Supplementary material

Supplementary Methods

Preparation of primary human neuro-glial culture

Cortical tissue was dissected from human embryos at 16 weeks of gestation (provided by Advanced Bioscience Resources, Alameda, CA) in DMEM containing 1% Pen/Strep. Single cells were dissociated by tripsinization, re-suspended in PR medium (DMEM, 10% bovine calf serum, 1% Pen/Strep, 1% sodium pyruvate, 1% L-glutamine; Gibco) and filtered using 70 μm cell strainer (BD Falcon). Live cells were separated by gentle centrifugation through 42.5% ficoll-paque/PBS (Fisher), resuspended in PR medium, and passed again through the cell strainer. Cells were washed twice and plated in PR medium on poly-L-lysine coated plates. The medium was changed to B27 maintenance medium (Neurobasal medium, 2% B27, 1% Pen/Strep, 1% sodium pyruvate; Gibco) 3 days later.

Oligonucleotides and transfections

Cells were transfected with 50 nM of 2'-O-MOE (**with either phosphate or phosphorothioate backbones)** control and anti-miR-10b oligos (Regulus) for miR-10b inhibition (1), and with hsa-miR-10b precursor and corresponding Negative Control #2 (Applied Biosystems) for over-expression using Lipofectamine 2000 (Invitrogen) as previously described (2). For RNAi experiments, siRNAs to Bim, TFAP2C, p16, p21,

and siGENOME RISC-Free Control siRNA (Dharmacon) were used at 20 nM concentrations. Oligo sequences are provided in Supplementary Table 1.

Analysis of miRNA and mRNA expression by qRT-PCR

RNA was extracted from cells or tissues using TRIzol (Invitrogen). Quantitative real-time RT-PCR (qRT-PCR) reactions were performed with TaqMan reverse transcription reagents and SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacture protocols. Normalization was performed on either GAPDH or actin mRNA levels. Primer sequences are provided in Supplementary Table 1. qRT-PCR for miRNA detection was performed with TaqMan miRNA assays (Applied Biosystems) and normalized to miR-16.

Cloning

For cloning of the miR-10b reporter, sense and anti-sense oligos (Supplementary Table 1) were annealed and ligated into psiCHECK-2 vector (Promega). For target validation, partial 3'UTRs (500-1500bp) containing putative miR-10b binding sites were cloned into the NotI and XhoI sites of psiCHECK-2, downstream of *Renilla* luciferase. Mutations were introduced in the potential miR-10b binding sites by PCR reactions with specific oligos, and *Pfu*Ultra II Fusion HS DNA Polymerase (Stratagene). Sequences of oligos used for cloning and mutagenesis are provided in Supplementary Table 1.

TUNEL staining

Mice were sacrificed, tumors removed and freshly frozen using tissue freezing medium and sectioned into 10 μm sections. Tumor sections were mounted on slides and analyzed using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) staining as described in the manufacturer's instructions (Promega). DNA/Nucleus was also stained using DAPI (4',6-diamidino-2-phenylindole). Stained sections were evaluated using fluorescent microscopy.

Western blotting

Following antibodies from Cell Signaling were used: anti-Cyclin D1 mouse monoclonal (1:2000), anti-Cyclin B1 mouse monoclonal (1:1000), anti-CDC2 rabbit polyclonal (1:1000), anti-Cleaved Caspase-3 (Asp175) rabbit polyclonal (1:1000), anti-Cleaved Caspase-7 (Asp198) rabbit polyclonal (1:1000), anti-LC3 rabbit monoclonal (1:1000), anti-p21 mouse monoclonal (1:1000), and anti-p16 rabbit polyclonal (1:1000); from Abcam: anti-CDK2 mouse monoclonal (1:1000), anti-Bim rabbit polyclonal (1:5000), anti-AP-2γ rabbit monoclonal (1:1000), and anti-Actin (1:5000); and from Santa Cruz: anti-HoxD10 (H-80) rabbit polyclonal (1:200).

Senescence

Cells were grown on coverslips in a 24-well plate, transfected and subjected to senescence assay by using Senescence β-Galactosidase Staining Kit (Cell Signaling) three days post-transfection.

