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# FOXA2 cooperates with mutant KRAS to drive invasive mucinous adenocarcinoma of the lung

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## Abstract

The endoderm-lineage transcription factor FOXA2 has been shown to inhibit lung tumorigenesis in *in vitro* and xenograft studies using lung cancer cell lines. However, FOXA2 expression in primary lung tumors does not correlate with an improved patient survival rate, and the functional role of FOXA2 in primary lung tumors remains elusive. To understand the role of FOXA2 in primary lung tumors *in vivo*, here we conditionally induced the expression of FOXA2 along with either of the two major lung cancer oncogenes, $EGFR^{L858R}$ or $KRAS^{G12D}$, in the lung epithelium of transgenic mice. Notably, FOXA2 suppressed autochthonous lung tumor development driven by $EGFR^{L858R}$, whereas FOXA2 promoted tumor growth driven by $KRAS^{G12D}$. Importantly, FOXA2 expression along with $KRAS^{G12D}$ produced invasive mucinous adenocarcinoma of the lung (IMA), a fatal mucus-producing lung cancer comprising ~5% of human lung cancer cases. In the mouse model *in vivo* and human lung cancer cells *in vitro*, FOXA2 activated a gene regulatory network involved in the key mucous transcription factor SPDEF and upregulated MUC5AC, whose expression is critical for inducing IMA. Co-expression of FOXA2 with mutant KRAS

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Author contributions

Y. Maeda, K. Tomoshige and M. Guo designed experiments. K. Tomoshige, W.D. Stuart, I.M. Fink-Baldauf and Y. Maeda performed experiments. M. Guo performed bioinformatical analyses. Y. Maeda, K. Tomoshige, W.D. Stuart, I.M. Fink-Baldauf analyzed data. M. Ito, T. Fukazawa, T. Tsuchiya, M. Okada, T. Nagayasu and T. Yamatsuji provided human specimens. All authors contributed experimental interpretation and manuscript writing.

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synergistically induced MUC5AC expression compared to that induced by FOXA2 alone. ChIP-seq combined with CRISPR interference indicated that FOXA2 bound directly to the enhancer region of *MUC5AC* and induced the H3K27ac enhancer mark. Furthermore, FOXA2 was found to be highly expressed in primary tumors of human IMA. Collectively, this study reveals that FOXA2 is not only a biomarker but also a driver for IMA in the presence of a *KRAS* mutation.

**Keywords**

Invasive mucinous adenocarcinoma of the lung (IMA); KRAS mutation; SPDEF; MUC5AC; MUC5B

## Introduction

Lung cancer is a devastating disease causing 1.76 million deaths worldwide every year (1). Recent advances in molecular genetics using human specimens revealed driver oncogenes and tumor suppressors that are responsible for causing lung cancer, including non-small cell lung cancer (NSCLC, the most common type) (2,3) and small cell lung cancer (SCLC) (4). These human molecular studies also revealed lung lineage-specific transcription factors such as NKX2–1 (also known as TTF-1) (3, 5–8) and SOX2 (2, 9) that initiate and/or promote lung tumorigenesis, which have also been validated by autochthonous mouse models (10–12). In addition to the genetic studies, comprehensive pathological studies have determined consensus biomarkers that define lung cancers, especially NKX2–1, that have been clinically used to identify primary non-mucinous lung adenocarcinoma (LUAD, the most prevalent pathological type of NSCLC) and SCLC (13, 14). Supporting the pathological observation that NKX2–1 is expressed in non-mucinous LUAD but not in mucinous LUAD (also known as Invasive Mucinous Adenocarcinoma of the lung [IMA] comprising ~5% of lung cancer cases), mutant KRAS (a driver oncogene most frequently seen in LUAD) along with the reduced expression of NKX2–1 resulted in the development of mucinous lung tumors (IMA) in autochthonous mouse models (10, 15), suggesting lung lineage-specific transcription factors are critically involved in lung tumor development and pathogenesis.

FOXA2 is a transcription factor that is expressed in the endodermal lineage, including normal lung epithelium (16). FOXA2 is also expressed in lung cancer, including NSCLC and SCLC (17). Reduction of FOXA2 using shRNA in an A549 human lung carcinoma line ($KRAS^{G12S}$;$CDKN2A^{del}$) increased the expression of EMT (Epithelial-Mesenchymal Transition) markers, cell migration and metastasis while induction of FOXA2 in an H446 human SCLC cell line ($TP53^{G154V}$;$RB^{null}$;$PTEN^{null}$) and an H1299 human NSCLC cell line ($NRAS^{Q61K}$;$TP53^{null}$), both of which are cell lines derived from metastatic sites, decreased the expression of the EMT markers, cell migration and metastasis *in vitro* and in a nude mouse model. FOXA2 did not influence proliferation in either the loss- or gain-of-function study in these cell lines (18). However, another report indicates that induction of FOXA2 in an H358 bronchioalveolar carcinoma cell line ($KRAS^{G12C}$;$TP53^{null}$; NSCLC cells), which are also derived from a metastatic site, suppressed growth of the cells by arresting proliferation and increasing apoptosis *in vitro* (19). In a study using a mouse lung cancer cell line ($Kras^{G12D}$; *Tp53* null; derived from a non-metastatic primary

tumor), reduction of *Foxa2* using shRNA also increased the growth of the cells on the subcutaneously transplanted site as well as increased the rate of lung metastasis in a nude mouse model (20). These cell line-based *in vitro* studies and xenograft models suggest that FOXA2 is a tumor suppressor especially for regulating metastasis. However, the expression level of FOXA2 in primary lung tumors does not significantly correlate with the survival outcome in human NSCLC cases (17, 20). In addition, FOXA2 has been shown to promote tumorigenesis in other cancers, including colon, pancreas, prostate and esophagus (21–24), suggesting that a further study using a different experimental model is required to elucidate the role of FOXA2 in lung tumorigenesis, especially for primary lung tumors. In the present study, we sought to determine the role of FOXA2 in autochthonous primary lung tumors by conditionally inducing FOXA2 in lung epithelium of a KRAS-mutant or an EGFR-mutant lung cancer transgenic mouse model. *KRAS* and *EGFR* mutations are the most prevalent genetic alterations seen in LUAD (1, 3). Consistent with the previous findings in the *in vitro* and xenograft mouse studies (18–20), FOXA2 suppressed the growth of autochthonous EGFR-mutant lung tumors; however, FOXA2 promoted the growth of autochthonous KRAS-mutant lung tumors. Importantly, FOXA2 along with mutant KRAS produced IMA-like mucinous lung tumors in this autochthonous mouse model. FOXA2 also induced the *in vitro* expression of mucous genes seen in IMA, including *MUC5AC* and *SPDEF*, in an H441 human lung adenocarcinoma cell line that carries a *KRAS* mutation ($KRAS^{G12V}$;$TP53^{R158L}$). Here, we demonstrate a novel context-dependent role of FOXA2 in autochthonous primary lung tumors developed in transgenic mice carrying an *EGFR* or a *KRAS* mutation.

## Materials and Methods

### Mice

[tetO]-*Foxa2* (rat *Foxa2*) mice were obtained from Jeffrey Whitsett and Gang Chen at Cincinnati Children's Hospital Medical Center (CCHMC) and the University of Cincinnati College of Medicine, Cincinnati, OH (25) and crossed with *Scgb1a1-rtTA;* [tetO]-$EGFR^{L858R}$ or *Scgb1a1-rtTA;*[tetO]-$Kras4b^{G12D}$ (26, 27) to develop *Scgb1a1-rtTA;* [tetO]-$EGFR^{L858R}$;[tetO]-*Foxa2* (FVB/N;B6;CBA mixed strain) or *Scgb1a1-rtTA;*[tetO]-$Kras4b^{G12D}$;[tetO]-*Foxa2* (FVB/N strain) as described previously (10). Transgenic mice were provided chow containing doxycycline (625 mg/kg chow) beginning at 4–5 weeks of age. Mouse maintenance and procedures were approved in accordance with the institutional protocol guidelines of Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee. See Supplementary data and Supplementary Tables S1–S4 for further mouse information.

### Human specimens

Paraffin sections for lung adenocarcinoma were obtained from Kawasaki Medical School, Okayama (approval # 1310) and Hiroshima University (approval # E-1919) in accordance with institutional guidelines for use of human tissue for research purposes. Written informed consent was obtained from all participants. Patients' information is summarized in Supplementary Table S5.

### Histology and immunohistochemistry

Staining (H&E, Alcian blue and immunohistochemistry) was performed using 5 μm paraffin-embedded lung sections as described previously (10). The antibody information is available in Supplementary data. The number of different types of tumors per H&E-stained section was counted in at least 3 mice of each group (see Supplementary Table S3 and S4 for details).

### Cell culture, lentivirus and/or retrovirus infection, siRNAs, CRISPRi, immunoblotting, co-immunoprecipitation, RNA-seq, TaqMan gene expression analysis

H441 and A549 human lung cell lines were obtained from ATCC on May 3rd, 2004 and July 26th, 2004, respectively (Manassas, VA). The BEAS-2B lung cell line was obtained from Thomas Korfhagen at CCHMC on April 27th, 2006. Cell line authentication was conducted by Genetica DNA Laboratories (Labcorp, Burlington NC) on December 3, 2010 for H441 and A549 cells and May 13, 2013 for BEAS-2B cells. Mycoplasma testing was performed by Universal Mycoplasma Detection Kit on December 8, 2022 (cat# 30–1012K, ATCC). Cell passage numbers used in this study were p81-p100 for A549 cells, p51-p84 for H441 cells and p24-p41 for BEAS-2B cells. Lentiviral vector delivering FOXA1, FOXA2 or NKX2–1 was made by inserting mouse *Foxa1*, rat *Foxa2* or rat *Nkx2-1* into the PGK-IRES-EGFP vector as described previously (10). CRISPR interference (CRISPRi; CRISPR/dCas9-KRAB) (28) lentiviral vector (pLV hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro; Plasmid #71236), pBabe (Plasmid #1764) and pBabe K-Ras 12V (human $KRAS^{G12V}$; Plasmid #12544) retroviral vectors were obtained from Addgene (Cambridge, MA). The siRNA-mediated knockdown analysis is described in Supplementary data.

