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Activation of β-catenin/TCF targets following loss of the tumor suppressor SNF5

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Abstract

The SWI/SNF chromatin remodeling complex is a master regulator of developmental cell fate decisions, although the key target pathways are poorly characterized. Here, we interrogated the contribution of the SWI/SNF subunit and tumor suppressor SNF5 to the regulation of developmental pathways using conditional mouse and cell culture models. We find that loss of Snf5 phenocopies β-catenin hyperactivation and that SNF5 is essential for regulating Wnt/β-catenin pathway target expression. These data provide insight into chromatin-based mechanisms that underlie developmental regulation and elucidate the emerging theme that mutation of this tumor suppressor complex can activate developmental pathways by uncoupling them from upstream control.

INTRODUCTION

Dynamic modulation of chromatin structure plays an integral role in eukaryotic transcriptional regulation. SWI/SNF chromatin remodeling complexes utilize the energy of ATP hydrolysis to remodel chromatin and mobilize nucleosomes at target genes, where they can contribute to both gene activation and repression. Specific combinations of SWI/SNF subunits are essential for lineage specification and appropriate development of numerous tissues ([1], [2]). At least five subunits of SWI/SNF are specifically and recurrently mutated in a

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AUTHOR CONTRIBUTIONS

E.L.M-B initiated the studies, conducted experiments, analyzed data and contributed to writing the manuscript. Y.M., E.J.T, Y-J.C. and C.S.T conducted experiments and analyzed data. S.L.P and W.S. supervised portions of the studies and assisted in the data analysis. C.W.M.R supervised the studies, assisted in the data analysis and contributed to the writing of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

variety of cancers suggesting a broad role for the complex in tumor suppression ([3]). However, key target pathways and regulatory mechanisms that underlie the role of the SWI/SNF complex in development and tumor suppression are poorly understood.

SNF5 is a core subunit of SWI/SNF complexes that possesses potent tumor suppressor activity ([4–6]). Biallelic inactivation of *SNF5* underlies the vast majority of malignant rhabdoid tumors (MRTs), highly aggressive pediatric cancers ([7, 8]). Inactivation of Snf5 in mice leads to rapid formation of cancers, demonstrating that *SNF5* is a *bona fide* tumor suppressor gene ([4, 9]). Intriguingly, *SNF5*-deficient tumors are diploid and genomically stable, suggesting that oncogenesis may substantially be driven by epigenetically altered expression of target genes ([10, 11]).

SNF5 serves a critical role in lineage specification ([9, 12–14]), suggesting that its regulation of developmental pathways may contribute to its tumor suppressor activity. Interestingly, the SWI/SNF ATPase subunit BRG1 (SMARCA4) has been implicated in regulation of the Wnt pathway and can physically interact with its downstream effector, β-catenin ([15–18]). Here we investigated the mechanism by which SNF5 contributes to lineage specification and developmental patterning using a limb bud model and elucidate relevance to its role as a tumor suppressor.

RESULTS AND DISCUSSION

To evaluate the contribution of SNF5 to developmental regulation and patterning *in vivo*, we utilized *Prx1-Cre* to conditionally inactivate Snf5 in developing limb mesenchyme at the onset of limb morphogenesis ([19]). Immunohistochemistry at E11.5 revealed that SNF5 was ubiquitously expressed in wild-type limbs (Figure 1A) but was lost in the mesenchyme of *Prx1-Cre; Snf5*$^{fl/fl}$ (hereafter referred to as *Snf5*$^{\bullet/\bullet}$) limb buds, with the residual positive cells likely representing surface epithelium and invading neural crest (Figure 1B). Since *Prx1-Cre* is expressed more uniformly in the forelimb than the hindlimb ([19]), we focused our subsequent analyses on forelimbs. Loss of SNF5 resulted in shortened, malformed limbs and agenesis of all limb bones from the scapula to the phalanges (Figure 1C–F). *Sox9*, a marker of the onset of cartilage development, from which bones of the limb ultimately form, was expressed correctly at E10.5, but was progressively lost over time in *Snf5*$^{\Delta/\Delta}$ limbs and was completely absent by E12.5 (Figure 1G, H).

