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Supplemental Information

A PRMT5-RNF168-SMURF2 Axis Controls H2AX Proteostasis

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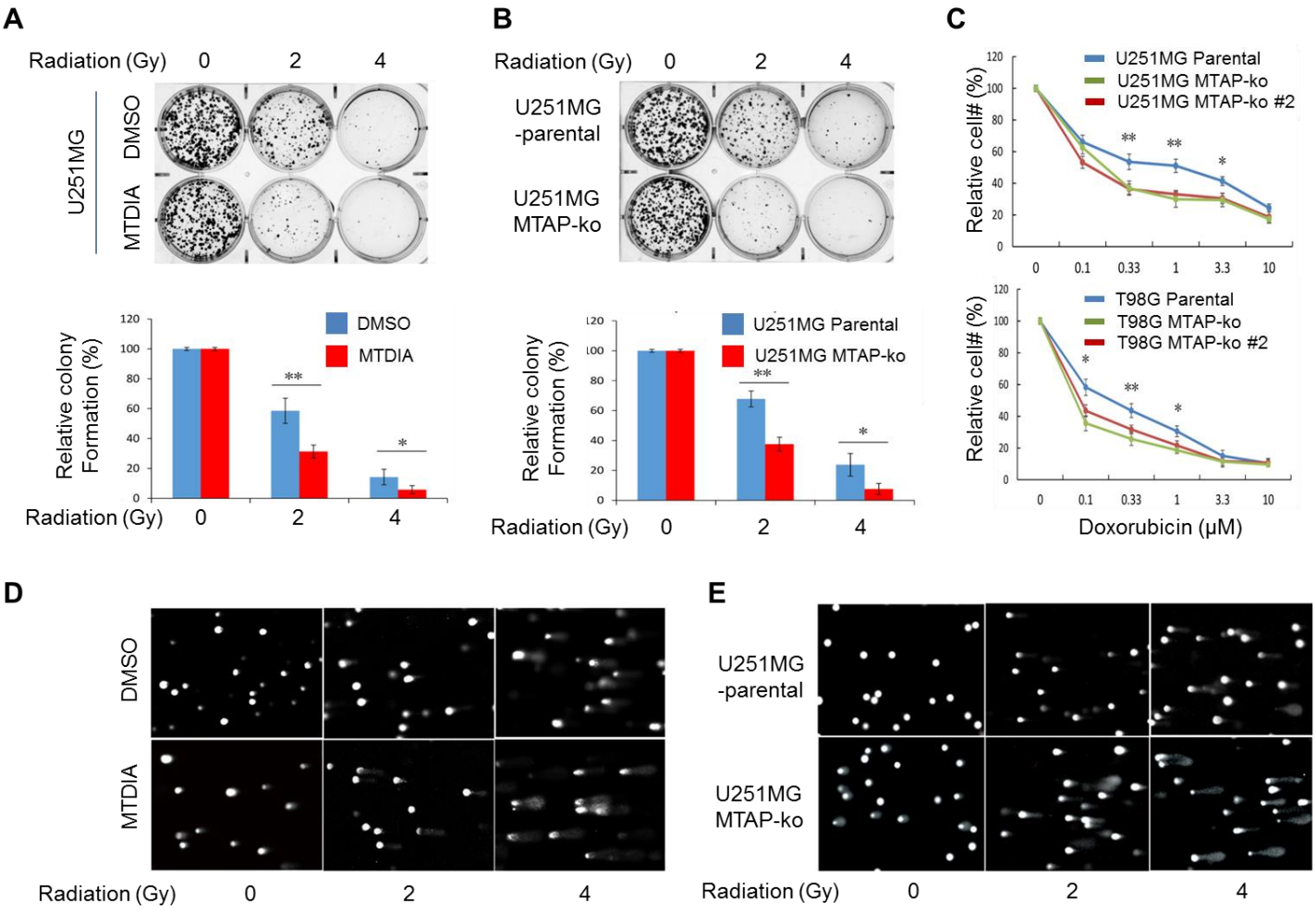

Figure S1: Related to Figure 1. Loss of MTAP function compromises GBM cells' response to DNA damage. (A) Colony formation assay for U251MG cells pre-treated with vehicle control or with MTDIA (3 μM, 24 hours) and then exposed to radiation (quantification of relative colony formation is shown in the bottom panel). **(B)** Colony formation assay for the parental U251MG cell line and its MTAP-ko derivative line (quantification of relative colony formation is shown in the bottom panel). **(C)** GBM cell lines (U251MG and T98G) and their matched MTAP-ko derivative lines (two derivatives for each parental cell line) were treated with Doxorubicin for three days, and relative numbers of viable cells were quantified by CCK-8 assay. **(D)** Images of alkaline comet assays for U251MG cell line treated with DMSO or with MTDIA for 24 hours and then exposed to radiation (quantification is shown in Fig. 1B). **(E)** Images of alkaline comet assays for parental U251MG and its MTAP-ko derivative line exposed to radiation (quantification is shown in Fig. 1C). Cells were allowed to recover for six hours after irradiation before alkaline comet assays were performed. *Student's t*-test was used in figure A and B; and ANOVA was used in figure C; * denotes $p<0.05$, and ** denotes $p<0.01$.

