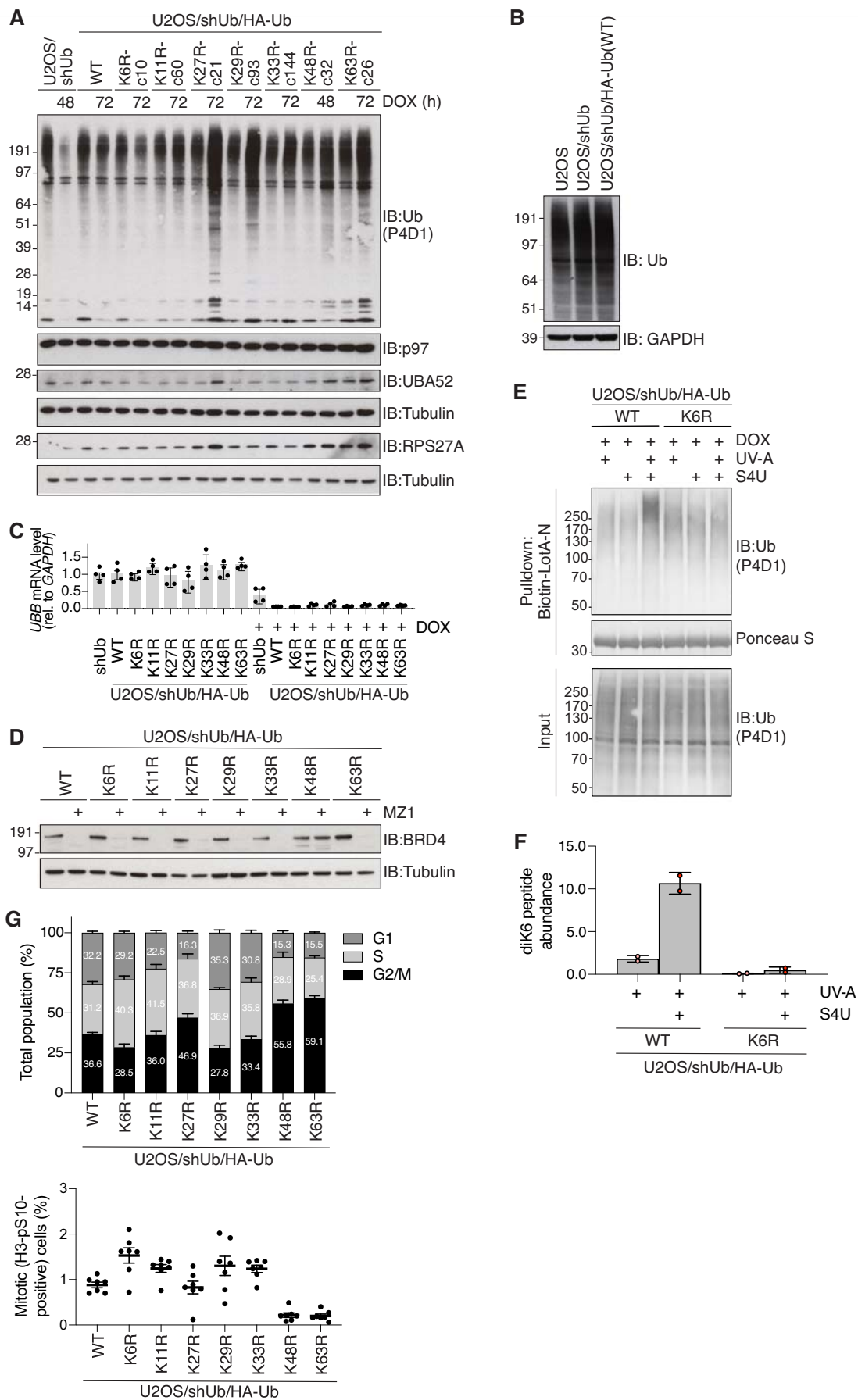
