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Supplementary Materials for

A DLL3-targeted antibody-drug conjugate eradicates high-grade pulmonary neuroendocrine tumor-initiating cells in vivo

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This PDF file includes:

Other Supplementary Material for this manuscript includes the following:
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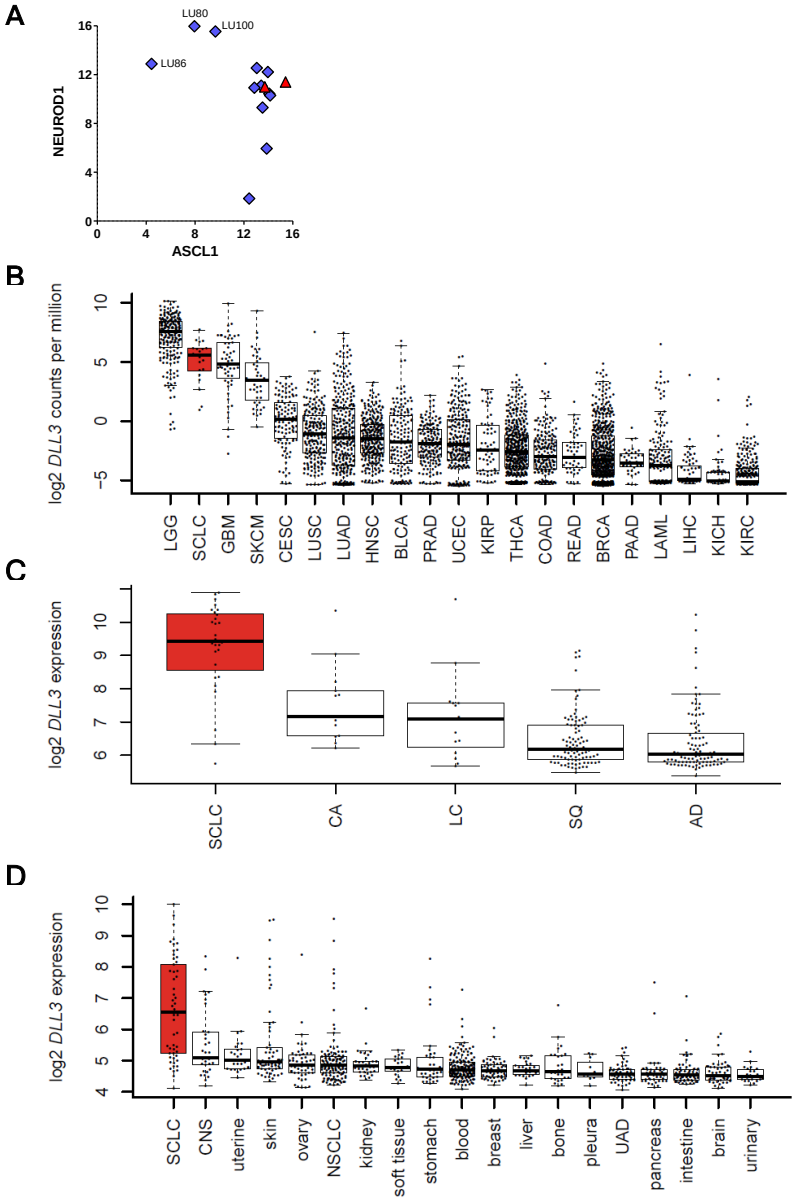
Table S5. DLL3 whole transcriptome metrics (provided as an Excel file).

