Supplemental Material and Methods

Virotrap Screening

The pMET7-GAG-TRAF7 construct was generated as described in (1). Virotrap experiments were performed as described in (2). Briefly, HEK293T cells were transfected pMET7-GAG-TRAF7 or pMET7-GAG-eDHFR together with either untagged, or FLAG-tagged VSV-G using polyethylenimine (Sigma-Aldrich). 36 hours after transfection, particles were purified from the supernatant using MyOne paramagnetic streptavidin beads (ThermoFisher Scientific) pre-loaded with 2 µg FLAG-Bio-M2 (Sigma-Aldrich). After washing with TRIS wash buffer (TWB: 20 mM Tris-HCl pH 7.5, 150 mM NaCl), particles were eluted using the FLAG peptide (Sigma Aldrich) in TWB and processed by amphipols lysis and precipitation under acidic conditions. After resuspension in triethylammonium bicarbonate (TEAB) buffer and boiling, proteins were overnight digested with sequencing-grade trypsin (Promega). After acidic precipitation of partially or undigested proteins by amphipols and acidification, the sample was analyzed by MS.

Of each sample, 5.0 µL was introduced into an LC−MS/MS system through an Ultimate 3000 RSLC nano LC (Thermo Fisher Scientific, Bremen, Germany) in-line connected to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). The sample mixture was first loaded with loading solvent (0.1% TFA in water/acetonitrile, 98/2 (v/v)) on a trapping column (made in-house, 100 µm internal diameter (I.D.) × 20 mm, 5 µm beads C18 Reprosil-HD, Dr. Maisch, Ammerbuch-Entringen, Germany). After flushing from the trapping column, the sample was loaded on an analytical column (made in-house, 75 µm I.D. × 200 mm, 1.9 µm beads C18 Reprosil-HD, Dr. Maisch) packed in the needle (pulled in-house). Peptides were eluted with a non-linear 60 min gradient from 98% solvent A (0.1% formic acid in MQ) reaching 55% solvent B (0.1% formic acid in water/acetonitrile, 20/80 (v/v)) in 50 min followed by an increase to 97% solvent B in 10 min at a flow rate of 250 nL/min. This was followed by

a 10 min wash reaching 99% solvent B and re-equilibration with solvent A. Column temperature was kept constant at 50°C (Butterfly nano-LC column oven, Phoenix S&T).

The mass spectrometer was operated in data-dependent, positive ionization mode, automatically switching between MS and MS/MS acquisition for the 16 most abundant peaks in a given MS spectrum. The source voltage was set to 2.5 kV and the capillary temperature was 250°C. One MS1 scan (m/z 375-1500, AGC target 3E6 ions, maximum ion injection time of 80 ms) acquired at a resolution of 60,000 (at 200 m/z) was followed by up to 16 tandem MS scans (resolution 15,000 at 200 m/z) of the most intense ions fulfilling predefined selection criteria (AGC target 1E5 ions, maximum ion injection time of 80 ms, isolation window of 1.5 m/z, fixed first mass of 145 m/z, spectrum data type: profile, underfill ratio 2%, intensity threshold 1.3E4, exclusion of unassigned, singly and >7 charged precursors, peptide match preferred, exclude isotopes on, dynamic exclusion time of 12 s). The HCD collision energy was set to 30% Normalized Collision Energy and the polydimethylcyclosiloxane background ion at 445.12002 Da was used for internal calibration.

The RAW Virotrap MS data were searched using MaxQuant (version 1.5.8.3) against the human Uniprot/SwissProt database, complemented with GAG, VSVG, and eDHFR protein sequences. The searches were performed with 4.5 ppm tolerance on precursor and 20 ppm tolerance on fragment mass, and with trypsin/P settings allowing up to two missed cleavages. Methionine oxidation and N-terminal acetylation formation were set as variable modifications. Minimum peptide length was set to 7, and maximum peptide mass was 4600 Da. PSM FDR and protein FDR were set to 0.01. Minimum peptides and minimum razor + unique peptides were set to 1. The searches were performed together with the eDHFR control samples to enable matching of MS spectra between runs. Contaminants and identifications against the reverse database hits were removed using the Perseus software (version 1.5.5.3).

