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Lysine acetyltransferase 5 in EGFR mutated non-small cell lung cancer

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LYSINE ACETYLTRANSFERASE 5 IN EGFR MUTATED NON-SMALL CELL LUNG CANCER

by

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DEDICATION

To my cousin Sylvia (1989 – 2014)

ACKNOWLEDGMENTS

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ABSTRACT

Histone modifications are crucial in activities such as transcriptional activation, gene silencing, and epigenetic cellular memory. In particular, lysine acetylation via lysine (K) acetyltransferases (KATs) has been implicated in cancer development. Interestingly, KAT5, also known as Tip60 (tat-interactive protein-60kDa), has been reported to possess both tumor promoting and tumor suppressing properties depending on the context of malignancy. Herein we report that KAT5 contributes to tumorigenesis in epidermal growth factor receptor (EGFR) mutated lung cancer, and *Kat5*-knockout mice models demonstrate significantly reduced lung tumor burden. To probe the aberrant modification of KAT5, we demonstrated that KAT5 binds to and is phosphorylated by oncogenic EGFR in co-immunoprecipitation experiments. Next, to investigate whether KAT5 is involved in cell proliferation and survival, H1975 cells harboring L858R-T790M double-activating mutations were transfected with doxycycline inducible short helical RNA (shRNA) targeting KAT5 (shKAT5). Following treatment, shKAT5 cells were observed to have suppressed proliferation rates. Pharmacological inhibition using TH1834, a known KAT5 inhibitor, also suppressed proliferation rates in shKAT5 cells; in contrast BEAS2B cells, an immortalized normal human bronchial cell line, surprisingly exhibited increased viability compared to transformed human lung H1975 cells. This finding supports KAT5's context-dependent role in in normal and abnormal cell homeostasis. To

further investigate KAT5 in lung tumorigenesis *in vivo*, we generated EGFR-mutant conditional *Kat5* knockout mice using a tetracycline-induced Cre/loxP system. Following doxycycline treatment for 10 weeks, isolated mice lungs for $EGFR^{TL}$/*CCSP-rtTA/Cre/*$Kat5^{F/F}$ possessed significantly lower tumor volume compared to $EGFR^{TL}$/*CCSP-rtTA/Cre/*$Kat5^{wt/F}$ and $EGFR^{TL}$/*CCSP-rtTA/Cre/*$Kat5^{wt/wt}$ mice lungs. Hemotoxylin and eosin staining showed no evident hyperproliferation in lungs isolated from $EGFR^{TL}$/*CCSP-rtTA/Cre/*$Kat5^{F/F}$ mice whereas lungs isolated from $EGFR^{TL}$/*CCSP-rtTA/Cre/*$Kat5^{wt/wt}$ and $EGFR^{TL}$/*CCSP-rtTA/Cre/*$Kat5^{wt/F}$ did, signifying that KAT5 has a potential regulatory role in cellular proliferation. RNA-Seq analysis of shKAT5 H1975 cells identified downstream targets involved in tumorigenic pathways. Subsequent quantitative polymerase chain reaction (PCR) of shKAT5 cells served to validate the reported targets. Taken together, these data offer insight into a KAT5 mediated oncogenic pathway that can provide novel therapeutic approaches in treating lung cancer.

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LIST OF ABBREVIATIONS

BIDMC ..... Beth-Israel Deaconess Medical Center

BU ..... Boston University

CCSP ..... Clara Cell Secretory Protein

cDNA ..... Complementary DNA

Cre ..... Cre Recombinase Protein

DMEM ..... Dulbecco's Modified Eagle Media

EGF ..... Epidermal Growth Factor

EGFR ..... Epidermal Growth Factor Receptor

GO ..... Gene Ontology

HAT ..... Histone Acetyltransferase

IgG ..... Immunoglobulin G

KAT ..... Lysine Acetyltransferase

KAT5 ..... Lysine Acetyltransferase 5

MTS ..... 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NSCLC ..... Non-Small Cell Lung Cancer

NuA4 ..... NuA4 Histone Acetyltransferase Complex

ORR ..... Objective Response Rate

p53 ..... Tumor Protein 53

PCR ..... Polymerase Chain Reaction

PFS ..... Progression Free Survival

PPEE ..... Posterior Probability of Equal Expression

RNA ..... Ribonucleic Acid

RPMI ..... Roswell Park Memorial Institute

SCLC ..... Small Cell Lung Cancer

SDS ..... Sodium Dodecyl Sulfate

rtTA ..... Reverse Tetracycline-Controlled Transactivator

TKI ..... Tyrosine Kinase Inhibitor

INTRODUCTION

This thesis serves to identify and assess the oncogenic role of lysine acetyltransferase 5 (KAT5), also known as tat-interactive protein-60KDA (Tip60), in lung tumorigenesis. The two predominant types of lung cancer are non-small cell lung cancer (NSCLC), which represents 85-90% of all reported cases, and small cell lung cancer (SCLC), which characterizes the remaining cases. The following introduction examines the social and clinical relevance of lung cancer in the context of epidermal growth factor receptor (EGFR) and KAT5 mutations.

Lung Cancer in Society and Clinics

Lung cancer is the most common cause of cancer related deaths worldwide. Even with the latest drugs in development, lung cancer still possesses a dismal 18% 5-year survival rate as a terminal illness at all stages of disease[1]. Currently, optimal treatment for tumors confined to the lung is surgical resection at early stages; however, patients with locally advanced or metastatic disease are not ideal candidates for surgery and necessitate other treatment. The current standard protocol for non-operable metastatic lung cancer is a combination of platinum-based chemotherapy and thoracic radiation, which only provides limited patient survivability[2]. Recent advances in genomic testing and development of targeted therapies have moved the odds for curing thoracic malignancies forward, but much work remains to be done[3].

Targetable Mutations in Cancer

The identification of targetable mutations in non-small cell lung cancer has provided individualized therapies to inhibit oncogenic signaling pathways. EGFR

mutations are one of most prolific in NSCLC, accounting for more than 60% of cases[4]. EGFR functions as a trans-membrane protein critical to intracellular signaling pathways. The receptor binds to activating ligands which leads to homo- or heterodimerization with HER2 and subsequent *trans*-phosphorylation of tyrosine residues that contribute in downstream signaling pathways for cellular proliferation, invasion, and angiogenesis[5]. The development of tyrosine kinase inhibitors (TKIs) has led to higher objective response rates (ORRs) and progression-free survival (PFS) compared to traditional cytotoxic therapies in patients with identifiable EGFR mutations[6]. However, acquired resistances from further genomic alterations such as the T790M point mutation in EGFR have reduced or halted therapeutic efficacy, leading to the need to characterize the underlying mechanisms for lung cancer development and progression in order to introduce novel therapeutic targets[7].

Lysine Acetyltransferases in Cancer

Aberrant epigenetic modifications in oncogenesis have long been an area of interest in cancer research. Lysine acetylation, a form of histone modification, has been associated with activating transcriptional processes in eukaryotic organisms. Acetylation on histones is catalyzed by proteins called histone acetyltransferases (HATs), which include a sub-type of enzymes known as lysine acetyltransferases (KATs)[8]. The three major acetyltransferase families are divided based on their protein sequence similarities: (1) GNAT (GCN5-related *N*-acetyltransferase), (2) MYST (Moz, Ybf2/Sas3, Sas2, and Tip60), and (3) CBP/p300 (CREB-binding protein/E1A binding protein p300)[9].

Acetylation unfolds heterochromatin to euchromatin by neutralizing the basic charge of lysine, rendering the nucleosome transcriptionally accessible and active.

