KSR1 regulates small-cell lung carcinoma tumor initiation and therapy resistance

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Abstract

Small-cell lung cancer (SCLC) is designated a recalcitrant cancer due to its five-year relative survival rate of less than 7%. First line SCLC treatment has not changed in the last 40 years. The NeuroD1 subtype of SCLC (SCLC-N) commonly harbors MYC amplifications and other hallmarks of aggressive behavior. Finding novel therapeutic options that effectively eliminate residual disease observed after initial response to therapy is essential to improving SCLC patient outcome. Tumor-initiating cells (TICs) are reported as the sanctuary population within the bulk tumor responsible for therapeutic resistance and relapse. In contrast to earlier studies in which ERK activation is reported to be inhibitory to growth and tumor development, we show that KSR1 signaling is conserved in SCLC-N and that it regulates tumor initiation through ERK. Thus, KSR1 function in SCLC-N serves as a novel model for understanding the role of KSR1-dependent signaling in normal and malignant tissues. We further show that KSR1 mediates cisplatin resistance in SCLC-N cells. CRISPR/Cas9-mediated KSR1 knockout causes a dramatic increase in sensitivity to cisplatin and is coincident with a marked decrease in TICs, indicating that targeting KSR1 might be selectively toxic to cells responsible for therapeutic resistance and tumor initiation. Our data show that KSR1, a molecular scaffold for the Raf/MEK/ERK signaling cascade, is critical for tumor initiation and clonogenicity, both *in vitro* and *in vivo* in the highly aggressive, metastatic and therapy resistant NeuroD1 subtype of SCLC. These findings shed light on a key distinct protein responsible for regulation in SCLC-N tumors, and a potential subtype specific therapeutic target.

Introduction

Small-cell lung carcinoma (SCLC) is an aggressive, lethal, and highly metastatic disease accounting for 13% of all lung cancers[1]. Predominately affecting heavy smokers, SCLC is also highly metastatic with 50%–80% of patients harboring metastases at the time of autopsy[2]. SCLC was categorized as a recalcitrant cancer in 2012 due to only modest improvements in SCLC detection, therapy, and survival over the past 40 years[3]. The five-year relative survival rates for patients with local, regional, and distant SCLC are 27%, 16%, and 3%, respectively (Cancer.net, ASCO, 2022). Currently, SCLC tumors are treated with first line (cisplatin or carboplatin, etoposide, plus an anti-PDL1 antibody), second line (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[4]. Despite extensive genomic and molecular analysis, few biochemical pathways in SCLC have yielded convincing therapeutic targets that might selectively disable SCLC with minimal toxicity to normal tissue. Identification of functionally significant pathways and proteins regulating growth and survival whose inhibition selectively disables the tumor's self-renewal capacity, could lead to the development of novel therapeutic strategies for SCLC, especially promoting a more durable treatment response and lesser incidences of recurrence.

Rigorous and innovative basic science using state-of-the-art genetically engineered mouse models (GEMM), and extensive cell lines painstakingly generated by multiple labs have led to key discoveries regarding the cells-of-origin and the common recurring mutations that underlie SCLC[5-12]. 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)[9,13]. A subpopulation of self-renewing pulmonary neuroendocrine cells (PNECs) give rise to SCLC-A tumors when TP53, Rb, and Notch mutations cause constitutive activation of stem cell renewal and altered deprogramming[14]. The transformed, self-renewing population within SCLC-A, which has a neuroendocrine origin, is characterized by high levels of CD24 and EPCAM and low levels of CD44[15]. Myc overexpression, which is common in aggressive SCLC, drives conversion of SCLC-A to SCLC-N[16]. Expression of NeuroD1 in SCLC-N is linked to the development of metastases and aggressive SCLC[17].

SCLC tumors have a small population of tumor initiating cells (TICs) essential for the initiation, long-term propagation, and metastatic ability of the tumor, as well as a bulk non-TIC population that is highly proliferative but inefficient at establishing new tumors *in vivo*[15]. TICs may be the sanctuary population within the bulk tumor responsible for therapeutic resistance in SCLCs[18-24]. Despite the negative implication of TICs in patient survival[25,26], very little is understood about the underlying mechanisms that support TIC formation and function.

Kinase Suppressor of Ras (KSR) proteins, namely Kinase suppressor of Ras 1 (KSR1) and KSR2 are molecular scaffolds that promote extracellular signal-regulated kinase (ERK) signaling through the mitogen-activated protein kinase (MAPK) pathway but have differing expression profiles and physiological functions[27,28]. KSR1 is required for oncogenic Ras-induced transformation[29,30], is overexpressed at the protein and the mRNA levels in human colorectal cancer (CRC) cell lines and is dispensable for normal cell survival[31]. *Ksr1*$^{-/-}$ knockout mice are fertile, developmentally normal, but are resistant to Ras-driven tumor formation[32]. KSR1 regulates Ras-dependent transformation and survival through both the Raf/MEK/ERK kinase cascade [29,30,33-36] and AMPK [27,33,37]. These observations suggest that KSR1 and the downstream effectors it modulates are essential for tumor formation but dispensable for normal tissues. Targeting these effectors should be selectively toxic to tumors. Our work reveals KSR1 regulates clonogenicity and tumor initiation in SCLC-N, both *in vitro* and *in vivo*. It also highlights KSR1 as a novel player in promoting therapy resistance in this aggressive and therapy resistant subtype

of NeuroD1 subtype of SCLC. This result defines a novel mechanism of tumor initiation in SCLC-N and a potential therapeutic vulnerability.

Materials and Methods

Cell culture

Human small-cell lung carcinoma cell lines H82, H524, HCC33 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.

