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Identification of a Wnt/Dvl/β-Catenin → Pitx2 Pathway Mediating Cell-Type-Specific Proliferation during Development

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Summary

Understanding the cell type-specific molecular mechanisms by which distinct signaling pathways combinatorially control proliferation during organogenesis is a central issue in development and disease. Here, we report that the bicoid-related transcription factor Pitx2 is rapidly induced by the Wnt/Dvl/β-catenin pathway and is required for effective cell-type-specific proliferation by directly activating specific growth-regulating genes. Regulated exchange of HDAC1/β-catenin converts Pitx2 from repressor to activator, analogous to control of TCF/LEF1. Pitx2 then serves as a competence factor required for the temporally ordered and growth factor-dependent recruitment of a series of specific coactivator complexes that prove necessary for *Cyclin D2* gene induction. The molecular strategy underlying interactions between the Wnt and growth factor-dependent signaling pathways in cardiac outflow tract and pituitary proliferation is likely to be prototypic of cell-specific proliferation strategies in other tissues.

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Introduction

Coordinated transcriptional regulation of cell proliferation, in addition to differentiation, is a central feature in development of all organs. Many tissue-restricted transcription factors mediate crucial steps in organogenesis, often functioning as the distal targets of regulatory signaling pathways. Organogenesis is controlled by families of signaling molecules including the Wnts, Sonic Hedgehog, BMPs, and FGFs, that control sequential proliferation and determination events (Hogan, 1996; Cadigan and Nusse, 1997; Martin, 1998). The downstream effects of the signaling pathways are modulated by the preprogramming of target cells, particularly the complement of transcription factors and coregulators present in each cell. Thus, it becomes important to determine roles for tissue-restricted factors in modulating specific aspects of general signaling pathways, particularly their relationship to proliferative events.

Signaling molecules of the Wnt family induce various cellular responses from cell proliferation to cell fate determination and terminal differentiation (Cadigan and Nusse, 1997; Ikeya et al., 1997). Wnt ligands bind to the Frizzled family of seven-transmembrane domain receptors) and coreceptors of the LRP/arrow family. This binding activates intracellular Dishevelled (Dvl), which in turn modulates the activity of the serine-threonine protein kinase glycogen synthase kinase 3β (GSK-3β). In the absence of Wnt signaling, β-catenin (*armadillo* in *Drosophila*) is found in a multi-protein complex containing adenomatous polyposis coli (APC), and axin, targeting β-catenin for ubiquitin-dependent degradation. Activation of Dvl inhibits GSK-3β within this complex leading to the stabilization and accumulation of β-catenin in the cytoplasm. Upon nuclear translocation, β-catenin interacts with members of the TCF/LEF family of DNA binding molecules to influence target gene expression (Boutros and Mlodzik, 1999). In addition to its role in mediating the classical Wnt pathway, *Drosophila* Dsh participates in planar cell polarity (Heslip et al., 1997) and Notch signaling pathways (Axelrod et al., 1996). Three highly related *Dvl* genes and proteins have been isolated in mice and humans, and their expression patterns overlapped significantly during mouse development (reviewed in Hamblet et al., 2002).

β-catenin is proposed to bind to the TCF/LEF family of transcription factors, changing them from repressors to activators of transcription primarily by displacing the groucho/TLE corepressor (Fisher and Caudy, 1998). However, given the expression patterns of the known TCF/LEFs, β-catenin may additionally bind to other tissue-restricted transcription factors to modulate specific aspects of Wnt signaling. A subset of organs where the Wnt signals provide critical regulation express a subfamily of *bicoid*-related factors, consisting of three *Pitx* factors that exert distinct roles in development. One factor, *Pitx2*, expressed in several tissues developmentally regulated by the Wnt pathway, was identified as one of the genes responsible for the human Rieger syndrome, an autosomal dominant condition (Semina

et al., 1996). *Pitx2*-deficient mice are characterized by failure of body-wall closure, arrest in turning, ocular defects, right pulmonary isomerism, altered cardiac position with valvular and atrial septation defects, and a block in early determination events in anterior pituitary gland and tooth organogenesis (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999).

In this manuscript, we report that induction of tissue-restricted competence factors, exemplified by Pitx2, acting in combination with general growth factor signaling pathways to mediate cell type-specific control of proliferation. The Wnt/Dvl/β-catenin→Pitx2 pathway operates in specific tissues to control proliferation by regulating expression of critical cell cycle control genes in G1. The Wnt pathway directly induces *Pitx2* and additional growth factor-dependent signaling results in dismissal of Pitx2-associated corepressors and mediates recruitment of specific coactivator complexes to growth regulatory target genes such as *Cyclin D2*.

Results

A Wnt/Dvl2/β-Catenin → *Pitx2* Pathway Is Essential for Cardiac Outflow Tract Development

In addition to the previously described nonseptated atrium and valvular deficiencies (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999), we noted that the *Pitx2*$^{-/-}$ mice that survive up to e14, invariantly exhibit major cardiac outflow tract abnormalities, which are best classified as persistent truncus arteriosus abnormalities (PTA), (~70%) and incomplete septation of the great arteries (~30%) that may develop with double outlet right ventricle (DORV) or transposition of the great arteries (TGA) (Figure 1A). Deletion of the *Dvl2* gene (Hamblet et al., 2002) resulted in a cardiac outflow tract phenotype similar to that of the *Pitx2*$^{-/-}$ mouse (Figure 1B). Thus, PTA, DORV, and TGA are observed in >60% of *Dvl2*$^{-/-}$ mice, shown in Figure 1B, serving as the cause of death. In the *Dvl2*$^{-/-}$ mice, there was a marked decrease in *Pitx2* expression in a population of cells, potentially cardiac neural crest, located in the fourth and sixth branchial arches and migrating to the cardiac outflow tract (Figure 1C), while *Pax3* positive cells were still present, suggesting that *Pitx2* is a downstream target of Dvl2-mediated pathways in cardiac outflow tract development.

If a Dvl-dependent pathway directly regulates *Pitx2* in cardiac neural crest cell development, then genetic interaction between *Dvl2* and *Pitx2* might be expected. More than 65% of *Dvl2*$^{+/-}$/*Pitx2*$^{+/-}$ mice exhibited unambiguous outflow tract abnormalities, particularly PTA (Figure 1D). Because Dvl can serve as component of pathways other than Wnt, we determined whether β-catenin is also required for outflow tract development. The expression of a *Wnt1/Cre* transgene, expressed in CNS/craniofacial regions and in cardiac neural crest (Jiang et al., 2000), permitted conditional deletion of floxed β-*catenin* alleles (Brault et al., 2001). Our examination of e10.5–e17.5 embryos with Wnt1/Cre-dependent deletion of floxed β-*catenin* alleles in the neural crest revealed the occurrence of cardiac outflow tract abnormalities in almost all embryos evaluated, particularly PTA (>88% penetrance) (Figure 1E). In situ hybridization revealed a marked decrease of *Pitx2* expression in the migrating cells of the fourth-sixth branchial arches in e11 mice, while *Pax3* positive cells were still present (Figure 1F). Thus, our genetic data suggest that *Pitx2* is a component of the Wnt/Dvl/β-catenin pathway.

Coexpression of Pitx2 and *Wnt-1-Cre*-activated β-gal (from ROSA26-lacZ reporter mice) was found in the migratory cell population of the fourth and sixth branchial arches of e10 embryos (Figure 1G), suggesting that Pitx2 is expressed in cardiac neural crest. Neural crest outgrowth cultures from e8.5 midotic to 3rd somite area were assessed for colocalization of Pitx2 and connexin 43 (Cx43), suggested to be a reliable marker of cardiac neural crest (Waldo et al., 1999). Twenty-nine percent of the cells examined in the stack of deconvolution microscopic images were Cx43^{+} and approximately one third of them coexpressed nuclear Pitx2 (Figure 1H). Pitx2, positive (blue) cells fail to migrate to the cardiac truncus at e9.5 Pitx2$^{-/-}$ mice (Figure 1I). Therefore, we suggest that mutation of the Pitx2 gene causes a defect in cardiac outflow tract based on events in cardiac neural crest, consistent with the phenotype of the *Wnt-1 Cre*/β-*catenin* gene deletion.

The Wnt/β-Catenin Pathway Induces *Pitx2* Gene Expression

We took advantage of the ability of lithium to suppress GSK-3β activity, an effect that mimics Wnt pathway activation (Hedgepeth et al., 1997), to examine whether *Pitx2* expression could be induced by β-catenin. After injection of lithium chloride (LiCl) (4 ng/g body weight) intraperitoneally to *Pitx2*$^{+/-}$ mothers, e9.75 *Pitx2*$^{+/-}$ heterozygous embryos (harvested 2 hr later) revealed a consistent, marked increase in *Pitx2*-driven LacZ expression of the bulbo-truncal junction region of the outflow tract of the heart (Figure 2A), as well as in other *Pitx2*-expressing organs, including pituitary, eye, and muscle (Figures 2A, 2B, and data not shown). These data further suggest that the Wnt/β-catenin pathway regulate *Pitx2* gene expression in several tissues.

