

## **STXBP4 drives tumor growth and is associated with poor prognosis through PDGF Receptor signaling in lung squamous cell carcinoma**

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## **Abstract**

**Purpose:** Expression of the  $\Delta N$  isoform of p63 ( $\Delta Np63$ ) is a diagnostic marker highly specific for lung squamous cell carcinoma (SCC). We previously found that Syntaxin Binding Protein 4 (STXBP4) regulates  $\Delta Np63$  ubiquitination, suggesting that STXBP4 may also be a SCC biomarker. To address this issue, we investigated the role of STXBP4 expression in SCC biology and the impact of STXBP4 expression on SCC prognosis.

**Experimental design:** We carried out a clinicopathological analysis of STXBP4 expression in 87 lung SCC patients. Whole transcriptome analysis using RNA-seq was performed in STXBP4-positive and STXBP4-negative tumors of lung SCC. Soft agar assay and xenograft assay were performed using overexpressing or knockdown SCC cells.

**Results:** Significantly higher levels of STXBP4 expression were correlated with accumulations of  $\Delta Np63$  in clinical lung SCC specimens (Spearman's rank correlation  $\rho=0.219$ ). Notably, STXBP4-positive tumors correlated with three important clinical parameters: T factor ( $P<0.001$ ), disease stage ( $P=0.030$ ) and pleural involvement ( $P=0.028$ ). Whole transcriptome sequencing followed by pathway analysis indicated that STXBP4 is involved in functional gene networks that regulate cell growth, proliferation, cell death and survival in cancer. Platelet-Derived Growth Factor Receptor alpha (PDGFR $\alpha$ ) was a key downstream mediator of STXBP4 function. In line with this, shRNA mediated *STXBP4* and *PDGFRA* knockdown suppressed tumor growth in soft agar and xenograft assays.

Conclusions: STXBP4 plays a crucial role in driving SCC growth and is an independent prognostic factor for predicting worse outcome in lung SCC. These data suggest that STXBP4 is a relevant therapeutic target for patients with lung SCC.

### **Translational Relevance**

$\Delta$ Np63 is a diagnostic marker highly specific for lung squamous cell carcinoma (SCC), but the regulation of p63 protein stability and the pathologic relevance of p63 in tumorigenesis remains unclear. We report here for the first time that Syntaxin Binding Protein 4 (STXBP4) expression increases the oncogenic potential of  $\Delta$ Np63, and is STXBP4 an independent negative prognostic marker for predicting poor outcome in lung SCC. Transcriptional analysis (RNA-seq) using Next Generation Sequencing in STXBP4-positive and STXBP4-negative lung SCC indicated that Platelet-Derived Growth Factor Receptor  $\alpha$  (PDGFR $\alpha$ ) is a key downstream mediator of STXBP4 function. The data suggest that STXBP4 is a new diagnostic marker in lung SCC, and STXBP4 might be a relevant therapeutic target for the treatment of patients with this disease.

## Introduction

Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all cases of lung cancer, and is mainly sub-classified into adenocarcinoma (AC) and squamous cell carcinoma (SCC) (1). Current treatment strategies for NSCLC include chemotherapy, depending on the histological tumor type, and targeted agents for patients whose tumors carry a specific targetable genomic alteration. Although there have been significant advances in the treatment of lung SCC, further improvements in prognosis are dependent upon the identification of SCC specific molecules or genomic alterations that can be used as therapeutic biomarkers and/or targets (2).

Several immunohistochemical markers have been investigated for their utility in distinguishing lung SCC from lung AC, including TTF-1, napsin A and CK5/6 and the  $\Delta N$  isoform of p63 ( $\Delta Np63$ ) (3-5). The latter is a highly specific marker for lung SCC and genomic regions containing the *TP63* gene are frequently amplified in a variety of SCCs, including lung, head and neck, bladder and cervical cancers (4, 6-8). Although these findings suggest that  $\Delta Np63$  is a lung SCC oncogene, the pathologic relevance of p63 in tumorigenesis remains unclear (9, 10).

Alternative splicing of the *TP63* gene generates transcripts encoding two opposing classes of proteins: one containing the transactivation domain (TAp63) and the

other lacking the domain ( $\Delta$ Np63) (11-13). Early studies showed that  $\Delta$ Np63 acts as a dominant-negative transcriptional repressor to inhibit p53- or TAp63-mediated transcription *in vitro* and *in vivo*, consistent with a potential oncogenic role for the  $\Delta$ Np63 isoform (12, 14). However, the  $\Delta$ Np63 isoform also has transcriptional activity that is independent of the second transactivation domain (15).

$\Delta$ Np63 is regulated in a coordinated manner by two scaffold proteins, Syntaxin Binding Protein 4 (STXBP4) and Receptor of activated kinase C1 (RACK1; encoded by the *GNB2L1*), which bind to  $\Delta$ Np63 (16, 17). STXBP4, originally identified as a glucose transporter, is localized on human chromosome 17q22 and plays a role in the translocation of transport vesicles from the cytoplasm to the plasma membrane (18, 19). While  $\Delta$ Np63 plays a role in maintaining the viability and proliferative capacity of basal epithelial cells, STXBP4 is a positive regulator of  $\Delta$ Np63 stability and is also crucial for keratinocyte proliferation (16, 20).

In this report, we focused on STXBP4 and its oncogenic function in lung SCC, with a particular emphasis on the interactions between STXBP4 and p63. We also addressed the relevance of STXBP4 expression to patient prognosis. Initially, we assessed the expression of STXBP4 and  $\Delta$ Np63 in SCC tumors by immunohistochemistry, and found that positive STXBP4 expression signified worse

Overall Survival (OS) and Progression-Free Survival (PFS). We further performed a genome-wide transcriptome analysis (RNA-seq) using Next Generation Sequencing (NGS) and found that Platelet-Derived Growth Factor Receptor  $\alpha$  (PDGFR $\alpha$ ) was positively correlated with the expression of STXBP4. In line with this, shRNA mediated depletion of PDGFR $\alpha$  suppressed the growth of a lung SCC cell line in soft agar and xenograft tumor assays, similar to the findings obtained when the expression of *STXBP4* or  *$\Delta Np63$*  were knocked down. Taken together, our data address the physiological role and diagnostic potential of STXBP4 in lung SCC, and suggest that PDGFR $\alpha$  may be a key mediator of STXBP4-mediated oncogenic activity.

## **Materials and Methods**

### **Cell Culture**

The human lung SCC cell lines, RERF-LC-Sq1 and EBC-1 were obtained from the Japanese Collection of Research Bioresources (JCRB). The cell lines were last authenticated by short tandem repeat (STR) analysis on December 22, 2015 (RERF-LC-Sq1), or on June 10, 2016 (EBC-1). RERF-LC-Sq1 cells were cultured in RPMI1640 with 10% fetal bovine serum (FBS), and EBC-1 cells were cultured in Eagle's minimal essential medium (EMEM) with 10% FBS at 37°C in a 5% CO<sub>2</sub> incubator.

### **Patients**

Human tissue specimens were surgically resected from 87 lung SCC patients at Gunma University Hospital and its affiliated hospitals between August 2003 and December 2010 (21). The main eligibility criteria were as follows: age 20 to 85 years; performance status based on Eastern Cooperative Oncology Group  $\leq 2$ ; estimated life expectancy  $\geq 3$  months; adequate hepatic, cardiac, renal, and bone marrow functions. The study was conducted in compliance with the Declaration of Helsinki, and was approved by the Institutional Review Board of the participating hospitals and



institutions. All patients provided written informed consent before registration. Tumor samples were stored at  $-80^{\circ}\text{C}$  until use.

### **Immunohistochemistry**

Immunohistochemical analysis was performed on formalin-fixed and paraffin-embedded SCC sections. The sections were de-paraffinized, blocked in PBS containing 5% FBS for 1 hr, and incubated overnight with diluted primary antibodies at  $4^{\circ}\text{C}$  in a humidified chamber. Staining reactions were developed using Vectastain universal ABC Kit (Vector Laboratories, Burlingame, CA) and then DAB Kit (Vector Laboratories) for immunohistochemistry. Meyer's hematoxylin (IHC world, Woodstock, MD) was used as a nuclear counterstain. STXBP4, p63,  $\Delta\text{Np63}$  levels were assessed by immunohistochemical staining and scored using a semi-quantitative method: 1  $\leq$  10%, 2 = 10-25%, 3 = 25-50%, 4 = 51-75% and 5  $\geq$  75% of positive cells. The tumors in which the stained cancer cells were scored as 3, 4, or 5 were defined as STXBP4-positive; 1 and 2 were defined as STXBP4-negative.

We used antibodies specific for p63 (4A4) (Santa Cruz Biotechnology, Dallas, TX) and STXBP4 (Abcam, Cambridge, MA). Rabbit polyclonal  $\Delta\text{Np63}$  antibody was previously described (16). CD147 (Santa Cruz Biotechnology) and mTOR (Cell

Signaling Technology, Danvers, MA) immunohistochemical staining was performed according to the procedures described in a previous report (22). The following diluted antibodies were used: p63 (1:100 dilution);  $\Delta$ Np63 (1:100 dilution); STXBP4 (1:100 dilution); CD147 (1:100 dilution); mTOR (1:80 dilution). Highly cellular areas of the sections were evaluated for Ki-67 expression. All epithelial cells with nuclear staining of any intensity were defined as high expression. Approximately 1,000 nuclei were counted on each slide. Proliferative activity was assessed as the percentage of Ki-67-stained nuclei (Ki-67 labeling index) in the sample. The median value of the Ki-67 labeling index was evaluated, and tumor cells with greater than the median value were defined as high expressors. The sections were assessed using light microscopy in a blind fashion by at least two of the authors.

### **Plasmids and antisense oligonucleotides**

Human cDNAs encoding FLAG-tagged or HA-tagged  $\Delta$ Np63 $\alpha$ , STXBP4 and PDGFR $\alpha$  were cloned into the LPCX retroviral expression vector (Takara Bio, Shiga, Japan). The sequences of the above constructs were verified using DNA sequencing. For siRNA experiments, 19 nucleotide siRNA duplexes with 3'dTdT overhangs were synthesized by Dharmacon (GE Dharmacon, Lafayette, CO). The siRNA

oligonucleotide sequences for Luciferase control (LUC),  $\Delta$ Np63, STXBP4 and PDGFR $\alpha$ , are described in the Supplementary Information. For siRNA transfection, RERF-LC-Sq1 or EBC-1 cells were transfected with 50 nM siRNA using DharmaFECT 1 siRNA transfection reagent (GE Dharmacon) according to the manufacturer's instruction. For shRNA experiments, the shRNAs for Luciferase (LUC),  $\Delta$ Np63, STXBP4 and PDGFR $\alpha$  oligonucleotides were cloned into the pLKO.1 puro lentivirus expression vector between Age I site and Eco RI site. The sequences of the above constructs were verified using DNA sequencing. The target sequences of the shRNA oligonucleotides are described in Supplementary Information.

