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The histone variant macroH2A suppresses melanoma progression through regulation of CDK8

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Abstract

Cancer is a disease consisting of both genetic and epigenetic changes. While increasing evidence demonstrates that tumour progression entails chromatin-mediated changes such as DNA methylation, the role of histone variants in cancer initiation and progression currently remains unclear. Here, we report that the histone variant macroH2A (mH2A) suppresses tumour

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Author Contributions A.K., M.S.G., K.R. and E.B. conceived ideas and experimental design. A.K. generated shRNA and overexpression cell lines and performed all *in vitro* and *in vivo* studies, DNA methylation analysis, microarray studies, qPCR and ChIP. M.S.G. performed immunoblots, IHC, and statistical analysis. L.K.C. performed IHC, immunoblots and *in vivo* studies and K.R. performed MNase studies. C.V. performed ChIP and P.O.E. and C.I.V. scored and evaluated all human and mouse tissues. S.M., M.F.S., and E.H. designed and performed mouse studies, G.L. and B.A.G. performed and analyzed Q-MS studies, and D.P. and I.O. contributed critical reagents. E.B. wrote the manuscript with contributions from all other authors.

Author Information Microarray data has been submitted to NCBI GEO (GSE19181). Reprints and permissions information is available at npg.nature.com/reprintsandpermissions.

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progression of malignant melanoma. Loss of mH2A isoforms, histone variants generally associated with condensed chromatin and fine-tuning of developmental gene expression programs¹⁻⁴, is positively correlated with increasing malignant phenotype of melanoma cells in culture and human tissue samples. Knockdown of mH2A isoforms in melanoma cells of low malignancy results in significantly increased proliferation and migration *in vitro* and growth and metastasis *in vivo*. Restored expression of mH2A isoforms rescues these malignant phenotypes *in vitro* and *in vivo*. We demonstrate that the tumour promoting function of mH2A loss is mediated, at least in part, through direct transcriptional up-regulation of CDK8. Suppression of CDK8, a colorectal cancer oncogene^{5, 6}, inhibits proliferation of melanoma cells, and knockdown of CDK8 in cells depleted of mH2A suppresses the proliferative advantage induced by mH2A loss. Moreover, a significant inverse correlation between mH2A and CDK8 expression levels exists in melanoma patient samples. Taken together, our results demonstrate that mH2A is a critical component of chromatin that suppresses the development of malignant melanoma, a highly intractable cutaneous neoplasm.

Histone variants replace conventional histones within the nucleosome and confer unique biological functions to chromatin^{1, 7, 8}. The H2A family is the most diverse and includes vertebrate-specific mH2A1 (splice variants mH2A1.1 and 1.2) and mH2A21, 8-11, which are generally associated with transcriptionally repressed chromatin^{12, 13}. However, mH2A is widely distributed throughout chromatin²⁻⁴ and exists in post-translationally modified forms^{8, 14}, suggesting additional unidentified functions for this variant.

Given increasing evidence for variant-mediated transcriptional control^{1, 8} and recent reports describing variants as prognostic markers in cancer^{15, 16}, we hypothesized that global alteration of variants could contribute to malignant melanoma - the most lethal form of skin cancer with rising incidence^{17, 18}. Its radial growth phase (RGP) is characterized by lateral melanocyte growth and vertical growth phase (VGP) by spread of melanoma cells into the dermis and subcutis, upon which, metastasis can occur¹⁹.

Using well characterised, paired series of murine and human melanoma cells lines, we probed the H2A variant profile. The murine B16 series represents cells of increasing metastatic potential²⁰ and the human series of a primary melanoma (WM115) and two subsequent skin metastases derived from this same patient (WM266-4 and WM165-1)²¹. In highly malignant cells of the murine and human series, a global decrease of mH2A1 and mH2A2 protein and mRNA was observed (Fig. 1a, Supplementary Fig. S1, S2). Analysis of histones from both series using multiplexed quantitative mass spectrometry (Q-MS)²² confirmed these findings (Supplementary Fig. S1). Furthermore, mH2A1 and mH2A2 loss was observed in a panel of primary and metastatic melanoma cells (Supplementary Fig. S1). Interestingly, a 1.5 to 3-fold increase in H2A.Z levels (often associated with promoters of active genes)^{1, 8} was also observed (Fig. 1a, Supplementary Fig. S3), implicating possible H2A variant exchange during melanoma progression. Consistent with a global loss of mH2A and increased H2A.Z levels, we observed highly decondensed chromatin in B16-F10 cells by micrococcal nuclease digestion (Supplementary Fig. S2).

