# Inhibition of the DAPKs-L13a axis prevents a GAIT-like motif-mediated HuR insufficiency in melanoma cells

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## 2. Materials and methods

### *2.1 Plasmids*

*Plasmids for transient expression*

The overall strategy is based on the anticipated use of a MS2-BioTRAP approach [1]. The *Tagged-IRES* plasmid was a gift from Marian Waterman (Addgene, #35570) and initially sequenced (Fasteris, Genesupport). To restore the first (5') of the four tandem stem-loops which was found mutated, and to replace the upstream XbaI (Dam methylated) with an XhoI site, the PCR-amplification product obtained from *Tagged-IRES* using the Q5 High-Fidelity DNA Polymerase (M0491S, NEB) and XhoI-containing FR101 and PmeI-containing FR102 primers (Table S1), was XhoI/PmeI digested (FD0694, FD1344, ThermoFisher) and back-ligated (quick ligation kit, M2200S, NEB) within the same sites into *Tagged-IRES*, which was also MfeI digested (FD0753, ThermoFisher) to distinguish fragments. The obtained *4MS2 corrected XhoI Tagged IRES* plasmid was consequently deleted of the complete luciferase sequence and of a partial fragment of the 5'UTR LEF1 sequence.

Full-length human HuR long 5'-UTR-CDS-PAS2-3'UTR was made synthetized (GeneArt Custom Gene Synthesis, Invitrogen, ThermoFisher) and obtained in the *pMA-RQ* backbone plasmid and entirely sequenced and digested at various sites for validation. *MS2 stem-loop HuR$^{CDS}$* plasmid was obtained by cloning the Bsp119I/XhoI-digested PCR-amplified product obtained from *pMA-RQ HuR long 5'UTR-CDS-PAS2-3'UTR* using the Q5 High-Fidelity DNA Polymerase and Bsp119I-containing FR103 and XhoI-containing FR104, into Bsp119I/XhoI-digested *4MS2 corrected XhoI Tagged-IRES*, which was consequently deleted from the remaining LEF1 5'UTR sequence. To obtain an *MS2 stem-loop empty vector*, the *4MS2 corrected XhoI Tagged-IRES* was Bsp119I/XhoI-digested and blunt ligated (quick blunting kit, E1201S, NEB). *MS2 stem-loop long 5'UTR-PAS1 3'UTR*, *MS2 stem-loop short 5'UTR-PAS1 3'UTR*, *MS2 stem-loop long 5'UTR-PAS2nm 3'UTR* (nm for PAS1 non-mutated), and *MS2 stem-loop short 5'UTR-PAS2nm 3'UTR* plasmids were obtained by the same strategy using FR105/106, FR107/106, FR105/108 and FR107/108 respectively. MS2 stem-loops-deleted expression plasmids for all these constructs were obtained by XhoI/NotI digestion (FD0594, ThermoFisher) and blunt ligation (XhoI site restored) and were named *HuR$^{CDS}$*, *long 5'UTR-PAS1 3'UTR*, *short 5'UTR-PAS1 3'UTR*, *long 5'UTR-PAS2nm 3'UTR* and *short 5'UTR-PAS2nm 3'UTR*. To obtain an MS2 stem-loops-deleted empty control plasmid named *vector*, the *MS2 stem-loop HuR$^{CDS}$* plasmid was Bsp119I/XhoI-digested, and blunt ligated.

mCherry constructs were obtained using the *pcDNA3.1-mCherry* plasmid, a gift from David Bartel [2] (Addgene, #128744) and a NEBuilder HiFi DNA Assembly Cloning Kit (E5520S, NEB). PCR amplifications were performed using the Q5 High-Fidelity DNA Polymerase. *mCherry long 5'UTR-PAS1 3'UTR* was obtained by recombination between a *long 5'UTR-PAS1 3'UTR* amplification product with FR110/111 and a *pcDNA3.1-mCherry* amplification product with FR112/109. *mCherry short 5'UTR-PAS1 3'UTR* was obtained by recombination between a *short 5'UTR-PAS1 3'UTR* amplification product with FR110/111 and a *pcDNA3.1-mCherry* amplification product with FR112/109. *mCherry long 5'UTR-PAS2nm 3'UTR* was obtained by BamHI (FD0054, ThermoFisher)/XhoI digestion of both *mCherry long 5'UTR-PAS1 3'UTR* and *long 5'UTR-PAS2nm 3'UTR* and subsequent ligation. *mCherry short 5'UTR-PAS2nm 3'UTR* was obtained by BamHI/XhoI digestion of both *mCherry short 5'UTR-PAS1 3'UTR* and *short 5'UTR-PAS2nm 3'UTR* and subsequent ligation. mCherry empty vector named *mCherry* was obtained by recombination between a *vector* amplification product with FR113/114 and a *pcDNA3.1-mCherry* amplification product with FR115/116.