Because development of the anterior pituitary gland is arrested in *Pitx2*$^{-/-}$ mice (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999), we investigated the effects of lithium in dissociated pituitary cells and organ culture. Addition of LiCl caused a rapid, marked increase in *Pitx2* expression in isolated e11.5 pituitaries cultured on collagen gel (data not shown) or primary cell cultures prepared from e10.5 pituitaries of *Pitx2*$^{+/-}$ mice. In the absence of LiCl, proliferating cells expressed low levels of the pituitary-specific marker, Lhx3 (data not shown), but displayed no significant *Pitx2* expression (as marked by LacZ) (Figure 2C). Addition of lithium to these cultures resulted in marked induction of *Pitx2*, clearly detected as β-gal expression in virtually every cell by 1–2 hr (Figure 2C, and data not shown). To further test a potential direct Wnt/Dvl → *Pitx2* pathway, we expressed a constitutively active form of β-catenin (β-catenin$_c$) that exhibits nuclear localization in the absence of Wnt signals (Aberle et al., 1997) with a selectable marker into the αT_3-1 pituitary cell line. Cells expressing β-catenin$_c$ exhibited a marked induction of the *Pitx2* gene expression (Figure 2D). Similar results

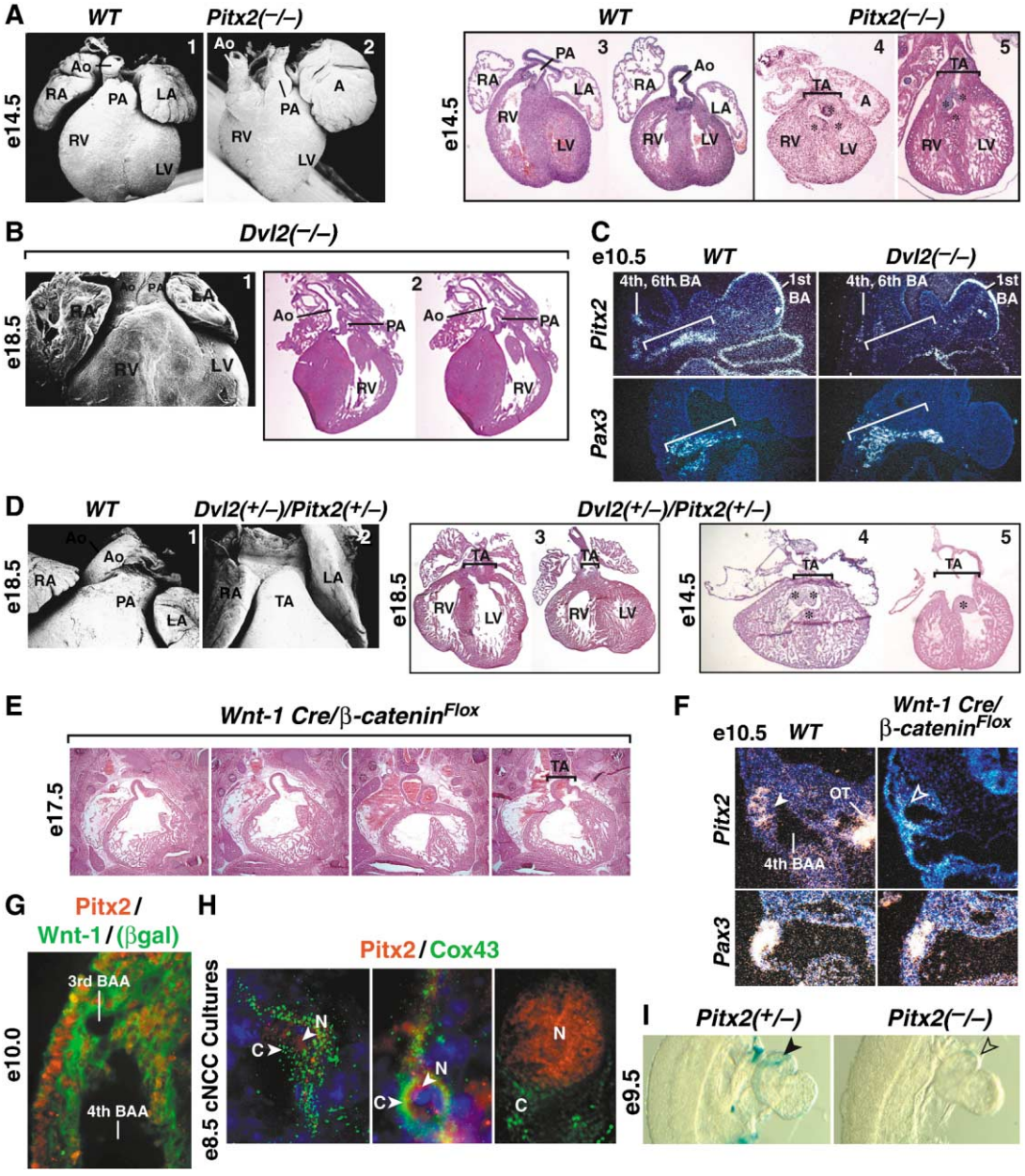

Figure 1. Genetic Evidence of *Pitx2*, *Dvl2*, and β-*Catenin* Roles in Cardiac Outflow Tract Development

(A) Scanning Electron Microscopy (SEM) (1,2) and hematoxylin and eosin H&E stained serial sections (3-5) from e14.5 hearts show PTA. Two different examples of PTA are shown (4,5), with three of the four cushions present seen in the single section shown (asterisk).

(B) SEM (1) and H&E staining (2) of heart revealed deficient positioning of the great arteries (DORV type) in e18.5 *Dvl2*$^{-/-}$ mice.

(C) *Dvl2*$^{-/-}$ mice failed to express *Pitx2* RNA, but do express *Pax3* transcripts in the fourth and sixth branchial arches (BA).

(D) *Pitx2*$^{+/-}$/ *Dvl2*$^{+/-}$ mutant mice exhibit cardiac outflow tract abnormalities. SEM (1-2) and H&E sections (3,5) of e18.5 (3) and e14.5 (4,5) hearts of these double heterozygotes.

(E) Serial sections of an e17.5 *Wnt-1 Cre*/β-*catenin*^{flox} gene-deleted heart characterized by common truncus.

(F) The *Wnt-1 Cre* /β-*catenin*^{flox} e10.5 mice exhibit marked decrease or absence of *Pitx2* expression in the fourth branchial arch.

(G) Pitx2 is expressed in Wnt-1 positive cells. Double labeling immunohistochemistry for Pitx2 and β-galactosidase, which detects Wnt-1 expression in e10.5 Wnt-1 Cre mice.

(H) Pitx2 is expressed in Connexin-43 positive cells. Double labeling immunohistochemistry and confocal microscopy was performed in primary branchial arch cultures from e8.5 mice for Pitx2 and Cx43 following migration of neural crest cells, with studies confirming colocalization of both proteins in the migrating cardiac neural crest cells.

(I) Pitx2-expressing cells are diminished in the e9.5 cardiac outflow tract of *Pitx2*$^{-/-}$ mice when compared with the Pitx2$^{+/-}$ littermates by LacZ whole-mount staining.

Ao = Aorta; PA = Pulmonary Artery; RA = Right Atrium; LA = Left Atrium; LV = Left Ventricle; RV =Right Ventricle; TA = Truncus Arteriosus; OT = outflow tract, C = cytoplasm, N = nucleus.

