

UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

The Role of the Salt-Inducible Kinase Family in Mediating LKB1-dependent Tumor Suppression in NSCLC

Permalink

<https://escholarship.org/uc/item/5m36g0z4>

Author

Galvez, Hector

Publication Date

2023

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of the Salt-Inducible Kinase Family in Mediating LKB1-dependent Tumor Suppression
in NSCLC

A Dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Biology

by

Hector M. Galvez

Committee in charge:

Professor Reuben J. Shaw, Chair
Professor Gen-Sheng Feng
Professor Randy Hampton
Professor Christian Metallo
Professor Alan Saghatelian

2023

©

Hector M. Galvez, 2023

All rights reserved.

The Dissertation of Hector M. Galvez is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2023

DEDICATION

This thesis work is dedicated to my wife, Lara, who provided the love, support, companionship, and encouragement needed to complete my studies; to my parents, who always reminded me that education is an invaluable resource to be pursued; and to my children, Bronson and Bonny, who provided me with a daily escape filled with unconditional love.

TABLE OF CONTENTS

DISSERTATION APPROVAL PAGE.....	iii
DEDICATION	iv
TABLE OF CONTENTS	v
LIST OF FIGURES	vii
LIST OF TABLES	xi
ACKNOWLEDGEMENTS.....	xii
VITA.....	xiv
ABSTRACT OF THE DISSERTATION.....	xv
CHAPTER 1: INTRODUCTION TO LKB1, THE AMPKRS, AND THE SIK KINASES	1
LKB1 IS A MASTER KINASE AND TUMOR SUPPRESSOR.....	1
LKB1 REGULATION OF THE AMPKRS	3
AMPK IS A MASTER ENERGY SENSOR.....	4
MARK KINASES CONTRIBUTE TO CYTOSKELETAL REMODELING AND EMT	6
BRSK KINASES ARE BRAIN SPECIFIC REGULATORS OF POLARIZATION	7
NUAK KINASES CONTRIBUTE TO CELLULAR POLARITY AND ADHESION	8
SNRK CONTRIBUTE TO ADIPOSITY MAINTENANCE AND INFLAMMATION	9
SIK KINASES CENTRAL TO TRANSCRIPTIONAL CONTROL AND GROWTH.....	9
LKB1 IN NON-SMALL CELL CANCER AND THERAPEUTICS	11
CHAPTER 2: THE AMPK-RELATED KINASES SIK1 AND SIK3 MEDIATE KEY TUMOR SUPPRESSIVE EFFECTS OF LKB1 IN NSCLC	16
ABSTRACT	16
INTRODUCTION.....	16
RESULTS.....	19
DISCUSSION.....	37

METHODS	42
ACKNOWLEDGMENTS.....	53
CHAPTER 3: SALT-INDUCIBLE KINASES MEDIATE LKB1-DEPENDENT TUMOR SUPPRESSION IN GENETICALLY ENGINEERED MOUSE MODELS OF NSCLC	88
ABSTRACT	88
INTRODUCTION.....	89
RESULTS.....	92
DISCUSSION.....	106
METHODS	111
ACKNOWLEDGEMENTS.....	116
REFERENCES	163

LIST OF FIGURES

Figure 1. 1: The LKB1-AMPKR Signaling Axis.....	13
Figure 1. 2: TCGA Data showing Alteration Frequency of LKB1 in Various Cancers	14
Figure 1. 3: Annotated Mutation sites on LKB1/STK11	15
Figure 2. 1: A lentiviral CRISPR-based screen targeting the AMPKR kinase family in human NSCLC cells reveals the SIK subfamily mediates tumor-suppressive phenotypes of LKB1.....	55
Figure 2. 2: CRISPR-based and genetically engineered mouse model (GEMM)-based mouse models of SIK family kinase inactivation in KRAS-driven NSCLC demonstrate acceleration of tumor growth upon SIK1 loss.	57
Figure 2. 3: Combined effect on tumor growth by genetic loss of Sik1 with CRISPR-mediated inactivation of Sik3 in a KRAS-driven mouse model of NSCLC.	61
Figure 2. 4: Transcriptional analysis of lung tumors derived from KSik1+sgSik3 and K+sgLkb1 mice reveal a shared core gene signature enriched for upregulation of genes involved in IL6-JAK-STAT and AP1 signaling pathways.	63
Figure 2. 5: Functional analysis of murine and human NSCLC cell lines bearing LKB1 or SIK kinase family inactivation reveals CRTC2-dependent induction of cytokine signaling and control of cell proliferation and growth under anchorage-free conditions.....	67
Supplemental Figure 2. 1: Characterization of A549 cells as a LKB1-dependent NSCLC to study effects of downstream targets on growth.	69
Supplemental Figure 2. 2: Loss of AMPK-related kinase Sik1, but not Mark4, accelerates Kras-driven lung tumor growth.	71
Supplemental Figure 2. 3: Analysis of pSECC sgRNAs in KP mice.....	72
Supplemental Figure 2. 4: Validation of Sik1 floxed allele in murine liver.	74
Supplemental Figure 2. 5: Supporting evidence that SIK1 is efficiently deleted in tumors from KSik1 mice. KSik1 tumors bear gene upregulation of LKB1-regulated transcripts.....	75
Supplemental Figure 2. 6: Analysis of tumor burden in K, KSik1, and KSik2 mice	77
Supplemental Figure 2. 7: Analysis of tumor burden in KP vs KPL vs KPSik1 mice	78
Supplemental Figure 2. 8: Proliferation and growth of GEMM tumor-derived KP cell lines deleted for SIK1 or LKB1.	79
Supplemental Figure 2. 9: Analysis of tumor burden in K vs K-Sik1 +/- sgRNAs against Sik1 and Sik3	80

Supplemental Figure 2. 10: Gene expression analysis of lung tumors isolated from K+sgTom, K+ sgLkb1, and KSik1+sgSik3 mice.	82
Supplemental Figure 2. 11: Deregulated AP1 and CREB signaling driven by SIK1 or LKB1 deficiency in 634T murine KP and human NSCLC cells.	84
Supplemental Figure 2. 12: SIK1/3 regulate IL-6/STAT signaling.....	85
Supplemental Figure 2. 13: Lack of kinase activity of SIK1 and SIK3 in cultured cells without LKB1	86
Figure 3. 1: Outline of Alleles in Genetic Mouse Models.	118
Figure 3. 2: Breeding Strategy to Generate the Combinatory Sik-floxed Mice.	119
Figure 3. 3: Validation of Combinatory Sik-Floxed Genetic Mouse Models by western blot.	120
Figure 3. 4: Bioluminescent imaging of early group Sik-null lung tumors reveals that loss of all three kinases matches growth kinetics of LKB1-null tumors in Kras-driven models.	122
Figure 3. 5: H&E histological sections of early group reveals how loss of all three Sik kinases enhance tumor growth and is the only Sik model that approaches KL model.	123
Figure 3. 6: Tumor area of the lung in KS model closely approached the KL model.	124
Figure 3. 7: Tumor sizes are similar after 12 weeks of tumor growth in all models with Sik1 inactivity.	125
Figure 3. 8: Increases in average tumor number in KS and KL models suggest they have higher ability to initiate tumors.	126
Figure 3. 9: Bioluminescent imaging of late group shows that the KS1/3 model has an intermediate phenotype.	127
Figure 3. 10: Percent tumor area in the KS1/3 models significantly increased compared to all other models 20 weeks post-cre.....	128
Figure 3. 11: Tumors sizes in the KSik1/3 models were significantly larger compared all other models 20 weeks post-cre.....	129
Figure 3. 12: Conditional Sik-floxed models show no differences in tumor number 20-weeks post cre.	130
Figure 3. 13: Longitudinal study of single floxed conditional Sik KO models take 30 weeks reach clinical endpoint.	131

Figure 3. 14: BrdU incorporation is highest in KL tumors despite similar KS tumor sizes and burden.	132
Figure 3. 15: BrdU incorporation is highest among the smallest KL tumors from the early group.	133
Figure 3. 16: Immunohistochemistry for neutrophil specific, neutrophil elastase, marker shows an increase in tumor associated neutrophils in KL but not KS models.	134
Figure 3. 17: KL tumors have high infiltration of neutrophils and is not mediated through the Siks.	135
Figure 3. 18: Expression of the Cxcl1/KC cytokine, the predominant neutrophil recruiting cytokine, is reduced after loss of the Sik kinases.	136
Figure 3. 19: Neutrophil recruiting cytokines, Cxcl2 (Mip2) and Cxcl5, expression is decreased in a Sik dependent manner.	137
Figure 3. 20: Model showing how the Lkb1-Sik axis regulates transcription by inhibiting two well studied transcriptional co-activators.	138
Figure 3. 21: Venn diagrams of up or down regulated differentially expressed genes from Sik genetic models.	139
Figure 3. 22: Volcano plots of DE expressed genes in tumors from Sik-genetic models....	140
Figure 3. 23: Principal component analysis (PCA) of the top 500 variable RNA-seq genes across tumors collected from the 7 genetic models.	141
Figure 3. 24: Heat map of top 500 most variable genes from Sik genetically engineered mouse models.	142
Figure 3. 25: Gene ontology analysis of 5-cluster heat map.	143
Figure 3. 26: Principal component analysis of Kaufman 16-Gene classifier in tumors from Sik-null genetic models.	144
Figure 3. 27: Heat map of Kaufman-16 gene classifier in Sik-null tumor genetic models. ..	145
Figure 3. 28: Heat map of epithelial to mesenchymal transition (EMT) gene signature proposed by Hollstein et al ¹² to be Lkb1-Sik regulated analyzed in genetic models.	146
Figure 3. 29: Heat map of genes categorized as hallmark epithelial to mesenchymal transition.	147
Figure 3. 30: Heat map of hallmark Il6-Jak-Stat gene set proposed by Hollstein et al ¹² to be Lkb1-Sik regulated analyzed in genetic models.	149

Figure 3. 31: Heat map of genes containing AP1 and CRE sites at their promoter region proposed by Hollstein et al ¹² to be Lkb1-Sik regulated analyzed in genetic models.....	150
Figure 3. 32: Heat map of bonafide CREB targets identified by RNA-seq and ChIP-seq in A549 lung tumor cell lines from Rodon et al ¹⁴¹ analyzed in genetic models.	151
Figure 3. 33: Curated heat map showing genes from cluster 1 which are upregulated in a Lkb1/Sik dependent manner, but remain unchanged in KP model [Cluster 1.1].....	152
Figure 3. 34: Gene Set enrichment analysis of 54 genes from cluster 1.1 show several polycomb repressive complex 2 (PRC2) subunit targets.	153
Figure 3. 35: Expression of PRC2 subunits, Eed and Ezh2, and countering demethylase UTX whose expression may modulate PRC2 complex activity.	154
Figure 3. 36: Western blot of PRC2 complex subunits in KP mouse lung cancer cell line clones with Lkb1 or Sik1/Sik2/Sik3 knockouts.	155
Figure 3. 37: Enrichment plot of Yap dependent genes show enrichment in KL tumor cells.	156
Figure 3. 38: Heat map of Yap-dependent genes identified from Yap-deleted EPCAM+ lung epithelial tissues.	157
Figure 3. 39: Ultra-seq screen <i>in vivo</i> target gene list and method of identification.....	158
Figure 3. 40: Schematic for ongoing Ultra-seq experiment targeting Lkb1/Sik-dependencies using <i>in vivo</i> Cas9 mouse models.....	159

LIST OF TABLES

Supplemental Table 2. 1: CRISPR sgRNA guide sequences.....	87
Table 3. 1: Cluster assignments from figure 3. 21 RNA-seq heat map.....	160

ACKNOWLEDGEMENTS

I would like to formally thank my advisor Dr. Reuben J. Shaw for giving me the opportunity to perform research in his lab over last 6 years. Without his persistence and dedication to the projects outlined in this thesis, and his trust in me to complete them, none of the data gathered here would be possible. Beyond the science, he has been a generous and compassionate mentor, allowing me the flexibility in lab to be able to grow my family during graduate school. I beyond grateful to him, the lab he has built, and the people he has brought together to perform great research.

I'd also like to thank my committee for their thoughtful guidance throughout the process of my Ph.D. studies. Christian Metallo, Alan Saghatellian, Randy Hampton, and Gen-Sheng Feng all provided great mentorship through the development of this thesis. Their outside perspective enhanced all aspects of my work, and their encouragement kept me motivated and engaged in the process.

Special thank you to all the current and past members of the Shaw lab. Post-docs Sébastien Herzig and Robert Svensson served as early mentors to my project in *de novo* lipogenesis dependencies in cancer, and were always up for leaving at lunch time to hit some golf balls. I feel lucky to have had the opportunity to work side by side with my fellow graduate students in the Shaw Lab Stephanie Curtis, Joshua Baumgart, Hoda Ahmed, and Anwesh Kamireddy. All through my studies, they were points of inspiration and perseverance, as we shared the “we can do this” attitude at our highest and lowest points. Post-docs Pablo Hollstein and Lillian Eichner were instrumental in the early design and implementation of the experiments outlined in this dissertation. Finally, thank you to Post-docs Elijah Trefts and Christopher Murray, who both provided critical experimental ideas and approaches in my projects later stages. This list is not exhaustive, and I would like to extend extreme gratitude to all current and past researchers in the Shaw lab, as well all the administration and

technician help through the years. I appreciate all the mentoring, support, and friendships made in the Shaw lab.

Beyond the lab, I could not have completed this Ph.D. without the support of my family and longtime friends. Since I was a child, my parents have promoted education as being the key to success, and they have inspired me with their strong work ethic. Special thanks to my wife Lara, whose encouragement was the fuel that propelled me every day, especially in the later stages of dissertation writing. Lastly, thank you to my circle of friends, new and old, which offered an escape from science.

Chapter 2, in full, is a reprint of the material as it appears in *Cancer Discover*, 2019. Hollstein, P. E., Eichner, L. J., Brun, S. N., Kamireddy, A., Svensson, R. U., Vera, L. I., Ross, D. S., Rymoff, T., Hutchins, A., Galvez, H. M., Williams, A. E., Shokhirev, M. N., Screatton, R. A., Berdeaux, R. & Shaw, R. J. The AMPK-related kinases SIK1 and SIK3 mediate key tumor suppressive effects of LKB1 in NSCLC. *Cancer Discov* 9, 1606–1627 (2019). On this publication, Pablo Hollstein was the primary author and the dissertation author was a co-author. Reuben J. Shaw directed and supervised the writing and oversaw the project which formed the basis for this chapter.

Chapter 3, in part is currently being prepared for submission for publication of the material. Galvez, H.G.; Murray, C.W.; Hollstein, P.E.; Baumgart, J.T.; Brun, S.N.; Ianniciello, A.; Rymoff, TJ; Ross, D.S.; Eichner, L.J.; Shaw, R.J. The dissertation author was the primary investigator and author of this material.

VITA

2016 Bachelor of Science in Biology, California State University San Marcos

2023 Doctor of Philosophy in Biology, University of California San Diego

PUBLICATIONS

1. Kumar A, Kuna R, **Galvez H**, Wessendorf-Rodriguez K, Green C, McGregor G, Cordes T, Shaw R, Svensson R, Metallo C. Inter-organelle crosstalk supports acetyl-coenzyme A homeostasis and lipogenesis under metabolic stress. *BioRxiv*. 2022 September; doi: <https://doi.org/10.1101/2022.09.24.509326>
2. Hollstein P, Eichner L, Brun S, Kamireddy A, Svensson R, Vera L, Ross D, Rymoff T, Hutchins A, **Galvez H**, Williams A, Shokhirev M, Screatton R, Berdeaux R, Shaw R. The AMPK-Related Kinases SIK1 and SIK3 Mediate Key Tumor-Suppressive Effects of LKB1 in NSCLC. *Cancer Discovery*. 2019 November; 9(11):1606-1627. PMID: PMC6825547
3. Aron M, Allen A, Kromer M, **Galvez H**, Vigil B, Jancovich J. Identification of essential and non-essential genes in *Ambystoma tigrinum* virus. *Virus Research*. 2016 June; 217:107-114. PMID: PMC4985175

ABSTRACT OF THE DISSERTATION

The Role of the Salt-Inducible Kinase Family in Mediating LKB1-dependent Tumor
Suppression in NSCLC

by

Hector M. Galvez

Doctor of Philosophy in Biology

University of California San Diego, 2023

Professor Reuben J. Shaw, Chair

Mutations in the Kirsten ras oncogene homolog from the mammalian ras gene family (KRAS) are often observed in patients suffering from several forms of cancers. These activating mutations in KRAS are suggested to be implicated in approximately 30% of non-small cell lung cancers (NSCLC). Among these patients with activating KRAS mutations, the most prevalent co-mutation occurs with the tumor suppressor serine/threonine kinase 11 (STK11), also known as Liver kinase B1 (LKB1). Co-mutations in KRAS and LKB1 make the

tumors hyperaggressive and modulates the sensitivity to various therapeutics. The function of LKB1 *in vivo* is diverse, and is central to cellular metabolism, polarity, and growth. LKB1 phosphorylates and activates 14 downstream kinases known as the AMPK-related kinases (AMPKRs), which upon several known and unknown stimuli, control these above-mentioned cellular processes. While several seminal findings have been made regarding KRAS-LKB1 (KL) driven NSCLC, one of the most significant findings showed loss of the salt-inducible kinase family (SIKs), specifically SIK1 and SIK3, enhanced KRAS driven tumor growth, and approached that of the loss of LKB1. Here we show how loss of all three SIK family members in Kras-driven (KS) genetically engineered murine models of NSCLC leads to enhanced tumorigenesis, and most closely resembles the KL tumor growth phenotype. We further characterize distinct biological overlaps between KL and KS tumors, to show to what extent the SIKs mediate Lkb1-dependent tumor biology and tumor suppression. Transcriptional analysis of these tumors revealed expression differences in several well characterized pathways, which changed in common in both KL and KS tumors. Ultimately we aimed to target several enriched pathways or individual genetic targets using an *in vivo* Cas9 screen, in order to uncover LKB1/SIK dependencies that can serve as future potential therapeutic or pharmacological targets for patients suffering from LKB1 mutations in NSCLC.

CHAPTER 1: INTRODUCTION TO LKB1, THE AMPKRS, AND THE SIK KINASES

LKB1 IS A MASTER KINASE AND TUMOR SUPPRESSOR

Cellular signaling is critical for the sensing of extracellular stimuli such as nutrient availability, pro-growth signals, inhibitory growth signals, and cell death signals, and passing this information to intracellular secondary messengers. One such way signals are propagated though the cell involves the phosphorylation of a protein target, which changes its structure and/or its activity. The transfer of phosphate groups are catalyzed by a class of enzymes called protein kinases. To date, over 500 unique kinases have been discovered, and they play a crucial role in most biological processes^{1,2}. One such kinase, whose biology is the focus of this dissertation, the liver kinase B1 (LKB1, also known as STK11), is a master kinase which phosphorylates and activates 14 downstream kinases in the Ca^{2+} /calmodulin-dependent protein kinase (CAMK) superfamily of kinases. LKB1's function as a kinase is dependent on its ability to form a heterotrimeric complex with the calcium binding protein-39 (MO25) and STE20 related adaptor (STRAD)(Figure 1.1)³. Studies have reported that LKB1 is phosphorylated at various regulatory sites by ataxia telangiectasia mutated serine/threonine kinase (ATM) at T366, proline-directed kinase at S325, or cAMP-dependent protein kinase A (PKA) and p90 ribosomal S6 protein kinase (RSK) at S431, however mutations to any of these sites have yet to inhibit LKB1's ability to phosphorylate and activate its downstream kinases⁴⁻⁶. Several autophosphorylation sites are present on LKB1 (T185, T189, T336, and S404), but similarly, mutations to these sites also led to no alterations to its kinase activity⁴⁻⁶. Rather, LKB1 function appears to be directly tied to the activity or direct interaction with MO25 and STRAD.

Direct interaction of MO25 and STRAD was identified in both yeast and mammalian cells by two-hybrid analysis and affinity purification, respectively⁶⁻⁸. STRAD and MO25 each have two

isoforms. STRAD α and STRAD β , two pseudokinases, have high sequence homology to other STE20 family members, yet lack the sequence preserving their kinase function required to phosphorylate target proteins⁷. MO25 α and MO25 β have no sequence homology to any other proteins, however they are highly conserved across species suggesting they serve a critical function⁹. LKB1 is activated by the binding of STRAD and MO25 in the cytoplasm^{6,7,10}. While the pseudokinase domain of STRAD lacks the ability to phosphorylate, its tertiary structure allows for its binding to the kinase domain of LKB1. Furthermore, it was noted that single residue mutations on LKB1 within the kinase domain was sufficient to abolish STRAD binding, and LKB1 activation¹⁰. Rather than bind directly to LKB1, MO25 interacts with the final three C-terminal residues on STRAD⁶. Although STRAD can bind LKB1 in the absence of MO25, affinity of STRAD:LKB1 is greatly enhanced in the presence of MO25⁶. Lastly, it was also discovered that the LKB1:STRAD:MO25 complex can form in the absence of the last 3 C-terminal residues of STRAD, suggesting that LKB1 can directly bind both subunits^{6,10}. Thus the LKB1:STRAD:MO25 complex, and its kinase activity, can be toggled by the relative abundance of each of the protein subunits.

LKB1:STRAD:MO25 complex activity may be regulated, in some unknown fashion, by the localization of LKB1 and/or MO25 and STRAD, since LKB1 retains a nuclear localization sequence (NLS), yet primarily phosphorylates its targets in the cytoplasm. It has been observed that LKB1 activity is elevated in conditions of anchorage independent growth (anoikis). While the mechanism remains unknown, increased activation of LKB1 is observed in soft agar, as well as cells detached with trypsin, EDTA, PBS, and mechanical shaking^{11,12}. Suggesting that anoikis alone, rather than a chemical treatment, increases LKB1 activity. C-terminal farnesylation localizes LKB1 to the cell membrane, where it is suggested to contribute to epithelial to mesenchymal transition (EMT), however, farnesylation alone does not impact its kinase activity¹³. Rather, membrane localization upon detachment, may be required to activate LKB1

via an unknown mechanism, directly connecting cellular membrane dynamics (such as those present during EMT) or an extracellular signal, to LKB1 signaling.

Mutations to LKB1 have been attributed to the development of the rare autosomal dominant disease Peutz-Jeghers Syndrome (PJS)^{14,15}. Patients suffering from PJS are characterized by the hyperpigmentation of the hands, lips, and feet, and have a high incidence of benign gastrointestinal hamartomas polyp formation^{14,15}. However, patients that suffer from this condition have a higher probability of developing many different forms of aggressive cancers later in life. Since the association between PJS and LKB1 has been made in the late 90s, LKB1 has been recognized as a powerful tumor suppressor. Mutations in LKB1 are observed in several cancers including adenocarcinomas, cervical cancers, cholangiocarcinomas and adrenocortical carcinomas (Figure 1.2)^{16,17}. Mutations to LKB1 in patients most often occur within the kinase domain, suggesting that these patients lose kinase function of LKB1 (Figure 1.3)^{16,17}. If the kinase function of LKB1 is required, it could be hypothesized that its ability of LKB1 to phosphorylate one or more of its downstream targets is critical to maintaining its tumor suppressive function. Thus, understanding how LKB1 functions as a kinase and how it regulates its downstream kinases is of critical importance. In particular, understanding how the phosphorylation or activation status of its targets mediated LKB1-dependent tumor suppression.

LKB1 REGULATION OF THE AMPKRS

LKB1 phosphorylates and activates 14 downstream kinases known as the AMPK-related kinases (AMPKRs), named after the prototypical member AMPK^{18,19}. The AMPKRs can be further divided by families, which contain high sequence and structural homology to each other, as well as to the other AMPKRs outside families. These include the SNRKs, BRSKs, NUAKs, MARKs, AMPK, and the SIKs. Phosphorylation of serine/threonine residues at the conserved T-loop

region mediates the AMPKRs activation¹⁹. The AMPKRs collectively govern critical cell processes including cell growth, metabolism, cell polarity, and transcriptional regulation.

AMPK IS A MASTER ENERGY SENSOR

The need to discover the function of LKB1 as a tumor suppressor led to the discovery of the prototypical AMPKR, AMP-activated protein kinase (AMPK), as a bonafide LKB1 target²⁰⁻²³. AMPK exists as a heterotrimeric complex, containing the catalytic subunit (AMPK α), and two regulatory subunits (AMPK β and AMPK γ)(Figure1.1). The AMPK complex can sense changes to the ATP:AMP ratio, and respond when they decrease, leading to the induction of energy producing processes and the inhibition to energy consuming processes. AMPK activation, either in response to energy depletion or allosteric activators, leads to increases in glycolysis, autophagy, and lipid β -oxidation, and the inhibition of glycogen storage, protein synthesis, and lipid synthesis²⁴. Changes to the ATP:AMP ratio have been noted to be affected by nutrient deprivation as well as hypoxia in culture²⁵. To date, none of the other 12 AMPKRs have been observed to become activated in response to energy stress^{19,26,27}.

Activation of AMPK by LKB1, leads to the phosphorylation and inhibition of the rate limiting steps in *de novo* lipogenesis (DNL) and cholesterol synthesis, acetyl-CoA carboxylase 1 (ACC1), ACC2, and HMG-CoA reductase (HMGCR)^{28,29}. Thus, diverting metabolite pools toward energy restoration processes and away from energy storage processes. Inhibition of cytosolic ACC1 is thought to provide the majority of DNL inhibition, while inhibition of the mitochondrial bound ACC2 depletes local sources of malonyl-CoA, an allosteric inhibitor to CPT1, the rate limiting step in β -oxidation^{28,30}. All leading to increases in fatty acid (FA) oxidation.

AMPK also activates autophagy as a mechanism to restore energy homeostasis. Direct phosphorylation of Unc-51 like Autophagy Activating Kinases (ULK1) at S555 under conditions of nutrient starvation is required for ULK1 dependent autophagy initiation and co-operates with

direct phosphorylation of mitochondrial fission factor (MFF) at S172^{31,32}. Ultimately, these two phosphorylation events activate global autophagy (ULK1), as well as primes mitochondria for mitophagy (MFF) by recruiting Dynamin-related protein 1 (Drp1), leading to mitochondrial fission. AMPK-ULK1 activation further phosphorylated Parkin to mediate Parkin-Pink1 induced mitophagy^{31,33}. These two processes work in concert to mitigate autophagic processes at the protein level, however, AMPK also controls autophagy via transcriptional regulation of the Coordinated Lysosomal Expression and Regulation (CLEAR) gene network, via Transcription Factor EB (TFEB) and Transcription Factor Binding to IGHM Enhancer 3 (TFE3). Transcriptional regulation of the CLEAR gene network is particularly important for supporting tumor growth *in vivo* using Kirsten ras oncogene homolog from the mammalian ras gene family (KRAS) driven genetic mouse models of NSCLC harboring floxed alleles to AMPK α 1 and AMPK α 2, such that loss led to decreases in tumor size^{34,35}. It had been hypothesized for years that AMPK was the main AMPKR that mediated LKB1-dependent tumor suppression, however the *in vivo* findings challenged these beliefs.

AMPK inhibits the mammalian target of rapamycin complex 1 (mTORC1) via direct phosphorylation and activation of the tuberous sclerosis complex subunit 2 (TSC2) tumor suppressor, directly linking the LKB1-AMPK signaling axis to cancer^{36,37}. Phosphorylation of TSC2 prevents heterodimerization with TSC1, its obligate partner for enzymatic activity, leading to the indirect hyperactivation of mTORC1 and its pro-growth signaling^{38,39}. In TSC2 null conditions, energy stress was still able to reduce mTORC1 activity, suggesting there was an AMPK-dependent regulatory step downstream of TSC2. Indeed, AMPK was also discovered to directly phosphorylate the mTORC1 subunit raptor, induce 14-3-3 binding, and decrease mTORC1 dependent signaling⁴⁰. These findings cemented AMPK as a regulator of not only metabolism, but of cell growth, and had all the make-up of being a tumor suppressor via the dual inhibition of mTOR. However, the requirement for AMPK to support lysosomal biogenesis

takes precedent over its ability to serve as an inhibitor of cell growth through mTOR inhibition in models of NSCLC. Further studies are needed to address whether AMPK has a tumor suppressive role in other tissues, especially those that rely heavily on the activity metabolic control of AMPK, such as the pancreas and liver.

MARK KINASES CONTRIBUTE TO CYTOSKELETAL REMODELING AND EMT

Of the 14 AMPKR kinases under the control of LKB1, 4 of them are part of the microtubule affinity-regulating kinase (MARK) family. Appropriately named, MARK1-4, these kinases are closely related to the *C.elegans* homolog *par-1* kinase, which has been observed to be required for maintaining cellular polarity in early embryonic development and oogenesis in *D. melanogaster*^{41,42}. Disruption in *lkb1* and *snfla* (which codes for AMPK in *drosophila*), was also sufficient to disrupt cell polarity during embryonic development, suggesting the LKB1 pathway in mammalian cells could regulate aspects of polarization^{43,44}. LKB1's effect on polarity has been directly connected to several mammalian tissues including colon cancer, lung cancer, and MDCK cell lines, as well as in pancreatic epithelium and neurons *in vivo*⁴⁵. Not all tissues required LKB1 to maintain apical/basal polarity, however, LKB1 may have been required for its establishment. The LKB1-MARK axis inhibits tubulin polymerization, and led to the hypothesis that the MARKs can potentially play a part in regulating LKB1 dependent cellular migration, in models of NSCLC, where LKB1 is lost or mutated^{46,47}. Indeed, LKB1 was found to inhibit epithelial to mesenchymal transition (EMT) through the MARK1/4 dependent phosphorylation of DIX Domain Containing 1 (DIXDC1) at S592, which subsequently inhibited Focal Adhesion Kinase (FAK)-induced expression of Snail, a hallmark marker for EMT⁴⁶. This finding was the first example of an AMPKR acting like a tumor suppressor *in vivo* by showing increases in lung colonization after orthotopic transplantation of shDixdc1 mouse cell lines⁴⁶. Several questions remain regarding the MARK kinases as tumor suppressors. While knockdown of Dixdc1 led to

increases in colonization in the lung, it failed to answer whether loss of the MARKs was enough to increase tumor burden in Kras driven genetic mouse models of NSCLC and approach the tumor burden of loss of Lkb1. In unpublished data from our lab, we have generated mouse models harboring floxed alleles to Mark1 and Mark4 (Mark1^{Flox/Flox}; Mark4^{Flox/Flox}) and crossed them with the Kras-driven model, however we observed no increases in tumor burden as compared to Kras alone. Suggesting that the LKB1-MARK axis, while important for inhibiting EMT, cellular migration, and growth in suspension, does not have the ability to enhance growth in the native lung setting upon its loss. It remains to be determined if the MARK kinases are partly required for mediating some portion of LKB1-dependent tumor suppression with cooperation of another AMPKR. Increases in FAK signaling is commonly observed in several forms of aggressive cancers and has been observed to play a role in promoting cell migration, growth, cell survival, and transcription⁴⁸⁻⁵⁴. It remains to be determined how crucial FAK signaling is to the initiation and progression of tumors in genetic mouse models of NSCLC harboring mutations to Lkb1.

BRSK KINASES ARE BRAIN SPECIFIC REGULATORS OF POLARIZATION

The BR Serine/Threonine Kinase (BRSK), also known as synapses of the amphoteric kinase (SAD), have two isoforms named BRSK1-2 (SADB-A, respectively). The BRSK are severely understudied, however they have been observed to play a role in neuronal polarization in mice⁵⁵. Specifically, unspecified neurites require LKB1-SAD signaling to mediate axonal fate and polarization after sensitization of extracellular signals such as Fgf, Wnt, semaphorin3A⁵⁶. Axon polarization also required PKA-dependent phosphorylation of Lkb1 at S431, suggesting that external factors are critical for LKB1 mediated polarization⁵⁷. While similar to the MARK in that they participate in cellular polarity, the BRSK have not been reported to contribute to cell migration or EMT. Expression of the BRSK is limited to the brain, as such, it is

highly unlikely that they would be key players in mediating LKB1-dependent tumor suppression⁵⁵.

NUAK KINASES CONTRIBUTE TO CELLULAR POLARITY AND ADHESION

The NUAK family kinase 1 and 2 (aka ARK5 and SNARK, respectively) remain understudied AMPKRs. However, the findings to date suggest the NUAK kinases may play a role in the progression of various cancers, and like the MARK kinases, in cellular polarity and cell migration^{11,58–60}. It was observed that the LKB1-NUAK axis can control myosin polymerization and effect cell adhesion¹¹. Expression of NUAK2 is induced by tumor necrosis factor (TNF α) within two hours and interacts with the myosin phosphatase MYPT-PP1 β complex in human embryonic kidney (HEK) 293 cells, suggesting a possible role in cell polarity or migration^{11,61}. NUAK1 contains three Gly-Ile-Leu-Lys (GILK) motifs (residues 399, 466, and 523) which when bound to directly to PP1 β , is stabilized and phosphorylates MYPT1 (S445, S472, and S910), at residues which match the consensus AMPK-motif commonly seen in AMPKRs and their substrates (R-X-X-(pS/pT-X-P and R-X-X-X-pS/pT-X-P)^{11,40,62}. Phosphorylation of MYPT1 by NUAK1 is thought to mediate its binding to 14-3-3, and suppression of MYPT1-myosin binding, ultimately inhibiting cellular detachment^{11,63}. Loss of the LKB1-NUAK axis, leads to MYPT activation, phosphorylation of myosin light chain, and promotes cell detachment in culture¹¹. Recently it was observed that Nuak1 played a critical role in mouse models of colorectal cancer, where in humans, high expression of NUAK1 is a sign of poor prognosis⁵⁹. In a *Villin-CreER*^{T2}, *APC*^{Flox/+}, *Kras*^{LSL:G12D}, *Nuak1*^{Flox/Flox} mouse model of colorectal cancer, Nuak1 was required for the formation of colonic polyps and participated in the NRF-2 oxidative stress pathway, particularly in those growths with high MYC expression^{59,60}. Paradoxically, the requirement for Nuak1 activity for colonic polyp formation is counter to the fact that polyps are observed in PJS patients which have lost LKB1, and ostensibly, NUAK activity. It's worth noting that alterations to LKB1 in

colorectal cancers only comprise about 2% (Figure 1.2), and unlike in lung adenocarcinomas where LKB1 is a strong tumor suppressor, the main tumor suppressor which drives growth may lead to the tissue specific requirement of the NUAKs.

SNRK CONTRIBUTE TO ADIPOSITY MAINTENANCE AND INFLAMMATION

By enlarge the most understudied AMPKR is the SNF Related Kinase (SNRK). To date, unlike all the other AMPKRs, no connections have been made between the function of SNRK and the development, maintenance, or enhancement of cancers or benign polyps. SNRK in plants has been linked to several metabolic pathways including sucrose and starch synthesis, nitrogen assimilation, and sterol synthesis⁶⁴. Highly expressed in mammalian adipose tissues, SNRKs have been shown to effect inflammation in brown adipose (BAT) and white adipose tissues (WAT) in heterozygous *Snrk* floxed mice (C57BL/6 *Snrk*^{flox/+} adiponectin-cre [WAT] UCP-1-Cre [BAT])^{65,66}. *Snrk*^{-/-} mice died after 24 hours after birth, suggesting that function of *Snrk* is critical during neonatal development⁶⁷. Heterozygous mice have impaired thermogenesis and decreased BAT deposits, suggesting that *Snrk* may regulative adipogenesis in mice in an ATP:AMP-independent fashion, opposite to the function of AMPK as an inhibitor to DNL . Loss of *Snrk* was also associated with adipocyte specific inhibition of insulin-stimulated AKT phosphorylation and glucose uptake⁶⁷. Ultimately loss of *Snrk* leads to increases in proinflammatory cytokines, decreases in glucose uptake, and results in local and systemic insulin resistance⁶⁸. It remains to be determined how SNRK activity contributes to cancer growth or maintenance, however direct connects have yet to be made.

SIK KINASES CENTRAL TO TRANSCRIPTIONAL CONTROL AND GROWTH

The Salt-Inducible Kinases (SIK) represent 3 of the 14 members of the AMPKRs. SIK1 (formally SIK) was discovered by looking for rapidly induced mRNAs upon feeding a diet high in

salt⁶⁹. However, it was later observed that SIK was also induced upon several other stimuli, including adrenocorticotrophic hormone, changes to sleep cycles, glucagon signaling, and loss of membrane potential^{69–72}. SIK2 (QIK) and SIK3 (QSK) were identified through the structural homology to SIK1 and were observed to have very similar catalytic domains^{73,74}. Like the other AMPKRs, LKB1 phosphorylates and activates the SIKs (T182 [SIK1], T175 [SIK2], and T221 [SIK3]) in the activation loop (T-loop) region, which is required for their catalytic function^{19,75}. Each of the SIKs contain several PKA phosphorylation sites outside the catalytic domains⁷⁶. Increases cellular levels of cyclic AMP, and activation of PKA, leads to 14-3-3 binding of the SIKs, and dephosphorylation of SIK substrates^{74,77–80}. To date, there are only two well described groups which are substrates of the SIKs, the CREB-regulated transcriptional co-activators (CRTC1, CRTC2, and CRTC3) and the class-II histone deacetylases (HDAC4, HDAC5, HDAC7, and HDAC9). The SIKs inhibit these two groups of enzymes, which upon phosphorylation, allows 14-3-3 binding and nuclear exclusion^{72,77,80–82}. Loss of LKB1 or SIK activity, either by genetic deletion or pharmacological inhibition (direct or through the activation of PKA), leads to dephosphorylation of the CRTCs and HDACs, nuclear translocation, and expression of their target genes^{83–88}. Translocation of the CRTCs facilitate the transcription of CREB-dependent genes, which while diverse, control aspects of proliferation, survival, and differentiation, among other processes⁸⁹. When dephosphorylated, the class-II HDACs bind to myocyte enhancer factor 2 (MEF2) and repress the expression of MEF2-dependent target genes, and simultaneously bind to and deacetylate FOXO transcription factors, which control transcription of gluconeogenic target genes^{80,86,90–92}. The SIKs have been proposed to effect various aspects of metabolism, however inconsistent modeling has led to confusion in the field. As mentioned, the SIKs catalyze very similar reactions, as a result, it remains unclear if remaining SIK kinases compensate for the loss of any single SIK allele which contributes to the metabolic phenotype observed. As mentioned prior, LKB1 kinase function is thought to contribute to its tumor suppressive function.

As a result, it was assessed if the SIK kinases themselves could mediate any of LKB1-dependent tumor suppression. Intratracheal transduction of a virus containing Cre-recombinase, Cas9 machinery, and a CRISPR guide against SIK3 (pSECC-sgSik3) into mice harboring *Kras*^{G12D} *Sik1*^{Flox/Flox}, led to an increase in tumor burden as compared to *Kras* mutations alone, and approached that of LKB1¹². These findings were confirmed in mice harboring a cre-inducible Cas9 allele (H11^{LSL:Cas9}), where virus containing sgRNAs to *Sik1* and *Sik3* revealed a similar phenotype⁹³. A similar SIK dependent, tumor suppressive, function was observed in a *GNAS*^{R201C} *Kras*^{G12D} driven model of pancreatic ductal adenocarcinoma, where sustained elevated levels of cAMP leads to PKA hyperactivation and downstream inhibition of the *Sik* kinases⁹⁴. While the LKB1-SIK axis seems to be the main axis of tumor suppression, there appears to be some tissue specificity. For example, in models of acute myeloid leukemia (AML), SIK2 and SIK3 phosphorylation of HDAC4, led to MEF2C target gene expression required for AML proliferation⁹⁵. Inhibition of the SIK kinases using the pan-SIK inhibitor HG-9-91-01 *in vitro*, or the *Sik3* inhibitor YKL-05-099 *in vivo*, was able to inhibit this MEF2C dependent growth effect, suggesting a role opposite to that observed in NSCLC models^{95,96}. Nevertheless, these studies highlight the role of the LKB1-SIK axis as it related to transcriptional control, as well as their regulation to growth in cancers. In the lung, disruption *Sik1* and *Sik3* only led to a partial phenotype when compared to loss of *Lkb1*, thus it remains to be determined if additional AMPKRs contribute to growth upon loss of *Sik1/3*, including *Sik2*. Or if *Lkb1* mediates tumor suppression through an unknown function, outside of the AMPKRs.

LKB1 IN NON-SMALL CELL CANCER AND THERAPEUTICS

Lung cancer remains the leading cause of cancer related deaths in both men and women⁹⁷. An estimated 238,000 new cases are expected in 2023, leading to upwards of 132,000 deaths⁹⁷. Of these new cases and deaths, 85% are of the common subtype NSCLC⁹⁸. Standard of care

treatment for NSCLC patients almost always includes cytotoxic chemotherapies such as cisplatin or carboplatin, as well as anti-PD-1/PDL-1 immune checkpoint blockade⁹⁹. Immune checkpoint blockade introduces antibodies to PD-1, PDL-1, and CTLA4, and prevents tumor induced inhibition of cytotoxic T-cells, thus allowing T-cell dependent tumor clearing¹⁰⁰. In humans, mutations to the LKB1 tumor suppressor are frequently observed in all histological subtypes of NSCLC. Inactivation of LKB1 is observed in about a third of lung adenocarcinomas (LUAD), and about 70% of mucinous bronchiolar adenocarcinomas^{101–103}. LKB1 mutations often come secondary to an initial tumor driver mutation. In the case of LUAD, the most common oncogenic driver is KRAS, which represents about 30% of all patient mutations, followed by EGFR (15%), BRAF (7%), and NF1 truncations (6%)¹⁰⁴. Among both KRAS and EGFR driven cancers, mutations to the tumor protein P53 (TP53) are most commonly co-mutated, however mutations to LKB1 are enriched in patients harboring KRAS mutations^{104,105}. Furthermore, KRAS-LKB1 co-mutations have a transcriptional signature which is unique to other common co-mutations in human NSCLC^{106,107}. While LKB1-KRAS mutations are one of the more commonly diagnosed forms of NSCLC, effective targeted therapies remain elusive. In fact, KRAS-LKB1 mutation have been shown to convey resistance to anti-PD-1/PDL-1 therapies, unlike KRAS-TP53 (KP) or K-only (K) tumors which showed sensitivity to immune checkpoint blockade¹⁰⁰.

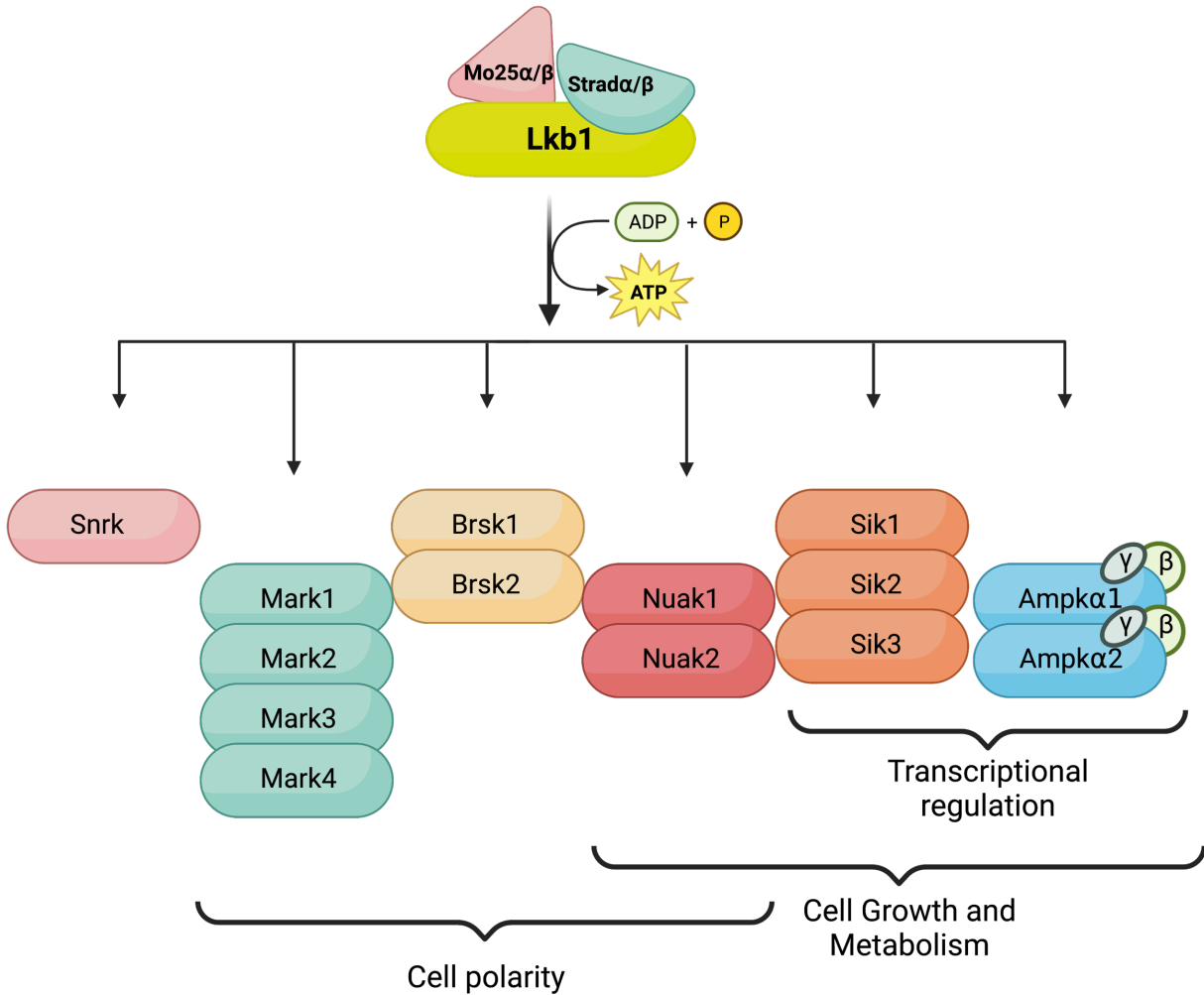


Figure 1. 1: The LKB1-AMPKR Signaling Axis.

Activation of the serine/threonine kinase LKB1 is dependent on formation of a heterotrimeric complex with MO25 and STRAD. This allows for the phosphorylates and activation of 14 downstream kinases known as the AMPK-related kinases (AMPKRs). The activity of the AMPKRs are diverse, and control various biological processes including cell polarity, cell growth, metabolism, and transcriptional regulation.

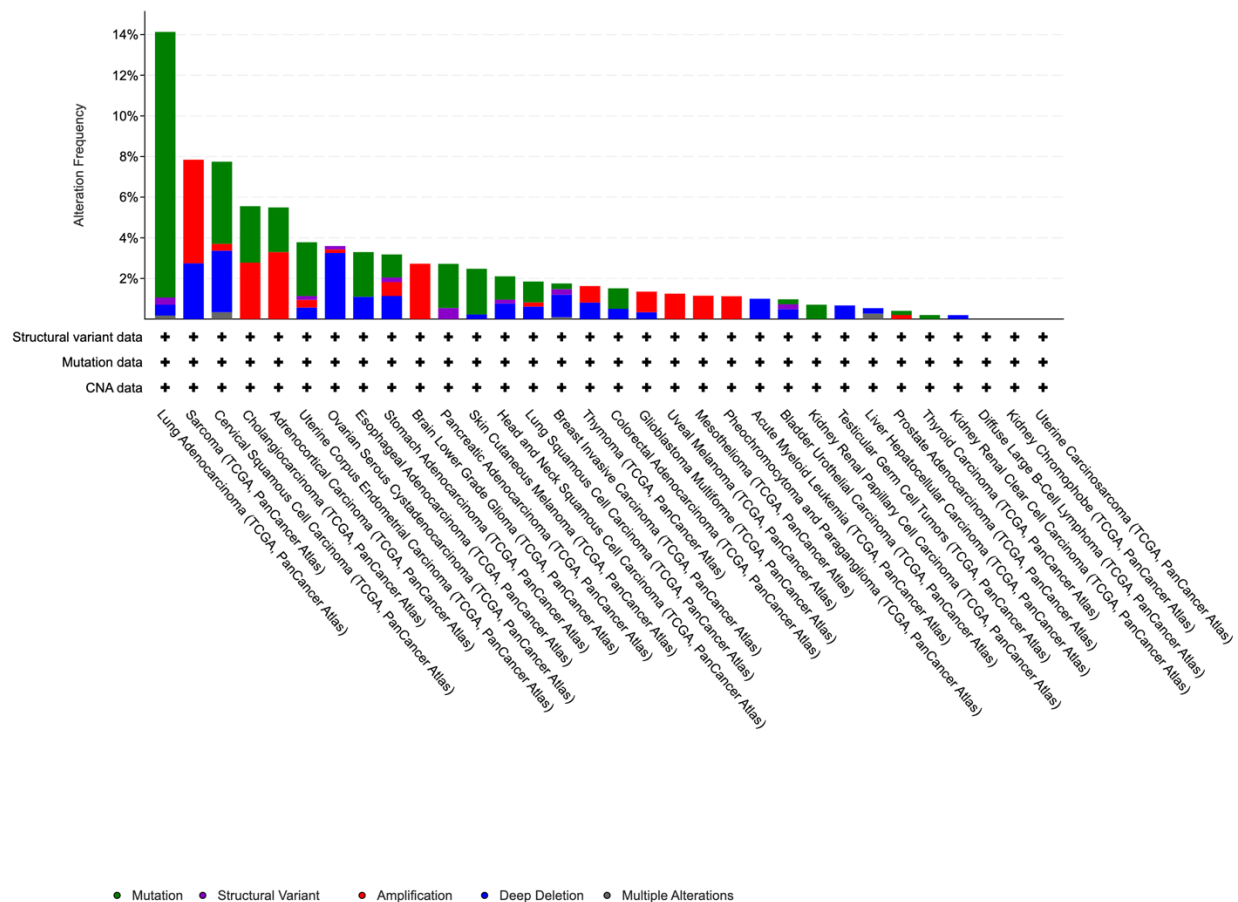


Figure 1. 2: TCGA Data showing Alteration Frequency of LKB1 in Various Cancers.

Mutations to LKB1 are common in several different types of human cancers. Alterations in LKB1 are highest in lung adenocarcinomas, showing mutations in approximately 14% of patients sampled by the TCGA. Similarly, cervical cancers, cholangiocarcinomas and adrenocortical carcinomas show mutations to LKB1 in >5% of sampled patients.

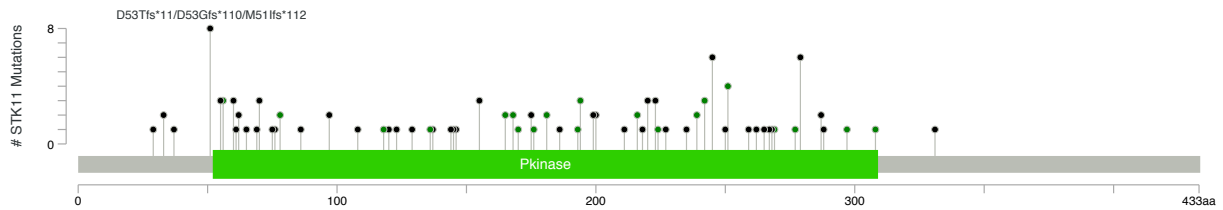


Figure 1. 3: Annotated Mutation sites on LKB1/STK11.

Mutations to LKB1 observed in TCGA samples often occur in the kinase domain. Mutations to the kinase domain suggests that its ability to function as a kinase, is critical to its tumor suppressor function. Since LKB1 phosphorylates and activates AMPKRs, it could be assumed that one or more of AMPKRs mediate its tumor suppressive function.

CHAPTER 2: THE AMPK-RELATED KINASES SIK1 AND SIK3 MEDIATE KEY TUMOR SUPPRESSIVE EFFECTS OF LKB1 IN NSCLC

Cancer Discovery, 2019

ABSTRACT

Mutations in the LKB1 (also known as STK11) tumor suppressor are the third most frequent genetic alteration in non-small cell lung cancer (NSCLC). LKB1 encodes a serine/threonine kinase that directly phosphorylates and activates 14 AMPK family kinases ("AMPKRs"). The function of many of the AMPKRs remains obscure, and which are most critical to the tumor-suppressive function of LKB1 remains unknown. Here, we combine CRISPR and genetic analysis of the AMPKR family in NSCLC cell lines and mouse models, revealing a surprising critical role for the SIK subfamily. Conditional genetic loss of Sik1 revealed increased tumor growth in mouse models of Kras-dependent lung cancer, which was further enhanced by loss of the related kinase Sik3. As most known substrates of the SIKs control transcription, gene-expression analysis was performed, revealing upregulation of AP1 and IL6 signaling in common between LKB1- and SIK1/3-deficient tumors. The SIK substrate CRTC2 was required for this effect, as well as for proliferation benefits from SIK loss.

INTRODUCTION

Twenty years ago, LKB1 (also known as STK11) was identified as the single gene responsible for the inherited cancer disorder Peutz–Jeghers syndrome¹⁵ and soon after was discovered to be the third most frequently mutated gene in non-small cell lung cancer (NSCLC), the leading cause of cancer-related death in the United States and worldwide^{101,102}. Mutations in LKB1 are found in 20% of patients with NSCLC and frequently coincide with activating KRAS mutations, prompting intense study of genetically engineered mouse models of LKB1 loss coupled with KRAS activation to understand how deregulation of these two genes drives tumor

formation and growth, and response to therapy in lung cancer^{108–113}. Studies of human patients and genetically engineered mouse models have shown remarkable convergence in determining that Kras;LKB1 (“KL”) tumors show a unique tumor biology that is distinct and separable from other subtypes of KRAS-driven NSCLC, such as tumors where KRAS mutations are coincident with p53 loss¹⁰⁶. One defining feature of KL tumors from a therapeutic standpoint is that these tumors exhibit primary resistance to a number of targeted therapeutics in both preclinical models and human patients^{108,114}, and very recent studies suggest they are also uniquely resistant to immune checkpoint inhibitors, thus making LKB1-mutant lung cancer a very common form of cancer for which treatment options are extremely limited^{100,115,116}. LKB1 is a serine/threonine kinase that activates a family of 14 downstream kinases, including the AMP-activated protein kinase (AMPK), which collectively are known to control cell metabolism, cell polarity, and growth^{3,19}. Despite advances in the understanding of what the targets and functions of some of the downstream effectors of these 14 kinases are in different tissues, it remains unknown which of the 14 LKB1-dependent kinases are most required for its tumor-suppressive activity in the lung. The best studied of these LKB1-dependent kinases is AMPK, which is a highly conserved master regulator of cell metabolism and growth that rewires metabolism and mTORC1 in response to low nutrient and energy conditions³. AMPK was hypothesized to be a lung tumor suppressor because it can indirectly suppress mTORC1 oncogenic signaling through the phosphorylation of a well-characterized downstream tumor suppressor, TSC2, and furthermore restrains pro-growth, pro-tumorigenic pathways that include inhibiting fatty-acid synthesis needed for cell growth¹¹². However, several recent studies utilizing mouse models of AMPK loss have suggested that AMPK plays a context-dependent role in cancer, as in some studies AMPK loss cooperated with mutational driver alterations to promote tumor formation in B- and T-cell lymphoma models^{117,118}, but in opposite fashion, loss of AMPK became detrimental to cancer cell survival in the context of several leukemia models^{119–121}. The requirement of AMPK for tumor-

cell survival fits with a wide body of literature on AMPK being required for cell survival under metabolic stress conditions; however, whether AMPK is pro- or antitumorigenic in the lung or any genetically engineered solid epithelial mouse model of cancer was completely unknown. In this context, we recently examined whether AMPK would be the central target of LKB1 in lung cancer³⁴. Unexpectedly, we found that AMPK deletion in KRAS-driven models of NSCLC did not mimic the effects of LKB1 loss, and instead suppressed tumorigenesis, being required for the outgrowth of KRAS-dependent tumors. Therefore, the identity of the critical kinase(s) downstream of LKB1 responsible for its tumor-suppressive functions in lung cancer remains unknown. Mutational data in The Cancer Genome Atlas (TCGA) database do not point to any of the 14 AMPK-related kinases (AMPKR) directly activated by LKB1 as being significantly mutated in lung cancer, and it has been assumed that redundancy among closely related kinases in this family could provide functional compensation, as nearly all of them contain one, two or even three very close homologs (see Fig. 1A). Although the degree of compensation among family members is not well characterized, one possibility is that compensation in tumor-suppressive functions among closely related AMPKR kinase family members mask their individual roles in tumor biology when analyzing their function in isolation, and thus may necessitate the disruption of multiple redundant family members to properly assess their role in tumor biology. The salt-inducible kinases (SIK1, SIK2, SIK3) are three such redundant kinases among the 14 AMPKRs dependent on LKB1 for their kinase activity. The SIKs are best known to control gene expression through direct inhibitory phosphorylation of the transcriptional regulators class IIa histone deacetylases (HDAC) and the CREB-regulated transcriptional coactivators (CRTC), thus controlling the transcriptional programs executed by these factors. The roles of SIKs in cancer remain very understudied. Among the SIKs, SIK1 had previously been described as a regulator of anoikis and suppression of metastatic potential in human breast cancer cells¹²². SIK2 was recently reported to play a pro-survival, oncogenic role in ovarian carcinoma cells¹²³ and to

cooperate with SIK3 in promoting the survival of myeloid leukemia cells⁹⁵, indicating that, like AMPK, perhaps SIK kinases can act in a pro-tumorigenic fashion and would not necessarily act as tumor suppressors; or at least exhibit context-dependent roles in different tissues. Most recently, the SIKs were shown to be critical targets inhibited by the oncoprotein GNAS through PKA-mediated phosphorylation. GNAS is mutationally activated in a subset of pancreatic adenocarcinomas, and suppression of the activity of the SIKs appeared to be necessary for tumor maintenance⁹⁴, thus indicating that in certain tissues they exert growth-restraining properties. Whether the SIKs have functions in mediating effects of LKB1 in lung cancer has not been explored. Using a combination of cellular systems and genetically engineered mouse models, we analyzed members of the AMPKR family and report here the unexpected finding that the AMPKR kinases SIK1 and SIK3 cooperatively mediate important tumor-suppressor functions of LKB1 in KRAS-driven lung cancer in mice.

RESULTS

CRISPR Analysis of the AMPKR Family in A549 Cells

Which of the 14 kinases of the AMPKR family downstream of the LKB1 tumor suppressor are most critical for mediating its effects in NSCLC remains unknown, despite years of intensive study. Given the potential redundancies among different members within subfamilies of the AMPKRs, we sought a cellular system where the tumor-suppressive effects of LKB1 could first be modeled as a surrogate toward eventual genetic modeling of these kinases on lung cancer in the mouse. To begin this analysis, we chose the LKB1-null human lung adenocarcinoma cell line A549. A549 cells show no difference in their growth rate and doubling time in standard full-media growth conditions when LKB1 cDNA is stably reconstituted (Supplementary Fig. S1A), yet show dramatic differences in tumorigenicity when orthotopically introduced into the lung by tail-vein injection (Supplementary Fig. S1B). We examined the expression of the 14 members of the

AMPKR family in A549 cells with or without stable LKB1 reconstitution, which revealed detectable mRNAs of all AMPKRs except MARK1 (Fig. 1A), indicating that many downstream kinases could be mediating the effects of LKB1 in these lung cancer cells. CRISPR/Cas9 was utilized to generate a panel of isogenic A549 cell lines, all of which bore stable retroviral reconstitution of wild-type LKB1 cDNA and were further genetically disrupted for different subfamilies of the 14 LKB1-dependent AMPKR kinases using single validated small guide RNAs (sgRNA) against each kinase via stable lentiviral expression. Lines were generated using distinct selectable markers to co-delete multiple members of each subfamily of AMPKRs. Immunoblotting confirmed loss of each AMPKR in its cognate cell line (Fig. 1B–D); however, SIK1 loss was confirmed via qPCR due to lack of sensitive antibodies able to robustly detect endogenous SIK1 under normal cellular conditions in human cells (Supplementary Fig. S1C). We additionally verified SIK1 disruption using Sanger sequencing and found a frequency of >80% in frameshift indels in the region surrounding the guide sequences in all cell lines targeted with SIK1 sgRNA guides (Supplementary Fig. S1D). Notably, despite not having an antibody to detect endogenous SIK1, we characterized a new antibody which recognizes the endogenous activation loop threonine phosphorylated by LKB1 in SIK1, SIK2, and SIK3 in human cells (Fig. 1E) and showed that this phosphorylation signal is correspondingly ablated in SIK knockout (KO) cells. In addition, as previously reported in other cell types¹⁹, here in A549 cells lacking LKB1, there was no evidence of phosphorylation of the activation loop of any of the three SIK family members. CRISPR KO of SIK1 and SIK3 resulted in a modest increase in SIK2 pT175, suggestive of cellular attempted compensation, although phosphorylation of CRTC3 was only modestly further band-shifted with loss of SIK1/2/3 as compared with SIK1/3 (Fig. 1D and E). Two features of epithelial cancer cells known to correlate well with in vivo tumorigenicity are the ability of cells to survive in suspension, indicative of anchorage-independent growth, and the ability of cells to form colonies when placed in 3-D matrix. Indeed, we observed an LKB1-dependent suppression of growth in A549

cells plated in nonadherent conditions, yet no LKB1-dependent effect over the same time frame in growth on standard adherent conditions (Supplementary Fig. S1E vs. S1F, first two columns). Analysis of cells reconstituted with LKB1 that were subsequently targeted to disrupt subsets of AMPKRs revealed that loss of AMPK α 1/ α 2, MARK1/4, MARK2/3, NUA1/2, BRSK1/2, or SNRK did not significantly alter the survival of A549 cells after 5 days in nonadherent conditions (Supplementary Fig. S1E). However, the combined loss of members of the SIK family showed a remarkable rescue of growth in nonadherent conditions, comparable with loss of LKB1 (Supplementary Fig. S1E). Notably, this was not observed in standard adherent conditions (Supplementary Fig. S1F). We next examined colony formation in soft agar in A549 cells, which revealed a nearly 80% suppression of colony growth when LKB1 was reconstituted (Fig. 1F and G). Unexpectedly, loss of AMPK α 1/ α 2, NUA1/2, MARK1/4, MARK2/3, BRSK1/2, or SNRK did not afford LKB1-reconstituted cells the ability to grow in soft agar (Fig. 1F and G). Analysis of all AMPKR subfamilies revealed very modest effects, except a remarkable full rescue of soft-agar colony formation when the entire SIK subfamily was genetically disrupted. Further study with cells lacking each SIK family member individually or lacking pairs of the SIKs revealed that of the three SIK family members, SIK1 mediated the greatest single effect on soft-agar colony formation (Fig. 1F and G). Loss of SIK2 had a minor effect and loss of SIK3 in particular synergized with loss of SIK1 (Fig. 1F and G). The relative importance of SIK1 and SIK3 to growth in soft agar matched the impact of SIK1, SIK2, or SIK3 loss on phosphorylation of CRTC3, one of the CRTC family of transcriptional coactivators that are targeted by SIKs (Fig. 1D). Genetic disruption of SIK1 or SIK3 alone, but not SIK2 alone, resulted in an increased abundance of the faster-mobility CRTC3 species (lower band), corresponding to a loss of phosphorylation. The observed redundancy among the three SIK family members matches what has been observed in studies of their function in mouse liver, where loss of all three unmasks strong phenotypes that are compensated when any one of the SIKs remains functional^{78,124}. Collectively, our findings

in A549 cells suggest that the SIK subfamily, and particularly SIK1, may be playing an unappreciated role in lung cancer tumor biology.

