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Targeted repression of *AXIN2* and *MYC* gene expression using designer TALEs

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ABSTRACT

Designer TALEs (dTALEs) are chimeric transcription factors that can be engineered to regulate gene expression in mammalian cells. Whether dTALEs can block gene transcription downstream of signal transduction cascades, however, has yet to be fully explored. Here we tested whether dTALEs can be used to target genes whose expression is controlled by Wnt/β-catenin signaling. TALE DNA binding domains were engineered to recognize sequences adjacent to Wnt responsive enhancer elements (WREs) that control expression of *axis inhibition protein 2* (*AXIN2*) and *c-MYC* (*MYC*). These custom DNA binding domains were linked to the mSin3A interaction domain (SID) to generate TALE–SID chimeric repressors. The TALE–SIDs repressed luciferase reporter activity, bound their genomic target sites, and repressed *AXIN2* and *MYC* expression in HEK293 cells. We generated a novel HEK293 cell line to determine whether the TALE–SIDs could function downstream of oncogenic Wnt/β-catenin signaling. Treating these cells with doxycycline and tamoxifen stimulates nuclear accumulation of a stabilized form of β-catenin found in a subset of colorectal cancers. The TALE–SIDs repressed *AXIN2* and *MYC* expression in these cells, which suggests that dTALEs could offer an effective therapeutic strategy for the treatment of colorectal cancer.
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1. Introduction

Advances in genome-editing technologies have dramatically improved the ability to manipulate expression of endogenous genes in mammalian cell lines. One technology involves the use of transcription activator-like effectors (TALEs) from the plant pathogenic bacteria of the genus *Xanthomonas* [1]. TALEs contain a central DNA binding domain that can be engineered to recognize specific DNA sequences within the mammalian genome. Designer tales (dTALEs) tether transcriptional activation or repression domains to TALE DNA binding domains. Effective dTALEs that target distal enhancer elements, proximal promoter regions, non-coding DNA regions and exons have been described [2–4]. The mammalian mSin3A interaction domain (SID) has been shown to be an effective transcriptional repressor domain for use in dTALEs [2]. The SID, first characterized from studies of the Mad transcription repressor, is a small amphipathic alpha helix that recruits the mammalian mSin3A/HDAC corepressor complex [5,6]. Whether dTALEs can be used to modulate expression of genes downstream of signaling pathways is an area of open research.

The Wnt/β-catenin signaling pathway is a critical regulator of tissue homeostasis, cellular proliferation, and stem cell biology [7]. A central component of this pathway is the β-catenin transcription coactivator and its levels and sub-cellular localization are tightly regulated. In the absence of extracellular Wnt ligand, cytosolic β-catenin associates with a multi-protein "destruction complex" that coordinates its phosphorylation and subsequent degradation by the proteasome. Under these conditions, T-cell factor transcription factors (TCFs) bound to Wnt responsive DNA elements (WREs) recruit transducin like enhancer (TLE) corepressor complexes to repress target gene expression [8]. In the presence of Wnt, the destruction complex is inactivated and β-catenin is translocated into the nucleus where it displaces TLE. β-Catenin/TCF complexes recruit additional chromatin modifying complexes to activate gene expression [8]. Mutations in components of the Wnt/β-catenin signaling pathway are found in approximately 90% of colorectal cancers (CRCs) [9]. These mutations cause accumulation of β-catenin in the nucleus and aberrant target gene expression.

AXIN2 and *MYC* are two well-characterized Wnt/β-catenin target genes [10–14]. AXIN2 is a component of the destruction

Abbreviations: TALE, transcription activator-like effector; SID, mSin3A interaction domain; AXIN2, axis inhibitor 2.

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complex and it thus serves in a negative feedback loop to control the duration of the Wnt response. The WREs that control *AXIN2* expression map to the 5′ promoter and regions downstream of the transcription start site [11–13,15,16]. MYC is a transcription factor that primarily activates expression of genes whose products drive cellular proliferation [17]. The WREs that control *MYC* expression are proximal to gene boundaries and also map several hundred thousand kilobases away from the transcription start site [10,14,18,19].

Here, we describe the generation and characterization of three TALE–SID fusion proteins targeting known WREs that control *AXIN2* and *MYC* gene expression. We demonstrate that the TALE–SIDs bind their targeted sequences and repress gene expression in HEK293 cells. Using a stable HEK293 system that mimics oncogenic Wnt/β-catenin signaling, we demonstrate that the TALE–SIDs also repress target gene expression in this setting. Together, these findings indicate that dTALEs can be used to modulate gene expression downstream of oncogenic Wnt/β-catenin signaling.

