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RHO GTPASE ACTIVITY MODULATES WNT3A/ β -CATENIN SIGNALING

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Abstract

Wnt proteins constitute a family of secreted signaling molecules that regulate highly conserved pathways essential for development and, when aberrantly activated, drive oncogenesis in a number of human cancers. A key feature of the most widely studied Wnt signaling cascade is the stabilization of cytosolic β -catenin, resulting in β -catenin nuclear translocation and transcriptional activation of multiple target genes. In addition to this canonical, β -catenin-dependent pathway, Wnt3A has also been shown to stimulate RhoA GTPase. While the importance of activated Rho to non-canonical Wnt signaling is well appreciated, the potential contribution of Wnt3A-stimulated RhoA to canonical β -catenin-dependent transcription has not been examined and is the focus of this study. We find that activated Rho is required for Wnt3A-stimulated osteoblastic differentiation in C3H10T1/2 mesenchymal stem cells, a biological phenomenon mediated by stabilized β -catenin. Using expression microarrays and real-time RT-PCR analysis, we show that Wnt3A-stimulated transcription of a subset of target genes is Rho-dependent, indicating that full induction of these Wnt targets requires both β -catenin and Rho activation. Significantly, neither β -catenin stabilization nor nuclear translocation stimulated by Wnt3A is affected by inhibition or activation of RhoA. These findings identify Rho activation as a critical element of the canonical Wnt3A-stimulated, β -catenin-dependent transcriptional program.

Keywords

Wnt; RhoA; β -Catenin; RhoGTPase; Ctgf; mesenchymal stem cell

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1. Introduction

Activation of signaling pathways by the Wnt family of secreted glycoproteins is central to a wide array of developmental events across all animal taxa, including cell proliferation, migration, the establishment of cell polarity, and the specification of cell fate [1,2]. Dysregulation of Wnt signaling is known to result in human disease [1,3], and aberrant Wnt activation is a critical step in oncogenesis in many human tumors [3,4]. Wnt ligands bind to cellular receptors to activate diverse signaling events, including β -catenin-dependent transcriptional induction (the canonical pathway), the stimulated release of intracellular calcium (the Wnt- Ca^{2+} pathway), and the initiation of the planar cell polarity/convergent extension pathway (the PCP pathway) [5–7].

There are ~20 members of the Wnt family in mammals that can be divided into two major categories: transforming and non-transforming. Transforming Wnts possess the ability to transform mammary epithelial cells and can induce secondary axis formation in *Xenopus*. Transforming Wnts generally activate the canonical, β -catenin-dependent signaling wherein Wnt stimulation regulates the fate of cellular β -catenin to ultimately induce transcription of target genes [8]. Cytosolic β -catenin is continuously degraded in unstimulated cells via phosphorylation-ubiquitination-coupled proteasomal degradation. Receptor binding of canonical Wnts inhibits this degradation resulting in the accumulation of stabilized β -catenin, which can then shuttle into the nucleus. Nuclear β -catenin interacts with members of the T-cell factor (TCF)/lymphoid enhancing factor (LEF) family of HMG-box transcription factors to induce expression of Wnt target genes.

Non-transforming Wnts neither transform cells nor induce secondary axis formation and are associated with stimulating the non-canonical Wnt- Ca^{2+} and PCP pathways. Wnt signaling in these pathways is β -catenin-independent. Non-transforming Wnts control cytoskeletal changes to affect movement and polarity and do so in part by promoting the activation of the Rho family of GTPases, including Rho and Rac, which are important regulators of cytoarchitecture, cell adhesion, transcriptional events, and cell cycle progression [9].

Wnt-induced activation of Rho GTPases is not restricted to non-transforming Wnts, however. The canonical Wnt1 in *Xenopus* was shown to trigger the activation of both Rho and Rac [10], and, in several studies, Wnt3A was reported to promote both canonical β -catenin-dependent signaling as well as the activation of RhoA [11–13]. While the importance of Wnt-triggered Rho activation to non-canonical signaling is well appreciated, its contribution to canonical signaling has not been examined.

In this study, we show that activated Rho is required for Wnt3A-stimulated osteoblastic differentiation in C3H10T1/2 mesenchymal stem cells, a process mediated by stabilized β -catenin [14]. Furthermore, the inhibition of Rho dramatically suppresses Wnt3A-stimulated β -catenin-dependent transcription of a subset of target genes in these cells. These results indicate that activated Rho modulates the transcriptional output and cellular consequences of the canonical pathway. We also show that the Rho activation does not affect β -catenin stabilization and nuclear accumulation, but rather is parallel to these signaling events and exerts its transcription modifying effects downstream of β -catenin stabilization and accumulation. These findings refine the Wnt3A signaling model to include Rho activation as a positive modifier of the Wnt3A-stimulated, β -catenin-dependent transcriptional program.

2. Materials and Methods

2.1 Antibodies and reagents

Mouse monoclonal anti-RhoA (sc-418) and anti-HA were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti- β -actin was from Cell Signaling, anti- β -catenin mouse monoclonal antibody and anti-RhoGDI were from BD Transduction Laboratories (San Jose, CA); anti-CREB was from Upstate (now Millipore, Billerica, MA). For western blots β -actin, HA, RhoA, β -catenin, and CREB antibodies were used at 1:1000 and the anti-RhoGDI was used at 1:5000. Tetanolysin was from Biomol (Plymouth Meeting, PA). Purified Wnt3A was purchased from Millipore (Billerica, MA) and R&D (Minneapolis, MN).

2.2 Plasmid constructs

The cDNA encoding HA-RhoA(GV) in pcDNA3.1 was obtained from the Missouri S&T cDNA Resource Center (<http://cdna.org>). The pSuper8X-TOPFlash reporter was from Randall Moon (U Washington, Seattle, WA). The pCtgf-luc reporter was provided by Eugene Chen (U Mich, Ann Arbor, MI). The pCtgf-trunc vector was made by digesting with MluI, which removes a 1.5 kb fragment from the 5' end of the promoter, and re-ligating. The pRL-TK and pHRG were from Promega. The GST-C3 construct was from Judith Meinkoth (UPenn, Philadelphia, PA), the C3 expression construct was a gift from Channer Der (UNC, Chapel Hill, NC), and the DN-TCF4 construct was from Bert Vogelstein [15]. A construct encoding axin Δ dix was from Harold Varmus (Sloan-Kettering, NY, NY), and the LRP5 Δ C construct was from Matthew Warman (Harvard, Boston, MA). HA-tagged p190RhoGAP in pKH3 was kindly provided by Ian Macara [16]. The β -catenin Δ N construct [17] was acquired from Eric Fearon via Addgene (www.addgene.org).