Proteome and Ubiquitome analyses

100 million cells were washed twice with PBS and lysed in 10ml urea lysis buffer containing 9 M urea and 20 mM HEPES pH 8.0. Samples were sonicated with 3 pulses of 15 s at an amplitude of 20% using a 3 mm probe, with incubation on ice for 1 minute between pulses followed by centrifugation for 15 minutes at 15,000 x g at room temperature. 10 mg of total protein per sample was used for further analysis. Proteins in each sample were reduced by incubating with 4.5 mM DTT for 30 minutes at 55˚C. Alkylation of the proteins was done by addition of 10 mM chloroacetamide for 15 minutes at room temperature. The samples were diluted with 20 mM HEPES pH 8.0 to a urea concentration of 4 M and the proteins were digested with lysyl endopeptidase (Wako, 1/100, w/w) for 4 hours at37°C. All samples were further diluted with 20 mM HEPES pH 8.0 to a final urea concentration of 2 M and proteins were digested with trypsin (Promega, 1/100, w/w) overnight at 37˚C. The resulting peptide mixtures was purified on Sep-Pak C18 cartridges (Waters), lyophilized for two days and re-dissolved in the immunoprecipitation buffer of the PTMScan® Ubiquitin Remnant Motif (K-ε-GG) Kit (Cell Signaling Technology). Aliquots corresponding to 200 µg of digested protein material were taken for shotgun proteomics analysis. Immunocapture of GG-modified peptides was then performed using the PTMScan Kit according to the manufacturer's instructions. Peptides were incubated with the antibody-bead slurry for 2 hours on a rotator at 4˚C. GG-modified peptides were later eluted in 10 µl 0.15% TFA and desalted on reversed phase C18 OMIX tips (Agilent). Purified GG-modified peptides were dried under vacuum in MS vials and stored at -20˚C until LC-MS/MS analysis.

For ubiquitome analysis, purified GG-modified peptides were re-dissolved in 20 µl loading solvent A (0.1% TFA in water/ACN (98:2, v/v)) of which 15 µl was injected for LC-MS/MS analysis. For proteome analysis, purified peptides for shotgun proteomics were re-dissolved in

20 ul solvent A, peptide concentration as determined on a Lunatic spectrophotometer and approximately 3 µg of peptides was injected for LC-MS/MS analysis. Both ubiquitome and proteome samples were analyzed on a Ultimate 3000 RSLCnano system in-line connected to a Q Exactive HF mass spectrometer (Thermo Scientific). The sample mixture was first loaded on a trapping column (made in-house, 100 µm I.D. x 20 mm length, 5 µm beads C18 Reprosil-HD, Dr. Maisch). After flushing from the trapping column, the sample was loaded on a reverse-phase column (made in-house, 75 µm I.D. x 200 mm length, 1.9 µm beads C18 Reprosil-HD, Dr. Maisch). Purified GG-modified peptides and peptides for shotgun analysis were loaded with loading solvent A and were separated with a non-linear 116 min or 140 min gradient, respectively, from 2% to 56% solvent B (0.1% formic acid in water/ACN 20:80 (v/v)) at a flow rate of 250 nl/min and at a constant temperature of 50°C. Following a 10 min wash reaching 97% solvent B, the column was re-equilibrated with solvent A (0.1% formic acid in water). For proteome analysis, the mass spectrometer was operated in data-dependent, positive ionization mode, automatically switching between MS and MS/MS acquisition for the 16 most abundant peaks in a given MS spectrum. One MS1 scan (m/z 375-1500, AGC target 3E6 ions, maximum ion injection time of 60 ms), acquired at a resolution of 60,000 (at 200 m/z) was followed by up to 16 tandem MS scans, acquired at a resolution of 15,000 (at 200 m/z) of the most intense ions fulfilling predefined selection criteria including an AGC target set at 1E5 ions, a maximum ion injection time of 80 ms, an isolation window of 1.5 m/z, an intensity threshold of 1.3E4, exclusion of unassigned and singly charged precursors and a dynamic exclusion time of 12 s. The normalized collision energy was set to 28% and the polydimethylcyclosiloxane background ion at 445.12003 Da was used for internal calibration. The ubiquitinome analysis was carried out in a similar way but with a maximum injection time of 120 ms and an intensity threshold of 8.3E3 as most important differences for peptide MS/MS selection. QCloud was used to control instrument longitudinal performance during the project.