Generation of pooled genomic KSR1 KO cell line H82

sgRNA sequences targeting KSR1 or non-targeting control (NTC) were inserted into pLentiCRISPRv2GFP (Addgene #82416). 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 H82 cells with 8 µg/mL Polybrene. GFP+ cells were selected by fluorescence-activated cell sorting after 72-96 hours.

Generation of inducible genomic KSR1 KO cell lines

sgRNA sequences targeting KSR1 or non-targeting control (NTC) were inserted into TLCV2-RB1 (pLentiCRISPRv2GFP modified with puromycin selection marker) (Addgene #87836). 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 added to H82, H524, and HCC33 cells. 48-h post-infection, cells were selected in 2 µg/mL Puromycin (Invitrogen). The cells were then treated with 2 µg/mL doxycycline for 72 hours, inducing Cas9-2A-eGFP. The GFP+ cells were finally selected by fluorescence-activated cell sorting.

Generation of KSR1 addback in inducible genomic KSR1 KO cell line H82

Murine KSR1 was cloned into MSCV-IRES-KSR1-RFP (Addgene #33337) and PEI transfected into HEK293T cells along with pUMVC retroviral packaging construct (Addgene #8449) and CMV5-VSVG envelope construct (Addgene #8454). Retrovirus containing media was harvested at 96-h and added to inducible KSR1 KO H82 cells with 8 mg/ml polybrene. RFP-selection was performed by fluorescence-activated cell sorting 48 h post-infection.

Generation of ERK-binding mutant genomic KSR1 KO cell line H82

A KSR1 transgene deficient in binding ERK due to engineered mutation in the DEF-domain, $KSR1^{AAAP}$ [29,34] was cloned into MSCV-IRES-KSR1-RFP and PEI transfected into HEK293T cells along with pUMVC retroviral packaging construct (Addgene # 8449) and CMV5-VSVG envelope construct (Addgene #8454). Retrovirus-containing media was harvested at 96 h and added to *KSR1* KO cells with 8 mg/ml polybrene. RFP-selection was performed by fluorescence-activated cell sorting 48 h post-infection.

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).

Colony Formation Assay

For colony formation assays, SCLC cells were dissociated by gentle pipetting. Cells were DAPI-stained for viability determination and live GFP+ cells were single cell sorted as one cell per well into a 96-well plate (Fisher #12556008) filled with regular media (200 µl/well). 50 µl fresh media was added to the wells once every week. Three-four 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.

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 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. 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 absence/presence of KSR1 by western blot. ELDA software was used to estimate TIC frequency for control and KSR1 KO 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 except for the 10 cells/well condition, for which 24 wells were seeded. Cells were cultured for 10-15 days, and wells with spheroids >100 µM were scored as spheroid positive. TIC frequency and significance between groups was calculated by ELDA website https://bioinf.wehi.edu.au/software/elda/ [38] To assess the effect of cisplatin on TIC frequency, cells were plated in the presence of the indicated dose of cisplatin and scored as positive/negative after 10-15 days as previously described.

Dose response Curve

Cells were seeded at 10,000 cells per well in 50 µl in the inner-60 wells of 96-well black-sided, clear-bottom culture plates (Fisher #237105) and allowed to incubate for 24 hours prior to drug treatment. After 24 hours, cells were treated with nine different nM drug concentrations (20, 100, 200, 500, 1000, 2000, 10000, 20000, 50000) with each column of the 96 well plate getting a particular concentration of drug for 72 hours prior to assessment of cell viability using CellTiter-Glo® 2.0. Data were analyzed by non-linear regression using Prism 9.

Multi-well Resistance Assay

Cells were plated at low density (1000-2000 cells/well) in replicate 96-well plates, and each plate was treated with the indicated doses of cisplatin. Wells were fed and assessed weekly for outgrowth, wells that were >50% confluent were scored as resistant to the given dose of cisplatin. Data are plotted as a Kaplan-Meier survival curve; significance was assessed by comparing Kaplan-Meyer Meier curves using Prism 9.

Results

KSR1 is required for clonogenicity in SCLC NeuroD1 subtype cell lines H82, H524 and HCC33.

SCLC tumors have a small population of tumor initiating cells (TICs) essential for the initiation, long-term propagation, and metastatic ability of the tumor, as well as a bulk non-TIC population that is highly proliferative but inefficient at establishing new tumors *in vivo*[15]. TICs may be the sanctuary population within the bulk tumor responsible for therapeutic resistance in SCLCs[18-24]. Despite the negative implication of TICs in patient survival[25,26], very little is understood about the underlying mechanisms that support TIC formation and function. As KSR1 but not KSR2 is expressed in the aggressive SCLC-N subtype[48] we tested the ability of KSR1 disruption to impair clonogenicity and TIC formation in these cells. KSR1 was targeted in three different SCLC-N cell lines H82, H524, and HCC33 by constitutive expression or DOX-induction of Cas9 and one of two distinct sgRNAs (CR1, CR2) **(Fig. 1, upper panels)**. Clonogenicity is an *in vitro* index of self-renewal, a key property of tumor initiating cells[15]. Clonogenicity was tested through single-cell colony forming assays and *in vitro* Extreme Limiting Dilution Analysis (ELDA)[15,38]. Following confirmation of KSR1 knockout by western blotting, control (NTC) and KSR1^{KO} cells were sorted by FACS and plated as single cells in 96-well plates to be analyzed for colony formation by CellTiter-Glo®. Robust colony formation was observed for the control SCLC-N cells that was significantly suppressed with KSR1 knockout in all 3 cell lines **(Fig. 1, lower panels)**. These data demonstrate that KSR1 is a significant contributor to the clonogenic properties of SCLC-N TICs.

KSR1 disruption inhibits TIC frequency in SCLC NeuroD1 subtype cells.

