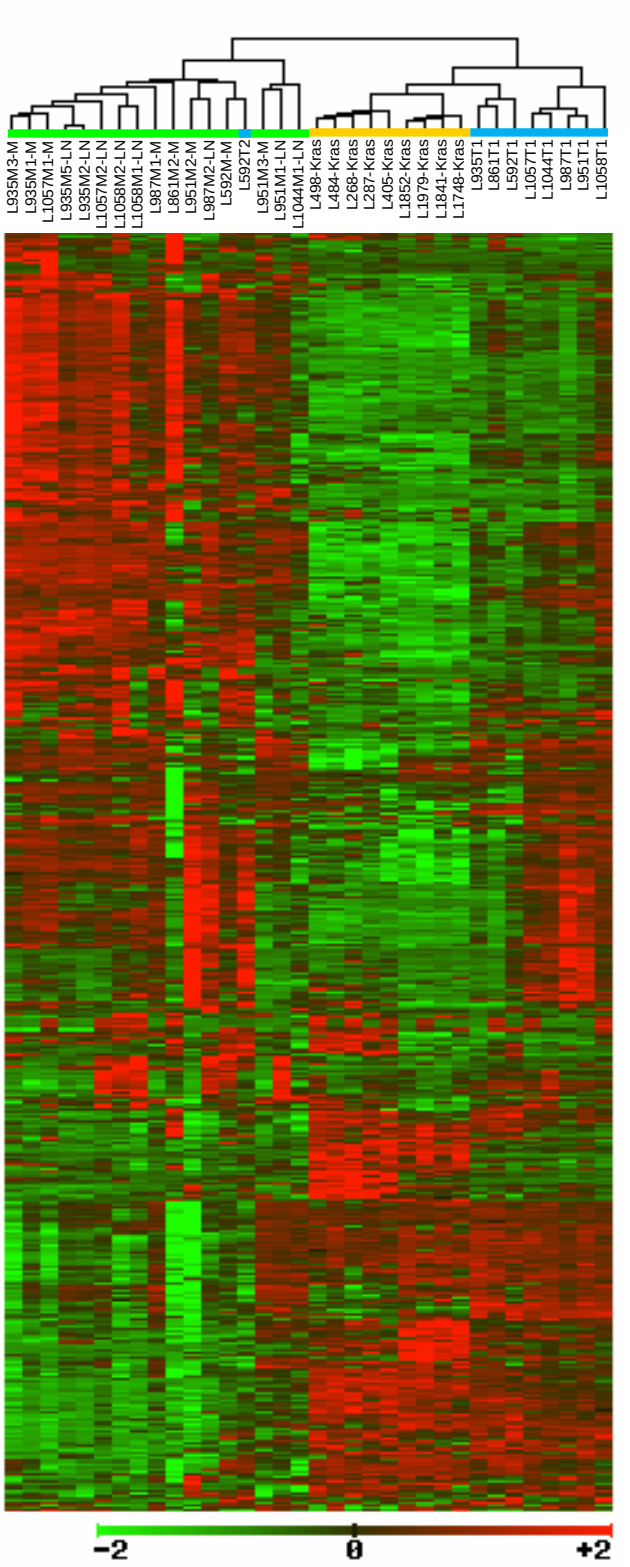
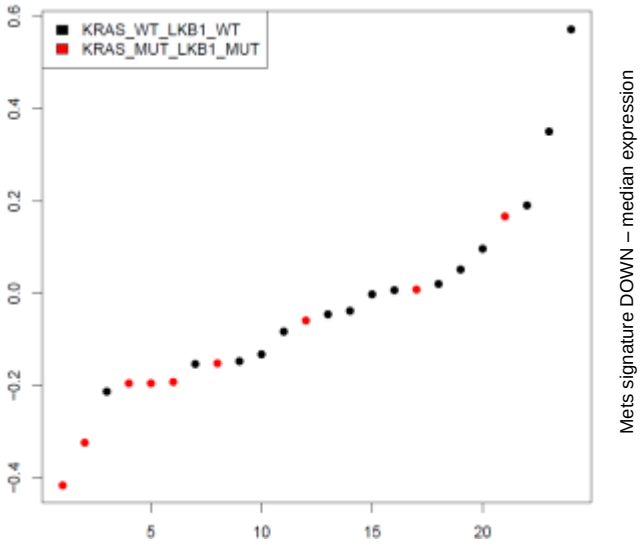
Inventory of Supplemental Information.

