THE ROLE OF NOTCH SIGNALING IN THE REGULATION OF PROLIFERATION AND CELL DIFFERENTIATION DURING EMBRYONIC AND POSTNATAL PITUITARY DEVELOPMENT

BY

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DISSERTATION

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ABSTRACT

The pituitary develops in two distinct waves in mice, one occurring during embryogenesis and a second taking place during early postnatal development. During both periods, progenitor cells proliferate and subsequently differentiate into hormone-producing cells. Maintaining the proper number of progenitors and producing the appropriate number and type of endocrine cells is essential to pituitary development and function. Although signals necessary for initial pituitary commitment and those required for pituitary hormone synthesis have been well characterized, pathways that regulate progenitor proliferation and cell fate remain unclear. Previous studies have implicated the Notch signaling pathway in these processes, both in the pituitary and in other developing tissues. In order to more specifically define the role of Notch signaling during different stages of pituitary development, we utilized pituitary-specific gain- and loss-of-function mouse models, in combination with chemical Notch inhibition.

Notch signaling is necessary for progenitor maintenance in many systems. Additionally, loss of the canonical Notch target *Hes1* in combination with loss of the pituitary specific Notch target, *Prop1,* results in premature differentiation of corticotropes. Based on these observations, we hypothesized that Notch signaling is sufficient to inhibit the differentiation of corticotropes and maintain these cells in a progenitor state. To test this hypothesis, we generated mice with persistent expression of activated Notch in POMC-expressing cells. We show that constitutive Notch expression prevents the differentiation of corticotropes and melanotropes, which also contain POMC. These cells instead maintain

progenitor markers. In addition, mRNA levels of transcription factors necessary for *Pomc* expression are downregulated in this model. Cells that aberrantly maintain progenitor markers are lost during early postnatal development, resulting in dysfunction of the hypothalamic-pituitary-adrenal axis. These findings confirm our hypothesis that Notch signaling is sufficient to prevent corticotrope differentiation.

Previous data have suggested that Notch signaling may be able to transcriptionally activate *Prop1*, which is necessary for *Pit1* transcription, which is necessary for GH, TSH and Prl synthesis. These findings, in combination with the observation that Notch signaling is sufficient to inhibit differentiation of POMC-expressing cells, led us to hypothesize that Notch signaling may regulate the balance between POMC lineage cells and Prop1/Pit1 lineage cells. Additionally, we hypothesized that alterations in Notch signaling may lead to changes in proliferation and progenitor maintenance, both well characterized roles of Notch. To address these hypotheses, we examined a mouse model with loss of *Notch2* prior to pituitary induction. In this model, we observe a decrease in Pit1-positive cell number and an increase in corticotrope cell number, suggesting Notch regulates the choice between these pituitary lineages. Additionally, we observe defects in proliferation and progenitor maintenance, but not until after birth, indicating *Notch2* is dispensable for these functions during embryonic development. Because of this observation, we examined the role of Notch signaling during postnatal development using a chemical inhibitor of Notch, which was specifically administered during postnatal. We observe an increase in *Pomc*

mRNA, a decrease in *Pit1* mRNA and a decrease in the number of proliferating cells after Notch inhibition. These findings demonstrate that Notch signaling is an important regulator of cell fate and proliferation in the postnatal pituitary. In order to identify targets that may be necessary for these functions, microarray analysis was performed comparing *Notch2* ablated mice to controls. Among the target mRNAs that were altered, many have been implicated in cell proliferation and a subset are involved in corticotrope cell signaling. These targets will be areas of future focus in the Raetzman laboratory. Taken together, these studies have defined a role for Notch signaling in the control of cellular proliferation, progenitor maintenance and specification during embryonic and postnatal pituitary development.

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CHAPTER 1**: Introduction**

The pituitary gland, often referred to as the master gland, is an important regulator of many physiological processes including growth, metabolism, fertility and the body's response to stress. The pituitary regulates these processes through the release of hormones, which act on target endocrine organs to regulate the subsequent release of their hormones. Proper pituitary development is necessary for pituitary function and the function of its target organs. Inappropriate development of the gland can lead to multiple disorders including Cushing's syndrome, gigantism and combined pituitary hormone deficiency. Additionally, pituitary tumors are present in roughly 12% of the population upon autopsy, while the presence of clinically apparent tumors is 1:1100 (Daly, et al. 2006; Fernandez, et al. 2010; Raappana, et al. 2010), indicating the pituitary is highly susceptible to defects in proper control of cell proliferation. Because of the prevalence of pituitary disease, developing both during embryogenesis and during postnatal gland homeostasis, it is of great importance to understand the mechanisms and signaling pathways that underlie pituitary cell proliferation and differentiation.

In mice, the pituitary is composed of three lobes: the posterior lobe (PL), the anterior lobe (AL), and the intermediate lobe (IL). The posterior lobe, which is of neural origin, contains pituicytes and axon terminals of magnocellular neurons. The AL contains five hormone-producing cell types: thyrotropes, somatotropes, lactotropes, corticotropes and gonadotropes, which produce the hormones thyroid stimulating hormone (TSH), growth hormone (GH), prolactin (PRL),

adrenocorticotropic hormone (ACTH) and gonadotropins (LH and FSH), respectively. The IL contains a sixth hormone-producing cell type, the melanotrope. Melanotropes secrete melanocyte-stimulating hormone (αMSH). αMSH and ACTH are both cleavage products of the prohormone Proopiomelanocortin (POMC) (Zhu, et al. 2007).

The AL and IL are derived from cells located in hypophyseal placode, which is found at the midline of the anterior neural ridge. The hypophyseal placode is of ectodermal origin and expresses the transcription factor *Foxg1*. At the earliest stages, the hypophyseal placode is in direct contact with neural tissue that will develop into the PL and hypothalamus. This ectoderm invaginates at embryonic day (e) 9.0, forming the primordial pituitary. At e11.0, the developing pituitary pinches off from the underlying oral ectoderm to form the structure known as Rathke's Pouch (RP). Throughout development, this tissue remains in close contact with the neural tissue that makes up the infundibulum (Rizzoti. 2010). Signals from this tissue are necessary for pituitary development. In fact, genetic ablation of this region in *Nkx2.1* knockout mice, results in failure of the pituitary to form. This phenotype is likely due, at least in part, to the loss of FGF morphogens that are normally secreted by cells in the infundibulum (Kimura, et al. 1996) (Figure 1.1).

RP contains all of the progenitor cells necessary to form the AL and IL (Zhu, et al. 2007; Rizzoti. 2010). These progenitor/stem cells express the factors SOX2, and later SOX9 (Fauquier, et al. 2008). Using *in vivo* lineage tracing experiments, it has been shown that SOX2 and SOX9-expressing cells can give

rise to all of the different cell types of the pituitary. They can carry out this function during embryogenesis and, at a lower rate, in adult mice (Andoniadou, et al. 2013; Rizzoti, et al. 2013). Several factors have been identified that are necessary for the proper proliferation of these progenitors. These signals include both extrinsic factors emanating from the surrounding ventral diencephalon and oral ectoderm, such as members of the FGF, BMP, SHH and Wnt families, and intrinsic pituitary signals, (Ericson, et al. 1998; Treier, et al. 1998) (Figure 1.1). Despite the identification of these signaling pathways, nothing is known about how a progenitor cell integrates and translates the signals it receives to alter proliferative behavior.

At e11.5, progenitor cells begin to undergo cell cycle exit, migrate ventrally towards the developing AL and begin to differentiate into hormone producing cells (Zhu, et al. 2007; Bilodeau, et al. 2009). Many current models of pituitary development propose hormone cells are specified in an ordered fashion, with each cell type exiting the cell cycle during a discrete period of time. Furthermore, it is widely accepted that cells exiting the cell cycle at the same time migrate to the same location in the pituitary. This process is thought to be coordinated by opposing gradients of morphogens in the infindibulum and ventral diencephalon and the underlying oral ectoderm (Treier, et al. 1998; Ericson, et al. 1998). However, recent evidence shows that all of the AL hormone cell types exit the cell cycle at the same time, with the majority of cells doing so between e11.5 and e13.5. The IL melanotropes exit the cell cycle shortly thereafter. Additionally, there was found to be no correlation between the timing of cell specification and

location in the gland (Davis, et al. 2011). Based on these observations, it remains unclear how cells in the same location in the pituitary, and therefore exposed to the same chemical gradients, are specified to become different cell types.

Although all hormone cells exit the cell cycle at roughly the same time, corticotropes are the first cell type to express markers of terminal differentiation, with *Pomc* mRNA detectable beginning at e11.5 (Japón, et al. 1994). Transcription of *Pomc* is dependent on several transcription factors including TPIT (TBX19), a T-box transcription factor. TPIT cooperates with PITX1, a homeodomain transcription factor, to regulate transcription of the *Pomc* gene. It does so by binding directly to the *Pomc* promoter (Lamolet, et al. 2001; Liu, et al. 2001). Mice deficient for *Tpit* fail to express POMC, although corticotrope cell commitment is unaffected (Pulichino, et al. 2003a; Pulichino, et al. 2003b). Similarly, human *TBX19* mutations can result in isolated ACTH deficiency, which can be attributed to lack of POMC in the pituitary (Pulichino, et al. 2003a; Vallette-Kasic, et al. 2004). Additionally, NeuroD1, a basic helix loop helix transcription factor, can also bind synergistically with PITX1 to the *Pomc* promoter and activate its transcription. Mice null for *Neurod1* show delayed expression of ACTH but eventually show normal hormone production, suggesting it may not be as critical as TPIT (Poulin, et al. 1997; Lamolet, et al. 2004). Other proteins, including the orphan nuclear receptor Nur77, also bind to response elements on the *Pomc* promoter to drive its transcription (Philips, et al. 1997a; Philips, et al. 1997b). Conversely, *Pomc* transcription is inhibited by the expression of BMPs (Nudi, et al. 2005), (Figure 1.1). While much is known about

the terminal differentiation of these cells, the signals controlling their specification remain unclear. Previous data from our lab show that loss of two Notch effector genes results in premature differentiation of corticotropes, suggesting Notch signaling may play a role in specification of these cells (Himes and Raetzman. 2009a).

The common precursor of somatotropes, lactotropes and thyrotropes begins to express the protein PIT1 at roughly e12.5. Prophet of Pit1 (PROP1) is required for the activation of *Pit1* transcription (Nasonkin, et al. 2004)**.** Ames Dwarf mice, which lack functional *Prop1,* show near complete loss of all cells of the PIT1 lineage (Gage, et al. 1996). PIT1 acts in concert with other molecules to transcriptionally regulate terminal differentiation markers in each of the three cell types. In thyrotropes, which are fully differentiated by e15.5, GATA2 acts synergistically with PIT1 to drive *Tshb* transcription (Kashiwabara, et al. 2009). Similarly, in lactotropes, which express PRL beginning after birth, PIT1 acts with estrogen receptor and Ets-1 to drive *Prl* transcription (Bradford, et al. 1996). Thyroid hormone and retinoic acid, acting through their respective receptors, stimulate growth hormone transcription, which is first observed at e17.5 (Palomino, et al. 1998). In addition, *Math3*, a downstream target of PIT1, is necessary for the expression of growth hormone releasing hormone receptor, which allows somatotropes to respond to stimulatory signals from the hypothalamus (Zhu, et al. 2006; Zhu, et al. 2007), (Figure 1.1).

A gap in knowledge remains regarding how pituitary progenitor cells balance proliferation and differentiation. Additionally, although factors involved in

regulating terminal differentiation and hormone synthesis in each of the pituitary cell types are well characterized, signals necessary for initial cell specification remain largely unclear. Based on previous findings in the pituitary and in other developing organs, we hypothesize that Notch signaling regulates the balance between proliferating progenitors and differentiated cells in the pituitary.

Notch signaling is an evolutionarily conserved pathway that is important for progenitor maintenance and cell fate choices. Signaling occurs between two adjacent cells, one containing the ligand and the other containing the Notch receptors. In mammals, there are two families of ligands, the Delta-like (Dll1, Dll3 and Dll4) and Jagged (Jag1 and Jag2) families, which can bind to the four Notch receptors (Notch1-4). Upon binding, a series of cleavages is triggered, that ultimately results in the release of the Notch intracellular domain (NICD). A member of ADAM metalloprotease family performs the first cleavage, while the second is dependent on gamma secretase. Once the NICD is released, it can then travel into the nucleus. Here it binds to RBPj, an essential Notch cofactor, and other proteins to create a complex, which binds to DNA and activates the transcription of target genes. Canonical Notch targets are members of the *Hes* and *Hey* families of basic helix loop helix transcription factors. These factors often repress the transcription of genes necessary for differentiation, thus maintaining the cell as a progenitor (Patten, et al. 2003; Kopan and Ilagan. 2009), (Figure 1.2).

In many developmental contexts, Notch signaling is necessary for the proliferation of progenitor cells, but is not sufficient alone to direct their

proliferation. However, there are select cases in which Notch can promote proliferation, such as in cerebellar granule neuron precursors (Solecki, et al. 2001). Additionally, activated Notch is thought to contribute to cancer initiation in patients with T-ALL (T-cell acute lymphoblastic leukemia), a pediatric leukemia that is often associated with mutations in *NOTCH1* (Pancewicz, et al. 2010).

In addition to its role in regulating cell proliferation, Notch signaling has been shown to be important for cell fate decisions. For example, in the intestine Notch signaling biases cells toward an enterocyte fate and away from a secretory fate during adulthood (Stanger, et al. 2005). Additionally, in the inner ear, Notch signaling controls the balance between hair cells and support cells through a mechanism that is thought to involve lateral inhibition. It is hypothesized that hair cells upregulate Notch ligands and activate Notch signaling in neighboring cells. This activation prevents these adjacent cells from becoming hair cells and biases them toward the support cell fate. A reduction in Notch signaling, therefore, results in an increase in hair cell number (Lanford, et al. 1999; Daudet and Lewis. 2005).

It remains unclear how Notch signaling influences cell fate choice and the balance between proliferation and differentiation in the developing pituitary. Expression analysis studies show that the receptors *Notch2* and *Notch3*, the ligands *Dll1* and *Jag1* and the Notch effectors *Hes1* and *Hey1* are all present as early as e9.5 in proliferating progenitors. As cells begin to express terminal differentiation makers and migrate towards the developing AL, the expression of these molecules diminishes (Raetzman, et al. 2004; Zhu, et al. 2006). Several

recent studies have used knockout and transgenic mouse models to determine the role this pathway plays in pituitary development. *Hes1* null mice have hypoplastic pituitaries with failure of melanotropes to form (Zhu, et al. 2006; Kita, et al. 2007; Raetzman, et al. 2007). This hypoplastic phenotype is a result of a decrease in pituitary proliferation observed throughout embryogenesis (Monahan, et al. 2009). However, the confounding problem with this study is lack of pituitary specificity in the *Hes1* knockout, making it impossible to differentiate the pituitary from the hypothalamic role of Notch. Additionally, postnatal proliferation is decreased in Ames Dwarf mice, which lack functional *Prop1* (*Prophet of Pit1*), a putative pituitary specific target of Notch signaling (Ward, et al. 2005; Zhu, et al. 2006). In these mice, cells of the PIT1 lineage (lactotropes, thyrotropes and somatotropes) fail to differentiate, and LH and FSH levels are reduced. However, corticotropes are unaffected, indicating their differentiation is likely controlled by an alternative mechanism. These phenotypes are also observed in humans with mutations in the *PROP1* gene (Wu, et al. 1998). Although corticotropes are unaffected in *Prop1*-deficient mice, specification and differentiation of this lineage may also involve Notch signaling because in a global knockout of both *Hes1* and *Prop1*, accelerated differentiation of corticotropes is observed (Himes and Raetzman. 2009b). This phenotype is not observed in either of the single mutants and indicates Notch signaling is required to prevent early differentiation of these cells. Similarly, if hypothalamic-pituitary biased embryonic stem cells are differentiated in culture, Notch needs to be inhibited in order achieve corticotrope fate (Suga, et al. 2011). These data suggest Notch signaling is necessary to

inhibit the differentiation of corticotropes. It remains unclear if Notch signaling is also sufficient to prevent the differentiation of corticotropes and closely related melanotropes, if it can promote proliferation in these cells and the mechanism by which Notch signaling executes these diverse actions. These studies are important to increase our understanding of the role of Notch signaling in the regulation of proliferation and binary cell fate in the pituitary.

In addition to the embryonic wave of pituitary proliferation and differentiation described in detail above, the pituitary undergoes a second wave of proliferation during the first three postnatal weeks in rodents. In rats, during this time, the pituitary expands 5-6 fold in volume (Carbajo-Pérez and Watanabe. 1990). This period of development has only begun to be studied and signaling pathways involved in proliferation and subsequent cell differentiation have not yet been identified. Additionally, the adult pituitary has a remarkable ability to respond to environmental cues and adjust cell number and size according to need. For example, after adrenalectomy or gonadectomy, the pituitary proliferates due to lack of negative feedback from target organs (Nolan, et al. 2004; Nolan and Levy. 2006). Similarly, during pregnancy, the pituitary expands in anticipation of the increased need for prolactin during lactation (Yin and Arita. 2000; Carretero, et al. 2003).

Although postnatal proliferation has been characterized (Carbajo-Pérez and Watanabe. 1990; Kominami, et al. 2003; Gremeaux, et al. 2012), the identity of the proliferating cell type (s) remains unclear. Differentiated, hormone-producing cells have the ability to proliferate during these periods, but they do so

at a very low rate, making them unlikely to be the sole contributor to pituitary growth (Taniguchi, et al. 2000; Taniguchi, et al. 2001; Taniguchi, et al. 2002a; Taniguchi, et al. 2002b). Pituitary progenitors are present at this stage, found lining the pituitary cleft, a remnant of the lumen of RP, and scattered throughout the AL. Although SOX2 expression decreases in cells in the parenchyma of the AL as the animal ages, progenitor cells remain surrounding the cleft throughout the life of the animal and maintain the ability to generate all pituitary endocrine cell types (Fauquier, et al. 2008; Rizzoti, et al. 2013; Andoniadou, et al. 2013). Additionally, it is hypothesized that a transit amplifying population is derived from these progenitor cells. We hypothesize that these transit amplifying and progenitor populations contribute to postnatal pituitary expansion and may be activated in response to environmental cues.

Because these postnatal and adult progenitors face similar fate choices as their embryonic counterparts, it is likely that similar signaling pathways will be involved. This progenitor population was shown to be enriched in Notch signaling components in postnatal mice and inhibition of Notch signaling in dissociated pituitary cells or in pituitary cell aggregates results in a decrease in the size and proliferation of the progenitor pool. Increased progenitor proliferation and number are observed when these cells are treated with Notch ligands (Chen, et al. 2006; Tando, et al. 2013). However, neither the mechanism accounting for these observations, nor the consequences of a reduction in the progenitor population *in vivo,* were investigated. Based on these findings and its known role in the pituitary during embryogenesis, we hypothesize Notch signaling may act to

maintain progenitor cells in the postnatal and adult pituitary and thus influences pituitary proliferation and cell fate specification.

Improper pituitary cellular proliferation or differentiation during either the embryonic or postnatal period can result in multiple pituitary disorders, including combined pituitary hormone deficiency and adenoma formation. It is, therefore, critical, to understand mechanisms controlling these processes. We hypothesize that Notch controls binary cell fate choice and proliferation during all stages of pituitary development. The studies outlined below aim to determine: 1) the role of Notch signaling in embryonic corticotrope development using a pituitary-specific gain- and loss-of-function approach, 2) if Notch is necessary for proliferation, progenitor maintenance and cell fate choice during postnatal pituitary expansion and 3) the identity of Notch targets that are important during postnatal pituitary development.

REFERENCES

Andoniadou, C.L., Matsushima, D., Mousavy Gharavy, S.N., Signore, M., Mackintosh, A.I., Schaeffer, M., Gaston-Massuet, C., Mollard, P., Jacques, T.S., Le Tissier, P., Dattani, M.T., Pevny, L.H., Martinez-Barbera, J.P., 2013. Sox2(+) Stem/Progenitor Cells in the Adult Mouse Pituitary Support Organ Homeostasis and have Tumor-Inducing Potential. Cell Stem Cell 13, 433-445.

Bilodeau, S., Roussel-Gervais, A., Drouin, J., 2009. Distinct Developmental Roles of Cell Cycle Inhibitors p57Kip2 and p27Kip1 Distinguish Pituitary Progenitor Cell Cycle Exit from Cell Cycle Reentry of Differentiated Cells. Molecular and cellular biology 29, 1895-908.

Carbajo-Pérez, E., Watanabe, Y.G., 1990. Cellular Proliferation in the Anterior Pituitary of the Rat during the Postnatal Period. Cell and tissue research 261, 333-8.

Carretero, J., Rubio, M., Blanco, E., Burks, D.J., Torres, J.L., Hernández, E., Bodego, P., Riesco, J.M., Juanes, J.A., Vázquez, R., 2003. Variations in the Cellular Proliferation of Prolactin Cells from Late Pregnancy to Lactation in Rats. Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft 185, 97-101.

Chen, J., Crabbe, A., Van Duppen, V., Vankelecom, H., 2006. The Notch Signaling System is Present in the Postnatal Pituitary: Marked Expression and Regulatory Activity in the Newly Discovered Side Population. Molecular endocrinology (Baltimore, Md) 20, 3293-307.

Daly, A.F., Rixhon, M., Adam, C., Dempegioti, A., Tichomirowa, M.A., Beckers, A., 2006. High Prevalence of Pituitary Adenomas: A Cross-Sectional Study in the Province of Liege, Belgium. The Journal of clinical endocrinology and metabolism 91, 4769-75.

Daudet, N., Lewis, J., 2005. Two Contrasting Roles for Notch Activity in Chick Inner Ear Development: Specification of Prosensory Patches and Lateral Inhibition of Hair-Cell Differentiation. Development (Cambridge, England) 132, 541-51.

Davis, S.W., Mortensen, A.H., Camper, S.A., 2011. Birthdating Studies Reshape Models for Pituitary Gland Cell Specification. Developmental biology .

Ericson, J., Norlin, S., Jessell, T.M., Edlund, T., 1998. Integrated FGF and BMP Signaling Controls the Progression of Progenitor Cell Differentiation and the

Emergence of Pattern in the Embryonic Anterior Pituitary. Development (Cambridge, England) 125, 1005-15.

Fauquier, T., Rizzoti, K., Dattani, M., Lovell-Badge, R., Robinson, I.C.A.F., 2008. SOX2-Expressing Progenitor Cells Generate all of the Major Cell Types in the Adult Mouse Pituitary Gland. Proceedings of the National Academy of Sciences of the United States of America 105, 2907-12.

Fernandez, A., Karavitaki, N., Wass, J.A.H., 2010. Prevalence of Pituitary Adenomas: A Community-Based, Cross-Sectional Study in Banbury (Oxfordshire, UK). Clinical endocrinology 72, 377-82.

Gage, P.J., Brinkmeier, M.L., Scarlett, L.M., Knapp, L.T., Camper, S.A., Mahon, K.A., 1996. The Ames Dwarf Gene, Df, is Required Early in Pituitary Ontogeny for the Extinction of Rpx Transcription and Initiation of Lineage-Specific Cell Proliferation. Molecular endocrinology (Baltimore, Md) 10, 1570-81.

Gremeaux, L., Fu, Q., Chen, J., Vankelecom, H., 2012. Activated Phenotype of the Pituitary Stem/Progenitor Cell Compartment during the Early-Postnatal Maturation Phase of the Gland. Stem cells and development 21, 801-13.

Himes, A.D., Raetzman, L.T., 2009a. Premature Differentiation and Aberrant Movement of Pituitary Cells Lacking both Hes1 and Prop1. Dev Biol 325, 151-161.

Himes, A.D., Raetzman, L.T., 2009b. Premature Differentiation and Aberrant Movement of Pituitary Cells Lacking both Hes1 and Prop1. Developmental biology 325, 151-61.

Japón, M.A., Rubinstein, M., Low, M.J., 1994. In Situ Hybridization Analysis of Anterior Pituitary Hormone Gene Expression during Fetal Mouse Development. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 42, 1117-25.

Kashiwabara, Y., Sasaki, S., Matsushita, A., Nagayama, K., Ohba, K., Iwaki, H., Matsunaga, H., Suzuki, S., Misawa, H., Ishizuka, K., Oki, Y., Nakamura, H., 2009. Functions of PIT1 in GATA2-Dependent Transactivation of the Thyrotropin Beta Promoter. Journal of molecular endocrinology 42, 225-37.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C.H., Ward, J.M., Gonzalez, F.J., 1996. The T/Ebp Null Mouse: Thyroid-Specific Enhancer-Binding Protein is Essential for the Organogenesis of the Thyroid, Lung, Ventral Forebrain, and Pituitary. Genes & development 10, 60-9.

Kita, A., Imayoshi, I., Hojo, M., Kitagawa, M., Kokubu, H., Ohsawa, R., Ohtsuka, T., Kageyama, R., Hashimoto, N., 2007. Hes1 and Hes5 Control the Progenitor Pool, Intermediate Lobe Specification, and Posterior Lobe Formation in the Pituitary Development. Molecular endocrinology (Baltimore, Md) 21, 1458-66.

Kominami, R., Yasutaka, S., Taniguchi, Y., Shinohara, H., 2003. Proliferating Cells in the Rat Anterior Pituitary during the Postnatal Period: Immunoelectron Microscopic Observations using Monoclonal Anti-Bromodeoxyuridine Antibody. Histochemistry and cell biology 120, 223-33.

Kopan, R., Ilagan, M.X.G., 2009. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. Cell 137, 216-33.

Lamolet, B., Pulichino, A.M., Lamonerie, T., Gauthier, Y., Brue, T., Enjalbert, A., Drouin, J., 2001. A Pituitary Cell-Restricted T Box Factor, Tpit, Activates POMC Transcription in Cooperation with Pitx Homeoproteins. Cell 104, 849-59.

Lamolet, B., Poulin, G., Chu, K., Guillemot, F., Tsai, M., Drouin, J., 2004. Tpit-Independent Function of NeuroD1(BETA2) in Pituitary Corticotroph Differentiation. Molecular endocrinology (Baltimore, Md) 18, 995-1003.

Lanford, P.J., Lan, Y., Jiang, R., Lindsell, C., Weinmaster, G., Gridley, T., Kelley, M.W., 1999. Notch Signalling Pathway Mediates Hair Cell Development in Mammalian Cochlea. Nature genetics 21, 289-92.

Liu, J., Lin, C., Gleiberman, A., Ohgi, K.A., Herman, T., Huang, H.P., Tsai, M.J., Rosenfeld, M.G., 2001. Tbx19, a Tissue-Selective Regulator of POMC Gene Expression. Proceedings of the National Academy of Sciences of the United States of America 98, 8674-9.

Monahan, P., Rybak, S., Raetzman, L.T., 2009. The Notch Target Gene HES1 Regulates Cell Cycle Inhibitor Expression in the Developing Pituitary. Endocrinology 150, 4386-94.

Nasonkin, I.O., Ward, R.D., Raetzman, L.T., Seasholtz, A.F., Saunders, T.L., Gillespie, P.J., Camper, S.A., 2004. Pituitary Hypoplasia and Respiratory Distress Syndrome in Prop1 Knockout Mice. Human molecular genetics 13, 2727-35.

Nolan, L.A., Levy, A., 2006. A Population of Non-Luteinising Hormone/Non-Adrenocorticotrophic Hormone-Positive Cells in the Male Rat Anterior Pituitary Responds Mitotically to both Gonadectomy and Adrenalectomy. Journal of neuroendocrinology 18, 655-61.

Nolan, L.A., Thomas, C.K., Levy, A., 2004. Pituitary Mitosis and Apoptotic Responsiveness Following Adrenalectomy are Independent of Hypothalamic Paraventricular Nucleus CRH Input. The Journal of endocrinology 181, 521-9.

Nudi, M., Ouimette, J., Drouin, J., 2005. Bone Morphogenic Protein (Smad)-Mediated Repression of Proopiomelanocortin Transcription by Interference with Pitx/Tpit Activity. Molecular endocrinology (Baltimore, Md) 19, 1329-42.

Palomino, T., Sánchez-Pacheco, A., Peña, P., Aranda, A., 1998. A Direct Protein-Protein Interaction is Involved in the Cooperation between Thyroid Hormone and Retinoic Acid Receptors and the Transcription Factor GHF-1. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 12, 1201-9.

Pancewicz, J., Taylor, J.M., Datta, A., Baydoun, H.H., Waldmann, T.A., Hermine, O., Nicot, C., 2010. Notch Signaling Contributes to Proliferation and Tumor Formation of Human T-Cell Leukemia Virus Type 1-Associated Adult T-Cell Leukemia. Proceedings of the National Academy of Sciences of the United States of America 107, 16619-24.

Patten, B.A., Peyrin, J.M., Weinmaster, G., Corfas, G., 2003. Sequential Signaling through Notch1 and erbB Receptors Mediates Radial Glia Differentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 23, 6132-40.

Philips, A., Lesage, S., Gingras, R., Maira, M.H., Gauthier, Y., Hugo, P., Drouin, J., 1997a. Novel Dimeric Nur77 Signaling Mechanism in Endocrine and Lymphoid Cells. Molecular and cellular biology 17, 5946-51.

Philips, A., Maira, M., Mullick, A., Chamberland, M., Lesage, S., Hugo, P., Drouin, J., 1997b. Antagonism between Nur77 and Glucocorticoid Receptor for Control of Transcription. Molecular and cellular biology 17, 5952-9.

Poulin, G., Turgeon, B., Drouin, J., 1997. NeuroD1/Beta2 Contributes to Cell-Specific Transcription of the Proopiomelanocortin Gene. Molecular and cellular biology 17, 6673-82.

Pulichino, A., Vallette-Kasic, S., Couture, C., Gauthier, Y., Brue, T., David, M., Malpuech, G., Deal, C., Van Vliet, G., De Vroede, M., Riepe, F.G., Partsch, C., Sippell, W.G., Berberoglu, M., Atasay, B., Drouin, J., 2003a. Human and Mouse TPIT Gene Mutations Cause Early Onset Pituitary ACTH Deficiency. Genes & development 17, 711-6.

Pulichino, A., Vallette-Kasic, S., Tsai, J.P., Couture, C., Gauthier, Y., Drouin, J., 2003b. Tpit Determines Alternate Fates during Pituitary Cell Differentiation. Genes & development 17, 738-47.

Raappana, A., Koivukangas, J., Ebeling, T., Pirilä, T., 2010. Incidence of Pituitary Adenomas in Northern Finland in 1992-2007. The Journal of clinical endocrinology and metabolism 95, 4268-75.

Raetzman, L.T., Ross, S.A., Cook, S., Dunwoodie, S.L., Camper, S.A., Thomas, P.Q., 2004. Developmental Regulation of Notch Signaling Genes in the Embryonic Pituitary: Prop1 Deficiency Affects Notch2 Expression. Developmental biology 265, 329-40.

Raetzman, L.T., Cai, J.X., Camper, S.A., 2007. Hes1 is Required for Pituitary Growth and Melanotrope Specification. Developmental biology 304, 455-66.

Rizzoti, K., Akiyama, H., Lovell-Badge, R., 2013. Mobilized Adult Pituitary Stem Cells Contribute to Endocrine Regeneration in Response to Physiological Demand. Cell Stem Cell 13, 419-432.

Rizzoti, K., 2010. Adult Pituitary Progenitors/Stem Cells: From in Vitro Characterization to in Vivo Function. Eur J Neurosci 32, 2053-2062.

Stanger, B.Z., Datar, R., Murtaugh, L.C., Melton, D.A., 2005. Direct Regulation of Intestinal Fate by Notch. Proceedings of the National Academy of Sciences of the United States of America 102, 12443-8.

Suga, H., Kadoshima, T., Minaguchi, M., Ohgushi, M., Soen, M., Nakano, T., Takata, N., Wataya, T., Muguruma, K., Miyoshi, H., Yonemura, S., Oiso, Y., Sasai, Y., 2011. Self-Formation of Functional Adenohypophysis in Three-Dimensional Culture. Nature 480, 57-62.

Tando, Y., Fujiwara, K., Yashiro, T., Kikuchi, M., 2013. Localization of Notch Signaling Molecules and their Effect on Cellular Proliferation in Adult Rat Pituitary. Cell Tissue Res 351, 511-519.

Taniguchi, Y., Yasutaka, S., Kominami, R., Shinohara, H., 2001. Proliferation and Differentiation of Pituitary Somatotrophs and Mammotrophs during Late Fetal and Postnatal Periods. Anatomy and embryology 204, 469-75.

Taniguchi, Y., Kominami, R., Yasutaka, S., Kawarai, Y., 2000. Proliferation and Differentiation of Pituitary Corticotrophs during the Fetal and Postnatal Period: A Quantitative Immunocytochemical Study. Anatomy and embryology 201, 229-34.

Taniguchi, Y., Yasutaka, S., Kominami, R., Shinohara, H., 2002a. Proliferation and Differentiation of Rat Anterior Pituitary Cells. Anatomy and embryology 206, 1-11.

Taniguchi, Y., Yasutaka, S., Kominami, R., Shinohara, H., 2002b. Mitoses of Thyrotrophs Contribute to the Proliferation of the Rat Pituitary Gland during the Early Postnatal Period. Anatomy and embryology 206, 67-72.

Treier, M., Gleiberman, A.S., O'Connell, S.M., Szeto, D.P., McMahon, J.A., McMahon, A.P., Rosenfeld, M.G., 1998. Multistep Signaling Requirements for Pituitary Organogenesis in Vivo. Genes & development 12, 1691-704.

Vallette-Kasic, S., Pulichino, A.M., Gueydan, M., Barlier, A., David, M., Malpuech, G., Deal, C., Van Vliet, G., de Vroede, M., Riepe, F., Partsch, C.J., Sippell, W., Berberoglu, M., Atasay, B., de Zegher, F., Kyllo, J., Donohoue, P., Dechelotte, P., Fassnacht, M., Noordam, K., Dunkel, L., Pigeon, B., Weill, J., Yigit, S., Brauner, R., Leger, J., Heinrich, J.J., Enjalbert, A., Brue, T., Drouin, J., 2004. A Neonatal Form of Isolated ACTH Deficiency Frequently Associated with Tpit Gene Mutations. Endocrine research 30, 943-4.