2.3 Cell lines, culture, transfections, and preparation of Wnt conditioned media

HEK293T cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Transfections were performed using LipofectAMINE2000 (Invitrogen) for HEK293T cells and FuGENE (Roche Applied Science, Indianapolis, IN) for C3H10T1/2 cells according to manufacturers' instructions. C3H10T1/2 cells were obtained from ATCC and grown in Basal Eagle Medium (BEM) with 4 mM L-glutamine and 10% FBS. To produce Wnt conditioned media (CM), L cells producing and secreting Wnt3A (kindly provided by Karl Willert, Stanford U) were grown to confluency in DMEM with 10% FBS and 1000 U/ml penicillin-1000 μ g/ml streptomycin (Gibco). After 3 days, media was harvested and filtered through a 0.45 μ m filter unit (Millipore). The Wnt3A CM and control CM were used at a 1:5 dilution with DMEM + 10% FBS unless otherwise indicated.

2.4 Preparation and use of C3 exoenzyme

The GST-C3 fusion protein was prepared by expression in the BL21DE3 strain of *E. coli* (Novagen) and bound to glutathione-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ) as described for other GST fusion proteins [18]. The C3 protein was cleaved from the glutathione beads by rocking overnight at 4 °C with 10 U Thrombin (Enzyme Research Laboratories, South Bend, IN) in 50 mM Tris-HCl pH 7.7, 14 mM β -mercaptoethanol, 150 mM NaCl, and 2.5 mM CaCl₂. The cleaved product was then dialyzed into phosphate buffered saline (PBS), visualized by SDS-PAGE with coomassie blue staining, and stored in aliquots at -80 °C. To inhibit Rho activity, cells were incubated with 2.5 mg/ml C3 exoenzyme in the presence of 20 U/ml tetanolysin, to permeabilize the cellular membrane.

2.5 Reporter Assays

C3H10T1/2 cells were seeded in 6-well plates, grown to 85% confluence, and transfected with a reporter construct (0.4 µg/well pCtgf-luc, pCtgf-trunc, or pSuper8X–TOPFlash) and 2 ng/well of the internal control plasmid, pHRG, along with the plasmids indicated in the figure legends. Five h after transfection, the media was exchanged for either control or Wnt3A CM diluted 1:5 in media. 18 h after treatment or transfection, in the case of untreated cells, cell lysates were prepared and analyzed using the Dual Luciferase Assay kit (Promega) according to the manufacturer's protocol. Transcriptional activity was measured using a TD 20/20 luminometer (Turner Designs, Sunnydale, CA) as relative light units (RLU), a ratio of firefly luciferase activity to *Renilla reniformis* luminescence.

2.6 Cytosolic β -catenin stabilization

C3H10T1/2 or HEK293T cells were incubated with Wnt3A CM or control CM for 3–4 h, after which they were washed with cold PBS and incubated on ice for 30 min in 0.25 ml hypotonic buffer [10 mM Tris, pH 7.4, 1 mM EDTA and protease inhibitor cocktail complete mini tablet (Roche)]. The cells were then scraped into 1.5 ml tubes, passed 5 times through a 27.5 gauge needle, and centrifuged at 100,000 X g for 45 min. Protein concentrations were measured by Bradford assay (Biorad) and 10 µg of protein from the supernatant (cytosol) were analyzed for the presence of β -catenin by immunoblot. Immunoblot bands were quantified using the Odyssey system from Li-Cor Biotechnology (Lincoln, NE) [19].

2.7 Nuclear β -catenin accumulation

HEK293T cells were grown in 6-well plates to 75% confluency and transfected with pcDNA3.1 or RhoA(GV) as indicated in the figures. After 5 h, the cells were incubated with Wnt3A CM or control CM for 16 h and harvested as described [20]. Briefly, cells were harvested in 1.5 ml PBS, centrifuged at 14,000 rpm for 10 s at room temperature and resuspended in 400 µl ice cold buffer A [10 mM HEPES pH 8, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 1:500 PI mix: 23 µg/ml phenylmethylsulfonyl fluoride, 11 µg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone, 11 µg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone]. Cells were placed on ice for 10 min, and then agitated by vortex for 10 s. Lysates were centrifuged at 14,000 rpm for 10 s at room temperature and the nuclear pellets incubated in 100 µl ice-cold buffer containing 20 mM HEPES pH 8, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitors to extract bound proteins. Lysates were then centrifuged at 14,000 rpm for 2 min at 4 °C to remove cellular debris and 25 µg of total protein from the supernatant was evaluated by SDS-PAGE and immunoblot analysis. The presence of the nuclear protein CREB was assessed to confirm the nuclear origin of the fractions analyzed and to assess protein loading.

2.8 Rho activation and alkaline phosphatase activity assay in C3H10T1/2 cells

For RhoA activation assays, C3H10T1/2 cells were plated in 10 cm plates at ~75% confluent, starved for 24 h, and treated with 100 ng/mL purified Wnt3A or vehicle for 5 min at 37 °C. Cells were lysed and activated RhoA was measured using GST-rhotekin pull-downs essentially as described [21] with minor modifications [22]. For alkaline phosphatase activity, 6-well plates containing C3H10T1/2 cells were grown to 75% confluence and treated either with 2.5 mg/ml C3 exoenzyme (dissolved in reduced sera media containing 2% FBS) in the presence of 20 U/ml tetanolysin (Biomol International) or with tetanolysin alone as a control. After 3 h incubation at 37 °C, the media was removed and replaced with 1:5 Wnt3A CM (diluted also in reduced sera media). Cells were then incubated for 96 h, after which they were stained with the Neat Hematology Stain Kit (Marketlab, Kentwood, MI) according to the manufacturer's instructions or assayed for alkaline phosphatase (AP) activity using the Alkaline Phosphatase Activity Assay Kit #1 (BRSC at SUNY Buffalo, A-110). Briefly, cells were washed once in PBS at 21

°C and lysed in 100 µl cell lysis solution provided by the manufacturer. The lysates were centrifuged at 16,000 X g for 3 min and 20 µl from each sample was added to the wells of a 96-well plate. The assay was initiated by the addition of 100 µl AP reagent (provided by the manufacturer), and the plate was incubated at 37 °C for 1 h according to manufacturer's instructions. The reactions were stopped by the addition of 20 µl 1 M NaOH, and the endpoint absorbance was read at 405 nm using a kinetic microplate reader (Molecular Devices). Protein levels from each sample were measured by Bradford assay (Biorad) and used to normalize the absorbance measurements.

