RAL GTPases mediate multiple myeloma cell survival and are activated independently of oncogenic RAS

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Supplementary Material

Supplementary Tables

Supplementary Table 1. Immunohistochemical analysis of RAL expression in non-malignant plasma cells and MGUS *in situ*. Data on immunohistochemical stainings of the two RAL isoforms in normal plasma cells (NPC, n=5) and MGUS plasma cells (n=10) are summarized. Plus signs indicate the grade of staining intensity (+ = weak, ++ = moderate, +++ = strong) in the given proportion of positive cells. Samples with RAL staining below detection limits are marked by 0. Because in those samples staining of tumor cells was negative, no intensity score is provided (marked with a dash).

Sample	RALA		RALB	
	% positive cells	intensity	% positive cells	intensity
NPC 1	0	-	0	-
NPC 2	0	-	0	-
NPC 3	0	-	0	-
NPC 4	0	-	0	-
NPC 5	10	+	0	-
MGUS 1	0	-	0	-
MGUS 2	0	-	0	-
MGUS 3	0	-	0	-
MGUS 4	0	-	0	-
MGUS 5	20	+	0	-
MGUS 6	100	+	0	-
MGUS 7	25	+	0	-
MGUS 8	0	-	0	-
MGUS 9	50	+	0	-
MGUS 10	10	-	0	-

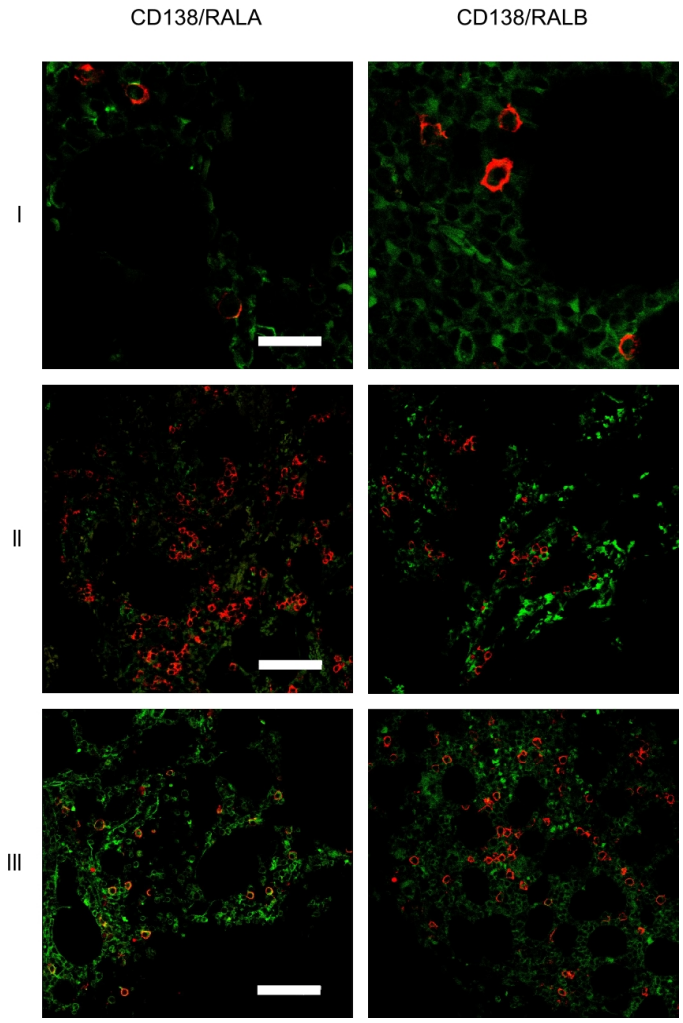
Supplementary Table 2. Immunohistochemical analysis of RAL expression in primary MM cells *in situ*. Data on immunohistochemical stainings of the two RAL isoforms in MM plasma cells (n=26) are summarized. Plus signs indicate the grade of staining intensity (+ = weak, ++ = moderate, +++ = strong) in the given proportion of positive cells. Samples with RAL staining below detection limits are marked by 0. Because in those samples staining of tumor cells was negative, no intensity score is provided (marked with a dash).

