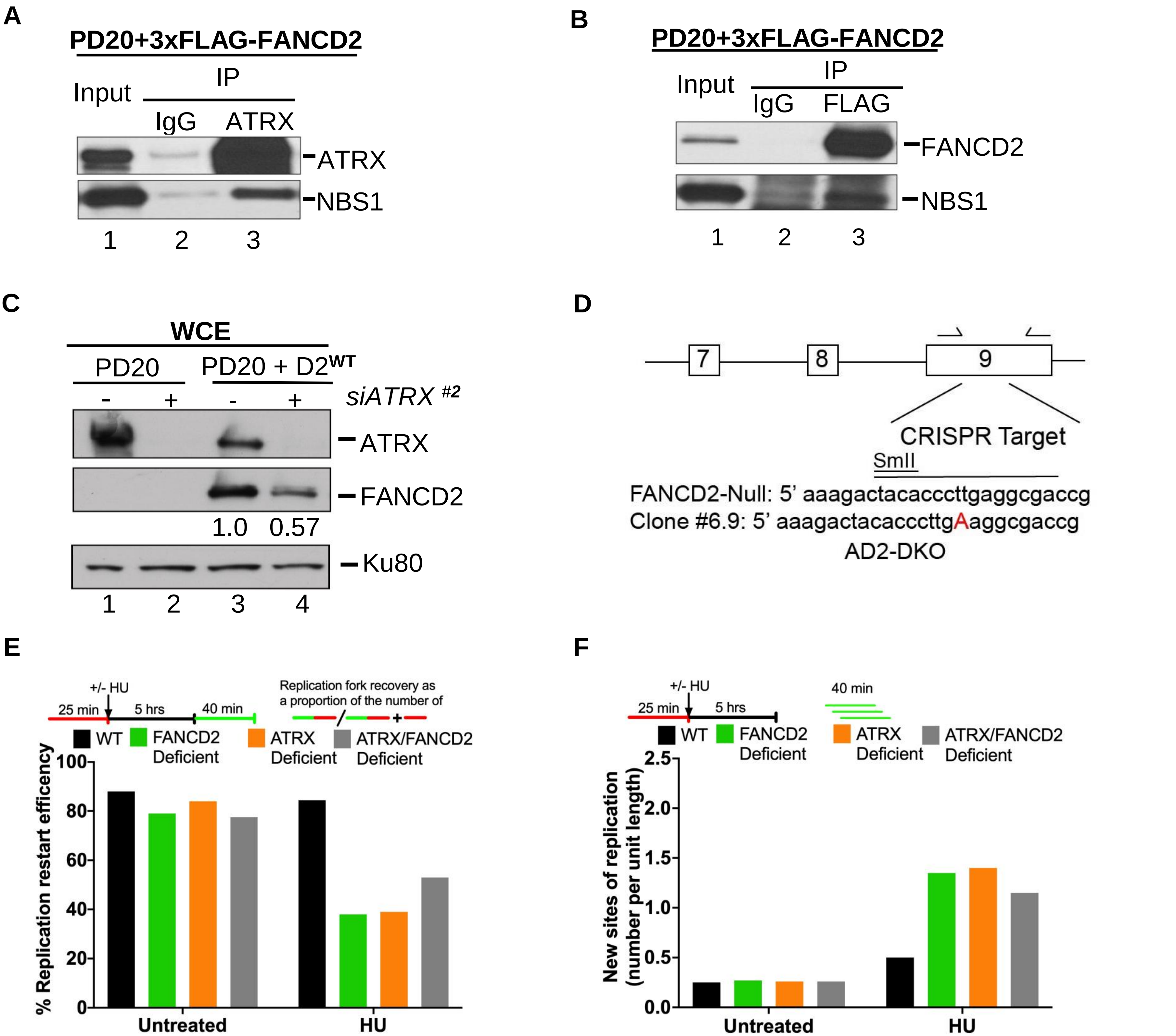
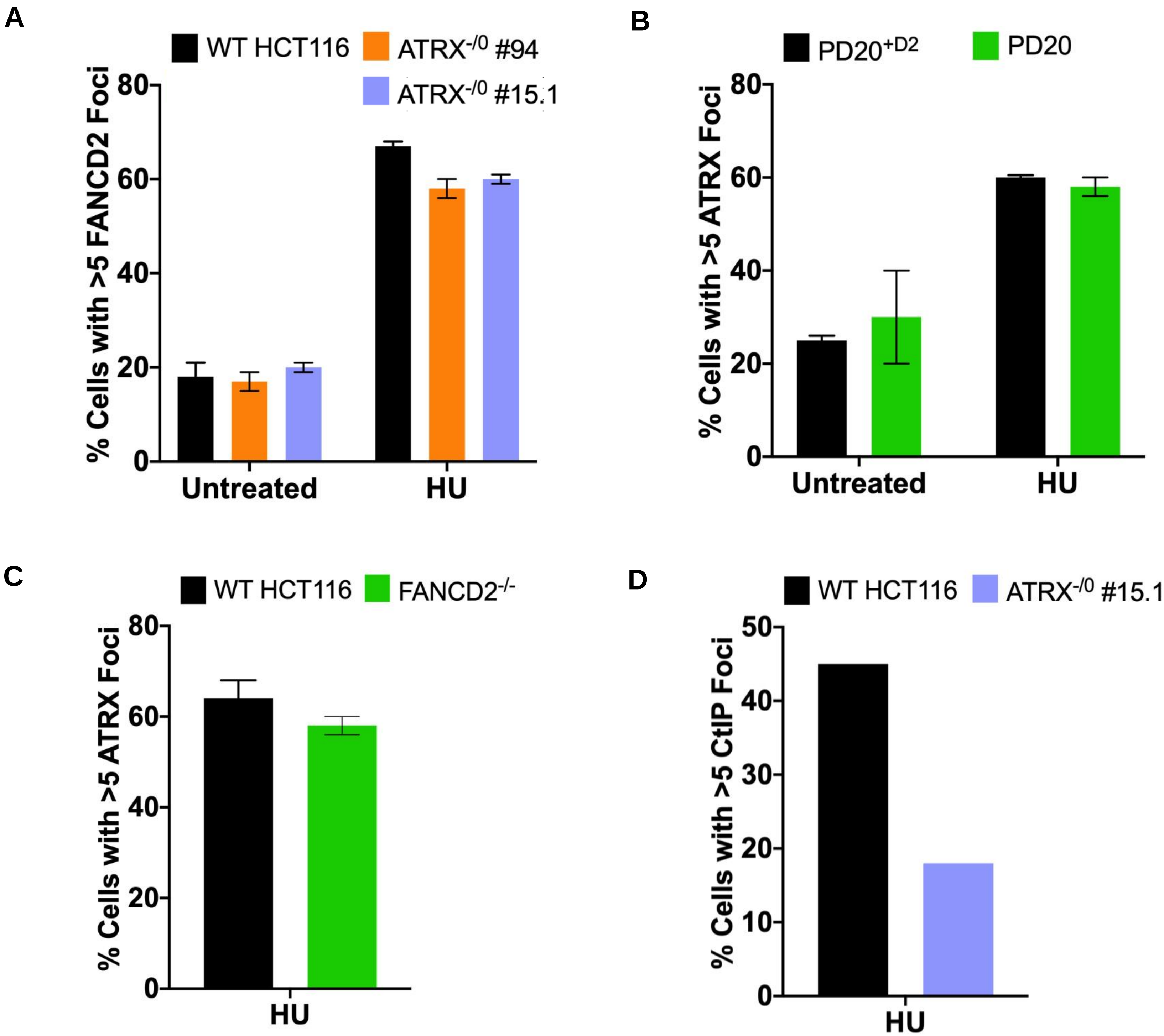
