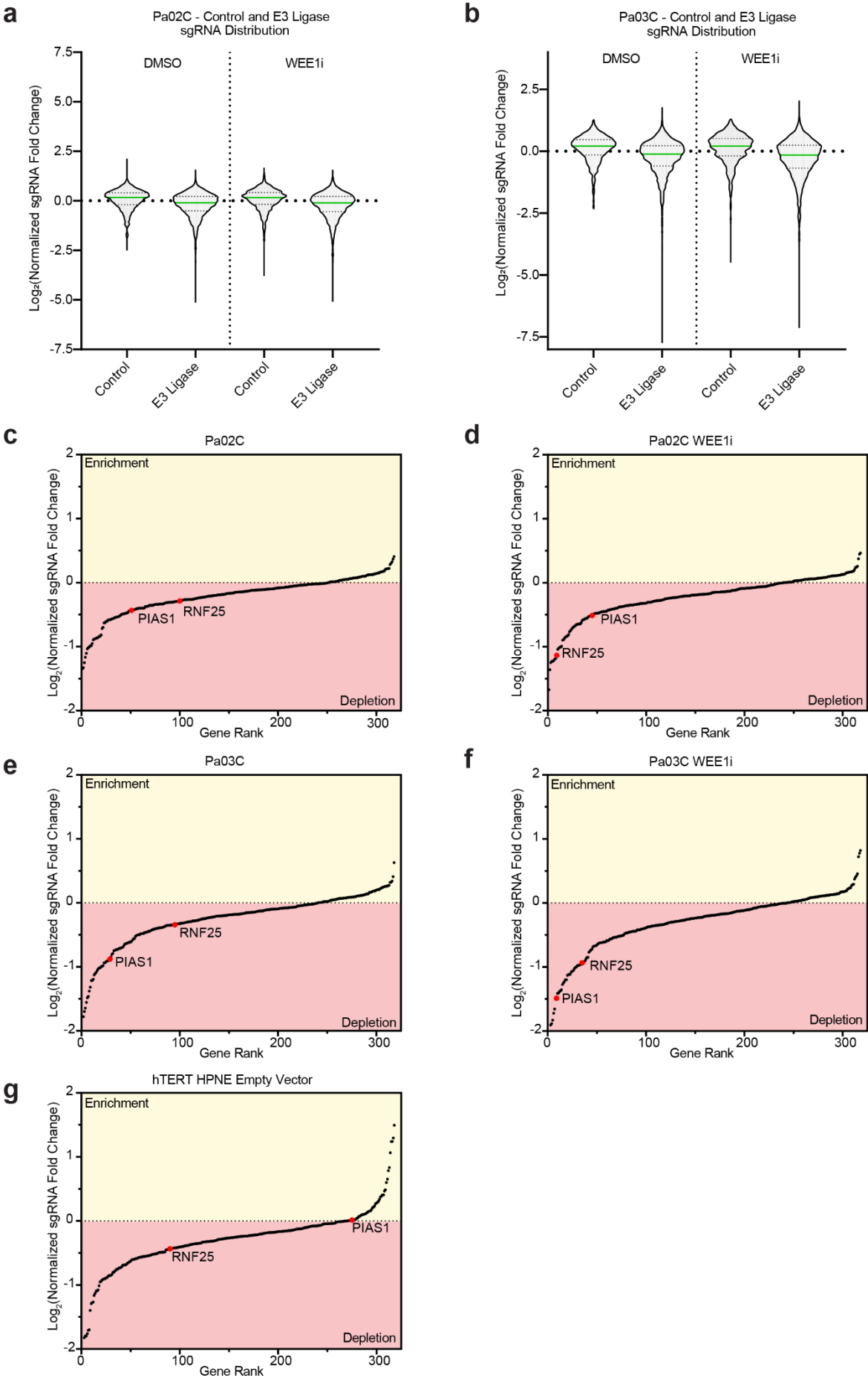


Supplementary Fig. 1



Supplementary Fig. 1: RNF25 and PIAS1 show increased gene ranks upon WEE1 inhibitor treatment in PDAC cell lines.

