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Supplemental Information

Activation of EPHA2-ROBO1 Heterodimer by SLIT2 Attenuates Non-canonical Signaling and Proliferation in Squamous Cell Carcinomas

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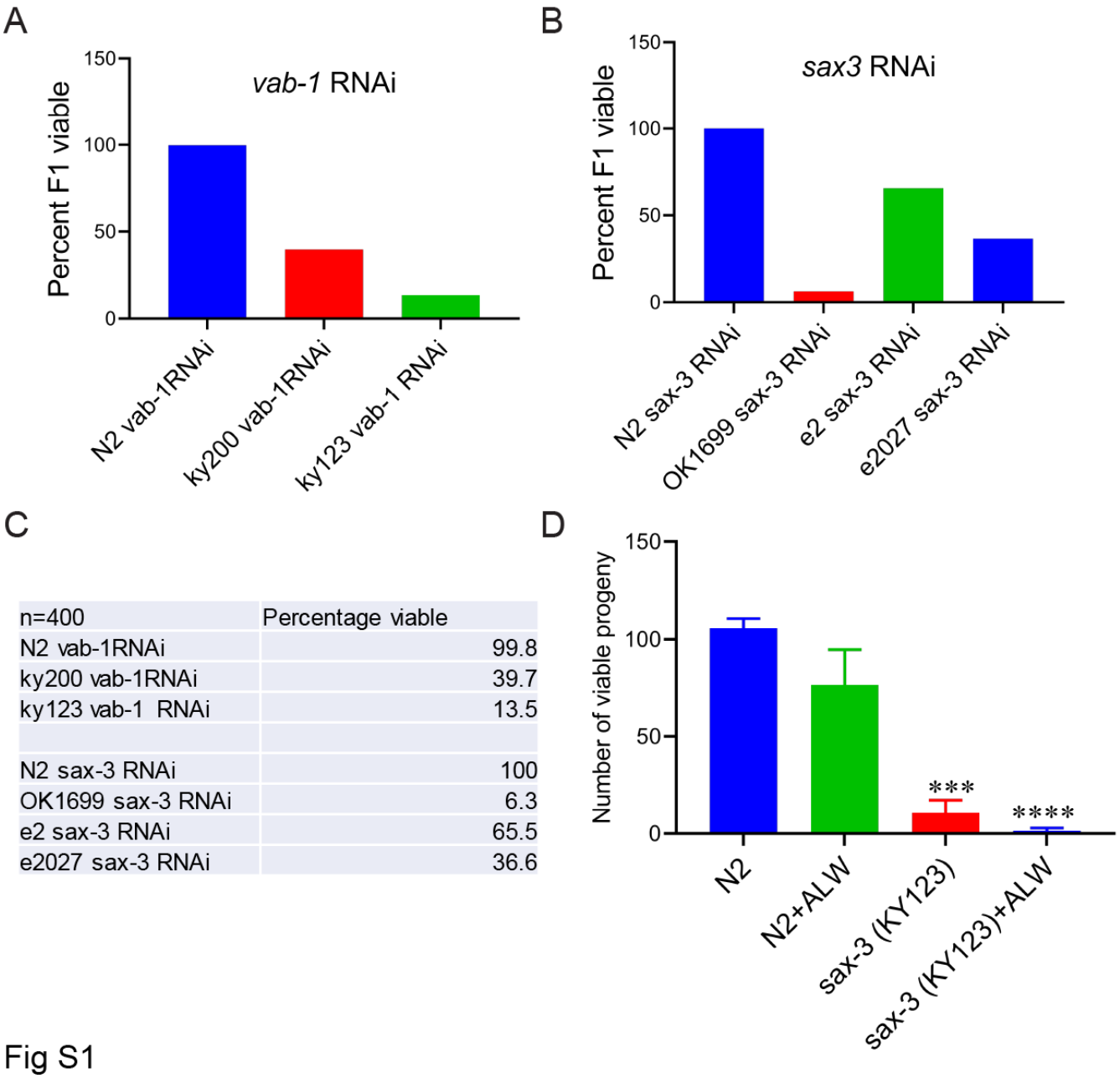
Figure S1: VAB-1 and SAX-3 pathways are synthetic lethal in *C. elegans*: (**A, and B**): RNA interference experiment of VAB-1(EPHA2) and SAX-3 (ROBO1) to determine embryonic lethality in comparison to wild type worms N2. (**C**): Table indicating F1 progeny which survived in (**A and B**) out of total worms. (**D**) The synthetic lethal phenotype is also seen with SAX-3 RNAi and use of an EPHA2 inhibitor ALW-II-41-27. The p-value was calculated using one-way ANOVA. N2+ALW-II-41-27 have significant worm death ($p < 0.0001$) in comparison to untreated N2 worms. Sax-3 RNAi+ALW-II-41-27 has more worm death than control ($p < 0.0001$). Biological replicates indicated in table. Data are represented as mean ± SD. Related to Fig. 1 and 2.



n=400	Percentage viable
N2 vab-1RNAi	99.8
ky200 vab-1RNAi	39.7
ky123 vab-1 RNAi	13.5
N2 sax-3 RNAi	100
OK1699 sax-3 RNAi	6.3
e2 sax-3 RNAi	65.5
e2027 sax-3 RNAi	36.6

Fig S1

Fig S2: Clinical significance of ROBO1 and EPHA2 expression. (**A**) LSCC overall survival comparison of low and high EPHA2 expressing patients, normalized to median expression level of ROBO1. (**B**) HNSCC overall survival comparison of low and high EPHA2 expressing patients, normalized to median expression level of ROBO1. (**C**) LSCC overall survival comparison of low and high ROBO1 expressing patients, normalized to median expression level of EPHA2. (**D**) HNSCC overall survival comparison of low and high ROBO1 expressing patients, normalized to median expression level of EPHA2. Hazard ratio and p-value calculated indicated in the plot. Related to Fig. 3.