KATs are subdivided based on the sequence of their catalytic domains. KAT5 is a founding member of the MYST family whose members function in chromatin remodeling, gene regulation, dosage compensation, DNA damage repair, and tumorigenesis[10]. KAT5 is a subunit in the NuA4 complex, which is recruited to target promoters by many transcription factors. However, KAT5 function in cancer development is complicated. Within the NuA4 complex, KAT5 acetylates nucleosomal histones H2A and H4 in addition to acting as a co-activator for transcriptional factors such as c-Myc and E2F[11]. In this context, KAT5 acts as a tumor initiator/promoter. However, KAT5 is also noted in p53 activation, commonly attributed to the induction of apoptosis[12]. Furthermore, KAT5 is required for the expression of KAI1, a tumor suppressor in the context of prostate cancer[13]. These context dependent roles of KAT5 suggest complex regulatory properties that can either promote or suppress tumorigenesis in cancer. This current study identifies KAT5 as a required component for malignant transformation in pulmonary epithelial cells and develops a preliminary understanding of the underlying mechanism in lung tumorigenesis.

METHODS

Mice

The experimental studies were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. *Kat5* conditional knockout mice for lung tumor analysis (*Kat5*$^{F/F}$) were generated by crossing lung-specific *EGFR-L858R-T790M* (*EGFR*^{TL}) transgenic mice with conditional *Kat5* tetracycline-inducible Cre-expressing mice (*TetOp-Cre/Kat5*$^{F/F}$) that targets pulmonary cells via clara cell secreting protein (CCSP) using a reverse tetracycline-controlled transactivator (rtTA) system. To induce *Kat5 EGFR*^{TL} expression and excise *Kat5*, mice were fed a doxycycline diet for 8-12 weeks (Envigo).

Cell Culture

A431, A549, BEAS2B, NCI-H1395, NCI-H1975, NCI-H3132, NCI-H358, HCC827, HCC98, and PC9 cells were grown at 37 ºC in a humidified incubator at 5% CO_2 in RPMI (Corning) media supplemented with 10% fetal bovine serum (Corning), 100 µg/ml penicillin and 100 units/ml streptomycin (Corning).

Generation of shKAT5 Cells

TRIPZ inducible Lentiviral KAT5 shRNA plasmids (Dharmacon) were used to generate retroviruses by transfection into HEK293T cells using the TransIT-X2 Dynamic Delivery System (Mirus). Retroviruses were then transduced into NCI-H1975 cells followed by puromycin selection (2.0 µg/ml) in Roswell Park Memorial Institute (RPMI) complete medium. Puromycin selection produced H1975 sh328, which harbors shRNA targeting

luciferase as a control. H1975 shKAT5 cell lines were generated using plasmids targeting different flanking regions of KAT5. To obtain KAT5 knockdown models, H1975 cells were cultured in the presence of doxycycline (1.0-2.0 μg/ml). KAT5 levels which were verified by quantitative PCR. Plasmid constructs can be found in Table 2.

Cellular Proliferation Assay

Cells (1×10^3) were seeded in 24-well plates in RPMI complete medium. The following day, cells were treated with doxycycline (2.0μg/ml) to induce shRNA over the course of 120 hours or were treated with TH1834. Cells were counted at 24, 72, and 120 hour marks. Media was changed every 48 hours. The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was used to assess cellular proliferation via a colorimetric method. The data was graphically displayed using GraphPad Prism (GraphPad Software).

RNA-Sequencing

RNA was isolated in triplicate from cells treated with doxycycline (0.5 μg/ml) after 48 hours. Strand-specific RNA libraries were generated, and sequencing was performed using single-end reads (50 base pairs, average 20 million reads per sample) on the BGISEQ-500 platform (BGI). Expressed genes for shKAT5 were compared to controls with a posterior probability of equal expression (PPEE) of a fold change of >1.5.

Quantitative PCR

Total RNA was isolated from NCI-H1975 shKAT5 cells with the use of an RNA isolation kit (Qiagen, RNeasy Mini Kit). Up to 3 μg of RNA was reverse transcribed to

cDNA following the protocol of Invitrogen Superscript™ II Reverse Transcriptase protocol (ThermoFisher Scientific, Cat#18064014). *ACTB* and *GAPDH* expression levels were used to normalize for differences in RNA input. For all genes of interest, iTaq™ Universal SYBR® Green Supermix (BIO-RAD) was used for gene expression analysis. mRNA expression levels were quantified in technical triplicates with 150 ng cDNA in a 15 µl reaction volume. Primers are shown in Table 1. Statistical significance was calculated with Student's *t*-test.

Table 1. Primers Used in Quantitative PCR for shKAT5 Knockdown Analysis

Gene	Primers	5' – 3'
ACTB	F	AGTGTGACGTGGACATCCGCAA
	R	ATCCACATCTGCTGGAAGGTGGAC
KAT5	F	AGGGAGTCAACGATCGCACGGGAGG
	R	CACAGACAGGGAGTCTTAGCCAGGG
MUC1	F	GTGCCCCCTAGCAGTACCG
	R	GACGTGCCCCTACAAGTTGG
TGFB1	F	AAGGACCTCGGCTGGAAGTG
	R	CCCGGGTTATGCTGGTTGTA
TGM2	F	CTGGGCCACTTCATTTTGC
	R	ACTCCTGCCGCTCCTCTTC

Western Blotting and Immunoprecipitation

Protein lysates were isolated from NCI-H1975 shKAT5 cells after treatment with doxycycline (2 µg/ml). Lysate total protein concentration was assessed by Bradford protein assay using spectrophotometry. For western blotting, lysates were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels of varying percentages and transferred onto polyvinylidene difluoride membranes (Millipore) to be probed by antibodies for the protein of interest.

For immunoprecipitation studies, 1 mg of total cell lysate was mixed with antibodies for the protein of interest or with normal mouse IgG antibodies and incubated with protein G Sepharose beads (GE Health Care Life Sciences). The beads were precipitated by centrifugation, washed three times with phosphate buffered saline (PBS), and suspended in buffer solution.

The antibodies used included those against: ß-actin (Santa Cruz, Cat#sc-47778); KAT5 (Santa Cruz, Cat#sc-166323); EGFR (Cell Signaling Technology, Cat#2232); FLAG (Sigma-Aldrich, Cat#F3165); phosphor-EGFR (Fisher Scientific, Cat#44-788-G); goat anti-rabbit IgG, HRP conjugate (BIO-RAD, Cat#1706515); goat anti-mouse IgG, HRP conjugate (BIO-RAD, Cat#1706516).

RESULTS

KAT5 Expression in Lung Cancer

Decreased levels of KAT5 expression have been correlated with reduced tumorigenic activity in various cancers. KAT5 expression was examined in various non-small cell lung cancer cell lines to assess its function in lung tumorigenesis. Western blot analysis showed all cell lines assessed expressed KAT5 (Fig. 1) including BEAS2B cells, which are an immortalized alveolar cell line. BEAS2B cells functioned as a control to assess basal KAT5 expression in non-tumorigenic cells compared to tumorigenic cells.

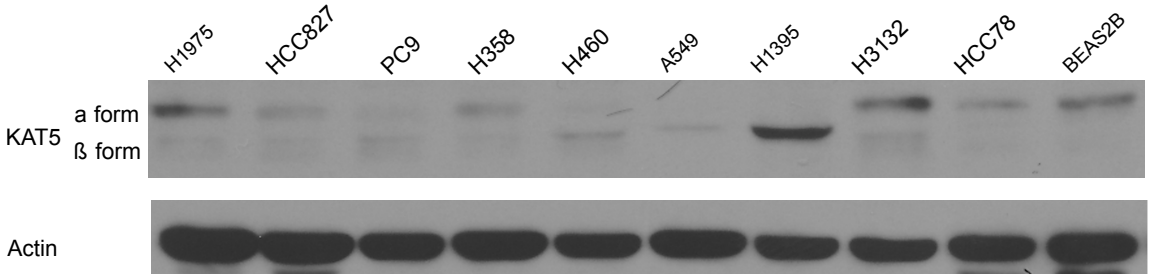

Figure 1. Expression of KAT5 Protein in Human Lung Cancer Cell Lines

KAT5 is expressed in lung cancer cell lines as well as an immortalized alveolar cell line, BEAS2B. α and β forms refer to two different splice variants of KAT5.

