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Original Article

RNAi screening identifies KAT8 as a key molecule important for cancer cell survival

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Abstract: Histone acetyltransferases (HATs) regulate many critical cancer events, including transcriptional regulation of oncogene and tumor suppressors, chromatin structure and DNA damage response. Abnormal expression of HATs has been reported in a number of cancers. However, cellular functions of HATs in cancer and molecular mechanisms remain largely unclear. Here, we performed a lentiviral vector-mediated RNAi screen to systematically address the function of HATs in lung cancer cell growth and viability. We identified 8 HATs genes involved in A549 cell viability. Further experiments showed that KAT8 regulates G2/M cell cycle arrest through AKT/ERK-cyclin D1 signaling. Moreover, KAT8 inhibition led to p53 induction and subsequently reduced bcl-2 expression. Our results demonstrate an important role of KAT8 in cancer and suggest that KAT8 could be a novel cancer therapeutic target.

Keywords: HATs, RNAi screen, KAT8, cyclin D1, p53, cell survival

Introduction

Histone acetylation, one of the most extensively characterized epigenetic modifications, is controlled by histone acetyltransferases (HATs) [1] and histone deacetylases (HDACs) [2]. HATs catalyze transfer of acetyl groups to NH2-terminal lysine residues in histones, which results in more open conformation of nucleosomes and increased accessibility of regulatory proteins to DNA, whereas HDAC activity causes chromatin condensation and transcriptional repression [3]. HDACs have been extensively studied and several HDAC inhibitors are currently in clinical trials as potential anticancer treatment. Vorinostat has been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma [4]. Recently, growing evidences suggest that in addition to HDAC inhibitors, HAT inhibitors may also have therapeutic potential [5]. It has been found that abnormal activities of HATs are linked to a number of human diseases, especially cancer. HATs are involved in many key cancer-related processes, including transcription regulation of a number of cancer-related genes, DNA damage response and repair, as well as DNA replication. Moreover, HATs gene expression profiles have been reported to be associated with pathological and clinical outcomes in breast cancer [6]. HATs are therefore promising cancer therapeutic targets.

Nonetheless, cellular functions of HATs in cancer and molecular mechanisms remain largely unclear. Although alteration of HAT activity has been found to be common in cancer, such as translocation of HAT genes in acute myelogenous leukemia [7, 8]; and mutations of p300 in human colorectal, gastric, breast, and pancreatic cancers [9, 10], the function of most HATs in cancer is unclear. Elucidation of function of different HATs in different cancer types will be helpful to better understand the consequences of histone and general protein acetylation and potentially provide novel therapeutic approaches for the treatment of cancer.

RNA interference (RNAi) genetic screens have been successfully used to identify genes involved in a phenotype [11]. To systematically address the function of HATs in lung cancer cell growth and viability, we carried out an RNAi screen for genes involved in the regulation of A549 lung cancer cell viability. We performed

the screen using a lentiviral short-hairpin RNA (shRNA) library targeting the entire human HATs. We reasoned that genes identified through the screen might provide insights into the value of HATs as therapeutic targets in lung cancer. Our results identified a number of HATs in A549 cell viability and revealed a functionally important pathway involving KAT8-mediated AKT/cyclin D1 signaling. KAT8 inhibition impairs AKT signaling, in turn reducing cyclin D1 expression. Our study provides a rational basis for identifying novel cancer therapeutic targets.

Materials and methods

Cell culture, antibody and shRNA lentiviral library

Human Embryonic Kidney cells 293T and human lung cancer cells A549 (ATCC, CCL-185) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. The following antibodies were used: BCL2, p53, CCND1, p-AKT, AKT, p-ERK, ERK, GAPDH.

A customized lentiviral shRNA library targeting 16 histone acetylase genes was purchased from 3D-HTS (China). Each gene in this library contained two pools of four distinct shRNA species targeting different sequences of the target transcript.

