Supplementary information for:

Mutant p53 gain-of-function induces epithelial-mesenchymal transition through modulation of the miR-130b-ZEB1 axis

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ONLINE SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Figure S1 shows that upregulation of mutant p53s induce expressions of metastatic markers.

Figure S2 shows that transient overexpression mutant p53 R248Q induces EMT phenotype.

Figure S3 shows that ZEB1 is a key downstream mediator in mutant p53 GOF-induced EMT.

Figure S4 shows that the p53 GOF mutants contribute to global repression of miRNA expression.

Figure S5 shows the suppressive effects of mutant p53s on miR-200c expression in HEC-50 cells

Figure S6 shows that p63 inhibition is not likely responsible for mutant p53 GOF-induced suppression of miR-130b.

Figure S7 shows that miR-130b interacts with ZEB1 mRNA.

Table S1 shows primers used for site-directed mutagenesis.

SUPPLEMENTARY METHODS

In vitro cell invasion assay

The cell line (2×10^4 cells) in 500 µl of serum-free medium was added to the upper chamber of a transwell plate. In the lower chamber, 750 µl of serum-free medium containing 15% FBS and 10 µg/ml bovine fibronectin (Invitrogen, Germany) were added. The cells were allowed to migrate through the intermediate membrane for 24 hours at 37°C. Membranes were then fixed with 10% neutral-buffered formalin and stained in 10% Giemsa solution. The cells attached to the lower side of the membrane were counted in ten high-powered (200×) fields under a microscope. Assays were performed in triplicate for each experiment, and each experiment was repeated three times.

MTT assay

The cell line (1×10^3 cells) was treated with Paclitaxel as indicated for 48 h. After this incubation, 10 µl of MTT solution (cell counting kit-8, Dojindo, Japan) was added into each well and the plates were incubated for additional 4 h at 37°C. The UV absorbance of each sample was then measured in a microplate reader at 450 nm. The experiment was performed in triplicate wells and repeated three times.

Sphere formation assay

Cells were plated in ultra-low attachment plates (Corning Inc., Corning, NY) at a density of 5,000 viable cells/ml and grown in serum-free Eagle's MEM medium (Sigma-Aldrich, UK) supplemented with N2 plus media supplement (Invitrogen, CA), 20 ng/ml epidermal growth factor (EGF), 20 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, CA), and 4 mg/ml heparin (Sigma-Aldrich, UK). The cells were grown for an additional 12 d after treatment and then the number of spheres was counted under a light microscope.

Quantitative real time RT-PCR of EMT and stemness markers

Total RNAs was reverse-transcribed using a Takara PrimeScript RT reagent kit (Takara, Kyoto, Japan). Quantitative real time PCR was performed with the Applied Biosystems 7300 real-time PCR system (Applied Biosystems) using the Takara SYBR Premix Ex Taq II (Takara, Kyoto, Japan). The primers used for *CD133*, *KLF4*, *NANOG*, *MDR-1*, *MRP-1*, *Osteopontin, MMP-2, MMP-9*, *ZEB1*, *BMI-1*, *Snail*, *E-cadherin,* and *GAPDH* were obtained from PrimerBank database (http://pga.mgh.harvard.edu/primerbank/).

Transient transfection

HEC-50 cells were transfected with a vector expressing mutant p53 R248Q or control vector using the Lipofectamine 2000 reagent (Invitrogen) and then harvested two days post-transfection for western blot, RNA extraction, or the cell invasion assay. Cells were transfected with *ZEB1* siRNA or control siRNA (50 nM, Ambion, TX) using siRNA transfection reagent (Santa Cruz, CA) to down-regulate ZEB1 levels in HEC-50 and HEC-1 cells. The cells were harvested 2 d after transfection.

RNA isolation and fluorescent labeling

Total RNA was extracted from cells using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. To prepare cellular miRNA, small RNA containing miRNA was isolated from total RNA using the RNeasy MinElute Cleanup Kit (QIAGEN Ontario, Canada). The isolated small RNA was subjected to direct labeling using the Platinum Bright 647 Infrared nucleic acid labeling kit (KREATECH, Amsterdam, Netherlands), purified from fluorescent-free substrates using KREApure columns (KREATECH), and subsequently used in hybridization.