H441 cells that stably express dCas9-KRAB were developed using the CRISPR/dCas9-KRAB lentiviral vector as we described previously (29). H441 cells that stably express dCas9-KRAB were infected with the PGK-IRES-EGFP lentiviral vector carrying rat *Foxa2* or empty control to develop H441 cells that stably express dCas9-KRAB with or without FOXA2 for the CRISPRi experiments. CRISPRi using these H441 cells were performed as described previously (29) by transiently transfecting synthetic sgRNA from the Invitrogen custom TrueGuide gRNA (sgRNA) ordering tool (ThermoFisher, Waltham, MA). Non-targeted gRNA (sgRNA) was used as a negative control (cat# A35526, ThermoFisher). H441 or BEAS-2B cells were infected with the lentiviral vector expressing FOXA1, FOXA2 and/or the retroviral vector expressing $KRAS^{G12V}$. A549 cells were infected with the PGK-IRES-EGFP lentiviral vector carrying rat *Foxa2*, rat *Nkx2-1* or empty control to develop A549 cells with or without ectopic FOXA2 or NKX2–1. The empty vectors were used as controls.

Protein and RNA were extracted as described previously (10). Co-immunoprecipitation analysis is described in Supplementary data. Immunoblotting assays were performed as described previously (10). The antibody information with RRID is available in Supplementary data. RNA-seq using biological triplicates were performed as described previously (29) except that RNA was obtained from H441 cells that were infected with lentivirus (Control or rat *Foxa2*). TaqMan gene expression analysis was performed as

described previously (10). The TaqMan probe information is available in Supplementary data.

### TCGA LUAD data and ChIP-seq analysis

Normalized gene expression data from TCGA LUAD RNA-seq datasets were retrieved from (3). ChIP-seq datasets were retrieved from GSE43252 (15), ENCODE using accession number ENCFF686MSH (ENCSR000BRE) (30, 31), GSE48930 (32) and our previous study (33). See Supplementary data for FOXA2 ChIP-seq analysis in H441 cells in details.

### Data availability

The RNA-seq and ChIP-seq data have been deposited in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE210121.

### Statistical analysis

Statistical differences were determined using two-tailed and unpaired Student's or Welch's *t*-test or the Kolmogorov-Smirnov test. Error bars represent SEM. The difference between two groups was considered significant when the *p*-value was $< 0.05$.

## Results

### FOXA2 suppresses growth of EGFR-mutant lung tumors but promotes growth of KRAS-mutant lung tumors

In order to assess the role of FOXA2 in autochthonous primary lung tumors, we conditionally induced the expression of FOXA2 along with either of the lung oncogenes $EGFR^{L858R}$ or $KRAS^{G12D}$ in the lung epithelium by generating triple transgenic mice (*Scgb1a1-rtTA*;*[tetO]-Kras4b*$^{G12D}$;*[tetO]-Foxa2* or *Scgb1a1-rtTA*;*[tetO]-EGFR*$^{L858R}$;*[tetO]-Foxa2*; hereafter *Kras*$^{G12D}$;*Foxa2* or *EGFR*$^{L858R}$;*Foxa2*) that express transgenes regulated by the tet-on system (Fig. 1A) (25–27). Consistent with previous reports, lung tumors developed in $EGFR^{L858R}$- (Fig. 1B–D; Supplementary Table S1) (27) or $KRAS^{G12D}$-expressing mice (Fig. 1E to G; Supplementary Table S2) (26) but not in mice expressing FOXA2 alone (Fig. 1B to G; Supplementary Table S1 and S2) (25). The number and volume of lung tumors that developed in $EGFR^{L858R}$ and FOXA2 co-expressing mice significantly decreased compared to those in the $EGFR^{L858R}$-expressing mice (Fig. 1B to D; Supplementary Table S1), consistent with previous *in vitro* and xenograft studies (18–20). In contrast, the volume (but not the number) of lung tumors developed in $KRAS^{G12D}$ and FOXA2 co-expressing mice was significantly promoted compared to that in $KRAS^{G12D}$-expressing mice (Fig. 1E to G; Supplementary Table S2). These *in vivo* results indicate that FOXA2 suppresses initiation and promotion of EGFR-mutant autochthonous lung tumors while FOXA2 promotes the growth of KRAS-mutant autochthonous lung tumors.

### FOXA2 along with mutant KRAS induces invasive mucinous adenocarcinoma of the lung (IMA)

In order to determine whether FOXA2 influences tumorigenesis through proliferation and/or apoptosis in the autochthonous lung tumors developed by $EGFR^{L858R}$ or $KRAS^{G12D}$, the

expression of PHH3 and caspase-3 was assessed. The expression of PHH3 (arrows), a marker for dividing cells, was frequently observed in lung tumors of mice expressing only $EGFR^{L858R}$ but not in the lungs of mice co-expressing $EGFR^{L858R}$ and FOXA2 (Supplementary Fig. S1A, S1B and Table S3). The expression of caspase-3, a marker for apoptosis, was not altered in the mice expressing only $EGFR^{L858R}$ compared to the mice co-expressing $EGFR^{L858R}$ and FOXA2 (Supplementary Fig. S1A, S1C and Table S3). Lung tumors were hardly seen in the mice co-expressing $EGFR^{L858R}$ and FOXA2 compared to the mice expressing only $EGFR^{L858R}$ (Supplementary Fig. S1D and Table S3). These results indicate that FOXA2 suppresses the growth of the EGFR-mutant lung tumors by reducing cell proliferation associated with tumor initiation by mutant EGFR, which is consistent with previous studies (19, 20) but not with another study (18) though these studies used isolated human KRAS-mutant cancer cell lines (18, 19) or a mouse *Kras*-mutant cancer cell line (20). In contrast, the expression of PHH3 (arrows) was induced in mice co-expressing $KRAS^{G12D}$ with FOXA2 compared to mice expressing only $KRAS^{G12D}$ (Fig. 2A and B; Supplementary Table S4). The expression of caspase-3 was not altered in mice co-expressing $KRAS^{G12D}$ and FOXA2 compared to mice expressing only $KRAS^{G12D}$ (Fig. 2A, C and Supplementary Table S4). In addition, lung tumors developed in mice co-expressing $KRAS^{G12D}$ and FOXA2 were comprised of more invasive adenocarcinoma cells than those developed in mice expressing only $KRAS^{G12D}$ (Fig. 2D). Importantly, 48% of lung tumors in mice co-expressing $KRAS^{G12D}$ with FOXA2 were mucinous lung tumors that stained with Alcian blue, the majority being located in the alveolar region of the lung (Fig. 2D; Supplementary Fig. S2 and Table S4). Mice expressing only $KRAS^{G12D}$ or $EGFR^{L858R}$ did not develop mucinous lung tumors (Fig. 2; Supplementary Fig. S1, Table S3 and S4). Consistent with Fig. 1 and Supplementary Table S1 and S2, lung tumors were not observed in mouse lungs overexpressing FOXA2 (Fig. 2, Supplementary Fig. S1, Table S3 and S4). These results indicate that FOXA2 promotes the growth of the KRAS-mutant lung tumors by inducing cell proliferation and a mucinous phenotype in the tumors.

## FOXA2 along with mutant KRAS induces biomarkers for IMA

Previously, we determined a gene signature for human invasive adenocarcinoma of the lung (IMA) by identifying the genes that are specifically expressed in both human and mouse IMAs (33). To characterize the mucinous lung tumors developed in mice co-expressing $KRAS^{G12D}$ with FOXA2 in the lung epithelium, we assessed the expression of selected IMA-signature genes (MUC5AC, MUC5B, AGR2, SPDEF, FOXA3 and HNF4A) along with NKX2–1, which is known to be deficient in human IMA (14). As shown in Fig. 3, these IMA-signature genes were expressed in the mucinous lung tumors developed by co-expression of $KRAS^{G12D}$ with FOXA2 while anti-mucous NKX2–1 was reduced compared to the other mouse groups (Control, *Foxa2* or $Kras^{G12D}$). HDM (house dust mite)-induced non-tumorigenic asthma-like mouse lungs (HDM) also express mucous genes and lack NKX2–1 as we previously reported (10); however, HDM lungs do not express HNF4A contrary to the mucinous lung tumors ($Kras^{G12D}$;*Foxa2*). The transgenic expression of FOXA2 alone induced goblet (mucous) cells accompanied by mucous genes though the goblet cells appeared immature compared to those seen in the mucinous lung tumors ($Kras^{G12D}$;*Foxa2*) or HDM lungs (Fig. 2, 3; Supplementary Fig. S3). Of note, although MUC5AC, AGR2 and SPDEF do not initiate lung tumor formation *in vivo*, they do promote

the growth of lung tumors (33, 34–36), suggesting that FOXA2 promotes the growth of KRAS-mutant lung tumors in part through MUC5AC, AGR2 and SPDEF. These results suggest that mucinous lung tumors developed by FOXA2 along with $KRAS^{G12D}$ mimic human IMA.

## FOXA2 binds to the locus of the mucus-transcription factor *Spdef* in $Kras^{G12D}$ lung tumors lacking *Nkx2-1 in vivo*

Snyder *et al*. have performed ChIP-seq to identify FOXA1/FOXA2 binding sites in $Kras^{G12D}$ lung tumors in the presence or absence of *Nkx2-1* (an antibody recognizing both FOXA1 and FOXA2 was used for their ChIP-seq) (15). Consistent with our previous report (10), $Kras^{G12D}$ in the absence of *Nkx2-1* induced mucinous lung tumors while $Kras^{G12D}$ alone induced non-mucinous lung tumors (15). Unexpectedly, the ChIP-seq data indicated that FOXA1/FOXA2 bound to the locus of *Muc5b* in the NKX2–1-positive lung tumors (non-mucinous lung tumors) but not in the NKX2–1-deleted lung tumors (mucinous lung tumors) (Fig. 4). FOXA1/FOXA2 did not bind to the locus of *Muc5ac* regardless of the expression of *Nkx2-1* (Fig. 4A). These results indicate that FOXA1/FOXA2 do not directly induce two major mucins *Muc5ac* or *Muc5b* in mucinous lung tumors in mice. However, the ChIP-seq data indicated that FOXA1/FOXA2 bound to the locus of *Spdef*, which is the key transcription factor that is indispensable for the expression of mucous genes, including *Muc5ac* and *Muc5b* (33, 37), in the NKX2–1-deleted mouse lung tumors (mucinous lung tumors) (Fig. 4B), suggesting that FOXA2 may directly induce the expression of *Spdef*, which may in turn increase the expression of *Muc5ac* and *Muc5b* in mucinous lung tumors in mice (Fig. 4C).

