Supplementary Materials for

**ARAF suppresses ERBB3 expression and metastasis in a subset of lung cancers**

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The PDF file includes:

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Other Supplementary Material for this manuscript includes the following:

- Tables S1 to S3
- Data file S1
Figure S1.
ARAF expression in lung cancer. (A) Low ARAF expression is correlated with poor prognosis of lung cancer patients (Lung adenocarcinoma) in GSE37745. Survival analysis was performed on high and low expression groups of ARAF for a total of 106 patients. P.value obtained was close to 0.05. Cut off of high low and low expression was chosen in the same way as for TCGA LUAD data. (B-C) Boxplots of ARAF expression based on different stages of cancer in TCGA LUAD and GSE37745 LUAD. Expression of ARAF in lung adenomacarcinoma not dependent on different tumor stages (D). Boxplots of ARAF expression based on age in TCGA LUAD. ARAF expression in lung cancer not dependent on gender.
Figure S2

A

| shARAF | +EV | +ARAF | +R362H | +S432A | +K336M |
|--------|-----|-------|--------|--------|--------|
|        |     |       | V5     | ARAF   |        |
| 75     |     |       |        |        |        |
| 75     |     |       |        |        |        |
| 48     |     |       |        |        |        |
| 48     |     |       |        |        |        |
| 48     |     |       |        |        |        |
| 48     |     |       |        |        |        |

IP: V5 (ARAF)

| V5 | ARAF |
|----|------|

kinase assay

C

| shCo | shARAF | +EV | +ARAF | +R362H |
|------|--------|-----|-------|--------|
| 75   |        |     |       |        |
| 63   |        |     |       |        |
| 115  |        |     |       |        |

ARAF

Vinculin

D

| V5 | ARAF |
|----|------|

**relative mRNA expression (ARAF)**

E

- **shARAF+EV**
- **shARAF+ARAF**
- **shARAF+R362H**

\[ 10^{0.2} \]

\[ 10^{1.0} \]

\[ 10^{2.0} \]

\[ 14 17 21 14 17 21 14 17 21 \]

\[ 1.0 \times 10^{0.2} \]

\[ 1.0 \times 10^{1.0} \]

\[ 1.0 \times 10^{2.0} \]

\[ 14 17 21 14 17 21 \]

\[ 0.0 \]

\[ 0.5 \]

\[ 1.0 \]

\[ 1.5 \]

\[ 2.0 \]

**relative mRNA expression (ARAF)**

F

- **shARAF**
- **+EV**
- **+ARAF**
- **+R362H**

\[ 1.0 \times 10^{0.2} \]

\[ 1.0 \times 10^{1.0} \]

\[ 1.0 \times 10^{2.0} \]

\[ 14d 17d 21d \]

\[ 1.0 \times 10^{0.2} \]

\[ 1.0 \times 10^{1.0} \]

\[ 1.0 \times 10^{2.0} \]

\[ 1.0 \times 10^{3.0} \]

\[ 14d 17d 21d \]

\[ 1.0 \times 10^{0.2} \]

\[ 1.0 \times 10^{1.0} \]

\[ 1.0 \times 10^{2.0} \]

\[ 1.0 \times 10^{3.0} \]

**relative mRNA expression (ARAF)**

G

- **shARAF**
- **shARAF-ARAF**
- **shARAF-R362**

\[ 10^{0.2} \]

\[ 10^{1.0} \]

\[ 10^{2.0} \]

\[ 10^{3.0} \]

\[ 0.0 \]

\[ 0.5 \]

\[ 1.0 \]

\[ 1.5 \]

\[ 2.0 \]

\[ 0.0 \]

\[ 0.5 \]

\[ 1.0 \]

\[ 1.5 \]

\[ 2.0 \]

\[ 0.0 \]

\[ 0.5 \]

\[ 1.0 \]

\[ 1.5 \]

