NatD promotes lung cancer progression by preventing histone H4 serine phosphorylation to activate Slug expression

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N-α-acetyltransferase D (NatD) mediates N-α-terminal acetylation (Nt-acetylation) of histone H4 known to be involved in cell growth. Here we report that NatD promotes the migratory and invasive capabilities of lung cancer cells in vitro and in vivo. Depletion of NatD suppresses the epithelial-to-mesenchymal transition (EMT) of lung cancer cells by directly repressing the expression of transcription factor Slug, a key regulator of EMT. We found that Nt-acetylation of histone H4 antagonizes histone H4 serine 1 phosphorylation (H4S1ph), and that downregulation of Nt-acetylation of histone H4 facilitates CK2α binding to histone H4 in lung cancer cells, resulting in increased H4S1ph and epigenetic reprogramming to suppress Slug transcription to inhibit EMT. Importantly, NatD is commonly upregulated in primary human lung cancer tissues where its expression level correlates with Slug expression, enhanced invasiveness, and poor clinical outcomes. These findings indicate that NatD is a crucial epigenetic modulator of cell invasion during lung cancer progression.
N-terminal acetylation (Nt-acetylation) is one of the most common protein covalent modifications in eukaryotes, occurring in 80–90% of soluble proteins in humans and 50–70% in yeast. This modification has a variety of biological roles, including regulation of protein degradation, protein–protein interactions, protein translocation, membrane attachment, apoptosis, and cellular metabolism. Nt-acetylation is catalyzed by N-α-acetyltransferases (NATs), which transfer the acetyl group from acetyl-coenzyme A (Ac-CoA) to the primary α-amino group of the N-terminal amino acid residue of a protein. In humans, six different NATs (NATα-NATf) have been identified to date based on their unique subunits and specific substrates. NATd (also termed Nat4 or Pat1) mediates the Nt-acetylation of histone H4 and H2A exclusively, differentiating it from all other Nat family members, which target various substrates. Natd contains only a single catalytic unit, Naa40p, and has no auxiliary subunit.

Natd was originally identified in yeast, but the human Natd ortholog has also been characterized. In yeast, loss of Natd or its acetyltransferase activity produced a synthetic growth defect showing increased growth sensitivity to various chemicals including 3-aminitriazole, an inhibitor of transcription. Natd was identified as a novel regulator of ribosomal DNA silencing during calorie restriction in yeast, which suggested that Natd might be critical for cell growth. In line with this, male mice lacking Natd in liver showed decreased fat mass, and were protected from age-associated hepatic steatosis. Natd is also linked to apoptosis of cancer cells. Intriguingly, in hepatocellular carcinoma, Natd was reported to enhance apoptosis, whereas in colorectal cells, depletion of Natd-induced apoptosis in a p53-independent manner.

Epithelial-to-mesenchymal transition (EMT) is a key cellular program by which cancer cells lose their cell polarity and adhesion, and gain the migratory and invasive capabilities of mesenchymal cells, which is closely associated with metastasis. Although this process was initially recognized during embryogenesis, it has been extended to cancer cell stemness, drug resistance, and immunosuppression during cancer progression. Recent studies have revealed interesting links between EMT and the control of the chromatin configuration resulting from histone modifications. However, the biological role of Nt-acetylation of histone by Natd during cancer progression involving EMT remains largely unknown.

In this study, we show that Natd-mediated N-α-terminal acetylation of histone H4 promotes lung cell invasion through antagonizing serine phosphorylation of histone H4 by CK2α. The results demonstrate a critical interplay between transcriptional and epigenetic control during lung cancer progression associated with EMT of cancer cells, thus suggesting that Natd could be a potential therapeutic target for lung cancer.

**Results**

**Natd expression associates with prognosis of lung cancer patients.** To investigate the clinical significance of Natd expression in patients with non-small cell lung cancer (NSCLC), we first examined Natd mRNA levels in human lung cancer tissues. Quantitative real-time PCR analysis showed that 69% (20/29) of lung cancer tissue samples showed significantly elevated Natd levels compared to adjacent normal tissue samples (Fig. 1a). We further examined expression of Natd by immunohistochemical staining (IHC) on two sets of human NSCLC tissue arrays containing 74 squamous carcinomas, 73 adenocarcinomas, and adjacent normal lung tissue controls (Supplementary Table 1). We found that Natd was significantly upregulated in both squamous carcinomas and adenocarcinomas compared with normal lung tissues (Fig. 1b, c). Notably, Natd expression correlated with higher grade lymph node status (Fig. 1d). Importantly, the Kaplan–Meier survival analysis showed that lung cancer patients with high Natd expression had shorter overall survival (Fig. 1e). These results indicate that Natd expression levels are upregulated in human lung cancer tissues and correlate with poor prognosis in lung cancer, suggesting that Natd may promote cancer cell invasion during malignant progression.

**Natd is required for lung cancer cell migration and invasion in vitro.** To determine the effect of Natd on cell growth and mobility, we generated two independent, stable Natd knockdown human lung cancer H1299 cell lines (Natd-KD1 and Natd-KD2 cells) using lentiviral vectors containing different specific shRNAs targeting Natd mRNA. Because shRNA KD2 produced a somewhat better knockdown (Fig. 2a), unless both Natd-KD1 and Natd-KD2 cells are indicated, only Natd-KD2 cells were used. Natd mRNAs in Natd-KD1 and Natd-KD2 cells were reduced to 30% of Natd mRNAs in the scrambled control (Scr) cells determined by quantitative real-time PCR (Fig. 2a), and decreased protein levels of Natd were confirmed by western blot analysis (Fig. 2b). Correspondingly, levels of Nt-acetylation of histone H4 (Nt-ac-H4) were also significantly reduced in Natd knockdown cells compared with the Scr cells (Fig. 2b). We found that Natd knockdown cells grew at a similar rate as the Scr cells (Supplementary Fig. 1a), and no difference in numbers of apoptotic cells or in cell cycle was found between knockdown and Scr cells (Supplementary Fig. 1b, c). These results suggest that Natd has no effect on cell growth and survival of lung cancer cells. However, in a wound healing assay, Natd knockdown cells migrated significantly more slowly than Scr cells (Fig. 2c). Consistently, time-lapse cell-tracking analysis confirmed our observation dynamically, and showed slower random motility of Natd knockdown cells compared with the Scr cells (Fig. 2d). Furthermore, results from the transwell assay showed that cell migratory and invasive capabilities of lung cancer cells were significantly reduced in Natd knockdown cells compared with the Scr cells (Fig. 2e, f). Similar results were also obtained with another human lung cancer cell line, A549, when Natd was knocked down (Supplementary Fig. 2a–c). Thus, these results indicate that Natd is crucial for lung cancer cell migration and invasion in vitro.

