SUPPLEMENTARY INFORMATION

The RNA transport factor PHAX is required for proper histone H2AX expression and DNA damage response.

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Supplementary Materials and Methods

Plasmids

Human PA28γ cDNA was synthesized by Eurofins Genomics (Tokyo, Japan) and cloned into pcDNA3 (Thermo Fisher Scientific, Waltham, MA), resulting in pcDNA3-PA28γ.

Cell cycle analysis

Cells were fixed with 70% ethanol, stained with FxCycle PI/RNase Staining Solution (Thermo Fisher Scientific), and analyzed by flow cytometry. Data was analyzed by FlowJo software (Tree Star, San Carlos, CA).

To synchronize the cell cycle, cells were cultured in thymidine-containing medium at 2.5 mM for 18 hrs, and then the medium was changed to normal medium. After 9-hr incubation, the cells were re-cultured in thymidine-containing medium at 2.5 mM for 16 hrs. The synchronization of cell cycle was confirmed by flow cytometry, as described above.

Northern blotting analysis

Northern blotting analysis was performed as previously described (Machitani et al. 2017). Total RNA was extracted from the cells with Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). Ten micrograms of total RNA per lane was loaded onto 15% polyacrylamide denaturing gel. After electrophoresis, bands of RNA were transferred to Hybond-N+ membranes (Roche, Mannheim, Germany). The membranes were then probed with ^{32}P-labeled synthetic oligonucleotides that were complementary to the sequence of human U1 snRNA, U7 snRNA, or 5.8S ribosomal RNA (U1: 5'-TTATGCAGTCGAGTTTCCCACATTT-3'; U7: 5'-CAGATTCCTGGTGAAAACTGG-3'; 5.8S: 5'-TCGAAGTGTCGATGATCAATGTGTC-3').

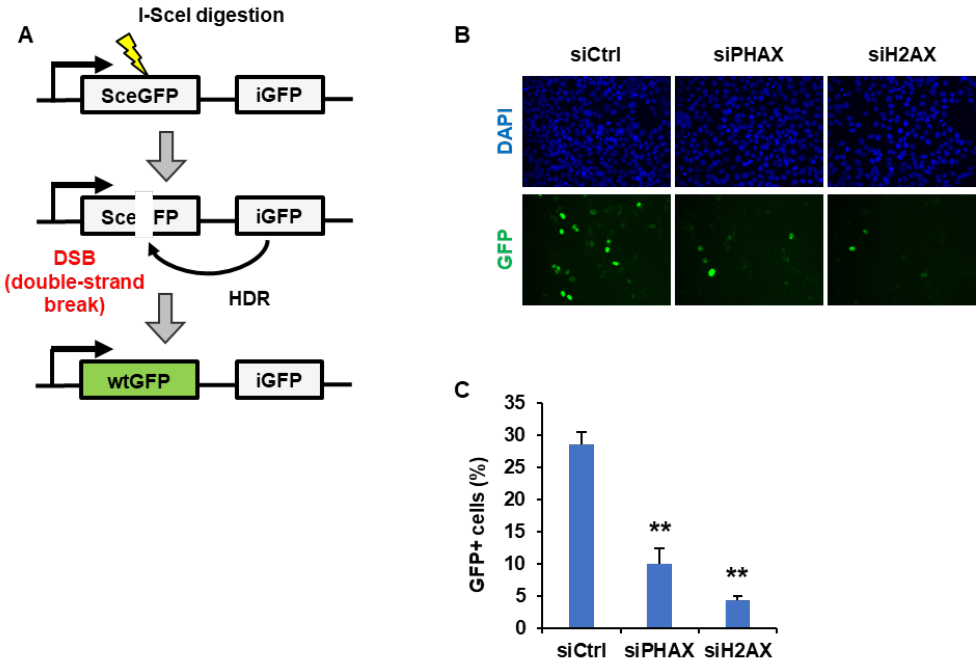

Fig. S1. Inhibition of DNA repair by knockdown of PHAX (related to Fig. 1).

(A) Reconstitution of the GFP gene by a homology-dependent repair (HDR). I-*Sce*I-mediated production of double-strand DNA breaks (DSBs) induces HDR, resulting in GFP expression. **(B,C)** U2OS cells were co-transfected with the indicated siRNAs and the reporter plasmid (pDRGFP) for DNA repair assay. Fluorescence microscopic images were obtained, and the numbers of GFP-positive cells were counted. Data are the means ± S.D. (n=4). ***P*<0.001.

