Expanded View Figures

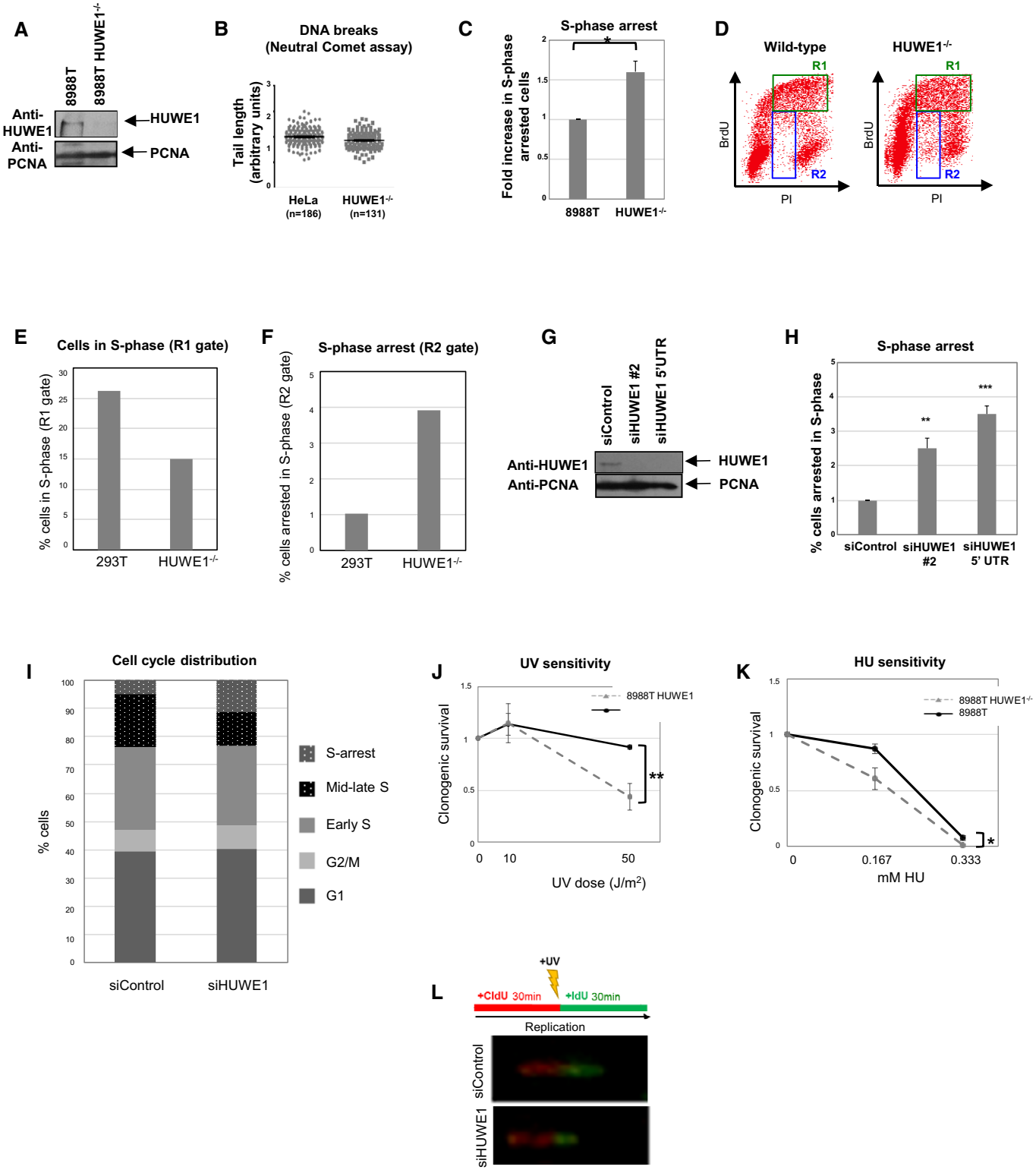


Figure EV1.

Figure EV1. Phenotypes of HUWE1-deficient cells.

A Western blot showing the absence of HUWE1 protein in 8988T cells subjected to CRISPR/Cas9-mediated HUWE1 deletion.

B HUWE1-knockout HeLa cells show no increase in Neutral Comet tail length. Cells were analyzed under normal growth conditions (no DNA damage treatment). The "*n*" numbers of comet tails analyzed (pooled from three independent experiments), as well as the mean ± SEM, are indicated on the graphs. We would like to mention that both normal and HUWE1-knockout cells showed no real "tails" but rather very short "smears", likely indicating that no DNA breaks are occurring in any of these cells. We measured those tails anyway, and present the result in this figure, in an attempt to provide a quantification of this result.