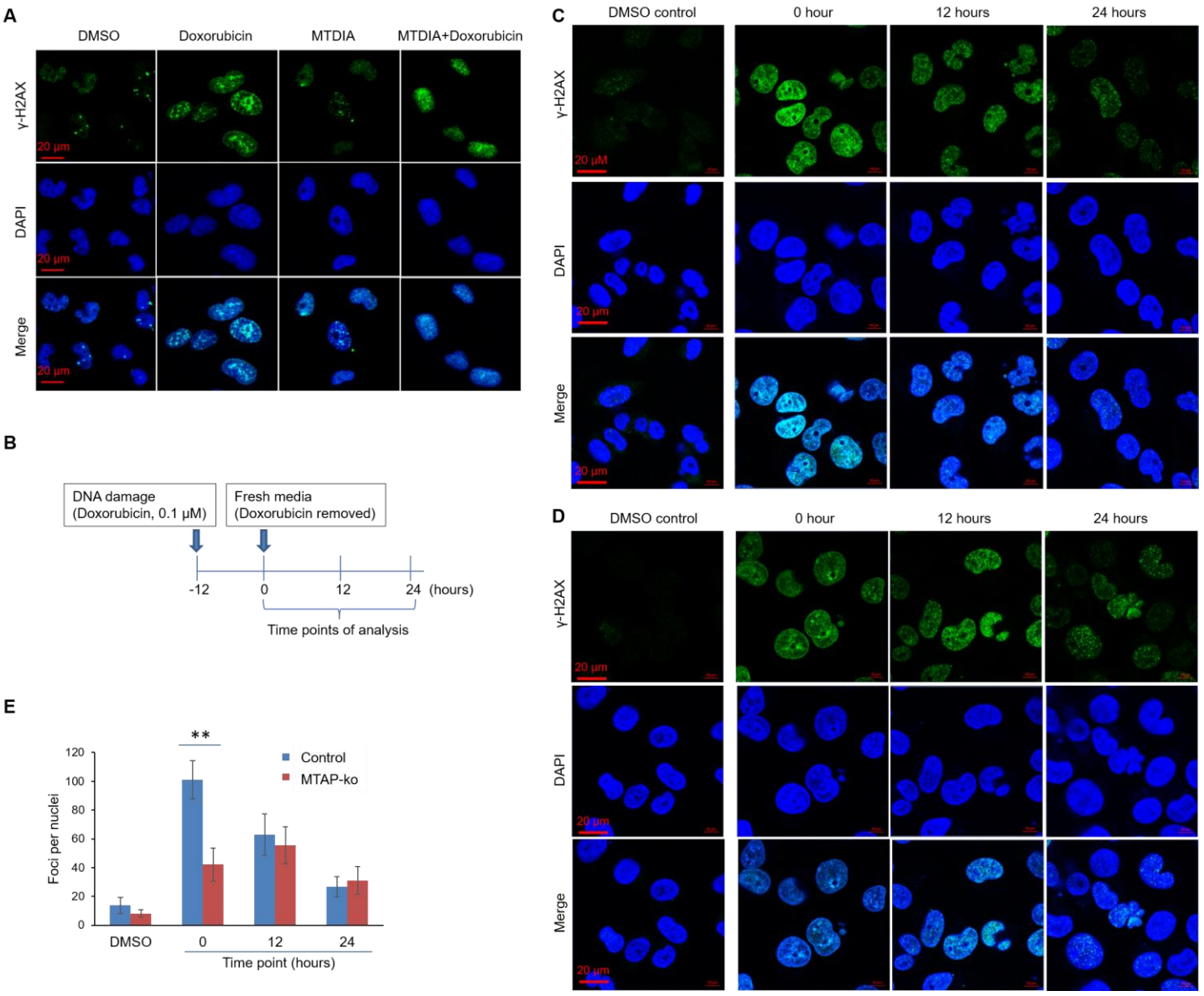

Figure S2: Related to Figure 1. Distinct γH2AX induction in MTAP-intact and MTAP-deficient cells in response to DNA damage stimulation. (A) U251MG cells, pre-treated with vehicle control (DMSO) or with MTDIA for 24 hours, were exposed to vehicle control or Doxorubicin (0.1 μM, 24 hours), and anti-γH2AX immunofluorescent staining was performed. **(B)** Schematic of the Doxorubicin treatment time course experiments in U251MG cells. **(C)** U251MG cell line (CRISPR control) and **(D)** its derivative, MTAP-ko line were treated as depicted in (B) and anti-γH2AX IF staining was performed. **(E)** Quantification of foci induction in (C) and (D), as assayed by ImageJ. **p<0.01.

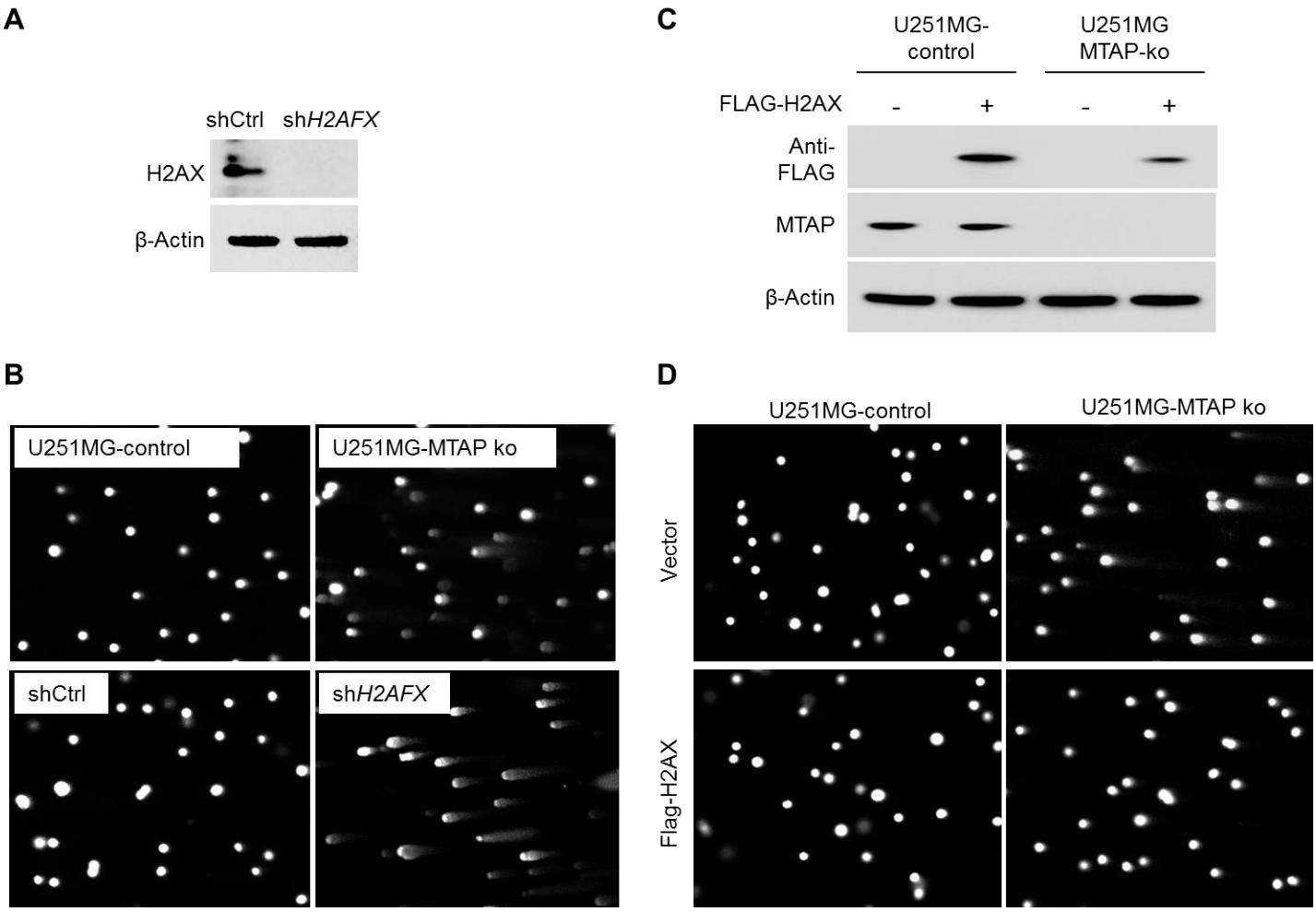

Figure S3: Related to Figure 2. Loss of H2AX led to higher levels of DSBs and restoration of H2AX in MTAP-deficient GBM cells mitigates MTAP loss-induced DSBs. (A) U251MG cell line, knockdown control (shCtrl) or *H2AFX* knockdown (sh*H2AFX*), were used for immunoblot to confirm the successful knockdown. **(B)** The same pair of cell lines, together with the U251MG-CRISPR control and the matched *MTAP*-ko lines, were used for alkaline comet assay. **(C)** U251MG cell lines (CRISPR control or MTAP-ko), without or with overexpression of exogenous H2AX, were used for immunoblot. **(D)** The same set of four cell lines were used for alkaline comet assay.