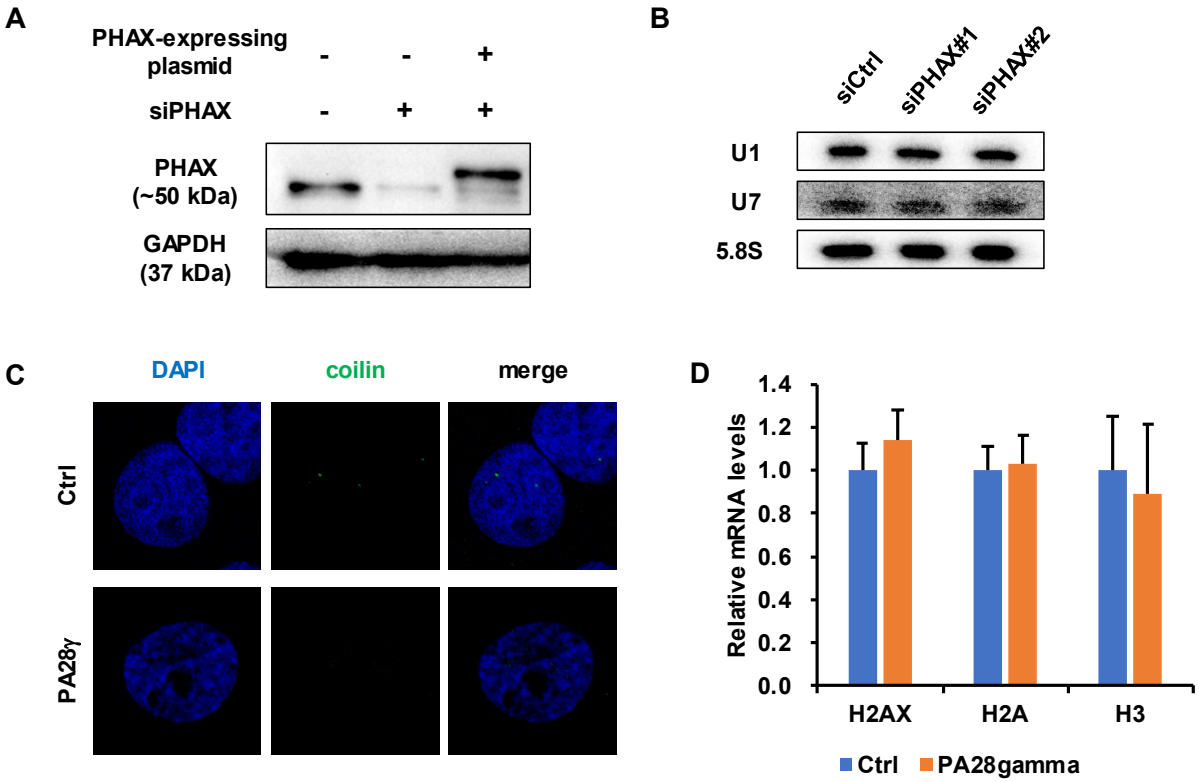
A
PHAX-expressing plasmid
siPHAX
PHAX (~50 kDa)
GAPDH (37 kDa)
B
siCtrl
siPHAX#1
siPHAX#2
U1
U7
5.8S
C
DAPI
coilin
merge
Ctrl
PA28γ
D
Relative mRNA levels
H2AX
H2A
H3
Ctrl
PA28gamma
E
siCtrl
siPHAX#1
siPHAX#2
G1:47.4%
S:26.4%
G1:52.8%
S:23.3%
G1:53.0%
S:22.8%
DNA content (PI)
F
Relative H2AX mRNA levels
Asyn
Syn
G
H2A (15 kDa)
H3 (15 kDa)
H2AX (15 kDa)
PHAX (~50 kDa)
α-tublin (52 kDa)