2. Materials and methods

2.1. Cell lines

The HEK293FT and Flp-In T-REx 293 cell lines were purchased from Invitrogen and maintained according to the manufacturer's guidelines.

2.2. Plasmids

The pGL3-basic and pGL3-promoter luciferase reporters were purchased from Promega, pME18-LEF was a gift from D. Ayer (University of Utah), and the *YAP1* luciferase reporter and the pcDNA3-β-catenin^{S45F} construct were previously described [20,21]. The TALEN plasmids that target *AXIN2* were obtained from Addgene (deposited by Dr. Keith Joung). The *MYC* TALE DNA binding domain was constructed using the TALE assembly kit (Addgene, deposited by Dr. Keith Joung) following the detailed instructions provided. The TALE1 and TALE2 plasmids were generated by removing the FokI nuclease as a BamHI-AgeI restriction fragment, filling in the 5′ overhangs with Klenow polymerase and ligating the blunt ends. Four copies of the SID were PCR-amplified from pUC57-SID4X (Addgene, deposited by Dr. Feng Zhang) and the products were sub-cloned into BamHI-AgeI digested TALE plasmids to generate the TALE–SIDs.

The *AXIN2* luciferase reporter plasmid was generated by PCR-amplifying a 787-bp fragment of the *AXIN2* gene from genomic HCT116 DNA that includes the TALE binding sites. The PCR product was sub-cloned into the pGL3 basic vector as a KpnI-NheI fragment. To generate the pcDNA5/FRT/TO-β-catenin^{S45F}-estrogen receptor (ER) expression plasmid, β-catenin^{S45F} cDNA was PCR-amplified from pcDNA3-β-catenin^{S45F}. The ER cDNA was amplified from pBabepuro-myc-ER (Addgene, deposited by Wafik El-Deiry). The resulting β-catenin^{S45F} and ER PCR products were ligated and cloned into the pcDNA5/FRT/TO expression plasmid (Invitrogen) using the restriction enzymes BamHI, KpnI, and ApaI. The *MYC* luciferase transgene, which contains approximately 8.6-kb of genomic DNA that encompasses *MYC*, was generated stepwise with two PCR fragments amplified from a bacterial artificial chromosome (BAC) that harbors human *MYC*. The firefly luciferase gene was inserted in-frame into the second exon of *MYC*. See Supplementary materials for additional details and oligonucleotide sequences used in plasmid construction.

2.3. Generation of the β-catenin^{S45F}-ER HEK293 cell line

These cells were generated using the Flp-In T-REx system (Invitrogen). Approximately 5×10^6 Flp-In T-REx 293 cells were transfected with 9 μg of pOG44 (Invitrogen), encoding the Flp recombinase, and 1 μg of pcDNA5/FRT/TO-β-catenin^{S45F}-ER using calcium phosphate precipitation. After 48 h, the transfected cells were selected with 100 μg/ml hygromycin for 2 weeks. The resistant cells were pooled and maintained in media containing 100 μg/ml hygromycin and 15 μg/ml blasticidin. To induce β-catenin^{S45F}-ER expression, the cells were treated with 1 μg/ml doxycycline (DOX) overnight. The following day, the cells were treated with 1 μM 4-hydroxytamoxifen (4-OHT) for 24 h to stimulate β-catenin^{S45F}-ER translocation into the nucleus.

2.4. Co-immunoprecipitation

Immunoprecipitations were performed as previously described [22] on 5×10^6 HEK293 cells transfected with 5 μg of the indicated TALE plasmids. Samples were pre-cleared with Protein A beads and 6 μg of rabbit anti-mouse IgGs (Jackson ImmunoResearch, 315-005-003) for 1 h at 4 °C. The samples were then incubated with 3 μg of anti-FLAG antibodies (Sigma, F1804) for 1 h at 4 °C followed by an overnight incubation with 6 μg of rabbit anti-mouse IgG at 4 °C.

