A β -catenin/TCF-coordinated chromatin loop at *MYC* integrates 5' and 3' Wnt responsive enhancers

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Aberrant *MYC* gene expression by the Wnt/ β -catenin pathway is implicated in colorectal carcinogenesis. Wnt/ β -catenin signaling stimulates association of the β -catenin coactivator complex with two Wnt responsive enhancers (WREs) located in close proximity to *MYC* gene boundaries. Each enhancer directly binds members of the TCF/Lef family of transcription factors that, in turn, recruit β -catenin. In a previous report, we showed that the downstream *MYC* enhancer (*MYC* 3' WRE) cooperated with the upstream enhancer (*MYC* 5' WRE) to activate expression of a heterologous reporter gene in response to Wnt/ β -catenin and mitogen signaling. Here we use chromatin conformation capture (3C) to show that the *MYC* 5' and 3' WREs are juxtaposed at the genomic *MYC* locus during active transcription. This *MYC* 5'3' chromatin loop is present in HCT116 human colorectal cancer cells that contain high levels of nuclear β -catenin and is absent in HEK293 cells that contain trace amounts of nuclear β -catenin. Depletion of functional β -catenin/TCF complexes blocks formation of the *MYC* 5'3' chromatin loop. Furthermore, we find that the chromatin loop is absent in quiescent cells, but is rapidly and transiently induced by serum mitogens in a β -catenin-dependent manner. Thus, we propose that a distinct chromatin architecture coordinated by β -catenin/TCF-bound WREs accompanies transcriptional activation of *MYC* gene expression.

colon cancer | chromatin conformation capture

The Wnt/ β -catenin signaling pathway plays a critical role in regulating cell proliferation, cell migration, and stem cell self-renewal in the gastrointestinal tract (1). β -Catenin is the key mediator of cellular responses to Wnt signaling, and its cytoplasmic/nuclear partitioning is tightly controlled. When Wnt is unavailable, cytoplasmic β -catenin associates with a multiprotein destruction complex that targets its degradation via the proteasome. Thus, in the absence of Wnt ligand, there is little, if any, nuclear β -catenin and Wnt/ β -catenin target genes are repressed by CtBP and Groucho/TLE corepressor complexes (2). These corepressors are tethered to target genes through interactions with members of the T-cell factor/lymphoid enhancer binding factor (TCF/Lef) family of transcription factors. In the presence of Wnt, cytoplasmic β -catenin is stabilized and subsequently translocated to the nucleus. Nuclear β -catenin binds to TCF/Lef, displaces the corepressor complexes, and recruits coactivator complexes including CBP/p300 acetyltransferases and MLL/Set methyltransferases to activate gene expression (2).

Mutations in components of the Wnt/ β -catenin pathway are among the earliest detected lesions during colorectal carcinogenesis (3). In most cases of colorectal cancer, missense mutations target the *adenomatous polyposis coli* (*APC*) gene and lead to synthesis of a truncated APC protein. Truncated APC has diminished capacity to coordinate degradation of cytoplasmic β -catenin and, as a result, β -catenin inappropriately accumulates in the nuclei of colonic epithelial and stem cells. Therefore, deregulation of target gene expression controlled by β -catenin drives cellular transformation. β -Catenin-dependent regulation of one target in particular, the *MYC* proto-oncogene, has been implicated as a key oncogenic event for colorectal tumorigenesis (4).

Vogelstein and colleagues were the first to realize the mechanistic link among Wnt/ β -catenin signaling, *MYC* expression, and

colorectal cancer (5). Using a screen to identify genes whose expression was dependent upon Wnt/ β -catenin in a human colon cancer cell line, He et al. found that *MYC* expression decreased when nuclear β -catenin was depleted (5). These authors then localized a Wnt-responsive enhancer element (WRE) to the 5' proximal promoter region of *MYC* and demonstrated that Wnt/ β -catenin signaling activated *MYC* gene expression through this element. This report spurred the discovery of additional *MYC* WREs. Using a computational approach termed the enhancer element localizer, Hallikas et al. identified 2 putative WREs that conferred tissue specific *MYC* expression (6). Recently, a fourth WRE was identified based on the proximity of the single nucleotide polymorphism rs6983267 to a consensus TCF4 binding site (7). This distal WRE, which localizes approximately 335 kb upstream from *MYC*, formed a chromatin loop with the promoter region of the *MYC* gene (8). Our group identified a fifth *MYC* WRE through an unbiased and genome-wide screen for β -catenin binding sites (9). This 3' WRE mapped 1.4 kb downstream from the *MYC* transcriptional stop site and is the principal regulatory element for *MYC* gene expression in response to Wnt/ β -catenin and mitogen signaling in human colon cancer cells.