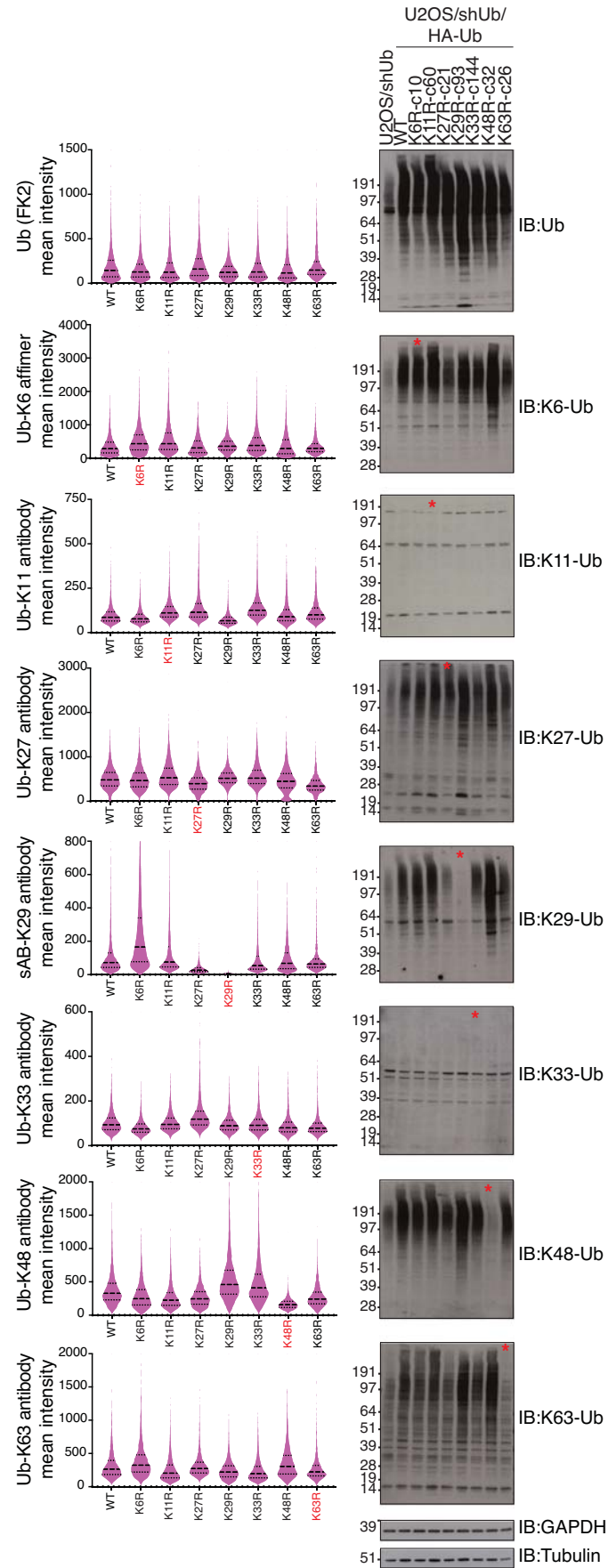