Autophagy assay

Glioma cells transfected with either control or miR-10b inhibitor were harvested 72 h post-transfection for Western blot analysis. Two forms of autophagic marker LC3, full LC3-I and cleaved LC3-II were detected by anti-LC3 antibody.

TCGA GBM data analysis

The TCGA miRNA and mRNA expression microarray data and metadata including survival information for GBM patients were downloaded from the following portal: http://tcga-data.nci.nih.gov/tcga/homepage.htm. The data were processed, normalized, matched and analyzed with customized R scripts (www.r-project.org). For miRNA expression, normalized level-2 data were directly used. For mRNA expression, level-1 raw data were normalized by RMA method and filtered using MAS5 detection calls to remove probes with signals below background levels. The lists of mRNAs correlated with selected miRNAs were further analyzed for pathway enrichment patterns using in-house tool PPEP analysis pipeline (3) and Gene Ontology database (GO, www.geneontology.org). The Kaplan-Meier survival analysis was performed using aggregate expression level of miR-10a and miR-10b computed as the sum of the z-scores of miR-10a and miR-10b for each sample. **Z-score reflects the expression of each miRNA in a sample relative to its expression in the whole sample set. For each miRNA, it was computed according to the following formula: Zi=(Xi-mean)/SD, where Xi is the normalized expression intensity (in log2) of the miRNA in sample i, mean is the mean expression intensity of this miRNA for all samples, SD is the standard deviation of expression intensity of the miRNA for all samples.**

Supplementary References

1. Davis S, Lollo B, Freier S, Esau C. Improved targeting of miRNA with antisense oligonucleotides. Nucleic Acids Res 2006;34:2294-304.
2. Gabriely G, Wurdinger T, Kesari S, *et al.* MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol 2008;28:5369-80.
3. Yi M, Mudunuri U, Che A, Stephens RM. Seeking unique and common biological themes in multiple gene lists or datasets: pathway pattern extraction pipeline for pathway-level comparative analysis. BMC Bioinformatics 2009;10:1-17.
4. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008;455:1061-8.

Supplementary Figures

Supplementary Fig. S1. TCGA dataset analysis of miR-10b expression in 261 GBM tumors and 10 normal brain specimens. miR-10b expression was measured as a normalized array signal and presented as a dot plot. The signal value of 9.7 corresponds to the background level.

Supplementary Fig. S2. Efficiency of miR-10b inhibition by 2'-*O*-MOE antisense inhibitor and comparison of the miR-10a and miR-10b inhibition effects on glioma cell growth. *A*, Efficiency of miR-10b inhibition as tested by qRT-PCR. A172, **U251, LN308, LN215, and LN464 glioma cells** were transfected with 50 nM anti-miR-10b 2'-*O*-MOE oligo and the corresponding control. The cells were harvested 48 h post-transfection, RNA was isolated, and miR-10b expression levels were assessed by TaqMan miRNA assays. Uniformly expressed miR-27a was used for normalization. *B*, Efficiency of miR-10b inhibition as assessed by luciferase reporter. Luciferase reporters containing miR-10b perfect binding site (miR-10b) or without it (Empty) were co-transfected with either miR-10b inhibitor or control oligonucleotides. Luciferase activities were measured 48 h post-transfection, and the ratio of Renilla/Firefly was calculated and plotted for each sample. Error bars represent SD, *p<0.05. *C*, **2'-*O*-MOE miR-10b inhibitor with phosphorothioate backbone inhibits glioma cell growth. Cell viability assay was performed on A172 and BT74 glioma cells transfected with either 2'-*O*-MOE with phosphorothioate (PS) backbone control (Control PS) or miR-10b inhibitor (Anti-miR-10b PS). Error bars represent SD; ***p<0.001, *p<0.05.** *D*, Effects of miR-10a and miR-10b inhibition on glioma cell growth. Cell viability assay was performed on

various glioma cells (A172, U251, and BT74) transfected with either control (Control), miR-10a inhibitor (Anti-miR-10a), or miR-10b inhibitor (Anti-miR-10b). Error bars represent SD for 3-6 independent transfections; *p<0.05, **p<0.001.