Supplementary Materials and Methods

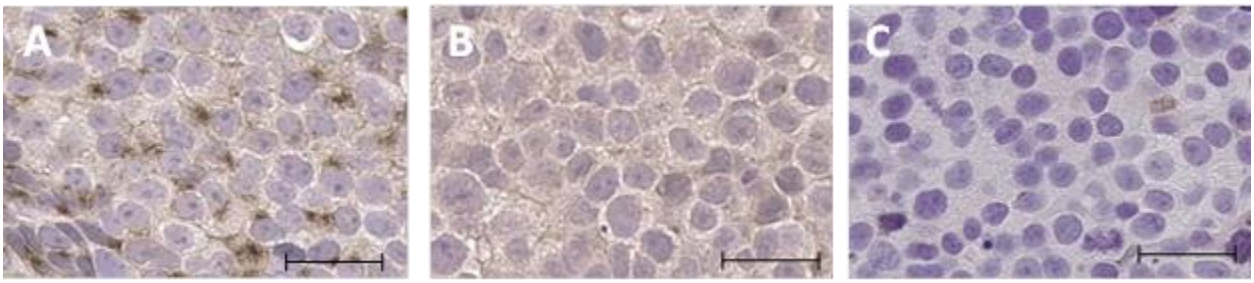
Biacore Affinity Determination. Human and cynomolgus DLL3 protein were produced as histidine fusion proteins in CHO cells stably transfected and selected for high expression using a pEE12.4-h*DLL3* or pEE12.4-c*DLL3* as described above. A histidine fusion of rat DLL3 was generated through transient transfection of pEE12.4-r*Dll3* into suspension CHO cells using polyethylenimine (Polysciences). Before the Biacore assay, each antigen was injected over a Superdex-200 (GE Healthcare) size exclusion chromatography column equilibrated with 200 mM arginine succinate buffer, pH 5.5, supplemented with 0.005% surfactant P-20 and 3 mM EDTA. The antigen was diluted in the identical buffer to concentrations of 25, 12.5, and 6.25 nM. Data were collected on a Biacore 2000 with a CM5 chip modified with the anti-human capture kit (GE Healthcare) as directed. Human IgG1 control was coated on flowcell 1 as a reference surface, and SC16 and SC16LD6.5 were coated on the test surfaces at 80-100RU. After antibody loading on the chip, antigen dilutions were injected at 5 µL/min for 120 seconds of association and 240 seconds of dissociation. Data were processed with BiaEval, aligning multiple antigen concentrations to baseline and analyzing the association and dissociation data. A fit to a 1:1 Langmuir binding model was performed to generate the kinetics constants (kon and koff) and to determine an apparent affinity.

Serum Stability of SC16LD6.5. SC16LD6.5 was spiked into human EDTA plasma at 100 µg/mL and incubated at 37ºC for up to 96 hours. Sample aliquots were frozen at -70ºC until analysis. The amount of conjugated SC16LD6.5 Ab in plasma was determined using a sandwich ELISA with mouse monoclonal antibodies specific for the linker-drug (LD6.5) and the idiotype of the therapeutic antibody (Conjugated Antibody Assay). The amount of total SC16LD6.5 Ab in plasma was determined using a sandwich ELISA with two mouse monoclonal antibodies specific

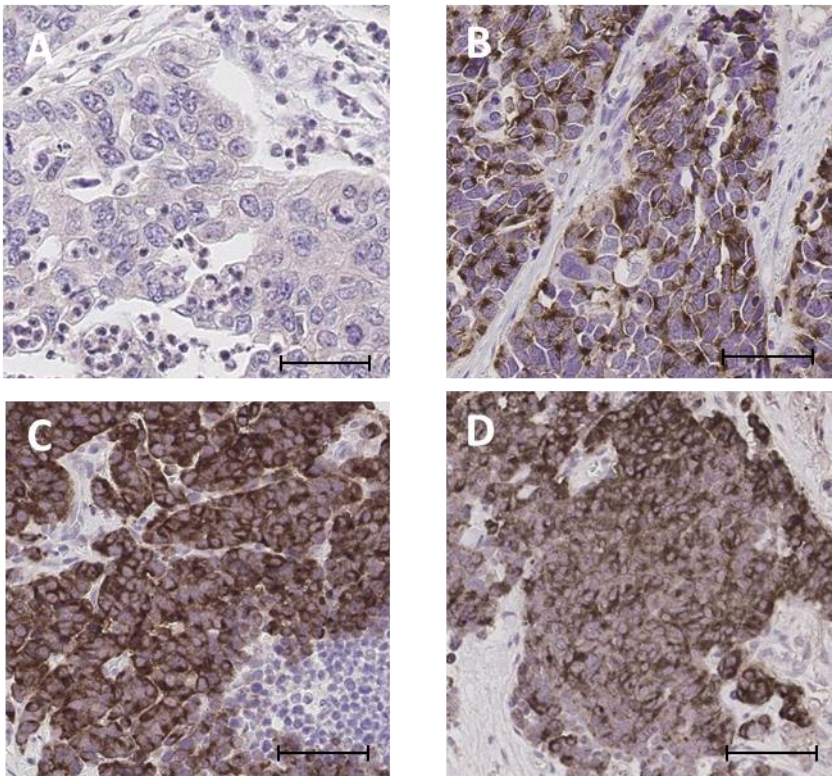
for the idiotype of the therapeutic antibody (Total Antibody Assay). The amount of analyte was determined by interpolating the obtained signals against those generated from a standard curve of SC16LD6.5 using a 4-parameter non-linear curve fitting algorithm. Free drug D6.5 was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) assay with electrospray ionization in Selected Reaction Monitoring (SRM) mode (Free Drug Assay). Analyte and deuterated internal standard (IS) peak areas were integrated, interpolated against a standard curve of D6.5, and normalized using the IS peak area (Tandem Laboratories).



