Kinase Suppressor of Ras 2 promotes small-cell lung carcinoma tumor initiation

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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) promotes the self-renewal and clonogenicity of SCLC TPCs. KSR2 is a molecular scaffold which promotes Raf/MEK/ERK signaling. 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 the colony forming ability of TPCs *in vitro* and their 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 novel effector promoting initiation of ASCL1 subtype SCLC tumors, and a potential subtype-specific therapeutic target.
Impact Statement

Manipulation of the molecular scaffold KSR2 in ASCL1-subtype small-cell lung cancer cells reveals its contribution to the formation and maintenance of tumor propagating cells via ERK signaling.
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 almost 40 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 30%, 18%, and 3%, respectively (American Cancer Society, 2024). 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)\(^6\),\(^12\). Recently, an additional subtype defined by expression of ATOH1 was identified\(^16\).

Lineage tracing and single cell RNA sequencing (scRNA-seq) in genetically modified mice showed that a rare PNEC subpopulation, NE\(^\text{stem}\) cells, actively responds to lung injury of the epithelia by expanding, migrating, and undergoing Notch-dependent transit amplification to regenerate the lost epithelium\(^17\). This effort additionally identified NE\(^\text{stem}\) as a cell-of-origin for SCLC following TP53, Rb and Notch mutation causing constitutive activation of stem cell renewal and altered deprogramming\(^17\). 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 the bulk non-TPC population is highly proliferative but incapable of establishing tumors \textit{in vivo}\(^18\). TPCs have been implicated in the initiation and growth of SCLC as well as therapy resistance\(^19\)-\(^25\). Tumor propagating cells are also implicated in epithelial-to-mesenchymal transition (EMT) and metastasis\(^25\). Their slower cycling and self-renewing ability enhances DNA repair, rendering these cells resistant to DNA damage-dependent chemo and radiation therapy\(^21\),\(^22\),\(^25\),\(^26\). 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 proteins KSR1 and KSR2 are molecular scaffolds for Raf/MEK/ERK signaling\(^27\),\(^28\). While KSR1 is widely expressed, KSR2 expression is restricted to the brain, pituitary, and adrenal glands, and neuroendocrine tissues\(^29\),\(^30\). KSR2 is abundantly expressed in the brain, and its disruption reduces body temperature, promotes cold intolerance, impairs glucose homeostasis, elevates fasting insulin and free fatty acid levels in the blood, and causes obesity\(^28\). ChIP-seq analysis also revealed KSR2 as a transcriptional target in human ASCL1 subtype SCLC cell lines\(^31\),\(^32\). KSR2 was identified as one of 24 druggable and overexpressed target genes of ASCL1 identified by Chip-seq, providing a rationale for studying the role of KSR2 in ASCL1 subtype SCLC\(^33\). Our work shows that KSR2 is expressed in PNECs and SCLC-A tumors and cell lines. Expression of KSR2 promotes the self-renewal and clonogenicity of SCLC-A TPCs and their tumor initiating capacity \textit{in vitro} and \textit{in vivo}. While KSR2 disruption is rescued by an intact KSR2 transgene, the inability of an ERK-binding deficient KSR2 construct to restore clonogenicity and tumor initiation implicates its scaffolding function in TPC formation. 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 tumors express KSR2.** ASCL1 subtype (SCLC-A) tumors can arise from PNECs\(^17\),\(^31\),\(^33\),\(^34\). PNECs are heterogeneous, including a small subpopulation termed NE\(^\text{stem}\) cells that respond to injury of the lung epithelia by expanding, migrating, and undergoing Notch-dependent transit amplification to regenerate the lost epithelium\(^17\). 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\(^17\). Analysis of human SCLC tumors 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 SCLC-A, but has varying expression in NEUROD1, POU2F3, and YAP1 subtypes (Fig. 1D).
RNAseq from the SCLC primary tumors shows KSR2 mRNA is preferentially expressed in SCLC-A compared to other subtypes, while KSR1 mRNA is detected across SCLC subtypes (Supplementary Table 1).