Supplementary Fig. S3. miR-10b does not afftect glioma cell invasion. The Matrigel invasion assay was performed on glioma cells (A172 and U251) transfected with either miR-10b mimic (miR-10b) or control oligonucleotide (Control) (for detailed protocol please see (2)). The experiment was performed in triplicates and data are represented as means ±SD.

Supplementary Fig. S4. Inhibition of miR-10b leads to senescence and accumulation of glioma cells in sub-G1. *A*, Induction of senescence by miR-10b inhibition. A172 cells were transfected with either control or miR-10b inhibitor, and 72 h later analyzed for accumulation of senescent cells. The arrow marks a cluster of senescent cells with enlarged cell morphology, exhibiting senescence-associated β-galactosidase activity. *B*, miR-10b inhibition results in accumulation of various glioma cells in sub-G1. Indicated glioma cells were transfected, fixed 72 h later, stained with PI, and DNA content was analyzed by FACS. p<0.05 for all comparisons.

Supplementary Fig. S5. Putative miR-10b binding sites and their evolutionary conservation within the 3'UTRs of predicted target mRNAs for Bim, TFAP2C, p16, and p21 (two potential sites). Stars indicate mutated nucleotides in corresponding reporters.

Supplementary Fig. S6. miR-10b inhibition up-regulates Bim, p21, and p16, but does not change HOXD10 expression in various glioma cells. *A*, Anti-miR-10b up-regulates miR-10b targets in different glioma cells. Cell lines indicated in italics were harvested 72 h post-transfection for Western blot analysis. Representative immunoblots for actin expression are shown for the loading control. *B*, miR-10b inhibition does not affect HOXD10 expression. U87 and LN308 glioma cells were transfected with anti-miR-10b or its corresponding control. The cells were harvested 72 h post-transfection, and lysed for protein isolation and Western blot analysis. Antibodies against HOXD10 (Santa Cruz; 1:200) were used for HOXD10 detection. Actin was used for the protein loading control.

Supplementary Fig. S7. miR-10b expression negatively correlates with TFAP2C and Bim mRNAs in a large set of GBM tumors. Correlation analysis was performed using TCGA mRNA and miRNA expression array datasets (4).

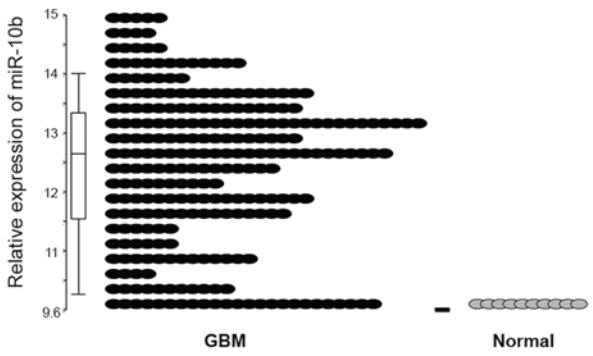
Supplementary Fig. S8. siRNAs to miR-10b targets efficiently inhibit protein expression and rescue the effects of miR-10b inhibition. *A*, A172 glioma cells were transfected with Bim siRNA, TFAP2C siRNA, p21, or control siRNA (Dharmacon), of which the sequences are provided in Supplementary Table S1. LN308 cells were transfected with p16 siRNA and the control siRNA. The cells were harvested 72 h post-transfection, the proteins isolated and their expression tested using western blot. Actin was used as a protein loading control. *B*, **Expression of miR-10b targets in the rescue experiment. Glioma LN308 cells were co-transfected with siRNA to Bim, p16, p21, TFAP2C, or**

control siRNA and anti-miR-10b oligo or its control, as indicated. Anti-miR-10b oligo or its control were also transfected without siRNAs (Mock). The cells were harvested 72 h post-transfection, the proteins isolated and their expression tested using western blot. Actin was used as a protein loading control. *C,* Silencing of miR-10b targets suppresses apoptosis caused by miR-10b inhibition. Glioma LN308 cells were co-transfected with siRNA to Bim, p16, p21, TFAP2C, or control siRNA and anti-miR-10b oligo or its control, as indicated. Anti-miR-10b oligo or its control were also transfected without siRNA (Mock). The cells were fixed 72 h following transfection, and stained with propidium iodide to examine DNA content. **Error bars represent SEM**; *p<0.05. *D*, p21 silencing rescues the decrease in S-phase population caused by miR-10b inhibition. Glioma A172 cells were co-transfected with anti-miR-10b or its control oligo, and either siRNA to p21 or control siRNA, or no siRNA (Mock), as indicated. The cells were fixed 48 h following transfection, and stained with propidium iodide to examine DNA content. Error bars represent SD; *p<0.05.