Results from work outlined in our previous study raised the possibility that the proximal *MYC* 5' and 3' WREs might associate physically during induction of *MYC* gene expression (9). Furthermore, β -catenin and TCF4 occupancy at the 3' WRE preceded occupancy at the 5' WRE, as cells exited quiescence and entered the G₁ stage. β -Catenin occupancy at the 5' promoter occurred before maximal *MYC* expression, consistent with its role in directly regulating induction of *MYC* transcription. These findings suggested that β -catenin might be delivered from the 3' to the 5' WRE via an intrachromosomal chromatin loop before the initiation of *MYC* gene transcription.

Results

A Chromatin Loop Containing the 5' and 3' WREs Is Present at *MYC* in HCT116 Cells. We hypothesized that β -catenin/TCF could coordinate a chromatin loop containing the 5' and 3' WREs at the *MYC* gene locus and tested this using the chromatin conformation capture (3C) technique (10, 11). 3C operates under the premise that if 2 distal regions of chromatin are associated via bridging proteins, this complex will be trapped when cells are fixed with formaldehyde. In the case of intrachromosomal interactions, this likely occurs via looping, with a protrusion of chromatin that does not directly participate in formation of the complex (Fig. 1A). The protruding chromatin is released by digesting it with a restriction endonuclease whose recognition motif appears frequently in the genome (Fig. 1B). The cleaved

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Fig. 1. Diagram of the 3C technique. The black line is chromatin, ovals are bridging proteins, triangles mark restriction enzyme recognition motifs, and arrows represent oligonucleotides used in PCR. (A) Formaldehyde crosslinks interacting DNA/protein complexes and results in a chromatin loop. Restriction endonuclease digestion releases the chromatin loop (B), compatible DNA ends are ligated (C), and purified 3C DNA is amplified using PCR (D).

DNA has compatible ends that can be ligated together (Fig. 1C). The ligated DNA is detected in a PCR using oligonucleotide primers whose design enables production of a product only if juxtaposition has occurred (Fig. 1D).

We initially chose to use the restriction enzyme BstY1 for 3C assays because it has 10 sites that localize to a 12-kb region containing the *MYC* locus (Fig. 2A). Eight PCR primer sets were designed that flank the BstY1 sites. Of these sets, for clarity, only the primers used in the 3C assays are depicted (Fig. 2A). To test

Fig. 2. A chromatin loop containing the 5' and 3' Wnt/β-catenin responsive enhancers is present at *MYC* in HCT116 cells. (A) Diagram of the human *MYC* locus with gray rectangles as exons, thick horizontal lines as introns, and thin horizontal lines as untranscribed DNA. The open rectangles labeled WRE are the 5' and 3' Wnt/β-catenin responsive enhancers (5, 9). An arrow above E1 marks the major *MYC* transcription start site. BstY1 restriction endonuclease sites are depicted by triangles and stunted arrows mark the position of oligonucleotides used in the 3C assays (labeled B1-B8). (B) Analysis by 3C of the *MYC* locus in HCT116 cells. A PCR product was generated with B4 and B8 only (labeled *MYC* 5'3'). LC is a loading control and S is a DNA standard. (C) A diagram of the *MYC* locus as in A except that triangles mark Xba1 restriction endonuclease sites and stunted arrows mark the position of x1, x2, and x3 oligonucleotides. (D) Same as B except that Xba1 was used in 3C reactions and purified DNA was amplified with x1 and x3.

whether these primers, sites, and BstY1 were appropriate for in vivo 3C assays, we first conducted in vitro assays using a bacterial artificial chromosome (BAC) harboring the human *MYC* locus. Chromatin conformation capture DNA was PCR-amplified with forward primers B1 through B6 coupled with reverse primer B7 or B8. Each primer set generated PCR products of the predicted size and approximately equivalent efficiencies, indicating that this strategy was suitable for 3C assays (Fig. S1A).

We then conducted 3C assays in asynchronous cultures of HCT116 human colorectal carcinoma cells. These cells contain high levels of nuclear β-catenin as a result of a mutation that stabilizes the β-catenin protein (12). β-Catenin/TCF4 complexes have been shown to associate with the *MYC* 5' and 3' WREs in these cells using the chromatin immunoprecipitation (ChIP) assay (9). Because we hypothesized involvement of the 3' WRE in the chromatin loop, we tested reverse primers B7 and B8 with various combinations of upstream primers B1 through B6. In addition, we amplified a region of *tubulin* to ensure equal amounts of 3C DNA were used in the PCR reactions. PCR product was obtained with primer set B4 and B8 only, and its generation depended upon the inclusion of BstY1 and DNA ligase to the 3C reactions (Fig. 2B). This 325-bp fragment was sequenced to confirm that it was the correct *MYC* product. This result suggested that 5' and 3' DNA elements are involved in a chromatin loop at the *MYC* gene.

