Kinase Suppressor of Ras 2 regulates small-cell lung carcinoma tumor propagating cells

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Abstract

Tumor propagating cells (TPCs) make up a small proportion of tumor cells responsible for self-renewal and long-term propagation of small-cell lung carcinoma (SCLC) tumors. Here, we show that Kinase Suppressor of Ras 2 (KSR2) is an important regulator of the self-renewing and clonogenic properties of SCLC cells. KSR2 is a molecular scaffold which promotes Raf/MEK/ERK signaling and AMPK signaling in SCLC. KSR2 is preferentially expressed in the ASCL1 subtype of SCLC tumors as well as the pulmonary neuroendocrine cells from which the SCLC tumors arise. The expression of KSR2 in SCLC and pulmonary neuroendocrine cells was previously unrecognized and serves as a novel model for understanding the role of KSR2-dependent signaling in normal and malignant tissues. Disruption of KSR2 in SCLC-A cell lines significantly reduces colony forming ability of TPCs *in vitro* and tumor initiating capacity *in vivo.* These data indicate that the expression of KSR2 is an essential driver of SCLC-A tumor propagating cell function, and therefore may play a role in SCLC tumor initiation. These findings shed light on a key distinct protein responsible for regulation in ASCL1 subtype SCLC tumors, and a potential subtype specific therapeutic target.

Introduction

Small-cell lung carcinoma (SCLC) affects current and former heavy smokers, accounting for 13% of all lung cancers[1]. There have been few improvements in SCLC detection, treatment, and survival in the past 30 years, leading to its classification as a recalcitrant cancer in 2012[1]. The five-year relative survival rates for SCLC patients with localized, regional, and distant disease are 27%, 16%, and 3%, respectively (American Cancer Society, 2021). Currently, SCLC tumors are treated with first line therapy (cisplatin or carboplatin plus anti-PDL1 antibody, atezolizumab), second (topotecan), and third line (PD1 antagonist, nivolumab) therapies. Although SCLC tumors are responsive to therapy initially, residual disease quickly develops resistance leading to the low five-year survival[2]. Substantial efforts have been made to characterize SCLC tumors and identify targets that may be selectively toxic to tumor cells while preserving normal lung tissue[3-7]. Rigorous and innovative basic science using state-of-the-art genetically engineered mouse models (GEMM), and an extensive set of cell lines have led to key discoveries regarding the cells-of-origin and the common recurring mutations that underlie SCLC[8-15]. These discoveries have yielded comprehensive genomic profiles and a durable classification of SCLC subtypes based on the differential expression of four key transcription factors, ASCL1 (SCLC-A), NeuroD1 (SCLC-N), Pou2F3 (SCLC-P) and Yap1 (SCLC-Y)[6, 12].

Lineage tracing and single cell RNA sequencing (scRNA-seq) in genetically modified mice showed that a rare PNEC subpopulation, NE^{stem} cells, actively responds to lung injury of the epithelia by expanding, migrating, and undergoing Notch-dependent transit amplification to regenerate the lost epithelium[16]. This effort additionally identified NE^{stem} as a cell-of-origin for SCLC following TP53, Rb and Notch mutation causing constitutive activation of stem cell renewal and altered deprogramming[16]. SCLC tumors have a small population of tumor propagating cells (TPCs) essential to the initiation, long-term propagation, and metastatic capacity of the tumor, while bulk non-TPC population are highly proliferative but incapable of establishing tumors *in vivo*[17]. TPCs have been implicated in initiation and growth of SCLC as well as therapy resistance[18-24]. Tumor propagating cells are also implicated in epithelial-to-mesenchymal transition (EMT) and metastasis[24]. Their slower cycling and self-renewing ability enhance DNA repair, rendering these cells resistant to DNA damage-dependent chemo and radiation therapy[20, 21, 24, 25]. Thus, SCLC TPCs offer a unique population within which to search for new targets, which in combination with current standard-of-care therapies may yield a durable and effective strategy for therapy.

Kinase Suppressor of Ras 2 (KSR2) is a molecular scaffold for Raf/MEK/ERK signaling, that also interacts with AMPK. AMPK is a critical intermediate of KSR2-dependent signals controlling cell and organismal metabolism[26, 27]. KSR2 is abundantly expressed in the brain, and disruption reduces body temperature, promotes cold intolerance, impairs glucose homeostasis, elevates fasting insulin and free fatty acid levels in the blood, and causes obesity[27]. Interestingly, ChIP-seq analysis of human ASCL1 subtype SCLC cell lines revealed KSR2 as a transcriptional target[28, 29]. KSR2 was identified as one of 24 druggable and overexpressed target genes of ASCL1 identified by Chip-seq, providing rationale for studying the role of KSR2 in ASCL1 subtype SCLC[30]. Our work reveals that KSR2 is expressed in PNECs as well as SCLC-A tumors and cell lines, although its role has not previously been determined. Expression of KSR2 promotes clonogenicity of SCLC-A TPCs and their tumor initiating capacity *in vitro* and *in vivo*. This result defines a novel mechanism of tumor initiation in SCLC-A and a potential therapeutic vulnerability.