Next, we performed immunohistochemistry (IHC) on ~115 human tissues ranging from benign nevi to metastatic melanoma (Tissue set 1, Supplementary Fig. S4). mH2A2

antibody was used for IHC, as it produced clear nuclear staining, and tissues were independently scored (0-3) by two blinded dermatopathologists with excellent inter-observer consistency (Kappa = 0.80). IHC demonstrated that while mH2A2 is abundant in melanocytes of benign nevi and RGP lesions, its expression is dramatically lost in >80% of VGP and metastatic melanomas ($P < 0.001$) (Fig. 1b, Supplementary Fig. S4). This suggests mH2A loss occurs during the critical RGP-VGP transition. IHC was also performed on 25 melanomas with known BRAF status (D.P. unpublished data)²³. An activating mutation of BRAF, V600E, is present in approximately 65% of melanomas²⁴. While this data set did not reveal a significant correlation between mH2A2 loss and V600E mutation, it produced similar mH2A2 results as the first cohort, as did a tissue microarray (Supplementary Fig. S4). Using fresh tissues, we observed significantly reduced levels of mH2A1 and mH2A2 mRNA in metastatic melanoma specimens as compared to nevi and primary melanocytes (Fig. 1c).

Due to the transcriptional downregulation of mH2A in human melanoma, combined with its re-expression in metastatic melanoma cells upon 5-Aza-2'-Deoxycytidine treatment (Supplementary Fig. S5), we hypothesized that DNA methylation may enable silencing of mH2A. Indeed, through extensive bisulfite sequencing analysis, we identified a region of the mH2A2 promoter that is significantly methylated in metastatic melanoma tissues and cell lines, but not in primary melanocytes, WM115 cells, or benign nevi (Fig. 1d; Supplementary Fig. S5).

Collectively, these findings prompted us to examine the functional consequences of mH2A loss. We established multiple stable shRNA lines in murine B16-F0 and F1 and human WM115 melanoma cells, targeting mH2A1, mH2A2, and control GFP (Supplementary Fig. S6, 7). Two shRNA-transduced cell lines from mouse and human were utilized for in-depth analysis: B16-F1 mH2A1_91 and mH2A2_25 and WM115 mH2A1_90 and mH2A2_05 (Supplementary Fig. S6, 7 for additional shRNA lines and isoform-specific knockdown).

Proliferation was examined in shRNA-expressing cells by colony formation and MTS cell viability assays. The loss of mH2A increases proliferation of murine and human melanoma cells (Fig. 2a, Supplementary Fig. S6, 7), as well as anchorage-independent growth of WM115 cells (Fig. 2b, Supplementary Fig. S6). In order to examine growth potential *in vivo*, B16-F1 shRNA cell lines were injected subcutaneously into mice; mH2A-deficient cells exhibited significantly enhanced tumour growth compared to controls (Fig. 2c). mH2A knockdown was confirmed by immunoblotting lysates from tumours (Supplementary Fig. S8).

Because cell motility contributes to metastasis and melanocytes originate from migratory neural crest cells, shRNA lines were analyzed for migratory behaviour. Loss of mH2A in murine and human cells enhanced migration through an 8 μ trans-well and the ability to close an artificial wound, as compared to control cells (Fig. 2d, Supplementary Fig. S6, 7). Next, shRNA cell lines were injected into the lateral tail veins of mice to assay metastatic potential. Fourteen days post-injection, mice were sacrificed and lungs dissected for macro- and microscopic histology (Fig. 2e). Lungs of mice injected with mH2A1_91 and mH2A2_25 cells showed a five- and thirty-fold increase, respectively, in the number of

macroscopic metastases as lungs from control mice (Fig. 2e). Hematoxylin and eosin (H&E) and Ki-67 staining revealed metastatic disease with proliferation, respectively, in mH2A shRNA-expressing tumours; mH2A knockdown in lungs was confirmed by IHC (Supplementary Fig. S8).