RNAi screening

A549 cells (2500 per well) were plated in 96 well plates and infected 24 hr later with shRNA library, screened as 32 pools of 128 shRNAs per pool in independent triplicates in the primary screening. At 120 hr following infection, the CellTiter One solution Cell Proliferation Assay (Promega, USA) was performed. The primary screen was completed in duplicate. Four distinct shRNA species targeting each gene were used to revalidate hits from the primary screening. A significance threshold of $P<0.05$ (Student's test) was used for each individual shRNA. Validation of RNAi gene silencing was measured by quantitative PCR.

Western blot analysis

Cells (6-well plates) were lysed with 300 μl SDS-lysis buffer supplemented with inhibitors cocktail, and the protein concentration was measured by BCA protein Assay. After adjusted to equivalent amounts, 30 μg of total protein from each sample were electrophoresed on an 8% SDS-PAGE gel and transferred onto PVDF membranes (Millipore). Membranes were blocked in 5% non-fat milk in TBST for 1 hr at room temperature, and then incubated with indicated antibodies for 1 hr at room temperature, following by incubated with an HRP-conjugated secondary antibody, eventually detected by ECL regent (Millipore).

Cell proliferation assay

For cell growth curve, cell proliferation was determined by CellTiter One solution Cell Proliferation Assay according to the manufacturer's instructions. Briefly, add 20 μl of One Solution Reagent into each well of the 96-well assay plate containing the samples in 100 μl of culture medium, then incubate the plate for 1-4 hours at 37°C in a humidified, 5% CO_2 atmosphere, record the absorbance at 490 nm using a 96-well plate reader.

Quantitative PCR: A549 cells were infected by distinct shRNA species for 96 hr, and then total RNA was isolated with TRIZOL reagent (Invitrogen). cDNA was generated using RT reagent Kit (TaKaRa). Specific cDNAs were quantitated by quantitative PCR using SYBY Green PCR kit (TaKaRa) on an CXF96 PCR system (BioRad) following the manufacturer's instruction. Gene expression was calculated relative to expression of GAPDH endogenous control and adjusted relative to expression in shControl-infected cells.

Flow cytometry: Cell cycle was measured with PI staining by FACS. Briefly, after infection for 120 hr, A549 cells were collected and washed with PBS. The cell pellet was resuspended by 2% PFA, and fix at room temperature for 30 min. PFA was moved, cell pellet was resuspended by 70% ethanol and Stored at -20°C for 2 h. Then spin cells out of fix, resuspend pellet in PBS with 200 μg/ml RNase A and incubate at 37°C for 1 h. PI was added to a final concentration of 50 μg/ml for 2 h before analysis on the flow cytometer.

Results

Loss of function screening for the identification of HATs involved in lung cancer cell proliferation

To identify components in cancer cell viability, we used a cultured cell assay and RNA interfer-

Table 1. The output of the RNAi screen

Gene symbol	Cell viability (% of shControl)	
	Average	*p*-Value
ATF2-1	71.17 ± 8.76	0.0025
ATF2-2	64.51 ± 4.60	0.0003
CIITA-1	95.71 ± 19.16	0.2900
CIITA-2	86.71 ± 4.80	0.0039
CREBBP-1	64.83 ± 9.90	0.0021
CREBBP-2	43.24 ± 4.72	0.0001
CSRP2BP-1	92.36 ± 8.64	0.0380
CSRP2BP-2	57.55 ± 2.08	0.0001
EP300-1	62.24 ± 13.34	0.0044
EP300-2	45.64 ± 6.48	0.0002
EPC1-1	61.61 ± 10.82	0.0021
EPC1-2	39.33 ± 8.06	0.0002
ESCO1-1	51.34 ± 6.47	0.0002
ESCO1-2	49.13 ± 5.63	0.0001
ESCO2-1	58.36 ± 9.85	0.0012
ESCO2-2	73.71 ± 1.93	0.0002
HAT1-1	48.47 ± 8.24	0.0004
HAT1-2	92.50 ± 20.45	0.2311
KAT2A-1	75.69 ± 4.99	0.0010
KAT2A-2	50.53 ± 8.22	0.0004
KAT2B-1	69.39 ± 11.20	0.0045
KAT2B-2	67.48 ± 2.78	0.0002
KAT5-1	52.52 ± 7.08	0.0003
KAT5-2	72.71 ± 9.71	0.0040
KAT8-1	99.14 ± 17.90	0.3837
KAT8-2	54.35 ± 3.72	0.0001
KAT7-1	99.00 ± 17.18	0.3614
KAT7-2	90.31 ± 17.66	0.1414
KAT6A-1	68.57 ± 13.17	0.0070
KAT6A-2	72.21 ± 6.71	0.0013
KAT6B-1	80.45 ± 13.30	0.0230
KAT6B-2	78.60 ± 7.10	0.0031