A tumor-initiating cell marker-free approach to measure clonogenicity is extreme limiting dilution analysis (ELDA), which is estimates stem cell frequency from limiting dilution analysis[38]. ELDA, when performed *in vitro* determines the stem cell frequency within the bulk tumor cell population based on whether a spheroid is formed or not formed at a variety of cell dilutions. An *in vitro* ELDA revealed a 3-fold dependency of H82 TICs on KSR1 expression. TIC frequency decreased from 1 TIC/3.36 tumor cells in control (NTC) to 1 TIC/11 tumor cells in *KSR1*^{KO} H82 cells **(Fig. 2A)**. This dependence upon KSR1 was replicated in SCLC-N cell line H524, where CRISPR targeting KSR1 also decreased TIC frequency 3-fold from 1 TIC/14.3 tumor cells in control (NTC) to 1 TIC/46.7 tumor cells in *KSR1*^{KO} H524 cells **(Fig. 2B)**, In SCLC-N cell line HCC33, depletion of KSR1 similarly decreased TIC frequency 3-fold from 1 TIC/2.65 tumor cells in control (NTC) to 1 TIC/8.70 tumor cells in *KSR1*^{KO} HCC33 cells **(Fig. 2C)**. Taken together, these data demonstrating a significant dependence of SCLC-N TICs on KSR1.

KSR1 regulates tumor initiation through ERK signaling in the SCLC NeuroD1 subtype.

To allay any concerns of off-target effects from the use of CRISPR-Cas9 targeting to establish KSR1^{KO} SCLC-N cell lines, a construct containing wildtype murine KSR1 that cannot be recognized by the sgRNAs (CR1, CR2) was expressed in *KSR1*^{KO} H82 cells. Rescue of KSR1 expression restored *in vitro* colony formation to wildtype levels in these cells **(Fig. 3B)**. The *in vitro* ELDA assays reveal a restoration of the TIC frequency to 1 TIC/2.68 tumor cells, comparable to the TIC frequency of 1 TIC/3.66 tumor cells in control (NTC) H82 cells **(Fig. 3C)**. These data confirm the on-target effects of our CRISPR targeting. The molecular scaffold Kinase Suppressor of Ras 1 (KSR1) contributes to TIC formation by promoting efficient signaling through the RAF/MEK/ERK kinase cascade[29,30,33-36]. To determine if KSR1-regulated ERK signaling pathway might be important in regulating tumor initiation in SCLC-N, a construct containing a transgene KSR1 deficient in binding ERK due to engineered mutation in the DEF-domain, KSR1^{AAAP} was expressed in *KSR1*^{KO} H82 cells[29,34]. Knockout of KSR1 reduces activation of ERK signaling in

H82 human SCLC-N cell line, that is rescued by the addition of KSR1 **(Fig. 3A)**. Furthermore, KSR1 deficient in binding ERK is unable to restore *in vitro* colony formation to wildtype levels in these cells **(Fig. 3B)**. Similarly, *in vitro* ELDA assays reveal a TIC frequency of 1 TIC/7.44 tumor cells for the ERK binding mutant cells, similar to the TIC frequency observed for the *KSR1*^{KO} cells, demonstrating that the mutant is also unable to restore the TIC frequency **(Fig. 3C)**. Under normal circumstances, all these cells (NTC, KSR1^{KO}, ERK MUTANT and RESCUE cells) grow at similar rates as determined by the growth curve, monitored over a period of a week **(Fig. 3D)**. These data show that KSR1 signaling is conserved in SCLC-N, suggesting that KSR1-dependent ERK signaling may contribute to SCLC-N tumor formation.

KSR1 disruption inhibits the tumor initiating capacity of SCLC-N cells *in vivo.*

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. To test the effect of KSR1 disruption *in vivo* using ELDA, control (NTC) and *KSR1*^{KO} H82 cells were injected with successive dilutions subcutaneously into NOD Prkdc^{em26Cd52}Il2rg^{em26Cd22}/NjuCr (NCG) mice. Tumors were monitored until one tumor reached 1 cm^2 and then all mice were sacrificed. The presence and absence of tumor formation was scored as “1” and “0” respectively. KSR1 disruption reduced frequency of TICs around 3-fold, from 1/62.5 control (NTC) H82 cells to 1/205.6 H82 cells with KSR1 knockout (KSR1^{KO}) (**Fig. 4**). These data indicate that KSR1 is a important effector of self-renewal and clonogenicity of SCLC-N TICs cells *in vivo*.

KSR1 disruption sensitizes SCLC NeuroD1 cell lines to cisplatin.

Considering the role of the TIC population in therapeutic resistance[2-8], and the contribution of KSR1 in promoting TIC formation, the effect of KSR1 disruption was tested on SCLC-N response to the first line chemotherapeutic, cisplatin. To establish the doses of cisplatin that inhibits survival (≥ EC50) in the SCLC-N cell lines, control (NTC) and *KSR1*^{KO} H82, H524, and HCC33 cells were treated with increasing doses of cisplatin and cell viability was assessed 72 hours after treatment **(Fig. 5A, B, C, upper panels)**. Dose response curves revealed that in all three SCLC-N cells, KSR1 disruption modestly but insignifcantly reduced the ED50. The doses of cisplatin that inhibited survival were higher in H82 than the other two SCLC-N cell lines, H524 and HCC33, which were more sensitive to the drug. This allowed us to use the same dose in each control and KO cell line to determine the concentration of cisplatin that caused prolonged growth arrest (> 6 weeks) prior to cell outgrowth in a majority of cell populations for each cell line, in a multi-well resistance assay[39]. For the assay, control (NTC) and *KSR1*^{KO} cells were plated at low density (3000 cells/well; <10% confluent) in multiple 96-well plates and each plate was either left untreated (to assess the time required for normal cell outgrowth) or treated weekly with a single inhibitory dose of cisplatin (ED85 dose for H82, ED75 for H524 and HCC33) for up to 14-15 weeks. All 96 wells of each plate were visually assessed weekly and wells that were ≥ 50% confluent were scored as resistant to that dose of cisplatin. Lack of wells being scored as resistant, indicates long term drug sensitivity. Percent growth inhibition was plotted as a Kaplan-Meyer curve. *KSR1*^{KO} prevented the outgrowth of resistant colonies in all the three SCLC-N cells. Cisplatin sensitivity was maintained for 11-12 weeks in *KSR1*^{KO} H82 cells, while more than 50% control cells showed complete resistance in 5-6 weeks for the H82 cells. For the more sensitive SCLC-N cell lines H524 and HCC33, cisplatin sensitivity was maintained for 12-13 weeks in *KSR1*^{KO} cells, while more than 50% control cells showed complete resistance in 5-6 weeks **(Fig. 5A, B, C, lower panels)**.