C Increased S-phase arrest in HUWE1-knockout 8988T cells, as measured using the BrdU/PI bi-dimensional flow cytometry assay. The average of three experiments, with standard deviations, is shown. *P*-value is 0.011 (calculated using the *t*-test two-tailed, equal variance).

D Representative BrdU/PI bi-dimensional flow cytometry profiles of wild-type and HUWE1-knockout 293T cells. Cells in mid- and late S-phase (DNA content >2 N and positive for BrdU) are labeled as R1 region. Cells with S-phase DNA content (between 2N and 4N) but negative for BrdU staining represent S-phase-arrested cells and are indicated as R2 region.

E, F Quantifications of R1 and R2 regions from the plot shown in (D), showing the proportions of cells in each region. While only one representative plot is shown, this result was reproduced in three independent experiments.

G Western blot showing that the siRNA oligonucleotides used are efficiently knocking down HUWE1 levels in 293T cells.

H Increased S-phase arrest in 293T cells following HUWE1 knockdown. The percentage of cells arrested in S-phase is shown. The average of five experiments, with standard deviations, is presented. The *P*-values shown (calculated using the *t*-test two-tailed, equal variance) indicate the statistical significance relative to siControl (0.0024 and 0.0001, respectively).

I Quantification of cell cycle distribution of control and HUWE1-depleted 293T cells, showing the altered distribution of mid-late S-phase vs. S-phase-arrested cells. Shown are G1 (BrdU-negative, 2N DNA content); G2/M (BrdU-negative, 4N DNA content); early S (BrdU-positive, 2N DNA content); mid-late S (BrdU-positive, >2N DNA content); S-arrest (BrdU-negative, DNA content between 2N and 4N). The quantification of one representative plot is shown, but this result was reproduced in many independent experiments.

J, K UV (J) and HU (K) sensitivity of HUWE1-knockout 8988T cells. The average of three experiments, with standard deviations, is shown. *P*-values (calculated using the *t*-test two-tailed, equal variance) are 0.0029 (J) and 0.0126 (K).

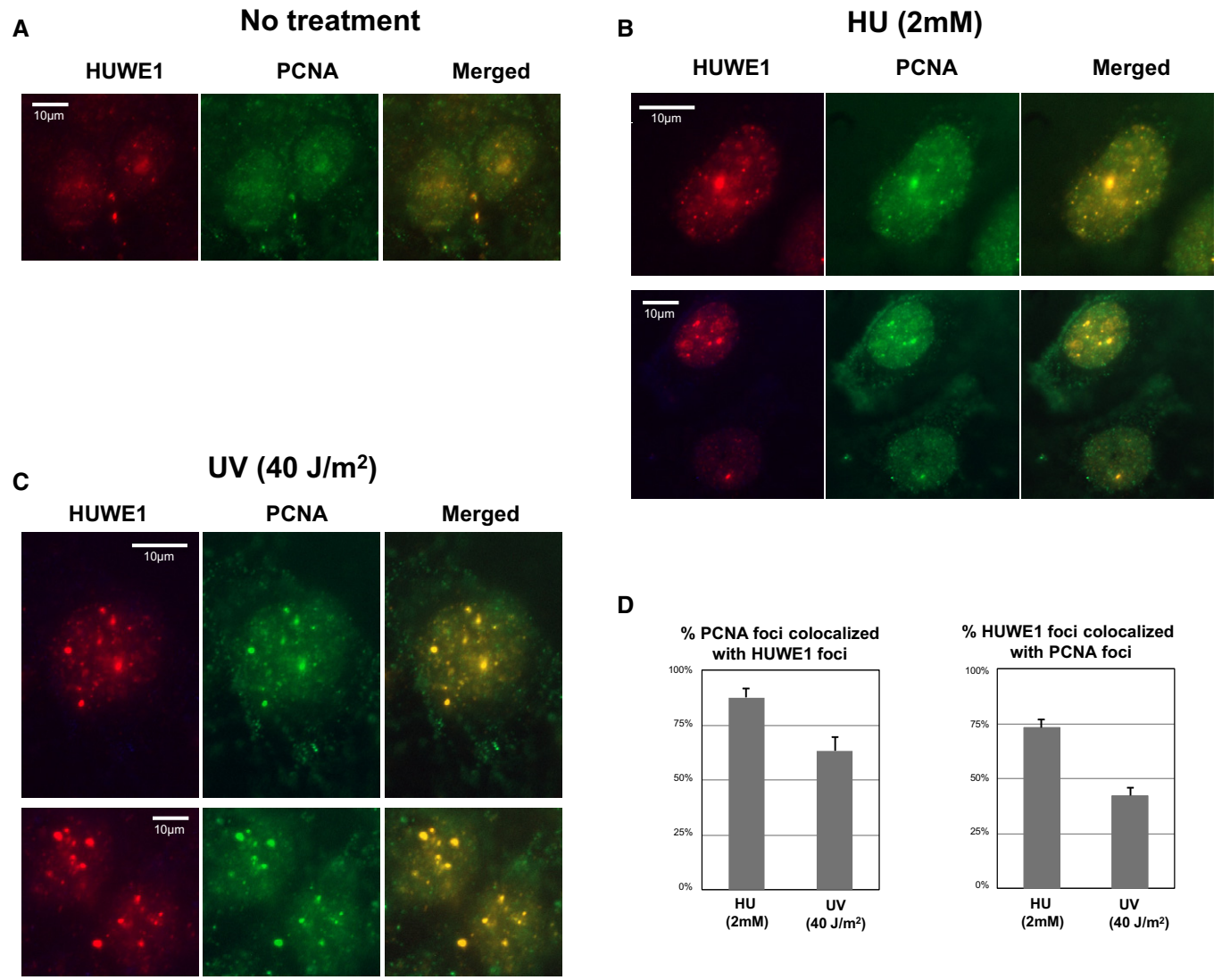
L Schematic of the experimental setup, including examples of micrographs, for the DNA fiber experiment.