ence (RNAi)-mediated disruption of gene function to systematically screen human HATs. A549 Cells were transfected with 32 different shRNAs targeting all human 16 histone acetylases in 96-well plates, with each gene contained two pools of four distinct shRNA species. Five days later, cell viability in each plate was measured to assess the effect of each shRNA pool on cell growth. The screening was repeated once with internal triplication. The output of the screen is described in **Table 1**. Pools were classified as hits if they decrease the percentage of cell viability by ≥ 45% compare to the negative control (**Figure 1**) and 8 positive genes were obtained (**Table 2**). Of the hits, infection of both of ESCO1 shRNA pools inhibited A549 viability with a similar efficiency (**Figure 1**). Notably, EP300 and KAT5 have been previously reported to be involved in cancer cell survival [12-14], indicating that our screening has little bias.

KAT8 inhibition decreases A549 cell viability

Among the hits, little is known about the function of KAT8 in tumorigenesis. We therefore next validated the effect of KAT8 inhibition on cell survival and explored the mechanism by which KAT8 regulates A549 cell survival. Considering the off-target effects of shRNAs, the inhibition of A549 viability induced by the knockdown experiments might be due to off-target effect. To exclude the possibility, we infected A549 cells with four different shRNA lentiviral vectors that comprise the positive shRNA pool. Of the four shRNA species, we observed more than 40% of growth inhibition in three shRNA-infected wells (**Figure 2A**), indicating that the observation of reduction in viability after KAT8 knockdown is unlikely to be the result of an off-target effect. Given that shKAT8-f has most inhibitory effects on A549 viability, we choose this shRNA vector for further functional experiments. Both qPCR and western blot analysis demonstrated a reduction of KAT8 expression in mRNA and protein expression levels after KAT8 silencing (**Figure 2B** and **2C**).

Reduction in KAT8 expression induces cell cycle arrest

To explore the mechanism by which KAT8 regulates cell proliferation, cell cycle distribution was evaluated using flow cytometric analysis. The results showed that knockdown of KAT8 in A549 cells caused a significant inhibition of cell cycle progression without induction of apoptosis (**Figure 3A**). Compare to the negative control, G_2/M phase after KAT8 inhibition increased from 14.96% to 28.41%. We also examined the effect of KAT8 knockdown on cell cycle-regulatory molecules. It is known that both AKT and ERK signaling regulates cyclin D1 expression at both transcription and protein levels, in turn