Results

Pulmonary neuroendocrine cells and ASCL1 subtype SCLC tumor express KSR2. ASCL1 subtype (SCLC-A) tumors can arise from PNECs[16, 28, 30, 31]. PNECs are heterogeneous, including a small subpopulation termed NE^{stem} cells that respond to lung injury of the epithelia by expanding, migrating, and undergoing Notch-dependent transit amplification to regenerate the lost epithelium[16]. Although KSR2 mRNA is not detectable in normal epithelial lung tissue (Fig. 1A), it is present in PNECs (Fig. 1B). SCLC tumors may arise from PNECs following TP53, Rb, and Notch mutations causing constitutive activation of stem cell renewal and altered deprogramming[16]. Analysis of human SCLC tumors in collaboration with Dr. Trudy Oliver (Huntsman Cancer Center, U. Utah) showed that ASCL1-high and MYC-low tumors[12] preferentially express KSR2 mRNA (Fig. 1C). Analysis of KSR2 mRNA by subtype reveals that KSR2 is highly expressed in the ASCL1 subtype of SCLC (SCLC-A), but has varying expression in NeuroD1, POU2F3, and Yap1 subtypes (Fig. 1D). By western blot analysis, SCLC-A cell lines show high expression of KSR2, while KSR2 is not detectable in the NeuroD1 subtype (Fig. 1E, 1F).

Depletion of Kinase Suppressor of Ras 2 reduces SCLC clonogenicity and self-renewal. A small population of tumor-initiating cells termed "tumor propagating cells" (TPCs)[17] are responsible for the long-term propagation and survival of the tumor, as well as therapy resistance[17]. These cells are essential for tumor initiation and metastasis and are characterized by high expression of CD24 and EpCAM, and, somewhat surprisingly, low expression of CD44[17], which is often associated with stem cells. A key property of TPCs is their ability to both self-renew and differentiate into the highly proliferative non-TPC population of the tumor. Therefore, identifying the vulnerabilities specific to TPCs will lead to better understanding of mechanisms to perturb SCLC tumor-initiating cells. Transplantation assays show that TPCs are a minor but highly tumorigenic subpopulation of SCLC cells. This population can be analyzed by isolating single cells and measuring colony forming activity, which is an index of clonogenicity and self-renewal[17, 32]. Mouse SCLC cell lines KP1 and KP3 were derived from spontaneous GEMM of SCLC with knockout of Rb, p53 (KP1) and Rb, p53 and p130 (KP3), replicate the ASCL1 subtype of SCLC tumors[6, 10]. KP1 and KP3 cells can be stained for TPC markers $CD24^{high}CD44^{low}EpCAM^{high}$ and isolated by fluorescence-activated cell sorting (FACS) (Fig. 2A). KP1 and KP3 cells expressing one of three dox-inducible shRNAs targeting KSR2 (sh5, sh6, sh7) were treated with or without doxycycline (Fig. 2B). Following downregulation of KSR2 expression, the cells were stained for TPC markers $CD24^{high}CD44^{low}EpCAM^{high}$, isolated by FACS and plated as single cells in 96-well plates to be analyzed for colony formation by CellTiter-Glo®. The proportion of TPCs was significantly reduced with Dox-induced RNAi of KSR2 (Fig. 2C). Robust colony formation, a measure of self-renewing capability of an individual cell[33-35], was observed in KP3 control TPCs. Viability was reduced by 91%, 58%, and 45% with KSR2 RNAi by sh5, 6, and 7, respectively, which is proportional to their effectiveness at targeting KSR2 (Fig. 2B, D). In KP1 cells, colony formation was assessed with or without Dox-induced RNAi by the most effective hairpin sh5 after TPC isolation, which significantly inhibited colony formation (Fig. 2B, E). These data demonstrate that KSR2 is a significant contributor to the clonogenic and self-renewing properties of SCLC TPCs.

KSR2 regulates ERK and AMPK in SCLC cells. To confirm on target effects of our inducible shRNA system for KSR2 knockdown, a construct containing wildtype KSR2 mutated to be resistant to binding hairpin sh5 (KSR2-R) was expressed in KP1 sh5 cells (Fig. 3A). Rescue of KSR2 expression restored colony formation to wildtype levels in KP1 sh5 cells confirming on target effects of hairpin sh5 (Fig. 3B). The Raf/MEK/ERK pathway and AMPK interact with, and are modulated by KSR2 in human and mouse cell lines [27, 36]. Knockdown of KSR2 reduces activation of ERK and AMPK signaling in human (Fig. 3C) and murine (Fig. 3D) SCLC cell lines. These data show that KSR2 signaling is preserved in SCLC, suggesting that KSR2-dependent AMPK and/or ERK signaling may contribute to SCLC formation or maintenance.