2.9 Microarray and quantitative RT-PCR analysis

For the expression microarray, C3H10T1/2 cells were grown to 80% confluence in 10 cm plates and treated for 2 h either with 2.5 mg/ml C3 exoenzyme (in reduced sera media) in the presence of 20 U/ml tetanolysin (Biomol International), or with tetanolysin alone for controls. Media was then changed to 1:5 dilutions of either control CM or Wnt3A-CM and cells were incubated for an additional 24 h. RNA was then harvested using the RNeasy kit (Qiagen) according to manufacturer's instructions for both microarray and real-time RT-PCR analysis. Microarrays were printed at the Duke Microarray Facility using the Genomics Solutions OmniGrid 300 Arrayer. The arrays contain the Operon Mouse Oligo set v3.0 (<http://omad.operon.com>), which possesses 31,769 70-mer probes representing 24,878 genes and 32,829 transcripts. RNA quality was ascertained using an Agilent 2100 bioanalyzer (Agilent technologies). Total RNA (10 µg) from each sample was hybridized to oligo(dT) primers at 65 °C and then incubated at 42 °C for 2 h in the presence of reverse transcriptase, Cy5-dUTP and -dCTP (for Wnt-treated samples) or Cy3-dUTP and Cy3-dCTP (for control treated samples), and a deoxynucleotide mix. NaOH was used to destroy residual RNA. Sample and reference cDNA were then pooled and purified with QIAquick Purification Column (Qiagen), mixed with 1X hybridization buffer [50% formamide, 5X saline-sodium citrate (Sigma), and 0.1% SDS], COT-1 DNA, and poly-deoxyadenylic acid to limit nonspecific binding, and heated to 95 °C for 2 min. This mixture was pipetted onto a microarray slide, and hybridized overnight at 42 °C on MAUI hybridization system (BioMicro Systems). The array was then washed at increasing stringencies and scanned on a GenePix 4000B microarray scanner (Axon Instruments). Genespring GX v7.3 (Agilent Technologies) was used to perform initial data filtering in which spots whose signal intensities below 50 in either the Cy3 or Cy5 channel were removed.

For RT-PCR analysis, C3H10T1/2 cells were transfected for 5 h, and then all cells were treated with control or Wnt3A CM, containing the indicated inhibitors: C3 exoenzyme or Y-27632. After 24 h, RNA was harvested using the RNeasy kit (Qiagen) according to manufacturer's instructions. One hundred ng of RNA from each sample was used for cDNA synthesis with the Accuscript High Fidelity RT-PCR system (Stratagene, La Jolla, CA), according to manufacturer's instructions. Real time PCR reactions to compare the relative levels of Wnt3A responsive genes were performed on a Stratagene Mx3005P instrument, using the following primers: *Ctgf*, 5'-CCCTAGCTGCCTACCGACTC-3' (fwd) and 5'-CATTCCACAGGTCTTAGAACAGGC-3' (rev), *Ned4*, 5'-AGGAGGAGAGACGATGGACTG-3' (fwd) and 5'-TAGGTGGGTTGGAATCGGCT-3' (rev), *Igf1*, 5'-AGATAACCAAATGTGCCGTGAT-3' (fwd), 5'-TGTCCCCACGATCTTCATCTT-3' (rev). Additionally, a primer set for the reference gene *Gapdh* was used to assess expression across all conditions to normalize any variations in template extraction, 5'-CCACTCACGGCAAATTCAAC-3' (fwd) and 5'-GTAGACTCCACGACATACTCA-3' (rev). 24 µl reactions, containing 12.5 µl SYBR Green Supermix (2x) (Stratagene), 0.125 µl 1:500 diluted Rox reference dye, 10.375 µl H₂O, 1 µl of 5 µM forward and reverse primer mix (1:1), and 1 µl of template cDNA from above, diluted 1:10, were placed in the wells of a 96-well plate. All conditions were evaluated in triplicate.

The thermal profile began with one 10 min incubation cycle at 95 °C, followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s, and completed with 1 cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 1 min. A standard curve with dilutions of cDNA template for each primer set was used to determine primer efficiency. Data were analyzed with MxPro QPCR software, version 3.00. Fold induction relative to reference gene was calculated using the method of Pfaffl [23].

3. Results

3.1 Inhibition of Rho function blocks Wnt3A-stimulated differentiation of C3H10T1/2 mesenchymal stem cells

Several groups have demonstrated that Wnt3A is capable of both stabilizing β -catenin and activating Rho [11–13]. To examine potential functional consequences of Wnt3A-stimulated Rho activation, we utilized the pluripotent C3H10T1/2 mesenchymal stem cell line. These cells undergo osteoblast-like differentiation when stimulated by Wnt3A, and only those Wnt ligands capable of triggering β -catenin accumulation have demonstrated an ability to induce differentiation [14]. As has been reported for several other cell types, treatment of C3H10T1/2 cells with Wnt3A results in β -catenin stabilization, β -catenin-dependent transcriptional induction, and simultaneous rapid activation of RhoA (Fig. 1A–C).

Wnt-treated C3H10T1/2 cells have been reported to undergo characteristic morphological changes, accompanied by expression of bone markers such as alkaline phosphatase, indicative of the differentiation response [14,24]. To assess the role of Rho in the Wnt3A-stimulated differentiation process, we pre-treated C3H10T1/2 cells with C3 exoenzyme derived from *C. botulinum*, which specifically inactivates RhoA, B, and C but has no effect on other Rho family proteins such as Rac or Cdc42 [25]. Consistent with the work of others, treatment of C3H10T1/2 cells with Wnt3A conditioned media (CM) resulted in the reported distinctive morphological changes, characterized by elongation of the cells, giving the appearance of vaguely nodular whorls (Fig. 2A). Strikingly, pre-treatment with C3 exoenzyme blocked the Wnt3A-induced morphologic changes (Fig. 2A), while cells treated with C3 alone appeared similar to untreated cells (data not shown). Since Rho inhibition could have morphologic effects that are independent of Wnt3A signaling, we also assessed osteoblast-like differentiation by measuring the induction of the early osteoblast marker, alkaline phosphatase [14]. Treatment of the C3H10T1/2 cells with Wnt3A CM resulted in a prominent, reproducible induction of alkaline phosphatase activity (Fig. 2B). In agreement with the morphologic data, inhibition of Rho markedly attenuated Wnt3A-stimulated alkaline phosphatase induction (Fig. 2B), while C3 treatment alone had no significant effect (data not shown). Collectively, these data indicate that Rho function is required for Wnt3A-stimulated differentiation in this stem cell line, a process previously determined to require β -catenin [14].

