SUPPLEMENTAL DATA, Liu et al.

In Vivo Selection of Highly Metastatic Human Ovarian Cancer Sublines Reveals Role for AMIGO2 in Intra-Peritoneal Metastatic Regulation

Supplemental Figure Legends

Supplemental Figure 1. Comparison of parental and IP3 sublines using phosphokinase array. OVCAR5 and OVCAR8 parental or IP3 cells were lysed and analyzed using the with Proteome Profiler Human Phospho-Kinase Array according to manufacturer's specifications. Log_2 fold change of phosphorylated proteins in the phospho-kinase array is shown.

Supplemental Figure 2. Quantitation of urinary-type plasminogen activator (uPA) activity. Net uPA activity in conditioned media from OVCAR5 and OVCAR8 parental and IP3 cells was quantified using a coupled assay to monitor plasminogen activation and the resulting plasmin hydrolysis of a colorimetric plasmin substrate (Val-Leu-Lys-p-nitroanilide, VLKpNA) by incubating plasminogen (0.3 uM) in 96 well microtiter plates in 20 mM HEPES buffer (pH 7.4) with conditioned medium (20 ul) at 37 ^{o}C. Following addition of VLKpNA (0.3 mM), plasminogen activation was quantified by monitoring the increase in absorbance at 405 nm using a Molecular Devices Thermomax microtiter plate reader. Assays were performed in triplicate.

Supplemental Figure 3. sgRNAs design of AMIGO2 genomic region. (A) Schematic map and **(B)** sequence of the four guide RNAs (green arrows) that target exon 3 of AMIGO2, as well as the primers (purple arrows) that were used for genomic PCR and Sanger Sequencing.

Supplemental Figure 4. ICE analysis of OVCAR5-IP3 cell lines generated by CRISPR/Cas9. **(A)** Genomic PCR performed on OVCAR5-IP3 cells transfected with four AMIGO2 sgRNAs/Cas9

RNP complexes. A single 568-bp PCR product was amplified spanning four guide RNAs in exon 3 of AMIGO2. **(B-D)** The representative outputs from the ICE analysis of OVCAR5-IP3-AMIGO2-KD Clone 2.6. The vertical dotted lines denote the cut site. **(B)** Left: The discordance plot for the edited (green) and the control (orange) trace files. The alignment window marks the region of the edited and control traces close together before the cut site. A CRISPR edit resulting in a jump in the discordance around the cut site (the inference window) and continuing to remain far apart after the cut site, which represents a high level of sequence discordance. Right: The Indel plot displays the inferred distribution of indels in the entire edited population of genome. **(C)** The sequence contribution specifies the inferred sequences present in edited population and relative proportions. **(D)** Sanger trace file segments spanning the region around the cut site and guide sequence from the control and the edited clones. The guide sequence is underlined in black, and the PAM sequence is marked by a dotted red underline in the control.

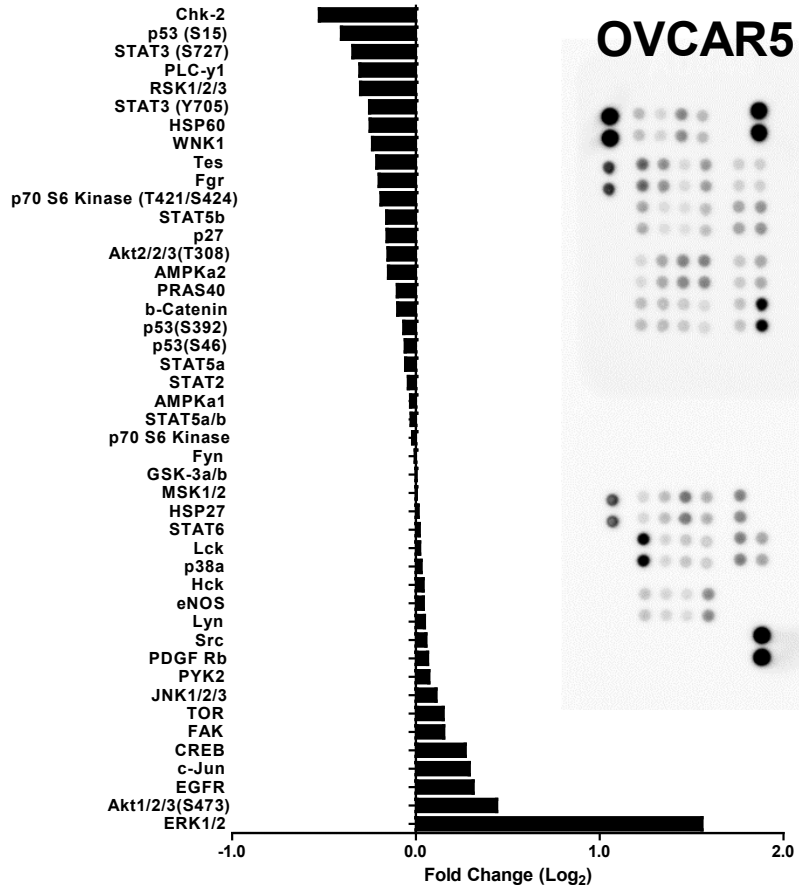
Supplemental Figure 5. Evaluation of cellular properties in AMIGO2-KD cells. (A) ALDEFLUOR assay. Parental OVCAR5 cells, OVCAR5-IP3, or OVCAR5-IP3-AMIGO2-KD cells ($2x10^5$), as indicated, were incubated with ALDEFLUOR reagent in the presence or absence of diethylaminobenzaldehyde (DEAB, negative control) at 37 ^oC for 35-55 min prior to analysis in a Beckman FC500 flow cytometer. ALDH bright and negative control (+DEAB) cells were quantified according to manufacturer's specifications. **(B)** Colorimetric ALDH assay. Cells ($1x10^6$) were lysed on ice, centrifuged to remove insoluble material and aliquots of supernatant (25ul of lysate adjusted to 50ul with assay buffer) were added to an equal volume of the reaction mixture followed by analysis of absorption at 450 nm using a Molecular Devices Thermomax microtiter plate reader. Absorbance measurements are compared to a standard curve (NADH) to yield ALDH activity values. Results represent the mean +/- SEM of five replicates. **(C)** Analysis of adhesion. Parental OVCAR5, OVCAR5-IP3 and OVCAR5-IP3-AMIGO2-KD cells were assayed for adhesion to type I collagen-coated wells. Cells ($1x10^5$) were added to wells

