Supporting Information

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SI Materials and Methods

Plasmids and siRNAs. A VSV-tagged *AP4* fragment was generated by amplification of the *AP4* cDNA from a full length cDNA clone (IRATp970D057D, RZPD, Berlin, Germany) using the primers AP4-Fwd: 5′-GGATCCGGAACCATGGAGTATTTCATG-GTGCCCA-3‘ and AP4-Rev: 5′: CGTCTAGATCCGG-GAAGCTCCCCGTCCCCCGACG-3‘. The amplified fragment was cloned into pcDNA3-VSV using BamHI and XbaI restriction sites to obtain pcDNA3-AP4-VSV and the *AP4* ORF (ORF) was verified by sequencing. Deletion of the AP4 basic region was achieved by amplification of two AP4 fragments using the primers AP4-Fwd (see above) and AP4-Del-Rev: 5′-CGAGATCTCTCCTGGTCCCGCTGAGTCTC-3′ or AP4-Del-Fwd: 5′-GCGGATCCATGCAGAGCATCAACGCGG-GATTC-3′ and AP4-Rev (see above). The resulting fragments were cloned into pcDNA3-VSV to create pcDNA3-AP4ΔBR. Site-directed mutagenesis of AP4 was achieved using the QuikChange II site directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer. Primers used for mutation of *AP4* are depicted in Table S1. To generate the episomal pRTS-1-AP4-VSV vector, the AP4-VSV ORF was isolated from pcDNA3-AP4-VSV and ligated into the pUC19-SfiI shuttle vector (2) via BamHI and NotI restriction sites. A SfiI fragment was ligated into pRTS 1 (2) via SfiI sites generating pRTS1-AP4-VSV. For generation of pBpuro-*AP4ER* vector, *c-myc* was removed by restriction with BamHI from pBpuro *c-mycER* (3) and the AP4 ORF was inserted and verified by sequencing.

The pRSM vector was generated by removing the H1 promoter from pRetroSuper (4) with a BglII-EcoRI restriction and ligation with a BamHI-EcoRI fragment containing the zinc-inducible pMT1-promoter of U265 (5). AP4-VSV was isolated from pcDNA3-VSV and inserted into the EcoRI site (blunt) of pRSM vector to generate pRSM-AP4.

pGL3b reporter plasmids containing the wild-type or mutant *p21* promoter (6) were a gift from Carme Gallego (Universitat de Lleida, Lleida, Spain). Site-directed mutagenesis of the CAGCTG sequences A1 and A2 (see Fig. 5*C*) was achieved using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer. Primers are shown in Table S1. pXP2-luc reporter plasmids containing the minimal c-MYC responsive region of the *p21* promoter and mutated derivatives (1) were a gift from Lars Gunnar Larsson (Karolinska Institute, Stockholm). The wild-type p53 expression vector pCEP4-p53 was described previously (7).

siRNAs were transfected at 10 nM final concentration using HiPerFect reagent (Qiagen). siRNA target sequences were as follows: AP4-specific siRNAs #1: 5′-GGUGCCCUCUUUG-CAACAU-3′, #2: 5‘-GUGAUAGGAGGGCUCUGUAG-3′, nonsilencing control siRNA NonS: 5′-UUGUCUUGCAUUC-GACUAAUU-3′.

Cell Lines/Culture and Reagents. Human diploid fibroblast (HDF), MCF-7, U-2OS, H1299, HaCaT, Phoenix A cell lines, and their derivatives were maintained in DMEM (Invitrogen) containing 10% FBS. U-937 cells and their derivatives were maintained in RPMI medium (Invitrogen) containing 10% FBS. DLD-1 colorectal cancer cells were maintained in McCoy's 5A Medium (Invitrogen) containing 10% FBS. RAT1-MYC-ER were cultured in DMEM with 8% FBS. All cell lines were cultivated in presence of 100 units/ml penicillin and 0.1 mg/ml streptomycin. Etoposide (Sigma) was dissolved in DMSO (40 mg/ml stock solution) and used at a final concentration of 20 μg/ml. ICI182,780 (Fulvestrant, Biotrend Chemikalien) was dissolved in DMSO (1 mM stock solution) and used at a final concentration of 1 μM. Recombinant Transforming Growth Factor Beta 1 (TGF-β1; R&D Systems) was reconstituted in 4 mM HCl with 1 mg/ml BSA (5 μg/ml stock solution) and used at a final concentration of 5 ng/ml. 4-Hydroxyltamoxifen (4-OHT, Sigma) was dissolved in ethanol (4 mM stock solution) and used at a final concentration of 200 nM. Doxycycline (Sigma) was dissolved in water (100 μg/ml stock solution) and used at a final concentration of 100 ng/ml. TPA (Axxora) was dissolved in ethanol and used at a final concentration of 10 nM.

Generation of Cell Lines. U-2OS osteosarcoma cells were transfected with pRTS1 or pEMI plasmids using FuGene reagents (Roche). After 48 h, cells were transferred into media containing 150 μg/ml hygromycin for 2 weeks. Homogeneity of the derived cell pools was tested by addition of 100 ng/ml doxycycline for 24 h and detection of mRFP by fluoresence microscopy.

For retrovirus production, Phoenix-A packaging cells were transfected with pBABE-AP4-ER vector or pRSM-AP4 or empty vector using calcium phosphate precipitation. Twenty-four hours after transfection, retrovirus-containing supernatants were harvested, passed through 0.45-μm filters, and used to infect HaCaT keratinocytes or U-937 myoblasts in the presence of polybrene (8 μg/ml) four times in 4 h intervals. After 48 h, cells were selected by growing them in the presence of 1.5 μg/ml puromycin for 10 days.

Western Blot Analysis and Antibodies. Cells were lysed in RIPA lysis buffer [50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 1% Nonidet P-40, 0.5% (wt/vol) sodium deoxycholate, 0.1% SDS, complete mini protease inhibitors (Roche)]. Lysates were sonicated and centrifuged at 16.060 $\times$ *g* for 15 min at 4°C. Per lane, 30–60 μg of whole-cell lysate was separated on 6% or 12% SDS-acrylamide gels and transferred on Immobilon PVDF membranes (Millipore). For immunodetection, membranes were incubated with antibodies specific for c-MYC (#1472–1, Epitomics or sc-764, Santa Cruz Biotechnology), AP4 (N-17, Santa Cruz), p53 (DO-1, Santa Cruz), p21 (Ab-11, NeoMarkers), Rb (G3–245, BD PharMingen), $p15^{Ink4b}$ (C-20, Santa Cruz), ER (gift from Goeffrey Greene, University of Rhode Island), VSV-tag (hybridoma clone p5d4), β-actin (A-2066, Sigma) and α-tubulin (DM 1A, T-9026, Sigma). Signals from HRP (horse-radish-peroxidase)-coupled secondary antibodies were generated by enhanced chemiluminescence (Perkin–Elmer Life Sciences) and recorded with a CCD camera (440CF imaging system, Eastman Kodak).

