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Supplementary Materials for

Inhibition of the translesion synthesis polymerase REV1 exploits replication gaps as a cancer vulnerability

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Figs. S1 to S6

Supplemental Figures:

Figure S1. Both WT and pro-TLS mutant S990A correct defects in replication restart due to FANCD1 deficiency.

(A) Cell survival assays with U2OS FANCD1 K/O cells complemented with FANCD1^{WT} (WT), empty vector (V) and the FANCD1-BRCA1 binding deficient, FANCD1^{S990A} (S990A) treated with increasing concentrations of MMC. Data represent the mean percent \pm SD of survival from three independent experiments.

(B) Western blot analysis with the indicated Abs of WCE from FANCD1 null FA-J cells complemented with FANCD1^{WT} (WT), empty vector (V) and the FANCD1-BRCA1 binding deficient, FANCD1^{S990A} (S990A), the FANCD1 helicase dead mutant (K52R) and the FANCD1-BRCA1 binding deficient and helicase dead (S990A+K52R) double mutant.

(C) Experimental schematic and quantification of CldU (red) tract length either after treatment with 20uM TLSi alone or under unchallenged conditions as observed in the U2OS FANCD1 K/O or FA-J complemented cells.

(D) Experimental schematic and the quantification of CldU (red) tract length after co-incubation with 0.5mM HU or 2J/m² of UV as observed in the FA-J complemented cells.

(E) Experimental schematic and quantification of CldU (red) tract length, percent CldU (red) tract connected with IdU (green), percent only IdU (green) and percent only CldU (red) tract as observed in the FA-J complemented cells.

(F) Experimental schematic and quantification of CldU (red) track length following 1h treatment with 0.5mM HU immediately followed by +/- S1 nuclease treatment and as observed in the FA-J complemented cells.

(G) Experimental schematic and quantification of CldU/IdU (red/green) ratio as observed in the FA-J complemented cells.

(H) Cell survival assays with PEO1 cells expressing V5 tagged - empty vector (V), FANCD1^{WT} (WT), and the FANCD1-BRCA1 binding deficient, FANCD1^{S990A} (pro-TLS) under increasing concentrations of cisplatin. Data represent the mean percent \pm SD of survival from three independent experiments. Experimental schematic and quantification of CldU (red) tract length after co-incubation with 0.5mM HU as observed in these cell lines.

For every DNA fiber assay analysis, each dot represents one individual fiber. Experiments were performed in biological triplicate with at least 100 fibers per replicate. Bars represent

the mean \pm SD. Statistical analysis were performed according to two-tailed Mann-Whitney test. All p values are further described in the statistical methods.

Figure S2. TLS polymerases overcome replication stress associated ssDNA gaps.

(A-B) Representative images (63X) of eGFP-pol-eta (pol- η) or eGFP-Rev1 foci as observed in U2OS FANCD1 K/O cells complemented with FANCD1^{WT} (WT) and the FANCD1-BRCA1 binding deficient, FANCD1^{S990A} (S990A) and U2OS cells expressing shRNA against NSC or p21 transiently transfected with eGFP-pol- η or eGFP-Rev1 and assessed either after no treatment (Unt), post 4h 15J/m² UV or post 48h 250nM MMC treatment. Around 300 cells were counted from multiple fields.

(C) Experimental schematic, representative images and quantification of the EdU (green) positive cells after varying doses of UV treatment as observed in U2OS FANCD1 K/O cells complemented with FANCD1^{WT} (WT - Control) and the FANCD1-BRCA1 binding deficient, FANCD1^{S990A} (S990A – pro-TLS). EdU staining was performed as described in Figure 2

(D-G) Experimental schematic, representative images and quantification of EdU (green) and ssDNA (red) positive cells following initial labeling with CldU for 48h followed by EdU treatment with or without 0.5mM HU +/- 20 μ M TLSi or +/- 5 μ M ATRi. EdU and ssDNA staining was performed as described in the Figure 2.

Experiments were performed in biological triplicate. Bars represent the mean \pm SD. Statistical analysis were performed according to two-tailed Mann-Whitney test. All p values are further described in the statistical methods.

Figure S3. TLS polymerases counteract oncogene induced replication stress response.

(A and C) Experimental schematic and representative images of EdU and ssDNA positive cells as previously described in Figure 3(B and E).

