Supplementary information for

Replication stress induces accumulation of FANCD2 at central region of large fragile genes

Yusuke Okamoto[1,2], Watal M. Iwasaki[3], Kazuto Kugou[4,#], Kazuki K. Takahashi[3], Arisa Oda[4], Koichi Sato[5,*], Wataru Kobayashi[5], Hidehiko Kawai[6], Ryo Sakasai[7], Akifumi Takaori-Kondo[2], Takashi Yamamoto[8], Masato T. Kanemaki[9,10], Masato Taoka[11], Toshiaki Isobe[11], Hitoshi Kurumizaka[5], Hideki Innan[3], Kunihiro Ohta[4], Masamichi Ishiai[1], Minoru Takata[1**]

[1] Laboratory of DNA Damage Signaling, Department of Late Effects Studies, Radiation Biology Center, Kyoto University, Kyoto Japan

[2] Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

[3] SOKENDAI (The Graduate University for Advanced Studies), Hayama, Japan

[4] Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan

[5] Laboratory of Structural Biology, Graduate School of Advanced Science and Engineering, Waseda University, Tokyo, Japan

[6] Department of Molecular Radiobiology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

[7] Department of Biochemistry I, School of Medicine, Kanazawa Medical University, Ishikawa, Japan.

[8] Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan

[9] Division of Molecular Cell Engineering, National Institute of Genetics, Research Organization of Information and Systems (ROIS), Mishima, Shizuoka 411-8540, Japan

[10] Department of Genetics, SOKENDAI, Mishima, Shizuoka 411-8540, Japan

[11] Department of Chemistry, Graduate School of Science and Engineering, Tokyo

Metropolitan University, Hachioji, Tokyo 192-0397, Japan
[#] Present address: Department of Frontier Research, Kazusa DNA Research Institute, Kisarazu, Japan
* Present address: Hubrecht Institute-KNAW & University Medical Center Utrecht, Utrecht, the Netherlands

** To whom correspondence should be addressed: Tel: +81-75-753-7563; Fax: +81-75-753-7564; E-mail: mtakata@house.rbc.kyoto-u.ac.jp