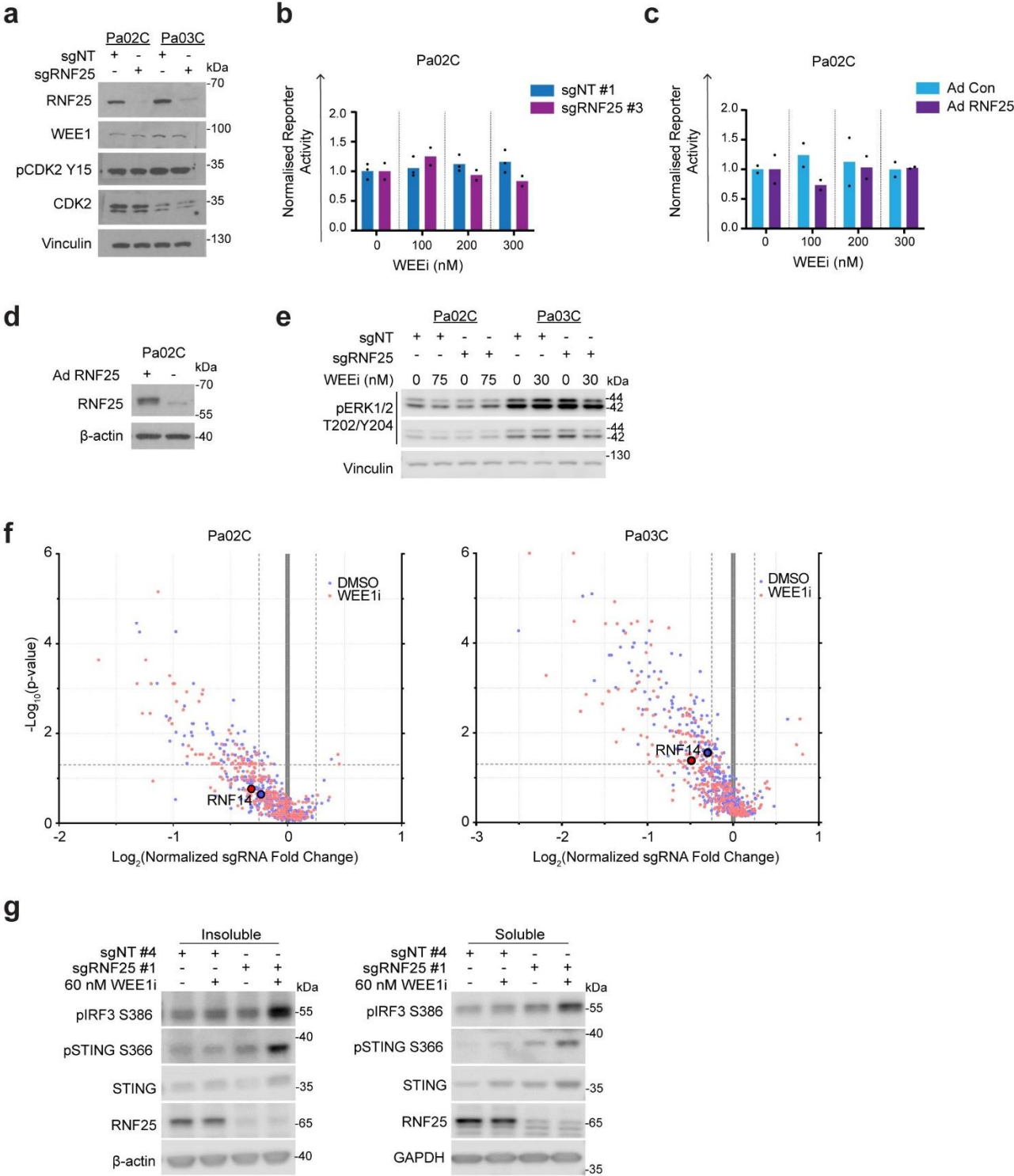
a, b Relative distribution of control non-targeting sgRNAs and RING-finger E3 ligase-directed sgRNAs in Pa02C and Pa03C cells after 20 population doublings $\pm$ WEE1 inhibitor treatment.

c, d, e, f Gene rank plots showing relative enrichment or depletion of RING-finger E3 ligase-directed sgRNAs in Pa02C and Pa03C cells after 20 population doublings $\pm$ WEE1 inhibitor treatment.

g Gene rank plot showing relative enrichment or depletion of RING-finger E3 ligase-directed sgRNAs after 20 population doublings in hTERT-immortalized normal human pancreatic epithelial cells (HPNE).

All data including sgRNA distribution violin and gene rank plots were gathered from at least n=3 biological replicates.

Supplementary Fig. 2



Supplementary Fig. 2: RNF25-dependent WEE1i-tolerance is unrelated to NF- κ B, ERK, or translation quality control.

a Immunoblot showing levels of WEE1, CDK2, and p-CDK2 in Pa02C and Pa03C sgNT and sgRNF25 cells.

b Effect of WEE1i on expression of NF κ B-responsive luciferase reporter activity in Pa02C sgNT and sgRNF25 cells.

c Effect of adenovirally-overexpressed HA-RNF25 on expression of NF κ B-responsive luciferase reporter activity in Pa02C cells.

d Immunoblots showing levels of RNF25 in HA-RNF25-overexpressing Pa02C cells.

e Immunoblot showing effect of WEE1i treatment on levels of active T202/Y204 dually-phosphorylated pERK in Pa02C and Pa03C sgNT and sgRNF25 cells after conditional treatment with WEE1i.