Cellular Proliferation and KAT5 Inhibition

NCI-H1975 cells harbor L858R-T790M double activating mutations in the EGFR gene that have been attributed to acquired drug-resistance in EGFR 1st - and 2nd – generation tyrosine kinase inhibitors for EGFR mutated non-small cell lung cancer. To avoid confounding variables from clonal effects, two clones, sh331 and sh342, harboring inducible-shRNA targeting KAT5 were selected and subject to cellular proliferation analysis. Following doxycycline treatment to suppress KAT5 expression, shKAT5 cells

proliferated more slowly compared to cells expressing control luciferase-shRNA (sh328) over a 72-hour treatment course (Fig. 2). H1975 wild-type cells that were treated with TH1834 (Fig. 3), a known KAT5 inhibitor, demonstrated slower cellular growth in an MTS assay as well (Fig. 4). On the other hand, TH1834 treatment increased viability of BEAS2B cells, suggesting that KAT5 may function in suppressing proliferation of transformed cells, but not normal cells.

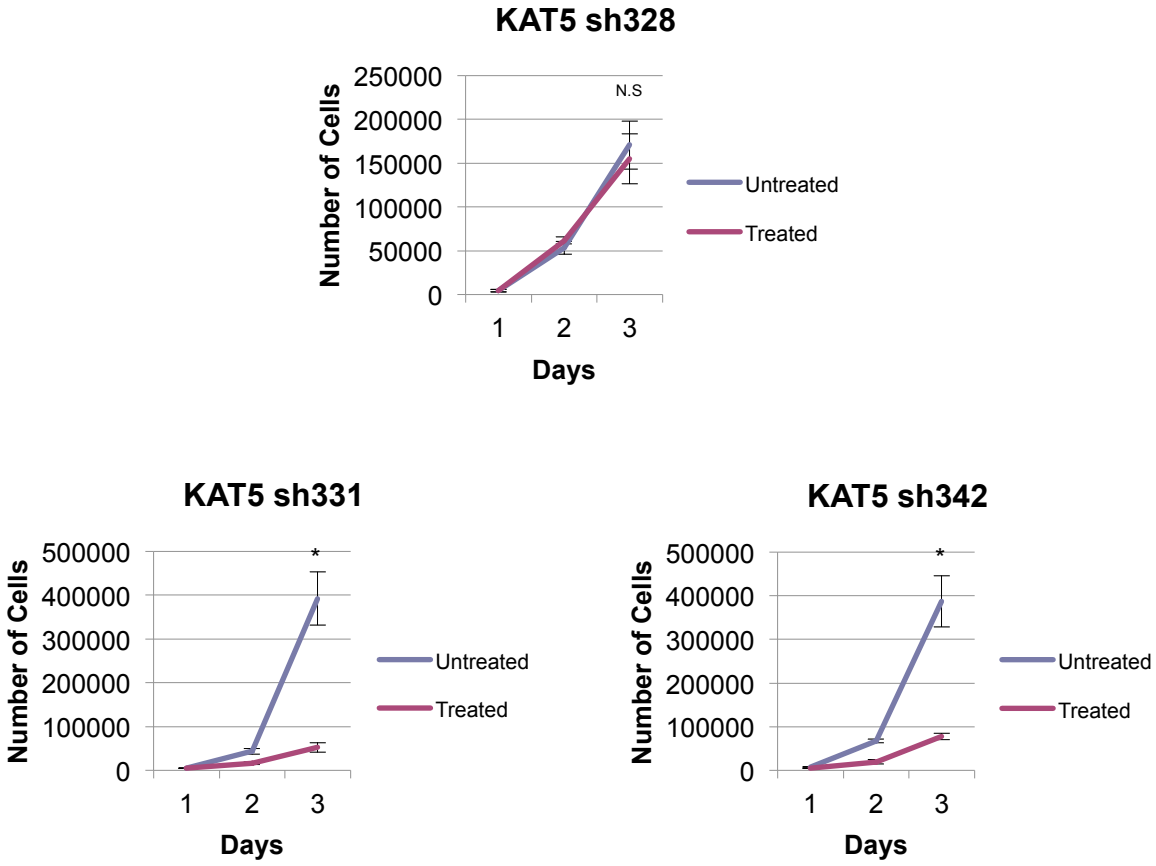

Figure 2. Quantification of shKAT5 Cells Treated with Doxycycline (2.0 µg/ml) Over 72-Hours

KAT5 sh328 functions as a control with shRNA targeting luciferase. KAT5 sh331 and sh342 possess shRNA targeting KAT5 to genetically deplete KAT5 in H1975 cells. Cells were counted at day 1 (24 h), day 2 (48 h), and day 3 (72 h) via 3 random fields per well with a microscope at 10X magnification. * indicates $p < 0.01$.

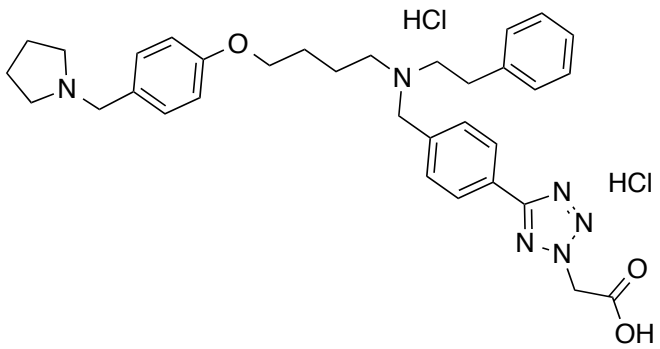

Figure 3. Histone Acetyltransferase Inhibitor TH1834

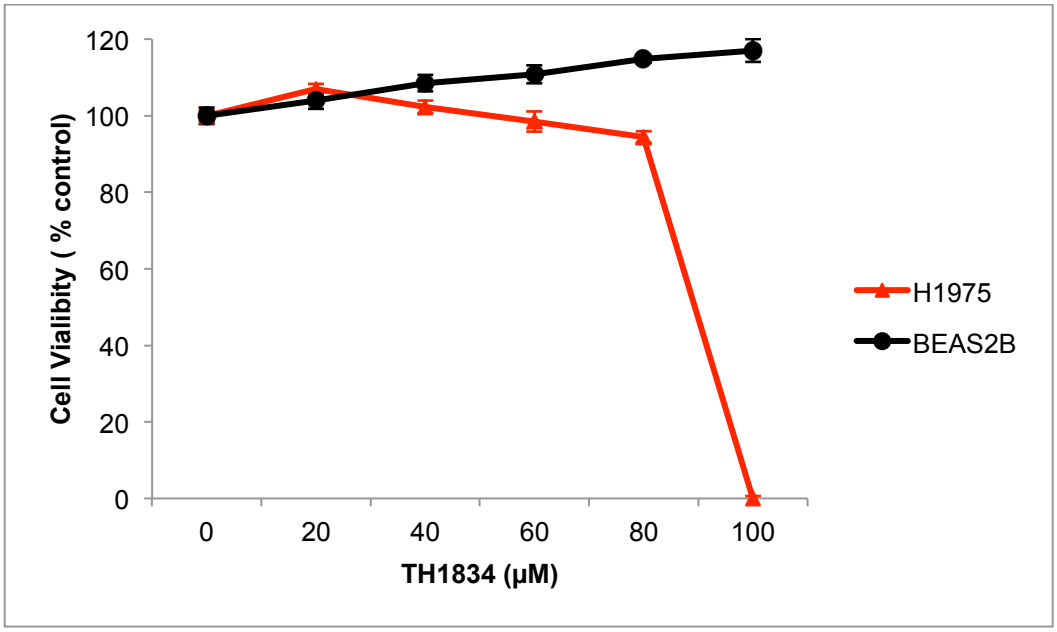

Figure 4. Pharmacological KAT5 Inhibition Decreases Cell Viability

H1975 and BEAS2B cells were treated with TH1834 for 72-hours in 96-well plates (3000 cells/well) and subject to an MTS assay.