Expanded View Figures

Figure EV1. Extended data related to Fig. 1.

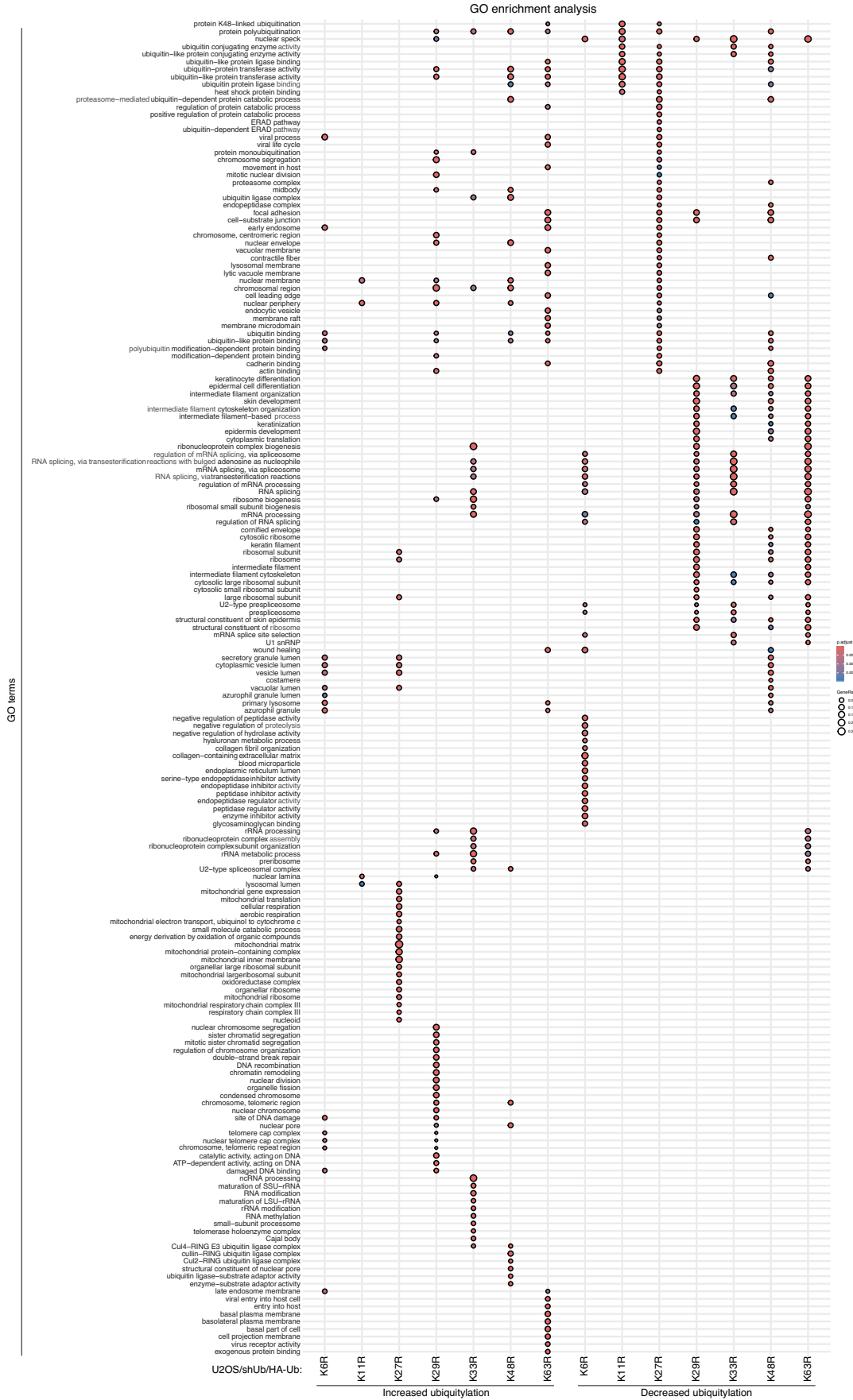
(A) Immunoblot (IB) analysis of U2OS/shUb and derivative U2OS/shUb/HA-Ub cell lines treated or not with DOX for the indicated times. (B) Immunoblot analysis of parental U2OS and derivative U2OS/shUb cell lines. (C) U2OS/shUb and derivative replacement cell lines were treated or not with DOX and mRNA levels were analyzed by RT-qPCR. Primers to GAPDH were used as a normalization control (data are technical duplicates of two independent experiments). (D) Immunoblot analysis of Ub replacement cell lines treated or not with the BRD4 PROTAC MZ1 (500 nM) overnight. (E) Immunoblot analysis of K6-linked Ub chains isolated by pulldown with the K6-Ub binder Biotin-LotA-N in the indicated DOX-treated Ub replacement cell lines exposed to UV-A (500 mJ/cm²) and/or 4-thiouridine (S4U, 25 mM), combined treatment of which leads to RNA-protein crosslink formation. (F) Abundance of K6 diGly peptide after OtUBD (total Ub) enrichment of indicated DOX-treated Ub replacement cell lines in (E) (mean \pm s.d.; $n = 2$ technical replicates). (G) Cell cycle analysis of indicated Ub replacement cell lines by QIBC. All Ub replacement cell lines were treated with DOX for 72 h, except Ub(K48R) cells that were treated for 48 h. Upper panel shows cell cycle fraction (mean \pm s.e.m.; $n = 4$ independent experiments; >1000 cells analyzed per condition). Lower panel shows mitotic fraction as determined by QIBC of H3-pS10 immunostaining (mean \pm s.e.m.; $n = 7$ independent experiments; >1000 cells analyzed per condition). Source data are available online for this figure.





**Figure EV2. Extended data related to Fig. 2.**

QIBC analysis (left) and immunoblot analysis (right) of DOX-treated Ub replacement cell lines using the indicated linkage-specific antibodies or binders (thick dashed lines, median; dotted lines, quartiles). Data were a single representative replicate from three independent experiments. Source data are available online for this figure.