Supplemental Figures

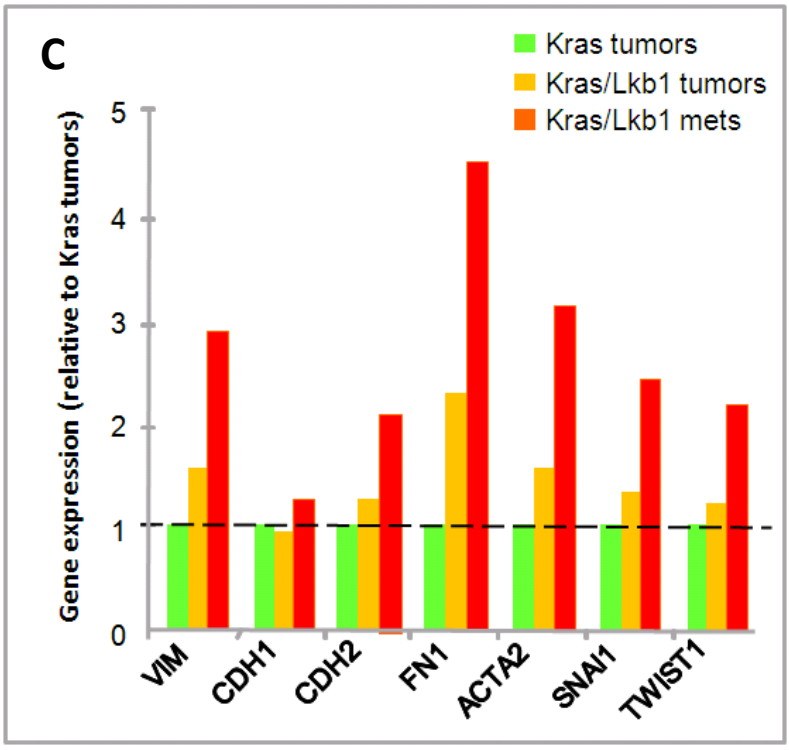


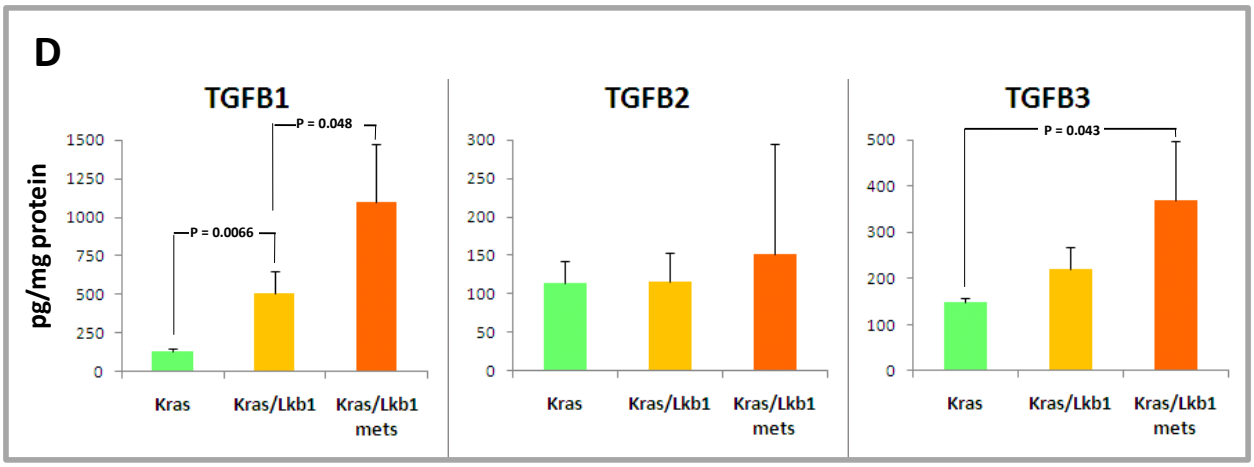
A



B

sion


C
Kras tumors
Kras/Lkb1 tumors
Kras/Lkb1 mets
Gene expression (relative to Kras tumors)
0
1
2
3
4
5
VIM
CDH1
CDH2
FN1
ACTA2
SNAI1
TWIST1


D
TGFB1
TGFB2
TGFB3
pg/mg protein
P = 0.048
P = 0.0066
P = 0.043
Kras
Kras/Lkb1
Kras/Lkb1 mets

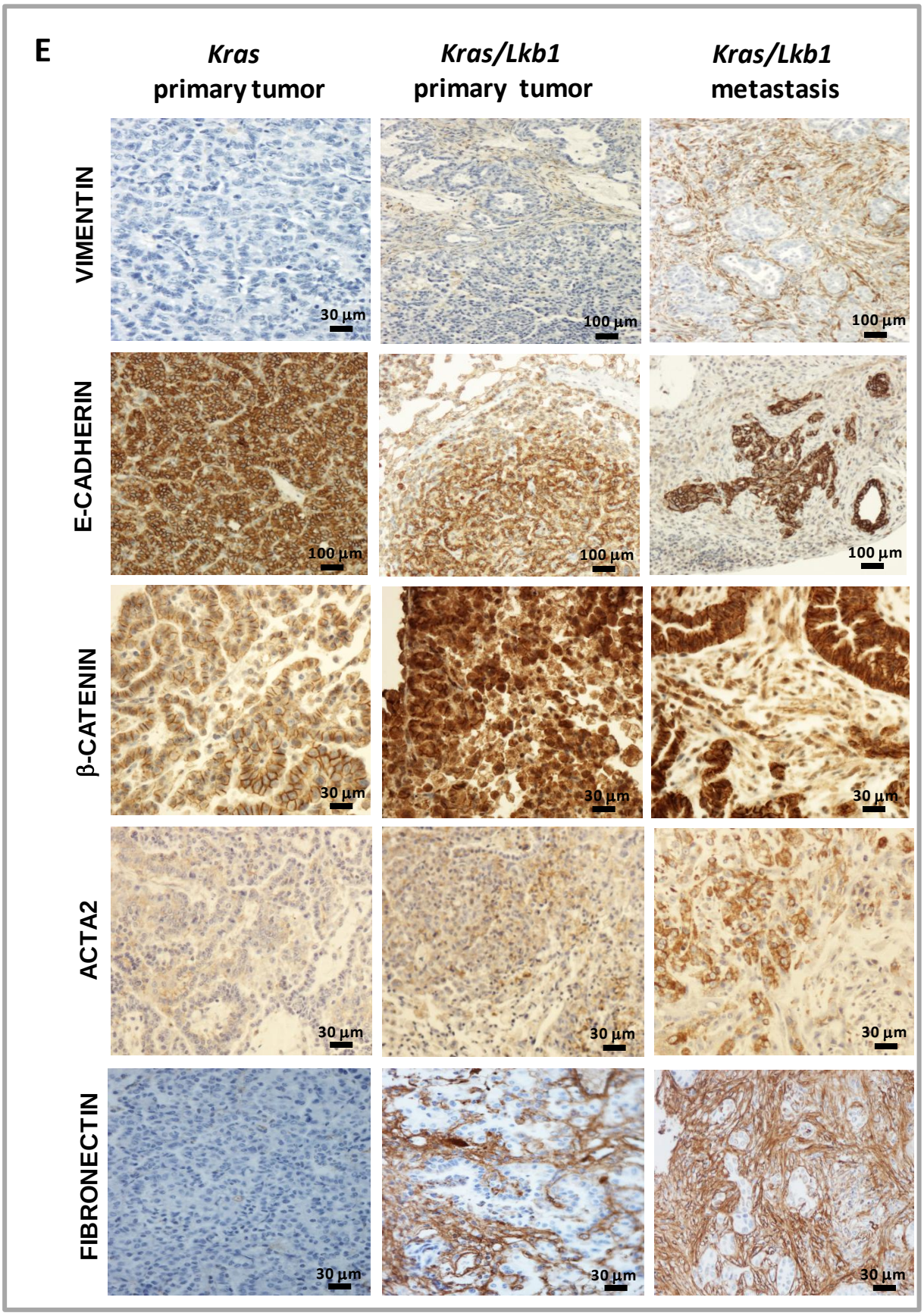

E
Kras
primary tumor
Kras/Lkb1
primary tumor
Kras/Lkb1
metastasis
VIMENTIN
E-CADHERIN
β-CATENIN
ACTA2
FIBRONECTIN
30 µm
100 µm

Figure S1. Related to Figure 1.