To independently validate these results, we asked whether the chromatin loop could be detected at *MYC* using a different restriction enzyme in 3C assays. We chose Xba1 because its recognition sites relative to *MYC* are located similarly to the BstY1 sites that detected the loop as described earlier (Fig. 2C). As was the case with BstY1, the expected PCR product was obtained in a ligase dependent manner (Fig. 2D). Thus, a chromatin loop is present at *MYC* in HCT116 cells and this loop is mediated by elements containing the 5' and 3' *MYC* WREs. For simplicity, we will refer to this interaction as the *MYC* 5'3' chromatin loop.

β-Catenin Is Required for the *MYC* 5'3' Chromatin Loop. We next determined whether β-catenin was required for the *MYC* 5'3' chromatin loop in HCT116 cells. To deplete β-catenin protein levels, we infected cells with a lentivirus expressing an shRNA targeting β-catenin mRNA. A lentivirus expressing an irrelevant shRNA was used as a control. Cells expressing the β-catenin-specific shRNA had much reduced levels of β-catenin protein compared with levels seen in control cells (Fig. 3A). To test whether nuclear levels were also reduced, we interrogated β-catenin binding to the 3' WRE using the ChIP assay. In control cells, levels of β-catenin binding to the 3' WRE were ninefold higher than background (Fig. 3B). In contrast, β-catenin binding to the *MYC* 3' WRE in cells expressing the β-catenin shRNA were only slightly above background. Therefore, both soluble and chromatin bound β-catenin levels are diminished by the β-catenin shRNA.

Depletion of β-catenin completely blocked formation of the *MYC* 5'3' chromatin loop (Fig. 3C). The loop was present in the control cells, indicating that its absence in the β-catenin shRNA treated cells was not a consequence of viral infection. We next attempted to rescue loop formation in β-catenin-depleted cells by overexpressing a cDNA encoding stabilized β-catenin. Expression of β-catenin restored the chromatin loop, implicating its involvement in bridging upstream and downstream *MYC* DNA elements (Fig. 3D). Together, these results indicate that β-catenin is required for the *MYC* 5'3' chromatin loop in a human colorectal cancer cell line.

TCF/Lef Transcription Factors Mediate the *MYC* 5'3' Chromatin Loop. TCF/Lef proteins recruit β-catenin to *MYC* 5' and 3' WREs to regulate *MYC* gene expression (9). To determine whether TCF/Lef is required for the *MYC* 5'3' chromatin loop, we used a plasmid encoding dominant-negative (dn) hLef1. dn hLef1 lacks

Fig. 3. β-Catenin is required for the *MYC* 5'3' chromatin loop in HCT116 cells. HCT116 cells were infected with a lentivirus expressing a shRNA directed against β-catenin (β-catenin shRNA) or a lentivirus expressing a control shRNA. (A) Western blot of whole cell protein extracts prepared from β-catenin shRNA and control shRNA-expressing cells. Blots were first probed with antibodies against β-catenin, and then with antibodies against tubulin, which served as a loading control. (B) Real-time PCR analysis of DNA fragments precipitated in a ChIP assay using an anti-β-catenin-specific antibody and control HCT116 cells (black bars) or β-catenin-depleted HCT116 cells (open bars). Oligonucleotides used to detect precipitated DNA were designed against the *MYC* 3' enhancer (3' WRE) or a control region 2.2 kb downstream from the 3' WRE (*MYC* distal) (9). Error bars are SEM. (C) Agarose gel of PCR products generated in 3C assays conducted in β-catenin depleted or control HCT116 cells as indicated. (D) Same as C, except 3C assays were conducted in β-catenin-depleted cells that were transfected with either β-catenin cDNA or control vector as indicated. For C and D, *MYC* 5'3' indicates the *MYC*-specific 3C products, LC is a loading control, and S is a DNA standard.

its amino-terminal β-catenin interaction domain and its overexpression competes with formation of transcriptionally competent β-catenin/TCF complexes (13–15). First, we tested whether dn hLef1 impaired recruitment of β-catenin to the 3' WRE. HCT116 cells were transfected with dn hLef1 and, 24 h later, β-catenin ChIP assays were conducted. As shown in Fig. 4A, the levels of β-catenin binding to the 3' WRE in cells expressing dn hLef1 was reduced compared with levels seen in control cells. We next conducted 3C assays and found that dn hLef1 expression impeded formation of the *MYC* 5'3' chromatin loop (Fig. 4B). These findings, together with those depicted in Fig. 3, implicate TCF/Lef members and β-catenin as bridging factors essential for interactions between the *MYC* 5' and 3' WREs.