Next, mH2A expression was stably restored in malignant B16-F10 and human WM266-4 and A375 cells. The core histone H2A, mH2A1 (1.2) and mH2A2 were fused to mCherry and functional stable lines generated (Supplementary Fig. S9, 11, 13). Ectopic expression of mH2A1.2 and mH2A2, but not H2A or mCherry alone, resulted in reduced proliferation (without evidence of apoptosis) and migration (Supplementary Fig. S10, 12, 14). Human A375 cells expressing the mCherry series were injected subcutaneously into the flanks of immunocompromised mice; expression of mH2A1.2 and mH2A2 suppressed growth (Supplementary Fig. S14). Furthermore, B16-F10 cells expressing mCherry fusions were injected into tail veins of mice; mH2A1.2 and mH2A2 significantly suppressed metastasis to the lungs (Fig. 2f, Supplementary Fig. S10).

Given the striking phenotypes of mH2A manipulation in melanoma cells, we hypothesized that loss of mH2A may alter the transcriptional state of proliferation- and metastasis-related genes. We performed gene expression profiling using Affymetrix microarrays with B16-F1 cells (mH2A1_91, mH2A2_25 and sh_GFP). As expected for mH2A's role in fine-tuning of gene expression², many genes showed < 2-fold change, (Supplementary Fig. S15, 16 and 17 for Venn diagrams, heatmaps, and Gene Ontology). Fifteen genes showed ≥ 2-fold change, common to both shRNA lines in two independent experiments, including Integrin alpha 4 (Itga4), transcriptional regulators CDK8 (Mediator complex component) and Cited1 (CBP/p300 transactivator) (Fig. 3a). In concordance with our data, Itga4 expression is inversely correlated with invasive potential of B16 cells²⁵ and expression profiling of human melanoma cells identified Cited1 loss in metastatic cells²⁶. CDK8, however, is a new player in melanoma malignancy.

In concordance, CDK8 mRNA and protein levels were elevated in both murine and human cells depleted of mH2A (Fig. 3a-c), as well as subcutaneous tumours and lung metastases derived from mH2A-deficient lines (Supplementary Fig. S18). Next we stably expressed RNAi-resistant mH2A2 and H2A in the B16-F1 mH2A2_24 shRNA line, which targets the 3'UTR of mH2A2. While CDK8 levels remained high in mH2A2_24 cells expressing H2A-mCherry, addition of mH2A-mCherry rescued CDK8 expression to B16-F1 levels (Fig. 3d). Moreover, CDK8 was downregulated in subcutaneous tumours derived from A375 cells expressing mH2A1- and mH2A2-mCherry (Fig. 3e, Supplementary Fig. S14).

Intrigued by transcriptional up-regulation of CDK8, a colorectal cancer oncogene^{5,6}, we enquired if CDK8 is a direct target of mH2A. Chromatin Immunoprecipitation (ChIP) analysis demonstrated that the CDK8 promoter is enriched in mH2A1-containing nucleosomes in B16-F1, but absent in B16-F10 cells and a control shRNA line (Fig. 3f, Supplementary Fig. S18). ChIP analysis of additional mH2A target genes (but not an intergenic locus or GAPDH) also revealed enrichment, and demonstrated that CDK8 is a highly enriched mH2A target gene (Fig. 3f).

By examining a panel of human cell lines, we observed high CDK8 protein levels in metastatic melanoma cells, comparable to that of colon cancer cells (Fig. 4a). We utilized shRNAs to deplete CDK8 from B16-F10 and human A375 and WM165-1 cells, which contain high levels of CDK8 (Fig. 4b, Supplementary Fig. S19, 20). The loss of CDK8 significantly reduced proliferation (Fig. 4b) mediated by G2/M arrest in human cells (Supplementary Fig. S20). Conversely, ectopic expression of CDK8 (and a kinase-defective mutant, D173A)5 in B16-F1 cells resulted in significantly increased proliferation (Supplementary Fig. S21). While proliferation in murine melanoma cells appears independent of CDK8's kinase activity, it may be consistent with recent studies demonstrating a kinase-independent role of CDK8²⁷.

