Supplementary Materials for

Ubiquitin-Fancd2 recruits Fan1 to stalled replication forks to prevent genome instability

Christophe Lachaud, Alberto Moreno, Francesco Marchesi, Rachel Toth, J. Julian Blow

& John Rouse

correspondence to: j.rouse@dundee.ac.uk

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Materials and Methods
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Materials and Methods

Mouse strains and husbandry

Animals were housed under specific pathogen free conditions in accordance with UK and EU regulations. All procedures were carried out in accordance with University of Dundee and United Kingdom Home Office regulations.

To generate Fan1$^{nd/nd}$ mice, a targeting vector was constructed to replace exon 13 on the murine Fan1 gene with a mutated exon where the codon for Lys975 was mutated to encode an alanine, and a puromycin resistance cassette (Fig. S1A). FRT sites were introduced so that the puromycin resistance cassette could be excised from the targeted locus. A thymidine kinase cassette was included at the 3' end of the targeting vector to enable selection against non-targeting random integration. The arms for the targeting vector - a short homology 5' arm of 4.0 kb and a long homology arm of 6.2 kb - were cloned by PCR using an appropriate BAC clone as template. All clones were fully sequenced to ensure that they contained no PCR generated errors before assembly into the final targeting vector (Fig. S1A). The linearized targeting vector was electroporated into E14 C57Bl6 Tac embryonic stem (ES) cells, followed by positive (G418) and negative (gancyclovir) selection using standard procedures. Resistant clones were screened for targeted integration by southern blotting and PCR (data not shown), and six targeted ES cell clones were isolated. These clones were injected into BALB/c blastocysts, which were then re-implanted into recipient female mice. Male chimeric mice showing a high percentage of ES cell contribution were bred to C57BL/6 female mice. After recovery, 8 injected blastocysts were transferred to each uterine horn of 2.5 days post coitum, pseudopregnant NMRI females. Chimerism was measured in chimeras (G0) by coat color contribution of ES cells to the BALB/c host (black/white). Highly chimeric mice were bred to strain C57BL/6 females. The C57BL/6 mating partners were mutant for the presence of a recombinase gene (Flp-Deleter), which then excised the puromycin resistance cassette leaving just a single FRT site between exon 13 and 14 of the Fan1 gene. Germline transmission was identified by the presence of black, strain C57BL/6, offspring (G1). Correct targeting and removal of puroycin resistance cassette

were verified by southern blotting and PCR. Mice were routinely genotyped from ear notch biopsies according to standard procedures. Genotyping with primers P1 (5' TCTGTTTCTCCCTTAATGTTTAGC) and P2 (5'CACAGTGTTAGATTCTGTCTGATACC) rise to a 159 bp band in wild type alleles and 234 bp in knockin alleles because of a residual FRT site in the knockin allele. Representative genotyping results are shown in Fig. S1B. Additionally, successful mutation of the K975 residue was verified by PCR using P3 (5'GGCAGAAGCACGCTTATCG) and P4 (5'GACAAGCGTTAGTAGGCACAT) primers. The generated 326 bp product was then cloned and sequenced.

Fancd2$^{-/-}$ mice described previously were a kind gift from Alan d'Andrea. All mice were backcrossed 4 times before analysis.

mRNA sequencing

Total RNAs were purified using RNeasy kit from Qiagen. Sequence at the interface between exon 13 and the following intron have been amplified by RT-PCR (Takara PrimeScript High Fidelity RT-PCR Kit) using primers P3 and P4 and clone into pSC-A before sequencing.

Isolation and immortalization of MEFs

13.5 dpc embryos were derived from timed matings between heterozygous parents. After decapitation, the heads were used for genotyping. The red organs were removed, the embryo was minced and resuspended in 1 ml trypsin and incubated at 37°C for 15 min before the addition of 10 ml growth medium. Cells were plated and allowed to attach over night before cells were washed with fresh medium to remove debris. When cells reached confluency they were split and re-plated and this was considered passage 1. All MEFs were immortalized using SV40 large T antigen.