In Vivo Lung-Specific Kat5 Knockout Mice

Juvenile mice (3-6 weeks of age) were subject to a doxycycline infused diet for 8-15 weeks. Lungs were isolated, weighed, and histologically analyzed. Genetic deletion of Kat5 was validated by PCR-based genotyping. Lungs isolated from $EGFR^{TL}$/CCSP-rtTA/Cre/$Kat5^{wt/wt}$ and $EGFR^{TL}$/CCS-rtTA/Cre/$Kat5^{wt/F}$ exhibited tumors 10 weeks after doxycycline treatment (Fig. 5). Histological analysis revealed tumor islets in the bronchial and alveolar space for $Kat5^{wt/wt}$ (Fig. 6II) and $Kat5^{wt/F}$ (Fig. 6III) mice lungs. However, lungs collected from $EGFR^{TL}$/CCSP-rtTA/Cre/$Kat5^{F/F}$ (Fig. 6IV) and CCSP-rtTa/Cre/$Kat5^{wt/wt}$ (Fig. 6I) mice exhibited similar features and appeared normal with no signs of hyperproliferation or tumor formation. Examination of gross appearance showed no difference in tumor growth as well (Fig. 7). Weight of mice right lungs supported the histological analysis and affirmed $EGFR^{TL}$/CCSP-rtTA/Cre/$Kat5^{wt/wt}$ and $EGFR^{TL}$/CCS-rtTA/Cre/$Kat5^{wt/F}$ to have heavier lungs compared to $EGFR^{TL}$/CCSP-rtTA/Cre/$Kat5^{F/F}$ and control CCSP-rtTa/Cre/$Kat5^{wt/wt}$ (Fig. 8). These results show complete excision of the *Kat5* gene resulted in loss of *Kat5* protein in oncogenic pulmonary cells reduced tumor burden. Importantly, it exposes a therapeutic opportunity by targeting KAT5 in EGFR mutated non-small cell lung cancer.

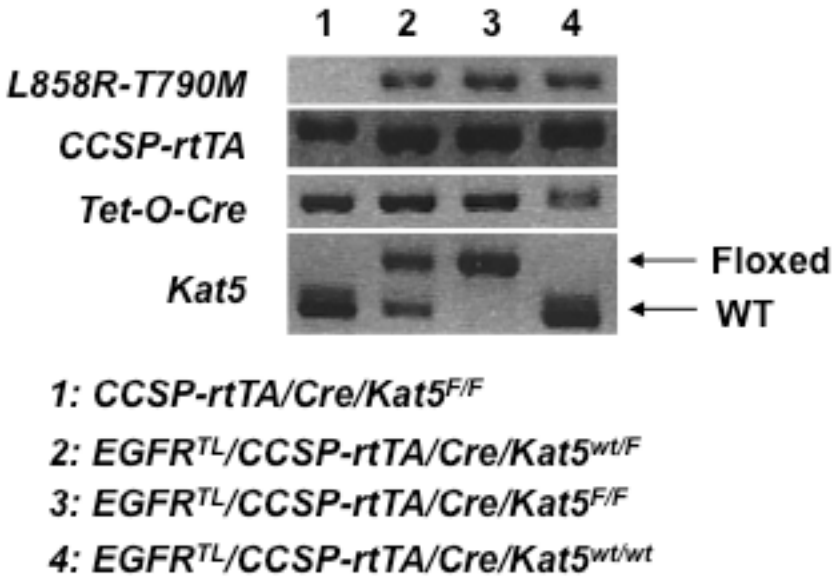

Figure 5. PCR-Based Genotyping Affirming Induced Genetic Variations in Mice

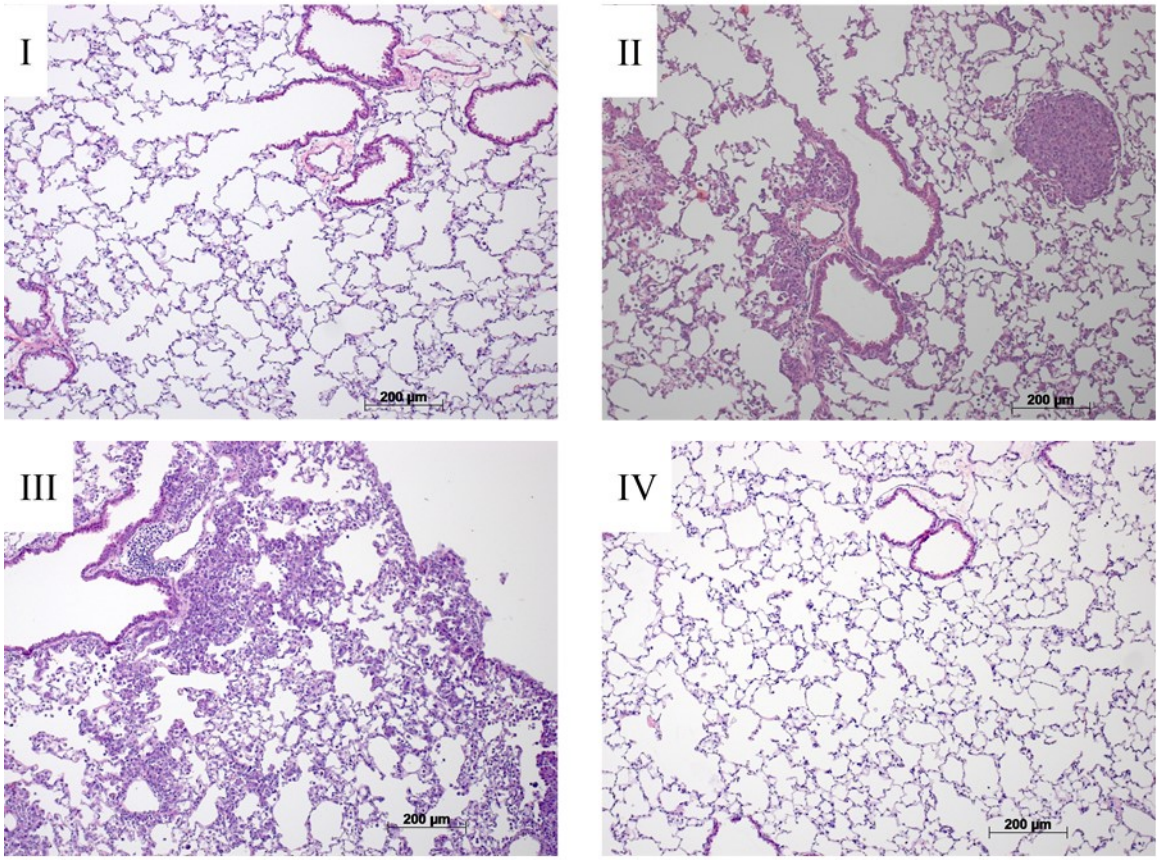

Figure 6. Kat5 Knockout Hinders Tumor Formation In Vivo

Hemotoxylin and eosin staining of lungs from indicated mice (I) CCSP-rtTa/Cre/Kat5$^{wt/wt}$ (II) EGFR^{TL}/CCSP-rtTA/Cre/Kat5$^{wt/wt}$ (III) EGFR^{TL}/CCS-rtTA/Cre/Kat5$^{wt/F}$ (IV) EGFR^{TL}/CCSP-rtTA/Cre/Kat5$^{F/F}$