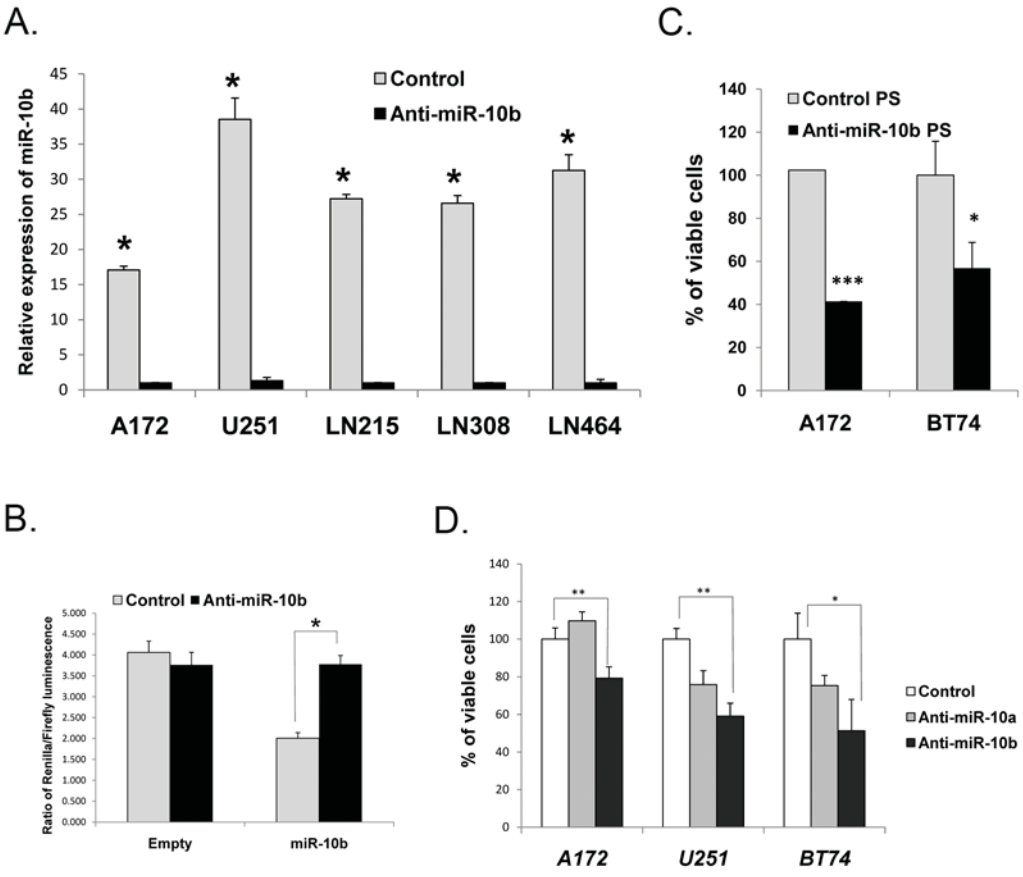
Supplementary Fig. S9. Inhibition of tumor growth and apoptosis following miR-10b inhibition by imaging. *A*, Bioluminescent tumor imaging following treatment with miR-10b inhibitor. U87-Fluc cells were implanted into nude mice and bioluminescent tumor images were taken at indicated time points (days: 7, 19, 27, and 37) following implantation. The images of typical control and anti-miR-10b treated mice are demonstrated. *B*, Glioma tumors treated with miR-10b inhibitor have increased TUNEL staining. **Tumors were injected with either control or miR-10b inhibitor for two days in a row. Two days later, mice were sacrificed, tumors removed, freshly**

frozen using tissue freezing medium and sectioned into 10 µm sections. Tumor sections were mounted on slides and analyzed using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) staining as described in the manufacturer's instructions (Promega). DNA/nuclei were also stained using DAPI (4',6-diamidino-2-phenylindole). Stained sections were evaluated using fluorescent microscopy.