Analysis of the Role of the SIK Family in Genetic KRAS-Dependent Lung Tumor Models Using CRISPR

On the basis of the findings in A549 cells, we sought to directly examine the role of SIKs in the well-studied genetically engineered mouse models of KRASG12D-dependent lung cancer¹²⁵. Given a lack of readily available conditional floxed alleles of the SIKs, to assess the contribution of Sik1 to tumor development we first turned to a recently described lentiviral vector allowing for co-expression of Cre recombinase with Cas9 and an sgRNA of choice, pSECC¹²⁶. pSECC lentiviruses can be delivered in vivo to the lungs of mice to induce CRISPR-mediated gene KO in combination with Cre-mediated activation of oncogenes and deletion of floxed alleles in a single cell (Fig. 2A). We began by generating pSECC vectors bearing three distinct validated RNA guides (sgRNAs) targeting Sik1 (Supplementary Fig. S2A). We first tested the effects of inactivating SIK1 via intratracheal delivery of functionally titered, equivalent amounts of pSECC viruses harboring sgRNAs against Sik1, along with sgRNAs targeting Lkb1 (Supplementary Fig. S2B) and control sgTom (nonspecific sgRNA) or sgLacZ^{126,127} sgRNAs, into KRASG12D mice containing a conditional Cre-activated reporter (ROSA26LSLluciferase), which allows for real-time, noninvasive tracking of tumorigenesis and tumor progression occurring in these mice. We quantitated tumor development using bioluminescence imaging (BLI) to measure the photon flux emitted from the lungs of these mice over time. This revealed significant acceleration of lung tumor development over the course of 22 weeks in mice treated with sgLkb1 (Fig. 2B). All three cohorts of KRAS mice treated with individual Sik1 sgRNAs displayed a modest, but consistent increase in tumor development over the control cohort by BLI over this time course. Of note, in a separate experiment, we additionally tested the effect of delivering pSECC viruses with two

validated Mark4 sgRNAs in a parallel group of KRASG12D mice, and found that targeting Mark4 alone in this model did not result in changes in tumorigenesis or progression in a manner distinguishable from control mice (Supplementary Fig. S2C). To specifically gauge the effects of pSECC-Sik1 viruses in these mice, we quantitated their tumor burden as a percent of tumor area over the total lung tissue area by analysis of hematoxylin and eosin (H&E)-stained lung sections of experimental and control cohorts (Fig. 2C). Although we observed a wide variability in the overall tumor burden per mouse within all groups (Supplementary Fig. S2D), when considering all animals in each cohort as individual sets, as expected, sgLkb1-treated animals clearly displayed a significant increase in tumor burden (20% and 16% for sgLkb1 guides A and B, 0.9% and 1.3% for control sgTom and sgLacZ animals, and 4%, 2%, and 2% for the Sik1 guides). Although the percent tumor burden on average was small in non-sgLkb1 animals at the 22-week endpoint, we corroborated the modest but consistent fold increase in the overall tumor burden of these mice with all three Sik1 sgRNA guides, ranging from 1.45 to 3.6 fold increase over the average of the control sgTom and sgLacZ cohorts. To more closely examine the effects that disrupting Sik1 had on tumors present in these mice, we analyzed the number and size of individual tumors in each mouse. Whereas the number of tumors per mouse did not reach above an average of about 9, among the non-sgLkb1 mice, the average size of individual tumors in all three cohorts of pSECC-Sik1 mice was significantly higher compared with control tumors, with a positive trend for all three guides used (8-, 3-, and 1.75-fold higher than control tumors for sgSik1 guides A, B, and C respectively; Fig. 2D). These observations represented a skew toward a higher number of larger tumors in sgSik1-treated animals: 20% and 11% of tumors were larger than 1 mm² in mice with sgSik1-A and sgSik1-B, respectively, vs. 0% of controls, and 60% (sgSik1-A), 82% (sgSik1-B), and 70% (sgSik1-C) of tumors were sized between 0.1 and 1 mm² compared with 42% for control tumors (Fig. 2E). Thus, targeting SIK1 consistently yielded a larger tumor size on average in cooperation with KRASG12D activation. Next, we aimed to

assess the impact of deleting Sik1 with pSECC-CRISPR viruses in KRASG12D tumors that were also conditionally deleted for the gene encoding the tumor suppressor p53 (KP). Notably, genetic loss of LKB1 on the KP background (KPL) greatly accelerates the rate of lung tumorigenesis, tumor growth, progression, metastasis, and tumor-related morbidity in mice compared with genetic KL and KP models³⁴. We reasoned that loss of SIKs in a cell-autonomous p53-null setting may likewise provide a more permissive background to better reveal the potential tumor-suppressive effects of SIK1 in models of NSCLC. In addition to targeting Sik1 or Lkb1 with pSECC in KP mice, we wanted to expand this analysis to include other SIK family members. We therefore developed sgRNAs to additionally target Sik2 and Sik3 (Supplementary Fig. S2B). BLI imaging across a 15-week time course revealed that in contrast to inactivation of Sik2 or Sik3, targeting Sik1 in KP mice resulted in an increased lung tumor burden compared with control mice (Fig. 2F, Sik1 mice only; Supplementary Fig. S3A and S3B, Sik1–3 mice). Indeed, tumor burden quantitation from H&E-stained slides from the lungs of KP mice treated with sgSik1 guides at study endpoint showed an average tumor burden of 23.7% (sgSik1-A), 16.1% (sgSik1-C), and 7.4% (sgSik1-B) compared with a burden of 4.6% and 7.1% of lung tumors induced by control sgTom or sgLacZ, respectively (Fig. 2G). The percent lung tumor burden in mice targeted for Sik2 and Sik3 inactivation on average did not differ from the burden seen in control animals. In contrast, the average of all three cohorts targeting Sik1 with pSECC-sgRNAs in the KP model showed the percent lung tumor burden average to be 18% in sgSik1 animals from an average of 7.2% in control animals (a 2.5-fold change; Supplementary Fig. S3C). At the individual level of KP mice treated with sgSik1 viruses, 5 of 10 animals showed tumor burden over 18%, which was the upper limit of burden in control KP mice, and two individuals presented with burden of 38% and 39%, falling inside the range of tumor burden of KP mice treated with sgLkb1 guides (26%–57%; Supplementary Fig. S3D). Quantitation of individual lung tumors revealed that mice treated with viruses harboring all three sgRNAs targeting Sik1 resulted in a significantly larger

tumor size compared with control-treated mice (Fig. 2H). The range of tumors larger than 1 mm² in mice treated with sgSik1 guides was 33%, 28%, and 15% depending on sgRNA guide, compared with 5% in control animals (Fig. 2I). Nonetheless, considerable variability in tumor size was observed in these models, making us examine whether some of the sgRNAs were more effective than others at inducing disruption of the locus in vivo, and/or whether a selection to observe out-of-frame indels in tumors at endpoint might be associated with specific guides. Using next-generation deep sequencing of barcoded and pooled primary tumors, we observed a greater percentage of frameshift indels with Sik1 guide A, as compared with Sik1 guides B and C (Supplementary Fig. S3E and S3F), which correlated directly with the extent of tumor burden associated with these guides (Supplementary Fig. S3A). Similarly, the more effective Lkb1 sgRNA, guide A, was also associated with a greater percentage of tumors bearing frameshift indels, indicative of a consistent selective advantage for tumors bearing Lkb1 guide A (Supplementary Fig. S3E and S3F vs. S3A).

Analysis of Genetic KRAS-Dependent Lung Tumor Models Using Floxed Sik1 and Sik2 Alleles

As a complementary and powerful alternative way to interrogate the function of SIKs in NSCLC, we turned to recently described conditional floxed alleles of Sik1¹²⁸ and Sik2⁸¹, which we bred into the KRAS-dependent mouse model to conditionally knock out expression of SIK1 and SIK2 (Fig. 2J). We first confirmed that excising Sik1 floxed alleles in mouse liver resulted in the modulation of known LKB1-dependent transcripts (Supplementary Fig. S4A and S4B), corroborating that some known CREB target genes in the liver were induced by conditional deletion of this Sik1 floxed allele. We next generated conditional floxed Kras;Sik1 (KSik1) and Kras;Sik2 (KSik2) mice and compared them against Kras (K) and Kras;Lkb1 (KL) NSCLC models. Lung tumor induction by delivery of lentivirus expressing Cre-recombinase efficiently

recombined *Sik1* and *Sik2* in lung tumors from the corresponding mice, and we further confirmed ablation of the expressed *Sik1* transcript in tumors from *KSik1* mice (Supplementary Fig. S5A and S5B), which additionally displayed deregulation of known *LKB1*-regulated transcripts (Supplementary Fig. S5C–S5E). *KSik1* mice showed an acceleration in tumor growth over *K* mice (Fig. 2K and L; Supplementary Fig. S6A–S6C), resulting in an increase from 8% to 12% in tumor burden over *K* controls (a 1.5-fold increase), which is within the range of tumor burden fold change observed in *KRAS* mice treated with sg*Sik1* guides, although this did not reach statistical significance. Intriguingly, 3 of 13 animals presented with high tumor burden, and two of them (37% burden each) matched the level of tumor burden observed in *KL* mice (32%–56%; Supplementary Fig. S6B), which was never observed in *K* mice, suggesting an estimation of about 15% to 20% of *KSik1* mice displaying very high lung tumor burden. Surprisingly, genetic loss of *Sik2* did not increase, and instead slightly reduced, tumor burden compared with *K* mice, suggesting that *SIK2* alone does not play a critical role in tumor suppression in the lung. Individual tumor analysis from *KSik1* mice (Fig. 2M; Supplementary Fig. S6D) revealed a highly significant difference in tumor size. Two percent of tumors in *K* mice were larger than 1 mm², whereas 9% of *KSik1* tumors were this size or larger (Fig. 2N).

Analysis of the Role of the SIK Family in Genetic KP Lung Tumor Models Using Floxed *Sik1*

Alleles

We next tested the effect of genetic ablation of *SIK1* in the KP model of NSCLC. *KPSik1* mice displayed an increase in tumor development compared with *KP* mice as measured by BLI (Fig. 2O; Supplementary Fig. S7A and S7B). Despite variability in tumor burden among individual mice (Supplementary Fig. S7C), 2 of 5 mice reached tumor burdens of 41% to 42% of the total lung area, which is similar to the tumor burden range of *KPL* mice (46%–62%), whereas *KP* mice had smaller burden (1%–27%; Supplementary Fig. S7D). As a cohort, *KPSik1* mice showed an

increase from 10% to 28% in tumor burden over KP mice (a 2.8-fold increase), compared with a tumor burden of 52% in the KPL mice (5.25-fold increase over KP; Fig. 2P), and this was consistent with the results observed in KP mice treated with Sik1 sgRNA guides (Supplementary Fig. S3B; 2.5-fold tumor burden increase of KP+sgSik1 over KP+sgControls). Analysis of individual tumors in KPSik1 mice, however, showed a significant increase in tumor size in these mice compared with KP (Fig. 2Q), and loss of SIK1 in this model increased the percentage of tumors larger than 1 mm² (18% tumors in KPSik1 vs. 7% in KP), and furthermore 2% of tumors in KPSik1 mice were (>10 mm²; Fig. 2R). To further ascertain the cell biological effect of SIK1 loss in lung cancer cells, we derived (n = 3) cell lines from KPSik1 tumors, first confirming that they expressed no detectable Sik1 transcript (Supplementary Fig. S8A), and functionally compared them to matched KP lines (n = 4) derived from the same genetic background. KPSik1 cells displayed an enhanced ability to form colonies from single cells in 2-D culture compared with control KP cells, and moreover grew into significantly larger colonies (Supplementary Fig. S8B–S8D). We next tested the ability of these cells to grow colonies in soft agar, and found that KPSik1 cells displayed an enhanced ability to grow both more and larger colonies in soft agar compared with KP cells (Fig. 2S; Supplementary Fig. S8E). Finally, we tested the dependency of SIK1 loss on the growth of these cells, and found that reexpression of the Sik1 cDNA significantly reduced their proliferative capacity (Supplementary Fig. S8F).

Targeted Coinactivation of SIK1 and SIK3 Accelerates Tumor Growth in KRAS-Driven NSCLC

Despite the enhanced tumor growth phenotype resulting from inactivation of SIK1, the loss of any individual SIK family kinase did not fully recapitulate the tumorigenic potential of LKB1 loss in our mouse models. SIK1, SIK2, and SIK3 share common substrates, and therefore it is likely that functional redundancy from the remaining SIK family members may mask the

ability to further reveal LKB1-like effects on tumor growth upon loss of an individual SIK, in agreement with our observations of targeted loss of SIK1 versus combinations of SIK1+3 and SIK1+2+3 loss in A549 cells (Fig. 1D–F). To test this hypothesis in vivo, we combined our genetic floxed KSik1 model with pSECC-mediated inactivation of Sik3 (Fig. 3A). This model uses Cre recombinase to activate KRAS and inactivate SIK1, and simultaneously delivers Cas9 and an sgRNA targeting SIK3 (sgSik3-B). Thus, this system effectively inactivates SIK1 and SIK3 simultaneously. Mice with this genetic combination efficiently yielded tumors that were deleted for Sik1 and further showed disruption of reading frames at the Sik3 sgRNA target site (Supplementary Fig. S9A–S9E). Remarkably, compounding the loss of these two SIK family members accelerated tumor growth as compared with K+sgTom mice or with cohorts that inactivated SIK1 or SIK3 singly, either using the genetic model (KSik1) or CRISPR-based targeting with sgRNAs targeting Sik1 or Sik3 (Fig. 3B–D, Supplementary Fig. S9B). Floxed KSik1 mice treated with pSECC-sgTom displayed an intermediate acceleration of tumor growth between KSik1+sgSik3 and K+sgTom mice, which was comparable to inactivation of SIK1 with sgRNAs. Furthermore, BLI imaging showed that all three conditions targeting SIK1 alone resulted in enhanced tumor growth in mice as compared with targeting SIK3 by itself. At study endpoint, tumor burden quantitation showed that KSik1+sgSik3 mice showed a significantly higher burden (10%) over control mice (1%), which was further reinforced upon tumor size quantitation (Fig. 3E and F). Use of deep sequencing of tumors to analyze the frequency of frameshift indels in the tumor DNA revealed a strong selection for Lkb1 sgRNA guide A (Supplementary Fig. S9C and S9D), similar to before (Supplementary Fig. S3E). However, now the increased percentage of tumors bearing a high proportion of frameshift indels could be clearly observed for the Sik3 sgRNA B, strikingly only in KRAS SIK1 mice and not in KRAS mice alone (Supplementary Fig. S9C and S9D). We interpret these results as potential evidence for a selective advantage of tumors inactivating SIK3 only when they already lack SIK1. Despite an apparent lower total tumor

burden in the cohort of KSik1+sgSik3 mice compared with K+sgLkb1 mice (Supplementary Fig. S9F and S9G), the average size of individual tumors between K+sgLkb1 and KSik1+sgSik3 mice was instead comparatively similar (average size >1 mm², difference in means between these two cohorts not significant) and both were significantly larger than tumors from control K+sgTom mice (Fig. 3F). Notably, mice with these two genotypes were the only ones with average tumor sizes above this threshold among all cohorts tested. We reasoned that enhanced cellular proliferation rates may underlie the dramatic increase in tumor size observed in these cohorts. BrdU positivity analysis showed that KSik1+sgSik3 tumors showed the highest average percent of BrdU incorporation among all cohorts tested (Fig. 3G), indicating that inactivation of multiple SIKs resulted in higher numbers of proliferating cells per tumor compared with control or single Sik-deleted tumors. Correlated with these observations, tumor size distribution analysis showed that KSik1+sgSik3 mice presented with the largest percent of tumors >10 mm² among cohorts (9% of tumors of this genotype, 5% observed in K+sgLkb1, 2% in KSik1 mice, 0% in all other cohorts; Fig. 3H). Together, our data indicate that the combined inactivation of SIK1 and SIK3 yielded a tumor phenotype characterized by a high percentage of actively dividing cells, resulting in an expanded fraction of large tumors similar in size to those observed upon loss of LKB1. We additionally assessed whether SIK1+3 loss would recapitulate the reported association of LKB1 loss with an elevated recruitment of tumor-associated neutrophils, a hallmark of KL lung adenocarcinomas¹¹⁴. Using IHC for the neutrophil marker Ly6G, we observed a significant increase in the percentage of neutrophils present in the KL tumors as compared with K tumors, and moreover this increase in neutrophil staining was also mirrored in the KSik1/3 tumors (Supplementary Fig. S9E). Together, these results suggest that loss of SIK1 and SIK3 in the mouse recapitulates histologic aspects of LKB1 loss in both tumor size and immune phenotype.

SIK-Mediated Control of Gene Expression

To determine which transcriptional programs driven by LKB1 inactivation are under the control of the SIKs, we performed transcriptional profiling using RNA sequencing (RNA-seq) on primary tumors plucked from K+PgkCre and K+pSECCsgTom (K), KSik1+Pgk-Cre (KSik1), KSik1+pSECCsgSik3-B (KSik1±3), and K+pSECCsgLkb1-A (KL-A) genotypes. Clustering all genes across all genotypes revealed discrete clusters of genes that were clearly coregulated in KL-A and KSik1±3 tumors (Supplementary Fig. S10A). Expanding these clusters (Supplementary Fig. S10A, red and green boxed insets), it is evident that Sik1+3 deletion induced the same magnitude of transcriptional deregulation as LKB1 inactivation, suggesting that the SIKs are the main targets of LKB1 in the normal suppression of these two specific subsets of genes. Notably, these clusters did not show deregulation in KSik1 tumors (Supplementary Fig. S10A), suggesting that coincident inactivation of SIK1+3 is necessary to reveal the LKB1-dependent transcriptional programs that are under SIK-mediated regulatory control. Moreover, when we unbiasedly clustered the data from K, KSik1±3, and KL-A tumors across all genes and samples, the samples clustered cleanly by genotype, confirming that genotype-dependent transcriptional changes dominated the dataset (Fig. 4A). We next used differential expression analysis (FDR <0.05, $\pm$ 1.5-fold) to define a KSik1±3 gene signature as compared with K tumors and, independently, a KL-A gene signature as compared with K tumors. We then identified the overlap between the KSik1±3 and KL-A gene signatures. Three hundred forty-four (38%) of the genes within the KL-A signature that were upregulated in KL-A tumors were also significantly upregulated in KSik1±3 tumors. Similarly, 627 (41%) of the genes with the KL-A signature that were downregulated in KL-A tumors were also significantly downregulated in KSik1±3 tumors (Fig. 4B). Together, we identified 971 genes commonly regulated by LKB1 or SIK1+3 inactivation (Fig. 4C). These data suggest that SIK1 and SIK3 together may be responsible for approximately 40% of the transcriptional changes induced upon LKB1 loss in primary lung tumors. We used gene set enrichment analysis (GSEA) to unbiasedly explore the pathways regulated by SIK1+3. We

considered the comparison of KSik1+3 tumors to K tumors, and queried this data against the “Hallmark” group of gene sets using GenePattern. We performed the same analysis considering KL-A tumors compared with K. We then compared the top enriched gene sets to determine which LKB1-dependent processes may be mediated by SIK1+3. Two gene sets related to well-established hallmarks of LKB1-mutant lung tumors, epithelial–mesenchymal transition (EMT) and IL6–JAK–STAT3 signaling, were among the top enriched gene sets, 1st and 8th, respectively, in the KSik1+3 data (Fig. 4D). In the KL-A data, these gene sets were the 6th and 7th most enriched. Metascape pathway analysis of the 344 genes upregulated in both KL-A and KSik1+3 tumors (Fig. 4B) confirmed a role for SIK1+3- and LKB1-dependent deregulation of the IL6 pathway, in that “IL6 family signaling” was identified as the second most significant term (Supplementary Fig. S10B). We combined the enriched genes from the GSEA plot with the genes comprised in the Metascape term to create an IL6 signaling gene list, shown in a heat map (Fig. 4D). The enriched genes from the EMT gene set are also depicted in a heat map (Fig. 4D). Thus, transcriptional analyses suggest that loss of SIK1+3 replicates effects on EMT and IL6 signaling pathways that are induced by LKB1 loss in lung tumors. We then extended our analysis of the 344 genes upregulated in both KL-A and KSik1+3 tumors (Fig. 4B). We queried this gene list in Enrichr. IL6 signaling or related JAK–STAT signaling were the top enriched pathways within this gene list across the Kyoto Encyclopedia of Genes and Genomes (KEGG), WikiPathways, Reactome, and BioCarta analyses (Fig. 4E; Supplementary Fig. S10C). This further implicates SIK1+3 in the regulation of the IL6 pathway. Homer motif enrichment analysis identified one statistically significant motif ($P < 1e-12$) within the promoters, defined as ± 2 kb of the transcriptional start site (TSS), of the 344 upregulated genes (Fig. 4F, top motif). This motif includes the recognition motif for the AP1 family of transcription factors, including AP1, JUND, FOS, FOSL1, and FRA2. Alignment of this motif to the JUND motif is pictured (Fig. 4F). This suggests that the AP1 family of transcription factors may be involved in driving a portion of the

LKB1- and SIK1+3-dependent transcriptional changes. A key established target of the SIKs is the CRTC family of transcriptional coactivators of the CREB transcription factor, which has also been reported to control AP1 activation^{129,130}. We found that both AP1 and CREB-dependent transcription became chronically upregulated by SIK1- or LKB1-dependent loss in cultured mouse and human NSCLC cells, in correlation with bioinformatic analysis of mouse tumors (Supplementary Fig. S10D–S10G, Supplementary Fig. S11A–S11D). To query this bioinformatically, we searched the promoters of the 344 upregulated genes common to KL-A and KSik1+3 tumors for AP1 and CRE motifs. Seventy-four percent of genes have at least one motif for CREB (CRE), AP1, or both in the promoter (Supplementary Fig. S10D). It follows that the genes involved in the biological processes that are deregulated upon LKB1 and SIK1+3 loss, including EMT and IL6 signaling, are likely to be under transcriptional control by the transcription factors whose activity are modulated in these conditions, potentially CREB and AP1. Indeed, of the 344 genes commonly upregulated in murine KRAS tumors by LKB1 loss and SIK1+3 loss, 55 genes contain both predicted elements in their promoters (Fig. 4G), 31 contain CRE elements (Supplementary Fig. S10E), and 170 contain AP1 elements (Supplementary Fig. S10F). Although only some of these motifs are likely to be functionally bound by CREB or AP1 in primary lung tumors, these data suggest that both CREB and AP1 may be involved in regulating a subset of the genes upregulated upon LKB1 and SIK1+3 loss. One well-studied promoter that contains functional AP1 and CREB binding sites is that of IL6¹³¹. As the enrichment analysis suggested, FPKM plots of the RNA-seq data from each tumor per genotype reveals transcriptional upregulation of nearly all steps of the canonical JAK–STAT pathway, from cytokines (IL6, IL33) to receptors (IL4RA, OSMR), core components (JAK3, STAT1, STAT3), coactivators (BCL3), and well known STAT targets (SOCS3; Fig. 4H). Thus, our data support the model that LKB1 loss in lung tumors induces IL6 signaling through inactivation of SIK1+3. To assess the overlap between the changes in gene expression by loss of Sik1+3 deletion in mouse tumors and genes regulated

by loss of LKB1 in human cells, we used GSEA to query the top 500 upregulated genes in SIK1+3 tumors and compared them against a ranked list of all genes differentially regulated by the presence or absence of LKB1 in human NSCLC A549 cells based on RNA-seq expression profiling (from A549-pBABE or A549+LKB1 cells represented in Figs. 1 and 5). We found there was a significant enrichment of genes upregulated in mouse tumors by SIK1+3 loss that were also found to be upregulated in human A549 cells compared with LKB1-reconstituted cells (Supplementary Fig. S10G, graph and first line in the summary table), which notably included IL6 and IL33. We also queried the set of 344 genes that were upregulated in common by SIK1+3 loss and LKB1 loss (Fig. 4B) to ask whether the set of genes that were commonly regulated by SIK1+3 loss and LKB1 loss in mouse tumors were also regulated by LKB1 in human NSCLC cells. GSEA showed a significant enrichment of this gene set when comparing genes differentially regulated by LKB1 in A549 cells (Supplementary Fig. S10G, second line in the summary table). Furthermore, this gene set was also found to be enriched in differential transcriptional profiles generated by RNA-seq of two other human NSCLC lines that we queried (H1355, H157) in the LKB1-null state versus LKB1 cDNA reexpression (Supplementary Fig. S10G), collectively indicating that SIK1+3 loss in mouse tumors induces transcriptional changes that are mirrored in part by LKB1 loss in the mouse and in human cells. These included genes associated with IL6 pathway signaling, which further raised the possibility that LKB1 may control IL6 signaling through SIK1/3 in human NSCLC cells.

SIKs Control IL6 and STAT Signaling via CRTC2

To further explore whether the gene-expression changes seen were due to cell-autonomous effects of loss of SIK1/3 signaling on transcription within the tumor cells themselves, as compared with elevated cytokine signaling in surrounding stroma or immune cells, including the elevated presence of tumor-associated neutrophils present in the

microdissected tumors, we turned to human A549 NSCLC cells in culture. Picking a limited number of well-known SIK1-regulated mRNAs (e.g., NR4A1, PDE4B), we examined a limited number of genes in the cytokine/JAK–STAT signaling pathway deregulated in the KL and KSik1+3 tumors. As seen in Supplementary Fig. S12A, mRNAs of some cytokines and cytokine receptors (IL10, IL15, OSMR) were suppressed by LKB1 reconstitution in A549 cells, and were largely reversed by SIK1/3 deletion in LKB1-expressing cells. As IL6-dependent signaling has been shown to be a common feature of other LKB1-deficient contexts¹³², we examined the cell autonomy of LKB1 effects on IL6. As a first examination, we created Lkb1 or Sik1/3 CRISPR KO murine KP cell lines using independent combinations of sgRNA guides targeting Sik1 and Sik3 in two independent KP lines, and profiled IL6 production by ELISA. In these cells, we observed significantly elevated IL6 production in both the Lkb1-deleted and Sik1/3-deleted KP cells (Fig. 5A). We next examined IL6 production in A549 cells with and without LKB1 reconstitution or Sik1/3 CRISPR deletion. Notably, A549 cells produced IL6 basally, which was strongly inhibited when LKB1 was restored (Fig. 5B), and this effect of LKB1 reintroduction was fully reversed upon deletion of SIK1/3. Given the presence of CRE and AP1 binding motifs in the IL6 promoter¹³¹, we next examined whether CRTC family members may mediate effects of LKB1 or SIK1/3 on IL6. First, we examined mRNA expression levels of the three members of the CRTC family in murine KP tumors as well as in A549 cells from RNA-seq data. In both mouse (Supplementary Fig. S12B) and human (Supplementary Fig. S12C) KRAS-mutant lung adenocarcinoma cells, CRTC2 was the most prominently expressed family member. Using two distinct sgRNAs, CRTC2 was deleted in A549 cells bearing LKB1 reconstitution and SIK1/3 deletion, which resulted in complete suppression of IL6 production (Fig. 5B). Given this requirement of CRTC2 for IL6 production, we examined whether gene expression controlled by SIK1/3 loss might similarly be dependent of CRTC2. We observed partial or full dependence on CRTC2 among genes well known to be CREB targets (NR4A2, NR4A3), but also among cytokine signaling (IL33, STAT1) and proliferation

genes (AREG, EREG; Fig. 5C). To examine whether these elevated mRNAs would translate into altered signaling pathways, we examined total STAT1 protein levels in our A549 cell series. A549 cells expressed STAT1 protein, which was reduced by LKB1 reconstitution, but that effect of LKB1 was reversed by SIK1/3 deletion (Fig. 5D; Supplementary Fig. S12D). The increase in total STAT1 protein (both α and β splice isoforms induced by loss of SIK1/3) was in turn reversed by deletion of CRTC2. In addition, SIK1/3 loss in the LKB1-proficient cells resulted in not only elevated STAT1 proteins but also increased basal phosphorylation of STAT1 at Y701 (Fig. 5D; Supplementary Fig. S12D), perhaps due to autocrine cytokines also being produced in parallel with STAT1 upon SIK1/3 deletion. This too was reversed by CRTC2 loss (Fig. 5D). We next examined whether CRTC2 was also a key target of SIK1/3 loss on growth in anchorage-free settings. First, we returned to growth and anoikis survival in detached conditions, and as observed earlier (Supplementary Fig. S1E), reexpression of LKB1 in A549 cells reduced their growth and survival in detachment, which was largely reversed by SIK1/3 loss, and this added growth from SIK deficiency in detached conditions was suppressed by CRTC2 loss (Fig. 5E). The enhanced growth in soft agar induced by SIK1/3 deficiency was also diminished by CRTC2 deletion (Fig. 5F), indicating that CRTC2-dependent control of transcription may be a major target of LKB1- and SIK1/3-dependent tumor suppression.

SIK-Mediated Effects Are Largely LKB1-Dependent

As we and others have previously demonstrated, despite LKB1 being a predominant upstream kinase for AMPK in most tissue types, most cell types also use CAMKK2 as an upstream kinase for the same activation-loop threonine that LKB1 phosphorylates, providing residual AMPK activity in LKB1-deficient cells, tumors, and tissues¹³³. Now armed with better tools and clear effects on a number of readouts, we sought to examine whether SIK family kinases might also retain catalytic activity independent of LKB1. Our first hint that this was not

the case came from use of the new activation- loop threonine phospho-specific antibody, which recognizes endogenous SIK1, SIK2, and SIK3 in A549 cells (Fig. 1E). Despite an apparent absence of pSIK1, pSIK2, or pSIK3 in the A549 cells which lack LKB1, we wondered whether an alternative kinase might be upregulated under detached conditions, given that this is a critical context for the SIKs and LKB1 activity. We next performed immunoblotting analysis to profile a number of targets of LKB1 and AMPK in A549 cells with or without LKB1 add-back, and/or with SIK1/2/3 deletion (Fig. 5G). This analysis again found no evidence of residual pSIK1, pSIK2, or pSIK3 in A549 cells without LKB1 restoration, unlike pAMPK, which was still residually phosphorylated in A549 cells, particularly in detached conditions, as was the AMPK substrate pRaptor (Fig. 5G). Furthermore, we performed in vitro kinase assays on SIK1 and SIK3 isolated from LKB1-containing or LKB1-deficient A549 cells, revealing a complete loss of SIK1 and SIK3 catalytic kinase activity in the LKB1-deficient cells, consistent with the activation- loop threonine being required for catalytic activation of these kinases (Supplementary Fig. S13A–S13D). To functionally address whether SIK1 and SIK3 may have tumor-suppressor functions independent of LKB1, we used A549 cells targeted for deletion of SIK1 or SIK3 individually or in combination and performed soft-agar colony formation. First, with single disruption of SIK1, or SIK2, or SIK3, there was no statistical increase in colony number in the LKB1-deficient cells (Supplementary Fig. S13E), unlike the benefit of SIK1 loss on soft- agar colony formation in the context of A549 cells bearing LKB1 (Fig. 1F and G). However, a modest increase (~1.8-fold) in soft-agar colony formation was observed when SIK1 and SIK3 were both removed from A549 cells (Supplementary Fig. S13F), compared with >4.5-fold increase in colony formation in the LKB1-replete A549 cells (Fig. 1F). The observed increase in soft-agar colonies when SIK1/3 are deleted in LKB1-null cells raises the possibility that these kinases may have some LKB1-independent scaffolding functions or roles not reliant on SIK1/3 kinase activity in these cells.

DISCUSSION

Here we have made the unexpected finding that the AMPK- related kinases SIK1 and SIK3 redundantly mediate some of the tumor-suppressor activity of LKB1 in KRAS-driven lung cancer. Notably, head-to-head analysis of the 14 LKB1- dependent AMPK family kinases in A549 cells revealed a unique role for the SIKs in suppressing soft-agar colony formation as well as susceptibility to cell death in detached conditions. In both assays, combined SIK1/SIK2/SIK3 deletion gave a phenotype most similar to LKB1 loss, and none of the other AMPKR subfamilies gave rise to similar effects on soft-agar colony formation or growth in detached conditions. Indeed, our previous genetic studies in mice indicate that AMPK α 1 and AMPK α 2 do not play a conventional tumor- suppressor role in KRAS-driven lung tumors and when deleted result in decreased tumor burden, in stark contrast to the robust tumor increases in models from loss of LKB1³⁴. In both K and KP models of NSCLC, loss of SIK1 accelerated tumorigenesis, albeit in a manner not fully penetrant to phenocopy LKB1 loss. It is worth noting that p53 and LKB1 are mutually exclusively mutated in the context of lung adeno- carcinomas with KRAS mutations in human patients, so the KPL mouse model is simply an additional tool for examining effects of LKB1 tumor-suppressor activity. Interestingly, LKB1 is co-mutated with p53 in human adenocarcinomas with wild-type KRAS, so this is a context worth exploring further in mouse models to define SIK-dependent effects. Notably, the K and KP models with pSECC-induced targeting of the SIKs or LKB1 generated only adenocarcinomas and we did not observe an increased incidence of squamous cell carcinoma (SCC) or adeno-squamous trans-differentiating lesions at the histologic level. An increased incidence of SCC cells has been reported as a hallmark of LKB1 loss in some mouse models^{109,110,113,129,134}. Although the reasons for this discrepancy are not resolved, we believe that some contributing factors could be potential differences in latency of tumor development at experimental endpoint among different models, the choice of strain back- ground, or the use of lentiviral Cre, which is thought to favor

the incidence of adenocarcinoma development in models of KRAS/LKB1 over adenoviral Cre¹³⁰, which may influence the frequency at which histologically defined squamous carcinomas may be able to develop and be detected in these models. Here we found that concomitant loss of SIK1 and SIK3 in KRAS-mutant tumors cooperatively accelerated lung tumorigenesis and promoted the ability of tumors to grow comparably with those observed by loss of LKB1 (Fig. 3). Furthermore, the loss of SIK1/3 recapitulated the reported phenotype observed in LKB1-mutant tumors denoting a significant accumulation of neutrophils within tumor areas. In our studies, we observed over a 3-fold increase in the number of infiltrating neutrophils in both LKB1- and SIK1/3-targeted tumors when compared with tumors from KRAS control mice by histologic quantification of the neutrophil marker Ly6G. An LKB1-dependent increase in tumor-associated neutrophil infiltration has been thought to contribute to the establishment of a proinflammatory, protumorigenic state in lung tumors bearing LKB1 mutations, and is characterized by an increase in the production and secretion of IL6 by tumor cells, which attracts neutrophils that exert immunosuppressive effects¹¹⁴. Future studies are needed to determine whether additional codeletion of Sik2 or a distinct AMPKR might further enhance the SIK1/SIK3 tumor and immune phenotypes. It will also be interesting to examine how combined floxed alleles of Sik3 might synergize with loss of SIK1 and activation of KRAS in the lung, because pSECC targeting is not fully penetrant (ref. 30; Supplementary Fig. S9C and S9D), and thus may limit the amount of synergistic signal that loss of SIK3 could provoke in this study. Independently, in elegant studies using CRISPR gene editing in Cas9-expressing mice, Murray and colleagues have also found cooperating roles for SIK1 and SIK3 as arbiters of tumor suppression in NSCLC⁹³. Together, these studies underscore critical functional roles which these two kinases share in mediating LKB1's tumor-suppressive function in the lung. Functional analysis of the three SIK genes in mammals has largely come from studies of their roles in metabolic tissues such as liver, adipose, and muscle, where they are known to phosphorylate and inhibit the CRTC family of

transcriptional coactivators as well as the class IIa family of histone deacetylases^{78,80,86,124,128,135} effects we observed here in A549 NSCLC cells (Fig. 5G). Studies of the role of the SIKs in different metabolic processes in metabolic tissues have been performed in genetic KO mice and in cell-culture systems, but the role of the SIKs in tumor cells is very underexplored. However, there are hints in the literature that SIK1 and/or SIK3 may behave as tumor suppressors in some contexts. Notably, very recently biochemical inhibition of the SIK subfamily was found to be a key driving force of PKA-dependent signals from oncogenic GNAS in pancreatic cancer⁹⁴. Moreover, an early shRNA screen for kinases involved in colony formation and tumorigenesis in mammary epithelial cells reported SIK1 as one of the top hits of the screen¹²². Depletion of *Sik1* using shRNAs or expression of a kinase-dead SIK1 led to increased metastatic spread in a transplant model, which was paralleled by TCGA data showing that reduced SIK1 mRNA correlated with poor clinical outcomes in breast cancer¹²². Given that the study of the SIKs to date has mainly been in metabolic tissues for their role in controlling transcriptional regulators, we performed transcriptional analysis on primary tumors from KRAS mice alone or those bearing loss of LKB1 or SIK1 and SIK3. Transcriptional profiling of primary tumors revealed that loss of LKB1 and loss of SIK1/3 in KRAS lung tumors trigger highly overlapped transcriptional responses, with 38% of the genes upregulated from LKB1 deficiency being upregulated by SIK1/SIK3 loss. Given that LKB1 activates 12 kinases in addition to SIK1 and SIK3, it is surprising that loss of just those two kinases would trigger such an overlapping extent of gene expression. Analysis of the 344 genes upregulated in common between LKB1 and SIK1/3 loss revealed many promoters bearing CREB and AP1 binding sites. Notably, both AP1 and CREB-dependent transcription have been tied to tumorigenesis before. Notably, studies on the role of the CRTC, perhaps the best-established substrates of the SIKs, have been common in a specific type of mucoid epithelial (MEC) tumors caused by translocations involving hyperactivation of CRTC function. Increased expression of both CREB and AP1 target genes has been studied in this

tumor context extensively¹³⁶. In some of the initial studies identifying the CRTC proteins, it was noted that the phorbol ester TPA can cause nuclear translocation of CRTC1¹³⁷ and that deletion of the AP1 binding sites in the IL8 promoter resulted in it being no longer responsive to CRTC1¹³⁸. Subsequent studies directly showed that CRTC1 is a coactivator for AP1 and its regulation of some AP1 targets is independent of any effects on CREB¹³⁶. Importantly, over- expression of CRTC1 synergized with c-Jun overexpression to drive growth in HeLa cells (which are LKB1-deficient), and siRNA to CRTC1 blocked the ability of overexpressed c-Jun to induce BrdU staining, directly implicating CRTC1 in cell proliferation¹³⁶. Indeed, we observed elevated BrdU in the SIK1/SIK3-deficient tumors, and colony formation in SIK1- deficient KP cells was greatly enhanced as well (Fig. 3F). Besides AP1-dependent effects on proliferation, recent studies of the CRTC1–MAML2 fusion in MEC tumors have found that CRTC1-dependent induction of AREG, an EGF family ligand, occurred in a manner dependent on CREB binding the AREG promoter¹³⁹, and AREG-dependent activation of EGFR was important for the tumorigenic potential of CRTC1–MAML2 fusion proteins in MEC tumors. Indeed, treatment of such tumors with the EGFR mAb cetuximab blocked growth of xenografts¹³⁹. Given the importance of AREG–EGFR to proliferation of these MEC tumors, it is notable that we found AREG was upregulated in this study in LKB1-deficient tumors in a SIK1/3–CRTC2-dependent manner. In addition, another CRTC transcriptional target, NEDD9, was reported to be important for LKB1-mediated tumor suppression¹⁴⁰. Recently, working with our colleagues, Rodón and colleagues¹⁴¹ reported the involvement of CRTC2 and the CREB target gene ID1 as important mediators of human NSCLC cell and tumor growth in LKB1-null settings, which further highlights the role of LKB1–CRTC–CREB signaling in the regulation of lung cancer cell growth. EMT was another well-noted LKB1-dependent effect on gene expression¹⁴², and is also highly upregulated in the SIK1/SIK3-deficient tumors. The single most common biological pathway upregulated at the mRNA level in common between LKB1 loss and SIK1/ SIK3 loss was IL6–JAK–STAT signaling. Very recently, two studies

describing conditional models of Peutz–Jeghers syndrome, the cancer predisposition syndrome exclusively driven by congenital mutations in LKB1, have drawn a consistent connection between LKB1 deficiency and upregulation of the IL6–JAK–STAT pathway in different lineages^{143,144}. Loss of LKB1 in T cells or intestinal stromal cells resulted in the secretion of the cytokines IL6/IL11, which in turn drove hyperactivation of JAK–STAT signaling in the stromal compartment of the intestine required for tumor growth. One group furthermore found that reduced expression of SIK1, MARK1, or MARK4 in fibroblasts was sufficient to induce production of IL11¹⁴³, which suggests that control of this proinflammatory axis may be a common target of SIK- and LKB1-dependent regulation in multiple tissues and cell types. We show here in both murine and human lung adenocarcinoma cell lines that SIK1/3 deletion provokes IL6 production to a similar extent as LKB1 deletion, and IL6 production is ablated if CRTC2 is deleted (Fig. 5A and B). It will be important to investigate whether inhibition of the IL6–JAK–STAT pathway could have therapeutic value in LKB1-mutated lung cancer, especially given that therapeutic options are so limited for this tumor type. Furthermore, it will be interesting to determine whether additional genes within our newly defined list have as yet unexplored targetable potential. Mechanistically, it is also noteworthy that both SIK1 and SIK3 were previously reported to regulate TLR4 signaling to NFκB via direct binding and regulation of TRAF6¹⁴⁵. Whether CREB, AP1, NFκB, or STAT-dependent gene expression underscores the primary resistance seen in KL mouse models and the striking resistance to immune checkpoint inhibitors seen in patients with LKB1 mutations remains to be determined, but several specific hypotheses can now be tested in future studies. Although unexpected, the finding that SIK1 and SIK3 mediate much of the tumor-suppressor function of LKB1 also opens new avenues of therapeutics aimed against the pathways directly regulated by SIK1 and SIK3. Given that only a handful of substrates are known for SIK1 and SIK3, there is much to be learned mechanistically about their function in lung cancer, which may have broad implications for resistance to

checkpoint inhibitors and the large fraction of NSCLC bearing LKB1 mutations, for which there are currently no robust therapeutic strategies.

METHODS

Cell Culture and Cell Lines

All cell lines were maintained in DMEM supplemented with 10% FBS and incubated at 37°C with 5% CO₂. All cell lines were tested for Mycoplasma once a month. A549 cells were from ATCC, and isogenic A549 lines reconstituted with pBABE-LKB1 wild-type (WT) were obtained by retroviral transduction under selection (hygromycin, 600 µg/mL). 634T KP cells were a gift from Kwok Wong (New York University). FVB-mouse lung tumor-derived KP and KPSik1 cells were isolated from freshly collected mouse tumors, minced, and digested with 1 mg/mL collagenase/dispase (Sigma) in DMEM + 10% FBS, and 1× penicillin/streptomycin for 2 hours under rotation at 37°C, and were subsequently cultured on DMEM with 10% FBS, 1× penicillin/streptomycin (Gibco), and 2.5 µg/mL Fungizone (Gibco), with media replacement every 48 hours over 6 days to establish sterile cultures. KPSik1 tumor-derived cell lines were confirmed to be SIK1 KO by qPCR of Sik1 transcript. For luciferase assays, 634T cells stably expressing sgTom, sgLKB1, and sgSIK1 guide RNAs were transfected with plasmids encoding AP1 target promoter and CREB-binding Cre promoter reporters [Cignal CRE (CCS002L), AP1 (CCS-011L) coupled to luciferase], according to the manufacturer's instructions.

In Vitro Kinase Assay

FLAG-tagged SIK1 WT cDNA or T182 activation-loop mutant (TA), or SIK3 WT or TA-mutant were expressed in A549-pBABE cells or A549-LKB1-reconstituted cells with pLenti-Neo. Equivalent amounts were immunoprecipitated (SIK1, FLAG-M2-resin, SIK3, Bethyl Laboratories A302-455A with Protein A beads) from cell lysates (3 mg/sample). SIK activity assays were

performed in a final volume of 50 μ L containing 15 μ L bead volume of protein kinase and 35 μ L of reaction mix, using 200 μ mol/L of Sakamoto-tide (CRTC2 peptide ALNRTSSDSALHRRR; MRC-PPU Reagents) as a substrate, with 50mmol/L HEPES, 1mmol/L EGTA, 10 mol/L magnesium acetate, and 2 μ mol/L [γ 32P]-labeled ATP. Control reactions lacked kinase using control immunoprecipitates, or lacked Sakamoto-tide in the presence of immunoprecipitated kinase. Reactions were incubated at 30 degrees for 20 minutes, and were terminated by spotting 40 μ L of reaction on P81 phosphocellulose paper followed by immediate immersion in 75 mmol/L orthophosphoric acid. Papers were washed three times for 10 minutes with 75 mmol/L orthophosphoric acid, and washed one final time for 5 minutes in acetone. Papers were air-dried, and incorporation of P-32 was quantitated by Cherenkov counting.

Soft-Agar Colony Growth Assay

KP/KPSik1 cells (0.02 million) or A549 cells (0.03 million) were mixed with agarose in 2 $\times$ growth medium (final concentration 0.3%), plated on top of a solidified layer of 0.6% noble agar in 6-well plates, and fed every 4 to 5 days with growth medium. Cells were collected after 3 weeks, stained with 0.02% Giemsa stain (Sigma-Aldrich 48900) for 20 minutes at room temperature, and then washed with water and stored at 4°C overnight for stain development. Plates were imaged using a LI-COR Odyssey CLx (LI-COR Inc.) and Image Studio Lite software (LI-COR Inc.). Analysis of colony number and colony size were performed using ImageJ software (NIH).

2-D Colony Formation Assay

One thousand cells were plated on 10-cm dishes in full media (10% FBS in DMEM). After 7 days, cells derived from KP and KPSik1 GEMMs were washed with PBS, fixed for 15 minutes in 10% formalin (EMD Millipore R04586-82) at room temperature, and stained for 20 minutes

with 0.25% crystal violet (Sigma C3886). Plates were washed, imaged, and analysis of colony number and colony size was performed using ImageJ software.

Survival/Proliferation Assays Cell

Survival under Detachment Conditions (Anoikis Resistance). A549 cells (5×10^5) were plated on solidified 1% noble agar-coated P60 plates in full-growth media to allow for growth in detached conditions. After 5 days, cells were collected, briefly incubated with TrypLE to produce single-cell suspensions, and transferred to 24-well plates for proliferation/survival analysis using the CyQuant Direct Cell Proliferation Assay according to the manufacturer's protocol (Invitrogen C35011). Briefly, cells were incubated in staining buffer for 1 hour at 37°C and analyzed on an Infinite M1000 Pro plate reader (Tecan). For quantification, values from LKB1 add-back and AMPKR KO cells were divided by values from control A549 cells and expressed as percentage of control. *Adherent Cells.* Five thousand A549, KP, or KPSik1 cells were plated in triplicate in 96-well plates and analyzed using CyQuant assay at time-zero, 24-hour, 48-hour, and 72-hour timepoints to infer relative cell proliferation. Values for each cell line were divided by values collected at time zero and are expressed as percent growth.

IL6 Quantification

To assess IL6 secretion by tumor cells, 0.5 mol/L A549 or KP CRISPR-modified cells were plated in duplicate in 6-well plates containing 1.5-mL of full media (10% FBS in DMEM). Supernatants were harvested after 48 hours and flash-frozen in liquid nitrogen before use. Human and mouse IL6 ELISA assays were performed per manufacturer's instructions (BD Biosciences). Briefly, plates were prepared overnight with IL6 antibody, and 100 μ L of each sample was loaded in duplicate in 96-well plates. As needed, supernatants were diluted and reassessed to stay within the standard range of detection of the assay.

CRISPR/Cas9 Studies

sgRNAs targeting human or mouse AMPKRs were selected using the optimized CRISPR design tool (<http://crispr.mit.edu>). Guides with high targeting scores and low probability of off-target effects were chosen, targeting the 1st or 2nd coding exon, or otherwise the first most common downstream exon for transcripts reported to produce multiple isoforms from searches of UniProt or Ensembl databases. At least two independent sgRNA sequences were selected for each gene. Oligonucleotides for sgRNAs were synthesized by IDT, annealed in vitro, and subcloned into either BbsI-digested pSpCas9(BB)-2A-puro (pX459) vector (Addgene no. 48139), and BsmBI-digested lentiCRISPRv.2-puro (Addgene 52961) lentiCas9-blast (Addgene 52962), or pSECC (Addgene no. 60820). Validation of guide specificity was assessed by Western blot analysis of low-passage A549 human or 634T murine cells transfected with control nontargeting guides, sgTom, or sgLacZ, or sgAMPKR guides with pX459 followed by transient selection (48 hours) with 2 µg/mL puromycin, or stable integration of pLentiCRISPRv.2 or lentiCas9-blast counterparts by lentiviral transduction with 0.45 µm-filtered viral supernatant supplemented with Polybrene followed by selection with 2 µg/mL puromycin or 10 µg/mL blasticidin. Two-dimensional proliferation assays, anoikis resistance assays, and colony growth in soft agar assays were performed with cells stably expressing sgRNAs from pLentiCRISPRv.2-puro and lentiCas9-blast. pSECC lentiviral vectors harboring identical mouse sgRNAs were utilized for large-scale viral production for in vivo delivery. Oligonucleotide sequences are listed in Supplementary Table S1.

Lentiviral Production and Titering

Lentiviruses made from pLentiCRISPRv.2, pSECC, and Lenti pGKCre (latter vector was a gift from Tyler Jacks, MIT) were produced by cotransfection of the lentiviral backbone

constructs and packaging plasmids psPAX2 (Addgene 12260) and pMD2.G (Addgene 12259). For low-scale preps, Lipofectamine 2000 (Thermo Fisher Scientific) was used as a transfection reagent at a ratio of 3:1 lipofectamine:DNA. For large-scale preps, the transfection reagent was 1 μ g/ μ L PEI, pH 4.5 (Polysciences #23966-2) at a ratio of 4:1 PEI:DNA). Viral supernatant was collected 48 and 72 hours post-transfection, concentrated by ultracentrifugation at 20,000 rpm for 120 minutes, and resuspended in small volumes of 1 $\times$ Hank's Balanced Salt Solution prior to aliquoting, flash-freezing, and storing at -80°C . Most large-scale viral preps were made in-house and were supplemented as needed with custom orders prepared by the Salk Institute GT3 Viral Vector Core or the University of Iowa Viral Vector Core. For titering, lentiviral preps for mouse experiments (pSECC and PGK-Cre) were functionally titered by transduction of a reporter line (293-LSL-GFP), which turns on expression of GFP upon Cre-mediated recombination and allows quantitation of functional titers derived from the percent of GFP-positive cells obtained by flow cytometry. The formula used to calculate transforming units/mL (TU/mL) was $\text{TU/mL} = (\text{percent GFP}^+ \text{ cells} \times \text{number of cells seeded}) / (\text{volume of virus used} \times \text{dilution fold factor})$. Briefly, 80,000 293-LSL-GFP cells were plated on 24-well plates on day 0 and transduced on day 1 in duplicate across four 2-fold serial dilutions of virus supernatant. After 48 hours, cells were collected, fixed, and analyzed by flow cytometry to calculate % GFP positivity with an LSR II (BD Biosciences). TU/mL titers for each virus were derived from the average TU/mL across the dilution series, assayed two independent times in duplicate wells for each virus. All viruses used together in a given experiment were titered in a batch, and were subsequently normalized to deliver an equal number of TU/mouse.

Mouse Studies

GEMM and CRISPR Models. All procedures using animals were approved by the Salk Institute Institutional Animal Care and Use Committee. All mice were maintained on the FVB/N

background. KRAS (KrasLSLG12D/+; R26LSL;luc/luc); KL (KrasLSLG12D/+; Lkb1fl/fl; R26LSL;luc/luc), KP (KrasLSLG12D/+; p53fl/fl;R26LSL;luc/luc), and KPL (KrasLSLG12D/+; Lkb1fl/fl;p53fl/fl;R26LSL;luc/luc) mice in FVB/N have been previously described^{34,111} and Sik1fl/fl and Sik2fl/fl conditionally floxed mice have also recently been described^{81,128}. In this study, Sik2fl/fl was crossed into the FVB/N K background for 8 generations and Sik1fl/fl into the FVB/N K and KP background for 4 generations before generating experimental mice. All experiments used a mixture of female and male mice. Experimental endpoint was defined across experiments as the time point at which the experimental cohorts of K+sgLkb1, KL, or KPL mice reached tumor burden of 10⁹ mean photon flux. Experiments were stopped at that time, and all mice in that experiment were collected at that point. All animals at experimental endpoint were included for analysis of lung tumor burden and tumor size analysis. No animals were excluded from longitudinal BLI measurements and graphs. Because of the different kinetics of tumor growth inherent in K and KP animals, experiments with K and KP animals were considered independent of each other, and thus specific amounts of virus were administered to each set of K and KP animals. Viruses were delivered by intratracheal intubation, according to the protocol of DuPage¹⁴⁶. Amounts of virus delivered per mouse are as follows: 75K TU/mL of pSECC for KRAS animals, 50K TU/mL pSECC for KP animals, 2 × 10⁶ TU/mL PGK-Cre to KRAS animals, 4 × 10⁵ for KP animals, and 4 × 10⁵ for KP animals. For intravenous lung tumor studies, 8-week-old female SCID Beige mice (PrkdcscidLystbg-J/ Crl:Fox Chase, catalog no. 250) were injected with 1 × 10⁶ luciferase-expressing A549 cells into the lateral tail vein. All mice were imaged via BLI 30 minutes after intravenous injection. BLI was performed once per week for a period of 52 days.

BLI

BLI was performed biweekly for all cohorts, with the exception of the floxed KP/KPSik1/KPL cohort, which was imaged weekly. All imaging was done using an IVIS Spectrum

(Caliper Life Sciences). Mice were injected intraperitoneally with 150 mg/kg d-luciferin (Caliper Life Sciences), anesthetized with isoflurane, and imaged both ventrally and dorsally 10 minutes post luciferin injection. The total lung photon flux for each animal was calculated by the combination of ventral and dorsal photon flux calculated within a region of interest encompassing the thorax.

IHC and Image Analysis

Lungs from mice were collected at each experimental endpoint as noted in the figures, and were fixed in formalin for 16 hours, transferred to 70% ethanol and paraffin-embedded (FFPE) at the Salk Institute Histology Core or Pacific Pathology (San Diego, CA). Five micron-thick sections were prepared and stained with H&E for tumor burden quantitation. For BrdU IHC, slides were deparaffinized and rehydrated, and antigen retrieval was performed for 30 minutes at high heat in citrate buffer. Endogenous peroxidase activity was quenched with hydrogen peroxide. Slides were blocked with 5% goat serum and incubated overnight with primary anti-BrdU antibody diluted in blocking buffer, and secondary antibody incubation was carried out according to the manufacturer's instructions (ImmPress HRP Reagent Kit, Vector Labs MP-7444). Staining was visualized with ImmPACT DAB peroxidase substrate (Vector Labs SK-4105), and further counterstained with hematoxylin, dehydrated through ethanol and xylenes, and mounted with Cytoseal 60 (Thermo Scientific). H&E-stained and immunostained slides were scanned using a PerkinElmer Slide Scanner (Panoramic MIDI Digital Slide Scanner) for further downstream analysis using inForm v2.1 image analysis software (Cambridge Research and Instrumentation).

Lung Tumor Burden

Total lung tumor burden was quantitated from H&E sections using inForm v2.1 image analysis software (Cambridge Research and Instrumentation) in an unbiased manner. In brief, the Trainable Tissue Segmentation method was trained to identify tumor, normal lung, vessel, and space. This program was then applied to all H&E images, and each of the resulting mapped images was then screened to verify that accurate tissue segmentation had occurred. The quantitation data from this analysis was then used to calculate the percentage of tumor area as normalized to total lung area (tumor area + normal lung area).

Tumor Size Quantitation

The size of each individual tumor was measured from H&E sections using morphometric analysis in Panoramic Viewer software (PerkinElmer), which calculates the size of each identified tumor by area in squared microns. The area of all tumors found in 5 lobes of each mouse was exported and compiled to plot every tumor per mouse. The data were plotted for each mouse on an x-axis and tumor size on the y-axis.

BrdU Positivity Analysis

For each tumor analyzed, image preparation and tissue and cell segmentation were performed using inForm to calculate the ratio of diaminobenzidine-positive nuclei out of the total number of hematoxylin-stained nuclei per tumor to derive a percent BrdU-positive score for each tumor. All individually cropped tumor images were analyzed simultaneously as a batch and verified for accuracy in a blinded manner to exclude extreme outlier images from final analysis.

Immunoblots

Protein lysates from cells were isolated in cold 1% NP-40 RIPA buffer supplemented with protease inhibitors (cOmplete, Roche) and phosphatase inhibitors (PhosStop, Roche). Lysates were normalized using a BCA protein assay kit (Pierce) and resolved on 8% SDS-PAGE gels.

Antibodies

For Western blotting, antibodies from Cell Signaling Technology were used diluted at 1:1,000 unless otherwise noted: LKB1 (#3047), AMPK α (#2532), pAMPK(T172) (#2535), pACCS79 (#3661), pRAPTOR (S792; #2083), BRSK1 (#5935, 1:500), BRSK2 (#5460), MARK2 (#9118), MARK3 (#9311), MARK4 (#4834, 1:500), pMARK activation loop (#4836), NUA1 (#4458), SIK2 (#6919), GAPDH (#5174, 1:10,000), NR4A1 (#3960), pHDAC4/5/7(S246; #3443), STAT3 (#9139), STAT1 (#14994S), pSTAT1-Y701 (#9167), pSIK family T-loop antibody (Abcam 199474, 1,000), SIK1: rabbit polyclonal 1-111 (kind gift from Rebecca Berdeaux, Salk Institute), SIK3 (human; Novus Biologicals, NBP2-47278), SIK3 (mouse; Bethyl Laboratories, A302455A), NUA2 (Novus Biologicals, NBP1-81880, 1:1,000), SNRK (ab173538, 1:500), CRT3 (ab91654, 1:1,000), CRT2 (Calbiochem ST1099, 1:10,000), pCRT2 (kind gift from Marc Montminy), FLAGM2-affinity gel (Sigma A2220). For IHC, BrdU (Abcam #6326, 1:200) and Ly-6G (BioLegend, clone 1A8, 1:200).

SIK1 PCR

To validate loss of Sik1 in lung tumors, DNA was isolated from flash-frozen tumor tissue derived from control K+sgTom, KSik1, and KSik1+sgSik3 tumors using the Quick-RNA Miniprep Kit (Zymo Research), and PCR was performed with primers to amplify the recombined Sik1 allele or primers to amplify the floxed allele as described in Nixon and colleagues¹²⁸.

mRNA Preparation and qPCR

mRNA from snap-frozen tumor samples was prepared using the Quick-RNA Miniprep Kit (Zymo Research), including DNase treatment. cDNA was synthesized from 2 µg of RNA using SuperScript III (Life Technologies), and qPCR was carried out with diluted cDNA, appropriate primers, and SYBR Green PCR master mix (Thermo Fisher Scientific) using a C1000 Thermal Cycler (Bio-Rad). Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method, using actin as an internal control.

mRNA-seq

Tumor RNA was isolated using the Quick-RNA MiniPrep Kit (Zymo Research), including a DNase treatment. RNA integrity numbers were determined using an Agilent TapeStation prior to library preparation. mRNA-seq libraries were prepared using the TruSeq RNA Library Prep Kit (version 2), according to the manufacturer's instructions (Illumina). Libraries were quantified, pooled, and sequenced by single-end 50 base pairs using the Illumina HiSeq 2500 platform at the Salk Next-Generation Sequencing Core. Raw sequencing data were demultiplexed and converted into FASTQ files using CASAVA (version 1.8.2). Libraries were sequenced at an average depth of 80 million reads per sample.

Deep Sequencing and Bioinformatics Analysis of Cas9 Target Loci

Tumor DNA was obtained by laser-capture microdissection at the Biophotonics Core at the Salk Institute. DNA was isolated from lung histology sections to represent all sgRNA guides used in this study and was prepared with the Arcturus PicoPure DNA Extraction Kit (ABI), with subsequent cleanup with AMPure beads (Agilent). For each targeted gene, a 300-bp amplicon centered around the PAM site was amplified using New England BioLabs Next 2X master mix. Sequencing libraries were prepared from 50–80 ng of tumor PCR product and barcoded with custom index R2 primers (kind gift from Patrick Hsu, Salk Institute). Individual libraries were

quantitated after electrophoresis with analytic quantitative ladder, and relative molar amounts of barcoded amplicons were pooled, cleaned up with AMPure beads and gel purified 2×. Final pooled libraries were characterized for size by TapeStation, quantitated by qPCR using KAPA Library Quantification Kits, and sequenced on an Illumina MiSeq machine with 300-bp end-paired reads. MiSeq reads were filtered by requiring an average Phred quality (Q score) of at least 30, as well as perfect sequence matches to barcodes. Data was demultiplexed with a custom script, and pair-end stitching was done with Pear. The python program CRIS.py v2 was applied to stitched data to assess endonuclease activity. CRIS.py output was used to quantify the edit rate, frameshift rate, and average indel size of all clones.

Bioinformatic Analysis for RNA-seq

Sequenced reads were quality-tested using the online FastQC tool (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) and aligned to the mouse mm10 genome using the STAR aligner version 2.4.0k. Raw gene expression was quantified across all annotated exons using HOMER¹⁴⁷, and differential gene expression was carried out using the edgeR package version 3.6.8 using duplicates to compute within-group dispersion and correcting for batch effects¹⁴⁸. Differentially expressed genes were defined as having an FDR < 0.05 and a log2 fold change >0.59. GSEA was carried out with the Gene-Pattern interface (<https://genepattern.broadinstitute.org>) using pre-ranked lists generated from FDR values, setting gene set permutations to 1,000 and using the Hallmark collection in MSigDB version5.0. Clustering was performed with Gene Cluster 3.0 and visualized by heat maps using Java TreeView (version 1.1.6r4). Area-proportional Venn diagrams were plotted using BioVenn¹⁴⁹. Metascape: <http://metascape.org>; Enrichr: <http://amp.pharm.mssm.edu/Enrichr>; Homer motif enrichment analysis: <http://homer.ucsd.edu/homer/motif/>.

Statistical Analyses

Statistical analyses described in each figure were all performed using GraphPad Prism 7.

ACKNOWLEDGMENTS

This study was supported by grants to R.J. Shaw from the NIH (R35CA220538, P01CA120964), the Samuel Waxman Cancer Research Foundation, and The Leona M. and Harry B. Helmsley Charitable Trust grant #2012-PG- MED002. NIH grants to R. Berdeaux also supported this work (R01DK092590 and R01AR059847). R.U. Svensson was supported by a postdoctoral fellowship from the American Cancer Society (ACS#124183-PF-13-023-01-CSM). L.J. Eichner is supported by a postdoctoral fellowship from the American Cancer Society (PF-15-037-01-DMC). S.N. Brun was supported by training grant 5T32CA009370 to the Salk Institute Cancer Center and 5F32CA206400. The Salk CCSG P30 CA014195 and Helmsley Charitable Trust supported the Functional Genomics Core and the Bioinformatics Core. We thank Thales Pappagiannakopoulos (New York University) and Francisco Sánchez-Rivera (Memorial Sloan Kettering Cancer Center) for advice regarding pSECC viral preparation. We additionally are grateful to Nasun Ha and the Salk Next-Generation Sequencing Core for helpful discussions; Marc Montminy, Ezra Wiater, and Patrick Hsu for assistance and discussions regarding MiSeq; Jill Meisenholder and Xavier Miralles Fusté for helpful advice with in vitro kinase reactions; and Jeanine Van Nostrand for help with intratracheal intubations. We thank Matthew Chun for help with transcription analysis in human NSCLC cells and Daniel Garcia for help with graphic design. We thank Chris Murray and Monte Winslow of Stanford for helpful discussions, and members of the Shaw lab for feedback and advice.

Chapter 2, in full, is a reprint of the material as it appears in: Hollstein, P. E., Eichner, L. J., Brun, S. N., Kamireddy, A., Svensson, R. U., Vera, L. I., Ross, D. S., Rymoff, T., Hutchins, A., Galvez, H. M., Williams, A. E., Shokhirev, M. N., Screatton, R. A., Berdeaux, R. & Shaw, R. J. The

AMPK-related kinases SIK1 and SIK3 mediate key tumor suppressive effects of LKB1 in NSCLC. *Cancer Discov* 9, 1606–1627 (2019). On this publication, Pablo Hollstein was the primary author and the dissertation author was a co-author. Reuben J. Shaw directed and supervised the writing and oversaw the project which formed the basis for this chapter.

Figure 2. 1: A lentiviral CRISPR-based screen targeting the AMPKR kinase family in human NSCLC cells reveals the SIK subfamily mediates tumor-suppressive phenotypes of LKB1.