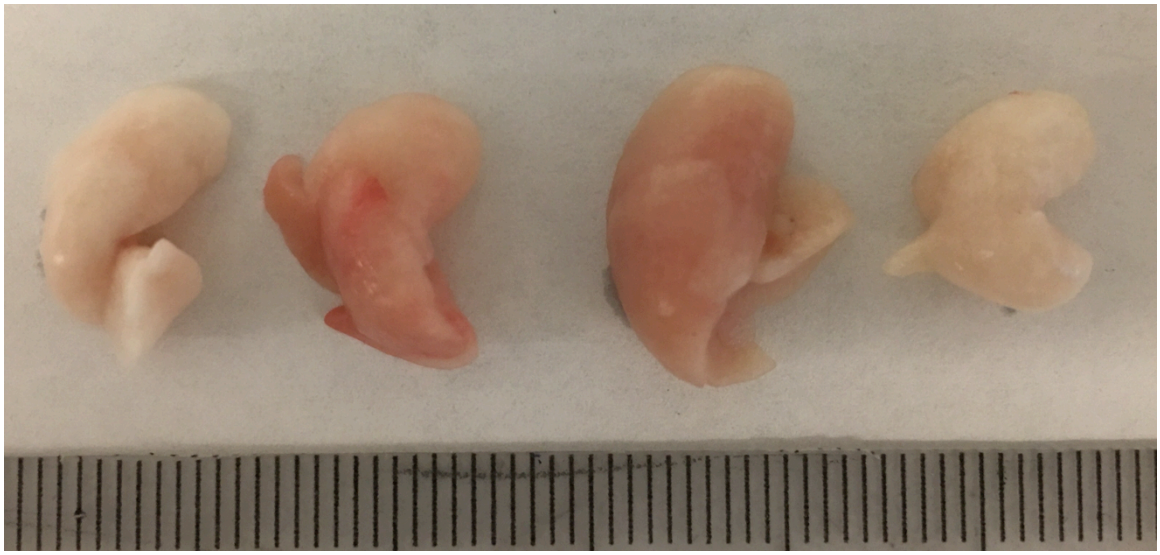

Figure 7. Gross Appearance of Right Lung of Mice

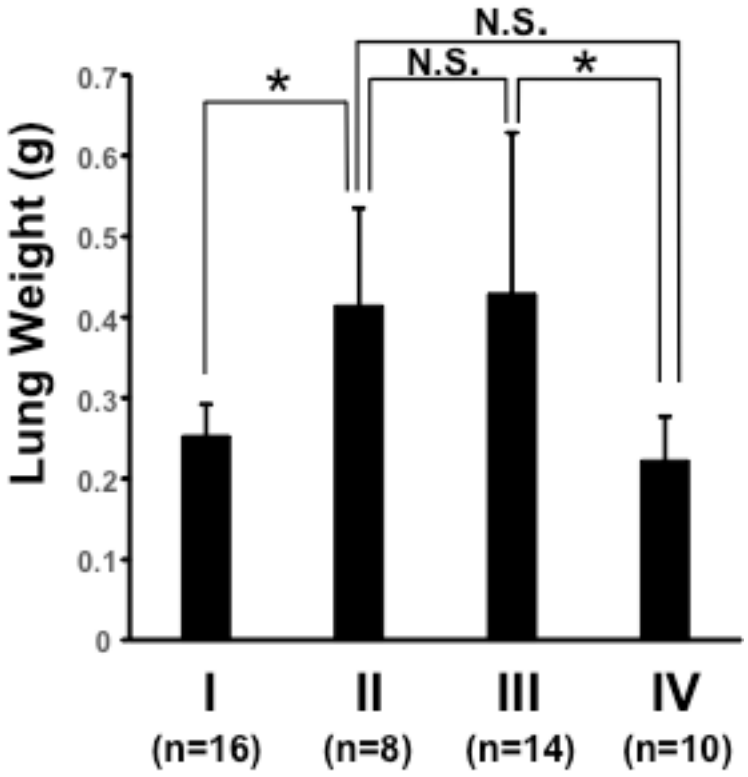

Figure 8. Lung Weight from Doxycycline Treatment of Mice

Weight of right lungs isolated from mice was measured. * indicates $p<0.01$ while N.S. signifies no significance was detected.

RNA-Sequencing

Three distinct H1975 shKAT5 cells (sh328, sh331, and sh342) were subjected to transcriptome analysis and sequencing. H1975 sh328 cells harbored an shRNA targeting luciferase, which functioned as a control. H1975 sh331 and sh342 were selected from a pool of nine candidates (Table 2). The selected clones harbored shRNA targeting KAT5 at different exons and exhibited optimal downregulation of KAT5 via quantitative PCR assessment. Gene ontology analysis provided a functional classification for genes of interest at a macro level (Fig. 9) by illustrating cellular processes correlated with a downregulation of *KAT5*. Raw RNA-sequencing data revealed 576 overlapped differential genes between sh331 and sh342 that possessed changes in gene expression of fold change greater than 1.5 compared to the control (Fig 10). Common genes associated with tumorigenesis and cell-cycle regulation such as *MUC1, TGFB1,* and *TGM2* were identified and subject to mRNA analysis to confirm the fold change observed in RNA-Seq.

Table 2. TRIPZ Lentiviral Vector Sequences

TRIPZ Inducible Lentiviral Vectors	
sh329	CACAGACCCTTTCCTCTTCTAC
sh330	TAACTCTTGTTCTTACGTC
sh331	TTCCATCAGAGCTGTCCTG
sh333	TGGGCTACTTCTCCAAGGAGAGAA
sh342	CGATTTTCGCTTCCGTCCT
sh343	TCATAGCTGAACTCGATCA
sh344	TATGGTCAAGGAAACACTT
sh345	TCACTAATCTCATTGATGG
sh346	CGTAGGTCACACTTGGTCA