Sample	Infiltration rate [%]	RALA		RALB	
		% positive tumor cells	intensity	% positive tumor cells	intensity
1	60	100	+++	< 5	+
2	80	90	++	10	+
3	5	n.a.	-	n.a.	-
4	70	90	+++	< 5	+
5	60	95	+++	15	+
6	80	95	+++	< 5	+
7	10	n.a.	-	n.a.	-
8	50	100	+++	< 5	+
9	5	n.a.	-	n.a.	-
10	80	25	++	10	+
11	20	90	++	< 5	+
12	80	100	+++	< 5	+
13	50	100	+++	30	+
14	25	90	+++	< 5	+
15	40	90	+++	5	+
16	90	95	+++	5	+
17	30	80	++	30	+
18	70	50	++	10	+
19	80	100	+++	< 5	+
20	40	90	++	20	+
21	15	80	++	30	+
22	90	100	+++	30	+
23	40	95	+++	10	+
24	70	90	+++	5	+
25	70	50	+	n.a.	-
26	50	90	++	20	+

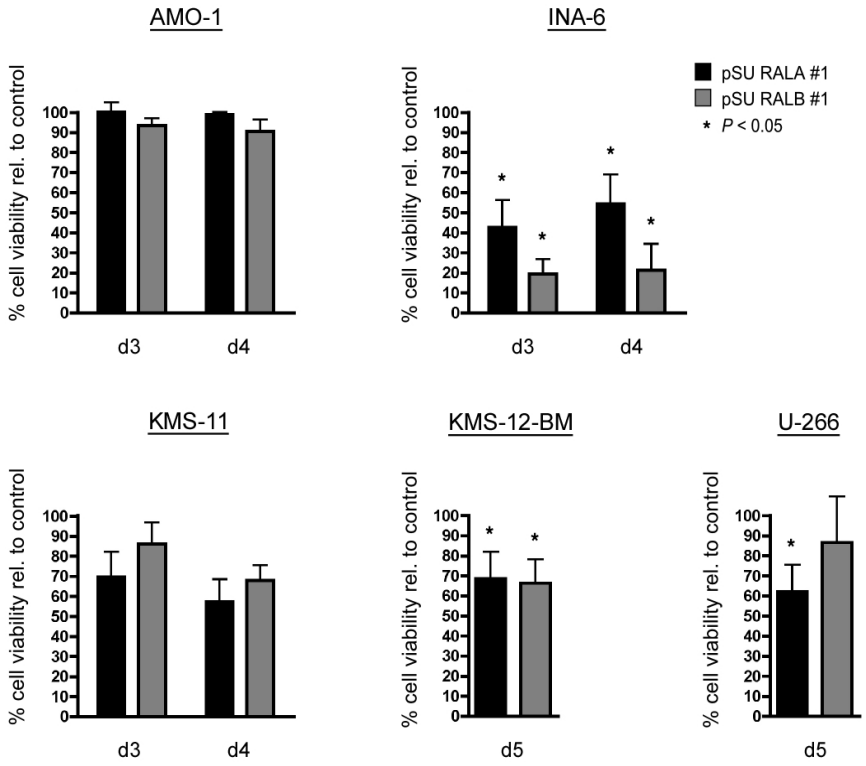
Supplementary Table 3. Effects of RAL knockdown on cell survival rates of HMCLs. RNAi-induced RALA and B knockdown in a panel of HMCLs led to a reduction in cell survival in most cell lines. Shown are mean values of cell viability rates of three independent experiments. Percentages were calculated relative to the respective empty vector control. Time of cell survival measurement after transfection via annexin V/PI staining is given for each cell line.