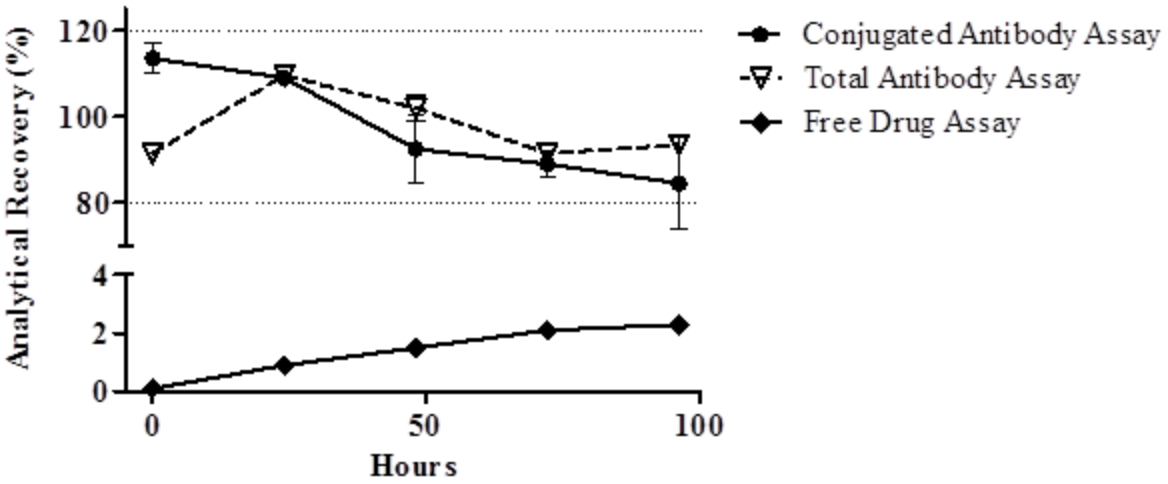
Supplementary Figure 1. Elevated expression of *DLL3* mRNA in SCLC. (**A**) Microarray data showing expression of *NEUROD1* versus *ASCL1* in SCLC (blue diamond) and LCNEC (red triangle) PDX. (**B**) Mapped reads to *DLL3* transcripts as determined by whole transcriptome RNA-sequencing in TCGA tumor samples and primary SCLC tumor samples (*11*). Low grade glioma (LGG), GBM (glioblastoma), SKCM (melanoma), CESC (cervical squamous cell and endocervical adenocarcinoma), LUSC (lung squamous cell carcinoma), LUAD (lung adenocarcinoma), HNSC (head and neck squamous cell carcinoma), BLCA (bladder carcinoma), PRAD (prostate adenocarcinoma), UCEC (uterine corpus endometrial carcinoma), KIRP (kidney renal papillary cell carcinoma), THCA (thyroid carcinoma), COAD (colon adenocarcinoma), READ (rectum adenocarcinoma), BRCA (breast carcinoma), PAAD (pancreatic adenocarcinoma), LAML (acute myeloid leukemia), LIHC (liver hepatocellular carcinoma), KICH (kidney chromophobe), and KIRC (kidney renal clear cell carcinoma). (**C**) Illumina BeadChip data of *DLL3* expression from the Clinical Lung Cancer Genome Project. CA (carcinoid), LC (large cell carcinoma), SQ (squamous cell carcinoma), and AD (adenocarcinoma). (**D**) Microarray data of *DLL3* expression in various cell lines from the Cancer Cell Line Encyclopedia. UAD (upper aerodigestive). Box and whiskers represent median, quartiles, and outliers, with every data point shown as a dot.



