Melanoma Differentiation Associated Gene-9/Syndecan Binding Protein (MDA-9/SDCBP) Promotes Hepatocellular Carcinoma (HCC)

Supplementary Materials and Methods

Construction of Lenti.MDA-9 and generation of stable MDA-9 overexpressing HCC cells: Lenti.MDA-9 was generated using the Gateway cloning system (Invitrogen). The human MDA-9 (SDCBP) ORF with a C-terminal HA tag was initially amplified from the Alb/MDA-9 transgenic construct, using primers 5′-CACCatgTCTCTCTATCCATC-3′ and 5′-tcaAGCGTAATCTGGAACATC-3′ (start and stop codons in lower case). Sequential directional cloning of the blunt-ended PCR product into the pENTR/D-TOPO and pLenti/CMV/Puro/DEST (w118-1) vectors was performed according to the Gateway protocols. pLenti.MDA-9 and packaging plasmids were transfected in HEK-293T cells using Lipofectamine 2000 transfection reagent (ThermoFisher, Catalog#: 11668019) and the supernatant was harvested to obtain Lenti.MDA-9. HuH-7 and HepG2 cells were infected with Lenti.MDA-9 and pooled clones were generated by selection with 1 and 2 μg/ml puromycin, respectively, for 2 weeks.

Cell proliferation assays: Hepatocytes ($1x10^4$ cells/well) were plated in a 96 well plate to measure proliferation by a standard MTT assay (1).

Colony formation assays: Human HCC cells (500 per 6 cm dish) were plated and cultured for 2 weeks. The colonies were fixed using 4% formaldehyde, stained with crystal violet and scored. Clonogenic efficiency was calculated as (number of colonies/number cells plated) x 100.

siRNA transfection assays: Control siRNA (Catalog#: D-001810-01-05) and siRNA for mouse Spp1 (Catalog#: L-043718-01-0005) and Ilk (Catalog#: L-040115-00-0005) were obtained from Horizon Discovery and were transfected into hepatocytes using PromoFectin-siRNA transfection reagent (PromoCell, Catalog#: PK-CT-2000-HEP-50) according to the manufacturer's instruction. The siRNAs used are pools of three different siRNAs targeting each gene.

NF-κB luciferase reporter assay: NF-κB luciferase reporter assay was performed in hepatocytes exactly as described (2). Experiments were performed in triplicates with two independent experiments.

Macrophage migration assay: Migration of macrophages toward conditioned medium (CM) was performed by a modified Boyden chamber (pore size: 5 μM, Corning, Catalog#: 3421) assay. WT macrophages ($1x10^5$ cells/well) were plated in serum-free DMEM on the upper chamber and CM was added to the bottom chamber. The macrophages were allowed to migrate for 48 h (3). Migrated macrophages across the membrane were fixed, stained with crystal violet, photographed and counted.

In vitro capillary-like tube formation assay: Tube formation by HUVEC was performed as described with minor modification (4). HUVECs were seeded in phenol red-free Matrigel-coated 96-well plates (Sigma, Catalog# E6909) at $2×10^4$ cells/well, treated with the CM collected from RIL-175 cells (positive control), WT and Alb/MDA-9 hepatocytes and incubated at 37°C for ~16 h. The tube structures were photographed after 12 h using a Zeiss Axio Observer Z1 microscope and the degree of network formation was measured as the total tube length per field (average of three fields) using ImageJ software.

References

1. Chen D, Yoo BK, Santhekadur PK, Gredler R, Bhutia SK, Das SK, Fuller C, et al. Insulin-like growth factor-binding protein-7 functions as a potential tumor suppressor in hepatocellular carcinoma. Clin Cancer Res 2011;17:6693-6701.

2. Robertson CL, Srivastava J, Siddiq A, Gredler R, Emdad L, Rajasekaran D, Akiel M, et al. Genetic deletion of AEG-1 prevents hepatocarcinogenesis. Cancer Res 2014;74:6184-6193.

3. Robertson CL, Mendoza RG, Jariwala N, Dozmorov M, Mukhopadhyay ND, Subler MA, Windle JJ, et al. Astrocyte Elevated Gene-1 Regulates Macrophage Activation in Hepatocellular Carcinogenesis. Cancer Res 2018;78:6436-6446.