Virus-based rescue experiments in MEFs

Viruses were produced by co-transfecting 293T cells with the relevant open reading frames cloned into pBABE vector, and with pCMV-VSV-G and pCMV-Gag-Pol

expression vectors to direct viral packaging. After 48 h the virus-containing supernatant was filtered through a 45 μm filter, mixed with polybrene (final concentration of 8 μg/ml) and added to MEFs. Three hours later the infection medium was replaced with fresh growth medium. The infection protocol was repeated 24 h later. MEFs were stably selected with puromycin (3μg/ml) 24 h after the second infection. Protein expression was tested by western blotting.

Clonogenic survival analysis

MEFs were seeded in triplicates in 10 cm dishes and were allowed to attach before treatment. Genotoxin was added to cells for 24 h before medium was replaced with fresh growth medium. After 10 days cells were washed, fixed and stained with Giemsa. The number of colonies with >100 cells were counted. For each genotype, cell viability of untreated cells was defined as 100%. Data are represented as mean ± SD from three independent experiments.

Analysis of chromosomal abnormalities

For the analysis of chromosomal abnormalities MEFs were left untreated, or were exposed to MMC (50 ng/ml) for 24 h or MMS (50 μM or 100 μM) for 20 h. In the case of HU, cells were treated with HU (1 mM) for 6 h followed by 6 h recovery. Colcemid (0.1 mg/ml) was added to the cells for 30 min. Cells were then trypsinized, washed and incubated with 75 mM KCl for 20 min at 37°C. Cells were subsequently fixed with 3:1 methanol:acetic acid at 4°C overnight before spreading. Spreads were stained with DAPI and 20 spreads were analyzed for chromatid breaks and radial chromosomes; at least two independent experiments were performed. Data are presented as chromosome abnormalities per metaphase.

DNA fiber assay

Cells were incubated with CldU (20μM; Sigma C6891) for 20 min, then quickly washed with PBS, before being given a 120 min pulse of IdU (100 μM; Sigma I7125) in the presence of HU (0.5 mM) added at the same time as IdU. In control experiments, cells were incubated with CldU for 20 min, followed by a 20 min pulse of IdU in the absence

of HU. Cells were harvested, lysed and spread to obtain single DNA molecules on microscope slides before standard immunofluorescence with antibodies against BrdU (Anti-BrdU Rat, BioRad OBT0030CX; Anti-BrdU Mouse, BD 357580). Images were acquired using an Olympus IX70 deltavision deconvolution microscope. An Olympus 63x oil immersion objective was used. Images were captured using a CCD camera. 100 fields or more from each condition were analysed. Data from microscopy experiments was analysed using Volocity 3-dimensional (3D) image analysis software (PerkinElmer). A standard protocol was designed within this programme for measuring the IdU-tracks length.

Cell culture

All cells were kept at 37°C under humidified conditions with 5% CO_2. U2OS and 293T cells were grown in DMEM, 10% FBS, L-glutamine and penicilin/streptomycin. For MEFs, medium was supplemented with Na-pyruvate and non-essential amino acids. Puromycin was used at 3 µg/ml for selection of complemented (virus-infected) MEFs, or stably transfected U2OS cells.

Cell lysis and immunoprecipitation

Cells were lysed in ice–cold buffer: (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 270 mM sucrose, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1% ß-mercaptoethanol) with protease inhibitors (Roche) and 50 U/ml of benzonase (Novagen).

Immunofluorescence-based detection of Fan1 foci

For indirect immunofluoresence analysis, cells were plated onto sterile coverslips and treated with either MMC (50ng/ml) or HU (10mM) for 6 hours. Coverslips were then rinsed in PBS and fixed using 4% paraformaldehyde for 10 min at room temperature. Cells were permeabilized with 0.2% Triton X–100 in PBS for 10 min at room temperature, washed several times in PBS and incubated in blocking solution (PBS containing 5% donkey serum, 0.1% fish skin gelatine and 0.05% Tween-20). Coverslips were incubated with primary antibodies against GFP (Abcam) in blocking solution for 1 h at room temperature. After washing in PBS, coverslips were incubated with the appropriate 488-conjugated secondary antibodies (Life Technologies) for 90 min. The

nuclei were stained with DAPI for 5 min and coverslips mounted on glass slides. Cells were imaged using a Zeiss 710 confocal microscope equipped with a 37°C environmental chamber and supplied with humidified 5% CO_2, a Zeiss Plan-Apochromat 63x 1.4NA oil immersion objective lens.