Ward, R.D., Raetzman, L.T., Suh, H., Stone, B.M., Nasonkin, I.O., Camper, S.A., 2005. Role of PROP1 in Pituitary Gland Growth. Molecular endocrinology (Baltimore, Md) 19, 698-710.

Wu, W., Cogan, J.D., Pfäffle, R.W., Dasen, J.S., Frisch, H., O'Connell, S.M., Flynn, S.E., Brown, M.R., Mullis, P.E., Parks, J.S., Phillips, J.A.(., Rosenfeld, M.G., 1998. Mutations in PROP1 Cause Familial Combined Pituitary Hormone Deficiency. Nature genetics 18, 147-9.

Yin, P., Arita, J., 2000. Differential Regulation of Prolactin Release and Lactotrope Proliferation during Pregnancy, Lactation and the Estrous Cycle. Neuroendocrinology 72, 72-9.

Zhu, X., Gleiberman, A.S., Rosenfeld, M.G., 2007. Molecular Physiology of Pituitary Development: Signaling and Transcriptional Networks. Physiological reviews 87, 933-63.

Zhu, X., Zhang, J., Tollkuhn, J., Ohsawa, R., Bresnick, E.H., Guillemot, F., Kageyama, R., Rosenfeld, M.G., 2006. Sustained Notch Signaling in Progenitors is Required for Sequential Emergence of Distinct Cell Lineages during Organogenesis. Genes & development 20, 2739-53.

FIGURES

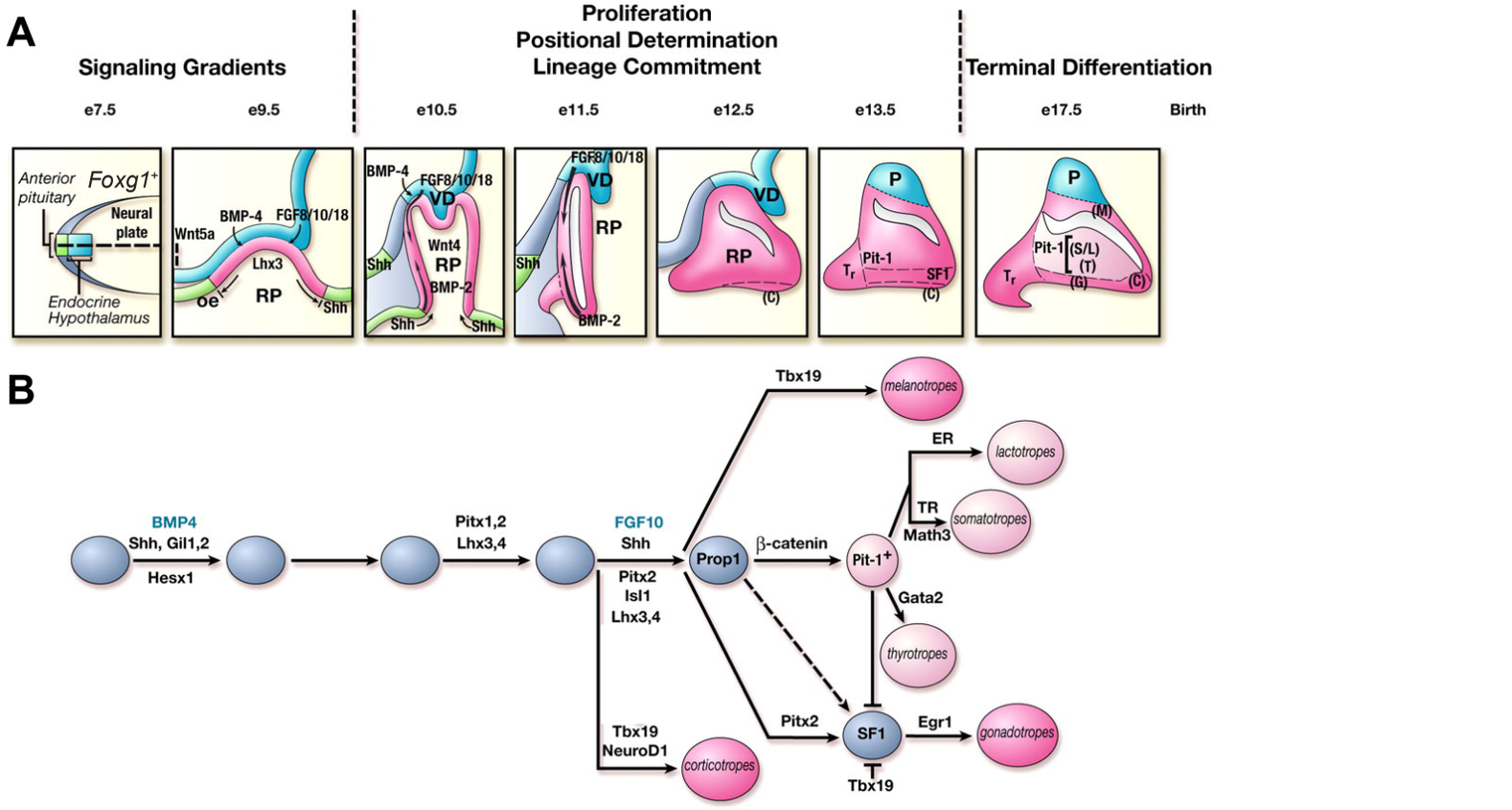

Figure 1.1. Schematic of pituitary development. The anterior and intermediate lobes of the pituitary develop from the most anterior portion of the neural ridge, which expresses *Foxg1*. The posterior lobe (P) develops from the neural tissue that is just adjacent to this region. At e9.5, induction of the pituitary relies on expression of Wnt5a, BMP-4, and FGF8/10/18 in the VD and SHH in the oral ectoderm. At this age, RP is marked by the expression of the transcription factor LHX3. By e11.5, RP pinches off from the underlying oral ectoderm to form a closed pouch. By e12.5, the emergence of differentiated cell types is evident, as shown by expression *Pomc* mRNA in corticotropes. Pit1^{+} cells, which are precursors of lactotropes, thyrotropes and somatotropes, and SF1+ cells, which are precursors to gonadotropes, are observed by e13.5. Melanotropes in the IL are evident by e16.5 and GH and TSHβ are expressed in the AL by e17.5 (A). The differentiation of corticotropes and melanotropes rely on the expression of Tbx19, with corticotropes also requiring expression of NeuroD1 for *Pomc* transcription. Expression of *Pit1* is reliant on Prop1, whereas each cell type of the Pit1 lineage requires additional transcription factors for hormone expression (ER for lactotropes, TR for somatotropes and Gata2 for somatotropes). Gonadotropes develop from SF1^{+} precursors and require Egr1 for hormone production (B). RP=Rathke's pouch, VD=Ventral diencephalon, T=Thyrotropes, S=Somatotropes, L=Lactotropes, C=Corticotropes, M=Melanotropes. (Adapted from Zhu et al, 2007).

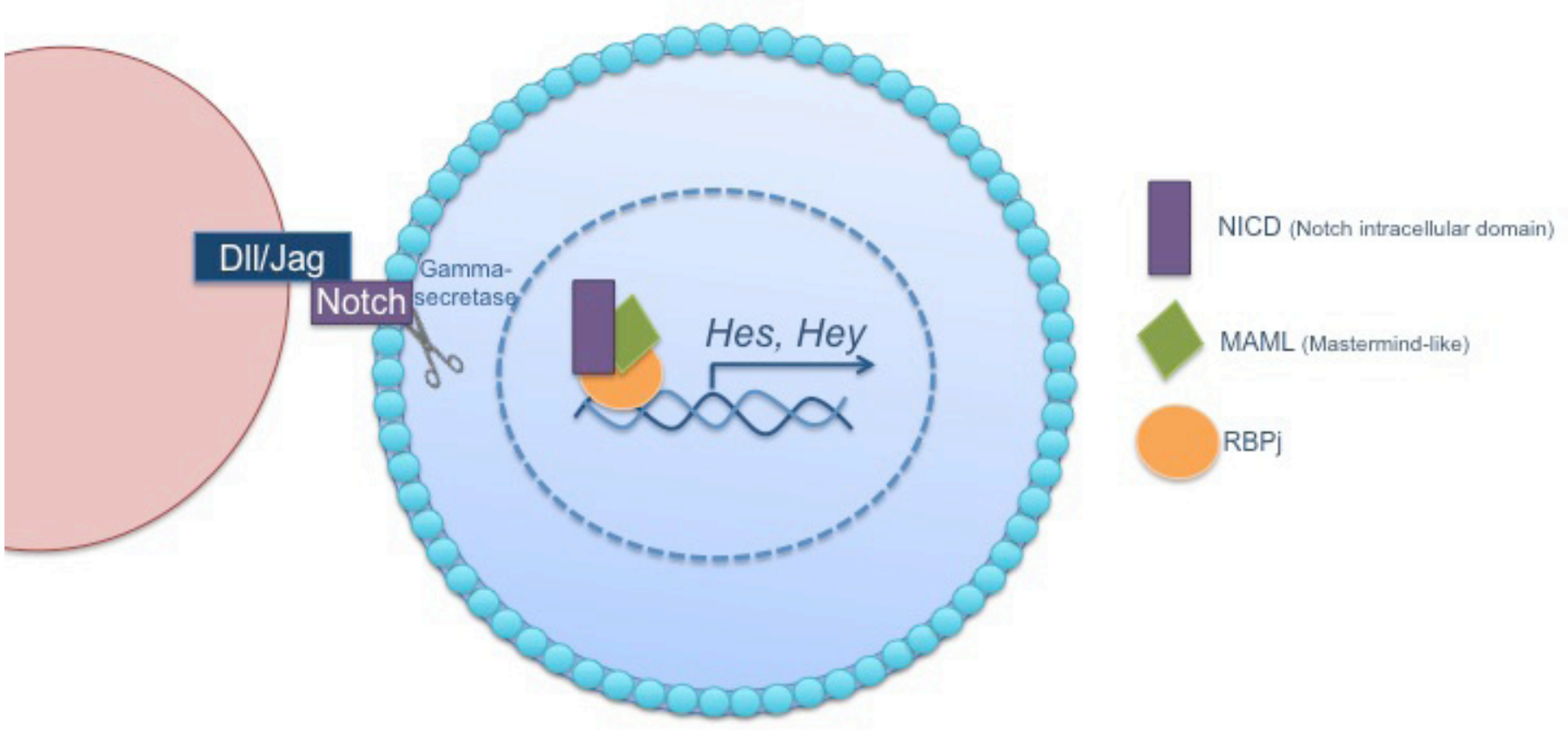

Figure 1.2. Schematic of Notch signaling. Notch signaling occurs between two adjacent cells, one that contains a ligand of the Delta-like and/or Jagged families and the other that contains the Notch receptor. Upon ligand binding, the intracellular domain of Notch is cleaved by gamma secretase. It can then translocate into the nucleus where it binds several cofactors. These cofactors include RBPj, which is the cofactor that binds directly to DNA and Mastermind-like, which serves as a scaffold for the recruitment of other coactivators. Activation of this cascade allows the NICD-containing complex to drive the transcription of downstream targets of the *Hes* and *Hey* families of transcriptional repressors. Dll=Delta-like.

CHAPTER 2: **Persistent Expression of Activated Notch inhibits corticotrope and melanotrope differentiation and results in dysfunction of the HPA axis**[1]**.**

ABSTRACT

The hypothalamic-pituitary-adrenal (HPA) axis is an important regulator of energy balance, immune function and the body's response to stress. Signaling networks governing the initial specification of corticotropes, a major component of this axis, are not fully understood. Loss of function studies indicate that Notch signaling may be necessary to repress premature differentiation of corticotropes and to promote proliferation of pituitary progenitors. To elucidate whether Notch signaling must be suppressed in order for corticotrope differentiation to proceed and whether Notch signaling is sufficient to promote corticotrope proliferation, we examined the effects of persistent Notch expression in *Pomc* lineage cells. We show that constitutive activation of the Notch cascade inhibits the differentiation of both corticotropes and melanotropes and results in the suppression of transcription factors required for *Pomc* expression. Furthermore, persistent Notch signaling traps cells in the intermediate lobe of the pituitary in a progenitor state, but has no effect on pituitary proliferation. Undifferentiated cells are eliminated in the first two postnatal weeks in these mice, resulting in a modest increase in CRH expression in the paraventricular nucleus, hypoplastic adrenal glands and decreased stress-induced corticosterone levels. Taken together, these findings

[1] This chapter appeared in its entirety in *Developmental Biology* and is referred to later in this dissertation as "Goldberg et al., 2011". Goldberg, L.B., Aujla, P.K., Raetzman, L.T., 2011. Persistent Expression of Activated Notch Inhibits Corticotrope and Melanotrope Differentiation and Results in Dysfunction of the HPA Axis. Dev Biol 358, 23-32. This article is reprinted with the permission of the publisher and is available from http://www.elsevier.com and using DOI: 10.1016/j.ydbio.2011.07.004

show that Notch signaling is sufficient to prevent corticotrope and melanotrope differentiation, resulting in dysregulation of the HPA axis.

INTRODUCTION

The hypothalamic-pituitary-adrenal (HPA) axis is critical to mediating the body's response to stress. It also functions to regulate many important homeostatic processes, including immune response and energy balance. Stressors, either immune or environmental, activate the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus to the anterior pituitary through the hypothalamo-hypophyseal portal system. CRH release stimulates the anterior pituitary to synthesize and release adrenocorticotropic hormone (ACTH), which acts on the adrenal glands to trigger the release of cortisol (corticosterone in mice) (Garren. 1968; Vale, et al. 1981). Increased cortisol release results in a number of different physiological effects, including an increase in glucose production and mobilization and an anti-inflammatory response (Munck, et al. 1984). In addition to increased glucose release, cortisol also exerts negative feedback on both the hypothalamus and pituitary, inhibiting further release of CRH and ACTH (Swanson, Simmons. 1989). Disruption in proper development or function at any level of the HPA axis can result in multiple disorders, including Cushing's syndrome and adrenal insufficiency, which are characterized by increased and decreased levels of cortisol, respectively (Drouin, et al. 2007).

The pituitary, a crucial component of the HPA axis, develops from an invagination of the oral ectoderm at embryonic day (e) 8.5 in mice. By e11.5, the developing pituitary pinches off from the underlying oral ectoderm, forming the structure known as Rathke's pouch (RP). RP contains all the progenitor cells that are necessary for formation of the anterior lobe (AL) and intermediate lobe (IL) of the pituitary (Burrows, et al. 1999; Rizzoti, Lovell-Badge. 2005). The AL contains five hormone producing cell types, which include thyrotropes, lactotropes, gonadotropes, corticotropes, somatotropes, and lactotropes. The IL houses a sixth hormone cell type, the melanotropes. Current models propose that pituitary hormone producing cells are specified such that each cell type differentiates during a discrete time during embryonic development (Pope, et al. 2006). Furthermore, it is widely accepted that cells exiting the cell cycle at the same time migrate to the same location in the pituitary (Dasen, Rosenfeld. 2001; Wagner, Thomas. 2007). This synchronized cell specification and migration is thought to be coordinated by opposing gradients of bone morphogenetic proteins (BMPs) in the underlying oral ectoderm and fibroblast growth factors (FGFs) in the ventral diencephalon (Ericson, et al. 1998; Treier, et al. 1998). However, recent evidence indicates that each AL hormone cell type exits the cell cycle at the same time, with the majority of cells doing so between e11.5 and e13.5. Furthermore, there is no apparent correlation between the timing of cell specification and cell placement within the gland (Davis, et al. 2011). Based on these observations, it remains unclear how undifferentiated progenitor cells

within the same region of the AL, exposed to the same chemical gradients, are specified to become different cell types.

Although all AL hormone cell types exit the cell cycle at the same time, corticotropes are the first to express hormone beginning at e12.5 (Japón, et al. 1994). Melanotropes, found in the IL, exit the cell cycle after AL hormone producing cells and express hormone beginning at e16.5 (Davis, et al. 2011). Terminal differentiation of corticotropes and melanotropes is characterized by the expression of pro-opiomelanocortin (POMC), a precursor of both ACTH, which is released by corticotropes, and melanocyte stimulating hormone (αMSH), which is produced by melanotropes.

Several studies have identified multiple transcription factors that activate or inhibit expression of POMC in the pituitary. These include TPIT (TBX19), (Lamolet, et al. 2001; Liu, et al. 2001), NEUROD1 (Poulin, et al. 1997; Lamolet, et al. 2004), Nur77 (Philips, et al. 1997a; Philips, et al. 1997b) and BMPs (Nudi, et al. 2005). While these factors are known to regulate *Pomc* transcription and thus the terminal differentiation of corticotropes and melanotropes, it remains unclear which signals are involved in the specification of these cells.

We hypothesize that the Notch signaling pathway, an essential player in progenitor maintenance and cell fate determination in many tissues, may play a critical role in the specification of corticotropes and melanotropes. The Notch ligands Delta-like1 and Jagged1, the Notch2 and Notch3 receptors, as well as downstream Notch effectors of the *Hes* and *Hey* families of genes, are present during development in proliferating progenitors in RP. As cells differentiate and

migrate towards the developing AL, the expression of these molecules is suppressed (Raetzman, et al. 2004; Zhu, et al. 2006). Additionally, the Notch1 and Notch2 receptors, as well as Hes and Hey transcription factors, are present in pituitary stem cells (Chen, et al. 2005; Chen, et al. 2006).

Studies have shown that loss of function manipulations of the Notch signaling pathway result in defects in corticotrope and melanotrope differentiation. In mice lacking *Hes1*, a canonical Notch target, *Tpit* mRNA expression was found to be increased (Zhu, et al. 2006), suggesting that Notch may be necessary to prevent early expression of this transcription factor. When both *Prop1*, a pituitary specific Notch target, and *Hes1* are lost, robust premature differentiation of corticotropes is observed (Himes, Raetzman. 2009). A similar, but more modest, acceleration of *Pomc* expression is seen in a pituitary-specific knockout of *Rbpj*, the primary mediator of Notch signaling (Zhu, et al. 2006). Taken together, these results suggest Notch signaling is necessary to prevent differentiation of corticotropes and may act as a negative regulator of early corticotrope specification. We hypothesize that Notch signaling is sufficient to prevent the differentiation of corticotropes and melanotropes and that Notch expression needs to be extinguished for cell differentiation to proceed.

In order to address this hypothesis, we examined the effects of persistent expression of activated Notch1 in POMC-expressing cells. We find that Notch signaling is sufficient to prevent differentiation of these cells, coincident with the suppression of the transcription factors *Tpit* and *Neurod1*. Additionally, we show that these undifferentiated cells maintain progenitor qualities but are eliminated

during the first two postnatal weeks. The loss of POMC expression in the pituitary results in severely hypoplastic adrenal glands and decreased stress-induced corticosterone levels. Our results indicate that Notch signaling is both necessary and sufficient to repress corticotrope differentiation and that alterations in Notch signaling could result in dysfunction of the HPA axis.

MATERIALS AND METHODS

Mice

Rosa^{Notch} floxed mice (Murtaugh, et al. 2003) were purchased from Jackson Laboratories (Bar Harbor, ME). A breeding colony was established and progeny were bred to *Pomc* Cre mice (Balthasar, et al. 2004), also purchased from Jackson Laboratories (Bar Harbor, ME). The resulting progeny all possess one Rosa^{Notch} floxed allele, with half also expressing Cre recombinase. To genotype the mice, tail biopsies were obtained and DNA was extracted using a salt-out method. PCR for the *Pomc* and Cre alleles was performed as previously described (Himes, Raetzman. 2009). PCR for the Rosa^{Notch} floxed allele was performed in a similar manner with the following exceptions. The primers used to detect this allele were 5'-AAA GTC GCT CTG AGT TGT TAT-3', 5'-TAA GCC TGC CCA GAA GAC T-3' and 5'-GAA AGA CCG CGA AGA GTT T G-3'. The samples underwent 35 cycles of denaturing at 95 C for 30 sec, annealing at 55 C for 30 sec, and elongation at 72 C for 60 sec, followed by 72 C for 5 min. All animals were housed in a facility with a 12 hour light-dark cycle and were maintained in accordance with the University of Illinois at Urbana-Champaign

Institutional Animal Care and Use Committee.

Immunohistochemistry

Mice were sacrificed at e16.5, p1, and p32 and embryos and pituitaries were fixed in 3.7% formaldehyde diluted in phosphate buffered saline (PBS). Samples were dehydrated through a series of graded ethanol, embedded coronally in paraffin, sectioned to a thickness of 6 microns and mounted on charged slides. For frozen section preparation used to detect CRH in the hypothalamus, p32 control and Tg brains were snap frozen in 2-methylbutane, cryoprotected overnight at 4°C in 30% sucrose diluted in PBS, and sectioned at 14μm.

Paraffin sections were deparaffinized, rehydrated and washed in PBS. Samples were then boiled in hot citrate solution (10uM citrate, pH6) for 10 minutes and cooled for 10 minutes (for all antibodies except POMC and CRH). Following antigen retrieval, samples were blocked in a solution containing 5% normal donkey serum diluted in immunohistochemical block which contains 3% bovine serum albumin and 0.5% TritonX-100 diluted in PBS. Frozen sections were fixed in 4% PFA for 30 mins, washed in PBS prior to application of blocking solution. For all immunohistochemistry, blocking was followed by an overnight incubation at 4°C with primary antibody diluted in immunohistochemical block. Primary antibodies used were raised against the following peptides: POMC (1:300, Dako, Carpinteria, CA), Sox2 (1:750, Millipore, Billerica, MA), Pax7 (1:500, Developmental Studies Hybridoma Bank, Iowa City, IA), ki67 (1:100, Dako, Carpinteria, CA), phospho-histone-H3 (1:500 Upstate Cell Signaling

Solutions, Lake Placid, NY) and CRH (1:1000, Millipore, Billerica, MA, USA). Slides were then incubated with biotin-conjugated rat (ki67), mouse (Pax7) or rabbit (POMC, Sox2, phospho-histone-H3) secondary antibody diluted in immunohistochemical block for one half hour at room temperature. This was followed by a series of washes and a thirty-minute incubation with a streptavidin-conjugated cy3 or dylight488 fluorophore. Secondary and streptavidin-conjugated antibodies were purchased from Jackson ImmunoResearch (West Grove, PA) and were used at a concentration of 1:200. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Sigma, St. Louis, MO) and visualized at 100x, 200x, or 400x magnification using a Leica DM2560 microscope. Photographs were taken using a Retiga 2000R color camera (Q-Imaging, Surrey, Canada) and acquired using Q-Capture Pro software (Q-Imaging). Images were processed using Adobe Photoshop CS4 (San José, CA).

Quantitative Reverse Transcriptase PCR

RNA was isolated from embryonic and postnatal whole pituitaries using an RNAqueous micro kit (Ambion, Austin, TX) as per manufacturers protocol. For p32 pituitaries, 0.5μg of RNA was synthesized into cDNA using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). For e16.5 and p1 pituitaries, RNA was isolated from individual pituitaries of each genotype and the total RNA from each pituitary was converted into cDNA. A no enzyme control was also prepared and used as a negative control. For quantitative RT-PCR 0.2μL of cDNA from p32 and p1 pituitaries and 0.5μL from e16.5 pituitaries was amplified using gene-specific primers and SYBR green mix

(Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad MyIQ real-time PCR machine. Data were analyzed with the change in cycle threshold (ΔCt) value method. Specifically, for each sample, the mean Ct of the gene of interest was calculated as a average of the duplicates of that sample. This normalized mean was subtracted from the mean Ct for *Gapdh* to obtain the ΔCt. The ΔΔCt was then calculated as the difference between the ΔCt for each Tg and each control mouse. The relative fold change of Tg mice as compared to control mice was calculated as $2^{-\Delta\Delta Ct}$. The error bars represent the standard error of the mean of the relative fold change. Statistical significance was determined using Student's *t* test.

Gene specific primers for real time RT-PCR include: *Gapdh* forward, GGT GAG GCC GGT GCT GAG TAT G; *Gapdh* reverse, GAC CCG TTT GGC TCC ACC CTT C; *Hes1* forward, CTC GCT CAC TTC GGA CTC; *Hes1* reverse, GTG GGC TAG GGA CTT TAC; *Hey1* forward, CAC GCC ACT ATG CTC AAT; *Hey1* reverse, CCT TCA CCT CAC TGC TCT G; *Hey2* forward, GAT TCC GAG AGT GCT TGA C; *Hey2* reverse, AGG TGC TGA GAT GAG AGA C; *Heyl* forward, GGA ACA ACA GAG AAT GAA C; *Heyl* reverse, CAG CAG TAG TGA GTA ACC; *Pomc* forward, GTT ACG GTG GCT TCA TGA CCT C; *Pomc* reverse, CGC GTT CTT GAT GAT GGC GTT C; *Tpit* forward, GATGCC AAG GAG AGA AAC C; *Tpit* reverse, AGC TTT TCT ATC AAA TTC ACT GA; *Neurod1* forward, GCCCAGCTTAATGCCATCTTTC; *Neurod1* reverese, AGC CAC AGT GGA TTC GTT TCC C; *Mash1* forward, TGG ACT TTG GAA GCA GGA TGG; *Mash1* reverse, TGA CGT CGT TGG CGA GAA ACA; *Myc* forward, TGA CCT AAC

TCG AGG AGG AGC TGG AAT C; *Myc* reverse, AAG TTT GAG GCA GTT AAA ATT ATG GCT GAA GC.

Reverse Transcriptase PCR

RNA and cDNA form p5, p7 and p9 pituitaries were prepared in the same way as described above with the exception that 0.33μL of cDNA was amplified. Amplification was performed using *Pax7* specific primers on a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Amplification was visualized using an ethidium bromide stained gel. Sequences of primers used: *Pax7* forward, GCA CAG AGG ACC AAG CTC AC; *Pax7* Reverse TGG TGG TGG GGT AGG TAG AG.

Corticosterone Assay and Adrenal Collection

p32 mice were subjected to one hour of restraint stress in a ventilated 50mL conical tube beginning at 1500h. At 1600h, mice were sacrificed using CO_2. Blood was collected by cardiac puncture and allowed to clot for 30 minutes in EDTA-coated tubes. Blood was spun down and plasma was collected and stored at -20°C until needed. A corticosterone Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI) was used to measure plasma corticosterone levels as per manufacturers protocol. All samples were run in duplicate and statistical significance was determined using Student's *t* test. Adrenals were dissected from p32 male and female mice. They were fixed overnight in 3.7% formaldehyde diluted in PBS, washed in PBS and photographed.

RESULTS

NICD expression in melanotropes and corticotropes activates Notch target genes

To determine the effect of aberrant Notch activity during corticotrope and melanotrope development, we generated a mouse model with persistent expression of the activated Notch1 intracellular domain (NICD) in POMC-expressing cells. This mouse was generated from the mating of a *Rosa*^{Notch} floxed mouse (Murtaugh, et al. 2003) to a mouse expressing Cre recombinase under the control of the *Pomc* promoter (Balthasar, et al. 2004). POMC is expressed in corticotropes in the AL and in melanotropes in the IL. Additionally, POMC is produced by hypothalamic neurons in the arcuate nucleus. However, these neurons do not innervate pituitary melanotropes or corticotropes and are formed through different mechanisms than pituitary cells (McNay, et al. 2006)(Oertel, et al. 1982; Léránth, et al. 1983; Mezey, et al. 1984; Kawano, Daikoku. 1987; Goudreau, et al. 1995). In the absence of Cre recombinase, a stop codon flanked by loxP sites prevents the expression of the single-copy NICD construct. However, when Cre recombinase is active, the stop codon is excised allowing for the constitutive expression of NICD in *Pomc*-expressing cells (Figure 2.1A). Although *Notch1* is not normally expressed during pituitary development, studies have shown that the intracellular domains of Notch1 and Notch2 can have similar transcriptional activity (Kraman, McCright. 2005). Furthermore, *Notch1* is expressed in the adult stem cell population (Chen, et al. 2005; Chen, et al. 2006), indicating it may play a role in cell fate choice or maintaining progenitor cells in the adult pituitary. Therefore, it is of interest to

determine the effects of persistent *Notch1* expression in pituitary cell specification during development and in postnatal progenitor maintenance.

Quantitative RT-PCR for *Hes1, Hey1, Hey2* and *Heyl* was performed on *Rosa*$^{Notch/+}$ (control) and *Rosa*$^{Notch/+}$; *Pomc-Cre* (Tg) pituitaries at e16.5 to determine if NICD overexpression results in an increase in any of the canonical Notch effector genes. The age e16.5 was chosen to begin analysis since it is the earliest developmental stage at which POMC expression is present in both corticotropes and melanotropes. Our results show that *Hes1* mRNA levels are unchanged between the two experimental groups. However, *Hey1*, *Hey2*, and *Heyl* are all significantly increased in Tg pituitaries as compared to their control counterparts (Figure 2.1B). These results indicate that overexpression of Notch in pituitary melanotropes and corticotropes is sufficient to produce a modest change in Notch effector mRNA levels.

Persistent expression of activated NICD is sufficient to prevent corticotrope and melanotrope terminal differentiation

Notch signaling is known to regulate the balance between proliferating progenitors and differentiated cells in many endocrine organs. Therefore, aberrant Notch signaling could possibly alter the size or morphology of the developing pituitary. To detect if any such morphological changes are present in Tg mice, control and Tg pituitary sections were stained with hematoxylin and eosin. At e16.5, the morphology and size of the control (Figure 2.2A) and Tg (Figure 2.2B) pituitaries appear similar, with all three lobes present in both experimental groups. The same is true at postnatal day (p) 1, when pituitaries of

control (Figure 2.2E) and Tg (Figure 2.2F) mice are histologically indistinguishable. Since Notch signaling is also implicated in cell differentiation in many tissues, we used immunohistochemistry to examine whether POMC expression is affected by persistently activated Notch expression at e16.5 and p1. At e16.5, control mice have POMC-positive cells in the IL and scattered throughout the AL (Figure 2.2C). At this same age, Tg mice have very few POMC-positive cells in either the AL or IL. (Figure 2.2D), indicating their differentiation is prevented by expression of NICD. This phenotype persists through p1, when almost no cells in the IL or AL of Tg mice are POMC-positive (Figure 2.2H), compared to the control (Figure 2.2G). *Pomc* mRNA levels of control and Tg e16.5 pituitaries were measured using quantitative RT-PCR to determine if this transcript is altered by persistent NICD expression. Similar to immunohistochemical observations, Tg mice have significantly lower levels of *Pomc* mRNA as compared to control littermates (Figure 2.2I). Taken together, these data show that constitutive NICD expression in melanotropes and corticotropes is sufficient to inhibit their differentiation and imply that it is critical that Notch signaling be suppressed in order for corticotrope and melanotrope differentiation to proceed.

Activated Notch inhibits the transcription of factors necessary for POMC expression

To better elucidate the mechanism by which Notch inhibits the differentiation of corticotropes and melanotropes, quantitative RT-PCR was used to examine the mRNA levels of the transcription factors *Tpit*, *Neurod1* and *Mash1*

in control and Tg mice at e16.5. *Tpit* and *Neurod1* have been shown to play a role in the activation of *Pomc* transcription, whereas *Mash1* appears to be restricted to *Pomc* containing cells, although it is not required for *Pomc* transcription (Poulin, et al. 1997; Lamolet, et al. 2001; Liu, et al. 2001; Pulichino, et al. 2003a; Pulichino, et al. 2003b; Lamolet, et al. 2004; McNay, et al. 2006). *Tpit*, *NeuroD1* and *Mash1* all show significantly lower mRNA levels in Tg mice as compared to their control counterparts, with *Tpit* showing the strongest suppression (Figure 2.3). Therefore, Notch signaling components may directly or indirectly affect the expression of factors important for terminal differentiation of corticotropes and melanotropes.

Undifferentiated IL cells remain SOX2-positive progenitors

Given that IL cells fail to differentiate when activated NICD is constitutively expressed, we hypothesized that these cells may retain markers of pituitary progenitors, such as SOX2 (Fauquier, et al. 2008). In control mice, SOX2-positive cells are seen in the IL in a thin layer lining the lumen of the pituitary (Figure 2.4, A, C, E and G). Conversely, in Tg mice, nearly every cell in the IL is positive for SOX2, indicating these cells maintain progenitor cell qualities (Figure 2.4, B, D, F and H). Pax7 is a putative marker of intermediate pituitary progenitors (Hosoyama, et al. 2010) and is expressed throughout the IL in control mice at p1 (Figure 2.4I), however a thin layer of cells lining the pituitary cleft are mostly Pax7-negative (Figure 2.4 K, M, O). Based on localization, they likely constitute SOX2-positive cells. In Tg mice, Pax7 is also restricted to the IL (Figure 2.4J). However, nearly every cell in the IL, including those lining the cleft,

expresses Pax7 (Figure 2.4, L, N, P). Taken together, these findings indicate that constitutive NICD expression results in an increased progenitor population in the IL.

Activated Notch leads to elimination of undifferentiated melanotropes and corticotropes

Morphology and size of adolescent (p32) control and Tg pituitaries were examined using hematoxylin and eosin in order to determine if persistent Notch expression affects pituitary morphology at later ages. In control pituitaries, a prominent IL is observed surrounding the PL (Figure 2.5A). In contrast, the IL of Tg mice is reduced to a single-cell thick layer surrounding the PL (Figure 2.5B). The AL and PL look similar in both experimental groups. POMC expression was analyzed at p32 to determine if the remaining cells in the IL of adolescent Tg mice express terminal differentiation markers. In control mice, nearly every cell in the IL expresses POMC (Figure 2.5C). However, in Tg mice, only a handful of the residual IL cells are POMC-positive (Figure 2.5D). In addition, very few POMC immunoreactive cells are observed in the AL of Tg mice (Figure 2.5F), as compared to control mice (Figure 2.5E). The near complete loss of POMC-expressing cells was confirmed by quantitative RT-PCR for *Pomc*, which shows dramatically lower levels of *Pomc* mRNA in Tg mice than in control littermates at p32 (Figure 2.5G). We also observe a reduction in *Crhr1* mRNA in Tg mice, further indicating there is a loss of both melanotropes and corticotropes (data not shown). To determine if the corticotropes and melanotropes are able to regenerate in older mice, pituitary sections from four-month-old control and Tg

mice were stained for POMC. Similarly, almost no POMC expression was observed in the AL or IL of Tg mice (data not shown). These results suggest that expression of activated Notch results in the elimination of undifferentiated melanotropes and corticotropes.

