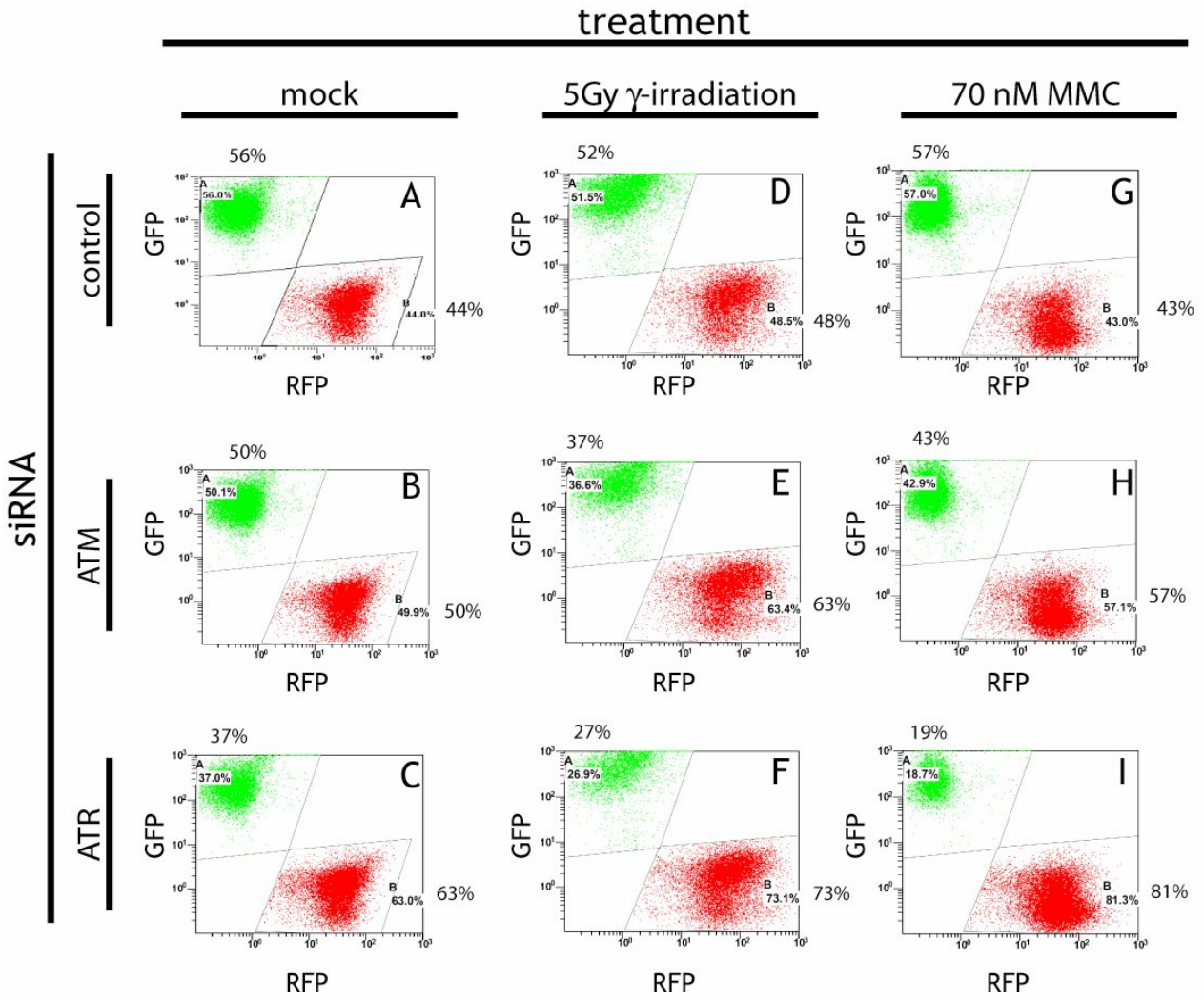