PAS1-mutated *mCherry long 5'UTR-PAS2 3'UTR* and PAS1-mutated *mCherry short 5'UTR-PAS2 3'UTR* constructs were obtained by site-directed mutagenesis (Q5 Site-directed mutagenesis kit, E0554S, NEB) using the FR117/118 on *mCherry long 5'UTR-PAS2nm 3'UTR* and *mCherry short 5'UTR-PAS2nm 3'UTR* respectively. PAS1-mutated *MS2 stem-loop long 5'UTR-PAS2 3'UTR* construct was obtained by site-directed mutagenesis using the FR117/118 on *MS2 stem-loop long 5'UTR-PAS2nm 3'UTR*. *MS2 stem-loop mCherry long 5'UTR-PAS2 3'UTR* was obtained by EcoRI/SacI (FD0274/FD1133, ThermoFisher) digestion of both *MS2 stem-loop long 5'UTR-PAS2 3'UTR* and *mCherry long 5'UTR-PAS1 3'UTR* and subsequent ligation.

*Hairpin (-)* mCherry long 5'UTR-PAS2 3'UTR and *hairpin (+)+A* mCherry long 5'UTR-PAS2 3'UTR mutants were obtained by site-directed mutagenesis of the *mCherry long 5'UTR-PAS2 3'UTR* using the FR119/120 and the FR121/122 respectively. The *MS2 stem-loop hairpin (-)* and *MS2 stem-loop hairpin (+)+A* constructs were obtained by BamHI/XhoI digestion of *MS2 stem-loop mCherry long 5'UTR-PAS2 3'UTR* and subsequent ligation

with the BamHI/XhoI digested product of *hairpin (-)* or *hairpin (+)+A* plasmids. All constructs described above were verified by digestion and sequencing.

*Plasmids for stable expression*

The *pQCXIP MS2-HB* plasmid was a gift from Marian Waterman (Addgene, #35573) [1]. The HuR coding DNA sequence (CDS) retroviral plasmid was obtained by cloning the NotI/EcoRI (FD0594, FD0274, ThermoFisher)-digested PCR-amplified product obtained from *pMA-RQ HuR long 5'UTR-CDS-PAS2-3'UTR* using the Q5 High-Fidelity DNA Polymerase and NotI-containing FR123 and EcoRI-containing FR124, into NotI/EcoRI-digested *pQCXIP MS2-HB.* The control *vector* retroviral plasmid was obtained upon NotI/EcoRI digestion and blunt ligation of *pQCXIP MS2-HB.* Both constructs were verified by digestion and sequencing.

*2.2 Cell lines and drugs*

The A375 *BRAFV600*-mutated cell line was purchased earlier from CLS Cell Lines Service GmbH and authentified, before being used for this project. The SK-MEL28 *BRAFV600*-mutated cell line (HTB-72) was previously purchased from ATCC. The A375 and SK-MEL28 cells were grown in DMEM media supplemented with 10% FBS, 2 mmol/l glutamine and 1% penicillin/streptomycin. The BRAF inhibitor vemurafenib (PLX4032, RG7204) was purchased from Selleckchem and dissolved at 10 mmol/l in dimethylsulphoxide (DMSO, storing concentration, renewed every six months). The DAPK inhibitor (4*Z*)-4-(3-Pyridylmethylene)-2-styryl-oxazol-5-one (CAS 315694-89-4, Calbiochem) [3] was purchased from Sigma-Aldrich and dissolved at 50 mmol/l in DMSO (storing concentration, light-protected until added to cells, stored less than six months).

*2.3 Adaptive regimens*

Adaptive regimens were conducted on the A375 and SK-MEL28 melanoma cells using the same protocol previously described [4]. Cells were exposed to incremental increases in vemurafenib during four weeks using a standardized mild selection protocol (week 1: 50 nM, week 2: 100 nM, week 3: 200 nM, week 4: 300 nM). This protocol was used in order to avoid cell mortality that might select mutated populations not reflecting the adaptive response and used in all experiments performed in this study with the exception of the experiment shown in Fig. 3A, in which the selection protocol was pushed further (week 5: 500 nM, week 6:600 nM) in order to maximize the slow/senescent growing phenotype of the cells and not miss differences in the expression level of the target genes being analyzed in the Co-IP experiment. For the DAPKi / adaptive regimens, cells were treated (in the treated arm) throughout the four weeks with the DAPK inhibitor at 5 µM (final concentration).

*2.4 Retrovirus/lentivirus-mediated stable expression*

Stable A375 cell line overexpressing HuR CDS ($A375^{HuR}$) or the control vector ($A375^{vector}$) cell lines were generated by retrovirus infection. Briefly, the HuR and vector corresponding retrovirus plasmids (section 2.1) were JetPRIME-transfected in the 293T packaging cells together with pBS-CMV-gagpol packaging plasmid (Addgene, #35614) and PGAG-VSVG plasmid. Supernatants were treated with Benzonase and filtered. Concentrated viral particles, obtained upon precipitation with PEG-it, were used to infect A375 cells (plated at $7.5x10^4$ / well in 24-well plates) followed by Puromycin (1 µg/ml) selection. Resistant cells were further amplified. RT-qPCR (FR137/138) was used to confirm HuR overexpression.

Stable A375 cell line expressing MS2-HB ($A375^{MS2HB}$) was generated following the same procedure using the pQCXIP MS2-HB retroviral plasmid. Cells were regularly tested by RT-qPCR (FR133/134) for MS2-HB expression. MS2-HB stable expression had no effect on cell expression distribution of mCherry constructs described in 2.1 (comparative transfections of A375 and $A375^{MS2HB}$ cells).