(B) Representative images and quantification of the colony formation assay as observed in FANCD1^{WT} or FANCD1^{S990A} complemented FANCD1 K/O U2OS cell lines stably infected with only vector (V) and as described in the Figure 3(C).

Experiments were performed in biological triplicate. Bars represent the mean \pm SD. Statistical analysis were performed according to two-tailed Mann-Whitney test. All p values are further described in the statistical methods.

Figure S4. TLS polymerase re-wiring of cancer cells promote unrestrained replication during stress.

(A) Experimental schematic and quantification of EdU (green) positive cells. Cells were either labeled for 30mins with EdU or for 2h with EdU and varying doses of HU. Cells were stained for EdU as previously described in Figure 2. Percent EdU positive cells were quantified from around 300 cells counted from multiple fields.

(B) Experimental schematic and quantification of EdU (green) and ssDNA (red) positive cells as previously described in Figure 4(A).

Experiments were performed in biological triplicate. Bars represent the mean \pm SD. Statistical analysis were performed according to two-tailed Mann-Whitney test. All p values are further described in the statistical methods.

Figure S5. FANCI promotes TLS pathway by suppressing the negative regulator, p21

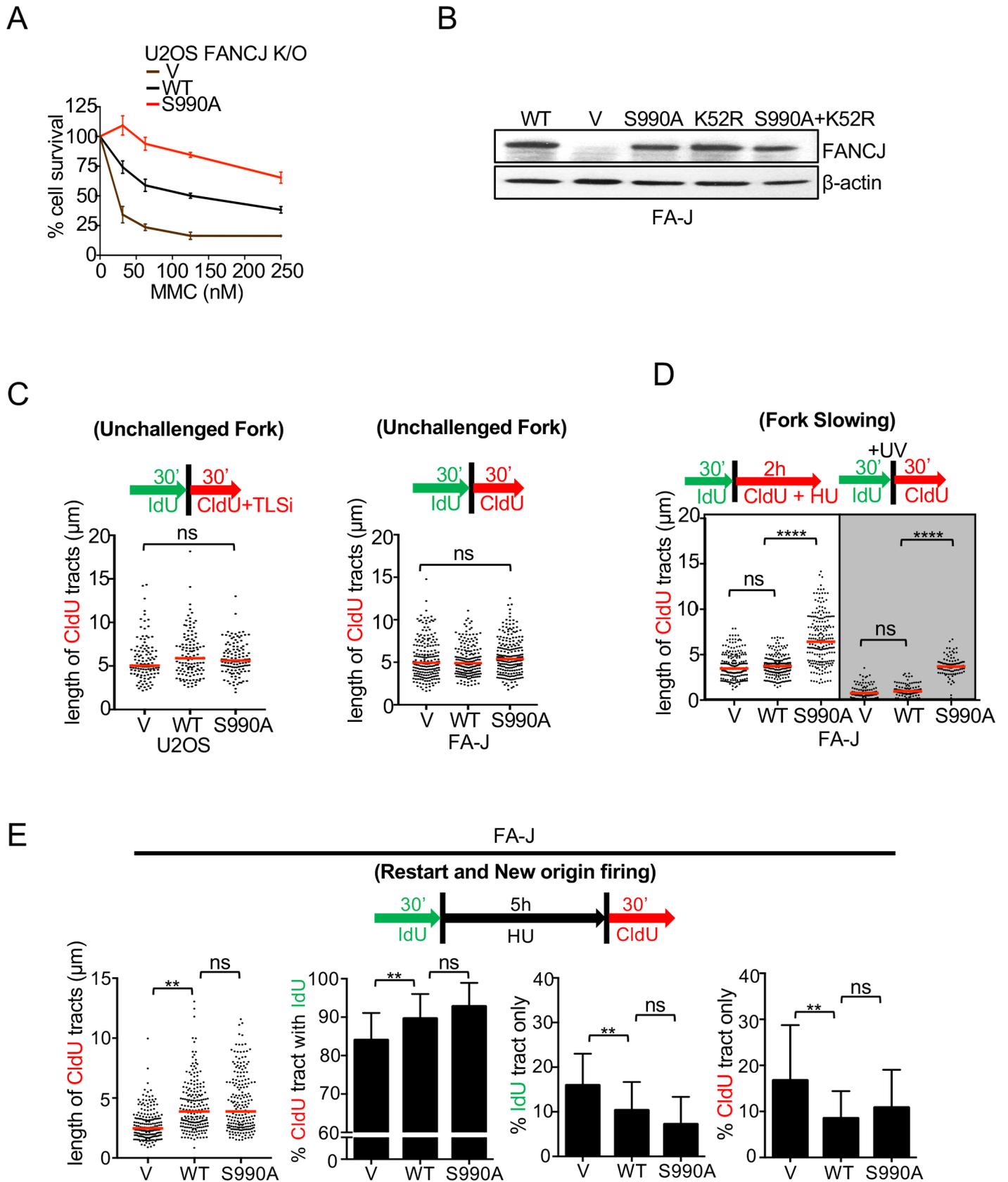
(A) Western blot analysis with the indicated Abs of WCE from HeLa CRISPR control and FANCI CRISPR knockout (K/O) cells and HeLa FANCI K/O cells expressing shRNA against NSC or p21. Experimental schematic and quantification of EdU (green) positive cells. Cells were either labeled for 30mins with EdU or 2h with EdU and 0.5mM HU. Cells were stained for EdU as previously described in Figure 2.