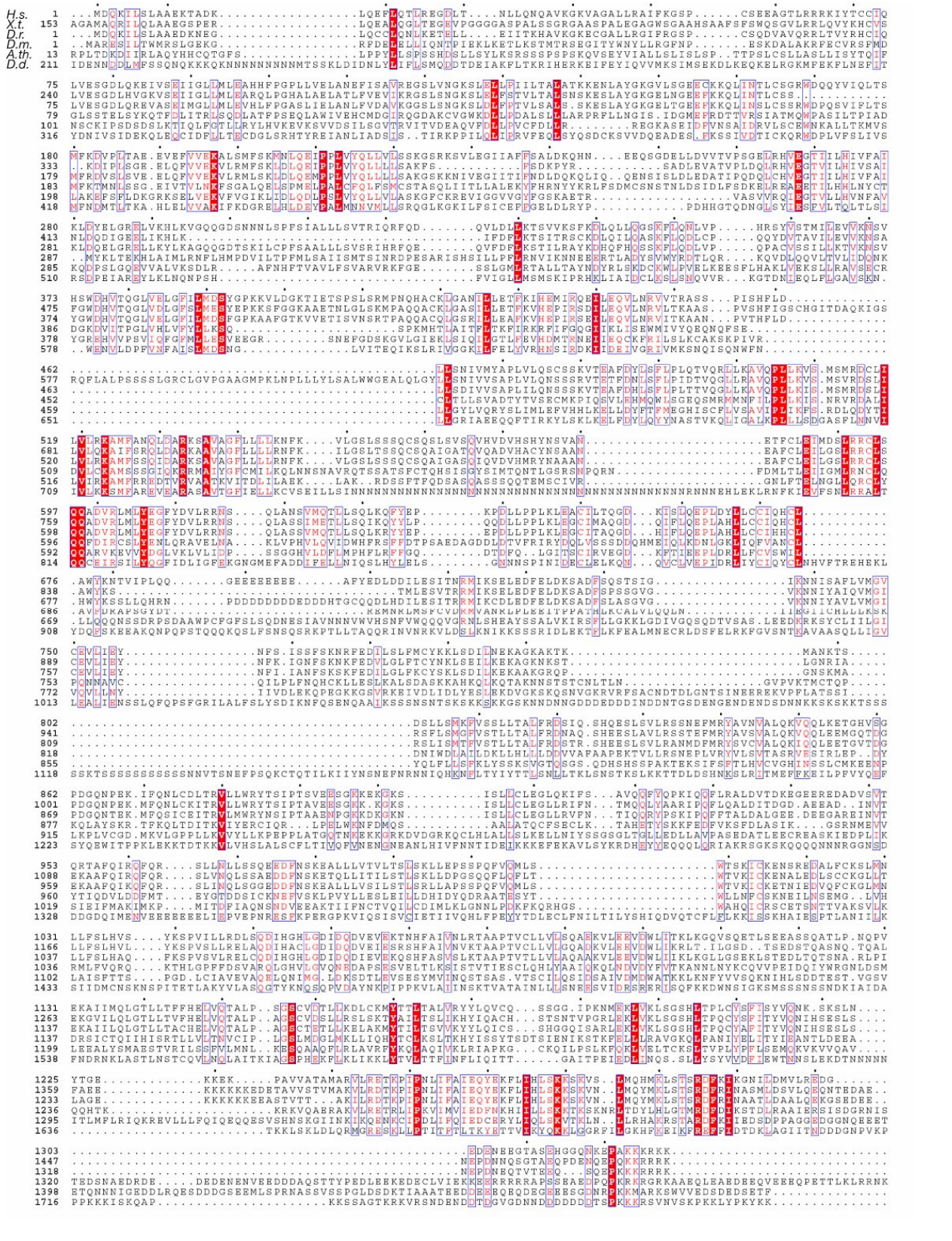
Supplementary Figures