**Figure EV3. Extended data related to Fig. 2.**

GO term analysis of cellular compartments enriched among proteins showing significantly up- or downregulated ubiquitylation in Ub(K-to-R)-replaced cells relative to Ub(WT)-replaced cells (Fig. 2B-H) ($n = 3$ technical replicates; unpaired two-tailed t-test, Benjamini-Hochberg corrected).

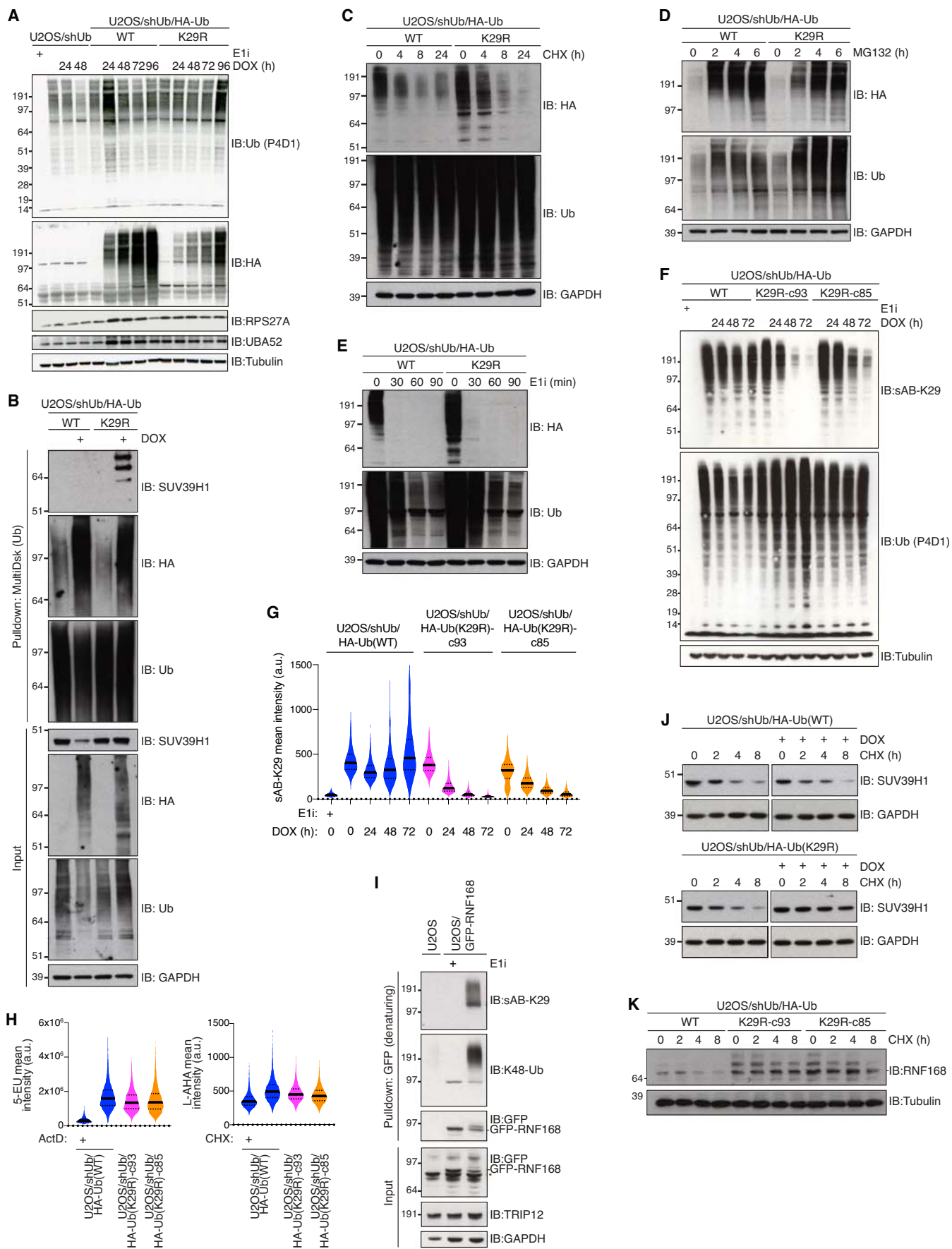


Figure EV4. Extended data related to Fig. 3. (A) Immunoblot analysis of U2OS/shUb, U2OS/shUb/HA-Ub(WT) and U2OS/shUb/HA-Ub(K29R)-c93 cell lines treated or not with DOX for the indicated times. Where indicated, cells were treated with Ub E1 inhibitor (E1i) for 1 h. (B) Immunoblot analysis of Ub conjugates isolated via MultiDsk pulldown from indicated Ub replacement cell lines treated or not with DOX. (C) Immunoblot analysis of DOX-treated Ub(WT) and Ub(K29R) cell lines treated or not with cycloheximide (CHX) for the indicated times. (D) As in (C), except that cells were treated with MG132 for the indicated times. (E) As in (C), except that cells were treated with MLN-7243 (E1i) for the indicated times. (F) As in (A), but using Ub(WT), Ub(K29R)-c93 and Ub(K29R)-c85 replacement cell lines. (G) QIBC analysis of Ub(WT) and Ub(K29R) replacement cell lines treated with DOX for the indicated times and immunostained with sAB-K29 (thick dashed line, median; dotted lines, quartiles). Data were a single representative replicate from three independent experiments (>1000 cells analyzed per sample). (H) QIBC analysis of DOX-treated Ub(WT) and Ub(K29R) replacement cell lines treated or not with the transcription inhibitor Actinomycin D (ActD) or the protein synthesis inhibitor cycloheximide (CHX), where indicated and stained for nascent RNA (upper) or nascent protein (lower panel) by detection of incorporated EU and AHA, respectively (thick lines, median; dotted lines, quartiles). Data were a single representative replicate from three independent experiments. (I) U2OS cells or a derivative cell line stably expressing GFP-RNF168 were treated or not with Ub E1i, subjected to GFP pulldown under denaturing conditions and immunoblotted with the indicated antibodies. (J) Immunoblot analysis of Ub(WT) and Ub(K29R) cell lines incubated or not with DOX for 72 h and treated with cycloheximide (CHX) for the indicated times. (K) Immunoblot analysis of DOX-treated Ub(WT) and Ub(K29R) cell lines treated or not with cycloheximide (CHX) for the indicated times. Source data are available online for this figure.

