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Supplemental Information

MicroRNA-135b Promotes Cancer Progression by Acting as a Downstream Effector of Oncogenic Pathways in Colon Cancer

Nicola Valeri, Chiara Braconi, Pierluigi Gasparini, Claudio Murgia, Andrea Lampis, Viola Paulus-Hock, Jonathan R. Hart, Lynn Ueno, Sergei I. Grivennikov, Francesca Lovat, Alessio Paone, Luciano Cascione, Khlea M. Sumani, Angelo Veronese, Muller Fabbri, Stefania Carasi, Hansjuerg Alder, Giovanni Lanza, Roberta Gafa', Mary P. Moyer, Rachel A. Ridgway, Julia Cordero, Gerard J. Nuovo, Wendy L. Frankel, Massimo Rugge, Matteo Fassan, Joanna Groden, Peter K. Vogt, Michael Karin, Owen J. Sansom, and Carlo M. Croce

SUPPLEMENTAL DATA

Table S1, related to Figure 1.

Detector	Fold Change	P.value
mmu-miR-135b	9.765223391	0.0000338
mmu-miR-183	4.361436068	0.000618646
mmu-miR-1983	3.718271128	0.000572434
mmu-miR-96	3.311077284	0.000371255
mmu-miR-149	3.21050229	0.000616606
mmu-miR-140	2.604837165	0.0000135
mmu-miR-2132	2.401003819	0.000163062
mmu-miR-136	0.485351897	0.000367735
mmu-miR-376b	0.457472323	0.000899622
mmu-miR-127	0.411045052	0.000275617
mmu-miR-139-5p	0.405449262	0.00017289
mmu-miR-192	0.388894958	0.00000758
mmu-miR-365	0.38447848	0.0000759
mmu-miR-133a	0.3659658	0.000224533
mmu-miR-194	0.364852234	0.00000124
mmu-miR-375	0.277780238	0.000536254
mmu-miR-137	0.252069058	0.0000139
mmu-miR-378	0.21037031	0.0000169
mmu-miR-1	0.187497059	0.000576732
mmu-miR-143	0.093908059	0.000508751
mmu-miR-145	0.090010695	0.000275879

List of miRs deregulated in cancer compared to normal tissues in the CPC;Apc mice. p value < 0.001. deregulation greater than two-fold. Red indicates miRs that are up-regulated, green those that are downregulated.

Table S2, related to Figure 1.

Detector	Fold Change	P.value
mmu-miR-31	10.69466	0.000688
mmu-miR-135b	4.36653	8.82E-08
mmu-miR-183	3.12414	0.000364
mmu-miR-182	2.623878	1.47E-05
mmu-miR-1983	2.577786	0.000353
mmu-miR-149	2.552777	0.000594
mmu-miR-96	2.519349	4.71E-05
mmu-miR-25	2.251941	5.68E-05
mmu-miR-18°	2.213416	8.98E-05
mmu-miR-20a+mmu-miR-20b	2.114987	2.02E-07
mmu-miR-106a+mmu-miR-17	2.096179	5.01E-11
mmu-miR-93	2.086193	3.21E-10
mmu-miR-19b	2.026195	0.000571
mmu-miR-101°	0.494073	0.000183
mmu-miR-133°	0.475076	0.000196
mmu-miR-127	0.432725	7.86E-05
mmu-miR-365	0.42945	3.56E-05
mmu-miR-9	0.366543	7.22E-05
mmu-miR-137	0.346919	0.000542
mmu-miR-1	0.298719	2.74E-05
mmu-miR-143	0.219351	2.77E-09
mmu-miR-145	0.217391	3.17E-09

List of miRs deregulated in cancer compared to normal tissues in the AOM/DSS mice. p value < 0.001. Deregulation greater than two-fold. Red indicates miRs that are up-regulated, green those that are down-regulated.

Table S3, related to Figure 1.

Sample_ID	hsa-mir-135b reads (cancer primary tissue)	hsa-mir-135b (matched normal tissue)
TCGA-A6-2671-01A-01T-1409-13	44	0
TCGA-A6-2680-01A-01T-1409-13	78	1
TCGA-A6-2684-01A-01T-1409-13	260	0
TCGA-A6-2685-01A-01T-1409-13	90	0
TCGA-AA-3520-01A-01T-0822-13	274	0
TCGA-AA-3525-01A-02T-0827-13	107	0
TCGA-A6-2683-01A-01T-0822-13	4393	0

Matched analysis of cancer and normal paired tissues from the TCGA miR-Seq dataset. No miR-135b reeds in normal compared to cancer tissues. Paired t test P value: 0.0028.

