Published in final edited form as:

Cancer Res. 2018 July 15; 78(14): 3834–3848. doi:10.1158/0008-5472.CAN-17-3571.

HP1 γ promotes lung adenocarcinoma by downregulating the transcription-repressive regulators NCOR2 and ZBTB7A

Hunain Alam¹, Na Li¹, Shilpa S. Dhar¹, Sarah J. Wu^{1,2}, Jie Lv^{3,4,5}, Kaifu Chen^{3,4,5}, Elsa R. Flores⁶, Laura Baseler^{7,8}, and Min Gyu Lee^{1,2}

¹Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, TX, USA.

²The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX, USA.

³Institute for Academic Medicine, The Methodist Hospital Research Institute, Houston, TX, USA.

⁴Department of Cardiovascular Sciences, Center for Cardiovascular Regeneration, The Methodist Hospital Research Institute, Houston, TX, USA.

⁵Weill Cornell Medical College, Cornell University, New York, NY, USA.

⁶Department of Molecular Oncology, Moffitt Cancer Center, Tampa, FL 33612, USA.

⁷Department of Veterinary Medicine and Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX, USA.

⁸Current address: University of British Columbia, School of Population and Public Health, 2329 West Mall Vancouver, British Columbia V6T 1Z4, Canada; Animal Health Centre, Ministry of Agriculture, 1767 Angus Campbell Rd., Abbotsford, British Columbia V3G 2M3, Canada.

Abstract

Lung adenocarcinoma (LUAD) is a major form of lung cancer, which is the leading cause of cancer death. Histone methylation reader proteins mediate the effect of histone methylation, a hallmark of epigenetic and transcriptional regulation of gene expression. However, their roles in LUAD are poorly understood. Here our bioinformatic screening and analysis in search of an LUAD-promoting histone methylation reader protein show that heterochromatin protein 1 γ (HP1 γ ; also called CBX3) is among the most frequently overexpressed and amplified histone reader proteins in human LUAD, and that high *HP1 γ* mRNA levels are associated with poor prognosis in LUAD patients. *In vivo* depletion of HP1 γ reduced K-Ras^{G12D}-driven LUAD and lengthened survival of mice bearing K-Ras^{G12D}-induced LUAD. HP1 γ and its binding activity to methylated histone H3 lysine 9 were required for the proliferation, colony formation, and migration of LUAD cells. HP1 γ directly repressed expression of the transcription-repressive regulators NCOR2 and ZBTB7A. Knockdown of NCOR2 or ZBTB7A significantly restored defects in proliferation, colony formation, and migration in HP1 γ -depleted LUAD cells. Low

Corresponding Author: Min Gyu Lee, Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: 713-792-3678, Fax: 713-794-3270, mglee@mdanderson.org.

The authors declare no potential conflicts of interest.

NCOR2 or *ZBTB7A* mRNA levels were associated with poor prognosis in LUAD patients and correlated with high *HP1* mRNA levels in LUAD samples. *NCOR2* and *ZBTB7A* downregulated expression of tumor-promoting factors such as *ELK1* and *AXL*, respectively. These findings highlight the importance of *HP1* and its reader activity in LUAD tumorigenesis and reveal a unique LUAD-promoting mechanism in which *HP1* downregulates *NCOR2* and *ZBTB7A* to enhance expression of pro-tumorigenic genes.

Keywords

Histone reader; Heterochromatin protein 1; lung adenocarcinoma; histone methylation; *NCOR2*; *ZBTB7A*

Introduction

Lung cancer is the leading cause of global cancer-related death in both men and women. The overall 5-year survival rate for lung cancer patients is low (about 18.1%). Lung adenocarcinoma (LUAD), the most prevalent histological subtype of lung cancer, accounts for approximately 40% of lung cancer cases. The molecular etiology of LUAD is diverse. For example, activating mutations and gene amplification of oncogenic kinases (e.g. *K-Ras*, *EGFR*, *MET*, and *ERBB2*) and inactivating alterations in tumor suppressor genes (e.g. *TP53* and *LKB1/STK11*) frequently occur in LUAD (1, 2). For the treatment of LUAD patients, much research has focused on kinase signaling. Kinase-targeted therapies (e.g., the EGFR inhibitor erlotinib) have been developed but had a limited success because of tumor recurrence. There is still a lack of well-defined molecular targets for treating LUAD. Therefore, there is a great need for a better mechanistic understanding of LUAD tumorigenesis.

Histone methylation is a key mark of epigenetic and transcriptional gene regulation and occurs at both lysine and arginine residues in histones. This modification plays an important role in regulating various biological processes, including cellular differentiation, stem cell maintenance, and cancer (3). Histone methylation is associated with either gene activation or silencing, depending on the modified sites, at the genome-wide levels. For example, methylation at histone H3 lysine 9 (H3K9) is generally coupled with gene silencing, whereas methylation at H3K4 is linked to gene activation (3). The levels of histone methylation can be dynamically regulated by histone methyltransferases and demethylases.

Histone methylation provides specific binding sites for 'readers' (also called binding modules). The reader-containing proteins mediate the effect of histone methylation. Such readers include chromodomain, Tudor, PHD (plant homeodomain), PWWP (Pro-Trp-Trp-Pro), bromo-adjacent homology (BAH), MBT (malignant brain tumor), WD40, ankyrin, and the zinc finger CW (zf-CW) (4). These readers are present in multiple types of proteins, including transcription factors, DNA-modifying enzymes, and histone-modifying enzymes. It has been known that deregulated histone methylation reader proteins contribute to cellular transformation and tumorigenesis. However, the pathogenic roles of histone methylation reader proteins in LUAD tumorigenesis are largely unknown.

Recently, multiple tumor-sequencing studies have compiled a list of mutations and genomic rearrangements in LUAD, providing great resources to screen potential candidate genes implicated in lung tumorigenesis. In search of an oncogenic histone methylation reader protein in LUAD, we performed *in silico* screening with use of several databases (e.g., The Cancer Genome Atlas [TCGA]) to determine which proteins of 124 histone methylation reader proteins undergo alterations in expression, DNA sequences, and copy numbers in LUAD. This screening led us to identify heterochromatin protein 1 γ (HP1 γ), an di- and trimethylated H3K9 (H3K9me2/3) reader protein (5–7), as one of the most frequently amplified and overexpressed histone methylation reader proteins in human LUAD. Because the *in vivo* tumor-promoting role of HP1 γ and its mechanism of action in LUAD were unclear, we chose to study the role of HP1 γ in LUAD. Our analysis showed that high HP1 γ protein and mRNA levels correlated with poor prognosis in lung cancer patients. *In vivo* HP1 γ knockdown in the K-Ras^{G12D} LUAD mouse model inhibited K-Ras^{G12D}-induced tumorigenicity and prolonged mouse survival. Our results from RNAi and rescue experiments demonstrated that HP1 γ and its H3K9me2/3-binding activity were required for the proliferation, anchorage-independent growth, and migration of LUAD cells. Our mechanistic results provided evidence that HP1 γ downregulates expression of the transcription-repressive regulators NCOR2 and ZBTB7A to upregulate expression of several tumor-promoting factors, such as AXL, PVT1, and ELK1. These findings provide a previously unknown mechanistic insight into the pathogenesis of LUAD and also suggest a rationale for targeting the binding activity of the prognostic biomarker HP1 γ in LUAD patients with few therapeutic options.

Materials and Methods

Samples, reagents, cell lines, and antibodies.

For immunohistochemical (IHC) experiments, human lung normal and tumor tissue microarrays (TMAs) were purchased from Biomax (LC951 and LC1291) and Imgenex (IMH-305). IHC analysis was performed on these TMAs as previously described (8). All lung cancer cell lines were procured from ATCC (Rockville, MD, USA), which verifies cell lines using short tandem repeat analysis, and were cultured within 15 times of passages in 2–3 months. Mycoplasma testing was conducted for cell lines using the mycoplasma detection kit Plasmotest™ (InvivoGen). Cell culture reagents and other chemicals were purchased from Gibco, Hyclone, Corning, Sigma-Aldrich and Fisher Bioreagents. The antibodies used for this study are listed in Supplementary Table S1.

Expression data and survival analysis.

TCGA, NCI Director's Challenge Consortium, and lung cancer transcriptomic datasets (SE37745 and GSE29013) were used for expression data and survival analysis (see Supplementary methods for details).