By western blot analysis, SCLC-A and -N cells express KSR1, but only SCLC-A cells express KSR2 (Fig. 1E, 1F).

**Depletion of Kinase Suppressor of Ras 2 reduces SCLC clonogenicity and self-renewal.** SCLC-A tumor propagating cells (TPCs) are essential for tumor initiation and metastasis and are enriched by selection for high expression of CD24 and EpCAM, but low expression of CD44. Transplantation assays show that TPCs are a minor but highly tumorigenic subpopulation of SCLC cells characterized by their colony forming activity *in vitro*, which measures their capacity for clonogenicity and self-renewal. Mouse SCLC cell lines KP1 and KP3 were derived from spontaneous mouse models of SCLC with knockout of Rb, p53 (KP1) or Rb, p53 and p130 (KP3), which replicate the ASCL1 subtype of SCLC tumors. KP1 and KP3 cells expressing one of three doxycycline (dox)-inducible shRNAs targeting Ksr2 (sh5, sh6, sh7) were treated with or without doxycycline (Fig. 2A). Human ASCL1 subtype SCLC cell line NCI-H209 (H209) expressing dox-inducible shRNA targeting Ksr2 (sh5) was also treated with or without doxycycline (Fig. 2A). Control (CTRL) and KSR2 knockdown (sh5) KP1 and H209 cells were stained for CD24highCD44lowEpCAMhigh (Fig. 2B, C). Dox-induced targeting of Ksr2 reduced the proportion of CD24highCD44lowEpCAMhigh TPCs detected by FACS in H209 cells from 42.4% to 0.4%, and in KP1 cells from 39.1 to 5.4% (Fig. 2D). Following downregulation of KSR2 expression, KP3 cells were stained for TPC markers CD24highCD44lowEpCAMhigh isolated by FACS and plated as single cells in 96-well plates to be analyzed for colony formation by CellTiter-Glo®. Robust colony formation, a measure of self-renewing capability of an individual cell, was observed in KP3 control TPCs. Viability was reduced by 58%, and 45% with Ksr2 RNAi by sh6, and sh7, respectively, which reflects their effectiveness at targeting Ksr2 (Fig. 2A). In KP1 cells, colony formation was assessed with or without dox-induced RNAi by hairpin sh5 after TPC isolation, which inhibited colony formation 86% (Fig. 2F). These data demonstrate that KSR2 is a critical effector of the clonogenic and self-renewing properties of SCLC TPCs.

**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. Stem cell frequency within the bulk tumor cell population is determined from the frequency of tumor-positive and tumor-negative injections arising from varying doses of xenografted cells. Dox-inducible shRNA targeted KP1 cells were injected with successive dilutions into NOD-PrkdcscidIl2rgtm1Wjl2rgtm1Wjl2tm1JmuCrl (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 cm² and then all mice were sacrificed. ELDA was performed by scoring each tumor that arose 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. 3, Table 1). These data indicate that KSR2 is a critical effector of tumor initiation of SCLC tumor propagating cells *in vivo*.

**Expression of a KSR2 transgene restores colony formation and increases TPC frequency**. To confirm on-target effect of our inducible shRNA system for KSR2 knockdown, a construct containing wildtype Ksr2 mutated to be resistant to binding hairpin sh5 (sh5RKS2) was expressed in KP1 sh5 cells (Fig. 4A). Rescue of KSR2 expression restored colony formation to wildtype levels in KP1 sh5 cells confirming the on-target effect of hairpin sh5 (Fig. 4B). In *vitro* ELDA was used to estimate the relative frequency of TPCs. KSR2 disruption reduced TPC frequency from 1/19 in control KP1 cells to 1/166 in KP1 cells with KSR2 KD (Fig. 4C, D). The expression of wildtype Ksr2 construct sh5RKS2 restored the TPC frequency to 1/18 (Fig. 4C, D). These data demonstrate the on-target action of the inducible Ksr2 shRNA system to regulate TPC abundance.