For the ERK activity live monitoring experiments, the stable $A375^{HuR}$ and $A375^{vector}$ cell lines were similarly transduced, except that a second generation psPAX2 packaging plasmids was used to complement the lentiviral pENTR-ERKKTRClover plasmid, a gift from Markus Covert [5] (Addgene, #59138). Since the cells were already Puromycin resistant, transduced cells were cell-sorted based on mClover expression and further amplified. Cells were regularly checked for green fluorescence.

*2.5 shRNA long-term L13a knockdown*

For long-term knockdown experiments, $A375^{MS2HB}$ cells (section 2.4) were chosen in order to perform both MS2-BioTRAP and RIP experiments in the same cells. Cells were seeded at $1.5x10^5$ per well in 1 ml medium free

of antibiotics w/o FBS in twelve-well plates. The next day, cells were infected o/n in 1 ml Polybrene containing medium (5 µg/ml) with $10^5$ IFU of either L13a shRNA (sc-97893-V) or control shRNA (sc-108080) lentiviral particles (Santa Cruz). Cell clones were obtained upon serial dilution and further amplified before being screened. RT-qPCR (FR131/132) was used to confirm L13a knockdown.

*2.6 Flow cytometry analysis and cell sorting*

For transient mCherry expression analyses, A375 cells were seeded at 4.5 x$10^5$ per well in six-well plates 24h before being transfected and maintained overnight in media without penicillin/streptomycin. Transfection were performed the next day using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Five µg of indicated plasmid DNA for the largest construct and lower amounts for smaller constructs were used in order to ensure an equimolar amount of plasmids being transfected (constructs sizes varying between 2791 and 6013 bp). Forty-eight hours post-transfection, cells were harvested in PBS following trypsinization, stained with DAPI (0.2 µg/ml, D1306, Invitrogen), filtered and analyzed on an Attune NxT optical configuration analyser (ThermoFisher) using a YL2 (561nm) laser with a 620/15 filter for mCherry expression analysis and a VL1 (405 nm) laser with a 440/50 filter for DAPI dead cells detection. Data were analyzed in the FlowJo software. Comparisons were performed on an equal number of live singlets. For the DAPKi experiments, cells were transfected with indicated plasmids and exposed 24h before being harvested (24h post-transfection) to the DAPKi at 5 µM (final concentration).

For cell sorting experiments, adapted (to vemurafenib) A375 cells were transfected in three six-well plates (18 wells) with the *mCherry long 5'UTR-PAS2 3'UTR* construct (section 2.1), in order to ensure, based on preliminary experiments, at least 3 to 5 x $10^5$ cells in each HuR low and high cell subpopulations. Cells were sorted on two different cell sorters (BD FACSAria II and Biorad S3) and were either frozen (-80°C) for subsequent RNA extraction or plated again at $10^5$ per well in six-well plates for exposure to actinomycin D (5 µg/ml, A9415, Sigma) and to vemurafenib (300 nM corresponding to the final concentration used in the adaptive regimen) during 4 and 8h. These cells were frozen in turn for subsequent RNA extraction and mRNA half-life determination (section 2.9). Final analysis was conducted on the cells obtained from one of the two cell sorting experiments for which the higher cell yield at the fastest speed (< 90 minutes, Biorad S3 in our hands) was obtained in order to reduce as much as possible any effect of vemurafenib withdrawal during the cell sorting procedure.

For HuR expression analyses, following trypsinization, cells were counted, washed (PBS), fixed in 1.5% formaldehyde solution (10 min at room temperature) and permeabilized with 100% ice-cold methanol (10 min). After being washed (x2, PBS. 1% BSA), cells were re-suspended in 100 µl PBS 1% BSA and incubated (30 min) with the alexa 488-conjugated anti-HuR mouse monoclonal 3A2 antibody (sc-5261, AF488, Santa Cruz, 1:20) or a similar concentration of alexa 488-conjugated isotype control (sc-3890, Santa Cruz), washed (x3, PBS) and resuspended in 200 µl of PBS. Filtered cells were analyzed on an Attune NxT optical configuration analyser (ThermoFisher) using a BL1 (488 nm) laser with a 526/52 filter. Data were analyzed in the FlowJo software. Comparisons were performed on an equal number of singlets.

*2.7 Clonogenic assays*

For clonogenic assays, cells were plated at 1x$10^3$ or 2x $10^3$ per well in six-well plates. For DAPKi experiments, DAPKi was added (or not) to cells when being seeded (5 uM). After 24h, cells were treated with vemurafenib (and DAPKi in corresponding experiments) at the indicated concentrations or DMSO. The medium with corresponding treatments was changed twice a week. After 2-3 weeks depending on the experiment, A375 cells were washed with PBS, fixed with 4% PFA and stained with 0.5% (w/v) crystal violet in 70% ethanol for 15 min. The wells were washed with distilled water, dried and photographed with a macro lens.

