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Authors
Yokoyama, Noriko N.
Pate, Kira T.
Sprowl, Stephanie
et al.

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A role for YY1 in repression of dominant negative LEF-1 expression in colon cancer

Noriko N. Yokoyama, Kira T. Pate, Stephanie Sprowl and Marian L. Waterman*

Department of Microbiology and Molecular Genetics, University of California, Irvine, Irvine, CA 92697, USA

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ABSTRACT

Lymphoid enhancer factor 1 (LEF-1) mediates Wnt signaling via recruitment of β-catenin to target genes. The *LEF1* gene is aberrantly transcribed in colon cancers because promoter 1 (P1) is a Wnt target gene and is activated by TCF–β-catenin complexes. A second promoter in intron 2 (P2) produces dominant negative LEF-1 isoforms (dnLEF-1), but P2 is silent because it is repressed by an upstream distal repressor element. In this study we identify Yin Yang 1 (YY1) transcription factor as the P2-specific factor necessary for repression. Site-directed mutagenesis and EMSA were used to identify a YY1-binding site at +25 in P2, and chromatin immunoprecipitation assays detected YY1 binding to endogenous *LEF1* P2. Mutation of this site relieves P2 repression in transient transfections, and knockdown of endogenous YY1 relieves repression of integrated P2 reporter constructs and decreases the H3K9me3 epigenetic marks. YY1 is responsible for repressor specificity because introduction of a single YY1-binding site into the P1 promoter makes it sensitive to the distal repressor. We also show that induced expression of dnLEF-1 in colon cancer cells slows their rate of proliferation. We propose that YY1 plays an important role in preventing dnLEF-1 expression and growth inhibition in colon cancer.

INTRODUCTION

Colorectal cancer initiation and progression frequently develops from overactive activation of Wnt signaling due to genetic mutation of one or more of the midstream components. The most common mutations are found in components of the APC (adenomatous polyposis coli)-dependent destruction complex. Loss of function mutations in APC or other components leads to stabilization and accumulation of β-catenin in the nucleus, and this results in constitutive and inappropriate, high levels of transcription of target genes such as *MYC*, *CCND1* (cyclin D1) and the Wnt-signaling transcription factor *LEF1* (1–3). About 80% of colon tumors exhibit aberrant activation of *LEF1* gene expression (4). Aberrant expression is due to inappropriate transcriptional activation of the *LEF1* locus and unbalanced overexpression of the Wnt- and growth-promoting isoform of *LEF1* relative to the Wnt- and growth-suppressing isoform of *LEF1*.

LEF1 is a member of the LEF/TCF family of transcription factors that mediate downstream events in the WNT-signaling pathway. When expressed in normal cells, the *LEF1* locus produces two protein isoforms from two different RNA polymerase II promoters. Promoter 1 (P1) produces a mRNA encoding a full length LEF-1 isoform (FL-LEF1) with a β-catenin-binding domain at the N-terminus and a DNA-binding domain near the C-terminus (5,6). This form acts as a Wnt- and growth-promoting form as its DNA-binding domain recognizes Wnt response elements (WREs) in target genes and its N-terminal domain recruits β-catenin to those target genes for activation. Promoter 2 (P2) produces a truncated mRNA that encodes a smaller form of LEF-1 missing the β-catenin domain. This isoform, dominant negative LEF-1 (dnLEF-1), retains the ability to bind to WRE but it cannot recruit co-activator β-catenin. Dn-LEF-1 therefore suppresses Wnt target gene activation and opposes the actions of FL-LEF-1 and other LEF/TCF factors. In the study here, we show that forced expression of dnLEF-1 slows colon cancer cell growth.

In colon cancer, *LEF1* is aberrantly transcribed and transcripts come exclusively from activation of P1 since P2 is silent. P1 is activated because the promoter is targeted for regulation by the Wnt pathway, and this mechanism is well defined (4). P2 is actively silenced, but the mechanism of this repression is unknown. The repression mechanism is important to define because interference with its function might enable expression of dnLEF-1 and therefore provide a balance to offset full-length LEF/TCF actions in cancer. We have

*To whom correspondence should be addressed. Tel: +1 949 824 2885; Fax: +1 949 824 8596; Email: mlwaterm@uci.edu

previously shown that P1 is aberrantly activated by TCF–β-catenin complexes that bind to WREs in the promoter. However, we have also identified WREs in P2 (4), and we find that P2 can also be activated by TCF–β-catenin complexes. Furthermore, the basal P2, which contains three of the identified WREs, is highly active in colon cancer cells. These data suggest that P1 and P2 have equal potential to be activated by the Wnt pathway. Nevertheless, *in vivo* chromatin immunoprecipitation (ChIP) studies showed that P1, but not P2, is directly occupied by TCF–β-catenin complexes in colon cancer cell lines (4). Thus, even though P2 has at least three WREs, these elements are not bound by TCF–β-catenin complexes, a feature consistent with its silence in colon cancer. A survey of chromatin acetylation surrounding the *LEF1* locus in the colon cancer genome revealed that P1 is highly acetylated and P2 is poorly acetylated, and interestingly, the drop in acetylation levels corresponds to a small region between the two promoters. We identified this region as a transcription repressor because highly active fragments of P2 can be strongly repressed by sequences from this upstream region (4). Mapping experiments located the repressor element to a 165-bp sequence between −1446 and −1281 relative to the P2 transcription start site (Figure 1). We also found that TCF–β-catenin complexes could recognize and bind the P2 promoter only if the repressor was deleted, suggesting that the repressor acts to displace or disallow TCF–β-catenin complexes from recognizing WREs (4). In contrast, the distal repressor does not prevent TCF–β-catenin complexes from binding to P1 WREs and activating transcription. Therefore, distal repressor action does not directly target TCF–β-catenin complexes for displacement. Here we investigate the hypothesis that the repressor indirectly interferes with TCF–β-catenin action by targeting a P2-specific protein or element.

Our investigation used site-directed mutagenesis and molecular biology to identify Yin Yang 1 (YY1) as the P2 factor necessary for distal repressor action on P2. YY1 is a ubiquitously expressed, multifunctional zinc finger transcription factor that belongs to the human GLI-Kruppel family of nuclear proteins (7–9). Depending on the context of cells and the environment, YY1 can be an activator, a repressor, or an initiator of transcription and it regulates a broad spectrum of genes. As a repressor, YY1 is known to recruit Polycomb Group complexes and initiate epigenetic changes in chromatin (e.g. H3K9me3, H3K27me3) (10–12). The versatility of YY1 is attributed to its flexibility in recognizing DNA in a variety of contexts including both distal and proximal promoter regions (7,8). In this study we identify a YY1-binding site in the core promoter region of *LEF1* P2. We show that YY1 is necessary for the distal element to functionally repress P2, that YY1 occupies silent *LEF1* P2 in the colon cancer genome, and that YY1 confers distal repressor action to P1 when its binding site is introduced into the core promoter. These data support the model that YY1 is the distinguishing feature of P2 that renders it susceptible to transcription repression in colon cancer. YY1 is a regulator of cell proliferation and development, and its overexpression and activation is linked to tumorigenesis in a wide range of human cancers, including colon cancer. Our study makes a first important link between YY1 actions and Wnt signaling in colon cancer.

MATERIALS AND METHODS

Sulforhodamine B cell growth assay

A 10-day growth curve experiment was performed with DLD-1 cells, which inducibly express dnLEF-1. Cells were plated on 96-well plates (5000 cells per well) as eight replicates for each experimental time point. Every 3 days cells were provided with fresh media containing either doxycycline (0.01 μg/ml) or water (mock treatment control). Cells were fixed and stained according to published protocols (13). Optical density readings were performed at 490 nm with Spectra Max 340 from Molecular Devices Corporation. Two independent growth curves in two different DLD-1 dnLEF-1 stable cell lines were carried out for 10 days, and the SD was generated from eight replicates.

Reporter plasmid constructs

Fusion of the 165-nt repressor element to different sequences of P2 plasmids [−27/+30, −27/+60, −77/+60 and −177/+6, previously described in Li *et al.* (4)] was achieved by PCR amplification of repressor sequences and ligation of the repressor fragment in front of the following P2 pGL2E reporters (**R**−27/+30, **R**−27/+60,

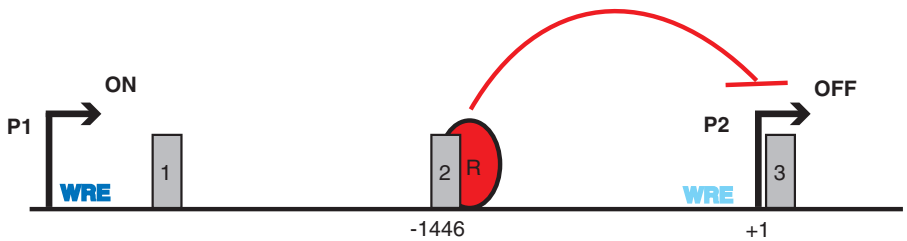


Figure 1. Two promoters in the *LEF1* locus. The LEF1 locus contains two RNA polymerase II promoters. One promoter (P1) produces an mRNA that encodes full-length LEF-1 protein. A second promoter in intron 2 (P2) produces an mRNA that encodes the shorter, dominant negative LEF-1 isoform. Both promoters contain *bona fide* WREs (blue WREs) that mediate activation by TCF–β-catenin complexes. P1 and P2 expression patterns are aberrant in colon cancer cells. P1 is aberrantly activated by TCF–β-catenin, whereas P2 is silenced by a distal repressor located between –1446 and –1281 nt upstream relative to the P2 transcription start site (red circle). The repressor targets a unique feature of basal P2 as P1 is not silenced by the repressor.

