UNIVERSITY OF CALIFORNIA, SAN DIEGO

REGULATION OF CELL LINEAGE DETERMINATION BY A *PAIRED*-LIKE HOMEODOMAIN

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The dissertation of Jessica Tollkühn is approved, and is acceptable in quality and form for publication on microfilm:

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ABSTRACT OF THE DISSERTATION

REGULATION OF CELL LINEAGE DETERMINATION BY A *PAIRED*-LIKE HOMEODOMAIN

by

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This dissertation describes the role of the transcription factor Prop1 in pituitary development. During pituitary development, six hormone-secreting cell types emerge from a common primordium, making it an ideal model system for the study of cell-lineage commitment and differentiation. This process is mediated through the actions of opposing signaling gradients, which induce transcriptional regulators in a distinct spatial and temporal fashion. Overlapping patterns of transcription factors regulate the proliferation of precursor cells and the generation of specific cell types. One of these factors, Prop1 is a *paired*-like, pituitary-specific homeodomain factor, expressed

transiently between e10.5 and birth. Genetic and biochemical analysis have established that Prop1 is required for both proper morphogenesis of the pituitary gland, and for the determination of the Pit1 lineage, which produces growth hormone, prolactin and thyroid-stimulating hormone. In addition to the loss of the *Pit1* expressing cell types, the Prop1 pituitary displays a striking dysmorphogenesis due to the apparent failure of cells to migrate from the pituitary lumen to populate the caudomedial portion of the anterior lobe. In this work, I will discuss how Prop1 regulates lineage determination in pituitary development.

Chapter 1 details the process of pituitary development, with a focus on the various signaling pathways and transcription factors that have been identified through genetic and biochemical analysis.

Chapter 2 presents results from both loss- and gain-of function genetic studies, in the form of the *Prop1* knockout mouse and a *Pit1-Prop1* transgenic line.

Chapter 3 describes the mechanism of *Pit1* gene regulation through the actions of Prop1 and the Wnt signaling effector, β-catenin.

Chapter 4 describes the identification of *Prop1* regulatory information through the use of *lacz* reporter transgenes. Cooperation between Prop1 and the Notch signaling pathway in the maintenance of a pleuripotent progenitor population will also be discussed.

Chapter 5 examines further the genetic programs mediated by Prop1 and β-catenin through microarray analysis of e12.5 pituitaries and concludes with a discussion of my findings and their implications.

Chapter 1: The Pituitary Gland As A Model For Cell-Type Determination

Introduction

Identifying molecular mechanisms that regulate cellular diversification during metazoan development has long been a central question in biology. The pituitary gland has proven to be an instructive model in which to investigate the appearance of specific cell types from a common primordium. Initial extrinsic signals from organized signaling centers in the form of secreted morphogens or transmembrane signaling receptors, give rise to cell-autonomous intrinsic signals. These early events regulate organ commitment and proliferation. Opposing dorsal and ventral signaling gradients induce spatially overlapping patterns of transcription factors, which then regulate the appearance and differentiation of individual cell types. In this chapter, I will give an overview of pituitary development, with a specific focus on the required signaling molecules and transcription factors that have been identified through genetic and biochemical analysis. The final section of the chapter introduces the homeodomain factor Prop1, whose role in pituitary development will be the focus of this dissertation.

The six endocrine cell types of the pituitary gland produce POMC (pro-opiomelanocortin), which is proteolytically cleaved to produce adrenocorticotropic hormone (ACTH) in corticotropes and melanocyte-stimulating hormone (MSH) in melanotropes, thyroid-stimulating hormone (TSH) in thyrotropes, growth hormone (GH) in somatotropes, prolactin (Prl) in lactotropes, and the gonadotropins; follicle stimulating hormone (FSH) and luteinizing hormone (LH) in gonadotropes. An

additional cell type, the rostral tip thyrotrope, is present only in the developing embryo [1-3]. These hormones act on peripheral target tissues to regulate homeostasis, stress response, reproduction, growth and lactation. Production and secretion of pituitary hormones is directed by the hypothalamus, which instructs the gland both through direct axonal projection into the posterior lobe, and through release of hypophysiotropic hormones that reach the intermediate and anterior lobes through the hypophyseal portal [4].

Signaling regulation

The pituitary gland is of dual embryonic origin. The posterior lobe arises from neuroectoderm, while the anterior and intermediate lobes develop from ectoderm. Beginning at e8.5, cells of the anterior pituitary placode in the oral ectoderm thicken and invaginate to form Rathke's pouch [4]. By e10.5, invagination is complete, and the overlying infundibulum begins to evaginate toward the nascent gland. Classical co-culturing experiments have shown that this direct contact with the developing ventral diencephalon is required for differentiation and survival of the pituitary [2, 5]. At e12.5, the gland has fully separated from the underlying oral ectoderm, and consists of a population of pleuripotent ectodermal cells surrounding a central lumen. Cells proliferate in the lumenal area, and then migrate out to populate the developing anterior lobe, where they begin to express their terminal markers between e13.5 and birth.

The use of mouse genetics has identified roles for many well known signaling pathways in pituitary development. Opposing dorsal and ventral signals regulate organ commitment, patterning and cell-type specification through induced gradients of

transcription factors. The first secreted signaling molecule to be expressed from the infundibulum is bone morphogenetic protein 4 (BMP4), at e8.5. In the absence of BMP4, the pouch is never formed [6]. It is thought that one of the functions of BMP4 is to maintain the expression of the LIM-homeobox *Islet1* within the gland, as *Isl1* null embryos also lack a pouch [7]. Other dorsal signals include members of the fibroblast growth factor family (FGF): FGF 8, 10 and 18, which are required for proliferation and commitment [7, 8]. These dorsal signals are balanced by a ventral signal of Sonic hedgehog, which is secreted by the oral ectoderm. Shh acts with FGFs to sustain expression of the LIM homeodomain *Lhx3* [1, 8]. Lhx3 is one of the first transcription factors to be expressed specifically in the pituitary gland, and its deletion results in the absence of almost all pituitary cell types, with the exception of a few corticotropes, which are the first lineage to terminally differentiate [9]. An intrinsic ventral to dorsal gradient of BMP2 initiates at e10.5, but extends to the entire pouch by e12.5 [7, 8].

Transcription factors

Two *bicoid*-related homeodomain factors, *Pitx1* and *Pitx2*, are expressed in overlapping patterns throughout the pituitary. *Pitx1* knockout mice display a loss of the terminal markers for differentiation of the thyrotropes and gonadotropes, and an increase in corticotropes, as well as additional defects in crainiofacial and hindlimb morphogenesis [10, 11]. Pitx2 is well-known for its role in left-right asymmetry. Targeted deletion of *Pitx2* results in failure of body-wall closure, right pulmonary isomerism, cardiac outflow tract abnormalities, and defects in pituitary, eye and tooth organogenesis [12-15]. *Pitx2*$^{-/-}$ pituitaries resemble those of the Lhx3 knockout, with

an arrest early in organogenesis at e10.5. The pouch never comes into contact with the infundibulum, so the proper signaling gradients are not established [14].

Isl1, Pitx1/2 and Lhx3/4 all play roles in early pituitary patterning and expansion. As development progresses, many other transcription factors are needed to specify the terminal differentiation of the various hormone-secreting cell types. Analysis of the Snell (*dw*) and Jackson (dw^J) allelic murine dwarf mutations established that Pit1, a POU transcription factor, is required for the activation of the secreted hormones and receptors expressed in somatotropes, lactotropes and thyrotropes [16, 17], which are collectively referred to as the Pit1 lineage. The POU family of transcription factors contain an amino-terminal POU specific domain and a carboxy-terminal POU homeodomain. Pit1 is also required for the survival and expansion of Pit1-lineage cells following birth [18, 19]. Both thyrotropes and lactotropes contain the protein αGSU, which is a required alpha-subunit for TSHβ in thyrotropes, as well as for FSHβ and LHβ in lactotropes. This shared subunit suggests a common ancestral precursor for these two cell types [2]. The zinc-finger factor GATA-2 is also expressed in both cell types, but at higher levels in gonadotropes. It is thought that competition between Pit1 and GATA-2 drives cell fate towards that of thyrotrope or lactotrope, respectively. Overexpression of GATA-2 under the *Pit1* promoter is sufficient to convert all Pit1 lineages to a gonadotrope fate, while ventral targeting of *Pit1* using the *αGSU* promoter results in a switch from gonadotropes to thyrotropes [20]. Gonadotropes also require the orphan nuclear receptor SF-1, [21-23]. The Tbox factor Tpit is selectively expressed in POMC-containing corticotropes and melanotropes and is capable of activating the *POMC* gene [24]. Although ectopic

expression of Tpit under the *αGSU* promoter is sufficient for production of ACTH in the rostral tip [24], mice with a targeted deletion of Tpit still express POMC, although in decreased numbers [25]. Other factors such as NeuroD1 and Nurr77 have been implicated in POMC regulation, but these experiments have been largely performed in cell lines [3]. As the corticotrope lineage is the first determined cell-type to appear, it is possible that it is the "default" cell-type that is generated in the absence of other specifying signals.

Reciprocal regulation by Prop1 and Hesx1

The Ames dwarf (*df*) mouse, has a similar phenotype to the Snell and Jackson mice, with a hypoplastic anterior lobe, although no *Pit1* transcripts are detectable, indicating that the Ames mutation is epistatic to Pit1 [26]. The gene responsible for the Ames phenotype was positionally cloned by genetically directed representational difference analysis (GDRDA) and termed *Prophet of Pit1* (*Prop1*). Prop1 is a pituitary-specific, *paired*-like homeodomain transcription factor. The *df* allele encodes a protein with a single point mutation (S83->P) in the α1 helix of the Prop1 homeodomain, dramatically reducing DNA binding activity. *Prop1* expression initiates in the dorsal part of Rathke's pouch at e10, peaking throughout the gland at e12.0. Transcripts are prevalent in the caudo-medial portion of the gland, at e13.5, the time at which *Pit1* expression is activated, but then show a marked decrease following e14.5 [27].

In addition to the loss of the three *Pit1* expressing cell types, the Ames pituitary displays a striking dysmorphogenesis, beginning at e13.5. The lumen undergoes a dramatic expansion at the expense of the anterior lobe. The basis of this

phenotype is unknown, but it has been postulated that the perilumenal cells are analogous to the ventricular zone of the developing nervous system. Final determination occurs upon migration away from the lumen. In the Ames pituitary, this process is defective, with the Pit1 lineages failing to leave the lumen. This then causes the expansion of the lumen, as well as a failure of determination. This model implicates Prop1 as controlling some aspect of asymmetric cell division or migration, but this has not yet been confirmed [27].

Another *paired*-like homeodomain factor essential for pituitary development is the repressor Hesx1. *Hesx1* is expressed broadly in early development, throughout many of the anterior, placodally-derived structures, including the eye, olfactory epithelium, forebrain and pituitary [28, 29]. Hesx1 is present throughout the oral ectoderm and invaginating Rathke's pouch, but levels decline around e12.5, with no detectable transcripts by e14.5. However, in the Ames mouse, Hesx1 can still be detected at this time [27]. The *Hesx1* expression pattern is reciprocal to that of *Prop1*, as the decrease of *Hesx* mirrors the rise in *Prop1* [30]. This reciprocity can be extended to functional actions of Prop1 and Hesx1 in transient transfection assays. Both Prop1 and Hesx1 are capable of binding to a palindromic *paired* binding site (PrdQ), both as homodimers, and as a heterodimer, with Prop1 always acting as an activator, and Hesx1 as a repressor in this assay [27, 30]. Repression by Hesx1 is mediated through a conserved eh-1 motif, which recruits members of the Groucho/TLE corepressor family [30].

Analysis of *Hesx1* knockout mice revealed the complete loss of the pituitary in 5% of the mutants. The remaining mice displayed either multiple invaginations or

extreme overgrowth of the gland. These phenotypes result from the expansion of FGF8/10 throughout the oral ectoderm. A negative feedback loop between these FGFs and Hesx is required for proper boundary formation during organogenesis. Interestingly, temporal misexpression of *Prop1*, under the *Pitx1* promoter also results in complete ablation of the pituitary. These results indicate that Hesx1 and Prop1 may reciprocally inhbit and activate genes involved in lineage commitment, as failure of repression in *Hesx1* mutants and premature activation in *Pitx1-Prop1* transgenics both block organogenesis [30]. The specific mechanisms utilized by Hesx1/Prop1 in regulating lineage commitment will be discussed in Chapter 3.

Conclusions

The last few years have seen many new insights into pituitary development, as conditional deletion strategies have elaborated upon initial findings from transgenic animals. The use of microarrays to identify changes in gene expression have revealed many additional factors and pathways that have not previously been considered in the pituitary. In addition, the technique of chromatin immunoprecipitation has made it possible to identify novel enhancer regions, and to investigate the temporal events linked to gene activation and repression. In this dissertation, I will discuss the application of these various methods to the study of the function of Prop1. Chapter 2 describes the creation and analysis of both *Prop1* null animals and Prop1 gain-of-function transgenics. Chapter 3 details the effects of pituitary-specific deletion of *β-catenin* on cell-type determination, and how this process is mediated by the actions of Prop1. In Chapter 4, I will address further the interplay between Wnt/β-catenin

signaling, and the actions of Prop1, with an emphasis on genomic data obtained from microarray analysis of both *Prop1* null and *β-catenin* null embryonic pituitaries. Finally, in Chapter 5, I will look at upstream of *Prop1*, and describe identification of *Prop1* regulatory information, generation of a *Prop1-Cre*, and the role of the Notch pathway and Prop1 in maintaining a proliferating precursor population.

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Chapter 2: Genetic Strategies for the Analysis of Prop1 Function

Introduction

The human *PROP1* gene shows over 90% homology with mouse *Prop-1* within the homeodomain (ref). Mutations in *PROP1* are the primary cause of familial combined pituitary hormone deficiency (CPHD) [1-3]. CPHD is defined by impaired production of growth hormone (GH) and one or more of the other five anterior pituitary hormones. A study of four CPHD families revealed deficiencies in LH and FSH production, in addition to decreased GH, prolactin and thyroid stimulating hormone. PROP1-defective patients from these families also fail to respond to gonadotropin-releasing hormone stimulation. In transient cotransfection assays and gel shifts, constructs containing human *PROP1* mutations displayed even weaker DNA binding activity than the Ames mutant protein [1]. These observations may indicate an additional function of Prop-1 that is not affected in the Ames mouse.

Results

Generation and analysis of Prop1 knockout mice

In order to resolve the discrepancy between human families with inactivating mutations of Prop1 and the phenotype of the Ames mouse, the *Prop1* knockout mouse was generated (Figure 2.1). As the *Prop1* gene is small, with only three exons, the targeting vector was designed to replace almost the entire Prop1 locus, leaving only 5' UTR and coding sequence for the first 8 amino acids. *In situ* analysis of pituitary markers demonstrated a complete recapitulation of the Ames phenotype in the *Prop1*

knockout. At e15.5, *Pit1* is completely absent. Thyrotropes are also lost, with the exception of the rostral tip thyrotropes, which are not dependent on Pit1. Corticotropes appear unaffected, in contrast to some previous reports indicating hypercortisolism in human patients with mutations in *Prop1* [4] (Figure 2.2). At e18.5, *Prop1* null mice appear to have a full complement of gonadotropes, as measured by *LHβ* levels (Figure 2.3). By six weeks of age, the *Prop1* -/- gland is quite hypoplastic, with such a limited amount of tissue, that it is difficult to analyze. The posterior lobe, however, appears normal. There are no somatotropes, as in the Ames mouse, but *POMC* and *αGSU* are both expressed. *LHβ* also continues to be expressed (Figure 2.4).

While these studies were being carried out, the *Prop1* knockout mouse was published by another lab [4]. This group found that in the 129 background, deficiency of TSH led to secondary endocrine problems, causing respiratory distress due to decreased surfactant production in the lung. These problems were not evident in the B6 background, presumably because this strain normally has more robust thyroid activity. Variability in gonadotrope function between different backgrounds was also seen. In the 129 background, LH levels appeared normal at e16.5 and 17.5, but was lost at e18.5. However, in the B6 outbred background, LH levels were normal [4].

As all of our analysis was carried out in the outbred B6 background, we observed no additional defects in the *Prop1* null mice when compared with the Ames. The loss of FSH and LH in human families with PROP1 mutations must therefore be due to the genetic and developmental differences between mice and humans. It is also possible that these gonadotrope deficiencies are secondary to the loss of the regulation of *TSHβ* by Pit1, as mice require thyroid hormone for proper gonadotrope function

[4]. We conclude that Prop1 exerts its effects solely through DNA binding, and that these effects are specific to the Pit1 lineage in the mouse.

Overexpression of Prop1 delays terminal differentiation

The peak expression of *Prop1* coincides with a burst of proliferation at e12.5. As the anterior lobe has been described as extremely hypoplastic in the Ames mice [5-7], BrdU labeling experiments were performed. BrdU was injected in wild-type and mutant mice at e12.5 and embryos harvested at birth. This extended incubation period should result in an increase in the number of BrdU-positive cells when proliferation is down, as once a cell has undergone three rounds of division, it is no longer detectable by the BrdU antibody. Staining with αBrdU on frontal sections showed approximately equal numbers of labeled cells (Figure 2.5). Therefore, there is no reduction in the number of cell divisions in the absence of Prop1. It appears that the loss of cells in the anterior lobe is not due to a decrease in cell number, but rather a mis-localization due to the failure of *Prop1* -/- cells to migrate from the lumen (Figure 2.6). Similar conclusions have been reached through the use of BrdU pulse-labeling at e12.5 and e14.5; after a 2 hour pulse at each time point, there was no change in the number of labeled cells in either wild-type or *Ames* mutant pituitaries [8].

Since the *Prop1* -/- pituitary has the same number of cells as the wild-type, the absence of Pit1 is not due to a loss of the *Pit1*-expressing population. As Prop1 levels decline as specific cell types appear, I hypothesized that Prop1 may play a role in the maintenance of a precursor population. To test this, I constructed a transgene expressing *Prop1* under the control of the *Pit1* promoter. Extending the expression of

Prop1 should delay terminal differentiation of the Pit1 lineage. Analysis at P0 shows that, while *Prop1* levels are quite low in wild-type mice, *Pit1-Prop1* positive transgenes display robust *Prop1* expression. Surprisingly, *Pit1* expression seems somewhat lower in transgenes, as does *TSHβ*. Interestingly, *GH* levels are more strikingly downregulated than those of *TSHβ*, with only a few cells producing signal (Figure 2.7, and data not shown).

Analysis of transgenes at 26 days of age reveals that this decrease in GH persists into adulthood, as mice are visibly smaller when compared with wild-type (Figure 2.8). In addition, transgenes also have a hypoplastic gland, similar to that found in the *Prop1* knock-out, but less severe. It is probable that the origin of this hypoplasia is similar to that in the Prop1 knockout, where the undifferentiated cells fail to mature and continue normal post-natal proliferation, and instead undergo apoptosis [9]. The role of Prop1 in defining a precursor population has also been suggested by results obtained from its over-expression under the *αGSU* promoter [10]. *αGSU* expresses in thyrotropes and gonadotropes, and *αGSU-Prop1* transgenes display a delay in gonadotrope differentiation, with normal *GHRHR* expression at birth, but no *FSHβ* or *LHβ*. Although hormone levels have recovered by 4 weeks of age, this early reduction in terminal markers, accompanied by normal levels of earlier cell determination genes, parallels the results from the *Pit1-Prop* transgene, with GHRHR replacing Pit1 as the "determination" mark. Also noteworthy is the mild hypothyroidism in *αGSU-Prop1* adults, another sign that differentiation is impaired [10]. It is interesting to note that neither *Pit1-Prop1* or *αGSU-Prop1* transgenes

express more *Pit1*. Although ectopic expression of Prop1 appears to delay differentiation in any lineage, early expression of Prop1 in developing gonadotropes does not result in activation of the *Pit1* gene, indicating that an additional signal is required. The origin of this signal, and the mechanism by which Prop1 activates the *Pit1* gene, will be discussed in the following chapter.

Discussion

In order to elucidate the role of Prop1 in pituitary development, we have performed both gain- and loss-of-function genetic studies. Abrogation of Prop1 protein produces the same phenotype as when Prop1 is present, but unable to bind DNA. This is in contrast to many human patients with mutations in PROP1, who have reduced levels of FSH and LH in addition to the loss of GH, TSH, and prolactin [1]. In fact, it has been suggested that decreased serum gonadotropins be used as a diagnostic marker for mutations in PROP1, rather than POU1F1 (Pit1) [4]. As no such deficiencies are found in outbred Prop1 knockout animals, it would seem that gonadotropin dysfunction in certain human families occurs secondarily due to other modifying genes. The finding that the *Prop1* knockout mouse appears indistinguishable from the *Ames*, indicates that Prop1 functions solely through direct interaction with DNA, although another interpretation is that the S83P mutation somehow disrupts the structure of the homeodomain; a region that could potentially be required for protein-protein interaction. This finding also raises the question of whether or not Prop1-positive progenitor cells contribute to the entire pituitary, as the

differentiation of non Pit1 cells appears unaffected by loss of Prop1, even though these cells have also failed to migrate from the lumen.

Although it has previously been established that the anterior lobe of Ames mutant pituitaries is hypoplastic at birth, BrdU-labeling experiments have demonstrated that there is no change in proliferation (Figure 2.5). Immunostaining of Ames mutant sagittal sections with α-Prop1, shows that the overall volume of the mutant gland is unchanged, and that it is only the position of the cells that is altered. Cells fail to migrate away from the lumen, and it becomes convoluted to such an extent that it is possible to detect Prop1-positive cells dorsal to the nascent posterior lobe (Figure 2.6). This migration defect has been characterized by sequential labeling of proliferating cells with IdU and BrdU at e11.5 and e12.5, respectively. Analysis at e14.5 shows retention of both labels within the lumen in Prop1-null pituitaries, while in wild-type animals, proliferating cells have begun to populate the anterior lobe [9].This migration defect will be discussed further in Chapter 4.

Prop1 is most highly expressed at e12.5, when the gland still consists of a population of pleuripotent ectodermal cells surrounding the lumen. As Prop1 plays no role in the proliferation of these cells, its function must somehow be related to the cell identity and determination events that commence at this time. Extending Prop1 expression under either the *Pit1* (Figure 2.7) or *αGSU* [10] promoters, results in a delay in cell-type differentiation. In the case of the *Pit1-Prop1* transgenes, this delay is never fully recovered from, as adult mice can be distinguished from wild-type by eye, due to their small size. In conclusion, our findings show that Prop1 is required in a specific

temporal window to hold cells in an undifferentiated state. The role of Prop1 in maintaining a precursor population will be discussed in greater detail in Chapter 4.

Methods

Prop1 knockout mice

Prop1 mutant mice were generated by targeted mutagenesis in ES cells to replace the entire coding sequence with a β-galactosidase/neomycin selection cassette (Figure 2.1), and correct targeting was established by Southern blotting with 5' and 3' external probes. Mutant mice were genotyped by PCR with oligos:

wild type tgcacctgatcccagctctaag/cgtgtgaacagttagggttgcc

mutant (lacz) caacgagacgtcacggaaaatgcc/ccaacagttgcgcagcctgaatg.

Histology

For immunostaining, embryos were fixed for 30 minutes in 4% PFA, then dehydrated in 20% sucrose, frozen in 1:1 OCT/Aquamount, and sectioned at 14 microns. Prop1 protein was detected with a guinea-pig anti-Prop1 antibody, made to the C' of Prop1 (aa129-225). Secondary antibody from Molecular Probes was AlexaFluor 594-conjugated, and nuclei were counterstained with DAPI. *In situ* hybridization was performed as previously described [11], on formalin-fixed 14 micron cryosections, using S-35 labeled antisense probes.

BrdU labeling

BrdU was injected into pregnant mice at 100mg/g body weight. Embryos were harvested at birth and treated as in other immunostaining experiments. BrdU was detected with an anti-BrdU antibody at 1:50 (ICN BIomedicals) and AlexaFluor 594-conjugated secondary.

Pit1-Prop1 ***transgenic mice***

The 15kb *Pit1* promoter [12, 13] was used in combination with a cassette containing a 5' rabbit β-globin intron, and a 3' human growth hormone polyadenylation signal, used as a splice acceptor for the mouse Prop1 cDNA. The transgene was linearized and removed from the pBluescript backbone with NotI. The transgene DNA was purified by agarose gel electrophoresis, electroelution and dialyzed overnight into microinjection buffer (7.5mM Tris, pH7.4,, 0.15mM EDTA). Pronuclear injection was performed by the transgenic core facility at UCSD. Founders were identifed by PCR analysis.

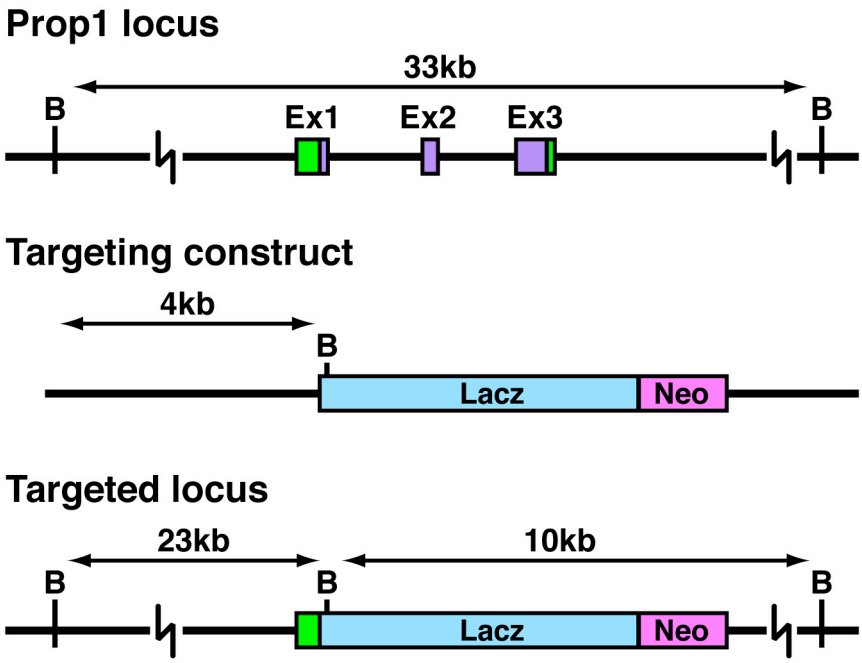

Figure 2.1. *Prop1* knockout.
The targeting vector was designed to replace the entire *Prop1* locus, with only the first 8 amino acids of coding sequence remaining. Digestion with BamHI produces a single 33kb wild-type fragment or two mutant fragments of 23kb (5') and 10kb (3').

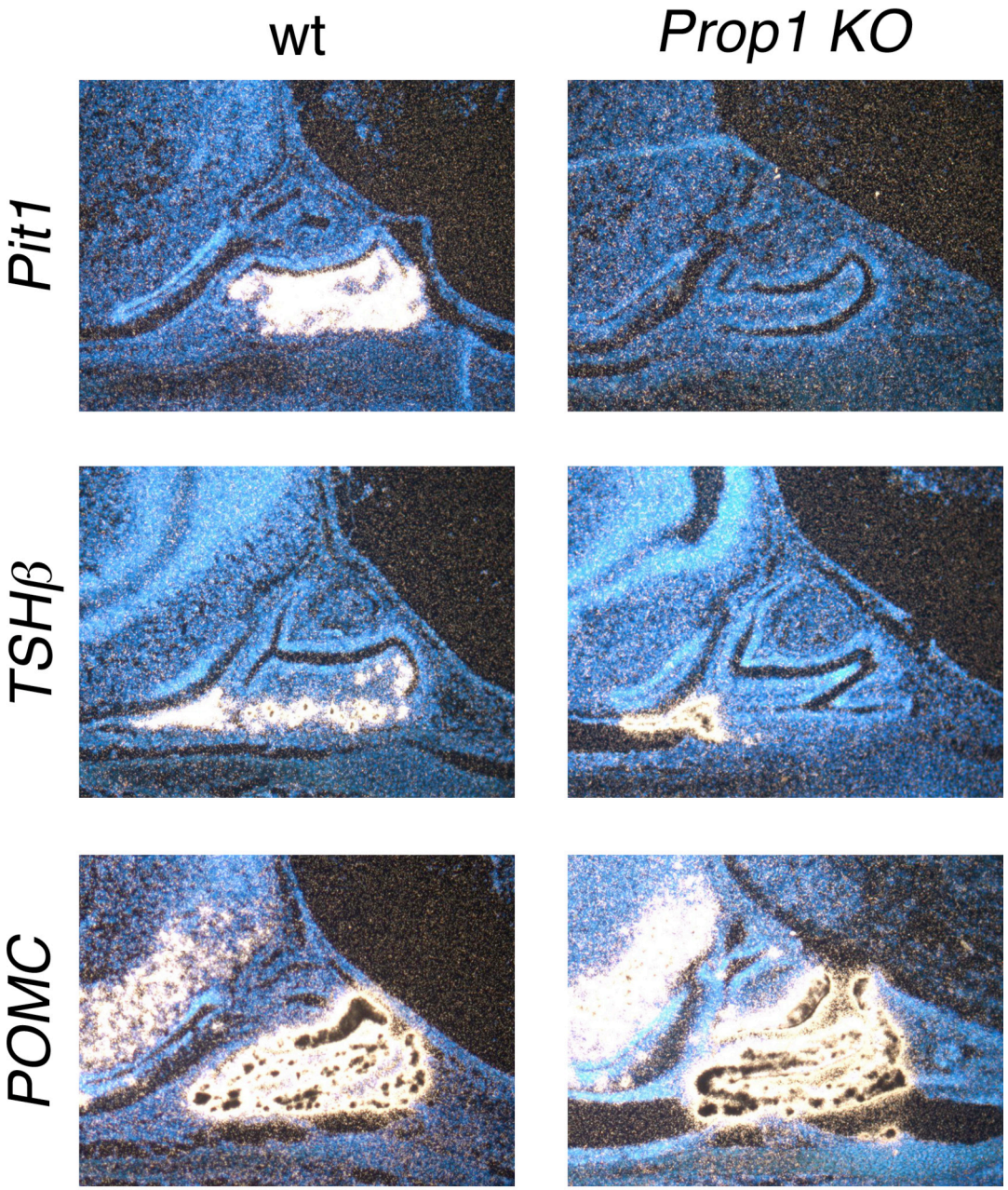

Figure 2.2: The *Prop1* knock-out mouse recapitulates the Ames phenotype. *In situ* analysis of sagittal sections from e15.5 wild-type and *Prop1* null embryos. *Pit1* is expressed normally in wild-type pituitaries, but not in pituitaries from *Prop1* knock-out animals. Similarly, the Pit1-dependent population of thyrotropes is also lost in *Prop1* mutants, as shown by *TSHβ* expression. The Pit1-independent thyrotropes in the rostral tip are unaffected, as are corticotropes and melanotropes, shown by expression of *POMC*.

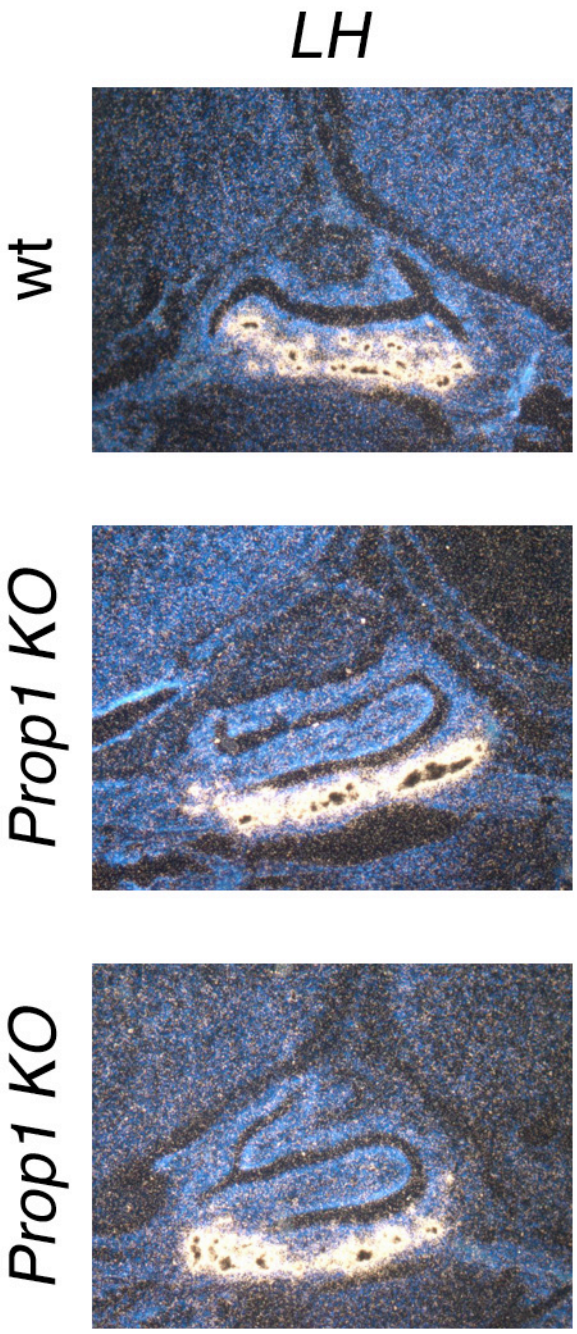

Figure 2.3: *LH* expression appears normal in e18.5 Prop1 null embryos.
In situ hybridization for *LH* reveals no detectable difference in levels in two different *Prop1* null animals, when compared with wild-type.