Supplementary Figures

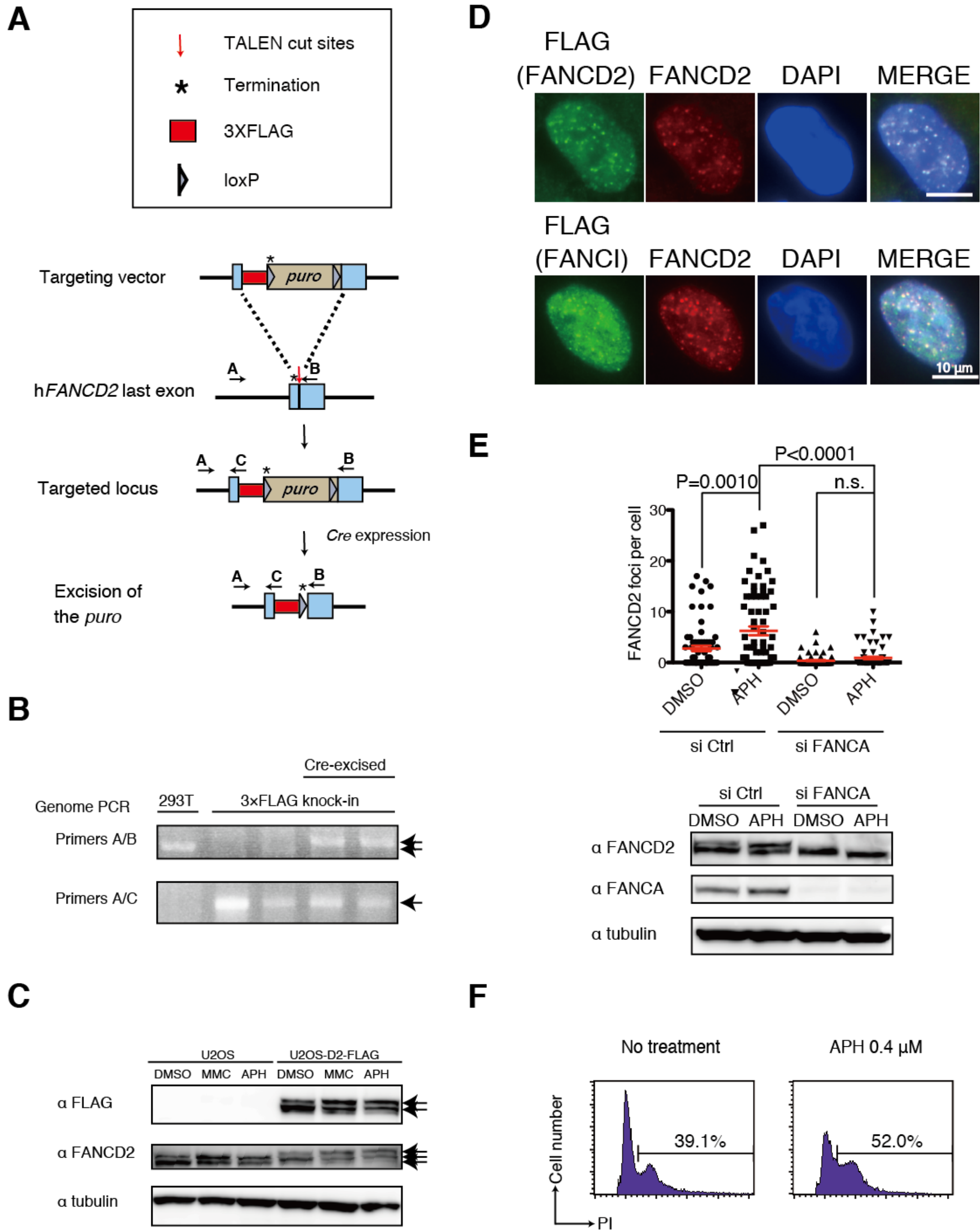

Figure S1

Supplementary Figure 1. Generation of U2OS-D2-FLAG cells using TALEN genome editing enzyme targeting the last exon of *FANCD2*, and the FANCD2-3xFLAG knock-in vector

(A) A schematic showing the strategy to knock in the 3xFLAG tag into the termination codon of *FANCD2* in U2OS cells.

(B) Obtained cell clones were verified by genomic PCR using primers indicated in (A).

(C) Parental U2OS and U2OS-D2-FLAG cells were subjected to western blotting using anti-FANCD2 and anti-FLAG antibodies. Cells were stimulated by the indicated reagents, and whole cell lysates were prepared and analyzed.

(D) Detection of FANCD2 and FANCI foci in U2OS-D2-FLAG or U2OS cells transiently transfected with FLAG-FANCI using anti-FLAG and anti-FANCD2. Cells were treated with 0.4 μM APH for 24 hr, fixed, and stained.

(E) *FANCA* depletion by siRNA abrogated FANCD2 foci and monoubiquitination in U2OS cells treated with 0.4 μM APH for 24 hr. Means ±SEM from n >50 nuclei are shown. P-values were calculated by unpaired, two-tailed Student's *t* test.

(F) Flow cytometric analysis of the cell cycle profile in U2OS cells treated with 0.4 μM APH for 24 hr. Cells were fixed in 70% Ethanol, stained with Propidium Iodide, and analyzed using a FACSCalibur flow cytometer (Becton-Dickinson).

