

KLF4 regulates adult lung tumor-initiating cells and represses K-Ras-mediated lung cancer

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Lung cancer is the leading cause of cancer-related mortality in both men and women worldwide. To identify novel factors that contribute to lung cancer pathogenesis, we analyzed a lung cancer database from The Cancer Genome Atlas and found that Krüppel-like Factor 4 (KLF4) expression is significantly lower in patients' lung cancer tissue than in normal lung tissue. In addition, we identified seven missense mutations in the *KLF4* gene. KLF4 is a transcription factor that regulates cell proliferation and differentiation as well as the self-renewal of stem cells. To understand the role of KLF4 in the lung, we generated a tamoxifen-induced *Klf4* knockout mouse model. We found that KLF4 inhibits lung cancer cell growth and that depletion of *Klf4* altered the differentiation pattern in the developing lung. To understand how KLF4 functions during lung tumorigenesis, we generated the *K-ras*^{LSL-G12D/+};*Klf4*^{fl/fl} mouse model, and we used adenovirus-expressed Cre to induce *K-ras* activation and *Klf4* depletion in the lung. Although *Klf4* deletion alone or *K-ras* mutation alone can trigger lung tumor formation, *Klf4* deletion combined with *K-ras* mutation significantly enhanced lung tumor formation. We also found that *Klf4* deletion in conjunction with *K-ras* activation caused lung inflammation. To understand the mechanism whereby KLF4 is regulated during lung tumorigenesis, we analyzed *KLF4* promoter methylation and the profiles of epigenetic factors. We found that Class I histone deacetylases (HDACs) are overexpressed in lung cancer and that HDAC inhibitors induced expression of KLF4 and inhibited proliferation of lung cancer cells, suggesting that *KLF4* is probably repressed by histone acetylation and that HDACs are valuable drug targets for lung cancer treatment.

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Lung cancer is the most commonly diagnosed cancer among men and women and the leading cause of cancer deaths in the United States.¹ Mutations in multiple genetic pathways, including *EGFR*, *TP53* and *K-RAS*, are linked to lung tumorigenesis. *KRAS* mutations are limited to non-small-cell lung cancer, which is the most prevalent form of lung cancer.² The K-ras protein belongs to a small GTP-binding protein family, which acquires transforming activity with specific mutations, such as a mutation at codon 12.^{3,4} These mutations result in a decrease in GTPase activity and constitutive activation of the K-ras signaling, which actively promotes proliferation and cell survival and ultimately leads to cancer. *KRAS* mutations are found in more than one-third of human lung adenocarcinomas. Somatic activation of the *K-ras* gene causes early-onset lung cancer in mice.⁴

Krüppel-like Factor 4 (KLF4) was initially identified as a zinc finger transcription factor enriched in the epithelium of intestine and skin.^{5,6} Later, it was found in a variety of other tissues, such as thymus, cornea, cardiac myocytes and lymphocytes.^{7–10} KLF4 has an important role in the development and cell differentiation.^{6,11,12} In normal lung tissue, KLF4 is expressed in fibroblasts and airway epithelial cells, and was found to be the most significantly altered lung gene at birth.¹³ KLF4 is downregulated in gastrointestinal cancers and has

been identified as a tumor suppressor in many types of cancer.^{14–16} As one of the four factors that induce pluripotent stem cells, KLF4 has a role in cell fate reprogramming and self-renewal of embryonic stem cells.^{17,18} *In vitro* studies indicated that KLF4 inhibits the growth of many cancer cell lines, including lung cancer cells.¹⁹ However, the function and regulation of KLF4 *in vivo* in the lung and lung cancer are still not known.

The cell lineage in the lung has been well studied. The major component of the trachea and the proximal conducting airways are basal secretory ciliated cells; bronchiolar and terminal bronchiolar epithelium are mainly composed of non-ciliated columnar Clara cells, and alveolar epithelium are composed of type I and type II cells (named AT1 and AT2 cells, respectively). After birth, AT2 cells function as stem cells in the lung.^{20,21} The self-renewal of AT2 cells is regulated by EGFR and K-ras signals.²¹ In patients, lung adenocarcinoma often stains positive for Clara cell marker CC10 (Clara cell antigen 10) and AT2 cell marker SP-C (surfactant protein C), which led to the hypothesis that Clara cells and AT2 cells might be cells of origin of lung adenocarcinoma. In a recent study, *K-ras* was activated by *polr2a*-Cre^{ER}, which led to tumors arising only in the lung. Although Cre recombination was detected in the alveoli, bronchioles and the bronchioalveolar

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Abbreviations: TCGA, The Cancer Genome Atlas; KLF4, Krüppel-like Factor 4; HDAC, histone deacetylases; SP-C, surfactant protein C; CC10, Clara cell antigen 10; BADJ, bronchioalveolar duct junction; IHC, immunohistochemistry; H&E, hematoxylin and eosin; NaBt, sodium butyrate; MPO, Myeloperoxidase

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duct junction (BADJ), only alveolar lesions progressed to more advanced adenomas and adenocarcinomas, which were composed exclusively of SP-C⁺ cells.²² In another study, *K-ras* was activated in either AT2 cells or Clara cells by adenovirus-expressed SP-C-Cre or CC10-Cre. Both AT2 cells and Clara cells had the ability to initiate malignant transformation, but the initiating cell type influences the type of tumors that arose.²³ These findings suggest that AT2 cells are the major origin of lung tumorigenesis.

In this study, we analyzed the function of KLF4 in lung homeostasis and lung cancer using novel mouse models. We also examined the mechanisms of KLF4 regulation in lung cancer.

Results

Klf4 is a putative tumor suppressor for lung cancer.