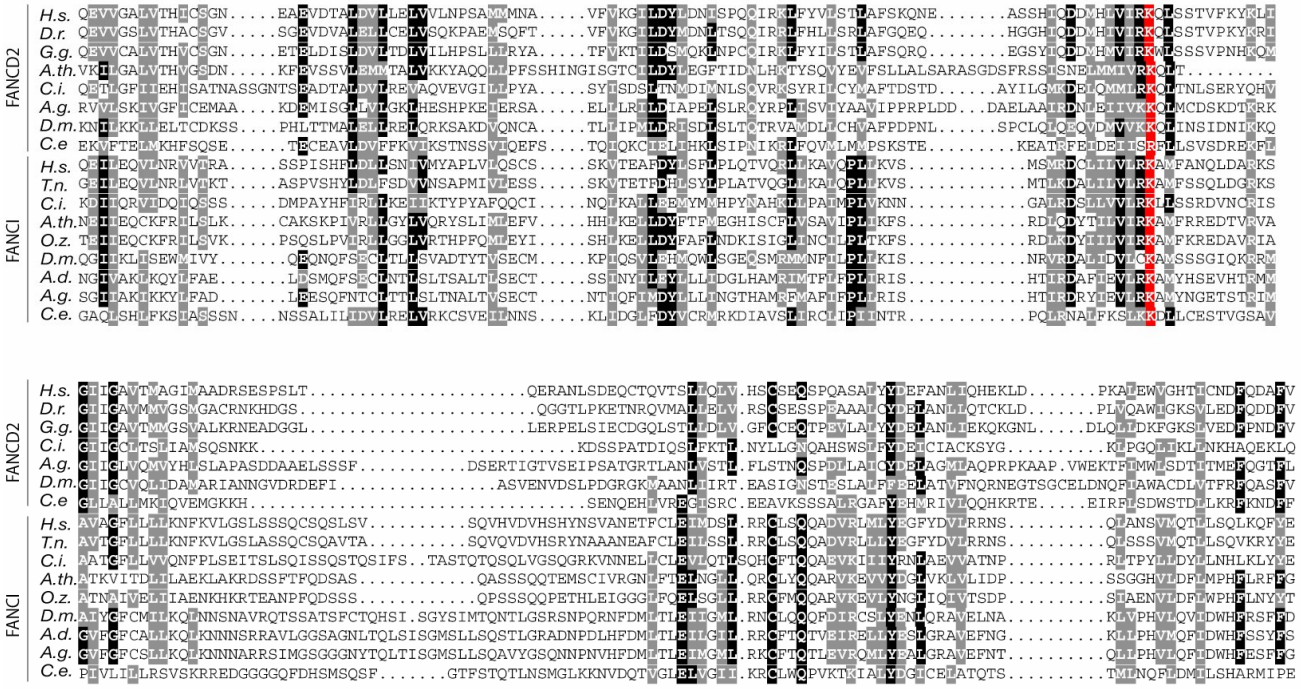


Supplementary Figure 1. MCA assay after ATM and ATR knockdown.

Raw data from the multicolor competition assay preformed as described in Figure 1 with cells that were depleted of ATM or ATR.

