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Nuclear AXIN2 represses *MYC* gene expression

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ABSTRACT

The β-catenin transcriptional coactivator is the key mediator of the canonical Wnt signaling pathway. In the absence of Wnt, β-catenin associates with a cytosolic and multi-protein destruction complex where it is phosphorylated and targeted for proteasomal degradation. In the presence of Wnt, the destruction complex is inactivated and β-catenin translocates into the nucleus. In the nucleus, β-catenin binds T-cell factor (TCF) transcription factors to activate expression of *c-MYC* (*MYC*) and *Axis inhibition protein 2* (*AXIN2*). AXIN2 is a member of the destruction complex and, thus, serves in a negative feedback loop to control Wnt/β-catenin signaling. AXIN2 is also present in the nucleus, but its function within this compartment is unknown. Here, we demonstrate that AXIN2 localizes to the nuclei of epithelial cells within normal and colonic tumor tissues as well as colorectal cancer cell lines. In the nucleus, AXIN2 represses expression of Wnt/β-catenin-responsive luciferase reporters and forms a complex with β-catenin and TCF. We demonstrate that AXIN2 co-occupies β-catenin/TCF complexes at the *MYC* promoter region. When constitutively localized to the nucleus, AXIN2 alters the chromatin structure at the *MYC* promoter and directly represses *MYC* gene expression. These findings suggest that nuclear AXIN2 functions as a rheostat to control *MYC* expression in response to Wnt/β-catenin signaling.

1. Introduction

The evolutionarily conserved Wnt/β-catenin signaling pathway controls cellular proliferation, and it is essential for tissue homeostasis [1]. The key mediator of this pathway is the β-catenin transcriptional co-activator [2]. In the absence of Wnt, β-catenin is targeted for proteasomal degradation by a multi-protein and cytoplasmic "destruction complex" that contains adenomatous polyposis coli (APC), axis inhibition proteins 1 and 2 (AXIN1 and 2), casein kinase 1α (CK1α), and glycogen synthase kinase-3β (GSK-3β). Under these conditions, T-cell factor (TCF) transcription factors bound to Wnt responsive enhancers (WREs) recruit transducin-like enhancer (TLE) corepressors to repress Wnt target gene expression. When Wnt ligands bind frizzled/low-density lipoprotein receptor-related protein 5/6 co-receptor complexes on the cell surface, the destruction complex is inactivated and cytoplasmic β-catenin levels are stabilized. β-Catenin then translocates to the nucleus, binds TCFs, and recruits histone-modifying complexes to activate target gene expression.

AXIN2 is a direct Wnt/β-catenin target gene and it functions in a negative feedback loop to control cytosolic β-catenin levels in response to Wnt signaling [3–5]. Mutations in *AXIN2* have been identified in a subset of mismatch repair-deficient colorectal cancers and these mutations block the ability of AXIN2 to negatively regulate Wnt/β-catenin signaling [6]. Thus, early studies ascribed the role of AXIN2 as a tumor suppressor. While AXIN2 is largely considered a cytoplasmic protein that acts in the destruction complex, it is also localized within the nucleus [7–9]. However, the function of AXIN2 within the nucleus is unknown.

Here, we investigate the role of nuclear AXIN2 in the regulation of Wnt/β-catenin target gene expression. After experimentally validating the specificity of a commercially available anti-AXIN2 antibody, we evaluated the subcellular distribution of AXIN2 within colonic epithelial cells and found that it localizes to both the cytoplasmic and nuclear compartments. By constitutively targeting AXIN2 to the nucleus, we demonstrate that a ternary AXIN2/β-catenin/TCF complex forms and that this complex directly represses expression of the Wnt/β-catenin target gene, *c-MYC* (*MYC*). Therefore, AXIN2 reinforces negative feedback regulation of the Wnt/β-catenin signaling pathway by targeting β-catenin in both the cytoplasm and the nucleus. Our findings support a role for AXIN2 functioning as a rheostat to directly control *MYC* gene expression in colorectal carcinoma cells (CRCs).