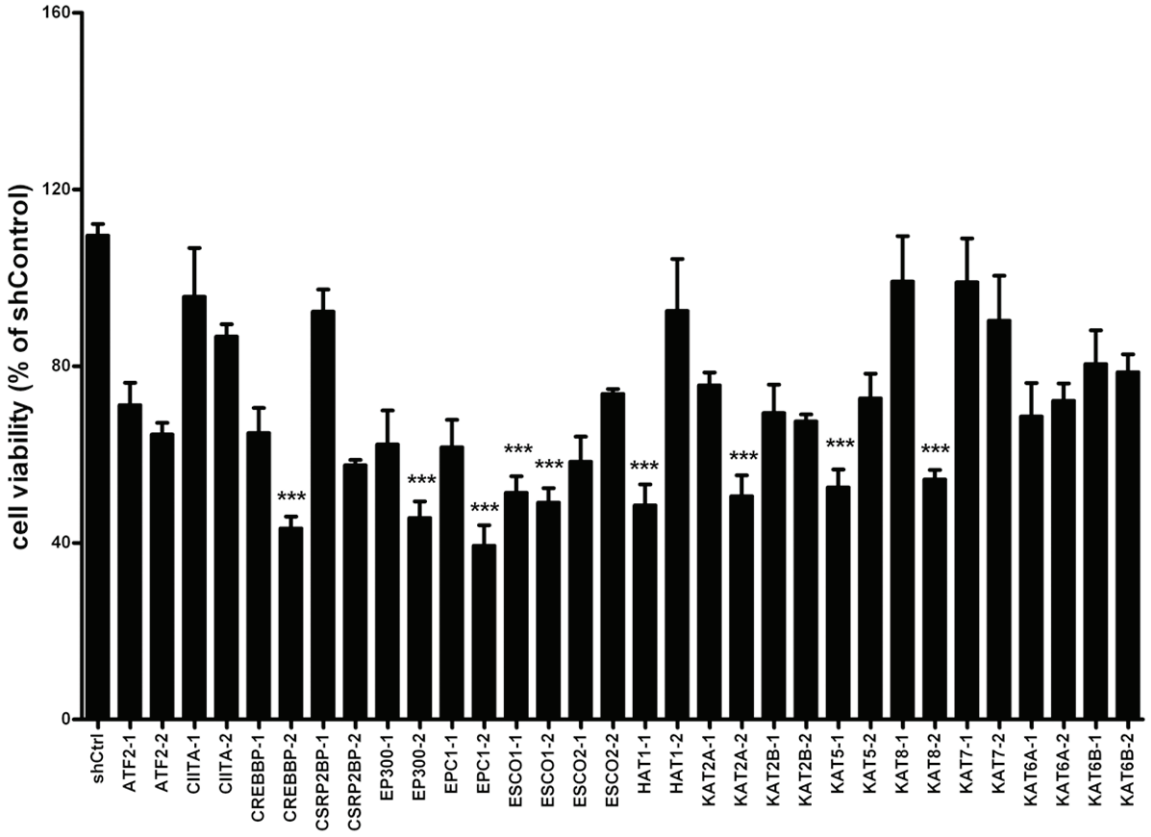

Figure 1. RNAi screening with a lentiviral shRNA library. A549 cells plated in 96-well plates were infected with lentiviral shRNA library targeting 16 histone acetylase genes, each gene in this library contained two pools (1 and 2) of four distinct shRNA species targeting different sequences of the target transcript. The screening was repeated in three independent plates at the same time. shControl was the negative control which was infected with the empty vector lentivirus. Cell viability was assessed after 5 days of infection using CellTiter-Glo Luminescent Cell Viability Assay (Promega). Error bars represent the standard error of the mean (SEM).

Table 2. 8 positive genes in the RNAi screening

shRNA pool	cell viability (% of control)	*p*-Value	Gene symbol	Entrez gene name	associated with cancer
CREBBP-2	43.24 ± 4.72	< 0.0001	CREBBP	CREB binding protein	[26]
EP300-2	45.64 ± 6.48	0.0002	EP300	E1A binding protein p300	[26]
EPC1-2	39.33 ± 8.06	0.0002	EPC1	enhancer of polycomb homolog 1 (Drosophila)	[27]
ESCO1-1	51.34 ± 6.47	0.0002	ESCO1	establishment of cohesion 1 homolog 1 (S. cerevisiae)	[28]
ESCO1-2	49.13 ± 5.63	0.0001	ESCO1	establishment of cohesion 1 homolog 1 (S. cerevisiae)	[28]
HAT1-1	48.47 ± 8.24	0.0004	HAT1	histone acetyltransferase 1	
KAT2A-2	50.53 ± 8.22	0.0004	KAT2A	K (lysine) acetyltransferase 2A	[26]
KAT5-1	52.52 ± 7.08	0.0003	KAT5	K (lysine) acetyltransferase 5	[26]
KAT8-2	54.35 ± 3.72	< 0.0001	KAT8	K (lysine) acetyltransferase 8	[18]

promoting G2/M phase progression [15, 16]. Consistent with this, we found that KAT8 silencing decreased phosphorylation of AKT at Ser473, as well as ERK signaling activity (**Figure 3C**). Importantly, KAT8 inhibition also induced a reduction in protein expression of cyclin D1