## FOXA2 induces IMA-signature genes in a *KRAS*-mutant human lung cancer cell line *in vitro*

Having determined that co-expression of mutant KRAS with FOXA2 induced IMA-signature mucous genes in autochthonous lung tumors in mice *in vivo*, we next sought to determine whether FOXA2 directly or indirectly induced these IMA-signature genes in human lung cancer cells that carry a *KRAS* mutation *in vitro*. First, we performed a gain-of-function study by infecting the H441 human lung papillary adenocarcinoma cell line ($KRAS^{G12V}$;$TP53^{R158L}$) with control lentivirus, lentivirus expressing *Foxa1* (mouse; 94% similarity to human *FOXA1*) or *Foxa2* (rat; 95% similarity to human *FOXA2*) and confirmed their protein expression using specific antibodies (Fig. 5A; Supplementary Fig. S4A). Using the H441 cells, mRNA-seq and TaqMan gene expression analyses were performed. As shown in Fig. 5B, 5C, Supplementary Fig. S5, Table S6 and S7, FOXA2 induced 42 genes that are highly expressed in human IMA compared to normal lung (33). Among the 42 genes, 12 genes, including *AGR2*, *BCAS1*, *CAPN5*, *CREB3L1*, *EHF*, *MMP7*, *MUC5AC*, *MUC5B*, *SLC44A4*, *SPDEF*, *TNS4* and *TOX3* (red highlighted, Fig. 5B, 5C; Supplementary Fig. S5, Table S6 and S7), were the IMA-signature mucous genes (143 genes) (33) highly expressed in both human and mouse IMA. Among these 12 genes, AGR2, EHF, MUC5AC, MUC5B and SPDEF are involved in mucus production (35, 38–41). Of note, FOXA2 alone did not affect the expression of anti-mucous transcription factor *NKX2-1* (Fig. 5C; Supplementary Table S7). We also performed a loss-of-function study using siRNAs targeting endogenous FOXA2 in A549

lung carcinoma cells (*KRAS*$^{G12S}$;*CDKN2A*$^{del}$), which indicated that FOXA2 is required for the expression of *MUC5AC*, *MUC5B* and *SPDEF* (Supplementary Fig. S6A, S6B and Table S8), consistent with the gain-of-function study results above. ChIP-seq analysis with the FOXA2 specific (no cross-reactivity with FOXA1) antibody (Fig. 5D; Supplementary Fig. S7A, S7B and Table S9) using H441 cells (*KRAS*$^{G12V}$;*TP53*$^{R158L}$) and A549 cells (ENCSR000BRE) (30, 31) indicated that FOXA2 bound to the loci of *MUC5* and *SPDEF* in both cell lines, suggesting that FOXA2 may directly induce the expression of *MUC5AC*, the most highly expressed human IMA mucous gene (33), in human lung carcinoma cells (Fig. 5D and E) contrary to mouse lung carcinoma cells (Fig. 4). Notably, 2 distinct non-coding regions at the *MUC5* locus (upstream of *MUC5AC* and intergenic region of *MUC5AC* and *MUC5B*) bound by FOXA2 were enhancer regions bound by SPDEF (a pro-mucous transcription factor), which we previously reported (Fig. 5D, top panel) (33). Importantly, co-immunoprecipitation experiments showed that FOXA2 interacted with SPDEF (Supplementary Fig. S8), indicating that the enhancer regions function as gene-regulatory hubs bound by multiple interacting transcription factors for the expression of *MUC5AC* and *MUC5B* (Fig. 5E). Of note, SPDEF did not bind to its own *SPDEF* locus as FOXA2 did (Fig. 5D, bottom panel).

## FOXA2 induces the expression of both *MUC5AC* and *MUC5B* through the upstream enhancer region of *MUC5AC*

CRISPR/Cas9 technology, including CRISPRi, has enabled researchers to assess the functional roles of non-coding regions bound by transcription factors, beyond mere binding knowledge obtained by ChIP-seq (29, 33). In order to validate whether the FOXA2-binding regions described above function as gene-regulatory regions to induce the expression of *MUC5AC* and/or *MUC5B*, we perturbed the enhancer regions using CRISPRi, which uses dCas9-KRAB and single-guide RNA targeting the enhancer regions (sgRNA#1 for the upstream enhancer region of *MUC5AC* [hereafter, *MUC5AC* enhancer region] or sgRNA#2 for the intergenic enhancer region between *MUC5AC* and *MUC5B* [hereafter, *MUC5B* enhancer region]), and assessed whether FOXA2-mediated induction of *MUC5AC* and/or *MUC5B* is functionally affected (Fig. 6A). Importantly, FOXA2-mediated induction of *MUC5AC* was repressed by CRISPRi targeting the *MUC5AC* enhancer region (sgRNA#1) but not the *MUC5B* enhancer region (sgRNA#2) in H441 cells (Fig. 6B; Supplementary Table S10), indicating that the *MUC5AC* enhancer region but not the *MUC5B* enhancer region is required for FOXA2-mediated induction of *MUC5AC* in H441 cells. Notably, FOXA2-mediated induction of *MUC5B* was repressed by CRISPRi targeting both the *MUC5AC* enhancer region (sgRNA#1) and the *MUC5B* enhancer region (sgRNA#2) in H441 cells, indicating that both enhancer regions are essential for the expression of *MUC5B* (Fig. 6B; Supplementary Table S10), which is consistent in part with the results shown by Helling et al. on the *MUC5B* enhancer region using A549 cells (42). Of note, CRISPRi targeting both regions did not influence the expression of pro-mucous transcription factors *SPDEF* or *FOXA3* (Supplementary Fig. S9), indicating the specificity of CRISPRi and direct regulation of *MUC5AC* and *MUC5B* by FOXA2 (not mediated by other pro-mucous transcription factors). These results suggest the *MUC5AC* enhancer region bound by FOXA2 has a dual role to regulate the expression of two genes (*MUC5AC* and *MUC5B*)

while the *MUC5B* enhancer region bound by FOXA2 has a single role to regulate the expression of only one gene *MUC5B* (Fig. 6C).

## Expression of mucous genes are primed by FOXA2 and amplified by mutant KRAS

Our current study demonstrates that FOXA2 alone induces the development of immature goblet (mucous) cells in the airways while co-expression of FOXA2 with mutant KRAS induces fully developed mucinous lung tumors in mice (Fig. 2, 3; Supplementary Fig. S3), suggesting that FOXA2 is a pro-mucous transcription factor, especially in the presence of mutant KRAS. In order to understand the role of FOXA2 or mutant KRAS in mucous gene regulation associated with the *in vivo* mucous phenotypes, we assessed chromatin modification at the locus of *MUC5AC* in the presence of FOXA2 and/or mutant KRAS ($KRAS^{G12V}$) using BEAS-2B human transformed bronchial epithelial cells. Consistent with the mouse results (Fig. 3), FOXA2 alone (*Foxa2*) moderately induced the mRNA expression of *MUC5AC* and *MUC5B* in the absence of mutant KRAS in BEAS-2B cells (Fig. 7A and B; Supplementary Fig. S10 and Table S11). Mutant KRAS alone ($KRAS^{G12V}$) did not induce the expression of *MUC5AC* or *MUC5B*, which is also consistent with the mouse data (Fig. 3). Notably, co-expression of FOXA2 with mutant KRAS ($KRAS^{G12V}$;*Foxa2*) synergistically induced the expression of *MUC5AC* and *MUC5B* compared to that induced by FOXA2 alone, indicating that mutant KRAS amplifies the expression of *MUC5AC* and *MUC5B* primed by FOXA2. Importantly, H3K27ac (an enhancer mark of histone) was detected at the upstream locus of *MUC5AC* in the BEAS-2B cells that express FOXA2 but not mutant KRAS (Fig. 7C; Supplementary Fig. S11A–S11C and Table S12), suggesting a novel role of FOXA2 as a pioneer factor that modifies chromatin by introducing H3K27ac at the locus of *MUC5AC*, which in turn allows mutant KRAS-induced transcriptional activators access to induce expression of *MUC5AC* (Fig. 7D).

## FOXA2 is highly expressed in human IMA

Next, in order to determine whether FOXA2 is associated with the mucous phenotype in human lung adenocarcinoma *in vivo*, we sought to determine by immunohistochemistry whether FOXA2 is expressed in human IMA (Supplementary Table S5). As shown in Fig. 8A, FOXA2 was expressed in the nucleus of mucinous lung tumor cells in human IMA. The expression of FOXA2 was seen in 71% of human IMA cases (Fig. 8B; considered positive when FOXA2 expression is seen in over 50% of IMA tumor cells in each case), which is similar to a previous pathological study (100% positivity reported) (43). This result suggests that FOXA2 expressed in mucinous tumor cells may drive the mucinous phenotype in human IMA. According to TCGA LUAD dataset (3), *KRAS* mutation did not affect the expression of *FOXA2* compared to *KRAS* wild type (Supplementary Fig. S12A–S12C); however, among *KRAS* mutations/*CDKN2A* loss co-occurring genomic alteration group (44), expression of *FOXA2* is significantly higher in IMA than non-IMA while the expression of *NKX2-1*, an anti-mucous transcription factor (10. 15), is significantly lower in IMA than in non-IMA (Fig. 8C). Of note, TCGA LUAD IMA cases with *KRAS* mutations are all associated with *CDKN2A* loss (3). Importantly, ectopic *Nkx2-1* significantly suppressed the expression of *FOXA2* in A549 cells ($KRAS^{G12S}$;$CDKN2A^{del}$) (Fig. 8D; Supplementary Table S13). These results suggest that genetic or epigenetic loss of

*NKX2-1* in LUAD with *KRAS* mutations/*CDKN2A* loss induces the expression of *FOXA2*, which drives IMA.