Abbreviations: APC, adenomatous polyposis coli; AXIN, Axis inhibition protein; TCF, T-cell factor; WRE, Wnt responsive enhancer; CRC, colorectal cancer.

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2. Materials and methods

2.1. Cell lines

HCT116, SW480, and SW620 human colorectal cancer (CRC) cell lines were purchased from ATCC and HEK293 cells were purchased from Invitrogen. These cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, 2 mM Glutamax, and 0.1 mg/ml streptomycin at 37 °C in 5% CO_2.

2.2. Plasmids

Generation of the *MYC* enhancer-driven firefly luciferase reporters and the pcDNA3-β-catenin S45F construct were described previously [10,11]. The pcDNA-MYC-TCF4 plasmid was obtained from Addgene (deposited by Dr. Bert Vogelstein). TOPflash and FOPflash luciferase reporters were purchased from Millipore. To generate the pcDNA3-NLS-AXIN2 expression vector, *NLS-AXIN2* was first amplified using pCMV6-entry-AXIN2 (Origene, RC210931) as the template and the indicated primers (Table S1) in standard PCR reactions containing Phusion polymerase (New England Biolabs). The SV40 nuclear localization sequence (NLS) was incorporated into the upstream primer such that it was in frame with the *AXIN2* coding sequence. The PCR product was subcloned as a BamHI–HindIII fragment into pCMV-Tag 2B (Agilent Technologies), which provided an amino-terminal FLAG epitope tag. The *NLS-FLAG–NLS-AXIN2* cDNA was then amplified by PCR, which incorporated a second SV40 NLS at the amino terminus of AXIN2. This PCR product was subcloned into pcDNA3 as a HindIII–XbaI fragment and this plasmid is referred to as pcDNA3-NLS-AXIN2 for simplicity. The protein produced from this plasmid is referred to as NLS-AXIN2.

2.3. Lentiviral shRNA-mediated knockdown of AXIN2

Lentiviral plasmids encoding a scrambled sequence (Ctrl.) and *AXIN2* shRNAs were obtained from Open Biosystems. Control and *AXIN2* shRNA lentiviruses were generated and used to transduce HCT116 and SW480 cells using protocols described previously [12]. AXIN2 protein levels were assessed 3 days after lentiviral transduction by Western blot analysis.

2.4. Immunohistochemistry and indirect immunofluorescence

A colon cancer tumor microarray was purchased from Imgenex (IMH-359) and stained using our published protocols with anti-AXIN2 (also known as conductin) primary antibodies (Santa Cruz, sc-20784, 1:250) [12]. AXIN2 expression in human CRC lines was detected by indirect immunofluorescence using protocols described previously [12]. The fixed cells were incubated with anti-AXIN2 antibodies (Santa Cruz, sc-20784, 1:50) overnight at 4 °C.

2.5. Cellular fractionation and Western blot analysis

Approximately 5×10^6 cells were fractionated into nuclear and cytoplasmic protein lysates as described previously [11]. Whole cell protein extracts were prepared using approximately 1.25×10^6 cells and Western blot analysis was conducted as described previously [13]. Each lane of an 8% polyacrylamide gel contained 20 μg of protein lysates and the blots were probed with the following primary antibodies: anti-AXIN2 (Santa Cruz, sc-20784, 1:250), anti-α-tubulin (Sigma, T9026, 1:1000), anti-histone H3 (Millipore, 07-690, 1:25000), anti-TCF4 (Millipore, 05-511, 1:1000), and anti-β-catenin (BD Transduction, 610154, 1:1000).

2.6. Luciferase assays

Luciferase assays were conducted as described previously [10]. Approximately 5×10^4 HCT116 cells were plated in quadruplicate wells in a 24-well plate. The following day the cells were transfected with 2 ng pLRL-SV40 *Renilla* luciferase (Promega), 100 ng of firefly luciferase reporter plasmid, 250 ng pcDNA3-NLS-AXIN2 or pcDNA3 (Invitrogen), and pBlueScript SK+ (Agilent Technologies) to obtain a 1 μg final DNA concentration using Lipofectamine 2000 (Invitrogen). For experiments involving HEK293 cells, the cells were seeded as above, but were instead transfected using calcium phosphate precipitation. Where indicated, transfection mixtures contained 125 ng pcDNA3-β-catenin S45F and/or 250 ng pcDNA-MYC-TCF4. Luciferase levels were measured 24 h after transfection using a firefly and *Renilla* luciferase assay kit (Biotium, 30005) and a GloMax 20/20 luminometer (Promega).

