KSR1 regulates small-cell lung carcinoma tumor initiation and cisplatin 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 changed modestly 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. Here we show that Kinase Suppressor of Ras 1 (KSR1), a molecular scaffold for the Raf/MEK/ERK signaling cascade is critical for clonogenicity and tumor initiation *in vitro* and *in vivo* in the highly aggressive, metastatic and therapy resistant NeuroD1 subtype of SCLC. Tumor-initiating cells (TICs) are reported as the sanctuary population within the bulk tumor responsible for therapeutic resistance and relapse. Previous studies concluded ERK activation was inhibitory to growth and tumor development. We show that signaling through KSR1 is conserved in SCLC-N and that it regulates tumor initiation through interaction with ERK. We further show that KSR1 mediates cisplatin resistance in SCLC-N cells. While 50% of control SCLC-N cells show resistance after 6 weeks of exposure to cisplatin, CRISPR/Cas9-mediated KSR1 knockout prevents resistance in >90% of SCLC-N cells. KSR1 KO also significantly enhances the ability of cisplatin to decrease SCLC-N TICs, indicating that targeting KSR1 might be selectively toxic to cells responsible for therapeutic resistance and tumor initiation. Thus, KSR1 function in SCLC-N serves as a novel model for understanding the role of KSR1-dependent signaling in normal and malignant tissues. These findings shed light on a key distinct protein responsible for regulation in SCLC-N tumors, and a potential subtype specific therapeutic target.

Impact

Genetic manipulation of the MAPK molecular scaffold, KSR1 in NeuroD1-subtype small-cell lung cancer cells reveals its contribution to cisplatin resistance in tumor initiation via ERK signaling.

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[1]. 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 therapy (cisplatin or carboplatin plus etoposide chemotherapy, anti-PDL1 antibody, atezolizumab), second line (topotecan), and third line (PD1 antagonist, nivolumab) therapies and for some patients thoracic radiation therapy[3]. 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[15]. In contrast, SCLC tumors also have 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) 1 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 but dispensable for normal cell survival[29-31]. *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]. KSR1 depletion suppressed transformation in Ras mutated colorectal cancer (CRC) cell lines and prevented drug-induced TIC upregulation in Ras mutation colon and non-small lung cancer cell lines [38,39]. 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.

Results

KSR1 is required for clonogenicity in SCLC-N.

SCLC tumors have been reported to harbor both a subpopulation of tumor initiating cells (TICs) as well as a bulk non-TIC population. In contrast to the TICs, which are crucial for initial establishment, long term propagation and metastatic ability of the tumors, the non-TIC population is highly proliferative but is inefficient at establishing new tumors *in vivo*[15]. In Ras mutated colorectal and non-small cell lung cancer cell lines, depletion of KSR1 suppressed transformation and prevented drug-induced TIC upregulation [38,39]. As KSR1 but not KSR2 is expressed in the SCLC-N subtype[40], 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 NCI-H82 (H82), NCI-H524 (H524), and HCC33 by constitutive expression or DOX-induction of Cas9 and one of two distinct sgRNAs (sg1, sg2) **(Fig. 1, upper panels)**. Clonogenicity is an in vitro index of self-renewal, a key property of TICs[15]. Clonogenicity was tested through single cell colony forming assays[15,41]. 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 three 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-N.

ELDA is a marker-free approach measuring TIC clonogenicity, which estimates stem cell frequency from limiting dilution analysis[42]. 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. *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)**. These data demonstrate a significant dependence of SCLC-N TICs on KSR1.

KSR1 regulates tumor initiation through ERK signaling in SCLC-N.

To test for off-target effects from the use of CRISPR-Cas9 targeting to establish KSR1 KO SCLC-N cell lines, a construct containing wildtype murine KSR1 (WTKSR1) that cannot be recognized by the sgRNAs (sg1, sg2) was expressed in KSR1 KO H82 cells **(Fig. 3A)**. 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 in WT KSR1 cells, comparable to the TIC frequency of 1 TIC/3.66 tumor cells in control (NTC) H82 cells **(Fig. 3C)**. These data confirm the specificity of KSR1 CRISPR targeting. The molecular scaffold KSR1 may contribute 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 encoding a KSR1 transgene deficient in binding ERK due to engineered mutation in the DEF-domain (KSR1AAAP) [29,34] was expressed in KSR1 KO H82 cells. Knockout of KSR1 reduces activation of ERK signaling as measured through phosphorylation of p90 ribosomal S6 kinase (RSK), key downstream substrates of ERK[43] in H82 human SCLC-N cell line, that is rescued by the addition of WT KSR1

transgene (Fig. 3A). Furthermore, KSR1 transgene deficient in binding ERK (KSR1AAAP) is unable to restore *in vitro* colony formation to WT 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 (KSR1AAAP) 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). Growth curves, monitored over one week show that control, KSR1^{KO}, KSR1AAAP and WT KSR1 cells grow at similar rates (Fig. 3D). These data show that KSR1 signaling through ERK signaling is conserved in SCLC-N, suggesting that KSR1-dependent ERK signaling may contribute to SCLC-N TIC 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*, control and KSR1 KO H82 cells were injected with successive dilutions subcutaneously into 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 an important effector of self-renewal and clonogenicity of SCLC-N TICs *in vivo*.