Cell-Based Reporter Assays. H1299 non-small cell lung cancer cells were transfected using FuGene Reagent (Roche) in 12-well plates with 15 ng of Renilla luciferase control reporter plasmid pRL, 600 ng firefly luciferase reporter constructs containing either wild-type or mutant *p21* promoter sequences (1, 6), 20 nM pcDNA-AP4-VSV (wild type or mutant AP4), or an equimolar amount of pcDNA3 backbone. For p53-mediated *p21* reporter activation, 400 ng firefly luciferase reporter constructs containing either wild-type or mutant *p21* promoter sequences (6), 250 ng pcDNA-AP4-VSV or an equimolar amount of pcDNA3 backbone and 0, 50 or 200 ng pCEP4-p53 or equimolar amounts of pCEP4 backbone were used. Firefly and Renilla luciferase activities were measured 36 h after transfection using the

Dual-luciferase assay (Promega). Firefly activity was normalized to Renilla luciferase activity.

Quantitative Real-Time PCR (qPCR). Total RNA was isolated by using the Total RNA Isolation System (Promega) or the RNeasy Mini Kit (Qiagen). cDNA was generated from 1 μg of total RNA per sample using anchored oligo(dT) primers (Reverse-iT First Strand Synthesis; ABgene). RT-qPCR was performed by using the LightCycler (Roche) and the FastStart DNA Master SYBR Green 1 kit (Roche) as described (8) or the Realplex 4 Mastercycler epgradientS (Eppendorf) and the Fast SYBR Green Master Mix (Applied Biosystems). The sequences of oligonucleotides used as qPCR primers are available in Table S2.

ChIP Assay. U-2OS and MCF-7 cells and their derivatives were cultured as described above. For cross-linking formaldehyde (Merck) was added to a final concentration of 1%. The reaction was stopped by addition of glycine at a final concentration of 0.125 M. Processing of cells was done as described in (9). Chromatin was sheared by sonication (Sonifier: Bandelin HD70 Sonoplus or BioRuptor, Biogenode) to generate DNA fragments with an average size of 700 bp. Preclearing and incubation with antibodies was performed as described in ref. 9 by using polyclonal c-MYC antibody (sc-764, Santa Cruz) or rabbit anti mouse IgG (M-7023 SIGMA) in case of MCF-7-PJMMR1-derived lysates or a monoclonal anti-VSV antibody (clone P5D4) or Protein G purified mouse IgG from mouse preimmune serum (S-7273, Sigma) for lysates from AP4-VSV-expressing U-2OS cells. Washing and reversal of cross-linking was performed as described (10). Purified DNA was first analyzed by amplification of a genomic fragment from chromosome 16q22. This amplification product was used to equalize DNA input into the qPCR reactions. Primer sequences used for ChIP are provided in Table S3.

BrdU Labeling for Detection of DNA Synthesis. DNA synthesis was monitored by incorporation of BrdU (10 μM; Roche) for 30 min at 37°C. Next, cells were harvested by trypsinization and centrifuged at 300 $\times$ *g* for 5 min. After washing with PBS, fixation was achieved by addition of ice-cold 70% ethanol and incubation for 30 min at −20°C. Fixed cells were resuspended in 0.1 mg/ml pepsin, and DNA was denatured by incubation in 2 M HCl for 30 min at room temperature. After centrifugation at 500 $\times$ *g*, neutralization was achieved by resuspending cells in 0.1 M $Na_2B_4O_7$. Cells were washed once with PBS and PTS buffer (PBS, 0.5% Tween 20, 2% FBS), respectively, and subsequently resuspended in 60 μl of PTS + 6 μl of anti-BrdU-FITC antibody (BD Biosciences PharMingen) or an appropriate isotype control IgG and incubated for 30 min at room temperature in the dark. Next, cells were washed two times with PTS and resuspended in 500 μl of PTS, 0.5 mg/ml RNaseA (Sigma), 50 μg/ml propidium iodide. After incubation for 30 min at room temperature, cells were analyzed by flow cytometry.

DNA Content Analyses by FACS. U-2OS cells were plated into T25 cell culture flasks and MCF-7 cells were plated into T25 cell culture flasks or 12-well plates. Floating cells and trypsinized cells were collected by centrifugation at 1,200 rpm (300 $\times$ *g*) for 7 min, fixed with ice-cold 70% ethanol, and stored overnight on ice. After washing with PBS, cells were incubated with FACS solution [PBS, 0.1% Triton X-100, 60 μg/ml propidium iodide (PI), 0.5 mg/ml DNase free RNase] at room temperature for 30 min. DNA content was determined by detection of propidium iodide fluorescence (FACSCalibur, Becton-Dickinson).

Proliferation Assay. MCF-7-PJMMR1 cells were plated in 12-well plates (1-ml culture volume). 200 μl of MTT solution [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide, 5 mg/ml in PBS, sterile-filtered] was added, and cells were incubated at 37°C for 4 h. 1 MTT triplex solution (500 μl, 10% SDS, 5% 2-butanol, 0.012 M HCl) was then added, and cells were incubated overnight at 37°C. OD570 and OD630 (background signal) were measured by using an ELISA reader (MRX Revelation, Dynex).

Indirect Immunofluorescence. Cells were fixed in 4% paraformaldehyde/PBS for 10 min, permeabilized in 0.2% Triton X-100 for 20 min, and blocked in 100% FBS for 1 h. For detection of ER-tagged AP4 protein, H222 antibody was used, which was kindly provided by Goeffrey Greene (University of Rhode Island) and as a secondary antibody Cy-conjugated AffiniPure Goat Anti-Rat IgG (112–165-143, Jackson ImmunoResearch Laboratories). Images were captured on an Axioplan2 microscope (Zeiss) equipped with a CCD camera (Monochrome No.70, Diagnostics Instruments) and the MetaVue software package (Universal Imaging).