2.7. Co-immunoprecipitation

Approximately 5×10^6 HEK293 cells were seeded in a 10 cm dish and transfected the next day using calcium phosphate precipitation with combinations of 3 μg pcDNA3-β-catenin S45F, 6 μg pcDNA-MYC-TCF4, and 6 μg pcDNA3-NLS-AXIN2 as indicated. Immunopreciptations were performed as described previously [14], except samples were incubated with 3 μg of anti-TCF4 antibodies (Millipore, 05-511) for 1 h at 4 °C with rocking followed by incubation with a 20 μl bed volume of Protein G beads (GE Healthcare) for 1 h at 4 °C. To examine the interaction between endogenous nuclear AXIN2 and β-catenin, 4×10^7 HCT116 cells were grown in 20 cm dishes and nuclei were prepared as described previously [11]. Proteins were immunoprecipitated by incubating the samples with 3 μg of anti-AXIN2 (Santa Cruz, sc-20784) or 3 μg of anti-rabbit IgG (Millipore, PP64B) antibodies overnight on a rotating platform at 4 °C.

2.8. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [15]. The following antibodies were used with 3 μg of each added per sample: anti-AXIN2 (Santa Cruz, sc-20784), anti-β-catenin (BD Transduction, 610154), anti-TCF4 (Millipore, 05-511), and 5 μl of anti-histone H3K4me3 (Active Motif, 39159). The precipitated DNA was analyzed by quantitative real-time PCR (qPCR) with the indicated primer sets (Table S1). A standard curve that was generated with serial dilutions of purified input DNA was used as a reference to quantify the precipitated DNA. The data was normalized to values detected using the control primer set and reported as relative levels of binding. For reactions involving overexpressed nuclear AXIN2, HCT116 cells were transfected with 10 μg pcDNA3-NLS-AXIN2 using FuGENE HD reagent (Promega). ChIP analysis was performed 24 h after transfection.

2.9. Real-time reverse-transcription PCR (qRT-PCR)

Approximately 5×10^6 HCT116 cells were transfected with 10 μg pcDNA3-NLS-AXIN2 and then serum starved for 48 h. After 4 h of treatment with media containing 10% FBS, total RNAs were isolated and cDNAs were synthesized using iScript cDNA synthesis kit (Bio-Rad) as described previously [12]. qRT-PCR was conducted as described previously using the indicated primer sets (Table S1) [12,15]. Expression values were obtained using a standard curve generated from sonicated and purified HCT116 genomic DNA. Values were normalized to *TUBULIN* and graphed as relative expression.

2.10. Statistics

Each experiment was repeated at least three times and statistical significance was calculated using Student's *t*-test.

3. Results

3.1. Subcellular localization of AXIN2 in colorectal carcinoma (CRC) cell lines and intestinal tissues

To evaluate the subcellular localization of AXIN2, we first examined the specificity of a commercially available anti-AXIN2 antibody. Western blot analysis of whole cell protein lysates prepared from HEK293 cells transiently transfected with pCMV-*AXIN2* confirmed that this anti-AXIN2 antibody recognizes exogenously expressed AXIN2 (Fig. 1A). We next assessed whether this antibody could detect endogenous AXIN2 in CRC cell lines. HCT116 and SW480 cells were transduced with lentiviruses expressing a scrambled sequence (Ctrl.) or four independent shRNAs that target *AXIN2*, and 3 days later, AXIN2 expression was assessed by Western blot analysis. Cells expressing AXIN2 shRNAs numbers 2 and 4 decreased AXIN2 protein levels relative to control (Fig. 1B). Having validated the specificity of the anti-AXIN2 antibody, we next determined the subcellular localization of AXIN2 in the HCT116, SW480, and SW620 CRC cell lines by cellular fractionation and Western blot analysis. AXIN2 was found in both cytoplasmic and nuclear fractions in each of the three cell lines tested (Fig. 1C). This distribution was confirmed by indirect immunofluorescence analysis on fixed cells (Fig. 1D). Finally, we probed a human tissue microarray that contained sections derived from uninvolved colonic mucosa, primary adenomas, and metastatic carcinomas. As expected, numerous cells within these tissues stained positively for AXIN2 in the cytoplasm (Fig. 1E, grey arrows). We also detected several clear examples of AXIN2 localizing within the nuclei of epithelial cells in sections prepared from both normal colonic mucosa and tumors (Fig. 1E, black arrows).

