Supporting Information

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SI Materials and Methods

RNA Isolation and miRNA Expression Analysis. Total RNA was isolated from SW620, IGR37, and SIHN-011B cells, before and after 5-aza-2′-deoxycytidine treatment, by TRIzol extraction (Invitrogen). miRNA microarray profiling was conducted as described (1). Briefly, 5 μg of total RNA was used for hybridization on a custom miRNA microarray platform containing quadruplicates of 389 human microRNA probes. These arrays contain gene-specific 40-mer oligonucleotide probes, spotted by contacting technologies and covalently attached to a polymeric matrix (1). The hybridized biotinylated transcripts were detected by streptavidin-Alexa Fluor 647 conjugation, scanned on an Axon 4000B microarray scanner, and analyzed by using GENEPIX PRO 6.0 (Axon Instruments). After background subtraction and normalization of average values of replicate spots of each miRNA, those that were differentially expressed in treated and nontreated cells were identified from significant *t* tests (Significance Analysis of Microarrays). Principal component analysis (PCA; Partek Genomic Suite) was performed to classify samples.

DNA Methylation Analyses. The miRNA sequences were analyzed by using miRBase (http://microrna.sanger.ac.uk/) and the University of California at Santa Cruz Human Genome Browser (http://genome.cse.ucsc.edu). The CpG Island Searcher Program (2) was used to determine which miRNAs were embedded in a CpG island, because it has been predicted that >90% of the human miRNA promoters are located 1,000 bp upstream of the mature miRNA (3). DNA methylation status was established by PCR analysis of bisulfite-modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated, cytosine to uracil. Two procedures were used. First, methylation status was analyzed by bisulfite genomic sequencing of both strands of the corresponding CpG islands. Eight independent clones were analyzed. The second analysis used methylation-specific PCR with primers specific for either the methylated or modified unmethylated DNA (Table S5).

Quantification of miRNAs with Real-Time PCR. TaqMan MicroRNA assays were used to quantify the level of mature miRNAs as described previously (4). Each reverse transcriptase (RT) reaction contained 6 ng of purified and DNase-treated (turbo DNA-free, Ambion) total RNA. Real-time PCRs included 2 μl of diluted RT product (1:15 dilution). Reactions were incubated in an Applied Biosystems 7900HT Fast Real-Time PCR system in 384-well plates. RNU19 was used to normalize the data. Total RNA was extracted from two independent experiments, and the real-time PCRs for each miRNA (10 μl) were performed in triplicate.

RACE. RACE was developed as previously described (5). We used the 5′ RACE System for Rapid Amplification of cDNA Ends Version 2.0 from Invitrogen according to the manufacturer's instruction. Briefly, 5 μg of total RNA from 5′-aza-2-deoxycytidine treated cells was reverse-transcribed into cDNA using SuperScript II RT reverse transcriptase and specific reverse primers (GSP1). cDNAs were amplified by PCR using Elongase Amplification System (Invitrogen) and other specific primers, first GSP2 and afterward GSP3, for nested amplification (Table S5). Specific PCR products were cloned into pGEMT vector (Promega), and, after transformation, multiple clones were sequenced.

Chromatin Immunoprecipitation Assay. Standard chromatin immunoprecipitation assays were developed as previously described (6). In brief, cells were treated with 1% formaldehyde for 15 min. Then, chromatin was sheared with a Bioruptor (Diagenode) to an average length of 0.4–0.8 kb for this analysis. The following antibodies were used: anti–trimethyl-K4 histone H3 (ab8580/ab1220; Abcam) and anti–acetyl H4 (06-598; Upstate Biotechnologies). PCR amplification was developed in 20 μl with specific primers for each of the analyzed promoters. Primers used are described in Table S5.

Mouse Xenograft and Metastasis Models. Four- to 5-week-old male athymic *nu/nu* mice (Charles River) were used in this study. Mice were anesthetized, and tumor cells were s.c. injected. A total of 3×10^6 cells of the c-shRNA-SIHN-011B cell line or those stably transfected with miR-148a or miR-34b/c were injected s.c. in both flanks of each animal ($n = 12$ for paired control/miR-148a, and $n = 10$ for control/miR-34b/c), respectively. Mice were weighed, and tumor width and length were measured every 5 days. Mice were killed 30 days after injection, and tumors from both groups were excised and weighed. The mean volume or tumor mass ± SEM were calculated. For experimental metastasis assays, 1×10^6 of c-shRNA control or stably miR-148a or miR-34b/c transfected cells were injected intravenously via the lateral tail vein ($n = 10$, 4- to 5-week-old male athymic nude mice per group). Mice were killed 40 days after injection, and the presence of macroscopic lung metastases was analyzed by H&E tissue staining.