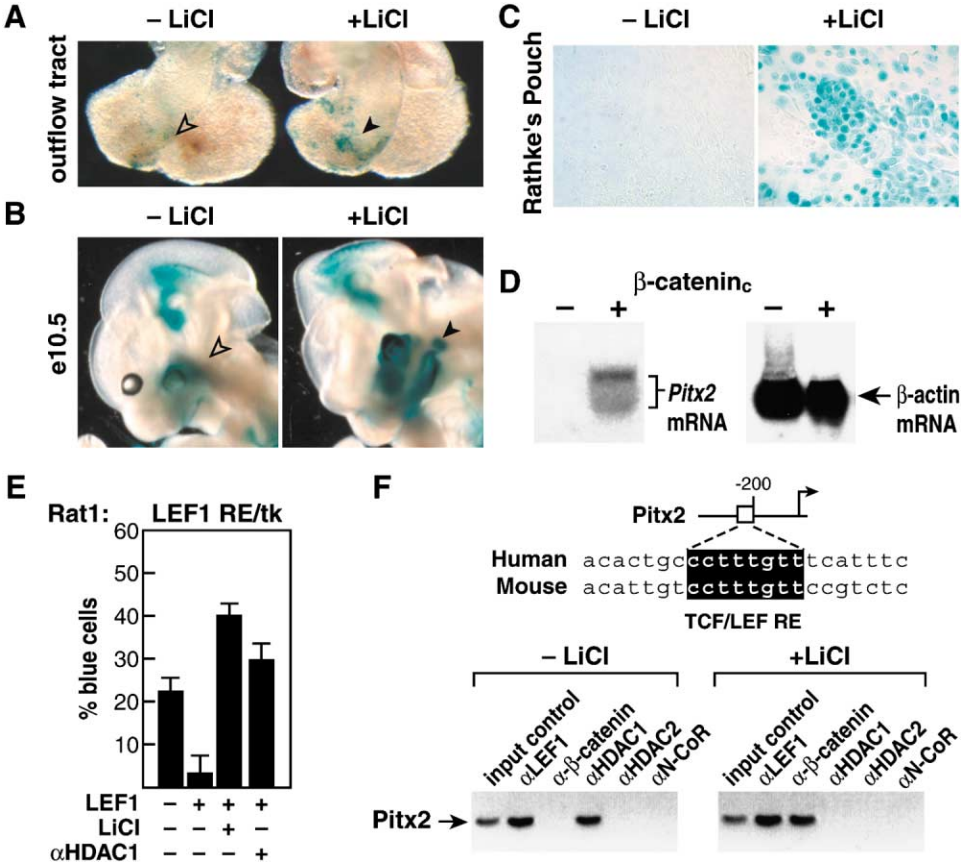

Figure 2. A Wnt/Dvl → Pitx2 Pathway

(A) In vivo treatment with lithium induces *Pitx2* expression in tissues including the developing cardiac outflow tract.

(B) Stimulation of Pitx2/β-gal expression in the anterior pituitary (arrow).

(C) Lithium-induced *Pitx2* expression in pituitary primary cell cultures.

(D) Constitutively active β-catenin (β-catenin$_c$) induces *Pitx2* gene expression in populations of stably transfected αT_3-1 pituitary cells.

(E) LEF1 acts as an HDAC1-dependent repressor, reversed by addition of lithium.

(F) β-catenin reverses LEF1/HDAC1 repression of *Pitx2* by dismissal of HDAC1. The sequence of the murine *Pitx2* promoter was determined, with conservation of two TCF/LEF responsive elements (RE). The effect of lithium on activation of β-catenin-dependent events was assessed in αT_3-1 cells using ChIP analysis. LEF1was detected on the *Pitx2* promoter, as was HDAC1, but not HDAC2 or N-CoR.

were obtained in a second *Pitx2*-expressing cell line (C2C12, data not shown). In the absence of lithium chloride, LEF1 can function as an inhibitor, relieved by either lithium chloride or nuclear microinjection of αHDAC1 IgG (Figure 2E) (Billin et al., 2000).

Interestingly, the 5′ regulatory regions of the human *Pitx2* gene contain perfect consensus TCF/LEF response elements. Addition of LiCl or β-catenin$_c$ expression caused an induction of the *Pitx2* promoter in transient transfection assays (data not shown). We cloned and determined the sequence of the murine *Pitx2* promoter and established that two TCF/LEF response elements were conserved in the mouse promoter, one identical with the human promoter sequence (Figure 2F). Chromatin immunoprecipitation (ChIP) assays were performed as previously described (Jepsen et al., 2000; Baek et al., 2002; Scully et al., 2000) using specific IgGs against β-catenin, LEF1, N-CoR and specific HDACs revealing that LEF1 was present on the *Pitx2* promoter (Figure 2F). In untreated cells, HDAC1 was detected on the *Pitx2* promoter, but HDAC2, β-catenin, and N-CoR were not. After lithium stimulation, HDAC1 was no longer detected, but β-catenin was now present on the *Pitx2* promoter (Figure 2F). These data suggest that *Pitx2* is a direct target of the Wnt/Dvl pathway, and single cell nuclear microinjection αLEF1 specifically blocks stimulation of a *Pitx2/LacZ* reporter by LiCl (data not shown).

Pitx2$^{-/-}$ Mice Exhibit a Proliferation Defect in Cardiac Neural Crest and Rathke's Pouch

To determine whether the actions of Pitx2 in cardiac neural crest reflected, at least in part, a proliferative defect, we performed BrdU pulse-labeling experiments on e9–e11 embryos and analyzed β-gal /BrdU colocalization. As shown in Figure 3A, there was a marked decrease in BrdU labeling of cells that normally express *Pitx2* migrating from the fourth and sixth branchial arches to the developing cardiac outflow tract in *Pitx2*$^{-/-}$ mice, as compared to their *Pitx2*$^{+/-}$ littermates. Similarly, BrdU pulse labeling of embryos carrying a *Wnt-1-Cre*-dependent deletion of the β-*catenin* alleles at e10.5 revealed a marked decrease of labeled cells in outflow tract (Figure 3B).

The absence of *Pitx2* causes developmental arrest of

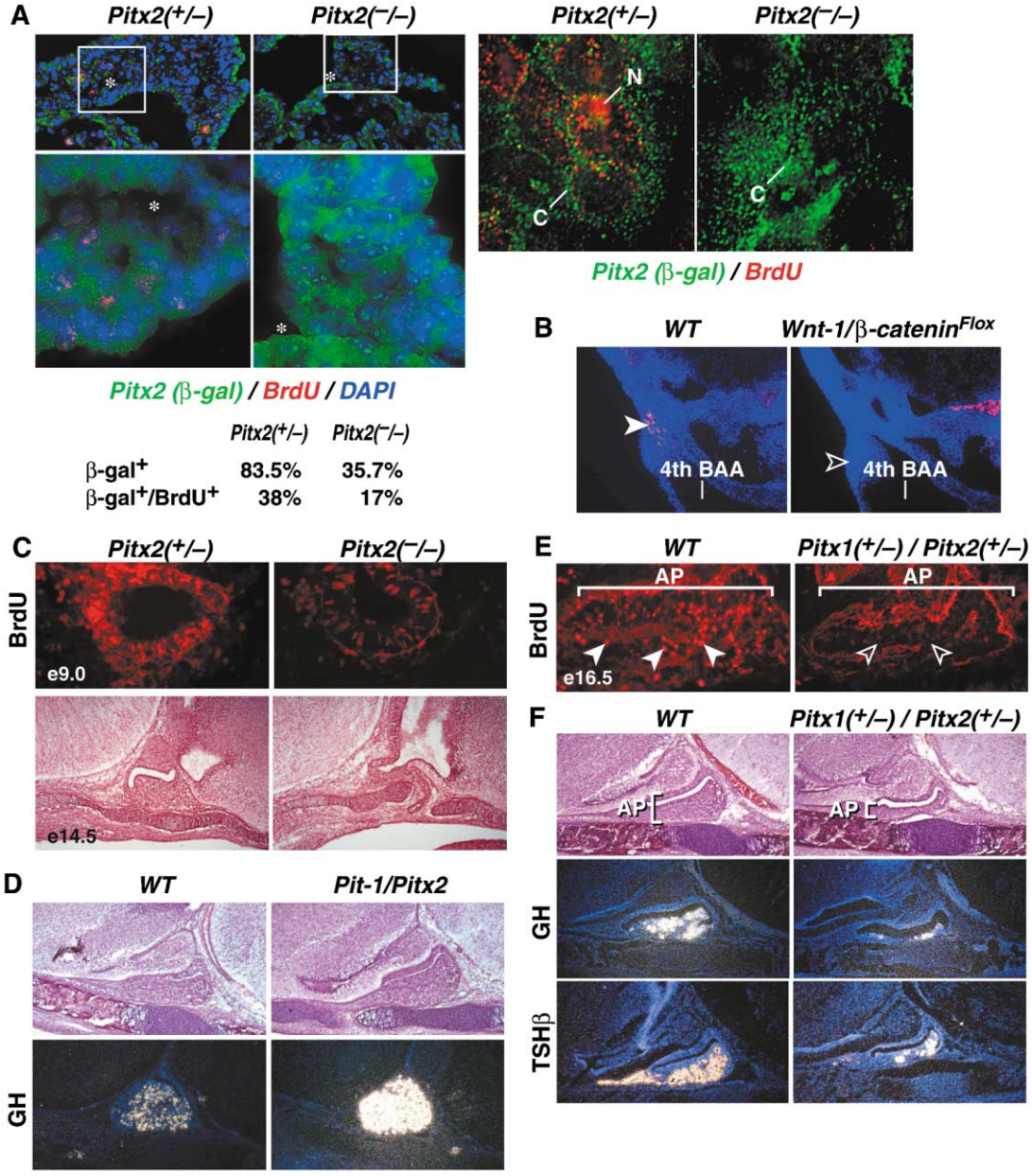

Figure 3. *Pitx2* Regulates Cell Proliferation during Cardiac and Pituitary Development

(A) Dramatic decrease of BrdU incorporation in *Pitx2*$^{-/-}$ cardiac outflow tract. Double labeling for BrdU (red) and β-galactosidase (green), showed a decrease of the Pitx2-positive proliferating cells in the cardiac outflow tract in mutant e9 mice compared to their heterozygote littermates. Boxed region indicates magnification of the same sagittal section.

