Published as: *Stem Cells*. 2016 August ; 34(8): 2169–2182.

# Barx2 and Pax7 regulate Axin2 expression in myoblasts by interaction with β-catenin and chromatin remodelling

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## Abstract

Satellite cells are the resident stem cells of skeletal muscle; quiescent in adults until activated by injury to generate proliferating myoblasts. The canonical Wnt signalling pathway, mediated by T-cell factor/Lymphoid Enhancer Factor (TCF/LEF) and β-catenin effector proteins, controls myoblast differentiation in vitro, and recent work suggests that timely termination of the Wnt/β-catenin signal is important for normal adult myogenesis. We recently identified the Barx2 and Pax7 homeobox proteins as novel components of the Wnt effector complex. Here we examine molecular and epigenetic mechanisms by which Barx2 and Pax7 regulate the canonical Wnt target gene Axin2, which mediates critical feedback to terminate the transcriptional response to Wnt signals. Barx2 is recruited to the Axin2 gene via TCF/LEF binding sites, recruits β-catenin and the coactivator GRIP-1, and induces local H3K-acetylation. Barx2 also promotes nuclear localization of β-catenin. Conversely, Pax7 represses Axin2 promoter/intron activity and inhibits Barx2-mediated H3K-acetylation via the corepressor HDAC1. Wnt3a not only induces Barx2 mRNA, but also stabilises Barx2 protein in myoblasts; conversely, Wnt3a potently inhibits Pax7 protein expression. As Barx2 promotes myogenic differentiation and Pax7 suppresses it, this novel posttranscriptional regulation of Barx2 and Pax7 by Wnt3a may be involved in the specification of differentiation-competent and -incompetent myoblast populations. Finally, we propose a model for dual function of Barx2 downstream of Wnt signals: activation of myogenic target genes in association with canonical myogenic regulatory factors, and regulation of the negative feedback loop that limits the response of myoblasts to Wnt signals via direct interaction of Barx2 with the TCF/β-catenin complex.

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Author contribution summary: J-AH performed the majority of in vitro analyses, TDTN generated stable cell lines, SC analysed Pax7 expression, SM performed bioinformatic analysis, RTY, MD and RME performed ChIP-seq, RM and HM jointly conceived and directed the project.

**Keywords**

Homeobox genes; Muscle stem cells; Myogenesis; Skeletal muscle; Transcription factors; Epigenetics; Cell signalling; Differentiation

## Introduction

Skeletal muscle repair is mediated by the essential resident muscle stem cell called the satellite cell, situated between the basal lamina and myofibre [1-4]. Quiescent in adult muscle [5-7], satellite cells are activated after injury and give rise to a population of proliferating myoblasts that can differentiate to repair or replace damaged myofibres [8, 9]. Myoblast differentiation involves activation of a multi-gene program whose products mediate such processes as cytoskeletal remodelling, migration, cell cycle regulation, adhesion and fusion.

The canonical Wnt signalling pathway plays critical roles in embryonic muscle development as well as being activated during adult muscle regeneration [10]. Canonical Wnt ligands promote myoblast differentiation in culture [11-13], and are suggested to be involved in the switch from proliferation to differentiation [14, 15]. Central effectors of the canonical Wnt signalling pathway are TCF/LEF factors and β-catenin. In the absence of Wnt signals, β-catenin is phosphorylated by a destruction complex including Axin1/Axin2 and glycogen synthase kinase 3 (GSK3), resulting in proteosomal degradation [16]. Meanwhile, TCF/LEF proteins repress target genes via interaction with Groucho/TLE co-repressors [17, 18] and HDAC1 [17, 19]. Upon binding of Wnt ligand, the destruction complex is disassembled and stabilised β-catenin translocates to the nucleus where it pairs with TCF/LEF proteins and histone acetyltransferases (HATs) [20-23] to activate Wnt-responsive genes [23-25].

Early work reported that canonical ligand Wnt3a increased activity of the myogenic regulatory factors (MRFs) MyoD and myogenin in terms of their ability to promote myogenic differentiation [26], and that Wnt3a could induce myogenic conversion of P19 cells by inducing expression of various regulators including MyoD [27] A very recent study [28] showed that Wnt3a induced myogenin at the protein level, and increased binding of myogenin to the myogenic target gene Follistatin; moreover activation of the canonical Wnt pathway induced precocious differentiation in adult muscle. While it is formally possible that TCF/β-catenin complexes directly regulate the suite of genes that mediate the structural and adhesion changes underlying differentiation, it is most likely that canonical Wnt signals (acting via TCF/β-catenin or other effectors), induce the expression and/or activity of key myogenic transcription factors including, although not limited to, MyoD and myogenin, that subsequently drive the downstream myogenic program.

Surprisingly a recent study found no defects in adult myogenesis in mice with satellite cell-specific deficiency of β-catenin [29], suggesting that β-catenin is redundant in this context [29]. The study did not show redundancy of the canonical Wnt ligand/receptor signalling complex, and it remains possible that so-called canonical Wnts can drive differentiation via β-catenin-independent processes. This study also showed that expression of constitutively active β-catenin in satellite cells impaired myogenesis, suggesting that while the activation

of β-catenin by Wnt signals is not required, its subsequent inhibition is critical for effective muscle regeneration [29]. Targets of Wnt signalling include several genes that mediate negative feedback, including Axin2, which promotes degradation of β-catenin.

We previously found that the Barx2 homeobox protein is upregulated early during myoblast differentiation, associates with muscle regulatory factors such as MyoD at differentiation-associated genes, and promotes pro-differentiation cell spreading, migration and adhesion [36-39]. Barx2 null mice have impaired postnatal muscle growth, maintenance and regeneration consistent with an important role of Barx2 in myoblast function [36]. We recently found that Barx2 is induced by Wnt3a in myoblasts and reasoned that Barx2, via its association with MRFs, may be part of the mechanism by which Wnt3a induces myogenic differentiation. However we also found that Barx2 can interact with β-catenin, suggesting that it may also be involved in other activities mediated by Wnt. In this study we determine that Barx2 is induced at the protein level by Wnt3a in a cell autonomous manner, and define the mechanisms by which Barx2 regulates the critical Wnt feedback gene Axin2. Moreover, we propose that Barx2 has a dual function downstream of Wnt signals: promoting the expression of myogenic differentiation genes via its association with MRFs and other myogenic effectors, and regulating the temporal response to the Wnt signal by inducing Axin2 in association with TCF/LEF-β-catenin complexes [40-42].

Pax7 is highly expressed in quiescent satellite cells and proliferating myoblasts [30] and downregulated during differentiation [31, 32]. Pax7 inhibits differentiation at least in part by inhibiting the expression and activity of MRFs [31, 33-35]. Here we show that Pax7 is potently inhibited by Wnt3a at the protein level, which may contribute to the ability of Wnt3a to induce differentiation. Moreover, we show that Pax7 blocks the ability of Barx2 to regulate Axin2, suggesting that Pax7 also has a dual function: suppressing differentiation through inhibition of MRFs such as myogenin [33], and also antagonizing the Wnt/β-catenin pathway.

## Methods

### Plasmids

The mouse Axin2 promoter/intron reporter construct (Axin2-luc) and the TOPflash reporter plasmid were from Addgene (plasmids 21275 and 12456) [40, 43]. Full-length mBarx2 and mPax7D in the pcDNA3 vector were previously described [12]. Constitutively active β-catenin and dominant-negative TCF4 (dnTCF4) cDNAs were obtained from Dr. Victor Korinek and subcloned into pcDNA3. HA-tagged mouse GRIP-1 in pSG5 vector was a gift from Dr. Michael Downes. The lentiviral Barx2 expression construct was generated using pTiger vector that was a gift from Professor Gary Nolan. Flag-tagged HDAC1-pcDNA3 was from Addgene (Plasmid 13820) [44].

