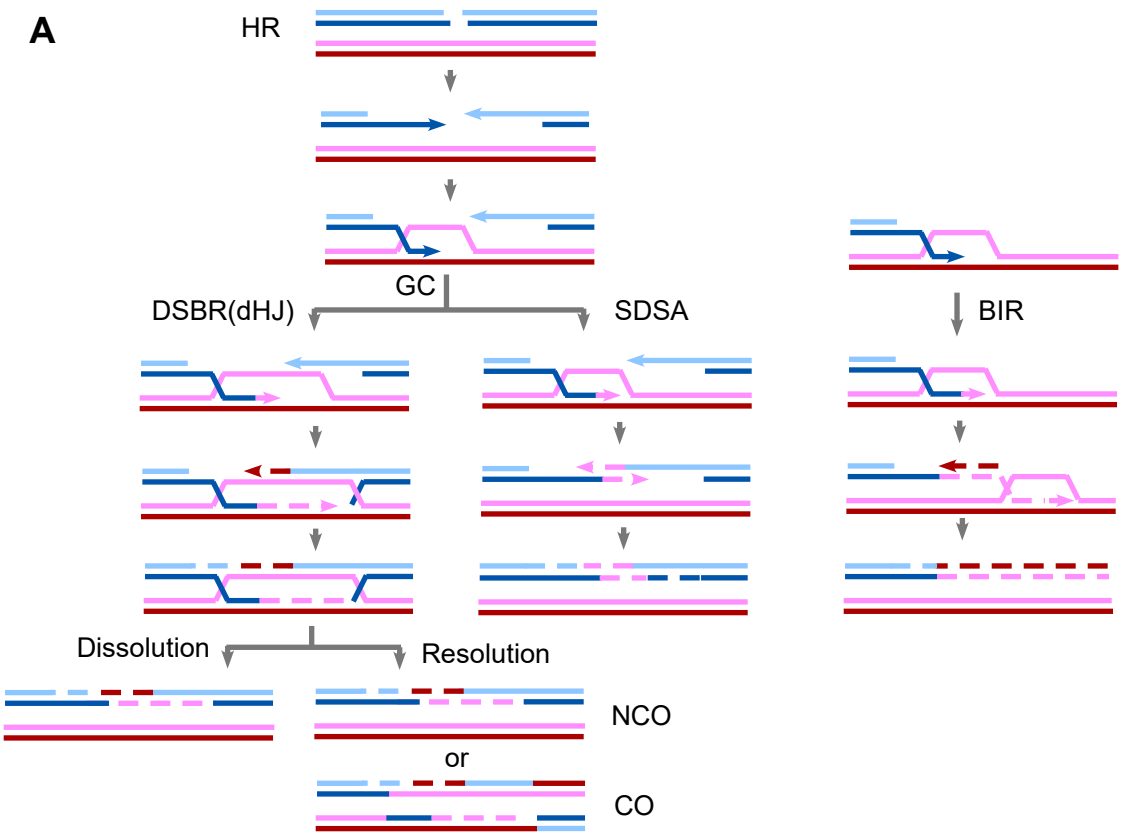
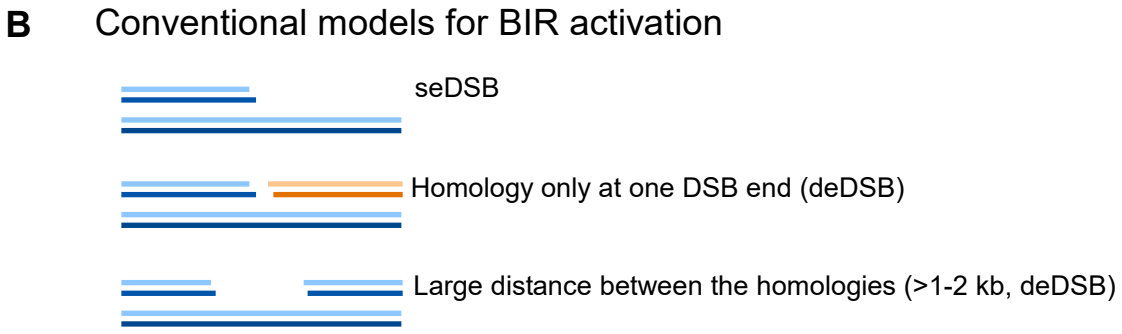
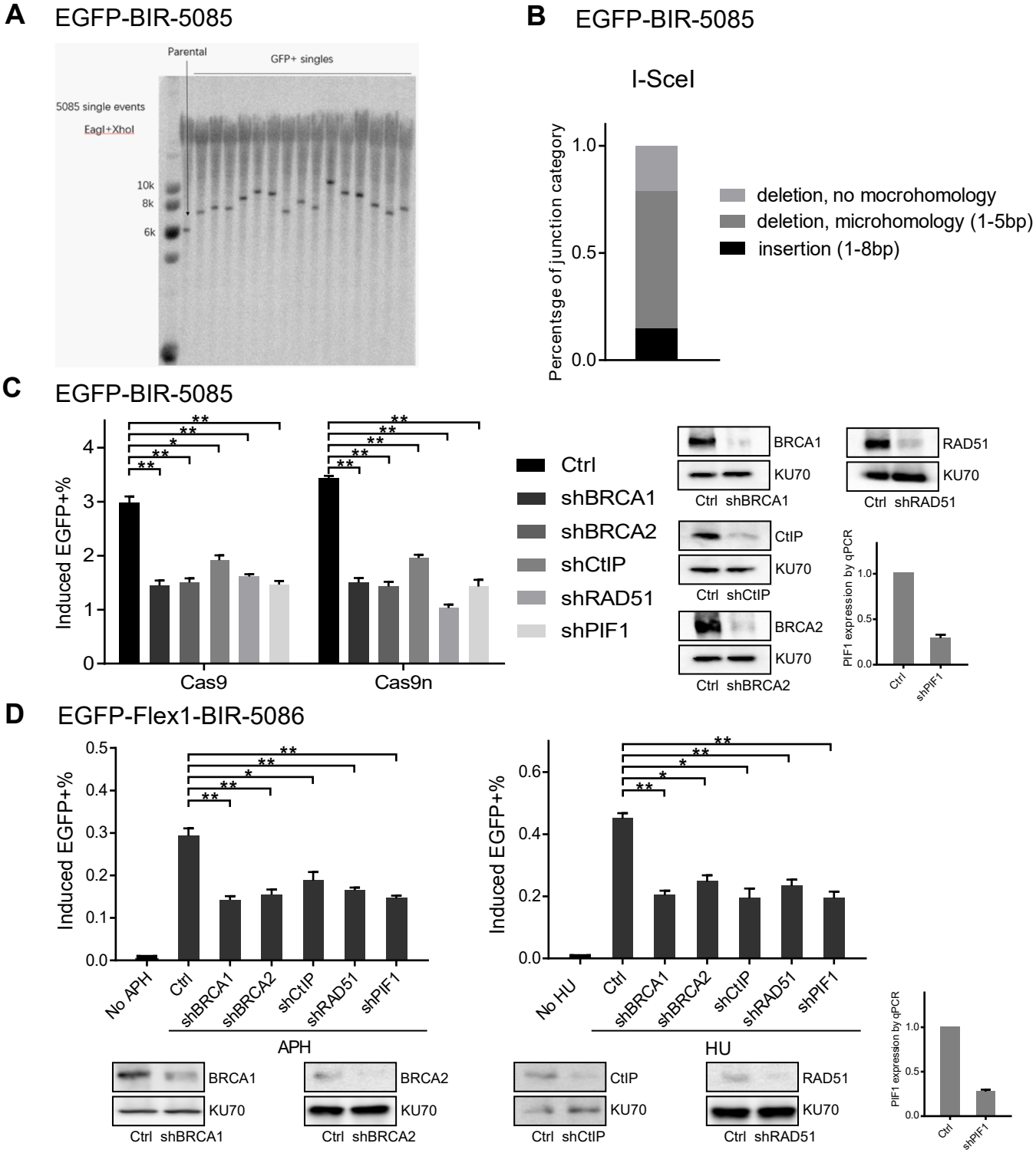
Appendix:

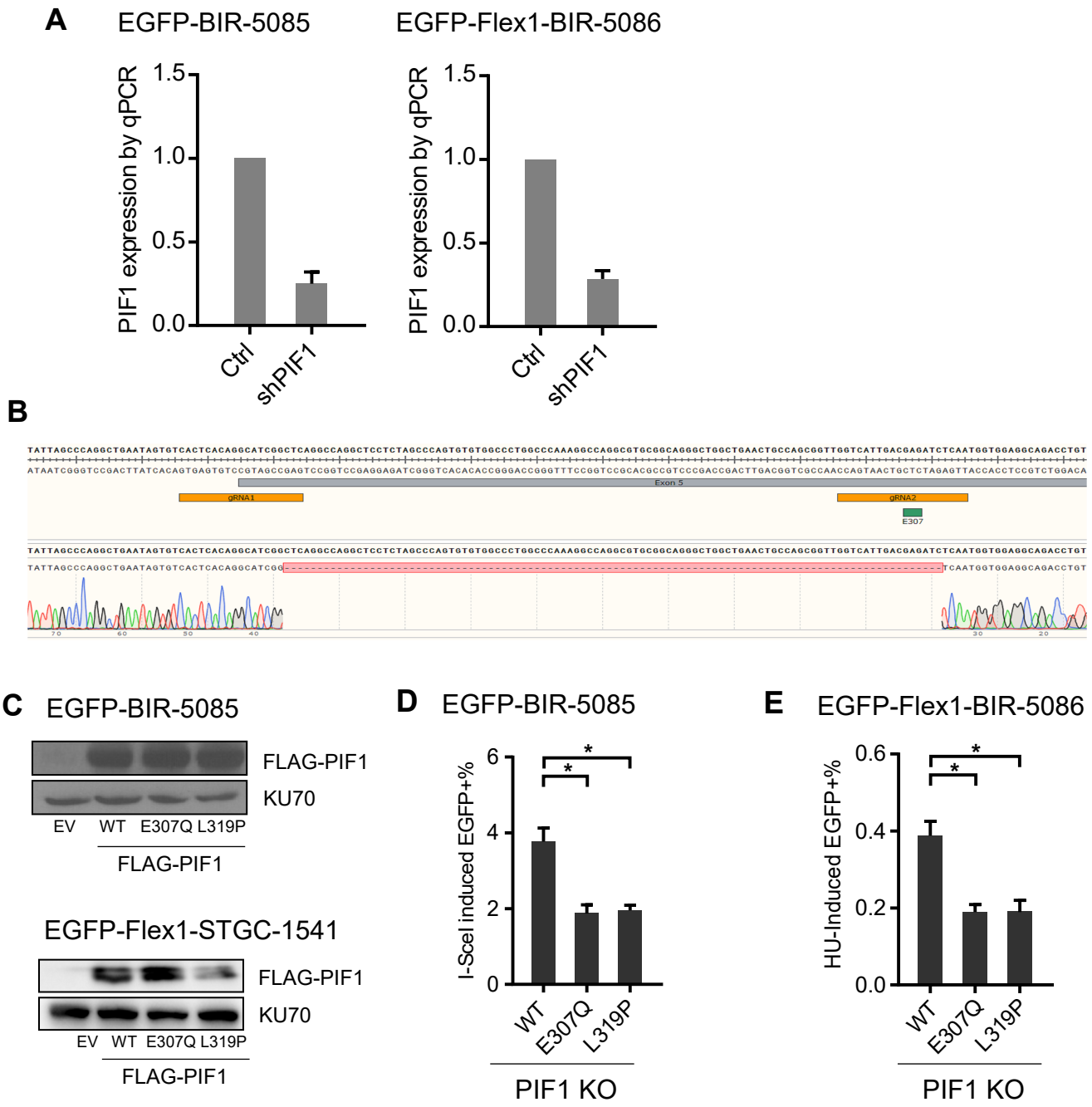




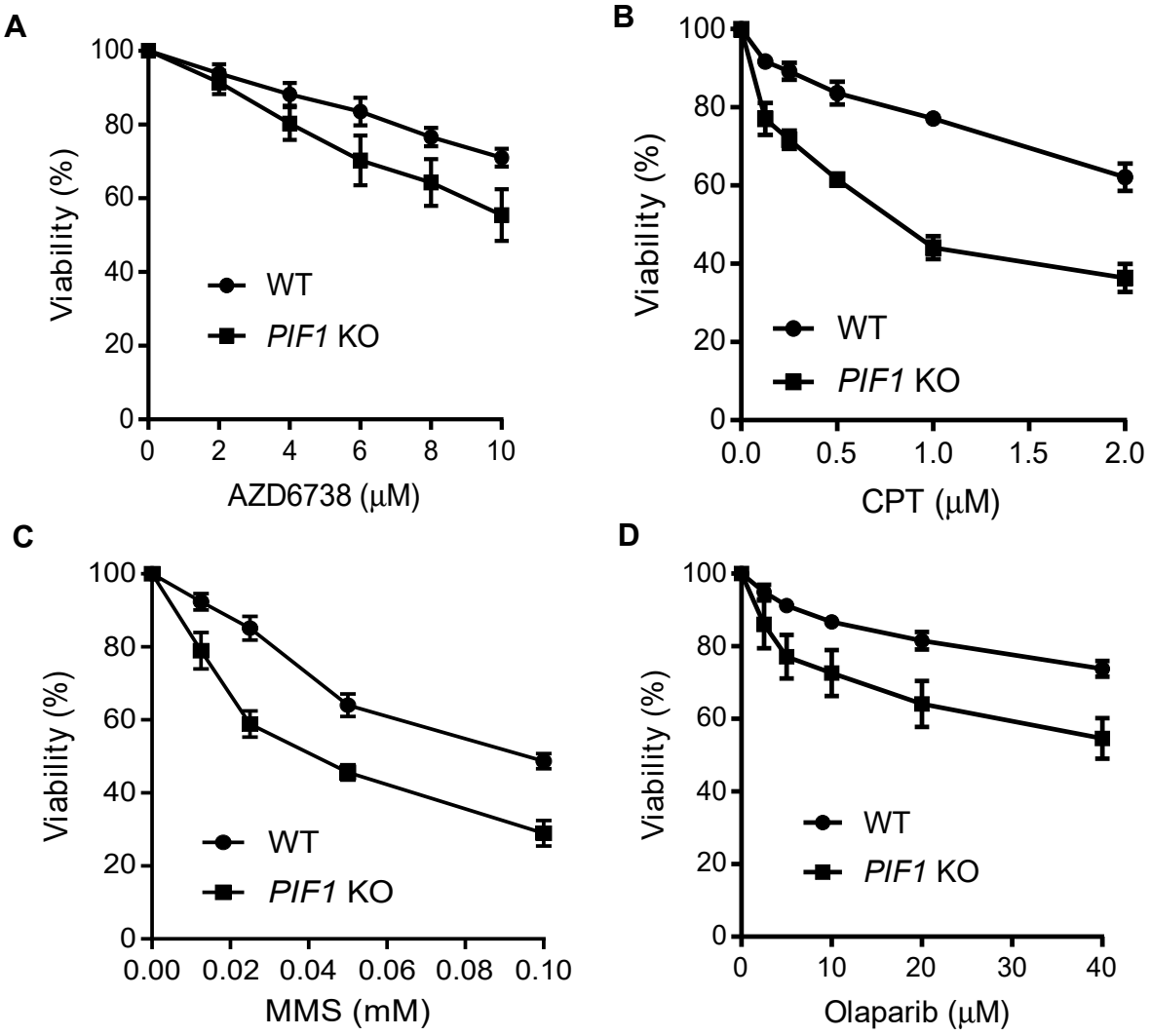
Appendix Fig S1. Pathways of homologous recombination (HR).
(A) Gene conversion (GC) is used when the two ends of a DSB both contain homology to the donor (left). Double-strand break repair (DSBR, also called dHJ) and synthesis-dependent strand annealing (SDSA) are the two subpathways of GC. Break-induced replication (BIR) is used when only one DSB end has homology to the donor (right). (related to Fig. 1A, 2C, 4A and 5A)
(B) Based on the study in yeast, upon endonuclease cleavage, BIR is activated at single-end DSB (seDSB), or at the DSBs with only one end containing homology to the donor or with homologies at both ends but more than 1-2 kb apart. deDSB: Double-end DSB. (related to Fig. 1A, 4G and 5A)



Appendix Fig S2. Repair analysis using the EGFP-BIR-5085 and EGFP-Flex1-BIR-5086 reporters.
(A) Southern blot analysis was performed on the EGFP positive single clones derived from U2OS (EGFP-BIR-5085) cells after I-SceI cleavage. Genomic DNA was digested by EagI+XhoI, and a ^{32}P-labelled CMV promoter fragment was used as the probe for hybridization. (related to Fig. 1C)
(B) The repair junctions of the BIR-EJ events (n=29) derived from U2OS (EGFP-BIR-5085) cells after I-SceI cleavage were analyzed. The percentage of deletions with or without microhomology and the percentage of insertions are shown. (related to Fig. 1C)
(C) U2OS (EGFP-BIR-5085, Dox-Cas9 or Dox-Cas9n) cells expressing shRNAs for BRCA1, BRCA2, CtIP and PIF1 or shRNA control (Ctrl) were incubated with 5 ug/ml Dox, and the percentage of EGFP positive cells was quantified by FACS analysis 2 days later (left). Expression of indicated genes was shown by Western blot or RT-qPCR (right).(related to Fig. 2D)