\[ 2.0 \]
Figure S2.
ARAF knockdown (shARAF+EV) and the kinase-deficient (shARAF+R362H, unable to form dimers) mutant convey high metastatic potential to A549 cells in nude mice. (A) Kinase activity of various kinase impaired ARAF mutants was studied in an *in vitro* kinase assay in A549 cells that were ARAF depleted (shARAF). Reconstituted ARAF (V5) was immunoprecipitated (IP) and blotted for MEK1 that was used as a substrate. Phosphorylation of the kinase dead MEK K97A substrate at S217/S221 was investigated by Western blot. t, total protein; p, phosphorylated protein. (B) A549 cells were depleted for ARAF, BRAF or CRAF and were subsequently cultured in soft agar for 2 weeks followed by crystal violet staining. Number of colonies was counted using Image J software. Cells transfected with shControl served as a control. (C) Representative Western blot showing ARAF levels in A549 control and ARAF-depleted cells as well as in ARAF-depleted A549 that were reconstituted with empty vector (+EV), wildtype ARAF (+ARAF) or with kinase-deficient ARAF mutant (R362H). (D) Same as in (C), but ARAF mRNA levels were analyzed by real-time PCR (n = 2-3). (E) Mice (n = 6 per group) were injected with 1x 10⁶ ARAF-depleted A549 stably expressing Luciferase reconstituted with either empty vector (EV) or wildtype ARAF (control) and kinase-deficient mutant (R362H), respectively (from left to right). Over the time course of 2-3 weeks post injection *in vivo* bioluminescence imaging was conducted after intraperitoneal injection of the luciferase substrate D-Luciferin (100µl, 40 mg/ml solution) and total counts were analyzed in Regions of Interest (ROI) at the indicated time points. Lung time course, *P* < 0.05, two-way ANOVA with uncorrected Fisher’s LSD (F) The graph shows the time course of individual mice. For mice with several metastases the ROIs per mouse were summed. (G) Representative bioluminescence images of all mice treated and analyzed as described in (E) 2 weeks post infection.
Figure S3.
Loss of ARAF leads to elevated AKT phosphorylation. (A) Human Phospho-Kinase Array was used for parallel detection of 43 kinase phosphorylation sites. Control and ARAF depleted A549 cells were seeded at a density of 2.5x10^6 and after 24h cell lysates were probed for activity of multiple kinases as described in the Material and methods section. Squared box indicates phosphorylation of AKT at Serine 473 in the two cell lines (shControl and shARAF). (B) Spot densities of phospho-proteins were quantified using ImageJ software and normalized to those of positive controls on the same membrane. ERK1/2 phosphorylation was decreased in ARAF-depleted cells, which served as proof of principle. (C) Western blotting of NCIH1437 cells showing that permanent knockdown of ARAF, but not of BRAF or CRAF, leads to Akt activation (T308, S473). Two different shRNAs targeting ARAF were employed t, total protein; p, phosphorylated protein. (D) Western blotting of A549 cells showing that transient knockdown of ARAF leads to increased AKT phosphorylation at S473. Two different siRNAs targeting ARAF were employed t, total protein; p, phosphorylated protein. (E) Anchorage-independent growth of A549 control and ARAF depleted cells was assessed upon treatment with the allosteric Akt inhibitor MK-2206 (1 μM and 5 μM). Two weeks after, colonies were stained with crystal violet and number of colonies was counted using Image J software. Cells treated with DMSO served as a control. Data are means ± SEM from three independent experiments. ***p < 0. 0001, one-way ANOVA with posthoc tests employing a correction of alpha according to Bonferroni. (F) Clonogenecity assay with A549 control and ARAF-depleted cells that were treated with the PI3K inhibitors GDC941 (1μM) and BKM 120 (5μM). Cells were fixed at 9 days, and stained with crystal violet to visualize cells that survived the treatment. Representative images are shown.
**Figure S4**