4. Leopold JA, Walker J, Scribner AW, Voetsch B, Zhang YY, Loscalzo AJ, Stanton RC, et al. Glucose-6-phosphate dehydrogenase modulates vascular endothelial growth factor-mediated angiogenesis. J Biol Chem 2003;278:32100-32106.

Supplementary Figure Legends

Figure S1. MDA-9 is overexpressed in human HCC. A. MDA-9/SDCBP gene is amplified in 8% of human HCC patients in TCGA database. Each bar represents one patient, and a fraction of the total 366 patients, which shows MDA-9/SDCBP amplification, is displayed in the figure. Figure created in cBioPortal. B. MDA-9/SDCBP copy number is significantly increased in human HCC *vs* normal liver in TCGA database. Figure created in Oncomine. C-D. MDA-9 mRNA (C) and protein (D) were analyzed by Q-RT-PCR and Western blot, respectively, in the indicated cell lines. Hc3716-hTERT is hTERT immortalized human fetal hepatocytes. All other cell lines are human HCC cells. Data represent mean ± SEM.

Figure S2. MDA-9 overexpression does not significantly affect body and liver weights and frequency of immune cell population in naïve aged mice. Body weight (A) and liver weight (B) of WT (N = 11) and Alb/MDA-9 (TG; N = 21) mice 18 months old. C-I. Livers were harvested from 18 months old WT (N = 6) and Alb/MDA-9 (N = 7) mice and were analyzed by high-dimensional flow cytometry. C. CD4 cell subsets. T_{EM}: effector memory cells; T_{CM}: central memory cells. D. CD8 cell subsets. E-F. Immune cell subsets. G. T regulatory cell populations, gated for PD1 or KLRG1. H. CD4 cell populations, gated for PD1 or KLRG1. I. CD8 cell populations, gated for PD1 or KLRG1. Black dots: WT mice, red dots: Alb/MDA-9 mice. Data represent mean ± SEM. *: $p<0.05$ vs WT.

Figure S3. Gating strategy of livers of mice aged for 18 months. Livers were analyzed by high-dimensional flow cytometry and gated as indicated. The gating was utilized in Figure S2.

Figure S4. Quantification of immunohistochemistry score (H score) for the indicated markers in WT and Alb/MDA-9 liver tumors. Corresponding to Fig. 2F. Data represent mean ± SEM. *: $p<0.01$.

Figure S5. Gating strategy of tumors and adjacent livers of DEN/PB-treated mice. Livers were analyzed by high-dimensional flow cytometry and gated as indicated. This gating was utilized in Figure 3C-F.

Figure S6. Heat maps of fluorescent markers used in high-dimensional flow analysis. Concatenated tSNE plots from Figure 3C-F with heatmap overlays of each fluorophore (identified in the bottom right corner of each plot) used in high-dimensional analysis and population identification (Figure 3D).

Figure S7. No significant differences were found in the 16 identified cell types in the adjacent livers of tumor-bearing WT (N = 4) and Alb/MDA-9 (N = 4) mice. A. Cell count from adjacent liver identified in Figure 3D. B. Cell count from adjacent liver for cell populations gated in supplemental Figure S5. C. CD4 cell subsets from adjacent liver. D. CD4 cell subsets from tumor tissue. Black dots: WT mice, red dots: Alb/MDA-9 mice. Data represent mean ± SEM.

