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Supplemental Information

Tumor Suppression of Ras GTPase-Activating Protein RASA5 through Antagonizing Ras Signaling Perturbation in Carcinomas

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Supplementary Information

Transparent Methods

Array-Comparative Genomic Hybridization (aCGH)

Whole-genome arrays of 1-Mb resolution with 3,040 BAC/PAC clones were kindly provided by Dr C. Langford at the Wellcome Trust Sanger Institute, Cambridge, UK (http://www.sanger.ac.uk/Projects/Microarrays/), with clone details listed in Ensembl database (www.ensembl.org/Homo sapiens/index.html). Array CGH was performed as described previously (Ying et al., 2006).

Cell lines, tumor samples and drug treatments

Multiple carcinoma cell lines and immortalized normal epithelial cell lines were purchased from ATCC and cultured under standard conditions. Human normal adult tissue RNA samples were purchased commercially. Archived DNA samples of paired Chinese ESCC (T) and adjacent surgical marginal tissues (N), NPC, normal nasopharynx and esophagus tissues, breast cancer tissues, and normal breast tissues were used (Jin et al., 2007).

For Aza treatment, cells were seeded at a density of 1×10^5 cells/ml and incubated overnight. Medium was then replaced with fresh medium containing Aza (Sigma-Aldrich, St Louis, MO) at a final concentration of 5-10 μM. Cells were allowed to grow for 72 h, with changing of Aza-containing medium every 24 h. For combined treatment of Aza with TSA, cells were treated with TSA (100 ng/ml in DMSO) for additional 24 h after Aza treatment. MEK inhibitor PD98059 (Sigma-Aldrich, St Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) to reach a concentration of 20 μM for cell treatment. MEK inhibitor U0126 (Cell Signaling, #9903) was dissolved in DMSO to reach a concentration of 10 μM for cell treatment.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). cDNA products were amplified with Go-Taq polymerase for 32 cycles (Promega, Madison, WI), with *GAPDH* as a control for RNA integrity. The sequences of primer sets and reaction systems was listed in Table S3, S4.

Bisulfite treatment and promoter methylation analysis

Bisulfite modification of DNA, MSP and BGS were carried out as previously described (Jin et al., 2007). The sequences of primer sets used for MSP and BGS were listed in Table S3. MSP was performed for 40 cycles using AmpliTaq Gold (Applied Biosystem, Foster City, CA). For BGS, amplified fragments were TA-cloned into PCR4-Topo vector (Invitrogen, CA) with 6-8 colonies randomly chosen and sequenced.

Construction of plasmids and mutagenesis

Primer sequences for cloning PCR were listed in Table S3. AccuPrime™ Taq DNA Polymerase was used for all PCR reactions. Full-length open reading frame (ORF) sequence of *RASA5* was cloned from the adult brain cDNA library by PCR with a Flag-tag attached to the N-terminus. The product was then digested and ligated into the BamHI/XbaI site of pEGFP-C1 (Clontech) or pcDNA3.1 expression vector (Invitrogen, CA).

Three truncated variants: RASA5$^{\Delta GAP}$ with deletion of GAP-G domain (aa 392-729), GAP-L domain (aa 373-1343) and RASA5$^{\Delta C2\text{-}GAP}$ deficient of C2-GAP tandem (aa 259-1343) were generated by site-directed PCR with pcDNA3.1-Flag-RASA5 plasmid as the template. Sequences of each insert were confirmed by sequencing.

For RASA5 silencing, cells were transfected with two different siRNAs targeting RASA5 and scrambled control siRNAs using Lipofectamine 3000 (Invitrogen, CA) according to the manufacturer's instruction. siRNA RASA5-1 was from Invitrogen: Stealth RNAi for RASA5 (Cat#: HSS113025) (UUA CAU AGA ACA GGU CUU UAC CAC C) with a Stealth RNAi negative control; siRNA RASA5-2 was purchased from OriGene (Cat#: SR305827).

Immunofluorescence assay

Cells were transfected with indicated plasmids using Lipofectamine™ 3000 reagent (Invitrogen). 24 h after transfection, cells were fixed in 4% paraformaldehyde in PBS and permeabilized with Triton X-100 at room temperature (RT). Cells were incubated with primary antibodies at 4 ^{o}C overnight. After three washes with PBS, rhodamine- or FITC, or Alexa Fluor® 488, or Alexa Fluor® 555-conjugated secondary antibodies were added and incubated for 1 h. Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI) (Calbiochem, San Diego, CA). Coverslips

were mounted in DABCO and subjected to analyze with fluorescence microscope (Olympus, Japan). Antibodies used were listed in Table S5.

Colony formation assay

For monolayer colony formation assay, cells were plated in a 12-well plate (2×10^5 cells/well). After overnight culture, cells were transfected with either RASA5-expressing plasmids or empty vectors, using Lipofectamine 3000 (Invitrogen, CA). 48 h after transfection, cells were replated with an appropriate density in 6-well plates with G418 (0.4 - 1.2 mg/ml) selection for 7-12 days. BT549 and T47D cells were plated for colony formation assay, and then transfected with RASA5 siRNAs or control siRNAs, and treated with the indicated inhibitors 20 μM PD98059 and 10 μM U0126. The culture medium with siRNAs and MEK inhibitors were incubated for an additional 10-14 days, changed with fresh medium containing siRNAs and MEK inhibitors every three days. Surviving colonies (≥ 50 cells per colony) were visualized with Gentian Violet staining (ICM Pharma, Singapore) and manually counted.

For colony formation assay in soft agar, transfections were undertaken as in monolayer colony formation assay. 48 h post-transfection, cells were resuspended in full medium containing 0.4 mg/ml G418 and 0.3% soft agar, and then seeded at a density of 4 $X10^3$ cells into a 24-well plate. Colonies (>50 cells) were counted and photographed after 10-20 days. All the experiments were performed in triplicate wells and repeated for three times independently.

Protein preparation and Western blot

Cells were transfected as described above. 48 h post-transfection, cells were lysed in RIPA buffer. A total of 50 μg of protein extracts were separated by SDS-PAGE and transferred to Nitrocellulose membranes. After probed with primary antibodies, the membrane was incubated with secondary antibodies and was visualized using the ECL detection system (GE Healthcare). Antibodies used were listed in Table S5. All the images were cropped around the known molecular weight of the interested band and representative blots were shown. Each blot repeated independently three times with similar results. Image J was used to quantify blots and the quantitative data presented by the graph, with error bars and significance calculations.

Luciferase assay

Cells were transfected with a plasmid either empty or expressing RASA5, along with SRE-luc and Renilla luciferase plasmid (pRL-SV40). Cell lysate was assayed for determining the firefly and Renilla luciferase activities in succession using a Dual Luciferase kit (Promega, Madison, WI). Luciferase activity of firefly was normalized against that of Renilla. Each assay was performed in triplicate and repeated for three times.

Wound healing assay

Cells grown on 12-well plates were carefully wounded using a sterile pipette tip, washed once with fresh medium, and then supplemented with fresh medium. After incubation for 24 h and 48 h, wound closure was monitored under a phase contrast microscope. Each assay was performed in triplicate and all experiments were repeated for three times.

Matrigel invasion assay

In vitro Matrigel invasion assays were performed with BD BioCoat Matrigel Invasion Chambers (Transwell) (BD Biosciences, Heidelberg) in 24-well plates. Briefly, transfected cells were resuspended in serum-free medium at a density of 2.5 x10^4 cells/ml. The GFR Matrigel inserts were rehydrated by incubating with 0.5ml warm culture medium for 2 hours, and then 0.5 ml of cell suspension was added to the inserts. Full medium with 10% serum was added to the bottom chamber as a chemoattractant. After incubation for 24 h at 37ºC, cells were fixed and stained with Gentian Violet. Data represented the results of three independent experiments.

Ras activity assay

Active levels of Ras-GTP were determined by affinity precipitation using GST-Raf-RBD (Upstate Biotechnology, Inc., NY), a GST fusion protein containing the Raf/Ras-binding domain. Cells were transfected with pcDNA3.1-Flag-RASA5 plasmid or empty vector, together with or without an oncogenic active form of H-Ras cDNA (Q61L mutant) in pUSEamp (Upstate Biotechnology, Inc., NY), using Lipofectamine 2000 (Invitrogen, CA). 48 h after transfection, cells were incubated in medium containing 10% serum for 5 minutes following overnight serum starvation. Then cells were lysed and incubated with 10ul of Raf-1 RBD agarose for 30 minutes at 4°C with gentle rocking. Agarose beads were collected by pulsing and bound proteins were eluted with

SDS/PAGE sample buffer, resolved on 12% acrylamide gels, and subjected to Western blot analysis with specific Ras antibody.

Statistical analysis

All statistical analyses were performed in excel and using SPSS version 21.0. All data were generated from at least three independent experiments and represented as means ± SEM. Gene expression data were analyzed using log2 transformed and normalized. Log-rank test was used to compare the survival distributions of cancer patient groups with different levels of *RASA5* expression or methylation. β-value ranging from 0 to 1 was a measure of DNA methylation level for completely unmethylated to completely methylated. The Spearman correlation coefficient between *RASA5* methylation and gene expression were calculated in excel. Overall survival curves were plotted using Kaplan-Meier analysis. Unpaired Student's t-test was used to compare two experiment groups and 1-way ANOVA (with Newman-Keuls post-hoc test) was used to compare groups of three or more. When $p < 0.05$, data were considered to be statistically significant for all experiments.

Supplementary figure legends

Figure S1. Identification of RASA5 as a target of the 6p21.3 deletion, related to Figure 1. (A) Representative results of 1-Mb array-CGH of CNE2 and 5-8F carcinoma cell lines. Normalized log2 signal intensity ratios from -1 to 1 are plotted. Each single BAC clone is represented by a dark blue colored dot. The BAC clone containing the RASA5 locus (bA175A4) is labeled with the red dot, and also indicated by red rectangle in the top cytoband of Chr. 6p. Middle panel, the 6p21.31-6p21.32 gene-rich region shown as in Ensembl Genome Browser (www.ensembl.org/index.html), with the RASA5 gene highlighted by red rectangle. (B) Expression pattern of all candidate genes within 6p21.31-6p21.32 in representative tumor cell lines by semi-quantitative RT-PCR, with 3 normal tissues (testis, larynx and esophagus) used as controls. ESCC, esophageal squamous cell carcinoma; NPC, nasopharyngeal carcinoma.

Figure S2. Analysis of RASA5 expression in human tissues, related to Figure 1 and Figure 2. (A) *RASA5* mRNA expression in normal human tissues from GTEx, BioGPS, and CGAP SAGE databases. (B) Bar graph illustrating RASA5 expression levels by IHC in human normal tissues. Data were retrieved from Human Protein Atlas database (http://proteinatlas.org, version 17). N, not detected; L, low; M, medium; H, high. (C) RASA5 protein expression in normal tissues and tumor cell lines by Western blot using anti-RASA5 (Ab2).

