

Restoring *LINC00673* expression triggers cellular senescence in lung cancer

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Heatmap of 479 ncRNAs with significant expression differences between non-matched normal lung and lung cancer ($n = 27$, corrected $P < 0.05$, $FC \geq 2$).

Supplementary Figure 2. Sex-specific expression ncRNAs and mRNAs. (A) The heatmaps depict the row-normalized gene expression patterns of male and female patients as detected by microarray hybridization of 27 normal and malignant lung tissues. (B) Six mRNAs and one ncRNA were statistically significantly regulated between the male and female samples (unpaired Student t test, corr. $P < 0.05$ (FDR) and $FC \geq 2$) which were all derived from the Y and X chromosomes, respectively.

Supplementary Figure 3. Expression levels of ncRNAs are lower as compared to mRNAs in normal and lung cancer. The average signal intensities (\log_2) of each ncRNA and mRNA were calculated for normal lung and lung cancer and the frequency of signal intensities were plotted in bins of 0.1 units of signal intensity for each group of samples separately for ncRNAs (red) and mRNAs (blue).

Supplementary Figure 4. Data for tumor : normal pairs from individual patients are depicted for elevated *LINC00673* levels validated by RT-qPCR analysis in two patient cohorts (cohort 1 overlapping with the microarray cohort) comprising matched tumor and normal samples. *PPIA* was used as reference gene and the mean ratios are shown in figure 1C.

Supplementary Figure 5. *LINC00673* is expressed in different cancer cell lines and cancers of different origin. (A) The relative *LINC00673* expression was determined in cell lines by RT-qPCR using two different primer pairs spanning exon 2-3 (E2-3) and located within exon 4 (E4), respectively, and normalized to *PPIA*. (B) *LINC00673* expression based on the reference sequence was extracted from TANRIC database for different normal and tumor tissues showing upregulation in multiple tumor entities. The statistical significance was determined per two-sided unpaired Student t test, with **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Figure 6. Correlation of *LINC00673* expression with lung cancer patient survival and clinipathological parameters. (A) *LINC00673* is neither a prognostic marker in TCGA lung adenocarcinoma (LUAD), nor in TCGA lung squamous cell carcinoma (LUSC). (B) *LINC00673* expression does not correlate significantly with LUAD stage, which is in contrast to LUSC. (C) *LINC00673* levels do not correlate with the smoking behavior in TCGA LUAD nor in TCGA LUSC. *LINC00673* expression was based on the linc00673 reference sequence and all presented analyses were performed with TANRIC.

Supplementary Figure 7. *LINC00673* is associated with cell proliferation. (A) Genes that positively correlate with *LINC00673* expression in the TCGA lung ADC set were retrieved with TANRIC database (correlation > 0.3) and functional enrichment analysis was performed with g:Profiler (using g:GOST with moderate filtering option). The transcription factor binding site enrichment based on TRANSFAC is shown. (B) *LINC00673* expression correlates with E2F1 protein levels in different lung cell lines ($n = 1$). The protein expression was determined by Western Blot using specific antibodies. The signals were monitored with the ECL ChemoCam Imager (Intas) and quantified with LabImage 1D software. (C) The RNA expression was determined by RT-qPCR. For comparison, both protein and RNA levels were normalized to β -actin and the IMR-90 values were set to 1 ($n = 1$) in (B) and (C). (E) *LINC00673* promoter region interacts with E2F1 according to available ChIP-Seq data. (F) WI-38 and NCI-H1299 cells expressing ER-E2F1 or an empty vector were treated with OHT (100 nM) for 24 h and *LINC00673* expression was determined by RT-qPCR ($n = 3$). *GAPDH* was used as reference gene. (G) NCI-H1299 cells expressing ER-E2F1 were left untreated or incubated with OHT (100 nM), CHX (10 μ g/mL) or both (OHT + CHX) for 8 h ($n = 2$). *LINC00673* expression was determined by RT-qPCR as described in E. (H) IMR-90 cells were serum starved for 4 days and the cell cycle block was released by adding complete medium containing 10% FBS. The cells were harvested at indicated time points and the gene expression was measured by RT-qPCR ($n = 3$). *PPIA* was used as reference gene. (I) A549 cells were serum starved for 3 days and the cell cycle block was released by adding complete medium containing 10% FBS. The cells were harvested at indicated time points and the gene expression was measured by RT-qPCR ($n = 6$). *PPIA* was used as reference gene. All data is shown as mean + SEM. Statistical significance was determined per two-sided unpaired Student t test, with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Figure 8. *LINC00673* knockdown impedes cell proliferation. (A) The relative *LINC00673* knockdown in A549 and Calu-3 cells 24 h and 36 h after transfection with two different 30 nM LNA longRNA GapmeRs (Exiqon) is shown. *GAPDH* was used as reference gene and *LINC00673* expression was normalized to the LNA longRNA GapmeR control A (Exiqon). The mean + SEM is shown ($n = 3$). (B) The relative *LINC00673* knockdown in A549 and Calu-3 cells 48 h after transfection with 3 nM or 10 nM *LINC00673* siPOOLs (custom designed by siTOOLS Biotech), respectively, is presented. RT-qPCR as in A ($n = 3$). In A and B the statistical significance was determined per two-sided unpaired Student t test, with **, $P < 0.01$; ***, $P < 0.001$. (C) IMR-90 cells were serum starved for 24 h and *LINC00673* was subsequently knocked down for 72 h using 0.3 nM siPOOLs. 4 days after serum starvation, cells were stimulated by adding complete medium containing 10% FBS. The cells were harvested at indicated time points and the gene expression was measured by RT-qPCR ($n = 3$). *PPIA* was used as reference gene and gene expression was normalized to the 0 h control values.

Supplementary Figure 9. Involvement of *LINC00673*-correlated genes in gene ontology biological processes in lung adenocarcinoma. All genes that positively correlated (> 0.3) with *LINC00673* expression in the TCGA lung ADC set were retrieved with TANRIC and functional enrichment analysis was performed with g:Profiler (using g:GOST with moderate filtering option). The clusters of gene ontology terms for the biological processes are shown ($P < 0.05$).

Supplementary Figure 10. *LINC00673* knockdown leads to changes in cell morphology. Pictures were taken with a Leica DM IRB microscope (10x objective) at 48 h after *LINC00673* knockdown in A549 (3 nM siPOOL each) and IMR-90 cells (0.3 nM siPOOL). The scale bars represent 100 μm .

Supplementary Figure 11. *LINC00673* depletion deregulates genes in IMR-90 cells. (A) Cell cycle and E2F1-regulated genes were determined by RT-qPCR at 48 h after *LINC00673* knockdown with siPOOLs (0.3 nM). *GAPDH* was used as reference gene ($n = 3$). The statistical significance was determined per two-sided unpaired Student t test, with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) SASP gene expression was determined by RT-qPCR as in C ($n = 3$).

Supplementary Figure 12. Expression of senescence markers upon *LINC00673* depletion. Immunofluorescence staining was performed 4 days after siPOOL-mediated *LINC00673* knockdown using specific antibodies (A549: 3 nM siPOOL, $n = 2$; IMR-90: 0.3 nM siPOOL, $n = 3$). Doxorubicin-treatment served as positive control. Pictures were taken with the Olympus Cell[^]R microscope (60x oil objective) and representative cells are shown.

Supplementary Figure 13. Knockdown efficiency and gene expression changes in lung cancer and lung fibroblast cells. Relative RNA levels were determined by RT-qPCR at 48 h after knockdown in A549 ($n = 3$) and IMR-90 cells ($n = 4$). For A549 cells, the reactions contained siPOOL negative control (5 nM) or a mix of 1 nM siPOOL p53, pRb and 3 nM siPOOL *LINC00673* supplemented with siPOOL negative control for a final concentration of 5 nM. For IMR-90 cells, the reactions contained siPOOL negative control (0.3 nM) or a mix of 0.3 nM siPOOL p53, pRb and *LINC00673* supplemented with siPOOL negative control for a final concentration of 0.9 nM. *GAPDH* was used as reference gene and gene expression was normalized to the siPOOL control. The mean + SEM is shown.

Supplementary Figure 14. Senescence bypass upon simultaneous overexpression of HPV type 16 E7 protein and *LINC00673* depletion in IMR-90 cells. For each condition, the number of senescent cells was determined by counting 200 cells in triplicates (the mean + SD of three independent counts are shown, $n = 1$). Pictures were taken with the Leica TCS SP5 microscope (10x objective) and representative pictures are shown. Scale bars represent 50 μ m.

Supplementary Figure 15. A549 cells adapted to heavy medium were treated with 3 nM of siPOOL *LINC00673*, whereas A549 cell adapted to light medium were treated with 3 nM of siPOOL negative control for 48 h prior sample preparation ($n = 2$). The correlation between both SILAC experiments is shown (Pearson correlation = 0.705). The ratios of heavy/light values are plotted.

Supplementary Figure 16. Validation of quantitative mass spectrometry hits by RT-qPCR. Relative RNA levels were determined by RT-qPCR at 48 h after siPOOL-mediated *LINC00673* knockdown in A549 cells (final concentration of 3 nM, $n = 3$). *GAPDH* was used as reference gene and the data represents the mean + SEM. The statistical significance was determined per two-sided unpaired Student t test, with *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Supplementary Figure 17. Depletion of p53 partially restores *LINC00673*-associated gene expression changes. Selected hits from mass spectrometry analysis were analyzed by RT-qPCR at 48 h after knockdown in A549 cells ($n = 3$). The reactions contained siPOOL negative control (5 nM) or a mix of 1 nM siPOOL p53, pRb and 3 nM siPOOL *LINC00673* supplemented with siPOOL negative control for a final concentration of 5 nM. *GAPDH* was used as reference gene and the data was normalized to the siPOOL control. The mean + SEM is shown.

Supplementary Figure 18. Gene expression was determined by RT-qPCR at 48 h after *UHRF1* and *PAI-1* knockdown with 0.3 nM siPOOL in IMR-90 cells (**A**) and 1 nM siPOOL in A549 cells (**B**), respectively. *GAPDH* was used as reference gene and the data was normalized to the siPOOL control ($n = 3$).

SUPPLEMENTARY TABLE LEGENDS

Supplementary Table 1: Microarray profiling of lncRNAs in lung adenocarcinoma

The linear fold change, the adjusted p-value (FDR Benjamini-Hochberg) and the log₂ signal intensities as well as the genomic re-annotation of the array is provided.

Supplementary Table 2: Correlation of *LINC00673*, E2F1 and Cyclin E

Pearson correlation coefficients calculated for RNA and protein levels are provided.

Supplementary Table 3: SILAC profiling after *LINC00673* knockdown

Cells were treated with LINC00673 siPOOLs (heavy, H) or the respective control (light, L) and subjected to proteome profiling by SILAC and mass spectrometry.

Supplementary Table 4: Differentially expressed proteins after *LINC00673* knockdown

Differentially expressed proteins identified by SILAC after LUACIR1 knockdown are listed along with their published links to senescence or their regulation by p53.

Supplementary Table 5: Gapmer and siPOOL sequences

Gapmer and siPOOL sequences are provided in 5'-3' direction.

Supplementary Table 6: Primer for qPCR

Primer sequences are provided in 5'-3' direction.

Supplementary Table 7: Primers for RACE

Primer sequences are provided in 5'-3' direction.

Supplementary Table 8: Primers for *in vitro* transcription and probe generation

Primer sequences are provided in 5'-3' direction.

SUPPLEMENTARY METHODS

Patient material

A total of 57 tumor and matched distant, normal (> 2 cm away from tumor) lung tissue samples were collected from NSCLC patients who underwent resection for primary lung adenocarcinoma at the Thoraxklinik Heidelberg, Germany. Tissue samples were snap-frozen within 30 min after resection and stored at -80°C until the time of analysis. All lung cancer specimens were diagnosed according to the 6th World Health Organization (WHO) classification for lung cancer by at least two experienced pathologists. Histomorphological subtyping was done according to the IASLC/ATS/ERS proposal. Only specimens with > 50% tumor cell amount were considered for this study. All resections were free of disease and considered complete (R0). None of the patients received tumor-specific therapy prior to resection. The study protocol was approved by the local Ethics Committee of the University Heidelberg, Germany.

Microarray screen

For lung cancer, 10 - 15 cryosections (10 - 15 µm each) of frozen tumor tissues were prepared for each patient for RNA extraction. The first and the last section of the series were stained with Hematoxylin and Eosin, and were reviewed by the study pathologist to determine the proportion of viable tumor cell, stromal cell, normal lung cell content, infiltrating lymphocytes and necrotic areas. Only sections with $\geq 50\%$ viable tumor cells were considered for RNA isolation and subsequent expression analyses. Matched normal tissues were macroscopically reviewed to be devoid of tumor. Frozen tumor cryosections were homogenized with the TissueLyser mixer-mill disruptor (2 x 3 min, 30 Hz; Qiagen, Hilden, Germany). Matched frozen normal lung tissue pieces were homogenized using a Micra D-8 rotor-stator homogenizer with DS-5/K1 (2 x 30 sec, 26,000 rpm; Micra, Müllheim, Germany). Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. For all samples, on-column DNase I digestion (Qiagen) was performed. The quantity of RNA was measured with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, MA, USA), and the quality of total RNA was assessed with an Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit (Agilent Technologies, Böblingen, Germany). Only RNA with 28S/18S > 1 and RIN > 6 were used. Using the SuperScript Indirect cDNA labeling system (Invitrogen, Karlsruhe, Germany) with anchored

oligo-dT primers, 7 µg RNA were labeled with Cy3 mono-reactive dye (Amersham Biosciences, GE Healthcare, Freiburg, Germany). The efficiency of cDNA synthesis and labeling was assessed on the NanoDrop before hybridization of the NCode Human Noncoding RNA Microarray (Invitrogen). Hybridization of the arrays was performed using the Agilent gene expression hybridization kit according to the manufacturer's protocol. The arrays were scanned on an Agilent scanner and the data was extracted using Agilent Feature Extraction Software (FE) 10.7.3.1.

Microarray statistical analyses

The microarray data analysis and statistics were done with GeneSpring 11.5.1 and SPSS 18. The microarray data was quantile normalized with minimum signal intensity (threshold 1.0). For quality control purposes, box plots of signal distribution of non-normalized and normalized datasets were compared and the correlation coefficients between the datasets calculated. To exclude outliers and technically impaired datasets, only samples were included into the study that displayed a correlation coefficient of at least 0.7 based on the assumption that the majority of the 39,186 features on the array (ncRNA and mRNA probes) should not be altered. The probe sets were grouped according to the coding potential of the respective transcripts (ncRNA or mRNA). For the analysis of expression intensity of the ncRNA vs. the mRNA group, the average signal intensity (log2) for each probe in each group (tumor vs. normal) was calculated and binned in 0.1 increments. The frequency of this average signal intensity was then normalized for the total number of probes representing ncRNAs (17,112 probes) or mRNAs (22,074 probes) and plotted against the signal intensity for ncRNAs and mRNAs separately. To discover differentially expressed genes between tumor and normal, unpaired t-tests were performed to calculate asymptotic significance values. The *P*-value was corrected for multiple testing using the False Discovery Rate (FDR) after Benjamini-Hochberg. A corrected *P*-value < 0.05 and a fold change ≥ 2.0 were considered as significant change. Hierarchical unsupervised clustering was generated using GeneSpring with the default parameters (Euclidean distance metric, Centroid linkage rule); row-normalized heatmaps of expression profiles were generated using the Heatmap Builder 1.0.