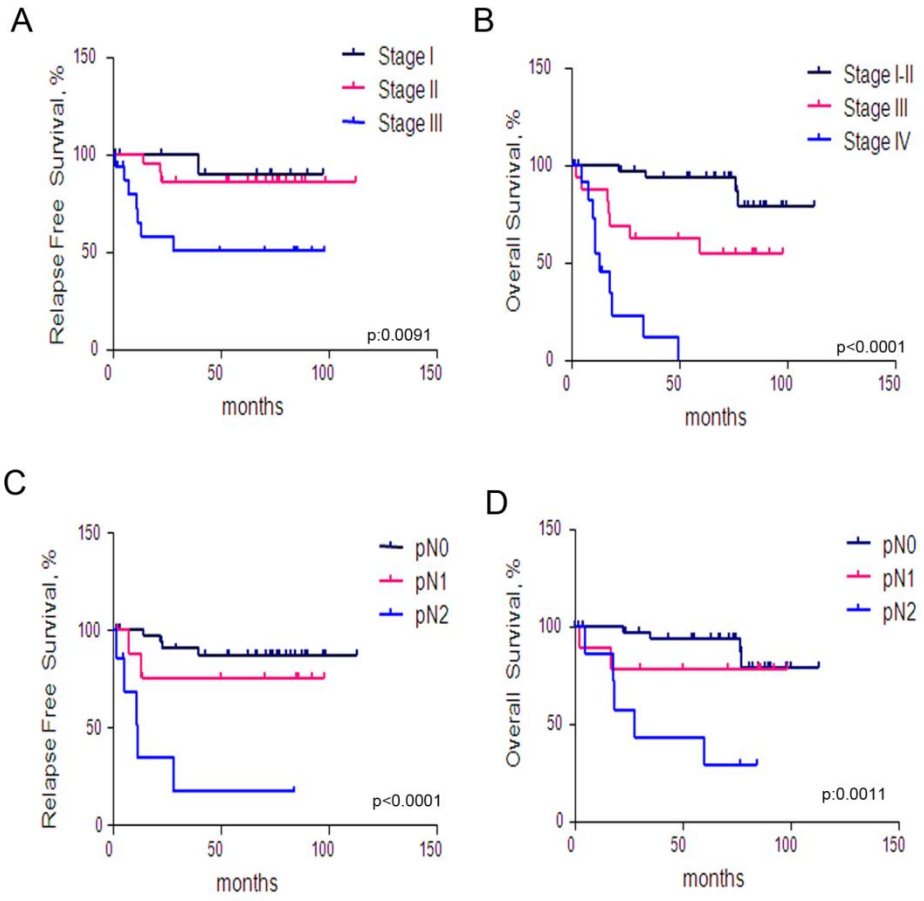

Figure S1, related to Figure 1. Prognostic Factors in Sporadic CRC.

Patient's prognosis according to tumor stage (A&B) and nodal status (C&D).

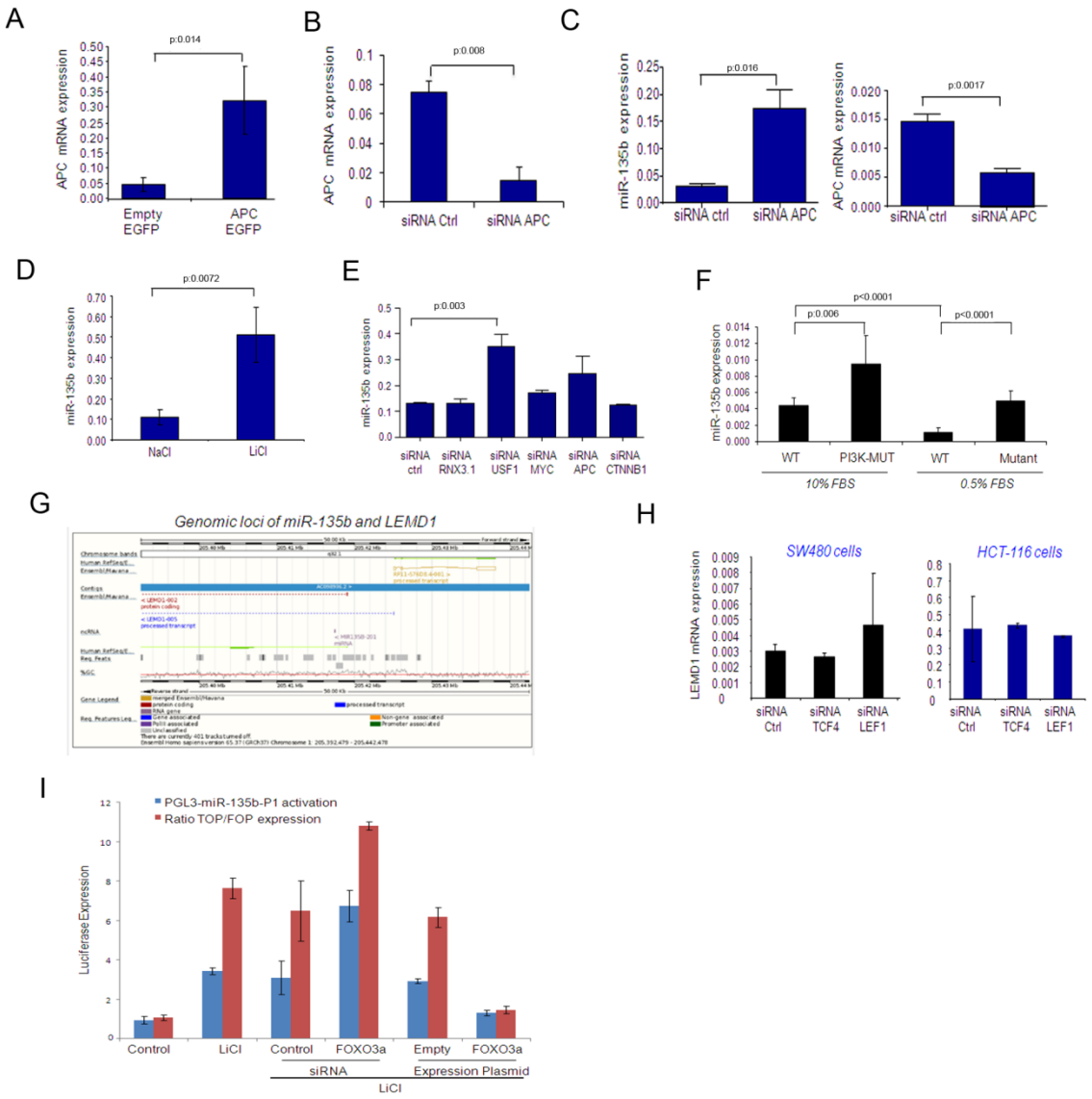

Figure S2, related to Figure 2. MiR-135b transcriptional regulation.

Hsa-miR-135b expression was assessed by real time PCR. The expression of miR-135b was normalized to that of RNU48, while that of other genes to GAPDH. Bars represent the mean and SD of 3 experiments, p values are reported within the figure.

(A) *APC* mRNA expression in SW480 cells transfected with an APC-encoding plasmid.

(B) *APC* mRNA expression in NCM460 cells transfected with *APC* siRNA.

(C) miR-135b (left) and APC (right) expression in NCM 356 cells transfected with APC siRNA.

(D) miR-135b expression after treatment with LiCl.

(E) miR-135b expression after transfection with selected siRNA targeting transcription factors downstream of the APC/β-catenin axis.