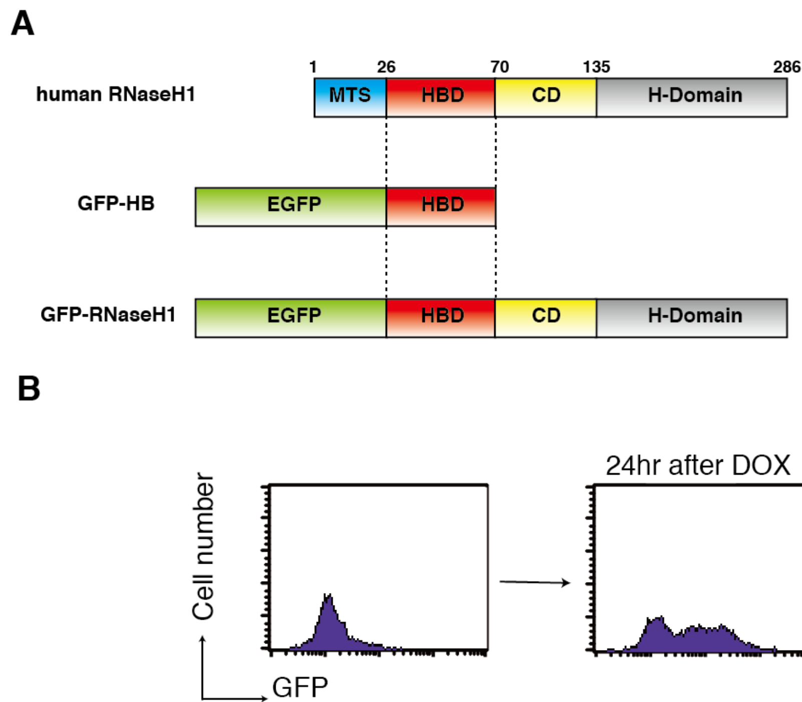

Figure S2

Supplementary Figure 2. Generation of U2OS stably expressing dox-inducible GFP-HBD

(A) Schematic depicting wild-type RNaseH1 and the HBD domain each fused with the GFP fragment.

(B) U2OS-D2-FLAG cells stably expressing GFP-HBD were induced by treatment with 2 μg/ml doxycycline for 24 hr. GFP expression levels were evaluated with a FACSCalibur flow cytometer (Becton-Dickinson).