Detection of G2 arrest by flow cytometry

Low passage, low confluence cultures of asynchronously growing MEFs were treated with MMC (50ng/ml) (Sigma-Aldrich) for 48 h. Following trypsinization, cells were collected by centrifugation, washed in PBS and fixed in ice-cold 70% ethanol. Cells were centrifuged, washed in PBS and treated for 15 min at 37°C with 50 μg/ml RNase A. Cells were stained at room temperature with propidium iodide (PI) solution (2 mM MgCl2, 10 mM PIPES buffer, 0.1 M NaCl, 0.1% Triton X-100, 0.01 mg/ml PI) and analysed on a FACS Calibur flow cytometer (BD Biosciences). Cell cycle profiles were created using FlowJo analysis software (Tree Star Inc., Ashland OR).

Edu incorporation detection by flow cytometry

Low passage, low confluence cultures of asynchronously growing MEFs were treated with HU (1mM, 2 h) prior treatment with Edu (0.5 mM, 2 h). Following trypsinization, cells were collected by centrifugation, washed in PBS and fixed in ice-cold 70% ethanol. Cells were centrifuged, washed in PBS and treated for 15 min at 37°C with 50 μg/ml RNase A. Cells were stained at room temperature with propidium iodide (PI) solution (2 mM MgCl2, 10 mM PIPES buffer, 0.1 M NaCl, 0.1% Triton X-100, 0.01 mg/ml PI) and analysed on a FACS Calibur flow cytometer (BD Biosciences). Cell cycle profiles were created using FlowJo analysis software (Tree Star Inc., Ashland OR).

Histopathology

Animals were euthanased by means of CO2 asphyxiation and a complete post mortem evaluation and dissection was performed. The following tissues were collected and fixed in 10% neutral buffered formalin: brain, salivary glands and mandibular lymph nodes, thymus, heart, lungs, stomach, pancreas, small and large intestine, pancreatic and mesenteric lymph nodes, spleen, liver, kidney, female reproductive system (ovaries and

uterus), male reproductive system (testes, epididymides, seminal vesicles and prostate), femur, with stifle joint and proximal tibia.

Following 48 hour fixation the tissues were trimmed and samples were processed to paraffin blocks. Sections (3 μm-thick) were stained with haematoxylin and eosin (HE) and evaluated by a veterinary pathologist unaware of the genotype of the animals.

Antibodies

Antibodies against mouse Fan1 were raised in sheep against the 300 first amino acid of mouse Fan1 fused to GST. Antibodies were affinity purified using immobilized antigen (S778D) and used at 0.5 mg/ml for 1h. Antibodies against mouse Fancd2 were raised in sheep against the full human FancD2 fused to GST (S099D) and used at 0.5 mg/ml for 12h at 4^{o}C. Anti-GAPDH (14C10) antibodies were purchased from Cell Signalling. All secondary antibodies were purchased from Pierce. Anti-GFP and anti-mCherry antibodies were purchased from Abcam. Commercial antibodies were used according to the manufactures instructions.

DNA constructs

The full-length coding regions for the relevant human proteins were generated by PCR using IMAGE consortium EST clones. Full-length coding regions for the relevant mouse proteins were generated by RT-PCR (Takara PrimeScript High Fidelity RT-PCR Kit). Point mutations were introduced by Quickchange mutagenesis. The sequence integrity was confirmed by sequencing analysis. Human and mouse cDNA were respectively cloned into pcdna5 FRT/TO.puro vector and pBabe.puro vectors.

Statistical analysis

Statistical significance calculated using one-way ANOVA (****, $p < 0.0001$) and Bonferroni's Multiple Comparison Test.

Reagents

All cDNA plasmids, antibodies and recombinant proteins generated for this study are available to request through our reagents website (https://mrcppureagents.dundee.ac.uk/).