A) Top 500 genes

B) DE genes – ARAFkd vs ctrl

C) GO:0018108 regulation of peptidyl-tyrosine phosphorylation

D) GO:0042060 wound healing

E) GO:0022409 positive regulation of cell-cell adhesion
Figure S4.
RNAseq analysis reveals differential gene expression in ERBB3 signaling upon ARAF knockdown in A549 cells. (A) Principal Component Analysis (PCA) of ARAF knockdown samples that were either reconstituted with control hairpin (shARAF+EV), wildtype ARAF (shARAF+ARAF) or kinase-deficient ARAF (shARAF+ARAFR362H) showed high similarity for the individual replicates. Depicted is the clustering of samples of the top 500 most variable genes. (B) Heatmap of the top 30 differentially expressed genes upon ARAF knockdown, where A549 cells that were depleted of ARAF are either reconstituted with control hairpin (shARAF+EV), wildtype ARAF (shARAF+ARAF) or kinase-deficient ARAF (shARAF+ARAF-R362H), respectively. Gene expression profiles were plotted as heatmaps (color-coded z-scores of regularized logarithm (rlog) transformed expression values). (C-E) Signature heatmaps for the differentially expressed genes associated with positive regulation of peptidyl-tyrosine phosphorylation (GO:0018108), wound healing (GO:0042060) and positive regulation of cell-cell adhesion (GO:0022409) were generated for ARAF depleted (shARAF+EV), ARAF WT (shARAF+ARAF) and kinase-impaired (shARAF+R362H) samples. Standardized values of the regularized-log transformed counts, after normalizing for library size, are plotted for each gene set.
Figure S5

A

B

C

TCGA Lung Adenocarcinoma (LUAD) – ERBB3 mRNA expression (normalized read counts, log2)

n=59
n=159
n=68
n=290
Normal
wt-KRAS Tumor
KRAS*
NA

D

ERBB3 mRNA expression (normalized read counts, log2)

n=59
n=194
n=33
n=290
Normal
wt-EGFR Tumor
EGFR*
NA

E

NCI-H441
NCI-H23

F

NCI-H1437
NCI-H1650

G

NCI-H292
NCI-H226

(TCGA Lung Adenocarcinoma (LUAD) – KRAS mutation)

p = 2.109e-12
p = 4.2e-07
p = 0.08946

p = 3.826e-05
p = 2.032e-14
p = 3.826e-05

NCI-H441
(KRAS G12V)
NCI-H23
(KRAS G12C)
Figure S5.

ARAF regulates ERRB3 expression in a cell type-dependent manner. (A) The graph represents RNaseq-derived normalized counts of control (shARAF+ARAF), ARAF knockdown (shARAF+EV) and kinase-deficient (shARAF+R362H) samples from two biological replicates of A549 cells. (B) ERBB3 expression in TCGA lung adenocarcinoma (LUAD) dependent on EGFR mutation status or (C) KRAS mutation status. (D) Lung adenocarcinoma cell lines NCI-H2122 with KRAS mutation G12C and Calu6 with KRAS mutation Q61K were stably depleted of ARAF by employing RNA interference and total levels of ERBB3 were assessed by Western blotting with vinculin serving as loading control. t- total protein. (E) 48h after inducing a siRNA-mediated knockdown of ARAF in the lung cancer cell lines NCI-H441 and NCI-H23, both of which have a KRAS mutation at codon 12, total ERBB3 levels were determined by Western blot analysis. t- total protein. (F) Lung adenocarcinoma cell lines H1650 and NCIH 1437 both wildtype for KRAS were transiently depleted for ARAF using two different ARAF targeting siRNAs and total levels of ERBB3 were assessed by Western blotting with vinculin serving as loading control. t- total protein. (G) NCI-H226 and NCI-H292, two more KRAS wildtype cell lines, were transfected with siControl or siARAF and after 48 h total ERBB3 levels were analysed by Western blotting. t- total protein.
**Figure S6**