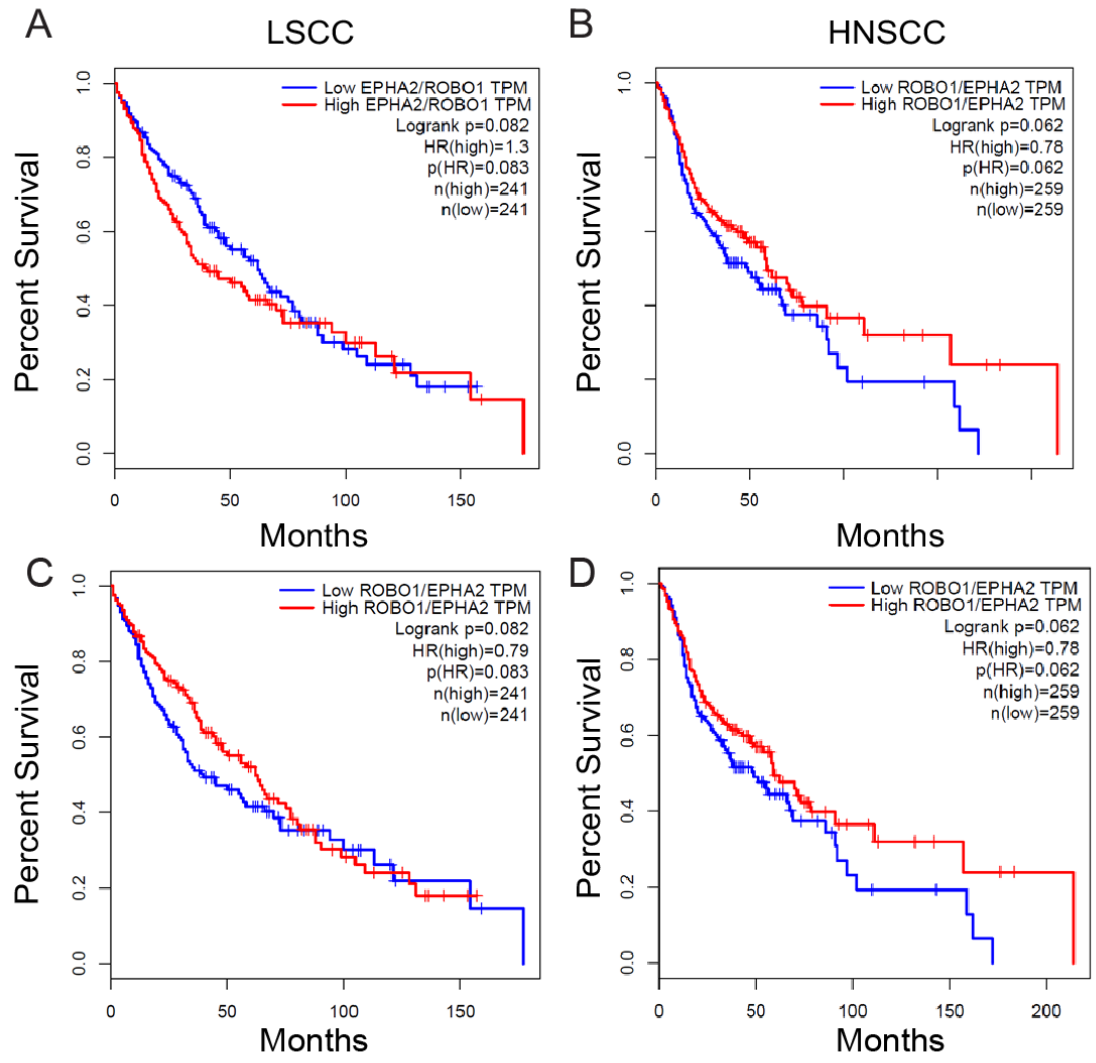
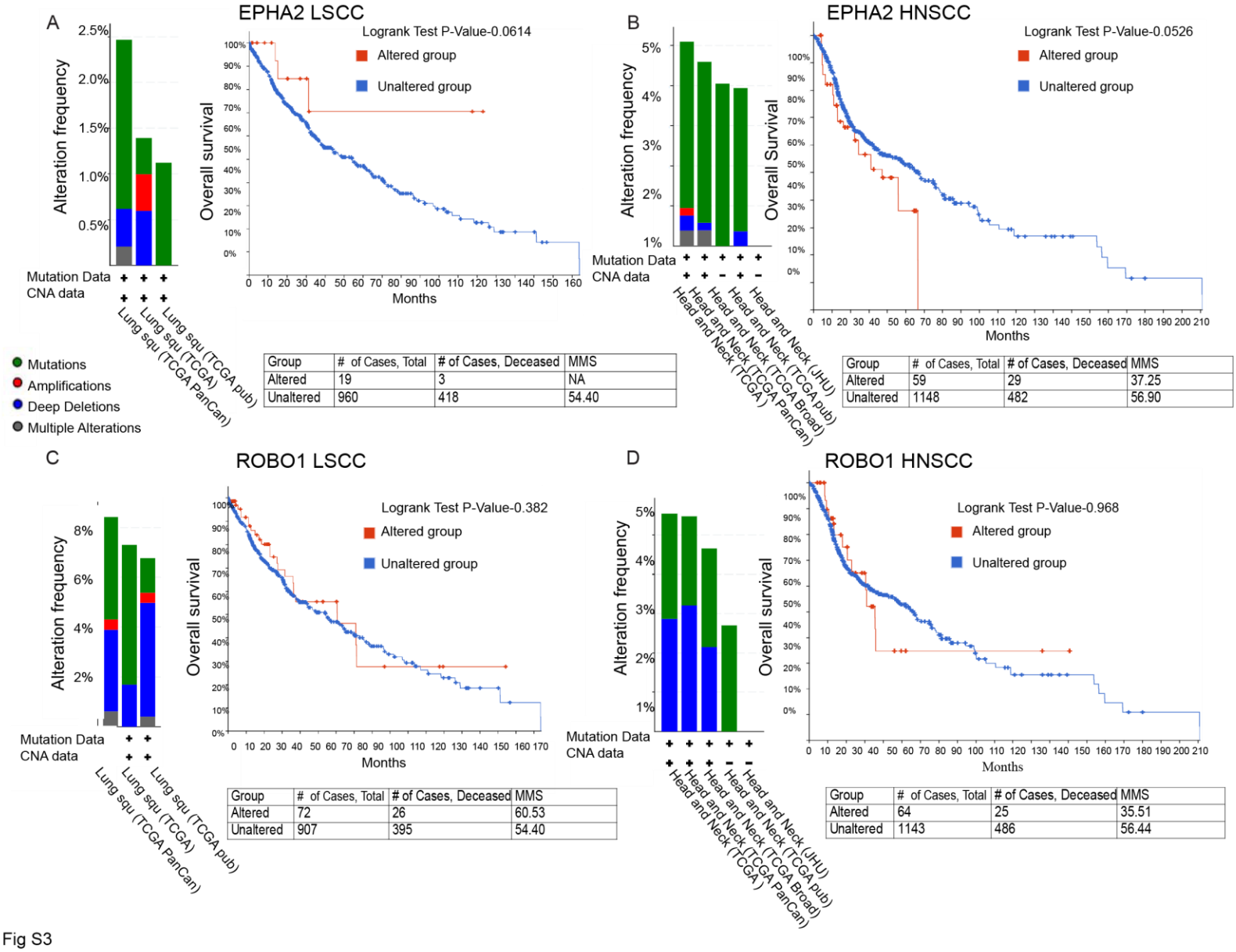