KSR2 disruption inhibits the tumor initiating capacity of murine SCLC cells *in vivo*. Extreme limiting dilution analysis (ELDA) is a software application optimized for estimating the stem cell frequency from limiting dilution analysis[37]. *In vivo* ELDA determines the stem cell frequency within the bulk tumor cell population from the frequency of tumor-positive and tumor-negative injections at a variety of transplant doses. The effect of KSR2 disruption *in vivo* was tested using ELDA[17]. Dox-inducible shRNA targeted KP1 cells were injected with successive dilutions into NOD-*Prkd*^{cem26Cd52}*Il2rg*^{em26Cd22}/NjuCrl (NCG) mice, and the mice were provided drinking water with sucrose, or sucrose plus doxycycline (2 mg/kg). Tumors were monitored until one tumor reached 1 cm^2 and then all mice were sacrificed. ELDA was performed by scoring all tumors in the control group as "1" and all tumors retaining knockdown in the doxycycline treated group as "1". The absence of tumor formation was scored as "0". KSR2 disruption reduced frequency of TPCs 10-fold, from 1/255 control KP1 cells to 1/2530 KP1 cells with KSR2 KD (Fig. 4, Table 1). These data indicate that KSR2 is a critical effector of self-renewal and clonogenicity of SCLC tumor propagating cells *in vivo*.

Discussion

Our data have identified Kinase Suppressor of Ras 2 as a novel regulator of tumor initiation in ASCL1 subtype SCLC cells, and therefore a potential subtype specific therapeutic vulnerability. Due to modest improvement in early detection, therapeutic options, or survival in the last 30 years, SCLC was categorized as a recalcitrant cancer in 2012[38]. Rigorous molecular characterization of SCLC tumors has yielded four recognized subtypes of SCLC based on expression of transcription factors ASCL1, NeuroD1, YAP1, and POU2F3[6, 8, 12, 13]. Approximately 69% of SCLC tumors are ASCL1-dominant while 17% are NeuroD1-dominant, and 14% are

double negative for ASCL1 and NeuroD1[39]. Currently, all small-cell lung carcinomas are treated with the same standard of care despite recent evidence that Myc-driven NeuroD1 tumors are responsive to distinct therapies from ASCL1 subtype tumors[40, 41]. Our work shows that KSR2 is a novel regulator of SCLC-A TPC clonogenicity, and a potential subtype specific therapeutic target.

We have demonstrated the ability of KSR2 to regulate AMPK and ERK activation in SCLC cell lines similar to its previously defined role in mouse tissues[27, 42]. Previous studies tested the efficacy of targeting Raf/MEK/ERK signaling in SCLC have had mixed results. Treatment with ERK inhibitor was found not to induce apoptosis in human SCLC-A cell lines H209 and H69[43], or reduce proliferation in murine SCLC-A cell line KP1 (data not shown). In contrast to these data, ARHGEF19 disruption, which leads to reduced Raf/MEK/ERK signaling significantly reduced SCLC-A cell line proliferation *in vitro* and in *in vivo* tumor xenografts[44]. Activation of Raf/MEK/ERK by endoplasmic reticulum (ER) stress has been reported to promote SCLC cell survival[45]. It has been proposed that Raf/MEK/ERK signaling may play an essential role in promoting metastasis of SCLC tumors[46]. CXCL12 has been shown to induce ERK activation in SCLC cells, which was correlated with increased invasion through extracellular matrix[47]. Although the role of ERK signaling in SCLCs is incompletely understood, it has been implicated in cell proliferation, differentiation, survival, and drug resistance[48] therefore, evaluating its potential role in regulating tumor initiation is necessary to understand the implications of targeting ERK signaling in SCLC tumors. Our study shows that KSR2, a molecular scaffold for the MAPK signaling pathway, is an important regulator of tumor initiating capacity. The relative contribution of KSR2-dependent AMPK and ERK activation to SCLC clonogenicity and tumor initiation has not yet been defined, and future studies will evaluate the KSR2-dependent mechanisms of supporting the function of the tumor-propagating cell population.

Kinase Suppressor of Ras 2 (KSR2) interacts with effectors of the Raf/MEK/ERK signaling cascade, calcineurin, and AMPK[27, 36, 42]. Through these interactions, KSR2 promotes phosphorylation of AMPK and ERK, and we have shown that disrupting KSR2 reduces clonogenicity of SCLC tumor-propagating cells. Previous studies in our lab showed that low and high levels of exogenous KSR2 promote proliferation of mouse embryonic fibroblasts (MEFs) in an ERK independent manner, and that high levels of KSR2 block ERK activation in response to PDGF treatment[26]. These data suggest that the role of KSR2 in promoting SCLC clonogenicity may be multifaceted. By targeting KSR2 rather than ERK or AMPK individually, we may see additional therapeutic benefit. KSR2 disruption coordinately reduces Raf/MEK/ERK signaling and AMPK signaling. Our future work aims to detail the relative contribution of KSR2-dependent Raf/MEK/ERK signaling and AMPK signaling in promoting self-renewal and clonogenicity in SCLC-A tumors.