KLF4 has been identified as a tumor suppressor for gastrointestinal cancers but was also suggested as an oncogene for breast cancer.^{24,25} *KLF4* mutations have been identified in colon cancer and meningiomas.^{14,26,27} To examine the function of KLF4 in lung cancer, we analyzed *KLF4* mutations in lung cancer patients from the The Cancer Genome Atlas (TCGA) adenocarcinoma database. In all, 9 out of 488 patients had *KLF4* mutations. Seven out of these nine mutations were found in the activation domain and the three zinc finger regions of the *KLF4* gene (Figure 1a). One

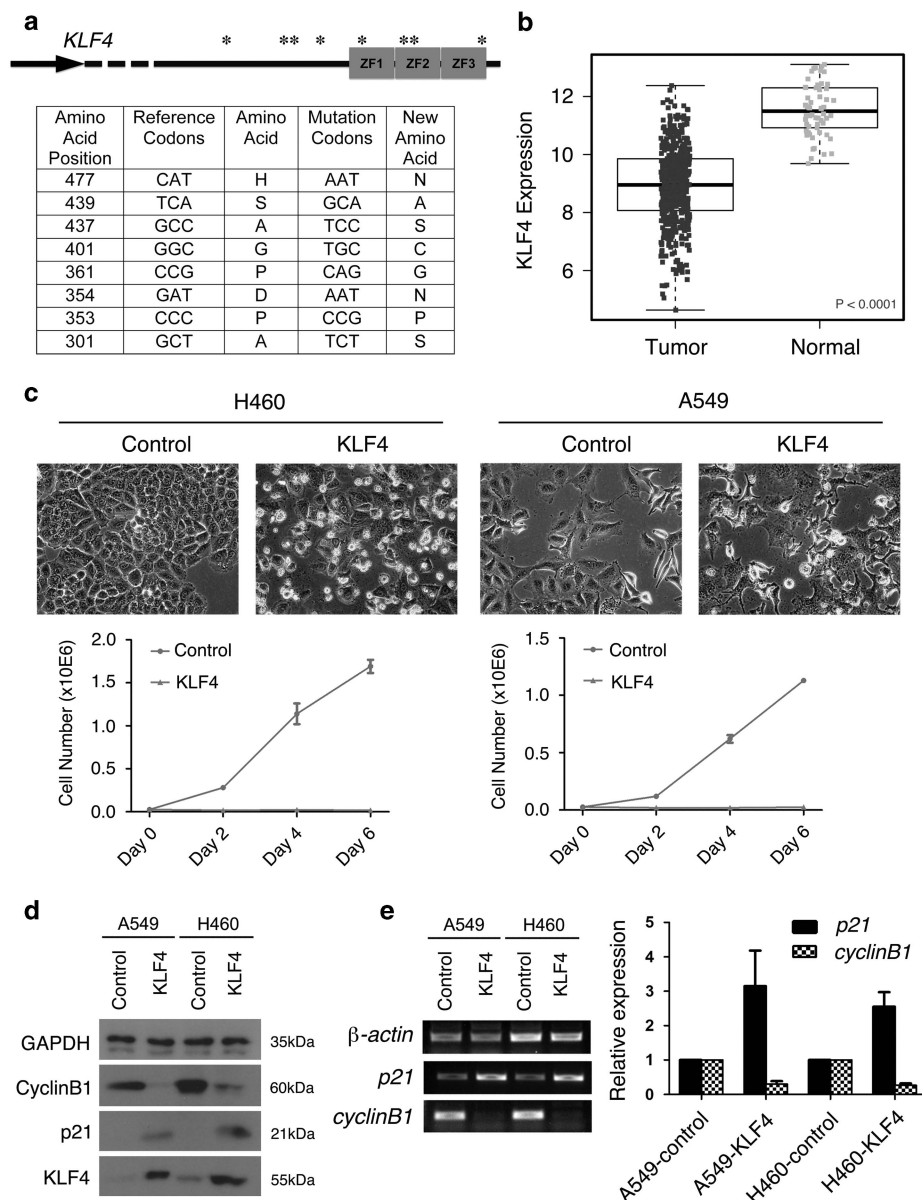


Figure 1 Klf4 is a putative tumor suppressor for lung cancer. (a) Schematic diagram and table list of mutational sites in the *KLF4* gene domains. ZF, zinc finger domain; ‘*’, Mutational sites, details of which are explained in the table below. (b) *KLF4* expression in normal and lung tumor tissues. The P value was calculated based on a linear mixed model. (c) Top: Morphology of lung cancer cells with ectopic *KLF4* expression. Middle and bottom: growth curve of lung cancer cell lines A549 and H460. ‘Control’, vector-virus-infected cells. (d and e) Western blot (d), semi-quantitative RT-PCR and quantification (e) of expression of *KLF4* target genes in lung cancer cell lines with the effect of overexpression of *KLF4*. Data are represented as mean ± S.D.

mutation was found in the intron and one was a silent mutation. We also analyzed the small-cell lung carcinoma data from TCGA and found only one case with KLF4 mutation (Supplementary Figure 1a).

KLF4 has the ability to repress cyclinB1 Luciferase reporter.^{15,28} To examine the relationship between mutations and functions of KLF4, we made seven KLF4 constructs containing each of the nine point mutations or containing two point mutations that are close together, and we tested their functions in cyclinB1 reporter assay. These mutations have no significant effect on KLF4 function in repressing cyclinB1 promoter activity (Supplementary Figure 1b).

To further examine the potential deregulation of KLF4 in lung cancer, we analyzed the expression level of *KLF4* mRNA in human normal lung versus lung tumor tissues from the TCGA adenocarcinoma database and found that *KLF4* expression was significantly lower in tumor tissues (Figure 1b, $P < 0.0001$). Analysis from the small-cell carcinoma database also indicated significantly lower level of KLF4 mRNA expression (Supplementary Figure 1c).

