Supporting Information

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SI Materials and Methods

Primary Tumors. Primary tumor RNAs were obtained as previously described (1).

Cell Lines and Transfection. HEK293 [American Type Culture Collection (ATCC); CRL-1573] and HCT116 (ATCC; CCL-247) cell lines were cultured with Iscove's modified Dulbecco's medium with 10% FBS. hsa-miR-483-3p precursor (Sanger accession no. MI0002467) and negative control 2-ribo-oligonucleotide were from Applied Biosystems/Ambion. siRNAs for MYC (L-003282-00-0005), β-catenin gene (CTNNB1) (L-003482-00), and scramble negative control (D-001810-10-20) were from Dharmacon. Transfection of miRNAs, siRNAs, and expression vectors was carried out with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's procedure.

Luciferase Assays and Vectors. The human 3′UTR of CTNNB1 was amplified by PCR using the primers indicated in Table S3 and cloned downstream of the firefly luciferase gene in the pGL3 control vector (Promega). Substitutions into the miR-483-3p binding sites of the CTNNB1 3′UTR genes were introduced by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions using the primers indicated in Table S3. As a reference, the pRLTK vector (Promega), which expresses Renilla luciferase, was used. Transfection was conducted in HEK293 and HCT116 cells cultured in 24-well plates; each well was cotransfected with 400 ng of pGL3 control vectors together with 40 ng of pRLTK reference vector (Promega) and 30 pmol of miR-483-3p or negative control 2. Twenty-four hours after transfection, *firefly* and *renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay (Promega). β-Catenin and upstream stimulating transcription factor 1 (USF1) expression vectors were generated by cloning β-catenin cDNA and USF1 cDNA into pIRES-Neo2 (Clontech) and pCMV-Tag (Agilent Technologies) vectors; see Table S3 for primer sequences. To test β-catenin nuclear activity, pGL3-OT and pGL3-OF luciferase-responsive vectors were used (2). pGL3-OT is a T-cell factor/lymphoid enhancer factor (TCF/LEF) protein family-responsive reporter; pGL3-OF is a negative control with a mutated TCF/LEF binding site. Each transfection was repeated at least twice in triplicate. To analyze the promoter of miR-483 we used pGL4 luc2 0.1 (Promega), where we cloned the SV40 enhancer downstream of the luciferase gene.

RNA Isolation and Quantitative Real-Time RT-PCR. Purification of total RNA using TRIzol reagent (Invitrogen) was carried out following the manufacturer's instructions. miR-483-3p, miR-483-5p, pri-miR-483, IGF2, β-catenin, β-actin, and RNU6B probes (Table S3) (Applied Biosystems) were used for mature miRNA, pri-miRNA, and mRNA gene quantification according the manufacturer's protocol. The retrotranscription step was conducted according to the manufacturer's protocol with a TaqMan MicroRNA Reverse Transcription Kit for miRNA and High Capacity cDNA Reverse Transcription Kit for genes and pri-miRNA quantification (Applied Biosystems). Quantitative real-time RT-PCR (qRT-PCR) analysis for the second intron of the IGF2 gene was carried out with Power SYBR Green PCR Master Mix (Applied Biosystems). To avoid genomic contamination in the quantification of IGF2 intron 2 and pri-miRNA-483, RNA samples were previously treated with DNase (TURBO DNA-free Kit; Ambion). The list of primers that we used herein is shown in Table S3. The levels of miRNA and mRNA were measured using Ct (threshold cycle). The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta Ct}$ (Applied Biosystems).