**Natd promotes lung cancer cell invasion in vivo.** To further investigate the effect of Natd on lung cancer cell invasion in an in vivo model, luciferase-labeled Scr or Natd knockdown A549 cells were injected into severe combined immunodeficiency (SCID) mice via tail vein. Tumor growth was assessed by bioluminescent (BLI) imaging on days 1, 4, 7, 14, and 28. Mice receiving Natd knockdown cells exhibited significantly reduced lung cancer growth signals (photon radiance) compared with the mice receiving Scr cells (Fig. 3a). The effect of Natd knockdown was evident as early as day 4, suggesting that Natd expression was critical for extravasation and invasion of lung cancer cells even at an early stage (Fig. 3a). In turn, the colonization of cancer cells was also significantly inhibited, as we found that the number of tumor nodules in mice received Natd knockdown cells was decreased threefold relative to mice received Scr cells on day 28 (Fig. 3b). These findings were confirmed by quantitation of bioluminescence intensity in lungs (Fig. 3c).

In addition, we have generated a stable murine Natd knockdown Lewis lung carcinoma (LLC) cell line (Supplementary Fig. 3a). In vitro migratory and invasive capabilities of LLC cells were significantly decreased in the Natd knockdown LLC cells compared with the Scr cells (Supplementary Fig. 3b, c). Consistently, mice injected with Natd knockdown LLC cells via tail vein developed significantly fewer tumor nodules compared

**References**

1. Natd contains only a single catalytic unit, Naa40p, and has no auxiliary subunit.
2. Natd was originally identified in yeast, but the human Natd ortholog has also been characterized.
3. Epithelial-to-mesenchymal transition (EMT) is a key cellular program by which cancer cells lose their cell polarity and adhesion, and gain the migratory and invasive capabilities of mesenchymal cells, which is closely associated with metastasis.
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**Supplementary Information**

The online version contains supplementary material available at http://www.nature.com/naturecommunications.

**Author contributions**

Conceptualization, X. Li; Data curation, X. Li, J. Wang, Y. Li, and Z. Liu; Formal analysis, X. Li, J. Wang, and Y. Li; Funding acquisition, X. Li; Investigation, X. Li, J. Wang, Y. Li, and Z. Liu; Methodology, X. Li, J. Wang, Y. Li, and Z. Liu; Writing – original draft, X. Li; Writing – review & editing, X. Li, J. Wang, Y. Li, and Z. Liu.
with the Scr cells measured after 30 days’ growth (Supplementary Fig. 3d), indicating that NatD knockdown markedly decreased the migratory and invasive ability of LLC cells. Consistently, in two orthotopic implantation models of lung cancers using human A549 and murine LLC cells, we found that the migration and invasion were significantly reduced in mice received NatD knockdown cells compared with the mice received Scr cells (Supplementary Fig. 9). These data indicate that the role of NatD is conserved between humans and mice, and that NatD has a critical role in promoting lung cancer cell invasiveness in vivo.

Silencing NatD suppresses cancer cell EMT by downregulating Slug. We next sought to determine how NatD controls the migratory and invasive phenotypes of cancer cells. In a TGF-β1-induced EMT experiment, we observed that Scr H1299 cells with an initial epithelial morphology developed a spindle-like appearance and mesenchymal morphology when treated with TGF-β1 (Fig. 4a). However, TGF-β1-treated NatD knockdown cells mostly retained their rounded epithelial morphology, and were largely, albeit incompletely, inhibited from undergoing EMT (Fig. 4a). This result suggests that NatD might be necessary for

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**Fig. 1** Upregulation of NatD in lung tissues correlates with enhanced invasiveness and poor prognosis of patients with lung cancer. 

a. Quantitative real-time PCR analysis of NatD mRNA levels normalized to GAPDH in lung carcinoma (LC) and matched normal tissues (NT); n = 29, two-tailed Student’s t-test; P = 0.0073 compared with matched normal tissue control. 

b. Representative images of H&E staining and immunohistochemical (IHC) staining of NatD in matched normal tissues (n = 147), human lung squamous carcinoma (n = 74), and lung adenocarcinoma (n = 73) tissue samples. Scale bars, 500 μm. 

c. Total IHC score of NatD in matched normal tissues (NT) and lung carcinoma (LC); mean ± s.d. of 147 pairs of tissue samples; two-tailed Student’s t-test, **P < 0.01 compared with matched normal tissue control. 

d. Percentage of lung cancer patients with high expression and low expression of NatD stratified according to lymph node status (N0 or N1–3) (n = 147); two-sided Pearson χ² test, *P < 0.05. 

e. Kaplan-Meier plots of overall survival of patients with lung cancer, stratified by NatD expression. Data were obtained from Kaplan-Meier plotter database49; log-rank test, P < 0.0001.
**Fig. 2** NatD is required for lung cancer cell migration and invasion in vitro. 

**a** Quantitative real-time PCR analysis of NatD mRNA levels normalized to GAPDH in scrambled control cells (Scr) and NatD-KD1 and NatD-KD2 cells. Results are shown as mean ± s.d. from three independent experiments. Two-tailed Student’s t-test was used. **P < 0.01 compared to Scr control.

