Oncogenic role of FGFR1 and vulnerability of RBL2-FGFR1 axis in small cell lung cancer development

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Abstract

The observation of recurrent fibroblast growth factor receptor 1 (FGFR1) amplification in small cell lung cancer (SCLC) raised the possibility of targeting the FGFR1 pathway to treat this aggressive disease. However, in vivo evidence for the significance of FGFR1 in SCLC development is lacking, and previous studies indicate a need for additional biomarkers to stratify patient tumours for anti-FGFR1 therapeutics. Here, we found that ectopic Fgfr1 expression in precancerous neuroendocrine cells (preSCs) increased cell growth in vitro and tumour formation in immune-compromised mice, results that coincided with transcriptomic changes indicative of altered differentiation and enhanced proliferation. Interestingly, Fgfr1 deletion suppressed tumour development in *Rb1/Trp53/Rbl2*-mutant mice but not in *Rb1/Trp53*-mutant mice. This Rbl2-dependent difference in phenotype suggests a functional link between this well-known tumour suppressor and FGFR1 signalling during SCLC development. Rbl2 knockout in preSCs selectively increased Fgfr1 expression while promoting tumour formation. Rbl2 loss also correlated with Fgfr1 induction in allograft tumours generated from preSCs carrying oncogenic mutations and primary tumours developed in the *Rb1/Trp53*-mutant mouse model. These results demonstrate the importance of enhanced FGFR1 and the vulnerability of the RBL2-FGFR1 axis for SCLC development.

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Introduction

Small cell lung cancer (SCLC) accounts for 13% of all lung cancers yet remains the deadliest type. The standard chemotherapy regimen of cisplatin and etoposide fails to improve overall patient survival, and the development of new therapies has been limited largely due to barriers and challenges inherent to SCLC research that recent advances are beginning to address [1, 2]. The recent discovery of recurrent alterations in the SCLC genome may lead to novel strategies for effective and early intervention [3-5]. While few of these genomic alterations are directly actionable, they are connected to molecular pathways that are targets of existing small molecule inhibitors. For instance, SCLC with *MYC* alterations demonstrated sensitivity to the aurora kinase inhibitor alisertib [6]. The link between MYC and the bromodomain and external family (BET) of bromodomain-containing proteins prompted clinical trials of a BET inhibitor that included SCLC patients [7].

FGFR1 amplification has been of significant interest among numerous alterations in the SCLC genome largely because the receptor tyrosine kinase critical for cell proliferation and survival can be modulated with small molecule inhibitors [4, 8-11]. It correlated with poor survival in SCLC patients with limited disease or following first-line chemotherapy; however, this correlation was weak due to the small sample sizes analyzed [8, 10]. Preclinical evidence suggests efficacy of inhibiting FGFR1 in SCLC cells with *FGFR1* copy number gain; however, high copy number and high mRNA and protein expression do not appear to be a strong predictor of drug response [12-14]. While

consistently pointing to a role for FGFR1 in SCLC, these studies highlight major gaps in the knowledge required to translate the concept of targeting the receptor tyrosine kinase to intervene in SCLC, including the absence of *in vivo* evidence for the significance of receptor signaling in tumor development and predictive biomarkers for response to receptor inhibition.

To characterize FGFR1 in SCLC development, we utilized genetically engineered mouse models (GEMM) in which adenoviral Cre (Ad-Cre)-driven conditional deletion of both *Rb1* and *Trp53*, mimicking the same set of alterations found in more than 90% of SCLC patient tumors, recapitulates most of the pathophysiological features of the human disease [15]. GEMMs facilitated determining the roles for SCLC recurrent alterations, including MYCL1, MYC, RBL2, and PTEN [6, 16-23]. In this study, we tested a model of FGFR1 amplification in precancerous neuroendocrine cells (preSCs) that transform into SCLC upon activation of oncogenic drivers [19, 24]. We tested the requirement of FGFR1 for SCLC development using genetically engineered mouse models. Our findings suggest an oncogenic role for FGFR1 in development and continuing expansion of a subset of SCLC.