Data analysis was performed with MaxQuant (version 1.6.1.0) using the Andromeda search engine with default search settings including a false discovery rate set at 1% on the PSM, peptide and protein level. Three different searches were performed to analyze the spectra from the GG-enriched samples and the shotgun samples separately. In all searches spectra were interrogated against the human proteins in the Uniprot/Swiss-Prot database.

The mass tolerance for precursor and fragment ions was set to 4.5 and 20 ppm, respectively, during the main search. Enzyme specificity was set as C-terminal to arginine and lysine, also allowing cleavage at proline bonds with a maximum of three missed cleavages. Variable modifications were set to oxidation of methionine residues, acetylation of protein N-termini and GG modification of lysine residues, while carbamidomethylation of cysteine residues was set as fixed modification. The minimum score for modified peptides was set to 30. Matching between runs was enabled and only proteins with at least one unique or razor peptide were retained. Proteins were quantified by the MaxLFQ algorithm integrated in the MaxQuant software. A minimum ratio count of two unique or razor peptides was required for quantification. Further data analysis was performed with the Perseus software after loading the proteinGroups and GG (K) sites tables from MaxQuant. Reverse database hits were removed as well as potential contaminants. The data of the protein groups table and was log2 transformed, proteins with less than three valid values in at least one group were removed and missing values were imputed from a normal distribution around the detection limit. The same steps were performed on the GG modified sites after expanding the sites table. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository.

Analysis of protein expression, activity, and interactions

Ubiquitinated proteins were purified as described previously in (3). Briefly, cells were lysed in the IP lysis buffer containing EDTA-free protease inhibitor cocktail (Roche). Cell lysates were

mixed with His-buffer A (PBS, pH 8.0, 6 M guanidinium-HCl, 0.1% NP-40 and 1 mM β-ME) and added to TALON Co^{2+} coated beads (Clontech) for 3 hours. The beads were washed one time with His-buffer A, three times with His-buffer B (PBS, pH 8.0, 0.1% NP-40, 5% glycerol, 20 mM imidazole), and then proteins were eluted from the beads with 500 mM imidazole solution for 30 minutes at room temperature.

Subcellular fractionation was performed according to manufacturer's protocol (ThermoFisher). Briefly, cells were scraped with ice-cold Cytoplasmic Extraction Buffer containing protease inhibitors, incubated at 4°C for 10 minutes and centrifuged at 500 g for 5 minutes at 4°C. The supernatant containing cytoplasmic fraction was collected. The pellets were incubated with ice-cold Membrane Extraction Buffer containing protease inhibitors at 4°C for 10 minutes. Samples were cleared by centrifuging at 3000 g for 5 minutes 4°C, and the supernatant containing membrane fraction was collected. Equal volumes of extracted protein lysates were loaded and separated by SDS–PAGE and detected by immunoblotting.

For immunoblotting, equivalent amounts of cell lysates were separated on 4-12% gradient gels (Invitrogen), transferred to nitrocellulose membranes, and incubated with the indicated antibodies. The signal was visualized by chemiluminescence (Amersham Pharmacia Biotech) using an automated digital developer.

RAS activity was assessed using the RAS activation ELISA assay Kit (EMD Millipore). Cells were washed twice with ice-cold TBS and scraped on ice in Lysis Buffer containing protease and phosphatase inhibitor cocktails (Roche) and centrifugated for 10 minutes 16,000 g at 4°C. Glutathione-coated 96-well plate was incubated with GST-tagged RAF1-RAS Binding Domain (RBD). After incubation with 10 µg of protein lysate, the plate was washed and then incubated with anti-RAS antibody (Millipore, Clone RAS10), followed by secondary HRP-conjugated antibody. The signal was detected after addition of Chemiluminescent Substrate and recorded using Victor X3 Multi-label Plate Reader (PerkinElmer).