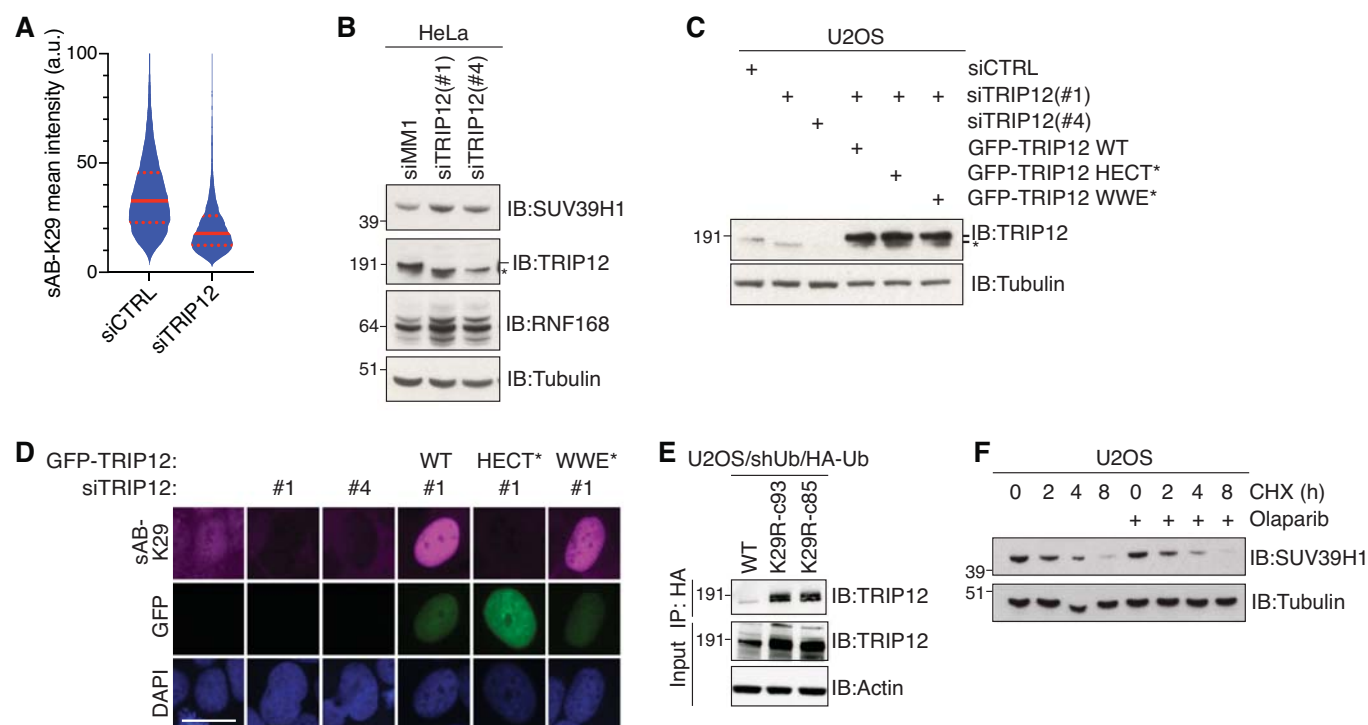


Figure EV5. Extended data related to Fig. 3.

(A) QIBC analysis of HeLa cells transfected with indicated siRNAs (thick line, median; dotted lines, quartiles). Data were a single representative replicate from three independent experiments (>1000 cells analyzed per sample). (B) Immunoblot of HeLa cells transfected with indicated siRNAs. (C) Immunoblot analysis of U2OS cells sequentially transfected with indicated siRNA and siRNA-resistant GFP-TRIP12 expression plasmids. (D) Representative images of individual cells from data shown in Fig. 3J. Scale bar, 25 μ m. (E) DOX-treated Ub(WT) and Ub(K29R) replacement cell lines were subjected to HA IP under denaturing conditions and immunoblotted with the indicated antibodies. (F) Immunoblot analysis of SUV39H1 in U2OS cells treated or not with cycloheximide (CHX) for the indicated times in the absence or presence of the PARP1 inhibitor Olaparib. Source data are available online for this figure.