Results and Discussion

FGFR1, despite its recurrent amplification in SCLC patient tumors, is not amplified in the GEMMs of SCLC [3, 20]. Instead, Affymetrix gene-chip revealed a significant increase in *Fgfr1* transcript levels in murine SCLC cells relative to preSCs [19]. RT-qPCR validated the increased level of Fgfr1 transcript, and immunoblot confirmed the increase at the protein level in murine SCLC cells and primary tumors relative to preSCs and normal lung (Fig. 1A). This increased Fgfr1 expression in tumor cells relative to precancerous cells suggest a role for the growth factor receptor-mediated signaling in SCLC development. To determine whether increased Fgfr1 promotes tumor development, we tested its ability to transform preSCs. Lentiviral expression of Fgfr1, mimicking gene amplification, in preSC (Fgfr1-preSCs) increased the number of colonies formed in soft agar compared to control preSCs infected with an empty lentiviral vector (Fig. 1B, C). Immunoblot showing increased phosphorylation of both Erk1/2 and Akt, two of main signaling mediators downstream of Fgfr1, in Fgfr1-preSC relative to control preSCs upon Fgf2 treatment suggest that amplified Fgfr1 render the cells more responsive to the ligand (Fig. 1B). Fgfr1-preSC formed tumors in the flanks of immune-compromised nude mice at a faster rate than control preSC (Fig. 1D). The allograft tumors generated from Fgfr1-preSC showed histological features of SCLC including high nuclear/cytoplasmic ratio in hematoxylin and eosin (H&E) staining and positive staining for neuroendocrine markers including Calcitonin gene-related peptide (CGRP) (Fig. 1E). These findings demonstrated that Fgfr1 overexpression drives SCLC development by promoting neoplastic transformation of precursors.

To gain insight into the mechanism of increased Fgfr1-driven tumorigenesis, we examined a large-scale change in the molecular network using RNA-sequencing to compare Fgfr1-preSCs versus control. To identify pathways and gene sets related to increased Fgfr1 in preSCs, we performed gene set enrichment analysis (GSEA) of the whole gene expression profile for the 40 "Hallmark" genes sets from MSigDB [25]. E2F_TARGETS, G_2M_CHECKPOINT, MYC_TARGETS v1 and v2, and MITOTIC SPINDLE were significantly enriched in Fgfr1-preSC (nominal $p<0.05$, FDR-adjusted q-value <0.2) (Fig. 1F; Supplementary Fig. 1). This enrichment of proliferation-related gene sets may underlie the increased colony-forming ability of cells *in vitro* and the accelerated tumor formation in the allograft model. We identified a set of 2369 differentially expressed (DE) genes (FDR-adjusted q-value <0.05) (Fig. 1G; Supplementary Fig. 2; Supplementary Table 1). Gene ontology (GO) analysis of these DE genes using DAVID Bioinformatics Resources [26] indicated not only enrichment of cell proliferation-related changes, including mitosis, but also of neuronal development and differentiation (Fig. 1H; Supplementary Table 2). Notably, altered neural differentiation was the main indication of annotated functions for top 199 DE genes whose expression changes two-fold or higher in Fgfr1-preSC relative to control preSCs as four of five GO terms enriched are 'axon genesis', 'neuron projection development/morphogenesis', 'cell morphogenesis involved in neuronal differentiation' (FDR<0.2) (Supplementary Table 2). This molecular state reflects the downregulation of genes involved in neural differentiated and synapse formation, including *Nefl*, *Tbr1*, *Dcx*, and *Chl1* (Supplementary Table 2). The other GO term 'positive regulation of protein kinase cascade' reflects the up-regulation of receptor signaling and intracellular

mediators, including *Fgfr1*, *Prex2*, and *Rhoa*. These and other up-regulated DE genes, including *Sox21* and *Gli3*, may also be involved in altering differentiation of preSCs because they are implicated in neural progenitor cells and neurogenesis (Fig. 1G; Supplementary Fig. 2) [27-32]. Sox21 is a mediator of Sox2-driven cellular reprogramming and upregulated in SCLC [5, 33, 34]. Gli3 is a mediator of hedgehog signaling that is important for SCLC [35, 36]. Together, these findings suggest that increased Fgfr1 promotes SCLC development by altering differentiation of precancerous precursor cells, in addition to enhancing cell proliferation.