**b** Western blot analysis of NatD and Nt-ac-H4 protein levels in scrambled, NatD-KD1, and NatD-KD2 cells. GAPDH and histone H4 served as loading controls. Blots are representative of three independent experiments.

**c** Representative images from wound healing assay of scrambled, NatD-KD1, and NatD-KD2 cells from three independent experiments (left panels). Wound healing assay results are quantified in the histogram (right panel). Results are shown as mean ± s.d. from three independent experiments. Two-tailed Student’s t-test was used. **P < 0.01 compared to Scr control.

**d** Representative images of the migration of scrambled, NatD-KD1, and NatD-KD2 cells in a time-lapse cell tracker migration assay from three independent experiments. Representative images of the migration (e) and invasion (f) of scramble, NatD-KD1, and NatD-KD2 cells with transwell assay from three independent experiments (top panel). Cell counts for the corresponding assays of at least four random microscope fields (×100 magnification). Cell migration and invasion are expressed as a percentage of control (bottom panel). Results are shown as mean ± s.d. from three independent experiments. Two-tailed Student’s t-test was used. **P < 0.01 compared to Scr control.
EMT. Loss of component molecules of cell adhesion and tight junctions is the hallmark of EMT in cancer. We then examined changes in expression levels of key EMT-related transcription factors and markers in lung cancer cells after NatD knockdown under basal conditions in the absence of TGF-β1. Quantitative real-time PCR showed that NatD knockdown increased the expression of the epithelial marker E-cadherin, but reduced the expression of mesenchymal markers, N-cadherin, and Vimentin (Fig. 4b). Interestingly, in terms of transcription factors, only the expression of Slug was significantly repressed in NatD
knockdown cells, whereas the expression of Twist1, Snail, Zeb1, or Zeb2 was not changed in this context (Fig. 4b; Supplementary Fig. 5a). The protein levels of E-cadherin, N-cadherin, Vimentin, and Slug were also altered consistently as determined by western blot analysis (Fig. 4c). Immunofluorescence staining experiments further confirmed that E-cadherin staining was significantly increased and N-cadherin was decreased in cell-to-cell junctions in NatD knockdown cells compared with the Scr cells (Fig. 4d).
We further found that NatD knockdown blocked changes of expression levels of EMT marker genes E-cadherin, N-cadherin, Vimentin, and transcription factor Slug in the presence of TGF-β1 relative to basal condition (Fig. 4b–d). In addition, we analyzed the expression of a spectrum of key proliferation-related and cell cycle-related genes, including CCND1, p21, p27, p57, p16, p18, p19, CHEK2, E2F1, CCND2, KRAS, PTEN, c-Myc, and PCNA in H1299 cells by quantitative real-time PCR27–29. We found that NatD knockdown did not affect the expression of these genes except for CCND2, a gene which may function in cell migration as well (Supplementary Fig. 4a)30. Taken together, these data indicate that NatD is mainly required for maintaining the mesenchymal phenotype, and its downregulation inhibits EMT of lung cancer cells. Consistent results were also obtained in murine LLC cells and human A549 cells; NatD knockdown increased the expression of E-cadherin and decreased the expression of Slug, N-cadherin, and Vimentin in LLC cells (Supplementary Fig. 5a, e, f) and in A549 cells (Supplementary Fig. 4b–d).

Slug is a critical transcriptional regulator of EMT that suppresses E-cadherin expression by direct binding to the CDH1 promoter31. Thus, we tested the possibility that enforced expression of Slug would compensate for NatD knockdown. As expected, migratory and invasive capabilities of NatD knockdown H1299 cells were restored by ectopic expression of Slug (Fig. 4e, f), which was accompanied by suppression of E-cadherin and increased expression of N-cadherin and Vimentin (Fig. 4g, h). Similar results were also obtained in NatD knockdown A549 cells in which Slug expression was ectopically enforced (Supplementary Fig. 4e–h). These results suggest that the ability of NatD to promote EMT likely involves activation of Slug expression.

To probe Slug expression in patients with NSCLC, we performed IHC staining on the same set of human NSCLC tissue arrays containing 74 squamous carcinomas, 73 adenocarcinomas, and adjacent normal lung tissue controls (Supplementary Table 1) with anti-Slug antibody. We found that Slug expression was also significantly upregulated in both squamous carcinomas and adenocarcinomas compared with the normal lung tissues (Supplementary Fig. 5b, c). More interestingly, the expression of Slug and NatD correlated well across all NSCLC samples analyzed (Fig. 4i). This is supported by the Kaplan–Meier survival analysis showing that lung cancer patients with high Slug expression had shorter overall survival (Supplementary Fig. 5d). These results further suggest that NatD may positively regulate Slug expression to promote cancer cell invasion during lung cancer progression.

**Regulation of Slug by NatD is acetyltransferase activity-dependent.** NatD is an α-terminal acetyltransferase that exclusively modifies histone H4 and H2A. To determine whether the regulation of Slug expression and EMT by NatD was acetyltransferase activity-dependent, we constructed a mutant form of NatD (NatDΔ) in which four amino acids (RRKG, aa147–150) located in the acetyl-CoA (Ac-CoA)-binding motif were deleted. Of note, the Ac-CoA binding motif is highly conserved from yeast to humans11. The loss of acetyltransferase activity of NatDΔ was confirmed in an in vitro acetylation assay of a histone H4 N-terminal peptide using 3H-Ac-CoA as an acetyl donor (Fig. 5a). Nat-ac-H4 was also assessed by western blot analysis with an anti-Nt-ac-H4 antibody (Fig. 5b; Supplementary Fig. 6a, b).