IHC experiments, IHC scoring, and immunofluorescence.

IHC experiments were performed as described previously (8). TMA samples (n = 73) with clear IHC staining and survival follow-up data of patients were scored on a scale of 0–5 based on the percentage of tumor cells stained: 0, negative; 1, 1%–10%; 2, 11%–25%; 3,

26%–50%; 4, 51%–70%; 5, >71%. The samples were further divided into low (score 0–3) and high (4) score categories. For immunofluorescence, Alexa 488-conjugated anti-mouse IgG and Alexa 568-conjugated anti-rabbit IgG secondary antibodies were used for detection, and images were captured using a laser confocal microscope.

Mouse strains and *in vivo* lung tumorigenesis study.

Briefly, shRNAs against mouse HP1 γ (mHP1 γ) were designed and cloned into U6-shRNA pgkCre vector (Addgene plasmid # 24971; provided by Tyler Jacks) (Supplementary Table S1). To induce tumors in the lungs of 6–8 week old K-*Ras*^{LSL-G12D} mice (Strain number 01XJ6, NCI mouse repository), each mouse was infected with 1×10^5 lentivirus particles that expressed either Cre alone (a control) or both Cre and shHP1 γ (shmHP1 γ -1-Cre) via an intratracheal intubation method. The mice were monitored for tumor growth for 12 to 14 months using micro-computed tomography (micro-CT). Mouse survival was compared between the control group and shmHP1 γ -1-Cre group. Humane end-points were used in the mouse experiments. The lungs were collected at necropsy and lung tumors were analyzed microscopically (see Supplementary methods for details).

Stable knockdown

For knockdown experiments, lentivirus-based, puromycin-resistant shRNAs were purchased from Sigma and Open Biosystem (Supplementary Table S1). The shRNA-infected cells were selected in puromycin-containing medium (1 μ g/ml). shLuciferase (shLuc)-infected cells were used as a control.

Quantitative RT-PCR, Western blot, and chromatin immunoprecipitation (ChIP) assays

The quantitative RT-PCR, Western blot, and ChIP assays were performed as previously described (8, 9). The primers and antibodies used for quantitative RT-PCR and ChIP assays are listed in Supplementary Table S1.

Cell proliferation, cell migration, and soft agar assays

Cell proliferation, cell migration, and soft agar assays were performed as previously described (8, 10).

Clonogenic cell survival assay

Cells (1×10^3) were plated onto six-well plates in triplicate. After 10–14 days passed, the cells were fixed with 4% paraformaldehyde, followed by staining with 0.5% crystal violet for 1 hour at room temperature. Stained cells were then washed with 1 x PBS, and images were captured.

Rescue and overexpression experiments.

For rescue experiments, human HP1 γ cDNA was cloned into the lentivirus vector pLenti6.3/V5-DEST (Thermo Fisher Scientific) using standard cloning methodology. To generate an H3K9me2/3-binding mutant of HP1 γ (mtHP1 γ), HP1 γ cDNA cloned in pLenti6.3/V5-DEST was mutated by site-directed mutagenesis. Then, shRNA-resistant HP1 γ and mtHP1 γ constructs were generated by introducing silent mutations into the target

site of shHP1 γ -16. The primer sequences used for mutagenesis are listed in Supplementary Table S1. Cells infected by pLenti6.3 were selected in blasticidin-containing medium (5 μ g/ml). For HP1 γ overexpression experiments, cells infected with pLenti6.3-GFP and pLenti6.3 vector were used as controls.

Microarrays.

The whole transcriptomic analysis was performed using Affymetrix GeneChip™ Human Genome U133 Plus 2.0 Arrays. Transcriptomic profile data of HP1 γ -depleted cells were compared with those of shLuc-infected cells. Gene ontology analysis was performed using DAVID functional annotation tools (<http://david.abcc.ncifcrf.gov>). The accession number for transcriptomic profile data reported in this paper is GEO: GSE111321.

Statistical analysis.

Student's t-test was used to determine the statistical significance of two groups of data. For correlation analysis, the chi-squared test was performed to calculate the level of significance. The log-rank method was used to test the statistical significance of survival data. Data are presented as means $\pm$ standard error of the mean (SEM; error bars). *P*-values less than 0.05 were considered statistically significant. *, *P*<0.05; **, *P*<0.01; and ***, *P*<0.001 indicate statistically significant differences.

Study approval.

The care and use of all mice was approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

Results

HP1 γ is frequently amplified and overexpressed in LUAD, and its high levels are linked to poor prognosis in LUAD patients

To identify a histone methylation reader protein with oncogenic function in LUAD, we first analyzed whether histone methylation reader proteins, including those in the WERAM database (<http://weram.biocuckoo.org/>) (11), are altered in TCGA human LUAD samples (Fig. 1A). For this analysis, we considered the following three types of alterations: copy number variations, mutations, and changes in mRNA expression. Of 124 histone methylation reader proteins that we analyzed using TCGA database, 58 proteins showed 8% alterations (Fig. 1B; the top 15 proteins are detailed in Supplementary Fig. S1A and B). We then determined whether these 58 proteins are upregulated in LUAD tumors compared with adjacent normal lung tissues. Of 58 reader proteins, 14 showed 1.5-fold upregulation in their mRNA levels (Fig. 1C; Supplementary Fig. S2; Supplementary Table S2). These top 14 hits were further subjected to Kaplan-Meier survival analysis using a publicly available microarray dataset of 443 lung adenocarcinoma patients (stages I, II, and III) from the NCI Director's Challenge Consortium for the Molecular Classification of Lung Adenocarcinoma. Of the 14 hits, data for 12 reader proteins, all except PYGO2 and CDYL2, were available, and high HP1 γ and MSH6 mRNA levels showed statistically significant shorter survival in LUAD patients (Fig. 1D; Supplementary Fig. S3). Median survival time of HP1 γ -high LUAD patients (21.2 $\pm$ 3.42 months) was significantly shorter than that of MSH6-high

LUAD patients (30.1 ± 13.0 months). In addition, results from TCGA database analysis demonstrated that alterations in *HP1 γ* but not *MSH6* correlated with shorter overall and disease-free survival in LUAD patients (Supplementary Fig. S4A–D). These results led us to choose HP1 γ as a putative oncogenic histone methylation reader protein for subsequent analyses.

To further determine the lung cancer relevance of HP1 γ , we assessed the association of HP1 γ levels with survival in lung cancer patients by analyzing several other lung cancer datasets. Our analysis demonstrated that high *HP1 γ* mRNA levels, but not high *MSH6* mRNA levels, were consistently associated with shorter survival in lung cancer patients. (Supplementary Fig. S5A–D). Interestingly, *HP1 γ* mRNA levels were also increased in lung squamous cell carcinomas (Supplementary Fig. S5E). In addition, IHC analysis showed that in contrast to only 12.5% (1/8) of normal lung samples, 32.9% (24/73) of lung cancer samples displayed high HP1 γ protein levels (Fig. 1E; Supplementary Fig. S5F; Supplementary Table S3). Survival analysis of these lung cancer patients ($n = 73$) demonstrated that patients with high HP1 γ protein levels survived shorter than did those with low HP1 γ protein levels (Fig. 1F; Supplementary Table S4). Together, these results indicate that high HP1 γ levels correlate with worse survival in lung cancer patients.

***In vivo* knockdown of HP1 γ in mouse lung impedes K-Ras^{G12D}–driven LUAD tumorigenicity and increases survival of mice bearing K-Ras^{G12D}–driven LUADs.**

To determine the importance of HP1 γ in *in vivo* LUAD tumorigenesis, we examined the effect of HP1 γ knockdown on K-Ras^{G12D}–driven mouse lung tumorigenesis. The K-Ras^{G12D} mouse model was used because it is a well-established LUAD mouse model (12) and HP1 γ overexpression partially overlapped with K-Ras mutations in LUAD samples (Supplementary Fig. S6A). To achieve HP1 γ knockdown *in vivo*, we employed the lentivirus-based U6-shRNA-pgkCre system (Supplementary Fig. S6B) (13). This lentiviral vector system not only expresses Cre recombinase under the pgk promoter to induce K-Ras^{G12D} expression by Cre-mediated deletion of the LSL (loxP-STOP-loxP) cassette but also produces shRNA under the U6 promoter to deplete a specific protein. We cloned four different shRNAs against mouse HP1 γ (shmHP1 γ –1, –2, –3 and –4) in the U6-shRNA-pgkCre vector (see Supplementary Table S1). Our analysis of knockdown efficiency of U6-shHP1 γ -pgkCre constructs in mouse lung 393P cells showed that shmHP1 γ –1 was the most effective shRNA against mouse HP1 γ (Supplementary Fig. S6C). Using HEK-293 cells containing a Cre-Reporter, we also determine whether Cre expressed from this vector is active. The Cre-Reporter HEK-293 cells were used because they normally express a green fluorescence protein (GFP) signal but, upon Cre-mediated deletion of GFP-Stop in loxP-GFP-Stop-loxP-RFP in the Cre-reporter, express red fluorescence protein (RFP). Our results showed that GFP signals switched to RFP signals after infection with lentiviruses containing U6-shHP1 γ -pgkCre, indicating that all constructs expressed active Cre (Supplementary Fig. S6D).