We show that KSR2 is preferentially expressed in the ASCL1-subtype of SCLC tumors as well as their cell of origin, PNECs. In the SCLC-A tumors derived from PNECs, ASCL1 is indispensable for tumor formation[13]. ChIP-seq analysis of ASCL1 subtype SCLCs revealed KSR2 as a transcriptional target[28, 29]. KSR2 was identified as one of 24 druggable and overexpressed target genes of ASCL1 identified by Chip-seq[30]. Interestingly, SCLC-A tumors can be converted to non-NE subtype tumors including NeuroD1 and YAP1 by overexpressing Myc[49]. The signaling pathways involved in Myc-driven tumor evolution remain largely undefined and the potential role of KSR2 in driving or maintaining the ASCL1 subtype is yet to be determined. Disruption of KSR2 expression in SCLC-A cell lines significantly reduce the proportion of tumor-propagating cells (TPCs), and the ability of SCLC TPCs to form colonies *in vitro*. Disruption of KSR2 reduces the TPC frequency *in vivo,* reducing tumor formation 10-fold. We demonstrated that KSR2 disruption reduces Raf/MEK/ERK signaling and AMPK activation, both of which potentially contribute to the effects on clonogenicity. Further characterization of the mechanisms through which KSR2 is promoting clonogenicity, and tumor initiation should reveal novel understanding of the regulation of tumor initiation in ASCL1 subtype SCLC tumors.

Materials and Methods

Cell culture

Murine small-cell lung carcinoma cell lines KP1 and KP3 were a gift from J. Sage (Stanford University). Human small-cell lung carcinoma cell lines H209 and H1963 were a gift of John Minna (UT Southwestern). The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and grown at 37°C with ambient

O_2 and 5 % CO_2. Cells were routinely tested for mycoplasma. No further authentication of cell lines was performed by the authors.

Generation of KSR2 shRNA knockdown cell lines

Individual SMARTvector human inducible lentiviral shRNAs targeting KSR2 expressed in piSMART hEF1a/TurboGFP vector were stably transfected into KP1, KP3, and H209 SCLC cell lines with PEI. Cells were selected for expression of the shRNAs using 0.25 ug/mL of puromycin. 48 hours after doxycycline induction, cells were selected again by flow cytometry sorting for GFP+ cells. Knockdown of KSR2 was confirmed by western blot. KSR2 cDNA (MSCV KSR2 IRES GFP) was made resistant to binding of hairpin sh5 by introducing three point mutations in the binding region. Point mutations were introduced using the QuikChange Lightning Site Directed Mutagenesis Kit (Agilent #210518) according to the manufacturer's protocol. MSCV KSR2 IRES GFP resistant to binding hairpin sh5 (sh5RKSR2) was transfected into HEK-293T cells using trans-lentiviral packing system (ThermoFisher Scientific). The virus was collected 48 hours post transfection and used to infect KP1 sh5 cells with 8 ug/mL Polybrene for 72 hours. KP1 sh5 cells expressing the sh5RKSR2 construct were selected for using flow cytometry sorting YFP+ cells. Presence of the sh5RKSR2 expression after doxycycline induced downregulation on endogenous KSR2 was confirmed via western blotting.

Cell lysis and western blot analysis

Whole cell lysate was extracted in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl, 1% NP-40, 0.5 % Na deoxycholate, 0.1 % Na dodecyl sulfate, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, and 1 X protease and phosphatase inhibitors aprotinin (0.5 U/mL), leupeptin (20 mM), and NA_3VO_4 (0.5 mM). The estimation of protein concentration was done using BCA protein assay (Promega #PI-23222, PI-23224). Samples were diluted using 1 X sample buffer (4 X stock, LI-COR #928–40004) with 100 mM dithiothreitol (DTT) (10 X stock, 1 mM, Sigma #D9779-5G). The protein was separated using 8–12% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with Odyssey TBS blocking buffer (LICOR-Biosciences #927–50003) for 45 min at room temperature, then incubated with primary antibodies (*Key Resources Table*) at least overnight at 4°C. IRDye 800CW and 680RD secondary antibodies (LI-COR Biosciences # 926–32211, # 926–68072) were diluted 1:10,000 in 0.1% TBS-Tween and imaged on the Odyssey Classic Scanner (LI-COR Biosciences).

Analysis of KSR2 expression in normal tissues

GTEx portal was used to display the relative expression of KSR2 mRNA (TPM) in brain-cortex and lung tissue. Neuroendocrine specific reporter, Chga-GFP was used to identify PNECs. GFP+ neuroendocrine cells from 3 mouse lungs were isolated by flow cytometry. qPCR was performed to measure mRNA expression of Ksr2, Crgp, Chga, Syp, and Spc in GFP+ neuroendocrine cells and GFP- lung epithelial cells.