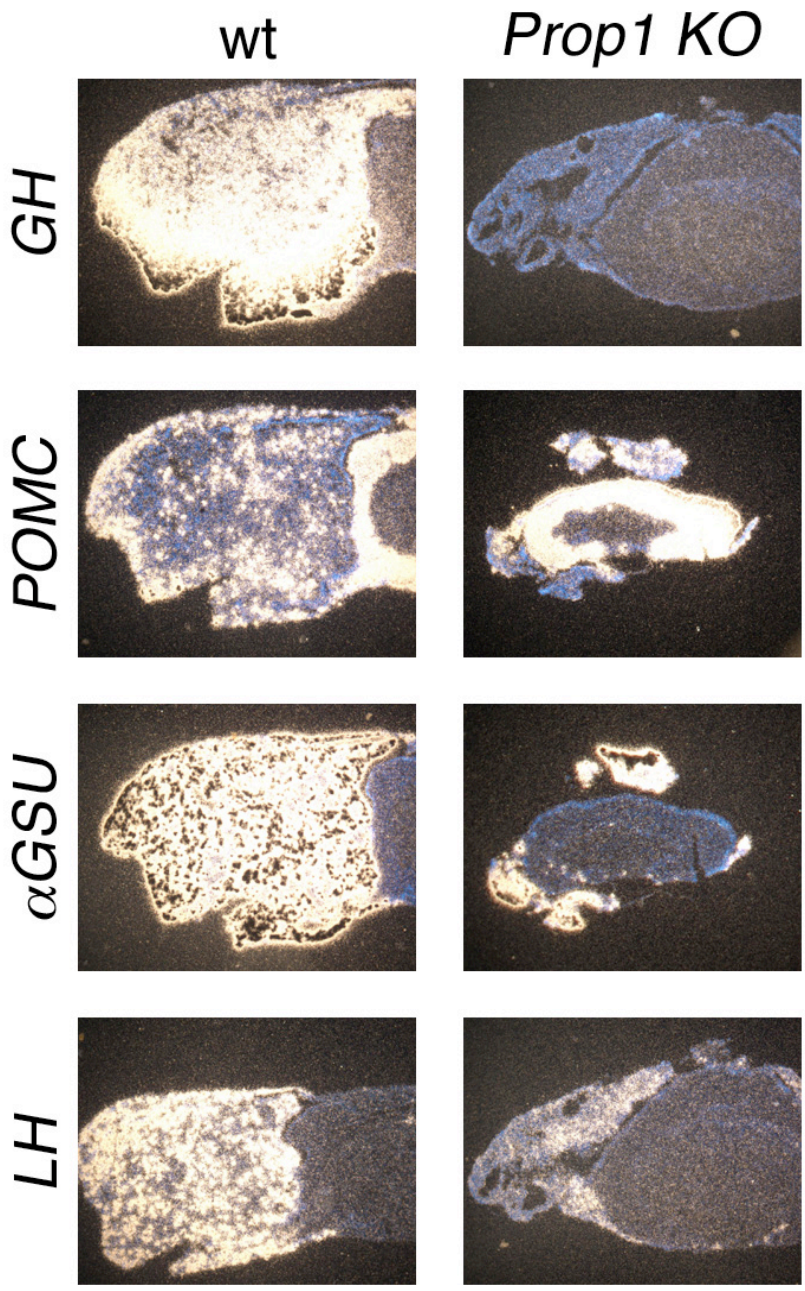

Figure 2.4: *Prop1* knock-out animals have a drastically reduced anterior pituitary.
In situ hybridization on frontal sections. At six weeks of age, the anterior lobe of the pituitary is quite small, but still expresses markers for cell types outside of the Pit1 lineage. *GH* is still absent, but *POMC*, *αGSU* and *LH* remain. The photographs of the *POMC* and *αGSU* probes on Prop1 knockouts were taken at 2.5x, while the rest used 5x.

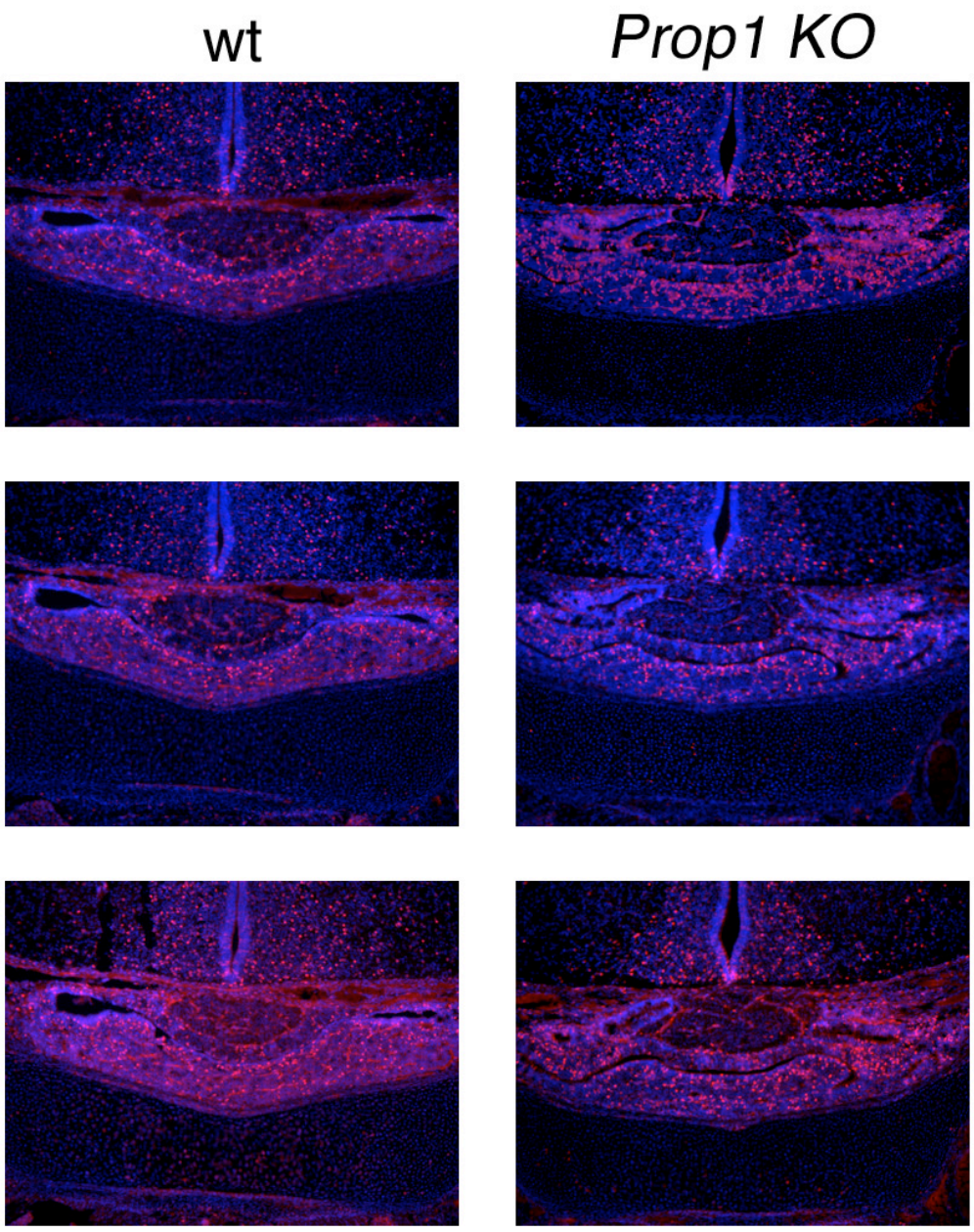

Figure 2.5: *Prop1* null pituitaries display no obvious defect in cell proliferation. Matched frontal sections of P0 wild-type and *Prop1* null mice. Pregnant mice were injected with BrdU at e12.5 of gestation. Retention of label indicates cells have divided less than three times since injection. *Prop1* null pituitaries do not appear to have significantly more labeled cells than wild-type.

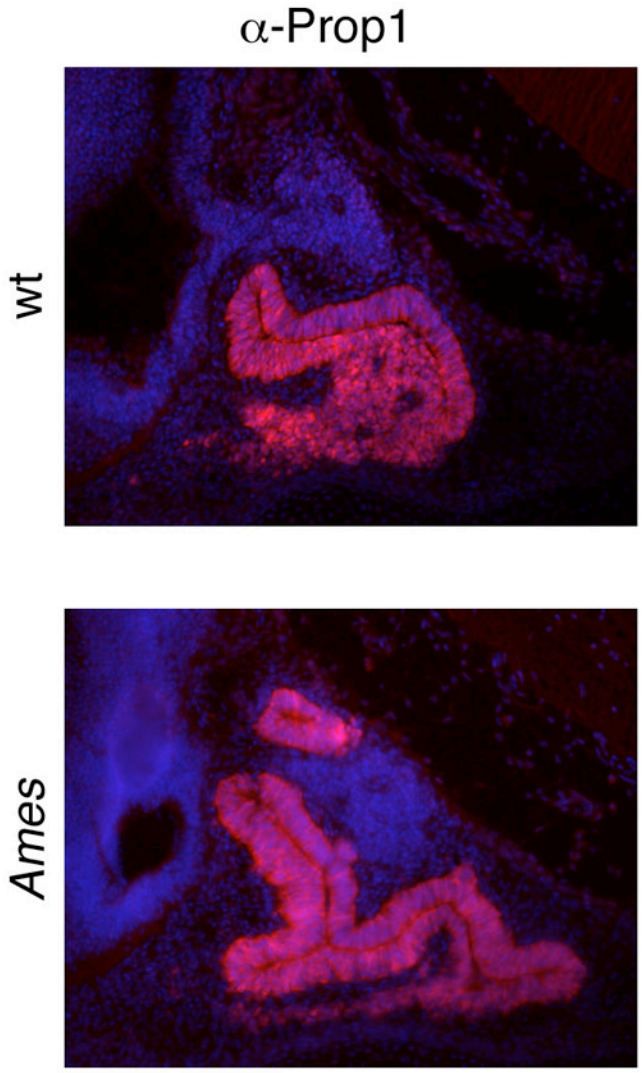

Figure 2.6: Ames mutant pituitaries display a striking dysmorphogenesis. Sagittal sections of e14.5 pituitaries from wild-type or Ames mice. Immunostaining with an antibody to Prop1 reveals a failure of cells to migrate from the lumen to populate the anterior lobe. The lumen is so convoluted that it pushes up dorsal to the posterior lobe.

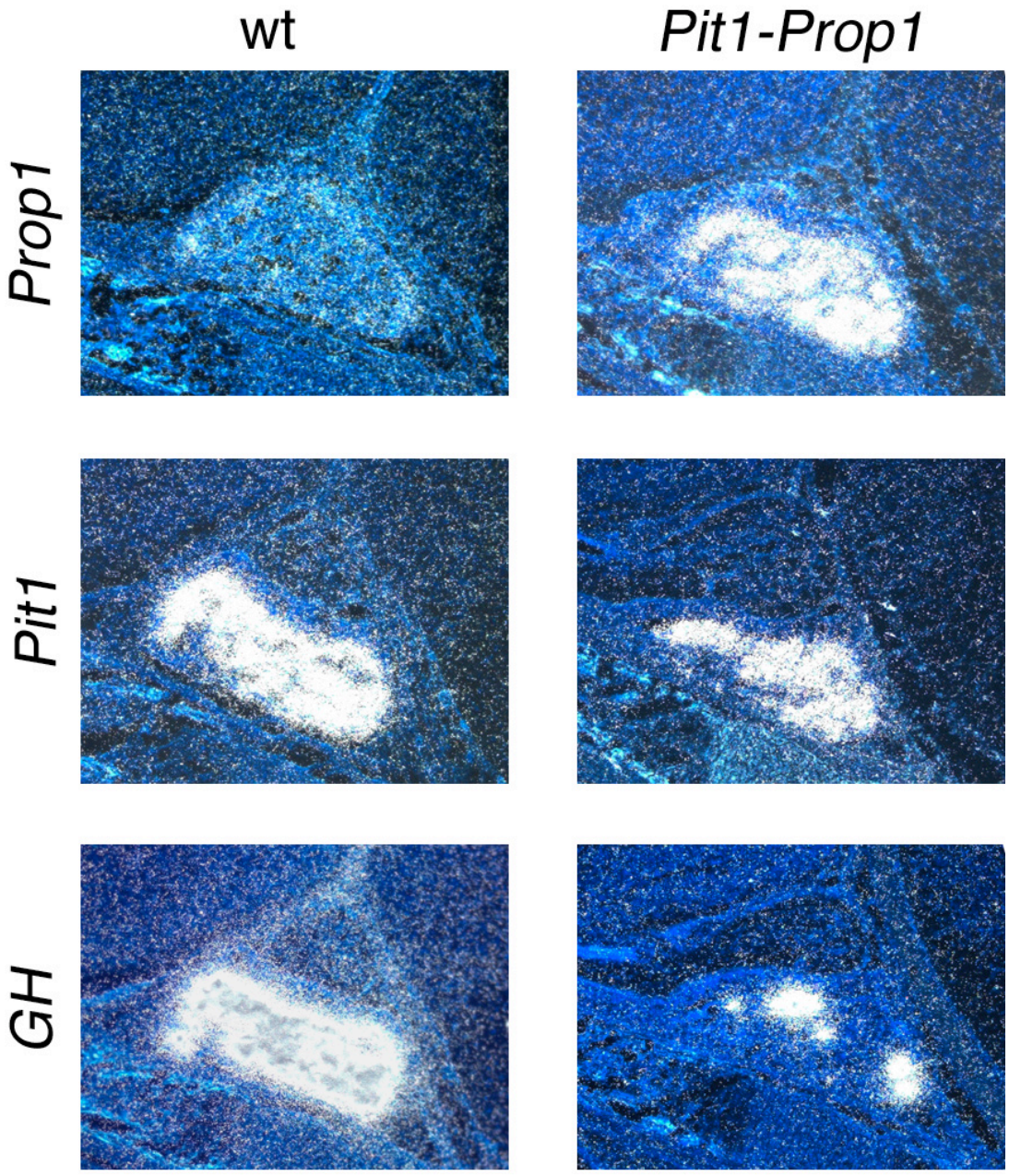

Figure 2.7: Overexpression of *Prop1* blocks terminal differentiation.
In situ analysis of P0 sagittal sections. In wild-type animals, *Prop1* expression has already declined, but the transgene is expressed robustly. *Pit1* expression is slightly decreased, and somatotrope number (*GH*)is dramatically reduced.

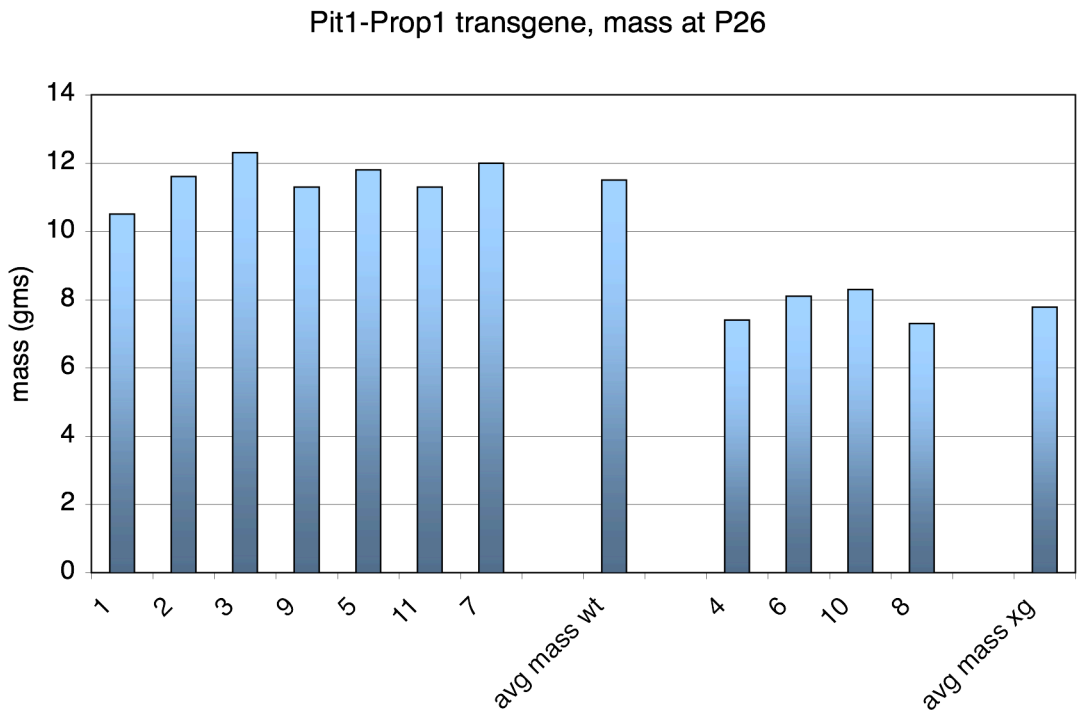

Figure 2.8: *Pit1-Prop* transgenic mice display post-natal dwarfism.
Mice carrying the *Pit1-Prop* transgene are approximately 30% smaller than wild-type littermates at 26 days of age. This size difference is easily detectable by eye.

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Chapter 3: A Prop1/Beta-Catenin Complex Dictates Cell Lineage Determination

Abstract

While the biological roles of canonical Wnt/β-catenin signaling in development and disease are well documented, understanding the molecular logic underlying the functionally distinct nuclear transcriptional programs mediating the diverse functions of β-catenin remains a major challenge. Here, we report an unexpected strategy for β-catenin-dependent regulation of cell lineage determination, based on interactions between β-catenin and a specific homeodomain factor, Prop1, rather than Lef/Tcfs. β-catenin acts as a binary switch to simultaneously activate expression of the critical lineage-determining transcription factor, *Pit1*, and to repress the gene encoding the lineage-inhibiting transcription factor, *Hesx1*, acting via TLE/Reptin/HDAC1 corepressor complexes. The strategy of functionally-distinct actions of a homeodomain factor in response to Wnt signaling is suggested to be prototypic of a widely-used mechanism for generating diverse cell types from pluripotent precursor cells in response to common signaling pathways during organogenesis.

Introduction

Among evolutionarily conserved signaling pathways, the pleiotropic effects of Wnt/β-catenin signaling functions are well established in biological processes including embryogenesis, tumorigenesis and stem cell biology [1-22]. Activation of

the canonical Wnt/β-catenin pathway stabilizes β-catenin protein levels, allowing relocation of β-catenin to the nucleus where it serves as a coactivator of the Lef/Tcf DNA binding factors, displacing HDAC and TLE corepressor complexes [23-30] and recruiting coactivators p300/CBP [31] and Brg1 for chromatin remodeling [32]. Many proteins are associated with cytoplasmic β-catenin for regulation of Wnt/ß-catenin pathway activities [33, 34], but the Lef/Tcf-family of transcription factors remain the sole focus as unambiguous DNA-binding partners for the diverse β-catenin-dependent nuclear transcription programs [35, 36]. Therefore, understanding whether additional transcriptional strategies are required to achieve the pleiotropic effects of the Wnt/β-catenin signaling pathway remains of major interest.

The development of the anterior pituitary gland provides an ideal model system for investigating signaling functions, because it sequentially progresses from a primordium of pluripotent ectodermal cells to a complex organ containing five distinct hormone-producing cell types: corticotropes, lactotropes, somatotropes, thyrotropes and gonadotropes. Early pituitary development at e9.0-e9.5 requires dorsal-ventral signals such as Sonic hedgehog, Fgf8/10 and Bmp4, and involves the actions of several homeodomain transcription factors expressed before or during the initial invagination of oral ectoderm that creates Rathke's pouch [37-39]. Later generation of somatotrope, lactotrope, and thyrotrope cell types depends on the function of a tissue-specific POU-class homeodomain transcription factor, Pit1 [40, 41]. The expression of *Pit1* is positively regulated by a paired-like homeodomain transcription factor, *Prophet of Pit1 (Prop1*) [42, 43], and negatively regulated by a second, highly-related,

paired-like factor, *Hesx1/Rpx* [44-47], which recruits Groucho/TLE and N-CoR co-repressors [48, 49].

Here, we report a strategy by which the Wnt/β-catenin pathway provides a key signal for determining cell lineages during pituitary development, with direct interactions between β-catenin and the tissue-specific homeodomain factor Prop1, rather than Lef/Tcfs, serving as the mechanism for transcriptional activation of the *Pit1* gene. A Prop1/β-catenin complex simultaneously represses expression of the *Hesx1 paired*-like homeodomain factor, via recruitment of TLE/Groucho, HDACs, and Reptin. Together, these results establish a transcriptional switching mechanism for β-catenin control of cell-fate determination, based on the actions of Prop1/β-catenin in both gene activation and gene repression.

Results

Temporal regulation of Wnt/ß-catenin activity dictates a specific pituitary cell lineage

Specific members of the Lef/Tcf transcription factor family exhibited distinct pituitary-specific expression patterns, with *Lef1* re-appearing at e13.5 in the anterior gland, following initial transient expression at e9.0 (Figure 3.1A). *Tcf3* and *Tcf4* were expressed during early stages of organ patterning but did not overlap with the initiation of *Lef1* and *Pit1* expression at e13.5 (Sup. Fig. 3.1A), suggesting distinct and non-overlapping roles with respect to Lef1. *Tcf1* expression was negligible in Rathke's pouch. Ten of the 19 murine *Wnt* genes were present in the developing e12.5 pituitary by semi-quantitative RT-PCR analyses (Sup. Fig. 3.1C). The Wnt target *Axin2* [50, 51] was expressed between e11.5-e15.5 (Figure 3.1A), suggesting a

temporally specific function of the Wnt-signaling pathway during pituitary organogenesis. Indeed, chromatin immunoprecipitation on microdissected Rathke's pouches at e12.5 revealed that β-catenin protein was associated with Lef/Tcf binding regions in the *Axin2* promoter [50, 51] (Figure 3.1B) and with a Wnt-responsive element conserved in the mouse/human *Lef1* promoter [52] at e13.5 (Figure 3.1B), providing direct evidence of transcriptional activity of the Wnt/β-catenin pathway during pituitary development.

We generated two *Cre*-expressing transgenic lines, *Pitx1/Cre* and *Pit1/Cre*, to modulate canonical Wnt/β-catenin signaling pathway activity by controlling expression of *β-catenin*, using a Cre/LoxP genetic strategy. Crossing with R26R LacZ reporter mice [53] revealed that the *Pitx1/Cre* transgene, controlled by –8kb *Pitx1* promoter [48, 54], exhibited efficient Cre-recombinase activity starting at e9.0 in all progenitors of Rathke's pouch that give rise to every cell type of the mature pituitary gland (Figure 3.1C, and Sup. Fig. 3.1C), while *Pit1/Cre*, controlled by –15kb *Pit1* promoter [54, 55], exhibited efficient Cre-recombinase activity only in the Pit1 lineage starting at e13.5 (data not shown). The Pitx1/Cre and Pit1/Cre transgenic mice were then crossed with conditionally active or inactive β-catenin mouse lines: the *β-catenin/loxP(ex2-6)* line deletes critical *β-catenin* coding exons, and consequently knocks out the Wnt/β-catenin pathway after Cre-dependent recombination [56], while the *β-catenin/loxP(ex3)* line generates a stable form of β-catenin protein, therefore leading to a constitutively active Wnt/β-catenin pathway after Cre recombination [57] (Figure 3.1C). The efficiency of these genetic approaches was verified directly by

assaying β-catenin protein levels (Figure 3.1C). The potential roles of β-catenin could then be examined with respect to various transcription factors expressed in temporally specific patterns during pituitary organogenesis (Figure 3.1D).

Following early loss of β-catenin function due to *Pitx1/Cre* the embryos exhibited relatively normal early pituitary development with only subtle morphological defects and a smaller gland along the lateral axis (Figure 3.2A), with unaltered expression of early homeodomain factors *Lhx3, Hesx1, Pitx2*, *Isl1,* and *Msx1* from e9.5 to e11.5 (Figure 3.2A-C and data not shown). Unexpectedly, *Pit1* gene expression was completely absent in the mutants, based on both *in situ* hybridization and qPCR analyses, whereas expression of *Prop1*, an upstream regulator of *Pit1*, was apparently not altered (Figure 3.2B). Hormone markers for the three Pit1-dependent cell types, somatotropes (*GH)*, thyrotropes (*TSHβ*), and lactotropes (*PRL)* were completely absent (Figure 3.2C,D) in the e17.5-P0 pituitary gland, consistent with the disappearance of *Pit1*. The gonadotropes, represented by *SF-1,* α*GSU* and *LHβ*, were still present, although in somewhat reduced numbers (Figure 3.2C, D and data not shown). The corticotrope/melanotrope lineage, represented by the lineage determining factor T-Pit/Tbx19 and the hormone POMC, appeared to be increased (Figure 3.2C, and data not shown). No increased apoptosis was observed from e9.5-e13.5 (data not shown). These data suggest that β-catenin activity is required for specific cell-lineage determination in the anterior pituitary gland, functioning epistatic to *Pit1* activation, potentially in parallel to Prop1.

In contrast to the dramatic consequence of *β-catenin* deletion with *Pitx1/Cre* transgenic mice, deletion of *β-catenin* specifically in the *Pit1* lineage starting at e13.5

using *Pit1/Cre* transgenic mice resulted in apparently normal expression of *Pit1* (Figure 3.2E), indicating that maintenance of later *Pit1* expression is independent of β-catenin activity. Pituitary cell differentiation was not affected in these mice, as determined by expression of *POMC, GH, αGSU* and *TSHβ* (Figure 3.2E and data not shown). Together, these temporally-controlled loss-of-function experiments (Figure 3.2) demonstrate that β-catenin in the developing pituitary functions specifically to initiate *Pit1* gene expression, but is not required for the subsequent terminal differentiation events of *Pit1* cell types. Indeed, during normal development, expression of *Axin2* and *Lef1,* both indicators of Wnt/β-catenin pathway activity, are diminished after e16.5 (Figure 3.1A).

Lef1/β-catenin does not induce* Pit1 *expression

Consistent with their dependence on the Wnt/β-pathway for expression, neither *Lef1* nor *Axin2* were detected in the caudomedial (*Pit1*-positive) field in *Pitx1/Cre* early loss-of-function pituitary glands (Figure 3.3A). However, rostral tip expression of *Axin2* persisted in mutants, suggesting that independent mechanisms were responsible for its expression in this region (Figure 3.3A, and data not shown). Comparison of *Pit1* and *Lef1* expression using adjacent sections from wild type embryos revealed that *Pit1* became robustly expressed before *Lef1* expression commenced. Furthermore, at e14.5, *Lef1* expression in the caudomedial domain of the anterior pituitary only partially overlapped the *Pit1* expressing cell field (Figure 3.3B), raising questions about any role of Lef1 in regulation of *Pit1*.

Indeed, in *Lef1* gene-deleted mice [58] *Pit1* gene expression, as well as *GH* and *TSHß* gene expression, were not reduced, but if anything, were elevated in the *Lef1*$^{-/-}$ embryos (Figure 3.3C,D). Expression of other Lef/Tcf factors was not altered in *Lef1* mutant pituitaries (data not shown). We next examined the presence of Lef1 on the *Pit1* gene regulatory regions during development, and found that at e14.5, Lef1 could be detected on the evolutionarily-conserved *Pit1* early enhancer (EE), located at -8kb, [42] but not the late, autoregulatory enhancer (LE) at -10.2kb. (Figure 3.3E); Tcf4 was not detected, consistent with the fact that it is not expressed at this timepoint (Sup Fig 3.1A). To investigate whether Lef1 was present on the active or inactive Pit1 gene regulatory sequences, we performed two-step chromatin immunoprecipitation. The first step was performed with anti-Ac K9-H3 IgG and the second step with either anti-Prop1 or anti-Lef1 IgGs (Figure 3.3F). These studies revealed that Prop1, but not Lef1, is present on the activated *Pit1* early enhancer, as marked by the presence of acetylated H3K9.

Thus, the expression of Lef1 actually somewhat attenuates *Pit1* gene expression, in contrast to the required actions of β-catenin for inducing *Pit1* expression. The Lef1-related factors Tcf3/Tcf4 are poor candidates for compensatory actions with Lef1 in negatively regulating *Pit1*, because their expression domains are clearly non-overlapping with *Lef1* and *Pit1* (Figure 3.1A, 3.3B and Sup Fig 3.1A). More importantly, previous analysis of *Tcf4* mutant mice revealed no change in the complement of mature cell types at e18.5, but instead an increase in pituitary size apparent at e14.5 [59]. Together, these observations raised the possibility that Wnt/β-

catenin might act in a Lef/Tcf-independent manner to achieve *Pit1* expression and cell lineage determination events.

A Prop1/β-catenin complex is required to induce* Pit1 *expression

Therefore, the function of Prop1 in *Pit1* gene activation [42] and its potential relationship with the Wnt/β-catenin pathway was investigated. We generated mice in which the *Prop1* gene was completely deleted and found that they fully recapitulated the essential features of *df* [42, 60], including loss of Pit1-dependent cell types and dysmorphogenesis of the anterior lobe (Figure 3.4A, Figure 2.2, 2.6). *Lef1* was not observed at e13.5 and e14.5 in the *Prop1*$^{-/-}$ anterior gland, but it was still expressed in the intermediate lobe (Figure 3.4A). *Axin2* remained expressed in the ventral aspect of the lumen and in the anterior pituitary in *Prop1*$^{-/-}$ pituitaries, indicating that some aspects of *Wnt/ß-catenin* signaling remained intact in the absence of *Prop1* (Figure 3.4A).

To investigate potential genetic interactions between *Prop1* and *ß-catenin*, we examined double heterozygous embryos generated by crossing *Prop1*$^{+/-}$ mice with conditionally-deleted *ß-catenin* heterozygotes, and observed a consistently diminished expression of *Pit1* in e14.5 double heterozygote embryos (Figure 3.4B), which provided evidence that *ß-catenin* and *Prop1* function in the same genetic pathway to activate *Pit1* gene expression. These genetic interactions were further supported by the observation of *in vitro* physical interactions between these two proteins (Figure 3.4C). The C-terminal 60aa of Prop1 interacted robustly with the full-length β-catenin protein in a GST pull-down protein-protein interaction assay (Figure 3.4C). The

homeodomain of Prop1 was capable of weaker interactions with β-catenin (Figure 3.4C). The β-catenin domain was mapped using a series of overlapping fragments, finding that a small region of β-catenin protein consisting of Armadillo repeat regions (5-9), corresponding to the Lef/Tcf interaction domain [61-63] was sufficient to mediate the interactions with Prop1 (Figure 3.4C and data not shown). Together, these genetic data demonstrate that β-catenin and Prop1 are both required to regulate *Pit1* gene expression. To establish that these interactions between Prop1 and β-catenin holoproteins occur in a cellular context, we performed co-immunoprecipitation in GHFT-1 cells, as demonstrated by α-β-catenin immunoblot of α-FLAG-Prop1 immunoprecipitate (Figure 3.4D). The recruitment of β-catenin to the PrdQ (consensus Prop1-binding) site was analyzed using an Avidin-Biotin Complex DNA Binding Assay (ABCD) [76]. Biotinylated oligos containing 2 PrdQ sites could specifically pull down β-catenin from Hela cells transfected with FLAG-Prop1 (Figure 3.4E).

We found that in transiently transfected pituitary cell lines in which the β-catenin pathway was stimulated, Prop1 activated the -10kb Pit1 promoter, which is sufficient for *Pit1* gene expression in transgenic mice [64] (Figure 3.4F). In support of a direct functional relationship between the β-catenin/Prop1 complex and *Pit1* gene expression, single cell nuclear microinjection assays demonstrated the requirement for β-catenin in activation of the early enhancer in the GHFT-1 pituitary cell line, which expresses endogenous *Pit1*, but not *Prop1* [65], and data not shown). Prop1 activated the early enhancer, and this activation was specifically blocked by anti ß-catenin IgG

or *ß-catenin* siRNA (Figure 3.4G, and data not shown). As a control for functional specificity, Pit1 actions on a Pit1 response element were ß-catenin-independent, and unaffected by anti-ß-catenin IgG or *β-catenin* siRNA (Figure 3.4H and data not shown). Synergistic activation by Prop1 and β-catenin was found using a minimal reporter construct under control of Prop1 regulatory elements (3x PrdQ) [42, 48] in HeLa cells that do not express Prop1. (Figure 3.4I).

Consistent with the model that β-catenin is a required coactivator for Prop1 (Figure 3.4J), as it is for Lef/Tcf, the transcriptional activity of Prop1/β-catenin complex on a PrdQ reporter was attenuated by over-expression of the β-catenin inhibitors Chibby and ICAT [66, 67]. Interestingly, cotransfection of Lef1 with *Prop1* and *β-catenin*$_c$ expression vectors produced even stronger inhibition of *PrdQ* reporter activity than Chibby or ICAT (Figure 3.4J). Lef1 was also capable of attenuating Prop1/β-catenin-dependent activation of a -10kb *Pit1* reporter (Figure 3.4K).

While the precise molecular mechanisms by which Lef1 diminishes Prop1/β-catenin-dependent *Pit1* gene activation *in vivo* remain incompletely defined, we have observed in cell culture that excess Lef1 can impair the recruitment of Prop1 to its cognate PrdQ sites in a co-transfection assay followed by chromatin immunoprecipitation and qPCR (Figure 3.4L).

Coordinated recruitment of regulatory complexes to* Pit1 *gene promoter and enhancers

Based on the developmental role of Prop1/β-catenin, analyses of factor/cofactor recruitment to regulatory regions of the *Pit1* gene [55, 64] by

chromatin immunoprecipitation assay using microdissected embryonic pituitaries were performed. These studies revealed that at e11.5, the diMe K4-H3, triMeK4-H3, and AcK9-H3 marks of activation were absent, but diMe K9-H3 was present on *Pit1* regulatory elements, consistent with an active repression of the *Pit1* gene at this time (Figure 3.5A, and data not shown). At e11.5, the *Pit1* early enhancer, which contains Prop1/Hesx1 homeodomain binding sites at –8kb [42], was occupied by the Hesx1 repressor and TLE, but Prop1 was not detected. By e12.5 the early enhancer was now occupied by Prop1, with a small residue of TLE1 still detected. However, there was no longer occupancy by Hesx1 at this time and a diMe K4-H3 mark was selectively present on the early enhancer (Figure 3.5B). By e13.5, the early enhancer and promoter were co-occupied by both Prop1 and β-catenin, with full dismissal of TLEs (Figure 3.5C), coinciding with initial *Pit1* gene activation. The diMe K4-H3, triMe K4-H3 and Ac K9-H3 marks associated with active promoters [68] were also present (Fig 5C and data not shown). In the adult, the *Pit1* gene promoter harbored the histone marks of gene activation (triMe K4-H3 and Ac K9-H3), with diMe K4-H3 now present on both the late and early enhancers (Figure 3.5D and data not shown). This temporal progression of histone modifications on regulatory regions of the *Pit1* gene correlates with the timing of β-catenin transcriptional activity and *Prop1* gene expression. Similarly, the loss of Hesx1 and TLE binding from e12.5-e13.5 tracks the attenuated expression of these factors preceding the induction of the *Pit1* gene [48].

Wnt/β-catenin activity inhibits* Hesx1 *expression

Expression of *Pit1* and subsequent differentiation of somatotropes, thyrotropes and lactotropes also depends on a concurrent attenuated expression of another *paired*-like homeodomain transcription factor, *Hesx1* [48]. Maintaining transcriptional repression of targets of the Hesx1/TLE complex is important for early pituitary development, and development of the organ can be ablated in *Hesx1*$^{-/-}$ embryos in specific genetic backgrounds, or when Hesx1-mediated repression is blocked by ectopic expression of Prop1 from the *Pitx1* promoter [48]. Examination of the *Hesx1* expression pattern in *Pitx1/Cre β-catenin*-gene deleted embryos by *in situ* hybridization and qPCR revealed that *Hesx1* expression persisted in the e14.5 anterior pituitary gland, whereas expression was normally already diminished in the wild-type littermates (Figure 3.6A). *Hesx1* expression was similarly extended in the *Prop1* mutant embryos [42, 43], and data not shown), suggesting the possibility that β-catenin and Prop1 together might subserve the inhibition of *Hesx1* expression at e11.5-e13.5.