KSR1 disruption enhances cisplatin toxicity towards SCLC-N TICs.

Given the contribution of KSR1 in promoting TIC formation and the role of TICs in therapeutic resistance[1,2,4-8], the effect of KSR1 disruption was tested on SCLC-N response to the first line chemotherapeutic, cisplatin. To establish the doses of cisplatin that inhibit survival (≥ EC50) in the SCLC-N cell lines, control 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 insignificantly 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. To understand KSR1-dependent TIC formation in context of cisplatin resistance, 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. 5A, lower panel). For the more sensitive cell lines, H524 and HCC33, as indicated by the dose response curves, *in vitro* ELDA was performed with increasing doses of cisplatin (ED25, ED50, and ED75) for the control 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 (Fig. 5B, C lower panel) .

KSR1 disruption sensitizes SCLC-N cells to cisplatin.

The ability of KSR1 KO to enhance the ability of three different doses of cisplatin to decrease TICs 3-fold in SCLC-N cells (Fig. 5A, B, C, lower panels) led us to test if KSR1 disruption has any effect on cisplatin resistance in these cells. Although the dose-response curves, that are typically performed over a short period of 72 hours, revealed that in all three SCLC-N cells, KSR1 disruption only modestly reduced the ED50, this allowed us to use the same dose in each control and KO cell line to determine long-term cisplatin-dependent effects in a multi-well resistance

assay [39] in a more long-term approach. Control and KSR1 KO cells were plated at low density (1000 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 scored weekly and wells that were ≥ 50% confluent were scored as resistant to that dose of cisplatin. An absence of resistant cells 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 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 13 weeks in KSR1 KO cells, while more than 50% control cells showed complete resistance in 5-6 weeks **(Fig. 6A, B, C,)**. Thus, the multi-well resistance assays **(Fig. 6)** and *in vitro* ELDAs **(Fig. 5)** indicate that a KSR1-mediated cisplatin resistance is coincident with KSR1-dependent TIC formation in SCLC-N cells.

Based on our evidence of that KSR1-regulated ERK signaling plays an important role in regulating tumor initiation in H82 cells **(Figs. 1,2,3)**, we determined the extent to which the interaction of KSR1 with ERK contributes to 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 (WT KSR1) or a mutated KSR1 construct unable to bind ERK (KSR1AAAP). While more than 70% control and rescue wells showed complete resistance in 8-9 weeks, only 10% of KSR1 KO wells and 25% of wells with KSR1 KO expressing the ERK binding mutant became cisplatin resistant **(Fig. 6D)**. These data indicate that the ability of KSR1-dependent signaling to promote cisplatin resistance in SCLC-N cells is dependent upon interaction with ERK.

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 only shown modest improvement in the last 40 years and has led to the classification of SCLC as a recalcitrant cancer in 2012[1]. Extensive genomic and molecular analysis have led to a robust 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 now been proposed[44,45]. Myc overexpression, which is common in aggressive SCLC, drives conversion of SCLC-A to SCLC-N [16]. In spite of such detailed molecular characterization, 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[16,46]. Tumor initiating cells (TICs) are a slow-growing subpopulation essential to tumor formation. TICs also serve as the reservoir of drug-tolerant persister (DTP) cells [18-24]. Therefore, killing TICs, not just bulk tumor, is a key to limiting tumor dissemination and improving SCLC therapy. 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.

KSR1 is a molecular scaffold, acting downstream of Ras and growth factor receptor tyrosine kinases coupled to Ras that promote extracellular 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 of tumor suppressor genes, T*p53* and *Rb*[47]. 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 cell lines. 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[48-51]. This apparent disparity may indicate 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 ERK scaffolding properties that combine with ERK signaling to promote TIC formation and maintenance. It is 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[52] . 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 to identify the upstream and downstream molecules of KSR1-dependent ERK signaling to support TIC formation and explore the possibility of combined MEK and ERK inhibition to overcome therapy resistance in the non-KRAS mutated SCLC tumors[53].