**KSR2 disruption inhibits the tumor initiating capacity of human SCLC cell lines *in vitro***. In *vitro* ELDA was used to test the effect of doxycycline (dox)-induced KSR2 disruption on human SCLC cell line H209. The TPC frequency of control H209 cells was reduced 4- to 25-fold from 1/3-1/5 in control cells to 1/12-1/76 in H209 sh5 cells (Fig. 5A, Supplementary Fig. 1A, B). KSR2 was also targeted using a CRISPR/Cas9 in conjunction with a KSR2 sgRNA (CR3), or non-targeting control (NTC) (Fig. 5B). The TPC frequency was reduced 3- to 12-fold from 1/10-1/39 in NTC to 1/112-1/140 in KSR2 KO cells (Fig. 5C, Supplementary Fig. 1C, D). These data indicate that KSR2 is a critical effector of tumor initiating capacity in human SCLC tumor propagating cells.
**KSR2-ERK interaction is necessary for SCLC clonogenicity.** KSR2 knockdown reduces activation of ERK (Fig. 6A). With serum starvation, control and KSR2 knockdown samples are reduced to a basal level of ERK activation (Fig. 6A). ERK can be stimulated with addition of serum in control cells, while KSR2 knockdown cells activate ERK poorly after serum addition (Fig. 6A). Treatment with calcium ionophore ionomycin also induces ERK activation in control cells, but KSR2 knockdown inhibits this response (Fig. 6A). Cells expressing sh5RKFIF570 rescue ERK activation in each condition (Fig. 6B). To assess the relative contribution of KSR2-dependent ERK signaling to the effect on ERK activation and clonogenicity, a construct containing a mutant KSR2 deficient in its ability to bind ERK (FIF570) was mutated to be resistant to binding hairpin sh5 and expressed in KP1 sh5 cells. Cells expressing sh5RFIF570 are unable to rescue ERK activation when endogenous KSR2 is depleted (Fig. 6C). Disruption of KSR2/ERK interaction prevents ERK activation in response to serum stimulus or treatment with ionomycin. Immunoprecipitation of the FLAG epitope tag on this construct and the full length KSR2 demonstrated reduced phospho-ERK associated with the ERK-binding mutant construct (sh5RFIF570) (Fig. 6D). Colony formation was significantly reduced in the sh5RFIF570 cells after dox-induced targeting of endogenous KSR2, suggesting that KSR2 interaction with ERK is necessary for the clonogenic capacity of SCLC TPCs (Fig. 6E). Disruption of KSR2 significantly reduced the number of colonies formed by TPCs (Fig. 6F). sh5RKFIF570 rescued colony formation (Fig. 6F), while sh5RFIF570 was unable to restore colony formation to KP1 cells (Fig. 6F). These data indicate that KSR2-dependent ERK signaling is an important contributor to colony formation by TPCs.

**Discussion**

Kinase Suppressor of Ras proteins KSR1 and KSR2 have unique and overlapping functions. Both function as scaffolds for Raf/MEK/ERK signaling promoting phosphorylation of ERK. KSR2 also interacts with calcineurin to promote calcium dependent ERK signaling. Calcineurin dephosphorylates KSR2 on specific sites in response to Ca2+ signals regulating KSR2 localization and activity. KSR2 is expressed in the brain, pituitary gland, and adrenal gland. We show that KSR2 is expressed in neuroendocrine cells and tissues. Min-6, a transformed pancreatic beta cell line, and NG108-15, a neuroblastoma/glioma fusion cell line express KSR2 at a detectable level on western blot. Depletion of KSR2 in these cell lines reduced proliferation but moderately decreased ERK activation in response to EGF stimulation.