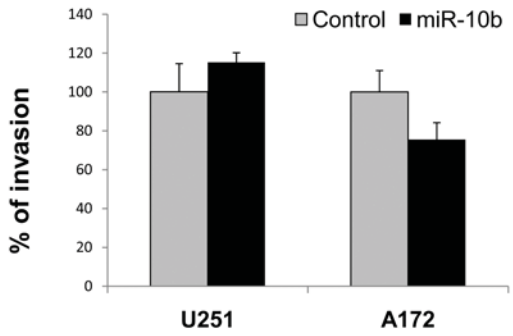
Supplementary Fig. S10. Expression of migration-related genes do not correlate with miR-10b expression in GBM patients. Significantly correlated genes ($p<0.0001$) were assessed for enrichment level of GO biological processes terms and the enrichment scores are presented in the form of heatmaps, showing the terms in rows. The gradient of red color shows the level of enrichment and black corresponds to no enrichment. Enrichment of all correlated (All), negatively correlated (Negative), and positively correlated (Positive) genes is demonstrated for two miR-10b probes (Probe A and Probe B) used on the TCGA arrays. Correlation for miR-21 and miR-10a is demonstrated for comparison.



Supplementary Figure S1.

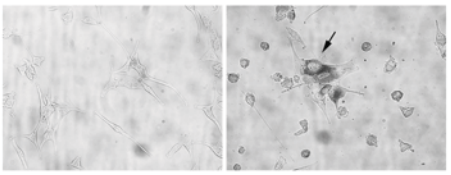


Supplementary Figure S2.

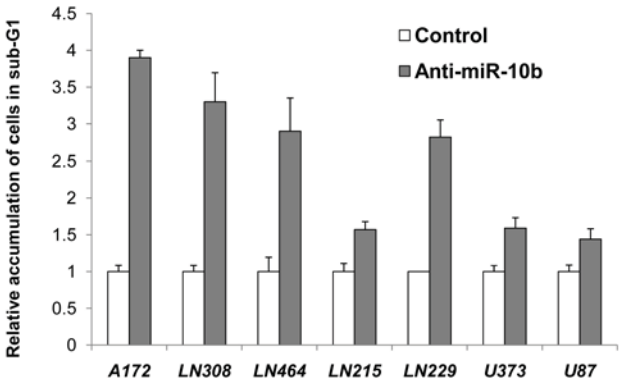


Supplementary Figure S3.

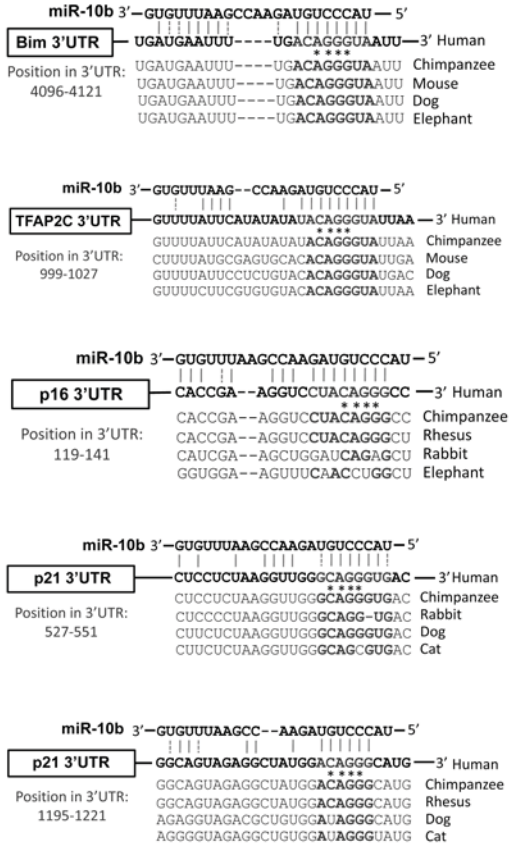
A.