(F) MiR-135b expression assessed by RT-PCR in DLD-1 isogenic human CRC cells with or without mutation of the PI3K after exposure to 10% or 0.5% of fetal bovine serum (FBS).

(G) Genomic loci of miR-135b and LEMD1.

(H) LEMD1 expression following transfection with selected siRNA in SW480 (left) and HCT-116 (right) cells.

(I) miR-135b promoter activation (blue bars) and ratio between TOPFLASH and FOPFLASH (red bars) luciferase activity following treatment with LiCl in presence/absence of FOXO3a modulation.

Table S4, related to Figure 3.

Group 1	Group 2	Day 1	Day 2	Day 3	Day 4	Day 5
WT miR scramble	WT miR-135b	0.3036	**0.0765**	**0.0120**	**<0.0001**	**0.0001**
	PI3K-MUT LNA control	**0.0200**	**0.0012**	**<0.0001**	**<0.0001**	**<0.0001**
	PI3K-MUT LNA anti-135b	0.0772	**0.0018**	**0.0001**	**<0.0001**	**<0.0001**
WT miR-135b	PI3K-MUT LNA control	0.5584	0.2967	0.1702	**0.0001**	**0.0038**
PI3K-MUT LNA control	PI3K-MUT LNA anti-135b	0.2397	**0.0465**	**0.03482**	**0.0011**	**0.0018**

List of t-tests for different class comparison in the cell viability assay in HCT-116 PI3K WT and mutant cells.

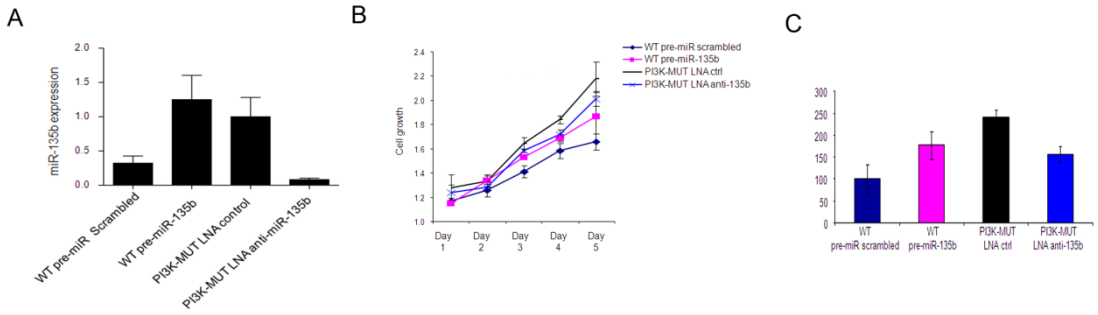

Figure S3, related to Figure 3. MiR-135b mediates PI3K activating mutation phenotype on cell proliferation and cell growth.

Bars represent the mean and SD of 3 experiments; p values are reported within the figure.

(A) miR-135b expression assessed by RT-PCR in isogenic human CRC cells transfected with pre-miR-135b, LNA anti-miR-135b and relative controls. The expression of miR-135b was normalized to that of RNU48.

(B) Isogenic PI3K WT and mutant DLD-1 cells were transfected with pre-miR-135b, LNA anti-miR-135b or relative controls. Cell viability was measured at selected time points. p values are reported in Table S5.

(C) Isogenic PI3K mutant and WT cells over-expressing miR-135b or anti-miR-135b were plated in soft agar and grown in low FBS conditions. Colonies greater than 2 mm in size were counted and quantitated.

Table S5, related to Figure 3.

Group 1	Group 2	Day 1	Day 2	Day 3	Day 4	Day 5
WT miR scramble	WT miR-135b	0.1227	**0.0223**	**0.0002**	**0.0036**	**0.0425**
	PI3K-MUT LNA control	**0.0445**	**0.0198**	**<0.0001**	**<0.0001**	**<0.0001**
	PI3K-MUT LNA anti-135b	**0.0276**	0.3299	**<0.0001**	**0.0010**	**<0.0001**
WT miR-135b	PI3K-MUT LNA control	**0.0201**	0.9358	**0.0003**	**<0.0001**	**0.0137**
PI3K-MUT LNA control	PI3K-MUT LNA anti-135b	0.4870	**0.0383**	**0.0166**	**0.0001**	**0.0244**

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List of t-tests for different class comparison in the cell viability assay in DLD-1 PI3K WT and mutant cells.