Exogenous β-Catenin Induces the *MYC* 5'3' Chromatin Loop in HEK293 Cells. Myc is a ubiquitously expressed transcription factor required for cellular proliferation. However, not all cells expressing Myc harbor mutations in components of the Wnt/β-catenin signaling pathway. We therefore determined whether the *MYC* 5'3' chromatin loop was present in a cell line that does not contain high levels of nuclear β-catenin. For these studies, we chose HEK293 human embryonic kidney cells. Consistent with other reports, we found little β-catenin in protein extracts isolated from purified HEK293 nuclei (Fig. 5A) (16, 17). However, transfecting these cells with the β-catenin S45F expression plasmid caused a dramatic increase in nuclear β-catenin (Fig. 5A). ChIP assays demonstrated that β-catenin overexpression resulted in increased β-catenin occupancy at the 5' and 3' WREs (Fig. 5B). We then conducted 3C assays in HEK293 cells overexpressing β-catenin or transfected with an empty vector control. Exogenous β-catenin expression induced the *MYC* 5'3' loop, whereas no loop was detected in the control cells (Fig. 5C). The Wnt/β-catenin pathway can also be stimulated in HEK293 cells

Fig. 4. TCF is required for the *MYC* 5'3' chromatin loop in HCT116 cells. (A) HCT116 cells were transfected with a plasmid encoding dominant negative human Lef1 (dn hLef1) or control plasmid, and ChIP assays with a β-catenin-specific antibody were conducted 24 h later. β-Catenin binding to the *MYC* 3' WRE or to a distal *MYC* region was assessed with specific oligonucleotides in a real-time PCR. Error is SEM. (B) Agarose gel of the PCR amplified and *MYC* specific 3C product (*MYC* 5'3'). Chromatin conformation capture assays were conducted in HCT116 cells 24 h following transfection with control or dn hLef1 plasmids. LC is a loading control.

by addition of Wnt3A or GSK3β inhibitors (16–18). However, after such treatments, we were unable to detect the *MYC* 5'3' chromatin loop. Treatment of HEK293 cells with these agonists caused a considerably lower level of nuclear β-catenin compared with that seen in cells expressing the β-catenin S45F cDNA (Fig. S2). However, agonist treatment did cause increased β-catenin occupancy at the *MYC* 5'3' WREs (Fig. S2). These results indicate that β-catenin is rate limiting for the *MYC* 5'3' chromatin loop in HEK293 cells and suggest that loop formation may require levels of nuclear β-catenin characteristic of colon cancer cells.

The *MYC* 5'3' Chromatin Loop Is Induced by Serum Mitogens. Transcriptional regulation of *MYC* expression is tightly controlled during the cell cycle (19–21). In quiescent cells or at G₀/G₁, *MYC* expression is repressed. As cells are stimulated to enter the cell cycle, Myc levels are dramatically induced during early to middle G₁, and then diminish in S phase. We conducted 3C assays in synchronized HCT116 cells to determine whether the *MYC* 5'3' chromatin loop is regulated during quiescence and G₁. The *MYC* 5'3' chromatin loop was absent in quiescent cells; however, the loop was detected after addition of serum for 1 h (Fig. 6). The interaction was transient as it was absent in cells cultured in serum for 2 h and 4 h even though there was abundant Myc mRNA at these times (Fig. 6 and ref 9). Induction of the loop coincided with recruitment of β-catenin to the 3' WRE (9), suggesting that β-catenin may initiate a chromatin structure conducive to looping.

Recent studies have demonstrated that mitogen and Wnt/β-catenin pathways intersect to control target gene expression (9,

Fig. 5. β-Catenin overexpression induces the *MYC* 5'3' chromatin loop in HEK293 cells. (A) HEK293 cells were transfected with a plasmid encoding β-catenin (+) or a control plasmid (-) and nuclei were isolated 24 h later. Protein extracts were prepared and β-catenin levels assessed by Western blot. Membranes were re-probed with antibodies specific for TBP, which served as a loading control. (B) ChIP assays, using an antibody specific for β-catenin, were conducted in HEK293 cells transfected with β-catenin or a control plasmid. β-Catenin binding to the *MYC* 5' and 3' WREs was interrogated using specific oligos and real-time PCR. Error is SEM. (C) Agarose gel of the PCR amplified and *MYC* specific 3C product. HEK293 cells were transfected as in A. LC is a loading control.