Assessing the effects of premature activation of β-catenin signaling on organ development by generating *Pitx1/Cre*:*CA-Cat* mutants (early gain-of-function) revealed that while initial organ commitment was normal at e9.5, there was complete absence of the gland in all mutants examined by e13.5 (12/12) (Figure 3.6B). *Lhx3*, a marker for the definitive Rathke's pouch that is essential for pituitary development, was initially expressed in the prospective Rathke's pouch at e9.5 in both controls and gain of function mutants (Figure 3.6C), but was not detected in Rathke's pouch of the *ß-catenin* gain-of-function mutant after e10.5 (Figure 3.6D), a phenotype similar to

that observed when Hesx1/TLE repression is absent [48]. The canonical Wnt/β-catenin target genes, *Axin2* and *Lef1*, were up-regulated at e9.5 *ß-catenin* gain-of-function mutant embryos as expected (Figure 3.6E). Consistent with the model that the Prop1/β-catenin complex negatively regulates *Hesx1* expression, we found that *Hesx1* was not expressed in e9.5 ß-catenin gain-of-function mutants (Figure 3.6C).

A bioinformatic analysis of the mouse and human *Hesx1* regulatory sequences revealed several conserved *paired*-like homeodomain binding sites (Figure 3.6F-H). Prop1 caused repression of reporters containing the conserved regions encompassing the Prop1 binding sites in transient transfection assays (Figure 3.6G). Chromatin immunoprecipitation of e12.5 pituitary glands demonstrated the presence of both ß-catenin and Prop1, but not Lef1, on these conserved *Hesx1* gene regulatory regions. ChIP also revealed that HDAC1, TLEs and Reptin were also present with Prop1/β-catenin on the *Hesx1* regulatory regions at e12.5 (Figure 3.6H). The functional significance of these putative corepressors was confirmed by use of single cell nuclear microinjection of specific antibodies (Figure 3.6I) or siRNAs (Figure 3.6J), against β-catenin, HDACs and TLEs, which reversed the repressive effects of the Prop1/β-catenin complex on the Hesx1 regulatory region. In contrast, αHDAC3 did not reverse repression. Thus, in addition to the role of Prop1/β-catenin in activation of the *Pit1* gene, our data suggest that the Prop1/β-catenin complex simultaneously acts to repress expression of *Hesx1* at e12.5 via recruitment of specific corepressor machinery, providing an essential regulatory event required for the progression of normal pituitary development.

Discussion

A novel DNA-binding partner for β-catenin in cell-lineage determination

In these studies, we have uncovered a novel transcriptional strategy that underlies β-catenin control of cell lineage determination in organogenesis, revealing the unexpected role of a tissue-specific homeodomain factor as the essential DNA binding transcription factor that recruits β-catenin in mediating the actions of the Wnt/β-catenin pathway, both for activation and for repression of specific gene targets in pituitary development. In addition to the common Lef/Tcf factors as the key DNA binding partners mediating many aspects of β-catenin activity, we established genetically and biochemically that β-catenin directly interacts with Prop1 and is a required coregulator for Prop1 transcription activity, causing initial activation of the cell-lineage determining factor *Pit1* at e12.5-e13.5, and simultaneously directing repression of the *Hesx1* repressor (Figure 3.7).

While some aspects of pituitary gland proliferation are regulated by Wnt4, Wnt5a, Pitx2, Aes1, and Tcf4 [54, 59, 69, 70], our findings suggest that other components of the Wnt signaling pathway serve as key signals for lineage determination events by exerting both positive and negative regulation on tissue-specific homeodomain factors. We find that Wnt/β-catenin signaling occurs in a specific developmental window between e11.5-e14.5 of pituitary development, where it is required for cell-type determination as cells leave their niche in the lumen of Rathke's pouch. These events reflect a precise contextual requirement for the β-catenin signal; if β-catenin is activated at e9.5, we find that Rathke's pouch is

completely destroyed, apparently through perturbations of the endogenous repression program mediated by Hesx1. Conversely, if the β-catenin signal is extended after initial Pit1 lineage determination, terminal cell type differentiation events within the lineage are inhibited (our unpublished data).

Our finding that Prop1 is a key nuclear mediator for the Wnt/β-catenin signaling provides a molecular insight into the mechanism by which Wnt signaling activity dictates cell lineage determination. In the skin, for example, β-catenin has proved to be essential for the decision of hair follicle stem cells to adopt the epithelial or follicular fate [12], apparently involving the actions of Lef1 and Tcf3 [11, 58, 71]. In contrast to the skin, the nuclear events downstream of Wnt/ β-catenin in pituitary cell lineage determination are not primarily mediated via Tcf/Lef, and our findings suggest that a subset of tissue-restricted homeodomain factors [70], and undoubtedly other classes of transcription factors, will prove to play key roles as DNA-binding mediators of β-catenin signaling in cell fate decisions.

Our analyses have also revealed a transcriptional repression function of the Prop1/β-catenin complex, important in mediating cell lineage determination based on promoter-specific repression of *Hesx1*. This appears to require a series of corepressors, including HDACs 1/2, Reptin and Groucho/TLEs. The selective participation of Reptin as a component of β-catenin-mediated repression is supported by its role in repression of Wingless signaling in *Drosophila* [72] and in suppression of a metastasis suppressor gene in prostate cancer [73].

Thus, investigation of the actions of β-catenin in cell lineage determination during pituitary organogenesis has uncovered a simple molecular logic for promoting cell lineage determination, significantly differing from the canonical Wnt/β-catenin pathway, based on the key role of β-catenin as the promoter-specific coactivator or corepressor of a tissue restricted DNA-binding transcriptional partner. It is likely that analogous molecular events function broadly in development and disease.

Methods

In situ hybridization

In situ hybridization was performed as described previously [74]. The antisense in situ probe for Axin2 was a gift from Dr. Wei Hsu (University of Rochester). Probe templates for Lef/Tcf factors were generated by PCR for the following nucleotides: Tcf1 245-1487 (NM_009331), Tcf3 356-1360 (NM_009332), Tcf4 212-1803 (NM_013865), Lef1 full length 990-2183 (NM_010703), Lef1 β-catenin binding domain 701-1193 (NM_010703).

Generation and analysis of transgenic animals and gene targeted mice

Genotype analysis of *ß-catenin/loxP(ex2-6)* and *ß-catenin/loxP(ex3)* mice [56, 57], *Lef1* mutant mice [58] and generation of transgenic lines using *Pitx1* and *Pit1* promoters [54], has been described previously. *Prop1+/-,Pitx1CreKO- ßCat+/-* trans-heterozygotes were obtained by crossing *Prop1+/-:Pitx1Cre* mice with *ß-catenin*$^{ex2\text{-}6/ex2\text{-}6}$ mice, and two controls and four trans-heterozygotes were processed for analysis. Prop1 mutant mice were generated by targeted mutagenesis in ES cells to replace the

entire coding sequence with a β-galactosidase/neomycin selection cassette (Figure 2.1), and correct targeting was established by Southern blotting with 5' and 3' external probes.

Transfection and nuclear microinjection assays

Cotransfection experiments were performed as described previously [55] in 293T and HeLa cells using 750ng of luciferase reporter, 100ng of pCMX expression plasmids and 500ng of pRSV*β*Gal as an internal control for transfection efficiency. Transfections of pituitary cell lines used Fugene6 (Roche) instead of calcium phosphate. The multimerized PrdQ/p36 luciferase reporter was described previously [42].

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed as previously described [75] on microdissected pituitaries, with a modified fixation time of 30 minutes in 2% paraformaldehyde. Approximately 12 e13.5 pituitaries, 15 e12.5 pituitaries, 25 11.5 pituitaries or one adult pituitary, were used for each antibody. Anti-Prop1 antibody was generated in guinea pigs against bacterially expressed C' of murine Prop1. Rabbit anti-Reptin was a generous gift from Otmar Huber (Institute of Clinical Chemistry and Pathobiochemistry, Berlin, Germany). Anti- diMeK4H3 and triMeK4H3 were obtained from Upstate Biotechnology, while anti-TLE, anti-Hesx1, anti-HDAC1 and anti-β-catenin were from Santa Cruz Biotechnology.

Co-immunoprecipitation

Hela cells were transfected with a Flag-tagged Prop-1 expression vector (15 μg / 10 cm plate); after 48 hrs the cells were harvested and nuclear extracts were prepared. 500 μg of nuclear extract were immunoprecipitated with 5 μg of either normal mouse IgG or anti-Flag antibody (SIGMA). The western blot was carried out with goat anti-β-catenin antibody from Santa Cruz Biotechnology.

GST-affinity purification and protein interaction studies

GST-Prop1 homeodomain (amino acids 51-131) and GST-Prop1 C-terminus (aa 129-225) fusion proteins for protein interactions were expressed in *E. coli* and purified from homogenized lysates with glutathione-agarose beads at 25 degrees C for 1 h. For interaction studies, immobilized GST-fusion proteins were then mixed with 293T-cell lysates containing overexpressed, tagged ß-catenin protein, or S35-methionine labeled ß-catenin protein fragments expressed *in vitro* using reticulocyte lysates (Promega TnT Quick Coupled Transcription/Translation System). Following SDS-PAGE and transfer to nitrocellulose, interacting proteins were visualized by Western blotting or autoradiography. Isotope-labeled ß-catenin fragments: N-terminus (aa 1-126), C-terminus (aa 601-781), Arm repeats 1-4 (aa 121-276), Arm repeats 5-9 (aa 270-483), Arm repeats 10-12 (aa 477-713).

Avidin-Biotin Complex DNA Binding Assay

The ABCD Assay was performed as described [76].

Quantitative PCR

Real-time PCR was performed on RNA extracted from wild-type and β-catenin knockout embryonic pituitaries at e13.5 (Pit1) and 14.5 (Hesx1). The data were normalized to GAPDH and are presented as fold change, with respect to the wild-type. All experiments were performed with two biological and two technical replicates. For oligo sequences, see supplementary methods.

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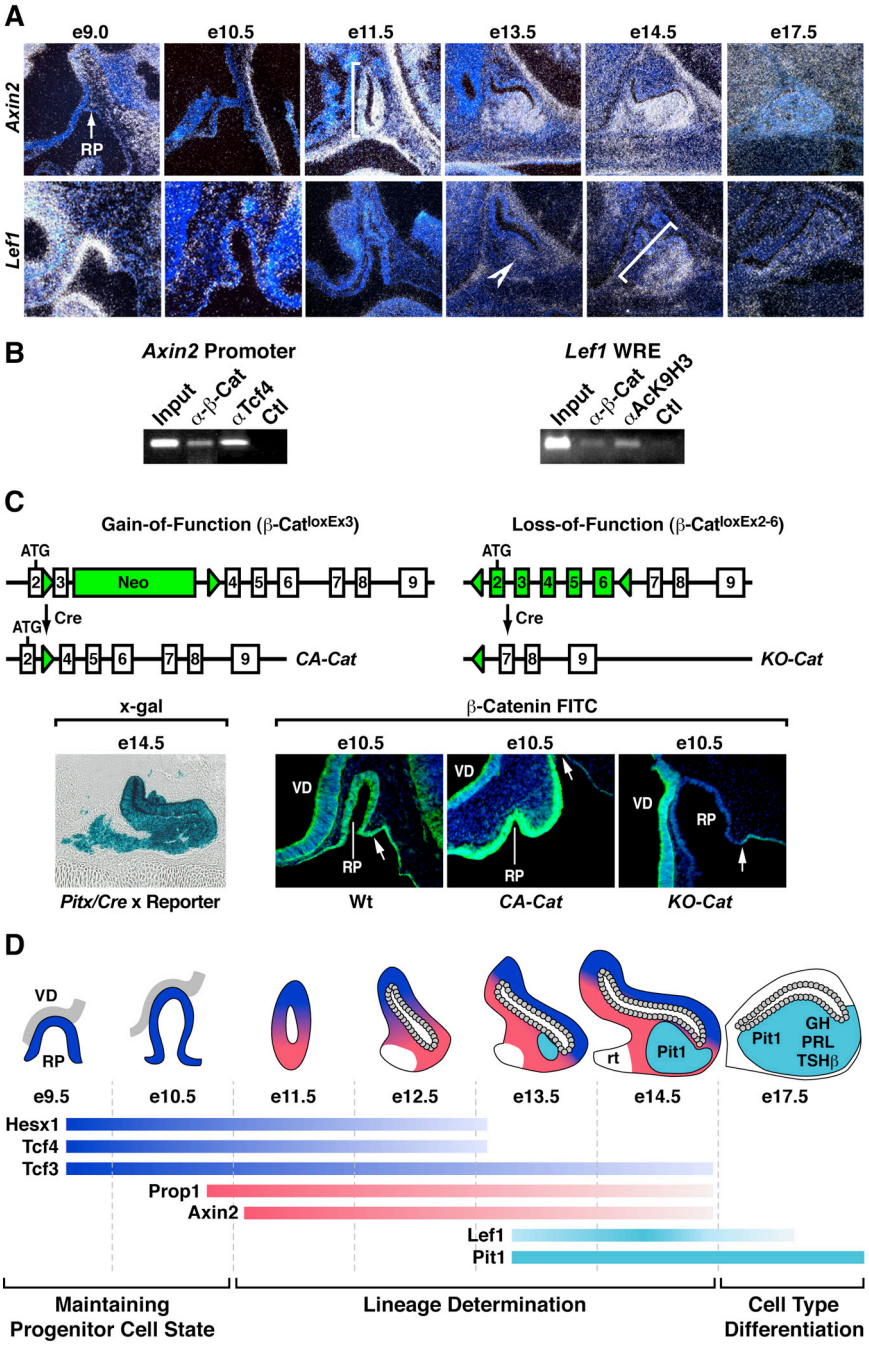
Chapter 3, in full, is a reprint of the material as it appears in *Cell* 2006: Olson LE*, Tollkuhn J*, Scafoglio C, Krones A, Zhang J, Ohgi KA, Wu W, Taketo MM, Kemler R, Grosschedl R, Rose DW, Li X, Rosenfeld MG, Homeodomain-mediated beta-catenin-dependent switching events dictate cell lineage determination. Cell 2006 May 5;125(3):593-605.

Figure 3.1. Temporal control of Wnt/β-catenin signaling in pituitary development.
A) *Axin2* expression (white signal by *in situ* hybridization) in the anterior pituitary gland during the specific e11.5-e14.5 developmental window; and dynamic *Lef1* expression at e9.0 in the presumptive Rathke's pouch (RP) epithelium, then extinguished from RP until e13.5 to reappear in a restricted caudomedial domain of the anterior pituitary (arrowhead). **B)** Chromatin immunoprecipitation (ChIP) on e12.5 and e13.5 pituitary glands using specific ß-catenin, acetylated histone H3, and Tcf4 antibodies, indicating binding to Wnt-responsive elements in the *Axin2* and *Lef1* promoters. **C)** Conditional β-catenin alleles for generating tissue-specific constitutively active (CA-Cat) [57] and knockout (KO-Cat) [56] forms of β-catenin. Activity of Pitx1/Cre transgene precedes the lineage of all cells of the pituitary gland at e14.5, assayed by analysis of R26R/lacZ activation (x-gal stain). Direct evidence of stabilization or knockout of β-catenin protein (FITC) in embryos with Pitx/Cre transgene and either wt/wt, wt/loxEx3, or loxEx2-6/loxEx2-6 β-catenin genotypes. Arrow indicates the caudal limit of nascent Rathke's pouch and Pitx/Cre activity. Neuroepithelium (ne), Rathke's pouch (RP). **D)** Coordinated expression of critical homeodomain factors (Hesx1, Prop1, Pit1), Lef/Tcf factors (Tcf3, Tcf4 and Lef1) and Axin2 in pituitary development. Cell types of the Pit1 lineage (light blue) express growth hormone (GH), prolactin (PRL), and thyroid stimulating hormone (TSHß). Ventral diencephalon (VD), Rathke's pouch (RP), rostral tip (rt).


A
e9.0
e10.5
e11.5
e13.5
e14.5
e17.5
Axin2
Lef1
RP
B
Axin2 Promoter
Input
α-β-Cat
αTcf4
Ctl
Lef1 WRE
Input
α-β-Cat
αAcK9H3
Ctl
C
Gain-of-Function (β-Catlox Ex3)
Loss-of-Function (β-Catlox Ex2-6)
ATG
Neo
Cre
CA-Cat
KO-Cat
x-gal
β-Catenin FITC
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Pitx/Cre x Reporter
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PRL
TSHβ
e9.5
e10.5
e11.5
e12.5
e13.5
e14.5
e17.5
Hesx1
Tcf4
Tcf3
Prop1
Axin2
Lef1
Pit1
Maintaining Progenitor Cell State
Lineage Determination
Cell Type Differentiation

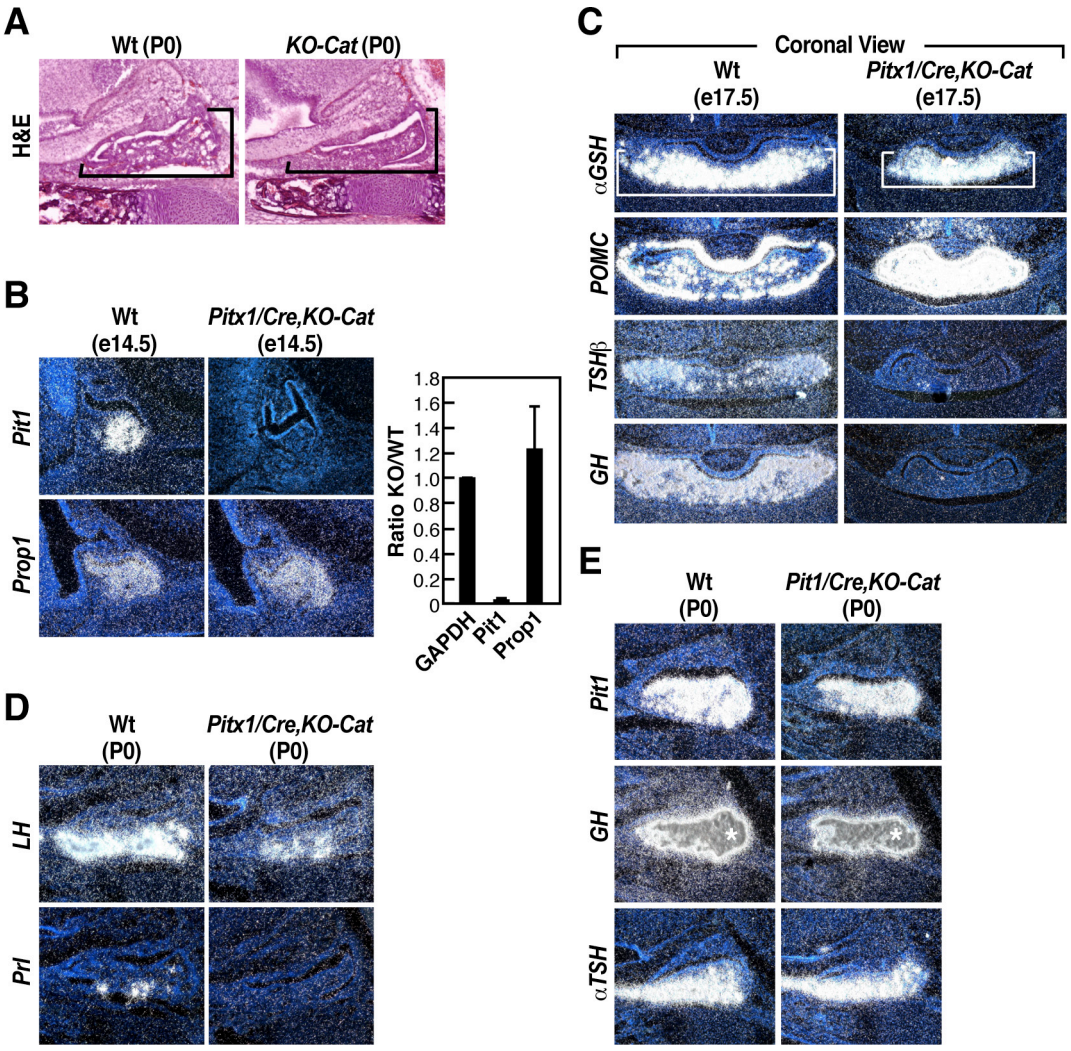


Figure 3.2. Wnt/β-catenin signaling is required for Pit1 lineage determination but is not required for cell-type differentiation.
All histology panels are sagittal sections of embryonic pituitary except for C, which shows frontal sections. **A)** Smaller anterior gland (bracket) and caudal expansion of lumen in a pituitary with early conditional knockout for β-catenin. **B)** As assessed by *in situ* hybridization (specific signal is white) *Pit1* is not expressed in early conditional knockout for β-catenin, but an epistatic factor, *Prop1*, is expressed normally. Quantitative PCR analysis of *Pit1* ratio shown. Data are represented as mean ± SEM. **C)** Smaller anterior gland (bracket) in early conditional knockouts, with expression of *αGSU*, a marker for ventral cell types, and abundant corticotropes (*POMC*), but loss of Pit1-dependent thyrotropes (*TSHβ*) and somatotropes (*GH*). **D)** Gonadotropes, represented by expression of lutenizing hormone (*LH*), are still present, but Pit1-dependent lactotropes (*PRL)* are absent in early conditional knockouts for β-catenin. **E)** Late conditional knockout of β-catenin following initial activation of *Pit1* does not affect *Pit1* autoregulation or cell differentiation.

Figure 3.3. Lef1 is not required for Pit1 expression and Pit1 lineage determination.
A) In early conditional knockouts for β−catenin, *Axin2* expression remains only in the rostral tip (rt), and *Lef1* is lost from both the intermediate lobe (il) and caudomedial (cm) region of the anterior gland. **B)** Wild-type expression of *Lef1* and *Pit1* in adjacent sections; at e13.5 or e14.5, *Lef1* is weaker and delayed relative to *Pit1*. **C)** In *Lef1* knockout pituitaries, *Pit1* expression is upregulated but *Prop1* is not changed. **D)** Loss of *Lef1* leads to increased levels of Pit1 target genes (*GH* and *TSHß*) expressed by Pit1 dependent thyrotropes and somatotropes, while other cell types (*POMC)* are apparently unchanged. **E)** ChIP analysis of e14.5 pituitaries demonstrating that Lef1 is recruited to the *Pit1* early enhancer (EE), but Tcf4 is not. **F**) Two-step ChIP in GHFT-1 cells with α-AcK9H3, followed by either α-Prop1 or α-Lef1. Prop1 is detected on the active endogenous *Pit1* early enhancer, while Lef1 is not recruited.

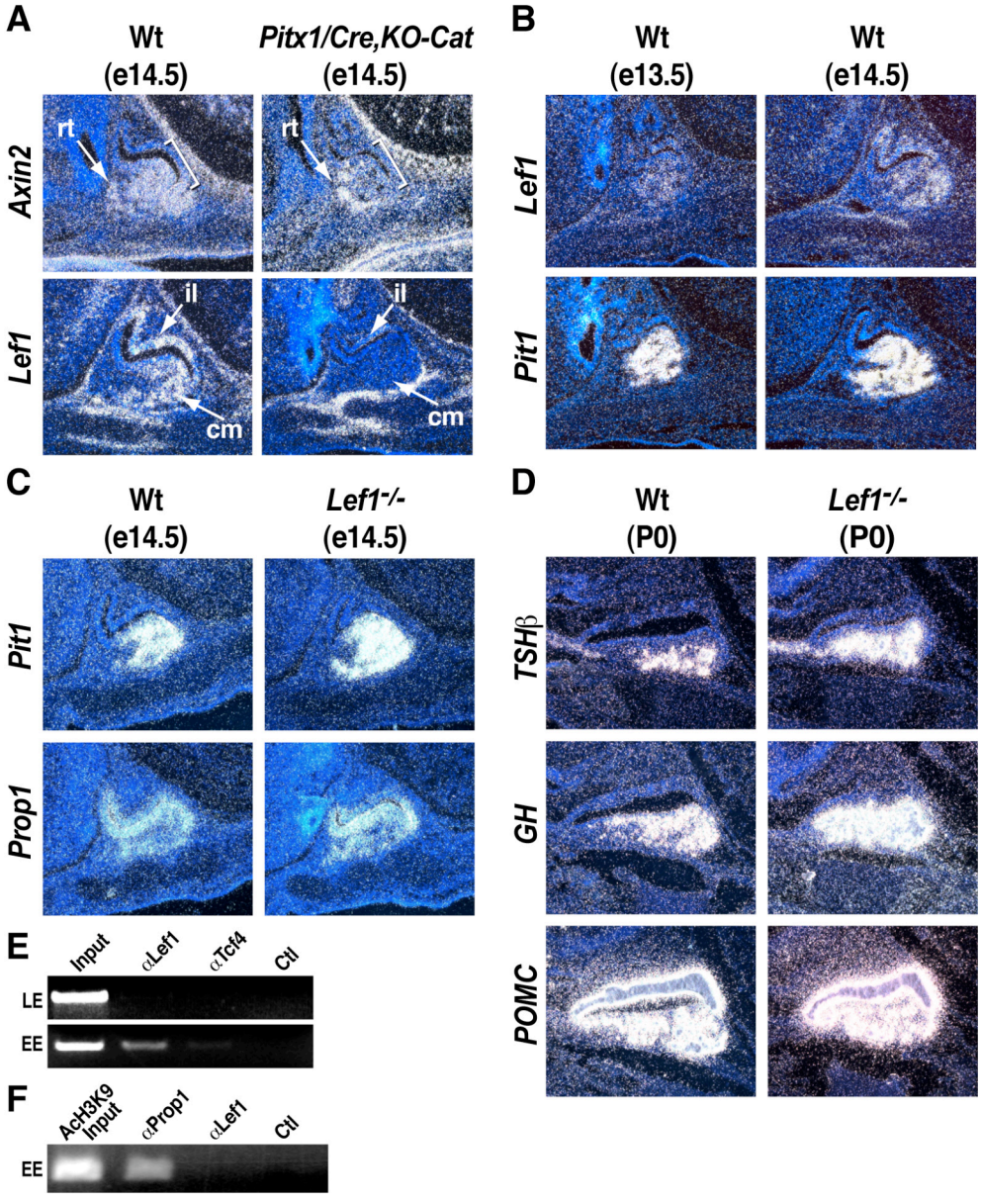
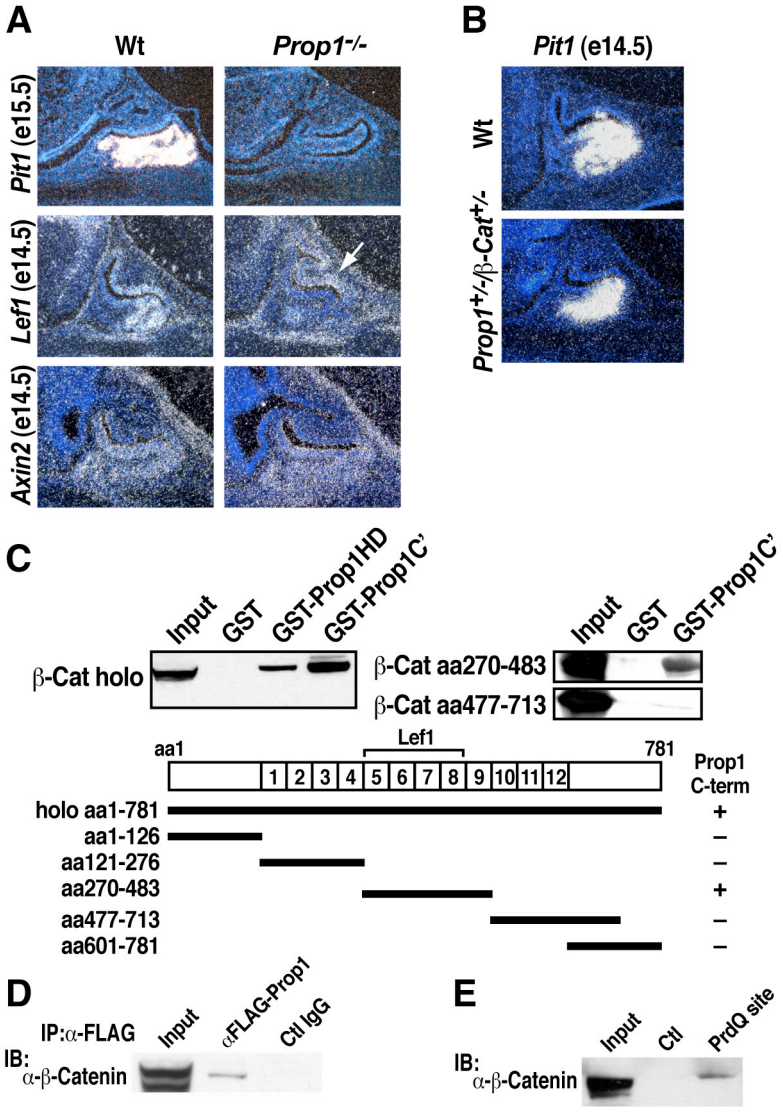

A
Wt
(e14.5)
Pitx1/Cre,KO-Cat
(e14.5)
Axin2
Lef1
rt
il
cm
B
Wt
(e13.5)
Wt
(e14.5)
Lef1
Pit1
C
Wt
(e14.5)
Lef1-/-
(e14.5)
Pit1
Prop1
D
Wt
(P0)
Lef1-/-
(P0)
TSHβ
GH
POMC
E
Input
αLef1
αTcf4
Ctl
LE
EE
F
AcH3K9
Input
αProp1
αLef1
Ctl
EE

Figure 3.4. Prop/β-catenin interactions activate the Pit1 early enhancer.
A) Prop1 gene is required for anterior pituitary expression of Pit1 and Lef1 in the caudomedial domain, but *Lef1* is still expressed in the intermediate lobe on e14.5-e15.0 (arrow), and *Axin2* does not require *Prop1*. **B)** Pituitaries doubly heterozygous for *Prop1* and *ß-catenin* show a reduced domain of *Pit1* expression. **C)** Left panel: Anti-Flag Western blotting for biochemical interactions between GST-Prop1 fragments (homeodomain and carboxyl-terminus) with Flag-tagged β-catenin. Right panel and lower panel: High-affinity interactions with the Prop1 carboxy-terminus occur through β-catenin armadillo repeats 5-9, similar to β-catenin/Lef1 interactions. **D)** Co-immunoprecipitation of FLAG-tagged Prop1 and β-catenin in GHFT-1 cells. **E)** Avidin-Biotin Complex DNA Binding Assay, demonstrating β-catenin binding to double-stranded oligonucleotides containing the PrdQ consensus Prop1 binding site.


A
Wt
Prop1-/-
Pit1 (e15.5)
Lef1 (e14.5)
Axin2 (e14.5)
B
Pit1 (e14.5)
Wt
Prop1+/-/β-Cat+/-
C
Input
GST
GST-Prop1HD
GST-Prop1C'
β-Cat holo
β-Cat aa270-483
β-Cat aa477-713
aa1
Lef1
781
1 2 3 4 5 6 7 8 9 10 11 12
Prop1 C-term
holo aa1-781 +
aa1-126 –
aa121-276 –
aa270-483 +
aa477-713 –
aa601-781 –
D
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Input
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Input
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IB: α-β-Catenin

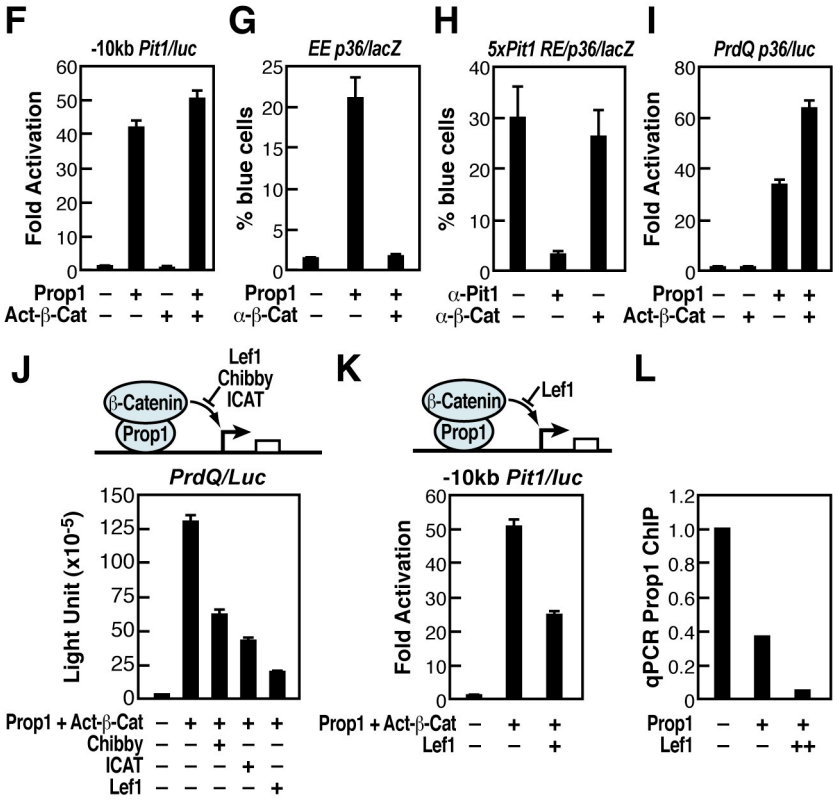


Figure 3.4 Prop/β-catenin interactions activate the Pit1 early enhancer. continued

F) In transfected pituitary cells (TαT1 or αT3), Prop1 and constitutively active β-catenin stimulate a luciferase reporter gene with –10kb of the *Pit1* gene. **G)** In microinjected pituitary cells (GHFT-1), Prop1 activates a lacZ reporter gene regulated by the -5/-8.5kb *Pit1* early enhancer with a minimal 36bp promoter from the *prolactin* gene, and Prop1 activation is blocked by coinjection of specific antibody against β-catenin. **H)** As a control for antibody specificity, a reporter gene with Pit1 binding sites is not blocked by coinjection of antibody against β-catenin (performed in GHFT-1 cells that express endogenous Pit1). **I)** In transfected HeLa cells, Prop1 and active β-catenin stimulate a luciferase reporter with 3x*paired*-like homeodomain binding sites (PrdQ). **J)** Chibby and ICAT block activation of a Prop1/β-catenin dependent reporter in HeLa cells, as does Lef1. **K)** Lef1 inhibits activation by Prop1/β-catenin of a reporter gene regulated by –10kb of *Pit1* gene enhancer/promoter in transfected pituitary cells (GHFT-1). **L)** Expression of Lef1 decreases Prop1 recruitment to the PrdQ reporter, as measured by quantitative PCR read-out of an α-Prop1 ChIP. Data are represented as mean ± SEM.