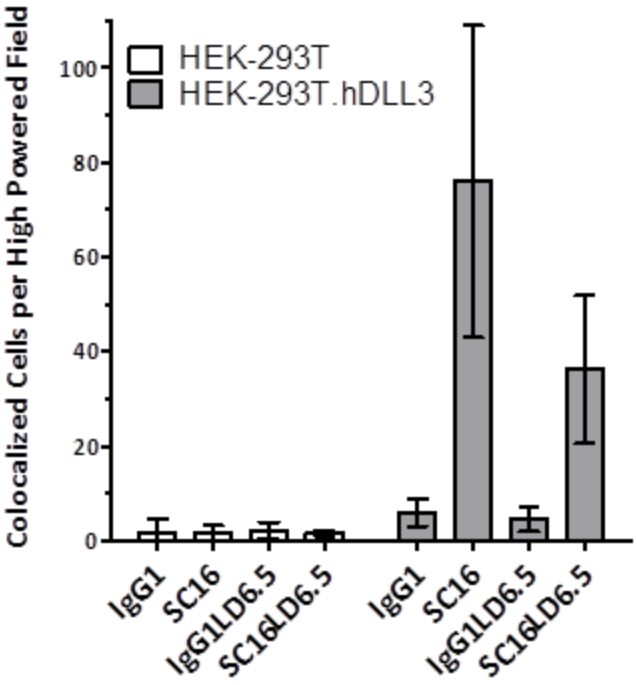
Supplementary Figure 2. Specificity of anti-DLL3 IHC antibody. (**A**) DLL3 IHC on LU37. (**B**) IHC staining in LU37 after DLL3 antibody is pre-incubated with DLL3-His protein shows specific competition of staining. (**C**) Isotype control IHC with IgG2A shows no staining on LU37. Scale bars are 20 μm.



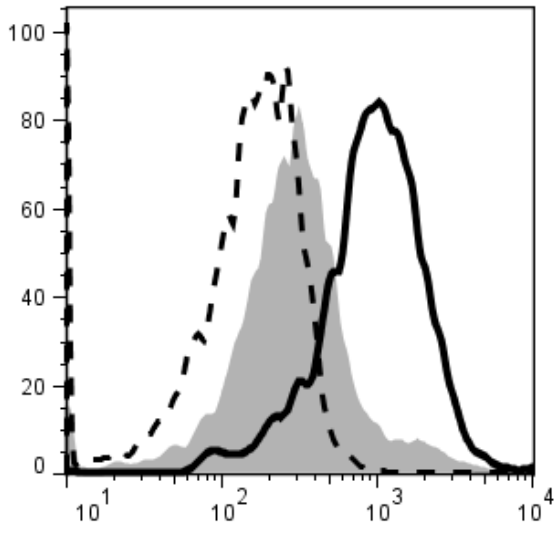
Supplementary Figure 3. DLL3 protein expression by IHC in representative samples from tissue microarrays. (**A**) NSCLC (H-score = 0). (**B**) LCNEC (H-score = 270). (**C**) Naïve SCLC (H-score = 260) and (**D**) recurrent/refractory SCLC (H-score = 190). Scale bars are 40 μm.