Cell line	pSU RALA#1	pSU RALB#1	Time after transfection
AMO-1	100 %	93 %	72 h
	99 %	90 %	96 h
INA-6	43 %	19 %	72 h
	54 %	21 %	96 h
KMS-11	70 %	86 %	72 h
	57 %	68 %	96 h
KMS-12-BM	69 %	66 %	120 h
L-363	33 %	71 %	72 h
	28 %	59 %	96 h
MM.1S	57 %	87 %	72 h
	42 %	79 %	96 h
U-266	48 %	82 %	120 h

Supplementary Figures

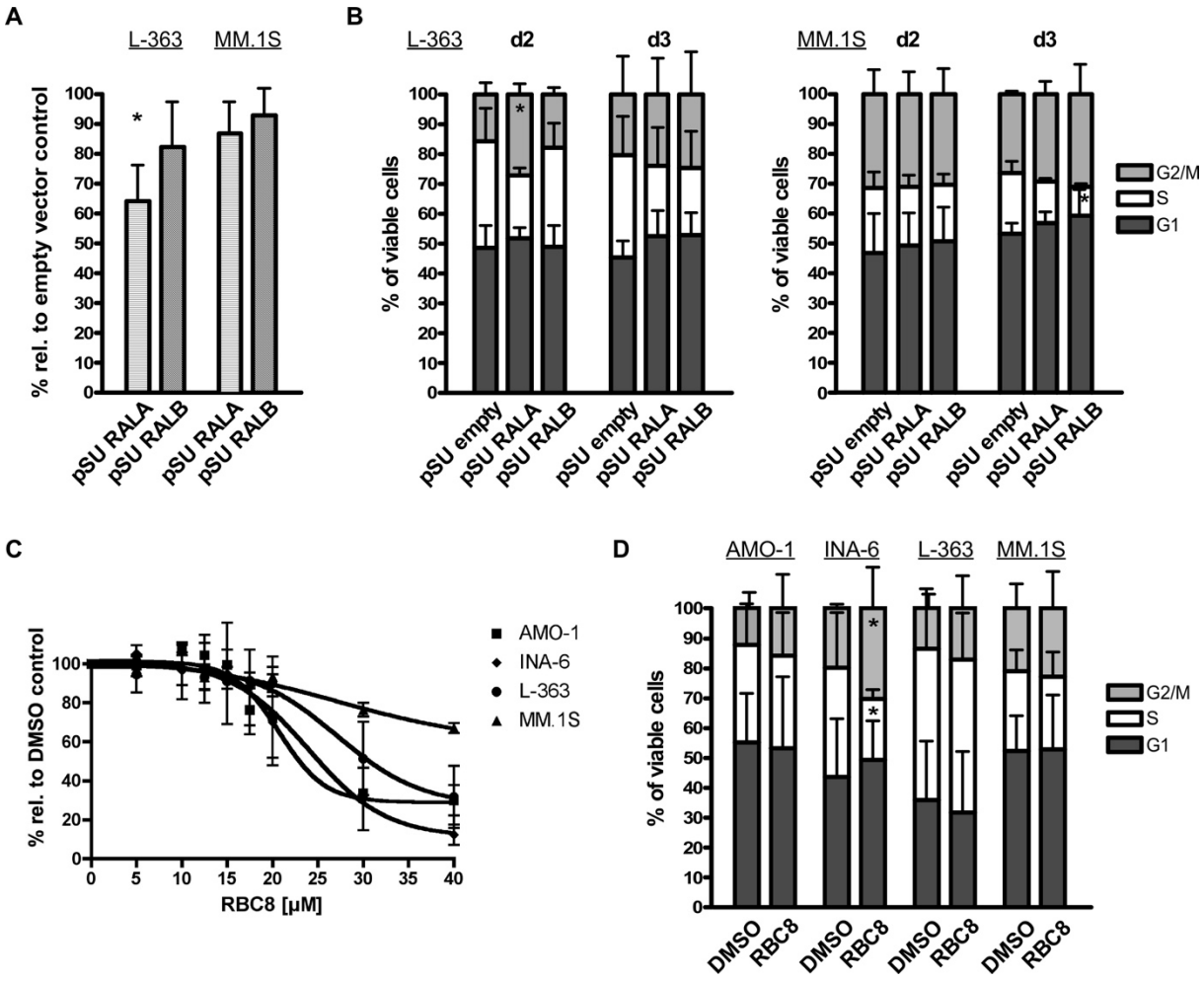


Supplementary Figure 1: Expression of the two RAL isoforms in normal plasma cells (NPC) and MGUS cells. *In situ* expression of RALA and RALB in bone marrow trephines of non-MM/non-MGUS patients (n=5) and MGUS patients (n=10). CD138 staining (red) as well as RALA or RALB staining (green) are shown for 3 different patients (I, II, III). Sample I (normal plasma cells, 400x magnification, scale bar: 25 µm) corresponds to sample number NPC 4 in Supplementary Table 1. Samples II and III (MGUS, 200x magnification, scale bar: 75 µm) correspond to sample numbers MGUS 1 and MGUS 6 in Supplementary Table 1, respectively.