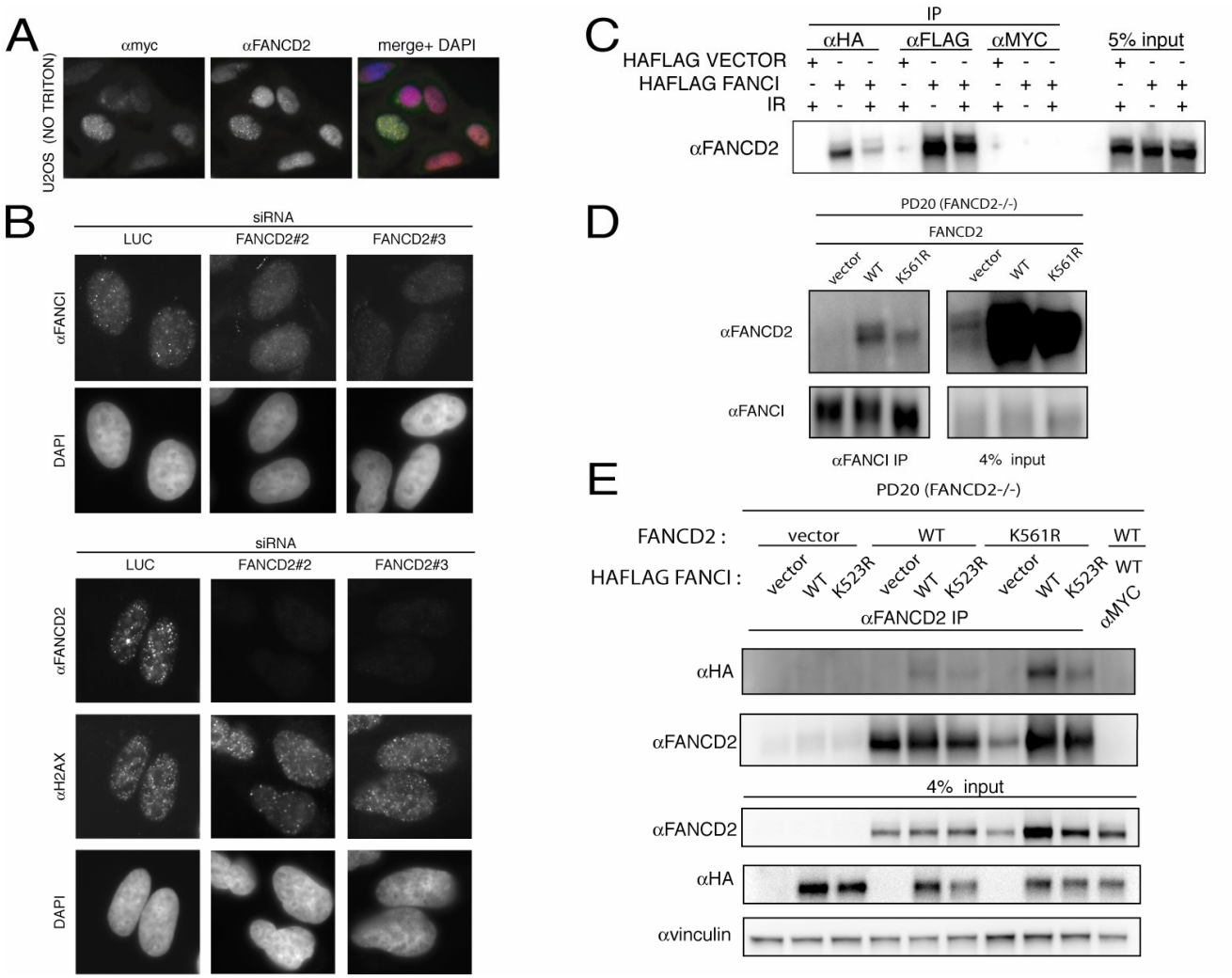

H.s.
X.t.
D.r.
D.m.
A.th.
D.d.

Supplementary Figure 2. FANCI conservation. Alignment of FANCI from *Homo sapiens (H.s.), Xenopus tropicalis (X.t), Danio rerio (D.r.), Drosophila melanogaster (D.m.), Arabidopsis thaliana (A.th.), and Dictyostelium discoideum (D.d).* Identities are highlighted in red.



Supplementary Figure 3. FANCI and FANCD2 conservation

Alignment of FANCD2 and FANCI from *Homo sapiens (H.s.), Danio rerio (D.r.), Gallus gallus (G.g.), Arabidopsis thaliana (A.th.), Ciona intestinalis (C.i.), Anopheles gambiae (A.g.), Drosophila melanogaster (D.m.), Caenorhabditis elegans(C.e.), Tetraodon nigroviridis (T.n.), Oryza sativa (O.z.), and Aedes aegypti (A.d.)*



Supplementary Figure 4. FANCI localization and interaction with FANCD2.