Although the initial approach to SCLC treatment varies substantially by stage(54), the five-year relative survival rates for patients with local, regional, and distant SCLC are 27%, 16%, and 3%, respectively (Cancer.net, ASCO, 2022). The standard first line chemotherapy regimen for limited stage SCLC is cisplatin–etoposide, which has not changed for the past three decades[54]. Patients with metastatic disease are treated with systemic chemotherapy with or without immunotherapy. Even though SCLC is initially exceptionally responsive to cytotoxic therapies, in the vast majority of these patients, these responses are transient and exhibit a high incidence of relapse, in which case second-line chemotherapy is, in general, much less effective than initial treatment[54,55]. This results in a median survival duration of only about a year for patients with metastatic disease[54].

Recent studies show that ‘persisters’, a subpopulation of drug-tolerant cells found in tumors, play a critical role in the development of drug resistance. Like the TICs, persisters are often able to maintain viability under therapy but are typically slow cycling or dormant, adding to the growing evidence of the reported shared identity of TICs and DTPs[56]. Thus, specifically targeting the TICs should prevent the development of resistance, but 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 considered treatment of SCLCs because they lack KRAS mutations or evidence of tumor-dependent RAS-mediated signaling, our data show that KSR1 is an important regulator of ERK-signaling 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 maintained for more than 12 weeks in KSR1 KO H82 cells. This dramatic increase in cisplatin sensitivity coincides with a KSR1-mediated decrease in TICs, indicating that targeting KSR1 might be selectively toxic to the TICs. Thus, this study reveals a new direction in the form of KSR1-targeted therapies that may help develop more durable therapeutic strategies for SCLC that ultimately improve patient survival.

Materials and Methods

Cell culture

Human small-cell lung carcinoma cell lines NCI-H82 (H82), NCI-H524 (H524), HCC33 were a gift of John Minna (UT Southwestern). The cells were cultured in RPMI 1640 (VWR #16750-106) 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 and confirmed to be free of contamination.

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 polyethylenimine (PEI) (Tocris #7854) 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-hour post-transfection and used to infect the H82 cells with 8 µg/mL Polybrene (Sigma #TR1003G). GFP+ cells were selected by fluorescence-activated cell sorting after 72-96 hours on a BD FACSAria II using FACSDiva software.

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 with polybrene. 48-hour post infection, cells were selected in 2 µg/mL Puromycin (Invivogen #antpr1). The cells were then treated with 2 µg/mL doxycycline (Sigma #D9891) for 72 hours, inducing Cas9-2A-eGFP. The GFP+ cells were finally selected by fluorescence-activated cell sorting.

Generation of ectopically expressed KSR1 construct in inducible genomic KSR1 KO cell line H82

Murine KSR1 (resistant to degradation by sgRNA sequences targeting human 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-hour 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 hour and added to KSR1 KO cells with 8 mg/ml polybrene. RFP-selection was performed by fluorescence activated cell sorting 48 hours 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 (LICORBiosciences #927–50003) for 45 min at room temperature, then incubated with primary antibodies 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 (Abcam #ab228549) 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 complete media (200 µl/well). 50 µl fresh media was added to the wells once every week. Three-four weeks later, clonogenicity was assessed by measuring luminescence using CellTiter-Glo 2.0 reagent (Promega #G9242) and 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 (ELDA)

***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). Cells were injected subcutaneously into the shoulders and flanks of six eight-week-old *Prkdc*^{em26Cd52}*Il2rg*^{em26Cd22}/NjuCrl (NCG) mice. Three replicates for each dilution were used. Injection sites were palpated biweekly to monitor for tumor growth and all mice 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 (https://bioinf.wehi.edu.au/software/elda/) [42]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/ [42]. To assess the effect of cisplatin on TIC frequency, cells were plated in the presence of the indicated dose of cisplatin (Sigma #232120) 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 doses of drug concentrations spanning a range of concentrations (20, 100, 200, 500, 1000, 2000, 10000, 20000, 50000 nM) with each column of the 96 well plate getting a particular concentration of drug for 72 hours prior to assessment of cell viability using CellTiterGlo® 2.0 reagent. Data were analyzed by non-linear regression using GraphPad Software, Inc.

Growth Curve

Cells were seeded at low density (500 cells/well) in 100ul in replicate 96-well plates (Fisher #237105), with each condition plated in a single column of the 96 well plate. Each replicate plate was reserved for a single day. Wells were fed with complete media and assessed daily for growth for a period of 9 days, through assessment of cell viability using CellTiterGlo® 2.0 reagent. Data were analyzed by non-linear regression using GraphPad Software, Inc.