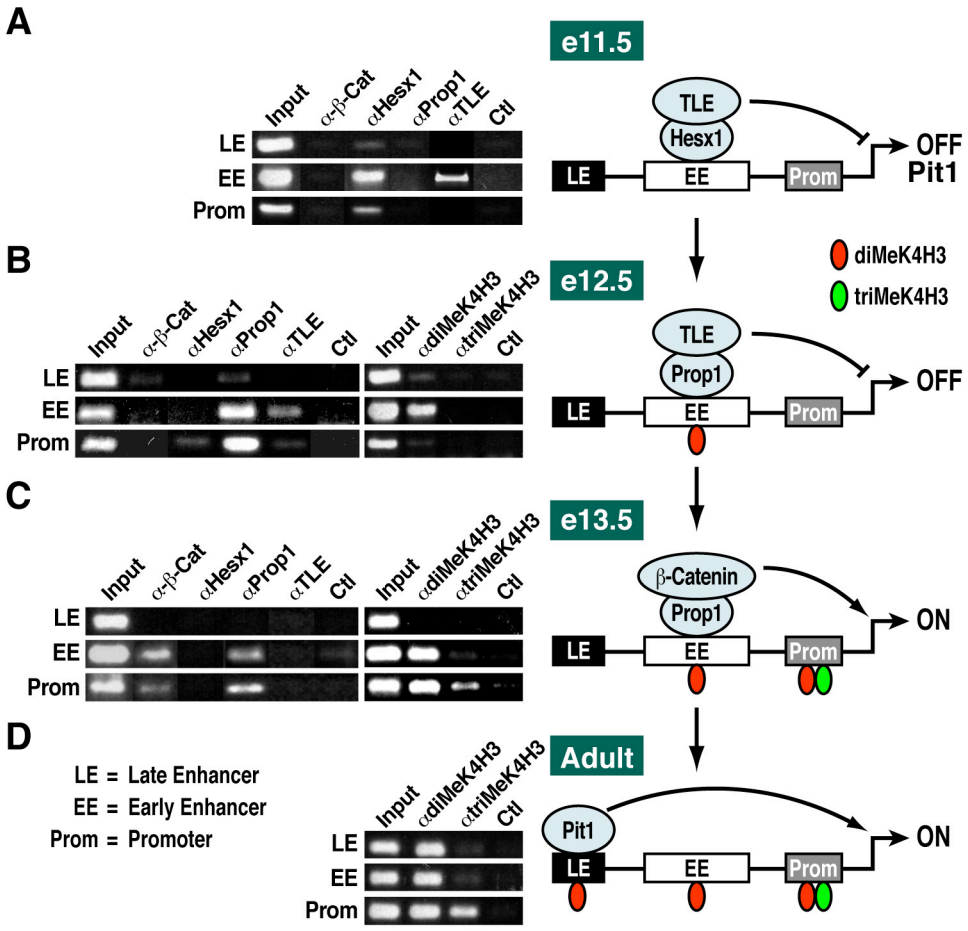
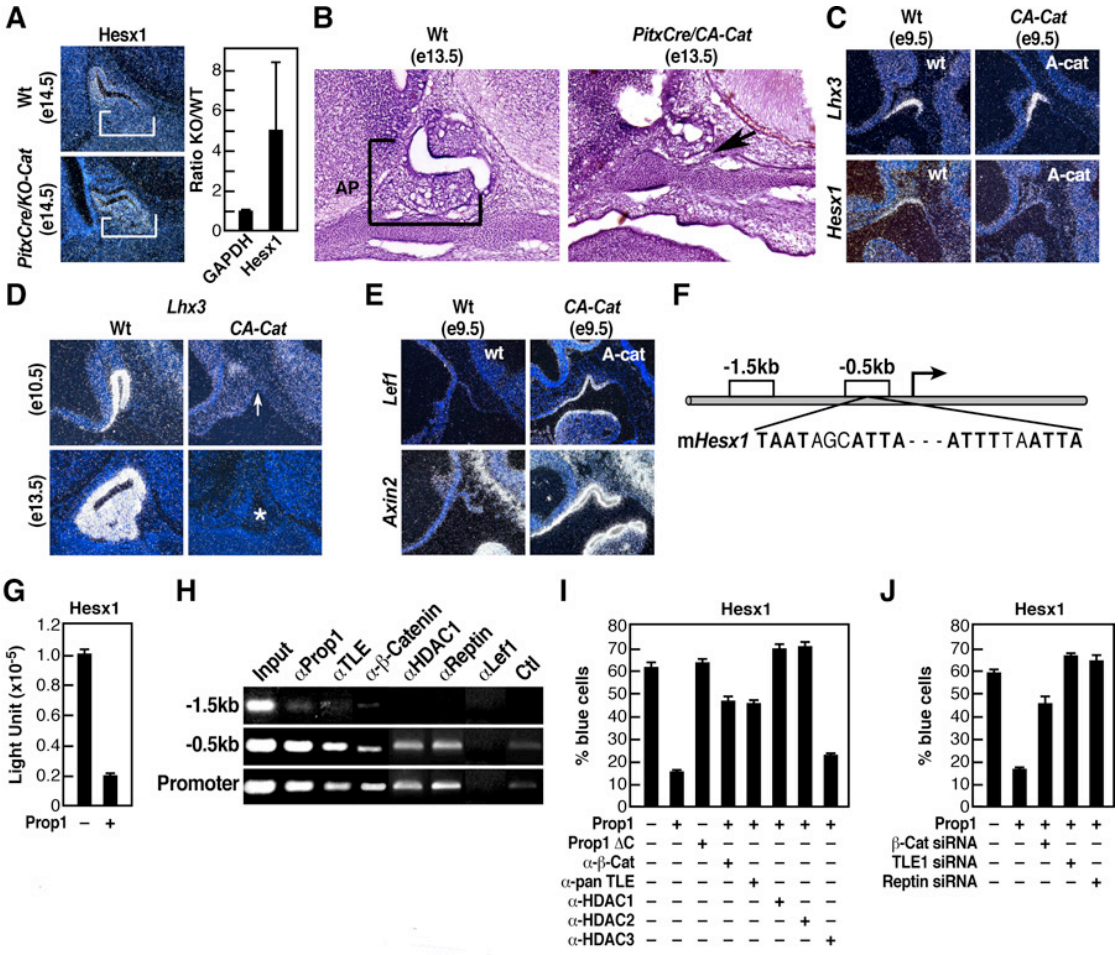


Figure 3.5. Coordinated recruitment of regulatory complexes to *Pit1* gene promoter and enhancers.
Left: ChIP assay and PCR detection of protein/chromatin interactions in microdissected embryonic pituitary glands, showing temporally ordered replacement of (**A)** transcriptional repressors at e11.5, by (**B)** Prop1 at e12.5, and (**C**) β-catenin at e13.5, in coordination with the appearance of histone activation marks (**B-D**). Right: Organization of the *Pit1* gene promoter and enhancers: -10.4kb late enhancer, -5 to –8.5kb early enhancer with *paired*-like homeodomain DNA sites, and –0.327kb promoter.

Figure 3.6. Prop1/β-catenin represses *Hesx1* repressor expression.
A) *Pitx1Cre/KO-Cat* knockout pituitaries exhibit ectopic expression of *Hesx1* (in situ hybridization signal is white) in the anterior gland (bracket), while in wild type littermates *Hesx1* is restricted dorsally or extinguished. **B)** Hematoxylin/eosin stained tissue sections, where early activation of ß-catenin in *Pitx1Cre/CA-Cat* mutants ablates pituitary development before e13.5. The arrow on the mutant panel indicates where the presumptive location of the anterior pituitary (AP). **C)** Expression of *Lhx3* is normal at e9.5 while *Hesx1* is repressed in *Pitx1Cre/CA-Cat* mutants. **D)** Loss of *Lhx3* expression in *Pitx1Cre/CA-Cat* e10.5 and13.5 pituitaries. **E)** Wnt target genes *Axin2* and *Lef1* are induced in *CA-Cat* mutants. **F)** The *Hesx1* 5' enhancer (enh) contains conserved *paired*-like homeodomain sites. **G**) In transfected GHFT-1 cells, Prop1 and β-catenin represses a luciferase reporter gene under control of the conserved *Hesx1* 5' enhancer and a heterologous thymidine kinase promoter. **H)** ChIP of the *Hesx1* gene from microdissected e12.5 pituitaries, showing spatially localized interactions of Prop1, TLE, HDAC1, Reptin and β-catenin at the *Hesx1* promoter and 5' enhancer at –0.5kb.), but not a negative control region at –1.5kb. **I,J**) Prop1 repression of *Hesx1* promoter required β-catenin, Reptin, TLEs, HDAC1, HDAC2, but not HDAC3 in a single cell nuclear microinjection assay using specific antibodies **(I)** and gene specific siRNAs **(J).**


A
Hesx1
Wt (e14.5)
PitxCre/KO-Cat (e14.5)
Ratio KO/WT
GAPDH
Hesx1
B
Wt (e13.5)
PitxCre/CA-Cat (e13.5)
AP
C
Wt (e9.5)
CA-Cat (e9.5)
Lhx3
Hesx1
wt
A-cat
D
Lhx3
Wt
CA-Cat
(e10.5)
(e13.5)
E
Wt (e9.5)
CA-Cat (e9.5)
Lef1
Axin2
F
-1.5kb
-0.5kb
mHesx1 TAATAGCATTA - - - ATTTTAATTA
G
Hesx1
Light Unit (x10-5)
Prop1
H
Input
αProp1
αTLE
α-β-Catenin
αHDAC1
αReptin
αLef1
Ctl
-1.5kb
-0.5kb
Promoter
I
Hesx1
% blue cells
Prop1
Prop1 ΔC
α-β-Cat
α-pan TLE
α-HDAC1
α-HDAC2
α-HDAC3
J
Hesx1
% blue cells
Prop1
β-Cat siRNA
TLE1 siRNA
Reptin siRNA

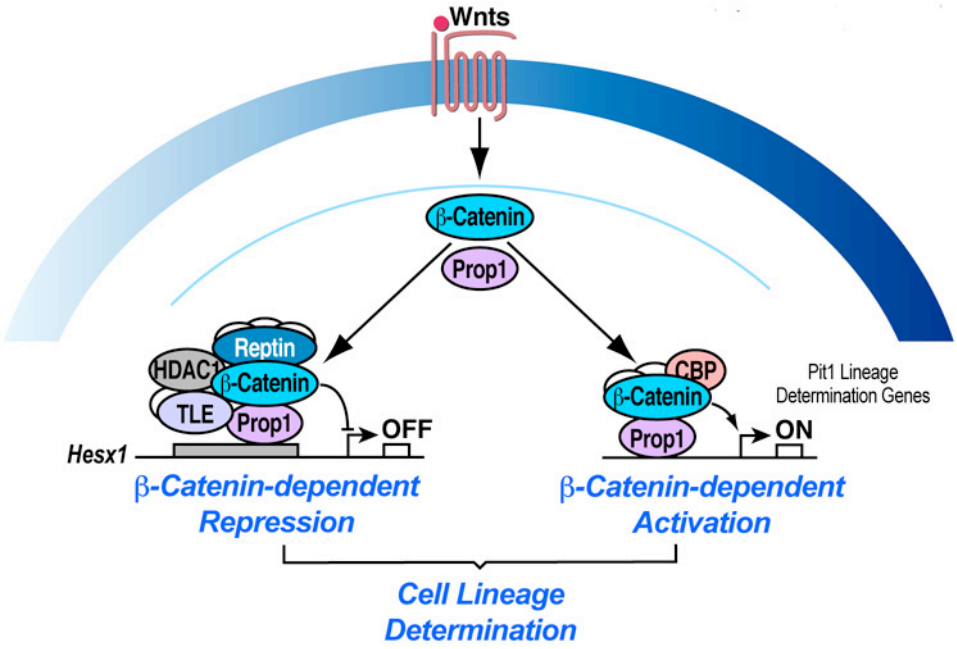
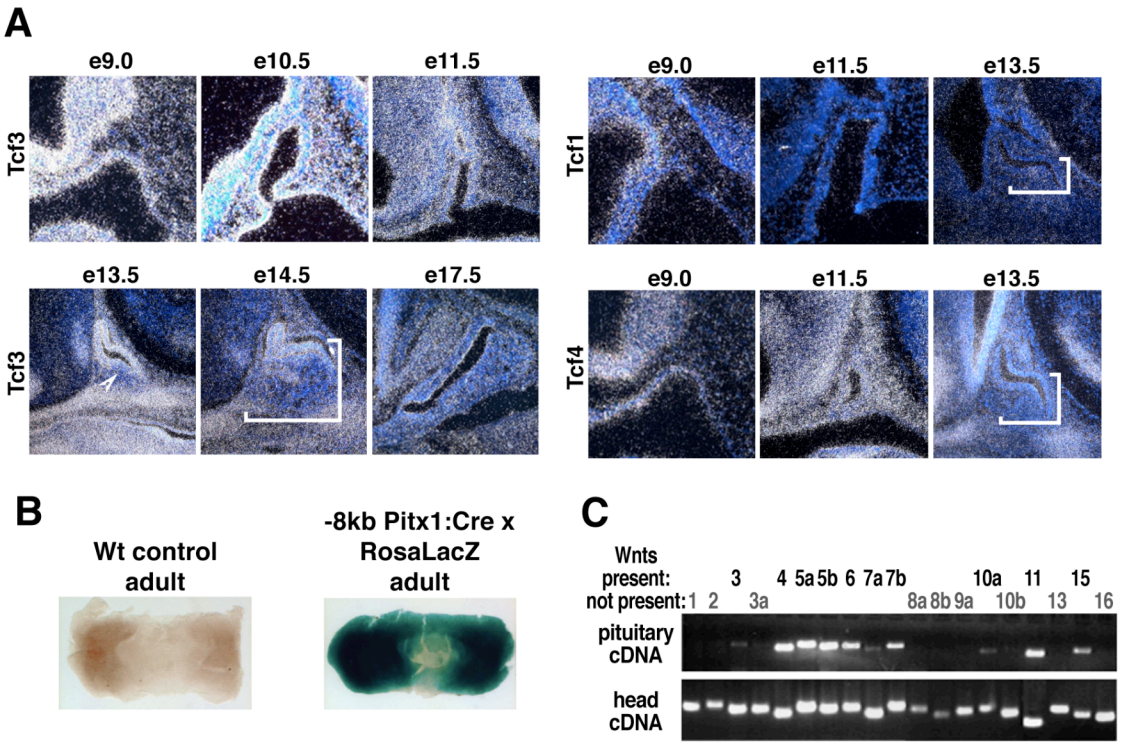


Figure 3.7. A model for Prop1 homeodomain mediated nuclear events downstream of Wnt/β-catenin signaling on distinct sets of target genes.
In response to a Wnt signal, received by pluripotent progenitor cells, stabilized β-catenin acts through direct interactions with the C-terminus of Prop1 to activate the *Pit1* gene, a lineage-determining factor in pituitary development. Lineage determination is also achieved through the repressive actions of Prop1 and β-catenin, with recruitment of TLE, HDAC and Reptin co-repressors to silence *Hesx1,* which maintains cellular pluripotence and inhibits cell fate decisions.



Supplementary Figure 3.1.
A) Ontogeny of Tcf factors in the developing pituitary. *Tcf3* is expressed early, but is restricted from the *Pit1*-expressing caudomedial area of the gland (arrowhead) and is gone by e17.5 *Tcf1* and *Tcf4* also show early expression, but are also in surrounding tissues and are markedly decreased by the time of *Pit1* activation. **B)** Determination of Wnt expression in e12.5 laser-captured Rathke's pouches or e12.5 embryonic heads as positive control, using semi-quantitative RT-PCR for all 19 vertebrate Wnts.
C) *Pitx1/Cre* mice express faithfully in every cell of the pituitary, as shown by crosses with the *R26R* reporter mice.

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Chapter 4: Regulation of Prop1 Expression

Introduction

Prop1 is the earliest transcription factor that is expressed solely in the pituitary, [1] yet despite extensive characterization of many signaling pathways in the gland, nothing is known about the activation of *Prop1*. In the *Prop1* knockout mouse, the *lacz* gene was inserted in the targeting vector with the intent of marking *Prop1* mutant cells. However, my analysis of tissue from knockout mice has revealed that *lacz* is not expressed. Furthermore, past attempts in the lab to create a *Prop1*-driven transgene with 6 kb of upstream sequence have failed (J. Dasen, unpublished data). In this chapter, I will describe the mapping of the *Prop1* regulatory information, and the generation of a *Prop1-Cre* transgenic line. Next, I will present data on the contribution of Prop1 to various cell lineages in the pituitary, which were obtained through crossing the *Prop1-Cre* transgene to the *ROSA26* reporter mice.

In the second part of the chapter, I will discuss the relationship between Prop1 and the Notch signaling pathway. Notch signaling works through direct cell-cell interactions to regulate patterning and morphogenesis in organisms from nematode to human [2]. In mammals, Notch signaling is mediated by interactions between the ligands Delta or Jagged, and the Notch receptor. Both ligands and receptor are single-pass transmembrane proteins, with extracellular EGF (epidermal growth factor) repeats. When ligand binds, it activates the Notch receptor, leading to a series of proteolytic cleavage events known as regulated intramembrane proteolysis. The Notch

intracellular domain (NICD), then translocates to the nucleus, where it acts as a transcriptional activator for the DNA-binding protein RBP-Jķ along with the co-activator Mastermind. In the absence of NICD, RBP-Jκ recruits corepressor complexes [3]. Known target genes of the Notch pathway include the Hes (hairy enhancer of split) family of bHLHs. Two of these proteins, Hes1 and Hes5, are known to play an inhibitory role in neurogenesis during cortical development by holding progenitor cells in an undifferentiated state, thereby preventing differentiation [4]. In pituitary development, *Hes1* is downregulated as cells undergo lineage commitment, suggesting that active Notch signaling may interfere with terminal differentiation [5]. Thus it seems that both Prop1 and Notch signaling work to promote the presence of an early undifferentiated progenitor population.

Results

Identification of Prop1 regulatory information

In an effort to identify the regulatory information required for the expression of *Prop1*, I constructed a reporter transgene consisting of the entire *Prop1* locus, plus an additional 6 kb upstream sequence, and 4 kb downstream sequence (Figure 4.1). A *lacz* cassette was fused to the third exon of Prop1. From one round of oocyte injection, I received three founders which carried the transgene. X-gal staining of e12.5 founders, followed by clearing, revealed that one of these three founders expressed *lacz* throughout the developing pituitary (Figure 4.1). Thus, it seems that the necessary information required for *Prop1* expression is contained somewhere within the 13 kb of *Prop1* genomic sequence in the reporter construct.

As the *lacz* insertion in the *Prop1* knockout is not expressed, I reasoned that one of the two *Prop1* introns must contain a required enhancer. A search on the UCSC Genome Browser revealed a highly conserved region in the first intron. The only other non-coding conserved region is located in the promoter. With this information, I designed my next reporter construct using only 2.2 kb of genomic sequence, comprising approximately 1 kb of promoter sequence, and the first exon and intron. As before, one round of injection produced 3 founders carrying the transgene, one of which gave strong, pituitary-specific expression (Figure 4.2). Therefore, this 2.2 kb construct contains regulatory information that is sufficient for the expression of *Prop1*.

The role of Prop1 in lineage determination

With this newly defined *Prop1* promoter, I generated a *Prop1-Cre* construct, in order to investigate Prop1 cell lineage analysis. *Prop1* is expressed throughout the anterior and intermediate lobes of the pituitary, with the exception of the rostral tip. However, only the Pit1 lineage appears to be affected by loss of Prop1 function. Are all pituitary cells descended from *Prop1*-expressing cells, and if so, why are the other lineages apparently normal even though they are equally mislocalized? To begin this investigation, I performed immunostaining experiments, double labeling with antibodies to Prop1 and either ACTH, αGSU or Lhx3 (Figure 4.3). Lhx3 is a LIM homeodomain factor expressed early in the developing gland, beginning at e9.5. Loss of Lhx3 produces early hypoplasia, and absence of most of the pituitary cell types [6, 7]. αGSU and ACTH are the only terminal markers expressed early enough to be

double-labeled with Prop1. At e14.5, αGSU is only expressed in the rostral tip thyrotropes, a region that is almost entirely separate from the Prop1 field (Figure 4.3). The origin of the rostral tip is unknown, as it appears to be the only population of cells capable of leaving the lumen in the Ames mutant. ACTH-expressing corticotropes are unaffected in the Ames, even though they are still trapped in the lumen. In addition, there does not appear to be any overlap with Prop1. Are these cells Prop1-negative because they have terminally differentiated, or because they never expressed *Prop1* to begin with? Either option seems possible, particularly when considering that losing Prop1 function does not compromise the ability of these cell types to progress normally in development, in contrast to the Pit1 lineages. In addition, these data confirm the hypothesis that Prop1 marks a progenitor population, as at e14.5, there is no co-localization with the early terminal markers, and Prop1 can no longer be detected by immunostaining by the time the rest of the gland differentiates.

In order to resolve the role of Prop1 in cell identity and lineage determination, it is necessary to be able to mark both *Prop1*-expressing cells, and their progeny. Injection of the *Prop1-Cre* construct into mouse oocytes produced 8 founders. The offspring of these founders were bred to the *ROSA26* reporter line, to determine the strength and specificity of expression. This strain carries a *beta-galactosidase* gene preceded by LoxP-flanked stop codons, inserted into the *Rosa26* locus that provides ubiquitous expression through the embryo [8]. Whole-mount analysis of P0 pups from each of the 8 founders revealed that 4 of the 8 Cre lines gave expression in the pituitary gland. Additional experiments were performed at earlier time-points with

lines 19 and 52, to insure that *Prop1-Cre* expression commenced as early as endogenous *Prop1*. Whole-mount x-gal staining of line 19 at e12.5, shows staining of the entire gland. Surprisingly, additional staining can be seen in the developing olfactory epithelium (Figure 4.4). This staining was not evident in the initial reporter mapping (Figure 4.1, 4.2). Sagittal sections of line 52 at e14.5 also show strong labeling of the entire pituitary gland. The migration of cells from the lumen into the surrounding mesenchyme can be clearly seen. Interestingly, the rostral tip also shows strong label, in contrast to immunostaining for Prop1 at the same age (Figure 4.3, 4.5). A similar result can be seen in frontal sections from P0 mice. At this time, cells have completed their migration from the lumen, and every differentiated cell type is present. The entirety of the anterior and intermediate lobes express *lacz* (Figure 4.6). This is the first evidence that every cell in the pituitary is descended from a Prop1-positive progenitor. Although there is no overlap between Prop1 and terminal markers from the non-Pit1 lineages, corticotropes, melanotropes and gonadotropes all originate from cells expressing *Prop1*. Final confirmation of the strength and efficacy of the *Prop1-Cre* line was achieved through crosses with the conditional β-catenin allele. As shown in Figure 5.7, deletion of *β-catenin* with the *Prop1-Cre* recapitulates the phenotype from the *Pitx1-Cre* crosses, in that there is a complete loss of Pit1. Therefore, this newly created *Prop1-Cre* should prove to be a useful genetic tool for the analysis of various signaling pathways in pituitary development.

Prop1 is a target of the Notch signaling pathway

I searched for conserved transcription factor binding sites using the TESS TRANSFAC database (http://www.cbil.upenn.edu/cgi-bin/tess/tess). A consensus RBP-Jκ sequence was found in the most conserved region (90%) of the first intron. To investigate whether RBP-Jκ was capable of binding to this region, electrophoretic mobility shift assays were performed using synthetic oligonucleotides representing the putative binding site and flanking regions. In vitro translated RBP-Jκ bound efficiently to the Prop1 intronic sequence, and this binding could be competed with cold oligo, or with another known RBP-Jκ binding site, but not with oligos in which the putative recognition sites had been mutated (Figure 4.8B).

In order to determine the relevance of this RBP-Jκ binding site *in vivo*, we looked at mice which have had Notch signaling ablated in the pituitary by conditional deletion of RBP-Jκ. Analysis of Prop1 expression in *Pitx1-Cre, RBP-Jκ*$^{f/f}$ mice, shows a dramatic decrease in Prop1 levels at e12.5, when Prop1 levels are at their peak. In contrast, at e11.5, Prop1 levels appear unchanged (Figure 4.8C). These results suggest that Notch signaling is required for the maintenance of Prop1 expression, but not for initial activation. As would be expected in mice with a Prop1 deficiency, Pit1 is also absent in *Pitx1-Cre, RBP-Jκ*$^{f/f}$ mice. However, in contrast to the Prop1 knockout mice, the *Pitx1-Cre, RBP-Jκ*$^{f/f}$ mice also display a striking increase in the number of corticotropes [5].

Interestingly, ectopic Notch signaling gives a similar phenotype to that of the *Pit1-Prop1* transgene. Expression of NICD under the 15 kb *Pit1* promoter produces

transgenes with post-natal dwarfism and pituitary hypoplasia, with reduction in somatotropes, lactotropes and thyrotropes (Figure 4.9, [5]). These observations indicate that sustained expression of activated Notch in Pit1-expressing precursors inhibits terminal differentiation of these cell types. *In situ* hybridization at e14.5 and e17.5 shows a dramatic increase in Prop1 levels (Figure 4.9). In addition, both Pit1-Prop1 and Pit1-NICD females are infertile (J. Tollkuhn, unpublished data, [5]).

Discussion

2.2 kb of Prop1 genomic sequence drives expression in all anterior pituitary cells.

In this chapter I have described the identification of *Prop1* regulatory information through the use of transgenic reporter mice. Surprisingly, 2.2 kb of genomic sequence is sufficient to direct expression throughout the developing Rathke's pouch. Using this newly defined "Prop1 promoter", I have generated *Prop1-Cre* mice, and validated the efficacy of this new Cre line by recapitulating the β-catenin loss-of-function phenotype described in Chapter 3. This *Prop1-Cre* should prove to be a valuable tool for targeted deletion within the pituitary gland, as the *Pitx1-Cre* that is currently used for such a purpose is expressed in many regions outside of the pituitary. Expression of *Pitx1-Cre* in the first branchial arch can result in mutants with jaw and palate defects, and indeed, our *β-catenin* mutants die at birth as they are unable to feed due to a complete lack of a lower jaw (L. Olson, unpublished data). In addition, as *Prop1-Cre* initiates expression at e10.5, a day later than *Pitx1-Cre*, it can be used to evaluate the role of various signaling pathways in determination

and differentiation, rather than in earlier organ commitment events. As discussed in Chapter 3, use of the *Pitx1-Cre* to constitutively activate β-catenin resulted in the complete loss of the pituitary gland, making it impossible to assess the contribution of Wnt signaling to later developmental events.

I have also used the *Prop1-Cre* to perform Prop1 lineage analysis. Crossing *Prop1-Cre* to *ROSA26* reporter mice produces a permanent change in the genome of *Cre*-expressing cells, so that all resulting progeny will continue to express *lacz*. The contribution of Prop1 to the various pituitary cell-types can then be assessed long after *Prop1* itself has ceased to express. My results indicate that every cell in the pituitary is descended from a Prop1-positive progenitor. In addition, it appears that *Prop1-Cre* is expressed in the olfactory epithelium. As olfactory x-gal staining can be seen in both line 19 and line 52, it can not be attributed to effects of random transgene insertion. Therefore, the 2.2 kb promoter must contain sequence that directs *Cre* expression to this region, which is surprising as both the 16 kb and the 2.2 kb reporter constructs expressed only within the developing pituitary. As the *Prop1-Cre*, *ROSA26* crosses show the location of where the *Cre* was expressed and the reporter mapping could only reveal where lacz was expressed at a specific moment in time, it is possible that ectopic expression of the reporters was missed, as it occurred before e12.5, the time at which the reporter transgenics were analyzed. The hypophyseal placode, from which the pituitary develops, and the olfactory placode are located in the same vicinity in the early embryos, and are the only two sensory placodes that originate in the absence of neural crest cells [9]. Perhaps there is some early transient expression of *Prop1* in the

olfactory placode, that has never been noticed before. What is more likely, however, is that in creating a 2.2 kb –driven *Cre*, I have removed a required repressor element from the genomic sequence. This has been seen before in the case of the *Hesx1* regulatory information, where *Hesx1* expression can be directed to the anterior neural ridge with as little as 568 bp of information. However, at later stages, rather than becoming fully restricted to Rathke's pouch, there is ectopic *Hesx1* expression within the hypothalamus. This ectopic expression is lost when an additional upstream element between -568 and -532 was included in the reporter [10]. Another potential cause of *Prop1-Cre* expression in the olfactory epithelium could be that the *Prop1* gene is located adjacent to a cluster of four olfactory receptor genes (http://genome.ucsc.edu/, chr11:50,700,124-50,790,587). Although the closest gene, *Olfr1378*, is located 25 kb away from the *Prop1* start site, there are no other genes in between. It is possible that the 2.2 kb of *Prop1* genomic sequence contains an enhancer for this cluster of olfactory receptor genes, thereby causing Cre expression within the olfactory epithelium. This seems the most plausible explanation, as by *in situ* hybridization at e10.5 onwards, *Prop1* itself can only be detected in the pituitary [11].

Prop1 is a direct target of the Notch signaling pathway.

As loss of Prop1 function appears to seriously affect only the Pit1 lineages, this raises the question of what Prop1 is doing in the other cell-types. The answer has come from studies on the Notch pathway and its role in maintaining a proliferating precursor population within the lumenal area of the pituitary [5]. Notch activity is

required within a precise temporal window to control lineage commitment of Pit1-positive precursors. The loss of Notch signaling results in a fate switch to corticotropes, as cells cease proliferation and begin migration from the lumen as early as e12.5 [5]. This finding re-enforces parallels between the developing pituitary and the central nervous system (CNS). Conditional deletion of *Notch1* in neural progenitors with *Nestin-Cre* results in precocious neuronal differentiation, while deletion in the telencephalon with a *FoxG1-Cre* driver gives fewer neurons due to depletion of the early progenitor pool. [12, 13].

Notch exerts its control over cell fate largely through regulation of Prop1. The *Pitx1-Cre, RBP-Jκ$^{f/f}$* mice display a dramatic decrease in Prop1 levels at e12.5, and this effect appears to be direct, as RBP-Jκ is capable of binding to a conserved CSL site within the required intronic enhancer of Prop1. Thus it seems that a combination of Prop1 and Notch signaling causes cells to undergo irreversible changes in gene expression or epigenetic status such that they are competent to assume a later cell fate. However, the latest cell-type to appear, the gonadotrope, is unaffected in either RBP-Jκ or Prop1 mutants, implicating the existence of an additional later signal. Intriguingly, the conditional β-catenin mutants also appear to have undergone a cell-fate switch to corticotropes, as seen by increased *POMC* signal (Chapter 3, Figure 3.2). This suggests synergy between Notch and Wnt signaling in pituitary development, as is seen in many other systems, such as somitogenesis, intestine, and hair follicle development [14-16].

Methods

Transgenic mice

Prop1 reporter mice: The initial 16 kb Prop1 reporter construct was made beginning with a 6kb *NotI/Xho* fragment of Prop1 upstream genomic sequence. The sequence spanning the first and second exons, up to an endogenous *XhoI* site was produced by PCR and ligated to the upstream sequence. The 2nd through 3rd exons (with no stop codon) were generated by PCR, using the sites *XhoI* and *ApaI*. A LacZ cassette, containing its own stop codon was fused to the end of the third PropI exon using *ApaI* and *SacII*. An additional 4kb of 3' genomic sequence was produced by PCR, using *SacII* and *NotI* sites. Thus, the entire 16 kb construct could be linearized from pBKS with *NotI*. The second reporter construct was made with a 2.2 kb *HindIII* fragment of Prop1 genomic sequence, comprising 1kb of upstream sequence, the first exon and intron, and part of the second exon. A lacz cassette containing it's own stop codon and poly-adenylation signal was fused to the *HindIII* fragment.

Pit1-NICD: The mouse Notch1 intracellular domain, amino acids 1744–2183, was generously provided by Dr. R. Kopan (Washington University, St. Louis, MO) [18]. A hemagglutinin (HA) tag was added at the C terminus of the NICD cDNA. The Notch1-ICD-HA ORF was inserted between a rabbit 0.65-kb b-globin intron and a 0.63 kb poly A fragment of the human growth hormone gene at the 3' end. The 15-kb Pit1 promoter was inserted 5' of this cassette, and transgenic animals were genotyped by PCR using primers. 5'-GCAACGTGCTGGTTATTGTGC-3' and 5'-CGGTCTGT CTGGTTGTGCAAGCTG-3'.

Prop1-Cre: The 2.2kb Prop1 HindIII fragment was used in combination with a 3' polyadenylation signal from human growth hormone as an acceptor for a bacteriophage P1 Cre-recombinase cDNA. Resulting transgenes were genotyped with the oligos 5'- GGAAATGGTTTCCCGCAGAAC-3', 5'-ACCCTGATCCTGGCAAT TTCG-3'. All transgenic mice were prepared for injection as described [19].

Floxed mouse lines and breeding

The floxed RBP-Jκ mice have been previously described [20]. Pituitary-specific deletion was achieved by crossing to *Pitx1-Cre* mice [21]. Prop1 lineage analysis was performed by crossing *Prop1-Cre* mice to ROSA26 homozygotes. To establish the efficacy of *Prop1-Cre*, line 52 was crossed to floxed β-catenin mice [22]

Histology

X-gal staining: e12.5 embryos were fixed in 4% paraformaldehyde for 30 min, washed 5x in PBS, and incubated overnight at 37°C in staining solution (5mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM MgCl2, 0.2% Triton X-100, 1mg/ml x-gal in PBS). Embryos were dehydrated in an ethanol series, and cleared in a 1:2 mix of benzyl alcohol and benzyl benzoate. e14.5 and P0 embryos were treated as for immunostaining, and the slides were incubated in x-gal solution overnight at 37°C. For immunostaining, embryos were fixed for 30 minutes in 4% PFA, then dehydrated in 20% sucrose, frozen in 1:1 OCT/Aquamount, and sectioned at 14 microns. Prop1 protein was detected with a guinea-pig anti-Prop1 antibody, made to the C' of Prop1 (aa129-225), and rabbit polyclonals to αGSU, ACTH, and

Pit1. Secondary antibodies from Molecular Probes were AlexaFluor 488- or 594-conjugated, and nuclei were counterstained with DAPI. In situ hybridization was performed as previously described [23], on formalin-fixed 14 micron cryosections, using S-35 labeled antisense probes.

Electrophoretic Mobility Shift Assay

EMSA experiments were performed as previously described [21]. RBP-Jκ was transcribed and translated using the TNT Quick Coupled Transcription/Translation System (Promega). In vitro translated proteins were incubated with 1x binding buffer (25mM Tris at pH 7.5, 50mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glyerol), DNA competitors, and 1 μg poly dIdC for 15 min on ice prior to adding probe. Probe was allowed to bind for 20 min at room temperature, and then protein-DNA complexes were resolved by electrophoresis. Oligonucleotides used for EMSA are 5'-CTTGAGCTCGTGGGAAAGGCTTGCC-3', 5'-GGCAAGCCTTTCCCACGAGC TCAAG-3' (Prop1 intron); 5'- CTTGAGCTCGTGaacAAGGCTTGCC-3', 5'-GGCAAGCCTTgttCACGAGCTCAAG-3' (Prop1 intron with mutations); and 5'-AAACACGCCGTGGGAAAAAATTTGG-3',5'-CCAAATTTTTTCCCACGGCGTG TTT-3' (RBP-Jκ binding site from Epstein-Barr virus C promoter region).