Figure S3. *RASA5* promoter is frequently methylated in primary carcinomas, related to Figure 2. (A) *RASA5* was downregulated and methylated in carcinoma cell lines, detected by semi-quantitative RT-PCR and MSP, but expressed in immortalized non-transformed normal epithelial cell lines (with names green underlined). M, methylated; U, unmethylated; Ca, cancer; CRC, colon cancer. (B) Expression pattern of RASA family members in cancer cell lines as examined by semi-quantitative RT-PCR. *GAPDH* has been used as an internal control for RNA integrity (not shown here). Ca, cancer; CRC, colon cancer. (C, D, E) Representative MSP analyses of *RASA5*

promoter methylation in primary carcinomas. (F) BGS results confirmed the dense methylation of RASA5 in primary NPC and ESCC tumor samples but not in normal tissue samples. M, methylated; U, unmethylated. N, paired tumor-adjacent normal tissues; T, tumor.

Figure S4. RASA5 reconstitution triggers tumor cell apoptosis and suppresses Ras signaling as a RasGAP, related to Figure 3-6. (A) Re-expression of RASA5 induced the activation of caspase-3. HNE1 and KYSE150 carcinoma cells were co-transfected with EGFP-caspase-3-sensor reporter plasmid, together with either *RASA5*-expressing or empty vector and inspected 48 hours after co-transfection. Original magnification, 400x (upper), 100x (lower). Scale bar 200 μm. (B) Quantitative analyses of caspase-3-acitivated cells. Data were mean ± SEM (N=3), asterisk indicated $p < 0.05$). (C) Assessed by dual-luciferase reporter system. Ectopic expression of *RASA5* in both HEK293 and KYSE150 cell lines significantly downregulated activities of TOPFlash and AP-1-Luc. Data were presented as mean ± SEM of three independent experiments via Student's t test and representative data were shown. *, $p < 0.05$. (D) Altered subcellular localizations of p-ERK1/2 in HONE1 carcinoma cells with RASA5 re-expression. HONE1 cells were transfected with RASA5-construct or empty vector and immunolabled for p-ERK1/2 (red). Original magnification, 400x. (E) Nuclear translocation of p-ERK1/2 in T47D cells depleted of RASA5 expression. T47D cells were transfected with control or RASA5 siRNA, and then immunolabled for p-ERK1/2 (red). DAPI counterstaining (blue) represented cell nuclei. Original magnification, 400x. Scale bar 200 μm.

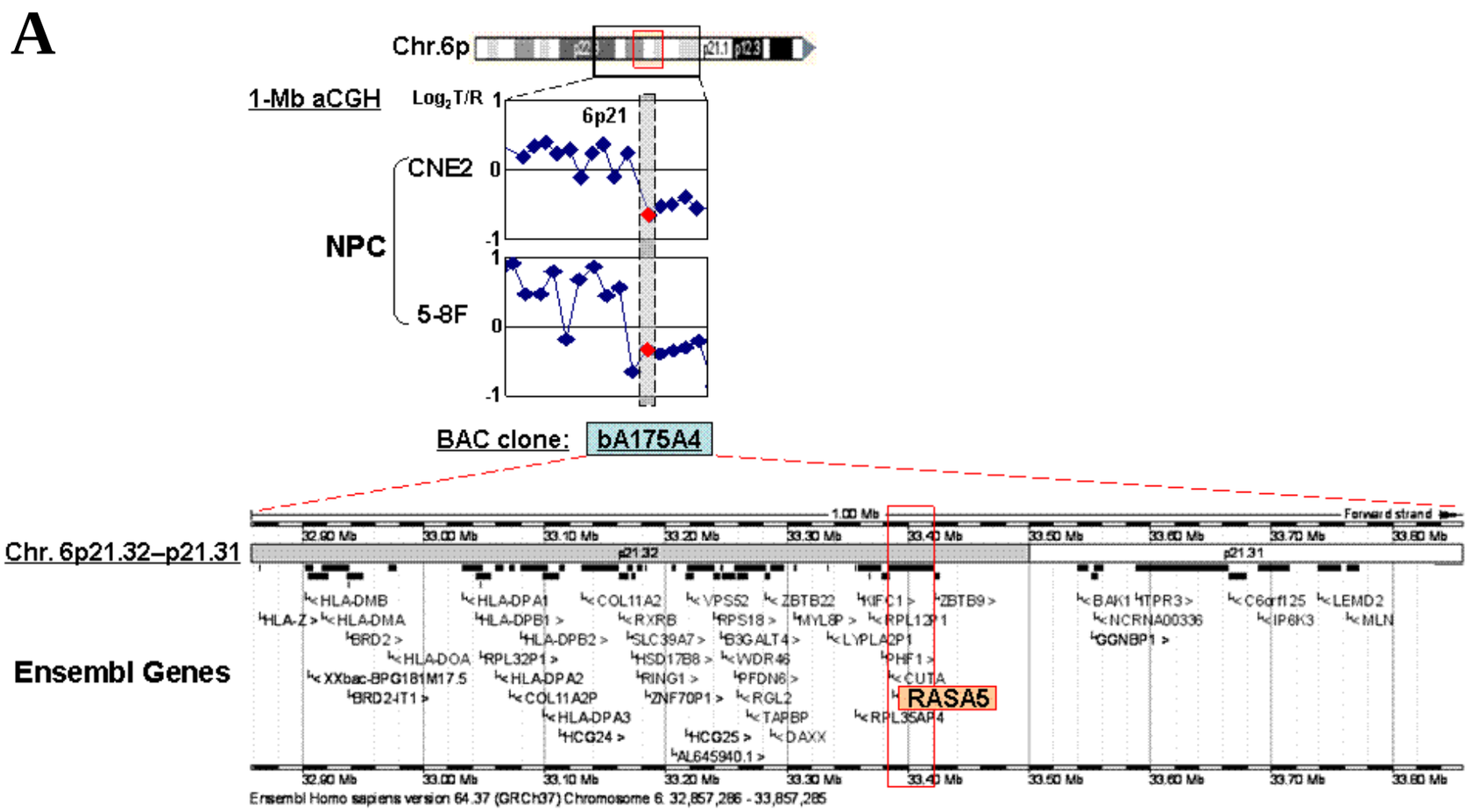
Figure S5. Knockdown of RASA5 enhances ERK phosphorylation levels, related to Figure 6. Graphs represent quantification of the phosphor-immunoblots normalized to corresponding total protein levels by Western blot in T47D and BT549 cells. Data were presented as mean ± SEM of three independent experiments via Student's t test. *, $p < 0.05$; **, $p < 0.01$.

Figure S6. The RASA5 protein sequence is evolutionarily well conserved among

different species, related to Figure 7. RASA5 amino acid sequences from different species (Homo sapiens, Bos Taurus, Rattus norvegicus, and Xenopus (Silurana) tropicalis) were compared and aligned using ClustalX (http://www.clustal.org/clustal2/). The only major difference in its C-terminal tail is emphasized with a red rectangle.

Figure S7. RASA5 inhibits the migration and F-actin stress fiber formation of carcinoma cells, related to Figure 8-9. (A) Wound healing assay showed reduced wound closure rate in RASA5-expressing cells compared to controls 24 hours after scratch wound made on confluent cell monolayer. Dash lines indicated wound edge. (B) Knockdown of RASA5 induced morphology change of T47D carcinoma cells from cobblestone-like to spindle-like. Original magnification, 400x. Scale bar 200 μm. (C) HONE1 and MB231 cells were transiently transfected with RASA5-EGFP construct or empty vector and subjected to immunofluorescence staining of F-actin stress fiber with Phalloidin (red). Arrows indicated EGFP+ cells. DAPI counterstaining (blue) represented cell nuclei. Original magnification, 400x. Scale bar 200 μm. (D) Immunofluorescence staining showed that RASA5 is located in cell-cell interaction regions and actin tails under the fluorescence microscope. Left, FITC green fluorescence of Flag-tagged RASA5; middle, DAPI-stained cell nuclei; right, merged images. Original magnification, 400x. Scale bar 200 μm.