Expression Analyses of miRNA Target Genes by Western Blot and Immunohistochemistry. Information of base-pairing comparison among miR-34b, miR-34c, and miR-148a and their targets sites in the 3′ UTR of c-MYC, E2F3, CDK6, and TGIF2 mRNA is available at Targetscan (www.targetscan.org/). Western blot was done as previously described (6). The membranes were immunoprobed with antibodies against C-MYC (1:500; Santa Cruz Biotechnology), E2F3 (1:1,000; Abcam), CDK6 (1:1,000; Cell Signaling), and TGIF2 (1:500; Abcam). An antibody against β-actin (1:5,000; Sigma) or nucleolin (1:1,000; Santa Cruz Biotechnology) was used as a loading control. C-MYC and CDK6 were immunohistochemically stained with a 1:1,500 dilution. Tissue microarrays were read and scored by a pathologist who had no knowledge of the clinical features.

Luciferase Reporter Assay. Standard luciferase reporter assays were developed as previously described (6). Luciferase constructs were made by ligating oligonucleotides containing the wild-type or mutant putative target site of the C-MYC, E2F3, CDK6, and TGIF2 3′ UTR into the multicloning site of the p-MIR Reporter Luciferase vector (Ambion). Cells were cotransfected in 24-well plates using Lipofectamine 2000 (Invitrogen) with 0.4 μg of firefly luciferase reporter vector containing the wild-type or mutant oligonucleotides, 0.04 μg of control vector (pRL-TK vector; Promega) containing *Renilla* luciferase, and 100 ng of miR-34b, miR-34c, or miR-148a precursor (Ambion). Firefly and *Renilla* luciferase activities were measured consecutively 48 h after transfection using *Renilla* for normalization in dual-luciferase assays (Promega). The experiments were performed in quadruplicate in three independent experiments. The mean luciferase levels ± SEM were calculated for each group.

1. Liu CG, *et al*. (2004) An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci USA* 101:9740–9744.
2. Takai D, Jones PA (2003) The CpG island searcher: A new WWW resource. *In Silico Biol* 3:235–240.
3. Zhou X, Ruan J, Wang G, Zhang W (2007) Characterization and identification of microRNA core promoters in four model species. *PLoS Comput Biol* 3:e37.
4. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM (2005) Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *RNA* 11:1737–1744.
5. Cai X, Hagedorn CH, Cullen BR (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA* 10:1957–1966.
6. Lujambio A, *et al*. (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res* 67:1424–1429.

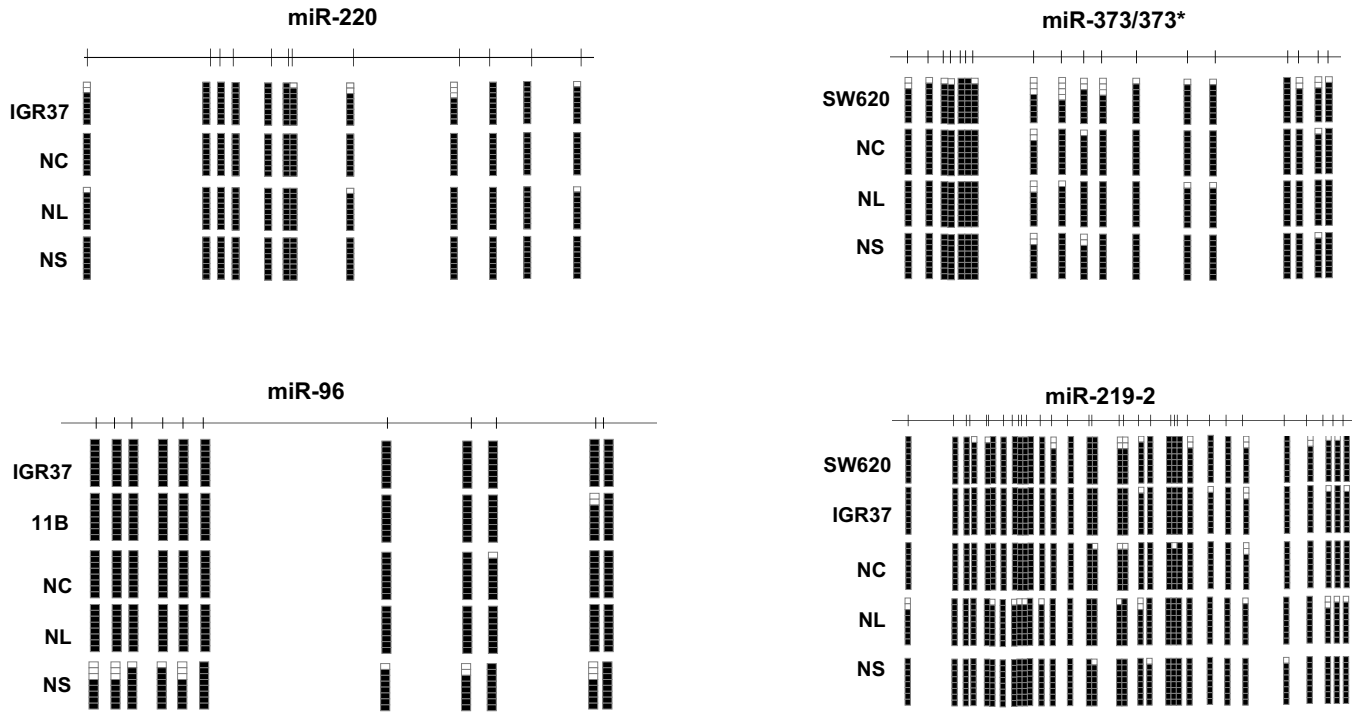

Fig. S1. Bisulfite genomic sequencing analyses of illustrative miRNAs that show a methylated CpG island in normal and cancer cells. Eight single clones are represented for each sample. The CpG island is depicted, and each vertical bar illustrates a single CpG. Black and white squares represent methylated and unmethylated CpG, respectively. NL, normal lymphocytes; NC, normal colon; NS, normal skin.