Here we show that disruption of KSR2 in SCLC-A reduces TPC clonogenicity and self-renewal in vitro, and tumor initiation in vivo. Further work demonstrates that the clonogenic capacity of TPCs depends upon the interaction of KSR2 with ERK. Activating Ras mutations are rare in SCLC tumors, therefore Raf/MEK/ERK signaling is likely activated by extracellular stimuli. The role of ERK signaling in SCLC is incompletely understood, but is implicated in cell proliferation, differentiation, survival, and drug resistance. Our data reveal its KSR2-dependent involvement in tumor initiation. This observation contrasts with previous studies that tested the efficacy of targeting Raf/MEK/ERK signaling in SCLC and concluded that ERK activation can be both pro- and anti-proliferative toward SCLC. Treatment with ERK inhibitor was found not to induce apoptosis in human SCLC-A cell lines H209 and H69. Our data are consistent with observations that SCLC-A cell line proliferation was significantly reduced in vitro and in vivo tumor xenografts by ARHGEF19 disruption and downstream reduced Raf/MEK/ERK signaling. Activation of Raf/MEK/ERK by endoplasmic reticulum (ER) stress has also been reported to promote SCLC cell survival. Raf/MEK/ERK signaling may play an essential role in promoting metastasis of SCLC tumors. CXCL12 induces ERK activation in SCLC cells, which correlates with increased invasion through extracellular matrix. These contrasting observations may suggest that the intensity and duration of ERK activation impacts SCLC cell fate.

The effect of KSR2 depletion on ERK activation is surprising as SCLC-A cells express both KSR2 and KSR1. We do not detect KSR1 compensation for KSR2 loss, suggesting that KSR2 is required for TPC function in SCLC-A cells. Our data suggest that either KSR2 is contributing to TPC function by promoting signaling that KSR1 cannot influence, or that KSR2 and KSR1 coordinate function to conduct efficient signaling. KSR1 and KSR2 are capable of heterodimerization with Raf, resulting in a conformational change of KSR proteins that allows phosphorylation of MEK. The dimerization of KSR proteins with Raf orients the Raf protein so that its catalytic site is not in close proximity to the phosphorylation site on MEK to complete the phosphorylation, which necessitates MEK phosphorylation by Raf through a trans interaction. KSR2-BRAF heterodimerization results in an increase of MEK phosphorylation via the KSR2-mediated relay of signal from BRAF to release the activation...
segment of MEK for phosphorylation\textsuperscript{47}. KSR2 can also homodimerize via the same interface that interacts with BRAF, however this creates a different quaternary structure when interacting with MEK and it’s unknown how this may affect the availability of MEK for phosphorylation\textsuperscript{47}. BRAF homodimers, or KSR1-BRAF heterodimers should generate a quaternary structure sufficient for availability of MEK for phosphorylation by an additional BRAF protein\textsuperscript{47}. Via the same interaction interface, it is possible that KSR1 and KSR2 heterodimerize. Our incomplete understanding of how KSR2 homodimers or KSR1-KSR2 heterodimers may impact MEK phosphorylation makes it difficult to predict the effect of KSR2 loss on MEK phosphorylation and downstream ERK activation. However, KSR2 depletion has a deleterious effect of ERK activation in SCLC-A cells. It is notable that we fail to detect KSR2 expression in the NEUROD1 subtype of SCLC, while they retain expression of KSR1 (Fig. 1E, F). This observation suggests that SCLC-N cells are capable of compensating for loss of KSR2 with KSR1 expression, or that transition to the NEUROD1 subtype SCLC results in upregulation of other compensatory signaling pathways that negate the dependency on KSR2 for ERK signaling.

ASCL1 and NEUROD1 drive distinct transcriptional profiles in SCLC\textsuperscript{13, 50, 51}. SCLC-A cells can be converted to SCLC-N cells by elevation of MYC\textsuperscript{51}. MYC amplification has been associated with poor patient outcome, therapy resistance, and tumor progression\textsuperscript{52-54}. KSR2 expression seems to be lost during transition from SCLC-A to SCLC-N. The initiation of switching from SCLC-A subtype to SCLC-N subtype is incompletely understood.