Based on our evidence of KSR1-regulated ERK signaling pathway playing an important role in regulating tumor initiation in H82 **(Figs. 1-2)**, we determined whether ERK is also important in regulating cisplatin resistance in SCLC-N. Multi-well resistance assays were performed with

control (NTC) H82 cells, *KSR1*^{KO} H82 cells or , *KSR1*^{KO} H82 cells expressing KSR1 (RESCUE) or a mutated KSR1 construct unable to bind ERK (ERK MUTANT)[29,34]. While more than 70% control and rescue wells showed complete resistance in 8-9 weeks, only 10% of *KSR1*^{KO} wells and 25% of well with *KSR1*^{KO} expressing the ERK binding mutant became cisplatin resistant **(Fig. 5D)**. These data indicate that the ability of KSR1-dependent signaling to promote cisplatin resistance in SCLC-N cells is dependent upon interaction with ERK.

KSR1 knockout enhances cisplatin toxicity toward SCLC-N tumor initiating cells.

Tumor initiating cells are associated with drug resistance[18-24].To understand if KSR1-dependent affects on cisplatin resistance are related to KSR1-dependent TIC formation, we performed an *in vitro* ELDA with the control (NTC) and *KSR1*^{KO} H82 cells with and without cisplatin (ED85). While the TIC frequency decreases by 3-fold in the absence of any drug treatment in control (1 TIC/2.82 tumor cells) vs *KSR1*^{KO} (1 TIC/8.34 tumor cells) H82 cells, cisplatin treatment reduced the TIC frequency greater than 20-fold from 1 TIC/8.34 tumor cells in *KSR1*^{KO} cells without treatment to 1 TIC/173.36 tumor cells in *KSR1*^{KO} cells with ED85 cisplatin treatment **(Fig. 6A)**. Since the other two SCLC-N cell lines, H524 and HCC33 are more sensitive to the drug as indicated by the dose response studies earlier, they do not survive the high ED85 drug dose. Hence, for H524 and HCC33, *in vitro* ELDA was performed with increasing doses of cisplatin (ED25, ED50 and ED75) for the control (NTC) and *KSR1*^{KO} cells. For each cell line, while there is an expected decrease in the TIC frequency of 3-4 fold with KSR1 disruption with each cisplatin dose, the TIC frequency decreased in a dose-dependent manner with increasing doses of cisplatin. The ability of KSR1 KO to enhance the ability of three different doses of cisplatin to decrease TICs 3-fold in H524 and HCC33 cells shows that a KSR1-mediated cisplatin resistance is coincident with KSR-dependent TIC formation in SCLC-N cells.

Discussion

Our data reveal an unexpected role of the Raf/MEK/ERK scaffold KSR1 in regulating ERK-dependent resistance to the first line chemotherapeutic cisplatin and in the formation and maintenance of tumor initiating cells. These data may yield translatable approaches for SCLC therapy which has shown modest improvement in the last 40 years and led to the classification of SCLC as a recalcitrant cancer in 2012[3]. Rigorous molecular characterization has led to key discoveries regarding the cells-of-origin and the common recurring mutations that underlie SCLC[5-12]. 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)[9,13]. Based on PDX studies, a fifth subtype based on the bHLH proneural transcription factor ATOH1, has also been proposed[40,41]. Myc overexpression, which is common in aggressive SCLC, drives conversion of SCLC-A to SCLC-N [16]. All SCLC subtypes 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[16,42]. SCLC tumors have a subpopulation of tumor initiating cells (TICs) essential for the initiation, long-term propagation, and metastatic ability of the tumor, as well as a bulk non-TIC population that is highly proliferative but inefficient at establishing new tumors *in vivo*[15]. TICs may be the sanctuary population within the bulk tumor responsible for therapeutic resistance in SCLCs[18-24]. Our data shows that KSR1 is a novel regulator of SCLC-N TIC clonogenicity both *in vitro* and *in vivo*, and a potential subtype specific therapeutic target.

Kinase suppressor of Ras 1 (KSR1) is a molecular scaffold, acting downstream of Ras and growth factor receptor tyrosine kinases coupled to Ras that promote extracellular signal-regulated kinase (ERK) signaling through the mitogen-activated protein kinase (MAPK) pathway[27,28]. Rather than activating Ras mutations, however, SCLC tumorigenesis is driven by loss of function mutations

to tumor suppressor genes, T*p53* and *Rb*[43]. The interaction of activated ERK with KSR1 is essential to its signaling capability[34]. The inability of mutated KSR1 transgenes that produce a molecular scaffold defect in ERK binding reveal the dependence of SCLC clonogenicity and spheroid formation on KSR1 regulation through ERK signaling in SCLC-N. This observation is surprising because activation of the RAF/MEK/ERK signaling pathway is reported to be detrimental to non-KRAS mutated SCLC tumor growth[44-48]. Explanations for the disparity include the possibility that KSR1-dependent ERK signaling is uniquely important for the TIC subpopulation or that KSR proteins mediate additional signals other than those conveyed by their scaffolding properties. It's also possible that KSR1-dependent signaling is specific to the NeuroD1 subtype. Although the role of ERK signaling in SCLCs is incompletely understood, it has been implicated in cell proliferation, differentiation, survival, and drug resistance[49] therefore, evaluating its potential role in regulating tumor initiation is necessary to understand the implications of targeting ERK signaling in SCLC tumors. Future work should be directed toward defining the mechanisms upstream and downstream of KSR1-dependent TIC formation and drug resistance.