### Cloning, Deletions and Mutagenesis

Barx2 and Pax7 homeodomain swapping was performed by PCR amplification of the homeodomain regions and insertion into Barx2 and Pax7 constructs from which the corresponding regions had been removed [12]. The TCF/LEF sites in the Axin2-luc reporter

construct (T2, T3, T4, T5, T6, T7, T8; nomenclature from [40]), were mutated by the introduction of three nucleotide substitutions into the core motif (ga(a/t) mutated to cgc). A homeobox binding site (HBS) cluster at the 5' end of Axin2-luc was also deleted. A detailed description of these manipulations is provided in Supporting Information Methods.

### Cell Line Culture

Cell lines were obtained from the American Type Culture Collection (ATCC). C2C12 cells were grown in DMEM with 20% fetal calf serum (FCS). HEK293T were grown in DMEM with 10% FCS at 37°C, 5% $CO_2$. L-cells stably expressing Wnt3a [45] were maintained in DMEM with 10% FCS and 1 mg/ml G418. Conditioned media (CM) was prepared as recommended by ATCC: briefly, the cells were split 1:10 from confluence and grown for 1 week in DMEM with no antibiotics, media was collected on day 4 and day 7, combined, filtered through a 40 μm membrane and stored at −20°C. CM was diluted with myoblast media (1:1 unless otherwise stated) before use.

### Primary Myoblast Cultures

Primary myoblasts were isolated from postnatal day 21 TP1-Venus or Barx2/TP1-Venus mice by immuno-FACS as previously described [12] and further detailed in Supporting Information Methods. TP1-Venus are an ICR transgenic strain (RIKEN BioResource Center (RBRC06137) [46, 47]. Barx2/TP1-Venus mice are a cross between Barx2 null [48] and TP1-Venus mice.

### Chromatin Immunopreciptiation and Co-immunoprecipitation

ChIP was performed using a modified MicroChIP protocol [49] in primary myoblasts or a stable C2C12 cell line carrying the TOPflash promoter/luciferase reporter, TOP-Puro, described previously [12]. In some ChIP experiments, cells were first transfected with GRIP-1 and HDAC1 siRNAs at 30 nM using Lipofectamine as recommended by the manufacturer. GRIP-1 and HDAC1 siRNAs were purchased from Santa Cruz Biotechnologies. Purified genomic DNA was analysed by quantitative PCR (qPCR) with primers that amplify the target regions or a control non-target locus (β2-microglobulin). Details are provided in Supporting Information Methods and primer sequences are in Supplementary Table 1. Co-immunoprecipitations were performed using recombinant proteins expressed in HEK293T cells as previously described [12, 50] and detailed in Supporting Information Methods.

### Transfections and Promoter assays

C2C12 cells seeded in 24-well plates at $3 \times 10^4$ cells/well were cotransfected with TOPflash, pGL3-Basic or Axin2-luc and combinations of expression plasmids (0.5 μg each plasmid unless otherwise stated) using Lipofectamine 2000 (Invitrogen). pRL-Null renilla-luciferase reporter (Promega) was included as an internal reference. Luciferase assays were performed 48 hours post-transfection using a Dual Luciferase assay kit (Promega). Transfections were performed in triplicate and repeated two to eight times; significance was assessed using Student's *t* test. Pax7 siRNAs designed based on [31] or universal negative control siRNAs (Integrated DNA Technologies, Coralville, Iowa, www.idtdna.com) were cotransfected at 30

nM with reporter and effector plasmids and assayed as described above. The Pax7-siRNAs have been used previously [12] and knockdown efficacy is shown in Supplemental Figure 1.

### RNA Isolation and Reverse Transcription PCR (qRT-PCR)

RNA was prepared from cells using TRIzol (Life Technologies, Carlsbad, California, www.lifetechnologies.com); after DNase treatment, cDNA was synthesized using NxGen M-MuLV reverse transcriptase (Lucigen, Wisconsin, www.lucigen.com) and random primers (New England Biolabs, Ipswich, Massachusetts, www.neb.com). Quantitative RT-PCR was performed using a Corbett Rotorgene (Qiagen, Venlo, Limburg, Netherlands, www.qiagen.com) and GoTaq SYBR green (Promega). Significance was assessed using Student's *t* test.

### Viral Packaging and Transduction

HEK293T cells seeded in 6-well plates at $4 \times 10^5$ cells/well were transfected with 1.125 μg viral DNA plasmid, 1.875 μg gag-pol and 1 μg VSV-G plasmids combined with 10 μl Lipofectamine 2000 (Invitrogen); media was replaced after 6 hours with myoblast media and cells grown a further 42 hours before collecting viral supernatant. Supernatant was centrifuged and polybrene (Sigma) added at 4 μg/ml final concentration. Spinfection of myoblasts was performed for 2 hours at 2500 rpm, 30°C. Media was replaced and cells grown for a further 48 hours.

### Immunofluorescence labelling

Glass coverslips were coated with 50 μg/ml rat-tail collagen for 1 hour at 37°C and seeded with C2C12 cells at $1 \times 10^4$ cells/well in 24-well plates before treatments. Transfection of C2C12 cells used a high efficiency Lipofectamine 2000 (Invitrogen) protocol: $2 \times 10^5$ cells in 6-well plates were transfected with 4 μg DNA and 20 μl Lipofectamine 2000 per well. Media was changed after 6 hours; 24 hours post transfection, cells were trypsinised and re-seeded on coated coverslips in 24-well plates at $1 \times 10^4$ cells/well. Cells were fixed with 3.7% formaldehyde in PBS for 10 minutes at room temperature, rinsed with PBS and permeabilised with 0.5% Triton-X for 5 minutes. Cells were then blocked with 1% BSA in PBS for 30 minutes before addition of antibodies. β-catenin or Barx2 (Santa Cruz Biotechnology) antibodies were applied at 4 μg/ml, overnight in a humidified chamber; fluorescently labelled secondary antibodies (Dylight-594 or Dylight-488; Vector Labs) were applied at 10 μg/ml for 2 hours. Nuclei were counterstained with 1 μg/ml DAPI for 5 minutes. Cells were imaged with an Olympus BX-50 microscope. Primary myoblast immunostaining was performed in collagen-coated wells and imaged in-well using an Olympus IX71 microscope.

## Results

### Barx2-mediated activation of Axin2 is regulated through TCF/LEF motifs

The Axin2 promoter/intron is regulated by Wnt signalling and contains 8 consensus TCF/LEF binding sites [40], as well as a cluster of putative homeobox binding sites (HBS, motif TAAT) (Figure 1A). We recently reported that Barx2 induced activity of the Axin2 promoter/intron region [12]. To assess the requirement for TCF/LEF vs HBS motifs in

Barx2-mediated activation, we performed extensive mutagenesis of the Axin2 promoter/intron (Axin2-luc) construct. We first truncated the 'full length' Axin2 construct to remove the cluster of HBS motifs at the 5'-end of the promoter adjacent to the distal T1 TCF/LEF motif; this did not diminish the ability of Barx2 to activate Axin2-luc. Simultaneous mutation of the T2, T3, T4 and T5 elements led to 50% decrease in both Barx2 and β-catenin mediated activation (Figure 1B). Further mutation of the T6 and T7/T8 sites did not reduce Barx2-mediated activation further, although it did abolish the residual activation by β-catenin. These data suggest that Barx2 activates the Axin2 promoter/intron region largely, but not exclusively through TCF/LEF motifs (T2-T5), in a similar manner to β-catenin, and not by the upstream HBS cluster. Generalizing this model, we found that Barx2-mediated activation of the cyclinD1 promoter also required only the TCF/LEF motif, and not an upstream HBS cluster (Supplemental Figure 2).