The expression of KSR2 in SCLC-A is thought to be driven by its defining transcription factor ASCL1, as KSR2 is a transcriptional target of ASCL1\textsuperscript{31, 32}. ASCL1 and KSR2 are also expressed in SCLC-A cell-of-origin, PNECs. A subpopulation of PNEC cells termed NE\textsuperscript{stem} cells actively responds to injury of the lung epithelia by expanding, migrating, and undergoing Notch-dependent transit amplification to regenerate the lost epithelium\textsuperscript{17}. Although KSR2 plays a role in SCLC TPC function, its role in normal PNECs is undetermined. KSR2 knockout mice have metabolic defects\textsuperscript{28, 29} but have no reports of abnormal lung pathology. Studies determining the effect of KSR2 manipulation on lung repair are required to determine if KSR2 plays a role in the function of PNECs in response to damage.

If KSR2 is dispensable for normal lung repair, it represents a potential therapeutic target for SCLC-A tumors. The discovery that cell surface proteins CD24, CD44, and EpCAM can be used to enrich and isolate SCLC-A TPCs significantly advanced investigation of potential therapeutic vulnerabilities within this population\textsuperscript{17}. Cisplatin treatment does not enrich TPCs indicating that TPCs are not a reservoir for drug tolerant persister cells\textsuperscript{17}, however TPCs are capable of repopulating tumors after treatment suggesting that they are not preferentially sensitive to the chemotherapeutic. Isolation of TPCs allows for interrogation of potential therapies that may be selectively detrimental to the TPC population. SCLC-N with elevated MYC are sensitized to Aurora kinase inhibition, suggesting the distinct transcriptional profiles of the SCLC subtypes are therapeutically relevant\textsuperscript{51}. Using TPC markers SCLC-A TPCs can be assayed for potential TPC and subtype specific vulnerabilities.

JQ1, an inhibitor of bromodomain and extra-terminal (BET) proteins, reduced the frequency of TPCs \textit{in vivo} and suppressed the ability of single TPCs to form colonies \textit{in vitro}\textsuperscript{17}. Our data demonstrate that KSR2 disruption reduces frequency of TPCs and reduces colony formation \textit{in vitro} and tumor initiation \textit{in vivo}. The effect of KSR2 on TPC clonogenicity is dependent on interaction with ERK, suggesting that ERK activation is necessary for TPC function. Together these results suggest that targeting of KSR2 or its effector ERK may be TPC-specific therapeutic strategies, and may create a more durable therapeutic response when used in conjunction with standard of care chemotherapies. This provides rationale for interrogating the effects of targeting KSR2 in a genetically engineered mouse models (GEMM) of SCLC-A\textsuperscript{11, 13, 55}. KSR2 disruption may work well in combination with cisplatin to target TPCs, reducing tumor burden and recurrence.

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\textsubscript{2} and 5% CO\textsubscript{2}. Serum starved cells were grown in above conditions, with serum removed for 16 hours prior to cell lysis. Serum shocked cells were grown normally followed by boosting FBS concentration to 50% for 15 minutes prior to cell lysis. Ionomycin (ThermoFisher Scientific l24222) treatment was given at a 1 uM dose for
15 minutes prior to cell lysis. All cells were routinely tested for mycoplasma. No further authentication of cell lines was performed by the authors.

Generation of KSR2 shRNA knockdown and CRISPR/Cas9 knockout cell lines

Individual SMARTvector human inducible lentiviral shRNAs targeting KSR2 expressed in pSMART 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 µg/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 YFP) and ERK binding deficient KSR2 cDNA (MSCV KSR2 FIF570 IRES YFP) 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. Sequencing of the construct confirmed the correct point mutations were made with no additional mutations. MSCV KSR2 IRES YFP resistant to binding hairpin sh5 (sh5RKSR2) and MSCV KSR2 FIF570 IRES YFP resistant to binding hairpin sh5 (sh5RFIF570) were 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 µg/mL Polybrene for 72 hours. KP1 sh5 cells expressing the sh5RKSR2 or sh5RFIF570 construct were selected for using flow cytometry sorting GFP+ cells. Presence of the sh5RKSR2 or sh5RFIF570 expression after doxycycline induced downregulation on endogenous KSR2 was confirmed via western blotting. To generate knockout cells, sgRNA sequences targeting KSR2 or non-targeting control (NTC) were inserted into lentiCRISPR v2 (Addgene #52961). The constructs were PEI transfected into HEK293T cells along with psPAX2 lentiviral packaging construct (Addgene #12259) and pMD2.G envelope construct (Addgene #12259). Lentivirus-containing media was harvested at 72-h and used to infect the H209 cells with 8 µg/mL Polybrene. Cells were selected for expression of the sgRNAs using 2 µg/mL of puromycin, and western blot was used to confirm KSR2 knockout.