Suppl fig.1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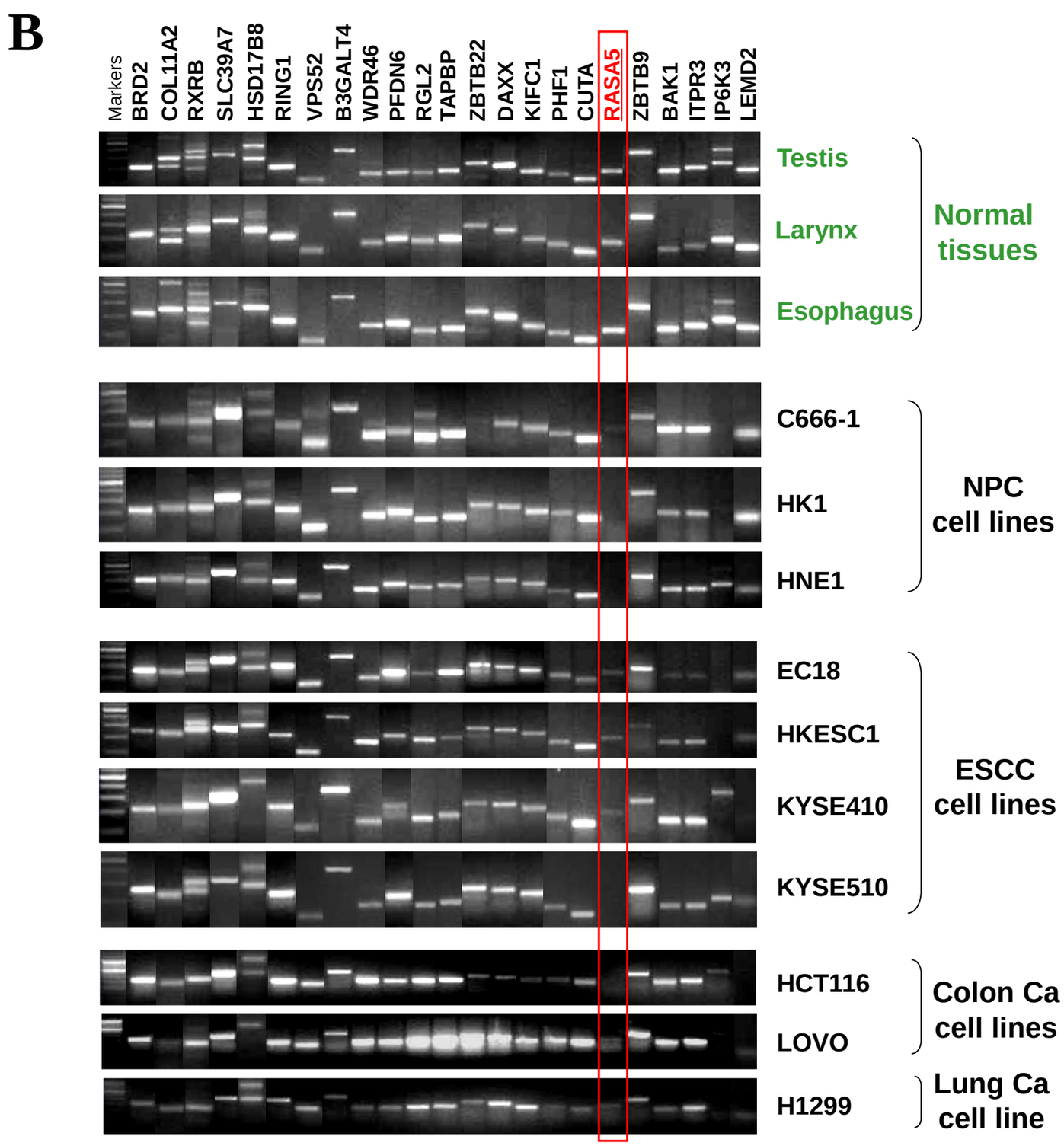
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ZBTB9
BAK1
ITPR3
IP6K3
LEMD2
Testis
Larynx
Esophagus
Normal tissues
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HK1
HNE1
NPC cell lines
EC18
HKESC1
KYSE410
KYSE510
ESCC cell lines
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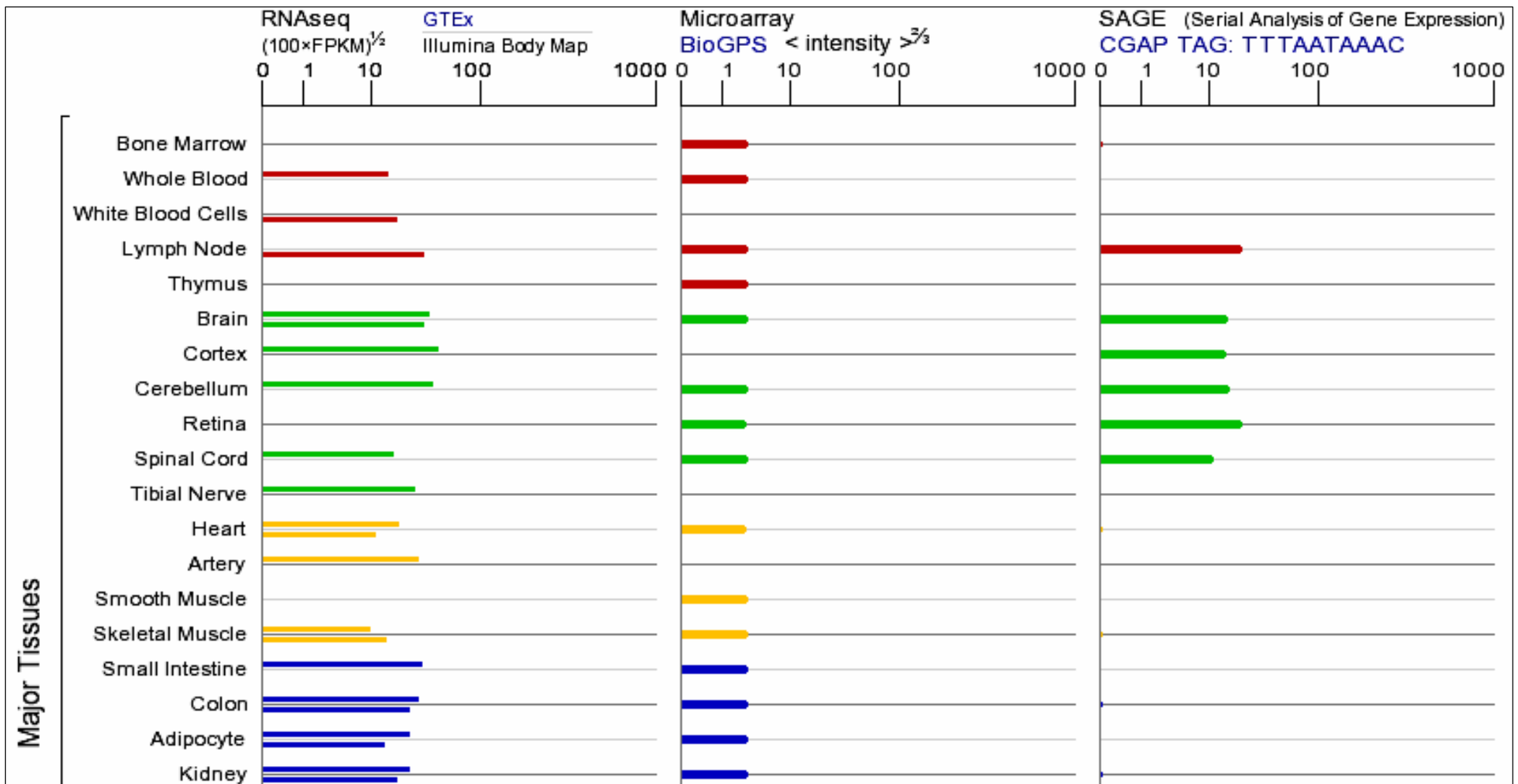
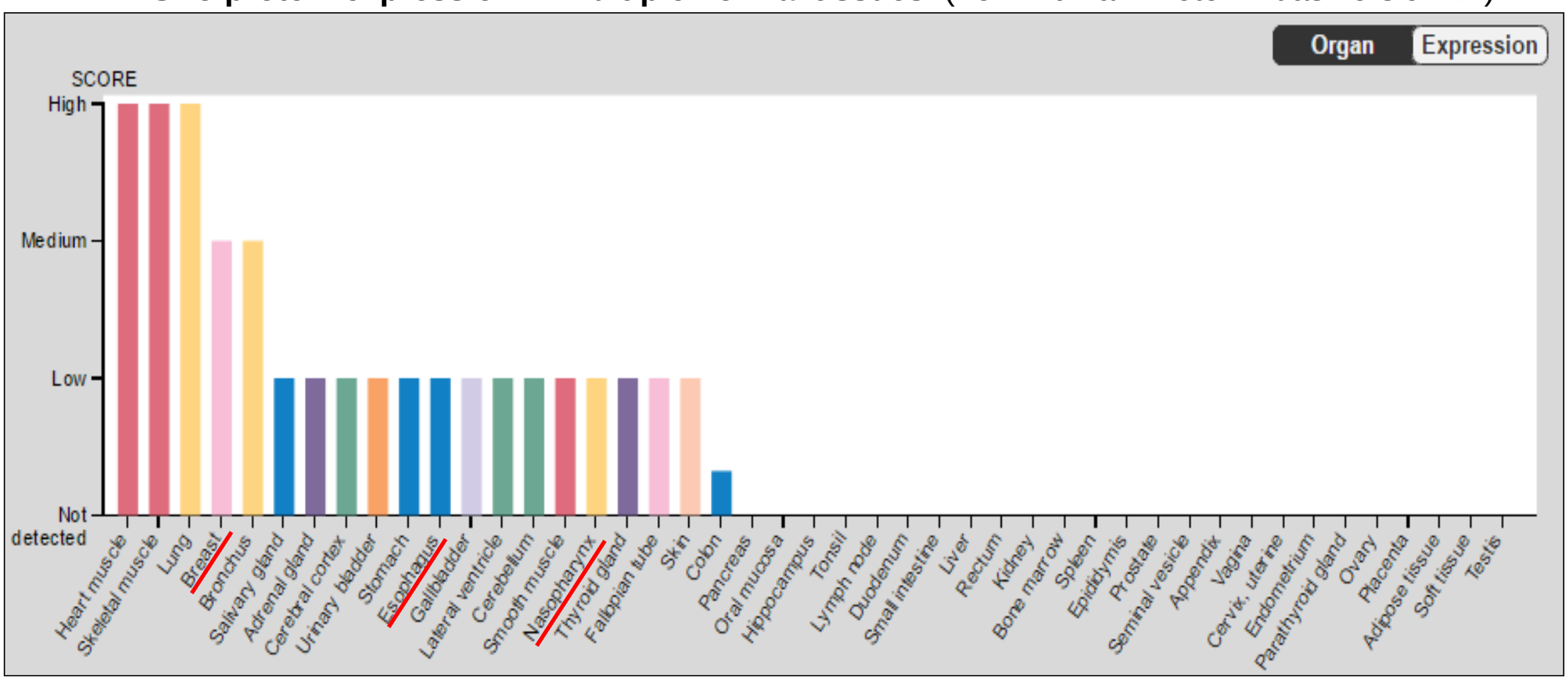
LOVO
Colon Ca cell lines
H1299
Lung Ca cell line

Suppl fig2

A ***RASA5* RNA expression in multiple normal tissues** (from GeneCards human gene database)



B **RASA5 protein expression in multiple normal tissues** (from Human Protein Atlas version 17)



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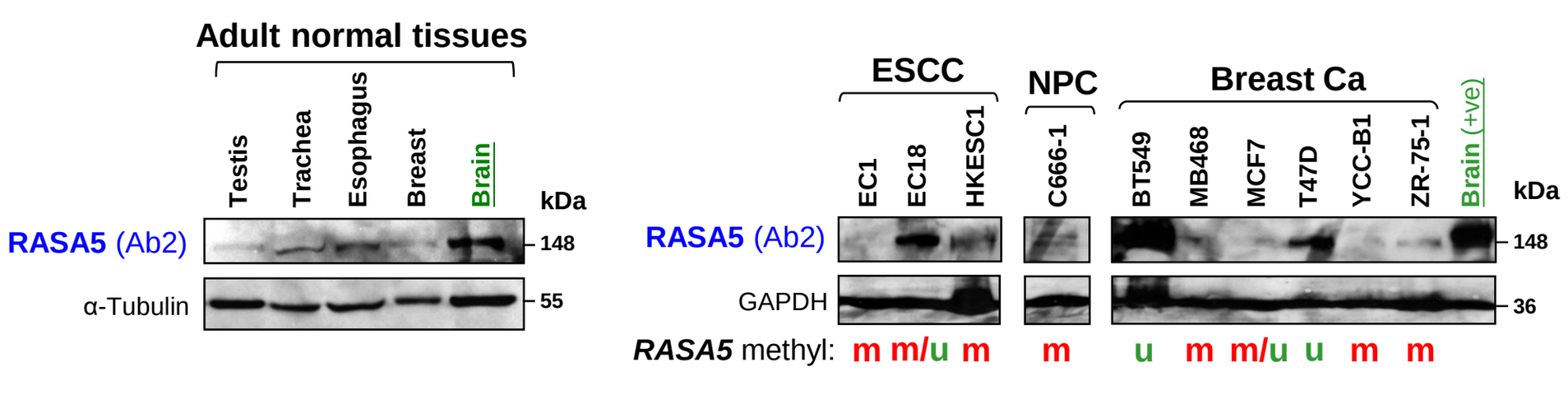
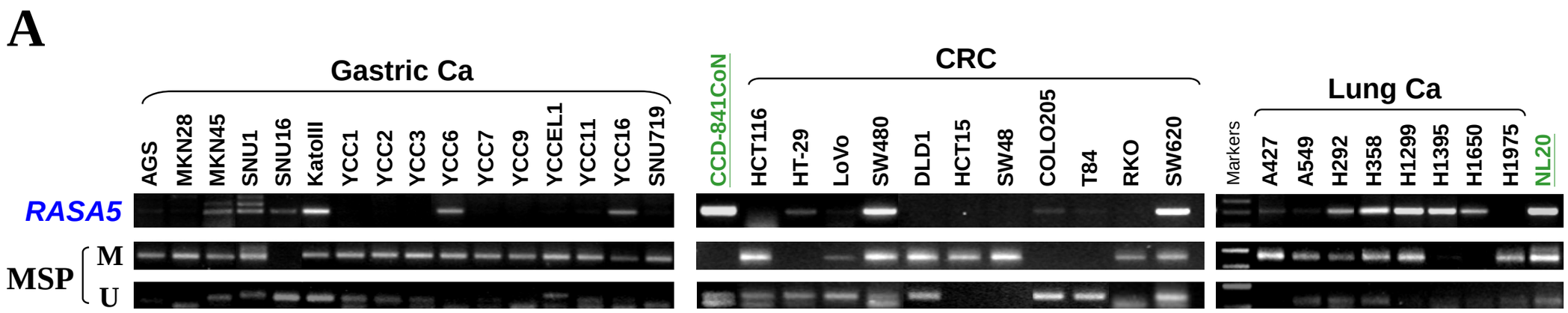