Protein Isolation, Immunoblot Analysis, and Immunoprecipitation. Cells were collected from six-well plates using trypsin-EDTA (Sigma) and dissolved in lysis buffer (M-PER Mammalian Protein Extraction Reagent; Thermo Scientific) freshly supplemented with complete protease inhibitor and phosphatase inhibitor mixtures 1 and 2 (Roche) in accordance with the manufacturer's procedure. Nuclear extracts were prepared by dissolving cells in nuclear and cytoplasmic extraction reagents (Thermo Scientific) following the manufacturer's procedure. After electrophoresis and blotting, primary antibodies (Cell Signaling; anti-CTNNB1 9562, anti-MYC 5605) (Santa Cruz Bioechnology; anti-USF1 sc-229) were incubated overnight at 4 °C and then peroxidase-conjugated anti-mouse or anti-rabbit antibodies were incubated for 1 h at room temperature. Detection was conducted by chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Scientific). β-Actin antibody (Cell Signaling; 4967) or vinculin antibody (Millipore; MAB3574) was used to normalize protein loading. To quantify Western blot signals, digital images of autoradiographs were acquired with a Fluor-S MultiImager, and band signals were quantified in the linear range of the scanner using specific densitometric software (Bio-Rad). Immunoprecipitation was carried out with 100 μg of nuclear extracts in 500 μL of binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 7.5) with 2 μg of USF1 (Santa Cruz Biotechnology; sc-229) or CTNNB1 antibody (Cell Signaling; 9562) for 2 h at 4 °C with gentle rotation. The immunoprecipitate was recovered with 25 μL of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) overnight at 4 °C with gentle rotation. Then the immunoprecipitate was washed in 0.1% Nonidet P-40 three times and analyzed by Western blot.

Electrophoretic Mobility Shift Assay. Protein/DNA binding was determined by electrophoretic mobility shift assay (EMSA) according to the manufacturer's procedure with biotinylated probes (Pierce; LightShift Chemiluminescent EMSA Kit); see Table S3 for oligonucleotide sequences. Samples were incubated with E-box wild-type probe or the mutant form and with 100× E-box wild-type unlabeled probe, and with specific polyclonal USF1 antibody (C-20, sc-229; Santa Cruz Biotechnology) before the addition of the probe. The DNA–protein complexes were resolved on 5% (wt/vol) nondenaturing acrylamide gels and visualized by exposure to autoradiographic films.

Pull-Down Experiment. Pull-down assay was performed with 10 nmol of recombinant proteins GST (ab85244; Abcam), GST-β-catenin (ab63175; Abcam), and His-USF1 (ab82069; Abcam). Reactions were carried out in binding buffer (150 mM NaCl, 20 mM Tris·HCl, pH 8, 1% Nonidet P-40, 1 mM EDTA), 1 mM PMSF, and protease inhibitors at 4 °C for 1 h of gentle rocking. The protein–protein complexes formed on the resin [Glutathione Sepharose 4 Fast Flow (Amersham) or Ni-NTA Agarose (Qiagen)] were brought down by centrifugation (500 × *g*). The resin was washed five times for 5 min at 4 °C with 1 mL of cold wash buffer (250 mM NaCl, 20 mM Tris·HCl, pH 8, 1% Nonidet P-40, 1 mM EDTA). The protein complexes were resolved on SDS/PAGE and immunoblotted, and detection was conducted by chemiluminescent metti l'ECL (Pierce).

DNA Purification and Methylation Analysis. Genomic DNA was isolated from cell lines and tissue by standard treatment with SDS and EDTA in the presence of 200 μg/mL of proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The methylation status of miR promoters was determined by bisulfite sequencing PCR (3). One microgram of genomic DNA was subjected to treatment with the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Specific primers for PCR and sequencing were designed through Methyl Primer Express v1.0 software (Applied Biosystems) (Table S3).

1. Veronese A, et al. (2010) Oncogenic role of miR-483-3p at the IGF2/483 locus. *Cancer Res* 70(8):3140–3149.
2. Suzuki H, et al. (2004) Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 36(4):417–422.
3. Olek A, Oswald J, Walter J (1996) A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res* 24(24):5064–5066.

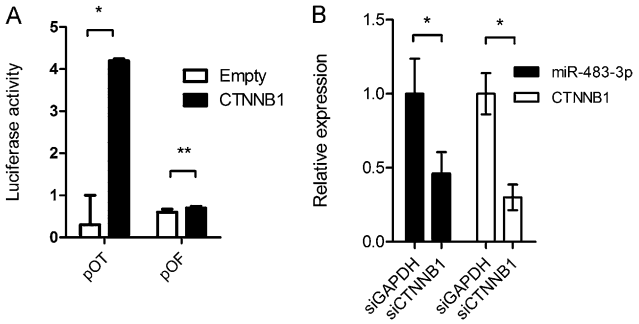

Fig. S1. (*A*) Ability to activate the luciferase reporter vector pGL3-OT containing the β-catenin/TCF-responsive element by exogenous expression of β-catenin. pGL3-OF, which carries a mutated β-catenin/TCF binding site, was used as a negative control. (*B*) miR-483-3p (black bars) and CTNNB1 (white bars) relative expression in an HCT116 cell line transfected with CTNNB1 and GAPDH siRNAs at 36 h from transfection. $*P < 0.02$, $**P > 0.02$.