3.2. Nuclear AXIN2 decreases Wnt/β-catenin reporter activity

We used the HEK293 cell line to determine the influence of nuclear AXIN2 on Wnt/β-catenin signaling. In the absence of Wnt, the cytoplasmic destruction complex is active in these cells, which causes reduced levels of β-catenin and AXIN2 in the nucleus (Fig. 2A). To study the role of AXIN2 in the nucleus, we engineered two SV40 large T-antigen nuclear localization sequences (NLSs) onto the amino-terminus of AXIN2, and when expressed in HEK293 cells, NLS-AXIN2 localized to the nucleus (Fig. 2B). We next tested whether nuclear AXIN2 regulated the activity of the Wnt/β-catenin-responsive luciferase reporter, TOPflash. Co-transfection of plasmids encoding TCF4 and stabilized β-catenin activated expression of TOPflash, but not the FOPflash control reporter (Fig. 2C). Addition of pcDNA3-NLS-AXIN2 to the transfection reaction diminished the levels of TOPflash activity driven by β-catenin/TCF4 complexes (Fig. 2C). These results indicate that nuclear AXIN2 negatively regulates β-catenin/TCF-dependent transcriptional activity.

3.3. Nuclear AXIN2 interacts with TCF4 indirectly through β-catenin

We next addressed potential mechanisms that might explain how nuclear AXIN2 was repressing β-catenin/TCF-dependent transcriptional activation. We first tested whether nuclear AXIN2 was altering total β-catenin levels or affecting β-catenin subcellular localization. In HCT116 cells, NLS-AXIN2 cells did not affect total β-catenin levels or β-catenin subcellular localization (Fig. 3A). We next tested whether endogenous AXIN2 and β-catenin interacted in the nucleus and found that β-catenin co-precipitated with AXIN2 in nuclear protein lysates prepared from HCT116 cells