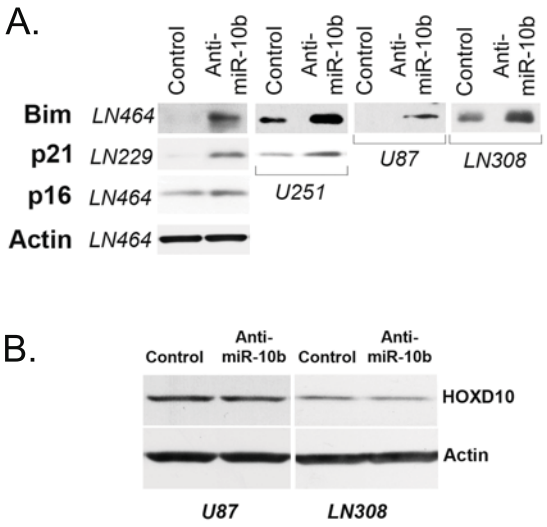
B.



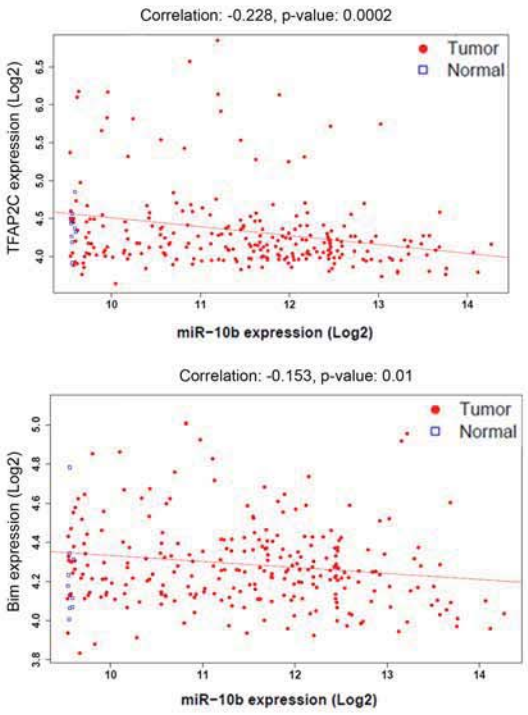
Supplementary Figure S4.



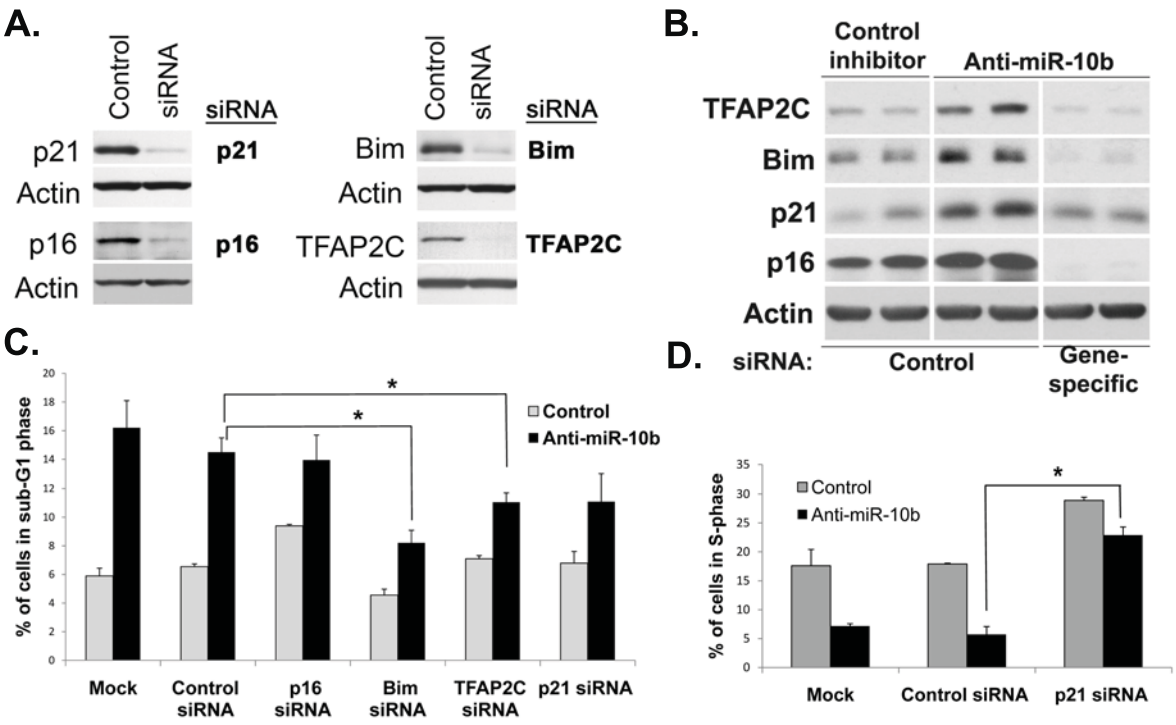
Supplementary Figure S5.