SILAC and Mass Spectrometry analysis

A total of 15 µg of lysate (1:1 mixed heavy and light sample of each experiment) was separated on a NuPAGE Novex 10% Bis-Tris protein gel (1.0 mm; Life Technologies, Darmstadt,

Germany) using NuPAGE MOPS SDS Running Buffer (Life Technologies) and stained with colloidal coomassie (Generon, Slough, UK). Mass spectrometric analysis was performed at the Core Facility for Mass Spectrometry and Proteomics of the ZMBH, Heidelberg, Germany. The samples were reduced with DTT, alkylated with iodoacetamide and digested with trypsin (Thermo Fisher Scientific, Dreieich, Germany) using a Digest pro MS liquid handling system (Intavis AG, Cologne, Germany) as previously described¹. Digested peptides were then extracted from the gel pieces with 50% acetonitrile/0.1% TFA, concentrated nearly to dryness in a SpeedVac vacuum centrifuge and diluted to a total volume of 30 µl with 0.1% TFA. 10 µl of the sample was analyzed by a nano HPLC system (Ultimate 300; Thermo Fisher Scientific) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Sample was loaded on a C18 Acclaim PepMap100 trap-column (Thermo Fisher Scientific) with a flow rate of 30 µl/min 0.1% TFA. Peptides were eluted and separated on an C18 Acclaim PepMap RSLC analytical column (75 µm x 250 mm) with a flow rate of 300 nl/min in a 90 min gradient of 3% buffer A (0.1% formic acid) to 40% buffer B (0.1% formic acid, acetonitrile). MS data were acquired with an automatic switch between a full scan and up to twenty data-dependent MS/MS scans. All results were searched in MaxQuant v1.5.3.30 using the databases UP000005640_9606.fasta, UP000005640_9606_additional.fasta (PSM FDR = 0.01, protein FDR = 0.01) and subsequent analyses were performed with Perseus Software v1.5.3.2. A good correlation between both SILAC experiments was observed and significantly deregulated proteins were identified ($P < 0.05$, Supplementary Figure 13).

Cell culture, transfections and retroviral infections

A549 (CCL-185), IMR-90 (CCL-186), WI-38 (CCL-75) and HEK293 cells were purchased from the American Type Culture Collection (ATCC), Calu-3 cells were a kind gift from Heike Allgayer (Experimental Surgery, Mannheim Medical Faculty of University of Heidelberg, Centre for Biomedicine and Medical Technology Mannheim (CBTM), Germany). A549 and Hek293 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 2 mM L-glutamine (Sigma-Aldrich). Calu-3 and IMR-90 cells were grown in Eagle's minimal essential medium (EMEM; PAN-Biotech, Aidenbach, Germany) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and non-essential amino acids. For the SILAC experiments, A549 cells were grown in heavy or light SILAC media (DMEM SILAC-

Lys8-Arg6-Kit by Silantes, Munich, Germany) supplemented with 200 µg/ml of L-Proline (Thermo Fisher Scientific) for at least 2 weeks prior transfection to reach an incorporation rate of > 95%. All cells were maintained in a humidified incubator at 37°C with 5% CO₂. The cell culture conditions and induction of ER-E2F1 in NCI-H1299 and WI-38 have been described previously ². For the knockdown studies, cells were reverse transfected using Lipofectamine RNAiMAX (Invitrogen) and processed at the indicated timepoints as required for the respective assays. When RNA isolation was performed, cells were transfected in 6-well or 60 mm dishes, and 100 mm dishes were used for serum starvation experiments. For luciferase assays, 40,000 Hek293 cells were seeded in 500 µl of media 24 hrs prior transfection. Transfection was carried out with a total of 1.2 µg of DNA (0.2 µg of psiCHECK2 and 1.0 µg of pCRII plasmid) and TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA) at a ratio of 1:3, then cells were lysed 48 hrs after transfection. GapmeRs were purchased from Exiqon (Vedbaek, Denmark) and sequences listed in Supplementary Table 5. The siPOOLS were designed and purchased from siTOOLS Biotech (Planegg, Germany). The final concentration of GapmeRs and siPOOLS used for knockdown studies and number of biological replicates performed are indicated in figure legends. Retroviral infections were performed as previously described ³. Briefly, the Phoenix amphotropic packaging cell line was transfected with either HPV type 16 E6, E7, E6/E7 or the empty control vector pLXSN. Viral supernatants were harvested 48 hrs following transfection, supplemented with 10% FBS and 6 µg/ml polybrene, and transferred onto IMR-90 cells. Infected IMR-90 cells were then selected using 400 µg/ml neomycin for seven days. Infected cell populations were then infected with H-RasV12 or an empty control vector then selected using 2.5 µg/ml puromycin for 3 days. The different cell lines generated were then transfected using siPOOL targeting *LINC00673* or non-targeting controls.

RNA extraction and RT-qPCR

Cells were lysed in TRIzol (Ambion, Thermo Fisher Scientific) at the indicated timepoints and the RNA was extracted according to the manufacturer's recommendations. RNA was subsequently treated with DNase I (Roche, Grenzach-Wyhlen, Germany) for 30 min at 37°C. 1 µg of DNase I-treated RNA was reverse transcribed using the RevertAid Reverse Transcriptase (Thermo Fisher Scientific) and random hexamer primers (Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time quantitative PCR reactions (RT-qPCR) were

performed using an Applied Biosystems StepOnePlus cycler in technical triplicates. Therefore, the cDNA was diluted 1:40 in distilled water and 4 µl were used in a 10 µl PCR reaction containing 2x Power SybrGreen Master Mix (Applied Biosystems, Thermo Fisher Scientific) and 0.2 µM each of the respective forward and reverse primers. The qPCR program was as follows: holding stage of 95°C for 10 min followed by a cycling stage of 95°C for 15 sec and 60°C for 30 sec repeated 40 times. *PPIA* or *GAPDH* housekeeping genes were used for normalization. The relative expression was determined according to the $2^{-\Delta C_t}$ and $2^{-\Delta\Delta C_t}$ methods. Primer sequences are listed in Supplementary Table 6.

Luciferase assays

The pCRII-LINC00673 plasmid was generated by cloning the full-length *LINC00673* sequence into a modified pCRII-TOPO vector (Life Technologies, Thermo Fisher Scientific). To generate the psiCHECK2-TP53(3'UTR) plasmid, the TP53 3'UTR sequence (NM_000546.5, nucleotides 1421-2591) was amplified by PCR and cloned into the psiCHECK2 vector (Promega, Mannheim, Germany) using SgfI and NotI restriction sites. The TP53 3'UTRΔ sequence contained a deletion of 273 nucleotides (2172-2444), thereby lacking the *LINC00673* interaction site. Luciferase assays were performed in quadruplicates for each sample with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions with the following modification. The cells were lysed in 150 µl of passive lysis buffer for 30 min at room temperature. The luminescence was measured with a luminometer (Fluoroskan Ascent FL, Thermo Fisher Scientific) and the ratio of Renilla to Firefly luciferase signal was analyzed.

Northern Blot

A total of 30 µg of DNase I-treated RNA from indicated cell lines were resuspended in 6.75 µl of distilled water and mixed with 3 µl of 10x MOPS buffer (200 mM MOPS, 80 mM sodium acetate, 10 mM EDTA, pH 7.0), 5.25 µl of 37% formaldehyde and 15 µl of formamide, heated 15 min at 65°C and transferred to ice. Next, the RNA was mixed with 3 µl of loading dye (95% of formamide, 0.05% of SDS, 0.05% of bromophenol blue) supplemented with 1/20 volume of 10 mg/ml ethidium bromide and separated on a 1% agarose gel (15 x 15 cm) containing formaldehyde and MOPS buffer. The RNA integrity was confirmed using a UV gel imager (Intas Science Imaging Instruments, Göttingen, Germany) and the gel soaked in 1 L of distilled water three times for 10 min. Next, the RNA was transferred to an Amersham Hybond N+ nylon

membrane (GE Healthcare, Freiburg, Germany) by capillary transfer overnight. The nylon membrane was dried and the RNA crosslinked with the UV Stratalinker 1800 using the autocrosslink mode (120,000 $\mu\text{J}/\text{cm}^2$; Stratagene, Agilent, Waldbronn, Germany). The Northern Blot probes were generated from gel-purified PCR products by *in vitro* transcription (primer sequences are listed in Supplementary Table 8). Therefore, 20 μl reactions containing 4 μl of 5x Reverse Transcriptase buffer (Fermentas, Thermo Fisher Scientific), 1 μl of an ATP, GTP, CTP mixture (10 mM each), 1 μl of gel-purified PCR product (100 ng/ μl), 0.5 μl of RiboLock RNase Inhibitor (40 U/ μl), 1 μl of Fermentas T7 RNA polymerase and 5 μl of [α - ^{32}P] UTP (100 μM ; Perkin Elmer, Rodgau, Germany) were incubated for 1 h at 37°C, and the PCR template was digested by adding 1 μl of DNase I (10 U/ μl ; Roche) for 15 min. The enzyme was inactivated by adding 2 μl of 0.5 M EDTA solution and the RNA probes were purified with illustra MicroSpin G25-columns (GE Healthcare) according to the manufacturer's instructions. The membranes containing the crosslinked RNA were subsequently incubated with 10 ml of Ambion ULTRAhyb Ultrasensitive Hybridization Buffer containing the probes for 14 hrs at 68°C in a hybridization oven under constant rotation. Membranes were then washed with 20 ml of low stringency wash buffer (2x SSC, 0.1% SDS, pH 7.0) and high stringency wash buffer (0.1x SSC, 0.1% SDS, pH 7.0) at 68°C, and the signal visualized by autoradiography. The membranes were stored at -80°C for the time of film exposure.

Western Blot and antibodies

Cells were lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with PhosStop (Roche) and EDTA-free protease inhibitor mixture (Roche). The protein concentration was determined using BCA assay. 10 or 20 μg of protein lysate were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Whatman Protran, Sigma-Aldrich). The primary antibodies and dilutions were as follows: β -actin (dilution: 1:20,000; catalog no. A2228, Sigma-Aldrich), GAPDH (dilution: 1:500; catalog no. sc-137179, Santa Cruz Biotechnology, Dallas, TX, USA), E2F1 (dilution: 1:1000; catalog no. 3742, Cell Signaling Technology, Danvers, MA, USA), p53 (dilution: 1:1000; catalog no. sc-126, Santa Cruz Biotechnology), P-p53 Ser-15 (dilution: 1:1000; catalog no. 9284, Cell Signaling Technology), pRb (concentration: 2 $\mu\text{g}/\text{ml}$; catalog no. 554136, BD Biosciences, Franklin Lakes, NJ, USA), PAI-1 (dilution: 1:200; catalog no. sc-5297, Santa Cruz Biotechnology), UHRF1 (concentration: 0.5 $\mu\text{g}/\text{ml}$; catalog no. ABE551, Merck Millipore, Darmstadt, Germany).

Cell viability, proliferation and apoptosis assay

For cell proliferation analyses of IMR-90 following serum starvation, 1800 cells were seeded per well and starved as described below. 4 days after starvation, cells were released by adding medium supplemented with 10% FBS and 10 μ l of the BrdU labeling reagent per well, and the assay was performed at timepoints indicated in the figure legend. All other transfections were carried out in a 96-well format in technical triplicates or quadruplicates using 3000-5000 cells per well. For the cell proliferation assay, black 96-well plates with a clear bottom were used. Cell viability was measured with the CellTiter-Glo Luminescent Cell Viability Assay (Promega), cell proliferation was quantified with the BrdU Cell Proliferation ELISA Kit (Roche, cells were labeled with BrdU for 6 h) and apoptosis was measured with the Caspase-Glo 3/7 luminescent assay (Promega). For the latter, cells were treated with 5 μ g/ml of actinomycin D (Sigma-Aldrich) as a positive control. All assays were carried out according to the manufacturer's instructions with the following modifications. For the cell viability assay, the media was removed from the cells at indicated timepoints after knockdown and 60 μ l of a 1:4 mixture of the CellTiter-Glo reagent in PBS was added. The cells lysis was carried out for 15 min at room temperature using an orbital shaker and 40 μ l of the lysate were transferred into white 96-well plates for readout. For the apoptosis assay, 50 μ l of a 1:1 mixture of Caspase-Glo reagent and PBS were added to each 96-well and the contents were mixed for 30 sec using an orbital shaker. The readout was performed after a 30 min incubation period at room temperature. The chemiluminescence was measured with a luminometer (Fluoroskan Ascent FL, Thermo Fisher Scientific).

Cell cycle analysis and serum starvation

For FACS analyses, 2×10^5 A549 cells were grown on 60 mm dishes and trypsinized 48 h and 72 h after reverse transfection. The cells were washed once with 3 ml PBS, spun down and resuspended in 100 μ l ice-cold PBS. 1 ml ice-cold 70% ethanol was added and cells were precipitated for at least 2 h at -20°C . Next, cells were collected by centrifugation at 500 - 1000 g for 5 min at 4°C and washed twice with 1 ml of ice-cold PBS. Afterwards, cells were resuspended in 200 μ l PBS containing 100 μ g/ml of RNase A (Sigma-Aldrich) and incubated for 30 min at 37°C . The cells were transferred to FACS tubes (BD Falcon 5 ml Round Bottom Polystyrene Test Tube with Cell Strainer Snap Cap) containing 300 μ l PBS with 66.7 μ g/ml propidium iodide (Sigma-Aldrich). All samples were sorted with the BD FACSCanto II at the

ZMBH Flow Cytometry & FACS Core Facility (Heidelberg, Germany). For the analysis, at least 20,000 gated events were considered per sample and FlowJo (V10) data analysis software was used. Cell cycle profiles for A549 cells were determined with the cell cycle function of FlowJo (V10). For serum starvation, 2.6×10^5 IMR-90 cells were seeded in 100 mm dishes and washed three times with PBS the next day before adding medium containing 0.2% FBS. When indicated, cells were transfected 24 h later and incubated for another 3 days. 4 days after starvation, cells were either directly processed for RNA isolation or released from the cell cycle block by adding medium supplemented with 10% FBS. The G0-phase arrest at 4 days after starvation was confirmed by FACS analysis (data not shown).