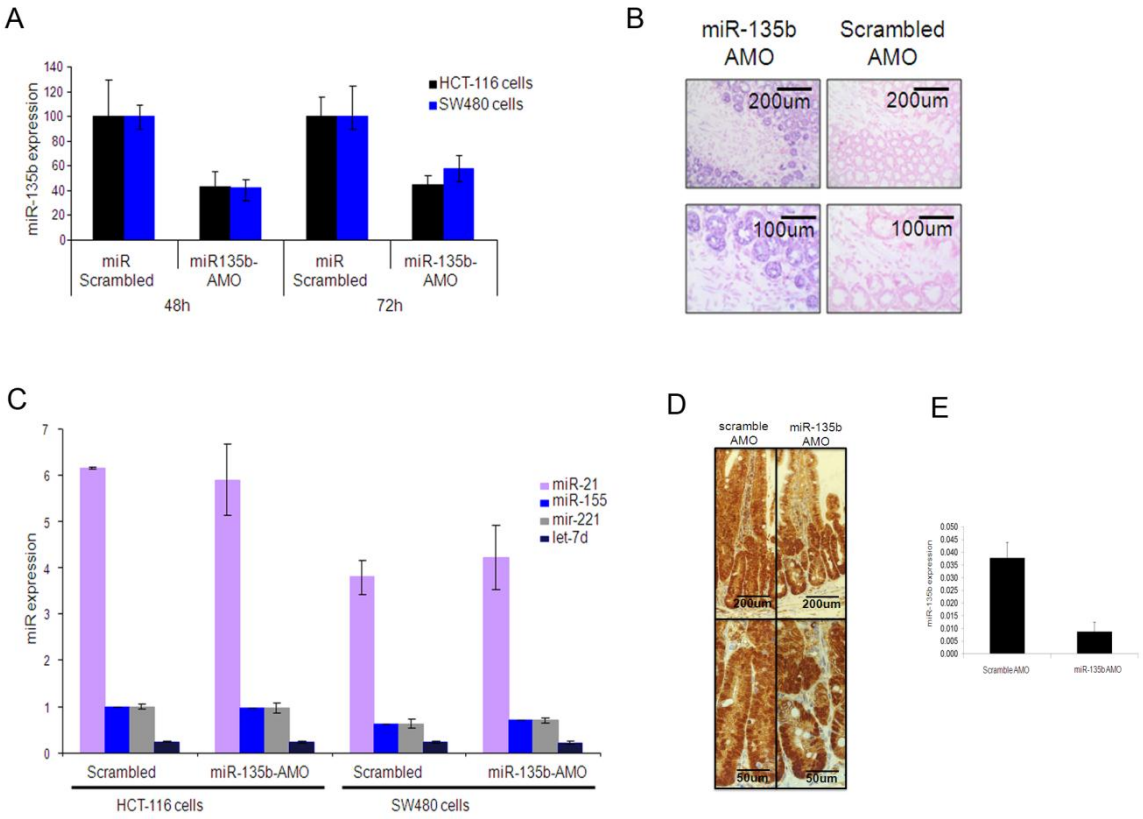

Figure S4, related to Figure 4. *In vitro* and *in vivo* analysis of 135b-AMO activity.

The expression of miR-135b was normalized to that of RNU48 or SNU234 for human and mice experiments respectively. Bars represent the mean and SD of 3 mice, p value <0.001.

(A) Human CRC cell lines were treated with a probe anti-miR-135b (miR-135b-AMO) or a scrambled probe (scrambled-AMO) and miR-135b expression assessed by RT-PCR.

(B) 8 week-old BL/6 wild type mice were treated with miR-135b-AMO. ISH was performed using an anti-anti-miR-135b (a specific LNA probe designed to recognize the 135b-AMO).

(C) Expression of miR-21, miR-155, miR-221 and let7-d was evaluated by RT-PCR to test 135b-AMO off-target effects.

(D) Nuclear localization of β-catenin in every cell within Apc-deficient crypts shown by immunohistochemistry at four days post Cre induction in the scramble and anti-miR135b-AMO treated mice.

(E) miR-135b expression in intestines of mice treated with miR135b-AMO or scramble-AMO.

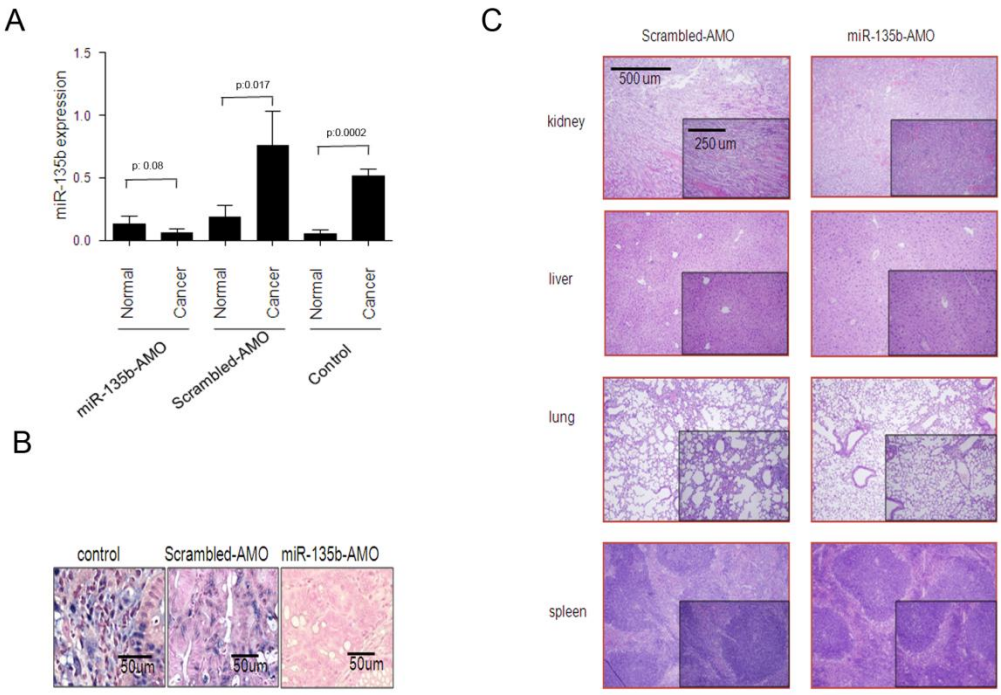

Figure S5, related to Figure 5. MiR-135b knockdown in the AOM/DSS model.

(A & B) miR-135b expression was assessed by RT-PCR (A) and ISH (B) in cancer tissues from AOM/DSS treated mice injected with miR-135b-AMO, scrambled-AMO or control. Bars represent the mean and SD of a paired analysis for tumor and matched normal tissue from 8 different mice for each group; p values are reported within the figure.

(C) Morphology of lungs, livers, kidneys and spleens from miR-135b-AMO and scrambled-AMO treated mice were compared by H&E.

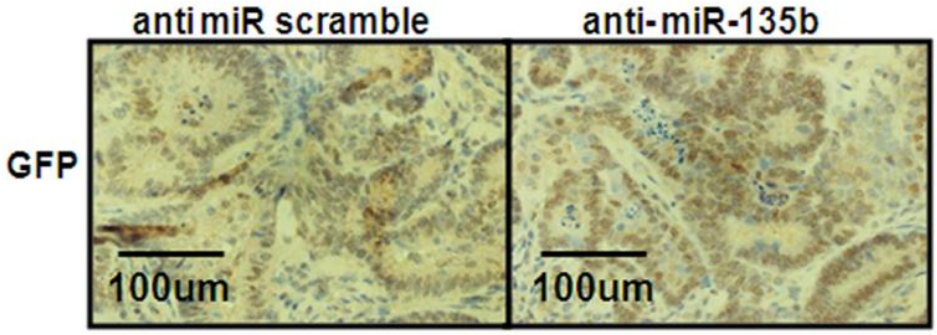

Figure S6, related to Figure 6. Lentivirus integration in tumor derived organoids.
GFP was assessed by Immunohistochemistry in xeno-transplanted organoids 8 weeks after transplantation.