(B and D) Representative images and quantification of the colony formation assay as observed in HeLa CRISPR control and FANCI CRISPR knockout (K/O) cells and HeLa FANCI K/O cells expressing shRNA against NSC or p21. The HeLa FANCI K/O shRNA p21 cells were either grown in absence or continuous presence of 20 μ M TLSi.

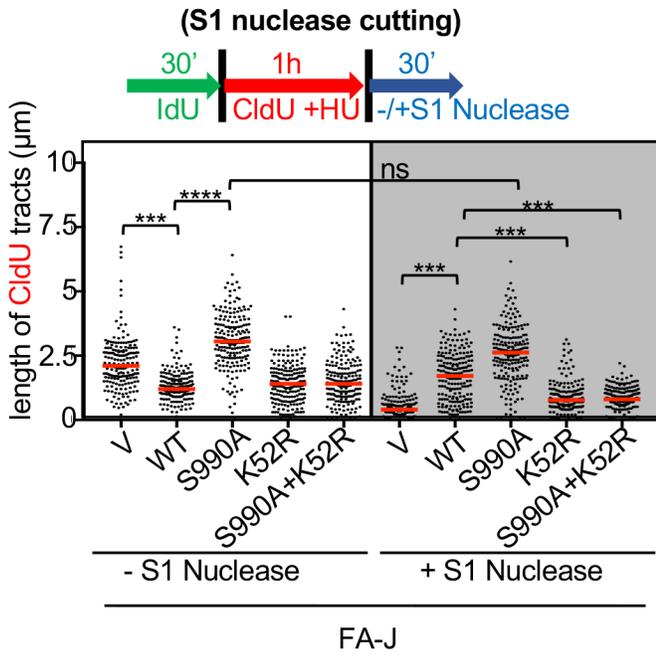
(C) Experimental schematic and quantification of EdU (green) and ssDNA (red) positive cells as previously described in Figure 4(A). EdU and ssDNA staining was performed as described in Figure 2.

Experiments were performed in biological triplicate. Bars represent the mean \pm SD. Statistical analysis were performed according to two-tailed Mann-Whitney test. All p values are further described in the statistical method.

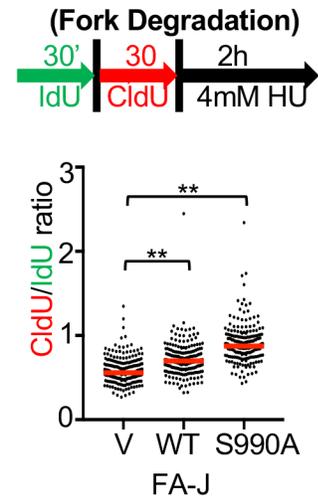
Figure S6. TLSi as a promising cancer therapy (A-D) Representative images and quantification of the colony formation assay after dose dependent treatment with ATRi (μM) and WEE1i (nM) alone or in combination with 20 μM TLSi across different cell lines. Experiments were performed in biological triplicate. Bars represent the mean \pm SD. Statistical analysis were performed according to two-tailed Mann-Whitney test. All p values are further described in the statistical methods.