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 protease and phosphatase inhibitors aprotinin (0.5 U/mL), leupeptin (20 mM), and Na3VO4 (0.5 mM). The estimation of protein concentration was done using BCA protein assay (Promega #P1–23222, 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 was analyzed for KSR2 expression based on high and low ASCL1 expression or high and low Myc expression. RNA sequencing data of human SCLC cell lines (DepMap Portal, Broad Institute MIT) was segregated by SCLC subtype and analyzed for KSR2 mRNA levels. RNA samples from SCLC (n = 69) and NSCLC-NE (n = 14) cell lines were analyzed by paired-end RNA sequencing as previously described (McMillan et al., 2018, Cell 173, 864–878; dbGaP study accession: phs001823.v1.p1). Briefly, reads were aligned to the human reference genome GRCh38 using STAR-2.7 (https://github.com/alexdobin/STAR [github.com]) and FPKM values were generated with cufflinks-2.2.1 (http://cole-trapnell-lab.github.io/cufflinks/[cole-trapnell-lab.github.io]). All data were then pooled, normalized to Transcripts Per Millions (TPM), and log-transformed. RNAseq from the SCLC primary tumors (George et al., 2015, Nature 524, 47–53) were included in the analysis.
Fluorescence Activated Cell Sorting
SCLC cell lines were incubated 20 minutes on ice in PBS with DAPI (3μM), 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 100 μm nozzle on a BD FACSaria II using FACSDiva software. Debris were excluded by gating on forward scatter area versus side scatter area. Doublets were excluded by gating on forward scatter area versus side scatter height. Viable cells were identified by exclusion of DAPI stained cells. CD24\textsuperscript{high}CD44\textsuperscript{low} cells were included by sequential gating followed by EpCAM\textsuperscript{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 100 μm nozzle on a BD FACSaria II. TPCs were sorted individually into 96 well plates filled with regular media (200 μl/well) containing DMSO or doxycycline (DOX) (1 μg/mL). 50 μL 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. CellTiter-Glo® readings greater than 300 Relative Luminescence Units (RLUs) in colony forming assays were considered colonies.

Extreme Limiting Dilution Analysis
In vivo
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 50 μl media (RPMI +10% FBS) and 50 μl 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 5% 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 1 cm. 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.

In vitro
Cells were seeded in 96-well plates (Fisher #12556008) at decreasing cell concentrations (1000 cells/well − 1 cell/well) at half log intervals (1000, 300, 100, 30, 10, 3, 1), 12 wells per condition. Cells were cultured for 14 days, and wells with spheroids >100 μM were scored as spheroid positive. TPC frequency and significance between groups was calculated by ELDA software.
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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 and KSR1 expression, with Actin loading control, in western blots of ASCL1 subtype SCLC cell lines and non-transformed human bronchial epithelial cells (HBEC). F) KSR2 and KSR1 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) Dox-inducible RNAi of Ksr2 in KP1 and KP3 cells from SCLC GEMM. Dox-inducible RNAi of KSR2 in human SCLC cell line H209. NTC, non-targeting control. FACS of H209 (B) and KP1 (C) cells for CD24highCD44low (left) and EPCAMhigh (right) with (sh5) and without (CTRL) dox-inducible RNAi of KSR2. D) Percent TPCs detectable by FACS in control or KSR2-targeted (sh5) H209 and KP1 cells. Clonogenicity of Dox-treated KP3 (E) and KP1 (F) SCLC TPCs with and without the indicated shRNAs targeting Ksr2. Colony viability was measured by CellTiter-Glo. ****, p<0.0001, n=3.