R−77/+60 and **R**−177/+60). All mutations in the P2 promoter constructs (−24mt, +10mt, +14mt, +19mt, +25mt, YY1mt) were generated by site-directed mutagenesis PCR methods (Strategene). Parallel mutations were introduced in two reporter plasmids, one without the repressor sequence and one with the repressor sequence (−27/+30 pGL2E and **R**−27/+30 pGL2E). The YY1 mutation was also generated in the −1446/+60 pGL2B P2 construct. The plasmid containing the repressor region upstream of *LEF1* P1 (**R**−P1) was previously described in Li *et al*. (4). To introduce a YY1 site in the P1 and **R**–P1 plasmids, a 3-nt mutation was introduced by site-directed mutagenesis. All plasmid constructs were verified by restriction enzyme digestion and DNA sequencing. A bacterial expression vector of His-tagged YY1 (in pGEX-4T-2) was a generous gift from G. Sui (Wake Forest University, Winston-Salem, NC, USA.).

Transient transfection assay

Colo320HSR cells (0.4×10^6), DLD-1 cells (0.4×10^6), SW480 cells (0.4×10^6) were all transfected with 0.5 μg of reporter plasmid and 0.1 μg of cytomegalovirus (CMV)- β-galactosidase plasmid using FuGENE 6 reagent according to the manufacture's protocol (Roche Molecular Biochemicals). Cells were harvested at 18–20 h post-transfection and assayed for luciferase activity. Luciferase activity values were normalized using β-galactosidase activity values. Fold activation was calculated as a ratio of luciferase levels from each reporter construct. In all cases, experiments were performed with duplicate samples, and at least three or more independent experiments were performed.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) reactions were carried out with 1 or 2 pmol (∼200 cps) of oligonucleotide (see sequence listed in Figure 4A) and 0.05–0.3 μg purified YY1 in a final reaction volume of 25 μl containing 15 mM HEPES (pH 7.9), 0.1% NP-40, 10% glycerol, 30 mM KCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.5 μg of poly(dI-dC), 18.8 μg BSA and 5 mM dithiothreitol. YY1 antibody (1–4 μg; Santa Cruz sc-7341x) or Rat IgG (Santa Cruz, negative control) was added to 0.15 μg of YY1 protein in gel shift buffer (above) for 1 h at 4°C, followed by addition of probe. Recombinant His-tagged YY1 protein was expressed in Rosetta cells, using induction with 0.15 M IPTG for 3.5 h at 37°C. Sonicated bacterial lysates were applied to a nickel His-trap column (Amersham) and protein was eluted according to the manufacturer's protocol. The following wild-type and mutated oligonucleotides were annealed with complementary strands: wild-type 5′-AAAGCATCCAGATGGAGGCCTCTAC-3′, mutant 5′-AAAGCATCCAGCGTGAGGCCTCTAC-3′.

ChIP

Cross-linking and lysate preparation was performed according to the Upstate Biotechnology protocol with modifications. Eight plates of 3×10^6 cells (Colo320 HSR cells, Colo320 −1446/+60 shScr and −1446/+60 shYY1) were cross-linked with 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped with the addition of 125 mM glycine and cold PBS for 10 min. Cross-linked cells were centrifuged at 1200 r.p.m. for 10 min at 4°C and washed with 1X PBS and subsequently resuspended in sodium dodecyl sulfate lysis buffer (Upstate) with protease inhibitors and 1 mM phenylmethylsulfonyl fluoride and incubated on ice for 10 min. Crossed-linked DNA was sheared by sonication to an average size of between 350 and 800 nt. YY1 antibody (Sc-7341x) was obtained from Santa Cruz Biotechnology, CA, USA. Antibody against pan-specific Histone (MAB052, detects Histone H1 and nucleosomal H2A, H2B, H3 and H4) was obtained from Chemicon International. Histone H3K9me3 antibody (ab8898) and Histone H3K27me3 antibody (ab6002) were obtained from Abcam. Magnetic beads carrying mouse and rabbit secondary antibodies were purchased from Dynal (Invitrogen, CA, USA). Primary antibodies (10 μg anti-YY1, 5 μg anti-Histone, 10 μg anti-H3K9me3, 10 μg anti-H3K27me3 or 10 μg IgG) were immobilized on magnetic beads in PBS/BSA overnight at 4°C. Chromatin (∼0.5 mg) was immunoprecipitated with 100 μl beads/antibody in 1% Triton-X, 0.1% DOC and protease inhibitor at 4°C overnight. Immunoprecipitates were washed eight times in RIPA buffer and eluted from the beads with elution buffer (10 mM Tris, pH8, 1 mM EDTA, 1% SDS) at 65°C for 15 min and treated with 283 mM NaCl for reverse cross-link. DNA was treated with proteinase K and subsequently phenol-chloroform extracted and ethanol precipitated. The primer pairs used for the PCR survey of YY1 occupancy of P2 were: 5′-CTGGTTTGCTGCTAAGCTATTTAAGAGAATA-3′ and 5′-TGTTCTCGGGATGGGTGGAGAAAG-3′. Primers for *ZNF609*: 5′-CTCTCTGCTCCTCCTGTTCG-3′ and 5′-CAATATCCACTTTACCAGAGT-3′.

Primers for *LEF1* intron3 (∼40.5 kb downstream of P2): 5′-TGCTCCACAGCATATGGCAGAAGTG-3′ and 5′-GGCTCATGGGGCCATGTTTCCT-3′.

Band intensities of the PCR products are presented as a ratio over the intensity obtained from total input supplied to each immunoprecipitation reaction (+Ab/Input).

The ChIP analysis of methylated histones was quantified by real-time PCR (qPCR). Enrichment was calculated as percentage of input DNA, and IgG was used as negative control and subtracted from enrichment values. Each qPCR point was performed in triplicates and the average was calculated from two independent ChIP analyses. qPCR primers as follows:

P2 Integrated: 5′-AAACAAATCTTTTATGTAGGGTCTGA-3′ and 5′-CTTTATGTTTTTGGCGTCTTCCA-3′.

Endogenous P2 (−108 to +82): 5′-AAACAAATCTTTTATGTAGGGTCTGA-3′ and 5′-GCATCATTATGTACCCGGAATAAC-3′

GAPDH: 5′-TACTAGCGGTTTTACGGGCG-3′ and 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′.

Cell culture

Colo320 HSR, DLD-1 and SW480 colon cancer cells were cultured in RPMI 1640 with 10% fetal bovine serum and

2 mM L-glutamine (RPMI complete). Colo320 −1446/+60 and −816/+60 stable cells were cultured in RPMI complete media supplemented with 200 μg/ml G418 (4). Colo320 −1446/+60 shYY1, −1446/+60 shScr, −816/+60 shYY1 and −816/+60 shScr cells were cultured in RPMI complete media supplemented with 200 μl/mg G418 (A.G. Scientific), 10 μg/ml blasticidin (InvivoGen), and 5 μg/ml puromycin (InvivoGen). DLD-1 TR7 cells (2×10^5, DLD-1 colon cancer cells expressing Tet repressor; generous gift from M. van de Wetering and H. Clevers) were transfected with a vector for Tet inducible dnLEF-1N (2 μg) by Effectene transfection reagent (QIAGEN). Stably transfected cells were selected in complete RPMI media containing 500 μg/ml Zeocin (InvivoGen) and 10 μg/ml Blasticidin (InvivoGen) and those cells that were resistant to Zeocin and Blasticidin were isolated as single colonies. These clonal cells were expanded into individual cell lines and screened for induction of dnLEF-1N protein expression by western blot analysis.

shRNA inducible cell lines

Colo320 −1446/+60 and −816/+60 stable cell lines were used as parental cell lines to establish stable cell lines that constitutively express the Tetracycline repressor (TR) and inducibly express shRNA. We first created a stable cell line that expresses the TR by transiently transfecting pcDNA6/TR (Invitrogen). Colo320 −1446/+60 and −816/+60 cells (2×10^6) were transfected with pcDNA6/TR (3 μg) by use of effectene transfection reagent (QIAGEN). Stably transfected cells were selected in media containing 200 μl/ml G418 and 10 μg/ml blasticidin and those cells that were resistant to G418 and blasticidin were pooled 3 weeks after the initial transfection. These new cell lines (−1446/+60TR and −816/+60TR) were used to create a second cell line that expresses shRNA from pSUPERIOR.puro vector (OligoEngine, Inc.). The shRNA sequence targeting human YY1 has been previously designed and validated (14). The template for shRNA expression was obtained by annealing oligonucleotides (5′-GATCCCCGGCAGAATTTGCTAGAATGTTCAAGAGACATTCTAGCAAATTCTGCCTTTTTA-3′ and 5′-AGCTTAAAAAGGCAGAATTTGCTAGAATGTCTCTTGAACATTCTAGCAAATTCTGCCGGG-3′). Annealed oligonucleotides were designed to carry *Bgl*II and *Hind*III compatible overhangs enabling them to be ligated into the *Bgl*II and *Hind*III linearized vector pSUPERIOR.puro. A mammalian scrambled oligonucleotide (MAMM-2; OligoEngine) was ligated into the pSUPERIOR.puro vector as a shRNA negative control (shScr). Colo320 −1446/+60TR and −816/+60TR were transfected with each of the shRNA (shYY1 and shScr) using Effectene following the manufacturer's protocol. Stable cell lines were selected with 200 μl/mg G418, 10 μg/ml blasticidin and 5 μg/ml puromycin. Stable cells that were resistant to G418, blasticidin and puromycin were pooled 3 weeks after transfection.

