Supplementary Material and Methods

Rapid decay of engulfed extracellular miRNA by XRN1 exonuclease promotes transient epithelial-mesenchymal transition

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Cell culture

The A549, NCI-H1975, NCI-H1299, NCI-H522 and SK-LU-1 cells were cultured according to the recommendations of the ATCC. A549 (human lung adenocarcinoma epithelial cell line, ATCC, number CCL-185), NCI-H441 (human lung adenocarcinoma epithelial cell line, ATCC, HTB-174), BEAS-2B (human normal lung epithelial cell line, ATCC, CRL-9609), MCF7 (human breast adenocarcinoma epithelial cell line, ATCC, HTB-22) and TPC1 (human thyroid papillary cancer cell line, kind gift from Pr M. Santoro, Napoli, Italia) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin (Life Technologies). PLB-985 (human myeloid cell line HL-60 subclone prone to neutrophil differentiation, kind gift from Pr S. Chollet-Martin, Paris, France), NCI-H1975 (human lung adenocarcinoma epithelial cell line, ATCC, number CRL-5908), NCI-H1299 (human lung carcinoma epithelial cell line, ATCC, number CRL-5803), NCI-H522 (human lung adenocarcinoma epithelial cell line, ATCC, number CRL-5810) and A375 (melanoma epithelial cell line, ATCC, number CRL-1619) cells were grown in RPMI-1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 µg/ml streptomycin (Life Technologies). SK-LU-1 (human lung adenocarcinoma epithelial cell line, ATCC, number HTB-57) cells were grown in Minimum Essential Media supplemented with 10% FBS, 1 mM sodium pyruvate, 100 µM MEM Non-Essential Amino Acids, 50 U/ml penicillin, and 50 µg/ml streptomycin (Life Technologies). All the cells were maintained for less than 25 passages at 37°C in a 5% CO_2 and humidified atmosphere. All cell lines were authenticated after the first amplification or subcloning (here, stable miR-223-3p clone selection) by

determining the genetic characteristics using PCR-single-locus-technology (Promega, PowerPlex 21 PCR Kit) and certified (Eurofins, Eurofins Genomics, Ebersberg, Germany). Testing for mycoplasma contamination was performed monthly using PlasmoTest™ - Mycoplasma Detection Kit (Invivogen).

Reagents. ***$DiIC_{16}$ staining.*** For lipid transfer experiments, PMN or PLB-985 cells were stained with $DiIC_{16}(3)$ (1,1'-Dihexadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate, Molecular Probes) at 2.5 mg/ml for 5 min at 37°C directly in culture media. Cells were then washed twice to remove the unbound dye in the medium and resuspended in the appropriate culture media for further experiments. ***TGF-β1 stimulation.*** 24 h after miR-223-3p transfection, A549 cells were starved overnight and then treated with TGF-β1 (PromoKine) at 10 ng/ml for 1 or 24 h. Cells were lysed for protein analysis as described below. ***N-acetyl-L-cysteine (NAC) treatment.*** Cells were treated with N-acetyl-L-cysteine, a ROS inhibitor, at 10 mM during invasion assays as described below.

Supernatant production (SPN) and extracellular vesicle (EVs) purification

SPN was isolated from PMN conditioned media after 300g centrifugation to remove cells and 2000g to remove cell debris. EVs were then isolated by ultracentrifugation at 100,000g for 70 min at 4°C. The pellet was either resuspended in phosphate-buffered saline (PBS) for culture cell experiments or in lysis buffer for protein analysis (1). The degree of purification and quality of EVs were analysed either by the Nanoparticle tracking analysis NanoSight LM10-HS (Malvern, Orsay, France) or by immunoblotting. NanoSight assays were performed according to the recommended protocols of the equipment's manufacturer. Briefly, 4 independent replicates of SPN or diluted EV preparations (about 10^9 particles/ml) in PBS were injected into the chamber. The specimens were tracked at room temperature for 80 sec. The shutter and gain were manually adjusted for optimal detection and were kept at this setting for all samples. The data were captured and analysed with NTA BUILD 127 software (version 2.2, Malvern Instruments Ltd, Malvern, UK). Three measurements of the same sample were taken. Four different preparations were analysed.

siRNA transient transfection

Cells were plated at 200,000 cells/well in 6-well plates. After 24 h, cells were transfected with the mirVana miRNA mimic Control or miR-223-3p at 17 nM or with the mirVana miRNA antagomiR Control or miR-223-3p at 68nM or with siRNA negative Control or Silencer Pre-designed siRNA XRN1, XRN2, DIS3, DIS3L, DIS3L2, PNPase (PNPT1), ERI1 at 50 nM using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. 48 h after transfection, cells were lysed for RNA or protein analysis as described below.

Immunoblotting

Immunoblotting was performed as described previously (2). Whole cell lysates (WCL) were extracted from cells using Laemmli lysis buffer (12.5mM Na_2HPO_4, 15% glycerol, 3% SDS). The protein concentration was measured with the DC Protein Assay (BIO-RAD) and 30µg of total protein was loaded onto 7.5% or 11% SDS-PAGE gels for electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). After 1 h of blocking with 5% bovine serum albumin or non-fat milk prepared in PBS-Tween buffer, the blots were incubated overnight at 4°C with antibodies against CD63 (clone EPR5702, 1:1000, Epitomics, ref 5756-1), CD53 (clone EPR4342(2), 1:1000, Epitomics, ref 3734-1), CD82 (clone EPR4112, 1:1000, Epitomics, ref 3757-1), FOXO1 (clone C29H4, 1:1000, Cell Signaling Technologies, ref #2880), E-CADHERIN (clone 4A2C7, 1:500, Invitrogen, ref 33-4000), SNAIL (clone C15D3, 1:1000, Cell Signaling Technologies, ref #3879), SLUG (clone A-7, 1:400, Santa Cruz Biotechnology, ref sc-166476), VIMENTIN (clone SP20, prediluted, 1:100, Abcam, ab27608), XRN1 (1:1000, Bethyl Laboratories, ref A-300-443A), XRN2 (1:1000, Bethyl Laboratories, ref A-301-103A), DIS3 (1:1000, GeneTex, ref GTX115645), DIS3L (1:1000, Bioss antibodies, ref bs-9052R), DIS3L2 (1:400, Santa Cruz Biotechnology, ref sc-132270), PNPase (1:400, Santa Cruz Biotechnology, ref sc-365049), ERI1 (1:400, Santa Cruz Biotechnology, ref sc-137089), PARP-1 (1:1000, Abcam, ref ab32071) and β-ACTIN (clone AC-40, 1:10000, Sidma-Aldrich, ref A3853) used as loading control. After 1 h of incubation with a horseradish peroxidase-conjugated secondary antibody (1:5000, Santa Cruz Biotechnologies), protein bands were visualized using an enhanced chemiluminescence detection kit (Millipore) with the imaging system, Syngene Pxi4 (Syngene).