A. Gene expression profiles of *Kras* tumors, *Kras/Lkb1* primary tumors as well as *Kras/Lkb1* metastasis were filtered to exclude probesets with signals present at background noise levels, and for probesets that do not vary significantly across samples. Genes and tumors were clustered using average linkage method with the uncentered correlation metric using Gepas analysis tool (www.gepas.org).

B. Correlation study of *KRAS* and *LKB1* status and murine Mets signatures in human lung adenocarcinoma samples. Downregulated genes in murine *Kras/Lkb1* metastases ("Mets DOWN" signature) showed a significant correlation with the human samples that are mutant for both *KRAS* and *LKB1* (p=0.029). No significant correlation was found for upregulated genes (data not shown).

C. Total RNA from *Kras* tumors, *Kras/Lkb1* primary tumors and *Kras/Lkb1* metastases (n=5) were reverse-transcribed and then analyzed using RT-PCR and TaqMan probes for indicated transcripts. Glyceraldhyde-3-phosphate dehydrogenase (GAPDH) was used as the reference control. Fold changes were expressed relative to *Kras* primary tumors.

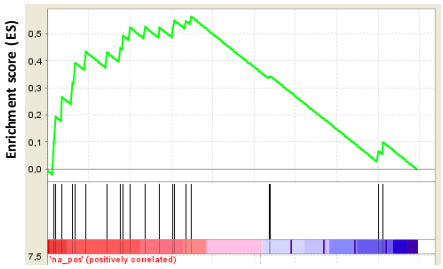
D. TGF-β multiplex bead analysis. Protein lysates of *Kras* primary tumors, *Kras/Lkb1* primary tumors and *Kras/Lkb1* metastases (n=5) were incubated with beads coated with specific antibodies for TGFB1, TGFB2 and TGFB3. Data is graphed as mean pg/100 mg of total protein ± SD.

E. Expression of EMT protein markers in murine tumors. Representative photomicrographs of sections of *Kras* primary tumors, *Kras/Lkb1* primary tumors and *Kras/Lkb1* metastases immunohistochemically stained with the indicated antibodies.

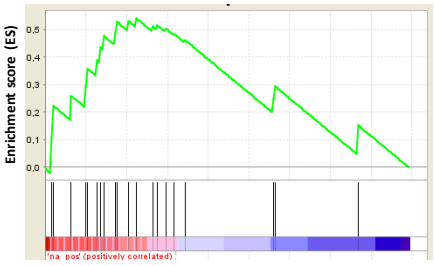
A

GS follow link to MSigDB	GS DETAILS	SIZE	ES	NES	NOM p-val	FDR q-val
SRC	Details ...	20	0.54	1.88	0.000	0.030
INSR	Details ...	8	0.58	1.60	0.000	0.275
EGFR	Details ...	14	0.47	1.57	0.038	0.223
PDGFRA	Details ...	7	0.65	1.52	0.054	0.214
LCK	Details ...	8	0.51	1.41	0.075	0.301
MET	Details ...	6	0.61	1.38	0.114	0.289
IGF1R	Details ...	5	0.64	1.38	0.152	0.254
PDGFRB	Details ...	8	0.50	1.25	0.214	0.348
PTK2	Details ...	13	0.41	1.18	0.261	0.401
ERBB2	Details ...	7	0.52	1.15	0.313	0.379
KIT	Details ...	7	0.42	1.00	0.457	0.550
ABL1	Details ...	6	0.42	0.91	0.571	0.692
JAK2	Details ...	7	0.36	0.90	0.585	0.659
RET	Details ...	8	0.29	0.72	0.818	0.895
FYN	Details ...	7	0.21	0.60	1.000	0.983