Western blot analysis

About 25 μg of DLD-1 Tet_induced dnLEF-1N lysates (dnLEF1 A and dnLEF1 B cells, Figure 2) were analyzed by western blot analysis. Anti-flag (F-3165 Sigma) and anti-LEF1 (#2286 Cell Signaling) antibodies were used to detect LEF-1 expression and anti-Lamin A/C (#2032 Cell Signaling) was used as loading control. Recombinant His-tagged YY1 protein (0.3 μg) was also analyzed by western blot (Figure 4A) using an anti-YY1 monoclonal antibody (Santa Cruz sc-7341x) Cell lysates of 1.5×10^5 Colo320 stable cells (–1446shScr, −1446shYY1, −816shScr, −816shYY1) treated with doxycycline (1 μg/ml) were analyzed for expression of YY1 by western blotting (Figure 5) with a polyclonal YY1 antibody (#2185 Cell Signaling) and secondary anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Monoclonal β-tubulin

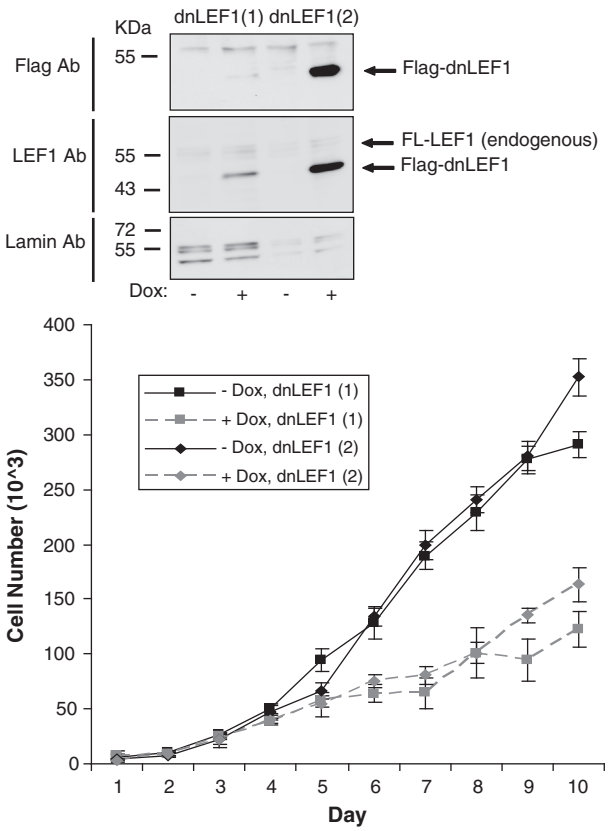


Figure 2. Overexpression of dnLEF-1 slows down DLD-1 colon cancer cell growth. The effect of doxycycline-induced expression of dnLEF-1 on DLD-1 colon cancer cell growth was monitored over a 10-day period in two different clonal stable cells lines [dnLEF1(1) and dnLEF1(2)]. Quantitation of cell number with or without doxycycline (0.01 μg/ml) was performed using a sulforhodamine B cell proliferation assay (colorimetric-based growth curve). Differences in the rate of cell growth appeared after 4 days after which the dnLEF-1 expressing cells grew at a significantly reduced rate. A representative graph is shown from two independent trials. Error bars depict SDs of the results obtained with eight replicates from one trial. The western blots above the cell growth assay show the induced levels of expression of flag-tagged dnLEF-1 protein after 24 h of doxycycline treatment. Endogenous LEF-1 is also detected with LEF1 antibody (FL-LEF-1). Lamin antisera is used for a loading control.

(Zymed laboratories Inc.) antibody was used as loading control. All blots were developed with SuperSignal West Dura Luminol/Enhancer reagent (Thermo Scientific).

Luciferase assay for stable cells

All four Colo320 stable cells (−1446/+60 shScr, −1446/+60 ShYY1, −816/+60 shScr, −816/+60 shYY1) were seeded in six-well plates (150 000 cells) 24 h prior to doxycycline treatment. Doxycycline (1 μg/ml) was added to the cultures to induce either shRNA YY1 or shScr and cells were harvested 0, 24, 36, 48 and 60 h after doxycycline treatment for luciferase and β-galactosidase assays. The luciferase activities were normalized to the β-galactosidase activities.

RESULTS

Expression of dnLEF1 attenuates colon cancer cell growth

Overexpression of dominant negative forms of TCFs can halt the growth of colon cancer cells via a strong stall in cell-cycle progression (15,16). If transcription silencing of *LEF1* P2 was disrupted and dnLEF-1 expressed, then dnLEF-1 might have a similar effect on colon cancer cells. We therefore investigated whether induced expression of dnLEF-1 could affect colon cancer cell growth. To test this we developed stable DLD-1 colon cancer cell lines with doxycycline inducible expression of flag-tagged dnLEF-1 protein. Using a colorimetric-based growth curve assay, we monitored cell growth over a 10-day period in the absence or presence of induced dnLEF-1 expression in multiple, independent clonal stable cell lines [two cell lines are shown in Figure 2 as dnLEF1 (1) and dnLEF1 (2)]. Western analysis confirmed the expression of dnLEF-1 within 24 h of doxycycline induction (Figure 2). Differences in the rate of cell growth appeared after 4 days, which was also when the cultures approached confluency. At the point of confluence (Day 5), the dnLEF-1 expressing cells continued to grow but at a significantly reduced rate (50%) compared with the mock-treated cells (Figure 2). This reduction in cell growth occurred regardless of dnLEF-1 induction levels, which varied between the two cell lines. These data suggest that repression of endogenous dnLEF-1 expression enables faster rates of colon cancer cell proliferation.

The P2 core promoter is a target of repression

To examine the mechanism of P2 repression by the upstream repressor region, we investigated whether repression is position dependent and whether intervening sequences between −1281 and the transcription start site are important. Placement of the 165-bp repressor element in different positions upstream of P2 in a luciferase reporter plasmid and transfection of these reporter plasmids into Colo320 colon cancer cells showed that repression was possible from any position upstream of P2 (Figure 3A). Deletion of sequences downstream of the transcription start site from +60 to +30 reduced repression from 8.9- to 2.7-fold. Overall however, the deletion study showed that the repressor element specifically targets core promoter sequences between −27 and +30.

The target of the repressor is an element at +25 relative to the start site of P2 transcription

We previously reported that distal repressor action is specific to P2 and has no effect on P1 either upstream or downstream (4). These observations imply that differences in the promoters make them sensitive or insensitive to repressor action. Given that the repressor acts on core P2 (−27/+30), we hypothesized that a factor(s) binding to sequences within this 57-nt region was important for communication with the distal repressor. To define the sequence elements in core P2 that are targeted by the repressor, multiple mutations were introduced between −27 and +30 by site-directed mutagenesis. We have previously shown that mutating the initiator-like element (–17) and the WRE that overlaps the transcription start site (+1) results in an inactive core P2 [(4); bold text Figure 3B P2 wild-type]. Therefore only nucleotides upstream and downstream of these essential elements were mutated. Each mutation was introduced into two reporter plasmids that differed by the presence or absence of the repressor element (−27/+30 pGL2 and **R**−27/+30 pGL2). P2 sequences and the mutations within −27 to +30 are shown in Figure 3B. These mutated reporter plasmids were then analyzed for promoter activity and repression by transient transfection in Colo320 colon cancer cells (Figure 3C). Overall, mutations in the core promoter had a negative effect on core P2 activity. This is expected since many known Polymerase II preinitiation components require interactions with key positions near transcription start sites. Mutation of +10 to +12 especially had a negative effect on P2 activity since the mutations are close to the initiator element. However, despite these effects, repression was still evident for most constructs as promoter activity was reduced to similar or lower levels compared to the wild-type repressed promoter (Figure 3C; dotted line, **R**−27/+30). The one exception was a mutation that altered 4 nt: +25 to +28 (TGGA to GTTC). Mutagenesis of these nucleotides had only modest effects on core P2 activity, yet almost completely prevented repression (**R**+25mt, Figure 3C). We conclude that a sequence element at +25 is recognized by a key factor for repression.