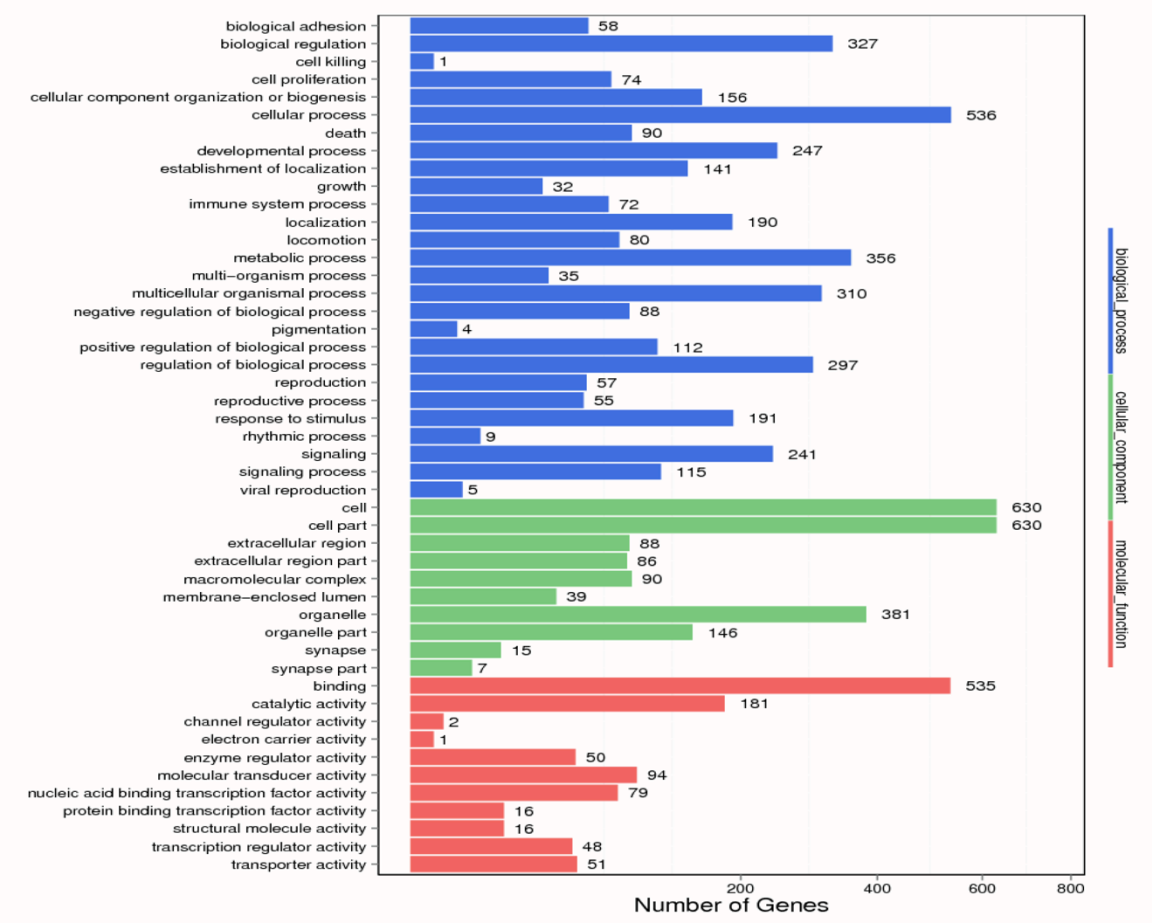

Figure 9. GO Functional Classification for H1975 shKAT5 Cells

Gene ontology analysis of RNA-sequencing results for H1975 shKAT5 (sh331 and sh342) cells show multiple cellular processes were affected upon genetic depletion of KAT5.

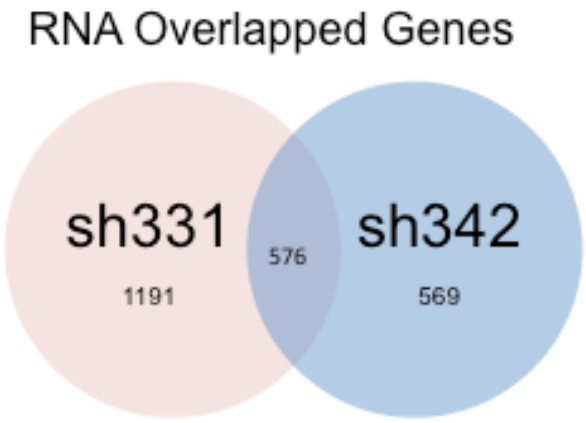

Figure 10. Venn Diagram Illustrating Overlapped Genes with 1.5 Fold Change or Greater

HAT Activity Assay and Immunoprecipitation

HEK239T cells were transfected with KAT5 and EGFR overexpression constructs to probe mutant EGFR activity in relation to KAT5 acetyltransferase activity. Cells co-transfected with both KAT5 and EGFR exhibited higher HAT activity compared to cells solely transfected with KAT5. HAT activity was observed to be the highest in cells transfected with KAT5 and mutant EGFR-L858R-T790M (Fig. 11). Addition of osimertinib, an EGFR tyrosine kinase inhibitor restored HAT activity for HEK293T cells transfected with KAT5 and mutant EGFR compared to wild-type HEK293T EGFR HAT levels.

Immunoprecipitation experiments showed KAT5 was tyrosine phosphorylated in HEK293T cells expressing KAT5 and EGFR constructs. The level of phosphorylation observed was higher in cells transfected with mutant EGFR-L858R-T790M than compared to cells transfected with wild-type EGFR, implying EGFR may be responsible for phosphorylating KAT5. EGF stimulation to cells co-transfected with wild-type EGFR enhanced KAT5 phosphorylation (Fig 12). Subsequently, afatinib, an EGFR tyrosine kinase inhibitor, was used to treat cells possessing EGFR and in cells possessing tyrosine-kinase inactive form of mutant EGFR, EGFR-KD (Fig. 13). Additionally, it is of interest to note that ß-catenin was observed to bind to KAT5 cells transfected with mutant EGFR (Fig. 14).

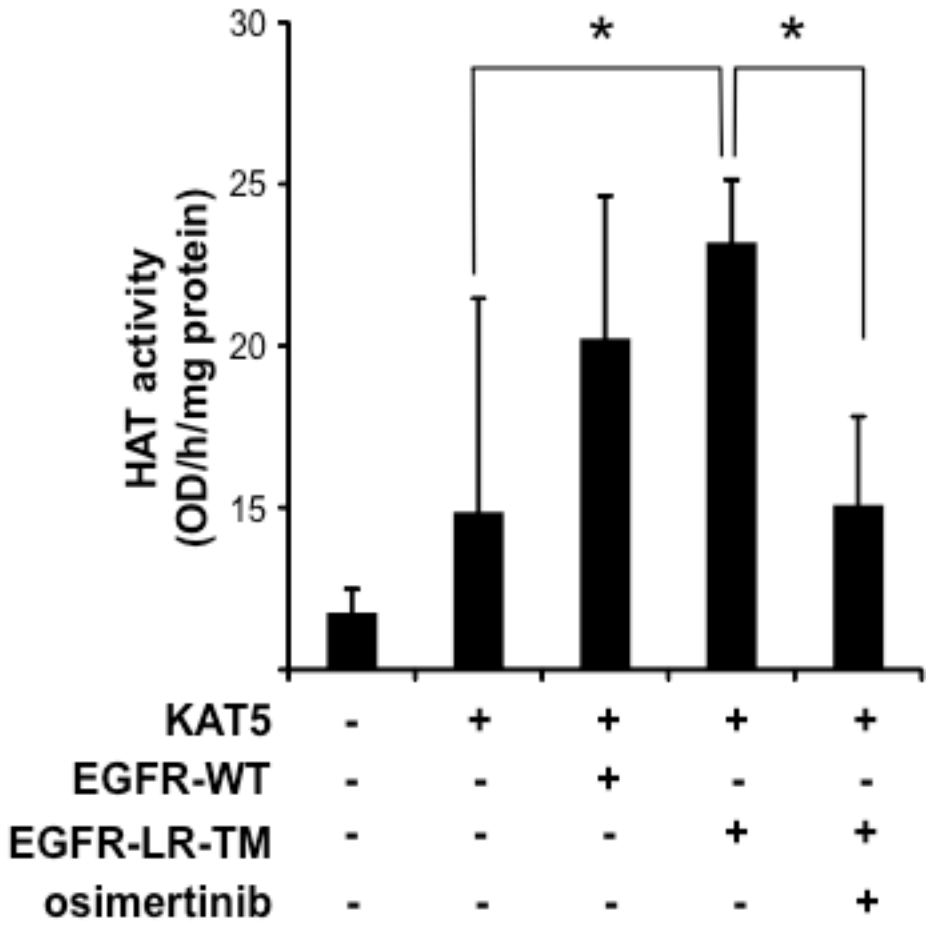

Figure 11. Inhibition of EGFR Tyrosine Kinase Activity with Osimertinib Decreases KAT5 Acetyltransferase Activity

HEK293T cells were transfected with a KAT5 expression construct plus either wild-type EGFR or mutant EGFR-L858R-T790M expression constructs. Nuclear extracts were subject to a colorimetric HAT activity assay.