***Fig. 4*** Silencing NatD suppresses cancer cell EMT by downregulating Slug. a Representative phase contrast images of Scr and NatD-KD H1299 cells treated with TGF-β1. Data are representative of three independent experiments. Scale bar, 100 μm. b Quantitative real-time PCR analysis of mRNA levels of indicated key EMT-related genes in Scr and NatD-KD H1299 cells normalized to GAPDH in the absence or presence of TGF-β1. Results are shown as mean ± s.d. of three independent experiments. Two-tailed Student’s t-test was used. **P < 0.01 or *P < 0.05 compared with the indicated control. c Western blot analysis of indicated protein levels in Scr cells, NatD-KD cells, and NatD-KD cells with enforced Slug expression (NatD-KD + Slug). (bottom panels) Cells were counted in at least four random microscopic fields (×100 magnification) for the corresponding assays; migration and invasion are expressed as a percentage of control. Results are shown as mean ± s.d. of three independent experiments. Two-tailed Student’s t-test was used. **P < 0.01 compared with the indicated control. g Quantitative real-time PCR analysis of the mRNA levels of NatD and indicated key EMT-related genes (normalized to GAPDH) in Scr cells, NatD-KD cells, and NatD-KD + Slug cells. Results are shown as mean ± s.d. of three independent experiments. Two-tailed Student’s t-test was used. **P < 0.01 or *P < 0.05 compared with Scr or indicated control. h Western blot analysis of indicated protein levels in Scr cells, NatD-KD cells, and NatD-KD + Slug cells. GAPDH served as a loading control. Data are representative of three independent blots. i Pearson correlation scatter plot of the H score of Slug and NatD in human lung carcinoma (n = 147); r = 0.6672, P < 0.0001
reduced enrichment levels of H4R3me2a and H4K5ac on the Slug promoter in NatD-KD cells compared with the Scr cells (Fig. 6b). Enrichment levels of H4R3me2a on the Slug promoter were unchanged in NatD-KD cells compared with the Scr cells (Fig. 6b). We also found significantly reduced enrichment levels of H3K4me3 and increased enrichment levels H3K27me3 in NatD-KD cells compared with the Scr cells (Fig. 6c). Of note, these changes in histone marks were consistent with down-regulation of Slug expression by NatD knockdown. Enrichment levels of H4S1ph, H3K4me3, and H3K27me3 on the promoters of Zeb1, Zeb2, Twist1, and Snail were unchanged although those of Nt-ac-H4 were reduced in NatD-KD cells compared with the Scr cells (Supplementary Fig. 7a–d). Importantly, an antagonistic relationship between Nt-ac-H4 and H4S1ph was dependent on the acetyltransferase activity of NatD (Fig. 6d, e). These results suggest that Nt-acetylation and phosphorylation of histone H4S1 are antagonistic, and histone mark Nt-ac-H4 can communicate with other histone modifications to co-ordinately modulate Slug gene expression.

**Downregulation of Nt-acetylation of histone H4 facilitates binding of CK2α to histone H4 in lung cancer cells.** We have shown that levels of histone marker H4S1ph were significantly increased when NatD was knocked down in lung cancer cells. Therefore, we wanted to determine whether CK2α, a catalytic subunit of CK2 responsible for triggering phosphorylation of histone H4S1ph, was upregulated due to NatD knockdown. Quantitative RT-PCR detection and western blot analysis showed no increase in the levels of either CK2α mRNA or protein in NatD knockdown cells compared with the Scr cells (Supplementary Fig. 8a, b). These results indicated that the increased levels of H4S1ph in NatD knockdown cells were due to elevated expression of CK2α. Thus, we suspected that the increased levels of H4S1ph in NatD knockdown cells were because more CK2α was being shuttled into the nucleus after NatD knockdown. To test this possibility, we performed a confocal immunofluorescence assay using specific anti-CK2α antibody in both NatD knockdown cells and Scr cells. We found that nearly 100% of CK2α in NatD knockdown cells was localized in the nucleus, as opposed to being mainly in the cytoplasm in Scr cells.

Fig. 5 Regulation of Slug by NatD is acetyltransferase activity-dependent. a (left) In vitro acetylation assay showing the catalytic activity of NatDΔ and wild-type NatD (CPM, counts per minute). Data are mean ± s.d. of three independent experiments; Student’s t-test, **P < 0.01 compared with wild-type NatD. (right) SDS-PAGE analysis of purified recombinant NatDΔ and wild-type NatD proteins from E. coli stained by Coomassie brilliant blue (CBB). MW, protein molecular weight markers. b (top) Western blot analysis of an H4 (1–31) peptide with anti-CK2α antibody in both ΔΔ and +ΔΔ cells. (bottom) Autoradiographic image showing products from in vitro acetylation assay using histones as substrates extracted from H1299 cells. Results are representative of three independent experiments. c (top) Autoradiographic image showing products from in vitro acetylation assay using histones as substrates extracted from H1299 cells. Results are representative of three independent experiments. (bottom) Histones shown by Coomassie blue staining. d Quantitative real-time PCR analysis of mRNA levels of Slug, E-cadherin, N-cadherin, and Vimentin normalized to GAPDH in H1299 cells overexpressing NatDΔ or wild-type NatD. Data are mean ± s.d. of three independent experiments; Student’s t-test, **P < 0.01 compared with the wild-type NatD. e Western blot analysis of indicated proteins from H1299 cells overexpressing NatDΔ or wild-type NatD. GAPDH and histone H4 served as loading controls. Data are representative of three independent experiments. f, g Representative images of the migration (e) and invasion (f) of H1299 cells overexpressing NatDΔ or wild-type NatD with transwell assay from three independent experiments (top panel). Cell counts for the corresponding assays of at least four random microscope fields (>100 magnification). Cell migration and invasion are expressed as a percentage of control (bottom panel). Results are shown as mean ± s.d. from three independent experiments. Two-tailed Student’s t-test was used. **P < 0.01 compared with the indicated control.
but in Scr cells only about 70% of CK2α was localized in the nucleus (Fig. 7a). There were no detectable levels of CK2α in the cytoplasm of NatD knockdown cells on western blots, consistent with the immunofluorescence assay (Fig. 7a, b). In contrast, in Scr cells, expression of CK2α was also detected in the cytoplasm as well as in the nucleus (Fig. 7a, b). These results provide evidence indicating that NatD knockdown resulted in re-localization of CK2α to the nucleus. This finding raised the question of what is the consequence of the movement of CK2α from the cytoplasm to the nucleus.