***Kras/Lkb1* vs *Kras* tumors (FDR = 0.234)**



***Kras/Lkb1* mets vs *Kras* tumors (FDR = 0.03)**



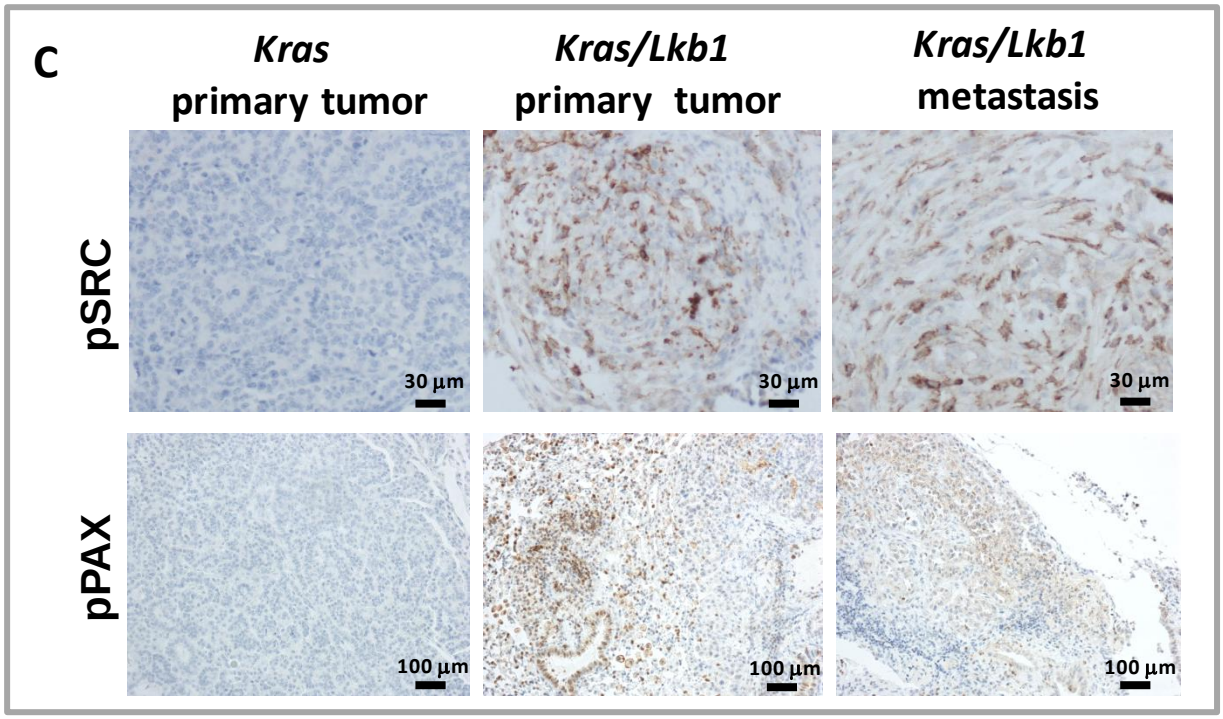
B

Phosphoproteins deregulated in *Kras/Lkb1* compared to *Kras* primary tumors

KEGG pathway	Protein
Leukocyte transendothelial migration	PXN, F11R, PIK3R1, PTK2, PTPN11, CLDN3, PECAM1, CLDN18
Jak-STAT signaling pathway	STAT3, STAT5A, STAT5B, PIK3R1, PTPN11, JAK1
ErbB signaling pathway	SRC, STAT5A, STAT5B, PIK3R1, PTK2, ABL1, SHC1
Tight junction	SRC, F11R, CLDN3, PRKCD, MYH9, CLDN18
Adipocytokine signaling pathway	STAT3, PTPN11, JAK1
Cell adhesion molecules (CAMs)	F11R, CLDN3, PECAM1, CLDN18
Focal adhesion	PXN, SRC, PIK3R1, PTK2, SHC1, ACTN1, TLN1, VCL
VEGF signaling pathway	PXN, SRC, PIK3R1, PTK2
Regulation of actin cytoskeleton	PXN, PIK3R1, PTK2, ACTC1, MYH9

Phosphoproteins deregulated in *Kras/Lkb1* mets compared to *Kras/Lkb1* primary tumors

KEGG pathway	Protein
ErbB signaling pathway	SRC, STAT5A, STAT5B, PTK2, ABL1, MAPK3, SHC1, MAPK1
Insulin signaling pathway	MAPK3, TSC1, SHC1, MAPK1
Regulation of actin cytoskeleton	PTK2, MAPK3, ACTC1, MYH10, MAPK1
Leukocyte transendothelial migration	CDH5, F11R, PTK2, PTPN11, CLDN3, PECAM1, CLDN18, NOX3
Natural killer cell mediated cytotoxicity	MAPK3, PTPN11, SHC1, MAPK1
Jak-STAT signaling pathway	STAT3, STAT5A, STAT5B, PTPN11, JAK1
Gap junction	SRC, MAPK3, TJP1, MAPK1
Tight junction	SRC, F11R, CLDN3, TJP1, MYH6, MYH10, CLDN18
Adherens junction	SRC, MAPK3, TJP1, LMO7, MAPK1
Cell adhesion molecules (CAMs)	CDH5, F11R, CLDN3, PECAM1, CLDN18
Acute myeloid leukemia	STAT3, STAT5A, STAT5B, MAPK3, CEBPA, MAPK1
Focal adhesion	SRC, PTK2, MAPK3, SHC1, MAPK1
VEGF signaling pathway	SRC, PTK2, MAPK3, MAPK1
Axon guidance	PTK2, ABL1, MAPK3, MAPK1
Dorso-ventral axis formation	MAPK3, ETS1, MAPK1
mTOR signaling pathway	MAPK3, TSC1, MAPK1


C
Kras
primary tumor
Kras/Lkb1
primary tumor
Kras/Lkb1
metastasis
pSRC
pPAX
30 µm
100 µm

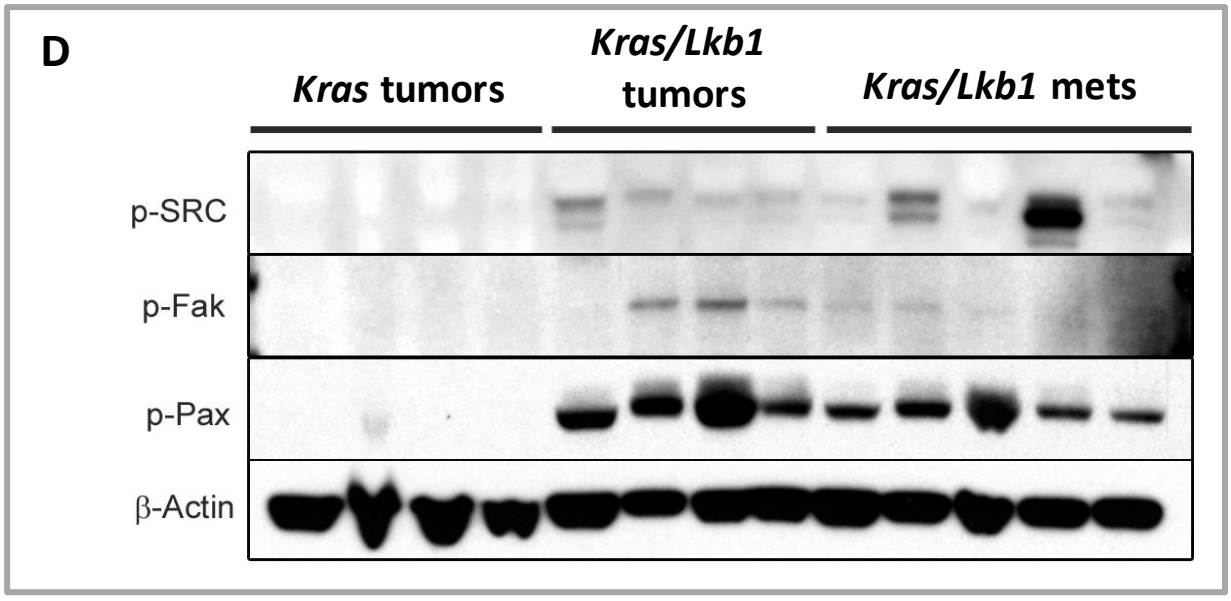

D
Kras tumors
Kras/Lkb1
tumors
Kras/Lkb1 mets
p-SRC
p-Fak
p-Pax
β-Actin

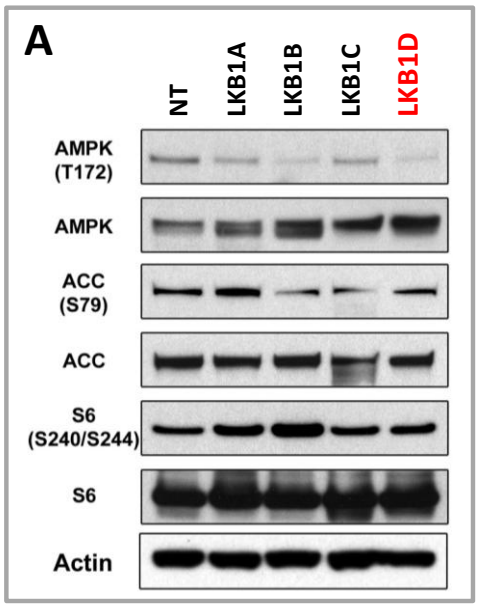
Figure S2. Related to Figure 2. Phosphoprotein profiling of murine tumors.