2.5. Cellular fractionation and Western blot analysis

Nuclear, cytoplasmic, and whole cell protein lysates were prepared as previously described [21]. Western blot analysis was performed as described [15] using the following primary antibodies: anti-HDAC1 (Abcam, ab7028, 1:1000), anti-FLAG (Sigma, F1804, 1:1000), anti-AXIN2 (Santa Cruz, sc-20784, 1:250), anti-α-tubulin (Sigma, T9026, 1:1000), and anti-histone H3 (Upstate, 06-755, 1:25,000).

2.6. Luciferase assays

Luciferase assays were conducted as described previously [14]. HEK293 cells were seeded in quadruplicate wells and transfected by calcium phosphate precipitation with each well receiving 2 ng of pLRL-SV40 *Renilla* luciferase (Promega), 100 ng of firefly luciferase reporter plasmid, 150 ng of TALE plasmid or pcDNA3 (Invitrogen), and pBlueScript SK+(Agilent Technologies) to a final DNA concentration of 1 μg. Where indicated, transfection reactions included 50 ng of pcDNA3-β-catenin^{S45F} and 50 ng of pME18-LEF.

2.7. Chromatin immunoprecipitation (ChIP)

ChIP was conducted as described previously [14,15] on 5×10^6 HEK293FT 48 h or 72 h after the cells were transfected with 5 μg of pcDNA3 or the TALE–SIDs, using calcium phosphate. For β-catenin^{S45F}-ER cells, ChIP was performed on 5×10^6 cells following treatment with or without DOX and 4-OHT. To precipitate the cross-linked chromatin, 3 μg of anti-FLAG (Sigma, F1804) or 5 μl of anti-histone H3K4me3 (Active Motif, 39159) antibodies were added to the samples. Oligonucleotide sequences for qPCR are available in Supplementary materials.

2.8. Quantitative and real-time reverse-transcription PCR (qRT-PCR)

A total of 5×10^6 HEK293FT or β-catenin^{S45F}-ER cells were transfected with 10 μg of pcDNA3 or 5 μg each of the TALE–SID plasmids. Total RNAs were isolated and cDNAs were synthesized 48 after transfection in HEK293FT cells or 72 h after transfection in β-catenin^{S45F}-ER cells as previously described [20]. Transcripts were measured by qRT-PCR as described previously [15,20] and the data was analyzed using the $2^{-\Delta\Delta CT}$ method. Oligonucleotide sequences used for qRT-PCR are available in Supplementary materials.

2.9. Immunohistochemistry

β-Catenin^{S45F}-ER expression was detected by staining fixed cells with anti-FLAG antibodies (Sigma, F1804, 1:1000) for 1 h at room temperature as previously described [20].

2.10. Statistics

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

3. Results

3.1. Generation of designer TALEs that target AXIN2

A common strategy to regulate expression of a gene of interest using designer TALEs is to target well-characterized DNA enhancer elements that control expression of that particular gene [2–4]. In this respect, *AXIN2* is an inherently difficult gene to target because its expression is potentially controlled by numerous enhancer elements in response to Wnt/β-catenin signaling [11,12,15,16]. In a previous study, we mapped regions across the genome of a human colorectal cancer cell line that bound β-catenin using ChIP-Seq [15]. We identified two highly enriched β-catenin-bound regions that mapped to the *AXIN2* transcription start site and first exon (Fig. 1A). We therefore reasoned that the region between these sites would serve as an appropriate target sequence for which to develop *AXIN2*-specific dTALEs. In addition, Reyon et al. generated a pair of FLAG-tagged TALE nucleases (TALENs) that targeted the first exon of *AXIN2*, which is positioned between the two β-catenin binding regions [23]. We obtained these TALENs, and replaced the FokI nuclease domain with 4 copies of the SID. We refer to these *AXIN2*-specific dTALEs as TALE1–SID and TALE2–SID (Fig. 1A).

3.2. The TALE–SIDs repress AXIN2 gene expression

Because the SID recruits the mSin3A/HDAC corepressor complex [5,6], we tested whether the TALE–SIDs interacted with endogenous HDAC1. Plasmids encoding TALE1–SID and TALE2–SID, or the TALE backbones as controls, were independently transfected into HEK293 cells and the expressed proteins were immunoprecipitated with anti-FLAG antibodies. In a Western blot analysis, we found that HDAC1 co-precipitated with TALE1- and TALE2-SIDs indicating that these chimeric proteins were capable of interacting with endogenous mSin3A/HDAC (Fig. 1B).