3.2 Wnt3A-stimulated, β -catenin-dependent induction of a subset of target genes is modulated by activated Rho

The data in Figure 2 suggest that Rho inhibition could affect Wnt3A-dependent induction of target genes responsible for differentiation. To assess the influence of Rho on Wnt3A-stimulated transcriptional induction, we performed expression microarray analysis on Wnt3A-treated C3H10T1/2 cells in the presence or absence of the Rho inhibitor C3 exoenzyme. RNA was harvested to generate cDNA, and the samples were hybridized to custom microarrays (see *Materials and Methods*). After data filtering to select probes producing signal above background, we found that hybridization with 11,686 of the probes showed signal sufficiently above background in each of the conditions to allow analysis. Among these probes, 337 demonstrated a greater than 2-fold increase in hybridization signal with cDNA from the

Wnt3A-treated cells relative to control-treated cells (Fig. 3A, Table 1, and Supplemental data 1).

Definitive annotations were available for 259 of these probes, which represent 235 distinct genes (Table 1; complete list in Supplemental data 1). Many of these genes showing Wnt3A-stimulated induction have been previously identified by others as Wnt3A targets in these cells, including *Ahr*, *Axin2*, *Bmp4*, *Cyr61*, *Ctgf*, *Hes1*, *Igfbp2*, *Nkd1*, *Omd*, *Tgfb3*, *Thbs1*, *Twist1*, *Wisp1* [26]. The parallel experiment in which cells were treated with Wnt3A after pre-treatment with C3 exoenzyme revealed that induction of a distinct subset of the Wnt3A target genes was diminished by the Rho inhibitor (Fig. 3A; complete list in Supplemental data 2). A scatter plot of the probes induced by Wnt3A versus Wnt3A plus C3 highlights the Rho-sensitive targets (open circles), for which Wnt3A induction is greater than 1.5 times the induction in the presence of Wnt3A+C3 (Fig. 3B). Both the Rho-sensitive genes (open circles) and Rho-insensitive genes (closed circles) included Wnt targets that were weakly and strongly induced (Fig. 3B). The Rho-sensitive genes represent a minority of the Wnt3A-induced genes (Fig. 3B), and a query of the Gene Ontology database (<http://www.geneontology.org>) using GATHER (<http://gather.genome.duke.edu>) [27] revealed that many of these targets are involved in development and morphogenesis, as well as regulation of cell growth and size (Supplemental data 3). Annotations were available for 64 of the 79 probes, representing 62 distinct genes. Each of these 62 genes was assigned to a functional category based on its individual gene ontology entry (Figure 3C; Table 2). The majority of the genes can be categorized as regulating metabolism, cell growth, signaling, or skeletal development and differentiation.

To validate and characterize the Rho-dependent regulation of Wnt3A target genes, quantitative RT-PCR was used. Initially, we verified the Wnt3A-dependent induction of several genes, including *Ahr*, *Axin2*, *Ctgf*, *Igfbp2*, *Pdgfr1c*, *Ier3*, and *Timp3* (data not shown). As illustrated in Figure 3D, Wnt3A target genes identified by the array as inhibited by C3, including *Ctgf* and *Ned44*, were shown to be inhibited by C3 using RT-PCR. Wnt target genes that were not inhibited by C3 on the array, such as *Igfbp4*, were shown not to be inhibited by C3 when assessed by RT-PCR (Fig. 3D). Similar RT-PCR results were obtained whether purified Wnt3A or Wnt3A CM was used (data not shown). These quantitative RT-PCR results confirm the array data and demonstrate that the full induction of a subset of Wnt3A-stimulated target genes requires Rho activation.

A more detailed analysis was conducted on one of these Rho-sensitive targets, *Ctgf*, (connective-tissue growth factor). The expression of *Ctgf* has been seen to occur at an early stage of Wnt3A-stimulated C3H10T1/2 differentiation and is thought to promote proliferation of the early osteoblast progenitor cells [28]. To confirm the contribution of canonical Wnt3A signaling to induction of this gene, a dominant negative TCF construct (DN-TCF4) was expressed prior to Wnt3A treatment. This TCF mutant lacks the β -catenin binding domain and has been shown to have a dominant-negative effect on canonical Wnt signaling [29]. As expected, Wnt3A-stimulated induction of *Ctgf* was substantially inhibited by expression of DN-TCF4 (Fig. 4A). To further characterize the role of Rho activation in induction of *Ctgf*, C3H10T1/2 cells were treated with a different Rho inhibitor, p190RhoGAP, which inactivates Rho by facilitating bound GTP hydrolysis. As expected, p190RhoGAP suppressed the Wnt3A induction of *Ctgf* (Fig. 4B). We also examined the impact of a well-characterized Rho effector, Rho-associated kinase (ROCK) [30], on Wnt3A-stimulated transcriptional induction. Inhibition of ROCK by the chemical inhibitor Y-27632 also resulted in an inhibition of Wnt3A-stimulated *Ctgf* induction (Fig. 4C). These findings confirm that the full induction of *Ctgf* stimulated by Wnt3A in these cells requires both β -catenin and activated Rho.

3.3 Activated RhoA does not stabilize cytosolic β -catenin nor affect β -catenin nuclear translocation

Since cytosolic stabilization and subsequent nuclear translocation of β -catenin is reported to be the primary mechanism by which canonical Wnts stimulate transcription [31], we assessed whether the effects of Rho on Wnt3A-stimulated transcription were due to effects on β -catenin stabilization and nuclear translocation. Accordingly, we stimulated cells with Wnt3A, isolated cytosol, and measured cytosolic β -catenin by quantitative immunoblot. As predicted, treatment with Wnt3A caused prominent cytosolic β -catenin accumulation (Fig. 5A and B). However, the presence of the Rho inhibitor C3 exoenzyme had no effect on the Wnt3A-promoted increase in β -catenin levels (Fig. 5A and B). We also determined the effects of a constitutively active RhoA on β -catenin stabilization by expressing a GTPase-deficient form of RhoA, RhoA(GV), and measured cytosolic β -catenin levels. RhoA(GV) expression did not affect cytosolic β -catenin stabilization stimulated by Wnt3A (Fig. 5A and B).