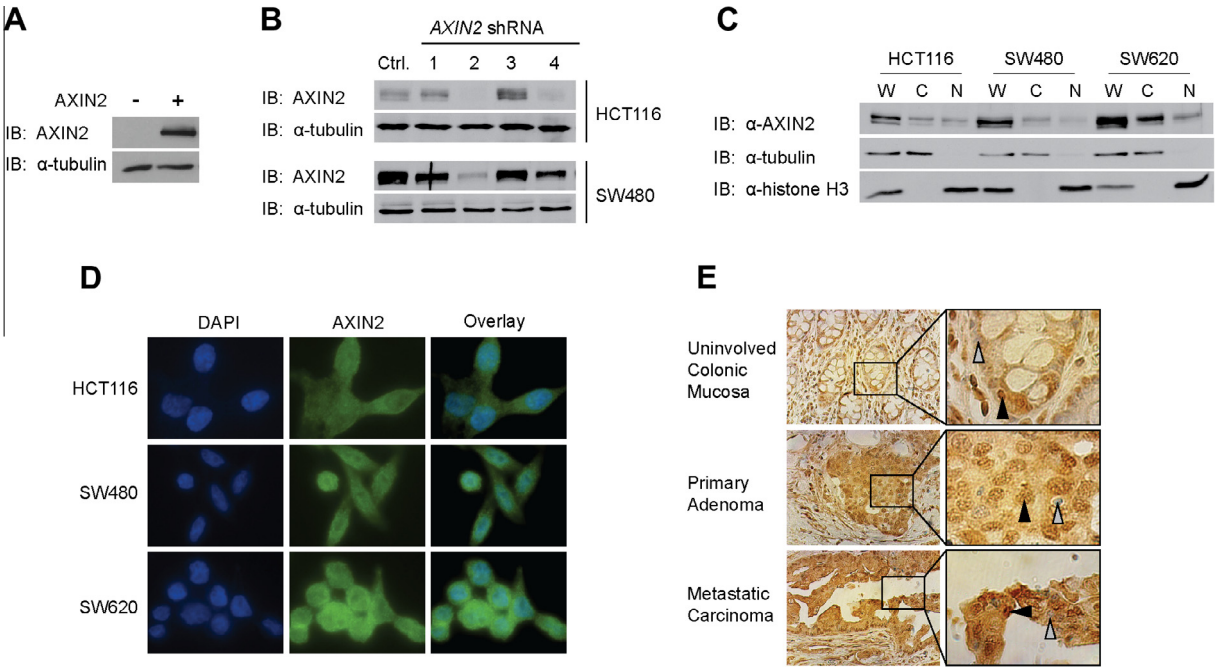

Fig. 1. Subcellular localization of AXIN2 in established human CRC cell lines and intestinal tissues. (A) Western blot analysis of protein lysates prepared from HEK293 cells that were transfected with pcDNA3-NLS-AXIN2. (B) Western blot analysis of protein lysates prepared from HCT116 and SW480 cells that were transduced with lentiviruses expressing a scrambled sequence (Ctrl.) or four independent shRNAs designed to target *AXIN2*. (C) Western blot analysis of whole cell [W], cytoplasmic [C], and nuclear [N] lysates prepared from HCT116, SW480, and SW620 cell lines. (D) Immunocytochemical analysis of AXIN2 subcellular localization in HCT116, SW480, and SW620 CRC cell lines. (E) Immunohistochemical analysis of human tissue microarrays prepared from uninvolved colonic mucosa, primary colorectal adenomas, and colorectal tumor metastases. Shown are representative images. Grey and black arrows identify cells with cytoplasmic and nuclear AXIN2 staining, respectively.

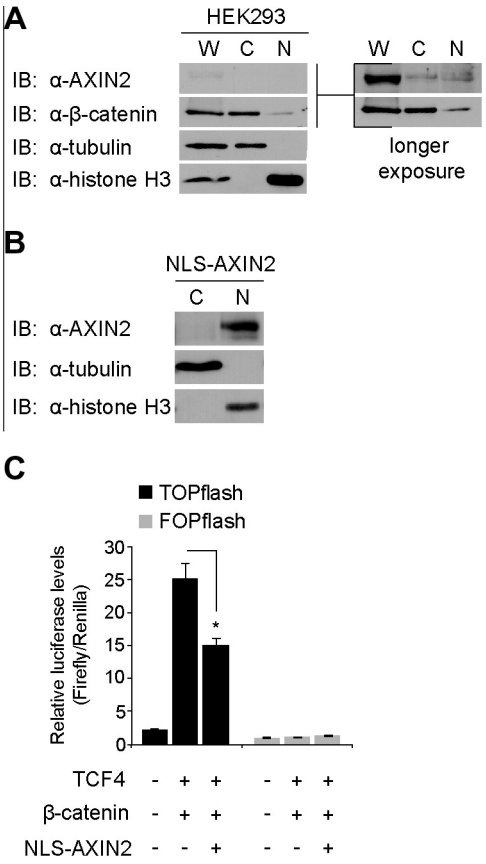

Fig. 2. Nuclear AXIN2 decreases the activity of a Wnt/β-catenin-responsive luciferase reporter. (A) Western blot analysis of whole cell [W], cytoplasmic [C], and nuclear [N] protein lysates prepared from HEK293 cells. (B) Western blot analysis of protein lysates prepared from cytoplasmic [C] and nuclear [N] compartments of HEK293 cells that were transfected with pcDNA3-NLS-AXIN2. (C) Luciferase reporter assays in HEK293 cells transfected with the Wnt-responsive TOPflash reporter or the control FOPflash reporter. Where indicated, cells were co-transfected with plasmids encoding TCF4, β-catenin S45F, and NLS-AXIN2. Data are represented as mean ± SEM ($n = 4$, $^*P < 0.05$).

