). To generate *FANCM*$^{-/-}$ / *MHF1*$^{-/-}$-double mutant cells, the same targeting vectors were transfected into *FANCM*$^{-/-}$ DT40 cells to disrupt MHF1 gene.

To perform complementation analysis, pcDNA3.1-Zeocin expression plasmid carrying Flag-tagged human MHF1 wildtype or its mutant form was transfected into *MHF1*$^{-/-}$ DT40 cells. 0.5 mg/ml Zeocin was used for selection. Western blotting was performed for selecting the clones that stably express protein.

To measure the SCE levels, DT40 cells (10^5/ml) were incubated in 10 μM BrdU for 2 cell cycles (18-22 hr) and added with colcemid (0.1μg/ml) for 2 hr as described previously(Sonoda et al., 1999).

To analyze FANCD2 focus formation, cells ($3x10^5$/ml) treated with or without MMC(50ng/ml, 20 hr) were fixed with 4% PFA, stained with chicken FANCD2 antibody and followed by Alexa Fluor 488-conjugated secondary antibody (Invitrogen) with DAPI counterstaining as described (Matsushita et al., 2005). Images were captured under Zeiss microscope with AXioCam digital camera and Axiovision software.

Budding yeast strains and MMS sensitivity assay

All strains are isogenic to the S288c background strain YKJM1 (*ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069::URA3).* Null yeast mutants were generated using the conventional PCR-based gene disruption cassette strategies. Yeast transformations were performed as described previously (Myung et al., 2001; Smith et al., 2004). Yeast extract peptone-dextrose (YPD) was purchased from KD medical and consists of the following ingredients per liter of water: 20g peptone, 10g yeast extract, 20g dextrose, 17g agar. For the qualitative MMS sensitivity assay, 10 mL of YPD was innoculated with 200 μL of a 2 mL overnight culture. Yeast cells were grown to mid-logarithmic phase at 30^{o}C (~3-4 hours), harvested, washed once in water, and resuspended in 100 μL water. 50 μL of each strain was added to either 1 mL of water or 1 mL of freshly made 0.1% MMS. Cells were incubated at 30^{o}C for 2 hours with shaking. Following incubation, cells were harvested, washed once in water, resuspended in 100 μL of water, and serially diluted 1:5 in water. 2 μL of each dilution were spotted onto non-selective YPD. Cell viability was visualized after 2 days (no treatment) or 3 days (MMS) of growth at 30^{o}C. The quantitative MMS sensitivity assay was similar to the qualitative assay, however cells were harvested, diluted, and single colonies were plated onto YPD following MMS treatment. Colonies were counted after 3 days of growth at 30^{o}C. Percent survival was calculated as the number colonies that

survived MMS treatment versus untreated controls. Results from three independent clones were averaged and standard deviations were calculated. *P* values were calculated with Microsoft Excel using the two-tailed, two-sample, equal variance Student's t-Test.

***S. pombe* strains and recombination assay**

The *S. pombe mhf2*Δ mutant contains a replacement of the *mhf2* orf with the kanMX4 selectable marker, and was obtained from the Bioneer collection of deletion mutants. The wild-type (MCW1433), *fml1*Δ*::natMX4* (MCW3061), *mhf2*Δ*::kanMX4* (MCW4515) and *fml1*Δ*::natMX4 mhf2*Δ*::kanMX4* (MCW4517) strains share the following genotype: *ura4-D18 his3-D1 leu1-32 arg3-D4 ade6-M375 int::pUC8/his3*$^{+}$*/RTS1 site A orientation 2/ade6-L469*. The direct repeat recombination assay has been described (Ahn et al., 2005). Recombinant frequencies are mean values from at least 15 colonies, and two-sample *t*-tests were used to assess whether differences were significant.

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