Other members of KLF family transcription factors may also be altered in lung cancers.²⁹ To evaluate the correlation of other KLFs in TCGA lung adenocarcinoma, we analyzed mRNA expression level of KLF1-17 in adenocarcinoma patients and found that in addition to KLF4, KLF1, 2, 6, 8, 9, 10, 11, 12, 13, 14, 15 and 17 were also significantly lower in tumor compared with normal tissues (Supplementary Figure 2).

To test the role of KLF4 in lung cancer cells, we ectopically expressed KLF4 by lentivirus-mediated delivering in the human lung cancer cell lines H460 and A549, respectively, and found that *KLF4* overexpression inhibited cell growth to a large extent (Figure 1c), which is consistent with the role of KLF4 as a tumor suppressor for lung cancer. We also infected the BEAS-2B (lung epithelial) cell line with KLF4-carrying lentivirus and tested the cell growth rate. We found that KLF4 overexpression had no significant effect on proliferation of these cells, probably because of the endogenous KLF4 expression (Supplementary Figure 3).

To test the molecular effects of *KLF4* overexpression in the lung cancer cell lines, we analyzed the protein expression of KLF4 and target genes by western blot (Figure 1d), and tested mRNA expression of KLF4 target genes by reverse transcriptase PCR (RT-PCR; Figure 1e). We found that *p21^{Cip1/WAF}* was upregulated, whereas *cyclin B1* was downregulated 48 h post transfection (Figures 1d and e), suggesting that KLF4 represses lung cancer cell growth by regulating cell cycle and cell proliferation. Deregulation of KLF4 could increase cell proliferation during lung tumorigenesis.

Klf4 ablation altered the differentiation pattern in mouse lung. To test the function of KLF4 in mouse lung, we generated the *Rosa-Cre⁺;Klf4^{fl/fl}* mice (Figure 2a), in which *Rosa-Cre* can be activated by tamoxifen. KLF4 is expressed in the epithelial cells of bronchioles as well as alveolar cells (Figure 2b). *Rosa26* locus is ubiquitously expressed in mouse embryos³⁰ and thus can actively drive tamoxifen-induced *Klf4* deletion in most cells in mouse lung (Figures 2b–d). Two types of cells have been suggested as the origins of lung cancer: AT2 cells and Clara cells.^{21–23,31,32}

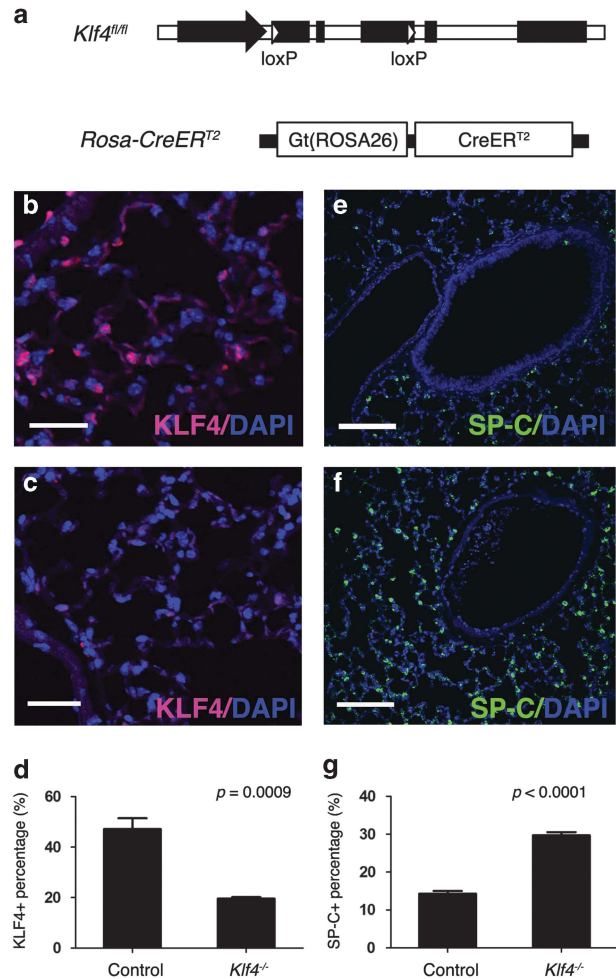


Figure 2 Klf4 ablation altered the differentiation pattern in mouse lung. (a) Genetic model of the *Rosa-Cre⁺;Klf4^{fl/fl}* mouse. LoXP elements allow Cre-induced *Klf4* deletion (top); and *Rosa26* drives CreER^{T2} expression in mouse lung (bottom). (b–d) Immunofluorescent staining for KLF4 (red) in wild-type mouse lung (b) and in *Klf4*-deleted mouse lung (c). (d) Statistical analysis of KLF4-positive cells in mouse lung. Data are represented as mean \pm S.D. (e–g) Immunofluorescent staining for SP-C in wild-type (e) and *Klf4*-deleted (f) mouse lung. (g) Statistical analysis of SP-C-positive cells in mouse lung. Data are represented as mean \pm S.D. Scale bars for (b and c): 30 μ m. Scale bars for (e and f): 100 μ m

SP-C is a marker for AT2 cells and CC10 is a marker for Clara cells. To detect the effects of *Klf4* deletion on AT2 cells in the lung, immunofluorescent staining was performed using SP-C antibody and we found that SP-C protein was increased in *Klf4*-deleted mouse lung tissue (Figures 2e–g). This result suggests that KLF4 has a role inhibiting differentiation of AT2 cell lineage and/or controlling the number of differentiated AT2 cells. To test the effect of *Klf4* deletion on other cell types, we stained the lung tissues with antibodies to AT1 cell marker Caveolin 1 (Cav1; Supplementary Figures 4a and b), Clara cell marker CC10 (Supplementary Figures 4c and d), as well as ciliated cell marker FoxJ1 (Supplementary Figures 4e and f). There was no significant difference in these cell markers. AT2 cells have been suggested as the origin of solid tumors in the lung.³³ Although bronchioalveolar stem cells (CC10⁺ SP-C⁺) were identified as the putative cells of origin of lung adenocarcinoma,³² more studies performed recently

reported that AT2 cells are the predominant cells of origin of adenocarcinoma that is driven by *K-ras* activation.^{21–23,31,34} Our results suggest that *Klf4* deletion could be a driving force of lung tumorigenesis through stimulating AT2 cells in mouse lung.