Tissue Samples and Immunohistochemistry (IHC). Twelve cases of sporadic colorectal carcinomas were retrieved from the archives of the Institute of Pathology (Ludwig Maxmilians University, Munich). All carcinomas were World Health Organization grade 2 or 3. Patients had given informed consent, and none of the patients had received cancer therapy before surgical resection of the lesions. For IHC, biopsies from diagnostic colonoscopies were used to ensure that the tumor tissue had been immediately fixed after removal in neutral 4% buffered formalin. Sections were deparaffinized in xylene followed by rehydration with solutions of decreasing ethanol concentration. Next, sections were pretreated by microwave (2 $\times$ 15 min) in target retrieval solution pH 6.1 (DakoCytomation) for AP4 and Ki67, TUF target unmasking fluid (DakoCytomation) for c-MYC and ProTags IV Antigen Enhancer (Quartett Immundiagnostika) for p21 detection. After quenching of endogenous peroxidase activity with 7.5% H_2O_2 for 10 min, the staining procedure was performed using the VECTASTAIN Elite ABC Kit (Vector Laboratories) in case of c-MYC antibody (dilution 1:20) and AP4 antibody (dilution 1:125) and the LSAB2 System-HRP (DakoCytomation) was used for Ki67 (dilution 1:60) and p21 antibodies (dilution 1:60) essentially according to the manufacturer's protocol. The streptavidin/peroxidase system combined with exposure to DAB+ (3,3′-diaminobenzidine) chromogen (DakoCytomation) for 3 min in the case of AP4 detection or exposure to AEC (β-amino-9-ethyl-carbazole) (Zytomed) reagent for 10 min for detection of c-MYC, Ki67, and p21 was used for visualization. Counterstaining was performed with hematoxylin before mounting of sections in an aqueous mounting medium (Aquatex, Merck). Images were captured on a Zeiss Axioskop 40 microscope (×200 magnification) connected to a Leica DC500 camera.

Generation of Recombinant Adenoviruses and Infection of Target Cells. The adenoviral vector AdGFP-AP4-VSV was constructed by ligation of AP4-VSV cDNA into the plasmid AdTrack-CMV (11) followed by electroporation together with the Adeasy-1 plasmid (11) and recombination in *Escherichia coli* (BJ5183 EC, Qbiogene). Viral production and amplification in 911 cells (12) and purification of the recombinant adenovirus were performed as described (11). The minimal amount of virus needed to reach >90% infection efficiency was determined by monitoring GFP signals with fluorescence microscopy. HaCaT and DLD-1 cells were infected in serum free medium with adenovirus for 3 h and an equal amount of medium containing 20% FBS was added subsequently.

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7. Hermeking H, *et al.* (1997) 14–3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* 1:3–11.
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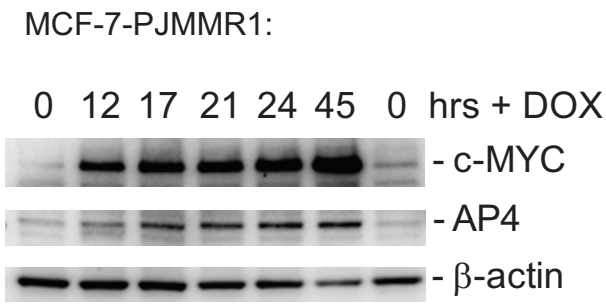

Fig. S1. AP4 expression is induced by c-MYC in MCF-7 cells. Protein lysates were prepared at the indicated time points. Expression of c-MYC, AP4 and β-actin was detected by immunoblotting.

