Supplemental Data

Article

MiR-221&222 regulate TRAIL-resistance and enhance tumorigenicity through PTEN and TIMP3 down-regulation.

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Supplemental Figures:

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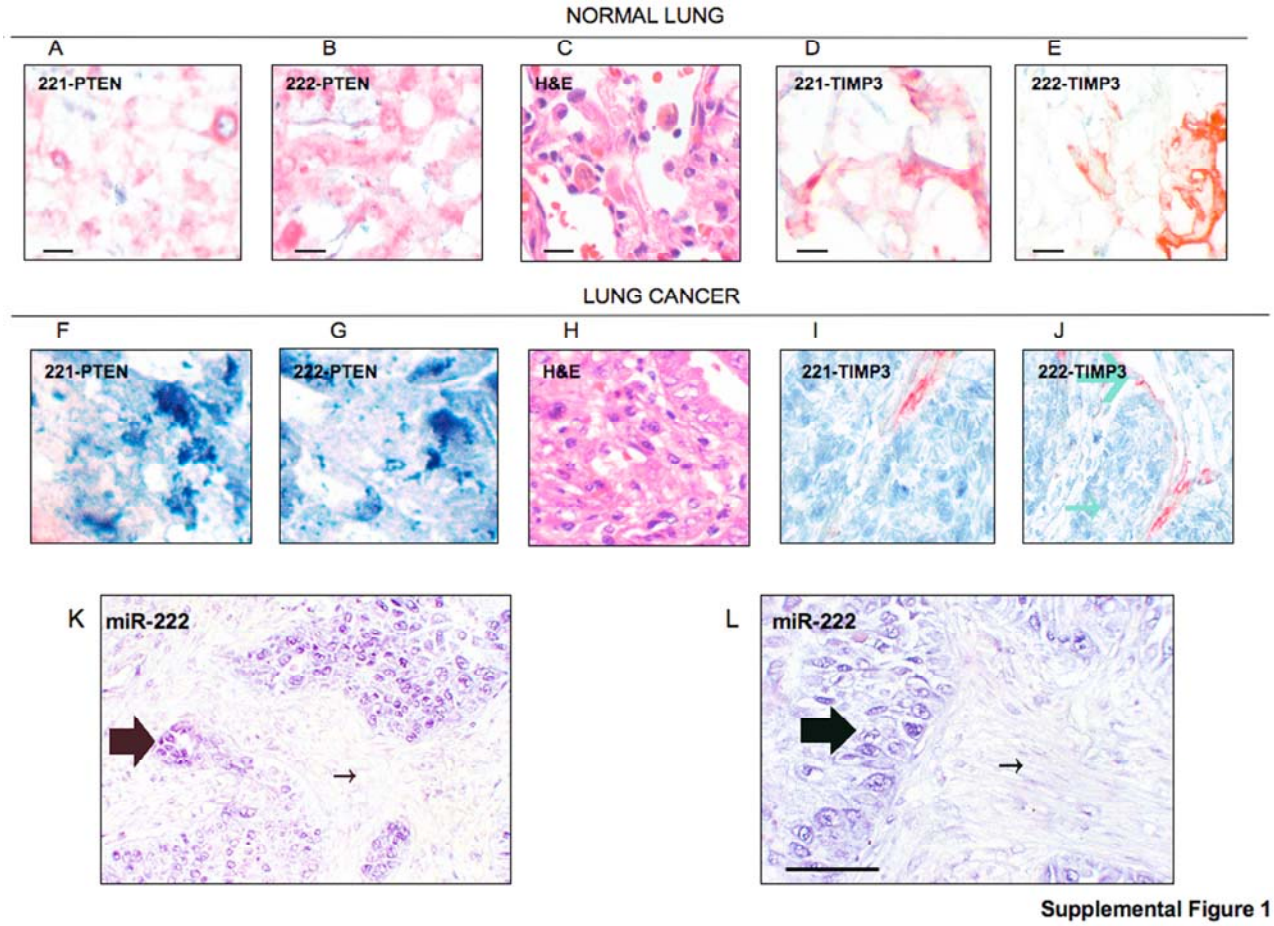
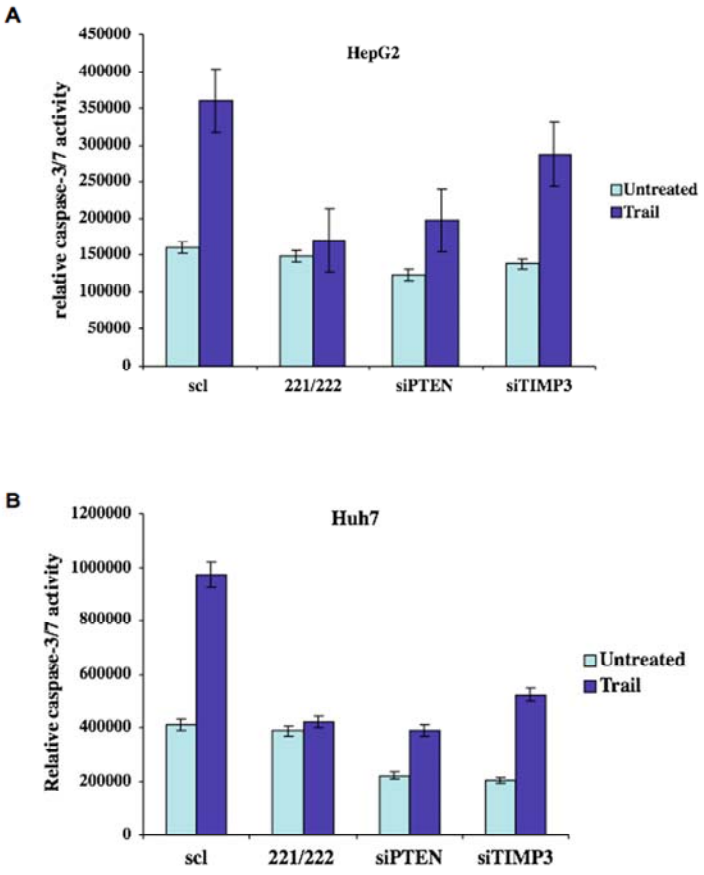