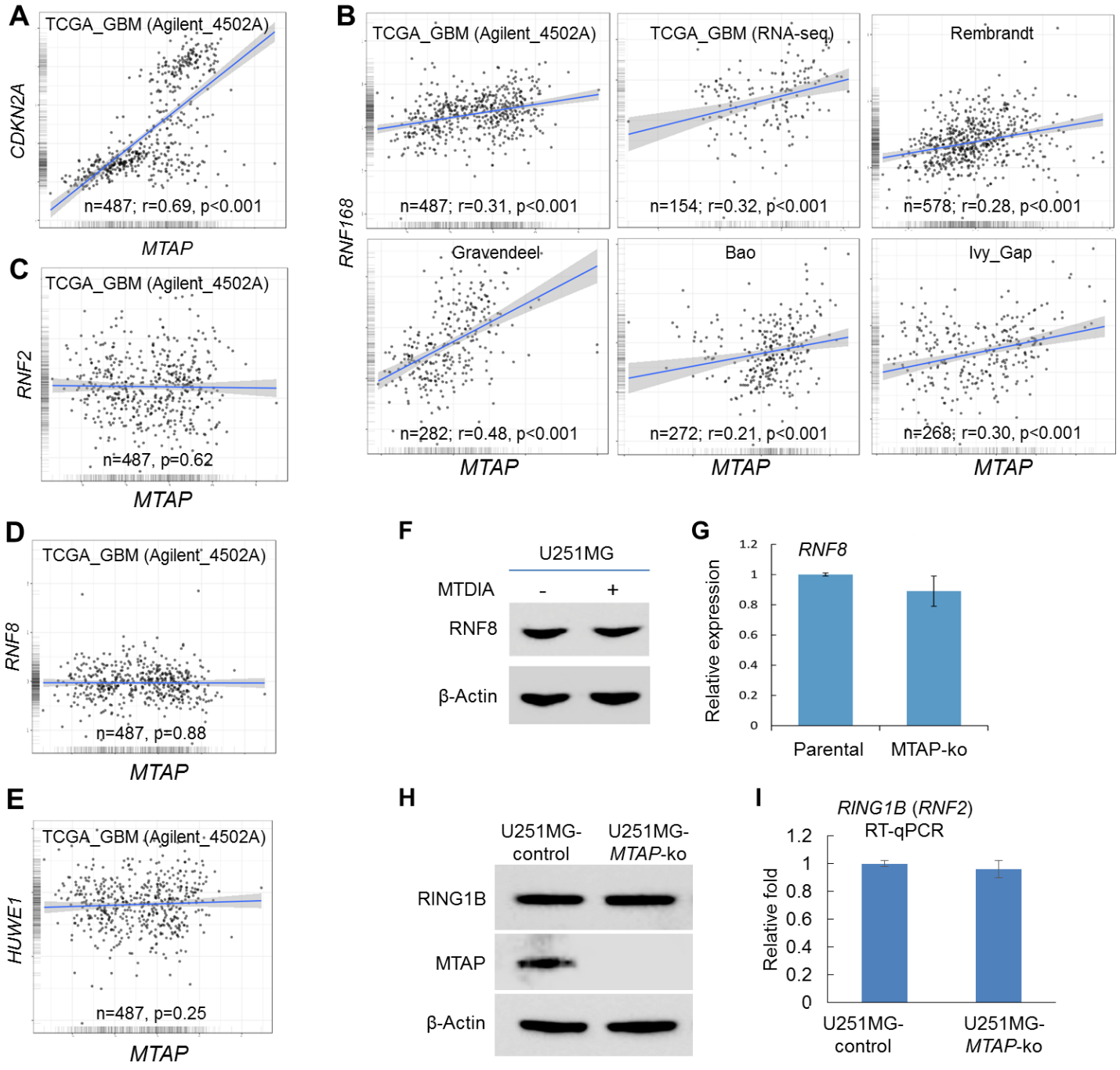

Figure S4: Related to Figure 3. *MTAP* expression is positively correlated with *RNF168* expression in GBM and in glioma samples, but loss of MTAP function has no effect on the expression of *RNF8* and *RNF2*. Gene expression data analysis was performed via the GlioVis data visualization tool web application, including the expression of **(A)** *CDKN2A*, **(B)** *RNF168*, **(C)** *RNF2*, **(D)** *RNF8*, and **(E)** *HUWE1*. For each analysis, dataset used, number of samples (n), Pearson's r value, and *p* value were denoted. **(F)** Immunoblot detection of RNF8 in U251MG cells treated with vehicle control or with MTDIA (3 μM, 24 hours). **(G)** RT-qPCR analysis of the *RNF8* transcript in the parental U251MG cell line or its MTAP-ko derivative line. **(H)** Isogenic pair of U251MG (CRISPR control) and U251MG-MTAP-ko cells were used for immunoblots using indicated antibodies. **(I)** The same pair of isogenic cell lines were used for RT-qPCR analysis of *RNF2* (*RING1B*) gene expression.