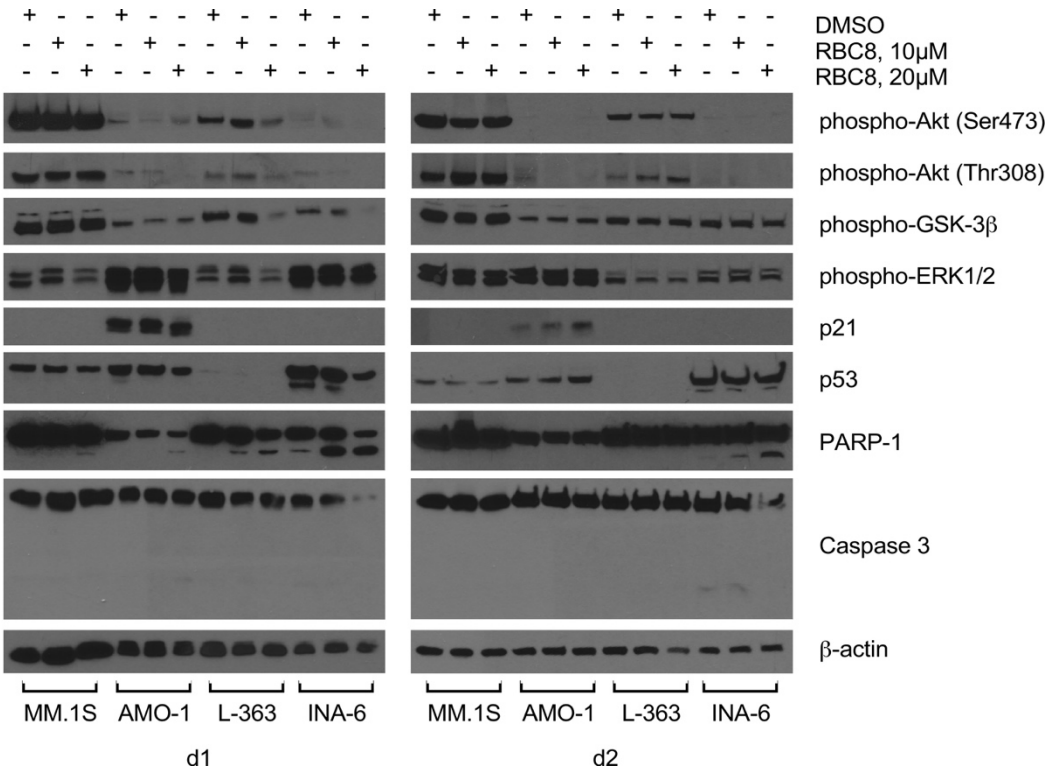


Supplementary Figure 2: Effects of RAL knockdown on MM cell survival.

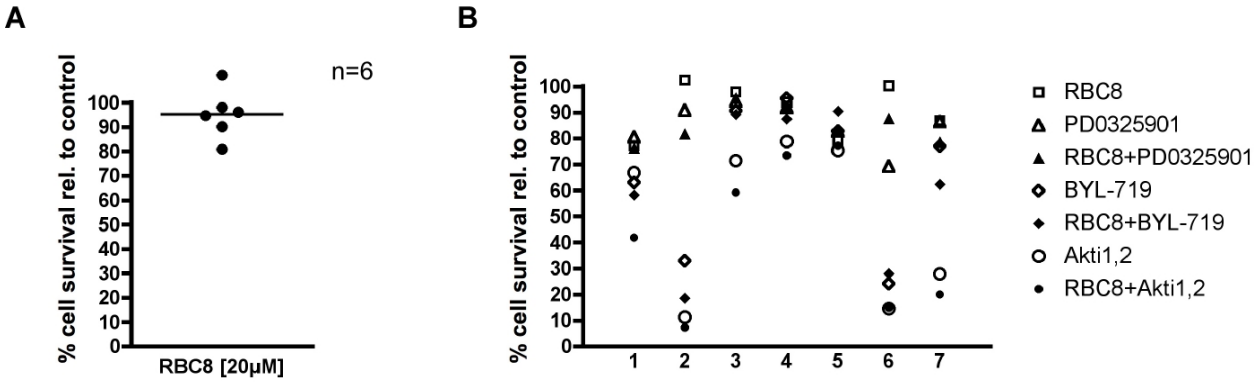
RALA and RALB knockdown was achieved with shRNA expression vector constructs for each RAL isoform and cell survival was assessed after 72 and 96 h in AMO-1, INA-6, KMS-11 cells or after 120 h in KMS-12 and U-266 cells. Percentages were calculated relative to the respective empty vector control. Cell viability was monitored by annexin V/PI staining. Means and s.d. are shown.