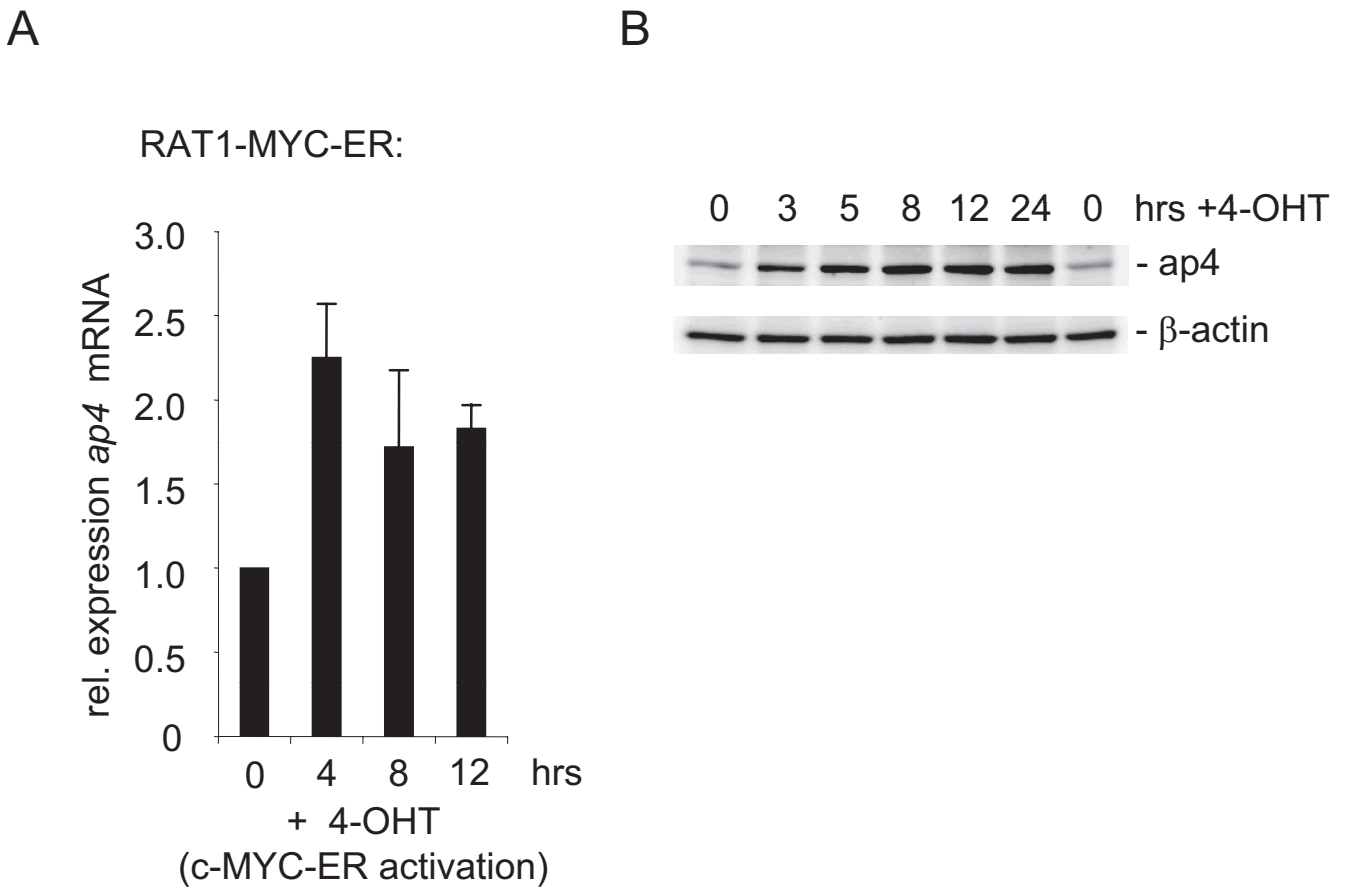

Fig. S2. *AP4* induction by c-MYC is evolutionary conserved. (*A*) RAT1-myc-ER cells were serum-deprived for 48 h. RNA was isolated at the indicated time points after treatment with 4-OHT and cDNA was analyzed for *ap4* expression by qPCR analysis. *EF1α* expression was used for normalization. (*B*) Immunoblot analysis of ap4 protein expression after addition of 4-OHT to serum deprived RAT-myc-ER cells.

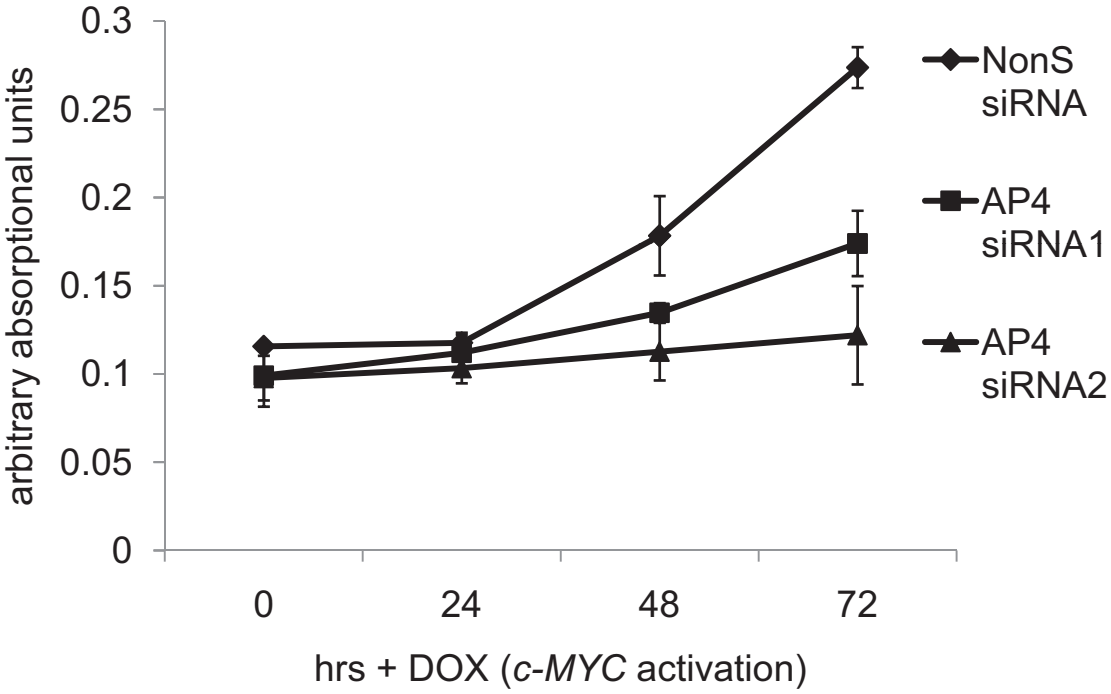

Fig. S3. Effect of *AP4* knockdown on c-MYC-mediated proliferation. MCF-7-PJMMR1 cells were transfected with the indicated siRNAs. 20 h after transfection cells were treated with the anti-estrogen ICI182,780 (1 μM) and 24 h later DOX (1 μg/ml) was added to induce ectopic expression of *c-MYC*. Quantification of viable cell numbers was performed using an MTT assay. Absorption units normalized to background are indicated. For each time point biological triplicates were measured. Error bars indicate standard deviations.

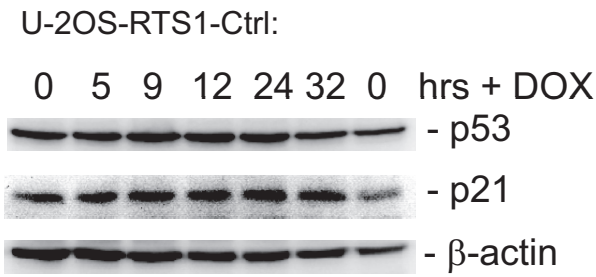

Fig. S4. Expression of p53, p21, and β-actin proteins was detected by immunoblot analysis after DOX treatment of U-2OS-RTS1-Ctrl cells.