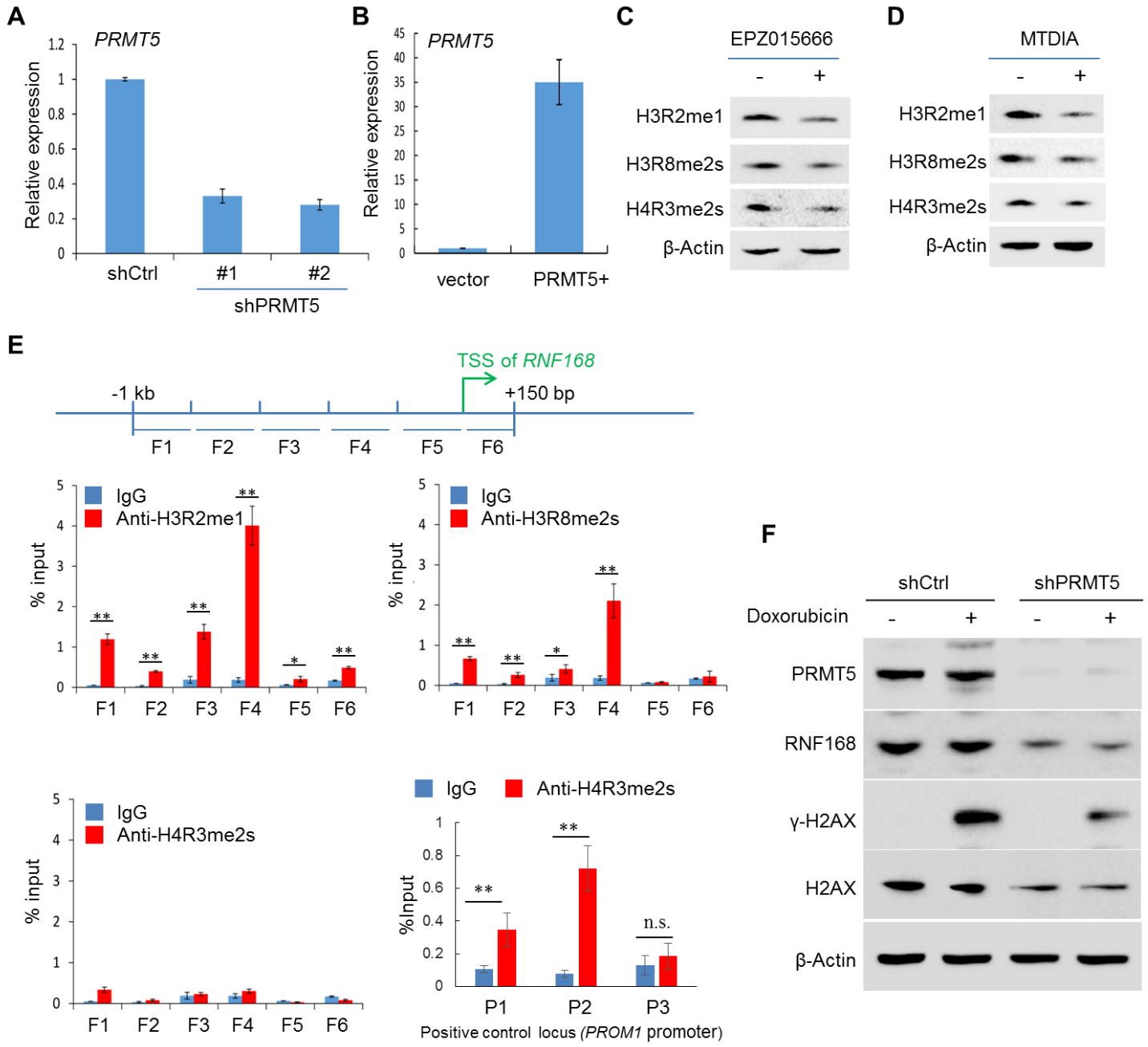

Figure S5: Related to Figure 4. PRMT5 maintains the transcription of *RNF168*. **(A)** RT-qPCR analysis of the *PRMT5* transcript level in the parental U251MG cell line and its derivative lines with knockdown of *PRMT5*. **(B)** RT-qPCR analysis of the *PRMT5* transcript level in the parental U251MG cell line and its derivative exogenous *PRMT5*-expressing line (PRMT5+). **(C-D)** U251MG cells treated with vehicle control, with 0.3 μM EPZ015666 (c), or with 3 μM MTDIA (d) for 24 hours; immunoblot was performed to determine histone methylation marks attributed to PRMT5 activity. **(E)** ChIP-qPCR analysis was performed to detect the indicated histone methylation marks, including transcription activation mark H3R2me1 and H3R8me2s, and transcription suppression mark H4R3me2s, in the promoter region of *RNF168* (note the slight variations in ChIP enrichments likely were due to different

control cell lines and antibody doses used). The *PROM1* promoter region was used as a positive control (as identified by our unpublished study) for anti-H4R3me2s ChIP. **(F)** U251MG cell line, knockdown control (shCtrl) or with *PRMT5* knockdown were treated with Doxorubicin (0.1 μM, 24 hours) and immunoblots with indicated antibodies were performed. Note no increased H2AX level was observed in response to Doxorubicin, and the *PRMT5* knockdown-induced reduction in H2AX levels in both untreated and treated cells.