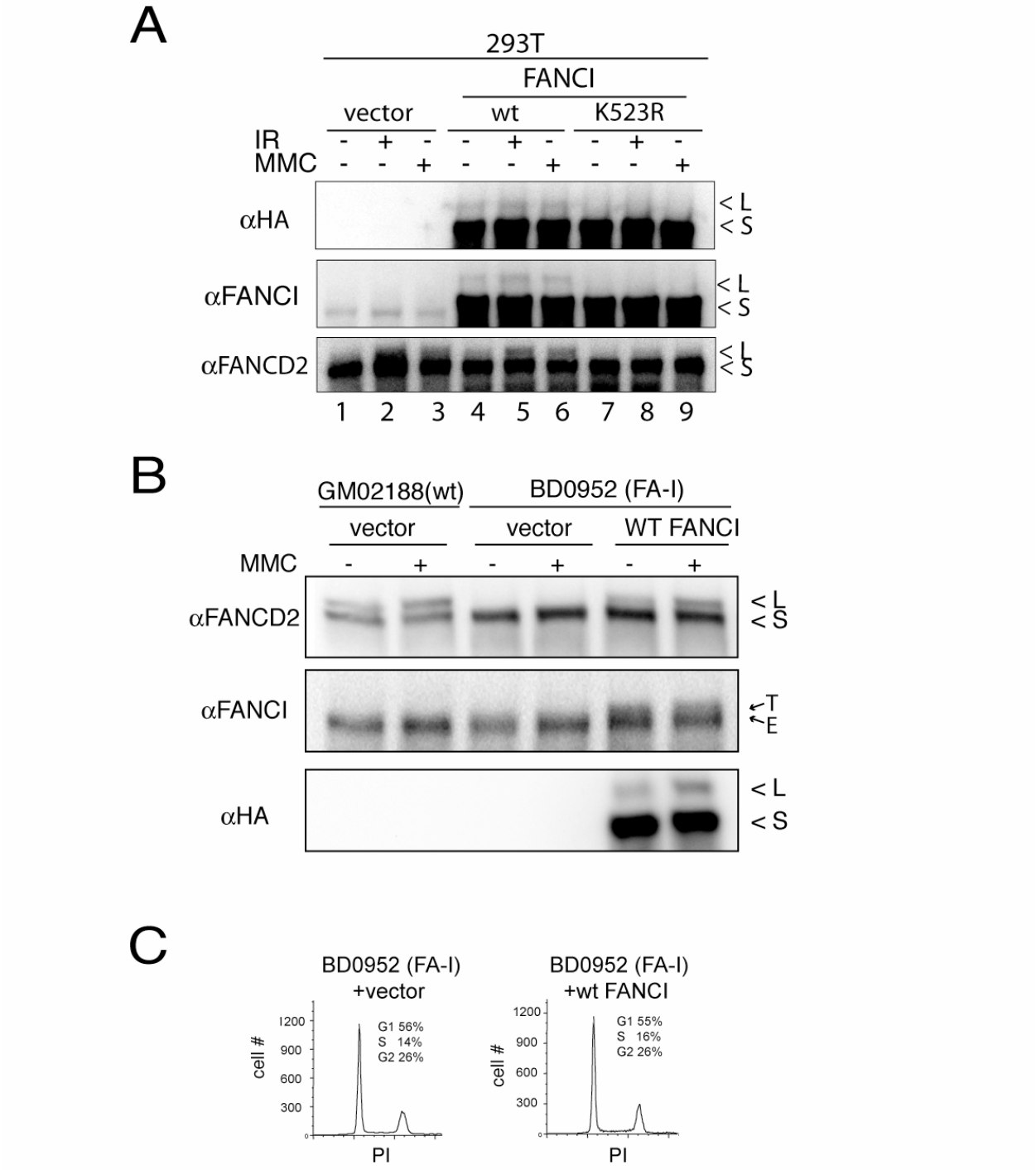
A. Localization of exogenous myc-tagged FANCI. U2OS cells were transduced with a Myc-tagged *FANCI*-carrying retrovirus and treated with 1 μM mitomycin C. 24 hours later cells were co-stained with 9E10 antibody (Myc) and a rabbit antibody against human FANCD2 without triton pre-extraction.

B. Localization of FANCI in cells transfected with 2 different siRNAs against FANCD2. U2OS cells were transfected with the indicated individual siRNAs against FANCD2 and treated with 1 μM mitomycin C. Twenty-four hours later following 0.5 % triton extraction the cells were stained with an antibody against FANCI, FANCD2, or H2AX.

