Supporting Information

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SI Materials and Methods

NanoString nCounter Assay. The NanoString nCounter Human miRNA Expression Assay Kit (http://www.nanostring.com) was used to profile more than 700 human and human–viral microRNAs (miRNAs) in U87 cells treated with Nutlin-3a (10 μM) and DMSO; 100 ng total RNA were used as input for nCounter miRNA sample preparation reactions. All sample preparation was performed according to the manufacturer's instructions (NanoString Technologies). Preparation of small RNA samples involves the ligation of a specific DNA tag onto the 3′ end of each mature miRNA. These tags are designed to normalize the Tms (melting temperatures) of the miRNAs as well as provide a unique identification for each miRNA species in the samples. The tagging is accomplished in a multiplexed ligation reaction using reverse complementary bridge oligonucleotides to direct the ligation of each miRNA to its designated tag. After the ligation reaction, excess tags and bridges are removed, and the resulting material is hybridized with a panel of miRNA tag-specific nCounter capture and barcoded reporter probes. Hybridization reactions were performed according to the manufacturer's instructions with 5 μL fivefold diluted sample preparation reaction. All hybridization reactions were incubated at 64 °C for a minimum of 18 h. Hybridized probes were purified using the nCounter Prep Station (NanoString Technologies) following the manufacturer's instructions to remove excess capture and reporter probes and immobilize transcript-specific ternary complexes on a sterptavidin-coated cartridge. Date collection was carried out on the nCounter Digital Analyzer (NanoString Technologies) following the manufacturer's instructions to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. For each assay, a high density (600 fields of view) was performed.

Western Blot Analysis. Samples were extracted in 15 mM Tris·Cl, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 0.1 mM DTT, 0.5% Triton X-100, 10 mg/mL leupeptin, and 0.5 mM PMSF. Total protein (50 μg) from each sample was separated on a 4–20% Tris·HCl Criterion precast gel (catalog number 345–0032; Bio-Rad) and transferred to a poly (vinylidene difluoride) filter (Millipore). The filter was blocked in 5% nonfat dry milk, incubated with the specific antibody, washed, probed with secondary antibody IgG conjugated to HRP (Santa Cruz Biotechnology), and developed with enhanced chemiluminescence (Amersham Pharmacia). Immunoblot analyses were performed using the following antibodies: TSC1 (LS-C36590; LifeSpan Bioscience), p53 (sc-53394; Santa Cruz Biotechnology), Mdm2 (sc-965; Santa Cruz Biotechnology), phospho-Mdm2 (catalog number 3521; Cell Signaling), c-MYC (1472-1; Epitomics), E2F1 (3240-1; Epitomics), total Akt (catalog number 9272; Cell Signaling), phospho-Akt (catalog number 4060; Cell Signaling), total-S6 (catalog number 2217; Cell Signaling), phospho-S6 (catalog number 2211; Cell Signaling), p21 (sc-817; Santa Cruz Biotechnology), and GAPDH (catalog number 2118; Cell Signaling). In knockdown gene expression, a commercial pool (Santa Cruz) of three target-specific 20- to 25-nt siRNAs was used.

RT-PCR. RNA was isolated from cell lines using TRIzol reagent (Invitrogen) as per the manufacturer's protocol. An aliquot of 5 μg RNA was then used for cDNA synthesis using the SuperScript first-strand cDNA synthesis kit (Invitrogen). RT-PCRs were carried out using ABI Prism 7900HT sequence detection systems with Applied Biosystems TaqMan gene expression assays [p21 (CDKN1A): Hs01121172_m1; MYC: Hs99999003_m1; E2F1: Hs00153451_m1; TP53: Hs00153349_m1; MDM2: Hs01066938_m1; TSC1: Hs01060648_m1].

RNA Extraction. Total RNA was extracted using TRIzol Reagent (catalog number 15596–018; Invitrogen) following the manufacturer's instruction. Specifically, the pellet obtained from 5×10^6 cells was lysed with 1 mL TRIzol solution. At the end of the extraction, the isolated RNA was dissolved in 35 μL RNase-free water and incubated for 10 min at 55 °C.