The effects of Rho activation and inhibition on nuclear accumulation of β -catenin were also assessed. As expected, Wnt3A stimulation caused accumulation of β -catenin in the nucleus (Fig. 5C and D). Pretreatment with C3 exoenzyme had no effect on this Wnt3A-stimulated nuclear accumulation (Fig. 5C and D), whereas nuclear accumulation was inhibited by overexpression of axin Δ dix, a soluble form of axin that will negatively regulate β -catenin stability when overexpressed [32], or LRP5 Δ C, a form of LRP5 that lacks the C terminal cytoplasmic domain and inhibits signaling by sequestering Wnt [33], as expected (Fig. 5E–F). Moreover, expression of the constitutively active RhoA(GV) did not induce nuclear accumulation of β -catenin. These data indicate that the Rho-mediated effects on Wnt3A-stimulated transcription are not due effects on β -catenin stabilization and nuclear translocation.

3.4 β -catenin and Rho can cooperate to regulate the transcriptional induction of Wnt3A target *Ctgf*

In addition to effects on β -catenin stabilization and localization, we also considered the possibility that RhoA may affect Wnt3A-dependent transcriptional induction by influencing specific regions of the target gene promoters. To examine this issue, we used a luciferase reporter, pCtgf, that contains ~2.1 kb from the 5' regulatory region of the human homolog of *Ctgf* [34] (Fig. 6A). Notably, in a previous study the pCtgf reporter was shown to be activated by Rho [35]. In agreement with these studies, expression of RhoA(GV) with the pCtgf reporter resulted in its robust stimulation (Fig. 6B). In addition, reporter activation was also triggered by Wnt3A treatment (Fig. 6C). Neither RhoA(GV) nor Wnt3A stimulated luciferase production from the parent vector pGL3 (data not shown).

Sequence analysis of the *Ctgf* promoter using TESS, a web-based tool to identify transcription factor binding sites (<http://www.cbil.upenn.edu/cgi-bin/tess>) [36] revealed 6 potential TCF/LEF binding sites, of which 5 are 5' to an internal MluI site in the promoter (Fig. 6A). To more precisely determine the regions of the promoter responsive to Rho and Wnt3A, we digested the reporter with MluI and re-ligated, which removed ~1.5 kb from the 5' end of the response element (pCtgf-trunc, Fig. 6A). While this truncated reporter also exhibited robust activation by RhoA(GV) (Fig. 6B), the response of pCtgf-trunc to Wnt3A was significantly diminished (Fig. 6C) relative to the full-length pCtgf. These data suggest that the Rho-dependent regulatory site is distinct from the Wnt/ β -catenin site(s) and support the hypothesis that both RhoA and β -catenin contribute in parallel to transcriptional regulation of *Ctgf* (Fig. 7).

To examine potential cooperation between Rho and β -catenin in regulating *Ctgf* transcription, we expressed activated RhoA in conjunction with a constitutively stabilized form of β -catenin that lacks the N-terminal phosphorylation site responsible for directing its degradation (β -

catenin Δ N) [17]. As illustrated in Figure 6D, expression of either RhoA(GV) or β -catenin Δ N stimulated transcription from pCtcf. Moreover, expression of β -catenin Δ N in conjunction RhoA(GV) significantly enhanced reporter activation. Collectively, these data support the hypothesis that *Ctcf* induction is regulated by both Wnt3A-stimulated β -catenin stabilization and Wnt3A-stimulated Rho activation.

4. Discussion

In this study, we show that Wnt3A-timulation of activated Rho is required for a complete β -catenin-dependent transcriptional and cellular response in C3H10T1/2 cells. In this mesenchymal stem cell line, Wnt3A-stimulated osteoblastic differentiation, a response previously demonstrated to require β -catenin [14], also requires Rho GTPase function. Furthermore, using expression microarray analysis and quantitative RT-PCR analysis, we show that Wnt3A-dependent induction of a subset of endogenous target genes is sensitive to inhibition of Rho. These novel results identify signaling convergence between Rho, which is conventionally thought of as non-canonical, and β -catenin, which is thought of as canonical. Furthermore, they imply that, for some Wnts, the distinction between these two signaling arms is less clear than formerly believed.

While the involvement of Rho in the Wnt3A transcriptional pathway is an unexpected finding, it is compatible with available data from Wnt3A knockout mice. The Wnt3A^{-/-} mice show characteristic loss of paraxial mesoderm [37]. Rho GTPases can play a role in mesoderm development, especially through their regulation of convergent extension movements that are important for appropriate mesoderm formation [38]. Thus, it is possible that these defects in the Wnt3^{-/-} mice are in part the result of a deficient Wnt3A-Rho pathway.

Another novel finding of this study is that the mechanism by which Rho functions in the Wnt3A pathway does not involve β -catenin stabilization, which is widely viewed as the primary mechanism through which Wnt signaling impacts gene expression. Inhibition of Rho by C3 exoenzyme does not affect Wnt3A-stimulated β -catenin stabilization or nuclear accumulation (Fig. 5), despite its potent inhibition of β -catenin-dependent transcription of some genes. Also, constitutively active RhoA does not impact total cytosolic or nuclear β -catenin levels (Fig. 5), despite its ability to enhance β -catenin-dependent activation of the pCtcf reporter. These results indicate that activated Rho modifies Wnt3A-stimulated, β -catenin-dependent transcription without affecting β -catenin stabilization or nuclear localization. A recent paper demonstrated that Wnt3A activation of a Rho-related GTPase, Rac1, can also positively regulate β -catenin-dependent transcription [39]. In contrast to our findings, however, that study showed that Rac1-JNK activation results in β -catenin phosphorylation and affects its nuclear localization. The idea of a regulator influencing β -catenin-dependent transcription without affecting β -catenin stabilization or localization is not without precedent, though. For example, two recent papers identified Wnt5A signaling cascades that inhibited β -catenin-dependent transcription without affecting β -catenin stabilization or localization [40,41].

Our findings suggest a model whereby Wnt3A-activated Rho potentiates the transcriptional activating capacity of stabilized β -catenin for a subset of genes (Fig. 7). Activation of Rho by Wnt3A may be mediated all or in part through the actions of Dvl (Fig. 7), which has been demonstrated previously to contribute to the stimulation of this small GTPase [10,42]. Once activated, the precise mechanism through which Rho augments β -catenin function is not clear, however, we provide evidence that the Rho effector ROCK plays a role. The data presented here also indicate that the promoter region of one of the Wnt targets contains β -catenin-independent, Rho-sensitive regulatory elements that promote transcriptional induction.