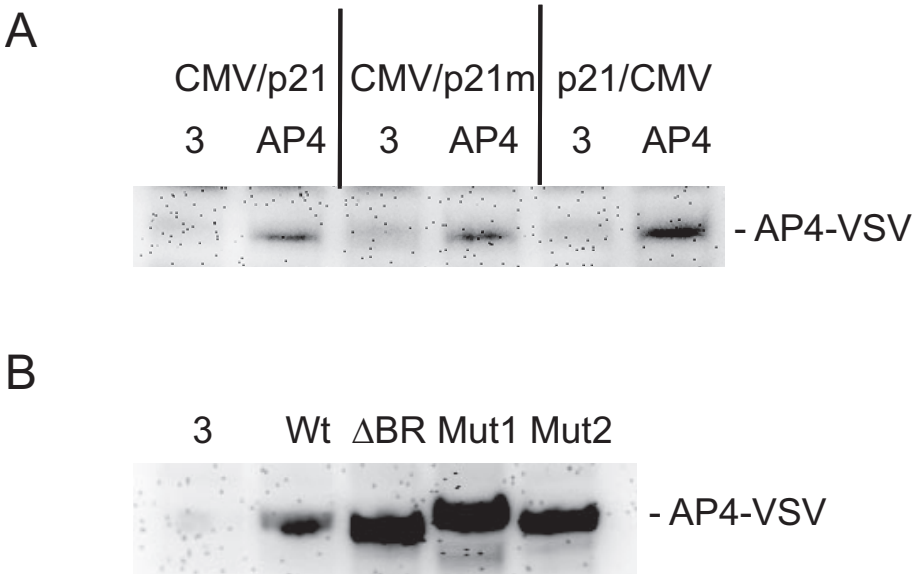

Fig. S5. Whole protein lysates from H1299 cells used for transient reporter assays were subjected to Western blot analysis. The amount of lysates was adjusted to Renilla luciferase expression levels detected by luminometric measurement (data not shown) to normalize for transfection efficiency. (*A*) CMV/p21, CMV/p21m(ut), p21/CMV: nomenclature according to Wu S *et al.* (1). (*B*) 3: pcDNA3-VSV; Wt: pcDNA3-AP4-VSV; ΔBR, Mut1 and Mut2: pcDNA3 vectors encoding for different VSV-tagged AP4 mutants.

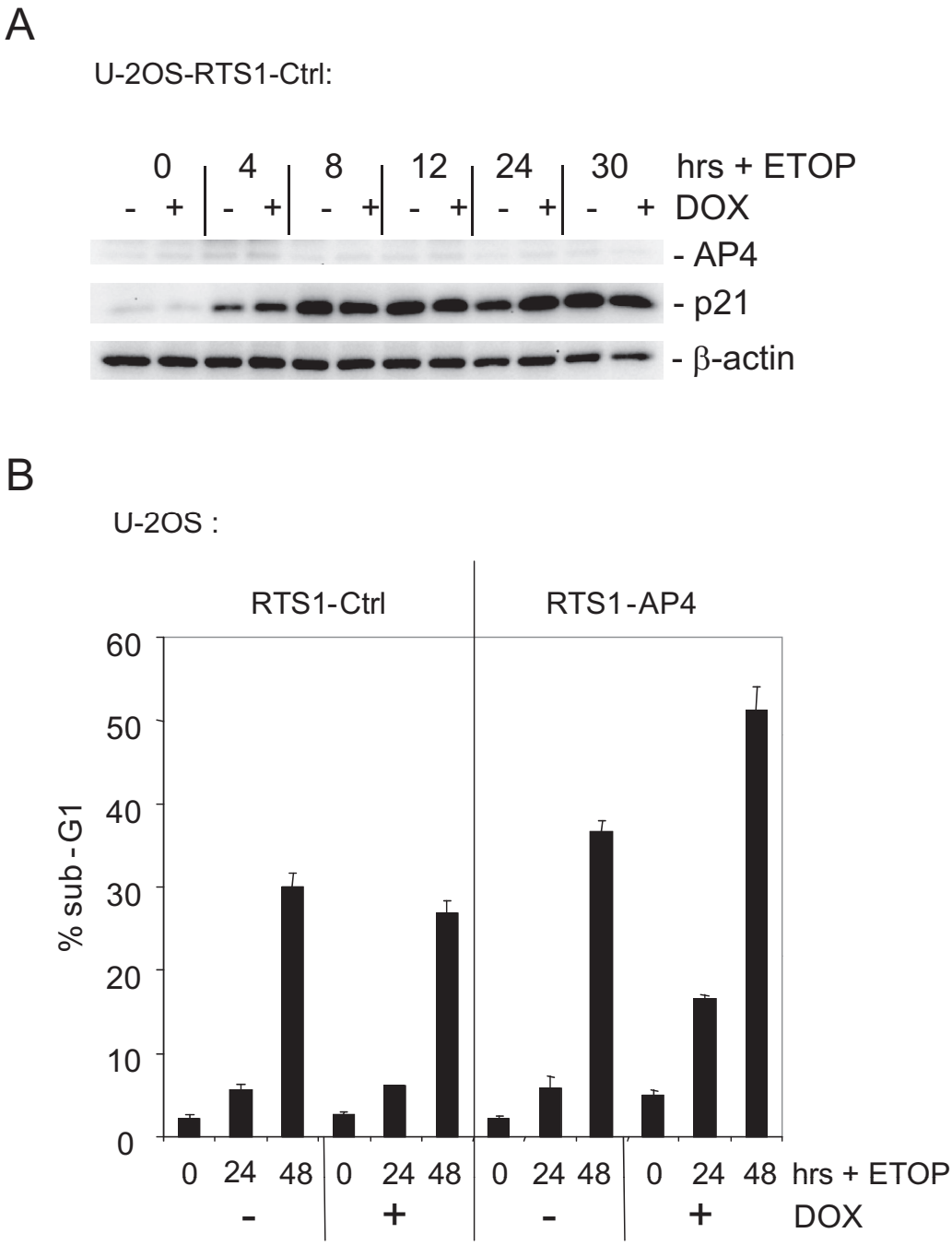

Fig. S6. (*A*) U-2OS cells expressing a control vector (RTS1-Ctrl) were left untreated or treated with DOX (100 ng/ml) for 12 h. After exposure to etoposide (ETOP, 20 μg/ml) for the indicated periods AP4-VSV, p21 or β-actin expression was detected by immunoblotting. (*B*) DOX (100 ng/ml) was added to U-2OS RTS1-AP4-VSV or U-2OS RTS1-Ctrl cells for 12 h. Then cells were treated with ETOP for the indicated durations and analyzed by flow cytometry. The diagram depicts the fraction of cells with subG$_1$ DNA content, which corresponds to cells undergoing apoptosis. The experiment was performed in triplicates. Standard deviations are depicted.

