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Supplemental Information

Sox2 Cooperates with Lkb1 Loss in a Mouse Model of Squamous Cell Lung Cancer

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Supplemental Data

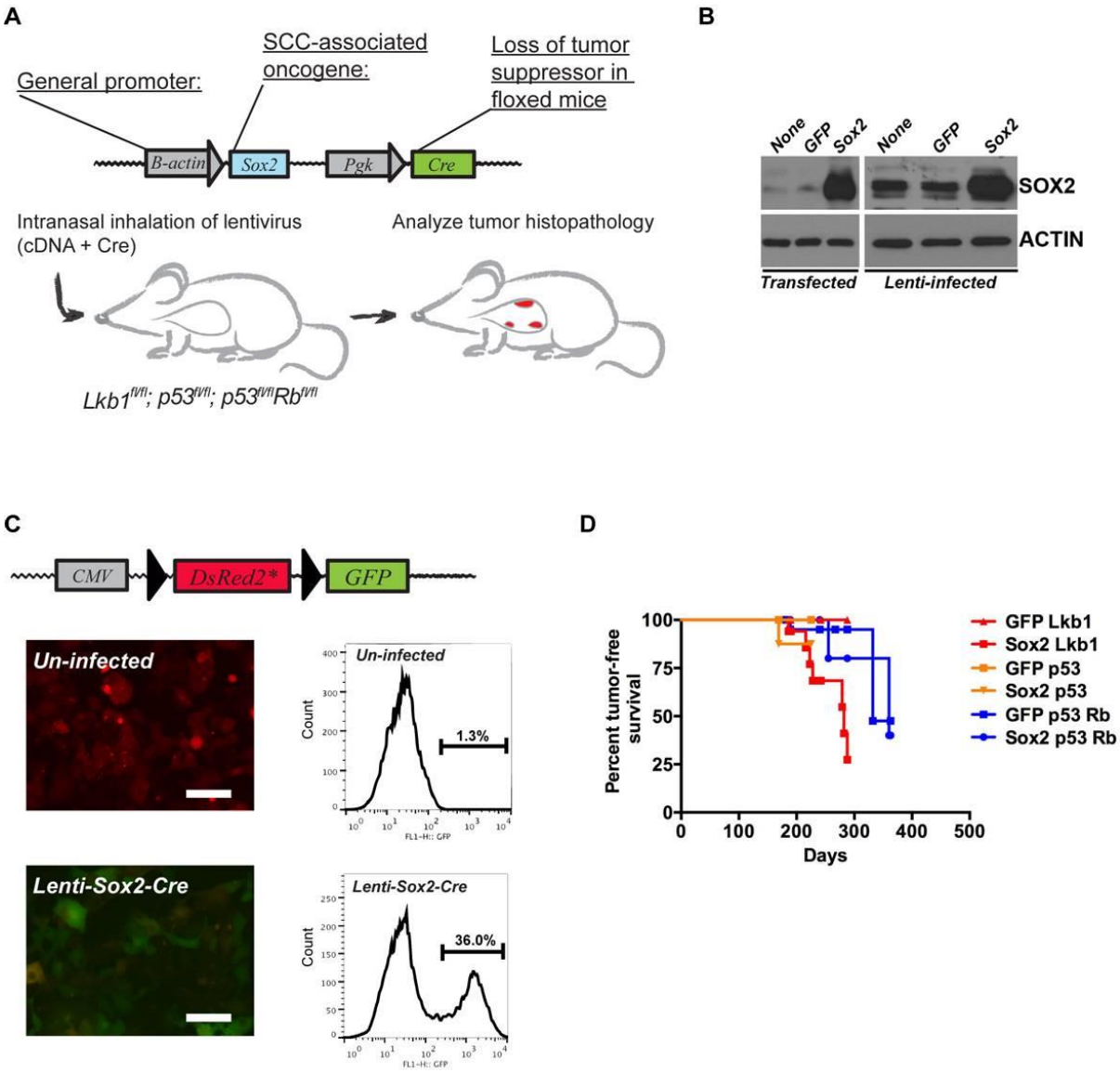

Figure S1. Lentiviral approach to identify combinatorial drivers of lung SCC, Related to Table 1 and Figure 1. (A) *Sox2* and *Cre recombinase* were delivered specifically to mouse lungs using intranasal inhalation of lentiviruses in the background of $Lkb1^{fl/fl}$, $p53^{fl/fl}$ or $p53^{fl/fl}Rb^{fl/fl}$ mice. A ubiquitous β-*Actin* promoter drives expression of *Sox2*, while a general *Pgk* promoter drives expression of *Cre recombinase*. (B) Immunoblot of Sox2 expression in