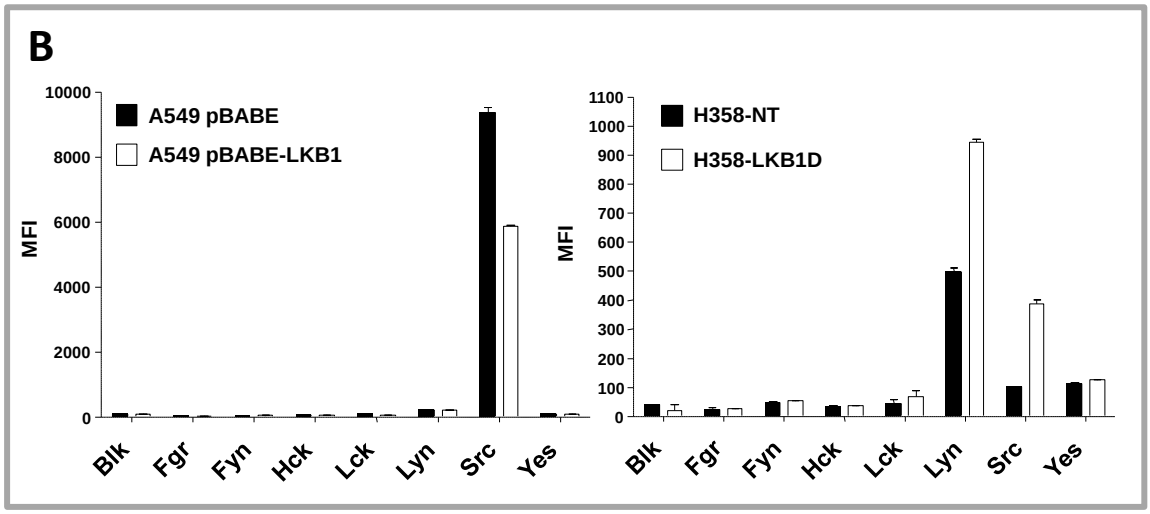
A. GSEA analysis of tyrosine kinase substrates sets confirms a significant enrichment of SRC substrates in *Lkb1* deficient murine tumors. Gene set enrichment analysis method was adapted to evaluate the enrichment of tyrosine kinase substrates sets in the murine phosphoprotein dataset. Log_2 ratios of *Kras/Lkb1* primary tumors versus *Kras* primary tumors and *Kras/Lkb1* metastases versus *Kras* primary tumors were calculated, ranked and loaded in GSEA program (www.broadinstitute.org/gsea). Left table: enrichment scores (ES), statistical significance of the enrichment (NOM p-val) and multiple hypothesis testing adjustment (FDR q-val) for the substrate sets tested. Right plots: location of the maximum enrichment score (ES) and the leading-edge subsets of Src substrates in *Kras/Lkb1* primary versus *Kras* tumors (detectable albeit not statistically significant enrichment) and *Kras/Lkb1* metastases versus *Kras* primary tumors (statistically significant enrichment, FDR<0.05).

B. KEGG classification of deregulated phosphotyrosine proteins in *Kras/Lkb1* tumors and metastases. Phosphoproteins that changed at least two fold or more in indicated comparisons, were classified into KEGG pathways using FatiGO software (www.babelomics.org).

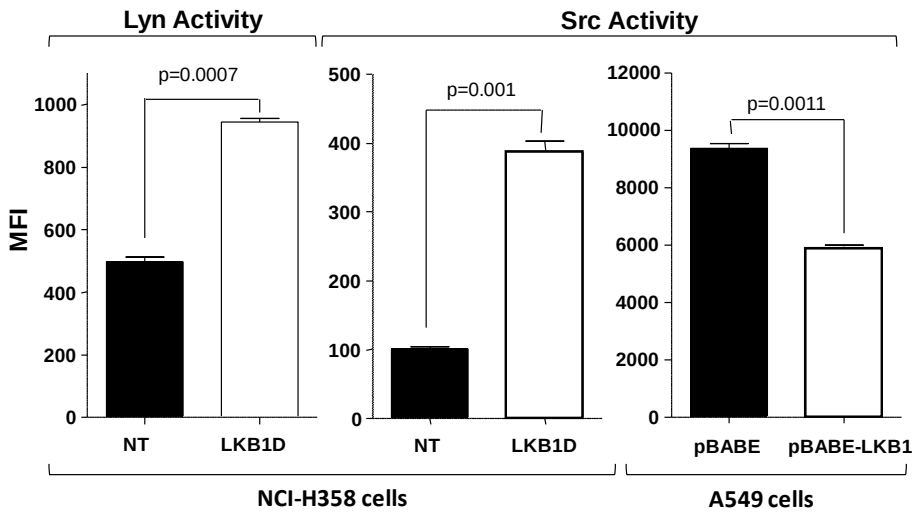
C. Src/FAK tyrosine hyperphosphorylation in *Kras/Lkb1* primary tumors and metastasis analyzed by immunohistochemistry. Representative photomicrographs of sections of *Kras* primary tumors, *Kras/Lkb1* primary tumors and *Kras/Lkb1* metastases immunohistochemically stained with the indicated phosphotyrosine specific antibodies for Src (Y416) and paxillin (Y118).