The observation that Nt-ac-H4 and H4S1ph are antagonistic, and that NatD knockdown results in additional shuttling of CK2α into the nucleus leading to significantly increased phosphorylation of H4S1, suggests that, in NatD-replete cells, Nt-acetylation of histone H4 may obstruct binding of CK2α to histone H4 preventing phosphorylation. To examine this possibility, we performed a peptide pulldown assay using C-terminal biotin-tagged 31 amino acid N-terminal peptides of histone H4 in which the Ser1 residue was either acetylated (pNt-ac-H4) or non-acetylated (pH4), or mutated to alanine (pH4S1A), or using C-terminal biotin-tagged 20 amino acid N-terminal peptides of histone H3 without N-terminal acetylation (pH3). We analyzed the eluates from pulldowns by western blot with an antibody against CK2α.

Histone modifications have an essential role in gene regulation. However, the function of N-α-terminal acetylation of histone H4 has remained uncertain even though this modification is abundant, and the corresponding enzyme NatD is highly conserved in eukaryotes3, 4. In this study, we show that NatD-mediated Nt-acetylation of histone H4 antagonizes serine phosphorylation to promote EMT in lung cancer. This process is depicted in the model shown in Fig. 8. High NatD expression in lung cancer samples was correlated with high Slug expression, enhanced invasiveness, and reduced patient survival. These findings suggest that NatD is a key epigenetic regulator of cell invasion during lung cancer progression.
A large body of evidence suggests that EMT is an important driver of cancer progression \(^{20-22}\). Histone modifications have been shown to link closely to EMT \(^{23, 24}\). To undergo EMT, cancer cells need to acquire epigenetic changes other than genetic changes \(^{23, 24, 35}\). This study demonstrated that NatD can trigger Nt-acetylation of histone H4 on the Slug promoter to promote EMT of lung cancer cells. These findings identify a new function for NatD in gene expression regulation, and extend our understanding of epigenetic regulation of EMT via Nt-acetylation of histone H4. Slug, a key regulator of EMT, has been identified as one of the major drivers of chemoresistance, and is associated with cancer stem cell properties \(^{36, 37}\). In our results, we found that
Slug is a direct epigenetic target of NatD to mediate EMT processes of lung cancer cells. More importantly, the expression levels of Slug correlate intimately with those of NatD in lung cancer tissues. These data suggest that NatD may also be linked to chemoresistance and cancer cell stemness which deserve further investigation in the future. We favor the hypothesis that Slug is the key regulator of EMT. However, given the capacity of NatD to regulate expression of multiple genes, we cannot at this point completely rule out the possibility that other genes directly regulated by NatD might also contribute to migration and invasion independent of Slug expression.

Interestingly, we observed that Nt-acetylation by NatD and phosphorylation of histone H4S1 by CK2α were antagonistic on the Slug promoter. Our results demonstrated that removal of Nt-acetylation facilitated nuclear accumulation of CK2α and its binding to histone H4 in lung cancer cells resulting in phosphorylation of histone H4 Ser1 by CK2α, suggesting that Nt-acetylation of histone H4 obstructs binding of CK2α. These data unveil a mechanistic switch from Nt-acetylation of histone H4 by NatD to phosphorylation of histone H4 Ser1 by CK2α, although the reason why NatD knockdown led to cytoplasm-to-nucleus shuttling of CK2α is currently unclear.

In addition to increased enrichment of H4S1ph, reduced Nt-acetylation of H4 also resulted in decreased enrichment of H4K5ac and H4R3me2a on the Slug promoter. Histone H4S1ph has been shown to have a temporal inverse relationship with histone H4K5/K8/K12ac during yeast sporulation and mammalian spermatogenesis, and is inhibitory to acetylation on histone H4K5/K8/K12ac during DNA damage. Thus, our results indicate that H4S1ph may act as a key histone mark mediating crosstalk between Nt-acetylation of H4 and acetylation and methylation of histone H4 tail. However, in yeast, loss of Nt-acetylation induces H4R3me2a, but not H4K5/K8/K12ac on ribosomal DNA, even though H4S1ph was not determined. It is likely that the communication between NatD-mediated Nt-acetylation of histone H4 and internal acetylation and methylation is context-dependent and gene-specific.

Identification of a reliable epigenetic biomarker and related mechanisms in lung cancer will provide new insights for diagnosis and prognosis. Previous studies demonstrated that the NatA complex or Naa10p (the catalytic subunit of the NatA complex) is associated with cancer, and is crucial for maintaining proliferation and ensuring survival of various cancer cells. Recently, NatD was shown to have an anti-apoptotic role in colorectal cancer cells through a p53-independent mechanism. Agreeing with these observations, we found that NatD has an important role in promoting cancer cell migration and invasion. Furthermore, NatD expression levels were significantly elevated in lung cancer tissues compared with adjacent normal tissues, and correlated inversely with patient survival, corroborating the view that NatD promotes lung cancer progression. Therefore, these data indicate that NatD might be a useful diagnostic and prognostic molecular marker in lung cancer.

In summary, this study demonstrates a novel link between NatD-mediated Nt-acetylation of histone H4 and lung cancer progression. We show that NatD-mediated Nt-acetylation of histone H4 antagonizes serine 1 phosphorylation of histone H4 to promote EMT of lung cancer cells through epigenetic control of Slug (Fig. 8). NatD is essential for lung cancer cells to maintain a mesenchymal phenotype and to promote invasion, thus highlighting NatD inhibitor as a potential early therapeutic intervention in lung cancer patients.

**Methods**

**Cell cultures and viral infection.** H1299 cells, A549 cells, LLC, and 293T cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). These cells were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide in DMEM with 10% FCS (Invitrogen).