Multi-well Resistance Assay

Cells were plated at low density (1000-2000 cells/well) in replicate 96-well plates (Fisher #237105), and each plate was treated with the indicated doses of cisplatin (Sigma #232120). Wells were fed with complete media 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 GraphPad Software, Inc.

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 p90RSK antibody: Cell Signaling 9344; 1:500 dilution
RSK1/RSK2/RSK3 antibody: Cell Signaling 9347; 1:1000 dilution
KSR1 sgRNA sequences:
sg1 5' TTGGATGCGCGGCGGGAAAG 3'
sg2 5' CTGACACGGAGATGGAGCGT 3'
NTC sgRNA sequence: 5' CCATATCGGGGCGAGACATG 3'
Plasmid catalog numbers listed in the sections above.

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Figures

Fig 1

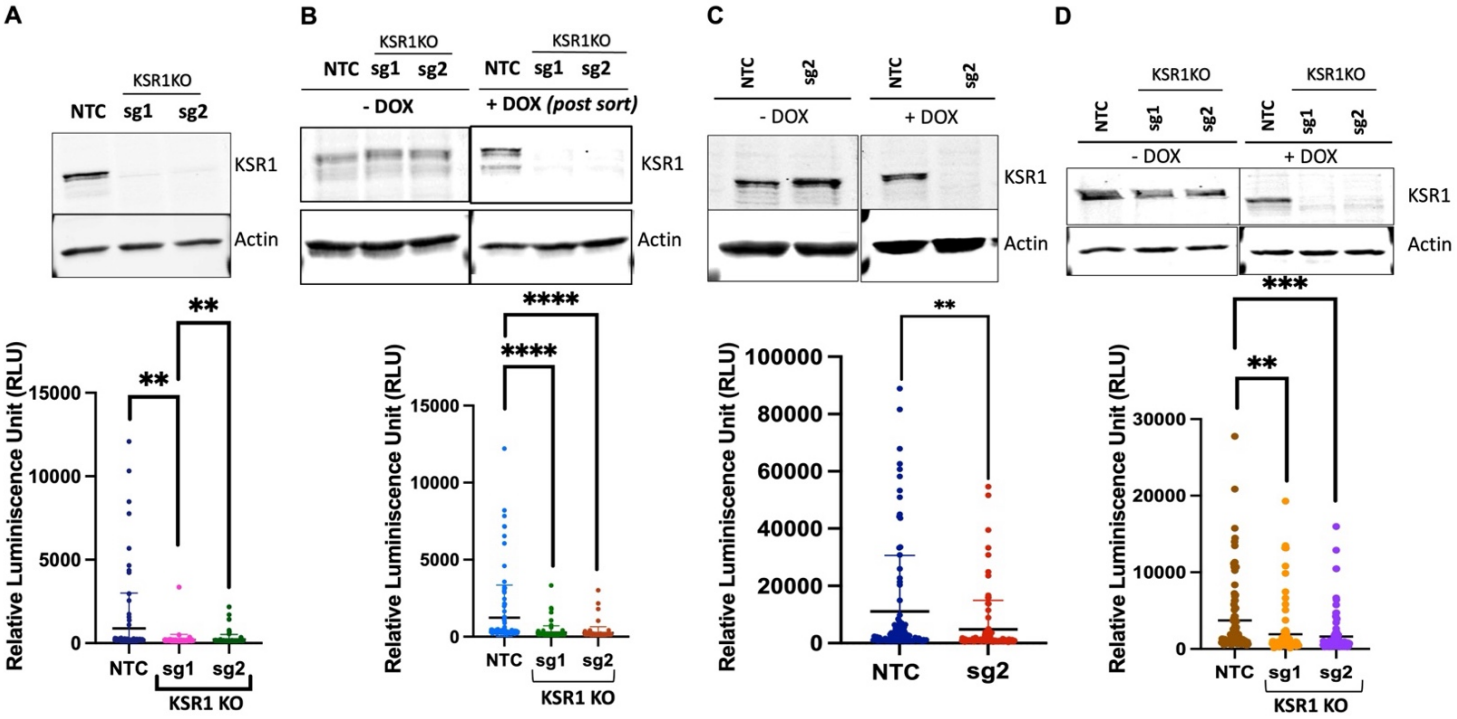
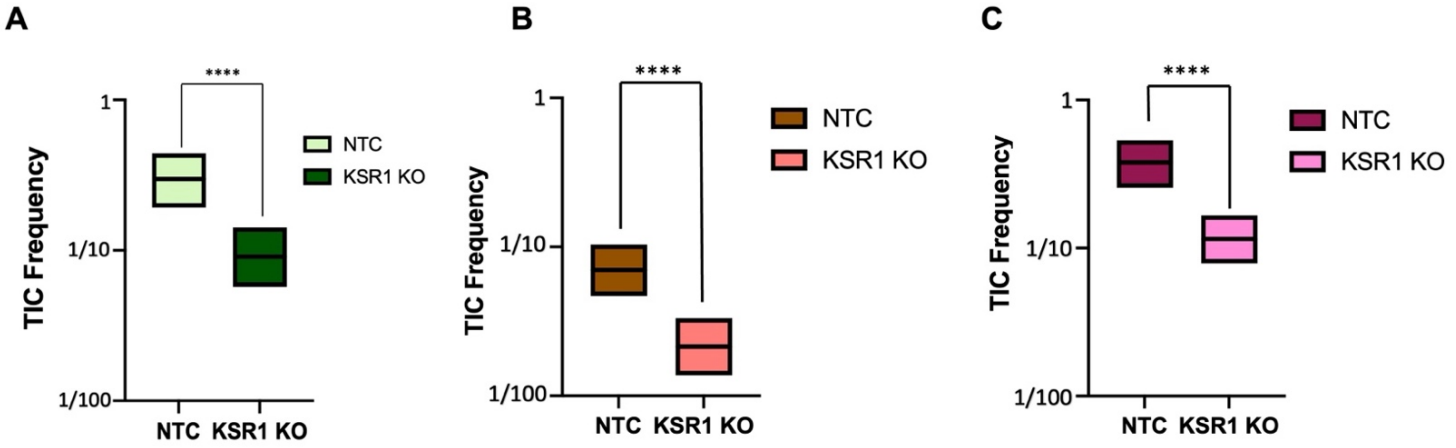