To dissect the relationship between mH2A and CDK8, we first depleted CDK8 in mH2A shRNA-expressing cells (B16-F1 and WM115 lines). Knockdown of CDK8 was able to suppress the enhanced proliferation induced by mH2A loss *in vitro* and *in vivo* (Fig. 4c, d; Supplementary Fig. S21, S22). Knockdown of Med12, a subunit of the CDK8 submodule of Mediator 27, showed a similar effect (Fig. 4d; Supplementary Fig. S22), suggesting that CDK8 functions within the Mediator subcomplex in melanoma.

Next, we performed qRT-PCR of mH2A and CDK8 in 36 melanoma specimens. This analysis demonstrated a statistically significant inverse correlation of mH2A2 and CDK8 at the mRNA level (Pearson's $r = -0.406$; $p = 0.014$; Fig. 4e). We performed IHC for CDK8 in human tissues previously scored for mH2A2 (Kappa = 0.58), and observed strong CDK8 staining (scored 2-3) in a large fraction of mH2A2 negative (scored 0) melanomas (29/38 = 76%; Supplementary Fig. S23). A similar trend was observed in a panel of human melanoma cell lines (Supplementary Fig. S23). Finally, by probing fresh benign nevus tissues, we observed high mH2A and low CDK8 protein levels (Supplementary Fig. S23). Collectively, these results strongly suggest that CDK8 is a major effector of mH2A-mediated melanoma progression.

Here, we demonstrate that mH2A is globally lost during melanoma progression. Similar findings have recently been described in lung cancer; mH2A1.1 is enriched in pre-cancerous senescent cells, but lost upon bypass of senescence¹⁶. However, the mechanism by which this occurs and its biological consequences remain unclear. Our study suggests mH2A loss in melanoma, mediated in part by DNA methylation, occurs after a potential senescence bypass (i.e. in a nevus), but rather during the critical RGP to VGP transition. Nevertheless, mH2A isoforms may serve as important biomarkers for melanoma, and/or other cancers.

The data presented here point towards a novel mechanism whereby CDK8 is regulated by the unique histone variant mH2A. We look forward to future studies focused on CDK8 function and its inhibition in melanoma. Our findings support emerging links between chromatin structure and cancer, and for the first time, demonstrate a direct role of mH2A in this process.

Methods Summary

Cell culture, plasmids, infections and RNAi

Detailed information is described in Methods.

Chromatin fractionation, acid extraction of histones and immunoblotting

Chromatin fractionation and acid extraction of histones performed as described¹⁴. Antibodies used for immunoblotting can be found in Methods.

Quantitative mass spectrometry

Q-MS performed as previously described²².

Immunohistochemistry, pathology and statistical analysis

Specimens were obtained from MSSM's Division of Dermatopathology (Project# HSD08-00565), NYU (IRB# 10362), and melanoma tissue microarray (Imgenex #IMH-369). Details on staining, pathology and statistical analyses described in Methods.

Clinical Specimens

Human specimens were collected at the time of surgery. Approval to collect melanoma specimens was granted by Mount Sinai Biorepository Cooperative and the NYU IMCG (Project #'s above). Approval to collect benign nevi was granted by MSSM's Division of Dermatopathology (Project # 08-0964).

Bisulfite sequencing

Performed according to manufacturer's instructions (Zymo Research). Details described in Methods.

Cell proliferation, migration and mouse injections

MTS performed according to manufacturer's instructions (Promega). Colony formation and soft agar assays performed as described²⁸. Trans-well migration assay described in Methods. *In vivo* metastasis assays performed as described²⁸. For subcutaneous injections, 2.5×10^5 B16-F1 cells were injected into 6-week old C57BL/6J mice and 2×10^6 A375 cells injected into NOG mice (Jackson Laboratories); tumour volume measured over a 14 and 20-day period, respectively.

Microarray hybridization, data analysis and hierarchical clustering

Microarray was performed using two biological replicates according to Affymetrix GeneChip protocol. Initial data extraction performed at the Microarray Shared Research Facility at MSSM. Heatmaps generated using Cluster and Tree View programs.