Microarrays

Total RNA of A549 cells transfected for 48 h with either the mirVana miRNA mimic Control or miR-223-3p was extracted using the RNeasy kit (Qiagen, Hilden, Germany). The integrity of the RNA was assessed using an Agilent BioAnalyzer 2100 (Agilent Technologies). RNA samples were then labelled and hybridized on 8×60K high density SurePrint G3 gene expression human Agilent microarrays following the manufacturer's instructions. Three or 4 biological replicates were performed for each experimental condition. The microarray experimental data were deposited in the NCBI GEO under the serial record number GSE81370. The data were quantile normalized using the Bioconductor package limma (3). Means of ratios from all comparisons were calculated and the moderated t-statistic of the limma package provided the per gene *p*-values. The Benjamini-Hochberg procedure was used to control the experiment-wise false discovery rate (FDR) from multiple testing procedures. Differentially expressed genes were analysed based on the miR-223-3p *versus* miR-Ctrl transfection in A549 cells. Down-regulated genes in miR-223-3p transduced A549 *versus* miR-Ctrl cells were selected based on a log2 average expression value superior or equal to 6, a log2 fold change value inferior or equal to -1, and an adjusted *p*-value inferior or equal to 0.05. MiRonTop (4) is an online java web tool (available at http://www.genomique.info:8080/miRonTop/index) that integrates DNA microarray data to identify the potential implication of miRNAs on a specific biological system. Data enrichment analysis was performed using EnrichR (5).

Immunofluorescence and confocal microscopy

Cells were grown to confluence and fixed in 4% paraformaldehyde for 20 min. After fixation, cells were permeabilised with a solution containing 0.3% Triton X-100 for 5 min. Cells were then incubated with primary antibodies against E-cadherin (1:50, BD Biosciences, ref 610182) overnight at 4 °C in a humidified chamber in a solution containing 0.03% Triton X-100, 0.2% gelatin and 1% BSA. Cells were washed and incubated with Alexa Fluor-conjugated secondary antibodies (1:500; Molecular Probes) for 1 h at room temperature and mounted in ProLong Diamond Reagent with DAPI (Molecular Probes). For actin staining, fixed cells were incubated with Texas Red-X phalloidin (1:1000; Molecular Probes) for 1 h at room temperature. Images were captured on a Zeiss LSM confocal microscope.

Proliferation assay using Cell Proliferation Kit II (XTT)

A colorimetric assay (XTT based, Roche Diagnostics, Germany, ref 11465015001) for the non-radioactive quantification of cell proliferation and viability was performed according to the manufacturer's instructions. Briefly, cells were plated at 6,200 cells/well in 96-well plates. After siRNA treatment (24 h or 48 h), cells were incubated with XTT labeling mixture for 4 h at 37°C. Then, the absorbance of the formazan product was measured using a microplate reader (EnVision) at 485 nm. All assays were performed in quadruplicate and were repeated three times in independent conditions. Data are presented as means ± SD.

Supplementary Table and Figure Legends

Supplementary Table S1. Transcriptome Analysis of cells transfected with miR-223-3p by principal Gene Ontology pathways (GO_Cellular_Component_2015) using EnrichR online tool. Only results with an adjusted p. value below 0.05 were taken into account.

Supplementary Figure S1. Characterization of PMN-derived EVs

(A-B) Effective lipid transfer from PMN (A) and SPN of PMN (B) into A549 recipient cells was revealed using fluorescent $DiIC_{16}$ (magnification 200x). (C) Quantification and evaluation of the size of EVs in SPN of PMN was performed by a Nanoparticle tracking analysis. (D) Quantification and size evaluation of EVs isolated by ultracentrifugation (UC) from SPN of PLB-985 cells was performed by a Nanoparticle tracking analysis. (D) EVs were isolated from the SPN of PLB-985 cells by UC. Enrichment of tetraspanin markers (CD63, CD53 and CD82), specific for immune cells, was analysed in the EV fraction (20 µg) and whole cell lysates (WCL, 20 µg) by immunoblotting. (F) Effective membrane content uptake of EVs isolated from SPN of PLB-985 cells in A549 recipient cells was revealed with fluorescent $DiIC_{16}$ (magnification 200x).

Supplementary Figure S2. Ex-miRNA are actively transferred to recipient cells

(A-F) Quantification of accumulation of ex-miR-223-3p in H1975, H1299, BEAS2B, H522 (lung), A375 (melanoma), and MCF7 (breast) cell lines, respectively. After co-culture with PMN overnight, cells were extensively washed to remove PMN and harvested. The levels of miR-223-3p were normalized using U6

snRNA. (G) Quantification of miR-143-3p expression in cell lines. (H) Relative quantification of the expression of ex-miR-143-3p in the H1299 cell line following co-culture with increasing numbers of PMN. (A-H) The levels of miR-223-3p were normalized using U6 snRNA. (I) Expression level of *MYC* and *GAPDH* mRNA in A549 cells treated with actinomycin D for 8 or 24 h. (J) Expression level of U6 snRNA in A549 cells treated with actinomycin D for 8 or 24 h. (A-J) Results are representative of 3 biological replicates, 'centre values' as mean and error bars as s.d.