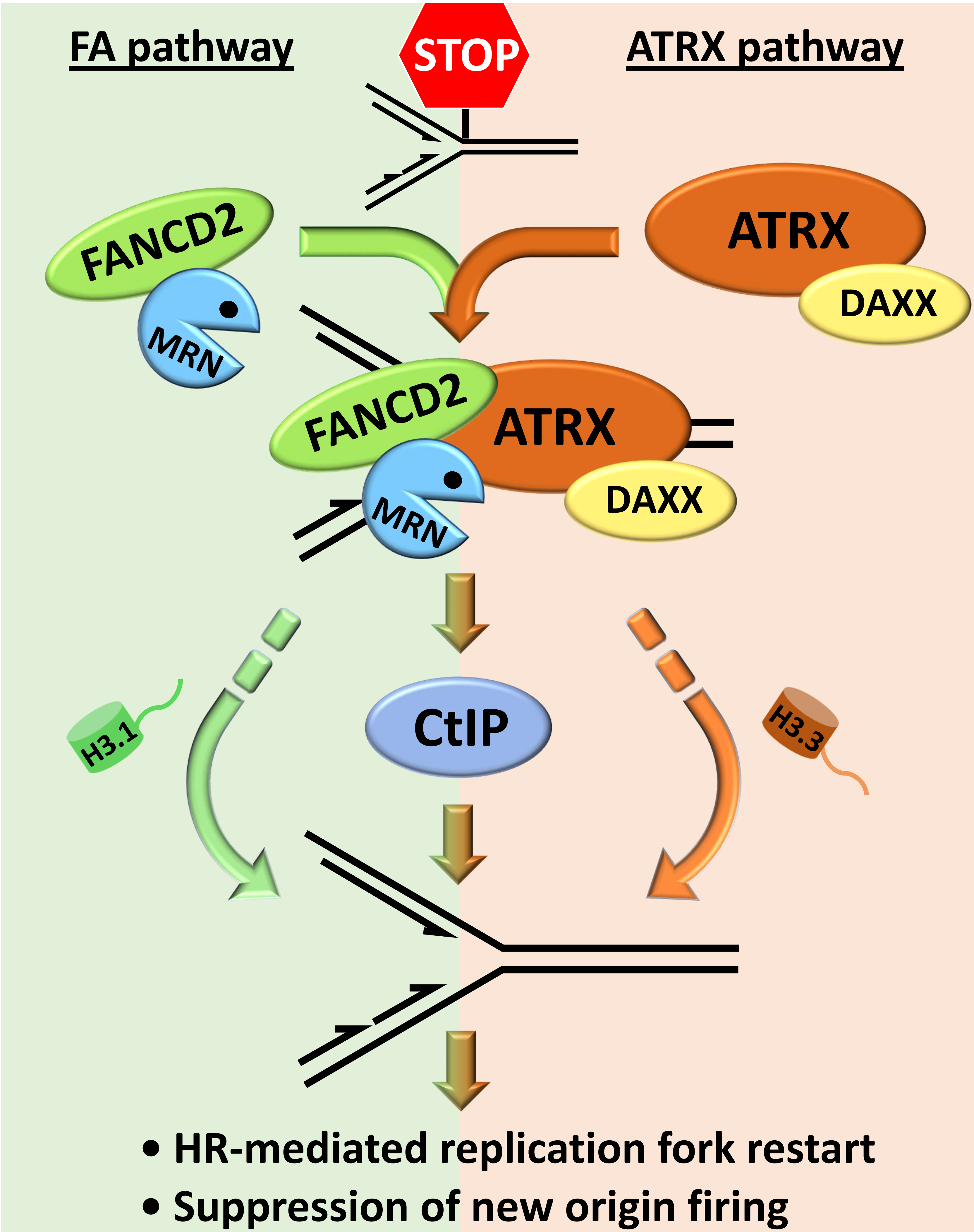
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