Inhibition of FGFR1 decreased the viability of several human SCLC cells (Supplementary Fig. 3) [13]. However, it remains unknown whether or not FGFR1 is important for SCLC development *in vivo*. To address this, we tested the effect of deleting *Fgfr1* on *Rb1/Trp53*-mutant model of SCLC development [15]. We infected *Rb1/Trp53* mice carrying additional floxed or wild type alleles of *Fgfr1* (*Rb1*$^{lox/lox}$; *Trp53*$^{lox/lox}$; *Fgfr1*$^{lox/lox}$ or *Fgfr1*$^{+/+}$) using intratracheal instillation of Ad-Cre and eight months later analyzed the lungs of the infected mice. Both groups of mice formed lung tumors with similar tumor burden (tumor area/lung area) and incidence (Fig. 2A), indicating that Fgfr1 did not influence SCLC development. However, the considerable variability in tumor incidence and latency in the *Rb1/Trp53* model could mask a moderate impact of Fgfr1 loss on tumor development. To further validate the impact of deleting Fgfr1, we repeated the *in vivo* experiment using *Rb1/Trp53/Rbl2*-mutant model in which a *Rb1* homolog, *Rbl2* (previously *p130*), is deleted together with *Rb1* and *Trp53* loss. This model mimics the loss or reduced expression of *Rbl2* in a subset of human SCLC tumors

[3, 37]. Deletion of *Rbl2* increased tumor incidence and shortened tumor latency compared to those of the *Rb1/Trp53* model, making *Rb1/Trp53/Rbl2* robust in determining potential tumor suppressive effects of genetic factor [3, 19, 21, 35]. Six months after Ad-Cre infection of these *Rb1/Trp53/Rbl2* mice with *Fgfr1*$^{lox/lox}$ or *Fgfr1*$^{+/+}$ alleles, we compared tumor development in the lungs. Interestingly, unlike the findings in the *Rb1/Trp53* model, the lungs of *Fgfr1*$^{\Delta/\Delta}$ *Rb1/Trp53/Rbl2* mice had reduced tumor burden compared to those of *Fgfr1*$^{+/+}$ *Rb1/Trp53/Rbl2* mice (Fig. 2B). To determine whether this tumor-suppressive effect is specifically related to *Fgfr1*, we tested the effect of deleting *Fgfr2* on tumor development using the *Rb1/Trp53/Rbl2* model and did not find significant phenotypic difference between the mice with or without *Fgfr2* (Fig. 2C). Furthermore, to exclude the possibility of incomplete recombination of the floxed alleles as a contributing factor to the phenotypes observed, genotyping PCR and immunoblot on four tumors randomly selected from three cohorts helped to verify the complete knockout of *Rb1*, *Trp53*, *Rbl2*, and *Fgfr1* in primary cells from all tumors but one tumor that retained one copy of *Fgfr1* floxed allele and expressed the protein at the level comparable to *Fgfr1*$^{+/+}$ tumor cells (Supplementary Fig. 4A). We acutely deleted Fgfr1 in these primary tumor cells with *Fgfr1*$^{lox/lox}$ by infecting them with Ad-Cre and tested their capacity for in vitro expansion and allograft formation (Fig. 3A, B). The infected *Fgfr1*$^{lox/lox}$ cells completely lost Fgfr1 expression (Supplementary Fig. 4B) and formed fewer colonies in soft agar culture and smaller tumors in the flanks of immune-competent mice than uninfected *Fgfr1*$^{lox/lox}$ cells or Cre-infected *Fgfr1*$^{+/+}$ cells (Fig. 3A, B). Immunoblot verified reduced expression of Fgfr1 in the allograft tumors and its complete loss in the primary cells (Supplementary Fig. 4C). These findings suggest that Fgfr1 is

important for SCLC tumors specifically driven by Rbl2 loss and the continuing expansion of SCLC tumor cells.