ChIP Assay. ChIP was performed as described in the work by de Belle et al. (1) with slight modifications. Cells (5×10^6) from U87 cells treated with MYC siRNA were fixed in 1% formaldehyde for 10 min at 37 °C for chromatin cross-link. Cells were washed with ice-cold 1× PBS, scraped in 1× PBS plus protease inhibitors, and collected by centrifugation. Cell pellets were resuspended in cell lysis buffer (50 mmol/L Tris·HCl, pH 8.0, 10 mmol/L EDTA, 1% SDS) plus protease inhibitors. The probes were sonicated 25× for 30 s with a Bioruptor sonicator (Diagenode) and pelleted. The supernatant was diluted with dilution buffer [17 mmol/L Tris, pH 8.0, 167 mmol/L NaCl, 1.2 mmol/L EDTA, 1.1% (vol/vol) Triton X-100, 0.01% (wt/vol) SDS]. DNA—protein complexes were immunoprecipitated using 5 μg anti-Myc antibody (Santa Cruz) or mouse 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 primers are listed in *SI Materials and Methods*, *Primers List*.

In Situ Hybridization and Immunohistochemistry. In situ hybridization (ISH) was carried out on deparaffinized human glioblastoma tissues, which includes a digestion in pepsin (1.3 mg/mL) for 30 min. The sequences of the probes containing the six dispersed locked nucleic acid-modified bases with digoxigenin conjugated to the 5′ end were miR-*25*-(5′) cattgcacttgtctcggtctga and miR-*32*-(5′) tattgcacattactaagttgca. The probe mixture and tissue miRNA were codenatured at 60 °C for 5 min followed by hybridization at 37 °C overnight and a low stringency wash in 0.2× SSC and 2% (wt/vol) BSA at 4 °C for 10 min. The probe–target complex was seen because of the action of alkaline phosphatase on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate. Negative controls included the use of a probe that should yield a negative result in such tissues. No counterstain was used, to facilitate colabeling for TSC1 and p53 proteins. After ISH for the miRNAs, the slides were analyzed for immunohistochemistry using the optimal conditions for TSC1 (1:800; cell conditioning for 30 min) and p53 (1:20; cell conditioning for 30 min). For the immunohistochemistry, we used the Ultrasensitive Universal Fast Red System from Ventana Medical Systems. In immunohistochemistry and ISH assay, the signal (+) indicates that at least 10% cancer cells with signal required for positive results are present in tissue.

Luciferase Reporter Vector. The 3′ UTRs of the human TSC1 and Mdm2 genes were PCR-amplified (primers are listed in *SI Materials and Methods*, *Primers List*). They were then cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega), giving rise to the p3′ UTR-TSC1 and p3′ UTR-MDM2 plasmids. These constructs were used to generate (by inverse PCR) the p3′ UTRmut-TSC1 and -Mdm2 plasmids (primers are listed in *SI Materials and Methods*, *Primers List*). U87 cells were cotransfected with 1 μg p3′ UTR-TSC1 or p3′ UTR-

Mdm2, p3′ UTRmut-TSC1 or p3′ UTRmut-Mdm2 plasmids, and 0.1 μg Renilla luciferase expression construct, pRL-TK (Promega), using Lipofetamine 2000 (Invitrogen). Cells were harvested 24 h after transfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

In case of promoter assay, miR-32 cluster promoters were amplified by PCR from genomic DNA (293T/17 cells; primers are listed in *SI Materials and Methods*, *Primers List*) and cloned into pGL3 basic vector (Invitrogen) using NheI-XhoI sites.

Statistical Analysis. Student *t* test and one-way ANOVA were used to determine significance. All error bars represent the SEM. Statistical significance for all of the tests, assessed by calculating the *P* value, was <0.05. Spearman correlation coefficient was calculated to test the association between miR-25 or -32 and MDM2 mRNA in glioblastoma samples ($n = 31$). The Kruskal–Wallis test was used to assess whether the two miRNAs are differentially expressed among normal brain and glioblastoma samples on the basis of the Bartlett test *P* value. The Kruskal–Wallis test was used for Bartlett test *P* values <0.0068 (miR-25) and <0.001 (miR-32). The in vivo anticancer effect of miRNA treatments with mice was assessed by plotting survival curves according to the Kaplan–Meier method, and groups were compared using the log rank test.