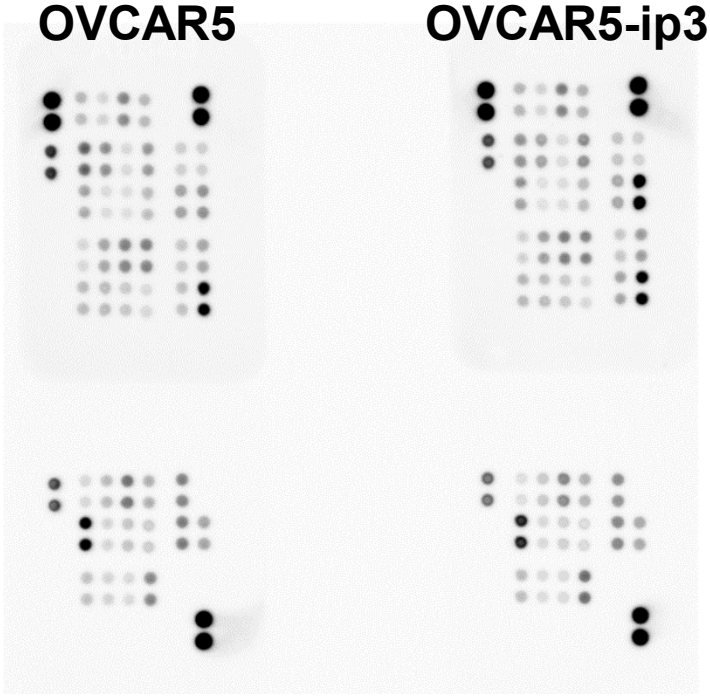
and allowed to adhere for 45 min, followed by washing to remove non-adherent cells and enumeration of adherent cells (minimum of 20 high-powered fields per condition). Adhesion is shown as % of control adhesion (24h time point). **(D)** Analysis of invasion. Parental OVCAR5, OVCAR5-IP3, and OVCAR5-IP3-AMIGO2-KD cells were assayed for invasive activity using a Boyden chamber (8-um pore) coated with Matrigel (10 ug). Cells ($2x10^5$) were added to the chamber in serum-free medium for 24-72h. At the termination of the assay, non-invading cells were removed from the top of the filter using a cotton swab. Diff-Quik was used to fix and stain filters and migrating cells adherent to the bottom of the filter were enumerated by counting a minimum of 20 high-powered fields. All experiments were performed in at least triplicate and results show mean +/- SEM.

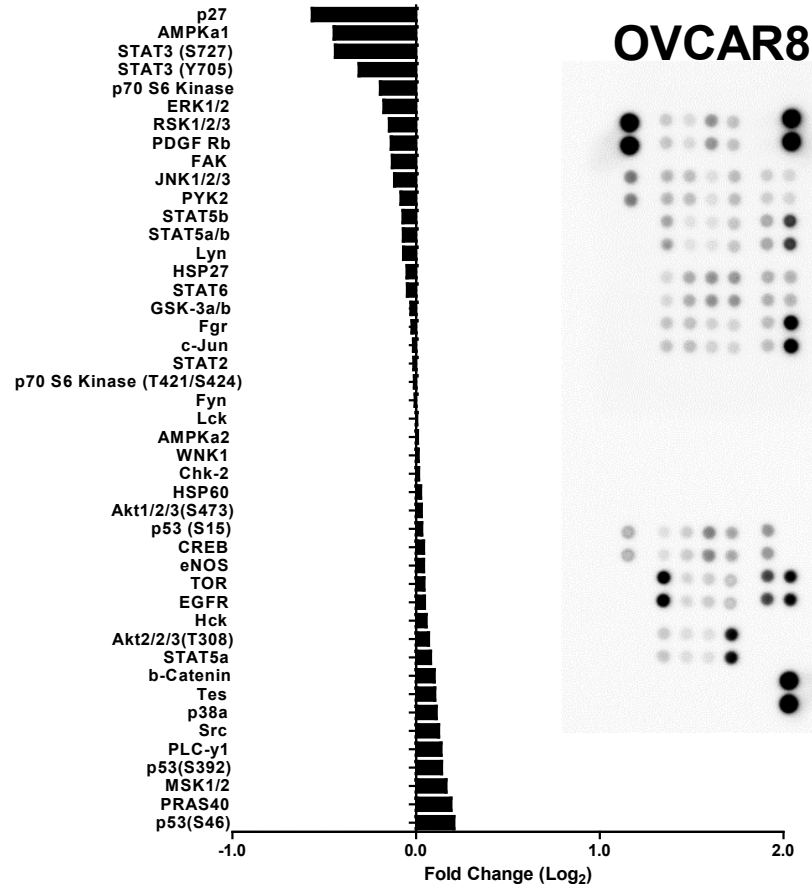
Supplemental Figure 6. AMIGO2 regulates multicellular aggregate dynamics.