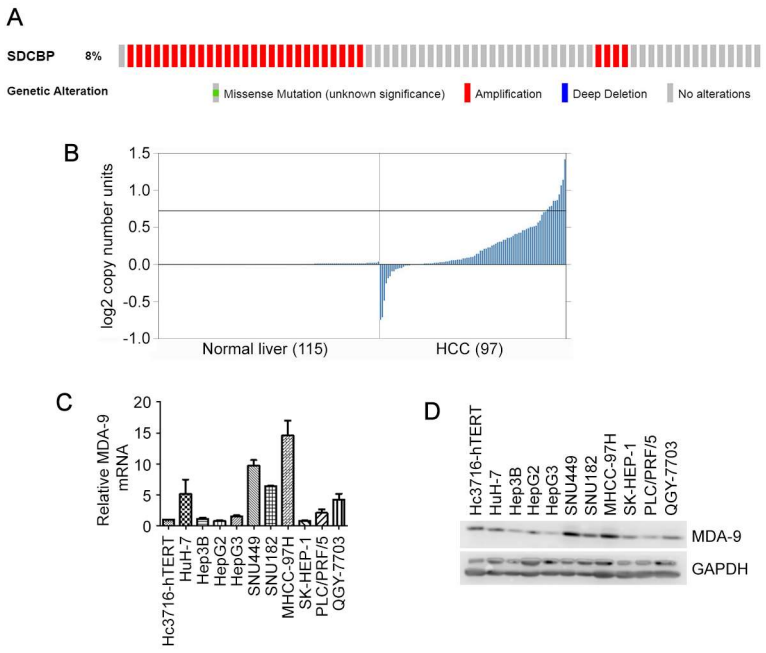
Figure S8. Spp1 induction by MDA-9 does not require PI3K activation. Alb/MDA-9 hepatocytes were treated with LY294002 (10 μM) for 24 h and Spp1 mRNA levels were analyzed by Q-RT-PCR. Data represent mean ± SEM.

Fig. S9. MDA-9-induced NF-κB and ILK pathways do not interact with each other. Alb/MDA-9 hepatocytes were treated for 48 h with either BMS-345541 (5 μM; left panel) or Cpd 22 (2.5 μM; right panel). Representative Western blot of the indicated proteins. β-Actin was used as loading control.

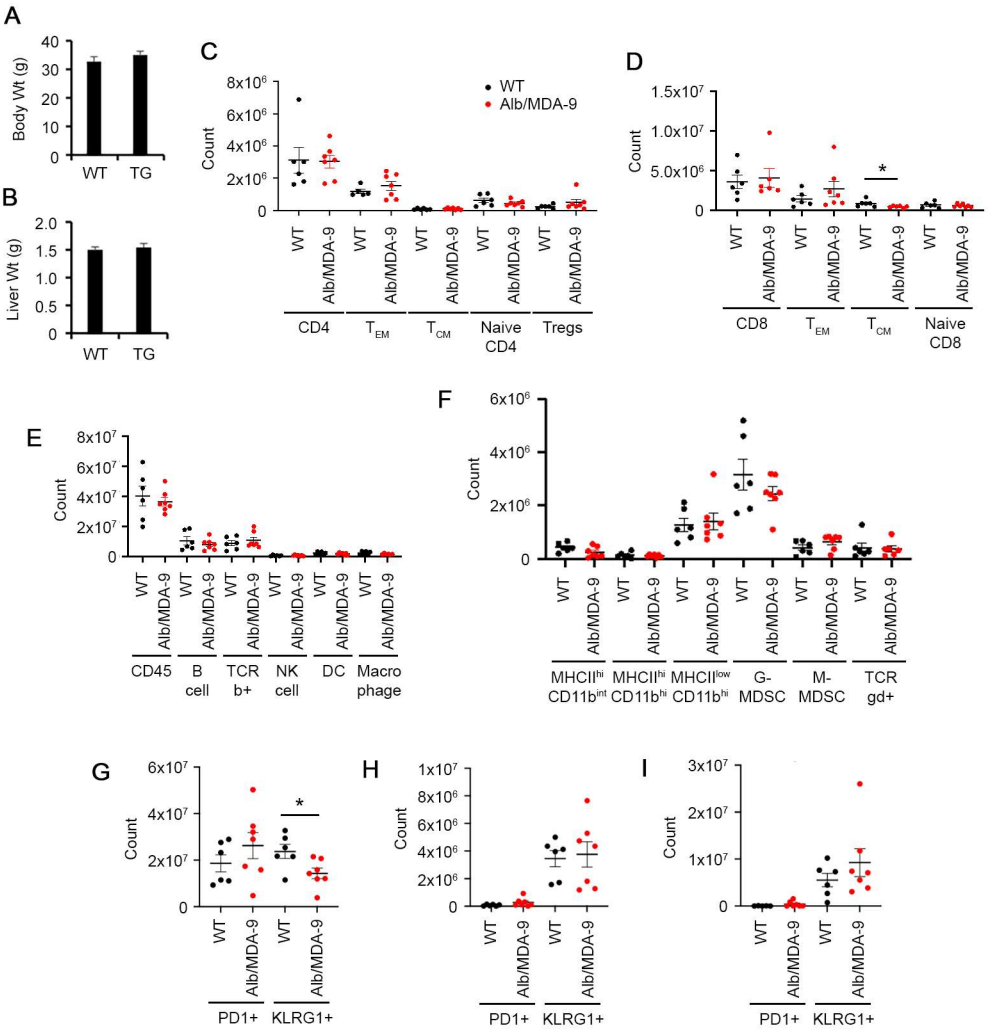
Figure S10. MDA-9 does not augment proliferation. A. Proliferation of WT and Alb/MDA-9 hepatocytes was measured by standard MTT assay at the indicated days. Data represent mean ± SEM. B. Western blot for the indicated proteins in the indicated cell lines. β-Actin was used as loading control. Asterisk indicates endogenous MDA-9 protein. C. Proliferation of HuH-7 and HuH-7-MDA-9 cells was measured by standard MTT assay at the indicated days. Data represent mean