(B) Absence of proliferating cardiac neural crest cells in the *Wnt-1-Cre*/β *-catenin*^{flox} mice in the fourth and sixth branchial arches of e11 *Wnt-1-Cre*/β-*catenin*^{flox} mice.

(C) Decreased BrdU incorporation in *Pitx2*$^{-/-}$ pituitaries, compared to wild-type littermate controls, following intraperitoneal injection at e9. Quantitation revealed 60%–75% reduction of BrdU positive cells. Reduction of the pituitary gland at e14.5 in the *Pitx2*$^{-/-}$ mice compared with the *Pitx2*$^{+/-}$ littermates.

(D) Effects of sustained expression of *Pitx2* under 15 kb of *Pit-1* gene regulatory information, founder analyses was performed at e17.5, revealing particular expansion of the growth hormone (GH) positive cells.

(E) Decreased BrdU incorporation in pituitaries from *Pitx1*$^{+/-}$/*Pitx2*$^{+/-}$ mice, compared to wild-type littermates following intraperitoneal injection at e16.5.

(F) In situ analysis of e18.5 *Pitx1*$^{+/-}$/*Pitx2*$^{+/-}$ mice exhibit a decrease in all pituitary cell types with particularly striking effects on growth hormone and thyrotrope producing cells. The anterior pituitary gland was markedly hypoplastic, as shown by H&E staining of adjacent sections (upper images).

AP = anterior pituitary; GH = growth hormone; TSHβ = thyrotrope stimulating hormone.

the pituitary gland by e10.5 (Gage et al., 1999; Lin et al., 1999), consistent with the shift in growth dependence of Rathke's pouch from extrinsic (FGFs, BMP4, Shh) to intrinsic signals (BMP2, Wnt) (Ericson et al., 1998; Treier et al., 1998). The arrest of organ development proved to be due to a failure of proliferation, with 65 to 70% reduction of the number of BrdU-labeled cells consistently observed in the pituitary gland from *Pitx2*$^{-/-}$ lineages compared to the wild-type littermate controls (Figure 3C). A similar reduction in the number of BrdU-labeled cells was seen in the body wall and limbs and no changes in apoptotic cells were detected in the developing pituitary of *Pitx2*$^{-/-}$ mice (data not shown). Conversely, in vivo overexpression of *Pitx2,* under the control of 15 kb *Pit-1* promoter (Treier et al., 1998), revealed hyperplasia of the anterior pituitary with one of the *Pit-1*-dependent cell lineages, the somatotrope (GH producing cell), particularly increased in number (~2-fold), with increased levels of growth hormone transcripts (Figure 3D).

Pitx2$^{+/-}$*/Pitx1*$^{+/-}$ mice displayed a marked decrease in BrdU incorporation on e16.5, resulting in hypoplastic anterior pituitary (Figure 3E). The growth defect involves all pituitary cell types, except perhaps the POMC lineage, with somatotrope proliferation being strikingly affected (Figure 3F and data not shown), and is similar to that previously described in *Wnt4*$^{-/-}$ mice (Treier et al., 1998).

Pitx2 Regulates Cell-Autonomous Proliferation Acting in G1

Based on genetic evidence linking *Pitx2* to proliferation in several organ systems, we next investigated whether *Pitx2* might serve a cell-autonomous role directly regulating proliferation of cells in which it is expressed. Because *Pitx2* is highly expressed in a subpopulation of Pax3^{+} migratory muscle precursor cells at e10.5 (Figure 4A), and in pituitary, we could address this issue in both the well-characterized C2C12 murine myoblast cell line, which can be induced to differentiate by withdrawal of mitogens (Blau et al., 1985), and in the pituitary cell line αT_3-1. *Pitx2* transcripts (data not shown) and Pitx2 protein (Figure 4B) were expressed in undifferentiated C2C12 cells, with decreasing levels after 3 days of differentiation. There was a reproducible 70%–80% decrease in BrdU incorporation in serum-treated cells microinjected with specific αPitx2, but not with preimmune IgG (Figure 4C). A similar result was obtained in αT_3-1 cells and SiRNA against Pitx2 transcripts and gave similar results in both cell types (data not shown). These effects were observed when EGF, IGF-1, and FGF were used to stimulate growth (data not shown). Serum or growth factor activation of AP1 was unaffected by injected αPitx2 (Figure 4D), indicating that this pathway is stimulated in a Pitx2-independent fashion. Using specific antiphospho ERK antibodies, we confirmed that normal activation of the signaling pathway through ERK phosphorylation was maintained in cells injected with αPitx2 (Figure 4E), suggesting the possibility that Pitx2 might exert effects on specific transcriptional targets, while not affecting the initial transduction of proliferative signals.

Highly expressed DNA binding transcription factors can exert transcriptional effects by transrepression; therefore, we determined the requirement for DNA binding and other domains of Pitx2 for its effects on cell proliferation. These experiments utilized the Pitx2$_{HDmut}$, a Pitx2a holoprotein in which three amino acids involved in major groove contacts in α3 DNA binding helix of the homeodomain were mutated (to alanine) to prevent sequence-specific DNA binding without altering homeodomain structure. We also evaluated Pitx2 ΔN, which deleted all sequences N-terminal to the homeodomain of Pitx2a, and Pitx2 ΔC, which deleted all sequences C-terminal to the homeodomain of Pitx2a. Neither Pitx2$_{HDmut}$, nor Pitx2 ΔN, were able to rescue the block of BrdU incorporation by αPitx2 IgG, while identical molar concentrations of nuclear microinjected Pitx2 holoprotein or Pitx2 ΔC rescued the growth inhibiting effects of αPitx2 (Figures 4F and 4G). Conversely, in the absence of αPitx2 IgG, the N-terminal domain of Pitx2 (Pitx2 N′) or homeodomain (Pitx2$_{HD}$), but not the C terminus (Pitx2 C′) or Pitx2a holoprotein, acted in a dominant-negative fashion, inhibiting BrdU incorporation in C2C12 cells (Figure 4F and data not shown). These data establish that the DNA binding function of Pitx2, and its N-terminal domain, are required for its effects on proliferation.

Nuclear microinjection of Pitx2 holoprotein did not further stimulate BrdU incorporation in Rat1 cells in which neither Pitx2 or Pitx1 are expressed (Figure 4H). Conversely, αPitx2 had no effect on proliferation in these cells (Figure 4H). These data demonstrate that inhibition of cell proliferation by αPitx2 is a specific effect observed only in cell types that normally express Pitx2. In order to estimate the time in the cell cycle, at which Pitx2 might act in regulating cell proliferation, synchronized C2C12 cells were serum-treated and the effect of nuclear microinjection of αPitx2, or preimmune, was assessed at various times following serum addition. αPitx2 effectively inhibited BrdU incorporation for 6–8 hr; however, by 9 hr after serum stimulation, αPitx2 was no longer effective at inhibiting BrdU (Figure 4I). Therefore, Pitx2 serves as a transcriptional regulator in early to late G1 in cells in which it is expressed.

Cyclin D2 Is a Pitx2 Target Gene

The next issue was whether Pitx2 might be involved in induction of specific growth control genes, particularly those regulated in early/mid G1. Examination of the *Cyclin D2* promoter revealed that it contained consensus bicoid binding sites (Figure 5A), as did the *Cyclin D1* and *c-Myc* promoters. Double-stranded oligonucleotides corresponding to the two most conserved Pitx2 cognate binding sites could be bound by bacterially expressed Pitx2a by EMSA (data not shown) and therefore potential Pitx2 targets.