### **Immunoblotting analysis**

Immunoblotting analysis was performed as previously described (23). In short, cells were solubilized with lysis buffer (20 mM sodium phosphate [pH 7.0], 125 mM NaCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM sodium fluoride, 0.1 mM Na<sub>3</sub>VO<sub>4</sub> and 1 mM phenylmethylsulfonyl fluoride) supplemented with Complete protease inhibitor cocktail (Roche, Penzberg, Germany), and homogenized by passage through a 20G needle. The eluates were then concentrated and separated by SDS-PAGE. Transfer to nitrocellulose membranes and screening using rabbit polyclonal

antibodies for  $\Delta$ Np63 and STXBP4 were carried out as previously described (24). We used antibodies specific for p63 (4A4),  $\Delta$ Np63 $\alpha$ , STXBP4, Phospho-PDGFR $\alpha$  (Tyr849) (Cell Signaling Technology), PDGFR $\alpha$  (Abcam), phospho-p38MAPK (Thr180/Tyr182) (Cell Signaling Technology), p38MAPK (Cell Signaling Technology) and  $\beta$ -Actin (Sigma-Aldrich, St. Louis, MO).

### **Genome-wide transcriptome analysis (RNA-seq) and real-time RT-PCR**

Total RNA was prepared from surgically resected samples using a RNeasy Mini kit (Qiagen, Hilden, Germany) after homogenizing with Mixer Mill MM400 (Qiagen). RNA quality was assessed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). High quality RNA (RNA integrity numbers > 7.0) from six STXBP4-positive and six STXBP4-negative samples were used for genome-wide transcriptome analysis (RNA-seq experiments). mRNAs were captured using a Dynabeads mRNA DIRECT Micro Purification Kit (Thermo Fisher Scientific, Waltham, MA). The mRNA was then used to generate sequencing libraries of barcoded fragments using the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) following the manufacturer's instructions. Libraries were sequenced on an Ion Proton System using four libraries per Ion PI Chip v2, Ion PI Template OT2 200 kit v3 and Ion PI

Sequencing 200 kit v3 (Thermo Fisher Scientific). BAM files generated by the Ion Proton System were converted to FASTQ files using bam2fastq software (v1.1.0, <https://gsl.hudsonalpha.org/information/software/bam2fastq>), and reads shorter than 21 nucleotides were removed. Quantitation of each gene was undertaken as previously described (25). Briefly, the reads were aligned to the UCSC reference human genome 19 (hg19) using a combination of Tophat2 (v2.0.11, <http://ccb.jhu.edu/software/tophat/index.shtml>), and the Bowtie2 (2.2.2.0, <http://bowtie-bio.sourceforge.net/index.shtml>) pipelines. The read counts were obtained using Partek Genomics Suite software (<http://www.partek.com/>). Differentially expressed genes were detected using edgeR software (26) and genes with a FDR < 0.50 ( $p < 0.01$ ) were analyzed by Ingenuity Pathway Analysis (Qiagen).

For real-time RT-PCR, relative RNA quantities were measured by Universal Probe Library set (Roche) with KAPA Master mix (KAPA Biosystems, Wilmington, MA) on a StepOne real-time PCR system (Thermo Fisher Scientific). The Universal Probe Library Human *ACTB* Gene Assay (Roche) was used for an endogenous normalization control. Sequence detection software was utilized for data analysis, and relative fold induction was determined by the comparative threshold cycle method using standard curves, which were generated by plotting the observed Ct values against the

standard dilutions of a positive control sample. In all experiments, the average of three independent reactions is shown with error bars indicating standard deviation. Gene expression data were downloaded from the Gene Expression Omnibus database (GSE84339).

### **Subcutaneous xenografts**

A total of  $5 \times 10^6$  lentivirally transduced or retrovirally expressed cells were injected subcutaneously into nude mice (BALB/c-nu/nu, CLEA Japan, Tokyo, Japan) and tumor size was measured after 20 days (RERF-LC-Sq1) or 14 days (EBC-1). All animal procedures were performed with the approval of the Animal Ethics Committee of Gunma University.

### **Anchorage-independent growth**

RERF-LC-Sq1 cells were transduced with lentiviruses carrying shRNAs for Luciferase (LUC),  $\Delta$ Np63, STXBP4 or PDGFR $\alpha$ . For soft agar assays, the cells were grown in triplicate for 12 days, after which anchorage-independent growth was quantified with a CytoSelect-96 kit (Cell Biolabs, San Diego, CA).

### **Statistical analysis**

Probability values ( $P$ -value)  $< 0.05$  indicated a statistically significant difference. Fisher's exact test was used to examine the association of two categorical variables. The correlation between different variables was analyzed using the nonparametric Spearman's rank test. Follow-up for the 87 patients was conducted using the patient medical records. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. The day of surgery was defined as the starting day for measuring postoperative survival. OS was determined as the time from tumor resection to death from any cause. PFS was defined as the time between tumor resection and first disease progression or death. Multivariate analyses were performed using a stepwise Cox proportional hazards model to identify independent prognostic factors. Statistical analysis was performed using JMP 8 (SAS) software.

## Results

### Survival outcomes according to STXBP4 and p63 expression

The clinicopathological features of the 87 patients included in this study are shown in Table 1. The median age of the patients was 72 (range 56 to 84), the majority of patients were male (92.0 %), and former or current smokers (98.9 %). All patients received radical surgery with evidence of pathological stage IA/B in 54.0 %, stage IIA/B in 26.4 %, and stage IIIA in 18.4 % of patients. Pleural involvement, lymphatic permeation and venous invasion were observed in 41 patients (47.1%), 47 patients (54.0%), and 40 patients (46.0%), respectively.

Frequently, lung SCCs exhibit simultaneous up-regulation of both TAp63 and  $\Delta$ Np63, and  $\Delta$ Np63 in particular, is a putative diagnostic marker for pulmonary SCC (10). To address the clinical significance of STXBP4 expression, we investigated whether high expression of this gene correlates with  $\Delta$ Np63 status. We found that 59.8 % (52/87) of all patients were STXBP4-positive, and STXBP4 expression was detected in those tumors that showed an accumulation of p63 (Fig. 1A).

Statistical correlation analysis between STXBP4 expression and clinicopathological features revealed that pathological local tumor factor stage (Disease stage), pathological tumor-node-metastasis (TNM) stage, and pleural involvement as a



local invasion factor, were correlated with STXBP4-positivity (Table 1). On the other hand, age, gender, and pathological differentiation were not correlated with STXBP4 expression. The expression of  $\Delta$ Np63 was significantly correlated with age and differentiation status, but not with clinical stage of disease. Remarkably, patient survival was significantly associated with T factor, disease stage, pleural involvement, and STXBP4 expression, as assessed by univariate analysis. Multivariate analysis confirmed that STXBP4 expression and disease stage were independent prognostic factors in lung SCC patients with poor OS and PFS (Table 2).

Kaplan-Meier analysis of OS and PFS according to STXBP4 expression revealed a statistically significant difference in OS and PFS between the patients who were STXBP4-positive compared with those who were STXBP4-negative (Fig. 1B and 1C). The five-year survival rate and median survival time for all patients were 50.2 % and 38.3 months (0.75 to 111.5 months), respectively. The median PFS and OS (21.2 months versus 52.2 months;  $P < 0.05$ ) were shorter in STXBP4-positive patients compared with STXBP4-negative patients (Fig. 1B and 1C). STXBP4-positive patients showed poor OS (log-rank  $P < 0.01$ ) and PFS (log-rank  $P < 0.01$ ) compared with those with STXBP4-negative patients. Interestingly, STXBP4 levels significantly predicted outcome in patients with tumors expressing high  $\Delta$ Np63 levels (Supplementary Fig. 1A

and 1B), while the complementary analysis showed that  $\Delta$ Np63 levels did not significantly predict OS ( $P = 0.35$ ) and PFS ( $P = 0.54$ ) in the STXBP4 high-expressing group (Supplementary Fig. 1C and 1D). Thus, these results indicate that STXBP4 could be an independent prognostic marker for predicting poor outcome in lung SCC.

We observed significantly higher levels of STXBP4 expression in those tumors that showed an accumulation of  $\Delta$ Np63 (Spearman's  $\rho = 0.219$ ;  $P < 0.05$ ), while among all p63 isoforms, no significant correlations were observed ( $P > 0.5$ ) (Fig. 1D). Interestingly, high mammalian target of rapamycin (mTOR), a major controller of growth and is often deregulated in cancer (27), was significantly correlated with STXBP4-positivity (Spearman's  $\rho = 0.220$ ;  $P < 0.05$ ), while other tumor markers, including CD147, a member of the immunoglobulin superfamily involved in angiogenesis ( $P < 0.5$ ), and Ki-67, a general marker for cell division ( $P < 0.1$ ), were not significantly correlated (Fig. 1D).

### **Transcriptional profiling and functional screening to identify possible downstream mediators of STXBP4**

Hierarchical cluster analysis after alignment of a total of 15,346 genes to the reference sequence, showed that STXBP4-positive and STXBP4-negative tumors had

distinctly different gene expression profiles (Fig. 2A). Among the differentially expressed genes ( $P$ -value  $< 0.05$ , False Discovery Rate (FDR)  $< 0.5$ ), we identified a total of 172 genes that were either significantly up-regulated (79 genes) or down-regulated (93 genes) in the STXBP4-positive tumors. These candidate genes potentially represent a network involved in STXBP4-mediated biology (Fig. 2B). To address this possibility in more detail, we carried out Ingenuity Pathway Analysis (IPA), which revealed that more than 30% of the affected genes were classified in the functional class of “Cell Death and Survival”. This finding supported our experimental observations that STXBP4 could be linked to the poor prognosis of lung SCC (Fig. 2C).

Additionally, other significant functional classes identified by IPA, including “Cellular Movement” and “Cell to Cell Signaling and Interaction”, may be relevant to the correlation of STXBP4-positivity with local tumor progression related to local tumor size (T factor) and disease stage (Fig. 2C). The canonical pathway analysis characterized two signaling pathways as the functional relationship of STXBP4-positivity. “Cellular Movement” and “Cell Morphology”, have been predicted as the most significantly activated canonical pathways (Supplementary Fig. 2).