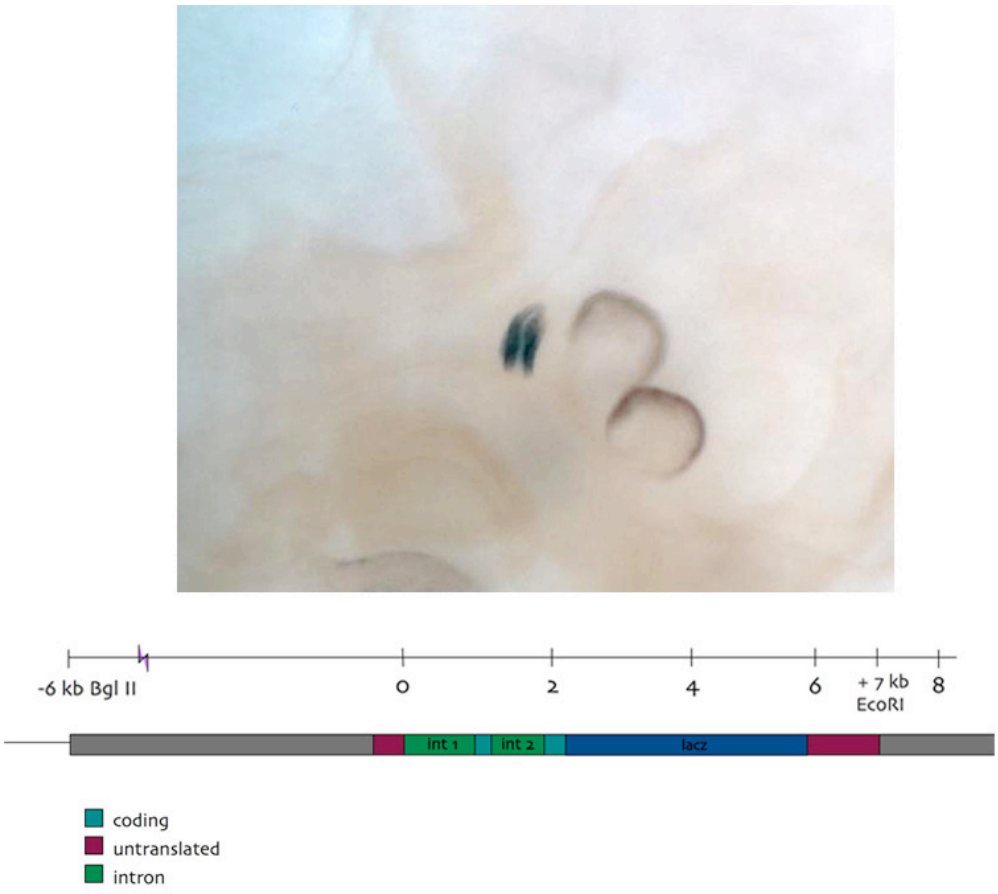

Figure 4.1 Expression of the 16kb *lacz* reporter.
Sagittal view through a cleared e12.0 embryo. X-gal staining shows lacz expression throughout the developing pituitary, although the ventral-most portion of Rathke's pouch is not yet marked, reflecting the dorsal to ventral gradient of endogenous *Prop1* expression.

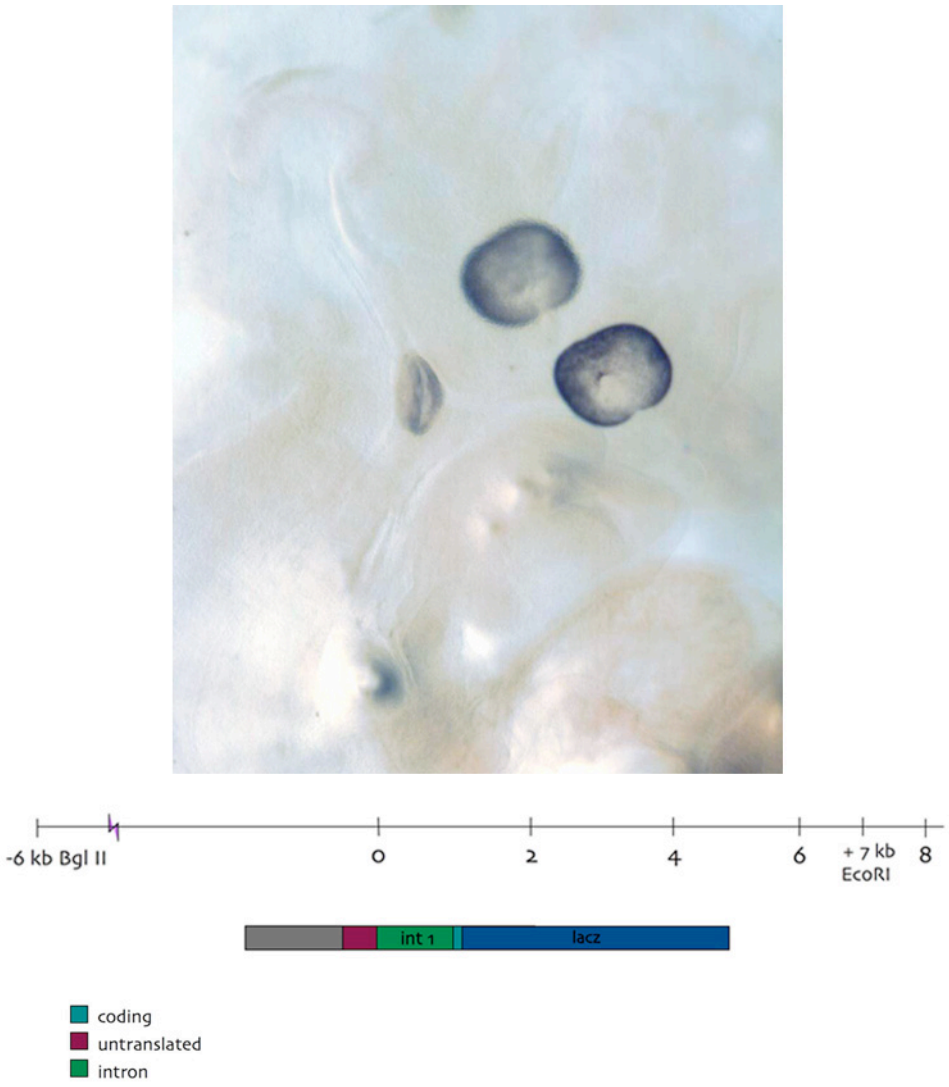

Figure 4.2 Expression of the 2.2kb *lacz* reporter.
Sagittal view of a cleared e12.5 embryo. 2.2kb of Prop1 genomic sequence is sufficient to drive expression of lacz specifically within the pituitary.

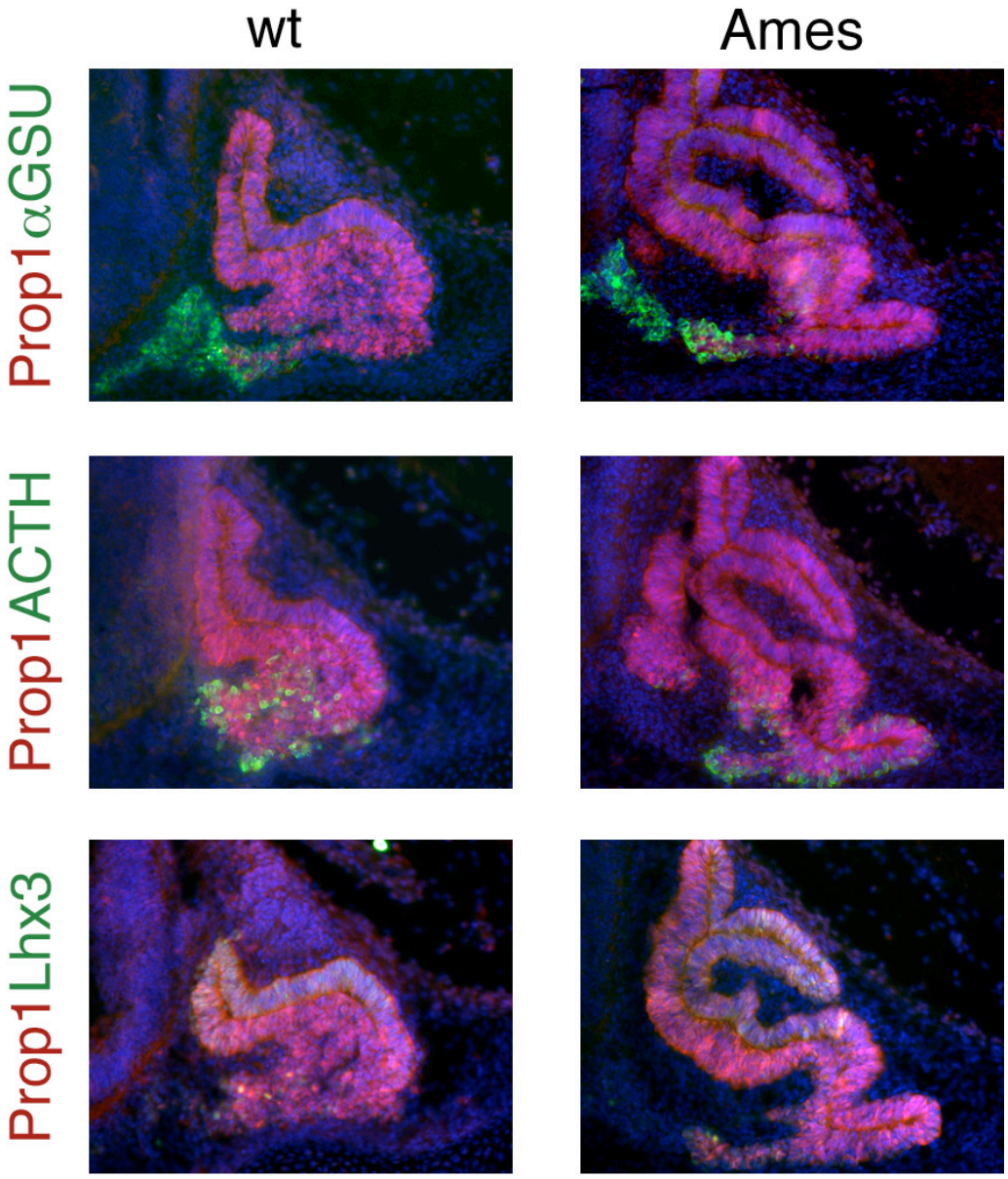

Figure 4.3 Prop1 is not expressed in terminally differentiated cells.
Double immunostaining with antibodies Prop1 and either αGSU or ACTH shows that Prop1 is excluded from both the rostral tip of the gland (αGSU) or from corticotropes (ACTH). In contrast, Prop1 overlaps with Lhx3, one of the first transcription factors to be expressed in the pituitary gland, before differentiation occurs.

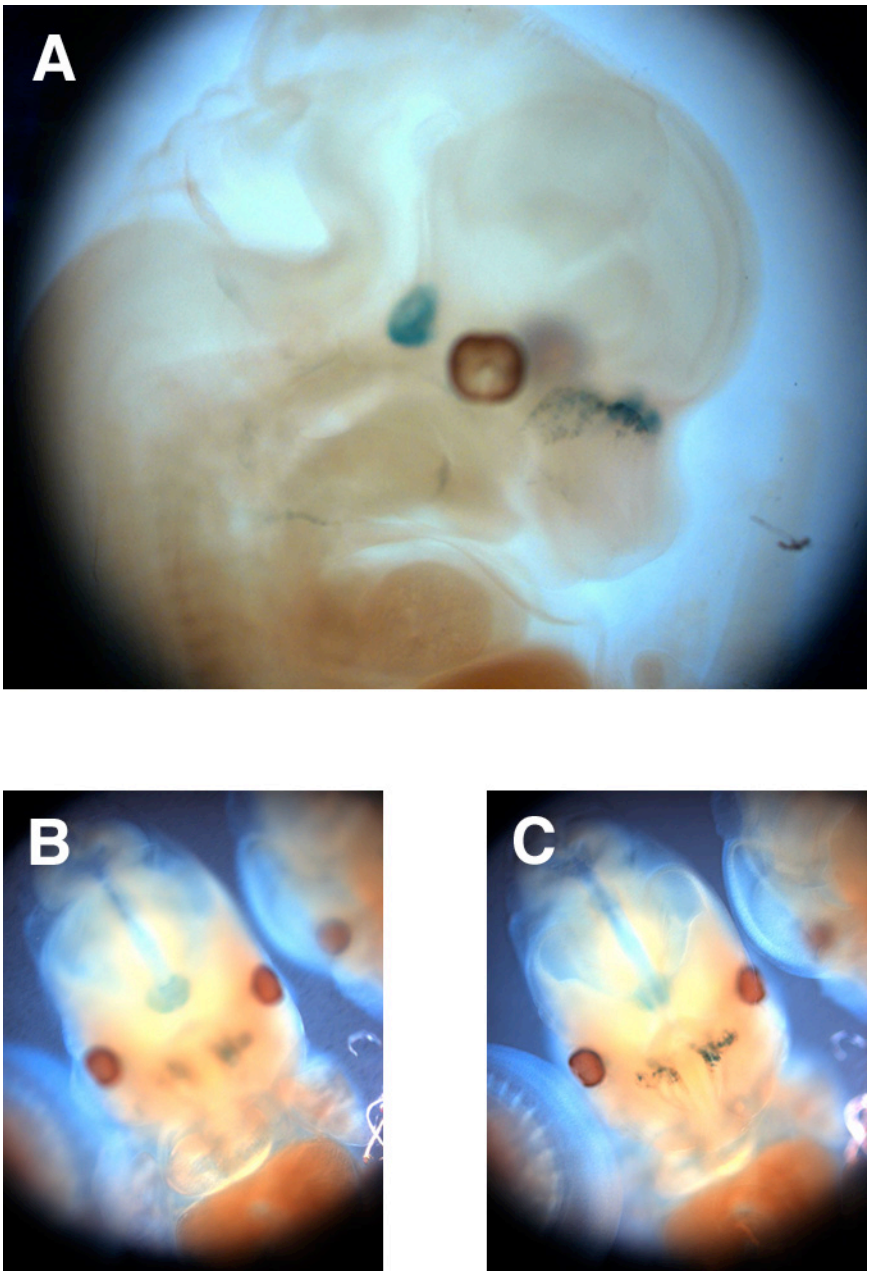

Figure 4.4 Prop1-Cre expresses throughout the pituitary at e12.5
Line19 was crossed to ROSA26 reporter mice. **A)** At e12.5, lacz expression can clearly be seen throughout the entire pituitary, but also in the developing olfactory epithelium. **B)** Frontal view, focused in the plane of the pituitary, showing blue color in the entire pouch. **C)** Frontal view, focused on the olfactory epithelium

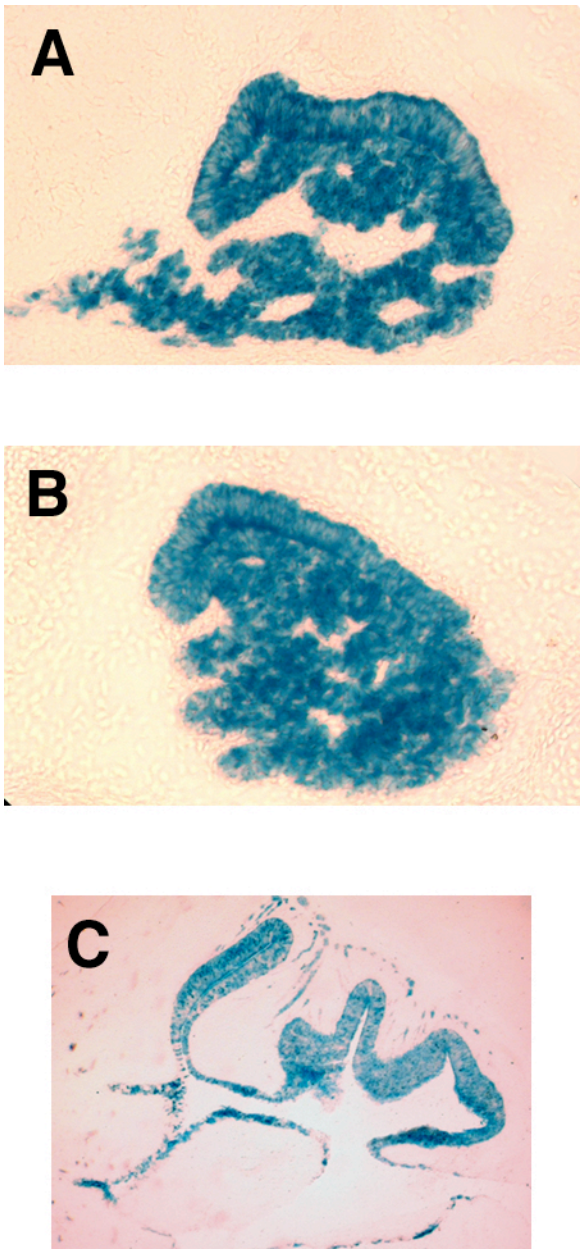

Figure 4.5 Prop1-Cre marks every cell in the pituitary at e14.5
A) Sagittal midline sections of e14.5 embryos from Prop1-Cre line 52 crossed to ROSA reporter mice. The migratory stream of cells leaving the lumen can be clearly seen. The rostral tip is also marked. **B)** Lateral section where the gland is more dense. **C)** Midline section of olfactory epithelium.

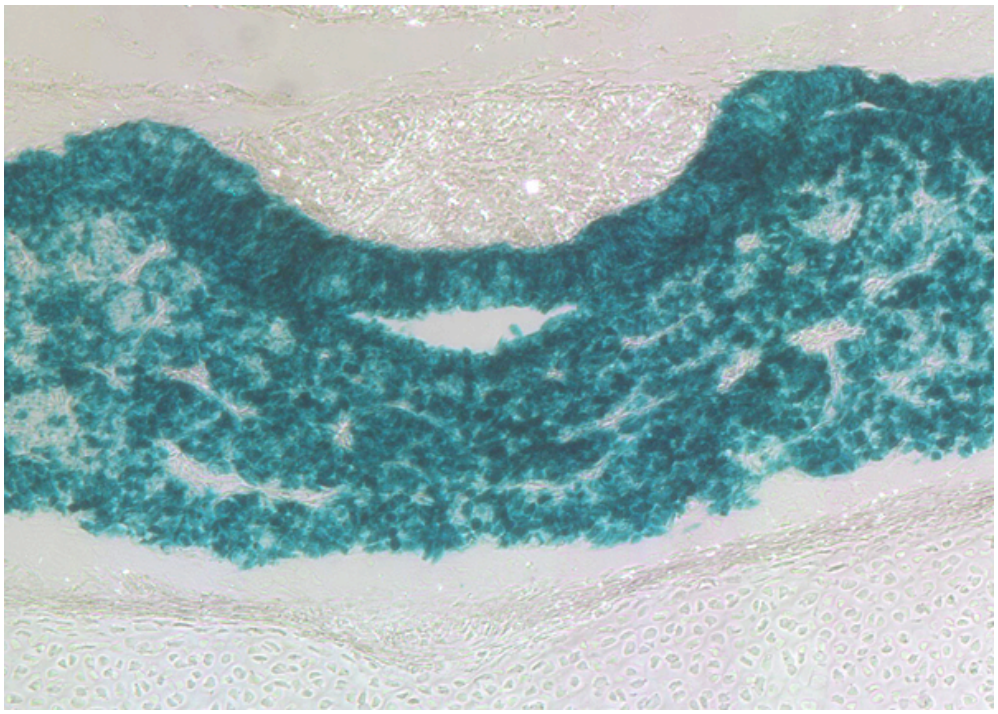

Figure 4.6 Prop1-Cre marks every cell in the pituitary at birth
Frontal section of P0 mouse pituitary. The entirety of the intermediate and anterior lobes express lacz, indicating that all cells have descended from *Prop1-Cre* expressing progenitors.

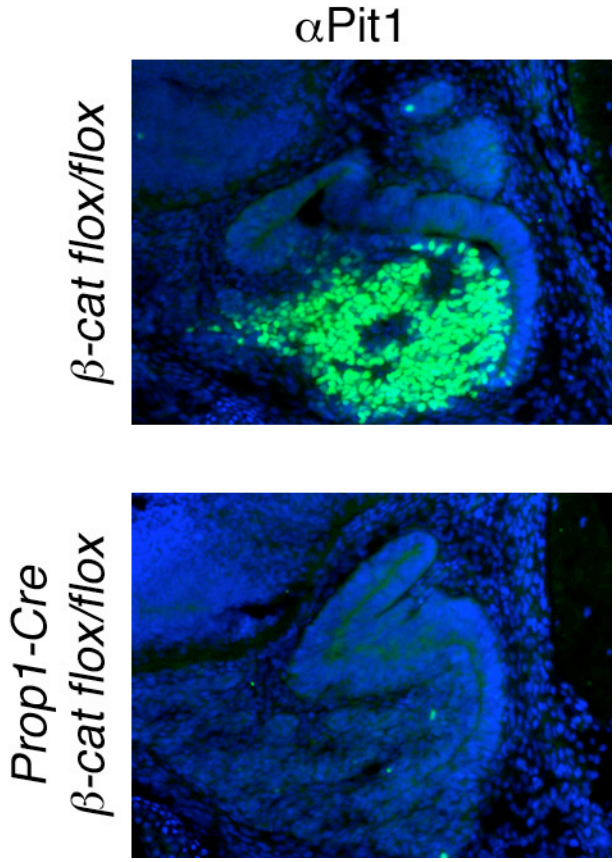

Figure 4.7 Conditional deletion of β-catenin with *Prop1-Cre* abrogates *Pit1* expression
Immunostaining for Pit1 in both *β-cat flox/flox*, and *Prop1-Cre*, *β-cat flox/flox* embryos at e14.5 demonstrates a recapitulation of the phenotype seen with conditional deletion using *Pitx1-Cre*. At e14.5, there is no Pit1 protein in embryos carrying the *Prop1-Cre*, thereby demonstrating its efficacy.

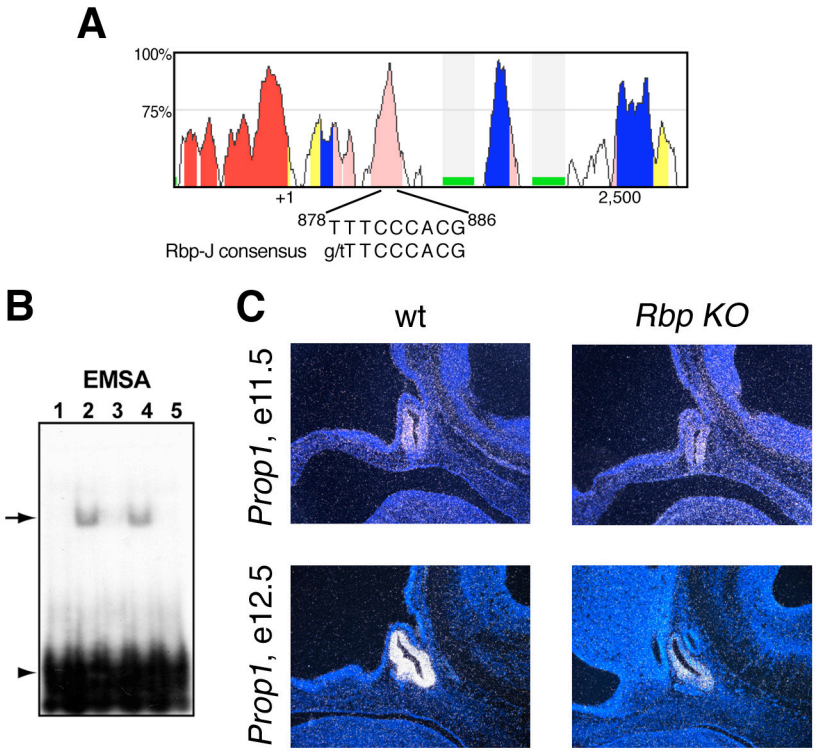

Figure 4.8 *Prop1* is a direct target of Notch signaling
A) Prop1 expression is significantly down-regulated in RBP-Jk, Pitx1-Cre mutant embryos at e12.5, but is unchanged at e11.5. **B)** Genomic DNA sequences of mouse and human Prop1 were compared using VISTA (red) promoters, (yellow) UTRs, (blue) exons, (pink) introns. Two evolutionarily conserved regions, the promoter and the first intron, show >75% homology. A putative RBP-Jk binding site is identified in the first intron. **C)** A 32P-labeled 25 bp oligonucleotide encompassing the putative RBP-Jk binding site was incubated in the absence (lane 1) or the the presence (lanes 2-5) of in vitro translated RBP-Jk and the competitors. Unlabeled oligonucleotides (lane 3), equivalent oligonucleotides where the putative RBP-Jk binding site was mutated (lane 4), or the oligonucleotides containing a RBP-Jk binding site from the Epstein-Barr virus C promoter region (lane 5) were used as competitors at 100x molar excess. The arrow indicates the shifted probed caused by RBP-Jk binding, and the arrowhead indicates free probe.

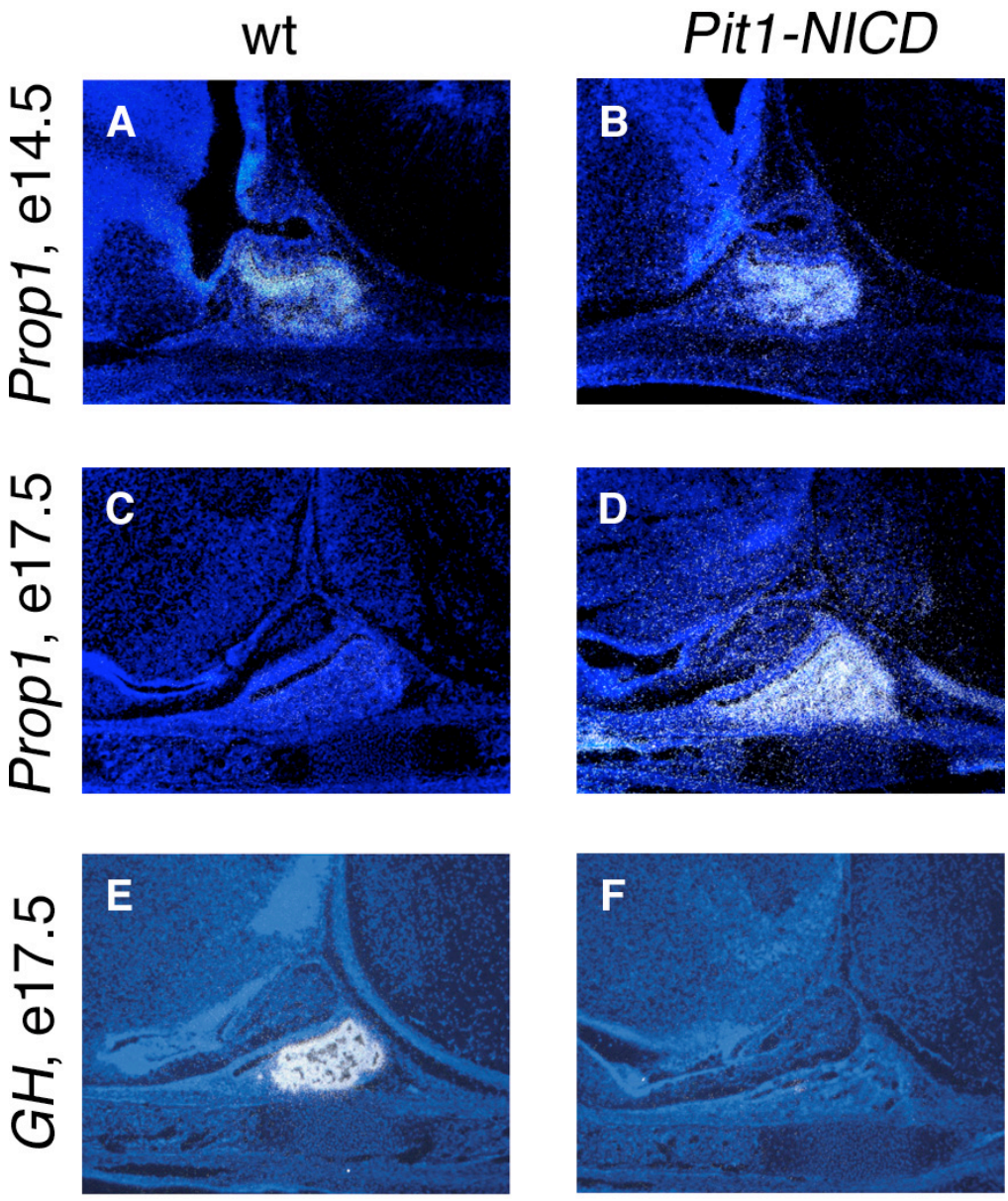

Figure 4.9 *Pit1-NICD* transgenes display an upregulation of Prop1 expression
Sagittal sections of e14.5, and e17.5 embryos show strong Prop1 expression in Pit1-NICD transgenes **(B, D)**, whereas in wild-type littermates, Prop is expressed primarily in luminal cells at e14.5 **(A)**, and is completely absent by e17.5 **C)**. As in the Pit1-Prop transgenes, GH expression is downregulated by Pit1-NICD (E,F).

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Chapter 5:Analysis of the Prop1 Genetic Program

Introduction

Chapter 3 has described the mechanism by which the Prop1/β–catenin complex activates the *Pit1* gene. Analysis of Lef1 knockout pituitaries indicates that β–catenin uses only Prop1 for this process, rather than its established DNA-binding partners, the Tcf/Lefs. In this chapter, I will investigate further the interplay between Prop1 and Lef1 through both biochemical experiments and analysis of the LefB mice, which act as a dominant-negative for all Tcf/Lef family members. Both Prop1 and β–catenin have additional roles in pituitary development, as seen in the genetic gain and loss of function studies detailed in Chapters 2 and 3. Early over-expression of either *Prop1* or *β–catenin* under *Pitx1* causes a complete loss of the gland, indicating the existence of additional common target genes besides *Pit1* that are required for proper cell identity during organogenesis. The *β–catenin* null pituitaries are hypoplastic and mildly dysmorphogenic, while *Prop1* null pituitaries possess a normal number of cells, but a very severe dysmorphogenesis. This chapter more thoroughly examines the genetic programs mediated by Prop1 and β–catenin through the use of microarray expression analysis. Results which demonstrate that additional homeodomain factors are also capable of inhibiting canonical Wnt/β–catenin signaling will also be presented. This finding indicates that the processes of differentiation, proliferation and cell migration mediated by Prop1 and β–catenin may prove to be relevant to development of other systems and in disease. The chapter will conclude with a

summary of the findings presented in the dissertation and their implications, as well as discussion of future directions of research.

Results

Tcf/Lef signaling is not required for Pit1 *expression*

Although we have clearly established that Lef1 is not required for *Pit1* expression, the question of redundancy with other Tcf/Lef factors remained. To address this point, we analyzed pituitaries from *LefB* mutant mice. The *LefB* allele was created by a targeted in-frame insertion of the *beta-galactosidase* gene into the *Lef1* locus. This insertion disrupts the HMG domain of Lef1, and interferes with its ability to bind DNA. The LefB protein is also capable of interfering with the function of other Tcf/Lef factors, both in transient transfection assays, and *in vivo* [1]. Hematoxylin and eosin staining at e16.5 shows that mutant pituitaries are somewhat larger, although the overall density of the gland has decreased (Figure 5.1). This is reminiscent of the pituitaries of Tcf4 knockout mice [2]. Immunostaining for Pit1 shows slightly sparser staining in the LefB mice when compared to wild-type, but the DAPI counterstain reveals that this is due only to the increased interstitial space (Figure 5.2). Thus, Pit1 levels are not affected by a block in all Tcf/Lef factors, further corroborating the initial conclusion from Chapter 3. Surprisingly, GH is entirely absent in the LefB mice (Figure 5.2). ACTH is not affected, indicating that this block in differentiation is specific to the Pit1 lineage.

Analysis of the Prop1 genetic program

Although the regulation of *Pit1* by Prop1 has been extensively characterized, there are clearly many other genes regulated by Prop1, as is evident from both the dysmorphogenic phenotype of the *Prop1* mutant, as well as the results from early and late overexpression of *Prop1*. Expressing *Prop1* under the *Pitx1* promoter, beginning at e9.0, results in the complete loss of the pituitary gland [3], while extending *Prop1* expression past e14.5 using the *Pit1* promoter causes a delay in differentiation (Chapter 2, Figure 2.7). In an effort to elucidate the genes responsible for these other effects of Prop1, I performed microarray analysis on microdissected e12.5 pituitaries from wild-type and *Prop1* mutant animals. At this time, Prop1 levels are highest, and there is no obvious difference between wild-type and mutant animals, so there is the greatest likelihood that the differentially expressed genes will be direct targets of Prop1. The complete list of known genes with significant changes is presented as Appendices 1 and 2. Components of the Wnt, Notch and BMP signaling pathways, as well as genes involved in cell adhesion and migration, are strongly represented (Table 5.1). I selected some of these genes for validation by quantitative RT-PCR. Downregulated genes are *Prickle1*, *Spock3*, *Id2* and *Corl1*. Prickle1 is one of three mammalian homologs to Drosophila Prickle, which is an component of the planar polarity pathway [4]. *Prickle1* has previously been shown to be highly expressed in pituitary at e12.5 [4]. Spock3 is a proteoglycan named for its sparc/osteonectin, cwcv and kazal-like domains, also called Testican-3. Spock3 can inhibit membrane type matrix metalloproteinases [5]. Id2 is a member of the Id family of bHLHs. Id proteins

lack a DNA binding domain, but interfere with transcription by forming dimers with E proteins, thereby sequestering them from other bHLHs that require E proteins for activity. In cortical development, Ids work to promote progenitor proliferation, and inhibit differentiation [6]. Corl1 is a corepressor expressed in the CNS, that can interact with and repress transcription by, the homeodomain factor Lbx1 [7]. Lbx1 is also capable of using TLE as a corepressor, similar to Prop1.

For upregulated genes, I chose *Dll1*, *Bmpr1b*, and *BMP-3*. Dll1 (Delta-like 1) is a single-pass transmembrane ligand for the Notch receptor. Bmpr1b (bone morphogenetic protein receptor 1b) is a receptor for the BMP members of the TGFβ superfamily of cell signaling molecules [8]. BMP-3 is an inhibitory BMP, as its actions oppose those of BMP-2 and 4, both of which are required for pituitary development. In Xenopus, BMP-3 can inhibit both BMP and activin signaling by binding to ActRIIb, a common receptor for both proteins. This binding inhibits the phosphorylation of receptor-associated Smad proteins, which normally act on other DNA-binding Smads, thereby transmitting the BMP signal to the nucleus. Thus, BMP-3 acts as a conventional BMP antagonist such as noggin or chordin [9].

Real-time PCR analysis corroborated the results from microarrays for every gene checked (Figure 5.3). In addtion, the relative levels of transcript normalized to the housekeeping gene L32, roughly correlate with the fold change numbers. For example, *Corl1* was the most strikingly downregulated on the array, with a fold change of -12.33. *Corl1* was also the most downregulated gene by real-time analysis, with an

average ΔΔCt of -4.015 cycles. Similarly, *Id2*, was the least changed gene in both assays, with a fold change of -3.09 and a ΔΔCt of -1.15 (Figure 5.3).