Fig. 6. The *MYC* 5'3' chromatin loop is transiently induced by serum. HCT116 cells were synchronized in G₀, and then released into the G₁ stage of the cell cycle by addition of serum for 1, 2, or 4 h. Chromatin conformation capture assays were performed and PCR products were analyzed by agarose gel electrophoresis. *MYC* 5'3' is the *MYC* 3C product and LC is a loading control.

16, 22–25). In fact, our previous work found that serum mitogens dramatically stimulated β -catenin and TCF4 binding to the *MYC* 3' WRE in colon cancer cells (9). Furthermore, depletion of nuclear β -catenin in colon cancer cells blocked mitogen-stimulated accumulation of *Myc* transcript and protein (9). We therefore sought to determine whether β -catenin was required for the serum-induced *MYC* 5'3' chromatin loop. We used the β -catenin shRNA and control HCT116 cells described earlier for these experiments. Consistent with our previous findings, β -catenin binding to the 3' WRE was induced as quiescent control cells entered G₁ (compare –serum vs. +serum in Fig. 7A). As expected, this binding was drastically reduced in β -catenin-depleted cells (Fig. 7A). Strikingly, β -catenin depletion blocked the mitogen-stimulated chromatin loop (Fig. 7B). Together, these experiments demonstrate that β -catenin/TCF complexes respond to Wnt and mitogen signaling pathways by coordinating a chromatin loop to stimulate *MYC* gene expression.

Discussion

Genome-wide screens for β -catenin and TCF4 binding sites in human colon cancer cells have allowed identification of direct target genes regulated by Wnt/ β -catenin signaling (26, 27). A common conclusion from these screens is that β -catenin and TCF4 occupy sites outside the 5' proximal promoter regions of protein-coding genes. Although it is likely that β -catenin/TCF4 binding to many proximal promoters directly regulates expression of adjacent genes, the function of other β -catenin/TCF4 binding regions is less clear. Previously, by using a serial analysis of chromatin occupancy screen for β -catenin binding sites in

colon cancer cells, we identified a strong β -catenin/TCF4 site that localized 1.4 kb downstream of the *MYC* transcriptional stop (9, 27). We showed that this binding site demarcated a WRE that cooperated with the promoter proximal *MYC* 5' WRE to regulate expression of heterologous luciferase plasmids. In this report, we find that the 5' and 3' *MYC* WREs interact via a chromatin loop and that this interaction requires β -catenin/TCF complexes.

Our findings strongly implicate β -catenin and TCF4 as bridging factors for the interaction between the 5' and 3' *MYC* WREs. However, neither β -catenin nor TCF proteins are known to form homodimers. In addition, structural analysis suggests that one β -catenin molecule cannot simultaneously interact with 2 TCF proteins (28, 29). How then might β -catenin/TCF complexes bound to both the 5' and 3' WREs form a bridge to mediate the *MYC* chromatin loop? One possibility is through recruitment of additional factors. One such accessory factor may be APC. APC is known to dimerize, shuttle between the cytoplasm and nucleus, and function as a β -catenin chaperone (30–32). Moreover, studies from Sierra and colleagues demonstrated that APC co-occupies the *MYC* promoter with β -catenin as synchronized C2C12 mouse myoblasts are stimulated with mitogens and LiCl (33). In this system, β -catenin and APC occupancy persisted as *Myc* transcript accumulated. Using a different model system, however, Sierra et al. provided evidence that APC was involved in the exchange of corepressors for coactivators at *MYC*, a finding more supportive of a repressive role (33). This latter experiment was not conducted in synchronized cells, however, and relied upon APC overexpression. HCT116 cells have WT APC which, as a result of its numerous protein interaction domains, may be capable of organizing macromolecular chromatin complexes necessary for the 5' and 3' WRE interaction. Most colon cancers have mutant APC, however, and future work will examine the role of APC, as well as other components of the β -catenin degradation complex, in mediating the assembly and disassembly of the *MYC* 5'3' chromatin loop.

Recently, Pomerantz et al. reported a long-range interaction between a novel WRE localizing 335 kb upstream of the *MYC* transcriptional start site and the 5' *MYC* promoter (8). Although it was not tested whether β -catenin and/or TCF were required for this particular chromatin loop, their findings, along with ours, suggest that the 5' promoter of *MYC* functions as a platform to receive β -catenin and TCF4 complexes bound to distal enhancers. Currently, the role for the –335 kb WRE-containing loop in regulating *MYC* gene expression is unclear (8, 34). It will be of interest to determine whether both loops simultaneously form at the *MYC* promoter or whether there is a hierarchy of regulatory interactions.