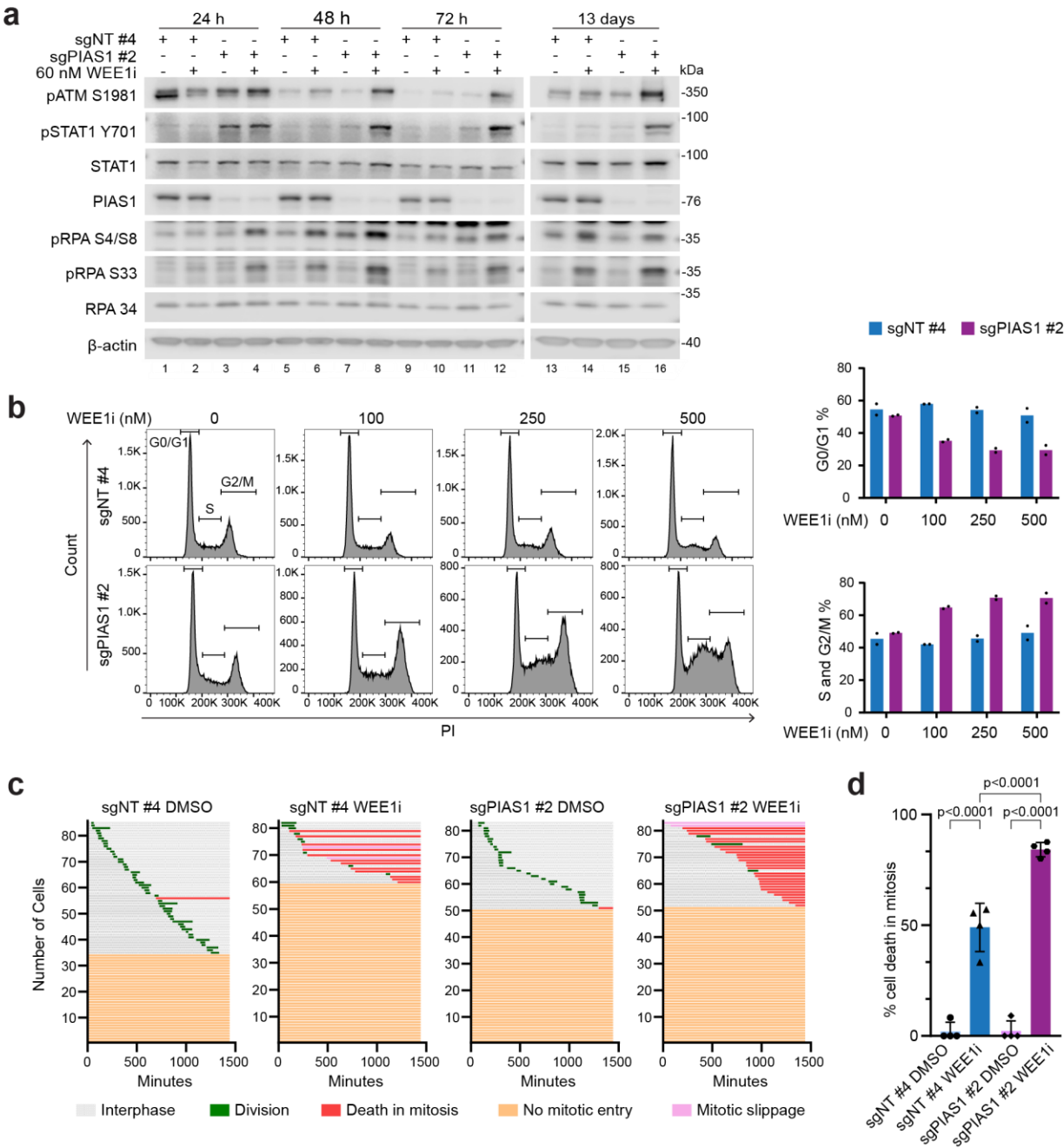
f Results of CRISPR screens showing dropout of RNF14-directed sgRNAs in relation to all other library sgRNAs in vehicle (DMSO)- and WEE1i-treated Pa02C and Pa03C cells. Log₂-transformed sgRNA abundance scores and statistical significance were calculated using Völundr. The two-sided two sample Kolmogorov–Smirnov test was used to calculate statistical significance from n=3 biological replicates.

g Immunoblot showing effect of WEE1i treatment on the indicated protein markers of the cGAS-STING pathway in Pa03C sgNT and sgRNF25 cells.