Additional target genes were validated by *in situ* hybridization. This method is complimentary to real-time PCR, as it shows exactly where in the pituitary the various transcripts are being generated, but is not very quantitative. At e12.5, *Robo2* is expressed more strongly in the ventral part of the gland. There is also expression throughout the presumptive palate, and in the ventral diencephalon. In the Prop1 null animals, the pituitary signal is lost, but *Robo2* persists just ventral to the pouch, in the area where cells should begin to migrate (Figure 5.4). *Crossveinless-2* (*Cv-2)*, a known potentiator of BMP signaling, [10] is present in very discrete areas in the ventral part of the gland, the nascent posterior lobe, and in locations of cartilage condensation (Figure 5.4). The three upregulated genes, shown in Figure 5.5, are expressed either at very low levels, or not at all in wild-type mice at e14.5 In *Prop1* knock-outs, *Patched2* marks a very specific section of the lumen that appears to correspond to the the most dorsal portion of the anterior lobe. *Hes5*, a target gene of the Notch pathway, is the most strongly changed gene analyzed by in situ. In wild-type pituitaries, its expression is primarily within lumenal cells, similar to the expression pattern of *Notch2* [11]. In the mutant, *Hes5* signal is very bright throughout the entire lumen, and the dysmorphogenesis can be clearly seen (Figure 5.5). *BMP-3* is also expressed in the lumen, although it is absent in both the intermediate lobe, and the dorsal-most portion of the anterior lobe where *Patched2* can be detected. *BMP-3* is entirely absent in wild-type pituitaries (Figure 5.5).

Comparison of Prop1 and beta-catenin genetic programs

The results from the LefB mice indicate an additional role for canonical Wnt signaling in the pituitary besides the induction of *Pit1*. To address this question, and to better understand the relationship between Prop1 and β–catenin, microarray analysis was performed on wt and *β–catenin* null e12.5 pituitaries. A complete list of changed genes is presented as Appendices 3 and 4. The first observation that can be made is that there are many more genes affected by loss of β–catenin than by loss of Prop1, which is consistent with β–catenin being the downstream effector of multiple Wnt signaling molecules, as well as the component of adherens junctions. As would be expected, there are many changes in levels of genes involved in cell cycle regulation and the Wnt pathway (Table 5.2). Several Wnt molecules are induced, while the Wnt/β–catenin target gene, *Axin2*, is downregulated. The *β–catenin* null pituitaries are substantially smaller than wild-type (Figure 3.2C), reflecting the role of canonical Wnt signaling in cell proliferation and self-renewal [12]. There is also a large group of genes involved in cell adhesion and migration, but surprisingly, very few of these genes are also changed in the *Prop1* knockout. Some dysmorphogenesis is apparent the caudal region of *β–catenin* mutant pituitaries (Figure 3.2A, B), but frontal sections reveal only a slightly enlarged lumen (Figure 3.2C), without the extensive convolution seen in *Prop1*-null animals (Figure 2.5). It is possible that this group of genes is part of a response to the loss of adherens junctions. β–catenin plays an essential role in the formation of this complex, coupling transmembrane E-cadherin to α-catenin and the actin cytoskeleton [13]. Tyrosine phosphorylation of β–catenin has been shown to

lead to loss of cell-adhesion, as well as an increase in transcriptional activity [13]. Interestingly, there are two different tyrosine phosphotases upregulated in the *Prop1* (Ptprd) and *β–catenin* knockouts, (Ptprq).

One of the more intriguing findings from the β–catenin array data is the number of general transcription enzymes and co-factors that are found, in addition to many sequence-specific factors. HP1gamma (-2.50) binds methylated H3K9 residues on both active and inactive chromatin, and is associated with transcriptional elongation [14]. Jmjd1a (-2.12) is a JmjC-domain containing protein, and these enzymes have recently been shown to demethylate histones specifically at trimethyl-H3K9 and trimethyl-H3K36 [15]. Phc2 is a member of the Polycomb group of transcriptional repressors that act to maintain silencing of clusters of homeotic genes during development [17]. Phc2 has been purified as a member of an E3 ubiquitin complex that is specific to H2A [16], and is required for silencing of Hox clusters in mouse development [17]. Upregulated genes include two methyl DNA-binding proteins, Mbd1 (1.85) and Zbtb33 (1.72), also known as Kaiso. In developing Xenopus embryos, Kaiso represses β–catenin target genes and this repression is relieved by binding of Kaiso to p120-catenin [18]. Other transcriptional regulators of note include a large number of zinc-finger containing proteins; two forkhead family members, *Foxp2* and *Foxg1*; the repressive factor *Engrailed*; two Nkx family members; and the orphan nuclear receptor *Nr2e1*, also known as *Tlx*.

Common targets of Prop1 and β-catenin

The similarity of both gain- and loss- of function phenotypes for Prop1 and β–catenin suggests the existence of other shared target genes in addition to *Hesx1* and *Pit1*. Table 5.3 consists of genes that have changed in the same direction in both knockouts, while Table 5.4 lists genes which are regulated in an opposing fashion. The most highly downregulated gene in both *Prop1* and *β–catenin* null pituitaries is *Prickle1*, a component of the planar cell polarity pathway (PCP). In Drosophila, the PCP pathway controls the orientation of hairs, bristles and ommatidia, while in vertebrates it mediates convergent extension movements required for gastrulation and neurulation, as well as orientation of hairs within the cochlea [19-21]. The PCP pathway contains two components of the canonical Wnt signaling pathway, the transmembrane receptor Frizzled, and the cytoplasmic signaling molecule Dishevelled, but does not utilize β–catenin. As the PCP pathway is associated with coordinated cell movements, it is possible that it may play a role in the migration of cells away from the lumen. *Robo2* is another gene that is downregulated in both knockouts. Robo receptors, and their ligand, Slit, are important mediators of axon guidance and cell migration. Robo2 has been well-studied in Drosophila, where it is required for migration of sensory neurons, compartmentalization of the visual system and cardiac morphogenesis [22-25]. In all of these systems, Robo2 works with Slit to maintain cell alignment and organization, suggesting that when Robo2 and Slit are co-expressed, they act as heterophilic cell adhesion molecules [23, 24]. Other genes associated with adhesion and migration that are downregulated with the loss of either

Prop1 or β–catenin include *Lmo7* [26], *endomucin* (*Emcn*) [27], *neuropilin2* (*Nrp2*) [28, 29], and *Flrt3* [30].

A surprising number of homeobox-containing genes were upregulated in both knockouts: *Dbx1*, *Dlx2*, *Lhx5*, *Isl2*, and *Onecut2*. None of these factors have been previously implicated in pituitary development. The HMG-box protein Sox2 is the only other transcription factor to appear on the list of common upregulated genes. Mice mutant for *Sox2* show perinatal lethality, but heterozygotes display a variety of pituitary defects, including reduced cell number, deficiencies in LH and GH levels, and a bifurcated pouch. *Sox2* is expressed in neural stem cells in the ventricular zone, and in the lumenal area of the pituitary, providing a molecular link between these two analogous systems [31]. The general increase in homeodomain protein expression could potentially be an attempt to compensate for the loss of Prop1/β–catenin function. This indicates that other homeodomains could function with β–catenin in a similar fashion to Prop1.

The last group of genes are changed in opposing directions in the two knockouts. This is a small cohort of genes, but it contains a few interesting transcription factors, adhesion/migration components and cell cycle regulators. There are also two more tyrosine phosphatases; *Ptprb* and *Ptpro*. Not only is tyrosine phosphorylation crucial for β–catenin regulation [13], but Ptpro can mediate axon guidance [32], specifically through de-phosphorylation of Eph receptor tyrosine kinases [33], thereby regulating their responsiveness to ephrin ligands. Ephs and ephrins also play a role in cell migration in many systems [34]. The homeodomain-

containing factor; Pax1, has already been shown to be a β–catenin target gene [35], while the bHLH Heyl is an established Notch effector which represses *GATA4* and *GATA6* in heart development [36]. *Scleraxis* (*Scx*), also a bHLH, functions downstream of FGF signaling and the MAP kinase cascade [37], and is a target of the Ets transcription factors Pea3 and Erm, both of which are highly expressed in the e12.5 pituitary [38, 39].

Reciprocal inhibition by Prop1 and Lef1

As shown in Chapter 3, Lef1 is capable of inhibiting the actions of Prop1/β–catenin on the *-10kb Pit1* promoter in transient transfection assays (Figure 3.4K). Deletion analysis of Lef1 effects on the PrdQ reporter demonstrates that the C' of Lef1, not the N', is required for this repressive function (Figure 5.6A). This implicates an additional level of complexity in Prop1/β–catenin/Lef1 interactions beyond the simple competition for β–catenin binding that was initially suggested, as the N' of Lef1 that is required for interaction with β–catenin is not needed for repression. This led us to ask if Prop1 is capable of directly interacting with Lef1. We used bacterially-expressed fragments of Prop1 fused to GST, and found that the Lef1:Prop1 interaction mapped to the B–box located at the extreme C-terminus of Lef1, and the homeodomain of Prop1 (Figure 5.6B, C). We next asked if Prop1 was capable of inhibiting the actions of Lef1 on its cognate sites, just as Lef1 can inhibit Prop1 activation of the PrdQ reporter. We utilized a *TopFlash* reporter, which consists of three consensus Tcf/Lef binding sites, and is strongly activated in the presence of Lef1

and β–catenin. Addition of Prop1 does indeed attenuate this activation (Figure 5.6D). Furthermore, in nuclear microinjection assays, Prop1 can inhibit the activation of the *CyclinD1* promoter, a known target of canonical Wnt signaling (Figure 5.6E) [40]. These data suggest that Prop1 and Lef1 can reciprocally inhibit each other's activities, possibly to regulate a switch between precursor proliferation and differentiation. To test if these effects could be functionally conserved, I co-transfected a series of homeodomain-containing proteins with β–catenin, Lef1 and the *TopFlash* reporter into 293T cells. In addition to Prop1; Pit1, Hesx1 and Pitx2 were all capable of inhibiting activation, but Lhx3, Sox2, Oct1, Prep2 and Pbx were not (Figure 5.7). In a microinjection assay in Hela cells, using the *CyclinD1* reporter, slightly different results were achieved. In this case, Pitx2 could no longer repress, but Sox2 and Pknox2 did repress (Figure 5.6F). Sox2 does not contain a homeodomain, rather it possesses an HMG DNA-binding domain, similar to Tcf/Lefs. Although it is not yet known whether the other homeodomain-containing factors are capable of direct binding to β–catenin, both Pitx2 and Pit1 can directly interact with Lef1 [41],(L. Olson, unpublished data), and Hesx1 can interact with Tcf3 (J. Tollkuhn, unpublished data). It is possible that the different results achieved with the *TopFlash* and *CyclinD1* reporters can be attributed to the expression of the various Tcf/Lef family members in the two different cell lines, since Hela cells lack Lef1. Regardless, it appears that many homeodomain family members are capable of inhibiting canonical Wnt signaling in a cell culture system. The extension of this finding to *in vivo* systems other than the pituitary will undoubtedly provide many insights into development and disease.

Summary and Future Directions

In this dissertation, I have described the role of the *paired*-like homeodomain, Prop1, as a regulator of cell-lineage determination in the developing pituitary gland. Genetic loss- and gain-of-function experiments established a defined temporal window for the actions of Prop1 in cell-type specification. Targeted deletion of *Prop1* results in the loss of the Pit1 lineage, and a severe dysmorphogenesis. Initiating *Prop1* expression one day early, by transgenic expression under the *Pitx1* promoter, causes a complete loss of the gland, while using *Pit1* as a driver to extend *Prop1* expression after birth dramatically reduces the number of somatotropes, causing dwarfism. Prop1 cooperates with β–catenin to regulate the activation of the lineage-determining factor *Pit1*, while simultaneously repressing the lineage-inhibiting factor, *Hesx1*. Microarray analysis of Prop1 and β–catenin null e12.5 pituitaries has elaborated upon both Prop1 and β–catenin genetic programs by identifying genes and pathways not yet explored in the pituitary, including the PCP pathway and a variety of cell migration molecules. Many of these target genes could provide insight into the newly established paradigm of β–catenin:homeodomain regulation of differentiation in organogenesis. Transgenic mapping of Prop1 regulatory information has made it possible to perform Prop1-lineage analysis through the use of a *Prop1-Cre* transgene. Identification of a conserved RBP-Jk binding site within this sequence led to an investigation into the relationship between Prop1 and the Notch signaling pathway. *Prop1* is a direct target of Notch signaling, and is required to maintain a proliferating progenitor population

within the lumen. This finding partially explains early or late misexpression of *Prop1* produces such severe defects; Prop1 confers identity of a specific stage of organogenesis between organ commitment and differentiation.

The newly identified *Prop1* promoter, can be used to ascertain the role of various genes and pathways suggested by the microarray analysis presented in Chapter 4, by targeting expression of either a transcription factor, such as Id2, or a signaling molecule like BMP3. Resulting transgenes can be rapidly analyzed as founders, or lines can be established for breeding to the *Prop1* knockout. In this way, the various aspects of the Prop1 genetic program, from lineage determination to cell migration, can be clearly delineated. I have already created a *Prop1-dTomato* transgene for the purpose of visualizing the migration of *Prop1*-expressing cells. DTomato is a variant of red fluorescent protein (RFP) that has been optimized for brightness and photostability [42]. Through imaging of e12.5 slice cultures, it will now be possible to observe cell migration away from the lumen in real-time. Additionally, an siRNA screen in primary cultures of *Prop1-dTomato* pituitaries can potentially identify novel regulators of *Prop1*. If successful, this screen should be very informative, as the signals responsible for the initial activation of *Prop1* are still unknown. The best two candidates are the BMP and FGF pathways, as both are required for pituitary formation, and act in a spatial and temporal fashion that is consistent with *Prop1* induction [43]. Crosstalk between the BMP, FGF and Wnt pathways is well-established in neural crest development, [44, 45], while in the pituitary, blocking BMP signaling through the use of an αGSU-dnBMPR transgene abrogates *Pit1* expression

[43]. Flrt3, a FGF target gene, is downregulated upon the loss of either Prop1 or β-catenin, as is Cv-2, a potentiator of BMP-signaling [10]. In Xenopus, XFLRT3 acts as a positive regulator of FGF signaling through interactions with FGF receptors [46]. These results suggest interplay between FGF, BMP and Wnt signaling in the developing pituitary. A true genetic experiment with the BMP pathway has yet to be performed in pituitary, although floxed BMP receptor alleles exist for crosses to either the *Pitx1-Cre* or *Prop1-Cre*. Crossing floxed *Bmpr1a* mice to *Pitx1-Cre* does not affect pituitary development (L. Olson, unpublished data), but the majority of these mice do not survive to adulthood, as their lower teeth overgrow and impair feeding (J. Tollkuhn, unpublished data). This result is probably due to the expression of Pitx1-Cre in the oral epithelium, but it somewhat contradicts a previous study where deletion of Bmprla in the epithelium using a K14-Cre arrested lower tooth development [47]. Double knockouts of Bmpr1a and Bmpr1b are currently being generated, and should prove to be informative, as the two receptors are redundant in spinal cord, retina and chondrogenesis [8, 48, 49]. The fastest way to analyze the contribution of the FGF pathway to Prop1 activation would be through the use of a dominant-negative Ets, expressed under *Pitx1*. As previously mentioned, Pea3 and Erm are both highly expressed in the developing pituitary [39], and they are also direct effectors of FGF signaling [38, 50, 51]. Finally, microarray expression analysis of pituitaries with attenuated BMP, FGF or Notch signaling could then be compared to the results from the Prop1 and β-catenin arrays to generate a detailed molecular description of signaling networks within the developing pituitary.

Methods

Microarray analysis

RNA was prepared from individual microdissected e12.5 pituitaries from Prop1 and ß-catenin wild-type and mutant animals. RNA quality was assessed using the Agilent Bioanalyzer 6000 Pico LabChip. 100ng of total RNA was labeled with Cy-3 or Cy-5 using the Agilent Low RNA Input Fluorescent Linear Amplification Kit. Labeled cDNA was hybridized to the Agilent 44K Whole Mouse Genome Array. Data was collected using the Agilent Microarray Scanner and Feature Extraction Software, using a Lowess option with spatial detrend. Normalized data were imported into Focus [52], to extract genes of interest with more confidence than through the use of fold-change only. Experiments were performed in triplicate, with litter-matched wild-type and mutant samples.

Quantitative PCR

Real-time PCR was performed on RNA extracted from wild-type and *Prop1* knockout embryonic pituitaries at e12.5. The data were normalized to L32 and are presented as fold change, with respect to the wild-type. All experiments were performed with two biological and two technical replicates. Oligos used were:

Dll1 gaaacaccagcctccacctga/agctgctcttctcggctccat

BMP-3 tcggaactgtgccaggaggta/cgctcgcactatgctctggat

Bmpr1b gttgacatcccacccaacacc/tcctgggactctgacattttgg

Prickle cctacatgcagaacgccaacc/aggaggacgtggaggacgaac

Spock3 tcgcaagtggagattttcgtga/catggacatccccatcgtcat

Id2 ggaaatcctgcagcacgtcat/gtccgtgttcagggtggtcag

Corl1 tgaggatgggctcactttgga/actctggaattcccgctccag

Pit1 aaggtgggagcaaacgaaagg/atctcctgcgaggaaggcttg

Prop1 ggagtcagcctttgggaggaa/cgctcttgcttccgttgctta

Histology

For immunostaining, embryos were fixed for 30 minutes in 4% PFA, then dehydrated in 20% sucrose, frozen in 1:1 OCT/Aquamount, and sectioned at 14 microns. Pit1, GH and ACTH were detected with rabbit polyclonal antibodies at 1:100. Secondary antibody from Molecular Probes was AlexaFluor 488-conjugated, and nuclei were counterstained with DAPI. In situ hybridization was performed as previously described [53], on formalin-fixed 14 micron cryosections, using S-35 labeled antisense probes.

GST-affinity purification and protein interaction studies

GST-Prop1 homeodomain (amino acids 51-131) and GST-Prop1 C-terminus (aa 129-225) fusion proteins for protein interactions were expressed in *E. coli* and purified from homogenized lysates with glutathione-agarose beads at 25 degrees C for 1 h. For interaction studies, immobilized GST-fusion proteins were then mixed with 293T-cell lysates containing overexpressed, HA-tagged Lef1 protein fragments: N-terminus (aa 1-278), Lef1°C (aa 1-371), Lef1ΔN (aa 272-397). Interacting proteins

were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose membrane, and visualized by Western blotting. After SDS-PAGE, interacting radiolabeled fragments were visualized by autoradiography.

Transfection and nuclear microinjection assays

Cotransfection experiments were performed as described previously [54] in 293T and HeLa cells using 750ng of luciferase reporter, 100ng of pCMX expression plasmids and 500ng of pRSV*ß*Gal as an internal control for transfection efficiency. The multimerized PrdQ/p36 luciferase reporter was described previously [55]. pCMX expression plasmids for mutant Prop1, Lef1 and ß-catenin were generated by PCR and sequenced to ensure their integrity. Microinjection of reporter plasmids and analysis were performed as previously described [3]. All transfection and microinjection experiments were performed at least twice.

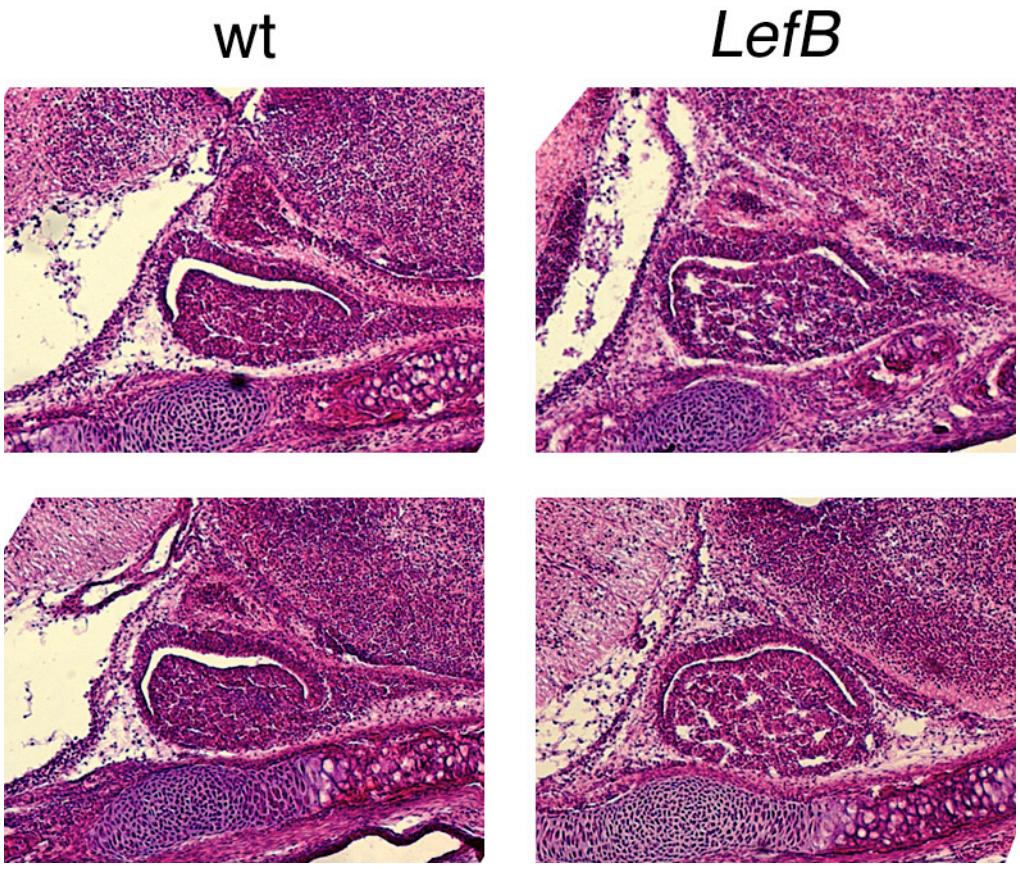


Figure 5.1 LefB mice display defects in pituitary shape
H&E staining of e16.5 wild-type and *LefB* mutant pituitaries. Although the cell number is the same in both mutants and wild-type, the LefB pituitaries appear larger due to increased interstitial space.

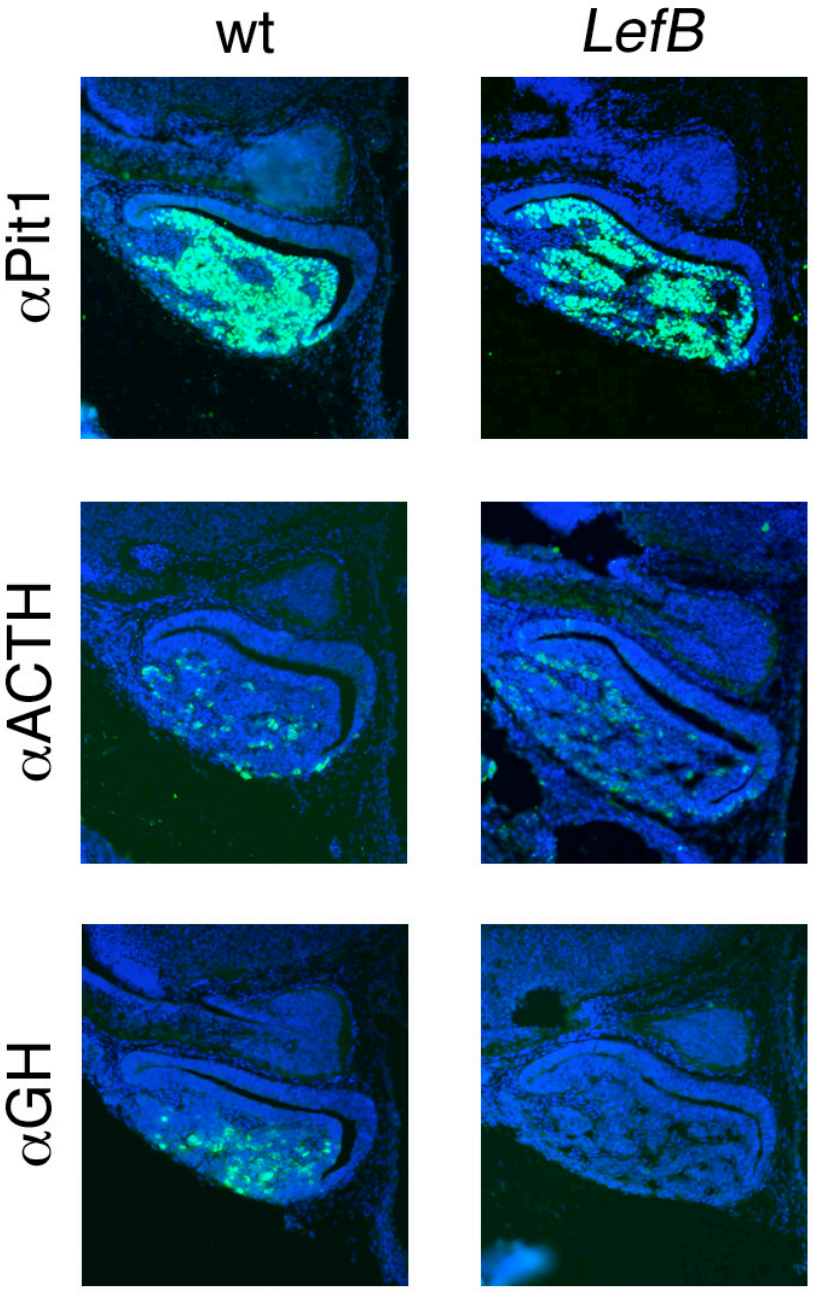


Figure 5.2: LefB pituitaries show a lineage-specific differentiation defect. Immunostaining of e16.5 sagittal sections. Pit1 levels appear normal, further confirming that Tcf/Lef factors are not required for Pit1 expression. There is also a normal complement of corticotropes, as indicated by ACTH staining. However, the somatotropes seem to be completely lost, as there is no GH detectable in the *LefB* mutant.

Table 5.1 Categories of genes regulated by Prop1

Wnt			
Gsc	-6.31	NM_010351	goosecoid
Lrp4	-2.03	NM_172668	low density lipoprotein receptor-related protein 4
Wnt9a	-1.74	BC066165	wingless-type MMTV integration site 9A
Wif1	1.77	NM_011915	Wnt inhibitory factor 1
Cdh1	1.90	NM_009864	cadherin 1
Fzd9	2.02	AK021164	frizzled homolog 9 (Drosophila)
Rspondin	2.50	NM_138683	thrombospondin type 1 domain containing gene
Notch			
Jag1	1.84	NM_013822	jagged 1
Hes5	2.26	NM_010419	hairy and enhancer of split 5 (Drosophila)
Dll1	4.30	NM_007865	delta-like 1 (Drosophila)
BMP			
Bmper	-4.70	NM_028472	BMP-binding endothelial regulator
Bmpr1b	1.79	NM_007560	bone morphogenetic protein receptor, type 1B
Bmp3	3.39	NM_173404	bone morphogenetic protein 3
adhesion/ migration			
Prickle1	-4.71	AK004304	prickle homolog 1 (Drosophila)
Pcdh8	-4.63	NM_021543	protocadherin 8
Cldn8	-2.82	NM_018778	claudin 8
Plxna2	-2.62	AK028562	plexin A2
Nrp2	-2.15	BC029876	neuropilin 2
Sema3c	-2.08	NM_013657	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
Cldn3	1.68	NM_009902	claudin 3
Itm2a	1.69	NM_008409	integral membrane protein 2A
Lama1	1.69	NM_008480	laminin, alpha 1
Dsc2	1.80	NM_013505	desmocollin 2
Pcdha9	1.82	NM_138661	protocadherin alpha 9
Pcdha6	1.88	NM_007767	protocadherin alpha 6
Cdh1	1.90	NM_009864	cadherin 1
Lum	2.04	NM_008524	lumican
Anxa1	2.16	NM_010730	annexin A1
Pcolce2	2.31	NM_029620	procollagen C-endopeptidase enhancer 2
Mt2	2.31	NM_008630	metallothionein 2 (Mt2)
Rgma	2.49	NM_177740	RGM domain family, member A
Slitrk6	2.51	NM_175499	SLIT and NTRK-like family, member 6

Table 5.1, continued Categories of genes regulated by Prop1

transcription			
Idb2	-3.09	NM_010496	inhibitor of DNA binding 2
Zim1	-2.88	NM_011769	zinc finger, imprinted 1
Sp5	-2.19	NM_022435	trans-acting transcription factor 5
Pknox2	-2.03	AK082952	PREP2 PROTEIN homolog
Idb1	-1.88	NM_010495	inhibitor of DNA binding 1
Gata3	-1.67	NM_008091	GATA binding protein 3
Zfp537	1.81	NM_172298	zinc finger protein 537
Prrx1	3.03	NM_011127	paired related homeobox 1
Elf5	3.87	NM_010125	E74-like factor 5
other signaling			
Rgs2	-6.32	NM_009061	regulator of G-protein signaling 2
Vegfc	-3.09	NM_009506	vascular endothelial growth factor C
Fgf5	1.58	NM_010203	fibroblast growth factor 5
Pdgfc	1.70	NM_019971	platelet-derived growth factor, C polypeptide
Il7	1.86	NM_008371	interleukin 7
Il22	2.20	NM_016971	interleukin 22
Ptch2	2.39	BC058397	patched homolog 2
Socs2	2.68	AK033206	cytokine inducible SH2-containing protein 2
Igsf1	3.07	NM_183335	immunoglobulin superfamily, member 1, transcript variant 3
Ptprd	1.57	AK080973	RIKEN full-length enriched library, clone:B530033

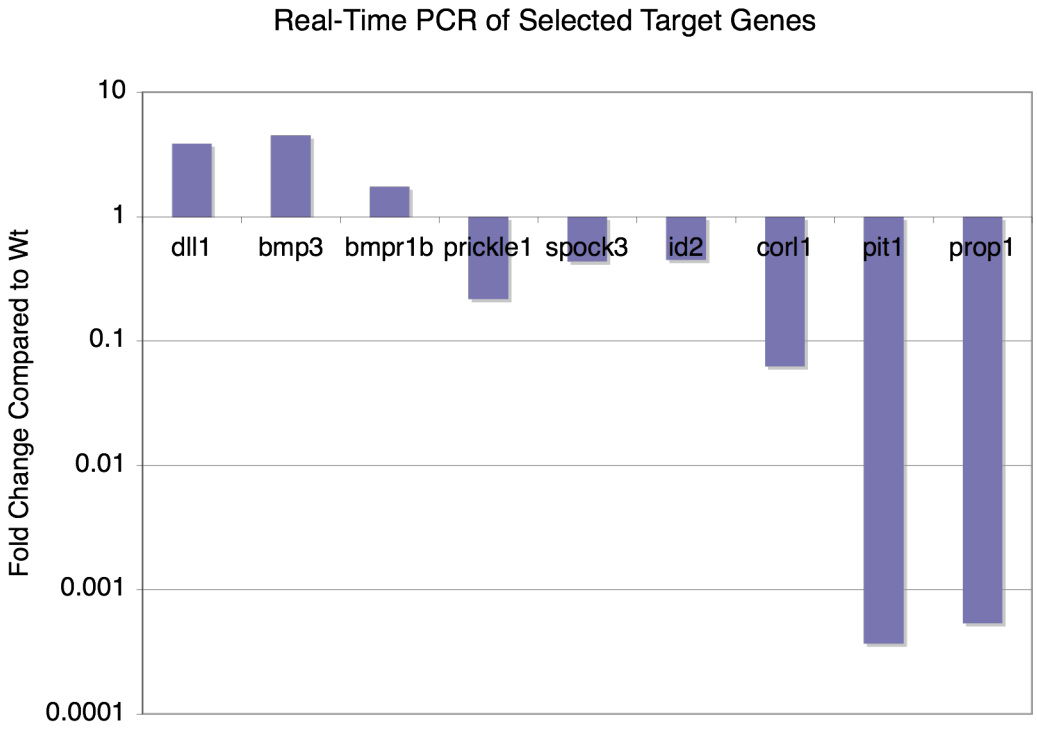


Figure 5.3: Quantitative RT-PCR validation of target genes from microarrays. Relative level of each transcript shown as fold change with respect to wild-type.

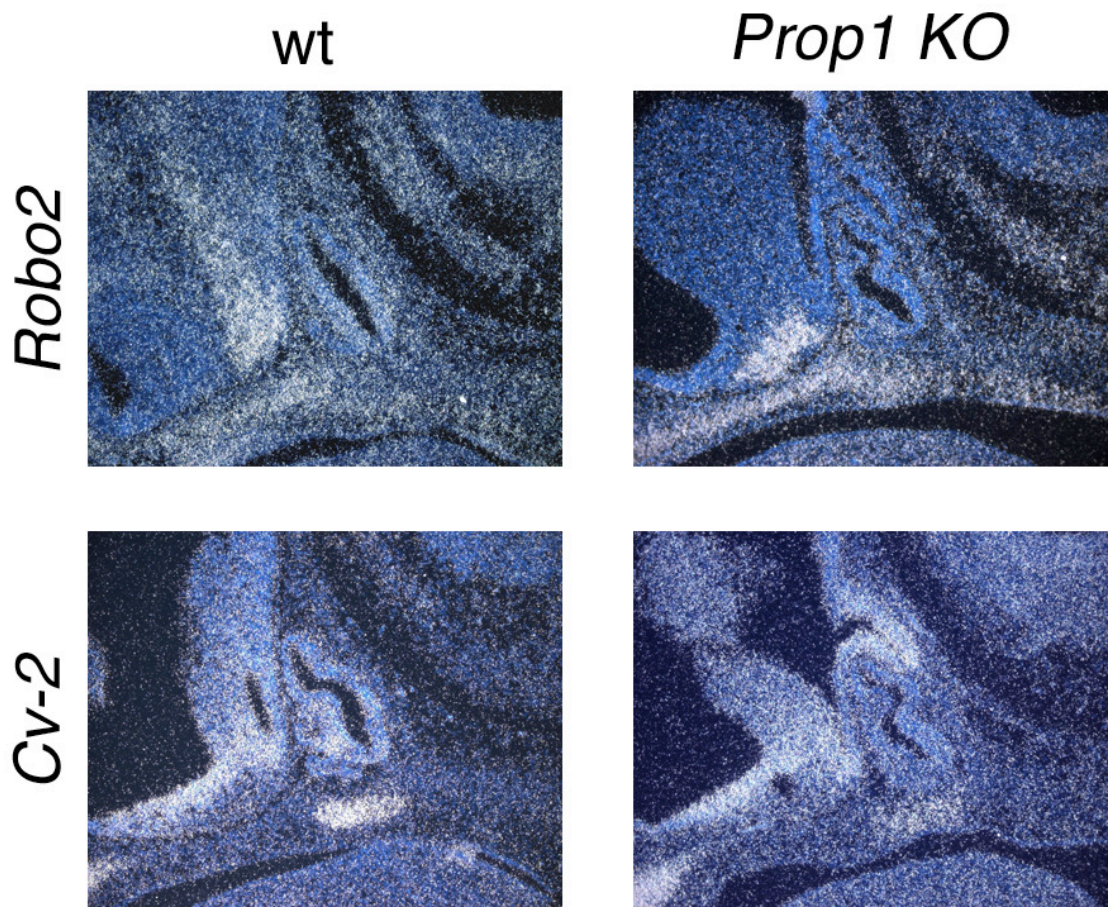


Figure 5.4: Validation of selected target genes by *in situ* hybridization.
Sagittal sections of e12.5 wild-type and *Prop1* mutant pituitaries. *Robo-2* is expressed throughout Rathke's pouch, as well as in the ventral diencephalon and condensing mesenchyme. Expression is lost in the pouch in *Prop1* null animals, but persists elsewhere. *Crossveinless-2* is expressed similarly, but at higher levels in the nascent posterior lobe and in developing bone. Both markers appear to be enriched ventrally within the gland.