Hybridization with DNA chips

Hybridization was carried out using Genopal-MICH07 DNA chips (Mitsubishi Rayon, Tokyo), in which 188 oligonucleotide DNA probes are adhered to a chip at 50° C. After hybridization, the DNA chips were washed with 2× SSC containing 0.2% SDS, and then hybridization signals were examined and analyzed using a DNA chip image analyzer according to the manufacturer's instructions (Mitsubishi Rayon). Chip analysis was repeated at least twice and hybridized signal intensities were analyzed as previously described. The DNA chip data were compared using KURABO custom analysis services (KURABO Industries, Osaka, Japan).

Enforced expression and knockdown of miR-130b

The precursor miR-130b, negative control precursor miRNA, anti-miR-130b, and control anti-miR (Ambion, TX) were transfected into EC cells using Lipofectamine 2000 (Invitrogen, CA) at a final concentration of 30 nM. The cells were then harvested 48 h post-transfection for analysis.

miRNA real time quantitative RT-PCR (*qRT-PCR)*

Total RNA containing small RNA was extracted from cell lines using the mirVana™ miRNA isolation kit (Ambion, TX). *qRT-PCR was* performed to quantify mature miRNA expression by NCode miRNA qRT–PCR analysis (Invitrogen, CA) according to the manufacturer's protocol. Forward primers used for qRT–PCRs were designed and synthesized by Invitrogen. GAPDH was used for normalization. Quantitative miRNA expression data were acquired and analyzed using an Applied Biosystems 7300 real-time PCR system (Applied Biosystems).

Site-directed mutagenesis

The 3′-UTR vector of *ZEB-1* containing an intact miR-130b recognition sequence was purchased from OriGene Technologies (Rockville, MD). A pGL3 construct containing *ZEB-1* 3′-UTR with point mutations in the seed sequence was constructed using a quick-change site-directed mutagenesis kit (Stratagene, CA). Mutations were created in the p53 binding site of pGL3-miR-130b with the quick-change site-directed mutagenesis kit (Stratagene, CA). The resulting construct was named Mutant pGL3-miR-130b. The sequences of the PCR primers used for site-directed mutagenesis are shown in the Supplementary Information (Supplementary Table S1).

Luciferase assay

Cells were seeded in 24-well plates at 5×10^4 cells per well the day before transfection. The luciferase reporter (100 ng) as well as miR-130b, control miRNA, anti-miR-130b, and control anti-miRNA (30 nM), and the pRL-CMV vector (10 ng) (Promega) were added to each well. Twelve hours after the transfection, the cells were treated with 5 μmol/l of Nutlin-3 (Sigma, MO) or DMSO for an additional 12 h and harvested for analysis using the dual-luciferase reporter assay system (Promega, WI).

Biotin-labeled miR-130b pull down assay

3′-Biotinylated miR-130b mimic (bio-miR-130b) and 3′-biotinylated control *Caenorhabditis elegans* miR-67 mimic (bio-cel-miR-67) were synthesized by Sigma-Aldrich. HEC-50 cells were transfected with bio-miR-130b or bio-cel-miR-67 mimic at a final concentration of 30 nM using Lipofectamine 2000 reagent (Invitrogen). Twenty-four hours later, whole-cell lysate was collected. Cell lysates were mixed with streptavidin-coupled Dynabeads (Invitrogen, Carlsbad, CA) and incubated at 4°C on rotator overnight. After the beads were washed thoroughly, the bead-bound RNA was isolated and subjected to RT followed by qPCR analysis. The mRNA levels were normalized to 5S rRNA**.** Fold enrichment of mRNAs by miR-130b over control cel-miR-67 was calculated.