Supplementary Figure S3. miR-223-3p transfer induced enhanced invasive properties

(A) Significance of the enrichment in miR-223-3p predicted targets among the downregulated transcripts (represented as –log10 (adjPVal)) according to the fold enrichment by MitonTop in microarray experiments of A549 cells transfected with miR-223-3p. (B) Identification of the Gene Ontology Cellular Component after analysis of DNA microarray of A549 cells transfected with miR-223-3p. (C-D) miR-223-3p-induced EMT. Modifications in EMT marker expression were analysed post TGF-β1 stimulation by immunoblotting at 1 h and by confocal microscopy at 24 h.

Supplementary Figure S4. Invasion assays

(A) *In vitro* invasion assay of H1975 cells co-cultured either with PMN or their supernatant produced in serum-free medium. (B) *In vitro* invasion assay of A549 cells co-cultured with SPN. N-acetyl-L-cysteine (NAC) was added to SPN to inhibit ROS. (C) *In vitro* invasion assay of A549 cells ectopically transfected with miR-223-3p. (A-C) Each dot corresponds to a biological replicate. ★ for $p<0.05$, ★★ for $p<0.01$.

Supplementary Figure S5. Ex-miRNA decay in recipient cells

(A-C) Relative quantification analysis of ex-miRNA-223-3p expression in H1299, SK-LU-1, MCF7 and TPC1 cells. Cells co-cultured with PMN overnight were harvested at the indicated times post initial washes (Time Washed, T.W.). (D) Relative quantification analysis of *FOXO1* mRNA in A549 cells. A549 cells co-cultured with PMN overnight were harvested at the indicated times post initial washes. (E) Relative quantification analysis of ex-miRNA-143-3p expression in A549 cells. A549 cells co-cultured with PMN

overnight were harvested at the indicated times post initial washes. (A-E) Results are representative of 3 biological replicates and expressed as ΔΔCT (linear expression). 'centre values' as mean and error bars as s.d.

Supplementary Figure S6. Exoribonuclease screening

(A) Expression levels of the different exonucleases after siRNA transfection in A549 cells. For XRN1, we also analysed the level of PARP to verify the absence of induction of cell death . (B) Relative quantification analysis of ex-miRNA-223-3p levels. siRNA transfected A549 cells were co-cultured with PMN overnight and harvested at the indicated times post initial washes (Time Washed, T.W.). Results are representative of 3 biological replicates 'centre values' as mean and error bars as s.d.

Supplementary Figure S7. Control experiments of XRN1 modulation

(A) Cell proliferation of cells treated with siXRN1 or siCtrl was monitored by using XTT kit. Results are representative of 3 biological replicates, 'centre values' as mean and error bars as s.d. (B) *In vitro* invasion assay of siXRN1 or siCtrl treated cells. Each dot corresponds to a biological replicate. (C) XRN1 expression stability in presence of Actinomycin D. (A and C) Results are representative of 3 biological replicates, (A-C) 'centre values' as mean and error bars as s.d.

Supplementary Figure S8. *XRN1* copy number variation and disease free survival

Analysis of the disease free survival of lung adenocarcinoma patients (TCGA_LUAD) with heterozygous loss of XRN1 gene in comparison with tumours with 2N or higher loci copy number variations (CNA) (p.value=0.0219, Hazard Ratio: 1.453, 95% CI of ratio: 1.065 to 2.168).