Table S6, related to Figure 6.

Week 1	Week 2	Week 3	Week 4	Week 5
0.092580682	**0.040063**	**0.050296**	**0.03497**	**0.0276337**

List of t-tests for different class comparison Apc/Pten xenograft experiment.

Table S7, related to Figure 6.

Day 6	Day 9	Day 12	Day 15	Day 18
0.380495	**0.038277**	**0.049294**	**0.047362654**	**0.04141142**

List of t-tests for different class comparison Apc/Kras/P53 xenograft experiment.

Table S8, related to Figures 7.

Detector	Fold Change	P.value
LCK	0.389407676	0.001356558
TGFBR2	0.610044021	0.00019909
E2F1	0.644618708	0.000194241
DAPK1	0.655562593	0.00911103
APC	0.671786237	0.007969989
ITGB1	0.680891133	0.000264439
CCNE1	0.697700211	0.001720298
ERBB3	0.70045955	0.002725399
CD44	0.70832554	0.001464214
FOSL2	0.724355164	0.001598557
MYC	0.737391241	0.001225426
CDKN2A	0.754864465	0.002437274
NF1	0.820655892	0.001035126
CASP2	0.827988072	0.00943856
CDC25B	0.854754978	0.001721084
TYMS	0.879967681	0.006887917
NUMA1	0.893306529	0.000171889
TP53	0.907023142	0.00153682
PGK1	0.921414688	0.00946697
IFNGR1	1.154157953	0.006356075
HSP90AB1	1.161040166	0.000717024
MET	1.248757391	0.000942007
PRKAR1A	1.256327673	0.001614072
CAV1	1.261251305	0.002378304
TGFBI	1.292219437	0.001548686
JUN	1.362735697	0.006689712
GADD45A	1.397467087	0.00385239
E2F3	1.407114666	0.000377653
STAT3	1.488736178	0.000960675
CTNNB1	1.583223152	0.003370135
CDKN1A	1.603793441	4.24E-05
BCL2L1	1.607592181	4.37E-06
ETS1	2.151658733	0.005275762
THPO	2.797732526	0.005369301
IL8	4.58189054	0.002107009

List of genes deregulated in NCM 460 cells over-expressing miR-135b compared to scrambled transfected cells. P value < 0.01.

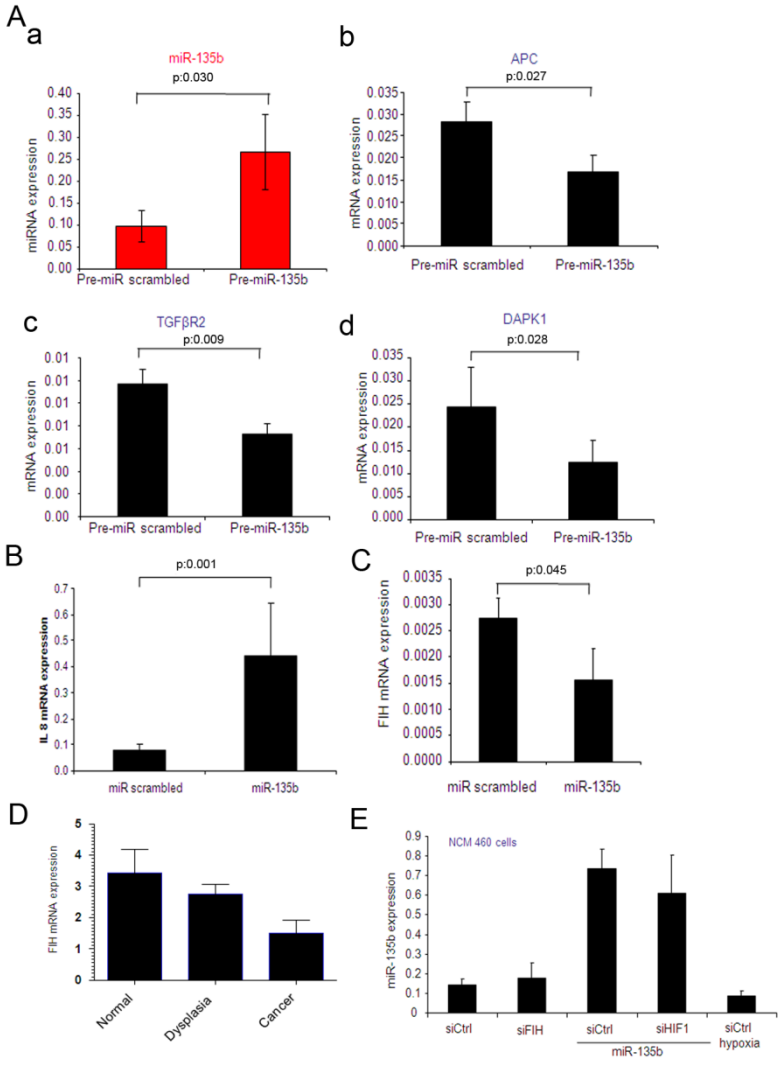

Figure S7, related to Figure 7. MiR-135b target validation.

The expression of miR-135b was normalized to that of RNU48, while that of other genes to GAPDH. Bars represent the mean and SD of 3 experiments, p values are reported within the figure.

(A) RT-PCR for miR-135b in NCM356 transfected cells (a). RT-PCR for *APC (b)*, *TGFβR2* (c) and *DAPK1* (d) expression in NCM356 cells over-expressing miR-135b compared to scrambled transfected cells .

(B) *IL8* mRNA expression after miR-135b over-expression in NCM 356 cells.

(C) *FIH* mRNA following miR-135b over-expression in NCM 356 cells.