In order to better elucidate the age at which the elimination of these cells occurs, TUNEL staining was performed at e16.5 and p1. No differences were observed between control and Tg pituitaries at either age (data not shown). This indicates that cell death is not initiated until later postnatal development in Tg mice. In order to determine the developmental window where IL cells are eliminated, *Pax7* RT-PCR was performed on p5, p7, and p9 control and Tg pituitaries (Figure 2.5H). Since previous results suggest *Pax7* is maintained in undifferentiated IL cells in Tg mice (see Figure 2.2J), this approach was an effective method to determine the age at which IL cells are no longer present. At p5, pituitaries from both control and Tg mice express roughly equal levels of *Pax7*, suggesting NICD-containing cells are still present at this age. By p7, *Pax7* levels in Tg mice appear to be suppressed as compared to control mice. In one p7 Tg mouse, *Pax7* levels are visibly reduced as compared to control, whereas another Tg animal shows near complete loss of *Pax7* pituitary mRNA. At p9, all Tg pituitaries examined contain no detectable levels of *Pax7* mRNA, whereas control pituitaries still maintain *Pax7* expression. These findings show that aberrant expression of NICD results in elimination of NICD-containing cells within the first two postnatal weeks.

Persistently activated NICD expression in POMC-expressing cells results in HPA axis dysfunction

The effects of NICD expression in corticotropes may be reflected in alterations throughout the HPA axis. Development and function of the adrenal cortex relies heavily on the release of ACTH from the pituitary (Estivariz, et al. 1982; Simpson, Waterman. 1988). Therefore, adrenal glands were compared between control and Tg adolescent mice at p32. Control mice have visibly larger adrenal glands than Tg counterparts in both sexes (Figure 2.5I). To determine the functional capabilities of the hypoplastic adrenals in Tg mice, stress-induced corticosterone levels were measured. Significant decreases in levels of corticosterone are observed in both male and female Tg mice as compared to control counterparts (Figure 2.5J).

Under normal conditions, both corticosterone released from the adrenal gland and ACTH released from the pituitary can influence CRH release at the level of the hypothalamus (Swanson, Simmons. 1989). Given that both corticosterone levels and ACTH-positive cells are decreased in Tg mice, we would expect that CRH levels in the hypothalamus would be increased as a result of positive feedback. To determine differences in CRH expression between Tg and control male mice at p32, immunohistochemical expression of CRH was examined in the PVN. Tg mice display a slight increase in CRH immunoreactivity in the medial parvocellular portion of the PVN (PaMP; Figure 2.6A), a region known to be highly responsive to stress (Renard, et al. 2010). As expected, without colchicine pretreatment to inhibit axonal transport of peptides, CRH-

positive cell bodies in control animals are hardly detectable, with some immunopositive processes present. In contrast, Tg animals display immunoreactive CRH-positive cell bodies as well as neuronal processes, indicating an increased level of CRH in this region (Figure 2.6B). These data are consistent with CRH localization in *Pomc* knockout mice (Yaswen, et al. 1999; Smart, et al. 2007) and, taken together with the decreased levels of corticosterone, show that Notch-induced loss of POMC expression in the pituitary leads to subsequent dysfunction of the HPA axis.

Activated NICD in melanotropes and corticotropes is not sufficient to promote their proliferation

Because Notch is known to promote proliferation in many cell types (Solecki, et al. 2001; Collesi, et al. 2008; Zhang, et al. 2008), we hypothesized it may have a similar role in corticotropes and melanotropes. We, therefore, examined the expression of proliferation markers in p1 control and Tg pituitaries. Ki67, a marker of all stages of the cell cycle, is observed throughout the AL, IL and PL in control mice (Figure 2.7A). No difference in expression pattern was observed in Tg mice (Figure 2.7B), indicating overall proliferation in *Pomc*-expressing cells is unaffected by the constitutive expression of NICD. Phospho-histone-H3 staining was also performed in order to determine if mitosis is altered in cells persistently expressing NICD. Again, no visible changes in expression were observed between control (Figure 2.7C) and Tg (Figure 2.7D) pituitaries at p1. Histological observations were confirmed by quantitative RT-PCR analysis of control and Tg p1 pituitaries, which showed no difference in *Myc* mRNA levels

between the two experimental groups (Figure 2.7E). These results indicate that activated NICD is insufficient to promote proliferation in undifferentiated *Pomc*-expressing cells.

DISCUSSION

Disruption in normal corticotrope development or function can result in various disorders, including isolated ACTH deficiency and Cushing's syndrome (Drouin, et al. 2007). Signals controlling the commitment and specification of corticotropes are not well understood, but the Notch signaling pathway has been implicated in these processes. We have previously demonstrated that global loss of *Hes1* and *Prop1*, two Notch effector genes, results in premature differentiation of corticotropes, indicating this pathway is necessary to prevent early differentiation of these cells (Himes, Raetzman. 2009). Given this finding, we hypothesized that Notch signaling is also sufficient to inhibit the differentiation of both corticotropes and closely related melanotropes. Using a mouse model in which activated Notch is persistently expressed in these two cell types, we were able to confirm this hypothesis.

As in many developing tissues, Notch signaling components are found in RP progenitors and in the adult progenitor population but are excluded from differentiated cells (Raetzman, et al. 2004; Zhu, et al. 2006). The presumptive pituitary progenitor population also contains the transcription factor SOX2, which is thought to be important in maintaining pluripotency and inhibiting cell differentiation (Episkopou. 2005; Masui, et al. 2007). SOX2 is expressed in

isolated adult murine pituitary progenitors and is mainly localized to a single cell layer lining either side of the pituitary cleft in the adult (Rizzoti. 2010). In cochlear development, suppression of Notch signaling results in a decrease in the expression of SOX2 and in increase in differentiated cells (Dabdoub, et al. 2008). It is possible that SOX2 is a direct target of the Notch signaling cascade because luciferase reporter assays have shown that NICD has the ability to activate transcription of the SOX2 promoter (Ehm, et al. 2010). It is, therefore, not surprising that we observe an increase in SOX2 immunoreactivity in pituitary cells that contain activated Notch. This increase in SOX2 may correlate with an increase in multipotent pituitary progenitor cells, and indicates that Notch signaling acts in a similar fashion in the pituitary as it does in other developing systems.

We observed that inappropriately specified cells in the pituitary expressing NICD do not reenter the cell cycle and are eliminated during the first two postnatal weeks. Persistent expression of activated Notch has been shown to induce apoptosis in multiple cell types, including intestinal and neural progenitors (Yang, et al. 2004; Fre, et al. 2005). Although we cannot rule out a role for activated Notch in inducing apoptosis, it is likely that these cells are eliminated because they are undifferentiated. Ames dwarf mice, which lack functional *Prop1,* have extensive apoptosis in aberrantly undifferentiated cells in the pituitary at p1 and p8 (Ward, et al. 2005). This data supports the hypothesis that undifferentiated pituitary cells may be more prone to cell death, particularly

during periods of significant postnatal proliferation (Carbajo-Pérez, Watanabe. 1990; Taniguchi, et al. 2002a; Taniguchi, et al. 2002b).

While Notch signaling in early development is known to influence the balance between proliferating progenitors and differentiated cells, it also directs cell fate determination at later stages of development in many systems. For example, Notch promotes cerebellar granule neuron precursor proliferation (Solecki, et al. 2001), but also promotes radial glia differentiation and inhibits granule neuron differentiation at later stages of development (Patten, et al. 2003). A similar dual role of Notch is seen in the intestine where Notch signaling inhibits stem cell differentiation during embryogenesis but biases cells toward an enterocyte fate and away from a secretory fate during adulthood (Stanger, et al. 2005). We found that that NICD is not sufficient to convert melanotropes and corticotropes to an alternate cell fate but instead traps them in a progenitor fate. This is especially clear in the IL where NICD-containing cells do not adopt another fate, but rather retain progenitor-like properties. However, since we are altering Notch signaling only after cells already committed to become melanotropes and corticotropes, it is possible that Notch influences cell fate at a different developmental stage as it does in other tissues discussed above.

Through both gain and loss of function studies, it is clear that Notch can influence terminal differentiation of *Pomc* containing cells (Zhu, et al. 2006; Dutta, et al. 2008; Himes, Raetzman. 2009). Molecules required for *Pomc* transcription, and therefore for the terminal differentiation of corticotropes and melanotropes, have been well characterized. These factors include TPIT (Liu, et al. 2001;

Pulichino, et al. 2003b) and NEUROD1 (Poulin, et al. 1997; Lamolet, et al. 2004). In contrast, BMPs have been shown to inhibit the expression of *Pomc in vitro* (Nudi, et al. 2005). While these factors are known to regulate *Pomc* transcription and thus the terminal differentiation of corticotropes and melanotropes, it remains unclear which signals are involved in the specification of these cells. Lineage specification is likely controlled in part by extrinsic signals, such as morphogens expressed in the oral ectoderm and ventral diencephalon surrounding the developing pituitary. However, cells located in the same region during development do not necessarily develop into the same cell type (Davis, et al. 2011), suggesting intrinsic properties are also likely involved. We hypothesize that Notch signaling may be one such intrinsic factor. We show that persistently expressing NICD in corticotropes and melanotropes results in suppression of *Tpit* and *Neurod1*, indicating their expression may be under the control of the Notch signaling cascade. It is also possible that the suppression of these transcription factors is a result of the progenitor-like nature of the cells, which are ectopically expressing SOX2, and is not a result of their direct regulation by NICD. Future studies will be necessary in order to elucidate if and how Notch signaling directly regulates these molecules. Taken together with the fact that *Prop1*, a direct transcriptional target of Notch signaling, is required for PIT1 expression and the subsequent differentiation of lactotropes, somatotropes, and thyrotropes (Gage, et al. 1996a; Gage, et al. 1996b; Zhu, et al. 2006), we propose a model by which the Notch signaling pathway regulates the choice between precursors of the corticotrope and PIT1 lineages during early pituitary cell specification (Figure 2.8)

This study is the first to show that Notch signaling is sufficient to inhibit melanotrope and corticotrope differentiation, resulting in loss of these cells and subsequent HPA axis dysfunction. This mouse model physiologically resembles a *Pomc* knockout mouse (Yaswen, et al. 1999) and highlights the importance of Notch signaling in the regulation of corticotrope ontogeny during HPA axis development. Our study indicates that subtle alterations in Notch signaling within POMC lineage cells during early embryonic development can lead to disruption of the HPA axis at the level of the hypothalamus, as well as the adrenal gland. Disruption of the HPA axis during development has significant physiological consequences on the body's response to stress, and can result in multiple disorders, including Cushing's syndrome and adrenal insufficiency (Drouin, et al. 2007). Our findings are not only important to the study of normal pituitary development, but also demonstrate how alterations in signaling within corticotropes can contribute to dysfunction along the entire HPA axis.

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REFERENCES

Balthasar, N., Coppari, R., McMinn, J., Liu, S.M., Lee, C.E., Tang, V., Kenny, C.D., McGovern, R.A., Chua, S.C., Elmquist, J.K., Lowell, B.B., 2004. Leptin Receptor Signaling in POMC Neurons is Required for Normal Body Weight Homeostasis. Neuron 6, 983-91.

Burrows, H.L., Douglas, K.R., Seasholtz, A.F., Camper, S.A., 1999. Genealogy of the Anterior Pituitary Gland: Tracing a Family Tree. Trends in endocrinology and metabolism: TEM 9, 343-352.

Carbajo-Pérez, E., Watanabe, Y.G., 1990. Cellular Proliferation in the Anterior Pituitary of the Rat during the Postnatal Period. Cell and tissue research 2, 333-8.

Chen, J., Crabbe, A., Van Duppen, V., Vankelecom, H., 2006. The Notch Signaling System is Present in the Postnatal Pituitary: Marked Expression and Regulatory Activity in the Newly Discovered Side Population. Molecular endocrinology (Baltimore, Md.) 12, 3293-307.

Chen, J., Hersmus, N., Van Duppen, V., Caesens, P., Denef, C., Vankelecom, H., 2005. The Adult Pituitary Contains a Cell Population Displaying stem/progenitor Cell and Early Embryonic Characteristics. Endocrinology 9, 3985-98.

Collesi, C., Zentilin, L., Sinagra, G., Giacca, M., 2008. Notch1 Signaling Stimulates Proliferation of Immature Cardiomyocytes. The Journal of cell biology 1, 117-28.

Dabdoub, A., Puligilla, C., Jones, J.M., Fritzsch, B., Cheah, K.S.E., Pevny, L.H., Kelley, M.W., 2008. Sox2 Signaling in Prosensory Domain Specification and Subsequent Hair Cell Differentiation in the Developing Cochlea. Proceedings of the National Academy of Sciences of the United States of America 47, 18396-401.

Dasen, J.S., Rosenfeld, M.G., 2001. Signaling and Transcriptional Mechanisms in Pituitary Development. Annual review of neuroscience 327-55.

Davis, S.W., Mortensen, A.H., Camper, S.A., 2011. Birthdating Studies Reshape Models for Pituitary Gland Cell Specification. Developmental biology .

Drouin, J., Bilodeau, S., Vallette, S., 2007. Of Old and New Diseases: Genetics of Pituitary ACTH Excess (Cushing) and Deficiency. Clinical genetics 3, 175-82.

Dutta, S., Dietrich, J., Westerfield, M., Varga, Z.M., 2008. Notch Signaling Regulates Endocrine Cell Specification in the Zebrafish Anterior Pituitary. Developmental biology 2, 248-57.

Ehm, O., Göritz, C., Covic, M., Schäffner, I., Schwarz, T.J., Karaca, E., Kempkes, B., Kremmer, E., Pfrieger, F.W., Espinosa, L., Bigas, A., Giachino, C., Taylor, V., Frisén, J., Lie, D.C., 2010. RBPJkappa-Dependent Signaling is Essential for Long-Term Maintenance of Neural Stem Cells in the Adult Hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 41, 13794-807.

Episkopou, V., 2005. SOX2 Functions in Adult Neural Stem Cells. Trends in neurosciences 5, 219-21.

Ericson, J., Norlin, S., Jessell, T.M., Edlund, T., 1998. Integrated FGF and BMP Signaling Controls the Progression of Progenitor Cell Differentiation and the Emergence of Pattern in the Embryonic Anterior Pituitary. Development (Cambridge, England) 6, 1005-15.

Estivariz, F.E., Iturriza, F., McLean, C., Hope, J., Lowry, P.J., 1982. Stimulation of Adrenal Mitogenesis by N-Terminal Proopiocortin Peptides. Nature 5865, 419-22.

Fauquier, T., Rizzoti, K., Dattani, M., Lovell-Badge, R., Robinson, I.C.A.F., 2008. SOX2-Expressing Progenitor Cells Generate all of the Major Cell Types in the Adult Mouse Pituitary Gland. Proceedings of the National Academy of Sciences of the United States of America 8, 2907-12.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., Artavanis-Tsakonas, S., 2005. Notch Signals Control the Fate of Immature Progenitor Cells in the Intestine. Nature 7044, 964-8.

Gage, P.J., Brinkmeier, M.L., Scarlett, L.M., Knapp, L.T., Camper, S.A., Mahon, K.A., 1996a. The Ames Dwarf Gene, Df, is Required Early in Pituitary Ontogeny for the Extinction of Rpx Transcription and Initiation of Lineage-Specific Cell Proliferation. Molecular endocrinology (Baltimore, Md.) 12, 1570-81.

Gage, P.J., Roller, M.L., Saunders, T.L., Scarlett, L.M., Camper, S.A., 1996b. Anterior Pituitary Cells Defective in the Cell-Autonomous Factor, Df, Undergo Cell Lineage Specification but Not Expansion. Development (Cambridge, England) 1, 151-60.

Garren, L.D., 1968. The Mechanism of Action of Adrenocorticotropic Hormone. Vitamins and hormones 119-45.

Goudreau, J.L., Falls, W.M., Lookingland, K.J., Moore, K.E., 1995. Periventricular-Hypophysial Dopaminergic Neurons Innervate the Intermediate but Not the Neural Lobe of the Rat Pituitary Gland. Neuroendocrinology 2, 147-54.

Himes, A.D., Raetzman, L.T., 2009. Premature Differentiation and Aberrant Movement of Pituitary Cells Lacking both Hes1 and Prop1. Developmental biology 1, 151-61.

Hosoyama, T., Nishijo, K., Garcia, M.M., Schaffer, B.S., Ohshima-Hosoyama, S., Prajapati, S.I., Davis, M.D., Grant, W.F., Scheithauer, B.W., Marks, D.L., Rubin, B.P., Keller, C., 2010. A Postnatal Pax7 Progenitor Gives Rise to Pituitary Adenomas. Genes & cancer 4, 388-402.

Japón, M.A., Rubinstein, M., Low, M.J., 1994. In Situ Hybridization Analysis of Anterior Pituitary Hormone Gene Expression during Fetal Mouse Development. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 8, 1117-25.

Kawano, H., Daikoku, S., 1987. Functional Topography of the Rat Hypothalamic Dopamine Neuron Systems: Retrograde Tracing and Immunohistochemical Study. The Journal of comparative neurology 2, 242-53.

Kraman, M., McCright, B., 2005. Functional Conservation of Notch1 and Notch2 Intracellular Domains. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 10, 1311-3.

Lamolet, B., Pulichino, A.M., Lamonerie, T., Gauthier, Y., Brue, T., Enjalbert, A., Drouin, J., 2001. A Pituitary Cell-Restricted T Box Factor, Tpit, Activates POMC Transcription in Cooperation with Pitx Homeoproteins. Cell 6, 849-59.

Lamolet, B., Poulin, G., Chu, K., Guillemot, F., Tsai, M., Drouin, J., 2004. Tpit-Independent Function of NeuroD1(BETA2) in Pituitary Corticotroph Differentiation. Molecular endocrinology (Baltimore, Md.) 4, 995-1003.

Léránth, C., Palkovits, M., Krieger, D.T., 1983. Serotonin Immunoreactive Nerve Fibers and Terminals in the Rat Pituitary--Light- and Electron-Microscopic Studies. Neuroscience 2, 289-96.

Liu, J., Lin, C., Gleiberman, A., Ohgi, K.A., Herman, T., Huang, H.P., Tsai, M.J., Rosenfeld, M.G., 2001. Tbx19, a Tissue-Selective Regulator of POMC Gene Expression. Proceedings of the National Academy of Sciences of the United States of America 15, 8674-9.

Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A.A., Ko, M.S.H., Niwa, H., 2007. Pluripotency Governed by Sox2 Via Regulation of Oct3/4 Expression in Mouse Embryonic Stem Cells. Nature cell biology 6, 625-35.

McNay, D.E.G., Pelling, M., Claxton, S., Guillemot, F., Ang, S., 2006. Mash1 is Required for Generic and Subtype Differentiation of Hypothalamic Neuroendocrine Cells. Molecular endocrinology (Baltimore, Md.) 7, 1623-32.

Mezey, E., Léránth, C., Brownstein, M.J., Friedman, E., Krieger, D.T., Palkovits, M., 1984. On the Origin of the Serotonergic Input to the Intermediate Lobe of the Rat Pituitary. Brain research 2, 231-7.

Munck, A., Guyre, P.M., Holbrook, N.J., 1984. Physiological Functions of Glucocorticoids in Stress and their Relation to Pharmacological Actions. Endocr. Rev. 1, 25-44.

Murtaugh, L.C., Stanger, B.Z., Kwan, K.M., Melton, D.A., 2003. Notch Signaling Controls Multiple Steps of Pancreatic Differentiation. Proceedings of the National Academy of Sciences of the United States of America 25, 14920-5.

Nudi, M., Ouimette, J., Drouin, J., 2005. Bone Morphogenic Protein (Smad)-Mediated Repression of Proopiomelanocortin Transcription by Interference with Pitx/Tpit Activity. Molecular endocrinology (Baltimore, Md.) 5, 1329-42.

Oertel, W.H., Mugnaini, E., Tappaz, M.L., Weise, V.K., Dahl, A.L., Schmechel, D.E., Kopin, I.J., 1982. Central GABAergic Innervation of Neurointermediate Pituitary Lobe: Biochemical and Immunocytochemical Study in the Rat. Proceedings of the National Academy of Sciences of the United States of America 2, 675-9.

Patten, B.A., Peyrin, J.M., Weinmaster, G., Corfas, G., 2003. Sequential Signaling through Notch1 and erbB Receptors Mediates Radial Glia Differentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 14, 6132-40.

Philips, A., Lesage, S., Gingras, R., Maira, M.H., Gauthier, Y., Hugo, P., Drouin, J., 1997a. Novel Dimeric Nur77 Signaling Mechanism in Endocrine and Lymphoid Cells. Molecular and cellular biology 10, 5946-51.

Philips, A., Maira, M., Mullick, A., Chamberland, M., Lesage, S., Hugo, P., Drouin, J., 1997b. Antagonism between Nur77 and Glucocorticoid Receptor for Control of Transcription. Molecular and cellular biology 10, 5952-9.

Pope, C., McNeilly, J.R., Coutts, S., Millar, M., Anderson, R.A., McNeilly, A.S., 2006. Gonadotrope and Thyrotrope Development in the Human and Mouse Anterior Pituitary Gland. Developmental biology 1, 172-81.

Poulin, G., Turgeon, B., Drouin, J., 1997. NeuroD1/beta2 Contributes to Cell-Specific Transcription of the Proopiomelanocortin Gene. Molecular and cellular biology 11, 6673-82.

Pulichino, A., Vallette-Kasic, S., Couture, C., Gauthier, Y., Brue, T., David, M., Malpuech, G., Deal, C., Van Vliet, G., De Vroede, M., Riepe, F.G., Partsch, C., Sippell, W.G., Berberoglu, M., Atasay, B., Drouin, J., 2003a. Human and Mouse TPIT Gene Mutations Cause Early Onset Pituitary ACTH Deficiency. Genes & development 6, 711-6.

Pulichino, A., Vallette-Kasic, S., Tsai, J.P., Couture, C., Gauthier, Y., Drouin, J., 2003b. Tpit Determines Alternate Fates during Pituitary Cell Differentiation. Genes & development 6, 738-47.

Raetzman, L.T., Ross, S.A., Cook, S., Dunwoodie, S.L., Camper, S.A., Thomas, P.Q., 2004. Developmental Regulation of Notch Signaling Genes in the Embryonic Pituitary: Prop1 Deficiency Affects Notch2 Expression. Developmental biology 2, 329-40.

Renard, G.M., Rivarola, M.A., Suarez, M.M., 2010. Gender-Dependent Effects of Early Maternal Separation and Variable Chronic Stress on Vasopressinergic Activity and Glucocorticoid Receptor Expression in Adult Rats. Dev. Neurosci. 1, 71-80.

Rizzoti, K., 2010. Adult Pituitary progenitors/stem Cells: From in Vitro Characterization to in Vivo Function. The European journal of neuroscience 12, 2053-62.

Rizzoti, K., Lovell-Badge, R., 2005. Early Development of the Pituitary Gland: Induction and Shaping of Rathke's Pouch. Reviews in endocrine & metabolic disorders 3, 161-72.

Simpson, E.R., Waterman, M.R., 1988. Regulation of the Synthesis of Steroidogenic Enzymes in Adrenal Cortical Cells by ACTH. Annual review of physiology 427-40.

Smart, J.L., Tolle, V., Otero-Corchon, V., Low, M.J., 2007. Central Dysregulation of the Hypothalamic-Pituitary-Adrenal Axis in Neuron-Specific Proopiomelanocortin-Deficient Mice. Endocrinology 2, 647-59.

Solecki, D.J., Liu, X.L., Tomoda, T., Fang, Y., Hatten, M.E., 2001. Activated Notch2 Signaling Inhibits Differentiation of Cerebellar Granule Neuron Precursors by Maintaining Proliferation. Neuron 4, 557-68.

Stanger, B.Z., Datar, R., Murtaugh, L.C., Melton, D.A., 2005. Direct Regulation of Intestinal Fate by Notch. Proceedings of the National Academy of Sciences of the United States of America 35, 12443-8.

Swanson, L.W., Simmons, D.M., 1989. Differential Steroid Hormone and Neural Influences on Peptide mRNA Levels in CRH Cells of the Paraventricular Nucleus: A Hybridization Histochemical Study in the Rat. The Journal of comparative neurology 4, 413-35.

Taniguchi, Y., Yasutaka, S., Kominami, R., Shinohara, H., 2002a. Mitoses of Thyrotrophs Contribute to the Proliferation of the Rat Pituitary Gland during the Early Postnatal Period. Anatomy and embryology 1-2, 67-72.

Taniguchi, Y., Yasutaka, S., Kominami, R., Shinohara, H., 2002b. Proliferation and Differentiation of Rat Anterior Pituitary Cells. Anatomy and embryology 1-2, 1-11.

Treier, M., Gleiberman, A.S., O'Connell, S.M., Szeto, D.P., McMahon, J.A., McMahon, A.P., Rosenfeld, M.G., 1998. Multistep Signaling Requirements for Pituitary Organogenesis in Vivo. Genes & development 11, 1691-704.

Vale, W., Spiess, J., Rivier, C., Rivier, J., 1981. Characterization of a 41-Residue Ovine Hypothalamic Peptide that Stimulates Secretion of Corticotropin and Beta-Endorphin. Science (New York, N. Y.) 4514, 1394-7.

Wagner, J., Thomas, P., 2007. Genetic Determinants of Mammalian Pituitary Morphogenesis. Frontiers in bioscience : a journal and virtual library 125-34.

Ward, R.D., Raetzman, L.T., Suh, H., Stone, B.M., Nasonkin, I.O., Camper, S.A., 2005. Role of PROP1 in Pituitary Gland Growth. Molecular endocrinology (Baltimore, Md.) 3, 698-710.

Yang, X., Klein, R., Tian, X., Cheng, H., Kopan, R., Shen, J., 2004. Notch Activation Induces Apoptosis in Neural Progenitor Cells through a p53-Dependent Pathway. Developmental biology 1, 81-94.

Yaswen, L., Diehl, N., Brennan, M.B., Hochgeschwender, U., 1999. Obesity in the Mouse Model of Pro-Opiomelanocortin Deficiency Responds to Peripheral Melanocortin. Nature medicine 9, 1066-70.

Zhang, X., Zheng, G., Zou, L., Liu, H., Hou, L., Zhou, P., Yin, D., Zheng, Q., Liang, L., Zhang, S., Feng, L., Yao, L., Yang, A., Han, H., Chen, J., 2008. Notch Activation Promotes Cell Proliferation and the Formation of Neural Stem Cell-Like Colonies in Human Glioma Cells. Molecular and cellular biochemistry 1-2, 101-8.

Zhu, X., Zhang, J., Tollkuhn, J., Ohsawa, R., Bresnick, E.H., Guillemot, F., Kageyama, R., Rosenfeld, M.G., 2006a. Sustained Notch Signaling in Progenitors is Required for Sequential Emergence of Distinct Cell Lineages during Organogenesis. Genes & development 19, 2739-53.

FIGURES

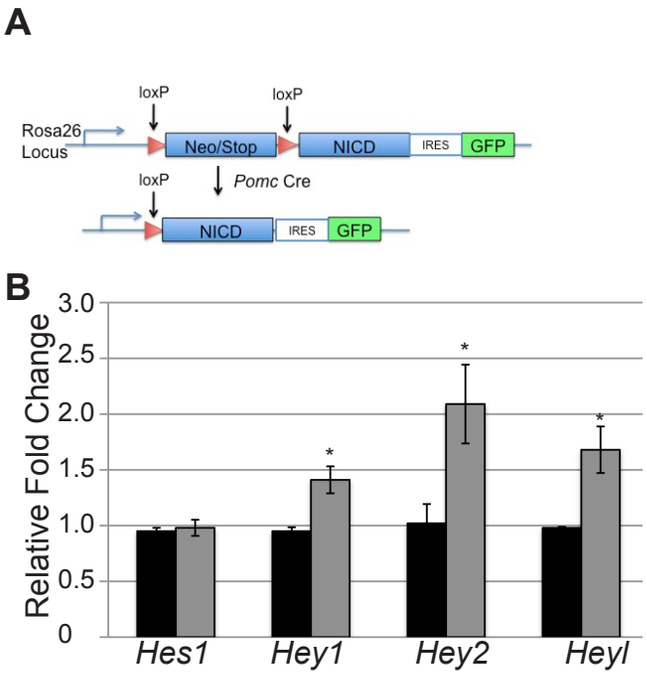

Figure 2.1. Persistent expression of NICD results in increases in mRNA levels of Notch targets. (A) Construct and mating scheme for activated NICD expression in *Pomc*-expressing cells. (B) Activated NICD results in modest increases in canonical target mRNA levels in Tg (gray bars) pituitaries as compared to control (black bars) at e16.5. n=3-5. *:p<0.05.

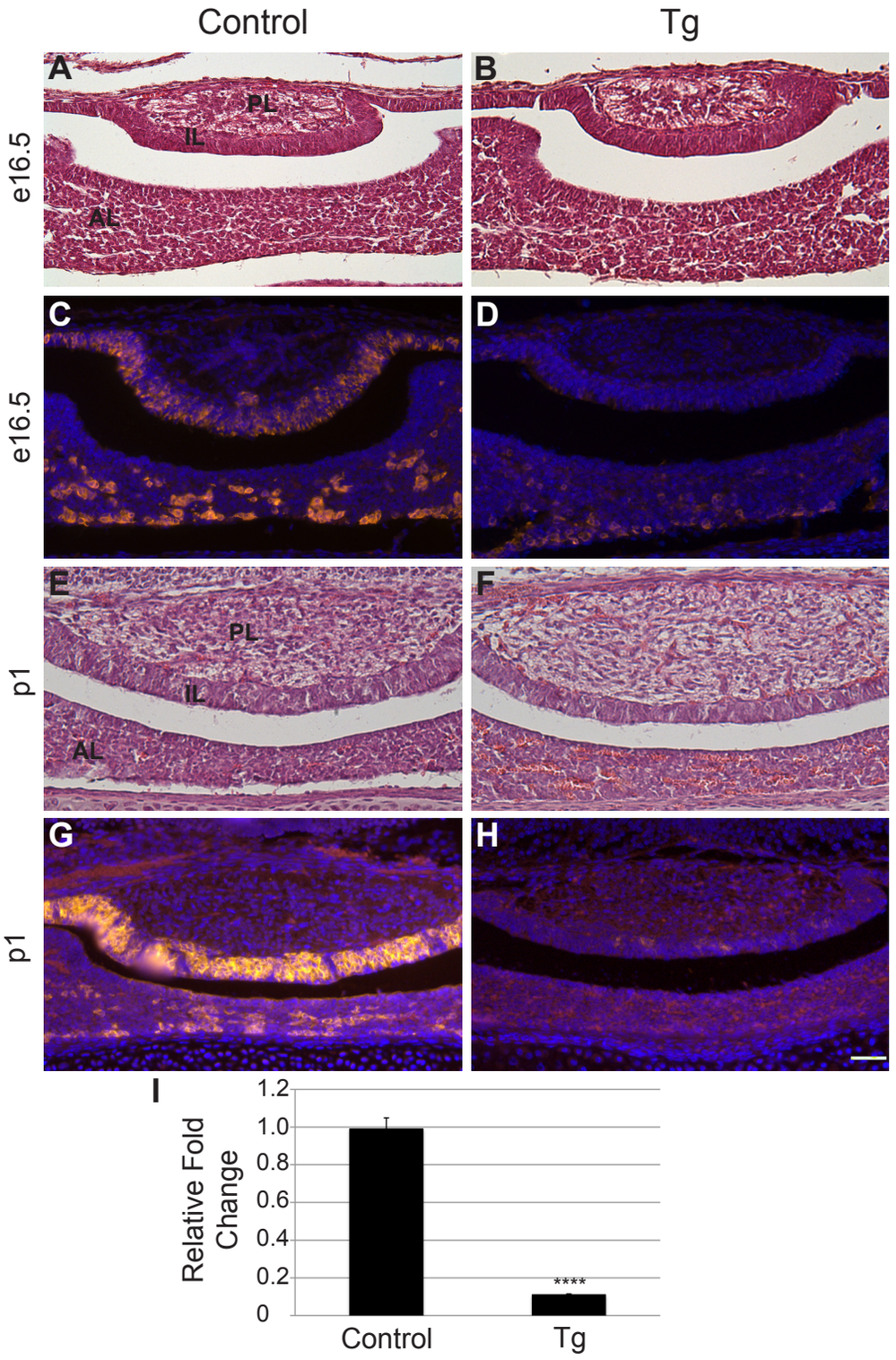

Figure 2.2. Persistent NICD expression inhibits the differentiation of melanotropes and corticotropes. Coronal sections of control (A) and Tg (B) e16.5 pituitaries were stained with hematoxylin and eosin. No differences in morphology were observed between the two groups. POMC immunoreactive cells (red) are found in the IL and AL of control (C) pituitaries at e16.5 and are diminished in both lobes of Tg (D) mice. Morphology is similar at p1 in control (E) and Tg (F) pituitaries. POMC expression (red) is observed in the AL and IL of control mice (G) and is markedly decreased in Tg pituitaries (H) at p1. RT-PCR at e16.5 reveals significant decreases in *Pomc* mRNA in Tg mice as compared to controls (I). Photos taken at 200x; Scale bars: 50μm. ****:p<0.0001. n=5-7 (immunohistochemistry) and n=3-5 (qRT-PCR). PL=posterior lobe. AL=anterior lobe. IL=intermediate lobe.