β-catenin antagonizes Sox9 during chondrogenesis and, notably, the consequences of Snf5 loss phenocopied the skeletal agenesis caused by forced overexpression of β-catenin in the developing limb ([20]). We therefore investigated whether SNF5 loss affects expression of β-catenin/Tcf targets. Expression of *Lmx1b*, which is activated in part by mesenchymal β-catenin ([21]) and normally restricted to the dorsal mesenchyme, was expanded into the ventral mesenchyme in SNF5-deficient limbs (Supplemental Figure 1). *Axin2*, a direct transcriptional target of β-catenin and reported to be the most reliable indicator of β-catenin activity ([22]), was elevated in SNF5-deficient limbs compared to littermate controls (Figure 1I, J). We next crossed the *Snf5*$^{fl/fl}$*; Prx1-Cre* mice to the *Topgal* reporter mice, in which TCF mediated repression of the β-galactosidase gene is relieved by binding of β-catenin ([23]). Unlike control limbs, where staining was restricted to the apical ectodermal ridge

(AER) and developing cartilage, SNF5- deficient limbs instead exhibited diffuse staining throughout the limb field (Figure 1K, L). Overexpression of β-catenin in the limb bud mesenchyme leads to premature regression of the AER ([21]). In *Snf5*$^{\Delta/\Delta}$ limbs at E10.5, anterior/posterior expression of *Fgf8*, an AER marker was truncated and subsequently lost by E11.5 (Figure 1M,N), a pattern identical to that in limbs overexpressing β-catenin ([21]). Collectively, these results demonstrate that inactivation of Snf5 in developing limb mesenchyme leads to aberrant activation or the Wnt pathway and leads to phenotypic defects consistent with Wnt/β-catenin overexpression.

We next sought to determine whether the Wnt pathway was active in SNF5 deficient cancers. We performed gene expression analysis of SNF5-deficient primary human CNS rhabdoid tumors (RT) and compared their expression of known gene signatures of WNT activation to primary medulloblastomas and normal cerebellum using Gene Set Enrichment Analysis (GSEA) on a previously published data set ([24])(Figure 2A and Supplemental Figure 3). This analysis identified that WNT targets are elevated in SNF5-deficient RTs compared to normal cerebellum. Further, this expression signature is similar to “Wnt type” medulloblastomas that contain activating mutations of the WNT pathway (Supplemental Figure 3).

We next assessed whether upregulation of the β-catenin pathway was directly attributable to SNF5 loss. We re-expressed SNF5 in a panel of five SNF5-deficient RT cell lines by viral transduction. As a positive control for SNF5 function, we monitored the expression of *P16*^{INK4A}, which has been shown to be upregulated when SNF5 is re-introduced into deficient cells ([25]). Expression of SNF5 resulted in downregulation of β-catenin target genes *AXIN2, APC, βTRCP, LEF1*, and *HDAC4*, while the control *P16*^{INK4A} was upregulated (Figure 2B and Supplemental Figure 4).

As a core member of the SWI/SNF complex, the direct contributions of SNF5 to pathway regulation putatively occur at the level of chromatin and transcription. To investigate the mechanism by which *SNF5* loss activates the Wnt pathway, we first investigated whether inhibition of the upstream Wnt signaling would have a functional effect upon the growth of SNF5 deficient cancer cells. We therefore treated the G401 MRT cell line with three inhibitors previously shown to act directly upon the canonical Wnt pathway including 1) a Porcupine inhibitor to block the Wnt ligand processing and secretion; 2) an antibody against the LRP6 receptor to which the Wnt ligand binds; and 3) a Tankyrase inhibitor that stabilizes the AXIN destruction complex that degrades β-catenin ([26], [27]). In order to capture effects upon either proliferation and colony forming ability of these cancer cells, we measured colony growth after treatment with each of the inhibitors. Notably, none of these Wnt pathway inhibitors impaired G401 colony formation (Figure 3A and data not shown). Further, depletion of β-catenin itself had no effect on colony formation or on expression of the β-catenin target *AXIN2* (Figure 3B, C). These results indicated that aberrant activation of β-catenin target genes in SNF5-deficient cells occurs independently of canonical Wnt pathway activation.