Glioblastoma Samples and Cell Lines. Human U87, U87 (Δ epidermal growth factor receptor), U251, and LNZTA3WT4 cell lines were grown in DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin. The LNZTA3WT4 cell line was derived from the glioblastoma cell line LN-Z308 that has no endogenous p53 gene expression because of an internal rearrangement of the endogenous p53 gene. This cell line contains a p53 encoding sequence that is under the control of tetracycline, and p53 is produced in the absence of the antibiotic and not produces in its presence. A total of 4 snap-frozen normal and 57 glioblastoma tissues were collected at San Filippo Neri Hospital in Italy. The human tissues were obtained and studied in strict adherence to the San Filippo Neri Hospital's protocol.

Cell Cycle Analysis. The cells were transfected with each miRNA (scrambled or miR-25 or -32) and then incubated for 48 h. For DNA content analysis, cells were fixed in methanol at −20 °C, washed again, rehydrated, resuspended in PBS containing 50 g/mL propidium iodide and 50 g/mL RNase A, and analyzed by flow cytometry (Becton Dickinson). Continuous variables were expressed as mean values ± SD.

1. de Belle I, Mercola D, Adamson ED (2000) Method for cloning in vivo targets of the Egr-1 transcription factor. *Biotechniques* 29:162–169.

Primers List. ***List of primers used for promoter assay of pre–miR-32.*** The numbers indicate promoter regions of pre-miR-32 in Fig. 2*E*.

For (−2,584): 5′-attgctagccctctgtcctacagccaagct-3′
For (−1,926): 5′-attgctagcgcatgctcctaaaatacaatgt-3′
For (−1,276): 5′-attgctagctttctcttgttctgctttctta-3′
For (−1,110): 5′-attgctagcagctgattgaaagcacatactc-3′
For (−812): 5′-attgctagcttttattatcaccttattttaa-3′
For (−626): 5′-attgctagcaggtgtttttttttgcagtttaag-3′
For (−326): 5′-attgctagcatatcagtgataccaactttta-3′
For (−83): 5′-attgctagctgattctgaataagtattcttct-3′
Rev (−5): 5′-attctcgagtaaaggatgcatgccactgactc-3′
Rev (−837): 5′-attgctagctgccagtatactatggaaccaag-3′

List of primers used for ChIP assay. Pre–miR-32 promoter.

For: 5′- caccatgatgccttctttca-3′
Rev: 5′-tgccagtatactatggaaccaaga-3′

CAD (positive control).

For: 5′-ccagttcccattggtgttgttgcc-3′
Rev: 5′-gagaggcgcatcacagagtgggataa-3′

List of primers used for luciferase assay of 3′ UTR. 3′ UTR of TSC1.

For: 5′-aattctagaggaatgatggtcaatcagtgtt-3′
Rev: 5′-acttctagacaagtgcttctcgggtaacct-3′

3′ UTR of Mdm2 (2137).

For: 5-aattctagaaccttcttggcctgggttac-3′
Rev: 5′-acttctagacttttcttccagctgcgttc-3′

3′ UTR of Mdm2 (4917).

For: 5′-aattctagagagcctccaatgagagcaac-3′
Rev: 5′-acttctagatcagcatccacccataaagc-3′

List of primers used for luciferase assay of 3′ UTR mutation. 3′ UTR of TSC1 mutation.

For: 5′-atggttgcgtcctttgaacctgaggccaaatttaatctttg-3′
Rev: 5′-caaagattaaatttggcctcaggttcaaaggacgcaaccat-3′

3′ UTR of Mdm2 mutation (2137).

For: 5′- tcacaaaaactttaaaagaatctcaaaaggttaggtgga-3′
Rev: 5′-tccacctaacctttgagattcttttaaagtttttgtga-3′

3′ UTR of Mdm2 mutation (4917).