IP experiments were performed from nuclear extracts (NE) of PD20+3xFLAG-FANCD2 (wild type) cells. (**A)** *NBS1 co-IPs with ATRX*. NEs (lane 1) were subjected to IP with mouse IgG (lane 2) or an anti-ATRX antibody (lane 3). NE and IP samples were analyzed for the presence of ATRX and NBS1 by western blot. (**B**) *NBS1 co-IPs with FANCD2*. NEs (lane 1) were subjected to IP with mouse IgG (lane 2) or an anti-FLAG antibody (lane 3). NE and IP samples were analyzed for the presence of FANCD2 and NBS1 by western blot. **(C)** Whole cell extracts (WCE) were prepared from PD20+D2 or PD20 cells that had been treated with control siRNA (lanes 1 and 3) or ATRX-#2 siRNA (lanes 2 and 4) and analyzed for the presence of ATRX and FANCD2. Ku-80, loading control. Immunoblot signals for FANCD2 in lanes 3 and 4 were analyzed by densitometry and normalized against Ku80 signals using ImageJ. The relative FANCD2 protein level values are provided underneath each corresponding lane. **(D)** *Schematic of the CRISPR/Cas9 targeting of FANCD2 exon 9 in HCT116 cells*. Sequencing data show a single base pair insertion (in red) in AD2-DKO clone #6.9. (**E-F**) FANCD2 and ATRX cooperate to promote replication fork restart and suppress new origin firing in a human fibroblast cell line after HU treatment. (**E**) *ATRX and FANCD2 cooperate to mediate replication fork restart.* Replication fork restart efficiencies were compared between WT (PD20+D2), FANCD2-deficient (PD20), ATRX-deficient (PD20+D2, siATRX) and FANCD2/ATRX double-deficient (PD20, siATRX) cells. (**F**) *ATRX and FANCD2 act in concert to suppress new origin firing during replication blockade.* The number of new replication sites originating during BioU labeling after HU treatment was compared between WT (PD20+D2), FANCD2-deficient (PD20), ATRX-deficient (PD20+D2, siATRX) and FANCD2/ATRX double-deficient (PD20, siATRX) cells.