(D) U2OS (EGFP-Flex1-BIR-5086) cells expressing shRNA control or indicated shRNAs were treated by 0.4 uM APH or 2 mM HU for 24 hr, and the percentage of EGFP positive cells was assayed by FACS analysis 3 days after drug removal (top). Expression of indicated genes was shown by Western blot or RT-qPCR (bottom).(related to Fig. 3C)

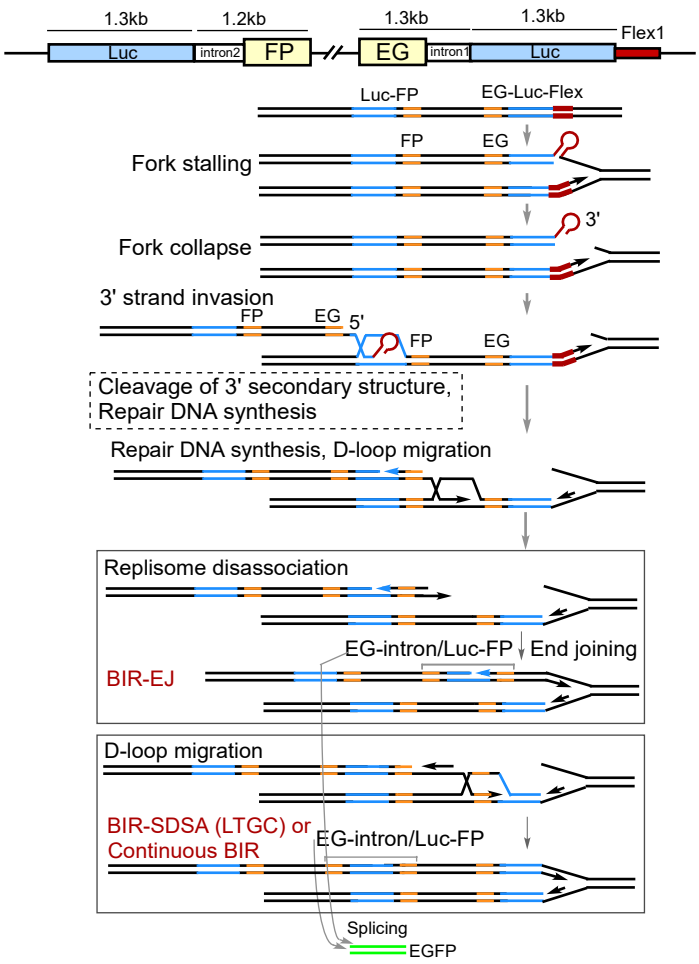


Appendix Fig S3. The function of PIF1 in BIR.
(A) PIF1 expression is quantified by qPCR in U2OS (EGFP-BIR-5085) cells (left, related to Fig. 1F) and U2OS (EGFP-Flex1-BIR-5086) cells (right, related to Fig. 3C) to determine the depletion effect of PIF1 shRNA.
(B) *PIF1* Knock-out (KO) by CRISPR with two sgRNAs (sgRNA1 and sgRNA2) targeting Exon 5 is verified by sequencing (related to Fig. 1G). E307: a key residue in the PIF1 helicase catalytic domain.
(C) Expression of FLAG-PIF1 WT, E307Q and L319P in U2OS (EGFP-BIR-5085) cells (top, related to Fig. 7G, 7H and 7I) or in U2OS (EGFP-Flex1-STGC-1541) cells (bottom, related to Fig. 5H) is shown by Western blot using anti-FLAG antibody.
(D) EGFP-BIR-5085 *PIF1*-KO cells expressing PIF1 WT or E307Q and L319P mutants were infected by lentiviruses expressing I-SceI. The percentage of EGFP positive cells was quantified by FACS analysis 4 days after I-SceI infection. (related to Fig. 7E)
(E) EGFP-Flex1-BIR-5086 *PIF1*-KO cells expressing PIF1 WT or E307Q and L319P mutants were treated with 2 mM HU for 24 hr. The percentage of EGFP positive cells after HU treatment was assayed by FACS analysis 3 days after HU removal. (related to Fig. 7F)