For: 5′- ctcaggctgagtacagtgcttggctcactgcaacctct-3′
Rev: 5′-agaggttgcagtgccaagcactgtactcagcctgag-3′

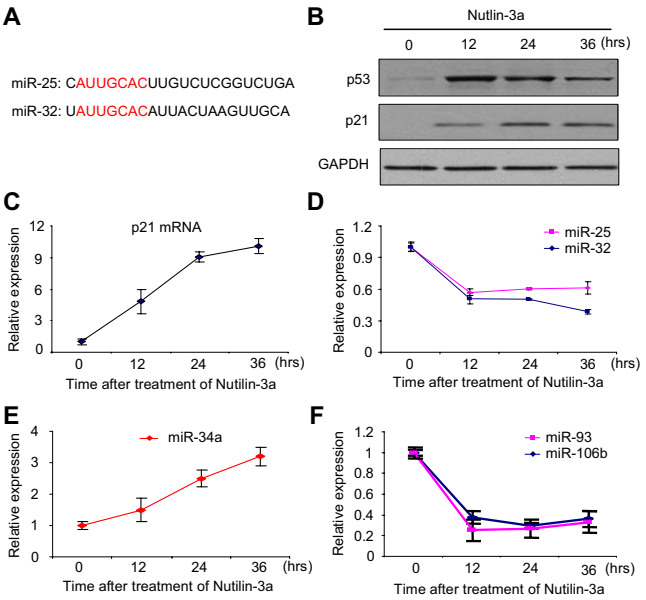

Fig. S1. miR-25 and -32 are repressed by p53. (*A*) Sequence homology between miR-25 and -32. Red color indicates the seed sequence of each miRNA. (*B*) p53 and p21 protein levels in response to p53 induction after the treatment of Nutlin-3a in U87 cells. (*C*) p21 mRNA levels were determined by quantitative RT-PCR (qRT-PCR) in p53-induced U87 cells. (*D–F*) miR-25 and -32, miR-34a, or miR-93 and -106b expression levels in p53-induced U87 cells were measured by qRT-PCR. We performed three biological experiments in triplicate.