The differential impact of inhibiting Fgfr1 in the presence or absence of *Rbl2* suggests a mechanistic link between Fgfr1 and Rbl2. While the mechanism of the tumor-selective increase of Fgfr1 is not known, the relationship between Rbl2 and Fgfr1 during SCLC development may be similar to the mechanistic link between them in the context of muscle cell differentiation in which Rbl2 represses *Fgfr1* expression by binding E2F transcription factors at the gene promoter [38]. To test whether *Rbl2* loss increases Fgfr1 expression in *Rb1/Trp53* cells, we inactivated *Rbl2* in preSCs using CRISPR/Cas9-mediated gene targeting and injected targeted cells subcutaneously in the flanks of athymic nude mice (Fig. 4A). *Rbl2*-targeted preSCs, despite the near complete loss of Rbl2, did not readily increase Fgfr1 compared to non-targeted control in culture. Intriguingly, however, the primary cells from the subcutaneous tumors, generated from *Rbl2*-targeted preSCs, drastically induced Fgfr1 expression compared to those derived from non-targeted preSCs (Fig. 4B). To determine whether this relationship between Rbl2 and Fgfr1 is limited to Rbl2-mutant cells, we surveyed various subcutaneous tumors driven by different oncogenic alterations and lung primary tumors, and found that Fgfr1 expression is specifically induced in the cells lacking Rbl2 (Fig. 4C, D). Likewise, Rbl2 expression was inversely correlated with Fgfr1 expression. These findings suggest that the inactivation of Rbl2 plays a role in activating Fgfr1-driven pathway for SCLC development.

In conclusion, this study for the first time demonstrates the role of FGFR1 in promoting SCLC development in vivo and the continuing growth of tumor cells and suggests a mechanistic link between RBL2 and FGFR1. The concept of targeting FGFR1 has been tested on human SCLC lines in culture and xenograft model; however, the tumor-suppressive effects of FGFR1 inhibitors on SCLC lines varied [13]. For instance, a recent comprehensive drug screening also showed that only one out of 63 SCLC lines responded to three FGFR1 inhibitors. This finding and others alike suggest that the gene amplification, copy number gain, and mRNA/protein overexpression generally correlate with response to inhibitors but may not necessarily be a robust predictor for therapeutic effect. Therefore, the selective induction of FGFR1 and the specificity of the tumor-suppressive effect of its inhibition in Rbl2 null background raises an intriguing possibility that RBL2 status could be a potential biomarker for predicting response to FGFR1-targeted therapy [39].

Materials and Methods

This section is described in Supplementary Information.

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Figure Legends

Figure 1. Increased Fgfr1 promotes SCLC development

A, RT-qPCR data showing expression of *Fgfr1* transcript normalized to *ARBP P0* in SCLC cells or in preSCs (n=3). Right, Immunoblot for Fgfr1 in whole and different cell types. Gapdh blot verifies equal loading of total proteins. **B,** immunoblot for total and phosphorylated Erk1/2 and Akt as well as Fgfr1 in control and Fgfr1-preSCs treated with FGF2 (20ng/mL) for 36 hours. β-actin immunoblot verifies equal loading of total proteins. **C,** Representative images of control and Fgfr1-preSCs in soft agar 4 weeks after seeding $1x10^4$ cells per well (12 well). Right, quantification of colonies (>0.2mm) in diameter (n=3). Scale bar: 0.5mm. **D,** Image of athymic nude mice (n=5), 40 days after subcutaneous injection of cells; Arrow points to tumor. Right, quantification of tumor development at end-point (>1.5cm), measured by tumor weight (mg) divided by latency (day). **E,** Images of the subcutaneous tumors derived from Fgfr1-preSC stained with hematoxylin-eosin (H&E) and, right, with antibodies for calcitonin gene-related peptide (CGRP) and DAPI for nuclei. Asterisk indicates non-tumor area negative for CGRP. **F,** A summary of top 4 gene sets enriched in Fgfr1-preSC, identified in Gene Set Enrichment Analysis (GSEA). **G,** Volcano plot showing differential gene expression between control and Fgfr1-preSC; red horizontal line indicates adjusted *p*-value 0.05, and red dots are genes whose changes are 2-fold or higher). **H,** A summary of 38 gene ontology (GO) terms enriched in the set of genes upregulated in Fgfr1-preSC relative to control (FDR<0.05). Scale bars: C, 5mm; D, 2cm; E, left: 200μm, right: 50μm. NES: normalized enrichment score; FDR: false discovery rate.