Supplemental Figure 1



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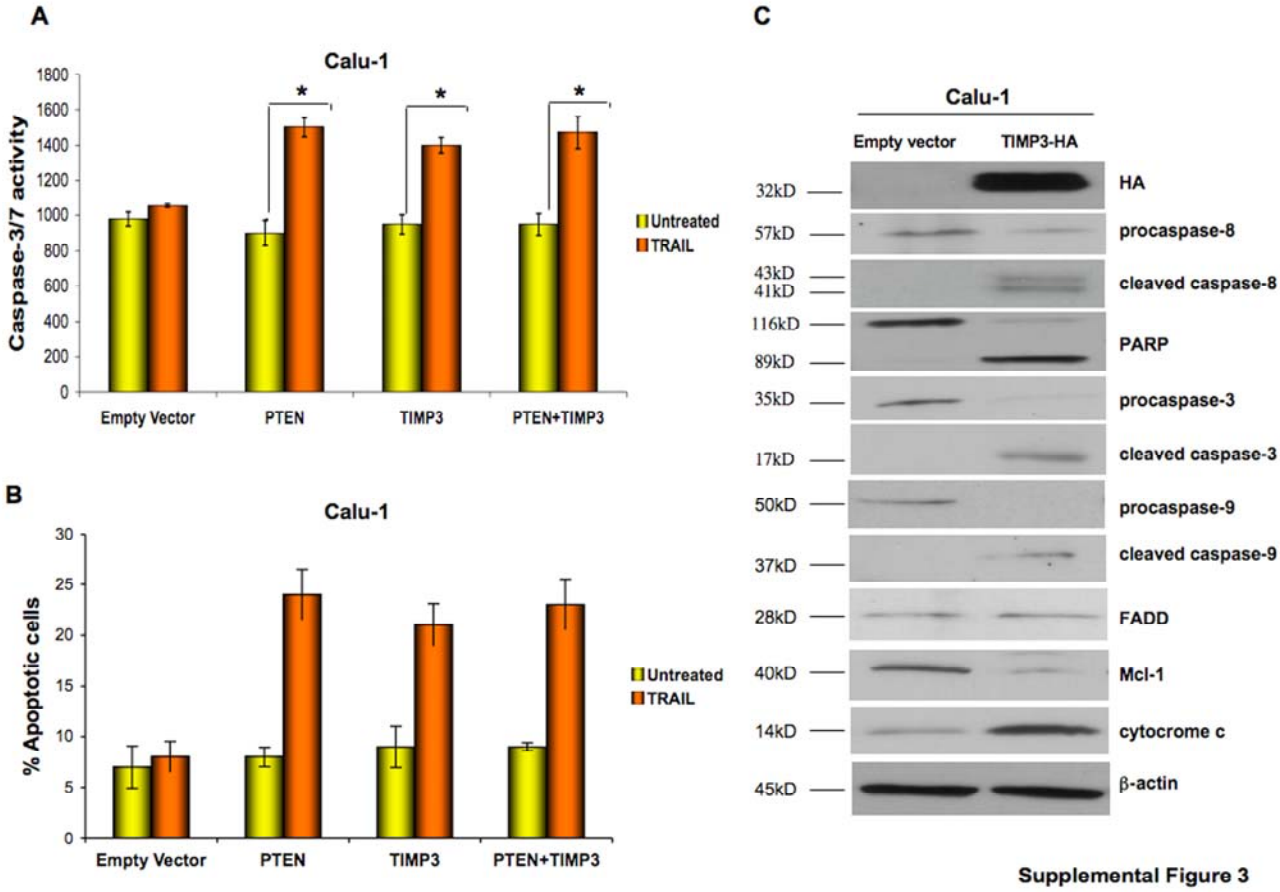

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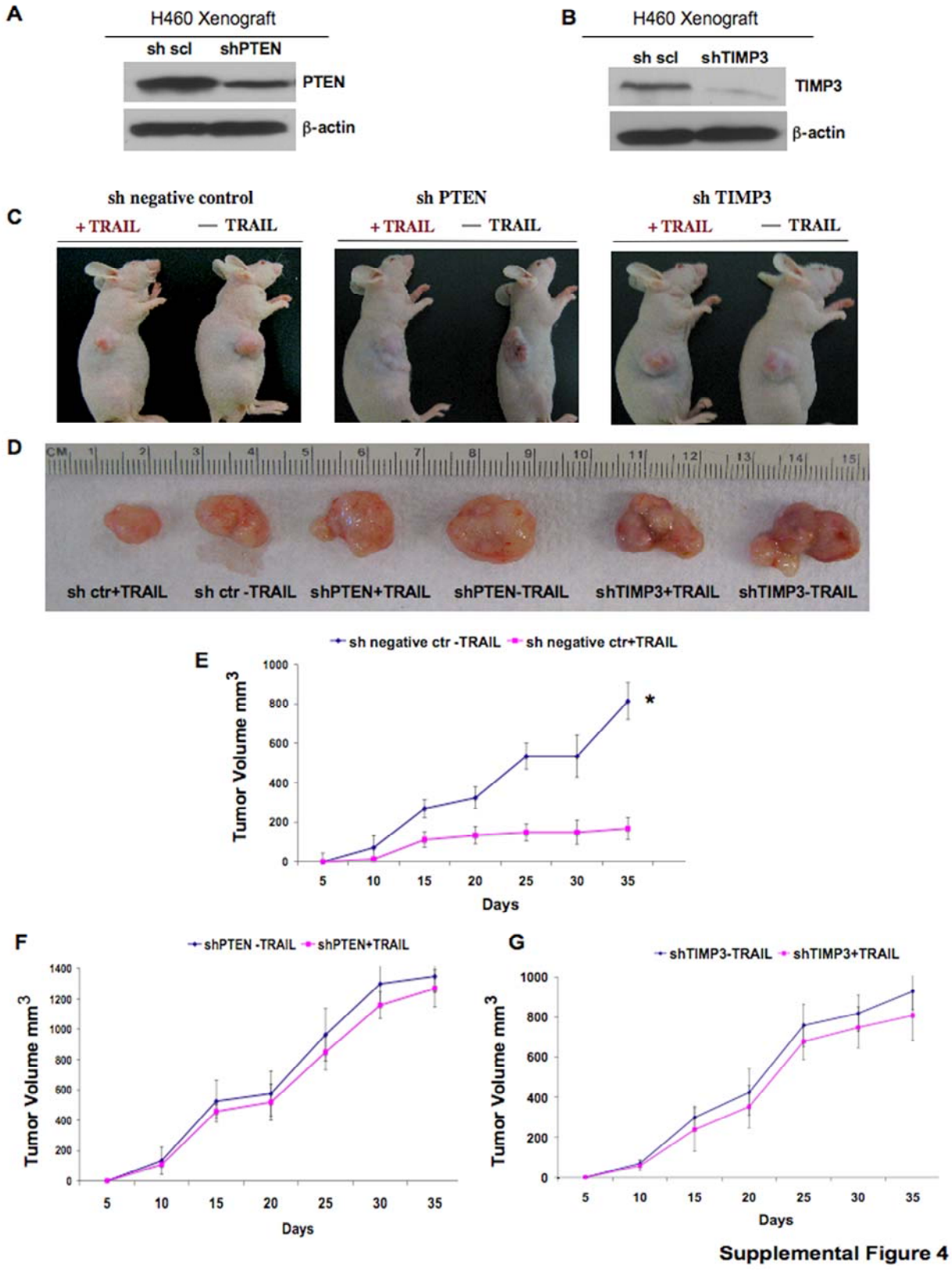
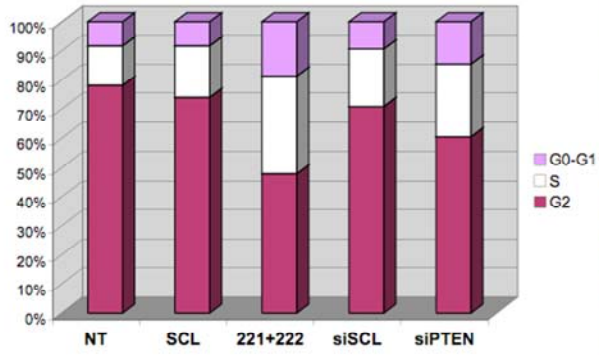