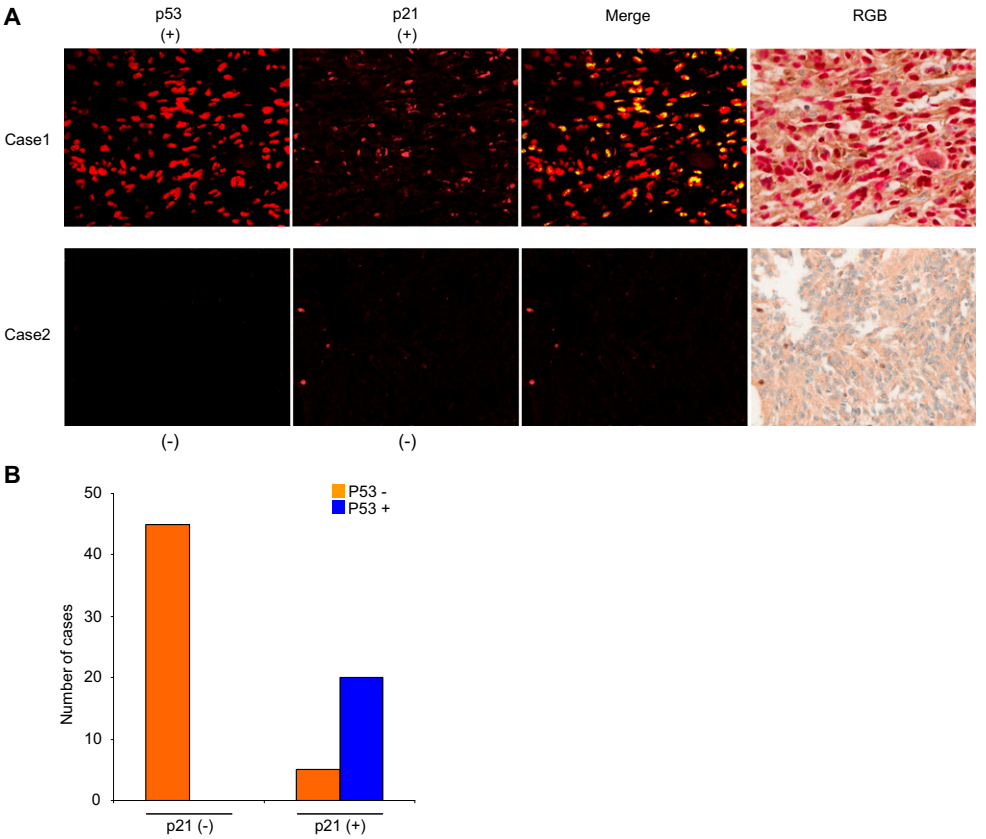

Fig. S2. p53 and p21 are coexpressed in glioblastoma multiforme. (*A*) Representative cases from 70 glioblastoma specimens in tissue microarrays were analyzed by immunohistochemical staining (red, p52; brown, p21; yellow, coexpression). (Scale bar: 20 μm.) (*B*) Graphs summarizing χ^2 analysis of immunohistochemical staining results.