SCLC sequencing analysis

RNA sequencing data from human primary tumor samples[12] was analyzed for KSR2 expression based on high and low ASCL1 expression or high and low Myc expression (with Trudy Oliver, Huntsman Cancer Institute). RNA sequencing data of human SCLC cell lines (CCLE, Broad Institute MIT) was segregated by SCLC subtype and analyzed for KSR2 mRNA levels.

Fluorescence Activated Cell Sorting

SCLC cell lines were incubated 20 minutes on ice in PBS with DAPI (3uM), PE-CD24 (1:400) PE-Cy7-CD44 (1:300) and APC-EpCAM (1:100). Cells were resuspended in PBS and flow cytometry of SCLC cell lines was performed using a 100um nozzle on a BD FACSAria II using FACSDiva software. Debris were excluded by gating on forward scatter area versus side scatter area. Doublet were excluded by gating on forward scatter area versus side scatter height. Viable cells were identified by exclusion of DAPI stained cells. $CD24^{high}CD44^{low}$ cells were included by sequential gating followed by $EpCAM^{high}$ TPCs. Compensation was performed using single stain and fluorescence- one (FMO) controls. Positive gates were set based on the negative unstained sample. Data were analyzed using FlowJo software.

Colony Formation Assay

For colony formation assays, SCLC cells were dissociated by gentle pipetting. Live TPCs were sorted using a 100µm nozzle on a BD FACSAria II. TPCs were sorted individually into 96 well plates filled with regular media

(200µl/well) containing DMSO or doxycycline (DOX) (1 ug/mL). 50 uL fresh media with or without DOX was added to the wells every 10 days. Three weeks later, colony numbers were assessed using CellTiter-Glo 2.0 reagent (Promega #G9242) and luminescence was measured (POLARstar Optima plate reader) according to the manufacturer's protocol.

In vivo Extreme Limiting Dilution Analysis

The viable cell number was assessed by replicate cell counts on a hemocytometer using Trypan Blue exclusion. Viable cell number was used to derive a titration of cell numbers for implantation. Cells were diluted in 50µl media (RPMI +10% FBS) and 50µl Cultrex PathClear BME (Trevigen # 3632-005-02). Six eight-week-old NCG mice were injected subcutaneously into the shoulders and flanks. 3 replicates for each dilution were used. Mice were provided drinking water with sucrose, or sucrose plus doxycycline (2 mg/kg). Injection sites were palpated biweekly to monitor for tumor growth and all mice were sacrificed when any one tumor reached 1cm. Tumors that formed were scored as 1 and the absence of tumor formation was scored as 0 for the extreme limiting dilution analysis (ELDA). Tumors that formed were analyzed for expression of KSR2 by western blot and tumors in the doxycycline treated group which did not maintain knockdown were scored as 0. ELDA software was used to estimate TPC frequency for control and doxycycline treated groups.

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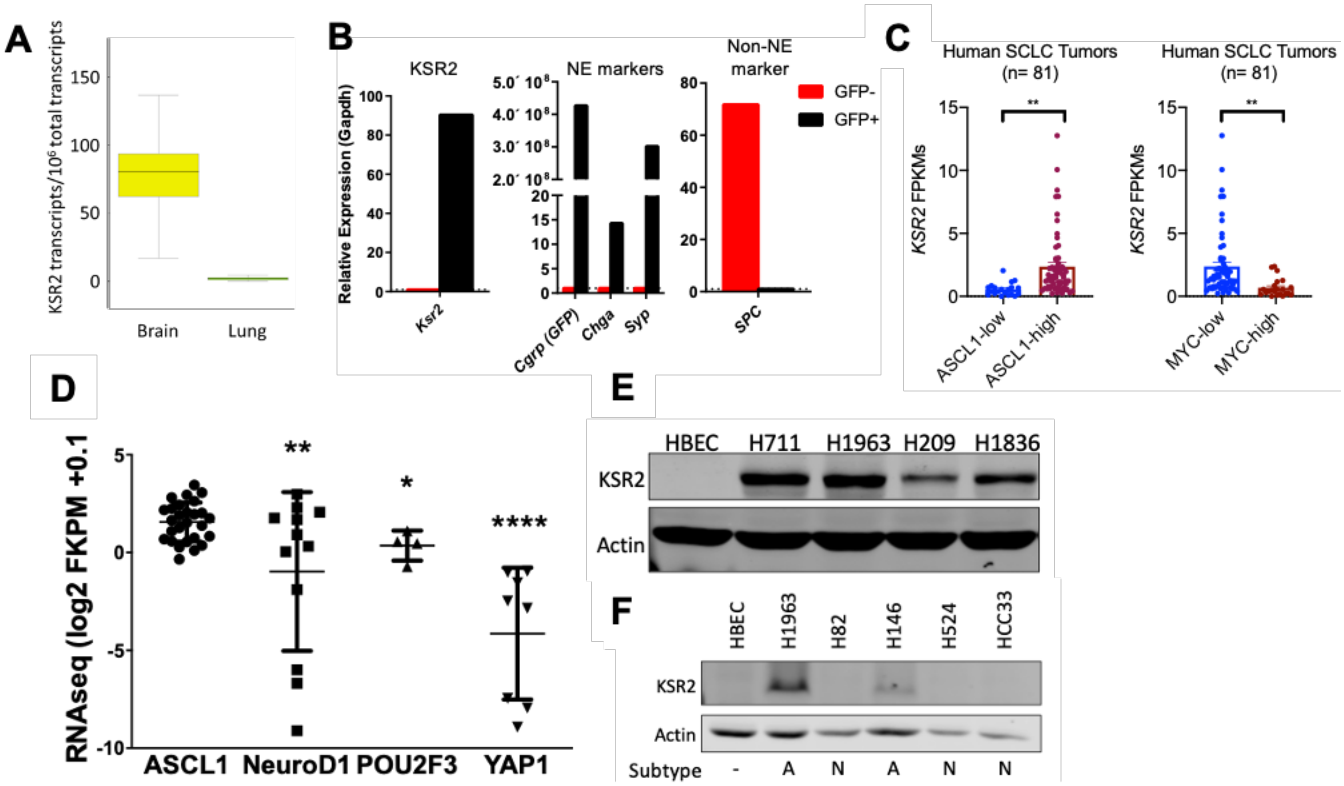
Figure Legends