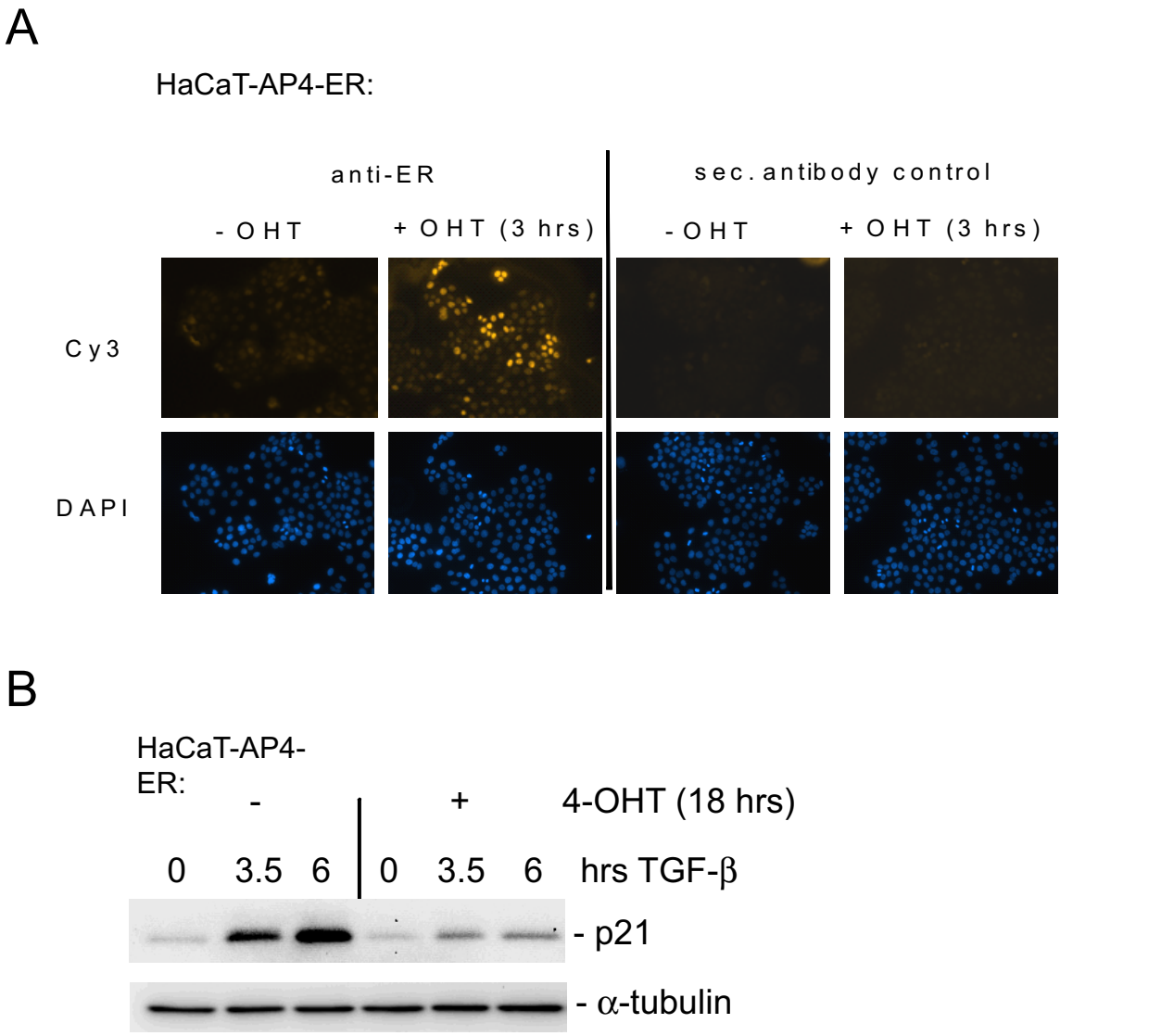

Fig. S7. Effect of AP4-ER activation on TGF-β signaling. (*A*) HaCaT cells stably expressing an AP4-ER fusion protein were treated with 200 nM 4-OHT for 3 h or left untreated. Subcellular localization of AP4-ER was detected by immunostaining using an anti-ER antibody. Signals generated by a Cy3-coupled secondary antibody were detected by fluorescence microscopy. 4-OHT treatment promoted the nuclear localization of AP4-ER and also the accessibility of AP4-ER to the anti-ER antibody resulting in stronger signals. (*B*) HaCaT cells stably infected with a retrovirus encoding AP4-ER were treated with 200 nM 4-OHT for 12 h. TGF-β (5 ng/ml) was added for the indicated periods. Expression of p21 and β-actin was determined by immunoblotting.