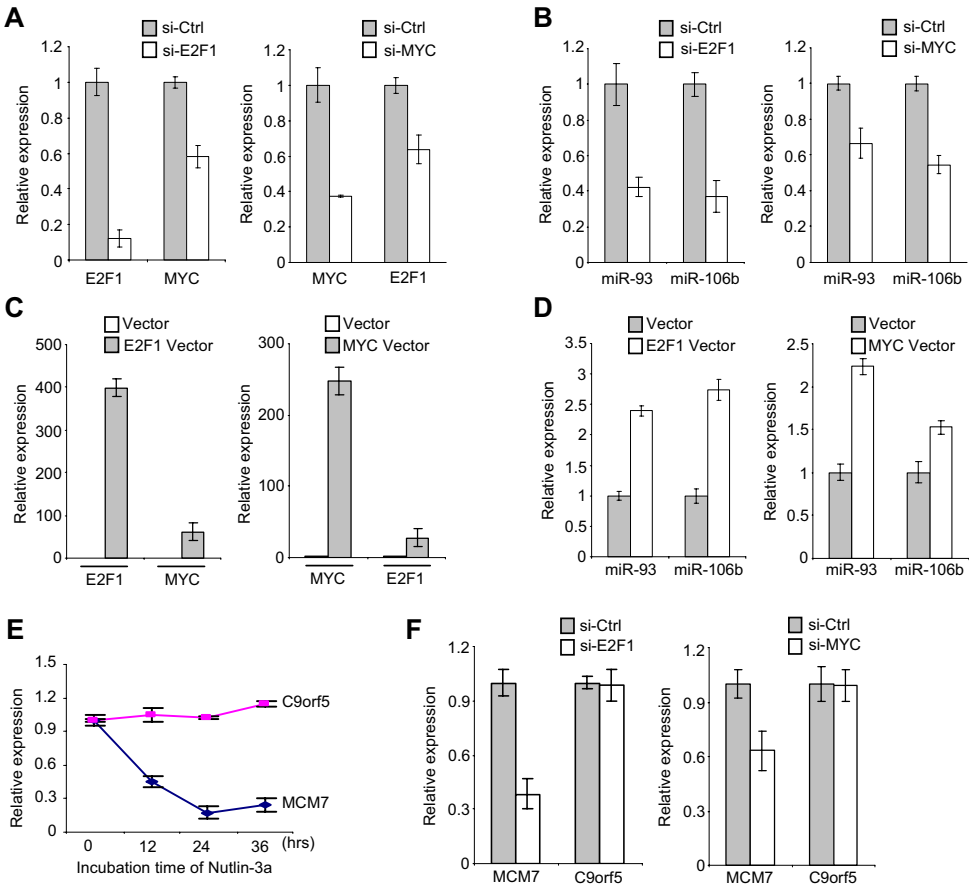

Fig. S3. E2F1 and MYC are transcriptional regulators of miR-25 and -32. (*A*) E2F1 and MYC mRNA levels in response to knockdown of E2F1 or MYC were measured by qRT-PCR. (*B*) miR-93 and -106b expression levels in E2F1 or MYC-silenced cells (U87). (*C*) E2F1 and MYC mRNA levels in E2F1- or MYC-overexpressed cells (U87). (*D*) miR-93 and -106b expression levels in response to overexpression of E2F1 or MYC in U87 cells. (*E*) C9orf5 and MCM7 mRNA levels in p53-activated U87 cells were determined by qRT-PCR. (*F*) In E2F1- or MYC-silenced cells, U87, MCM7, and C9orf5 mRNA levels were measured by qRT-PCR. (*A–F*) Data are presented as mean ± SD. We performed three biological experiments in triplicate.

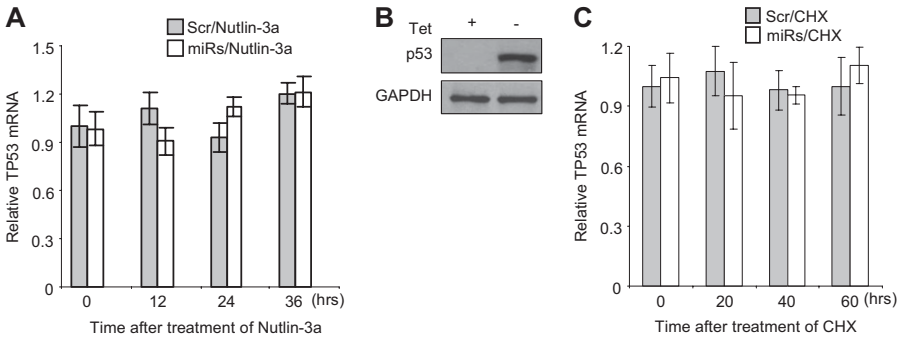

Fig. S4. miR-25 and -32 stabilize p53 protein to induce cell cycle arrest and inhibit cell proliferation. (*A*) p53 mRNA levels in U87 cells transfected with miR-25 and -32 or scrambled oligonucleotides (100 nM) after treatment of Nutlin-3a. (*B*) p53 protein levels in p53-inducible cells, LNZTA3WT4, with or without tetracycline. (C) p53 mRNA levels in U87 cells transfected with miR-25 and -32 or scrambled (100 nM) after treatment of cycloheximide (CHX). Data are presented as mean ± SD. We performed three biological experiments in triplicate.