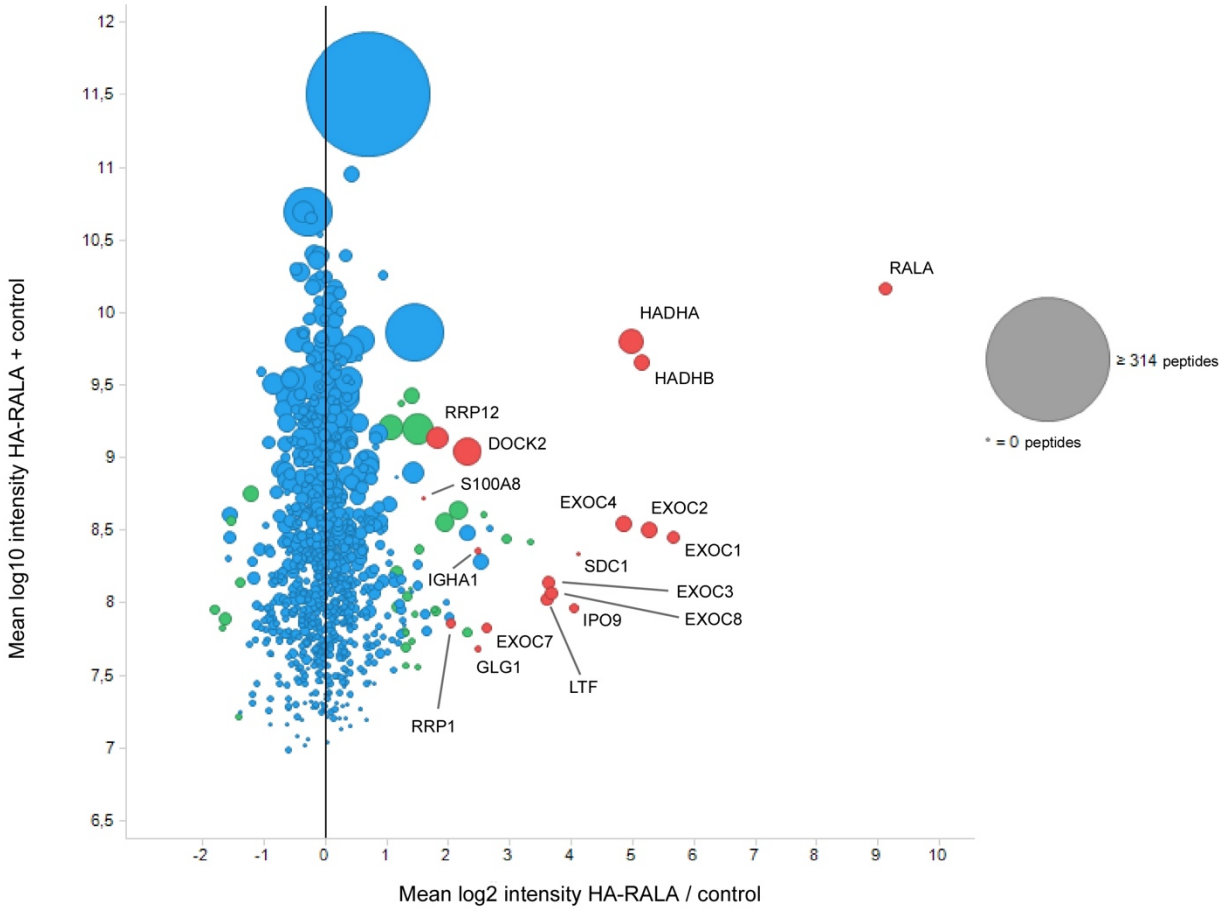
Supplementary Figure 3: Effects on cell metabolism, proliferation and cell cycle distribution after RAL knockdown or pharmacological RAL inhibition. (A) L-363 and MM.1S cells were transfected with shRNA expression plasmids targeting RALA or RALB and purified cells were cultured for 3 d before metabolic activity assessment (Alamar Blue assay). **(B)** Cell cycle distribution as determined by BrdU/PI assay after RAL knockdown. **(C)** Dose-effect curves for RBC8 in different MM cell lines as measured by Alamar Blue metabolic activity assay after 3 d of drug treatment. **(D)** Cell cycle distribution in MM cell lines treated with 20 µM of RBC8 for 3 d. Drug-induced changes were most pronounced in INA-6 cells. Cells treated with DMSO served as control. Means and s.d. are shown; * indicates statistical significance ($P < 0.05$).



