SUPPLEMENTARY INFORMATION

Selective Requirement of H2B N-Terminal Tail for

p14ARF-Induced Chromatin Silencing

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SUPPLEMENTARY MATERIALS AND METHODS

Antibodies and constructs

Antibodies used in this study are as follows: Flag, B23, Lamin A/C and Actin antibodies from Sigma; Tubulin antibody from Signaling Technology; HA, β-catenin, and p14ARF antibodies from Santa Cruz Biotechnology; hnRNP-U, ARF-BP1, HMGB1, H2B, H2B AcK20, AcH3 and AcH4 antibodies from Abcam.

To generate the plasmid for mammalian expression of H2B N-terminal domain, cDNA sequence encoding amino acids 1-37 of human H2B was PCR amplified and subcloned into the NotI-EcoRI sites of pBluescript SK+ (pBS-1xnH2B). The NsiI-EcoRI fragment of H2B tail cDNA from pBS-1xnH2B was inserted into PstI-EcoRI-digested pBS-1xnH2B to generate the plasmid containing two copies of H2B tail cDNA (pBS-2xnH2B). The plasmid containing four copies of H2B tail cDNA (pBS-4xnH2B) was generated by inserting two copies of H2B tail cDNA into the PstI-EcoRI sites of pBS-2xnH2B. The same procedure was followed to insert four copies of H2B tail cDNA into pBS-4xnH2B, and the final eight copies of H2B tail cDNA were transferred into the EcoRI-NotI sites of pIRES (Clontech) containing N-terminal Flag and HA tags to create pIRES-8xnH2B. pG5ML601-14 plasmid was constructed as follows: the DNA fragment (G5ML) bearing Gal4 binding sites, adenovirus major late core promoter and G-less cassette was isolated from EcoRI digestion of pG5ML array plasmid (1). To construct pG5ML601-7, the G5ML fragment was ligated into
the EcoRI site of p601-7 containing seven direct repeats of the 207 bp 601 nucleosome positioning sequence. The EcoRI site at the 3' end of pG5ML601-7 was removed by site-directed mutagenesis. Finally, the 601-7 fragment generated by PCR amplification from p601-7 was cut with EcoRI and ApoI and subcloned into the EcoRI site at the 5' end of pG5ML601-7 to create pG5ML601-14 as illustrated in Supplemental Figure 2A. For construction of GST-fusions of HDAC1 and H2B tail deletion mutants, PCR products were prepared with primers spanning the corresponding cDNA sequences and inserted into the BamHI and EcoRI sites of pGEX-4T1 (GE Healthcare). Bacterial expression constructs of RPL5 and B23 were generated by PCR tagging of the corresponding cDNAs and subcloning them into pET11d and pET15b (Novagen) in frame with 5’ Flag and hexa-His sequences, respectively. Expression plasmids encoding H2B Δ1-15 and H2B Δ1-26 were constructed by subcloning the corresponding cDNA fragments into the NdeI and BamHI sites of pET11b (Novagen). All point mutations in H2B expression constructs were made by using the QuikChange mutagenesis kit (Stratagene). Expression vectors encoding p14ARF, hnRNP-U and hnRNP-H1 were generated by inserting cDNAs into the NdeI and BamHI sites of pET11d with an NH2-terminal Flag tag. The cDNA for hnRNP-K was subcloned into the Ncol and AvrII sites of pET-duet vector (Novagen). pGEX-4T1-β-catenin and ARF-BP1 baculovirus were described previously (2,3). Expression vectors for core histones, Gal4-VP16 and GST-histone tails were as described (4-6). Further details of plasmid constructions are available.
upon request.