F



G



H

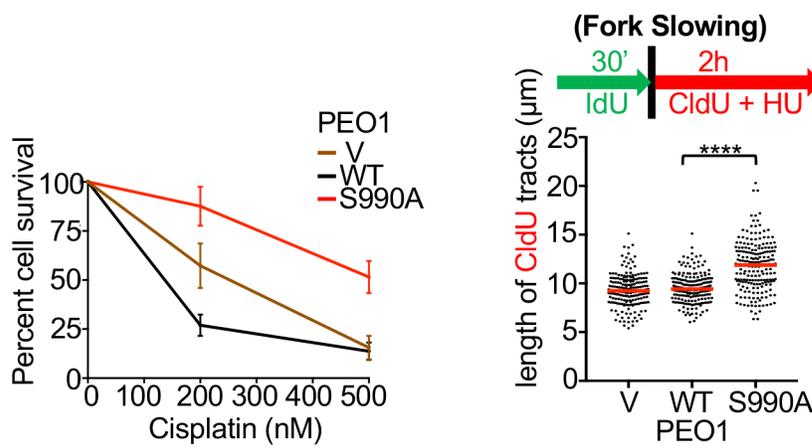


Fig S2

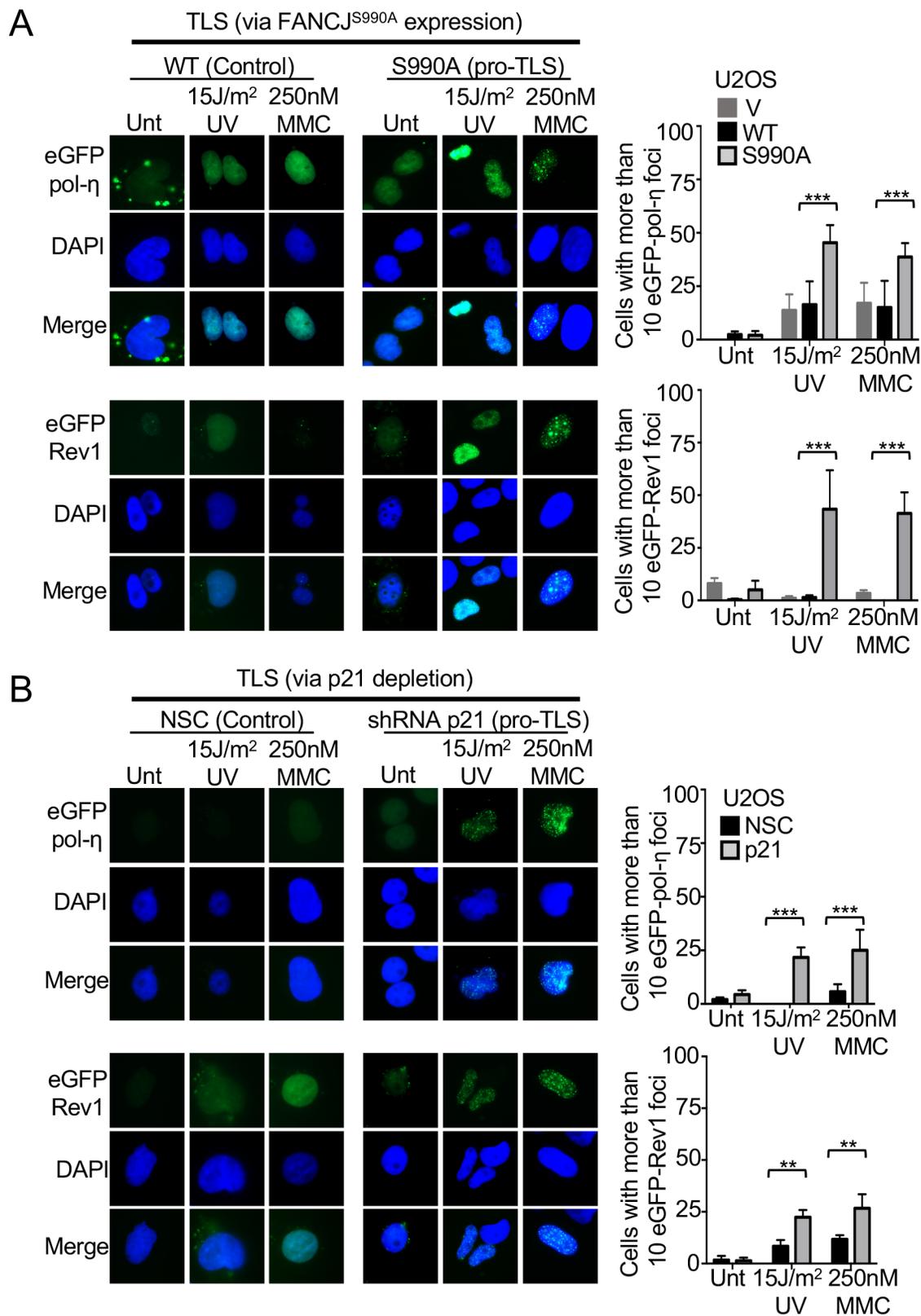