References

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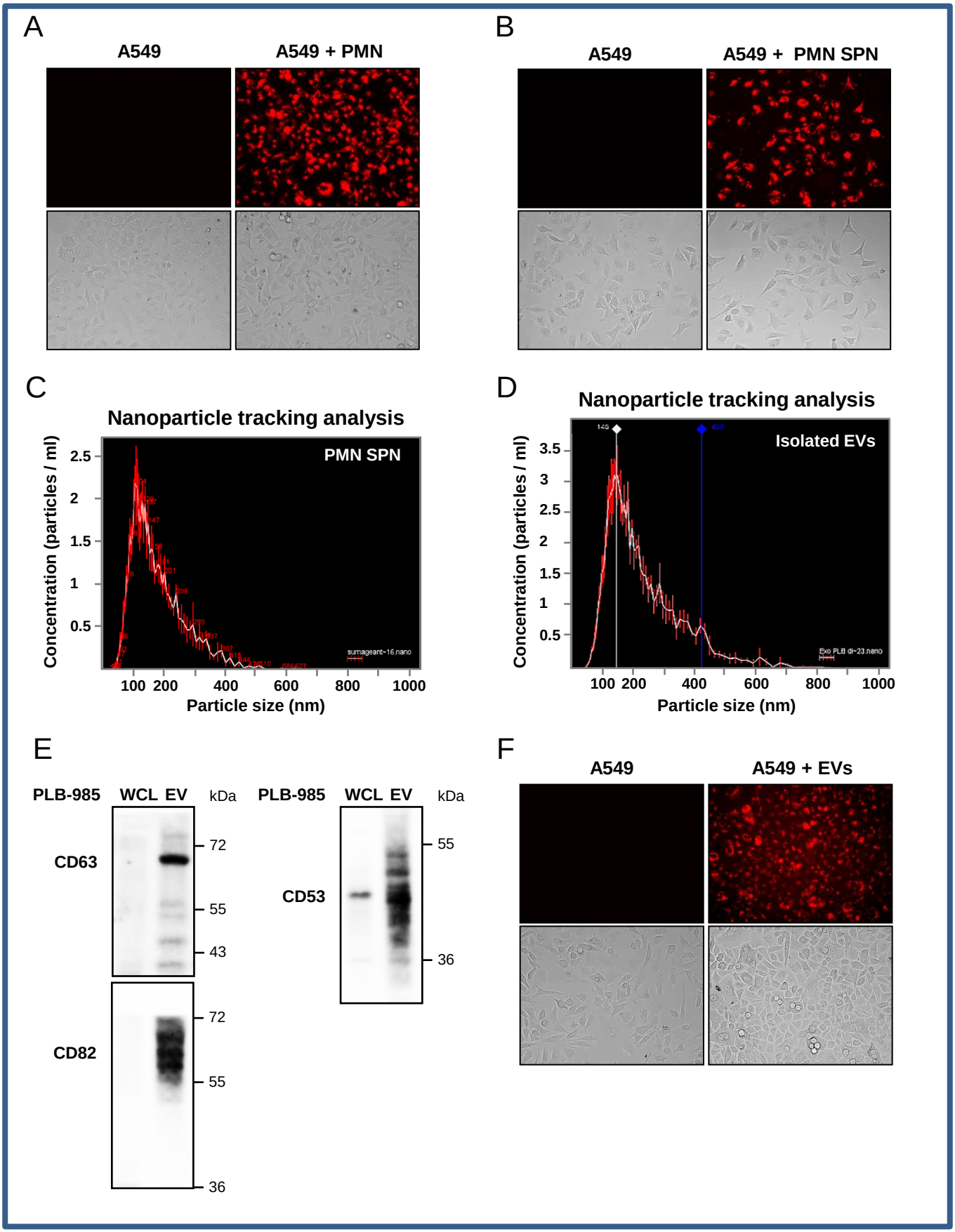
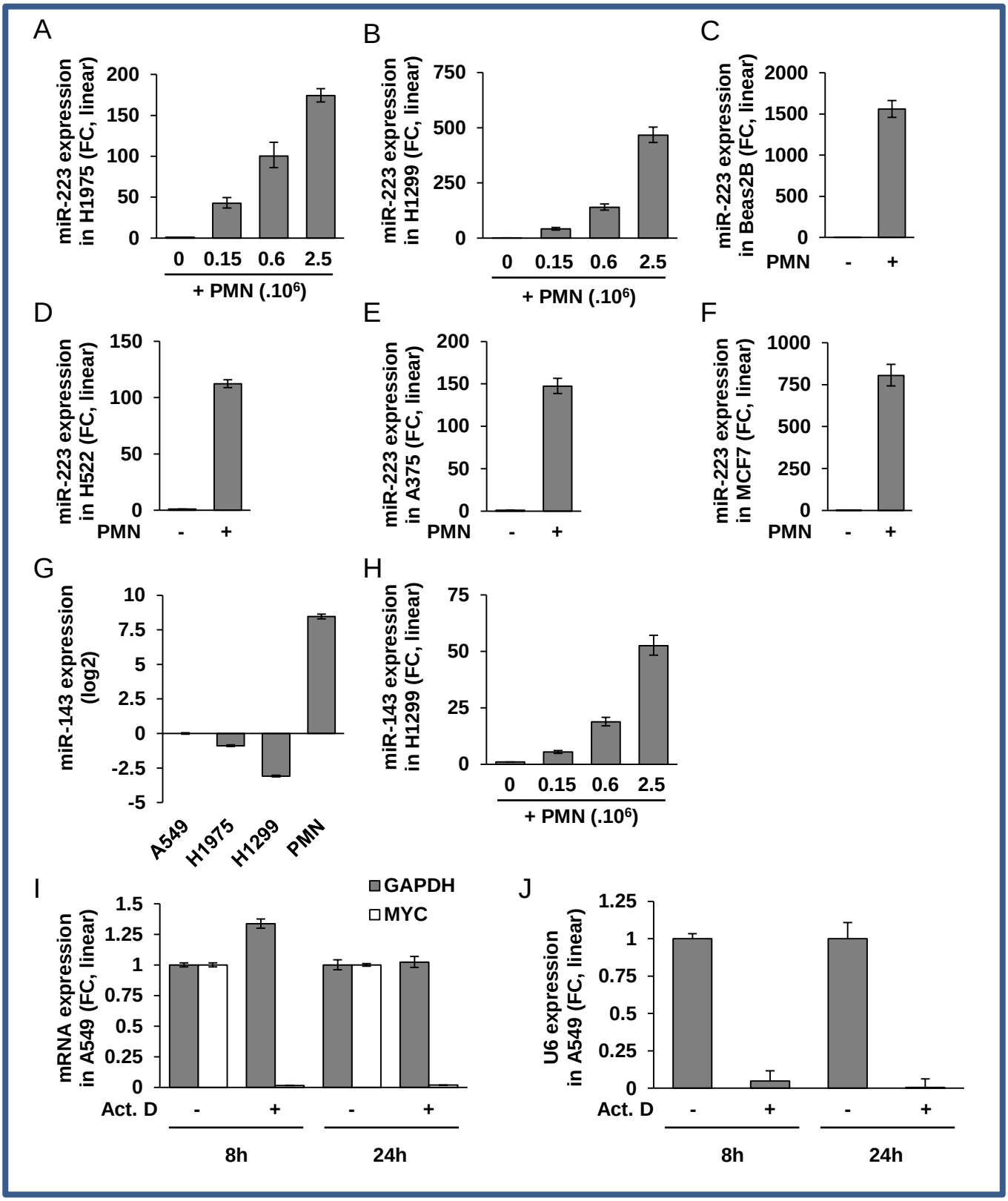
Supplementary Table S1