C. 293T cells stably transduced with a HA-FLAG FANCI retrovirus, were treated with 10 Gy of γ-IR. 1 mg total protein was immunoprecipitated with HA, FLAG or Myc antibodies. The immunoprecipitates were analyzed by western blotting with a rabbit anti-FANCD2 antibody.

D. Interaction of FANCD2 and endogenous FANCI. 0.5 mg total protein from PD20 fibroblasts expressing WT or K561R allele of FANCD2 were immunoprecipitated with anti-FANCI antibody (BL1000) under non-damaged conditions. The immunoprecipitates were analyzed by western blotting with a rabbit anti-FANCD2 or rabbit anti-FANCD2 antibody.

E. Interaction of FANCD2 and FANCI; FANCD2 IP. 0.5 mg total protein from PD20 fibroblasts expressing WT or K561R allele of FANCD2 and also expressing HAFLAG-tagged WT or K523R allele was immunoprecipitated with anti-FANCD2 antibodies under non-damaged conditions. The immunoprecipitates were analyzed by western blotting with a rabbit anti-FANCD2 or mouse anti-HA antibody

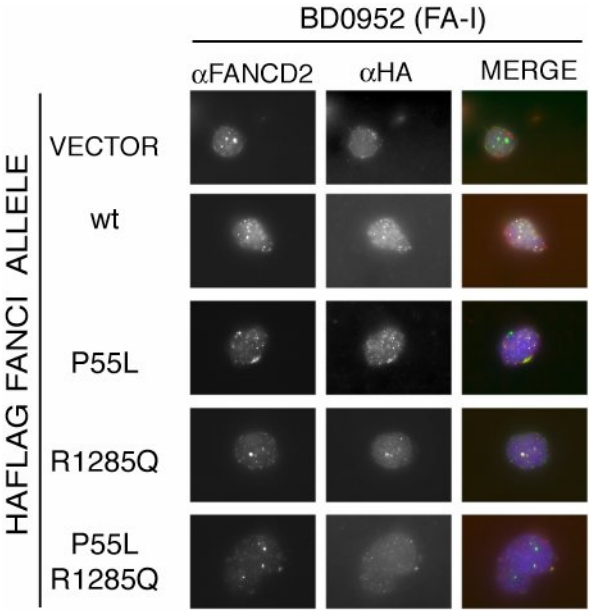


Supplementary Figure 5. FANCI ubiquitination

A. Lack of ubiquitination of K523R FANCI. 293T cells were stably transduced with HA-FLAG-tagged WT or K523R FANCI alleles. 8.5 hours after 15Gy IR or 1μM MMC treatment cells were harvested and lysed in Laemmli buffer.

B. Complementation of FANCD2 ubiquitination defects in FA-I cells by expression of WT FANCI. A similar experiment to the one in Figure 6A to show that the WT HA-tagged FANCI becomes ubiquitinated. The exposure for the western blot performed with the FANCI antibody is not high enough to see the long form of FANCI in this blot. However, the transduced form of the protein is identifiable (T for tagged) since it runs slightly slower than the endogenous (E) form. The long form of FANCI is visible when probed with an antibody recognizing HA tag.

C. Cell cycle analysis of BD0952 complemented with an empty vector or with WT FANCI. Cells stably transduced with HA-tagged WT FANCI or with empty vector were stained with PI and the cell cycle stage was assessed by flow cytometry.



Supplementary Figure 6

Localization of WT, P55L, R1285Q, and P55L, R1285Q mutant proteins in BD0952 (FA-I) cells. BD0952 transduced with the above alleles of *FANCI* were treated with 100 nM MMC and 24 hours were processed for immunofluorescence. Note that BD0952 that were not complemented, still contained some FANCD2 foci. However they were much fewer in number

and they were large and amorphous unlike the foci that formed after complementation with the WT or P55L FANCI allele. It remains to be determined what structures these foci represent.