The Wnt3A-activation of Rho/ROCK not only is required for the transcription of genes that drive osteoblastic differentiation in pluripotent C3H10T1/2 mesenchymal stem cells, as we show here, but also may actively provide a block to prevent passage of these cells down other differentiation pathways, namely adipogenesis. Indeed, Wnt3A has been reported to inhibit adipogenesis in these cells [43]. Moreover, adipogenesis in 3T3-L1 pre-adipocytes is inhibited by ligand-dependent Rho activation and is significantly enhanced by inhibition of ROCK activity [44]. Thus, it is tempting to speculate that the activation of Rho/ROCK induced by Wnt3A serves as a molecular switch to stimulate passage down one differentiation pathway (osteoblastic) and to repress passage down another (adipogenic).

In addition to mesenchymal stem cells, activation of a Wnt/Rho/ROCK pathway may be an important molecular switch governing cell fate decisions in other types of stem cells. For example, Wnts are known to play an important role in embryonic stem (ES) cell survival and pluripotency [45]. Notably, several recent reports have shown that human and monkey ES cells treated with the ROCK inhibitor, Y-27632, display a marked reduction in cell death after dissociation and an increase in maintenance of pluripotency [46–49]. Thus, it may be that a Wnt/Rho/Rock pathway influences cell fate decisions in ES cells as well. While we have identified Wnt3A-activated, Rho-sensitive genes in mesenchymal stem cells important for osteoblastic differentiation, it is possible that different cells/tissues will exhibit distinct subsets of Rho-sensitive Wnt targets and/or differing abilities to activate Rho in response to Wnt. This scenario provides a potential mechanism for allowing distinct cellular responses to the same Wnt ligand.

The placement of Rho as an important component in Wnt3A-stimulated β -catenin-dependent signaling has intriguing implications for possible cross-regulation of the pathway, not only in stem cells, but other cell types as well. This potential crosstalk may have particular relevance in disease states, especially cancer, that include or require aberrant Wnt signaling. Elevated Wnt activity is observed in a number of human cancers [3,4], and Rho activation has been linked to advancing stage and grade of many types of cancer [50]. It is possible that amplification of Wnt signals provides a mechanism through which Rho can modulate tumor behavior, in addition to its well-characterized effect on cell motility. In this regard, it is notable that lysophosphatidic acid (LPA), an agent that triggers Rho activation, recently has been demonstrated to impact cell proliferation in colon cancers in a fashion dependent upon the β -catenin signaling pathway [51]. A detailed understanding of the mechanisms of canonical Wnt signaling is required to unravel the complexities of the pathway, including its cross-regulation. The identification of Rho as a positive mediator of the pathway may provide an important link to other signaling networks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard abbreviations

CM, conditioned media; Ctgf, connective tissue growth factor; Dvl, dishevelled; FBS, fetal bovine serum; Fzd, frizzled; GST, glutathione S-transferase; LEF, lymphoid enhancing factor; PBS, phosphate buffered saline; PCP, planar cell polarity; TCF, T cell factor.

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Figure 1.

Characterization of Wnt3A signaling in C3H10T1/2 cells. **(A)** C3H10T1/2 cells were grown to 75% confluence in 6-well dishes and then treated with Wnt3A conditioned media (CM) or control CM for 3 h prior to lysis and isolation of the cytosolic fractions. 10 μ g of protein from these fractions were subjected to SDS-PAGE and immunoblot analysis to detect endogenous β -catenin. The data is from a single experiment that is representative of at least five experiments. **(B)** C3H10T1/2 cells were grown to 75% confluence and transfected with Super8X TOPFlash and phRG internal control reporter. 5 h after transfection, cells were treated with control CM or Wnt3A-M and incubated a further 18 h prior to lysis and measurement of luciferase activity. The data shown are the mean and standard error from four separate

determinations in a single experiment and are representative of at least four experiments. (C) C3H10T1/2 cells were grown to 75% confluence in 10 cm plates, starved overnight, and then treated with 100 ng/ml recombinant purified Wnt3A or vehicle alone for 5 minutes. Activated RhoA was isolated using GST-rhotekin pull down assay. The immunoblot is representative of two independent experiments and the data shown in the graph represent the mean and standard error of these experiments.

Figure 2.

Inhibition of Rho by C3 exoenzyme blocks Wnt3A-stimulated differentiation of C3H10T1/2 mesenchymal stem cells. **(A)** C3H10T1/2 cells were treated either with 2.5 mg/ml C3 exoenzyme in the presence of 20 U/ml tetanolysin (for cellular membrane permeabilization) or with tetanolysin alone (non-C3-treated cells). Following this pretreatment, cells were incubated with Wnt3A CM or control conditioned CM as indicated and then fixed and stained as described in *Materials and Methods*. A representative image for each condition is shown. **(B)** C3H10T1/2 cells were treated either with C3/ tetanolysin or tetanolysin alone (non-C3-treated cells) for 3 h prior to incubation with control CM or Wnt3A CM for a further 96 h incubation. Alkaline phosphatase activity was measured as described in *Materials and*

Methods and is reported as arbitrary units/ μ g total protein. The data shown are the mean and standard error from three separate determinations from a single experiment and are representative of at least 4 different experiments.

Figure 3.

Expression microarray analysis of Wnt3A-stimulated C3H10T1/2 cells reveals subset of Rho-sensitive genes. C3H10T1/2 cells were grown to 75% confluence in 10 cm plates and treated either with C3/tetanolysin or tetanolysin alone followed by a further incubation in either control CM or Wnt3A CM for 24 h as described in *Materials and Methods*. RNA was then harvested, and cDNA was generated, labeled, and hybridized to custom microarrays. **(A)** Venn diagram indicating total number of gene probes detectable with signal above background under all conditions (outer circle), the number from that group showing ≥ 2 -fold increase in signal in the Wnt3A-treated group relative to control (middle circle), and the number from the Wnt3A-stimulated group showing sensitivity to C3 exoenzyme, defined as Wnt3A fold-induction $\geq 1.5\times$ induction of the same probe following treatment with Wnt3A plus C3. **(B)** For each of the 337 probes showing induction by Wnt3A, the fold-induction in the presence of Wnt3A is plotted versus fold-induction with Wnt3A + C3. The outer bold lines represent where Wnt3A induction would be either 1.5 times induction with Wnt3A + C3 or 1.5 times less; the middle thin line represents Wnt3A-stimulated genes that are unaffected by C3 treatment. The Rho sensitive gene probes are represented by open circles (o); the Rho-insensitive gene probes are represented by closed circles (•). The data are representative of two independent experiments. **(C)** Genes represented by probes (62 genes total) showing sensitivity to C3 were classified into the functional groups indicated in the figure based on the individual gene ontology entry for