Figure 2. Deletion of Fgfr1 suppresses SCLC development in vivo

A-C, Representative images of H&E stained sections of different SCLC models and, right, quantification of tumor burden (tumor area/lung area). (A) *Fgfr1*$^{+/+}$ *vs. Fgfr1*$^{\Delta/\Delta}$ *Rb1/Trp53* (n=7 and n=5, respectively) (B) *Fgfr1*$^{+/+}$ *vs. Fgfr1*$^{\Delta/\Delta}$ *Rb1/Trp53/Rbl2* (n=7 and n=9, respectively) (C) *Fgfr2*$^{+/+}$ *vs. Fgfr2*$^{\Delta/\Delta}$ *Rb1/Trp53/Rbl2* (n=8 and n=4, respectively). Arrows and arrowheads indicate tumors and small lesions, respectively. n.s.: not significant. Scale bars: 5mm.

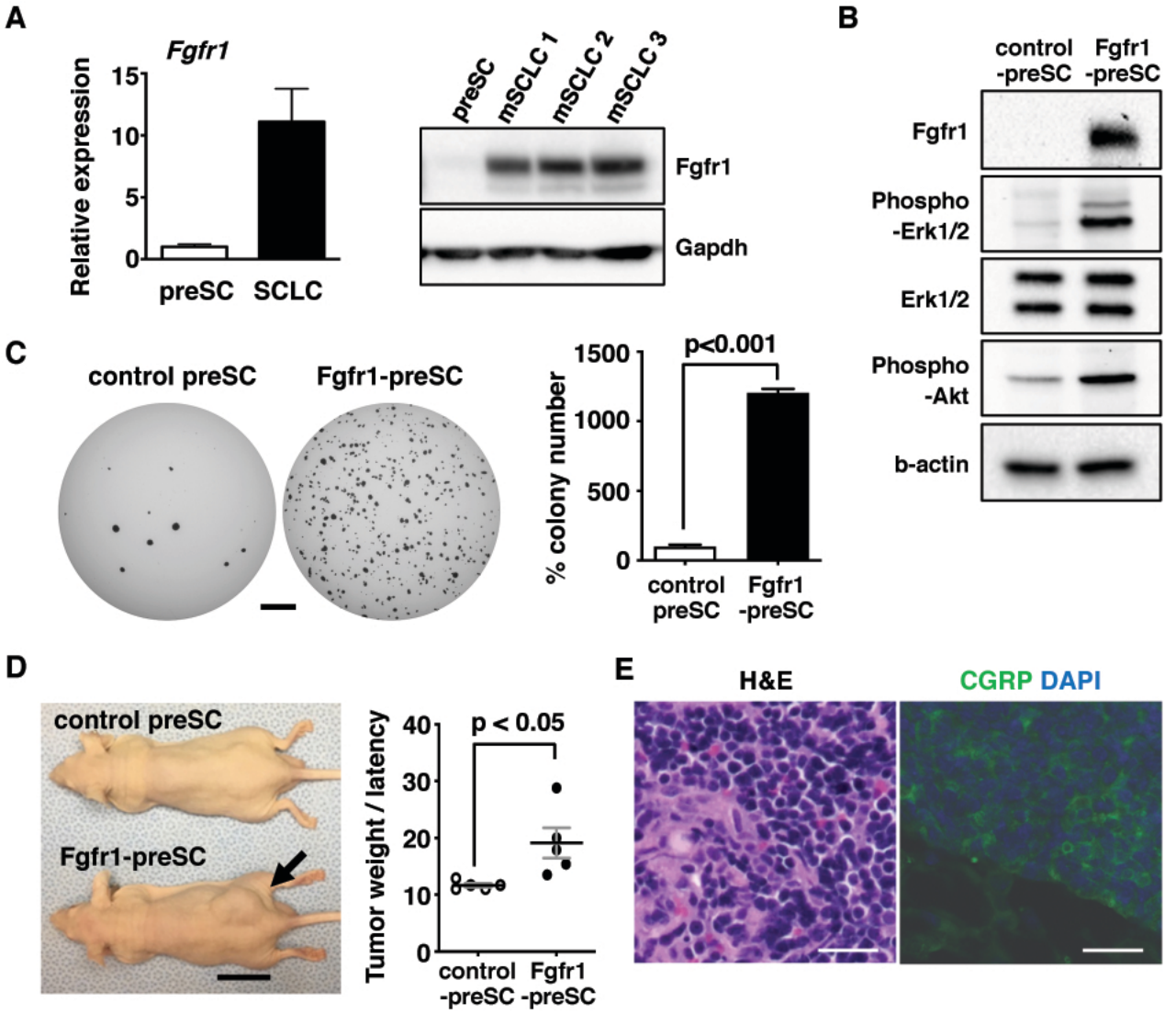
Figure 3. Fgfr1 is important for the continuing growth of SCLC cells.