Many functions have been suggested for chromosomal looping. These include developmental regulation of gene clusters by locus control regions (35), production of transcription factories where multiple genes are concomitantly expressed (36), coordination of cytokine transcription (36, 37), and even formation of repressive chromatin structures to silence expression (38). Our data indicates that formation of the *MYC* 5'3' chromatin loop accompanies Wnt/ β -catenin and mitogen activation of *MYC* gene expression in human colon cancer cells.

Finally, we propose that the *MYC* 3' WRE may function as an “onco-enhancer” where the *MYC* 5'3' chromatin loop is driven by pathogenic levels of nuclear β -catenin. We define an onco-enhancer as a DNA element whose primary role is to promote tumorigenesis. Several characteristics of Wnt/ β -catenin signaling, *MYC* expression, and colorectal carcinogenesis support this idea. First, the deregulation of Wnt/ β -catenin signaling that characterizes the vast majority of colorectal cancers results in elevated levels of nuclear β -catenin and *MYC* expression. We found in colon cancer cells that β -catenin preferentially binds the *MYC* 3' WRE compared with the *MYC* 5' WRE (9). Second, almost all of

Fig. 7. The serum induced *MYC* 5'3' chromatin loop requires β -catenin. (A) Confluent control or β -catenin depleted HCT116 cells were synchronized by serum deprivation, and ChIP assays were conducted using an anti- β -catenin-specific antibody in quiescent cells (–serum) or cells exposed to medium containing serum for 1 h (+serum). β -Catenin occupancy of the *MYC* 3' WRE was detected by real-time PCR analysis. Error is SEM. (B) Control and β -catenin-depleted HCT116 cells were synchronized and either kept quiescent (–serum) or released into the cell cycle for 1 h (+serum). Chromatin conformation capture assays were conducted and products were detected using *MYC* 5'3' loop-specific primers. Products amplified with *tubulin* primers served as a loading control (LC).

the genetic abnormalities that contribute to cancer-associated *MYC* gene expression lie in noncoding DNA regulatory elements and not in the protein-coding portion of *MYC*. Because chromosomal translocations analogous to those that characterize hematological malignancies, such as Burkitt lymphoma, are not common for colorectal cancers, it is possible that the *MYC* 3' WRE acquired the role of deregulating *MYC*. Third, the 3' WRE overlaps with a downstream DNase I hypersensitivity region that was described in a colon cancer cell line (39). This cell line-specific attribute is consistent with a role for the hyperactive Wnt/ β -catenin signaling and *MYC*-dependent processes that characterize colon carcinogenesis. Finally, the biological and tissue-specific responses to *Myc* levels are highly dose-dependent (40). *Myc* is intimately involved in normal cellular proliferation, and a modest increase in its expression causes hyperplasia in some tissues. However, cells senesce or undergo apoptosis in response to more elevated (approximately 15 fold higher) *Myc* levels. It is possible that physiological levels of *Myc* are controlled by proximal promoter elements and that carcinogenic levels of *Myc* result from complexes that are assembled at the upstream -335 kb WRE and 3' *MYC* WRE that are then juxtaposed to the transcription start site.

Materials and Methods

Cell Culture. HCT116 and HEK293 cells were cultured as described by Yochum et al. (9). Synchronizing HCT116 cells in the cell cycle was achieved using a previously described protocol (9, 22).

Chromatin Conformation Capture. Chromatin conformation capture was conducted as described by Palmer et al. (41) with some modification. Crosslinked chromatin was digested with either 20 μ L (200 U) of BstY1 or 10 μ L (200 U) Xba1 (New England Biolabs) overnight at 37°C. The purified 3C DNA was dissolved in water and 50 ng was amplified by PCR. The primer sequences used in BstY1 experiments were B1, CACCTCAGGTGATGTACAC; B2, AAATGCTCTATTCCTTACAC; B3, CATCTAGAGTAGAGTGCTCG; B4, TGTAGTAATTCACGAGAGGC; B5, ACCGAGGAGAATGTCAAGAGGC; B6, GAATCTGCCCCAGTTGATGG; B7, CTACAGA-TAAGTTACATAACC; and B8, CTGTACATTCTCCAGCTGG. The *MYC* 5'3' chromatin loop was detected using B4 and B8. The primer sequences used in Xba1 experiments were $\times$ 1, CTACCGCATTTCTGACAGC; $\times$ 2, AAGAAGTTGGCATTGGC; and $\times$ 3, CCAGG-TAGG-TCTCATCTCC. The *MYC* 5'3' loop was detected using $\times$ 1 and $\times$ 3. A region of *tubulin*, which served as a loading control, was amplified with GGGGCTGGGTAATGGCAA and TGGCACTGCTCTGGGTTCC. Cycling pa-

rameters were 94°C for 2 min. followed by 42 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s After a final extension of 3 min at 72°C, PCR products were resolved on a 1.4% agarose/1 $\times$ TAE gel. The *MYC* 5'3' chromatin loop was also detected using the 3C protocol reported previously (35).