Barx2 contains a central homeodomain (HD) and adjacent 17 amino acid Barx basic region (BBR) that together (HDBBR) mediate DNA-binding [51]. The C-terminal domain mediates transactivation whilst the N-terminal region has both activation and repression domains [12, 48, 51]. Barx2 constructs containing either the N-terminal region and HDBBR (NHDBBR) or the C-terminal region and HDBBR (HDBBRC) could activate the Axin2-luc reporter, although HDBBRC was more potent (Figure 1C). Progressive truncation of the C-terminus decreased activation and the HDBBR alone was insufficient to activate the reporter. A Barx2 construct lacking the homeodomain (ΔHD) was unable to activate the reporter (Figure 1C). Barx2 and β-catenin were also able to induce Axin2-luc activity in COS7 cells which are unable to undergo myogenic conversion (not shown), suggesting that no muscle-specific co-factors are required.

### Barx2 binds to the Axin2 intronic enhancer and promotes recruitment of β-catenin

We used ChIP to determine whether exogenous Barx2 is recruited to the Axin2 promoter/intron region following transfection of C2C12 cells. A stable C2C12 cell line carrying an integrated TOPflash promoter-luciferase reporter (TOP-Puro) was used so that recruitment of Barx2 to TOPflash [12] could be used as a reference. In cells transfected with Barx2, ChIP with Barx2 antibodies produced an average of 2.3-fold enrichment of the Axin2 T3 region, while ChIP with β-catenin antibodies produced 3-fold enrichment (relative to IgG-precipitated controls) (Figure 2A). Thus exogenously expressed Barx2 recruited β-catenin to the Axin2 intronic enhancer in the absence of β-catenin over-expression or stabilization by Wnt treatment. No Axin2 enrichment was observed with when cells were transfected with empty vector. As a control, recruitment of exogenously expressed constitutively-active β-catenin was demonstrated using the same β-catenin antibodies. Cells transfected with the Barx2 ΔHD construct showed no enrichment of the Axin2 intronic enhancer after ChIP with Barx2 or β-catenin antibodies; suggesting that the homeodomain is essential for recruitment. We also confirmed that Barx2 was not recruited to the HBS cluster located upstream of the T3 motif in the same ChIP samples (Figure 2A). Wnt3a induces endogenous Barx2 expression in primary myoblasts [12]. To assess whether endogenous Barx2 is recruited to the TCF/LEF sites in the Axin2 intronic enhancer, chromatin immunoprecipitation (ChIP) was performed in primary myoblasts that had been treated with either control L-cell conditioned media (CM) or Wnt3a-CM for 24 or 48 hours. ChIP with Barx2 antibodies at 48

hours post-Wnt3a-treatment produced ~3-fold enrichment of DNA corresponding to the Axin2 T3 and T4/5 regions, indicating that endogenous Barx2 binds to the intronic enhancer after its induction by Wnt3a in primary myoblasts (Figure 2B). No enrichment was observed at 24 hours post Wnt3a-treatment, consistent with the relatively slow rate of Barx2 induction. Contrastingly, ChIP with β-catenin antibodies at 24 and 48 hour time points produced 3- to 7-fold enrichment respectively of Axin2 intronic enhancer DNA (Figure 2B). ChIP with either Barx2 or β-catenin antibodies in the L-cell control condition gave no enrichment of Axin2 intronic enhancer DNA, consistent with low endogenous levels of these factors in standard myoblast culture conditions (e.g. Figure 2C, E).

### Over-expression of Barx2 leads to accumulation of β-catenin in the nucleus

Wnt3a increased total β-catenin in C2C12 cells as observed by an increase in cellular immunofluorescence (Figure 2C) and by western blot (see Figure 6H). Wnt3a also increased the ratio of nuclear:cytoplasmic β-catenin, with Wnt3a treated cells containing on average 16-fold more β-catenin in the nucleus relative to the cytoplasm (Figure 2D). These results are consistent with previous reports [52, 53]. We next examined the effect of Barx2 on β-catenin expression and localization by immunofluorescence staining in C2C12 cells transfected with a Barx2 expression plasmid (Figure 2E). In cells expressing high levels of Barx2 we observed a significant increase in nuclear β-catenin compared to control cells. On average, $Barx2^{high}$ cells had 7.6-fold more nuclear:cytoplasmic β-catenin than $Barx2^{low/negative}$ cells (Figure 2F), suggesting that Barx2 sequesters β-catenin in the nucleus.

### Pax7 antagonizes Barx2 and β-catenin mediated induction of Axin2 activity

We recently reported that Pax7 could suppress the TOPflash reporter gene through a previously unknown interaction with β-catenin [12], but it was unclear how this activity was relevant to natural target genes. Here we tested the ability of Pax7 to modulate Axin2 promoter/intron activity. In contrast to its effect on the TOPflash promoter (Figure 3A), transfection of Pax7 alone had no impact on basal Axin2-luc activity (Figure 3B). However, co-transfection of Pax7 with Barx2 or β-catenin reduced their ability to activate Axin2-luc to about half (Figure 3B). Conversely, knockdown of Pax7 by co-transfection of Pax7-siRNA increased Barx2- and β-catenin-mediated activation of the Axin2-luc up to 2-fold (Figure 3C). Pax7-ChIP analysis showed that Pax7 was recruited to the Axin2 T3 region in C2C12 cells following over-expression (Figure 3D). Efficacy of the Pax7 antibody in ChIP was demonstrated by showing the recruitment of Pax7 to the Myf5 −57kb regulatory enhancer element [54].

To better understand the roles that the Barx2 and Pax7 homeodomains (HD) play in this differential regulation of Wnt target promoters, we swapped the homeodomain and the Barx basic region from Barx2 (Barx2HDBBR) with the homeodomain of Pax7 (Pax7HD), and vice versa, to generate two chimeric transcription factors: Barx2/Pax7HD and Pax7/Barx2HDBBR. We examined the effects of these chimeric factors on both Axin2-luc and TOPflash. Chimeric Barx2 containing the Pax7HD (Barx2/Pax7HD) activated both reporters (Figure 3A, B), but less so than wildtype (WT) Barx2. Chimeric Pax7 containing the Barx2HDBBR (Pax7/Barx2HDBBR) was still repressive of basal TOPflash activity; however, less so than WT Pax7 (Figure 3A). In co-transfections, Pax7/Barx2HDBBR

reduced Barx2-mediated activation of TOPflash by ~12-fold but, unlike WT Pax7, did not completely abolish activation (Figure 3A). In contrast, Pax7/Barx2HDBBR did not significantly reduce Barx2-mediated activation of Axin2-luc (Figure 3B). These data suggest that the Barx2 and Pax7 homeodomains serve largely in recruitment to TCF/LEF motifs, while adjacent domains determine the degree of target-gene specific activation or repression.