A and **B,** Representative images of *Fgfr1*$^{lox/lox}$ and *Fgfr1*$^{+/+}$ *Rb1/Trp53/Rbl2* cells in soft agar 3 weeks after being infected with Ad-Cre and seeded at a density of $1x10^4$ cells per well (12 well). Right, quantification of colonies (>0.2mm in diameter; n=3). **C,** Plot of volumes of tumors (n=6) generated from subcutaneous injection of *Fgfr1*$^{lox/lox}$ *Rb1/Trp53/Rbl2* cells with or without Ad-Cre infection. Right, quantification of tumor weights at the end point (tumor size >1.5cm in any dimension).

Figure 4. Fgfr1 is selectively induced by Rbl2 loss.

A, Schematic of CRISPR-mediated targeting of preSCs and generation of subcutaneous tumors from targeted preSCs or control. **B,** Immunoblots for Fgfr1 and Rbl2 in control preSC and *Rbl2*-targeted preSC in culture and, right, in the primary cells of subcutaneous tumors. **C** and **D,** Immunoblots for Fgfr1 and Rbl2 in the subcutaneous tumors generated from control and various targeted preSCs (C) and in the primary lung tumors (D)

developed in the *Rb1/Trp53*-mutant GEMM. Asterisk in D indicates a non-specific band. β-actin blot verifies equal loading of total proteins.