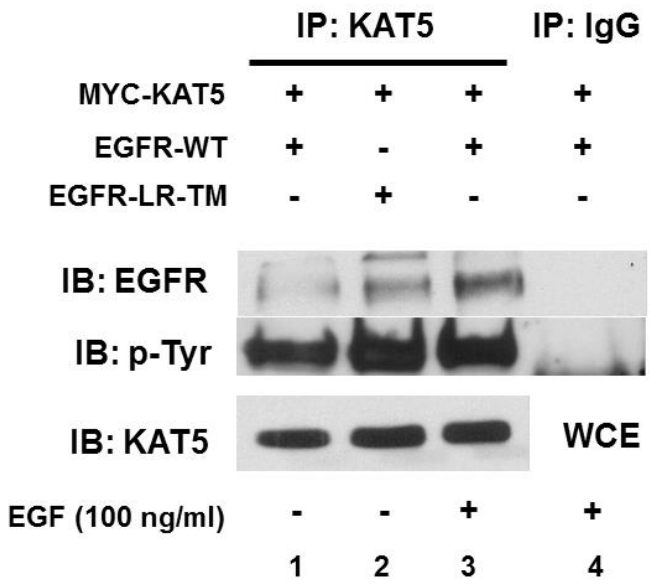

Figure 12. EGF Stimulation Releases KAT5 Bound to EGFR

A431 cells were serum-stared overnight and treated with 100 ng/ml EGF for 2 hours. Cell extracts were co-immunoprecipitated with anti-KAT5 antibody. WCE indicates whole cell extracts.

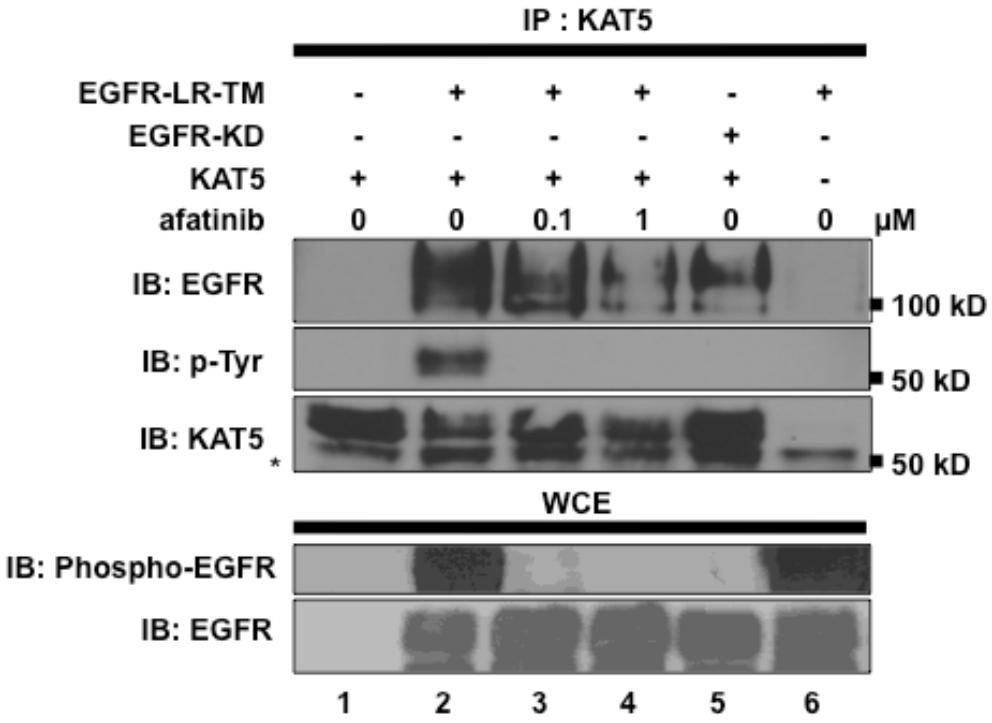

Figure 13. Oncogenic EGFR Binds and Phosphorylates KAT5. * indicates heavy chain (ß form) of KAT5

HEK293T cells were transfected with a KAT5 expression construct and either a mutant EGFR expression construct containing L858R and T790M mutations (EGFR-LR-TM) or an EGFR-KD construct. Extracts were immunoprecipitated with an anti-KAT5 antibody. * indicates heavy chain.

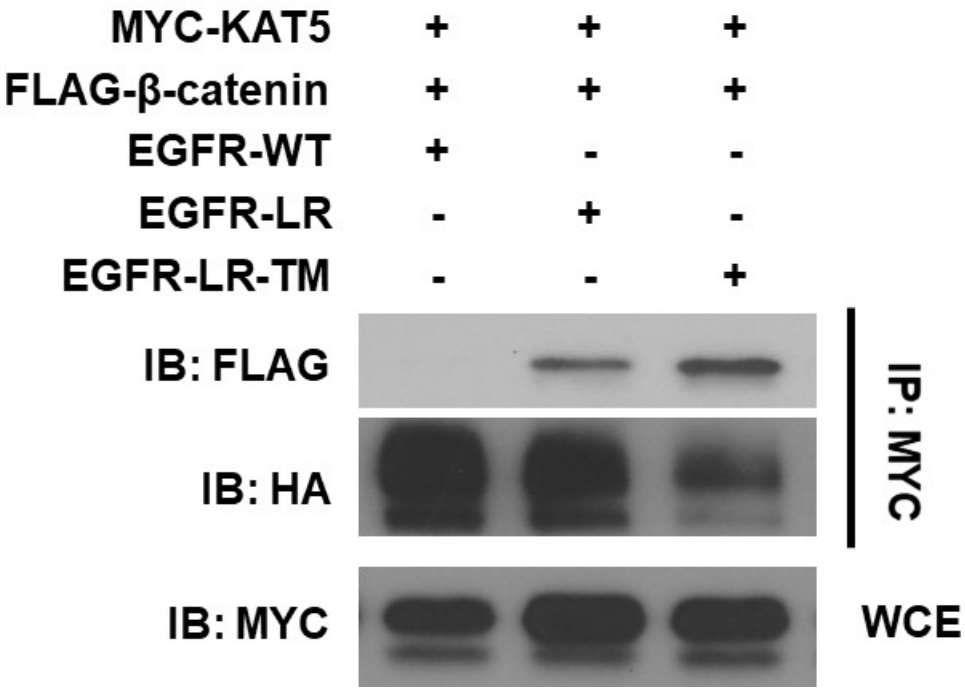

Fig 14. KAT5 Binds ß-catenin in cells expressing mutant EGFR

HEK293T cells were co-transfected with KAT5 and ß-catenin expression constructs along with EGFR constructs. Extracts were immunoprecipitated using a MYC antibody.

Quantitative PCR

PCR targets were identified from RNA-Sequencing data that possessed a change of greater than 1.5 fold. Samples were assessed in technical triplicates and normalized to ß-actin for relative comparison (Fig. 15).

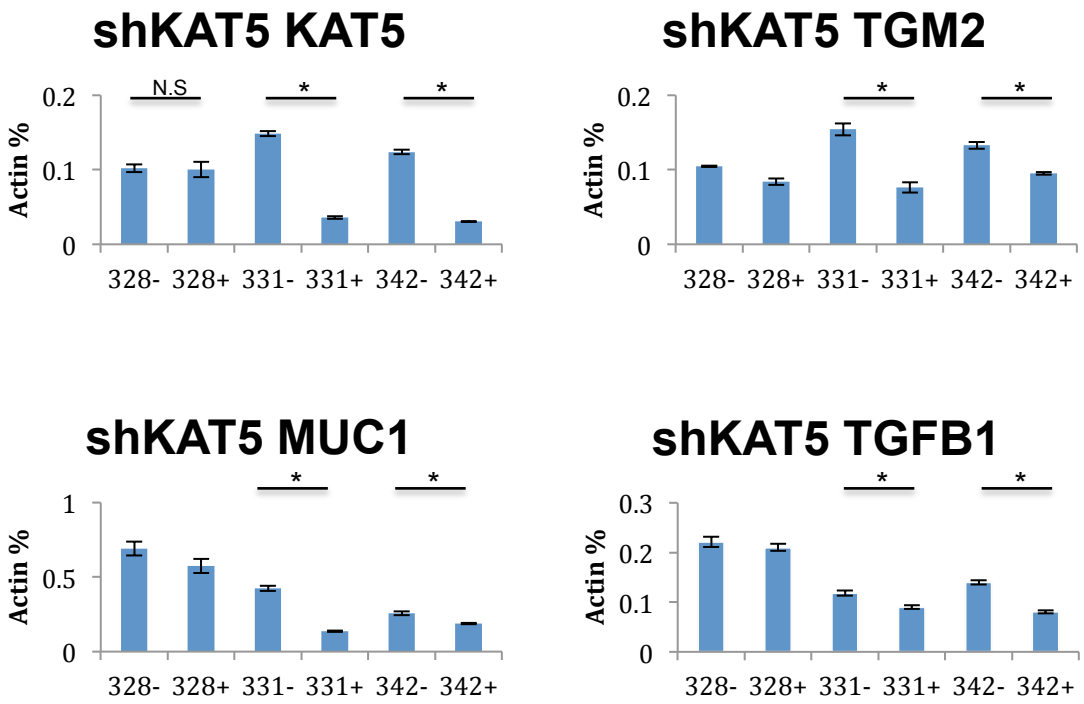

Figure 15. Relative mRNA Quantities in H1975 shKAT5 With and Without Doxycycline Treatment