The *Cyclin D1* promoter was reported to be regulated by constitutively active β-catenin in human colon cancer cells based on TCF/LEF binding elements (Shtutman et al., 1999, Tetsu and McCormick, 1999). The *Cyclin D2* promoter does not contain recognized TCF/LEF sites, raising the possibility that its induction is dependent on other classes of transcription factors, such as Pitx2. Consistent with this possibility the addition of lithium, even in the absence of serum, caused some increase in *Pitx2* levels in αT_3-1 or C2Cl2 cells (not shown) (Figure 5A). *Cyclin D2* transcripts were rapidly (30–60 min) and

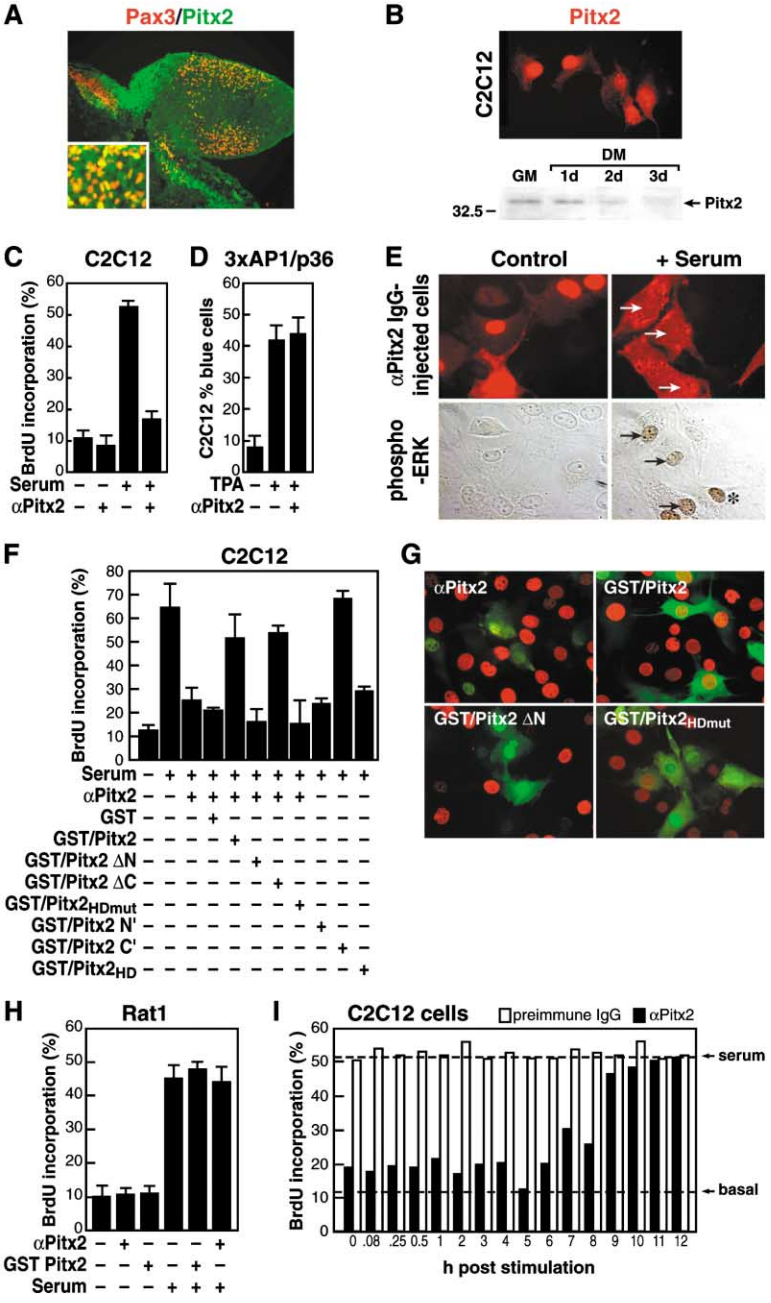

Figure 4. Pitx2 Modulates Cell Growth in Murine C2C12 Myoblasts

(A) Pitx2 and Pax3 coexpression in migratory precursor myoblasts assessed by immunofluorescence.

(B) Pitx2 expression in C2C12 cells assessed by immunofluorescence (upper), and Western blot (lower) showing a decrease in Pitx2 levels after differentiation. GM, growth medium; DM, differentiation medium.

(C) Single cell nuclear microinjection of αPitx2 in C2C12 cells caused a 75%–85% decrease on serum-stimulated BrdU incorporation.

(D) Response to stimulation by AP1 elements. Microinjections of αPitx2 IgG in C2C12 cells failed to alter TPA (10^{-6} M)-dependent induction of an AP1-dependent reporter. Results are in ± SEM with >300 cells microinjected in each condition; similar results were observed in two additional experiments.

(E) Staining of C2C12 cells with α phospho ERK1-specific IgG signaling revealed identical nuclear localization of phospho ERK1 in injected (arrows) or noninjected (asterisk) cells in response to serum. Upper images show the injected cells stained by rhodamine; the DAB staining quenches the nuclear fluorescence (upper right image).

(F) Role of DNA binding and N-terminal domains in Pitx2-dependent proliferation events. The ability of purified, bacterially expressed GST fusion proteins containing αPitx2 holoprotein (GST/Pitx2), N terminally deleted Pitx2a (GST/Pitx2ΔN), C terminally deleted Pitx2a (GST/Pitx2 ΔC), or Pitx2a HD_{mut} harboring a 3 aa mutation in a α3 helix of the homeodomain (R45A, V47A, N51A), eliminating specific DNA binding, were evaluated for ability to rescue αPitx2 IgG-inhibited BrdU incorporation. Injection of GST Pitx2a N′ or GST Pitx2 HD, GST Pitx2a, and GST Pitx2 C′ were also evaluated in the absence of αPitx2.

(G) Photomicrographs of representative fields of the experiment presented in (F), showing injected cells (fluorescein), and nuclear staining with αBrdU IgG (rhodamine).

(H) Proliferation of Rat1 cells was unaltered by Pitx2 expression. Rat1 cells, which do not contain detectable *Pitx1* or *Pitx2* transcripts or protein, fail to exhibit any growth stimulation on nuclear microinjection of GST/Pitx2, nor any inhibition of BrdU incorporation with αPitx2 IgG.

(I) Effects of αPitx2 IgG are maintained until late G1. C2C12 cells were subjected to nuclear microinjection with either preimmune or αPitx2 IgG at indicated times after initial serum stimulation, with BrdU incorporation assessed after 14 hr. αPitx2 inhibited BrdU incorporation until ~8–9 hr after serum addition.

robustly induced upon lithium and serum treatment of either C2C12 cells (Figure 5A), or αT_3-1 (not shown), with either treatment alone being insufficient for effective activation (not shown). Using ChIP analysis in either cell type, we noted very little, if any, Pitx2 present on the *Cyclin D2* promoter in cells maintained in the absence of lithium or serum, addition of serum alone caused some appearance of Pitx2 on the *Cyclin D2* promoter, and a marked Pitx2 recruitment occurred rapidly in response to lithium. Consistent with binding of Pitx2 in the presence of serum HDAC1 were detected, but HDAC 2,3,4,5, or 6 were not. HDAC1 was dismissed on treatment with LiCl (Figure 5A, and data not shown), consistent with the β-catenin-dependent induction of *Pitx2* and dismissal of HDAC1. However, simultaneous activation of both the Wnt/β-catenin and growth factor-dependent pathways, showed a marked increase in histone H3/H4 acetylation and recruitment of Pol II (Figure 5A). Thus, the Wnt/β-catenin and growth factor-dependent pathways functioned combinatorially in the activation of the *Cyclin D2* promoter. Virtually identical ChIP data was observed in both C2C12 and αT_3-1 cell lines.

Expression of a reporter under control of the *Cyclin D2* promoter was specifically blocked in a single cell

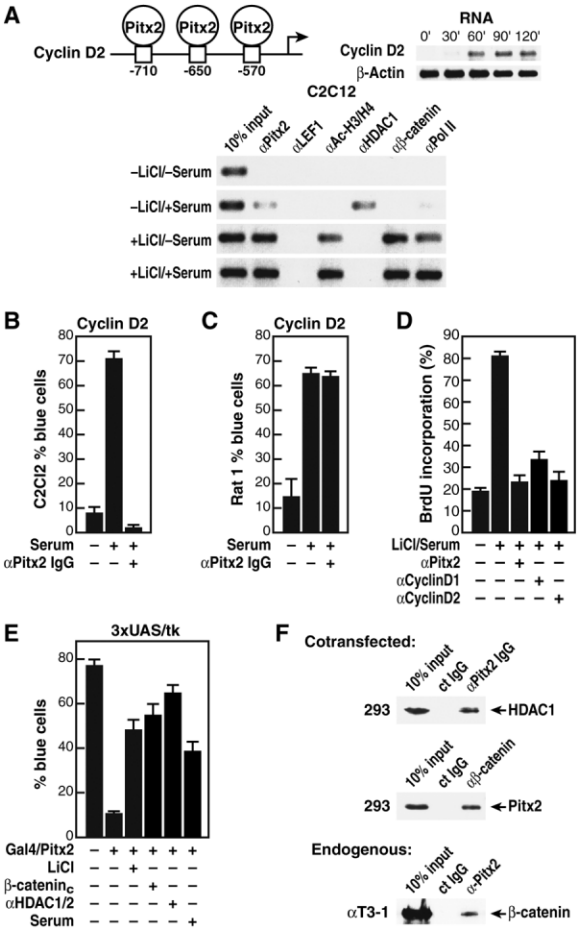

Figure 5. *Pitx2* Regulates Specific G1 Growth-Control Genes

(A) Induction of *Cyclin D2* by 30–60 min lithium and serum treatment. ChIP assay was performed using specific αPitx2, αLEF1, αβ-catenin, αacetylated histone H3/H4, or αPol II IgGs.