Supplementary Experimental Procedures

Tissue culture conditions

The adherent cell lines were grown in Dulbecco Modified Eagle medium (DMEM) supplemented with 100 units of penicillin per ml, 0.1 mg streptomycin per ml, L-glutamine (2 mM), non-essential amino acids (0.1mM), and 10% or 15% (v/v) FBS (Invitrogen) depending on the cell line, and lymphoblastoid lines were grown in RPMI with the same supplementation. Retroviral transduction of the lymphocytes was performed by spinning 1x10^6 with a freshly-collected virus supplemented with 8 μg of polybrene per ml of supernatant at 2500 rpm for 45 minutes at room temperature.

Preparation of cells for the Multicolor Competition Assay and assay conditions

U2OS cells were infected with MSCVgfp or MSCVdsRed retrovirus. Without selection, the cells were sorted using the Aria Sorter (BD) for cells with intermediate expression. The gfp cells grew slightly faster than the rfp cells and this was taken into account when calculating the changes in survival due to treatment with DNA damaging agents. The concentration of Mitomycin C (Sigma) was chosen to result in about 50% survival of non-transfected cells, which was about 70 nM MMC for U2OS cells.

siRNAs

Target sequences were as follows. siRNAs were purchased from Invitrogen unless otherwise

stated:

lacZ (Qiagen) AACGTACGCGGAATACTTCGA

FANCI (Qiagen) CTGGCTAATCACCAAGCTTAA

USP1 (Qiagen) TCGGCAATACTTGCTATCTTA)

ATM: GCGCAGTGTAGCTACTTCTTCTATT, GGGCCTTTGTTCTTCGAGACGTTAT,

GCAACATTTGCCTATATCAGCAATT

ATR: GGGAAATAGTAGAACCTCATCTAAA, GGTCTGGAGTAAAGAAGCCAATTTA,

CCACCTGAGGGTAAGAACATGTTAA

FANCI #1: TCTCCTCAGTTTGTGCAGATGTTAT

FANCI #2: GGCAGCTGTGTGGACACCTTGTTAA

FANCI #3: GCTGGTGAAGCTGTCTGGTTCTCAT

FANCD2 #2: TTAGTTGACTGACAATGAGTCGAGG

FANCD2#3: AATAGACGACAACTTATCCATCACC

BRCA1:AAATGTCACTCTGAGAGGATAGCCC, TTCTAACACAGCTTCTAGTTCAGCC,

TAGAGTGCTACACTGTCCAACACCC

FANCA: GGAAGATATCCTGGCTGGCACTCTT, CCAGCATATTCAGGAGGCCTTACTA,

TCCCTCCTCACAGACTACATCTCAT

Primers

KIAA1794 cDNA cloning primers CCGCTCGAGGACCAGAAGATTTTATCTCTAGCAG

and CCGGTTAACTTAACTCAGGCATTTCATTTATTTT

1st coding exon primers: TTCAGGATTATTTTGGTTAGGTTA and

GGTCACAAATGCCCTCAAG

3rd coding exon primers: TCAAAGCCCTTAACCATTGC and TGCCATCTTACCTCCAGCAT

36th coding exon primers: TCTTGATCTGATGACCTGAACC and

GTCGGGGCAACTTCATAGGAT.

K523R mutation primers: GCTTGATACTTGTCCTTCGGCGAGCTATGTTTGCCAACCAGC and GCTGGTTGGCAAACATAGCTCGCCGAAGGACAAGTATCAAGC.