*2.8 Reverse transcription-quantitative PCR and 3'RACE*

Total RNA was purified from cultured cells using a Quick-RNA Miniprep Kit, (Zymoresearch, R1055) according to the manufacturer's instructions. A DNAse digestion step on column was used as recommended to remove genomic DNA contamination (Zymoresearch, E1010). Recovered RNA was quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific). Reverse transcription was carried out using Improm-II reverse transcriptase (Promega, A3802) and random hexamer primers (Promega, C1181) at 42°C for 1h. Recombinant RNasin Ribonuclease inhibitor was added to the reaction (Promega, N2515). cDNA samples were analyzed by quantitative PCR using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, A25776) and a StepOnePlus real Time PCR system (Applied Biosystems). Primers used for qPCR are listed in Table S1. The

expression of targets was normalized to GAPDH (FR127/128) or UFD1L (FR129/130). Importantly, we screened a large number of housekeeping genes (GAPDH, UFD1L, HPRT1, ACTB, RPLP0, TBP, B2M, 18S) and found that GAPDH and UFD1L were the most stable ones; however, regarding experiments in which parental cells were compared to adapted cells (adaptive regimen to vemurafenib), we noticed that UFD1L expression was even more stable than GAPDH, UFD1L was therefore used thereafter in these experiments. All qPCR reactions regarding HuR transcripts detection were initially validated for their efficiency and single signal amplification, and were size verified (agarose gel migration) and sequenced.

For 3' RACE analyses, the reverse transcription reaction was performed using a 3' RACE Adapter (FR145) initially obtained from the FirstChoice™ RLM-RACE Kit (AM1700M, ThermoFisher) and thereafter synthetized, which includes an oligo(dT) sequence in addition to a sequence specific for the outer reverse primer used in the subsequent qPCR reactions (FR150). 3'RACE RT-qPCR (FR148/150, FR149/150) analyses were validated by transfection of the PAS variants constructs (section 2.1) and further sequencing of the amplicons (FR151/153 and FR152/153). Sequencing of the 3' RACE-generated amplicons (FR146/150 and FR147/150) was also used to confirm the ESTs-based annotated HuR PAS1 and PAS2 cleavage sites in endogenous transcripts.

*2.9 mRNA half-life determination*

For endogenous transcripts mRNA half-life determination, at the end of the adaptive regimen, cells were plated at 7x $10^5$ in six-well plates the day before exposure to actinomycin D at the final concentration of 5 µg/ml and total RNA was extracted at the indicated time point using the Quick-RNA Miniprep Kit. RT-qPCR was performed with HuR CDS (FR137/138), PAS1 (FR139/140), PAS2 (FR141/142) and PAS4 (FR143/144) primers. The data was normalized to the UFD1L RT-qPCR (FR129/130) signal (section 2.8) and plotted as a function of the actinomycin D treatment time and fitted to a single linear regression function. For exogenous transcripts mRNA half-life determination, cells were transfected as described (section 2.6) with the indicated constructs, and 48h later or following cell sorting, exposed to actinomycin D. RNA extraction was performed as described above followed by RT-qPCR analysis using mCherry (FR125/126) primers. mCherry amplification was chosen in order to ensure a similar amplification efficacy among transcripts being compared. c-myc mRNA half-life (FR135/136) was measured as a positive control (short half-life). The data was normalized to the GAPDH or the UFD1L RT-qPCR signal.

*2.10 RNA-seq analyses and CAGE-seq data visualisation.*

A375 cells were submitted to the adaptive regimen described in 2.3. Parental and adapted cells were maintained in culture in independent biologic triplicates. Following RNA extraction, RNA was quantified by a Qubit (Life Technologies) and RNA integrity was assessed with a Bioanalyser (Agilent Technologies). The Illumina TruSeq stranded total RNA (Ribo-Zero Gold) kit was used for library preparation with 500 ng of total RNA as input. Library molarity and quality were assessed with the Qubit and TapeStation (Agilent Technologies). Paired-end 100 sequencing was performed on an Illumina HiSeq4000 with the TruSeq SBS chemistry. FastQ reads were mapped to the ENSEMBL reference genome (GRCh38.89) using STAR version 2.4.0j with standard settings, except that any reads mapping to more than one location in the genome were discarded ($m = 1$). A unique gene model was applied to quantify reads per gene. Briefly, the model considers all annotated exons of all annotated protein coding isoforms of a gene to create a unique gene where the genomic region of all exons are considered coming from the same RNA molecule and merged together. All reads overlapping with the exons of each unique gene model were reported using featureCounts version 1.4.6-p1. BAM and BAM.BAI files were imported to Integrative Genome Viewer (IGV) for visualisation. Similarly, BAM and BAM.BAI files available from CAGE-seq databases (FANTOM 5 CAGE database) [6] were imported in IGV and compared with the UCSC Genome Browser database.

https://fantom.gsc.riken.jp/5/datafiles/reprocessed/hg38_v8/basic/

https://genome-euro.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr19%3A8005595%2D8005660&hgsid=287286161_er133dD0WdFqgVOKa7vVatYDNmwV