To determine whether the TALE–SIDs could regulate *AXIN2* gene expression, we initially conducted a series of luciferase reporter assays. A 787-bp fragment of *AXIN2* that contains the TALE–SID binding sites was inserted upstream of the luciferase gene in the pGL3-basic plasmid. This fragment also contains the TCF consensus-binding site, ATCAAAG. The *AXIN2* reporter was transfected into HEK293 cells with and without plasmids that express human Lef1 (LEF) or a β-catenin^{S45F} protein that contains a serine to phenylalanine substitution at amino acid 45 found in a subset of colon cancers [24]. β-Catenin^{S45F} and LEF activated expression of the pGL3-*AXIN2* reporter relative to control (Fig. 1C). Co-transfection of plasmids encoding either TALE1–SID or TALE2–SID repressed β-catenin^{S45F}/LEF-dependent luciferase activity (Fig. 1C). In contrast, the TALE–SIDs only modestly affected luciferase gene expression in

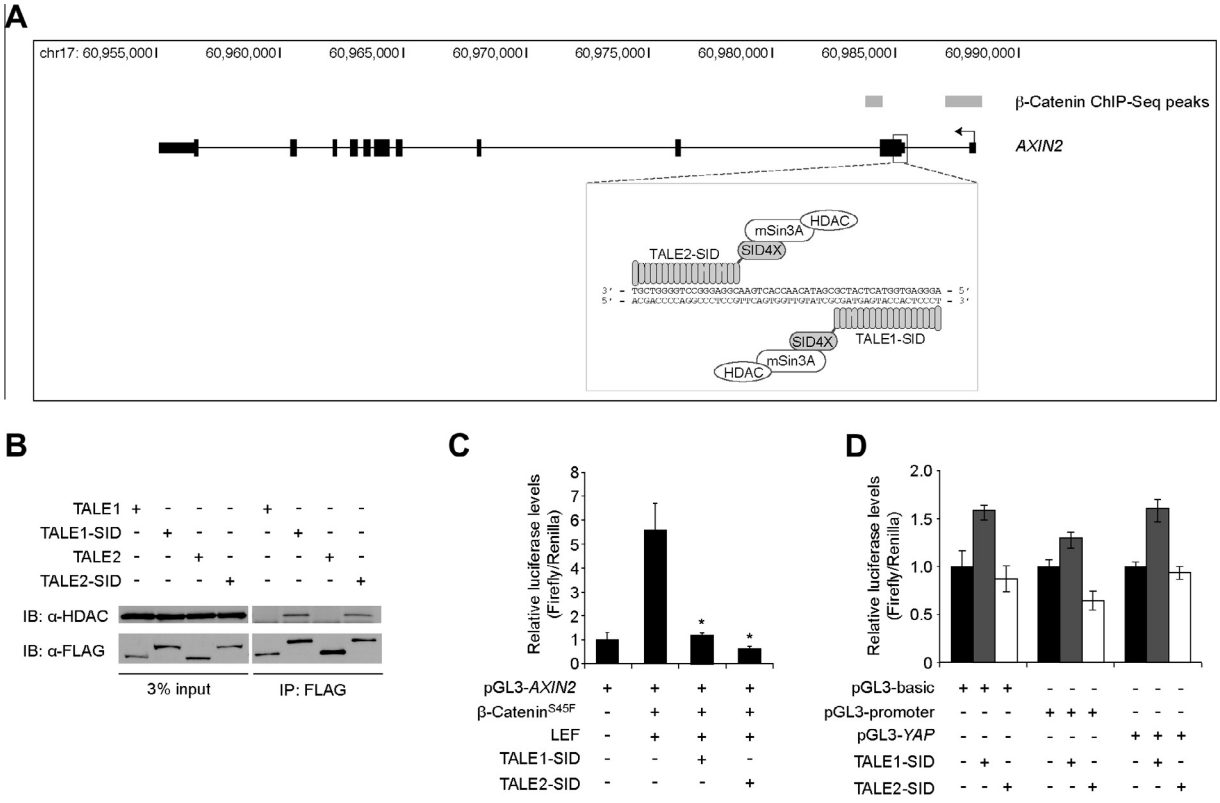

Fig. 1. TALE–SIDs that bind *AXIN2* associate with HDAC1 and repress *AXIN2*-luciferase reporter activity. (A) Schematic of the *AXIN2* gene locus with the transcription start site on the right side of the figure. Gray rectangles indicate the β-catenin-bound regions identified in a β-catenin ChIP-Seq screen [15]. A diagram of the TALE–SIDs is boxed below and shown is their target sequence within the first exon of *AXIN2*. (B) Western blot analysis of protein lysates prepared from HEK293 cells transfected with the indicated FLAG-tagged TALE constructs and immunoprecipitated with anti-FLAG antibodies. (C) Luciferase reporter assays in HEK293 cells transfected with a pGL3-basic plasmid that contains a 787-bp fragment of the *AXIN2* gene encompassing the TALE binding sites inserted upstream of the luciferase gene. Where indicated, cells were co-transfected with plasmids encoding β-catenin^{S45F}, LEF, TALE1–SID, and TALE2–SID. Error is SEM (*$P < 0.05$). (D) Luciferase reporter assays in HEK293 cells transfected with the indicated pGL3 luciferase plasmids and plasmids encoding TALE–SIDs.

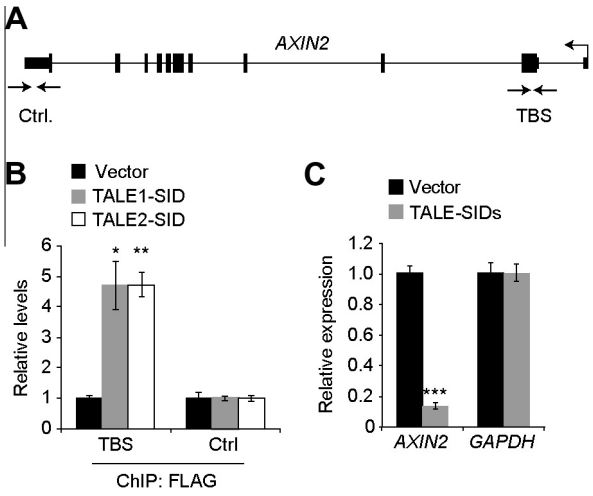