Fig. 1. A) KSR2 mRNA in brain tissue and lung epithelial tissue (GTEx Portal). B) GFP^+ cells isolated from the lungs of mice expressing GFP from the promoter for the neuroendocrine (NE)-specific *Crgp.* mRNA expression of KSR2 (*left*), neuroendocrine markers (Chga, Syp) (*middle*), and lung epithelial marker surfactant protein C (SPC) (*right*). C) KSR2 expression in human ASCL-1 subtype SCLC tumors (*left*) and tumors with elevated Myc expression (*right*) from RNA-seq analysis of human SCLC tumors. **, $p<0.01$ D) KSR2 RNAseq (DepMap) in SCLC segregated by subtype. *$p=0.03$; **$p<0.004$; ****$p<0.0001$. E) KSR2 expression, with Actin loading control, in western blots of ASCL1 subtype SCLC cell lines and non-transformed human bronchial epithelial cells (HBEC). F) KSR2 expression, with Actin loading control, in western blots of ASCL1(A) or, NeuroD1 (N) subtypes SCLC lines and non-transformed human bronchial epithelial cells (HBEC).

Fig. 2. A) FACS of KP1 cells for $CD24^{high}$ $CD44^{low}$ (left) and $EPCAM^{high}$ (right). B) Dox-inducible RNAi of KSR2 in KP1 cells from SCLC GEMM. NTC, non-targeting control. Actin, loading control. C) Percent TPCs detectable in control or KSR2-targeted (sh5) KP1 cells. Clonogenicity of Dox-treated KP3 (D) and KP1 (E) SCLC TPCs with and without the indicated shRNAs targeting KSR2. Colony viability was measured by CellTiter-Glo. ****, $p<0.0001$, $n=3$.

Fig. 3. A) KSR2 cDNA resistant to the sh5 shRNA (KSR2-R) or the empty expression vector were introduced into KP1 cells expressing the Dox-inducible sh5 shRNA. HDAC2, loading control. B) Clonogenicity of KP1 SCLC TPC colonies within wildtype (WT), dox-induced RNAi of KSR2 (sh5) and dox-induced RNAi of KSR2 + KSR2-R. Colony viability was measured by CellTiter-Glo. ****, $p<0.0001$. C-D) Dox-inducible RNAi of KSR2 in human SCLC (H209) (C) and KP1 cells (D) from SCLC GEMM. Actin, loading control. HDAC2, loading control.