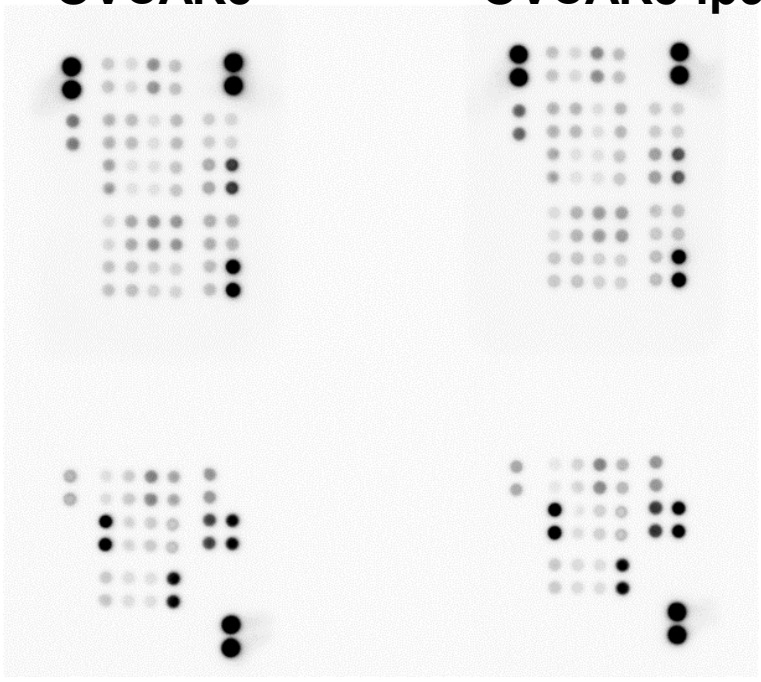
(A) MCAs were generated via the hanging drop method by seeding cells at a density of 1000 cells per 20 µl droplet onto the lid of culture dishes. Dishes were gently inverted and allowed to aggregate for 2 days. MCAs were imaged using Echo Revolve hybrid microscopy in bright field and fluorescence modes. Shown are parental OVCAR5 cells, OVCAR5-IP3, OVCAR5-IP3-AMIGO2-KD or OVCAR5-IP plus anti-AMIGO2 antibody. **(B,C)** MCA areas (μm^2) were measured using ImageJ and statistical analysis was performed using one-way ANOVA. Experiments were performed in triplicate and results show mean +/- SEM.