### Barx2 and Pax7 alter histone modifications at the Axin2 intronic enhancer

Activating and repressive histone modifications at the Axin2 T3 region were examined using ChIP with antibodies recognizing histone H3 Lys acetylation (H3Kac), histone H3 Lys4 tri-methylation (H3K4me3) and histone H3 Lys27 tri-methylation (H3K27me3) in primary myoblasts as well as in our TOP-Puro C2C12 stable cell line (allowing us to simultaneously assess histone modifications at the Axin2 gene and the integrated TOPflash promoter). Interestingly, the histone modification signature at the Axin2 T3 region differed in these two cell types in basal growth conditions (Figure 4A). In C2C12 cells the levels of activating marks H3Kac and H3K4me3 were very low, whereas repressive H3K27me3 was enriched. Conversely, in primary myoblasts, the T3 region was enriched for H3Kac and H3K4me3 and showed a low level of H3H27me3, suggesting that it is in a more accessible state in these cells. Consistent with this chromatin signature, the basal Axin2 mRNA level was higher in unstimulated primary myoblasts than in C2C12 cells (Supplemental Figure 3). In contrast to Axin2, the integrated TOPflash reporter in TOP-puro C2C12 cells was enriched for active histone marks (Figure 4A). In both primary myoblasts and C2C12 cells, Wnt3a stimulation dramatically increased activating histone modifications and decreased repressive modifications at the Axin2 intronic enhancer (Figure 4B). The integrated TOPflash reporter exhibited similar changes.

Transfection of either β-catenin or Barx2 in TOP-Puro C2C12 cells increased H3Kac levels at the Axin2 T3 region and at the TOPflash reporter (Figure 4C). Transfection of Barx2 ΔHD had no effect on histone acetylation consistent with its lack of activity in Axin2-luc reporter assays and inability to recruit β-catenin to the Axin2 intronic enhancer. Transfection of Pax7 into TOP-Puro C2C12 cells did not alter the basal level of H3KAc at the Axin2 T3 region; however co-transfection of Pax7 with Barx2 reduced the ability of Barx2 to increase H3K acetylation. Similar effects were seen at the TOPflash promoter (Figure 4C). Thus Barx2 promotes histone acetylation at Wnt-responsive promoters, and Pax7 antagonizes this function.

### The histone acetyltransferase GRIP-1 synergizes with Barx2 in regulation of Axin2 promoter/intron activity and is recruited to TCF/LEF elements

We screened several HAT co-activators, including GRIP-1 and CBP, for their ability to synergistically activate the Axin2-luc reporter construct with Barx2 (not shown). Of those screened, only GRIP-1 synergized with both Barx2 and β-catenin (Figure 5A). Co-immunoprecipitation assays showed that Barx2 and GRIP-1 have an affinity for each other in detergent lysates of transfected cells (Figure 5B). ChIP analysis showed that GRIP-1 was greatly enriched at both the endogenous Axin2 T3 site and the integrated TOPflash promoter following Wnt3a-treatment in C2C12 cells (Figure 5C). Over-expression of either Barx2 or β-catenin also resulted in enrichment of GRIP-1 at both loci (Figure 5C). We then tested

whether GRIP-1 was required for Barx2 to induce histone acetylation by inhibiting GRIP-1 expression using siRNA. Co-transfection of GRIP-1 siRNA with the Barx2 cDNA blocked the ability of Barx2 to increase H3Kac levels at the Axin2 intronic enhancer as indicated by ChIP analysis (Figure 5F). Hence GRIP-1 is likely to be involved in H3K acetylation at Axin2 and possibly other β-catenin-TCF/LEF target genes.

### Pax7 associates with the histone deacetylase HDAC1

Pax7 inhibits the ability of Barx2 to induce activating histone modifications at TCF/LEF target promoters (Figure 4C). Due to the known involvement of HDAC1 in repressive TCF/LEF complexes [17, 19], we asked whether Pax7 might associate with HDAC1. Co-transfection of HDAC1 significantly inhibited Barx2 and β-catenin-mediated activation of both TOPflash and Axin2-luc reporter constructs. Furthermore, when coexpressed with HDAC1, Pax7 became repressive to basal Axin2-luc activity (Figure 5D); expression of HDAC1 alone had no significant effect on basal activity. Co-immunoprecipitation revealed that Pax7 and HDAC1 have an affinity for each other in detergent lysates of transfected cells (Figure 5E). To determine whether HDAC1 was required for Pax7 to inhibit the histone acetylation induced by Barx2, we inhibited HDAC1 expression using siRNA and performed ChIP analysis. As in previous experiments (Figure 4C), co-transfection of Pax7 with Barx2 reduced the ability of Barx2 to increase H3Kac levels at the Axin2 intronic enhancer (Figure 5F). However transfection of HDAC1 siRNA together with Pax7 and Barx2 rescued the ability of Barx2 in induce H3K acetylation (Figure 5F). Thus HDAC1 is required for Pax7 to block the activating effects of Barx2 on chromatin at the Axin2 locus.

### Wnt3a indirectly induces Barx2 mRNA expression in myoblasts

We previously determined that Barx2 mRNA is induced by Wnt3a in cells and *in vivo* [12]. To further understand the mechanism of induction, we assessed the kinetics of Barx2 induction over a time-course of Wnt3a treatment. There was no change in Barx2 mRNA level after 12 hours of Wnt3a stimulation, but there was 14-fold increase by 24 hours and 25-fold by 48 hours. By comparison, Axin2 mRNA was induced 94-fold by 12 hours post-Wnt stimulation and maintained that level over 48 hrs of continuous Wnt3a stimulation (Figure 6A).

In cancer cells Axin2 mRNA has been reported to have a short half-life dropping rapidly to baseline levels after the Wnt signal is removed [55], likely explaining its oscillatory expression pattern during somitogenesis [56]; to our knowledge the stability of Axin2 in myoblasts has not been examined. It was also unknown whether Barx2 mRNA remained stable after induction. To assess this, we performed a Wnt-pulse experiment: myoblasts were incubated with Wnt3a for 12 hours, followed by 36 hours without ligand. The level of Barx2 mRNA continued to rise following removal of Wnt3a, although it did not reach the same level as with continuous Wnt3a stimulation (2-fold at 24 hours, 8-fold at 48 hours). By comparison, Axin2 mRNA levels, which were rapidly induced by Wnt3a, dropped to baseline after removal of Wnt3a (equivalent to the control condition at the same time point) (Figure 6A). When myoblasts were treated with cycloheximide (CHX) (Figure 6B) following the 12 hour Wnt3a pulse, the increase of Barx2 mRNA was greatly inhibited, suggesting that translation of intermediate factors (rather than simply stabilization of β-

catenin) was required for the induction of Barx2 mRNA. Moreover, lentiviral overexpression of constitutively active β-catenin in primary myoblasts did not increase Barx2 mRNA levels; whereas it potently increased Axin2 mRNA levels (Supplemental Figure 4). Taking these data together with our observations that neither Wnt3a nor β-catenin could activate a series of Barx2 promoter-luciferase constructs up to −3kb from the TSS (not shown), we propose that Wnt3a induces Barx2 mRNA indirectly, not by binding of β-catenin to the Barx2 promoter, but via intermediate factors that may bind at sites distal to the promoter. Identifying these factors and their sites of action within the Barx2 genomic locus will require further study.