(D) *FIH* mRNA expression in cancer compared to dysplastic and normal tissues in IBD-associated CRC.

(E) miR-135b expression following transfection with different siRNA in NCM 460 cells.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Cultures and Transfections

SW620, SW480, HCT-116, DLD-1 and RKO colorectal cancer (CRC) cells (American Type Culture Collection ATCC, Manassas, VA) were cultured in RPMI 1640 (Gibco, Carlsbad, CA). PI3KCA mutant and WT HCT-116 and DLD-1 cells (kind gift from Prof. Bert Vogelstein, Johns Hopkins University, Baltimore, MD, USA) were grown in McCoy's 5A modified medium (Gibco, Carlsbad, CA). All cells were supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) plus antibiotics. For serum starvation, cells were grown to 70–80% confluence, washed once with PBS, and incubated for 19 h in McCoy's 5A modified medium containing 0.5% FCS. Normal epithelial colon cell lines NCM356 and NCM460 (InCell, San Antonio, TX) were cultured in M3:10TM medium. Mouse embryo fibroblasts were provided by Prof. Peter K. Vogt (The Scripps Research Institute, La Jolla, CA, USA) and cultured in 10% DMEM (Gibco, Carlsbad, CA) with 10% FCS (Omega, Tarzana, CA), 292 ug/mL l-glutamine, 200 ug/mL G418 plus antibiotics. Cells were examined for mycoplasma contamination periodically and were always found negative. Cell transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's protocol. For over-expression studies, specific miRNA or control precursor oligonucleotides were purchased from Ambion (Austin, TX) and used at 20 nM. ON-TARGETplus siRNA pools and relative controls were purchased from Thermo Fisher Scientific (Lafayette, CO). For silencing experiments, miRCURY LNA™ anti-miR-135b or control miRCURY knockdown probes (Exiqon, Vedbaek, Denmark) were used at 25 nM. EGFP-*APC* and empty control vectors were a gift from Prof. Joanna Groden (OSU, OH, USA). The PI3K inhibitor LY294002 was purchased at Cell Signaling (Beverly, MA) and used as described previously at 10 μM. Dasatinb (Selleck Chemicals, Houston, TX) was used as previously described at 100 nmol/L. AS703026, a MEK1-2 inhibitor (Selleck Chemicals Houston, TX), was used at 10 μmol/L.

Real time PCR for mature miRNAs and genes

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Mature miRNAs expression was assessed by single-tube TaqMan MicroRNA Assay, while the expression of mRNAs of interest was evaluated by the Gene Expression analysis using Syber-Green and Taq-man Probes. For Syber-Green, RNA was treated with RNase-free DNase I (Qiagen Valencia CA). One microgram of RNA was reverse-transcribed to cDNA, and quantitative real-time PCR was performed with specific primers for *IL8*, *VEGF*, *DAPK1*, *FIH*, *APC* and *TGFβR2* (list of primers available upon request). TaqMan Probes were as follow: *TGFBR2* (Hs00234253_m1), *HIF1AN* (Hs00215495_m1), *DAPK1*

(Hs00234489_m1). MiRNA expression was normalized to that of *RNU44* and *RNU48*. Gene expression was normalized to *GAPDH*. All retro-transcriptase (RT) reactions, including no-template controls and RT minus controls, were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems, Carlsbad, California). Each sample was tested in triplicate.

Western Blotting

For immunoblotting analysis, cells were lysed with ice-cold Cell Lysis Buffer plus protease inhibitor (Cell Signaling Technology Inc., Danvers, MA). Equivalent amounts of protein were resolved and mixed with 4X SDS-PAGE sample buffer, electrophoresed in a 4–20% and 7.5% linear gradient Tris-HCL Criterion Precast Gels (Bio-Rad Hercules CA), and transferred to nitrocellulose or PVDF membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20, and were incubated with primary and secondary antibodies according to the manufacturer's instructions. The following antibodies were used: Phospho-Akt Pathway Sampler Kit (Cell Signaling cat. no. 9916); Anti-DAP Kinase 1 rabbit (Sigma cat. no. D1319); Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling cat. no. 9101S); p44/42 MAPK (Erk1/2) (Cell Signaling cat. no. 9102); FIH (Cell Signaling cat. no. D19B3); β-Catenin (Cell Signaling cat. no. 9562); p21 Waf1/Cip1 (12D1) Rabbit (Cell Signaling cat. no. 2947); TGF-β Receptor II (K105) (Cell Signaling cat. no. 3713) GAPDH (Santa Cruz, CA)..

Luciferase Assay

The predicted miRNA binding sites in the 3'-UTR of *FIH*, *TGFβR2* and *APC* were cloned downstream of the firefly luciferase gene as follows. Complimentary DNA (cDNA) from NCM460 cells was amplified by PCR using specific primers (primer sequences available upon request). The product was then digested with *SpeI* and *SacII* (New England Biolabs, Ipswich, MA) and inserted into the *pGL3* control vector (Promega, Madison, WI), previously modified to harbor the SpeI and SacII sites immediately downstream of the stop codon of the firefly luciferase gene. The *DAPK1-3'UTR* plasmid was purchased at SwitchGear Genomics (Menlo Park, CA) and transfected as previously described (Iliopoulos et al, 2011). Mutant plasmids harboring a deletion in the miR-135b seed region were prepared for each target gene using QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA). NCM460 cells were co-transfected in 12-well plates with 1 μg of pGL3 firefly luciferase reporter control vector, 0.1 μg of the phRL-SV40 control vector (Promega, Madison, WI), and 20 nM pre-miR-135b or control precursors. Firefly and Renilla luciferase activities were measured consecutively by using the Dual

Luciferase Assay (Promega, Madison, WI) 24 h after transfection. For promoter luciferase assays a 2547bp area upstream of the miR-135b pre-miR was sub-cloned in PGL3-Promoter vector (Promega). Plasmid with mutant or WT promoter were used for transfection in NCM460 cells. Promoter activation was induced by LiCl treatment for 24h.