± SEM. D. Colony formation assay in HuH-7 and HuH-7-MDA-9 cells. Data represent mean ± SEM. E. Proliferation of HepG2 and HepG2-MDA-9 cells was measured by standard MTT assay at the indicated days. Data represent mean ± SEM. F. Colony formation assay in HepG2 and HepG2-MDA-9 cells. Data represent mean ± SEM. G. Representative IHC staining of the indicated markers in DEN/PB-treated WT and Alb/MDA-9 liver tumors. Magnification: 400x. Scale bar: 20 μm. H. Quantification of immunohistochemistry score (H score) for the indicated markers in WT and Alb/MDA-9 (TG) liver tumors. Corresponding to Fig. S10G. Data represent mean ± SEM. *: $p<0.01$.

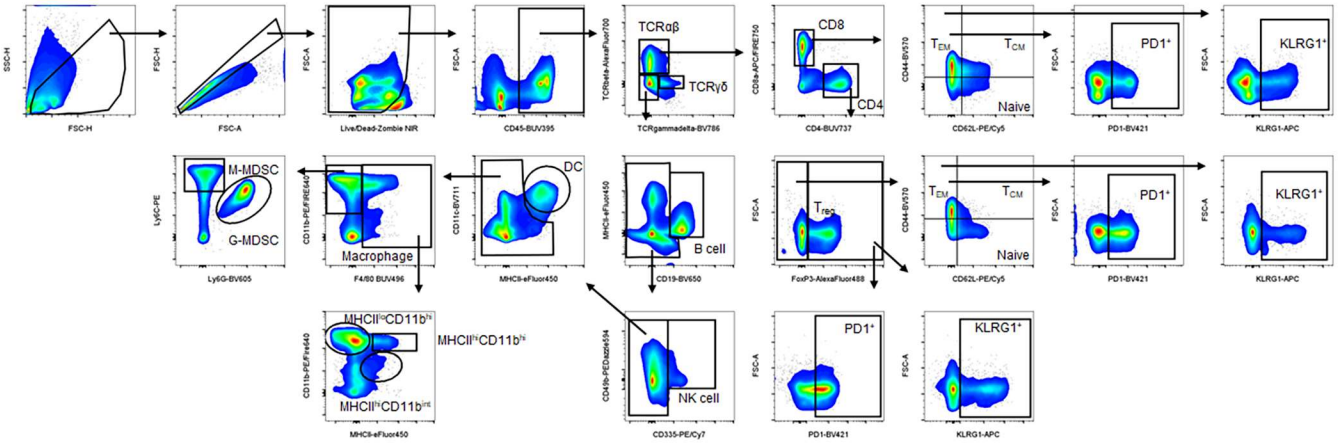
Manna et al. Fig. S1



Manna et al. Fig. S2



Manna et al. Fig. S3



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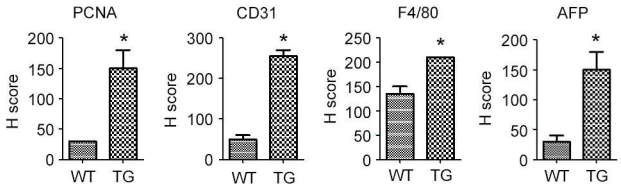

