SUPPLEMENTARY INFORMATION

Caspase-9b drives cellular transformation, lung inflammation, and lung tumorigenesis.

Minjung Kim[1*], Ngoc T. Vu[1], Xue Wang[1], Gamze B. Bulut[2], Min-Hsuan Wang[3], Cora Uram-Tuculescu[4], Raghavendra Pillappa[4], Sungjune Kim[3], Charles E. Chalfant[1,5,6,*,#]

SUPPLEMENTARY MATERIALS AND METHODS

Viral production: All plasmid vectors were generated with standard gateway cloning methods (1). PCR fragments of Myc-tagged wild-type (WT) and AT/GG mutant of C9b (Mut) CDS (2) flanked with attB1 and attB2 were cloned into pDONR223 (Invitrogen) using BP clonase (Invitrogen) to generate pEntry clones. To generate lentiviral expression plasmids, these inserts in pEntry clones were mobilized into pLEX304 (addgene #25890, blasticidin) via LR clonase (Invitrogen). As a control, eGFP (addgene #5301) is cloned into pLEX304. In addition, 3-way LR clonase gateway reaction (1) is performed by mixing pBEG R2-iCre T2A Luciferase-L3 (addgene #48992) and pLEG R1-R3 (addgene #48956) with pEntry-eGFP, -WT, or - AT/GG Mut C9b to allow polycistronic expression(3) of eGFP, WT, or Mut C9b with Cre. To produce virus, 293T cells are transfected with expression viral vectors together with psPAX2 (addgene #12260) and pMD2.G (addgene #12259) using Polyethylenimine (PEI, Sigma). The supernatant of the 293T cells was harvested at 48 and 72hr post-transfection and passed through a 0.45 μm filter before concentration by centrifugation as described (4). Viral titer is determined using p24 ELISA kit (Takara) according to the manufacturer's instruction.

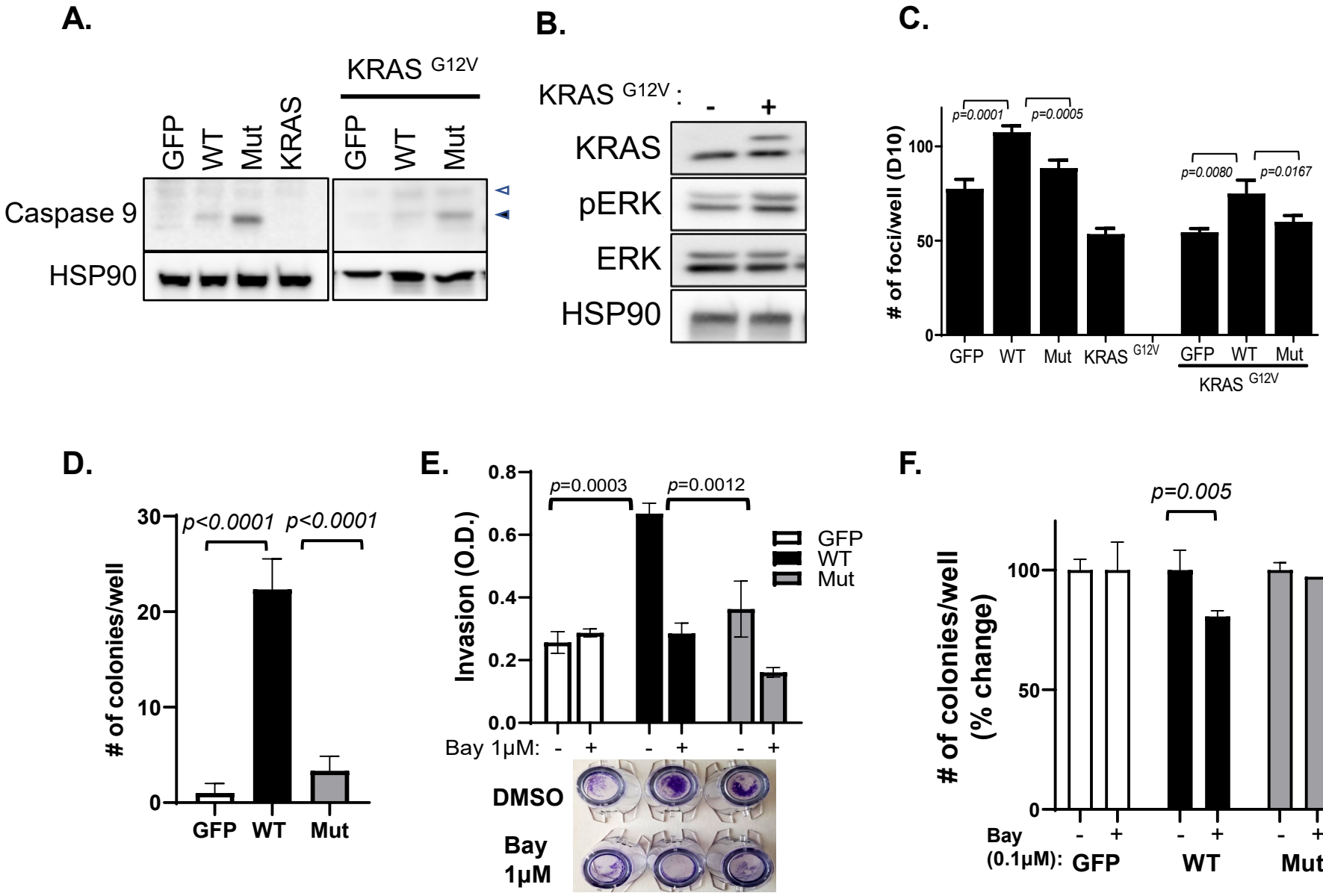
Quantitative RT-PCR (RT-qPCR): All RT-qPCRs were performed as previously described (2,5). The relative mRNA expression of human *IL-6* (forward (f): 5'-ACTCACCTCTTCAGAACGAATTG-3', reverse (r): 5'-CCATCTTTGGAAGGTTCAGGTTG-3', annealing temperature at 54 degree)

and human *CXCL1* (f: 5'-AGTGGCACTGCTGCTCCT-3', r: 5'-TGGATGTTCTTGGGGTGAAT-3', annealing temperature at 56 degree) was normalized to β-Actin. Mouse *Cxcl1* (f: 5'-ACTGCACCCAAACCGAAGTC-3', r: 5'- TGGGGACACCTTTTAGCATCTT-3') and *Cxcl2* (f: 5'-GAGCTTGAGTGTGACGCCCCCAGG-3', r: 5'- GTTAGCCTTGCCTTTGTTCAGTATC-3') was normalized to R15 (f: 5'-CTTCCGCAAGTTCACCTACC-3', r: 5'- TACTTGAGGGGGATGAATCG-3').

Mouse Cytokine array: The levels of cytokines, chemokines, and acute-phase proteins in the plasma were measured with Proteome Profiler Mouse Cytokine Array Kit Panel A from R&D systems according to the manufacturer's protocol using plasma samples pooled from 4 mice (at 1:1:1:1 ratio) in each group (control and C9b). The array data were quantitated using ImageJ (NIH), normalized to the reference spot on the same blot to determine the ratio (C9b to control).