Inspection of the +25 sequence element revealed a match to the consensus binding site for the ubiquitously expressed YY1 transcription factor (CAGATGGAG). This factor is known to mediate both transcription activation or transcription repression (7,8). To determine if the putative YY1 consensus sequence is important for P2 repression, we introduced a more precise mutation to destroy the core YY1-binding site at +24 and +25 (YY1mt, Figure 3D). Wild-type P2 (P2 WT), the +25mt, and the new YY1mt reporter plasmids (with or without the repressor element) were analyzed for promoter activity and repression by transient transfection in three colon cancer cell lines (Colo320, DLD-1 and SW480, Figure 3D). Consistent with the previous result, mutating the core promoter region had a negative effect

on core P2 activity compared to the wild-type P2 reporter. However, mutating the putative YY1-binding site resulted in either partial (+25mt) or complete (YY1mt) relief of repression when comparing the mutants with the repressor to the mutants without the repressor (compare YY1mt with **R**-YY1mt in Colo320, gray bars, Figure 3D). Although we observed the greatest relief of repression in Colo320 cells, the promoter activity profiles for all three cell lines were similar.

We also introduced the YY1 mutation (YY1 mt) in the more physiologically relevant −1446/+60 reporter plasmid where the repressor element is at the native location and analyzed promoter activity by transient transfection in colon cancer cell lines (Figure 3E). For all three cell lines, introduction of the 2-nt mutation at the YY1 consensus sequence resulted in relief of P2 repression (3- to 7-fold). We conclude that a YY1 element between +21 and +28 is necessary for full repressor function.

YY1 binds to the +25 region of P2 *in vitro* and *in vivo*

To determine if YY1 could bind to the +25 element, EMSA was performed with partially purified, recombinant His-tagged YY1 protein and radiolabeled wild-type and mutant probes encoding (+13 to +37) of P2 (Figure 4A). Purified, intact and pure His-tagged YY1

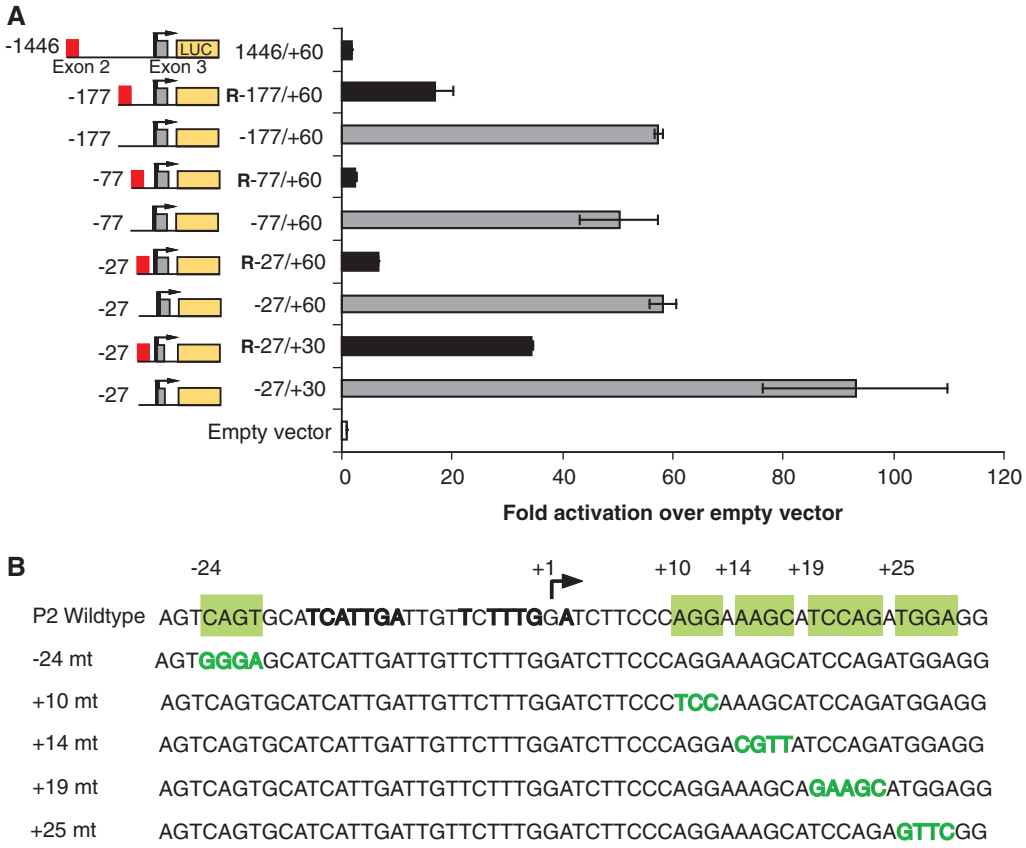
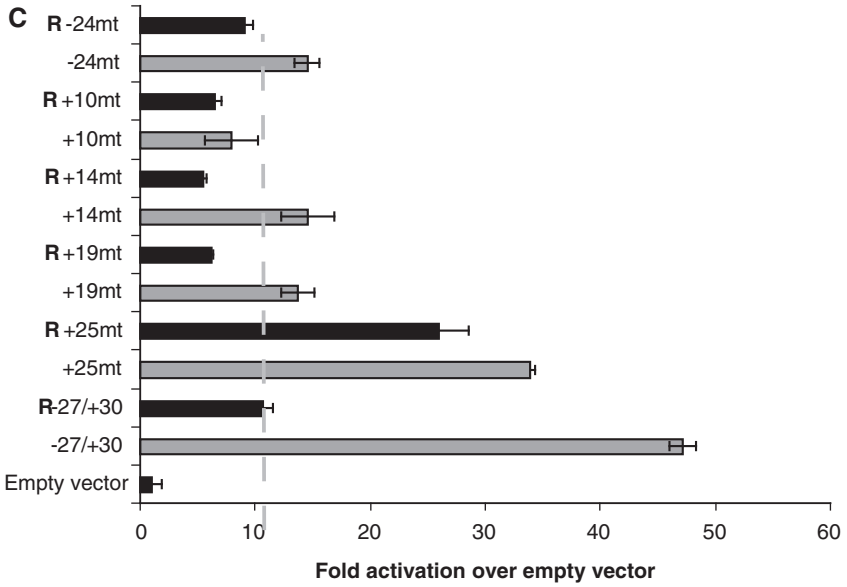
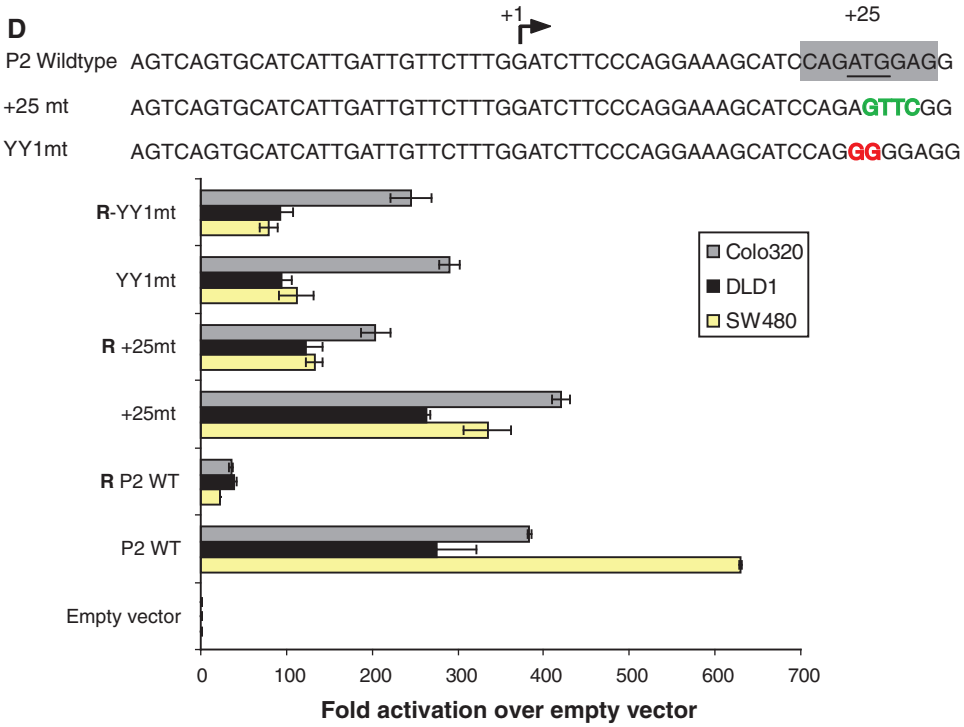