Suppl fig 3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YCC16
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HCT15
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COLO205
T84
RKO
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Lung Ca
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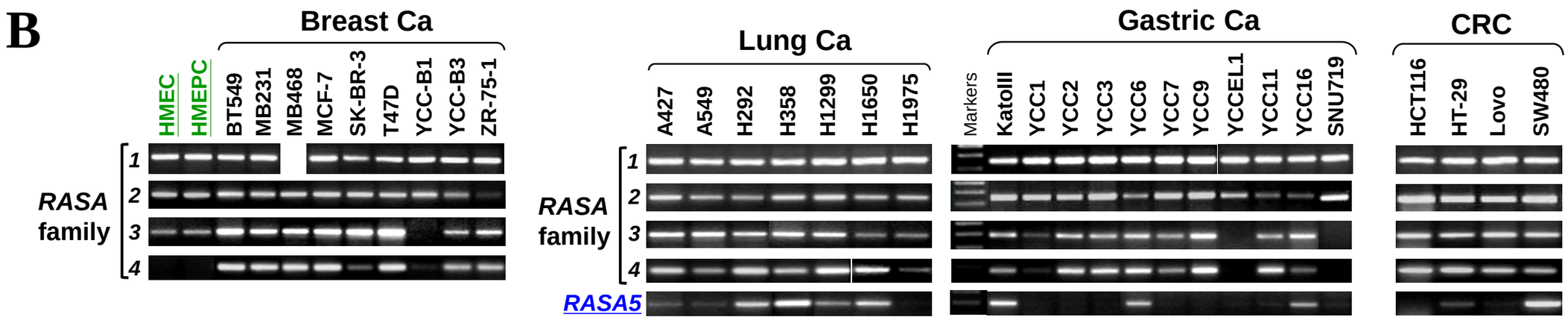
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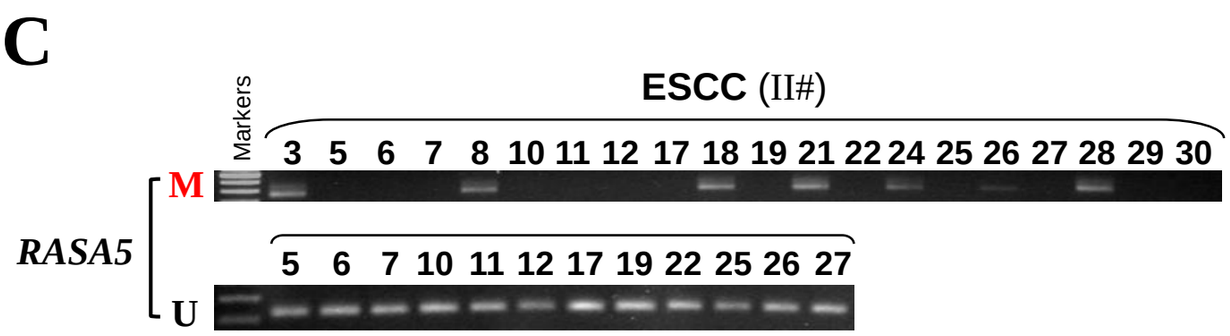
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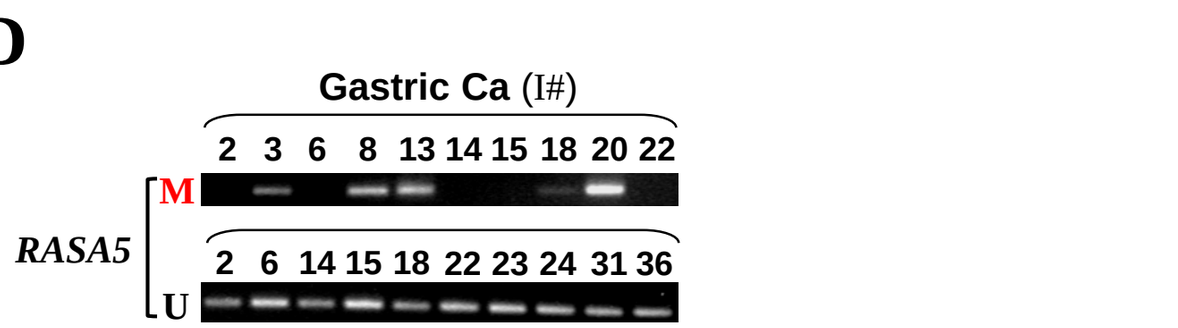
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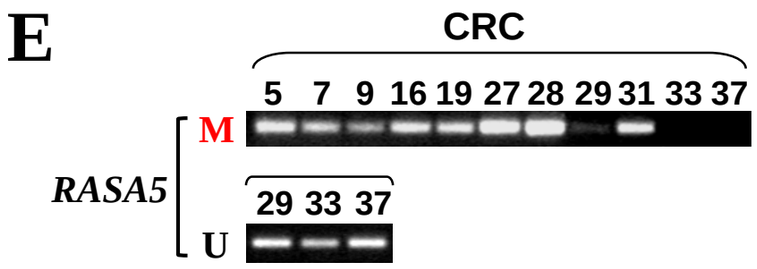
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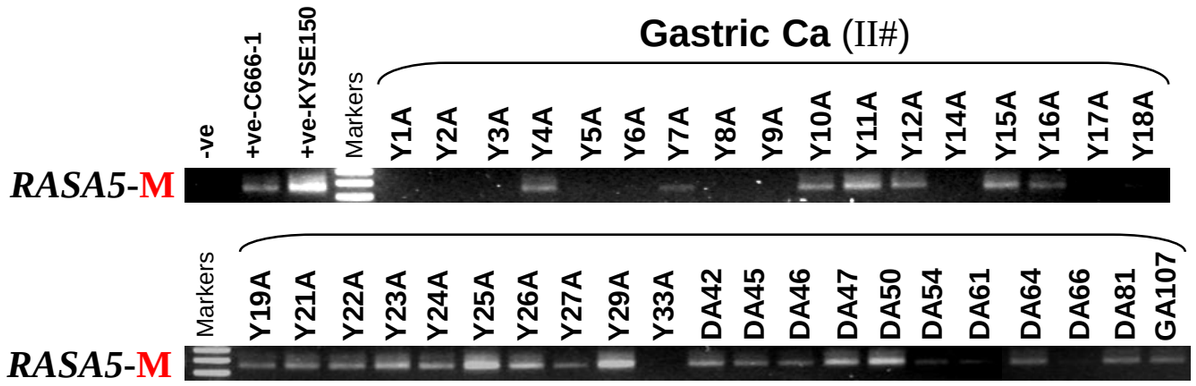
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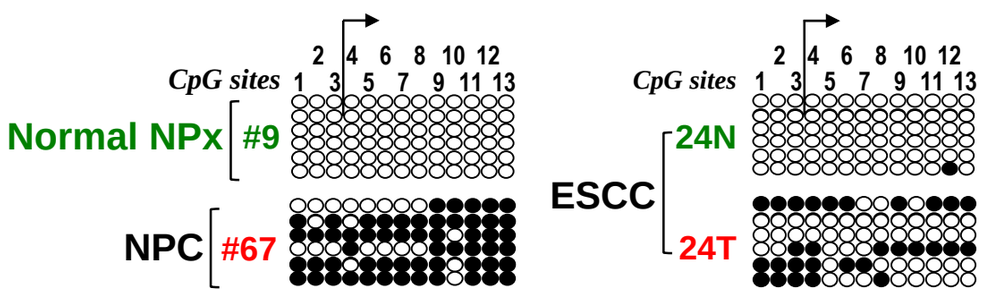
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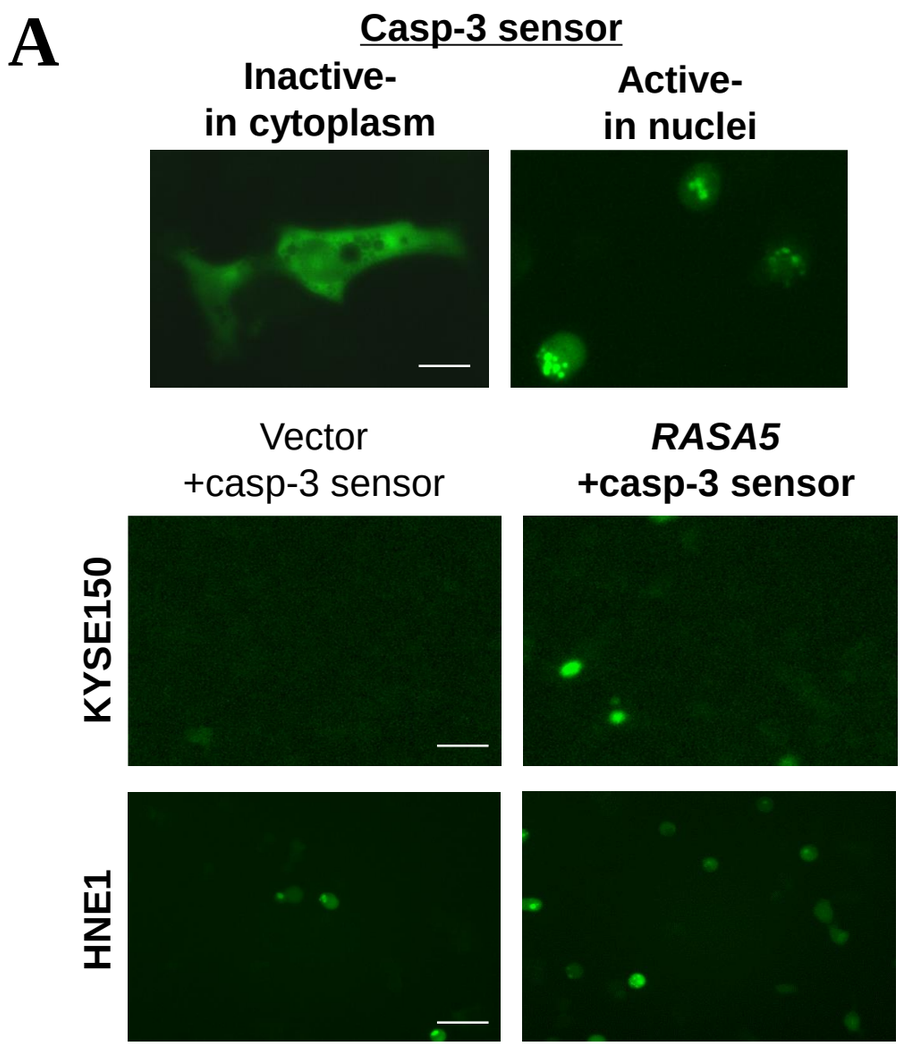
NPC
#67
ESCC
24N
24T