Reference

1. C. MacKay *et al.*, Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. *Cell* **142**, 65-76 (2010).

Supplementary figures

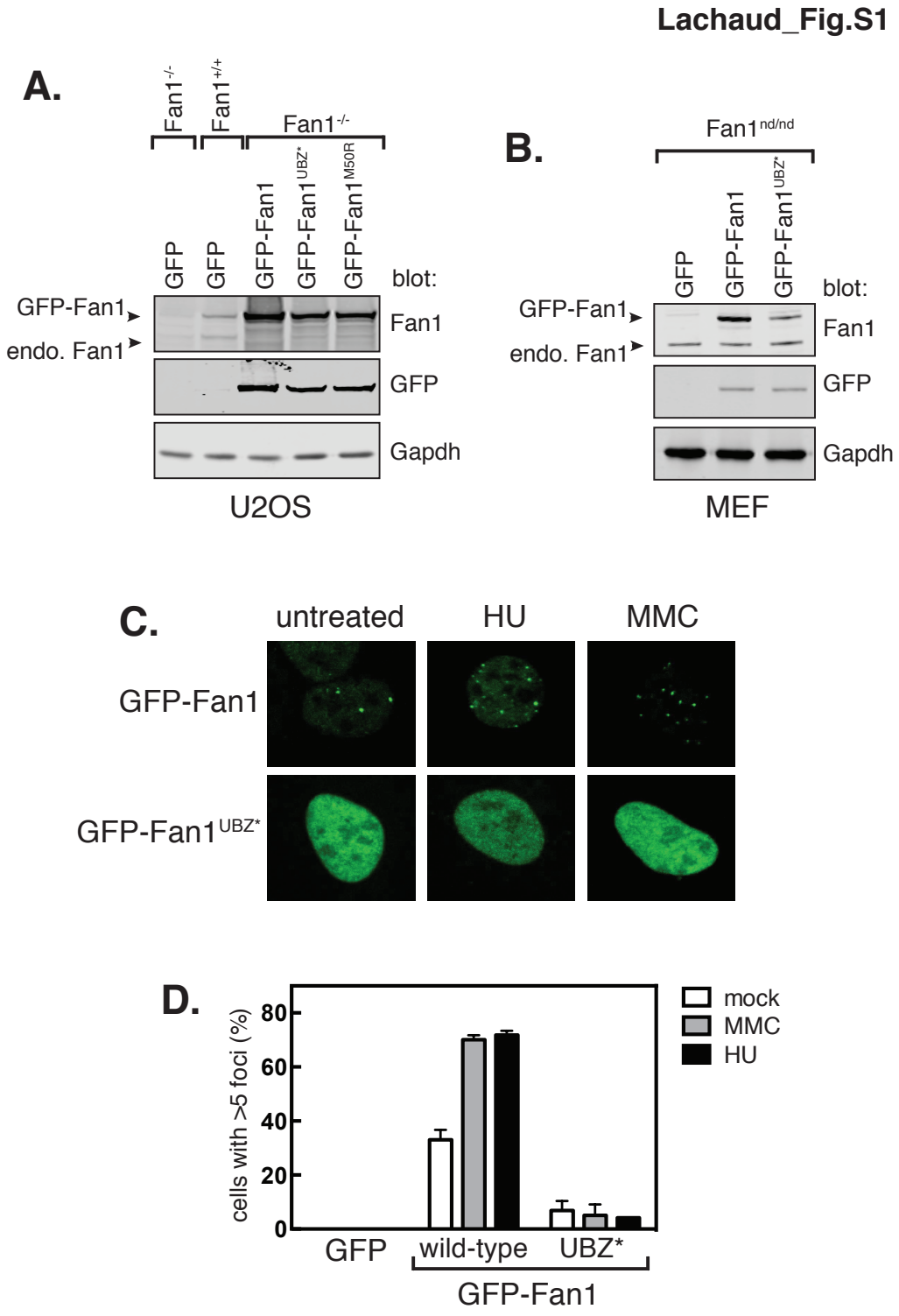

Figure S1: Murine Fan1 UBZ mutation abolishes Fan1 foci

A. Extracts of U2OS Fan1$^{-/-}$ cells stably expressing GFP or GFP-tagged forms of Fan1, Fan1-UBZ* (C44A+C47A) or Fan1 M50R, and extracts of parent U2OS cells (Fan1$^{+/+}$) expressing GFP were blotted with the antibodies indicated. **B.** Extracts of Fan1$^{nd/nd}$ MEFs stably expressing GFP or GFP-tagged forms of Fan1 or Fan1-UBZ* (C44A+C47A) were blotted with the antibodies indicated. **C.** Representative images of foci formed by GFP-tagged murine Fan1 or the Fan1 C44A+C47A (UBZ*) mutant in U2OS cells 6h after 6 h exposure to the genotoxins indicated. **D.** The proportion of cells with greater than five Fan1 foci per cell was counted in 100 cells, in three independent experiments.