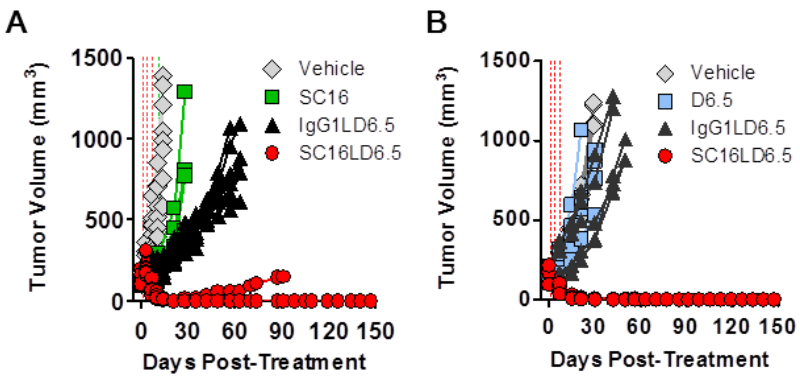
Supplementary Figure 4. In vitro plasma stability of SC16LD6.5. Stability of SC16LD6.5 in human serum was quantified to measure concentrations of conjugated and total antibody by ELISA and free drug, D6.5, by LC-MS/MS. Interpolated concentrations of the 3 analytes were back-calculated to nominal analyte concentrations spiked into the samples to obtain a percent analytical recovery.



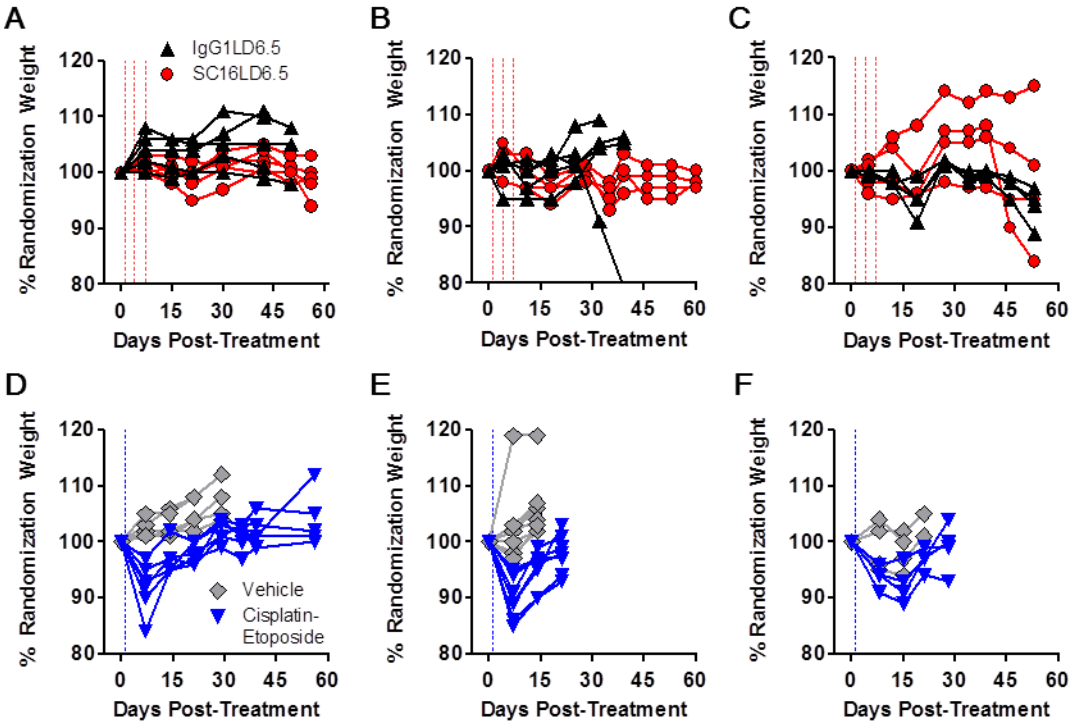
Supplementary Figure 5. Enumeration of cells with localization of human antibody in the late endosome.