Suppl fig 4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in cytoplasm
Active-
in nuclei
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+casp-3 sensor
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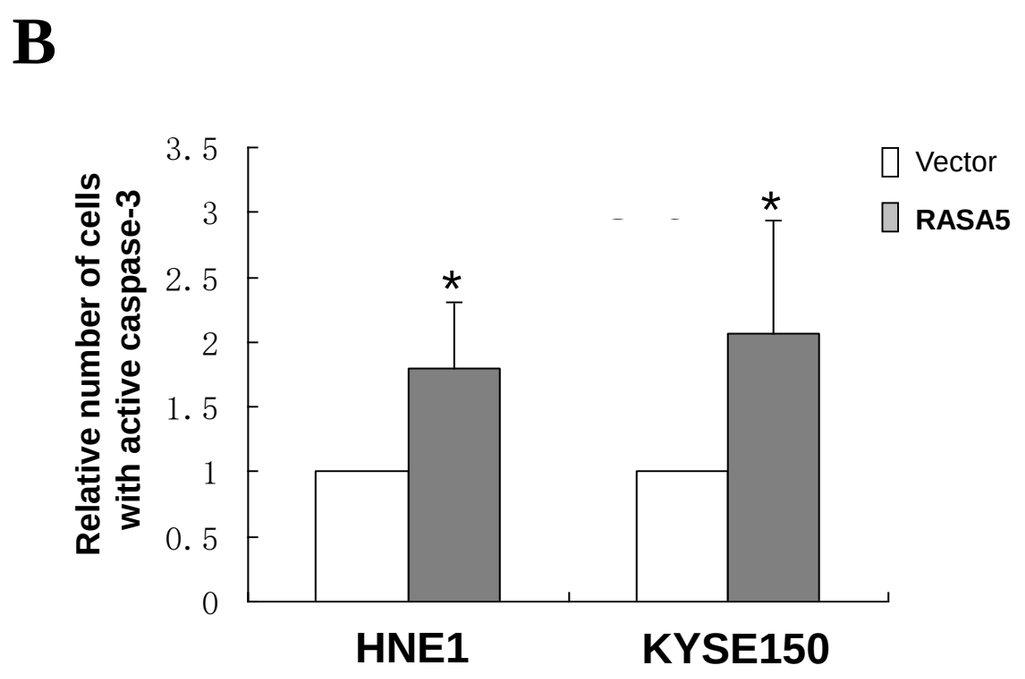
+casp-3 sensor
KYSE150
HNE1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Relative number of cells
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2.5
2
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*
*
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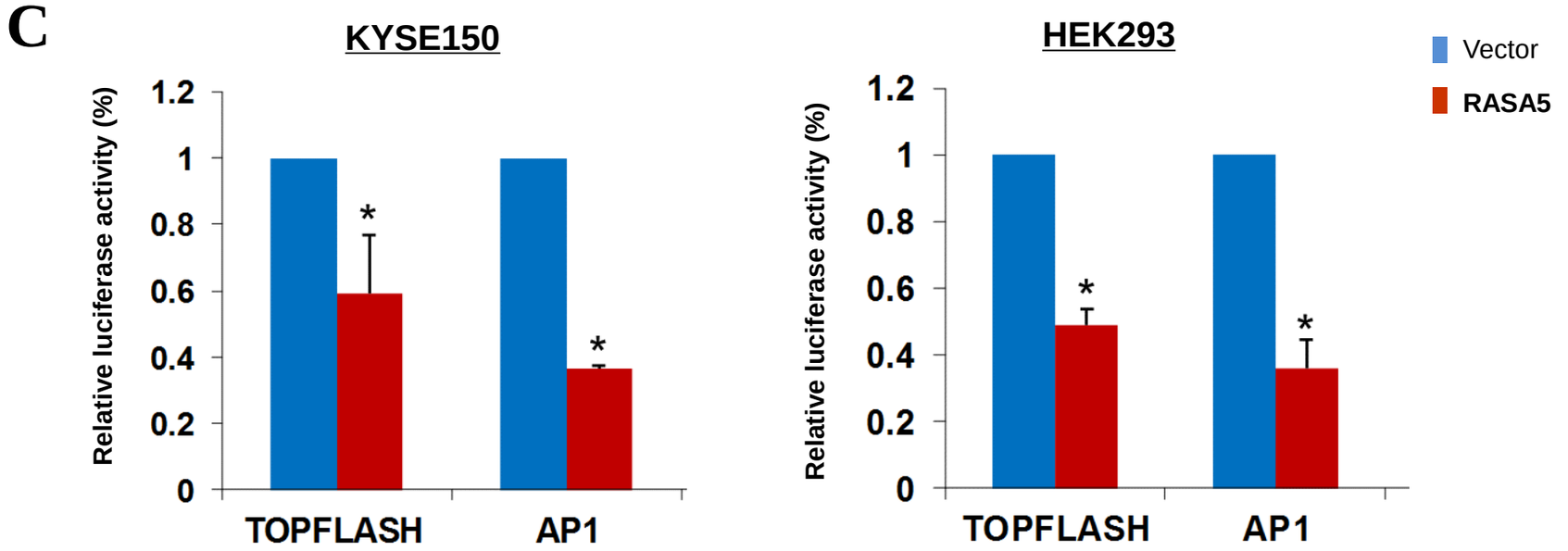
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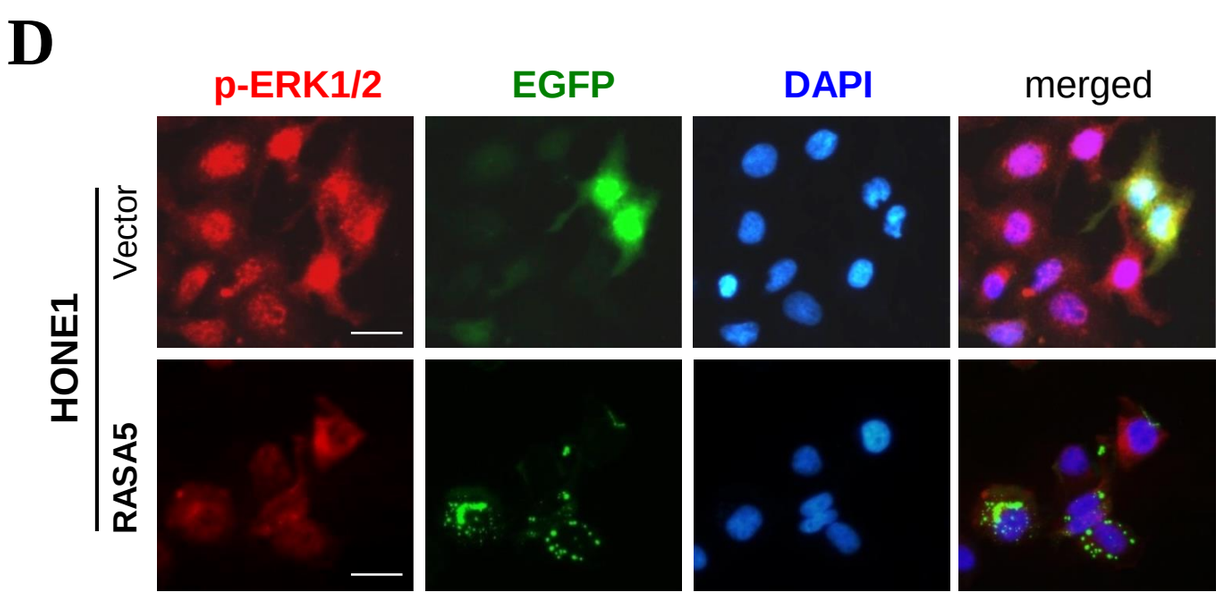