each gene. The percent of the total Rho-sensitive genes in each functional category is listed below the category. **(D)** C3H10T1/2 cells were grown to 75% confluence in 6-well dishes and treated either with C3/tetanolysin or tetanolysin alone, followed by further incubation with 100 ng/ml purified Wnt3A or vehicle for 24 h. RNA was harvested and quantitative RT-PCR was performed using primers for the genes designated in the figure as described in *Materials and Methods*. Results were calculated as fold induction relative to the reference gene (*Gapdh*) and normalized, with 100% defined as maximal induction by Wnt3A, which was typically 7, 3, and 5 for *Ctgf*, *Ned4*, and *Igfbp4*, respectively. The data represent the mean and standard error of two independent experiments.

Figure 4.

Induction of the endogenous Wnt3A target gene, *Ctgf*, is blocked by inhibition of both TCF/LEF signaling and Rho activation. **(A and B)** C3H10T1/2 cells were transfected with either empty vector, DN-TCF4, or p190RhoGAP constructs as indicated. 5 h after transfection, cells were treated with control CM or Wnt3A CM and incubated for an additional 24 h. Cells were then harvested, RNA was extracted, and quantitative RT-PCR was performed for each condition using primers specific for *Ctgf*, as described in *Materials and Methods*. **(C)** C3H10T1/2 cells were treated for 24 h in control CM or Wnt3A CM plus either 10 μ M Y-27632 or DMSO vehicle, as indicated. Quantitative RT-PCR was performed as above. All results are reported as percentage of Wnt3A CM stimulated induction in the presence of vector (A & B) or vehicle (C), relative to the reference gene (*Gapdh*). One hundred percent induction was typically ~7–10 fold. The data represent the mean and standard error from three separate determinations and are representative of at least 3 independent experiments (A and C) or 2 independent experiments (B).

Figure 5.

RhoA activation or inhibition does not affect cytoplasmic stabilization or nuclear translocation of β -catenin. **(A)** Cells were transfected with the constructs indicated in B, as described in *Materials and Methods*. After 24 h, cells were treated with control CM or Wnt3A CM for 4 h prior to lysis and isolation of the cytosolic fractions. 10 μ g of protein from these fractions were subjected to SDS-PAGE and immunoblot analysis to detect endogenous β -catenin. **(B)** Cytosolic β -catenin detected by immunoblot in panel A was quantified using the Odyssey® system from Li-Cor as described in *Materials and Methods*. Results shown are normalized to RhoGDI, a protein loading control, with 100% defined as the stabilization after Wnt3A treatment. The data are the mean and standard error from three separate determinations from a single experiment and are representative of at least three experiments. **(C)** Cells were transfected with the vectors indicated in D, then, after 5 h, treated as indicated with control CM or Wnt3A CM for an additional 16 h. Cell nuclei were isolated then extracted in high salt buffer as described in *Materials and Methods*. 25 μ g of protein from each of the indicated nuclear fractions were analyzed by SDS-PAGE and subsequent immunoblot to detect endogenous β -catenin. **(D)** Nuclear β -catenin detected by immunoblot in panel C was quantified and normalized to CREB levels as in (B) and are the mean and standard error from three separate determinations from a single experiment and are representative of at least three experiments. **(E)** Cells were transfected with the constructs indicated in F, then, after 5 h, treated as indicated with control CM or Wnt3A CM for an additional 16 h. Cell nuclei were isolated then extracted in high salt buffer as described in *Materials and Methods*. 25 μ g of protein from each of the indicated nuclear fractions was analyzed by SDS-PAGE and subsequent immunoblot to detect endogenous β -catenin. **(F)** Nuclear β -catenin was quantified as in (D) and the data represent the mean and standard error from two separate experiments.

Figure 6.

Isolating the RhoA sensitive element in the *Ctgf* promoter. **(A)** Diagram representing the pCtgf reporter (top) and the truncated reporter (pCtgf-trunc, bottom), from which ~1.5 kb of the hCtgf regulatory element was removed by MluI digestion, as described in *Materials and Methods*. The arrowheads represent the approximate position of TCF/LEF binding sites (see text). **(B)** Cells were transfected with the pCtgf or pCtgf-trunc reporters, phRG internal control reporter, and RhoA(GV) constructs as indicated in the figure, lysed 18 h later, and luciferase activity was measured as described in *Materials and Methods*. Results are reported as fold change relative to vector. The data shown are the mean and standard error from four separate determinations in a single experiment and are representative of at least six independent experiments. **(C)** Cells were transfected with the pCtgf or pCtgf-trunc reporters, along with phRG internal control reporter. 5 h after transfection, cells were incubated with control CM or Wnt3A CM for 18 h prior to lysis and the measurement of luciferase activity. Results are reported as fold change relative to control CM treatment. The data shown are the mean and standard error from seven separate determinations. * $P < 0.03$ **(D)** Cells were co-transfected with the pCtgf reporter, phRG internal control reporter and the indicated constructs, lysed 18 h later, and luciferase activity was measured as described above. Luciferase results are reported as RLU as described in *Materials and Methods*. Western blots were performed on lysates used

to measure luciferase activity. The data are the mean and standard error from six separate determinations from a single experiment and are representative of at least two independent experiments. ** $P < 0.0001$

Figure 7.