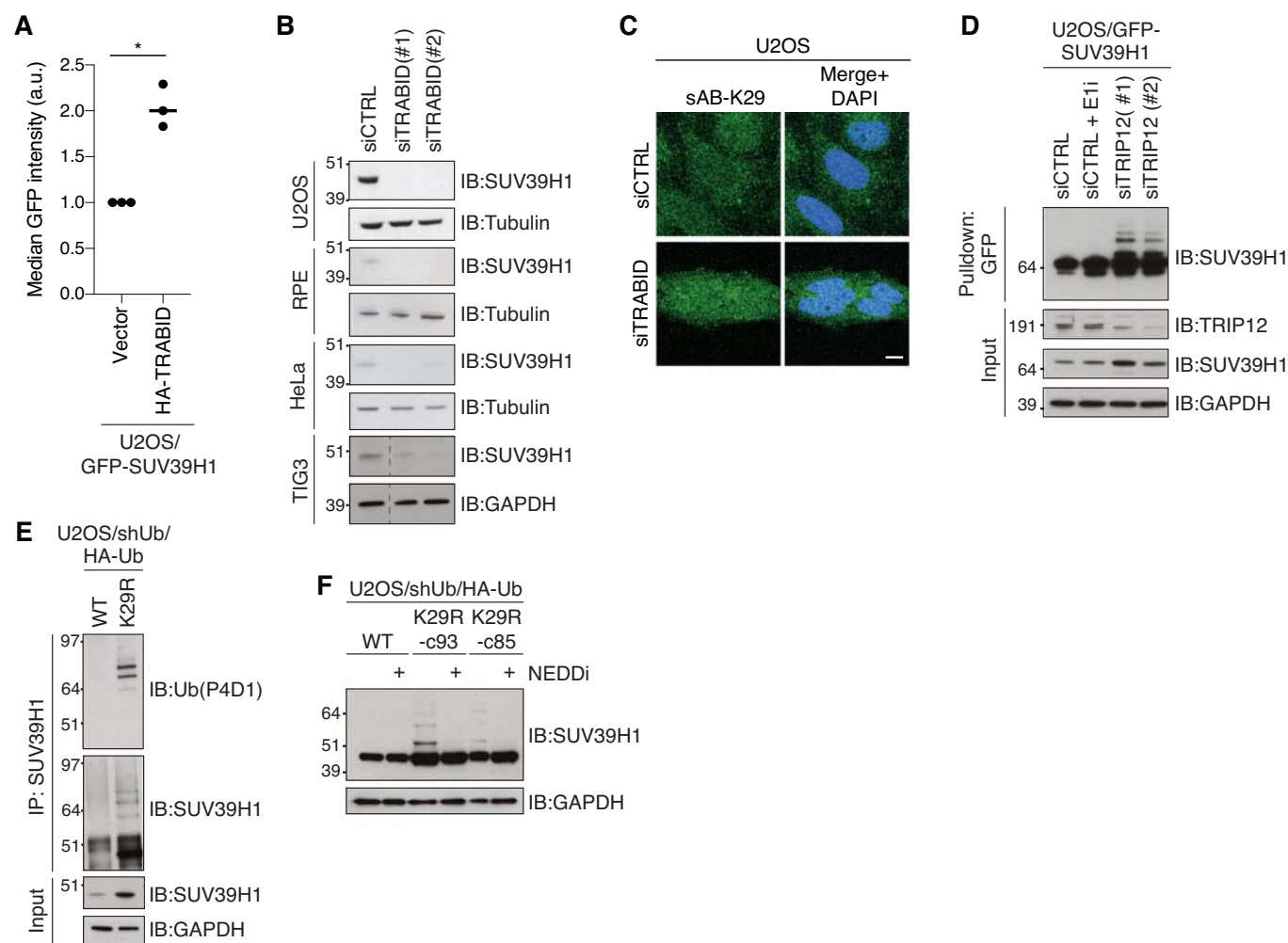