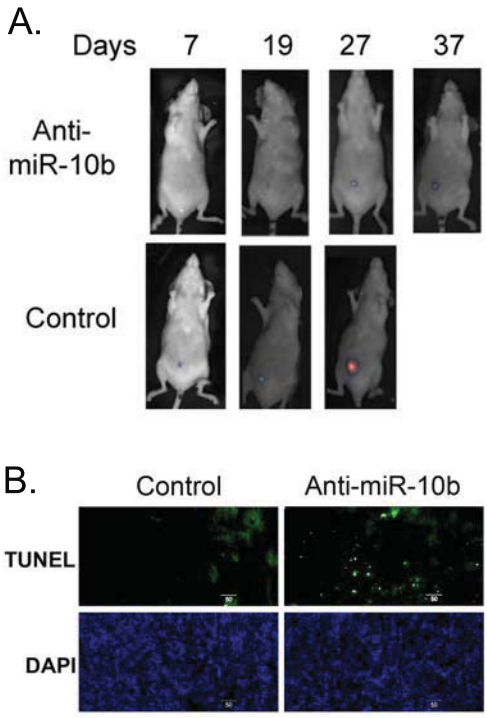
Supplementary Figure S6.



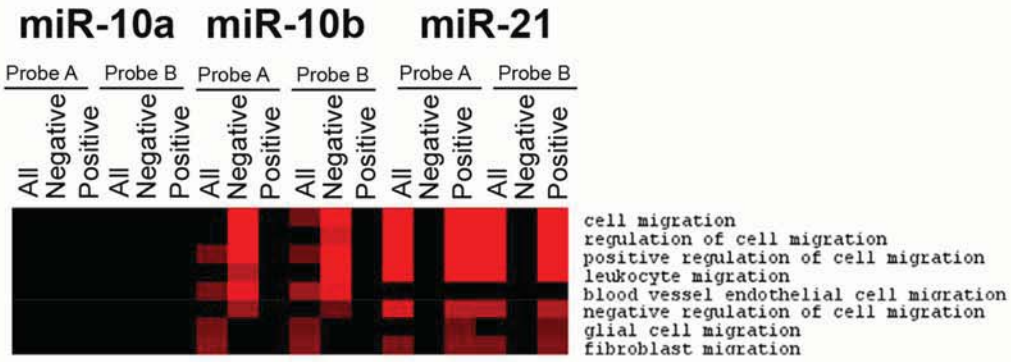
Supplementary Figure S7.



Supplementary Figure S8.



Supplementary Figure S9.



Supplementary Figure S10.