Term	Overlap	Adjusted P-value	Z-score	Combined Score	Genes
anchoring junction (GO:0070161)	34/419	1,15E-05	-2,37	27,00	SCARB2;KRT80;RALA;AHNAK;SDC4;CTNND1;TWF1;CIB2;TENC1;NDRG1;CSRP1;CDH2;EPB41L2;G3BP1;DAG1;FLNB;CD59;RAC1;PDLIM7;TGM2;TNS1;ITGA3;CAV1;ITGA1;RRAS2;PLEKHA7;ABI2;DLG5;PKP2;EVL;NF2;AJUBA;LIMS2;ARF6
adherens junction (GO:0005912)	33/405	1,15E-05	-2,35	26,71	SCARB2;RALA;AHNAK;SDC4;CTNND1;TWF1;CIB2;TENC1;NDRG1;CSRP1;CDH2;EPB41L2;G3BP1;DAG1;FLNB;CD59;RAC1;PDLIM7;TGM2;TNS1;ITGA3;CAV1;ITGA1;RRAS2;PLEKHA7;ABI2;DLG5;PKP2;EVL;NF2;AJUBA;LIMS2;ARF6
cell-substrate adherens junction (GO:0005924)	26/358	1,26E-03	-2,35	15,66	SCARB2;RALA;AHNAK;SDC4;CIB2;TWF1;TENC1;CSRP1;CDH2;EPB41L2;G3BP1;DAG1;FLNB;CD59;RAC1;PDLIM7;TGM2;TNS1;ITGA3;CAV1;ITGA1;RRAS2;EVL;AJUBA;LIMS2;ARF6
cell-substrate junction (GO:0030055)	26/362	1,26E-03	-2,34	15,64	SCARB2;RALA;AHNAK;SDC4;TWF1;CIB2;TENC1;CSRP1;CDH2;EPB41L2;G3BP1;DAG1;FLNB;CD59;RAC1;PDLIM7;TGM2;TNS1;ITGA3;CAV1;ITGA1;RRAS2;EVL;AJUBA;LIMS2;ARF6
focal adhesion (GO:0005925)	25/352	1,77E-03	-2,29	14,52	SCARB2;RALA;AHNAK;SDC4;TWF1;TENC1;CSRP1;CDH2;EPB41L2;G3BP1;DAG1;FLNB;CD59;RAC1;PDLIM7;TGM2;TNS1;ITGA3;CAV1;ITGA1;RRAS2;EVL;AJUBA;LIMS2;ARF6
cell-cell junction (GO:0005911)	24/335	1,88E-03	-2,25	14,11	KRT80;AHNAK;HEG1;MAGI2;CTNND1;KIAA1462;TWF1;ANK3;MPP5;ATP1B1;CLDN2;NDRG1;PLEKHA7;SIRT2;CLDN4;CDH2;ABI2;PODXL;DLG5;PKP2;DAG1;SYNPO;AJUBA;CGNL1
extracellular vesicular exosome (GO:0070062)	102/2717	1,62E-02	-2,13	8,77	SCARB2;CYFIP2;TPMT;SLC23A1;CLSTN1;PITPNA;CTNND1;SERPINE1;CISD1;FNBP1L;CLDN2;ANTXR1;SLC4A4;AKR7A2;EFEMP1;CDH2;ARHGDIA;STMN1;DAG1;KIFAP3;HIST1H2AC;TGM2;HGD;ANXA13;SHISA5;SARS;PDZK1;ATP1B1;CMPK1;SLC26A2;TMED10;LXN;SDC4;CNP;AK2;ARL1;NEDD4L;DRG1;TMEM27;PODXL;EPB41L2;ALDH3B1;NEU1;MAP4;ATP6V1D;DECR1;GFPT1;GNG12;ACADSB;C6ORF120;COPS8;MAGEF1;ETFB;PRSS23;ANXA8L2;EEA1;CSRP1;PRADC1;SUMO3;CHMP1B;TIMP2;AOX1;RAC1;SERPINB1;ITGA3;ANXA3;ITGA1;RRAS2;GFRA1;RHOF;PTGR1;SRP9;SLC7A5;MBLAC2;MRAS;RRM2B;ALDH1A1;ANG;MFGE8;ARF6;MTPN;RALA;DDC;PDXK;AHNAK;DLST;FURIN;UQCR10;FLNB;CD59;RPL15;UGT1A6;CYB5A;NQO1;GALNT4;GPR56;SCLT1;LAMB1; PDZK1IP1;MPP5;PLEKHA7;XPNPEP3
cell-cell adherens junction (GO:0005913)	07/49	2,36E-02	-2,06	7,73	CDH2;ABI2;DLG5;CTNND1;DAG1;NDRG1;PLEKHA7
cell projection membrane (GO:0031253)	14/196	4,71E-02	-2,18	6,66	PEX19;ITGA3;IFIT5;TWF1;PDZK1;ANTXR1;SGCE;SH3YL1;TMEM27;PODXL;RAC1;ATP7A;SLC17A3;ARF6
lamellipodium (GO:0030027)	10/111	4,71E-02	-2,06	6,29	CDH2;ABI2;PODXL;DPYSL3;CTNND1;DAG1;EVL;NF2;RAC1;AJUBA