Fig. 2. The TALE–SIDs repress *AXIN2* gene expression. (A) Diagram of the *AXIN2* gene locus with the positions of the primer sequences used in the ChIP assays indicated by the opposing arrows. TBS: TALE binding site, Ctrl.: control. (B) ChIP analysis using anti-FLAG antibodies of HEK293 cells transfected with plasmids encoding FLAG-tagged TALE1–SID, TALE2–SID, or pcDNA3 as a control. The precipitated DNA was measured using qPCR analysis with *AXIN2*-specific oligonucleotides. (C) Analysis of *AXIN2* and *GAPDH* transcript levels in HEK293 cells transfected with the TALE–SIDs or pcDNA3 as a control. In (B) and (C) error is SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

control experiments conducted with luciferase reporters that lacked the *AXIN2* recognition element (Fig. 1D) [20].

We next determined whether the TALE–SIDs could repress expression from the endogenous *AXIN2* gene in HEK293 cells. First, we determined whether the TALE–SIDs bound their expected target site. We conducted ChIP assays with FLAG antibodies in transiently transfected cells and found that both TALE1–SID and TALE2–SID displayed enriched binding to their target sites within *AXIN2* relative to a control site that mapped within the 3′ untranslated region (Fig. 2A and B). A qRT-PCR analysis of transcripts found that cells transfected with both TALE–SIDs contained 5-fold lower levels of *AXIN2* expression relative to control (Fig. 2C). There was no difference in the levels of *GAPDH* transcripts in this experiment indicating that the TALE-repressors did not indiscriminately repress gene transcription. Together, these results indicate that the TALE–SIDs can access their target sites and repress *AXIN2* expression from its chromosomal locus.

3.3. The TALE–SIDs repress AXIN2 expression induced by oncogenic β-catenin

Due to the difficulties of transfecting human CRC cell lines at high efficiency, we established a clonal HEK293 cell line that expresses β-catenin^{S45F} in a doxycycline (DOX)- and tamoxifen (4-OHT)-dependent manner. Using a homologous recombination-based system (see Section 2), we inserted a cDNA into a single