Figure 3. An element in core promoter P2 is necessary for repression. (**A**) The 165-nt distal repressor (red box) was fused to P2 at different upstream positions (from –177 to –27 relative to the start site of transcription). Promoter activities were analyzed by luciferase reporter assays in Colo320 colon cancer cells. All promoter fragments were sensitive to repression, therefore, the repressor specifically targets core P2 sequences between –27 and +30. Data are derived from duplicate samples, and the results shown represent one of four replicate experiments. Fold activation was calculated as a ratio of luciferase levels from each reporter construct relative to the promoterless reporter vector. Error bars represent the spread between duplicate samples. (**B**) Multiple mutations of core P2 were generated by site-directed mutagenesis. P2 sequences from –27 to +30 are shown. Each mutation was introduced into pairs of luciferase reporter plasmids that differed only by the presence of the distal repressor element at –27 (pGL2E **R**–27/+30 and pGL2E –27/+30). Black bolded nucleotides show the essential initiator sequence (–17) and the transcription start site (+1). Green bolded nucleotides show the five different mutated sequences. (**C**) Luciferase assays of the mutated P2 reporter plasmids are shown. P2 reporter plasmids were transiently transfected into Colo320 cells. Mutations of core promoter sequences reduced P2 activity in all cases (gray bars), whereas only the mutation at +25 relieved repression by the distal repressor (black bars). Luciferase activity is reported as activation over that for a promoterless reporter vector. Data are derived from duplicate samples, and the results shown represent one of three replicate experiments. Error bars represent the spread between duplicate samples. (**D**) A mutation to destroy the YY1-binding site at +24 and +25 (AT to GG, in red) was introduced by site-directed mutagenesis. The mutation was introduced in luciferase reporter plasmid pGL2E **R**−27/+30 and pGL2E −27/+30 (P2 WT and **R** P2 WT respectively). Wild-type P2 and mutated P2 reporter plasmids were transiently transfected into three different colon cancer cell lines (Colo320, DLD-1 and SW480). Data shown are from a representative experiment out of three trials; error bars represent the spread of duplicate samples. The mutation at the YY1-binding site resulted in partial relief of repression in all three cell lines. (**E**) A YY1-binding site mutation was also introduced in the more physiologically relevant reporter plasmid pGL2B −1446/+60. Both wild-type −1446/+60 P2 and −1446/+60 YY1mt reporter plasmids were transiently transfected into three different colon cancer cell lines (Colo320, DLD-1, and SW480). Data are derived from three replicate experiments and error bars represent SDs of the data. For all three cell lines, introduction of the YY1-binding site mutation showed relief of P2 repression.





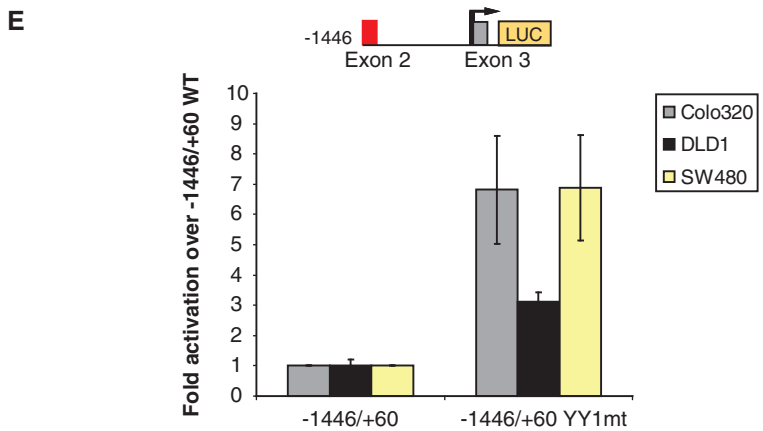


Figure 3. Continued.

protein is shown in Coomassie stained SDS–PAGE gel as well as in western blot analysis probed with anti-YY1 antibody (Figure 4A). Results from the EMSA assay showed that YY1 binds to the +25 wild-type sequence but not to the mutant probe. Addition of YY1 monoclonal antibody to the EMSA-binding reaction blocked binding, showing that it is YY1 protein that is specifically binding to the +25 region and not an irrelevant protein from the bacterial extracts (Figure 4A, far right). A faster migrating form is not affected by YY1 monoclonal antibody.

We next performed ChIP assays to assess whether endogenous YY1 binds to the +25 region of endogenous *LEF1* P2. ChIP assays were performed using monoclonal YY1 antibody to pull down cross-linked YY1/DNA complexes from Colo320 colon cancer cells. Occupation of YY1 over the core P2 promoter region was detected with primers that amplify the genomic region from −188 to +144 relative to P2. A primer set that detects intron3 of the *LEF1* gene (~40.5-kb downstream of P2) and a set that detects an unrelated region of the genome (*ZNF609*) were used as negative controls for the assay. Immunoprecipitation data is represented as the percent of PCR product amplified from the immunoprecipitates over the total amount of product amplified from the input (input set at 100). A pan-specific histone antibody

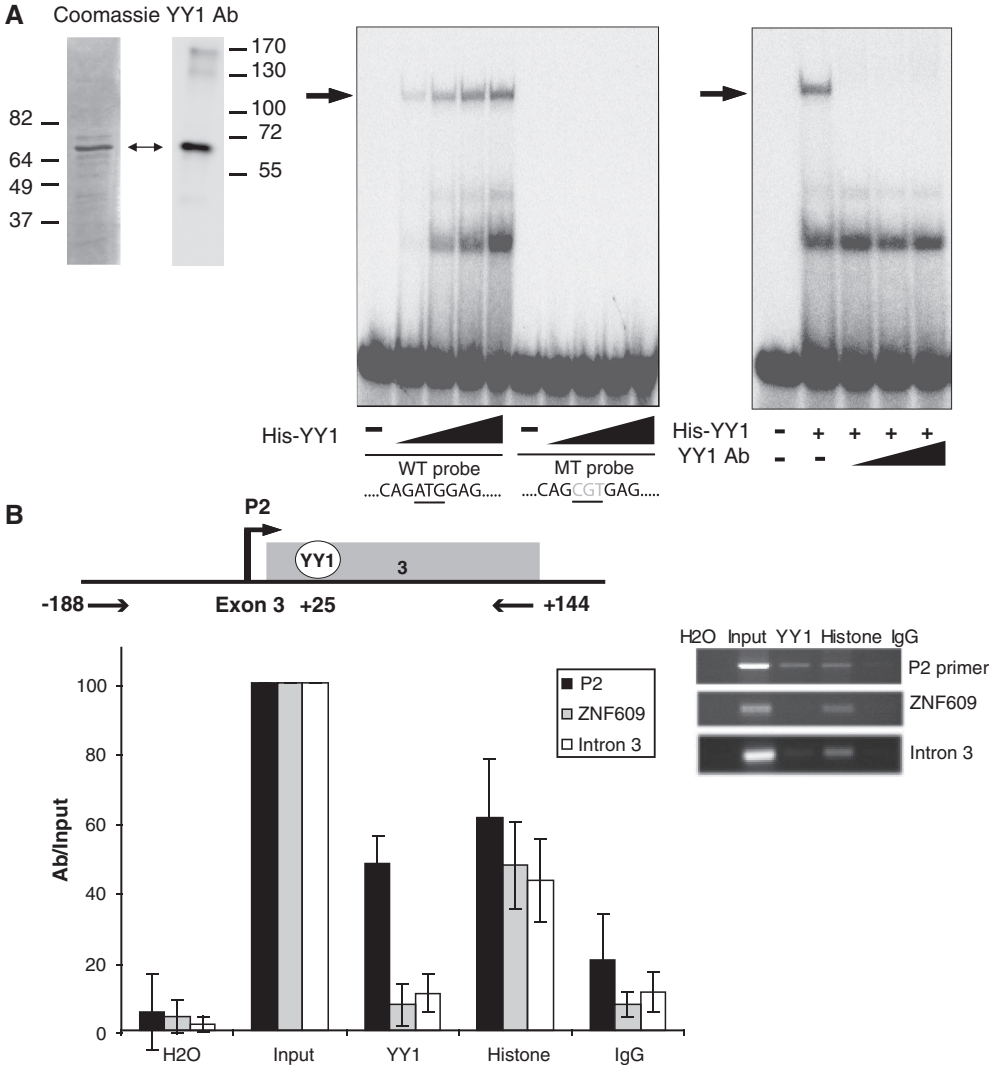


Figure 4. YY1 binds to the +25 region of P2 *in vitro* and *in vivo*. (**A**) EMSA was performed with a ^{32}P-labeled P2 probe containing the putative YY1 wild-type sequence (-CAGATGGAG-) or a mutated sequence (-CAGCGTGAG-) and purified recombinant human His-tagged YY1 protein. Purified His-tagged YY1 is shown in both Coomassie stained SDS–PAGE gel and YY1 probed western blot. Protein was added in increasing amounts (0.05, 0.1, 0.15 and 0.3 μg) (left EMSA). To confirm binding specificity, 1, 2 or 4 μg YY1 monoclonal antibody was included in the EMSA assay with 0.15 μg purified YY1 protein and ^{32}P-labeled P2 probe (right EMSA). YY1 antibody blocked formation of the larger complex, but not the faster migrating complex. Arrows point to the YY1 bound probe. (**B**) ChIP assay was performed with Colo320 cells lysates. Chromatin from formaldehyde-crosslinked Colo320 cells was immunoprecipitated with monoclonal YY1 and pan-specific Histone antibodies. ChIP immunoprecipitates were analyzed by PCR primers that detect the core P2 region (−188 to +144), intron3 of the *LEF1* gene, and an unrelated region of the genome (*ZNF609*). Data are derived from two to three independent experiments and error bars represent standard deviations. The difference between YY1 and IgG occupancy was significant for the core P2 region ($P < 0.05$) but insignificant for intron3 or *ZNF609* (Student's *t* test). Representative gels for each primer set are shown.