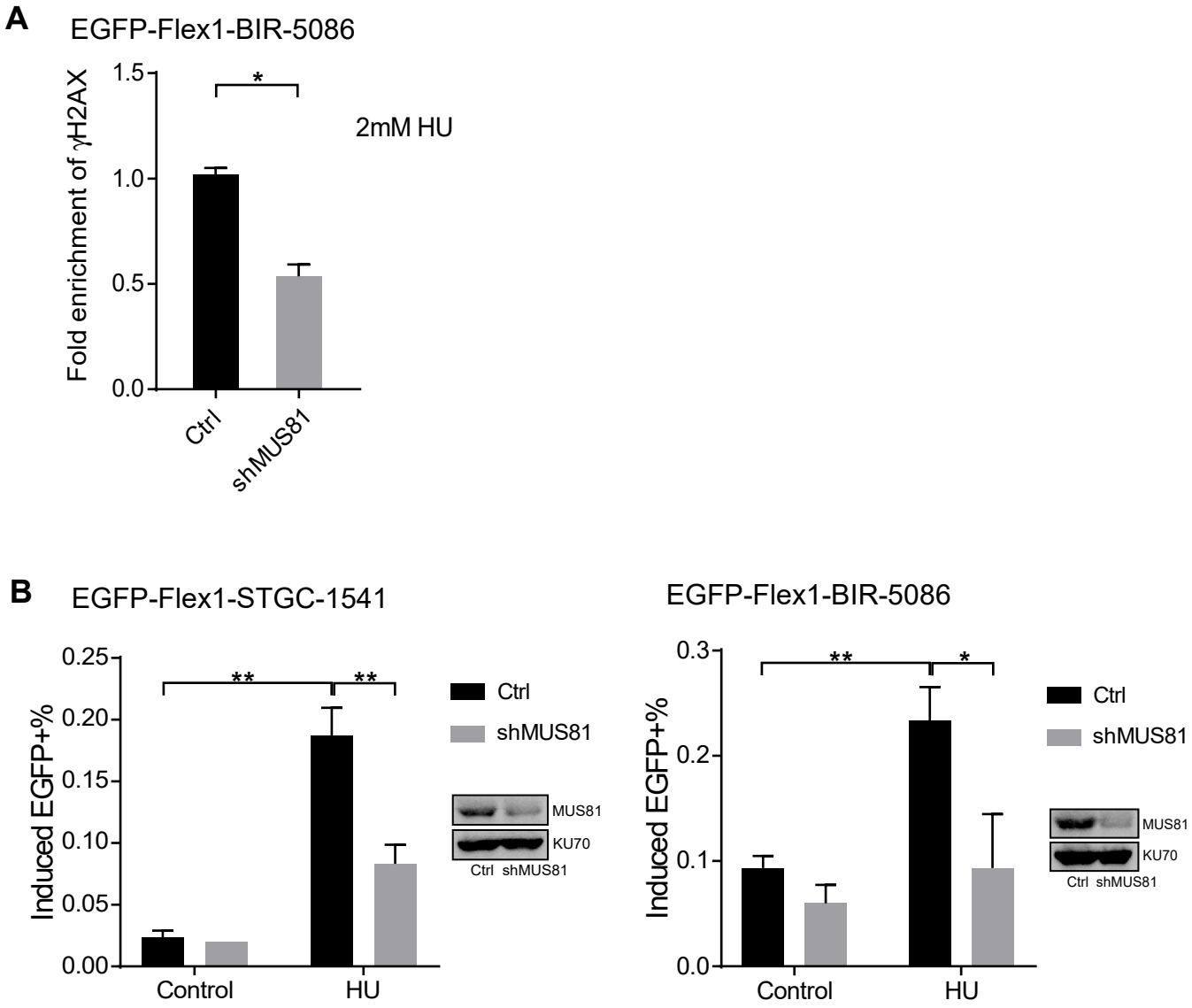


Appendix Fig S4. PIF1-deficient cells are sensitive to drugs that disturb DNA replication. U2OS WT or *PIF1*-KO cells were treated with the indicated concentrations of AZD6738 (A), CPT (B), MMS (C) or Olaparib (D) for 72 hr, and the cell viability assay was performed. (related to Fig. 2A)