We therefore considered the possibility that β-catenin targets were being aberrantly de-repressed, rather than activated. In the absence of nuclear β-catenin the TCFs/LEF interact

with co-repressor complexes to prevent permissive expression of β-catenin targets. We thus tested whether the co-factor TCFs are still capable of binding to their consensus sequence in the absence of SNF5. We inactivated *Snf5* in *Snf5*$^{fl/fl}$ MEFs and performed chromatin immunoprecipitation (ChIP) analysis of TCF4 at well-characterized target promoters relevant in murine development ([28], [29]). Loss of SNF5 concomitantly decreased the ability of TCF4 to bind to the *Axin2, Msx1*, and *Myc* loci and increased gene expression of these targets, suggesting that SNF5 normally facilitates TCF4 binding to its target loci to induce repression (Figure 4A). As Wnt pathway targets are tissue and lineage-specific (with the exception of AXIN2 as a universal target) we examined whether target loci relevant in human cancer had altered TCF binding after re-introduction of SNF5 into G401 MRT cells by ChIP analysis ([30]). Introduction of SNF5 resulted in increased binding of TCF4 to targets assessed and was accompanied by a decrease in target gene expression (Figure 4B). To further interrogate TCF4 function in SNF5-deficient cells, we introduced a dominant negative form of TCF4 (TCF4ΔN) into the G401 MRT cell line. TCF4ΔN lacks the β-catenin interaction domain but retains the DNA binding domain and hence acts as a constitutive repressor. Expression of TCF4ΔN in G401 cells had no effect on the expression of TCF targets, further indicating that TCF4 cannot act as an efficient repressor in the absence of SNF5 (Supplemental Figure 2).

In this report, we demonstrate a novel mechanism of regulation of Wnt/β-catenin targets by the SWI/SNF chromatin remodeling subunit SNF5. Previous studies assessing the role of individual SWI/SNF subunits in Wnt signaling have yielded seemingly paradoxical results. In one study in *Drosophila melanogaster*, loss of the ortholog of mammalian BAF250, BRG1/BRM, or BAF155/170 proteins de-repressed Wnt/Wingless target genes without an increase in Armadillo, the fly β-catenin ([31]). However, in a different study that used the fly eye as a model system, it was found that Brm haploinsufficiency relieved the rough eye phenotype associated with overexpression of Armadillo ([32]). In T-cells, the role of Brg1 in the regulation of β-catenin/TCF targets has been similarly unclear ([33]). In endothelial cells Brg1 loss led to downregulation of the Wnt pathway through the transcriptional regulation of Wnt target genes and a subset *Fzd* receptors ([15], [17]). Collectively, these results begin to elucidate a model whereby the Swi/Snf complex can act on the same pathway in a tissue- and context-dependent manner to specifically regulate the WNT/β-catenin pathway by modulating both activation and repression. Disruption of this regulation via loss of the SNF5 tumor suppressor can then drive tumor formation. Given the recent finding that BRG1 (SMARCA4) is specifically mutated in human medulloblastomas, but only in the subgroup that also contain activating mutations in the WNT/β-catenin pathway ([34]), it is tempting to speculate that rather than preventing WNT signaling, mutation of this SWI/SNF subunit may cooperate to drive WNT signaling in these cancers..

Mutations in genes encoding SWI/SNF subunits are increasingly being identified at high frequency in a wide variety of cancer types. However, the mechanisms underlying oncogenesis have been unclear. Via exome sequencing of 35 human SNF5-mutant primary rhabdoid tumors with matched normal controls, we recently showed that despite their highly aggressive nature, MRT contain a remarkably simple genome with loss of SNF5 being essentially the sole recurrent event ([35]). Indeed, in two of the cancers there were no other identified mutations at all. These results demonstrate that high mutation rates are

dispensable for the genesis of cancers driven by SNF5 loss and suggest that the oncogenic drive provided by SNF5 loss is likely exerted at the epigenetic/chromatin level via perturbation of several pathways that ultimately cooperate to facilitate transformation. In addition to regulation of the WNT/β-catenin pathway, we have previously identified epigenetic antagonism between SWI/SNF and Polycomb complexes, and a role for SNF5 in regulation of the Hedgehog pathway. Collectively, this raises the possibility that the role of the complex in tumor suppression is derived from its contribution to the regulation of several caner related pathways. The mechanistic findings we report here also have substantial therapeutic implications. Our results reveal that mutation of a SWI/SNF subunit can uncouple pathway activation from dependence upon upstream molecules that otherwise modulate pathway activity. Consistent with these findings, we recently showed that activation of the Hedgehog pathway caused by SNF5 loss rendered a Smoothened antagonist targeting the upstream Hh pathway useless ([14]). Similarly, we have shown here that SNF5 loss uncouples Wnt pathway targets from canonical pathway control and renders existing inhibitors ineffective at blocking target gene expression. This likely occurs because mutation of *SNF5* affects control of target genes directly at the level of chromatin, distal to the role of canonical pathway regulators. If the mechanism that drives tumor formation following mutation of other SWI/SNF subunits, including *ARID1A, PBRM1, ARID2, BRD7* and *BRG1* is similar, our work suggests that elevated target expression will be a poor predictor of response to targeted pathway inhibitors. Given that the sole detected genetic driver event in these cancers is the absence of the SNF5 tumor suppressor, development of effective targeted therapeutics will likely to require an understanding of the pathways activated by this loss and the mechanisms by which altered chromatin structure contributes to this activation.