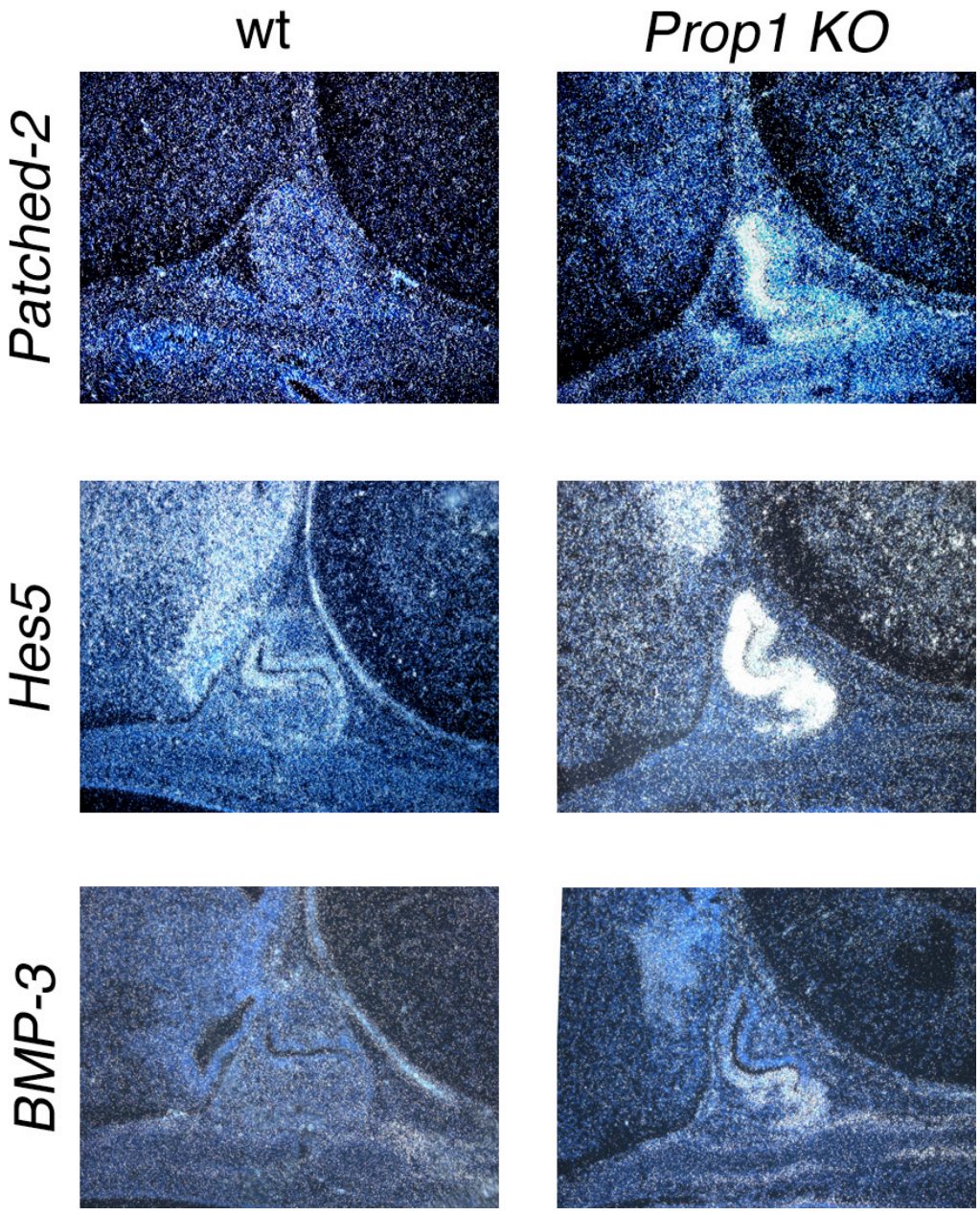


Figure 5.5: Validation of selected target genes by *in situ* hybridization.
Sagittal sections of e14.5 wild-type and *Prop1* mutant pituitaries. Patched-2 does not appear to be strongly expressed in wild-type, but does appear in the lumen of *Prop1*-null animals. *Hes5* levels are highest in the lumen, but are quite low in wild-type compared with mutant. *BMP-3* is not detectable in wild-type, but is expressed in what would be the anterior lobe of the mutant gland. Curiously, *BMP-3* appears to be restricted from the region where *Patched-2* is detected.

Table 5.2 Categories of genes regulated by β-catenin

signaling			
Axin2	-2.33	NM_015732	axin2
Kazald1	1.57	NM_178929	Kazal-type serine protease inhibitor domain 1
Akap6	1.59	BC057114	A kinase (PRKA) anchor protein 6
Fmn	1.64	X62379	formin (isoform IV)
Sgpl1	1.65	NM_009163	sphingosine phosphate lyase 1
AK085088	1.69	AK085088	A kinase (PRKA) anchor protein (gravin) 12
Wnt3a	1.70	NM_009522	wingless-related MMTV integration site 3A
Egfr	1.73	AK049452	epidermal growth factor receptor
Dner	1.81	NM_152915	delta/notch-like EGF-related receptor
Shh	1.84	NM_009170	sonic hedgehog
Drctnnb1a	1.86	NM_053090	down-regulated by Ctnnb1, a
Wnt4	1.87	NM_009523	wingless-related MMTV integration site 4
Wnt9a	1.89	BC066165	wingless-type MMTV integration site 9A
Tcfl5	1.91	NM_178254	transcription factor-like 5 (basic helix-loop-helix)
Ptprq	1.93	AK041657	hypothetical Fibronectin type III domain/ Fibronectin type III repeat containing protein
Tieg3	1.94	NM_178357	TGFB inducible early growth response 3
cell cycle			
Cdc6	-2.89	BC052434	cell division cycle 6 homolog (S. cerevisiae)
Mybl1	-2.64	NM_008651	myeloblastosis oncogene-like 1 (Mybl1)
Mre11a	-2.54	NM_018736	meiotic recombination 11 homolog A (S. cerevisia
Orc4l	-2.45	NM_011958	origin recognition complex, subunit 4-like (S. cerevisiae)
Rag1	-1.86	NM_009019	recombination activating gene 1 (Rag1)\
Cdkn1c	1.63	NM_009876	cyclin-dependent kinase inhibitor 1C (P57)
Cdkn3	2.68	AK033341	weakly similar to CYCLIN-DEPENDENT KINASE INHIBITOR 3
pituitary			
Ghsr	-2.60	NM_177330	growth hormone secretagogue receptor
Cdh22	2.03	NM_174988	cadherin 22
Pomc1	5.76	NM_008895	pro-opiomelanocortin-alpha
zfp			
Zfp426	-2.30	AK048284	ZFP61P (FRAGMENT) homolog
Zfp92	-2.17	NM_009566	zinc finger protein 92
Zmat4	-2.04	NM_177086	(9630048M01Rik), mRNA [NM_177086]
Zfp533	-1.68	AK033075	hypothetical U1-like zinc finger/Zinc finger, C2H2 type containing protein
Zfp72	-1.66	U29508	KRAB-zinc finger protein 74
1700049G17Rik	-1.63	AK006741	similar to zinc finger protein 254 [Homo sapiens]
Pogz	-1.54	AK122288	mRNA for mKIAA0461 protein
Zfp26	1.61	M36514	zinc finger protein (mkr3)
AK028967	1.69	AK028967	similar to ZINC FINGER PROTEIN 60
Zfp192	1.75	AK011044	zinc finger protein 192
Zfp386	4.28	AK089216	unclassifiable, full insert sequence. [AK089216]

Table 5.2 continued Categories of genes regulated by β-catenin

adhesion/ migration			
Lamc3	-2.76	NM_011836	laminin gamma 3 (Lamc3)
Cspg2	-2.56	AK034871	chondroitin sulfate proteoglycan 2
Cldn1	-2.38	NM_016674	claudin 1
AK035727	-2.32	AK035727	slit homolog 3 (Drosophila)
Sema4d	-2.00	NM_013660	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D
Pcsk5	-1.88	AK032736	proprotein convertase subtilisin/kexin type 5
Cdgap	-1.80	NM_020260	Cdc42 GTPase-activating protein
Unc5b	-1.60	NM_029770	unc-5 homolog B
Sema4f	-1.51	NM_011350	sema domain, immunoglobulin domain (Ig), TM domain, and short cytoplasmic domain
Spna1	1.59	NM_011465	spectrin alpha 1
Serpini1	1.60	NM_009250	serine (or cysteine) proteinase inhibitor, clade I, member 1
Cts3	1.62	NM_026906	cathepsin 3
Mamdc1	1.65	AK089133	hypothetical protein, full insert sequence
Serpinb12	1.66	NM_027971	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 12
Astn1	1.67	NM_007495	astrotactin 1
Sema5b	1.69	AK129362	mRNA for mKIAA1445 protein
Mfng	1.70	NM_008595	manic fringe homolog (Drosophila)
Ncam2	1.84	NM_010954	neural cell adhesion molecule 2
Glycam1	1.92	NM_008134	glycosylation dependent cell adhesion molecule 1
Adam10	1.93	NM_007399	a disintegrin and metalloprotease domain 10
Mmp24	1.96	AB021226	membrane-type-5 matrix metalloproteinase
Pcdh8	2.33	AK006960	hypothetical protein, full insert sequence
Cspg3	3.53	NM_007789	chondroitin sulfate proteoglycan 3
transcription			
Erg	-2.32	AK078113	ets-related gene ERG, transcript variant 1
Foxp2	-2.00	NM_053242	forkhead box P2
Eya1	-1.95	BC066860	eyes absent 1 homolog (Drosophila)
Fhl4	-1.68	NM_010214	four and a half LIM domains 4
Mga	1.59	AK032570	Max dimerization protein 5
Nkx2-6	1.59	NM_010920	NK2 transcription factor related
Ndp52	1.61	AK010816	weakly similar to NDP52 [Bos taurus]
Homez	1.69	AK129361	mRNA for mKIAA1443 protein
Nkx2-4	1.82	AF202039	homeobox transcription factor
Sox3	1.86	NM_009237	SRY-box containing gene 3
Tcfl5	1.91	NM_178254	transcription factor-like 5 (basic helix-loop-helix)
Trim8	1.92	BC024694	tripartite motif protein 8
Sall3	1.92	NM_178280	sal-like 3 (Drosophila)
Nr2e1	2.08	NM_152229	nuclear receptor subfamily 2, group E, member 1
En1	2.38	NM_010133	engrailed 1
Tox	2.39	AK051947	hypothetical HMG1/2 (high mobility group) box containing protein
Foxg1	4.20	NM_008241	forkhead box G1

Table 5.2 continued Categories of genes regulated by β-catenin

transcription enzymes			
Cbx3	-2.50	AK017958	chromobox homolog 3 (Drosophila HP1 gamma)
Adprh	-2.25	NM_007414	ADP-ribosylarginine hydrolase
Jmjd1a	-2.12	AK129204	mRNA for mKIAA0742 protein
Arid5b	-1.90	NM_023598	AT rich interactive domain 5B (Mrf1 like)
Amd1	-1.88	NM_009665	S-adenosylmethionine decarboxylase 1
Tcerg1	-1.85	NM_019512	transcription elongation regulator 1 (CA150)
Gtf2h2	-1.65	NM_022011	general transcription factor II H, polypeptide 2
Phc2	-1.62	AK051465	POLYHOMEOTIC 2 homolog [Mus musculus]
Rpap1	1.66	NM_177294	RNA polymerase II associated protein 1
Zbtb33	1.72	NM_020256	zinc finger and BTB domain containing 33
Mbd1	1.85	NM_013594	methyl-CpG binding domain protein 1
map kinase			
Spred1	-1.75	NM_033524	sprouty protein with EVH-1 domain 1
Dusp9	-1.70	NM_029352	dual specificity phosphatase 9
Trp73	1.62	AK014503	transformation related protein 73
Map4k5	1.99	AK052373	mitogen-activated protein kinase kinase kinase kinase 5
Rapgef4	2.04	NM_019688	Rap guanine nucleotide exchange factor (GEF) 4
Rab2b	3.22	NM_172601	RAB2B, member RAS oncogene family

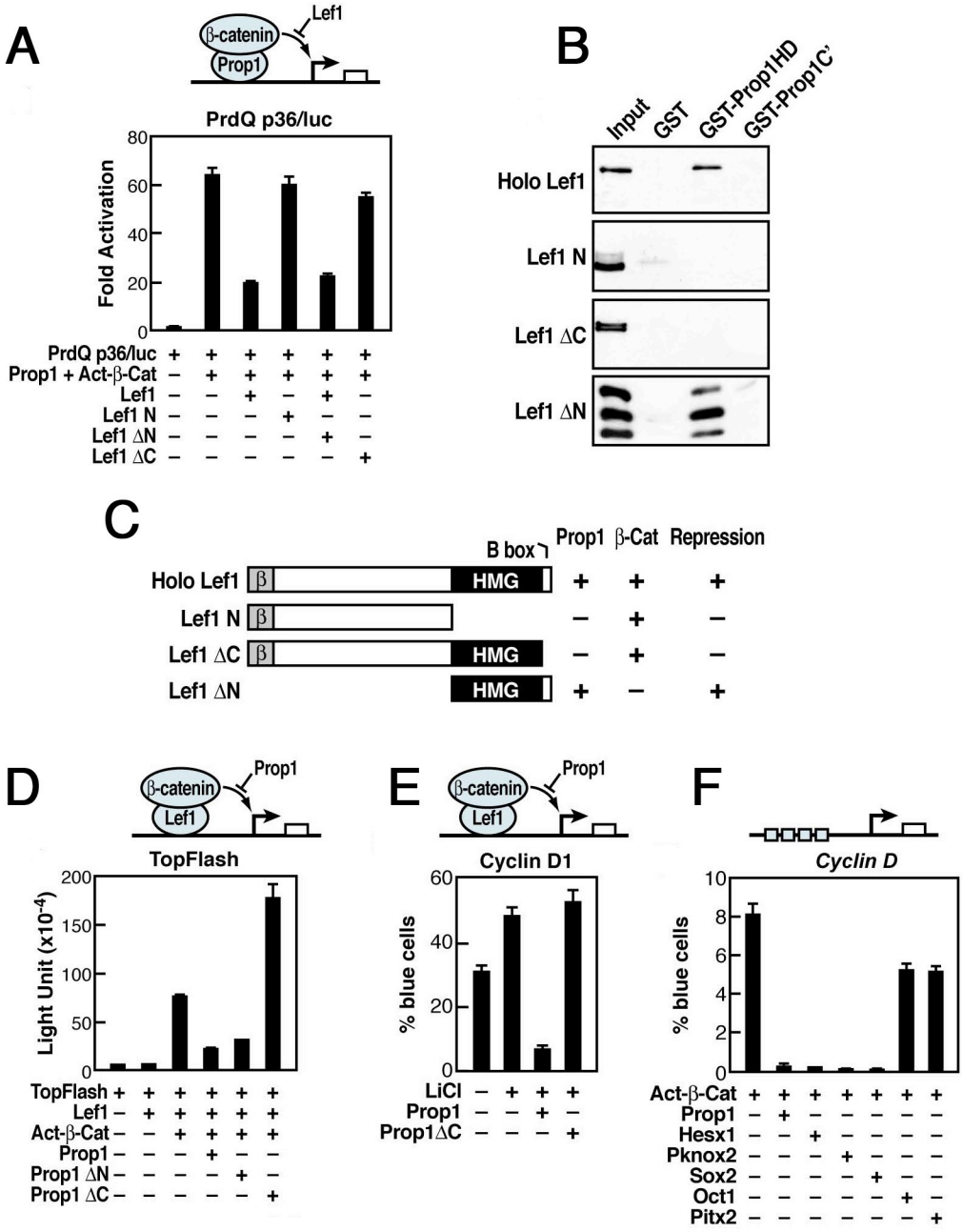
Table 5.3 Genes changed in the same direction in *Prop1* and *β-catenin* knockouts

	Prop1 KO	β-catenin KO
Prickle1	-4.71	-2.29
Nefl	-6.64	-2.41
Rgs2	-7.83	-2.31
XM_147935	-4.71	-2.29
Msx1	-2.11	-2.21
AK054314	-1.98	-2.07
Emcn	-1.56	-2.05
Stim2	-1.98	-1.98
Lmo7	-1.74	-1.87
Robo2	-2.61	-1.86
Pms1	-1.44	-1.81
Plk2	-1.50	-1.77
Rab38	-1.58	-1.75
Rbpms	-1.80	-1.68
Idb2	-3.07	-1.61
Cilp	-2.49	-1.60
Dhrs3	-1.87	-1.57
Lrp4	-2.11	-1.54
Cv-2	-4.36	-1.52
Inpp4b	-2.99	-1.51
Nrp2	-2.24	-1.49
Elovl7	-3.36	-1.46
Flrt3	-1.44	-1.46
Rprm	-1.84	-1.43
Hoxb3	-1.77	-1.41
Gsn	-1.59	-1.38
Myh3	2.99	4.63
Elavl3	2.17	2.85
Dbx1	1.90	2.60
Myh8	1.80	2.47
Dlx2	1.99	2.24
Neb	1.99	2.18
Lhx5	2.17	2.17
Slc7a5	1.62	1.98
AV152162	4.00	1.84
Idb4	1.98	1.82
Tnnt1	1.68	1.79
Myh7	2.02	1.72
Sox2	1.76	1.71
Igsf1	3.21	1.65
Scrn1	2.12	1.59
Ckb	1.79	1.55
Isl2	2.10	1.54
Scn5a	2.61	1.49
Onecut2	1.57	1.49
Lrig1	1.98	1.43
Fzd9	2.05	1.35

Table 5.4 Genes changed in opposing directions in *Prop1* and *β-catenin* knockouts

	Prop1 KO	β-catenin KO
Ank1	-1.49	3.55
Unc5a	-1.57	2.42
Nsg2	-1.49	2.01
Ccne1	-1.82	2.00
Ptpro	-1.51	1.91
AK077083	-1.40	1.90
Wnt9a	-1.74	1.89
Kctd8	-3.61	1.78
Cdkn1c (p57)	-1.84	1.63
Scx	-1.39	1.60
Bace2	-1.63	1.36
Aldh1a3	4.25	-2.74
Rnf28	1.74	-2.10
Plac8	3.01	-1.90
Amd1	1.70	-1.88
Ptprb	1.58	-1.87
Trpc3	2.42	-1.81
Myocd	2.06	-1.77
Aldh1a7	2.65	-1.75
BC060737	1.82	-1.74
Cpeb2	1.89	-1.73
Tmem26	2.00	-1.71
Colec12	1.73	-1.60
Anxa1	2.22	-1.52
Tacr3	3.69	-1.50
Socs2	2.85	-1.45
AK052617	2.63	-1.45
Sat1	2.26	-1.45
Heyl	1.59	-1.41
Pax1	1.67	-1.41
Ptch2	2.40	-1.34

Figure 5.6 Lef1 inhibits gene activation by a Prop1/β-catenin complex, and Prop1 inhibits Lef1/ß-catenin.
A) In transfected HeLa cells, a Lef1 fragment containing the HMG domain and carboxyl-terminus (LefΔN), is sufficient to block Prop1/ß-catenin activation, and the Lef1 ß-catenin interacting domain, located at the extreme N-terminus, is not required. **B)** Western blotting for detection of HA-tagged Lef1 fragments affinity purified from 293T cell extracts using bacterially expressed GST-Prop1 fragments. A Lef1 fragment consisting of the HMG domain and carboxyl-terminus (LefΔN) is sufficient for interaction with the homeodomain of Prop1. **C)** Summary of interactions by Lef1 fragments with Prop1 or ß-catenin, and Lef1 fragments that disrupt Prop1/ß-catenin complex formation or repression. **D)** In transfected HeLa cells, Prop1 fragments containing the carboxyl-terminus (Full Prop1, Prop1ΔN) are strong inhibitors of TopFlash activity, but a Prop1 fragment missing the carboxyl-terminus (Prop1ΔC) actually stimulates TopFlash. **E)** In microinjected GHFT-1 cells, coinjection of Prop1 blocks the induction of a *cyclin D1*/lacZ reporter gene by LiCl, but a Prop1 fragment without the carboxyl-terminus does not interfere. **F)** In Hela cells, β-catenin activity on the *CyclinD1* promoter is blocked by microinjection of Prop1, Hesx1, Pknox2 and Sox2


A
β-catenin
Lef1
Prop1
PrdQ p36/luc
Fold Activation
80
60
40
20
0
PrdQ p36/luc + + + + + +
Prop1 + Act-β-Cat – + + + + +
Lef1 – – + – + –
Lef1 N – – – + – –
Lef1 ΔN – – – – + –
Lef1 ΔC – – – – – +
B
Input
GST
GST-Prop1HD
GST-Prop1C'
Holo Lef1
Lef1 N
Lef1 ΔC
Lef1 ΔN
C
B box
Prop1
β-Cat
Repression
Holo Lef1
Lef1 N
Lef1 ΔC
Lef1 ΔN
HMG
D
β-catenin
Lef1
Prop1
TopFlash
Light Unit (x10⁻⁴)
200
150
100
50
0
TopFlash + + + + + +
Lef1 – + + + + +
Act-β-Cat – – + + + +
Prop1 – – – + – –
Prop1 ΔN – – – – + –
Prop1 ΔC – – – – – +
E
β-catenin
Lef1
Prop1
Cyclin D1
% blue cells
60
40
20
0
LiCl – + + +
Prop1 – – + –
Prop1ΔC – – – +
F
Cyclin D
% blue cells
10
8
6
4
2
0
Act-β-Cat + + + + + + +
Prop1 – + – – – – –
Hesx1 – – + – – – –
Pknox2 – – – + – – –
Sox2 – – – – + – –
Oct1 – – – – – + –
Pitx2 – – – – – – +

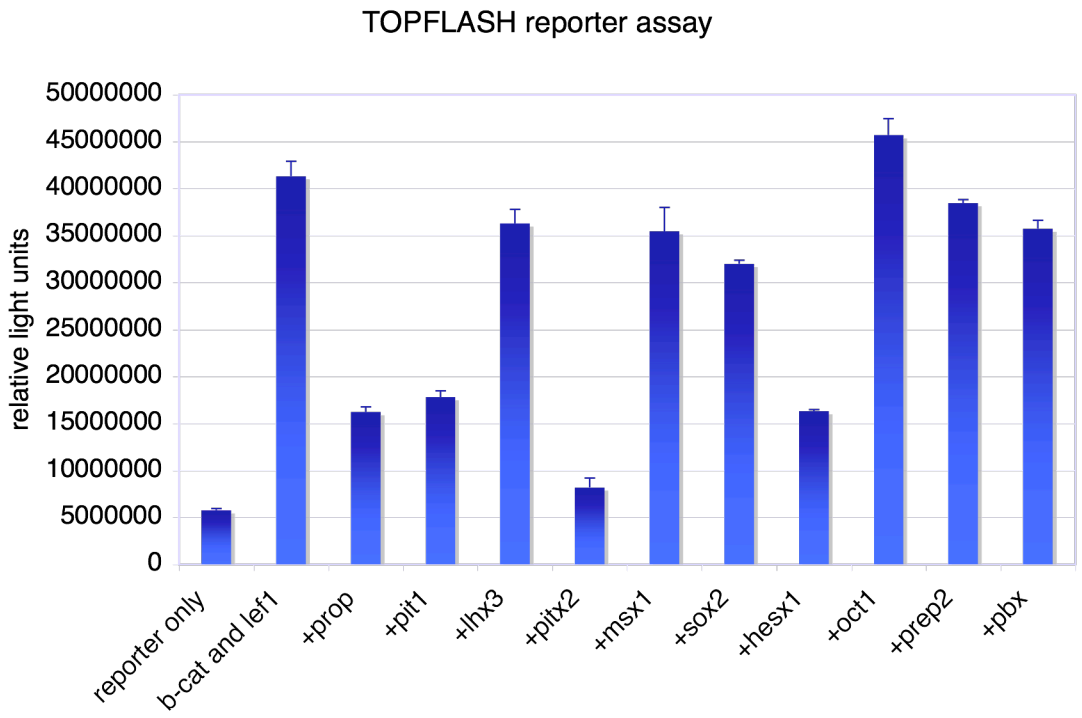


Figure 5.7: Other homeodomain-containing factors can also repress canonical Wnt signaling.
Transient transfection assay in 293T cells. Lef1 and constitutively-active β-catenin activate the TOPFLASH luciferase reporter. This activation can be partially blocked by the addition of Prop1. Pit1, Pitx2 and Hesx1 also inhibit activation, while Lhx3, Msx1, Sox2, Prep2 and Pbx do not. All of these transcription factors are expressed in the developing pituitary.

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Appendix 1: Downregulated genes in *Prop1* null e12.5 pituitaries, $p \leq 0.01$

Symbol	fold change	Accession #
Gpr64	-12.71	NM_178712
Corl1	-12.33	NM_172446
Gsta2	-8.81	NM_008182
Rgs2	-7.83	NM_009061
Nfl-Chd4	-7.15	AK039580
Spock3	-6.91	NM_023689
Nefl	-6.64	NM_010910
Rgs2	-6.32	NM_009061
Gsc	-6.31	NM_010351
BY713104	-6.29	BY713104
Stac	-6.18	NM_016853
Ptprd	-4.81	XM_489051
Slc6a15	-4.80	NM_175328
Aldh1a2	-4.78	NM_009022
Prickle1	-4.71	AK004304
Bmper	-4.70	NM_028472
Cart	-4.66	NM_013732
Pcdh8	-4.63	NM_021543
March	-4.45	NM_175188
Bmper	-4.36	NM_028472
Ptger2	-4.35	NM_008964
Cart	-4.18	NM_013732
Fbp1	-4.02	NM_019395
C630013B14Rik	-3.98	AK083106
Gsta1	-3.54	NM_008181
Crlf1	-3.54	NM_018827
Galnt13	-3.52	AK047871
D230019N24Rik	-3.52	AK051927
Kctd8	-3.43	NM_175519
2610028F08Rik	-3.36	NM_172815
Elovl7	-3.29	NM_029001
Hcn1	-3.28	NM_010408
Edil3	-3.20	NM_010103
Mgl1	-3.18	NM_010796
Vegfc	-3.09	NM_009506
Idb2	-3.09	NM_010496
Aim1	-3.07	NM_172393
Avpr1a	-3.07	NM_016847
Idb2	-3.03	NM_010496
Pdyn	-3.00	NM_018863
Peg3	-2.97	NM_008817
Inpp4b	-2.92	XM_134427
BC051045	-2.89	BC051045
Zim1	-2.88	NM_011769
Slc9a2	-2.88	AK077026

Appendix 1 continued

Symbol	fold change	Accession #
Gsta1	-2.82	NM_008181
Cldn8	-2.82	NM_018778
TC975474	-2.79	TC1262853
Prickle1	-2.75	BC023970
Slc38a4	-2.74	NM_027052
9630018L10Rik	-2.71	NM_177346
Snap91	-2.66	NM_013669
AK027992	-2.65	AK027992
Hs3st1	-2.64	NM_010474
D6Ertd253e	-2.64	NM_178608
Plxna2	-2.62	AK028562
Sgk	-2.61	NM_011361
BC064813	-2.61	BC064813
Robo2	-2.61	NM_175549
1700091G21Rik	-2.59	XM_203404
D930038M13Rik	-2.58	BC026627
Papln	-2.58	NM_130887
Gem	-2.57	NM_010276
Galnt13	-2.55	NM_173030
A830082K12Rik	-2.49	AK044036
Nef3	-2.48	NM_008691
Myo6	-2.47	NM_008662
Pmaip1	-2.46	NM_021451
Plxna2	-2.40	NM_008882
A930021H16Rik	-2.40	NM_174850
Klrg1	-2.40	NM_016970
AK048225	-2.39	AK048225
Cilp	-2.35	NM_173385
9430010M06Rik	-2.33	AK020409
H2-M3	-2.32	NM_013819
Aqp4	-2.29	NM_009700
Atp1b1	-2.28	NM_009721
Alk	-2.26	NM_007439
Cbln2	-2.26	NM_172633
Igsf4d	-2.25	NM_178721
2810457I06Rik	-2.19	NM_176860
Sp5	-2.19	NM_022435
AK044114	-2.18	AK044114
BC025076	-2.15	BC025076
E130306M17Rik	-2.15	NM_177271
Nrp2	-2.15	BC029876
C030033F14Rik	-2.13	BC079850
C130076O07Rik	-2.12	NM_176930
Msx1	-2.11	NM_010835
Car8	-2.11	NM_007592

Appendix 1 continued

Symbol	fold change	Accession #
Sema3c	-2.08	NM_013657
Gch1	-2.08	NM_008102
4930506F14Rik	-2.07	AK029954
Slc16a3	-2.06	NM_030696
Adra2a	-2.05	AK046802
Pknox2	-2.03	AK082952
Lrp4	-2.03	NM_172668
Mgll	-2.02	NM_011844
Adra2a	-2.02	AK046802
AK048479	-2.01	AK048479
Stim2	-1.98	BC043455
6430514L14Rik	-1.96	NM_029784
Entpd3	-1.95	NM_178676
Aadac	-1.95	NM_023383
Glce	-1.94	AK043361
C330012K04Rik	-1.93	AK082774
1700054O13Rik	-1.93	NM_026096
A530017D24Rik	-1.92	AK040705
Cables1	-1.92	NM_022021
1810045K07Rik	-1.92	NM_026432
Ccne1	-1.90	NM_007633
3100002B05Rik	-1.90	AK008026
Usp29	-1.90	NM_021323
Nptx2	-1.89	NM_016789
M17518	-1.89	M17518
1110014L15Rik	-1.88	AK003710
Idb1	-1.88	NM_010495
Ppp1r3b	-1.88	NM_177741
Ldh1	-1.88	NM_010699
P4ha2	-1.87	NM_011031
C130076O07Rik	-1.86	AK122252
Dhrs3	-1.86	NM_011303
Bace2	-1.85	NM_019517
Cdkn1c	-1.84	NM_009876
Myrip	-1.84	NM_144557
Rprm	-1.84	NM_023396
Nef3	-1.83	AK048964
1620401E04Rik	-1.83	NM_175329
Ccne1	-1.82	NM_007633
Gch1	-1.82	NM_008102
Ldh1	-1.81	NM_010699
Spats2	-1.81	NM_139140
AK079373	-1.81	AK079373
Rbpms	-1.80	AF148511
CK790536	-1.80	CK790536
Crlf1	-1.78	NM_018827

Appendix 1 continued

Symbol	fold change	Accession #
Mlf1	-1.78	NM_010801
Aldoa-ps1	-1.77	AK016920
A930041G11Rik	-1.77	NM_177033
Ripk5	-1.77	AK050542
B430201A12Rik	-1.77	AK046595
Hoxb3	-1.77	NM_010458
Rab27a	-1.76	NM_023635
Mgll	-1.76	NM_011844
6230415J03Rik	-1.76	AK078026
BC057022	-1.75	NM_001004180
AK083432	-1.75	NM_001010988
Crtac1	-1.74	NM_145123
BE651658	-1.74	BE651658
Wnt9a	-1.74	BC066165
Ubqln2	-1.72	NM_018798
Zfp291	-1.72	AK049219
2600017H02Rik	-1.71	NM_025915
Dscam	-1.70	NM_031174
AF229056	-1.69	AF229056
Stim2	-1.69	AF328907
Tdrd3	-1.69	AK078326
Pkp1	-1.68	NM_019645
Pcp4l1	-1.67	AY304481
Pdzrn3	-1.67	NM_018884
Gata3	-1.67	NM_008091
TC1031811	-1.67	TC1247844
AK085018	-1.65	AK085018
4930452B06Rik	-1.64	NM_028934
AK028982	-1.63	AK028982
AI427515	-1.63	NM_173016
Bnip3	-1.62	NM_009760
Dgkg	-1.61	NM_138650
Msrb	-1.61	NM_029619
4930553I04Rik	-1.60	AK016105
Osbpl1a	-1.60	AB017026
9330147L21Rik	-1.59	AK079070
Mllt2h	-1.58	NM_133919
Slc16a4	-1.53	NM_146136
AK122498	-1.52	AK122498

Appendix 2: Upregulated genes in *Prop1* null e12.5 pituitaries $p \leq 0.01$

Symbol	fold change	Accession #
Nt5e	10.54	NM_011851
D630004D15Rik	7.31	AK052608
4930511E03Rik	5.95	AK019674
Arhgef6	4.67	NM_152801
Dll1	4.30	NM_007865
Aldh1a3	4.25	NM_053080
AV152162	4.00	AV152162
Elf5	3.87	NM_010125
Tacr3	3.63	NM_021382
Tmprss2	3.53	NM_015775
Bmp3	3.39	NM_173404
TC947782	3.33	TC1344701
Igsf1	3.21	NM_183336
E130309F12Rik	3.14	NM_178756
Siat8d	3.09	NM_009183
Igsf1	3.07	NM_183335
Prrx1	3.03	NM_011127
Plac8	3.01	NM_139198
Kcnn2	2.99	NM_080465
Siat8d	2.95	NM_009183
Slc27a2	2.93	NM_011978
E030025L21Rik	2.92	NM_207531
E330037M01Rik	2.87	AK033305
Adrb1	2.85	AK039569
A_51_P281078	2.84	A_51_P281078
Socs2	2.83	NM_007706
E030025L21Rik	2.82	NM_207531
Cyp2a5	2.76	BC046605
Klhl13	2.74	NM_026167
Cidea	2.71	NM_007702
D130067I03Rik	2.71	NM_172485
Socs2	2.68	AK033206
Il22	2.66	NM_016971
Mbldc1	2.64	NM_178728
2610034M16Rik	2.63	AK011672
Aldh1a7	2.56	NM_011921
Scn5a	2.55	AK085822
Slitrk6	2.51	NM_175499
Rspondin	2.50	NM_138683
AK049610	2.50	AK049610
Rgma	2.49	NM_177740
B230112C05Rik	2.49	AK050937
Myh4	2.47	AJ278733
6030440P17Rik	2.45	BC068185
2810458H16Rik	2.43	AK013364