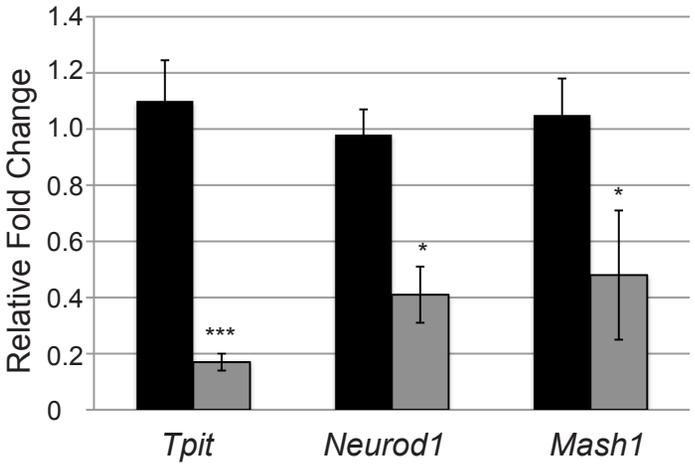

Figure 2.3. Transcription factors regulating *Pomc* expression are decreased in pituitaries of NICD expressing mice at p1. *Tpit*, *Neurod1* and *Mash1* mRNA levels are significantly reduced in Tg mice (gray bars) as compared to control (black bars) littermates at e16.5 determined by qRT-PCR. * $p<0.05$. ***: $p<0.001$. n=3-5.

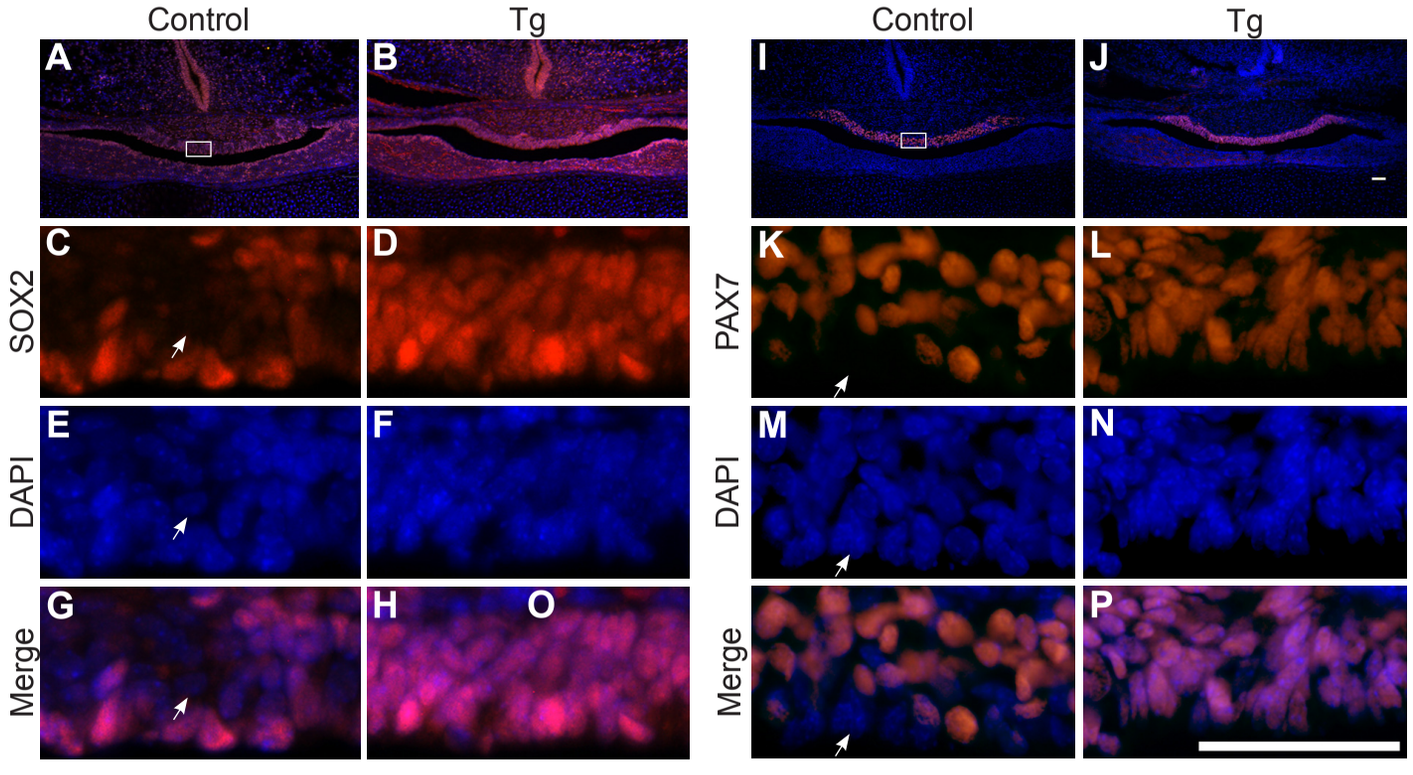

Figure 2.4. IL cells containing NICD express markers of pituitary progenitors. SOX2 (red) is expressed at p1 in the pituitary of control (A) and Tg (B) mice. In control animals, SOX2 immunoreactivity (red) is mostly observed lining the cleft of the pituitary (C, G), with nuclei stained with DAPI (blue; E, G). Although there are also scattered SOX2 immunoreactive cells in the IL, many cells are not SOX2 immunoreactive (compare E (nuclei stained with DAPI) and G). In contrast, nearly every cell in the IL of Tg mice is immunoreactive for SOX2 (D, compare F (nuclei stained with DAPI) and H). Expression of Pax7, a marker of intermediate progenitors was also examined. Pax7 immunoreactive cells (red) are observed in the IL of control (I) and Tg mice (J) at p1. Many cells lining the pituitary cleft at p1 do not express Pax7 in control mice (K, O with nuclei stained with DAPI (blue; M,O), arrows show Pax7-negative cell). Nearly every cell in the IL of Tg mice is immunoreactive for Pax7 (L, compare N (nuclei stained with DAPI) and P). Photos taken at 100x (A, B, I and J), 400x (C-H, K-P). White box (A and I) indicates where higher magnification photos were taken. Scale bars (A-P): 50μm. n=4-5.

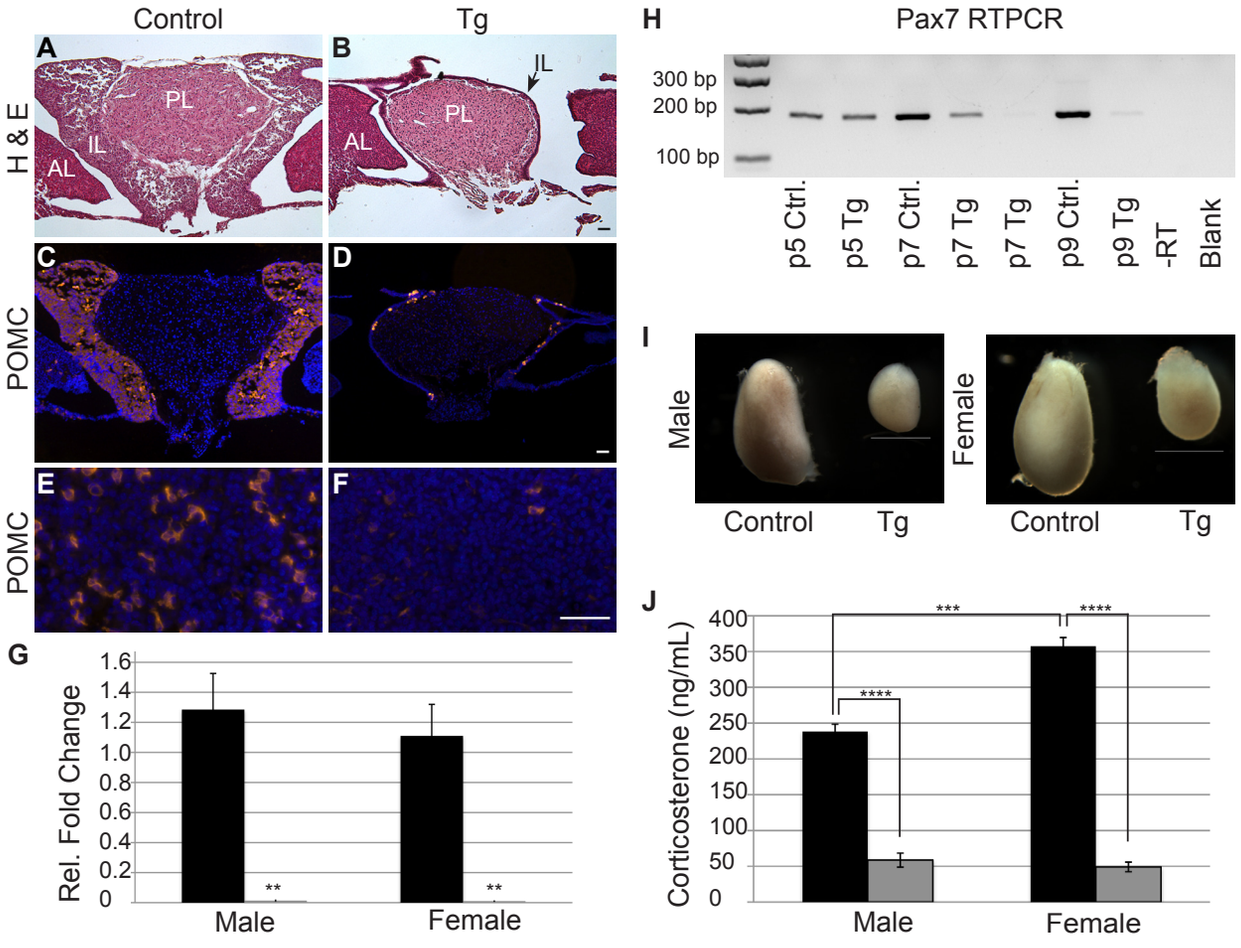

Figure 2.5. Undifferentiated cells in Tg mice are incompatible with postnatal survival, resulting in adrenal dysfunction. Adolescent (p32) pituitaries were stained with hematoxylin and eosin to visualize morphology. Control (A) animals have a prominent IL, whereas Tg (B) animals have a very thin layer of IL cells (arrow) surrounding the PL. Nearly all of the IL lobe cells are immunoreactive for POMC (red) in the control (C) mice. In contrast, very few POMC-positive cells are observed in the IL of Tg mice (D). The same is true in the AL where POMC immunoreactive corticotropes are abundant in control mice (E) and nearly absent in Tg pituitaries (F). Similarly, qRT-PCR for *Pomc* reveals a significant decrease in *Pomc* mRNA in Tg adolescent pituitaries (gray bars) as compared to control littermates (black bars) in both males and females (G). *Pax7* mRNA, which is exclusive to the IL in the pituitary, is present at p5 in both experimental groups and decreases dramatically by p9 in Tg pituitaries, indicating IL cells are no longer present at this age in Tg animals. Adrenals (I) of Tg males and females are reduced in size as compared to control mice. Stress-induced corticosterone levels (J) are significantly decreased in male and female Tg mice (gray bars) when compared to control mice (black bars). Photos taken at 100x (A-D) and 400x (E & F). Scale bars: 50μm (A-F) and 1mm (I). **: p<0.01. ***: p<0.001. ****: p<0.0001. n=3-5 (RT-PCR), n=5 (immunohistochemistry) and n=4 (corticosterone assay). PL=posterior lobe, AL=anterior lobe, IL=intermediate lobe.

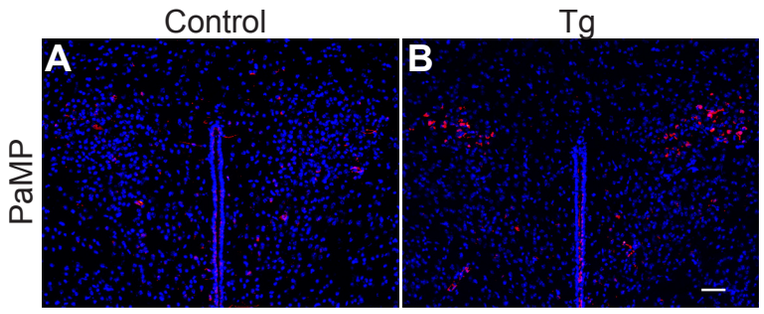

Figure 2.6. Corticotropin releasing hormone (CRH) expression in the paraventricular (PVN) hypothalamus of male mice at p32. CRH immunoreactivity appears lower in control mice (A) compared to Tg mice (B) in the medial parvocellular portion of the PVN (PaMP). Scale bar: 50μm. n=3.

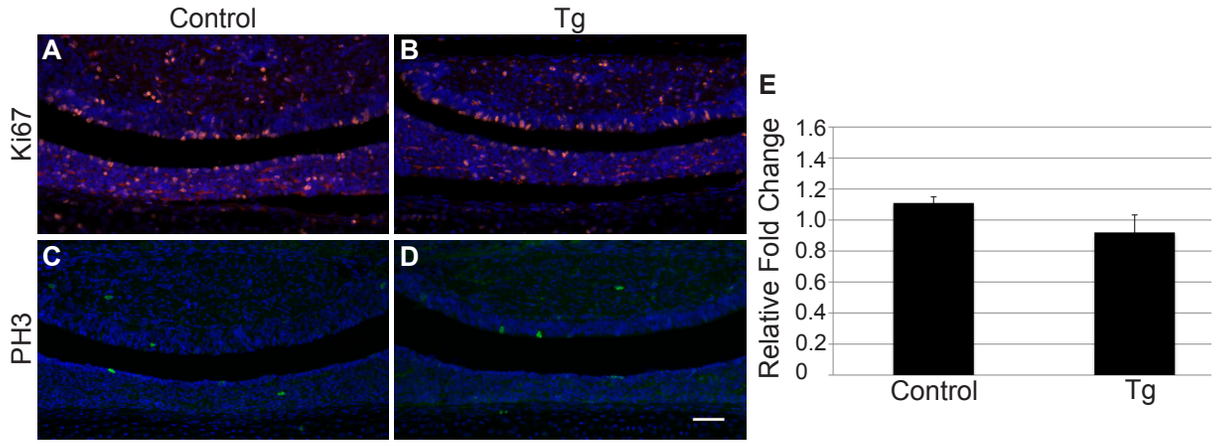

Figure 2.7. Proliferation is unaffected by persistent NICD expression. Ki67 immunohistochemistry was performed on p1 control (A) and Tg pituitaries (B) and no difference in immunoreactivity (red) was observed. Similarly, no difference in Phopho-histone-H3 immunoreactivity (green) was observed between control (C) and Tg (D) pituitaries at p1. Nuclei are visualized with DAPI staining (blue; A-D). Additionally, qRT-PCR showed no change in Myc mRNA levels between control and Tg pituitaries at p1 (E). Photos taken at 200x. Scale bars: 50μm. n=4-5 (immunohistochemistry) and n=4 (RT-PCR).

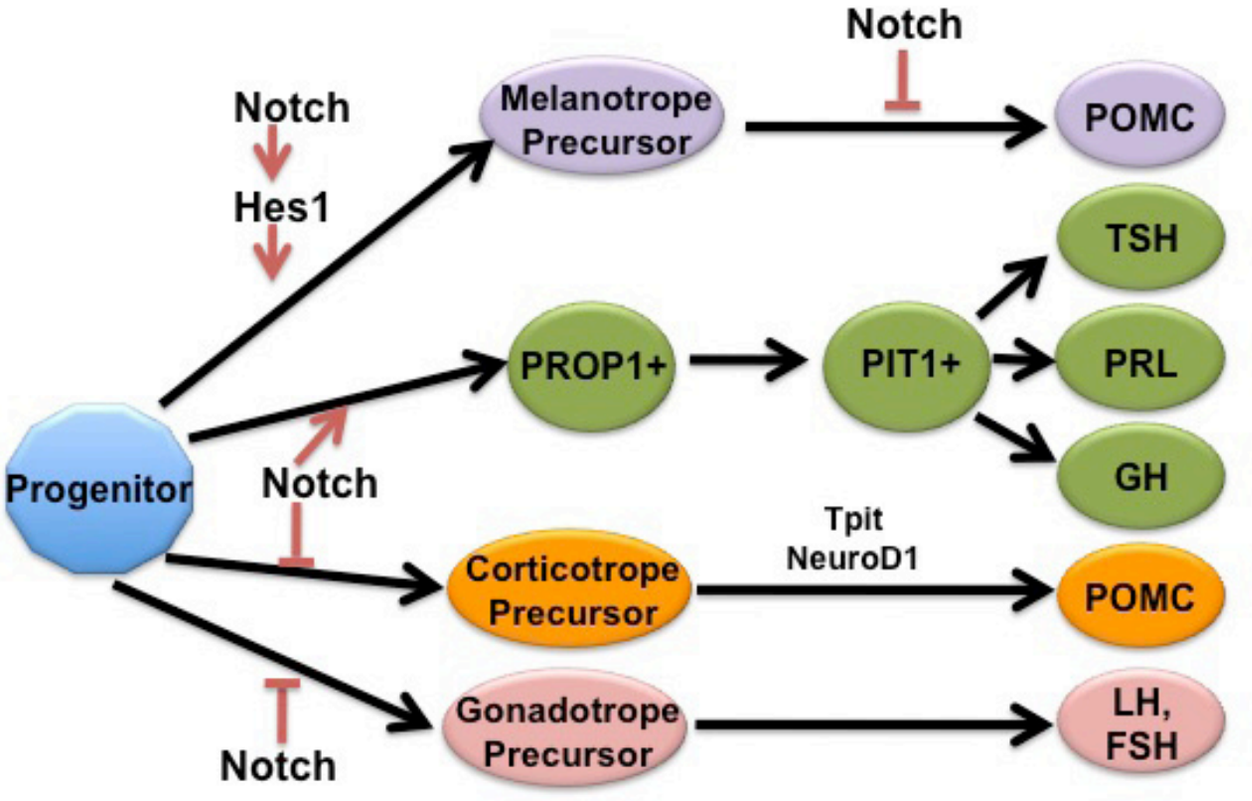

Figure 2.8. A proposed model for the role of Notch signaling in pituitary cell specification. Notch signaling must be repressed for corticotrope, melanotrope and gonadotrope differentiation to occur. In addition to repressing differentiation, Notch signaling is also necessary for the activation of PROP1, which is required for the emergence of the PIT1 lineage. Hes1, another Notch effector molecule, is necessary for the specification of melanotropes.

CHAPTER 3: **Notch signaling in postnatal pituitary expansion: proliferation, progenitors and cell specification**[1].

ABSTRACT

Mutations in *Prop1* account for up to half of the cases of combined pituitary hormone deficiency that result from known causes. Despite this, few signaling molecules and pathways that influence *Prop1* expression have been identified. Notch signaling has been linked to *Prop1* expression, but the developmental periods during which Notch signaling influences *Prop1* and overall pituitary development remain unclear. To test the requirement for Notch signaling in establishing the normal pituitary hormone milieu, we generated mice with early embryonic conditional loss of *Notch2* (cKO) and examined the consequences of chemical Notch inhibition during early postnatal pituitary maturation. We show that loss of *Notch2* has little influence on early embryonic pituitary proliferation, but is crucial for postnatal progenitor maintenance and proliferation. Additionally, we show that Notch signaling is necessary embryonically and postnatally for *Prop1* expression and robust Pit1 lineage hormone cell expansion, as well as repression of the corticotrope lineage. Taken together, our studies identify temporal and cell-type specific roles for Notch signaling and highlight the importance of this pathway throughout pituitary development.

[1] This chapter has been accepted in its entirety for publication in *Molecular Endocrinology.* Nantie, L.B., Himes, A.D., Getz, D. and Raetzman, L.T., 2014. Notch Signaling in the postnatal pituitary: proliferation, progenitors and cell specification. *In press*.

INTRODUCTION

Hypopituitarism, including combined pituitary hormone deficiency (CPHD), has a prevalence of 45.5 per 100,000 individuals (Regal, et al. 2001). Approximately 50% of known causes of CPHD result from mutations in the transcription factors *PROP1* and *PIT1* (Cogan, et al. 1998; Wu, et al. 1998; Fluck, et al. 1998; Kelberman, et al. 2009; Prince, et al. 2011). PROP1 is necessary for the transcription of *PIT1*, which is required for growth hormone (GH), thyroid stimulating hormone (TSHβ) and prolactin (PRL) expression. Therefore, patients with mutations in *PROP1*, have loss of these hormones. Although PROP1 is not necessary for gonadotrope or corticotrope cell differentiation, many patients also present with reductions in gonadotropin (LH and FSH) levels and acquired adrenocorticotropic (ACTH) deficiency (Pernasetti, et al. 2000; Agarwal, et al. 2000; Bottner, et al. 2004). Mouse models of *Prop1* deficiency have been fundamental to our understanding of the etiology of CPHD. Ames Dwarf ($Prop1^{df}$) mice, which harbor a spontaneous point mutation in *Prop1*, and genetically engineered $Prop1^{null}$ mice both recapitulate the phenotype of humans with *PROP1* mutations (Sornson, et al. 1996; Gage, et al. 1996a; Nasonkin, et al. 2004).

Although the importance of PROP1 in pituitary development and disease is well understood, few genes that regulate PROP1 expression have been elucidated. Identifying these factors is important, as mutations in upstream regulators may be responsible for additional genetic causes of CPHD. One pathway that has been implicated in the control of *Prop1* is the Notch signaling pathway. In a mouse model of pituitary-specific loss of the essential Notch cofactor, *Rbpj*, a reduction in *Prop1* transcript, detected by in situ hybridization, is observed. This correlates with reduced

expression of *Pit1*, *Gh* and *Tshb.* Furthermore, it was shown that RBPJ can bind to a canonical element within the first intron of the *Prop1* gene, indicating the control of *Prop1* by the Notch signaling pathway may be direct (Zhu, et al. 2006). Interestingly, Ames Dwarf mice have reduced levels of the receptor Notch2, although increased levels of the *Dll1* ligand and target *Hey1*, suggesting that PROP1 may also regulate Notch signaling (Raetzman, et al. 2004; Mortensen, et al. 2011).

The Notch signaling pathway is an evolutionarily conserved pathway that is critical to the development of many organs, where its most characterized role is in progenitor maintenance. This pathway requires cell-to-cell contact between one cell containing the ligand and the other containing the Notch receptor. Activation of the cascade in the receptor-containing cell, results in cleavage of the intracellular domain (NICD) of the receptor. The NICD can then translocate into the nucleus and, in complex with Mastermind-like and RBPJ, drive the transcription of downstream targets. These targets are basic helix-loop-helix transcriptional repressors that inhibit the expression of pro-differentiation genes, thus maintaining the progenitor state of the cell (reviewed in (Kopan and Ilagan. 2009)). Notch2 and *Notch3* are expressed in the embryonic pituitary and global loss of the Notch effector gene, *Hes1*, results in reduced proliferation, pituitary hypoplasia and loss of αMSH cells in the intermediate lobe (IL) (Raetzman, et al. 2004; Zhu, et al. 2006; Raetzman, et al. 2007; Monahan, et al. 2009). In contrast, overexpression of Notch receptors can inhibit the differentiation of gonadotropes, melanotropes and corticotropes (Raetzman, et al. 2006; Goldberg, et al. 2011). However, the contribution of individual Notch receptors during pituitary development, as well as the effects of pituitary-specific loss of these receptors, remains unclear.

In addition to its expression during embryonic development, Notch signaling components are present in the adult pituitary. Expression of these factors are restricted to the cells lining the pituitary cleft, which correlates with the localization of adult pituitary progenitors (Tando, et al. 2013). *In vitro* treatment of side population progenitor cells from the pituitary of adult mice or dissociated whole pituitary cells from adult rats with the Notch inhibitor DAPT, results in a decrease in the number and proliferation of progenitor cells. Conversely, *in vitro* treatment with a soluble Notch ligand, using the same experimental systems, results in increased progenitor cell number and proliferation (Chen, et al. 2006; Tando, et al. 2013). It is unclear whether similar effects would be observed *in vivo*, as cell-to-cell contact is an important aspect of the Notch signaling cascade.

Studies show that the pituitary undergoes a second wave of proliferation and cell differentiation during the first three weeks after birth in rodents, during which it expands greatly in size (Carbajo-Pérez and Watanabe. 1990). Some of this expansion is due to increases in cell volume, but progenitor and differentiated cells also proliferate during this period (Taniguchi, et al. 2000; Taniguchi, et al. 2001; Taniguchi, et al. 2002a; Taniguchi, et al. 2002b; Gaston-Massuet, et al. 2011). Additionally, the number of progenitor cells greatly decreases after postnatal day (p) 21 in mice, indicating these cells may play an important role in this phase of expansion (Gremeaux, et al. 2012). The constellation of Notch signaling components expressed and whether they are important for proliferation and differentiation during this time is unclear.

In order to further dissect the role of Notch signaling during these critical windows of development, we used both genetic and chemical inhibition of Notch signaling

throughout early development and specifically during postnatal gland expansion. In order to examine the contribution of *Notch2* during embryonic pituitary development, we generated a mouse with conditional loss of *Notch2* (cKO), beginning at embryonic day (e) 8.5 in *Foxg1*-expressing cells (Wang, et al. 2010). Loss of *Notch2* does not appear to greatly affect overall embryonic pituitary development, but does result in decreased expression of *Prop1*. Rather, we show that Notch signaling components are highly expressed during the early postnatal period and both acute chemical Notch inhibition and *Notch2* cKO mice display decreases in pituitary proliferation and alterations in hormone cell proportions. These changes parallel a substantial decrease in *Prop1* expression. Taken together, these studies further solidify *Prop1* as a bona fide Notch target and identify a critical role for Notch signaling in postnatal pituitary development. Further studies will be necessary to identify whether altered Notch signaling could be a contributing factor in CPHD.

MATERIALS AND METHODS

Mice

For Notch signaling pathway component analysis, wildtype mice of mixed genetic background were used. For *in vitro* and *in vivo* DAPT treatment studies, CD-1 mice (Charles River, Wilimington, MA), from a breeding colony maintained in our laboratory, were used. For all studies, p1 is considered to be the day of birth. All animals were housed in a facility with a 12 hour light-dark cycle and were maintained in accordance with the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

In order to generate *Notch2* cKO mice, *Notch2*$^{fl/fl}$ mice (McCright, et al. 2006) were obtained from Jackson Laboratory (Bar Harbor, ME) and bred to *Foxg1*$^{+/cre}$ mice, (Hebert and McConnell. 2000) maintained on a 129 background, also purchased from Jackson Laboratory. The resulting *Notch2*$^{+/fl}$, *Foxg1*$^{+/cre}$ mice were bred to *Notch2*$^{+/fl}$, *Foxg*$^{+/+}$ littermates to obtain cKO animals. To genotype the mice, tail biopsies were obtained and DNA was extracted using a hot shot or salt-out method. PCR for the *Cre* and *Notch2* alleles was performed as previously described (Himes and Raetzman. 2009) using published primer sequences (Hebert and McConnell. 2000; McCright, et al. 2006).

DAPT injections

Beginning at p2, CD1 mice were injected daily for 3 days with 100mg/kg DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-Butyl Ester, Gamma Secretase Inhibitor IX, Millipore, Billerica, MA) diluted in Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) or an equivalent volume of DMSO alone. Pituitaries and whole heads were harvested 24h after last injection. For studies examining hormone cell fate choice, mice were injected with 100mg/kg DAPT or an equivalent volume of DMSO for five days beginning on p3. Pituitaries were harvested on p12, five days after last injection. For bromodeoxyuridine (BrdU) studies, mice were injected subcutaneously with 0.1mg/g body weight BrdU (Sigma-Aldrich, St. Louis, MO), diluted in water, 30 minutes prior to harvest.

Pituitary explant culture and DAPT washout assay

Pituitaries were dissected from p3 CD1 mice, rinsed with PBS and transferred to a 96 well plate. Culture media was made by adding 1% Penicillin Streptomycin (Fisher,

Fairlawn, NJ) and 10% fetal bovine serum (HyClone, Logan, UT) to DMEM/F-12 media (Cellgro, Manassas, VA). 10μM DAPT dissolved in DMSO (Millipore) or an equivalent amount of DMSO (Cambridge Isotope Laboratories, Andover, MA) was added to the culture media from the beginning of culture. After 24h in culture, media was spiked with DAPT or DMSO (an equivalent amount to what was added at the beginning of culture) and pituitaries were cultured for an additional 24h. For washout assays, media was removed from pituitaries, pituitaries were rinsed and DAPT-free, DMSO-containing media or 10μM DAPT-containing media was added. Pituitaries were harvested 6h later.

Immunohistochemistry

Whole e12.5 embryos, heads of postnatal mice and pituitaries were fixed in 3.7% formaldehyde (Sigma Aldrich, St. Louis, MO) diluted in phosphate buffered saline (PBS) and embedded in paraffin for sectioning. Slides were deparaffinized and rehydrated. For immunohistochemical detection of SOX2, SOX9, Ki67, PH3, BrdU and Pit1 samples were boiled in 10 mM citrate solution, pH6 for 10 minutes and cooled for 15 minutes. Next, all samples were blocked in a solution containing 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) diluted in immunohistochemical block, which contains 3% bovine serum albumin (Jackson Immunoresearch) and 0.5% TritonX-100 (Sigma Aldrich, St. Louis, MO) diluted in PBS. Primary antibodies used were raised against the following peptides: SOX2 (1:2000, Millipore, Billerica, MA), phospho-histone-H3 (PH3, 1:500, Upstate Cell Signaling Solutions, Lake Placid, NY), Ki67 (1:500 BD Biosciences, San Jose, CA) SOX9 (1:500, Millipore, Billerica, MA), Pit1 (1:4000, kind gift of Dr. Simon Rhodes), POMC (1:1000, DAKO, Carpinteriam, CA), GH, TSHβ and LHβ (1:1000, Dr. A.F. Parlow and the National Hormone and Pituitary

Program, University of California, Los Angeles) and BrdU (1:200, AbD Serotec, Raleigh, NC). Slides were then incubated with biotin-conjugated rat (BrdU), mouse (Ki67) or rabbit (Pit1, GH, TSHβ, POMC, LHβ, SOX2, SOX9 and PH3) secondary antibody. This was followed by incubation in a streptavidin-conjugated cy3 fluorophore. Biotin and streptavidin-conjugated antibodies were purchased from Jackson ImmunoResearch and were used at a concentration of 1:500. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Life Technologies, Grand Island, NY) and visualized at 50x 100x, 200x, or 400x magnification using a Leica DM2560 microscope. Photographs were taken using a Retiga 2000R color camera (Q-Imaging, Surrey, Canada) and acquired using Q-Capture Pro software (Q-Imaging). Images were processed using Adobe Photoshop CS4 (San José, CA).

Pit1, POMC, LHβ BrdU quantification

In order to quantify cells incorporating BrdU, three equally spaced slides containing three sections each, were immunohistochemically stained for BrdU. For Pit1, POMC and LHβ cell number quantification, 5-6 equally spaced slides containing two sections each, were immunohistochemically stained using their respective antibodies. For BrdU, the number of positive cells in the AL and IL were counted and the area of the AL and IL was measured. For Pit1, POMC and LHβ quantification, the number of positive cells in the AL was counted and the area of the AL was measured. Additionally, for all cell counts, a ratio of cells per area was determined for each group by counting the number of DAPI cells per area. This ratio was used to calculate the total cells in the each pituitary section. The number of BrdU-, Pit1-, POMC- or LHβ-positive cells was divided by the calculated total cells in each pituitary, giving the percent of

immunopositive cells. Statistical significance was determined using Student's *t* test.

AL Volumetric Approximation

For volumetric approximation, every third slide, containing two sections each, of p6 control and cKO mice was stained with DAPI. The area of the AL was measured for each stained section using ImageJ (NIH, Bethesda, MD). In order to obtain an approximation of the AL volume, this area was multiplied by the thickness of the section and by three, to account for slides in between stained slides.

In Situ Hybridization

Slides were deparaffinized, rehydrated and incubated in PBS containing 0.3% triton-X 100 (Sigma-Aldrich, St. Louis, MO) for 10 minutes and permeabilized using proteinase K (Life Technologies, Foster City, CA) for fifteen minutes at 37°C. Samples were then post-fixed using 3.7% formaldehyde (Sigma-Aldrich) and acetylated for ten minutes in a solution containing 0.25% acetic anhydride (Sigma-Aldrich) diluted in triethanolamine (Sigma-Aldrich) buffer. Following acetylation, sections were incubated in hybridization solution at 55-58°C for a minimum of two hours. DNA Digoxigenin-UTP (DIG) labeled probes for the *Prop1* (Cushman, et al. 2001), *Notch2* (kind gift of Dr. Shelley Ross, Raetzman, et al. 2004)), *Hes1* (kind gift of Dr. Ryoichiro Kageyama), *Hey1* (kind gift of Dr. Mannfred Gessler) or *Ghrh* (kind gift of Drs. Iain Robinson and Paul LeTissier) were denatured and applied to slides for overnight incubation at the hybridization temperature. Next, samples were treated with a solution of 50% formamide (Sigma-Aldrich) for one hour at the hybridization temperature, followed by blocking and then application of anti-DIG Fab fragments for one to two hours at room temperature. Slides were then washed, treated with Chromagen buffer solutions of

increasing pH and developed at room temperature overnight in Chromagen buffer containing NBT/BCIP. Dig-labeled nucleotides, anti-dig antibody and NBT/BCIP were purchased from Roche (Mannheim, Germany).

Quantitative Reverse Transcriptase PCR

RNA was isolated from embryonic and postnatal whole pituitaries using an RNAqueous micro kit (Ambion, Austin, TX) as per manufacturers protocol. For p21 and adult pituitaries, 0.5μg of RNA was synthesized into cDNA using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). For e16.5 and p1, p5 and p10 and p12 pituitaries, the total amount of RNA from each pituitary was converted into cDNA. A no Reverse Transcriptase enzyme control was also prepared and used as a negative control. For quantitative RT-PCR 0.1-0.33 μL of cDNA was amplified using gene-specific primers and SYBR green mix (Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad MyIQ real-time PCR machine. Data were analyzed with the change in cycle threshold (ΔCt) value method (Goldberg, et al. 2011). Statistical significance was determined using Student's *t* test. For a list of gene specific primers, see Table 3.1.

RESULTS

Loss of *Notch2* does not affect proliferation or corticotrope differentiation at e12.5.