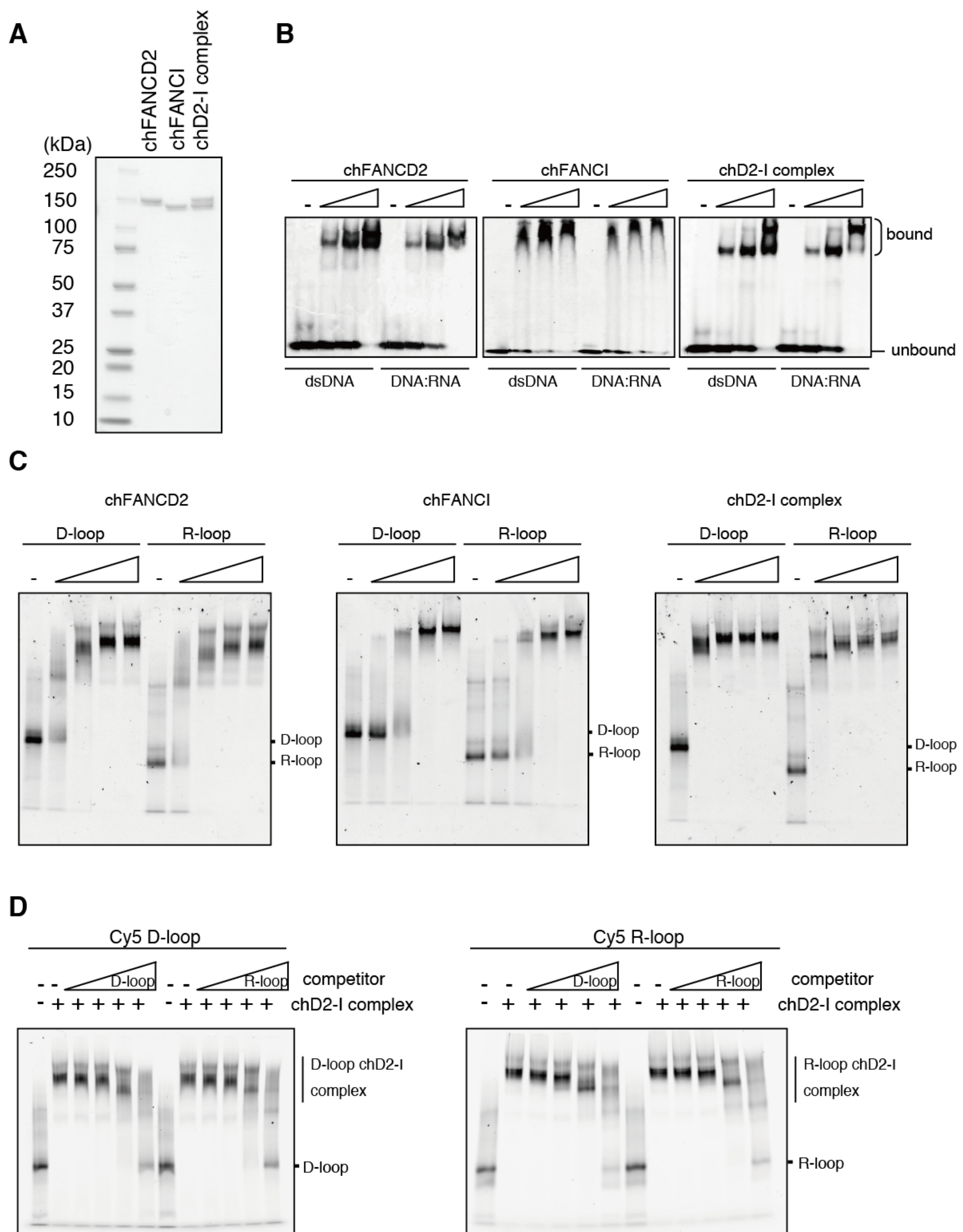

Figure S3

Supplementary Figure S3. FANCD2 and the D2-I complex binding of D-loop or R-loop *in vitro*

(A) The purified chicken FANCD2 and FANCI proteins. FANCD2 and FANCI were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining.

(B) Gel shift assay with FANCD2, FANCI and the D2-I complex with dsDNA or DNA:RNA hybrid. Equimolar amounts of the synthetic dsDNA (5 μM) as well as DNA:RNA hybrid (5 μM) were incubated with increasing amounts of FANCD2 (0, 0.1, 0.2, and 0.4 μM), FANCI (0, 0.6, 1.2, and 1.8 μM), or the D2-I complex (0, 0.05, 0.10, and 0.20 μM) at 37°C for 10 min. The samples were then separated by 3.5 % PAGE in 0.2 x TBE buffer, and the bands were visualized by SYBR Gold staining.

(C) Gel shift assay with FANCD2, FANCI and the D2-I complex with D-loop or R-loop. Equimolar amounts of the synthetic D-loop (5 μM) as well as R-loop (5 μM) were incubated with increasing amounts of FANCD2 or FANCI (0, 0.1, 0.2, 0.4, and 0.6 μM) or the D2-I complex (0, 0.1, 0.2, 0.3, and 0.4 μM) at 37°C for 10 min. The samples were then separated by 3.5 % PAGE in 0.2 x TBE buffer, and the bands were visualized by SYBR Gold staining.

(D) Competition assay with D-loop and R-loop. Cy5-labeled D-loop (2.5 μM) or R-loop (2.5 μM) and D2-I complex (0.2 μM) were incubated with increasing amounts of unlabeled competitor D-loop (2.5, 5.0, 12.5, and 25 μM) or R-loop (2.5, 5.0, 12.5, and 25 μM) at 37°C for 10 min. The samples were then separated by 3.5 % PAGE in 0.2 x TBE buffer, and the bands were detected by Amersham Typhoon (GE Healthcare).