We next infected lungs of 6- to 8-week-old K-Ras^{LSL-G12D} mice with shmHP1 γ –1-Cre or control (only Cre expression) viruses using an intratracheal intubation method and monitored tumor growth in mouse lungs at 3 and 9 months post-infection using micro-CT

(Fig. 2A). Tumor sizes were significantly smaller in the shHP1 γ -1-Cre group of mice than in the control group (Fig. 2B and C). At 12 months post-infection, the average lung tumor burden in the shHP1 γ -1-Cre group was about one-half of that in the control group (Fig. 2D and E; Supplementary Fig. S7A). Moreover, the percentage of the lung effaced by tumors was analyzed and microscopically scored. As shown in Fig. 2F, the shHP1 γ -1-Cre group of mice had a lower percentage of their lung affected (grades I and II) than did the control group (grades II and III). Consistent with slower growth of HP1 γ -depleted tumors, fewer Ki-67-expressing cells were present in HP1 γ -depleted tumors than in control tumors (Fig. 2G and H). IHC analysis for the well-known LUAD marker TTF1/NKX2-1 showed that lung tumors from both groups expressed TTF1, confirming that the K-Ras^{G12D} model develops LUAD (Supplementary Fig. S7B). In addition, HP1 γ expression in K-Ras^{G12D} mice was higher in tumor cells than in adjacent normal lung cells (Supplementary Fig. S7C). Notably, shHP1 γ -1-Cre mice had significantly longer survival times than did control mice. Median survival times of shHP1 γ -1-Cre and control mice were 354 and 286 days, respectively (Fig. 2I). These results indicate that *in vivo* depletion of HP1 γ inhibits LUAD tumorigenicity in the K-Ras^{G12D} mouse model.

HP1 γ and its H3K9me2/3-binding ability are necessary for the proliferation, colony formation, and migration of LUAD cells.

Oncogenic proteins commonly play a critical role in the proliferation, anchorage-independent colony formation, and migration of cancer cells. To investigate whether HP1 γ is required for these cellular properties of LUAD cells, we examined the effect of HP1 γ knockdown on such cellular properties of H1792 cells (an LUAD cell line with relatively high HP1 γ levels) using the two most effective shRNAs (shHP1 γ -16 and shHP1 γ -17) of five HP1 γ shRNAs tested (Supplementary Fig. S8A–C). HP1 γ knockdown in H1792 cells reduced cell proliferation and cell cycle S phase but increased subG1 phase (Supplementary Fig. S8D and E). In addition, HP1 γ depletion highly inhibited colony formation in the clonogenic cell survival assay, anchorage-independent colony formation in soft agar, and cell migration in Boyden's chamber assay (Supplementary Fig. S8F–H). We confirmed the inhibitory effect of HP1 γ knockdown on the proliferation, colony formation, and migration of LUAD cells using another HP1 γ -high LUAD cell line, H23 (Supplementary Fig. S8A and S9A–E). These results indicate the importance of high HP1 γ levels for the proliferation, anchorage-independent growth and migration in LUAD cells. In line with these results, HP1 γ overexpression using a lentiviral system increased the sizes of anchorage-independent colonies of H460 cells (a large cell lung cancer cell line with a relatively low HP1 level) in soft agar (Supplementary Fig. S9F–H).

To examine whether the H3K9me2/3-binding activity of HP1 γ is necessary for the proliferation, colony formation, and migration of LUAD cells, we generated an shHP1 γ -resistant HP1 γ lentivirus construct and introduced a valine-to-methionine mutation at position 32 (V32M) into the HP1 γ construct using site-directed mutagenesis. It should be noted that the V32M mutation ablates H3K9me2/3-binding ability of HP1 γ (7). We then infected HP1 γ -depleted cells with lentiviruses expressing shHP1 γ -resistant wild type HP1 γ or its H3K9me2/3-binding mutant (mtHP1 γ). Protein levels of HP1 γ and mtHP1 γ ectopically expressed in HP1 γ -depleted cells were similar to endogenous levels of HP1 γ in

shLuc-treated cells, although their ectopically expressed mRNA levels were much higher than endogenous HP1 γ mRNA levels (Fig. 3A and B). Interestingly, H3K9me3 levels were not changed by HP1 γ knockdown or exogenous expression of HP1 γ and mtHP1 γ (Fig. 3B). Importantly, HP1 γ but not mtHP1 γ rescued defects in the proliferation, colony formation in clonogenic cell survival assay, migration, and anchorage-independent colony formation of HP1 γ -depleted cells (Fig. 3C–F). These results suggest that the tumor-promoting function of HP1 γ is dependent largely on its H3K9me2/3-binding activity.

HP1 γ enhances the proliferation, colony formation, and migration of LUAD cells by directly repressing *NCOR2* and *ZBTB7A* expression.

To delineate the molecular mechanisms by which HP1 γ may regulate cell proliferation, colony formation, and cell migration, we compared the whole transcriptomic profiles between HP1 γ -depleted (shHP1 γ -16 and shHP1 γ -17) and control H1792 cells (shLuc) using Affymetrix microarray. Our analysis showed that genes upregulated and downregulated by shHP1 γ -16 significantly overlapped with those upregulated and downregulated by shHP1 γ -17, respectively (Fig. 4A). Gene ontology analysis showed that several gene expression programs, including apoptosis and negative regulation of cell proliferation, were upregulated by HP1 γ knockdown. In contrast, other gene expression programs, such as cell migration and positive regulation of cell proliferation, were downregulated by HP1 γ knockdown (Fig. 4B). These results corroborated the inhibitory effects of HP1 γ knockdown on the proliferation, colony formation, and migration of LUAD cells.

Interestingly, AXL, PVT1, ELK1, and ADP-ribosylation factor 1 (ARF1) were the top four tumor-promoting factors downregulated by HP1 γ knockdown (Fig. 4A and C). The receptor tyrosine kinase AXL is overexpressed in multiple types of cancer, and its inhibition blocks tumor growth in a mouse model (14). The long non-coding RNA PVT1 has been found to be upregulated in multiple types of cancer and to promote tumorigenesis (15). ELK1 is an ETS-domain-containing transcription factor that promotes tumor progression in bladder cancer (16). In addition, AXL and PVT1 are associated with enhanced proliferation and migration of lung cancer cells (17, 18). ARF1 is a member of the Ras superfamily of small GTPases that may enhance cell proliferation and cancer progression (19).

Although it has been reported that its levels in the gene body may be positively associated with gene transcription (20), HP1 γ often acts as a transcriptional corepressor (21, 22). Therefore, we reasoned that HP1 γ indirectly enhances expression of tumor-promoting factors by repressing tumor-suppressive transcriptional repressors (or corepressors). We thus searched for transcription-repressive regulators that may act as tumor suppressors among the first 200 genes that are upregulated by HP1 γ knockdown. Our search showed that nuclear receptor corepressor 2 (*NCOR2*; also called SMRT), zinc finger and BTB domain containing 7A (*ZBTB7A*; also called LRF, Pokemon, and FBI-1), and ETS Homologous Factor (*EHF*; also called ESE-3) were the top three transcription-repressive regulators with putative tumor-suppressive function (Fig. 4A and C), although their functions in LUAD tumorigenesis are not clear. *NCOR2* acts as a transcriptional corepressor that interacts with the histone deacetylase 3 (*HDAC3*) and regulates chromatin structure and genome stability (23).