Fig 2

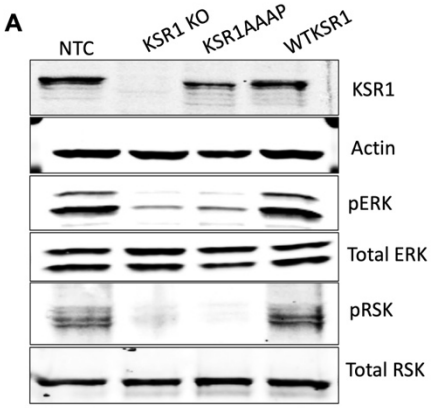


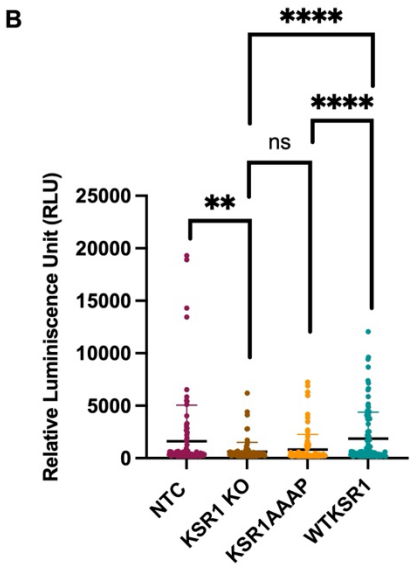
Group	TIC Estimate
NTC	1/3.36
KSR1 KO	1/11.03
Chisq	P value
14.5	0.000142

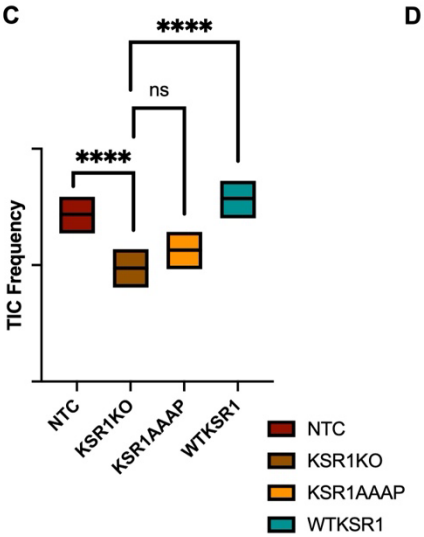
Group	TIC Estimate
NTC	1/14.3
KSR1 KO	1/46.7
Chisq	P value
24.3	$8.45e^{-7}$

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
KSR1AAAP	1/7.44
WTKSR1	1/2.68
Chisq	P value
34.8	1.34e-7