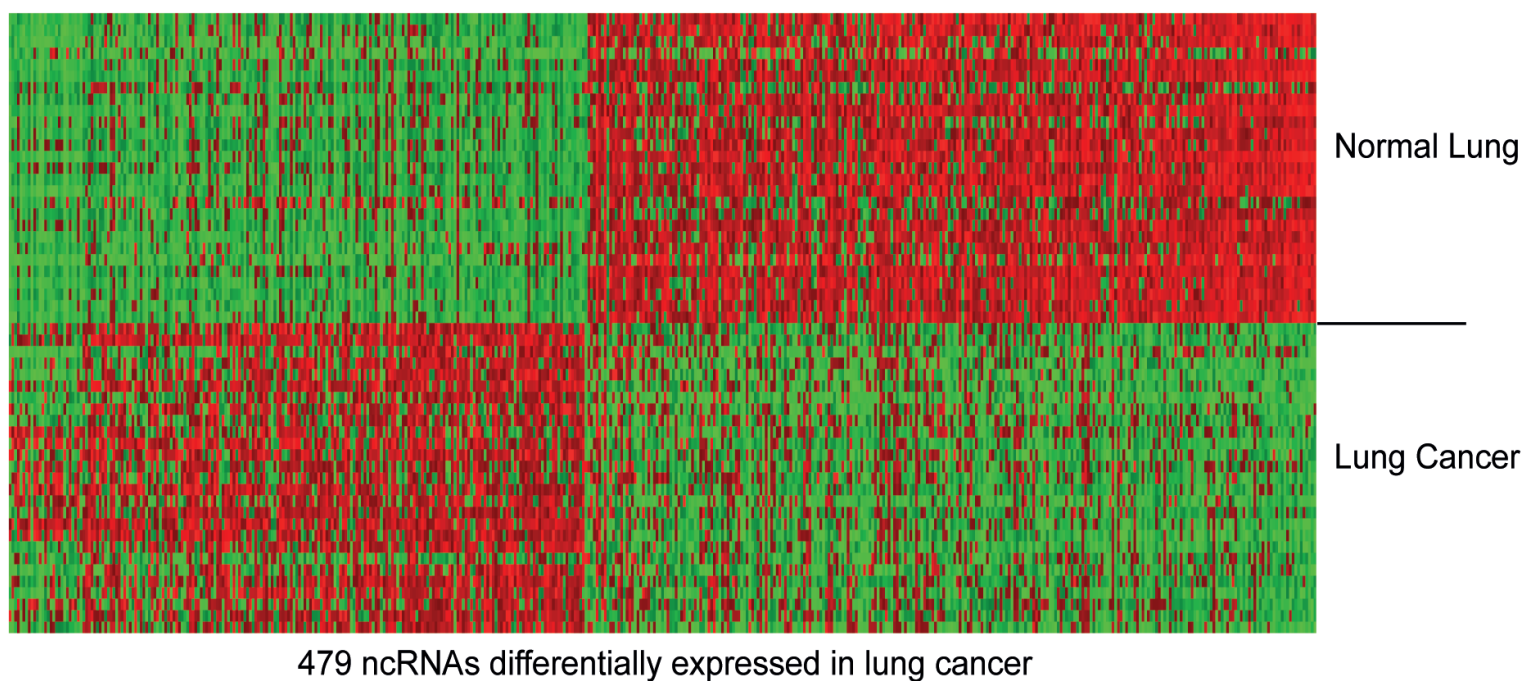
Immunofluorescence staining

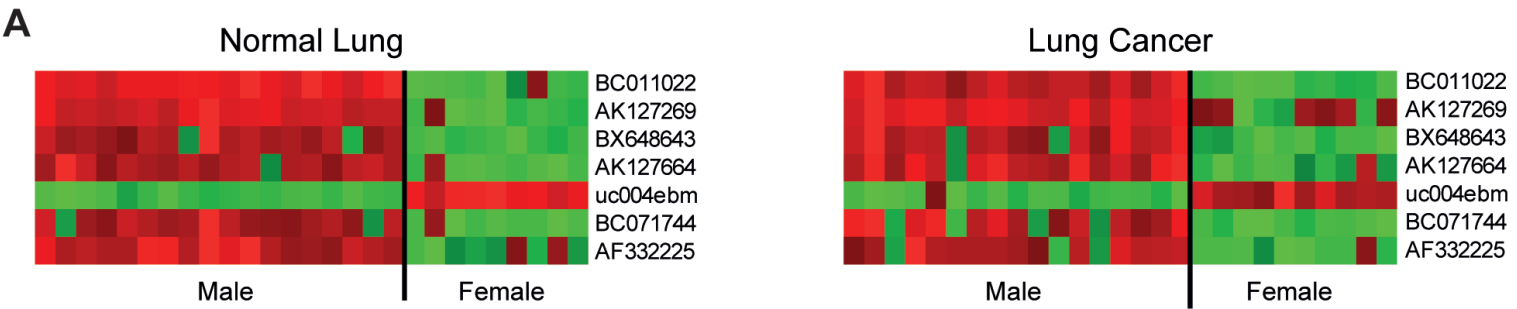
5×10^4 A549 cells and 1×10^5 IMR-90 cells were grown on coverslips in 60 mm dishes and fixed 4 days after reverse transfection with 4% paraformaldehyde-PBS for 10 min at room temperature. Cells were washed twice with PBS and permeabilized for 5 min with PBS containing 0.5% Triton X-100. Next, coverslips were washed once with PBS and blocked for 20 min with 2% BSA in PBS-Triton X-100 (0.1%). The primary antibodies H3K9me3 (dilution: 1:500; catalog no. ab8898, Abcam, Cambridge, UK) and γ -H2AX (dilution: 1:250; catalog no. 05-636, Merck Millipore) were diluted in 2% BSA-PBS-Triton X-100 (0.1%) and applied for 1-1.5 h at room temperature. The coverslips were washed five times with PBS-Triton X-100 (0.1%) and incubated with the secondary antibody Alexa Fluor 594 goat anti-rabbit IgG (dilution: 1:500; catalog no. A11037, Thermo Fisher Scientific) or Alexa Fluor 594 goat anti-mouse IgG (dilution: 1:500; catalog no. A11032, Thermo Fisher Scientific) for 1 h at room temperature in PBS-Triton X-100 (0.1%) containing 300 nM DAPI. Next, coverslips were washed five times with PBS-Triton X-100 (0.1%), then briefly in PBS and mounted on cover slides using Mowiol. Microscopy pictures were taken with the Olympus Cell^R microscope using the 60x oil objective.

SUPPLEMENTARY REFERENCES

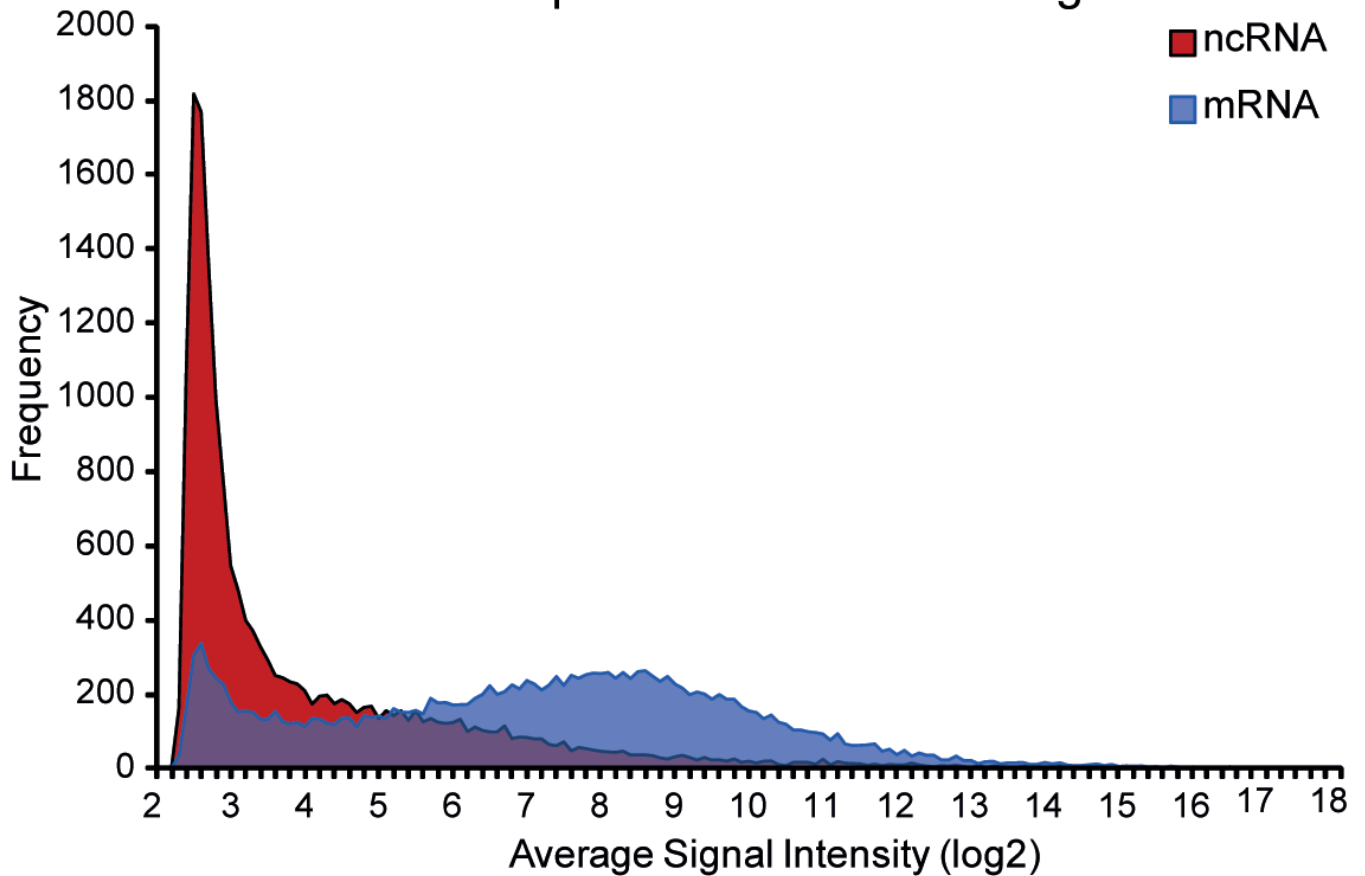
- 1 Catrein I, Herrmann R, Bosserhoff A, Ruppert T (2005). Experimental proof for a signal peptidase I like activity in *Mycoplasma pneumoniae*, but absence of a gene encoding a conserved bacterial type I SPase. *The FEBS journal* **272**: 2892-2900.
- 2 Feldstein O, Nizri T, Doniger T, Jacob J, Rechavi G, Ginsberg D (2013). The long non-coding RNA ERIC is regulated by E2F and modulates the cellular response to DNA damage. *Molecular cancer* **12**: 131.
- 3 Mallette FA, Richard S (2012). JMJD2A promotes cellular transformation by blocking cellular senescence through transcriptional repression of the tumor suppressor CHD5. *Cell reports* **2**: 1233-1243.