Fig. S2. Suppression of H2AX expression by knockdown of PHAX (related to Figs. 2 and 3). **(A)** U2OS cells were co-transfected with siPHAX and a PHAX-expressing plasmid. After 48-hr incubation, PHAX protein levels were determined by western blotting analysis. **(B)** U2OS cells were transfected with the indicated siRNAs. After 48-hr incubation, the indicated RNA levels were determined by northern blotting analysis. **(C,D)** U2OS cells were transfected with a control plasmid (pcDNA3) or a PA28γ-expressing plasmid (pcDNA3-PA28γ). After 48-hr incubation, cells were fixed and immunostained for coilin. Cajal bodies (CBs) were observed in the form of nuclear dots (C). The indicated mRNA levels were determined by qRT-PCR analysis (D). **(E)** U2OS cells were transfected with the indicated siRNAs. After 48-hr incubation, cells were stained by propidium iodide (PI) and cell cycle properties of the cells were analyzed by flow cytometry. **(F)** U2OS cells were transfected with the indicated siRNAs, followed by synchronization of the cell cycle by a double-thymidine block. H2AX mRNA levels were determined by qRT-PCR analysis. **(G)** U2OS cells were transfected with the indicated siRNAs. After 48-hr incubation, the indicated protein levels were determined by western blotting analysis. Data are the means ± S.D. (n=3-4). $^{**}P<0.001$.