Fig S2

Fig S3: Different mutations and associated survival for EPHA2 and ROBO1 in LSCC and HNSCC. (**A and C**) LSCC: EPHA2 and ROBO1 distribution of different mutations and their associated survival. (**B and D**) HNSCC: EPHA2 and ROBO1 distribution of different mutations and their associated survival. Related to Fig. 3.



Group	# of Cases, Total	# of Cases, Deceased	MMS
Altered	19	3	NA
Unaltered	960	418	54.40

Group	# of Cases, Total	# of Cases, Deceased	MMS
Altered	59	29	37.25
Unaltered	1148	482	56.90

Group	# of Cases, Total	# of Cases, Deceased	MMS
Altered	72	26	60.53
Unaltered	907	395	54.40

Group	# of Cases, Total	# of Cases, Deceased	MMS
Altered	64	25	35.51
Unaltered	1143	486	56.44

Fig S3

Fig S4: Caspase activity plot to all proliferation plots represented in Fig. 6. (A, B and C) SW900, SK-MES-1 and H2170, LSCC cell lines caspase activity plot after treatment with SLIT2 (2μg/ml) and SLIT2+EGF(0.5μg/ml) panel 1; SLIT2 (2μg/ml), ALW-II-41-27 (2μM) SLIT2+ALW-II-41-27 panel 2 and ensartinib 3.461μM and SLIT2+Ensartinib combination treatment panel 3. For each experiment, 6-8 biological replicates were examined. Data are represented as mean ± SD. The p-value was calculated using One-way (ANOVA). See also Table 3 for p-values. Related to Fig. 6.