## Discussion

Since FOXA2 expression was observed in human lung cancer specimens (e.g., https://www.proteinatlas.org/ENSG00000125798-FOXA2/pathology/lung+cancer), the role of FOXA2 in lung cancer has been studied by multiple groups using *in vitro* cell culture and *in vivo* xenograft mouse models. However, the role of FOXA2 in genetically engineered mouse models (GEMMs) that induce autochthonous lung tumors has not been well studied. Here, using a tet-on 'gain-of-function' system, we conditionally induced FOXA2 along with oncogenic mutant KRAS or mutant EGFR in lung epithelium in GEMMs and determined the role of FOXA2 in autochthonous lung tumors driven by mutant KRAS or mutant EGFR. Although ectopic expression of FOXA2 in human lung cancer cell lines that were derived from metastatic sites (H446, H1299 and H358 cells) inhibited tumorigenicity of the cells (18, 19), transgenic expression of FOXA2 in a GEMM promoted KRAS mutant-driven autochthonous primary lung tumors and suppressed EGFR mutant-driven autochthonous primary lung tumors in our study. These results suggest that FOXA2 functions as a tumor promoter or a tumor suppressor depending on the presence of different driver oncogenes (e.g., KRAS mutant vs EGFR mutant) and tumor locations (e.g., primary or metastatic), which is reminiscent of the role of NKX2–1 as a context-dependent lung tumor promoter or tumor suppressor (45). A sequential transgenic mouse model that induces FOXA2 before or after mutant KRAS or mutant EGFR expression will further delineate how FOXA2 influences the initiation and/or promotion of KRAS-mutant or EGFR-mutant lung tumorigenesis. The use of knock-in mice carrying alveolar type II cell-specific *Sftpc*-Cre or club cell-specific *Scgb1a1*-Cre other than our rat *Scgb1a1*-rtTA transgenic mouse model targeting broad lung epithelial cells may further elucidate the precise origin of mucinous tumor cells influenced by FOXA2 (46). Notably, Camolotto et al. reported using a GEMM 'loss-of-function' model that ubiquitous PGK-Cre-mediated co-deletion of *Foxa1* and *Foxa2* suppressed growth of autochthonous KRAS-mutant primary lung tumors (47), which is in part consistent with our 'gain-of-function' results that FOXA2 promotes the growth of KRAS mutant-lung tumors (Fig. 1). Of note, FOXA1 also significantly induced the expression of *SPDEF*, *MUC5AC* and *MUC5B* in H441 cells. Interestingly, FOXA1 was a major inducer of *MUC5B* while FOXA2 was a major inducer of *MUC5AC* (Supplementary Fig. S13 and Table S14), suggesting that co-induction of FOXA1 and FOXA2 along with mutant KRAS may further drive invasive mucinous adenocarcinoma of the lung.

FOXA2 has also been shown to be a pro-mucous transcription factor or anti-mucous transcription factor depending on the context. Conditional co-deletion of *Foxa1* and *Foxa2* in developing (non-tumorigenic) mouse gastrointestinal (GI) tract reduced goblet (mucous) cell differentiation accompanied by the decreased expression of *Muc2* and the increased expression of *Muc5ac* (48); however, conditional deletion of *Foxa2* in asthmatic (non-tumorigenic) mouse lung induced goblet (mucous) differentiation accompanied by the increased expression of *Muc5ac* (25). These previous studies indicate that FOXA2 suppresses goblet (mucous) cell differentiation in part by repressing the expression of MUC5AC in non-tumorigenic lung. However, *in vitro* loss-of-function studies by us

(Supplementary Fig. S6A, S6B and Table S8) and others (42) in A549 human lung carcinoma cells (*KRAS*$^{G12S}$;*CDKN2A*$^{del}$) indicated that knockdown of FOXA2 reduced the expression of mucous genes, including *SPDEF*, *MUC5AC* and *MUC5B*. Consistent with these *in vitro* loss-of-function studies, our present *in vivo* and *in vitro* gain-of-function study by ectopic expression of *Foxa2* in the presence of mutant *Kras* in the adult GEMM, H441 human lung papillary adenocarcinoma cells (*KRAS*$^{G12V}$; *TP53*$^{R158L}$) and BEAS-2B human transformed bronchial epithelial cells (transformed by adenovirus-12 SV40 hybrid virus) indicated that FOXA2 in the presence of mutant KRAS induced tumorigenic goblet (mucous) cells accompanied by the expression of IMA-related genes, including *MUC5AC* and *MUC5B*, in mice and in human cells (Fig. 2, 3, 5–8). For therapeutic interest for IMA, IL23A is a potential target gene among the FOXA2-induced IMA genes since IL23A-IL23R pathway is therapeutically targetable by antibody (49). Further studies are required to elucidate the role of FOXA2 in a different context in normal and diseased lungs (e.g., lung cancer with different KRAS or EGFR mutations, NRG fusions or non-tumorigenic chronic diseased lungs; Supplementary Fig. S12A–S12D).

Since FOXA1 and FOXA2 can bind both nucleosome-bound and nucleosome-free DNA targets, FOXA1 and FOXA2 are considered to be pioneer transcription factors that allow the subsequent binding of other transcription factors at nearby sites (50). In our study, the mRNA expression of *MUC5AC* and *MUC5B* primed by FOXA2 was further induced by mutant KRAS in BEAS-2B bronchial epithelial cells; however mutant KRAS alone did not prime the expression (Fig. 7B). Our data demonstrate that ectopic expression of FOXA2 alone is sufficient to introduce the H3K27ac enhancer mark at the locus of *MUC5AC* (Fig. 7C), suggesting that FOXA2 not only binds both nucleosome-bound and nucleosome-free DNA targets but also recruits chromatin modifiers to mark gene-regulatory enhancers at the target loci. Most of the studies investigating the role of FOXA1 and FOXA2 as pioneer transcription factors have been performed using cells derived from liver, breast and prostate. FOXA2 may associate differently with chromatin in lung-originating cells; however, further studies looking at different histone modifications at loci of genes expressed in lung are required to understand the precise role of FOXA2 as a pioneer transcription factor and/or a recruiter for chromatin modifiers in the lung.

The association of FOXA2 expression in primary lung NSCLC with patients' survival has been assessed by immunohistochemistry using paraffin-embedded NSCLC samples (17) and analysis of mRNA-seq data from TCGA lung adenocarcinoma datasets (20). Although previous reports using lung cancer cell lines suggest that FOXA2 is a tumor suppressor especially in the context of metastasis (18–20), the mRNA or protein expression of FOXA2 in primary lung tumors does not significantly correlate with poor survival of such lung cancer patients (17, 20). Our present data suggests that FOXA2 is a context dependent tumor influencer driven by distinct oncogenes and tumor suppressors (e.g., *CDKN2A*, *TP53* and/or *STK11/LKB1*) (44) just as NKX2–1 also functions in a different manner depending on the presence of such oncogenes (e.g., mutant KRAS or mutant EGFR) (45). Assessing the expression of FOXA2 based on different driver oncogenes and tumor suppressors is required to understand whether FOXA2 influences survival in lung cancer patients. In addition, since FOXA2 suppresses itself in an auto-inhibitory fashion (Supplementary Fig. S4B and S4C),

assessing FOXA2 at the protein level might be more accurate than assessing FOXA2 at the mRNA level to discern the role of FOXA2 in patient survival.

In summary, using transgenic model mice that develop autochthonous lung tumors, we identified that FOXA2 suppresses EGFR-mutant lung tumors whereas FOXA2 promotes KRAS-mutant lung tumors. Notably, FOXA2 in the presence of mutant KRAS induces mucinous lung tumors *in vivo*. The expression of FOXA2 protein is also significantly associated with human IMA. In human lung adenocarcinoma cells, FOXA2 bound to the two enhancer regions at the *MUC5* (*MUC5AC* and *MUC5B*) locus and induced the expression of both *MUC5AC* and *MUC5B in vitro*. Notably, FOXA2 but not mutant KRAS introduced the H3K27ac enhancer histone mark to the upstream enhancer region of *MUC5AC*, which indicates that FOXA2 primes the expression of both *MUC5AC* and *MUC5B* through this enhancer (Supplementary Fig. S14). Our present study along with the previous studies by others indicates that FOXA2 promotes KRAS-mutant primary lung tumors and suppresses EGFR-mutant primary lung tumors; and may inhibit lung tumor metastasis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Significance

FOXA2 expression combined with mutant KRAS drives invasive mucinous adenocarcinoma of the lung by synergistically promoting a mucous transcriptional program, suggesting strategies for targeting this lung cancer type that lacks effective therapies.