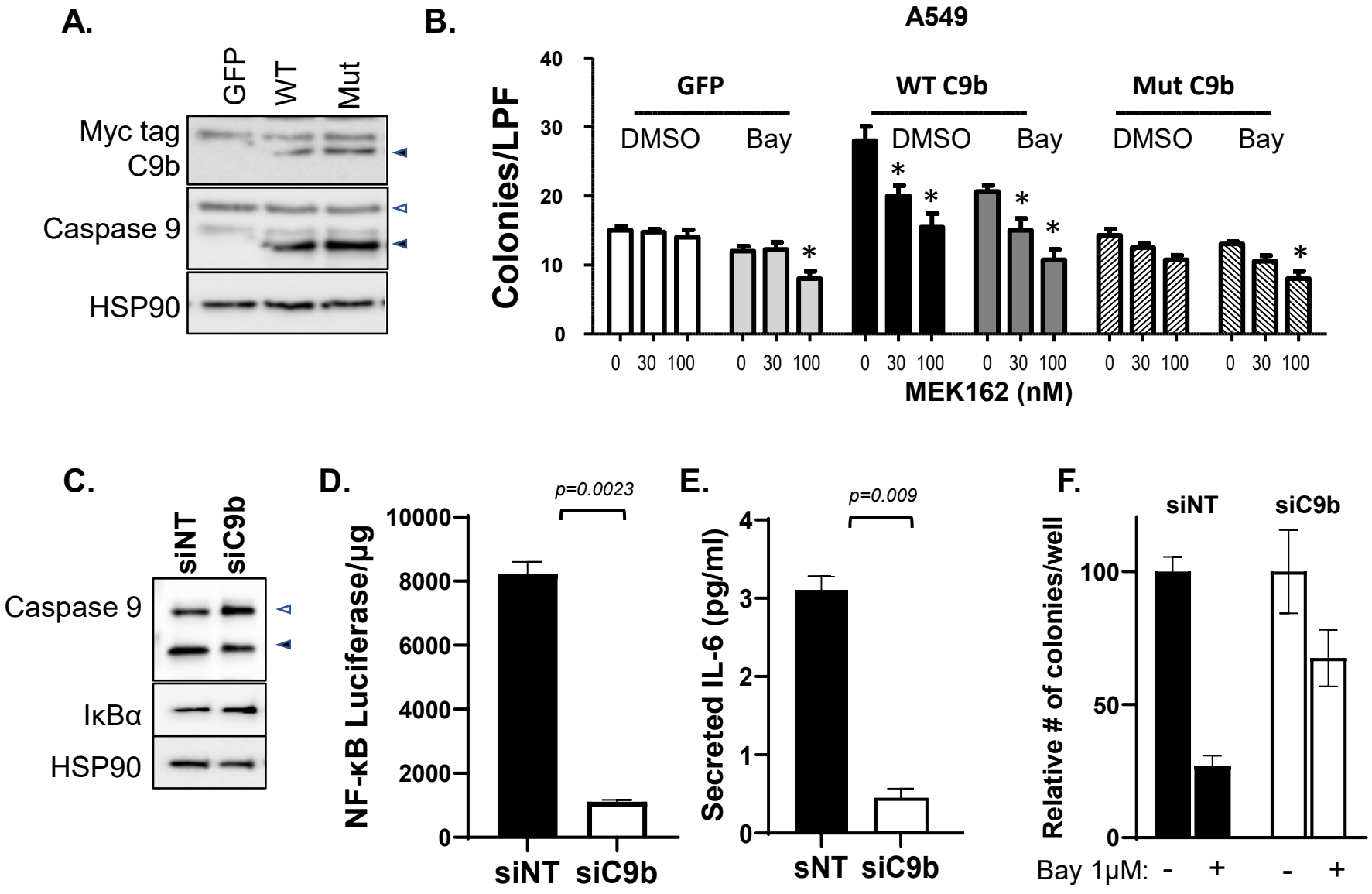
IL-6 enzyme-linked immunosorbent assays (ELISA): The levels of IL-6 in the plasma were determined with an IL-6 mouse ELISA kit (ThermoFisher) following the manufacturer's instruction. using 100 µl of plasma (diluted in sample diluent at 1:1) or standard was added into the wells pre-coated with anti-mouse IL-6 antibody.

SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

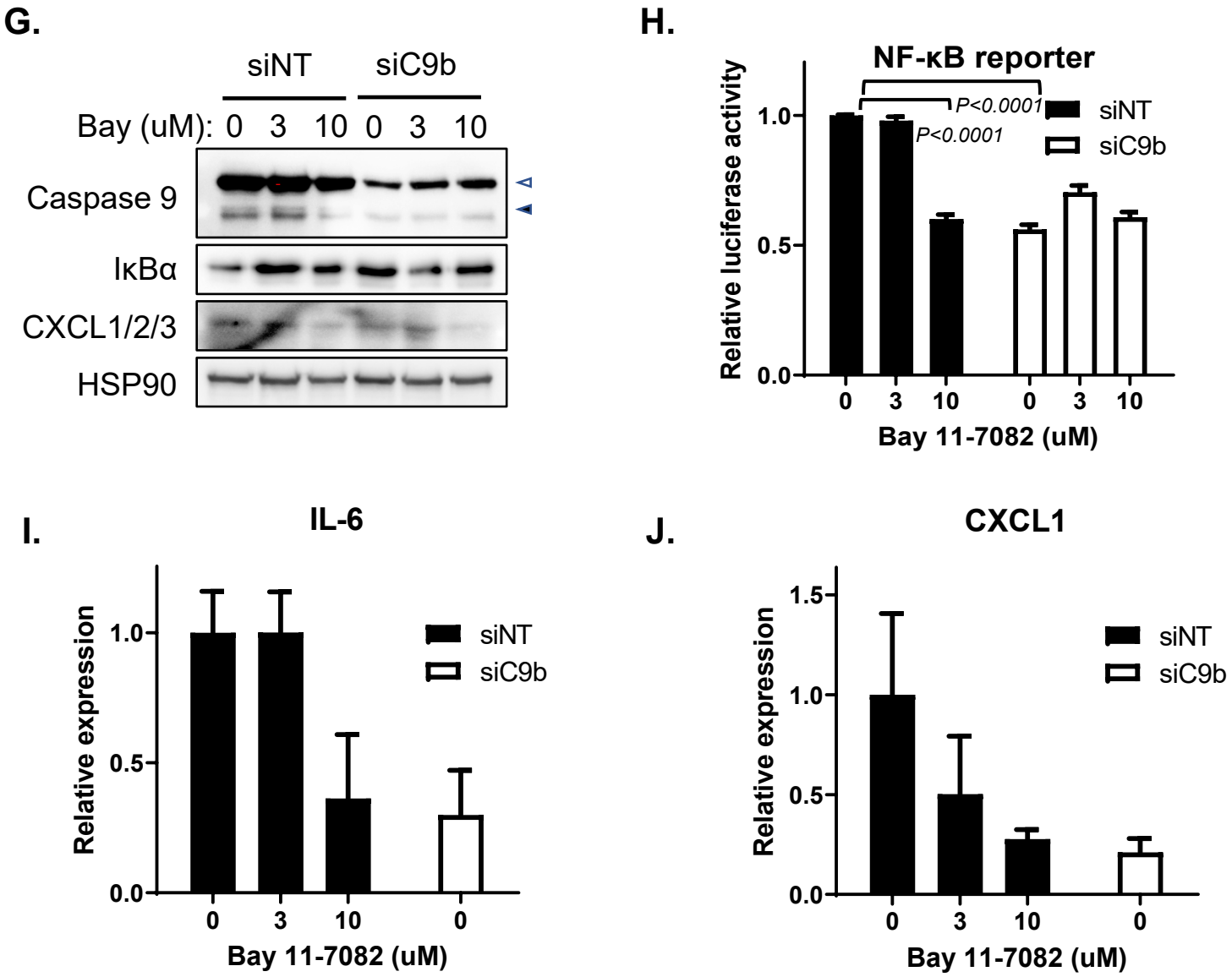

A.
KRAS G12V
GFP
WT
Mut
KRAS
GFP
WT
Mut
Caspase 9
HSP90
B.
KRAS G12V : - +
KRAS
pERK
ERK
HSP90
C.
# of foci/well (D10)
p=0.0001
p=0.0005
p=0.0080
p=0.0167
GFP
WT
Mut
KRAS G12V
GFP
WT
Mut
KRAS G12V
D.
# of colonies/well
p<0.0001
p<0.0001
GFP
WT
Mut
E.
Invasion (O.D.)
p=0.0003
p=0.0012
GFP
WT
Mut
Bay 1μM: - + - + - +
DMSO
Bay 1μM
F.
# of colonies/well (% change)
p=0.005
Bay (0.1μM): - + - + - +
GFP
WT
Mut

Suppl Fig. S1.

Supplementary Figure S1. A&B. Western blot analyses of MLE12 and MLE12-K (with *KRAS* ^{G12V}) cells expressing GFP, wild type (WT), or AT/GG mutant (Mut) C9b with indicated antibodies. KRAS ^{G12V} expression increased pERK (**B**). The empty and filled triangles denote full-length C9a and C9b proteins, respectively. **C.** Clonogenic assays. The Graph shows the number of clonal foci per well on D10. **D.** Number of colonies of MLE12-K cells per well grown in soft agar on D75. **E.** Absorbance (O.D.at 570nm) of crystal violet dye extracted from MLE12-K cells expressing GFP, WT, or Mut C9b invaded through Matrigel at 22hr per well (seeded in triplicates). Cells were treated with DMSO or Bay 11-7082 (1 μm). **F.** Normalized number of clonal foci in clonogenic assays. Cells were treated with DMSO or Bay 11-7082 0.1 μM for 48 hrs followed by growth in complete media for 10 days. WT C9b expression sensitized cells to NF-κB inhibition. Data in the graph are means ± SD; n = 3 each. Adjusted *p* values are determined by ANOVA Tukey's multiple comparison test.


A.
GFP
WT
Mut
Myc tag C9b
Caspase 9
HSP90
B.
A549
GFP
WT C9b
Mut C9b
DMSO
Bay
Colonies/LPF
MEK162 (nM)
C.
siNT
siC9b
Caspase 9
IκBα
HSP90
D.
p=0.0023
NF-κB Luciferase/µg
siNT
siC9b
E.
p=0.009
Secreted IL-6 (pg/ml)
sNT
siC9b
F.
siNT
siC9b
Relative # of colonies/well
Bay 1µM:

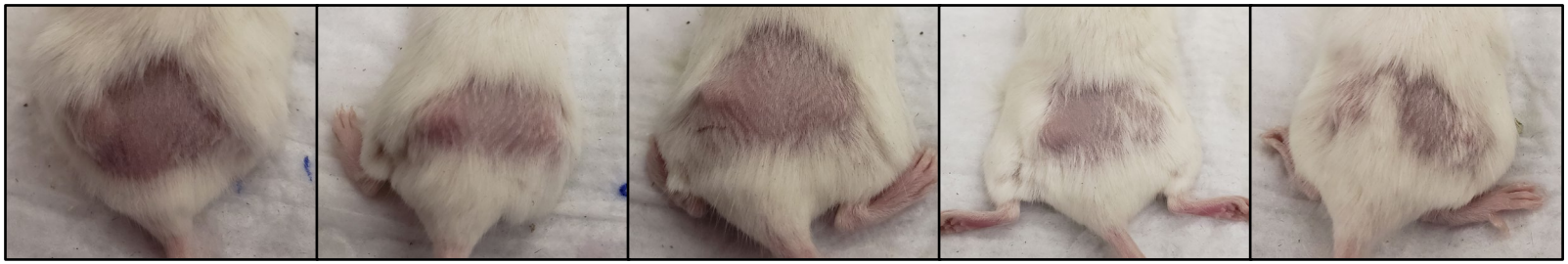
Suppl Fig. S2


G.
siNT
siC9b
Bay (uM): 0 3 10 0 3 10
Caspase 9
IκBα
CXCL1/2/3
HSP90
H.
NF-κB reporter
P<0.0001
P<0.0001
siNT
siC9b
Relative luciferase activity
1.0
0.5
0.0
0 3 10 0 3 10
Bay 11-7082 (uM)
I.
IL-6
Relative expression
1.0
0.5
0.0
siNT
siC9b
0 3 10 0
Bay 11-7082 (uM)
J.
CXCL1
Relative expression
1.5
1.0
0.5
0.0
siNT
siC9b
0 3 10 0
Bay 11-7082 (uM)