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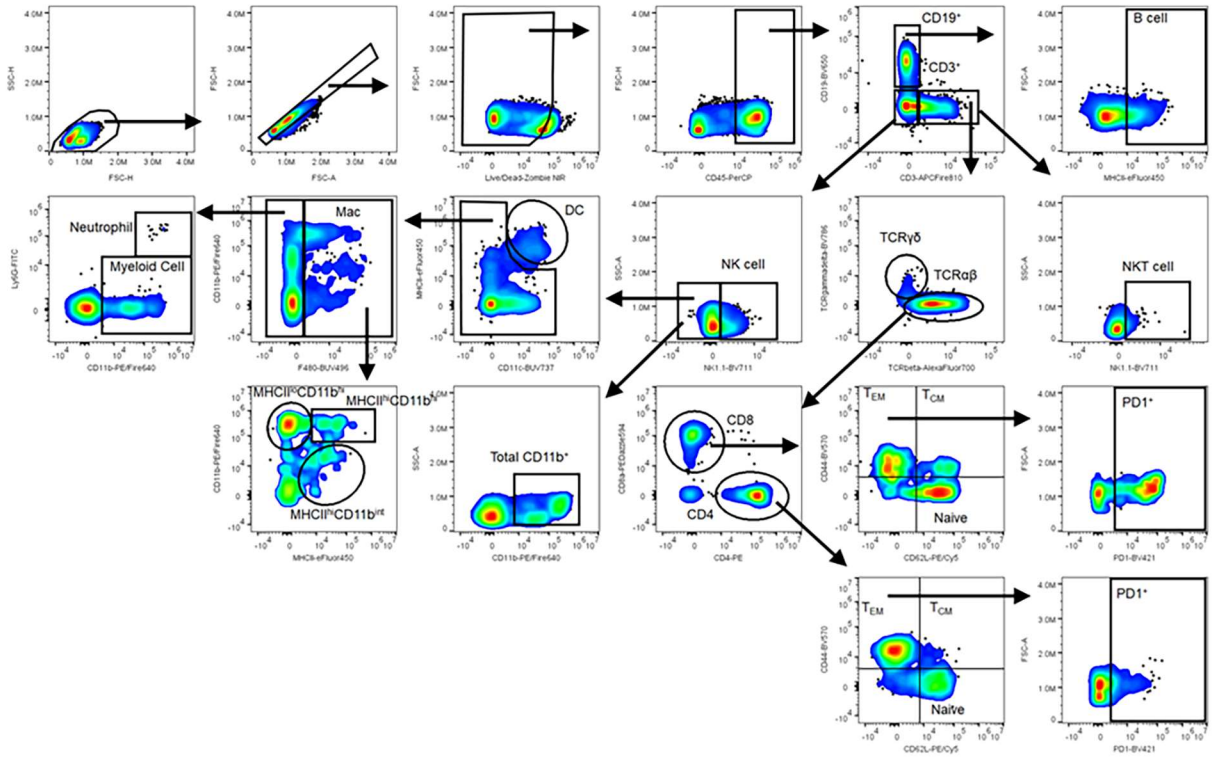

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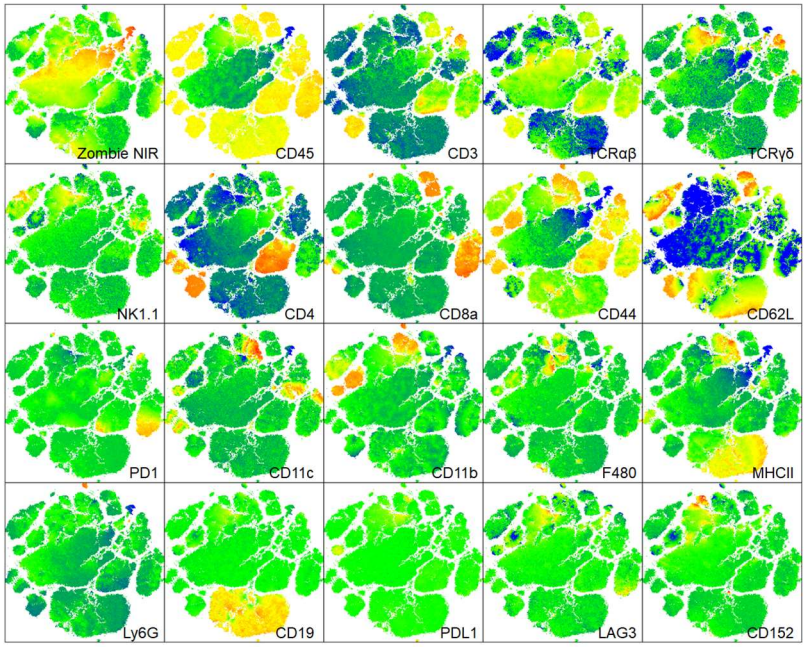