(B) Role of Pitx2 in *Cyclin D2* expression. C2C12 cells (shown), or αT_3-1 cells (not shown) were subjected to single cell nuclear microinjection with αPitx2 IgG and a construct encoding a LacZ reporter under the control of the *Cyclin D2* promoter.

(C) No difference was recorded in *Cyclin D2* expression by αPitx2 IgG in Rat-1 cells, in which Pitx2 is not expressed.

(D) Microinjection of αPitx2, αCyclin D1, or αCyclin D2 IgG inhibits BrdU incorporation in C2C12 cells.

(E) Pitx2 can function as a β-catenin-regulated repressor, Gal4/Pitx2 caused repression of UAS/tk reporter in Rat1 cells, using the single cell nuclear microinjection assay. Addition of lithium chloride, serum, expression of constitutively active β-catenin (β-catenin$_c$), or αHDAC1 reproducibly overcame the repression of UAS/tk reporter by Gal4/Pitx2.

(F) Pitx2 interacts with HDAC1 based on coimmunoprecipitation from cell extracts prepared from 293T cells transfected with HA-Pitx2 and Flag-HDAC1 expression plasmids. Pitx2 interacts with β-catenin in cell extracts prepared either from 293T cells transfected with HA-Pitx2 and β-catenin expression plasmids, or by coimmunoprecipitation from untransfected, lithium-treated αT_3-1 cells (shown) or C2C12 cells (not shown).

nuclear microinjection assay in C2C12 cells by αPitx2 IgG, further supporting the direct requirement for Pitx2 in *Cyclin D2* gene activation in these cell types (Figure 5B). In contrast, in Rat 1 cells, which do not express Pitx2, αPitx2 had no effect on expression of the *Cyclin D2* promoter (Figure 5C). Similarly, there was no effect of expressing Pitx2 N terminus (data not shown). Nuclear microinjection of αCyclin D2 and αCyclin D1 IgG significantly inhibited serum-dependent BrdU incorporation in C2C12 cells, consistent with their roles in G1 progression (Figure 5D).

Actions of β-Catenin in Pitx2 Derepression

The rapid induction of the *Cyclin D2* gene raises the question of what additional machinery is required for Pitx2-dependent activation. Homeodomain activators can serve as repressors, dependent upon promoter context and culture conditions, based on interactions with specific corepressor complexes (Pinsonneault et al., 1997; Xu et al., 1998; Asahara et al., 1999; Scully et al., 2000). Consistent with these observations, Pitx2 or a Gal4/Pitx2 fusion protein exhibited repressor activity on target genes in Rat1 cells (Figure 5E and data not shown), even though Pitx2 clearly serves as an activator of growth regulatory genes, as shown here, and on other promoters (Amendt et al., 1998, Bach et al. 1997). Treatment with lithium chloride, microinjection of constitutive β-catenin, HDAC1 IgG, or the addition of serum all acted to reverse this repressive function.

Immunoprecipitation from cells transfected with a *Pitx2* expression vector revealed the ability of Pitx2 to robustly interact with β-catenin and with HDAC1, even at endogenous levels of Pitx2 and β-catenin (Figure 5F), analogous to interactions between TCF/LEF1 and β-catenin. Because β-catenin has been reported to interact with HDAC1 (Billin et al., 2000), we evaluated the potential effects of β-catenin on the enzymatic activity of HDAC complexes by coimmunoprecipitation assay, finding that HDAC1 activity was inhibited 4–5-fold by β-catenin, while the activities of HDAC2, HDAC3, HDAC4, and HDAC5 were not affected (data not shown).

Sequential Recruitment of Required Pitx2 Coactivators

We next wished to define the coactivator machinery required for Pitx2-dependent activation of these growth control genes (Hermanson et al., 2002). Both Pitx2 and β-catenin were highly recruited to the Cyclin D2 promoter at 30 min and remained bound at all time points evaluated (Figure 6A). We examined the potential recruitment of specific components of several well-characterized coactivator complexes, at various times after lithium and serum stimulation. Based on our previous observation (Bach et al., 1997) that Pitx2 was capable of interactions with NLI/Ldb/CLIM coactivators, initially identified by interactions with LIM homeodomain factors (Agulnick et al., 1996; Bach et al., 1997; Jurata et al., 1998; Jurata and Gill, 1997), we evaluated their potential recruitment on the *Cyclin D2* promoter. NLI/Ldb/CLIM was recruited by 30 min, in lithium/serum-treated cells, at much decreased levels at 60–90 min, and again robustly at 120 min (Figure 6A). CBP/p300 were clearly recorded at 30 min but declined markedly by 60 min and subsequently were undetectable (Figure 6A).

Based on the continued acetylation of histone H3 and H4 after CBP/p300 was dismissed (Figure 6A), we examined the potential recruitment of the MYST family members of histone acetyltransferases. As shown in Figure

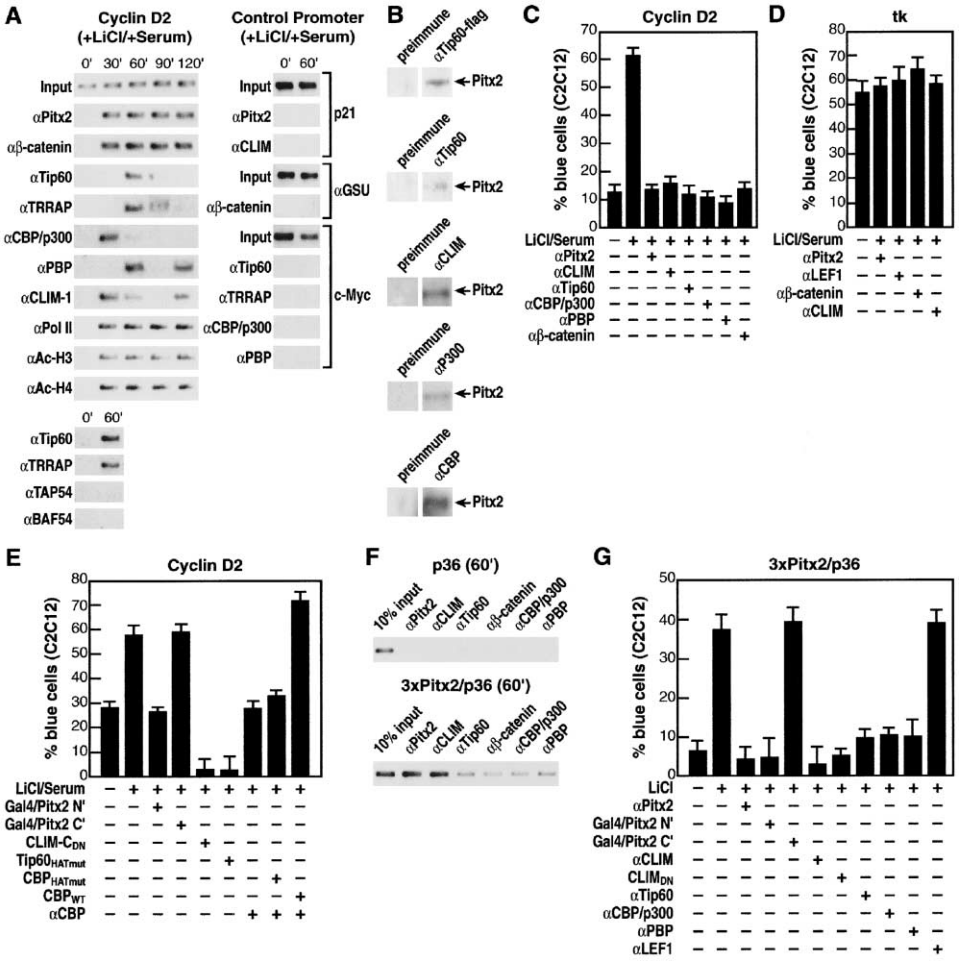

Figure 6. Recruitment of Coactivators to the Cyclin D2 Promoter

(A) Recruitment of coactivators on *Cyclin D2* promoter in C2C12 cells, after stimulation with lithium and serum revealing that specific coactivators were recruited in a temporally specific fashion. Examples of controls are shown on the right.

(B) Pitx2 interacts with Tip 60, CBP/300, and CLIM in coimmunoprecipitation assay from C2C12 cells both in transfected cells (αFlag) and at endogenous levels in αT_3-1 cells.

(C) The role of coactivators on activation of the *Cyclin D2* promoter. C2C12 cells were microinjected with specific antibodies against Pitx2, CLIM, Tip60, CBP/p300, PBP, and a construct in which the *Cyclin D2* promoter drives a LacZ reporter.

(D) Failure of these same antibodies to alter expression of tk/LacZ reporter with single cell nuclear microinjection in C2C12 cells.

(E) Ability of dominant-negative forms of Pitx2 (Pitx2 N terminus), NCI/Ldb/CLIM (CLIM C terminus), or Tip 60 HAT_{mut} expressed as CMV transcription units, to inhibit *Cyclin D2*.