At e12.5, the morphology of the developing pituitary appears similar between control (Figure 3.1A) and *Notch2* cKO mice (Figure 3.1B). Additionally, proliferation appears to be largely unchanged between control (Figure 3.1C) and cKO (Figure 3.1D) pituitaries. POMC immunostaining reveals few positives cells in control (Figure 3.1E)

and cKO mice (Figure 3.1F), indicating there is no apparent alteration in corticotrope number when *Notch2* is lost, contrary to what is observed in the *Prop1*, *Hes1* double knockout (Himes and Raetzman. 2009). Because no striking phenotype is observed in *Notch2* cKO mice, we performed in situ hybridization for *Hey1* in control and cKO mice to determine if a canonical Notch signaling target is reduced as expected. In control mice, at e12.5, *Hey1* is expressed throughout the developing pituitary (Figure 3.1G). In cKO mice, there is a marked reduction, although not complete loss, of *Hey1* expression throughout the developing pituitary (Figure 3.1H). Because Notch signaling has been shown to potentially regulate *Prop1*, we examined *Prop1* expression in control and cKO developing pituitaries. In control mice, *Prop1* is expressed throughout Rathke's pouch (RP) and is restricted away from the ventral portion of the developing pituitary where differentiated cells are housed (Figure 3.1I). In cKO mice, *Prop1* appears to be reduced throughout RP (Figure 3.1J). Taken together, these data suggest that *Notch2* is not necessary for proliferation during early development, but is necessary for *Prop1* expression.

Characterization of Notch signaling components during postnatal expansion phase of pituitary development

The pituitary undergoes two distinct waves of cell proliferation and differentiation, one occurring during embryogenesis and a second that takes place during the first three postnatal weeks in the mouse (Zhu, et al. 2007; Gremeaux, et al. 2012). Although it appears *Notch2* is not necessary for early pituitary development to proceed normally, the role of Notch signaling during the second wave of proliferation and differentiation has yet to be elucidated. In order to address this question, we first investigated the

spatial and temporal expression patterns of Notch signaling components during this critical window to determine if this pathway is active during this time. We used qRT-PCR to determine the relative levels of Notch family members during this time (e16.5, p1, p5, p10, p21) and compared these levels to those observed in the adult pituitary (Figure 3.2A-3.2C). These data show that Notch signaling components are present during this wave of pituitary expansion and their mRNA levels are, in general, at higher levels than those found in adult pituitaries.

In order to determine the mRNA localization of Notch targets and receptors, we performed in situ hybridization for *Notch2*, *Hey1* and *Hes1* in p5 coronal sections. We find that *Notch2* mRNA is mainly restricted to some, but not all cells surrounding the pituitary cleft, particularly in the area where the intermediate and anterior lobes intersect. There are also *Notch2*-positive cells scattered throughout the anterior lobe (AL, Figures 3.3A and 3.3B). This expression pattern correlates with the localization of the population of pituitary progenitors containing SOX2 (Fauquier, et al. 2008). A similar expression pattern is observed for *Hey1* (Figures 3.3C and 3.3D), with positive cells seen lining the cleft and in scattered cells in the AL. *Hes1* appears to be in fewer cells than *Notch2* and is restricted to the AL side of the pituitary cleft (Figures 3.3E and 3.2F). Recent studies have shown that PROP1 is also present in cleft cells after birth (Garcia-Lavandeira, et al. 2009; Tando, et al. 2013).

These findings indicate that Notch signaling components are present during early postnatal pituitary development and are dynamic, warranting further investigation of the role of this signaling pathway during this period. We examined the expression of Notch receptors in *Notch2* cKO mice at p1 and find that *Notch2* is specifically reduced,

although not completely lost (Figure 3.3G). Additionally, we find significant reductions in mRNA levels of Notch targets, especially *Prop1* (Figure 3.3H), making the cKO mouse a good model to investigate the effects of loss of *Notch2* at this age.

Notch2 is necessary for the maintenance of pituitary progenitors after birth

Our analysis shows that *Notch2* is maintained in cleft cells after birth, indicating it may have a role in progenitor maintenance during the postnatal wave of pituitary development. We, therefore, examined SOX2 expression at p1 in control and cKO mice. At p1, SOX2 is normally expressed in most cells lining the cleft of the pituitary on both the AL and IL sides. In addition, SOX2 is also found in cells scattered throughout the IL and the AL. (Figure 3.4A, 3.4C and 3.4E). In cKO mice, none of the cells lining the pituitary cleft on the IL side are positive for SOX2. Instead, SOX2-immunoreactive cells are found in the dorsal IL, at the IL-posterior lobe (PL) border (Figure 3.4B and 3.4D). Interestingly, cells in the AL appear to be largely unaffected in localization or number in the cKO (Figure 3.4B and 3.4F). The expression pattern of SOX9, another progenitor marker, is similar to that of SOX2. In control mice at p1, SOX9-positive cells are found lining the pituitary cleft and scattered throughout the IL and AL, as well as in many cells in the PL (Figures 3.4G, 3.4I and 3.4K). In cKO mice, SOX9 is not found in cells lining the cleft in the IL, but, similar to SOX2 localization, is restricted to cells in the dorsal IL (Figures 3.4H and 3.4J). In addition, in cKO mice, the number of SOX9-immunoreactive cells lining the cleft and in the parenchyma of the AL appears to be reduced (Figures 3.4H and 3.4L, arrows indicate SOX9-negative cleft cells). In support of these observations, mRNA levels of *Sox2* and *Sox9* are both significantly decreased in cKO

mice as compared to controls (Figure 3.4M). Taken together, these results show that *Notch2* is necessary for the maintenance and localization of progenitor cells at p1.

Loss of *Notch2* results in a progressive decrease in pituitary progenitor number

To assess whether progenitor cells are maintained in the *Notch2* cKO throughout early postnatal expansion, we stained p5 control and cKO pituitaries for SOX2 and SOX9. In control mice, SOX2 expression in the IL is mostly limited to cells lining the cleft (Figure 3.4N and 3.4P). In cKO mice, fewer SOX2-expressing cells remain in the IL than what is observed at p1. The cells that do remain are still present at the IL-PL border (Figure 3.4O and 3.4Q), rather than lining the cleft. On the AL side of the cleft, SOX2 is present in nearly every cell in control mice (Figure 3.4N and 3.4R), and appears to be expressed in fewer cells in cKO mice (Figure 3.4O and 3.4S, arrows). In addition to SOX2-positive progenitors lining the cleft, there are clusters of SOX2-expressing cells in the AL parenchyma (Figure 3.4T). Although these clusters are still present in cKO mice, they appear to be reduced (Figure 3.4U). In control mice, SOX9 expression follows a similar pattern in the IL to that of SOX2 (Figure 3.4V and 3.4X). Likewise, SOX9 is only expressed in few cells in the IL of cKO and these cells are present only in the dorsal IL (Figure 3.4W and 3.4Y). As with SOX2, SOX9 is expressed in nearly every cell lining the cleft in the AL in control mice (Figure 3.4V and 3.4Z). However, SOX9 expression appears to be lost in many AL cleft cells in cKO mice (Figure 3.4W and 3.4A', arrows). SOX9 is expressed in clusters in the AL parenchyma in control mice (Figure 3.4B'). In contrast, very few, if any, clusters are observed in the AL parenchyma of cKO mice, and the SOX9-positive cells that are present appear to be morphologically different than their control counterparts (Figure 3.4C'). Taken together,

these data suggest that *Notch2* is necessary to maintain and expand the progenitor population during postnatal pituitary maturation.

Loss of *Notch2* results in decreased pituitary proliferation at p1 and decreased AL volume at p6

Although proliferation is not affected at e12.5 when Notch signaling is reduced, we observe a disruption of progenitor cells at p1 and p5. Therefore, we examined whether these changes in progenitor cell number and localization correlate with changes in postnatal proliferation. In control mice, Ki67-immunoreactive cells are observed throughout the AL, IL and PL (Figure 3.5A). In the IL, Ki67 expression is found throughout, especially in cells lining the cleft (Figure 3.5C). In cKO mice, there appears to be an overall reduction in the number of Ki67-expressing cells in the AL and a striking reduction of immunopositive cells in the IL (Figures 3.5B and 3.5D). Additionally, an apparent decrease in cells in mitosis is also observed in the cKO as evidenced by a reduction in the number of cells expressing phospho-histone H3 (PH3, Figure 3.5F) as compared to control (Figure 3.5E). Furthermore, *mKi67* and *Ccne1* mRNA levels are reduced in cKO pituitaries. This is coincident with an increase in *Cdknc1* (p57) mRNA (Figure 3.5G), a cell cycle inhibitor shown to be necessary for pituitary progenitors to exit the cell cycle before differentiating (Bilodeau, et al. 2009). Together, these data indicate that *Notch2* is an important regulator of pituitary proliferation at p1.

Because we observe decreased proliferation at p1, we examined approximate AL volume at p6 to determine how these changes in proliferation may result in changes in pituitary size. We find that at p6 the AL of cKO mice (Figures 3.5J and 3.5K) appears hypoplastic compared to control (Figures 3.5H and 3.5I). Additionally, the approximate

volume of the cKO AL is significantly reduced as compared to control (Figure 3.5L). These findings suggest that reduction in *Notch2* can lead to a hypoplastic pituitary as early as p6.

Chemical Notch inhibition results in decreased postnatal proliferation *in vivo*

In *Notch2* cKO mice, we do not observe proliferation defects until after birth, indicating Notch signaling may be specifically important for proliferation that occurs postnatally. In order to confirm the postnatal changes we observe in *Notch2* ckO mice are due to direct actions postnatally, we injected mice during early postnatal development with the gamma secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-Butyl Ester). DAPT inhibits cleavage of the Notch intracellular domain, thus preventing activation of the downstream pathway (Dovey, et al. 2001; Sastre, et al. 2001; Geling, et al. 2002). Mice were injected with 100 mg/kg DAPT or vehicle control (DMSO) daily between p2 and p4 and pituitaries were harvested 24 hours after last injection (Figure 3.6A). This time period was chosen because, based on expression analysis, many Notch signaling components appear to be most highly expressed between p1 and p5. To determine the efficacy of Notch inhibition in DAPT-injected mice, mRNA levels of the canonical Notch targets *Hes1* and *Hey1* and the putative pituitary-specific Notch target *Prop1* were examined in whole pituitaries. We see a significant decrease in all targets examined, with *Prop1* levels being most highly suppressed after DAPT treatment as compared to vehicle-treated controls (Figure 3.6B).

Because Notch signaling is known to be important for proliferation in many developing organs and because we observe decreases in proliferation at p1 in *Notch2*

cKO mice, we examined proliferation by quantifying the percent of BrdU positive cells in the AL and IL of pituitaries of DAPT-treated mice and compared these values to vehicle controls. In vehicle-injected mice there are an average of 5.51% (+/- 0.20%) of cells that are positive for BrdU (Figure 3.6C) as compared to 2.98% (+/- 0.26%) of cells in pituitaries of DAPT-treated mice (Figure 3.6D). Additionally, we find a reduction in mRNA levels of *mKi67*, which is present in cells during all phases of the cell cycle, and in *Ccnd1*, which is important for transition from G_1 to S phase, in mice treated with DAPT (Figure 3.6E). This is coincident with an increase in *Cdknc1* (p57) mRNA (Figure 3.6F). These observations show that Notch signaling contributes to postnatal pituitary proliferation *in vivo* and are similar to those seen in *Notch2* cKO mice at p1.

In vitro chemical Notch inhibition reveals direct effects on the pituitary

To elucidate whether the effects observed in the *in vivo* DAPT injection study are consistent with direct effects on the pituitary, we employed an *in vitro* organ culture system (Figure 3.6G). We examined whether proliferation in DAPT-cultured pituitaries is affected in an analogous manner to that seen in DAPT-injected mice. Similarly, we find a significant decrease in mRNA levels of *mKi67* in pituitaries treated with DAPT as compared to vehicle treated pituitaries after 48h in culture (Figure 3.6H). Taken together, the *Notch2* cKO and acute DAPT treatment studies implicate Notch signaling in maintaining at least a subset of progenitors and allowing their proliferation during postnatal development.

To identify potentially direct targets of Notch signaling, we performed a 6h washout assay, using the *in vitro* explant system. Targets that are altered after DAPT treatment, but recover within the 6h period, are considered to be likely direct targets of

the Notch signaling pathway (Weng, et al. 2006; Chadwick, et al. 2009; Wang, et al. 2011). We observe significant reductions in mRNA levels of the Notch targets *Hes1*, *Hey1* and *Prop1*, with *Prop1* levels again being the most decreased after culture in the presence of DAPT. Six hours after washout, the mRNA levels of the canonical Notch targets *Hes1* and *Hey1* have recovered to levels comparable to those in vehicle-treated pituitaries. *Prop1* levels recover to levels that are statistically similar to vehicle-treated pituitaries, albeit not as complete of a response as *Hes1* or *Hey1* (Figure 3.6I). This further suggests that *Prop1* is likely a direct Notch target and show that Notch signaling can influence *Prop1* expression after birth.

Loss of *Notch2* results in decreased Pit1 cell number and increased POMC number at p1

In addition to its role in progenitor maintenance and proliferation, Notch signaling has been shown to be a critical regulator of cell fate in many systems (Ohtsuka, et al. 2001; Murtaugh, et al. 2003; Patten, et al. 2003; Siveke, et al. 2008). In order to determine whether Notch signaling has a similar role in the pituitary, we examined the fate of Pit1-lineage cells, which contain GH, TSH and PRL, as well as corticotropes and gonadotropes at p1 in control and *Notch2* cKO mice. GH is normally expressed in the AL of control mice (Figure 3.7A) and appears to be reduced in cKO pituitaries (Figure 3.7B). TSHβ appears throughout the AL in control mice (Figure 3.7C) and appears to be largely unchanged in cKO pituitaries (Figure 3.7D). In parallel, we examined mRNA levels of *Gh, Tshb* and *Pou1f11* (*Pit1*). We observe a significant reduction in cKO mRNA levels of *Pou1f1* and *Gh*, with *Tshb* levels statistically similar between control and cKO mice (Figure 3.7Q). To determine if the observed changes in mRNA levels

correlates with changes in cell number, we quantified the % of Pit1-positive cells in the AL of control and cKO mice. In control mice 44.35% (+/- 1.59%) of AL cells are positive for Pit1, compared with only 26.97% (+/- 2.85%) in cKO mice (Figure 3.7T), suggesting *Notch2* is, at least in part, necessary to obtain the proper number of Pit1-positive cells.

POMC is expressed in melanotropes in the IL and corticotropes in the AL in both control (Figure 3.7E) and cKO (Figure 3.7F) mice and appears to be slightly increased in the AL of cKO mice as compared to control. Additionally, we observe a trend towards an increase in mRNA levels of *Tpit* and *Pomc*, which are present in both corticotropes and melanotropes, in cKO mice. To more specifically explore corticotrope and melanotrope lineages, we examined expression levels of genes that are specific to each cell type (Budry, et al. 2012). We show an increase in levels *Crhr1*, which is expressed in corticotropes, in cKO pituitaries. Conversely, no changes in mRNA levels of *Pax7*, a melanotrope-specific marker, are observed in cKO mice (Figure 3.7R). In order to determine if these changes in mRNA are coincident with changes in cell number, we quantified the number of POMC-positive cells in the AL. Control mice have 11.87% (+/- 0.40%) POMC-expressing cells in the AL. cKO mice have a significantly increased number of POMC-containing AL cells (15.49% +/- 0.83%) (Figure 3.7T), suggesting that *Notch2* is an important factor regulating corticotrope number.

LHβ is found in few cells scattered throughout the ventral pituitary in control mice (Figure 3.7G) and the number of LHβ-positive cells does not appear to be altered in cKO pituitaries (Figure 3.7H). Although the number of LHβ-immunopositive cells does not appear to be changed in cKO mice, *Lhb* mRNA levels are significantly increased in cKO pituitaries as compared to control. Additionally, *Nr5a1*, a transcription factor

expressed only in gonadotropes in the pituitary is significantly increased in cKO at p1, while *Gnrhr* shows a trend towards an increase (Figure 3.7S). To determine if these changes in mRNA levels correlate with changes in LHβ-positive cell number, we quantified the percentage of AL cells that express this hormone. We find no difference between control (2.95% +/- 0.20%) and cKO mice (2.99% +/- 0.08%) (Figure 3.7T), suggesting regulation of the gonadotrope cell number is likely independent of *Notch2.*

Mice with conditional loss of *Notch2* have reduced GH and TSHβ at p6

In order to examine how loss of *Notch2* affects expansion of the pituitary hormone lineages during postnatal development, we immunohistochemically examined the expression of pituitary hormones at p6. Similar to what was observed at p1, GH appears to be reduced in cKO pituitaries (Figure 3.7J) as compared to control pituitaries (Figure 3.7I). In contrast to p1, TSHβ appears to be strongly reduced in cKO mice at p6 (Figure 3.7L) as compared to control mice (Figure 3.7K). POMC is present in the IL and AL of control mice (Figure 6M) and appears to be slightly increased in the AL of cKO mice (Figure 3.7N), mirroring the qRT-PCR results and cell counts observed at p1. LHβ is present in scattered AL cells of control mice (Figure 3.7O) and cKO mice (Figure 3.7P) and appears similar between the two groups. Taken together, these data show that *Notch2* is necessary to control the number of Pit1-positive cells and POMC-positive cells during postnatal pituitary expansion.

In order to eliminate the possibility that the reduction in *Gh* is a result of the presence of the Cre transgene alone, we examined *Gh* mRNA in control mice with (*Notch2*$^{+/+}$ *Foxg1*$^{+/cre}$ or *Notch2*$^{+/fl}$ *Foxg1*$^{+/cre}$) and without (*Notch2*$^{+/+}$ *Foxg1*$^{+/+}$, *Notch2*$^{+/fl}$ *Foxg1*$^{+/+}$ or *Notch2*$^{fl/fl}$ *Foxg1*$^{+/+}$) the Cre transgene. No differences were observed

between the two groups in *Gh* levels (Figure 3.8A) at p1. Additionally, although the *Foxg1* Cre should not be expressed in the developing hypothalamus and thus *Notch2* expression should not be altered there, we examined whether reduced GH expression could be due to unexpected alterations in *Ghrh* in the hypothalamus in the cKO mice. In control mice, *in situ* hybridization reveals *Ghrh* mRNA is present in cells lateral to the third ventricle in the arcuate nucleus (Figure 3.8B). A similar pattern of expression is observed for cKO mice (Figure 3.8C). These data suggest that the phenotype of the *Notch2* cKO mice is likely due to the pituitary intrinsic effects of loss of *Notch2*.

Postnatal chemical Notch inhibition results in decreased mRNA levels of Pit1-lineage hormones and increased *Pomc* mRNA levels

Because alterations in pituitary hormones were observed when *Notch2* is lost from early embryogenesis, we investigated whether Notch signaling is also specifically important for postnatal pituitary cell fate choice. In order to examine this question, we injected mice with DAPT daily from p3 through p7 and harvested whole pituitaries on p12 in order to allow changes in differentiated cells to manifest (Figure 3.9A). In mice treated with DAPT, we observe a significant decrease in mRNA levels of *Pou1f1*, *Prl* and *Tshb*. Interestingly, although part of the Pit1 lineage, mRNA levels of *Gh* are unchanged after DAPT treatment. *Pomc* mRNA levels are increased after DAPT treatment, while *Lhb* mRNA levels appear unaffected (Figure 3.9B). In order to determine whether these alterations are maintained after DAPT treatment, we injected mice with DAPT daily from p3 through p7 and harvested their pituitaries on p32 (Figure 3.9C). We show that mRNA levels of *Pou1f1* and *Prl* remain decreased in DAPT-treated mice, whereas all other hormones are unchanged (Figure 3.9D). These findings show

that Notch signaling is necessary for proper mRNA levels of *Pou1f1, Tshb*, *Prl,* and *Pomc* and suggest a role for Notch in postnatal pituitary cell fate choice, similar to what was uncovered in the *Notch2* cKO mouse.

DISCUSSION

Taken together, our studies have defined key roles for Notch signaling during postnatal development. We show that Notch signaling is required for pituitary proliferation and progenitor maintenance during postnatal pituitary expansion, but appears to be less important for these processes during embryonic development. Furthermore, during both periods of development, we show that Notch signaling regulates the balance between the Pit1 and POMC lineages.

We have identified *Notch2* as an important regulator of postnatal pituitary progenitor placement and number. Pituitary stem/progenitor cells contain the markers SOX2 and SOX9 and have been shown to contribute to postnatal pituitary homeostasis during both normal and stressed conditions (Rizzoti, et al. 2013; Andoniadou, et al. 2013). Therefore, it is critical that an appropriate number of progenitor cells are maintained. We show that *Notch2* cKO mice have progressive loss of this progenitor population after birth, suggesting that Notch signaling is necessary for maintenance of these cells. Loss of SOX2 in the embryonic pituitary results in impaired proliferation, pituitary hypoplasia and a reduction in cells of the Pit1 lineage (Jayakody, et al. 2012), mirroring what is observed in *Notch2* cKO mice. Additionally, overexpression of activated Notch in POMC-expressing cells results in increased SOX2 expression in the IL (Goldberg, et al. 2011), suggesting Notch may have the ability to induce SOX2

expression. Both *Sox2* and *Sox9* are direct Notch targets in neural progenitors *in vivo* (Li, et al. 2012), indicating the potential for Notch to directly control these factors in the pituitary.

Loss of *Notch2* results in differential effects in AL and IL progenitors. IL progenitors are misplaced at p1, similar to what is observed with conditional loss of the Notch pathway components *Numb* and *Numblike* (Moran, et al. 2011) and are nearly absent by p5. This is likely due to the fact that they lack the proper progenitor niche. In contrast, AL progenitors appear to be correctly localized but decreased in number by p5. These differences may be due to the heterogeneity of progenitor populations observed throughout the pituitary (Garcia-Lavandeira, et al. 2009; Rizzoti. 2010). Additionally, we show that *Notch2* is not uniformly expressed in all progenitors, which may contribute to differences observed in different populations. These differential effects in progenitor populations may also be a contributing factor to the changes in hormone producing cell number observed at p1, as some lineage-committed or intermediate progenitors may have altered ability to expand.

We show that reductions in *Notch2* have little effect on embryonic pituitary proliferation, but that *Notch2* is required for proliferation during postnatal pituitary expansion. This is in contrast to studies showing reduced proliferation in the embryonic pituitary of *Hes1* knockout mice (Zhu, et al. 2006; Raetzman, et al. 2007; Kita, et al. 2007; Monahan, et al. 2009). This indicates that the *Hes1* null phenotype may be due to factors extrinsic to the pituitary or that the reduced but detectable presence of *Hes1* and other Notch signaling components, including *Notch3,* in *Notch2* cKO mice is sufficient to maintain embryonic proliferation. Additional studies examining pituitary-specific loss of

Hes1 will be required to further explore this question. Although we do not observe alterations in proliferation in cKO mice at e12.5, we see a striking decrease in proliferation at p1. We see a similar reduction in proliferation in mice treated postnatally with the chemical Notch inhibitor DAPT. This correlates with studies showing decreased proliferation in adult dissociated pituitary cells *in vitro* when treated with DAPT (Chen, et al. 2006; Tando, et al. 2013). However, it remains unclear if Notch signaling directly controls proliferation or alters the ability of stem cells to respond to proliferative signals.

We show that loss of *Notch2* results in alterations in cell fate, a phenomenon that has been described in multiple contexts, including pancreatic and central nervous system development (Ohtsuka, et al. 2001; Murtaugh, et al. 2003; Patten, et al. 2003; Siveke, et al. 2008). Specifically, after loss of *Notch2*, we detect a decrease in Pit1 cell number. This finding is similar to that observed in a conditional pituitary knockout of *Rbpj*, an essential Notch cofactor (Zhu, et al. 2006). Additionally, we have extended these observations showing that alterations in hormone producing cell number are maintained during postnatal expansion in *Notch2* cKO mice and that the Pit1 lineage is similarly affected after postnatal DAPT treatment. It is likely that the changes observed in the Pit1 lineage in both models are due to reductions in *Prop1* levels as Prop1 is necessary for differentiation of cells of the Pit1 lineage (add reference here). However, it is also likely that the levels of *Prop1* in *Notch2* cKO and DAPT-injected mice are sufficient to allow for some expression of Pit1, as we do not observe a complete absence of this lineage. Taken together, our results show that reduced levels of *Notch2*, or of Notch signaling components in general, result in specific and incomplete reduction in Pit1 and hormones of the Pit1 lineage. This suggests that other signaling pathways or

molecules are also important development and function of Pit1 lineage hormones. Such factors may include GATA2, which helps to maintain *Tshb* expression (Kashiwabara, et al. 2009) and *Math3*, which is required for somatotrope maturation (Zhu, et al. 2006).

In addition to the decrease observed in Pit1 lineage cells when Notch is reduced, we find that the POMC lineage is also affected by reductions in Notch signaling. In *Notch2* cKO mice, we observe an increase in the percent of POMC-containing AL cells and an increase in mRNA levels of *Crhr1*, which is restricted to corticotropes, in *Notch2* cKO mice. These changes are paralleled by an increase in *Pomc* mRNA in mice treated with the chemical Notch inhibitor, DAPT. A similar increase in POMC-expressing cells is observed in mice with loss of both *Hes1* and *Prop1* and in mice with pituitary-specific loss of *Rbpj* (Zhu, et al. 2006; Himes and Raetzman. 2009). Additionally, in order to obtain ACTH-producing cells from embryonic stem cells, these cells must first be incubated with DAPT (Suga, et al. 2011). This is confirmed by the observation that persistent expression of activated Notch in corticotropes results in POMC repression (Goldberg, et al. 2011). Taken together, these data all suggest that Notch signaling must be inhibited for corticotrope differentiation to proceed. Interestingly, the IL POMC-expressing melanotropes appear to be less affected by alterations in Notch signaling in our model. This may be due to the fact that we observe no changes in *Pax7*, a pioneer factor that dictates melanotrope fate by altering chromatin accessibility (Budry, et al. 2012). Additionally, despite these changes in corticotrope and Pit1 lineage cell number, our data suggest the gonadotrope lineage differentiates independently of Notch signaling. Taken together, these data strongly suggest that *Notch2* may help to regulate

the balance between the Pit1 and POMC lineages. This role may be direct or may be a result of the altered ability of different progenitor populations to expand.

Our studies have defined *Notch2* as a critical regulator of *Prop1* expression. We show that loss of *Notch2* leads to decreased expression of *Prop1* at both e12.5 and p1 and that *in vivo* and *in vitro* exposure to the chemical Notch inhibitor DAPT results in strong reductions in mRNA levels of *Prop1*. A recovery in *Prop1* mRNA levels *in vitro* is observed six hours after DAPT washout, mirroring the recovery seen in the canonical Notch targets *Hes1* and *Hey1*. Recent work has shown that RBPJ can bind to the first intron of the *Prop1* gene, further solidifying *Prop1* as a direct Notch target (Zhu, et al. 2006). Additionally, the phenotype observed in *Notch2* cKO mice is similar, although less severe, to that observed in *Prop1*-deficient mice, with both showing a reduction in hormones of the Pit1-lineage and inability of the pituitary to appropriately expand after birth (Gage, et al. 1996a; Gage, et al. 1996b; Nasonkin, et al. 2004; Ward, et al. 2005). Although some aspects of the phenotype observed when *Notch2* is lost are similar to that of *Prop1* mutants, we do not see increased adhesion and inability of cells to migrate out of Rathke's pouch in *Notch2* cKO mice, as is seen in mice with loss of *Prop1* (Ward, et al. 2005). These differences indicate that the levels of *Prop1* in *Notch2* cKO mice may be sufficient to prevent cell migration defects or that other Notch targets are able to compensate for defects observed when *Prop1* is lost.

Genetic causes of CPHD include mutations resulting in hypomorphic *SOX2* expression and loss of function mutations in *PROP1* and *PIT1* (Wu, et al. 1998; Kelberman, et al. 2009; Castinetti, et al. 2012). Our studies show that reductions in Notch signaling can result in alterations in expression of all of these factors during

embryonic and postnatal development. Furthermore, we show that loss of *Notch2* can lead to hypoplastic pituitary as early as p6, indicating that the Notch signaling pathway is an important regulator of pituitary size and cell fate. Based on these studies, it may be advisable to consider whether *Notch2* or other members of the Notch signaling pathway are altered in patients with CPHD that have no known genetic mutations.

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REFERENCES

Agarwal, G., Bhatia, V., Cook, S., Thomas, P.Q., 2000. Adrenocorticotropin Deficiency in Combined Pituitary Hormone Deficiency Patients Homozygous for a Novel PROP1 Deletion. J Clin Endocrinol Metab 85, 4556-4561.

Andoniadou, C.L., Matsushima, D., Mousavy Gharavy, S.N., Signore, M., Mackintosh, A.I., Schaeffer, M., Gaston-Massuet, C., Mollard, P., Jacques, T.S., Le Tissier, P., Dattani, M.T., Pevny, L.H., Martinez-Barbera, J.P., 2013. Sox2(+) Stem/Progenitor Cells in the Adult Mouse Pituitary Support Organ Homeostasis and have Tumor-Inducing Potential. Cell Stem Cell 13, 433-445.

Bilodeau, S., Roussel-Gervais, A., Drouin, J., 2009. Distinct Developmental Roles of Cell Cycle Inhibitors p57Kip2 and p27Kip1 Distinguish Pituitary Progenitor Cell Cycle Exit from Cell Cycle Reentry of Differentiated Cells. Molecular and cellular biology 29, 1895-908.

Bottner, A., Keller, E., Kratzsch, J., Stobbe, H., Weigel, J.F., Keller, A., Hirsch, W., Kiess, W., Blum, W.F., Pfaffle, R.W., 2004. PROP1 Mutations Cause Progressive Deterioration of Anterior Pituitary Function Including Adrenal Insufficiency: A Longitudinal Analysis. J Clin Endocrinol Metab 89, 5256-5265.

Budry, L., Balsalobre, A., Gauthier, Y., Khetchoumian, K., L'honore, A., Vallette, S., Brue, T., Figarella-Branger, D., Meij, B., Drouin, J., 2012. The Selector Gene Pax7 Dictates Alternate Pituitary Cell Fates through its Pioneer Action on Chromatin Remodeling. Genes Dev 26, 2299-2310.

Carbajo-Pérez, E., Watanabe, Y.G., 1990. Cellular Proliferation in the Anterior Pituitary of the Rat during the Postnatal Period. Cell and tissue research 261, 333-8.

Castinetti, F., Reynaud, R., Saveanu, A., Barlier, A., Brue, T., 2012. Genetic Causes of Combined Pituitary Hormone Deficiencies in Humans. Ann Endocrinol (Paris) 73, 53-55.

Chadwick, N., Zeef, L., Portillo, V., Fennessy, C., Warrander, F., Hoyle, S., Buckle, A., 2009. Identification of Novel Notch Target Genes in T Cell Leukaemia. Molecular cancer 8, 35.

Chen, J., Crabbe, A., Van Duppen, V., Vankelecom, H., 2006. The Notch Signaling System is Present in the Postnatal Pituitary: Marked Expression and Regulatory Activity in the Newly Discovered Side Population. Molecular endocrinology (Baltimore, Md) 20, 3293-307.

Cogan, J.D., Wu, W., Phillips, J.A.(., Arnhold, I.J., Agapito, A., Fofanova, O.V., Osorio, M.G., Bircan, I., Moreno, A., Mendonca, B.B., 1998. The PROP1 2-Base Pair

Deletion is a Common Cause of Combined Pituitary Hormone Deficiency. The Journal of clinical endocrinology and metabolism 83, 3346-9.

Cushman, L.J., Watkins-Chow, D.E., Brinkmeier, M.L., Raetzman, L.T., Radak, A.L., Lloyd, R.V., Camper, S.A., 2001. Persistent Prop1 Expression Delays Gonadotrope Differentiation and Enhances Pituitary Tumor Susceptibility. Human molecular genetics 10, 1141-53.

Dovey, H.F., John, V., Anderson, J.P., Chen, L.Z., de Saint Andrieu, P., Fang, L.Y., Freedman, S.B., Folmer, B., Goldbach, E., Holsztynska, E.J., Hu, K.L., Johnson-Wood, K.L., Kennedy, S.L., Kholodenko, D., Knops, J.E., Latimer, L.H., Lee, M., Liao, Z., Lieberburg, I.M., Motter, R.N., Mutter, L.C., Nietz, J., Quinn, K.P., Sacchi, K.L., Seubert, P.A., Shopp, G.M., Thorsett, E.D., Tung, J.S., Wu, J., Yang, S., Yin, C.T., Schenk, D.B., May, P.C., Altstiel, L.D., Bender, M.H., Boggs, L.N., Britton, T.C., Clemens, J.C., Czilli, D.L., Dieckman-McGinty, D.K., Droste, J.J., Fuson, K.S., Gitter, B.D., Hyslop, P.A., Johnstone, E.M., Li, W.Y., Little, S.P., Mabry, T.E., Miller, F.D., Audia, J.E., 2001. Functional Gamma-Secretase Inhibitors Reduce Beta-Amyloid Peptide Levels in Brain. J Neurochem 76, 173-181.

Fauquier, T., Rizzoti, K., Dattani, M., Lovell-Badge, R., Robinson, I.C.A.F., 2008. SOX2-Expressing Progenitor Cells Generate all of the Major Cell Types in the Adult Mouse Pituitary Gland. Proceedings of the National Academy of Sciences of the United States of America 105, 2907-12.

Fluck, C., Deladoey, J., Rutishauser, K., Eble, A., Marti, U., Wu, W., Mullis, P.E., 1998. Phenotypic Variability in Familial Combined Pituitary Hormone Deficiency Caused by a PROP1 Gene Mutation Resulting in the Substitution of Arg-->Cys at Codon 120 (R120C). J Clin Endocrinol Metab 83, 3727-3734.

Gage, P.J., Brinkmeier, M.L., Scarlett, L.M., Knapp, L.T., Camper, S.A., Mahon, K.A., 1996a. The Ames Dwarf Gene, Df, is Required Early in Pituitary Ontogeny for the Extinction of Rpx Transcription and Initiation of Lineage-Specific Cell Proliferation. Molecular endocrinology (Baltimore, Md) 10, 1570-81.

Gage, P.J., Roller, M.L., Saunders, T.L., Scarlett, L.M., Camper, S.A., 1996b. Anterior Pituitary Cells Defective in the Cell-Autonomous Factor, Df, Undergo Cell Lineage Specification but Not Expansion. Development (Cambridge, England) 122, 151-60.