Oncogenic signaling of ERBB3 and influence of ARAF on transcription factor binding. (A) Western blot analysis of A549 control (shCo) and ARAF-depleted cells (shARAF) after treating the cells with the ERBB3 inhibitor sapitinib for 4 h (1 µM and 5 µM). ERBB3 phosphorylation as well as phosphorylation of AKT at S473 were determined. Samples were loaded on to the same gel, transferred and the membrane was developed for various antigens. For better visualization, lanes were cut out between the presented samples from a single gel. t, total protein; p, phosphorylated protein. (B) Control- and ARAF-depleted A549 cells were cultured in soft agar in presence or absence of the ERBB2/3 inhibitor sapitinib (1 or 5 µM) for 1 week. The average size of colonies was quantified using Fuji/ Image J software after the colonies were stained with Hoechst and photographed. Data are means ± SEM from three independent experiments. **P < 0.01, one-way ANOVA with Bonferroni post-hoc test. (C) Signature heatmaps for the differentially expressed genes associated with positive regulation of transcription factor binding (GO:0008134) were generated for ARAF depleted (shARAF+EV), ARAF WT (shARAF+ARAF) and kinase-impaired (shARAF+R362H) samples. Standardized values of the regularized-log transformed counts, after normalizing for library size, are plotted for each gene set.
Figure S7

**A**

Potential binding sites for KLF5 and other TFs identified in RNA seq

| ID     | Motif ID | Sequence | Logos | Peaks | Strand | Gene name |
|--------|-----------|----------|--------|-------|--------|-----------|
| KLF5   | H10480.1  | GGGGGGGGGGCCCCCCCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
**Figure S7.**

**ARAF regulates transcription factor binding.** (A) p-value ranked overview on potential binding sites for KLF5 and other transcription factors upregulated in ARAF knockdown control cells (shARAF+EV) and ARAF knockdown cells reconstituted with kinase-deficient mutant (shARAF+R362H), respectively, based on RNAseq analysis. (B) KLF5 and other transcription factors and their location within the human ERBB3 promoter region (-1500 to translation start site). (C) Western blotting of A549 control (shCo) and ARAF depleted (shARAF) cells showing that stable knockdown of ARAF leads to increased ERBB3, KLF5 and FOXC1 expression. t- total protein. (D) Control (shCo) and ARAF-depleted (shARAF) A549 cells were transfected with siRNAs to specifically knockdown KLF5 and FOXC1. Knockdown of ARAF, KLF5, FOXC1 was confirmed by Western blot analysis and total ERBB3 protein level were analyzed with vinculin serving as loading control. t- total protein.
Figure S8

ARAF regulates ligand-induced phosphorylation of AKT in an ERBB3-dependent manner. 

(A) Western blot analysis of A549 control (shCo) and ARAF-depleted cells (shARAF) that were stimulated with NRG1 (50 ng/ml and 100 ng/ml), bFGF (50 ng/ml and 100 ng/ml) and EGF (100 ng/ml) for 20 min. The induction of activating phosphorylations of EGFR, ERBB3, AKT and ERK1/2 were determined. p- phosphorylated protein, t-total protein

(B) A549 cell line obtained and authenticated from a second source (DSMZ) was stably transduced with empty control (EV) or ARAF shRNA (shARAF) and cells were stimulated for 24h after seeding with indicated concentrations of hNRG1 or EGF for 20 min. Phosphorylation levels of ERBB3 (Tyr1289) and AKT (S473) were monitored with vinculin serving as loading control.

(C) Lung adenocarcinoma cell lines H1650 and NCIH 1437 (D) were stably transfected with shRNA targeting ARAF and treated with the indicated concentrations of hNRG1 and EGF for 20min. Lysates were subjected to Western blotting and probed for the indicated proteins with vinculin serving as loading control. 

t- total protein, p- phosphorylated protein
Figure S9

(A) Control (shCo) and ARAF-depleted (shARAF) A549 cells were treated with 1µM trametinib for 1h, 24h and 48h. Total ERBB3 levels were monitored by Western blot analyses. Phosphorylation levels of ERK1/2 were tested to validate the inhibitor with vinculin serving as loading control. (B) Control (shCo) and ARAF-depleted (shARAF) A549 cells were treated as in (A) and ERBB3 mRNA levels were determined by RT-PCR over time (n=3).

Additional Supplementary materials

Data file S1: Original images of uncropped western blots used to prepare various figures presented in the manuscript

Supplementary tables 1-3: Results of RNA-seq analyses (shControl Vs shARAF, shARAF vs shARAF+ARAFR362H and sh Control Vs shARAF+ARAFR362H) to identify factors differentially regulated by ARAF and its kinase activity