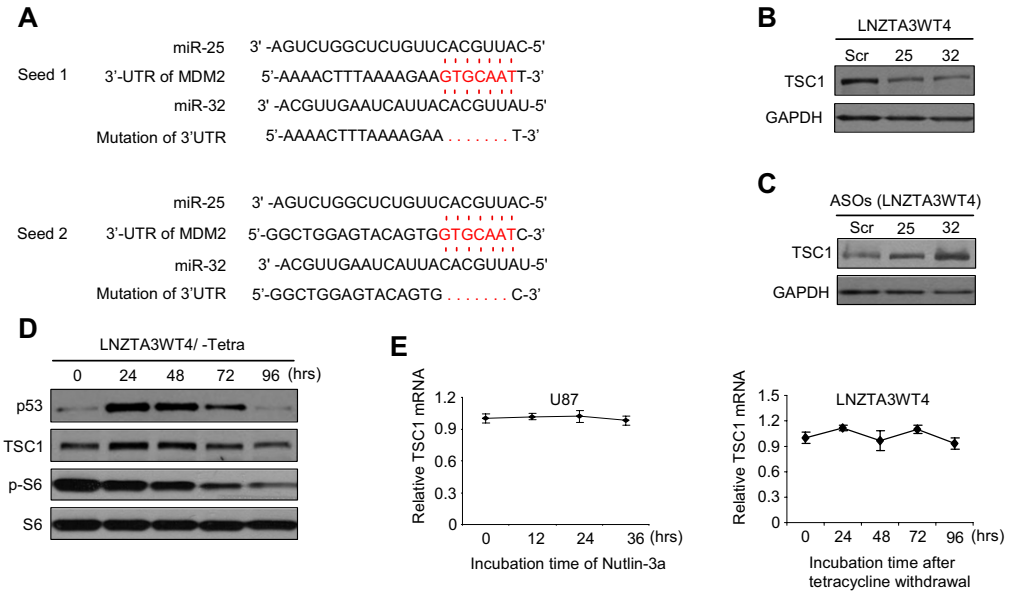

Fig. S5. miR-25 and -32 target Mdm2 and TSC1. (*A*) Mdm2 3′ UTR contains two predicted miR-25 and -32 binding sites. The sites of target mutagenesis are indicated in red. (*B* and *C*) TSC1 protein levels in LNZTA3WT4 cells transfected with pre–miR-25 and -32 or antioligonucleotides (ASOs). (*D*) The levels of TSC1 protein and mTOR activity (p-S6) were determined in p53-activated cells, LNZTA3WT4, by Western blot analysis. (*E*) TSC1 mRNA levels in p53-activated cells, U87 and LNZTA3WT4, were measured by qRT-PCR; Nutlin-3a (10 μM) treatment in U87 cells and tetracycline withdrawal in LNZTA3WT4 cells for indicated times are shown. Data are presented as mean ± SD. We performed three biological experiments in triplicate.

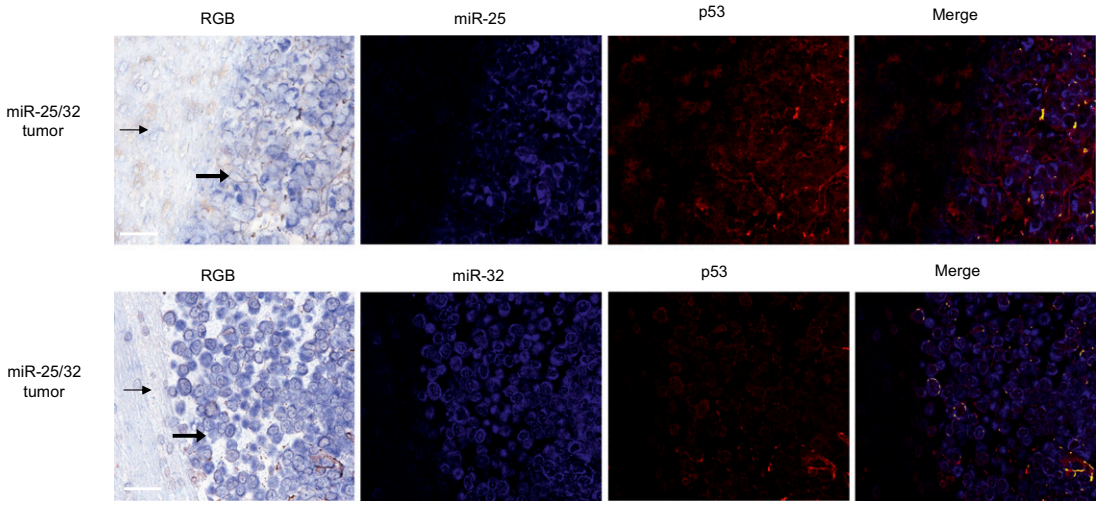

Fig. S6. Coexpression assay between p53 and miR-25 or -32 in miRNA-transfected tumors. The small arrow shows the normal mouse brain, and the large arrow shows the human brain cancer. (Scale bar: 20 μm.)