The human lung cancer cell lines were recently authenticated by Genetic Testing Biotechnology Corporation (Suzhou, China) using short tandem repeat (STR) profiling. No cell line used in this paper is listed in the database of commonly
misidentified cell lines maintained by the International Cell Line Authentication Committee (iCLAC). All lines were found to be negative for mycoplasma contamination.

The small interfering RNA (siRNA) target sequences for RNA interference of NatD were inserted into the Xhol/Hpal sites in the pLL3.7 lentiviral vector according to the manufacturer’s recommendations (American Type Culture Collection, USA). The oligonucleotides with the following sequences were synthesized by RibioBio Co. Ltd (Guangzhou, China).

Holographic Imaging, Sweden). For cell migration assays, 5 × 10⁵ cells were seeded on the upper surface that did not pass through the 8-μm pore-size carbonate filter. Thermophoresis was measured for 30 s on a NanoTemper Monolith NT.115 (NanoTemper Technologies GMBH) using 60% LED power and 20% laser power. Dissociation constants were calculated by NanoTemper Analysis 1.5.41 software using the mass action equation (K₆₅ formula).

Cell viability and invasion assays. The in vitro viability of H1299 cells was measured using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer’s protocol. Flow cytometric analysis of apoptosis was assessed by Annexin V and PI staining using the Annexin V-APOC Apoptosis Detection Kit (KeyGEN BioTECH, China) according to the manufacturer’s guide. For cell cycle analysis, cells were harvested and fixed at 4°C overnight with 70% ethanol. Cells were washed twice with PBS, and their DNA was stained using a Cell Cycle Detection Kit (KeyGEN BioTECH, China). The samples were analyzed by flow cytometry (Becton Dickinson, NJ, USA), and results were analyzed with FlowJo software according to the manufacturer’s instructions. For the wound healing assay, cells were plated to confluence in a 6-well plate, and the cell monolayer was scratched using a pipette tip. Representative photos were taken using a digital camera mounted on an inverted microscope (Olympus) at indicated times. Live cell imaging was performed using the HoloMonitor M4 time-lapse cytometer (Phase Holographic Imaging, Sweden). For cell migration assays, 5 × 10⁵ cells were seeded into the upper chamber of the transwell apparatus (Corning Costar) in serum-free medium, and medium supplemented with 15% FBS was added to the bottom chamber. After 12 h, the cells on the upper surface that did not pass through the 8-μm pore-size polycarbonate filter were removed using a moistened cotton swab; the adherent cell lines maintained by the International Cell Line Authentication Committee (ICLAC). All lines were found to be negative for mycoplasma contamination.

For overexpressing Slug, human -UTR was cloned by DNA sequencing. Expression and purification of recombinant proteins and generation of anti-NatD-acetylation antibody against NatD-ac-h4. Human NatD cDNA was cloned into pGE66p-1 vector, and expression of full-length protein was induced in E. coli BL21 (DE3) by IPTG. The GST-tag was removed by treatment with PreScission protease (GE Healthcare Life Sciences). The mutant NatD (lacking RRK at amino acids 147–150) was constructed using a site-directed mutagenesis kit (SBS Genetech, China). The oligonucleotides used to introduce the deletion were: 5′- TTGGAGACGGCCTGGAGGGATGCT-3′ and its complementary DNA. Expression and purification of NatD were as described for NatD. Human CK2α cDNA (amino acids 1–335) was cloned into pET28a vector at unique SalI and BamHI sites. All clones were confirmed by DNA sequencing. Expression and purification of CK2α were performed according to the manufacturer’s protocol (Takara). No IG-specific antibody was generated by immunization of rabbits using Nat-a4-H4 peptide (amino acids 1–14) conjugated to KLH (Keyhole limpet hemocyanin) as an antigen. Subsequently, the IgG fraction from serum was purified by GenScript, Nanjing, China (Supplementary Fig. 6).

In vitro acetylation assays. Purified wild-type NatD or NatDΔ was incubated with either a C-terminal biotinylated histone H4 peptide (amino acids 1–31) or histones purified from H1299 cells, plus 2 μM 13C-3H-Acetyl-CoA (Amersham) as the acetyl donor in a mixture of 20 μl acetyltransferase buffer (50 mM Tris-HCl pH 8.2, 100 μM EDTA, 10% Glycerol, 1 mM DTT) for 2 h at 37°C. Half of the sample of C-terminal biotinylated histone H4 peptide (amino acids 1–31) and the acetylated histones were resolved on a 15% (w/v) SDSPAGE gel, stained with Coomassie blue, dried, and subjected to autoradiography.

Western blot analysis and protein interaction studies. Cellular proteins were extracted by RIPA lysis buffer at high salt concentration (420 mM NaCl), and western blot analysis was performed in accordance with standard protocols. Scans of enhanced chemiluminescence (ECL) films showing uncropped blots are presented in Supplementary Fig. 6. For autoradiography, slides were incubated with the aid of ImageJ software (NIH). Measurements were performed on more than 30 cells from three independent experiments.

RNA isolation and quantitative RT-PCR. Total RNA from tissue samples and cultured cells was extracted using TRIzol reagent (Invitrogen). cDNAs were synthesized with a HiScript 1st Strand cDNA Synthesis Kit (Vazyme Biotech, China). Quantitative RT-PCR was performed using a FastStart Universal SYBR Green Master (Roche) according to the manufacturer’s instructions in a Rotor-Gene 6000 (Corbett Research) in a final volume of 20 μl. Cycling conditions were 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Each reaction was performed in triplicate. The primer sequences for RT-PCR are listed in Supplementary Table 2 and Table 3.