All reporter activity data (b, c) represent means from duplicate dishes and are representative of n=2 biological replicates.

Source data are provided as a Source Data file.

Supplementary Fig. 3



Supplementary Fig. 3: PIAS1 promotes S-phase and G2/M progression in WEE1i-treated PDAC cells.

a Immunoblot showing effect of WEE1i treatment on levels of the indicated DNA damage markers in Pa03C sgNT and sgPIAS1 cells at the indicated timepoints.

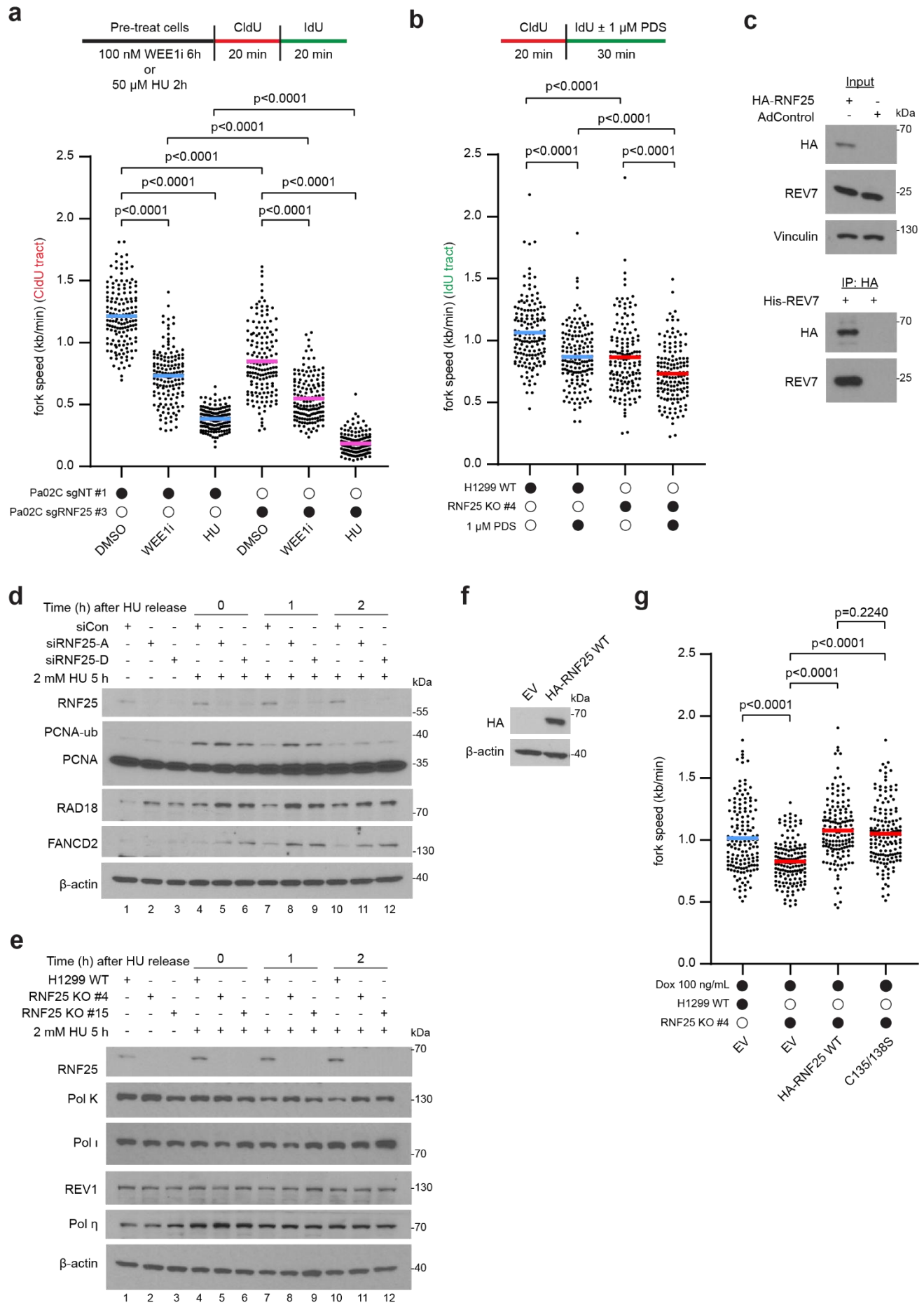
b Flow cytometry analyses showing effect of WEE1i treatment on cell cycle profiles of Pa03C sgNT and sgPIAS1 cells. The cell cycle analyses for the sgNT cells shown here are the same profiles shown for sgNT cells in Fig. 2b. These Pa03C cells were transduced with non-targeting sgRNAs and are wild-type for both RNF25 and PIAS1. All the cell cycle analyses shown in Fig. 2b and Supplementary Fig. 3b were performed at the same time. These data represent means from duplicate dishes and are representative of n=2 biological replicates.