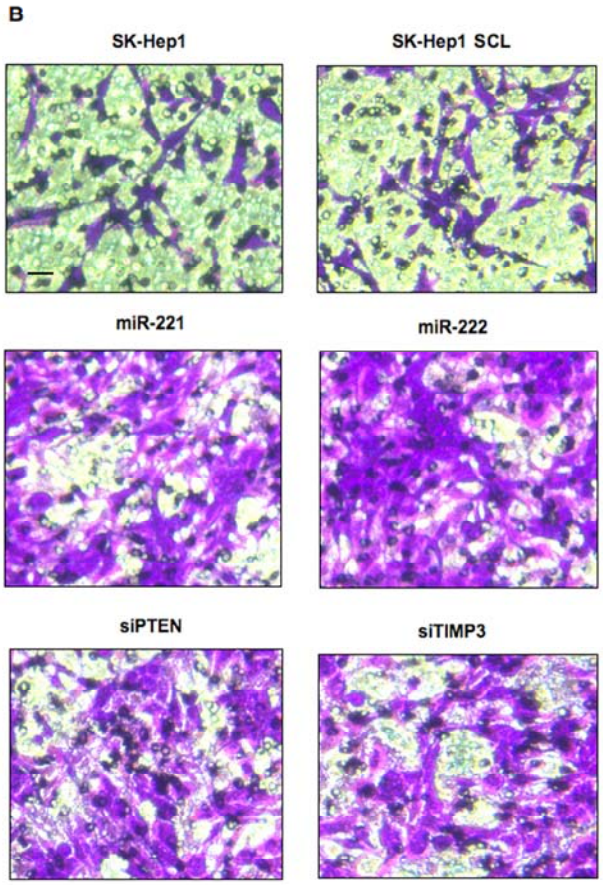
Supplemental Figure 4

A

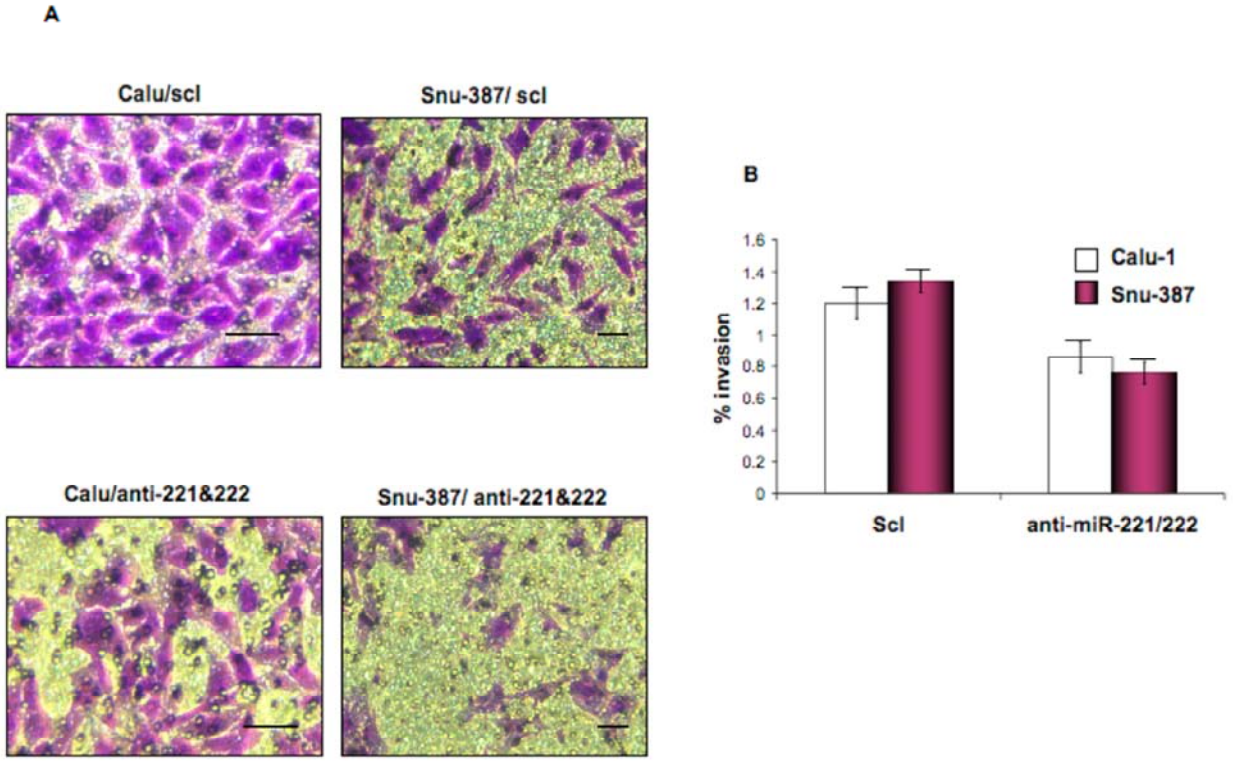


	NT	SCL	221+222	siSCL	siPTEN
G0-G1	75	71	46	68	58
S	13	17	32	19	24
G2	8	8	18	9	14

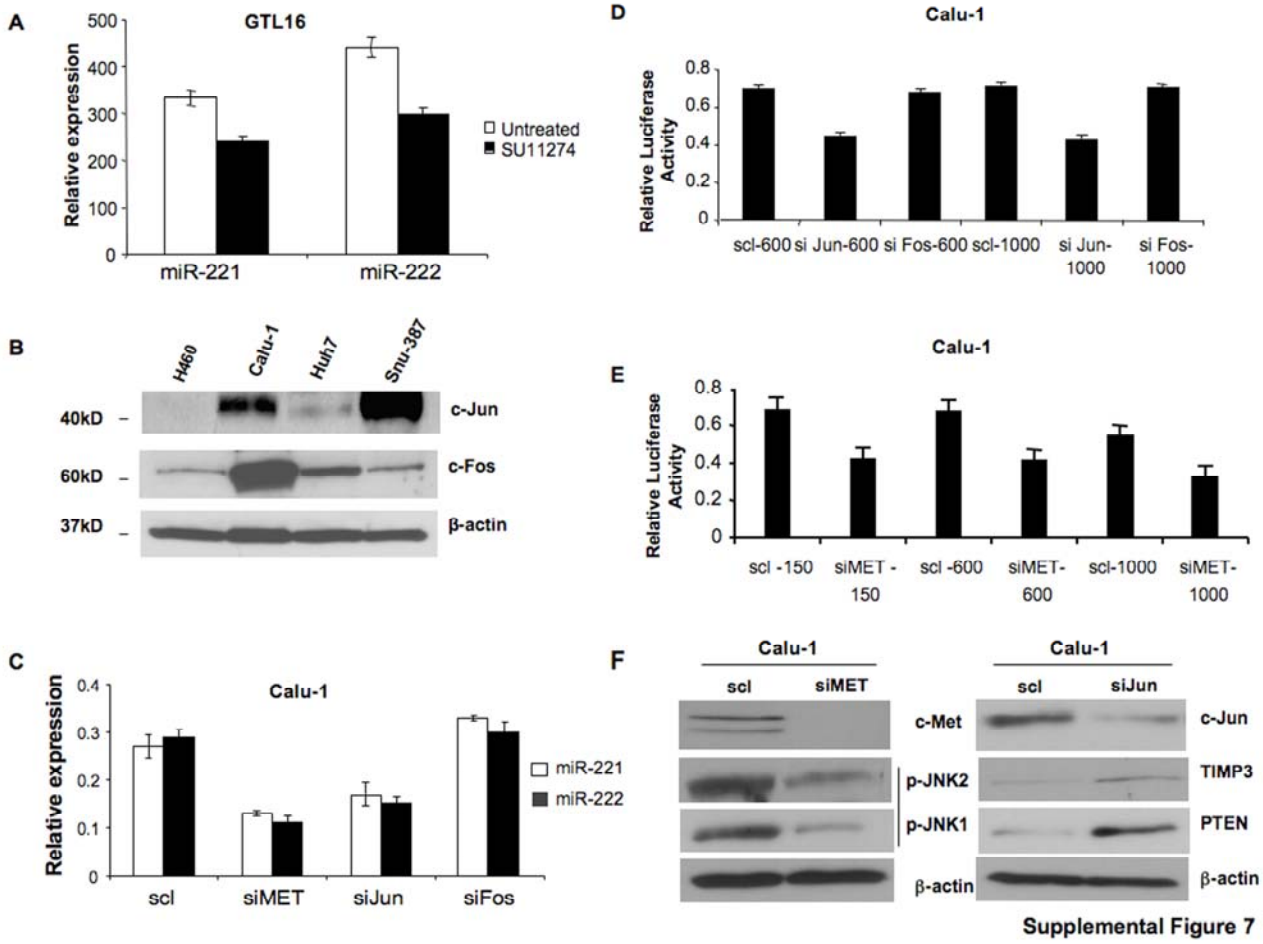
B



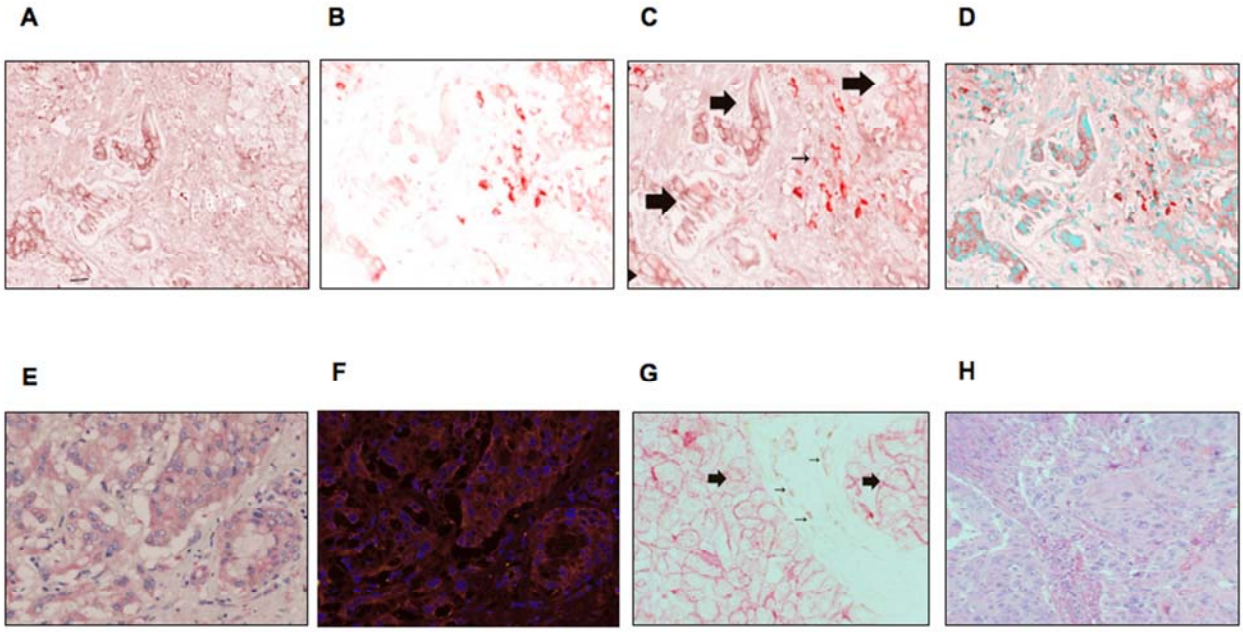
Supplemental Figure 5



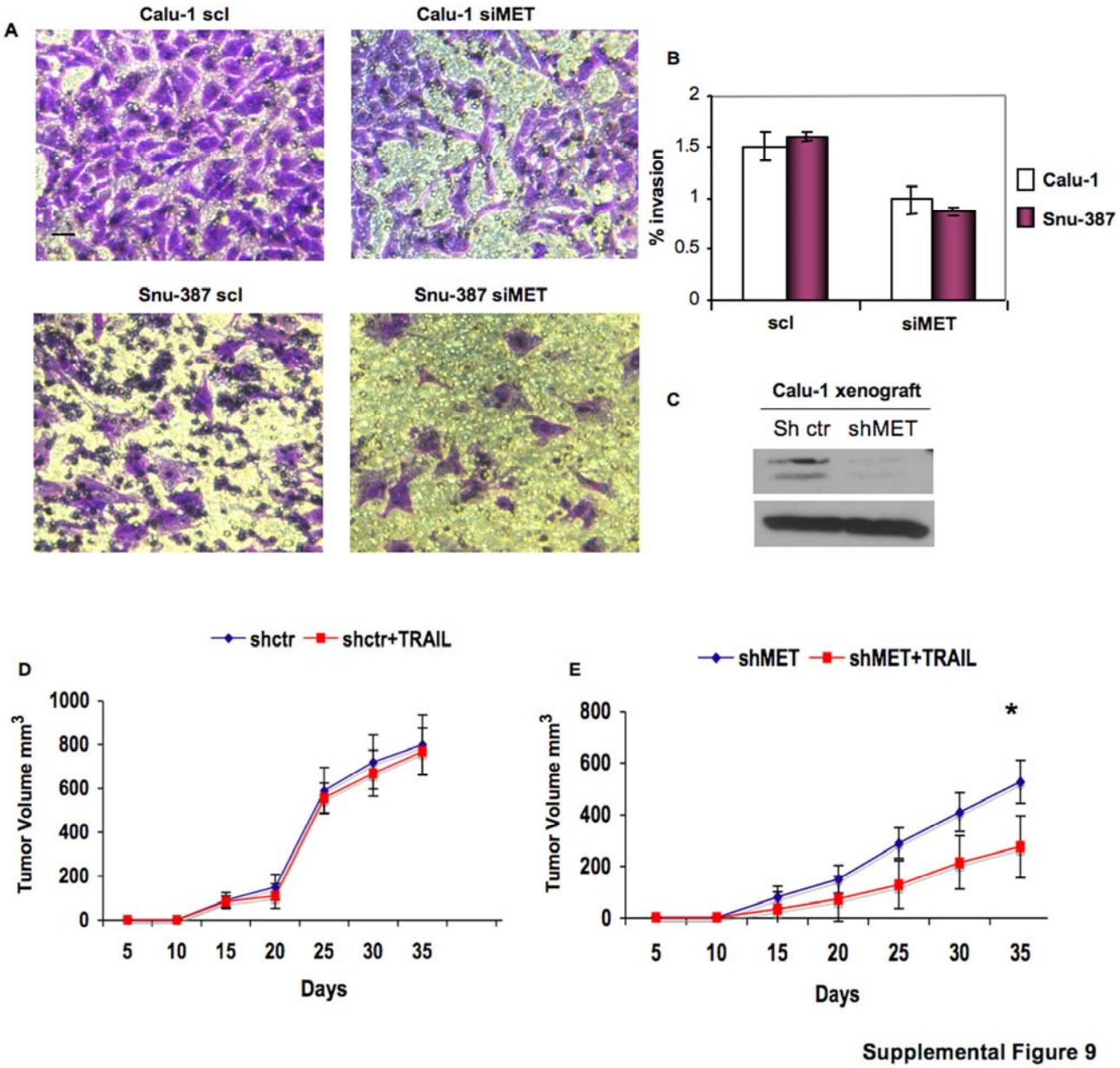
Supplemental Figure 6



Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure 9

Supplemental Figure 1

IHC and ISH of miR-221/222 and PTEN/TIMP3 in lung cancers and the adjacent benign tissues. MiR-221/222 (blue) and PTEN/TIMP3 (red) expression were inversely related in lung cancers and the adjacent normal lung tissues. These tissues were analyzed for miR-221 and miR-222 expression by in situ hybridization, followed by immunohistochemistry for PTEN and TIMP3 as described in the " Supplemental Experimental Procedures". The majority of cancer cells were positive for miR-221 and miR-222 and negative for PTEN (F-G) and TIMP3 (I-J). Conversely, the normal cells were negative for miR-221/222 (A-B-D-E) and positive for PTEN and TIMP3.

Note that in several cancers (I and J) miR-221/222 expression was evident with TIMP3 expression; however the miRNA expression was evident in the cancer cells and the TIMP3 expression in the surrounding cells in the desmoplastic tissue. C-H H&E. Small arrow miR-221-222, big arrow TIMP3. 