Chromatin immunoprecipitation (ChiP) assay. ChiP assays were performed with H1299 cells in accordance with standard protocols. Normal rabbit IgG served as the control. ChiP samples were analyzed by quantitative real-time PCR using the FastStart Universal SYBR Green Master (Roche). A standard curve was prepared for each set of primers using serial titration of the input DNA. The percentage of ChiP DNA was calculated relative to the input DNA from primer-specific standard curves using the Rotor-Gene 6000 Series Software 1.7. The primer sequences for ChiP are listed in Supplementary Table 4. Antibodies used were: H45115 (Abcam; ab14723), H4K5ac (Millipore; CS204381), H4K8ac (Millipore; CS204357, 1:1000), H4K12ac (Millipore; 06-1352, 1:1000), H4R3me2a (Active Motif; 39705, 1:1000), H4R3me2s (Abcam; ab5932, 1:1000), H3K18ac (Abcam; ab14723, 1:1000), CK2α (ab26974, 1:2000), H2B (ab70774, 1:2000), A01455, 1:2000), and Hsp70 (GenScript; A01236, 1:1000). Peptide pulldown assays were performed according to standard protocols. Briefly, we coupled streptavidin beads to 2 μg C-terminal biotin-tagged 31-mer N-terminal peptides of histone H4 and to acetylated H4 (aa 1–44), as well as 31-mer non-acetylated 20-mer terminal peptides of non-acetylated histone H3. The resulting streptavidin-coupled peptides were incubated with H1299 cellular extracts prepared with high salt extraction (420 mM NaCl). We eluted specifically bound protein from stringently washed beads, separated the samples by SDS-PAGE, and visualized proteins by western blot with anti-CK2α antibody.

For MST analysis, purified recombinant CK2α proteins were labeled with the Monolith NT.647-NHS. Labeled proteins were used at a concentration of 100 nM in PBS pH 7.4 containing 0.05% TWEEN-20. The concentration of peptides of either histone H4 (aa 1–31) or Nt-acetylated histone H4 (aa 1–31) ranged from 10 nM to 500 nM. The combined solution of labeled proteins and peptides were incubated for 5 min and transferred into silicon-treated capillaries. Thermophoresis was measured for 30 s on a NanoTemper Monolith NT.115 (NanoTemper Technologies GMBH) using 60% LED power and 20% laser power. Dissociation constants were calculated by NanoTemper Analysis 1.5.41 software using the mass action equation (K₆₅ formula).

Immunofluorescence and confocal microscopy. For immunofluorescence assays, cells were fixed with 4% formaldehyde for 5 min at room temperature. After washing cells 3 times in PBS with 0.1% Triton X-100, cells were blocked with 3% BSA for 30 min. Cells were incubated with primary antibody (E-cadherin, N-cadherin, or CK2α) for 1 h at room temperature. Following washes with PBS 0.1% Triton X-100, cells were incubated with a secondary antibody (Alexa Fluor 555 from Cell Signaling Technology; 4431 or Vecto Laboratories, TI-2000) for 1 h at room temperature. Following washes with PBS 0.1% Triton X-100, cells were stained with DAPI (Sigma) and visualized by immunofluorescence microscopy (Nikon). Sub-cellular distribution of CK2α was analyzed by confocal scanning microscopy (Olympus FV10i). The relative intracellular distribution of CK2α in non-tumor and tumor sample was calculated using software ImageJ (National Institutes of Health) where the nuclear/cytoplasmic (n/c) ratio by measuring the intensity of the signals in each cellular compartment with the aid of ImageJ software (NIH). Measurements were performed on more than 30 cells from three independent experiments.
7. Forte, G. M., Pool, M. R. & Stirling, C. J. N-terminal acetylation inhibits protein targeting to the endoplasmic reticulum. PLoS Biol. 9, e1001073 (2011).

8. Tran, J. C. et al. Mapping intact protein isoforms in discovery mode using top-down proteomics. Nature 480, 254–8 (2011).

9. Mullien, J. R. et al. Identification and characterization of genes and mutants for an N-terminal acetyltransferase from yeast. EMBO J. 8, 2607–75 (1989).

10. Magin, R. S., Lissczak, G. P. & Marmorestein, R. The molecular basis for histone H4- and H2A-specific amino-terminal acetylation by NatD. Structure 25, 332–41 (2011).

11. Hole, K. et al. The human N-alpha-acetyltransferase 40 (HnAat40/NatD) is conserved from yeast and N-terminal acetylates histones H2A and H4. PLoS ONE 6, e24713 (2011).

12. Song, O. K., Wang, X., Waterborg, J. H. & Sternglanz, R. An N-alpha-acetyltransferase responsible for acetylation of the N-terminal residues of histones H4 and H2A. J. Biol. Chem. 278, 38109–12 (2003).

13. Polevoda, B., Hoskins, J. & Sherman, F. Properties of Nat4, an (N-alpha)-acetyltransferase of Saccharomyces cerevisiae that modifies N terminals of histones H2A and H4. Mol. Cell Biol. 29, 2913–24 (2009).

14. Schià, V., Molina-Serrano, D., Kyrkaïou, D., Hadjianiotiou, A. & Kirmizis, A. N-alpha-terminal acetylation of histone H4 regulates arginine methylation and ribosomal DNA silencing. PLoS Genet. 9, e1003805 (2013).

15. Liu, Z. et al. Patt1, a novel protein acetyltransferase that is highly expressed in liver and downregulated in hepatocellular carcinoma, enhances apoptosis of hepatoma cells. Int. J. Biochem. Cell Biol. 41, 2528–37 (2009).

16. Liu, Y. et al. Liver Patt1 deficiency protects male mice from age-associated but not high-fat-diet-induced hepatic steatosis. J. Lipid Res. 53, 358–67 (2012).

17. Pavlou, D. & Kirmizis, A. Depletion of histone N-terminal-acetyltransferase Naa40 induces p53-independent apoptosis in colorectal cancer cells via the mitochondrial pathway. Apoptosis 21, 298–311 (2016).

18. Thiery, J. P., Acolque, F., Nieto, M. & Nieto, K. M. Epithelial-mesenchymal transitions in development and disease. Cell 139, 871–90 (2009).