c Cell fate maps showing effect of WEE1i-treatment on interphase and mitotic progression of Pa03C sgNT and sgPIAS1 cells, as determined using live cell imaging experiments.

d Quantification of results from (c) showing effect of WEE1i-treatment on mitotic cell death in Pa03C sgNT and sgPIAS1 cells. All data represent mean $\pm$ SD from n=4 separate cell populations. Ordinary one-way ANOVA with Tukey's multiple comparisons test was performed to analyze statistical significance.

Source data are provided as a Source Data file.

Supplementary Fig. 4



Supplementary Fig. 4: RNF25 promotes fork movement in response to replication stress

a Pa02C sgNT and sgRNF25 cells were treated as indicated before conducting the DNA fiber fork movement assay. CldU was added to cells after drug treatments without washing.

b H1299 WT and RNF25 KO cells were used in the DNA fiber fork movement assay, with PDS added during the second pulse.

c HA-RNF25 WT was expressed in H1299 WT cells using adenoviral vectors. 1 µg of purified His-REV7 was added to whole cell lysates before immunoprecipitation with anti-HA beads.

d H1299 WT cells were transfected with control siRNA and two independent siRNAs targeting RNF25 for 48 h before being treated with 2 mM HU for 5 h, then washed and nuclease-released chromatin fraction timepoints taken at 0, 1, and 2 h after release from HU for analysis by immunoblotting.

e H1299 WT and two independent RNF25 KO clones were treated as described in (d).

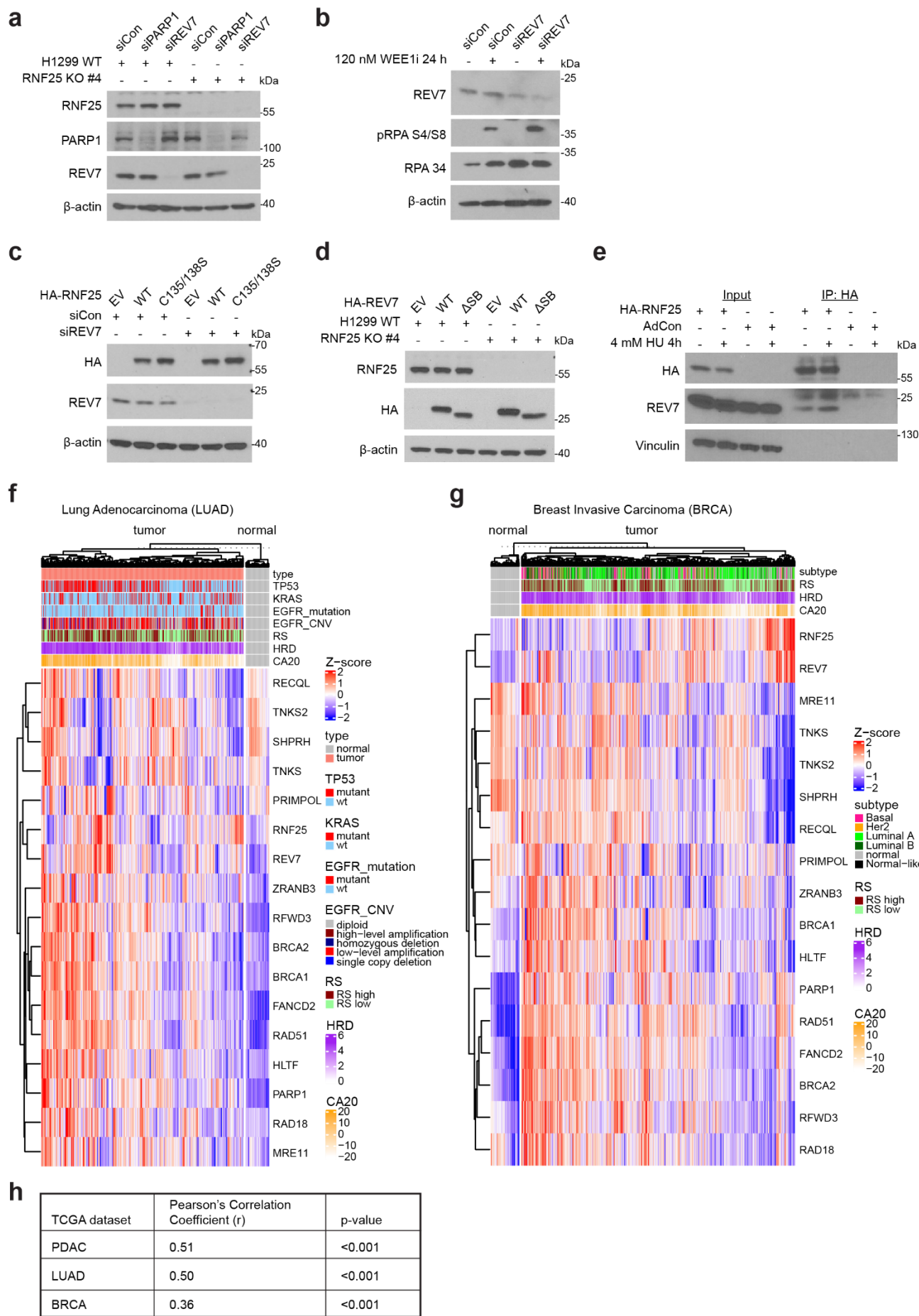
f Immunoblots showing validation of ectopic HA-RNF25 expression in the SIRF assay shown in Fig. 4e.