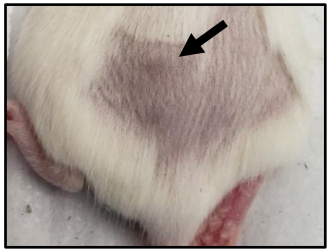
Suppl Fig. S2

Supplementary Figure S2. C9b activates NF-κB pathway and cooperates with RAS/MEK/ERK pathway in A549 (*KRAS* ^{G12C} mutant) cells. A-B. WT or Mut C9b expression in A549 cells was confirmed by immunoblotting with anti-Myc tag and Caspase 9 (C9) antibody (**A**). HSP90 serves as a loading control. **B.** Number of colonies per LPF view grown in soft agar on day 21 of A549 cells with GFP, WT or Mut C9b expression (mean +/-SD). Cells were continuously treated with DMSO, Bay 11-7082 (IKK inhibitor, 10uM), and/or MEK162 (MEK inhibitor, 30 and 100 nM). Media were changed twice a week. Bay 11-7082 suppressed soft agar colony growth of A549 cells with WT C9b expression, which were further suppressed by the combined treatment of Bay11-7082 with MEK162. Treatments that caused statistically significant changes compared to DMSO control are marked with * ($p<0.05$). **C-F.** siRNA mediated knock-down of C9b expression (siC9b) in A549 cells (transduced with luciferase reporter driven by minimal Thymidine kinase promoter with 4 consecutive NF-κB consensus sequences (Addgene # 49343)). Reduced C9b expression suppressed NF-κB-driven luciferase activity (**D**) and decreased secreted IL-6 (one of the NF-κb downstream targets) in conditioned media (**E,** IL-6 Proquantum assay, ThermoFisher) and increased IκBα (**C**). **F.** Normalized number of clonal foci per well of A549 cells with siNT or siC9b in clonogenic assays. Cells were treated with DMSO or Bay 11-7082 1 μM for 24 hrs followed by growth in 10% serum containing media for 10 days. C9b knock-down desensitized cells to NF-κB inhibition. **G-J.** Bay 11-7082 treatment (18hr in serum free media) of A549 cells with siNT suppressed NF-κB-driven luciferase activity (**G**) and mRNA expression of IL-6 (**I**) and CXCL1 (**G** and **J**), NF-κb downstream targets to the level comparable to those observed in A549 with siC9b. Data are means ± SD; n = 3 each. Adjusted *p* values are determined by ANOVA Tukey's multiple comparison test. The empty and filled triangles denote full-length C9a and C9b proteins, respectively.

WT



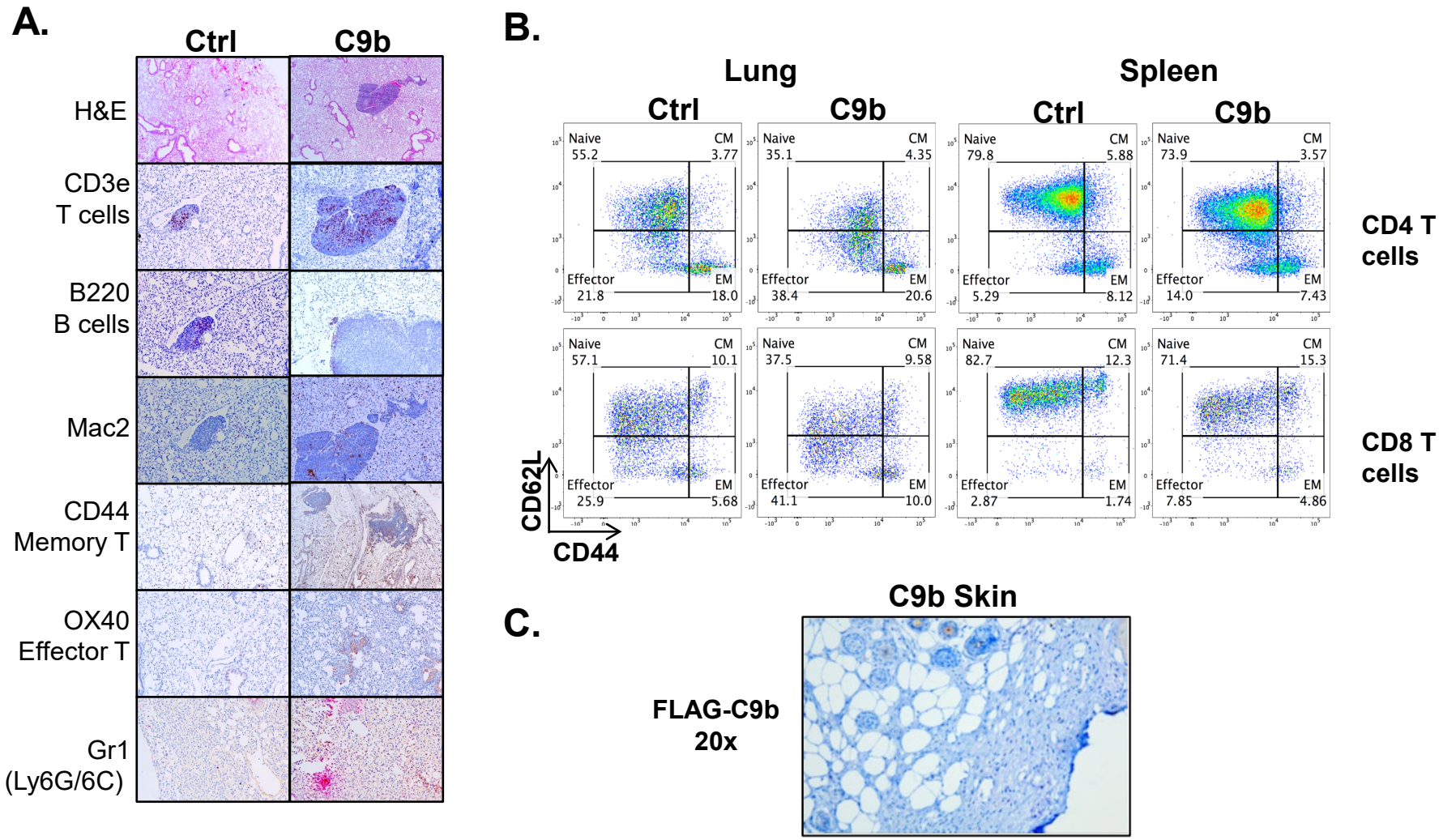
Mut



on D30 post-implantation

Suppl Fig. S3.

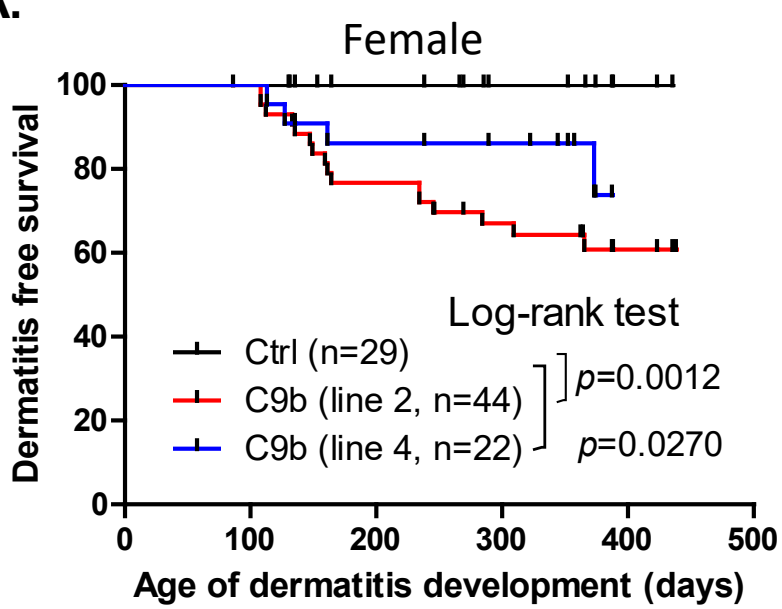
Supplementary Figure S3. Images of subcutaneous tumors on SCID mice implanted with HBEC-3KT/KP cells with WT or Mut C9b on day 30 post implantation.