92 human lung carcinomas were analyzed. K-L Correlation of miRNA-221/222 expression and histology in the lung. MiR-221 and -222 showed equivalent distribution patterns in this squamous cell carcinoma of the lung. Panel K shows a strong signal (large arrow) in the nests of tumor cells that are infiltrating the adjacent fibrotic lung tissue. Note that the signal shows a cytoplasmic and nuclear membrane based localization in the cancer cells (panel L, higher magnification). In comparison, only rare benign cells express miR-222 in the adjacent fibrotic tissue (small arrow) which is being invaded by the cancer cells. Scale bars indicate 25 μm.

Supplemental Figure 2

Caspase 3/7 activity in HepG2 and Huh7 cells after miR-221&222 upregulation or PTEN/TIMP3 knockdown (A-B) For caspase 3/7 activity detection, cells were cultured in 96-well plates, transfected with 100nM miR-221 and miR-222 for 72 h. After 48 h from transfection cells were treated with TRAIL 400ng/ml for 24h and analyzed using Caspase-Glo 3/7 Assay kit according to the manufacturer's instructions. HepG2 and Huh7 cells became resistant to TRAIL-inducing apoptosis after miR-221&222 forced expression or PTEN/TIMP3 downregulation. Data are presented as ± SD.

Supplemental Figure 3

TIMP3 overexpression induces apoptosis in Calu-1 TRAIL resistant cells. (A) Caspase 3/7 activity in Calu-1 cells after PTEN, TIMP3 and PTEN/TIMP3 upregulation. Cells were cultured in 96-well plates, transfected with PTEN, TIMP3 or both for 72 h. After 48 h from transfection cells were

treated with TRAIL 400ng/ml for 24h and analyzed using Caspase-Glo 3/7 Assay kit according to the manufacturer's instructions. Calu-1 cells became sensitive to TRAIL-inducing apoptosis after PTEN, TIMP3 or both PTEN/TIMP3 overexpression. (B) Effects of PTEN and