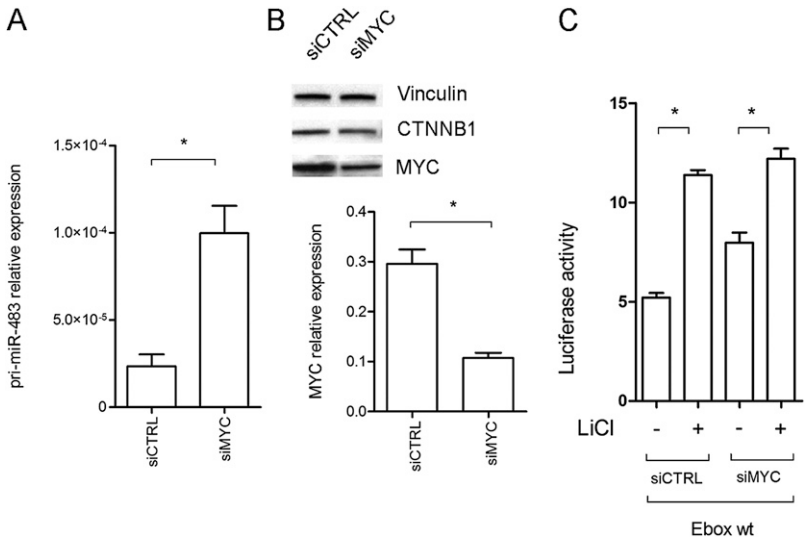

Fig. S2. MYC transcription factor represses miR-483 transcription. (*A*) Pri-miR-483 expression in HEK293 cells transfected with siRNA control (siCTRL) and a specific siRNA for MYC (siMYC). (*B*) On the same samples, the MYC and CTNNB1 proteins were assessed by Western blot to ensure MYC silencing and that CTNNB1 was not involved in this experiment (*Upper*). (*Lower*) MYC mRNA expression by qRT-PCR. (*C*) Luciferase activity of wild-type (wt) pGL4E_Ebox with and without treatment with LiCl in HEK293 cells transiently knocked down for MYC by siRNA (siMYC). Firefly luciferase activity was normalized on Renilla luciferase activity of the cotransfected pGL4R vector. $*P < 0.02$.

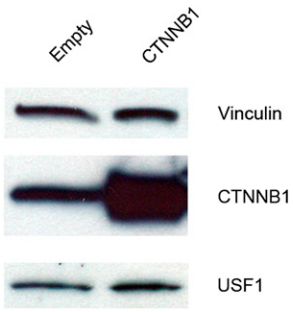

Fig. S3. Western blot analysis of β-catenin and USF1 in HEK293 cells transfected with the β-catenin expression vector. Vinculin was used as protein load control.

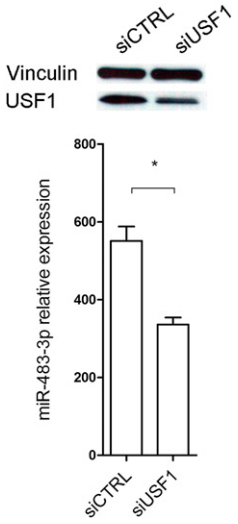

Fig. S4. Western blot analysis of USF1 protein in HepG2 cells transfected with USF1 siRNA at 72 h from transfection (*Upper*) and the corresponding miR-483-3p relative expression (*Lower*). *$P < 0.02$.

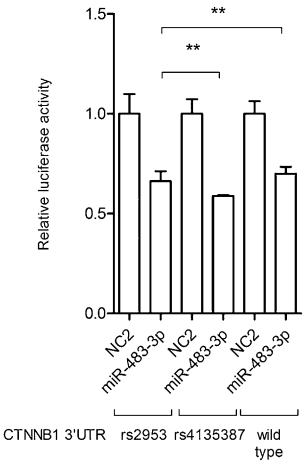

Fig. S5. CTNNB1 3′UTRs regulate luciferase activity dependent on miR-483-3p in HEK293 cells independently of the SNPs rs2953 and rs4135387 when compared with the wild-type reference sequence. Firefly luciferase activity was normalized on Renilla luciferase activity of the cotransfected pRL-TK vector. **$P > 0.02$.