P55L mutation primer CTTCAAAGGTTCCCTCTGCTCTGAGGAAGCTGG

R1285Q mutation GCTCAGCACCTCACAAGACTTCAAGATCAAAGG

Immunofluorescence

Cells grown on autoclaved cover slips were rinsed in PBS and were fixed in 3.7% (w/v) formaldehyde (Sigma) diluted in PBS for 10 minutes at room temperature. Cells were washed once with PBS, permeabilized in 0.5% (v/v) NP40 in PBS for 10 minutes, washed again in PBS, and blocked with PBG (0.2% [w/v] cold fish gelatin, 0.5% [w/v] BSA in PBS) for 20 minutes. Coverslips were incubated for 2 hours at room temperature or at 4^{o} C overnight in a humidified chamber with a primary antibody and after washing 3 times for 5 minutes in PBG, then were incubated with the appropriate secondary antibody. After three additional washes in PBG, the coverslips were embedded in Vectashield (Vector Laboratories) supplemented with DAPI. Triton pre-extraction was performed by incubating cells for 5 minutes at room temperature with 0.5% Triton in PBS. Cells were fixed and processed as above. Images were captured with Axioplan2 Zeiss microscope with a AxioCam MRM Zeiss digital camera supported by Axiovision 4.5 software. Any co-staining experiments included proper controls to exclude crossing of signal between channels.

Radioresistant DNA synthesis assay

RDS assays to evaluate the intra-S phase checkpoint were done as described previously (Silverman et al., 2004). Briefly, U2OS cells were transfected with control siRNA or siRNAs against KIAA1794 (combination of 3 siRNAs, approximately 30 nM of each) using oligofectamine (Invitrogen). 24 hours later, medium containing 10 nCi/mL of [methyl-14C] thymidine (Amersham, CFA532) was added and cells were incubated for 24 hours. Then, medium without label was added for 24 hours. The cells were then irradiated (Cesium 137 source) with 5-15 Gy. Following a 30-minute incubation at 37 degrees, the cells were pulse labeled with 2.5 uCi/mL [methyl-3H] thymidine (Amersham, TRK758) for 20 minutes and then washed twice with medium containing 2.5 mM cold thymidine (no serum). Cells were harvested by trypsinization and TCA precipitation was performed on Whatman glass microfibre filters (GF/C, 25mM, Fisher) using a vacuum manifold. Following an ethanol wash, the filters were dried and counted using a liquid scintillation counter (Beckman LS6000). The ratio of ^{3}H counts per minute to ^{14}C counts per minute, corrected for those counts per minute that were the result of channel crossover, were a measure of DNA synthesis.

HR assay

Five or 10 pfu of adenovirus per cell was used since this level of virus resulted in 100% infection but had no visible deleterious effects on cells. Events were gated to exclude any doublets. Both gated and non-gated analysis gave similar results.

G2/M assay image acquisition

Plates were imaged on an automated ImageXpress Micro (Molecular Dynamics) at 10X and the mitotic index was calculated using the MetaExpress Software package. An average of 1000 cells

was counted per well. Wells scoring above control levels were visually inspected to verify accurate scoring by the software.

Cell cycle analysis

For cell cycle analysis, collected cells were resuspended in 100 µl (PBS). While vortexing, 2 ml of ice cold 70% (v/v) ethanol were added drop-wise and the suspension was stored at 4^{o}C at least overnight. 30 min before FACS, cells were spun down, resuspended in propidium iodine (PI) mix (500 µl PBS, 10 µl RNase [of stock solution of 10 mg/ml], 25 µl PI [of stock solution of 1 mg/ml]), and analyzed using LSR2 (Becton Dickinson). Cell cycle analysis was performed using FlowJo.

Immunoprecipitations

For immuoprecipitations, cells were lysed in TBS (20 mM Tris +150 mM NaCl) supplemented with 0.5% NP-40, protease Inhibitors (Roche), 1 mM PMSF, 5 mM NaF, 5 mM Na3VO4 and 50U of Benzonase (Novagen) per ml of lysis buffer. Experiment shown in Supplementary Figure 4C was done without addition of benzonase. 1 mg protein extract was incubated with 2 µg of the indicated antibody and 5 µl of Protein A/G PLUS-Agarose (Santa Cruz). Following three washes in lysis buffer, the immunoprecipitates were eluted in tris-Glycine SDS sample buffer and size-fractionated on a Tris-Glycine gel (Invitrogen). Streptavidin immunoprecipitation under denaturating conditions was performed as described (Tagwerker et al., 2006) except the His-purification step was omitted. Streptavidin sepharose (GE Healthcare) was used with lysis and wash buffer consisting of 8 M urea, 200 mM NaCl, 100 mM Tris pH 8, 0.5% SDS, 0.5% NP40.