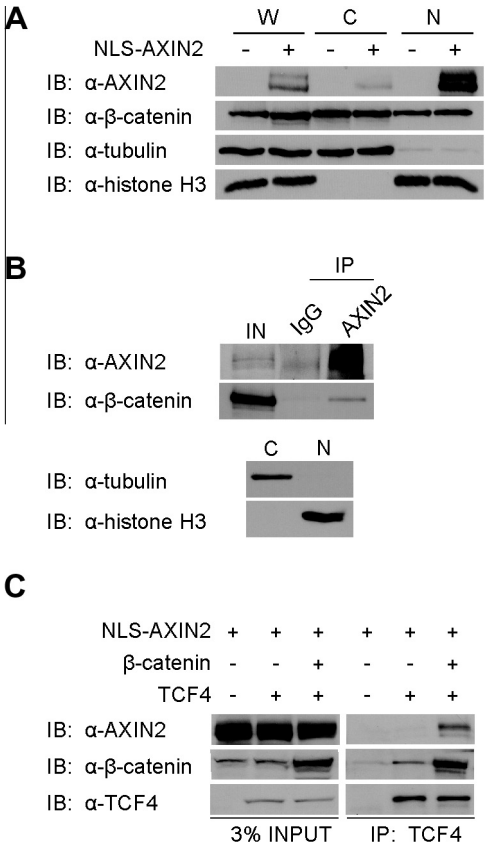

Fig. 3. β-Catenin bridges AXIN2 to TCF4. (A) Western blot analysis of proteins prepared from whole cell [W], cytoplasmic [C], and nuclear [N] compartments of HCT116 cells expressing NLS-AXIN2. (B) *Top*, Co-immunoprecipitation/Western blot analysis of proteins precipitated with anti-AXIN2 antibodies in nuclear lysates prepared from HCT116 cells. *Bottom*, Western blot analysis of cytoplasmic [C] and nuclear [N] fractions using α-tubulin and histone H3 antibodies. (C) Co-immunoprecipitation/Western blot analysis of TCF4 interacting proteins. HEK293 cells were transfected with plasmids expressing the indicated cDNAs and TCF4 was precipitated with anti-TCF4 antibodies.

(Fig. 3B). Because β-catenin has independent and non-overlapping binding surfaces for AXIN2 and TCF [16], we explored whether β-catenin could simultaneously interact with these two proteins. To test this possibility, we conducted co-immunoprecipitation/Western blot analyses of transiently transfected HEK293 cells. In the absence of exogenous β-catenin, TCF4 failed to precipitate AXIN2 (Fig. 3C). However, when β-catenin, TCF4 and NLS-AXIN2 were co-expressed, TCF4 precipitated exogenous AXIN2. These findings suggest that β-catenin bridges AXIN2 to TCF4.

3.4. Nuclear AXIN2 binds the MYC gene locus and alters the chromatin architecture to decrease MYC expression

Given that nuclear AXIN2 can interact with a β-catenin/TCF4 complex, we next tested whether AXIN2 regulates expression of the β-catenin target gene, *MYC*. We first determined whether nuclear AXIN2 regulated the activity of a *MYC* luciferase reporter [10,17]. This reporter contains the *MYC* 5′ WRE, a well-established WRE that maps within the *MYC* proximal promoter region, upstream of the luciferase gene (Fig. 4A). As a control, we used a reporter that contains mutations in the two TCF4 motifs required for *MYC* responsiveness to Wnt/β-catenin signaling within this enhancer [10,17]. Expression of nuclear AXIN2 in the HCT116 cell line reduced luciferase expression driven by the wild-type, but not the mutant *MYC* reporter (Fig. 4A).