Cell Death analysis

Propidium iodide (PI) staining: Cells were detached with trypsin, washed with cold phosphate-buffered saline (PBS)–5% FCS and then fixed in 70% ethanol for 24 h. After washing with PBS, cells were incubated with 1 μg/ml PI for 3 h at 25°C before FACS analysis by Coulter Epics XL flow cytometer (Beckman Coulter, Fullerton, CA). Cells were considered apoptotic when their DNA content was <2 N. For detection of caspase-3/7 activity, cells were plated in 10 cm dishes transfected with pre-miR-135b, siRNA to p21 or TGFβR2. The day after transfection cells were harvested and re-plated in 96-well plates in quadruplicate treated with 10 μM TGF and analyzed using a Caspase-Glo 3/7 Assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Focus formation assay in soft agar

HCT116 transfected with pre-miR-135b, LNA anti-miR-135b or relative controls were plated in triplicate at 5000 cells/ml in top plugs consisting of McCoy's 5A modified medium containing various FCS concentrations and 0.4% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine). After two weeks, the colonies were photographed and counted.

SRC-MEFs were infected with lentiviruses expressing anti-miR-135b hairpins or empty controls. MEFs were counted and plated as described above and GFP was monitored over time with the use of EVOS microscopy (LifeTechnology). Total colonies were counted after 4 weeks.

Cell viability assays.

Cell viability was examined with 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheniltetrazolium bromide (MTS)-Cell Titer 96 Aqueous One Solution Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's protocol. Metabolically active cells were detected by adding 20 μl of MTS to each well. After 1 h of incubation, the plates were analyzed in a Multilabel Counter (Bio-Rad).

Tube Formation Assay

NCM 460 cells were transfected with pre-miR-135b or pre-miR-control under starvation. 16 hr after transfection, media from both experiments was collected and used to culture HUVEC Matrigel (BD PharMingen; 12.9 mg/mL)–coated LabTek (55,000 cells per

chamber). Results are expressed as number of tube-like structures per field (magnification, ×200).

Animals and Tumor Induction

RNAs from CpC;Apc (matched tumor and normal), Azoxymethane (AOM)/Dextran Sulphate Sodium (DSS) (matched cancer and normal), wild type (untreated normal epithelium) and short-term (3 to 5 days) DSS-treated mice (inflamed epithelium) for the initial microRNA expression screening were provided by Prof. Michael Karin (UCSD, San Diego, CA, USA). RNA from intestinal epithelium of long term [3 cycles (1 weeks each) over a 78 days period) DSS-treated mice was collected at OSU. All mice were C75BL/6 strain mice. For *in vivo* silencing experiments, C57BL/6 mice were obtained from The Jackson Laboratory. All mice were maintained in filter-topped cages on autoclaved food and water at OSU according to NIH guidelines, and all experiments were performed in accordance with OSU and NIH guidelines and regulations. CAC was induced as described previously (Grivennikov et al., 2009). Briefly, on day 1, mice were injected intra-peritoneally (i.p.) with 12.5 mg/kg Azoxymethane (AOM; National Cancer Institute) and maintained on regular diet and water for 7 days. After 7 days, mice received water with 2.5% dextran sulfate sodium (DSS; MP Biomedicals, molecular weight 35,000–50,000 kDa) for 7 days. After this, mice were maintained on regular water for 14 days and subjected to two more DSS treatment cycles. 2'-O-methoxyethyl-3'cholesterol conjugated oligonucleotides (Lennox and Behlke 2011) specific to target miR135b (135b-AMO) or a non-targeting sequence (scrambled-AMO) were synthetized by Girindus America Inc. (Cincinnati OH).

Mice were treated as follows:

AOM + DSS + Scrambled-AMO (Scrambled-AMO group) (n=8) C57BL/6 8 week-old mice were treated with AOM i.p. at day 0 and received 2.5% DSS water according to the above mentioned schedule. Anti-miR-control was given twice a week for the entire treatment (22 injections over a 11 weeks period) at 50 mg/kg.

AOM + DSS + miR-135b-AMO (135b-AMO treatment group) (n=8) C57BL/6 8 week-old mice were treated with AOM i.p. at day 0 and received 2.5% DSS water according to the above mentioned schedule. Anti-miR-135b was given twice a week for the entire treatment (22 injections over a 11 weeks period) at 50 mg/kg.

AOM + DSS (Mock Group) (n=8) C57BL/6 8 week-old mice were treated with AOM i.p. at day 0 and received 3% DSS water according to the above mentioned schedule. No anti-miRs were administered.

Macroscopic tumors were counted and measured with a calliper. Tumors and matched normal adjacent tissues from the distal colon were taken as a tissue sample and snap-

frozen in liquid nitrogen, maintained in Trizol or fixed in 10% neutral buffered formalin for 24 h and transferred to 70% ethanol for subsequent paraffin embedding and histological analysis. The clinical course of disease was followed daily by measurement of body weight and monitoring for signs of rectal bleeding or diarrhoea.