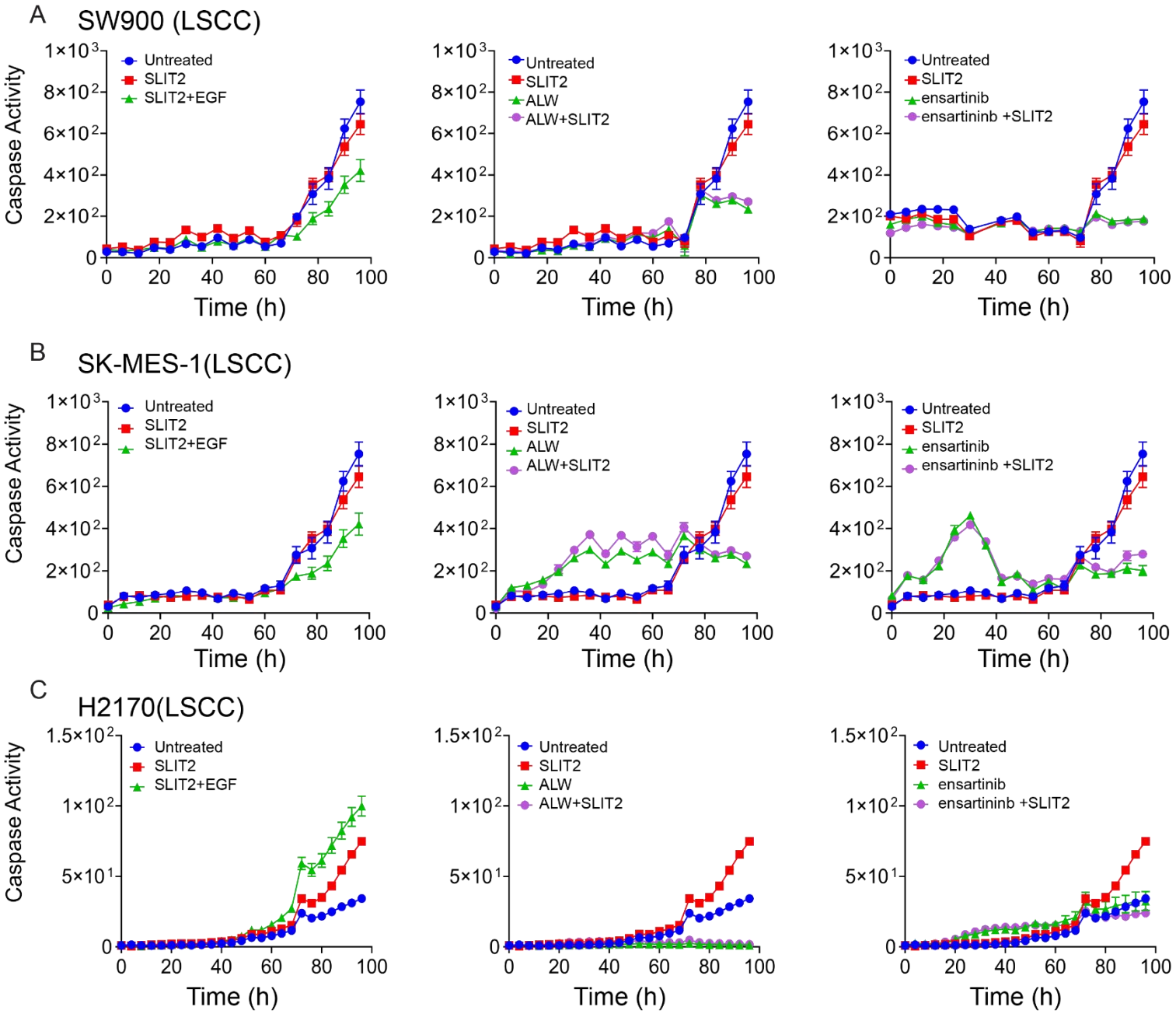


Fig S4

Fig S5: Caspase activity plot to all proliferation plots represented in Fig. 7. (A, B and C) SCC1, SCC6 and SCC90, HNSCC cell lines caspase activity plot after treatment with SLIT2 (2μg/ml) and EGF(0.5μg/ml) panel 1; SLIT2 (2μg/ml), ALW-II-41-27 (2μM) and SLIT2+ALW-II-41-27 panel 2 and ensartinib 1.79 μM and SLIT2+ensartinib combination treatment panel 3. For each experiment, 6-8 biological replicates were examined. Data are represented as mean ± SD. The p-value was calculated using One-way (ANOVA). See also Table 3 for p-values. Related to Fig. 7.

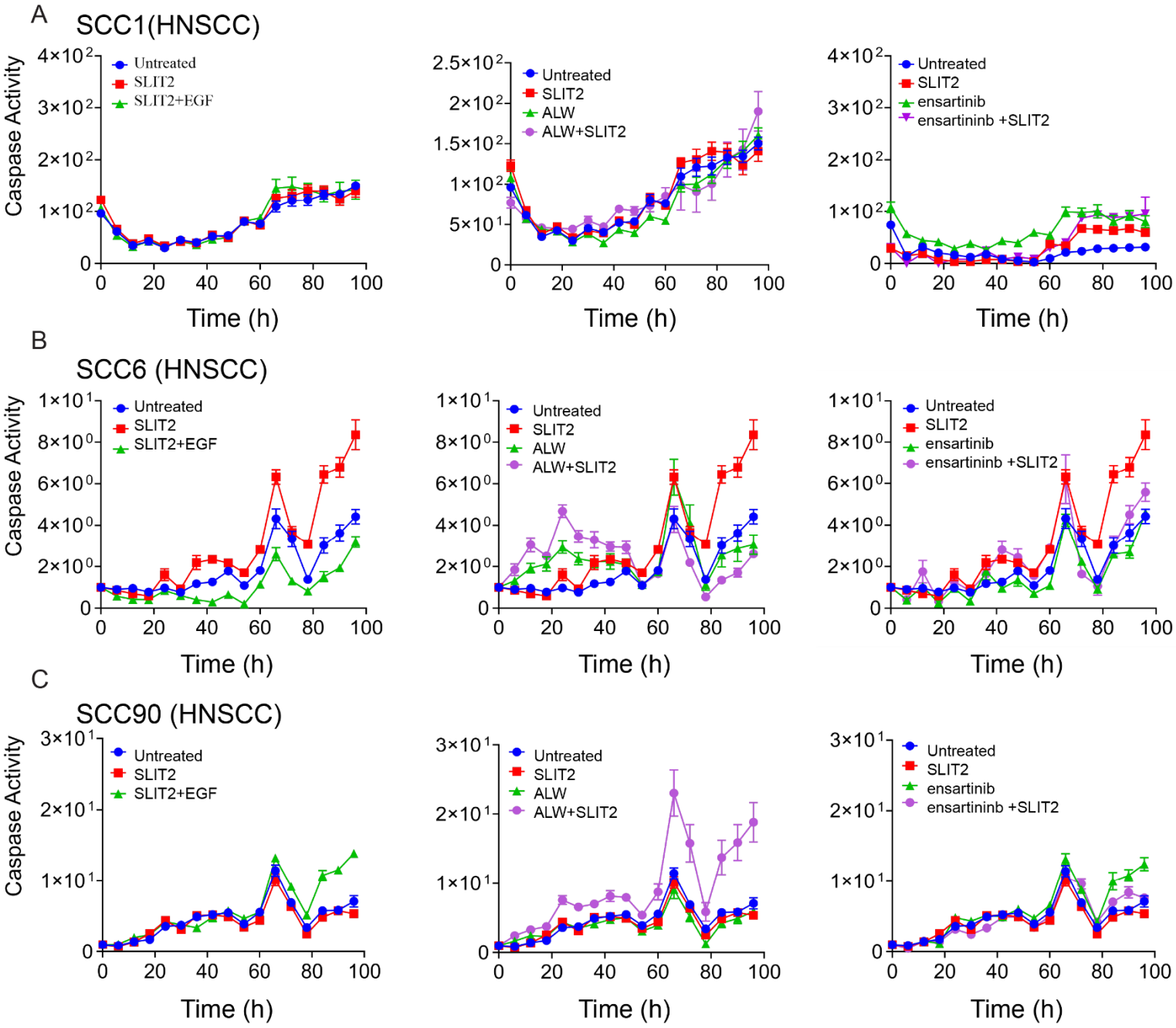
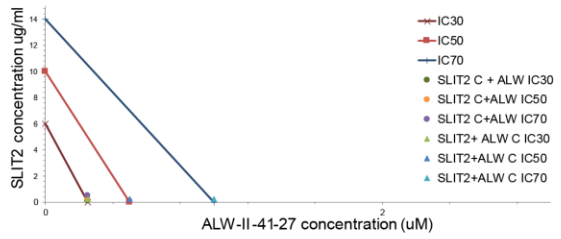