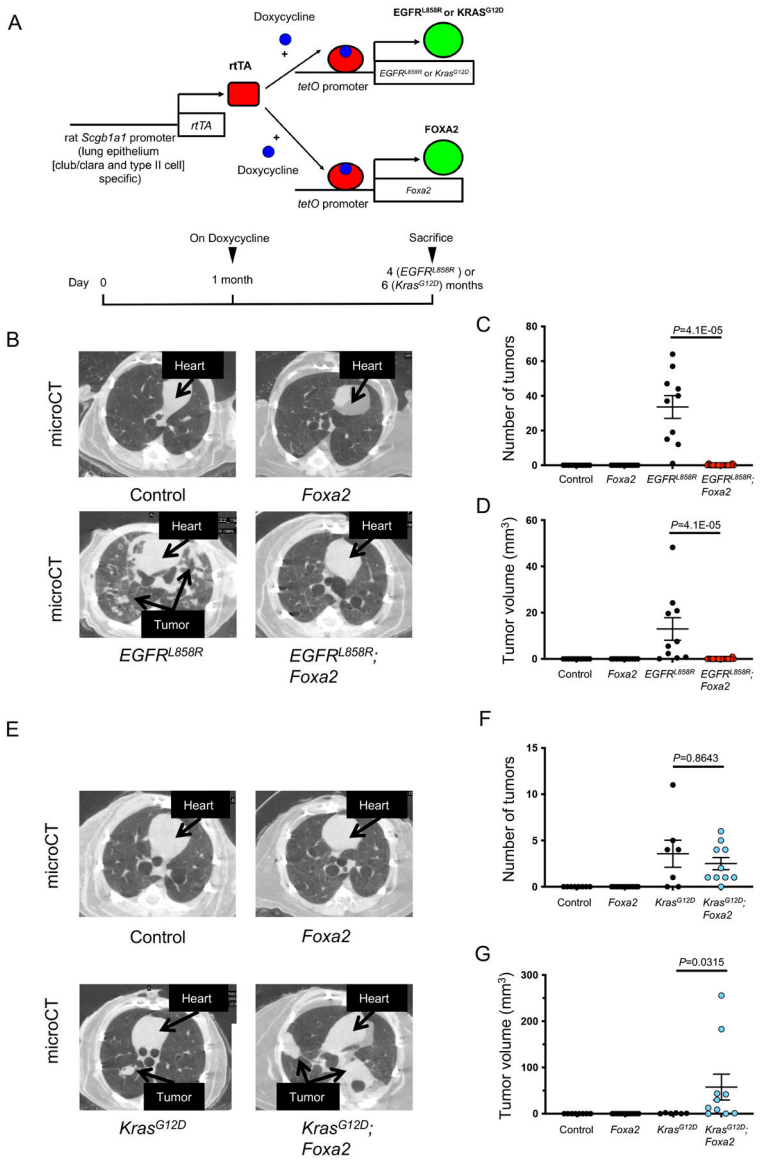


**Figure 1. *EGFR*$^{L858R}$-autochthonous lung tumors are suppressed by FOXA2 whereas *Kras*$^{G12D}$-lung autochthonous tumors are promoted by FOXA2 in mice.**

**A,** Upper panel: Schematic view of the transgenic mouse model that conditionally induces the expression of *EGFR*$^{L858R}$ or *Kras*$^{G12D}$ along with *Foxa2* in lung epithelial cells, including airway club cells and alveolar type 2 cells, using the tetO-inducible system upon doxycycline administration. Bottom panel, Timeline for doxycycline administration and sacrifice.

**B,** Representative microCT images detecting mouse lung tumors induced by EGFR$^{L858R}$ and/or FOXA2 that were conditionally induced in lung epithelium.

**C,** Shown are per mouse tumor number counted using microCT images ($n \geq 10$). FOXA2 significantly suppressed the number of lung tumors developed by EGFR$^{L858R}$ (Kolmogorov-Smirnov test).

**D,** Tumor volume per mouse measured using microCT images ($n \geq 10$) is shown. FOXA2 significantly suppressed the volume of lung tumors developed by $EGFR^{L858R}$ (Kolmogorov-Smirnov test).

**E,** MicroCT images detecting mouse lung tumors developed by $KRAS^{G12D}$ and/or FOXA2 that were conditionally induced in lung epithelium.

**F,** Per mouse tumor numbers counted using microCT images ($n \geq 6$) are shown. FOXA2 did not influence the number of lung tumors developed by $KRAS^{G12D}$ (Kolmogorov-Smirnov test).

**G,** Tumor volume per mouse measured using microCT images ($n \geq 6$) is shown. FOXA2 significantly induced the volume of lung tumors developed by $KRAS^{G12D}$ (Kolmogorov-Smirnov test).

Data are presented as mean +/− SEM.

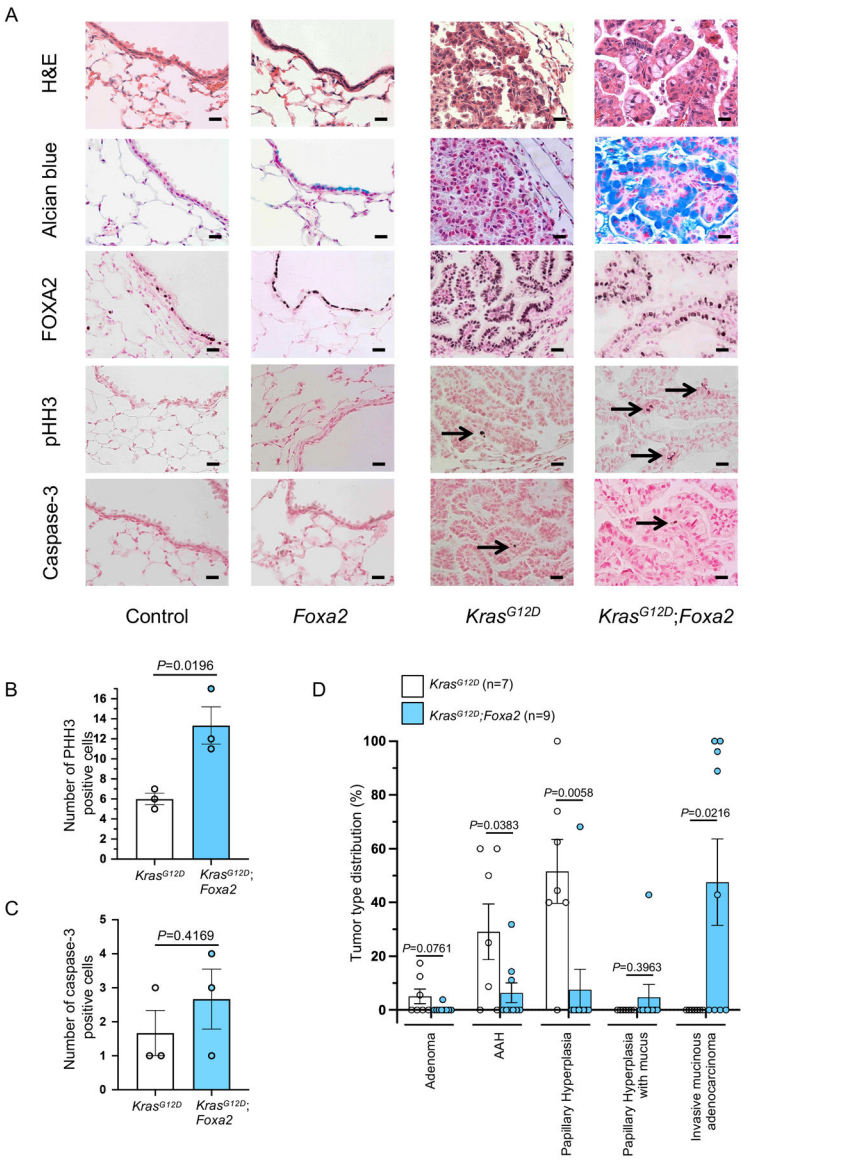


**Figure 2. FOXA2 promotes the proliferation of KRAS$^{G12D}$-lung tumor cells in mice.**

**A,** Analysis of histology and immunohistochemistry on mouse lungs that conditionally expressed FOXA2 and/or KRAS$^{G12D}$ was performed. Alcian blue staining was performed to detect mucins. Staining of PHH3 (phosphohistone H3; a marker for proliferation) and caspase-3 (a marker for apoptosis) was performed to assess whether FOXA2 influenced proliferation and apoptosis of KRAS$^{G12D}$-lung tumors. Black dots in the nuclei indicate the expression of FOXA2. H&E, hematoxylin and eosin. X-axis indicates mouse genotype. Scale bar: 20 μm.

**B,** Number of PHH3 positive cells in lungs of different mouse genotypes was counted in 3 different views per section per mouse. Number of PHH3 positive cells was significantly increased in lungs of *Kras$^{G12D}$;Foxa2* mice compared to those of *Kras$^{G12D}$* mice ($n = 3$ mice).

**C,** Number of caspase-3 positive cells in lungs of different mouse genotypes was counted in 3 different views per section per mouse. Caspase-3 (a marker for apoptosis) was not altered in lungs of different mouse genotypes ($n = 3$ mice).

**D,** Differences in histology of mouse lung tumors developed by $KRAS^{G12D}$ and/or FOXA2 were assessed. Invasive mucinous adenocarcinoma was significantly induced by FOXA2 in the presence of $Kras^{G12D}$ ($n \geq 9$). AAH: Atypical adenomatous hyperplasia.

All tests are unpaired, two-tailed Student's *t*-tests. Data are presented as mean +/− SEM.