Garcia-Lavandeira, M., Quereda, V., Flores, I., Saez, C., Diaz-Rodriguez, E., Japon, M.A., Ryan, A.K., Blasco, M.A., Dieguez, C., Malumbres, M., Alvarez, C.V., 2009. A GRFa2/Prop1/Stem (GPS) Cell Niche in the Pituitary. PLoS One 4, e4815.

Gaston-Massuet, C., Andoniadou, C.L., Signore, M., Jayakody, S.A., Charolidi, N., Kyeyune, R., Vernay, B., Jacques, T.S., Taketo, M.M., Le Tissier, P., Dattani, M.T., Martinez-Barbera, J.P., 2011. Increased Wingless (Wnt) Signaling in Pituitary

Progenitor/Stem Cells Gives Rise to Pituitary Tumors in Mice and Humans. Proc Natl Acad Sci U S A 108, 11482-11487.

Geling, A., Steiner, H., Willem, M., Bally-Cuif, L., Haass, C., 2002. A Gamma-Secretase Inhibitor Blocks Notch Signaling in Vivo and Causes a Severe Neurogenic Phenotype in Zebrafish. EMBO Rep 3, 688-694.

Goldberg, L.B., Aujla, P.K., Raetzman, L.T., 2011. Persistent Expression of Activated Notch Inhibits Corticotrope and Melanotrope Differentiation and Results in Dysfunction of the HPA Axis. Dev Biol 358, 23-32.

Gremeaux, L., Fu, Q., Chen, J., Vankelecom, H., 2012. Activated Phenotype of the Pituitary Stem/Progenitor Cell Compartment during the Early-Postnatal Maturation Phase of the Gland. Stem cells and development 21, 801-13.

Hebert, J.M., McConnell, S.K., 2000. Targeting of Cre to the Foxg1 (BF-1) Locus Mediates loxP Recombination in the Telencephalon and Other Developing Head Structures. Dev Biol 222, 296-306.

Himes, A.D., Raetzman, L.T., 2009. Premature Differentiation and Aberrant Movement of Pituitary Cells Lacking both Hes1 and Prop1. Developmental biology 325, 151-61.

Jayakody, S.A., Andoniadou, C.L., Gaston-Massuet, C., Signore, M., Cariboni, A., Bouloux, P.M., Le Tissier, P., Pevny, L.H., Dattani, M.T., Martinez-Barbera, J.P., 2012. SOX2 Regulates the Hypothalamic-Pituitary Axis at Multiple Levels. J Clin Invest 122, 3635-3646.

Kashiwabara, Y., Sasaki, S., Matsushita, A., Nagayama, K., Ohba, K., Iwaki, H., Matsunaga, H., Suzuki, S., Misawa, H., Ishizuka, K., Oki, Y., Nakamura, H., 2009. Functions of PIT1 in GATA2-Dependent Transactivation of the Thyrotropin Beta Promoter. Journal of molecular endocrinology 42, 225-37.

Kelberman, D., Rizzoti, K., Lovell-Badge, R., Robinson, I.C., Dattani, M.T., 2009. Genetic Regulation of Pituitary Gland Development in Human and Mouse. Endocr Rev 30, 790-829.

Kita, A., Imayoshi, I., Hojo, M., Kitagawa, M., Kokubu, H., Ohsawa, R., Ohtsuka, T., Kageyama, R., Hashimoto, N., 2007. Hes1 and Hes5 Control the Progenitor Pool, Intermediate Lobe Specification, and Posterior Lobe Formation in the Pituitary Development. Molecular endocrinology (Baltimore, Md) 21, 1458-66.

Kopan, R., Ilagan, M.X.G., 2009. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. Cell 137, 216-33.

Li, Y., Hibbs, M.A., Gard, A.L., Shylo, N.A., Yun, K., 2012. Genome-Wide Analysis of N1ICD/RBPJ Targets in Vivo Reveals Direct Transcriptional Regulation of Wnt, SHH, and Hippo Pathway Effectors by Notch1. Stem cells (Dayton, Ohio) 30, 741-52.

McCright, B., Lozier, J., Gridley, T., 2006. Generation of New Notch2 Mutant Alleles. Genesis (New York, N Y : 2000) 44, 29-33.

Monahan, P., Rybak, S., Raetzman, L.T., 2009. The Notch Target Gene HES1 Regulates Cell Cycle Inhibitor Expression in the Developing Pituitary. Endocrinology 150, 4386-94.

Moran, T.B., Goldberg, L.B., Serviss, S.L., Raetzman, L.T., 2011. Numb Deletion in POMC-Expressing Cells Impairs Pituitary Intermediate Lobe Cell Adhesion, Progenitor Cell Localization, and Neuro-Intermediate Lobe Boundary Formation. Mol Endocrinol 25, 117-127.

Mortensen, A.H., MacDonald, J.W., Ghosh, D., Camper, S.A., 2011. Candidate Genes for Panhypopituitarism Identified by Gene Expression Profiling. Physiol Genomics 43, 1105-1116.

Murtaugh, L.C., Stanger, B.Z., Kwan, K.M., Melton, D.A., 2003. Notch Signaling Controls Multiple Steps of Pancreatic Differentiation. Proceedings of the National Academy of Sciences of the United States of America 100, 14920-5.

Nasonkin, I.O., Ward, R.D., Raetzman, L.T., Seasholtz, A.F., Saunders, T.L., Gillespie, P.J., Camper, S.A., 2004. Pituitary Hypoplasia and Respiratory Distress Syndrome in Prop1 Knockout Mice. Human molecular genetics 13, 2727-35.

Ohtsuka, T., Sakamoto, M., Guillemot, F., Kageyama, R., 2001. Roles of the Basic Helix-Loop-Helix Genes Hes1 and Hes5 in Expansion of Neural Stem Cells of the Developing Brain. The Journal of biological chemistry 276, 30467-74.

Patten, B.A., Peyrin, J.M., Weinmaster, G., Corfas, G., 2003. Sequential Signaling through Notch1 and erbB Receptors Mediates Radial Glia Differentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 23, 6132-40.

Pernasetti, F., Toledo, S.P., Vasilyev, V.V., Hayashida, C.Y., Cogan, J.D., Ferrari, C., Lourenco, D.M.,Jr, Mellon, P.L., 2000. Impaired Adrenocorticotropin-Adrenal Axis in Combined Pituitary Hormone Deficiency Caused by a Two-Base Pair Deletion (301-302delAG) in the Prophet of Pit-1 Gene. J Clin Endocrinol Metab 85, 390-397.

Prince, K.L., Walvoord, E.C., Rhodes, S.J., 2011. The Role of Homeodomain Transcription Factors in Heritable Pituitary Disease. Nat Rev Endocrinol 7, 727-737.

Raetzman, L.T., Ross, S.A., Cook, S., Dunwoodie, S.L., Camper, S.A., Thomas, P.Q., 2004. Developmental Regulation of Notch Signaling Genes in the Embryonic Pituitary: Prop1 Deficiency Affects Notch2 Expression. Developmental biology 265, 329-40.

Raetzman, L.T., Cai, J.X., Camper, S.A., 2007. Hes1 is Required for Pituitary Growth and Melanotrope Specification. Developmental biology 304, 455-66.

Raetzman, L.T., Wheeler, B.S., Ross, S.A., Thomas, P.Q., Camper, S.A., 2006. Persistent Expression of Notch2 Delays Gonadotrope Differentiation. Molecular endocrinology (Baltimore, Md) 20, 2898-908.

Regal, M., Paramo, C., Sierra, S.M., Garcia-Mayor, R.V., 2001. Prevalence and Incidence of Hypopituitarism in an Adult Caucasian Population in Northwestern Spain. Clin Endocrinol (Oxf) 55, 735-740.

Rizzoti, K., Akiyama, H., Lovell-Badge, R., 2013. Mobilized Adult Pituitary Stem Cells Contribute to Endocrine Regeneration in Response to Physiological Demand. Cell Stem Cell 13, 419-432.

Rizzoti, K., 2010. Adult Pituitary Progenitors/Stem Cells: From in Vitro Characterization to in Vivo Function. The European journal of neuroscience 32, 2053-62.

Sastre, M., Steiner, H., Fuchs, K., Capell, A., Multhaup, G., Condron, M.M., Teplow, D.B., Haass, C., 2001. Presenilin-Dependent Gamma-Secretase Processing of Beta-Amyloid Precursor Protein at a Site Corresponding to the S3 Cleavage of Notch. EMBO Rep 2, 835-841.

Siveke, J.T., Lubeseder-Martellato, C., Lee, M., Mazur, P.K., Nakhai, H., Radtke, F., Schmid, R.M., 2008. Notch Signaling is Required for Exocrine Regeneration After Acute Pancreatitis. Gastroenterology 134, 544-55.

Sornson, M.W., Wu, W., Dasen, J.S., Flynn, S.E., Norman, D.J., O'Connell, S.M., Gukovsky, I., Carrière, C., Ryan, A.K., Miller, A.P., Zuo, L., Gleiberman, A.S., Andersen, B., Beamer, W.G., Rosenfeld, M.G., 1996. Pituitary Lineage Determination by the Prophet of Pit-1 Homeodomain Factor Defective in Ames Dwarfism. Nature 384, 327-33.

Suga, H., Kadoshima, T., Minaguchi, M., Ohgushi, M., Soen, M., Nakano, T., Takata, N., Wataya, T., Muguruma, K., Miyoshi, H., Yonemura, S., Oiso, Y., Sasai, Y., 2011. Self-Formation of Functional Adenohypophysis in Three-Dimensional Culture. Nature 480, 57-62.

Tando, Y., Fujiwara, K., Yashiro, T., Kikuchi, M., 2013. Localization of Notch Signaling Molecules and their Effect on Cellular Proliferation in Adult Rat Pituitary. Cell Tissue Res 351, 511-519.

Taniguchi, Y., Yasutaka, S., Kominami, R., Shinohara, H., 2001. Proliferation and Differentiation of Pituitary Somatotrophs and Mammotrophs during Late Fetal and Postnatal Periods. Anatomy and embryology 204, 469-75.

Taniguchi, Y., Kominami, R., Yasutaka, S., Kawarai, Y., 2000. Proliferation and Differentiation of Pituitary Corticotrophs during the Fetal and Postnatal Period: A Quantitative Immunocytochemical Study. Anatomy and embryology 201, 229-34.

Taniguchi, Y., Yasutaka, S., Kominami, R., Shinohara, H., 2002a. Mitoses of Thyrotrophs Contribute to the Proliferation of the Rat Pituitary Gland during the Early Postnatal Period. Anatomy and embryology 206, 67-72.

Taniguchi, Y., Yasutaka, S., Kominami, R., Shinohara, H., 2002b. Proliferation and Differentiation of Rat Anterior Pituitary Cells. Anatomy and embryology 206, 1-11.

Wang, H., Zou, J., Zhao, B., Johannsen, E., Ashworth, T., Wong, H., Pear, W.S., Schug, J., Blacklow, S.C., Arnett, K.L., Bernstein, B.E., Kieff, E., Aster, J.C., 2011. Genome-Wide Analysis Reveals Conserved and Divergent Features of Notch1/RBPJ Binding in Human and Murine T-Lymphoblastic Leukemia Cells. Proceedings of the National Academy of Sciences of the United States of America 108, 14908-13.

Wang, Y., Martin, J.F., Bai, C.B., 2010. Direct and Indirect Requirements of Shh/Gli Signaling in Early Pituitary Development. Developmental biology 348, 199-209.

Ward, R.D., Raetzman, L.T., Suh, H., Stone, B.M., Nasonkin, I.O., Camper, S.A., 2005. Role of PROP1 in Pituitary Gland Growth. Molecular endocrinology (Baltimore, Md) 19, 698-710.

Weng, A.P., Millholland, J.M., Yashiro-Ohtani, Y., Arcangeli, M.L., Lau, A., Wai, C., Del Bianco, C., Rodriguez, C.G., Sai, H., Tobias, J., Li, Y., Wolfe, M.S., Shachaf, C., Felsher, D., Blacklow, S.C., Pear, W.S., Aster, J.C., 2006. C-Myc is an Important Direct Target of Notch1 in T-Cell Acute Lymphoblastic Leukemia/Lymphoma. Genes & development 20, 2096-109.

Wu, W., Cogan, J.D., Pfäffle, R.W., Dasen, J.S., Frisch, H., O'Connell, S.M., Flynn, S.E., Brown, M.R., Mullis, P.E., Parks, J.S., Phillips, J.A.(., Rosenfeld, M.G., 1998. Mutations in PROP1 Cause Familial Combined Pituitary Hormone Deficiency. Nature genetics 18, 147-9.

Zhu, X., Gleiberman, A.S., Rosenfeld, M.G., 2007. Molecular Physiology of Pituitary Development: Signaling and Transcriptional Networks. Physiological reviews 87, 933-63.

Zhu, X., Zhang, J., Tollkuhn, J., Ohsawa, R., Bresnick, E.H., Guillemot, F., Kageyama, R., Rosenfeld, M.G., 2006. Sustained Notch Signaling in Progenitors is Required for Sequential Emergence of Distinct Cell Lineages during Organogenesis. Genes & development 20, 2739-53.

TABLES AND FIGURES

Gene	Forward Primer	Reverse Primer
Gapdh	GGTGAGGCCGGTGCTGAGTATG	GACCCGTTTGGCTCCACCCTTC
Dll1	TAACCTCGTTCGAGACCTCAAGGG	TGGCACTTGGTGTCACGTTTGC
Jag1	TGTAGCCAGAAAGTGACTGGTTG	TGTAGCCAGAAAGTGACTGGTTG
Jag2	TGGACACGAAAGCGCAGGAAAG	ACTGGTTGTTGGCGCTCTCATC
Notch1	TTCCATGATTGCCTCCTGCAGTGG	CAGGATCAGTGGAGTTGTGCCATCATGCA
Notch2	TGCCAATACTCCACCTCTC	TCCACTGACACTGCTTCC
Notch3	GAAGACCCTTGCCACTCAG	CCACGGAGACAACGACAG
Notch4	GAGGACCTGGTTGAAGAATTGATC	TGCAGTTTTTTCCCTTTTATCCC
Hes1	CTCGCTCACTTCGGACTC	CTCGCTCACTTCGGACTC
Hey1	CACGCCACTATGCTCAAT	CCTTCACCTCACTGCTCTG
Hey2	GATTCCGAGAGTGCTTGAC	AGGTGCTGAGATGAGAGAC
Heyl	GGAACAACAGAGAATGAAC	CAGCAGTAGTGAGTAACC
Prop1	CCAGAACCGCAGGGCTAAG	GGCTATCGGCTGGAGAAGTG
Sox2	GGAGAAAGAAGAGGAGAGAG	CTGGCGGAGAATAGTTGG
Sox9	AGCCGACTCCCACATTTCCTCC	GAGATTGCCCAGAGTGCTCGCCC
mKi67	CCAGGGATCTCAGCGCAATTACAG	GGATAGGACAGAGGGCCACATTC
Ccne1	GGCTCCGACCTTTCAGTCCGC	TGGGTCTTGCAAAAACACGGCC
Cdkn1c	TCCATCACCAATCAGCCAGCAGAA	ATCGCTGGAGGCCAAGCGTTC
Ccnd1	CGCGCGTACCCTGACACCAAT	CTCCTCACAGACCTCCAGCATCCA
Pou1f1	AGCTGAGCAGGTCGGAGCTTTGT	GGAAGGCTTGCTGTGCTCCCC
Gh	AACTGAAGGACCTGGAAGAG	GGCGTCAAACTTGTCATAGG
Tshb	TGGTCAGGCAGTAGGCACACTCT	GTGGGTGGAGAAGAGTGAGC
Tpit	GATGCCAAGGAGAGAAACC	AGCTTTTCTATCAAATTCACTGA
Pomc	GTTACGGTGGCTTCATGACCTC	CGCGTTCTTGATGATGGCGTTC
Crhr1	TTTGCTGCCCTGCACCTTTAGAC	AGTGACAGAAAGCTGGCTTGGC
Pax7	GCACAGAGGACCAAGCTCAC	TGGTGGTGGGGTAGGTAGAG
Gnrhr	ATGATGGTCGTGATTAGC	ATTGCGAGAAGACTGTGG
Nr5a1	AAATTCCTGAACAACCACAGC	GCATCTCAATGAGAAGGTTG
Lhb	CCCAGTCTGCATCACCTTCAC	GAGGCACAGGAGGCAAAGC

Table 3.1. Primers used for qRT-PCR analysis.

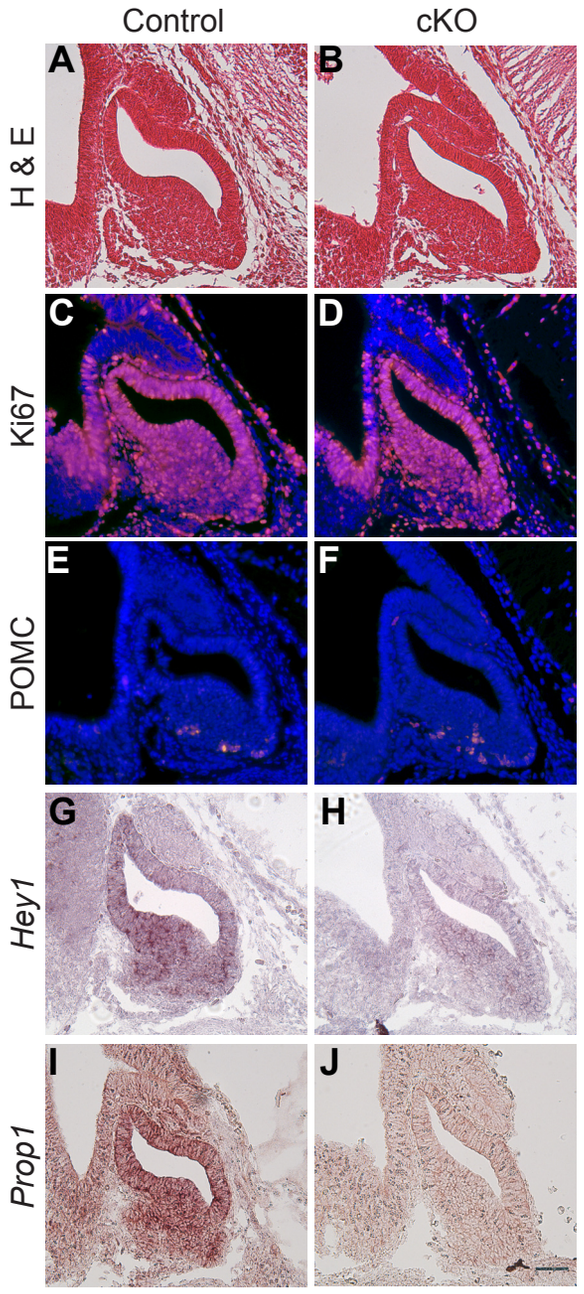

Figure 3.1. Conditional loss of *Notch2* results in decreased *Prop1* mRNA at e12.5. Sagittal sections of control (A) and cKO (B) e12.5 pituitaries were stained with hematoxylin and eosin and developing pituitaries appear roughly the same shape and size. Ki67 immunostaining is similar between control (C) and cKO (D) pituitaries. POMC is expressed in few cells in the pituitaries of control (E) and cKO (F) mice. The canonical Notch target *Hey1* is expressed in the developing pituitary in control mice (G) and appears reduced in cKO mice (H). *Prop1* is expressed in control mice (I) and appears reduced in cKO mice (J). Photos taken at 200x. Scale bar: 50μm. n=4-6.

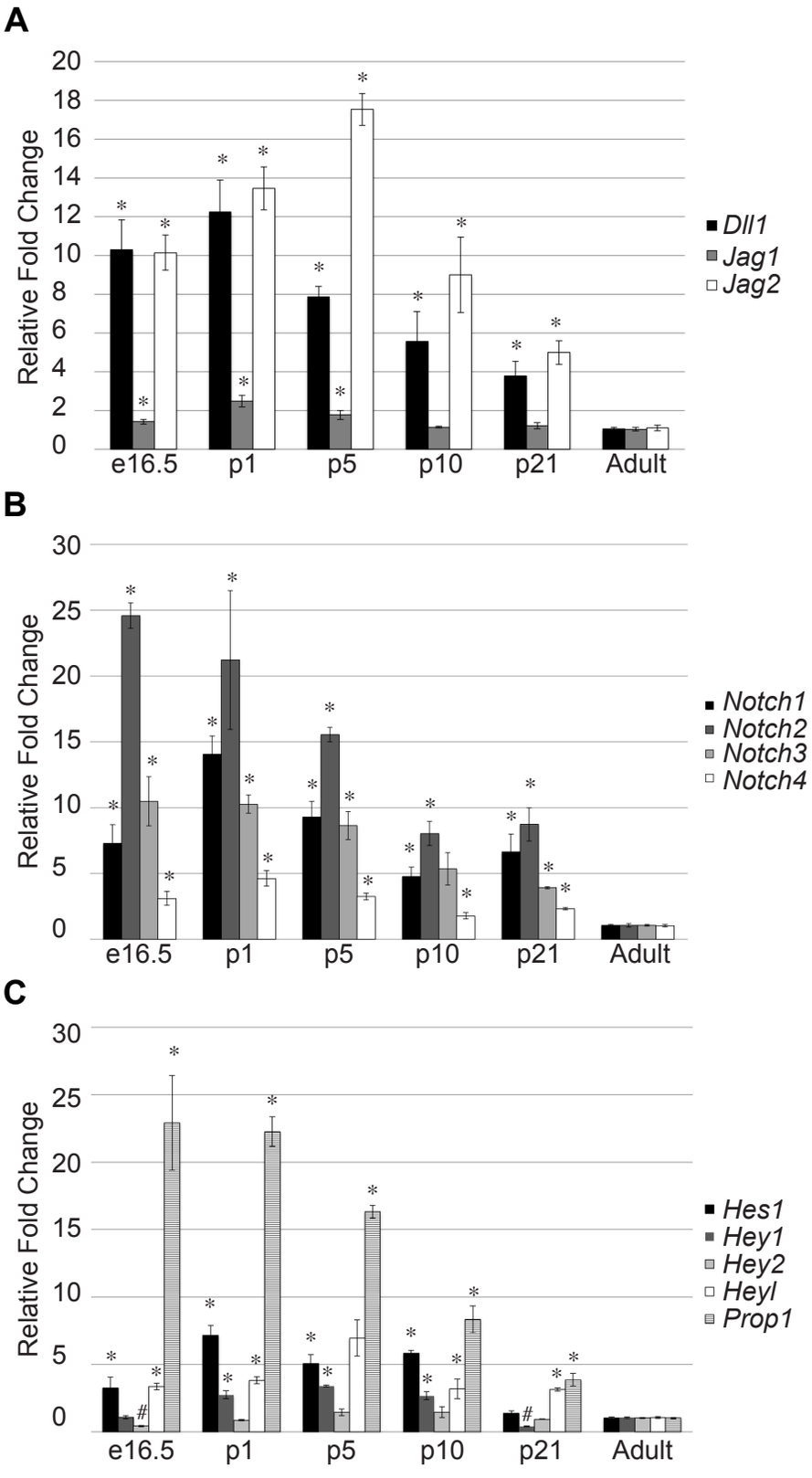

Figure 3.2. Characterization of mRNA levels of Notch family members in the pituitary during early postnatal development. mRNA levels of Notch ligands (A), receptors (B) and targets (C) were measured at e16.5, p1, p5, p10 and p21 and were compared to levels found in adult pituitaries. *:p< 0.05, significantly increased compared to adult control. #:p< 0.05, significantly decreased compared to adult control. n=4.

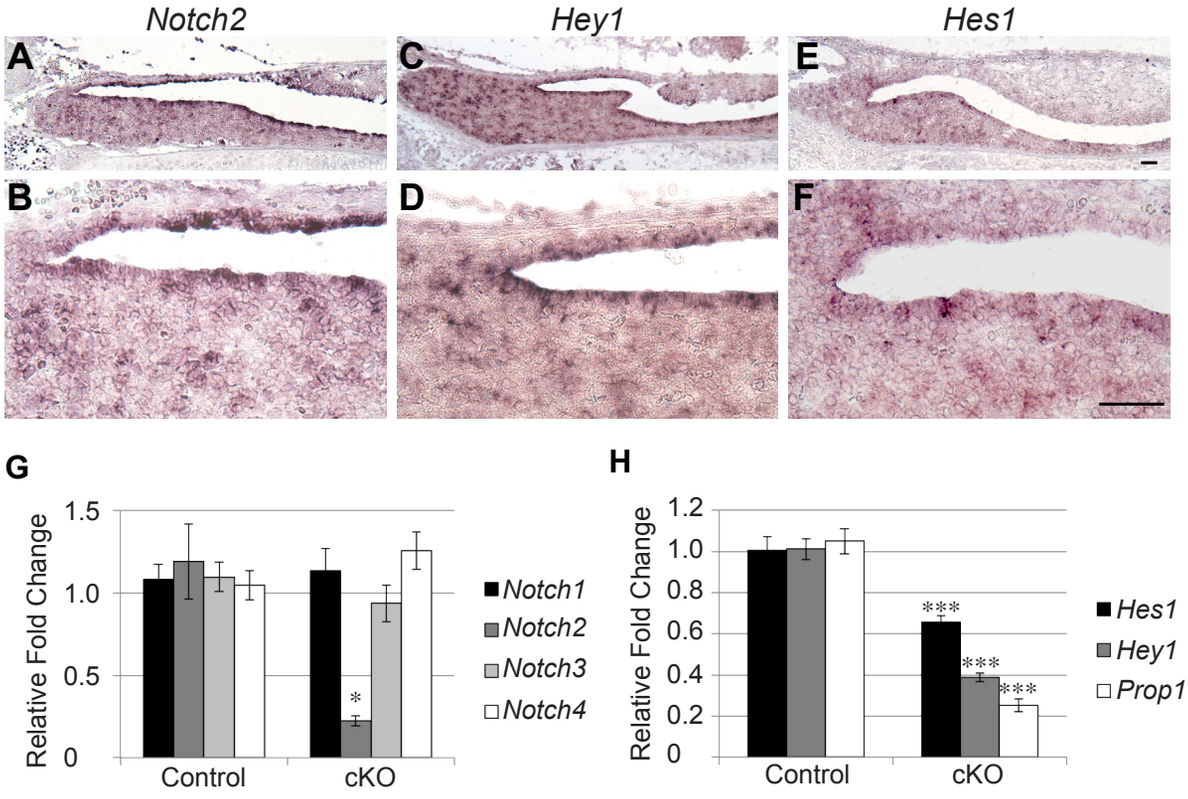

Figure 3.3. Localization of Notch family members in the pituitary at p5. *In situ* hybridization for *Notch2* (A and B), *Hey1* (C and D) and *Hes1* (E and F) reveals that Notch signaling components are mainly localized to cells surrounding the pituitary cleft. qRT-PCR comparing control and *Notch2* cKO pituitaries at p1 shows specific and significant reductions in mRNA levels of *Notch2* (G), *Hes1*, *Hey1*, and *Prop1* (H). *:p< 0.05. ***:p< 0.001. Scale bar: 50µm. n=3-4 (in situ hybridization) and n=4 (qRT-PCR).

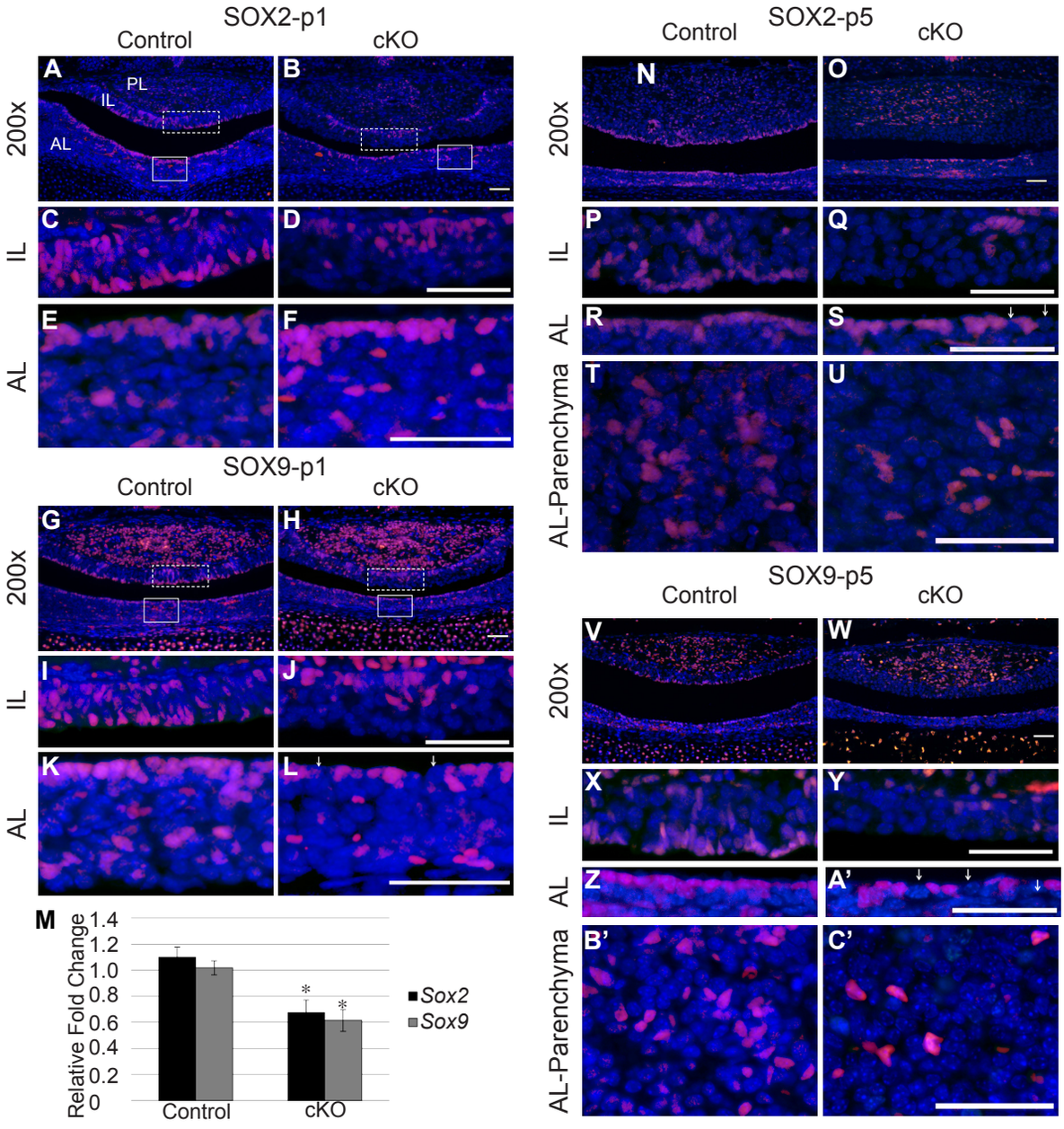

Figure 3.4. Loss of Notch2 results in misplacement of pituitary progenitors at p1 and decreased progenitor number at p5. SOX2 is expressed at p1 in cells lining the pituitary cleft and in scattered cells in the intermediate lobe (IL) and anterior lobe (AL) of control mice (A, C and E). In contrast, in cKO mice, SOX2 is only present in cells in the dorsal IL and is absent from IL cleft cells (B and D), while AL expression of SOX2 appears to be unaffected in cKO mice (B and F). SOX9 follows a similar expression pattern to that of SOX2 in control mice (G, I and K). SOX9 is restricted to dorsal IL cells (H and J) and appears to be reduced in the AL (H and L) of cKO mice. mRNA levels of *Sox2* and *Sox9* are reduced in whole pituitaries of cKO mice at p1 as compared to control (M). SOX2 is expressed in cells lining the cleft in the AL and IL in control mice at p5 (N,P,R). In cKO mice, SOX2 containing cells in the IL and the AL are reduced (O,Q,S). In the AL parenchyma, SOX2 cells are seen in clusters in control mice (T) and appear reduced in cKO mice (U). SOX9 is also expressed in cells lining the cleft in the AL and IL in control mice (V, X, Z) and has similar reductions in cKO mice (W,Y,A') as those seen with SOX2. In control mice, SOX9 is seen in clusters in the AL parenchyma (B'). These clusters are not observed in cKO mice (C'). Photos taken at 200x (A, B, G, H, N, O, V, W), and 400x (C-F, I-L, P-U and X-C'). White boxes (A, B, G and H) indicate where higher magnification photos were taken. Arrows (L, S and A') indicate SOX9 and SOX2-negative cleft cells. Scale bars: 25μm (E, F, K, L) and 50μm (A-D, G-J, N-C'). *:p< 0.05. n=6-8 (histology) and n=4-9 (qRT-PCR). AL=anterior lobe. IL=intermediate lobe.