Klf4 deletion facilitates lung tumor formation and progression. To study the role of KLF4 in mouse lung tumorigenesis, we established another mouse model by crossing the *Klf4*^{fl/fl} line with the *K-ras*^{LSL-G12D/+} line. In this model, activation of *K-ras*^{G12D} can occur simultaneously with *Klf4* deletion in the lung. *K-RAS* mutations are found in more than one-third of human lung adenocarcinomas. Somatic activation of the *K-ras* gene causes early-onset lung cancer in mice.⁴ Adenovirus-expressed-Cre recombinase was delivered to *Klf4*^{fl/fl}, *K-ras*^{LSL-G12D/+} or *K-ras*^{LSL-G12D/+}; *Klf4*^{fl/fl} mouse models by nasal administration, to induce *Klf4* deletion, *K-ras* activation or both in mouse lung (Figure 3a). As indicated by hematoxylin and eosin (H&E) staining, tumor tissue appeared in *K-ras*^{LSL-G12D/+}; *Klf4*^{fl/fl} mice as soon as 8 weeks after Cre-induction. Immunofluorescent staining

indicated that *Klf4* was depleted in these tumors (Figure 3b), suggesting that loss of tumor-suppressing function of KLF4 contributed to tumor formation in these mice.

To test the effect of loss of function of KLF4, along with the effect of *K-ras*^{G12D} mutation in lung tumorigenesis, we infected the mouse models (control, *Klf4*^{fl/fl}, *K-ras*^{LSL-G12D/+} or *K-ras*^{LSL-G12D/+}; *Klf4*^{fl/fl} mice) with Cre-adenovirus and tested the tumor formation in the lungs of these mice. By 8 weeks post infection, diffuse hyperplasia and a few isolated lesions were observed in *K-ras*^{LSL-G12D/+} mouse lung, whereas more hyperplasia and adenoma were observed in *K-ras*^{LSL-G12D/+}; *Klf4*^{fl/fl} mice (Figures 4a and c). Both immunohistochemistry (IHC) staining and immunofluorescent staining show that SP-C-positive AT2 cells were increased in lung tumor tissue from *K-ras*^{LSL-G12D/+}; *Klf4*^{fl/fl} mice, compared with normal tissue in wild-type mice (Figure 4b), consistent with the *Rosa-Cre*⁺; *Klf4*^{fl/fl} model in Figures 2e–g. These results suggest that *Klf4* deletion synergized with *K-ras* activation to facilitate tumor formation in the lung. Our findings are consistent with the previous finding that *Klf4* deletion sufficiently initiated carcinoma development upon *K-ras* activation in mouse tongue.³⁵

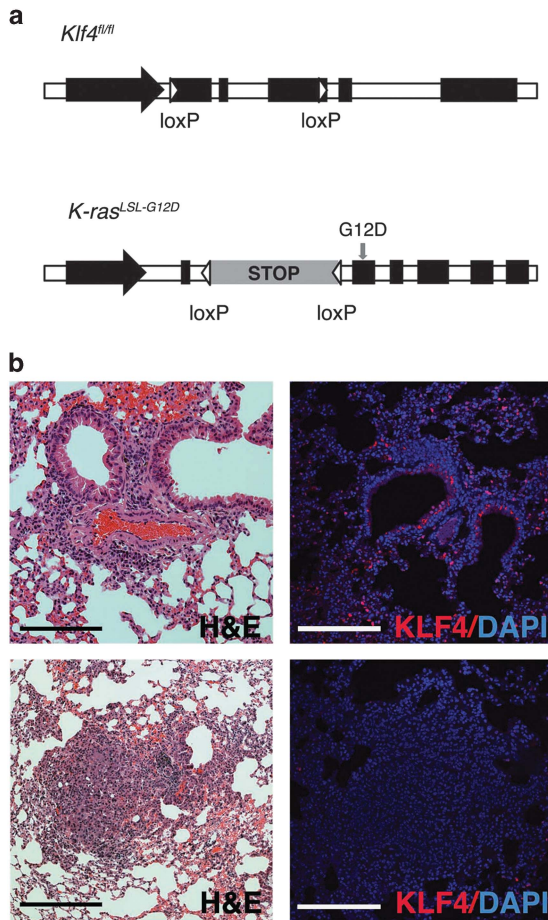


Figure 3 Establishing the genetic model of the *K-ras*^{LSL-G12D/+}; *Klf4*^{fl/fl} mouse. (a) LoxP elements allow Cre-induced *Klf4* deletion or G12D mutation-mediated *K-ras* activation. (b) H&E staining and KLF4 immunofluorescent staining in normal tissue from wild-type mice (top) and in tumor tissue from *K-ras*^{LSL-G12D/+}; *Klf4*^{fl/fl} mice (bottom) tissue in the lung, indicating loss of *Klf4* expression in lung tumor. Scale bars: 100 μ m

Multiple mechanisms of KLF4 downregulation in lung cancer. KLF4 mutations were identified in fewer than 2% of the total patients (Figure 1a), suggesting that genetic mutation is not the main cause of KLF4 downregulation in lung cancer. We also analyzed DNA methylation on the *KLF4* gene in lung adenocarcinoma patients from the TCGA database. Although a small portion of lung cancer tissues contains *KLF4* promoter methylation, there was no significant correlation between *KLF4* expression and methylation in lung tumor tissues (Figure 5a, $P=0.8$). As KLF4 levels were significantly reduced in lung cancer tissues (Figure 1b), there must be other major factors that repress *KLF4* expression during lung tumorigenesis.