Supplementary Figure 2

(**A**) *FANCD2 foci formation occurs independently of ATRX during normal DNA replication and following replication stress*. HCT116 WT cells and two different $ATRX^{-/0}$ clones (#94 and #15.1) were untreated or treated with 2 mM HU for 20 hr and analyzed for the presence of FANCD2 foci. Nuclei with > 5 foci were considered positive for FANCD2 foci formation. (**B and C)** ATRX foci formation occurs independently of FANCD2 during normal DNA replication and following replication stress. (**B**) PD20+D2 (FANCD2-proficient) and PD20 (FANCD2-deficient) fibroblast cells were untreated or treated with 2mM HU for 20 hr and analyzed for the presence of ATRX foci. Nuclei with > 5 foci were considered positive for ATRX foci formation. **(C)** Same experiment as in **B**, but performed in HCT116 WT and $FANCD2^{-/-}$ cells. **(D, accompanies Main Fig 4B)** *ATRX is required for the HU-inducible CtIP foci formation*. HCT116 WT cells and a second $ATRX^{-/0}$ clone (#15.1) were treated with 2 mM HU for 20 hours and analyzed for the presence of CtIP foci. Nuclei with > 5 foci were considered positive for CtIP foci formation.

Supplementary Figure 3

Model of the physical and functional FA-ATRX pathway interactions during the restart of HU-stalled replication forks.

Supplementary Table 1

Summary of all P-values for results shown in Main Figures 2 to 6.

Supplementary Table 1

Figure	**Samples**	**p-value (two tales)**
2B	WT vs $D2^{-/-}$ Day 5	0.001
	WT vs $ATRX^{-/0}$ Day 5	0.011
	WT vs $AD2^{DKO}$ Day 5	0.0001
	$ATRX^{-/0}$ vs $AD2^{DKO}$ Day 5	0.001
	$D2^{-/-}$ vs $AD2^{DKO}$ Day 5	0.016
2C	WT vs $D2^{-/-}$ 150uM HU	0.000149
	WT vs $ATRX^{-/0}$ 150uM HU	0.022412
	WT vs $AD2^{DKO}$ 150uM HU	0.010017
	$ATRX^{-/0}$ vs $D2^{-/-}$ 150uM HU	ns
	$ATRX^{-/0}$ vs $AD2^{DKO}$ 150uM HU	ns
	$D2^{-/-}$ vs $AD2^{DKO}$ 150uM HU	ns
	$D2^{-/-}$ vs $AD2^{DKO}$	ns
3B	WT vs $D2^{-/-}$	ns
	WT vs $ATRX^{-/0}$	ns
	WT vs $AD2^{DKO}$	ns
	$ATRX^{-/0}$ vs $D2^{-/-}$	ns
	$ATRX^{-/0}$ vs $AD2^{DKO}$	ns
	$D2^{-/-}$ vs $AD2^{DKO}$	ns
	WT vs $D2^{-/-}$ +HU	0.00063
	WT vs $ATRX^{-/0}$ +HU	0.00029
	WT vs $AD2^{DKO}$ +HU	0.00227
	$ATRX^{-/0}$ vs $D2^{-/-}$ +HU	ns
	$ATRX^{-/0}$ vs $AD2^{DKO}$ +HU	ns
	$D2^{-/-}$ vs $AD2^{DKO}$ +HU	0.01368