Table S1. miRNAs differentially expressed in Nutlin- vs. no Nutlin-treated cells

Detector	Mean + Nutlin	Mean − Nutlin	SD + Nutlin	SD − Nutlin	*P* value	Fold change
hsa-miR-1292	12.471392	19.150033	0.97233	0.95719	0.00106212	0.651247
hsa-miR-760	12.869514	21.675136	1.236815	1.51929	0.00172923	0.593745
hsa-miR-376c	118.24395	84.121762	5.481979	2.59294	0.0028263	1.405629
hsa-miR-582–5p	11.918647	14.607637	0.516777	0.50292	0.00296647	0.815919
hsa-miR-19a	58.742371	81.133032	1.518295	3.8368	0.00439659	0.724025
hsa-miR-20a	536.84426	969.58799	82.8604	117.676	0.00866299	0.553683
hsa-miR-489	6.9899673	10.087333	0.704852	0.85339	0.00912682	0.692945
hsa-miR-211	8.5796761	11.631986	0.824543	0.81539	0.0103496	0.737593
hsa-miR-106a	69.109866	101.77045	6.973513	9.52334	0.01077435	0.679076
hsa-miR-1979	81.7924	107.81187	7.617492	7.90809	0.01484198	0.758659
hsa-miR-210	52.075561	28.770635	8.209873	6.33523	0.01985723	1.810025
hsa-miR-136	68.281265	37.724137	8.257694	10.779	0.01989421	1.810015
hsa-miR-323–3p	14.220654	7.9563872	1.430242	2.21626	0.02015021	1.787325
hsa-miR-25	44.197682	98.103479	8.302841	18.6335	0.02333454	0.450521
hsa-miR-769–3p	5.6437573	11.452057	1.646571	2.16122	0.02343266	0.492816
hsa-miR-32	18.241074	27.490693	2.682329	3.6111	0.02691275	0.663536
hsa-miR-203	21.561205	31.264152	3.066789	3.85228	0.02909752	0.689646
hsa-miR-298	7.2306864	10.963624	1.511647	1.09814	0.0298465	0.659516
hsa-miR-24	917.50417	780.24385	51.10708	20.0265	0.03021795	1.17592
hsa-miR-483–5p	15.147752	10.475191	1.318186	2.02992	0.03621486	1.44606
hsa-miR-1321	10.567507	17.277498	2.329555	2.92601	0.0384247	0.611634
hsa-miR-218	22.876567	13.431465	2.532339	4.18094	0.03843153	1.703207
hsa-miR-320d	10.72213	5.7149827	2.245007	1.47647	0.0394909	1.876144
hsa-miR-635	22.088031	17.233315	1.262631	2.1741	0.0399814	1.281705
hsa-miR-34a	1,463.1898	1,001.5261	186.0068	46.492	0.04309231	1.46096
hsa-miR-125a-5p	960.36328	833.75647	59.03419	35.8969	0.04409294	1.151851
hsa-miR-624	10.381405	8.2842691	0.072968	0.81898	0.04626079	1.253147
hsa-miR-339–5p	9.3206729	16.754376	1.553843	3.42267	0.04651721	0.556313
hsa-miR-221	3,124.6042	2,350.7846	333.4859	99.914	0.04693757	1.329175
hsa-miR-1308	81.534362	61.217258	9.788702	7.27875	0.04927971	1.331885
has-miR-2277	15.207929	24.994851	3.095093	4.78886	0.04992844	0.608442

NanoString assay with U87 cells treated with 10 μM Nutlin-3a overnight (biological triplicate) and DMSO (biological triplicate) using the genes that vary the most between samples. miRNAs were sorted by *P* value of the univariate *t* test at the nominal 0.05 level of the univariate.

Table S2. Summary of immunohistochemistry and ISH assay in tissue microarrays

Tumor type	Grade	p53/p21 (case)	miR-25 and -32 (case)
GBM	4	–/– (45)	–/– (3), –/+ (1), +/+ (40), +/– (1)
GBM	4	–/+ (5)	–/– (1), –/+ (0), +/+ (4), +/– (0)
GBM	4	+/+ (20)	–/– (16), –/+ (0), +/+ (3), +/– (1)
GBM	4	+/– (0)	–/– (0), –/+ (0), +/+ (0), +/– (0)

Clinical specimens in tissue microarrays, including tumor type and grade, and results of immunohistochemistry of p53 and p21 and ISH of miR-25 and -32 are shown. GBM, glioblastoma multiforme.