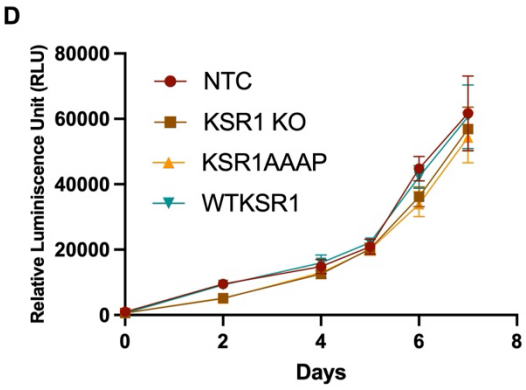
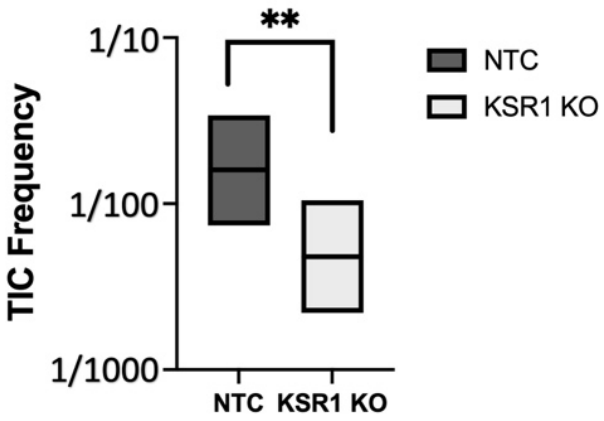
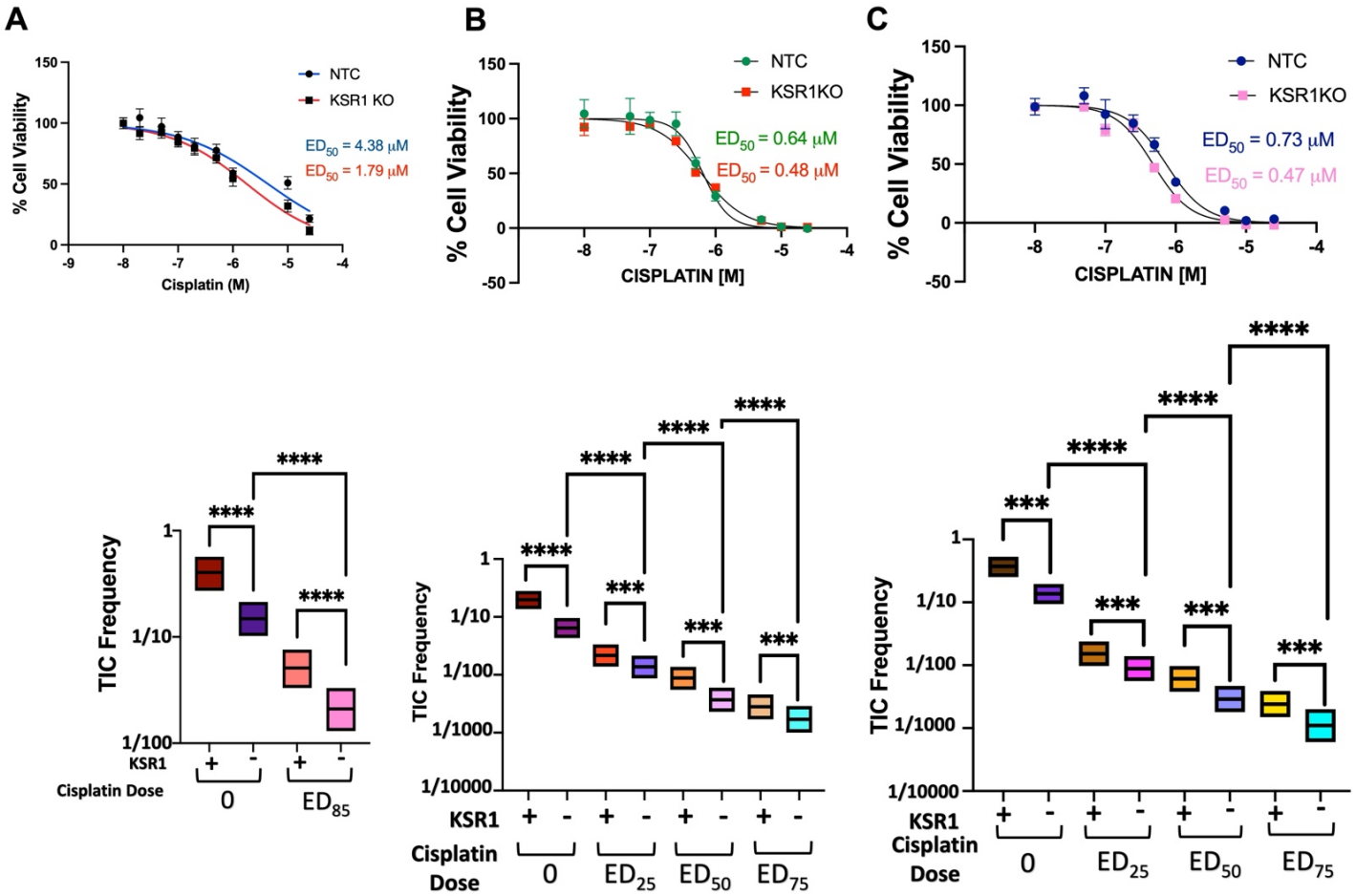