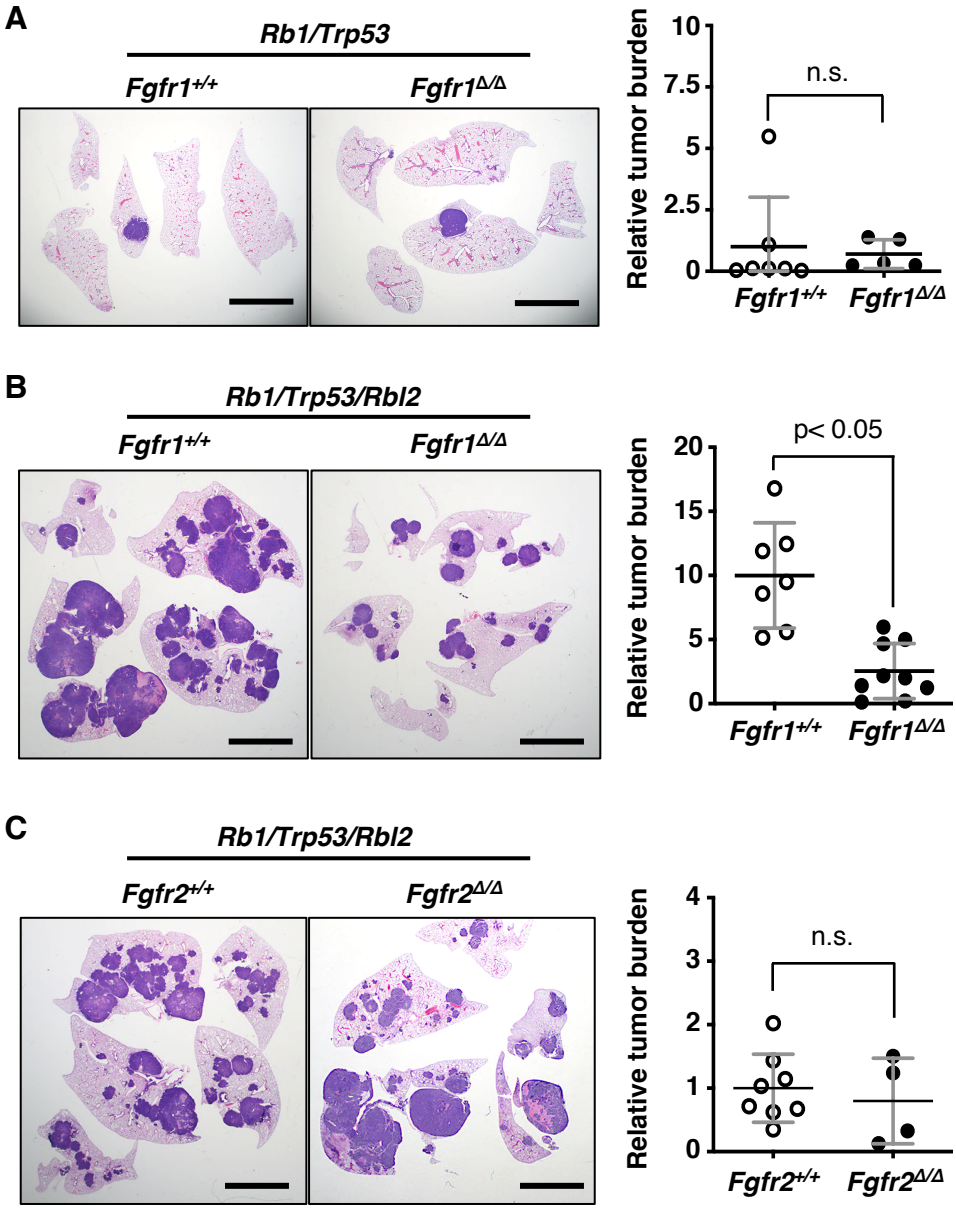
F

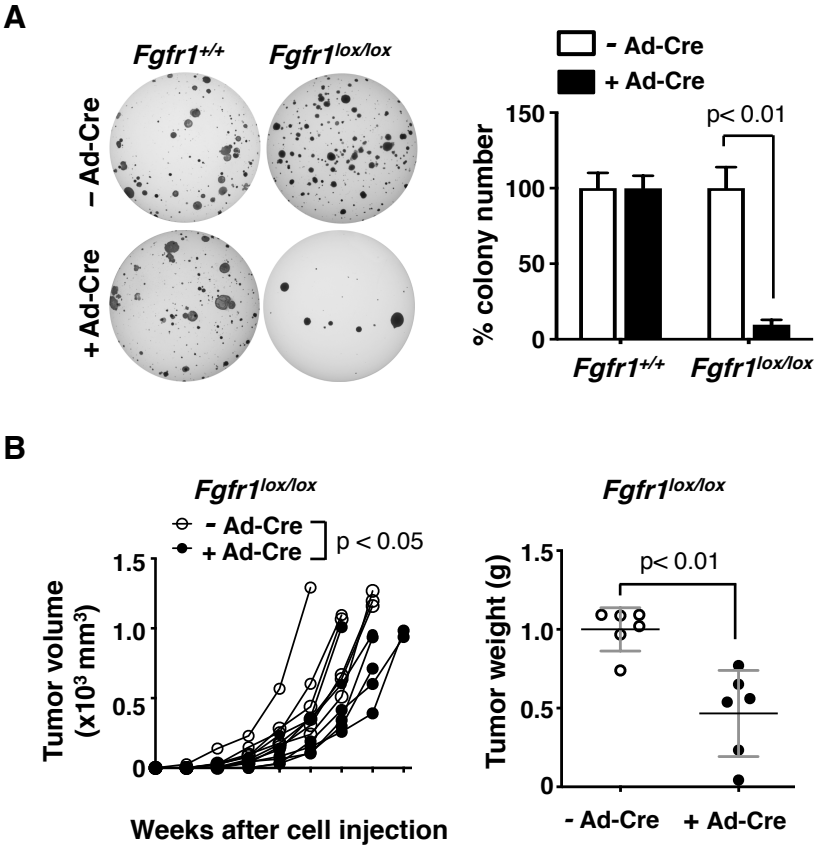
GSEA Gene Sets	NES	Nom. *p*-val	FDR *q*-val
G_2M_checkpoint	1.71	0.0204	0.0178
E2f_targets	1.80	0.0204	0.0194
MYC_targets_v1	1.63	0.0214	0.0287
MYC_targets_v2	1.63	0.0208	0.0389
Mitotic spindle	1.46	0.0754	0.178