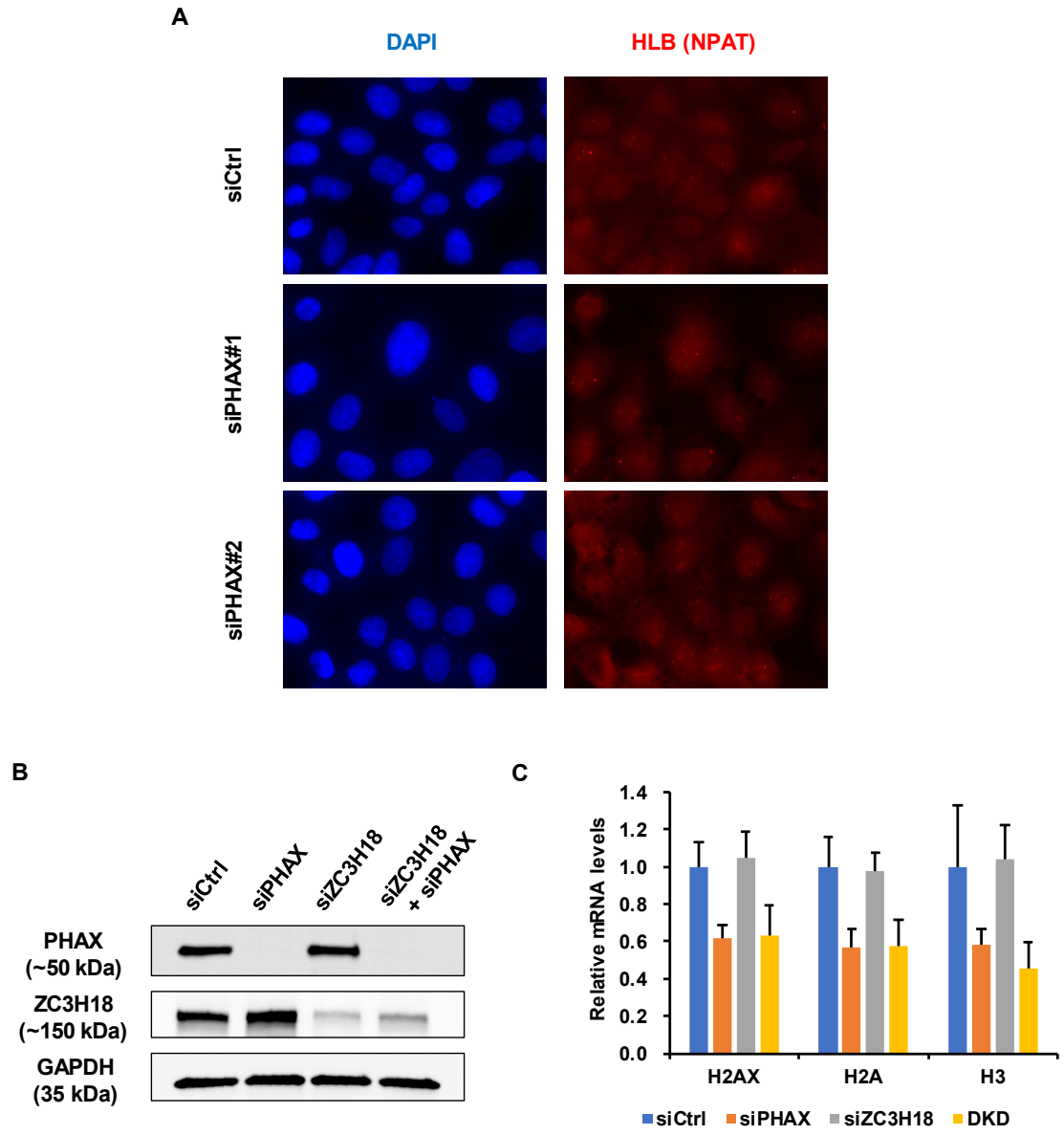

Fig. S3. Suppression of histone mRNA transcription by knockdown of PHAX (related to Fig. 4).

(A) U2OS cells were transfected with the indicated siRNAs. After 48-hr incubation, cells were fixed and immunostained for NPAT. Histone locus bodies (HLBs) were observed in the form of nuclear dots. **(B,C)** U2OS cells were transfected with the indicated siRNAs. After 48-hr incubation, the indicated protein (B) and mRNA (C) levels were determined by western blotting analysis and qRT-PCR analysis, respectively. Data are the means ± S.D. (n=4).

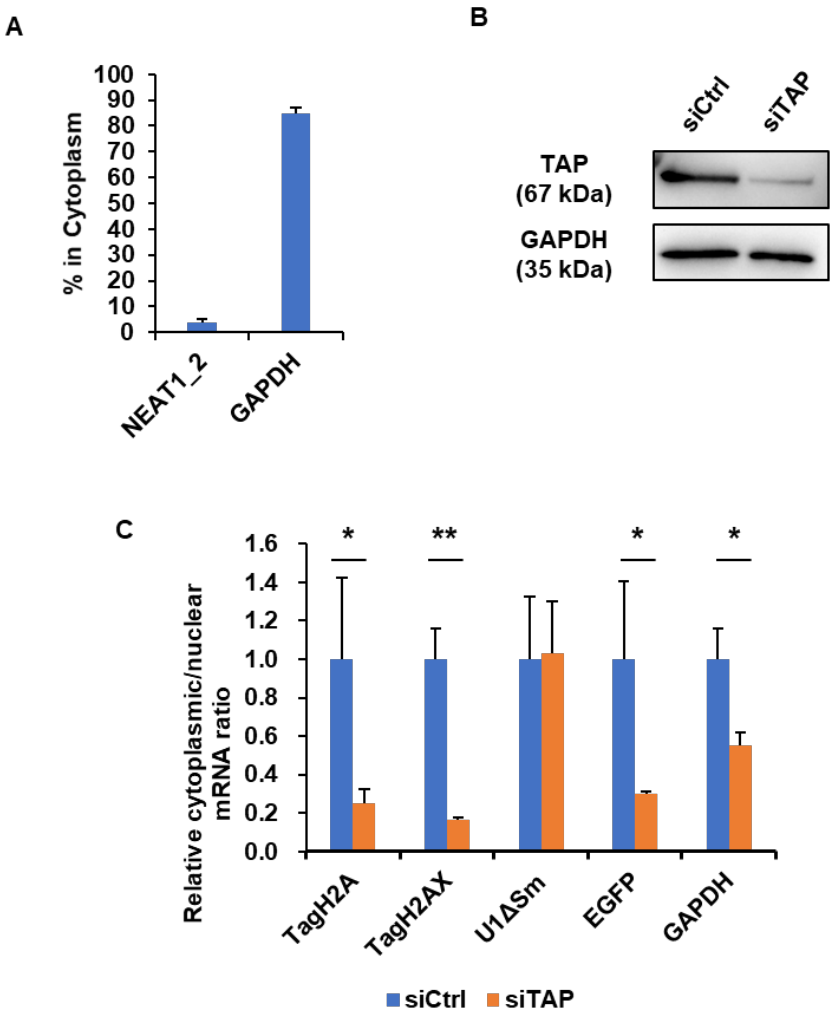

Fig. S4. Reduction in histone mRNA export efficiencies by knockdown of PHAX (related to Fig. 5).