Supplementary Figure 4: Effect of pharmacological RAL inhibition on survival and apoptosis signaling. MM.1S, AMO-1, L-363, and INA-6 were incubated with RBC8 and harvested for Western blotting after 1 and 2 days. INA-6 cells – which are most sensitive to RBC8 – show PARP-1 cleavage. DMSO served as control. Western Blots are representative of 3 independent experiments.



Supplementary Figure 5: Pharmacological RAL inhibition in primary MM cells. **(A)** Primary MM cells were treated with 20 µM of RBC8. Cell survival was measured by annexin V/PI staining after 72 h of treatment. RBC8 treatment did not lead to greater reduction of cell survival in any of the patient samples. **(B)** Combined blockade of RAL and PI3K/Akt or MEK/MAPK signaling in primary MM cells. Cells were treated for 72 h with 20 µM of RBC8, 1 µM of PD0325901, 10 µM of BYL-719, 10 µM of Akti-1,2 and the combination of RBC8 with one of the other drugs. Combination of RBC8 treatment with PD0325901, BYL-719 or Akti-1,2 inhibition led to a mild reduction of cell survival in a subset of primary MM samples.



Supplementary Figure 6: Mass spectrometric analysis of RAL interaction partners.

MM.1S cells stably expressing HA-RALA were used for Co-IPs of HA-RALA. Control Co-IPs were carried out with empty vector control cells lacking HA-RALA expression. Label-free quantification (LFQ) shows mean log2 transformed protein ratios of two replicates. Unspecifically captured proteins (blue) have log2 intensity ratios of HA-RALA/control of close to 0. Specific interactions partners (green (potential outlier) and red (extreme outlier)) have positive log2 intensity ratios of HA-RALA/control. 48 potential interaction partners were identified, including 17 extreme outliers (red). Size of data points represents number of identified peptides.

Supplementary Methods

Culture of MM cell lines and preparation of primary MM cells

The human MM cell line (HMCL) MM.1S was obtained from LGC Biolabs (Wesel, Germany; ATCC CRL-2974). INA-6 cells were a gift from Martin Gramatzki (Kiel, Germany). Other HMCLs were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany).

Reagents

RBC8 and PD0325901 were obtained from Selleck Chemicals (Houston, TX, USA). BYL-719 and MK2206 were purchased from Active Biochem (Bonn, Germany), and Akti-1,2 was from Merck (Darmstadt, Germany). Control incubations were made with dimethyl sulfoxide.

Immunohistochemical stainings of bone marrow biopsies

Consecutive sections of specimens with confluent plasma cell infiltrates were stained with the following antibodies: anti-CD138 (1:100, clone MI15, Dako Agilent, Waldbronn, Germany), RALA (mouse monoclonal, 1:800, no. ABD-048, Jena Bioscience, Jena, Germany) and RALB (rabbit polyclonal, 1:100, no. 3523, Cell Signaling Technology, Frankfurt am Main, Germany). All MGUS cases and specimens with reactive plasma cells or a more diffuse infiltration of malignant plasma cells were analyzed by double-immunofluorescence (CD138/RALA and CD138/RALB) using the same antibodies with slightly adjusted concentrations in conjunction with fluorochrome-coupled secondary antibodies.