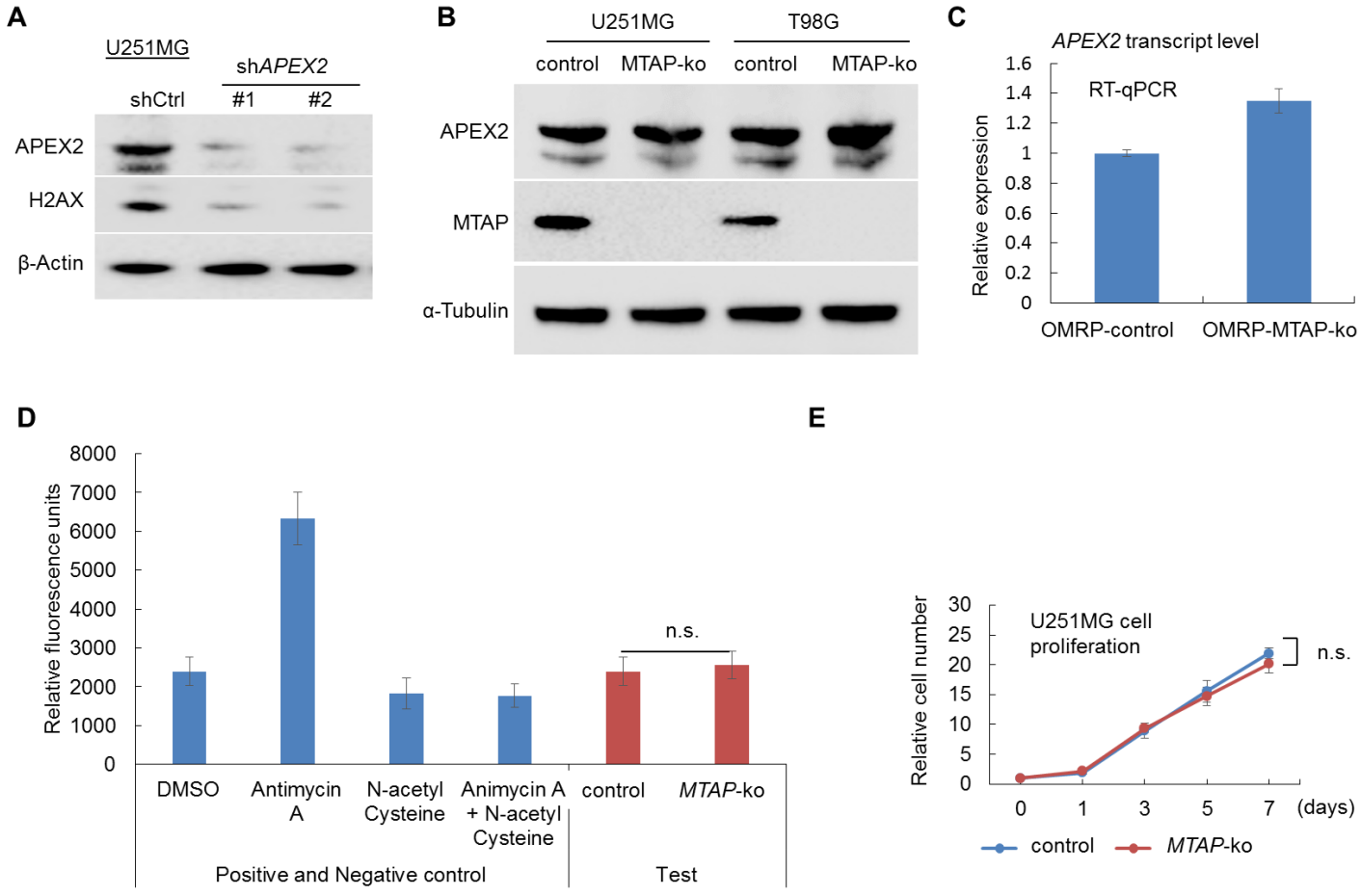

Figure S6: Related to Figure 5. MTAP loss does not affect the expression of APEX2, another protein that can regulate H2AX abundance, and does not affect the ROS level in and the proliferation of GBM cells in vitro. (A) U251MG cell lines without or with *APEX2* knockdown were used for immunoblots. **(B)** Isogenic U251MG and T98G cell lines (CRISPR control or MTAP-ko) were used for immunoblots. **(C)** OMRP cell line (CRISPR control) and its MTAP-ko derivative were used for RT-qPCR analysis of *APEX2* expression. **(D)** U251MG cell lines, CRISPR control or *MTAP*-ko were used for ROS measurement. Parental U251MG cells treated with indicated agents were used as positive and negative controls for the ROS assay. **(E)** Proliferation of the isogenic pair of U251MG cell lines, CRISPR control or *MTAP*-ko, was measured by CCK8 assay. n.s.: no significance.

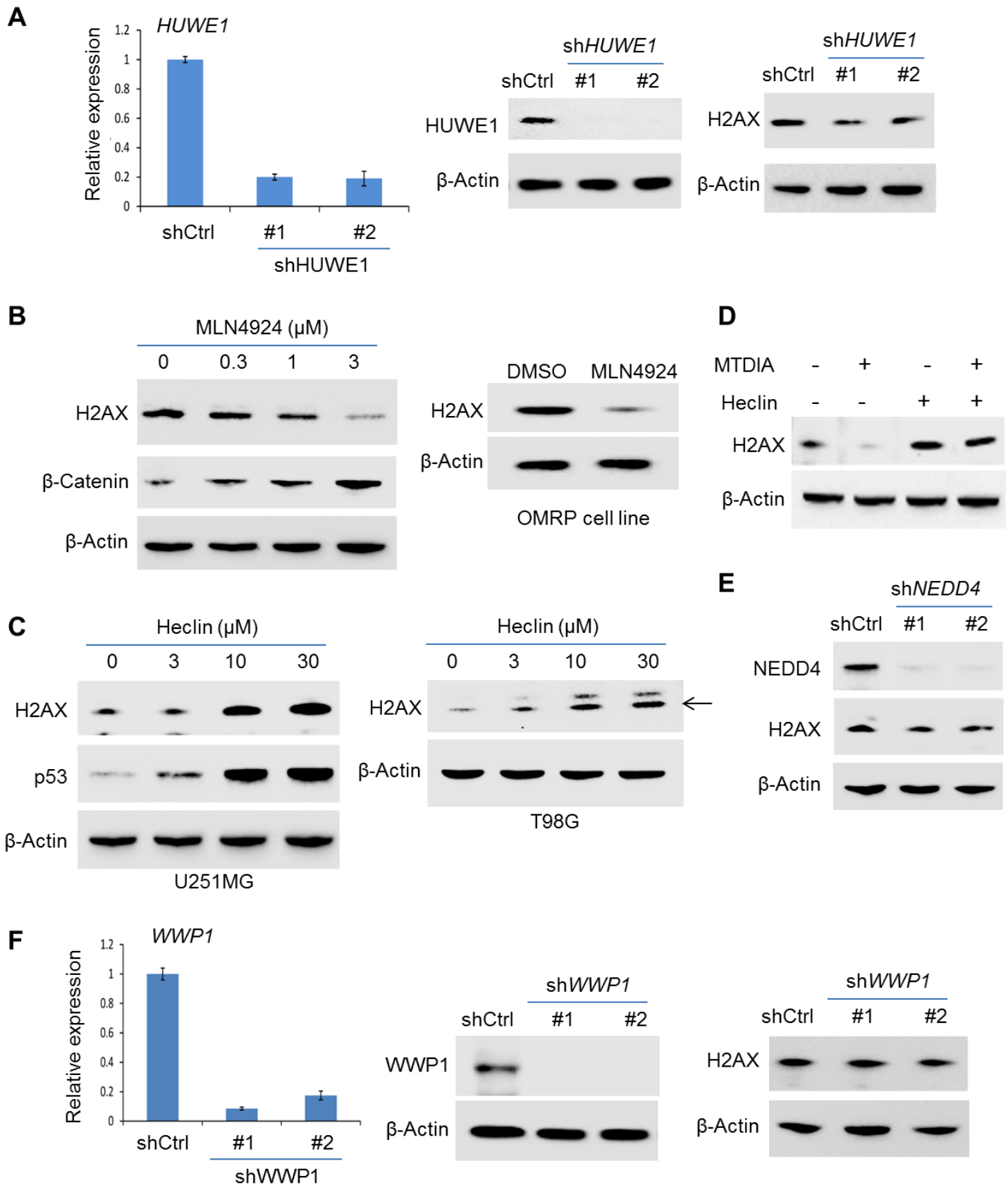

Figure S7: Related to Figure 6. Determining the effects of HECT-type E3 ligases on H2AX abundance. (A) RT-qPCR analysis of the *HUWE1* transcript level in the parental U251MG cell line and its derivative lines with knockdown of *HUWE1* (left panel); and immunoblot analysis of HUWE1