*2.11 Western blot analysis*

Generally, cell lysates were prepared using RIPA buffer (PBS, 1% IGEPAL®, 0,5% sodium deoxycholate, 0.1% SDS) supplemented with fresh protease (cOmplete mini EDTA-free tablets, Roche) and phosphatase inhibitors (phosSTOP, Roche) complemented with Na3VO4 10 mM, NaF50 mM for phospho stainings. Cells were washed with PBS and scrapped in cold RIPA buffer. Recovered homogenates were chilled on ice, vortexed, chilled again and sonicated, the same cycle was then repeated followed by high-speed centrifugation. Recovered supernatants were quantified for protein content using Bio-Rad DC kit (500-0116). Protein samples (5-12 µg depending on the experiment) were resolved on 4-12% polyacrylamide Bis-Tris gel (NuPAGE, ThermoFisher) and transferred to nitrocellulose membrane using an iBlot® Dry Blotting system (Invitrogen, life technologies), according to the manufacturer's instructions. After saturation (PBS, 0.1% Tween, 5% BSA or milk, 30 min), the membranes were incubated either for 1h or o/n with the primary antibody (see below). The membranes were then incubated for 45 min with a secondary goat anti-mouse or goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, catalogue n°170-6516 and 170-6515 respectively, 1:3000), washed and developed using SuperSignal WestPico Plus chemiluminescent substrate (ThermoFisher) according to manufacturer's instructions and the ImageQuant LAS4000 (GE Healthcare).

For most conducted western blot analyses in which the adaptive response was potentially modulated (HuR stable overexpression, L13a knockdown and vemurafenib/DAPKi combined chronic exposure), cells were washed with serum-free medium and synchronized o/n in their medium containing 0.1% serum and ± vemurafenib at the final concentration used in the adaptive regimen. Similarly, DAPKi (5 µM) was ± added in DAPKi experiments. This conditioning is justified to avoid any additional perturbation induced by vemurafenib or DAPKi withdrawal. The next morning, cells were treated in some experiments with vemurafenib at the indicated concentration; in that case, DAPKi treatment was maintained during the six-hour stimulation in corresponding conditions in the DAPKi experiments. The following primary antibodies were used: alpha Tubulin (mouse, DM1A, Abcam, ab-7291, 1:5000), EPRS (rabbit, polyclonal, epitope recognized maps to a region between residue 950 to 1000 of human EPRS, Bethyl Laboratories, A303-959A-1, 1:1000); following antibodies were purchased from Santa Cruz and used at 1:1000: HuR (mouse, 3A2, 5261), HSP90α/β (mouse, F-8, 13119), p53 (mouse, DO-1, 126), L13a (mouse, C-11, 390131), ZIPK (mouse, C-3, 514223); following antibodies were purchased from Cell Signaling and used at 1:1000: MEK1/2 (mouse, L38C12, 4694), phospho-Ser217/221-MEK1/2 (rabbit, 41G9, 9154), ERK (rabbit, polyclonal, 9102), phospho-Thr202/Tyr204-ERK (rabbit, 20G11, 4376).

*2.12 Protein half-life determination*

Parental and adapted A375 and parental SK-MEL28 melanoma cells were plated at 4x $10^5$ per well in six-well plates the day before being exposed to cycloheximide (100 µg/ml) for indicated time points. Adapted cells were maintained in medium containing vemurafenib at the final concentration used in the adaptive regimen. The cycloheximide ± vemurafenib-containing medium was renewed at 24h. Cells lysates were prepared as described (section 2.11). Western blots were performed with the HuR 3A2 antibody. Tubulin stainings were performed as loading control. p53 expression was used as a positive control (short half-life).

*2.13 Co-immunoprecipitation and LC-MS analysis*

The conditioning of A375 cells for co-immunoprecipitation is described above (section 2.3). The co-immunoprecipitation was performed using the anti-L13a antibody (section 2.11) and the Pierce™ MS-Compatible Magnetic IP kit (90409, ThermoFisher) according to the manufacturer's instructions. The adaptive regimen was performed on biologic duplicates. A mouse IgG was used as a negative control (Santa-Cruz, 2025). Following protein dosage of cell lysates, a total of 1 mg of protein was immunoprecipitated with 5 µg of L13a and control antibodies. For ESI-LC-MSMS analyses, samples were dried under speed-vacuum and resuspended in 100 µl of 6M urea in 50 mM ammonium bicarbonate (AB). 2µl of Dithioerythritol (DTE) 50 mM were added for reduction during 1h at 37°C. Alkylation was performed by adding 2 µl of iodoacetamide 400 mM during 1h at room temperature in the dark. Urea concentration was reduced by addition of 500 µl of AB. Samples were digested using 10 µl of freshly prepared trypsin (Promega) at 0.1 µg/µl in AB, overnight at 37°C. After being dried under speed-vacuum, samples were desalted with a C18 microspin column (Harvard Apparatus, Holliston, MA, USA) according to the manufacturer's instructions and completely dried under speed-vacuum before being stored at -20°C. Before ESI-LC-MSMS analyses, samples were diluted in 20 µl of loading buffer (5% $CH_3CN$, 0.1% FA). 4 µl were injected on column. The LC-ESI-MS/MS was performed on an Orbitrap Fusion Lumos Tribrid mass

