Regulated subset of G₁ growth-control genes in response to derepression by the Wnt pathway


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Pitx2 is a bicoid-related homeodomain factor that is required for effective cell type-specific proliferation directly activating a specific growth-regulating gene cyclin D2. Here, we report that Pitx2, in response to the Wnt/β-catenin pathway and growth signals, also can regulate c-Myc and cyclin D1. Investigation of molecular mechanisms required for Pitx2-dependent proliferation, in these cases, further supports a nuclear role for β-catenin in preventing the histone deacetylase 1-dependent inhibitory functions of several DNA-binding transcriptional repressors, potentially including E2F4/p130 pocket protein inhibitory complex, as well as lymphoid enhancer factor 1 and Pitx2, by dismissal of histone deacetylase 1 and loss of its enzymatic activity. Thus, β-catenin plays a signaling-integrating role in Wnt- and growth factor-dependent proliferation events in mammalian development by both derepressing several classes of repressors and by activating Pitx2, regulating the activity of several growth control genes.

The Wnt pathway induces various cellular responses from cell proliferation to cell fate determination and terminal differentiation (1–3). The binding of Wnt ligands to receptors activates intracellular Dishevelled (Dsh in Drosophila, Dvl in vertebrates), which, in turn, modulates the activity of the GSK-3β signals. In the absence of Wnt signaling, β-catenin (armadillo in Drosophila) is found in a multiprotein complex containing adenosomatous polyposis coli and axin, targeting β-catenin for degradation. Activation of Dsh/Dvl leads to the stabilization and accumulation of β-catenin in the cytoplasm. On translocation, β-catenin interacts with members of the T cell factor (TCF)/lymphoid enhancer factor (LEF) family of DNA-binding molecules to influence target gene expression (4, 5), although recent evidence in Drosophila indicates that β-catenin can regulate gene transcription by selective nuclear export of regulatory proteins (6).

β-Catenin is proposed to bind to the TCF/LEF family of transcription factors, changing them from repressors to activators of transcription (7, 8). In part, because the expression patterns of the known TCF/LEFs probably are not broad enough to explain all of the activities of the Wnts expressed in the development, we investigated whether β-catenin may bind to other tissue-restricted transcription factors to modulate specific aspects of Wnt signaling.

Recently, we reported that a Wnt/β-catenin → Pitx2 pathway operates in several specific tissues to control proliferation by regulating expression of cyclin D2 gene in G₁ (9). The Wnt pathway directly induces Pitx2 and, with additional growth factor-dependent signaling, dismisses Pitx2-associated corepressors and mediates a temporarily specific, sequential recruitment of specific coactivator complexes, which includes a factor Ldb1/NL1/CLIM, previously identified as a coactivator of LIM homeodomain factors (10, 11), and Tip60, a member of the MYST family of coactivators (12, 13), required for activation of the E2F-independent G₁ growth regulatory target gene Cyclin D2.

Pitx2 is a member of a subfamily of bicoid-related factors (14, 15). Pitx2, expressed in several tissues, initially was identified as one of the genes responsible for the human Reiger syndrome as well as occasional abnormal cardiac and pituitary development (16).

Here, we report that Pitx2 can regulate additional G₁ cell cycle control genes including c-Myc and cyclin D1. We find that β-catenin not only reverses the repressive function of LEF1 and Pitx2 but also can overcome the repressive actions of the E2F4-related pocket protein p130 and histone deacetylase 1 (HDAC1), potentially providing an additional component in mediating Wnt-dependent growth and developmental programs. Together, these results suggest that the nuclear response to Wnt-dependent signaling events in target tissues involves a specific antirepressive role for β-catenin in integrating the transcriptional response as well as an activation role of Pitx2.

Materials and Methods

Antibodies. Anti-Pitx2 IgG was generated from guinea pigs (9). The following antibodies were obtained from Santa Cruz Biotechnology: anti-β-catenin, HDAC1, HDAC2, Tip60, TCFs, and LEF-1. Antiacetylated histone H3 and H4 antibodies were from Upstate Biotechnology (Lake Placid, NY), and anti-RNA polymerase II antibody was from Berkeley Antibody (Richmond, CA).