(F) Ability of Pitx2 to recruit specific coactivator complexes, comparing a minimal promoter (p36 LacZ) transcription unit and the same transcription unit containing a 3 × Pitx2 response element.

6A, Tip60 recruitment was not evident at 30 min but was recruited to the *Cyclin D2* promoter at 60 min in response to addition of lithium and serum and TRRAP was also detected in a similar temporal pattern, consistent with recruitment of a TRRAP/Tip60 complex. Tip60 is present in many complexes, including the DNA repair complex (Ikura et al., 2000), which does not effectively produce histone H3 acetylation complexes. The absence of TAP54 and BAF53 suggests that the recruited Tip60 complex is distinct from the repair complex (Figure 6A and data not shown). The recruited TRRAP/Tip60 complex is dismissed by 120 min. In addition to the ability of Pitx2 to interact with β-catenin (Figure 5F), immunoprecipitation experiments in C2C12 cells revealed a specific Tip60/Pitx2 interaction at endogenous levels of each protein (Figure 6B), as well as interactions between Pitx2 and NLI/Ldb/CLIM and CBP (Figure 6B). These data are consistent with Pitx2-dependent recruitment of distinct coactivator complexes. As reflected by the presence of PBP/TRAP 220/DRIP 205, the TRAP/DRIP/ARC complex (reviewed in Hampsey and Reinberg, 1999), also appeared to be recruited by 60 min, dismissed at 90 min, and again robustly present at 120 min (Figure 6A).

To evaluate the potential roles of each of the serially recruited complexes in activation of the *Cyclin D2* promoter, single cell nuclear microinjection assays were performed in C2C12 cells (Figure 6C). Intriguingly, specific blocking of antibodies against CBP/p300, Tip60, NLI/Ldb/CLIM, and TRAP/DRIP/ARC entirely inhibited

Cyclin D2 promoter activity, as did anti-β-catenin IgG (Figure 6C). Therefore, the temporally specific recruitment of specific coactivator complexes to the *Cyclin D2* promoter upon binding of Pitx2, appears to be required for activation of the *Cyclin D2* gene. As a further control, we show that injection of IgGs did not affect other transcription units (e.g., tk or CMV promoters), confirming promoter specificity (Figure 6D and data not shown).

To utilize an independent method to assess the functional roles of these cofactors, we expressed dominant-negative forms of these factors. The N terminus of Pitx2 is required for its actions in proliferation (Figure 4F) and can itself serve as a dominant-negative for proliferation in C2C12 cells (Figure 4F). We also observed that Gal4 DBD/Pitx2a N terminus fusion protein inhibited actions of Pitx2 holoprotein in transient cotransfection assays (data not shown), and we therefore used this protein as a dominant-negative, showing that Gal4/Pitx2aN′ selectively blocked *Cyclin D2* activation in C2C12 cells (Figure 6E). In contrast, Gal4/Pitx2a C′ had no effect (Figure 6E). Further, the C-terminal activation domain of CLIM/Ldb/NLI, fused to Gal4 DBD, which inhibits actions of the holoprotein, also blocked *Cyclin D2* activation (Figure 6E). To further test that the antibody block was specific and did not merely reflect removal of complexes, we performed a rescue experiment with CBP. In the presence of αCBP, expression of wild-type CBP fully rescued *Cyclin D2* expression, while expression of CBP harboring point mutations that abolished HAT function (CBP_{HATmut}) (Korzus et al., 1998) did not (Figure 6E).

Because ChIP assay cannot determine whether cofactors are recruited by a specific DNA binding transcription factor, we wished to confirm that the factors shown to be bound to the *Cyclin D2* promoter in ChIP assays and to interact with Pitx2 by coimmunoprecipitation could be directly recruited by Pitx2 and required for its activation and function by an independent assay. We therefore used ChIP analysis in C2C12 cells to evaluate the recruitment of these factors to a minimal promoter containing a 3 × Pitx2 response element (Figure 6F). We found that, dependent upon the Pitx2 DNA binding sites, Pitx2 was recruited along with Tip60, NLI/Ldb/CLIM, CBP/p300, PBP, and β-catenin, supporting the model that these cofactors can, indeed, be directly recruited to the *Cyclin D2* promoter by Pitx2, although other DNA binding factors are likely to participate in this recruitment. Single cell nuclear microinjection of each cofactor-specific antibody, or expression of dominant-negative forms of these cofactors on the Pitx2 response element-dependent promoter, revealed that the factors required for activation of the *Cyclin D2* promoter were also required for activation of the Pitx2 site-dependent promoter (Figure 6G).

Discussion

The Wnt/Dvl/β-Catenin → Pitx2 Pathway in Cardiac Outflow Tract Development

In this manuscript, we have demonstrated that pathway-specific induction of a tissue-restricted transcription factor can provide a critical requirement for proliferation in specific cell types, providing a cell-specific code for regulating growth. The *Pitx2* gene provides a striking example because its deletion causes a failure of cell type-specific proliferation at specific stages of development, defining a Wnt-dependent pathway regulating proliferative events in cardiac outflow tract, pituitary, muscle, and other tissues. This role of Pitx2 may extend to several other tissues, including colon and tooth bud. Our data are consistent with the postulate that the Wnt/β-catenin → Pitx2pathway regulates cardiac outflow tract development, in addition to its pivotal role in brain and craniofacial development (Brault et al., 2001, McMahon et al., 1992, Megason and McMahon, 2002). We have demonstrated that Wnt signaling, in acting upstream of *Pitx2*, directly induces *Pitx2* gene expression, based on the recruitment of LEF1 to evolutionary-conserved sites in the *Pitx2* gene 5′-regulatory regions, with a regulated exchange of HDAC1 for β-catenin occurring on these *Pitx2* sites. *Pitx2* is expressed in the cardiac neural crest as these cells migrate from the dorsal neural tube through the fourth and sixth branchial arches, and *Pitx2*-expressing cells exhibit defective proliferation in both *Wnt-1 Cre*/*β-catenin*^{flox} and *Pitx2*$^{-/-}$ mice. This leads to a marked diminution of cells arriving in the outflow tract and consequent lack of septation of the great arteries. Thus, we have documented a genetic and biochemical linkage between *Pitx2,* as a critical target of the Wnt pathway with respect to cardiac outflow tract and pituitary proliferation.

Because multiple components of the Wnt/Dvl/β-catenin → Pitx2 pathway are dosage sensitive for these events, these findings suggest a common etiology for the highly prevalent multigenic cardiac outflow tract abnormalities that occur in the human population (Chien, 2000; Srivastava and Olson, 2000). The events in cardiac outflow tract development appear to be similar to those in pituitary and muscle, where biochemical analyses and primary cell cultures reveal that the Wnt → β-catenin pathway directly induces *Pitx2* gene expression, based on the recruitment of LEF1 sites in the *Pitx2* gene 5′-regulatory regions (Figure 7).

The genetic linkage between the Wnt/Dvl2 pathway and *Pitx2* in cardiac outflow tract proliferation is consistent with previous data (Thomas et al., 1991; Treier et al., 1998, Serbedzija et al., 1996) that established in vivo proliferative roles for Wnt factors in pituitary organ development. Our data suggest that in pituitary, muscle, and cardiac neural crest cells, the Wnt pathway serves as a major signaling mechanism regulating *Pitx2* gene expression.

A Wnt/Dvl/β-Catenin → Pitx2 Pathway Regulates Cell Type-Specific Proliferation

Activation of the Wnt pathway results in rapid recruitment of the *Pitx2* gene and binding of Pitx2 to promoters of specific growth control genes. This linkage between the Wnt pathway and *Pitx2* gene expression provides an insight into the molecular mechanisms of cell type-specific proliferation, based on the required actions of Pitx2 to activate specific, critical growth-control gene targets acting in G1. Based on in vivo studies, as well as the actions in pituitary and muscle cell models, *Pitx2* is required for normal proliferation when expressed in heterologous cells; *Pitx2* can actually inhibit proliferation (Wei and Adelstein, 2002). We speculate that this may