293T cells transiently transfected or lentivirally-infected with no vector (none), *Lenti-GFP-Cre* (GFP) or *Lenti-Sox2-Cre* (Sox2). ACTIN serves as loading control. (C) HEK293T cells stably express a *Lox-DsRed2(stop)-Lox-GFP* cassette such that GFP expression is an indication of successful Cre-mediated recombination. Black triangles in the cartoon indicate LoxP sites. * indicates a stop codon within *DsRed2*. Left panels: overlay image (green and red fluorescence) of un-infected or *Lenti-Sox2-Cre*-infected HEK293T cells. Scale bar represents 20 μM. Right panels: Viral titer (10^7) was calculated by quantifying # of GFP-positive cells by flow cytometry. HEK293T reporter cells were infected with and without *Lenti-GFP-Cre* or *Lenti-Sox2-Cre* and after 72 hours, GFP expression was quantified by flow cytometry and used to calculate viral titer. (D) Tumor-free survival curve from mice of genetic combinations indicated in Table 1. Mice were sacrificed upon detection of tumors by microCT.

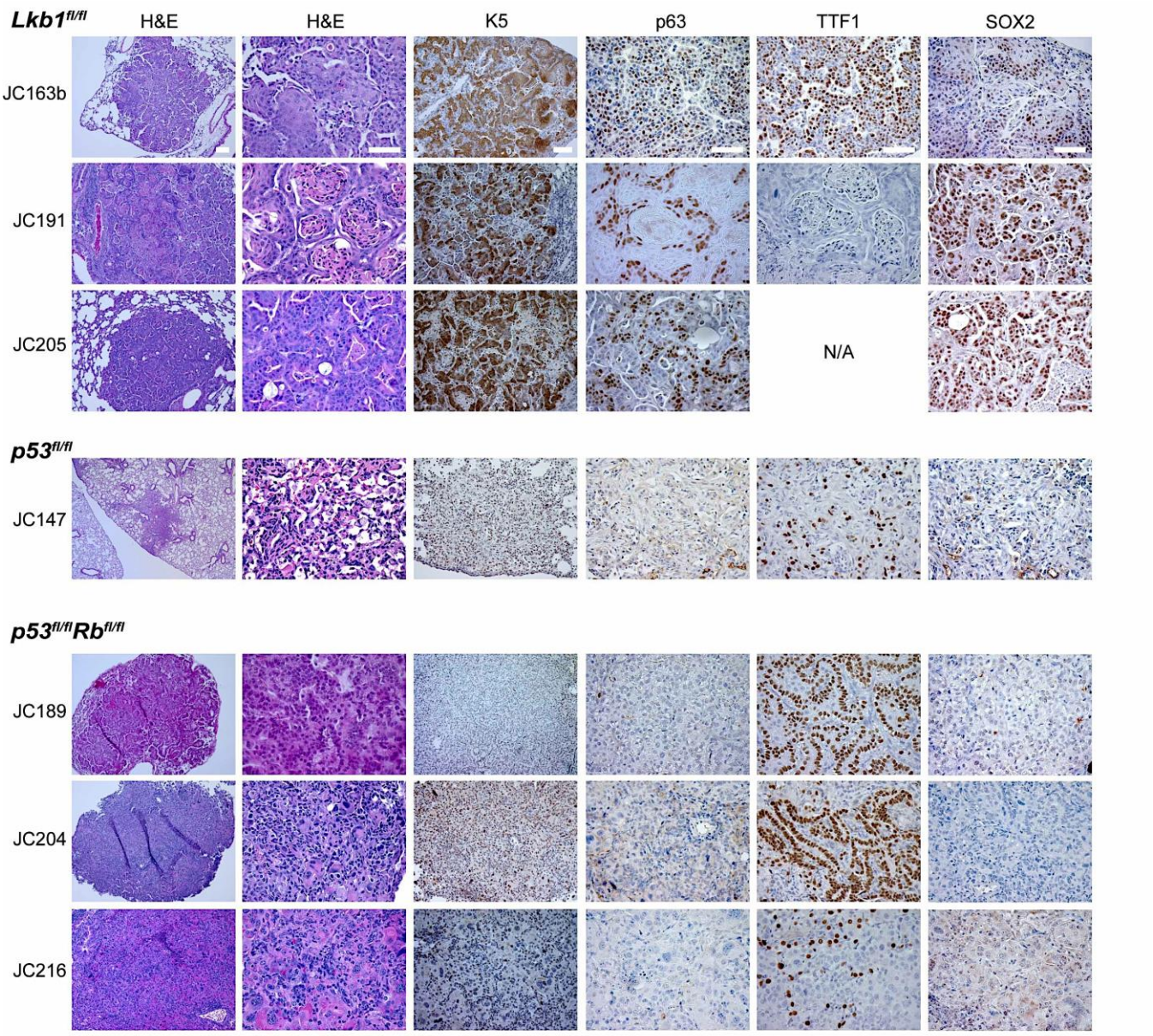

Figure S2. *Lenti-Sox2 Lkb1*$^{fl/fl}$ tumors express Sox2 and squamous biomarkers, Related to Figure 2. Representative H&E (10X, 40X), K5 (20X), p63 (40X), TTF1 (40X) and Sox2 (40X) IHC images from lung tumors of indicated mice from Table 1, grouped by genetic background (*Lkb1*$^{fl/fl}$, *p53*$^{fl/fl}$, or *p53*$^{fl/fl}$*Rb*$^{fl/fl}$). Mouse identification number (i.e. JC163b, etc.) corresponds to tumors described in Table 1. "N/A" indicates insufficient tissue available for IHC. Scale bar represents 50μm. Positive staining is brown, which is cytoplasmic for K5 and nuclear for Sox2, p63 and TTF1.

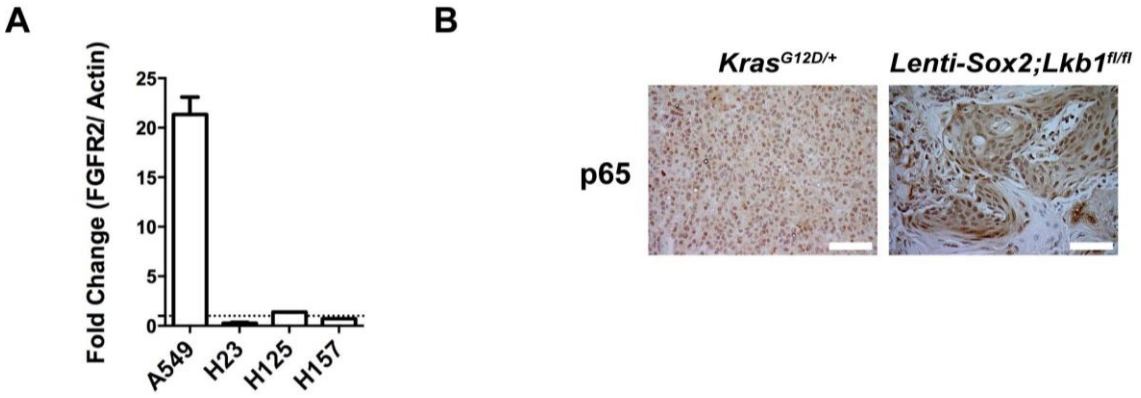

Figure S3. Signaling pathway activation related to *Lenti-Sox2 Lkb1*$^{fl/fl}$ tumors, Related to Figure 3.