Fig. S2. Bisulfite genomic sequencing analyses of miR-9-1 and miR-9-2 that show cancer-specific CpG island hypermethylation. Eight single clones are represented for each sample. The CpG island is depicted, and each vertical bar represents a single CpG. Black and white squares represent methylated and unmethylated CpG, respectively. NL, normal lymphocytes; NC, normal colon; NS, normal skin.

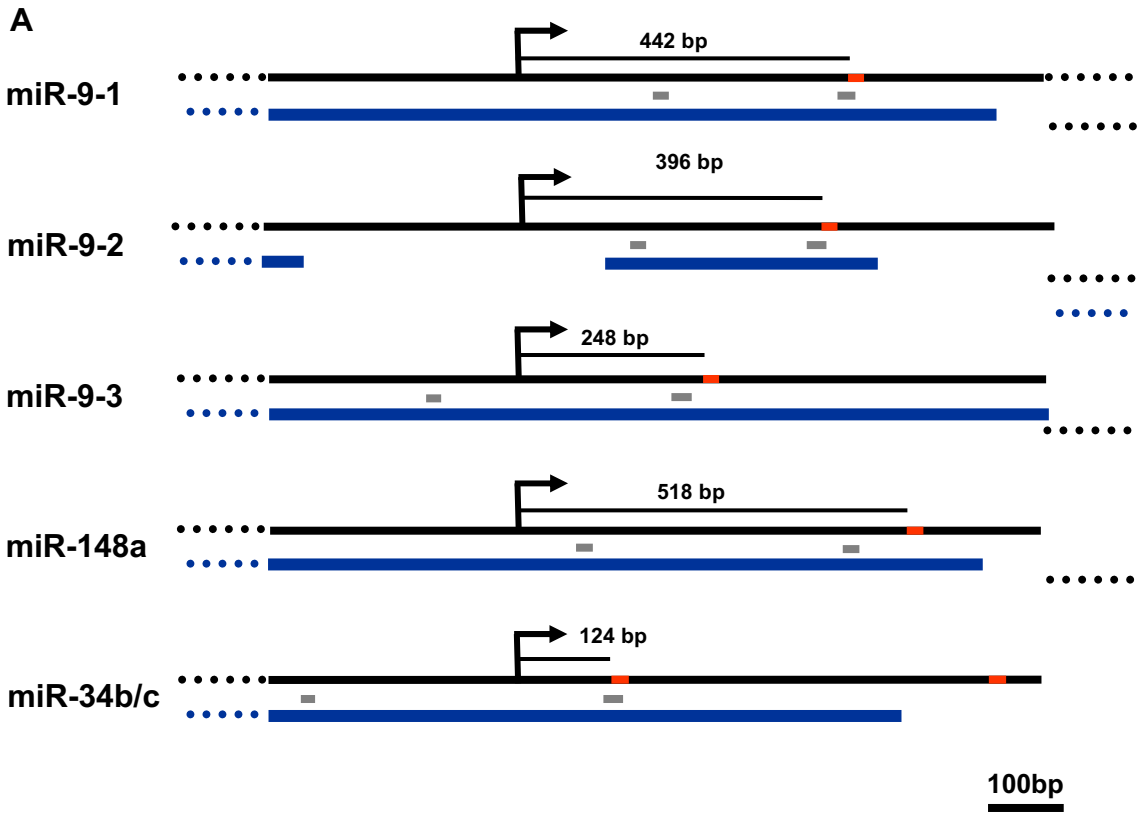

Fig. S3. 5′ RACE analyses of the five miRNAs in 5-aza-2′-deoxycytidine-treated cells. Black arrows represent the putative transcriptional start site. Red lines indicate the position of the mature miRNAs, and gray lines represent the location of the bisulfite sequencing primers. Blue lines depict the CpG islands.

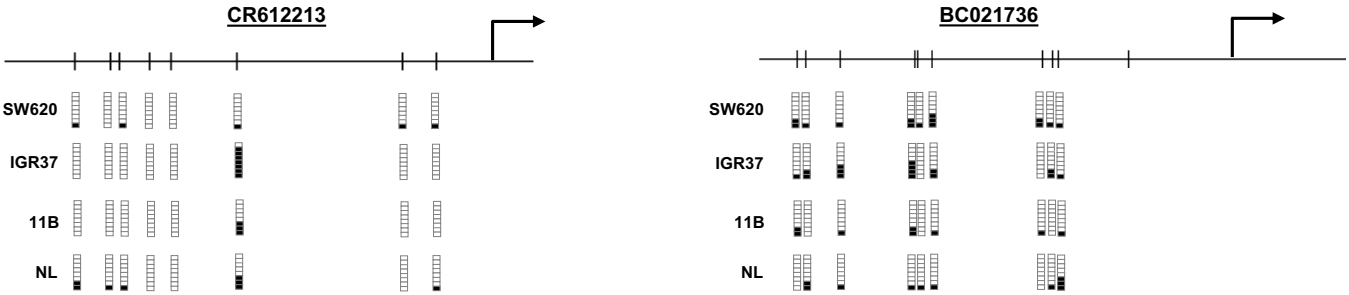

Fig. S4. Bisulfite genomic sequencing analyses of CR612213 and BC021736 5′ ends. Eight single clones are represented for each sample. Each vertical bar represents a single CpG. The black arrow represents the transcriptional start site. Black and white squares represent methylated and unmethylated CpGs, respectively. NL, normal lymphocytes.