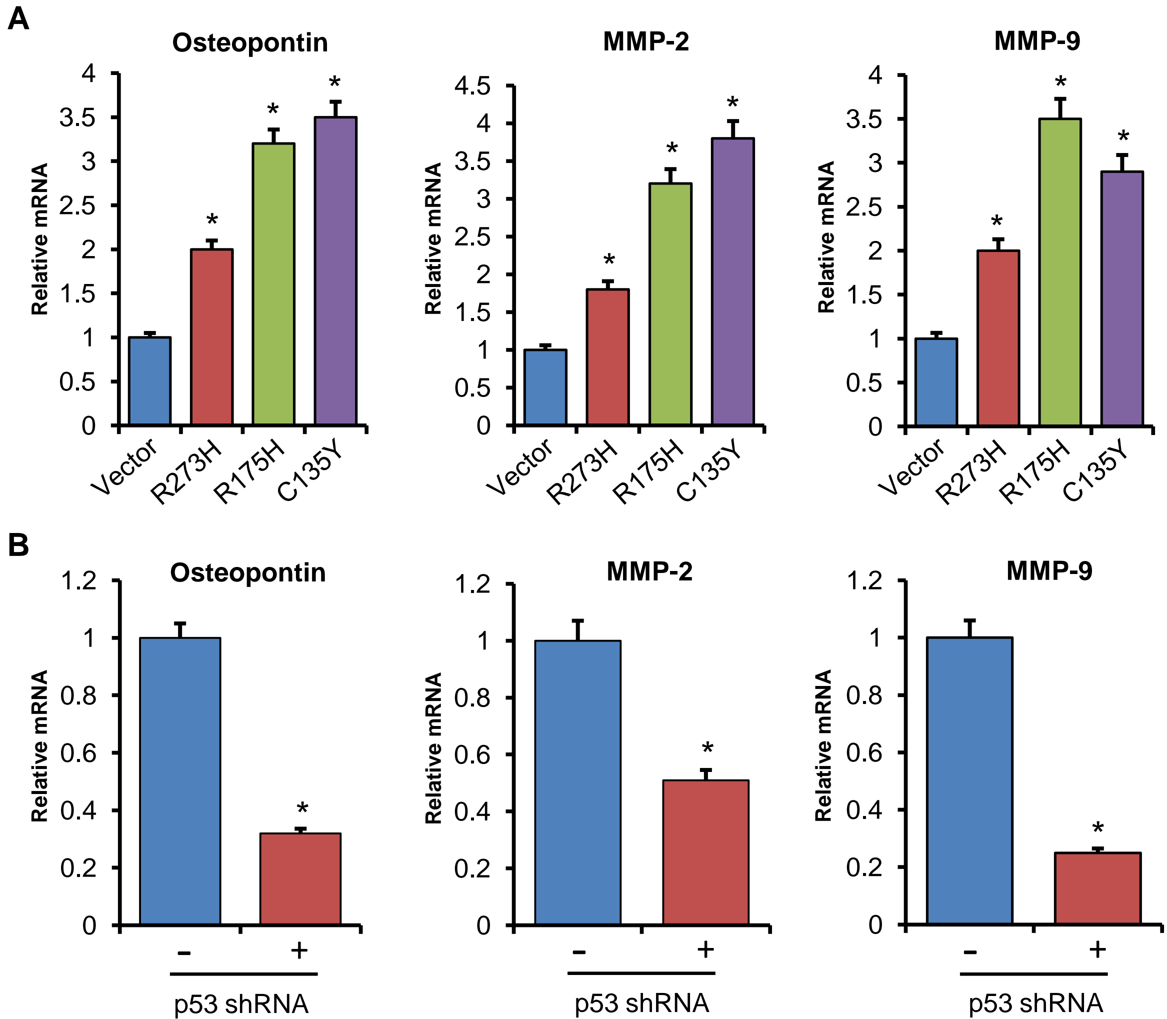

Figure S1. Upregulation of mutant p53s induce expressions of metastatic markers. Relative mRNA expression of metastatic markers (normalized to GAPDH) in HEC-50 cells transfected with control or mutant p53 vectors (**A**), and in HEC-1 cells transfected with control or p53 shRNA vectors (**B**) determined by *qRT-PCR* (mean ± s.d.; n = 3; $^{*}P < 0.01$).