spectrometer (ThermoFisher Scientific) equipped with an easy nLC1200 liquid chromatography system (ThermoFisher Scientific). Peptides were trapped on an Acclaim pepmap100, C18, 3µm, 75µm x 20mm nano trap-column (ThermoFisher Scientific) and separated on a 75 µm x 500 mm, C18 ReproSil-Pur (Dr. Maisch GmBH), 1.9 µm, 100 Å, home-made column. The analytical separation was run for 90 min using a gradient of H2O/FA 99.9% /0.1% (solvent A) and CH3CN/FA 80% /0.1% (solvent B). The gradient was run as follows: 0-5 min 95 % A and 5 % B, then to 65 % A and 35 % B in 60 min, and finally to 5% A and 95% B in 10 min with a stay for 15 min at this composition. Flow rate was of 250 nl/min. Data-dependant analysis was performed with MS1 full scan at a resolution of 120'000 FWHM followed by as many subsequent MS2 scans on selected precursors as possible within 3 second maximum cycle time. MS1 was performed in the Orbitrap with an AGC target of 4 x 105, a maximum injection time of 50 ms and a scan range from 400 to 2000 m/z. MS2 was performed in the ion-trap with an AGC target at 1 x 104 and a maximum injection time of 35 ms. Isolation window was set at 1.2 m/z and 30% normalised collision energy was used for higher-energy collisional dissociation. Dynamic exclusion was set to 20s.

Peak lists (MGF file format) were generated from raw data using the MS Convert conversion tool from ProteoWizard. The peaklist files were searched against the Human reference proteome database (Uniprot, reviewed, release 2022_03, 20361 entries) and an in-house database of common contaminant using Mascot (Matrix Science, London, UK; version 2.6.2). Trypsin was selected as the enzyme, with one potential missed cleavage. Precursor ion tolerance was set to 10 ppm and fragment ion tolerance to 0.6 Da. The Mascot search was validated using Scaffold 5.1.2 (Proteome Software). Peptide identifications were accepted if they could be established at a greater than 8.0 % probability to achieve an FDR less than 0.1 % by the Percolator posterior error probability calculation. Protein identifications were accepted if they could be established at greater than a 98.0 % probability to achieve an FDR less than 1.0 % and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Based on duplicated results, the Normalized Spectral Abundance Factor (NSAF) was used for quantification. For immunoblot analysis, both the input total cell lysates and immunoprecipitated samples were analyzed as described (section 2.11) using the indicated antibodies.

*2.14 Ribonucleoprotein immunoprecipitation (RIP)*

For EPRS RIP experiments, we used the previously reported protocol [7] (see the reference for the composition of buffers). shCtrl and L13a knockdown A375 cells were transfected according to the protocol described (section 2.6) with the *mCherry long 5'UTR-PAS2 3'UTR* (WT named in figures *hairpin (+))* and *hairpin (-)* constructs as well as the empty *vector* (section 2.1). The mCherry constructs were chosen to ensure a similar final RT-qPCR quantification of transcripts based on mCherry expression detection. After 48h, cells were trypsinized and counted. Pellets from $2x10^6$ cells were washed with 1 ml ice-PBS and stored at -80°C. Preswollen Protein A (Dynabeads Protein A for Immunoprecipitation, Invitrogen, 10001D) were prepared in NT2 buffer (75 µl per sample) with repeated washes, suspended in NT-2 buffer and tumbled o/n at +4°C with 5 µg of the anti-EPRS antibody (section 2.11) or a control antibody (Invitrogen, 31887). The next morning, bead/antibody slurries for each sample were centrifuged (15 s at 5000xg) and repeatedly washed (x6) in the NT-2 buffer and suspended in 900 µl of NET-2 buffer. Thawed cell pellets were mixed with 1 volume (equivalent to the volume of the pellet) of polysome lysis buffer (PLB), kept on ice, vortexed regularly and centrifuged (20,000xg for 10min, +4°C). 100 µl of the upper layer of the lysates were then mixed with previously prepared bead /antibody containing NET-2 buffer (900 µl) and quickly centrifuged. 100 µl of the samples were kept as "total RNA" analysis and the remaining were tumbled o/n at +4°C. The next morning bead samples were washed six times with ice cold NT-2 buffer and resuspended in 150 µl of Proteinase K buffer. Similarly, the total RNA samples were mixed with 36 µl of NT-2 buffer to which 15 µl of 10% SDS and 9 µl of Proteinase K were added. IP and total RNA samples were incubated at 55°C. After 30 min, samples were quickly centrifuged and mixed with an equal volume (150 µl) of buffer-saturated acid phenol-chloroform (pH 4.5) with isoamyl alcohol, vortexed and centrifuged (20,000xg for 10 min at +4°C). The aqueous upper layer was removed and mixed with 150 µl of chloroform, vortexed and centrifuged (10,000g for 10 min at RT). The supernatants were recovered and mixed with 50 µl of 5M ammonium acetate, 15 µl of 7.5 M LiCl, 1.25 µl of 20 mg/ml glycogen and 1 ml of cold 100% ethanol and frozen for 30 min at -80°C. Samples were then again centrifuged (20,000g for 30 min at +4°C) and the obtained RNA pellets were washed with 80% ethanol, centrifuged (20,000g at +4°C), dried in a vacufuge concentrator and resuspended in 15 µl of RNAase-free water. We initially analyzed the samples with RT-qPCR following the protocol described (section 2.8) and determined the optimal CT values (linear amplification range) for each target gene (CT values of 23 for mCherry and L13a

and of 27 for UFD1L). The PCR reactions shown in Fig 3G were performed using these optimal CT values. Samples were run on an agarose gel for relative visual quantification. These visual and more illustrative results reflected the qPCR results initially obtained.