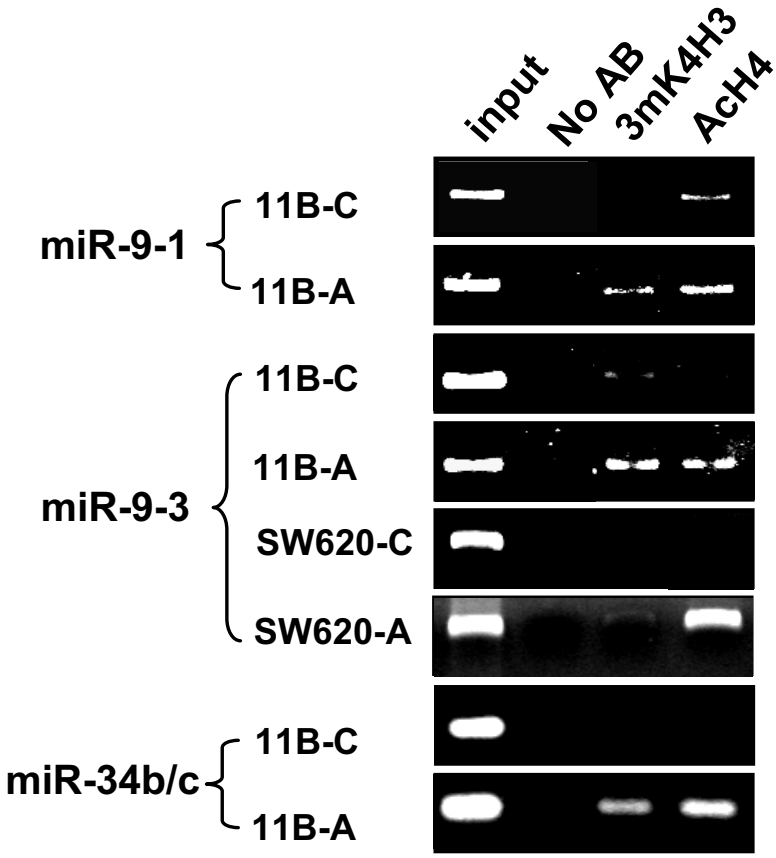

Fig. S5. Chromatin immunoprecipitation assay for histone modification marks in the miRNA-associated CpG islands (examples in miR-9-1, miR-9-3, and miR-34b/c) in untreated (c) and 5-aza-2′-deoxycytidine-treated (A) cells. The presence of miRNA methylation is associated with the lack of histone modifications linked to transcriptional activity, such as acetylation of histones H4 (AcH4) and trimethylation of Lys-4 of histone H3 (3mK4H3), whereas the opposite scenario is observed when DNA demethylation events are present by pharmacologic treatment with a DNA-demethylating agent. No AB, no antibody.