ZBTB7A is a transcriptional repressor that belongs to the POZ/BTB and Krüppel (POK) transcription factor family (24, 25). It has been reported that NCOR2 and ZBTB7A may have pro-tumorigenic or tumor-suppressive functions in a tissue-dependent manner (see Discussion). EHF is a transcriptional repressor for a set of genes containing ETS/AP-1-binding sites and can have a tumor-suppressive function (26, 27).

Our quantitative RT-PCR data and Western blot analysis confirmed that NCOR2, ZBTB7A, and EHF levels in H1792 cells were upregulated by HP1 γ depletion (Fig. 4C and D). Similar to this, *NCOR2*, *ZBTB7A*, and *EHF* mRNA levels in H23 cells were increased by HP1 γ knockdown (Supplementary Fig. S10A–C). We also performed chromatin immunoprecipitation (ChIP) experiments to examine whether *NCOR2*, *ZBTB7A*, and *EHF* are occupied by HP1 γ . Our ChIP results showed that HP1 γ was significantly enriched at the proximal promoters of *NCOR2*, *ZBTB7A* and *EHF* genes (Fig. 4E–G), indicating that *NCOR2*, *ZBTB7A*, and *EHF* genes are directly repressed by HP1 γ in LUAD cells. However, HP1 γ knockdown did not have any significant effect on H3K9me3 levels at these genes (Supplementary Fig. S10D–F).

To determine whether the tumor-promoting function of HP1 γ in LUAD cells is dependent on HP1 γ -mediated repression of *NCOR2*, *ZBTB7A*, and *EHF* expression, we first selected the most effective shRNAs against NCOR2, ZBTB7A and EHF (shNCOR2–1, shZBTB7A–1, and shEHF–1) in H1792 cells (Supplementary Fig. S11A). We then examined the effects of NCOR2, ZBTB7A and EHF knockdown on the proliferation, colony formation, and migration of HP1 γ -depleted H1792 cells. For this, we compared these cellular characteristics among H1792 cells treated with five groups of shRNAs: 1) shLuc, 2) shHP1 γ –16, 3) shHP1 γ –16 + shNCOR2–1, 4) shHP1 γ –16 + shZBTB7A–1, and 5) shHP1 γ –16 + shEHF–1. Knockdown efficiencies of HP1 γ , NCOR2, ZBTB7A, and EHF in these five groups of cells were assessed by quantitative RT-PCR and Western blot analysis (Fig. 5A and B). As shown in Fig. 5C–E, knockdown of NCOR2 or ZBTB7A substantially restored defects in the proliferation, colony formation in clonogenic cell survival assay, and migration of HP1 γ -depleted cells, whereas knockdown of EHF had no significant effect on such cellular characteristics of HP1 γ -depleted cells. These results indicate that HP1 γ -mediated transcriptional repression of *NCOR2* and *ZBTB7A* is important for the cellular function of HP1 γ that enhances the proliferation, colony formation, and migration of LUAD cells.

Interestingly, ectopic expression of HP1 γ but not mtHP1 γ in HP1 γ -depleted H1792 cells substantially downregulated *NCOR2* and *ZBTB7A* mRNA levels while restoring *AXL* and *PVT1* mRNA levels (Supplementary Fig. S11B and C). These results indicate that HP1 γ 's binding activity is required for HP1 γ -mediated regulation of these genes.

Low NCOR2 or ZBTB7A levels are associated with increased cell proliferation and migration, high HP1 γ levels in LUAD tumors, and poor prognosis in LUAD patients.

To determine whether HP1 γ has a negative effect on *NCOR2* and *ZBTB7A* expression in our mouse model, we compared NCOR2 and ZBTB7A levels between K-Ras^{G12D} lung tumors and mHP1 γ -depleted K-Ras^{G12D} tumors using IHC analysis. NCOR2 and ZBTB7A levels were increased by *in vivo* HP1 γ knockdown (Fig. 6A). To assess whether there is any

anti-correlation between *HP1γ* and either *NCOR2* or *ZBTB7A* expression in human tumor samples, we analyzed the LUAD dataset ($n = 357$) in TCGA database. This analysis showed that *HP1γ* mRNA levels inversely correlated with *NCOR2* and *ZBTB7A* levels (Fig. 6B and C). Similar results were obtained from our analysis using the NCI Director's Challenge Consortium dataset (Supplementary Fig. S11D and E). These results support the notion that transcriptional repression of *NCOR2* and *ZBTB7A* by *HP1γ* contributes to the tumor-promoting function of *HP1γ* in LUAD.

To assess the roles of *NCOR2* and *ZBTB7A* in regulating tumor-relevant characteristics of LUAD cells, we examined the effects of their knockdown on colony formation and migration of LUAD cells. Knockdown of *NCOR2* or *ZBTB7A* significantly increased colony formation and migration of H1792 cells (Fig. 6D–G). Interestingly, *NCOR2* and *ZBTB7A* mRNA levels were significantly downregulated in TCGA LUAD samples ($n = 357$) compared with adjacent normal samples ($n = 54$) (Fig. 6H), and low *NCOR2* and *ZBTB7A* mRNA levels correlated with worse survival in the LUAD patients (Fig. 6I and J; Supplementary Fig. S11F and G). Analysis of TCGA database showed that *NCOR2* and *ZBTB7A* genes in LUAD had approximately 12% and approximately 6% mutations, respectively, which contained loss-of-function mutations (Supplementary Fig. S12A and B). These results suggest that *NCOR2* and *ZBTB7A* have tumor-suppressive functions in LUAD.

Expression of the tumor-promoting factors *AXL*, *PVT1*, and *ELK1* in LUAD cells requires *HP1γ*-mediated downregulation of *NCOR2* and *ZBTB7A*.

As mentioned above, *AXL*, *PVT1*, *ELK1*, and *ARF1* have tumor-promoting functions. In line with this, their high mRNA levels correlated with worse survival in LUAD patients (Fig. 7A–C; Supplementary Fig. S12C). In our effort to understand how *HP1γ* upregulates expression of the oncogenes *AXL*, *PVT1*, *ELK1*, and *ARF1*, we determined whether the transcription-repressive regulators *NCOR2* and *ZBTB7A* whose genes are repressed by *HP1γ* downregulate expression of these oncogenes. Specifically, we examined the effect of *NCOR2* or *ZBTB7A* knockdown on expression of these oncogenes in H1792 cells. *NCOR2* knockdown increased *ELK1* mRNA levels (Fig. 7D) and *ZBTB7A* knockdown upregulated *AXL* and *PVT1* expression (Fig. 7E). In contrast, knockdown of *EHF* did not show any significant effect on *AXL*, *PVT1*, *ELK1* and *ARF1* mRNA levels (Fig. 7F), consistent with the above results (Fig. 5C–E). Interestingly, our ChIP results showed that *ZBTB7A* was recruited to the promoter regions of *AXL* and *PVT1* while *NCOR2* was located to the *ELK1* promoter region in H1792 cells (Fig. 7G–I). These results indicate that expression levels of *AXL*, *PVT1* and *ELK1* but not *ARF1* are directly downregulated by *NCOR2* or *ZBTB7A*.

To determine whether expression of *AXL*, *PVT1* and *ELK1* is dependent on *HP1γ*-mediated repression of *NCOR2* and *ZBTB7A* expression, we examined whether *NCOR2* or *ZBTB7A* knockdown increases expression of these oncogenes in *HP1γ*-depleted cells. Basically, we compared *AXL*, *PVT1*, *ELK1*, and *ARF1* expression among H1792 cells treated with the following four groups of shRNAs: 1) shLuc, 2) sh*HP1γ*-16, 3) sh*HP1γ*-16 + sh*NCOR2*-1, and 4) sh*HP1γ*-16 + sh*ZBTB7A*-1. In *HP1γ*-depleted cells, *NCOR2* knockdown restored *ELK1* mRNA levels, and *ZBTB7A* depletion rescued *AXL* and *PVT1* mRNA levels (Fig.

7J–L). In contrast, NCOR2 and ZBTB7A knockdown did not affect *ARF1* expression (Supplementary Fig. S12D). These results suggest that HP1 γ positively and indirectly regulates expression of oncogenes, such as *AXL*, *PVT1*, and *ELK1*, by directly downregulating expression of *NCOR2* and *ZBTB7A*.