*2.15 RNA pull-down / MS2-BioTRAP*

Overall, we used a reported protocol [1]. shCtrl A375$^{MS2HB}$ cells and MS2-HB non-expressing cells (technical negative control) were transfected according to the protocol described (section 2.6) with the tagged stem-loops-containing RNAs-expressing constructs (section 2.1) i.e. the *MS2 stem-loop mCherry long 5'UTR-PAS2 3'UTR* (WT named in figures *hairpin (+))*, the *MS2 stem-loop hairpin (-)* and the *MS2 stem-loop empty vector*. The mCherry constructs were chosen in order to distinguish in the final western blot analyses, exogenous from endogenous HuR expression. A minimum of twelve wells (two six-well plates) were transfected for each variant in order to obtain, based on preliminary experiments, strong enough final protein signals. After 48h, cells were washed with cold PBS, immersed in 450 µl of PBS (per well) and UV cross-linked on ice in a 1800 Stratalinker (400 mJ/cm$^2$). Cells were then harvested by scraping and collected in a cold tube by centrifugation. Cell pellets (recovered from twelve wells) were lysed in 600 µl of native lysis buffer (100mM NaCl, 50mM Tris, 5mM $MgCl_2$, 10% glycerol, 0,5% Igepal, 5mM EDTA, 5mM EGTA, RNasin 40U/µl, 1mM PMSF, 50mM NaF, 0,1mM Na3VO4, 0,5mM β-mercaptoethanol, protease inhibitor tablet and phosSTOP phosphatase inhibitors), left on ice (5 min at 1$^{st}$ wash, 2 min at 2$^{nd}$ wash) and vortexed, twice, sonicated, left on ice (5 min at 1$^{st}$ wash, 2 min at 2$^{nd}$ wash) and vortexed, twice, and finally high-speed centrifuged (10min, +4°C). Protein dosage was performed on supernatants in order to proceed to affinity purification on 2 mg of total recovered protein.

For affinity purification, 800 µl (for six samples / conditions) of streptavidin-coated beads (Dynabeads M-280, 11205D, ThermoFisher) were prepared as follows: beads were mixed with 1 ml of cold PBS in a 1.5 ml microfuge tube, washed first with 800 µl of PBS, twice with 1 ml of 0,1M NaOH, 0,05M NaCl, DEPC $H_2O$ cold solution (solution A, tumbled for 2 min), then with 800 µl of 0.01M NaCl, DEPC $H_2O$ cold solution (solution B, tumbled for 2 min) and resuspended in 800 µl of solution B. Subsequently, beads were washed three times in 1ml of cold native lysis buffer (tumbled for 2 min at each wash) and resuspended in 800 µl of native lysis buffer. Each 2 mg of total recovered protein was then mixed with 100 µl of prepared beads and tumbled for 5 min at +4°C. Unbound supernatants were removed and stored at -20°C. Recovered beads were further washed six times with 2 ml of cold native lysis buffer (tumbled for 2 min at each wash). Each sample was resuspended in Laemmli solution + 1x reducing agent (NuPAGE sample reducing agent, NP0004, ThermoFisher) and denatured at 70°C (10 min). Magnet was applied and elution products were stored at -20°C for western blot analysis.

Western blot analyses were performed on both mRNA-bound and total proteins. To improve detection, L13 antibody was used at 1:250 on mRNA-bound-loaded membranes. Importantly, mRNA-bound HuR was analyzed as a negative control not being affected in its interaction with its own hairpin (-)-mutated transcript (see Fig. S3 legend); but since its endogenous signal was also detected in the *MS2 stem-loop empty vector*-transfected cells above the background signal observed in the MS2-HB-non-expressing cells (technical background), HuR interaction with the MS2 stem-loops SV40 PAS backbone-generated transcript (biological background), could not be excluded. Nevertheless, the HuR endogenous signal was a valid assessment of the "general" technical and biological background to ensure that the mRNA-bound L13a signal in the hairpin (+) / and the hairpin (-) /-transfected MS2-HB cells could be compared. Moreover, considering that in an experiment as the one shown in Fig. 3I, the technical background in MS2-HB non-expressing cells is very low, the signal observed in MS2-HB expressing cells can almost entirely be attributed to mRNA-bound proteins; since (i) the occupation at any site on mRNAs (regardless of being or not on HuR mRNA sequence), by exogenous and endogenous mRNA-bound HuR protein species is assumed to occur proportionally to their respective concentration, and (ii) in the *MS2 stem-loop hairpin (+)* (or *MS2 stem-loop hairpin (-))*-transfected cells, the endogenous species constitute only a fraction of the mRNA-bound HuR proteins, a similar HuR endogenous signal between the *MS2 stem-loop empty vector*-transfected cells and the *MS2 stem-loop hairpin (+)* (or *MS2 stem-loop hairpin (-)*)-transfected cells, indicates that the amount of bound HuR endogenous species is far more important in these latter.