ChIP. ChIP was conducted as previously described (15). Real-time PCR was used to detect isolated DNA fragments as described (15) with the following modifications: 2 $\times$ iQ SYBR Green Supermix (170-8882; Bio-Rad) was used in reactions and a MyIQ Single Color Real-Time PCR machine (Bio-Rad) was used to amplify and detect products. Primers GCTCAGCTTTGCCCTTGTGG and AACACCTTCCCATTCCCAAGTG were used to amplify the *MYC* 3' WRE, GAATAGGGGGCTTCGCTCTG and CGTCCTTGCTCGGGTGTGTGAAG were used to amplify the *MYC* 5' WRE region, and AACGGCTGCTTTCTGTCTCC and ATGTGGATGCTGCTGTCTGGAA were used to amplify the *MYC* control region. Real-time PCR data are represented as fold binding relative to control.

Cellular Fractionation and Western Blot. Preparation of nuclear lysates and Western blot analysis was conducted as described (15). Dilutions of antibodies used are: 1:1,000 β -catenin (BD Transduction), 1:10,000 α -tubulin (Sigma), and 1:1,000 TATA-binding protein (TBP; Millipore).

Plasmids and Transfections. Plasmids pCDNA3.1 β -catenin S45F and pME18-deltaN67-hLef1 (dn hLef1) were previously described (15). For experiments involving HEK293 cells, 5 μ g of plasmid was introduced into 2×10^6 cells using Lipofectamine 2000 (Invitrogen). For experiments involving HCT116 cells, 5 μ g of pCDNA3.1 β -catenin S45F was electroporated into 5×10^6 cells using the Nucleofector kit V (Amaxa). ChIP, 3C, and Western blot experiments were conducted 24 h after transfection.

Lentivirus and β -Catenin shRNA. The lentiviral plasmid (pLKO.1) encoding the β -catenin shRNA was obtained from Open Biosystems (cat. TRCN000003845). HEK293 FT (Invitrogen) cells were transiently cotransfected with the pLKO.1- β -catenin shRNA plasmid and the ViralPower Packaging Mix (Invitrogen) using Eugene6 reagent (Roche). As a negative control, shRNA virus was produced using the MISSION Non-Target shRNA Control Vector (Sigma-Aldrich). A detailed protocol for lentivirus production and infection of target cells is available at Addgene (<http://www.addgene.org/>).