MATERIALS AND METHODS

Snf5 Knockdown and Excision

Primary MEFs were harvested from E13.5 embryos. Cre was introduced into cells via retroviral infection with pBabe-puror-Cre retroviral supernatant two times at 4 h intervals. Cells were stably selected in medium containing puromycin (2.5 μg/ml) 48 h after infection.

Mouse Strains

Crosses were performed between strains carrying the floxed *Snf5* allele ([9]), the *Prx1-Cre* transgene ([19]) and. the *Topgal* reporter ([23]). All mice were maintained on a mixed genetic background at the Harvard School of Public Health. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC).

In Situ Hybridizations

Whole-mount in situ hybridizations were performed as described ([36]). Non-radioactive digoxygenin (DIG) labeled probes were generated according to the manufacturer (Roche). All probe templates used in this study were generously provided by Clifford Tabin (Harvard Medical School).

Cell lines

TTC642, and TM87 were the kind gift of Dr. Bernard Weissman, University of North Carolina. BT12 and BT16 were the kind gift of Dr. David James, University of San Francisco. G401 was obtained from ATCC.

X-gal Staining of Embryos

Dissected embryos were placed in 2% paraformaldehyde/Pipes buffer at 4°C for 30 minutes. Embryos were washed with cold PBS twice, then washed with concentrated rinse buffer (sodium phosphate, sodium deoxycholate, magnesium chloride and NP-40), Embryos were stained with X-gal (Roche) per manufacturers specifications.

RNA Extraction and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions and reverse-transcribed by the Reverse Transcription System (Promega). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and the iCycler thermocycler (Bio-Rad). Primer sequences are available upon request. Relative mRNA expression was calculated by the formula $2^{-(C_T \text{ of sample} - C_T \text{ of } \beta\text{-actin})}$, where C_T (cycle count) is the threshold cycle value. The following primer pairs were used in the qPCR following chip: Mouse *AXIN2* F 5'CTCGCATACCTCCCTTCC3', R 5'TTCCAGCAGTCACTAGGC3'; Mouse *MSX1 F 5'* GATCGGAGAATCCAAGTAGCTAC 3', R 5' GACAGTGGAGTTTGAGACCTACTC 3'; Mouse *MYC* F 5' CAAGCTTTAATTAGCTTAACACA 3', R 5' GGAGCCTGCAG AGACCCTA 3'; Human *AXIN2* F 5'TTTCCTCTCCTCCCAGTTG3', F 5'AAGTTGA GCCTACAGTGATTAG3'; Human *HDAC4* 5' TGAAAGCACCGCTCATTCTCTGTG 3', R 5' GCTGCCTTAAACTTGGCATCAAAGG 3'.

Immunoblots

The following antibodies were used: β-catenin (Sigma-Aldrich, C2206), SNF5 (Bethyl, A301-087A), β-Actin-HRP (Abcam, 20272-200), and HRP-conjugated secondary antibodies (Jackson Immunoresearch)

SNF5 Reintroduction-Expression Analysis

SNF5-deficient G401 cells were transduced using either pBabe-puro^r-FLAG-SNF5 (generously provided by Robert Kingston, Massachusetts General Hospital) or pBabe-puro^r-Empty two times at 4 h intervals followed by selection in puromycin (1μg/ml) for 48 h. Cells were then harvested 48 h later for RNA or protein analysis.

Chromatin immunoprecipitation

Chromatin immunoprecipitations (ChIP) was performed as described ([13]), using the anti-TCF4 antibody (sc-8631), Santa Cruz Biotechnology.