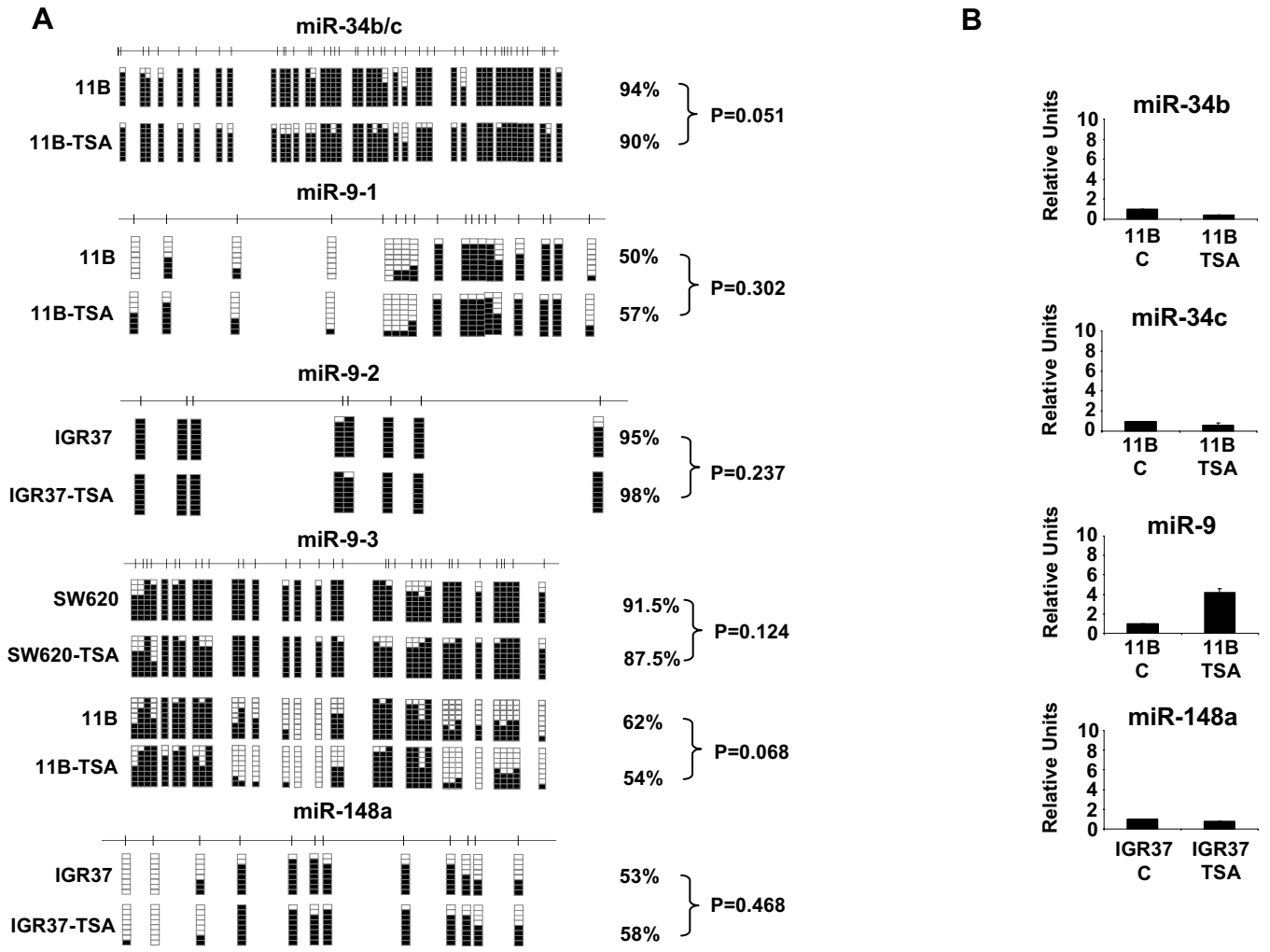

Fig. S6. (*A*) Bisulfite genomic sequencing analyses of the five miRNAs that show cancer-specific CpG island hypermethylation, before and after treatment with trichostatin A (TSA) in the corresponding cell lines. Eight single clones are represented for each sample. The CpG island is depicted, and each vertical bar illustrates a single CpG. Black and white squares represent methylated and unmethylated CpG, respectively. The percentage of methylation and the significance after χ^2 test are represented. There are no significant differences in the DNA methylation levels. (*B*) Expression analyses of mature miRNAs by qRT-PCR in methylated metastatic cell lines in untreated cells (c) and upon treatment with TSA. No significant differences were observed.