TIMP3 on cell death. Calu-1 cells were transfected either with PTEN and TIMP3 plasmids. After 48 h from the transfection cells were treated with 400 ng/ml of Super-Killer TRAIL for 24 hours. Apoptosis was evaluated by Annexin-V. Percentage of apoptotic cells increases after PTEN and TIMP3 upregulation. (C) Western Blots in Calu-1 cells after TIMP3 overexpression. Fifty micrograms of total extract was loaded onto SDS-PAGE polyacrilammide gels and membranes were blotted with the indicated antibodies. TIMP3 overexpression activates both the extrinsic and intrinsic apoptotic pathways. Error bars indicate ± SD. *p < 0.001 by t test.

Supplemental Figure 4

Effects of PTEN and TIMP3 silencing on tumorigenicity of H460 cells in vivo. (A-B) Western blots showing PTEN and TIMP3 expression in H460 xenografts after shPTEN and shTIMP3 stable transfection. 35 days from the injection mice were sacrificed and tumors were analyzed by western blot. (C-D) Comparison of tumor engraftment sizes of sh control, shPTEN and shTIMP3 in H460 cells 35 days from the injection in nude mice and after treatment with vehicle (PBS) or TRAIL. PTEN and TIMP3 knockdown increases TRAIL resistance in vivo. The images show average-sized tumors from among five of each category. (E-F-G) Growth curve of engrafted tumors in nude mice injected with H460 cells stable transfected with sh control, sh PTEN and shTIMP3. Data are presented as ± SD. *p <0.001.

Supplemental Figure 5

Ectopic expression of miR-221 and miR-222 affects the cell cycle distribution and migration/invasion capabilities of Sk-Hep1 cells.