Discussion

In the current study, our bioinformatic screening and analysis of more than 120 histone methylation reader proteins with use of several databases identified HP1 γ as one of the most frequently overexpressed and amplified histone methylation reader proteins in human LUAD samples. Our analysis of a cohort of lung tumor samples and several databases demonstrated that high HP1 γ protein and mRNA levels were associated with worse survival in lung cancer patients, indicating that HP1 γ may be a prognostic marker for LUAD patients. Our results also indicate that the proliferative, colony-forming, and migratory abilities of LUAD cells are dependent on HP1 γ and its H3K9me2/3-binding activity. Furthermore, *in vivo* knockdown of HP1 γ in the K-Ras^{G12D} LUAD mouse model decreased K-Ras^{G12D}-driven lung tumorigenesis while increasing mouse survival. Thus, our findings indicate that HP1 γ , along with its reader activity, is necessary for LUAD tumorigenesis.

Three members in the HP1 family (HP1 α , HP1 β , and HP1 γ) are conserved from *Drosophila* to mammals and play a critical role in diverse biological processes, including gene regulation, chromosome segregation, heterochromatin formation and maintenance, and DNA repair (28, 29). HP1 α and HP1 β are present largely in heterochromatin but HP1 γ localizes in both heterochromatin and euchromatin (29, 30). Although their roles in LUAD have not been well characterized, the HP1 family of proteins has been linked to tumorigenesis of other cancer types. For example, HP1 α is upregulated in several cancer types (e.g., breast, prostate, pancreatic and uterine cancer), and high HP1 α levels are required for cell proliferation (31). HP1 β promotes tumor growth by enhancing androgen receptor's transcriptional activity in prostate cancer (32). On the contrary, low HP1 α and HP1 β levels correlate with cancer metastatic phenotype (33, 34). Therefore, it is possible that HP1 α and HP1 β may have pro-tumorigenic or tumor-suppressive functions depending on cancer stage. HP1 γ has been shown to be upregulated in different types of tumors, including prostate, colorectal, esophageal, cervical, breast and lung cancer (35–37). It has been reported that HP1 γ represses the cyclin-dependent kinase inhibitor *p21* in colon cancer cells and enhances prostate cancer progression by repressing expression of *miR-451a* (36, 38). HP1 γ knockout mice study has reported that HP1 γ may be not required for normal somatic cell proliferation although it positively regulates cell cycle of primordial germ cells (39). However, the *in vivo* tumorigenic role of HP1 γ and its mode of action in cancer cells, especially lung cancer cells, have been poorly documented. Using a genetically engineered mouse model (i.e., the well-established K-Ras^{G12D} LUAD mouse), our current study demonstrated the *in vivo* requirement of HP1 γ in LUAD tumorigenesis. In addition, our results revealed that HP1 γ directly downregulated expression of the transcription-repressive regulators NCOR2 and ZBTB7A to indirectly upregulate expression of tumor-promoting factors (e.g., *AXL*, *PVT1*, and *ELK1*). We furthermore showed that NCOR2 repressed *ELK1* expression while ZBTB7A downregulated expression of *AXL* and *PVT1* (Fig. 7D–I).

Thus, our findings, distinct from other HP1 γ studies, provide unique epigenetic and mechanistic insights into LUAD pathogenesis (Fig. 7M).

In addition to H3K9 methylation readers, the H3K9 methyltransferases appear to regulate tumorigenesis. For example, G9a has been reported to repress the cell adhesion molecule Ep-CAM and to promote cell invasion and metastasis of lung cancer (40). Equally interesting, H3K9 methylation has been linked to silencing of tumor-suppressor genes, such as *p16^{INK4A}* (41). Therefore, it is possible that an H3K9 methylation-epigenetic system, including writers and readers, cooperates to promote lung tumorigenesis.

HP1 γ has been associated with gene silencing of multiple genes, such as E2F- and Myc-responsive genes in G₀ cells and the HIV gene (21, 22). In contrast, HP1 γ is enriched in the gene body, and its levels positively correlate with gene activity (20, 42). In the latter studies, HP1 γ was linked to gene activation via transcriptional elongation and co-transcriptional RNA splicing. In the current study, we showed that HP1 γ occupied and repressed expression of transcription-repressive regulator genes, such as *NCOR2* and *ZBTB7A*, in LUAD cells (Fig. 4). Moreover, our results showed that *NCOR2* and *ZBTB7A* mRNA levels inversely correlated with HP1 γ mRNA levels in human LUAD datasets (Fig. 6B and C). Although we cannot exclude the possibility that HP1 γ could directly activate the transcription of some tumor-promoting factors in LUAD cells, our results support the notion that HP1 γ -mediated transcriptional repression of tumor-suppressor genes contributes to the progression of LUAD.

The transcriptional repressor *ZBTB7A* has a tumor-suppressive function that downregulates oncogenic glycolytic genes (24), and *ZBTB7A* undergoes mutations that disrupt the anti-proliferative function of *ZBTB7A* in leukemia (43). However, *ZBTB7A* has an oncogenic function for lymphoma and represses expression of the tumor-suppressor gene *p14^{ARF}* (25). Similar to *ZBTB7A*, the transcriptional corepressor *NCOR2* appears to have pro-tumorigenic and anti-tumorigenic functions. *NCOR2* is downregulated in multiple myeloma (44) and its loss is linked with the neoplastic transformation of non-Hodgkin's lymphoma (45). However, increased *NCOR2* levels are associated with faster recurrence of estrogen receptor α -positive breast tumors (46). It is possible that *NCOR2* and *ZBTB7A* may function as tumor suppressors or oncogenic proteins in a tissue-dependent manner. In the current study, tumor-suppressive functions of *NCOR2* and *ZBTB7A* in lung are supported by the following results. First, *NCOR2* or *ZBTB7A* knockdown rescued, at least in part, defects in the proliferation, colony formation and migratory abilities of HP1 γ -depleted LUAD cells (Fig. 5). Second, *NCOR2* or *ZBTB7A* knockdown significantly increased colony formation and migration of LUAD cells (Fig. 6D–G). Third, our TCGA analysis showed that *NCOR2* and *ZBTB7A* mRNA levels were downregulated in LUADs compared with their adjacent normal samples (Fig. 6H). Fourth, low *NCOR2* and *ZBTB7A* mRNA levels correlated with shorter survival in human LUAD patients (Fig. 6I and J). Fifth, *in vivo* HP1 γ knockdown upregulated *NCOR2* and *ZBTB7A* levels in mouse K-Ras^{G12D} lung tumors (Fig. 6A) and *NCOR2* and *ZBTB7A* mRNA levels were inversely associated with *HP1 γ* mRNA levels in human LUAD datasets (Fig. 6B and C). Finally, our database analysis showed that *NCOR2* and *ZBTB7A* underwent loss-of-function of mutations in LUAD samples (Supplementary Fig. S12A and B). For these reasons, it could be concluded

that HP1 γ promotes lung tumorigenesis, at least in part, by repressing expression of *ZBTB7A* and *NCOR2*.

Histone methylation reader proteins are frequently dysregulated in cancer. Such dysregulation includes recurrent mutations within the methylation reader domains, oncogenic fusion proteins containing readers, and overexpression of histone reader proteins. For instance, the H3K4me3/2-binding PHD in the inhibitor of growth 1 (ING1) is mutated in various tumors, including breast cancer and melanoma (47). Examples of oncogenic fusion proteins containing readers include fusion proteins between nucleoporin-98 and H3K4me3-binding PHD fingers in the PHD finger-containing proteins JARID1A and PHF23, which cause acute myeloid leukemia in mice (48). As reported here, HP1 γ is an example of a reader protein that is overexpressed in LUAD and is a prognostic marker necessary for LUAD tumorigenesis. In addition to dysregulation in histone methylation reader proteins, alterations of their partner proteins affect their function and tumorigenesis. For example, a recent study from our laboratory has shown that ZYMND8 (also called RACK7) acts as a reader protein for the dual histone mark H3K4me1-H3K14ac via its PHD/bromodomain and suppresses metastasis-linked genes in cooperation with the H3K4 demethylase JARID1D (9). Because JARID1D is frequently deleted or downregulated in prostate tumors and their metastases (9, 10), the gene-repressive function of ZYMND8 at metastasis-linked genes may be impaired in these tumors. Similar to this, it is possible that the tumor-promoting activity of HP1 γ is regulated by its partner proteins. With respect to HP1 γ -interacting proteins, it has been shown that the chromatin remodeler CHD4 is isolated as an HP1 γ -interacting protein (49) and has an oncogenic function by epigenetically suppressing multiple tumor-suppressor genes (50). Thus, it would be interesting to examine in the future whether CHD4 might cooperate with HP1 γ for tumorigenesis.