A, Left, schematic of the AMPK-Related (AMPKR) kinase family tree branch. Right, reads per kilobase million (RPKM) plot showing the relative mRNA expression of the AMPKR kinase family in human A549 (LKB1-null) and LKB1 wild-type (WT)-reconstituted cells. B–D, Western blots on lysates from A549-LKB1 WT cells treated with lentiviral small guide RNAs (sgRNA) targeting the AMPKR family. Control sgRNA-treated A549 (LKB1-null) and A549-LKB1 WT cells also shown. B, Western blots of lysates from A549-LKB1 WT cells targeted to co-delete NUAK1+NUAK2 (left) and SNRK (right). C, Western blot of lysates from A549-LKB1 WT cells targeted to co-delete MARK1+MARK4, MARK2+MARK3, and BRSK1+BRSK2. D, Left, Western blots of lysates from A549-LKB1 WT cells targeting AMPK catalytic subunits $\alpha 1/2$. Right, Western blots of lysates from A549-LKB1 WT cells targeting the SIK subfamily, either with individual guides against SIK1, SIK2, and SIK3, or using combinations of guides to co-delete SIK1+SIK2, SIK1+SIK3, and SIK1+2+3. E, Western blots on lysates from A549-pBABE and A549-LKB1 WT cells targeting SIK1 + SIK3 or SIK1+SIK2+SIK3. SIKs are phosphorylated only in the presence of LKB1, and cell lines targeted for SIK deletion show loss of phosphorylation only on the targeted SIK proteins, validating deletion in these lines. F, Soft-agar colony formation of AMPKR-deficient cells. Average colony number after 3 weeks of growth in soft agar is shown. Assays were done two times in triplicate wells, and the values represent the average of independently generated cell lines for a given AMPKR as described in Supplementary Fig. S1E. *, $P < 0.05$; ****, $P < 0.0001$ by ANOVA and post hoc Student t test. G, Representative images of soft-agar colony growth of A549 (LKB1-null) cells, A549 LKB1-WT (“L”) cells, and AMPKR-deficient A549 LKB1-WT cells.

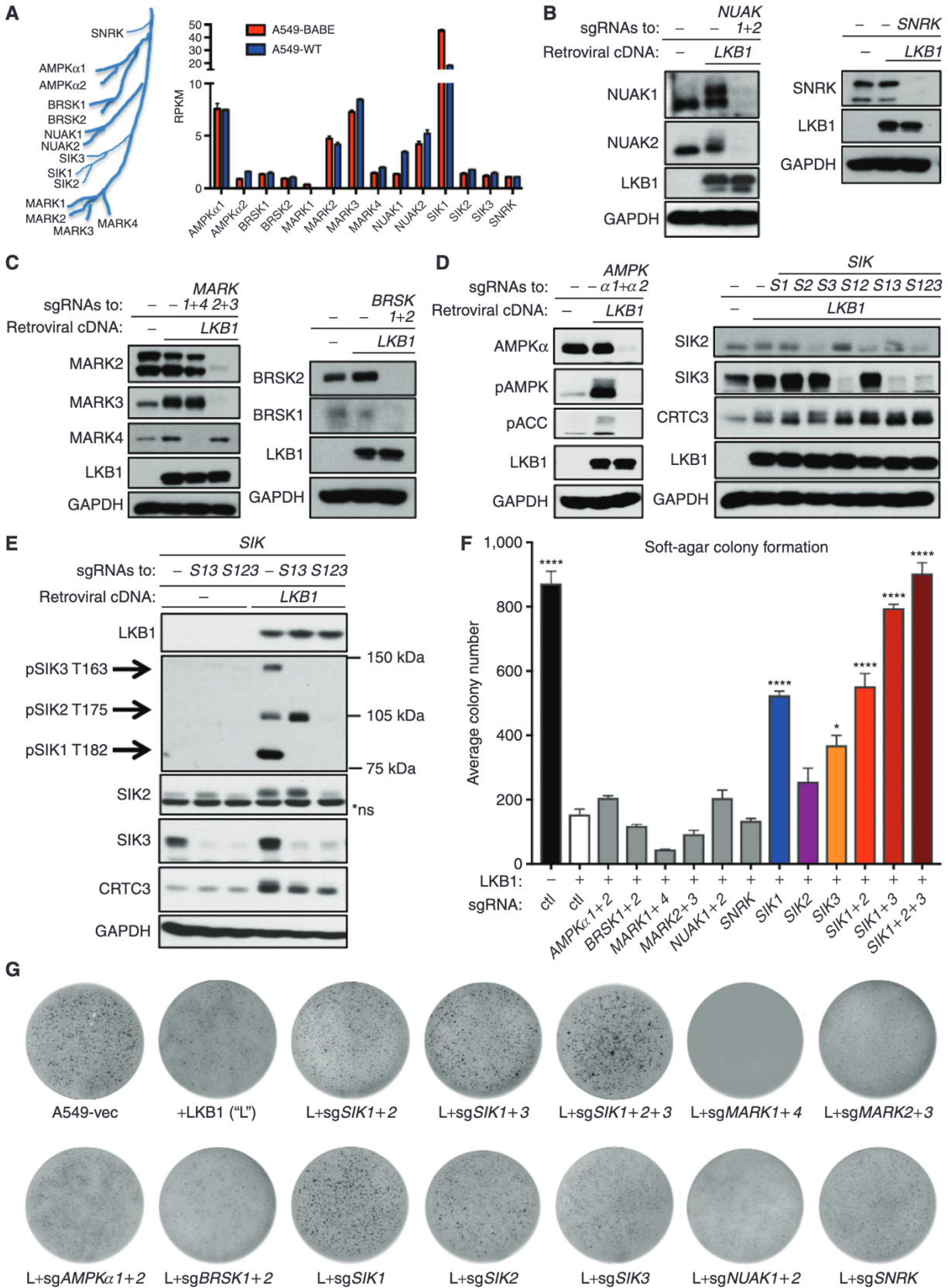


Figure 2. 2: CRISPR-based and genetically engineered mouse model (GEMM)-based mouse models of SIK family kinase inactivation in KRAS-driven NSCLC demonstrate acceleration of tumor growth upon SIK1 loss.

A, Schematic of the experimental design using pSECC lentivirus to deliver Cre-recombinase, Cas9, and an sgRNA of choice as a single payload to the lungs of KRASG12D mice (K) and KRAS-p53 floxed mice (KP) after intratracheal virus delivery. B–E, Analysis of inactivating Sik1 or Lkb1 with pSECC-sgRNA viruses in the lungs of K mice. B, Longitudinal BLI data from induced tumors in K mice treated with pSECC control (sgTom, N = 4 or sgLacZ in C, N = 3), sgLkb1 (N = 10), and three independent sgSik1 viruses (N = 11). Average bioluminescence (photon flux) at each imaging time point is shown for each cohort. The last point shown indicates study endpoint. C, Quantitation of tumor burden. Tumor area was calculated as a percent of total lung area from hematoxylin and eosin (H&E)-stained sections for each mouse. The average tumor burden for each cohort is shown as a fraction of tumor area/total lung. D, Quantitation of individual tumor size in lungs of K mice treated with pSECCcontrol (sgTom) and pSECC-sgSik1 viruses. The area of individual tumors was quantitated in mm² from H&E sections for each mouse and are shown in a dot plot per cohort for each sgRNA guide. Each dot represents a single tumor, with a bar denoting the average tumor size per cohort. E, Distribution in tumor size observed in pSECC-control and pSECC-sgSik1-treated mice. Individual tumors from all mice in each cohort were binned by size and are shown as a percent for each size bin in a pie chart. F–I, Analysis of inactivating the SIK kinase family or LKB1 with pSECC-sgRNA viruses in the lungs of KP mice. F, Longitudinal BLI data from KP mice treated with control pSECC virus or pSECC viruses harboring sgRNA guides to target mouse Lkb1 (N = 6), Sik1 (N = 10), Sik2 (N = 9), and Sik3 (N = 7). Average bioluminescence (photon flux) at each imaging time point is shown for each cohort. The last point shown indicates study endpoint. G, Tumor burden analysis for all pSECC-treated cohorts of KP mice. Tumor area was calculated as a percentage of total lung area. The average tumor burden for each cohort treated with individual sgRNA guides is shown as a fraction of tumor area/total lung. H, Quantitation of individual tumor size (area in mm²) in pSECC control and pSECC-sgSik1-treated KP mice. The number of tumors per cohort treated with individual sgRNA guides targeting Sik1 is shown. Each dot represents an individual tumor, with a bar denoting the average tumor size per cohort. I, Tumor size distribution in KP mice treated with control and pSECC-Sik1 viruses. Individual tumors were binned by size and are represented as percentages of different size bins shown in a pie chart. All values in (B–I) are expressed as means $\pm$ SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001 determined by Student t test.

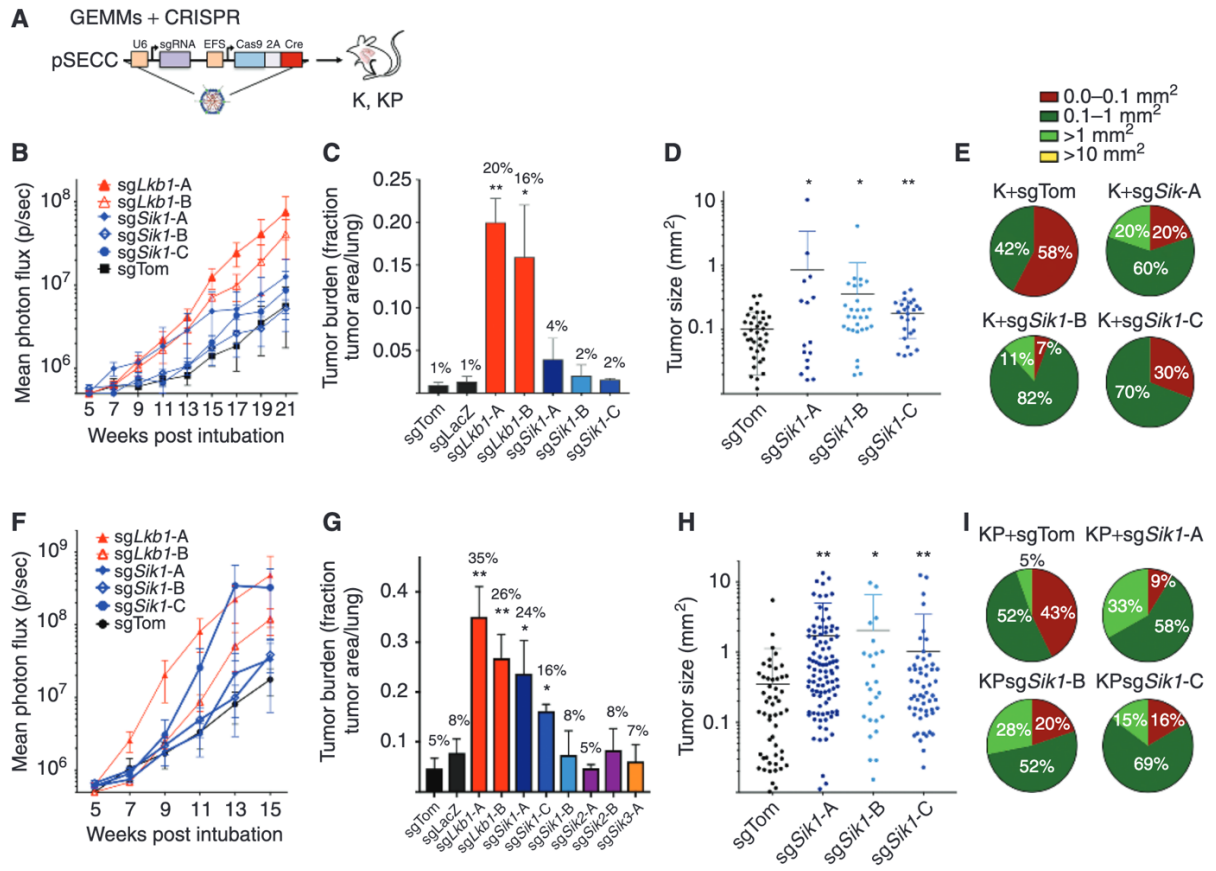


Figure 2. 2: CRISPR-based and genetically engineered mouse model (GEMM)-based mouse models of SIK family kinase inactivation in KRAS-driven NSCLC demonstrate acceleration of tumor growth upon SIK1 loss, Continued.

J, Schematic of the experimental design using GEMM mice with floxed alleles of *Sik1*, *Sik2*, and *Stk11* (LKB1) in a conditional KRASG12D background (KSik1, KSik2, KL), and *Sik1* and *Stk11* in a conditional KP genetic background (KPSik1 and KPL). Genetic conditional activation of *Kras* and conditional KO of the listed genes in the lung is generated by Cre-recombinase-mediated loxP recombination after intratracheal delivery of pGK-Cre-virus. K–N, Analysis of tumor growth, endpoint tumor burden, and tumor size distribution in floxed K, KSik1, KSik2, and KL mice. K, Longitudinal BLI data from induced lung tumors in floxed K (n = 13), KSik1 (n = 12), KSik2 (n = 6), and KL (n = 6) mice after PGK-Cre-mediated recombination. Average bioluminescence (photon flux) at each imaging time point is shown for each cohort. The last point shown indicates study endpoint. L, Tumor burden analysis of floxed K, KSik1, KSik2, and KL mice. Tumor area was calculated as a percent of total lung area per mouse, and shown as the average fraction of tumor/lung for each cohort. M, Quantitation of tumor size (area in mm²) in lung tumors from floxed K and KSik1 mice. Each dot represents an individual tumor, with a bar denoting the average tumor size per cohort. N, Tumor size distribution in floxed K and KSik1 mice. Individual tumors were binned by size and are represented as percentages of different size bins shown in a pie chart. O–R, Analysis of tumor growth, endpoint tumor burden, and tumor size distribution in floxed KP and KPSik1 mice. O, Longitudinal BLI data from induced lung tumors in floxed KP (n = 8), KPSik1 (n = 5), and KPL (n = 5) mice after PGK-Cre-mediated recombination. Average bioluminescence (photon flux) at each imaging time point is shown for each cohort. The last point shown indicates study endpoint. P, Tumor burden analysis of floxed KP, KPSik1, and KPL mice. Tumor area was calculated as a percent of total lung area per mouse, and shown as the average fraction of tumor/lung for each cohort. Q, Quantitation of tumor size (area in mm²) in individual lung tumors from GEMM KP and KPSik1 mice. Each dot represents an individual with a bar denoting the average tumor size per cohort. R, Tumor size distribution in GEMM KP and KPSik1 mice. Individual tumors were binned by size and represented as percentages of different size bins shown in a pie chart. S, Colony growth in soft agar of mouse tumor-derived KPSik1 and KP cells lines. Data represent the combined average of n = 4 independent KP lines and n = 3 independent KPSik1 lines from assays performed in triple wells two times. Graph shows average colony number at a threshold to detect large colonies (50-pixel range). All values in (K–S) are expressed as means ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.001 determined by Student t test).

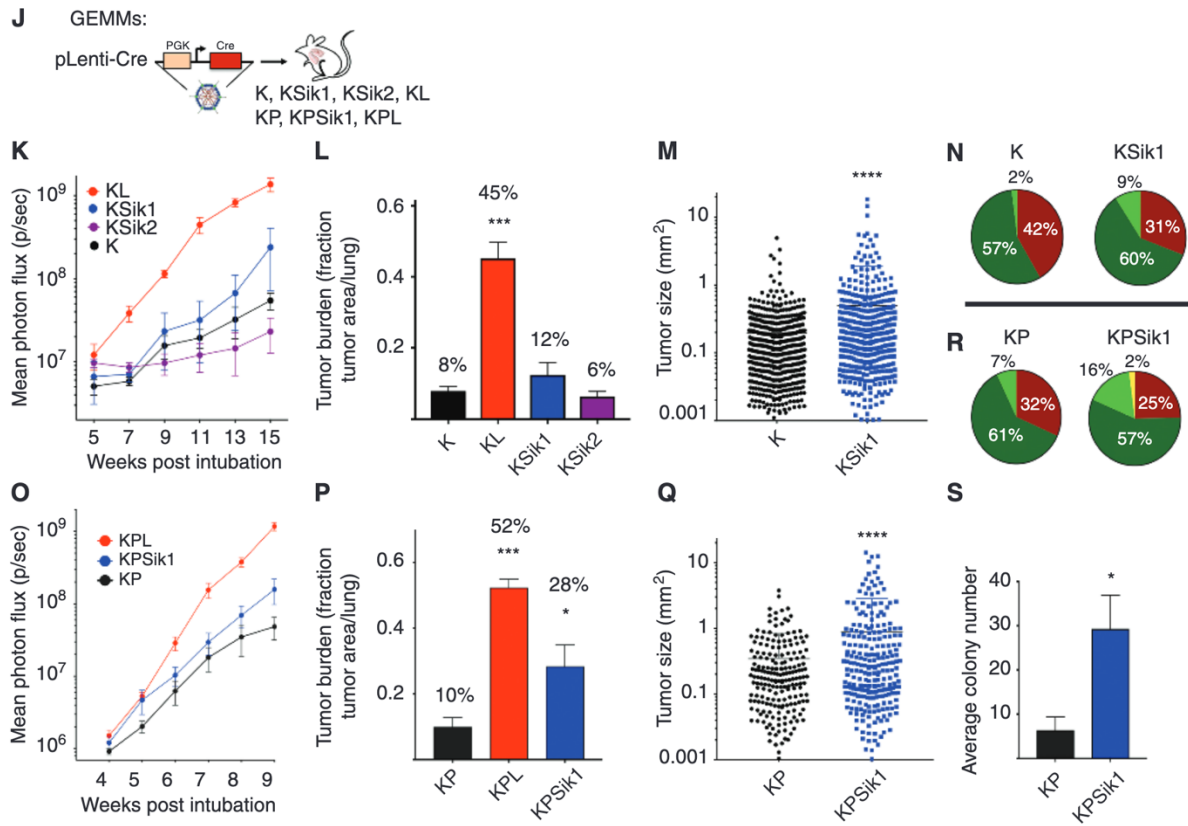


Figure 2. 3: Combined effect on tumor growth by genetic loss of Sik1 with CRISPR-mediated inactivation of Sik3 in a KRAS-driven mouse model of NSCLC.

A, Schematic of the experimental design using pSECC lentivirus to deliver Cre-recombinase, Cas9 and a Sik1 or Sik3 sgRNA as a single payload to the lungs of KRASG12D mice (K) and Kras-Sik1 floxed mice (KSik1) after intratracheal virus delivery. B, Longitudinal BLI data from induced lung tumors in GEMM KSik1 mice treated with pSECC control (sgTom; n = 7) or pSECC-sgSik3-B (n = 7), and K mice treated with pSECC control (sgTom; n = 7), sgLkb1 (n = 3 at study endpoint), or two sgRNA viruses targeting Sik1: sgSik1-D, (n = 7), sgSik1-E (n = 5), or Sik3: sgSik3-B (n = 9), and sgSik3-C (n = 6). Average bioluminescence (photon flux) at each imaging time point is shown for each cohort. The last point shown indicates study endpoint. C, BLI data of lung tumors from study endpoint for K and KSik1 mice treated with control sgTom and sgSik guides. Each dot represents the endpoint BLI value for each mouse overlaid on a bar graph denoting the mean for each group. D, Overlay of BLI images at study endpoint for K+sgTom, KSik1+sgTom, and KSik1+sgSik3 tumor-bearing mice. Shown are n = 6 images for mice with the highest amounts of luminescence emitted from lung tumors within each cohort. n = 2 images from K+sgLkb1 mice are shown for comparison. Scale bar represents photons/sec/cm²/sr. E, Tumor burden analysis of K+sgTom, KSik1+sgSik3, KSik1+sgTom, and K+sgSik1 and K+sgSik3 mice. Tumor area was calculated as a percent of total lung area per mouse, and shown as the average fraction of tumor/lung for each cohort. F, Quantitation of tumor size (area in mm²) in individual lung tumors from K+sgLkb1, K+sgTom, KSik1+sgSik3, KSik1+sgTom, and K+sgSik1, and K+sgSik3 mice. Each dot represents an individual with a bar denoting the average tumor size per cohort. G, BrdU positivity in lung tumors from K+sgLkb1, K+sgTom, KSik1+sgSik3, or sgTom, and K+sgSik1 or sgSik3 mice. Data are displayed as percent BrdU+ cells over total cell number for each tumor. Each dot represents a single tumor within each cohort. H, Lung tumor size distribution in KRAS (K) and KSik1 mice treated with control or sgSik3 pSECC viruses (KSik1 mice), or control and sgLkb1, sgSik1, and sgSik3 pSECC viruses (K mice). Individual tumors were binned by size and represented as percentages of different size bins shown as a pie chart. All values in (B–H) are expressed as means $\pm$ SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.001 determined by Student t test).

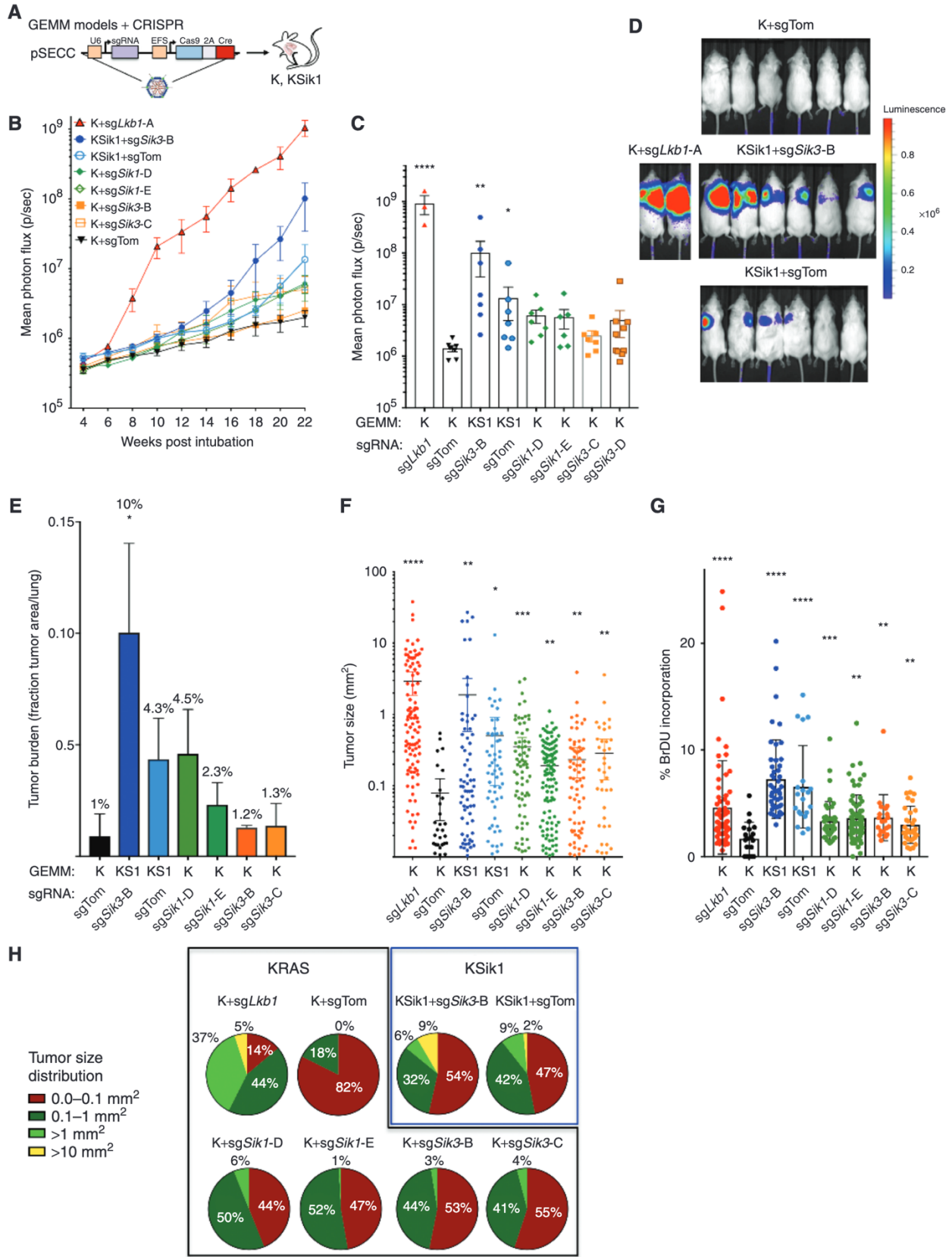


Figure 2. 4: Transcriptional analysis of lung tumors derived from KSik1+sgSik3 and K+sgLkb1 mice reveal a shared core gene signature enriched for upregulation of genes involved in IL6–JAK–STAT and AP1 signaling pathways.

A, Transcriptional profile heat map denoting unbiased clustering of mouse tumors from the following genotypes: K+sgTom (KTom; n = 9), KSik1+sgSik3 (n = 6), and KL-A (n = 10). Tumor RNA was analyzed by high-throughput wholetranscriptome sequencing (RNA-seq). Samples clustered by genotype in an unbiased manner. B, Differential expression analysis on tumors from A (using fold change $\pm \log_2 0.59$, FDR <0.05 cutoffs) reveals that 38% of upregulated genes and 41% of downregulated genes that are LKB1-dependent respond in the same, statistically significant manner to SIK1+SIK3 inactivation. C, Cluster of the 971 genes commonly regulated by inactivation of LKB1 or SIK1+3 inactivation identified in B. D, GSEA plots from KSik1+sgSik3 versus KTom data queried against the “Hallmark” group of gene sets. Six gene sets were independently identified in both the K+sgLkb1 and KSik1+3 dataset queries. Two of these gene sets, shown in D, represent biological processes well established to be induced upon LKB1 deletion. The EMT signature was the top enriched gene set, IL6_JAK_STAT3_Signaling was eighth most enriched gene set. EMT heat map shows FPKMs for all enriched genes in EMT GSEA plot. IL6 heat map shows FPKMs for all enriched genes in IL6 GSEA plot combined with Metascape IL6 category genes (Supplementary Fig. S10B).

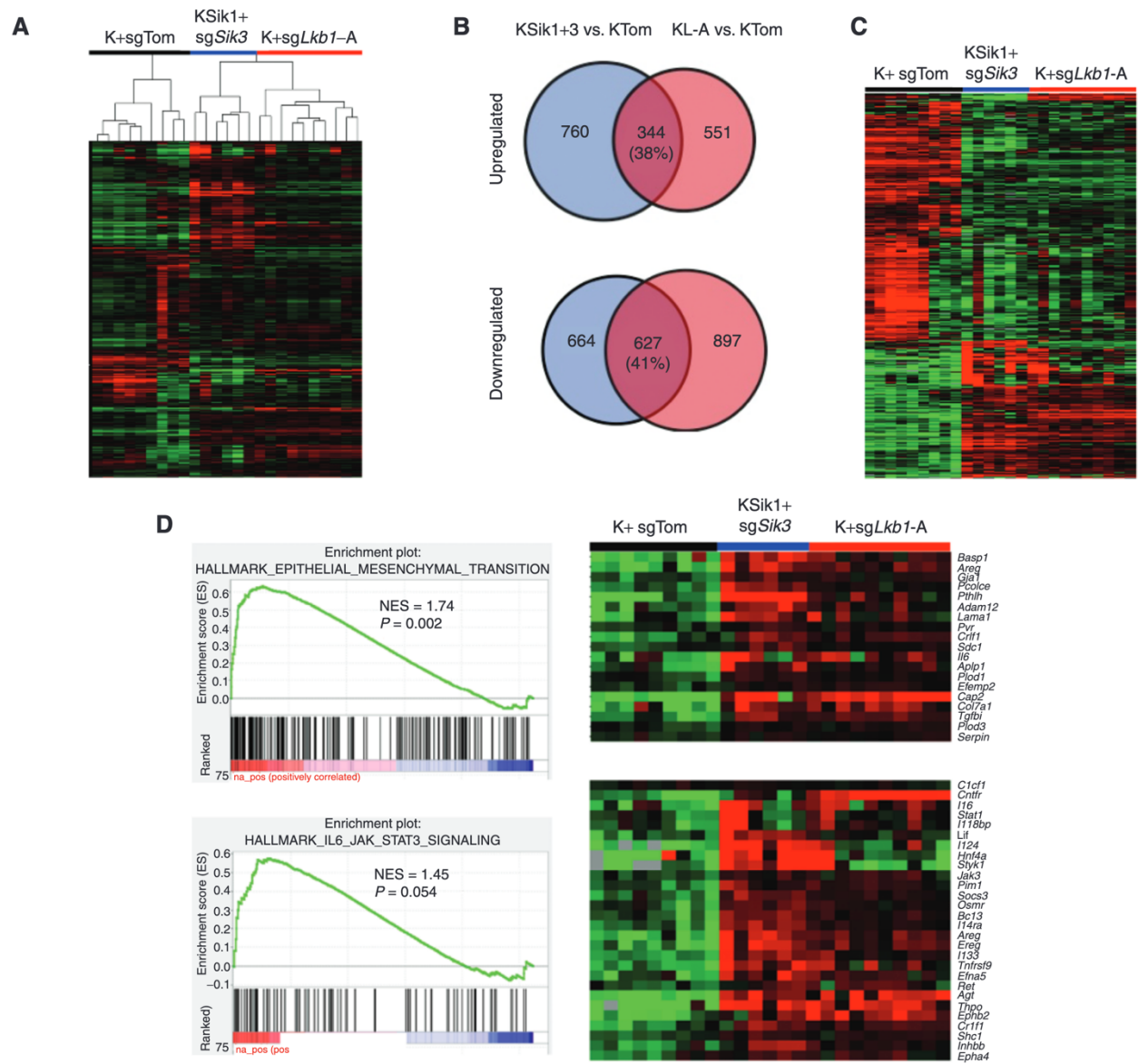
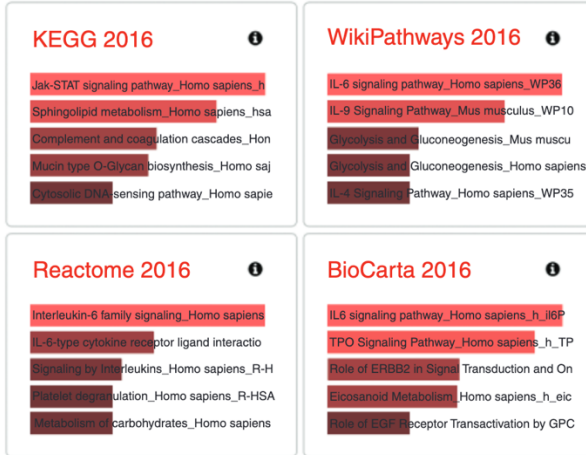


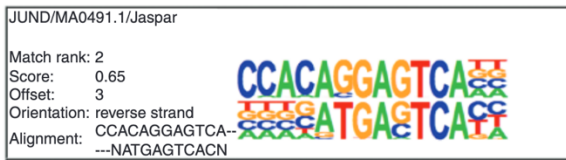
Figure 2. 4: Transcriptional analysis of lung tumors derived from KSik1+sgSik3 and K+sgLkb1 mice reveal a shared core gene signature enriched for upregulation of genes involved in IL6–JAK–STAT and AP1 signaling pathways, Continued.

E–G, Analysis of the 344 upregulated genes common to both K+sgLkb1 and KSik1+sgSik3. E, Enrichr analyses identify IL6 pathway enrichment only within upregulated genes common to K+sgLkb1 and KSik1+sgSik3. F, Homer de novo motif enrichment analysis on the upregulated genes common to K+sgLkb1 and KSik1+sgSik3. The only significantly enriched motif ($P = 1e-12$) is shown. The enriched motif corresponds to the JUND/FOS/FOSL1/AP1/FRA2 motif; JUND motif is pictured. G, Heat map of genes with AP1+CRE elements in the promoter (± 2 kb of TSS). H, FPKM plots for genes from the IL6–JAK–STAT3 signaling pathway. Cytokine (Il6, Il33), receptor (Il4ra), core component (Jak3, Stat3), coactivator (Bcl3, Osmr), and canonical downstream STAT3 transcriptional target (Socs3) mRNAs were all upregulated in both LKB1-deficient and SIK1+SIK3-deficient tumors.

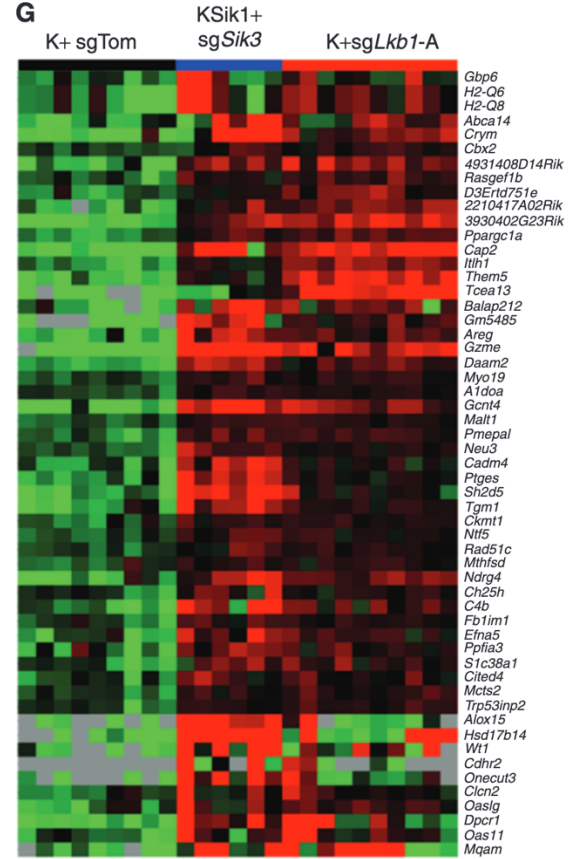
E



F



G



H

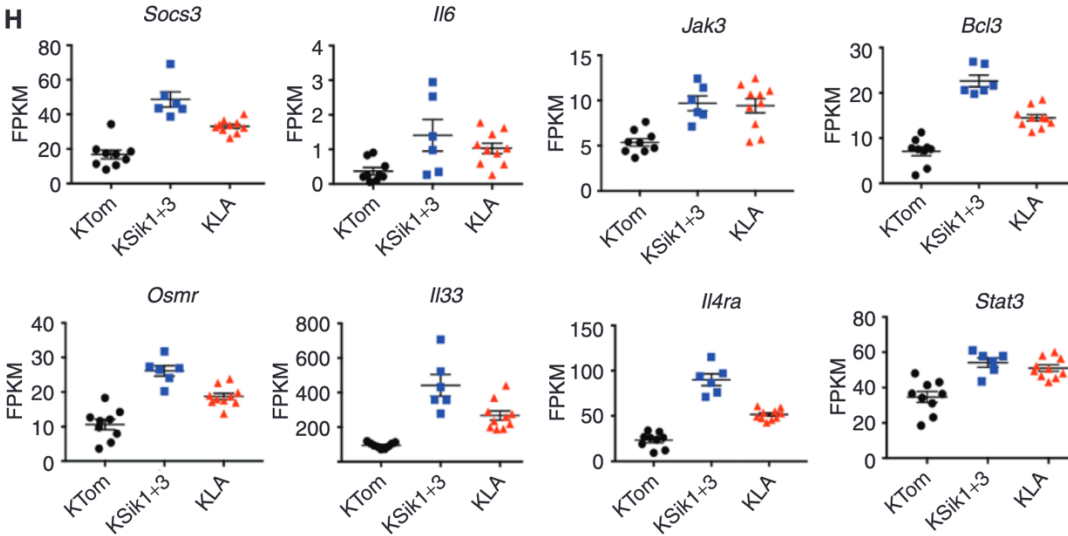
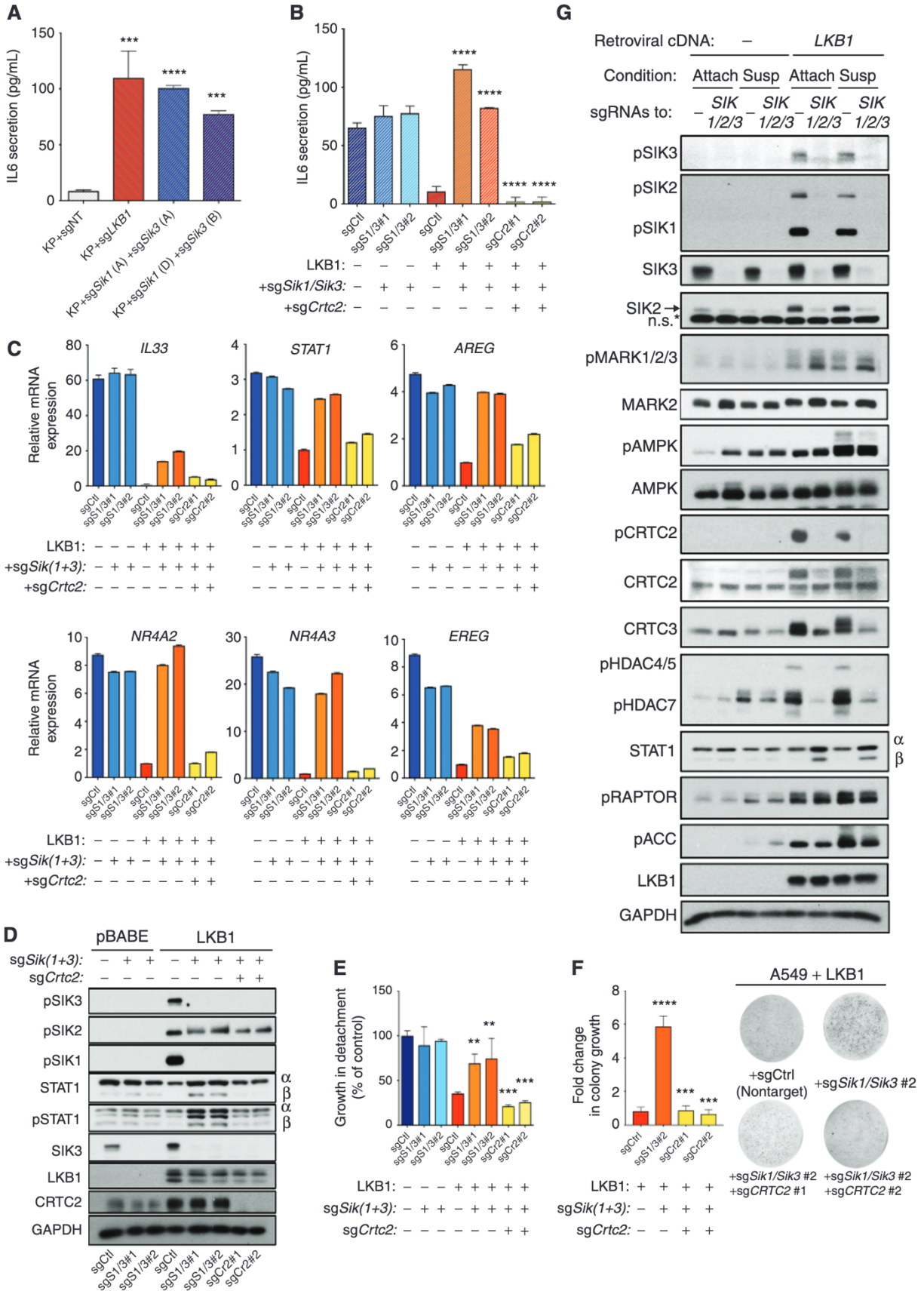


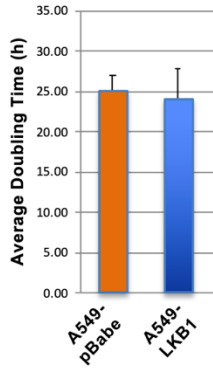
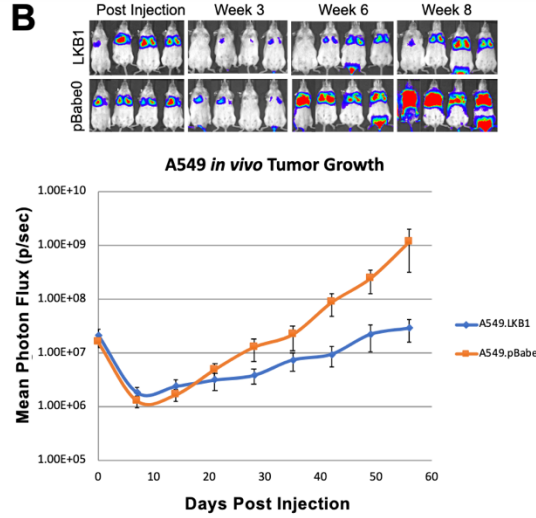
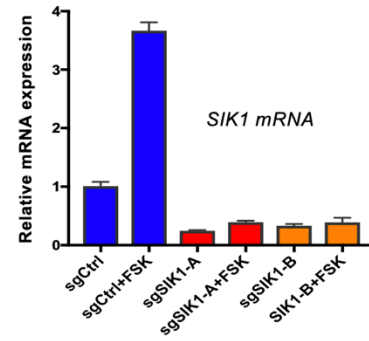
Figure 2. 5: Functional analysis of murine and human NSCLC cell lines bearing LKB1 or SIK kinase family inactivation reveals CRTC2-dependent induction of cytokine signaling and control of cell proliferation and growth under anchorage-free conditions.

A, ELISA analysis of IL6 secretion in mouse KP lines. KP cell lines were transduced with a sgRNA guide targeting *Lkb1* or with two independent sets of guides to target *Sik1* and *Sik3* simultaneously. 5×10^5 control or KO cells were seeded in 6-well plates and supernatant was collected at 48 hours to quantitate the amount of IL6 released by the cells into the media. B, ELISA analysis of IL6 secretion in human A549 NSCLC cells. Two independent cell lines bearing distinct guides to simultaneously target *Sik1* and *Sik3* were generated each in pBABE cells (LKB1-null) and in cells reconstituted with *Lkb1* cDNA. In addition, the LKB1-reconstituted sgSik1/3 cell line #2 was further targeted with one of two guides to target expression of *Crtc2* or a control guide. 5×10^5 cells from each genotype were seeded and supernatant was collected at 48 hours, and IL6 secretion was quantitated by ELISA. C, Relative expression of genes commonly regulated by LKB1, SIK1+3 kinases, and CRTC2. qPCR was performed on pBABE and LKB1-expressing A549s, $\pm$ sgSik1+3 and $\pm$ sgCrtc2 guides to assess modulation of SIK1+3 and CRTC2-dependent transcription. For all genes examined, mRNA expression was plotted relative to LKB1-expressing control line (A549 LKB1 + sgCtl). D, Immunoblots from sgSIK1+3 A549 (control or LKB1-reconstituted) and sgSIK1+3+sgCrtc2 cell lines were assessed for expression and phosphorylation status of SIKs. E, Proliferation of cells under anoikis (attachment-free) conditions. Control pBABE or LKB1-expressing A549 cells expressing guides targeting *Sik1* + *Sik3* $\pm$ sgCrtc2 guides were challenged with detachment conditions for 5 days. The number of surviving cells was quantitated with CyQuant staining. Data are means of triplicate wells $\pm$ SD. F, Left, fold change in colony growth in soft agar of LKB1-expressing A549 cells expressing guides targeting *Sik1* + *Sik3* $\pm$ sgCrtc2 guides. Colonies were counted at four weeks and the data are shown as the average of two biological replicates of triplicate wells for each genotype $\pm$ SEM. Right, representative images of colony formation in soft agar at experimental endpoint. G, Western immunoblots of lysates from LKB1-null pBABE or LKB1-expressing A549s, further expressing sgRNA guides targeting all three SIKs. Lysates were collected from cells under adherent and suspension conditions. Immunoblots depict phosphorylation status of the SIKs and additional AMPKRs (AMPK, MARKs), direct SIK substrates (CRTC2s, HDACs), and AMPKR targets or downstream transcriptionally regulated genes.



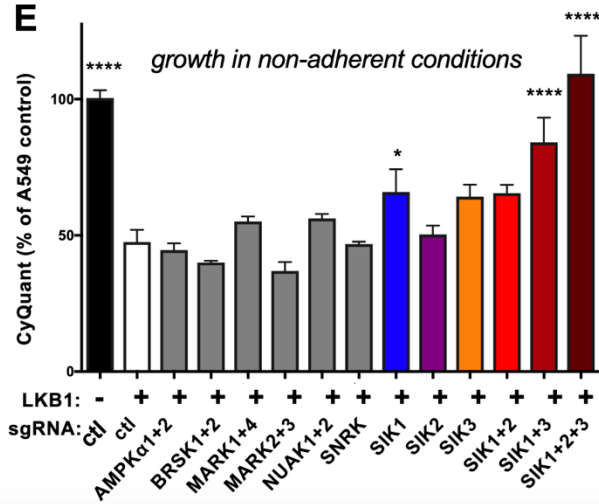
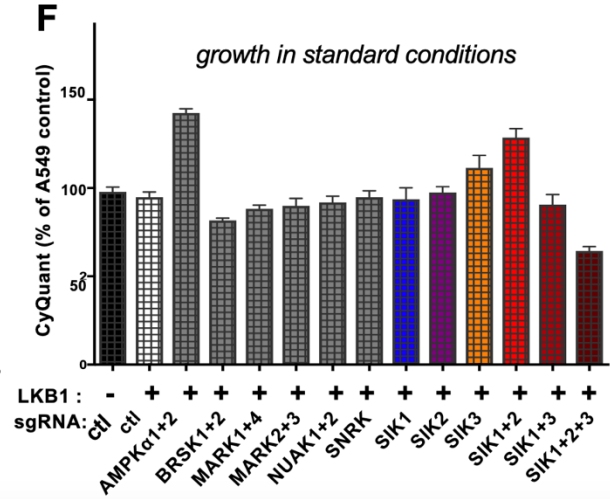
Supplemental Figure 2. 1: Characterization of A549 cells as a LKB1-dependent NSCLC to study effects of downstream targets on growth.

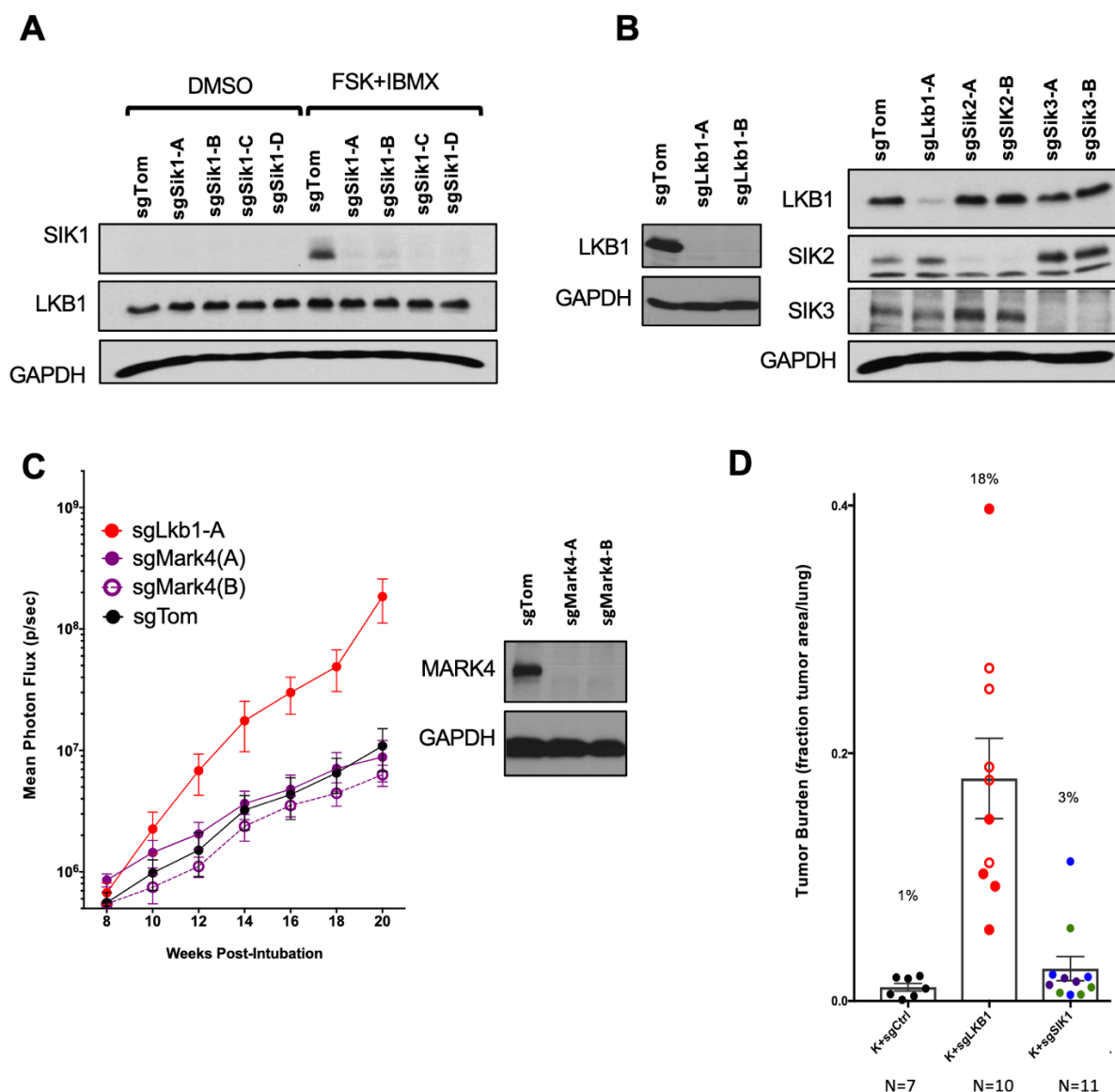
(A) A549 cells were stably infected with retrovirus bearing pBabe (vector) or pBabe-wild-type LKB1 cDNA. Doubling time of cells growing in subconfluent full media conditions in tissue culture. (B) Lung colonization of luciferase expressing A549-pBabe and A549+LKB1 WT cells from panel A. Top: Representative Bioluminescent Images (BLI) of mice. Cells were orthotopically introduced via tail-vein injection of NOD-SCID mice and lung tumor burden was followed by bioluminescent imaging. Imaging 30 minutes after tail-vein injection (post-injection) revealed that comparable cell numbers of each cell line were already found in the lung of each recipient mouse. Bottom: Longitudinal chart of BLI imaging depicting lung colonization of cells. (C) qPCR of SIK1 in A549+LKB1 cells expressing SIK1 sgRNAs. These parental A549-SIK1 cells were further used to generate lines with loss of multiple SIK kinases, as used in E and F. Forskolin treatment was used a positive control to induce SIK1 mRNA in control A549 cells. (D) Average frequency of frameshift indels at the CRISPR cutting site in polyclonal pools of A549 cell lines of indicated genotype after targeting SIK1 with sgSik1 guides. N=12 single PCR amplicons were subcloned from each line and sequenced to calculate the average indel rate. (E) Cyquant measurement of DNA content reflecting cell survival and growth under non-adherent conditions (anoikis survival) of AMPK α -deficient A549-LKB1 cells after 5 days in suspension. Data are plotted as percent of control A549-LKB1 WT cells and represents the combined average of independently-generated biological replicates as noted: 3 independent cell lines for A549-LKB1, 3 cell lines for SIK1 and SIK2, 2 cell lines each for SIK3, SIK1+2, SIK1+3, MARK2+3 and SNRK. AMPK α , MARK1+4, and NUAK1+2 had single lines. Each individual cell line was assayed two times in triplicate wells. All values are expressed as means $\pm$ s.e.m. *P<0.05, **** P<0.0001 by ANOVA and post-hoc students ttest. (F) Cyquant measurement of DNA content 2 days after equal numbers of indicated isogenic A549 cells were plated in standard adherent cell culture conditions. Mean $\pm$ s.e.m.

A**B****C****D**

Frequency of frameshift indels:

A549-pBabe+sgSIK1	83.3%
A549-LKB1+sgSIK1	91.6%
A549-pBabe+sgSIK1/Sik3	83.3%
A549-pBabe+sgSIK1/Sik2/Sik3	83.3%
A549-LKB1+sgSIK1/Sik3	81.8%
A549-LKB1+sgSIK1/Sik2/Sik3	90.0%

E**F**

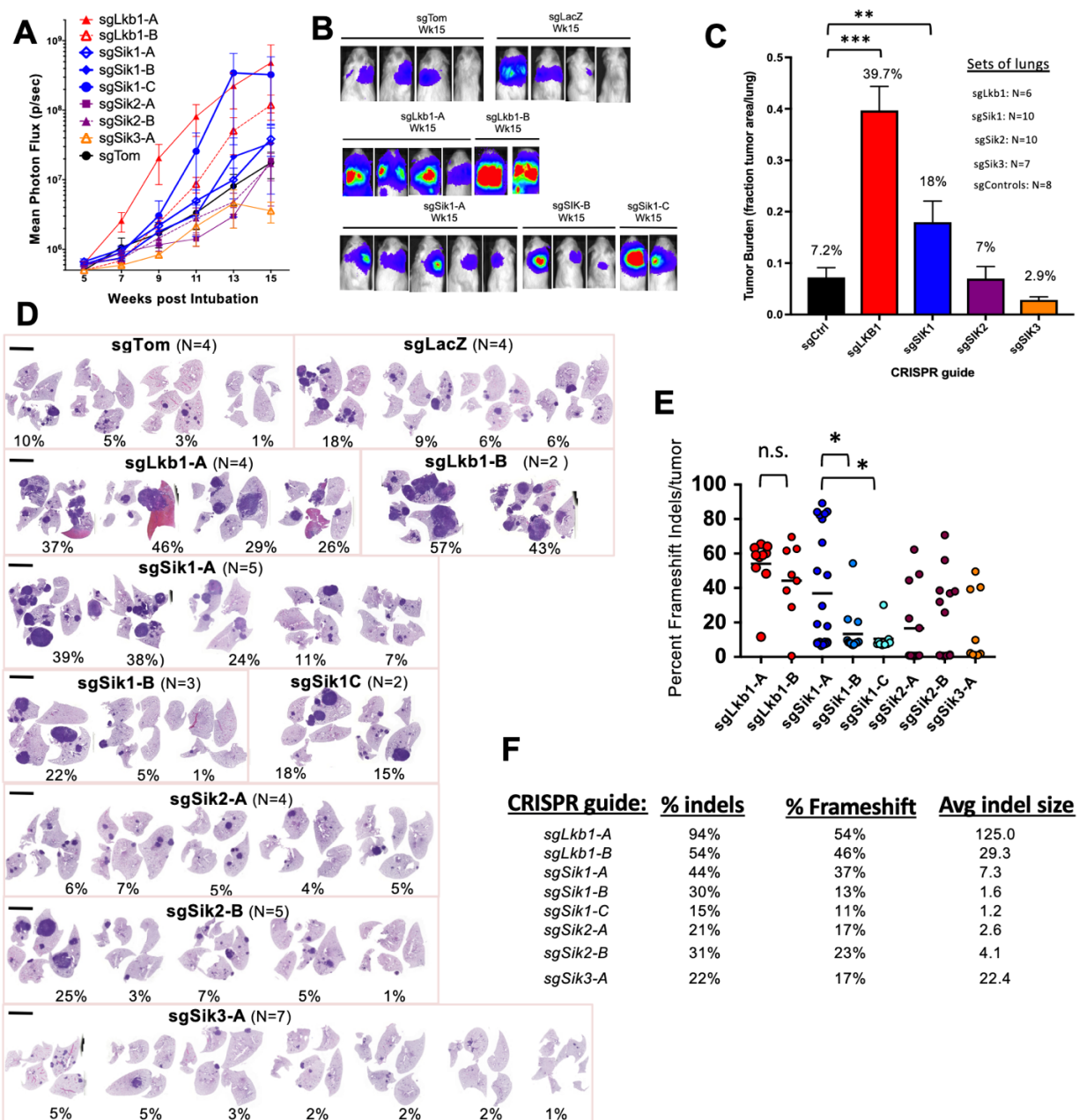


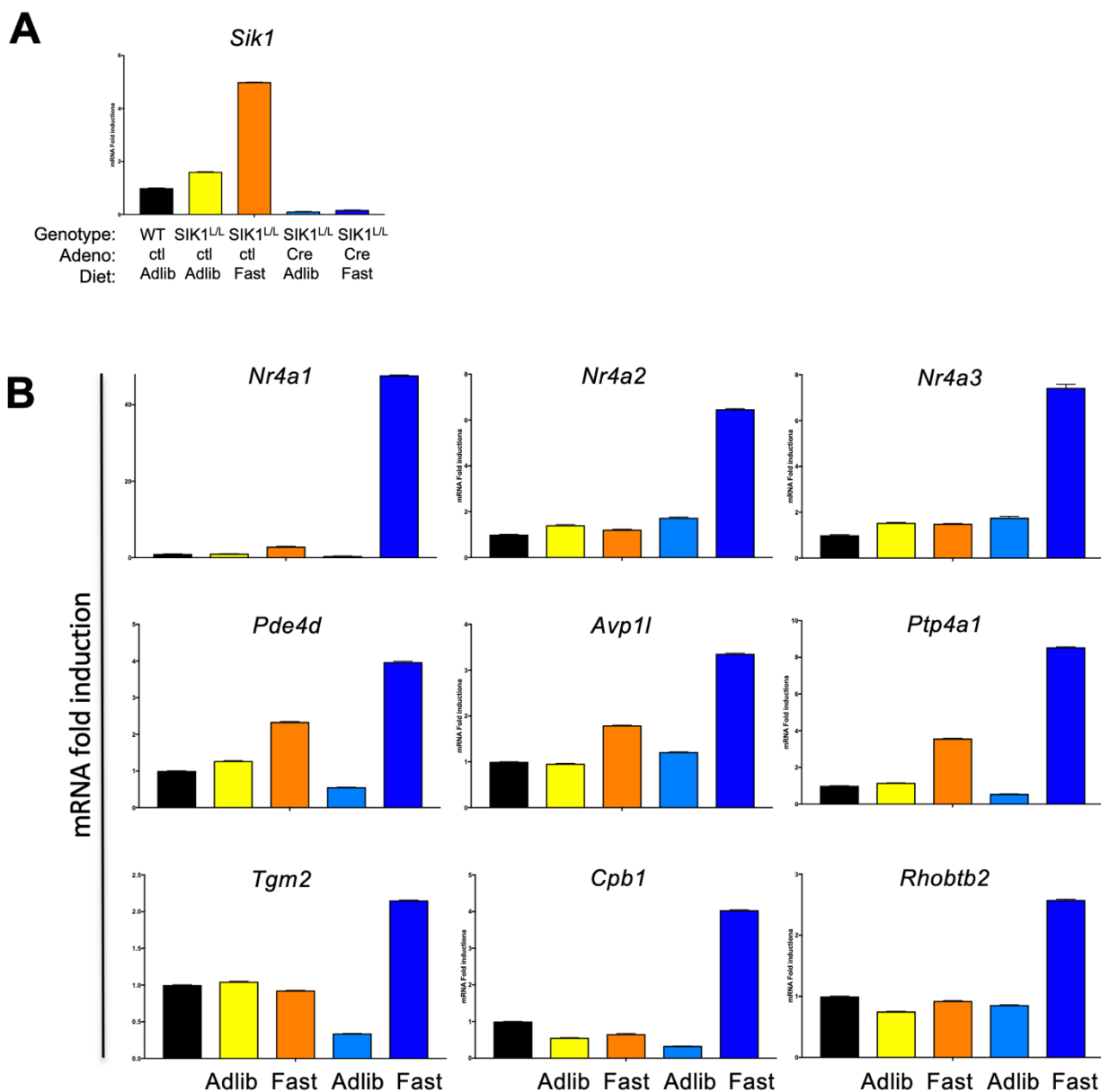
Supplemental Figure 2: Loss of AMPK-related kinase Sik1, but not Mark4, accelerates Kras-driven lung tumor growth.

(A) Western blot of lysates from 634T KP mouse NSCLC cells validating sgRNAs targeting Sik1 which were cloned into pSECC. Forskolin treatment elicits upregulation and stabilization of SIK protein to detectable levels by Western blot. (B) Western blot of lysates from 634T KP mouse NSCLC cells validating sgRNAs targeting Lkb1, Sik2 and Sik3 which were cloned into pSECC. (C) Left: Longitudinal bioluminescent imaging (BLI) of mice intubated with pSECC virus harboring control, sgLkb1, or two independent guides targeting Mark4 over 20 weeks. sgTom (N=6), sgMark4-A: (N=5). sgMark4B (N=5). sgLkb1-A: (N=5). Right: Western blot of lysates from 634T KP cells validating sgRNAs targeting Mark4 which were cloned into pSECC. (D) Tumor burden at endpoint of K mice which received control, sgLkb1 or sgSik1 guides. Bar graph shows the aggregate mean tumor burden by genotype (Figure 2C), overlaid with a dot plot. Each dot represent a mouse. sgTom (N=4) and sgLacZ (N=3): filled black circles. sgLkb1-A: filled red circles (N=5). sgLkb1-B: open red circles (N=4). sgSik1-A: filled blue circles (N=4). Sik1- B: filled green circles (N=4). sgSik1-C: filled purple circles (N=3).

Supplemental Figure 2. 3: Analysis of pSECC sgRNAs in KP mice

(A) Longitudinal BLI data from KP mice treated with control pSECC virus or pSECC viruses harboring sgRNA guides to target mouse Lkb1, Sik1, Sik2 and Sik3. Data from (Figure 2F), but overlaid with mice treated with sgSik2 and sgSik3 sgRNAs. Average bioluminescence (photon flux) at each imaging time point is shown for each cohort. The last point shown indicates study endpoint. (B) Overlay of BLI images at study endpoint for KP mice treated with pSECC sgTom, sgLacZ, sgLkb1-A, sgLkb1-B, sgSik1-A, sgSik1B, sgSik1-C showing luminescence emitted from lung tumors within each cohort. (C) Aggregate tumor burden for each genotype in Figure 2G is expressed as a fraction of tumor/total lung at endpoint from lungs of KP mice which received sgControl (sgTom+sgLacZ), sgLkb1(A+B), sgSik1 (A+B+C), sgSik2 (A+B), or sgSik3 (A) guides. * $P \leq 0.05$ ** $P \leq 0.01$, *** $P \leq 0.001$ by student's t test. (D) H&E-stained lung sections of KP mice induced with pSECC sgControl, sgLkb1, sgSik1, sgSik2 and sgSik3 at endpoint. Number of mice per genotype is shown. Scale bars, 2000 μm . (E) Percent frameshift indels at the target site of Lkb1, Sik1, Sik2, and Sik3 in tumor DNA isolated by laser-capture microdissection. Each dot represents one sequenced lung tumor, generated by intubation of mice with pSECC virus bearing the targeting guide denoted in the graph. Anova with post-hoc T-Test. * $P \leq 0.05$ (F) Summary table of the average total, frameshift and avg indel size frequency in sequenced tumors from the designated genotypes.