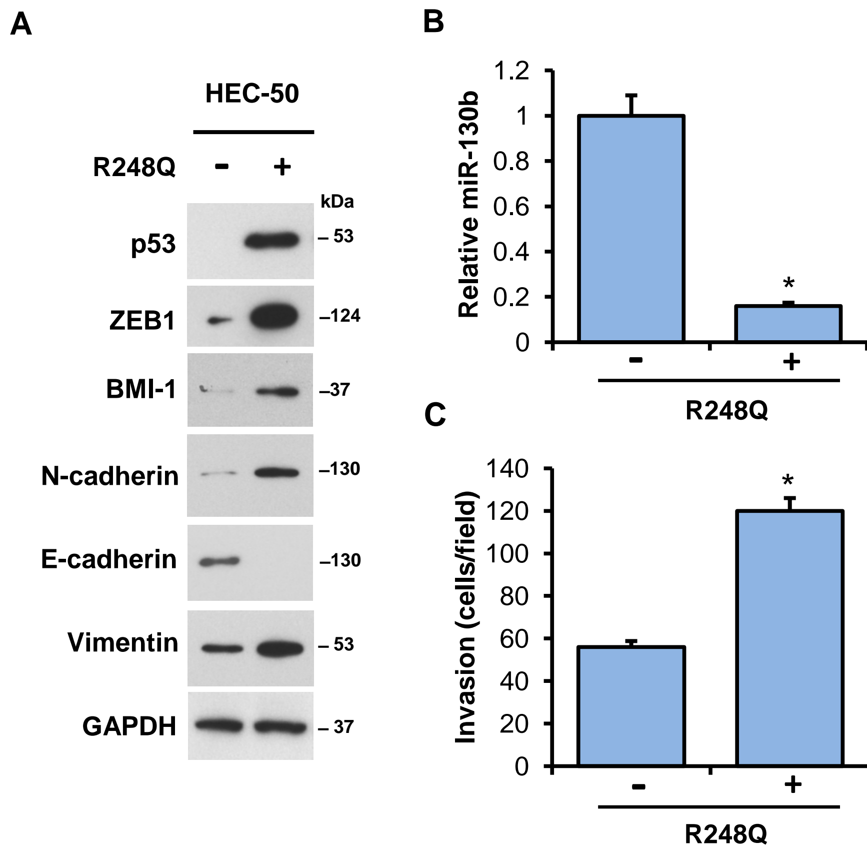

Figure S2. Transient overexpression mutant p53 R248Q induces EMT phenotype. (**A**) Western blot analysis for p53 and EMT markers in HEC-50 cells expressing mutant p53 R248Q or control vector. (**B, C**) qRT-PCR (**B**) and invasion assays (**C**) of HEC-50 cells after transfection of R248Q (mean ± s.d.; n = 3; $^{*}P < 0.01$).