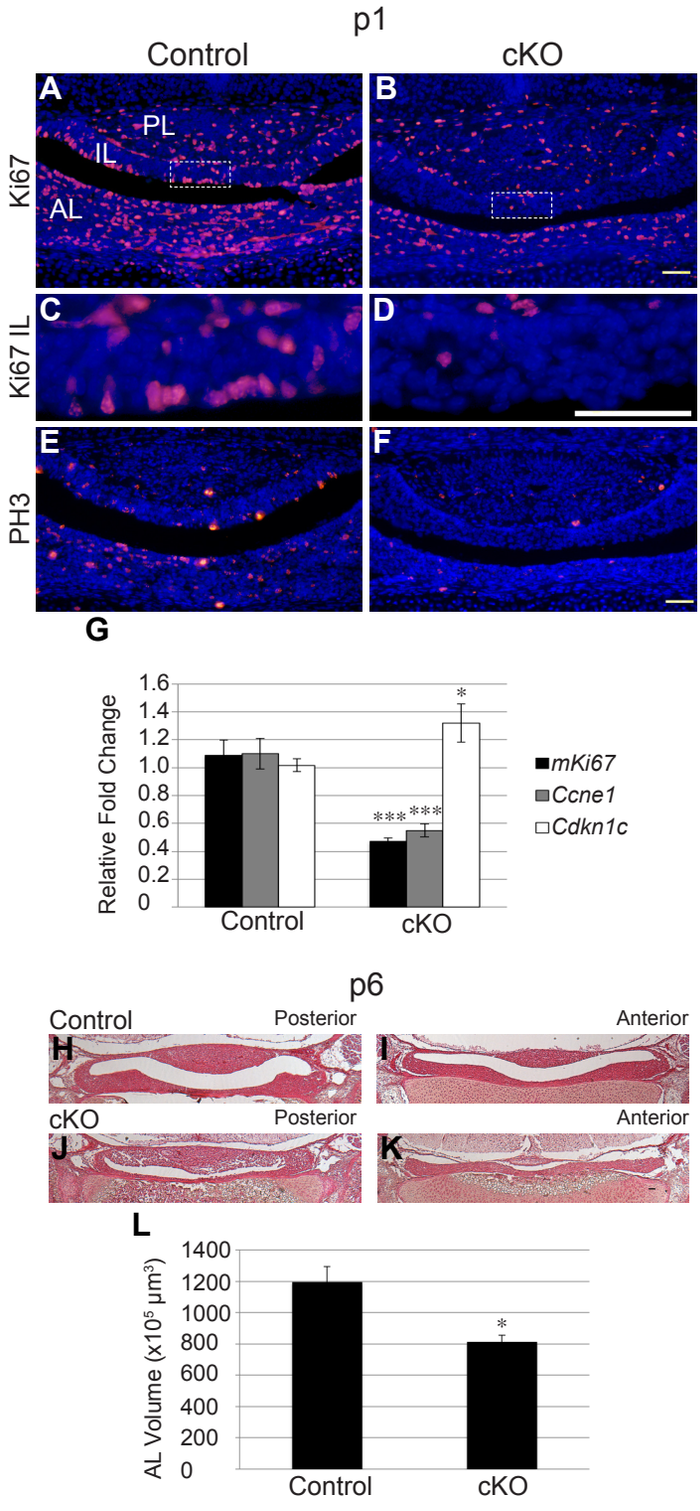

Figure 3.5. Decreased proliferation is observed in mice with conditional loss of Notch2, resulting in a hypoplastic pituitary by p6. Ki67 immunoreactive cells are found in the AL and IL of control (A and C) pituitaries at p1. The number of Ki67-immunopositive cells appears to be reduced in both lobes of cKO (B and D) mice. Phospho-histone H3 (PH3) expression is scattered throughout the pituitary at p1 in control (E) mice and is markedly decreased in cKO (F) pituitaries. qRT-PCR in whole pituitaries at p1 reveals a significant decrease in mRNA levels of *mKi67* and *Ccne1* and a significant increase in *Cdkn1c* mRNA in cKO mice as compared to controls (G). Hematoxylin and eosin staining reveals a hypoplastic AL at p6 in cKO mice (J and K) as compared to control mice (H and I). Volume of AL at p6 is decreased in cKO mice. Photos taken at 50x (H-K), 200x (A, B, E and F) and 400x (C and D). White box (A and B) indicates where higher magnification photos were taken. Scale bars: 50µm. *:p< 0.05. ***:p<0.001. n=6-7 (immunohistochemistry), n=4-7 (qRT-PCR) and n=3-4 (AL volume approximation). PL=posterior lobe. AL=anterior lobe. IL=intermediate lobe.

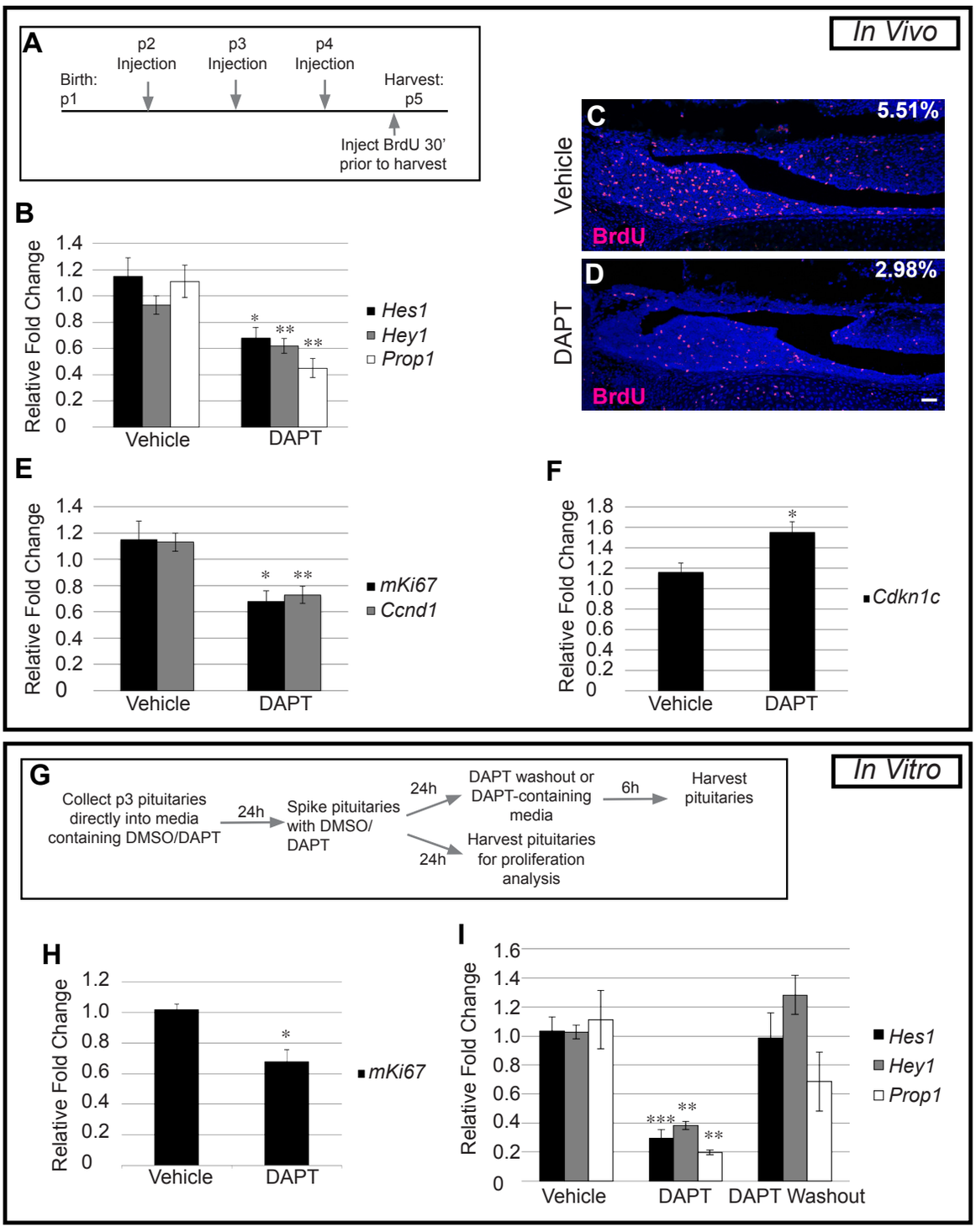

Figure 3.6. Postnatal Notch inhibition results in decreased proliferation and identifies Prop1 as a direct target. Experimental design for in vivo DAPT injection (A). Subcutaneous injection with DAPT results in a decrease in whole pituitary mRNA levels of Hes1, Hey1 and Prop1 as compared to vehicle-injected mouse pituitaries (B). BrdU-positive cells (red) are present throughout the pituitaries of vehicle-treated mice (C, 5.51% ± 0.20%) and are significantly reduced (p=0.0001) in pituitaries of DAPT-treated mice (D, 2.98% ± 0.26%). mRNA levels of mKi67 and Ccnd1 in whole pituitaries are decreased after DAPT injection as compared to vehicle injection (E). Conversely, mRNA levels of Cdkn1c are increased in the whole pituitary of DAPT-injected mice (F). Experimental design for in vitro DAPT treatment and DAPT washout assay (G). After 48h in culture, mRNA levels of mKi67 are significantly decreased compared to vehicle-treated controls (H). mRNA levels of Hes1, Hey1 and Prop1 are reduced after DAPT treatment and recover after 6h DAPT washout (I). Photos taken at 100x. Scale bars: 50μm. *:p< 0.05. **:p<0.01. ***:p<0.001. n=4 (immunohistochemistry), n=5-8 (qRT-PCR in vivo) and n=4-8 (qRT-PCR in vitro).

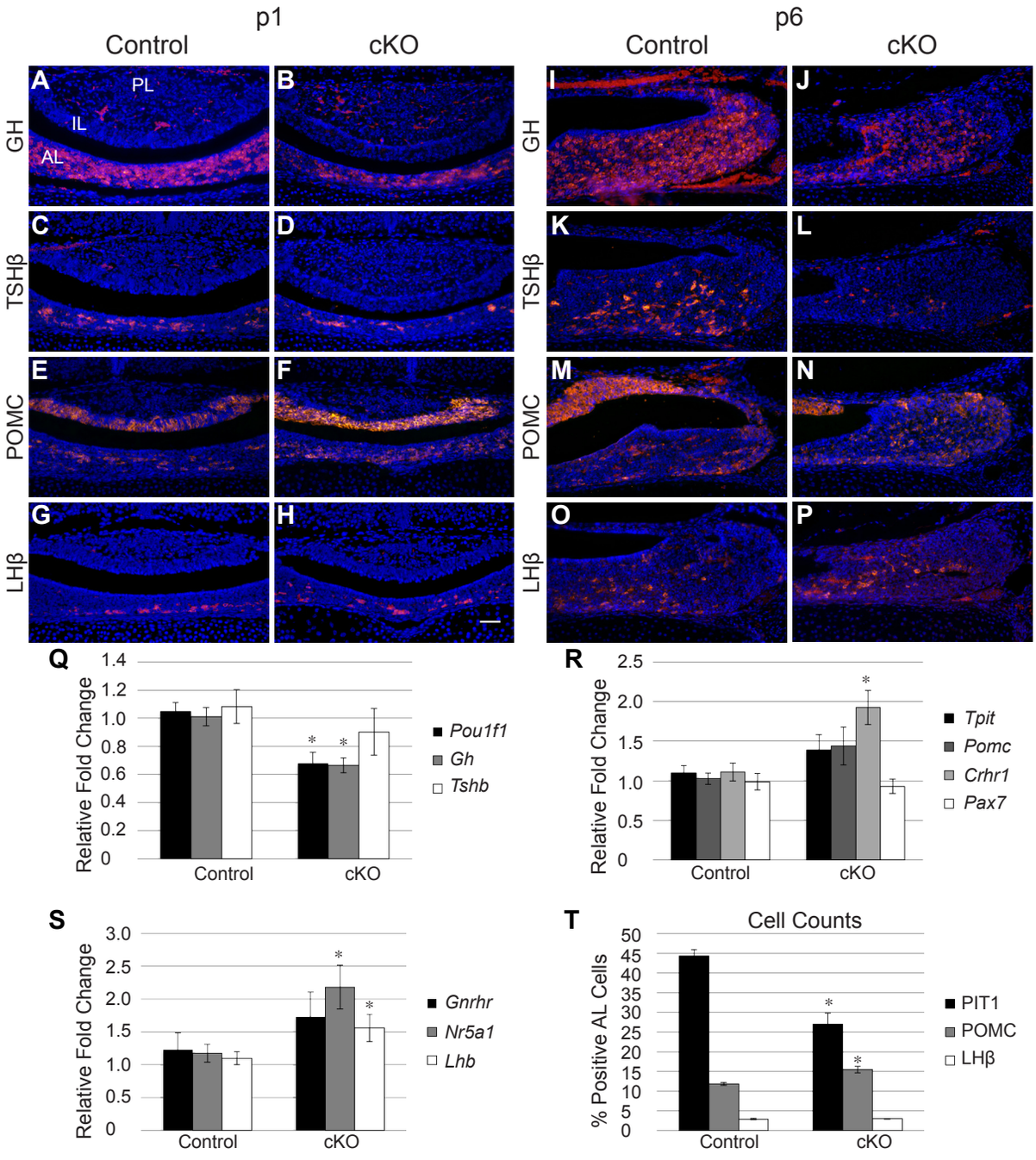

Figure 3.7. Conditional loss of Notch2 results in decreased expression of Pit1-lineage hormones and increased expression of POMC. GH immunopositive cells are found scattered throughout the AL of control (A) mice and appear to be reduced in pituitaries of cKO (B) at p1. TSHβ is localized to the AL of control (C) and cKO (D) mice. POMC immunoreactive cells are found in the AL and the IL of control (E) pituitaries and appear slightly increased in the AL of cKO mice (F). LHβ expression is confined to the AL and appears similar between control (G) and cKO (H) mice. At p6, GH is present in the AL of control (I) mice and appears reduced in cKO mice (J). Similarly, TSHβ is strongly reduced in cKO mice (L) as compared to control (K). POMC is present in the IL and AL of control mice (M) and appears slightly increased in the AL of cKO mice (N). LHβ appears similar between control (O) and cKO (P) pituitaries. qRT-PCR analysis at p1 reveals a significant decrease in *Pou1f1* (*Pit1*) and *Gh* mRNA, but no change in *Tshb* mRNA between control and cKO mice (Q). Additionally, p1 mRNA levels of *Tpit* and *Pomc* show a trend towards an increase in cKO mice, whereas *Crhr1* is significantly increased and *Pax7* is unchanged (R). mRNA levels of *Nr5a1*, and *Lhb* are significantly increased in pituitaries, while *Gnrhr* is trending towards an increase at p1 (S). The percent of Pit1-, POMC- and LHβ-positive cells in the AL was quantified in control and cKO mice and the percent of Pit1-positive cells was found to be deceased in cKO mice, whereas the percent of POMC-immunoreactive cells is increased (T). *: $p < 0.05$. Photos taken at 200x. Scale bars: 50μm. n=5-7 (histology), n=4-7 (qRT-PCR) and n=3-5 (cell counts). PL=posterior lobe. AL=anterior lobe. IL=intermediate lobe.

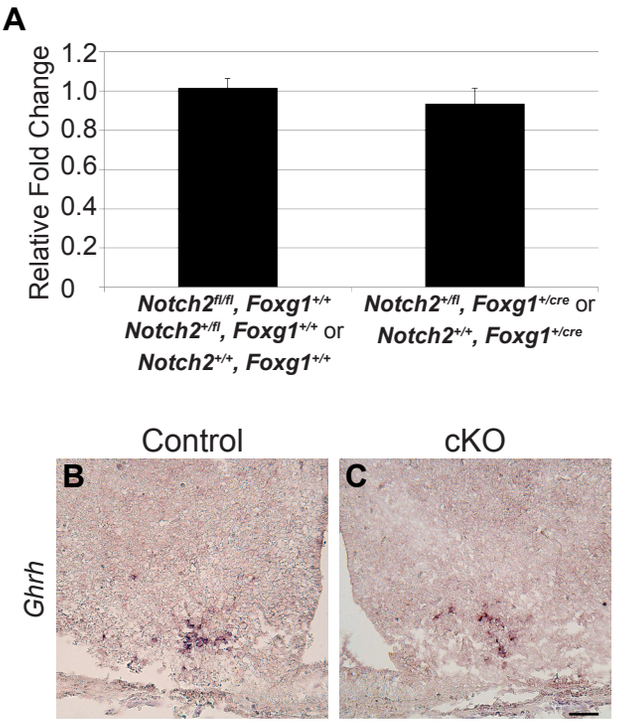

Figure 3.8. *Foxg1* Cre mice do not display altered pituitary development or ability to influence hypothalamic Ghrh neuron number. Gh mRNA levels in whole pituitaries of p1 mice with and without a Cre transgene were similar (A). Ghrh mRNA expression is similar in the arcuate nucleus between control (B) and cKO (C) mice at p1. Photos taken at 200x. Scale bars: 50μm. n=3 (in situ hybridization) and n=5-8 (qRT-PCR).

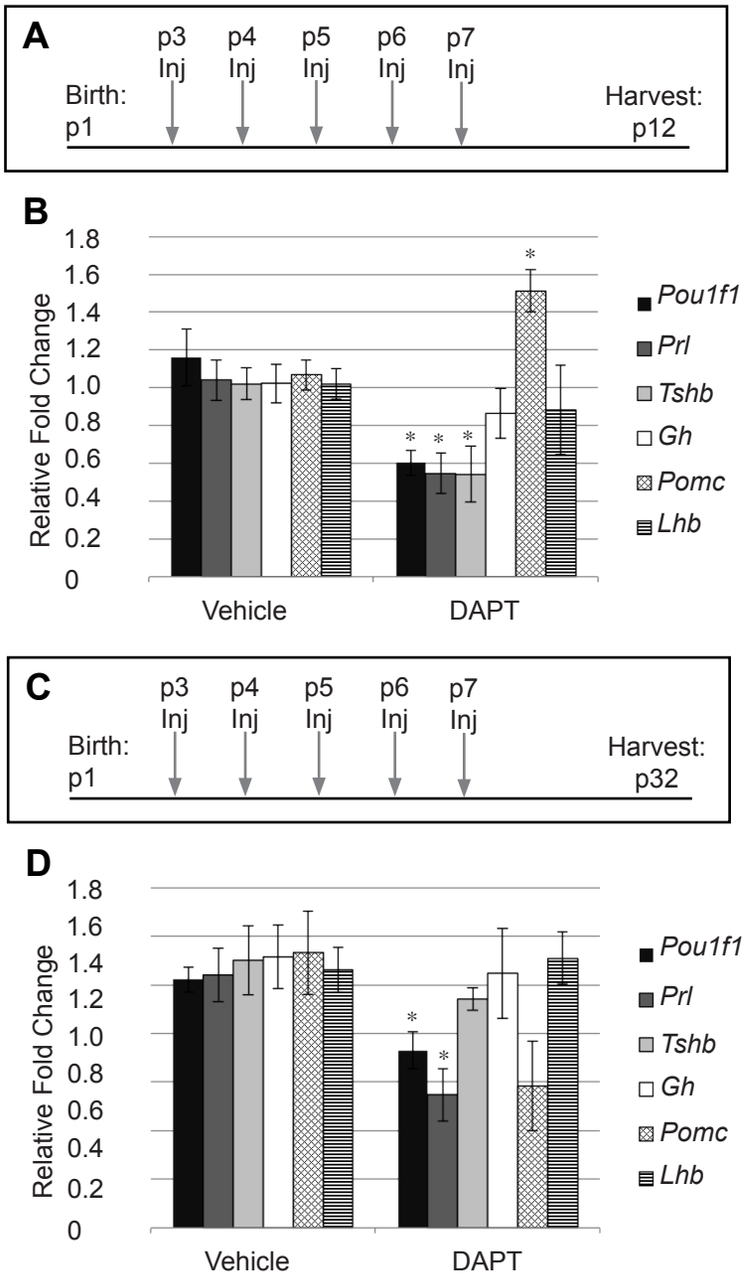

Figure 3.9. Postnatal chemical Notch inhibition results in decreased mRNA levels of *Pou1f1* (*Pit1*), Prl, and Tshb and increased levels of *Pomc*. Experimental design for *in vivo* subcutaneous DAPT injection (A). mRNA levels of *Pou1f1*, *Prl* and *Tshb* are significantly decreased and mRNA levels of *Pomc* are significantly increased in whole pituitaries after DAPT injection as compared to vehicle-treated mice. mRNA levels of *Gh* and *Lhb* are unchanged between the two groups (B). Experimental design for *in vivo* subcutaneous DAPT injection longer paradigm (C). mRNA levels of *Pou1f1* and *Prl* are decreased in whole pituitaries after DAPT injection as compared to vehicle-treated mice. mRNA levels of all other hormones are unchanged between the two groups (D). *: $p < 0.05$. n=3-5. *: $p < 0.05$. n=3-5.

CHAPTER 4: **Notch signaling regulates proliferation and cell fate determination genes in the pituitary**

ABSTRACT

During embryonic and postnatal development, pituitary progenitors proliferate and differentiate in response to internal and external cues. Specific factors that are important for regulating these processes remain unclear. Previous work shows that Notch signaling components are elevated during embryonic and postnatal pituitary development and help to regulate proliferation and corticotrope cell number (Chapters 2 and 3). To identify transcriptional targets through which Notch regulates these processes, microarray analysis was performed to compare the pituitaries of control and *Notch2* cKO. Gene ontology analysis shows that many of the altered transcripts in *Notch2* cKO mice are implicated in cell division events, including mitosis, replication and cell cycle control. Additionally, we identified a subset of genes that are changed in *Notch2* cKO mice that are important for corticotrope differentiation and signaling. One such gene, *Hsd11b1*, which encodes for the 11β-HSD1, is an important factor in regulating local corticosteroid levels, which are known to affect corticotrope number. We confirmed that *Hsd11b1* is decreased in *Notch2* cKO mice and in mice or pituitary explants treated postnatally with DAPT, a chemical Notch inhibitor. Scanning the *Hsd11b1* promoter for candidate regulatory sequences of Notch signaling reveals three Hey1 binding sites, suggesting a mechanism by which the Notch pathway may control expression of this enzyme. Expression analysis shows that *Hsd11b1* is expressed at very low levels at e16.5 and

increases by p1. Additionally, we show that 11β-HSD1 is mostly present in POMC-expressing melanotropes and corticotropes. This study is the first to show that Notch signaling can alter *Hsd11b1* levels and suggests a mechanism by which Notch may influence corticotrope cell number and pituitary proliferation.

INTRODUCTION

All pituitary endocrine cells are derived from a common pool of progenitors that express the stem cell markers SOX2 and SOX9 (Fauquier, et al. 2008; Andoniadou, et al. 2013; Rizzoti, et al. 2013). During early postnatal development, these progenitor cells remain highly proliferative and integrate intrinsic and extrinsic signals to help them choose between proliferation and endocrine differentiation (Gaston-Massuet, et al. 2011; Gremeaux, et al. 2012). Intrinsic factors that influence postnatal pituitary proliferation include cell cycle control protein Cdk4 (Jirawatnotai, et al. 2004) and Wnt signaling mediator β-catenin (Gaston-Massuet, et al. 2011). Pituitary progenitors can also proliferate in response to external cues. For example, adult mice that have either been adrenalectomized or treated with estrogen have increased pituitary progenitor proliferation (Nolan and Levy. 2006; Rizzoti, et al. 2013). Altered external hormonal signaling can also lead specific changes in differentiated pituitary hormonal cell number. For example, adrenalectomy leads specifically to an increased number of corticotropes (Nolan, et al. 2004). Conversely, treatment with the glucocorticoid receptor agonist, dexamethasone, results in a reduction in corticotrope number (Nolan and Levy. 2006). These changes in corticotrope cell

number are a direct reflection of the pituitary's ability to regulate cell number in response to signals from both the hypothalamus and the adrenals. In both studies, levels of corticosteroids, which are normally produced by the adrenals are altered, leading to a change in pituitary corticotrope number. Our studies show that Notch signaling is important for both progenitor proliferation and differentiated cell balance, suggesting it may be an important point of integration for intrinsic and extrinsic cues.

Notch signaling is an evolutionarily conserved pathway that functions to maintain cells in a progenitor state by repressing transcription of pro-differentiation factors (Louvi and Artavanis-Tsakonas. 2006). In conjunction with this role, reductions in Notch signaling often result in decreased proliferation (van Es, et al. 2005). In more rare instances, such as in T-cell acute lymphoblastic leukemia, excessive Notch signaling can actively promote proliferation (Pancewicz, et al. 2010). Multiple studies have sought to identify direct Notch targets that may account for Notch's influence on proliferation. Specifically, Notch signaling has been shown to directly regulate transcription of cell cycle regulatory genes, including *Ccnd1* (Cohen, et al. 2010) *Ccnd3* (Joshi, et al. 2009) and *Cdnk1* (Georgia, et al. 2006). These factors are considered direct targets since the Notch intracellular domain and cofactor, RBPJ, can bind directly to their promoters to influence transcription. Indirect Notch/RBPJ targets, which are controlled by binding of downstream Notch effectors (Hes or Hey proteins) at their promoters, have also been shown to regulate cell cycle modulators (Murata, et al. 2005). In the developing pituitary, loss of the Notch target gene *Hes1*

results in increased expression of the cell cycle inhibitors p21, p27 and p57, suggesting that Notch signaling may control similar factors in the pituitary (Monahan, et al. 2009). Additionally, mice with conditional loss of *Notch2* have reduced proliferation and altered levels of cell cycle molecules (Chapter 3). Other factors that are regulated by Notch and are important for pituitary proliferation have yet to be identified.

Notch signaling also regulates cell fate choice in multiple systems, including the pancreas and the nervous system (Ohtsuka, et al. 2001; Ge, et al. 2002; Murtaugh, et al. 2003; Siveke, et al. 2008). In the pituitary, reduced levels of Notch signaling result in an increased number of corticotropes and a decreased number of somatotropes, thyrotropes and lactotropes (Chapter 3). Additionally, when Notch is persistently expressed, differentiation of melanotropes and corticotropes is inhibited (Chapter 2). Identification of novel Notch targets will be important to understand how Notch mediates cell fate choices in the pituitary. The following studies were designed as a preliminary analysis of the transcriptome regulated by Notch, with the goal of determining the mechanism by which Notch influences pituitary proliferation and differentiation.

MATERIALS AND METHODS

Mice

For expression analysis, wildtype male and female mice of mixed genetic background were used. For *in vitro* and *in vivo* DAPT treatment studies, CD-1 mice (Charles River, Wilimington, MA), from a breeding colony maintained in our

laboratory, were used. For all studies, p1 is considered to be the day of birth. All animals were housed in a facility with a 12 hour light-dark cycle and were maintained in accordance with the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

In order to generate *Notch2* cKO mice, *Notch2*$^{fl/fl}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME) and bred to *Foxg1*$^{+/cre}$ mice, maintained on a 129 background, also purchased from Jackson Laboratory. The resulting *Notch2*$^{+/fl}$, *Foxg1*$^{+/cre}$ mice were bred to *Notch2*$^{+/fl}$, *Foxg*$^{+/+}$ littermates to obtain cKO animals. To genotype the mice, tail biopsies were obtained and DNA was extracted using a hot shot or salt-out method. PCR for the *Cre* and *Notch2* alleles was performed as previously described using published primer sequences (Chapter 3).

Agilent Array

Pituitaries of female control and *Notch2* cKO p1 mice were collected and stored in RNAlater (Life Technologies, Grand Island, NY) until processing. Two samples from female mice of the same genotype were pooled together and RNA was extracted using the RNAqueous Micro Kit (Life Technologies). RNA was then sent to the Roy G. Carver Biotechnology Center (University of Illinois at Urbana-Champaign), where cDNA was synthesized and spotted on an Agilent 4x44k mouse whole genome array (Agilent Technologies, Santa Clara, CA). Four arrays were run for each genotype. Data analysis was performed by Jenny Drnevich (Bioinformatics Unit, University of Illinois at Urbana, Champaign).

Quantitative Reverse Transcriptase PCR (qRT-PCR)

RNA was isolated from embryonic and postnatal whole pituitaries using an RNAqueous micro kit (Ambion, Austin, TX) as per manufacturers protocol. For p21 and adult pituitaries, 0.5μg of RNA was synthesized into cDNA using the ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). For e16.5 and p1, p5 and p10 and p12 pituitaries, the total amount of RNA from each pituitary was converted into cDNA. A no Reverse Transcriptase enzyme control was also prepared and used as a negative control. For quantitative RT-PCR 0.1-0.33 μL of cDNA was amplified using gene-specific primers and SYBR green mix (Bio-Rad Laboratories, Hercules, CA) on a Bio-Rad MyIQ real-time PCR machine. Data were analyzed with the change in cycle threshold (ΔCt) value method (Goldberg, et al. 2011), using *Gapdh* levels to normalize the data. Statistical significance was determined using Student's *t* test. The sequences of primers used are as follows: *Hsd11b1* forward: TCT TCC ATG ACG ACA TCC AC, *Hsd11b1* reverse: GAG TAG GGA GCA ATC ATA GG, *Gapdh* Forward: GGT GAG GCC GGT GCT GAG TAT G, *Gapdh* Reverse: GAC CCG TTT GGC TCC ACC CTT C.

DAPT injections

Beginning at p2, CD1 mice were injected daily for 3 days with 100mg/kg DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-Butyl Ester, Gamma Secretase Inhibitor IX, Millipore, Billerica, MA) diluted in Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) or an equivalent volume of DMSO alone. Pituitaries and whole heads were harvested 24h after last injection.

For longer-term studies, mice were injected with 100mg/kg DAPT or an equivalent volume of DMSO for five days beginning on p3. Pituitaries were harvested on p12 or p32.

Pituitary explant culture and DAPT washout assay

Pituitaries were dissected from p3 CD1 mice, rinsed with PBS and transferred to a 96 well plate. Culture media was made by adding 1% Penicillin Streptomycin (Fisher, Fairlawn, NJ) and 10% fetal bovine serum (HyClone, Logan, UT) to DMEM/F-12 media (Cellgro, Manassas, VA). 10μM DAPT dissolved in DMSO (Millipore) or an equivalent amount of DMSO (Cambridge Isotope Laboratories, Andover, MA) was added to the culture media from the beginning of culture. After 24h in culture, media was spiked with DAPT or DMSO (an equivalent amount to what was added at the beginning of culture) and pituitaries were cultured for an additional 24h.

Immunohistochemistry

Whole heads of p1 mice and pituitaries were fixed using a Zinc fixative (BD, Franklin Lakes, NJ) and embedded in paraffin for sectioning. Slides were deparaffinized and rehydrated. Samples were boiled in 10 mM citrate solution, pH6 for 10 minutes and cooled for 15 minutes. Next, all samples were blocked in a solution containing 5% normal donkey serum (Jackson Immunoresearch, West Grove, PA) diluted in immunohistochemical block, which contains 3% bovine serum albumin (Jackson Immunoresearch) and 0.5% TritonX-100 (Sigma Aldrich, St. Louis, MO) diluted in PBS. Primary antibodies used were raised against the following peptides: 11β-HSD1 (1:100, Abcam, Cambridge, UK), Pax7

(1:500,Developmental Studies Hybridoma Bank, University of Iowa), Pit1 (1:2000, kind gift of Dr. Simon Rhodes) and POMC (1:1000, Dako, Carpinteria, CA). Slides were then incubated with a biotin-conjugated mouse (Pax7) or rabbit (POMC, Pit1 and11β-HSD1) secondary antibody. This was followed by incubation in a streptavidin-conjugated cy2 or cy3 fluorophore. Biotin and streptavidin-conjugated antibodies were purchased from Jackson ImmunoResearch and were used at a concentration of 1:500. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, Life Technologies, Grand Island, NY) and visualized at 50x 100x, 200x, or 400x magnification using a Leica DM2560 microscope. Photographs were taken using a Retiga 2000R color camera (Q-Imaging, Surrey, Canada) and acquired using Q-Capture Pro software (Q-Imaging). Images were processed using Adobe Photoshop CS4 (San José, CA).

RESULTS

A microarray comparing control and *Notch2* cKO p1 pituitaries was performed to identify targets that may contribute to defects observed in proliferation and corticotrope cell number in *Notch2* cKO mice. 1310 mRNAs were found be differentially expressed (p<0.10) between control and *Notch2* cKO mice. Of these genes, we show that members of the Notch signaling pathway are altered, as expected (Table 4.1). In order to identify pathway or global processes that may be changed, we used DAVID to group the altered genes using gene ontology terms. We find that the majority of top terms changed are related to

proliferation. These include DNA replication, cell cycle and M phase. In addition, we find alterations in transcripts implicated in cell fate commitment and gland development (Table 4.2). Because many of the altered gene products are involved in proliferation, we next examined the array data more closely and identified a subset of cell cycle-associated genes and genes known to influence proliferation that are downregulated when *Notch2* is lost (Table 4.3). These genes include *Cdca2*, *Cdca5*, *Cdca*, *Ccna1*, *Ccnf* , *Pcna* and *Mki67*. These findings are in agreement with our findings showing that overall proliferation and mRNA levels of *mKi67* are reduced in *Notch2* cKO mice. Future studies validating these changes will be performed in an independent sample set. Additionally, further work will be aimed at determining whether these genes are direct Notch targets in the pituitary.

Additionally, we have identified a subset of differentially expressed genes that may be involved in the regulation of corticotrope differentiation, cell number and/or signaling. These factors include *Tbx19*, *Avpr1b* and *Crhr1*, which are upregulated and *Hsd11b1*, which is significantly downregulated in *Notch2* cKO mice (Table 4.4). *Tbx19*, *Avpr1b* and *Crhr1* are not generally expressed until after corticotrope differentiation has occurred. Therefore, they may be upregulated as a byproduct of the increase in corticotrope cell number and are not direct factors in the regulation of cell number or differentiation. For this reason, we chose to focus the remainder of this study on how alterations in *Hsd11b1*, the gene that encodes for 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), may affect corticotrope cell number and how this gene may be

regulated by Notch. 11β-hydroxysteroid dehydrogenase type 1 is an enzyme that converts inactive cortisone to its active form, cortisol (11-dehydrocorticosterone to corticosterone in mice). This conversion helps to locally increase active cortisol, which could then activate the glucocorticoid receptor. Changes in glucocorticoid signaling have been shown to affect corticotrope cell number during development, (Schmidt, et al. 2009) making this enzyme a possible mechanism by which Notch signaling may influence corticotrope number.

In order to confirm our microarray results showing that *Hsd11b1* is downregulated in *Notch2* cKO mice, we performed quantitative RT-PCR comparing control and cKO male and female pituitaries at p1. In agreement with the array data, we observe a significant decrease in *Hsd11b1 in Notch2* cKO mice (Figure 4.1A). Furthermore, when p3 pituitary explant cultures are treated *in vitro* with the Notch inhibitor DAPT for 48 hours, a significant reduction in *Hsd11b1* is observed (Figure 4.1B). In order to determine if similar effects are observed *in vivo*, we explored whether *Hsd11b1* is altered in several paradigms in which mice were injected with DAPT. In the first paradigm, (shown in chapter 3, figure 3.5A) we show that when mice are injected with 100/mg/kg DAPT on p2, p3 and p4 and then harvested on p5, pituitary mRNA levels of *Hsd11b1* are decreased (Figure 1C). In contrast, if mice are injected with DAPT daily from p3 through p7 and harvested on p12 (Figure 4.1D) or p32 (Figure 4.1E), no significant change is observed in *Hsd11b1* levels, suggesting levels may be able to recover during the period in which DAPT is not injected. Together, these data,

which examine the effects of both genetic and chemical Notch inhibition, suggest that *Hsd11b1* is a target of Notch signaling.