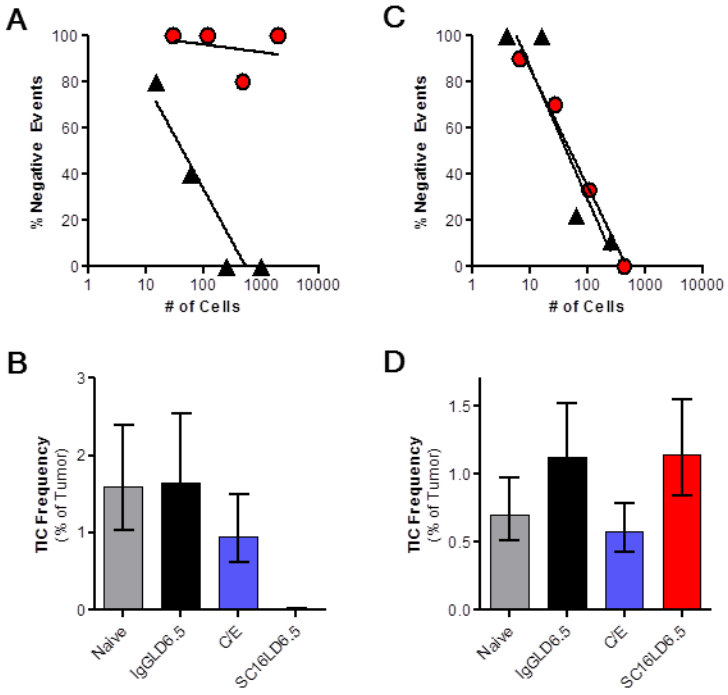
Supplementary Figure 6. DLL3 knockdown confirmation by flow cytometry. Dissociated LU37 tumor cells transduced with DLL3 shRNA (dashed line) or control LU37 cells (solid line) were labeled with AF647-anti-DLL3 or AF647-isotype control (gray filled) and measured by flow cytometry. Live human cells are displayed.



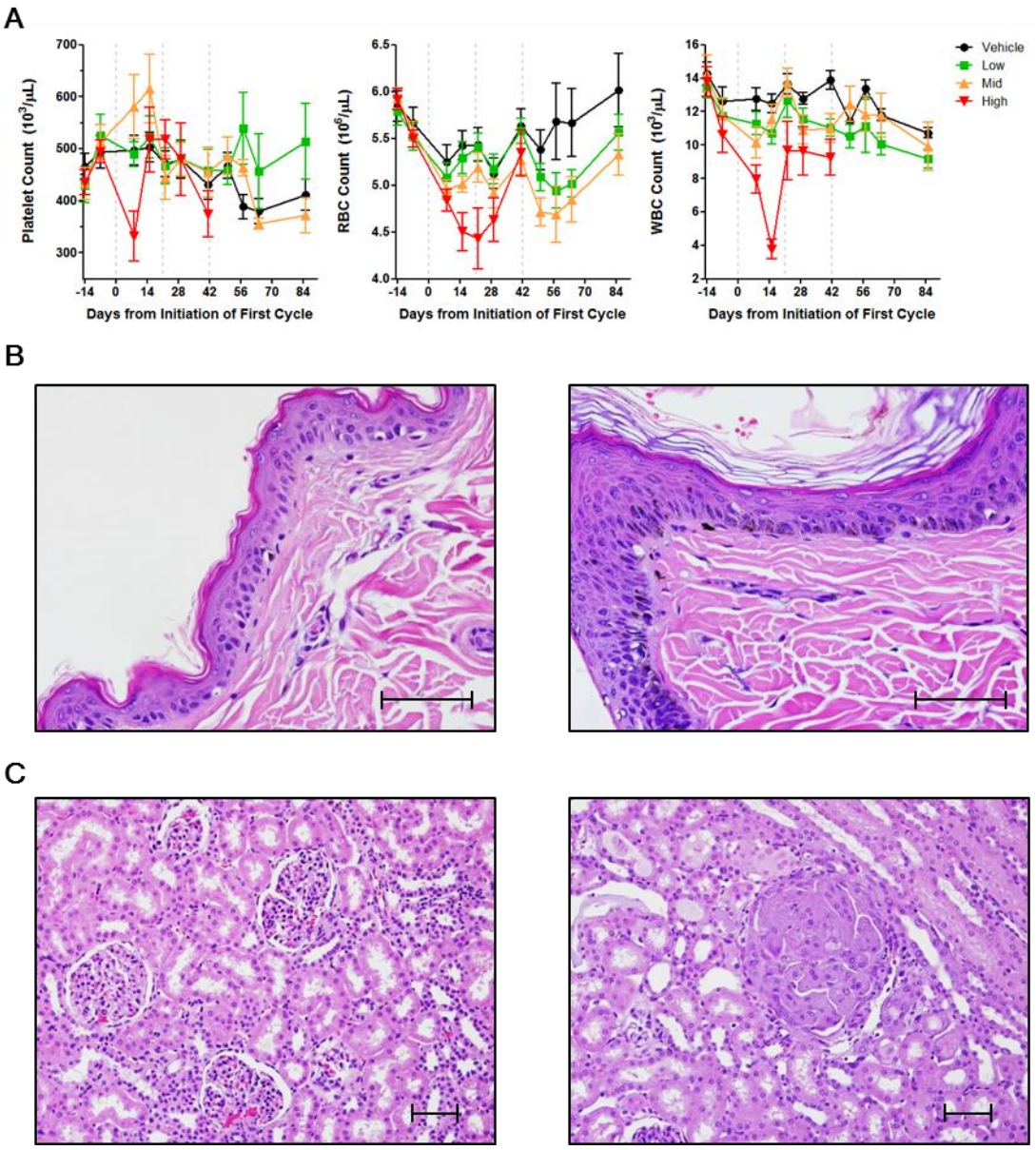
Supplementary Figure 7. In vivo efficacy of SC16LD6.5, naked SC16 antibody, and free toxin, D6.5. (**A-B**) Mice bearing (**A**) DLL3-expressing SCLC LU117 or (**B**) SCLC LU64 PDX tumors were treated with vehicle (5% glucose/saline, Q4Dx4), 30 mg/kg SC16 naked antibody [Q4Dx4, (**A**) only], 1 mg/kg IgG1LD6.5 (Q4Dx3), 1 mg/kg SC16LD6.5 (Q4Dx3), or 0.02 mg/kg D6.5 [Q4Dx3, (**B**) only].