3C	WT vs D2$^{-/-}$	ns
	WT vs ATRX$^{-/0}$	ns
	WT vs AD2^{DKO}	ns
	ATRX$^{-/0}$ vs D2$^{-/-}$	ns
	ATRX$^{-/0}$ vs AD2^{DKO}	ns
	D2$^{-/-}$ vs AD2^{DKO}	ns
	WT vs D2$^{-/-}$ +HU	0.00227
	WT vs ATRX$^{-/0}$ +HU	0.00288
	WT vs AD2^{DKO} +HU	0.02518
	ATRX$^{-/0}$ vs D2$^{-/-}$ +HU	ns
	ATRX$^{-/0}$ vs AD2^{DKO} +HU	ns
	D2$^{-/-}$ vs AD2^{DKO} +HU	0.00728
3D	WT vs D2$^{-/-}$ +HU	0.00205
	WT vs ATRX$^{-/0}$ +HU	0.00224
	WT vs AD2^{DKO} +HU	0.00681
	ATRX$^{-/0}$ vs D2$^{-/-}$ +HU	ns
	ATRX$^{-/0}$ vs AD2^{DKO} +HU	ns
	D2$^{-/-}$ vs AD2^{DKO} +HU	0.00589
	WT vs D2$^{-/-}$ +HU +Mirin	ns
	WT vs ATRX$^{-/0}$ +HU +Mirin	ns
	WT vs AD2^{DKO} +HU +Mirin	0.03137
	ATRX$^{-/0}$ vs D2$^{-/-}$ +HU +Mirin	ns
	ATRX$^{-/0}$ vs AD2^{DKO} +HU +Mirin	ns
	D2$^{-/-}$ vs AD2^{DKO} +HU +Mirin	0.00303
3E	WT vs D2$^{-/-}$ +HU	0.00108
	WT vs ATRX$^{-/0}$ +HU	0.00204

	WT vs AD2-DKO +HU	0.00049
	ATRX$^{-/0}$ vs D2$^{-/-}$ +HU	ns
	ATRX$^{-/0}$ vs AD2^{DKO} +HU	ns
	D2$^{-/-}$ vs AD2^{DKO} +HU	0.00165
	WT vs D2$^{-/-}$ +HU +Mirin	ns
	WT vs ATRX$^{-/0}$ +HU +Mirin	ns
	WT vs AD2^{DKO} +HU +Mirin	0.0333
	ATRX$^{-/0}$ vs D2$^{-/-}$ +HU +Mirin	ns
	ATRX$^{-/0}$ vs AD2^{DKO} +HU +Mirin	ns
	D2$^{-/-}$ vs AD2^{DKO} +HU +Mirin	0.00150
4B	WT vs D2-/- +HU	0.00119
	WT vs ATRX-/0 +HU	0.00031
	WT vs AD2-DKO +HU	0.00122
	ATRX-/0 vs D2-/- +HU	ns
	ATRX-/0 vs AD2-DKO +HU	ns
	D2-/- vs AD2-DKO +HU	ns
4C	WT vs D2$^{-/-}$	0.0053
	WT vs ATRX$^{-/0}$	0.02528
	WT vs AD2^{DKO}	0.01945
	ATRX$^{-/0}$ vs D2$^{-/-}$	ns
	ATRX$^{-/0}$ vs AD2^{DKO}	ns
	D2$^{-/-}$ vs AD2^{DKO}	ns
4E	siControl vs siFANCD2	0.00414
	siControl vs siATRX	0.00233
	siControl vs siFANCD2+siATRX	0.00427