Quantitative PCR and Chromatin Immunoprecipitation

qPCR performed in triplicate on Stratagene Opticon 2 using FastStart SYBR Green Mix (Roche). Expression levels normalized to TATA Binding Protein (TBP) or GAPDH. ChIP assays performed using Magna ChIP™ Kit (Millipore) as per manufacturer's instructions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

Methods

Cell culture, plasmids and infections

Murine B16 and human WM266-4, A375 and HCT116 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. All other melanoma cells were grown in Tu2% media (80% MCDB 153 media, 20% Leibovitz's L-15 media, 2% FBS, 5µg/ml bovine insulin, 1.68mM CaCl₂). Human melanocytes were grown in Medium 254 (Invitrogen). Lentiviral plasmids encoding shRNAs against murine and human mH2A1, mH2A2, GFP (control) and murine CDK8 were obtained from Open Biosystems (Thermo Fisher Scientific). Med12 and CDK8 shRNAs were kindly provided by Joaquin Espinosa (University of Colorado, Boulder) and Addgene (CDK85). RNAi sequences are listed in Supplementary Table 1. cDNA encoding human H2A, rat mH2A1.2 and human mH2A2 were amplified and cloned into the retroviral vector N-Cherry-LPC (gift of M. Narita; Cambridge, UK). Plasmids expressing CDK8 (pBabe.puro.CDK8 and CDK8-KD5) were obtained from Addgene. Infections were carried out using standard procedures.

Chromatin fractionation, acid extraction of histones and immunoblotting

Chromatin fractionation and acid extraction of histones performed as described¹⁴. Whole cell extracts were generated by lysing cells directly in Laemmli loading buffer, followed by sonication (for tumor tissue), and boiled extensively. The following antibodies were used for immunoblotting: mH2A1 (Millipore 07-219); mH2A211; H3 C-terminal (Abcam ab1791 or Millipore 05-928); H4 (Millipore 05-858); H2A.X (Millipore 07-627); H2A.Z (Millipore 07-594); CDK8 (Santa Cruz sc-1521); Cited1 (Abcam ab15096); DsRed (Clontech 632496); and Actin (Sigma A5441).

Quantitative mass spectrometry

Bulk acid extracted histones were derivatized by treatment with propionyl anhydride as described²². Histones were stable isotope-labeled using *d*₁₀-propionic anhydride

(Cambridge Isotope Laboratories, Inc., Andover, MA). On-line HPLC separation of peptides was followed by LC-MS/MS using LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA) as described²². All data was manually inspected for quantification and MS/MS interpretation. Two independent experiments using biological replicates were performed.

Immunohistochemistry, pathology and statistical analysis

Specimens were obtained from MSSM's Division of Dermatopathology via Institutional Review Board approval (Project # HSD08-00565) and NYU (IRB#10362). Primary RGP melanoma (Breslow thickness <1.0mm) and VGP melanoma (Breslow thickness >1.0mm) were examined. IHC was also performed on BRAF-genotyped melanoma (NYU) and melanoma Tissue Microarray (Imgenex IMH-369). IHC performed as per manufacturer instructions (Vector Laboratories, USA). In brief, five micron sections from formalin-fixed paraffin-embedded (FFPE) specimens were deparaffinized, incubated for antigen retrieval with Vector Citrate-Based Antigen Unmasking Solution (Vector Laboratories H-3300) in microwave for 10 min, exposed to 0.3% hydrogen peroxide to block endogenous peroxidase activity, blocked with Vector Normal Horse Serum (2.5%) x 20 minutes, incubated with mH2A211 (1:350-1:500) prepared in 0.1% BSA and incubated at 4°C overnight. Slides were subsequently developed using Vector imPRESS Universal Kits Anti-Mouse/Rabbit Ig or Anti-Goat Ig (Vector Laboratories MP-7500 or MP-7405), Vector DAB Peroxidase Substrate Kit as the chromagen (Vector Laboratories SK-4100) and Harris Hematoxylin (Sigma HHS32) for counterstaining. Slides then sealed and mounted with Permount (Sigma SP15) and randomized for subsequent blinded review. Two independent dermatopathologists (P.O.E. and C.I.V.) scored specimens for extent of melanocyte nuclear staining (0- 3). Slides were compared with H&E sections and all slides stained with H3 (Abcam ab1791 or Millipore 05-928; both at 1:200) for tissue quality control. For CDK8 staining, mH2A negative melanomas were stained (Santa Cruz sc-1521; 1:200 and sc-13155; 1:15), followed by randomization and scoring. All statistical analyses were conducted using SPSS 14 software (SPSS Inc., Chicago). Average staining score was used for analyses and inter-observer consistency between dermatopathologists assessed with Kappa-Coefficient. Statistical significance of mH2A2 scores first assessed using the non-parametric Kruskal-Wallis one-way analysis of variance test, followed by two-sided Mann-Whitney U-tests.