We next determined whether AXIN2 binds to the *MYC* 5′ WRE embedded within the endogenous *MYC* locus in HCT116 cells using chromatin immunoprecipitation (ChIP) assays [10,15]. In addition to β-catenin and TCF4, we detected AXIN2 binding to this element, whereas little binding was detected to a control element that lacks TCF motifs and maps far upstream from the *MYC* transcription start site (Fig. 4B and C). To determine whether nuclear AXIN2 alters β-catenin binding at the *MYC* 5′ WRE, HCT116 cells were transfected with NLS-AXIN2 and ChIP analyses were performed the following day. NLS-AXIN2 occupied the *MYC* 5′ WRE, and it did not alter β-catenin binding (Fig. 4D). However, NLS-AXIN2 reduced levels of histone H3 that is trimethylated on lysine 4 (H3K4me3) at the *MYC* promoter (Fig. 4D). This histone modification demarcates the promoters of transcriptionally active genes in general [18] and it has been shown to localize to the *MYC* promoter under conditions where *MYC* is expressed [10,19]. Finally, NLS-AXIN2 reduced *MYC* gene expression 2-fold relative to control (Fig. 4E). Taken together, these results suggest that a nuclear AXIN2/β-catenin/TCF4 complex directly represses transcription of the Wnt/β-catenin target gene, *MYC*.

4. Discussion

The Wnt/β-catenin signaling pathway is subjected to both positive and negative feedback regulation [1,2]. AXIN2 is a direct Wnt/

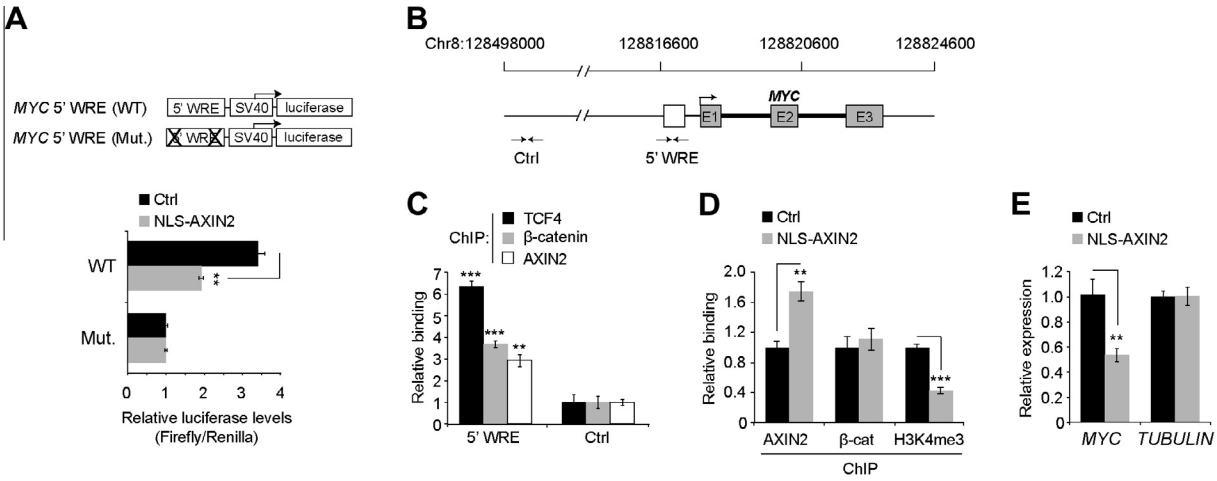

Fig. 4. AXIN2 alters the chromatin structure at the *MYC* promoter and decreases *MYC* expression. (A) Luciferase assays of HCT116 cells transfected with wild-type or mutant *MYC* 5′ WRE-containing luciferase reporters in the presence or absence of pcDNA3-NLS-AXIN2. (B) Diagram of the *MYC* gene locus with the *MYC* 5′ WRE represented as a white box, *MYC* exons as gray boxes, and introns as thick black lines. The positions of PCR primer sets used to interrogate DNA elements precipitated in the ChIP assays are indicated by opposing arrows. Control (Ctrl.) is a region approximately 287 kb upstream from the *MYC* transcription start site. (C) ChIP analysis of TCF4 (black bars), β-catenin (gray bars), and AXIN2 (white bars) binding to the *MYC* 5′ WRE in HCT116 cells. (D) ChIP analysis of AXIN2, β-catenin and H3K4me3, in HCT116 cells transfected with pcDNA3 (Ctrl.) or pcDNA3-NLS-AXIN2. (E) Real-time reverse transcription PCR (qRT-PCR) analysis of *MYC* expression in control or HCT116 cells expressing NLS-AXIN2. (C–E) Data are represented as mean ± SEM ($n = 4$, $^{**}P < 0.01$, $^{***}P < 0.001$).