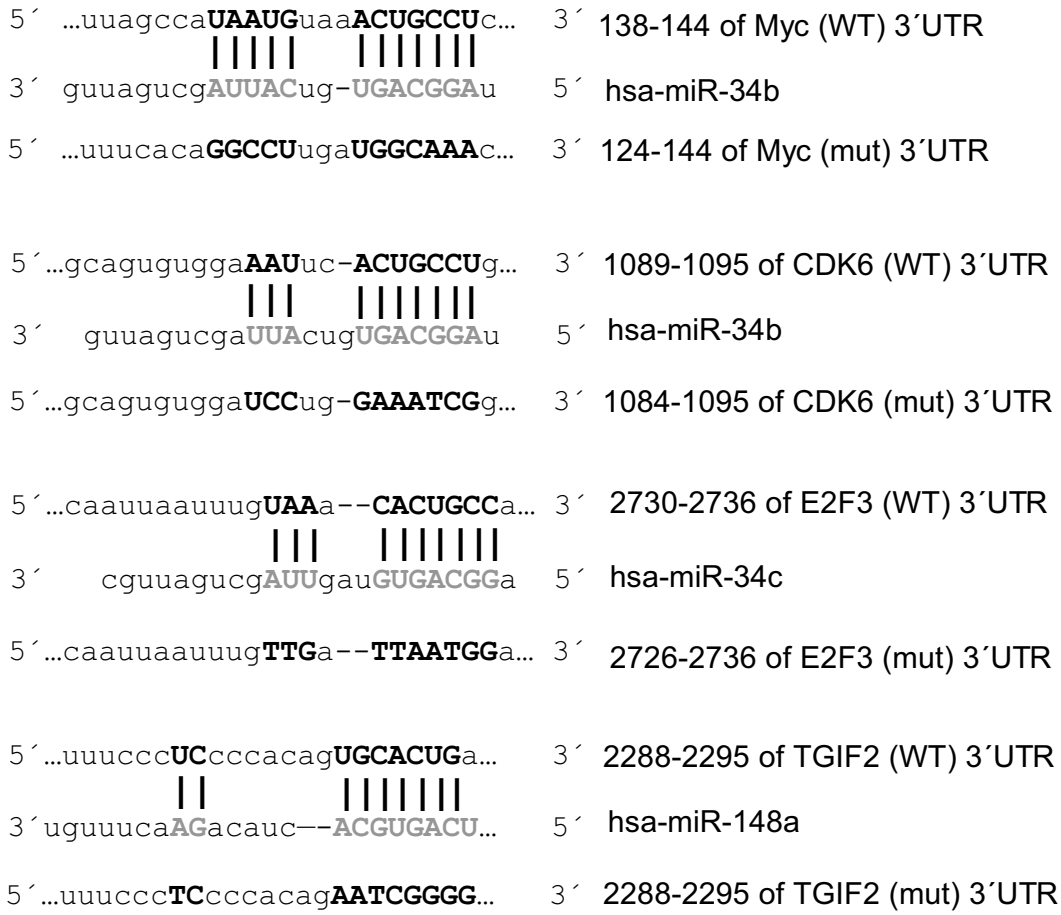

Fig. S7. Complementary sites between miR-34b and CDK6 and C-MYC, between miR-34c and E2F3, and between miR-148 and TGIF2. The capital and bold letters identify perfect base matches according to the TARGETSCAN 4.1 software. The base pairing between the miRNAs and the mutant target site is also shown.

Table S1. Fifty-seven different miRNAs up-regulated ≥2-fold with minimal basal expression in untreated cells

miRNA	SW620	IGR37	11B
nsa-miR-100		2.2	
nsa-miR-101-1		2.57	
nsa-miR-101-2		2.57	
nsa-miR-106a		2.16	
nsa-miR-126	2.9	2.3	2.8
nsa-miR-126*	2.4	2.5	2.6
nsa-miR-132	2	4.26	
nsa-miR-135-1			2.28
nsa-miR-145	8.17		
nsa-miR-147			2.08
nsa-miR-148a		2.8	
nsa-miR-148b		2.56	
nsa-miR-150			3.56
nsa-miR-151			2.05
nsa-miR-152		2.58	
nsa-miR-155	30	2.05	
nsa-miR-15b		2.14	
nsa-miR-17		3.25	
nsa-miR-181a		2.27	
nsa-miR-192		2.19	2.07
nsa-miR-193a		2.2	2.55
nsa-miR-193b		2.2	2.55
nsa-miR-196-1			3.47
nsa-miR-19a		2.4	
nsa-miR-205			2.12
nsa-miR-21		3.23	
nsa-miR-210		2.9	
nsa-miR-212		3.03	2.22
nsa-miR-213			3.87
nsa-miR-215			3.47
nsa-miR-218-2		5.58	2.22
nsa-miR-219-1	2.15	2.47	
nsa-miR-219-2	2.37	2.13	
nsa-miR-220		3.05	
nsa-miR-221		2.37	
nsa-miR-222		2.1	
nsa-miR-27b		2.09	
nsa-miR-29b-2		2.43	
nsa-miR-301	3.1		3.24
nsa-miR-30a		2.4	
nsa-miR-32		2.88	2.29
nsa-miR-324	2.87		3.71
nsa-miR-34b			3.9
nsa-miR-34c			5.38
nsa-miR-361	2.32		
nsa-miR-373	2.46		
nsa-miR-373*	3.4		
nsa-miR-425			2.21
nsa-miR-429	2.1		6.97
nsa-miR-494			2.04
nsa-miR-516			2.86
nsa-miR-7-2		2.29	
nsa-miR-9-1			2.54
nsa-miR-92	2.56		
nsa-miR-9-2		4.42	
nsa-miR-9-3	2	2.87	
nsa-miR-96		3.46	2.46

Table S3A. Frequency of CpG island hypermethylation of the metastasis-associated miRNAs in human malignancies and normal tissues

Cells	miR-148A	miR-34b/c	miR-9-1	miR-9-2	miR-9-3
			Tumor type		
Colon	14/36 (39)	13/37 (35)	12/37 (32)	6/37 (16)	31/37 (84)
Lung	20/36 (56)	34/63 (54)	22/42 (52)	26/47 (55)	31/58 (53)
Breast	38/71 (53)	50/72 (69)	23/40 (57)	22/40 (56)	42/72 (58)
Head and neck	4/17 (24)	1/12 (8)	8/17 (47)	4/9 (44)	5/15 (33)
Melanoma	29/59 (49)	18/72 (25)	32/89 (36)	55/86 (64)	11/65 (17)
			Normal		
Lymphocytes	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
Colon	0/8 (0)	0/15 (0)	0/16 (0)	0/12 (0)	2/13 (15)
Lung	0/4 (0)	0/4 (0)	0/4 (0)	1/4 (25)	0/4 (0)
Breast	0/4 (0)	0/4 (0)	0/4 (0)	0/4 (0)	0/4 (0)
Skin	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)
Melanocytes	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)

Data in parentheses are percentages.

Table S3B. The distribution of cases related to methylation and positive immunostaining

Target gene	Staining	miR-34b/c unmethylated	miR-34b/c methylated	*P* value
C-MYC	−	34	5	0.0001
	+	6	20	
CDK6	−	21	4	0.040
	+	10	8	

P value was calculated by using Pearson's χ^2 test.