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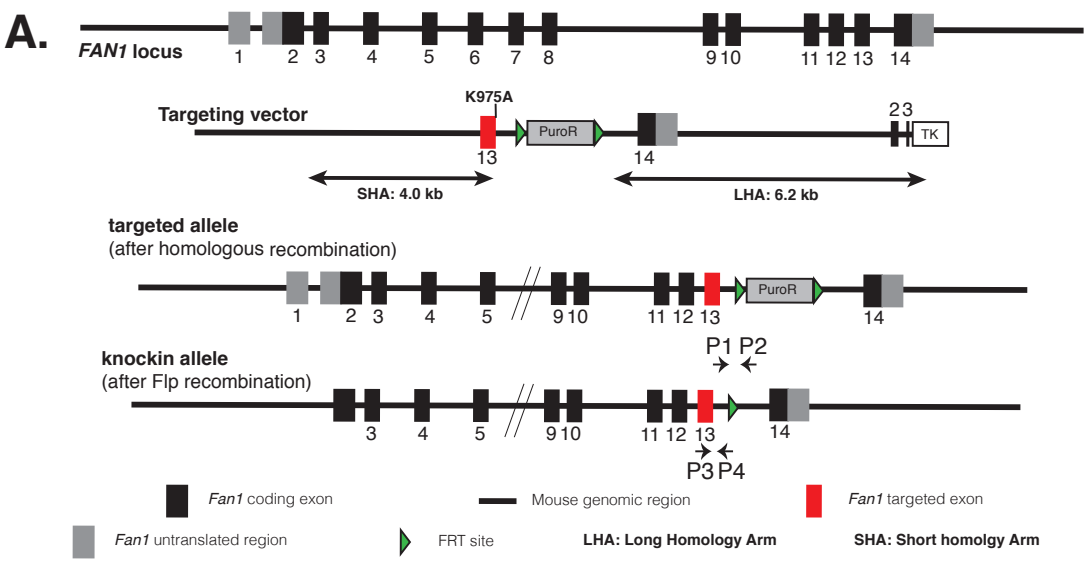