and H2AX in U251MG cell line and its derivative line with knockdown of *HUWE1* (right panel). **(B)** U251MG cells were treated with MLN4924 for 24 hours before immunoblot was performed to measure the abundance of H2AX (β-catenin served as a control to validate the expected MLN4924-mediated inhibition of E3 ubiquitin ligases); In the right panel, OMRP cells were treated with MLN4924 (1 μM) before immunoblots were performed. **(C)** U251MG (left panel) and T98G (right panel) cells were treated with Heclin for 24 hours before immunoblot was performed to measure the abundance of H2AX (p53 served as a control to validate the expected Heclin-mediated inhibition of E3 ubiquitin ligases). **(D)** U251MG cells were treated with MTDIA (3 μM) for 24 hours, in the presence of vehicle control or Heclin (10 μM), and immunoblot was performed to detect the H2AX protein level. **(E)** U251MG control cell line or derivative lines with knockdown of *NEDD4* (shNEDD4) were used for immunoblot to determine the level of H2AX (anti-NEDD4 immunoblot confirmed the expected gene knockdown). (**F)** RT-qPCR analysis of the *WWP1* transcript level in the parental U251MG cell line and its derivative lines with knockdown of *WWP1* (left panel)*;* and immunoblot analysis of WWP1 and H2AX in U251MG cell line and its derivative lines with knockdown of *WWP1* (middle and right panels).

A

No.	Energy(-)		Rotation			Translation	
[match]							
1	40	357	46	142	-5.43	16.79	58.29
2	37	342	38	165	1.07	16.79	58.29
3	37	243	74	33	-5.43	16.79	58.29
4	37	261	163	230	-5.43	16.79	58.29
5	36	61	105	342	-5.43	16.79	58.29
6	36	135	93	165	-5.43	16.79	58.29
7	36	271	115	50	-11.93	16.79	64.79
8	36	357	46	142	-5.43	16.79	45.29
9	36	342	38	165	1.07	10.29	58.29
10	36	57	105	333	1.07	16.79	64.79

B

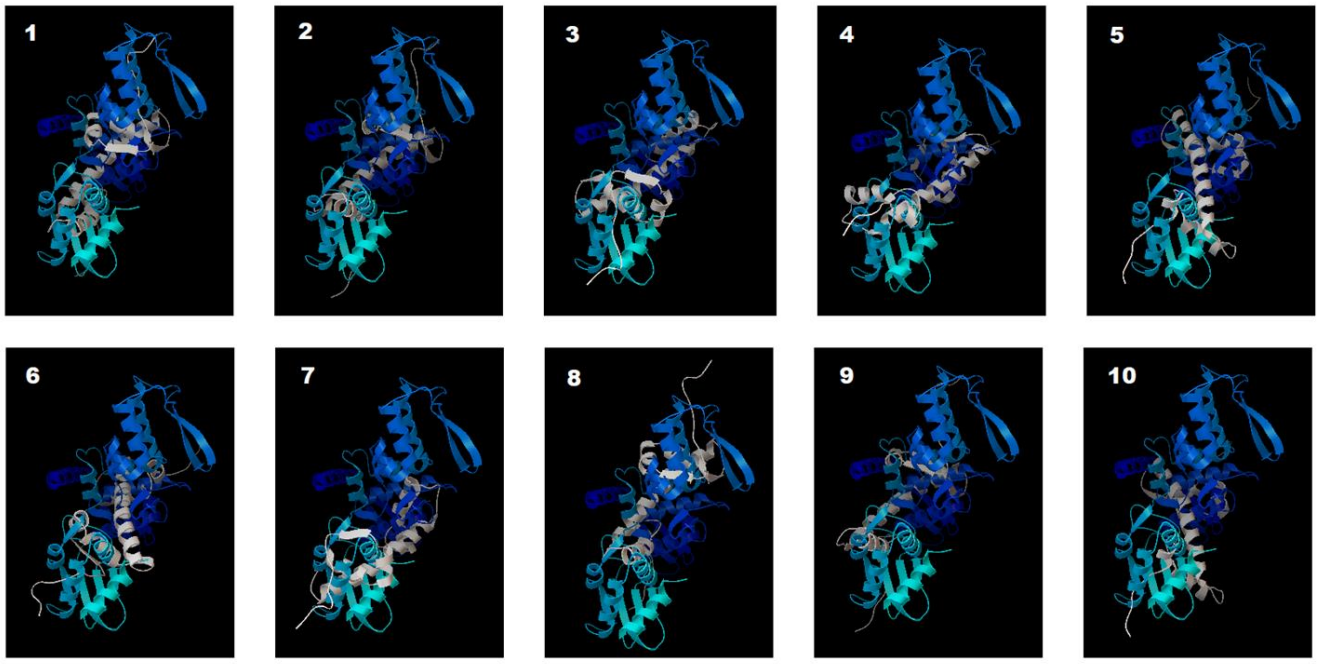

Figure S8: Related to Figure 6. Top ten predicted SMURF2 HECT domain - H2AX protein binding models. **(A)** Ten models ranked by the lowest to the highest thermodynamic free energy. **(B)** Snapshots of the 3D structures for the ten models. More details for Model #1 and color codes used are shown in Figure 6G.