Alamar Blue mitochondrial activity assay

After performing RAL knockdown or treatment with RBC8, cells were seeded in triplicates in a 96-well format and incubated with 20 µl of Alamar Blue solution (Resazurin, R7017-5G, Sigma-Aldrich, Deisenhofen, Germany). After an incubation time between 4 to 6 h, the color reaction was measured with a microplate reader and calculations were performed according to the manufacturer’s protocol.

Cell cycle analysis

For cell cycle analysis, cells were seeded in a 24-well format, incubated with 2 mM of 5-bromo-2'-deoxyuridine (BrdU) for 5 h and stored in 70 % ethanol at -20°C over night. Subsequent incubation with 2 N HCl followed by $Na_2B_4O_7$ and BrdU staining using an allophycocyanin (APC)-conjugated anti-BrdU antibody (BioLegend, San Diego, CA, USA) was performed according to the manufacturer's instructions. Cells were counterstained with propidium iodide (PI) and measured with flow cytometry.

Construction of shRNA expression vectors

Target sequences used for the generation of shRNA expression vectors were: 5'-AGACAGGTTTCTGTAGAAG-3' (Human *RALA#1*),[36]
5'-ACAGAGCTGAGCAGTGGAA-3' (Human *RALA#2*),[36]
5'-GACTATGAACCTACCAAAG-3' (Human *RALB#1*),[36]
5'-AAGCTGACAGTTATAGAAA-3' (Human *RALB#2*),[37]
5'-GTTGGAGCTGCTGGCGTAG-3' (Human *KRAS* as mutated in cell line MM.1S (G12A))[8] and 5'-GTTGGAGCAGATGGTGTTG-3' (Human *NRAS* as mutated in cell line INA-6 (G12D)).[8] Oligonucleotides for pSU-based shRNA expression constructs were purchased from Sigma-Aldrich (Deisenhofen, Germany) and annealed and cloned according to standard protocols.

Western analysis

Antibodies against RALA (no. 3526), RALB (no. 3523), pan-Akt (no. 9272), phospho-Akt (no. 4058 (Ser473) and no.2965 (Thr308)), ERK1/2 (no. 9102), phospho-ERK1/2 (no. 9101) and phospho-GSK-3β (no. 9336) were purchased from Cell Signaling Technology (Frankfurt am Main, Germany). Anti-KRAS (sc-30) and anti-NRAS antibodies (sc-31) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The anti α-tubulin antibody (no. 03568) was purchased from Biozol (Eching, Germany), the anti β-actin antibody (no. A5316) was from Sigma-Aldrich (Deisenhofen, Germany). Secondary antibodies specific for mouse (no. 115-036-003), rabbit (no. 111–036-045) and rat (no. 112-036-062) were purchased from Jackson ImmunoResearch Laboratories (Newmarket, UK).

RNA sequencing analysis

Cells were harvested two days after electroporation and RNA was isolated using the NucleoSpin RNA-Kit (no. 740955.250; Macherey-Nagel, Düren, Germany) according to the instructions. Extracted total RNA was subjected to integrity checks using a Bioanalyzer 2100 (Agilent, Waldbronn, Germany). The median RNA integrity number was 10.0 (range 9.6 – 10.0). RNA sequencing (RNA-Seq) libraries were prepared with the TruSeq stranded mRNA kit (Illumina, Munich, Germany), pooled equimolarly and subjected to single-end 75nt sequencing using a NextSeq 500 (Illumina, Munich, Germany). Library preparation and sequencing was performed at the Core Unit SysMed of the University of Würzburg. Residual adapter sequences present in sequencing reads were removed with Cutadapt version 1.12 and reads were aligned to the human reference sequence GENCODE v24 using STAR version 2.5.2b. Differential gene expression analysis was carried out in the R (v3.3.1)/Bioconductor (v3.3) environment using the DESeq2 package v1.12.3.