Tumour Organoids

Intestinal fragments containing tumours from Apc, Apc/Pten, Apc/Kras, Apc/Pten/Kras and Apc/Kras/P53 genotypes were incubated in 2 mmol/L EDTA chelation buffer for 60 minutes on ice. Following washing with cold chelation buffer, most of the normal intestinal epithelial cells were detached, while tumour cells remained attached to the mesenchyme. Next, the adenoma fragments were incubated in digestion buffer (Dulbecco's modified Eagle medium with 2.5% fetal bovine serum, penicillin/streptomycin [Invitrogen], 75 U/mL collagenase type IX [Sigma, St Louis, MO], 125 µg/mL dispase type II [Invitrogen]) for 30 minutes at 37°C. The tumour fragments were allowed to settle down under normal gravity for 1 minute, and the supernatant was collected in a 50-mL Falcon tube, pelleted, and washed with PBS. Isolated adenoma cells were centrifuged at 150–200g for 3 minutes to separate adenoma from single cells. Single cells were embedded in Matrigel on ice (growth factor reduced, phenol red free; BD Biosciences) and seeded in 48-well plates (500 crypts/fragments or 1000 single cells per 25 µL of Matrigel per well). The Matrigel was polymerized for 10 minutes at 37°C, and 250 µL/well basal culture medium (advanced Dulbecco's modified Eagle medium/F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, Glutamax, 1× N2, 1× B27 [all from Invitrogen], and 1 mmol/L N-acetylcysteine [Sigma]) was overlaid containing the following optimized growth factor combinations: murine EGF and noggin as previously described (Sato et al., 2011). For lentiviral infection, cells were infected as previously described (Koo et al.; 2011) using anti-miR-135 or empty vectors from SBI Mountains View CA) and selected using puromycin. 3 days after infection, GFP positive puromycin resistant spheres were picked manually under the microscope and transfer to new plates. For mice xenograft experiments, spheres were counted and 12 mice for each genotype were injected into the left flank with 50 spheres in 100ul of matrigel. Tumours were measured twice a week. Tumor volume was measured at the beginning of treatment and then once twice a week. The estimated tumor volume (V) was calculated by the formula $V = W2 \times L \times 0.5$, where W represents the largest tumor diameter in centimeters and L represents the next largest tumor diameter. The individual relative tumor volume (RTV) was calculated by $RTV = Vx/V1$ where Vx is the volume in cubic millimeters at a given time and V1 is the volume at baseline (Valeri et al.; 2010). For Immunofluorescence, spheres were removed from matrigel using BD cell recovery solution according to protocol (30 min on ice). They were fixed with 4%PFA for 10 min at room temperature subsequently and washes

twice with ice cold PBS. Spheres were permeabilized with 0.2% Triton-100 in PBS for 10 min on ice, washed with PBS and blocked with 3%BSA, 0.1% Tween-20 in PBS for one hour at room temperature on a roller. Cells were stained with GFP (Abcam AB13970-100) antibody 1:200 over-night at 4C. Washed 3 times 5 min with PBS and incubated with secondary antibody and Rhodamin coupled Phalloidine for 1h at room temperature. The sheres were washed and mounted in Vecta shield H1000 containing DAPI. Pictures were taken with the Zeiss LSM 710 microscope.

Rapid Apc inactivation in $Apc^{fl/fl}$ mice

To induce recombination, mice were given daily intraperitoneal injections of β-napthoflavone (80 mg/kg) as previously described (Sansom et al., 2004). At each time point, mice were killed and intestines removed and flushed with water. Intestines were dissected as follows: The proximal 7 cm was mounted en face, fixed overnight in methacarn (methanol, chloroform, and acetic acid; 4:2:1 ratio), and paraffin embedded. The following 3 cm was opened and placed into RNA later (Sigma), ensuring that all mesentery and Peyers patches were removed. The following 5 cm was divided into 1-cm lengths, bundled using surgical tape, and then fixed in 4% formaldehyde at 4°C for no more than 24 h before processing. Samples for Western analysis, frozen sections, and electron microscopy were then taken from the next 5 cm of intestine, and the remainder was fixed in methacarn. Apoptosis and mitotic index were scored from hematoxylin-and-eosin-stained sections as previously described (Sansom et al., 2004). Apoptosis was independently confirmed by immunohistochemical staining with an antibody against active caspase 3 (1:750; R&D systems). For BrdU labeling, mice were injected with 0.25 mL of BrdU (Amersham), and staining was performed using an anti-BrdU antibody conjugate (Roche) at 1 part in 50.

Histological Analysis

Colons were examined using 4 μm thick, 200 μm step serial sections stained with hematoxylin and eosin. For TUNEL assay, an In Situ Cell Death Kit (Roche, Indianapolis, Indiana) was used according to the manufacturer's recommendations. MicroRNA detection was performed on colon cancer tissues from mice intestines or human colon cancer sections by *in situ* hybridization (ISH) as previously described (Nuovo 2010). The negative controls included omission of the probe and the use of a scrambled LNA probe. Immunohistochemistry for Ki-67 was performed as previously described at the OSU comparative pathology core facility.

Statistical Analysis

Expression graphs and Wilcoxon matched-pairs tests were used to analyze differences in microRNA expression between tumors and paired non-tumorous tissue for all quantitative RT-PCR data using Graphpad Prism 5.0 (Graphpad Software Inc, San Diego, California). Results of statistical analyses are expressed as mean ± SD unless indicated otherwise. Comparisons between groups were performed using the two-tailed Student's t test. A p value <0.05 was considered significant. Graphpad Prism version 5.0 was used for Pearson correlations and survival analysis correlation.

SUPPLEMENTAL REFERENCES

Iliopoulos, D., Rotem, A. and Struhl, K. (2011). Inhibition of miR-193a expression by Max and RXRα activates K-Ras and PLAU to mediate distinct aspects of cellular transformation. Cancer Res. *71*, 5144-53.

Koo, B.K., Stange, D.E., Sato, T., Karthaus, W., Farin, H.F., Huch, M., van Es, J.H. and Clevers, H. (2011) Controlled gene expression in primary Lgr5 organoid cultures. Nat Methods. *9*:81-3.

Lennox, K.A. and Behlke, M.A. (2011). Chemical modification and design of anti-miRNA oligonucleotides. Gene Therapy *18*, 1111–1120.

Nuovo, G.J. (2010). In situ detection of microRNAs in paraffin embedded, formalin fixed tissues and the co-localization of their putative targets. Methods. *52*, 307-15.

Sato, T., Stange, D.E., Ferrante, M., Vries, R.G., Van Es, J.H., Van den Brink, S., Van Houdt, W.J., Pronk, A., Van Gorp, J., Siersema, P.D. and Clevers, H. (2008). Long-term expansion of epithelial rganoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology. *141*, 1762-72.

Valeri, N., Gasparini, P., Braconi, C., Paone, A., Lovat, F., Fabbri, M., Sumani, K.M., Alder, H., Amadori, D., Patel, T., Nuovo, G.J., Fishel, R. and Croce, C.M. (2010). MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2). Proc Natl Acad Sci U S A.*107*:21098-103.