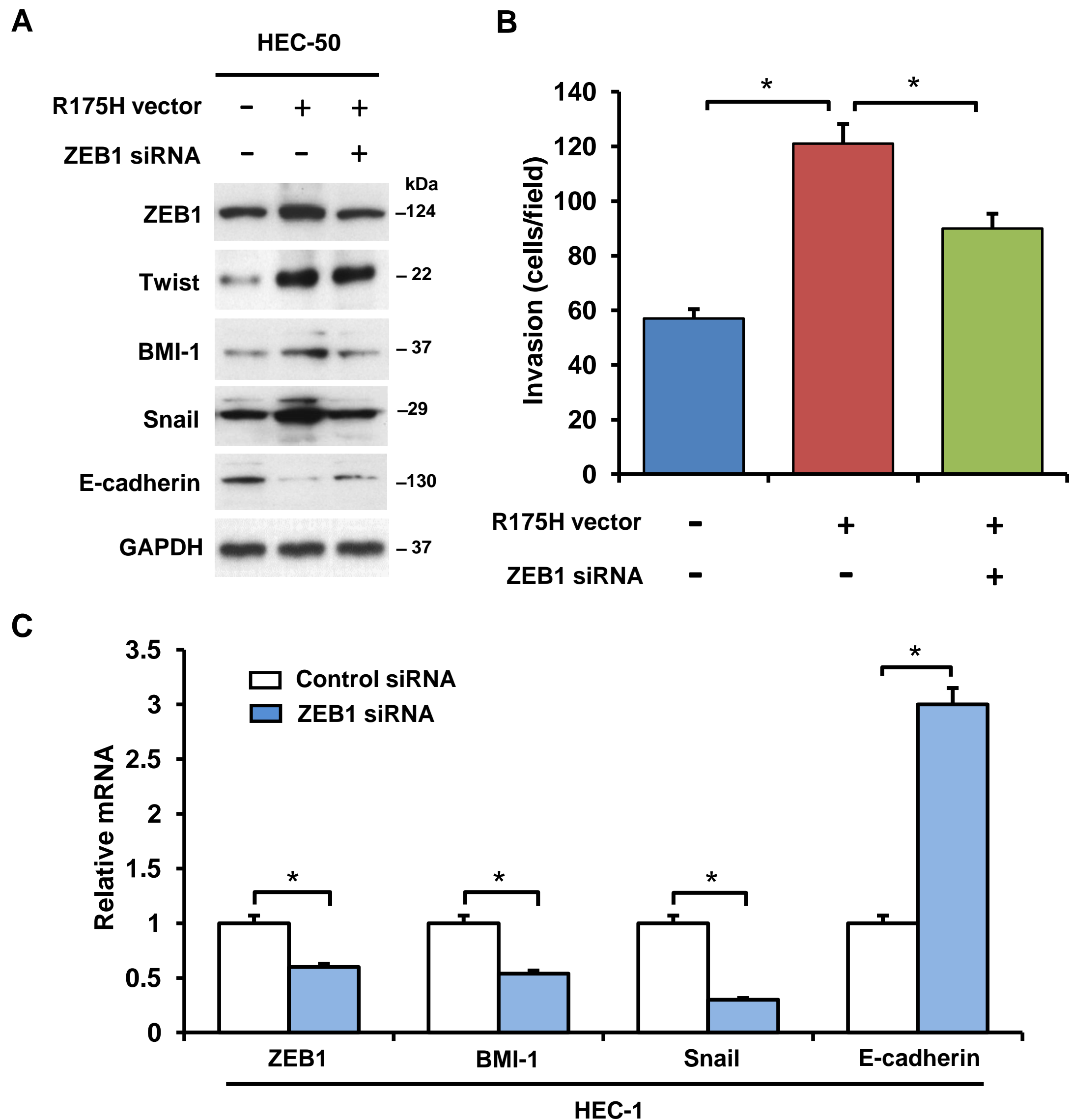

Figure S3. ZEB1 is a key downstream mediator in mutant p53 GOF-induced EMT. (**A, B**) Western blot analysis for EMT markers (**A**), and invasion assays (**B**) in HEC-50 cells expressing the indicated vectors and siRNA (mean ± s.d.; n = 3; $^{*}P < 0.01$). (**C**) qRT-PCR for EMT markers in HEC-1 cells transfected with ZEB1 siRNA or control siRNA (mean ± s.d.; n = 3; $^{*}P < 0.01$).