IPA revealed that STXBP4-positivity was also correlated with the expression of growth factor receptors and components of downstream pathways. Among these genes

listed in descending order of normalized expression, *PDGFRA* was a significant up-regulated gene (FDR < 0.1) (Fig. 2D and 2E), and a most relevant candidate for addressing the growth of STXBP4-positive lung SCC cells (Supplementary Fig. 3). PDGFR $\alpha$  is a receptor tyrosine kinase that is a critical regulator of growth and proliferation of certain cell types during embryonal development (28, 29). In subsequent experiments described later in this study, PDGFR $\alpha$  proved to be a key mediator of STXBP4 oncogenic activity.

### **STXBP4 regulates PDGF-PDGFR signaling in lung SCC**

PDGF and PDGFR isoforms have important functions in the regulation of growth and survival of certain cell types (28, 29), and upregulation of PDGF-PDGFR signaling drives tumor cell growth. Indeed, the oncogenic properties of mutated or amplified *PDGFR $\alpha$*  have been studied in several tumor types, and PDGFR $\beta$  has been linked to not only tumor angiogenesis via paracrine effects, but also cancer metastasis (30, 31).

We measured mRNA expression levels by real-time RT-PCR in a total of 52 available samples from lung SCC patients for which high quality RNA was available (RIN > 2.0). The mRNA levels of *STXBP4* were also correlated with  *$\Delta Np63$*  mRNA

levels in those 52 samples (Supplementary Fig. 4A). Interestingly, we observed that *PDGFRA* expression was significantly up-regulated in STXBP4-positive lung SCC samples compared with STXBP4-negative samples ( $P < 0.05$ ) (Fig. 3A and 3B). On the other hand, *PDGFRB*, *VEGFR1 (FLT1)*, *VEGFR2 (KDR)* and *VEGFR3 (FLT4)* were consistently, but not significantly, up-regulated in STXBP4-positive lung SCC samples. STXBP4-positive samples defined by immunohistochemistry also had high *STXBP4* mRNA expression levels compared with STXBP4-negative samples, and interestingly, *PDGFRA* mRNA levels were also significantly correlated with *STXBP4* mRNA levels (Fig. 3C). Additionally, analyses of The Cancer Genome Atlas (TCGA) datasets of lung SCC (n = 488), supported in part, our finding that *PDGFRA* mRNA levels are significantly correlated with *STXBP4* mRNA levels ( $P = 0.015$ ) (Supplementary Fig. 4B).

To confirm the enhanced expression levels of *PDGFRA* in STXBP4 high expressing tumors, the lung SCC cell line, EBC-1, was transduced with STXBP4 and  $\Delta$ Np63 $\alpha$  retroviruses. As shown in Fig. 3D and 3E, the STXBP4 transduced stable clones showed high induction levels of both *PDGFRA* mRNA level (Fig. 3D) and PDGFR $\alpha$  protein levels (Fig. 3E), consistent with our findings in 52 resected patient lung SCC samples (Fig. 3B and 3C). Correspondingly, high expressing STXBP4 cells

had elevated  $\Delta$ Np63 protein levels, but not mRNA levels. Additionally, the relative increase in *PDGFRA* mRNA expression is more marked in *STXBP4* low-expressing EBC-1 cells compared with *STXBP4* high-expressing RERF-LC-Sq1 cells (Supplementary Fig. 5A and 5B). The results indicate that *STXBP4* regulates PDGFR $\alpha$  expression in lung SCC, most likely in a  $\Delta$ Np63-dependent manner, and also suggest that *STXBP4* may serve as a new SCC biomarker.

#### ***STXBP4*-depletion represses lung SCC tumor growth *in vivo***

We next examined the oncogenic role of *STXBP4* in regulating the expression of PDGFR $\alpha$  in a lung SCC cell line using a loss-of-function approach. Two independent siRNAs against *STXBP4* (*STXBP4*#1 and *STXBP4*#2) were transfected into the lung SCC cell line, RERF-LC-Sq1, and a siRNA targeting luciferase (siLUC) was used as a control. The *STXBP4* knockdown cells showed low expression of *STXBP4*, which correlated with down-regulation of both *PDGFRA* mRNA (Fig. 4A) and PDGFR $\alpha$  protein levels (Fig. 4B), consistent with our findings in resected patient lung SCC samples.

In order to evaluate the functional relevance of *STXBP4* and PDGFR $\alpha$  expression during tumor formation, we monitored the colony formation of

RERF-LC-Sq1 cells lentivirally transduced with STXBP4,  $\Delta$ Np63 or PDGFR $\alpha$  shRNAs. As shown in Fig. 4C, PDGFR $\alpha$ , STXBP4 or  $\Delta$ Np63 knockdown in RERF-LC-Sq1 cells, led to decreased anchorage-independent colony formation in soft agar. Subcutaneous transplantation of *PDGFRA* or *STXBP4* knockdown clones into immunodeficient mice resulted in suppressed tumor formation compared with control luciferase shRNA xenografts (Fig. 4D). Additionally, the knockdown effect of *STXBP4* and suppression of tumorigenesis are more marked in high *STXBP4* expressing RERF-LC-Sq1 cells compared with low *STXBP4* expressing EBC-1 cells (Fig. 4 and Supplementary Fig. 6). These data suggest that downregulation of *STXBP4* decreases PDGFR $\alpha$  expression and suppresses tumor formation.

Overall, our results indicate that STXBP4 has oncogenic activity both *in vitro* and *in vivo*, and further suggest that STXBP4 could be a critical driver of tumor propagation through regulating the PDGFR $\alpha$  pathway.

## Discussion

We demonstrated that STXBP4 expression in clinical specimens was closely associated with T factor ( $P < 0.001$ ), disease stage ( $P = 0.030$ ), and pleural involvement ( $P = 0.028$ ). Furthermore, univariate and multivariate analysis indicated that STXBP4 expression was an independent prognostic factor for OS and PFS. While p63 is essential for normal epidermal stratification and the proliferative potential of epithelial stem cells,  $\Delta$ Np63 is thought to maintain the proliferative potential of basal regenerative cells, including stem cells, in skin, thymus, breast, prostate, and urothelial stratified epithelium (20, 32-36). STXBP4 can physically interact with  $\Delta$ Np63 and is indispensable for stabilizing  $\Delta$ Np63, which is consistent with a putative diagnostic role for STXBP4 in lung SCC.

Polymorphisms of *STXBP4/COX11* (rs6504950; AA/AG-genotype) were associated with a significantly decreased risk of carcinogenesis in a meta-analysis of breast cancer patients (37). Although functional assessments of these polymorphisms were not undertaken, and the extent of loss of STXBP4 function in tumors was not studied, the data suggested that STXBP4 could play a role in carcinogenesis and tumor progression in breast cancer patients. Our study provides diagnostic role of STXBP4 alongside  $\Delta$ Np63 in lung SCC.



The pathological function of STXBP4 in human cancers remains unclear. However, STXBP4 can physically interact with p63 and is indispensable for stabilizing  $\Delta Np63$  even in normal conditions (16). Consistent with STXBP4 localization in both the nucleus and cytoplasm, it has been suggested that nuclear STXBP4 has p63-mediated functions, and that cytoplasmic STXBP4 could facilitate other functions in a p63-independent manner (16). In fact, our data indicated that STXBP4 induction partially increased tumor growth even in the absence of elevated  $\Delta Np63$  (Supplementary Fig. 7A), and that PDGFRA induction also partially increased tumor growth even in *STXBP4* knockdown cells (Supplementary Fig. 7B). Thus, STXBP4 may contribute to the susceptibility and severity of cancer in a p63-dependent and independent manner. Indeed, amplification and overexpression of *p63* has frequently been observed in a variety of SCCs, including lung cancers and head and neck cancers (8, 38). However, p63 expression is decreased during progression to invasion and metastasis of lung, breast and bladder cancer, and loss of p63 expression is associated with worse prognosis in some cases (35, 39, 40). It could be the balance between the TA isotype (tumor suppressive) and  $\Delta N$  isotype (oncogenic), as well as the tissue context, which is critical for proliferation and differentiation in both epithelial stem cells and cancer stem cells.

Global transcriptome profiling using next-generation sequencing technologies has become more common for comprehensive gene expression analysis to explore novel regulators and target genes in different types of cancers. In this report, genome-wide transcriptome analysis identified mediators of STXBP4 activity, including PDGFR $\alpha$ , which contributes to cell growth and metastasis in a p63-dependent manner. PDGF family proteins consist of several disulfide-bonded, dimeric isoforms (PDGF AA, PDGF AB, PDGF BB, PDGF CC and PDGF DD) that bind in a specific pattern to two related receptor tyrosine kinases, PDGFR $\alpha$  and PDGFR $\beta$  (41). PDGFR $\alpha$  homodimers bind to all PDGF isoforms except those containing PDGF D (42). A number of different signaling pathways, including mTOR (Fig. 1D) and MAPK (Fig. 3E), are initiated by activated PDGF receptors, and stimulate cell growth, actin reorganization, migration, and differentiation (43, 44).

PDGF receptors are expressed at low levels in normal lung epithelial cells, however, increased PDGFR $\alpha$  expression has been reported in lung cancer. PDGFR $\beta$  expression is observed mainly in stromal cells, but also in the sarcomatoid type of NSCLC (45). Based on recent evidence, inhibition of the p53/NF- $\kappa$ B complex by mutant gain of function p53 enhances PDGFR $\beta$  expression and promotes metastasis in a subset of pancreatic cancers (31). In addition, the interaction of mutant p53 with p63 regulates

the expression of p63 target genes to enhance invasion and metastasis (46). Hence, the oncogenic activity of mutant p53 is a consequence of the physical association between mutant p53 and the p53 family members, p63 and p73.

Treatment strategies for lung cancer are based on the assumption that an individual patient's cancer is purely of one subtype. Since many cancers are heterogeneous and relatively resistant to chemotherapy or radiation, there is strong interest in molecular-targeted therapies based on tumor biology. In particular, targeted agents that inhibit the epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) are approved for the treatment of NSCLC harboring genetic alterations in the genes encoding these proteins (47). EGFR inhibitors, such as erlotinib and gefitinib, are only effective against NSCLCs with *EGFR* mutations, which occur almost exclusively in lung AC. Similarly, the recently identified *EML4-ALK* rearrangement, which predicts susceptibility to the targeted agent crizotinib, also occurs only in lung AC. Unfortunately, therapeutic advances in the treatment of lung SCC have lagged behind those for AC (48). Therefore, the capacity to distinguish between lung AC and SCC is particularly important for the effective use of novel targeted therapies to treat patients with these NSCLC subtypes.