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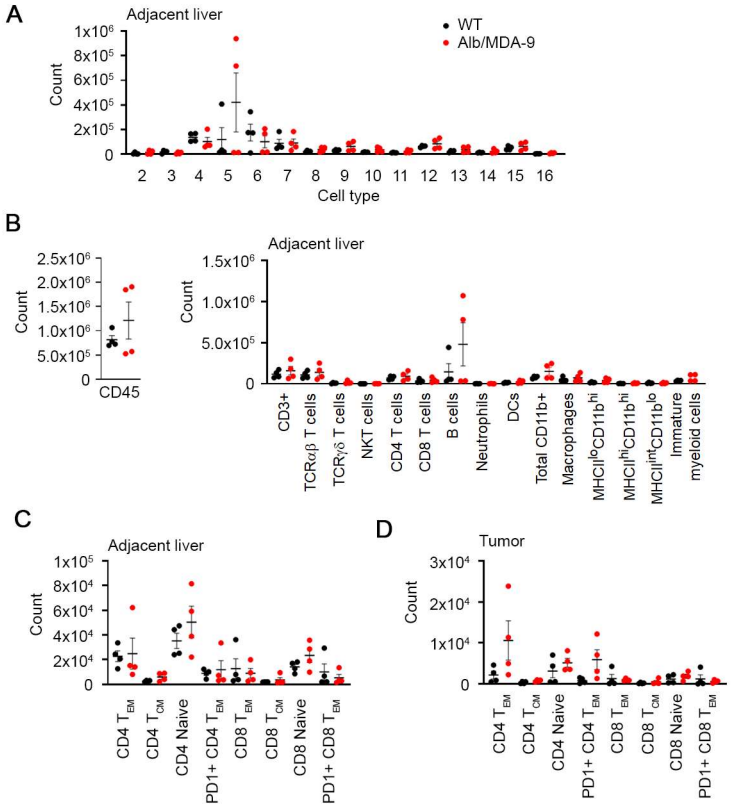

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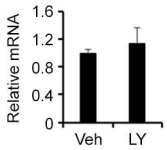

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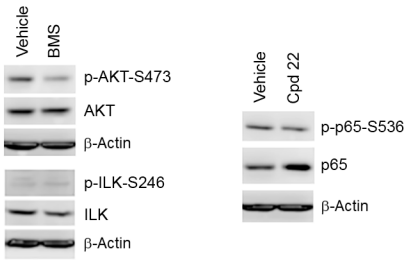