Fig. 4. Quantification (left) and raw data (right) of *in vivo* extreme limiting dilution analysis of KP1 cells without and with Dox-induced RNAi of KSR2. **, $p=0.00698$.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100
50
0
Brain
Lung
B
KSR2
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Ksr2
NE markers
Cgrp (GFP)
Chga
Syp
Non-NE marker
SPC
GFP-
GFP+
C
Human SCLC Tumors (n= 81)
KSR2 FPKMs
ASCL1-low
ASCL1-high
MYC-low
MYC-high
D
RNAseq (log2 FKPM +0.1
ASCL1
NeuroD1
POU2F3
YAP1
E
HBEC
H711
H1963
H209
H1836
KSR2
Actin
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Actin
Subtype
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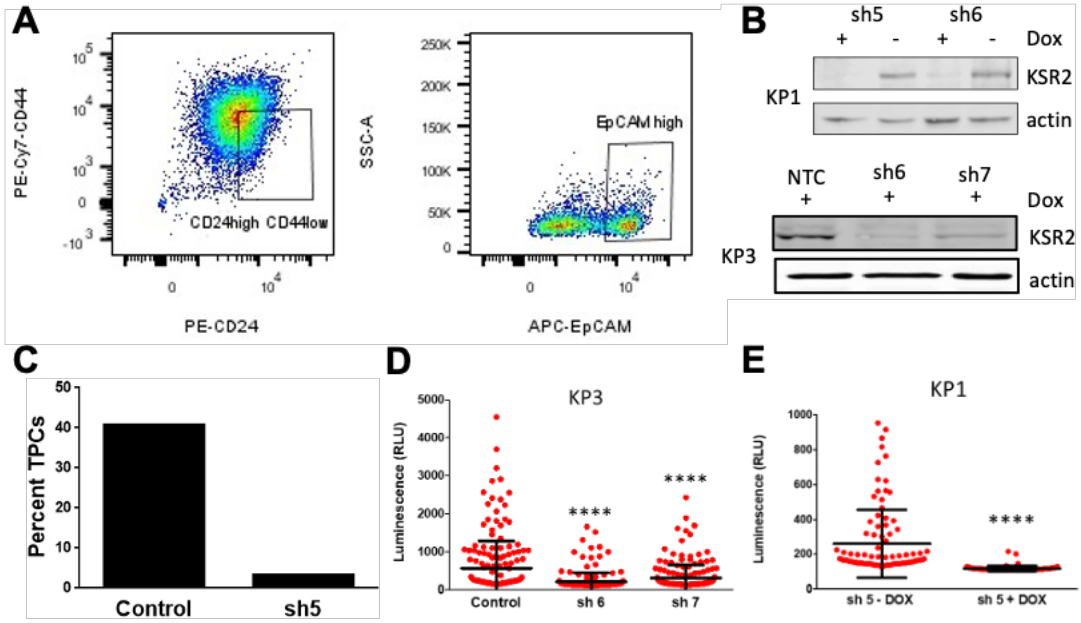
N
A
N
N

Fig. 1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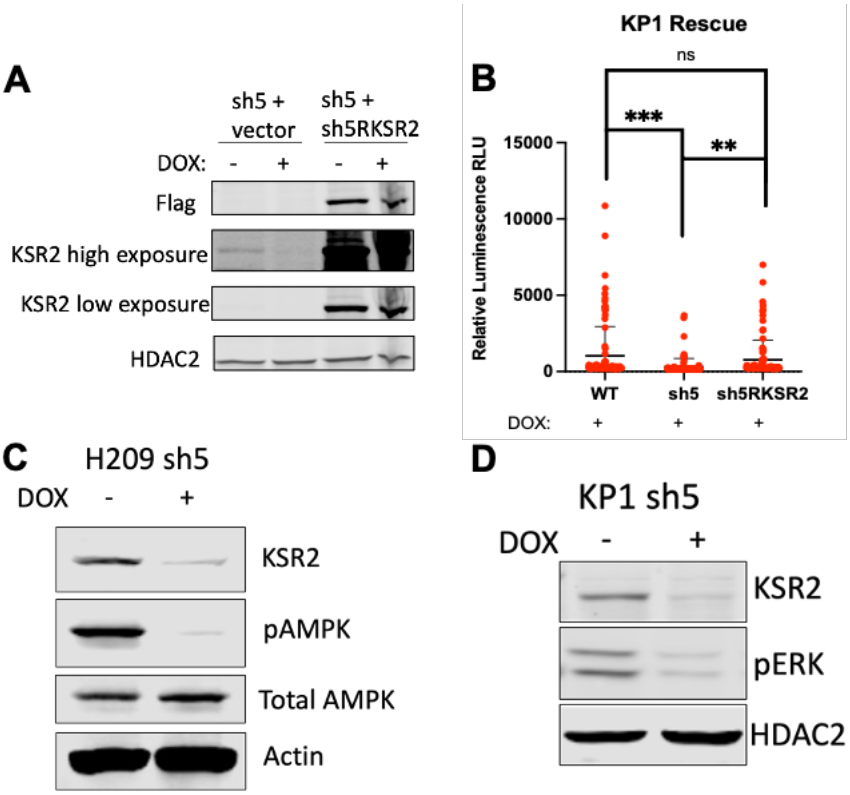
CD24high CD44low
PE-CD24
SSC-A
EpCAM high
APC-EpCAM
B
sh5
sh6
Dox
KP1
KSR2
actin
NTC
sh6
sh7
KP3
C
Percent TPCs
Control
sh5
D
KP3
Luminescence (RLU)
****
Control
sh 6
sh 7
E
KP1
sh 5 - DOX
sh 5 + DOX