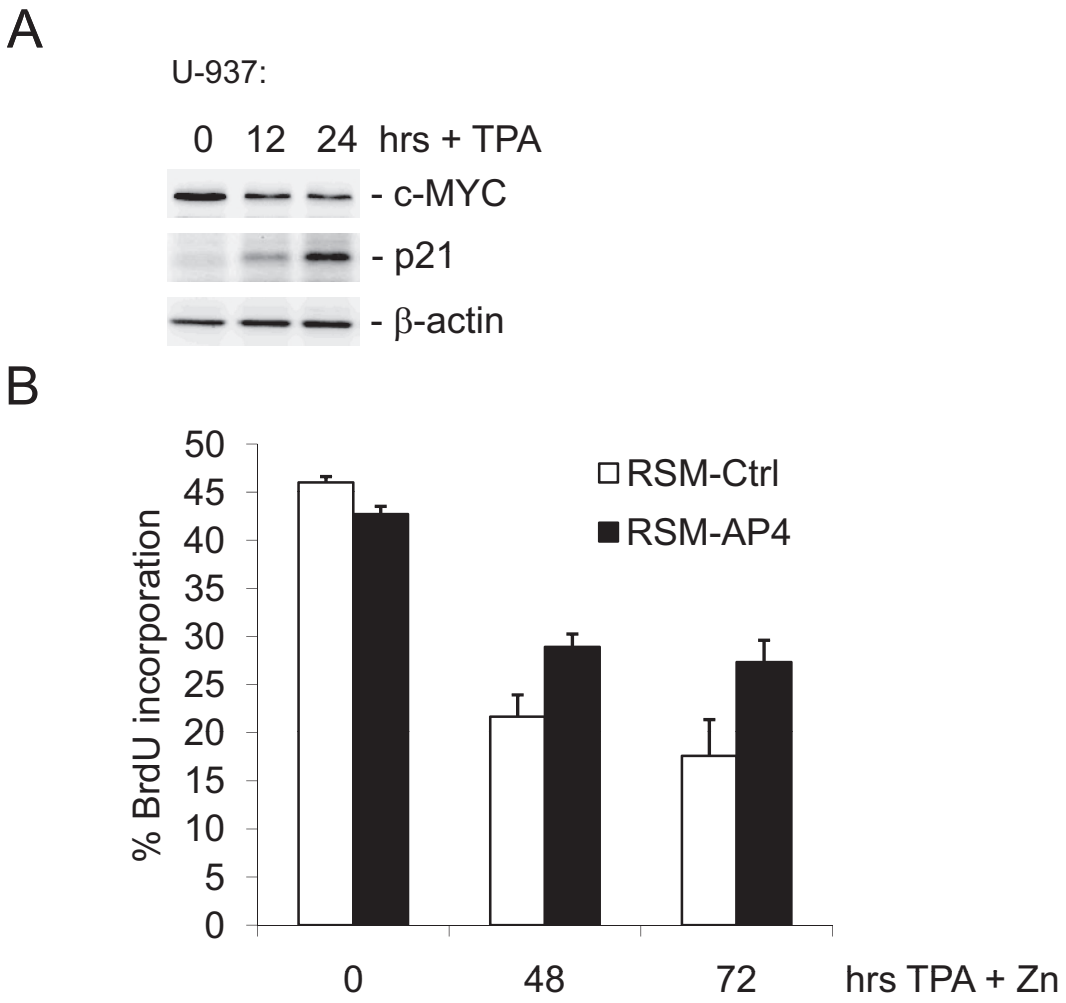

Fig. S8. Effect of AP4 on differentiation-associated cell cycle arrest. (*A*) U-937 cells were treated with 10 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) for the indicated periods and expression of c-MYC, p21 and, as a control for equal loading, β-actin was determined by immunoblotting. (*B*) U-937 RSM-AP4 or RSM-Ctrl cells were treated with 10 nM TPA and 100 μM zinc sulfate for the indicated periods and DNA synthesis (BrdU incorporation: 30 min, 10 μM) was determined by flow cytometry. The percentages of BrdU-incorporation represent mean values of three independent experiments. Error bars are depicted and represent standard deviations.

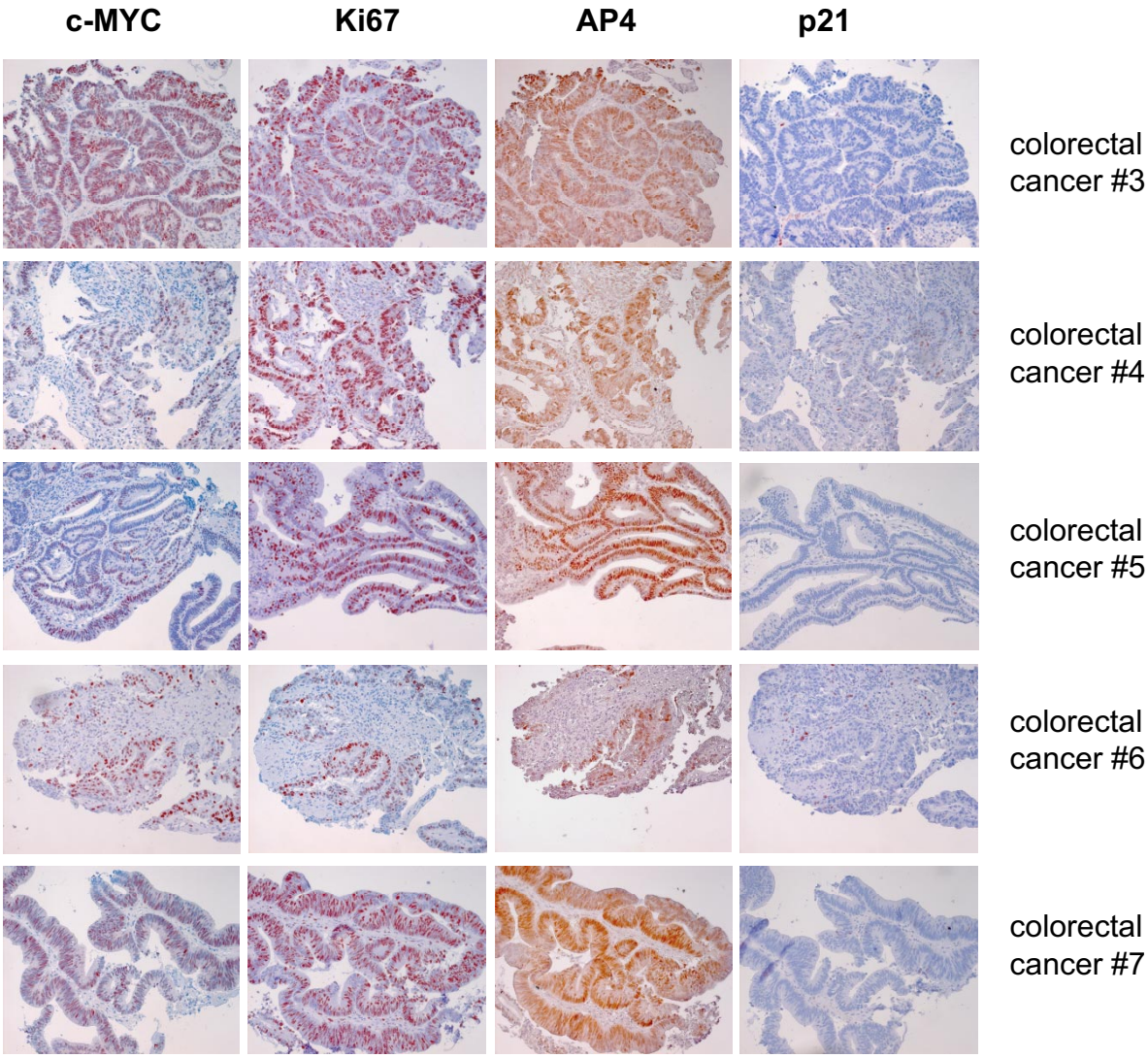

Fig. S9. Sections of endoscopic biopsies derived from primary tumors of 10 patients are depicted. Consecutive paraffin sections were stained with antibodies directed against c-MYC, Ki67, AP4 or p21. Magification: ×200.