Inhibition of the PDGFR $\alpha$  signaling pathway by treatment with a neutralizing

PDGFR $\alpha$  antibody, MEDI-575, had minimal effect on tumor cell proliferation in preclinical models of NSCLC (49). Lung SCC histology also identified patients at a higher risk of bleeding during treatment with bevacizumab, a monoclonal anti-VEGF antibody (50). Thus, more studies are required to determine whether specific inhibition of PDGF receptors, without inhibition of VEGF receptors, is of any benefit for lung cancer patients. These issues highlight the growing importance of accurate identification of NSCLC subtypes for assigning patients to appropriate histology-based therapies and the triage of tissue for appropriate molecular studies.

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## Figures Legends

**Figure 1.** STXBP4 expression is correlated with p63 expression and poor prognosis in lung SCC. (A) Representative immunohistochemical staining of a lung SCC. STXBP4 immunostaining demonstrates a nuclear and cytoplasmic pattern with a score of 5. Scale bars are 200  $\mu$ m. (B, C) Kaplan-Meier analysis of Overall Survival (OS) and Progression Free Survival (PFS) defined according to STXBP4 expression. A statistically significant difference in OS and PFS was observed between the STXBP4-positive patients and those with low STXBP4 expression [OS,  $p = 0.0056$ (A); PFS,  $p = 0.0068$  (B)].  $P$ -values were obtained by log-rank test. (D) Spearman's rank correlation was performed based on the expression levels of STXBP4 and  $\Delta$ Np63.

**Figure 2.** Gene expression profiling of clinical samples from lung SCC patients. (A) A cluster diagram of RNA-seq data from six pairs of STXBP4-positive and STXBP4-negative samples. The color bars represent relative expression levels: Red indicates higher than average expression and blue indicates lower than average expression. (B) 79 significantly differentially expressed upregulated genes and 93 downregulated genes ( $P < 0.05$  and FDR  $< 0.5$ ) were identified. (C) Functional analysis of differentially expressed genes was performed by

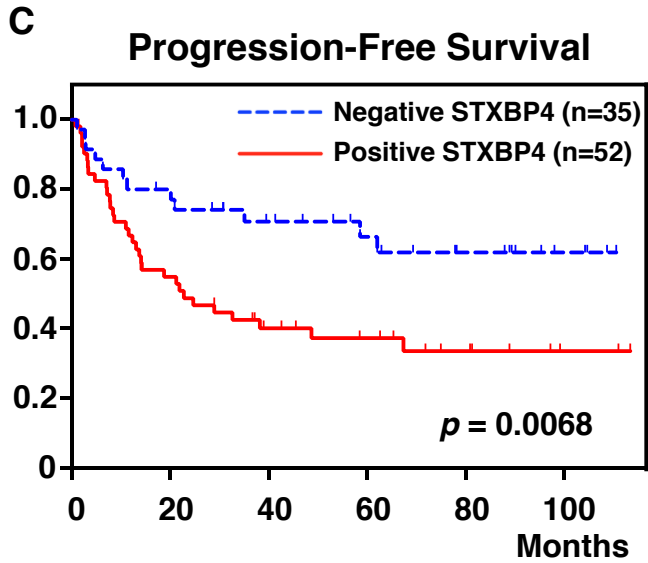
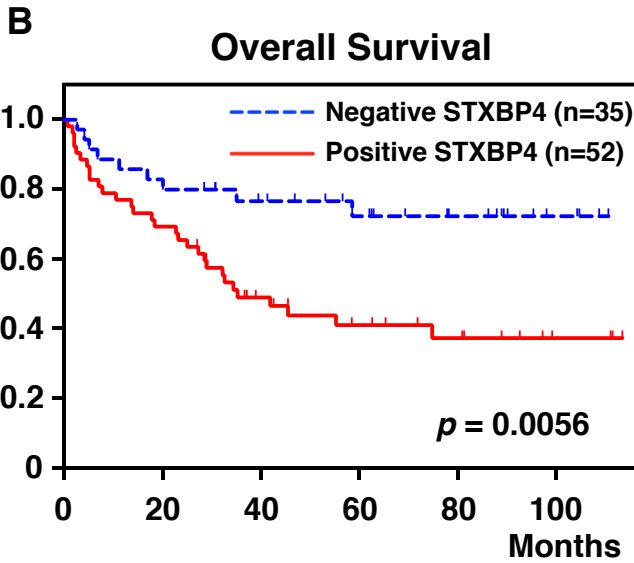
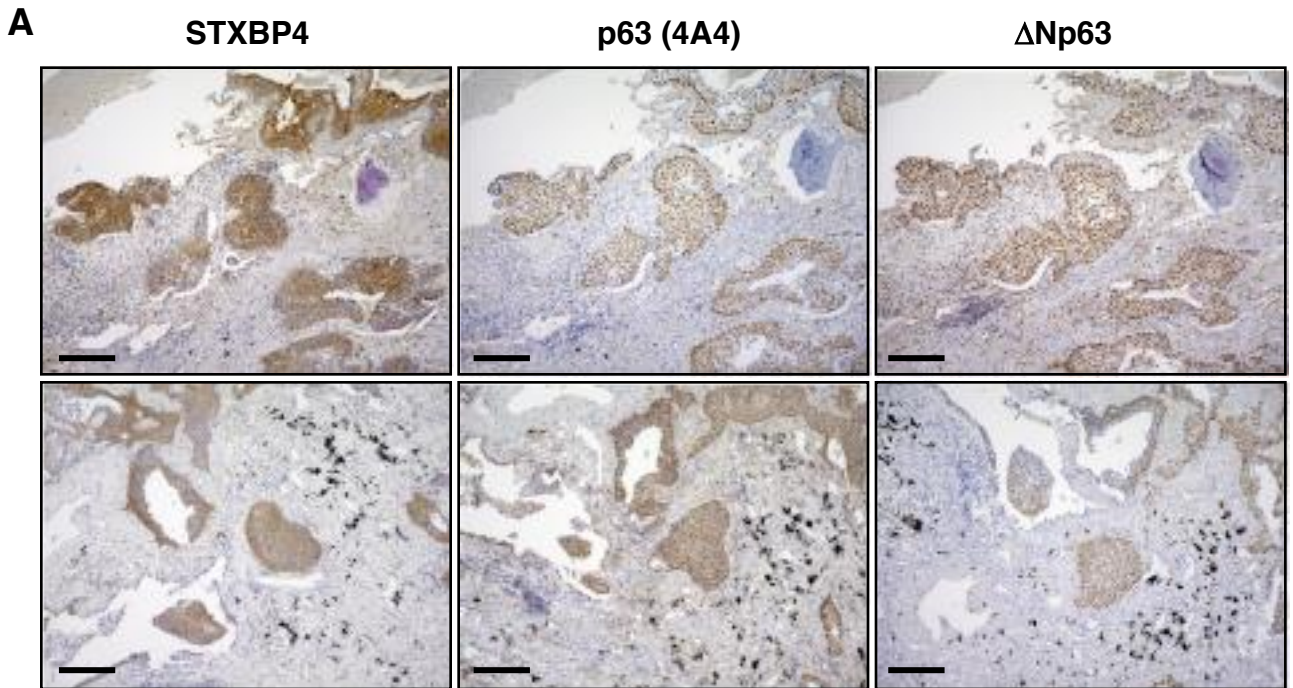
Ingenuity Pathway Analysis. (D) The genes are listed in descending order of normalized expression. \*FDR < 0.1. (E) A cluster diagram of PDGFR and VEGFR expression from RNA-seq analysis. The color bars represent relative expression levels: red indicates higher than average expression and blue indicates lower than average expression.

**Figure 3.** *PDGFRA* expression is significantly upregulated in STXBP4-positive samples from lung SCC Patients. (A) A cluster diagram of relative expression of *PDGFRs* and *VEGFRs* by real-time RT-PCR analysis. Tumor specimens were collected from lung SCC patients with surgical resection. A total of 52 high RNA integrity number (RIN > 2.0) STXBP4-positive samples (n=34) and STXBP4-negative samples (n=18), were used for transcriptome profiling by real-time RT-PCR. (B) *PDGFRA* mRNA was significantly upregulated in STXBP4-positive lung SCC samples. Relative mRNA levels of *PDGFRs* and *VEGFRs* of the STXBP4-positive samples (n=35) and STXBP4-negative samples (n=19); \**P* < 0.05. (C) Scatter plot of relative mRNA expression levels of *PDGFRA* and *STXBP4*. (D) STXBP4 or  $\Delta$ Np63 $\alpha$  induces *PDGFRA* expression in a lung SCC cell line, EBC-1. The cells were retrovirally transduced with empty vector control (Mock),  $\Delta$ Np63 $\alpha$  or STXBP4. The mRNA levels of *PDGFRA*,

*STXBP4* or  $\Delta Np63$  were determined by real-time RT-PCR. (E) EBC-1 cells transduced as in (D), were subjected to immunoblotting using antibodies for phospho-PDGFR $\alpha$  (p-PDGFR $\alpha$ ), PDGFR $\alpha$ , phospho-p38MAPK (p-p38MAPK), p38MAPK, *STXBP4*,  $\Delta Np63$  or  $\beta$ -Actin.

**Figure 4.** *STXBP4*-depletion inhibits lung SCC tumorigenesis and modulates PDGF signaling *in vivo*. (A) The lung SCC cells, RERF-LC-Sq1, were treated with siRNAs for Luciferase (siLUC) as a control, *STXBP4*#1, *STXBP4*#2,  $\Delta Np63$  or PDGFR $\alpha$ . Total RNAs were quantified by real-time RT-PCR analysis and the induction levels of *PDGFRA* were determined by the relative Ct method. (B) RERF-LC-Sq1 cells depleted as in (A), were subjected to immunoblotting using anti-PDGFR $\alpha$ , *STXBP4*,  $\Delta Np63$  or  $\beta$ -Actin antibodies. (C) The growth of RERF-LC-Sq1 cells after shRNA mediated PDGFR $\alpha$ , *STXBP4* or  $\Delta Np63$  knockdown was monitored by soft agar colony formation assays. Standard deviations (SD) are plotted. \* $P < 0.05$ . (D) Representative images of xenografts from subcutaneously transplanted with lentivirally shRNA transduced Luciferase as a control (shLUC), *Stxbp4*,  $\Delta Np63$  or PDGFR $\alpha$  knockdown RERF-LC-Sq1 cells (n = 6 for each knockdown). The results of six independent injections of

knockdown cells are shown. Twenty days after implantation, the length (L) and width (W) of the tumor mass were measured, and the tumor volume (TV) was calculated using the equation:  $TV = (L \times W^2)/2$ . \* $P < 0.05$ .