The inhibition of the binding activities of oncogenic histone readers can be an attractive strategy to block their function. For example, the inhibition of the BET family of readers that recognize histone lysine acetylation has been shown to be a promising therapeutic strategy. Because our results indicate that HP1 γ 's reader activity is required for LUAD tumorigenicity, abrogating the interaction of HP1 γ with H3K9me2/3 using small molecule inhibitors may be relevant to therapeutic intervention for the treatment of LUAD. In summary, our findings provide new insights into how overexpression of a tumor-promoting histone reader protein represses expression of tumor suppressors to promote LUAD tumorigenicity and also highlight the clinical relevance of an HP1 γ -regulated mode of mechanism to LUAD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Kenneth L. Scott and Tyler Jacks for providing their reagents, and Zhenbo Han, Su Zhang, and Charles Kingsley for their technical assistance. We are also thankful to Michael Worley (Department of Scientific Publications, The University of Texas MD Anderson Cancer Center) for the editorial assistance. Experimental and technical support were provided by Sequencing and Microarray Facility, Small Animal Imaging Facility, and Histopathology Core Lab at The University of Texas MD Anderson Cancer Center (the NIH Cancer Center Support

Grant P30CA016672). This work was supported by grants to M. G. Lee from the National Institutes of Health (NIH; R01 CA157919, R01 CA207109, and R01 CA207098), the Cancer Prevention and Research Institute of Texas (RP140271) and the Center for Cancer Epigenetics at The University of Texas MD Anderson Cancer Center, by a grant to E. R. Flores from the NIH (R35CA197452), and by a fellowship to H. Alam from the Odyssey program at The University of Texas MD Anderson Cancer Center.

REFERENCES

1. Imielinski M, Berger AH, Hammerman PS, Hernandez B, Pugh TJ, Hodis E, et al. Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell*. 2012;150:1107–20. [PubMed: 22980975]
2. Cancer Genome Atlas Research N. Comprehensive molecular profiling of lung adenocarcinoma. *Nature*. 2014;511:543–50. [PubMed: 25079552]
3. Martin C, Zhang Y. The diverse functions of histone lysine methylation. *Nat Rev Mol Cell Biol*. 2005;6:838–49. [PubMed: 16261189]
4. Musselman CA, Lalonde ME, Cote J, Kutateladze TG. Perceiving the epigenetic landscape through histone readers. *Nat Struct Mol Biol*. 2012;19:1218–27. [PubMed: 23211769]
5. Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, et al. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*. 2001;410:120–4. [PubMed: 11242054]
6. Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*. 2001;410:116–20. [PubMed: 11242053]
7. Jacobs SA, Taverna SD, Zhang Y, Briggs SD, Li J, Eissenberg JC, et al. Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. *EMBO J*. 2001;20:5232–41. [PubMed: 11566886]
8. Wagner KW, Alam H, Dhar SS, Giri U, Li N, Wei Y, et al. KDM2A promotes lung tumorigenesis by epigenetically enhancing ERK1/2 signaling. *J Clin Invest*. 2013;123:5231–46. [PubMed: 24200691]
9. Li N, Li Y, Lv J, Zheng X, Wen H, Shen H, et al. ZMYND8 Reads the Dual Histone Mark H3K4me1-H3K14ac to Antagonize the Expression of Metastasis-Linked Genes. *Mol Cell*. 2016;63:470–84. [PubMed: 27477906]
10. Li N, Dhar SS, Chen TY, Kan PY, Wei Y, Kim JH, et al. JARID1D Is a Suppressor and Prognostic Marker of Prostate Cancer Invasion and Metastasis. *Cancer Res*. 2016;76:831–43. [PubMed: 26747897]
11. Xu Y, Zhang S, Lin S, Guo Y, Deng W, Zhang Y, et al. WERAM: a database of writers, erasers and readers of histone acetylation and methylation in eukaryotes. *Nucleic Acids Res*. 2017;45:D264–D70. [PubMed: 27789692]
12. DuPage M, Dooley AL, Jacks T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc*. 2009;4:1064–72. [PubMed: 19561589]
13. Young NP, Jacks T. Tissue-specific p19Arf regulation dictates the response to oncogenic K-ras. *Proc Natl Acad Sci U S A*. 2010;107:10184–9. [PubMed: 20479239]
14. Pacciez JD, Vogelsang M, Parker MI, Zerbini LF. The receptor tyrosine kinase Axl in cancer: biological functions and therapeutic implications. *Int J Cancer*. 2014;134:1024–33. [PubMed: 23649974]
15. Cui M, You L, Ren X, Zhao W, Liao Q, Zhao Y. Long non-coding RNA PVT1 and cancer. *Biochem Biophys Res Commun*. 2016;471:10–4. [PubMed: 26850852]
16. Kawahara T, Shareef HK, Aljarah AK, Ide H, Li Y, Kashiwagi E, et al. ELK1 is up-regulated by androgen in bladder cancer cells and promotes tumor progression. *Oncotarget*. 2015;6:29860–76. [PubMed: 26342199]
17. Shieh YS, Lai CY, Kao YR, Shiah SG, Chu YW, Lee HS, et al. Expression of axl in lung adenocarcinoma and correlation with tumor progression. *Neoplasia*. 2005;7:1058–64. [PubMed: 16354588]
18. Yang YR, Zang SZ, Zhong CL, Li YX, Zhao SS, Feng XJ. Increased expression of the lncRNA PVT1 promotes tumorigenesis in non-small cell lung cancer. *Int J Clin Exp Pathol*. 2014;7:6929–35. [PubMed: 25400777]

19. Boulay PL, Schlienger S, Lewis-Saravalli S, Vitale N, Ferbeyre G, Claing A. ARF1 controls proliferation of breast cancer cells by regulating the retinoblastoma protein. *Oncogene*. 2011;30:3846–61. [PubMed: 21478909]
20. Vakoc CR, Mandat SA, Olenchock BA, Blobel GA. Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. *Mol Cell*. 2005;19:381–91. [PubMed: 16061184]
21. Ogawa H, Ishiguro K, Gaubatz S, Livingston DM, Nakatani Y. A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. *Science*. 2002;296:1132–6. [PubMed: 12004135]
22. du Chene I, Basyuk E, Lin YL, Triboulet R, Knezevich A, Chable-Bessia C, et al. Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. *EMBO J*. 2007;26:424–35. [PubMed: 17245432]
23. You SH, Lim HW, Sun Z, Broache M, Won KJ, Lazar MA. Nuclear receptor co-repressors are required for the histone-deacetylase activity of HDAC3 in vivo. *Nat Struct Mol Biol*. 2013;20:182–7. [PubMed: 23292142]
24. Liu XS, Haines JE, Mehanna EK, Genet MD, Ben-Sahra I, Asara JM, et al. ZBTB7A acts as a tumor suppressor through the transcriptional repression of glycolysis. *Genes Dev*. 2014;28:1917–28. [PubMed: 25184678]
25. Maeda T, Hobbs RM, Merghoub T, Guernah I, Zelent A, Cordon-Cardo C, et al. Role of the proto-oncogene Pokemon in cellular transformation and ARF repression. *Nature*. 2005;433:278–85. [PubMed: 15662416]
26. Cangemi R, Mensah A, Albertini V, Jain A, Mello-Grand M, Chiorino G, et al. Reduced expression and tumor suppressor function of the ETS transcription factor ESE-3 in prostate cancer. *Oncogene*. 2008;27:2877–85. [PubMed: 18037958]
27. Tugores A, Le J, Sorokina I, Snijders AJ, Duyao M, Reddy PS, et al. The epithelium-specific ETS protein EHF/ESE-3 is a context-dependent transcriptional repressor downstream of MAPK signaling cascades. *J Biol Chem*. 2001;276:20397–406. [PubMed: 11259407]
28. Lomberk G, Wallrath L, Urrutia R. The Heterochromatin Protein 1 family. *Genome Biol*. 2006;7:228. [PubMed: 17224041]
29. Maison C, Almouzni G. HP1 and the dynamics of heterochromatin maintenance. *Nat Rev Mol Cell Biol*. 2004;5:296–304. [PubMed: 15071554]
30. Minc E, Courvalin JC, Buendia B. HP1gamma associates with euchromatin and heterochromatin in mammalian nuclei and chromosomes. *Cytogenet Cell Genet*. 2000;90:279–84. [PubMed: 11124534]
31. De Koning L, Savignoni A, Boumendil C, Rehman H, Asselain B, Sastre-Garau X, et al. Heterochromatin protein 1alpha: a hallmark of cell proliferation relevant to clinical oncology. *EMBO Mol Med*. 2009;1:178–91. [PubMed: 20049717]
32. Shiota M, Song Y, Yokomizo A, Tada Y, Kuroiwa K, Eto M, et al. Human heterochromatin protein 1 isoform HP1beta enhances androgen receptor activity and is implicated in prostate cancer growth. *Endocr Relat Cancer*. 2010;17:455–67. [PubMed: 20308360]
33. Yi SA, Ryu HW, Lee DH, Han JW, Kwon SH. HP1beta suppresses metastasis of human cancer cells by decreasing the expression and activation of MMP2. *Int J Oncol*. 2014;45:2541–8. [PubMed: 25201136]
34. Kirschmann DA, Lininger RA, Gardner LM, Seftor EA, Otero VA, Ainsztein AM, et al. Down-regulation of HP1Hsalpha expression is associated with the metastatic phenotype in breast cancer. *Cancer Res*. 2000;60:3359–63. [PubMed: 10910038]
35. Takanashi M, Oikawa K, Fujita K, Kudo M, Kinoshita M, Kuroda M. Heterochromatin protein 1gamma epigenetically regulates cell differentiation and exhibits potential as a therapeutic target for various types of cancers. *Am J Pathol*. 2009;174:309–16. [PubMed: 19056850]
36. Liu M, Huang F, Zhang D, Ju J, Wu XB, Wang Y, et al. Heterochromatin protein HP1gamma promotes colorectal cancer progression and is regulated by miR-30a. *Cancer Res*. 2015;75:4593–604. [PubMed: 26333808]