Figure EV2. Co-immunofluorescence experiments showing co-localization of HUWE1 and PCNA.

A–C Representative immunofluorescence micrographs of U2OS cells untreated (A), treated with 2 mM HU for 24 h (B), or exposed to 40 J/m^2 UV and analyzed 2 h later (C).

D Quantifications showing the percentage of PCNA foci co-localizing with HUWE1 foci and, reciprocally, the percentage of HUWE1 foci co-localizing with PCNA foci. Only cells with more than five foci were analyzed. The average of three experiments, with standard errors, is shown. In total, 191 HUWE1 and 156 PCNA foci were counted.

E HUWE1-knockout cells corrected with wild-type or PIP mutant were subjected to immunofluorescence using HUWE1 and PCNA antibodies. Similar to the endogenous protein, Myc-HUWE1 wild-type forms UV- and HU-induced chromatin foci. In contrast, the PIP-box mutant does not co-localize with PCNA after damage exposure. Shown are representative micrographs, as well as quantifications of the percentage of PCNA foci co-localizing with HUWE1 and, reciprocally, the percentage of HUWE1 foci co-localizing with PCNA, in wild-type and PIP mutant-corrected cells. Only cells with more than five foci were analyzed. The average of three experiments, with standard errors, is shown. A total number of foci counted were as follows: for the HU experiment: 162 HUWE1-WT foci with 144 PCNA foci; 196 HUWE1-FF foci with 163 PCNA foci. For the UV experiment: 146 HUWE1-WT foci with 121 PCNA foci; 101 HUWE1-FF foci with 78 PCNA foci.


A
No treatment
HUWE1
PCNA
Merged
10μm
B
HU (2mM)
HUWE1
PCNA
Merged
10μm
C
UV (40 J/m2)
HUWE1
PCNA
Merged
10μm
D
% PCNA foci colocalized with HUWE1 foci
100%
75%
50%
25%
0%
HU (2mM)
UV (40 J/m2)
% HUWE1 foci colocalized with PCNA foci
100%
75%
50%
25%
0%
HU (2mM)
UV (40 J/m2)