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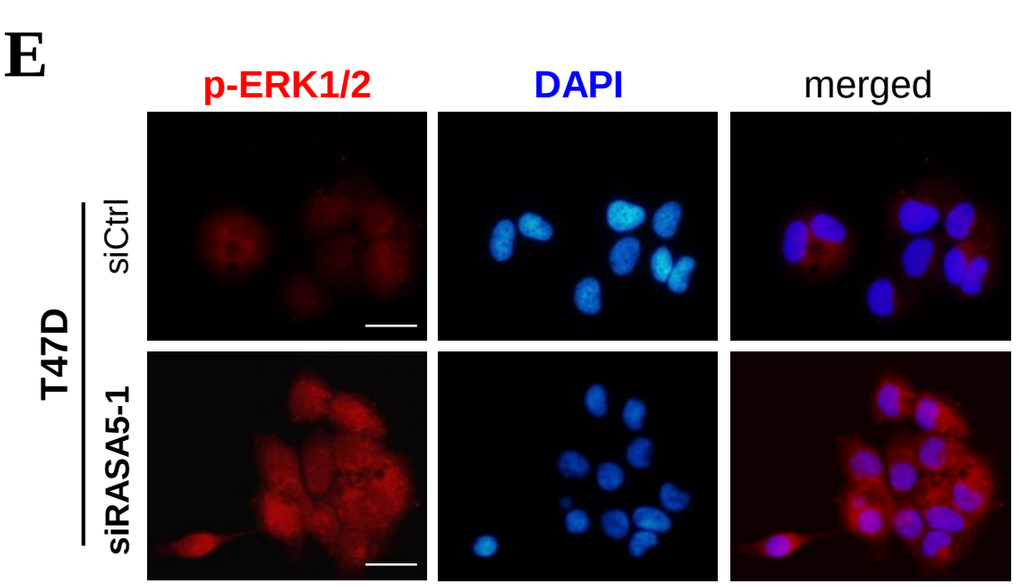
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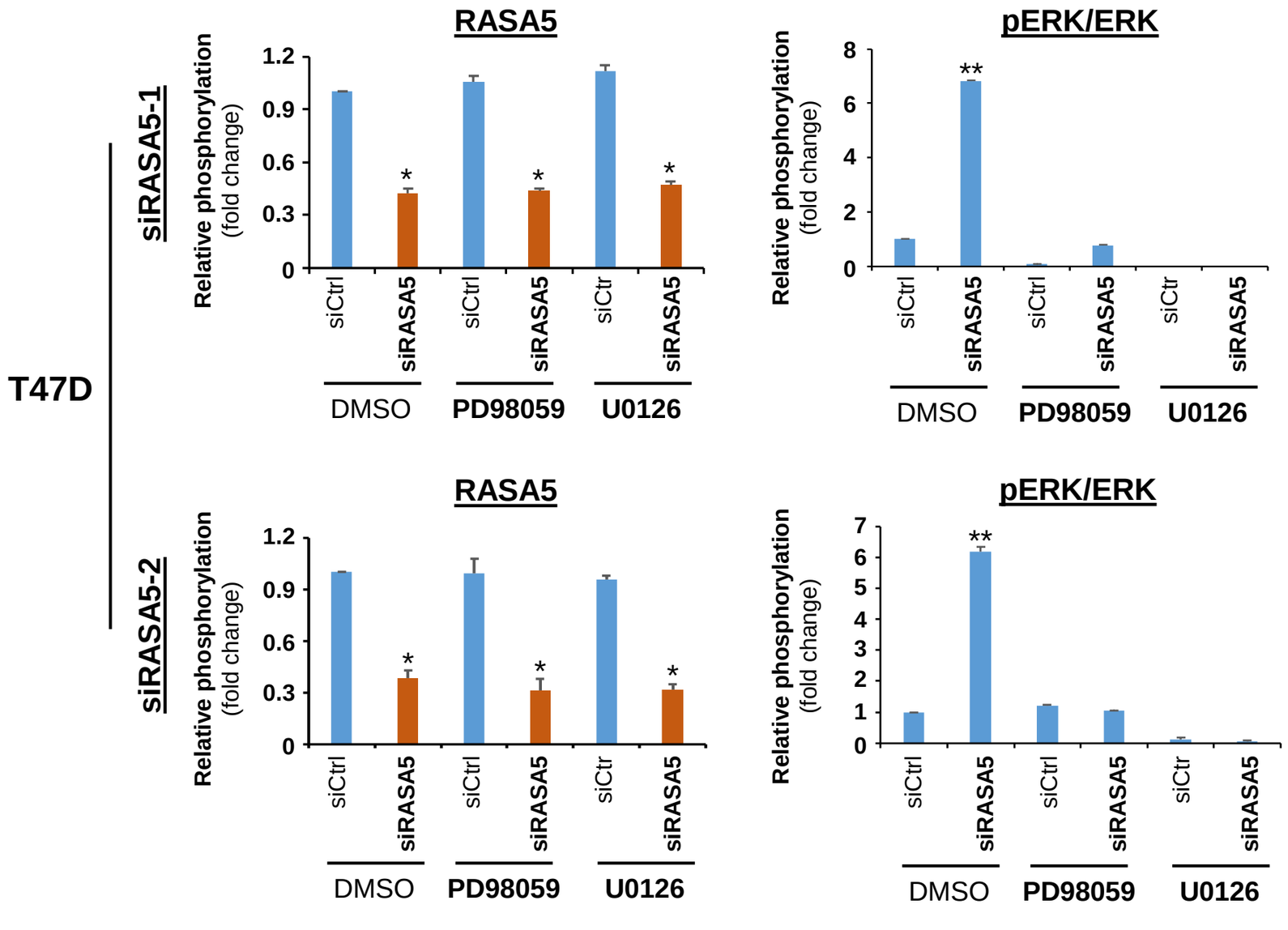
Suppl fig 5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siRASA5-2
RASA5
pERK/ERK
Relative phosphorylation
(fold change)
siCtrl
siRASA5
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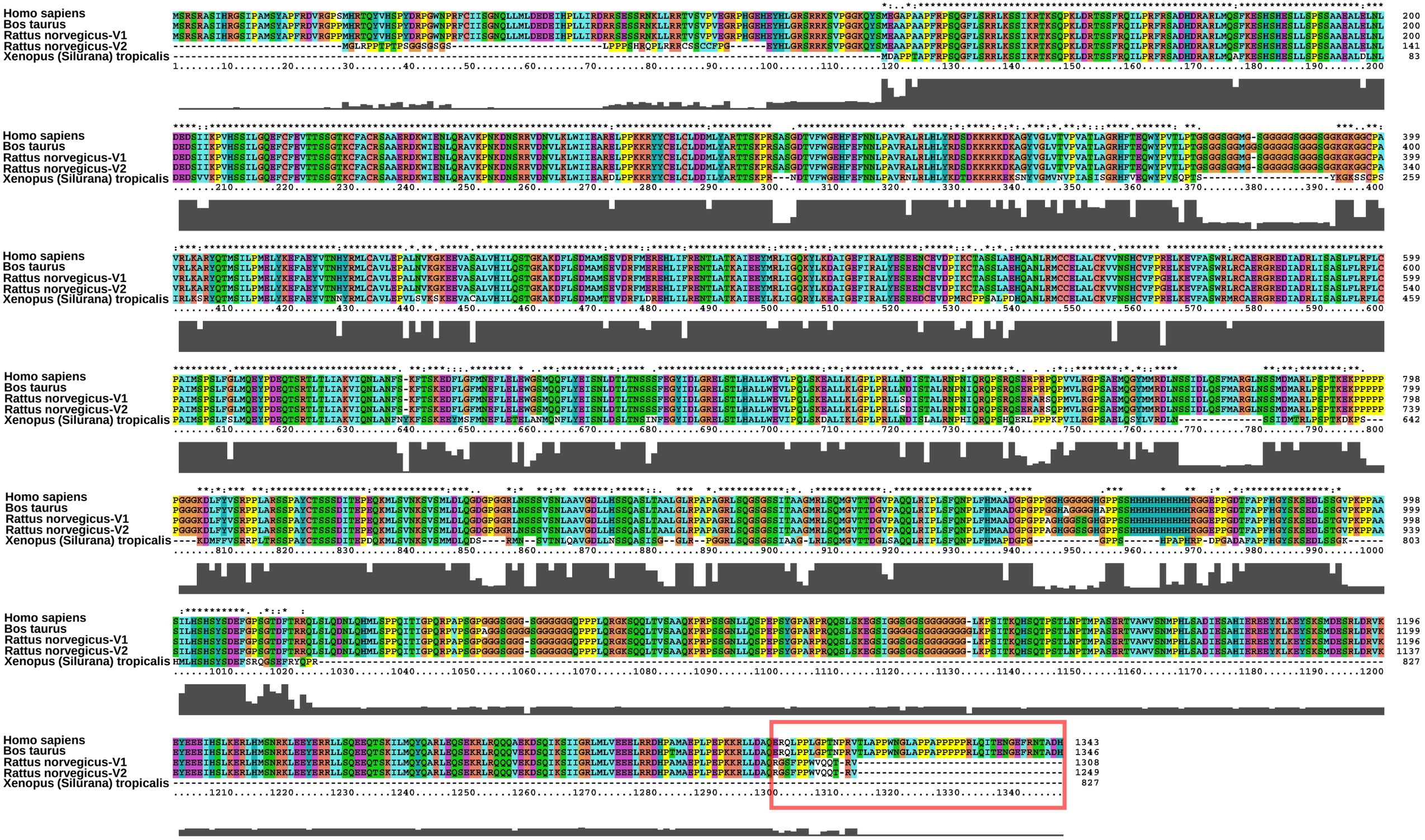
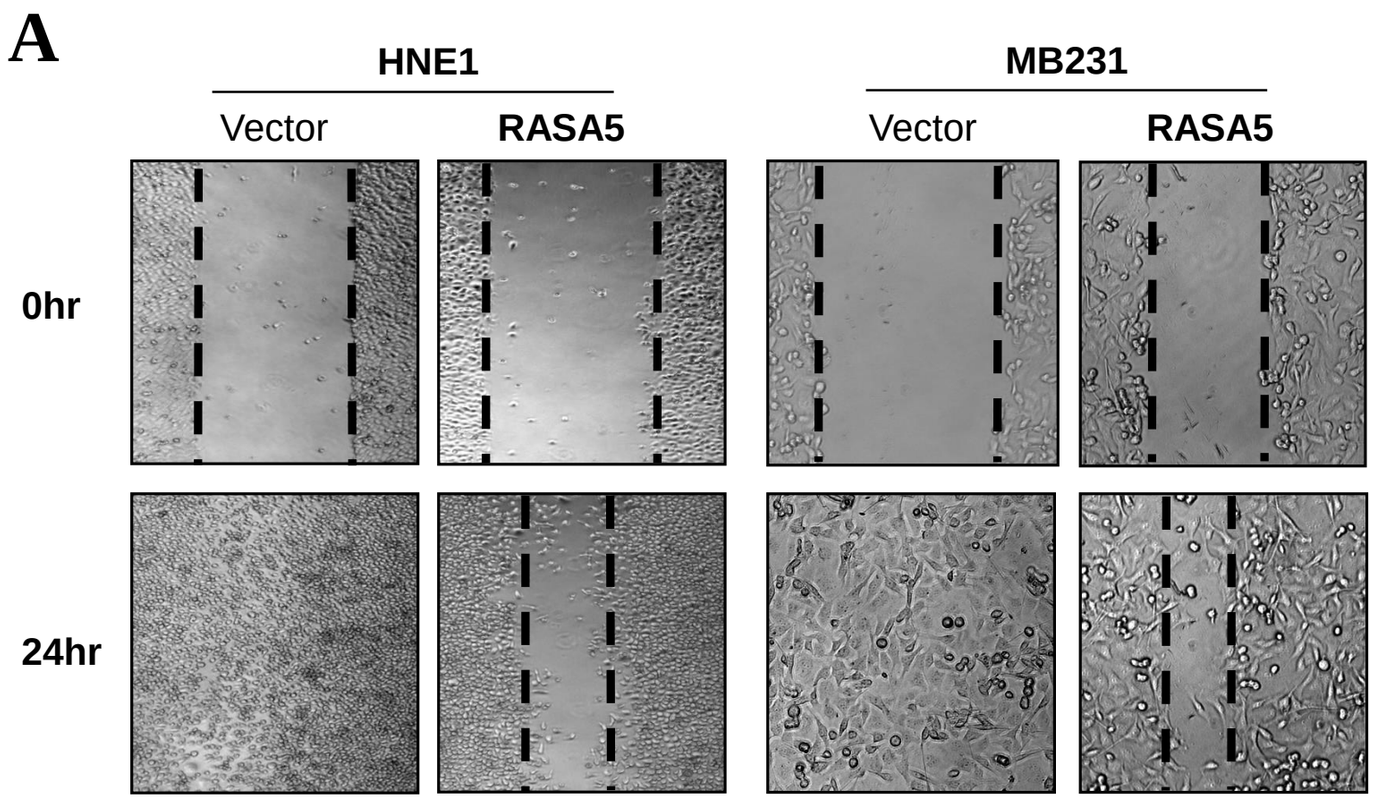
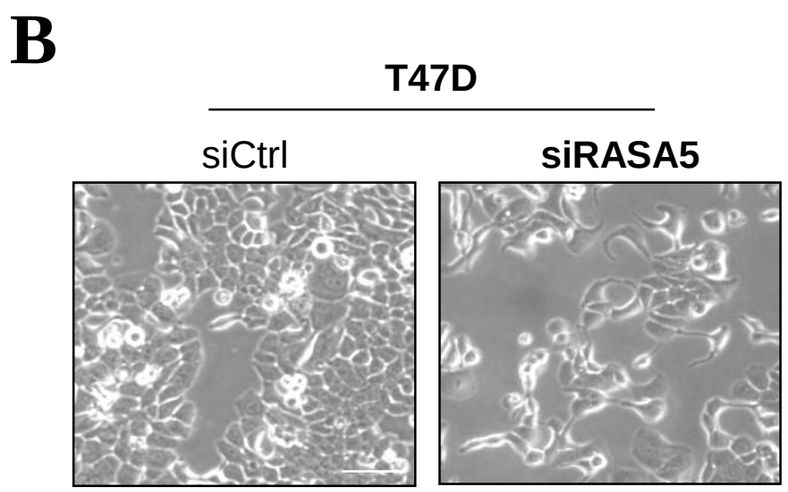
PD98059
U0126
BT549
siRASA5-1
siRASA5-2
RASA5
pERK/ERK
Relative phosphorylation
(fold change)
siCtrl
siRASA5
DMSO
PD98059
U0126