Histone modifications bear important roles in regulating cell proliferation and tumor growth. We have reported that KLF4 interacts with histone acetyltransferase and regulates histone acetylation.^{28,36} KLF4 also interacts with histone deacetylases (HDACs) and regulates gene expression.³⁷ To identify the major mechanisms of how KLF4 is regulated in the process of lung tumorigenesis, we analyzed the profile of the lung cancer database from TCGA and studied the expression levels of HDACs in both normal and lung tumor tissues. Among HDACs 1–11, expression levels of HDACs 1, 2, 3 and 8, which belong to Class I HDACs, were increased in lung tumor tissues compared with normal lung tissues ($P<0.0001$), whereas expression of HDACs 4, 5 and 7, which belong to Class IIa HDACs, were decreased (Figure 5b, and Supplementary Figure 5b, $P<0.0001$). These findings are consistent with the previous observation that Class I and Class II HDACs are differentially expressed in cancers, and that the Class IIa HDACs exert a dual role in cancer.³⁸

To test the hypothesis that HDACs inhibit *KLF4* expression in lung cancer, we treated the lung cancer cell A549 as well as HEK293T cells with HDAC inhibitors and analyzed the expression of *KLF4* and its target genes by RT-PCR and real-time PCR. We found that KLF4 was induced in cells treated with several HDAC inhibitors, including suberoylanilide

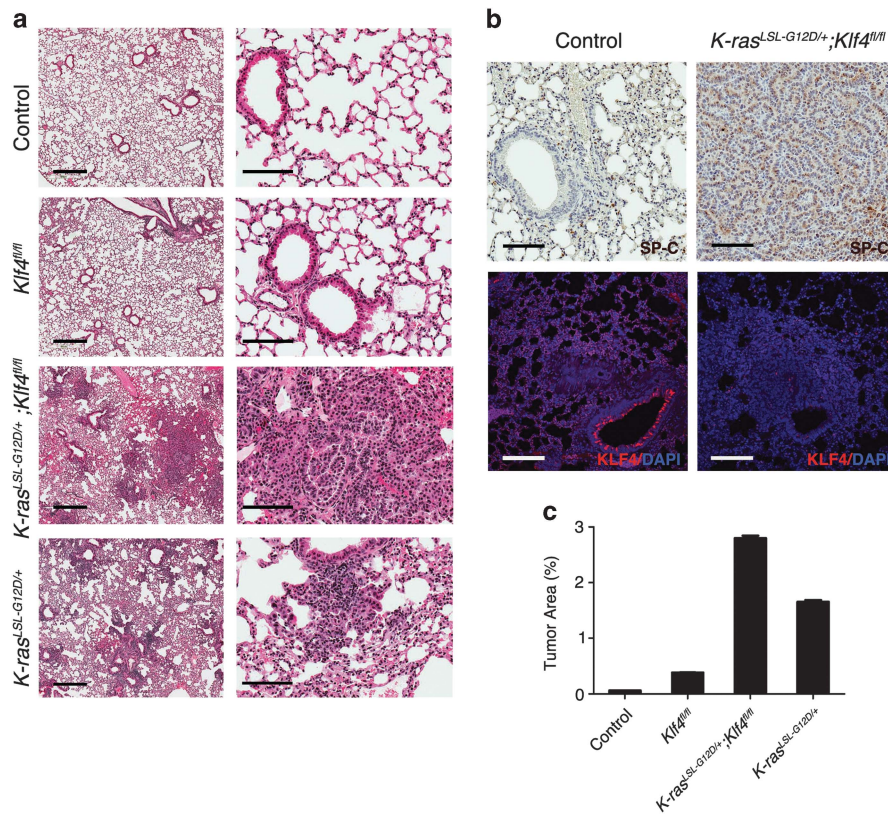


Figure 4 *Klf4* deletion facilitates lung tumor formation and progression. (a) H&E staining of the lung tissue from wild-type mice (control), the *K-ras^{LSL-G12D/+}* mice, *Klf4^{fl/fl}* mice and *K-ras^{LSL-G12D/+};Klf4^{fl/fl}* mice by 8 weeks post infection. Scale bars from left to right: 2 mm and 100 μ m. (b) IHC staining of SP-C (top) and immunofluorescent staining of KLF4 (red, bottom) showing that SP-C tends to increase in tumor tissue from *K-ras^{LSL-G12D/+};Klf4^{fl/fl}* mice than in normal tissue from wild-type mice. Scale bars: 100 μ m. (c) Statistical analysis of the percentage of tumor area by 8 weeks post infection (based on H&E staining). Data are represented as mean \pm S.D.

hydroxamic (SAHA) and Trichostatin A (TSA), which are inhibitors of Class I, II and IV HDACs; and sodium butyrate (NaBt) and valproic acid (VPA) (Figure 5c), which are inhibitors of Class I and IIa HDACs.³⁹ The cell proliferation assay also demonstrated that HDAC inhibitors repressed the growth of A549 and H460 cells (Figure 5d), which is consistent with their role in inducing *KLF4* expression.

Studies have found that HDAC1 and KLF4 interact to regulate human myeloid leukemia cell proliferation.⁴⁰ In order to study the correlation between HDACs and KLF4 in lung cancer cells, we did the luciferase reporter assays using GKLF-pGL2-Luciferase containing KLF4 promoter reporter.⁴¹ We found that HDAC3 has an inhibitory role on KLF4 promoter activity (Supplementary Figure 6).