Supplementary Figure S1



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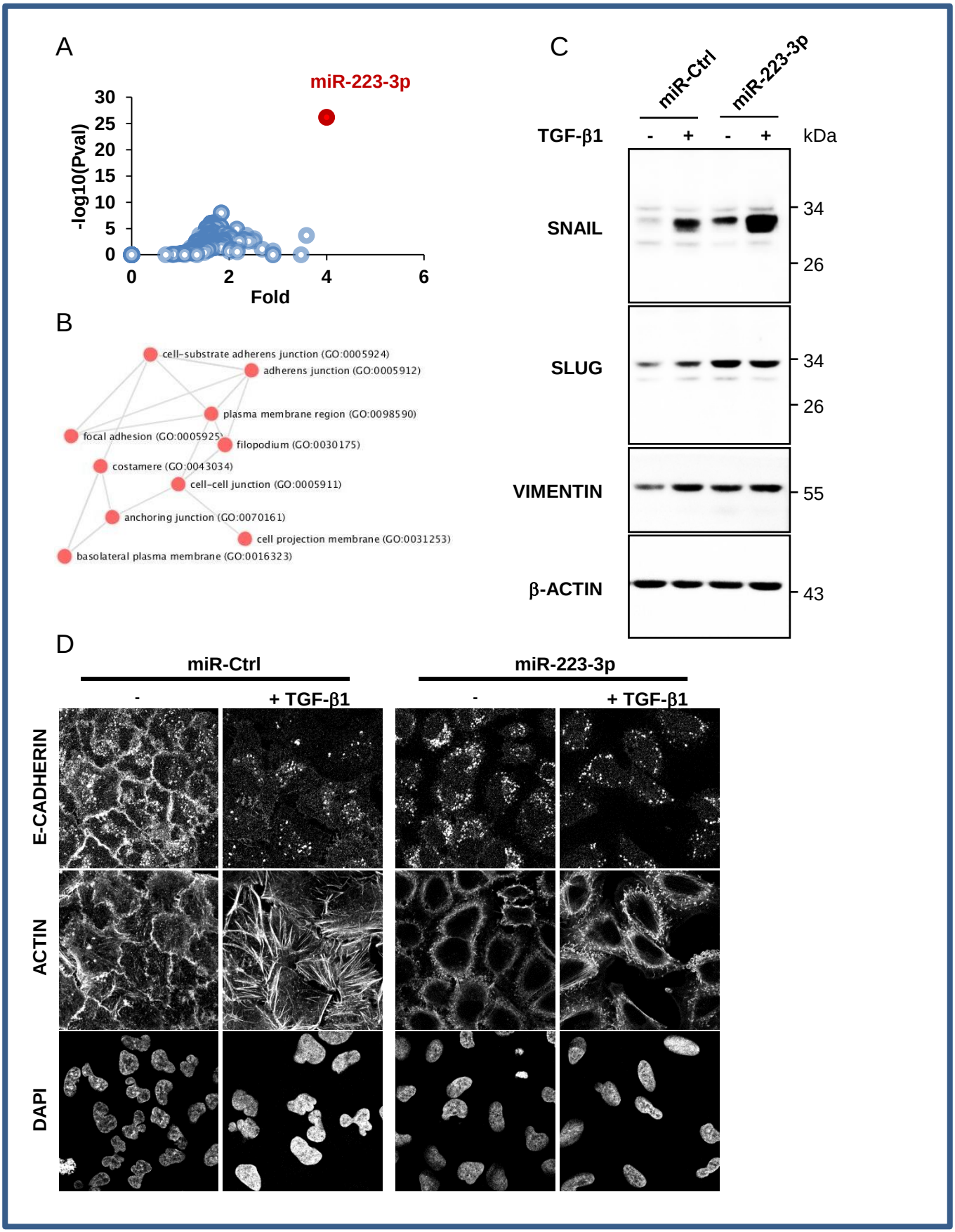