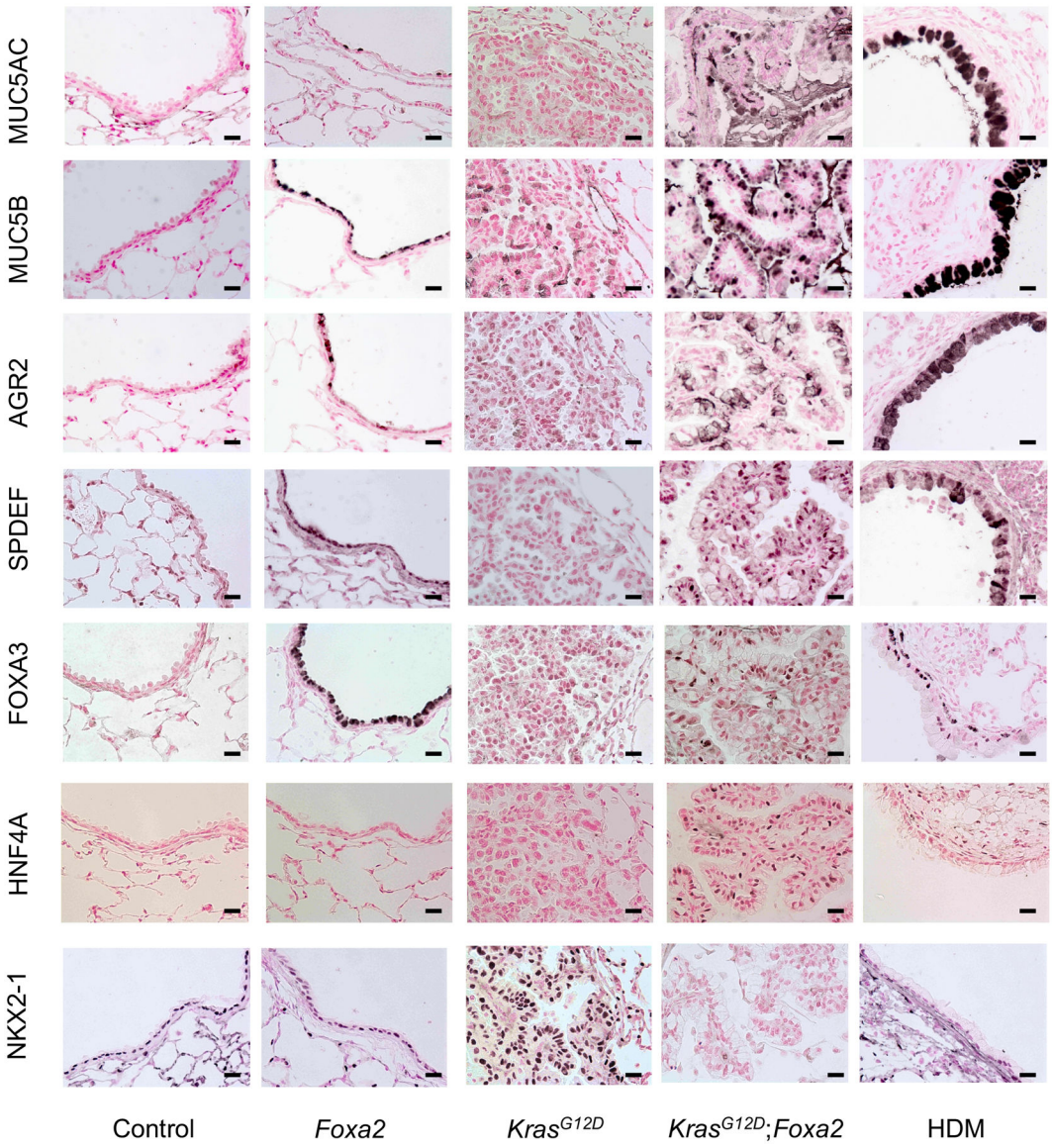


**Figure 3. FOXA2 together with mutant KRAS induces mucinous lung tumors in mice.**

Immunohistochemistry detecting mucous genes in mouse lungs conditionally induced to express FOXA2 and/or $KRAS^{G12D}$ was performed as described in Materials and Methods. HDM (house dust mite)-challenged mouse lungs (an asthma model) are used as a reference for non-tumorigenic lungs that produce mucus. Co-expression of FOXA2 and $KRAS^{G12D}$ induced mucinous lung tumors accompanied by the expression of markers for human invasive mucinous adenocarcinoma of the lung (IMA). X-axis indicates mouse genotype. Scale bar: 20 μm.

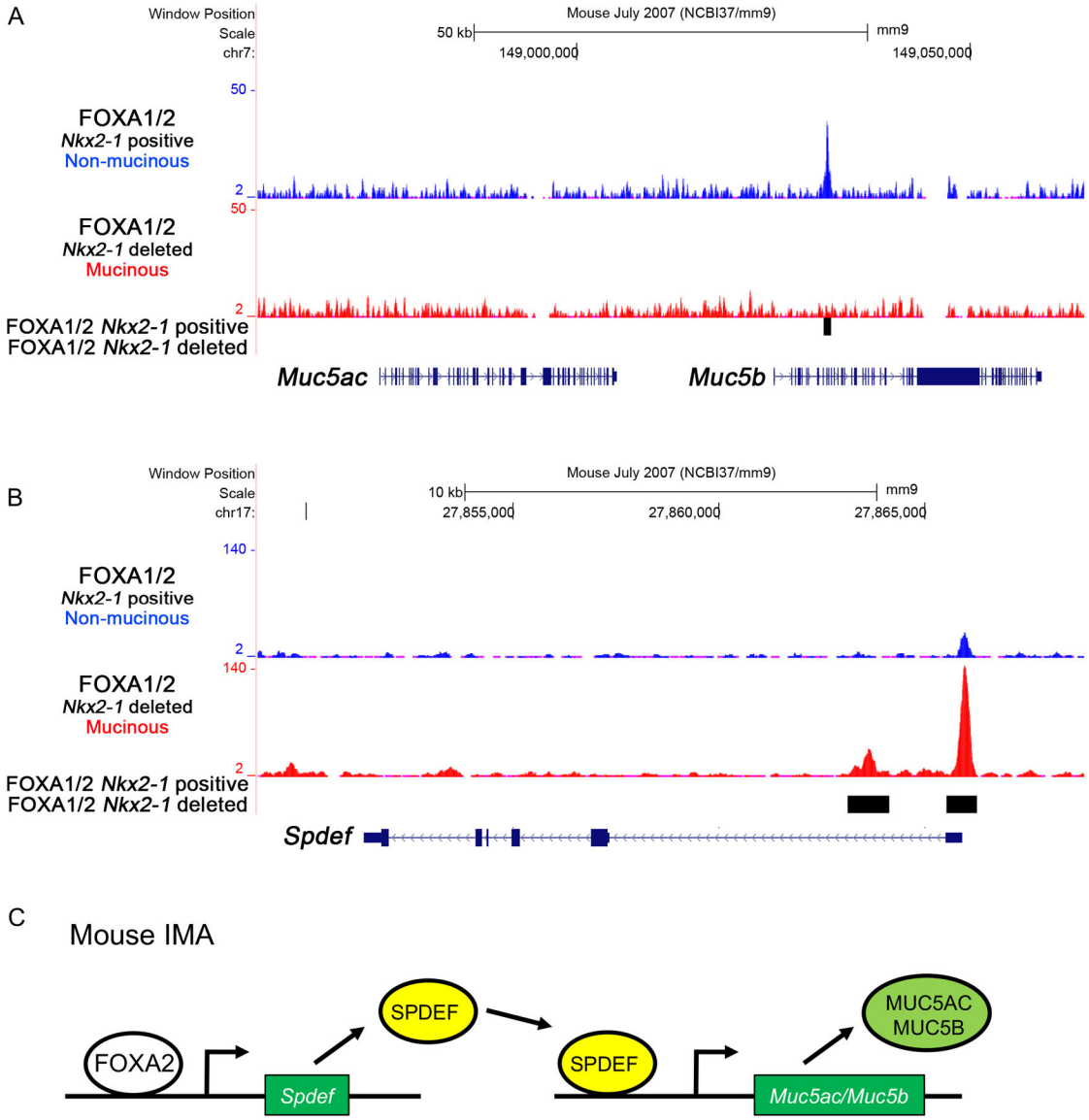


**Figure 4. FOXA2 binds to the locus of *Spdef* in mucinous lung tumors in mice.**

**A,** ChIP-seq data (15) using an antibody that recognizes FOXA1 and FOXA2 (FOXA1/2) indicated that FOXA1 and FOXA2 bound to the locus of *Muc5b* in non-mucinous *Kras*$^{G12D}$-mouse lung tumors (*Nkx2-1* positive) but not in the mucinous *Kras*$^{G12D}$-mouse lung tumors (*Nkx2-1* deleted).

**B,** ChIP-seq data using the antibody described above indicated that FOXA1 and FOXA2 bound to the upstream and intronic regions of *Spdef* gene in mucinous *Kras*$^{G12D}$-mouse lung tumors (*Nkx2-1* deleted) but not in non-mucinous *Kras*$^{G12D}$-mouse lung tumors (*Nkx2-1* positive).

**C,** Schematic indicates a proposed model of the mechanism by which FOXA2 induces two major IMA mucin genes *MUC5AC* and *MUC5B* in mice through induction of the mucus-transcription factor SPDEF.