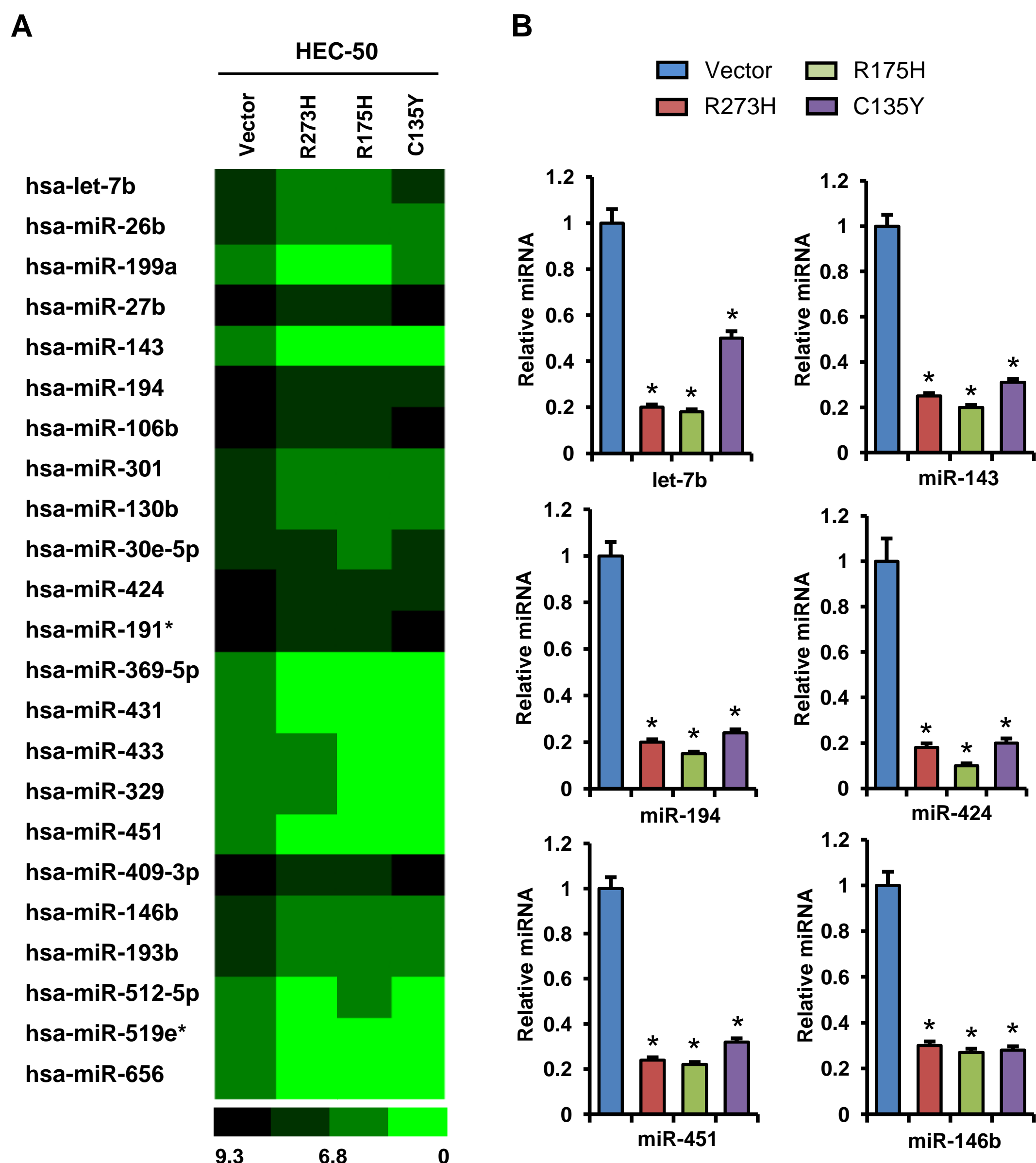

Figure S4. The p53 GOF mutants contribute to global repression of miRNA expression. (**A**) Heat map showing the expression of miRNAs that are downregulated in mutant p53s expressing HEC-50 cells when compared with control cells. The bar code on the bottom represents the color scale of the log 2 values. Dark green denotes high signal and light green denotes low signal. (**B**) Relative miRNA expression (normalized to GAPDH) in HEC-50 cells transfected with mutant p53 constructs or control construct, determined by *qRT-PCR* (mean ± s.d.; n = 3; $^{*}P < 0.01$).