Micrococcal nuclease assays

Cells were counted (Beckman Coulter particle counter) and evenly aliquoted (2.5×10^6 cells per MNase time point). Each sample treated with 1/150 unit of micrococcal nuclease (Sigma) for 2, 5, 7 or 10 minutes at 37°C, and stopped with 1mM EGTA. Samples were centrifuged at 10K RPM ($10'$) and DNA extracted using DNeasy Blood and Tissue Kit (Qiagen). Equal amounts of DNA were resolved on 1% agarose gel and stained with ethidium bromide.

Aza treatment and bisulfite DNA methylation analysis

Cells were treated with 10 μ m 5-Aza-2'- Deoxycytidine and harvested at days 2 and 4. Fresh medium containing 5-Aza-2'- Deoxycytidine was added at day 2 for harvesting at day 4. For

bisulfite DNA methylation analysis, DNA from cells and tissues was prepared with DNeasy blood and tissue kit (Qiagen). Bisulfite treatment was performed with EZ DNA methylation kit (Zymo Research) according to the manufacturer's instructions (2 µg of DNA was used in the bisulfite reaction). Following bisulfite conversion, DNA was amplified by PCR in triplicate, pooled, cloned into pGEMT (Promega) and sequenced using SP6 universal primer. Primers used for amplifying mH2A2 promoter are as follows: M2-CG2-F: GTTTAGTTTTGGGGAAAGTTTTATGT and M2-CG2-R: TAAAAAAATTACTCAACCTCATCC. The online tool QUMA (<http://quma.cdb.riken.jp/>) was used for bisulfite sequencing analysis²⁹.

Cell proliferation, soft agar and migration assays

MTS proliferation kit used according to manufacturer's instructions (Promega). Absorbance values (490 nm) were recorded on at least triplicate samples using a BIOTEK Microplate Reader. Colony assays performed by seeding cells at low density and allowing growth for 10 days. Colonies were fixed, stained with crystal violet and counted. Soft agar performed essentially as described²⁸. Briefly, cells were plated in Tu2% media with 0.33% (w/v) noble agar on top of a 0.5% noble agar layer. After three weeks, colonies were stained, photographed and counted in five different fields using an inverted microscope. Cell migration was measured by trans-well assay (8-µm pores from Corning, Inc). Cells were suspended in serum-free medium, and DMEM supplemented with 10% FBS used as chemoattractant. For assays with WM115 cells, the lower surface of the trans-well was pre-coated with fibronectin (Sigma, 100 µg/mL for 30min at 37°C). Cells that migrated after 18 hours were stained with Diff-Quick Stain Kit (Dade Behring) and counted in five different fields using an inverted microscope. Wound healing assays were performed as described²⁸.

Statistics

All results are presented as the mean ± s.d or s.e.m as indicated. Statistical analyses performed by calculating p-values using unpaired student t-test (two tailed), unless indicated otherwise.

Flow Cytometry

Cells were harvested, washed in phosphate-buffered saline (PBS), and fixed in ice-cold 70% ethanol. Propidium iodide staining was carried out using Cycletest Plus staining kit following manufacturers instructions (Becton Dickinson Inc.). For apoptosis studies, cells were analyzed by flow cytometry by Annexin V staining using Apoptosis detection kit (R&D systems).