### Wnt3a regulates Barx2 at the protein level in myoblasts

Primary myoblasts treated for 48 hours with Wnt3a showed an increase in Barx2 protein by fluorescence-immunostaining (Figure 6C). When assessed on a population basis, Wnt-treated myoblasts showed an average of 2-fold increase in Barx2 protein immunoreactivity (Figure 6D). However, the Barx2 staining was highly heterogeneous (Figure 6E), prompting us to examine if there is cell-autonomous posttranscriptional regulation of Barx2. Primary myoblasts were transduced with a Barx2-lentiviral construct and Barx2 protein was examined at a single cell level by immunostaining. Very few cells in the Barx2-lentivirus-transduced population were strongly immunoreactive for Barx2, whereas when GFP-lentivirus was prepared and transduced under identical conditions almost every cell was GFP-bright. Moreover, the level of Barx2 mRNA and GFP mRNA measured in the Barx2- and GFP-lentivirus transduced myoblast populations respectively was comparable suggesting similar transduction efficiency (Supplemental Figure 5A), and transduction of HEK293T cells with the Barx2 lentivirus produced expression in almost every cell (Supplemental Figure 5B). This suggested that in a primary myoblast population, accumulation of Barx2 protein was inhibited in many of the cells expressing Barx2 mRNA. To assess the role of the proteasome in Barx2 protein regulation in myoblasts, we treated Barx2-transduced myoblasts for 8hrs with proteasome-inhibitor MG312, and then immunostained for Barx2 protein. We found a dramatic increase in both the number of cells expressing Barx2 protein and the level of expression per cell (Figure 6F, G) suggesting that Barx2 is regulated by proteosomal turnover. We next examined whether Wnt3a affected Barx2 protein stability: myoblasts transduced with the Barx2-lentivirus and treated with Wnt3a for 24 hours showed 2-fold increase in the level of Barx2 staining per cell (Figure 6F). Although Wnt3a induces endogenous Barx2 mRNA (Figure 6A), there was no increase in Barx2-immunoreactivity in untransduced cells after only 24 hours Wnt3a treatment; thus we conclude that increased immunoreactivity in Barx2-transduced cells after Wnt3a treatment is due to stabilization of the heterologously-expressed protein. Overall, we conclude that Wnt3a induces Barx2 mRNA expression indirectly, and helps to stabilize Barx2 protein. Moreover, Barx2 protein is regulated in a cell autonomous manner in myoblasts by proteasomal turnover.

We recently reported ~2-fold decrease in Pax7 mRNA in primary myoblast treated for 24hrs with Wnt3a [12]. As Pax7 has been previously shown to be regulated at the protein level in myoblasts [33], we assessed whether Pax7 might also be post-transcriptionally regulated by Wnt3a. The level of Pax7 protein in primary myoblasts was assessed after 24 hours of

Wnt3a treatment by immunoblotting. Pax7 protein was undetectable in extracts from these cells after 24hrs of Wnt3a treatment (Figure 6H) suggesting that Pax7 is potently post-transcriptionally inhibited by Wnt3a.

## Discussion

Canonical Wnt signalling is activated during adult muscle regeneration *in vivo* [10] and canonical ligands (e.g. Wnt3a, Wnt1, Wnt4) promote myoblast differentiation *in vitro* [11-14]. Intriguingly however, recent work [29] shows that loss of β-catenin in satellite cells has no effect on adult muscle repair, suggesting that there may be β-catenin-independent pathways for so-called canonical Wnt ligands to affect myogenesis. In contrast, overexpression of constitutively active β-catenin (that cannot be degraded via the destruction complex) was found to impair regeneration, with myoblasts trapped in a prolonged differentiation phase leading to small myofibres and fibrosis [29]. Hence our emerging understanding is that while canonical Wnts are active during muscle regeneration, it is the negative feedback to attenuate the Wnt/β-catenin signal that is critical for effective regeneration [29]. Negative feedback is regulated at several levels including at the ligand-receptor complex by Wif1, Sfrps, and Dkk, and at the destruction complex via Axin2 [16, 57, 58].

Here we show that Wnt3a induces Barx2 protein, and that Barx2 works with β-catenin to promote induction of Axin2, suggesting that Barx2 is part of the Wnt feedback loop in myoblasts. Consistent with this idea, the muscle phenotype resulting from expression of constitutively-active β-catenin *in vivo* is similar to that of loss of Barx2: after injury there is impaired/prolonged differentiation, and fibrosis [29, 36]. Whether lack of Barx2 disrupts the timely attenuation of the Wnt signal via Axin2 *in vivo* is an ongoing investigation.

### Mechanism of action of Barx2 and Pax7 at the Axin2 promoter

Overexpression of Barx2 increased nuclear β-catenin and induced β-catenin recruitment to the Axin2 intronic enhancer in the absence of a Wnt signal. A role for Barx2 in sequestering of β-catenin in the nucleus is consistent with a previous report that Msx2 could promote β-catenin nuclear localization in fibroblasts [59]. The Barx2 homeodomain was necessary, although not sufficient, for recruitment of Barx2 and β-catenin to TCF/LEF sites. Domain swapping experiments suggest that the homeodomains of Barx2 and Pax7 are partially interchangeable and may be primarily involved in recruitment to TCF/LEF sites, while the flanking N and C-terminal domains determine whether recruitment mediates activation or repression. Notably, Pitx2 has also been reported to interact with β-catenin via its homeodomain [60] and this may be a general feature of homeodomain proteins that interact directly with Wnt signalling effectors.

Changes in histone acetylation induced by Barx2 required the presence of GRIP-1. GRIP-1 was previously reported to interact with β-catenin [61, 62] and is expressed in myoblasts with expression increasing during differentiation [63]. Interestingly, while Wnt3a dramatically remodelled chromatin at the Axin2 locus to an accessible state as indicated by increased H3K4 acetylation (H3K4ac) and methylation (H3K4me3) and decreased H3K27 methylation (H3K27me3), neither Barx2 nor β-catenin could fully recapitulate these

chromatin effects, mediating little or no change in histone H3 methylation (not shown). Similarly, a previous study reported no change in H3K4me3 at the Axin2 promoter/intron in myoblasts following β-catenin stabilization by a GSK inhibitor [64]. These findings suggest that the epigenetic changes induced by Wnt signalling at target loci are not mediated exclusively by the β-catenin effector complex, but that they involve other as yet unknown effectors and mechanisms. Whether this observation is relevant to the apparent redundancy of β-catenin (but not Wnt signals *per se*) for myogenesis *in vivo* remains to be determined.

Pax7 was recruited to Axin2 TCF/LEF sites and inhibited Barx2 and β-catenin mediated gene activation at least in part by inhibiting histone acetylation. HDAC1 was reported to associate with TCF/LEF members [17, 19] and is down-regulated by Wnt3a [65]. Based on our new data we propose that Pax7 may retain HDAC1 (and possibly other corepressors) at TCF/LEF sites in a manner that is dominant over Barx2 and β-catenin-mediated co-repressor dismissal/coactivator recruitment. This idea is consistent with our previous observation that exogenous Pax7 inhibited TOPflash activity even in the presence of Wnt3a [12], suggesting that high levels of Pax7 maintain the balance of positive and negative co-factors at TCF/LEF sites in favour of a repressive state.

### Distinct roles for Barx2 in induction of differentiation and Wnt signalling feedback

We previously showed that Barx2 can coregulate differentiation-associated genes in association with MRFs and MADS box proteins (MyoD, SRF, MEF2) [38]. Hence induction of Barx2 protein by Wnt3a is likely to promote differentiation through its association with these classical myogenic regulators. With this new study, we now show that Barx2 is also an important part of the feedback loop that constrains Wnt signalling in myoblasts by regulating Axin2. In this context, it appears to work through direct interaction with β-catenin-TCF complexes. Thus our working model (Figure 7) for the role of Barx2 in the Wnt response is a dual function model: Barx2 induction promotes differentiation of a competent subpopulation of myoblasts, and is also important for negative feedback to terminate the Wnt response; these functions are mechanistically distinct involving different transcriptional partners.