Table S5. Primers used in this study

miRNA	Sequence
Primers used for genomic bisulfite sequencing (BS) and methylation-specific PCR (MSP) analyses	
BS-219-1-s	GGGGATAAGATGTTTATTAGGG
BS-219-1-as	CTTTAACTCTCCCTAAACCCTC
BS-210-s	TGGTTGAGGGATTAGGTTATTT
BS-210-as	AAAAATTAACCCCACCCC
BS-152-s	GAGAGGAGGTTTGTTTTGAGTT
BS-152-as	ACTCCACTCCCAAACAAATATT
BS-92-s	TGGGGATATTTGAYGTTAG
BS-92-as	CACCTTAAAAACCAAAAAAACT
BS-425-s	TTGAGTTGTTTGGGTTTTAGTTT
BS-425-as	CACTAAACRAACACRACATTC
BS-132/212-s	YGGGTYGTTTTTAGTAATAGTT
BS-132/212-as	TAAACTATTACCTCCAATTCCC
BS-9-1-s	GGTTTTTGTYGTGTTTGTATT
BS-9-1-as	TAACTTTATAAAAACTCCACACCA
BS-9-2-s	YGGAATAAATTTTGAAGGTAAT
BS-9-2-as	TAACTTTATAAAAACCAATACACTCA
BS-9-3-s	GTGTTTTAYGTGAGTTTYGG
BS-9-3-as	CACTCATACAACTAAATAACCAAAAAA
BS-34b/c-s	GGTTGGGAATTGAAGTTTG
BS-34b/c-as	TCAACTAATAACACTACCTACAAACC
BS-148a-s	TTTTTTAGATGGGAAGGTAAAAAG
BS-148a-as	TCCTCCAAAAATTTCTTCCTAC
BS-373-s	TTGGGGAAGGGAAGGGGGTTTT
BS-373-as	CCTACCTCAACCTCCCAAATAAC
BS-220-s	TTATGTTGTTGGGGATTTATTG
BS-220-as	ACCAACTAAACACCAACCTACA
BS-219-2-s	GATTTTAGGTTGGGGGTAGG
BS-219-2-as	CACAAATATCCAACCACAATTC
BS-7-2-s	AGTTGGAGTGAGTTGTGATTGT
BS-7-2-as	AAAAAAAAAAACTACCCCCATT
BS-96-s	GAGTGGGTAGGTTAGGAGTAGG
BS-96-as	AAAAACCTTACATCCCTACACC
BS-27b-s	GGGATTTTGTTTTTAAGGGTTA
BS-27b-as	ACAAATCAACATACCAAAAACC
BS-126-s	TAAATAGTTTTGGTTGTGTTTGG
BS-126-as	CRTCTTCCAAAATACTACRCT
BS-193b-s	TTTGGTATTTTGGTGAGATGAA
BS-193b-as	CTATCCAAAATAACCCCAAAAA
BS-193a-s	TYGTGTAATTTTTGGAGGGT
BS-193a-as	AACTTTATAAACCAATTAATCCRACA
BS-218-2-s	GGTGGGGTATAAGGGTAGTAG
BS-218-2-as	CTCCTACCCTCCTACCTTTAAT
BS-494-s	GAAGGTTGTTTGGATTTATGG
BS-494-as	ATCACCTCCAAAAAAATTCC
BS-192-s	TAGTTTTGGTTGGGAAAGAAAT
BS-192-as	AAAATATCCCATACCCACTCAC
MSP-9-1-ms	TTTTATTTTCGTTGACGGGC
MSP-9-1-mas	CCCGCCTCCTAACTACTATCG
MSP-9-1-us	TTTTTTTATTTTTGTTGATGGGT
MSP-9-1-uas	CCCACCTCCTAACTACTATCACC
MSP-9-2-ms	TTTCGTTTGGATGTTTTAGTC
MSP-9-2-mas	AAAATACTTACCGCGCTTAA
MSP-9-2-us	AGATTTTGTTTGGATGTTTTAGTT
MSP-9-2-uas	CAAAATACTTACCACACTTAAAA
MSP-9-3-ms	GGTGTTAGGACGTACGGAAC
MSP-9-3-mas	TACCCGAATCCTAAAACGC
MSP-9-3-us	GGTGTTAGGATGTATGGAAT
MSP-9-3-uas	TACCCAAATCCTAAAACAC
MSP-34b/c-ms	TTTAGTTACGCGTGTTGTGC
MSP-34b/c-mas	ACTACAACTCCCGAACGATC
MSP-34b/c-us	TGGTTTAGTTATGTGTGTTGTGT
MSP-34b/c-uas	CAACTACAACTCCCAAACAATCC
MSP-148a-ms	TAGGGGAGGTTTCGTAAAGC
MSP-148a-mas	CACGAAAACGAATATTCGAAA
MSP-148a-us	TTTTAGGGGAGGTTTTGTAAAGT