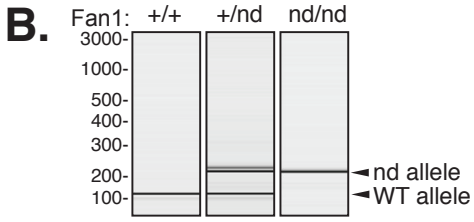

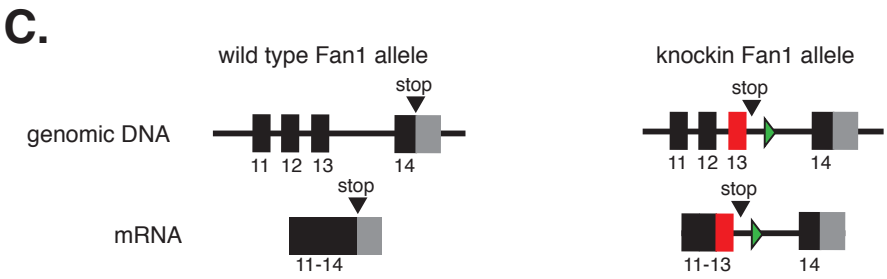

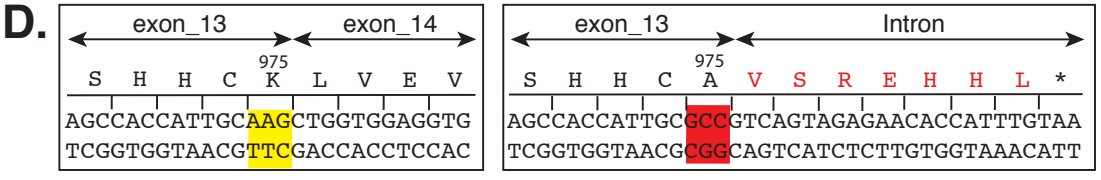

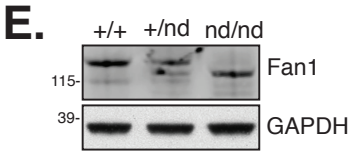

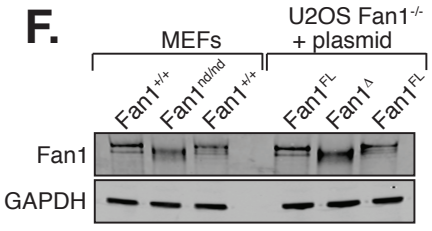

Figure S2: Generating Fan1 nuclease-defective mice

A. Generation of Fan1 nuclease-defective mice (see Methods below). A strategy was devised to introduce a mutation in exon 13 of the murine Fan1 gene that would replace Lys975 with an alanine residue. The black/grey rectangles represent exons and the green triangles represent FRT sites. The knock-in allele containing the K975A mutation in exon 13 is illustrated as a red rectangle. The knock-in allele can be detected by genotyping using PCR primers P1 and P2, which are represented by black arrows. **B.** Genomic DNA extracted from tail biopsies were analyzed by PCR with the oligonucleotides P1 and P2 indicated in A. Results are displayed as an electronic gel picture. PCR with primers P1 and P2 yields a 159 bp band with the wild type Fan1 allele, and 234 bp with the knockin allele because of a residual FRT site in the knockin allele. **C.** Effect of Fan1 knockin mutation on Fan1 pre-mRNA splicing. **D.** The Fan1 knockin mutation changed K975 to A, but also inhibited splicing of exon 13 with exon 14 (the last Fan1 exon), resulting in retention of the intervening intron in the mRNA. As a result A975 in the knockin allele is followed by seven intron-encoded amino acids and a stop codon, concomitant with deletion of the last 41 amino acids of Fan1. The 41 amino acids deleted includes several residues essential for catalysis such as Asp 981 and Arg 982 (*1*). **E.** Western blot of MEF extracts from mice of the indicated genotypes. **F.** Human U2OS $Fan1^{-/-}$ cells were transfected with full length (FL) murine Fan1, or a truncated form of Fan1 (Fan1Δ) corresponding to the truncated protein that results from inhibition of splicing of exon 13 (see D). Extracts were subjected to SDS-PAGE and western blotting, in parallel with extracts of $Fan1^{+/+}$ and $Fan1^{nd/nd}$ MEFs.

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A. Genomic DNA sequence: FAN1$^{+/+}$

CTGTCCTCAGTGGTGTGTGCAGGCGCCTGGCTGCTGACTTTCGGCACTGCCGAGGGG
GCCTCCCAGACTTGGTGGTGTGGAATTCTCAGAGCCACCATTGC**AAG**GTCAGTAGAG
AACACCATTTGTAATTTCAGCAGCAGCTATGTCACAGTTCACGTGCTGGACATTTTC
TTCTGACTGTTGGAAGGATCAAATTCCTTGATAGCCACCAGGCTGAGGAGCTGGGGT
GCAAGCAAGACAGGCAGTCTCATGTCAGCCCATCATGGAGGTGATAGTCGACCAGTA
CAACATTCTGTTACTAGAACAAGCCACTAGCTGCGAGGCAC

Genomic DNA sequence: FAN1$^{nd/nd}$

CTGTCCTCAGTGGTGTGTGCAGGCGCCTGGCTGCTGACTTTCGGCACTGCCGAGGGG
GCCTCCCAGACTTGGTGGTGTGGAATTCTCAGAGCCACCATTGC**GCC**GTCAGTAGAG
AACACCATTTGTAATTTCAGCAGCAGCTATGTCACAGTTCACGTGCTGGACATTTTC
TTCTGACTGTTGGAAGGATCAAATTCCTTGATAGCCACCAGGCTGAGGAGCTGGGGT
GCAAGCAAGACAGGCAGTCTCATGTCAGCCCATCATGGAGGTGATAGTCGACCAGTA
CAACATTCTGTTACTAGAACAAGCCACTAGCTGCGAGGCAC