(A) *FGFR2* mRNA levels in human lung cancer cell lines analyzed by real time RT-PCR and normalized to *ACTIN*. Data represents fold change in gene expression following 48 hr of SOX2 expression from Tet-On cells induced by doxycycline relative to un-induced cells. (B) Representative 40x IHC for NF-κB p65 in *Kras*$^{G12D/+}$ mouse lung adenocarcinomas and mouse *Lenti-Sox2 Lkb1*$^{fl/fl}$ tumors. Scale bar represents 50 μM.

Supplemental Experimental Procedures

Antibodies

The following antibodies were used for immunohistochemistry and immunoblotting:

Antibodies	Dilution	Companies	Cat. ID
Actin	1:1000	Sigma	A2066
p63	1:200	Santa Cruz Biotechnology	SC-8431
FGFR2	1:50	Santa Cruz Biotechnology	SC-6930
Krt5	1:2000	Covance	PRB-160P
Krt14	1:1000	Covance	PRB-155P
TTF1	1:200	Epitomics	EP1584Y
Sox2	1:250	EMD Millipore	MAB4423
NF-κB p65	1:50	Abcam	ab28835
Phospho-4E-BP1(Thr37/46)	1:800	Cell Signaling	2855
Phospho-AMPKα (Thr172)	1:50	Cell Signaling	2535
Phospho-Stat3 (Tyr705)	1:200	Cell Signaling	9145
Phospho-Akt	1:50	Cell Signaling	4060
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	1:400	Cell Signaling	4370
Nemo	1:1000	Santa Cruz Biotechnology	SC-8330
PARP	1:1000	Cell Signaling	9532

Western blotting

Total protein lysates or nuclear and cytoplasmic fractions prepared as previously described were separated via SDS-PAGE and transferred to a PVDF membrane (Oliver et al., 2010; Oliver et al., 2011). Membranes were blocked for 1 hr, followed by overnight incubation with primary antibodies at 4°C. Membranes were washed at room temperature in PBS-T. Mouse and rabbit HRP-conjugated secondary antibodies (Jackson ImmunoResearch, 1:10,000) were incubated for 1 hr at room temperature. For detection, membranes were exposed to WesternBright HRP Quantum substrate (Advansta) and detected on Hyblot CL film (Denville Scientific Inc).