(A) Flow cytometric distributions of Sk-Hep1 cells transfected with empty vector,miR-221&222, siRNA PTEN. The average of three independent experiments is reported. (B) miR-221&222 regulate cell migration ability in Sk-Hep1 cells. Transwell insert chambers with 8-μm porous membrane were used for the assay. After transfection cells were washed with PBS and 150,000 cells were added to the top chamber in serum-free media. The bottom chamber was filled with media containing 10% FBS. To quantify migrating cells, cells on the top chamber were removed by using a cotton-tipped swab, and the migrated cells were fixed in PBS, 25% glutaraldehyde and

stained with Crystal Violet stain. Four random fields were counted. Scale bar indicates 25 μm. The magnification is the same for all the panels.

Supplemental Figure 6

2'-O-me-anti-mR-221&222 reduce cell migration and invasion ability of Calu-1 and Snu-387 cells. (A) Transwell insert chambers with 8-μm porous membrane were used for the assay. After transfection cells were washed with PBS and 50,000 cells were added to the top chamber in serum-free media. The bottom chamber was filled with media containing 10% FBS. To quantify migrating cells, cells on the top chamber were removed by using a cotton-tipped swab, and the migrated cells were fixed in PBS, 25% glutaraldehyde and stained with Crystal Violet stain. Five random fields were counted. MiR-2221&222 knockdown reduce Calu-1 and Snu-387 cells migration. (B) miR-221&222 influence Calu-1 and Snu-387 cell invasion ability. Histogram reports the percentage of cells that invaded through Matrigel-coated membrane after transfection with negative control miRNA, anti-miR-221, or anti-miR-222. Data are presented as ± SD of 3 separate determinations. Scale bars indicate 25 μm.

Supplemental Figure 7

c-Jun binds to miR-221/222 promoter determining its activation.

(A) qRT-PCR in GTL-16 cells after MET inhibition by using the MET inhibitor SU11274. MiR-221&222 were downregulated of about 40%, as compared with the untreated cells. (B) c-Jun and c-Fos expression levels in four different cell lines. 50 μg of total lysates were loaded onto a 12% polyacrilammide gel. Calu-1 and Snu-387 showed high c-Jun expression, Huh7 low expression levels and in H460 c-Jun expression was absent. c-Fos expression level is very high in Calu-1 cells, lower in all the other cell lines. (C) qRT-PCR on Calu-1 cells after MET, c-Jun, and c-Fos downregulation. Total 5ng of RNA in 10 μl PCR was used. TaqMan ΔC_T values were converted into absolute copy numbers using a standard curve from synthetic lin-4 miRNA. Data are expressed as the relative expression of the different miRs, compared to U44 and U48 rRNA. MiR-221&222 are downregulated after MET and c-Jun but not c-Fos knockdown by siRNAs, demonstrating that c-Jun is the transcription factor responsible for miR-221&222 activation. (D-E) Luciferase assays in Calu-1 cells after cotransfection with reporter plasmid harboring different sites of miR-221&222 promoter (-150,-600,-1000) and siRNA MET, siRNA c-Jun, siRNA c- Fos. MET and c-Jun siRNAs but not c-Fos siRNA, were able to decrease miR-221&222 luciferase activity. (F) Western blots after c-Met and c-

Jun silencing. MET knockdown reduces JNK1/2 phosphorylation. c-Jun silencing gives rise to an increased expression of PTEN and TIMP3. Data are presented as ± SD.

Supplemental Figure 8

PTEN, TIMP3 and MET co-labeling. IHC and ISH were performed on 72 lung tumor samples. (A) IHC of c-Met alone (brown), (B) TIMP3 alone (red) (C) and c-Met/TIMP3 colabeling in lung cancers, (D) nuclei as demonstrated by a hematoxylin counterstain. c-Met is expressed only in the cancer cells (big arrows) whereas TIMP3 is expressed in the surrounding benign cells (small arrows). Note that when c-MET is present TIMP3 is absent and viceversa. (E) Colocalization of c-Met (red) and TIMP3 (brown) in hepatocellular carcinoma (60 tumor samples were analyzed). Note that the tumor cells express c-Met and that TIMP3 expression is not evident. The panel also shows the hematoxylin stained features of the cancer, marked by multiple invasive nests in a desmoplastic stroma. (F) The same field analyzed by the Nuance system, with fluorescent red representing c-Met, fluorescent yellow representing TIMP3, and fluorescent green cells co-labeled with c-Met and TIMP3. As evident, no cancer cells co-label with c-Met and TIMP3. (G) Colocalization of c-Met and PTEN in lung carcinoma. The c-Met stain (red) shows the cell membrane pattern typical for c-Met in the cancer cells (large arrows). Adjacent to them is the stroma, with its benign fibroblasts and macrophages that express PTEN (brown - small arrow) but not cMET. (H) H&E. Scale bar indicates 25 μm. The magnification is the same for all the panels.

Supplemental Figure 9

MET silencing reduces cell migration and invasion in Calu-1 and Snu-387 cells and enhances TRAIL sensitivity in vivo.

(A) Transwell insert chambers with 8-μm porous membrane were used for migration assay. After transfection cells were washed with PBS and 50.000 cells were added to the top chamber in serum-free media. The bottom chamber was filled with media containing 10% FBS. To quantify migrating cells, cells on the top chamber were removed by using a cotton-tipped swab, and the migrated cells were fixed in PBS, 25% glutaraldehyde and stained with Crystal Violet stain. Five random fields were counted. (B) MET influences Calu-1 and Snu-387 cell invasion ability. Histogram reports the percentage of cells that invaded through Matrigel-coated membrane after transfection with siRNA negative control or siRNA MET. Data are

expressed as mean ± standard error of 3 separate determinations. (C) Western blots showing MET expression in Calu-1 xenografts after shMET stable transfection. 35 days from the injection mice were sacrificed and tumors were analyzed by western blot. (D-E) Growth curve of engrafted tumors in nude mice injected with Calu-1 cells stable transfected with sh control and shMET. Data are presented as ± SD. *p <0.01. Scale bar indicates 25 μm. The magnification is the same for all the panels.

Supplemental Experimental Procedures:

Western Blot Analysis.

Total proteins from NSCLC and HCC cells were extracted with radioimmunoprecipitation assay (RIPA) buffer (0.15mM NaCl, 0.05mM Tris- HCl, pH 7.5, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate and 1% Nonidet P40). Sample extract (50 μg) was resolved on 7.5–12% SDS–polyacrylamide gels (PAGE) using a mini-gel apparatus (Bio-Rad Laboratories) and transferred to Hybond-C extra nitrocellulose. Membranes were blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20, incubated overnight with primary antibody, washed and incubated with secondary antibody, and visualized by chemiluminescence. The following primary antibodies were used: anti-PTEN, anti-c-Jun, anti-p-c-Jun, anti—Fos, anti-p-JNK, anti-MMP3, anti-Mcl-1 (Santa Cruz), anti-TIMP3 (Millipore) anti-PI3K (BD Biosciences), anti-ERKs, anti-phospho ERKs, anti-AKT, anti-p-AKT, anti-GSK3b, anti-p-GSK3b (Ser9), anti-PAK1 anti-caspase-8,-3 and-9, anti-PARP, anti-cytochrome c (Cell signaling) and anti-MMP9, anti-FADD (Abcam), anti-β-actin antibody (Sigma). A secondary anti-rabbit or anti-mouse immunoglobulin G (IgG) antibody peroxidase conjugate (Chemicon) was used.