**Fig. 3.** 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.

**Fig. 4.** A) A Ksr2 cDNA resistant to the sh5 shRNA (sh5RKSR2) or the empty expression vector were introduced into KP1 cells expressing the Dox-inducible sh5 shRNA. B) Clonogenicity of KP1 SCLC TPC colonies within wildtype (WT), dox-induced RNAi of Ksr2 (sh5) and dox-induced RNAi of Ksr2 + expression of shRNA-resistant Ksr2 (sh5RKSR2). Colony viability was measured by CellTiter-Glo. ****, p<0.0001. C-D) In vitro ELDA of KP1 sh5 or sh5RKSR2 KP1 cells with dox-induced RNAi of KSR2 was used to determine the estimated frequency of TPCs. ****, p<0.0001.

**Fig. 5.** A) In vitro ELDA was used to determine the proportion of TPCs in H209 cells with dox-induced RNAi of KSR2 (sh5) cells. B) Western blot of H209 cells following CRISPR/Cas9 expression with non-targeting (NTC1) control gRNA or gRNA (CR3) targeting KSR2. C) In vitro ELDA of H209 non-targeting control (NTC) cells or KSR2-targeted (KSR2KO) cells. ****, p<0.0001, (one representative image is shown of an experiment completed in triplicate).

**Fig. 6.** Western blot showing phosphorylation of ERK in KP1 sh5 (A), sh5RKSR2 (B), and sh5RFIF570 (C) cells with and without dox-induced KSR2 depletion with no treatment (NT), serum starvation, serum shock, or ionomycin treatment. D) Immunoprecipitation of FLAG epitope-tagged wildtype KSR2 (sh5RKSR2) and ERK-binding mutant KSR2 (sh5RFIF570). E) Clonogenicity of KP1 SCLC TPC colonies with dox-induced RNAi of Ksr2 (sh5) and dox-induced RNAi of Ksr2 + expression of hairpin resistant Ksr2 (sh5RKSR2) or hairpin resistant ERK-binding mutant Ksr2 (sh5RFIF570). F) Quantification of colonies greater than 300 RLU per 96/well plate, sample size is as indicated, ****, p<0.0001.
Fig. 1

A

KSR2 transcripts/10^6 total transcripts

Brain  Lung

B

Relative Expression (Graph)

KSR2

GFP+ GFP-

C

Non-NE marker

GFP- GFP+

KSR2 FPKM

Cancer Type

Non-NE marker

GFP- GFP+

KSR2 FPKM

Cancer Type

Fig. 1
Fig. 2
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         | 7.28         |
P value        | 0.00698      |
Fig. 4

A

|          | sh5 + vector | sh5 + sh5RKS2 |
|----------|--------------|---------------|
| DOX:     | -            | -             |
| Flag     | 1            | 0.5           |
| KSR2 high exposure | 1            | 0.5           |
| KSR2 low exposure | 1            | 0.5           |
| HDAC2    | 1            | 0.5           |

B

Relative luminescence RLU

C

TPC Frequency

D

| Group            | TPC Estimate |
|------------------|--------------|
| sh5              | 1/19.14      |
| sh5 + DOX        | 1/166.37     |
| sh5RKS2          | 1/8.73       |
| sh5RKS2 + DOX    | 1/17.97      |
Fig. 5

**A**

![Graph showing TPC frequency with DOX: + and -](image)

| Group      | TPC Estimate |
|------------|--------------|
| Control    | 1/5.49       |
| KSR2 KD    | 1/49.86      |
| **Chisq**  | **56.4**     |
| **P value**| **5.84e-14** |