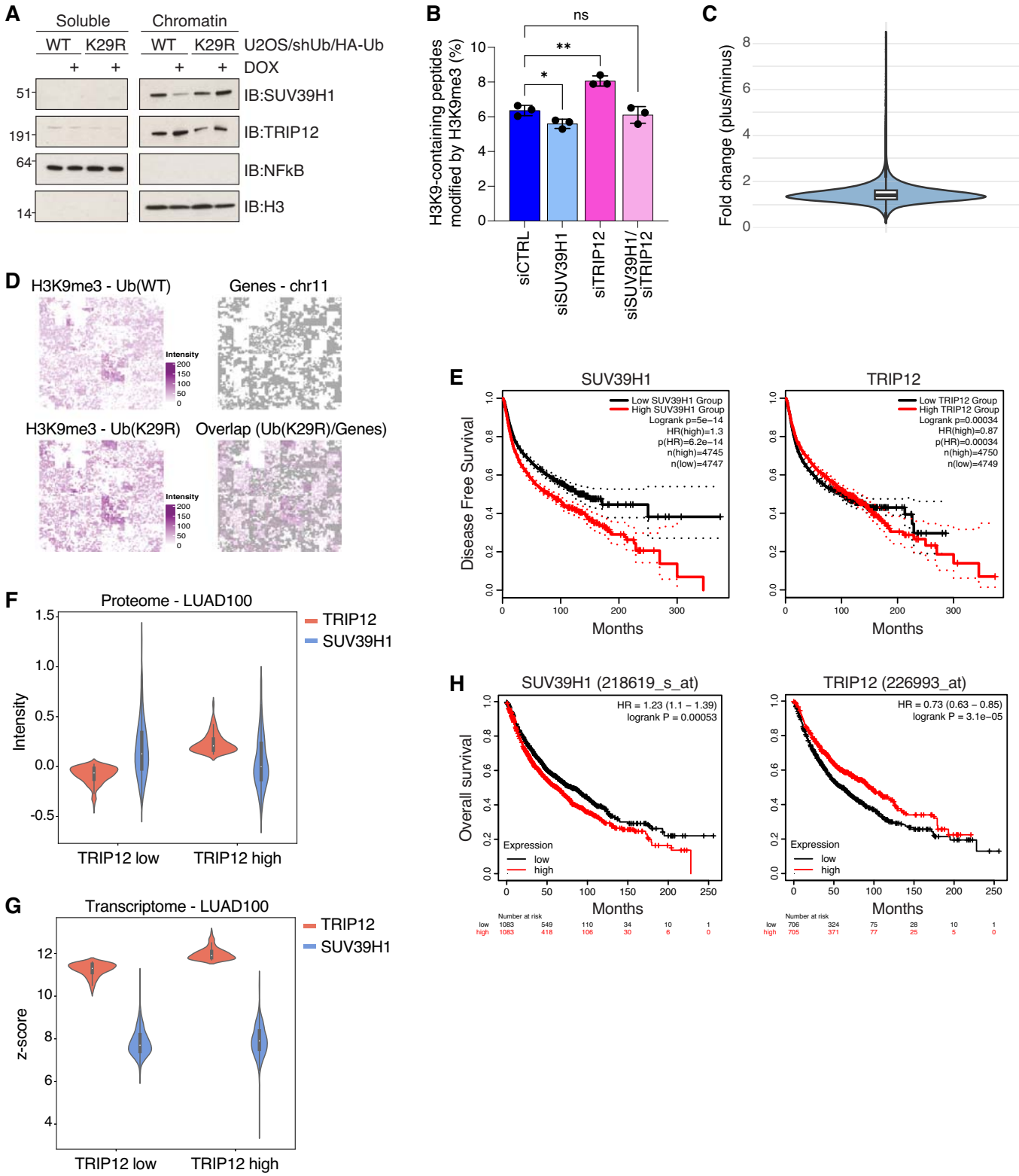