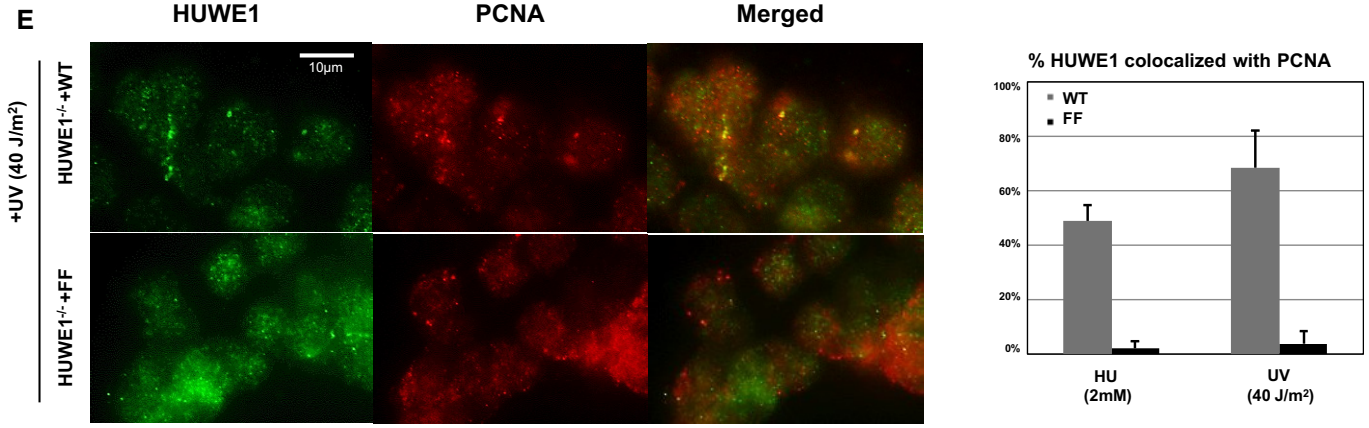

E
HUWE1
PCNA
Merged
+UV (40 J/m2)
HUWE1-/-+WT
HUWE1-/-+FF
10μm
% HUWE1 colocalized with PCNA
WT
FF
100%
80%
60%
40%
20%
0%
HU (2mM)
UV (40 J/m2)

Figure EV2.