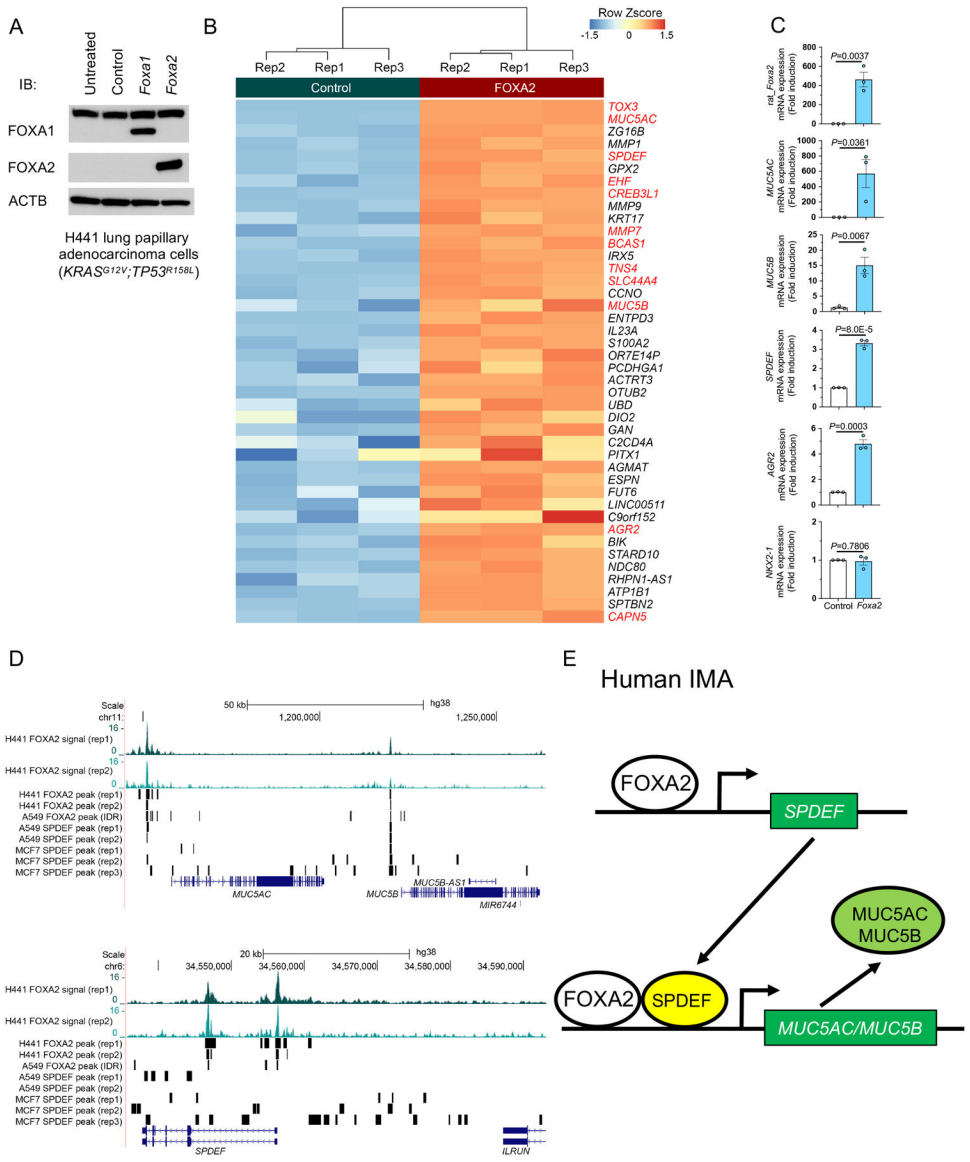


**Figure 5. FOXA2 binds to the loci of mucous genes (e.g., *MUC5AC* and *SPDEF)* and induces their transcriptional expression in human lung adenocarcinoma cells.**

**A,** Immunoblotting (IB) was performed using antibodies against FOXA1 or FOXA2. H441 cells were infected with a lentiviral vector carrying mouse *Foxa1* or rat *Foxa2* and cell extracts were used for IB. Control, an empty lentiviral vector. Shown are representative images from three independent experiments.

**B,** RNA-seq was performed using RNAs from H441 cells expressing ectopic FOXA2 as described in A ($n = 3$). Control, an empty lentiviral vector. Shown are FOXA2-induced genes that are highly expressed in human invasive mucinous adenocarcinoma of the lung (IMA). Red indicates genes that are also significantly expressed in mouse IMA.

**C,** TaqMan gene expression analysis was performed using RNAs as described in B. Results are expressed as mean +/− SEM of biological replicates for each group. Control, an empty lentiviral vector. $P < 0.05$ versus control was considered significant ($n = 3$, unpaired, two-tailed Student's *t*-test). Gene expression was normalized by comparison with the constitutive

expression of *GAPDH*. The expression of selected mucous genes identified by the RNA-seq in B was confirmed. Of note, the expression of ectopic rat *Foxa2* was induced as expected while the endogenous human *FOXA2* was reduced (See Supplementary Fig. S4B). The expression of anti-mucous gene NKX2–1 was not altered by ectopic rat *Foxa2*.

**D,** Shown is the combined analysis of ChIP-seq datasets. Each genome browser (*MUC5AC*, *MUC5B* loci and *SPDEF* locus) indicates bam files demonstrating FOXA2 binding in H441 cells at the top panel and bed files indicating FOXA2 or SPDEF binding in H441 (lung papillary adenocarcinoma), A549 (lung carcinoma) and MCF7 (breast adenocarcinoma) cells at the bottom panel.

**E,** Schematic indicates a proposed model of the mechanism by which FOXA2 induces two IMA major mucin genes *MUC5AC* and *MUC5B* in humans directly and indirectly through the induction of the mucus-transcription factor SPDEF.

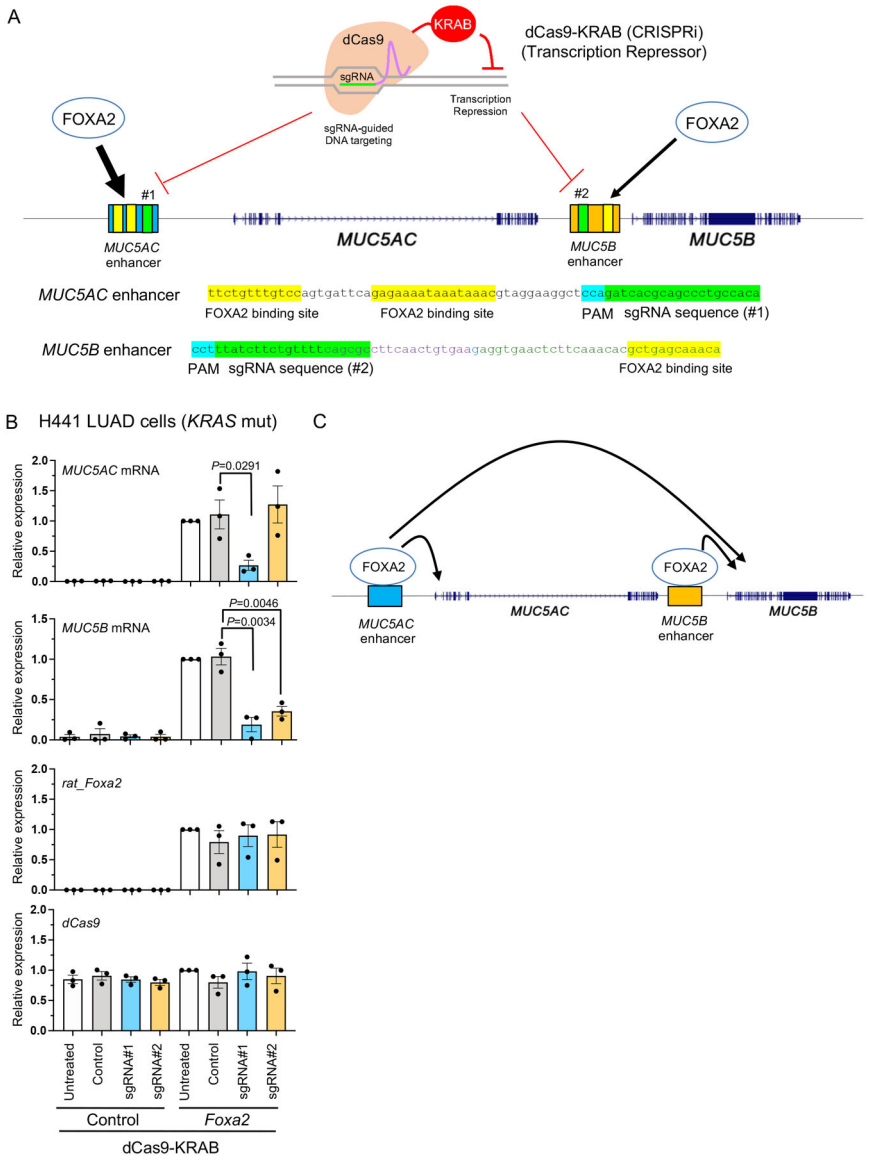


**Figure 6. FOXA2 induces the expression of both *MUC5AC* and *MUC5B* through the upstream enhancer regions of *MUC5AC*.**

**A,** Upper panel indicates two enhancer regions at the upstream region (#1) of *MUC5AC* and at the upstream region (#2) of *MUC5B* (between *MUC5AC* and *MUC5B*) that are targeted by CRISPRi, in which dCas9 (deactivated Cas9)-KRAB repressor is recruited to a region (#1 or #2) selected by sgRNA sequences (green highlighted) located at presumed FOXA2 binding sites (yellow highlighted). Lower panel indicates sgRNA target sequences (green) and adjacent PAM sequence (blue) and presumed FOXA2 binding sequences (yellow). **B,** TaqMan gene expression analysis indicates that a synthetic sgRNA targeting a region at the upstream region (#1) of *MUC5AC* significantly represses the FOXA2-mediated induction of *MUC5AC* and *MUC5B* in H441 KRAS-mutant lung adenocarcinoma cells that stably express dCas9-KRAB along with FOXA2 (Control is an empty vector). A synthetic sgRNA targeting an upstream region (#2) of *MUC5B* significantly represses the FOXA2-mediated induction of *MUC5B* but not that of *MUC5AC*. Non-targeted synthetic sgRNA

is used as control for sgRNAs targeting the loci of *MUC5AC* or *MUC5B*. Results are expressed as mean +/− SEM of 3 biological replicates for each group (Unpaired, two-tailed Student's *t*-test).

**C,** Schematic indicates a proposed model of the mechanism by which FOXA2 induces two IMA major mucin genes *MUC5AC* and *MUC5B* in human lung cancer cells through the upstream region of *MUC5AC* (*MUC5AC* enhancer) and the intergenic region between *MUC5AC* and *MUC5B* (*MUC5B* enhancer).