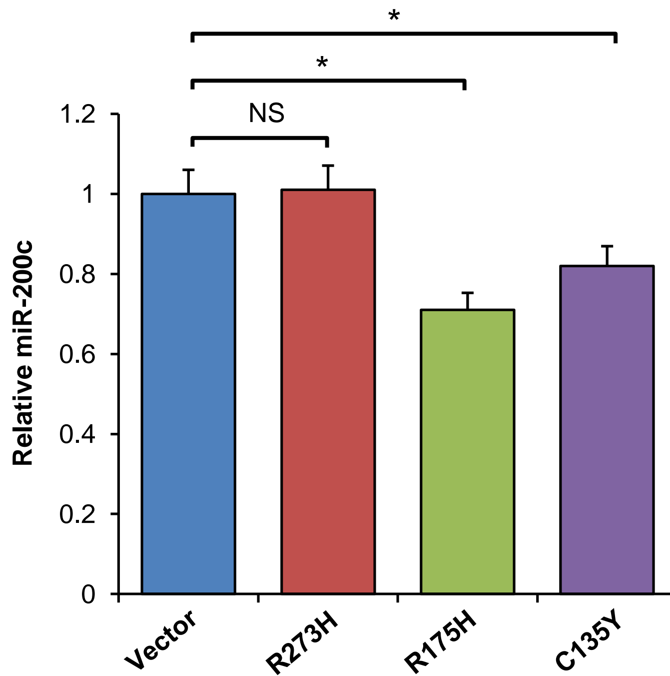

Figure S5. Overexpression of mutant p53 R175H and C135Y, but not R273H, decrease the expression of miR-200c in HEC-50 cells.

Relative miR-200c expression (normalized to GAPDH) in HEC-50 cells transfected with mutant p53 constructs or control construct, determined by qRT-PCR (mean ± s.d.; n = 3; NS: Not Significant; $^{*}P < 0.01$).