g Fork movement DNA fiber assay in H1299 RNF25 KO cells expressing RNF25 WT and RNF25 C135/138S.

For DNA fiber assays in (a), (b), (g), a total of 150 fibers were measured per condition and statistics analyzed using a two-tailed Mann-Whitney test. Means are indicated with bars. Results in these figures are representative of n=2 biological replicates.

Source data are provided as a Source Data file.

Supplementary Fig. 5



Supplementary Fig. 5: RNF25 and REV7 expression are significantly correlated.

a Immunoblots showing validation of REV7 and PARP1 knockdown in the DNA fiber assay shown in Fig. 6a, b.

b Pa03C sgNT cells were transfected with indicated siRNAs before treatment with WEE1i and immunoblotted with antibodies indicated.

c Immunoblots validating ectopic expression of HA-RNF25 WT and HA-RNF25 C135/138S, and knockdown of REV7 in the SIRF assay shown in Fig. 6d.

d Immunoblots validating ectopic expression HA-REV7 WT and HA-REV7 Δ SB mutant in the SIRF assay shown in Fig. 6e.

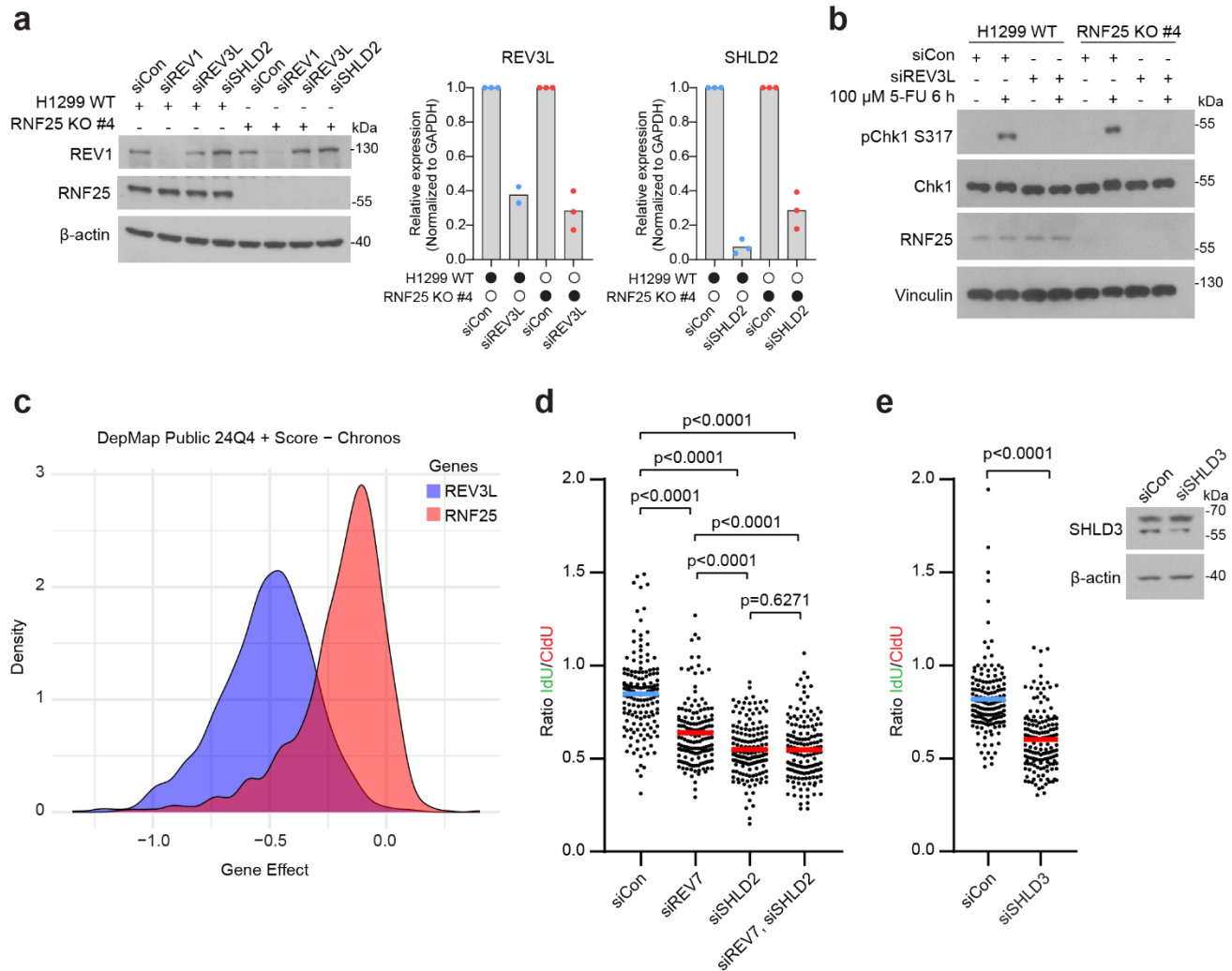
e HA-RNF25 WT was expressed in H1299 WT cells using adenoviral vectors before being treated with HU and immunoprecipitated with anti-HA beads.

f, g Heatmaps depicting relative mRNA expression of DNA replication and DNA damage repair factors in LUAD (f) and BRCA (g) patient tumor samples from TCGA. Supervised clustering was performed to separate normal and tumor samples. The heatmap in panel (f) shows mutation status of genes that are commonly altered in LUAD. Other classifiers shown include tumor subtype (shown for BRCA), and genome maintenance related features (RS – Replication Stress signature, HRD – Homologous Recombination Deficiency score, CA20 – Centrosome Amplification signature).