In vivo metastasis assay and subcutaneous injections

In vivo metastasis assays were performed as described²⁸. Briefly, 2×10^5 B16-F1 cells (stably transduced with sh_GFP, mH2A1_91 and mH2A2_25 shRNA) and 1.5×10^5 B16-F10 cells (stably transduced with mCherry, H2A-mCherry, mH2A1.2- and mH2A2-mCherry) were injected intravenously in BALB/c mice. Mice injected with B16-F1 cells were sacrificed 14 or 21 days post-infection (performed in duplicate with similar results; 6-8 mice per group). Mice injected with B16-F10 cells were sacrificed 10 days post-injection

(n= 6-9 per group). Lungs were removed and fixed, isolated and discrete pigmented lung surface lesions were counted. For subcutaneous injections, 2.5×10^5 of B16-F1 cells stably infected with shRNAs were injected in the flanks of 6-week old C57BL/6J mice (Jackson Laboratories). Mice were injected (6-9 per group) and measurements taken over 12-14 days. 2×10^6 A375 cells expressing mCherry series were injected into NOG mice (NOD/Shi-scid/IL-2R γ null, Jackson Laboratories), and measurements taken over 21 days. Tumour volume estimated by $V = (a^2 \times b)/2$ where a is the short axis and b is the long axis of the tumour. Tissues from all assays were paraffin-embedded and 5 μ m sections H&E stained. Experiments were conducted under protocol #080901-01 approved by NYU Institutional Animal Care and Use Committee.

Microarray Hybridization and Data Analysis

Microarray samples were processed in the Microarray Shared Research Facility at MSSM and performed on two biological replicates. Total RNA was isolated from cells using RNeasy column purification per manufacturer's protocol (Qiagen). The quality of the RNA was evaluated using the Agilent BioAnalyzer RNA nano assay. Briefly, 150ng of total RNA was reverse transcribed using T7-poly(dT) primer and converted into double-stranded cDNA. The cDNA was used as a template for subsequent in vitro transcription with biotin-labeled UTP at 37°C for 16 h using Genechip 3' IVT express kit (Affymetrix). The resulting biotin-labeled cDNA was chemically fragmented, made into hybridization cocktail and hybridized to the Mouse Genome 430 Plus 2.0 arrays (Affymetrix) according to the Affymetrix GeneChip protocol. The array images were generated through a high-resolution GeneChip Scanner 3000 7G (Affymetrix), then converted to digitized data based on MAS 5.0 within the GeneChip Operating Software (GCOS). Spike-in controls and percentage of present ('P') call generated were used for data quality control. Data analysis was performed as follows: *Normalization* – All chip data was scaled to have an average signal intensity of 150. *Comparison analysis*- Comparison analysis based on MAS 5 was performed for each pair (sh_GFP compared to mH2A1_91 and sh_GFP compared to mH2A2_25) on both data sets. The compared data was subjected to the following arbitrary filters to improve data reliability: 1) Detection call – only probes that had at least one 'P' call in the pair were retained. 2) Signal intensity – only probes that showed signal intensity ≥ 100 in at least one of the pair were retained. 3) Fold change – probes that showed log fold-change ≥ 2 in at least one data set were retained for further analysis. 4) Concordance analysis was performed to reduce false positive selection between shRNA lines and the two independent microarray experiments: direction and fold-change in gene regulation had to be 100% match to qualify as altered genes. Gene list was annotated by submission to Netaffx annotation center within Affymetrix website (<http://www.affymetrix.com>), which periodically updates the integrated information for each gene across multiple public genome databases.

Hierarchical clustering, generation of heat maps and GO analysis

A portion of the filtered subset of data was used for additional analysis. Cluster Analysis was performed by unsupervised hierarchical clustering on the log-transformed data with Gene Cluster 3.0 (<http://bonsai.ims.u-tokyo.ac.jp/~mdphoon/software/cluster/index.html>) by using the correlation (uncentered) similarity metric and centeroid linkage clustering method.

The resulting tree-images were visualized using Java TreeView. GO analysis was performed using DAVID Bioinformatics Resources30 (<http://david.abcc.ncifcrf.gov/home.jsp>).

Quantitative PCR and Chromatin Immunoprecipitation

Total RNA was extracted using RNeasy kit (Qiagen). Reverse transcription performed with SuperScript II (Invitrogen) using oligo dT. qPCR reactions performed in triplicate on Stratagene Opticon 2 using Fast start SYBR Green Mix (Roche). Expression levels normalized to TATA Binding Protein (TBP) in mouse cells and GAPDH in human cells; or relative to B16-F1 sh_GFP for gene target expression. Each qPCR performed using two independent biological replicates. Primer sets used for qRT-PCR listed in Supplementary Table 2. ChIP assays performed using Magna ChIP™ Kit (Protein G; Millipore) as per manufacturer's instructions. IPs performed with antibodies against mH2A1 (Millipore 07-219), H3 (Abcam ab1791), and control IgG (Millipore 12-370). ChIP signal represented as percentage of H3; calculated by $100 \times 2^{-(Ct_{H3} - Ct_{Antibody})}$. Primers used for ChIP-qPCR are listed in Supplementary Table 3.