Heat map from microarray screen of normal lung and lung ADC patients

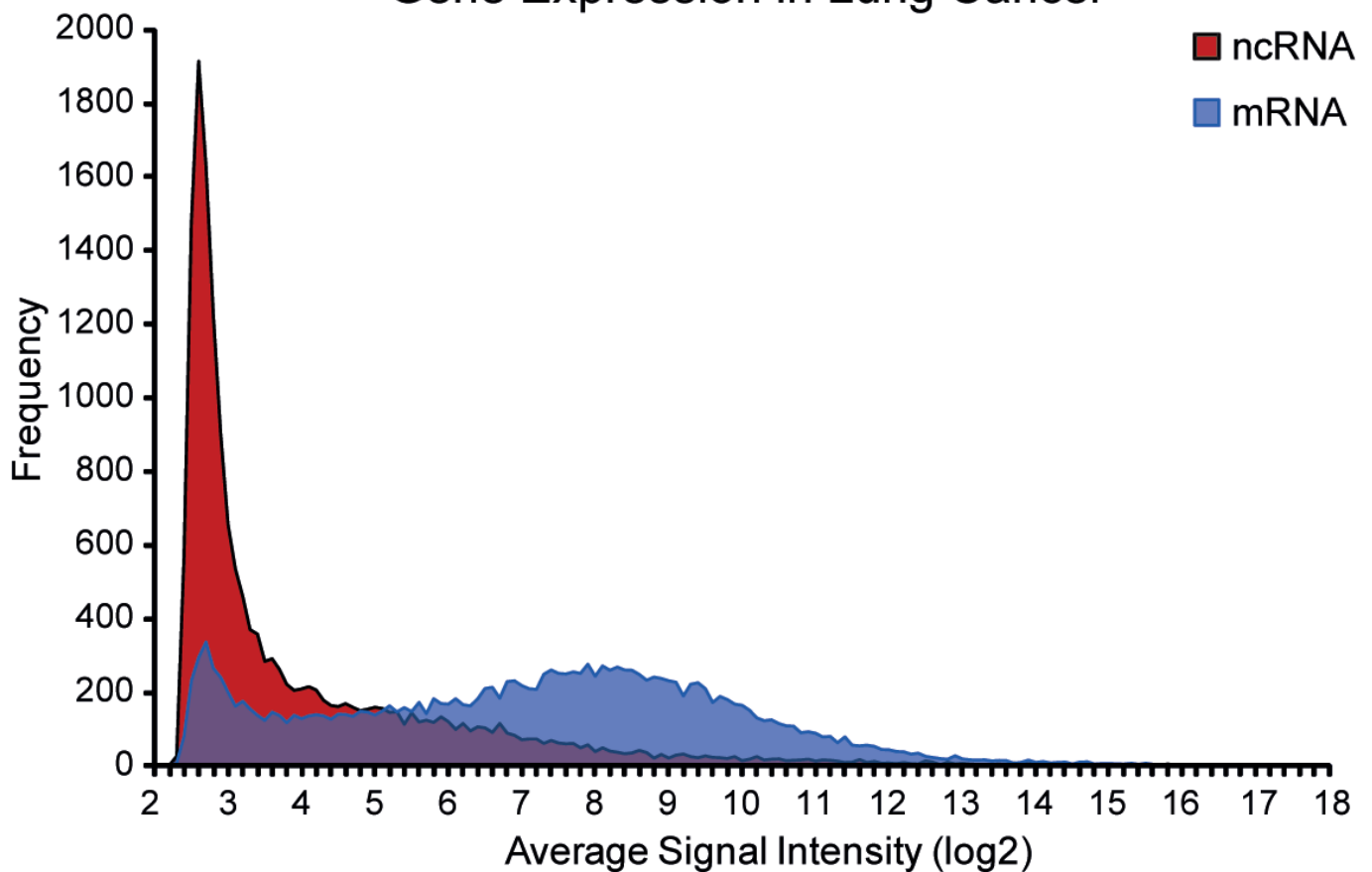


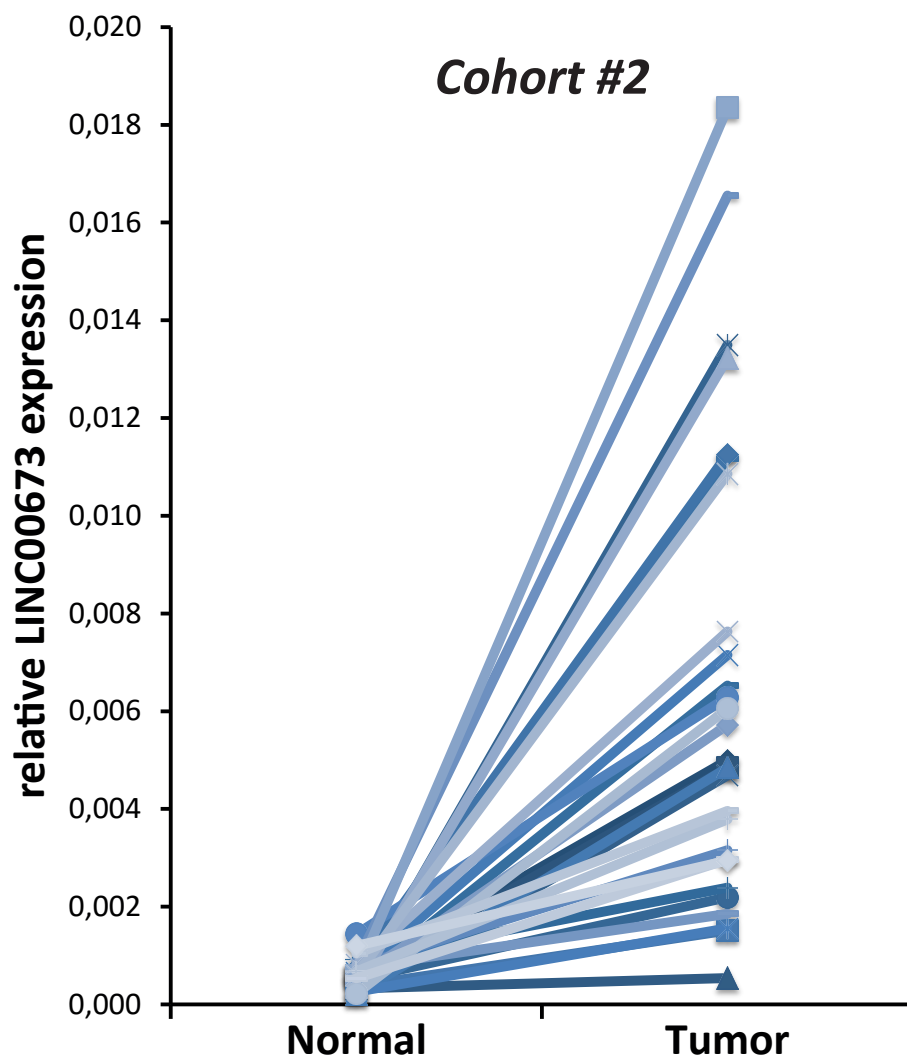
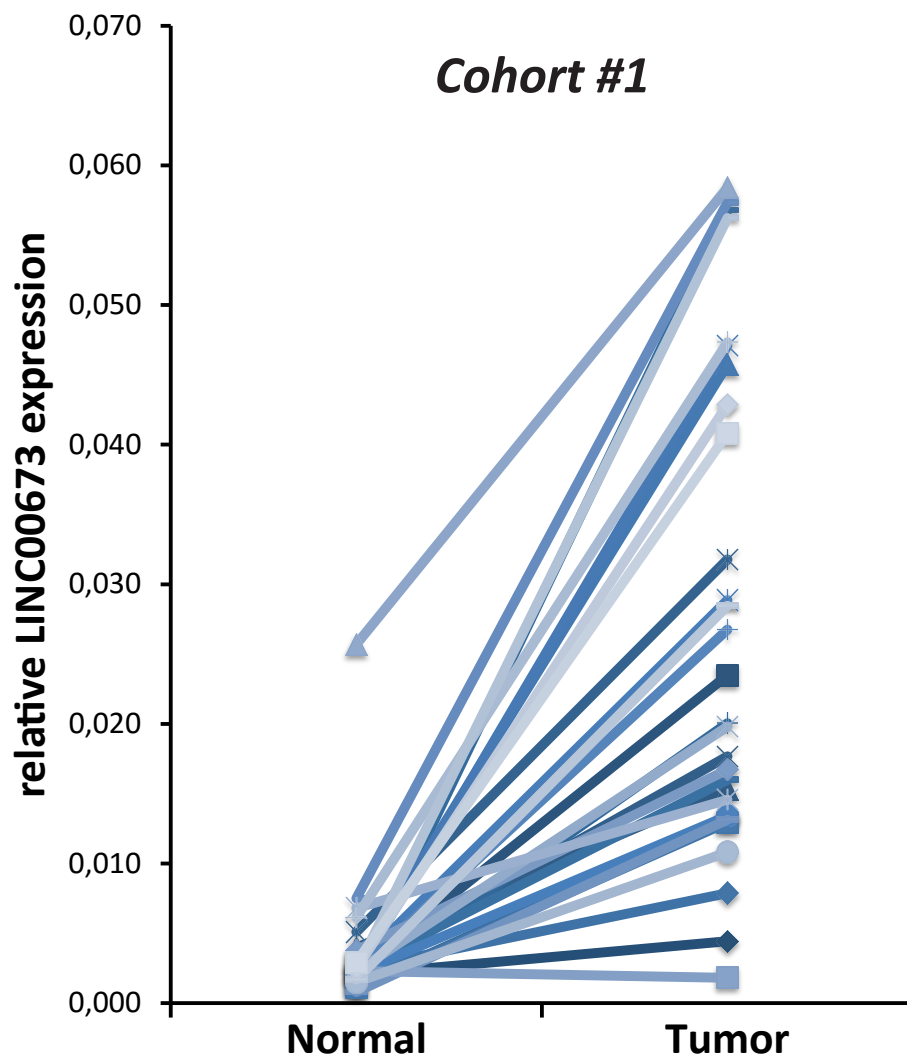


Gene Expression in Normal Lung

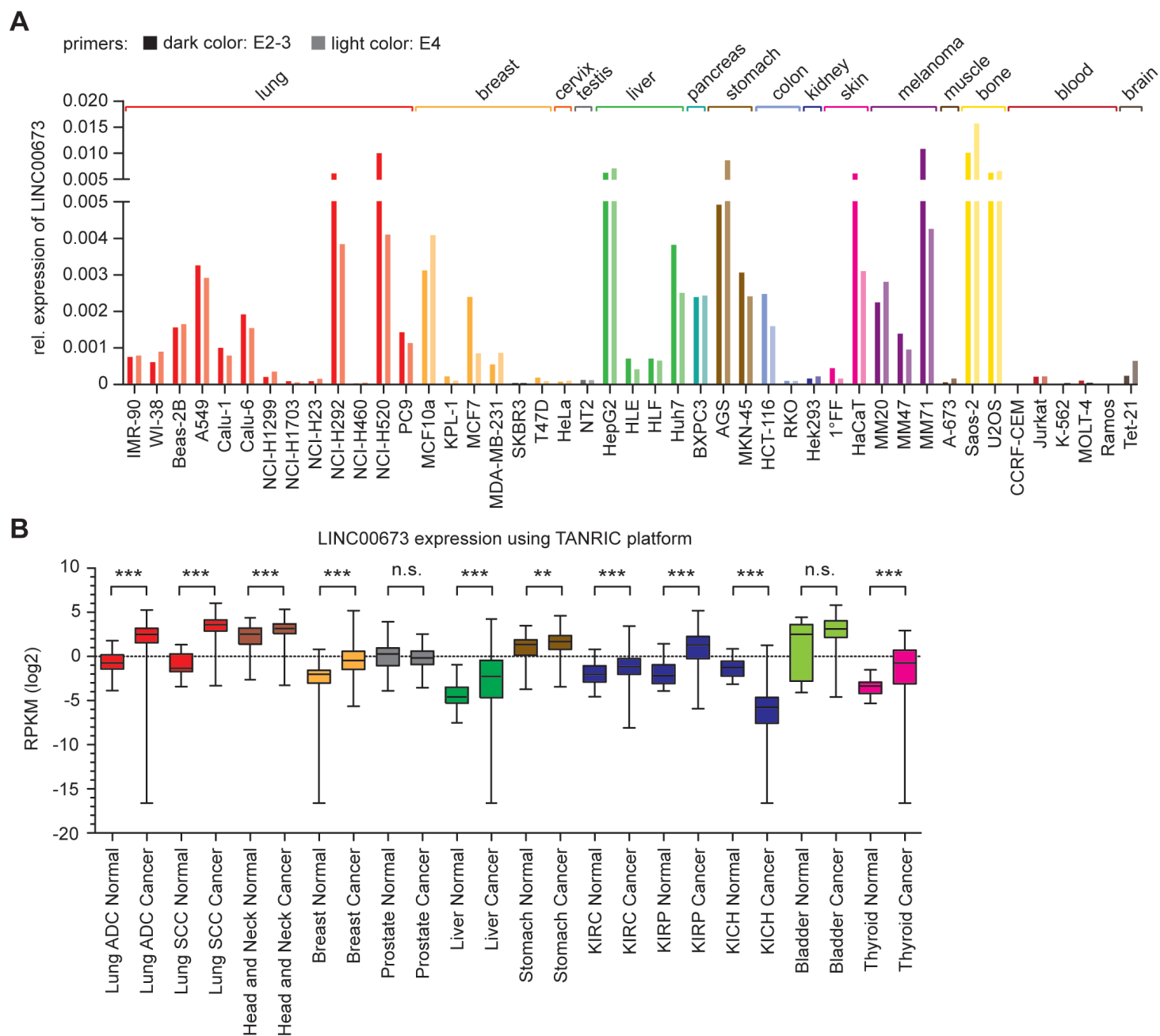


Gene Expression in Lung Cancer





Supplementary Figure 4

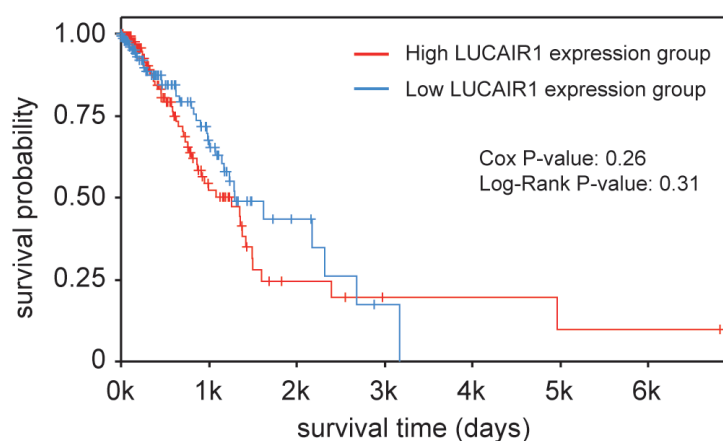


Supplementary Figure 5

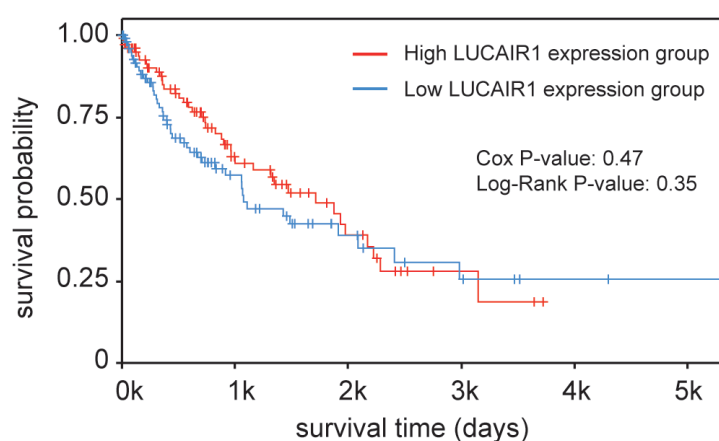
A

Correlation of LINC00673 expression with patient survival using TANRIC

TCGA-LUAD:

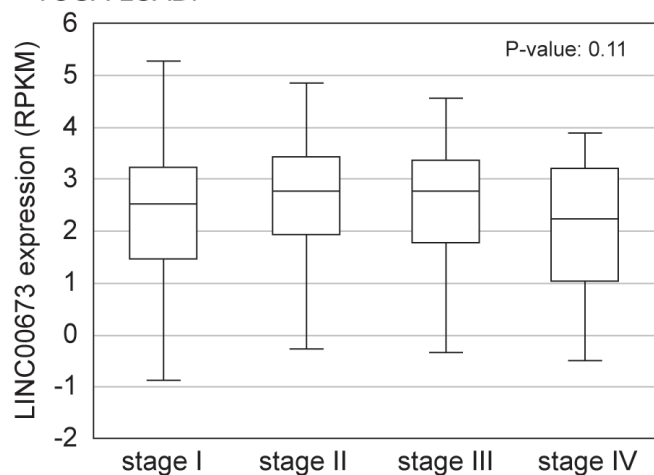


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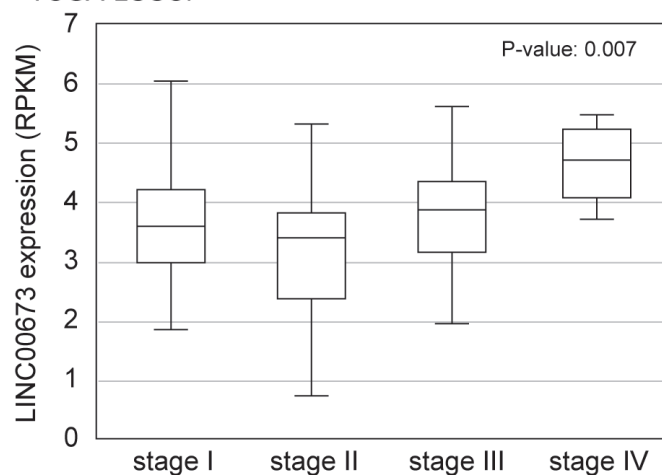
**B**

Correlation of LINC00673 expression with lung cancer stage using TANRIC

TCGA-LUAD:

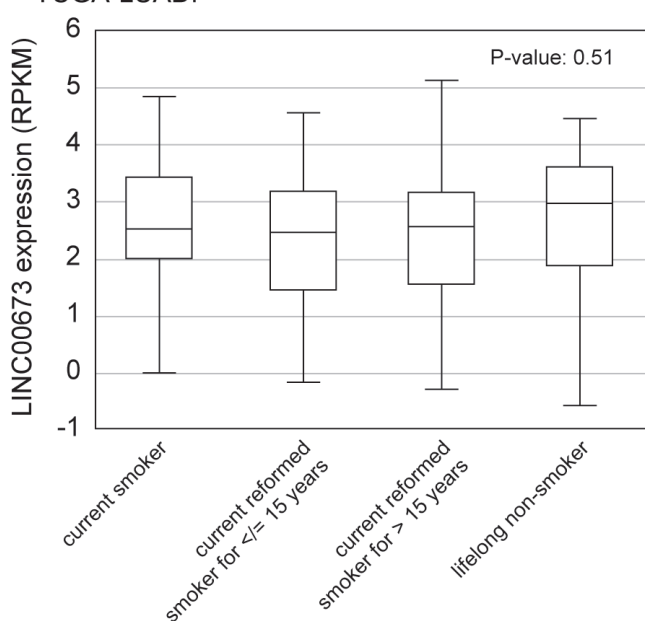


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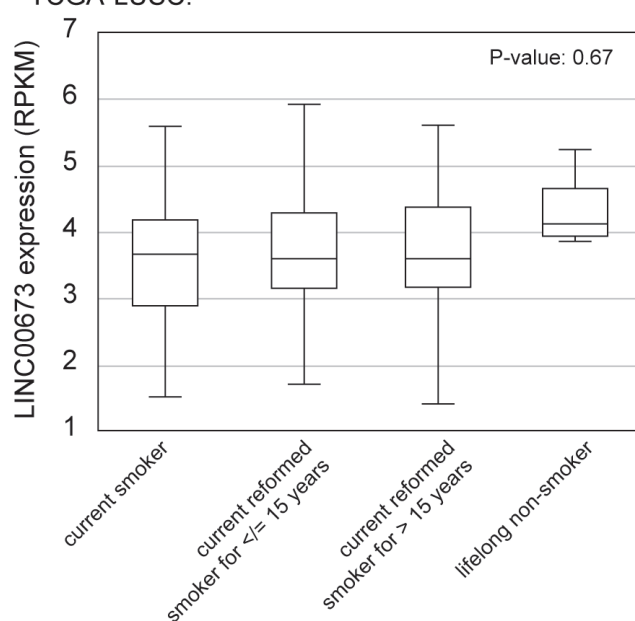
**C**

Correlation of LINC00673 expression with smoking status using TANRIC

TCGA-LUAD:



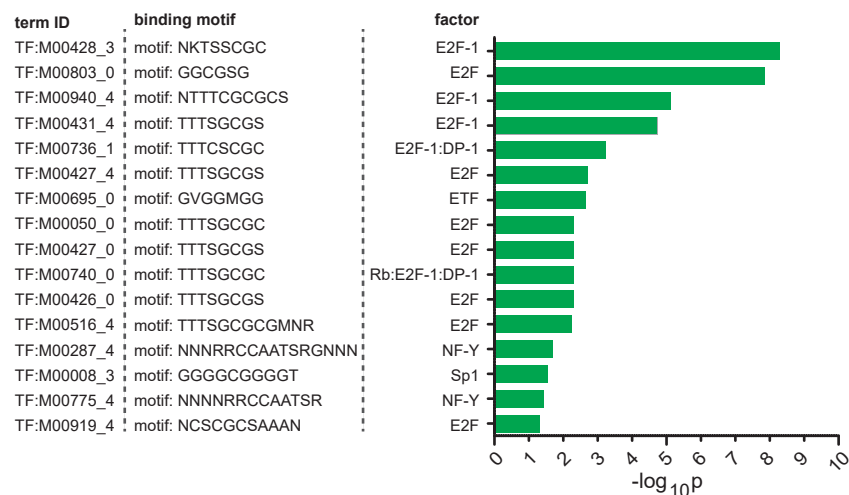
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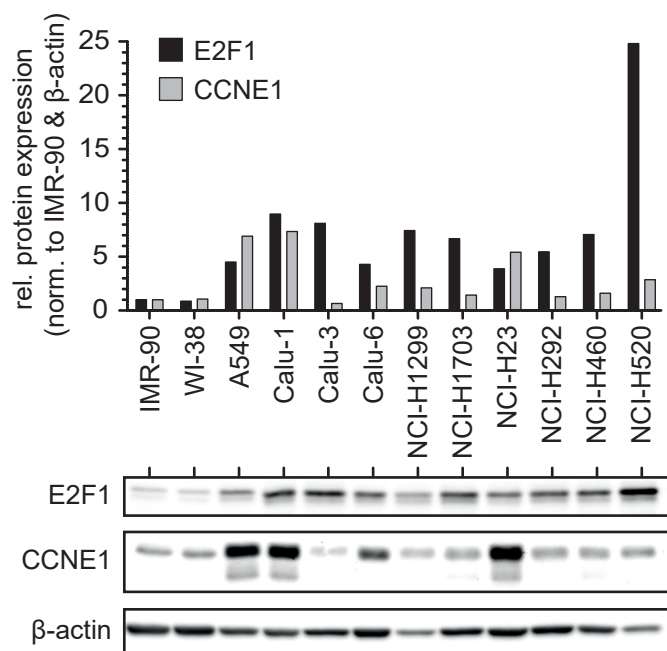
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Functional enrichment results based on (TRANSFAC TFBS database)

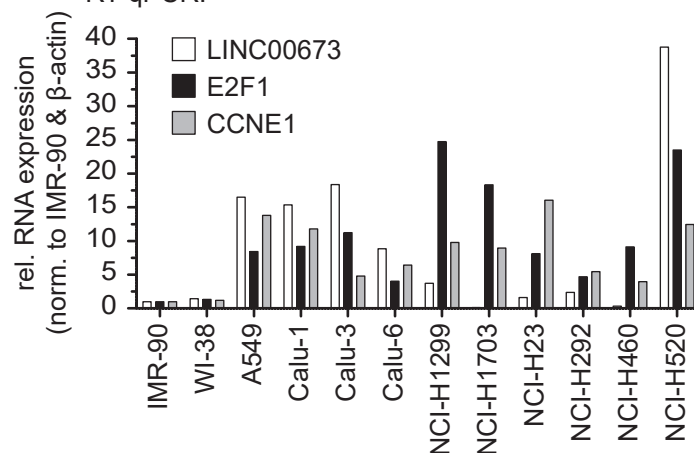
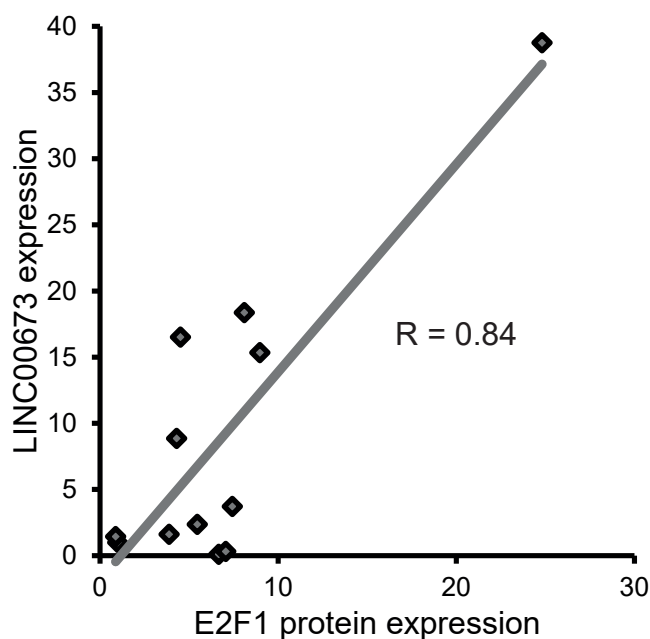
TCGA LUAD data

**B**

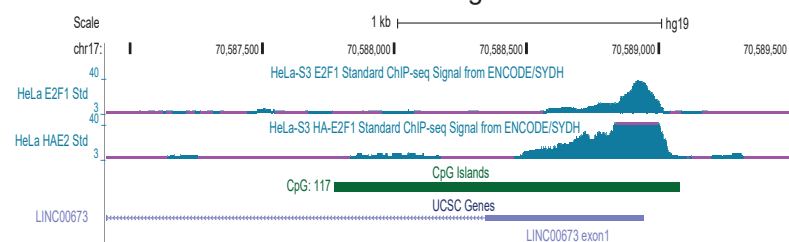
Western Blot and Quantification:

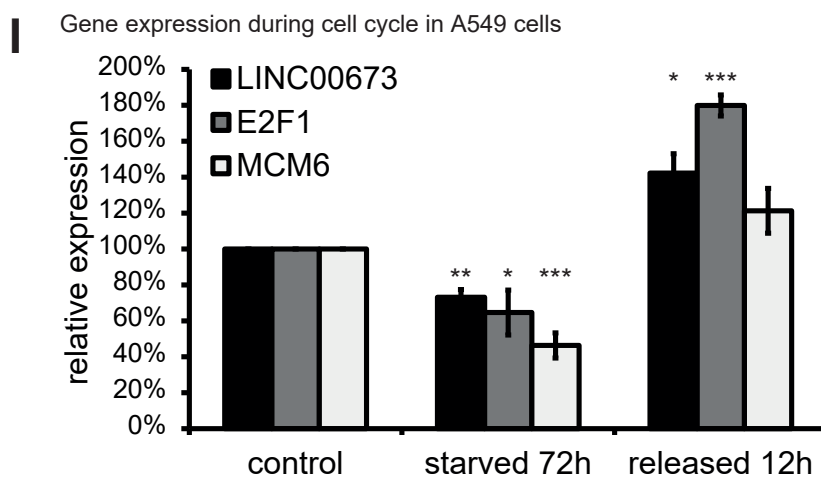
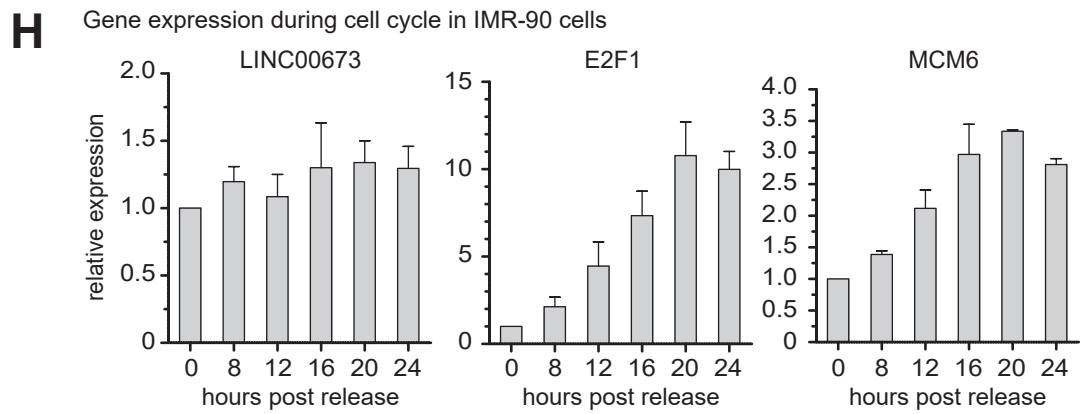
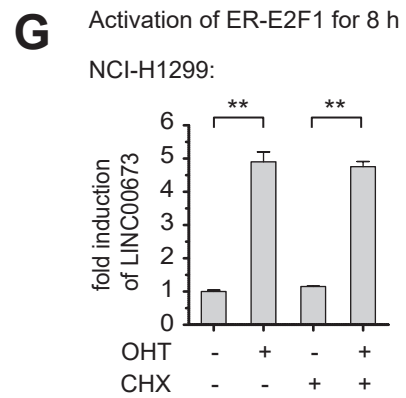
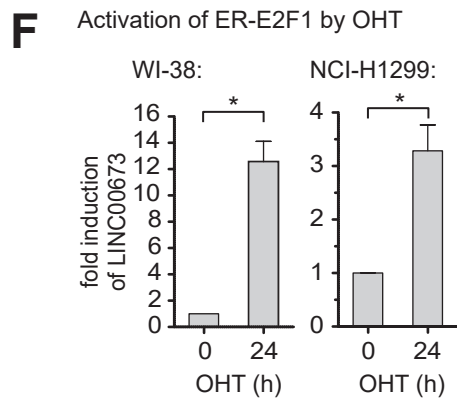
**C**

RT-qPCR:

**D****E**

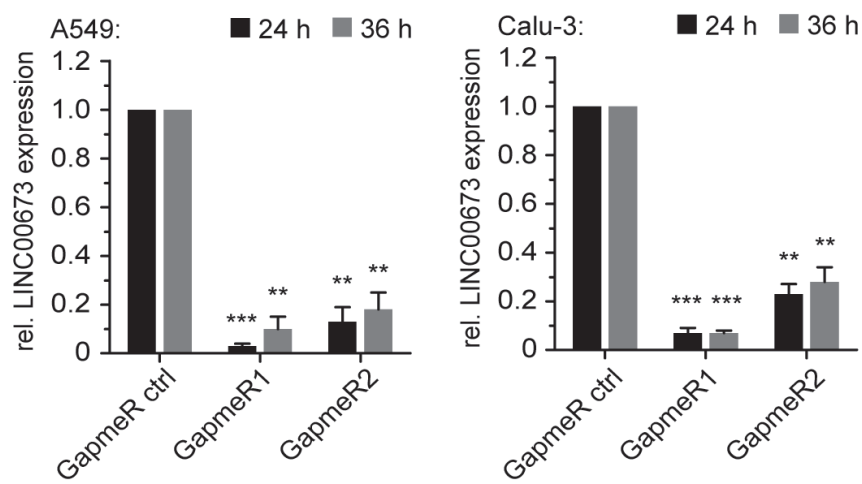
UCSC Genome Browser - E2F1 binding site from ChIP data



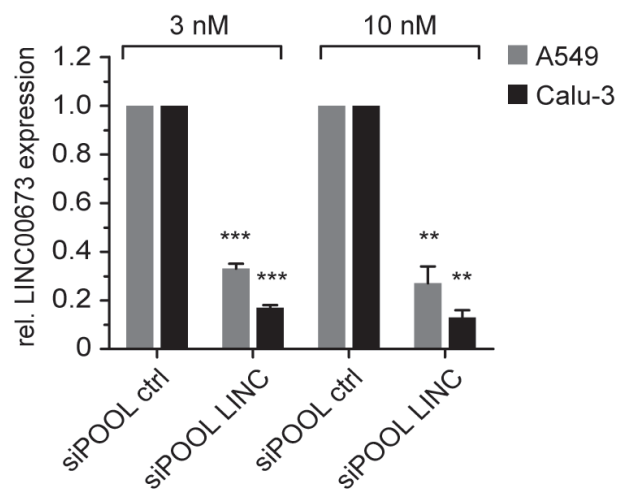
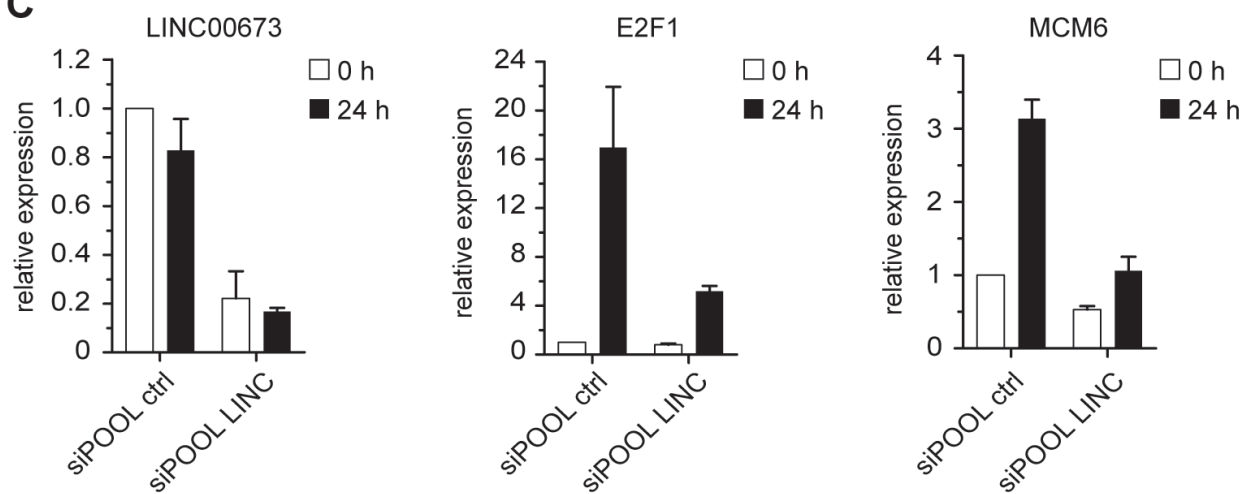