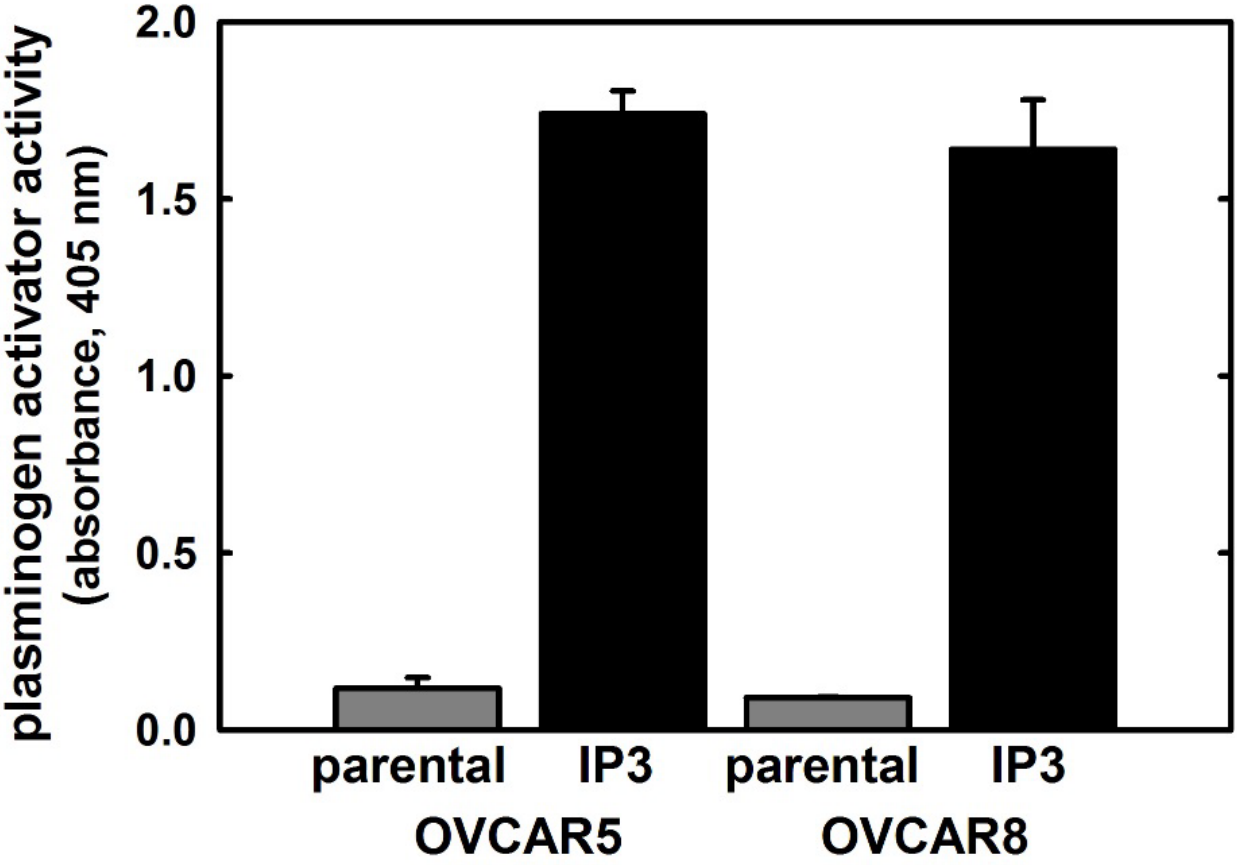
Liu et al., Suppl. Fig 1



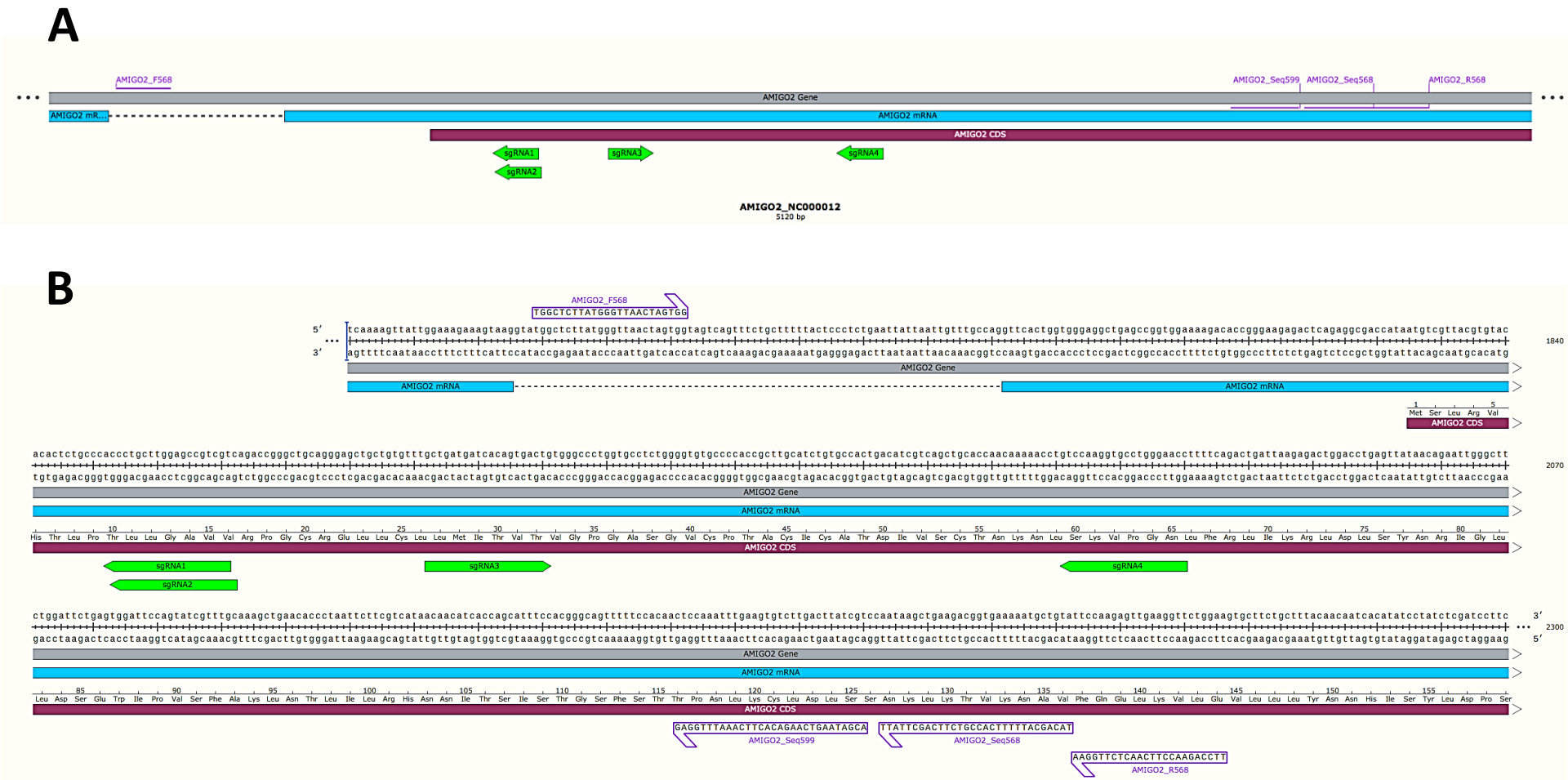