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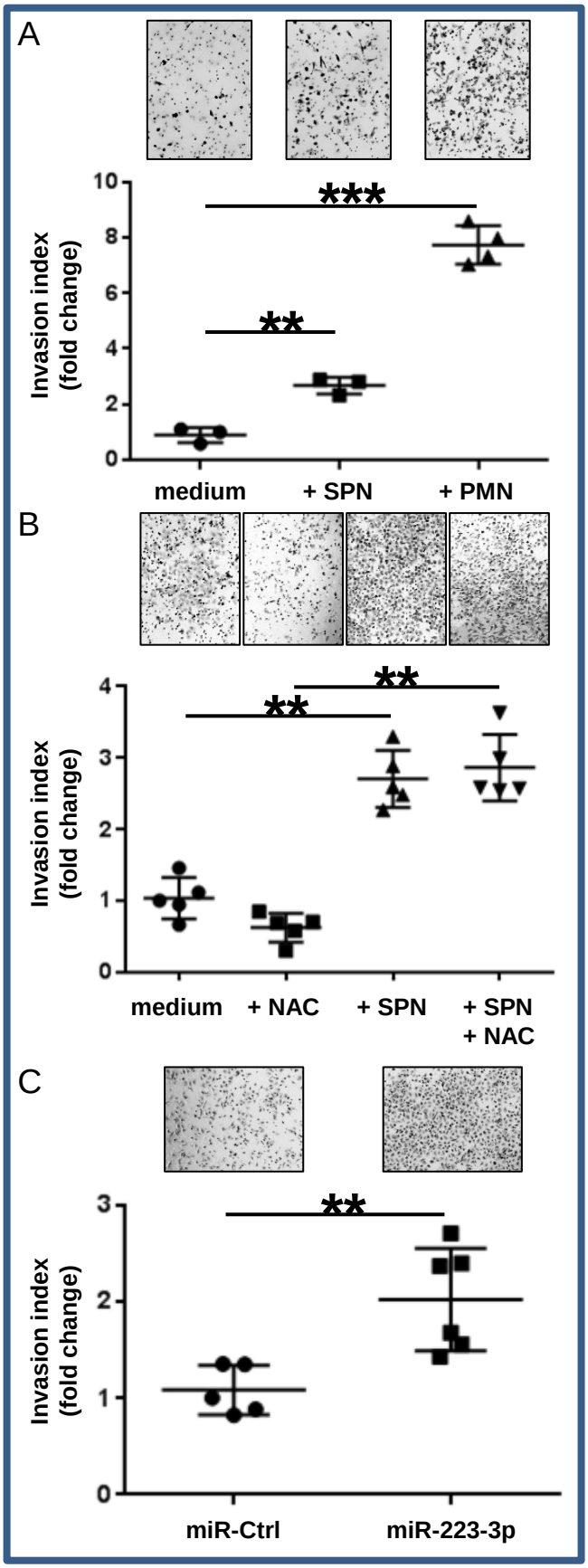
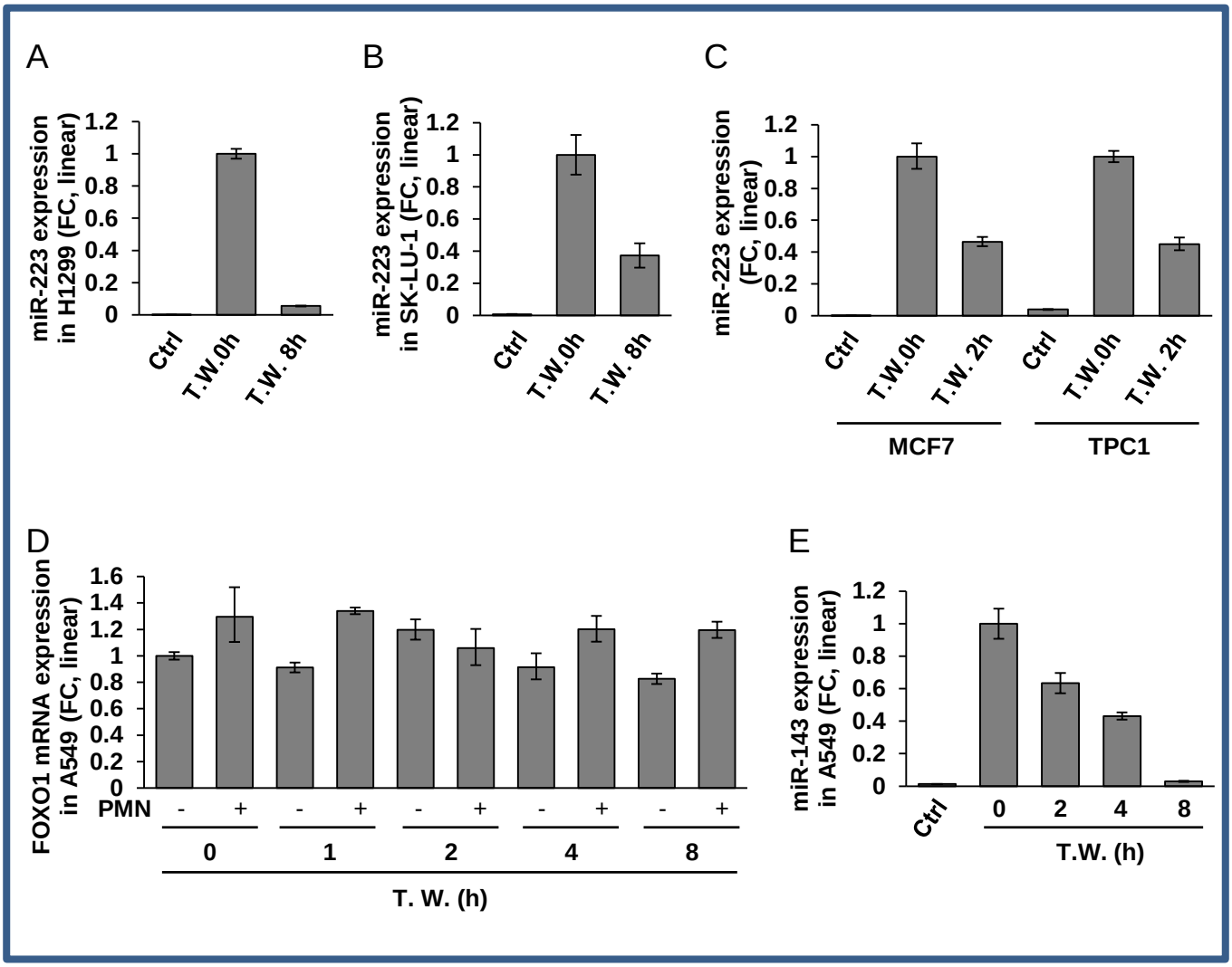