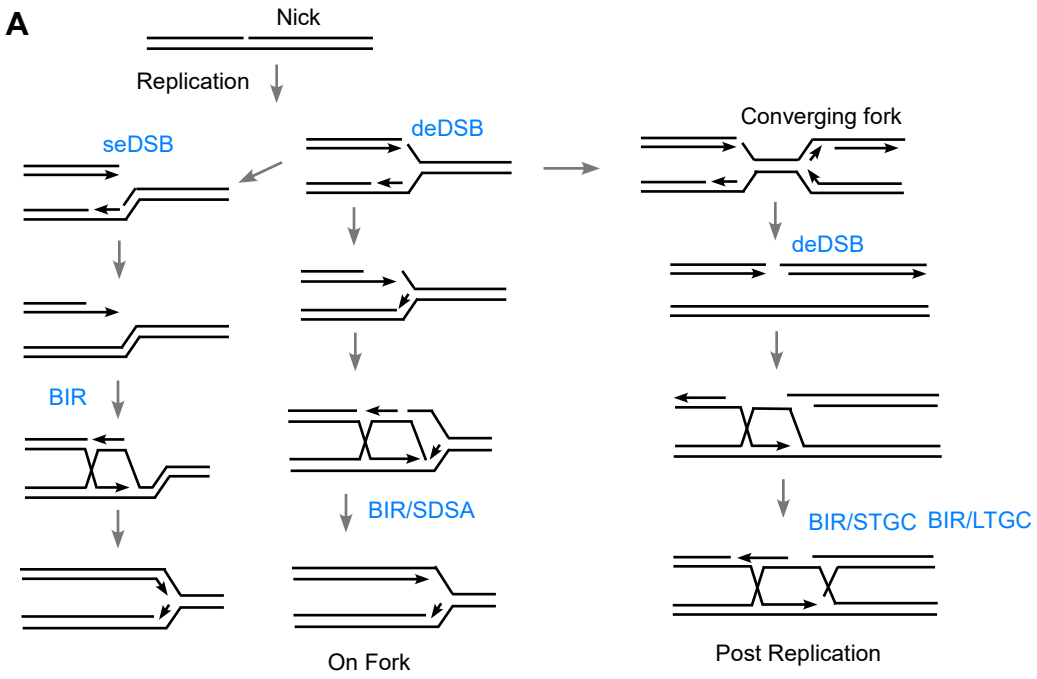
EGFP-Flex1-BIR-5086

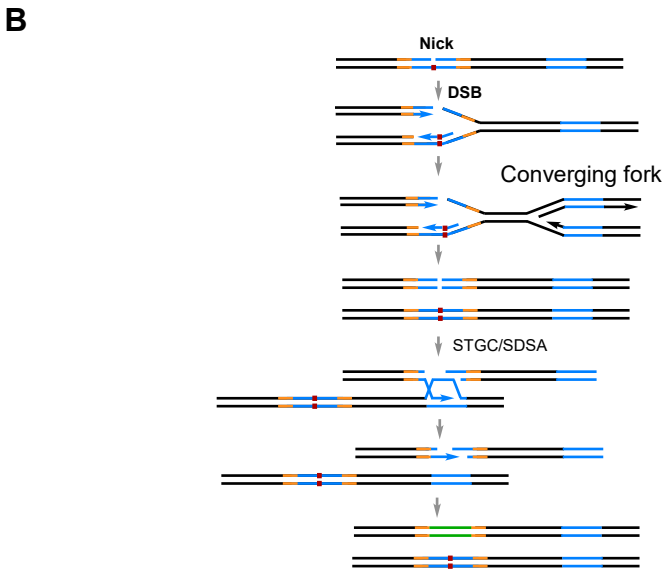


Appendix Fig S5. Schematic drawing of the EGFP-Flex1-BIR-5086 reporter. Upon replication stress, Flex1 forms DNA secondary structure on forks and causes DSB formation. The repair steps leading to the BIR-EJ and BIR-SDSA events that would produce EGFP positive cells are shown. (related to Fig. 3A)

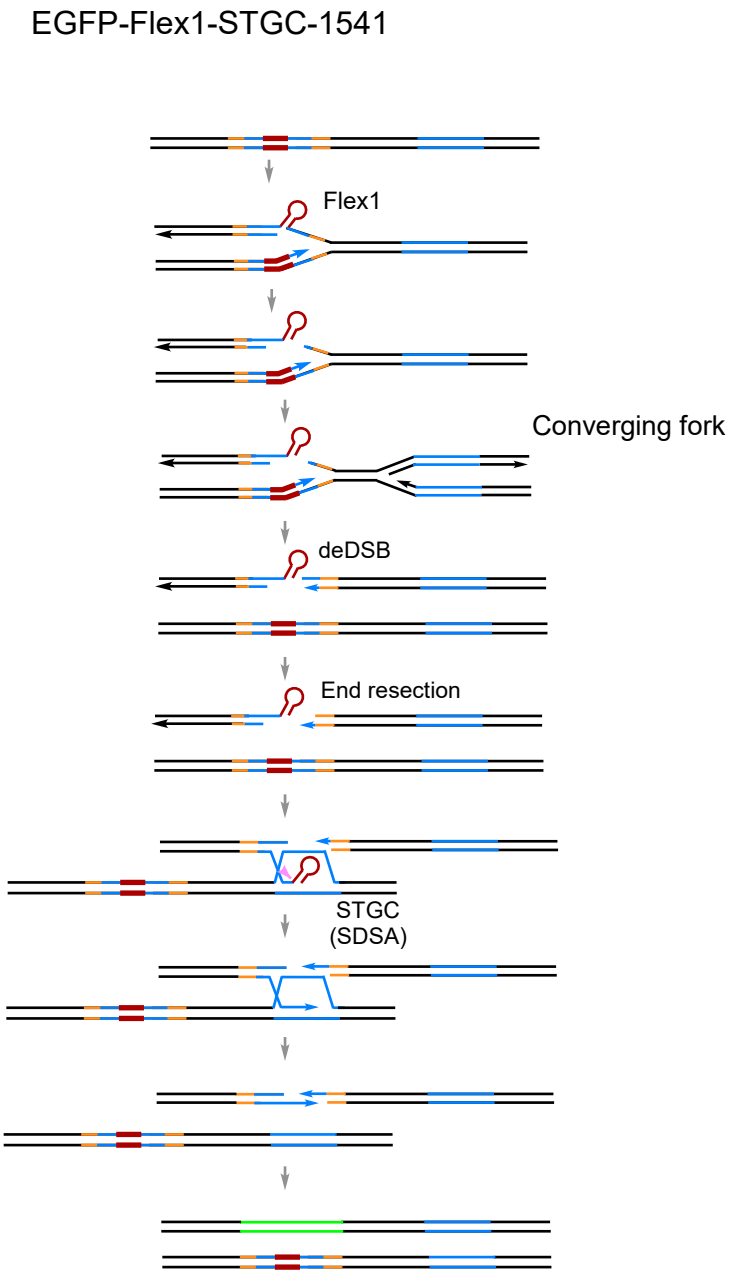


Appendix Fig S6. MUS81 is required for DSB formation at Flex1 upon replication stress.
(A) Enrichment of γH2AX at Flex1 site was quantified by anti-γH2AX ChIP analysis in U2OS (EGFP-Flex1-BIR-5086) cells expressing MUS81 shRNA or control shRNA after HU treatment (2 mM, 24 hr). (related to Fig. 3B)
(B) U2OS (EGFP-Flex1-STGC-1541 or EGFP-Flex1-BIR-5086) cells expressing MUS81 shRNA or control shRNA were treated with or without 2 mM HU for 24 hr, and the percentage of EGFP positive cells by HU treatment was assayed by FACS analysis 3 days after HU removal.(related to Fig. 3B)