that detects all four core, nucleosomal histones was used as a positive control for the cross-linking and immunoprecipitation, and IgG serum was used as a negative and background control. We observed YY1 specifically binding to the core P2 promoter as YY1 antibody was nearly as effective as histone antibody in the immunoprecipitation of core P2 sequences (Figure 4B; 50 versus 60%). Based on these results, we conclude that the YY1 transcription factor specifically binds to the core P2 promoter. Since mutation of the YY1-binding site at +25 interferes with promoter repression and YY1 binds to this sequence both *in vitro* and *in vivo*, these correlative data strongly suggest that YY1 is the functional protein target of the repressor. To more directly test for the importance of YY1 in P2 repression, we carried out two functional studies: a knockdown of endogenous YY1 to test for effects on P2 repression and epigenetic modifications to chromatin, and introduction of the YY1-binding site into a heterologous promoter to test for the ability of this site to confer repressor sensitivity.

YY1 is necessary for P2 repression

To knockdown endogenous YY1 in colon cancer cells we created an inducible shRNA YY1 system in two Colo320 colon cancer cell lines which have P2 luciferase reporter plasmids integrated into the genome [−816/+60 and −1446/+60, (4)]. Using luciferase as a readout of the integrated reporters, we previously showed that in the context of the colon cancer genome and authentic chromatin structures, the upstream repressor acts on P2 to repress transcription and prevent TCF–β-catenin complexes from occupying their cognate WREs (4). A co-integrated plasmid with the β-galactosidase reporter gene driven by the CMV promoter was used as a normalization control to compare luciferase activities between the two cell lines. Thus, normalized luciferase levels in the −816/+60 cell line is high because the integrated P2 reporter is missing the repressor element (Figure 5B). In contrast, normalized luciferase activities in the −1446/+60 reporter line are lower because the repressor is present (Figure 5A). If YY1 is an essential core P2 factor for repressor action, then knockdown of endogenous YY1 should relieve repression of the integrated −1446/+60 P2-luciferase reporter, but should have no effect on the −816/+60 P2 reporter. To test this hypothesis we established a TR system for inducible shRNA YY1 (shYY1) knockdown in the −816, and −1446 reporter lines ('Materials and methods' section). We also made complementary stable cell lines that have an inducible scrambled shRNA (shScr) as a negative control (Figure 5). In addition, the co-integrated β-galactosidase reporter plasmid was used as an internal negative control for YY1 knockdown. Doxycycline was added to the cultures of these newly derived sub-lines to induce either shRNA YY1 or scrambled sequences and cells were harvested 0, 24, 36, 48 and 60 h after treatment. Western analysis of the −1446/+60 inducible shYY1 cells showed significant reduction of YY1 protein expression by 36 h, whereas the scrambled shScr negative control did not affect YY1 even after 60 h of doxycycline treatment. For the −816/+60 shYY1 inducible cells, we observed leaky expression of the YY1-specific shRNA, as YY1 protein levels were partly reduced at 0 h even before doxycycline treatment. However, we observed further reduction of YY1 protein during the 60 h doxycycline time course. Since each of the stable cell lines have the luciferase and β-galactosidase reporter plasmids integrated in the cellular genome, we assayed extracts from the doxycycline-treated cells for both luciferase and β-galactosidase activities. Luciferase assays showed that knockdown of endogenous YY1 in the stable cell line with the P2 repressor (−1446/+60) led to partial up-regulation of P2 activity. No change in P2 activity was observed with induction of scrambled shRNA (shScr) demonstrating that expression changes were YY1-specific. We did not observe significant up-regulation or down-regulation of the P2 reporter without the repressor (−816/+60) when either YY1 or scrambled shRNAs were induced. Taken together, results from the knockdown studies show that YY1 is necessary for P2 repression.

To elucidate the mechanism of YY1-mediated repression, we investigated the pattern of chromatin modifications associated with gene inactivity and YY1 repression (10–12,17). Histone H3K9me3 and H3K27me3 are commonly linked to Polycomb group activities (10–12) and we tested for the presence of these marks on the P2 promoter in the presence and absence of YY1. We performed ChIP analysis with lysates from the previously described −1446/+60 shYY1 and −1446 shScr cell lines with or without doxycycline induction of YY1 knockdown. We used two sets of primers to detect the repressive marks on P2: a set that detects repressive marks on only the P2 integrated reporter plasmid (Figure 5C, P2 Integrated) and another set that detects repressive marks on the endogenous *LEF1* P2 locus (Figure 5C, P2 Endogenous). A GAPDH primer set was used as the negative control. In the cell line containing YY1 protein (–1446 shScr), we observed enrichment of the repressive histone-tail mark H3K9me3 on both the integrated and endogenous P2 compared to the GAPDH promoter. However, H3K9me3 was much higher in the integrated P2 compared to the endogenous locus. We did not detect significant enrichment of histone H3K27me3 marks on P2. In the cell line with reduced YY1 protein (–1446 shYY1), the high levels of H3K9me3 were drastically reduced on the integrated P2 locus. There was little-to-no change of H3K9me3 on the endogenous locus. Therefore, from this study we detected differential levels of histone methylation between the *LEF1* integrated P2 and endogenous locus, and found that YY1 is necessary for methylation of integrated P2, but not for endogenous P2.

YY1 confers repressor regulation to *LEF1* P1

If YY1 is the essential core P2 factor that confers susceptibility to the upstream repressor, then introduction of a YY1-binding site into a heterologous promoter should confer a similar susceptibility. To test this we introduced YY1-binding sequences into Promoter 1 (P1) of the *LEF1*