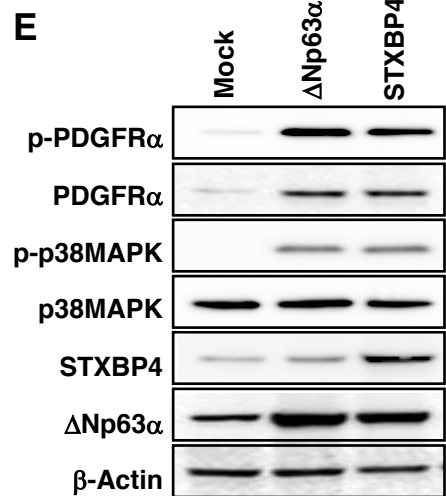
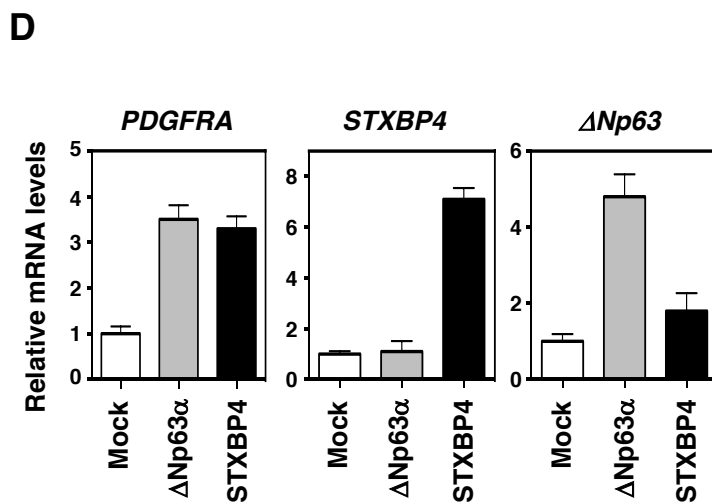
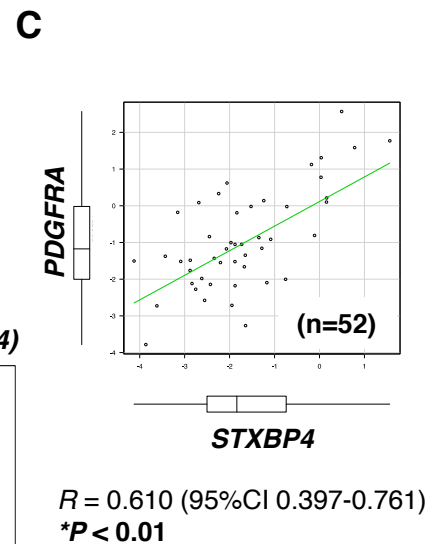
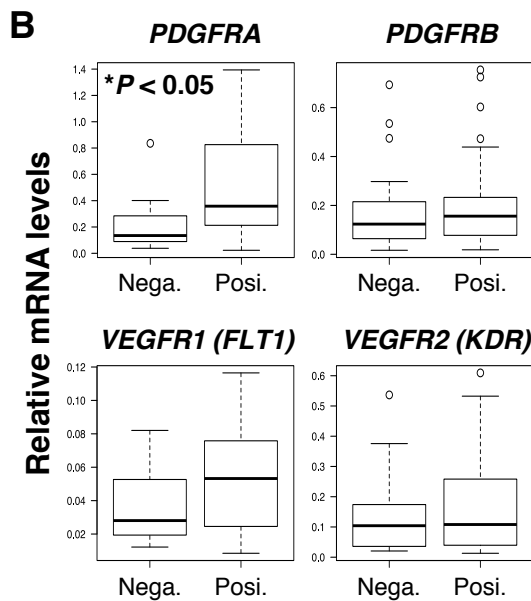
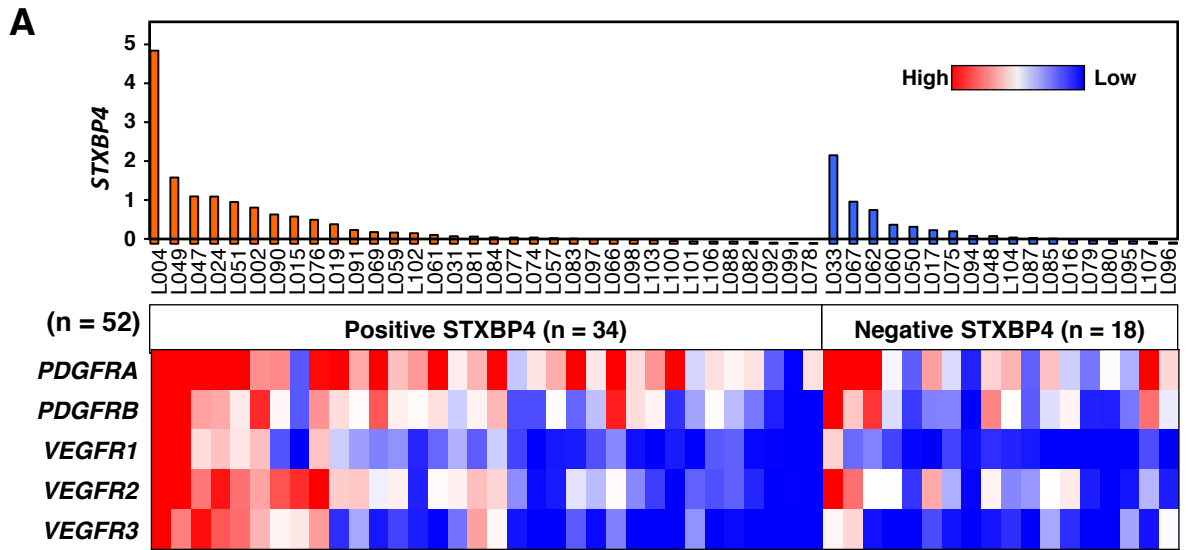


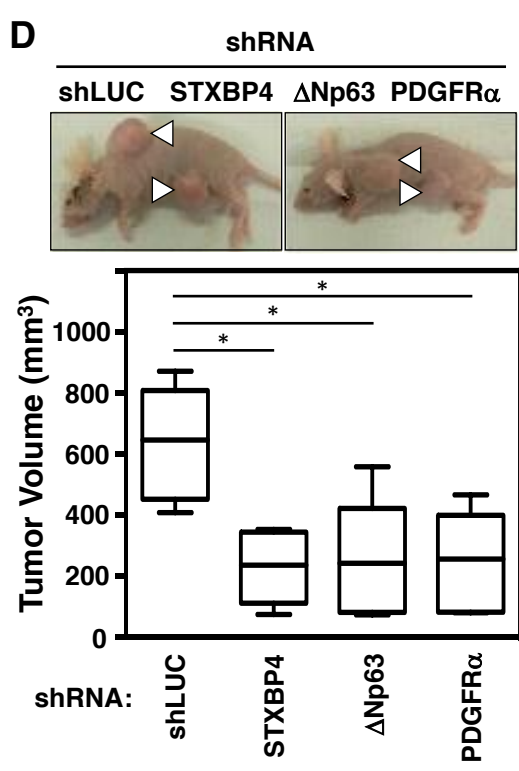
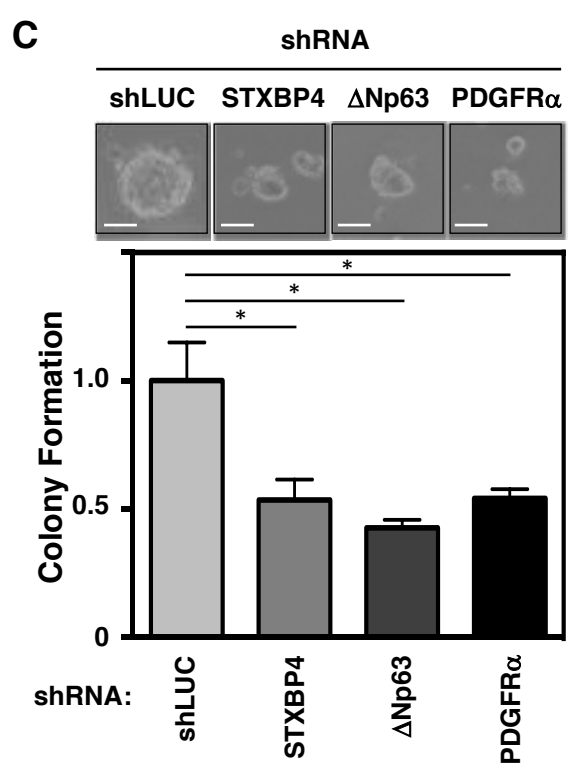
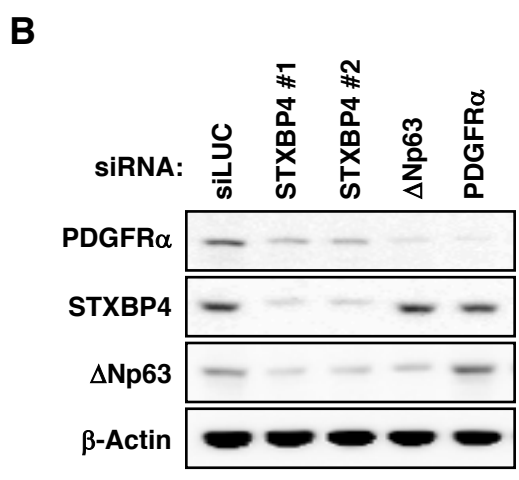
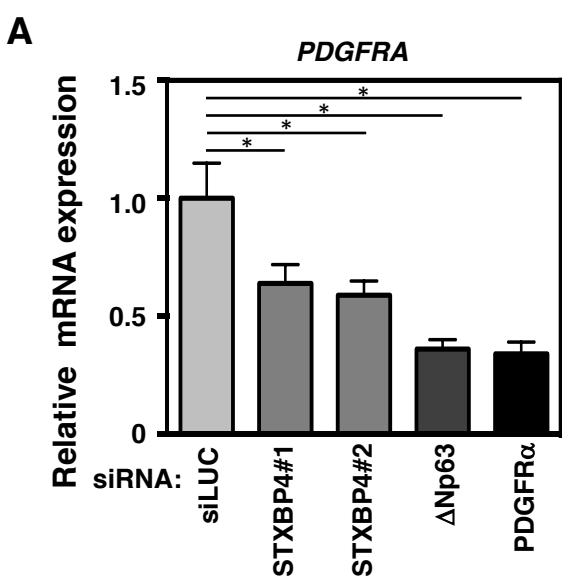
**D**

vs. STXBP4	$\Delta$ Np63	p63	mTOR	CD147
Spearman's $\rho$	0.219	0.088	0.220	0.144
<i>P</i> -value	<b>0.042</b>	0.417	<b>0.041</b>	0.183