Table S1. Mutational status of Wnt/β-catenin genes is associated with miR-483-3p expression

Sample	miR-483-3p relative expression	IGF2 relative expression	Ratio miR-483-3p/ IGF2	CTNNB1 exon 3 status	APC status (GeneBank accession no. NM000038 from nt 3361–4840)	AXIN 1 status (GeneBank accession no. NM003502 from nt 745–2186)
HCC_01	0.02	0.03	0.9	0	0	0
HCC_02	0.09	1.55	0.1	GAC→TAC_Asp32→Tyr	0	NC
HCC_03	0.15	0.04	3.8	0	0	0
HCC_04	0.16	12.47	0.0	0	0	0
HCC_05	0.21	0.17	1.3	0	NC	NC
HCC_10	1.12	0.14	7.7†	0	0	NC
HCC_12	1.32	0.67	2.0	NC	0	NC
HCC_13	1.44	0.12	11.7†	0	0	0
HCC_14	2.20	17.90	0.1	0	0	0
HCC_16	2.92	0.22	13.3*	0	0	NC
HCC_17	3.47	3.58	1.0	0	0	0
HCC_18	3.54	3.18	1.1	0	0	0
HCC_20	4.47	18.02	0.2	NC	NC	NC
HCC_21	6.63	0.94	7.0†	0	del 3719–3740	0
HCC_22	9.55	4.15	2.3	0	0	0
HCC_23	11.63*	10.89	1.1	TCT→CCT_Ser37→Pro	0	0
HCC_25	15.73*	3.01	5.2†	TCT→TAT_Ser37→Tyr	0	0
HCC_26	24.98*	0.32	77.1†	CAT→CCT_His36→Pro	0	0
HCC_28	55.52*	17.62	3.2	0	0	0
HCC_29	66.03*	1.26	52.3†	0	0	del 1024–1027 nt
HCC_30	69.23*	12.29	5.6†	GAC→GGC_Asp32→Gly	0	0
HCC_31	116.16*	11.78	9.9†	0	0	0
HCC_33	374.10*	1050.30	0.4	0	0	NC
HCC_34	388.40*	63.37	6.1†	0	NC	CCT→ACT_Pro486→Thr

Twenty-four HCC samples analyzed for IGF2 and miR-483-3p expression and for the mutational status of CTNNB1 exon 3, APC, and Axin genes are listed. APC, adenomatous polyposis coli; NC, not classified; 0, not mutated.

*miR-483-3p expression values greater than the considered cutoff (10).

†miR-483-3p/IGF2 expression ratio values greater than the considered cutoff (5).

Table S2. Methylation status of the three CpG dinucleotides close to the CTCF binding site

Cell line	miR-483-3p relative expression	Methylation status CpG 1 (%)	Methylation status CpG 2 (%)	Methylation status CpG 3 (%)
H460	0.015	0	50	0
SNU 423	0.022	0	50	0
PLC/PRF/5	0.029	0	0	0
SNU 449	0.042	0	50	50
MDA MB231	0.23	75	100	50
K562	0.639	25	50	NC
SW 480	0.697	0	0	NC
SNU 387	0.759	25	50	NC
SKHEP 1	0.789	0	0	0
HEK293	1	50	100	100
MCF7	3.543	75	100	100
HEPG2	23.87	100	100	100
HUH 7	44.985	100	100	100
HEPG2-C3A	51.464	75	100	100

Table S3. Oligonucleotide sequences used for cloning, qRT-PCR, mutational analysis, site-directed mutagenesis, bisulfite sequencing, and EMSA