Fig 4



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

Fig 5



Group	TIC Estimate
NTC	1/2
KSR1 KO	1/7
NTC + ED_{85} Cisplatin	1/19
KSR1 KO + ED_{85} Cisplatin	1/46
P Value $3.32e^{-65}$	Chiseq 302

Group	TIC Estimate
NTC	1/5
KSR1 KO	1/16
NTC + ED_{25} Cisplatin	1/46
KSR1 KO + ED_{25} Cisplatin	1/72
NTC + ED_{50} Cisplatin	1/114
KSR1 KO + ED_{50} Cisplatin	1/265
NTC + ED_{75} Cisplatin	1/348
KSR1 KO + ED_{75} Cisplatin	1/558

Group	TIC Estimate
NTC	1/2
KSR1 KO	1/5
NTC + ED_{25} Cisplatin	1/42
KSR1 KO + ED_{25} Cisplatin	1/71
NTC + ED_{50} Cisplatin	1/102
KSR1 KO + ED_{50} Cisplatin	1/212
NTC + ED_{75} Cisplatin	1/255
KSR1 KO + ED_{75} Cisplatin	1/491

Fig 6

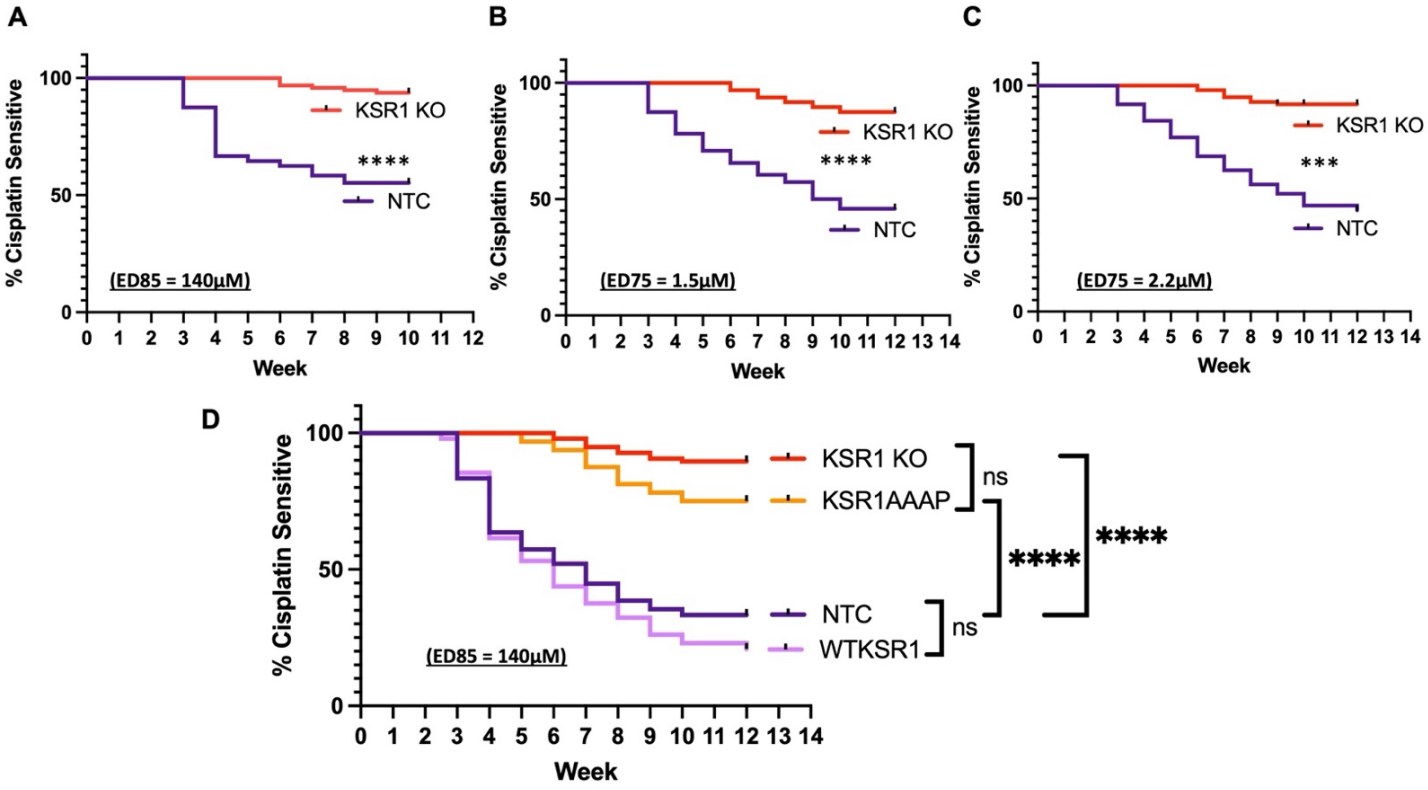

A
% Cisplatin Sensitive
100
50
0
KSR1 KO
****
NTC
(ED85 = 140µM)
0 1 2 3 4 5 6 7 8 9 10 11 12
Week
B
% Cisplatin Sensitive
100
50
0
KSR1 KO
****
NTC
(ED75 = 1.5µM)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week
C
% Cisplatin Sensitive
100
50
0
KSR1 KO
***
NTC
(ED75 = 2.2µM)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week
D
% Cisplatin Sensitive
100
50
0
KSR1 KO
KSR1AAAP
ns
****
****
NTC
WTKSR1
ns
(ED85 = 140µM)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Week

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 control (NTC) and sg1, sg2 gRNAs targeting KSR1. (lower) Clonogenicity of H82 cells without NTC and with constitutive sg1- or sg2-mediated KSR1 disruption.
(B) (upper) Western blot of untreated and DOX-treated H82 cells with DOX-inducible expression of Cas9 and NTC or KSR1-targeting (sg1, sg2) gRNAs. (lower) Clonogenicity of H82 cells without NTC and with (sg1, sg2) DOX-inducible targeting of KSR1.
(C) (upper) Western blot of untreated and DOX-treated H524 cells with DOX-inducible expression of Cas9 and NTC or KSR1-targeting sg2 gRNA. (lower) Clonogenicity of H524 cells without NTC and with DOX-inducible sg2 targeting of KSR1.
(D) (upper) Western blot of untreated and DOX-treated HCC33 cells with DOX-inducible expression of Cas9 and NTC or KSR1-targeting sg1 or sg2 gRNAs. (lower) Clonogenicity of HCC33 cells without NTC and with DOX-inducible sg1 or sg2 targeting of KSR1. Blots are representative of three independent experiments. **, $p<0.01$; ****, $p<0.0001$.