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Figure 1. mH2A loss correlates with increasing melanoma malignancy

a, Melanoma cells probed for H2A variants; core histones used for loading. **b**, IHC of human tissue with mH2A2 (left), and histone H3 (right). 20x and 40x shown. mH2A2 visualized using DAB (brown) and hemotoxalin (blue). Arrows depict mH2A2 staining in non-melanocytic cells. **c**, qRT-PCR of mH2A1 and mH2A2 in benign nevi and melanocytes (brown circles) and metastatic melanoma (black squares); $P < 0.0001$. **d**, DNA methylation of mH2A2 promoter in nevi ($n=6$, 10-12 clones per nevus) and metastatic melanoma tissues ($n=7$, 10-14 clones per tissue); 16 CpG sites shown. Open circles (unmethylated), black circles (methylated); P-values determined by Mann-Whitney U-test.

Figure 2. mH2A depletion and ectopic expression alter malignant properties of melanoma cells *in vitro* and *in vivo*

a, Colony assay of B16-F1 shRNA-expressing cells. Quantified (below); *P < 0.0000005. Mean ± s.e.m; (n=4). **b**, Soft agar assay of WM115 shRNA-expressing cells. Quantified (below); *P < 0.0005. Mean ± s.d; (n=4). **c**, Tumour volume (mm³) post-subcutaneous injections of B16-F1 shRNA cells; *P < 0.05 at day 14. Mean ± s.e.m; (n=10 mice per group). **d**, Trans-well migration assay in B16-F1 and WM115. Quantification below, *P < 0.0001 Mean ± s.d; (n=5). **e**, Representative lungs of B16-F1 shRNA cells injected intravenously, 14 days post-injection. Mean ± s.e.m; nodules/lung/mouse shown (n=6-8 mice per group). **f**, Representative lungs of B16-F10 mCherry cells injected intravenously, 10 days post-injection. Quantified (right); *P < 0.005. Mean ± s.e.m; (n=6-9 mice per group).

Figure 3. Microarray and ChIP analysis identify CDK8 as a direct mH2A-regulated gene in melanoma

a, Heat map representing gene expression changes (2-fold) in B16-F1 mH2A-deficient cells. qRT-PCR of CDK8 (bottom); Mean $\pm$ s.d; (n=3). **b**, Immunoblots of CDK8 in murine shRNA lines; actin for loading. **c**, Immunoblot and qRT-PCR of CDK8 in human shRNA lines; Mean $\pm$ s.d; (n=3). **d**, CDK8 and DsRed immunoblots of mH2A2_24 line expressing H2A- and mH2A2-cherry; asterisk depicts fusion proteins. **e**, CDK8 qRT-PCR analysis in A375 subcutaneous tumours; Mean $\pm$ s.d; (n=3). **f**, mH2A1 ChIP analysis of the -1kb position from TSS for CDK8, PACS2, ATP5G1, and GAPDH; intergenic control. IgG used as control antibody; Mean $\pm$ s.d; (n=3).

Figure 4. CDK8 is a major effector of mH2A loss

a, Melanocytes, metastatic melanoma, and colon cancer cells probed for CDK8; actin for loading. **b**, Immunoblot of A375 cells expressing CDK8 shRNA (left), MTS assay (right), Mean $\pm$ s.d; (n=5). **c**, Tumour volume (mm³) post-subcutaneous injections; *P < 0.05 at day 12. Mean $\pm$ s.e.m; (n=9 mice per group). **d**, MTS assay of WM115 mH2A1_90 line co-expressing CDK8 or Med12 shRNAs, Mean $\pm$ s.d; (n=5). **e**, qRT-PCR of mH2A2 and CDK8 in 36 melanoma tissues (from 30 patients; Supplementary Table S4); Pearson's $r = -0.406$ with $p = 0.014$. Mean $\pm$ s.d; (n=3).