Discussion

As an important regulator in cell differentiation and proliferation, KLF4 is essential in maintaining homeostasis and morphology in the lung. The *Rosa-Cre⁺;Klf4^{fl/fl}* mouse model study provided evidence that KLF4 is functional in maintaining the number and differentiation pattern of SP-C-positive AT2 cells in the mouse lung during normal development (Figure 2). In addition, the *K-ras^{LSL-G12D/+};Klf4^{fl/fl}* mouse model study showed evidence that depletion of *Klf4* facilitates lung tumor formation and progression through controlling the pattern of cell differentiation and proliferation (Figures 1 and 4). AT2 cells

have been identified as stem cells in adult lung.²⁰ The self-renewal of AT2 cells can be induced by activated *K-ras in vivo*.²¹ Our data suggest that *Klf4* deletion-induced AT2 cells are potential tumor-initiating cells leading to lung adenocarcinoma (Figures 2 and 4b). The tumor-suppressor role of KLF4 was further supported by the findings that KLF4 was significantly downregulated in human lung cancers (Figures 1a and b).

The *K-ras* mouse models have been used to identify the origins of lung cancer. The earliest findings suggested that a rare population of cells located at the BADJ is CC10/SP-C double positive, which is capable of expanding in early *K-ras* mutant tumors induced by adenoviral-CMV-Cre, thus considered the origin of adenocarcinomas.³² Later studies in mice with activated *K-ras* in SP-C⁺ or CC10⁺ cells using conditional Cre^{ER} knock-in alleles indicated that tumors arose only in the alveoli, although recombination occurred throughout the bronchioles, the BADJ and the bronchioalveolar stem cells, which are CC10/SP-C double-positive cells.^{21,31} We found that KLF4 deletion increased the number of AT2 cells and enhanced *K-ras*-mediated lung tumorigenesis (Figures 2e–g and 4), consistent with the findings that AT2 cells are the major origin of lung cancer²² and that *K-ras* regulates self-renewal of AT2 cells.²¹

Compared with lesions at 8 weeks post infection, more adenomas and adenocarcinomas arose at 16 weeks in *K-ras^{LSL-G12D/+};Klf4^{fl/fl}* mice, whereas only trace amount of

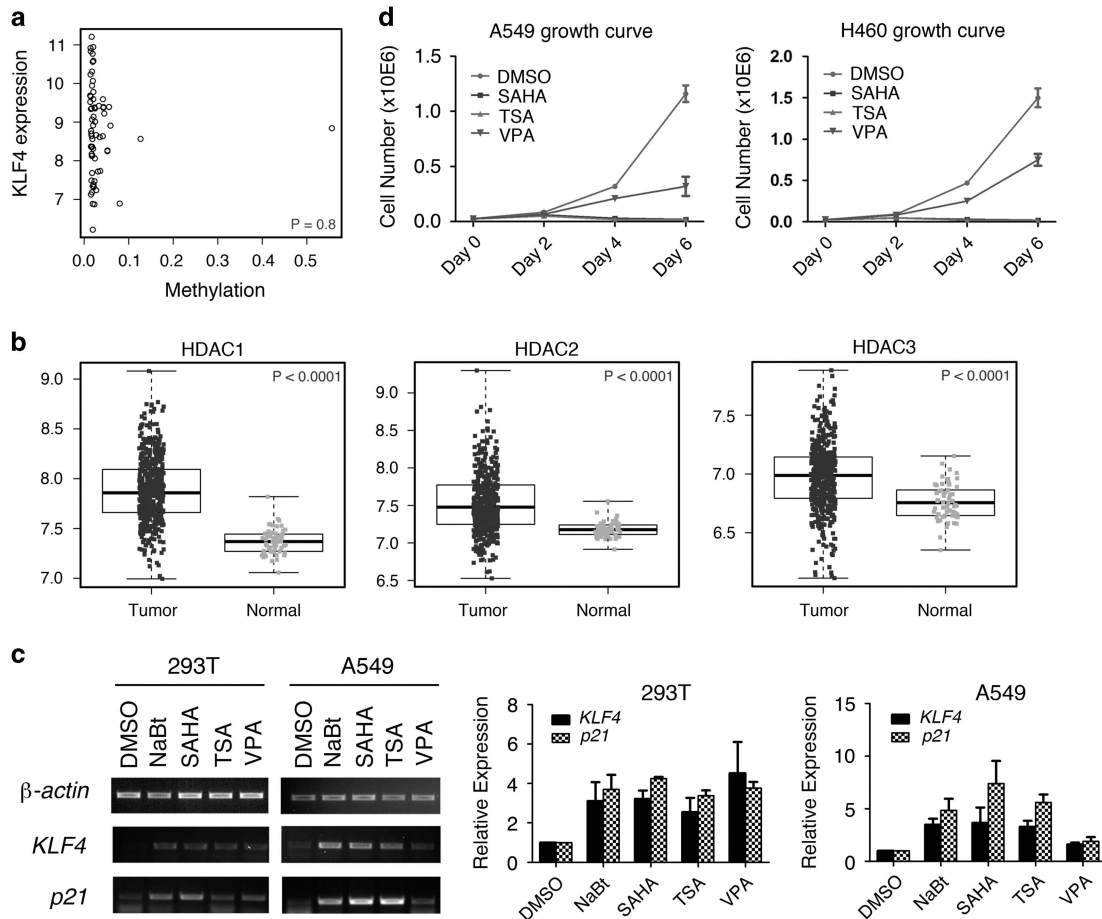


Figure 5 Multiple mechanisms of KLF4 downregulation in lung cancer. (a) Correlation between *KLF4* expression and methylation in lung adenocarcinoma patients. Pearson's correlation coefficient was -0.03 ($P = 0.8$, 95% confidence interval -0.27 to 0.21). (b) HDAC expression in normal lung and lung tumor tissues from TCGA lung adenocarcinoma samples. P values were calculated based on linear mixed models. (c) Semi-quantitative RT-PCR (left) and real-time PCR (right) testing expression of *KLF4* and target gene *p21*^{Cip1/WAF1} in HEK293T and A549 cell lines when treated with HDAC inhibitors. Data are represented as mean \pm S.D. (d) Growth curve of A549 and H460 cell lines when treated with HDAC inhibitors