**Table 1. Patient's demographics according to STXBP4 expression**

Variables	STXBP4			ΔNp63			p63		
	High No. (%)	Low No. (%)	P-value	Positive No. (%)	Negative No. (%)	P-value	Positive No. (%)	Negative No. (%)	P-value
Age			0.42			0.051			0.22
≤ 65yr	9 (10.3)	9 (10.3)		10 (11.5)	8 (9.2)		16 (18.4)	2 (2.3)	
> 65 yr	43 (50.5)	26 (29.9)		20 (23.0)	49 (56.3)		51 (58.6)	18 (20.7)	
Sex			0.70			0.69			< 0.99
Male	47 (54.0)	33 (37.9)		27 (31.0)	53 (60.9)		61 (70.1)	19 (21.8)	
Female	5 (5.7)	2 (2.3)		3 (3.4)	4 (4.6)		6 (4.6)	1 (3.4)	
Differentiation			0.080			<b>0.034</b>			0.24
Well or Moderately	43 (49.4)	23 (26.4)		27 (31.0)	39 (44.8)		53 (60.9)	13 (15.0)	
Poorly	9 (10.3)	12 (13.8)		3 (3.4)	18 (20.7)		14 (16.1)	7 (8.0)	
T factor			<b>&lt; 0.001</b>			0.33			<b>&lt; 0.001</b>
T1	8 (9.2)	19 (21.8)		7 (8.0)	20 (23.0)		27 (31.0)	0 (0.0)	
T2-3	44 (50.6)	16 (18.4)		23 (26.4)	37 (42.5)		40 (46.0)	20 (23.0)	
N factor			> 1			> 1			0.28
N0	36 (41.4)	25 (28.7)		21 (24.1)	40 (46.0)		49 (56.3)	12 (13.8)	
N1-2	16 (18.4)	10 (11.5)		9 (10.3)	17 (19.5)		18 (20.7)	8 (9.2)	
Disease stage			<b>0.030</b>			0.37			0.20
I	23 (26.4)	24 (27.6)		14 (16.1)	33 (37.9)		39 (44.8)	8 (9.2)	
II-III	29 (33.3)	11 (12.6)		16 (18.4)	24 (27.6)		28 (32.2)	12 (13.8)	
Pleural Involvement			<b>0.028</b>			0.66			0.29
Positive	30 (34.5)	11 (12.6)		13 (14.9)	28 (32.2)		30 (34.5)	11 (13.8)	
Negative	22 (25.3)	24 (27.6)		17 (19.5)	29 (33.3)		37 (42.5)	9 (10.3)	
Lymphatic permeation			0.83			> 1			0.61
Positive	29 (33.3)	18 (20.7)		16 (18.4)	31 (35.6)		35 (40.2)	12 (13.8)	
Negative	23 (26.4)	17 (19.5)		14 (16.1)	26 (29.9)		32 (36.8)	8 (9.2)	
Vascular invasion			0.39			> 1			> 1
Positive	26 (29.9)	14 (16.1)		14 (16.1)	26 (29.9)		31 (35.6)	9 (10.3)	
Negative	26 (29.9)	21 (24.1)		16 (18.4)	31 (35.6)		36 (41.4)	11 (12.6)	

\*P-values were obtained by Fisher's exact test.

†Clinical stage at the time of initial diagnosis was determined according to the seventh edition of General Rule for Clinical and Pathological Record of Lung Cancer (2010), the Japan Lung Cancer Society.

**Table 2. Univariate and multivariate survival analysis in all patients**

Variables	Overall Survival				Progression-Free Survival			
	Univariate		Multivariate		Univariate		Multivariate	
	5-yrs rate (%)	p-value	HR (95% CI)	p-value	5-yrs rate (%)	p-value	HR (95% CI)	p-value
Age		0.853		0.504		0.965		0.452
≤ 65yr	49		1.32		44		1.34	
> 65 yr	55		(0.60 - 3.21)		51		(0.64 - 3.06)	
Sex		0.579		0.690		0.215		1.90
Male	55		1.25		51		1.90	
Female	38		(0.36 - 3.26)		21		(0.63 - 4.55)	
Differentiation		0.206				0.447		
WD	68				54			
MD/PD	50				48			
T factor		<b>0.011</b>				<b>0.011</b>		
T1	75				75			
T2-3	44				44			
N factor		0.207				<b>0.011</b>		
No	57				56			
N1-2	43				29			
Disease Stage		<b>0.006</b>		<b>0.033</b>		<b>0.002</b>		<b>0.002</b>
I	70		2.17		67		2.94	
II - III	42		(1.06 - 4.24)		29		(1.49 - 5.67)	
Lymphatic permeation		0.448				0.182		
Positive	50				44			
Negative	62				54			
Vascular invasion		0.239				0.365		
Positive	49				48			
Negative	57				51			
Pleural involvement		<b>0.014</b>				<b>0.049</b>		
Positive	41				41			
Negative	65				56			
STXBP4		<b>0.005</b>		<b>0.028</b>		<b>0.006</b>		<b>0.040</b>
Positive	41		2.24		37		2.02	
Negative	72		(1.09 - 5.10)		66		(1.03 - 4.24)	
ΔNp63		0.362				0.458		
Positive	51				45			
Negative	61				54			
p63		0.523				0.111		
Positive	53				51			
Negative	54				36			

## Supplementary Information

### RNA interference

For siRNA experiments, 19 nucleotide siRNA duplexes with 3' dTdT overhangs were synthesized by Dharmacon (GE healthcare, Lafayette, CO). For siRNA transfection, cells were transfected with 50 nM siRNA using DharmaFECT 1 siRNA transfection reagent (Dharmacon) according to the manufacturer's instructions. The siRNA oligonucleotide sequences were as follows: Luciferase control (siLUC), 5'-CUUACGCUGAGUACUUCG A -3';  $\Delta$ Np63, 5'- CAATGCCCAGACTCAATTT -3'; STXBP4#1, 5'- CCTGGAGGAGACTGTTATA -3'; STXBP4#2, 5'-CCGACAACATTCAGCCAGAAA -3'; PDGFR $\alpha$ , 5'- CGAGACTCCTGTAACCTTA -3'; XAGE1B, 5'- GCGUCAAGGUGAAGAUAAU -3'; DPP4, 5'-UCAGUAAAGAGGCGAAGUA -3'; EPHA3, 5'- GAUCGGACCUCCAGAAAUA -3'.

For shRNA experiments, shRNAs for Luciferase (LUC),  $\Delta$ Np63, STXBP4 and PDGFR $\alpha$  oligonucleotides were annealed at 90°C for 15 min, 70°C for 30 min, 25°C for 30 min, and then cloned into the pLKO.1 puro lentiviral shRNA expression vector between Age I - Eco RI sites. All constructs were verified by DNA sequencing (Genewiz). Oligonucleotide target sequences used for shRNAs were follows:  $\Delta$ Np63, 5'- CAATGCCCAGACTCAATTT -3'; STXBP4, 5'- CCTGGAGGAGACTGTTATA -3'; PDGFR $\alpha$ , 5'- CGAGACTCCTGTAACCTTA -3'.

### Quantitative real-time PCR

Primers used were as follows: *STXBP4*, forward 5'-GGCCCATTTGGTATATATTCAGG -3' and reverse 5'- GGCTTCAAACGACCATCCT

-3'; *ΔNp63*, forward 5'- GGAAAACAATGCCCAGACTC -3' and reverse 5'-  
CTGCTGGTCCATGCTGTTC -3' ; *PDGFRA*, forward 5'-  
CCACCTGAGTGAGATTGTGG -3' and reverse 5'-  
TCTTCAGGAAGTCCAGGTGAA -3'; *PDGFRB*, forward 5'-  
CATCTGCAAAACCACCATTG -3' and reverse 5'-  
GAGACGTTGATGGATGACACC -3'; *VEGFR1 (FLT1)*, forward 5'-  
CAGCATACTCACTGTTCAAGG -3' and reverse 5'-  
CCACACAGGTGCATGTTAGAG -3'; *VEGFR2 (KDR)*, forward 5'-  
GCTCAAGACAGGAAGACCAAG -3' and reverse 5'- GGTGCCACACGCTCTAGG  
-3'; *VEGFR3 (FLT4)*, forward 5'- AAGATGTTTGCCCAGCGTAG -3' and reverse 5'-  
GCACTGTGGCATGAGGTCT -3'.

## Supplementary Figures Legends

**Supplementary Figure 1.** STXBP4 expression is correlated with poor prognosis in patients with tumors expressing high  $\Delta$ Np63 levels. (A, B) A total of 87 samples of lung SCC were classified into 2 subgroups based on the expression of  $\Delta$ Np63, high  $\Delta$ Np63 (n=30) and low  $\Delta$ Np63 (n=57). And then, Kaplan-Meier analysis defined according to STXBP4 expression. A statistically significant differences in OS and PFS were observed among the patients [OS (A),  $P < 0.01$ ; PFS (B),  $P < 0.01$ ].  $P$ -values were obtained by log-rank test. (C, D) A total of 87 samples of lung SCC were classified into 2 subgroups based on the expression of STXBP4, high STXBP4 (n=52) and low STXBP4 (n=35). And then, Kaplan-Meier analysis defined according to  $\Delta$ Np63 expression.  $P$ -values were obtained by log-rank test [OS (AC),  $P = 0.99$ ; PFS (BD),  $P = 0.84$ ].

**Supplementary Figure 2.** Causal networks associated with STXBP4 expression in lung SCC. The canonical pathway analysis characterized two signaling pathways as the functional relationship of STXBP4-positivity, including “Cellular Movement” and “Cell Morphology”.



**Supplementary Figure 3.** Depletion of PDGFR $\alpha$  suppresses the growth of lung SCC. The lung SCC cells, RERF-LC-Sq1, were treated with siRNAs for Luciferase (siLUC) as a control, PDGFR $\alpha$ , XAGE1B, DPP4 or EPHA3. The cell growth rate was measured at 72 hrs after siRNA transfection using CCK-8 reagent (Dojindo, Tokyo, Japan) according to the manufacturer's instruction.

**Supplementary Figure 4.** *PDGFRA* mRNA was significantly correlated with *STXBP4* in the gene expression profiles of lung SCC patients. (A) Scatter plot of relative mRNA expression levels between  $\Delta Np63$  and *STXBP4*. A total of 52 available samples with high RNA integrity number (RIN > 2.0) were used for transcriptome profiling by real-time RT-PCR. (B) A total of 488 lung SCC cases in the datasets of The Cancer Genome Atlas (TCGA), were classified into 2 groups based on the expressions of *STXBP4* mRNAs (High: high *STXBP4*, Low: low *STXBP4*). The z-score of each gene expression was analyzed. *P*-value was obtained by Student's T-test.