A.
Ctrl
C9b
H&E
CD3e
T cells
B220
B cells
Mac2
CD44
Memory T
OX40
Effector T
Gr1
(Ly6G/6C)
B.
Lung
Spleen
Ctrl
C9b
Ctrl
C9b
Naive
CM
Effector
EM
55.2
3.77
21.8
18.0
35.1
4.35
38.4
20.6
79.8
5.88
5.29
8.12
73.9
3.57
14.0
7.43
57.1
10.1
25.9
5.68
37.5
9.58
41.1
10.0
82.7
12.3
2.87
1.74
71.4
15.3
7.85
4.86
CD4 T cells
CD8 T cells
CD62L
CD44
C.
C9b Skin
FLAG-C9b
20x

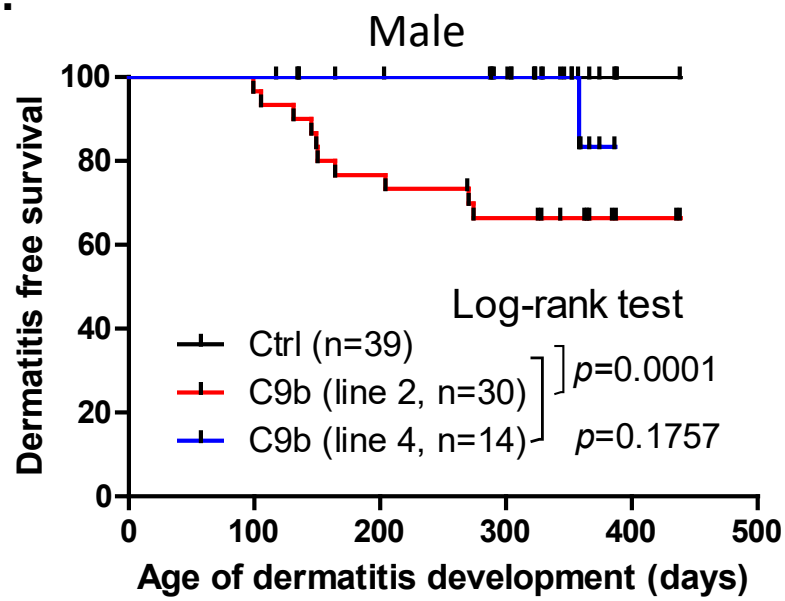
Suppl Fig. S4

Supplementary Fig. S4. A. H&E (5X) and immunohistochemical staining with indicated antibodies (10X) to detect each immune cell type present in lungs of control vs. C9b mice. **B.** FACS analyses of lungs and spleens of control and C9b mice show increased effector/effector memory cells both in CD4+ and CD8+ T cell populations. **C.** Immunohistochemical staining with FLAG antibody to show FLAG-tagged C9b is not expressed in the skin of C9b mice (20x).

A.



B.

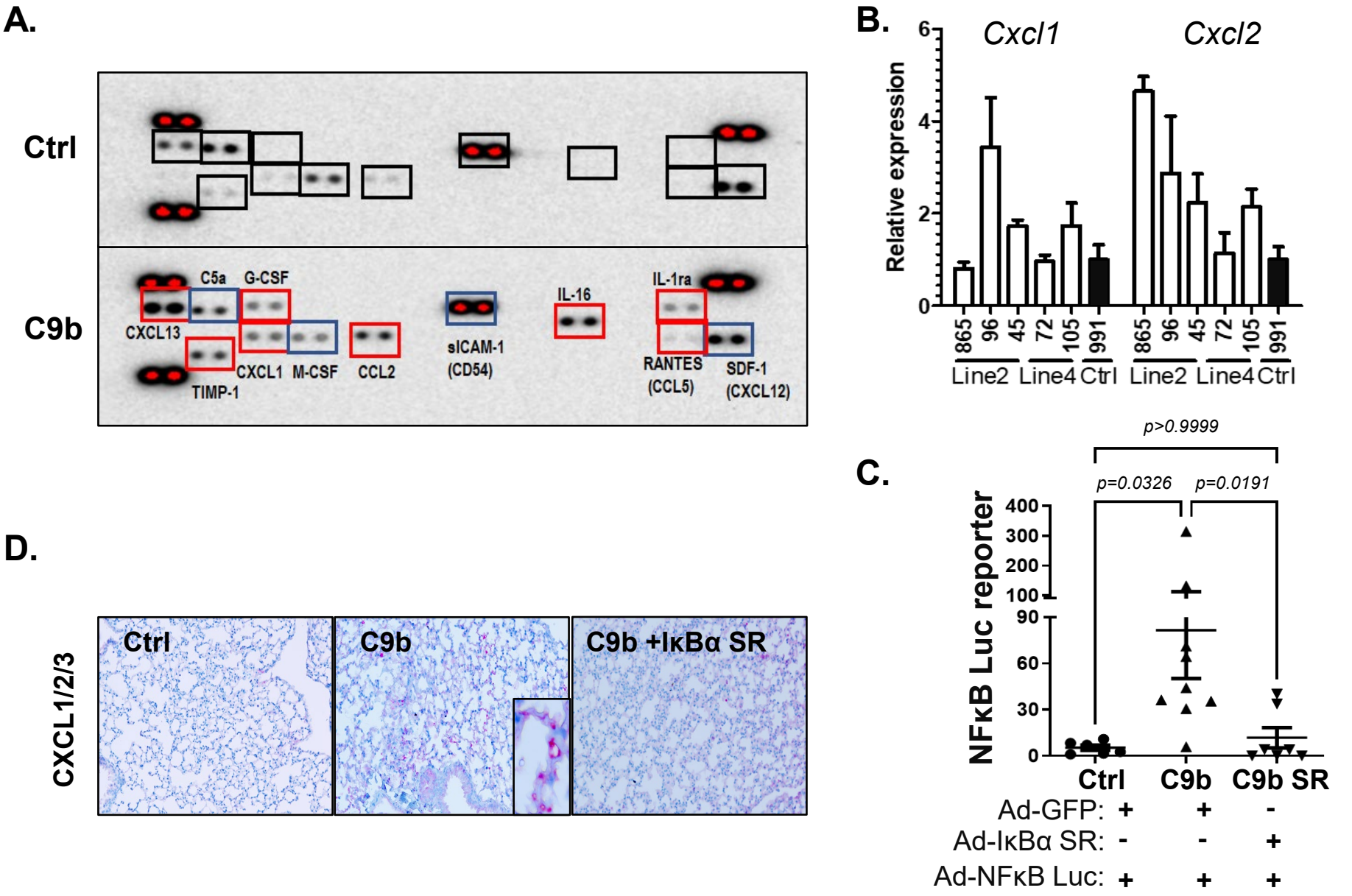


C.