Supplementary Table S1. Oligonucleotide sequences

Primers used for cloning	
miR-10b sense	TCG AAT GAC CCA CAA ATT CGG TTC TAC AGG GTA CTC GAG CTG C
miR-10b anti-sense	GGC CGC AGC TCG AGT ACC CTG TAG AAC CGA ATT TGT GGG TCA T
p21 3'UTR Fv	ACT GCT CGA GAC AGG AAG CCT GCA GTC C
p21 3'UTR Rv	ACTAGCGGCCGCAGTCTAGGTGGAGAAACG
p16 3'UTR Fv	ACTGCTCGAGAGAACCAGAGAGGCTCTG
p16 3'UTR Rv	ACTAGCGGCCGCTTGAGCTTTGGTTCTGCC
Bim 3'UTR Fv	ACTGCTCGAGGTGTGATGTGTCCTACTG
Bim 3'UTR Rv	ACTAGCGGCCGCCAGCAGTATTGCACAAG
TFAP2C 3'UTR Fv	ACTGCTCGAGGTTAGGAGAGTAGGGAAG
TFAP2C 3'UTR Rv	ACTAGCGGCCGCTTACAGGCAGAGAGTTC
Primers used for mutagenesis	
p21 d-mut1 Fv	CTC CTC TAA GGT TGG GAC CAG TGA CCC TGA AGT GAG CAC AGC
p21 d-mut1 Rv	GCT GTG CTC ACT TCA GGG TCA CTG GTC CCA ACC TTA GAG GAG
p21 d-mut2 Fv	GCA GTA GAG GCT ATG GAG CCA GCA TGC CAC GTG GGC
p21 d-mut2 Rv	GCC CAC GTG GCA TGC TGG CTC CAT AGC CTC TAC TGC
TFAP2C mut Fv	CAT ATA TAT AGC CCG TAT TAA GAA TTA AGA GGA TGC TGG GCT CTG
TFAP2C mut Rv	CAG AGC CCA GCA TCC TCT TAA TTC TTA ATA CGG GCT ATA TAT ATG
Bim mut Fv	GCA CTG ATG AAT TTT GAC CTC CTA ATT GCC ACT TTA CTT GTG C
Bim mut Rv	GCA CAA GTA AAG TGG CAA TTA GGA GGT CAA AAT TCA TCA GTG C
ARF mut Fv	CAT CAG TCA CCG AAG GTC CTA GCC AGC CAC AAC TGC CC
ARF mut Rv	GGG CAG TTG TGG CTG GCT AGG ACC TTC GGT GAC TGA TG
Primers used for qRT-PCR	
GAPDH Fv	CCTGCACCACCAACTGCTTAG
GAPDH Rv	TGGCATGGACTGTGGTCATG
Actin Fv	CGAGAAGATGACCCAGATCTG
Actin Rv	CGTCACCGGAGTCCATCAC
Bim Fv	CAG TTT CCC TGG CTT ACT TGT GTT
Bim Rv	GTA TTG CAC AAG TAA AGT GGC AAT TAC
TFAP2C Fv	GAA ACA GTG CGT TGA GTG TAC AGA T
TFAP2C Rv	AAC AGA GCC CAG CAT CCT CTT A
p21 Fv	TCC TCA TCC CGT GTT CTC CTT
p21 Rv	AGG AGG AAG TAG CTG GCA TGA A
p16 Fv	GCC CAA CGC ACC GAA TAG
p16 Rv	CGC TGC CCA TCA TCA TGA
Oligonucleotide sequences for miRNA expression modulation	
Control 2'-O-MOE ASO PO	ACATACTCCTTTCTCAGAGTCCA
miR-10a 2'-O-MOE ASO PO	CACAAATTCGGATCTACAGGGTA
miR-10b 2'-O-MOE ASO PO	CACAAATTCGGTTCTACAGGGTA
Control 2'-O-MOE ASO PS	ACATACTCCTTTCTCAGAGTCCA
miR-10b 2'-O-MOE ASO PS	ACAAATTCGGTTCTACAGGGTA
Pre-miR Negative Control #2 (Applied Biosystems)	CCGAAUCGUAAGCAGUACAtt
Pre-miR has-miR-10a (Applied Biosystems)	UACCCUGUAGAUCCGAAUUUGUG
Pre-miR has-miR-10b (Applied Biosystems)	UACCCUGUAGAACCGAAUUUGUG
siRNA sequences	
Control siRNA	Dharmacon proprietary information Catalog Number: D-001220-01

Bim siRNA	GACCGAGAAGGUAGACAAUUGDdTdT
p21 siRNA	CUUCGACUUUGUCACCGAGdTdT
TFAP2C siRNA	AAGGUCCCAUUUCCAUGACCAUU
p16 siRNA	CGCACCGAAUAGUUACGGUdTdT