Luciferase assay.

DNA fragments containing the putative regulatory region upstream to miR-222/-221 (from +1~-150 nt, +1~-600, +1~-1000 (Di Leva et al. unpublished) (+1 position corresponds to the 5' terminus of miR-222 hairpin) were amplified and cloned in pGL3basic (Promega). Meg01 cells were transfected with Lipofectamine 2000 (Invitrogen), 1.0 μg of pGL3basic empty vector or of pGL3 containing the above genomic fragments, 200 ng of Renilla luciferase expression construct pRL-TK (Promega) and MET, c-Jun, c-Fos siRNAs. After 48h, cells were lysed and assayed with Dual Luciferase

Assay (Promega) according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

The primers utilized for the cloning were the followings: (Di Leva et al., unpublished data):

-1000pGL3b Forw: 5` gctagccctagccaccttatcgaaaatagcattcc 3`

-600 pGL3b Forw: 5` gctagcctgacatgctagtgagcacctgc 3`

-150 pGL3b Forw: 5`gctagcccagaggttgtttaaaattacgta 3`

miR-222 pGL3b Rev: 5`ctcgagagctgggtgatcctttgccttctg 3`

Real-time PCR

Real-time PCR was performed using a standard TaqMan PCR Kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems). The 10 µl PCR reaction included 0.67 µl RT product, 1 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.2 mM TaqMan probe,1.5 mM forward primer and 0.7 mM reverse primer. The reactions were incubated in a 96-well plate at 95 ˚C for 10 min, followed by 40 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min. All reactions were run in triplicate. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The comparative CT method for relative quantization of gene expression (Applied Biosystems) was used to determine miRNA expression levels. The y axis represents the $2^{(-\Delta CT)}$, or the relative expression of the different miRs. miRs expression was calculated relative to U44 and U48 rRNA and multiplied by 10^4. Experiments were carried out in triplicate for each data point, and data analysis was performed by using software (Bio-Rad).

RNA extraction and Northern blotting

Total RNA was extracted with TRIzol solution (Invitrogen), according to the manifacturer's instructions and the integrity of RNA was assessed with an Agilent BioAnalizer 2100 (Agilent, Palo Alto, CA, USA). Northern blotting was performed as described by Calin et al., 2002. The oligonucleotides used as probes were the complementary sequences of the mature miRNA (miRNA registry): miR-221, 5'-GAAACCCAGCAGACAATGTAGCT-3';miR222,5'GAGACCCAGTAGCCAGATGTAGCT-3'.

Antisense inhibition of miRNA expression.

2'-O-methyl (2'-O-me) oligoribonucleotides were synthesized by Fidelity. The sequences of 2'-O-me-anti-miR-221 and 2'-O-me-anti-miR-222 are as follows: 5'- gaaacccagcagacaauguagcuL and 5'-gagacccagtagccagatgtagctL. 2'-O-me-GFP miR (5'- aaggcaagcugacccugaaguL) was used as control. Cells were grown in six well plate (1.7×10^6 per well) for 24 h and transfected 100 nmoli/L/well of 2'-O-me-oligoribonucleotides using lipofectamine 2000. RNA and proteins were extracted after 72h from the transfection.

Cell death and cell proliferation quantification

Cells were plated in 96-well plates in triplicate and incubated at 37°C in a 5% CO2 incubator. Super-Killer TRAIL (Alexis Biochemicals) was used for 24–48 h at 400 ng ml $^{-1}$. Cell viability was examined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer's protocol. Metabolically active cells were detected byding 20 µl of MTT to each well. After 1 h of incubation, the plates were analyzed in a Multilabel Counter (Bio-Rad Laboratories). Apoptosis was assessed using Annexin V–FITC apoptosis detection kits followed by flow cytometric analysis and caspase 3/7 activity. Cells were seeded at 1.8×10^6 cells per 100mm dish, grown overnight in 10% FBS/RPMI, washed with phosphate-buffered saline (PBS) and then treated for 24 h with 400 ng/ml TRAIL. Following incubation, cells were washed with cold PBS and removed from the plates by trypsinization. The resuspended cells were washed with cold PBS and stained with FITC-conjugated annexin V antibody according to the manufacturer's instructions (Roche Applied Science). Cells (5×10^5 per sample) were then subjected to flow cytometric analysis. Flow cytometry analyses were done as described (Garofalo et al., 2007). The fraction of H460 cells treated with TRAIL was taken as the apoptotic cell population. The percentage of apoptosis indicated was corrected for background levels found in the corresponding untreated controls. Statistical analysis was done using two sample t test, assuming equal variance, and P value was calculated based on two-tailed test. For detection of caspase 3/7 activity, cells were cultured in 96-well plates and treated with TRAIL 400ng/ml and analyzed using Caspase-Glo 3/7 Assay kit (Promega) according to the manufacturer's instructions. Continuous variables are expressed as mean values± standard deviation (s.d.).

Chromatin Immunoprecipitation.

Chromatin immunoprecipitation was performed as described by de Belle et al., 2000 with slight modifications. Cells (5×10^6) from H460, Calu-1 and Snu-387 cell lines were fixed in 1% formaldehyde for 10 min at 37°C. Cells were washed with ice-cold 1× PBS, scraped in 1×PBS plus protease inhibitors, and collected by centrifugation. Cell pellets, resuspended in cell lysis buffer [50 mmol/L Tris-HCl (pH 8.0),10 mmol/L EDTA, and 1% SDS] plus protease inhibitors, were then sonicated. DNA-protein complexes were immunoprecipitated using 5 μg of the anti-c-Jun antibody (Santa Cruz) or with rabbit polyclonal IgG control (Zymed). Cross-links in the immunoprecipitated chromatin were reversed by heating with proteinase K at 65°C overnight, and DNA was purified by the MinElute Reaction Cleanup column (Qiagen) and resuspended in water. The purified chromatin was subjected to PCR and the products were analyzed by gel electrophoresis using 2% agarose.
The following primers were used:
Region1F: 5` GATGTGGAGAATAGATACCTTTGAG 3`
Region1R: 5` GGCACTGCCTACAAACCAGAGCATA3`
Region2F: 5` GTCACTCAGTCAGTATCTGTTGGA 3`
Region2R: 5` GTGTGTAATTCAAGGTAAAGTTTTC3`

Anti-PTEN and anti-TIMP3 siRNAs transfection.