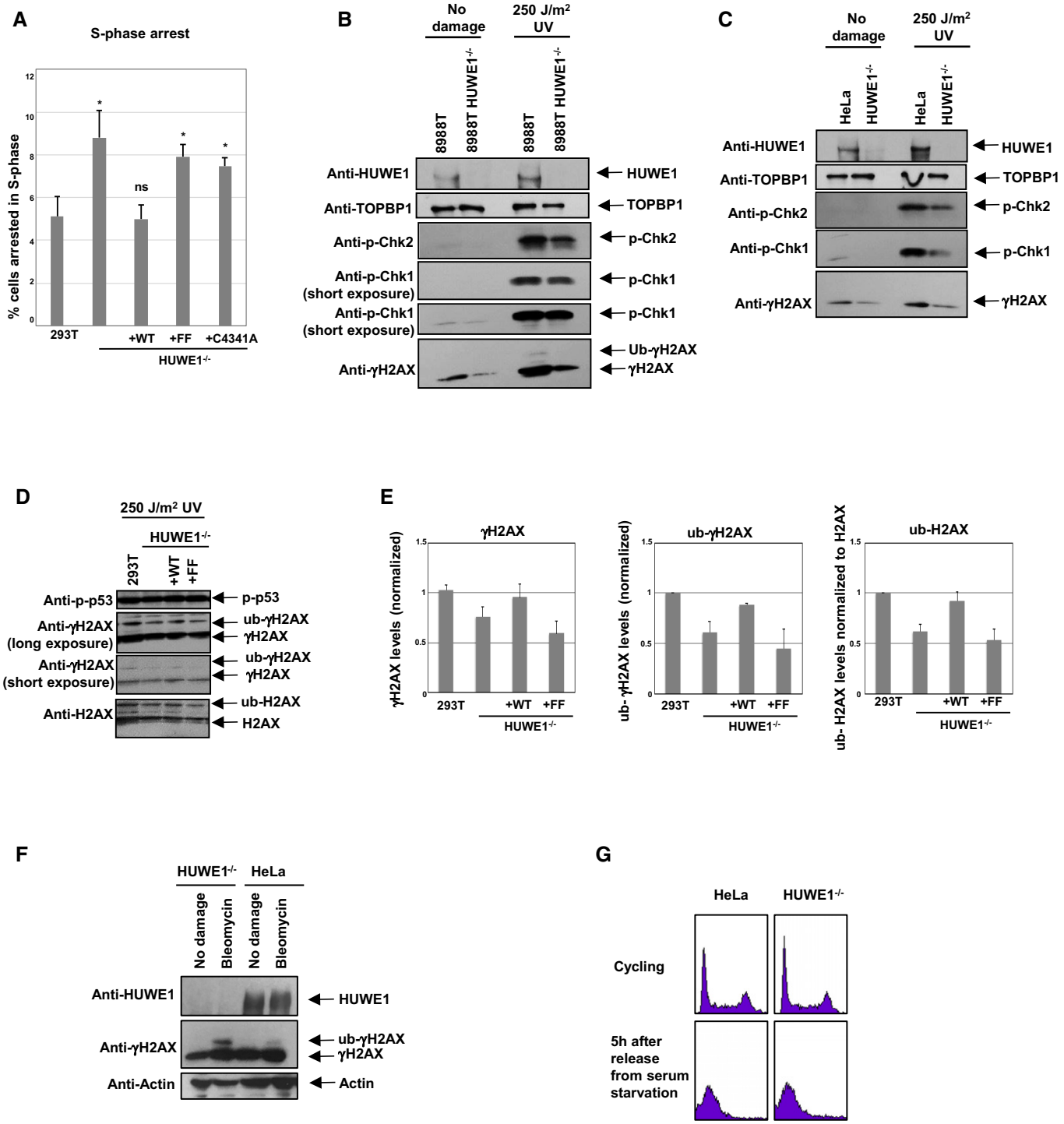


Figure EV3.

Figure EV3. Impact of HUWE1-knockout on replication stress signaling and H2AX ubiquitination.

A Correction of the S-phase arrest phenotype by wild-type but not PIP mutant HUWE1. The same data as in Fig 6E are shown, but this time the percent of cells in S-phase is plotted. Shown is the average of three independent experiments ± SD. The *P*-values shown (calculated using the *t*-test two-tailed, equal variance), indicate the statistical significance relative to 293T (0.015, 0.865, 0.011, and 0.016, respectively).

B, C Western blots showing reduced γH2AX and phospho-Chk1 in HUWE1-knockout 8988T (B) and HeLa (C) cells.

D Western blots showing reduced H2AX modification in HUWE1-knockout 293T cells, corrected by wild-type but not PIP mutant HUWE1.

E Quantification of γH2AX (against input control), γH2AX ubiquitination (against un-ubiquitinated γH2AX), and H2AX ubiquitination (against unmodified H2AX). The average of three experiments is shown. Error bars represent standard deviations.

F HUWE1 deletion does not reduce H2AX ubiquitination in G1. HeLa cells (wild-type or HUWE1-knockout) were arrested in G0 by serum starvation for 24 h, than released in normal media. Cells were analyzed 5 h later, when they reached G1. Cells were either treated with 5 μM bleomycin for the last 2 h of the release or left untreated.

G Flow cytometry profile (PI staining) of cells at the harvest time point (5 h after release from serum starvation). The plot indicates that cells are uniformly in G1. For comparison, the normal profile of cycling cells is also presented.