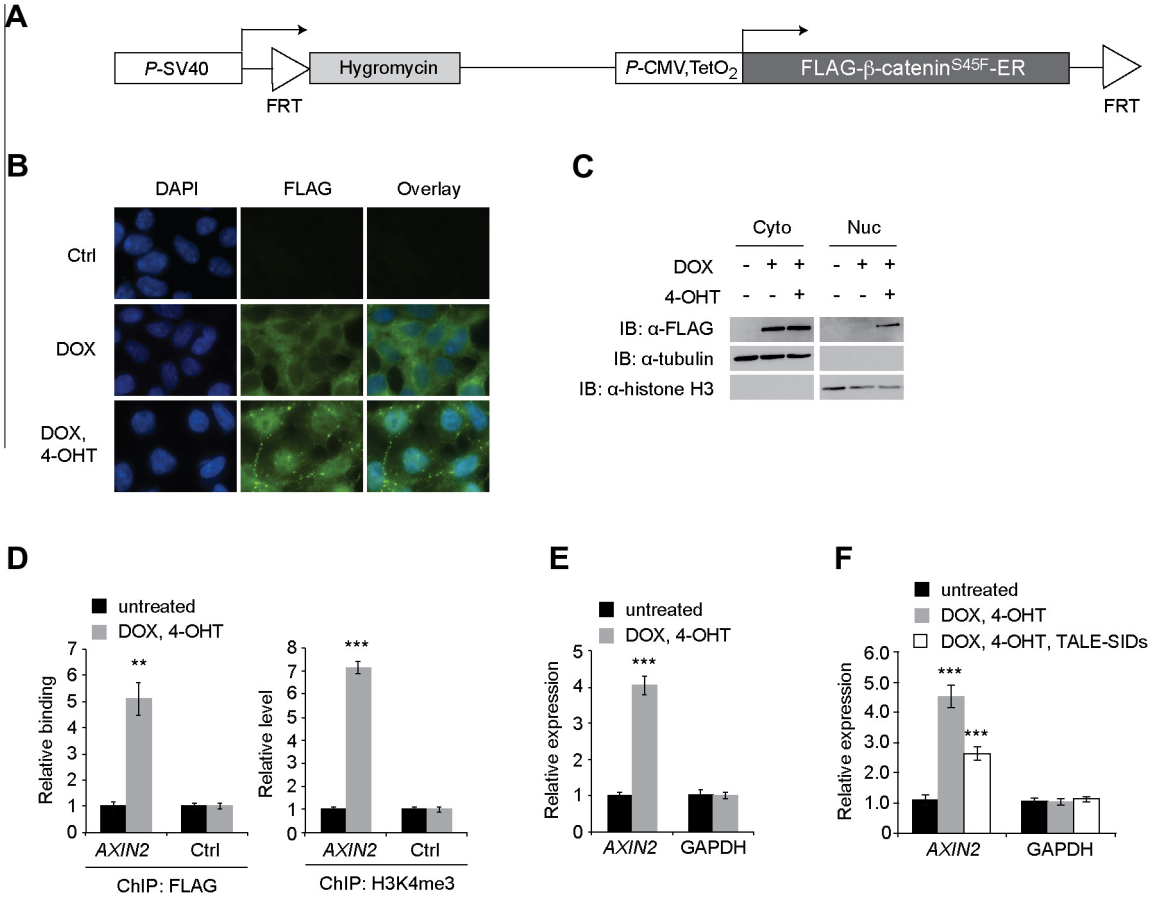

Fig. 3. The TALE–SIDs repress *AXIN2* expression induced by oncogenic β-catenin. (A) Diagram of the transgene inserted into Flp-In T-REx HEK293 cells. FRT; Flp-recombinase recognition target. $TetO_2$; Two copies of the tetracycline regulated operator sequence. (B) Indirect immunofluorescence analysis of HEK293-β-catenin^{S45F}-ER cells that were treated with DOX or DOX and 4-OHT. β-Catenin^{S45F}-ER expression was detected using anti-FLAG antibodies. (C) Western blot analysis of protein lysates prepared from cytoplasmic (Cyto) and nuclear (Nuc) compartments of HEK293-β-catenin^{S45F}-ER cells treated with DOX and 4-OHT as indicated. (D) ChIP analysis of FLAG-tagged β-catenin^{S45F}-ER binding and H3K4me3 levels at the *AXIN2* promoter, and the *AXIN2* 3′ UTR as a control, following the indicated treatments. (E) Analysis of *AXIN2* and *GAPDH* expression following β-catenin^{S45F}-ER induction and nuclear translocation. (F) As in (E) except cells were transfected with the TALE–SID plasmids where indicated. In (D), (E), and (F), error is SEM ($^{**}P < 0.01$, $^{***}P < 0.001$).