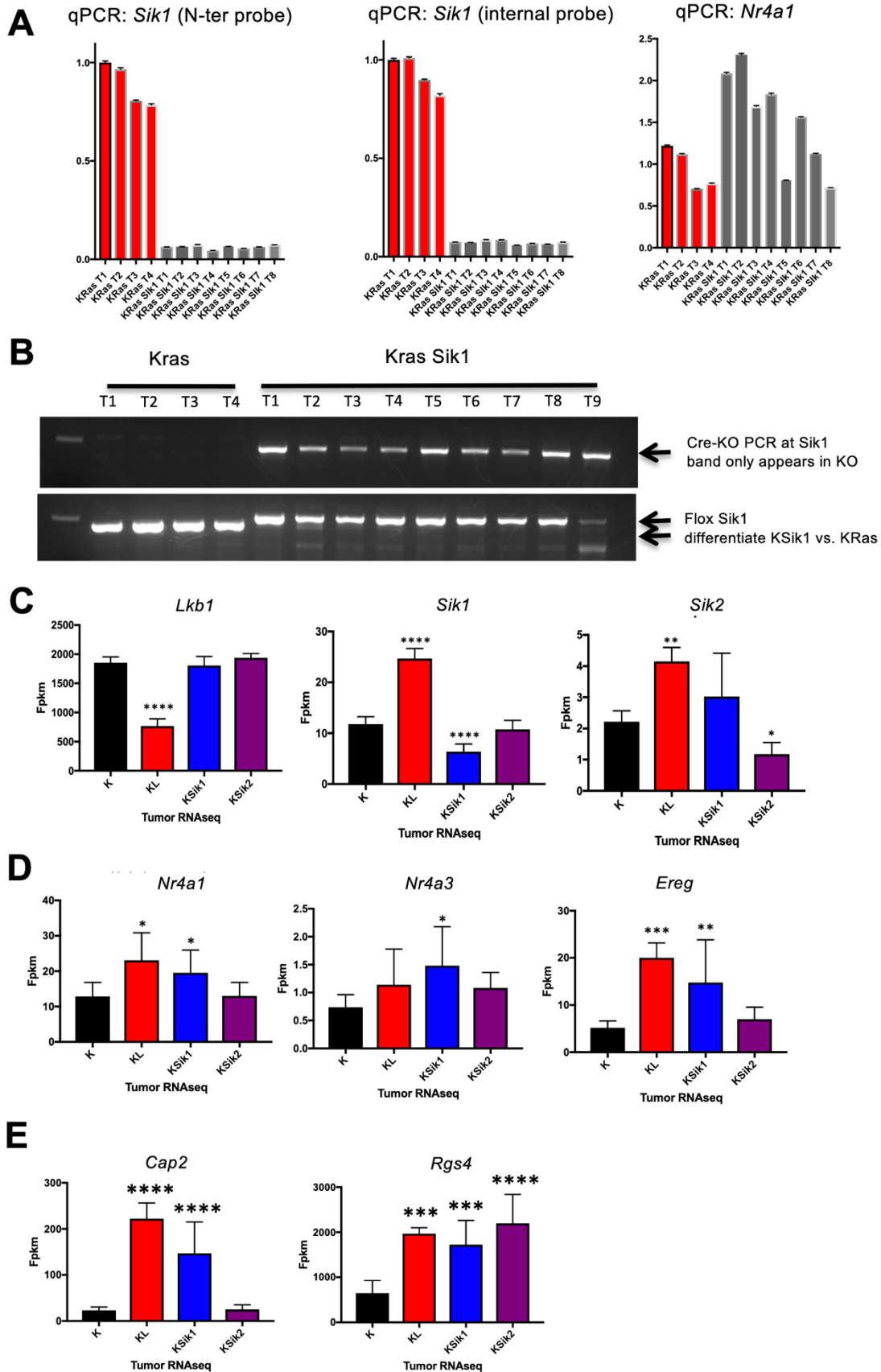


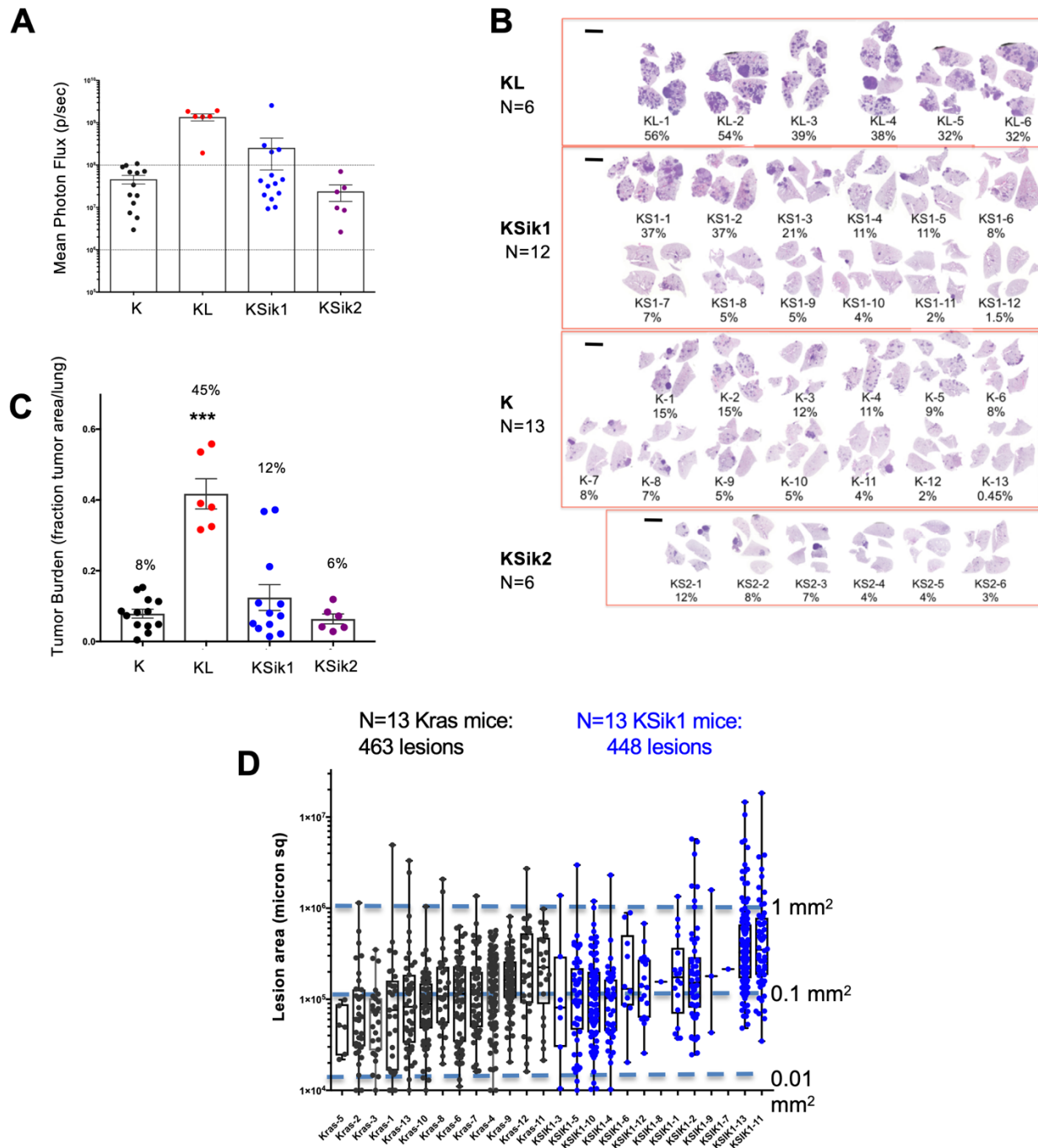
Supplemental Figure 2. 4: Validation of *Sik1* floxed allele in murine liver.

Deletion of *Sik1* in liver results in fasting-induced upregulation of known *Lkb1*-regulated mRNA transcripts. RNA extracted from whole liver under fasting vs. adlib conditions. (A) qPCR of *Sik1* mRNA from mouse livers transduced with control Adenovirus in WT mice (black) or floxed *Sik1* mice (yellow and orange), or Adeno-Cre virus-in floxed *Sik1* mice (light blue, dark blue) under Adlib or fasted (16hr) conditions as noted in the figure. (B) qPCR of known *LKB1*-regulated targets *Nr4a1-3*, *Pde4d*, *Avp1l*, *Ptp4a1*, *Tgm2*, *Cpb1*, *Rhobtb2* from liver RNA of WT and conditional *Sik1* KO mice as described in (A).

Supplemental Figure 2. 5: Supporting evidence that SIK1 is efficiently deleted in tumors from KSik1 mice. KSik1 tumors bear gene upregulation of LKB1-regulated transcripts.

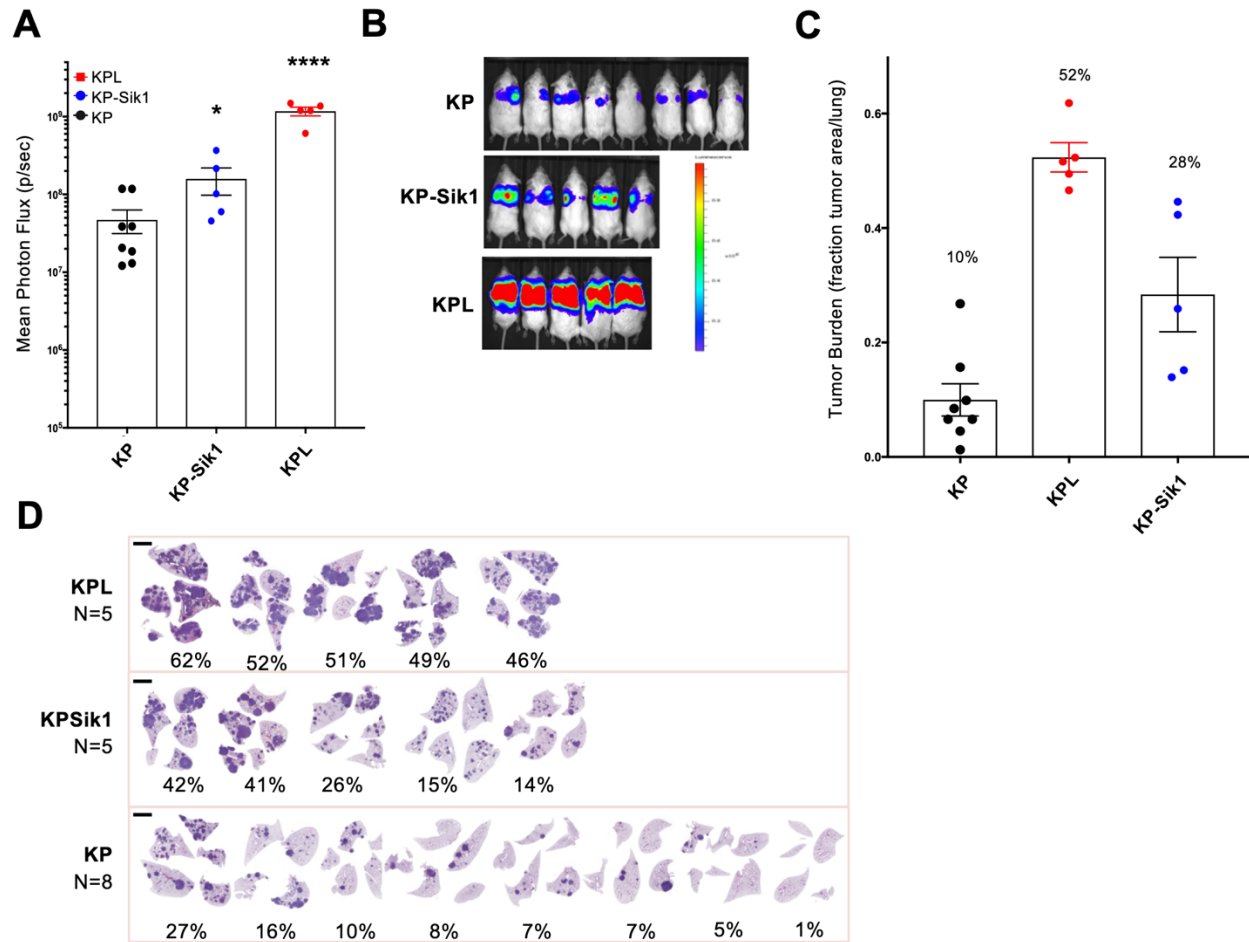
(A) qPCR of *Sik1* mRNA and canonical LKB1-regulated CREB target *Nr4a1* from control and KSik1 lung tumors. (B) Top: Agarose gel image of PCR showing recombination at the floxed SIK1 allele in tumors from KSik1 mice. Bottom: PCR showing *Sik1* flox allele status in tumors from *Kras* and KSik1 mice. Top band, homozygous floxed *Sik1*. Lower band, WT allele. (C) FPKM RNA expression plots from KL, KSik1 and KSik2 tumors showing depletion of respective transcripts. (D-E) FPKM plots of LKB1-regulated genes in lung tumors from KL, KSik1 and KSik2 mice. * $P \leq 0.05$ ** $P \leq 0.01$, *** $P < 0.001$, **** $P \leq 0.0001$ by student's t test.





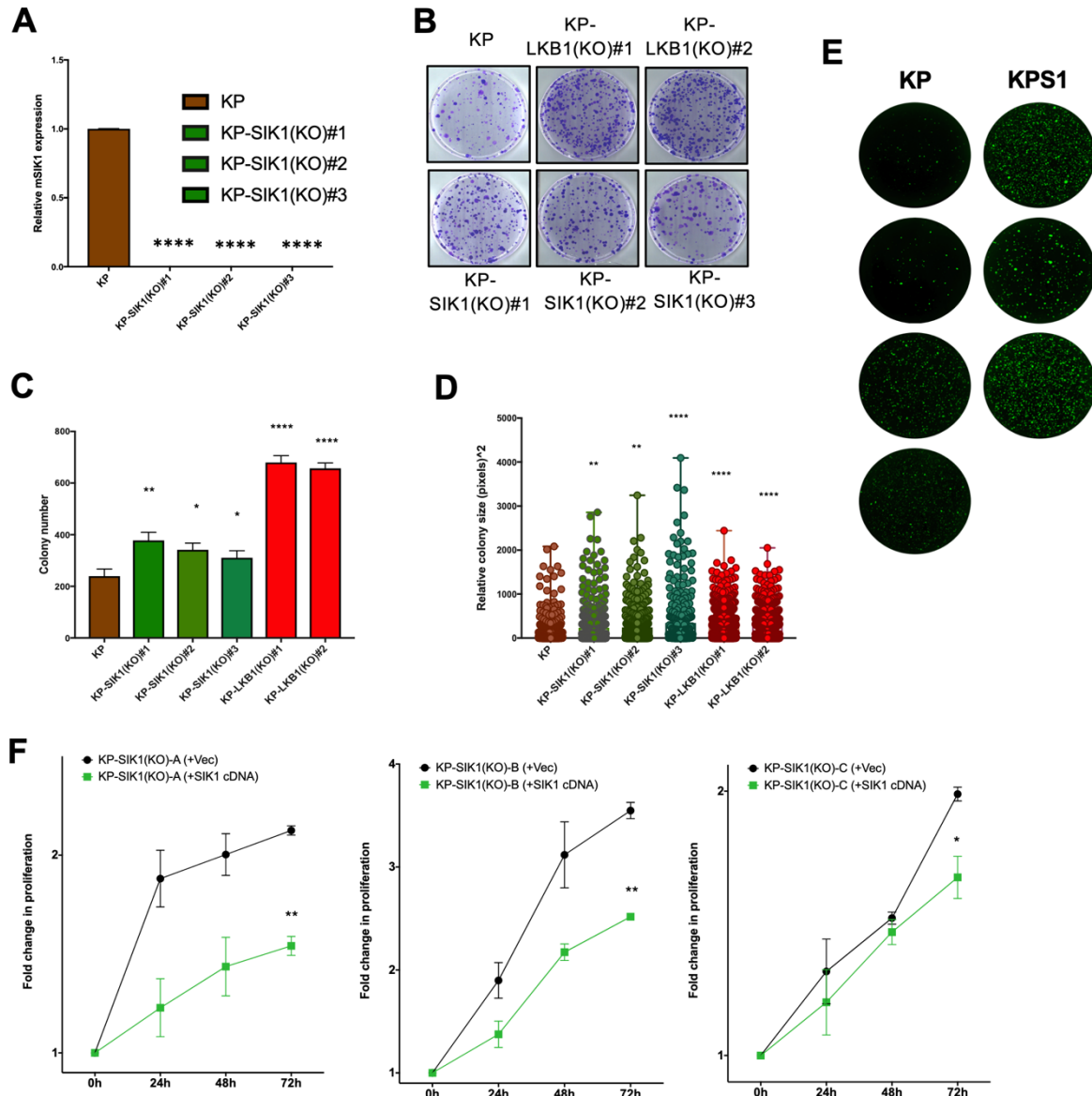
Supplemental Figure 2. 6: Analysis of tumor burden in K, KSik1, and KSik2 mice

(A) BLI data at endpoint for K, KSik1, KSik2, and KL mice from Figure 2K. Bar graph denotes mean $\pm$ s.e.m. Each dot represents an individual mouse. * $P \leq 0.05$ **** $P \leq 0.0001$ by student's t test. (B) H&E-stained lung sections of K, KL, KSik1, KSik2 mice at endpoint. Number of mice per genotype is shown. Percent tumor burden per mouse is noted. Scale bars, 2000 μ m. (C) Bar graph shows the mean tumor burden as a fraction of tumor/total lung area by genotype (Figure 2L), overlaid with a dot plot. Each dot represent a mouse. (D) Individual tumor lesions plotted by mouse for K and KSik1 mice. Each box and whiskers plot shows the range of tumor sizes observed in each mouse. Each dot represent a tumor.



Supplemental Figure 2. 7: Analysis of tumor burden in KP vs KPL vs KPSik1 mice

(A) BLI data at endpoint for KP, KPSik1 and KPL mice. Data are from Figure 2O. Bar graph denotes mean $\pm$ s.e.m. Each dot represents a mouse. * $P \leq 0.05$ **** $P \leq 0.0001$ by student's t test. (B) Overlay of BLI images at study endpoint for KP, KPSik1, and KPL mice showing luminescence emitted from lung tumors within each cohort. Scale bar represents photons/sec/cm²/sr. (C) Bar graph shows the mean tumor burden as a fraction of tumor/total lung area by genotype (Figure 2P), overlaid with a dot plot. Each dot represent a mouse. (D) H&E-stained lung sections of KP, KPSik1 and KPL mice at endpoint. Number of mice per genotype is shown. Percent tumor burden per mouse is noted. Scale bars, 2000 μ m.

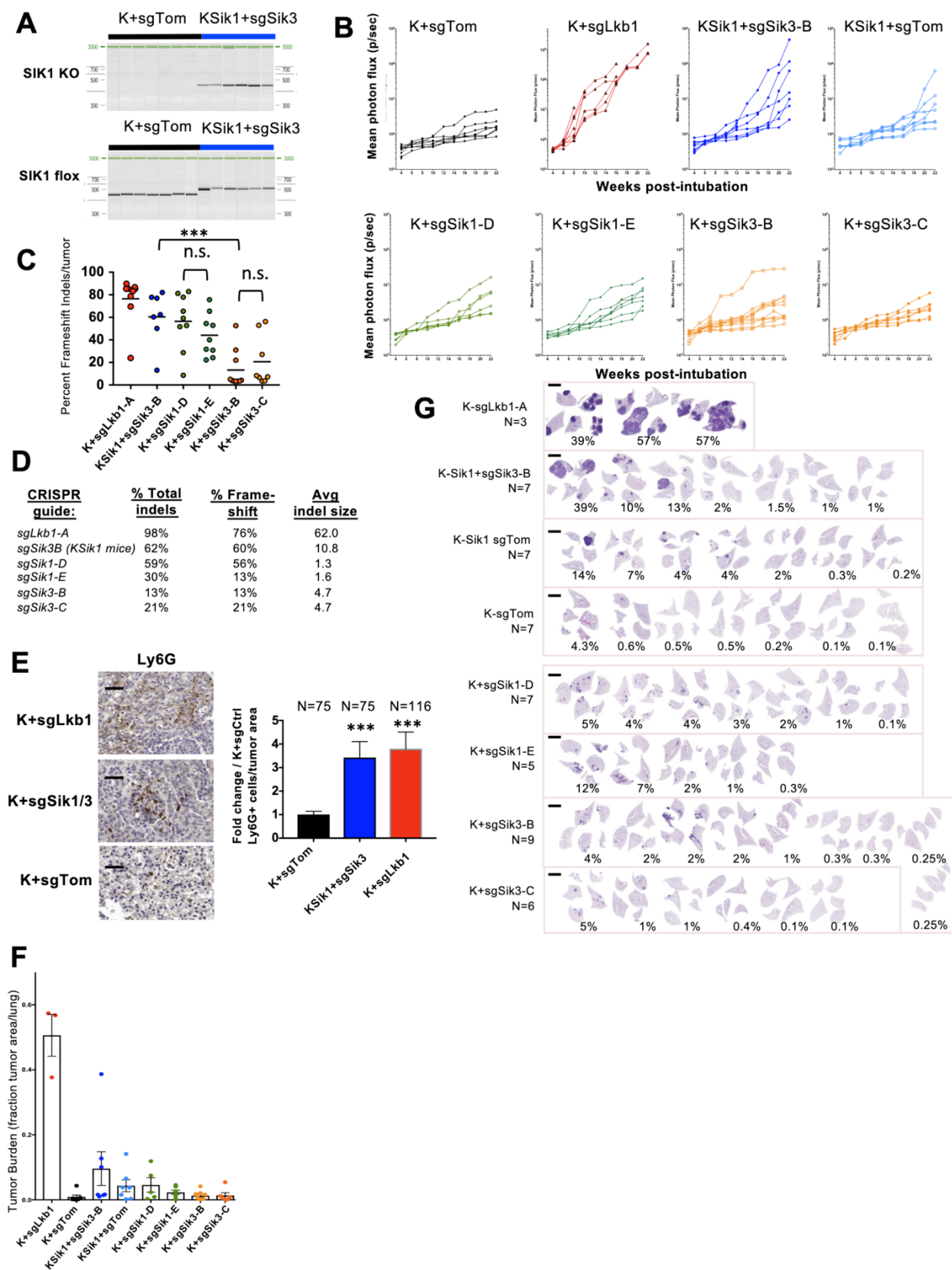


Supplemental Figure 2. 8: Proliferation and growth of GEMM tumor-derived KP cell lines deleted for SIK1 or LKB1.

(A) qPCR of Sik1 transcript in control KP and N=3 Sik1 KO KPSik1 cell lines. **** $P < 0.0001$ by student's t test. (B) Single-cell dilution 2D colony formation assay of KP, KPSik1 and KPL (LKB1 KO) mouse tumor-derived cell lines. Representative images of crystal-violet stained colonies after 7-day growth. (C) Average colony number from single cell 2D assay of control KP, KPSIK1 and KPL lines after 7-day growth. Shown is mean $\pm$ s.e.m of two experiments. * $P < 0.05$, ** $P \leq 0.01$, **** $P < 0.0001$ by ANOVA and post hoc student's t test. (D) Average colony size of control KP, KPSIK1 and KPL lines grown in single cell 2D assays. Each dot represent a colony, plotted by size. Y axis: relative size in pixels. Each dot represent a colony. , ** $P \leq 0.001$, **** $P < 0.0001$ by student's t test. (E) Representative images of soft agar colony growth of KP and KPSik1 tumor-derived lines after 3 weeks of growth. False coloring showing green colonies over black background (F) Introduction of SIK1 cDNA reduces proliferation of KPSIK1 cell lines over a 72hrs time course. Data is shown as fold change in proliferation over T=0. Each time point is the mean fold change $\pm$ s.e.m ** $P \leq 0.001$, * $P < 0.05$ by student's t test.

Supplemental Figure 2. 9: Analysis of tumor burden in K vs K-Sik1 +/- sgRNAs against Sik1 and Sik3

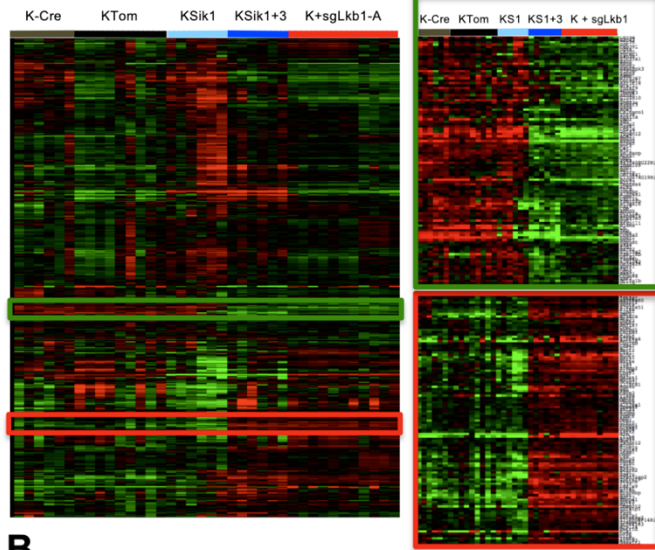
(A) Top: PCR depicting Sik1 allele recombination (band present) in tumors from KSik1+sgSik3 mice. Bottom: PCR denoting floxed allele status of Sik1 in WT (lower band) in K+sgTom tumors or homozygous floxed Sik1 alleles (upper band) in KSik1+sgSik3 tumors. (B) Longitudinal BLI charts for individual mice in the experiment from Figure 3A. (C) Percent frameshift indels at the target site of Lkb1, Sik1 and Sik3 in tumor DNA isolated by laser- capture microdissection. Each dot represents one sequenced lung tumor within the cohort of Figure 3A generated by pSECC bearing the indicated targeting guide. *** $P \leq 0.001$ (D) Summary table of the average total, frameshift and avg indel size frequency in sequenced tumors from the designated genotypes. (E) Representative images of anti-Ly6G (neutrophil marker) immunohistochemical staining in lung tumor sections from K, KSik1+sgSik3 and K-sgLkb1 mice. Scale bar = 100um. Graph represents the average number of tumor-infiltrating +Ly6G cells as a percent of total number of cells/quantitation field/tumor. N = tumors quantitated. *** $P < 0.001$ by student's t test following ANOVA of KSik1+3 or K-sgLKB1 compared to K-Control (sgTom) tumors. (F) Average tumor burden of mice from Figure 3, overlayed with a dot plot in which each dot represents an individual mouse in the cohort. (G) H&E stained lung section from each group (Figure 3). Number of animals in each cohort (N =) is shown. Percent tumor burden per mouse is noted.



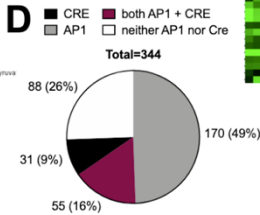
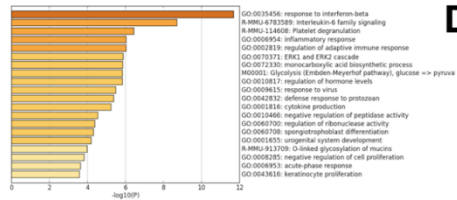
Supplemental Figure 2. 10: Gene expression analysis of lung tumors isolated from K+sgTom, K+sgLkb1, and KSik1+sgSik3 mice.

(A) For a distinct, definable set of genes, SIK1+3 inactivation replicates the same transcriptional changes induced by LKB1 loss. Right panel: Heat map of clusters common to KSik1+sgSik3 and K+sgLkb1. (B) Metascape analysis of all upregulated genes common to K+sgLkb1 and KSik1+sgSik3 (compared to K+sgTom). (C) Enrichr analysis of the 627 downregulated genes common to K+sgLkb1 and KSik1+sgSik3 identified in Figure 4B. (D) Within the 344 upregulated K+sgLkb1 and KSik1+sgSik3 common overlap genes, 16% contain both CRE and AP1 recognition motifs within the promoter as defined by +/-2 kb of the TSS. (E) Heatmap of genes with CRE elements in the promoter from Figure S10D. (F) Heatmap of genes with AP1 elements in the promoter from Figure S10D. (G) GSEA analysis: Graph and Table 1st line: Top 500 Upregulated genes in KSik1+3 tumors used as a gene set to search for enrichment across differentially expressed genes defined by LKB1 loss/LKB1 reconstitution in A549 human cells. Lines 2-5: 344 upregulated genes by KSik1+3 and Lkb1 loss in mouse tumors, used as a gene set to look for enrichment across differentially regulated genes defined by LKB1 loss/LKB1 reconstitution in human cell lines.

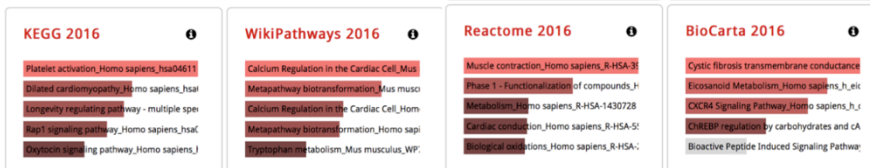
A



B

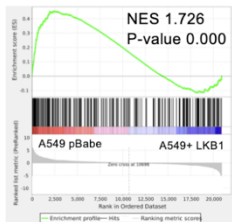


C



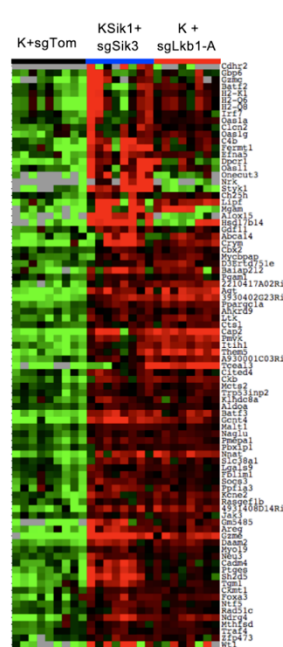
G

Gene Set 1: top 500 genes upregulated in KSik1+3. Gene set 2: 344 genes up in both mouse KSik1+sgSik3 or Lkb1 loss in mouse tumors

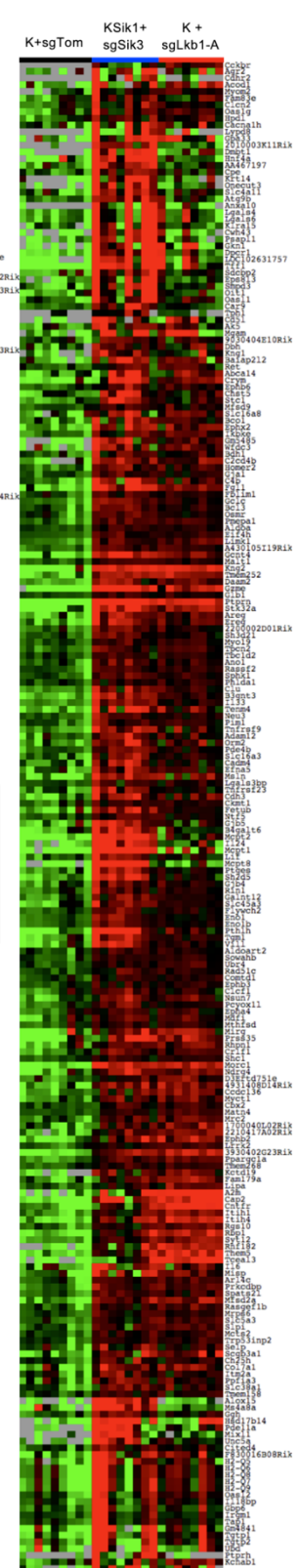


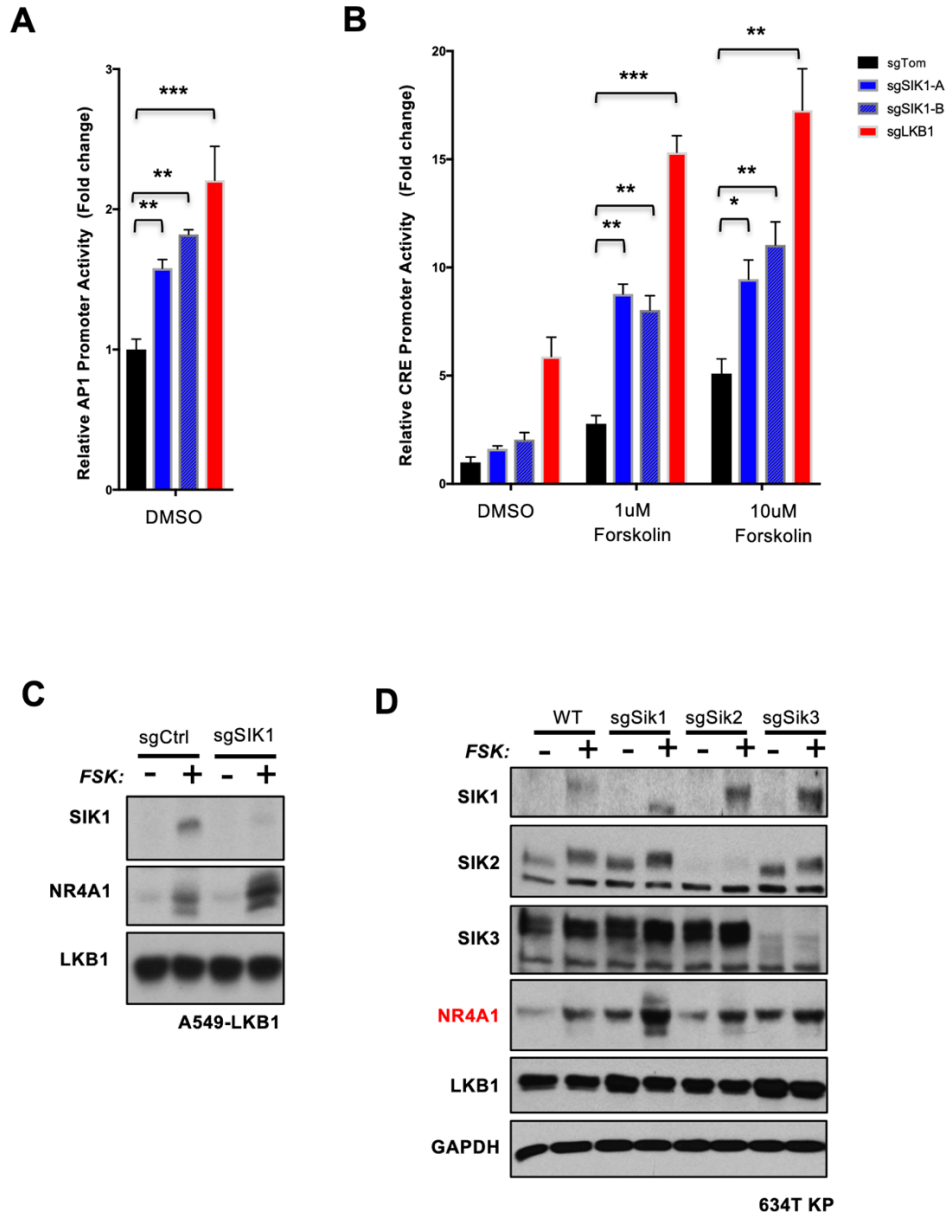
Cell line	Rank Log2FC	Gene Set	ES	NES	FDR	Source
A549	pBabe/LKB1	1. UP KSik1+3_only	0.452	1.726	0.00	this study
A549	pBabe/LKB1	2. UP KSik1+3+L-A	0.453	1.708	0.00	this study
H1355	pBabe/LKB1	2. UP KSik1+3+L-A	0.367	1.421	0.00	this study
H157	pBabe/LKB1	2. UP KSik1+3+L-A	0.469	1.644	0.00	this study
A549	pBabe/LKB1	2. UP KSik1+3+L-A	0.348	1.3311	0.0325	Kaufman et al., 2014

E



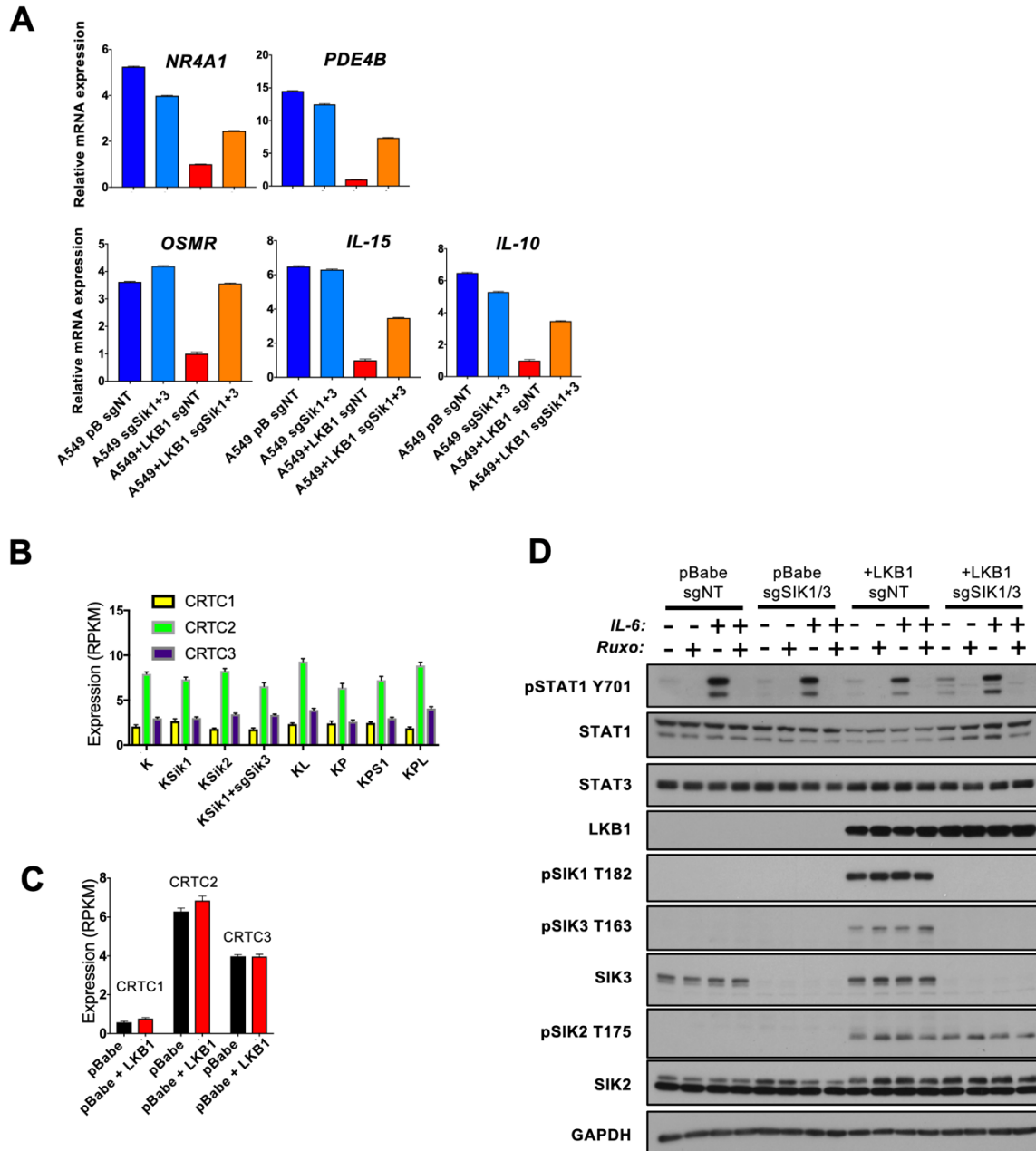
F





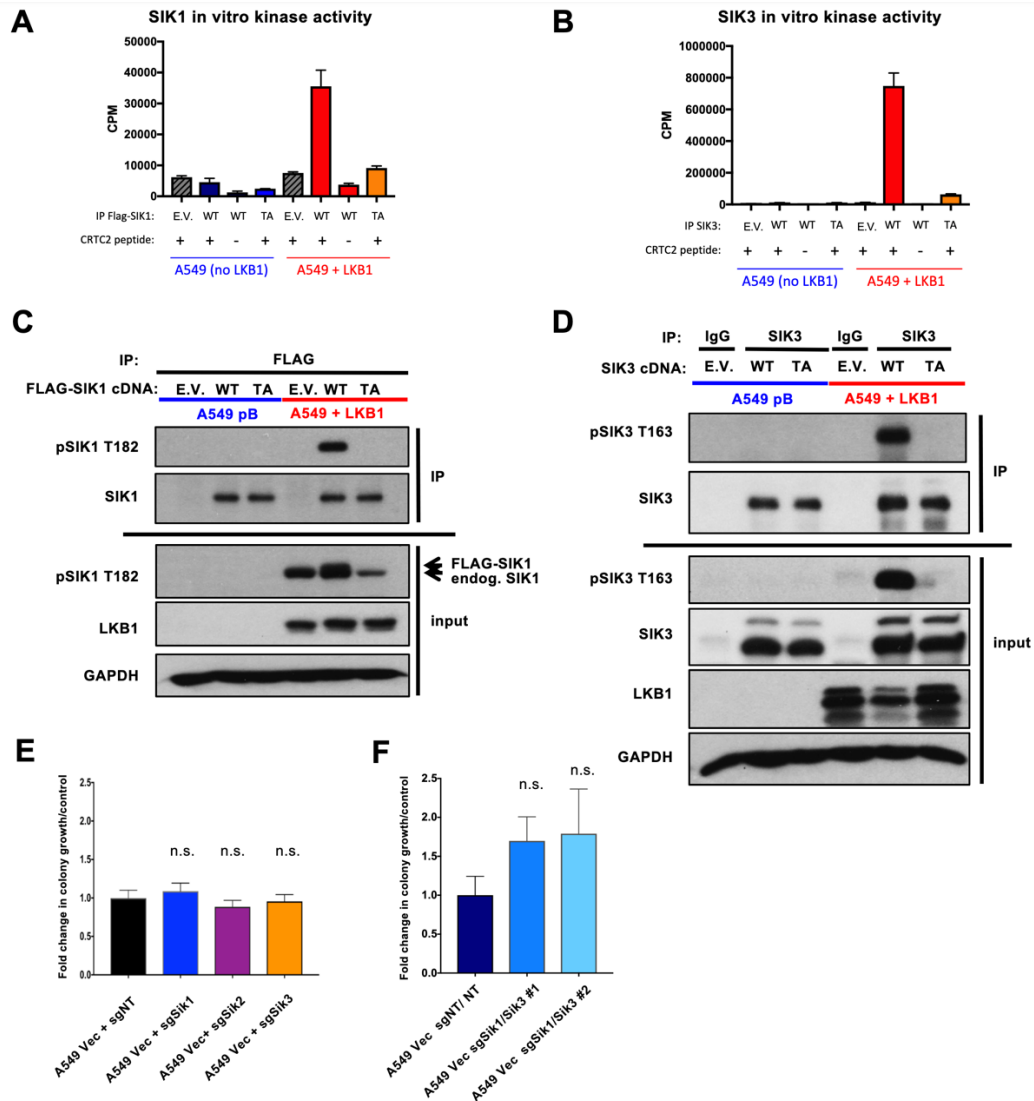
Supplemental Figure 2. 11: Deregulated AP1 and CREB signaling driven by SIK1 or LKB1 deficiency in 634T murine KP and human NSCLC cells.

(A) Luciferase reporter assay of AP1 promoter at steady state in mouse 634T KP cells expressing control, sgSik1 or sgLkb1 guides. Data is displayed as fold change over control $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ determined by ANOVA and post hoc student's t test. (B) Luciferase reporter assay of CRE promoter after 6hr forskolin treatment of 634T KP cells expressing control, sgSik1 or sgLkb1 guides. Data is displayed as fold change over control $\pm$ s.e.m., with P values determined by student's t test. (C) Western blot of lysates from A549 LKB1 WT cells expressing control and sgSIK1. Forskolin treatment induces expression of the canonical CREB target NR4A1, further amplified upon loss of SIK1. (D) Western blot of control or forskolin-stimulated 634T KP mouse cells expressing sgRNAs against Sik1, Sik2, Sik3. NR4A1 upregulation by forskolin treatment is detected most highly upon conditions where SIK1 is depleted.



Supplemental Figure 2. 12: SIK1/3 regulate IL-6/STAT signaling.

(A) Relative mRNA expression of LKB1- and SIK1/3- regulated genes from the mouse KSik1+3 tumor gene signature, recapitulated in A549-Vector (pBabe) and A549+LKB1 cells $\pm$ sgSIK1/3. Graphs show qPCR from human cell line RNA as the average $\pm$ s.e.m of two experiments performed in triplicate. (B) Average RPKM mRNA expression of CRTCs (cAMP-regulated transcriptional co-regulators) in KRas and LKB1-pathway driven mouse lung tumors as denoted by genotype in the graph. Mean $\pm$ s.e.m of $n=5-7$ tumors per genotype. (C) Average RPKM mRNA expression levels of CRTCs in human A549-Vector (pBabe) and A549+LKB1 NSCLC cells. Mean $\pm$ s.e.m of triplicate samples sequenced per cell line. (D) Immunoblots of pSTAT1 Y701, STAT1, STAT3 and pSIK1/2/3 activation loop in A549-Vector (pBabe) and A549+LKB1 cells $\pm$ sgSIK1/3 at normal conditions or after IL-6 stimulation (20ng/ml, 1hr), Ruxolitinib (JAK inhibitor) treatment (5uM), or a combination of IL-6+Ruxolitinib.



Supplemental Figure 2. 13: Lack of kinase activity of SIK1 and SIK3 in cultured cells without LKB1

(A) In vitro kinase assay of immunoprecipitated, FLAG-tagged WT SIK1 or activation-loop mutant (TA) SIK1 from A549 pBabe (LKB1 null) or A549+LKB1 cells, using CRTC2 peptide as a substrate. E.V.: Empty vector. Data is mean $\pm$ s.d, n=3. (B) In vitro kinase assay of exogenously-expressed WT or TA-mutant SIK3 immunoprecipitated with SIK3 antibody from A549 pBabe or A549+LKB1 cells, using CRTC2 peptide as a substrate. Data is mean $\pm$ s.d, n=3. (C) Top: Western blots of immunoprecipitated FLAG-tagged WT or TA SIK1 used in (S13A). Lower panel, pre-IP input lysates. (D) Top: Western blots of immunoprecipitated WT or TA SIK3 used in (S13B). Lower panel, pre-IP input lysates. (E) Colony growth in soft agar of A549-Vector (pBabe) cells targeting Sik1, Sik2, or Sik3 by lentiCRISPR transduction. Data depicted as fold change in growth over control cells expressing Cas9 and a non-targeting guide and plotted as mean $\pm$ s.e.m. from 3 independent experiments plated in triplicate and statistics determined by one way ANOVA. (F) Colony growth in soft agar of A549-Vector (pBabe) cells targeting Sik1 and Sik3 simultaneously, using independent sets of guides (cell lines #1 and #2). Data is depicted as fold change in growth over control cells expressing Cas9 and non-targeting guides under dual selection.

Supplemental Table 2. 1: CRISPR sgRNA guide sequences

Guide Name	HUMAN	Guide Sequence
Guide1	<i>AMPKa1</i>	CTGGTGTGGATTATTGTCAC
Guide1	<i>AMPKa2</i>	ACGTTATTTAAGAAGATCCG
Common guide	<i>AMPKa1/a2</i>	CACGACGGGCGGGTGAAGAT
Guide1	<i>BRSK1</i>	GGGTGAGCTATTCGACTACC
Guide2	<i>BRSK1</i>	CCGAGACGTGCTCCAGAACC
Guide1	<i>BRKS2</i>	ATCTCAATGCCCCGCCGCGC
Guide2	<i>BRKS2</i>	GCCGTGCCGGTTCAGCATCG
Guide1	<i>MARK2</i>	GTTGCGGCCCGAATCATGT
Guide2	<i>MARK2</i>	GGCAAAATTACCTTGCCAA
Guide1	<i>MARK3</i>	TCTAGAGTGCCGCGTTTAAT
Guide2	<i>MARK3</i>	ACTGCCACTTGATTTCGGGG
Guide1	<i>MARK4</i>	TTGAGGTTGTCCCGTCGAA
Guide2	<i>MARK4</i>	ACAGCTCCGGGGCGGCATAT
Guide1	<i>NUAK1</i>	TGCTATAAAATCCATTCTGA
Guide2	<i>NUAK1</i>	TGATGCCGCTTACCCCGTG
Guide1	<i>NUAK2</i>	AAGATCTGATGCACATACGG
Guide2	<i>NUAK2</i>	GTCCTTCCGGATTGACTTGA
Guide1	<i>SNRK</i>	CACTCTTAGACACATGGGA
Guide2	<i>SNRK</i>	GCCACACACCAACATGAAA
Guide1	<i>SIK1</i>	GACAGTACTCCACGGCCGAC
Guide2	<i>SIK1</i>	CGACCATCACATCGTCCACC
Guide1	<i>SIK2</i>	AATAATCGATAAGTCTCAGC
Guide2	<i>SIK2</i>	CTTCTCGGTAGATTTTCTCA
Guide1	<i>SIK3</i>	AGTTCAGGTGCAGCATAGGG
Guide2	<i>SIK3</i>	GTTCAAGGTGCAGCATAGGGA
Guide 1	<i>CRTC2</i>	TGATGGACATCGGCTCCACC
Guide 2	<i>CRTC2</i>	ACAGGCCCAAAAACGCGA

Control-1	<i>Tom</i>	GGCCACGAGTTCGAGATCGA
Control-2	<i>LacZ</i>	TGCGAATACGCCACGCGAT
Control-3	NT (Nontarget)	GCGAGGTATTCGGCTCCGCG

Guide Name	Mouse	Guide Sequence
Guide-A	<i>Lkb1</i>	CGTACGAGCCCTCCCGAGC
Guide-B	<i>Lkb1</i>	GACTCCGAGACCTTATGCCGC
Guide-A	<i>Sik1</i>	CGTCGTAAAAGCCCACCCGG
Guide-B	<i>Sik1</i>	CCGGGTGGGCTTTTACGACG
Guide-C	<i>Sik1</i>	ACGTTTAGATTCTAGCAATC
Guide-D	<i>Sik1</i>	GATCATGTCGGAGTTCAGTG
Guide-E	<i>Sik1</i>	TCTGGAGAAGATCTACCGGG
Guide-A	<i>Sik2</i>	CTACGACATCGAGGGCACGC
Guide-B	<i>Sik2</i>	GGTGGGGTTCTACGACATCG
Guide-A	<i>Sik3</i>	GCGAGCGGCCATCGACCCGC
Guide-B	<i>Sik3</i>	CTCGTAGTAGCCGATGCGAG
Guide-C	<i>Sik3</i>	TGGCAGAGAAGGAAGCTCGA
Guide-D	<i>Sik3</i>	GTTCAAACAGATCGTCACAG
Guide-A	<i>Mark4</i>	CGAGTTTCGATCGTTGCCCG
Guide-B	<i>Mark4</i>	CCCGGGCAACGATCGAAACT

CHAPTER 3: SALT-INDUCIBLE KINASES MEDIATE LKB1-DEPENDENT TUMOR SUPPRESSION IN GENETICALLY ENGINEERED MOUSE MODELS OF NSCLC

ABSTRACT

Lkb1, a serine/threonine kinase, is the most commonly mutated tumor suppressor in human Kras mutant non-small cell lung cancer (NSCLC). Dysregulation in LKB1s kinase function is thought to mediate its ability to perform as a tumor suppressor, and thus suggests that LKB1s downstream targets (known as the AMPK-related kinases [AMPKRs]) mediate its tumors suppressive function. Previously reported using a CRISPR based strategy, the Sik family kinases (specifically Sik1 and Sik3) were noted to mediate some of Lkb1's tumor suppressive function. In Kras driven mouse models, genetic loss of Sik1 combined with CRISPR targeting of Sik3, led to an intermediate phenotype which approached the growth of loss of Lkb1 (KL model). Targeting led to increases in Il6-Jak-Stat signaling, EMT expression signatures, and Creb and AP1 target gene expression, and was in agreement with Lkb1 loss¹². In the studies outlined here, we build on previous CRISPR based approaches, and have developed genetic conditional knockout models of all the Sik kinases (Sik1, Sik2, and Sik3). Redundancies in catalytic activity and compensation of the Sik kinases has been reported, and as a result, we generated crosses of all of the Sik kinases to produce combinatory floxed models. Growth in these models when compared to Kras was dependent on the degree to which all of the Sik kinases were deleted. Loss of any single Sik allele led to no considerable growth above Kras, co-deletion of Sik1 and Sik3 had an intermediate phenotype, and loss of all three Sik kinases (KS model) matched loss of Lkb1 (KL model). The KS model had significant overlap in upregulated and downregulated genes when compared to the KL model. Increases in expression of previously reported Sik dependent EMT, STAT, and CREB gene sets were consistent in the newly generated genetic models of Sik-loss. However the new genetic models revealed that the Sik models cannot recapitulate the commonly observed increases in tumor associated neutrophils seen in the Lkb1

model. PRC2 and Creb target genes were among the top enriched genes in common between KS and KL tumors. Independently, we observed Yap target genes increased in both KL and KS models, potentially contributing to the pro-growth nature of these genetic alterations.

INTRODUCTION

Human lung cancer is the leading cause of cancer related deaths in men and women in the United States⁹⁷. Antismoking efforts in combination with advancements in cancer treatments have helped to decrease total deaths, however patients continue to have only an average 20% survival after 5 years from the time of detection^{97,150}. The two main histological types of lung cancer are small cell lung cancer and non-small cell lung cancer (NSCLC), the latter which encompasses approximately 85% of all lung cancer diagnoses^{97,150,151}. Treatment for patients with NSCLC includes surgical removal, cytotoxic chemotherapies, immune checkpoint blockade treatment, and targeted/personalized treatment based on histological and tumor profile⁹⁸. The serine-threonine kinase (STK11), better known as liver-kinase B1 (LKB1), is commonly mutated in 1 in 5 patients with NSCLC^{101,102}. Despite affecting such a large portion of individuals, targeted therapies for patients with LKB1 mutations have remained out of reach. In addition, the mechanisms underlying LKB1's tumor suppressive function has remained unknown until recent, offering a new avenue for potential future therapeutic opportunities. Our lab has recently shown that the tumor suppressive function of LKB1 is not mediated through AMP-activated protein kinase (AMPK), a downstream kinase involved in energy homeostasis, but rather regulated through the salt-inducible kinases (SIK) kinases^{12,34,93}. The research described here improves on previous studies in the lab, which focus on the SIK kinases as key mediators of LKB1-dependent tumor suppression.

LKB1 was originally identified as the main mutational driver in patients suffering from Peutz–Jeghers syndrome (PJS)^{14,15}. Patients with PJS, an autosomal dominant hereditary

disorder, are characterized by the development of melanin pigmentation of the skin and gastrointestinal hamartomatous polyposis and are highly susceptible to malignant formations across tissues at an early age^{14,15,152}. Later studies revealed that LKB1 served as a tumor suppressor of sporadic cancers, which primarily manifests as NSCLC, but has also been observed in pancreatic and melanomas, however, rarely^{101,110,153}. Hemizygous loss of chromosome 19p (which contains LKB1), hypermethylation of the LKB1 promoter, and indiscriminate loss of LKB1 expression have all been implicated in aberrant sporadic carcinomas of various tissues^{154–156}. NSCLC-associated mutations of LKB1 are clustered to its kinase domain, suggesting that dysregulated phosphorylation of one or more of its downstream substrates is required for tumorigenesis, highlighting the need for extensive studies of targets of LKB1.

Significant advancements toward identifying the mechanisms behind LKB1-dependent tumor suppression were made when it was discovered that LKB1, in a complex with MO25 and STRAD, directly activates AMPK α 1/2 by phosphorylation of Thr172 within its activation loop^{21–23}. Soon thereafter it was observed that AMPK itself was able to inhibit the mammalian target of rapamycin complex 1 (mTORC1) via direct phosphorylation and activation of the tuberous sclerosis complex subunit 2 (TSC2) tumor suppressor, directly linking the LKB1-AMPK signaling axis to cancer^{36,37}. Phosphorylation of TSC2 prevents heterodimerization with TSC1, its obligate partner for enzymatic activity, leading to the indirect hyperactivation of mTORC1 and its pro-growth signaling^{38,39}. In TSC2- null conditions, energy stress was still able to reduce mTORC1 activity, suggesting there was an AMPK-dependent regulatory step downstream of TSC2. Indeed, AMPK was also discovered to directly phosphorylate the mTORC1 subunit raptor, induce 14-3-3 binding, and decrease mTORC1 dependent signaling⁴⁰. These findings cemented AMPK as a regulator of not only metabolism, but of cell growth, and led to the hypothesis that LKB1 tumor suppressive function was mediated through AMPK. Recently our lab reported an

unexpected breakthrough finding showing that LKB1-dependent tumor suppression was not mediated through AMPK, but instead AMPK was required for growth in genetically engineered mouse models of NSCLC³⁴. *In vivo* Cre-recombinase driven conditional expression of mutant KRAS^{LSL-G12D/+}; R26^{LSL-Luc/Luc} (K), a common alteration in NSCLC, combined with loss of both AMPK α 1/2 (KA), led to decreases tumor burden and tumor size when compared to the well-established KRAS^{LSL-G12D/+}; LKB1^{lox/lox}; R26^{LSL-Luc/Luc} (KL) controls³⁴. It was further confirmed that AMPK activity was required for TFEB/TFE3-dependent upregulation of the CLEAR network lysosomal genes in order to support tumor growth³⁴.

LKB1 also phosphorylates and activates 12 other downstream substrates, comprised of 6 potentially redundant families, named the AMPK-related kinases (AMPKR) for their sequence homology to the prototypical AMPK. The AMPKR's are known to be involved in cell polarity, metabolism, and growth, however studies of their tumor suppressive function have remained limited^{3,19}. Given the unforeseen finding relating to AMPK, emphasis was placed on determining which AMPKRs are crucial to LKB1-dependent tumor suppression. Among these AMPKRs are the SIKs, which we recently reported on using a CRISPR-based approach to target SIK1, SIK2, and SIK3 in mouse models harboring K mutations¹². Analysis of growth in soft agar after loss of SIK1, SIK2, or SIK3 approached that of LKB1-null lines, with even stronger effects coming when combinations of the SIKs were deleted, showing an additive or synergistic effect¹². *In vivo*, conditional deletion of on the SIKs in the lung was achieved by use of the pSECC vector: a lentiviral vector containing an sgRNA targeting a gene of interest, Cas9, and Cre-recombinase. pSECC vectors with guides against SIK1, SIK3, and LKB1, were delivered via intratracheal intubation into K and K SIK1^{lox/lox} (KS1) mice. SIK1 and SIK3 disruption in the lung were observed to work cooperatively *in vivo* and mediate some of the LKB1-dependent tumor suppression¹². Tumors from the K SIK2^{lox/lox} (KS2) mice grew significantly slower when compared to KS1 and K mice, suggest a potential oncogenic function when SIK1 and SIK3 remain intact.

In the studies outlined here, we improve on previous CRISPR-based strategies to target the Sik Kinases *in vivo*, which have variable targeting efficiencies based on sgRNA targeting location and sequence. We have built upon the KS1 and KS2 floxed mouse models and have generated a Sik3^{flox/flox} conditional KO allele, which has been crossed in the Kras^{LSL:G12D} model. The AMPKRs have been suggested to have redundant activity, with AMPKRs having overlapping targets. As result, we further crossed Sik alleles to each other, to find which combinations of Sik loss best capture Lkb1-dependent tumor suppression. These genetically engineered mouse models are widely considered to be the gold standard for the determination of gene function in models of NSCLC and will further validate the SIKs as bona fide tumor suppressors.

RESULTS

Generation of the Genetic Models of the Salt-Inducible Kinases

While previous studies have highlighted the potential role of the Sik kinases in mediating Lkb1-dependent tumor suppression in mouse models of NSCLC, they did so using a CRISPR Cas9 based strategy^{12,93}. Effective and reproducible targeting *in vivo* using Cas9 is highly variable, leading to heterogeneous tumor composition within an individual animal, and upwards of 46% escapers (those tumors which effectively recombine LoxP sites to induce tumors growth, but fail to delete their sgRNA targeted gene)¹⁵⁷. To overcome these challenges, we have built upon the previously described Sik1^{Flox/Flox} and Sik2^{Flox/Flox} alleles, which were crossed to the Kras^{LSL:G12D} (K) model as well as the Kras^{LSL:G12D}; Trp53^{Flox/Flox} (KP) model, and have generated a Sik3^{Flox/Flox} allele (additionally all models contain a homozygous ROSA26^{LSL:Luciferase} allele allowing bioluminescent imaging)^{12,81,128}. Mice containing the Sik3^{Flox/Flox} allele were crossed with the K model, a well described model for the study of Kras-driven NSCLC¹²⁵ (Figure 3.1). In previous studies, co-deletion of Sik1 and Sik3 led to the largest effect on tumor growth, suggesting that the Sik kinases can compensate for each other upon loss of a single allele^{12,93,158}. For this reason,

we intercrossed the $Kras^{LSL:G12D};Sik1^{FloxFlox};ROSA26^{LSL:Luciferase}$ (KSik1), KSik2, and KSik3 models, to generate combinations of the Sik alleles: KS1/2, KS1/3, KS2/3 and KS1/2/3 (aka KS)(Figure 3.2). Floxed alleles of the Sik kinases leads to loss of exons containing the T-activating loops, required for kinase function (Figure 3.1). Models were confirmed by tail vein injection (TVI) of Adenovirus containing Cre-recombinase (Ad-Cre) or an Ad-Green fluorescent protein (Ad-GFP) control. TVI of 5×10^{11} viral units of adeno-virus (Ad-Cre or Ad-GFP) was introduced into K, KS1, KS2, KS3, KS1/2, KS1/3, and KS mice. TVI of Adenovirus leads to liver accumulation and effective cre-recombination¹⁵⁹. Loss of each Sik kinase was assessed by western blot of phosphorylated Sik. Indeed, we observe decreases in phospho-Sik signal for each of the genetic models (Figure 3.3). Generation of these models allows us to assess to what degree the Sik kinases mediate Lkb1-dependent tumor suppression.

The salt-inducible kinases are key mediators of Lkb1-dependent tumor suppression in Sik-combinatory models.

Upon generating and validating the single and combinatory Kras-driven Sik-floxed mouse models, we assessed their growth differences. K, KSik1, KSik3, KS1/S2, KS1/S3, KS, and KL mice were transduced by introduction of lentivirus containing Pgk-Cre (1.5×10^5 Transforming Units [TFU]/mouse in 75uL sterile saline) by intratracheal injection directly into the lung. Tumors in the experimental cohort (K, n=14; KSik1, n=14; KSik3, n=16; KS1/S2, n=8; KS1/S3, n=13; KS, n=5; and KL, n=8) were initiated on the same day. At 5-weeks post-cre, we quantified tumor growth non-invasively by bioluminescent imaging (BLI) on an IVIS (Caliper) *in vivo* imager and continued bi-weekly until endpoint (Figure 3.4). By 9-weeks post-cre, the faster growing KS1/S3, KS, and KL models showed separation in BLI compared to the K control strain. Lkb1-null tumors at 12-weeks post-cre, which were expected to grow the fastest of all the models, reached endpoint (described by having a mean photon flux [photons/s] $\geq 10^9$). To our surprise, the KS

model which lost all three Sik kinases had similar BLI measurements as the Lkb1-null model at 12-weeks post-cre. It's worth noting that BLI measurements of the KS model were consistently like KL BLI along the time course of the experiment. Co-deletion of Sik1 and Sik3 (KS1/S3) had an intermediate phenotype at 12-weeks, which had higher tumor burden compared to K, and approached KL tumor burdens. Unexpectedly, tumors lacking Sik1 and Sik2 led to no measurable tumors after 12 weeks. And loss of Sik1 or Sik3 alone failed to add any enhancement beyond Kras tumors. These findings support previous findings that Sik1 and Sik3 contribute the most to Lkb1-dependent tumor suppression. However, it was previously reported that Sik2 was required for growth of the tumors. In fact, we see that Sik2 loss has a dual role, in that its loss reduces growth with co-loss of Sik1, and enhances growth with co-loss of Sik1 and Sik3.

Given the nonuniform numbers of mice within each genotype, and the differences in growth rates between each model, we evenly split the experimental cohort into an “early” and “late” group along BLI values at week 12, to not bias tumor sizes. Experimental endpoint for the early group was dictated by the KL and KS models, in which lungs of all mice within these genotypes were collected. Half of all other models made up the late group, and were allowed to continue to grow until the fastest growing model reached a $BLI \geq 10^9$.

***In vivo* imaging and histological analysis reveals the Sik kinases are bonafide tumor suppressors.**

Upon reaching experimental endpoint, mice received a 4-hour Bromodeoxyuridine (BrdU) pulse by intraperitoneal (IP) injection before being euthanized. Lungs were formalin (10%) inflated, fixed overnight, and submitted for paraffin embedding and sectioning. Hematoxylin and eosin (H&E) stained slides from mice in the early group were analyzed for tumor metrics (Figure 3.5). Percent tumor area of the lung (tumor area/total lung area) was highest in Lkb1-null tumors as expected (Figure 3.6). Consistent with the BLI data, only the KS model had elevated %tumor

area compared to K tumors. Percent tumor area appeared to be slightly lower in the KS model compared to KL, however was not statistically significant. While the total burden was elevated in the KS and KL model, we next assessed whether there were differences in tumor size and tumor number, which could reveal the reason for elevated burden. A comparison of tumor sizes revealed that all models which have dysregulated Sik1 activity (KSik1, KS1/S3, KS, and KL), all had very similar tumor sizes (Figure 3.7). Tumors in the KSik3 model were significantly smaller, while tumors from K and KS1/S2 each only had 1 tumor, and as a result, did not provide a proper comparison. It's worth noting that in Hollstein et al, loss of Sik1 led to an increased proportion of larger tumors ($>1\text{mm}^2$) in K and K-Trp53-null models¹². If loss of Sik1 is the main contributor to tumor growth, it is consistent with our findings on tumor size. Tumor number was significantly elevated in the KL and KS models, with on average over 10 tumors being counted per each cross section (Figure 3.8). Tumor number in all other models remained significantly lower than the KS and KL models, despite received the same TFU. The data suggests that the KS and KL model may have a high initiation rate compared to the other models, or the tumors in the other models were too small to detect at 12-weeks.

Mice in the late group, which included mice from all models except KL and KS, were imaged for an additional 8 weeks after euthanizing the early group. At 20-weeks post cre, the KS1/S3 model was able to reach experimental endpoint of having an average BLI $\geq 10^9$ (Figure 3.9). The KS1/S3 model was the only model in the late group that grew more aggressively than K. The KSik1 and KSik3 models grew similar to K, and consistent findings of the early group, the KS1/S2 model never developed tumors. Again, highlighting the dual role of Sik2 in the context of Kras-driven NSCLC models. Analysis of histological H&E sections revealed dual loss of Sik1 and Sik3 were the only models which led to an increase in tumor area (~20%) compared to K (~3%) (Figure 3.10). All other models showed no increases in tumor burden. Unlike the early group in which tumor sizes were not a major contributor to burdens, we see that tumors from

the KS1/S3 model were significantly larger than all other models (Figure 3.11). Indeed, the main factor driving differences in burden is tumor size alone, as there were no noticeable difference in tumor number between any of the models in the late group, other than the KSik1/2 model which had very low tumor numbers (Figure 3.12).