Colony Formation Assays

G401 cells were seeded in growth medium at 600 cells/well into 6-well plates. Sixteen hours after plating, compounds were added at the indicated concentrations. Medium was

replenished every four days until colony formation was observed. Colonies were stained by a solution of 2 mg/ml crystal violet in buffered formalin and imaged using a HP Scanjet G4050 scanner.

Expression Analysis

Gene set enrichment analysis (GSEA) was performed as previously described (Subramanian and Tamayo et al PNAS 2005), using a previously published dataset of 11 ATRT samples, 194 primary medulloblastomas and 12 normal cerebellum samples([24]).Three independent GSEA analyses were performed using the "CGP: chemical and genetic perturbations (3398 gene sets)", "CP: Canonical pathways (1452 gene sets)", and "C6: oncogenic signatures (189 gene sets)" (for more detailed description of gene sets, please refer to http://www.broad.mit.edu/gsea/msigdb). Medulloblastoma subgroups were identified as previously described (Cho et al. JCO 2011) and "WNT subgroup" medulloblastomas are highlighted in Supplemental Figure 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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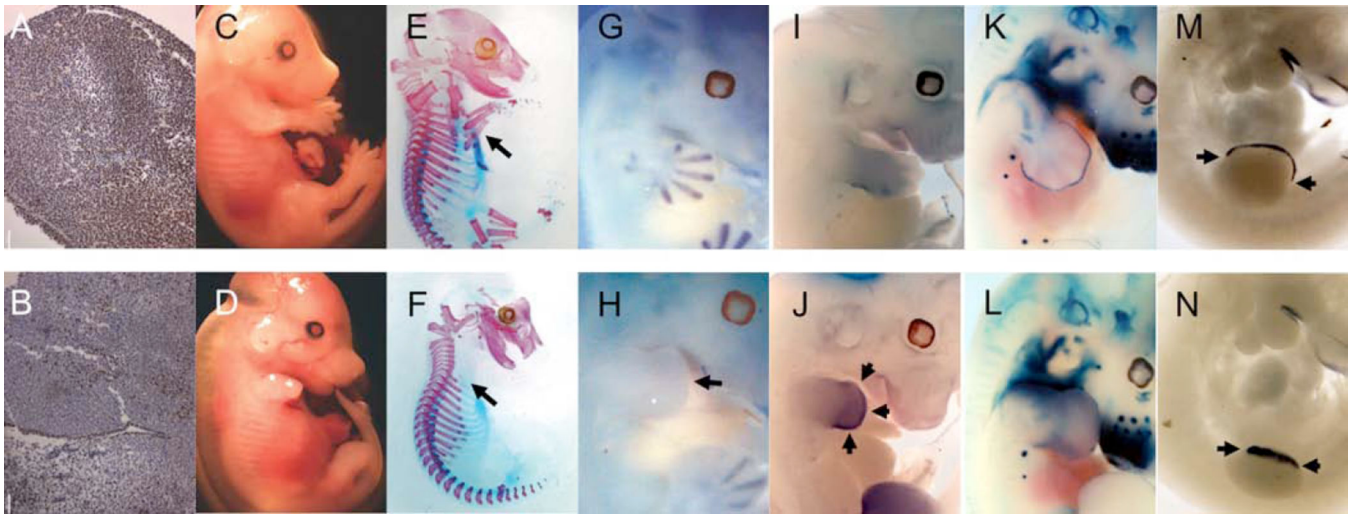

Figure 1. Loss of Snf5 leads to skeletal agenesis, loss of chondrogenic markers, and upregulation of β-catenin targets in the developing limb

The *Prx1-Cre* transgene was used to inactivate Snf5 in the developing limb. Immunohistochemistry for Snf5 from control (A) and $Snf5^{-/-}$ (B) limbs at E11.5. Gross morphology (C, D) and cartilage and skeletal preparations (E, F) of control and $Snf5^{-/-}$ embryos respectively. RNA *in situ* hybridization against *Sox9 (G, H), Axin2 (I, J), and Fgf8* (M, N) in control and $Snf5^{-/-}$ embryos. $Snf5^{fl/+}$; *Prx1*-Cre and $Snf5^{fl/fl}$; *Prx1*-Cre were crossed to the Topgal reporter strain to monitor β-catenin activity in limbs during development (K, L).