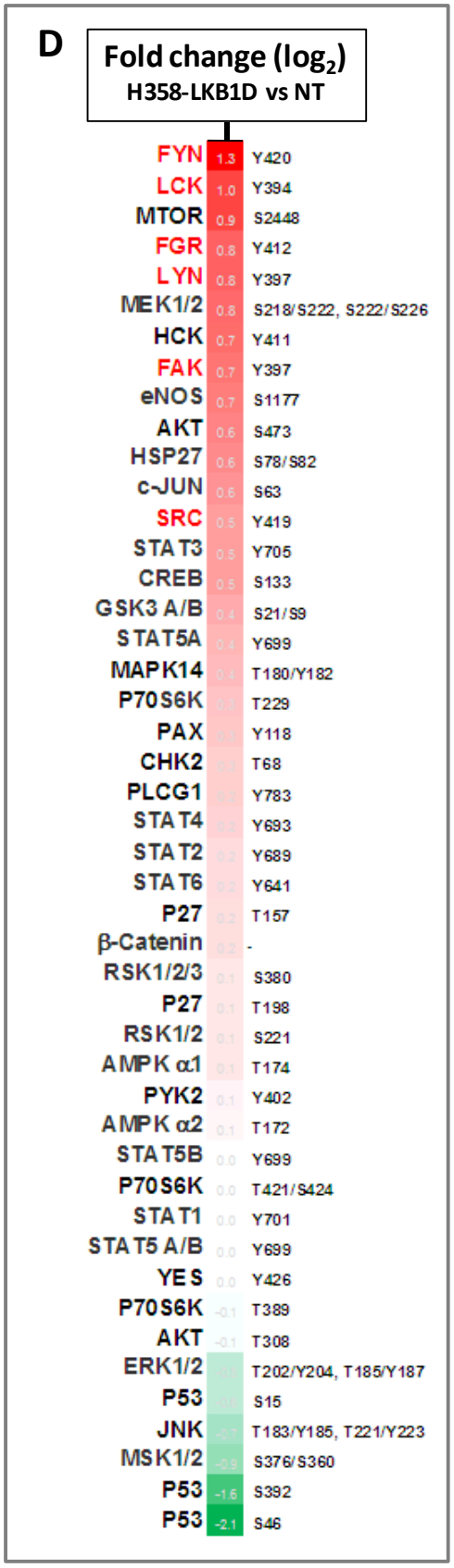
D. Tyrosine phosphorylation of Src, FAK and paxillin in *Kras/Lkb1* murine tumors. Whole cell extracts of *Kras* primary tumors, *Kras/Lkb1* primary tumors, and *Kras/Lkb1* metastases were immunoblotted with the indicated antibodies specific for Src (Y416), FAK (Y576/577), and paxillin (Y118), and β-Actin.

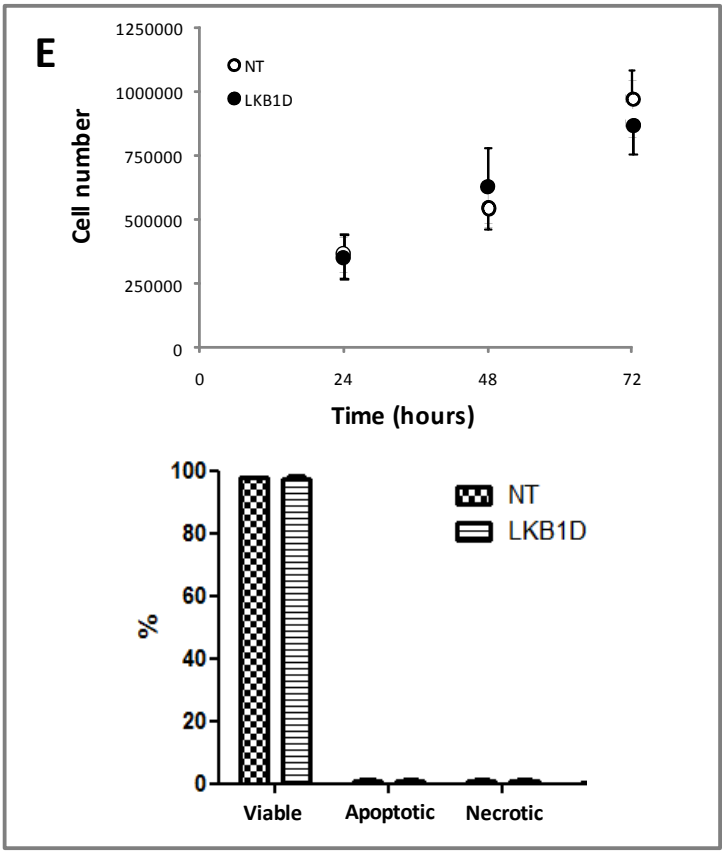

A
NT
LKB1A
LKB1B
LKB1C
LKB1D
AMPK (T172)
AMPK
ACC (S79)
ACC
S6 (S240/S244)
S6
Actin

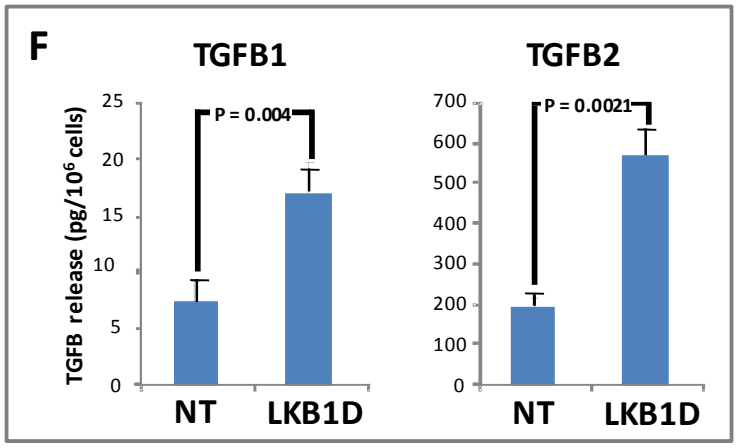

B
A549 pBABE
A549 pBABE-LKB1
MFI
10000
8000
6000
4000
2000
0
Blk
Fgr
Fyn
Hck
Lck
Lyn
Src
Yes
H358-NT
H358-LKB1D
MFI
1100
1000
900
800
700
600
500
400
300
200
100
0
Blk
Fgr
Fyn
Hck
Lck
Lyn
Src
Yes

C


Lyn Activity
Src Activity
MFI
p=0.0007
p=0.001
p=0.0011
1000
800
600
400
200
0
500
400
300
200
100
0
12000
10000
8000
6000
4000
2000
0
NT
LKB1D
NT
LKB1D
pBABE
pBABE-LKB1
NCI-H358 cells
A549 cells