From the *in vivo* study, it was clear that the KS and KL models were the fastest growing and led to the highest tumor burdens at only 12 weeks (Figure 3.4). The KS1/S3 model revealed an intermediate phenotype which outgrew the K model, and reached endpoint at 20 week (Figure 3.9). While no differences were observed in the KSik1 or KSik3 single floxed models compared K at 20 weeks, we asked if these single Sik floxed models would add any enhancement to growth within the time that it takes for K tumors to reach clinical endpoint (approximately 30-35 weeks). For this longitudinal study, we transduced mice from K, KSik1, KSik2, KSik3, and KL models with Pgk-Cre (1.5×10^4 TFU; K, n=16; KSik1, n=15; KSik2, n=21; KSik3, n=21; KL, n=20). Since the KSik2 model was not represented in the first experiment, KL mice were included to assess KSik2 burdens at 12 weeks. Consistent with the first experiment, none of the models, including KSik2, reached the tumor burden of the KL model measured by BLI (Figure 3.13). By 29 weeks, we observed a small increase in tumor burdens in the KSik1 and KSik2 models, however the differences were not significant (Figure 3.13). These findings suggest that the loss of any single Sik allele adds no enhancement to growth. The Sik kinases have been previously reported to catalytically compensate for each other, and these finding support this idea, with multiple Sik kinases needing to be lost in order to match loss of Lkb1.

Analysis of Lkb1-dependent biological features in Sik genetic mouse models

Several laboratories have reported unique features of KL tumor biology including cell proliferation rate and immunological phenotypes. Specifically, loss of Lkb1 enhances the rate at which tumor cells divide. Since tumor growth in Sik-null tumors appears to closely match loss

Lkb1 loss, we asked if some of these previously reported-on features were conserved in the Sik KO setting. In order to address proliferation rates, we took advantage of the use of BrdU incorporation into the lung tumors. BrdU is a synthetic thymidine analog that incorporates into dividing cells during the DNA synthesis (S-phase) stage of the cell cycle. Presence of the incorporated BrdU base can be assessed by immunohistochemical (IHC) analysis using an anti-BrdU antibody. As noted, mice were pulsed with BrdU (100mg/kg) for 4 hours before harvesting. IHC analysis of sections was performed, and tumor %BrdU positivity (BrdU positive cells/total tumor cells) was calculated and quantified. From the early group, KL tumors had an average of 5% BrdU positivity, and to our surprise, KS tumors did not share this similar elevated BrdU incorporation (Figure 3.14). To our surprise, the few KSik1 tumors (n=9), had elevated BrdU incorporation, however it is unclear if these 9 tumors were enough to have a proper representation of the BrdU incorporation in this model. The Sik3 model showed a bi-modal effect, with most tumors showing no incorporation of BrdU, but three tumors showing relatively high levels of BrdU. Lastly, the KS1/S3 model showed similar BrdU incorporation to the KS model, suggesting this may be a true effect after loss of the Sik kinases (Figure 3.14). In the late group, while no increases in tumor sizes were observed between KSik1, KSik3, and K, there is a modest increase in BrdU incorporation in these Sik models compared to K (Figure 3.14). And while the KS1/S3 model had the largest burden in the late group, there was no significant increase in BrdU incorporation compared to KSik1 and KSik3, and only a modest increase compared to K (Figure 3.14).

Since tumor sizes are drastically different in these models, we questioned whether there was a correlation between tumor size and BrdU incorporation. One would expect the largest tumors, to have the highest BrdU incorporation. Unexpectedly, we observed that the smallest tumors appeared to have the highest BrdU levels. This was the case in the KL tumors, where we noticed the highest levels of BrdU irrespective of size (Figure 3.15). As tumors increased in size,

there was a noticeable decrease in BrdU incorporation, and less variance between genetic models.

Loss of *Lkb1* in genetic models of NSCLC have also been reported to have unique effects on immune cell recruitment. Specifically, genetic ablation of *Lkb1* correlated with increases in tumor associated neutrophils (TAN), which are thought to contribute to T-cell suppression¹¹⁴. We then asked if TAN were accumulating in our KL genetic mouse models as compared to K, and if the *Sik*-null genetic models recapitulated this effect. IHC analysis of neutrophil elastase (NE), a neutrophil specific secreted protein, showed a significant increase in the presence of NE positive cells in KL lung tumors compared to K (Figure 3.16). To our surprise, little to no NE was present in K lung tumors or KS tumors (Figure 3.16). NE was quantified from histological cross sections in all early and late lung samples. The only model which displayed increases in tumor specific NE-positive cells was the KL model (Figure 3.17). All other models, in both the early and the late group, failed to show these increases.

We then hoped to identify the specific cytokines or chemokines driving neutrophil recruitment in the KL model, and those which are absent or reduced after loss of the *Sik* kinases. *Cxcl1* (c-x-c motif ligand 1, aka KC), *Cxcl2* (aka *Mip2*), and *Cxcl5* are all thought to be the main chemokines which activate and mediate the recruitment of neutrophils in mice^{160,161}. These secreted tumor chemokines bind to neutrophil receptor *Cxcr2* leading to activation and recruitment. RNA-seq analysis of tumors showed no increases in expression of any these chemokines in the KL model compared to K (Figure 3.18 and Figure 3.19). Loss of the *Sik* kinases led to significant decreases in *Cxcl1* mRNA levels in a *Sik* dependent manner, with KS tumors having the lowest *Cxcl1* expression (Figure 3.18). Similarly, *Cxcl2* and *Cxcl5* expression was *Sik* dependent, such that the KS tumors had the lowest expression (Figure 3.19). If these chemokines are responsible for the recruitment of these TAN, then the RNA-seq data agrees with IHC analysis

of NE positivity results in the KS model. However, its surprising that no increases in the KL tumors were observed.

RNA-seq analysis of the Sik-null tumors reveals significant transcriptional overlap with KL tumors.

Hyperactive Kras mutations are among the most common tumor initiating oncogenes in human NSCLC^{104,106}. Transcriptional analysis of all NSCLC patient samples in The Cancer Genome Atlas (TCGA) harboring activating Kras mutations has revealed that samples unbiasedly clustered into three distinct groups, and were represented by those harboring co-mutations with P53, CDKN2A, and LKB1¹⁰⁴. The LKB1-SIK axis also phosphorylates and inhibits two well-known families of transcriptional co-activators, the Class-IIa HDACs and the CRTC, such that loss of LKB1-SIK activity leads to loss of 14-3-3 binding and nuclear translocation, where they can facilitate the expression of their cognate transcription factors target genes (Figure 3.20)¹⁵⁸. Given the SIK kinases direct regulation of transcription, we asked to what degree do the SIK kinases capture the LKB1 transcriptional program.

Tumors were initiated by intratracheal intubation of Pgk-Cre in K, KSik1, KSik3, KS1/S3, KS, KL, and KP genetic models (3.0×10^5 for K, KSik1, and KSik3 and 1.5×10^5 TFU in KS1/3, KS, KL, and KP). Tumors in mice were allowed to grow until reaching experimental endpoint ($BLI \geq 10^9$), and which point mice were euthanized and tumors microdissected from lungs. RNA was purified from 6 tumors per genetic model (excluding KS; 18 tumors) and were of similar size (approximately 2mm in diameter). Libraries were prepared and sequenced by the NGS core, and initial analysis performed by the Bioinformatics Core at Salk Institute. Differential gene expression (DGE) analysis showed that the Sik kinases induced gene expression changes as compared to Kras and appeared to be dose dependent (Fold change [FC]=1.5; False discovery rate [FDR]=0.05). That is, the number of differential expressed (DE) genes were lowest in the

KSik1 and KSik3 models, followed by the KS1/S3 model, and highest in the KL and KS models (Figure 3.21). Additionally, significant overlap was observed between Sik models, with the majority of DE genes in the single floxed models being captured in the double and triple floxed models. Only 9 genes (Rps2, Hmga1, Hmga1-rs1, Atp6v0c, Atp6v0c-ps2, Paqr4, D17H6S56E-5, Ntn3, and Sema4f) were found to be upregulated in common between all models (Excluding KP), and 11 genes downregulated (Raet1a, Raet1b, Raet1d, Raet1c, Raet1e, H60b, Adtrp, Gm10825, Banp, Vwa7, and Shisa2) in common between models (Figure 3.21). Gene set enrichment analysis of these gene regulated in common yielded no significant findings. However, from the 11 downregulated genes, several participate in aspects of the major histocompatibility complex 1 (MHC1) activity or complex. Volcano plots showing pairwise comparisons between Kras and all other models showed several reoccurring upregulated genes, including two ribosomal 40S and 60S subunits Rps2 and Rps18 (Figure 3.22). In KS1/S3, KS, and KL models, Slc2a13 and Cntfr (as well as Rps2 and Rps18) were most significantly upregulated in common (Figure 3.22). There was little overlap in down regulated genes with the highest significance or magnitude of change (Figure 3.22). Dimensional reduction analysis of RNA-seq samples was performed and plotted across principle component 1 (PC1) and PC2 (32% and 16% variance, respectively) (Figure 3.23). Clustering across PC1 alone separated samples into 4 distinct groups: 1)KP; 2)K, KS1 and KS3; 3)KS1/S3 and KL; 4)KS. As a result, it appears that the genes included in PC1 describe those genes most effected by the loss of the Siks. Clustering across PC2 separated KS1/S3 and KL tumors, suggesting the genes in PC2 describe features unique to Lkb1 tumors (Figure 3.23). Interestingly, KS tumors lied between KS1/S3 and KL across PC2, highlighting the fact that Lkb1 has many other functions outside of the activity though the Sik kinases, and that expression of those genes contributing to distribution across PC2 (Figure 3.23).

Hierarchical clustering of the top 500 most variable genes in this data set revealed that most samples clustered by genotype (Figure 3.24). K-means clustering into 5 clusters showed

unique sets of genes that were mostly upregulated (cluster 1; 132 genes) or down regulated (cluster 5; 102 genes) in common between KL and KS models (Figure 3.24). In both cluster 1 and 5, no differences were observed between K and KP, and suggests the genes in these clusters are not simply those genes which are dictated by tumor size, but instead Lkb1/Sik dependent.

Gene ontology (GO) term analysis was performed on Figure 3.24 clusters to attempt to uncover unique biological features controlled by the Sik kinases. Clusters 1, 2, 3, and 4 showed enrichment of genes involved in responses to metal ions, intermediate filament organization, amino sugar catabolic processes, and vitamin transport, respectively (Figure 3.23). The most significant enrichment was detected in cluster 5, which showed enrichment of genes involved in responses to interferon beta, responses to interferon gamma, responses to protozoan, and antigen processing and presentation of peptide antigens (Figure 3.23). Decreases in expression of these genes is in agreement with previous reported findings demonstrating the immunological cold phenotype of Lkb1-null tumors, and it appears this is, in part, contributed to by the Sik kinases.

Analysis of previously described gene sets highlight transcriptional similarities between KL and KS tumors

LKB1 is highly studied, and several groups have reported distinct LKB1 dependent gene signatures. One of the most stringent gene sets was reported by Kauffman et al., where they identified a 16-gene classifier which was able to predict LKB1 mutations in lung adenocarcinoma¹⁰⁷. We performed a PCA analysis of the Kaufman-16 in our Sik-null tumors to assess if they were Lkb1-null “like”. Dimensional reduction allowed us to score each tumor from -1 to 1, with 1 being most Lkb1-null “like”. K, KSik1, and KSik3 tumors had the lowest scores (Figure 3.26). Tumors from the KS1/S3 model had higher scores than K, and were very similar to KL scores (Figure 3.26). Surprisingly, KS tumors had the highest Kaufman-16 score, suggesting

that the 16-gene classifier is better at predicting KS tumors. Assessment of the expression of each of the genes revealed the higher scores were as a result of elevated expression of each of the classifiers (Figure 3.27). For most genes that are upregulated in the KL model, expression is higher in the KS model (Figure 3.27).

Overlaps in gene expression between KL tumors and Sik-null tumors have already been shown. In Hollstein et al, it was shown that loss of Lkb1 or Sik1 and Sik3 led to upregulation in a gene set of Hallmark EMT genes¹². In our genetic models, we observe a similar increase in these same genes (Figure 3.28). Similar to the observation made in the Kaufman-16 gene classifier, expression of the genes that are upregulated in KL tumors are even higher in KS tumors (Figure 3.28). Most of the genes described in the Hollstein et al. EMT gene set also remain lowly expressed in KP tumors, suggesting that expression is indeed Lkb1/Sik mediated (Figure 3.28). Outside of the Hollstein EMT gene set, there are several other Hallmark EMT genes. We also assessed if those genes are induced in the KL and Sik-null models. Surprisingly, the majority of the genes in the GSEA Hallmark EMT gene signature are upregulated in an Lkb1-Sik dependent manner (Figure 3.29). However, only about half appear to be specific to KL/KS tumors, as about half of these genes are upregulated in the KP model (Figure 3.29). These observations suggest Lkb1, primarily through the Siks, induce an extensive transcriptional EMT program.

Loss of Sik1 and Sik3 or Lkb1 was also shown to increase the secretion of Il6, activate the Il6-Jak-Stat pathway, and induce Stat target genes¹². Most of the genes reported previously are induced in the KL and KS model, and remain low in KP tumors (Figure 3.30). Areg and Ereg, two Egf-like ligands that signal through Egfr, are the only two genes upregulated in KS/KL and KP (Figure 3.30).

Lkb1-Sik axis directly inhibits the CREB1 transcriptional coactivator Crtc2 which itself has been suggested to activate the AP1 transcription factor^{129,130}. Hollstein et al investigated if loss of Sik1 and Sik3 or Lkb1 led to increases in expression of genes containing AP1 and CRE

in their promoters. Here we tested if their identified AP1+CRE gene set was upregulated in our genetic models for Sik loss. Loss of Sik1 and Sik3 appears to be enough to induce the expression of the AP1+CRE target genes, as KS1/S3 expression pattern matches KL tumors for most genes in this gene set (Figure 3.31). Again, KS tumors have higher expression than either KL or KS1/S3 (Figure 3.30). Lastly, while most of these genes are upregulated in an Lkb1-Sik dependent manner, about half of the AP1+CRE genes are increased in the KP model, and don't appear to be specific to Lkb1(Figure 3.30).

One of the well-established targets of the Sik kinases, Crtc2, has been proposed to behave as an oncogene. In orthotopic models of NSCLC using A549 (LKB1 null) cells, it was observed that knockdown or knockout of CRTC2 led to significant decreases in lung implantation and lower tumor burdens¹⁴¹. Additionally, they reported a CREB target gene set (49 genes), by performing ChIP-seq on CRTC2 and cross analyzed it with RNA-seq from A549 vs A549+LKB1. In Sik-null tumors, we hypothesize the Crtc family is hyperactive. If Crtc2 is mediating a significant portion of Sik-mediated tumor suppression, we hypothesized that the Rodon-49 gene set would be significantly high in KL and Sik-null tumors. To our surprise, only about 20% of genes behaved in a Lkb1-Sik dependent manner (Figure 3.32). Several genes were high in K tumors as compared to KP, suggesting that those tumors had already high Crtc2 activity. It's unclear if any of the Rodon-49 Crtc2 targets directly contribute to tumor growth.

LKB1 control of PRC2 and Hippo pathway may contribute Sik mediated tumor growth

Tumor growth in the KS model is significantly elevated, suggesting that the Sik kinases are the main Lkb1 targets that mediate its tumor suppressive function. High transcriptional overlap was observed between tumors from KL and KS and was dependent on the number of Sik kinases being lost (DE genes in KS>KS1/S3>KS1 or KS3). As a result, we asked whether the transcriptional output of the KS model correlated with previously described pathways observed

in the KL model. Since the Sik kinases are known to be transcriptional repressors, we focused our attention to the genes in Cluster 1 (Figure 3.24). From Cluster 1, which had the highest number of gene upregulated in common between Lkb1 and the Sik's, we generated a hand curated heat map, Cluster 1.1 (Figure 3.33). The genes in this new cluster 1.1 were selected to match the prototypical Sik target gene, which assumed that the Sik kinases are catalytically redundant and compensate for each other. These genes are lowly expressed in K, increase slightly in KSik1 and KSik3 (or remain unchanged), are moderately increased in expression upon co-deletion of Sik1 and Sik3, and highly expressed upon loss of all 3 Sik kinases or Lkb1. Additionally, we only included those genes whose expression remained unchanged in the KP model vs K, in order to highlight only those genes which were Lkb1/Sik dependent (Figure 3.33). As the Siks are able to inhibit Creb target genes through phosphorylation of the Crtc's, we first asked if the genes in Cluster 1.1 could be described by merely being Creb targets. Cross referencing with a genome-wide assessment of those genes which contain CREB binding sites at their promoters (an indication of being a CREB target) or binding of Crtc2 by ChIP-seq, revealed that only about 20% (11 out of 54) of the genes could be possible Creb/Crtc targets (Figure 3.33)^{141,162}. As it appeared this highly Sik regulated cluster were not solely Creb targets, we then performed an unbiased gene set enrichment analysis on cluster 1.1. The analysis was matched with several gene sets including Suz12 targets, EED targets, and PRC2 targets^{163,164} (Figure 3.34). Interestingly, Suz12 and EED are both subunits in the PRC2 complex, which acts to methylate histone H3 at lysine (K) 27 in order to repress gene expression of their target genes. If the increases in expression of the genes in cluster 1 are through PRC2 activity, it would suggest that PRC2 is activated by the Sik kinases, and that loss of the Sik kinases or Lkb1 would lead to PRC2 activity loss and decreases in H3K27 methylation. In fact, it has been previously reported that lung tumors in the KL model have decreases in EED protein levels, and as a result, decreases in PRC2 activity read out by decreases in H3K27 methylation¹¹³. Analysis

of the mRNA in our Sik genetic models revealed no decreases in the message level of Eed, Ezh2, or its complimentary demethylase, Utx (Figure 3.35). Ribonucleoprotein (RNP) CRISPR KO cell lines containing Lkb1 or Sik KO were also assessed for differences in PRC2 subunit protein levels, and western blots performed. No differences were observed in the Lkb1 or the Sik KO cell lines to either EED, Ezh2, or Suz12 (Figure 3.37). H3K27 trimethylation (me3) was assessed but the results were inconsistent within each genetic alteration. However, it is worth noting that H3K27me3 was only detected in the NT and LKB1 KO, and not in any of the 5 Sik KO cell lines (Figure 3.36).

We then proceeded to investigate potential pro-growth pathways that the Sik kinases may control. The Hippo YAP/TAZ pathway is commonly implicated in a variety of malignancies, and which manifests with nuclear localized hyperactive Yap^{165,166}. In drosophila, the Sik kinases 2 and 3 have been reported to be involved in the regulation of the Hippo pathway as it relates to wing growth¹⁶⁷. Hippo was first discovered in Drosophila and is one of the main regulators of cell cycle exit and apoptosis¹⁶⁷. Beyond the Sik kinases, Lkb1 has been thought to mediate Hippo signaling particularly through two AMPKRs, the MARKs and AMPK¹⁶⁸⁻¹⁷⁰. We first asked if we observe a YAP signature in our KL model. To do this, we first identified a yap gene signature from Yap deleted mouse lung epithelial cells¹⁷¹. Upon generation of the Yap dependent gene set, we performed GSEA analysis against KL or K tumors. Interestingly, Yap dependent genes were heavily enriched in KL tumors as compared to K (Figure 3.37). Of the Yap dependent genes that are upregulated in a Lkb1/Sik dependent manner, it appears that about half of them are also upregulated in the KP tumors, with only about 15 genes appearing to be solely controlled by Lkb1 and the Sik kinases (Figure 3.38).

DISCUSSION

Here we present data on newly generated conditionally floxed mouse models of the Sik kinases. We generated a Sik3 conditionally floxed mouse in the FVB/N background, and crossed it to mice containing the Kras^{LSL:G12D} allele, named the KSik3 model. Further intercrossing of KSik1, KSik2, and KSik3, led to the generation of KS1/S2, KS1/S3, and KS1/S2/S3 (KS) models for the study of Lkb1-dependent NSCLC. Intratracheal injection of viral-cre led to transduction and tumor initiation in these models. As expected, we observed significant increases in tumor growth rate in the KL model when compared to K alone. And no significant differences in growth were observed between K, KSik1, KSik2, or KSik3 models. Consistent with previous findings, the KS1/S3 model had an intermediate phenotype, with tumors that grew larger than K tumors but slower than KL tumors. To our surprise, the KS model matched growth of the KL model by BLI, suggesting that Sik2 loss in the KS1/S3 model further enhances growth rate. However, the role of Sik2 in these models appears to be paradoxical, as no tumor initiation was observed in the KS1/S2 model, while KSik1 models produced small tumors. Lkb1 and Sik2 has been reported to be required for growth in models acute myeloid leukemia (AML), as they support MEF2C target gene expression required for proliferation^{95,96}. In Hollstein et al, it was observed that the KSik2 model imparted growth deficiency, while in the data we reported here in a longitudinal study of 29 weeks, KSik2 tumors grew very similar to K tumors. Sik1 is the predominantly expressed allele in the lung epithelia, followed by Sik3, then Sik2. Given the redundancies of the Sik kinases, it would appear that Sik2 only enhances tumor growth if Sik1 and Sik3 activity are lost. It's worth noting that Sik1, Sik2, and Sik3 mRNA increase after loss of any of the single Sik alleles. However, Sik2 mRNA does not appear to dramatically increase in the KS1/S3 model. This could potentially explain why the KS1/S2 model did not generate tumors, as Sik3 expression could be induced to allow for Sik3 to mobilize and compensate for the loss of Sik1 and Sik2. As opposed

to when Sik1 and Sik3 are lost, maintained low levels of Sik2 are unable to adequately compensate.

Histological analysis showed the scope of differences between models, as the KL and KS mice had significant higher tumor burdens as compared any other model. Burdens appeared to be primary driven by differences in tumor number at 12 weeks, and not tumor size. In a normal experiment, we would expect no differences in tumor number, as all mice received equal viral particles. However, this may suggest that the KL and KS models have significantly higher abilities to initiate tumors. One hallmark of LKB1-null tumors is that they are considered immunologically “cold”, as they don’t have any resident T-cells and fail to respond to immune checkpoint blockade therapies^{100,106,172}. Additionally, we see that both the KS and KL models have significant decreases in immune related genes such as those involved in the response to interferon beta, interferon gamma, and those genes involved in peptide and antigen presentation. I would be worth identifying the activity of those genes, as they relate to tumor initiation.

Unlike tumor burdens assessed at 12 weeks, in which differences of burden could be attributed to tumor number, tumors at 20 weeks appeared to differ in tumor size. Co-deletion of Sik1 and Sik3, which had an intermediate phenotype compared to KL tumors, had tumors which were larger than other models at 20 weeks. Surprisingly, 20-week KS1/S3 tumors were larger than KS and KL tumors at 12 weeks. This further suggests there are differences, which loss of Sik2 exacerbates, which enhance the ability of tumors to initiate at early timepoints. Its unclear if the role of Sik2 merely enhances the genes already up in KS1/3, or increases the expression of a unique set of genes. We analyzed the unique genes upregulated by Sik2 by comparing KS1/3 and KS transcriptional profiles. Top GSEA hits showed enrichment of genes involved in EMT, mitotic spindle formation, and myogenesis. It remains to be determined how, if all, these specific pathways enhance initiation of tumors.

While there are several factors in common between the KL and KS model, several differences remain. For example, while tumor burdens matched by BLI, there still appeared to be differences in burden by comparison of histological sections. While it appears the majority of the tumor suppressive function is mediated by the Siks, other AMPKRs may have a role that indirectly contribute to tumor growth. The AMPKRs effect polarity and metabolism, which may mediate tumor growth benefits in already established tumors. It's clear by measuring BrdU incorporation that Lkb1 loss contributes to the highest incorporation in the smallest tumors. As an example, KL tumors have been suggested to be dependent on CPS1 upregulation in order to maintain pyrimidine pool for nucleotide synthesis¹⁷³. However, upregulation of Cps1 in the KS model is not observed, potentially contributing to its decreased levels of nucleotide incorporation rates, and slightly lower burdens.

As mentioned, one of the main phenotypes that has been reported in the KL model are the increases in TANs and decrease in tumor associated macrophages. We found that the KS model is not able to capture this effect, possibly as a result of decreases in expression of the recruiting cytokines Cxcl1, Cxcl2, and Cxcl5. Complete immune cell profiling is required in these tumors, and may better reveal how much of Lkb1 immunophenotype is captured by the Sik kinases. However, it could be assumed that Cxcl cytokine expression is mediated by an AMPKR other than the Sik kinases. It has been suggested that the MARK kinases, in addition to regulating cell polarity and EMT, may regulate the expression of several cytokines and chemokines including various interleukin family members and CXCLs¹⁷⁴.

Differential gene expression analysis between all models revealed the PRC2 complex as a potential direct or indirect target of the Sik kinases. Unfortunately, we could not recreate the previously reported findings demonstrating decreases in PRC2 complex subunit EED protein level in KL (or KS tumors)¹¹³. However, several labs have reported differences in PRC2 activity as a result of Lkb1 manipulation. For example, it has been suggested that AMPK can

phosphorylate and inhibit EZH2 at T311, leading to PRC2 target demethylation, and target gene expression¹⁷⁵. However, this mechanism is counter to decreases PRC2 activity noted in KL tumors. Our data here, which shows increases in PRC2 target genes, suggests the Siks are required to activate PRC2, which is in agreement with previous published data in KL tumors¹¹³. Chromatin modifiers, including several PRC2 components, were also identified in a genome wide CRISPR screen which looked for enrichment of sgRNAs after restoring Lkb1, and compared them to Lkb1-deficient conditions. Within the top 20 hits, were Eed and Suz12¹⁷⁶. Suggesting inactivation of the PRC2 complex may enhance tumor growth. Of interest, is performing a ChIP-seq analysis of H3k27me3 in K, KS, and KL tumors to identify potential pro-growth, PRC2 mediated, gene signatures.

Our ultimate goal was to uncover a pro-growth pathway or mechanisms by performing RNA-seq analysis, and finding genes in common between KS and KL models. Transcriptional analysis revealed several pathways that were consistent with previous reports, including upregulation of EMT genes, Il6-Jak-Stat pathway genes, and Creb targets. However, unbiased gene set analysis revealed no pathways directly involved in growth. For this reason we combed literature and identified one potential pathway, which regulated growth, and has been implicated in the KL model: the hippo pathway. We identified a Yap gene set from lung epithelia in genetic mouse models harboring floxed alleles to Yap¹⁷¹. As expected, and consistent with previously reported data sets, Yap target genes were upregulated in KL tumors^{169,177}. Knockdown of MARK4 has been observed to reduce LATS2 phosphorylation, and increase Yap nuclear accumulation, suggesting that the Mark kinases control Lkb1-mediated hyperactive Yap¹⁶⁹. Others have reported that AMPK can directly phosphorylate LATS2 under energy stress at S61, S94, and T119, which subsequently leads to YAP phosphorylation and inactivation¹⁷⁸. While both of these findings may contribute to LKB1-dependent Yap activity in the respective cell lines in which they were studied, the Marks and Ampk showed no tumor promoting effects in lung cancer cell lines

grown in anchorage independent settings or *in vivo*, respectively^{12,34,93}. Our data set here argues that activated Yap, and enhancement to tumor growth, is mediated mostly by the Sik kinases rather than the previously suggested Mark kinases or AMPK¹⁶⁹.

Future direction

In this study, we developed a new model for the study of Lkb1 dependent tumor suppression. We have found that genetic loss of all three Sik kinases resulted in similar growth and transcriptional output as the KL model. However, several questions remain to be answered in these models. In our growth studies, we observed no enhancement to growth in KSik1, KSik2, or KSik3 models compared to K alone. However, one difference between these models and the KS or KL model, is that they appeared to initiate significantly less tumors at 12 weeks. In order to assess whether loss of any of the Sik kinases impart any enhancements to growth, we took advantage of the more aggressive KP model, which has high tumor initiation independent of Lkb1 status. We have since crossed all Sik floxed alleles into the KP background and will be assessing growth phenotype differences. The data from this study will show if loss of any single Sik allele contributes more than others, to tumor promotion. Additionally, the KPL model (p53 flox crossed with Lkb1 flox) has reported an extremely aggressive growth phenotype and a unique histological phenotype known as mucinous subtype, in which adenocarcinomas are thought to dedifferentiate into globular type cells which secrete mucin. We hope to assess the Sik contribution to the mucinous subtype by the use of the KPS model (p53 flox; Sik flox). These studies are currently on going.

Transcriptional analysis reported here also revealed several potential Lkb1 dependencies. Currently we are working to address whether any upregulated genes in common between KL and KS tumors can serve as Lkb1 dependencies. Using a previously described *in vivo* Cas9 screen, we will hope to identify target genes which disrupt Lkb1 or Sik dependent

growth¹⁵⁷. We have identified hundreds of genes which are upregulated in a SIK and LKB1 dependent manner and which may be involved in Lkb1/Sik-dependent tumor initiation or growth. To narrow down our gene list to 100 genes, we performed several transcriptional analyses across sample types, identified and removed essential genes, and favored those with previously reported mechanisms or those which have shown Lkb1-dependencies (Figure 3.39). We are currently working on generating pooled barcoded lentivirus containing sgRNAs to target these 100 genes. Ultimately, our goal is to use our RNA-seq data set to identify genes whose expression is Lkb1 or Sik dependent, target them using the *in vivo* *KL; H11^{LSL:Cas9}* mouse model, and ultimately be able to directly tie it back to growth and/or RNA-seq phenotype observed (Figure 3.40).

METHODS

Genetically Engineered Mouse Models for Conditional KO

All animal usage and procedures were approved by the Salk Institute Institutional Animal Care and Use Committee (IACUC). Animal husbandry services, including, but not limited to, cage changes, daily well-being checks, and veterinary services were provided by the Salk Institutes Animal Research Department (ARD). For all tumor bearing models, mice were kept on an FVB/N background. Mice containing the Sik1^{floxed} and Sik2^{floxed} alleles were provided from the Berdeaux and Screaton Labs, respectively, where their generation has been previously reported^{81,128}. The Sik1^{floxed} and Sik2^{floxed} were crossed with FVB/N Kras^{LSL-G12D}; Rosa26^{LSL-Luciferase} for >8 generations, before they were used in experiments, or crossed with other mice containing floxed alleles of the Sik Kinases. The Sik3 conditional knockout mouse model generated in this study, was imported via three male founder mice which harbored a KO-First Allele (C57BL/6N-Atm1Brd Sik3tm1a (EUCOMM) Hmgu/WtsiOrl). These mice were acquired from Wellcome Trust Sanger Institute. Mice originated from a EUCOMM ES

clone (HEPD0744_6_B02) containing gene targeting cassettes placed at introns 4 and 5 of SIK3, which were generated using previously described methods¹⁷⁹. Mice containing the SIK3 KO-First Allele were crossed with ACTBFLPe B6J (JAX stock #005703) females which express FLP1 recombinase gene allowing for the deletion of the Frt-flanked LacZ reporter and Neomycin cassette. Resulting progeny were sequenced for a tm1c conditional ready allele, containing *loxP* sites flanking the critical exon 5 of the endogenous *Sik3* allele with deletion of LacZ. Mice containing the tm1c allele were backcrossed into in FVB/N mice for 4 generations allowing for the homozygosity of the *Sik3*^{flax/flax} (*Sik3*) allele and cross out for Frt. Further crossing of *Sik3* into *Kras*^{LSL-G12D} mice was performed to generate *KSik3* experimental mice.

mRNA-Sequencing

RNA samples were purified from single tumors ranging from 1-3mm in diameter. RNA was purified using the *Quick*-RNA Miniprep Kit from zymo research (R1054). RNA sample concentrations were calculated using a nanodrop and normalized. Sample libraries were prepared by the Salk Institutes Next Generation Sequencing Core. There, RNA samples were quality controlled using Quibit and tapestation, maintaining RIN values ≥ 8 . 500ng of stranded mRNA was used as input for library prep, and prepared following Illuminas manufacturers instruction and guide. Samples were sequenced on the HiSeq 4000 platform and configured for single end 50 cycles at an average depth of 30M reads per sample.

Bioinformatic mRNA-Sequencing Analysis

Sequenced reads were quality-tested using FASTQC (v0.11.8)¹⁸⁰ and mapped to the mm10 mouse genome using the STAR aligner (v2.5.3a)¹⁸¹ with default parameters. Raw or TPM (Transcripts Per Kilobase Million) gene expression was quantified across all the exons of RefSeq genes with *analyzeRepeats.pl* in HOMER (v4.11.1)¹⁴⁷, which used the top-expressed isoform as

proxy for gene expression. Differential gene expression was performed with the raw gene counts using the R package, DESeq2 (v1.24.0)¹⁸², using replicates to compute within-group dispersion. Differentially expressed genes were defined as having a false discovery rate (FDR) <0.05 and an absolute fold-change (FC) >1.5 when comparing two experimental conditions. Analysis was performed by the Razavi Newman Integrative Genomics and Bioinformatics Core (IGC) at the Salk Institute.

Packaging and Concentrating Lentivirus

Large scale preps of HIV-pGK-Cre lenti-virus used for intratracheal transduction of non-Cas9 genetic mice was packaged and concentrated by Iowa Vector Lab at the University of Iowa Carver College of Medicine (pGK-Cre plasmid was a kind gift from Tyler Jacks).

Intratracheal Intubations for Viral Transductions

Genetic recombination, and induction of tumorigenesis, is achieved by intratracheal intubation of lenti-viral containing PGK-Cre directly into the lung cavity. Mice are anesthetized by intraperitoneal injection of sterilized ketamine/xylazine, and placed on an intubation platform such that the mouse is resting with its head pointed upward with its chest perpendicular to the intubation platform. Fiber-lights are positioned directly onto the chest cavity of the mouse. Needleless Exel Safelet IV Catheters are inserted gently into the trachea, and is confirmed by the observation of white light visualized by looking through the opening of the catheter. Upon proper catheter insertion, 75uL of viral containing solution of known titer is injected into catheter and inhaled into the lung of the mouse. Mice were either given 1.5×10^5 PFU for KL m Catheter is removed from the trachea, and mouse is then immediately placed on a heating pad for recovery, where they are monitored for the duration of the recovery period. Once recovered, mice are quarantined for a 72-hour period to avoid viral cross contamination, then returned to the main

colony. All viral utilizing procedures are conducted in the CRAF Biohazard Suite in a biosafety hood.

Bioluminescent *In Vivo* Imaging

Tumorigenesis in transduced mice was monitored beginning at 5-weeks post transduction with Lentivirus containing CRE recombinase, a timeline consistent with previous observations in the lab. After 5 weeks, *in vivo* imaging was performed in an IVIS Spectrum (Caliper Life Sciences) Bioluminescent Imager. Mice were injected intraperitoneally with 150mg/kg D-Luciferin while anesthetized with isoflurane. Total photon flux (photons/second; a measure of tumor burden) was calculated by measuring and combining dorsal and ventral images, after exposure for 5 minutes on each side. Mean photon flux was calculated within a region of interest. Mice were imaged biweekly for all experiment, unless otherwise noted. Experimental endpoint is dictated by an average total photon flux of 1×10^9 photons/second.

Immunohistochemistry and Image Analysis

Mice were collected at endpoints dictated at the end of each figure. Upon euthanasia, lungs were inflated with 10% formalin solution, and incubated in formalin for 16 hours at room temperature. Following fixing, lung sections were transferred to 70% ethanol and submitted to Pacific Pathology (San Diego, CA) for paraffin-embedding (FFPE). 5 micron-thick sections were taken after cutting 50 microns into the FFPE blocks and transferred to charged slides. H&E slides were processed and stained by Pacific Pathology. For IHC, slides were deparaffinized, rehydrated, and antigen retrieval was performed for 10 minutes (or 30 minutes for BrdU IHC) in high temperature citrate buffer (10mM citrate, pH 6.0). Peroxidase activity in tissues was blocked by 3% H₂O₂ in MeOH for 10 minutes. Tissues were blocked in 5% serum, then incubated in

serum containing primary antibodies overnight. Vector Labs ImmPress HRP Reagent Kit was used as the secondary antibody, per manufacturers instructions, followed by staining with DAB- peroxidase substrate. Upon DAB quenching, slides were counterstained with hemotoxin, dehydrated with ethanol and xylazine, and cover slip mounted with Cytoseal 60. Slides were scanned using a Panoramic MIDI Digital Slide Scanner from PerkinElmer or an Olympus VS-120 Virtual Slide Scanning Microscope at the Salk Institute Waitt Advanced Biophotonics Core.

Digital Pathology Analysis

Analysis of scanned H&E and IHC sections was performed using the QuPath open source software¹⁸³. *Tumor burden quantification:* Histological H&E sections were analyzed individually, and used to quantitate burdens. Tumor burdens were quantified by creating annotations of single tumors using the want tool in QuPath. Normal tissues were differentiated from tumor tissue by observing cell morphology and nuclei density (tumors stain darker with with H&E staining). Tumor burdens for each mouse were calculated by the sum of all tumor annotations divided by the total area of the lung, giving %Tumor area. *Tumor number and size:* Tumor annotations measurements were exported from QuPath to excel, which provided the tumor areas (mm²). Annotations were counted to reveal total tumor number per mouse. *BrdU positivity analysis:* For tumors that were stained for BrdU, annotations of tumors were made as described above. Within tumor annotations, positive cell detection was performed in QuPath. Cell positive parameters were assessed across 3 different slides to confirm proper detection of BrdU+ cells. The same Cell positive parameters were applied to all sections, and %BrdU positive cells was exported for statistical analysis.

Statistical Analysis

Statistical analysis was performed using Prism9 statistical software. All graphs shown are reported with standard error of the mean (SEM).

Immunoblots

Lysates from cell lines grown in culture were prepared by washing monolayer with cold PBS-/- . Cells were isolated in cold lysis buffer (20mM Tris-HCL [pH 7.5], 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton-X, 2.5 mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na₃VO₄, cOmplete protease inhibitor cocktail), then lysates clarified by centrifugation. Clarified lysates were normalized by BCA protein assay kit (Peirce). Lysates were resolved on SDS-PAGE gels ranging from 8-10% acrylamide. Protein gels were wet transferred onto polyvinylidene difluoride (PVDF) membrane, blocked in 5% milk, then primary antibodies added diluted in 5% bovine serum albumen (BSA) and incubated at 4°C overnight with rocking. Upon washings with TBST, membranes were incubated with horseradish peroxidase (HRP)-linked anti-rabbit/mouse antibodies for 1 hour. Membranes were visualized by ECL-HRP detection (ECL: p-coumaric acid, luminol, and H₂O₂) on autoradiography film, and scanned for digital analysis.

ACKNOWLEDGEMENTS

This study was supported by grants to R.J.S. from the National Institutes of Health (NIH) (R35CA220538, P01CA120964, and R01DK080425) and the Leona M. and Harry B. Helmsley Charitable Trust grant no. 2012-PG-MED002. Over the course of this study, H.M. Galvez was supported by the Molecular Biological Approaches to Endocrinology T32 (T32DK007541) and Molecular and Cellular Genetics T32 (5T32GM007240). Partial funding was provided by the H.A. and Mary K. Chapman Charitable Trust and the Aileen S. Andrew Foundation. This work was supported by the NGS Core Facility of the Salk Institute with funding from NIH-NCI CCSG: P30

014195, the Chapman Foundation and the Helmsley Charitable Trust. This work was supported by the The Razavi Newman Integrative Genomics and Bioinformatics Core Facility of the Salk Institute with funding from NIH-NCI CCSG: P30 014195, and the Helmsley Trust. Special thanks to Jingting Yu from the Bioinformatics Core for her help with running the RNA-seq analysis. We are greatly appreciative to the Animal Resources Department for the maintenance and well-being of all the animals in this study.

Chapter 3, in part is currently being prepared for submission for publication of the material. Galvez, H.G.; Murray, C.W.; Hollstein, P.E.; Baumgart, J.T.; Brun, S.N.; Ianniciello, A.; Rymoff, T.J.; Ross, D.S.; Eichner, L.J.; Shaw, R.J. The dissertation author was the primary investigator and author of this material.

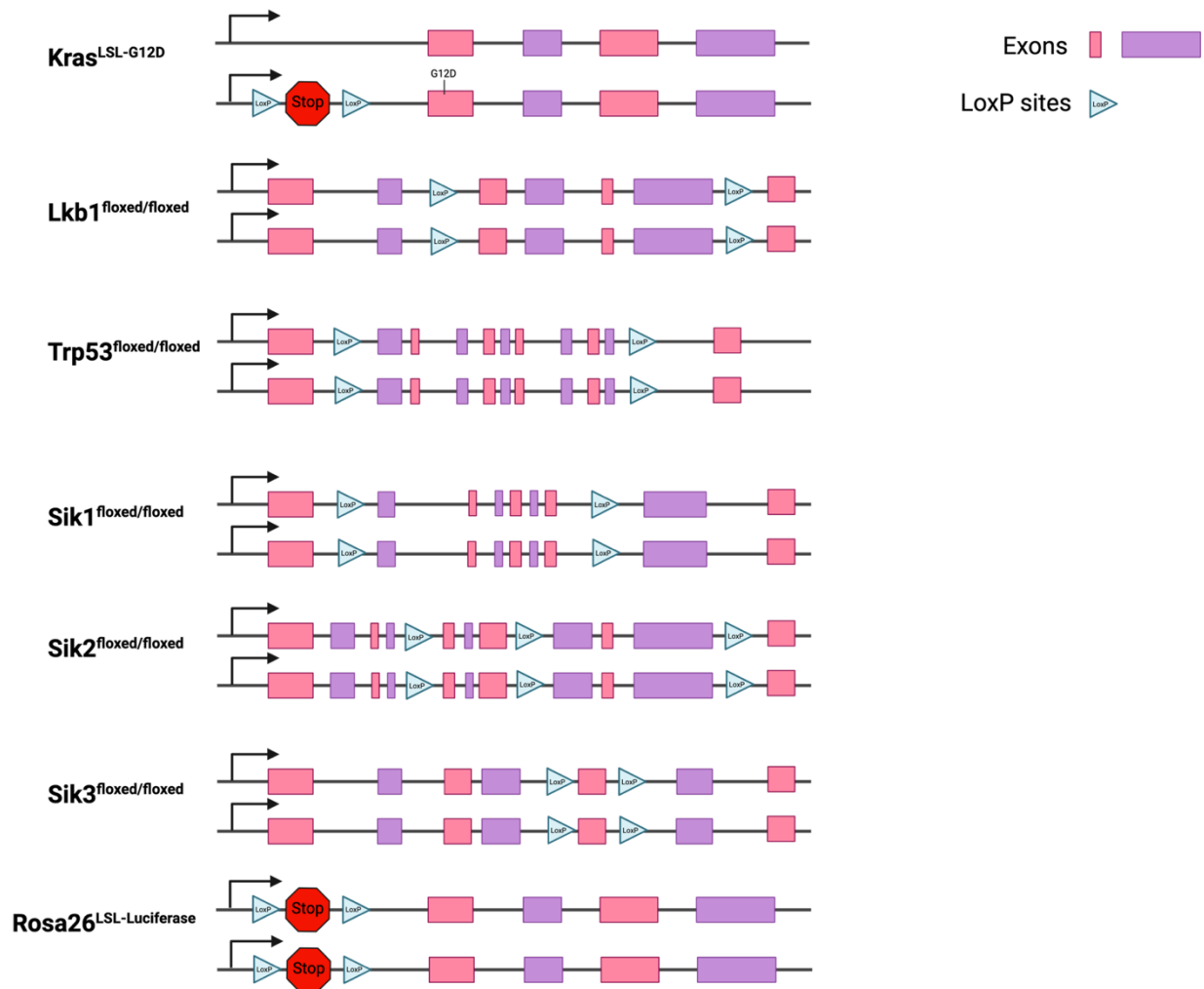


Figure 3. 1: Outline of Alleles in Genetic Mouse Models.

The following outlines the engineered alleles crossed into mouse models. Modification of each allele is dependent on introduction of Cre-recombinase delivered by lenti-viral vector. Kras^{LSL-G12D} expresses a single wild type (WT) allele. Cre-recombinase removes the stop sequence and allows the expression of the mutant constitutively active mutant allele. Lkb1, Trp53, Sik1, Sik2, and Sik3 all have loxP sites flanking critical exons 3-6, 2-10, 2-7, 5-7, and 5, respectively. The activating T-loop falls within the excised exons of all Sik alleles, a region critical to its kinase function. Luciferase is only expressed in cells which recombine with cre as both alleles contain stop sequences.

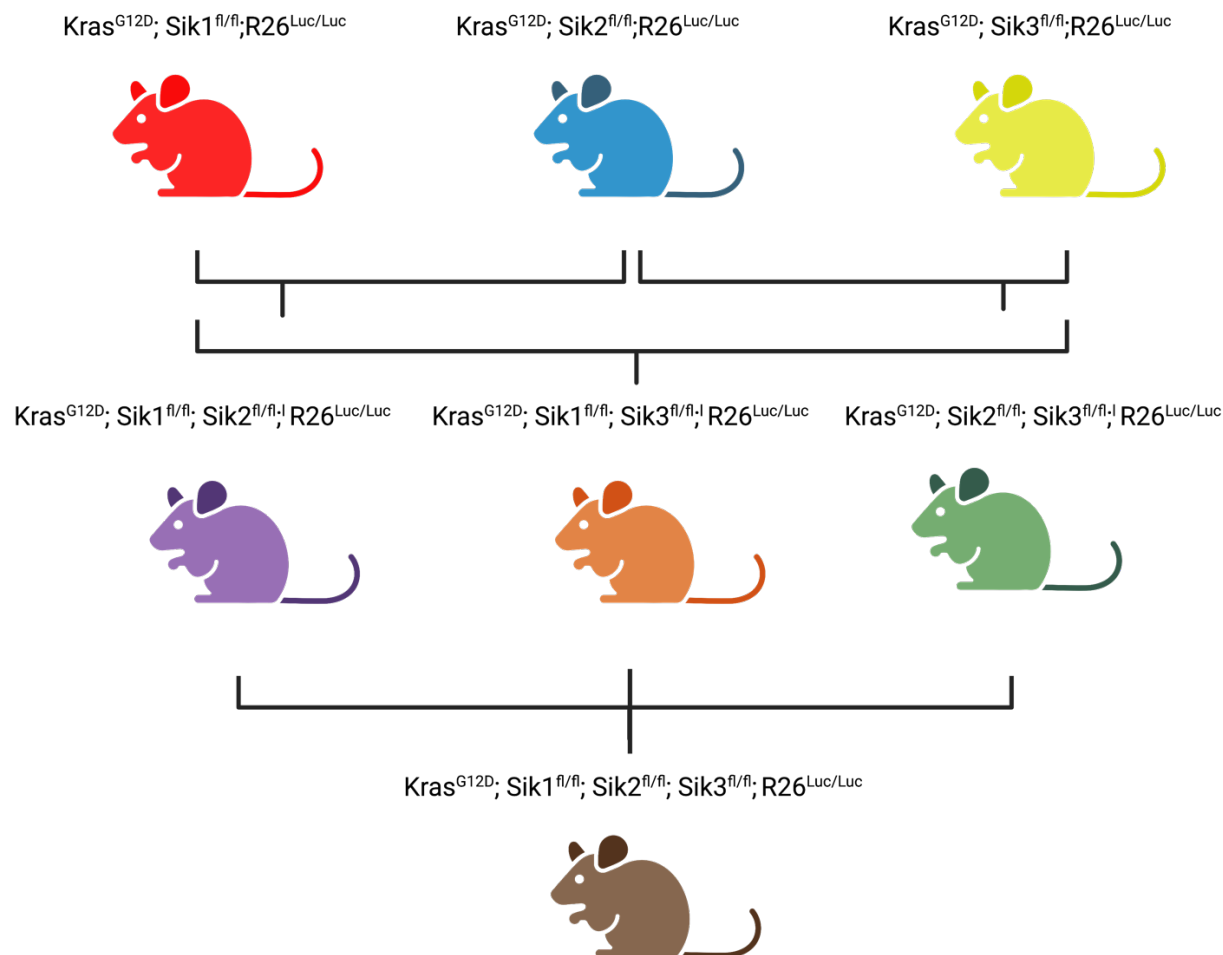
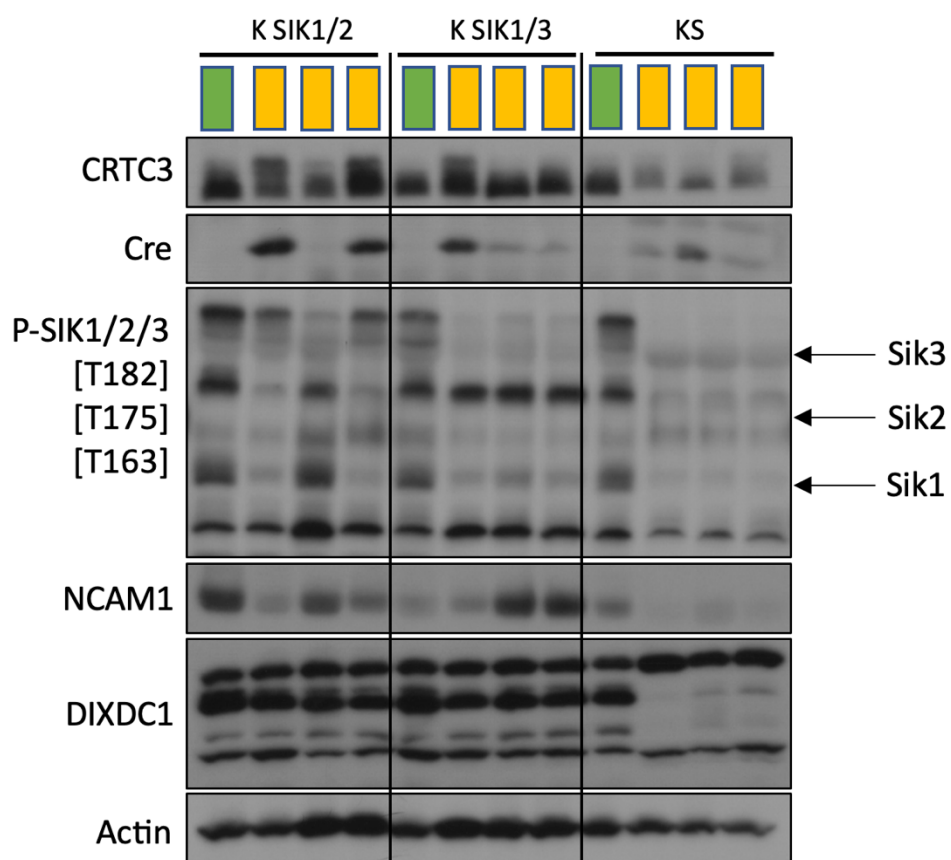
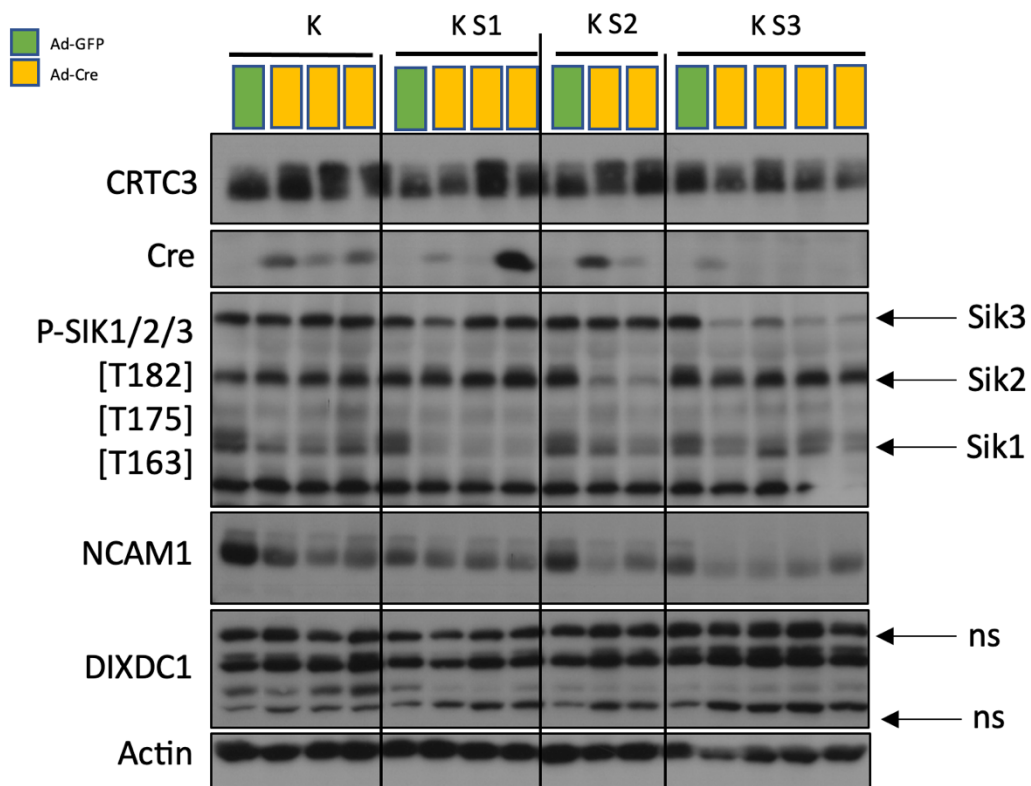


Figure 3. 2: Breeding Strategy to Generate the Combinatorial Sik-floxed Mice.

Since the Sik kinases are thought to be catalytically redundant, we hypothesized that multiple Sik kinases needed to be lost to recapitulate Lkb1 loss. We generated the full set of combinations of Sik loss by crossing mice harboring single floxed alleles to each other. KSik1, KSik2, and KSik3 mice were used to generate the KS1/2, KS1/3, and KS2/3 models. Further Crossing of KS1/3 to the KSik2 mouse was done to generate the KS1/2/3 (KS) mouse.

Figure 3. 3: Validation of Combinatory Sik-Floxed Genetic Mouse Models by western blot.

Western immunoblots of combinatory Sik-floxed models which were tail vein injected with 5×10^{11} viral units of adeno-virus containing Cre or GFP. Lysates were generated from liver tissues. Phosphorylated Sik kinases were used as proxy to measure total Sik levels as the Sik kinases are constitutively phosphorylated by Lkb1. All single floxed genetic models behaved as expected, losing phospho-Sik signal of Sik1, Sik2, and Sik3 in the KSik1, KSik2, and KSik3 models, respectively, upon cre injection. Similarly, the combinatory alleles also showed loss of their respective targeted Sik alleles, culminating in loss of all three Sik alleles in the KS model. CRTC3 band shifting was expected in Sik KO conditions, however, this was not observed in a predictable manner upon Sik loss. Decreases in CRTC3, Dixdc1, and Ncam1 were noted upon loss of all three Sik kinases, suggesting that loss of the Sik kinases inhibits the action of an unknown transcription factor.



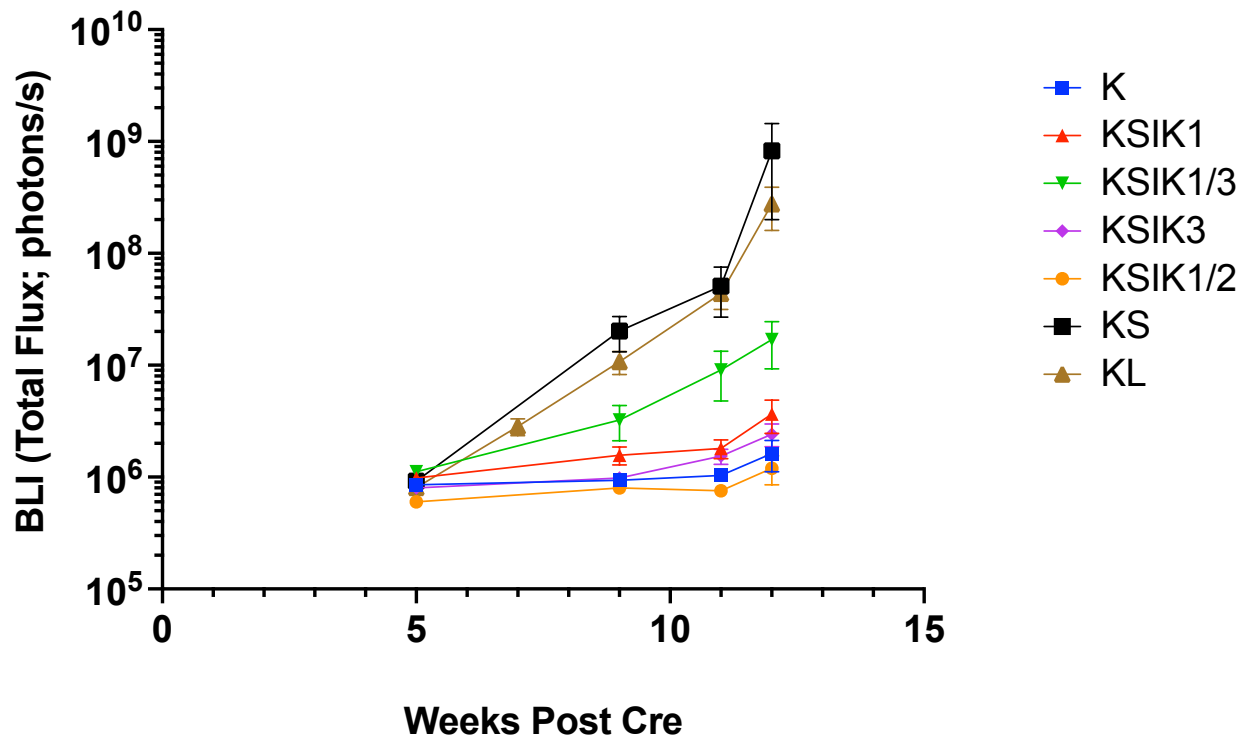


Figure 3. 4: Bioluminescent imaging of early group Sik-null lung tumors reveals that loss of all three kinases matches growth kinetics of LKB1-null tumors in Kras-driven models.

BLI data is displayed as the average photon flux (photon/second) per genotype at each timepoint. Mice were imaged biweekly. Genetic models containing floxed alleles to single or combinations of Sik alleles were intubated with Lenti-virus containing Cre. KL and KS models reached experimental endpoint at 12 weeks, and had very similar growth kinetics measure by bioluminescent imaging (BLI; photons per second). Similar to previously reported studies, co-deletion of Sik1 and Sik3 led to an intermediate growth phenotype, which approached KL tumor size. Unexpectedly, model of co-deletion of Sik1 and Sik2 led to no tumor initiation, despite tumors initiating in Sik1-null models. However, loss of Sik1 or Sik3 led to no increases in tumor burden over loss of Kras alone.

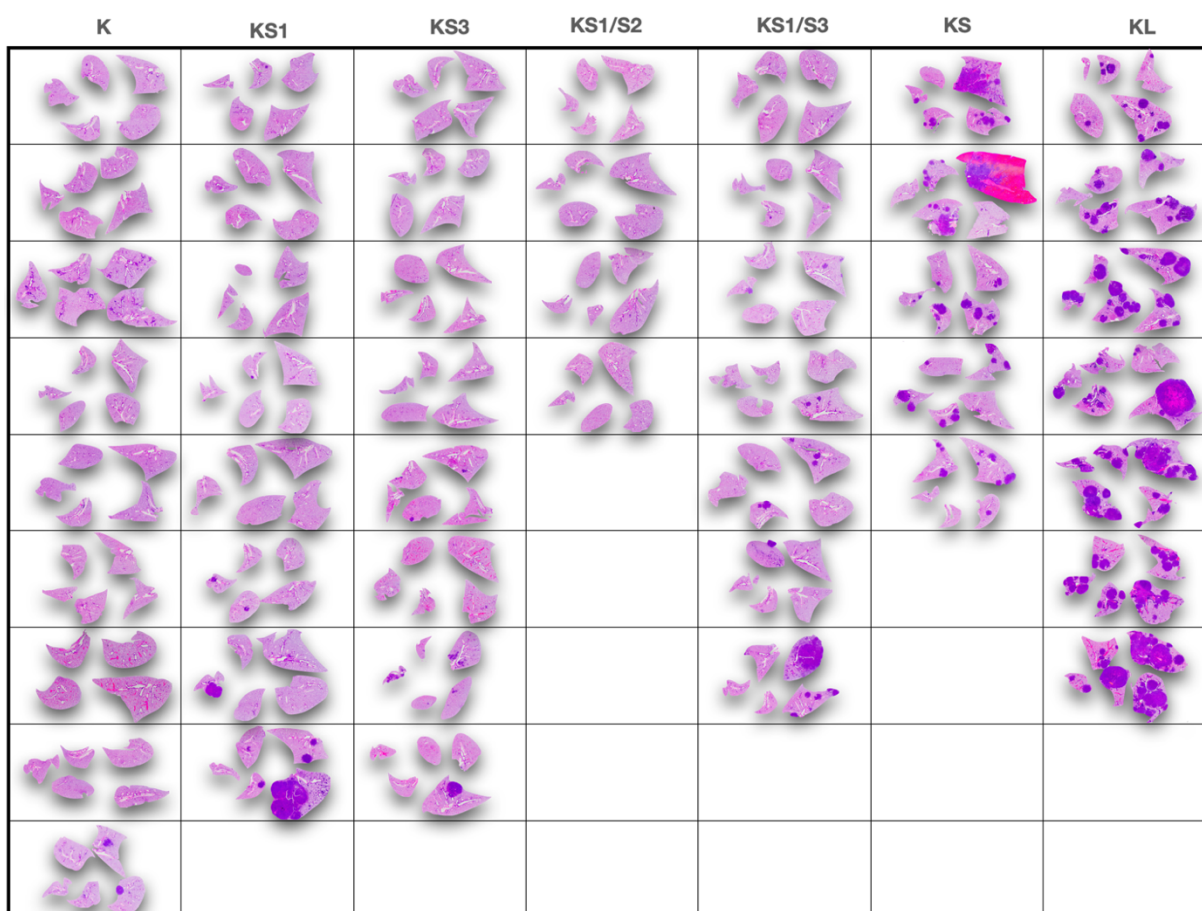


Figure 3. 5: H&E histological sections of early group reveals how loss of all three Sik kinases enhance tumor growth and is the only Sik model that approaches KL model.

H&E sections of mice of all genetic models transduced with lenti-cre. Mice harboring tumors were collected at 12-weeks post cre and lungs paraffin embedded for histological analysis. Tumor burden is observably low in K, KS1, and KS3 models, however, a single mouse outlier in KS1 and KS3 grew a large tumor. KS1/2 lungs have no observable tumor formation, matching what was observed by BLI (Figure 3.4). Surprisingly, the KS model had the highest observable tumor burden of the Sik-null models, and most closely approached the KL model.