Contribution of Rho to canonical Wnt3A signaling. Cytosolic β -catenin is continuously degraded in unstimulated cells via a process initiated by its binding to a complex of proteins that includes axin, the adenomatous polyposis coli (APC) protein and glycogen synthase kinase 3 β (GSK3 β), termed the β -catenin destruction complex. This complex interacts with β -catenin to induce its phosphorylation, which targets it for ubiquitination and degradation by the proteasome. Wnt binding by Fzd and a co-receptor, LRP5/6, activates a positive regulator of the pathway, disheveled (Dvl), which in turn inactivates the destruction complex and allows the accumulation of cytoplasmic β -catenin [52]. Stabilized β -catenin can then shuttle into the nucleus and bind to members of the T-cell factor (TCF)/lymphoid enhancing factor (LEF)

family of transcription factors to induce expression of target genes [53]. Wnt3A binding to Fzd and LRP5/6 also leads to the activation of RhoA GTPase in a manner that depends, at least in part, upon Dvl and possibly other pathways yet to be defined. Our results show that activated RhoA, through the stimulation of ROCK, potentiates the β -catenin-dependent induction of target genes via unknown intermediates indicated by the dashed line. The full activation of these genes, which comprise a subset of the β -catenin targets, is dependent on both β -catenin and Rho and is required for Wnt3A-driven cellular consequences, including osteoblastic differentiation.

Table 1

Partial list of target genes induced by Wnt3A in C3H10T1/2 cells.

2–3 fold induction	3–5 fold induction	>5 fold induction
<p> <i>Acpl2 Actb Adpgk Ahcy11 Alcam Ambn Ankrd29 Apbb1ip</i> <i>Apob48r Arntl Asf1b Aurka Bcat1 Bdkrb1 Bub1 C1qtnf1 Cald1</i> <i>Cav2 Ccdc80 Ccna2 Ccnb1 Ccnd1 Cdca3 Cdca5 Cdca8 Cenpa</i> <i>Ckap2l Cks2 Cnn2 Col12a1 Coq10b Cox4i2 Csrp2 Ctps Cx43</i> <i>Cyp39a1 Deblid2 Dusp14 Ect2 Ehbp1 Epdr1 Etfdh Fat1 Fbn2 Fosl2</i> <i>Fscn1 Fzd1 Gadd45 Golt1b Gprasp1 Gpsm1 Grk5 H2an H2afx</i> <i>Herpud1 Hip1 Hist1h3g Hist2h4 Hspb1 Hsd17b12 Htra1 Id3 Ier3</i> <i>Ier5 Ilk Incenp Inf2 Irak2 Irx5 Jub Kank2 Kifc3 Kpna2 Lars Lmnb1</i> <i>Lrrc58 Lrrk1 Lox Mad2l1 March3 Mcm5 Metrn1 Mthfd2 Mustn1</i> <i>Mxd3 Ncapg Ncaph Nedd9 Neil3 Npy1r P2ry5 P4ha2 Pbk Pde5a</i> <i>Pdlim5 Pdlim7 Pfkp Pkmyt1 Plat Plk1 Plk4 Pvr13 Prc1 Pvr13 Prx1</i> <i>Ppp3r1 Prr11 Psat1 Pttg1 Pvr13 Racgap1 Radil Rdh10 Reck Rho8</i> <i>Rock2 Rplp1 Rraga Samhd1 Scyl1bp1 Sec24a Shcgp1 Slc6a9</i> <i>Slc20a2 Slit2 Smc2 Snta1 Spc24 Stambpl1 Steap2 Stk12 Svit Sulf1</i> <i>Tagln2 Tera Tes Tk1 Tmeff1 Tmpo Tmsb4x Tnfrsf11b Trb3 Ttyh3</i> <i>Tubb6 Twist1 Vldlr Wisp1 Xpo7 Zmiz1</i> </p>	<p> <i>Acta2 Anxa1 Anxa3 Bok Bpgm Cav1</i> <i>Ccdc32 Cdkn3 Col8a1 Ddah1 Dpysl3</i> <i>Dusp1 Emd Enpp2 Fam26e Fhl1 Fhl3</i> <i>Flrt2 Foxd1 Grb14 Hes1 Hspb6</i> <i>Hist1h1b Klhdc8a Lmcd1 Klj5 Myl9</i> <i>Mtap Npr3 Olfm13 Palld Pcsk6 Pdgfc</i> <i>Plekh2 Pls3 Plxna2 Pmp2 Prx2</i> <i>Rapgef3 Rel11 Slc1a5 Slc1a6 Slc7a5</i> <i>Slit2 Smad6 Smad7 Smurf2 Sorbs1</i> <i>Stk17b Thbs1 Thbs2 Timp3 Tnfrsf12a</i> <i>Top2a Tpm1 Tpm4 Wisp2</i> </p>	<p> <i>Acta3 Aldh1a7 Ahr Bmp4</i> <i>Ccdc11 Ccny Cdkn2b</i> <i>Ctgf Cyr61 Fabp5 Fgf18</i> <i>Igfbp2 Igfbp4 Nedd4</i> <i>Nkd1 Nsg1 Omd Tgfb3</i> <i>Timp1 Tll5</i> </p>

nal classification of Rho-sensitive Wnt3a target genes.

Growth	Cell Signaling	Cytoskeleton	Bone/Skeletal Development & Differentiation	Cell Cycle	Transport	Transcription	Other	Nuclear	Muscle	Development & Morphogenesis
Myo2 Myo11 Myo19 Myo20 Myo21 Myo22 Myo23 Myo24 Myo25 Myo26 Myo27 Myo28 Myo29 Myo30 Myo31 Myo32 Myo33 Myo34 Myo35 Myo36 Myo37 Myo38 Myo39 Myo40 Myo41 Myo42 Myo43 Myo44 Myo45 Myo46 Myo47 Myo48 Myo49 Myo50 Myo51 Myo52 Myo53 Myo54 Myo55 Myo56 Myo57 Myo58 Myo59 Myo60 Myo61 Myo62 Myo63 Myo64 Myo65 Myo66 Myo67 Myo68 Myo69 Myo70 Myo71 Myo72 Myo73 Myo74 Myo75 Myo76 Myo77 Myo78 Myo79 Myo80 Myo81 Myo82 Myo83 Myo84 Myo85 Myo86 Myo87 Myo88 Myo89 Myo90 Myo91 Myo92 Myo93 Myo94 Myo95 Myo96 Myo97 Myo98 Myo99 Myo100	Ankrd29 Ccdc11 Gpsm1 Nkx1 Npy1r Rraga Trb3	Acta2 Actg2 Acta3 Elbbp1 Fat1 Tull5	Col12a1 Ctgf Fgf18 Npr3 Omd Trnfrsf11b	Ccnb1 Ect2 Nedd9 Plkl1 Tgfb3	Chac1 Mxi1 Slc1a6 Slc7a5	Hes1 Klf5 Lmcd1 Trtb3	Nei3 Rel1 Serpine1 Thbs1	Hist1h3g Lmnb1 Tmpo	Myl9 Tpm1	Dpysl3 Plxna2