Fig S5

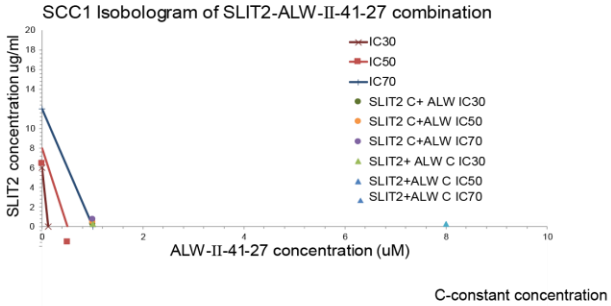
Fig S6: Combination index; EphrinA1 treatment in SW900 and SCC1; SCC104 proliferation and caspase activity plot; Fig. 4B immunofluorescence negative control. Combination index Isobolograms.: (**A, and B**). SW900 (LSCC) Isobologram and table of combination index values for SLIT2-ALW-II-41-27 treatment and SLIT2-ensartininb treatment for IC30, 50 and 70. (**C, and D**). SCC1 (HNSCC) Isobologram and table of combination index values for SLIT2-ALW-II-41-27 treatment and SLIT2-ensartininb treatment for IC30, 50 and 70. (**E**) SW900 (LSCC) and SCC1 (HNSCC) proliferation plot comparing ephrinA1 and SLIT2 treatment. Eight biological replicates were used. P-value was calculated using one-way ANOVA. Both the plots show there is no effect of ephrinA1 treatment. But the inhibition in proliferation after SLIT2 treatment is significant ($p < 0.0001$). Data are represented as mean ± SD. (**F**) SCC104 proliferation and caspase activity plot after SLIT2 (2μg/ml) and SLIT2+EGF (0.5μg/ml) treatment p-value was calculated using One-way ANOVA. Data are represented as mean ± SD. Eight biological replicates were used. (**G**) Negative controls for **Fig. 4B** showing only secondary Alexa Flour 547 (red) staining and secondary Alexa Flour 488 (green) staining. Images were taken at 40X magnification. Scale bar =10μm. Related to Fig. 6 and 7.

A



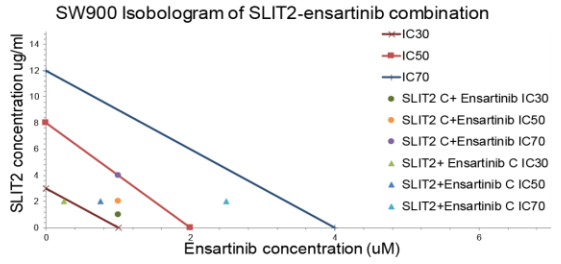
COMBINATION I (SLIT2 +ALW)	CI Values
IC	CI
30	1.02
50	0.53
70	0.29

C



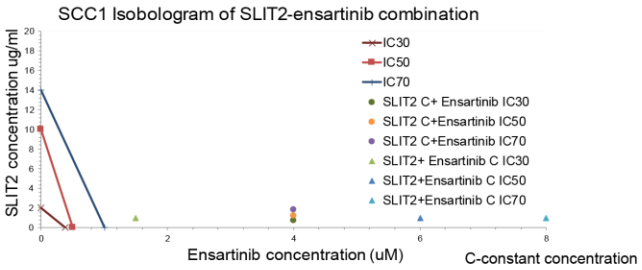
COMBINATION I (SLIT2+ALW)	CI Values
IC	CI
30	8.04
50	2.06
70	1.06

B



COMBINATION I (SLIT2+ENSR)	CI Values
IC	CI
30	1.33
50	0.75
70	0.58

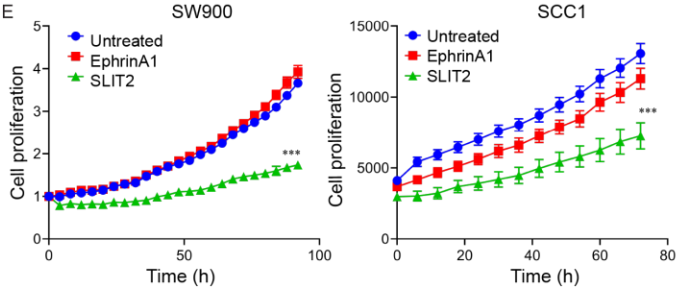
D



COMBINATION I (SLIT2+ENSR)	CI Values
IC	CI
30	11.04
50	8.12
70	4.13

CI=COMBINATION INDEX	
CI>1 Antagonism	
CI=1 Additive effect	
CI<1 synergism	

E



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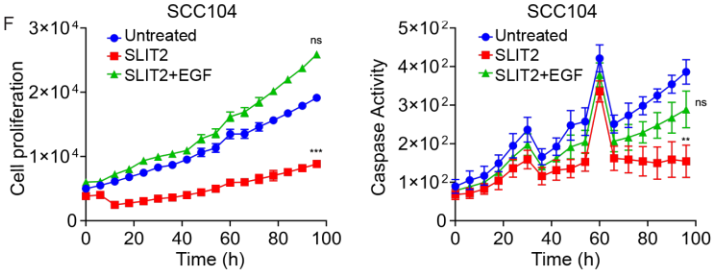