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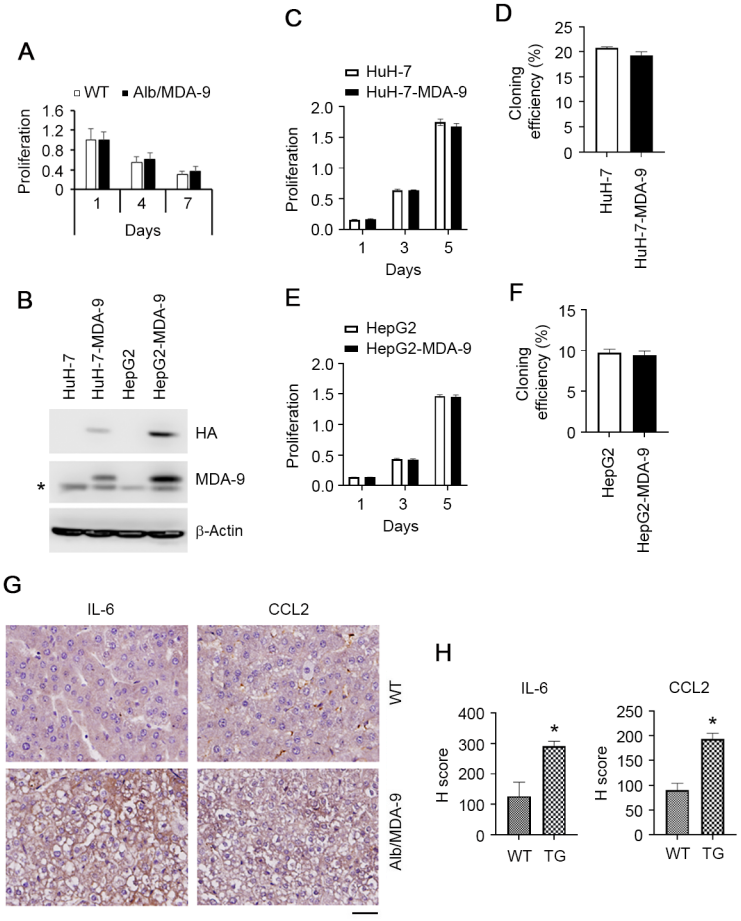

Table S1. Probes for q-RT-PCR obtained from ThermoFisher (Catalog# 4331182)

Gene name	Species	Assay ID
MDA-9/SDCBP	Human	hs01045460_g1
GAPDH	Human	hs02758991_g1
Mda-9/Sdcbp	Mouse	Mm00489742_m1
Spp1	Mouse	Mm00436767_m1
Tnfa	Mouse	Mm00443258_m1
Il1b	Mouse	Mm00434228_m1
Il6	Mouse	Mm00446190_m1
Il8	Mouse	Mm00441263_m1
Gapdh	Mouse	Mm99999915_g1

Table S2. Antibodies (Ab) used in Western blot and immunohistochemistry (IHC)

Primary Ab	Species	Dilution	Manufacturer (catalog #)	Western blot	IHC
MDA-9/SDCBP	Mouse	1:500	Abnova (H00006386-M01)	x	
HA	Rabbit	1:500	Cell Signaling (3724S)	x	
HA	Mouse	1:2000	Biolegend (901503)	x	
AKT	Rabbit	1:1000	Cell Signaling (9272S)	x	
p-AKT-S473	Rabbit	1:1000	Cell Signaling (4060S)	x	
p44/42 MAPK	Rabbit	1:1000	Cell Signaling (4695S)	x	
p-44/42 MAPK-T202/Y204	Rabbit	1:1000	Cell Signaling (4377S)	x	
SRC	Rabbit	1:1000	Cell Signaling (2108S)	x	
p-SRC-Y416	Rabbit	1:1000	Cell Signaling (2101S)	x	
ILK	Mouse	1:500	Invitrogen (MA5-17099)	x	
p-ILK-S246	Rabbit	1:500	Millipore/Sigma (AB1076)	x	
p65 NF-κB	Rabbit	1:1000	Cell Signaling (8242S)	x	
p-p65-S536	Rabbit	1:1000	Cell Signaling (3033S)	x	
E-cadherin	Rabbit	1:1000	Cell Signaling (3195S)	x	
β-Actin	Mouse	1:1000	Cell Signaling (3700S)	x	
GAPDH	Mouse	1:1000	Santa Cruz (sc-166545)	x	
MDA-9/SDCBP	Mouse	1:500	Abnova (H00006386-M01)		x
PCNA	Mouse	1:20000	Cell Signaling (2586S)		x
CD31	Mouse	1:200	Dako (M0823)		x
F4/80	Rat	1:100	Bio-Rad (MCA497G)		x
AFP	Rabbit	1:500	Dako (A0008)		x
IL-6	Rabbit	1:100	Abcam (ab208113)		x
CCL2	Rabbit	1:50	Abcam (ab7202)		x