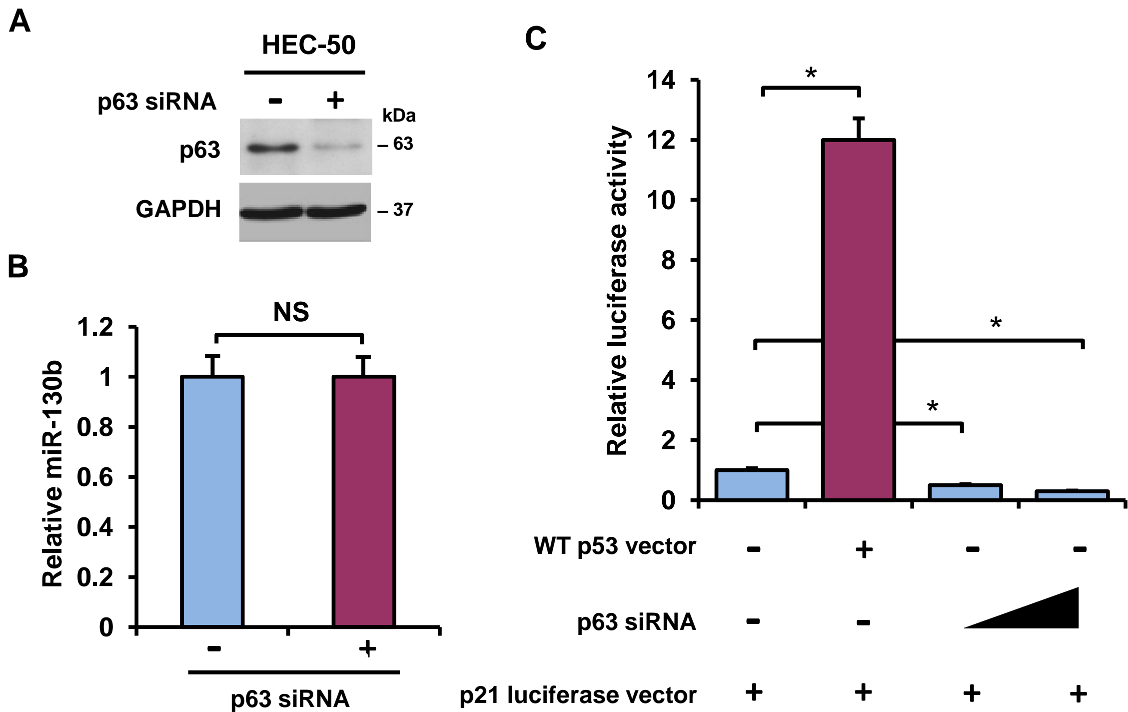

Figure S6. p63 inhibition is not likely responsible for mutant p53 GOF-induced suppression of miR-130b. (**A**) Western blot analysis for p63 in HEC-50 cells transfected with p63 siRNA or control siRNA. (**B**) qRT-PCR for miR-130b in HEC-50 cells transfected with p63 siRNA or control siRNA (mean ± s.d.; n = 4; NS: Not Significant). (**C**) Luciferase assay for p21 in HEC-50 cells transfected with the indicated vectors and siRNA (mean ± s.d.; n = 3; $^{*}P < 0.01$). All qPCR or luciferase values were normalized to GAPDH or Renilla activity, respectively.

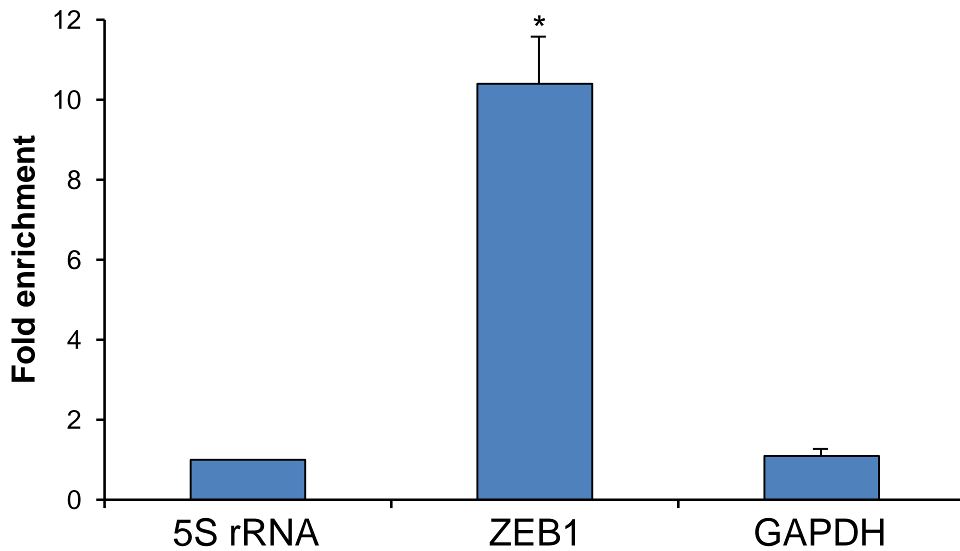

Figure S7. miR-130b interacts with ZEB1 mRNA. HEC-50 cells were transfected with bio-miR-130b or bio-cel-miR-67. The immunoprecipitated RNA was analyzed by qRT-PCRs normalized to 5S rRNA. Data represent fold changes of the mRNA levels in bio-miR-130b-transfected cells in comparison with bio-cel-miR-67-transfected cells. (mean ± s.d.; n = 3; $^{*}P < 0.01$).

Table S1. Primers used for site-directed mutagenesis

Primer Name	Sequence (5' – 3')	
ZEB1 miR-130b 3′-UTR mutagenesis	Forward	GAGAACTTCTGTCTTACAAAATTCCCTTCAC
	Reverse	GTGAAGGGAATTTTGTAAGACAGAAGTTCTC
Mutant pGL3-miR-130b 1	Forward	ACTACCTGAGGCTGGGTATAGTGGCTCATGCCTTT
	Reverse	AAAGGCATGAGCCACTATACCCAGCCTCAGGTAGT
Mutant pGL3-miR-130b 2	Forward	AGGCTGGGTATAGTGGCTTATACCTTTGGGAGGCAGAG
	Reverse	CTCTGCCTCCCAAAGGTATAAGCCACTATACCCAGCCT