This model for Barx2 function fits into a broader paradigm that we propose for the functions of Wnt3a in myogenesis: i.e. canonical Wnt signals induce the myogenic program via inducing the expression/activity of core myogenic transcription factors; in contrast TCF/β-catenin complexes are mainly involved in regulating Wnt signalling feedback through targets such as Axin2. In support of this model, we have used ChIP-seq to compare activating histone modifications in primary myoblasts with and without Wnt3a treatment, and found that the set of genes showing an active histone signature after Wnt3a (increased H3Kac and H3K4me3) was robustly enriched for Gene Ontology categories associated with myogenic differentiation including “striated muscle cell differentiation”, “contractile fiber”, “myofibril” and “sarcomere’ (Supplemental Figure 6). That is, Wnt3a appears to induce the mechanochemical effectors of differentiation. However Motif analysis showed that these ‘myogenic’ gene promoters are not enriched in TCF/LEF motifs; instead they were highly enriched in binding sites for classical myogenic regulators: specifically MEFs and MRFs (Supplemental Figure 6). This genome-wide data is also consistent with recent report that

the induction of Follistatin by Wnt3a involves increased recruitment of myogenin to the Follistatin promoter [28].

Based on the heterogeneity of Barx2 protein induction by Wnt3a (indicating cell autonomous regulation), it is reasonable to suggest that a subpopulation of myoblasts is inherently competent of responding to Wnt signals by increasing levels/activity of Barx2 and other myogenic regulators. This inherent competence vs incompetence might be determined by the level of Pax7 protein in the cell, as suggested by previous work showing that Pax7 inhibits myogenin at the protein level [33]. We also found that Wnt3a can inhibit Pax7 protein, however both the mechanism of this regulation and how it may contribute to the transition from a high-Pax7/Wnt-refractory/differentiation-incompetent state to a low-Pax7/Wnt-responsive/differentiation-competent state [14, 15, 37], remain under investigation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

This work was supported by National Institutes of Health Grant 5R01AR053163 (to H.P.M. and R.M.) and by a grant from the Association Francaise contre les Myopathies (to H.P.M.). The study was also supported by an Australian Research Council Fellowship FT110100573 (to R.M.), and a Flinders Foundation grant (to R.M.).

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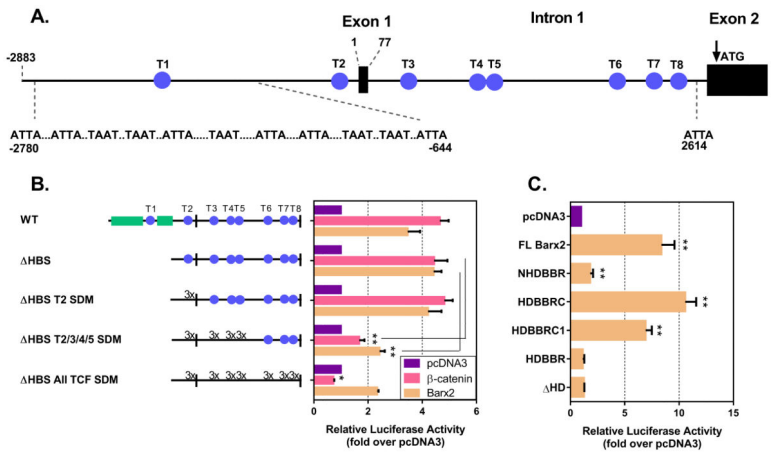


**Figure 1.**

Barx2 activates the Axin2 promoter via TCF/LEF motifs **(A):** Schematic of the Axin2-luc construct. Circles represent TCF/LEF binding sites designated T1-T8. **(B):** Luciferase analyses using deleted or mutated Axin2-luc constructs. Regulation by Barx2 does not require HBS motifs (ΔHBS) but involves TCF/LEF sites, as is β-catenin activation. Mutation of TCF/LEF sites T2 through T5 reduces Barx2 and β-catenin activation by half; additional mutations abolished remaining β-catenin activation. Left: schematic of mutated Axin2-luc constructs. Rectangles represent HBS clusters and circles represent TCF/LEF sites. Triple mutations are indicated by 3x. Right: Axin2-luc activities in C2C12 cells. **(C):** Analysis of deletion constructs shows that the homeodomain (HD) and Barx2 basic region (BBR) are essential for Barx2-mediated activation of Axin2-luc. Luciferase activities are expressed as the mean *firefly/Renilla* luciferase ratio relative to that of pGL3-basic vector (Luc) and normalised over pcDNA3 transfection, which was set to a value of 1. Data of at least two independent experiments performed in triplicate. Error bars represent SEM. * $p < 0.05$ and ** $p < 0.001$.

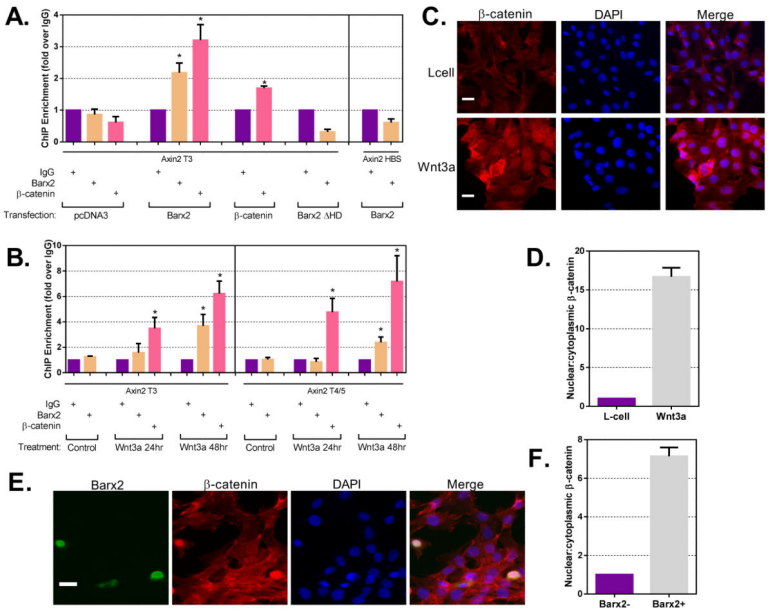


**Figure 2.**

Barx2 is recruited to TCF/LEF motifs and promotes nuclear translocation and recruitment of β-catenin. **(A,B):** ChIP on chromatin from the TOPPuro C2C12 line (A) or primary myoblasts (B) after the indicated treatments/transfections. Barx2 binds to the T3 and T4/5 sites in Axin2 after 48 hours Wnt3a treatment in primary myoblasts, or transfection of FL Barx2, but not Barx2 ΔHD in TOPPuro cells. No recruitment is seen with control L-cell or 24 hour Wnt3a treatment, or with transfection of pcDNA3. β-catenin is recruited at the T3 and T4/5 sites after 24 or 48 hour Wnt3a treatment and also after transfection of β-catenin or Barx2, but not after transfection of pcDNA3. Data are the ratio of PCR amplification values at the Axin2 locus over a control locus (β2-microglobulin) and are subsequently normalised to ChIP with preimmune serum, set to a value of 1. **(C):** Immunofluorescence labelling of β-catenin (red) and DAPI (blue) in C2C12 cells after 48 hours treatment with L-cell or Wnt3a CM. Scale bar represents 25 μm. **(D):** Average mean fluorescence ratio of nuclear:cytoplasmic β-catenin in (C) in Wnt3a treated cells relative to control treated. n=45 cells per condition. **(E):** Immunofluorescence labelling of Barx2 (green), β-catenin (red) and DAPI (blue) following Barx2 transfection in C2C12 cells. Scale bar represents 25 μm. **(F):** Average mean fluorescence ratio of nuclear:cytoplasmic β-catenin in (E) in Barx2-positive cells relative to Barx2-negative cells. n=45 cells per condition. Error bars represent SEM. * $p < 0.05$.