The five-year relative survival rates for patients with local, regional, and distant SCLC are 27%, 16%, and 3%, respectively (Cancer.net, ASCO, 2022). Currently, SCLC tumors are treated with first line (cisplatin or carboplatin, etoposide, plus an anti-PDL1 antibody), 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[4]. Despite extensive genomic and molecular analysis, few biochemical pathways in SCLC have yielded convincing therapeutic targets that might selectively disable SCLC with minimal toxicity to normal tissue. TICs serve as the sanctuary population within the bulk tumor responsible for therapeutic resistance and relapse[50]. There are currently no drugs selectively targeting TICs. *KSR1* disruption sensitizes SCLC-N to the first line chemotherapy drug, cisplatin. Although MEK/ERK inhibitors are not routinely used for treatment of SCLCs because of a lack of KRAS mutation, our data show that KSR1 is an important regulator of stemness in SCLC-N. Multi-well resistance assays show *KSR1*^{KO} prevented the outgrowth of resistant colonies in all the three SCLC-N cells, with cisplatin sensitivity being maintained for more than 12 weeks in *KSR1*^{KO} H82 cells. This dramatic increase in cisplatin sensitivity is shown to be a result of a KSR1-mediated decrease in TICs, indicating that targeting KSR1 might be selectively toxic to the TICs, the subset of cells known to be responsible for therapeutic resistance. Future studies will examine the effect of *KSR1*^{KO} on cisplatin resistance in the native tumor environment using an orthotopic *in vivo* tumor model**.**

Our data show that KSR1 is required for viability of the clonogenic and self-renewing tumor-initiating population essential for SCLC formation and that disruption of KSR1 may enhance the efficacy of first line chemotherapeutics used to treat SCLC with specific lethality directed towards the tumor-initiating subpopulation within SCLC. This study is an initial step towards identification of functionally significant pathways whose inhibition selectively impairs the tumor's self-renewal capacity, and could lead to the development of novel therapeutic strategies for SCLC, promoting a more durable treatments and reduced recurrence.

Key Resources

KSR1 antibody: Abcam ab68483, 1:750 dilution
β-actin antibody: Santa Cruz Biotechnology sc-47778, 1:1000 dilution
pERK1/2 antibody: Cell Signaling 9101; 1:500 dilution
ERK1/2 antibody: Cell Signaling 9102; 1:1000 dilution
KSR1 sgRNA sequences:
CR1 5' TTGGATGCGCGGCGGGAAAG 3'
CR2 5' CTGACACGGAGATGGAGCGT 3'
NTC sgRNA sequence: 5' CCATATCGGGGCGAGACATG 3'

Plasmid catalog numbers listed in the sections above.

Funding Information

This work was supported by NIH award GM121316, Lung Cancer Research Program (LCRP) award LC210123, and a Nebraska DHHS LB506 award to R.E.L.
The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

Acknowledgements

The authors thank the UNMC Flow Cytometry Research Facility for help isolating the GFP+ SCLC cells. We declare no conflicts of interest.

Figures

Fig 1

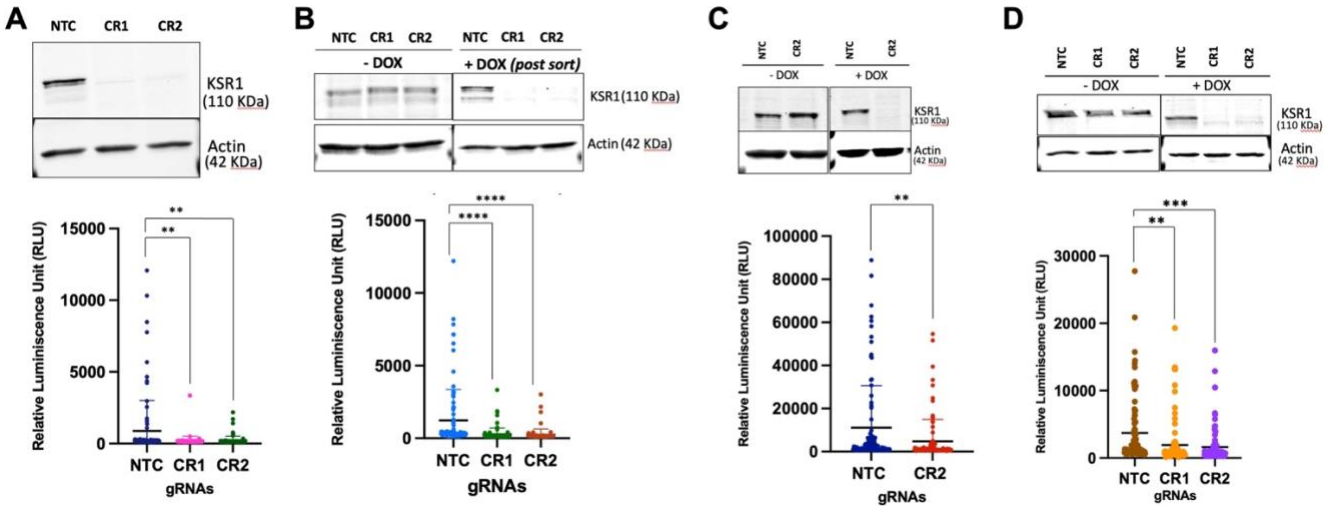
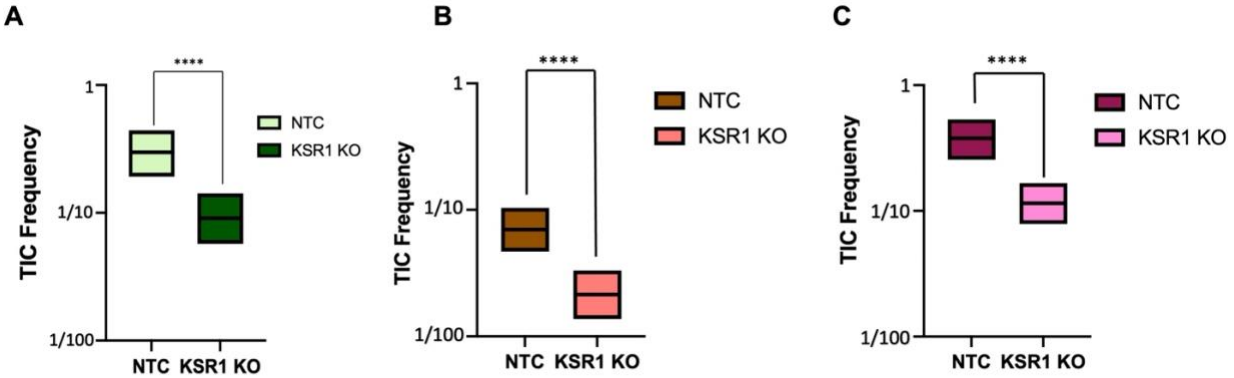