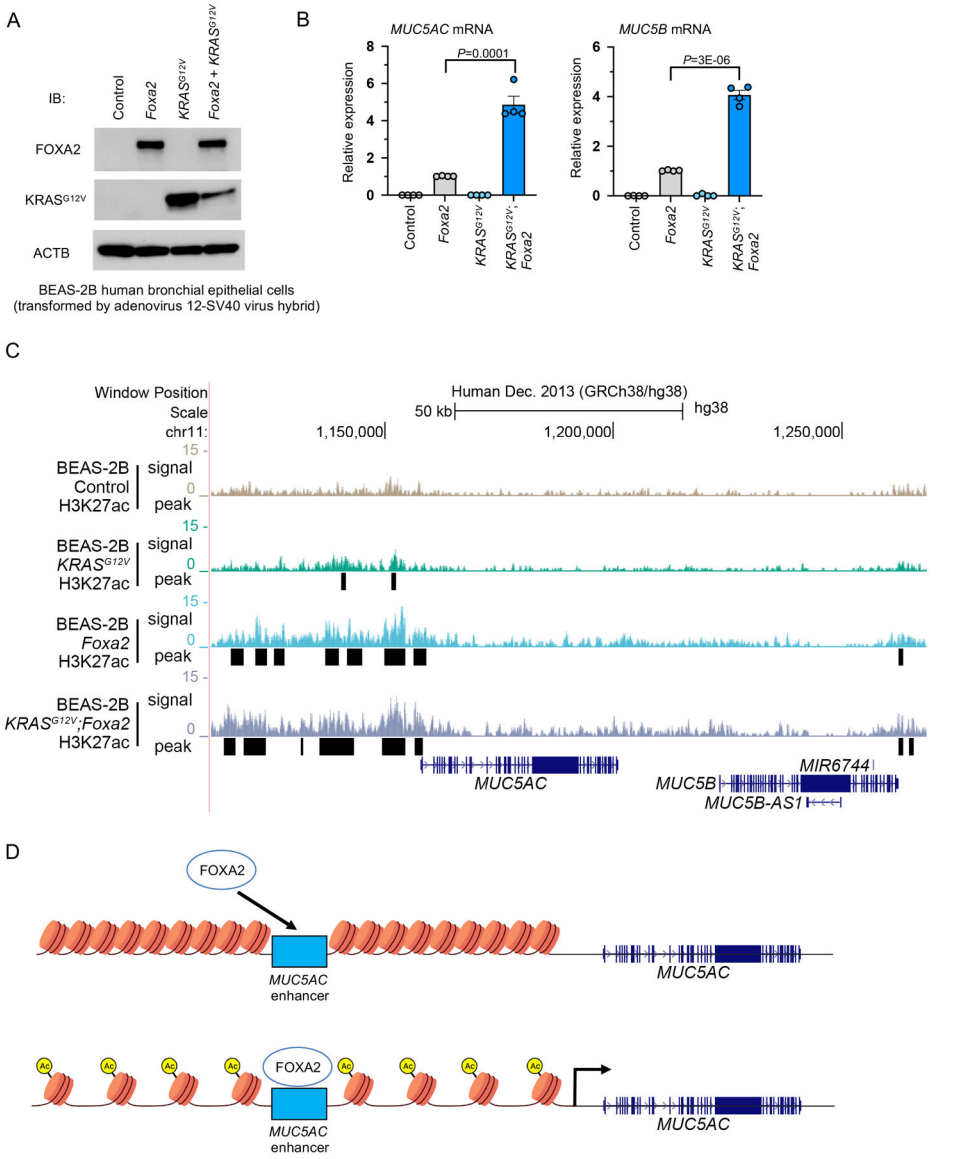


**Figure 7. Mutant KRAS amplifies the expression of *MUC5AC* that is primed by FOXA2.**

**A,** Immunoblotting (IB) was performed using antibodies against FOXA2 or KRAS$^{G12V}$. BEAS-2B human transformed bronchial epithelial cells were infected with a lentiviral vector carrying rat *Foxa2* and/or a retroviral vector carrying human *KRAS$^{G12V}$* and cell extracts were used for IB. Controls are empty lentiviral and retroviral vectors. Shown are representative images from four independent experiments.

**B,** TaqMan gene expression analysis indicates that the expression of *MUC5AC* and *MUC5B* is primed by FOXA2 and enhanced by a mutant KRAS (KRAS$^{G12V}$). BEAS-2B cells were infected with a lentiviral vector carrying rat *Foxa2* and/or a retroviral vector carrying human *KRAS$^{G12V}$* and RNA was extracted for analysis. Results are expressed as mean +/− SEM of 4 biological replicates for each group. Controls are empty lentiviral and retroviral vectors. $P < 0.05$ versus control was considered significant (Student's *t*-test). Gene expression was

normalized by comparison with the constitutive expression of *GAPDH* (P values are from unpaired, two-tailed Student's *t*-tests).

**C,** ChIP-seq using an antibody against H3K27ac (an enhancer mark of histone) was performed using BEAS-2B cells that ectopically expressed FOXA2 and/or $KRAS^{G12V}$ as described in A. Shown is the genome browser demonstrating H3K27ac gain compared to control at the *MUC5AC* locus in BEAS-2B cells expressing FOXA2 or $KRAS^{G12V}$;FOXA2 but not $KRAS^{G12V}$ alone.

**D,** Schematic indicates a proposed model of the mechanism by which FOXA2 induces two IMA major mucin genes *MUC5AC* and *MUC5B* in human lung cancer cells by initiating H3K27ac enhancer marks at the upstream region of *MUC5AC*. The figure drawings were created in part with BioRender.com.

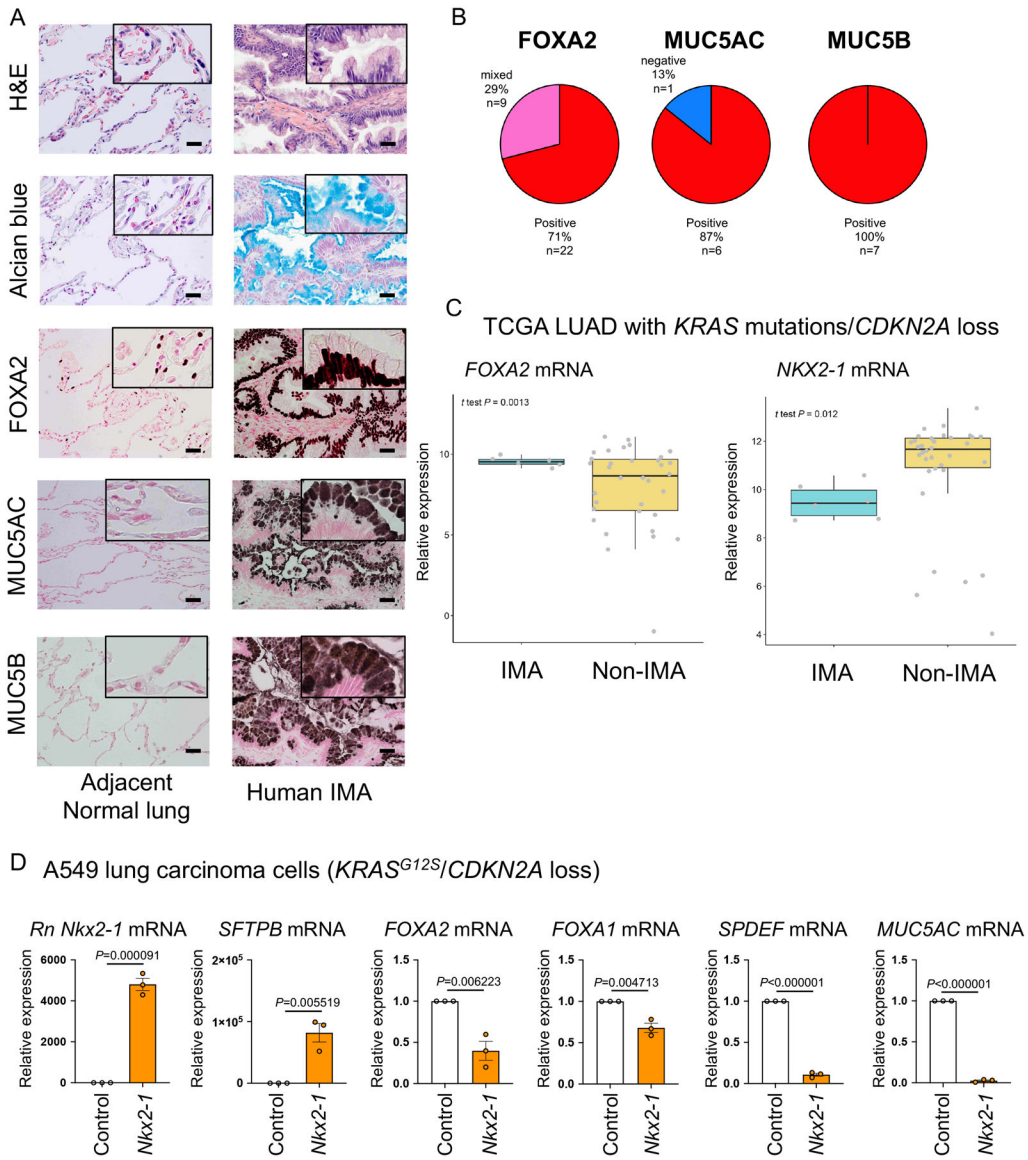


**Figure 8. FOXA2 is highly expressed in human invasive mucinous adenocarcinoma of the lung (IMA).**

**A,** Immunohistochemistry was performed using human IMA and adjacent normal lung tissues. FOXA2 expressed in the nucleus was induced in mucinous tumor cells (right panels) compared to normal lung epithelial cells (left panels). MUC5AC and MUC5B expressed in cytoplasm and secreted in lumen was also induced in mucinous tumor cells (right panels) compared to normal lung epithelial cells (left panels). H&E, hematoxylin and eosin. Alcian blue staining detects mucins. Scale bar: 20 μm.

**B,** Pie charts indicate the expression ratio of FOXA2, MUC5AC or MUC5B in human IMA. FOXA2 was expressed in the majority of human IMA. Mixed indicates that the expression of FOXA2 in IMA was less than 50% (See Supplementary Table S5).

**C,** Normalized and log2-transformed mRNA expression of *FOXA2* (left panel) and *NKX2-1* (right panel) in IMA or Non-IMA in TCGA LUAD with *KRAS* mutations/*CDKN2A*

loss co-occurring genomic alteration group is shown. $P < 0.05$ was considered significant (Unpaired, two-tailed Welch's *t*-test).

**D,** TaqMan gene expression analysis was performed using RNAs from A549 cells infected with a lentiviral vector carrying rat *Nkx2-1*. Control, an empty lentiviral vector. Results are expressed as mean +/− SEM of 3 biological replicates for each group. Gene expression was normalized by comparison with the constitutive expression of *GAPDH*. $P < 0.05$ was considered significant (Unpaired, two-tailed Student's *t*-test).