h Results of Pearson's Correlation Coefficient test for RNF25 and REV7 expression in the indicated TCGA datasets. The coefficient r value and p -value are indicated.

Source data are provided as a Source Data file.

Supplementary Fig. 6



Supplementary Fig. 6: Polζ has functions independent of RNF25

a Immunoblot validating knockdown of REV1 protein (left panel) and results of qPCR analysis validating knockdown of REV3L and SHLD2 mRNAs in the DNA fiber assay shown in Fig. 7a, 7b.

b H1299 WT and RNF25 KO cells were transfected with siREV3L for 48 h, then treated with 5-FU before being collected for immunoblot.

c Density plot of CRISPR gene effect scores for RNF25 and REV3L in 1178 cell lines (DepMap 24Q4 - Chronos).

d, e H1299 WT cells were transfected with indicated siRNAs for 48 h before conducting the DNA fiber fork degradation assay. A total of 150 fibers were measured per condition and statistics analyzed using a two-tailed Mann-Whitney test. Means are indicated with bars. These figures are a representative experiment from n=2 biological replicates.

Source data are provided as a Source Data file.

Supplementary Table 1

Gene	Log2(normalized sgRNA fold change)	Corrected p-value	–Log10 p-value (K-S test)
UBE2I	-1.34	5.30E-05	4.276
TRAIP	-1.33	3.50E-04	3.456
RNF113A	-1.25	2.32E-03	2.634
TRIM28	-1.17	6.54E-04	3.184
RBBP6	-1.10	3.58E-03	2.447
MSL2	-1.03	3.00E-03	2.522
NSMCE1	-1.02	8.88E-03	2.051
RANBP2	-1.01	1.70E-02	1.769
COP1	-0.99	2.20E-05	4.658
TRIM61	-0.99	3.19E-01	0.496
RNF4	-0.95	5.76E-03	2.240
TRIM64B	-0.89	1.67E-03	2.779
RNF168	-0.88	2.46E-02	1.608
BARD1	-0.88	5.76E-03	2.240
PCGF7P	-0.87	4.43E-02	1.353
NSMCE2	-0.86	6.54E-04	3.184
RCHY1	-0.86	1.40E-02	1.853
MARCH6	-0.84	5.76E-03	2.240
TRAF2	-0.84	5.76E-03	2.240
RNF8	-0.82	5.76E-03	2.240