**Supplementary Figure 5.** *PDGFRA* expression is upregulated in *STXBP4*-transduced lung SCC cells. (A) *STXBP4* or  $\Delta Np63\alpha$  induces *PDGFRA* expression in lung SCC cell line, RERF-LC-Sq1. The cells were retrovirally

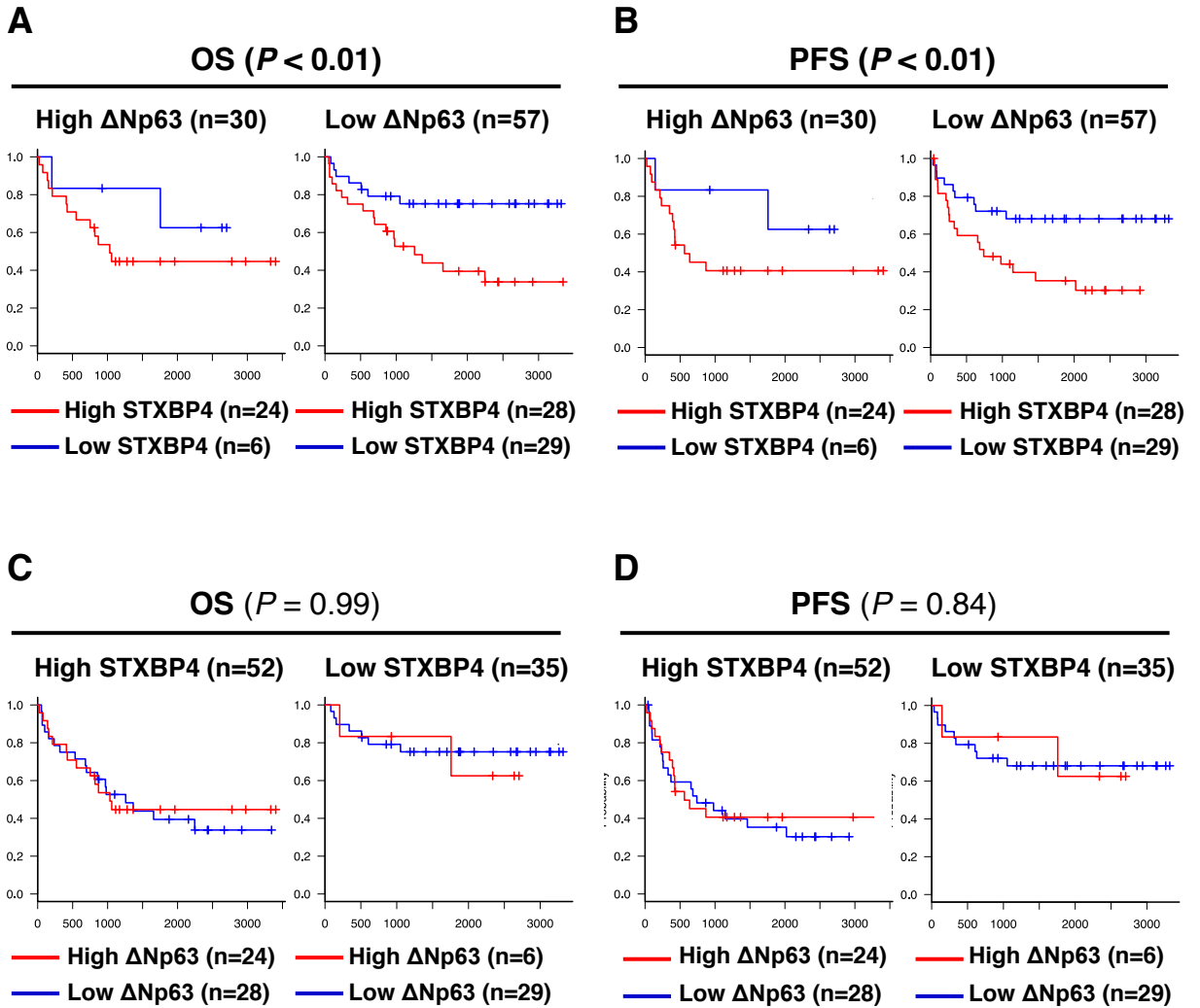
transduced with empty vector control (Mock),  $\Delta$ Np63 $\alpha$  or STXBP4. The mRNA levels of *PDGFRA* were determined by real-time RT-PCR. (C) RERF-LC-Sq1 cells transduced as in (B), were subjected to immunoblotting using indicated antibodies.

**Supplementary Figure 6.** STXBP4-depletion inhibits SCC tumorigenesis and modulates PDGF signaling *in vivo*. (A) The lung SCC cell line, EBC-1, were treated with siRNAs for Luciferase (siLUC) as a control, STXBP4,  $\Delta$ Np63 or PDGFR $\alpha$ . Total RNAs were quantified by real-time RT-PCR analysis and the induction levels of *PDGFRA* were determined by the relative Ct method. (B) EBC-1 cells depleted as in (A), were subjected to immunoblotting using indicated antibodies. (C) The growth of EBC-1 cells after shRNA mediated STXBP4,  $\Delta$ Np63 or PDGFR $\alpha$ , knockdown was monitored by soft agar colony formation assays. Standard deviations (SD) are plotted. \* $P < 0.05$ . (D) Representative images of xenografts from subcutaneously transplanted with lentivirally shRNA transduced Luciferase as a control (shLUC), STXBP4,  $\Delta$ Np63 or PDGFR $\alpha$  knockdown EBC-1 cells (n = 6 for each knockdown). The results of six independent injections of knockdown cells are shown. Fourteen days after implantation, the length (L) and width (W) of the tumor mass were measured,

and the tumor volume (TV) was calculated using the equation:  $TV = (L \times W^2)/2$ .

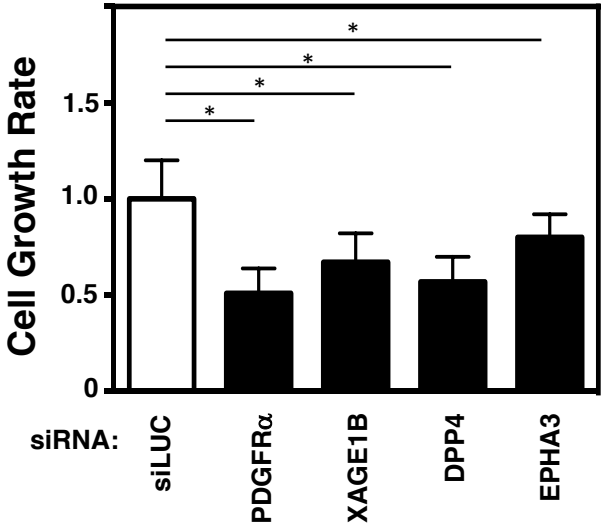
\* $P < 0.05$ .

**Supplementary Figure 7.** STXBP4 promotes tumorigenesis through PDGFRA in lung SCC. (A) STXBP4 promotes SCC tumorigenesis through PDGFR $\alpha$  in a  $\Delta Np63$ -dependent manner. The growth of STXBP4 expressing EBC-1 cells after depletion of luciferase as a control (shLUC),  $\Delta Np63$  or PDGFR $\alpha$ , were monitored by soft agar colony formation assays. Standard deviations (SD) are plotted. \* $P < 0.05$ . (B) STXBP4-depletion induces SCC tumorigenesis through PDGFR $\alpha$  in lung SCC cells. The growth of STXBP4-depleted RERF-LC-Sq1 cells after induction of PDGFR $\alpha$  was monitored by soft agar colony formation assays.

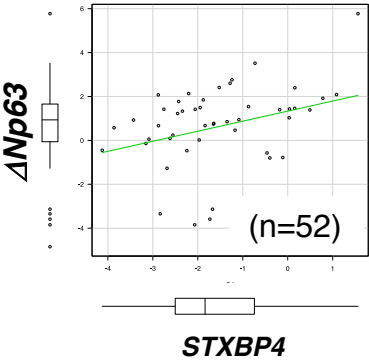


## Otaka et al. Supplementary Figure 2

Network	Molecules in Network	Score	Focus Molecules	Top Diseases and Functions
1	26s Proteasome, Alpha catenin, ASF1B, ATF5, BAG1, BCL3, BSCL2, CALB1, CD3, CLDN3, CXCL8, EPHA3, estrogen receptor, Gm-csf, GREM1, Hdac, HIF3A, Histone h4, HLA-DOB, Hsp70, Mapk, MMP11, MT1E, NFkB (complex), Nr1h, NT5E, RUNX3, SAA1, SFRP1, SLPI, STEAP4, THBS2, TNFRSF25, TSHZ3, Ubiquitin	39	23	Organismal Injury and Abnormalities, Reproductive System Disease, Cellular Movement
2	ADCY5, AKAP12, Akt, APOBEC3A, APOL1, AQP3, BCAT1, CEBPD, Cg, CLDN1, COL11A1, DHRS3, DSG1, DUSP9, ERK, FSH, GNLY, IgG, Igm, Immunoglobulin, Interferon alpha, IVL, KIT, KLK11, LANCL2, LCN2, Lh, MZB1, P38 MAPK, PDE5A, Pkc(s), SPIB, SPRY1, TCR, Vegf	36	22	Dermatological Diseases and Conditions, Cell Morphology, Hereditary Disorder
3	C4A/C4B, COL4A3, COL4A4, Collagen Alpha1, Collagen(s), DPP4, ERK1/2, FBN1, Focal adhesion kinase, FUT8, GPR39, H19, IGF2, ITGB1, KSR2, Laminin, LDL, Mek, MFAP5, OLR1, p85 (pik3r), PDGF BB, PDGFRA, PI3K (complex), PLC gamma, PROX1, RAPGEF2, Ras, SLC2A3, STAT5a/b, TIMP2, Tnf receptor, UBD, VCAN, VEGFC	34	21	Cellular Movement, Immune Cell Trafficking, Cardiovascular System Development and Function