diffuse hyperplasia was found in *Klf4*^{fl/fl} and *K-ras*^{LSL-G12D/+} mouse lung (Supplementary Figure 7a). The difference over time may be due to high dose of virus that we used (2.5×10^7 PFU), which may cause diffusion or absence of lesions in mouse lung.⁴² The average number of nodules of lung adenoma and adenocarcinoma in each lung lobe was greatly higher in *K-ras*^{LSL-G12D/+};*Klf4*^{fl/fl} mice compared with control mice by 16 weeks (Supplementary Figure 7b).

KLF4 has multiple functions. It acts as a transcription factor by binding to the promoters of its target genes, such as *cyclin B1* and *p21*^{Cip1/WAF1}. It is a transcription activator as well as a repressor. In addition, it can bind other transcription factors and regulate their activity. KLF4 may regulate lung homeostasis and tumorigenesis through multiple mechanisms. For example, it has been reported that the stem cell marker Bmi1 have critical roles in lung tumorigenesis in *K-ras*^{G12D} mice.⁴³ Bmi1 is critical for lung tumorigenesis and stem cell expansion. In the absence of oncogenic K-ras, Bmi1 deficiency led to impaired proliferation and self-renewal capacity of bronchioalveolar stem cells.⁴³ Our previous finding suggests that KLF4 inhibits Bmi1 expression in colorectal tumorigenesis.⁴⁴ Using

the mouse model from our current study, IHC staining of Bmi1 in the lung tissue showed that Bmi1 is overexpressed in tumor tissue in the *K-ras*^{LSL-G12D/+};*Klf4*^{fl/fl} mouse (Supplementary Figure 8). Both Bmi1 and AT2 cell markers were increased in the tumors, suggesting that KLF4 deletion induced tumor-initiating cells during lung tumorigenesis.

KLF4 was first defined as required for establishing the barrier function of the skin.⁴⁵ Studies have shown that KLF4 has a role in mediating proinflammatory signaling in macrophages,^{46,47} and it regulates anti-inflammatory cytokines such as interleukin-10.⁴⁸ Chronic lung inflammation has been associated with tumorigenesis in the lung. As one of the most common genetic changes in lung cancer, *K-ras* activation induces a robust inflammatory response.⁴⁹ Based on observations from H&E staining, Adenovirus-Cre administration induced certain areas of inflammatory infiltrates in the lung (Figure 4a). Myeloperoxidase (MPO) is a marker for neutrophils, which are important members of the innate immune system, and which are highly recruited to the site of inflammation during the immune response. We stained the lung tissues for MPO and compared the percentage of MPO⁺

cells. Although adenoviral infection could induce inflammation, which is associated with bronchiolar hyperplasia,²² *Klf4* deletion and *K-ras* mutation in combination had greatly enhanced the inflammatory response (Supplementary Figure 9). Moreover, higher intensity of MPO staining cells in *K-ras^{LSL-G12D/+};Klf4^{fl/fl}* mice are mainly localized at the tumor regions (Supplementary Figures 9g and h). The above data indicate that *Klf4* depletion triggered lung inflammation, which may contribute to lung tumorigenesis in the mice. Our findings suggest that KLF4 has an important role in protecting lung tissue from inflammation and inflammation-associated tumorigenesis.

Our study also demonstrated that mutations and methylation are not the major mechanisms that cause the down-regulation of KLF4 in lung cancer (Figures 1a and 5a, Supplementary Figures 1a, b and 5a), whereas analysis of HDACs in lung cancer database and lung cancer cells revealed an important mechanism by which KLF4 is regulated in lung tumorigenesis (Figure 5 and Supplementary Figures 5 and 6). HDACs are grouped in four families.⁵⁰ Among these, type III HDACs are known as sirtuins. The expression profiles of sirtuins in the lung cancer patients have not been analyzed in this study. HDAC6 belongs to group IIb along with HDAC10, and it has unique structural and functional features.^{51,52} We also treated cells with an HDAC6 inhibitor, ACY-1215,⁵³ but found no change in *KLF4* expression (not shown). It is very interesting that all four members of Type I HDACs were significantly increased in human lung cancers (Figure 5b and Supplementary Figure 5b). HDAC3 has an inhibitory role on KLF4 promoter activity in HEK293T cells. In A549 cells, the effect was not significant (Supplementary Figure 6). This could be due to the high level of HDAC3 expression in lung cancer cells or because of the redundant function of other HDACs. Previous studies found that KLF4 recruits HDAC3 to the cyclinB1 promoter and that KLF4 synergized with HDAC3 in repressing the cyclinB1 promoter activity.²⁸ This could be another mechanism that Class I HDAC inhibitors can regulate KLF4 and its target genes. The studies of HDAC inhibitors (Figures 5c and d) indicate that HDAC inhibitors inhibit lung cancer cell growth at least partially through inducing KLF4 expression. As discovered in many other types of malignancy,^{54,55} it is of potential importance to develop specific type I HDAC inhibitors targeting lung cancer-initiating cells.

In addition to KLF4, other members of KLF family can be altered in lung cancers²⁹ (Supplementary Figure 2). It is of great interest to further study the function of other KLF transcription factors in lung tumor initiation and progression.