Supplementary Table 2

Gene	Log2(normalized sgRNA fold change)	Corrected p-value	–Log10 p-value (K-S test)
UBE2I	-1.67	2.77E-04	3.558
NSMCE1	-1.36	2.77E-04	3.558
MSL2	-1.25	2.77E-04	3.558
TRAIP	-1.23	5.97E-04	3.224
TRIM28	-1.23	9.41E-04	3.026
RNF8	-1.22	4.38E-03	2.359
RNF113A	-1.19	9.41E-04	3.026
TRIM61	-1.18	1.66E-01	0.780
RNF25	-1.13	3.00E-06	5.523
NSMCE2	-1.04	7.75E-04	3.111
RNF4	-1.02	5.97E-04	3.224
TRIM49	-1.01	1.59E-02	1.798
COP1	-1.00	3.33E-04	3.478
RNF168	-0.99	7.75E-04	3.111
MARCH6	-0.90	9.41E-04	3.026
RANBP2	-0.89	5.70E-03	2.244
TRIM64B	-0.87	3.35E-02	1.475
RBBP6	-0.85	2.04E-02	1.691
ANAPC11	-0.84	2.40E-02	1.619
TRAF2	-0.83	1.69E-02	1.772

Supplementary Table 3

Gene	Log2(normalized sgRNA fold change)	Corrected p-value	–Log10 p-value (K-S test)
UBE2I	-2.53	6.70E-05	4.174
TRIM28	-1.78	1.00E-05	5.000
RNF4	-1.70	3.00E-06	5.523
MIB1	-1.64	5.19E-04	3.285
RNF113A	-1.56	8.60E-05	4.066
NSMCE1	-1.48	1.81E-04	3.742
NSMCE2	-1.46	2.59E-04	3.587
TRIM61	-1.38	1.60E-03	2.796
BARD1	-1.32	3.60E-05	4.444
RNF216	-1.21	2.60E-04	3.585
BRCA1	-1.21	5.19E-04	3.285
TRAIP	-1.19	1.95E-04	3.710
TRAF7	-1.13	7.50E-05	4.125
RNF8	-1.10	7.93E-04	3.101
ANAPC11	-1.08	8.45E-03	2.073
TRIM11	-1.06	3.61E-03	2.443
SENP6	-1.03	2.31E-03	2.637
ZNRF2	-1.02	7.06E-04	3.151
MNAT1	-1.02	3.64E-04	3.439
MARCH6	-1.00	3.64E-04	3.439

Supplementary Table 4

Gene	Log2(normalized sgRNA fold change)	Corrected p-value	–Log10 p-value (K-S test)
UBE2I	-2.40	0.00E+00	6.000
MIB1	-2.21	3.14E-04	3.503
NSMCE1	-1.91	2.00E-06	5.699
TRIM28	-1.88	4.40E-05	4.357
NSMCE2	-1.83	3.66E-03	2.437
TRIM11	-1.72	2.00E-03	2.700
RNF113A	-1.66	3.97E-04	3.401
ANAPC11	-1.50	5.57E-03	2.254
PIAS1	-1.49	4.40E-05	4.357
TRAF7	-1.41	1.49E-03	2.827
RNF4	-1.41	4.30E-05	4.367
RNF216	-1.38	1.90E-05	4.721
TRIM61	-1.37	9.25E-03	2.034
RNF8	-1.35	1.29E-03	2.890
MARCH6	-1.27	5.90E-05	4.229
SENP5	-1.25	1.29E-02	1.890
TRAIP	-1.23	3.14E-04	3.503
RNF126	-1.20	1.29E-03	2.890
COP1	-1.20	6.81E-04	3.167
BARD1	-1.13	3.14E-04	3.503

Supplementary Table 1-4: Top 20 depleted RING-finger E3 ubiquitin/SUMO ligase sgRNAs in Pa02C and Pa03C CRISPR Cas9 screens.

CRISPR screen results showing relative sgRNA depletion or enrichment in Pa02C/Pa03C control (Supplementary Table 1, 3) and WEE1i-treated groups (Supplementary Table 2, 4). The two-sided two sample

Kolmogorov–Smirnov test statistic and its corrected p-value are shown compared to the distribution of 1,000 control sgRNAs. These data were gathered from $n=3$ biological replicates.