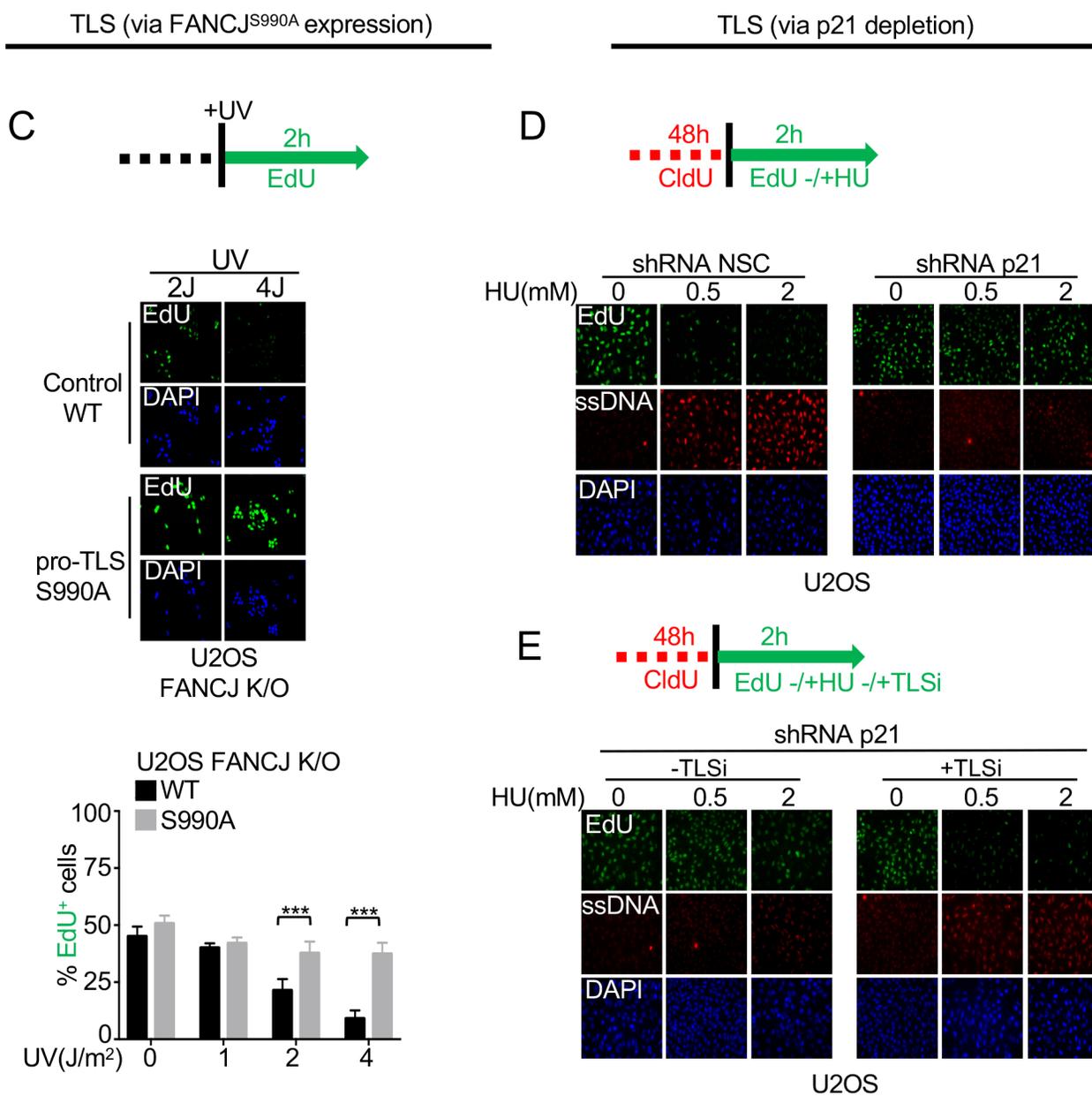
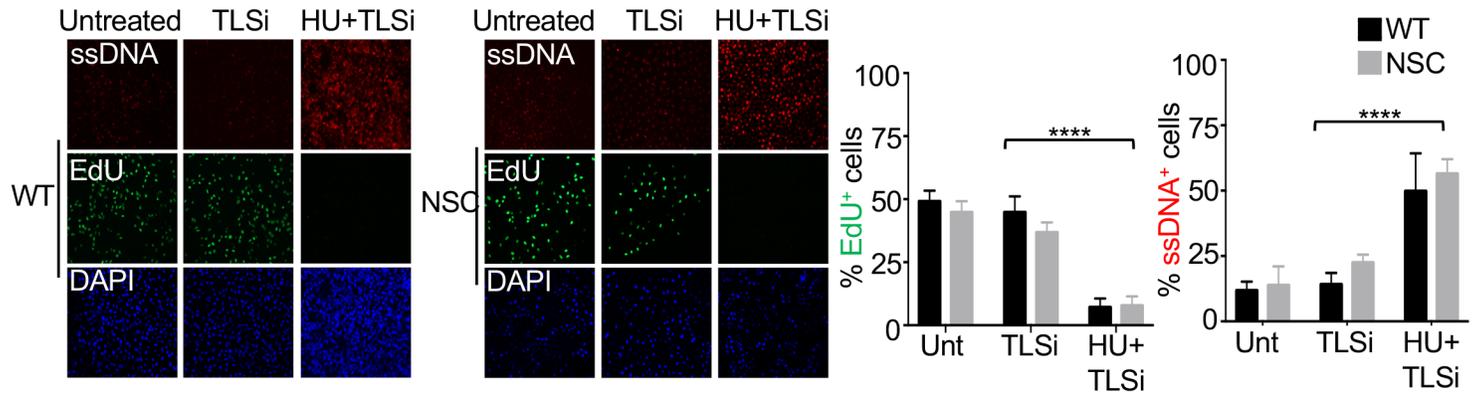