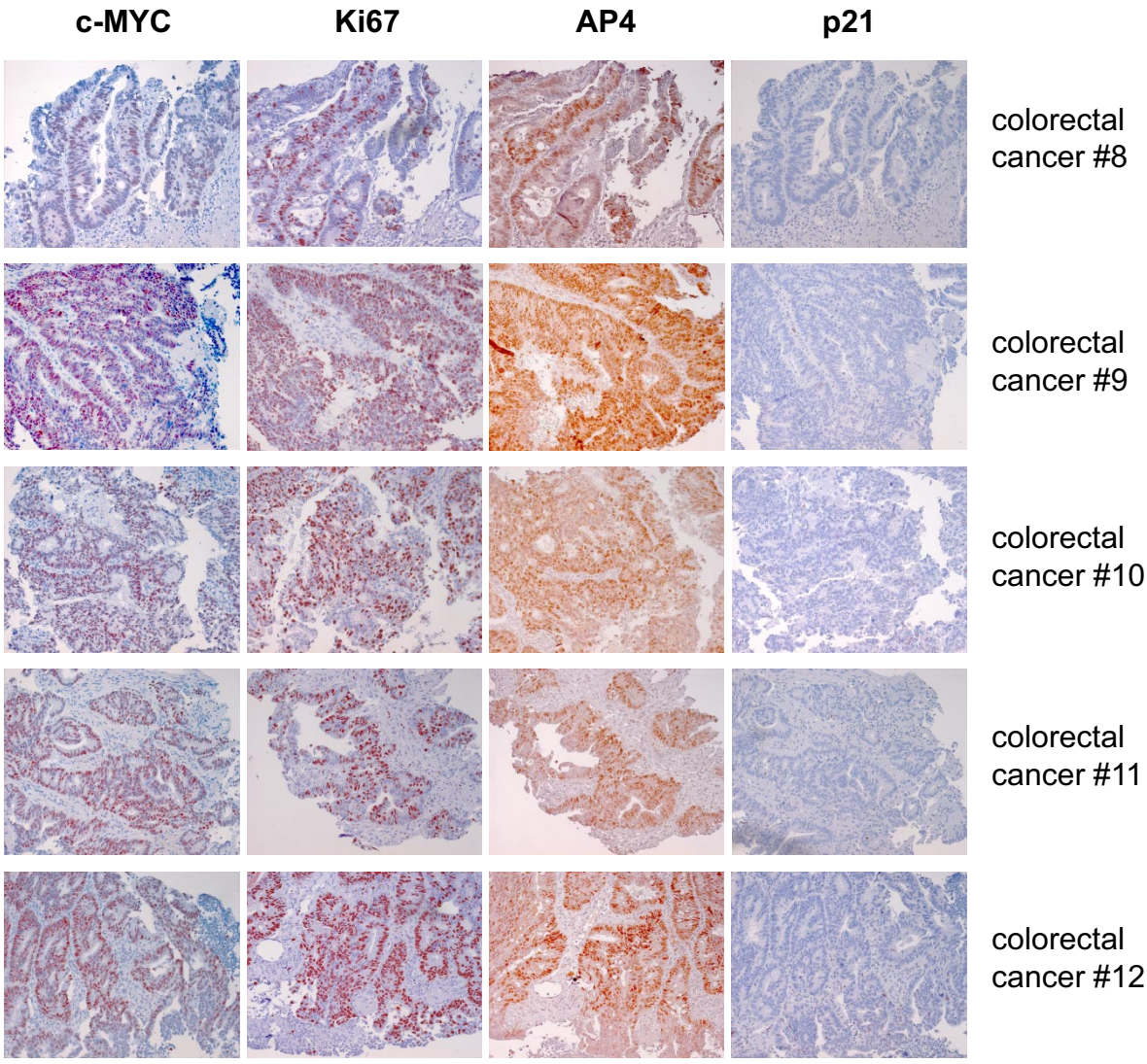

Fig. S9. Continued.

Table S1. Oligonuclotides used for site-directed mutagenesis

Name	Sequence (5′-3′)
p21 A2-Fwd	GAACTCGGCCAGGCTGAGCTTGCTCGGCG
p21 A2-Rev	CGCCGAGCAAGCTCAGCCTGGCCGAGTTC
p21 A1-Fwd	GTCATCCTCCTGATCTTTTGAATTCCATTGGGTAAATCCTTGC
p21 A1-Rev	GCAAGGATTTACCCAATGGAATTCAAAAGATCAGGAGGATGAC
AP4mut1-Fwd	CAGCGGGACCAGGAGCAGCAGATTCAGCAGGAGATCGCCAACAGC
AP4mut1-Rev	GCTGTTGGCGATCTCCTGCTGAATCTGCTGCTCCTGGTCCCGCTG
AP4mut2-Fwd	GATCGCCAACAGCAACGAGCAGCAACAGATGCAGAGCATCAACG
AP4mut2-Rev	CGTTGATGCTCTGCATCTGTTGCTGCTCGTTGCTGTTGGCGAT

Table S2. Oligonuclotides used for qPCR analyses

Name	Sequence (5′-3′)
p21 Fwd	GGCGGCAGACCAGCATGACAGATT
p21 Rev	GCAGGGGGCGGCCAGGGTAT
AP4 Fwd	GCAGGCAATCCAGCACAT
AP4 Rev	GGAGGCGGTGTCAGAGGT
β-actin Fwd	TGACATTAAGGAGAAGCTGTGCTAC
β-actin Rev	GAGTTGAAGGTAGTTTCGTGGATG
rat *ap4* Fwd	CTTCCTCCCACCACATCAAT
rat *ap4* Rev	TGCGGACAGACTTCACGATA
rat *EF1α* Fwd	CACACGGCCCACATAGCAT
rat *EF1α* Rev	CACGAACAGCAAAACGACCA

Table S3. Oligonuclotides used for qChIP analyses

Name	Position relative to the transcription start site (+1)	Sequence (5′-3′)
p21 promoter Fwd	−324	TGTGTCCTCCTGGAGAGTGC
p21 promoter Rev	−218	CAGTCCCTCGCCTGCGTTG
p21 intron 1 Fwd	+2146	AACAAGGGTTTGCGTTTCTG
p21 intron 1 Fwd	+2310	TCGGGAGTTCAAGACAGGAC
AP4 intron 1 Fwd1	+582	CGCGACGTTTGTAAATTGC
AP4 intron 1 Fwd1	+726	CTCAGATCCCGAGGAAGGA
AP4 intron 1 Fwd2	+1550	GAGGTGGGCGTTCTACGG
AP4 intron 1 Rev2	+1790	GGTTGGGCAGGAGTGTCTAC
AP4 intron 6 Fwd	+14760	TCTCAGTGGTTCGTCCCTGT
AP4 intron 6 Rev	+14861	GGAGGCGGTGTCAGAGGT
16q22 Fwd	—	CTACTCACTTATCCATCCAGGCTAC
16q22 Rev	—	ATTTCACACACTCAGACATCACAG