Immunohistochemistry

Mice were sacrificed by carbon dioxide asphyxiation and lungs were inflated with PBS, fixed overnight in normalized buffered formalin (NBF), and transferred to 70% ethanol. Paraffin-embedded lung lobes were sectioned at 4 μm and stained with H&E for tumor pathology. Sections were dewaxed, rehydrated and subjected to high temperature antigen retrieval, 20 min boiling in a pressure cooker in 0.01 M citrate buffer, pH 6.0. Slides were blocked in 3% H202 for 15 min, blocked in 5% goat serum in PBS/0.1% Tween-20 for 1 hr, and stained overnight in 5% goat serum in PBS/0.1%Tween-20 with primary antibodies. A HRP-conjugated secondary antibody (Vector Laboratories) was used at 1:200 dilution in 5% goat serum in PBS-Triton, incubated for 45 min at room temperature, followed by DAB staining (Vector Laboratories). All staining was performed with Sequenza coverplate technology. p4EBP1, pAMPKα and pStat3 antibodies were diluted in SignalStain® Antibody diluent as recommended by Cell Signaling Technology and SignalStain® Boost (HRP, Rabbit) secondary antibody was used.

Flow cytometry

Cells were harvested with trypsin, washed twice in PBS and pelleted. Cells were reconstituted in PBS/1% BSA and analyzed on a BD FACScan flow cytometer. Live cells were gated based on forward and side scatter analysis and analyzed using FlowJo software.

Cell Lines

A549, H460, H157 and H125 were obtained from ATCC. A549 was maintained in DMEM/10%FBS and H460, H157 and H125 were maintained in RPMI/10%FBS. Human *SOX2* cDNA was cloned into Lenti-Tet-On vectors and viruses were prepared as described.

Stable inducible lines were created following two rounds of viral infection and selection in blasticidin. SOX2 was induced with 0.5 μM of doxycycline and cells were harvested at indicated time points. A549 (adeno), H23 (adeno) and H157 (squamous) carry *LKB1* mutations/deletions, H125 (adeno-squamous) has wildtype *LKB1* (Sanger COSMIC database).

PCR analysis of tumors

Genomic DNA was extracted and purified from macro-dissected frozen or formalin-fixed paraffin embedded (FFPE) lung tumor samples using DNeasy (Qiagen, #69504) or QIAamp DNA FFPE Tissue Kit (Qiagen, #56404), respectively. Frozen lung samples were homogenized prior to DNA extraction. For FFPE samples, 5μm aniline blue stained sections were cut from each tumor sample and tumors were carefully micro-dissected with a sterile scalpel and used for DNA extraction. DNA was quantified using NanoDrop 8000 and Qubit PicoGreen dsDNA High Sensitivity Kit. Genomic DNA from the tumor samples was then used to detect *Lkb1* recombination. PCR was carried out using the following primers; R1 5'-CTGTGCTGCCTAATCTGTCG-3', F2 5'-TTCACCATCCCTTGTGACTG-3' and F4 5'-ATCGGAATGTGATCCAGCTT-3'. Primers R1 and F2 detect the *Lkb1* floxed and wildtype alleles while R1 and F4 detects the recombined *Lkb1*.

Real time RT-PCR

RNA was isolated by TRizol (Invitrogen) as described (Oliver et al., 2010) and 1μg of total RNA was used to generate cDNA using Iscript cDNA synthesis kit (Bio-Rad). Real time PCR was performed using primers against human *FGFR2* with Sybr Green supermix (Bio-Rad) in a 20μl reaction in triplicate on a Bio-Rad CFX96 Real Time PCR machine. Analysis

was performed using Bio-Rad CFX manager software and expression values were based on 10-fold serial dilutions of standards and normalized to *ACTIN* levels.

Primers include:

Gene	Forward	Reverse
Actin	TATTGGCAACGAGCGGTTCC	GGCATAGAGGTCTTTACGGATGTC
FGFR2	CGGCCTCTATGCTTGTACTG	CGGTGTCATCCTCATCATCTC

Statistical Analysis

GraphPad Prism was used to perform statistical analysis. Error bars represent mean +/- SEM. Fisher's Exact test was used for statistical significance in the analysis of contingency tables in Table 1. For the statistical analysis of the IHC stains, column analysis was performed by Student's unpaired t test with Welch's correction. p value of < 0.05 was considered statistically significant.