Supplementary Figure S4



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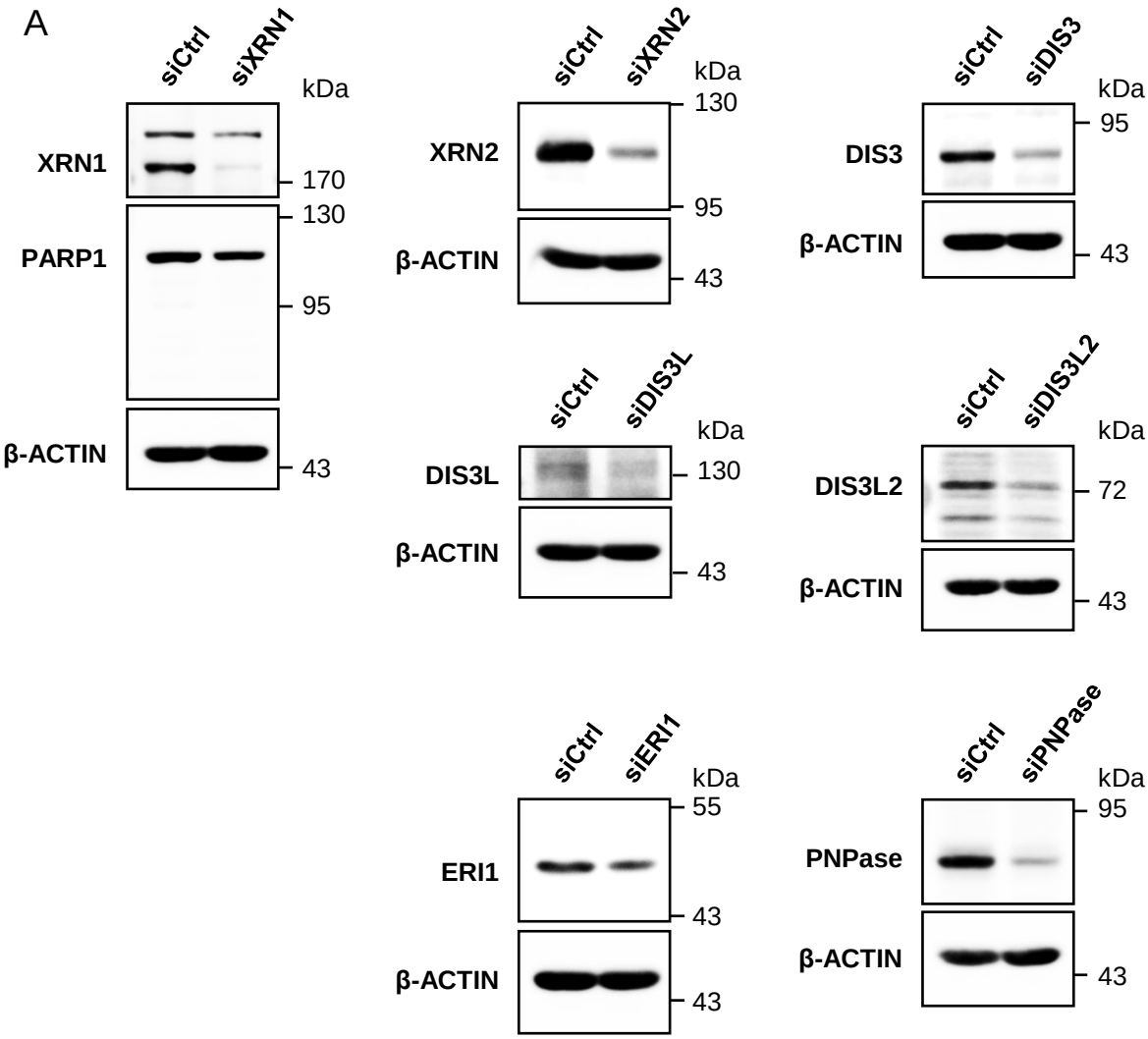
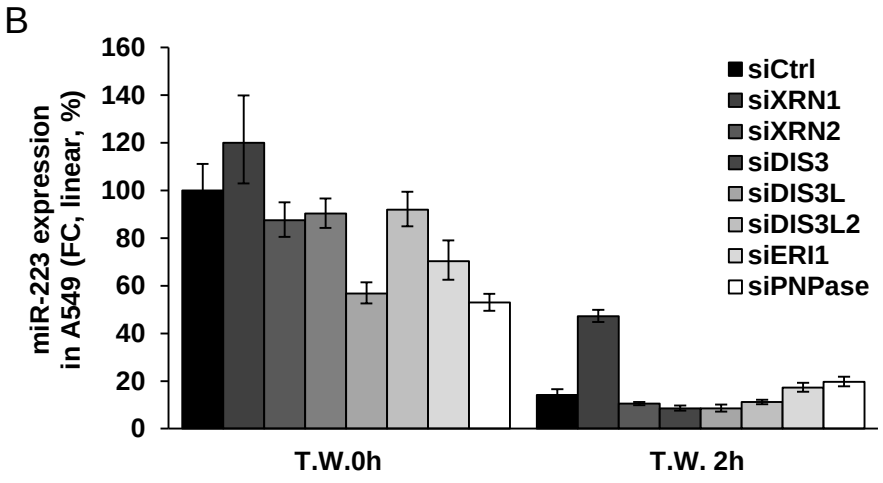
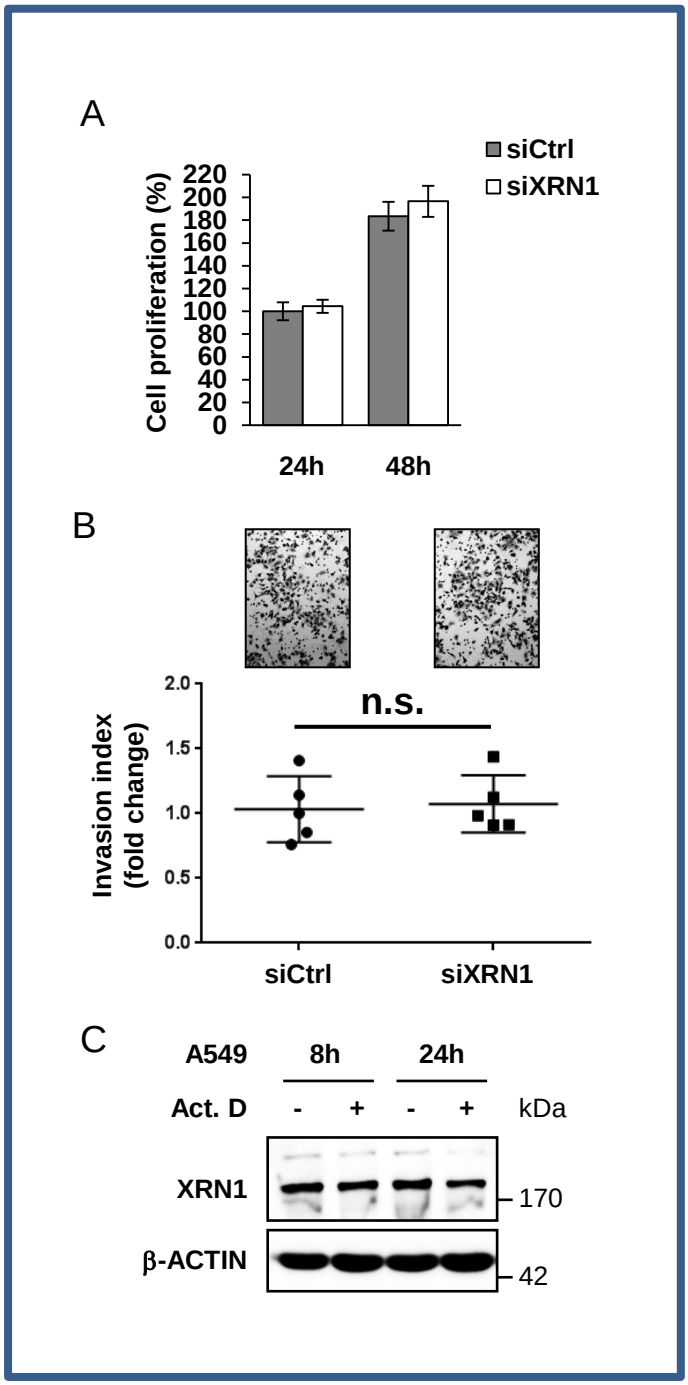
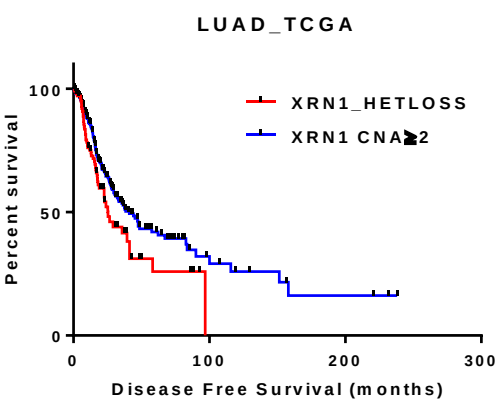




Supplementary Figure S6



Supplementary Figure S7



Disease Free Survival	XRN1_HETLOSS	XRN1 CNA≥2
Median survival (months)	25.36	41

Supplementary Figure S8