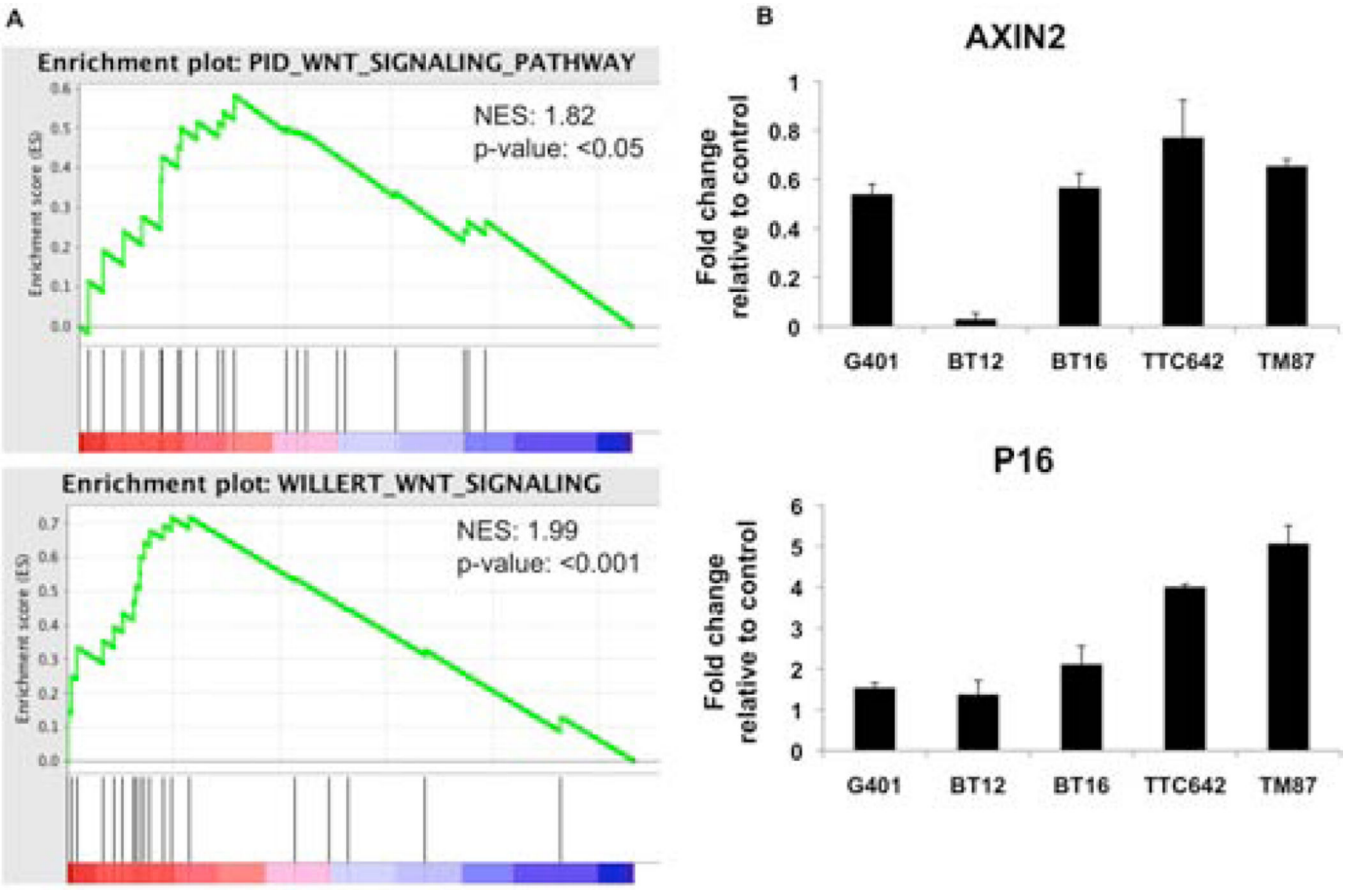

Figure 2. The Wnt/β-catenin pathway is active in SNF5-deficient cancers

Gene set enrichment analysis (GSEA) plot of genes upregulated in response to WNT activation (from ([37]) and ([38]), respectively) using expression data from MRT compared to primary medulloblastomas and normal cerebellum (A). Expression analysis of β-catenin/TCF target *AXIN2* and known downstream effector of SNF5, *P16*, in G401, Bt12, BT16, TTC642, and TM87 MRT cell lines(B).