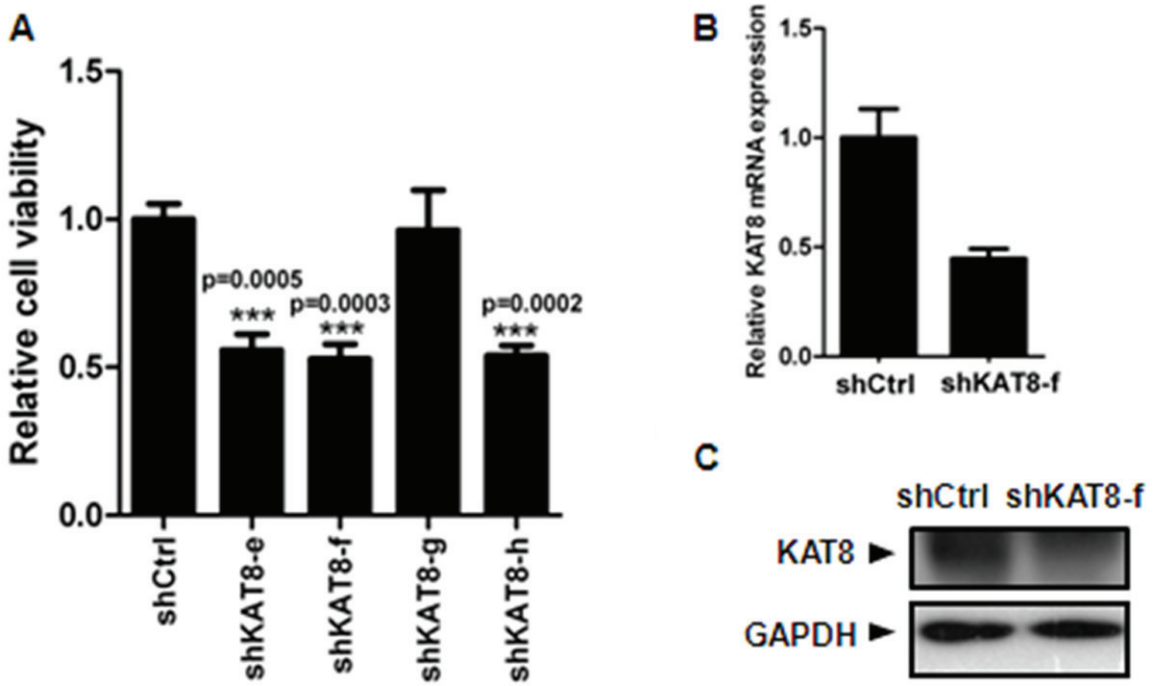

Figure 2. Validation of KAT8 effect on cell viability. A: KAT8-2 shRNA pool which was a positive hit from screening was revalidated with four distinct shRNA species (numbered as e, f, g and h). A549 cells were infected with four individual shRNA for 5 days respectively, and then cell viability was assessed. ***p < 0.001 compared to shControl (Student's t test). Error bars represent the standard error of the mean (SEM). shKAT8-f caused the most potent cell viability inhibition. B: KAT8 mRNA levels quantified by qPCR following infection of shKAT8-f into A549 cells. C: KAT8 protein levels assessed by western blot analysis of lysates prepared from A549 cells infected with shKAT8-f.

(**Figure 3B**). These results suggested that KAT8 inhibition induces G2/M arrest by down regulating cyclin D1 expression via AKT and ERK signaling.