**Preparation of recombinant proteins**

Recombinant histones were expressed in E. coli Rosetta 2 (DE3) pLysS cells (Novagen) and refolded to octamers as described (7,8). GST-histone tails were prepared as described previously (6). GST-HDAC1 and GST-β-catenin proteins were expressed and purified in a similar way using affinity chromatography on glutathione-Sepharose 4B beads. If necessary, the GST tag was removed by thrombin digestion (Novagen) according to the manufacturer’s protocol. Flag-B23, Flag-hnRNP-U, Flag-hnRNP-H1, His-RPL5 and His-hnRNP-K were expressed in E. coli Rosetta 2 (DE3) pLysS and purified with M2 agarose or Ni-NTA resin (Novagen) according to standard protocols. His-ARF-BP1 was expressed in insect (Sf9) cells using a baculovirus vector and purified by Ni-NTA and Q-Sepharose (Amersham Biosciences) affinity chromatographies. Full length p14ARF and p14ARF fragments were expressed in E. coli BL21 (DE3) pLysS (Novagen) and purified by a three-step procedure using M2 affinity, SP-Sepharose (Amersham Biosciences) and Q-Sepharose chromatographies.

**Cell growth assays**

The NARF-E6 cells were transfected with empty vector or vectors expressing H2B tails for
24 h and further mock-treated or treated with 1 mM IPTG for the indicated time. Cell viability was measured by trypan blue staining analysis. All reactions were performed in triplicate.

**Chromatin immunoprecipitation (ChIP)**

For ChIP assays, mock-treated or IPTG-treated NARF-E6 cells were incubated in culture media containing 1% formaldehyde for 15 min at room temperature, and crosslinking was stopped by addition of 2.5 M glycine to a final concentration of 0.125 M. After two washes with cold PBS, cells were lysed in ice cold lysis buffer 1 (150 mM HEPES, pH7.3, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, and 0.25% Triton X-100) for 10 min and washed with the lysis buffer 2 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, and 200 mM NaCl). Nuclei were collected by centrifugation, suspended in sonication buffer II (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Na-deoxycholate, and 0.1% SDS), and sonicated to shear the genomic DNA in the range between 150 - 300 bp. Sonicated lysates were cleared after addition of 1% of Triton X-100, and aliquots of the supernatants were used for immunoprecipitation with various antibodies immobilized on protein A/G-PLUS agarose. For p14ARF, HDAC1 and β-catenin ChIP, the beads were washed two times with the sonication buffer II, once with the LiCl wash buffer II (20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 50 mM LiCl, 0.1% NP-40, and 0.1% Na-
deoxycholate), and once with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). For H2B, H2B AcK20, AcH3 and AcH4 ChIP, the beads were washed two times with the sonication buffer II, once with the high salt sonication buffer II containing 500 mM NaCl, once with the LiCl wash buffer I (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, and 0.5% Na-deoxycholate), and once with TE buffer. The DNA samples were recovered using Qiagen kit after reversal of crosslinking at 65°C, and analyzed by qPCR. All samples were run in triplicate, and results were averaged.
Supplementary Figure S1. Purification of nH2BIFs. (A) Schematic summary of purification of nH2BIFs. Nuclear extracts from H2B tail-expressing cells were initially purified with anti-Flag affinity chromatography, and the eluted factors were further purified with anti-HA affinity chromatography as described under the Materials and methods. The purified proteins were separated in 10% SDS-PAGE and subjected to immunoblotting with anti-HA antibody. Lane 1, mock purification with regular HeLa nuclear extracts; lane 2, Flag-HA-nH2B immunoprecipitation with nuclear extracts prepared from H2B tail-expressing
HeLa cells. NLS: Nuclear localization signal. (B) Subcellular localization of ectopic H2B tails. Cytoplasmic (C) and nuclear (N) extracts were prepared as described recently (9), and subjected to immunoblot analysis with anti-Flag antibody. Tubulin and Lamin A/C were used as markers for cytoplasmic and nuclear fractions, respectively.
**Supplementary Figure S2.** In vitro transcription assay. (A) Outline of in vitro transcription assay. The G5ML601-280G array template contains the central three nucleosome length (594 bp) fragment bearing Gal4 binding sites (G5), adenovirus major late core promoter (ML) and G-less cassettes flanked on either side by seven repeats of 207 bp 601 nucleosome positioning sequences. Detailed procedures for chromatin reconstitution and transcription assay were described under the Materials and methods. PIC, preinitiation complex; NTPs, nucleotide triphosphates; Nuc ext, Nuclear extract; AcCoA, acetyl-CoA; nH2BIFs, H2B tail-interacting factors; and Txn, Transcription. (B) Micrococcal nuclease (MNase) digestion of the G5ML601 nucleosome arrays. Nucleosome arrays were reconstituted on the G5ML601 array DNA fragment and digested with MNase (4 mU) for 5 min to generate a ladder of partial digestion products. Lane 1, an 123 bp DNA size marker.
(M); lane 2, nucleosome arrays containing wild-type H2B; lane 3, nucleosome arrays containing H2B mutant lacking the first 26 amino acids. The predicted mobility of nucleosomal DNA is indicated to the right by arrows.
Supplementary Figure S3. Preparation of recombinant nH2BIFs. Recombinant proteins were prepared as described in the Supplementary Materials and methods and analyzed by SDS-PAGE and Coomassie blue staining. Asterisks indicate nonspecific bands.
Supplementary Figure S4. Inhibition of p14ARF activity by histone acetylation.  