Figure EV6. Extended data related to Fig. 4.

(A) QIBC analysis of U2OS/GFP-SUV39H1 cells transfected or not with HA-TRABID expression construct. Data shown is fold change of mean of average nuclear GFP intensity relative to untransfected cells ($n = 3$ independent experiments; $*p < 0.05$, two-tailed t -test). (B) Immunoblot analysis of indicated cell lines transfected with non-targeting control (CTRL) or TRABID siRNAs. (C) Representative images of U2OS cells transfected with the indicated siRNAs and immunostained with sAB-K29. (D) U2OS/GFP-SUV39H1 cells transfected with siRNAs and treated with Ub E1 inhibitor, where indicated, were subjected to GFP pulldown under denaturing conditions and immunoblotted with indicated antibodies. (E) DOX-treated Ub(WT) and Ub(K29R) replacement cell lines were subjected to SUV39H1 IP and immunoblotted with indicated antibodies. (F) Immunoblot analysis of DOX-treated Ub(WT) and Ub(K29R) replacement cell lines treated or not with NEDDylation inhibitor for 4 h. Source data are available online for this figure.



◀ **Figure EV7. Extended data related to Fig. 5.**

(A) Immunoblot analysis of soluble and chromatin-enriched fractions of Ub(WT) and Ub(K29R) replacement cell lines treated or not with DOX. (B) Quantitative proteomic analysis of H3K9me3 abundance (H3K9me3 peptides unmodified at K14) in U2OS cells treated with indicated siRNAs ($n = 3$ biological replicates; $**p < 0.01$; $*p < 0.05$; ns non-significant, unpaired two-tailed t -test). (C) Violin plot showing genome-wide distribution of H3K9me3 fold change values per 10 kb genomic bin, calculated from spike-in normalized H3K9me3 ChIP-seq signal in Ub(K29R)-replaced cells (+DOX) relative to non-replaced Ub(K29R) cells (–DOX). Each point represents a non-overlapping genomic bin, and fold changes were computed as the ratio of normalized signal between the two conditions. (D) Hilbert analysis depicting the spatial localization of H3K9me3 across chromosome 11 in DOX-treated Ub(WT) and Ub(K29R) replacement cell lines. Overlap panel depicts the signal from Ub(K29R) cells over genes on chromosome 11. (E) Kaplan–Meier analysis of disease-free survival stratified by expression of SUV39H1 (left) or TRIP12 (right) in a pan-cancer cohort. Dotted lines represent a 95% confidence interval. Analysis was performed using GEPIA2 on 33 cancer types. Hypothesis evaluation was performed with the log-rank test. Hazard ratios were calculated according to the Cox proportional-hazards model. (F) LUAD-100 proteome levels of indicated proteins stratified by relatively high and low TRIP12-expressing samples. Data were shown as the log2 ratio of reporter ion intensity. (G) LUAD-100 transcriptome levels stratified by relatively high and low TRIP12-expressing samples. (H) Kaplan–Meier analysis of overall survival in non-small cell lung cancer (NSCLC) stratified on SUV39H1 (left) or TRIP12 (right) expression. Analysis was performed using the KMPlotter tool. Hazard ratios and p values were calculated through Cox regression. Source data are available online for this figure.