Fig 2

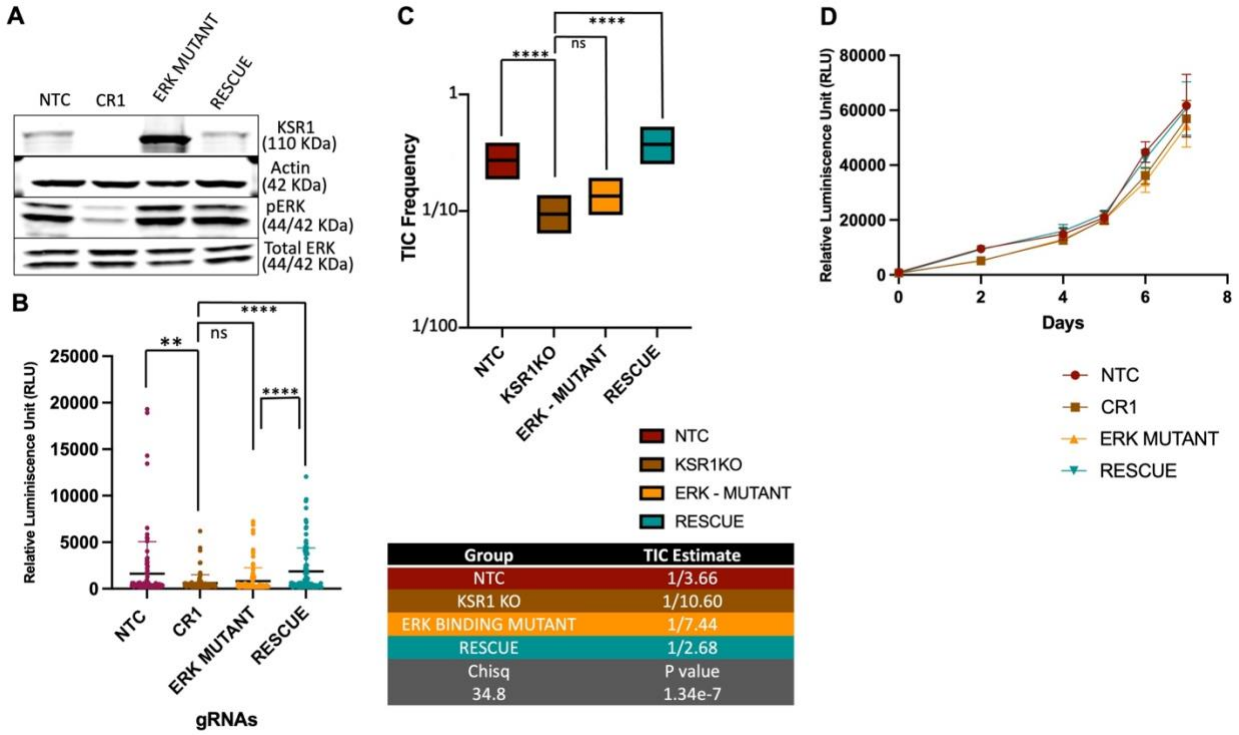


Group	TIC Estimate
NTC	1/3.36
KSR1 KO	1/11.03
Chisq	P value
14.5	0.000142

Group	TIC Estimate
NTC	1/14.3
KSR1 KO	1/46.7
Chisq	P value
24.3	$8.45e^{-7}$

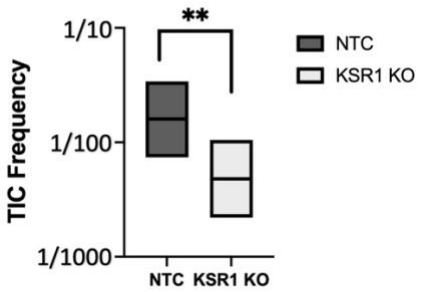
Group	TIC Estimate
NTC	1/2.65
KSR1 KO	1/8.70
Chisq	P value
23	$1.58e^{-6}$