Supplementary Table 1. Oligonucleotides used in this study.

TALEN	
primer	sequence
KD12-106	5’- CAGGCGGCCGCGGTGGGAGGGTTGCTCAAGCCCAGGAGG -3’
KD12-107	5’- AAGAATTCATCAGAGTCATCATAACTCTCATCACTATC -3’
KD12-108	5’- TTGAATTCTAGACCCCAGATAAATTGTTGCCTGCTTCT -3’
KD12-109	5’- TAGTCGACCTATACAGGGGGCTGAGACAGGAGAA -3’
ChIP qPCR	
primer	sequence
KD15-215	5’- GAGGCTAGCACCAAGGTCTG -3’
KD15-216	5’- ACTGGCTGAGGCTACCAGAA -3’
KD15-282	5’- GAGGCCCAGGACACATAGAA -3’
KD15-283	5’- GCAACAGGCTAACATGCAGA -3’
KD15-221	5’- TGTTGCGAAGAACCTGTGAG -3’
KD15-222	5’- ACTCCTATGGCTCTGCTGGA -3’
KD15-375	5’- CAGCCAGCACTCCTTCTCAA -3’
KD15-376	5’- CTCTGTGGAGAAGCCAAGCA -3’
KD15-247	5’- CGGGAGACATCCTCGAAATA -3’
KD15-248	5’- CCTAGCACCAGGTCTTGCTG -3’
KD15-385	5’- GCCTATACCGGAAGACTGTGTG -3’
KD15-386	5’- GGCTGCTCAGAAGACCACTTTA -3’
siRNA	
siRNA	sequence
FANCD2	5’- CAGAGUUUGCUUCACUCUCUATT -3’
FANCA	5’- AAGGGUCAAGAGGGAAAAAUA -3’
Luciferase	5'- UCGAAGUAUUCCGCGUACGTT -3'
DNA:RNA hybrid binding assay	
oligonucleotide	sequence
DNA forward	5’- ATCGATGTCTCTAGACAGCTGCTCAGGATTGATCTGTAATGGCCTGGGA -3’
DNA reverse	5’- TCCCAGGCCATTACAGATCAATCCTGAGCAGCTGTCTAGA GACATCGAT -3’
RNA forward	5’- AUCGAUGUCUCUAGACAGCUGCUCAGGAUUGAUCUGUAAUGGCCUGGGA -3’
RNA reverse	5’- UCCCAGGCCAUUACAGAUCAAUCCUGAGCAGCUGUCUAGAGACAUCGAU -3’
D-loop or R-loop binding and competition assay	
oligonucleotide	sequence
Oligo DNA A	5’- CATTGCATATTTAAAACATGTTGGAAGGCTCGATGCATGCTGATAGCCTACTAGTGCTGCTGGCTTTCAAATGACCTCTTATCAAGTGAC -3’
Cy5-Oligo DNA A	Cy5'- CATTGCATATTTAAAACATGTTGGAAGGCTCGATGCATGCTGATAGCCTACTAGTGCTGCTGGCTTTCAAATGACCTCTTATCAAGTGAC -3’
Oligo DNA B	5'- GTCACTTGATAAGAGGTCATTTGAATTCATGGCTTAGAGCTTAATTGCTGAATCTGGTGCTGGGATCCAACATGTTTTAAATATGCAATG -3’
Oligo DNA C	5'- CTGCTACGATGCTAGTCGTAGCTCGGCAGTCGTAGCAGGTTCCCAGCACCAGATTCAGCAATTAAGCTCTAAGCCATGAA -3’
Oligo RNA D	5'- CUGCUACGAUGCUAGUCGUAGCUCGGCAGUCGUAGCAGGUUCCCAGCACCAGAUUCAGCAAUUAAGCUCUAAGCCAUGAA -3’