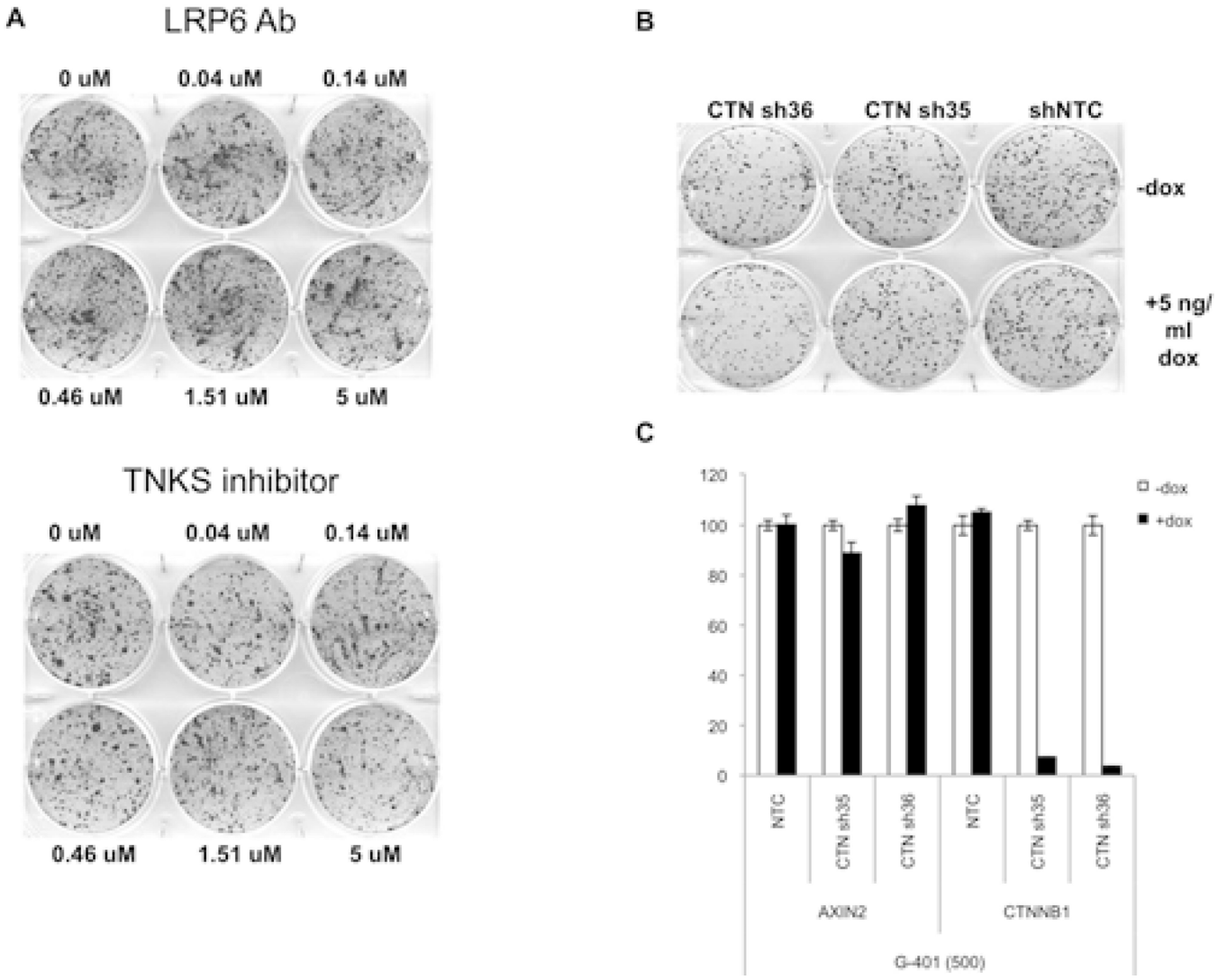

Figure 3. Chemical inhibition of the Wnt pathway in SNF5-deficient MRTs

Colony growth assay of MRT cells in the presence of the labeled Wnt pathway inhibitors (A). Colony growth assay of MRT cells expressing shRNAs against β-catenin (B). Knock-down of β-catenin by Doxycycline-inducible shRNA in MRT G401 cells (C).