Immunoprecipitations and HDAC Enzymatic Assays. Coimmunoprecipitation and other protein-interaction studies were performed as described (12). Fluorogenic histone deacetylase substrate [Boc-Lys(Ac)-AMC] was purchased from Calbiochem. From immunoprecipitates, HDAC enzymatic assay was done by using fluorogenic HDAC substrate as described (17).

Single-Cell Nuclear Microinjection Assays. Microinjection assays were carried out as described (9). Affinity-purified αPitx2 IgG was used. Each experiment was performed on three independent coverslips consisting of 1,000 cells. Where no experimental antibody was used, preimmune IgGs were coinjected, allowing the unambiguous identification of injected cells in addition to serving as a negative control.

Chromatin Immunoprecipitation (ChIP) Assays. For the ChIP assay, αT-Tα, a murine pituitary cell line, and C2C12 myoblast cell line were used, and LiCl (10 mM) was added for 1 h before harvest. Cells were washed twice with PBS and cross-linked with 1% formaldehyde for 10 min at room temperature. Cross-linked cells were treated as described (12). Cells then were resuspended in 0.3 ml of lysis buffer (50 mM Tris-HCl, pH 8.1/1% SDS/10 mM EDTA/protease inhibitors) and sonicated three times for 10 sec followed by centrifugation for 10 min. The average size of sheared fragments was expected to be ~300–1,000 bp. Immuno-

Abbreviations: LEF, lymphoid enhancer factor; TCF, T cell factor; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; N-cor, nuclear receptor corepressor; β-catenin, constitutively active β-catenin; RAR, retinoic acid receptor.

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occupied by the activators E2F1 and Ets2 (21). In contrast, in cells where the tors or repressors (20). For example, in proliferating myeloid cells where the c-Myc gene is expressed, the c-Myc promoter is occupied by the activators E2F1 and Ets2 (21). In contrast, in

terminally differentiated cells in which the c-Myc gene is repressed, the c-Myc promoter instead is occupied by the E2F repressor E2F4, the cell type-restricted ets repressor METS, and the E2F4-associated pocket proteins p107 and p130 that function as cell cycle-dependent coresspressors (22, 23). Furthermore, previous studies of the human c-Myc promoter suggested that it is a target of the Wnt pathway, and it contains upstream functional TCF/LEF-binding sites (24).

Indeed, ChIP analysis with primers that span the E2F sites and the adjacent Pitx2 sites revealed that Pitx2 was bound to c-Myc regulatory sequences in C2C12 cells (Fig. 1B). As expected, some HDAC1 also was present on the promoter under these cultures (Fig. 1B). In addition, binding of LEF1, but not TCF1, TCF3, or TCF4, could be detected further 5′ in the c-Myc promoter (data not shown), consistent with previous suggestions of c-Myc as a Wnt-regulated gene (24). Therefore, Pitx2, as well as LEF1, binds to the c-Myc promoter in specific cell types and may participate in the recruitment of β-catenin and the dismissal of HDAC1 upon activation of the Wnt pathway. In addition, in transient cotransfection assays in αT3–1 pituitary cells or C2C12 myoblast cells, Pitx2 could stimulate the c-Myc promoter (−1,100/+500), −2- to 2.5-fold, comparable to the effects of addition of lithium, requiring the presence of the three defined Pitx2 sites (data not shown). When the single-cell nuclear microinjection assay with the c-Myc reporter was used, serum-dependent stimulation was abolished by αPitx2 IgG in C2C12 cells (Fig. 1C). These data led us to further investigate the potential interactions and roles of β-catenin in the action of Pitx2 and determine whether a more general antirepressor function of β-catenin might serve functions in growth stimulation by the Wnt pathway.