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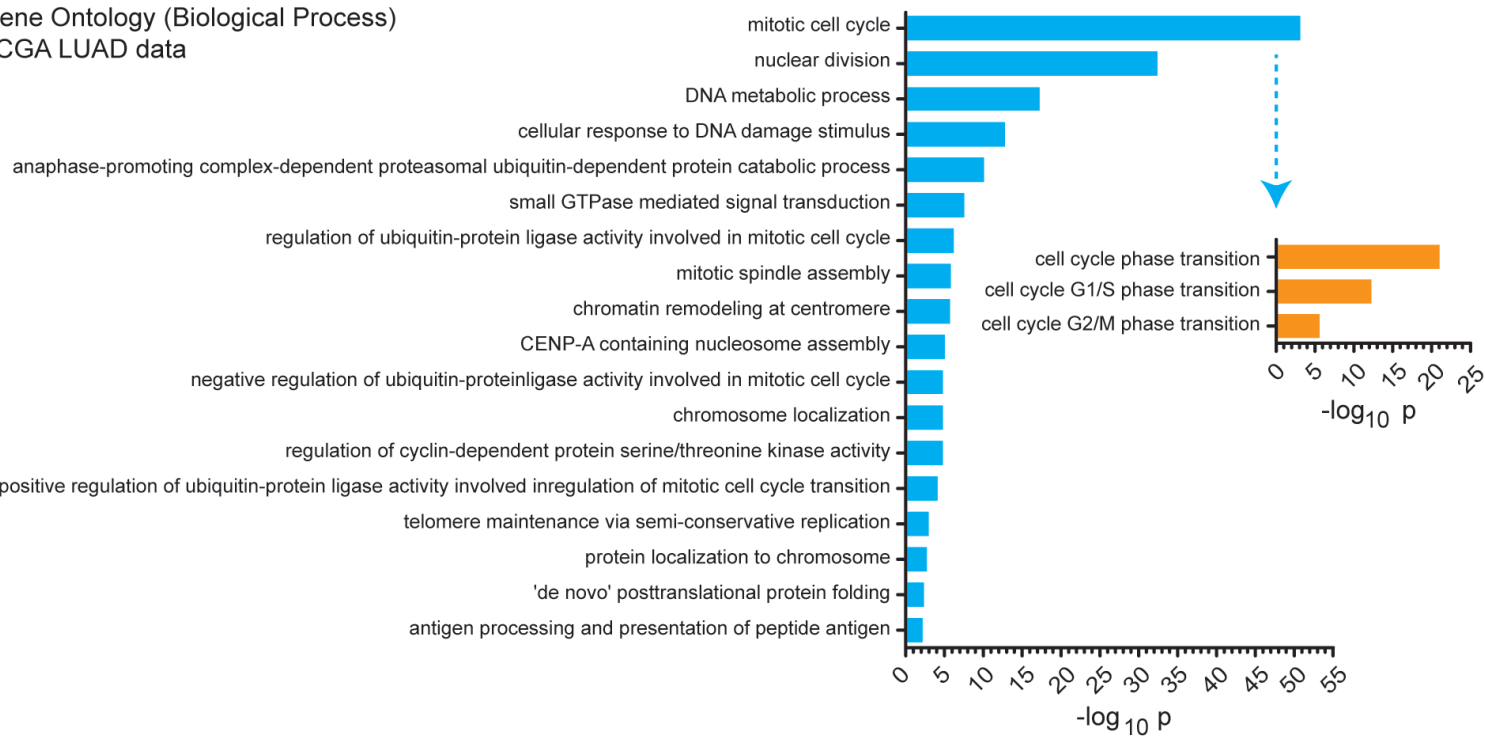
Knockdown efficiency with GapmeRs

**B**

Knockdown efficiency with siPOOLs

**C**

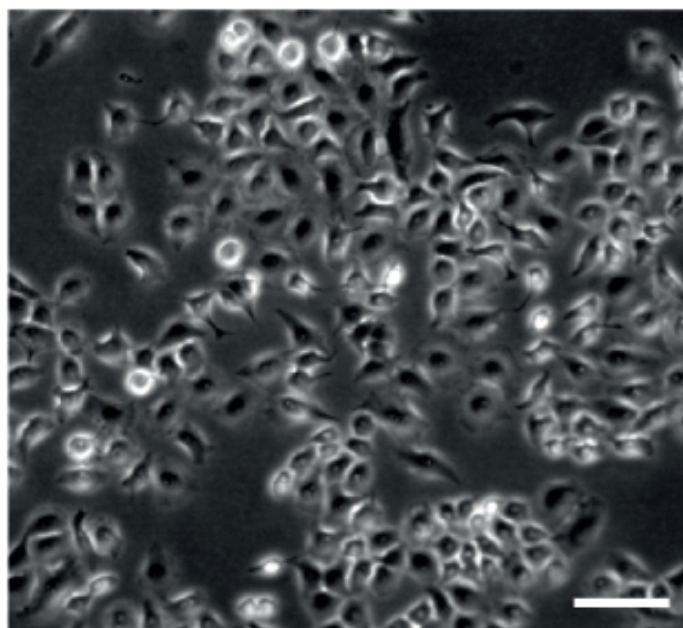
Gene Ontology (Biological Process)
TCGA LUAD data



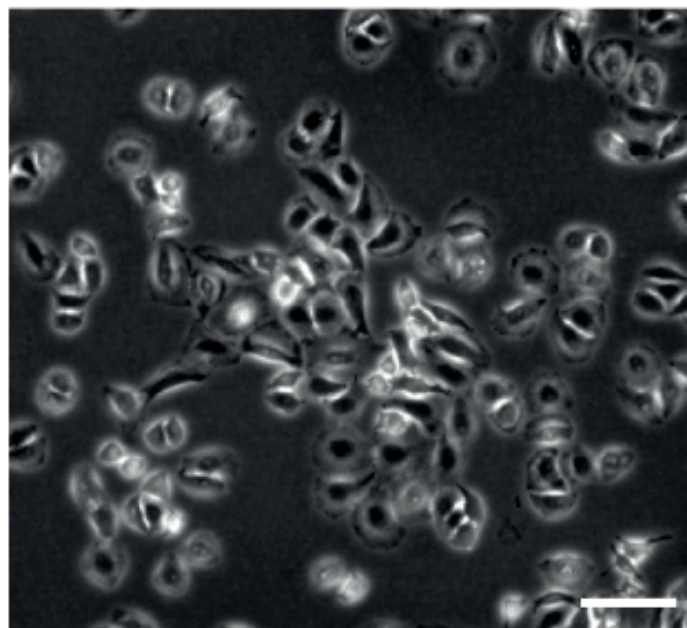
Supplementary Figure 9

A549

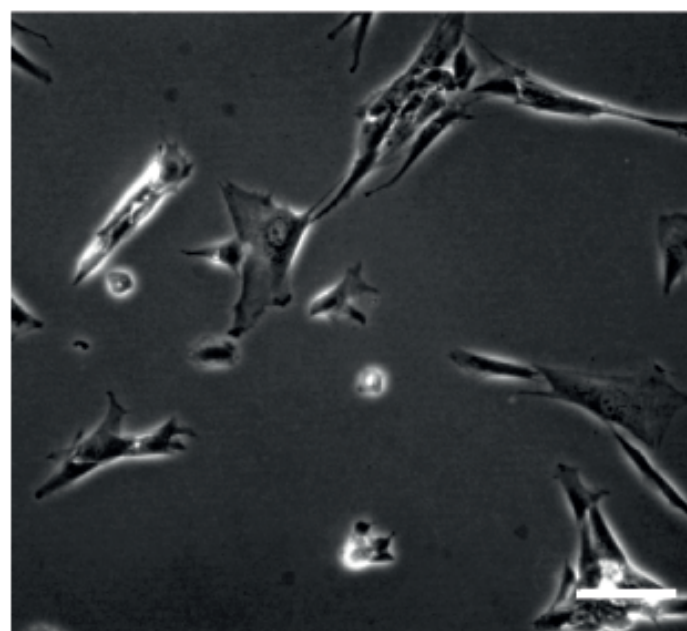
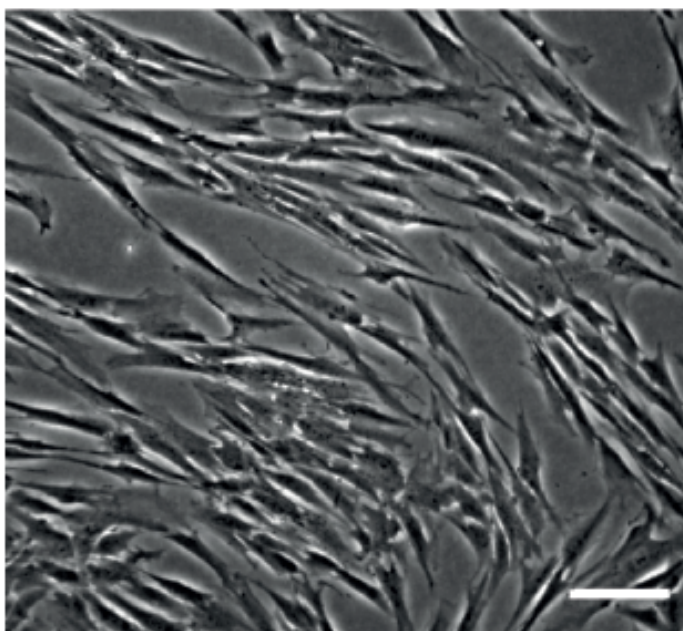
siPOOL control