D
Fold change (log2)
H358-LKB1D vs NT
FYN 1.3 Y420
LCK 1.0 Y394
MTOR 0.9 S2448
FGR 0.8 Y412
LYN 0.8 Y397
MEK1/2 0.8 S218/S222, S222/S226
HCK 0.7 Y411
FAK 0.7 Y397
eNOS 0.7 S1177
AKT 0.6 S473
HSP27 0.6 S78/S82
c-JUN 0.6 S63
SRC 0.5 Y419
STAT3 0.5 Y705
CREB 0.5 S133
GSK3 A/B 0.4 S21/S9
STAT5A 0.4 Y699
MAPK14 0.4 T180/Y182
P70S6K 0.3 T229
PAX 0.3 Y118
CHK2 0.2 T68
PLCG1 0.2 Y783
STAT4 0.2 Y693
STAT2 0.2 Y689
STAT6 0.2 Y641
P27 0.2 T157
β-Catenin 0.2 -
RSK1/2/3 0.1 S380
P27 0.1 T198
RSK1/2 0.1 S221
AMPK α1 0.1 T174
PYK2 0.1 Y402
AMPK α2 0.1 T172
STAT5B 0.0 Y699
P70S6K 0.0 T421/S424
STAT1 0.0 Y701
STAT5 A/B 0.0 Y699
YES 0.0 Y426
P70S6K -0.1 T389
AKT -0.1 T308
ERK1/2 -0.7 T202/Y204, T185/Y187
P53 -0.7 S15
JNK -0.7 T183/Y185, T221/Y223
MSK1/2 -0.9 S376/S360
P53 -1.6 S392
P53 -2.1 S46


E
Cell number
1250000
1000000
750000
500000
250000
0
NT
LKB1D
0
24
48
72
Time (hours)
%
100
80
60
40
20
0
NT
LKB1D
Viable
Apoptotic
Necrotic


F
TGFB1
TGFB2
TGFB release (pg/10^6 cells)
25
20
15
10
5
0
P = 0.004
700
600
500
400
300
200
100
0
P = 0.0021
NT
LKB1D
NT
LKB1D

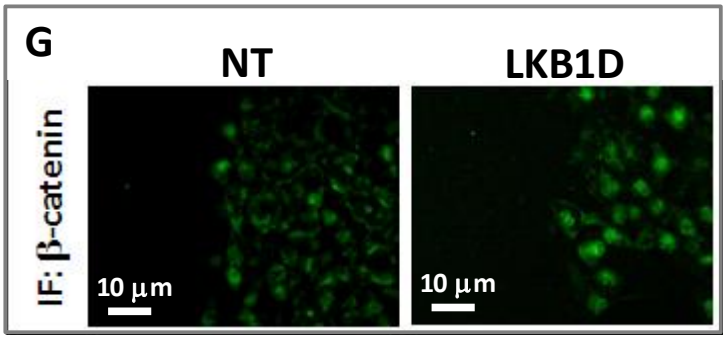

G
NT
LKB1D
IF: β-catenin
10 µm
10 µm

Figure S3. Related to Figure 4.

A. Western blot of protein lysates from NCI-H358 cells transduced with non-targeting (NT) or *LKB1* shRNA (LKB1D) were performed with specified antibodies (ACC = Acetyl-CoA carboxylase).

B. Tyrosine phosphorylation status of SRC family members were carried out in NCI-H358 cells stably expressing shRNA to LKB1 (LKB1D) or a non-specific shRNA (NT), and A549 cells stably expressing pBabe empty or pBabe-LKB1 wild type as described in Methods. Data is graphed as mean of 3 replicates ± SD.

C. Magnification of histograms shown in B, focused on predominant SRC family members in each cell line.

D. Phosphoprotein profile of NCI-H358 cells analyzed by protein arrays.

NCI-H358 cells stably expressing shRNA to *LKB1* (LKB1D) or a non-specific shRNA (NT) were subjected to a kinase array as described in materials in methods. Densitometric analysis was performed and normalized for background and array lot variations. Differences were expressed as Log_2 ratio and depicted as a heatmap, demonstrating the activation of Src family kinases and FAK, as well as MEK and AKT, in *LKB1* knockdown cells. In each array, each kinase was assayed in duplicate (two dots).

E. Cell proliferation and cell death in NCI-H358 lung cancer cells. NCI-H358 cells transduced with non-targeting (NT) or *LKB1* shRNA (LKB1D) were counted every 24 hours during 3 days. Annexin V and propidium iodide staining of apoptotic and/or necrotic cells were performed at 72 hours. Data is graphed as mean of 3 replicates ± SD.

F. TGF-β release analyzed by multiplex bead assay. *In vitro* secretion of TGFB1, TGFB2 and TGFB3 (below detection limit, not shown) were carried out in NCI-H358 cells stably expressing shRNA to *LKB1* (LKB1D) or a non-specific shRNA (NT). Conditioned media were collected after

24h of cell culture and incubated with beads coated with specific antibodies for TGFB1, TGFB2 and TGFB3 as described in Methods. Data is graphed as mean of 3 replicates (pg/10^6 cells ± SD). **G.** β-catenin immunostaining in NCI-H358 lung cancer cells. Confluent monolayers of NCI-H358 cells transduced with non-targeting (NT) or *LKB1* shRNA (LKB1D) were scratched, incubated for 12 hours and fixed and stained with a specific antibody for activated β-catenin as described in materials and methods. Representative photographs captured with a fluorescence microscope showed nuclear localization of β-catenin in NCI-H358 lacking *LKB1*.

Supplemental Tables

Table S1. Related to Figure 1. Gene sets used in gene set enrichment analysis.

See Excel file

Table S2. Related to Figure 6. Histological evaluation of *Kras/Lkb1* mice treated with Dasatinib.

	n	Lymph node/distant metastases
Untreated mice	10	7 (70%)
Dasatinib treated mice	5	0
***P* value**		0.026

Table S3. Related to Figure 6.

GSEA analysis of primary tumors from Kras/Lkb1 mice daily treated with Dasatinib, AZD/BEZ or AZD/BEZ/Dasatinib for 48 hours with doses indicated in Figure 6. Columns show enrichment results for each comparison. A positive or negative enrichment score (NES) represent respectively an up or down-regulation of the gene signature in the first class for each comparison.