Doxycycline treatment effectively reduces KAT5 by approximately 4-fold compared to untreated cells. * signifies a $p < 0.05$.

DISCUSSION

The role of KAT5 in lung tumorigenesis is complex and at times, paradoxical. It is a multi-functional protein that affects a diverse variety of targets including transcriptional machinery, cell cycle regulators, and checkpoint inhibitors. The increase in viability observed in BEAS2B cells after HAT inhibitor treatment affirms the multi-faceted role KAT5 retains in lung tissue. The findings suggest KAT5 retains its tumor suppressive role in normal bronchial tissue; yet, in transformed cells, a novel oncogenic process may be occurring that characterizes the tumorigenic properties of KAT5.

Our initial results demonstrate that KAT5 is involved in the development of lung tumors both *in vitro* and *in vivo.* The regulatory nature of KAT5 prompts the need to identify downstream genes and/or signals that may be responsible for lung tumorigenesis. KAT5 is known to be phosphorylated by a variety of different kinases such as CDC2, GSK3, cAbl, and p38α[14–16]. However, relatively little is known about EGFR phosphorylating KAT5. The co-immunoprecipitatin of KAT5 and EGFR suggests that a protein complex may be forming. Further investigation is warranted to determine the role and properties of this complex.

Previously, ß-catenin signaling had been shown to be required for EGFR induced lung cancer development where deletion of *Ctnnb1* significantly inhibited tumor formation in mutant EGFR-driven lung cancer mouse models[17]. The binding between KAT5 and ß-catenin observed in immunoprecipitation (Fig 13) and association between KAT5 and EGFR suggests KAT5 may be involved in Wnt/ß-catenin signaling in mutant

EGFR lung cancer. Studies demonstrating KAT5 to be a coactivator of TCF-ß-catenin signaling further support an oncogenic EGFR pathway mediated by a KAT5-ß-catenin complex[18]. Our data indicates a structure is being formed between KAT5 and ß-catenin; whether that structure is directly responsible for tumorigenic consequences or influences downstream signaling remains to be elucidated.

Lung-specific Kat5 knockout EGFR-L858R-T790M-induced lung cancer mice showed tumor formation to be nearly completely suppressed when mutant EGFR and Kat5 deletion were induced simultaneously. However, it is still unknown whether KAT5 is required for tumor maintenance. Lung weight measured between $EGFR^{TL}$/CCSP-rtTA/Cre/$Kat5^{wt/wt}$ and $EGFR^{TL}$/CCSP-rtTA/Cre/$Kat5^{wt/F}$ mice have no significant difference while there is a drastic reduction observed in $EGFR^{TL}$/CCSP-rtTA/Cre/$Kat5^{F/F}$. The complete knockout of Kat5 in mice demonstrated the only notable change in tumor volume compared to 50% Kat5 knockout mice (Fig. 6), which indicates there may exist a targetable therapeutic level of KAT5 for lung tumor treatment.

At the same time, the significant changes derived from *in vivo* doxycycline-induced models can be related to toxicity related to rtTa, Cre-recombinase, and doxycycline present in the mice[19]. Airspace enlargement without inflammation may occur in CCSP-rtTa mice[20]. Cre-recominbase has been attributed to G2/M cell cycle arrest, and the combination of Cre and doxycycline may contribute and enhance overall toxicity[21]. Still, these three reagents are essential for *in vivo* modeling of EGFR lung tumorigenesis and numerous controls have been implemented to ensure accurate data interpretation. Should toxicity concerns affect study groups, then bi-transgenic mice with

floxed Kat5 can be used to avoid chronic exposure to Cre-recombinase by administration of Cre-expressing recombinant adenoviruses[22].

HATs are generally recruited to target promoters by transcription factors[23]. Systematic identification of major effector genes downstream of KAT5 was conducted with RNA-Sequencing to assess the regulatory effect of KAT5 on relevant targets. Quantitative PCR assessment for KAT5 mRNA levels reveals a nearly 4-fold downregulation occurring using an inducible knockdown system for cell lines sh331 and sh342 (Fig. 15). Three potential targets, MUC1, TGFB1, and TGM2, demonstrated decreased mRNA expression levels following treatment, indicating the decrease in KAT5 expression is correlative to these respective genes. MUC1 and TGM2 have been shown to mechanistically contribute to tumor growth via ß-catenin and EGFR pathways as well as inducing drug resistance, making these attractive targets for examining a relationship to KAT5[24–27]. Similar to KAT5, the role of TGFB1 in cancer is complex and paradoxical, but its role in cancer is undeniable and warrants further investigation to clarify its specific role in relation to KAT5 and lung cancer[28].

A recent study confirmed the tumor suppressive properties of KAT5 in lung cancer models where elevated levels of KAT5 was attributed to tumor regression[29]. However, the cells used in the study for *in vitro* modeling, A549 and H1299, do not harbor EGFR mutations, but rather RAS and p53 mutations, respectively. These genomic discrepancies further support our initial hypothesis that KAT5 behaves differently under niche genomic perturbations.

In conclusion, our results show inhibition of KAT5 or potential combination therapies with EGFR TKIs could be attractive strategies to treat non-small cell lung cancer. The tyrosine phosphorylation of KAT5 by EGFR offers a possible biochemical mechanism for the tumorigenic activity of KAT5 as well as another therapeutic option. RNA-Sequencing identified numerous oncogenic targets that provide further opportunities to elucidate KAT5-dependent tumorigenic pathways. With lung cancer contributing to a vast amount of worldwide cancer-related deaths, it is an imperative to find new molecular targets to better optimize treatment protocol for patients with this disease.

LIST OF JOURNAL ABBREVIATIONS

Am. J. Respir. Cell Mol. Biol.	American Journal of Respiratory Cell and Molecular Biology
Biochim. Biophys. Acta	Biochimica et Biophysica Acta
CA. Cancer J. Clin.	CA: A Cancer Journal for Clinicians
Cancer Res.	Cancer Research
Cell Death Dis.	Cell Death & Disease
Eur. J. Cancer	European Journal of Cancer
Genes Dev.	Genes & Development
Int. J. Biochem. Cell. Biol.	International Journal of Biochemistry & Cell Biology
J. Cancer	Journal of Cancer
J. Thorac. Oncol.	Journal of Thoracic Oncology
Microbiol. Mol. Biol. Rev.	Microbiology and Molecular Biology Reviews
Mol. Cell	Molecular Cell
Mol Cell Biol	Molecular and Cellular Biology
Nat. Protoc.	Nature Protocols
Nat. Rev. Clin. Oncol.	Nature Reviews Clinical Oncology
NEJM	New England Journal of Medicine
Open Biochem. J.	The Open Biochemistry Journal
Trends Cell Biol.	Trends in Cell Biology
Trends Mol. Med.	Trends in Molecular Medicine

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