**B**

H209

NTC1  CR3

KSR2

β actin

**C**

![Graph showing TPC frequency with NTC and KSR2KO](image)

| Group   | TPC Estimate |
|---------|--------------|
| NTC     | 1/10.7       |
| KSR2KO  | 1/131.8      |
| **Chisq** | **40.2**    |
| **P value** | **2.29e-10** |
| 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) | NCI-H209    |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | NCI-H1963   |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | NCI-H711    |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | NCI-H196    |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | NCI-H1836   |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | NCI-H82     |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | NCI-H146    |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | NCI-H524    |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | HCC33       |                        |
| Cell line (Homo sapiens)          | Small-cell lung carcinoma | Obtained from John Minna (UT Southwestern) | HBEC        |                        |
| Transfected construct             | piSMART hEF1a/TurboGFP-KSR2 shRNA 5 | Dharmaco | Cat# V3SH11252-225640245 | GGAGCAAAATCCACGAGTT |
| Transfected construct             | piSMART hEF1a/TurboGFP-KSR2 shRNA 6 | Dharmaco | Cat# V3SH11252-227968197 | GCACATCGACGACGCTC |
| Transfected construct             | piSMART hEF1a/TurboGFP-KSR2 shRNA 7 | Dharmaco | Cat# V3SH11252-228320208 | CCATCAAGCAGGTTTTT |
| Transfected construct             | lentiCRISPR v2-KSR2 CR3 | Addgene | Plasmid #52961 | KSR2_3: 5' CCGAATCGTCGATGTCG CA 3' |
| Transfected construct | lentiCRISPR v2- NTC | Addgene | Plasmid #52961 | NTC: 5' CCATATCGGGGCGAGACA TG 3' |
|------------------------|---------------------|---------|----------------|----------------------------------|
| Recombinant DNA reagent | sh5RKSR2           | (adapted from Fernandez et al. 2012)²⁷ | MSCV KSR2 IRES YFP | QuikChange Lightning Mutagenesis: Forward 5' CGCTGCACAGGAGTAAGT CCCAGTAATTCCAGCTCGG G 3' Reverse 5'CCCGAGCGTGAAATTCAT GGGACTTACTCTCTGTGCAG CG 3' |
| Recombinant DNA reagent | sh5RFIF570          | (adapted from Fernandez et al. 2012)²⁷ | MSCV KSR2 FIF570 IRES YFP | QuikChange Lightning Mutagenesis: Forward 5' CGCTGCACAGGAGTAAGT CCCAGTAATTCCAGCTCGG G 3' Reverse 5'CCCGAGCGTGAAATTCAT GGGACTTACTCTCTGTGCAG CG 3' |
| Sequence based reagent | Ksr2 (PCR primer)   | (Guo et al. 2017)²⁹ | Forward primer 5’- TGGATGTCCGAAAGGAAGTC-3’ Reverse primer 5’- CTTCCTCACGGGTCTCAGCG-3’ |
| Sequence based reagent | Crgp (PCR primer)   | (Lim et al. 2017)³⁶ |
| Sequence based reagent | Chga (PCR primer)   | (Lim et al. 2017)³⁶ |
| Sequence based reagent | Syp (PCR primer)    | (Lim et al. 2017)³⁶ |
| Sequence based reagent | Spc (PCR primer)    | (Lim et al. 2017)³⁶ |
| 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               | p44/42 MAPK (Erk1/2), rabbit polyclonal | Cell Signaling | Cat# 9102 | WB 1:1000 |
| Antibody               | Anti-Flag (M2), mouse monoclonal | Sigma | Cat# F3165 | WB 1:1000 |
| Antibody                        | Description                                                                 | Supplier       | Cat#              | Assay |
|--------------------------------|-----------------------------------------------------------------------------|----------------|------------------|-------|
| Antibody                       | Anti-HDAC2 (Y461), rabbit monoclonal                                        | Abcam          | ab32117          | WB: 1:1200 |
| Antibody                       | APC anti-mouse CD326 (EpCAM), mouse monoclonal                              | Biolegend      | 118213           | FACS: 1:100 |
| Antibody                       | PE anti-mouse CD24, mouse monoclonal                                        | Biolegend      | 101807           | FACS: 1:400 |
| Antibody                       | PE Cyanine7 anti-mouse/human CD44, mouse monoclonal                          | Biolegend      | 103029           | FACS: 1:300 |
| Nuclear Stain                  | DAPI (4’,6-Diamidino-2-Phenylindole, Dilactate)                              | Biolegend      | 422801           | FACS: 3 uM |

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