Table S3. Antibodies used in Multiplex immunofluorescent staining

Primary antibody	Company (catalog #)	Primary antibody dilution	Opal	Opal dilution	Secondary antibody dilution
Rat-anti-Gr-1	Biolegend (108402)	1:200	520	1:600	Rat Biocare ready to use
Rabbit-anti-CD4	Cell Signaling (25229)	1:100	480	1:100	1:5
Rabbit-anti-CD11b	Abcam (ab13357)	1:6000	690	1:150	1:5
Rabbit-anti-CD8α	Cell Signaling (98941)	1:100	570	1:100	1:2.5
Rabbit-anti-FOXP3	Cell Signaling (12653)	1:200	620	1:150	1:5
Rabbit-anti-pan cytokeratin	Bioss (BS1712R)	1:200	TSA-DIG, 780	TSA-DIG 1:75 Opal 780 1:50	1:5

Table S4. Antibodies used in high-dimensional flow cytometry analysis

Target	Fluorophore	Main Laser	Clone	Company (catalog #)
CD45	BUV395	UV	30-F11	BD Fisher (bdb564279)
F4/80	BUV496	UV	T45-2342	BD Fisher (bdb750644)
CD4	BUV737	UV	GK1.5	BD Fisher (bdb612761)
PD1	BV421	Violet	RMP1-30	Biolegend (109121)
MHCII	eFlour 450	Violet	M5/114.15.2	eBiosciences (48-5321-82)
CD44	BV570	Violet	IM7	Biolegend (103037)
Ly6G	BV605	Violet	IA8	Biolegend (127639)
CD19	BV650	Violet	6D5	Biolegend (115541)
CD11c	BV711	Violet	N418	Biolegend (117349)
TCR-gd	BV786	Violet	GL3	BD Fisher (bdb744117)
Ly6C	PE	Yellow/Green	HK1.4	Biolegend (128008)
CD49b	PE Dazzle 594	Yellow/Green	DX5	Biolegend (108924)
CD62L	PE/Cy5	Yellow/Green	Mel-14	Biolegend (104410)
CD11b	PE/Fire 640	Yellow/Green	M1/70	Biolegend (101280)
CD335 (NK46)	PE/Cy7	Yellow/Green	29A1.4	eBiosciences (25-3351-82)
TCRb	AF700	Red	H57-597	Biolegend (109224)
Live/Dead	Zombie NIR	Red		Biolegend (423106)
CD8	APC/Fire 750	Red	53-6.7	Biolegend (100765)
KLRG1	APC	Red	2f1/klrg1	Biolegend (138412)
FoxP3	AF488	Blue	150D	Biolegend (320012)

Table S5. Tumor burden in WT and Alb/MDA-9 mice

Mouse #	Genotype	Tumor Diameter (mm)						
		<1	1	1-3	3-5	5-10	10	>20
986	WT	52	7	2	1	----	----	----
987	WT	13	----	----	----	----	----	----
125	WT	6	1	----	----	----	----	----
993	WT	22	1	----	----	----	----	----
990	WT	54	----	2	----	----	----	----
131	WT	31	1	3	----	----	----	----
134	WT	34	4	----	----	----	----	----
994	WT	9	----	----	----	----	----	----
995	WT	4	----	----	----	----	----	----
999	WT	10	----	1	----	----	----	----
1000	WT	95	----	----	----	----	----	----
988	Alb/MDA-9	43	5	1	2	----	----	----
989	Alb/MDA-9	90	2	6	----	----	----	----
126	Alb/MDA-9	24	16	26	20	5	----	----
124	Alb/MDA-9	39		1	----	----	----	----
123	Alb/MDA-9	34	5	----	----	----	----	----
991	Alb/MDA-9	79	----	8	----	----	----	----
992	Alb/MDA-9	24	10	----	----	----	1	1
127	Alb/MDA-9	114	10	4	----	----	----	----
129	Alb/MDA-9	31	3	----	----	----	----	----
128	Alb/MDA-9	79	5	2	----	----	----	----
130	Alb/MDA-9	94	2	----	2	----	1	----
133	Alb/MDA-9	21	2	----	1	----	----	----
136	Alb/MDA-9	28	2	1	----	----	----	----
137	Alb/MDA-9	6	----	----	----	----	----	1
135	Alb/MDA-9	29	----	1	1	----	----	----
997	Alb/MDA-9	46	18	12	1	----	----	----
998	Alb/MDA-9	135	2	8	----	2	----	----