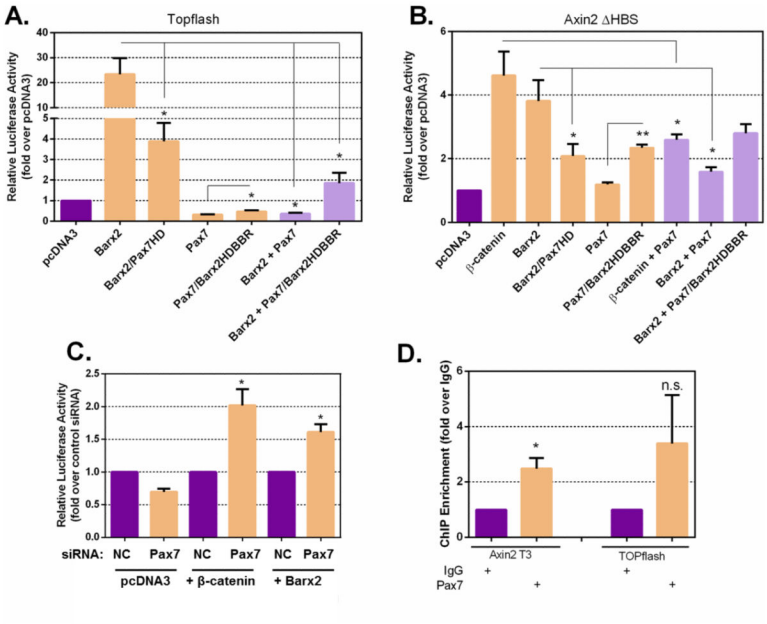


**Figure 3.**

Barx2 and Pax7 have opposing roles in regulation of TOPflash and the Axin2 promoter/intron. (**A,B**): TOPflash and ΔHBS Axin2-luc activity were assayed in C2C12 cells after cotransfection of Barx2 and Pax7 or constructs in which the homeodomains of Barx2 and Pax7 were swapped. **(C):** Knockdown of Pax7 increases the ability of β-catenin and Barx2 to activate ΔHBS Axin2-luc. Barx2 or β-catenin were cotransfected with either negative control siRNA or Pax7 siRNA. Luciferase activities were assayed 48 hours post transfection and expressed as the mean *firefly/Renilla* luciferase ratio relative to that of pGL3-basic vector (Luc) and normalised over pcDNA3 vector (A,B) or negative control siRNA (C), which were set to a value of 1. Data are average of at least three independent experiments in triplicate. **(D):** ChIP shows that Pax7 is recruited to the TOPflash reporter and the Axin2 T3 site in the TOP-Puro line after Pax7 transfection. Data are PCR amplification values for the appropriate loci normalised to amplification values for a control non-target locus (β2-microglobulin). Enrichment values for each antibody were subsequently normalised to the mock ChIP with preimmune IgG, set to a value of 1. Error bars represent SEM. * $p < 0.05$ and ** $p < 0.001$.

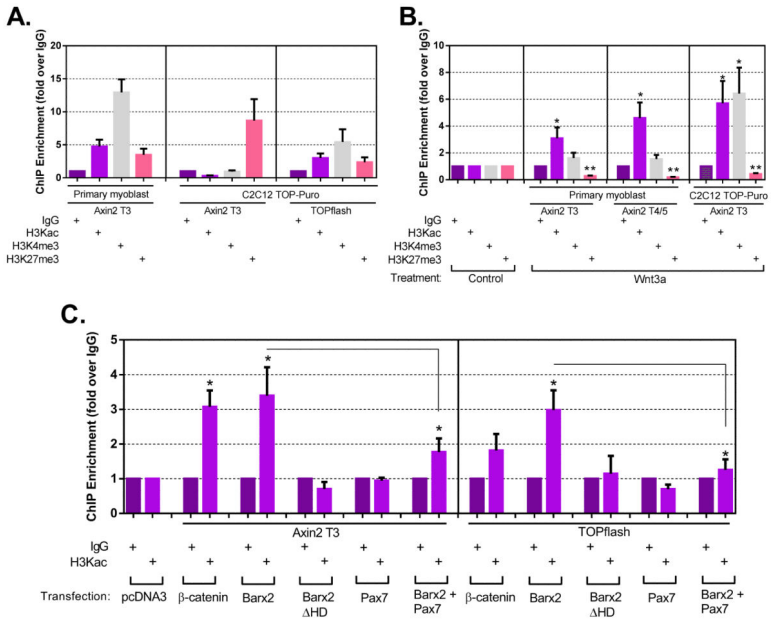


**Figure 4.**

Wnt signalling and Barx2 alter histone modifications and correlate with distinct epigenetic states of the TOPflash and Axin2 promoter/intron. **(A):** ChIP on primary myoblasts or the stable TOP-Puro line with antibodies recognising acetylated histone H3 (H3Kac), trimethylated histone H3K4 (H3K4me3) and trimethylated histone H3K27 (H3K27me3). **(B):** ChIP on primary myoblasts shows that 48 hour Wnt3a-treatment enriches H3Kac and H3K4me3 and reduces H3K27me3 at the Axin2 promoter/intron. **(C):** ChIP on transfected TOPpuro cells shows that β-catenin and Barx2, but not Barx2 ΔHD, increase H3Kac at the Axin2 and TOPflash loci. Pax7 transfection had no effect on H3Kac levels, but cotransfection of Barx2 and Pax7 decreased enrichment of H3Kac relative to Barx2 alone. Data are the ratio of PCR amplification values at the relevant locus over a control locus (β2-microglobulin) and are subsequently normalised to ChIP with preimmune serum, set to a value of 1. Data are the average of at least two independent experiments. Error bars represent SEM. * $p < 0.05$ and ** $p < 0.001$.