(A) RNA was extracted from the nuclear and cytoplasmic fractions of U2OS cells, and the indicated mRNA levels were determined by qRT-PCR analysis. **(B)** U2OS cells were transfected with siTAP. After 48-hr incubation, the indicated levels were similarly determined. **(C)** U2OS cells were transfected with siTAP, followed by transfection with plasmids expressing the indicated genes. After 3-hr incubation, the indicated mRNA levels in nucleus and cytoplasm of the cells were similarly determined. Data are the means ± S.D. (n=3). **P*<0.05, ***P*<0.01.

Suppl. Table S1

The antibodies used in this study

No.	antigen	type	Company
1	p53	mouse	Santa Cruz Biotechnology, Santa Cruz, CA
2	p53 (S392)	rabbit	Abcam, Cambridge, UK
3	α-tublin	rabbit	Abcam, Cambridge, UK
4	p-ATM	mouse	Rockland Inc, Limerick, PA
5	p-DNA-PKcs	rabbit	Abcam, Cambridge, UK
6	H2AX	rabbit	Cell Signaling Technology, Danvers, MA
7	gH2AX	rabbit	Cell Signaling Technology, Danvers, MA
8	H2A	rabbit	Cell Signaling Technology, Danvers, MA
9	H3	rabbit	Abcam, Cambridge, UK
10	GAPDH	rabbit	Abcam, Cambridge, UK
11	RNAPII-CTD	mouse	Abcam, Cambridge, UK
12	Coilin	mouse	Abcam, Cambridge, UK
13	ZC3H18	rabbit	Sigma-aldrich, St Louis, MO
14	PHAX	rabbit	Abcam, Cambridge, UK
15	PHAX	mouse	previously described (Ohno et al. 2000)
16	TAP	rabbit	previously described (McCloskey et al. 2012)

Suppl. Table S2

The primers and probes used in this study

No	name	purpose	sequence
1	H1-F	qRT-PCR	CCGGCTATGATGTGGAGAAA
2	H1-R		GTGCCTTTCGTTTGCACCAG
3	H2A-F		GGCTCGGGACAACAAGAAGAC
4	H2A-R		CTGGGCGATGGTGACTTTG
5	H3-F		CTGCCTTTTCAGCGTCTGGT
6	H3-R		GCACAGGTTGGTGTCCTCAA
7	H2AX-F		AGTGTACCTGGCGGCAGTGCT
8	H2AX-R		GGATGATTCGCGTCTTCTTGTTG
9	GAPDH-F		ATGAGAAGTATGACAACAGCCTCAA
10	GAPDH-R		AGTCCTTCCACGATACCAAAGTT
11	b-actin-F		ACGAGGCCCAGAGCAAGAGAGG
12	b-actin-R		TCTCCATGTCGTCCCAGTTGGT
13	c-myc-F		GGCTCCTGGCAAAAGGTCA
14	c-myc-R		CTGCGTAGTTGTGCTGATGT
15	hnRNPH1-F		ATTCAAAATGGGGCTCAAGGTAT
16	hnRNPH1-R		GTGTCAGGACTATTTGGACCAG
17	JTV1-F		GTTGAAAGCTGCAGTTGATGGCCT
18	JTV1-R		GCACTGAATTCAAGTCCAGCGCAT
19	PEX1-F		AAAGCTGAGCTCTTTGGGAGGAGT
20	PEX1-R		AGAGACATCAGCTGCCGAGACAAA
21	NEAT1-F		CAATTACTGTCGTTGGGATTTAGAGTG
22	NEAT1-R		TTCTTACCATACAGAGCAACATACCAG
23	U1snRNA-F		ACTTACCTGGCAGGGGAGATAC
24	U1snRNA-R		ACATCCGGAGTGCAATGGATAA
25	U2snRNA-F		ATCGCTTCTCGGCCTTTTG
26	U2snRNA-R		CCATTTAATATATTGTCCTCGG
27	U6snRNA-F		CTCGCTTCGGCAGCACATATAC
28	U6snRNA-R		ACGCTTCACGAATTTGCGTGTC
29	U7snRNA-F		GCATAAGCTTAGTGTTACAGCTCTTTTAGAATTTGTC
30	U7snRNA-R		CGTAGAATTCAGGGGCTTTCCGGTAAAAAGCCAG
31	7SKsnRNA-F		GGTCTTCGGTCAAGGGTATAC
32	7SKsnRNA-R		GTGTCTGGAGTCTTGGAAGC