(A) Acetylation status of native core histones. Core histones were prepared from HeLa cells and subjected to immunoblot analysis using antibodies against each of the acetylated core histones (lane 1). Recombinant histones were used as a negative control (lane 2). 

(B) Loss of p14ARF activity on nucleosome arrays containing native histones. In vitro transcription assays were as described in Figure 1E, but p14ARF was added prior to p300 in all reactions. Nucleosome arrays containing native histone octamers were included (lanes 1-5).
Supplementary Figure S5. Preparation of recombinant proteins.  (A) Preparation of intact and H2B tailless octamers. Recombinant histone octamers containing all intact histones (lane 1) or the indicated tailless H2B (lanes 2 and 3) were prepared as described in the Supplementary Materials and methods and analyzed by 15% SDS-PAGE stained with Coomassie blue.  (B) Preparation of p14ARF deletion mutants. Two different regions of p14ARF proteins were prepared as described in the Supplementary Materials and methods and analyzed by 15% SDS-PAGE.
Supplementary Figure S6. Interaction of p14ARF with H2B deletion mutant. (A)

p14ARF binding properties to GST-H2B tail deletion mutant. p14ARF was applied to a GST or GST-nH2B Δ16-26, and pull-down assays were performed as described in Figure 2B. A schematic diagram of H2B tail deletion mutant is shown on the top panel with the position of the deletion. (B) p14ARF binding properties to H2B-tailless nucleosomes. Nucleosomes containing intact H2B or H2B Δ16-26 were reconstituted on biotinylated 207 bp 601 fragments and immobilized on Streptavidin agarose beads. Nucleosome-p14ARF interactions were determined as described in the legend to Figure 2C.
Supplementary Figure S7. Expression of p14ARF and ectopic H2B tails. (A) Validation of p14ARF expression. NARF-E6 cells were mock-treated or treated with 1 mM IPTG for 48 h, and p14ARF expression from an IPTG-inducible promoter was analyzed by qRT-PCR (left panel) and immunoblotting (right panel). (B) Expression of ectopic H2B tails. NARF-E6 cells were transfected with empty vector (Con) or H2B tail expression vector
(nH2B) for 24 h and mock-treated (-p14ARF) or treated with 1 mM IPTG (+p14ARF) for 48 h. The expression of ectopic H2B tails was confirmed by immunoblotting. (C) Subcellular localization of p14ARF and histone N-terminal tails. NARF-E6 cells were transfected with the indicated plasmids and treated with IPTG as in Supplementary Figure S6B. Cytoplasmic (C) and nuclear (N) extracts were prepared as described recently (9), and subjected to immunoblotting with anti-p14ARF and anti-Flag antibodies. Tubulin and Lamin A/C were used as markers for cytoplasmic and nuclear fractions, respectively.
Supplementary Figure S8. Acetylation of wild type and mutant H2B nucleosomes.  (A)