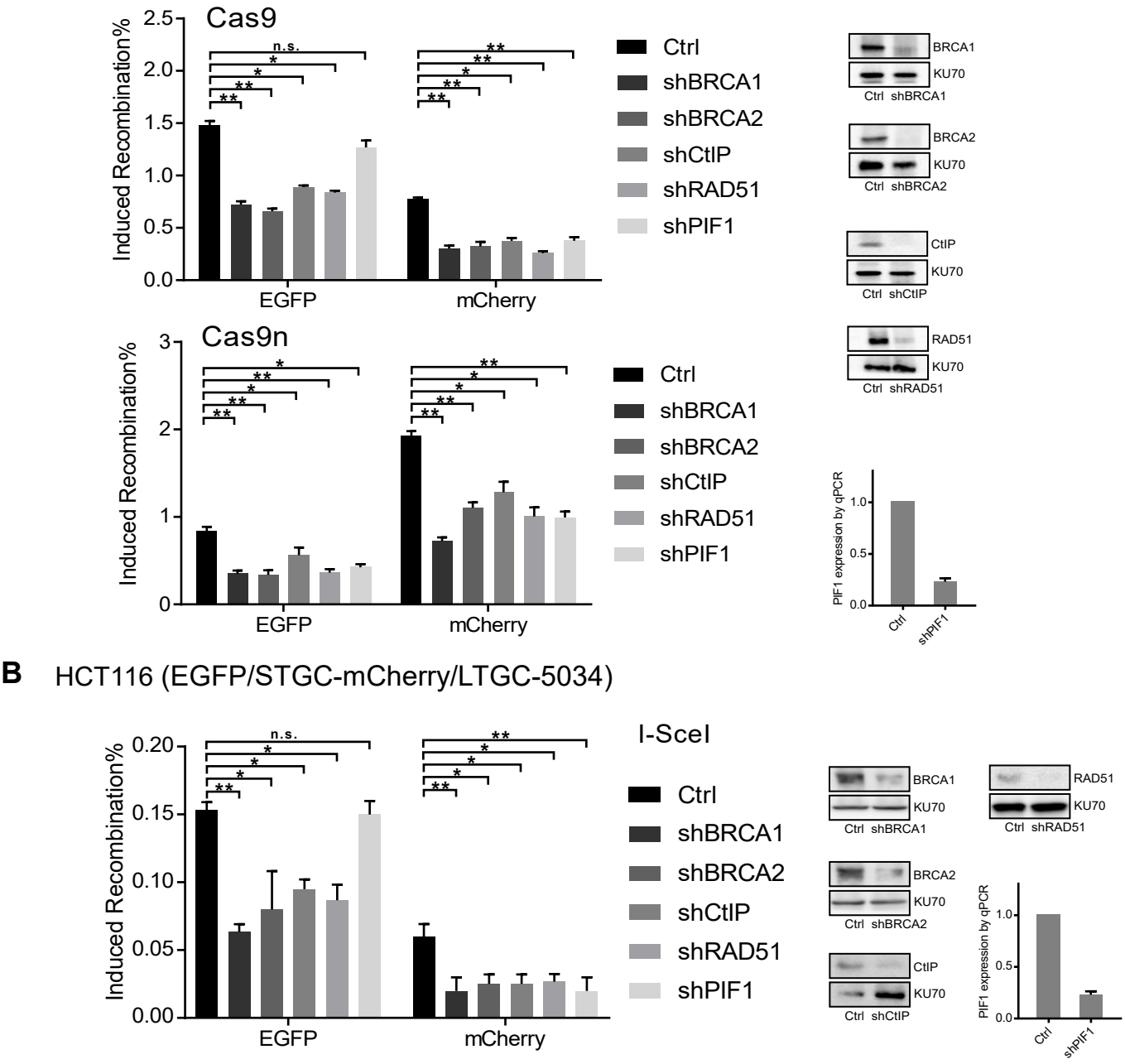




Appendix Fig S7. BIR is used to repair DSBs that are converted from nicks at replication forks.
(A) Proposed models for repair of DSBs converting from nicks at collapsed replication forks (see text for details). (related to Fig. 4A)
(B) DSBs converted from nicks on replication forks in EGFP-STGC-1731 reporter could be repaired by STGC/SDSA after adjacent fork convergence. (related to Fig. 4B)



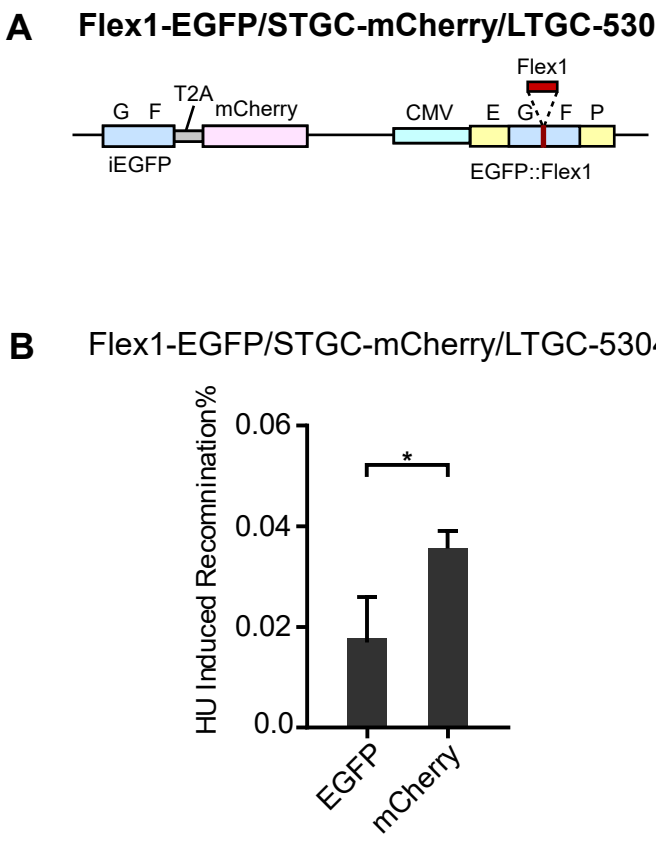
Appendix Fig S8. DSB repair at Flex1 upon fork collapse.
Collapsed fork at Flex1 in EGFP-Flex1-STGC-1541 reporter can be repaired by STGC after adjacent fork convergence to generate deDSBs (related to Fig. 4D). Pink arrow: endonuclease cleavage to remove Flex1.



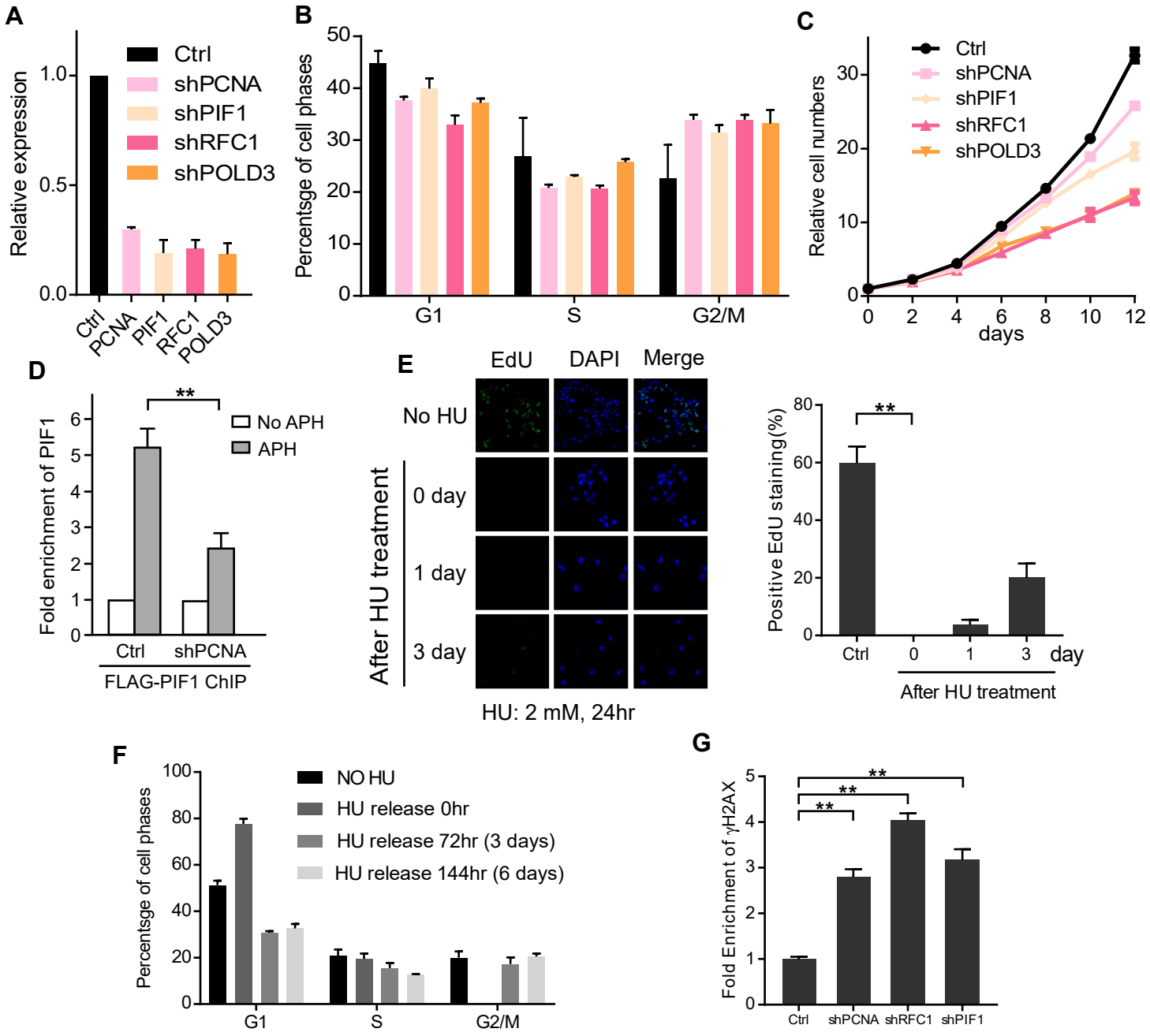
Appendix Fig S9. Analysis of STGC and LTGC in the EGFP/STGC-mCherry/LTGC-5034 reporter.