Fig S2
Cont



**Fig S2
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G

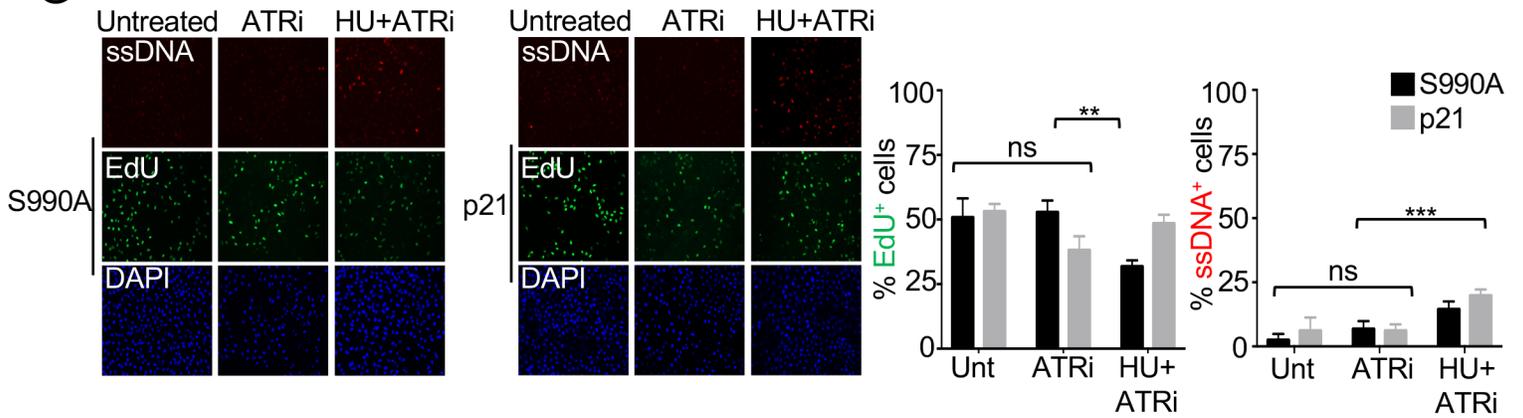


Fig S3