G

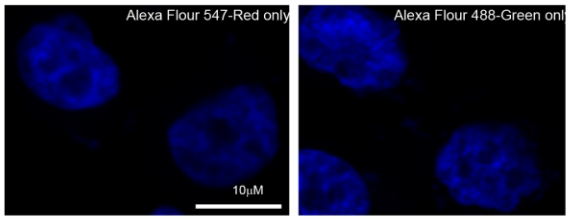

Fig S6

Supplementary tables

Supplementary table 2: Cell Lines. All HNSCC cell lines were obtained and validated by the following sources. HPV status of the following cell lines has been previously validated (Kimple RJ, 2013; Brenner J C, 2010). Related to Fig. 2.

Cell line	Sources	Culture condition
SCC-15 SCC-25	American Type Culture Collection	DMEM/F12 (1:1), 10% FBS, 400ng/ml, hydrocortisone, penicillin (100 units/mL), streptomycin (100 mg/mL)
93-vu-147T	Dr. Robert Ferris, with permission of Dr. Hans Joenje, VU Medical Center, Amsterdam, Netherlands	DMEM with 4.5 g/dL glucose, 10% FBS, penicillin (100 units/mL), streptomycin (100 mg/mL)
SCC4	DSMZ-German Collection of Microorganisms and Cell Cultures GmbH	
UD-SCC-2	Dr. Thomas Carey, with permission of Dr. Henning Bier, Technical University Munich, Munich, Germany	
UPCI: SCC-090	American Type Culture Collection	
TU-138	Dr. Jennifer Grandis, UCSF	DMEM/F12 (1:1)
HN30	Dr. Ravi Salgia, City of Hope	DMEM, 10% FBS, penicillin (100 units/mL), streptomycin (100 mg/mL) and NEAA
UM-SCC-1 UM-SCC-6	Millipore	EMEM medium supplemented with NEAA, 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml)
SCC-1483	Dr. Lawrence Marnett, Vanderbilt University	DMEM with 4.5 g/dL glucose, 10% FBS, 1% hydrocortisone, penicillin (100 units/mL), streptomycin (100 mg/mL)
UM-SCC-47 UM-SCC-104 UM-SCC-22B	Millipore	

Supplementary table 3: Oligonucleotides List For: Forward Primer; Rev: Reverse Primer. Related to Fig. 1, 4 and 5.

shEPHA2#1 For	caccGCGTATCTTCATTGAGCTCAAtcaagagTTGAGCTCAATGAAGATACGC
shEPHA2#1 Rev	aaaaGCGTATCTTCATTGAGCTCAActcttgaTTGAGCTCAATGAAGATACGC
shEPHA2#2 For	caccTCGGACAGACATATAGGATATtcaagagATATCCTATATGTCTGTCCGA
shEPHA2#2 Rev	aaaaTCGGACAGACATATAGGATATctcttgaATATCCTATATGTCTGTCCGA
shROBO1 #1 For	caccGCAGAAATACAGTCACATTATtcaagagATAATGTGACTGTATTTCTGC
shROBO1#1 Rev	aaaaGCAGAAATACAGTCACATTATctcttgaATAATGTGACTGTATTTCTGC
shROBO1 #2 For	caccTGACACATGACGCCAGATAAAtcaagagTTTATCTGGCGTCATGTGTCA
shROBO1 #2 Rev	aaaaTGACACATGACGCCAGATAAActcttgaTTTATCTGGCGTCATGTGTCA
EPHA2 coding For	aaaaactcgagATGGAGCTCCAGGCAGCCCGC
EPHA2 coding Rev	aaaaaggtaccGATGGGGATCCCCACAGTGTTCACC
ROBO1 coding For	ttttagatctATGATTGCGGAGCCCGCTCACTT
ROBO1 coding Rev	ttttcccgggcctgctgctgcGCTTTCAGTTTCCTCTAATTCTTCATTAT
ROBO1 FLAG Rev	ctagagtcgcggccgctTCACTTGTCGTCATCGTCTTTGTAGTCtgctgctgcGCTTTCAGTTTCC
EPHA2 HA Rev	atgatctagagtcgcggccgcTCAAGCGTAATCTGGAACATCGTATGGGTAtgctgctgcGATGGGC
EPHA2 K645R	CCGGTGGCCATCAGGACGCTGAAAGCCGG
ROBO1 Y932F	GAAGAGAAACGGACTTactagtACCttcGCGGGTATCAGAAAAGTAAC
ROBO1 Y1073F	ATCAGGGCAGCCTACTCCTttcGCCACCACTCAGCTCATC
vab-1 RNAi For	ttttggtaccGTTCTTGTTCCACGTGTCGTC
vab-1 RNAi Rev	tttagatctCCACATTCCACAAGTACATCC
sax-3 RNAi For	ttttggtaccTTCCGAAGTGAGTCTCTTCTC
sax-3 RNAi Rev	tttagatctCACCACCAACAATCGAGCATG
	For: Forward Rev: Reverse

Supplementary table 4: Antibodies List. Related to Fig. 1,2 and 5.