(A) U2OS (EGFP/STGC-mCherry/LTGC-5034, Dox-Cas9 or Dox-Cas9n) cells expressing shRNAs for BRCA1, BRCA2, CtIP, RAD51 and PIF1 or shRNA vector (Ctrl) were incubated with 5 ug/ml Dox, and the percentage of EGFP or mCherry positive cells was assayed by FACS analysis 2 days later (left). Expression of indicated genes was shown by Western blot or RT-qPCR (right). (related to Fig. 5A)

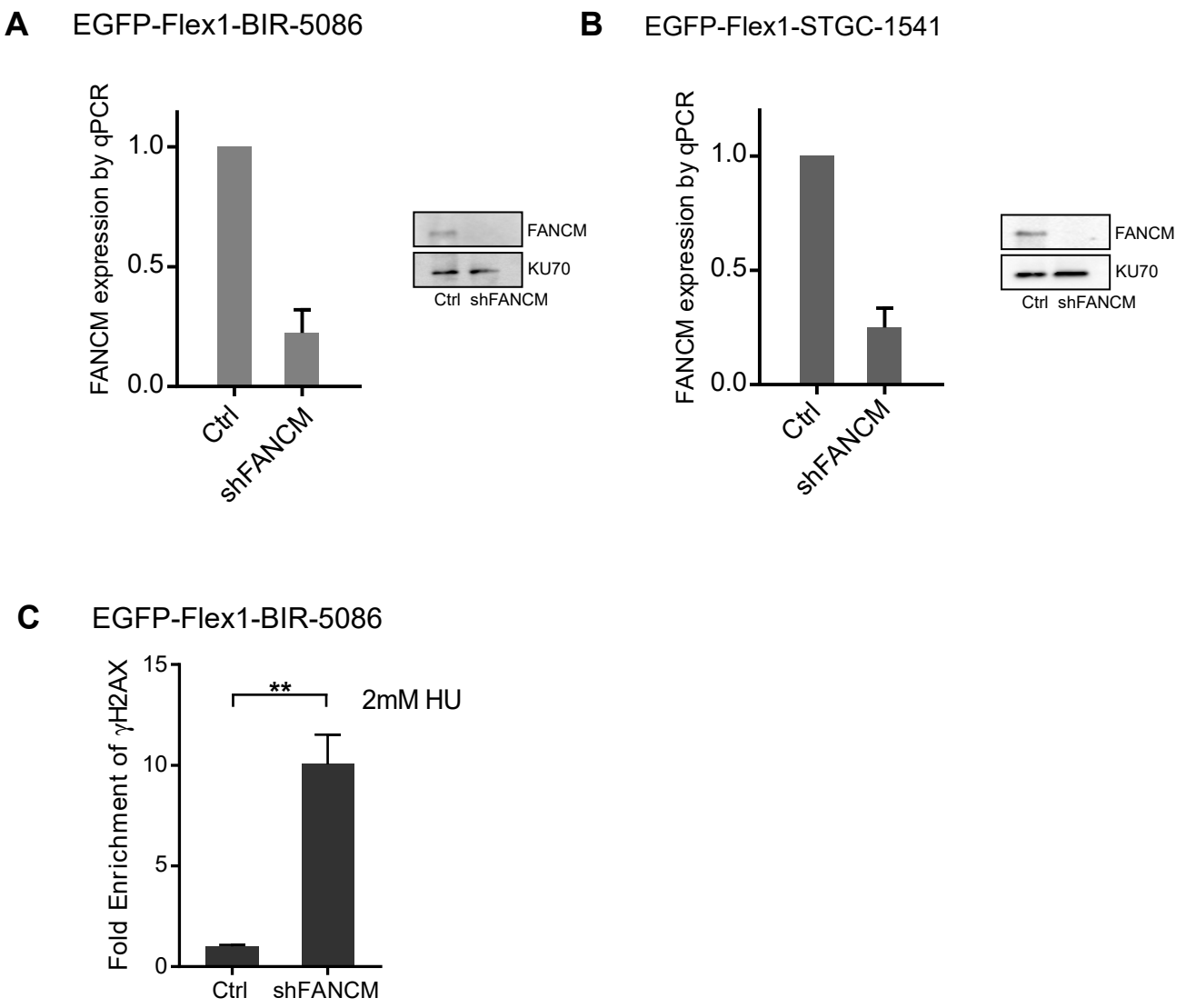
(B) HCT116 (EGFP/STGC-mCherry/LTGC-5034) cells were infected by lentivirus expressing indicated shRNAs followed by I-SceI lentivirus infection. The percentage of EGFP or mCherry positive cells was quantified by FACS analysis 4 days later (left). Expression of indicated genes was shown by Western blot or RT-qPCR (right). (related to Fig. 5A)