B. RT-PCR analysis of mRNA from Fan1$^{nd/nd}$ mouse kidney, using Fan1 primers P3 and P4 (see Fig. S1A)

CTGTCCTCAGTGGTGTGTGCAGGCGCCTGGCTGCTGACTTTCGGCACTGCCGAGGGG
GCCTCCCAGACTTGGTGGTGTGGAATTCTCAGAGCCACCATTGC**GCC**GTCAGTAGAG
AACACCATTTG***TAA***TTTCAGCAGCAGCTATGTCACAGTTCACGTGCTGGACATTTTC
TTCTGACTGTTGGAAGGATCAAATTCCTTGATAGCCACCAGGCTGAGGAGCTGGGGT
GCAAGCAAGACAGGCAGTCTCATGTCAGCCCATCATGGAGGTGATAGTCGACCAGTA
CAACATTCTGTTACTAGAACAAGCCACTAGCTGCGAGGCAC

Fig. S3; Sequencing around Fan1 knockin mutation in Fan1$^{nd/nd}$ mice

A. Genomic DNA extracted from ear notch biopsies of Fan1$^{+/+}$ and Fan1$^{nd/nd}$ mice was subjected to PCR analysis with the primers P3 and P4 (shown in Fig. S1A). Primer P3 lies within exon 13 and primer P4 within the following intron. The resulting PCR products were TA-cloned into plasmid pSC-A (Invitrogen), and sequenced backwards and forwards. The raw DNA sequences of the cloned inserts are shown. The codon corresponding to K975 is highlighted in red. The primer sequences are highlighted in yellow. **B.** Total RNA extracted from the kidneys of Fan1$^{+/+}$ and Fan1$^{nd/nd}$ mice was subjected to reverse transcription followed by PCR with the primers P3 and P4. This yielded a product of 326 bp using mRNA from Fan1$^{nd/nd}$ mice but not from wild-type mice – P4 anneals to the intron after exon 13, which is spliced out of the wild-type Fan1 mRNA. The PCR product from Fan1$^{nd/nd}$ mice was TA-cloned into plasmid pSC-A, and sequenced backwards and forwards. The sequence of the cloned insert is shown. The sequences annealing to primers P3 and P4 are highlighted in yellow. Exon 13 is highlighted in green. The codon corresponding to A975 is highlighted in red. The intron that lies between exon 13 and 14 is highlighted in blue.

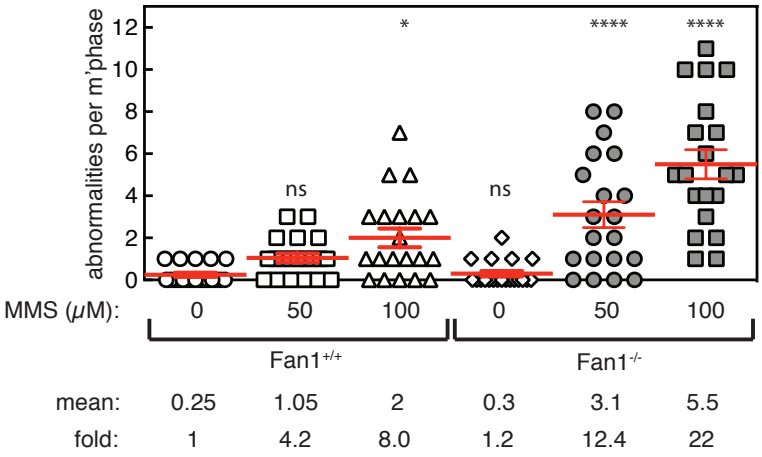

Figure S4: Increased chromosome abnormalities after MMS treatment of Fan1$^{-/-}$ U2OS cells

Chromosome abnormalities per metaphase were counted in human U2OS Fan1$^{-/-}$ cells, or parental U2OS cells (Fan1$^{+/+}$) exposed to the indicated concentrations of MMS for 20 h.

