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2006

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REGULATION OF CELL LINEAGE DETERMINATION BY
A PAIRED-LIKE HOMEODOMAIN

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biomedical Sciences

by

Jessica Tollkühn

Committee in charge:

Professor Michael G. Rosenfeld, Chair
Professor Richard D. Kolodner
Professor Pamela L. Mellon
Professor James W. Posakony
Professor Anthony Wynshaw-Boris

2006
The dissertation of Jessica Tollkühn is approved, and is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2006
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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Geoff Rosenfeld, for providing me with a phenomenal graduate experience. Also, many members of the Rosenfeld Lab, past and present, particularly Dr. Kristen Jepsen for being a fabulous mentor, both scientifically and for life in general; Dr. Lorin Olson, who gave me a great deal of guidance and support; Dr. Jeremy Dasen, whose work provided the foundation for much of my research; Dr. Wei Wu, who generated the Prop1 knockout; Dr. Jean Lozach for analysis of the microarray data; Havilah Taylor, for exceptional assistance with animal husbandry and the UCSD Transgenic Core for generating all my mice.

I would not have gotten to graduate school without the support of the following people: my undergraduate advisor, Dr. Lisa Urry, who introduced me to developmental biology and academic research; Dr. Blanche Shamoon and Dr. AB Jefferson supervised my internship at Chiron Corporation and taught me how to clone; everyone at Mills College; and of course my wonderful family, who have always encouraged my interest in science. I’m finally here!

CURRICULUM VITA

2000    B.A.  Biochemistry and Molecular Biology, Mills College,
Oakland, CA

2006    Ph.D., Biomedical Sciences University of California San Diego, CA

Publications


Abstracts

   Selected for an oral presentation


ABSTRACT OF THE DISSERTATION

REGULATION OF CELL LINEAGE DETERMINATION BY

A PAIRED-LIKE HOMEODOMAIN

by

Jessica Tollkühn

Doctor of Philosophy in Biomedical Sciences
University of California, San Diego, 2006

Professor Michael G. Rosenfeld, Chair

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 (dwJ) 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 thyro trope 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 \( \alpha GSU \) 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 \( \alpha 1 \) 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 inhibit 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.
References


Chapter 2: Genetic Strategies for the Analysis of Prop1 Function

Introduction

The human \textit{PROP1} gene shows over 90\% homology with mouse \textit{Prop-1} within the homeodomain (ref). Mutations in \textit{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 \textit{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

\textit{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 \textit{Prop1} knockout mouse was generated (Figure 2.1). As the \textit{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. \textit{In situ} analysis of pituitary markers demonstrated a complete recapitulation of the Ames phenotype in the \textit{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 $LH\beta$ 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. $LH\beta$ 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.
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 tgcacctgatccagctct/cgtgtgaacagttaggtgct
mutant (lacz) caacgagacgtcacggaaaatgcc/ccaacagttgcagctgaatg.

**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 identified 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.
References


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/β-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 thyro trope 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 corepressors [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/β-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, αGSU 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 TSHβ 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/β-catenin signaling remained intact in the absence of Prop1 (Figure 3.4A).

To investigate potential genetic interactions between Prop1 and β-catenin, we examined double heterozygous embryos generated by crossing Prop1+/− mice with conditionally-deleted β-catenin heterozygotes, and observed a consistently diminished expression of Pit1 in e14.5 double heterozygote embryos (Figure 3.4B), which provided evidence that β-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 β-catenin IgG
or β-catenin siRNA (Figure 3.4G, and data not shown). As a control for functional specificity, Pit1 actions on a Pit1 response element were β-catenin-independent, and unaffected by anti-β-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 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 Pitl regulatory elements, consistent with an active repression of the Pitl gene at this time (Figure 3.5A, and data not shown). At e11.5, the Pitl 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 Pitl 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 Pitl 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 Pitl 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 Pitl 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 β-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 β-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 β-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 β-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 β-catenin/loxP(ex2-6) and β-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- βCat+/− trans-heterozygotes were obtained by crossing Prop1+/−,Pitx1Cre mice with β-cateninex2-6/ex2-6 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 pRSVβGal 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 β-catenin protein, or S35-methionine labeled β-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 β-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.

Acknowledgments

We thank Janet Hightower and Marie Fisher for assistance with figure and manuscript preparation, Havilah Taylor for help in maintaining the mouse colony, Forrest Liu for help in generating Prop1 knockout mice, and the UCSD transgenic core facility for assistance in generating transgenic mice. L.E.O. and J.T. were supported by NIH training grants, X.L. was supported by a Research Career Award NIDDK DK 064744, and M.G.R. is an investigator of the Howard Hughes Medical Institute.

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 β-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 (TSHβ). Ventral diencephalon (VD), Rathke’s pouch (RP), rostral tip (rt).
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 TSHβ) 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.
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 β-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.
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 3xpaired-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 β-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 and 13.5 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).
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 pluripotency 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.
References


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-Jκ 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-Jκf/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-Jκf/f mice. However, in contrast to the Prop1 knockout mice, the Pitx1-Cre, RBP-Jκf/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-Jκf/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 *Apal*. A LacZ cassette, containing its own stop codon was fused to the end of the third Prop1 exon using *Apal* 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’-CGGTCTGTCTGGTTGTGCAAGCTG-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’- GGAAATGGTTTCCGCAGAAC-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% glycerol), 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’-CTTGAGCTCGTGGAAGGGCTTGCC-3’, 5’-GGCAAGCCCTTCCCCACGAGC-3’ (Prop1 intron); 5’-CTTGAGCTCGTGaeAAGGCTTGCC-3’, 5’-GGCAAGCCCTTgtCACGAGCTCAAG-3’ (Prop1 intron with mutations); and 5’-AAACACGCGCTGGAAAAATTTGG-3’,5’-CCAAAATTTTTTCCCCACGGCGTGTTT-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 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).
References


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 ActRIIib, 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 addition, 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 beta-catenin, microarray analysis was performed on wt and beta-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 beta-catenin than by loss of Prop1, which is consistent with beta-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/beta-catenin target gene, Axin2, is downregulated. The beta-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 in the caudal region of beta-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. beta-catenin plays an essential role in the formation of this complex, coupling transmembrane E-cadherin to alpha-catenin and the actin cytoskeleton [13]. Tyrosine phosphorylation of beta-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 \(-10kb\ \text{Pit1}\) 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 \(\beta\)-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 \textit{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/Lef1. 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
Flt3, 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 Bmpr1a 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 β-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 gaaacaccagctccacctga/agctgctcttctcggctccat
- BMP-3 tcggaactgtgccaggagta/cgctcgcactatgctctggat
- Bmpr1b gttgacatcccacccaaaccc/tctctgttccttgatctgtcttttg
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 pRSVßGal 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

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### Table 5.1, continued Categories of genes regulated by Prop1

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<td>AK080973 RIKEN full-length enriched library, clone:B530033l</td>
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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

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Table 5.2 continued Categories of genes regulated by β-catenin

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Table 5.2 continued Categories of genes regulated by $\beta$-catenin

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Figure 5.6 Lef1 inhibits gene activation by a Prop1/β-catenin complex, and Prop1 inhibits Lef1/β-catenin.

A) In transfected HeLa cells, a Lef1 fragment containing the HMG domain and carboxyl-terminus (LefΔN), is sufficient to block Prop1/β-catenin activation, and the Lef1 β-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 β-catenin, and Lef1 fragments that disrupt Prop1/β-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.
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.
References


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

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Appendix 4: Upregulated genes in $\beta$-catenin null e12.5 pituitaries, $p \leq 0.01$

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