**Linkage of β-Catenin to HDAC1 and Nuclear Receptor Corepressor (N-CoR) Repressive Actions.** Many homeodomain activators have been shown to serve as repressors dependent on promoter
context and culture conditions, based on their interactions with a specific corepressor (25). For example, Pit-1 can recruit, directly or indirectly, an N-CoR-containing corepressor that is required for cell type-specific gene restriction events (26, 27). Consistent with these findings, Pitx2 or a Gal4/Pitx2 fusion protein can function as a repressor on a Pitx2 RE/tk or a UAS/tk promoter (Fig. 2A and data not shown), even though it is clearly an activator on many promoters (28). Based on the ability of the Wnt pathway to reverse TCF/LEF-dependent repression, we evaluated whether activation of this pathway could reverse Pitx2-dependent repression.

We found that either lithium or constitutively active β-catenin (β-catenin) caused repression in MEFs from N-CoR mice (Fig. 2B), a dominant-negative form of Pitx2 (9) that is capable of disrupting N-CoR or HDAC complexes (29), by using an IgG against HDAC1 or HDAC2, but not HDAC3, HDAC4, HDAC5, or HDAC6, and, to a lesser extent, αN-CoR IgGs relieved repression by Gal4/Pitx2 in a single-cell nuclear microinjection assay in Rat1 cells. (B) N-CoR and β-catenin interactions. Based on isolation of β-catenin in a yeast two-hybrid screen with RDIII of N-CoR, immunoprecipitation experiments were performed in HCT116 colorectal tumor cells, revealing robust interactions, by using specific α-catenin and αN-CoR (Upper). Commonly, a common repressor domain III of protein can function as a repressor on a Pitx2 RE promoter (Fig. 2A). N-CoR and HDAC levels were equivalent in all immunoprecipitates, as assessed by using Flag-IgG. (E and F) Failure of β-catenin to reverse β-RAR- or Pit-1-dependent repression. Gal4/Pit-1 repression or β-RAR repression on a DR5-dependent lacZ reporter could not be overcome by expression of β-catenin, or administration of lithium. αN-CoR reversed both β-RAR- and Pit-1-dependent repression. All specific cell nuclear microinjection experiments are mean ± SEM with >300 cells microinjected, and experiments were repeated independently a minimum of three times.

Fig. 2. Dismissal of HDAC activity by β-catenin. (A) Cointegration of specific IgGs against HDAC1 or HDAC2, but not HDAC3, HDAC4, HDAC5, or HDAC6, and, to a lesser extent, αN-CoR IgGs relieves repression by Gal4/Pitx2 in a single-cell nuclear microinjection assay in Rat1 cells. (B) N-CoR and β-catenin interactions. Based on isolation of β-catenin in a yeast two-hybrid screen with RDIII of N-CoR, immunoprecipitation experiments were performed in HCT116 colorectal tumor cells, revealing robust interactions, by using specific α-catenin and αN-CoR (Upper). Commonly, a common repressor domain III of protein can function as a repressor on a Pitx2 RE promoter (Fig. 2A).
and HDAC5 were not affected (Fig. 2D). Interestingly, the ability of β-catenin to inhibit the associated deacetylase activity of HDAC1, as well as N-CoR, is consistent with the actions of αHDAC1 and α-N-CoR to potentially reverse Pitx2-dependent repression.

We did not observe reversal of the repressive actions of Pit-1 or unliganded retinoic acid receptor (RAR) by LiCl or β-catenin, in similar nuclear microinjection assays (Fig. 2E and F), whereas α-N-CoR did reverse repression, revealing specificity of the antirepressor action of β-catenin, based on recruitment to Pitx2 but not to Pit-1 or RAR.