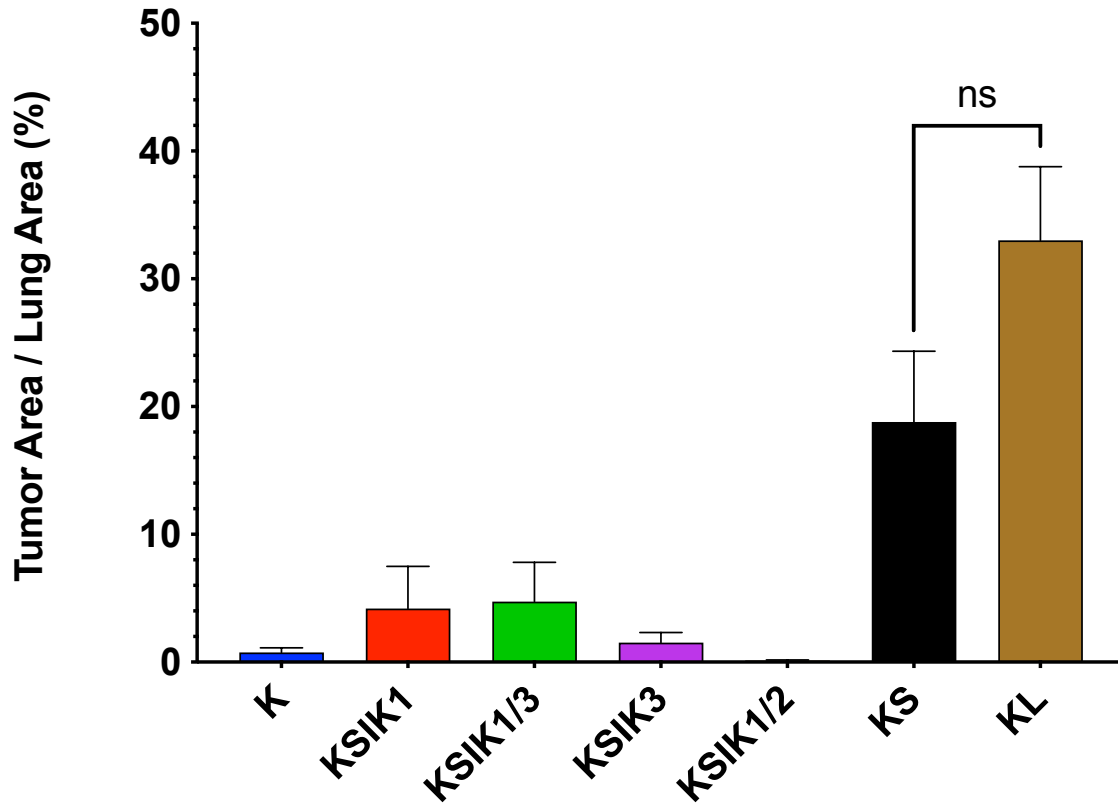


Figure 3. 6: Tumor area of the lung in KS model closely approached the KL model.

Data is presented as the average tumor area (mm²) divided by the total lung per mouse section. Error bars are displayed $\pm$ SEM. Tumor area of the lung was calculated by dividing the tumor area by the total lung area of the early group. Analysis of the histological section revealed that the KS model had a tumor burden most closely matching loss of Lkb1. Tumor areas of all other models remained less than 5%, while the KS and KL models reach >20% tumor area.

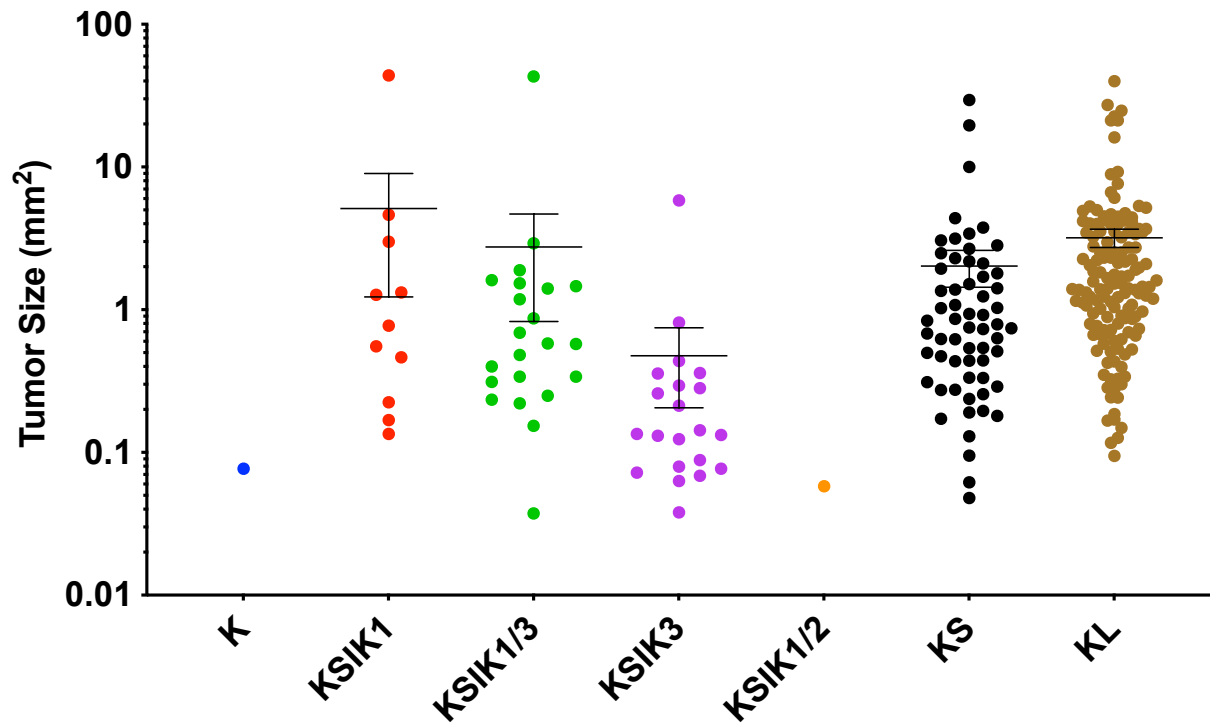


Figure 3. 7: Tumor sizes are similar after 12 weeks of tumor growth in all models with Sik1 inactivity.

Quantification of tumor sizes (mm²). Each dot represents a single tumor. Error bars are displayed $\pm$ SEM. Tumor sizes were calculated by measuring histological cross-sections of early group. Average tumor sizes were very similar in all models which had Sik1 inactivity (except KSIK1/2 in which only 1 small tumor was observed). In previous studies, loss of Sik1 led to a higher proportion of larger tumors within samples, potentially suggesting Sik1 dictates tumor size in these models.

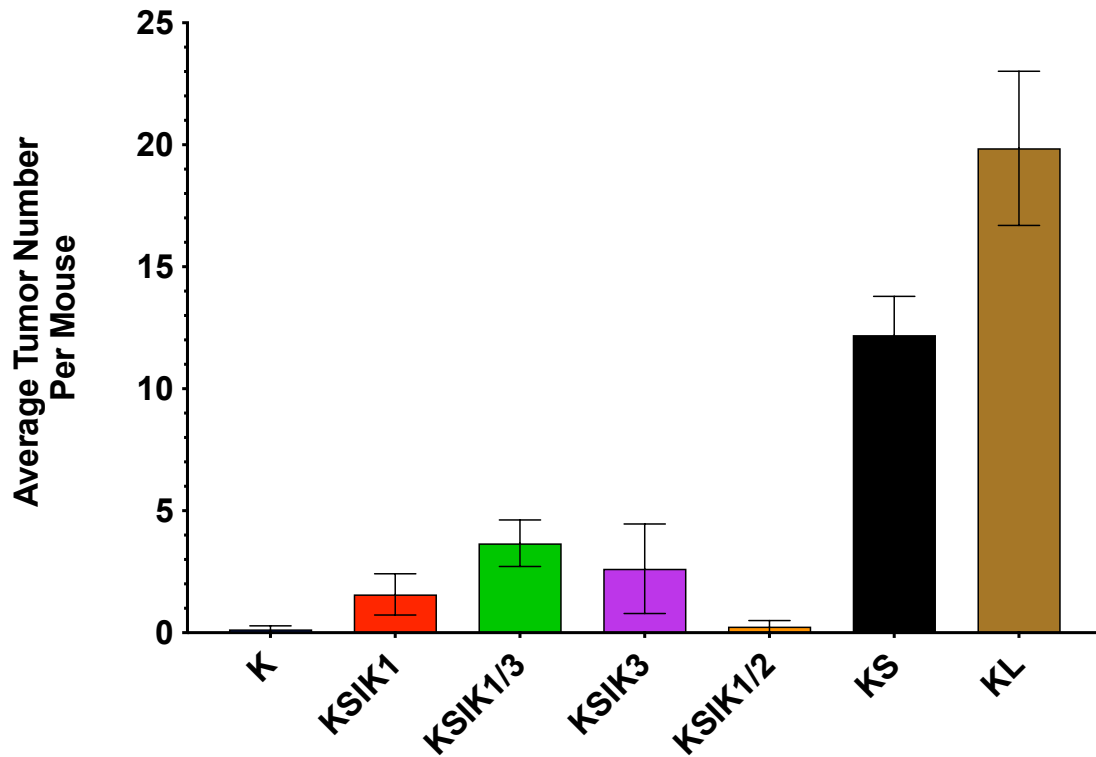


Figure 3. 8: Increases in average tumor number in KS and KL models suggest they have higher ability to initiate tumors.

Data is shown as the average number of tumors counted per each genotype. Error bars are displayed $\pm$ SEM. Tumors were counted in histological cross sections of the early group. All mice received equal viral tumor forming units (TFUs). As a result, an increase in tumors counted in KL and KS models suggest a higher ability to initiate tumors by 12 weeks post-cre. Since tumor sizes were similar across all models lacking Sik1 activity, the main contributor to tumor burden appears to come from the number of initiated tumors.

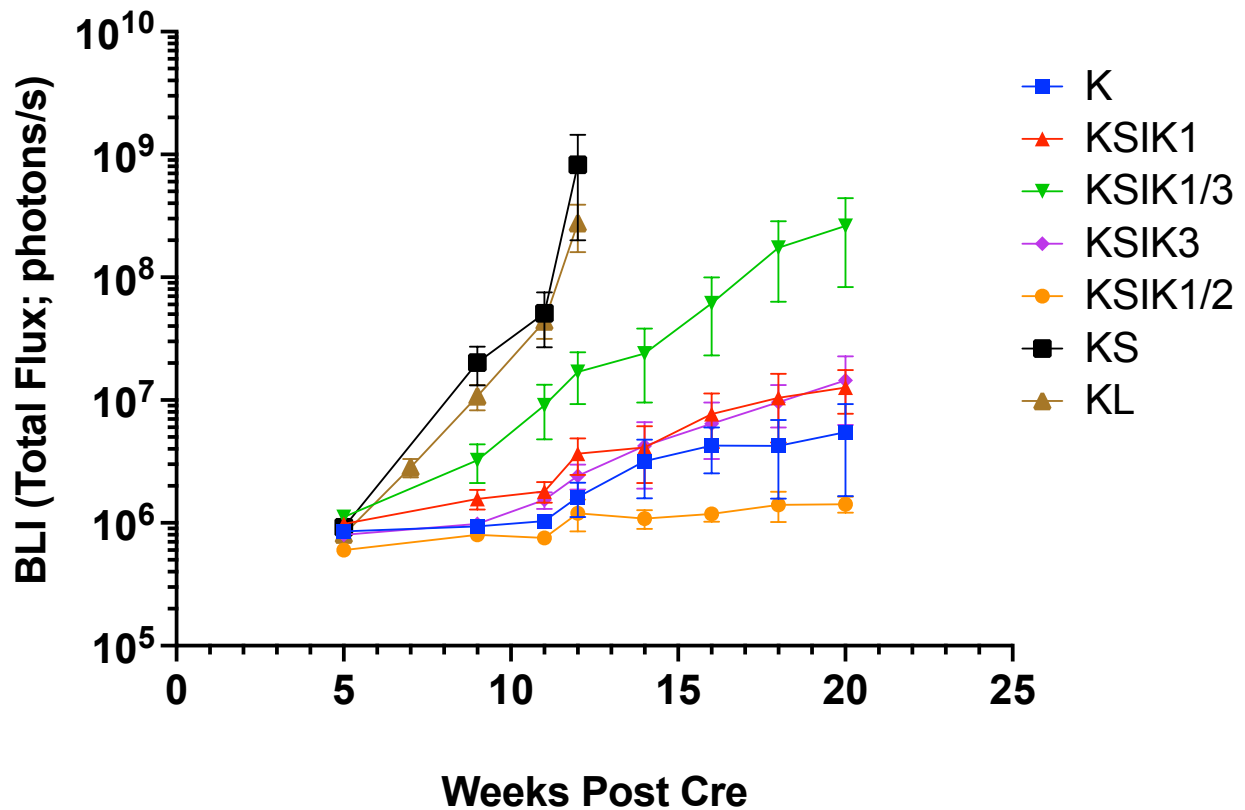


Figure 3. 9: Bioluminescent imaging of late group shows that the KS1/3 model has an intermediate phenotype.

BLI data is displayed as the average photon flux (photon/second) per genotype at each timepoint. Mice were imaged biweekly. BLI imaging for the late group continued for an additional 8 weeks after the early group was collected (20 weeks total). Even after 20 weeks, the KS1/2 model failed to initiate tumors. No differences in BLI was observed comparing K, KS1, or KS3. However, co-deletion of Sik1 and Sik3 continued to grow faster than the single-allele or Kras model. Interestingly, it took an additional 8 weeks for the KS1/3 model to reach the growth of the KS or KL models.

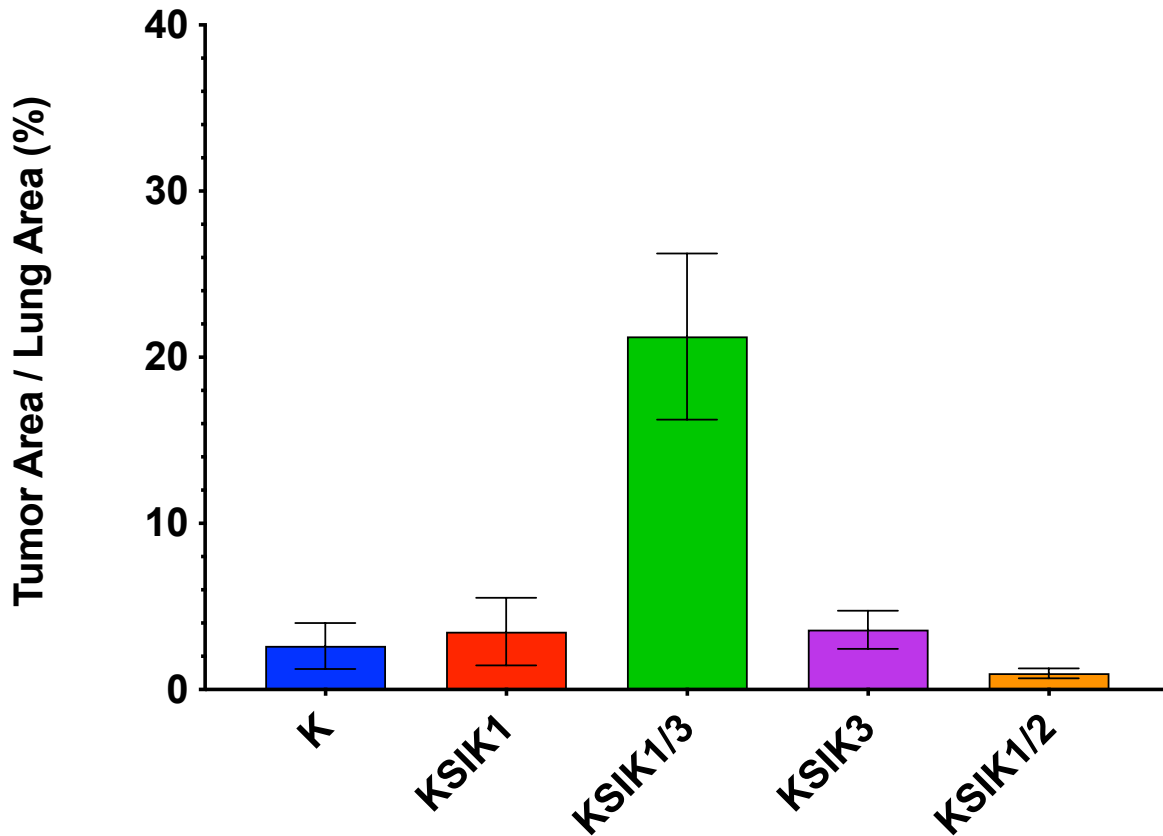


Figure 3. 10: Percent tumor area in the KS1/3 models significantly increased compared to all other models 20 weeks post-cre.

Data is presented as the average tumor area (mm^2) divided by the total lung per mouse section. Error bars are displayed $\pm$ SEM. After 20 weeks post-cre, lung histological cross sections showed that KS1/3 mice had significantly higher tumor burdens (~20%). Despite growing for 20 weeks, all other models had an average of ~3% tumor area. Similar to the early group, the KSik1/2 model had lower burdens compared to the Kras model.

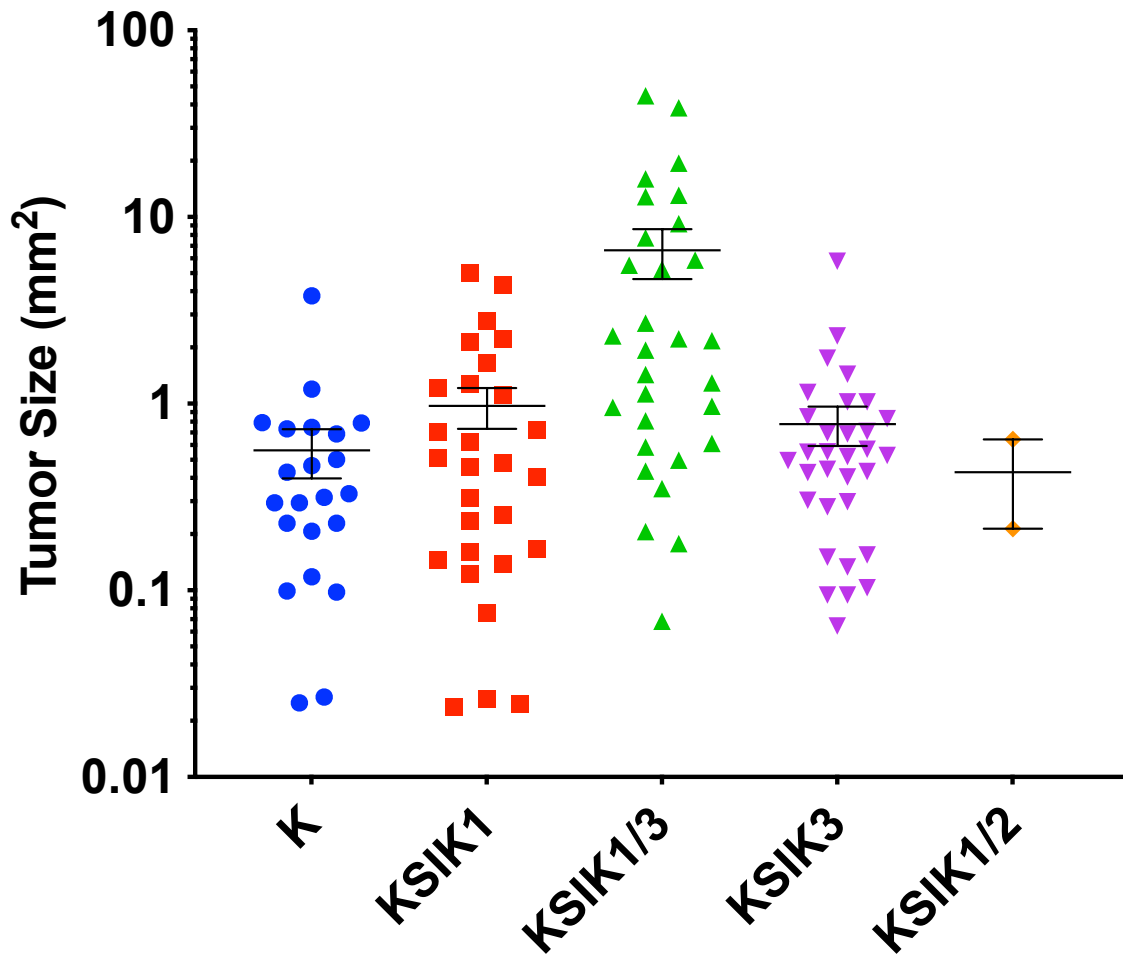


Figure 3. 11: Tumors sizes in the KSik1/3 models were significantly larger compared all other models 20 weeks post-cre.

Quantification of tumor sizes (mm²). Each dot represents a single tumor. Error bars are displayed $\pm$ SEM. Analysis of histological cross sections showed that tumors lacking Sik1 and Sik3 (KSik1/3) grew significantly larger than any other model. Suggesting that in addition to controlling tumor initiation, also control processes that dictate tumor size. Tumors from KSik1 and KSik3 sections were only slightly larger than Kras tumors. In the KSik1/2 mice, only 2 tumors were detected. Unlike the early group, in which all models with inactivated Sik1 had similar tumor sizes, Sik1/3 tumors grew larger. This suggests that tumors in K, KSik1, KSik3 and KSik1/3 grow at a similar rate up to 12 weeks post-cre, with higher initiation of the KSik1/3 tumors. Beyond 12 weeks, loss of Sik1 and Sik3 enhances tumor growth beyond that observed in KSik1 and KSik3 alone.

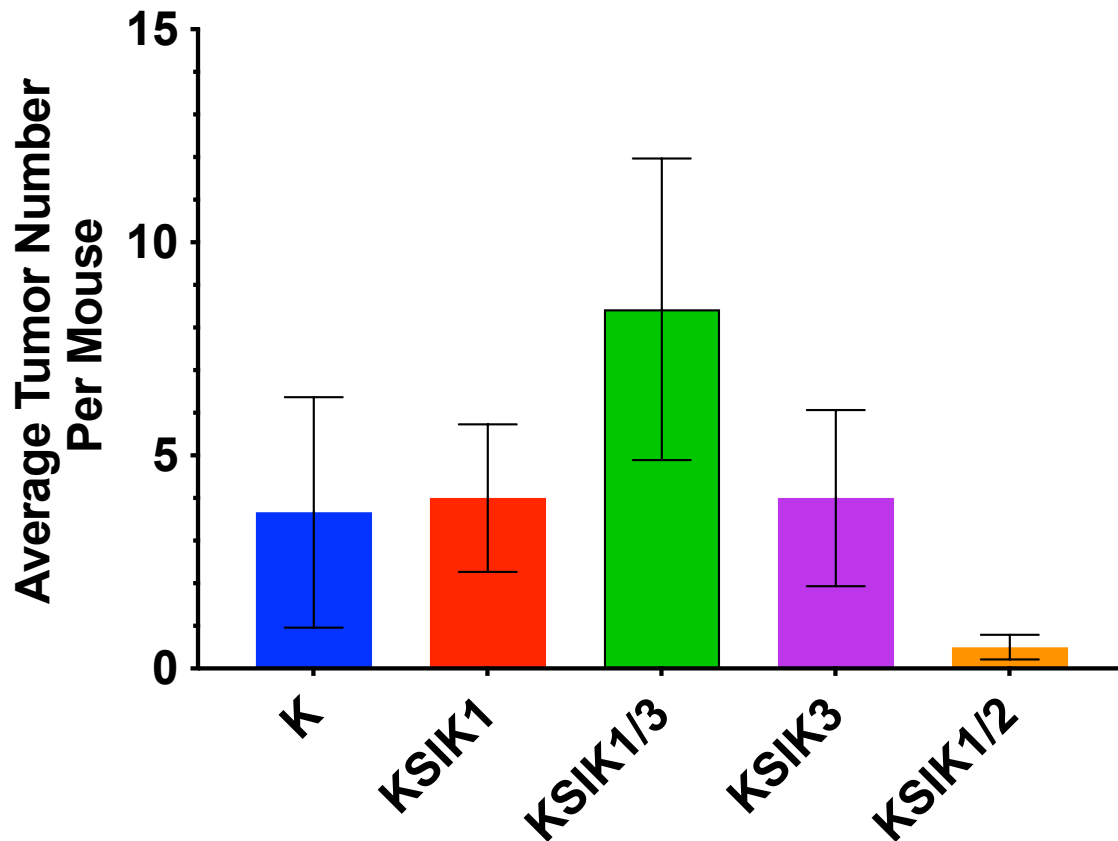


Figure 3. 12: Conditional Sik-floxed models show no differences in tumor number 20-weeks post cre.

Data shown as the average number of tumors counted per each genotype. Error bars are displayed $\pm$ SEM. No statistical differences was observed. Counting of tumors from histological cross sections showed similar tumor numbers in all models in the late group. This suggests that all models have very similar rates of tumor initiation. Unlike the differences observed in the early group analysis, which showed higher tumor numbers in the KS and KL models, similar tumor numbers here highlights the ability for the KSIK1/3 to grow faster and larger when assessing tumor burdens (Figure 3.9 and 3.10).

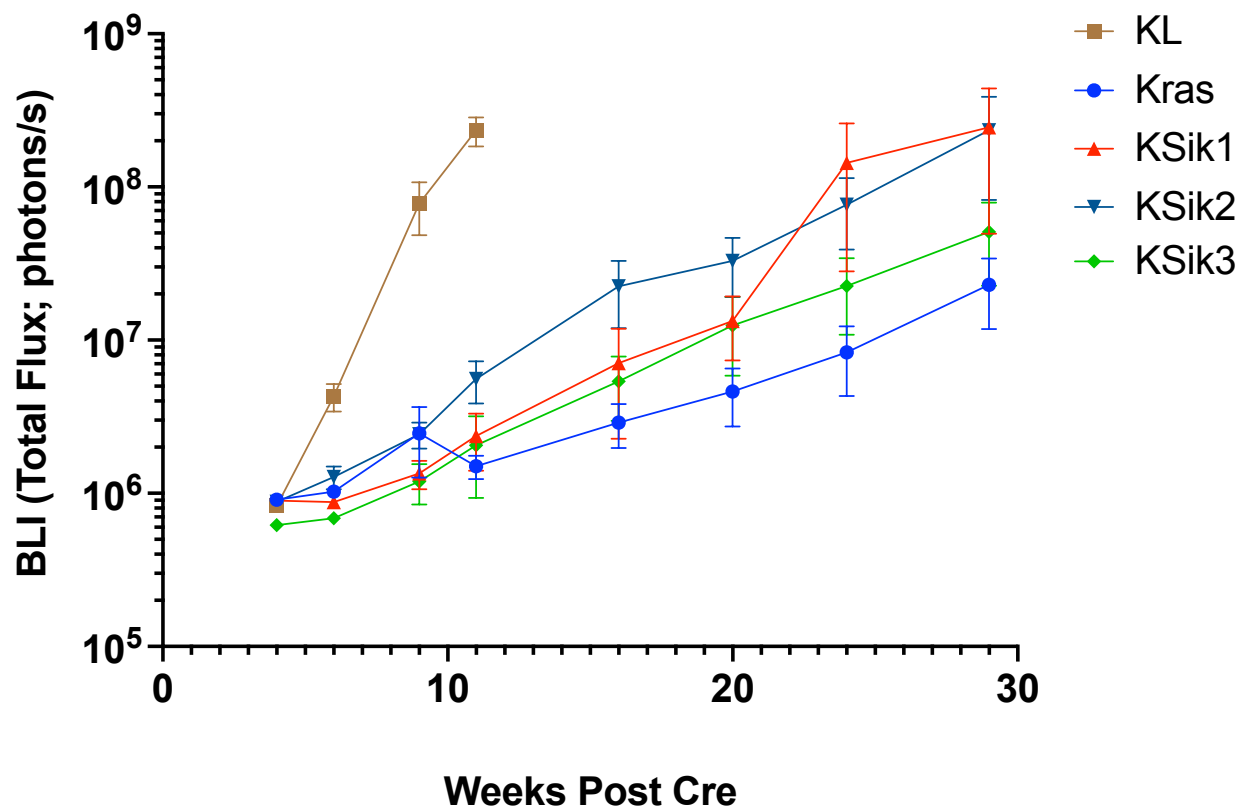


Figure 3. 13: Longitudinal study of single floxed conditional Sik KO models take 30 weeks reach clinical endpoint.

BLI data collected at each respective timepoint consists of the average BLI of all mice within that genotype. Mice were imaged bi-weekly beginning at 4 weeks post-cre. Experimental endpoint is defined as mice which reach BLI=10⁹. Single floxed Sik conditional KO models were analyzed in a longitudinal study. As expected, mice harboring KL mutations reached endpoint by 12 weeks post-cre. At that timepoint, only the KSik2 model showed a small increase in BLI signal as compared to K. After 30 weeks, conditional floxed models began to approach the BLI level of Lkb1 at 12 weeks. No differences in BLI was observed in KSik3 mice. A slight increase was observed in KSik1, potentially mediated by increases in tumor size. Surprisingly, the KSik2 model had a similar BLI to KSik1, despite previous reports that Sik2 loss is detrimental to tumor growth.

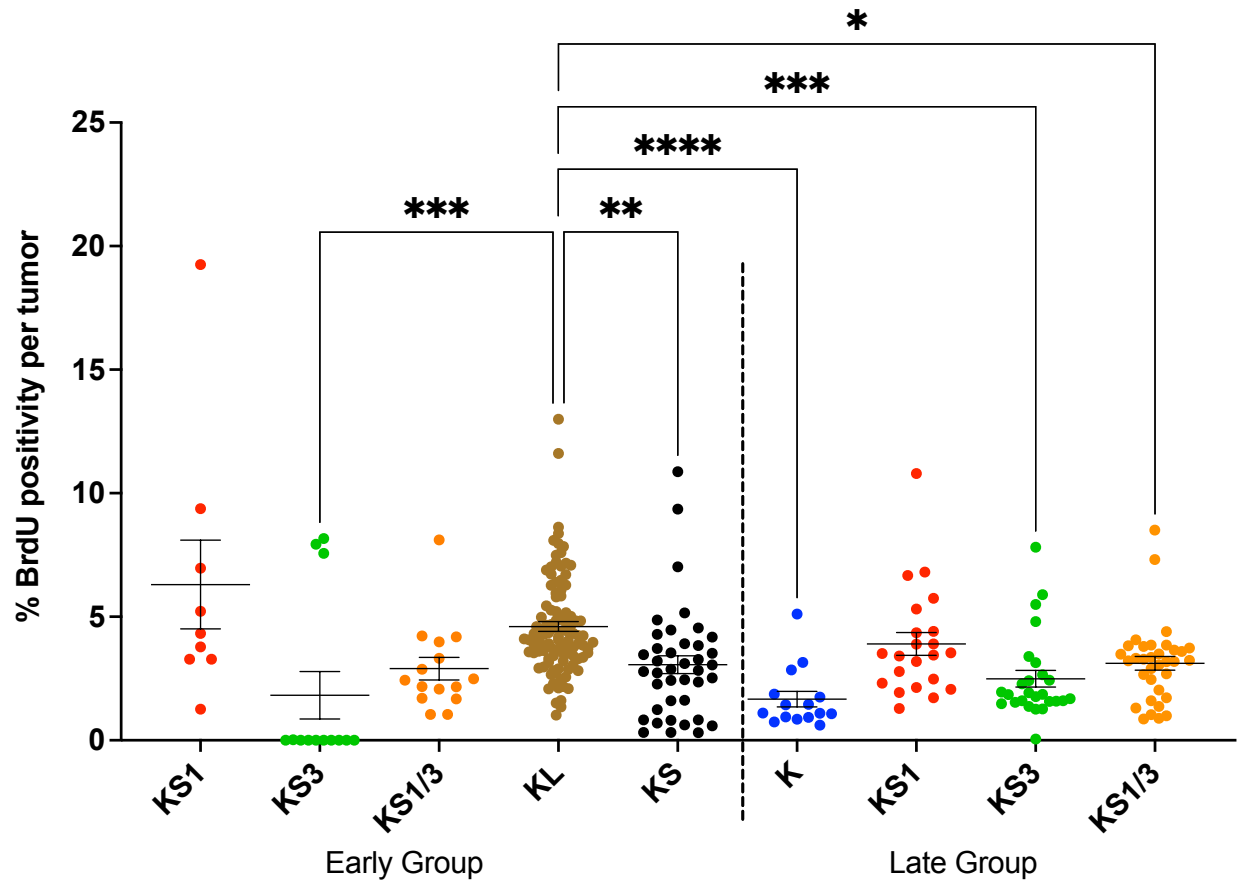


Figure 3. 14: BrdU incorporation is highest in KL tumors despite similar KS tumor sizes and burden.

BrdU positivity in lung tumors from all genetic models in early and late groups. Each data point is an individual tumor, presented as the percent of cells positive for BrdU out of the total cells in each tumor. Error bars are displayed $\pm$ SEM. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). Higher percentages were observed in KL tumors as compared to all models in the early group except the KSik1 model, which had high variability. Only 3 KSik3 tumors had high detectable BrdU positivity. Analysis of the late group showed that the KSik1, KSik3, and KSik1/3 tumors had higher average BrdU positivity compared to Kras alone.

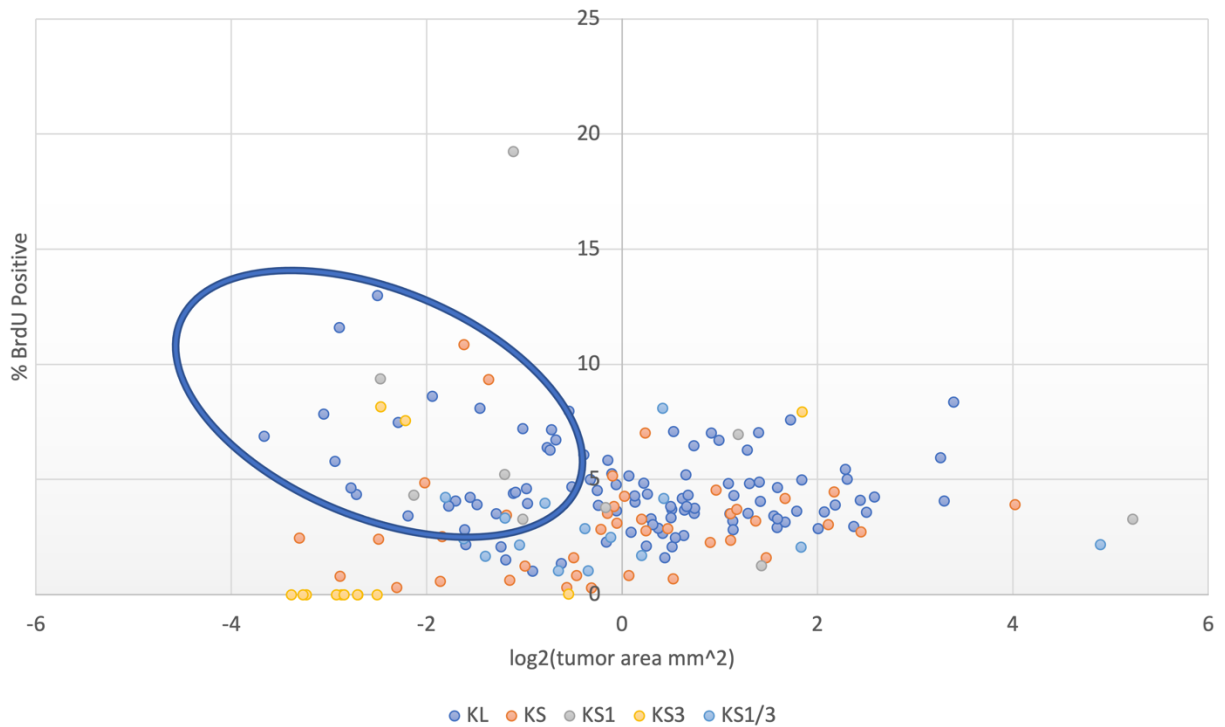


Figure 3. 15: BrdU incorporation is highest among the smallest KL tumors from the early group.

BrdU positivity in lung tumors in genetic models from the early group (12 weeks post-cre). Each data point denotes an individual tumor assessed for %BrdU vs $\log_2(\text{tumor area mm}^2)$ and colors designate genotype. Interestingly, all small KL tumors had significantly higher BrdU incorporation, and larger KL tumors had lower levels compared to their smaller counterparts. Tumors from KS model had consistent BrdU levels across all sizes.

Neutrophil elastase

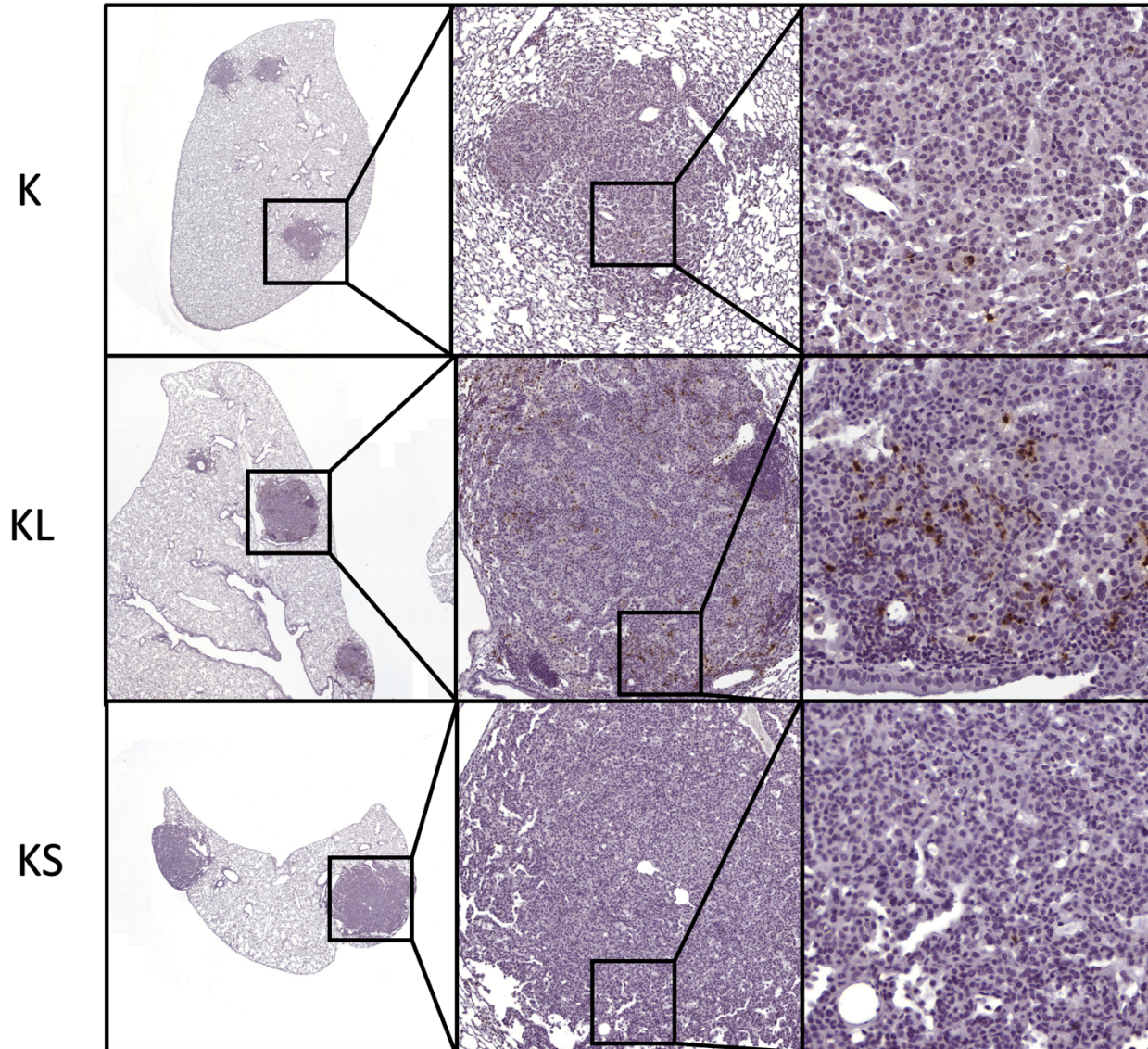


Figure 3. 16: Immunohistochemistry for neutrophil specific, neutrophil elastase, marker shows an increase in tumor associated neutrophils in KL but not KS models.

Data shows immunohistochemistry images of K, KS, and KL mouse lungs stained for neutrophil elastase (cell signaling technologies cat.90120; 1:400 dilution). Images generated at 15x, 40x, and 400x magnification. Increases in tumor associated neutrophils was observed in KL mice, a phenotype previously reported. These increases were not observed in the KS model which had no detectable neutrophils.

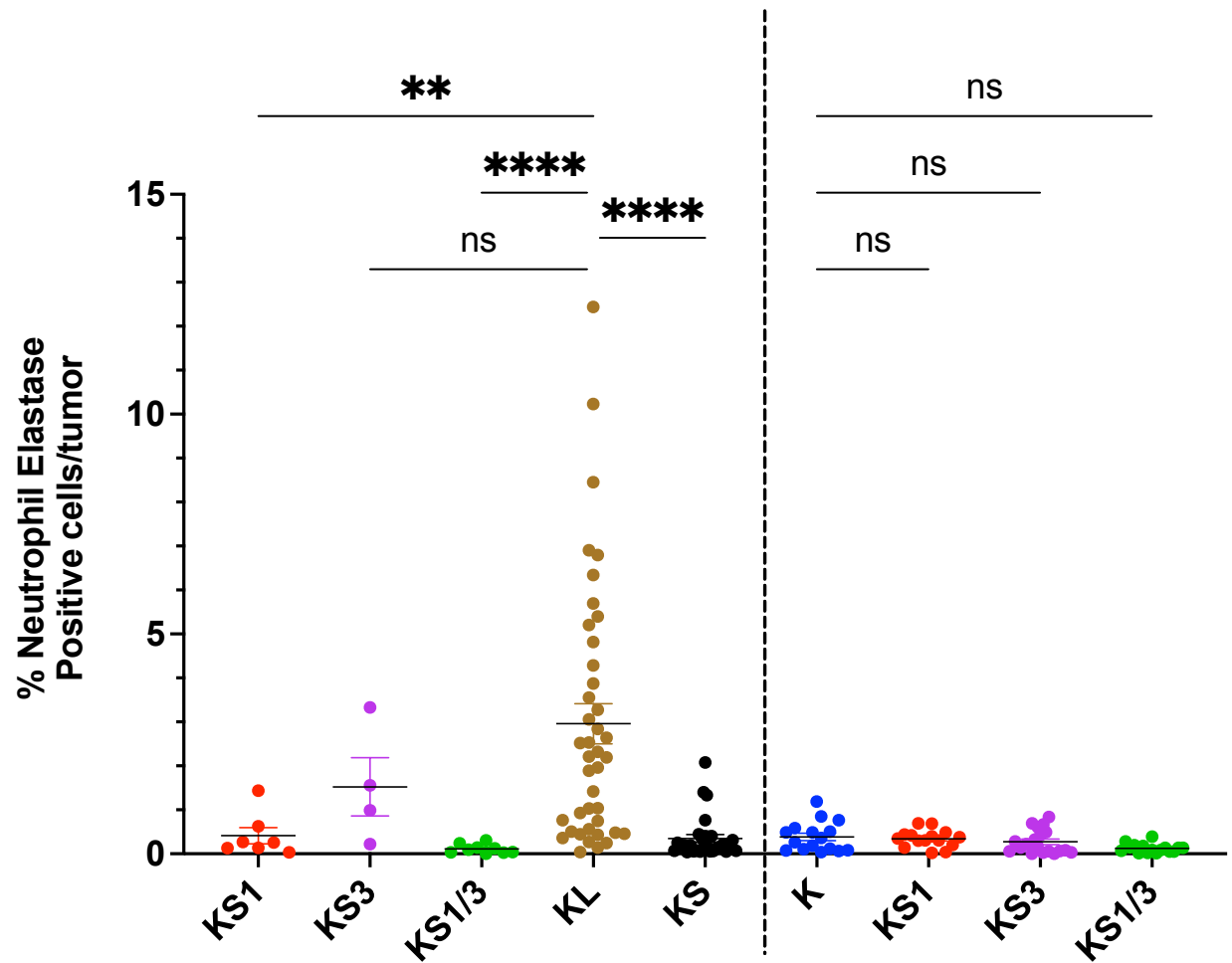


Figure 3. 17: KL tumors have high infiltration of neutrophils and is not mediated through the Siks.

Data presented as the number of neutrophils detected divided by the number of cells in the tumor (%neutrophil elastase). Each dot represents a single tumor in each genotype. Error bars are displayed $\pm$ SEM. (** $P < 0.01$; **** $P < 0.0001$). Neutrophil infiltration seemed to be completely dependent on loss of Lkb1. Surprisingly, KS1/3 tumors had little to no tumor infiltrating neutrophils, as has been previously reported. It appears that the Sik kinases are required for neutrophil recruitment.

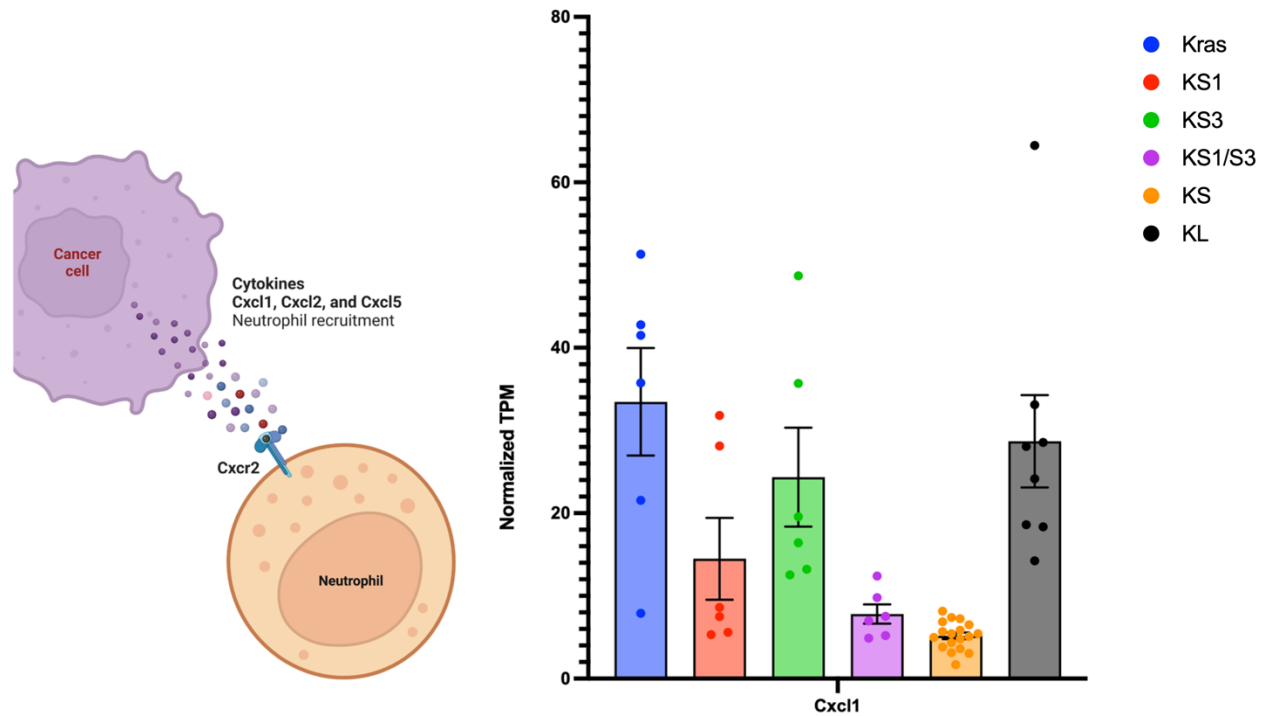


Figure 3. 18: Expression of the Cxcl1/KC cytokine, the predominant neutrophil recruiting cytokine, is reduced after loss of the Sik kinases.

Bar graphs represent the average normalized transcripts per million (TPM) of Cxcl1 (aka KC) from mouse lung tumors. Each dot represents the Cxcl1 TPM of an individual tumor analyzed by RNAseq. Error bars are displayed $\pm$ SEM. Expression of Cxcl1 appears to be Sik-dependent, such that loss of the Sik kinases, leads to decreases in Cxcl1 transcripts. Cxcl1 appears to be mostly Sik1 dependent, however loss of all three Sik kinases has the lower expression of Cxcl1, and suggests Sik2 and Sik3 might compensate for the loss of Sik1.

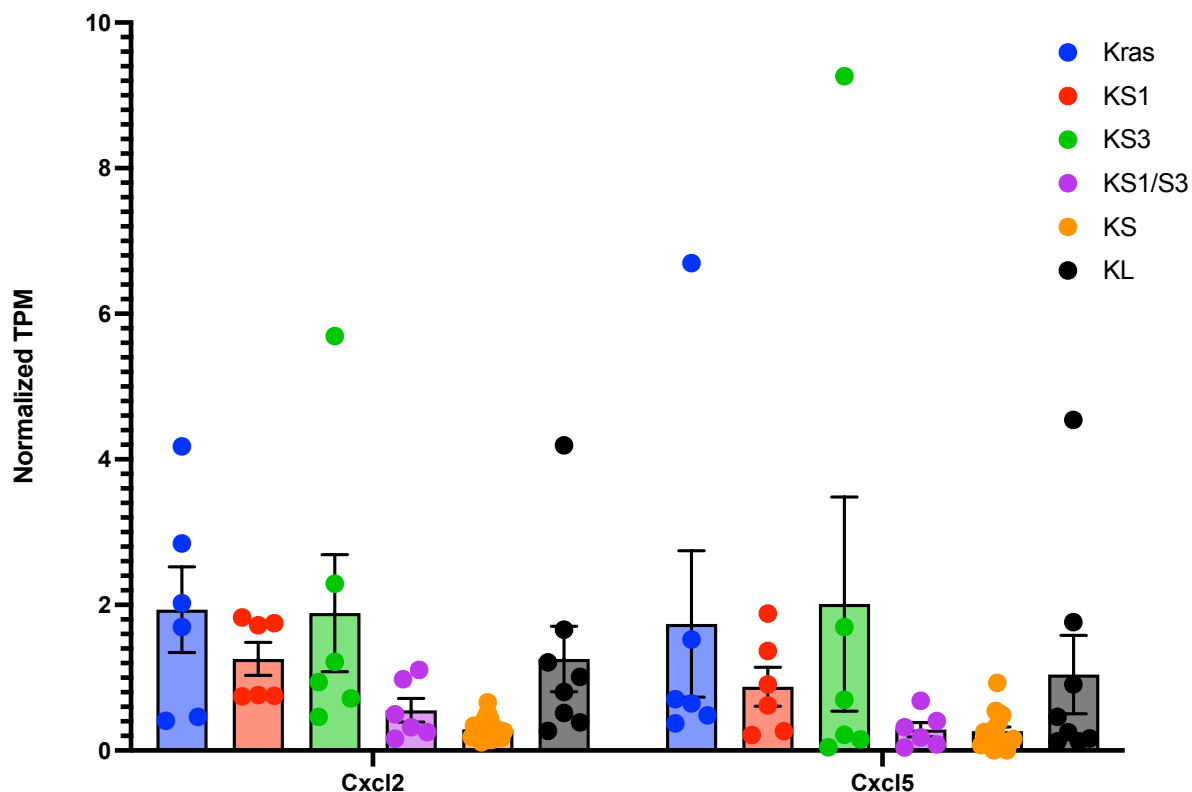


Figure 3. 19: Neutrophil recruiting cytokines, Cxcl2 (Mip2) and Cxcl5, expression is decreased in a Sik dependent manner.

Bar graphs represent the average normalized transcripts per million (TPM) of Cxcl2 (aka Mip2) or Cxcl5 from mouse lung tumors. Each dot represents the Cxcl1 or Cxcl5 TPM of an individual tumor analyzed by RNAseq. Error bars are displayed $\pm$ SEM. Similar to Cxcl1, expression of Cxcl2 and Cxcl5 is drastically reduced upon loss of the Sik kinases, with the strong effects observed in the KSik1/3 or KS models.

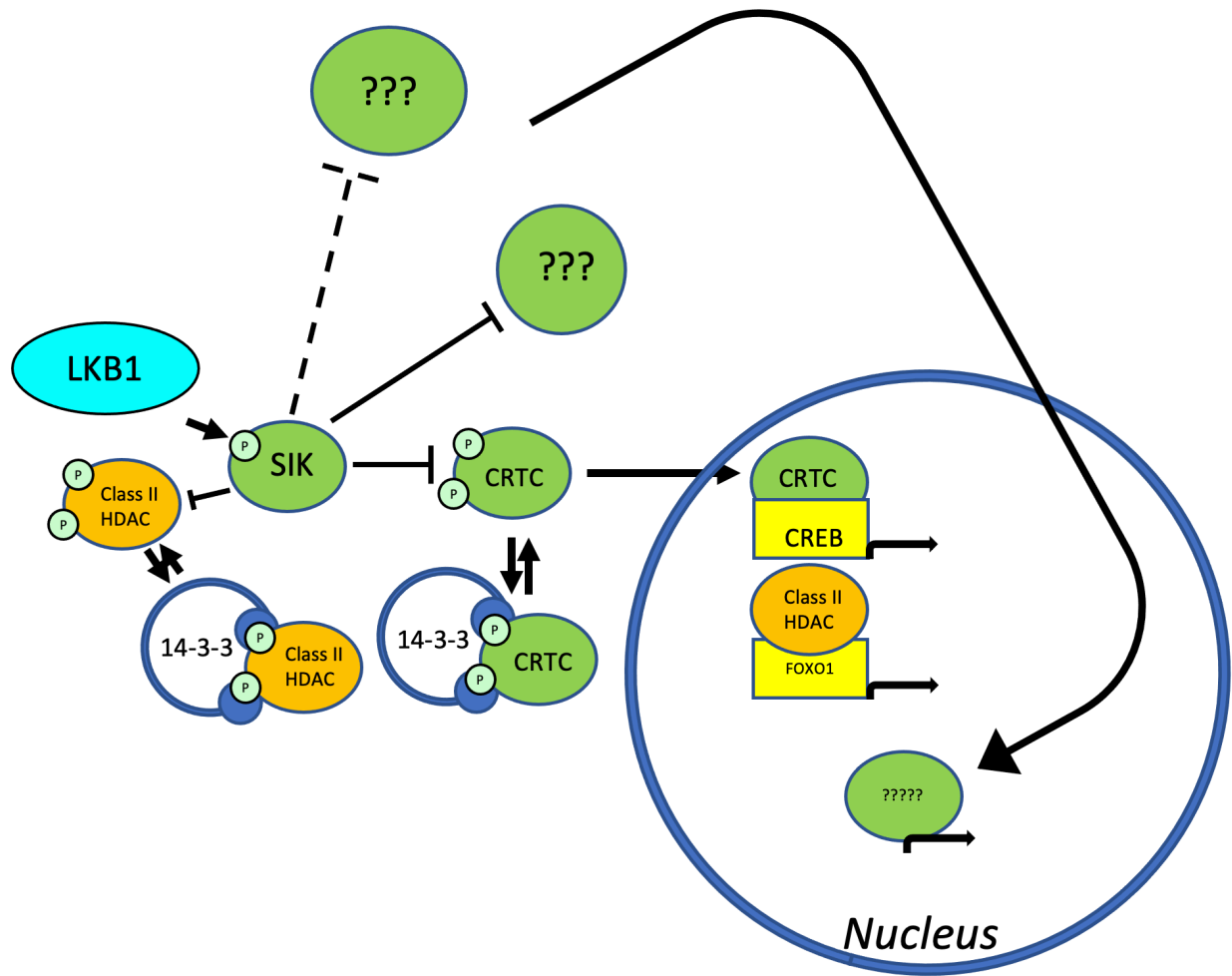


Figure 3. 20: Model showing how the Lkb1-Sik axis regulates transcription by inhibiting two well studied transcriptional co-activators.

Phosphorylation and activation of the salt-inducible kinases leads to the downstream phosphorylation and inhibition of the Crtc family and the class-IIa Hdacs (Hdac4, Hdac5, and Hdac7 in mouse lung). Phosphorylation leads to 14-3-3 binding, and cytosolic sequestration. Loss or inhibition of the Lkb1-Sik axis leads to Crtc and class-II Hdac nuclear translocation where they can express their target genes. It remains to be determined if the Sik kinases, either through direct phosphorylation or through an alternative pathway, regulate other proteins 14-3-3 binding and activity.

	Genes up (FDR 0.5, FC1.5)	Genes Down (FDR 0.5, FC1.5)
DE_Tumor_genotype_KSIK1_vs_KRAS	202	220
DE_Tumor_genotype_KSIK3_vs_KRAS	73	64
DE_Tumor_genotype_KSIK1_KSIK3_vs_KRAS	715	825
DE_Tumor_genotype_KS123_vs_KRAS	1757	1941
DE_Tumor_genotype_KL_vs_KRAS	1116	1049
DE_Tumor_genotype_KL_vs_KS123	1942	1396

Overlap of Genes Up-Regulated

Overlap of Genes Down-Regulated

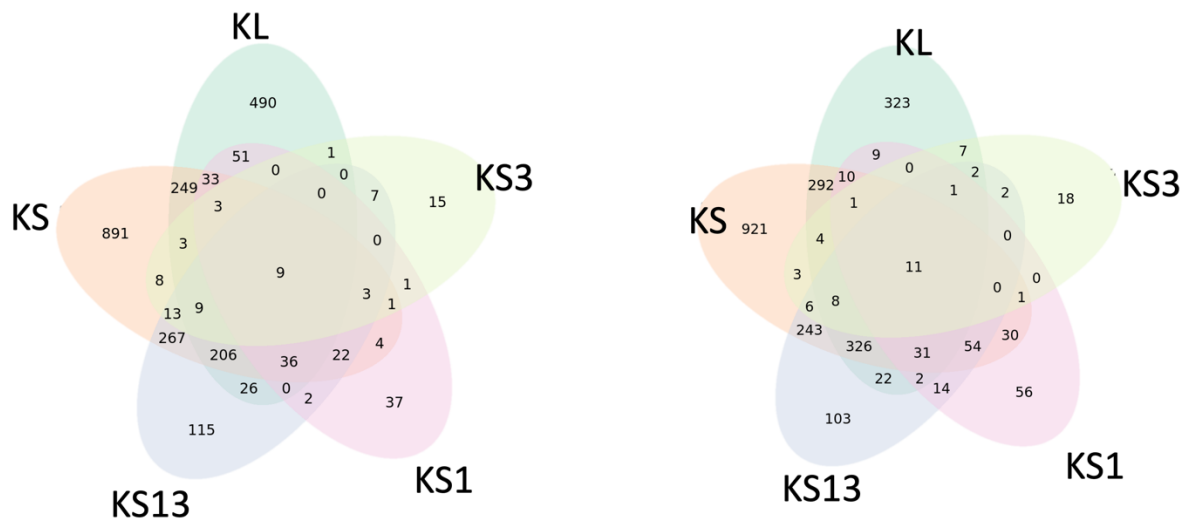


Figure 3. 21: Venn diagrams of up or down regulated differentially expressed genes from Sik genetic models.

Data is presented as the number of overlapped differentially expressed (DE) genes from tumors between each genotype compared to Kras^{G12D} tumors (FC1.5; FDR0.05). For both up and down regulated genes, loss of all three Sik kinases (KS) led to the most DE genes, followed by KL, KSik1/3, KSik1, and KSik3.

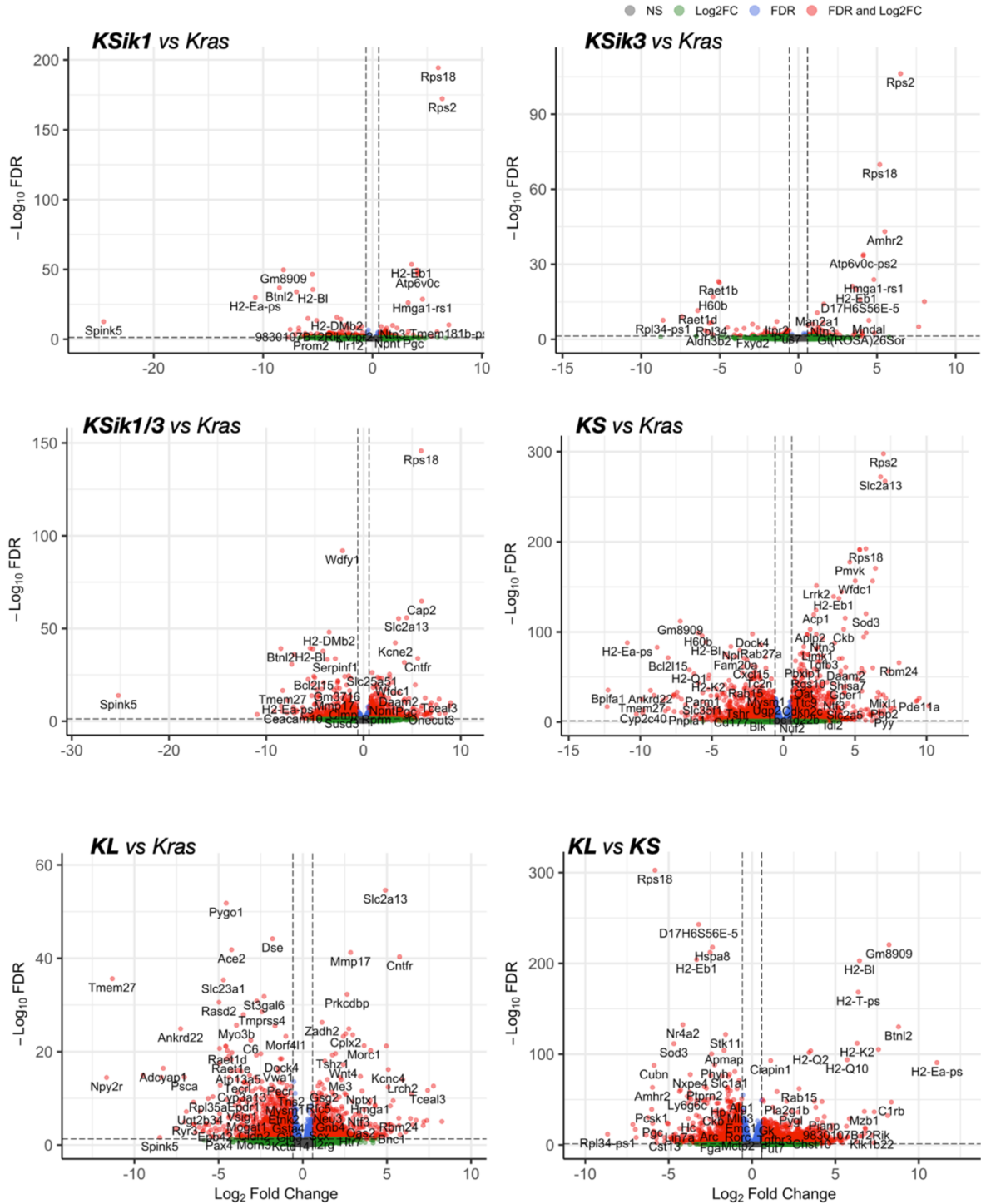


Figure 3. 22: Volcano plots of DE expressed genes in tumors from Sik-genetic models

Each volcano plot shows the DE genes as pairwise comparisons to *Kras* and a comparison of *KL* vs *KS* in the bottom right plot. Genes of interest in red have a $\text{FDR} > 0.05$ and a $\text{FC} > 1.5$.

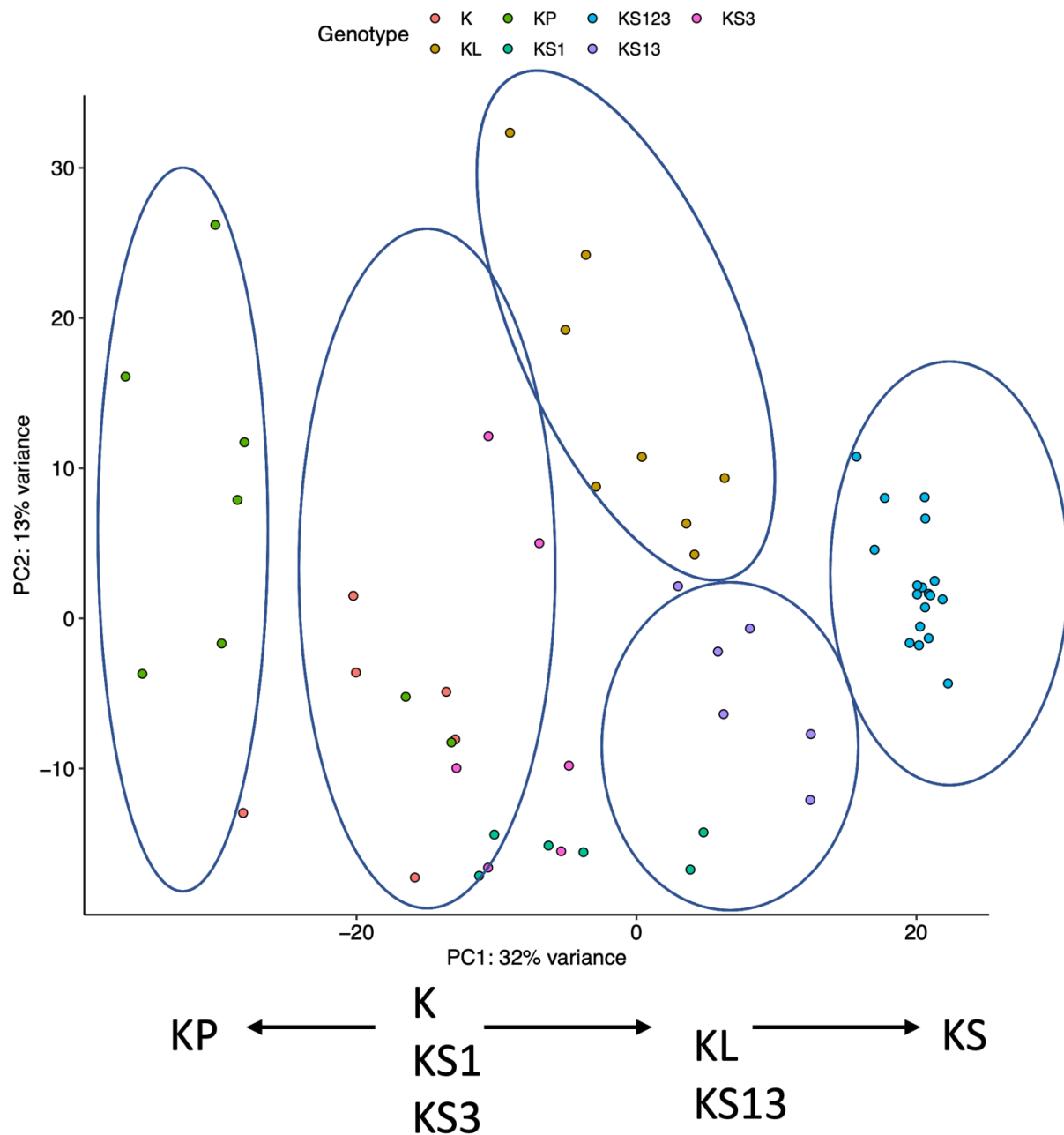


Figure 3. 23: Principal component analysis (PCA) of the top 500 variable RNA-seq genes across tumors collected from the 7 genetic models.