37. Zhou J, Bi H, Zhan P, Chang C, Xu C, Huang X, et al. Overexpression of HP1gamma is associated with poor prognosis in non-small cell lung cancer cell through promoting cell survival. *Tumour Biol.* 2014;35:9777–85. [PubMed: 24981246]
38. Chang C, Liu J, He W, Qu M, Huang X, Deng Y, et al. A regulatory circuit HP1gamma/miR-451a/c-Myc promotes prostate cancer progression. *Oncogene.* 2018;37:415–26. [PubMed: 28967902]
39. Abe K, Naruse C, Kato T, Nishiuchi T, Saitou M, Asano M. Loss of heterochromatin protein 1 gamma reduces the number of primordial germ cells via impaired cell cycle progression in mice. *Biol Reprod.* 2011;85:1013–24. [PubMed: 21778144]
40. Chen MW, Hua KT, Kao HJ, Chi CC, Wei LH, Johansson G, et al. H3K9 histone methyltransferase G9a promotes lung cancer invasion and metastasis by silencing the cell adhesion molecule Ep-CAM. *Cancer Res.* 2010;70:7830–40. [PubMed: 20940408]
41. Bachman KE, Park BH, Rhee I, Rajagopalan H, Herman JG, Baylin SB, et al. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell.* 2003;3:89–95. [PubMed: 12559178]
42. Smallwood A, Hon GC, Jin F, Henry RE, Espinosa JM, Ren B. CBX3 regulates efficient RNA processing genome-wide. *Genome Res.* 2012;22:1426–36. [PubMed: 22684280]
43. Hartmann L, Dutta S, Opatz S, Vosberg S, Reiter K, Leubolt G, et al. ZBTB7A mutations in acute myeloid leukaemia with t(8;21) translocation. *Nat Commun.* 2016;7:11733. [PubMed: 27252013]
44. Ghoshal P, Nganga AJ, Moran-Giuati J, Szafraniek A, Johnson TR, Bigelow AJ, et al. Loss of the SMRT/NCOR2 corepressor correlates with JAG2 overexpression in multiple myeloma. *Cancer Res.* 2009;69:4380–7. [PubMed: 19417136]
45. Song L, Zlobin A, Ghoshal P, Zhang Q, Houde C, Weijzen S, et al. Alteration of SMRT tumor suppressor function in transformed non-Hodgkin lymphomas. *Cancer Res.* 2005;65:4554–61. [PubMed: 15930272]
46. Smith CL, Migliaccio I, Chaubal V, Wu MF, Pace MC, Hartmaier R, et al. Elevated nuclear expression of the SMRT corepressor in breast cancer is associated with earlier tumor recurrence. *Breast Cancer Res Treat.* 2012;136:253–65. [PubMed: 23015261]
47. Baker LA, Allis CD, Wang GG. PHD fingers in human diseases: disorders arising from misinterpreting epigenetic marks. *Mutat Res.* 2008;647:3–12. [PubMed: 18682256]
48. Wang GG, Song J, Wang Z, Dormann HL, Casadio F, Li H, et al. Haematopoietic malignancies caused by dysregulation of a chromatin-binding PHD finger. *Nature.* 2009;459:847–51. [PubMed: 19430464]
49. Rosnoblet C, Vandamme J, Volkel P, Angrand PO. Analysis of the human HP1 interactome reveals novel binding partners. *Biochem Biophys Res Commun.* 2011;413:206–11. [PubMed: 21888893]
50. Xia L, Huang W, Bellani M, Seidman MM, Wu K, Fan D, et al. CHD4 Has Oncogenic Functions in Initiating and Maintaining Epigenetic Suppression of Multiple Tumor Suppressor Genes. *Cancer Cell.* 2017;31:653–68 e7. [PubMed: 28486105]

Significance

Direct epigenetic repression of the transcription-repressive regulators NCOR2 and ZBTB7A by the histone reader protein HP1 γ leads to activation of pro-tumorigenic genes in lung adenocarcinoma.

Figure 1: HP1 γ levels are upregulated in lung adenocarcinoma and high HP1 γ levels correlate with shorter survival in LUAD patients.

(A) An *in silico* screening strategy for the search of putative oncogenic histone methylation reader proteins. LUAD, Lung adenocarcinoma. (B) Bar graph showing alterations in the 58 histone methylation reader proteins in LUAD samples in TCGA database. The cutoff value for alterations was 8%. (C) Fourteen histone methylation reader proteins with >1.5 -fold upregulation in LUAD tumors ($n = 357$) compared with adjacent normal tissue samples ($n = 54$) in TCGA dataset. P-value for the 14 reader proteins, <0.05 . T, tumor; N, normal. (D)

Kaplan-Meier survival rate analysis on the basis of *HP1 γ* mRNA levels (probe set, 200037_s_at) using NCI LUAD dataset. Tumor samples ($n = 443$) were divided into *HP1 γ* -high or *HP1 γ* -low mRNA groups by using a cutoff value of the mean plus 2 standard deviations. (E) Representative images of IHC staining of HP1 γ in normal lung and lung tumors. Scale bars, 50 μ m. (F) Survival analysis of lung cancer patients on the basis of HP1 γ protein levels (see also Supplementary Table S3 and S4). HP1 γ protein levels in lung cancer samples in TMAs were determined by IHC staining ($n = 73$).

Figure 2: HP1 γ is required for *in vivo* growth of K-Ras^{G12D}-driven LUAD tumors. (A) A scheme for monitoring the effect of *in vivo* knockdown of HP1 γ on K-Ras^{G12D}-driven lung tumorigenesis. The lungs of 8-week-old mice were infected by an intratracheal intubation of shmHP1 γ -1-Cre lentiviruses, which express both Cre recombinase and shRNA targeting mouse HP1 γ . Lentiviruses with an empty vector expressing only Cre were used as a control. (B) Micro-CT-scan images of mouse lungs infected with shmHP1 γ -1-Cre (n = 3) and control (n = 3) viruses at 3 months and 9 months post-infection. (C–H) The effect of *in vivo* HP1 γ knockdown on K-Ras^{G12D}-driven lung tumorigenesis. Lung tumors

in mice infected with control viruses were compared with those in mice infected with shmHP1 γ -1-Cre viruses. Representative images of massive lung tumors in the control group and of tiny lung tumors in the shmHP1 γ -1-Cre group are shown; tumors are encircled by dotted green lines (**C**). Representative images of H&E-stained lung lobes effaced by lung tumors in the control and shmHP1 γ -1-Cre groups are shown (**D**). The percentages of tumor area per lobe in control (n = 10) and shmHP1 γ -1-Cre (n = 10) groups were quantified (**E**). Tumor grades, based on tumor size, in control (n = 4) and shmHP1 γ -1-Cre (n = 4) groups were scored (**F**). IHC staining data for HP1 γ and the cell proliferation marker Ki-67 in lung tumors from control and shmHP1 γ -1-Cre groups are shown (**G**). Ki-67-positive cells in eight random fields of three different tumors of control and shmHP1 γ -1-Cre groups were quantified (**H**). (**I**) Kaplan-Meier survival analysis of the control group of mice (n=17) and the shmHP1 γ -1-Cre group of mice (n=14). Scale bars, 7 mm (**D**) and 100 μ m (**G**).