**β-Catenin Causes a Dismissal of HDAC1 Associated with Pocket Proteins from the c-Myc Promoter.** Based on the complex regulation between E2F factor exchanges and the known association of HDAC1 with p130 (31), we next evaluated the potential association of E2F1 or E2F4 with β-catenin. Interestingly, communoprecipitation assays revealed a robust, selective interaction between β-catenin and E2F4, whereas little if any interaction was observed between β-catenin and E2F1 (Fig. 3A). Furthermore, the presence of β-catenin virtually eliminated the deacetylase activity normally associated with the immunoprecipitated E2F4/p130 complex, because communoprecipitation of E2F4, p107, or p130 in the presence of β-catenin, caused a striking inhibition of associated enzymatic activity (Fig. 3B and C). If HDAC1 enzymatic activity is a critical component of E2F4-dependent repression, then αHDAC1 IgG might be expected to increase expression of αMyc promoter-driven reporters. Indeed, under serum-free conditions for 48 h in C2C12 cells, we found that nuclear microinjection of αHDAC1, but not α-N-CoR, caused a stimulation of the c-Myc promoter-driven reporter similar to that obtained with serum (Fig. 3D).

Therefore, we wanted to determine whether β-catenin caused a dismissal of HDAC1 or p130 from the c-Myc promoter in response to activation of the Wnt/β-catenin pathway. To evaluate this issue, the effects of activation of the β-catenin pathway were evaluated in C2C12 cells by using the ChIP assay. C2C12 cells were placed under serum-free conditions for 48 h to synchronize cells, and then either serum or lithium or both were added for 1–16 h before harvest for ChIP assay. Consistent with serum-dependent regulation of Pitx2 gene expression in C2C12 cells, Pitx2 was not detected at 1 h on the c-Myc promoter in C2C12 cells under the serum-free conditions (Upper) but was detected in serum-treated cells (Lower). Under both conditions, E2F4, p130, and HDAC1, but not β-catenin, were detected. However, 1 h after lithium addition, HDAC1 was no longer detected and β-catenin was present on the promoter.

Regulation of Cyclin D1 and Cyclin D2 by the Wnt Pathway in Human Colon Cancer Cells. Regulation of cyclin D1 by the Wnt/β-catenin pathway has been reported in human colon cancer cells based on...
the presence of LEF/TCF sites in the promoter (32), raising the question of which cell-specific factor might operate in these cells. In response to lithium alone or lithium and serum, β-catenin is recruited to the cyclin D1 promoter (data not shown). However, we found that Pitx2 is expressed robustly in human colon cancer cells, consistent with its expression in developing colon and block of colon development in Pitx2−/− mice (data not shown). We find that Pitx2 is recruited to both the cyclin D2 and cyclin D1 promoters in colon cancer cells (HCT116) but that, in these cells, in contrast to C2C12 cells, the cyclin D1 promoter binds LEF1 (Fig. 4 A and B), consistent with previous reports (18). Antibodies against Pitx2, coactivators of Pitx2 including CBP/p300 and Tip60 (9), or Gal4/Pitx2-N′ block the cyclin D2 activation in these cells from the single-cell nuclear microinjection assay, indicating that Pitx2 again seems to serve as a cell-specific factor involved in G1 progression (Fig. 4C), as is the case for pituitary and muscle we reported recently (9).

Discussion

In this article, we have probed further the molecular mechanism by which a cell-restricted DNA-binding transcription factor, Pitx2, serves as an important modulator of growth control genes, indicating that c-Myc and cyclin D1 as well as cyclin D2 can serve as target genes. Using well studied C2C12 muscle cell and αT3-1 pituitary cell lines, we suggested that inhibitory E2F4, Pitx2, and LEF1 might all serve as synergistic regulated repressors that, upon binding β-catenin, have dismissal of corepressors including HDAC1.

Investigation of Pitx2-dependent gene regulation has revealed an additional level of control of Pitx2 by the Wnt pathway that may represent its effects on cellular proliferation events. As is the case for many homeodomain factors, Pitx2 can act alternatively as a repressor and/or activator depending on promoter context or cell type. The Wnt pathway not only induces Pitx2 gene expression via β-catenin pathway as we reported (9) but also reverses its ability to function as a repressor. This effect is conferred, in part, by actions of HDAC1 and, possibly to some extent, by N-CoR. These data are consistent with the hypothesis that β-catenin can modulate Pitx2 repressor function based on direct interactions, causing both dis-