Fig 3



Group	TIC Estimate
NTC	1/3.66
KSR1 KO	1/10.60
ERK BINDING MUTANT	1/7.44
RESCUE	1/2.68
Chisq 34.8	P value 1.34e-7

Fig 4



GROUP	TIC ESTIMATE
NTC	1/62.5
KSR1 KO	1/205.6
Chisq 7.66	P value 0.00564

Fig 5

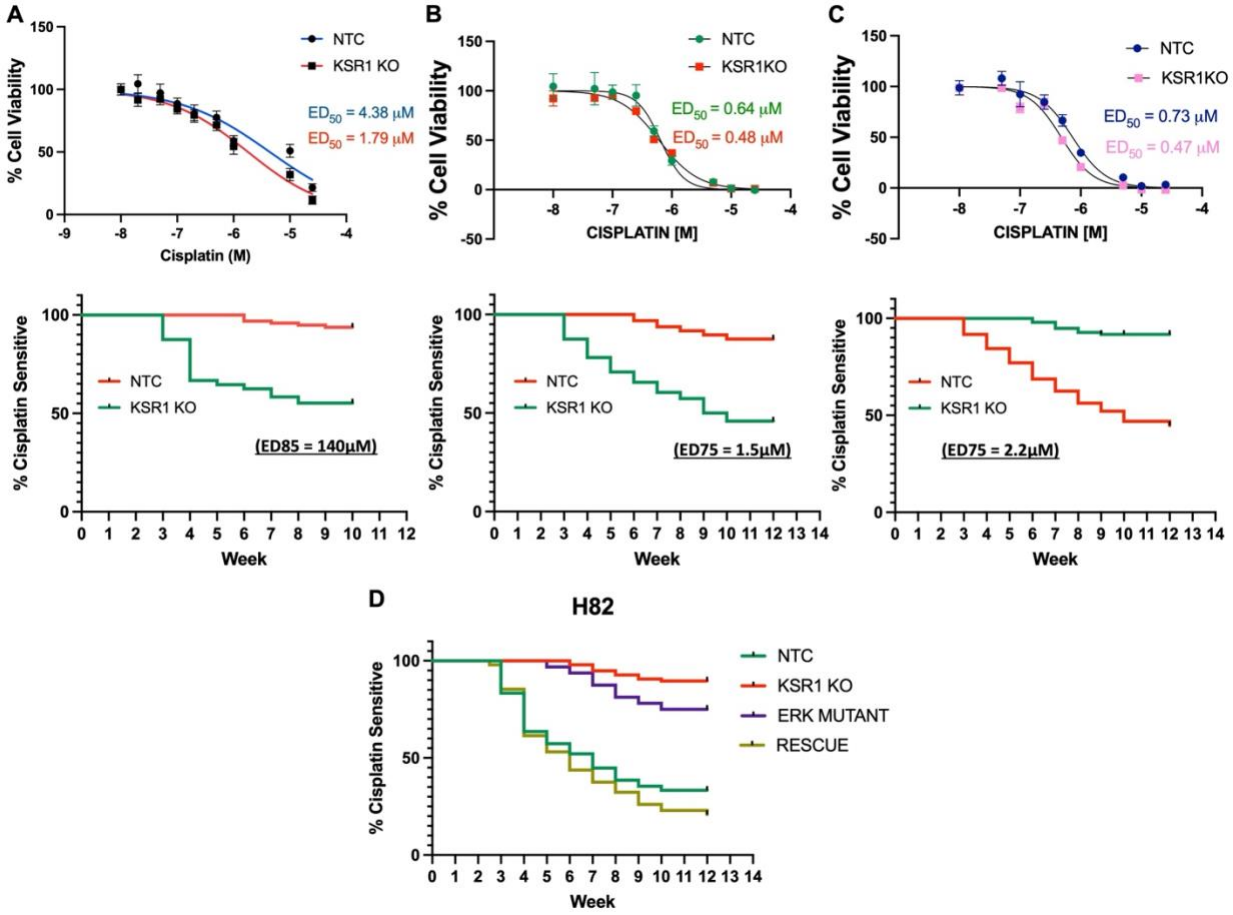
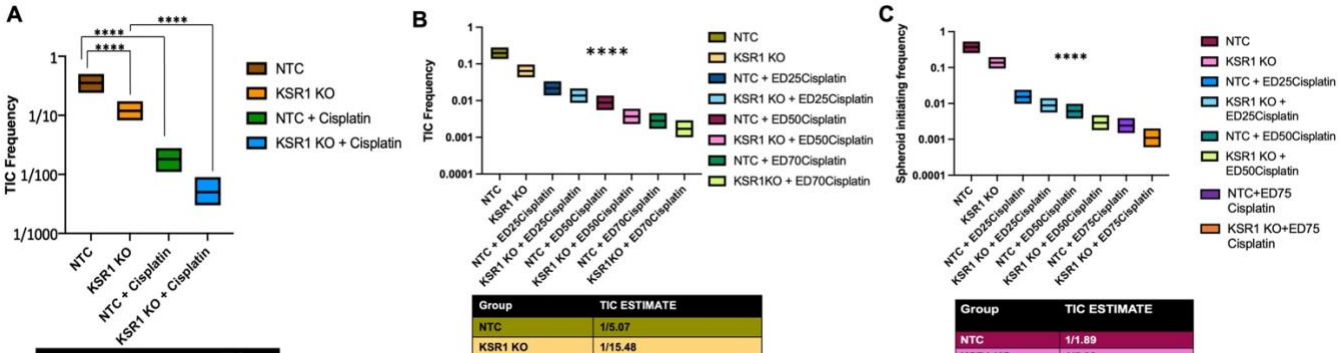