KAT8 inhibition regulates the expression of apoptotic regulators

As shown in **Figure 3**, KAT8 inhibition induced down-regulation of both AKT and ERK signaling, indicating that reduced A549 viability after KAT inhibition is due to impaired AKT and ERK signaling. In addition, it had been reported that KAT8 could acetylated p53 at K120. Depletion of KAT8 blocks p53 K120 acetylation and therefore induces an upregulation of p53 activity [17]. We next analyzed the changes in the levels of p53 proteins upon KAT8 inhibition. Western blot analysis showed that KAT8 silencing in A549 cells upregulated p53 expression (**Figure 4**). P53 has been shown to regulate the expression of many BH3-only family members. We found that knockdown of KAT8 expression impaired bcl-2 protein expression, suggesting that KAT8 inhibition reduces cell viability via p53-mediated pathway.

Discussion

Systematic investigation of HATs function by RNAi screening

RNAi screening is a powerful tool for targeted knockdown of specific genes. Such perturbation of gene expression allows for the systematic query of gene function. Furthermore, RNAi screens can be used to identify novel cancer therapeutic targets as well as the regulators of cancer-related phenotypes [11]. In this study, to our knowledge, it is the first time to use a direct RNAi screening-based approach to systematically study the effect of all human HATs on lung cancer cells growth. Using this approach, we identified many HATs which could regulate lung cancer cell viability. In our screening, 7 of the 8 hit genes have been reported to be abnormally expressed in cancers (**Table 2**). For some genes, primary genomic changes such as amplifications, deletions, point mutations or translocations of them have been detected. Additionally, an altered expression profile has also been reported in some cases. Of the hits, EP300 has been previously report-

A

	G_0/G_1 (%)	S (%)	G_2/M (%)
shCtrl	73.41	10.90	14.96
shKAT8	51.34	19.50	28.41

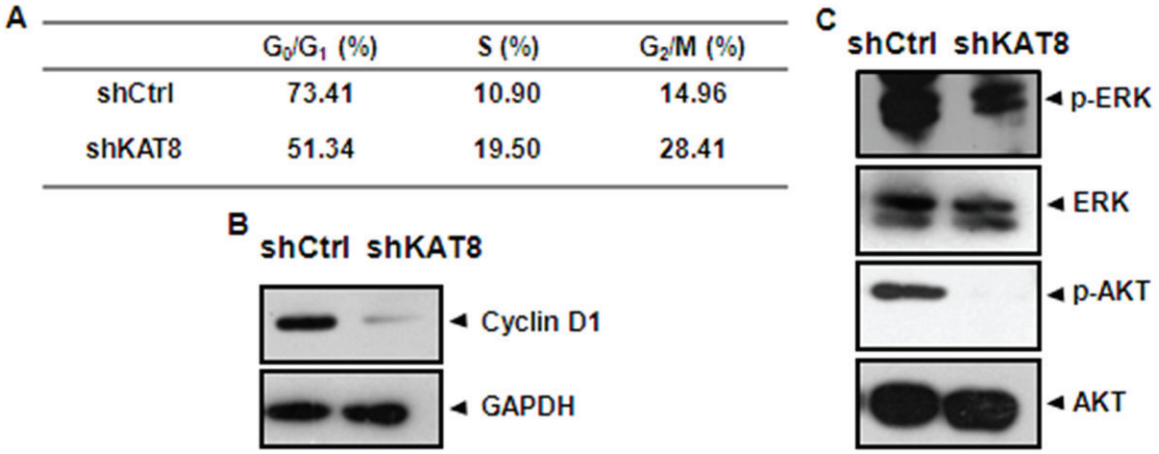

Figure 3. KAT8 silencing induces cell cycle arrest. A: A549 Cells were infected with either shKAT8-f or shControl for 5 days. Cell cycle profiles were assessed by propidium iodide (PI) staining using flow cytometric analysis. The percentage of cells distribution in different cell cycle phase was in the table. B: Cyclin D1 was determined by western blot assay after the A549 cells infected with shKAT8-f for 5 days. C: Silencing of KAT8 decreased ERK1/2 phosphorylation and AKT phosphorylation at Ser473. Western blot analysis of lysates prepared from A549 cells infected with KAT8 shRNA for 5 days. An antibody recognizing phospho and total ERK1/2, phospho and total AKT was used with GAPDH as a loading control.