Each dot designates a single tumor analyzed by RNAseq. Most genetic models can be identified by clustering by variance across PC1 (32%) as labeled. Samples with loss of no, or a single Sik, allele cluster closely across PC1. Loss of Sik1 and Sik3 or Lkb1 moves clusters up PC1. And loss of all the Sik kinases clusters even further along PC1. P53 loss (KP) move in the opposite direction as Lkb1.

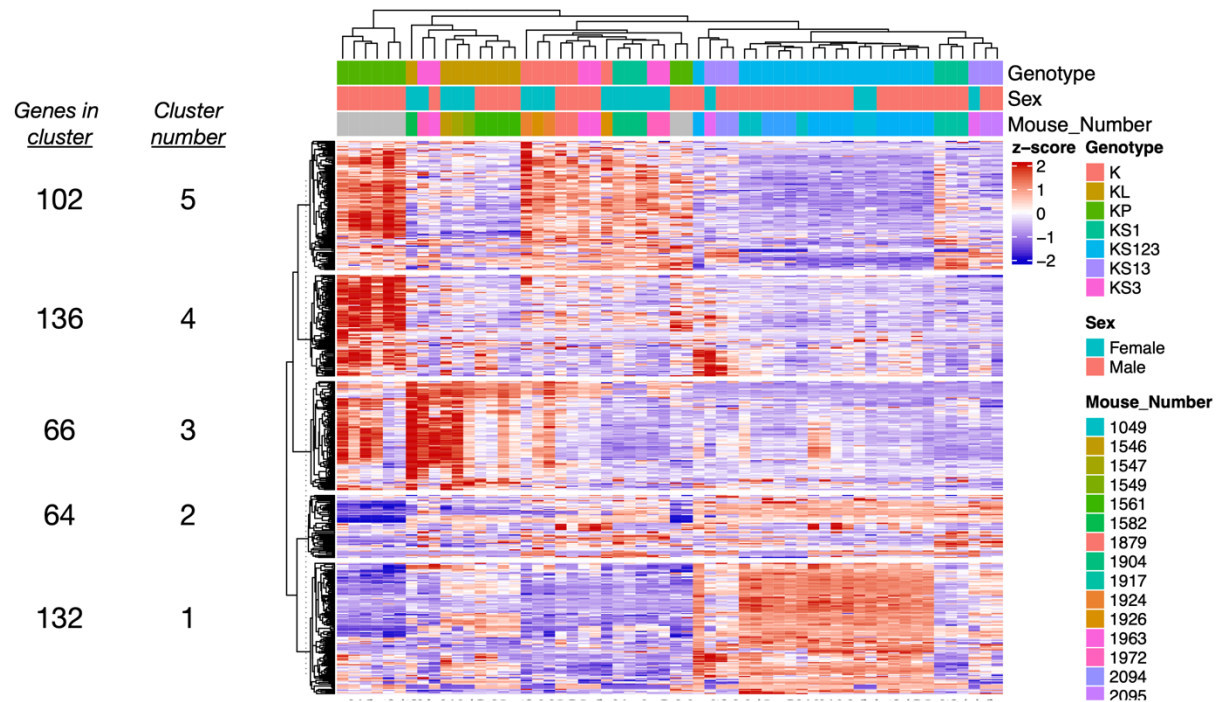


Figure 3. 24: Heat map of top 500 most variable genes from Sik genetically engineered mouse models.

Data presented shows a heat map of the top 500 most variable genes from tumors of Sik genetic models. Each column represents a single tumors RNAseq profile. Tumors collected at experimental endpoint ($BLI \leq 10^9$), of similar size (approximately 2mm in diameter). Heat map presented as the hierarchical clustering and K-means clustering of Z-scores (Fold change [FC] 1.5; False discover rate [FDR] 0.05).

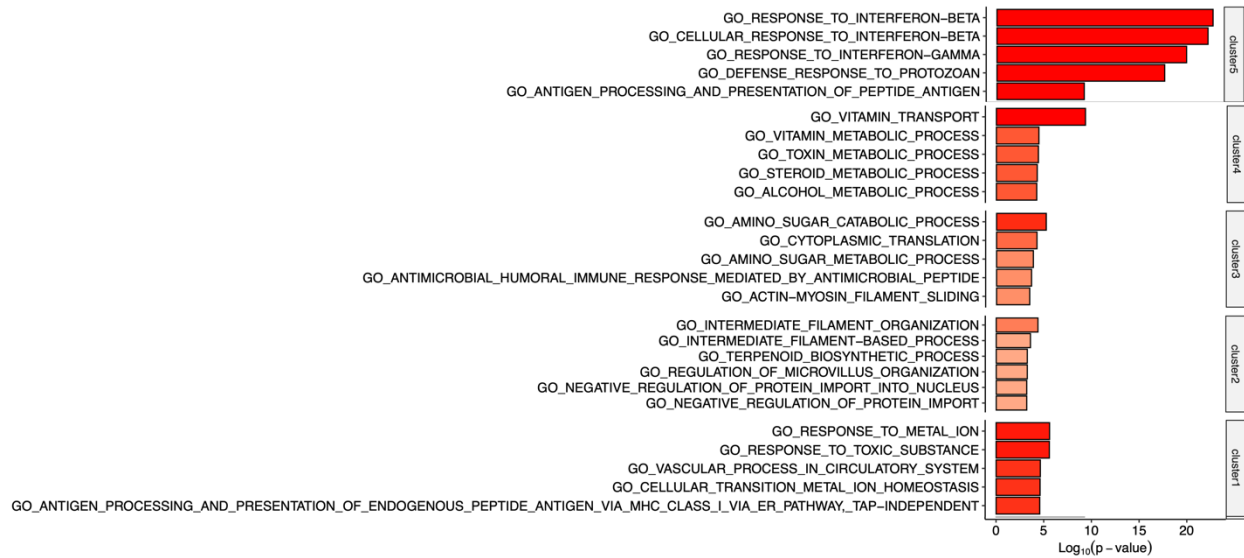


Figure 3. 25: Gene ontology analysis of 5-cluster heat map.

Data shows the top gene ontologies from the 5-cluster heat maps with p-values shown as the $\text{Log}_{10}(\text{p-value})$. Ordered from cluster 5 to cluster 1, from top to bottom, respectively. Genes within each cluster are available in 'Table 3.1'.

Kaufman 16-Gene Classifier

<i>AVPI1</i>	<i>FGA</i>	<i>MUC5AC</i>	<i>SIK1</i>
<i>BAG1</i>	<i>GLCE</i>	<i>PDE4D</i>	<i>TACC2</i>
<i>CPS1</i>	<i>HAL</i>	<i>PTP4A1</i>	<i>TESC</i>
<i>DUSP4</i>	<i>IRS2</i>	<i>RFK</i>	<i>TFF1</i>

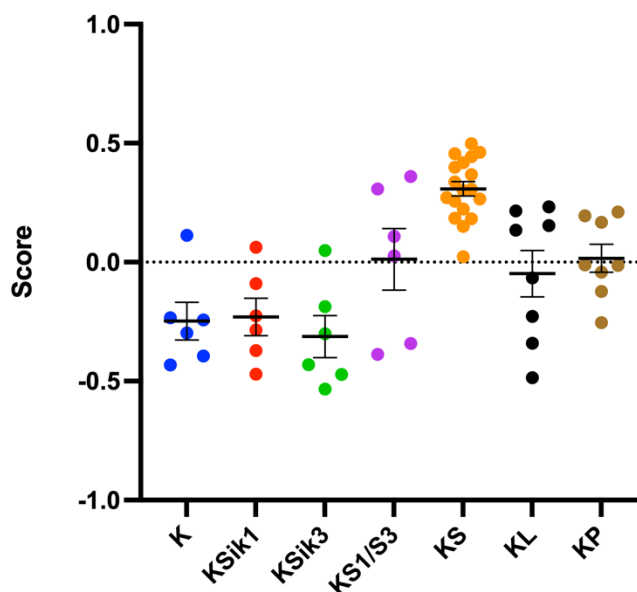


Figure 3. 26: Principal component analysis of Kaufman 16-Gene classifier in tumors from Sik-null genetic models.

Data shown is of a principal component analysis of the Kaufman 16-gene classifier of Sik-null tumors to assess if the Sik tumors had similar transcriptional overlap to KL tumors. Each dot represents a single tumor analyzed of each respective genotype. Tumors are scored on a range from -1 to 1, with -1 having the lowest correlation to the 16-gene classifier, and 1 having the tightest possible correlation to the classifier.

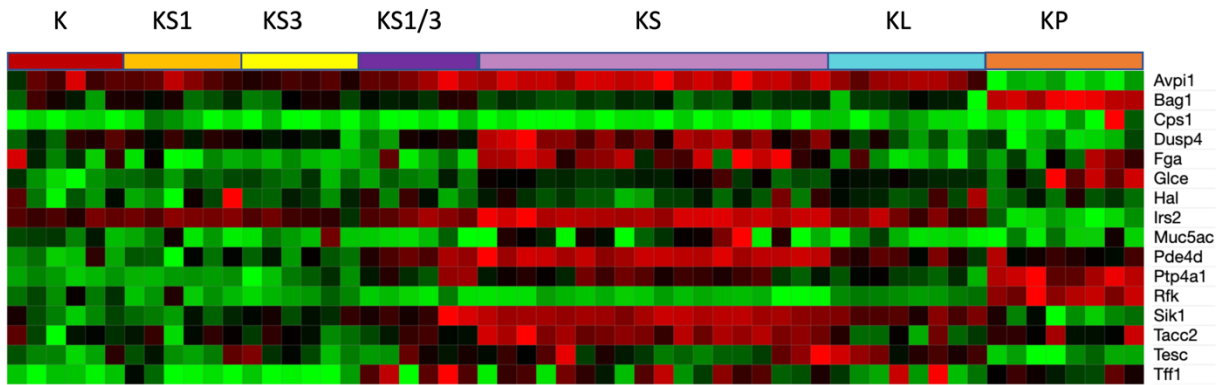


Figure 3. 27: Heat map of Kaufman-16 gene classifier in Sik-null tumor genetic models.

Data presented is a heat map of RNA-seq data. Each column shows an individual tumors expression (Z-score) of each gene per row. Kaufman et al. suggested the following 16-gene classifier accurately predicts LKB1 mutations in lung adenocarcinoma. Here we show that the Sik kinases are able to induce the expression of several of the genes. In fact, the genes under the control of LKB1, generally respond with greater magnitude in the KS model.

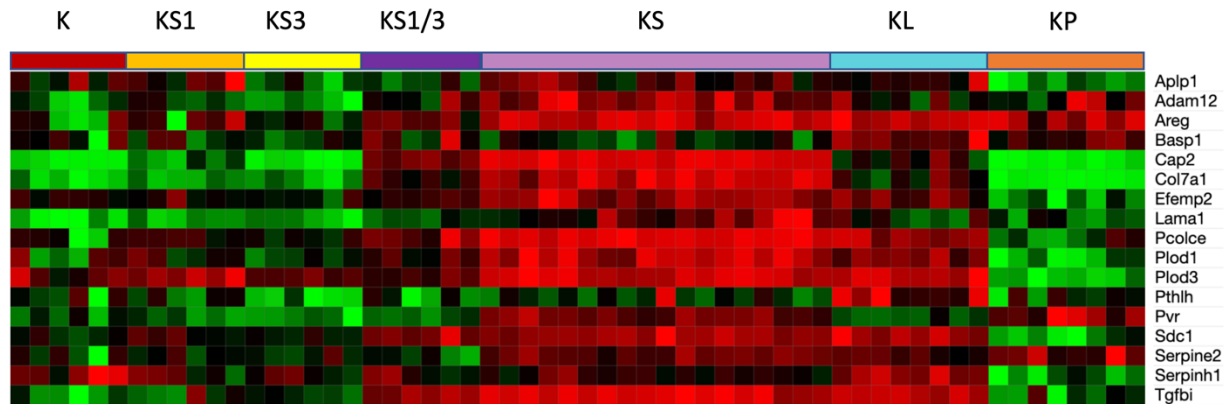


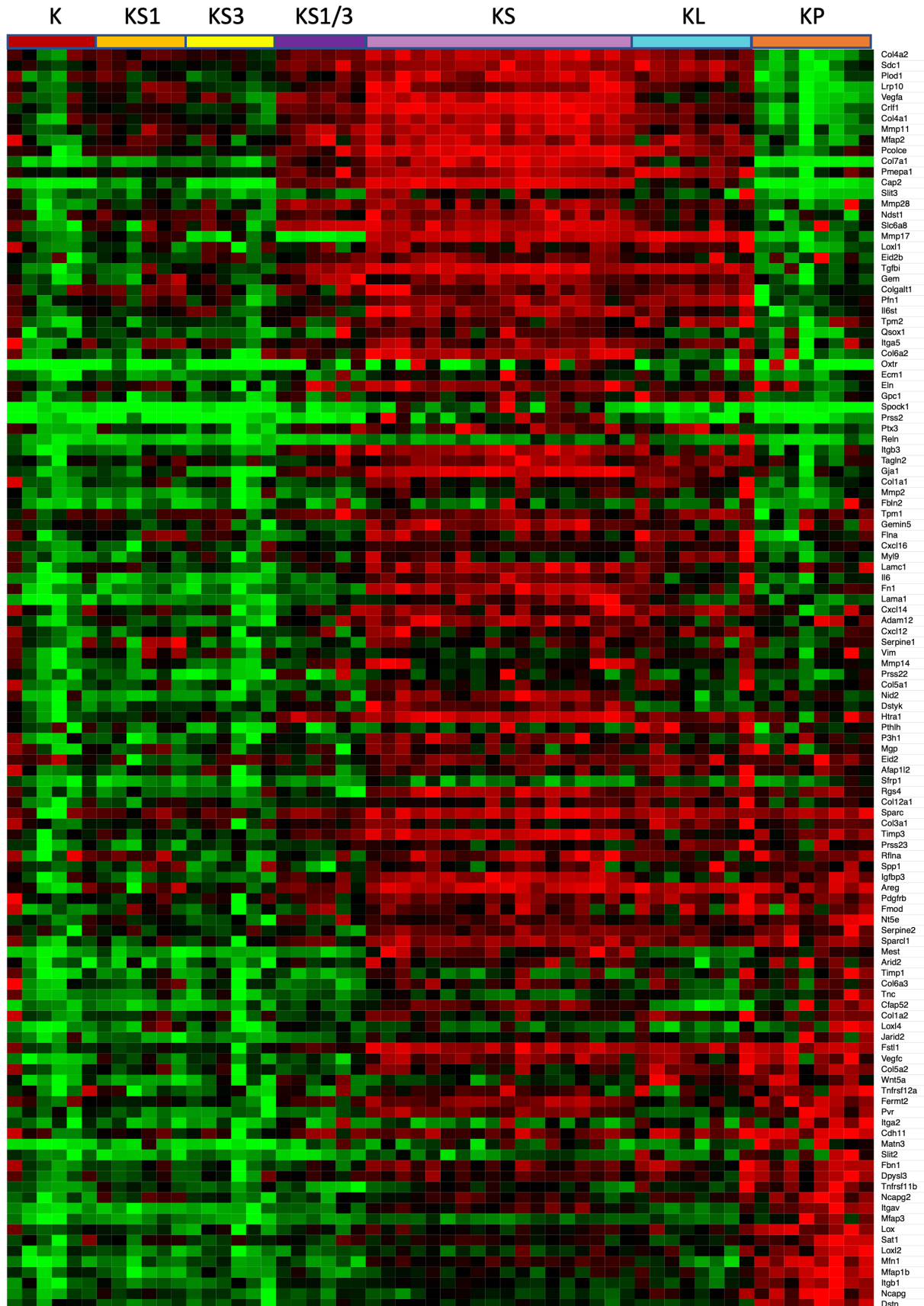
Figure 3. 28: Heat map of epithelial to mesenchymal transition (EMT) gene signature proposed by Hollstein et al¹² to be Lkb1-Sik regulated analyzed in genetic models.

Data presented is a heat map of RNA-seq data. Each column shows an individual tumors expression (Z-score) of each gene per row. Hollstein et al proposed the following 17 genes to involved in epithelial to mesenchymal transition (EMT), and transcripts to be regulated by Lkb1 and the Sik's¹². Indeed, we see that in our models, the genes are Lkb1 dependent. Interestingly, expression in the KS model is higher than in the KL model, suggesting that the Sik kinases may have some activity in the absence of Lkb1. While some of these EMT genes are up-regulated by loss of P53 (KP), the majority of this genes follow as being regulated by Lkb1.

Figure 3. 29: Heat map of genes categorized as hallmark epithelial to mesenchymal transition.

Data presented is a heat map of RNA-seq data. Each column shows an individual tumors expression (Z-score) of each gene per row. Gene set taken from the Broad Institutes Gene Set Enrichment Analysis (GSEA) molecular signatures database¹⁸⁴. Most genes within this gene set are regulated by the Lkb1-Sik axis. Not all genes within the gene set are unique to Lkb1-Sik loss, and appear to increase in expression upon loss of P53, suggesting they are involved in maintaining or promoting tumor growth. However, approximately 1/3 of genes are only up regulated in the KL/KS models.

GSEA Human Gene Set: [HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION]



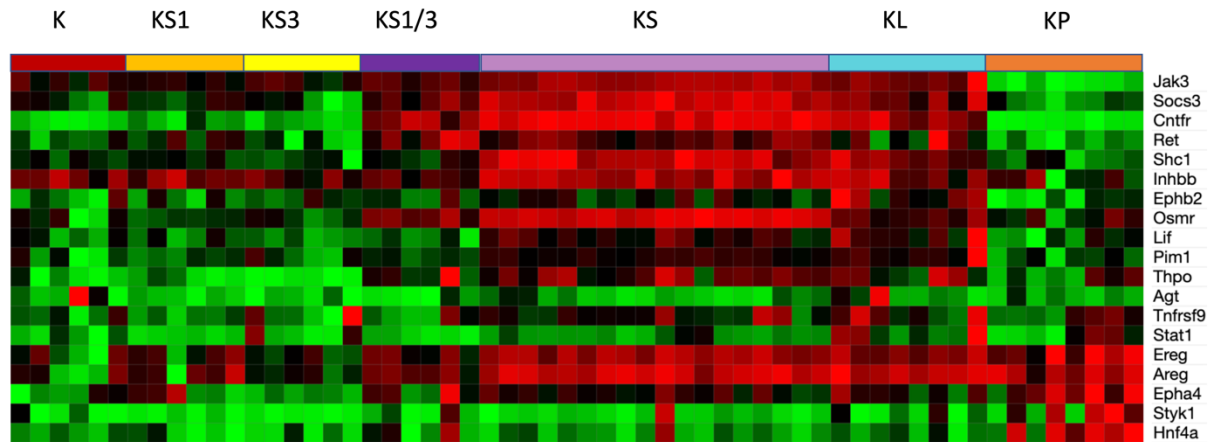


Figure 3. 30: Heat map of hallmark Il6-Jak-Stat gene set proposed by Hollstein et al¹² to be Lkb1-Sik regulated analyzed in genetic models.

Data presented is a heat map of RNA-seq data. Each column shows an individual tumors expression (Z-score) of each gene per row. The following 19 genes were reported to be Lkb1-Sik dependent, however only half appear to be up-regulated upon loss of the Lkb1-Sik axis. Interestingly, loss of Lkb1-Sik leads to the unique increase is Gp130 ligand Lif, as well as Gp130 subcomponents of the Gp130 receptor Cntfr and Osmr, offering a possible dependency for Lkb1 tumors.

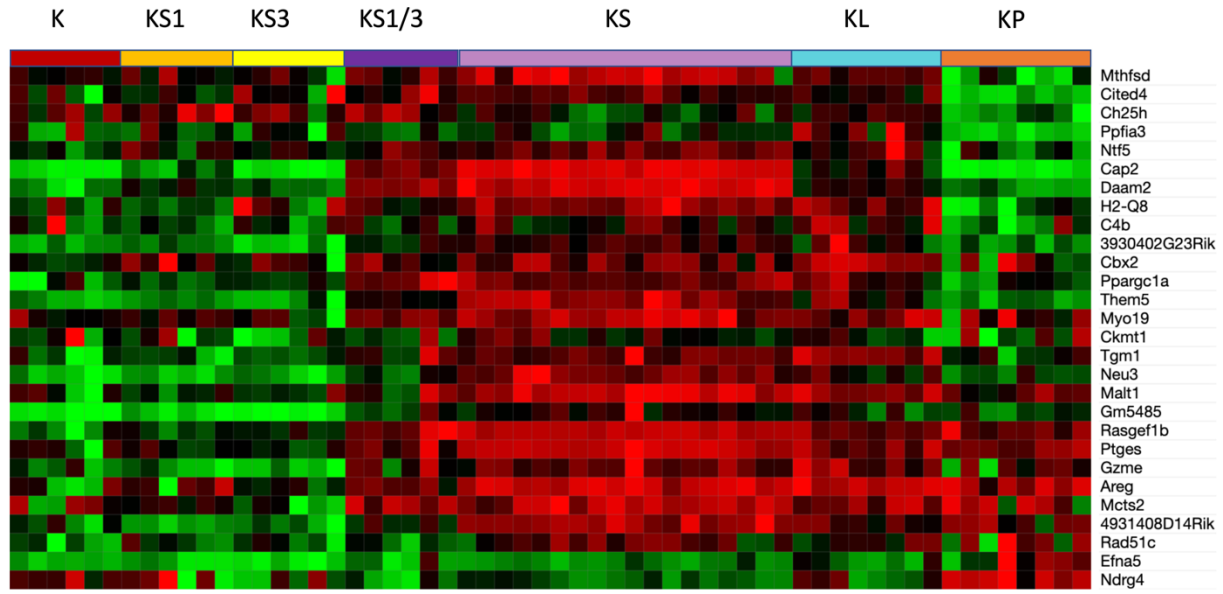


Figure 3. 31: Heat map of genes containing AP1 and CRE sites at their promoter region proposed by Hollstein et al¹² to be Lkb1-Sik regulated analyzed in genetic models.

Data presented is a heat map of RNA-seq data. Each column shows an individual tumors expression (Z-score) of each gene per row. Hollstein et al proposed the following 28 genes to contain AP1 and CRE binding sites at their promoters, and transcripts to be regulated by Lkb1 and the Sik's. Using genetic models, we see that most genes are Lkb1-Sik dependent. However, only half are unique to Lkb1-Sik, with several being up-regulated by loss of P53.

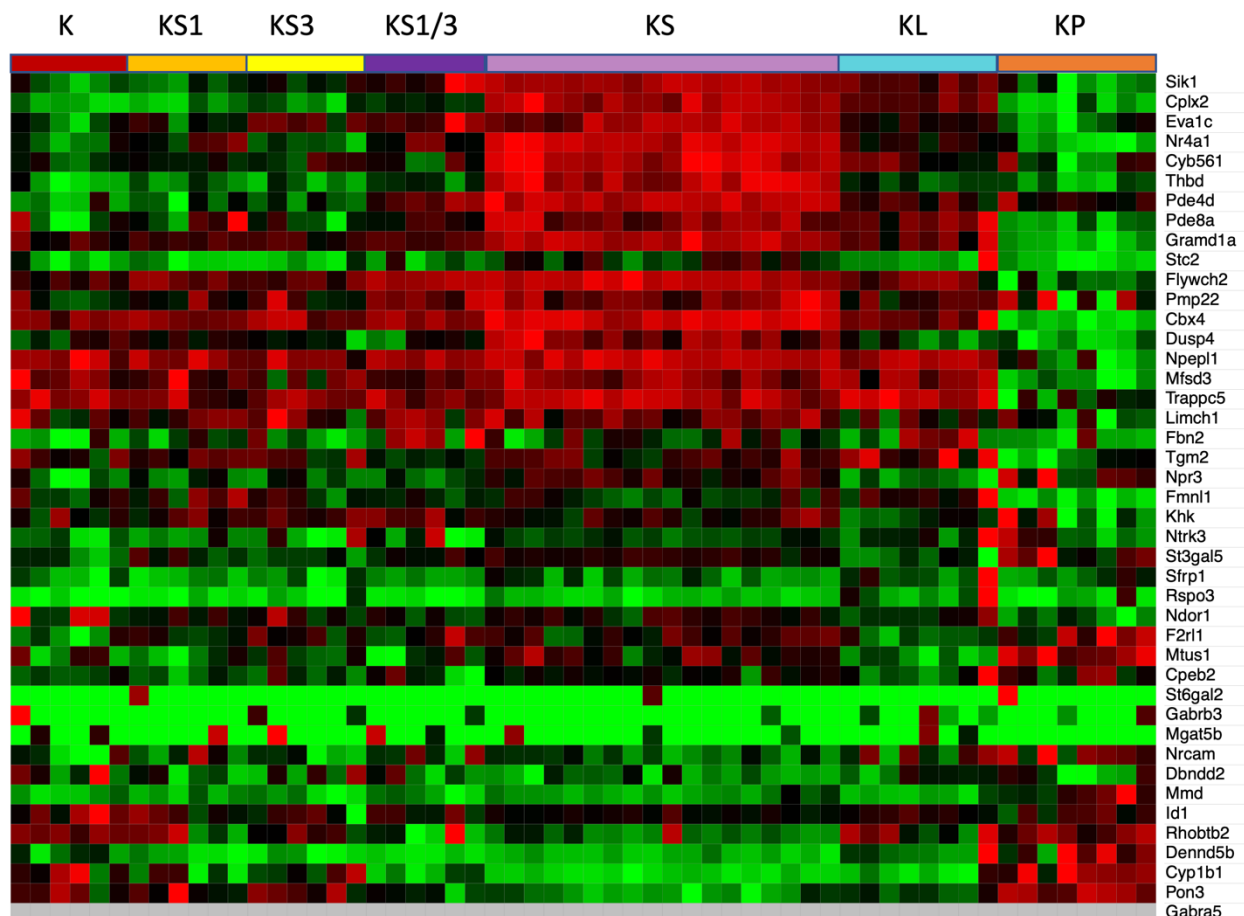


Figure 3. 32: Heat map of bonafide CREB targets identified by RNA-seq and ChIP-seq in A549 lung tumor cell lines from Rodon et al¹⁴¹ analyzed in genetic models.

Data presented is a heat map of RNA-seq data of Sik genetic models. Each column shows an individual tumors expression (Z-score) of each gene per row. The following gene set was identified by RNA-seq cross-referenced to ChIP-seq comparing A549-pBabe to A549-LKb1^{WT}, which had CRTc2 binding. Less than half of the genes in increased in a Lkb1-Sik dependent manner.

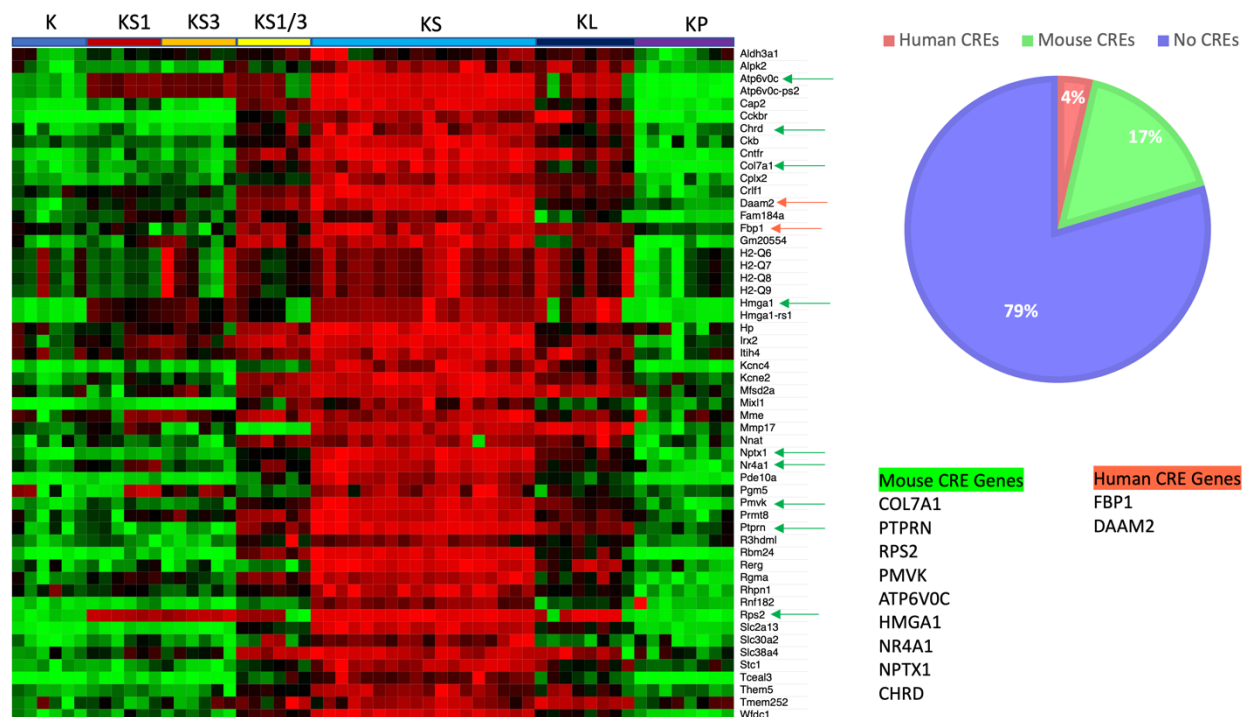


Figure 3. 33: Curated heat map showing genes from cluster 1 which are upregulated in a Lkb1/Sik dependent manner, but remain unchanged in KP model [Cluster 1.1]

Data presented is a heat map of RNA-seq data of Sik genetic models. Each column shows an individual tumors expression (Z-score) of each gene per row. The following heat map was hand curated from the genes in cluster 1 (Figure 3.21). Genes were included in the gene set if transcripts per million followed the pattern: K, KP, KSik1, KSik3<<KSik1/3<<KS, KL. Approximately 20% of genes in cluster 1.1 are CREB targets, suggesting that other pathways are contributing to increases in gene expression.

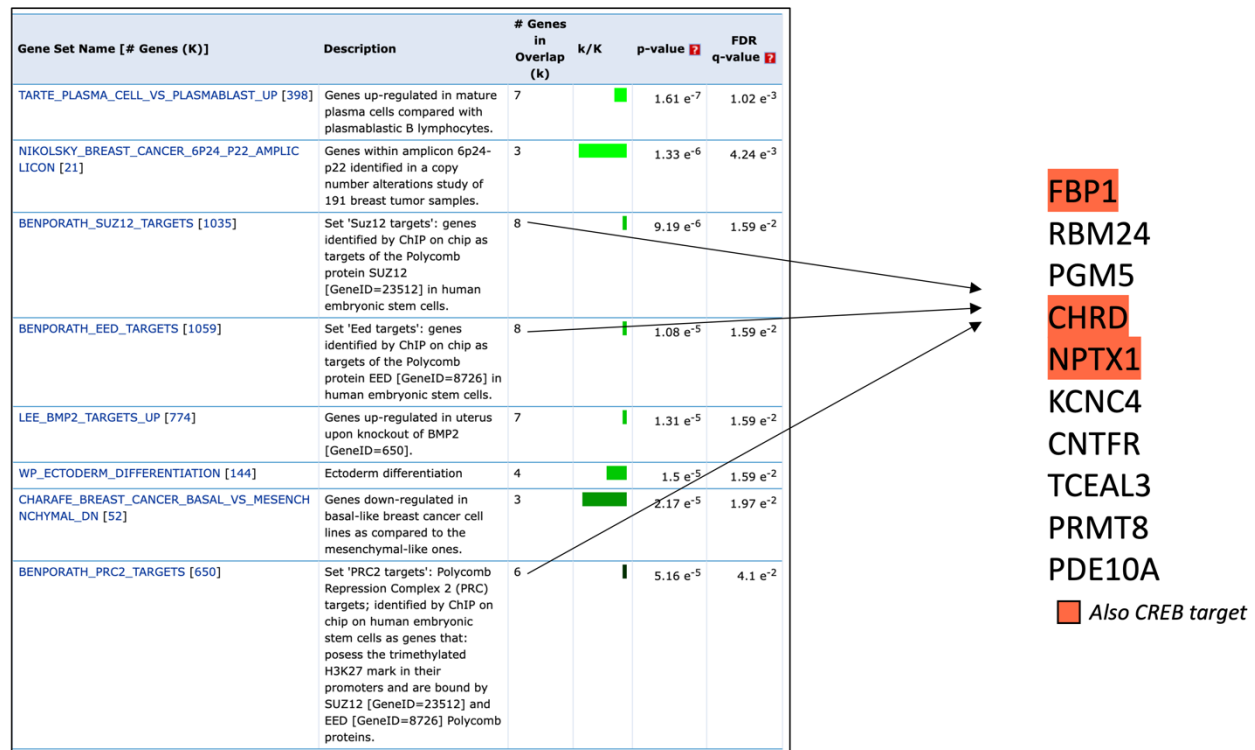


Figure 3. 34: Gene Set enrichment analysis of 54 genes from cluster 1.1 show several polycomb repressive complex 2 (PRC2) subunit targets.

Overlaps between hallmark and curated gene sets and cluster 1.1 (54 genes) were analyzed. Several genes were reported to be PRC2 target genes, suggesting that the Lkb1-Sik axis may regulated PRC2 activity, leading to increases in target gene expression. Suz12, a PRC2 complex subunit, was by far the most reoccurring term from genes up-regulated outside of cluster 1.1.

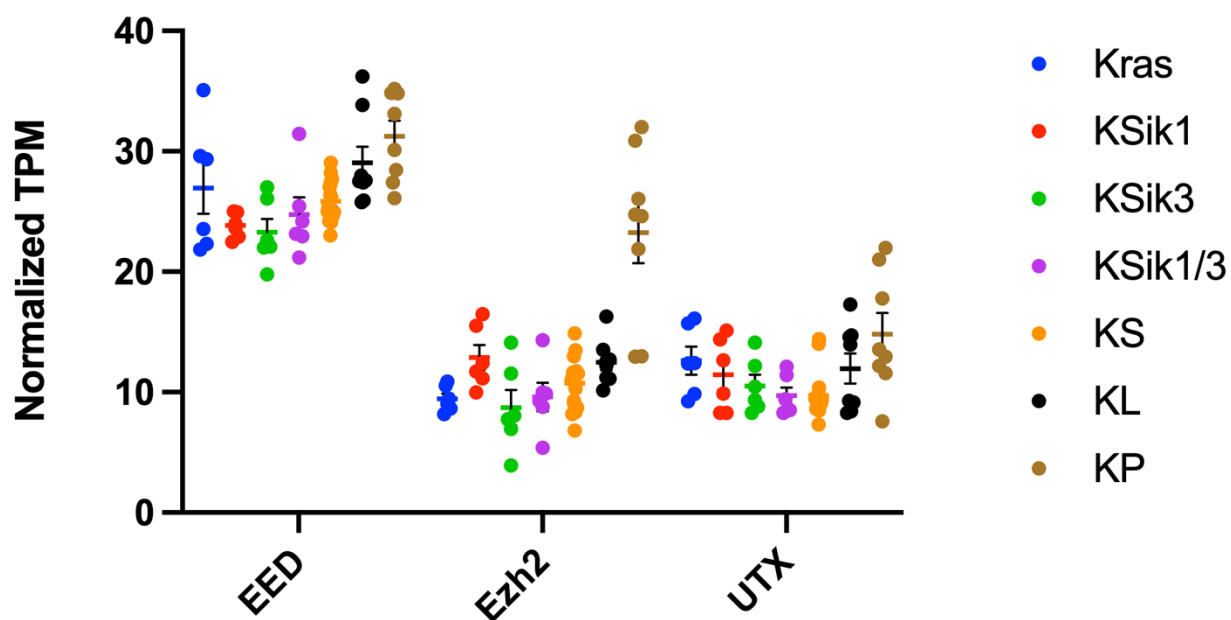


Figure 3. 35: Expression of PRC2 subunits, Eed and Ezh2, and counteracting demethylase UTX whose expression may modulate PRC2 complex activity.

Data shows the normalized TPM values of tumors from genetic mouse models. Each dot represents a single tumors expression of EED, Ezh2, or UTX (aka Kdm6a). The PRC2 complex is thought to be inhibited in adenocarcinomas by the reduction in EED protein levels, which facilitates the transition to squamous cell carcinoma. A similar decrease in protein of the counter demethylase UTX is observed when EED is decreased. In our models, we do not see decreases in expression in any of the PRC2 complex subunits upon Lkb1/Sik loss.

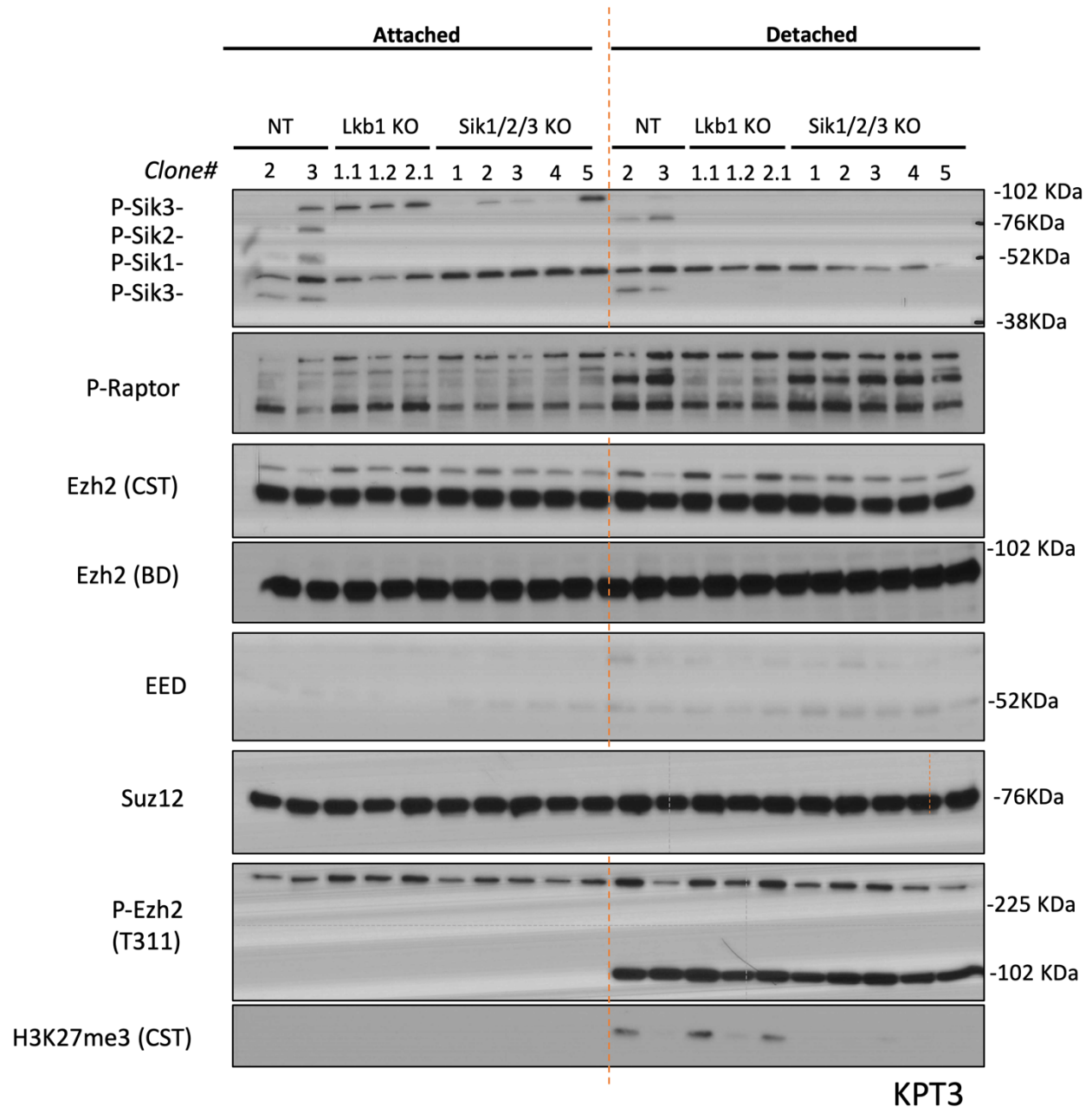


Figure 3. 36: Western blot of PRC2 complex subunits in KP mouse lung cancer cell line clones with Lkb1 or Sik1/Sik2/Sik3 knockouts.

The mouse KPT3 cell line was generated from tumors in the *in vivo* GEMM model. Western blot shows clones of the KPT3 cell line that were generated by CRISPR ribonucleoprotein (RNP) electroporation and single cell isolation of NT, Lkb1 KO, and Sik1/Sik2/Sik3 KO. Cells were grown in attached conditions or under detached conditions before lysed were generated. No major differences in PRC2 complex protein levels were detected in a Sik or Lkb1 dependent manner.

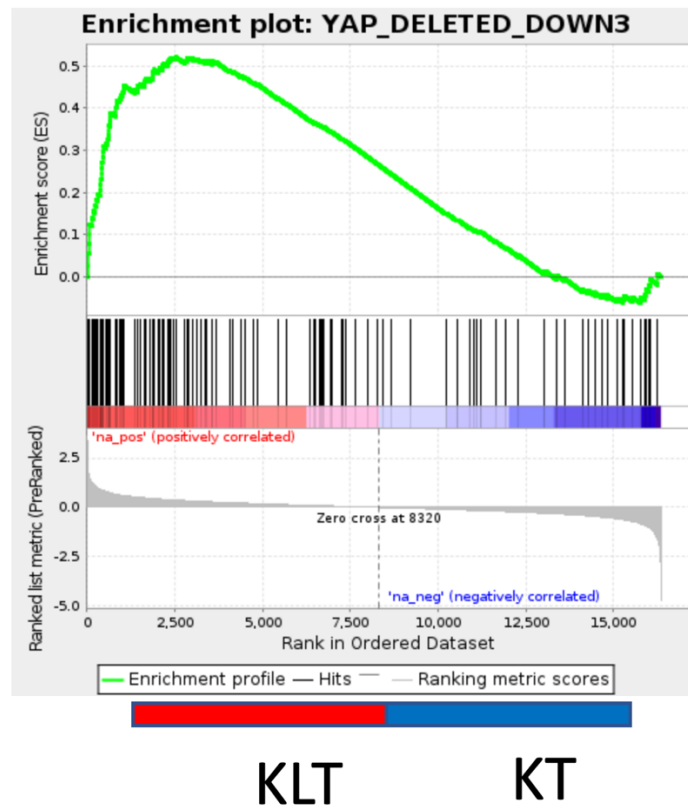


Figure 3. 37: Enrichment plot of Yap dependent genes show enrichment in KL tumor cells.

Yap dependent genes in the lung epithelial were found by analyzing Gokey et al RNA-seq data from Yap-deleted EPCAM+ sorted cells from genetic mouse models¹⁷¹. Enrichment analysis of the Yap dependent genes was performed in KL and K tumor cells sorted with tdTomato (KLT and KT, respectively). Enrichment of the Yap-dependent genes was observed in the KL model, suggesting an enhancement in Hippo pathway target genes.

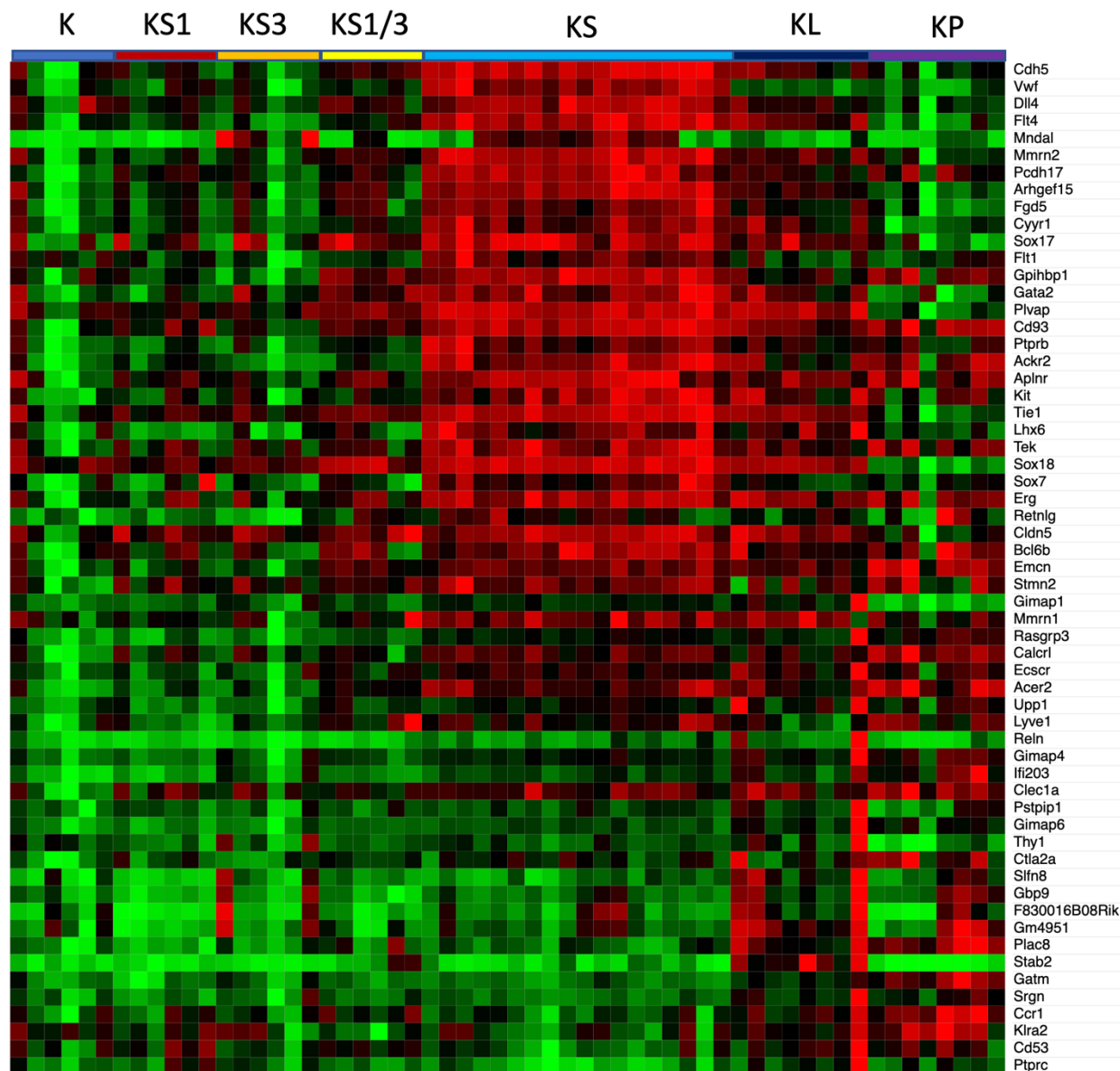


Figure 3. 38: Heat map of Yap-dependent genes identified from Yap-deleted EPCAM+ lung epithelial tissues.

Data presented is a heat map of RNA-seq data of Sik genetic models. Each column shows an individual tumors expression (Z-score) of each gene per row. Genes listed are the same genes identified from Figure 3.34. Over half of the genes identified as being Yap target genes are up regulated in the KS and KL models.

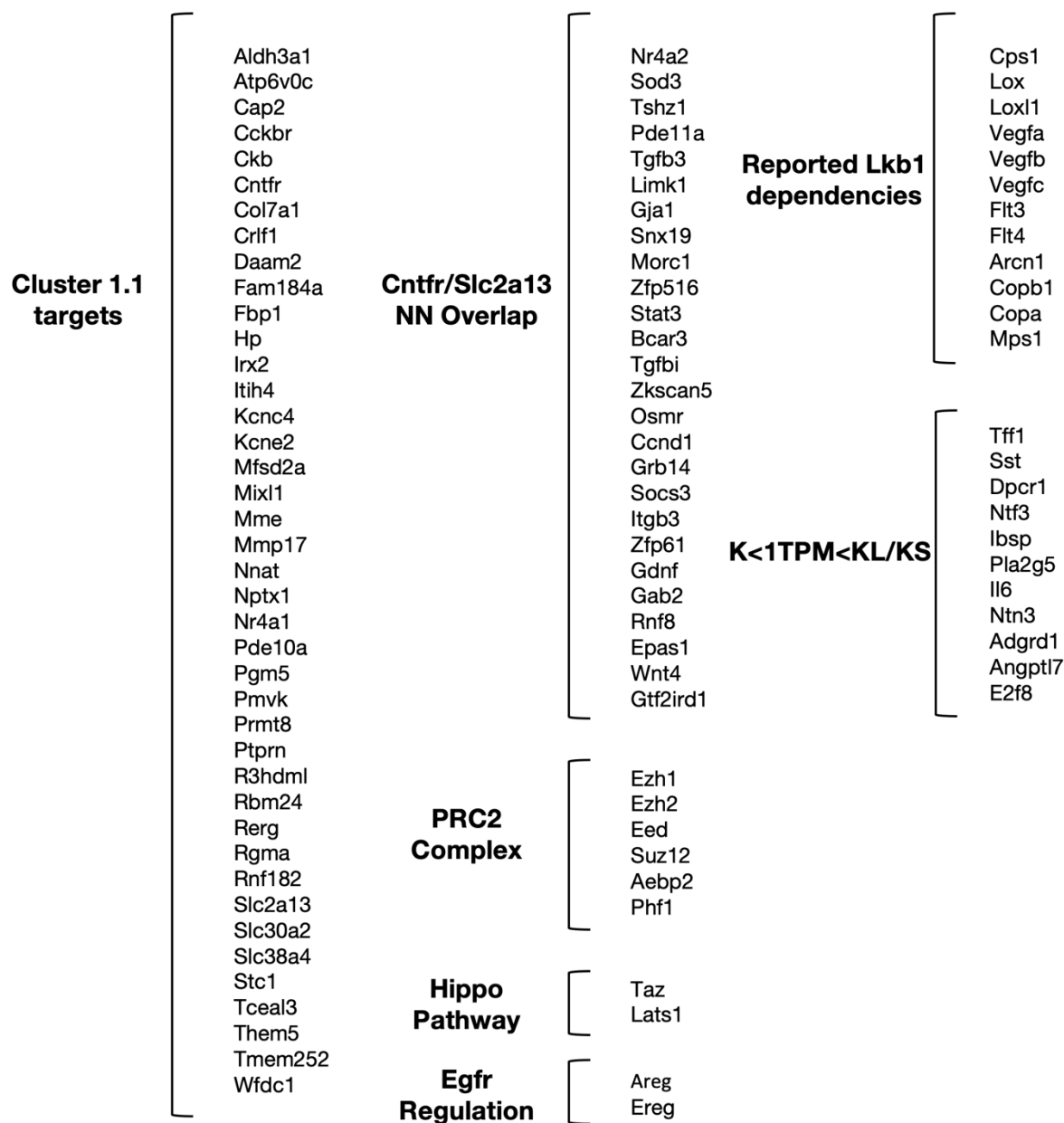


Figure 3. 39: Ultra-seq screen *in vivo* target gene list and method of identification

Shown are the genes which will be targeted using an *in vivo* Cas9 screen. Genes were identified by appearing in Cluster 1.1, nearest neighbor analysis of Cntfr/Slc2a13 which showed prototypical Lkb1/Sik transcriptional dependencies, genes in the PRC2 complex, hippo pathway intermediates, Egfr-like ligands, previously reported Lkb1 growth dependencies, and genes which dramatically change expression in an Lkb1/Sik dependent manner.

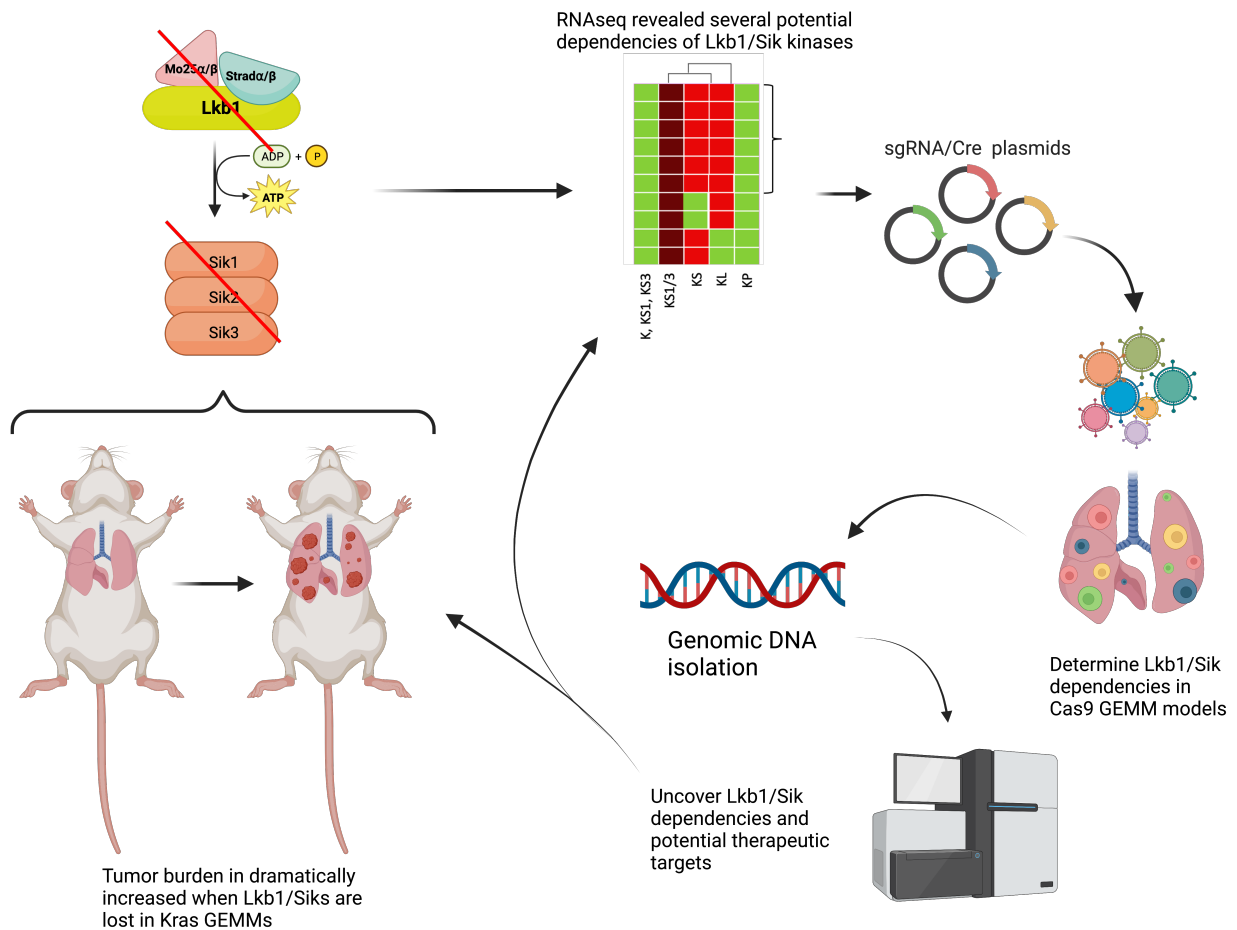


Figure 3. 40: Schematic for ongoing Ultra-seq experiment targeting Lkb1/Sik-dependencies using *in vivo* Cas9 mouse models.

Shown is the goal to identify Lkb1/Sik dependencies. We have identified several genes which may be involved in the initiation and progression of Lkb1/Sik tumors from our RNA-seq data set (Figure 3.39). For each of these identified genes, we will be generating 3 sgRNA which, which ultimately will be used to target *in vivo* at initiation using an sgRNA/Cre vector in KL H11^{LSL:Cas9} mouse models. Pooled and barcoded lentiviral particles will be transduced to initiated tumors, lungs collected, and DNA isolated and deep sequenced to identify the relative barcode densities within each sample.

Table 3. 1: Cluster assignments from figure 3. 21 RNA-seq heat map.

Cluster 5	Cluster 4	Cluster 3	Cluster 2	Cluster 1
Dlk1	Fbp2	Gm19990	Gabrp	Arc
Chl1	Stra8	Rpl3	Uty	Mt1
H2-DMb2	Prom2	Zfp33b	Eif2s3y	Mt2
H2-K2	Gpc6	Gt(ROSA)26Sor	Ddx3y	Ntn5
H2-T-ps	Xpnpep2	Ang4	Kdm5d	Sec1
H2-Ea-ps	Acpp	Ang3	Gzme	Scand1
Btnl2	Fut2	Ang5	Mcpt8	Rps3a1
Pianp	Cd177	Rpl29	Gcnt4	Tpt1
H2-Q10	Ceacam10	Tma7	Misp	Junb
Gm8909	Vsig2	Actc1	Fbln2	D17H6S56E-5
H2-Q1	Fam83a	Foxj1	Tenm4	H2-Eb1
H2-BI	Adam28	AU040972	Alpi	Rps18
H2-Q2	Grem1	Ccdc153	Lgals6	Amhr2
C1s2	Aass	Scgb3a2	Lgals4	Hmga1
C1rb	Ptprz1	Cyp2s1	Krt14	Hmga1-rs1
Awat1	Prr16	Lyz1	Mcpt2	Atp6v0c
H2-Eb2	Ryr3	Agt	Tff1	Atp6v0c-ps2
Ly6i	Akr1c18	Prlr	Mcpt1	Rps2
H2-Oa	Lce3f	Crct1	Dmbt1	Itih4
Pigr	P2ry4	Cyp2c40	Anxa10	Aldh3a1
Cd79a	Olfm5	Cpne4	Krt20	Mme
Mzb1	Amph	Xist	F5	Klf15
Pou2af1	Bpifa1	Ly6k	Mcpt-ps1	Rprm
Lax1	Tpd52l1	Marco	Pkp1	Rgma
Gimap7	Apoc1	Snurf	Anxa8	Fam184a
Ms4a1	Slc23a1	Snrpn	Ltf	Irx2
Cxcl13	Gsta3	Necab1	Agr2	Gm20554
Pdcd1	Gdpd2	C77370	Tff2	Irx1
Cd8a	Gc	Cox6a2	Gkn1	Lrg1
Cd8b1	Peg10	Psca	Porcn	6030419C18Rik
Dnase1l3	AU015836	Ly6d	AI506816	Pgm5
Apol10b	Tceal8	Pkdcc	Hmgcs2	Cbr2
Apol6	Bex4	Ivl	Clca1	Cyp2f2
Penk	Bex1	Ndufs5	St8sia3	Slc6a15
C1s1	St6galnac3	Cyp4a12b	Lypd8	C2cd4b
Spib	Pf4	Spag11b	Add2	Cyp4a12a
Ccl20	Stambpl1	Fabp1	2210407C18Rik	Ptprn2
Dtx1	Ankrd22	Ms4a8a	Onecut2	Cftr
Cxcr5	Tspan12	Nrn1	Cdh6	H2-Q5
Fcrl1	Rasd2	Lipo3	Hmga2	H2-Q9
Tnfrsf13c	Ace2	Vnn1	Oit1	H2-Q7
Bank1	Ros1	Cyp3a13	Sprr2a3	H2-Q8
Bcl11a	Npy2r	Smim6	Lman1l	H2-Q6
Siglecg	Slc2a3	Kcnk2	Gcnt3	Tmem252
Blk	Bcl2l15	Epdr1	Cdhr2	Rbp1
Fgf23	Stk26	Chia1	Gpa33	Chrd
Pla2g2d	Parm1	Rab15	Apoc2	Ptprn

Table 3. 1: Cluster assignments from figure 3. 21 RNA-seq heat map (*continued*).

Tnfrsf9	Atp13a4	Npl	Apoc4-apoc2	R3hdm1
A2m	Slc35f1	Nphs2	Itga2	Morc1
Ltb	Gja3	Hsd3b2	Cwh43	Mixl1
Ptprcap	Tceanc	Selenbp2	Aldh1a3	Msln
H2-Q4	Rbp4	Krt79	Hnf4a	Stc1
Gbp11	Tmem27	Fpr2	Ctse	Dpcr1
Gbp10	Adcyap1	Chil3	Nrk	Fgl1
Cxcl11	Slc2a2	Bex2	Scara3	Col6a2
Gbp2b	Scel	Acox1	Nkain1	Slc30a2
Gbp8	Tmed6	Xlr3a	Fermt1	Prmt8
Cxcl9	Akr1c19	Hspa1a	Cfi	Hp
Ubd	Slc16a12	Fosb	Spr1a	Rhpn1
Cxcl10	P2rx2	Rpph1	Cldn6	Nr4a1
Gbp2	Ddc	Cckar	Dnajc22	Nr4a3
Gbp3	Sox6	Myh6	Pthlh	Epor
Gbp5	Alb	Rpl35a	Scn8a	Col7a1
Gbp6	Habp2	Rpl34-ps1	Kng2	Crlf1
Nkg7	Raet1c	Rpl34		Ckb
Zbp1	Raet1e	Rplp1		Nptx1
Gbp9	Adgrg7			Pde10a
Gbp4	H60b			Slc2a13
Ccl8	Raet1d			Tceal3
Nlrc5	Raet1b			Pmvk
F830016B08Rik	Raet1a			Wfdc1
Tgtp1	Rnf128			Cap2
Iigp1	Rnf183			Rbm24
Gm4841	Apln			Kcne2
Irgm2	Cyp1b1			Daam2
Gm12250	Adtrp			Il10
Tgtp2	Cldn2			Pbp2
Igtp	Serpinf1			Gucy2c
Gm12185	Zfp618			Tub
Gm4951	Fras1			Eno3
Oas3	Zmat4			Gper1
Oasl2	Dpyd			Rimbp2
Ido1	Tfap2b			Cacng3
Cacna1h	Mogat1			Map3k15
Saa3	Cd59a			Pcsk1
Angptl7	Bcan			Enpp6
Upk3b	Papss2			Nr4a2
Ryr2	Vil1			Pde11a
Capn6	Apoa1			Kif1a
Ccl24	Sptssb			Sod3
Itln1	Rab3b			Cbs
Chil4	Tpsab1			Shisa7
C2	Dsg2			Nnat
Slc26a4	Moxd1			Slc38a4
Cfb	Dsc2			Mfsd2a
Serpina3g	Mboat2			Rerg

Table 3. 1: Cluster assignments from figure 3. 21 RNA-seq heat map (*continued*).