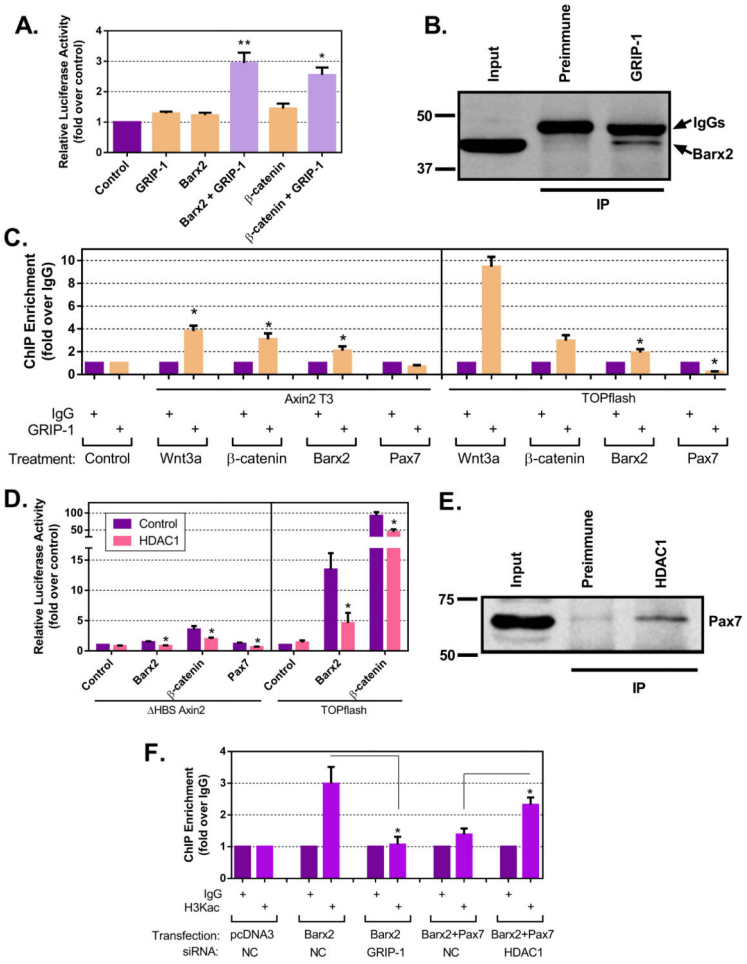


**Figure 5.**

GRIP-1 synergizes with Barx2 in regulation of Axin2-luc activity and is required for Barx2 mediated histone acetylation. HDAC1 is required for antagonism of Barx2 function by Pax7. **(A):** Cotransfection of Barx2 or β-catenin with GRIP-1 produces synergistic activation of ΔHBS Axin2-luc. **(B):** Myc-tagged Barx2 and GRIP-1 were co-immunoprecipitated using anti-GRIP-1 antibodies and blotted with anti-myc antibodies. **(C):** GRIP-1-ChIP on TOPPuro cells shows that GRIP-1 is recruited to both TOPflash and the Axin2 T3 site after 48 hour Wnt3a treatment or transfection with Barx2 or β-catenin but not Pax7. Data are the ratio of PCR amplification values at the relevant locus over a control locus (β2-microglobulin) and are subsequently normalised to ChIP with preimmune serum, set to a value of 1. **(D):** Cotransfection of HDAC1 repressed Barx2 and β-catenin mediated activation of TOPflash and ΔHBS Axin2-luc. **(E):** Myc-tagged Pax7 and HDAC1 were coimmunoprecipitated using anti-HDAC1 antibodies and blotted with anti-myc antibodies. **(F):** H3Kac-ChIP analysis after co-transfection of Barx2 with control or anti-GRIP-1 siRNA shows that GRIP-1 is required for Barx2 to induce H3K acetylation at the Axin2 locus. H3Kac-ChIP analysis after co-transfection of Barx2 and Pax7 with control or anti-HDAC1 siRNA shows that HDAC1 is important for Pax7 to inhibit the ability of Barx2 to induce H3K acetylation at the Axin2 locus. Error bars represent SEM. * $p < 0.05$ and ** $p < 0.001$.

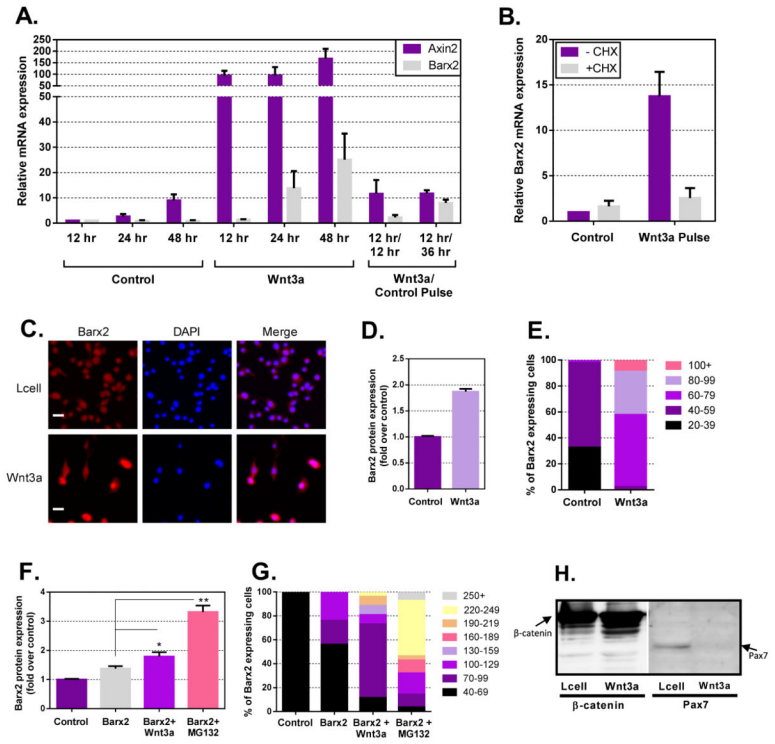


**Figure 6.**

Wnt3a indirectly induces Barx2 mRNA and protein in primary myoblasts. **(A):** Primary myoblasts were treated with L-cell or Wnt3a CM for different time periods and Axin2 and Barx2 mRNA levels measured. Data are the average of two independent experiments in duplicate. **(B):** Primary myoblasts were cultured with Wnt3a CM for 12 hours (Wnt-pulse), followed 36 hours in control L-cell CM. Addition of cycloheximide (CHX) following the Wnt-pulse blocked Barx2 induction. Data are the average of two independent experiments in duplicate. **(C):** Immunofluorescence labelling shows increased Barx2 protein (red) in primary myoblasts after 48 hours Wnt3a treatment. Scale bar represents 25 μm. Blue-DAPI **(D,E):** Mean fluorescence of Barx2 in (C) calculated as population average (D) or on a cell-by-cell basis (E) quantified by ImageJ. Representative images and quantification are from one of two independent experiments that gave the same result; n=45 cells per condition. **(F,G):** Expression of exogenously-expressed Barx2 was increased by addition of Wnt3a (24 hours) or the proteasome inhibitor MG132 (8 hours). Barx2 expression is calculated as population average (F) and on a cell-by-cell basis (G) quantified by ImageJ. n=45 cells per condition. Error bars represent SEM. * $p < 0.05$ and ** $p < 0.001$. **H.** Immunoblotting shows the increase in β-catenin and decrease in Pax7 protein levels (arrows) after 24 hours of Wnt3a treatment in primary myoblasts. The panels probed with β-catenin (left) and Pax7 (right) antibodies are from the same blot but were exposed for different times.

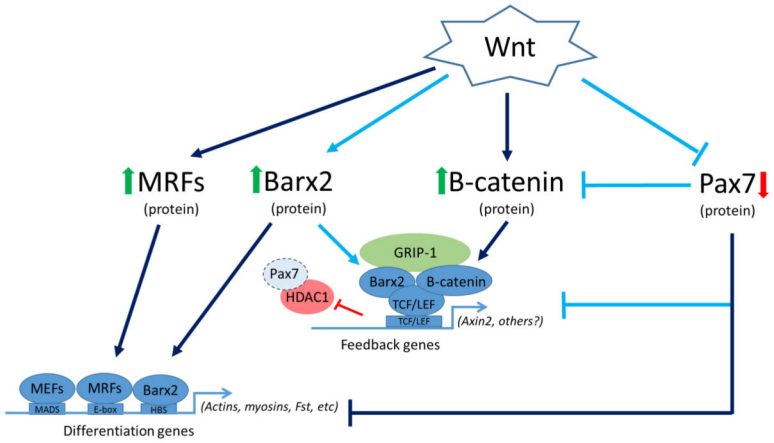


**Figure 7.**

Model for the dual function of Barx2 downstream of Wnt/β-catenin signalling. Barx2 protein is induced by Wnt3a, and in turn partners with core myogenic regulators to induce genes associated with the differentiated state. The model is based in part on published work showing that Wnts increase the activity/protein levels of various MRFs [26, 28], and our studies showing that Barx2 binds and/or cooperates transcriptionally with MRFs and MADS box factors SRF and MEF2 [37] to help activate myogenic genes. Barx2 also associates with β-catenin-TCF/LEF complexes and GRIP-1 to regulate non-myogenic targets such as the negative feedback gene Axin2, and possibly other direct Wnt target genes yet to be defined. Pax7 can also have a dual function, negatively regulating myogenesis through inhibition of MRFs such as myogenin [31] as well as regulating Wnt feedback via Axin2. Pax7 may function to block dismissal of co-repressors such as HDAC1 from the TCF/LEF complex. Hence, Wnt-mediated inhibition of Pax7 protein may be necessary both for myogenesis to proceed and also for appropriate Wnt feedback. Dark blue connectors indicate previously known regulatory connections and light blue connectors indicate new regulatory connections.