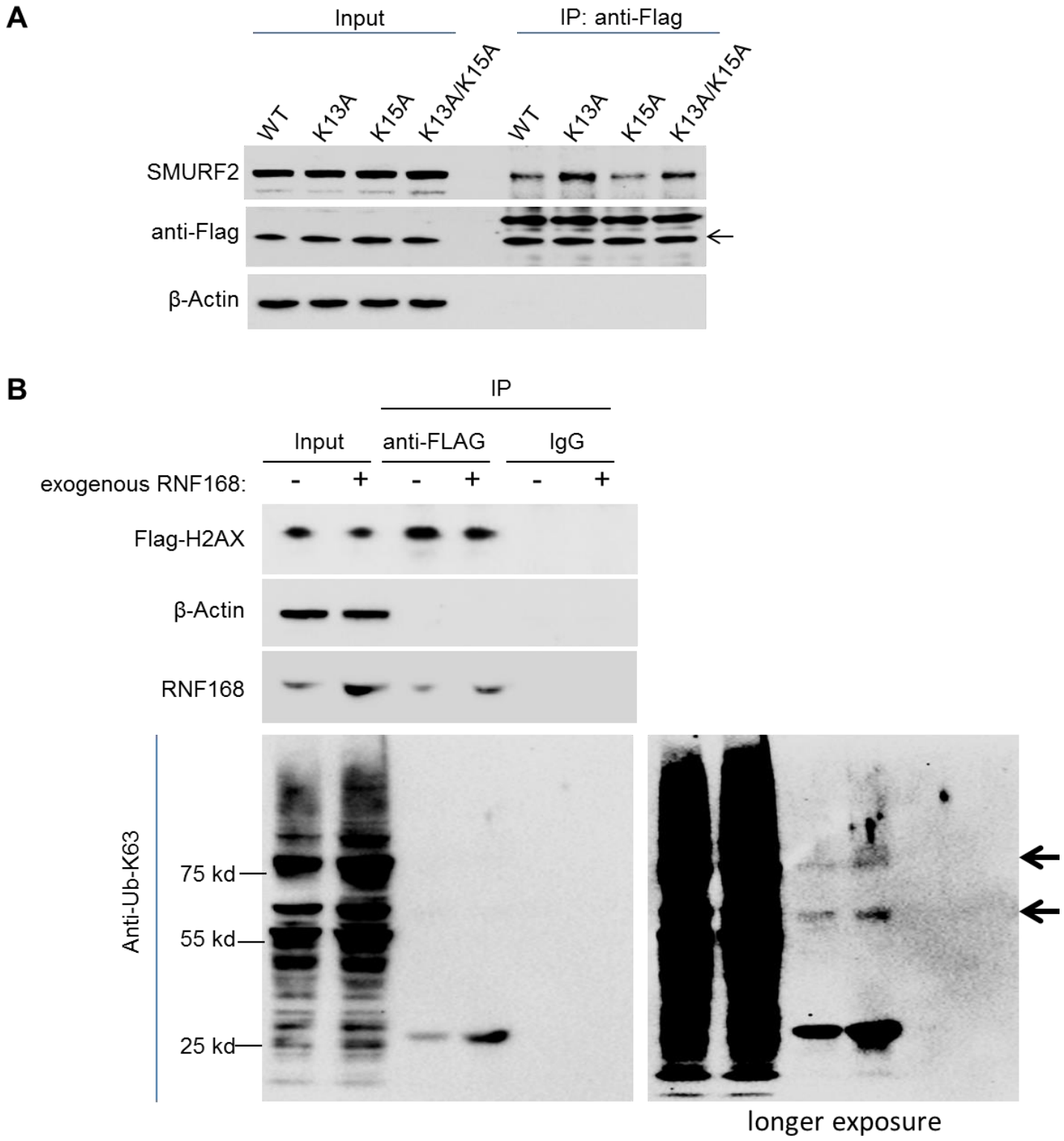

Figure S9: Related to Figure 7. H2AX-K13A mutant has enhanced interaction with SMURF2, and H2AX displays higher level of Ub-K63 poly-ubiquitination upon RNF168 overexpression. **(A)** U251MG cell lines expressing exogenous Flag-tagged H2AX or its derivative mutant (K13A, K15A, and K13A/K15A) were used for anti-Flag co-immunoprecipitation and immunoblot analysis was performed to determine the interaction of each H2AX variant with SMURF2. **(B)** U251MG cell lines

expressing exogenous Flag-tagged H2AX, without or with exogenous RNF168 overexpression, were used for anti-Flag IP and immunoblots.

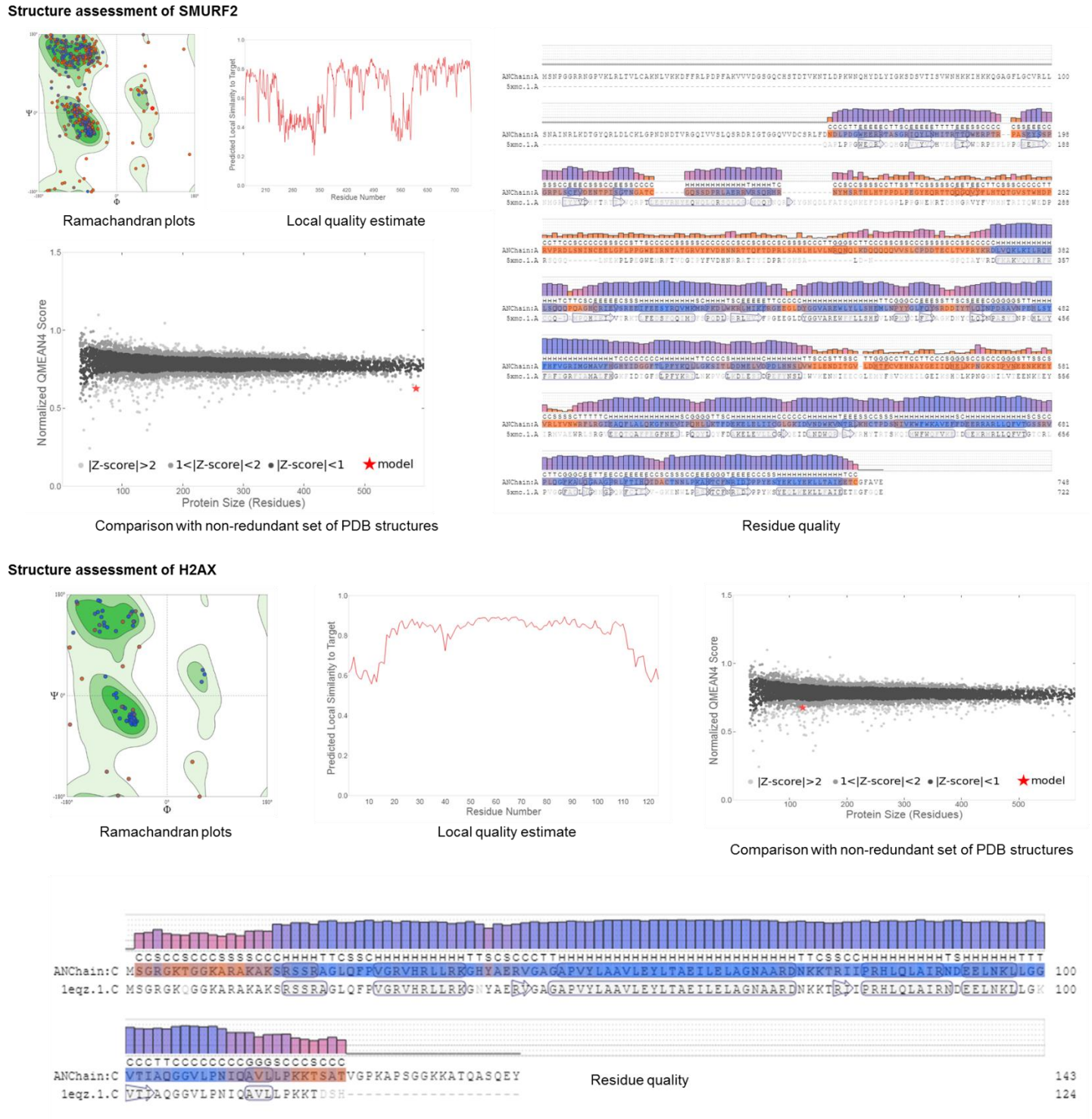

Figure S10: Related to Figure 6G. Structure assessment of SMURF2's HECT domain and H2AX. The top and the bottom panels show the structure assessment for SMURF2's HECT domain and for H2AX, respectively.