Figure 3: HP1γ and its binding activity are essential for the proliferation, colony formation and migration of LUAD cells.

(A and B) Analysis of mRNA levels of ectopically expressed HP1γ and mtHP1γ and endogenous HP1γ by quantitative RT-PCR (A) and Western blot analysis of HP1γ, mtHP1γ, H3K9me3, and H3 levels (B). In HP1γ-depleted H1792 cells, *HP1γ* and *mtHP1γ* were ectopically expressed using a lentivirus system. (C) The effect of ectopic expression of HP1γ and mtHP1γ on the proliferation of HP1γ-depleted H1792 cells. (D) The effect of ectopic expression of HP1γ and mtHP1γ on the colony formation ability of HP1γ-depleted

H1792 cells in a clonogenic cell survival assay. **(E and F)** The effect of ectopic expression of HP1 γ and mtHP1 γ on the migration **(E)** and anchorage-independent colony growth **(F)** of HP1 γ -depleted H1792 cells. Black scale bars: 200 μm ; white scale bars, 400 μm .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4: HP1γ directly represses NCOR2 and ZBTB7A expression while upregulating expression of oncogenes, such as AXL, PVT1, and ELK1.

(A) Venn diagrams and heat maps for genes upregulated (>1.5-fold) or downregulated (<0.5-fold) by HP1γ knockdown. RNA was isolated from HP1γ-depleted H1792 cells (shHP1γ-16 and shHP1γ-17) and control (shLuc) H1792 cells. The mRNA levels were assessed by Affymetrix Human Genome U133 plus 2.0 Array in duplicate. (B) Ontology analysis of genes upregulated or downregulated by HP1γ knockdown. The functional annotation tool DAVID was used. (C) Analysis of the effect of HP1γ knockdown on *EHF*, *NCOR2*,

ZBTB7A, *AXL*, *PVT1*, *ARF1* and *ELK1* mRNA levels using quantitative RT-PCR. **(D)** Western blot analysis of NCOR2, ZBTB7A and EHF protein levels in HP1 γ -depleted (shHP1 γ -16 and shHP1 γ -17) H1792 cells. **(E–G)** Chromatin levels of HP1 γ at the *NCOR2* **(E)**, *ZBTB7A* **(F)**, and *EHF* **(G)** genes. Schematic representations of individual genes are shown (top panels). Quantitative ChIP assay was performed using shLuc-infected cells (control) and HP1 γ -depleted cells. Arrows indicate the primer sites for PCR amplification of ChIP-enriched DNAs. TSS, transcription start site.

Figure 5: Knockdown of NCOR2 or ZBTB7A substantially rescues the proliferation, colony formation, and migration of HP1γ-depleted LUAD cells.

(A and B) Analysis of relative *HP1γ*, *NCOR2*, *ZBTB7A*, and *EHF* mRNA (A) and protein (B) levels in H1792 cells that were treated with the following five groups of shRNA-containing viruses: 1) shLuc, 2) shHP1γ-16, 3) shHP1γ-16 and shNCOR2-1, 4) shHP1γ-16 and shZBTB7A-1, and 5) shHP1γ-16 and shEHF-1. (C–E) The effect of NCOR2 or ZBTB7A knockdown on the colony formation (C), proliferation (D), and migration (E) of HP1γ-depleted H1792 cells. shLuc-infected cells were used as controls. Scale bars, 200 μm.

Figure 6: Low NCOR2 or ZBTB7A levels are linked to enhanced cell proliferation and migration, high HP1γ levels in LUAD tumors, and poor prognosis in LUAD patients. (A) IHC levels of NCOR2 and ZBTB7A in K-Ras^{G12D} lung tumors and mHP1γ-depleted K-Ras^{G12D} tumors. (B and C) Scatter plots showing an inverse correlation between *HP1γ* mRNA levels and either *NCOR2* (B) or *ZBTB7A* (C) mRNA levels in TCGA LUAD sample dataset. (D and E) The effect of NCOR2 knockdown on colony formation (D) and migration (E) of H1792 cells. (F and G) The effect of ZBTB7A knockdown on colony formation (F) and migration (G) of H1792 cells. (H) Box plots showing downregulation of *NCOR2* (left

panel) and *ZBTB7A* (right panel) mRNA levels in LUAD tumor samples ($n = 357$) compared with their adjacent normal samples ($n = 54$) in TCGA dataset. T, tumor; N, normal. **(I and J)** The Kaplan-Meier survival analysis showing the correlation of low *NCOR2* **(I)** and *ZBTB7A* **(J)** mRNA levels with shorter survival in LUAD patients. The auto cutoff was used to divide samples into low and high groups in the KM Plotter database (<http://kmplot.com/analysis>). *NCOR2* cutoff, 2870 in the range between 578 and 10323; *ZBTB7A* cutoff, 788 in the range between 259 and 20494; *NCOR2* probe set, 207760_s_at; *ZBTB7A* probe set, 226554_at; black scale bars, 100 μm **(A)** and 200 μm **(E and G)**.

Figure 7: HP1 γ indirectly upregulates expression of the oncogenes *AXL*, *PVT1*, and *ELK1* by downregulating *NCOR2* and *ZBTB7A* expression.

(A–C) Kaplan-Meier survival analysis of *AXL* (A), *PVT1* (B), and *ELK1* (C) levels in LUAD datasets. The auto cutoff was used to divide samples into low and high groups in the KM Plotter database (<http://kmplot.com/analysis>). *AXL* cutoff, 136 in the range between 5 and 677; *PVT1* cutoff, 454 in the range between 12 and 15881; *ELK1* cutoff, 13 in the range between 2 and 524; *AXL* probe set, 202685_s_at; *PVT1* probe set, 1558290_a_at; *ELK1*, 220802_at. (D–F) The effect of *NCOR2* (D), *ZBTB7A* (E), or *EHF* (F) knockdown on

AXL, *PVT1*, *ELK1* and *ARF1* mRNA levels in H1792 cells. (G–I) Chromatin levels of ZBTB7A at the *AXL* (G) and *PVT1* (H) genes and of NCOR2 at the *ELK1* gene (I). Schematic representations of individual genes are shown (top panels). Arrows indicate the primer sites for PCR amplification of ChIP-enriched DNAs. TSS, transcription start site. (J–L) The effect of NCOR2 or ZBTB7A knockdown on *AXL* (J), *PVT1* (K), and *ELK1* (L) mRNA levels in HP1 γ -depleted H1792 cells. H1792 cells were treated with the following four groups of shRNA-containing viruses: 1) shLuc, 2) shHP1 γ -16, 3) shHP1 γ -16 and shNCOR2-1, and 4) shHP1 γ -16 and shZBTB7A-1. shLuc-treated cells were used as controls. For analysis of mRNA levels in J–L, quantitative RT-PCR was used. (M) A hypothetical model for the molecular mechanism underlying the tumor-promoting function of HP1 γ in LUAD. HP1 γ interacts with H3K9me3 at the *NCOR2* and *ZBTB7A* promoters and represses *NCOR2* and *ZBTB7A* expression. NCOR2 downregulates *ELK1* expression, and ZBTB7A represses expression of *AXL* and *PVT1*. HP1 γ -mediated repression of *NCOR2* and *ZBTB7A* increases expression levels of tumor-promoting factors (e.g., *AXL*, *PVT1*, and *ELK1*) to enhance the proliferation, migration, and tumorigenic growth of LUAD cells.