Materials and Methods

Animal studies. Mouse experiments were performed under the approval by the Institutional Animal Care and Use Committees of the University of Kentucky (2009-0604). Mice of Cre-inducible *Klf4^{fl/fl}* were crossed with mice of the Cre-inducible *K-ras^{LSL-G12D/+}* strain. All mice used for experiments were on C57BL/6 genetic background. Mice were genotyped by PCR using genome DNA samples from mice tails. Adenovirus Ad5CMVCre-eGFP was from Gene Transfer Vector Core at the University of Iowa. Adenovirus was used to induce *Klf4* deletion and/or *K-ras* mutation by intranasal infection technique.⁵⁶ Mice were killed 8–16 weeks after the virus infection for analysis. For the *Klf4^{fl/fl}*, Ad5CMVCre-eGFP induces the 'flox' of critical exons of *Klf4* gene. And for *K-ras^{LSL-G12D/+}*, Ad5CMVCre-eGFP induces the 'flox' of the 'stop' element ('LSL'), to activate the mutant *K-ras^{G12D}* expression.

Cell culture and proliferation assay. Human lung cancer cell lines A549 and H460 were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin. For proliferation assay, cells were plated at $\sim 2.5 \times 10^4$ cells per well in 12-well plates and counted at appropriate times using the cell viability analyzer (Beckman Coulter, Indianapolis, IN, USA, Vi-Cell XR). For proliferation assay in Figure 1c, cells were infected with vector-carrying or KLF4-carrying lentivirus, and equal numbers of cells were seeded to 12-well plates 18 h post infection, thus counted as day 0.

Western blotting. Cells were lysed in the appropriate volume of lysis buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1% glycerol, 50 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100, with protease inhibitors). The following antibodies were used: mouse anti-GAPDH (GeneTex, Irvine, CA, USA, GT239), rabbit-anti-KLF4¹⁵ and Bmi1 (Epitomics, Burlingame, CA, USA, S2983).

RT-PCR and real-time PCR. A549 and H460 cells were plated at $\sim 2 \times 10^5$ cells per well in a six-well plate to be infected by vector-carrying or KLF4-carrying lentivirus. HEK293T and A549 cells were treated with lentivirus or the following molecules: DMSO (control, 1:1000 in volume); sodium butyrate (5 mM); suberoylanilide hydroxamic (10 μ M); Trichostatin A (1 μ M); VPA (1 mM). After 48 h of incubation, RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcriptase PCR (RT-PCR) was performed as described previously.¹⁵

Real-time RT-PCR reactions were carried out using SYBR Green PCR master mix reagents (Thermo, Waltham, MA, USA) on the ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Thermal cycling was conducted at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min, then the melt curve: 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The relative quantification of gene expression for each sample was analyzed by the Δ Ct method. The following primers were used: *β -actin*, 5'-CAA CCGCAGAGAAGATGAC-3' and 5'-AGGAAGGCTGAAGAGTG-3'; *p21^{Cip1/WAF1}*, 5'-CGACTGTGATGCGCTAATGG-3' and 5'-AGAAGATCAGCCGGCGTTTG-3'; *cyclin B1*, 5'-GCAGCACCTGGCTAAGAATG-3' and 5'-GCCACAGCCTTGGC TAAATC-3'; KLF4, 5'-AGAGGAGCCCAAGCCAAAG-3' and 5'-CGTCCAGT CACAGTGGTAAG-3'.

H&E staining and IHC staining. H&E staining was performed based on standard protocol by the Markey Cancer Center Biospecimen and Tissue Procurement Shared Resource Facility at the University of Kentucky. For IHC staining, the following antibodies were used: rabbit anti-KLF4,¹⁵ goat anti-CC10 (T-18, Santa Cruz, Dallas, TX, USA, sc-9772), rabbit anti-SP-C (FL-197, Santa Cruz, sc-13979), mouse anti-FoxJ1 (eBioscience, San Diego, CA, USA, 14-9965), mouse anti-Cav1 (7C8, NovusBio, Littleton, CO, USA, NB100-615SS), rabbit anti-Bmi1 (Epitomics, Burlingame, CA, USA, S2983), rabbit anti-Myeloperoxidase (Acris, San Diego, CA, USA, AP23272PU-N). Slides were scanned by the Aperio ScanScope XT and analyzed by nuclearv9 algorithm in the Aperio Analysis software (Leica Biosystems, Buffalo Grove, IL, USA).

Immunofluorescent staining. Deparaffinized slides were incubated with antigen retrieval buffer and blocking solution with 5% goat serum in PBST for 20 and 10 min, respectively. Slides then were incubated with primary antibody overnight at 4 °C, followed by secondary antibody for 1 h at room temperature. Rhodamine-Tyramide and FITC-Tyramide were used to amplify signals from the secondary antibodies, according to the previous protocol.⁵⁷ Primary antibodies used include rabbit anti-KLF4¹⁵ and rabbit anti-SP-C (FL-197, Santa Cruz, sc-13979). The secondary antibody used was goat anti-rabbit-HRP (Jackson, West Grove, PA, USA, 111-035-003). Nuclei were stained by DAPI (Sigma, St. Louis, MO, USA). The glass slides were mounted with coverslips, viewed and photographed with an Olympus FW1000 confocal microscope.

Statistical analysis. Normalized RNAseq(V2) gene expression data were downloaded from TCGA and log₂-transformed. The following numbers of patients were included: 488 lung adenocarcinoma versus 58 normal specimens; 501 lung small-cell carcinoma versus 51 normal specimens. The expressions of *KLF4* and HDACs in tumor and normal samples were compared based on linear mixed models, which accounted for the correlation between tumor and normal samples from the same individual. DNA methylation data (beta values) from 65 lung adenocarcinoma specimens and 65 lung small-cell carcinoma specimens were

downloaded from TCGA. Pearson's correlation coefficient was used to assess the correlation between *KLF4* mRNA expression and methylation in lung adenocarcinoma specimens and in lung small-cell carcinoma specimens. Statistical significance was defined as $P < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

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