siPOOL LINC

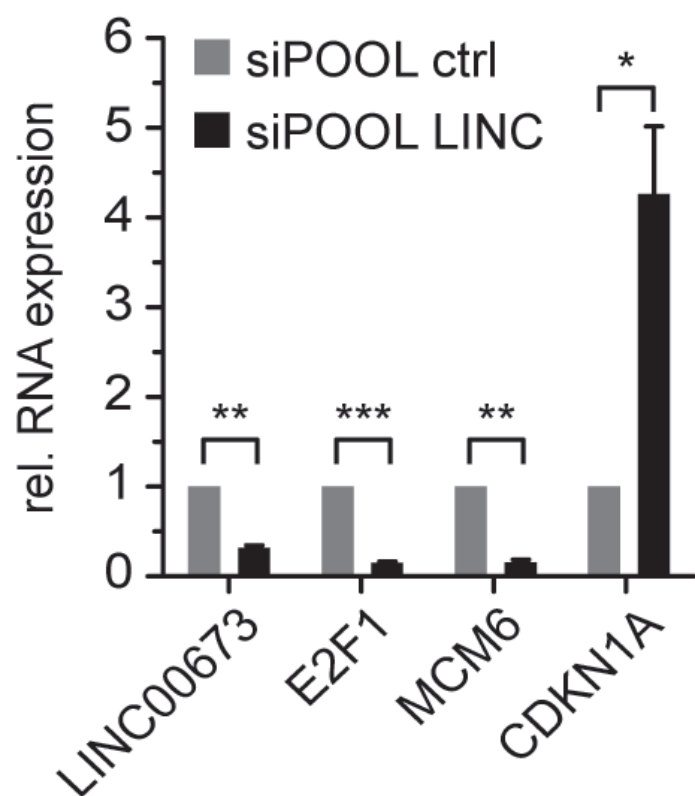


IMR-90

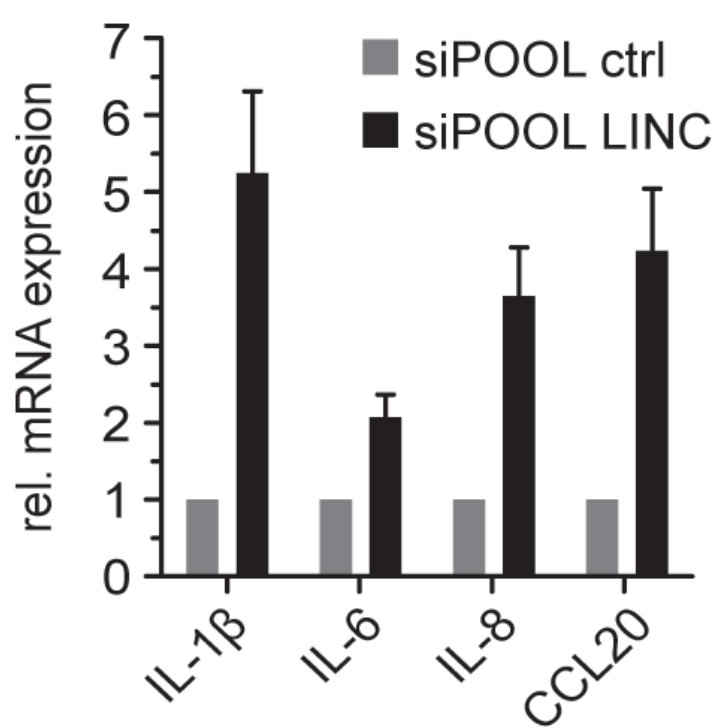


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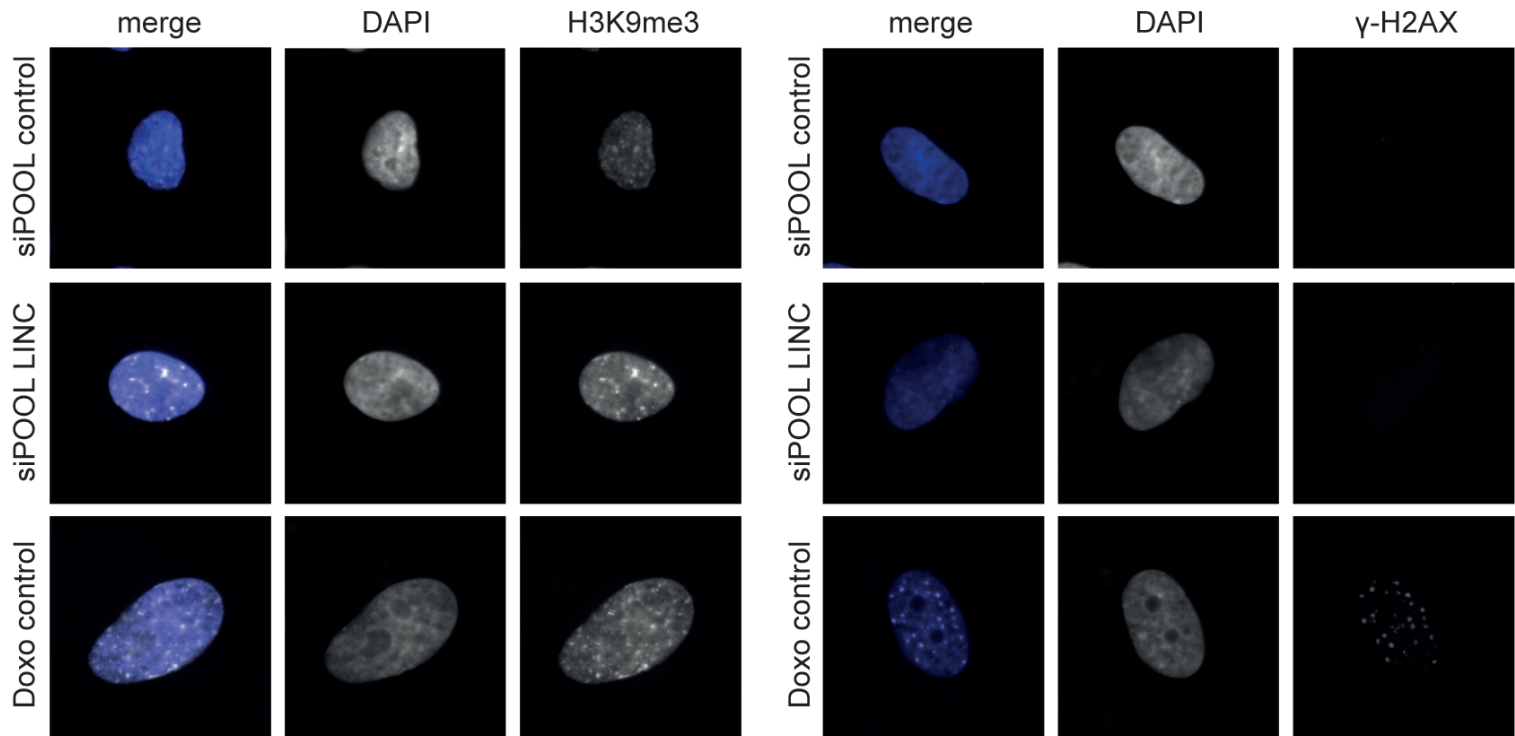
Cell cycle gene expression