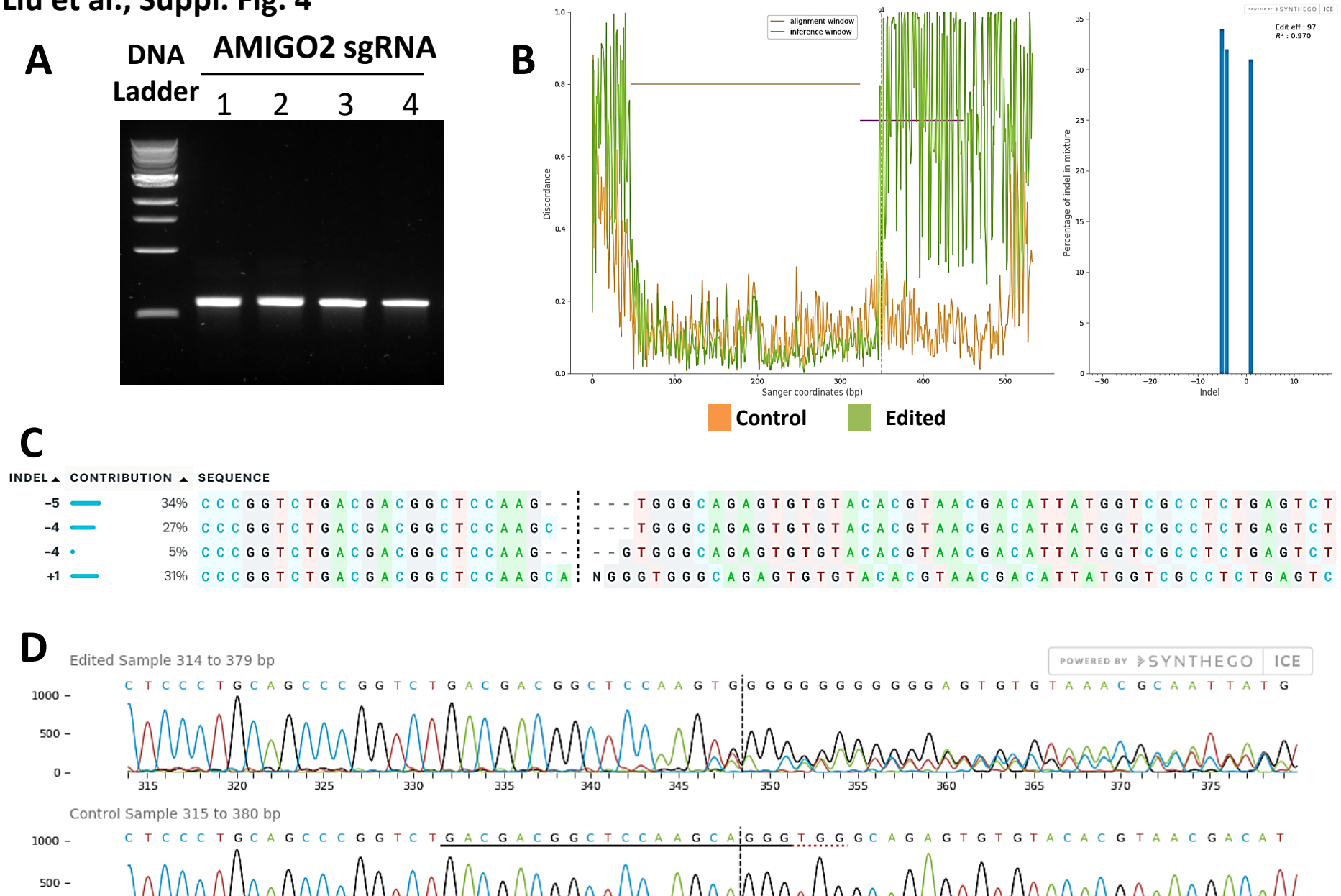


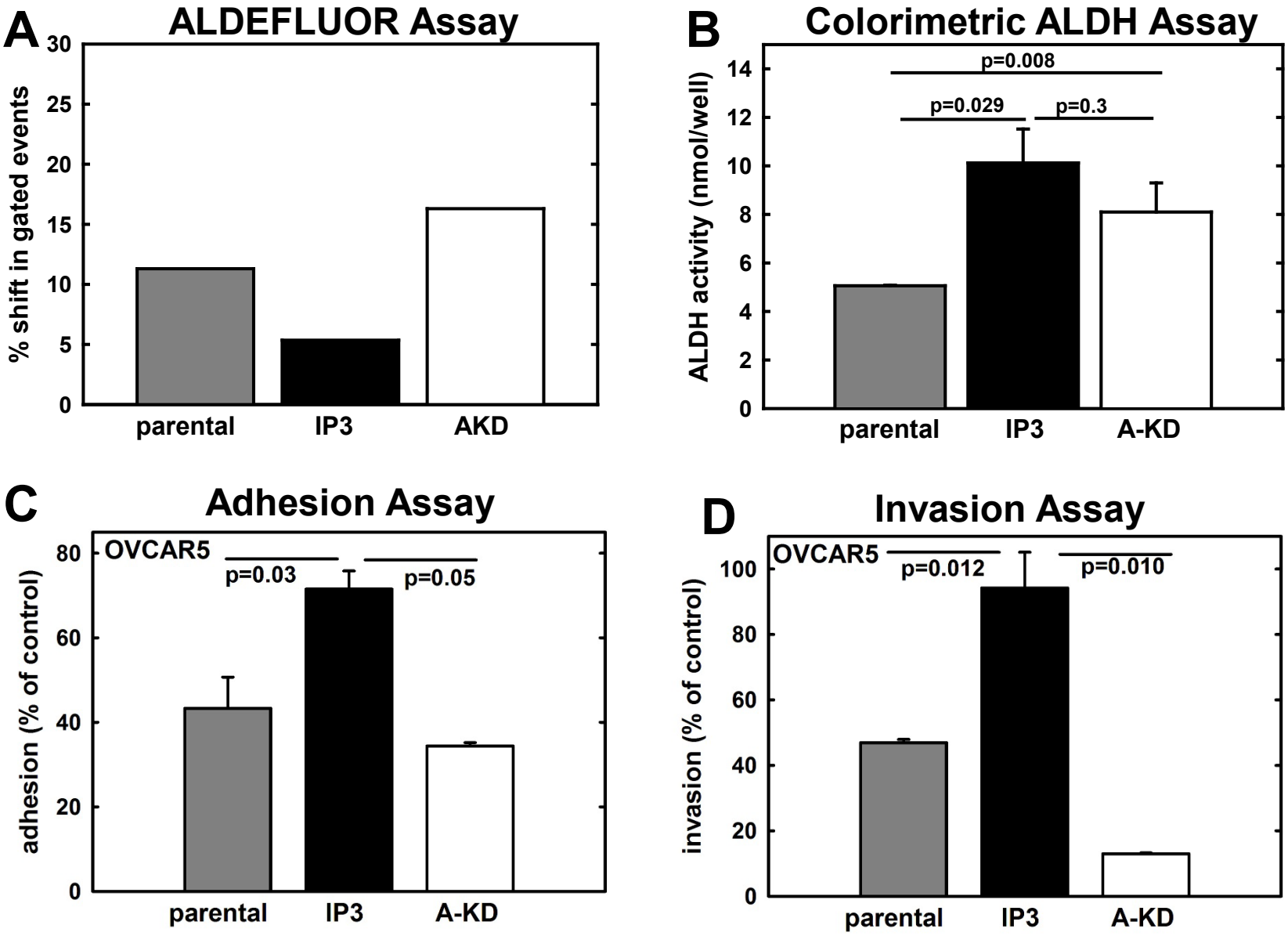

plasminogen activator activity
(absorbance, 405 nm)
2.0
1.5
1.0
0.5
0.0
parental
IP3
parental
IP3
OVCAR5
OVCAR8