Appendix 2 continued

Symbol	fold change	Accession #
AK052617	2.42	AK052617
Trpc3	2.41	NM_019510
Ptch2	2.39	BC058397
Cyp2a5	2.36	NM_007812
Tnfrsf19	2.35	NM_013869
Pcolce2	2.31	NM_029620
Mt2	2.31	NM_008630
Fut9	2.29	NM_010243
Cadps2	2.29	NM_153163
Lrrc4	2.28	NM_138682
Hes5	2.26	NM_010419
AK035605	2.26	AK035605
AK048149	2.26	AK048149
BC038167	2.22	XM_196478
TC994616	2.22	TC1263612
Gas1	2.21	NM_008086
Cyp2a4	2.20	NM_009997
Il22	2.20	NM_016971
5330426P16Rik	2.20	AK019912
Rit2	2.17	NM_009065
AK037005	2.16	AK037005
Anxa1	2.16	NM_010730
St6gal2	2.15	AK129462
Ccl11	2.14	NM_011330
Pou3f3	2.13	NM_008900
AK047440	2.12	AK047440
Kcna4	2.12	NM_021275
Scrn1	2.11	AK129084
Dmn	2.11	NM_201639
A830059I20Rik	2.11	NM_021427
AK029214	2.10	AK029214
Kif21a	2.09	AK047208
5730589L02Rik	2.08	AK039357
TC996139	2.08	TC1262550
Myocd	2.07	NM_146386
Thsd2	2.06	NM_028351
Khdrbs2	2.06	AK048524
Bnc1	2.05	NM_007562
Trpc1	2.05	AK005144
Lum	2.04	NM_008524
Fzd9	2.02	AK021164
Myh7	2.00	NM_080728
Slc16a11	2.00	NM_153081
Tnfaip2	2.00	NM_009396
2810002N01Rik	2.00	AK047131

Appendix 2 continued

Symbol	fold change	Accession #
AK052617	2.42	AK052617
Trpc3	2.41	NM_019510
Ptch2	2.39	BC058397
Cyp2a5	2.36	NM_007812
Tnfrsf19	2.35	NM_013869
Pcolce2	2.31	NM_029620
Mt2	2.31	NM_008630
Fut9	2.29	NM_010243
Cadps2	2.29	NM_153163
Lrrc4	2.28	NM_138682
Hes5	2.26	NM_010419
AK035605	2.26	AK035605
AK048149	2.26	AK048149
BC038167	2.22	XM_196478
TC994616	2.22	TC1263612
Gas1	2.21	NM_008086
Cyp2a4	2.20	NM_009997
Il22	2.20	NM_016971
5330426P16Rik	2.20	AK019912
Rit2	2.17	NM_009065
AK037005	2.16	AK037005
Anxa1	2.16	NM_010730
St6gal2	2.15	AK129462
Ccl11	2.14	NM_011330
Pou3f3	2.13	NM_008900
AK047440	2.12	AK047440
Kcna4	2.12	NM_021275
Scrn1	2.11	AK129084
Dmn	2.11	NM_201639
A830059I20Rik	2.11	NM_021427
AK029214	2.10	AK029214
Kif21a	2.09	AK047208
5730589L02Rik	2.08	AK039357
TC996139	2.08	TC1262550
Myocd	2.07	NM_146386
Thsd2	2.06	NM_028351
Khdrbs2	2.06	AK048524
Bnc1	2.05	NM_007562
Trpc1	2.05	AK005144
Lum	2.04	NM_008524
Fzd9	2.02	AK021164
Myh7	2.00	NM_080728
Slc16a11	2.00	NM_153081
Tnfaip2	2.00	NM_009396
2810002N01Rik	2.00	AK047131

Appendix 2 continued

Symbol	fold change	Accession #
AK087708	1.99	AK087708
Srpr	1.98	BC012512
Txk	1.98	NM_013698
AK045549	1.98	AK045549
Clic6	1.98	NM_172469
0610031P22	1.97	BC079863
Myh7	1.97	NM_080728
AV241894	1.97	XM_486240
3110006E14Rik	1.96	BC058543
C230066G23Rik	1.96	NM_175288
Chst9	1.96	NM_199055
Lrig1	1.95	NM_008377
AK087224	1.95	XM_488910
Ell2	1.95	BC006925
Iigp1	1.95	NM_021792
Moxd1	1.95	NM_021509
Tmem26	1.95	NM_177794
Fzd7	1.94	NM_008057
Ramp3	1.93	NM_019511
Pvrl3	1.91	NM_021495
6030440P17Rik	1.90	AK082256
Cdh1	1.90	NM_009864
Dusp10	1.89	NM_022019
Gria1	1.88	NM_008165
Ttid	1.88	NM_021484
Prkcq	1.88	NM_008859
Pcdha6	1.88	NM_007767
TC982071	1.87	TC1362874
Il7	1.86	NM_008371
CA316846	1.86	CA316846
AI390726	1.85	AI390726
Slc16a1	1.85	NM_009196
Jag1	1.84	NM_013822
A_52_P860487	1.83	A_52_P860487
Pcdha9	1.82	NM_138661
B130023L16Rik	1.82	AK045060
Clic6	1.81	NM_172469
Zfp537	1.81	NM_172298
Dsc2	1.80	NM_013505
E130309F12Rik	1.80	NM_178756
BC060737	1.80	NM_172872
Bmpr1b	1.79	NM_007560
Ckb	1.79	NM_021273
BC052446	1.79	BC052446
3110004L20Rik	1.79	AK013991

Appendix 2 continued

Symbol	fold change	Accession #
Wif1	1.77	NM_011915
Pak3	1.77	NM_008778
2610001E17Rik	1.77	NM_026439
2810442I22Rik	1.76	AK031677
Baiap1	1.76	NM_010367
Smpx	1.76	NM_025357
Sox2	1.76	NM_011443
Klhl13	1.76	AK053073
Esrrg	1.76	NM_011935
Has2	1.75	NM_008216
AK079617	1.75	AK079617
H2-Bl	1.74	NM_008199
Rnf28	1.74	AJ278734
Al848705	1.73	AK030326
NAP051592-1	1.72	NAP051592-1
Rit2	1.72	NM_009065
BQ952480	1.72	AK078889
Xdh	1.71	NM_011723
Pdgfc	1.70	NM_019971
Amd1	1.70	NM_009665
Itm2a	1.69	NM_008409
Lama1	1.69	NM_008480
5330426P16Rik	1.69	AK035047
Rhoe	1.69	NM_028810
Cldn3	1.68	NM_009902
Lgals7	1.67	BM939644
LOC234413	1.67	XM_134273
Aldh1a3	1.67	BC026667
Dio3	1.66	NM_172119
Fnbp2	1.65	BC058252
AK079288	1.65	AK079288
Aurkb	1.64	NM_011496
AK047328	1.62	AK047328
1500005K14Rik	1.61	AK033150
1700022C02Rik	1.61	NM_025495
Ppp1r1a	1.60	NM_021391
Fgf5	1.58	NM_010203
Ptprd	1.57	AK080973
Slco3a1	1.56	NM_023908
2310043D08Rik	1.54	NM_029617

Appendix 3: Downregulated genes in *β-catenin* null e12.5 pituitaries, p ≤ 0.01

Symbol	fold change	Accession #
NAP046044-1	-7.65	NAP046044-1
9930032E11Rik	-6.45	AK036977
8430437B07Rik	-5.85	AK018468
Olfr631	-5.07	NM_146959
Eif2s3y	-4.89	NM_012011
Uty	-3.50	AF057367
AK030263	-3.08	AK030263
Cdx2	-3.08	NM_007673
AK087291	-3.05	AK087291
Adh6a	-3.04	AK028114
Myoz2	-3.01	NM_021503
AK048933	-2.96	AK048933
2810038L03Rik	-2.96	XM_484853
NAP101006-001	-2.91	TC1277049
Cdc6	-2.89	BC052434
AK081945	-2.88	AK081945
Abca5	-2.88	NM_147219
AK039751	-2.87	AK039751
1110020C17Rik	-2.82	AK003851
Wdfy1	-2.82	BC025226
D5Ertd798e	-2.81	AK041660
5730469D23Rik	-2.80	NM_172712
Gucy1a3	-2.76	NM_021896
BC034507	-2.76	BC034507
Lamc3	-2.76	NM_011836
Aldh1a3	-2.74	NM_053080
9230106K09Rik	-2.71	AK036475
AK076725	-2.70	AK076725
AK035823	-2.69	AK035823
E130008O17Rik	-2.66	AK086191
6430709H04Rik	-2.66	XM_489560
Knsl7	-2.66	NM_010620
Nfl-Chd4	-2.65	AK039580
Mybl1	-2.64	NM_008651
9630019E01Rik	-2.60	AK051384
Ghsr	-2.60	NM_177330
Steap	-2.60	NM_027399
1200015N20Rik	-2.59	NM_024244
Oog4	-2.57	NM_173773
Cspg2	-2.56	AK034871
AK042501	-2.55	AK042501
Mre11a	-2.54	NM_018736

Appendix 3 cont

Symbol	fold change	Accession #
D830029A09Rik	-2.50	AK052897
Cbx3	-2.50	AK017958
Orc4l	-2.45	NM_011958
Nefl	-2.41	NM_010910
AK048332	-2.41	AK048332
AK079054	-2.41	AK079054
AK037506	-2.41	AK037506
AK079776	-2.40	AK079776
AK039177	-2.40	AK039177
AK085909	-2.39	AK085909
Spata5l1	-2.39	XM_485181
Cldn1	-2.38	NM_016674
4831416G18Rik	-2.36	NM_172788
AK087306	-2.35	AK087306
2610312B22Rik	-2.33	AK050782
Axin2	-2.33	NM_015732
AK080781	-2.33	AK080781
AK052328	-2.33	AK052328
AK035727	-2.32	AK035727
Erg	-2.32	AK078113
Rgs2	-2.31	NM_009061
A430034D21Rik	-2.31	AK039940
AK041794	-2.31	AK041794
AK043060	-2.30	AK043060
Zfp426	-2.30	AK048284
4930564G21Rik	-2.30	AK016220
Accn5	-2.30	NM_021370
Prickle1	-2.29	AK004304
AK082313	-2.29	AK082313
AK037171	-2.29	AK037171
2810049E08Rik	-2.28	AK012929
A130054J05Rik	-2.28	AK078376
Ap1s3	-2.27	NM_183027
Robo2	-2.26	AK084163
Adprh	-2.25	NM_007414
C030007I01Rik	-2.25	AK021055
9330178D15	-2.23	XM_489032
AK046042	-2.23	AK046042
Msx1	-2.21	NM_010835
CB236504	-2.21	CB236504
2810021B07Rik	-2.20	NM_025479
Ccl27	-2.20	NM_011336
AK048558	-2.20	AK048558
E130113K22Rik	-2.19	AK040888
AK080348	-2.19	AK080348
2900082C11Rik	-2.19	AK013809
AI528035	-2.19	AI5280

Appendix 3 cont

Symbol	fold change	Accession #
AK090297	-2.18	AK090297
AK029179	-2.18	AK029179
Zfp92	-2.17	NM_009566
Adam1b	-2.17	NM_172125
Gucy1a3	-2.15	NM_021896
AK046742	-2.14	AK046742
4933435N07Rik	-2.12	AK017073
Hs3st1	-2.12	NM_010474
Jmjd1a	-2.12	AK129204
AK049432	-2.12	AK049432
AK085875	-2.12	AK085875
A830073O21Rik	-2.12	NM_177118
Prickle1	-2.12	BC023970
2810489O06Rik	-2.11	NM_175386
1700003G18Rik	-2.10	AK005637
Rnf28	-2.10	AJ278734
XM_356691	-2.08	A_52_P587462
AK054314	-2.08	AK054314
A430057O09	-2.08	NM_183305
AK036034	-2.07	AK036034
2310044G17Rik	-2.07	NM_173735
Ttyh1	-2.06	AK081274
Cbln1	-2.06	NM_019626
Zmat4	-2.04	NM_177086
Gcn1l1	-2.03	AK037425
Tmlhe	-2.01	AK040228
A230092J17Rik	-2.00	AK039069
Foxp2	-2.00	NM_053242
Sema4d	-2.00	NM_013660
Slc40a1	-1.99	NM_016917
AI607873	-1.99	AK054438
Eif2s3x	-1.98	NM_012010
AJ315977	-1.98	AJ315977
AK045221	-1.97	AK045221
NAP025923-1	-1.97	NAP025923-1
B230220B15Rik	-1.97	NM_177246

Appendix 3 cont

Symbol	fold change	Accession #
A930005N03Rik	-1.96	AK085497
AK078992	-1.96	AK078992
Eya1	-1.95	BC066860
A930019J01Rik	-1.95	AK020879
XM_140524	-1.95	XM_123311
Phldb2	-1.95	NM_153412
Daam1	-1.94	AK033683
1700129L13Rik	-1.93	AK007317
3110001E11Rik	-1.93	AK013941
AK051799	-1.92	AK051799
Hpgd	-1.91	NM_008278
AK079917	-1.90	AK079917
Plac8	-1.90	NM_139198
LOC382109	-1.90	NM_198674
Arid5b	-1.90	NM_023598
Stim2	-1.90	BC043455
9530091C08Rik	-1.89	NM_177159
Lmo7	-1.89	AK129231
BE945021	-1.89	BE945021
Rtn3	-1.88	NM_001003934
Pcsk5	-1.88	AK032736
Amd1	-1.88	NM_009665
AK036708	-1.88	AK036708
AK049694	-1.87	AK049694
Cstf2	-1.87	NM_133196
C030048B12Rik	-1.87	AK081292
Rag1	-1.86	NM_009019
Gucy1b3	-1.86	NM_017469
Rab28	-1.85	AK052624
Tkt	-1.85	AK050793
AI593442	-1.85	NM_178906
Tcerg1	-1.85	NM_019512
AY648975	-1.84	XM_486093
Ncf1	-1.84	NM_010876
BC022771	-1.84	BC022771
AK049314	-1.84	AK049314
AK045332	-1.83	AK045332
1700028E10Rik	-1.83	AK006454
Txndc3	-1.83	NM_181591
AK045155	-1.82	AK045155
C130002K18Rik	-1.82	AK047829
Hspb8	-1.81	NM_030704
AK052507	-1.81	AK052507
1600029O10Rik	-1.81	BC021340

Appendix 3 cont

Symbol	fold change	Accession #
Cdgap	-1.80	NM_020260
BU937074	-1.80	BU937074
2310043D08Rik	-1.79	NM_029617
D230038C21	-1.79	AK052031
Myo5c	-1.79	AK033571
Unc13a	-1.79	NM_021468
Kifc5a	-1.78	NM_053173
AK082597	-1.78	AK082597
Kcnd2	-1.77	NM_019697
Afm	-1.77	NM_145146
Plk2	-1.77	NM_152804
Slc22a4	-1.77	NM_019687
AK048372	-1.76	AK048372
AK079268	-1.76	AK079268
AK053113	-1.76	AK053113
Tnfrsf14	-1.76	NM_178931
D14Abb1e	-1.75	BC037390
Spred1	-1.75	NM_033524
H2-T3	-1.75	NM_008208
AK047015	-1.75	AK047015
Hist2h2aa2	-1.75	AK028129
AK045942	-1.74	AK045942
AK077097	-1.74	AK077097
Aim1	-1.74	NM_172393
A430035B10Rik	-1.73	AK039608
Kif2a	-1.73	NM_008442
Aldh1a7	-1.73	NM_011921
Phf8	-1.73	NM_177201
1700034I23Rik	-1.72	NM_028494
Heph	-1.72	NM_181273
Rgs2	-1.72	NM_009061
Klra2	-1.72	NM_008462
3110007P09Rik	-1.72	AK049616
AK047096	-1.72	AK047096
AK076185	-1.72	AK076185
E030011K20Rik	-1.71	AK053134
Slc37a2	-1.71	NM_020258
AK047503	-1.71	AK047503
Flt1	-1.71	D88690
9430034D17Rik	-1.71	AK020455
BE989180	-1.71	AK086839
LOC268782	-1.71	XM_193784
Icsbp1	-1.71	NM_008320
Dusp9	-1.70	NM_029352

Appendix 3 cont

Symbol	fold change	Accession #
4931427F14Rik	-1.70	AK029691
BC060737	-1.70	NM_172872
5830411G16Rik	-1.69	AK122228
E130009M08Rik	-1.69	AK086561
Kifc5a	-1.69	NM_053173
4933404M02Rik	-1.69	NM_025744
Zfp533	-1.68	AK033075
Fhl4	-1.68	NM_010214
Ifi16	-1.68	NM_008329
AK076969	-1.68	NM_001004158
Cts7	-1.68	NM_019539
Polq	-1.68	NM_029977
Cd80	-1.68	NM_009855
Rbpms	-1.68	AF148511
AI647632	-1.68	AI647632
C530050I23Rik	-1.68	AK049797
5730507H05Rik	-1.68	AJ237585
AK038723	-1.68	AK038723
Tomm34	-1.67	NM_025996
Zcchc8	-1.67	NM_027494
AI595560	-1.67	AI595560
D7Ertd413e	-1.67	NM_177468
Pkhd1l1	-1.66	NM_138674
AK041774	-1.66	AK041774
3830431G21Rik	-1.66	XM_126991
BC049650	-1.66	BC049650
Olfr1158	-1.66	NM_146645
Zfp72	-1.66	U29508
D5Ertd579e	-1.66	AK083736
Pde4a	-1.66	AK089349
F630043A04Rik	-1.66	NM_198605
Olfr173	-1.66	NM_147000
9630005B12Rik	-1.65	NM_013862
Rhag	-1.65	NM_011269
Snap91	-1.65	AK051176
A130079P16Rik	-1.65	AK034599
Gtf2h2	-1.65	NM_022011
Cd86	-1.65	NM_019388
BC020188	-1.64	NM_199222
AK087409	-1.64	AK087409
BC065119	-1.64	BC065119
AK045164	-1.64	AK045164
XM_150222	-1.64	AK021030
Ddx10	-1.64	AK045032

Appendix 3 cont

Symbol	fold change	Accession #
Stc1	-1.63	NM_009285
AK046926	-1.63	AK046926
D230015J17Rik	-1.63	AK085005
AK083952	-1.63	AK083952
1700049G17Rik	-1.63	AK006741
C030019I05Rik	-1.63	NM_177075
LOC243881	-1.63	XM_145466
Limd1	-1.63	NM_013860
AK080274	-1.63	AK080274
MGC86034	-1.63	NM_001001184
Hel308	-1.63	AK052427
5730593N15Rik	-1.63	NM_175263
Hmmr	-1.63	NM_013552
BQ178288	-1.63	BQ178288
AK046811	-1.63	AK046811
BC002288	-1.62	BC002288
Phc2	-1.62	AK051465
Peo1	-1.62	NM_153796
A730069N07Rik	-1.62	AK043205
NAP042466-1	-1.62	NAP042466-1
Serpinb3c	-1.62	NM_009126
Phf14	-1.62	AK031170
Oprk1	-1.62	NM_011011
9430085L16Rik	-1.61	AK020505
A2m	-1.61	NM_175628
BC058175	-1.61	BC058175
ENSMUST00000 059895	-1.61	NP061531
AK032756	-1.61	AK032756
AK029775	-1.61	AK029775
9630045K08Rik	-1.61	AK044594
2310046G15Rik	-1.60	AK015592
AK043345	-1.60	AK043345
BC024969	-1.60	AB093268
Unc5b	-1.60	NM_029770
NAP069724-1	-1.59	NAP069724-1
9430047G12Rik	-1.59	AK034849
Gp2	-1.59	NM_025989
Rps24	-1.59	AK052480
Bcl6b	-1.58	NM_007528
Trpc1	-1.58	AK038676
AK052047	-1.58	AK052047
Ttc17	-1.57	AK018641
Clecsf8	-1.57	NM_010819
NAP053724-1	-1.57	NAP053724-1
AW060766	-1.57	NM_198033

Appendix 3 cont

Symbol	fold change	Accession #
NAP115494-1	-1.57	NAP115494-1
1500031M22Rik	-1.55	NM_025447
AK040835	-1.54	AK040835
A730012O14Rik	-1.54	NM_177882
Cfh	-1.54	NM_009888
Mobkl2c	-1.54	NM_175308
Pogz	-1.54	AK122288
W07899	-1.54	W07899
Pdcd11	-1.53	AK086428
E130102H24Rik	-1.53	XM_149469
4930419G24Rik	-1.53	AK015168
Bmper	-1.52	NM_028472
BC024683	-1.52	BC024683
1700009P03Rik	-1.51	NM_134077
Sema4f	-1.51	NM_011350

,

Appendix 4: Upregulated genes in *β-catenin* null e12.5 pituitaries, p ≤ 0.01

Symbol	fold change	Accession #
Fabp7	6.24	NM_021272
Pomc1	5.76	NM_008895
A_51_P462771	5.72	A_51_P462771
Myh3	5.64	M11154
Myh3	5.56	XM_354614
Zfp386	4.28	AK089216
Foxg1	4.20	NM_008241
Cspg3	3.53	NM_007789
Nrxn1	3.26	AK080992
AI854517	3.24	XM_489189
Rab2b	3.22	NM_172601
8030498J20Rik	3.12	AK033346
Nrxn1	3.06	NM_020252
AK080521	2.98	AK080521
AK032707	2.93	AK032707
Rog	2.87	NM_021397
A430107O13Rik	2.75	AK040587
Dbx1	2.74	NM_001005232
Elavl3	2.69	NM_010487
Ank1	2.69	NM_031158
Ttyh1	2.69	AK011493
Cdkn3	2.68	AK033341
Caskin1	2.64	NM_027937
A630057N01Rik	2.58	AK079103
A330104H05Rik	2.57	NM_029972
Rab6b	2.57	NM_173781
AK048142	2.57	AK048142
AK047060	2.56	AK047060
AK052789	2.53	AK052789
AK037532	2.50	AK037532
E130013N09Rik	2.45	BC060623
Cacnb4	2.45	NM_146123
Pou3f1	2.44	NM_011141
Unc5a	2.43	NM_153131
Prg4	2.42	AB034730
Olfr1160	2.42	NM_146649
Rtn1	2.42	NM_153457
XM_132608	2.42	XM_355782
AK036096	2.41	AK036096
9030624L02Rik	2.40	NM_172759
Tox	2.39	AK051947
En1	2.38	NM_010133
AK030677	2.38	AK030677
2310005G13Rik	2.37	NM_183281

Appendix 4 cont

Symbol	fold change	Accession #
BC055455	2.36	BC055455
ENSMUST00000059414	2.35	A_52_P2659
4932413O14Rik	2.34	NM_177230
Pdha2	2.34	NM_008811
Pcdh8	2.33	AK006960
AK038314	2.31	AK038314
Stmn4	2.31	NM_019675
Pdgfc	2.29	AK081347
AK050863	2.28	AK050863
AK076715	2.28	AK076715
AK051353	2.28	AK051353
Viaat	2.28	NM_009508
Atp6v1g3	2.24	NM_177397
NAP042509-1	2.23	NAP042509-1
2900024P20Rik	2.23	AK036188
Olfr420	2.22	NM_146305
Arhgef7	2.21	AK129064
2900002J02Rik	2.20	AK013470
Sntg1	2.19	NM_027671
Lhx5	2.19	NM_008499
LOC192950	2.19	BC072589
Dlx2	2.18	NM_010054
2310007B03Rik	2.18	NM_172411
2410007B07Rik	2.17	AK010436
9430031J16Rik	2.17	AK129372
Akap6	2.16	AK129115
AW146020	2.16	AK077263
Cd3z	2.16	BC052824
Igfbpl1	2.16	NM_018741
AK044114	2.14	AK044114
AK084416	2.14	AK084416
AK082172	2.14	AK082172
4933408M05Rik	2.14	AK016741
A330035P11Rik	2.13	NM_176990
4930521A18Rik	2.13	AK015858
Olfr109	2.12	NM_146835
Elavl4	2.09	NM_010488
Lrrc4b	2.08	NM_198250
AK047917	2.08	AK047917
Nr2e1	2.08	NM_152229
Ank1	2.07	NM_031158
BC070439	2.07	BC070439
LOC278676	2.07	NM_194347
2810404F18Rik	2.06	NM_178626
AK084077	2.06	AK084077

Appendix 4 cont

Symbol	fold change	Accession #
1200013F24Rik	2.05	AK045433
Mogat1	2.05	NM_026713
Rapgef4	2.04	NM_019688
Cdh22	2.03	NM_174988
Mast1	2.03	NM_019945
AK043066	2.02	AK043066
Nsg2	2.01	NM_008741
D830013H12	2.01	NM_177860
2210008A03Rik	2.00	NM_027299
AK129129	2.00	AK129129
1700034J05Rik	2.00	AK076643
E430013K19Rik	2.00	AK088349
Inpp5f	2.00	NM_178641
Ccne1	2.00	NM_007633
Map4k5	1.99	AK052373
Olfr585	1.99	NM_147087
Rtn4r	1.98	NM_022982
2900055D14Rik	1.97	AK007197
Ttc12	1.97	NM_172770
1700108J01Rik	1.97	AK018955
Tmprss5	1.96	NM_030709
Mmp24	1.96	AB021226
AK086690	1.96	AK086690
AK054460	1.95	AK054460
Calml4	1.95	NM_138304
Pde2a	1.95	NM_001008548
BC030462	1.95	BC030462
Krtap2-4	1.95	AK017442
AK044612	1.94	AK044612
Tieg3	1.94	NM_178357
NAP043441-1	1.94	NAP043441-1
D430014P18Rik	1.94	AK052420
AK037334	1.93	AK037334
Adam10	1.93	NM_007399
Ptprq	1.93	AK041657
AK082734	1.92	AK082734
Pon1	1.92	NM_011134
Sall3	1.92	NM_178280
Gzmf	1.92	NM_010374
AK084172	1.92	AK084172
Glycam1	1.92	NM_008134
Trim8	1.92	BC024694
AK049625	1.92	AK049625
Tcfl5	1.91	NM_178254

Appendix 4 cont

Symbol	fold change	Accession #
Kif27	1.91	AK015814
BC048780	1.91	BC048780
BC033596	1.90	BC061510
Olfr1233	1.90	NM_146972
AK052881	1.89	AK052881
Gm362	1.89	XM_141720
AK082439	1.89	AK082439
Bcat1	1.89	AK036309
Calb1	1.89	NM_009788
Fstl5	1.89	NM_178673
Wnt9a	1.89	BC066165
5830404H04Rik	1.88	AK086520
Olfr878	1.88	NM_146798
D730046L02Rik	1.88	AK028832
Calcr	1.88	NM_007588
AW125753	1.88	NM_029007
Fnbp1	1.87	NM_019406
Wnt4	1.87	NM_009523
2610029G23Rik	1.87	NM_026312
Prss21	1.87	NM_020487
D230015P20Rik	1.86	AK076444
AK089910	1.86	AK089910
Catnd2	1.86	NM_008729
Sox3	1.86	NM_009237
Drctnnb1a	1.86	NM_053090
Ppp1r14c	1.85	AK082372
B3galt2	1.85	NM_020025
Mbd1	1.85	NM_013594
A930019D19Rik	1.85	AK020875
NAP123597-1	1.84	NAP123597-1
Shh	1.84	NM_009170
Ncam2	1.84	NM_010954
AV152162	1.84	AV152162
4930438M06Rik	1.84	AK015338
AK040273	1.84	AK040273
Pte2b	1.83	NM_134247
Mtmr1	1.83	NM_016985
Actn2	1.83	NM_033268
AK086770	1.83	AK086770
LOC209380	1.83	NM_001001318
Nkx2-4	1.82	AF202039
5330421F07Rik	1.82	NM_175272
4732466D17Rik	1.82	BC025830

Appendix 4 cont

Symbol	fold change	Accession #
AK085642	1.82	AK085642
Gpr37	1.82	NM_010338
9230106F14Rik	1.82	AK020318
Ptger3	1.82	NM_011196
4921524P20Rik	1.81	NM_026641
Idb4	1.81	NM_031166
Baalc	1.81	NM_080640
BC057019	1.81	BC057019
AK051634	1.81	AK051634
Sult2a2	1.81	NM_009286
Dner	1.81	NM_152915
AK089653	1.81	AK089653
Klhl15	1.80	AK078112
Zc3hav1	1.80	NM_028864
AK085146	1.80	AK085146
C030016D13Rik	1.80	NM_175354
Dner	1.80	NM_152915
Tex16	1.80	NM_031382
TC975512	1.80	XM_133543
8030491N06Rik	1.79	XM_355968
Bnipl	1.79	NM_134253
Upf2	1.79	XM_140801
Gm573	1.79	BB315740
BB064041	1.79	A_52_P692499
Npy	1.79	NM_023456
6430604M11Rik	1.78	AK032585
4930511M06Rik	1.78	AK015760
C130099A20Rik	1.78	NM_153420
Ptger3	1.78	D17406
Apaf1	1.78	NM_009684
4930583C14Rik	1.78	NM_029472
D11Ertd686e	1.78	XM_110968
1500016L03Rik	1.78	AK018772
Pscd2	1.78	NM_011181
Tnnt1	1.77	NM_011618
4931405B09Rik	1.77	AK016430
Mllt4	1.77	XM_139743
8430427H17Rik	1.77	AK029844
5330416C01Rik	1.77	NM_207528
AK029197	1.76	AK029197
AK086095	1.76	AK086095
2900093J19Rik	1.76	AK013847
Scap2	1.76	AK043556
BC030396	1.76	NM_173862

Appendix 4 cont

Symbol	fold change	Accession #
Slc1a3	1.75	NM_148938
AK046642	1.75	AK046642
2900006F19Rik	1.75	AK013491
2700086A05Rik	1.75	AK053996
AK040321	1.75	AK040321
Ccne1	1.75	NM_007633
Zfp192	1.75	AK011044
Pglyrp1	1.75	NM_009402
AK086071	1.75	AK086071
AK053735	1.75	AK053735
BC033596	1.75	BC061510
AK086210	1.74	AK086210
1700094J05Rik	1.74	AK019782
Olfr1079	1.74	NM_146407
D330034A10Rik	1.73	AK052344
AK049871	1.73	AK049871
Rgs6	1.73	NM_015812
Egfr	1.73	AK049452
Mettl4	1.73	AK010837
A230057D06Rik	1.72	AK082411
Tbxas1	1.72	NM_011539
BQ044049	1.72	BQ044049
Olfr916	1.72	NM_146784
AJ409488	1.72	AJ409488
Trpc4	1.72	NM_016984
Zbtb33	1.72	NM_020256
A730046J19Rik	1.72	AK044075
AK078757	1.71	AK078757
A630026F04	1.71	AK041635
4930429F11Rik	1.71	AK015235
Prph1	1.71	NM_013639
Nsccn1	1.71	NM_010940
Sox2	1.71	NM_011443
Sec8l1	1.71	AK014499
AK052532	1.71	AK052532
AK044500	1.70	AK044500
AK052728	1.70	AK052728
XM_131595	1.70	AK006456
Nol4	1.70	NM_199024
4930485B16Rik	1.70	BC046559
Agtpbp1	1.70	NM_023328
Mfng	1.70	NM_008595
F9	1.70	M23109
Gabrb3	1.70	NM_008071
Wnt3a	1.70	NM_009522

Appendix 4 cont

Symbol	fold change	Accession #
AK035760	1.70	AK035760
Xpo6	1.69	AK088433
AK085088	1.69	AK085088
AK085112	1.69	AK085112
Homez	1.69	AK129361
5031420N21Rik	1.69	AK019875
4930513O06Rik	1.69	AK015788
BC049666	1.69	BC049666
AK082238	1.69	AK082238
AK028967	1.69	AK028967
4921506M07Rik	1.69	XM_126947
Sema5b	1.69	AK129362
D030001E08	1.69	AK050671
AK041856	1.69	AK041856
AK036030	1.69	AK036030
8030491N06Rik	1.68	XM_355968
9130002C22Rik	1.68	NM_029000
5730419A17Rik	1.68	AK017574
4932441J04Rik	1.67	AK034221
Astn1	1.67	NM_007495
Vps54	1.67	NM_139061
AK079230	1.67	AK079230
C230088H06Rik	1.67	AK089842
U29423	1.67	U29423
Rnf128	1.67	NM_023270
Dock8	1.66	BC055295
Serpinb12	1.66	NM_027971
Rpap1	1.66	NM_177294
4921508M14Rik	1.66	AK014845
AK035709	1.66	AK035709
A230062G08Rik	1.66	NM_173765
BC002112	1.66	BC002112
Vit	1.66	NM_028813
AK051847	1.66	AK051847
0610009B14Rik	1.66	AK002366
AK077314	1.66	AK077314
2700063G02Rik	1.66	AK012475
AK079066	1.65	AK079066
AW124722	1.65	XM_139515
Cadps	1.65	NM_012061
A530090P03Rik	1.65	NM_177155
AW125753	1.65	NM_029007
Adam26	1.65	NM_010085

Appendix 4 cont

Symbol	fold change	Accession #
Sgpl1	1.65	NM_009163
AK040234	1.65	AK040234
Churc1	1.65	AK006573
B130011D17Rik	1.65	AK044907
NP064182	1.65	NP064182
Mamdc1	1.65	AK089133
Igsf1	1.65	NM_183336
Fmn	1.64	X62379
NAP123115-1	1.64	NAP123115-1
AK031060	1.64	AK031060
Gys2	1.63	NM_145572
TC1062658	1.63	TC1311251
Cdkn1c	1.63	NM_009876
6530413N01Rik	1.63	NM_026380
Cd109	1.62	NM_153098
Cts3	1.62	NM_026906
Ckb	1.62	NM_021273
Ubie	1.62	NM_001002900
Trp73	1.62	AK014503
Hesx1	1.62	NM_010420
A830020B06Rik	1.62	NM_175564
AK088163	1.61	AK088163
A930005N03Rik	1.61	AK020813
Zfp26	1.61	M36514
Speer3	1.61	NM_027650
Eno3	1.61	NM_007933
NAP112605-1	1.61	NAP112605-1
AK076351	1.61	AK076351
V1rc2	1.61	NM_053232
Ndp52	1.61	AK010816
Serpini1	1.60	NM_009250
NAP047273-1	1.60	NAP047273-1
Magee1	1.60	NM_053201
AK051469	1.60	AK051469
BC048651	1.60	NM_207258
Olfr1080	1.60	NM_146409
Akap6	1.59	BC057114
9430041C03Rik	1.59	NM_133894
Blnk	1.59	NM_008528
Mga	1.59	AK032570
Scrn1	1.59	AK129084
AK037411	1.59	AK037411
Spna1	1.59	NM_011465

Appendix 4 cont

Symbol	fold change	Accession #
Nkx2-6	1.59	NM_010920
AK031182	1.59	AK031182
5730523P12Rik	1.58	AK017785
4930412F09Rik	1.58	AK015119
Olfml2a	1.58	NM_172854
2900041M22Rik	1.57	XM_484098
BB542257	1.57	AK084962
Kazald1	1.57	NM_178929
BQ043793	1.57	BC056175
AK048654	1.57	AK048654
Slc2a3	1.57	NM_011401
NAP028752-1	1.56	NAP028752-1
Klra15	1.56	NM_013793
6430701C03Rik	1.56	AK046541
4632403M07Rik	1.56	AK014547
Myl4	1.56	NM_010858
Xcr1	1.56	NM_011798
AK047066	1.55	AK047066
AK041441	1.55	AK041441
B230118I11Rik	1.55	AK080796
Klrc3	1.55	NM_021378
Ndrg3	1.55	NM_013865
AK087274	1.54	AK087274
Nedl1	1.54	AK033922
AK037313	1.54	AK037313
Scn3a	1.53	L42337
2310079L17Rik	1.53	AK028574
9630061B06Rik	1.53	AK036364
Gm1012	1.52	NM_201367
4931407G18Rik	1.52	NM_027631