33	Fluc-F		CGCAGCTTGCAAGACTATAAGATTCA
34	Fluc-R		AAGTTGCTTAGGTCGTACTTGTCGATG
35	Rluc-F		CAAGGAGAAGGGCGAGGTTA
36	Rluc-R		TGTAGTTGCGGACAATCTGGA
37	tag-F		CAGAATTCTGATCGCGCTTCTCGTTG
38	tagH2A-R		GAGACTTAGACTTGGCGCGT
39	tagH2AX-R		CACTGGGAACTGGAGGCC
40	H2AA-349-F	ChIP	TTTGGTGACGGCTTTCTTGG
41	H2AA-349-R		TACCGCTCTGCTTGCGTTCT
42	H2AA-73-F		GCTTGAGCCAATCAAAGTGCTC
43	H2AA-73-R		CAGCGATAGTAGTCACCGAGAGAAA
44	H2AA+263-F		GGCTCGGGACAACAAGAAGAC
45	H2AA+263-R		CTGGGCGATGGTGACTTTG
46	H2AA+749-F		CCACACAGTCACAAACACCAG
47	H2AA+749-R		CATAGCCTGCGGAAACCACT
48	H2AX-322-F		TCTAGGTGGCCTCAGACTCC
49	H2AX-322-R		ACGCAGACTGCCAACTATCC
50	H2AX-89-F		GGAGGCGGGTATTGGAGAAA
51	H2AX-89-R		AACACTAGAACAGACGCCCG
52	H2AX+220-F		AGTGTACCTGGCGGCAGTGCT
53	H2AX+220-R		GGATGATTCGCGTCTTCTTGTTG
54	H2AX+1166-F		CAGGCCTTTCACATCAGCTCTC
55	H2AX+1166-R		GGAGGAAGATGTGCCTGTTACCAA
56	GAPDH-466-F		CACCCTGCCCTCAATATCCC
57	GAPDH-466-R		TTCATTCCATCCAGCCTGGG
58	GAPDH+73-F		GAGCAGTCCGGTGTCACTAC
59	GAPDH+73-R		GCCCCCTCCCCTTTCTTTC
60	GAPDH+1662-F		ATTTCCACCGCAAAATGGCC
61	GAPDH+1662-R		CCCGGTGACATTTACAGCCT
62	GAPDH+3485-F		GACTGAGGCTCCCACCTTTC
63	GAPDH+3485-R		ACATCACCCCTCTACCTCCC

64	U1 probe	Northern blotting analysis	TTATGCAGTCGAGTTTCCCACATTT
65	U7 probe		TTCTAAAAGAGCTGTAACAC
66	5.8S probe		TCGAAGTGTCGATGATCAATGTGTC
67	H1Cp-F	pGL4-H1Cp	TACTCGAGCCTTGTCCCAGTCCACAGTT
68	H1Cp-R		GGAAGCTTGAGCAGTCTCGGACATGTTG
69	H2AXp-F	pGL4-H2AXp	TACTCGAGATCCTGTTTCCTGGCATGGG
70	H2AXp-R		GGAAGCTTCGCAGTGTAACTGCTGTCG
71	GAPDHp-F	pGL4-GAPDHp	TACTCGAGAGTTCCCCAACTTTCCCGC
72	GAPDHp-R		GGAAGCTTAGAAGATGCGGCTGACTGTC
73	H2Ap-H2A-F	pUC118-H2Ap-H2A	CTCGCTCGCTATACGCTCAA
74	H2Ap-H2A-R		TTGTCGACCCCAGCTAGTTTCTGTGGGG
75	H2AXp-H2AX-F	pUC118-H2AXp-H2AX	TATCTAGAATCCTGTTTCCTGGCATGGG
76	H2AXp-H2AX-R		TTAAGCTTCTGAAGGTGTTGTGTGTGCG
77	tagH2Amut-F	pUC118-H2Ap-tagH2A	AGATGATCGCGCTTCTCGTTGGGGCGTGTTGCTGCAATTTGATGC
78	tagH2Amut-R		AGAACCACATTTCTAGGGCTGC
79	tagH2AXmut-F	pUC118-H2AXp-tagH2AX	ATGATCGCGCTTCTCGTTGGGGTGTTTGAGCCGTCGTGCTTC
80	tagH2AXmut-R		CTAGAACAGACGCCCGCC

References

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