Serpina3f	Vsig1			Rnf182
AW112010	Afp			Cplx2
Jchain	Gkn2			Kcnc4
Serpina3h	Serpinb5			Cckbr
Ifi202b	Iqgap2			Cntfr
Xaf1	Aqp3			Alpk2
	Fam84a			Them5
	Gkn3			Fbp1
	Guca2a			Ibsp
	Cybrd1			Steap4
	BC048546			Mmp17
	Dcpp3			Adgrd1
	Dcpp2			M1ap
	Dcpp1			Spink5
	Bche			Pdlim3
	Fhit			Hapln1
	Shisa2			Mest
	Lrm1			Rpgrip1
	Folr1			Scara5
	Cxcl5			Cubn
	Egln3			Ly6g6c
	B4galt6			Rragd
	Ifi2712b			Fgg
	Vcan			Fga
	Tpbp			Pon1
	Vcam1			Hc
	Calml4			Proz
	Unc13c			1110019D14Rik
	Anks4b			2610507I01Rik
	Atp8a2			Kcnf1
	Tm4sf20			Camk2b
	Akr1c12			Muc5b
	Otc			Scgb1a1
	Cym			Rec8
	Apob			Fxyd4
	Rbm46			Retnla
	Lrat			
	Fam19a4			
	Chic1			
	Fmo1			

REFERENCES

1. Hunter, T. Protein kinases and phosphatases: The Yin and Yang of protein phosphorylation and signaling. *Cell* 80, 225–236 (1995).
2. Eid, S., Turk, S., Volkamer, A., Rippmann, F. & Fulle, S. KinMap: a web-based tool for interactive navigation through human kinome data. *Bmc Bioinformatics* 18, 16 (2017).
3. Shackelford, D. B. & Shaw, R. J. The LKB1–AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer* 9, 563–575 (2009).
4. Sapkota, G. P., Kieloch, A., Lizcano, J. M., Lain, S., Arthur, J. S., Williams, M. R., Morrice, N., Deak, M. & Alessi, D. R. Phosphorylation of the protein kinase mutated in Peutz-Jeghers cancer syndrome, LKB1/STK11, at Ser431 by p90(RSK) and cAMP-dependent protein kinase, but not its farnesylation at Cys(433), is essential for LKB1 to suppress cell growth. *J Biological Chem* 276, 19469–82 (2001).
5. Sapkota, G. P., Boudeau, J., Deak, M., Kieloch, A., Morrice, N. & Alessi, D. R. Identification and characterization of four novel phosphorylation sites (Ser31, Ser325, Thr336 and Thr366) on LKB1/STK11, the protein kinase mutated in Peutz-Jeghers cancer syndrome. *Biochem J* 362, 481 (2002).
6. Boudeau, J., Baas, A. F., Deak, M., Morrice, N. A., Kieloch, A., Schutkowski, M., Prescott, A. R., Clevers, H. C. & Alessi, D. R. MO25 / interact with STRAD / enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. *Embo J* 22, 5102–5114 (2003).
7. Baas, A. F., Boudeau, J., Sapkota, G. P., Smit, L., Medema, R., Morrice, N. A., Alessi, D. R. & Clevers, H. C. Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD. *Embo J* 22, 3062–3072 (2003).
8. Brajenovic, M., Joberty, G., Küster, B., Bouwmeester, T. & Drewes, G. Comprehensive Proteomic Analysis of Human Par Protein Complexes Reveals an Interconnected Protein Network*. *J Biol Chem* 279, 12804–12811 (2004).
9. Karos, M. & Fischer, R. Molecular characterization of HymA, an evolutionarily highly conserved and highly expressed protein of *Aspergillus nidulans*. *Molec. Gen. Genet.* 260, 510–521 (1999).
10. Boudeau, J., Scott, J. W., Resta, N., Deak, M., Kieloch, A., Komander, D., Hardie, D. G., Prescott, A. R., Aalten, D. M. F. van & Alessi, D. R. Analysis of the LKB1-STRAD-MO25 complex. *J Cell Sci* 117, 6365–6375 (2004).
11. Zagórska, A., Deak, M., Campbell, D. G., Banerjee, S., Hirano, M., Aizawa, S., Prescott, A. R. & Alessi, D. R. New Roles for the LKB1-NUAK Pathway in Controlling Myosin Phosphatase Complexes and Cell Adhesion. *Sci Signal* 3, ra25 (2010).

12. Hollstein, P. E., Eichner, L. J., Brun, S. N., Kamireddy, A., Svensson, R. U., Vera, L. I., Ross, D. S., Rymoff, T. J., Hutchins, A., Galvez, H. M., Williams, A. E., Shokhirev, M. N., Screatton, R. A., Berdeaux, R. & Shaw, R. J. The AMPK-Related Kinases SIK1 and SIK3 Mediate Key Tumor-Suppressive Effects of LKB1 in NSCLC. *Cancer Discov* 9, 1606–1627 (2019).
13. Wilkinson, S., Hou, Y., Zoine, J. T., Saltz, J., Zhang, C., Chen, Z., Cooper, L. A. D. & Marcus, A. I. Coordinated cell motility is regulated by a combination of LKB1 farnesylation and kinase activity. *Sci Rep-uk* 7, 40929 (2017).
14. Hemminki, A. The molecular basis and clinical aspects of Peutz-Jeghers syndrome. *Cell Mol Life Sci Cmls* 55, 735–750 (1999).
15. Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., Bignell, G., Warren, W., Aminoff, M., Höglund, P., Järvinen, H., Kristo, P., Pelin, K., Ridanpää, M., Salovaara, R., Toro, T., Bodmer, W., Olschwang, S., Olsen, A. S., Stratton, M. R., Chapelle, A. de la & Aaltonen, L. A. A serine/threonine kinase gene defective in Peutz–Jeghers syndrome. *Nature* 391, 184–187 (1998).
16. Gao, J., Aksoy, B. A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S. O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., Cerami, E., Sander, C. & Schultz, N. Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. *Sci Signal* 6, pl1 (2013).
17. Cerami, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., Jacobsen, A., Byrne, C. J., Heuer, M. L., Larsson, E., Antipin, Y., Reva, B., Goldberg, A. P., Sander, C. & Schultz, N. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. *Cancer Discov* 2, 401–404 (2012).
18. Jaleel, M., McBride, A., Lizcano, J. M., Deak, M., Toth, R., Morrice, N. A. & Alessi, D. R. Identification of the sucrose non-fermenting related kinase SNRK, as a novel LKB1 substrate. *Febs Lett* 579, 1417–1423 (2005).
19. Lizcano, J. M., Göransson, O., Toth, R., Deak, M., Morrice, N. A., Boudeau, J., Hawley, S. A., Udd, L., Mäkelä, T. P., Hardie, D. G. & Alessi, D. R. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *Embo J* 23, 833–843 (2004).
20. Hong, S.-P., Leiper, F. C., Woods, A., Carling, D. & Carlson, M. Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc National Acad Sci* 100, 8839–8843 (2003).
21. Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Mäkelä, T. P., Alessi, D. R. & Hardie, D. G. Complexes between the LKB1 tumor suppressor, STRADA β and MO25 α/β are upstream kinases in the AMP-activated protein kinase cascade. *J Biology* 2, 28 (2003).
22. Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G. D., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M. & Carling, D. LKB1 Is the Upstream Kinase in the AMP-Activated Protein Kinase Cascade. *Curr Biol* 13, 2004–2008 (2003).

23. Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A. & Cantley, L. C. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc National Acad Sci* 101, 3329–3335 (2004).
24. Trefts, E. & Shaw, R. J. AMPK: restoring metabolic homeostasis over space and time. *Mol Cell* 81, 3677–3690 (2021).
25. Laderoute, K. R., Amin, K., Calaoagan, J. M., Knapp, M., Le, T., Orduna, J., Foretz, M. & Viollet, B. 5'-AMP-Activated Protein Kinase (AMPK) Is Induced by Low-Oxygen and Glucose Deprivation Conditions Found in Solid-Tumor Microenvironments. *Mol Cell Biol* 26, 5336–5347 (2006).
26. Al-Hakim, A. K., Göransson, O., Deak, M., Toth, R., Campbell, D. G., Morrice, N. A., Prescott, A. R. & Alessi, D. R. 14-3-3 cooperates with LKB1 to regulate the activity and localization of QSK and SIK. *J Cell Sci* 118, 5661–5673 (2005).
27. Sakamoto, K., Göransson, O., Hardie, D. G. & Alessi, D. R. Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. *Am J Physiol-endoc M* 287, E310–E317 (2004).
28. O'Neill, H. M., Lally, J. S., Galic, S., Thomas, M., Azizi, P. D., Fullerton, M. D., Smith, B. K., Pulinilkunnil, T., Chen, Z., Samaan, M. C., Jorgensen, S. B., Dyck, J. R. B., Holloway, G. P., Hawke, T. J., Denderen, B. J. van, Kemp, B. E. & Steinberg, G. R. AMPK phosphorylation of ACC2 is required for skeletal muscle fatty acid oxidation and insulin sensitivity in mice. *Diabetologia* 57, 1693–1702 (2014).
29. Carling, D., Zammit, V. A. & Hardie, D. G. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. *Febs Lett* 223, 217–222 (1987).
30. Abu-Elheiga, L., Brinkley, W. R., Zhong, L., Chirala, S. S., Woldegiorgis, G. & Wakil, S. J. The subcellular localization of acetyl-CoA carboxylase 2. *Proc National Acad Sci* 97, 1444–1449 (2000).
31. Egan, D. F., Chun, M. G. H., Vamos, M., Zou, H., Rong, J., Miller, C. J., Lou, H. J., Raveendra-Panickar, D., Yang, C.-C., Sheffler, D. J., Teriete, P., Asara, J. M., Turk, B. E., Cosford, N. D. P. & Shaw, R. J. Small Molecule Inhibition of the Autophagy Kinase ULK1 and Identification of ULK1 Substrates. *Mol Cell* 59, 285–297 (2015).
32. Toyama, E. Q., Herzig, S., Courchet, J., Lewis, T. L., Losón, O. C., Hellberg, K., Young, N. P., Chen, H., Polleux, F., Chan, D. C. & Shaw, R. J. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. *Science* 351, 275–281 (2016).
33. Hung, C.-M., Lombardo, P. S., Malik, N., Brun, S. N., Hellberg, K., Nostrand, J. L. V., Garcia, D., Baumgart, J., Diffenderfer, K., Asara, J. M. & Shaw, R. J. AMPK/ULK1-mediated phosphorylation of Parkin ACT domain mediates an early step in mitophagy. *Sci Adv* 7, eabg4544 (2021).

34. Eichner, L. J., Brun, S. N., Herzig, S., Young, N. P., Curtis, S. D., Shackelford, D. B., Shokhirev, M. N., Leblanc, M., Vera, L. I., Hutchins, A., Ross, D. S., Shaw, R. J. & Svensson, R. U. Genetic Analysis Reveals AMPK Is Required to Support Tumor Growth in Murine Kras-Dependent Lung Cancer Models. *Cell Metab* 29, 285-302.e7 (2019).
35. Young, N. P., Kamireddy, A., Nostrand, J. L. V., Eichner, L. J., Shokhirev, M. N., Dayn, Y. & Shaw, R. J. AMPK governs lineage specification through Tfeb-dependent regulation of lysosomes. *Gene Dev* 30, 535–552 (2016).
36. Inoki, K., Zhu, T. & Guan, K.-L. TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival. *Cell* 115, 577–590 (2003).
37. Shaw, R. J., Bardeesy, N., Manning, B. D., Lopez, L., Kosmatka, M., DePinho, R. A. & Cantley, L. C. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* 6, 91–99 (2004).
38. Corradetti, M. N., Inoki, K., Bardeesy, N., DePinho, R. A. & Guan, K.-L. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Gene Dev* 18, 1533–1538 (2004).
39. Saxton, R. A. & Sabatini, D. M. mTOR Signaling in Growth, Metabolism, and Disease. *Cell* 168, 960–976 (2017).
40. Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E. & Shaw, R. J. AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint. *Mol Cell* 30, 214–226 (2008).
41. Tomancak, P., Piano, F., Riechmann, V., Gunsalus, K. C., Kemphues, K. J. & Ephrussi, A. A *Drosophila melanogaster* homologue of *Caenorhabditis elegans* par-1 acts at an early step in embryonic-axis formation. *Nat Cell Biol* 2, 458–460 (2000).
42. Shulman, J. M., Benton, R. & Johnston, D. S. The *Drosophila* Homolog of *C. elegans* PAR-1 Organizes the Oocyte Cytoskeleton and Directs oskar mRNA Localization to the Posterior Pole. *Cell* 101, 377–388 (2000).
43. Lee, J. H., Koh, H., Kim, M., Kim, Y., Lee, S. Y., Karess, R. E., Lee, S.-H., Shong, M., Kim, J.-M., Kim, J. & Chung, J. Energy-dependent regulation of cell structure by AMP-activated protein kinase. *Nature* 447, 1017–1020 (2007).
44. Martin, S. G. & Johnston, D. S. A role for *Drosophila* LKB1 in anterior–posterior axis formation and epithelial polarity. *Nature* 421, 379–384 (2003).
45. Hezel, A. F. & Bardeesy, N. LKB1; linking cell structure and tumor suppression. *Oncogene* 27, 6908–6919 (2008).
46. Goodwin, J. M., Svensson, R. U., Lou, H. J., Winslow, M. M., Turk, B. E. & Shaw, R. J. An AMPK-Independent Signaling Pathway Downstream of the LKB1 Tumor Suppressor Controls Snail1 and Metastatic Potential. *Mol Cell* 55, 436–450 (2014).

47. Kojima, Y., Miyoshi, H., Clevers, H. C., Oshima, M., Aoki, M. & Taketo, M. M. Suppression of Tubulin Polymerization by the LKB1-Microtubule-associated Protein/Microtubule Affinity-regulating Kinase Signaling*. *J Biol Chem* 282, 23532–23540 (2007).
48. Canel, M., Serrels, A., Frame, M. C. & Brunton, V. G. E-cadherin–integrin crosstalk in cancer invasion and metastasis. *J Cell Sci* 126, 393–401 (2013).
49. Fan, H., Zhao, X., Sun, S., Luo, M. & Guan, J.-L. Function of Focal Adhesion Kinase Scaffolding to Mediate Endophilin A2 Phosphorylation Promotes Epithelial-Mesenchymal Transition and Mammary Cancer Stem Cell Activities in Vivo *. *J Biol Chem* 288, 3322–3333 (2013).
50. Li, X.-Y., Zhou, X., Rowe, R. G., Hu, Y., Schlaepfer, D. D., Ilić, D., Dressler, G., Park, A., Guan, J.-L. & Weiss, S. J. Snail1 controls epithelial–mesenchymal lineage commitment in focal adhesion kinase–null embryonic cells. *J Cell Biol* 195, 729–738 (2011).
51. Frisch, S. M., Schaller, M. & Cieply, B. Mechanisms that link the oncogenic epithelial–mesenchymal transition to suppression of anoikis. *J Cell Sci* 126, 21–29 (2013).
52. Cance, W. G. & Golubovskaya, V. M. Focal Adhesion Kinase Versus p53: Apoptosis or Survival? *Sci Signal* 1, pe22 (2008).
53. Shibue, T., Brooks, M. W., Inan, M. F., Reinhardt, F. & Weinberg, R. A. The Outgrowth of Micrometastases Is Enabled by the Formation of Filopodium-like Protrusions. *Cancer Discov* 2, 706–721 (2012).
54. Mitra, S. K., Hanson, D. A. & Schlaepfer, D. D. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Bio* 6, 56–68 (2005).
55. Kishi, M., Pan, Y. A., Crump, J. G. & Sanes, J. R. Mammalian SAD Kinases Are Required for Neuronal Polarization. *Science* 307, 929–932 (2005).
56. Barnes, A. P., Lilley, B. N., Pan, Y. A., Plummer, L. J., Powell, A. W., Raines, A. N., Sanes, J. R. & Polleux, F. LKB1 and SAD Kinases Define a Pathway Required for the Polarization of Cortical Neurons. *Cell* 129, 549–563 (2007).
57. Shelly, M., Cancedda, L., Heilshorn, S., Sumbre, G. & Poo, M. LKB1/STRAD Promotes Axon Initiation During Neuronal Polarization. *Cell* 129, 565–577 (2007).
58. Courchet, J., Lewis, T. L., Lee, S., Courchet, V., Liou, D.-Y., Aizawa, S. & Polleux, F. Terminal Axon Branching Is Regulated by the LKB1-NUAK1 Kinase Pathway via Presynaptic Mitochondrial Capture. *Cell* 153, 1510–1525 (2013).
59. Port, J. L. F., Muthalagu, N., Raja, M., Ceteci, F., Monteverde, T., Kruspig, B., Hedley, A., Kalna, G., Lilla, S., Neilson, L., Brucoli, M., Gyuraszova, K., Tait-Mulder, J., Mezna, M., Svambaryte, S., Bryson, A., Sumpton, D., McVie, A., Nixon, C., Drysdale, M., Esumi, H., Murray, G. I., Sansom, O. J., Zanivan, S. & Murphy, D. J. Colorectal tumors require NUAK1 for protection from oxidative stress. *Cancer Discov* 8, CD-17-0533 (2018).

60. Monteverde, T., Tait-Mulder, J., Hedley, A., Knight, J. R., Sansom, O. J. & Murphy, D. J. Calcium signalling links MYC to NUA1. *Oncogene* 37, 982 (2017).
61. Yamamoto, H., Takashima, S., Shintani, Y., Yamazaki, S., Seguchi, O., Nakano, A., Higo, S., Kato, H., Liao, Y., Asano, Y., Minamino, T., Matsumura, Y., Takeda, H. & Kitakaze, M. Identification of a novel substrate for TNF α -induced kinase NUA2. *Biochem Biophys Res Commun* 365, 541–547 (2008).
62. Scott, J. W., Norman, D. G., Hawley, S. A., Kontogiannis, L. & Hardie, D. G. Protein kinase substrate recognition studied using the recombinant catalytic domain of AMP-activated protein kinase and a model substrate. *J Mol Biol* 317, 309–323 (2002).
63. Koga, Y. & Ikebe, M. A Novel Regulatory Mechanism of Myosin Light Chain Phosphorylation via Binding of 14-3-3 to Myosin Phosphatase. *Mol Biol Cell* 19, 1062–1071 (2008).
64. Halford, N. G. & Hey, S. J. Snf1-related protein kinases (SnRKs) act within an intricate network that links metabolic and stress signalling in plants. *Biochem J* 419, 247–259 (2009).
65. Li, Y., Nie, Y., Helou, Y., Ding, G., Feng, B., Xu, G., Salomon, A. & Xu, H. Identification of Sucrose Non-Fermenting-Related Kinase (SNRK) as a Suppressor of Adipocyte Inflammation. *Diabetes* 62, 2396–2409 (2013).
66. Li, J., Feng, B., Nie, Y., Jiao, P., Lin, X., Huang, M., An, R., He, Q., Zhou, H. E., Salomon, A., Sigrist, K. S., Wu, Z., Liu, S. & Xu, H. Sucrose Nonfermenting-Related Kinase Regulates Both Adipose Inflammation and Energy Homeostasis in Mice and Humans. *Diabetes* 67, 400–411 (2018).
67. Li, J., An, R., Lai, S., Li, L., Liu, S. & Xu, H. Dysregulation of PP2A-Akt interaction contributes to Sucrose non-fermenting related kinase (SNRK) deficiency induced insulin resistance in adipose tissue. *Mol Metab* 28, 26–35 (2019).
68. Lu, Q., Ma, Z., Ding, Y., Bedarida, T., Chen, L., Xie, Z., Song, P. & Zou, M.-H. Circulating miR-103a-3p contributes to angiotensin II-induced renal inflammation and fibrosis via a SNRK/NF- κ B/p65 regulatory axis. *Nat Commun* 10, 2145 (2019).
69. Wang, Z., Takemori, H., Halder, S. K., Nonaka, Y. & Okamoto, M. Cloning of a novel kinase (SIK) of the SNF1/AMPK family from high salt diet-treated rat adrenal1. *FEBS Lett* 453, 135–139 (1999).
70. Lin, X., Takemori, H., Katoh, Y., Doi, J., Horike, N., Makino, A., Nonaka, Y. & Okamoto, M. Salt-Inducible Kinase Is Involved in the ACTH/cAMP-Dependent Protein Kinase Signaling in Y1 Mouse Adrenocortical Tumor Cells. *Mol Endocrinol* 15, 1264–1276 (2001).
71. Feldman, J. D., Vician, L., Crispino, M., Hoe, W., Baudry, M. & Herschman, H. R. The Salt-Inducible Kinase, SIK, Is Induced by Depolarization in Brain. *J Neurochem* 74, 2227–2238 (2000).
72. Jagannath, A., Butler, R., Godinho, S. I. H., Couch, Y., Brown, L. A., Vasudevan, S. R., Flanagan, K. C., Anthony, D., Churchill, G. C., Wood, M. J. A., Steiner, G., Ebeling, M., Hossbach,

M., Wettstein, J. G., Duffield, G. E., Gatti, S., Hankins, M. W., Foster, R. G. & Peirson, S. N. The CRTCL-SIK1 Pathway Regulates Entrainment of the Circadian Clock. *Cell* 154, 1100–1111 (2013).

73. Horike, N., Takemori, H., Katoh, Y., Doi, J., Min, L., Asano, T., Sun, X. J., Yamamoto, H., Kasayama, S., Muraoka, M., Nonaka, Y. & Okamoto, M. Adipose-specific Expression, Phosphorylation of Ser794 in Insulin Receptor Substrate-1, and Activation in Diabetic Animals of Salt-inducible Kinase-2*. *J Biol Chem* 278, 18440–18447 (2003).

74. Katoh, Y., Takemori, H., Horike, N., Doi, J., Muraoka, M., Min, L. & Okamoto, M. Salt-inducible kinase (SIK) isoforms: their involvement in steroidogenesis and adipogenesis. *Mol Cell Endocrinol* 217, 109–112 (2004).

75. Darling, N. J., Toth, R., Arthur, J. S. C. & Clark, K. Inhibition of SIK2 and SIK3 during differentiation enhances the anti-inflammatory phenotype of macrophages. *Biochem J* 474, 521–537 (2017).

76. Takemori, H., Katoh, Y., Horike, N., Doi, J. & Okamoto, M. ACTH-induced Nucleocytoplasmic Translocation of Salt-inducible Kinase Implication in the protein kinase A-activated gene transcription in mouse adrenocortical tumor cells * 210. *J Biol Chem* 277, 42334–42343 (2002).

77. Sonntag, T., Vaughan, J. M. & Montminy, M. 14-3-3 proteins mediate inhibitory effects of cAMP on salt-inducible kinases (SIKs). *Febs J* 285, 467–480 (2018).

78. Patel, K., Foretz, M., Marion, A., Campbell, D. G., Gourlay, R., Boudaba, N., Tournier, E., Titchenell, P., Pegg, M., Deak, M., Wan, M., Kaestner, K. H., Göransson, O., Viollet, B., Gray, N. S., Birnbaum, M. J., Sutherland, C. & Sakamoto, K. The LKB1-salt-inducible kinase pathway functions as a key gluconeogenic suppressor in the liver. *Nat Commun* 5, 4535 (2014).

79. MacKenzie, K. F., Clark, K., Naqvi, S., McGuire, V. A., Nöehren, G., Kristariyanto, Y., Bosch, M. van den, Mudaliar, M., McCarthy, P. C., Pattison, M. J., Pedrioli, P. G. A., Barton, G. J., Toth, R., Prescott, A. & Arthur, J. S. C. PGE2 Induces Macrophage IL-10 Production and a Regulatory-like Phenotype via a Protein Kinase A–SIK–CRTCL Pathway. *J Immunol* 190, 565–577 (2013).

80. Berdeaux, R., Goebel, N., Banaszynski, L., Takemori, H., Wandless, T., Shelton, G. D. & Montminy, M. SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. *Nat Med* 13, 597–603 (2007).

81. Sakamaki, J.-I., Fu, A., Reeks, C., Baird, S., Depatie, C., Azzabi, M. A., Bardeesy, N., Gingras, A.-C., Yee, S.-P. & Srean, R. A. Role of the SIK2–p35–PJA2 complex in pancreatic β -cell functional compensation. *Nat Cell Biol* 16, 234–244 (2014).

82. Katoh, Y., Takemori, H., Doi, J. & Okamoto, M. IDENTIFICATION OF THE NUCLEAR LOCALIZATION DOMAIN OF SALT-INDUCIBLE KINASE. *Endocr Res* 28, 315–318 (2002).

83. Clark, K., MacKenzie, K. F., Petkevicius, K., Kristariyanto, Y., Zhang, J., Choi, H. G., Pegg, M., Plater, L., Pedrioli, P. G. A., McIver, E., Gray, N. S., Arthur, J. S. C. & Cohen, P. Phosphorylation of CRTCL by the salt-inducible kinases controls the interconversion of

classically activated and regulatory macrophages. *Proc National Acad Sci* 109, 16986–16991 (2012).

84. Screatton, R. A., Conkright, M. D., Katoh, Y., Best, J. L., Canettieri, G., Jeffries, S., Guzman, E., Niessen, S., Yates, J. R., Takemori, H., Okamoto, M. & Montminy, M. The CREB Coactivator TORC2 Functions as a Calcium- and cAMP-Sensitive Coincidence Detector. *Cell* 119, 61–74 (2004).

85. Altarejos, J. Y. & Montminy, M. CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. *Nat Rev Mol Cell Bio* 12, 141–151 (2011).

86. Henriksson, E., Säll, J., Gormand, A., Wasserstrom, S., Morrice, N. A., Fritzen, A. M., Foretz, M., Campbell, D. G., Sakamoto, K., Ekelund, M., Degerman, E., Stenkula, K. G. & Göransson, O. SIK2 regulates CRTCs, HDAC4 and glucose uptake in adipocytes. *J Cell Sci* 128, 472–486 (2014).

87. Sonntag, T., Moresco, J. J., Vaughan, J. M., Matsumura, S., Yates, J. R. & Montminy, M. Analysis of a cAMP regulated coactivator family reveals an alternative phosphorylation motif for AMPK family members. *Plos One* 12, e0173013 (2017).

88. Luo, Q., Viste, K., Urdy-Zaa, J. C., Kumar, G. S., Tsai, W.-W., Talai, A., Mayo, K. E., Montminy, M. & Radhakrishnan, I. Mechanism of CREB recognition and coactivation by the CREB-regulated transcriptional coactivator CRTC2. *Proc National Acad Sci* 109, 20865–20870 (2012).

89. Mayr, B. & Montminy, M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Bio* 2, 599–609 (2001).

90. Mihaylova, M. M., Vasquez, D. S., Ravnskjaer, K., Denechaud, P.-D., Yu, R. T., Alvarez, J. G., Downes, M., Evans, R. M., Montminy, M. & Shaw, R. J. Class IIa Histone Deacetylases Are Hormone-Activated Regulators of FOXO and Mammalian Glucose Homeostasis. *Cell* 145, 607–621 (2011).

91. Chan, J. K. L., Sun, L., Yang, X.-J., Zhu, G. & Wu, Z. Functional Characterization of an Amino-terminal Region of HDAC4 That Possesses MEF2 Binding and Transcriptional Repressive Activity*. *J Biol Chem* 278, 23515–23521 (2003).

92. Linden, A. M. van der, Nolan, K. M. & Sengupta, P. KIN-29 SIK regulates chemoreceptor gene expression via an MEF2 transcription factor and a class II HDAC. *Embo J* 26, 358–370 (2007).

93. Murray, C. W., Brady, J. J., Tsai, M. K., Li, C., Winters, I. P., Tang, R., Andrejka, L., Ma, R. K., Kunder, C. A., Chu, P. & Winslow, M. M. An LKB1–SIK Axis Suppresses Lung Tumor Growth and Controls Differentiation. *Cancer Discov* 9, CD-18-1237 (2019).

94. Patra, K. C., Kato, Y., Mizukami, Y., Widholz, S., Boukhali, M., Revenco, I., Grossman, E. A., Ji, F., Sadreyev, R. I., Liss, A. S., Screatton, R. A., Sakamoto, K., Ryan, D. P., Mino-Kenudson, M., Castillo, C. F., Nomura, D. K., Haas, W. & Bardeesy, N. Mutant GNAS drives pancreatic

tumourigenesis by inducing PKA-mediated SIK suppression and reprogramming lipid metabolism. *Nat Cell Biol* 20, 811–822 (2018).

95. Tarumoto, Y., Lu, B., Somerville, T. D. D., Huang, Y.-H., Milazzo, J. P., Wu, X. S., Klingbeil, O., Demerdash, O. E., Shi, J. & Vakoc, C. R. LKB1, Salt-Inducible Kinases, and MEF2C Are Linked Dependencies in Acute Myeloid Leukemia. *Mol Cell* 69, 1017–1027.e6 (2018).

96. Tarumoto, Y., Lin, S., Wang, J., Milazzo, J. P., Xu, Y., Lu, B., Yang, Z., Wei, Y., Polyanskaya, S., Wunderlich, M., Gray, N. S., Stegmaier, K. & Vakoc, C. R. Salt-inducible kinase inhibition suppresses acute myeloid leukemia progression in vivo. *Blood* 135, 56–70 (2020).

97. Siegel, R. L., Miller, K. D., Wagle, N. S. & Jemal, A. Cancer statistics, 2023. *Ca Cancer J Clin* 73, 17–48 (2023).

98. Govindan, R., Page, N., Morgensztern, D., Read, W., Tierney, R., Vlahiotis, A., Spitznagel, E. L. & Piccirillo, J. Changing Epidemiology of Small-Cell Lung Cancer in the United States Over the Last 30 Years: Analysis of the Surveillance, Epidemiologic, and End Results Database. *J Clin Oncol* 24, 4539–4544 (2006).

99. Arbour, K. C. & Riely, G. J. Systemic Therapy for Locally Advanced and Metastatic Non–Small Cell Lung Cancer. *Jama* 322, 764–774 (2019).

100. Skoulidis, F., Goldberg, M. E., Greenawalt, D. M., Hellmann, M. D., Awad, M. M., Gainor, J. F., Schrock, A. B., Hartmaier, R. J., Trabucco, S. E., Gay, L., Ali, S. M., Elvin, J. A., Singal, G., Ross, J. S., Fabrizio, D., Szabo, P. M., Chang, H., Sasson, A., Srinivasan, S., Kirov, S., Szustakowski, J., Vitazka, P., Edwards, R., Bufill, J. A., Sharma, N., Ou, S.-H. I., Peled, N., Spigel, D. R., Rizvi, H., Aguilar, E. J., Carter, B. W., Erasmus, J., Halpenny, D. F., Plodkowski, A. J., Long, N. M., Nishino, M., Denning, W. L., Galan-Cobo, A., Hamdi, H., Hirz, T., Tong, P., Wang, J., Rodriguez-Canales, J., Villalobos, P. A., Parra, E. R., Kalhor, N., Sholl, L. M., Sauter, J. L., Jungbluth, A. A., Mino-Kenudson, M., Azimi, R., Elamin, Y. Y., Zhang, J., Leonardi, G. C., Jiang, F., Wong, K.-K., Lee, J. J., Papadimitrakopoulou, V. A., Wistuba, I. I., Miller, V. A., Frampton, G. M., Wolchok, J. D., Shaw, A. T., Jänne, P. A., Stephens, P. J., Rudin, C. M., Geese, W. J., Albacker, L. A. & Heymach, J. V. STK11/LKB1 Mutations and PD-1 Inhibitor Resistance in KRAS-Mutant Lung Adenocarcinoma. *Cancer Discov* 8, CD-18-0099 (2018).

101. Sanchez-Cespedes, M., Parrella, P., Esteller, M., Nomoto, S., Trink, B., Engles, J. M., Westra, W. H., Herman, J. G. & Sidransky, D. Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer Res* 62, 3659–62 (2002).

102. Ding, L., Getz, G., Wheeler, D. A., Mardis, E. R., McLellan, M. D., Cibulskis, K., Sougnez, C., Greulich, H., Muzny, D. M., Morgan, M. B., Fulton, L., Fulton, R. S., Zhang, Q., Wendl, M. C., Lawrence, M. S., Larson, D. E., Chen, K., Dooling, D. J., Sabo, A., Hawes, A. C., Shen, H., Jhangiani, S. N., Lewis, L. R., Hall, O., Zhu, Y., Mathew, T., Ren, Y., Yao, J., Scherer, S. E., Clerc, K., Metcalf, G. A., Ng, B., Milosavljevic, A., Gonzalez-Garay, M. L., Osborne, J. R., Meyer, R., Shi, X., Tang, Y., Koboldt, D. C., Lin, L., Abbott, R., Miner, T. L., Pohl, C., Fewell, G., Haipek, C., Schmidt, H., Dunford-Shore, B. H., Kraja, A., Crosby, S. D., Sawyer, C. S., Vickery, T., Sander, S., Robinson, J., Winckler, W., Baldwin, J., Chirieac, L. R., Dutt, A., Fennell, T., Hanna, M., Johnson, B. E., Onofrio, R. C., Thomas, R. K., Tonon, G., Weir, B. A., Zhao, X., Ziaugra, L., Zody,

- M. C., Giordano, T., Orringer, M. B., Roth, J. A., Spitz, M. R., Wistuba, I. I., Ozenberger, B., Good, P. J., Chang, A. C., Beer, D. G., Watson, M. A., Ladanyi, M., Broderick, S., Yoshizawa, A., Travis, W. D., Pao, W., Province, M. A., Weinstock, G. M., Varmus, H. E., Gabriel, S. B., Lander, E. S., Gibbs, R. A., Meyerson, M. & Wilson, R. K. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 455, 1069 (2008).
103. Osoegawa, A., Kometani, T., Nosaki, K., Ondo, K., Hamatake, M., Hirai, F., Seto, T., Sugio, K. & Ichinose, Y. LKB1 Mutations Frequently Detected in Mucinous Bronchioloalveolar Carcinoma. *Jpn J Clin Oncol* 41, 1132–1137 (2011).
104. Skoulidis, F. & Heymach, J. V. Co-occurring genomic alterations in non-small-cell lung cancer biology and therapy. *Nat Rev Cancer* 19, 495–509 (2019).
105. Sanchez-Cespedes, M. A role for LKB1 gene in human cancer beyond the Peutz–Jeghers syndrome. *Oncogene* 26, 7825–7832 (2007).
106. Skoulidis, F., Byers, L. A., Diao, L., Papadimitrakopoulou, V. A., Tong, P., Izzo, J., Behrens, C., Kadara, H., Parra, E. R., Canales, J. R., Zhang, J., Giri, U., Gudikote, J., Cortez, M. A., Yang, C., Fan, Y., Peyton, M., Girard, L., Coombes, K. R., Toniatti, C., Heffernan, T. P., Choi, M., Frampton, G. M., Miller, V., Weinstein, J. N., Herbst, R. S., Wong, K.-K., Zhang, J., Sharma, P., Mills, G. B., Hong, W. K., Minna, J. D., Allison, J. P., Futreal, A., Wang, J., Wistuba, I. I. & Heymach, J. V. Co-occurring Genomic Alterations Define Major Subsets of KRAS-Mutant Lung Adenocarcinoma with Distinct Biology, Immune Profiles, and Therapeutic Vulnerabilities. *Cancer Discov* 5, 860–877 (2015).
107. Kaufman, J. M., Amann, J. M., Park, K., Arasada, R. R., Li, H., Shyr, Y. & Carbone, D. P. LKB1 Loss Induces Characteristic Patterns of Gene Expression in Human Tumors Associated with NRF2 Activation and Attenuation of PI3K-AKT. *J Thorac Oncol* 9, 794–804 (2014).
108. Chen, Z., Cheng, K., Walton, Z., Wang, Y., Ebi, H., Shimamura, T., Liu, Y., Tupper, T., Ouyang, J., Li, J., Gao, P., Woo, M. S., Xu, C., Yanagita, M., Altabef, A., Wang, S., Lee, C., Nakada, Y., Peña, C. G., Sun, Y., Franchetti, Y., Yao, C., Saur, A., Cameron, M. D., Nishino, M., Hayes, D. N., Wilkerson, M. D., Roberts, P. J., Lee, C. B., Bardeesy, N., Butaney, M., Chirieac, L. R., Costa, D. B., Jackman, D., Sharpless, N. E., Castrillon, D. H., Demetri, G. D., Jänne, P. A., Pandolfi, P. P., Cantley, L. C., Kung, A. L., Engelman, J. A. & Wong, K.-K. A murine lung cancer co-clinical trial identifies genetic modifiers of therapeutic response. *Nature* 483, 613–617 (2012).
109. Han, X., Li, F., Fang, Z., Gao, Y., Li, F., Fang, R., Yao, S., Sun, Y., Li, L., Zhang, W., Ma, H., Xiao, Q., Ge, G., Fang, J., Wang, H., Zhang, L., Wong, K., Chen, H., Hou, Y. & Ji, H. Transdifferentiation of lung adenocarcinoma in mice with Lkb1 deficiency to squamous cell carcinoma. *Nat Commun* 5, 3261 (2014).
110. Ji, H., Ramsey, M. R., Hayes, D. N., Fan, C., McNamara, K., Kozlowski, P., Torrice, C., Wu, M. C., Shimamura, T., Perera, S. A., Liang, M.-C., Cai, D., Naumov, G. N., Bao, L., Contreras, C. M., Li, D., Chen, L., Krishnamurthy, J., Koivunen, J., Chirieac, L. R., Padera, R. F., Bronson, R. T., Lindeman, N. I., Christiani, D. C., Lin, X., Shapiro, G. I., Jänne, P. A., Johnson, B. E., Meyerson, M., Kwiatkowski, D. J., Castrillon, D. H., Bardeesy, N., Sharpless, N. E. & Wong, K.-K. LKB1 modulates lung cancer differentiation and metastasis. *Nature* 448, 807–810 (2007).

111. Shackelford, D. B., Abt, E., Gerken, L., Vasquez, D. S., Seki, A., Leblanc, M., Wei, L., Fishbein, M. C., Czernin, J., Mischel, P. S. & Shaw, R. J. LKB1 Inactivation Dictates Therapeutic Response of Non-Small Cell Lung Cancer to the Metabolism Drug Phenformin. *Cancer Cell* 23, 143–158 (2013).
112. Svensson, R. U., Parker, S. J., Eichner, L. J., Kolar, M. J., Wallace, M., Brun, S. N., Lombardo, P. S., Nostrand, J. L. V., Hutchins, A., Vera, L., Gerken, L., Greenwood, J., Bhat, S., Harriman, G., Westlin, W. F., Harwood, H. J., Saghatelian, A., Kapeller, R., Metallo, C. M. & Shaw, R. J. Inhibition of acetyl-CoA carboxylase suppresses fatty acid synthesis and tumor growth of non-small-cell lung cancer in preclinical models. *Nat Med* 22, nm.4181 (2016).
113. Zhang, H., Brainson, C. F., Koyama, S., Redig, A. J., Chen, T., Li, S., Gupta, M., Garcia-de-Alba, C., Paschini, M., Herter-Sprie, G. S., Lu, G., Zhang, X., Marsh, B. P., Tuminello, S. J., Xu, C., Chen, Z., Wang, X., Akbay, E. A., Zheng, M., Palakurthi, S., Sholl, L. M., Rustgi, A. K., Kwiatkowski, D. J., Diehl, J. A., Bass, A. J., Sharpless, N. E., Dranoff, G., Hammerman, P. S., Ji, H., Bardeesy, N., Saur, D., Watanabe, H., Kim, C. F. & Wong, K.-K. Lkb1 inactivation drives lung cancer lineage switching governed by Polycomb Repressive Complex 2. *Nat Commun* 8, ncomms14922 (2017).
114. Koyama, S., Akbay, E. A., Li, Y. Y., Aref, A. R., Skoulidis, F., Herter-Sprie, G. S., Buczkowski, K. A., Liu, Y., Awad, M. M., Denning, W. L., Diao, L., Wang, J., Parra-Cuentas, E. R., Wistuba, I. I., Soucheray, M., Thai, T., Asahina, H., Kitajima, S., Altabef, A., Cavanaugh, J. D., Rhee, K., Gao, P., Zhang, H., Fecci, P. E., Shimamura, T., Hellmann, M. D., Heymach, J. V., Hodi, F. S., Freeman, G. J., Barbie, D. A., Dranoff, G., Hammerman, P. S. & Wong, K.-K. STK11/LKB1 Deficiency Promotes Neutrophil Recruitment and Proinflammatory Cytokine Production to Suppress T-cell Activity in the Lung Tumor Microenvironment. *Cancer Res* 76, 999–1008 (2016).
115. Biton, J., Mansuet-Lupo, A., Pécuchet, N., Alifano, M., Ouakrim, H., Arrondeau, J., Boudou-Rouquette, P., Goldwasser, F., Leroy, K., Goc, J., Wislez, M., Germain, C., Laurent-Puig, P., Dieu-Nosjean, M.-C., Cremer, I., Herbst, R., Blons, H. F. & Damotte, D. TP53, STK11 and EGFR Mutations Predict Tumor Immune Profile and the Response to anti-PD-1 in Lung Adenocarcinoma. *Clin Cancer Res* 24, clincanres.0163.2018 (2018).
116. Rizvi, H., Sanchez-Vega, F., La, K., Chatila, W., Jonsson, P., Halpenny, D., Plodkowski, A., Long, N., Sauter, J. L., Rekhtman, N., Hollmann, T., Schalper, K. A., Gainor, J. F., Shen, R., Ni, A., Arbour, K. C., Merghoub, T., Wolchok, J., Snyder, A., Chaft, J. E., Kris, M. G., Rudin, C. M., Socci, N. D., Berger, M. F., Taylor, B. S., Zehir, A., Solit, D. B., Arcila, M. E., Ladanyi, M., Riely, G. J., Schultz, N. & Hellmann, M. D. Molecular Determinants of Response to Anti-Programmed Cell Death (PD)-1 and Anti-Programmed Death-Ligand (PD-L)-Ligand 1 Blockade in Patients With Non-Small-Cell Lung Cancer Profiled With Targeted Next-Generation Sequencing. *J Clin Oncol* 36, JCO.2017.75.338 (2018).
117. Faubert, B., Boily, G., Izreig, S., Griss, T., Samborska, B., Dong, Z., Dupuy, F., Chambers, C., Fuerth, B. J., Viollet, B., Mamer, O. A., Avizonis, D., DeBerardinis, R. J., Siegel, P. M. & Jones, R. G. AMPK Is a Negative Regulator of the Warburg Effect and Suppresses Tumor Growth In Vivo. *Cell Metab* 17, 113–124 (2013).

118. Houde, V. P., Donzelli, S., Sacconi, A., Galic, S., Hammill, J. A., Bramson, J. L., Foster, R. A., Tsakiridis, T., Kemp, B. E., Grasso, G., Blandino, G., Muti, P. & Steinberg, G. R. AMPK β 1 reduces tumor progression and improves survival in p53 null mice. *Mol Oncol* 11, 1143–1155 (2017).
119. Chan, L. N., Chen, Z., Braas, D., Lee, J.-W., Xiao, G., Geng, H., Cosgun, K. N., Hurtz, C., Shojaee, S., Cazzaniga, V., Schjerven, H., Ernst, T., Hochhaus, A., Kornblau, S. M., Konopleva, M., Pufall, M. A., Cazzaniga, G., Liu, G. J., Milne, T. A., Koeffler, H. P., Ross, T. S., Sánchez-García, I., Borkhardt, A., Yamamoto, K. R., Dickins, R. A., Graeber, T. G. & Müschen, M. Metabolic gatekeeper function of B-lymphoid transcription factors. *Nature* 542, 479–483 (2017).
120. Kishton, R. J., Barnes, C. E., Nichols, A. G., Cohen, S., Gerriets, V. A., Siska, P. J., Macintyre, A. N., Goraksha-Hicks, P., de Cubas, A. A., Liu, T., Warmoes, M. O., Abel, E. D., Yeoh, A. E. J., Gershon, T. R., Rathmell, W. K., Richards, K. L., Locasale, J. W. & Rathmell, J. C. AMPK Is Essential to Balance Glycolysis and Mitochondrial Metabolism to Control T-ALL Cell Stress and Survival. *Cell Metab* 23, 649–662 (2016).
121. Saito, Y., Chapple, R. H., Lin, A., Kitano, A. & Nakada, D. AMPK Protects Leukemia-Initiating Cells in Myeloid Leukemias from Metabolic Stress in the Bone Marrow. *Cell Stem Cell* 17, 585–596 (2015).
122. Cheng, H., Liu, P., Wang, Z. C., Zou, L., Santiago, S., Garbitt, V., Gjoerup, O. V., Iglehart, J. D., Miron, A., Richardson, A. L., Hahn, W. C. & Zhao, J. J. SIK1 Couples LKB1 to p53-Dependent Anoikis and Suppresses Metastasis. *Sci Signal* 2, ra35 (2009).
123. Ahmed, A. A., Lu, Z., Jennings, N. B., Etemadmoghadam, D., Capalbo, L., Jacamo, R. O., Barbosa-Morais, N., Le, X.-F., Group, A. O. C. S., Vivas-Mejia, P., Lopez-Berestein, G., Grandjean, G., Bartholomeusz, G., Liao, W., Andreeff, M., Bowtell, D., Glover, D. M., Sood, A. K. & Bast, R. C. SIK2 Is a Centrosome Kinase Required for Bipolar Mitotic Spindle Formation that Provides a Potential Target for Therapy in Ovarian Cancer. *Cancer Cell* 18, 109–121 (2010).
124. Itoh, Y., Sanosaka, M., Fuchino, H., Yahara, Y., Kumagai, A., Takemoto, D., Kagawa, M., Doi, J., Ohta, M., Tsumaki, N., Kawahara, N. & Takemori, H. Salt-inducible Kinase 3 Signaling Is Important for the Gluconeogenic Programs in Mouse Hepatocytes. *J Biol Chem* 290, 17879–17893 (2015).
125. Chen, Z., Fillmore, C. M., Hammerman, P. S., Kim, C. F. & Wong, K.-K. Non-small-cell lung cancers: a heterogeneous set of diseases. *Nat Rev Cancer* 14, 535–546 (2014).
126. Sánchez-Rivera, F. J., Papagiannakopoulos, T., Romero, R., Tammela, T., Bauer, M. R., Bhutkar, A., Joshi, N. S., Subbaraj, L., Bronson, R. T., Xue, W. & Jacks, T. Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature* 516, 428–431 (2014).
127. Platt, R. J., Chen, S., Zhou, Y., Yim, M. J., Swiech, L., Kempton, H. R., Dahlman, J. E., Parnas, O., Eisenhaure, T. M., Jovanovic, M., Graham, D. B., Jhunjhunwala, S., Heidenreich, M., Xavier, R. J., Langer, R., Anderson, D. G., Hacohen, N., Regev, A., Feng, G., Sharp, P. A. &

Zhang, F. CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. *Cell* 159, 440–455 (2014).

128. Nixon, M., Stewart-Fitzgibbon, R., Fu, J., Akhmedov, D., Rajendran, K., Mendoza-Rodriguez, M. G., Rivera-Molina, Y. A., Gibson, M., Berglund, E. D., Justice, N. J. & Berdeaux, R. Skeletal muscle salt inducible kinase 1 promotes insulin resistance in obesity. *Mol Metab* 5, 34–46 (2016).

129. Xu, C., Fillmore, C. M., Koyama, S., Wu, H., Zhao, Y., Chen, Z., Herter-Sprie, G. S., Akbay, E. A., Tchaicha, J. H., Altabef, A., Reibel, J. B., Walton, Z., Ji, H., Watanabe, H., Jänne, P. A., Castrillon, D. H., Rustgi, A. K., Bass, A. J., Freeman, G. J., Padera, R. F., Dranoff, G., Hammerman, P. S., Kim, C. F. & Wong, K.-K. Loss of Lkb1 and Pten Leads to Lung Squamous Cell Carcinoma with Elevated PD-L1 Expression. *Cancer Cell* 25, 590–604 (2014).

130. Gilbert-Ross, M., Konen, J., Koo, J., Shupe, J., Robinson, B. S., Wiles, W. G., Huang, C., Martin, W. D., Behera, M., Smith, G. H., Hill, C. E., Rossi, M. R., Sica, G. L., Rupji, M., Chen, Z., Kowalski, J., Kasinski, A. L., Ramalingam, S. S., Fu, H., Khuri, F. R., Zhou, W. & Marcus, A. I. Targeting adhesion signaling in KRAS, LKB1 mutant lung adenocarcinoma. *Jci Insight* 2, e90487 (2017).

131. Luo, Y. & Zheng, S. G. Hall of Fame among Pro-inflammatory Cytokines: Interleukin-6 Gene and Its Transcriptional Regulation Mechanisms. *Front Immunol* 7, 604 (2016).

132. Hollstein, P. E. & Shaw, R. J. Inflamed T cells and stroma drive gut tumors. *Science* 361, 332–333 (2018).

133. Hardie, D. G., Ross, F. A. & Hawley, S. A. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Bio* 13, 251–262 (2012).

134. Mukhopadhyay, A., Berrett, K. C., Kc, U., Clair, P. M., Pop, S. M., Carr, S. R., Witt, B. L. & Oliver, T. G. Sox2 Cooperates with Lkb1 Loss in a Mouse Model of Squamous Cell Lung Cancer. *Cell Reports* 8, 40–49 (2014).

135. Park, J., Yoon, Y.-S., Han, H.-S., Kim, Y.-H., Ogawa, Y., Park, K.-G., Lee, C.-H., Kim, S.-T. & Koo, S.-H. SIK2 Is Critical in the Regulation of Lipid Homeostasis and Adipogenesis In Vivo. *Diabetes* 63, 3659–3673 (2014).

136. Canettieri, G., Coni, S., Guardia, M. D., Nocerino, V., Antonucci, L., Magno, L. D., Screaton, R., Screpanti, I., Giannini, G. & Gulino, A. The coactivator CRTC1 promotes cell proliferation and transformation via AP-1. *Proc National Acad Sci* 106, 1445–1450 (2009).

137. Bitteringer, M. A., McWhinnie, E., Meltzer, J., Iourgenko, V., Latario, B., Liu, X., Chen, C. H., Song, C., Garza, D. & Labow, M. Activation of cAMP Response Element-Mediated Gene Expression by Regulated Nuclear Transport of TORC Proteins. *Curr Biol* 14, 2156–2161 (2004).

138. Iourgenko, V., Zhang, W., Mickanin, C., Daly, I., Jiang, C., Hexham, J. M., Orth, A. P., Miraglia, L., Meltzer, J., Garza, D., Chirn, G.-W., McWhinnie, E., Cohen, D., Skelton, J., Terry, R., Yu, Y., Bodian, D., Buxton, F. P., Zhu, J., Song, C. & Labow, M. A. Identification of a family of

cAMP response element-binding protein coactivators by genome-scale functional analysis in mammalian cells. *Proc National Acad Sci* 100, 12147–12152 (2003).

139. Chen, Z., Chen, J., Gu, Y., Hu, C., Li, J.-L., Lin, S., Shen, H., Cao, C., Gao, R., Li, J., Ha, P. K., Kaye, F. J., Griffin, J. D. & Wu, L. Aberrantly activated AREG–EGFR signaling is required for the growth and survival of CRTC1–MAML2 fusion-positive mucoepidermoid carcinoma cells. *Oncogene* 33, 3869–3877 (2014).

140. Feng, Y., Wang, Y., Wang, Z., Fang, Z., Li, F., Gao, Y., Liu, H., Xiao, T., Li, F., Zhou, Y., Zhai, Q., Liu, X., Sun, Y., Bardeesy, N., Wong, K., Chen, H., Xiong, Z. & Ji, H. The CRTC1–NEDD9 Signaling Axis Mediates Lung Cancer Progression Caused by LKB1 Loss. *Cancer Res* 72, 6502–6511 (2012).

141. Rodón, L., Svensson, R. U., Wiater, E., Chun, M. G. H., Tsai, W.-W., Eichner, L. J., Shaw, R. J. & Montminy, M. The CREB coactivator CRTC2 promotes oncogenesis in LKB1-mutant non-small cell lung cancer. *Sci Adv* 5, eaaw6455 (2019).

142. Carretero, J., Shimamura, T., Rikova, K., Jackson, A. L., Wilkerson, M. D., Borgman, C. L., Buttarazzi, M. S., Sanofsky, B. A., McNamara, K. L., Brandstetter, K. A., Walton, Z. E., Gu, T.-L., Silva, J. C., Crosby, K., Shapiro, G. I., Maira, S.-M., Ji, H., Castrillon, D. H., Kim, C. F., García-Echeverría, C., Bardeesy, N., Sharpless, N. E., Hayes, N. D., Kim, W. Y., Engelman, J. A. & Wong, K.-K. Integrative Genomic and Proteomic Analyses Identify Targets for Lkb1-Deficient Metastatic Lung Tumors. *Cancer Cell* 17, 547–559 (2010).

143. Ollila, S., Domènech-Moreno, E., Laajanen, K., Wong, I. P. L., Tripathi, S., Penttimikko, N., Gao, Y., Yan, Y., Niemelä, E. H., Wang, T. C., Viollet, B., Leone, G., Katajisto, P., Vaahtomeri, K. & Mäkelä, T. P. Stromal Lkb1 deficiency leads to gastrointestinal tumorigenesis involving the IL-11–JAK/STAT3 pathway. *J Clin Invest* 128, 402–414 (2017).

144. Poffenberger, M. C., Metcalfe-Roach, A., Aguilar, E., Chen, J., Hsu, B. E., Wong, A. H., Johnson, R. M., Flynn, B., Samborska, B., Ma, E. H., Gravel, S.-P., Tonelli, L., Devorkin, L., Kim, P., Hall, A., Izreig, S., Loginicheva, E., Beauchemin, N., Siegel, P. M., Artyomov, M. N., Lum, J. J., Zogopoulos, G., Blagih, J. & Jones, R. G. LKB1 deficiency in T cells promotes the development of gastrointestinal polyposis. *Science* 361, 406–411 (2018).

145. Kim, S. Y., Jeong, S., Chah, K.-H., Jung, E., Baek, K.-H., Kim, S.-T., Shim, J.-H., Chun, E. & Lee, K.-Y. Salt-Inducible Kinases 1 and 3 Negatively Regulate Toll-Like Receptor 4-Mediated Signal. *Mol Endocrinol* 27, 1958–1968 (2013).

146. DuPage, M., Dooley, A. L. & Jacks, T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc* 4, 1064–1072 (2009).

147. Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., Cheng, J. X., Murre, C., Singh, H. & Glass, C. K. Simple Combinations of Lineage-Determining Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and B Cell Identities. *Mol Cell* 38, 576–589 (2010).

148. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139–140 (2010).
149. Hulsen, T., Vlieg, J. de & Alkema, W. BioVenn – a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *Bmc Genomics* 9, 488 (2008).
150. Groot, P. M. de, Wu, C. C., Carter, B. W. & Munden, R. F. The epidemiology of lung cancer. *Transl Lung Cancer Res* 7, 220–233 (2018).
151. Inamura, K. Lung Cancer: Understanding Its Molecular Pathology and the 2015 WHO Classification. *Frontiers Oncol* 7, 193 (2017).
152. Lier, M. G. F. van, Westerman, A. M., Wagner, A., Looman, C. W. N., Wilson, J. H. P., Rooij, F. W. M. de, Lemmens, V. E. P. P., Kuipers, E. J., Mathus-Vliegen, E. M. H. & Leerdam, M. E. van. High cancer risk and increased mortality in patients with Peutz–Jeghers syndrome. *Gut* 60, 141 (2011).
153. Koivunen, J. P., Kim, J., Lee, J., Rogers, A. M., Park, J. O., Zhao, X., Naoki, K., Okamoto, I., Nakagawa, K., Yeap, B. Y., Meyerson, M., Wong, K.-K., Richards, W. G., Sugarbaker, D. J., Johnson, B. E. & Jänne, P. A. Mutations in the LKB1 tumour suppressor are frequently detected in tumours from Caucasian but not Asian lung cancer patients. *Brit J Cancer* 99, 245–252 (2008).
154. Contreras, C. M., Gurumurthy, S., Haynie, J. M., Shirley, L. J., Akbay, E. A., Wingo, S. N., Schorge, J. O., Broaddus, R. R., Wong, K.-K., Bardeesy, N. & Castrillon, D. H. Loss of Lkb1 Provokes Highly Invasive Endometrial Adenocarcinomas. *Cancer Res* 68, 759–766 (2008).
155. Contreras, C. M., Akbay, E. A., Gallardo, T. D., Haynie, J. M., Sharma, S., Tagao, O., Bardeesy, N., Takahashi, M., Settleman, J., Wong, K.-K. & Castrillon, D. H. Lkb1 inactivation is sufficient to drive endometrial cancers that are aggressive yet highly responsive to mTOR inhibitor monotherapy. *Dis Model Mech* 3, 181–193 (2010).
156. Esteller, M., Avizienyte, E., Corn, P. G., Lothe, R. A., Baylin, S. B., Aaltonen, L. A. & Herman, J. G. Epigenetic inactivation of LKB1 in primary tumors associated with the Peutz-Jeghers syndrome. *Oncogene* 19, 164–168 (2000).
157. Rogers, Z. N., McFarland, C. D., Winters, I. P., Naranjo, S., Chuang, C.-H., Petrov, D. & Winslow, M. M. A quantitative and multiplexed approach to uncover the fitness landscape of tumor suppression in vivo. *Nat Methods* 14, 737–742 (2017).
158. Darling, N. J. & Cohen, P. Nuts and bolts of the salt-inducible kinases (SIKs). *Biochem J* 478, 1377–1397 (2021).
159. Akagi, K., Vooijs, M., Valk, M. V. der, Giovannini, M., Berns, A., Sandig, V. & Strauss, M. Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res* 25, 1766–1773 (1997).

160. Sawant, K. V., Sepuru, K. M., Lowry, E., Penaranda, B., Frevert, C. W., Garofalo, R. P. & Rajarathnam, K. Neutrophil recruitment by chemokines Cxcl1/KC and Cxcl2/MIP2: Role of Cxcr2 activation and glycosaminoglycan interactions. *J Leukocyte Biol* 109, 777–791 (2021).
161. Sawant, K. V., Poluri, K. M., Dutta, A. K., Sepuru, K. M., Troshkina, A., Garofalo, R. P. & Rajarathnam, K. Chemokine CXCL1 mediated neutrophil recruitment: Role of glycosaminoglycan interactions. *Sci Rep-uk* 6, 33123 (2016).
162. Zhang, X., Odom, D. T., Koo, S.-H., Conkright, M. D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E., Kadam, S., Ecker, J. R., Emerson, B., Hogenesch, J. B., Unterman, T., Young, R. A. & Montminy, M. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *P Natl Acad Sci Usa* 102, 4459–4464 (2005).
163. Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S. & Mesirov, J. P. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc National Acad Sci* 102, 15545–15550 (2005).
164. Mootha, V. K., Lindgren, C. M., Eriksson, K.-F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstråle, M., Laurila, E., Houstis, N., Daly, M. J., Patterson, N., Mesirov, J. P., Golub, T. R., Tamayo, P., Spiegelman, B., Lander, E. S., Hirschhorn, J. N., Altshuler, D. & Groop, L. C. PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34, 267–273 (2003).
165. Pan, D. The Hippo Signaling Pathway in Development and Cancer. *Dev Cell* 19, 491–505 (2010).
166. Ramos, A. & Camargo, F. D. The Hippo signaling pathway and stem cell biology. *Trends Cell Biol* 22, 339–346 (2012).
167. Wehr, M. C., Holder, M. V., Gailite, I., Saunders, R. E., Maile, T. M., Ciirdaeva, E., Instrell, R., Jiang, M., Howell, M., Rossner, M. J. & Tapon, N. Salt-inducible kinases regulate growth through the Hippo signalling pathway in Drosophila. *Nat Cell Biol* 15, 61–71 (2013).
168. DeRan, M., Yang, J., Shen, C.-H., Peters, E. C., Fitamant, J., Chan, P., Hsieh, M., Zhu, S., Asara, J. M., Zheng, B., Bardeesy, N., Liu, J. & Wu, X. Energy Stress Regulates Hippo-YAP Signaling Involving AMPK-Mediated Regulation of Angiomotin-like 1 Protein. *Cell Reports* 9, 495–503 (2014).
169. Mohseni, M., Sun, J., Lau, A., Curtis, S., Goldsmith, J., Fox, V. L., Wei, C., Frazier, M., Samson, O., Wong, K.-K., Wong, K.-K., Kim, C. & Camargo, F. D. A genetic screen identifies an LKB1-MARK signalling axis controlling the Hippo-YAP pathway. *Nat Cell Biol* 16, 108–117 (2014).
170. Mo, J.-S., Meng, Z., Kim, Y. C., Park, H. W., Hansen, C. G., Kim, S., Lim, D.-S. & Guan, K.-L. Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway. *Nat Cell Biol* 17, 500–510 (2015).

171. Gokey, J. J., Snowball, J., Sridharan, A., Sudha, P., Kitzmiller, J. A., Xu, Y. & Whitsett, J. A. YAP regulates alveolar epithelial cell differentiation and AGER via NFIB/KLF5/NKX2-1. *Isience* 24, 102967 (2021).
172. Bonanno, L., Zulato, E., Pavan, A., Attili, I., Pasello, G., Conte, P. & Indraccolo, S. LKB1 and Tumor Metabolism: The Interplay of Immune and Angiogenic Microenvironment in Lung Cancer. *Int J Mol Sci* 20, 1874 (2019).
173. Kim, J., Hu, Z., Cai, L., Li, K., Choi, E., Faubert, B., Bezwada, D., Rodriguez-Canales, J., Villalobos, P., Lin, Y.-F., Ni, M., Huffman, K. E., Girard, L., Byers, L. A., Unsal-Kacmaz, K., Peña, C. G., Heymach, J. V., Wauters, E., Vansteenkiste, J., Castrillon, D. H., Chen, B. P. C., Wistuba, I., Lambrechts, D., Xu, J., Minna, J. D. & DeBerardinis, R. J. CPS1 maintains pyrimidine pools and DNA synthesis in KRAS/LKB1-mutant lung cancer cells. *Nature* 546, 168–172 (2017).
174. Deng, J., Wen, C., Ding, X., Zhang, X., Hou, G., Liu, A., Xu, H., Cao, X. & Bai, Y. LKB1-MARK2 signalling mediates lipopolysaccharide-induced production of cytokines in mouse macrophages. *J Cell Mol Med* 24, 11307–11317 (2020).
175. Wan, L., Xu, K., Wei, Y., Zhang, J., Han, T., Fry, C., Zhang, Z., Wang, Y. V., Huang, L., Yuan, M., Xia, W., Chang, W.-C., Huang, W.-C., Liu, C.-L., Chang, Y.-C., Liu, J., Wu, Y., Jin, V. X., Dai, X., Guo, J., Liu, J., Jiang, S., Li, J., Asara, J. M., Brown, M., Hung, M.-C. & Wei, W. Phosphorylation of EZH2 by AMPK Suppresses PRC2 Methyltransferase Activity and Oncogenic Function. *Mol Cell* 69, 279-291.e5 (2018).
176. Pierce, S. E., Granja, J. M., Corces, M. R., Brady, J. J., Tsai, M. K., Pierce, A. B., Tang, R., Chu, P., Feldser, D. M., Chang, H. Y., Bassik, M. C., Greenleaf, W. J. & Winslow, M. M. LKB1 inactivation modulates chromatin accessibility to drive metastatic progression. *Nat Cell Biol* 23, 915–924 (2021).
177. Zhou, W., Zhang, J. & Marcus, A. I. LKB1 tumor suppressor: Therapeutic opportunities knock when LKB1 is inactivated. *Genes Dis* 1, 64–74 (2014).
178. Wang, W., Xiao, Z.-D., Li, X., Aziz, K. E., Gan, B., Johnson, R. L. & Chen, J. AMPK modulates Hippo pathway activity to regulate energy homeostasis. *Nat Cell Biol* 17, 490–499 (2015).
179. Skarnes, W. C., Rosen, B., West, A. P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A. O., Thomas, M., Harrow, J., Cox, T., Jackson, D., Severin, J., Biggs, P., Fu, J., Nefedov, M., Jong, P. J. de, Stewart, A. F. & Bradley, A. A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 474, 337–42 (2011).
180. Andrews, S. A Quality Control Tool for High Throughput Sequence Data. (2010). at <<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>>;
181. Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M. & Gingeras, T. R. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21 (2013).
182. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550 (2014).

183. Bankhead, P., Loughrey, M. B., Fernández, J. A., Dombrowski, Y., McArt, D. G., Dunne, P. D., McQuaid, S., Gray, R. T., Murray, L. J., Coleman, H. G., James, J. A., Salto-Tellez, M. & Hamilton, P. W. QuPath: Open source software for digital pathology image analysis. *Sci Rep-uk* 7, 16878 (2017).
184. Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J. P. & Tamayo, P. The Molecular Signatures Database Hallmark Gene Set Collection. *Cell Syst* 1, 417–425 (2015).