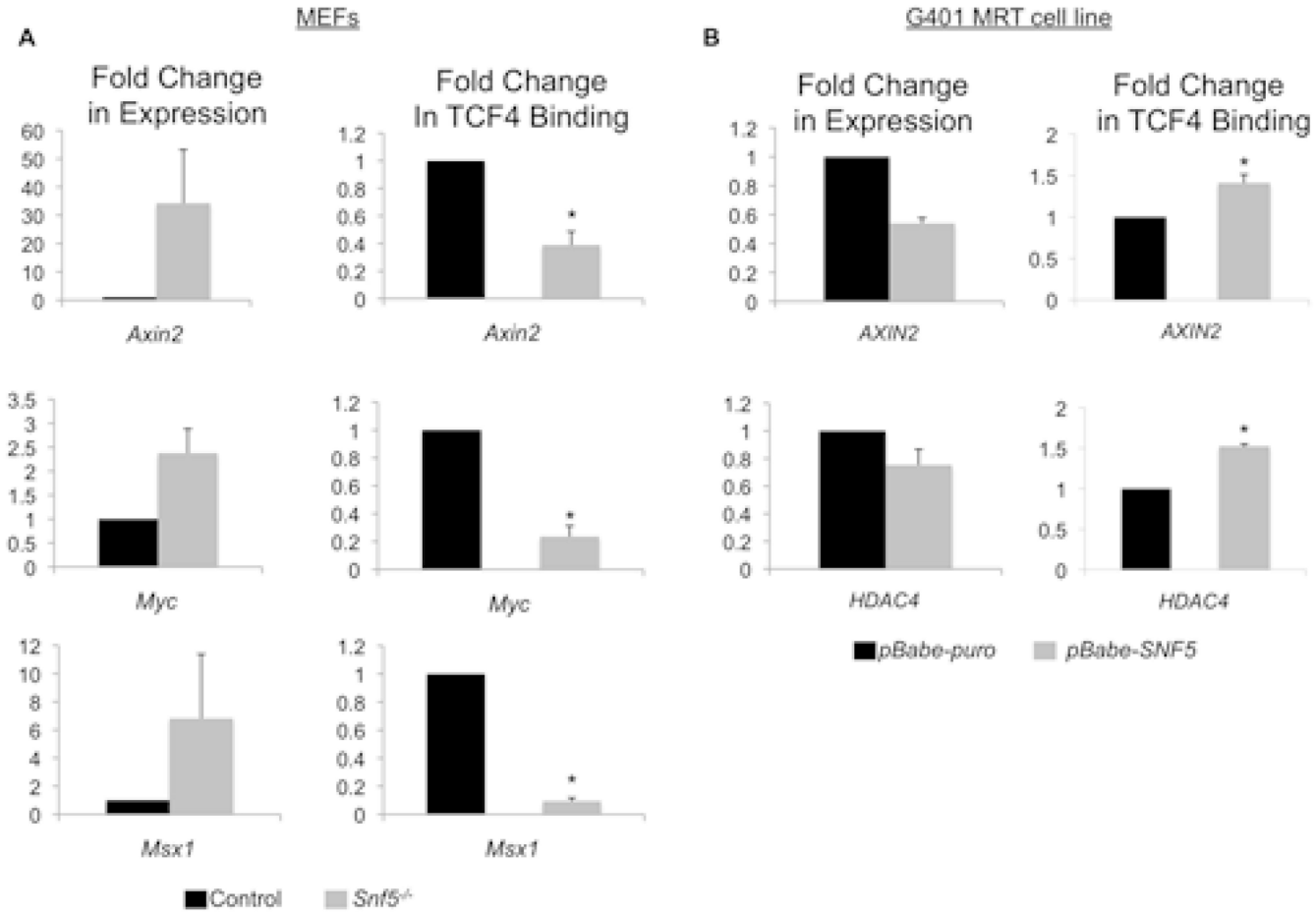

Figure 4. SNF5 acts downstream in the Wnt pathway to regulate β-catenin/TCF targets

Gene expression and chromatin immunoprecipitation of TCF4 and at the *Axin2, Myc*, and *Msx1* loci in control (black bars) and *Snf5*-deleted (grey bars) MEFs (A). Gene expression and chromatin immunopcrecipitation of TCF4 at the *AXIN2* and *HDAC4* promoters in control (black bars) and *SNF5*-reintroduced (grey bars) G401 cells (B). Asterisk indicate $P<0.05$.