	3Gene	Name	Sequence (5′-3′)
Gene cloning	CTNNB1	CTNNB1_215F	ATGGCTACTCAAGCTGATTTGATGG
	CTNNB1	CTNNB1_2560R	ACAGGTCAGTATCAAACCAGGC
	CTNNB1_3′UTR	bCAT_2677F_Xba	GttctagaTGCCACAAAAACAGGTAT
	CTNNB1_3′UTR	bCAT_3193R_Xba	GttctagaTTCAAAGCAAGCAAAGTC
	USF1	USF1_BamHI_F	caaggatccCCCCCTCACAGAGAGATGAA
	USF1	USF1_EcoRI_R	caagaattcGGATTAGGCTGTTGCTCCTG
qRT-PCR (SYBR Green)	IGF2	IGF2_3044F	TCCTCCCTGGACAATCAGAC
	IGF2	IGF2_5965R	AGAAGCACCAGCATCGACTT
	IGF2	IGF2_5965F	AAGTCGATGCTGGTGCTTCT
	IGF2	IGF2_7866R	CGGAAACAGCACTCCTCAA
	IGF2	IGF2_6907F	GTGAAATGGGCTCACAGGAT
	IGF2	IGF2_7252R	AGCAATGCTCAGCTGGAAG
	IGF2	IGF2_7846F	CGTTGAGGAGTGCTGTTTCC
	IGF2	IGF2_8307R	GGACTGCTTCCAGGTGTCAT
	IGF2_3′UTR	IGF2-3	CTTGGACTTTGAGTCAAATTGG
	IGF2_3′UTR	IGF2-2	CGGGGATGCATAAAGTATGAG
	18S	18s_F	CTGCCCTATCAACTTTCGATGGTAG
	18S	18s_R	CCGTTTCTCAGGCTCCCTCTC
qRT-PCR (TaqMan probe)	IGF2-INS	Hs01005963_m1	
	CTNNB1	Hs00355045_m1	
	MYC	Hs00153408_m1	
	β-actin	Hs00357333_g1	
	Pri-miRNA hsa-mir-483	Hs03293803_pri	
	hsa-miR-483–5p	Assay ID 002338	
	hsa-miR-483–3p	Assay ID 002339	
	RNU6B	Assay ID 001093	
Mutational analysis	CTNNB1	BCAT3	AAAATCCAGCGTGGACAATGG
	CTNNB1	BCAT2	TGTTCTTGAGTGAAGGACTGA
	APC	APC_3361F	TTTTGGACAGCAGGAATGTG
	APC	APC_4091R	TGCTGGATTTGGTTCTAGGG
	APC	APC_4072F	CCCTAGAACCAAATCCAGCA
	APC	APC_4840R	TGTTGGCATGGCAGAAATAA
	Axin1	Axin1_745F	CAGGCCACTATGGAGGAAAA
	Axin1	Axin1_1583R	CTCTCAGGGTTCTCCTCGTG
	Axin1	Axin1_1429F	ATGGAGGAGGAAGGTGAGGA
	Axin1	Axin1_2186R	AGGGACAAGGGTCTGGAGTT
Site-directed mutagenesis	miR-483 (promoter)_Ebox site	483_Ebox-mut_F	CACCTGACACTCTTAGGTCTGGATCTTTACCACC
	miR-483 (promoter)_Ebox site	483_Ebox-mut_R	GGTGGTAAAGATCCAGACCTAAGAGTGTCAGGTG
	CTNNB1	CTNNB1_483mut_F	AACAATACAAATGGATTTTGTCGCCTCCTCAAGAA GTGAAGAATG
	CTNNB1	CTNNB1_483mut_R	CATTCTTCACTTCTTGAGGAGGCGACAAAATCCAT TTGTATTGTT
	miR-483 (promoter)_CTCF_BS1	CTCF_mut1F	CTCCTGCTcactggCTGATaGCAtCTGCCCTTTGGC
	miR-483 (promoter)_CTCF_BS1	CTCF_mut1R	cAGaTGCtATCAGccagtgAGCAGGAGCCCCATCAC
	miR-483 (promoter)_CTCF_BS2	CTCF_mut2F	CCTTTGGCAAGGTGGAGCCCCCAGCGACCTTCCC
	miR-483 (promoter)_CTCF_BS2	CTCF_mut2R	CCACCTTGCCAAAGGGCAGGTGCCATCAGCCGGA
Bisulfite sequencing PCR	miR-483 (promoter)	483MetF2	GGTAGGAAGTGGTATTGTAGGG
	miR-483 (promoter)	483MetR2	ACCCCCACAAAAAAAACTACT
EMSA probes	miR-483 (promoter)	483_Ebox wt	ACCTGACACTCACCACGTGACATCTTTACCACC
	miR-483 (promoter)	483_Ebox mut	ACCTGACACTCTTAGGTCTGGATCTTTACCACC