**B**

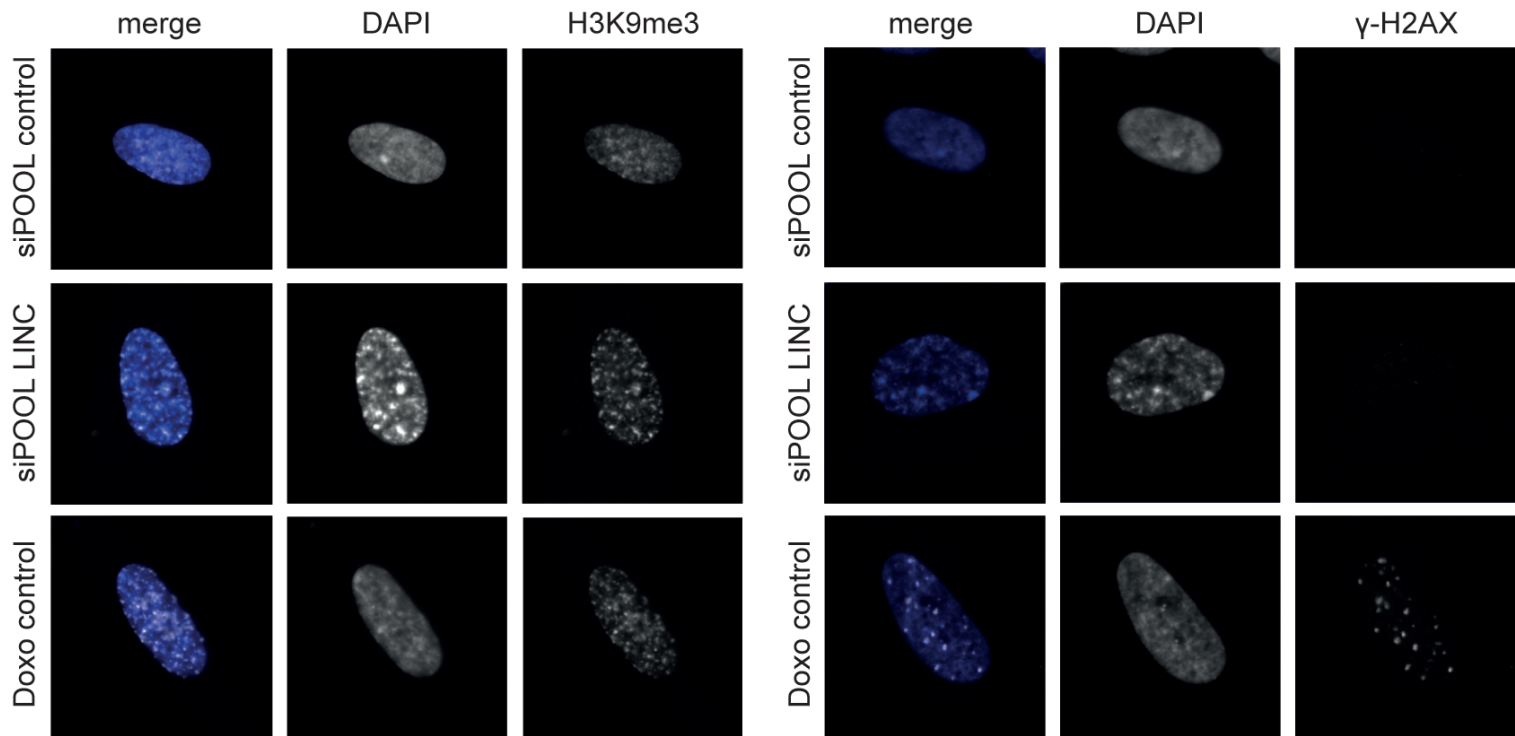
Induction of SASP genes

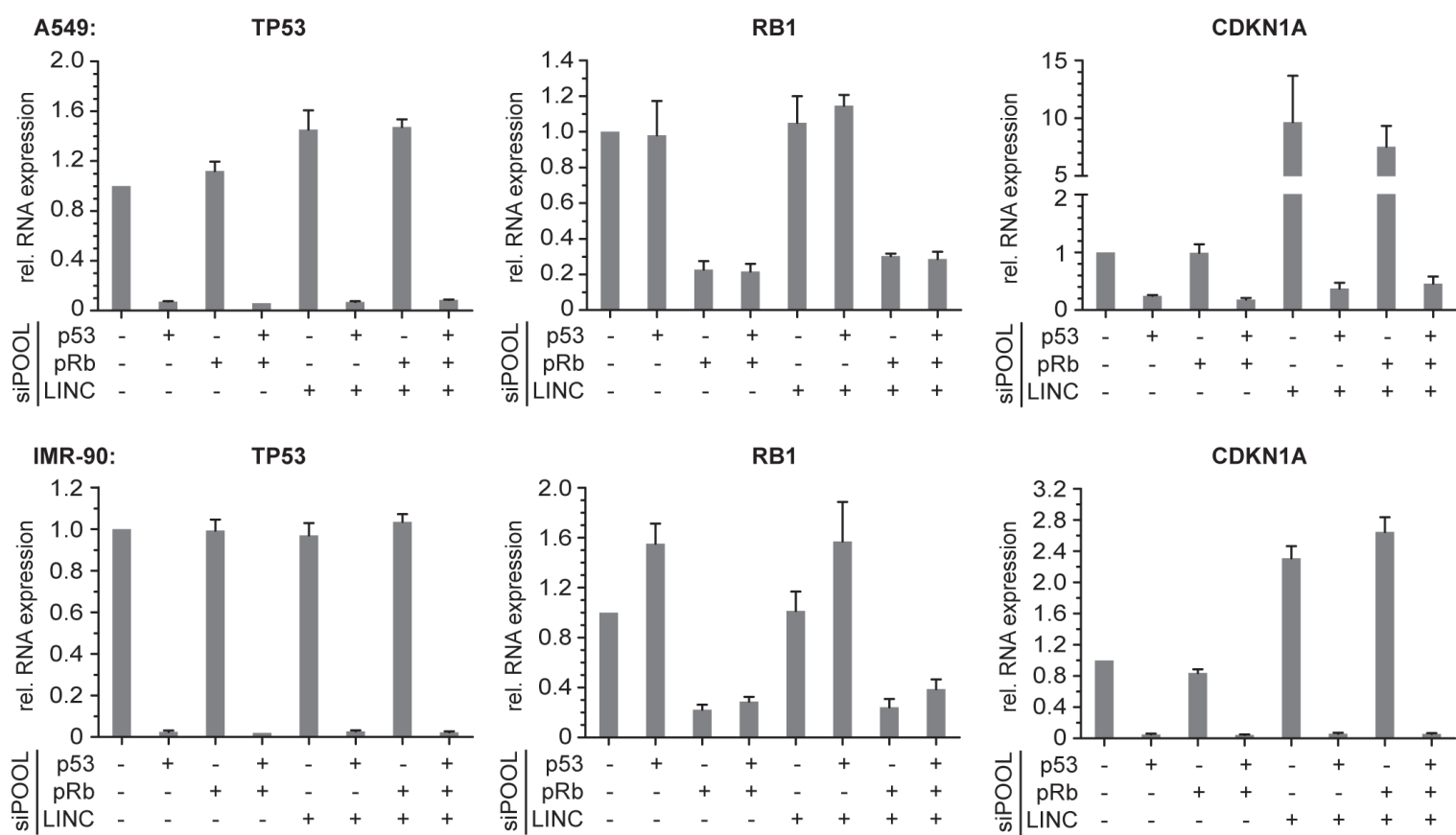


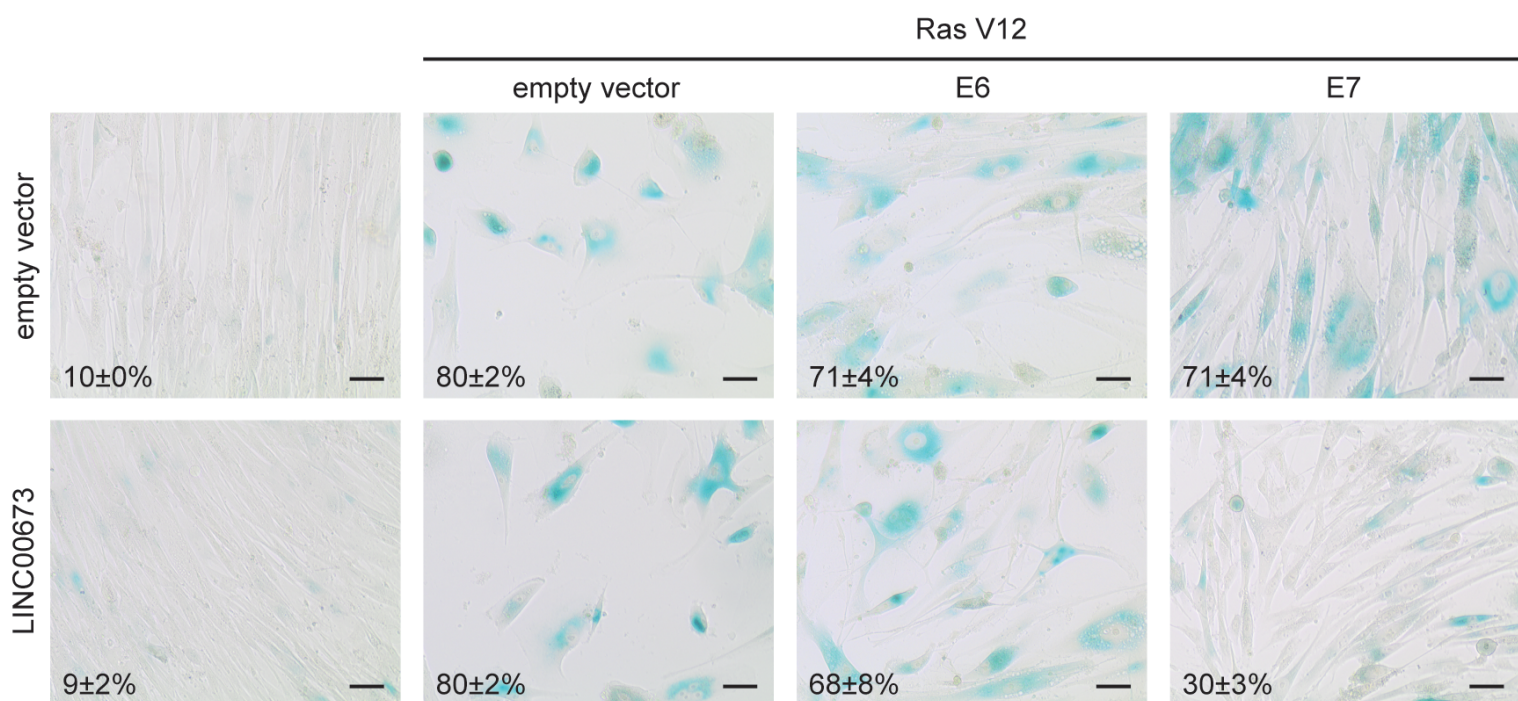
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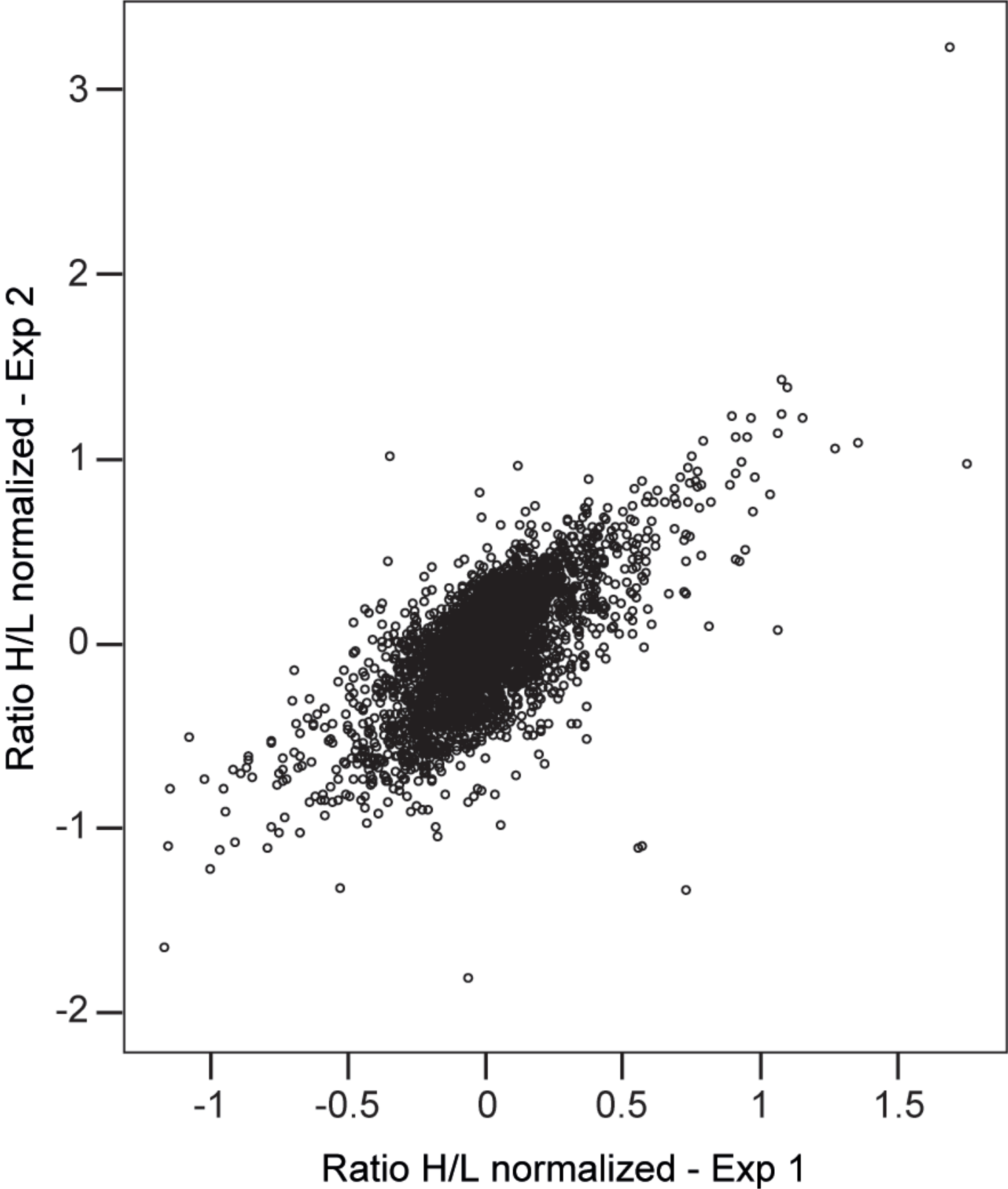
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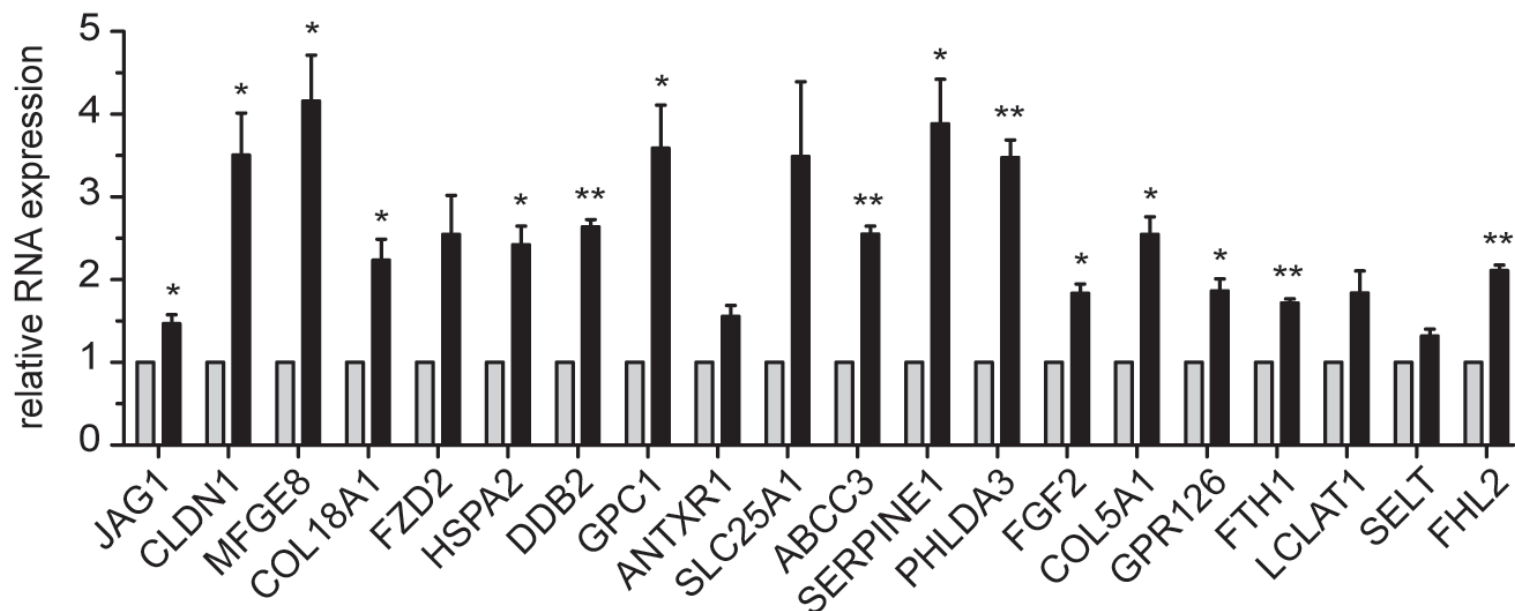


Scatter Plot:

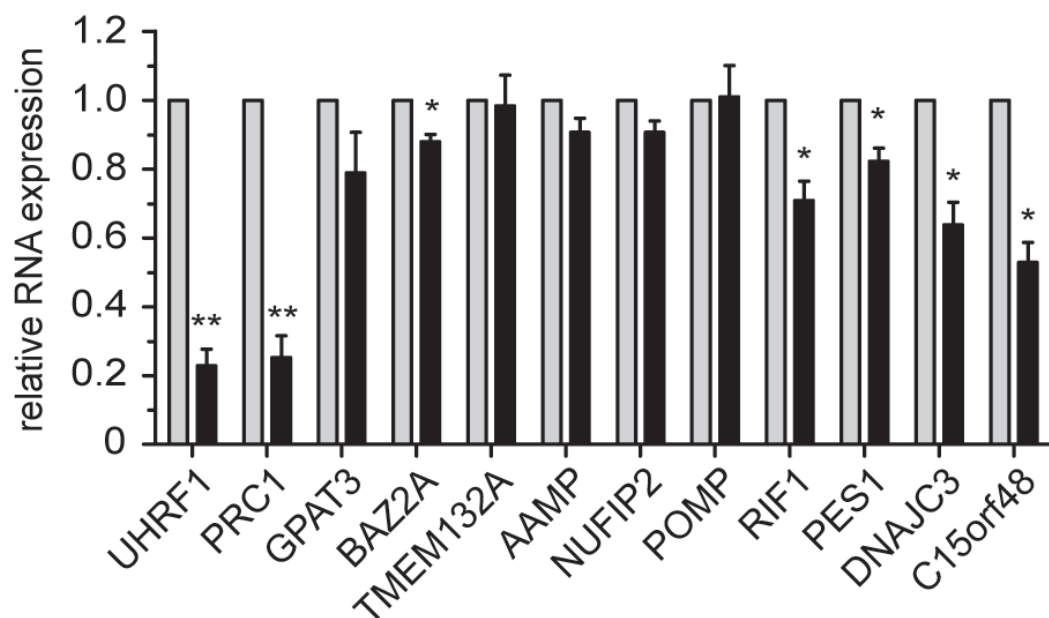


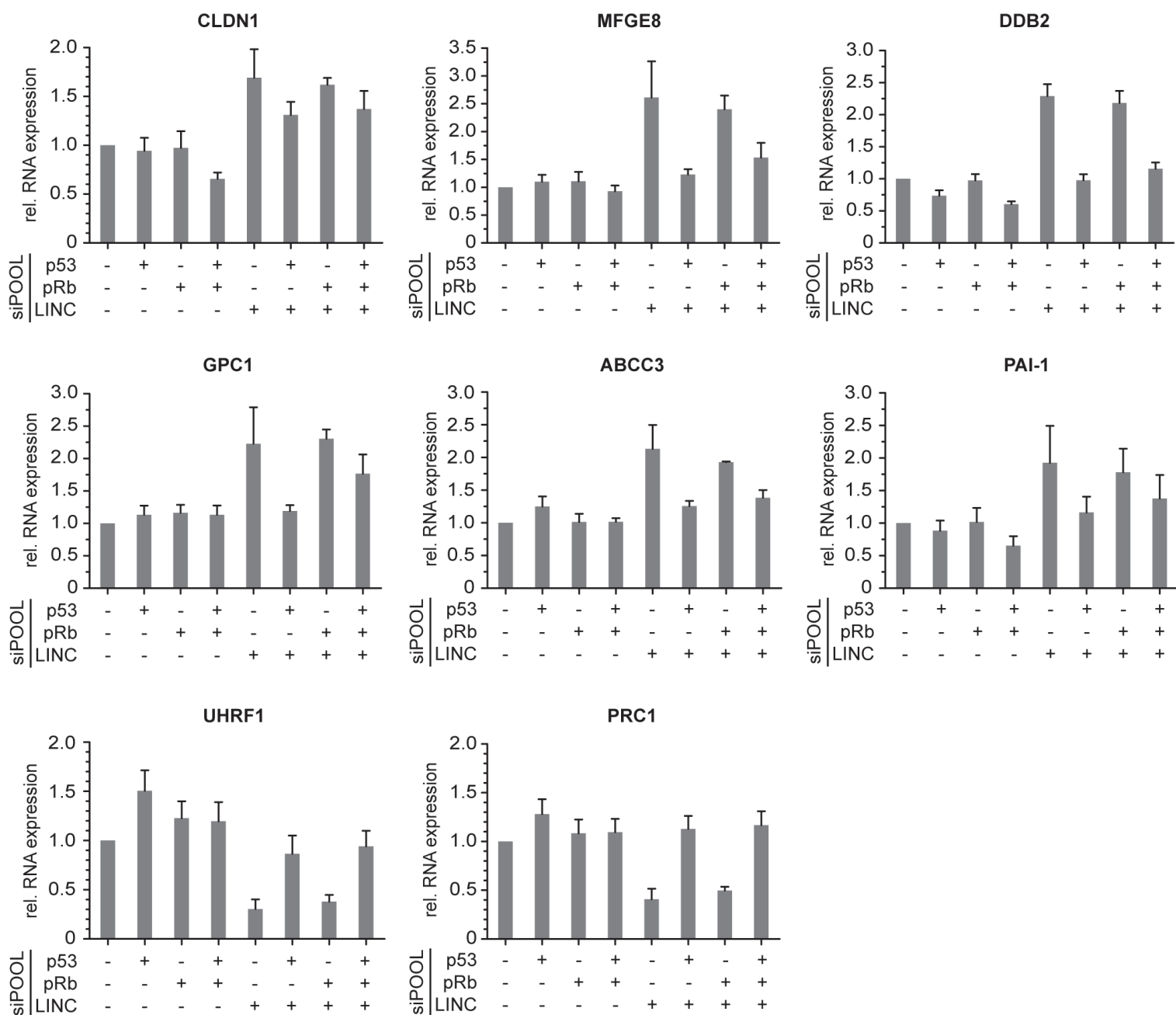
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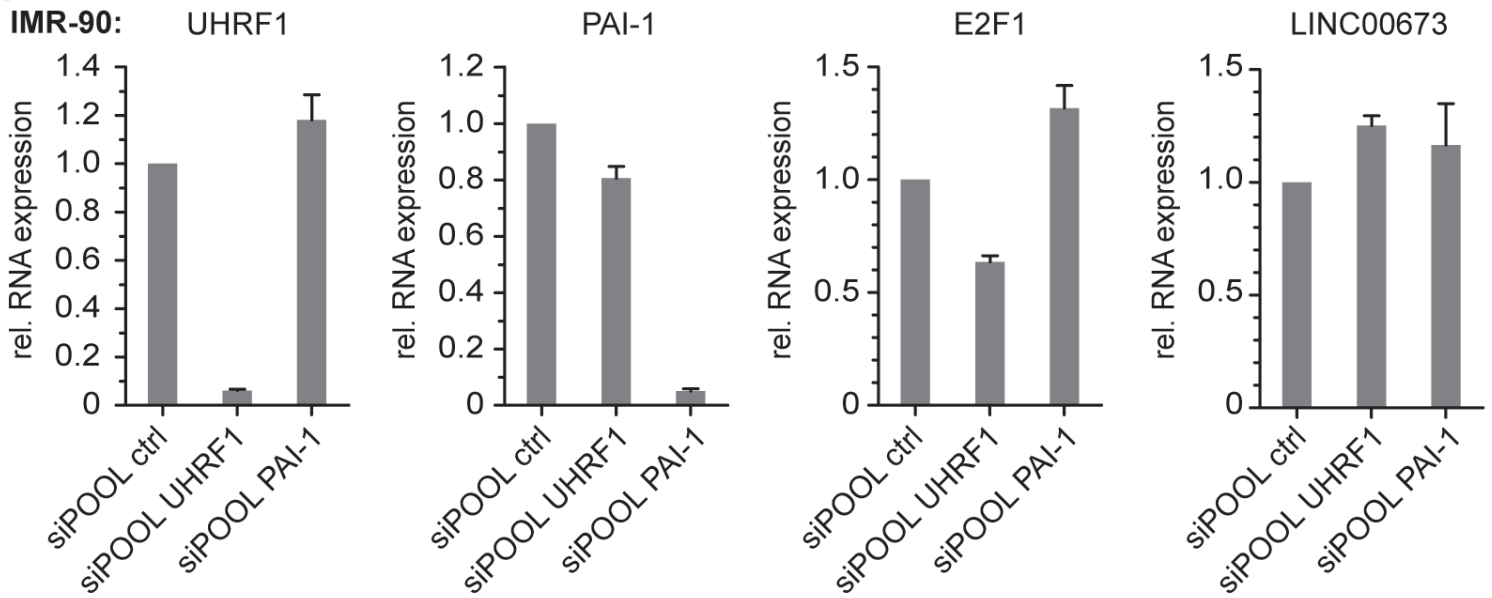
RT-qPCR analysis of genes upregulated by quantitative mass spectrometry

**B**

RT-qPCR analysis of genes downregulated by quantitative mass spectrometry





A**B**