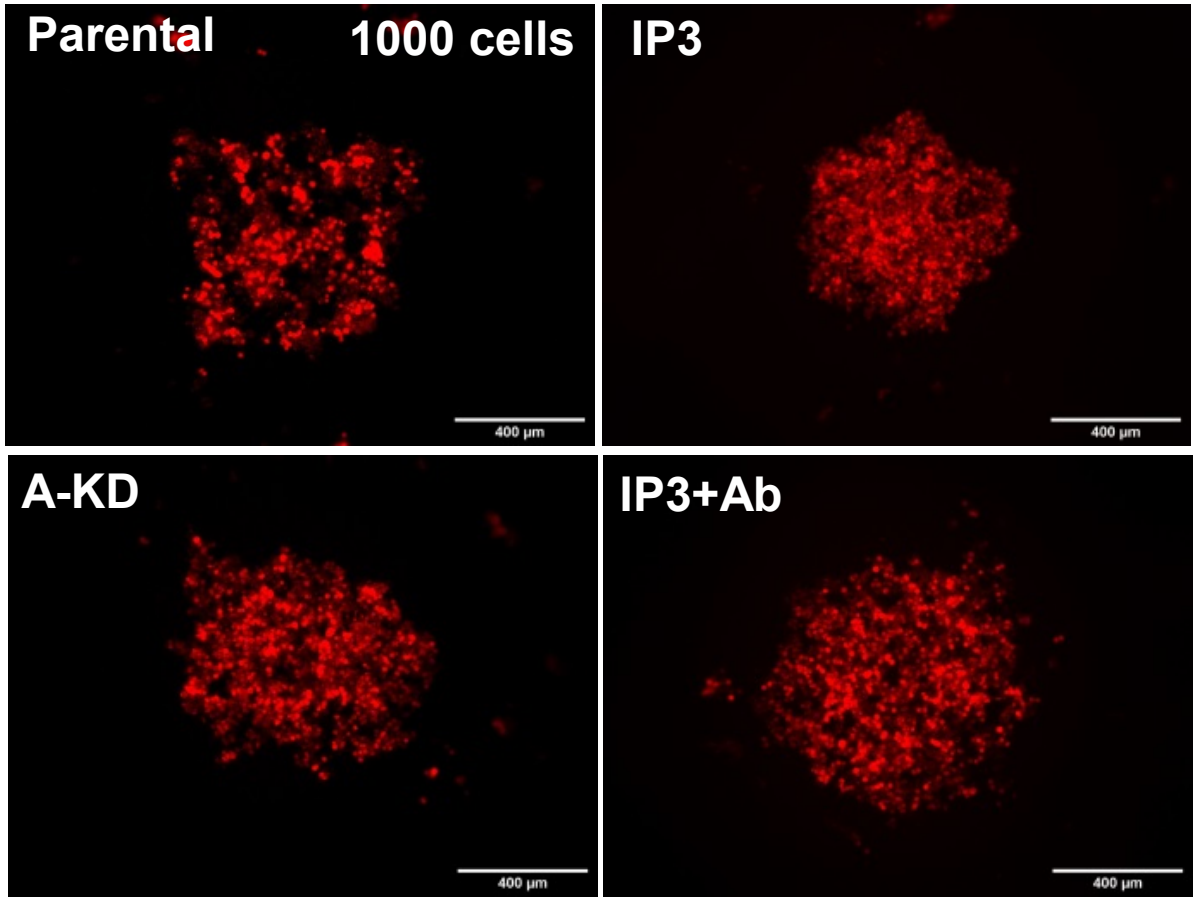
Liu et al., Supplemental Fig. 3

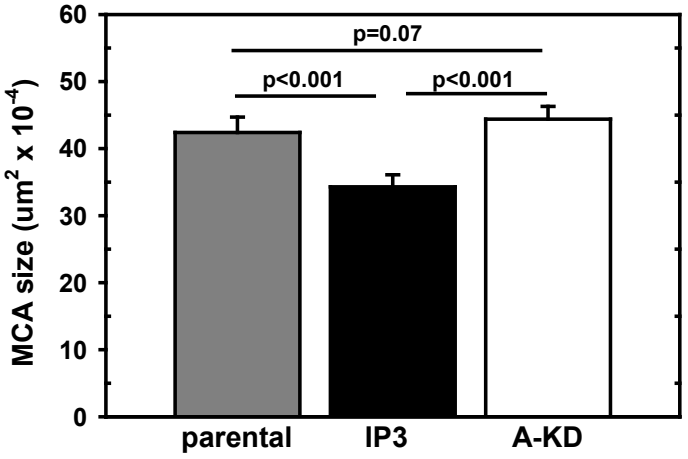


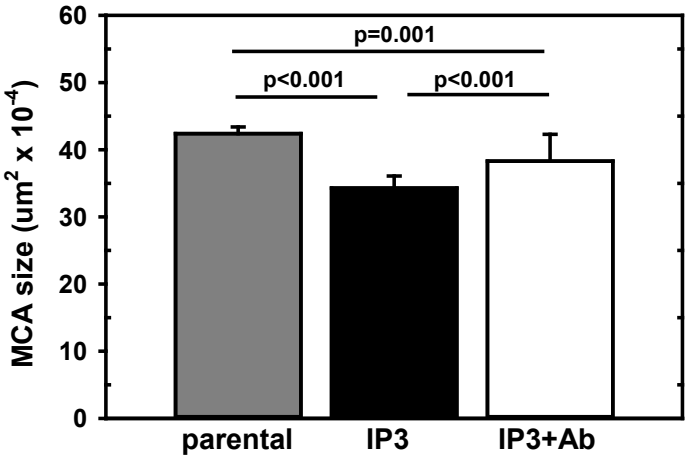
Liu et al., Suppl. Fig. 4




A
ALDEFLUOR Assay
% shift in gated events
0
5
10
15
20
25
30
parental
IP3
AKD
B
Colorimetric ALDH Assay
ALDH activity (nmol/well)
0
2
4
6
8
10
12
14
p=0.008
p=0.029
p=0.3
parental
IP3
A-KD
C
Adhesion Assay
OVCAR5
p=0.03
p=0.05
adhesion (% of control)
0
20
40
60
80
parental
IP3
A-KD
D
Invasion Assay
OVCAR5
p=0.012
p=0.010
invasion (% of control)
0
20
40
60
80
100
parental
IP3
A-KD


Parental
1000 cells
IP3
A-KD
IP3+Ab
400 µm


MCA size (um^2 x 10^{-4})
60
50
40
30
20
10
0
p=0.07
p<0.001
p<0.001
parental
IP3
A-KD


MCA size (um^2 x 10^{-4})
60
50
40
30
20
10
0
p=0.001
p<0.001
p<0.001
parental
IP3
IP3+Ab

Supplemental Table 1: qPCR primers for validation of investigated genes and reference genes.