Antigen	Company	Cat#
FLAG Clone M2	Sigma	F1804
b-actin	Sigma	A5441
EPHA2	Santa Cruz	SC924
EPHA2 pS897	Cell signaling	6347
EPHA2 pY588	Cell signaling	12577
ROBO1	Invitrogen	PA5-29917
AKT pS473	Cell signaling	9271
EGFR pY1068	Cell signaling	2236

HA	Cell signaling	3724
ROBO1	Protein Tech	20219-1-AP

Transparent Methods

Cell Culture and Reagents

All NSCLC cell lines were from American Type Culture Collection (ATCC) (Manassas, VA, USA). NSCLC cell lines H2170, SK-MES-1, SW900 and the nonmalignant and immortalized control cell line BEAS-2B, were cultured in RPMI 1640 medium (Gibco/BRL) supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine and 1% penicillin-streptomycin. HEK293 cells were cultured in DMEM medium (Gibco/BRL) supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine and 1% penicillin-streptomycin. All HNSCC cell lines were obtained from indicated sources (**Supplementary Table 2**). All cell lines were cultured at 37°C with 5% CO_2. ALW-II-41-27 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). ROBO1, EphrinA1, soluble EPHA2 and SLIT2 were purchased from R&D Systems (Minneapolis, MN, USA). EGF ligand was purchased from Stemcell Technology (Vancouver, Canada).

C. elegans RNAi and drug treatment

Culture and handling of *C. elegans* were carried out as described (Stiernagle, 2006). *vab-1* mutants (*OK1699, e2 and e2047*) and sax-3 mutants (*ky200 and ky123*) were obtained from *Caenorhabditis* Genetic Center. RNAi knockdown was carried out by bacterial feeding method (Kamath et al, 2001). Single wild type or mutant L3 worm were placed onto L4440 *E. coli* expressing either no RNA, or dsRNA targeting *vab-1* or *sax-3*. Total number of viable and dead F1 embryos were scored. For treatment with ALW-II-41-27, the indicated amount of drug or DMSO was added to 0.5 ml of base agar in 12-well plates and allowed to diffuse for 2 h. Feeding bacteria OP50 were then added on top of agar. A single L4 worm was then placed into an individual well. Viable F1 worms were then scored.

siRNA, shRNA, DNA vectors, transfection and cloning

shRNA plasmids were constructed by inserting annealed oligonucleotide pairs targeting EPHA2, ROBO1 or luciferase into pJR288 as described (Pang et al., 2018). The shRNA targeting EPHA2 and ROBO1 were transfected using jetPRIME® using manufacturer's protocol (Polyplus transfection, 67400 Illkirch, France). We used $2X10^5$ cells in a 6-well and transfected with 2μg of shRNA plasmid DNA. Transfection was done for indicated time points. Non-targeting control pool siRNA (catalog no. D-001810) and SMARTpool siRNA targeting EPHA2 (catalog no. L-003116) were purchased from Dharmacon, Inc. and were used at a final concentration of 15 nmol/L siRNA with Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA). ROBO1 or EPHA2 expression vectors were

constructed by fusing PCR fragments containing full length ROBO1 or EPHA2 upstream to eGFP, mCherry, mClover3, mRuby3, HA or FLAG sequences. All expression vectors were based on peGFP-N3 (Clontech/Takara, Mountain View, CA, USA) with CMV promoter replaced by a eF1a promoter and eGFP replaced by mCherry, mClover3 or mRuby3. Point mutations were introduced by Q5 site directed mutagenesis kit according to manufacture protocol (New England Biolabs, Ispwich, MA, USA). All constructs were confirmed by DNA sequencing. Oligonucleotides used are listed in **Supplementary Table 3**.

Immunoblotting and immunoprecipitation

Cell lysates for immunoblotting were prepared by scraping cells and lysing them using RIPA buffer. Lysates were run on 4–15% or 4-20% Mini-protean TGX gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto Immobilon^{TM} membranes (MilliporeSigma, Burlingon, MA, USA) or Turboblot system (Bio-Rad). Blots were blocked using 5% nonfat dry milk in TBST for 1 h and incubated with primary antibodies (listed in **Supplementary Table 4**) overnight at 4°C. After washing 3 times in TBST, blots were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The blots were then washed three times and immuno-reactive bands were detected by WesternBright ECL (Advansta, San Jose, CA, USA) or Azure Radiance (Azure) and imaged with ChemiDoc MP Imager (Bio-Rad) or Azure C600 (Azure). For co-immunoprecipitation assays, plasmids expressing EPHA2-HA and ROBO1-FLAG were cotransfected into HEK293 cells. Cells were collected 48 h post-transfection and lysed by IP buffer (PBS + 1% triton with HALT protease and phosphatase inhibitor cocktails) (ThermoFisher, Waltham, MA, USA). Lysates were adjusted to 1 mg/ml by IP buffer and protein complexes were immunoprecipitated by anti-FLAG magnetic beads (MilliporeSigma, Burlington, MA) or anti-HA magnetic beads (ThermoFisher) at 4^0C for 4 h. Immunoprecipitated complexes were detected by immunoblotting.