	ALL		Female		Male	
Ctrl	n=0/68 (0%)		n=0/29 (0%)		n=0/39 (0%)	
Line 2	n=26/74 (35%)	*p<0.0001*	n=16/44 (36.4%)	p=0.0012	n=10/30 (33.3%)	p=0.0001
Line 4	n=5/36 (13.9%)	*p=0.0029*	n=4/22 (18.2%)	p=0.0270	n=1/14 (7%)	p=0.1757

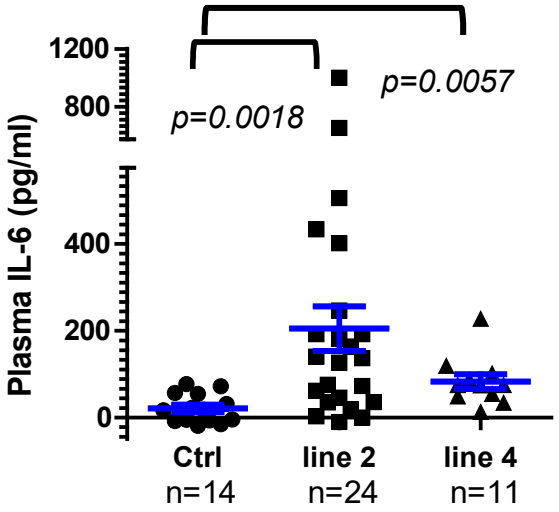
Suppl Fig. S5

Supplementary Fig. S5. A-B. Kaplan-Meier dermatitis free survival curves for female (**A**) and male (**B**) mice for each group. **C.** A summary table of dermatitis-free survival for each group for female, male and all combined mice. *p* values (log-rank test) compared to the control group are shown.

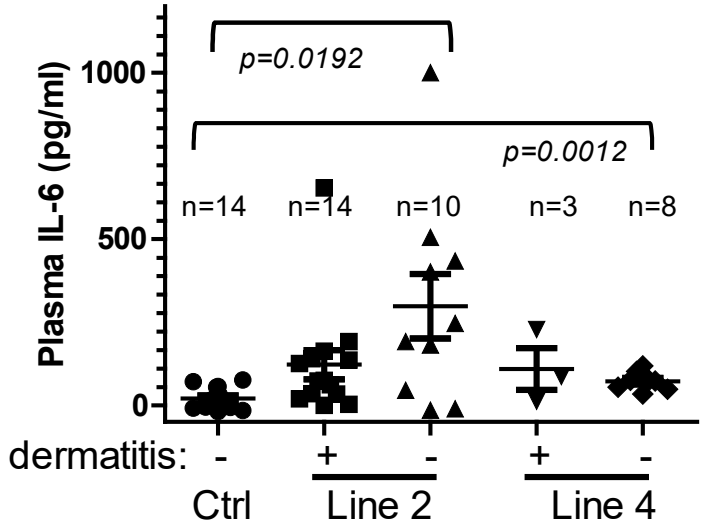

A.
Ctrl
C9b
C5a
G-CSF
CXCL13
CXCL1
M-CSF
CCL2
TIMP-1
sICAM-1
(CD54)
IL-16
IL-1ra
RANTES
(CCL5)
SDF-1
(CXCL12)
B.
Cxcl1
Cxcl2
Relative expression
6
4
2
0
865
96
45
72
105
991
Line2
Line4
Ctrl
C.
p>0.9999
p=0.0326
p=0.0191
NFκB Luc reporter
400
300
200
100
90
60
30
0
Ctrl
C9b
C9b SR
Ad-GFP: + + -
Ad-IκBα SR: - - +
Ad-NFκB Luc: + + +
D.
CXCL1/2/3
Ctrl
C9b
C9b +IκBα SR

Suppl Fig. S6

E.



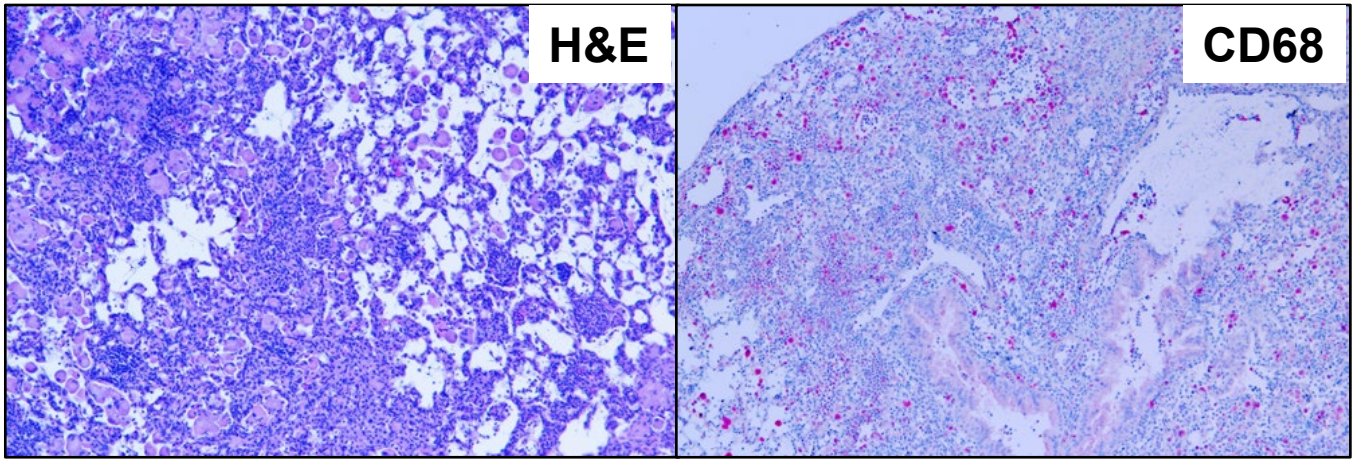
F.