Table S2: Related to STAR Methods. Oligonucleotides used in the study.

shRNA sequences:

Name	Label	Sequence
PRMT5#1	TRCN0000107085	CCGGCCTCAAGAACTCCCTGGAATACTCGAGTATTCCAGGGAGTTCTTGAGGTTTTTG
PRMT5#2	TRCN0000107086	CCGGGCCCAGTTTGAGATGCCTTATCTCGAGATAAGGCATCTCAAACTGGGCTTTTTG
RNF168#1	TRCN0000034134	CCGGCGACACTTTCTCCACAGATATCTCGAGATATCTGTGGAGAAAGTGTCGTTTTTG
RNF168#2	TRCN0000034136	CCGGCGTGGAACTGTGGACGATAATCTCGAGATTATCGTCCACAGTTCCACGTTTTTG
RNF8#1	TRCN0000003437	CCGGGCCTGAATGTACTATGTTTAACTCGAGTTAAACATAGTACATTCAGGCTTTTT
RNF8#2	TRCN0000003441	CCGGCCAAAGAATGACCAAATGATACTCGAGTATCATTTGGTCATTCTTTGGTTTTT
HUWE1#1	TRCN0000344528	CCGGCCTAGGCTGCAGGACTAATATCTCGAGATATTAGTCCTGCAGCCTAGGTTTTTG
HUWE1#2	TRCN0000344529	CCGGTTTGATGTCAAGCGCAAATATCTCGAGATATTTGCGCTTGACATCAAATTTTTG
SMURF2#1	TRCN0000003475	CCGGCGGTACAAGTCACATTTCATTCTCGAGAATGAAATGTGACTTGTACCGTTTTT
SMURF2#2	TRCN0000003477	CCGGCGCCTCAAAGACACTGGTTATCTCGAGATAACCAGTGTCTTTGAGGCGTTTTT
NEDD4#1	TRCN0000007550	CCGGGCCTTTCTCTTGCCTGCATATCTCGAGATATGCAGGCAAGAGAAAGGCTTTTT
NEDD4#2	TRCN0000007554	CCGGCGGTTGGAGAATGTAGCAATACTCGAGTATTGCTACATTCTCCAACCGTTTTT
WWP1#1	TRCN0000003395	CCGGATTGCTTATGAACGCGGCTTTCTCGAGAAAGCCGCGTTCATAAGCAATTTTTT
WWP1#2	TRCN0000003398	CCGGCCTCTCAAATTCATAACAGTTCTCGAGAACTGTTATGAATTTGAGAGGTTTTT
H2AFX	TRCN0000073278	CCGGCGTTGGCTTCTGAACTGGAATCTCGAGATTCCAGTTCAGAAGCCAACGTTTTTG
Non-targeting control		CCGGGCGCGATAGCGCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCTTTTT

sgRNA sequences:

control sgRNA (LentiCRISPREv2)	CACCgtatcctgacctacgcgctg	AAACcagcgcgtaggtcaggatac
exon1 sgRNA	CACCGGCTCATCTCACCTTCACGG	AAACCCGTGAAGGTGAGATGAGCC
exon6 sgRNA	CACCGTCTGCCCGGGAGCTAAAACG	AAACCGTTTTAGCTCCCGGGCAGAC

Primers for RT-qPCR:

Gene name	Forward (F) or Reverse (R)	Sequence (5’ to 3’)
ACTB	F	GATTCCTATGTGGGCGACGA
	R	AGGTCTCAAACATGATCTGGGT
H2AFX	F	AGGCCTCCCAGGAGTACTAAGA
	R	CTGAAGCGGCTCAGCTCTTT
RNF168	F	GGCGAGTTTATGCTGTCCCT
	R	GCCGCCACCTTGCTTATTTC
MTAP	F	TCTTGTGCCAGAGGAGTGTG
	R	ACCGAAACTGCTTCCTCGTG
PRMT5	F	ACTAGTCATCCCGGAGAAGCA
	R	GCGTATTCCAGTCCCTTCCT
SMURF2	F	TGCCCAGGGATCTTAGCAAC
	R	CTGAACCAGGTCTCGCTTGT
NEDD4	F	ATTAGAGCCTGGCTGGGTTG
	R	CACGTTGTGCTTGCAGTTGA
WWP1	F	TGCAGCTCATCTCCAACCATAGAA
	R	GGCTCAGATGCGAGTGGTTT
RNF8	F	GCAGGAGCATTGGGCTCTAA
	R	GCACAGTTCAAGGTGACAGC
HUWE1	F	AGCCTGACCTGAGTGGGTTA
	R	ACTCGCACTTTCCAATGTTCC
GAPDH	F	GGAGCGAGATCCCTCCAAAAT
	R	GGCTGTTGTCATACTTCTCATGG

Primers for constructing H2AX mutants:

Mutant site	Forward (F) or Reverse (R)	Sequence (5’ to 3’)
K13A	F	GCGCGACTTGGCCGCGGCGCGGGCCTTG
	R	CAAGGCCCGCGCCGCGGCCAAGTCGCGC
K15A	F	CGACGAGCGCGACGCGGCCTTGGCGCGG
	R	CCGCGCCAAGGCCGCGTCGCGCTCGTCG
K13A and K15A (double)	F	CGACGAGCGCGACGCGGCCGCGGCGCGGGCCTTG
	R	CAAGGCCCGCGCCGCGGCCGCGTCGCGCTCGTCG

Primers for constructing mutant SMURF2:

Mutant site	Forward (F) or Reverse (R)	Sequence (5’ to 3’)
1001T-A	F	CGATTTAAAACTAAATGCTAGTTAGCAGACAGCCGAGGATCT
	R	AGATCCTCGGCTGTCTGCTAACTAGCATTTAGTTTTAAATCG

ChIP-qPCR primers:

Promoter region	Forward (F)	Sequence (5’ to 3’)

	or Reverse (R)	
F1: -1000- -801	F	TGGACCTGAACTCCTGG
	R	AGAGCATTCAGGCTGGGTA
F2: -800- -601	F	CAGCAGCCTCAAAACAACTT
	R	ACTGTTTTATCCAGGCTGGA
F3: -600- -401	F	GAGACCCCAATTCTTTTATC
	R	AATACAAAAATTAGCTGGGC
F4: -422- -177	F	ATGCCCAGCTAATTTTTG
	R	GACCTTTCCCTGAGATT
F5: -200- -1	F	TCGCAGAAATCTCAGGGAAA
	R	GCTAGCAACGTCACAACC
F6: +1-+150	F	GCTTGTCCGGTGGCT
	R	CAGCATAACTTCCGCTTTACC