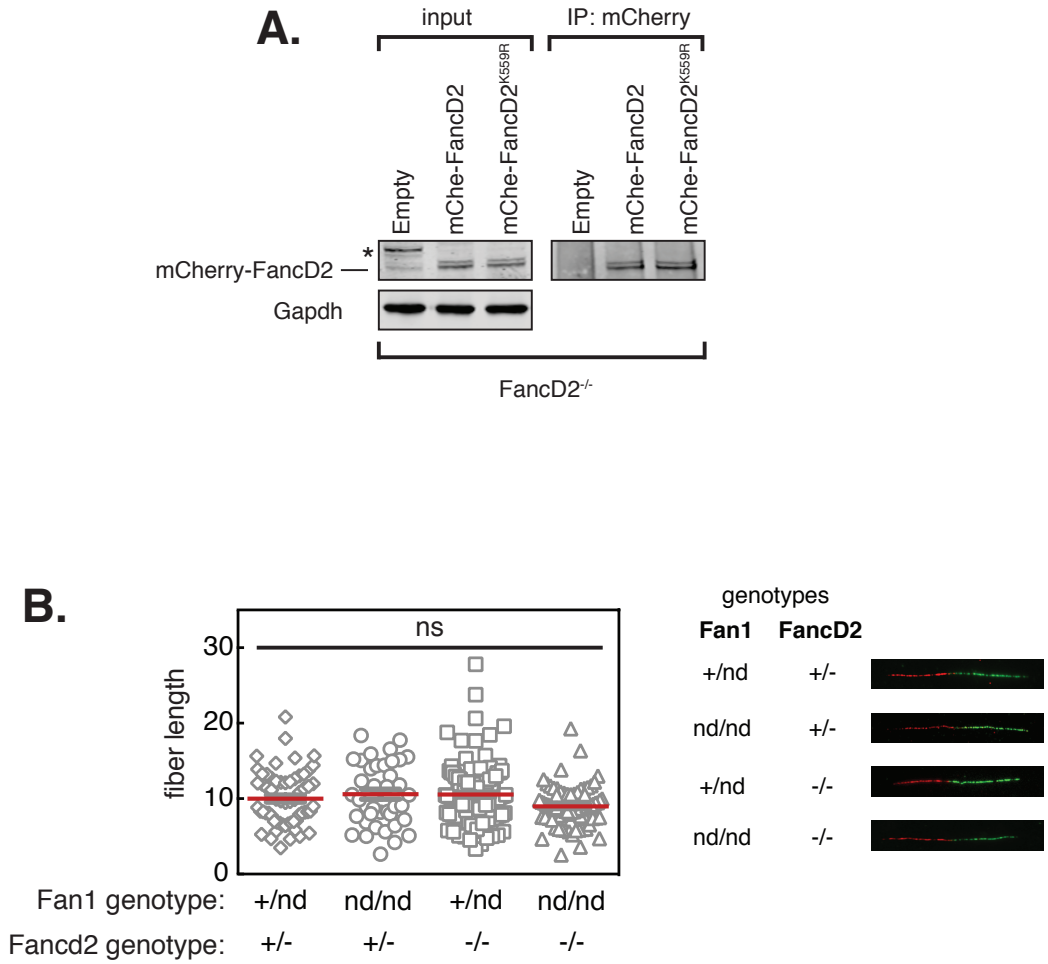

Figure S5: Fan1/Fancd2 does not affect progression of forks before stalling

A. Extracts of Fancd2$^{-/-}$ MEFs stably expressing mCherry, mCherry-Fancd2 or mCherry-Fancd2-K561R were blotted with the antibodies indicated. Asterisk denotes non-specific band. **B.** Dot plots of ldU replication tracks in MEFs of the genotypes indicated that were not treated with HU. Representative CldU and IdU replication tracks are shown (right panels). Red lines represent mean track length.

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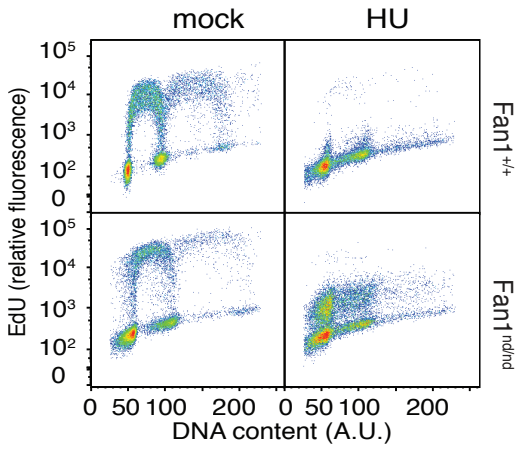

Figure S6: Edu incorporation in Fan1$^{nd/nd}$ MEFs after HU treatment

Fan1$^{+/+}$ and Fan1$^{nd/nd}$ MEFs were treated with HU (1 mM; 2 h) follow by EdU treatment (30 min). After fixation, cells were stained with propidium iodide and subjected to FACS to monitor DNA content and EdU incorporation.