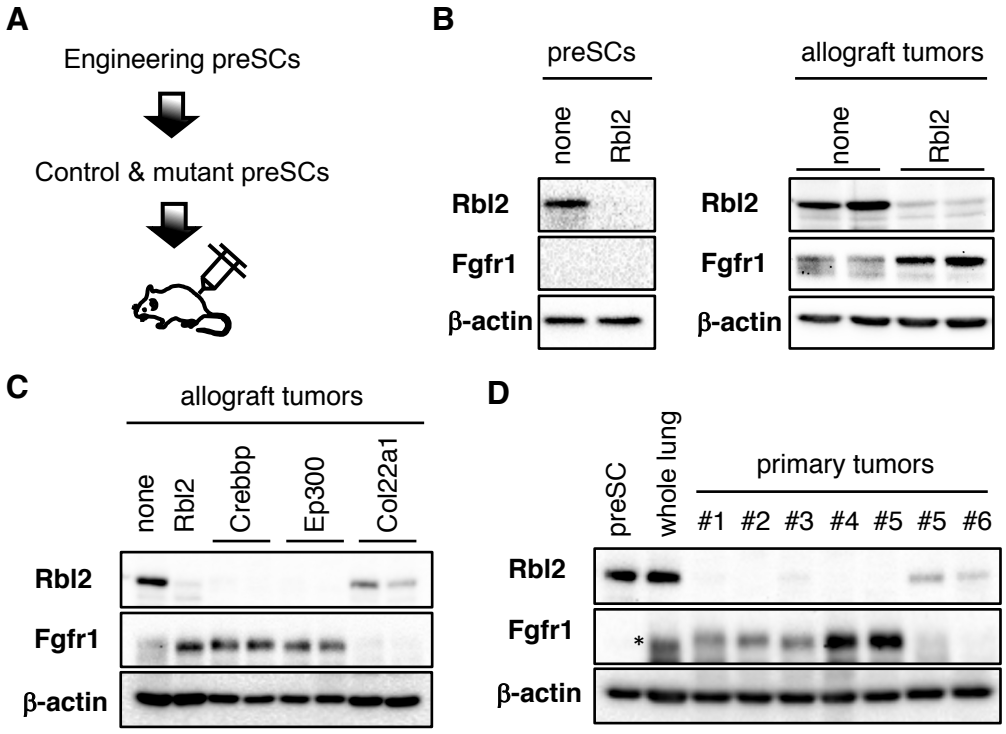
G

H

GO terms enriched in Fgfr1-preSC relative to control preSC	Go terms #
Cell cycle & mitosis	11
Neuronal differentiation & morphogenesis	8
Axon guidance & migration	5
Organelle fission & localization	5
Translation & RNA processing	4
Regulation of kinase cascade & phosphate metabolism	3
Other	2


A
Rb1/Trp53
Fgfr1+/+
Fgfr1Δ/Δ
Relative tumor burden
10
7.5
5
2.5
0
n.s.
Fgfr1+/+
Fgfr1Δ/Δ
B
Rb1/Trp53/Rbl2
Fgfr1+/+
Fgfr1Δ/Δ
Relative tumor burden
20
15
10
5
0
p< 0.05
Fgfr1+/+
Fgfr1Δ/Δ
C
Rb1/Trp53/Rbl2
Fgfr2+/+
Fgfr2Δ/Δ
Relative tumor burden
4
3
2
1
0
n.s.
Fgfr2+/+
Fgfr2Δ/Δ


A
Fgfr1+/+
Fgfr1lox/lox
– Ad-Cre
+ Ad-Cre
- Ad-Cre
+ Ad-Cre
p< 0.01
% colony number
150
100
50
0
Fgfr1+/+
Fgfr1lox/lox
B
Fgfr1lox/lox
- Ad-Cre
+ Ad-Cre
p < 0.05
Tumor volume (x10³ mm³)
1.5
1.0
0.5
0
Weeks after cell injection
Fgfr1lox/lox
p< 0.01
Tumor weight (g)
1.5
1.0
0.5
0
- Ad-Cre
+ Ad-Cre


A
Engineering preSCs
Control & mutant preSCs
B
preSCs
none
Rbl2
Rbl2
Fgfr1
β-actin
allograft tumors
none
Rbl2
Rbl2
Fgfr1
β-actin
C
allograft tumors
none
Rbl2
Crebbp
Ep300
Col22a1
Rbl2
Fgfr1
β-actin
D
preSC
whole lung
primary tumors
#1 #2 #3 #4 #5 #5 #6
Rbl2
Fgfr1
*
β-actin