Mass spectrometry-based interactome analysis

MM.1S cells were transfected with 20 µg/ml of Sleeping Beauty transposon plasmid (containing the HA-RALA sequence and a puromycin resistance gene), 30 µg/ml of transposase expression plasmid (pCMV-SB100, Addgene, Cambridge, MA, USA) and 10 µg/ml of pEGFP-N3. Stably transfected cells were selected with 0.5 µg/ml of puromycin and expanded in cell culture. HA-RALA protein was isolated by immunoprecipitation of $3x10^7$ stably transfected MM.1S cells using anti-HA magnetic beads (no. 88836, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Empty vector-transfected MM.1S cells served as negative control.

Proteins were eluted in NuPAGE LDS sample buffer (Life Technologies), reduced with 50 mM DTT at 70 °C for 10 min, alkylated with 120 mM iodoacetamide at room temperature for 20 min, and separated on NuPAGE Novex 4-12 % Bis-Tris gels (Life Technologies) in MOPS buffer according to manufacturer's instructions. Gels were washed three times for 5 min with water and stained for 45 min with Simply Blue™ Safe Stain (Life Technologies). After washing with water for 2 h, each gel lane was cut into 15 slices.

For in-gel digestion, the excised gel slices were destained with 30% acetonitrile, shrunk with 100% acetonitrile, and dried in a vacuum concentrator. Trypsin digest

was performed overnight at 37 °C in 0.05M NH_4HCO_3 (pH 8), using 0.1 µg of protease per slice. Peptides were extracted from the gel slices with 5% formic acid.

NanoLC-MS/MS analyses were performed on an Orbitrap Fusion (Thermo Scientific) equipped with a PicoView Ion Source (New Objective) and coupled to an EASY-nLC 1000 (Thermo Scientific). Peptides were loaded on capillary columns (PicoFrit, 30 cm x 150 µm ID, New Objective) packed with ReproSil-Pur 120 C18-AQ 1.9 µm (Dr. Maisch), and separated with a 30 min linear gradient from 3% to 30% acetonitrile and 0.1% formic acid at a flow rate of 500 nl/min.

Both MS and MS/MS scans were acquired in the Orbitrap analyzer with a resolution of 60,000 for MS scans and 15,000 for MS/MS scans. HCD fragmentation with 35% normalized collision energy was applied. A Top Speed data-dependent MS/MS method with a fixed cycle time of 3 s was used. Dynamic exclusion was applied with a repeat count of 1 and an exclusion duration of 120 s; singly charged precursors were excluded from selection. Minimum signal threshold for precursor selection was set to 50,000. Predictive AGC was used with a target value of 5e5 for MS scans and 5e4 for MS/MS scans. EASY-IC was used for internal calibration.

For raw data file processing, database searches and quantification, MaxQuant version 1.5.7.4 was used.[39] The search was performed against the *H. sapiens* reference proteome database (Uniprot) and, additionally, a database containing common cell culture contaminants. The search was performed with tryptic cleavage specificity with three allowed miscleavages. Protein identification was under control of the false-discovery rate (<1% FDR on protein and peptide level). In addition to MaxQuant default settings, the search was performed allowing the following variable modifications: Protein N-terminal acetylation, Gln to pyro-Glu formation (N-terminal Gln), and oxidation (Met). For protein quantitation, the LFQ intensities were used.[38] Proteins with less than two identified razor/unique peptides were dismissed.

Further data analysis was performed using R scripts developed in-house. LFQ intensities were used and missing LFQ intensities in the control samples were imputed with values close to the baseline. Data imputation was performed with values from a standard normal distribution with a mean of the 5% quantile of the combined log10-transformed LFQ intensities and a standard deviation of 0.1. For the identification of significantly co-immunoprecipitated proteins, mean log2 transformed protein ratio were calculated from the two replicate experiments and boxplot outliers were identified in intensity bins of at least 300 proteins. Log2 transformed protein

ratios of CoIP *versus* control with values outside a 1.5x (potential) or 3x (extreme) interquartile range (IQR), respectively, were considered as significantly co-immunoprecipitated.