chromosomal locus that encodes a FLAG-tagged β-catenin^{S45F}-estrogen receptor (ER) fusion protein downstream of a CMV promoter under control of two tetracycline operator sequences (Fig. 3A). Treating these cells with DOX induces β-catenin^{S45F}-ER expression; however, the ER sequesters it in the cytoplasm (Fig. 3B and C). When cells are treated with DOX and 4-OHT, 4-OHT binds the ER receptor causing a conformational change and subsequent translocation of β-catenin^{S45F} into the nucleus (Fig. 3B and C). Using FLAG antibodies in ChIP assays, we found that nuclear β-catenin^{S45F} bound the *AXIN2* gene promoter and induced levels of trimethylated histone H3 on lysine 4 (H3K4me3), which is a histone modification that correlates with actively transcribed genes (Fig. 3D) [25]. Nuclear accumulation of β-catenin^{S45F} also increased expression of *AXIN2* indicating that the β-catenin^{S45F}-ER fusion protein was functioning appropriately (Fig. 3E). Finally, we found that transfection of the TALE–SIDs reduced *AXIN2* mRNA levels in these cells, indicating that they are capable of repressing *AXIN2* expression driven by oncogenic β-catenin (Fig. 3F).

3.4. Targeting *MYC* expression with a TALE–SID

To determine whether this strategy can also be used to modulate expression of an additional Wnt/β-catenin target gene, we designed TALE3–SID which recognizes a sequence adjacent to the promoter proximal *MYC* 5′ WRE (Fig. 4A) [10]. TALE3–SID specifically repressed β-catenin^{S45F}/LEF-regulation of a *MYC*-luciferase reporter transgene (Fig. 4B and C). In addition, using ChIP assays and qRT-PCR analysis, we found that TALE3–SID bound its target sequence and repressed *MYC* transcription in transiently transfected HEK293 cells (Fig. 4D and E). Finally, TALE3–SID reduced levels of *MYC* expressed in HEK293 β-catenin^{S45F}-ER cells that were treated with DOX and 4-OHT (Fig. 4F). These results indicate that as was the case for *AXIN2*, *MYC* gene expression can also be targeted using a custom dTALE.

4. Discussion

TALE proteins can be engineered to bind specific sequences in the mammalian genome [1]. By linking the TALE DNA binding protein to well-characterized and portable transcription regulatory domains, designer TALEs can be used to specifically modulate target gene expression [2–4]. In agreement with Cong et al., our findings here indicate that the SID is a potent and effective transcription repression domain that is suitable for use in mammalian systems [2].

One limitation of designer TALEs is that their efficacy can vary depending on whether they target promoter regions, enhancer elements or non-coding DNA regions. Cong et al. found that TALE–SIDs that bound proximal promoter regions led to a 3-fold decrease and a 4-fold decrease in *SOX2* and *CACNA1C* expression, respectively [2]. Our TALE–SIDs that targeted the first exon of *AXIN2*, resulted in a 5-fold decrease of *AXIN2* expression in HEK293 cells. However, for *MYC*, the TALE3–SID designed to target the proximal promoter caused only a 30% decrease in *MYC* gene expression in these cells. It is probable that the strategy of using multiple independent dTALES simultaneously would lead to greater target gene repression. Additional work is needed to optimize dTALE technology to achieve gene-silencing capabilities typically seen when using siRNA and shRNA-based systems.

Using a novel HEK293 cell line that we generated, we found that TALE–SIDs were capable of repressing *AXIN2* and *MYC* expression that is induced by oncogenic β-catenin. However, in this system, the TALE–SIDs caused only a 2-fold decrease and a 20% decrease in *AXIN2* and *MYC* expression, respectively. One possible explana-