Suppl Fig. S6

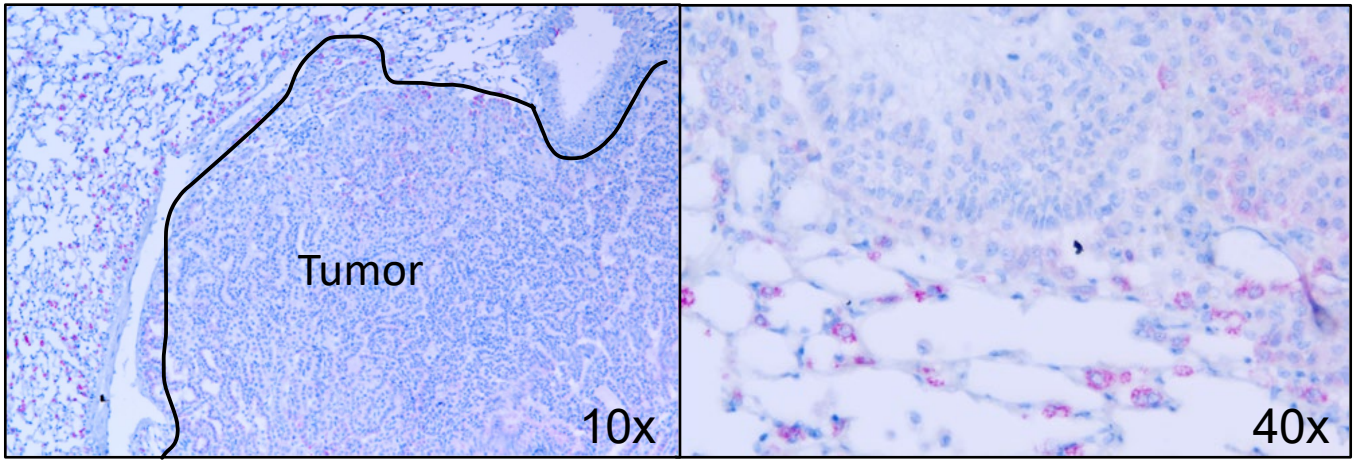
Supplementary Fig. S6. A. Scanned images of mouse cytokine arrays with plasma samples pooled from 4 mice in each group. Cytokines/chemokines with increased signal in C9b mice are marked with red box and ones with decreased signal in blue quantified by image J. **B.** Relative mRNA level of *Cxcl1* and *Cxcl2* in lungs of Line 2 and Line 4 C9b mice normalized against C9b negative control measured by real-time qRT-PCR (ddCt method). **C.** NF-κB promoter activity measured by luciferase activity (Promega) on day 7 following intratracheal injection of Ad-NF-κB reporter with Ad-GFP control or Ad-IκBα supper repressor (SR) (Vector BioLabs, all 2.5X10^7 PFU per mouse) into control or C9b mice. **D.** Immunohistochemical staining of CXCK1/2/3 (Groα/β/γ, Santacruz biotechnology) of lungs of control (left) and C9b (middle) mice with control Ad-GFP and C9b (right) with Ad- IκBα SR. Inset in the middle panel shows staining in the lung epithelial cells. **E-F.** Plasma levels of IL-6 (pg/ml) were measured by ELISA in Control, Line 2, and Line 4 mice. Data are means ± SEM from 2 independent experiments.

A.



B.

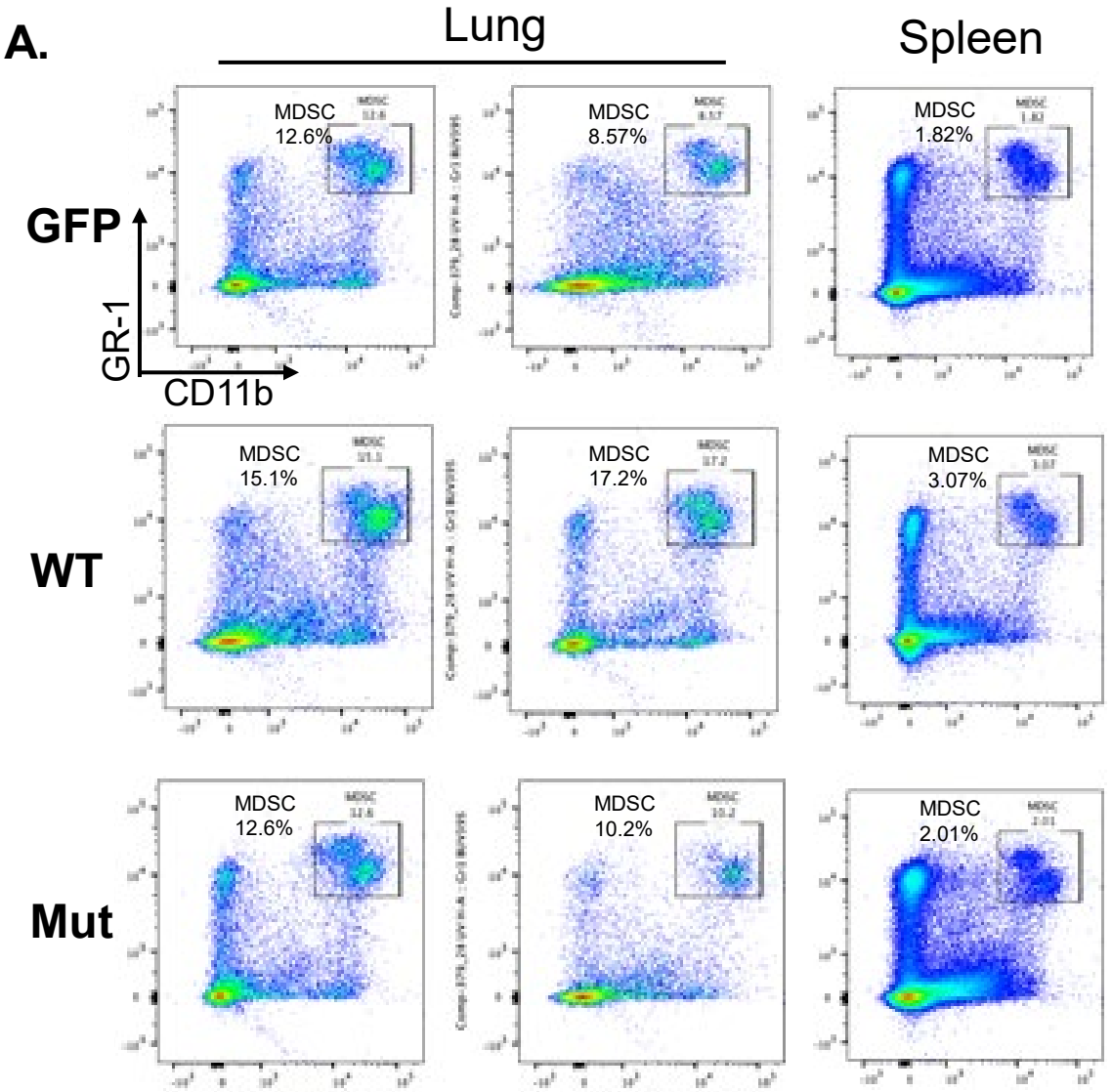
IL6



Suppl Fig. S7

Supplementary Fig. S7. A. Lungs of C9b mice show features similar to those observed in smoking-related lung diseases with severe inflammation associated with increased intra-alveolar infiltration of macrophages (CD68+) and lymphocytes (n=2/20 in line 2 and n=1/13 in line 4 mice). **B.** Immunohistochemical staining with anti-IL6 antibody showed strong IL6 signal in type II pneumocytes but not in tumor cells.

A.