β-catenin target gene, and as a component of the destruction complex, it functions to negatively regulate Wnt signaling. In the present study, we demonstrate that nuclear AXIN2 forms a ternary complex with β-catenin and TCF to directly repress *MYC* gene expression. Thus, as is the case for cytoplasmic AXIN2, nuclear AXIN2 participates in a negative feedback loop to control expression of Wnt/β-catenin target genes.

Despite the identification of AXIN2 as a nuclear protein, it is unknown how AXIN2 translocates to the nucleus or how it's subcellular localization is regulated. As we detected AXIN2 within the nucleus of SW480 and SW620 CRC cells that harbor mutant *APC* alleles [20], it is unlikely that APC is a key mediator of AXIN2 nuclear import. Moreover, we found that NLS-AXIN2 did not affect β-catenin subcellular localization. These findings argue that interactions between AXIN2 and β-catenin do not regulate their nuclear import. It is, therefore, unlikely that β-catenin functions as a molecular chaperone to shuttle AXIN2, but rather, AXIN2 forms a subcomplex with β-catenin once both proteins have translocated into the nucleus. As is the case with its paralog, AXIN1, numerous nuclear localization and nuclear export signal sequences have been identified within AXIN2 [9]. Future studies will determine which of these sequences are required for targeting and retaining AXIN2 within the nucleus in response to Wnt/β-catenin signaling.

The dominant model in the literature that AXINs negatively regulate Wnt signaling was recently challenged [21]. Lui et al. found that concomitantly diminishing AXIN2 and AXIN1 levels in CRC cell lines decreased, rather than increased, Wnt/β-catenin reporter activity and they suggested that the nuclear pool of AXIN may be responsible for this phenotype [21]. Our results indicate that the nuclear pool of AXIN2 negatively regulates Wnt/β-catenin signaling. Differences in experimental approaches might account for these discrepancies. The small interfering RNA (siRNA) approach used by Lui et al. affects both cytoplasmic and nuclear pools of AXIN, whereas our study specifically addressed the role of AXIN2 in the nucleus.

Nuclear APC sequesters β-catenin from TCF transcriptional complexes and functions to exchange co-activator complexes with co-repressor complexes at the *MYC* gene [19,22]. Given this relationship, we hypothesized that nuclear AXIN2 might function similarly to influence β-catenin/TCF transcriptional complexes. While we did not detect a change in β-catenin binding at the *MYC* promoter in response to nuclear AXIN2, we did observe an increase in AXIN2 binding and a decrease in H3K4me3 levels. We, therefore, propose a model to explain how nuclear AXIN2 regulates Wnt/β-catenin signaling. When the Wnt pathway is activated, or in CRC cells with hyperactive Wnt signaling, β-catenin associates with TCF at WREs to activate expression of *MYC* and *AXIN2*. Sustained Wnt signaling leads to heightened AXIN2 protein levels and AXIN2 translocation into the nucleus where it associates with β-catenin/TCF complexes to repress *MYC* gene expression.

Based upon our findings, we suggest that nuclear AXIN2 functions as a rheostat to control *MYC* expression. In stem cells, this mechanism would reinforce the "just right" amount of Wnt signaling required to promote proliferation and self-renewal [23]. In intestinal cancers driven by aberrant Wnt/β-catenin signaling, nuclear AXIN2 might function to sustain oncogenic *MYC* expression and dampen the apoptotic response that is caused by elevated *MYC* levels [24]. Our findings, therefore, support the further development of compounds that stabilize AXIN, such as XAV939, for the treatment of Wnt/β-catenin-driven intestinal cancers [25].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.089.

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