For the initial LC-MS MS2-BioTRAP analysis, adapted to vemurafenib A375 cells were transfected with either the *MS2 stem-loop mCherry long 5'UTR-PAS2 3'UTR* plasmid (section 2.1) or with the untagged equivalent (*mCherry long 5'UTR-PAS2 3'UTR*, negative control) and the subsequent procedure was performed as described above, except that following the final wash with cold lysis buffer, samples were further washed three times in ice-cold 50 mM $NH_4HCO_3$ and resuspended in 20 µl ice-cold 50 mM $NH_4HCO_3$ before being prepared for ESI-LC-MSMS analysis as described in section 2.13. LC-ESI-MS/MS analysis was performed with a similar (to section

2.13) but adapted protocol on a Q-Exactive HF Hybrid Quadrupole-Orbitrap Mass-Spectrometer (ThermoFisher Scientific) equipped with an Easy nLC 1000 liquid chromatography system. Data analysis was performed as described in section 2.13.

*2.16 ERK activity live monitoring-kinase translocation reporter assay*

A375$^{HuR}$ and A375$^{vector}$ cells stably-expressing ERK-KTR (section 2.1 and 2.4) were plated at 1.5 x$10^4$ in 96-well flat clear bottom black polystyrene plates (Corning n°3603) pre-coated with 10 ug/ml of fibronectin as described [5]. The next day, cells were washed and stained with SPY650-DNA (sc501, Spirochrome) diluted at 1/1000 in imaging medium containing 10% of FBS (100 µl per well). Cells were not synchronized to maintain physiologic heterogeneity of ERK activity at steady-state. Images were acquired on a ImageXpress widefield scanner device (Molecular devices) with temperature controller with $CO_2$ and humidity delivery, every 5 min during 24h (7 images per well at 20X), even though the final analysis was performed on 400 min of image acquisition (see below). For mClover signal measurements, an excitation filter with 475/28 nm transmission and an emission filter with 520/35 nm transmission were used. For SPY650 nucleus signal detection, an excitation filter with 637/12 nm transmission and an emission filter with 692/40 nm transmission were used. Brightfield images were also acquired at the same frequency. Initial image/data acquisition was performed in the MetaMorph software (Molecular devices) and were first exported and visualized in Imaris 9.8. Selected frames were further exported in QuPath-03.2. Analyses were performed on images acquired within a 400 min timeframe during the first 8 h of live monitoring in order to avoid as much as possible overlapping cells due to confluency and additional uncontrolled effects of culturing conditions and phototoxicity. To avoid any bias, analyses were performed on all single cells that were detected in 15 cells containing areas. The areas were chosen as to contain the smallest number of cells either dying or undergoing cell division within the 400 min timeframe. Signal quantification was performed in manually depicted region of interest of the same size (ROI) in the nucleus and the cytoplasm of cells at each frame. Data were further imported to a MATLAB script for background subtraction, cytoplasmic ROI mean intensity / nuclear ROI mean intensity (ratio) calculation and graph generation. Final images and movies were generated in the Fiji software.

*2.17 RNA secondary structure prediction and sequence alignments*

For RNA secondary structure prediction, both free energy minimization and the frequency in the Boltzmann-weighted ensemble of structures of the centroid [8] were estimated using the Vienna RNA Websuite / RNA webserver. Diagrams were generated using Forna (force-directed RNA). Predictions were also performed using the RNAstructure, 6.4 package (Mathews lab) particularly to predict structures common to multiple sequences. Sequence alignments were performed using Clustal Omega (EMBL-EBI) and were further hand curated.

## References

[1] B.P. Tsai, X. Wang, L. Huang , et al., Quantitative profiling of in vivo-assembled RNA-protein complexes using a novel integrated proteomic approach, Mol. Cell. Proteomics 10 (2011) M110 007385.
[2] B. Kleaveland, C.Y. Shi, J. Stefano, A network of noncoding regulatory RNAs acts in the mammalian brain, Cell 174 (2018) 350-362.
[3] M. Okamoto, K. Takayama, T. Shimizu, et al., Structure-activity relationship of novel DAPK inhibitors identified by structure-based virtual screening, Bioorg. Med. Chem. 18 (2010) 2728-2734.
[4] R. Merat, A. Bugi-Marteyn, L.J. Wrobel, et al., Drug-induced expression of the RNA-binding protein HuR attenuates the adaptive response to BRAF inhibition in melanoma, Biochem. Biophys. Res. Commun. 517 (2019) 181-187.
[5] S. Regot, J.J. Hughey, B.T. Bajar, et al., High-sensitivity measurements of multiple kinase activities in live single cells, Cell 157 (2014) 1724-1734.
[6] I. Abugessaisa, S. Noguchi, A. Hasegawa, et al., FANTOM5 CAGE profiles of human and mouse reprocessed for GRCh38 and GRCm38 genome assemblies, Scientific Data 4 (2017) 170107.
[7] S. Jayaseelan, F. Doyle, S. Currenti, et al., RIP: An mRNA localization technique, Meth. Mol. Biol. 714 (2011) 407-422.
[8] Y.E. Ding, C.Y. Chan, C.E. Lawrence, RNA secondary structure prediction by centroids in a Boltzmann weighted ensemble, RNA 11 (2005) 1157-1166.