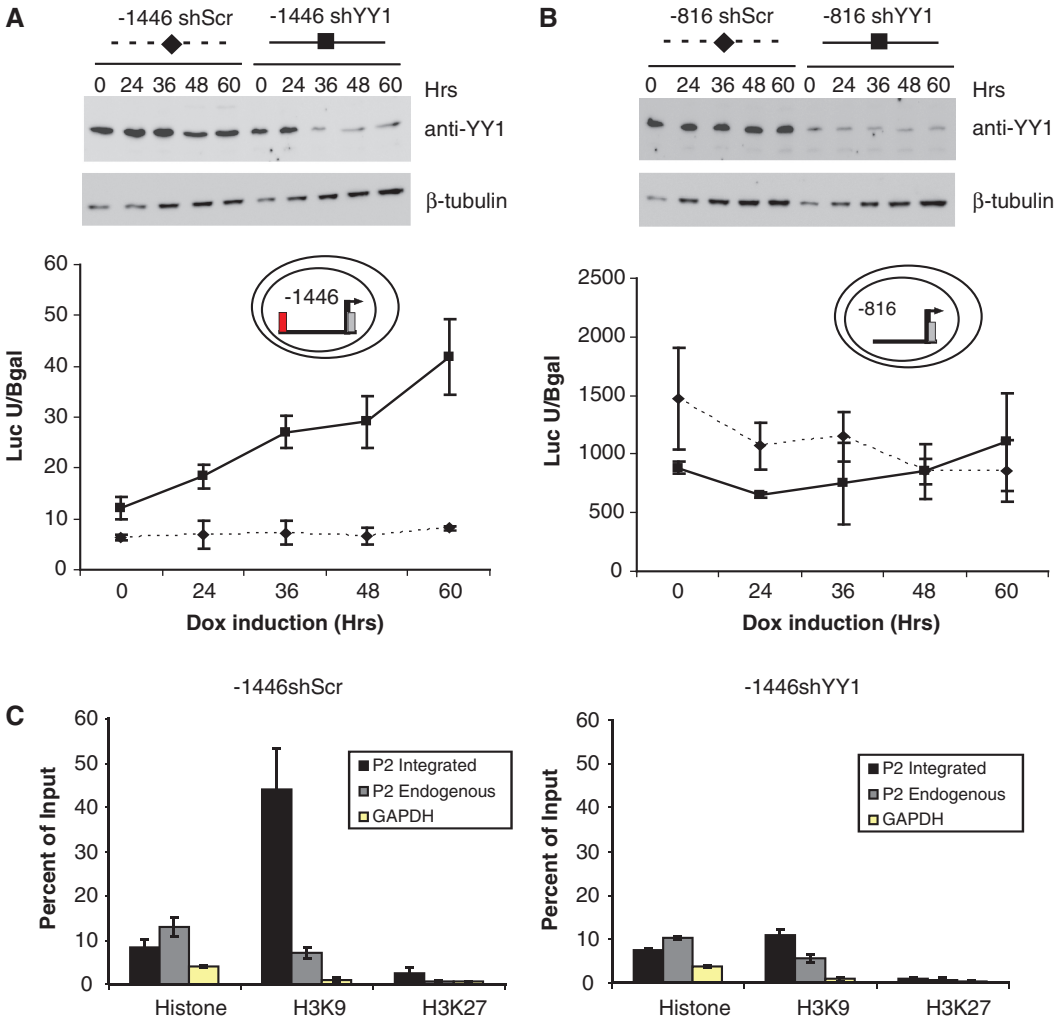


Figure 5. YY1 knockdown relieves P2 repression. Inducible shRNA YY1 (shYY1) and shRNA Scrambled (shScr) stable cell lines were derived from (**A**) Colo320 −1446/+60 and (**B**) −816/+60 luciferase reporter lines ('Materials and Methods' section). Knockdown was induced with 1 μg/ml of doxycycline and cells were harvested 0, 24, 36, 48 and 60 h after treatment and processed for western blot analysis and luciferase assays. Doxycycline induction of YY1-specific shRNA leads to knockdown of YY1 protein (western insets; –1446 shYY1 and –816 shYY1), whereas induction of a scrambled, negative control shRNA has no effect (−1446/+60 shScr and −816/+60 shScr). Anti β-tubulin antibody was used to control for loading in the western blot analysis. All four cell lines carry *LEF1* P2 luciferase and CMV-β-galactosidase reporter plasmids integrated in the cellular genome, therefore, we assayed extracts from the doxycycline-treated cells for both luciferase and β-galactosidase activities. β-galactosidase activity was used to normalize luciferase light units. The data are represented as ratios of luciferase light units to β-galactosidase units, and the error bars reflect standard deviation of the data from three independent assays. (**C**) ChIP assay was performed with −1446/+60 reporter integrated Colo320 stable cells with induction of either shScr or shYY1 plasmid to reduce YY1 protein levels. Chromatin from formaldehyde-crosslinked cells (either shScr or shYY1) was immunoprecipitated with pan-specific Histone, polyclonal H3K9me3, monoclonal H3K27me3 and IgG antibodies. ChIP immunoprecipitates were analyzed by qPCR primers that detect integrated P2 (−108 to +60), endogenous P2 (−108 to +82), and primer set that detects the promoter of the *GAPDH* gene. Data are derived from two independent ChIP experiments and qPCR was performed in triplicate.

gene. We have previously shown that *LEF1* P1 is highly active in colon cancer and is regulated by TCF–β-catenin complexes. We have also shown that *LEF1* P1 is insensitive to the repressor element as its placement upstream of P1 has no effect on transcription (4). Like P2, the P1 promoter is a TATA-less RNA polymerase II promoter with multiple WREs that activate transcription. However unlike P2, P1 is highly GC-rich, its essential core promoter sequences are different, and importantly, there are no YY1-binding sites in the promoter (Figure 6A). Therefore, we used this promoter to test whether introduction of a single YY1-binding site is sufficient to make P1 sensitive to the repressor region. Site-directed mutagenesis was used to alter the nucleotide sequence at three positions to create a YY1-binding site at +25 within core P1. These changes were introduced both in the presence and absence of the upstream repressor element (Figure 6B, **R**–P1 YY1 and P1 YY1 respectively), and promoter activities were assessed by transient transfection into three different colon cancer cell lines (Colo320 cells, DLD-1 and SW480). Similar to our previously reported findings, wild-type P1 activity was not affected by the repressor (Figure 6B, compare P1 to **R**–P1) and introduction of a YY1 site had a minimal effect (Figure 6B, compare P1 to P1 YY1). Most importantly however, the modified P1 promoter was now repressor sensitive. We observed

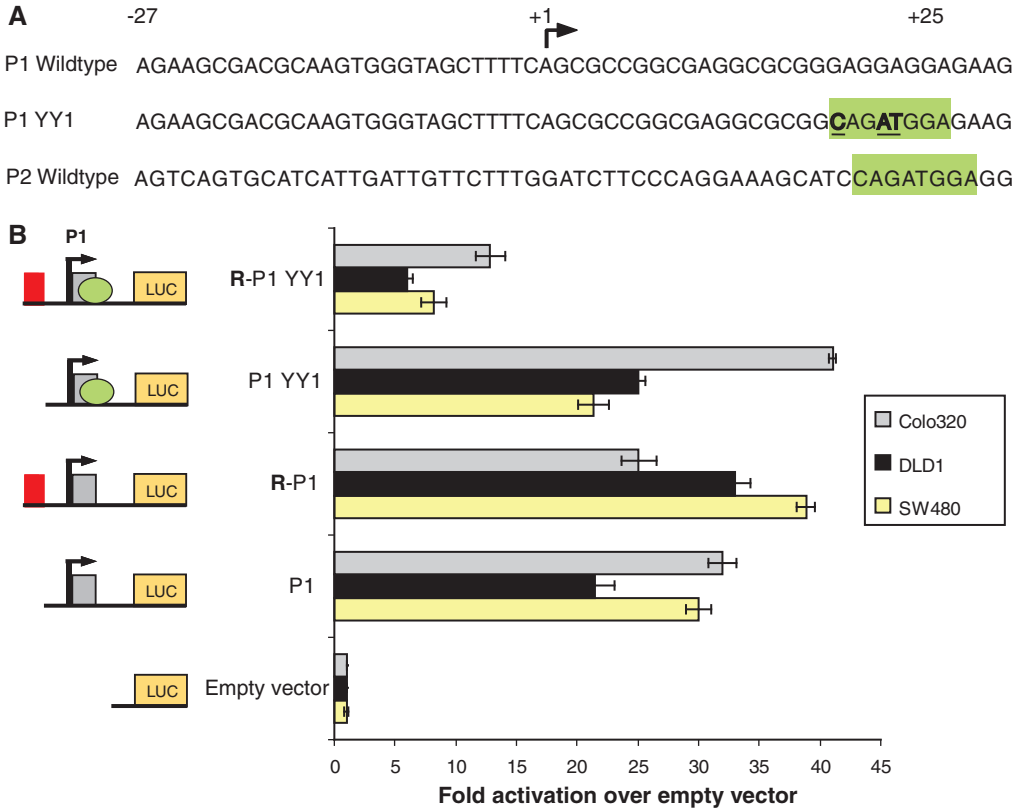


Figure 6. Introduction of a YY1-binding site confers repressor sensitivity to Promoter 1. (**A**) Wild-type P1 sequences (−27/+30) and a mutant P1 with an introduced YY1-binding site (boxed in green) are shown. For comparison, wild-type sequence of core P2 with its native YY1-binding site is shown. Site-directed mutagenesis was used to introduce the YY1-binding site into core P1 between +19 and +22 of two luciferase reporter plasmids that differed only by the presence and absence of the distal repressor element (P1 YY1 and **R**–P1 YY1). (**B**) Transient transfections of the indicated reporter plasmids were performed in Colo320, DLD-1 and SW480 colon cancer cell lines. The distal repressor element is indicated by a red box, the introduced YY1-binding site by a green oval and the core P1 promoter by a grey box. Luciferase activity is reported as fold activation over that for a promoterless reporter vector. Data are derived from duplicate samples, and the results shown are a representative of at least three experiments. Error bars represent the spread of duplicate samples.

2.5- to 5-fold repression of P1 in the presence of the repressor depending on the cell line (Figure 6B, **R**–P1 YY1), a level similar to the 3-fold repression of core P2 shown in Figure 3A. These results suggest that the repressor is specifically interacting with YY1 to repress core promoter activities, and they show that YY1 sites are sufficient to confer repressor susceptibility. Based on the findings from these two functional studies, we conclude that the upstream repressor requires YY1 to actively repress P2 and that YY1 is the unique, distinguishing feature between P1 and P2.