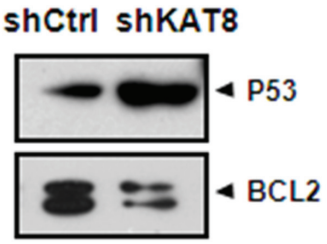

Figure 4. KAT8 silencing induces p53 proapoptotic signaling. Silencing of KAT8 induced p53 expression and decreased BCL2 expression. Western blot analysis of lysates prepared from A549 cells infected with KAT8 shRNA for 5 days. An antibody recognizing p53 and BCL2 were used with internal control as shown in the Figure 3B.

ed to be involved in transcriptional regulation of s-phase specific histone genes regulating cell growth [13]. KAT5, also known as Tip60, could either act as a tumor suppressor or promote oncogenesis, depending on the context. Therefore, RNAi screening is an unbiased tool to be used for identifying the therapeutic value of HATs in cancer therapy.

KAT8 inhibition reduces A549 viability via AKT-mediated regulation of cyclin D1 expression

KAT8, known as hMOF/MYST1/KAT8 in humans, has been shown to be essential for embryogenesis and genome stability in mammals. Notably, it has recently been correlated with lung cancer, primary breast carcinoma and medulloblastoma [18]. In this study, we first found that KAT8 silencing decreased lung cancer cell viability and induced cell cycle arrest. Knockdown of KAT8 inhibited the expression of cyclin D1, the core G2/M cell cycle-regulatory molecule. Cyclin D1 is induced to high levels in a Ras-dependent way during S phase to G2 phase. Akt prevents cyclin D1 degradation by regulating GSK3 activity, while ERK signaling has been reported to regulate cyclin D1 production at both mRNA and protein levels. Consistent with this, we found that inhibition of KAT8 by shRNA impairs both ERK and Akt signaling, suggesting that KAT8 inhibition might regulate both degradation and transcription of cyclin D1 expression.

KAT8-mediated p53 proapoptotic signaling

P53 plays an important role in tumorigenesis and cancer therapy [19]. In most cases, p53 is induced due to the phosphorylation at multiple sites by protein kinase upon stress [20-22]. In this study, we found KAT8 reduction significantly induced p53 expression. Previous reports demonstrate that p53 can be acetylated at K120 by hMOF and TIP60 [23, 24] in response to genotoxic stress, leading an inhibition of p53 protein degradation and subsequent p53 induction. Given that KAT8 is a histone acetyltransferase, p53 induction is unlikely due to a direct inhibition of acetylation by KAT8 inhibition. It is therefore interesting to investigate whether p53 induction after KAT8 inhibition is due to acetylation modification and to know how KAT8 regulates p53 acetylation.

P53 was reported to regulate apoptosis via BH3-only family members. In most cases, p53 is phosphorylated and subsequently induces bax induction [25]. However, we did not find an upregulation of bax after KAT8 inhibition (data not shown). Interestingly, we found a reduction of bcl-2, which is consistent with previous report that p53 induces decreases in the expression of bcl-2 in leukemia cells. It is worthy to study whether this selective regulation of bcl-2 by p53 is due to the different regulation of p53 activity by KAT8 from phosphorylation-mediated modulation of p53 activity.

In summary, we used a loss-of-function RNAi screening approach to systematically explore the cellular function of HATs on cell viability in lung cancer cells. We identified KAT8 as a modifier of cell growth and established its primary mechanism, including regulation of p53-bcl-2 axis and AKT/ERK-cyclin D1 signaling. Our work suggests that KAT8 could be a new therapy target for lung cancer therapies.

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