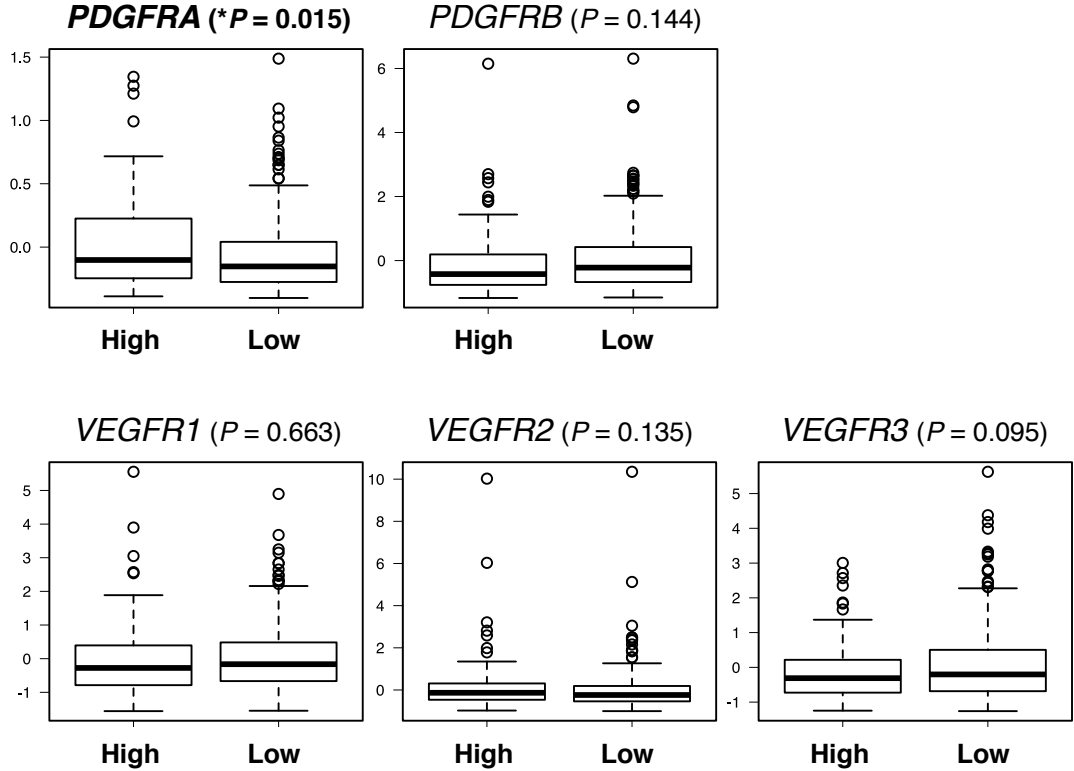


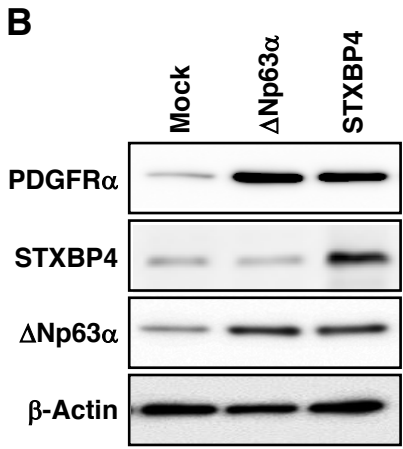
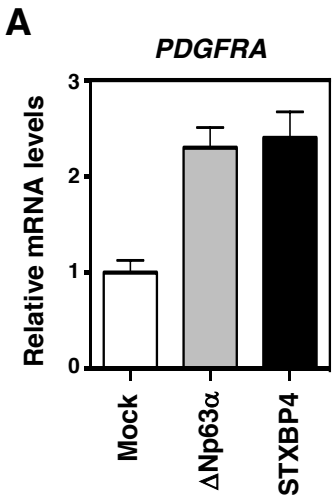
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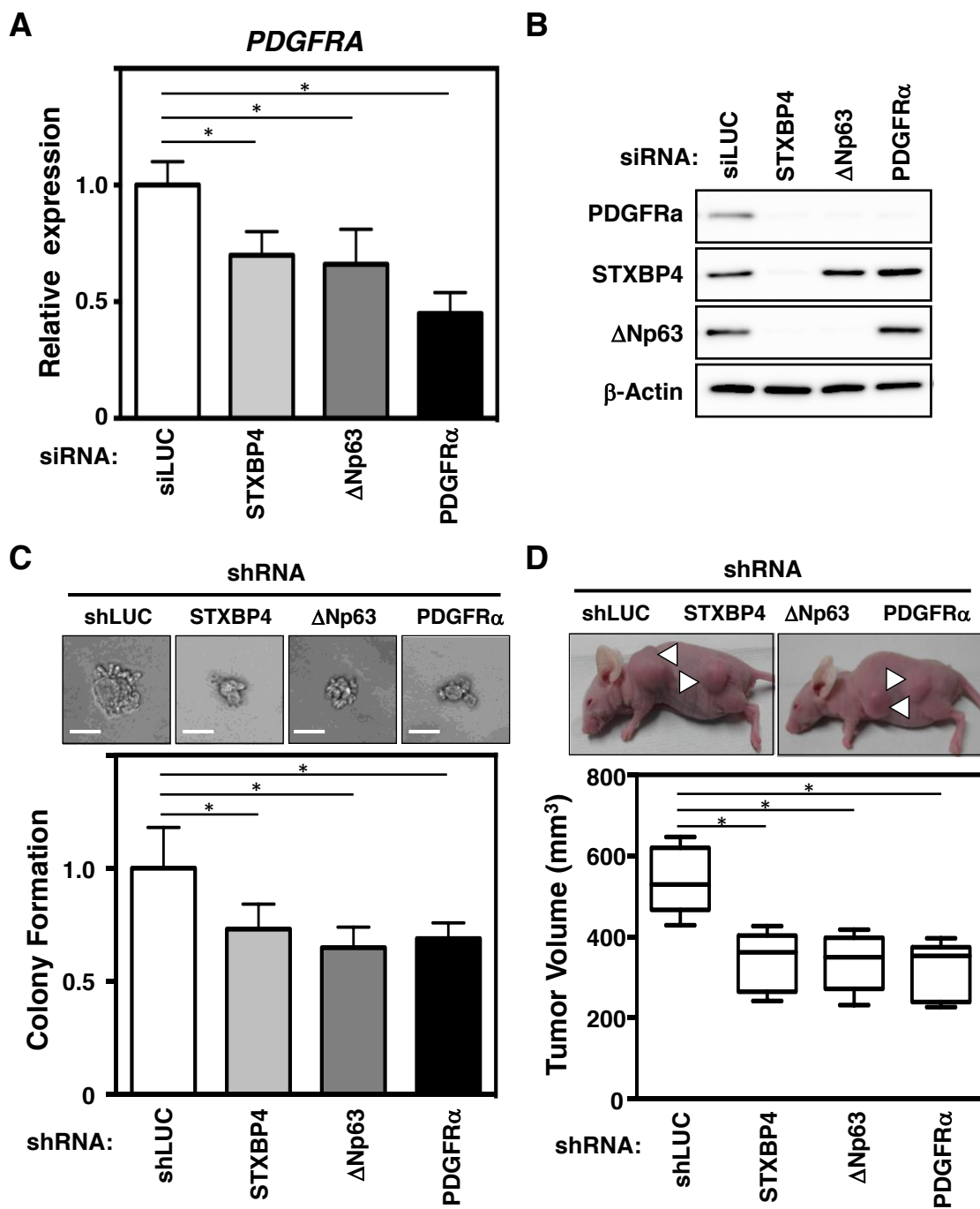
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 $*P = 0.032$

**B**

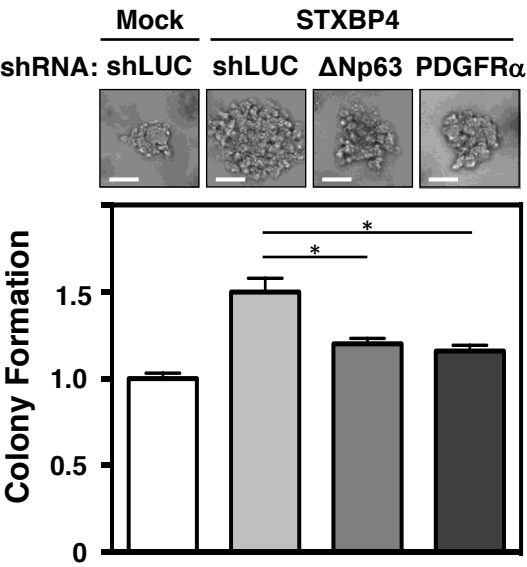




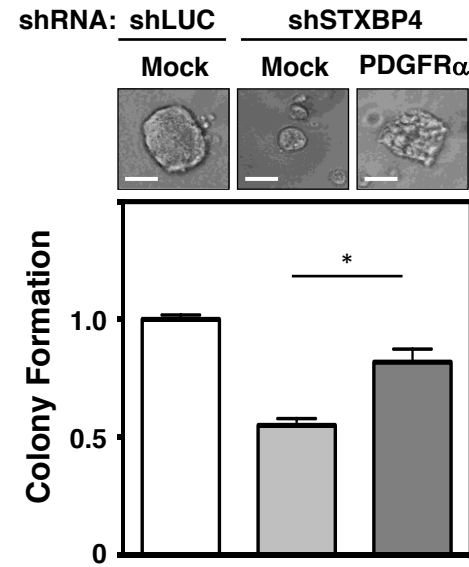




**A**



**B**



## Email

<b>Manuscript #</b>	CCR-16-1815R
<b>Title</b>	STXBP4 drives tumor growth and is associated with poor prognosis through PDGF Receptor signaling in lung squamous cell carcinoma
<b>Corresponding Author</b>	Dr. Susumu Rokudai (Gunma University)
<b>Date:</b>	2017-01-09 12:18:24
<b>Last Sent:</b>	2017-01-09 12:18:24
<b>Created By:</b>	Redacted
<b>From:</b>	bill.siegmann@aacr.org
<b>To:</b>	srokudai@gunma-u.ac.jp
<b>CC:</b>	Redacted
<b>Subject:</b>	Decision Rendered: CCR-16-1815R

Dear Dr. Rokudai:

I am pleased to inform you that your manuscript entitled, "STXBP4 drives tumor growth and is associated with poor prognosis through PDGF Receptor signaling in lung squamous cell carcinoma," has been accepted for publication in Clinical Cancer Research. You will receive electronic page proofs via e-mail in approximately 4 weeks. If your contact information is expected to change, please reply to this email with your updated information.

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Email

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Sincerely,  
Marc Ladanyi  
Senior Editor  
Clinical Cancer Research

[SAMPLE HIGHLIGHT]

The transmembrane mucin antigen MUC-1 is upregulated and deglycosylated in a large proportion of human cancers. To explore its role in screening, Moore, et al, designed a multi-modal MR/NIRF imaging probe, targeting underglycosylated MUC-1. The probe was highly specific to uMUC-1-positive versus non-neoplastic or uMUC-1-negative cancer cell lines, and in vivo MR/optical images of tumor bearing animals showed accumulation of the probe. This multi-modal targeted imaging probe successfully localized to uMUC-1 expression tumors, and thus shows promise as the basis of a clinically relevant tool in screening cancer patients and in possibly monitoring therapeutic efficacy.

\*\*\*\*\*

Reviewer #2 (Reviewer Comments to the Author):

This reviewer read this paper revised thoroughly and feel that this work presented herein is well studied and improved to publish it in Clinical Cancer Research. Thank you.