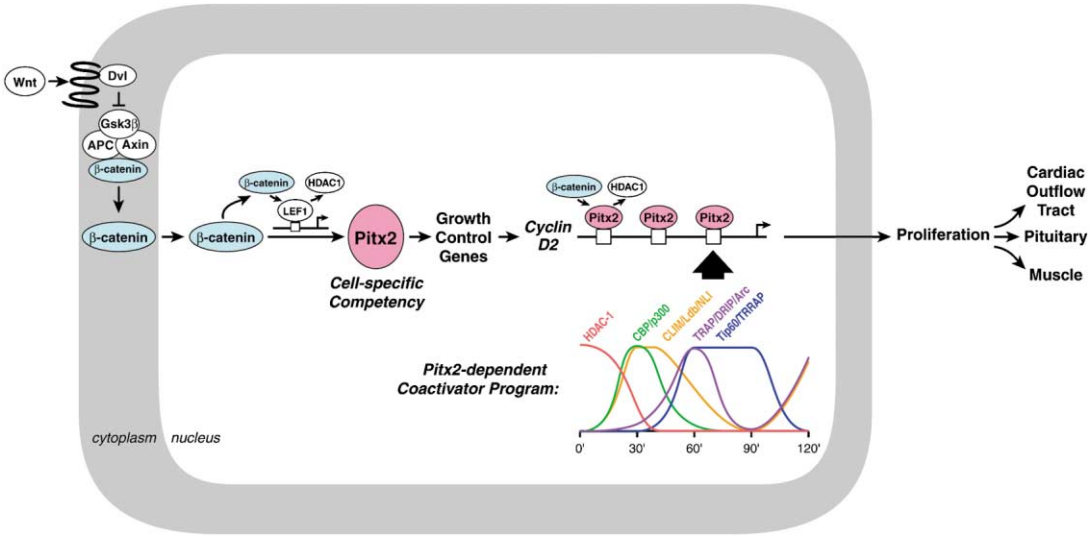

Figure 7. Model of a Wnt/Dvl/β-Catenin → Pitx2 Pathway Regulating Cell Proliferation

Pitx2 serves as a direct LEF-1 dependent target of a Wnt/Dvl/β-catenin pathway, and binds to the *Cyclin D2* promoter, as well as growth-promoting genes. With growth factor stimulation, Pitx2 serially recruits a series of coactivator complexes indicated in the schematic diagram that includes NLI/Ldb/CLIM and Tip60/TRRAP factors, all of which have proved to be required for stimulation of these promoters. An additional level of regulation appears to be provided by β-catenin interactions with Pitx2 (illustrated on the *Cyclin D2* promoter), causing dissociation of HDAC1, serving as a derepression strategy.

occur by squelching coregulatory factors required by DNA binding transcription factors that exert analogous functions to Pitx2. To subserve its proliferative effects, *Pitx2* must bind to its cognate DNA sites and requires an N-terminal activation domain, but not the C terminus, which is consistent with a previous report of inhibitory effects of the Pitx2 C terminus (Amendt et al., 1998).

Together, our data suggests that three independent events underlie Pitx2-dependent activation of cell type-specific proliferation: Wnt-dependent activation of Pitx2; Wnt and growth factor-dependent relief of Pitx2 repression function; and serial recruitment of a series of specific coactivator complexes that act in a promoter-specific manner, analogous to effects of β-catenin on LEF1 (Billin et al., 2000).

While it is possible that Pitx2 may influence the cell cycle at more than one stage, we have shown that Pitx2 exerts key actions in early to mid G1 and stimulates expression of specific growth control genes exemplified by *Cyclin D2*. The *Cyclin D2* promoter intriguingly has not been found to harbor functional E2F sites (Ren et al., 2002). Pitx2 thus provides a direct nuclear target for interactions between the Wnt and growth factor pathways in mediating cell type-specific regulation of growth control gene expression.

In a fashion reminiscent of the regulation of the HO locus in yeast (Cosma et al., 1999), there is a sequential recruitment of specific, required coactivator complexes that include the CBP/p300 complex, the NLI/Ldb/CLIM coactivators of LIM homeodomain factors, and the TRAP/DRIP/ARC complexes. We suggest that there is a requirement for the actions of initially recruited complexes in the subsequent recruitment or actions of later components, in light of the roles of the serially recruited complexes in HO gene regulation during cell cycle (Cosma et al., 1999). DNA binding factors other than Pitx2 are likely to also mediate cofactor complex recruitment to the *Cyclin D2* promoter.

The role of Tip60 is consistent with the actions of Esa1P, a Tip60 homolog in yeast, that acetylates histone H4 and is required for normal cell cycle (Howe et al., 2001). The HAT functions of both CBP/p300 and Tip 60 appear to be required for *Cyclin D2* activation, whereas the serial recruitment of distinct acetyltransferases may be required for acetylation of specific histone residues, as well as to modify non-histone transcriptional components.

These data point to an intriguing cell-specific model for coordinated regulation of specific growth control genes, with E2F-independent regulation of *Cyclin D2* occurring in response to signal pathways that induce or control cell-specific factors. In turn, activation of *Cyclin D2*, as well as *Cyclin D1*, is required for inactivation of Rb-related pocket proteins that inhibit transcription on a series of growth control genes. Induction of *Cyclin D2* in cell types not expressing Pitx2 has been recently reported to be sensitive to c-Myc, which interacts with a TRRAP complex (Ikura et al., 2000; Bouchard et al., 2001).

Implications for Regulation of Proliferation by Cell-Type Specific Factors

While the findings presented in the manuscript provide a molecular explanation for the synergistic actions of the Wnt pathway and growth factors in stimulating proliferation in Pitx2-expressing cells, they also raise the question of whether factors analogous to Pitx2 function in other cell types. We suggest that in cell types that do not express Pitx2, other factors, including other homeodomain proteins, substitute for the actions of Pitx2 in activation of specific growth-regulating genes, serving as distinct competence factors, perhaps requiring,

at least in part, different cofactors than those recruited by Pitx2. The events described for the Wnt/Dvl/β-catenin → Pitx2 pathway that mediate synergistic regulation of cell type-specific proliferation in G1 are likely to be prototypic for similar cell type-specific control of proliferation by distinct signaling pathways.

Experimental Procedures

Genetic Manipulations

Pitx2$^{+/-}$ mice (Lin et al., 1999) of 129/Sv background were crossed with *Pitx1*$^{+/-}$ mice of 129/Sv background and *Pitx2*$^{+/-}$ C57BL6 with *Dvl2*$^{+/-}$ mice of 129/Sv/NIH Black Swiss background (Hamblet et al., 2002) were used to generate double heterozygous animals. The generation of transgenic mice was produced by inserting *Pitx2a* cDNA into a vector containing a 15 kb mouse *Pit-1* promoter and founders were analyzed at e 17.5.

Antibodies, Immunohistochemistry, and In Situ Hybridization

Mouse Pitx2 protein produced in the Baculovirus system according to standard procedures was used to produce αPitx2 IgG polyclonal antisera guinea pigs. Pax3 (Gross et al., 2000) rat polyclonal was used at 1:400 dilution. The following commercially available antibodies were used: β-galactosidase, rabbit polyclonal (Cappel, 1:100); BrdU, rat polyclonal (Jackson, 1:500); Connexin 43, rabbit polyclonal (Zymed, 1:100); β-catenin, HDAC1, HDAC2, Tip60, TCFs, and LEF-1 rabbit polyclonal (Santa Cruz). Hybridization with ^{35}S-labeled antisense RNA probes was performed as previously described (Jepsen et al., 2000).

Single Cell Nuclear Microinjection Assays

Microinjection assays were carried out as described (Kamei et al., 1996). Affinity purified αPitx2 IgG was used. Each experiment was performed on three independent cover slips consisting of 1000 cells. Where no experimental antibody was used, preimmune IgGs were coinjected, allowing the unambiguous identification of injected cells in addition to serving as a preimmune control. BrdU incorporation experiments were conducted in serum-starved C2C12 cells. GST fusion proteins purified from bacterial extracts were microinjected at ~100 μg/ml into the nucleus of cells with fluorescein-conjugated dextran as a carrier, resulting in the introduction of ~10,000 molecules of fusion protein/cell. Cells were then treated with or without serum and a series or recombinant growth factors in the presence of BrdU labeling solution (Amersham) for 14 hr after fixation.

Chromatin Immunoprecipitation Assays

For the chromatin immunoprecipitation assay, αT_3-1 a murine pituitary cell line or C2C12 myoblast cell lines were utilized, and LiCl (10 mM) was added for 1 hr prior to harvest. Cells were washed twice with PBS and crosslinked with 1% formaldehyde for 10 min at room temperature. Crosslinked cells were treated as previously described (Scully et al., 2000; Baek et al., 2002). For PCR, the following promoter specific primers were used: *Pitx2* forward 5′ GACCAG TGGCAGGAGCTAG-3′; reverse 5′ AAAGCCTCACCCGGGATCA-3′, *c-Myc* forward 5′ GCTTGGCCCCAAAAAGAAGGG-3′, reverse 5′ AGAGCTGCCTTCTTAGGTCG′, *Cyclin D2*, forward 5′ GGCATAACC TTTATCCCTGGTTT-3′, reverse 5′ AACCCCATGGATTCCTATTG ATT-3′, αGSU forward 5′ GTCCTCAGA ATC ACCTCATACCTT, reverse 5′ ATTGACCCTTACACAAAACATCCT, p21 forward 5′ CTC TGGGAAGCCAGAAGTTGTT, reverse 5′ GGTCCAGTCCCTGCATCT AAGT.

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