CDC42 activation assay was performed according to the manufacturer's protocol (Cytoskeleton, Inc). Cell were washed with cold PBS and lysed in Cell Lysis Buffer supplemented with Protease Inhibitor Cocktail (Cytoskeleton, Inc) and centrifugated for 2 min 10,000 g at 4°C. 400 µg of cell lysates was mixed with GST-tagged PAK-PBD agarose beads (Cytoskeleton, Inc) and incubated for 60 minutes at 4°C. The beads were washed with Wash Buffer once and eluted by boiling for 5 minutes in 2x Laemmli sample buffer. Eluted proteins were separated by SDS–PAGE and detected by immunoblotting.

Live imaging

To perform live imaging, cells were plated on Nunc™ Glass Bottom Dishes 27mm (Thermo Fisher) and transfected with tdTomato-Lifeact-7 using Lipfectamine 3000 (Thermo Fisher). For image acquisition, we used a Nikon TiE inverted A1R (+ HD resonant scanning upgrade) microscope with a 40x Oil objective (NA 1,3) equipped with an Okolab incubator at 37°C and 5% CO_2. The setup was controlled by NIS-Elements (NIS 5.11.01 build 1368a, Nikon Instruments Europe B.V.). The length, size, and the number of protrusions per cell per time point were analyzed using a so-called general analysis protocol (GA2) in NIS-elements (NIS 4.50.00 build 1117, Nikon Instruments Europe B.V.) using standard image processing procedures. In short, the images were deconvolved, and transfected cells were identified using an intensity threshold. Objects touching image borders and not fitting the expected cell size, like clusters or debris, were excluded. Consequently, images were segmented to identify the complete cells with the protrusions, the rough cell outline, and the protrusions separately. Complete cells were identified using a threshold and by closing holes in the objects. The rough outline of the cells was identified using a combination of smoothing, erosion, and dilation steps together with size exclusion filters to remove isolated parts of protrusions. The protrusions were selected by the differential of the rough outline and the complete cells. The intersection of the

protrusions and a dilated mask of the rough cell outline yielded the number of protrusions per cell.

Bioinformatic analyses

mRNA expression levels were estimated by RSEM (4) using GENCODE reference annotation of the human genome (GRCh38), version 22 (Ensembl 79). Differential expression analysis was performed using the DESeq2 R package. The significance was determined by FDR<0.01 (Figure 1) or FDR<0.05 (Figure 6) and fold change>1.5 (GEO Series GSE156211).

The expression microarray data of meningiomas were previously published in (5) (GEO Series GSE84263). The *KLF4* expression probe was compared between mutation groups using two-tailed Student's t-test. We also used The R package Limma was used to identify the differentially expressed genes. FDR<0.05 and a fold change>1.5 were used as cutoff to determine differentially expressed genes. Differentially expressed genes were used as input for the IPA core pathway analysis.

To rank the enrichment of the pathways among the multi-omics dataset, we created the overall enrichment score. For each pathway, the overall enrichment score was calculated by: $\frac{\text{Sum}(-\log10(\text{P value}))}{1/n}$, where sum(-log10(P value)) is the total sum of the -log10(P value) from IPA of all datasets used, n is the number of datasets of which the pathway is significantly enriched. The P value < 0.01 was used to determine the significance of the pathway enrichment. If the pathway was not significantly enriched in a dataset, the -log10(P value) was set to 0.

For identification of *TRAF7* mutations, paired-end reads have been aligned against the human reference genome hg19 using BWA (6). The SAMtools (7) was used to convert and sort the sam files output from BWA to bam files. The mpileup command from SAMtools further converted the sorted bam files to the pileup format as input for the germline SNPs calling using Varscan2 (8). SnpEff (9) was used to annotate the SNPs and predict the functional consequence of these SNPs.

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