Fig. 2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sh5 + sh5RKSR2
DOX: - + - +
Flag
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HDAC2
B
KP1 Rescue
ns
***
**
Relative Luminescence RLU
15000
10000
5000
0
WT
sh5
sh5RKSR2
DOX: + + +
C
H209 sh5
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pAMPK
Total AMPK
Actin
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KP1 sh5
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pERK
HDAC2

Fig. 3

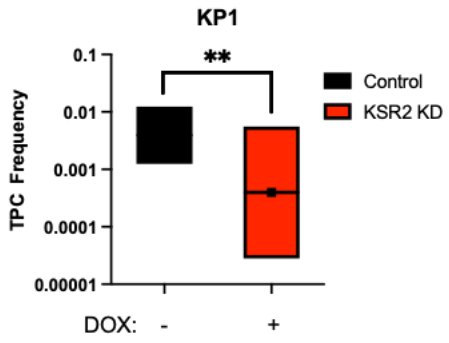

Table 1. ELDA KP1 cells ± KSR2

Cells	Mice	Tumor	Group
10	3	0	control
50	3	1	control
100	3	2	control
200	3	3	control
500	3	1	control
10	3	0	dox
50	3	0	dox
100	3	1	dox
200	3	0	dox
500	3	0	dox

Group	TPC Estimate
Dox	2530
control	255

Chisq	P value
7.28	0.00698

Fig. 4

Key Resources

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Cell line (*Mus musculus*)	Small-cell lung carcinoma	Obtained from Julien Sage (Stanford University)	KP1	
Cell line (*Mus musculus*)	Small-cell lung carcinoma	Obtained from Julien Sage (Stanford University)	KP3	
Cell line (*Homo sapiens*)	Small-cell lung carcinoma	Obtained from John Minna (UT Southwestern)	H209	
Cell line (*Homo sapiens*)	Small-cell lung carcinoma	Obtained from John Minna (UT Southwestern)	H1963	
Transfected construct	piSMART hEF1a/TurboGFP-KSR2 shRNA 5	Dharmacon	Cat# V3SH11252 -225640245	GGAGCAAATCCCACGAGTT
Transfected construct	piSMART hEF1a/TurboGFP-KSR2 shRNA 6	Dharmacon	Cat# V3SH11252 -227968197	GCACATCAGTCAGACGCTC
Transfected construct	piSMART hEF1a/TurboGFP-KSR2 shRNA 7	Dharmacon	Cat# V3SH11252 -228320208	CCATCAAGCACAGGTTTTC
Recombinant DNA reagent	sh5RKSR2	This paper	MSCV KSR2 IRES YFP	QuikChange Lightning Mutagenesis: Forward 5' CGCTGCACAGGAGTAAGT CCCAGTAATTCCAGCTCGG G 3' Reverse 5'CCCGAGCGTGAATTCAT GGGACTTACTCCTGTGCAG CG 3'
Sequence based reagent	Ksr2 (PCR primer)	(Guo et al. 2017)[42]		Forward primer 5′-TGGATGTCCGAAAGGAAGT C-3′ Reverse primer 5′-CTTCTCCACGGTCTCACAC A-3′
Sequence based reagent	Crgp (PCR primer)			
Sequence based reagent	Chga (PCR primer)			
Sequence based reagent	Syp (PCR primer)			
Sequence based reagent	Spc (PCR primer)			

Antibody	Anti-B actin, mouse monoclonal	Santa Cruz	Cat# 47778	WB 1:1000
Antibody	Anti-KSR2 (MO8), mouse monoclonal	Abnova	Cat# H00283455-M08	WB 1:1000
Antibody	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10), mouse monoclonal	Cell Signaling	Cat# 9106	WB 1:1000
Antibody	Anti-phospho-AMPKα(Thr172)(40H9), rabbit monoclonal	Cell Signaling	Cat# 2535	WB: 1:1000
Antibody	Anti-AMPKα, rabbit polyclonal	Cell Signaling	Cat# 2532	WB 1:1000
Antibody	Anti-Flag (M2), mouse monoclonal	Sigma	Cat# F3165	WB 1:1000
Antibody	Anti-HDAC2 (Y461), rabbit monoclonal	Abcam	Cat# ab32117	WB: 1:1200
Antibody	APC anti-mouse CD326 (EpCAM), mouse monclonal	Biolegend	Cat# 118213	FACS: 1:100
Antibody	PE anti-mouse CD24, mouse monoclonal	Biolegend	Cat# 101807	FACS: 1:400
Antibody	PE Cyanine7 anti-mouse/human CD44, mouse monoclonal	Biolegend	Cat# 103029	FACS: 1:300
Nuclear Stain	DAPI (4',6-Diamidino-2-Phenylindole, Dilactate)	Biolegend	Cat# 422801	FACS: 3 uM

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