	siControl vs siRAD51	0.00920
	siATRX vs siFANCD2	ns
	siATRX vs siFANCD2+siATRX	ns
	siATRX vs siRAD51	0.00016
	siFANCD2 vs siFANCD2+siATRX	ns
	siFANCD2 vs siRAD51	0.00065
	siFANCD2+siATRX vs siRAD51	0.00173
5B	WT vs D2 Deficient	ns
	WT vs siDAXX	ns
	WT vs D2 Deficient + siDAXX	ns
	D2 Deficient vs siDAXX	ns
	D2 Deficient vs D2 Deficient + siDAXX	ns
	siDAXX vs D2 Deficient + siDAXX	ns
	WT vs D2 Deficient +HU	0.00002
	WT vs siDAXX +HU	0.00157
	WT vs D2 Deficient + siDAXX +HU	0.00002
	D2 Deficient vs siDAXX +HU	ns
	D2 Deficient vs D2 Deficient + siDAXX +HU	0.00326
	siDAXX vs D2 Deficient + siDAXX +HU	ns
5C	WT vs D2 Deficient	ns
	WT vs siDAXX	ns
	WT vs D2 Deficient + siDAXX	ns
	D2 Deficient vs siDAXX	ns
	D2 Deficient vs D2 Deficient + siDAXX	ns

	siDAXX vs D2 Deficient + siDAXX	ns
	WT vs D2 Deficient +HU	0.002269
	WT vs siDAXX +HU	0.002882
	WT vs D2 Deficient + siDAXX +HU	0.007286
	D2 Deficient vs siDAXX +HU	ns
	D2 Deficient vs D2 Deficient + siDAXX +HU	0.025182
	siDAXX vs D2 Deficient + siDAXX +HU	ns
6C	WT vs $D2^{-/-}$	ns
	WT vs $+FANCD2^{WT}$	ns
	WT vs $+FANCD2^{231R}$	ns
	WT vs $FANCL^{-/-}$	ns
	$D2^{-/-}$ vs $+FANCD2^{WT}$	ns
	$D2^{-/-}$ vs $+FANCD2^{231R}$	ns
	$D2^{-/-}$ vs $FANCL^{-/-}$	ns
	$+FANCD2^{WT}$ vs $+FANCD2^{231R}$	ns
	$+FANCD2^{WT}$ vs $FANCL^{-/-}$	ns
	$+FANCD2^{231R}$ vs $FANCL^{-/-}$	ns
	WT vs $D2^{-/-}$ +HU	0.00273
	WT vs $+FANCD2^{WT}$ +HU	ns
	WT vs $+FANCD2^{231R}$ +HU	0.00373
	WT vs $FANCL^{-/-}$ +HU	ns
	$D2^{-/-}$ vs $+FANCD2^{WT}$ +HU	0.00837
	$D2^{-/-}$ vs $+FANCD2^{231R}$ +HU	ns
	$D2^{-/-}$ vs $FANCL^{-/-}$ +HU	0.00288
	$+FANCD2^{WT}$ vs $+FANCD2^{231R}$ +HU	0.00373
	$+FANCD2^{WT}$ vs $FANCL^{-/-}$ +HU	ns

	+FANCD2^{231R} vs FANCL$^{-/-}$ +HU	0.002
6D	WT vs D2$^{-/-}$	ns
	WT vs +FANCD2^{WT}	ns
	WT vs +FANCD2^{231R}	ns
	WT vs FANCL$^{-/-}$	ns
	D2$^{-/-}$ vs +FANCD2^{WT}	ns
	D2$^{-/-}$ vs +FANCD2^{231R}	ns
	D2$^{-/-}$ vs FANCL$^{-/-}$	ns
	+FANCD2^{WT} vs +FANCD2^{231R}	ns
	+FANCD2^{WT} vs FANCL$^{-/-}$	ns
	+FANCD2^{231R} vs FANCL$^{-/-}$	ns
	WT vs D2$^{-/-}$ +HU	0.01613
	WT vs +FANCD2^{WT} +HU	ns
	WT vs +FANCD2^{231R} +HU	0.02381
	WT vs FANCL$^{-/-}$ +HU	ns
	D2$^{-/-}$ vs +FANCD2^{WT} +HU	0.01507
	D2$^{-/-}$ vs +FANCD2^{231R} +HU	ns
	D2$^{-/-}$ vs FANCL$^{-/-}$ +HU	ns
	+FANCD2^{WT} vs +FANCD2^{231R} +HU	0.002