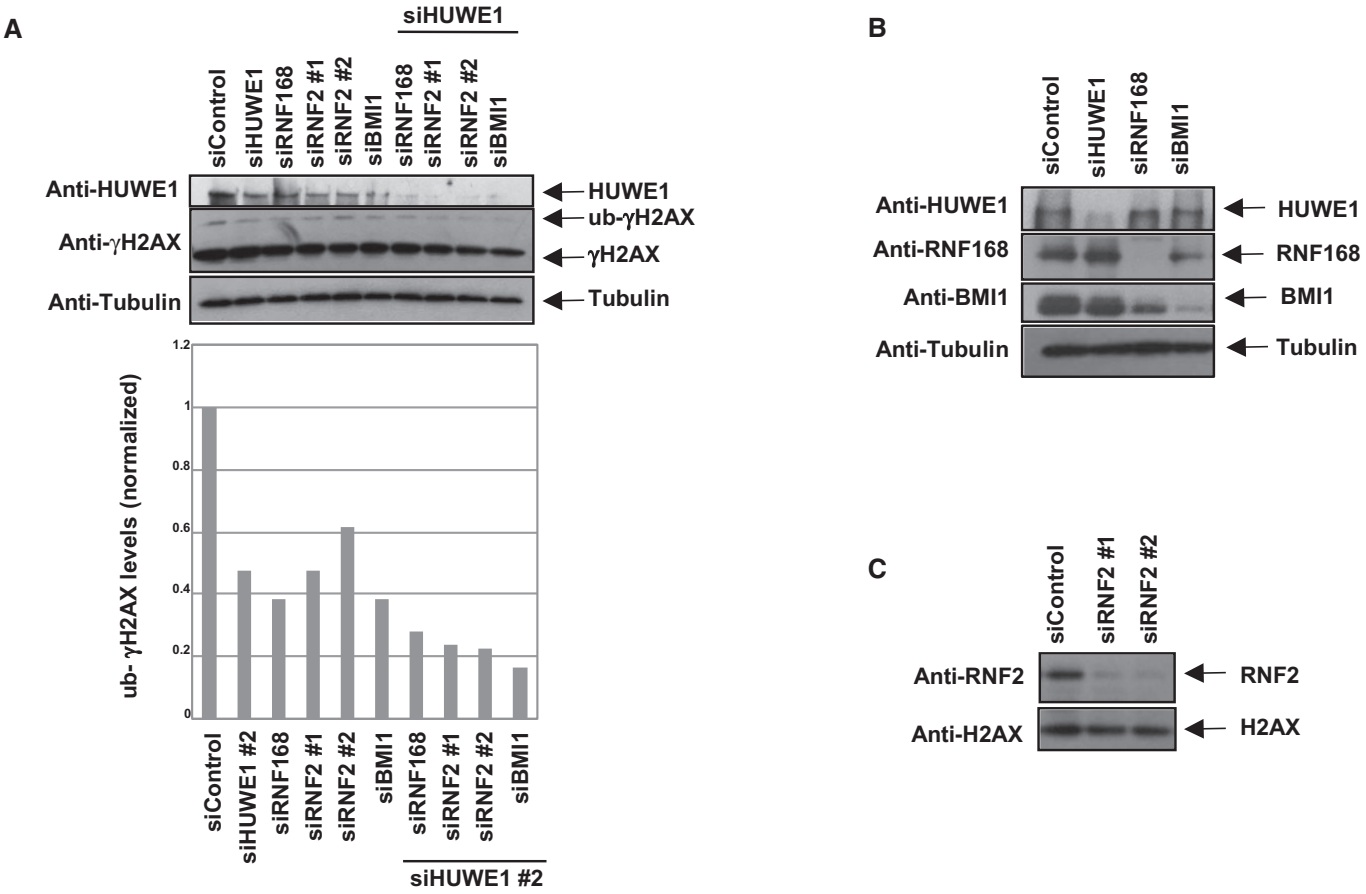
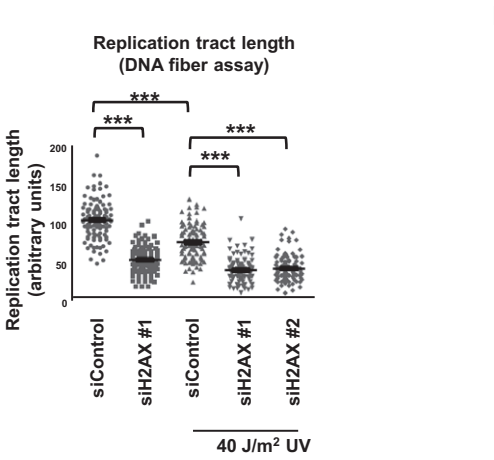


Figure EV4. Co-depletion of HUWE1 and other H2AX ubiquitin ligases enhances the H2AX ubiquitination defect.

A Representative Western blot experiment in HeLa cells. A quantification of this experiment is also shown. This result was replicated in three independent experiments.

B, C Western blots showing the efficient depletion of H2AX ubiquitin ligases.



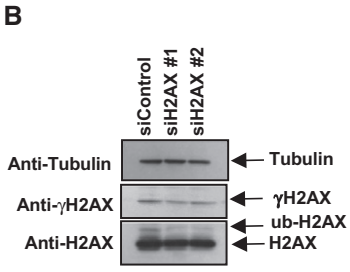


Figure EV5. H2AX knockdown reduces replication tract length.

A DNA fiber assay using HeLa cells with H2AX knockdown. Cells were either treated with 40 J/m^2 UV (analyzed 2 h later) or left untreated. The "*n*" numbers of samples are 101, 101, 106, 92, and 100, respectively (pooled from three independent experiments). *P*-values (calculated using the *t*-test two-tailed, equal variance) are 1.19×10^{-40} (siControl vs. siH2AX, no damage samples); 5.6×10^{-15} (siControl, no damage vs. UV samples); 1.1×10^{-20} (siControl vs. siH2AX #1, UV samples); 9.01×10^{-32} (siControl vs. siH2AX #2, UV samples).

B Western blot of HeLa cells showing the impact of H2AX siRNA oligonucleotides.