Fig 6



Group	TIC Estimate
NTC	1/2.82
KSR1 KO	1/8.34
NTC + Cisplatin	1/54.27
KSR1 KO + Cisplatin	1/173.36
Chisq 302	P value $3.32e^{-65}$

Group	TIC ESTIMATE
NTC	1/5.07
KSR1 KO	1/15.48
NTC + ED25 Cisplatin	1/46.17
KSR1 KO + ED25 Cisplatin	1/72.64
NTC + ED50 Cisplatin	1/113.59
KSR1 KO + ED50 Cisplatin	1/265.33
NTC + ED75 Cisplatin	1/348.05
KSR1 KO + ED75 Cisplatin	1/558.48

Group	TIC ESTIMATE
NTC	1/1.89
KSR1 KO	1/5.09
NTC + ED25 Cisplatin	1/41.79
KSR1 KO + ED25 Cisplatin	1/71.36
NTC + ED50 Cisplatin	1/102.05
KSR1 KO + ED50 Cisplatin	1/212.46
NTC + ED75 Cisplatin	1/255.19
KSR1 KO + ED75 Cisplatin	1/491.25

Figure Legends

Fig. 1. KSR1 is required for clonogenicity in SCLC NeuroD1 cell lines H82, H524, HCC33.
(A) (upper) Western blot of H82 cells following CRISPR/Cas9 expression with non-targeting (NT) control gRNAs and gRNAs (CR1, CR2) targeting KSR1. (lower) Clonogenicity of H82 cells without (NT) and with constitutive (CR1, CR2) KSR1 disruption.
(B) (upper) Western blot of untreated and DOX-treated H82 cells with DOX-inducible expression of Cas9 and non-targeting (NT) control or KSR1-targeting (CR1, CR2) gRNAs. (lower) Clonogenicity of H82 cells without (NT) and with (CR1, CR2) DOX-inducible targeting of KSR1.
(C) (upper) Western blot of untreated and DOX-treated H524 cells with DOX-inducible expression of Cas9 and non-targeting (NT) control or KSR1-targeting (CR2) gRNA. (lower) Clonogenicity of H524 cells without (NT) and with (CR2) DOX-inducible targeting of KSR1.
(D) (upper) Western blot of untreated and DOX-treated HCC33 cells with DOX-inducible expression of Cas9 and non-targeting (NT) control or KSR1-targeting (CR1, CR2) gRNAs. (lower) Clonogenicity of HCC33 cells without (NT) and with (CR1, CR2) DOX-inducible targeting of KSR1.
**, $p<0.01$; ****, $p<0.0001$, (one representative image shown of experiment completed in triplicate, n=3)

Fig. 2. KSR1 is required for tumor initiation in SCLC NeuroD1 cell lines H82, H524, HCC33.
The proportion of TICs in SCLC-N cells was determined by *in vitro* Extreme Limiting Dilution Analysis (ELDA) of non-targeting control (NTC) and KSR1-targeted ($KSR1^{KO}$) H82 **(A)**, H524 **(B)**, HCC33 **(C)** cells for 7-9 days. ****, $p<0.0001$, (one representative image shown of experiment completed in triplicate, n=3)

Fig. 3. KSR1 interaction with ERK is required for H82 TIC formation.
(A) Western blot of H82 cells without (NTC) and with (CR1) DOX-inducible targeting of KSR1, KSR1 ERK binding MUTANT and RESCUE (KSR1 added back to $KSR1^{KO}$ cells), showing pERK/Total ERK levels.
(B) Clonogenicity of H82 cells without (NTC) and with (CR1) DOX-inducible targeting of KSR1, KSR1-ERK binding MUTANT, and RESCUE cells.
(C) The proportion of TICs in H82 cells was determined by *in vitro* Extreme Limiting Dilution Analysis (ELDA) of non-targeting control (NTC) and KSR1-targeted ($KSR1^{KO}$), KSR1-ERK binding MUTANT and RESCUE H82 cells for 7-9 days.
(D) Growth curve of non-targeting control (NTC) and KSR1-targeted ($KSR1^{KO}$), KSR1-ERK binding MUTANT and RESCUE H82 cells monitored for 7-9 days.
**, $p<0.01$; ****, $p<0.0001$, (one representative image shown of experiment completed in triplicate, n=3)

Fig. 4. KSR1 knockout inhibits SCLC-N tumor initiation in vivo.
The proportion of TICs in H82 cells was determined by *in vivo* Extreme Limiting Dilution Analysis (ELDA) of non-targeting control (NTC) and KSR1-targeted ($KSR1^{KO}$) cells. **, $p<0.01$, n=3 for each cell dilution

Fig. 5. KSR1 knockout sensitizes SCLC-N cells to cisplatin.
(upper) Cisplatin dose response curve with non-targeting control (NT), and KSR1 knockout ($KSR1^{KO}$) H82 **(A)**, H524 **(B)**, HCC33 **(C)** cells.
(lower) Multi-well resistance assay of NTC and $KSR1^{KO}$ lines treated continuously with the indicated ED85/ED75 concentrations of cisplatin and scored weekly. Wells with >50% cell confluency were deemed resistant to cisplatin.

(D) Multi-well resistance assay of NTC and KSR1^{KO}, ERK binding MUTANT and RESCUE H82 cells, (one representative image shown of experiment completed in triplicate, n=3)

Fig. 6. KSR1 regulates cisplatin resistance in SCLC NeuroD1 subtype cell line H82, H524 and HCC33.

The proportion of KSR1 dependent TICs in SCLC-N cells treated with Cisplatin was determined by *in vitro* Extreme Limiting Dilution Analysis (ELDA) of non-targeting control (NTC) and KSR1-targeted (KSR1^{KO}) H82 **(A)**, H524 **(B)** and HCC33 **(C)** cells using the indicated doses for 7-9 days. ****, $p<0.0001$.

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