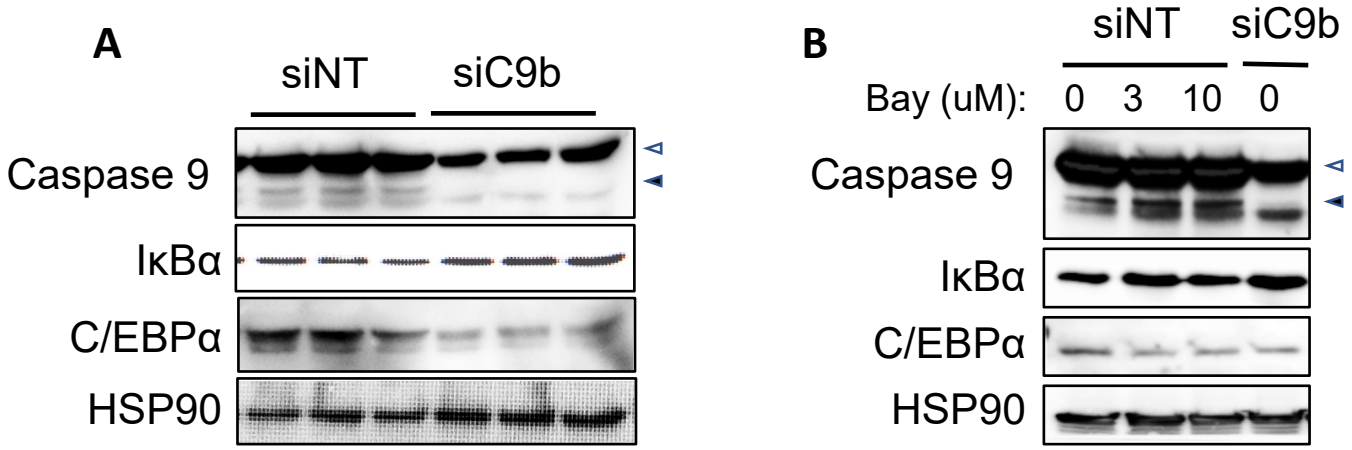
B.

CD11b+ Gr1+ MDSCs (%)

	Lung	Spleen
GFP	12.6 8.57	1.82
WT	15.1 17.2	3.07
Mut	12.6 10.2	2.01

Suppl Fig. S8

Supplementary Fig. S8 FACS analyses of lungs and spleens of *KRAS* $^{G12D\ L/+}$ mice with intratracheal injection of lentiviral particles containing GFP-, WT-, or mutant C9b-Cre(T2a)Luc. CD11b+ Gr1+ MDSCs are increased in the lungs of these mice by WT C9b expression compared to GFP control or Mut C9b expression.


A
siNT
siC9b
Caspase 9
IκBα
C/EBPα
HSP90
B
siNT
siC9b
Bay (uM):
0
3
10
0
Caspase 9
IκBα
C/EBPα
HSP90

Suppl Fig. S9

Supplementary Fig. S9. Western blot analyses of A549 cells transfected with siNT or siC9b with indicated antibodies. **A.** Reduced Caspase 9b expression via siRNA (siC9b) reduced C/EBPα level in A549 cells compared to siNT (non-targeting) control. **B.** NF-κB/IKK inhibitor Bay 11-7082 treatment reduced C/EBPα level in A549 cells. The empty and filled triangles denote full-length C9a and C9b proteins, respectively

SUPPLEMENTARY REFERENCES

1. Geiling B, Vandal G, Posner AR, de Bruyns A, Dutchak KL, Garnett S*, et al.* A modular lentiviral and retroviral construction system to rapidly generate vectors for gene expression and gene knockdown in vitro and in vivo. PLoS One **2013**;8:e76279
2. Vu NT, Park MA, Shultz MD, Bulut GB, Ladd AC, Chalfant CE. Caspase-9b Interacts Directly with cIAP1 to Drive Agonist-Independent Activation of NF-kappaB and Lung Tumorigenesis. Cancer Res **2016**;76:2977-89
3. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet **2001**;357:539-45
4. Sung H, Kanchi KL, Wang X, Hill KS, Messina JL, Lee JH*, et al.* Inactivation of RASA1 promotes melanoma tumorigenesis via R-Ras activation. Oncotarget **2016**;7:23885-96
5. Vu NT, Park MA, Shultz JC, Goehe RW, Hoeferlin LA, Shultz MD*, et al.* hnRNP U enhances caspase-9 splicing and is modulated by AKT-dependent phosphorylation of hnRNP L. J Biol Chem **2013**;288:8575-84

Supplemental table 1. List of key reagents and resoruces

Antibodies

Target	Vendor	Cat #	RRID	Usage
Caspase 9	Enzo Life Sciences	AAM-149E	RRID:AB_311717	WB
Caspase 9	Cell Signaling Technology	9508	RRID:AB_2068620	IHC
CXCL1/2/3	Santa Cruz Biotechnology	sc-365870	RRID:AB_10859555	IHC, WB
FLAG	Sigma	F1804	RRID:AB_262044	WB, IHC
Gr1 (Ly6G/6C)	Novus Biological	NBP2-00441	RRID:AB_2909793	IHC
HMGA2	Thermo Fisher Scientific	PA5-21320	RRID:AB_11155799	IHC
HSP90	Cell Signaling Technology	4877	RRID:AB_2233307	WB
IL6	Thermo Fisher Scientific (Bioss)	BS-0782R	RRID:AB_10859871	IHC
IκBα	Cell Signaling Technology	4814	RRID:AB_390781	WB, IHC
Myc-Tag	Cell Signaling Technology	2276	RRID:AB_331783	WB, IHC
p63	Santa Cruz Biotechnology	sc-25268	RRID:AB_628092	IHC
pERK	Cell Signaling Technology	4370	RRID:AB_2315112	WB, IHC
RIP1	Cell Signaling Technology	3493	RRID:AB_2305314	WB, IP
SOX2	Sigma (Millipore)	AB5603	RRID:AB_2286686	IHC
SP-C	Santa Cruz Biotechnology	sc-13979	RRID:AB_2185502	IHC
Synaptophysin	Santa Cruz Biotechnology	sc-55507	RRID:AB_630273	IHC
β-actin	Cell Signaling Technology	3700	RRID:AB_2242334	WB

Cell lines

Name	Vendor	Cat #	RRID
HBEC3-KT	ATCC	CRL-4051	RRID:CVCL_X491
MLE12	ATCC	CRL-2110	RRID:CVCL_3751
A549	ATCC	CCL-185	RRID:CVCL_0023
293T	ATCC	CRL-3216	RRID:CVCL_0063

Tools

Name	RRID
Image J	RRID:SCR_003070
FlowJo	RRID:SCR_008520
GraphPad Prism	RRID:SCR_002798

Experimental Models: Organisms/Strains

Name	RRID
KRAS G12D L/+ mice	RRID:IMSR_JAX:008179

Plasmids

Name	RRID
pLEX304	RRID:Addgene_25890
pBEG R2-iCre T2A Luciferase-L3	RRID:Addgene_48992
pLEG R1-R3	RRID:Addgene_48956
psPAX2	RRID:Addgene_12260
pMD2.G	RRID:Addgene_12259

Abbreviations

WB: western blot, IHC: immunohistochemistry, IP: immunoprecipitation

ATCC: American Type Culture Collection