See Excel file

Supplemental experimental procedures

Gene expression profiling

Total RNA from the primary tumors and metastases was extracted by Trizol (Invitrogen) and purified with an RNeasy kit (Qiagen). Synthesis of cRNA and hybridization to Mouse Expression Array 430A 2.0 chips or Mouse Gene 1.0 ST chips were performed following Affymetrix protocols (Affymetrix, Inc.). Probe-level intensity data files in the CEL format were pre-processed using Robust Multichip Average program (rmaexpress.bmbolstad.com). Gene-expression data were filtered using low stringency, pre-defined criteria: probe set intensity (32 in all samples) and dynamic variation (more than twofold over the entire sample set). After filtering, probes representing the same genes were collapsed into a single value, and standardized by taking the median value for each gene across sample set. Unsupervised hierarchical clustering was performed using Gepas Analysis suite (www.gepas.org). Two-sided t-tests were used to determine significant differences in gene expression between mice cohorts. False positives associated with multiple hypothesis testing were calculated with the False Discovery Rate method. Differentially expressed genes were $\log_2$ transformed, mean-centered and depicted using the GenePattern software (genepattern.broadinstitute.org)

Western blot

Whole-cell lysates (WCL) were prepared in lysis buffer (Cell Signaling Technology) supplemented with protease and phosphatase inhibitor cocktails (Calbiochem). Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce)

and equivalent amounts (20 µg) were subjected to SDS-PAGE on 4% to 12% Bis-Tris gradient gels.

Phosphopeptide immunoprecipitation, LC-MS/MS and phosphopeptide analysis.

Soluble protein extracts from cell cultures and tumor tissues were digested with Trypsin, and phosphorylated peptides were isolated using slurries of phosphotyrosine mouse monoclonal immobilized antibody. For LC-MS/MS analysis, mass spectra were collected with an LTQ-Orbitrap hybrid mass spectrometer (Thermo Scientific), and evaluated using TurboSequest in the Proteomics Browser package (v.27, rev.13). Searches were performed against the NCBI human database, released on September 5, 2006 and containing 34,180 protein sequences, in both forward- and reversed-sequence directions. The false positive assignment rate was approximated by taking the ratio of the reversed database assignments to the forward database assignments after filtering the initial SEQUEST search results based on XCorr (≥ 1.5) and mass accuracy (± 10 ppm). Each MS/MS spectrum arises from a parent ion observed during a survey MS scan was linked to the intensity of that parent ion at its chromatographic apex, essentially measuring the abundance of the peptide in the sample. An intensity of 20,000 counts was estimated when the peptide is not detected in a particular sample.

Invasion assay.

Assays were performed in Boyden chambers with matrigel precoated 8-µm pore filter inserts for 24-well plates (Cell Biolabs Inc.). H358 cells ($5x10^5$) in 200 µl of serum-free medium were added to the upper chamber with the filters precoated with

Matrigel. The lower chamber was filled with 300 µl of medium containing 10% fetal bovine serum as attractant. After 48 hours of incubation at 37°C, cells on the underside of the filter were fixed, stained, and extracted with 10% citric acid, and the optical density was measured at 560 nm by spectrophotometer for quantification of cell number.

Cell adhesion assay.

H358 cells were seeded at $5x10^5$ cells in wells coated with collagen I or collagen IV or BSA coated multi-well plates (Cell Biolabs, Inc.). Cells were allowed to adhere to the matrices for 90 minutes, washed three times with PBS with calcium and magnesium and then fixed and stained following manufacturer's instructions.

Magnetic resonance imaging and tumor volume measurement.

MRI was done with a 4.7T Bruker Avance horizontal bore system equipped with a 200-mm inner diameter gradient set capable of 30 G cm^{-1} gradient strength. Tumor-bearing mice were anesthetized with 1% isoflurane in an oxygen-air mixture. Respiratory and cardiac rates of the mice were monitored with Biotrig Software. We compared tumor volumes of mice treated with different therapies using a two-sided Student's exact *t*-test.

Antibodies

Target	Phosphosite	Company	Cat. #	Application	Remarks
LKB1		Cell Signaling	3050	WB	(27D10) Rabbit mAb
Src		Cell Signaling	2108	WB	
Src	Y416	Cell Signaling	2101	WB/IHC	Phospho-Src Family
FAK		Cell Signaling	3285	WB	
FAK	Y576/577	Cell Signaling	3281	WB	
E-Cadherin		Cell Signaling	4065	WB	
Vimentin		BioVision	3634-100	WB	
Paxillin	Y118	Cell Signaling	2541	WB/IHC	
β-Actin		Cell Signaling	4967	WB	
AMPK		Cell Signaling	2603	WB	(23A3) Rabbit mAb
AMPK	T172	Cell Signaling	2535	WB	(40H9) Rabbit mAb
ACC		Cell Signaling	3676	WB	(C83B10) Rabbit mAb
ACC	S79	Cell Signaling	3661	WB	
S6		Cell Signaling	2217	WB	(5G10) Rabbit mAb
S6	S240/S244	Cell Signaling	2215	WB	
Beta-catenin		Cell Signaling	9587	IHC	
Beta-catenin		Millipore	05-665	IF	(8E7) Mouse mAb
Fibronectin		Lab Vision	RB-077-A0,	IHC	
ACTA2		Novus Biologicals	NB110-55432	IHC	
E-Cadherin		Cell Signaling	3195	IHC	
Vimentin		DAKO	M7020	IHC	VIM3B4 Mouse mAb
Pan-cytokeratin		DAKO	IR053	IHC	

Real time PCR TaqMan probes (Applied Biosystems)

Gene	TaqMan probe
Twist1	Mm00442036_m1
Cdh1	Mm01247350_m1
Vim	Mm00449201_m1
Cdh2	Mm00483208_m1
Fn1	Mm01263588_m1
Acta2	Mm01546133_m1
Snai1	Mm01249564_g1

shRNA constructs (Openbiosystems)

Designation	Sequence	RNAi Consortium number
NT	CAACAAGATGAAGAGCACCAA	-
LKB1A	GAGTGTGCGGTCAATATTTAT	TRCN0000000407
LKB1B	GATCCTCAAGAAGAAGAAGTT	TRCN0000000409
LKB1C	GAAGAAGAAGTTGCGAAGGAT	TRCN0000000410
LKB1D	CATCTACACTCAGGACTTCAC	TRCN0000000411