miRNA	Sequence
MSP-148a-uas	ACACAAAAACAAATATTCAAAACT
Primers used for cloning the flanking regions of selected miRNAs and containing BamHI and HindIII restriction sites	
pSIL-148a-s	AAAAGGATCCGAACACACCTGCAGGAAGAA
pSIL-148a-as	AAAAAAGCTTCTGGCGTCTGGAGCACTG
pSIL-34b/c-s	AAAAGGATCCGGACCGTCCGGGAGCTG
pSIL-34b/c-as	AAAAAAGCTTGCACATTGATGATGCACAGG
Primers used for obtaining the WT or mutant (mut) target site of target genes, containing MluI and HindIII restriction sites	
pLUC-myc-WT-s	CGCGTGAAAGATTTAGCCATAATGTAAACTGCCTCAAATA
pLUC-myc-WT-as	AGCTTATTTGAGGCAGTTTACATTATGGCTAAATCTTTC
pLUC-myc-mut-s	CGCGTGAAAGTTTTTCACAGGCCTTGATGGCAAACAAATA
pLUC-myc-mut-as	AGCTTATTTGTTTGCCATCAAGGCCTGTGAAAAACTTTCA
pLUC-E2F3-WT-s	CGCGTATAGCAATTAATTTGTAAACACTGCCAGAATACTTTCTAGCTGCA
pLUC-E2F3-WT-as	AGCTTGCAGCTAGAAAGTATTCTGGCAGTGTTTACAAATTAATTGCTATA
pLUC-E2F3-mut-s	CGCGTATAGCAATTAATTTGTTGATTAATGGAGAATACTTTCTAGCTGCA
pLUC-E2F3-mut-as	AGCTTGCAGCTAGAAAGTATTCTCCATTAATCAACAAATTAATTGCTATA
pLUC-CDK6-WT-s	CGCGTAAGAAGCAGTGTGGAAATTCACTGCCTGGGACAA
pLUC-CDK6-WT-as	AGCTTTGTCCCAGGCAGTGAATTTCCACACTGCTTCTTA
pLUC-CDK6-mut-s	CGCGTAAGAAGCAGTGTGGATCCTGGAAATCGGGGACAA
pLUC-CDK6-mut-as	AGCTTTGTCCCCGATTTCCAGGATCCACACTGCTTCTTA
pLUC-TGIF2-WT-s	CGCGTGGGATTTTCCCTCCCCACAGTGCACTGAGCAATGGA
pLUC-TGIF2-WT-as	AGCTTCCATTGCTCAGTGCACTGTGGGGAGGGAAAATCCCA
pLUC-TGIF2-mut-s	CGCGTGGGATTTTCCCTCCCCACAGAATCGGGGGCAATGGA
pLUC-TGIF2-mut-as	AGCTTCCATTGCCCCCGATTCTGTGGGGAGGGAAAATCCCA
Gene-specific primers (GSPs) used for 5′ RACE analyses	
miR-9-1-GSP1	AAGGGACACGAGTGGAGTTG
miR-9-1-GSP2	GGGGAGGGTGAAGAGAGAAA
miR-9-1-GSP3	ACGACAGAGACCGAAAAAGG
miR-9-2-GSP1	CTCTTGCCAGACTCCAGGTC
miR-9-2-GSP2	TACTTGCCGCGCTTAAGATT
miR-9-2-GSP3	CGGCTAAAACATCCAAACGA
miR-9-3-GSP1	GGCTCTGTGGCACTCATACA
miR-9-3-GSP2	AGAAACGGGCCTCCCTTAG
miR-9-3-GSP3	GAGGGGATGGACAGACACAC
miR-148a-GSP1	ACCAAACGTGCTGTCTTCCT
miR-148a-GSP2	CCGATTCGACAAATTCTGGT
miR-148a-GSP3	CTTCCTTCCTACCCGTCCTC
miR-34b/c-GSP1	CAGGCAATTCATTGGTTGAG
miR-34b/c-GSP2	CAGGCATCTTCTCTCGAAGG
miR-34b/c-GSP3	CTCGGACCCCATTTCACC
Primers used for ChIP analyses	
chip-miR-9-1-s	TGTCCCTTCCCTCCTACTCC
chip-miR-9-1-as	TGAAGACTCCACACCACTCA
chip-miR-9-2-s	CGCACACACACAAACAGAAA
chip-miR-9-2-as	CTCTTGCCAGACTCCAGGTC
chip-miR-9-3-s	GGGAGCAGGGGAGAAATG
chip-miR-9-3-as	GAGGGGATGGACAGACACAC
chip-miR-148a-s	CGACCAGGAATGGGTTCTAA
chip-miR-148a-as	GCCCAACAGAAATGGTGTTT
chip-miR-34b-s	CTGGCGTGAAGGAAGTGG
chip-miR-34b-as	CCATGACCCCCAGGAGTG
Primers used for genomic bisulfite sequencing of CR612216 and BC021736	
BS-CR612216-s	TTGAAAGATTGTGGAAATTAAAA
BS-CR612216-as	ATATATTTCTCCCACTTCCACA
BS-BC021736-s	TTTGAGATGGAGTTTTGTTTTG
BS-BC021736-as	TCCCAACACTTTAAAAAATCAA

Other Supporting Information Files

Table S2 (PDF)
Table S4 (PDF)