19. Yang, J. & Weinberg, R. A. Epithelial-mesenchymal transition: the crossroads of development and tumor metastasis. Dev. Cell 14, 818–29 (2008).

20. Ye, X. & Weinberg, R. A. Epithelial-mesenchymal plasticity: a central regulator of cancer progression. Trends Cell Biol. 25, 675–86 (2015).

21. Zheng, X. et al. Epithelial–mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. Nature 527, 525–30 (2015).

22. Fischer, K. R. et al. Epithelial–mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. Nature 527, 472–6 (2015).

23. Stadler, S. C. & Allis, C. D. Linking epithelial-mesenchymal-transition and epigenetic modifications. Semin. Cancer Biol. 22, 404–10 (2012).

24. Tam, W. L. & Weinberg, R. A. The epigenetics of epithelial-mesenchymal plasticity in cancer. Nat. Med. 19, 1438–49 (2013).

25. Thiery, J. P. & Sleeman, J. P. Complex networks orchestrate epithelial-mesenchymal transitions. Nat. Rev. Mol. Cell Biol. 7, 131–42 (2006).

26. De Craene, B. & Berra, G. Regulatory networks defining EMT during cancer initiation and progression. Rev. Cancer 13, 97–110 (2011).

27. Maddika, S. et al. Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. Drug Resist. Updat. 10, 13–29 (2007).

28. Antoni, L., Sodha, N., Collins, I. & Garrett, M. D. CHK2 kinase: cancer susceptibility and cancer therapy—two sides of the same coin? Nat. Rev. Cancer 7, 925–36 (2007).

29. Hirao, A. et al. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science 287, 1824–1827 (2000).

30. Ladam, P. et al. Loss of a negative feedback loop involving p37 and cyclin D2 is required for p38-mediated migration in transformed mammary epithelial cells. Mol. Cancer Res. 11, 1412–1424 (2013).

31. Peinado, H., Olmeda, D. & Cano, A. Tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer 7, 415–28 (2007).

32. Cheung, W. L. et al. Phosphorylation of histone H4 serine 1 during DNA damage repair requires casein kinase II in S. cerevisiae. Curr. Biol. 15, 656–60 (2005).

33. Rank, G. et al. Identification of a PRMT5-dependent repressor complex linked to silencing of human fetal globin gene expression. Blood 116, 1585–92 (2010).

34. Ermolaeva, M. V., Boldyreff, R., Issinger, O. G. & Niefind, K. Crystal structure of a C-terminal deletion mutant of human protein kinase CK2 catalytic subunit. J. Mol. Biol. 330, 925–34 (2003).

35. Dawson, M. A. & Kouzarides, T. Cancer epigenetics: from mechanism to therapy. Cell 150, 12–27 (2012).

36. Kurniawan, N. K. et al. Snail2 and Slug mediate radioreistance and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer cells. Stem Cells 27, 2059–68 (2009).

37. Tsukasa, K. et al. Slug contributes to gemcitabine resistance through epithelial-mesenchymal transition in CD133(+) pancreatic cancer cells. Hum. Cell 28, 167–74 (2015).

38. Govin, J. et al. Systematic screen reveals new functional dynamics of histones H3 and H4 during gametogenesis. Genes Dev. 24, 1772–86 (2010).
39. Utley, R. T., Lacoste, N., Jobin-Robitaille, O., Allard, S. & Cote, J. Regulation of NuA4 histone acetyltransferase activity in transcription and DNA repair by phosphorylation of histone H4. *Mol. Cell Biol.* 25, 8179–90 (2005).

40. Lee, C. F. et al. hNaa10p contributes to tumorigenesis by facilitating DNMT1-mediated tumor suppressor gene silencing. *J. Clin. Invest.* 120, 2920–30 (2010).

41. Hua, K. T. et al. N-alpha-acetyltransferase 10 protein suppresses cancer cell metastasis by binding PIX proteins and inhibiting Cdc42/Rac1 activity. *Cancer Cell* 19, 218–31 (2011).

42. Kalvik, T. V. & Arnesen, T. Protein N-terminal acetyltransferases in cancer. *Oncogene* 32, 269–76 (2013).

43. Zhao, Q. et al. PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. *Nat. Struct. Mol. Biol.* 16, 304–11 (2009).

44. Iwase, S. et al. ATRX ADD domain links an atypical histone methylation recognition mechanism to human mental-retardation syndrome. *Nat. Struct. Mol. Biol.* 18, 769–76 (2011).

45. Wienken, C. J., Baaske, P., Rothbauer, U., Braun, D. & Duhr, S. Protein-binding assays in biological liquids using microscale thermophoresis. *Nat. Commun.* 1, 100 (2010).

46. Liu, M. et al. Heterochromatin protein HP1gamma promotes colorectal cancer progression and is regulated by miR-30a. *Cancer Res.* 75, 4593–604 (2015).

47. Kuniyasu, H. et al. CD10 enhances metastasis of colorectal cancer by abrogating the anti-tumoural effect of methionine-enkephalin in the liver. *Gut* 59, 348–56 (2010).

48. Blackhall, F. H. et al. Prevalence and clinical outcomes for patients with ALK-positive resected stage I to III adenocarcinoma: results from the European Thoracic Oncology Platform Lungscape Project. *J. Clin. Oncol.* 32, 2780–7 (2014).

49. Gyorffy, B., Surowiak, P., Budczies, J. & Lanczky, A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. *PLoS ONE* 8, e82241 (2013).

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Author contributions

J.L., A.C., Y.D., M.L., Ying W., Yadong W., M.N., B.Y., T.G., X.L., Z.X., and C.M. performed experiments; C.W. and M.F. purified proteins; H.D., M.K., and C.L. performed peptide binding assays; Y.S. and J.W. provided pathology expertise; K.Z., C.-Y.Z., D.C.S.H., and C.D.A. provided ideas, reagents, and discussion; R.T., C.K.Z., J.W., and Q.Z. designed the project and wrote the manuscript.

Additional information

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