Supplementary Figure 8. In vivo tolerability of SC16LD6.5. (**A**-**F**) Mice bearing (**A**, **D**) SCLC LU64, (**B**, **E**) SCLC LU86, or (**C**, **F**) LCNEC LU37 PDX tumors were treated with (**A**–**C**) 1 mg/kg ADCs (IgG1LD6.5 or SC16LD6.5) on a Q4Dx3 regimen, or with (**D**-**F**) vehicle (saline) or standard-of-care chemotherapy.



Supplementary Figure 9. Elimination of TICs by SC16LD6.5. (**A**, **C**) The frequency of no tumor growth after serial transplantation of (**A**) SCLC LU95 PDX and (**C**) SCLC LU80 PDX tumor cells in limiting dilutions is shown for IgGLD6.5 (black triangles) and SC16LD6.5 (red circles) cohorts. (**B**, **D**) The frequency of TIC was estimated for (**B**) LU95 and (**D**) LU80 by Poisson distribution statistics using tumor growth frequencies within each cohort.



Supplementary Figure 10. Off-target toxicities observed in nonhuman primates. Cynomologus monkeys (n = 10 per cohort) were treated with vehicle or SC16LD6.5 at a low, medium, or high dose on a Q21Dx3 dose regimen, followed by a 6 week recovery period. The high dose was not tolerated for more than 1 cycle, and these animals were euthanized at day 42. (**A**) Platelets were only impacted at the high dose level, whereas red blood cells (RBC) and white blood cells (WBC) were impacted at both the medium and high dose level. In contrast to vehicle-treated control animals (left panels), (**B**) skin thickening and hyperpigmentation was most evident in some animals at the high dose level (right panel), as was (**C**) mild kidney degeneration (right panel). Scale bars are 50 µm.

Supplementary Table 1. DLL3 normal tissue expression.