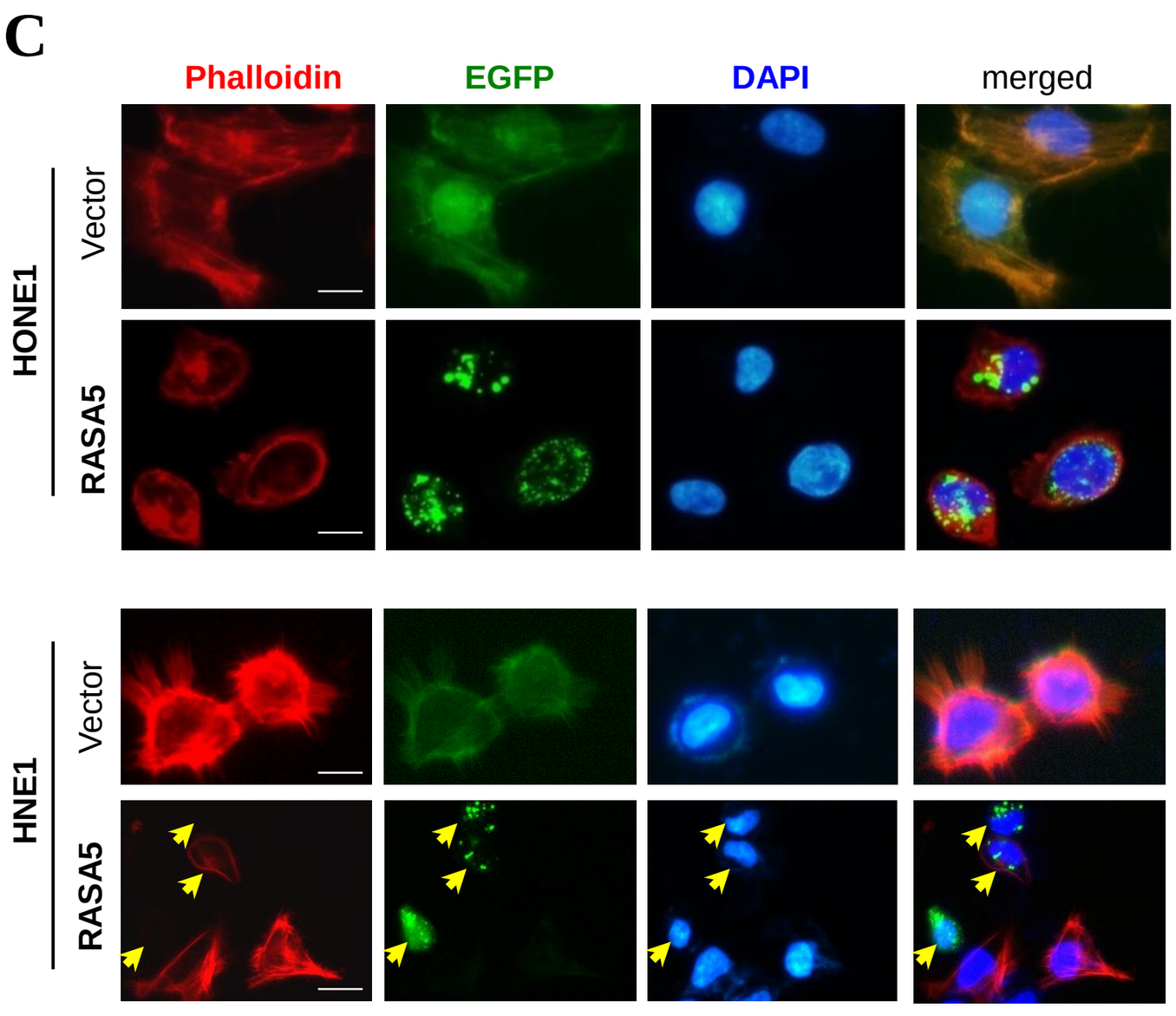
Suppl fig 6


Homo sapiens
Bos taurus
Rattus norvegicus-V1
Rattus norvegicus-V2
Xenopus (Silurana) tropicalis

Suppl fig 7


A
HNE1
MB231
Vector
RASA5
Vector
RASA5
0hr
24hr


B
T47D
siCtrl
siRASA5


C
Phalloidin
EGFP
DAPI
merged
HONE1
Vector
RASA5
HNE1
Vector
RASA5

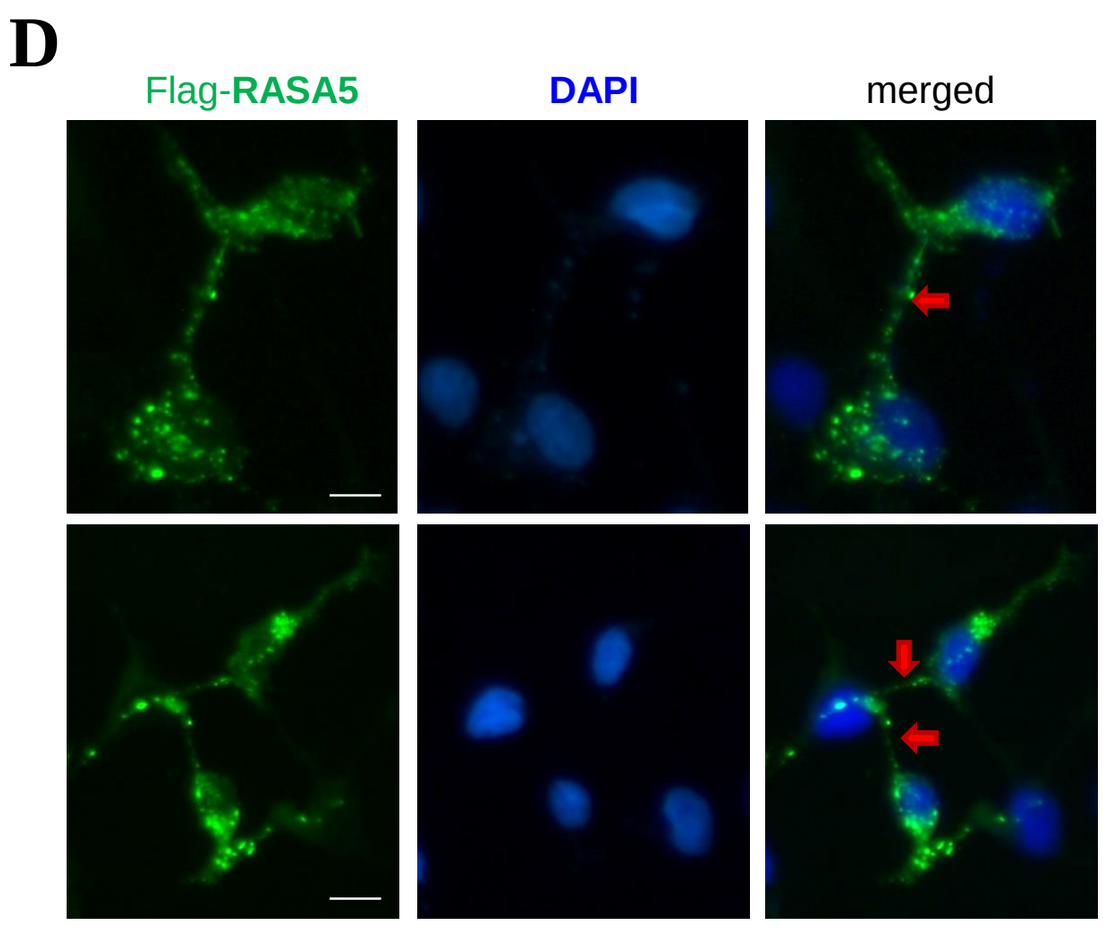

D
Flag-RASA5
DAPI
merged

Table S1. Summary of other genes within 6p21.3 deletion and their predicted functions, related to Figure 1.

Gene	Description	Predicted function
BRD2	Bromodomain containing 2	Involved in transcription complexes and acetylated chromatin during mitosis.
RPL32P1	Ribosomal protein L32 pseudogene 1	No CCDS
COL11A2P	Collagen, type XI, alpha 2 pseudogene	No CCDS
COL11A2	Collagen, type XI, alpha 2	Mutations in this gene are associated with type III Stickler syndrome.
RXRB	Retinoid X receptor, beta	Increasing both DNA binding and transcriptional function.
SLC39A7	Solute carrier family 39 (zinc transporter), member 7	Zinc cannot passively diffuse across cell membranes and requires specific transporters, such as SLC39A7.
HSD17B8	Hydroxysteroid (17-beta) dehydrogenase 8	Regulating the concentration of biologically active estrogens and androgens.
RING1	Ring finger protein 1	Acting as a transcriptional repressor interacts with the polycomb group proteins.
ZNF314P	Zinc finger protein 314 pseudogene	No CCDS
HTATSF1P	HIV-1 Tat specific factor 1 pseudogene	No CCDS
VPS52	Vacuolar protein sorting 52 homolog (S. cerevisiae)	Involved in tetrameric Golgi-associated retrograde protein complex.
RPS18	Ribosomal protein S18	S13P family of ribosomal proteins.
WDR46	WD repeat domain 46	Possessing WD40 domain, found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly.
PFDN6	Prefoldin subunit 6	A subunit of the heteromeric prefoldin complex that chaperones nascent actin and alpha- and beta-tubulin chains pending their transfer to the cytosolic chaperonin containing TCP1 complex.
RGL2	Ral guanine nucleotide dissociation stimulator-like 2	Possessing Ras guanyl-nucleotide exchange factor activity.
ZBTB22	Zinc finger and BTB domain containing 22	Interacting with BDP1, a subunit of TFIIIB.
B3GALT4	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4	Encoding type II membrane-bound glycoproteins with diverse enzymatic functions using different donor substrates.
DAXX	Death-domain associated protein	Interacting with apoptosis antigen Fas, centromere protein C, and transcription factor erythroblastosis virus E26 oncogene homolog 1.
TAPBP	TAP binding protein (tapasin)	Transporting antigenic peptides across the endoplasmic reticulum membrane.
PHF1	PHD finger protein 1	Zinc finger-like PHD (plant homeodomain) finger.
RPL12P1	Ribosomal protein L12 pseudogene 1	No CCDS
RPL35AP4	Ribosomal protein L35a pseudogene 4	No CCDS
KIFC1	Kinesin family member C1	Important in a cargo-transport system.
CUTA	CutA divalent cation tolerance homolog (E. coli)	Undergoing an unusual transfer into the secretory pathway and affecting the folding, oligomerization, and secretion of acetylcholinesterase.
ZBTB9	Zinc finger and BTB domain containing 9	
GGNBP1	Gametogenetin binding protein 1	No CCDS
BAK1	BCL2-antagonist/killer 1	Localized to mitochondria, inducing apoptosis; interacting with the tumor suppressor P53 after exposure to cell stress.
ITPR3	Inositol 1,4,5-triphosphate receptor, type 3	Encoding a receptor for inositol 1,4,5-trisphosphate, a second messenger that mediates the release of intracellular calcium.
IP6K3	Insitol hexaphosphate kinase 3	Encoding a protein that belongs to the inositol phosphokinase family.
LEMD2	LEM domain containing 2	Regulating kinase signaling in myoblast differentiation.
MLN	Motilin	Regulating gastrointestinal contractions and motility; producing the mature peptide and a byproduct referred to as motilin-associated peptide.