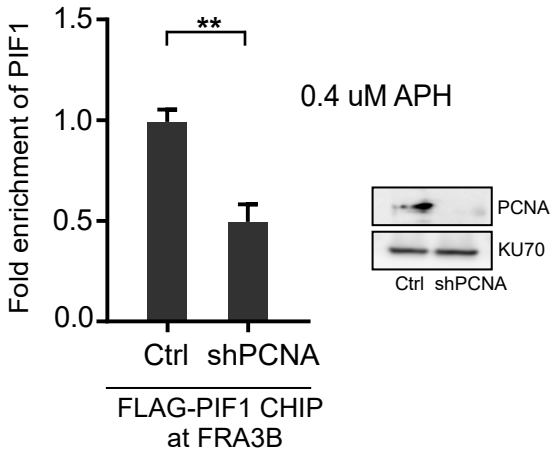
Appendix Fig S10. Analysis of STGC and LTGC in the Flex1-EGFP/STGC-mCherry/LTGC-5304 reporter.
(A) Schematic drawing of Flex1-EGFP/STGC-mCherry/LTGC-5304 reporter. (related to Fig. 5A)
(B) U2OS (Flex1-EGFP/STGC-mCherry/LTGC-5304) cells were treated with 2 mM HU for 24hr, and the percentage of EGFP or mCherry positive cells was determined by FACS analysis 3 days after HU removal. (related to Fig. 5B)



Appendix Fig S11. The role of PIF1, POLD3, PCNA and RFC1 in prevention of DSB formation.
(A and B) qPCR was performed to show expression levels of indicated proteins (A) and cell cycle profile was determined on day 6 following expression of indicated shRNAs (B) in U2OS (EGFP-Flex1-STGC-1541) cells. (related to Fig. 5D and 5E)
(C) Growth curve was plotted in U2OS (EGFP-Flex1-STGC-1541) cells after expressing of indicated shRNAs. (related to Fig. 5D and 5E)
(D) Enrichment of FLAG-PIF1 at Flex1 site in U2OS (EGFP-Flex1-STGC-1541) was performed by anti-FLAG ChIP when PCNA was depleted by shRNA using shRNA vector as a control (Ctrl). (related to Fig. 5G)
(E) U2OS cells were treated with or without 2 mM HU for 24 hr, and incubated with EdU for indicated time following HU removal. EdU incorporated was detected and quantified using Click-iT EdU Imaging Kit with Alexa Fluor 488 Azides. (related to Fig. 2B)
(F) Cell cycle profiles of U2OS (EGFP-Flex1-BIR-5086) were determined before or after HU (2 mM HU for 24 hr) treatment followed by HU removal for indicated time. (related to Fig. 3B)
(G) Enrichment of γH2AX at Flex1 in U2OS (EGFP-Flex1-STGC-1541) expressing PCNA, RFC1, PIF1 shRNA was determined by Anti-γH2AX ChIP analysis after treatment with 2 mM HU for 24 hr using shRNA vector as a control. (related to Fig. 4E and Fig.5E)



Appendix Fig S12. The role of FANCM in protecting CFS-ATs.
(A and B). The expression of FANCM is quantified by qPCR and Western blot in U2OS (EGFP-BIR-5086) cells (A, related to Fig. 6A) and in U2OS (EGFP-Flex1-STGC-1541) cells (B, related to Fig. 6B).
(C) Enrichment of γH2AX at Flex1 site in U2OS (EGFP-Flex1-BIR-5086) cells expressing FANCM shRNA was calculated by Anti-γH2AX ChIP analysis using shRNA vector as a control. (related to Fig. 6A)



Appendix Fig S13. Recruitment of PIF1 to CFS is dependent on PCNA.
Enrichment of FLAG-PIF1 at FRA3B site was determined by anti-FLAG ChIP after APH (0.4 uM, 20hr) treatment when PCNA was depleted by shRNA using shRNA vector as a control. Expression of PCNA was shown by Western blot. (related to Fig. 7E)

EGFP-BIR-5085

	Total	BIR-EJ (>0.9kb,<3.8kb)	BIR-SDSA (≥3.8kb)
I-SceI	30	29 (96.7%)	1 (3.3%)
Cas9	47	45 (95.7%)	2 (4.3%)
Cas9n	39	12 (30.7%)	27 (69.3%)

EGFP-BIR-5085 *PIF1*-KO

	Total	BIR-EJ (>0.9kb,<3.8kb)	BIR-SDSA (≥3.8kb)
I-SceI	41	41 (100%)	0 (%)
Cas9	45	45 (100%)	0 (%)

EGFP-Flex1-BIR-5086

	Total	BIR-EJ (>0.9kb,<3.8kb)	BIR-SDSA (≥3.8kb)
APH(0.4uM, 20hr)	38	0 (%)	38 (100%)

EGFP/STGC-mCherry/LTGC5034

	mCherry Total	BIR-EJ (>1.1kb,<2.2kb)	BIR-SDSA (≥2.2kb)
Cas9	76	7 (9.2%)	69 (90.8%)
Cas9n	48	0 (%)	48 (100%)

Appendix Fig S14. Analysis of BIR track length in different reporters.
Single EGFP or mCherry positive clones derived from indicated reporter cell lines after cleavage by I-SceI, Cas9 or Cas9n or APH (0.4uM, 20hr) treatment as indicated were analyzed by PCR sequencing of genomic DNA for BIR track length. The percentage of the events using BIR-EJ and BIR-SDSA with the range of track length is indicated. (related to Fig. 1C, 2E and 5C)

Appendix Table S1. sgRNA list:

Name	Sequence (5’ to 3’)
PIF1 KO sgRNA1	TCACTCACAGGCATCGGCTC
PIF1 KO sgRNA2	GGTCATTGACGAGATCTCAA
sgRNA-5085(for EGFP-BIR-5085)	AGTAGGAATTCAGTTACGCT
sgRNA-5034(for EGFP-STGC-1731 and EGFP/STGC-mCherry/LTGC-5034)	CTTCAAGCTTTAGGGATAAC

Appendix Table S2. shRNA list:

Gene	Targeting Sequence (5’ to 3’)
RAD51	AAGCUAUGUUCGCCAUUAAUG
POLD3	CAAUUAGUGGUUAGGGAAAAG
PIF1	GAAGACAGGUGCTCCGGAAGC
FANCM	GAACAAGAUUCCUCAUUACUU
RFC1	GAAGGCGGCCUCUAAAUCAAA
PCNA	GUGGAGAACUUGGAAAUGGAA
MUS81	GGGAGCACCUGAAUCCUAAUG
BRCA1	UGAUAAAGCUCCAGCAGGAAA
BRCA2	GAAACGGACUUGCUAUUUACU
CTIP	GAGCAGACCUUUCUCAGUAUA
RAD52	GAUGUUGGUUAUGGUGUUAGU

Appendix Table S3. RT-qPCR primer:

primer	Sequence (5’ to 3’)
PIF1-F	CGAGCCTAGCACAGAAGCC
PIF1-R	CCCAGGATTCGCTTTAGCAG
POLD3-F	CAAGGAAACGAAAACAGAGGC
POLD3-R	TTCACTGCTTGTTCTGAGTCC
FANCM-F	GCTTATTGTTCCGCTTGGTG
FANCM-R	TCAAAGAACGAGCAAATGATTCC
RFC1-F	CATCGCCAAGCAATTACAGC
RFC1-R	TTTCTCCCGCTTCTGTGTC
PCNA-F	CTAGCCTGACAAATGCTTGC
PCNA-R	AGGAAAGTCTAGCTGGTTTCG
HPRT-F	CTGGCGTCGTGATTAGTGAT
HPRT-R	CTCGAGCAAGACGTTCAGTC