Preparation of wild type and mutant H2B octamers.  H2B was mutated at four major
acetylation sites (K5, K12, K15 and K20), either individually or in combination, and used to
reconstitute histone octamers.  (B) Nucleosome HAT assays.  G5ML nucleosomes (200 ng)
containing wild type (lanes 1 and 2) or acetylation site-mutated (lanes 3 and 4) H2B were
reconstituted and incubated with Gal4-VP16 (15 ng), p300 (20 ng) and acetyl-CoA (10 μM).
Histone acetylation was analyzed by immunoblotting using antibodies specific for individual
acetylated histones.
Supplementary Figure S9. ChIP analysis of the Cyclin E1 locus. ChIP assays using NARF-E6 cells were essentially as described in Figure 5B, but at the Cyclin E1 gene. Numbers indicate the positions of the central base pair of each amplicon relative to the transcription start site. The primers used in the assays are listed in Supplementary Table S2.
### SUPPLEMENTARY TABLES

**Supplementary Table S1.** Primer sequences for qRT-PCR

| Primer  | Strand | Primer sequence (5’ → 3’)          |
|---------|--------|------------------------------------|
| p14ARF  | Forward| TGTCAGGTGACGGATGTAGC               |
|         | Reverse| CAAGATCTCGGAACGGCTCT               |
| AKT1    | Forward| TGCAGCATCGCTTTCTTTG               |
|         | Reverse| GTGGGCTGAGCTTTCTTTCT              |
| Cyclin E1| Forward| CAGCCAAAACTTTGAGGAAATCTA          |
|         | Reverse| TCCTGAACAAGCTCCATCT               |
| CHD5    | Forward| AATGGTGACAAAGAGGAAGATGAC          |
|         | Reverse| GTGTGCAACTCCGTGAAAG               |
| eIF4E3  | Forward| CCGCAGCAGATGATGAG                 |
|         | Reverse| CCCACTAAAGAGGCATTTACA             |
| RUNX2   | Forward| CCAACCCACGAATGCACTATC             |
|         | Reverse| TAGTGATGTTG                       |
| GAPDH   | Forward| GGCCTCCAAGGAGTAAGACC              |
|         | Reverse| AGGGGAGATTCAGTGTTGG               |
### Supplementary Table S2. Primer sequences for ChIP analysis

| Gene  | Region | Strand | Primer sequences (5’ → 3’) |
|-------|--------|--------|---------------------------|
| AKT1  | 470    | Forward| AGCTGGTGTCATCAGGTTAGG     |
|       |        | Reverse| CTTGCTCACCTCCCCATTC       |
|       | 224    | Forward| CAGGAGGTTTTTGAGGCTTG      |
|       | Reverse|        | TCCCCAGACTAGGAAAGGAGG      |
|       | 20     | Forward| AGTGCTCCCACCTTACTTG       |
|       | Reverse|        | CGCTGGAGACAAAGAGAGG       |
|       | -398   | Forward| AGTGCAAAGCAACCCCTTTTG     |
|       | Reverse|        | CAGCCAGCTTAGACGCTCTC      |
|       | -670   | Forward| GGGGACATCCAGAGGTCTTT      |
|       | Reverse|        | GGGCTCTGACGACACTGAG       |
|       | -941   | Forward| AGGAGCCCTTTGCAAAACAG      |
|       | Reverse|        | GCCTGCCCCATACAAGCA        |
|       | -1337  | Forward| CACGGACGAAAGTTGTAGC       |
|       | Reverse|        | CCAAGACAGGAAGCGACTTC      |
| Cyclin E1 | 236   | Forward| GGACAAGACCTTGAGTC         |
|         | Reverse|        | CCTAGTGTCCTCCCTCAC        |
|         | -72    | Forward| GGTGGAGGGGACACTGAG        |
|         | Reverse|        | CAGTACCCCTCCCCCTTT        |
|         | -262   | Forward| GCGAAAGGGGAAGGGGTA        |
|         | Reverse|        | GCTCCCTCGCATCCCTGTGGA     |
|         | -542   | Forward| CAGCCTGAGCAACATAGCAA      |
|         | Reverse|        | TCCACAGCTCCTCTGTCTTT      |
|         | -758   | Forward| TCTTCAGAGCCAGGAAGG        |
|         | Reverse|        | TTTGGTGCTTTACTTCA         |
|         | -1158  | Forward| CAGCCTGAGCAACATAGCAA      |
|         | Reverse|        | TCCACAGCTCCTCTGTCTTT      |
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