Cell viability assays

LSCC and HNSCC cells were labelled with m-Kate2 (red fluorescence) and stable cell lines were generated using puromycin selection. Labeled cells were seeded in 96 well plates for 24 h, followed by ligand treatment: SLIT2 (2μg/ml), EGF (0.5μg/ml) ensartinib and ALW-II-41-27 IC50 doses (**Table 4).** The caspase activity was monitored using Caspase green 3/7 reagent. Cells were imaged every 6 h for 96 h and their proliferation rates and caspase activity were plotted. Cell counting Kit 8 (Dojindo Molecular Technologies, catalog no. CK04) was used to determine relative numbers of viable cells 72 h after post transfection with shRNA targeting EPHA2 and ROBO1 in LSCC siRNA targeting EPHA2 (siEPHA2) and ROBO1 (siROBO1) in HNSCC.

Immunohistochemistry, immunofluorescence staining and live cells microscopy

Human lung cancer TMAs (LC642) were purchased from Biomax, Inc. (Rockville, MD, USA). EPHA2 was stained with anti-EPHA2 antibody (C-20, Santa Cruz Biotechnology, Dallas, TX, USA), 1:200 for 30 min, and ROBO1 was stained with anti-ROBO1 antibody

(PA5-29917, Invitrogen), 1:200 for 30 min. Each pair of stained TMAs was registered in Visiopharm before exporting a down sampled image. In FIJI, color deconvolution was used to extract the DAB staining (as grayscale) from each aligned TMA image, followed by pseudo-coloring the stains red or green. The staining was performed by the pathology/solid tumor core of The City of Hope National Medical Center. Each TMA was reviewed and scored by a pathologist on a scale of 0 to 3: 0+, no staining, no expression; 1+, weak staining, low expression; 2+, moderate staining, moderate expression; and 3+, strong staining, high expression. Scores of 0 and 1 were designated as low expression and scores of 2 and 3 were designated as high expression of EPHA2 or ROBO1. The resultant values of individual core for EPHA2 and ROBO1 scores were plotted as a pie chart using GraphPad Prism 7 software. (listed in **Supplementary Table 1**)

For immunofluorescence staining, cells were seeded in a Lab-Tek II Chamber Slide (ThermoFisher) or Number 1 cover slips in a 24-well plate for 24 to 48 h, then fixed by 1% formaldehyde in PBS for 20 min at room temperature, permeabilized by PBS containing 0.1% Tween and 0.25 % Triton X-100. After three washes with PBS, fixed cells were blocked with 5% FBS in PBS for 1 h at room temperature. Primary antibodies were then added and incubated overnight at 4^0C. Primary antibodies were removed, and the slides were washed 5 times with PBS. Alexa Flour 488 or Alexa Flour 547 conjugated secondary antibodies and Hoechst 33342 dye (ThermoFisher) or DAPI for staining nuclei were then added and allowed to incubate for 2 h at room temperature. The slides/coverslips were then washed five times with PBS and mounted in Prolong Gold Antifade reagent (ThermoFisher).

For live cell imaging, transfected cells were plated onto 35 mm Delta TPG dish (Bioptechs, Butler, PA, USA) for 24 h. The dishes were then placed on temperature controlled microscopic stage that was connected to CO_2 supply. All images were acquired on a Zeiss LSM880 confocal microscope and analyzed by Zen software (Zeiss USA, Thornwood, NY, USA).

Proximity Ligation Assay (PLA)

To perform a complete Duolink® PLA *in situ* experiment we used three primary antibodies (PLA, Immunofluorescence validated) that recognize EPHA2, ROBO1 or SLIT2 epitopes. The starter kit from SIGMA supplies all other necessary reagents for Duolink® PLA reactions, which include a pair of PLA probes (Anti-Rabbit PLUS and Anti-Mouse MINUS), red detection reagents, wash buffers, and mounting medium. The primary antibodies used came from the same species as the Duolink® PLA probes for EPHA2/ROBO1 or EPHA2/SLIT2 PLA (one mouse and one from rabbit species). Analysis was carried out using standard immunofluorescence assay technique. We used a confocal microscope (LSM880) to capture images. For the quantification of this staining the confocal images were extracted (multichannel to single channel), and a binary image was generated. The binary image was thresholded using FIJI software (Otsu). The average intensity was measured and plotted to compare the binding of the two proteins assayed.

Combination Index

For combination index (CI) calculation, LSCC (SW900) and HNSCC (SCC1) Incured cell lines were seeded in 96-well plate with 5000 cells per well. Three biological replicates (three 96-well plates for each drug combination) were used. For both cell lines, two drug combination were used SLIT2/ALW-II-41-27 and SLIT2-ensartinib. The drugs were used in linear dilution series with dilution factor of 2. SLIT2 doses ranged from 0μg/ml-6μg/ml and ALW-II-41-27 and ensartinib from 0μM-8μM. The plates were read at 72 h using the IncuCyte Live Cell Analysis System to measure live cells (Incurred object count per well). We then use an R package called SynergyFinder (He et al., 2018) to find the nature of drug-drug interaction (i.e. if they work in synergy or antagonistically or non-interactively). For this purpose, the drug response matrix is supplied to the mentioned package, which then uses several models namely Highest Single Agent (Berenbaum, 1989), Loewe additivity (Loewe, 1953), Bliss independence (Bliss, 1939) and Zero Interaction Potency (Yadav et al., 2015) to quantify the degree of drug synergy. The dose response matrix was used to calculate individual CI values for IC30, IC50 and IC70 drug treatments. The output values were used to plot Isobolograms using the following formula.

$$CI = \frac{IC50\ (A)\ pair}{IC50\ (A)} + \frac{IC50\ (B)\ pair}{IC50\ (B)}$$

Statistical analysis

One-way ANOVA, non-linear regression or simple T-test were performed to calculate significance between data sets as indicated with each result or figure legend. A level of significance of $p < 0.05$ was chosen. Data are presented as mean with standard deviation of the mean (± STD) in all figures in which error bars are shown. Graphs were generated using GraphPad Prism 7 software.

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