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- Sancho E, Batlle E, Clevers H (2004) Signaling pathways in intestinal development and cancer. *Annu Rev Cell Dev Biol* 20:695-723.
- Mosimann C, Hausmann G, Basler K (2009) Beta-catenin hits chromatin: regulation of Wnt target gene activation. *Nat Rev Mol Cell Biol* 10:276-286.
- Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. *Cell* 61:759-767.
- Sansom OJ, et al. (2007) *Myc* deletion rescues *Apc* deficiency in the small intestine. *Nature* 446:676-679.
- He TC, et al. (1998) Identification of c-MYC as a target of the APC pathway. *Science* 281:1509-1512.
- Hallikas O, et al. (2006) Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. *Cell* 124:47-59.
- Tuupanen S, et al. (2009) The common colorectal cancer predisposition SNP rs6983267 at chromosome 8q24 confers potential to enhanced Wnt signaling. *Nat Genet* 41:885-890.
- Pomerantz MM, et al. (2009) The 8q24 cancer risk variant rs6983267 shows long-range interaction with *MYC* in colorectal cancer. *Nat Genet* 41:882-884.
- Yochum GS, Cleland R, Goodman RH (2008) A genome-wide screen for beta-catenin binding sites identifies a downstream enhancer element that controls c-Myc gene expression. *Mol Cell Biol* 28:7368-7379.
- Dekker J (2006) The three 'C's of chromosome conformation capture: controls, controls, controls. *Nat Methods* 3:17-21.
- Dekker J, Rippe K, Dekker M, Kleckner N (2002) Capturing chromosome conformation. *Science* 295:1306-1311.
- Ilyas M, Tomlinson IP, Rowan A, Pignatelli M, Bodmer WF (1997) Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc Natl Acad Sci USA* 94:10330-10334.
- Billin AN, Thirlwell H, Ayer DE (2000) Beta-catenin-histone deacetylase interactions regulate the transition of LEF1 from a transcriptional repressor to an activator. *Mol Cell Biol* 20:6882-6890.
- Hecht A, Litterst CM, Huber O, Kemler R (1999) Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. *J Biol Chem* 274:18017-18025.
- Yochum GS, Cleland R, McWeeney S, Goodman RH (2007) An antisense transcript induced by Wnt/beta-catenin signaling decreases E2F4. *J Biol Chem* 282:871-878.
- Gan XQ, et al. (2008) Nuclear Dvl, c-Jun, beta-catenin, and TCF form a complex leading to stabilization of beta-catenin-TCF interaction. *J Cell Biol* 180:1087-1100.
- Li J, Wang CY (2008) TBL1-TBLR1 and beta-catenin recruit each other to Wnt target-gene promoter for transcription activation and oncogenesis. *Nat Cell Biol* 10:160-169.
- Major MB, et al. (2007) Wilms tumor suppressor WT1 negatively regulates WNT/beta-catenin signaling. *Science* 316:1043-1046.
- Kelly K, Siebenlist U (1985) The role of c-myc in the proliferation of normal and neoplastic cells. *J Clin Immunol* 5:65-77.
- Kelly K, Siebenlist U (1986) The regulation and expression of c-myc in normal and malignant cells. *Annu Rev Immunol* 4:317-338.
- Walker W, Zhou ZQ, Ota S, Wynshaw-Boris A, Hurlin PJ (2005) Mnt-Max to Myc-Max complex switching regulates cell cycle entry. *J Cell Biol* 169:405-413.
- Toualbi K, et al. (2007) Physical and functional cooperation between AP-1 and beta-catenin for the regulation of TCF-dependent genes. *Oncogene* 26:3492-3502.
- Hasselblatt P, Gresh L, Kudo H, Guinea-Viniegra J, Wagner EF (2008) The role of the transcription factor AP-1 in colitis-associated and beta-catenin-dependent intestinal tumorigenesis in mice. *Oncogene* 27:6102-6109.
- Nateri AS, Spencer-Dene B, Behrens A (2005) Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. *Nature* 437:281-285.
- Sancho R, et al. (2009) JNK signalling modulates intestinal homeostasis and tumorigenesis in mice. *EMBO J* 28:1843-1854.
- Hatzis P, et al. (2008) Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. *Mol Cell Biol* 28:2732-2744.
- Yochum GS, et al. (2007) Serial analysis of chromatin occupancy identifies beta-catenin target genes in colorectal carcinoma cells. *Proc Natl Acad Sci USA* 104:3324-3329.

28. Huber AH, Nelson WJ, Weis WI (1997) Three-dimensional structure of the armadillo repeat region of beta-catenin. *Cell* 90:871–882.
29. Poy F, Lepourcelet M, Shivdasani RA, Eck MJ (2001) Structure of a human Tcf4-beta-catenin complex. *Nat Struct Biol* 8:1053–1057.
30. Henderson BR (2000) Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat Cell Biol* 2:653–660.
31. Joslyn G, Richardson DS, White R, Alber T (1993) Dimer formation by an N-terminal coiled coil in the APC protein. *Proc Natl Acad Sci USA* 90:11109–11113.
32. Neufeld KL, Zhang F, Cullen BR, White RL (2000) APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export. *EMBO Rep* 1: 519–523.
33. Sierra J, Yoshida T, Joazeiro CA, Jones KA (2006) The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. *Genes Dev* 20:586–600.
34. Pomerantz MM, et al. (2009) Evaluation of the 8q24 prostate cancer risk locus and MYC expression. *Cancer Res* 69:5568–5574.
35. Tolhuis B, Palstra RJ, Splinter E, Grosveld F, de Laat W (2002) Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol Cell* 10:1453–1465.
36. Cai S, Lee CC, Kohwi-Shigematsu T (2006) SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat Genet* 38:1278–1288.
37. Hadjur S, et al. (2009) Cohesins form chromosomal cis-interactions at the developmentally regulated IFNG locus. *Nature* 460:410–413.
38. Horike S, Cai S, Miyano M, Cheng JF, Kohwi-Shigematsu T (2005) Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome. *Nat Genet* 37: 31–40.
39. Mautner J, et al. (1995) Identification of two enhancer elements downstream of the human c-myc gene. *Nucleic Acids Res* 23:72–80.
40. Murphy DJ, et al. (2008) Distinct thresholds govern Myc's biological output in vivo. *Cancer Cell* 14:447–457.
41. Palmer MB, Majumder P, Green MR, Wade PA, Boss JM (2007) A 3' enhancer controls snail expression in melanoma cells. *Cancer Res* 67:6113–6120.