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* ELDA of non-targeting control (NTC) and KSR1-targeted (KSR1 KO) H82 **(A)**, H524 **(B)**, HCC33 **(C)** cells for 7-9 days. Graphs are representative of three independent experiments. ****, $p<0.0001$.

Fig. 3. KSR1 interaction with ERK is required for H82 TIC formation.
(A) Western blot of H82 cells without (NTC) and with (KSR1 KO) DOX-inducible targeting of KSR1, KSR1AAAP (KSR1 mutant unable to bind to ERK) and WTKSR1 (KSR1 added back to KSR1 KO cells), showing pRSK/ Total RSK and pERK/Total ERK levels.
(B) Clonogenicity of H82 cells without (NTC) and with (KSR1 KO) DOX-inducible targeting of KSR1, KSR1AAAP, and WTKSR1 cells.
(C) The proportion of TICs in H82 cells was determined by *in vitro* Extreme Limiting Dilution Analysis (ELDA) of non-targeting control (NTC), KSR1-targeted (KSR1 KO), KSR1AAAP and WTKSR1 H82 cells for 7-9 days.
(D) Growth curve of non-targeting control (NTC), KSR1-targeted (KSR1 KO), KSR1AAAP and WTKSR1 H82 cells monitored for 7-9 days.
Results are representative three independent experiments. **, $p<0.01$; ****, $p<0.0001$.

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

Fig. 5. KSR1 regulates cisplatin resistance in SCLC NeuroD1 subtype cell line H82, H524 and HCC33.
(upper) Cisplatin dose response curve with non-targeting control (NTC), and KSR1 knockout (KSR1 KO) H82 **(A)**, H524 **(B)**, HCC33 **(C)** cells.
(lower) The proportion of KSR1 dependent TICs in SCLC-N cells treated with Cisplatin was determined by *in vitro* 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. ****, $p<0.0001$.

Fig. 6. KSR1 knockout sensitizes SCLC-N cells to cisplatin.
Multi-well resistance assay of NTC and KSR1 knockout (KSR1 KO) H82 **(A)**, H524 **(B)**, HCC33 **(C)** cells 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, KSR1 KO, KSR1AAAP and WTKSR1 H82 cells, (one representative image shown of experiment completed in triplicate, n=3)

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