Table S2. Summary of *RASA5* methylation in carcinomas and normal cell lines, related to Figure 2.

Samples	Promoter methylation (%)
Carcinoma cell lines	
Esophageal	18/18
Nasopharyngeal	5/5
Hypopharyngeal	1/1
Breast	6/9 (67%)
Lung	6/8 (75%)
Gastric	15/16 (94%)
Colon	9/10 (90%)
Immortalized normal epithelial cell lines	
Het1A, NE1, NE3, NE083, NP460 HMEC, HMEpC, NL20, CCD841con	1/9 (11%)
Primary carcinomas	
ESCC paired primary tumor (T)	12/20 (60%)
ESCC (II#)	12/49 (25%)
NPC (OCT#)	18/22 (82%)
Breast	13/19 (68%)
Colon (I#)	8/11 (73%)
Gastric Ca (I#)	6/14 (43%)
Gastric Ca (II#)	26/38 (68%)
Normal tissues	
Normal esophageal epithelial tissues	0/7
ESCC paired normal tissues (N)	2w/20 (10%)
Normal nasopharyngeal tissues	0/8
Normal breast tissues	0/7
Breast adjacent tissues	0/5

W: weak methylation.

Table S3. Sequences of other primers used in this study, related to Figure 2, 5 and 9.

PCR	Primers	Sequence (5'-3')	Cycles
RT-PCR	*NANOG*F	ATGAGTGTGGATCCAGCTTG	30
	*NANOG*R	CCTGAATAAGCAGATCCATGG	
	*NESTIN*F	TGGCACACATGGAGACGTC	30
	*NESTIN*R	AGCGATCTGGCTCTGTAGG	
	*ABCG2*F	CAGTGTCACAAGGAAACACC	30
	*ABCG2*R	GAGACCAGGTTTCATGATCC	
	*OCT4*F	AAGGAGAAGCTGGAGCAA	30
	OCT4R	GAGGGTTTCTGCTTTGCAT	
	*MCL1*F	AGTTGTACCGGCAGTCGCTG	30
	*MCL1*R	CTAGGTCCTCTACATGGAAG	
	*c-MYC*F	CTCTCCGTCCTCGGATTCTC	30
	*c-MYC*R	GCCTCCAGCAGAAGGTGATC	
	*KLF4*F	TCCCATCTTTCTCCACGTTC	30
	*KLF4*R	TCCAGGAGATCGTTGAACTC	
	*CD44*F	TGGACAAGTTTTGGTGGCAC	30
	*CD44*R	GGTGCTATTGAAAGCCTTGC	
	*GAPDH*F	GATGACCTTGCCCACAGCCT	23
	*GAPDH*R	ATCTCTGCCCCCTCTGCTGA	
MSP	*RASA5*m4	CGTTTTTTTTTTTTTTCGGTTGTC	40
	*RASA5*m2	CTAAAAAAAACATAAAACATCGCG	
	*RASA5*u11	GCGGTTTTTTTTTTTTTTTTTTTGTTTTTTT	40
	*RASA5*u22	GCGCTAAAAAAAACATAAAACATCACA	
BGS	*RASA5*BGS5	GTAGGGGTGGGGGTTGTAG	40
	*RASA5*BGS6	CCTCTAAAAAAAACATAAAACATC	
Cloning	*RASA5*CF	GCTGGATCCGACACCATGGATTACAAGGATGACGACGATAAGAGCAGGTCTCGAGCCTCC	15
	*RASA5*CR	GATGTCTAGACTAGTGGTCTGCGGTGTTTC	
	*RASA5ΔGAP-L*F	GCTGGATCCGACACCATGGATTACAAGGATGACGACGATAAGAGCAGGTCTCGAGCCTCC	
	*RASA5ΔGAP-L*R	GATGTCTAGACTACCCACTGCCTGTTGGCAG	
	*RASA5ΔGAP-G*F	TACCGGACTCAGATCTCGAGCGCCACCATGGATTACAAGGATGACGACGATAAGATGAGCA GGTCTCGAGCCTCCATC	
	*RASA5ΔGAP-G*R	TACCGTCGACTGCAGAATTCCTAGTGGTCTGCGGTGTTTCGGAAC	
	*RASA5ΔC2-GAP*F	GCTGGATCCGACACCATGGATTACAAGGATGACGACGATAAGAGCAGGTCTCGAGCCTCC	
	*RASA5ΔC2-GAP*R	GATGTCTAGACTAGCGGCTGTTGTCCTTGTTG	

Table S4. Primers for screening 6p21.3 genes by semi-quantitative RT-PCR, related to Figure 1**.**

Gene	Forward (5'-3')	Reverse (5'-3')
RASA5	GTCCTATGC CCCCTTCAGAG	GTCCTATGCCCCCTTCAGAG
BRD2	CTTGCGGTCAAGATGCTGCA	TTGGGATTGGACACCTCCG
COL11A2	ATGGAGCGGTGCAGCCGC	GCCACATCAGCTGGACAGAT
RXRB	AGAAATGCATTGTGGGGTCG	AAGGGATGGAGCTGTTGAAG
SLC39A7	GATCTGCAAGAGGACTTCCA	GCTGCTGAGATCAGCACTGT
HSD17B8	TCTCAGCTCCAGAACCGACT	ACTTGTTCCAGCAGGCACCT
RING1	ACTGAGTCTGTATGAGCTGC	CAGTCAGAGCAGAATCTGTG
VPS52	CTCAGATATGGAGGAGGAAG	CATCCACTTCATCCAGGATG
RPS18	GCGAGTACTCAACACCAACA	TTGGTGAGGTCAATGTCTGC
WDR46	GACAAACTTCAGACCAAGAG	CTTAGAGATCCGAGACTTCT
PFDN6	TACCTTCCAGAGAGTGAGAC	GTGCTTCAAGTTTCTGCCTC
RGL2	GAGTCGTACTGAGCAGCTTC	GGATCAAGAGGTCGATATTG
ZBTB22	AGGTTGCTGCTCTGGCCG	CTGCAGACGCTGCTGATTGA
B3GALT4	AACCAGGAAGCTTGCAGTGG	CCAGCTCTGATACCAGTTCA
DAXX	AGAAGATGAAGCAGCTGCT	GAGAGTGGGCACGTTGCTG
TAPBP	ATGAAGTCCCTGTCTCTGCT	ACACTGAGATAGAGCTCAGG
PHF1	TCAAGATGTGCTGGCCAGAT	TCTCAGAGCGACAGACACAA
KIFC1	TTCTCTTCCACTGCATTCCC	GGCCATCTTCCATCTGGTCA
CUTA	GTCTCTGCAGCCTTTGTTAC	CTCACTGTCTTCCTCGATCT
ZBTB9	AGACCTCTGTGGGCACTGT	AGGCAGCAGCTAACACTGCT
BAK1	AGGAGTGCGGAGAGCCTG	GAGGTAAGGTGACCATCTCT
ITPR3	GTCAATGGCTTCATCAGCAC	TCCTTGTCCTGCTTAGTCTG
IP6K3	GCACATGAGCGTGATGAAGT	GACTCTGTGGAGACCTTGAA
LEMD2	CTCTCTCGGCTTCTGCTCT	TCTCACAGTCCACTGGCAAT
MLN	AAGATGGTATCCCGTAAGGC	CTCCGCAGGGTCTACAGG

Table S5. Antibodies used in this study, related to Figure 1-3 and Figure 5-8.

Antibodies	Source	Identifier
anti-mouse IgG F(ab)2 antibody	DAKO	F0313
anti-mouse IgG-Alexa Fluor 555-F(ab')2 antibody	Cell Signaling	4409
anti-rabbit IgG-Alexa Fluor 555-F(ab')2 antibody	Cell Signaling	4413
anti-mouse IgG-Alexa Fluor 488-F(ab')2 antibody	ThermoFisher	A-11059
anti-rabbit IgG-Alexa Fluor 488-F(ab')2 antibody	ThermoFisher	A-11070
anti-mouse IgG-HRP	DAKO	P0161
anti-rabbit IgG-HRP	DAKO	P0448
α-tubulin	Lab Vision	MS-581
AKT (pan)	Cell Signaling	4691
b-actin (AC-74)	Sigma-Aldrich	A2228
cleaved caspase-3	Cell Signaling	9661
cleaved PARP	Cell Signaling	9541
E-Cadherin	Cell Signaling	4065
ERK1/2 (p44/42 MAPK)	Cell Signaling	4695
Fibronectin	Santa Cruz	sc-9068
Flag	Sigma-Aldrich	F3165
GAPDH	Millipore	MAB374
MEK1/2	Cell Signaling	4694
phosphor-AKT (Ser473)	Cell Signaling	4060
phosphor-Cofilin (Ser3)	Cell Signaling	3313
phosphor-ERK1/2 (Thr202/Tyr204)	Cell Signaling	9101
phosphor-MEK1/2 (Ser217/221)	Cell Signaling	9121
phosphor-SAPK/JNK (Thr183/Tyr185)	Cell Signaling	9251
phospho-Rac1/cdc42 (Ser71)	Cell Signaling	2461
RASA5 (Ab1)	ThermoFisher	PA1-046
RASA5 (Ab2)	Epitomics	2477-S
Rhodamine phalloidin	Invitrogen	R415
Vimentin	Sigma-Aldrich	V6630

Reference

Jin, H., Wang, X., Ying, J., Wong, A.H., Cui, Y., Srivastava, G., Shen, Z.Y., Li, E.M., Zhang, Q., Jin, J.*, et al.* (2007). Epigenetic silencing of a Ca(2+)-regulated Ras GTPase-activating protein RASAL defines a new mechanism of Ras activation in human cancers. Proc Natl Acad Sci U S A *104*, 12353-12358.

Ying, J., Li, H., Seng, T.J., Langford, C., Srivastava, G., Tsao, S.W., Putti, T., Murray, P., Chan, A.T., and Tao, Q. (2006). Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. Oncogene *25*, 1070-1080.