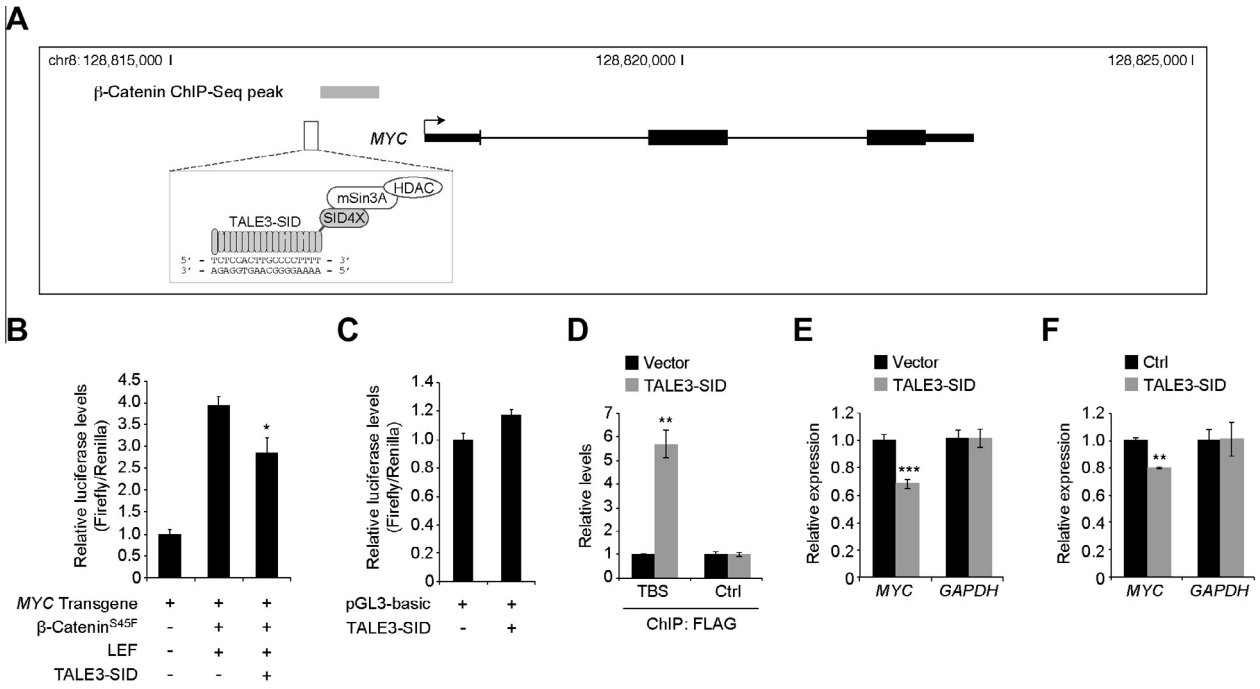

Fig. 4. A TALE–SID that targets *MYC*. (A) Diagram of the *MYC* genomic locus with a β-catenin ChIP-Seq peak represented by the gray rectangle and the TALE3–SID binding site indicated below. (B) Luciferase reporter assays conducted in HEK293 cells using a plasmid that contains a 8.6-kb *MYC* transgene with the firefly luciferase gene inserted into the *MYC* second exon. Where indicated, cells were co-transfected with plasmids encoding β-catenin^{S45F}, LEF, and TALE3–SID. (C) Control luciferase reporter assays using the promoter-less pGL3-basic construct. (D) ChIP analysis using anti-FLAG antibodies of HEK293 cells transfected with plasmids encoding FLAG-tagged TALE3–SID, or pcDNA3 as a control. The precipitated DNA was measured using qPCR analysis and *MYC*-specific oligonucleotides that annealed to the TALE binding site (TBS) or a control region (Ctrl.). (E) Analysis of *MYC* and *GAPDH* transcripts in HEK293 cells transfected with TALE3–SID or pcDNA3 as a control. (F) As in (E) except HEK293 β-catenin^{S45F}-ER cells were used that were treated with DOX and 4-OHT. In (B), (D), (E), and (F), error is SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

tion for this observation is that nuclear β-catenin is binding several enhancer elements that have been shown to regulate *AXIN2* and *MYC* and that the TALE–SIDs are unable to fully counteract this transcriptionally permissive state [10–16,19]. Future experiments will use the HEK293-β-catenin^{S45F}-ER cell line and additional TALE-effector proteins such as those linked to the LSD-1 histone demethylase [26], to evaluate how epigenetic regulatory mechanisms and TALE proteins interface to regulate target gene expression.

In summary, our results support the versatility of TALE-based strategies to target genes whose expression is elevated by oncogenic Wnt/β-catenin signaling. With the advance of hierarchical ligation-based assembly methods and fast ligation-based automatable solid-phased (FLASH) systems, custom TALE DNA binding domains can readily and rapidly be designed to recognize virtually any DNA sequence in the mammalian genome [23,27]. Thus, TALE technology should be pursued as a potential therapeutic strategy for the treatment of diseases such as colorectal cancer.

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.2014.03.077.

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