Cells were cultured to 80% confluence and transiently transfected using Lipofectamine 2000 with 100 nM anti-PTEN or with 100nM anti-TIMP3 SMARTpool siRNAs or control siRNAs (Dharmacon), a pool of four targetspecific 20–25 nt siRNAs designed to knock down gene expression.

MiRNA locked nucleic acid in situ hybridization of formalin fixed, paraffin-embedded tissue section.

In situ hybridization (ISH) was carried out on deparaffinized human lung and liver tissues using previously published protocol (Nuovo et al.,2009), which includes a digestion in pepsin (1.3 mg/ml) for 30 minutes. The sequences of the probes containing the six dispersed locked nucleic acid (LNA) modified bases with digoxigenin conjugated to the 5' end were: miR-221-(5') GAAACCCAGCAGACAATGTAGCT; miR-222(5') ACCCAGTAGCCAGATGTAGCT. The probe cocktail and tissue miRNA were co-denatured at 60˚C for 5 minutes, followed by hybridization at 37 ˚C overnight and a low stringency wash in 0.2X SSC and 2% bovine serum albumin at 4˚C for 10 minutes. The probe-target complex was seen due to the action of alkaline phosphatase on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP). Negative controls

included the use of a probe which should yield a negative result in such tissues. No counterstain was used, to facilitate co-labeling for PTEN, TIMP3 and MET proteins. After in situ hybridization for the miRNAs, as previously described (Nuovo et al., 2009), the slides were analyzed for immunohistochemistry using the optimal conditions for PTEN (1:800, cell conditioning for 30 minutes), TIMP3 (1:1300, cell conditioning for 30 minutes) and MET (1:20, cell conditioning for 30 minutes). For the immunohistochemistry, we used the Ultrasensitive Universal Fast Red system from Ventana Medical Systems. We used normal liver and lung tissues as controls for these proteins. The percentage of tumor cells expressing PTEN, TIMP3 and miR-221&222 was then analyzed with emphasis on co-localization of the respective targets (miR-221 or -222 and either PTEN or TIMP3).

Materials.

Media, sera and antibiotics for cell culture were from Life Technologies, Inc. (Grand Island, NY, USA). Protein electrophoresis reagents were from Bio-Rad Laboratories (Richmond, VA, USA) and western blotting and ECL reagents from GE Healthcare (Piscataway, NJ, USA). All other chemicals were from Sigma (St Louis, MO, USA).

Lung and liver cancer samples and cell lines.

Human Calu-1 and A549 cell lines were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS) and with 2mM L-glutamine and 100Uml^{-1} penicillin–streptomycin.Hel299,H460,A459,H1975,H1299,H1573,H23,PLCRF15, SNU-387, Snu-423,Snu-475 cell lines were grown in RPMI containing 10% heat-inactivated FBS and with 2mM L-glutamine and 100Uml^{-1} penicillin–streptomycin. Sk-hep1, Hep-G2, HepG2C3A, Hep3B, Huh7 were grown in MEM supplemented with 10% fetal bovine serum, 2mM L-glutamine and 100Uml^{-1} penicillin–streptomycin. Normal Hepatocytes were grown in Hepatocytes growth medium (Sciencell) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1% of hepatocyte growth supplement (HGS) and 100Uml^{-1} penicillin–streptomycin.

Migration assay

Transwell insert chambers with 8-μm porous membrane (Greiner bio-one) were used for the assay. Cells were washed three times with PBS and added to the top chamber in serum-free media. The bottom chamber was filled with media containing 10% FBS. Cells were incubated for 24 h at 37°C in a 5% CO_2 humidified incubator. To quantify migrating cells, cells on the top chamber were removed by using a cotton-tipped swab, and the migrated cells were fixed in PBS, 25% glutaraldehyde and stained with Crystal Violet stain, visualized under a phase-contrast microscope, and photographed. Cristal violet-stained cells were moreover solubilized in acetic acid and methanol (1:1) and absorbance was measured at 595 nm. The results are means of three independent experiments ± S.D.

Invasion Assay

H460 and SK-Hep-1 cells were placed into the top chamber of a BD Falcon HTS FluoroBlok insert with a membrane containing 8-μm pores (BD Biosciences) in 300 μL of serum-free Dulbecco's modified Eagle medium in triplicate. The inserts were placed into the bottom chamber wells of a 24-well plate containing Dulbecco's modified Eagle medium media and fetal bovine serum (10%) as chemoattractant. Cells that migrated through the pores of the membrane to the bottom chamber were labeled with 8 μg/mL calcein-AM (Molecular Probes, Eugene, OR) in phosphate-buffered saline (PBS) for 30 minutes at 37°C. The fluorescence of migrated cells was quantified using a fluorometer at excitation wavelengths of 485 nm and emission wavelengths of 530 nm and expressed as arbitrary fluorescence units. Data are expressed as mean ± standard error of 4 separate determinations.

PTEN and TIMP3 plasmids.

PTEN and TIMP3 cDNAs were obtained from H460 cells RNA by using the one step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. The PCR fragments were amplified by using the following primers:

NotI-TIMP3-HA: 5` gcggccgcatgacccctggctcgggctcatcgtgct 3`

BglII-TIMP3-HA: 5` agatctcagggtctggcgctcaggggtctgt 3`

NotI-PTEN-HA: 5` gcggccgcatgacagccatcatcaaagagatcgttag 3`

XbaI-PTEN-HA: 5` tctagaggtgttttatccctcttgataaaaaaaaattca 3`

and then cloned in pCRUZ-HA (Santa Cruz) after digestion with NotI-XbaI (PTEN) or NotI-BglII (TIMP3). All vectors were controlled by sequencing.

Target analysis

Bioinformatic analysis was performed by using these specific programs: Targetscan[1], Pictar[2] and RNhybrid[3].

[1]http://www.targetscan.org/

[2] http://pictar.bio.nyu.edu/

[3] http://bibiserv.techfak.uni-bielefeld.de/

Supplemental References:

Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., et al. (2002). *Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemi*a. Proc Natl Acad Sci U S A. 99,15524-9.

De Belle, I., Mercola, D., Adamson, E.D. (2000). *Method for cloning in vivo targets of the Egr-1 transcription factor*. Biotechniques 29,162-9.

Garofalo, M., Romano, G., Quintavalle, C., Romano, M.F., Chiurazzi, F., Zanca, C., Condorelli, G.(2007). *Selective inhibition of PED protein expression sensitizes B-cell chronic lymphocytic leukaemia cells to TRAIL-induced apoptosis.* Int. J. Cancer 120,1215-22

Nuovo, G.J., Nana-Sinkam, P., Elton, T., Croce, C,, Volinia, S,, Schmittgen, T.(2009). *A methodology for the combined in situ analyses of the precursor and mature forms of microRNAs and correlation with their putative targets*. Nature Protocols 4, 107-115.