DISCUSSION

Wnt signaling is important for regulating self-renewal, survival, proliferation and developmental cell fates and LEF-1 is one of four LEF/TCFs that can mediate these regulatory actions. Because both P1 (FL-LEF-1) and P2 (dnLEF-1) have *bona fide* WREs in their promoters, it is tempting to speculate that Wnts coordinately regulate transcription of both *LEF1* promoters and in so doing establish a system of positive and negative regulatory feedback. Indeed both *LEF1* mRNA isoforms are dynamically expressed during Wnt-dependent lymphocyte differentiation and peripheral activation of CD8+ T lymphocytes (18). Wnt signaling is also important for proliferation and differentiation of epithelial cells in crypts of the intestine, but *LEF1* is not normally expressed in this tissue. Instead, *LEF1* expression is turned on during intestinal carcinogenesis. *LEF1* mRNA appears at the adenomatous stage and stays on through the carcinoma and metastatic stages (4,19–21). We have previously shown that this expression depends entirely on the Wnt pathway and activated TCF–β-catenin complexes binding to WREs in the *LEF1* P1 promoter (4,19). The study here explored our previous finding that *LEF1* P2 is not activated in these cancers because it is transcriptionally repressed and its promoter WREs are unoccupied (4). The biological outcome of differential promoter activity is an unbalanced pattern of *LEF1* transcription where only the Wnt-promoting FL-LEF-1 form is expressed and the Wnt-opposing dnLEF-1 form is not expressed. Not only is it important to understand the mechanisms that set up this pattern of expression, but it may be important for understanding expression changes observed for other LEF/TCF family members, and other genes with multiple, differentially expressed promoters.

In normal intestinal cells, two predominant TCF transcription factors are expressed: full-length TCF-4 (FL-TCF-4) and dominant negative TCF-1 (dnTCF1) (1,20,22,23). Just as we observe for LEF-1 expression in developing lymphocytes, expression of the two forms of TCFs that oppose each other also provides a balance between the activities of Wnt-promoting and Wnt-suppressing forms. Interestingly, we have discovered that the growth suppressing form of dnTCF-1 is not present in colon cancer (24). Instead its expression has been replaced by FL-TCF1. The *TCF7* locus (which encodes TCF-1 protein) is very similar to the *LEF1* locus in that a P1 promoter produces FL-TCF-1 mRNA and an intronic, alternative P2 promoter produces dnTCF-1 mRNA. Thus, the observed switch in TCF-1 expression during colon carcinogenesis is most likely due to a switch in promoter activities. A fundamental question to address then is how it is that expression of all the dominant negative forms of LEF/TCFs are suppressed in colon cancer. Our work to define the mechanisms that govern aberrant LEF-1 expression is the first approach to answer this question. In the current study, we show that *LEF1* P2 is silent in colon cancer cells because the upstream repressor element (165 bp) and the core promoter factor YY1 cooperate to repress P2 transcription.

YY1 is a multifunctional protein that binds to a large number of cellular and viral genes (7,8). The YY1-binding site at +25 in *LEF1* P2, (5′-CAGATGGAG-3′), is a strong match to the known YY1 consensus binding site [C(ga)G(t)C(ta)CATNT(a)T(gc)]. Using *in vitro* and *in vivo* methods, we confirmed that YY1 binds to the +25 region. In fact, there are other general characteristics of YY1 binding that apply to the site in *LEF1* P2. YY1 motifs are overrepresented in core promoters and genome wide ChIP–chip studies in HeLa cells reveal that RNA polymerase II promoters are highly enriched for YY1-binding sites (25). More than 3% of the RNA polymerase II promoters had detectable YY1 motifs (756 genes) and many of these motifs occurred ~30 nt downstream of the transcription start site in a short 5′ untranslated region (5′ UTR) (25). These motifs often overlapped the translation start codon of the open reading frame. These characteristics are similar to the YY1 element in *LEF1* P2, which has the core YY1 site at +25 within a short P2 5′ UTR. Since these features appear to be shared among hundreds of RNA Polymerase II promoters, the transcription regulatory principles that we uncover for *LEF1* P2 may also apply to a larger set of YY1-connected promoters.

Two functional assays were performed to learn about the mechanism of YY1 in P2 repression. A knockdown experiment that depleted endogenous YY1 protein resulted in partial activated expression of the integrated luciferase reporter genes in a dose dependent manner, but only if the upstream repressor was present (Figure 5). Thus, continuous binding of YY1 to the +25 element is required for P2 repression of the integrated reporters. Also, placing the repressor element upstream of *LEF1* P1, and introducing a single YY1-binding site in an analogous position with only a 3-nt change conferred repression (Figure 6). Because wild-type *LEF1* P1 is not normally responsive to the repressor, it was the introduction of the YY1-binding site that enabled communication between the repressor and the core promoter for transcriptional silencing.

We also analyzed endogenous *LEF1* expression upon YY1 knockdown using northern and western blot assays. We did not detect any change in the expression of FL-LEF1 or dnLEF-1 mRNA or protein over the course of the 72 h experiment (data not shown). Thus, even though knockdown of YY1 is sufficient to activate an integrated P2 luciferase reporter gene, it is not sufficient to activate endogenous P2, at least in this time frame. Either loss of YY1 is not sufficient and another factor must be eliminated for re-activation of P2, or YY1 is no longer necessary for the maintenance of a silent *LEF1* P2 promoter. It is also possible that our partial YY1 knockdown did not reduce protein levels low enough for relief of repression. A longer time-course of knockdown was not possible as the cells could not tolerate YY1 reduction and began to die at 72 h.

We also performed ChIP assay to detect repressive histone marks H3K9me3 and H3K27me3 in the presence and absence of YY1. Interestingly, we observed high enrichment of H3K9 methylation on the integrated P2 when YY1 was present and dramatic reductions when YY1 was eliminated (Figure 5C). This result was consistent with our YY1 knockdown P2 luciferase assay when we detected partial reactivation of integrated P2 plasmid. Endogenous P2 was methylated, although not as high as integrated P2, but methylation was not reduced as a result of YY1 knockdown. This may explain why we do not observe reactivation of the endogenous P2 locus.

How is YY1 involved in P2 repression? YY1 is an extremely versatile transcription factor. YY1 binds and bends DNA and it can bind to RNA (26). YY1 can recruit regulatory proteins for either transcription activation or repression depending on the context [for review see (7,8)]. In the case of repression, YY1 acts as a Polycomb group (PcG) protein and recruits chromatin modifying co-repressors such as histone deacetylases and histone methyl transferases (7,11,27–29). In some cases of repression, YY1 action is required transiently at the beginning of the silencing process and is not necessary at later stages when a silent epigenetic signature of histone methylation and de-acetylation has been established (30,31). Perhaps the inability to reactivate the endogenous *LEF1* P2 locus is due to stable, YY1-independent-maintenance of silencing. If so, then our stable cell lines with the integrated luciferase plasmids report on early events of repression where YY1 is involved whereas endogenous P2 represents the permanent silent state maintained by epigenetic marks rather than YY1. This second layer of epigenetic repression maintains strong silencing such that reduction of YY1 no longer has an effect. A similar finding was recently reported for human *HOXD11* and *12* genes, which are repressed by YY1 and Polycomb group related proteins recruited to a distal repressor. YY1 and co-factors are involved in the initial repression of *HOXD11, HOXD12* during differentiation of human

mesenchymal stem cells into either osteoblasts or adipocytes (31). Interestingly, YY1 was no longer associated with the repressor region in the differentiated osteoblasts, even though repression was still maintained.

Based on what is known about YY1-mediated repression and the data presented here, we propose a model for the role of YY1 in *LEF1* P2 repression. First, YY1 binding to P2 is necessary to recruit the upstream repressor to act upon the core promoter. We cannot say that YY1 is sufficient, because even though a single, introduced YY1 site is enough to make P1 repressor-susceptible, other common components of the P1 and P2 core promoters, or accessory components of the RNA polymerase II pre-initiation complex may also be necessary. Also, YY1 is a DNA-bending protein and may use the bending feature to facilitate interactions of proteins on either side of its binding site to efficiently recruit the upstream repressor complex (32). Second, YY1 is a PcG protein and we therefore propose that repressor recruitment results in the association of chromatin modifying PcG complexes and changes in chromatin acetylation and methylation. Indeed, while *LEF1* P1 chromatin is very highly acetylated in colon cancer nuclei, these levels change significantly over the next 5000 bp where a sharp drop in acetylation begins at the upstream repressor region and continues through *LEF1* P2 (4). Alteration of the local chromatin structure could negatively affect the ability of TCF–β-catenin complexes to recognize WREs or the ability of core promoter factors to activate the general transcriptional machinery.

The predicted outcome of YY1 action on *LEF1* is silencing of dnLEF-1 expression, allowing FL-LEF-1 and other FL-TCFs to act unopposed in their regulation of Wnt target genes. As such, YY1 appears to positively affect Wnt signaling. While our study is the first to link the actions of transcription factor YY1 to Wnt signaling, YY1 expression and activity have been previously associated with tumorigenesis in a wide range of human cancers including human colorectal cancer where it is overexpressed (7,33). Indeed, we find that YY1 knockdown is lethal to colon cancer cells. It will be important to determine just how tightly linked oncogenic Wnt signaling and YY1 actions are in colon cancer and whether this applies to other tumor types. Additionally, since the YY1 motif is overrepresented in human promoters, it is possible that a subset of these are subject to repressor actions similar to *LEF1* P2. It is therefore important to define the repressor element and the complexes and protein(s) that associate with it for YY1-linked regulation.

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