Gene Symbol	Gene Name	Accession Number	Chromosomal Localization	Forward and Reverse Primer (5'→3')	Amplicon Size (bp)
AMIGO2	adhesion molecule with Ig like domain 2	NM_181847	12q13.11	5'-AGAGACTCAGAGGCGACCAT-3'	104
				5'-ATCAGCAAACACAGCAGCTC-3'	
COL6A3	collagen type VI alpha 3 chain	NM_004369	2q37.3	5'-CTGTTGCAGGCAAATGTAAAG-3'	121
				5'-CACAGGCTTCCCATCTAAAG-3'	
FN1	fibronectin 1	NM_001306129	2q35	5'-GGAGATTCATGGGAGAAGTATG-3'	115
				5'-GACCACTTGAGCTTGGATAG-3'	
IFI6	interferon alpha inducible protein 6	NM_022873	1p35.3	5'-CCTTCTTGGCCTAACTCTTC-3'	137
				5'-CAGTGTACTATGTTCGCATCT-3'	
KISS1	KiSS-1 metastasis suppressor	NM_002256	1q32.1	5'-CCCACCCTCTGGACATT-3'	133
				5'-GAGTTCATCTTGGTGAGAAGAG-3'	
PLAU	plasminogen activator, urokinase	NM_001145031	10q22.2	5'-GCAGAGACACTAACGACTTC-3'	125
				5'-CCAGCTCTTACTCACACTTAC-3'	
PODXL	podocalyxin like	NM_001018111	7q32.3	5'-GCTGTAGTAGCTCTGATGAAAT-3'	122
				5'-CAGACTGTGAGGAAGGAATTAG-3'	
SEC11C	SEC11 homolog C, signal peptidase complex subunit	NM_033280	18q21.32	5'-GAAGGACGAGACATTCCAATAG-3'	115
				5'-ACAAGCCTCTATCATCAACTTC-3'	
SERPINE2	serpin family E member 2	NM_006216	2q36.1	5'-GGAACACTGTACTGAGGAATG-3'	117
				5'-GAACTGGACAAACAGCAAATAC-3'	
SPINT2	serine peptidase inhibitor, Kunitz type 2	NM_021102	19q13.2	5'-GAGGGATTGACTCGGATTTG-3'	120
				5'-GGAGCCTCCTAGAGGATTT-3'	
HPRT1	hypoxanthine phosphoribosyltransferase 1	NM_000194	Xq26.2-q26.3	5'-CTGGAAAGAATGTCTTGATTGTG-3'	104
				5'-GACCTTGACCATCTTTGGATTA-3'	
HSP90AB1	heat shock protein 90 alpha family class B member 1	NM_001271969	6p21.1	5'-AAGAGAGCAAGGCAAAGTTTGAG-3'	120
				5'-TGGTCACAATGCAGCAAGGT-3'	

Supplemental Table 2: AMIGO2 guide RNA sequences

Label	Amigo2 Guide RNA Sequence
Amigo2_sgRNA1	A*C*G*ACGGCUCCAAGCAGG*G*U*
Amigo2_sgRNA2	G*A*C*GACGGCUCCAAGCAG*G*G*
Amigo2_sgRNA3	G*C*U*GAUGAUCACAGUGAC*U*G*
Amigo2_sgRNA4	G*G*U*UCCCAGGCACCUUGG*A*C*

N*N*N* indicate 2'-O-methyl analogues and 3'-phosphorothioate internucleotide linkages.

Supplemental Table 3: AMIGO2 primers for genomic DNA PCR and Sanger Sequencing

Application	Primer Name	Primer Sequence (5'-3')	Amplicon size (bp)
Genomic PCR	AMIGO2_F568	TGGCTCTTATGGGTTAACTAGTGG	568
	AMIGO2_R568	TTCCAGAACCTTCAACTCTTGGAA	
Sequencing	AMIGO2_Seq568	TACAGCATTTTTCACCGTCTTCAGCTTATT	/
	AMIGO2_Seq599	ACGATAAGTCAAGACACTTCAAATTTGGAG	/

Supplemental Table 4: Summary of AMIGO2 sgRNAs-edited OVCAR5-IP3 single cell sorting for clonal expansion

AMIGO2 sgRNAs-edited OVCAR5-IP3	sgRNA2	sgRNA3
Wells of sorted single cell (in 3 plates of 96-well plates)	288	288
Wells with live cells	173	168
% of well have > 1 cell	11%	59%
% of well have single cell that died	68%	20%
% of well have live single cell clones	36 (21%)	36 (21%)