Normal Tissue	RNA-Seq/Fig. 1A (rpkm_transcript)	RT-PCR/Fig. 1B (relative expression)	Microarray/Fig. 1F (intensity value)	ELISA/Fig. 2C (ppm)
Colon	0.03	20.6	6.7	<LLOQ
Heart	0.14	1.9	12.6	0.92
Kidney	0.11	0.56	10.3	<LLOQ
Liver	0.04	0.34	6.3	<LLOQ
Lung	0.07	1.99	7.5	<LLOQ
Pancreas	0.04	3227	8.7	<LLOQ
Ovary	0.02	4.14	11.9	
Adipose		0.39		
Bladder		0.16		<LLOQ
Brain		3882074		<LLOQ
Breast		0.58	25.1	<LLOQ
Cervix		0.18		
PBMC		1.4	26.7	
Placenta		0.14		
Prostate		0.19		<LLOQ
Testes		0.48		
Thymus		14.0		
Thyroid		2.3		
Adrenal gland		0.16		0.42
Esophagus		7584		<LLOQ
Skeletal Muscle		0.7		<LLOQ
Skin		1.09	6.8	<LLOQ
Small intestine		1.13		<LLOQ
Spleen		4.69	2.6	<LLOQ
Stomach		2.22	21.6	<LLOQ
Trachea		0.59		<LLOQ
Eye				<LLOQ
Lymph node				<LLOQ
Pituitary gland				<LLOQ
Spinal cord				<LLOQ
Artery				<LLOQ
Gallbladder				<LLOQ
Esophageal-gastric junction				<LLOQ
Nerve, peripheral				<LLOQ
Nerve, sciatic				<LLOQ

<LLOQ – below lower limit of quantitation, 0.37 ppm

Supplementary Table 2. DLL3 microarray expression in PDX.

PDX	Tumor type	Microarray/Fig. 1F (intensity value)
LU64	SCLC	3058
LU73	SCLC	2610
LU80	SCLC	572
LU86	SCLC	1820
LU95	SCLC	3516
LU100	SCLC	212
LU117	SCLC	2530
LU124	SCLC	7206
LU129	SCLC	4153
LU149	SCLC	3490
LU150	SCLC	3040
LU222	SCLC	4513
LU242	SCLC	2957
LU37	LCNEC	10053
LU240	LCNEC	6653

Supplementary Table 3. Biacore affinity characterization of SC16 and SC16LD6.5 binding to human, cyno, and rat DLL3.

Antigen Species (test article)	kon (1/Ms)	koff (1/s)	Rmax (RU)	Kd (M)
Human (SC16)	$2.9x10^{6}$	$7.6x10^{-3}$	41	$2.6x10^{-9}$
Human (SC16LD6.5)	$2.5x10^{6}$	$5.6x10^{-3}$	43	$2.3x10^{-9}$
Cyno (SC16)	$4.0x10^{6}$	$4.3x10^{-3}$	51	$1.1x10^{-9}$
Cyno (SC16LD6.5)	$3.2x10^{6}$	$3.3x10^{-3}$	57	$1.0x10^{-9}$
Rat (SC16)	$1.2x10^{6}$	$9.5x10^{-3}$	34	$7.8x10^{-9}$
Rat (SC16LD6.5)	$1.3x10^{6}$	$5.7x10^{-3}$	29	$4.6x10^{-9}$

Supplementary Table 4. LU64 TIC frequency determination.

Treatment	Implanted cell number	Tumor frequency
Naive	3	0/5
Naive	15	0/2
Naive	75	4/4
Naive	375	3/5
Naive	1875	4/4
SOC	2	0/8
SOC	3	1/3
SOC	10	1/10
SOC	15	0/4
SOC	50	1/8
SOC	75	3/3
SOC	200	2/8
SOC	375	4/5
SOC	1875	3/3
IgG1LD6.5	3	0/8
IgG1LD6.5	15	2/14
IgG1LD6.5	60	4/5
IgG1LD6.5	75	7/10
IgG1LD6.5	250	4/5
IgG1LD6.5	375	10/10
IgG1LD6.5	1000	4/4
SC16LD6.5	20	0/7
SC16LD6.5	30	0/5
SC16LD6.5	100	3/9
SC16LD6.5	120	1/4
SC16LD6.5	500	7/14
SC16LD6.5	2000	2/4
SC16LD6.5	2500	7/9