In order to bioinformatically determine if the Notch cofactor RBPJ or canonical Notch targets have the potential to bind to the promoter of *Hsd11b1*, we scanned the *Hsd11b1* promoter for known RBPJ, Hes1 or Hey1 binding motifs. We identified three Hey1 binding sites within 5kb upstream of the *Hsd11b1* transcriptional start site (Figure 4.2). We did not identify any potential binding regions for either RBPJ or Hes1 using this approach. The discovery of these binding sites implies that *Hsd11b1* may be downstream of Notch signaling and, if so, may be transcriptionally controlled by Hey1. Further work investigating this possibility will be a future area of focus in the Raetzman laboratory.

In addition to investigating whether *Hsd11b1* may be a direct Notch target, we have also begun to examine the expression pattern of *Hsd11b1* during development. This analysis will reveal during which periods the pituitary may be sensitive to alterations in *Hsd11b1* and other glucocorticoid signaling components. We show that the mRNA levels of *Hsd11b1* in the pituitary are very low (near the limits of detection) at e16.5. They rise by p1 to levels similar to those seen in the adult. Interestingly, at p5, a time during which the pituitary is undergoing cell expansion and differentiation, the levels of *Hsd11b1* are significantly increased in comparison to adult. By p9, the levels have decreased to levels similar to those observed in the adult and at p21 the levels are, again, increased (Figure 4.3). These results suggest that *Hsd1b11* begins to become

more highly expressed in the pituitary of mice after birth and is expressed at dynamic levels throughout postnatal expansion.

To determine where in the pituitary 11β-HSD1 is expressed, we performed immunohistochemistry in p1 pituitaries. We find that 11β-HSD1 is expressed in many cells of the IL and in scattered cells of the AL (Figures 4.4A). The expression pattern in the IL appears to closely resemble that of POMC, which is expressed in IL melanotropes. Because these cells express the transcription factor Pax7 (Budry, et al. 2012), we performed a double immunostain to determine whether 11β-HSD1 is expressed in Pax7-expressing cells. We show that 11β-HSD1 is expressed in many, but not all, Pax7-expressing cells and does not appear to be expressed in any Pax7-negative cells in the IL (Figures 4.4B). In order to confirm this observation, we performed colocalization studies with POMC. A similar result was observed in the IL, with 11β-HSD1 present in most, but not all, POMC-expressing melnanotropes (Figure 4.4C). POMC is also expressed in AL corticotropes and we find that the majority of corticotropes express 11β-HSD1 (Figure 4.4D). However, there are many 11β-HSD1-positive cells in the AL that do not colocalize with POMC. To help determine the identity of these cells, we performed double immunohistochehmistry for 11β-HSD1 and Pit1, a transcription factor present in somatotropes, thyrotropes and lactotropes. We show that a small subset of Pit1-positive cells also express 11β-HSD1 (Figure 4.4E). These findings show that, at p1, 11β-HSD1 is confined mainly to POMC-producing cells, suggesting it acts directly in cells that are responsive to glucocorticoid signaling.

DISCUSSION

The pituitary contains multiple endocrine cell types that are all derived from a common progenitor population. It is important for function of the gland, and thus of pituitary target organs, that the proper number of each cell type is formed. If too few or too many of any one population is formed, endocrine dysfunction can occur. In *Notch2* cKO mice, we observe an increase in the number of POMC-expressing corticotropes. Additionally, microarray analysis revealed a decrease in expression levels of *Hsd11b1*. *Hsd11b1* encodes the 11β-HSD1 enzyme, which converts corticosteroids to their active form, allowing them to bind to and activate the GR. Previous studies have shown that alterations in glucocorticoid signaling can result in changes in corticotrope number. Specifically, when GR is lost in POMC-expressing cells in the pituitary, an increased number of corticotropes is observed at p6 (Schmidt, et al. 2009). Additionally, after adrenalectomy in adult rats, an increase in proliferation and corticotrope number is observed (Nolan, et al. 2004). Conversely, when adrenalectomized rats are treated with dexamethasone, a GR agonist, an increase in ptiuitary apoptosis and a decrease in pituitary proliferation are observed (Nolan and Levy. 2006). These data show that the pituitary is able to regulate cell number in response to alterations in GR signaling and that the corticotrope POMC cell lineage is especially sensitive to these alterations. These data may help to explain the phenotype of increased corticotrope number in *Notch2* cKO mice, as these mice likely have decreased signaling through the GR due to decreased levels of *Hsd11b1*.

11β-HSD1 is a key metabolic enzyme whose dysregulation has been implicated in obesity and type 2 diabetes. Mouse models with overexpression of 11β-HSD1 develop many features of metabolic syndrome, including obesity, glucose insensitivity, insulin resistance and hyperlipidemia. Humans with metabolic syndrome also exhibit increased levels of 11β-HSD1. Because of these observations, 11β-HSD1 is a promising target for the treatment of metabolic syndrome and multiple studies have explored the effectiveness of 11β-HSD1 inhibitors in the clinic [reviewed in (Pereira, et al. 2012)]. Although much research has been focused on how this enzyme is dysregulated in metabolic syndrome and how it may be a useful target to combat such problems, little is known about how 11β-HSD1 expression regulated. We present data showing that mice with reduced Notch signaling have significantly reduced levels of *Hsd11b1,* suggesting this pathway may be involved in its regulated expression. There is evidence to suggest that, in other systems, levels of Notch signaling can influence metabolic processes. Previous studies have shown that both hypo- and hyper- activated Notch signaling induce a glycolytic phenotype in breast cancer cells (Landor, et al. 2011) and persistent expression of *Notch1* promotes insulin resistance and induces expression of glucose-6-phosphotase (Valenti, et al. 2013). These studies show that Notch signaling has the ability to regulate multiple metabolic processes and contribute to the notion that Notch may regulate metabolic enzymes in the pituitary.

We have identified three Hey1 binding sites within 5kb upstream of the *Hsd11b1* transcriptional start site. These identified sites indicate that Notch target

gene products may have the ability to regulate directly the expression of *Hsd11b1*. Hey1 and other canonical Notch downstream effectors have been characterized as transcriptional repressors. Therefore, if Hey1 regulates *Hsd11b1* directly, it would be expected that a reduction in Hey1 would result in an increase in *Hsd11b1* mRNA levels. Surprisingly, we observe a decrease in *Hsd11b1* levels when *Notch2* is lost and *Hey1* is downregulated. This relationship suggests that if Hey1 were to directly regulate *Hsd11b1*, it may be acting as a transcriptional activator. Although uncommon, previous studies have identified activation roles for Hey1. A study that utilized microarray and chromatin immunoprecipitiation identified a subset of targets that are activated by Hey proteins (Heisig, et al. 2012). Additionally, Hey1 has been implicated in the activation of p53, although the mechanism remains unclear (Huang, et al. 2004; Ban, et al. 2008). Transcriptional activation roles for Hes factors, which are also Notch targets and members of the basic helix-loop-helix family, have also been described. Specifically, in human fibroblast cells, Hes1 activates transcription of the human acid-glucosidase (GAA) gene and Hes1 and Hes5 have the ability to activate transcription of STAT-3 (Kamakura, et al. 2004). These studies provide precedent for the ability of Notch canonical targets to activate other genes.

Our data shows that 11β-HSD1 is expressed mainly in corticotropes and melanotropes. Previous findings show that Notch signaling components, including *Hey1*, are expressed in cells surrounding the pituitary cleft and in scattered AL cells, in a pattern mirroring that of the progenitor markers SOX2 and SOX9 (Chapter 3). Because Notch signaling is not likely active in melanotropes

and corticotropes, it is unlikely that the downstream Notch effector, Hey1 is able to directly regulate *Hsd11b1* expression. Future studies will be necessary to identify a target which is both under direct Notch control and that can directly control corticotrope number.

11β-HSD1 is an important metabolic regulator that has been implicated in the development and progression of metabolic syndrome. Therefore, understanding how this enzyme is controlled is of great importance. We show that reductions in Notch signaling results in decreased levels of *Hsd11b1*, suggesting that the Notch signaling pathway may be involved in its regulation. Future studies in the Raetzman laboratory will be aimed at determining whether *Hsd11b1* is a direct target of Notch signaling and how alterations in this enzyme and other glucocorticoid signaling factors regulate corticotrope cell number during early postnatal pituitary expansion.

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REFERENCES

Andoniadou, C.L., Matsushima, D., Mousavy Gharavy, S.N., Signore, M., Mackintosh, A.I., Schaeffer, M., Gaston-Massuet, C., Mollard, P., Jacques, T.S., Le Tissier, P., Dattani, M.T., Pevny, L.H., Martinez-Barbera, J.P., 2013. Sox2(+) Stem/Progenitor Cells in the Adult Mouse Pituitary Support Organ Homeostasis and have Tumor-Inducing Potential. Cell Stem Cell 13, 433-445.

Ban, J., Bennani-Baiti, I.M., Kauer, M., Schaefer, K.L., Poremba, C., Jug, G., Schwentner, R., Smrzka, O., Muehlbacher, K., Aryee, D.N., Kovar, H., 2008. EWS-FLI1 Suppresses NOTCH-Activated p53 in Ewing's Sarcoma. Cancer Res 68, 7100-7109.

Budry, L., Balsalobre, A., Gauthier, Y., Khetchoumian, K., L'honore, A., Vallette, S., Brue, T., Figarella-Branger, D., Meij, B., Drouin, J., 2012. The Selector Gene Pax7 Dictates Alternate Pituitary Cell Fates through its Pioneer Action on Chromatin Remodeling. Genes Dev 26, 2299-2310.

Cohen, B., Shimizu, M., Izrailit, J., Ng, N.F.L., Buchman, Y., Pan, J.G., Dering, J., Reedijk, M., 2010. Cyclin D1 is a Direct Target of JAG1-Mediated Notch Signaling in Breast Cancer. Breast cancer research and treatment 123, 113-24.

Fauquier, T., Rizzoti, K., Dattani, M., Lovell-Badge, R., Robinson, I.C.A.F., 2008. SOX2-Expressing Progenitor Cells Generate all of the Major Cell Types in the Adult Mouse Pituitary Gland. Proceedings of the National Academy of Sciences of the United States of America 105, 2907-12.

Gaston-Massuet, C., Andoniadou, C.L., Signore, M., Jayakody, S.A., Charolidi, N., Kyeyune, R., Vernay, B., Jacques, T.S., Taketo, M.M., Le Tissier, P., Dattani, M.T., Martinez-Barbera, J.P., 2011. Increased Wingless (Wnt) Signaling in Pituitary Progenitor/Stem Cells Gives Rise to Pituitary Tumors in Mice and Humans. Proc Natl Acad Sci U S A 108, 11482-11487.

Ge, W., Martinowich, K., Wu, X., He, F., Miyamoto, A., Fan, G., Weinmaster, G., Sun, Y.E., 2002. Notch Signaling Promotes Astrogliogenesis Via Direct CSL-Mediated Glial Gene Activation. Journal of neuroscience research 69, 848-60.

Georgia, S., Soliz, R., Li, M., Zhang, P., Bhushan, A., 2006. p57 and Hes1 Coordinate Cell Cycle Exit with Self-Renewal of Pancreatic Progenitors. Developmental biology 298, 22-31.

Goldberg, L.B., Aujla, P.K., Raetzman, L.T., 2011. Persistent Expression of Activated Notch Inhibits Corticotrope and Melanotrope Differentiation and Results in Dysfunction of the HPA Axis. Dev Biol 358, 23-32.

Gremeaux, L., Fu, Q., Chen, J., Vankelecom, H., 2012. Activated Phenotype of the Pituitary Stem/Progenitor Cell Compartment during the Early-Postnatal Maturation Phase of the Gland. Stem cells and development 21, 801-13.

Heisig, J., Weber, D., Englberger, E., Winkler, A., Kneitz, S., Sung, W.K., Wolf, E., Eilers, M., Wei, C.L., Gessler, M., 2012. Target Gene Analysis by Microarrays and Chromatin Immunoprecipitation Identifies HEY Proteins as Highly Redundant bHLH Repressors. PLoS Genet 8, e1002728.

Huang, Q., Raya, A., DeJesus, P., Chao, S.H., Quon, K.C., Caldwell, J.S., Chanda, S.K., Izpisua-Belmonte, J.C., Schultz, P.G., 2004. Identification of p53 Regulators by Genome-Wide Functional Analysis. Proc Natl Acad Sci U S A 101, 3456-3461.

Jirawatnotai, S., Aziyu, A., Osmundson, E.C., Moons, D.S., Zou, X., Kineman, R.D., Kiyokawa, H., 2004. Cdk4 is Indispensable for Postnatal Proliferation of the Anterior Pituitary. J Biol Chem 279, 51100-51106.

Joshi, I., Minter, L.M., Telfer, J., Demarest, R.M., Capobianco, A.J., Aster, J.C., Sicinski, P., Fauq, A., Golde, T.E., Osborne, B.A., 2009. Notch Signaling Mediates G1/S Cell-Cycle Progression in T Cells Via Cyclin D3 and its Dependent Kinases. Blood 113, 1689-1698.

Kamakura, S., Oishi, K., Yoshimatsu, T., Nakafuku, M., Masuyama, N., Gotoh, Y., 2004. Hes Binding to STAT3 Mediates Crosstalk between Notch and JAK-STAT Signalling. Nat Cell Biol 6, 547-554.

Landor, S.K., Mutvei, A.P., Mamaeva, V., Jin, S., Busk, M., Borra, R., Gronroos, T.J., Kronqvist, P., Lendahl, U., Sahlgren, C.M., 2011. Hypo- and Hyperactivated Notch Signaling Induce a Glycolytic Switch through Distinct Mechanisms. Proc Natl Acad Sci U S A 108, 18814-18819.

Louvi, A., Artavanis-Tsakonas, S., 2006. Notch Signalling in Vertebrate Neural Development. Nature reviews Neuroscience 7, 93-102.

Monahan, P., Rybak, S., Raetzman, L.T., 2009. The Notch Target Gene HES1 Regulates Cell Cycle Inhibitor Expression in the Developing Pituitary. Endocrinology 150, 4386-94.

Murata, K., Hattori, M., Hirai, N., Shinozuka, Y., Hirata, H., Kageyama, R., Sakai, T., Minato, N., 2005. Hes1 Directly Controls Cell Proliferation through the Transcriptional Repression of p27Kip1. Mol Cell Biol 25, 4262-4271.

Murtaugh, L.C., Stanger, B.Z., Kwan, K.M., Melton, D.A., 2003. Notch Signaling Controls Multiple Steps of Pancreatic Differentiation. Proceedings of the National Academy of Sciences of the United States of America 100, 14920-5.

Nolan, L.A., Levy, A., 2006. A Population of Non-Luteinising Hormone/Non-Adrenocorticotrophic Hormone-Positive Cells in the Male Rat Anterior Pituitary Responds Mitotically to both Gonadectomy and Adrenalectomy. Journal of neuroendocrinology 18, 655-61.

Nolan, L.A., Thomas, C.K., Levy, A., 2004. Pituitary Mitosis and Apoptotic Responsiveness Following Adrenalectomy are Independent of Hypothalamic Paraventricular Nucleus CRH Input. The Journal of endocrinology 181, 521-9.

Ohtsuka, T., Sakamoto, M., Guillemot, F., Kageyama, R., 2001. Roles of the Basic Helix-Loop-Helix Genes Hes1 and Hes5 in Expansion of Neural Stem Cells of the Developing Brain. The Journal of biological chemistry 276, 30467-74.

Pancewicz, J., Taylor, J.M., Datta, A., Baydoun, H.H., Waldmann, T.A., Hermine, O., Nicot, C., 2010. Notch Signaling Contributes to Proliferation and Tumor Formation of Human T-Cell Leukemia Virus Type 1-Associated Adult T-Cell Leukemia. Proceedings of the National Academy of Sciences of the United States of America 107, 16619-24.

Pereira, C.D., Azevedo, I., Monteiro, R., Martins, M.J., 2012. 11beta-Hydroxysteroid Dehydrogenase Type 1: Relevance of its Modulation in the Pathophysiology of Obesity, the Metabolic Syndrome and Type 2 Diabetes Mellitus. Diabetes Obes Metab 14, 869-881.

Rizzoti, K., Akiyama, H., Lovell-Badge, R., 2013. Mobilized Adult Pituitary Stem Cells Contribute to Endocrine Regeneration in Response to Physiological Demand. Cell Stem Cell 13, 419-432.

Schmidt, M.V., Sterlemann, V., Wagner, K., Niederleitner, B., Ganea, K., Liebl, C., Deussing, J.M., Berger, S., Schutz, G., Holsboer, F., Muller, M.B., 2009. Postnatal Glucocorticoid Excess due to Pituitary Glucocorticoid Receptor Deficiency: Differential Short- and Long-Term Consequences. Endocrinology 150, 2709-2716.

Siveke, J.T., Lubeseder-Martellato, C., Lee, M., Mazur, P.K., Nakhai, H., Radtke, F., Schmid, R.M., 2008. Notch Signaling is Required for Exocrine Regeneration After Acute Pancreatitis. Gastroenterology 134, 544-55.

Valenti, L., Mendoza, R.M., Rametta, R., Maggioni, M., Kitajewski, C., Shawber, C.J., Pajvani, U.B., 2013. Hepatic Notch Signaling Correlates with Insulin Resistance and Nonalcoholic Fatty Liver Disease. Diabetes 62, 4052-4062.

van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., Clevers, H., 2005. Notch/Gamma-Secretase Inhibition Turns Proliferative Cells in Intestinal Crypts and Adenomas into Goblet Cells. Nature 435, 959-963.

TABLES AND FIGURES

Notch Signaling Pathway

Ligands			
Gene	Gene Name	Fold Change	p value
Cntn1	contactin 1	1.739	0.0220
Dll3	delta-like 3	1.427	0.0231
Jag2	jagged 2	1.423	0.0306
Targets			
Gene	Gene Name	Fold Change	p value
Dtx4	deltex 4	-1.435	0.0354
Hey1	hairy/enhancer-of-split related with YRPW motif 1	-2.045	0.0499
Dtx1	deltex 1	1.327	0.0665
Hes1	hairy and enhancer of split 1	-1.293	0.0810
Cofactors			
Gene	Gene Name	Fold Change	p value
Rbpjl	recombination signal binding protein for immunoglobulin kappa J region-like	-1.234	0.0744

Table 4.1. Notch ligands, targets and cofactors changed in *Notch2* cKO mice, as identified by microarray analysis. Four independent samples from control and cKO mice were used to determine significnace.

Gene Ontology (GO) Terms Identified as Changed in *Notch2 cKO Mice*

GO Term	Number of Changed Genes	p value
Cell Cycle	81	$1.4x10^{-13}$
DNA replication	30	$2.7x10^{-9}$
M Phase	40	$8.0x10^{-8}$
Cell Fate Commitment	18	0.0027
Gland Development	20	0.011

Table 4.2. Pathways, grouped by gene ontology term, found altered in *Notch2* cKO mice. Analyzed using DAVID (Database for Annotatin, Visualization and Integrated Discovery) bioanalysis software.

Proliferation

Gene	Gene Name	Fold Change	p value
Cdca2	cell division cycle associated 2	-1.368	0.0373
Cdca5	cell division cycle associated 5	-1.498	7.01×10^{-14}
Cdca7	cell division cycle associated 7	-1.442	0.0282
Cdca8	cell division cycle associated 8	-1.248	0.0638
Cdc6	cell division cycle 6	-1.308	0.0339
Ccna1	cyclin A1	-1.221	0.0013
Ccnf	cyclin F	-1.368	0.0373
Cdk1	cyclin dependent kinase 1	-1.401	0.0976
Pcna	proliferating cell nuclear antigen	-1.350	0.0411
Mki67	antigen identified by monoclonal antibody Ki67	-1.619	0.0321
Mybl2	myeloblastosis oncogene-like 2	-1.421	0.0231

Table 4.3. Proliferation factors changed in *Notch2* cKO mice, as identified by microarray analysis. Four independent samples from control and cKO mice were used to determine significnace.

Corticotrope/Melanotrope Differentiation or Signaling

Gene	Gene Name	Fold Change	p value
Hsd11b1	hydroxysteroid 11-beta dehydrogenase 1	-2.851	2.88×10^{-16}
Tbx19	T-Box 19	1.540	0.0231
Avpr1b	arginine vasopressin receptor 1B	1.769	0.0354
Crhr1	corticotropin releasing hormone receptor 1	1.389	0.0796

Table 4.4. Corticotrope/Melanotrope factors changed in *Notch2* cKO mice, as identified by microarray analysis. Four independent samples from control and cKO mice were used to determine significnace.

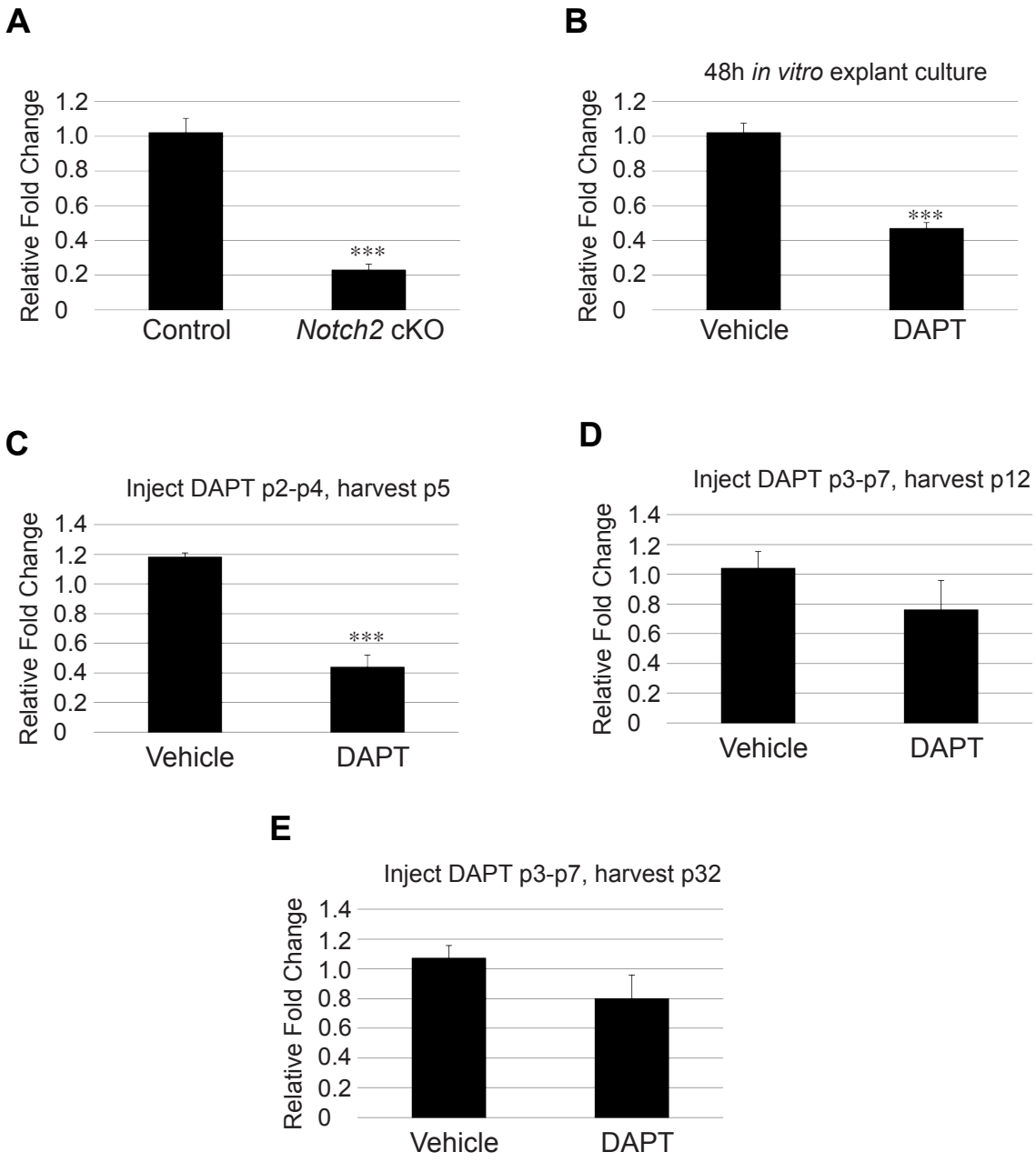

Figure 4.1. Reductions in Notch signaling result in a decrease in *Hsd11b1* mRNA levels. Mice with conditional loss of *Notch2* (*Notch2* cKO) have significantly decreased pituitary mRNA levels of *Hsd11b1* at p1 as compared to control (A). p3 pituitary explants treated with *in vitro* with 10μM DAPT have signficantly reduced mRNA levels of *Hsd11b1* as compared to DMSO controls (B). Mice injected subcutaneously with 100mg/kg DAPT from p2 through p4 and dissected 24 hours later show a significant reduction in *Hsd11b1* pituitary mRNA levels as compared to control (C). Mice injected with 100mg/kg DAPT from p3-p7 and harvested on p12 (D) or p32 (E) do not show differences in pituitary mRNA levels of *Hsd11b1.* n=3-8. ***:p<0.001.

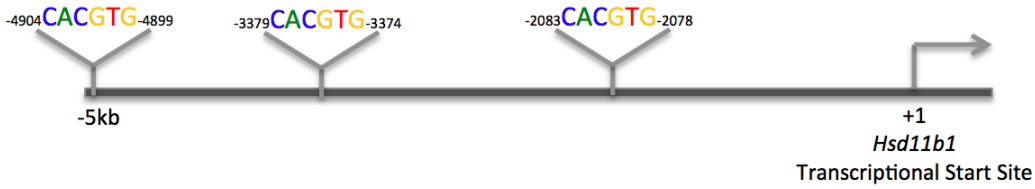

Figure 4.2. Schematic showing positions of Hey1 consensus binding sites within 5kb upstream of the *Hsd11b1* transcriptional start site.

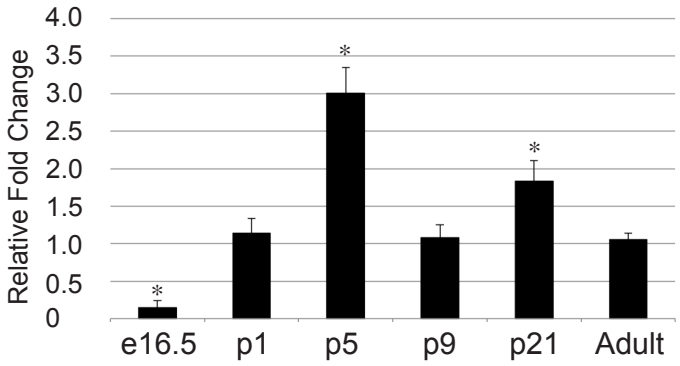

Figure 4.3. *Hsd11b1* mRNA begins to be more highly expressed in the mouse pituitary after birth and is dynamic throughout postnatal development. All levels are compared to adult. n=3-4. *:$p<0.05$.

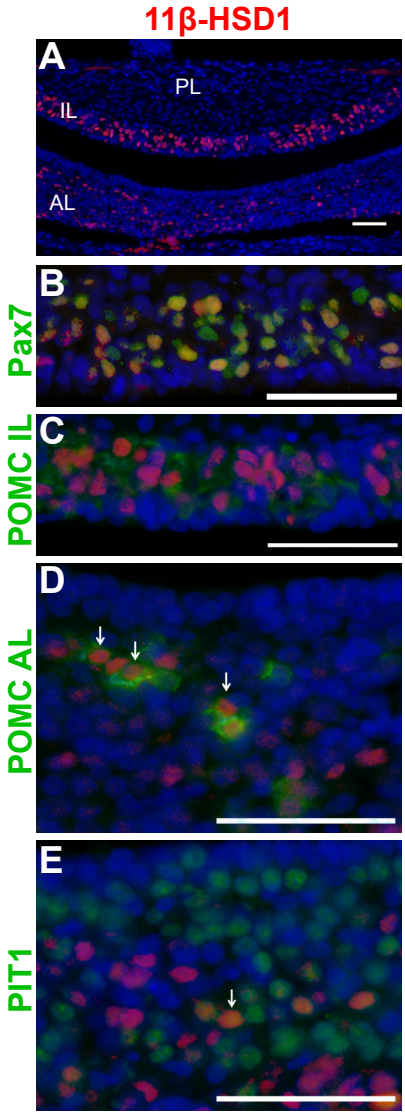

Figure 4.4. 11β-HSD1 (red) is expressed in melanotropes, corticotropes and few Pit1 l positive cells. Coronal section showing 11β-HSD1 expression in the IL and AL of wildtype mice (A). Most IL melanotropes contain Pax7 and some, but not all also express 11β-HSD1 (B). 11β-HSD1 is expressed in many POMC-containing melanotropes (C). Many, but not all, corticotropes express 11β-HSD1 (D). Few Pit1 cells contain 11β-HSD1 (E). Arrows indicate colocalized cells. Photos taken at 200x (A) and 400x (B-E). Scale bar: 50μm. n=2-3.

CHAPTER 5: **Conclusions/Discussion**

The pituitary sits at the center of the endocrine system, acting as a relay between the hypothalamus and various target organs. Improper development or function of the pituitary can lead to many disorders, including combined pituitary hormone deficiency (CPHD), Cushing's syndrome, isolated ACTH deficiency, acromegaly and dwarfism (Daly, et al. 2006; Drouin, et al. 2007; Castinetti, et al. 2012). An understanding of how cell number and cell fate are regulated in the pituitary is essential to our understanding of these disorders, as many of them are characterized by inappropriate cell number or absence of one or more endocrine cell types. The studies described in Chapters 2 through 4 have greatly contributed to the understanding of how Notch signaling regulates pituitary cell fate and cell number and how alterations in Notch signaling can lead to pituitary dysfunction. These findings are summarized in figure 5.1.

Notch signaling is an evolutionarily conserved pathway that functions to maintain cells in a progenitor state (Kopan and Ilagan. 2009). In many systems it has also been shown to regulate the choice between differentiated cell lineages, most notably in the nervous system where it influences the choice between neurons and glia (Ge, et al. 2002; Patten, et al. 2003). Studies have shown that Notch signaling components are expressed in the developing pituitary as early as e10.5 (Raetzman, et al. 2004; Zhu, et al. 2006). Mice with loss of the Notch effector *Hes1* have reduced pituitary proliferation and absence of the melanotrope lineage (Raetzman, et al. 2007; Monahan, et al. 2009). When *Hes1* is lost in conjunction with *Prop1*, early corticotrope differentiation is observed

(Himes and Raetzman. 2009). Conversely, *Notch2* prevents differentiation when overexpressed in gonadotropes (Raetzman, et al. 2006). These studies provide insight into the roles of Notch during embryonic pituitary development. The present studies seek to build upon and expand these observations by examining how Notch signaling acts to regulate proliferation and cell specification in various temporal and cell type-specific scenarios. Specifically, we show that persistent expression of activated Notch inhibits the differentiation of melanotropes and corticotropes and results in dysfunction of the hypothalamic-pituitary-adrenal axis (Goldberg, et al. 2011)Chapter 2). Additionally, we show that Notch signaling is necessary for postnatal pituitary proliferation, progenitor maintenance and cell fate choice (Chapter 3). In order to mechanistically understand how Notch influences these processes, we have identified targets of Notch signaling in the pituitary and have begun to interpret how these factors function in the pituitary (Chapter 4).

Persistent Notch signaling prevents the differentiation of corticotropes and melanotropes (Goldberg, et al. 2011)

Isolated adrenocorticotropic hormone (ACTH) deficiency is a rare condition in which corticotropes either fail to form or fail to produce ACTH. It can be caused by mutations in the *TPIT* (*TBX19)* gene and the congenital form of the disease is often fatal before diagnosis is made (Pulichino, et al. 2003; Vallette-Kasic, et al. 2004). We show that constitutive expression of activated Notch in melanotropes and corticotropes inhibits their differentiation and causes a phenotype similar to that seen in patients with isolated ACTH deficiency.

Because of this, it may be important to screen patients with ACTH deficiency to determine whether mutations in regulatory regions of Notch may be involved in this disease. Furthermore, this mouse model may be a good system in which to study isolated ACTH deficiency, as it closely mimics the human condition.

A major finding of this study is the observation that melanotropes and corticotropes fail to regenerate when ablated by persistent Notch expression. We show that by p7, the melanotrope-specific transcription factor *Pax7* (Budry, et al. 2012) is no longer expressed and that by p30, the IL is reduced to a very thin layer of cells containing only progenitor cell markers. Additionally, we do not observe POMC expression in the AL, suggesting a similar elimination of the corticotrope population. Interestingly, recent studies have shown that the melanotrope population appears to mainly regenerate from progenitor cells, whereas corticotropes regenerate using self-duplication (Langlais, et al. 2013). In our model, progenitor cells are not affected, yet we still observe failure of melanotropes to form and/or expand. It is possible that this is due to rapid activation of Notch in newly born melanotropes, which may prevent them from differentiating. Future studies aimed at understanding how hormone cells regenerate from progenitor cells and/or self-regenerate will be important to our understanding of the differences observed in these models.

Notch2 is necessary for postnatal proliferation, progenitor maintenance and cell differentiation

This study is the first to identify a specific signaling pathway that is important during postnatal pituitary expansion. Although studies have shown how

pituitary proliferation and progenitor number change during the first three postnatal weeks in mice (Carbajo-Pérez and Watanabe. 1990; Gaston-Massuet, et al. 2011; Gremeaux, et al. 2012), no study has examined which factors are in regulating these processes. Mice with loss of *Notch2* at the onset of embryonic pituitary development do not display defects until p1. These mice have a progressive loss of progenitor cells in the postnatal period, accompanied with a reduction in overall pituitary proliferation. Previous studies have shown that the number of progenitor cells is elevated during the postnatal period (Carbajo-Pérez and Watanabe. 1990; Gaston-Massuet, et al. 2011; Gremeaux, et al. 2012). This increased number of progenitors may be necessary to support the large expansion that occurs in the pituitary shortly after birth in mice. During this time, it is hypothesized that the pituitary begins to respond to external signaling, which is thought to influence cell proliferation and specification. We show that Notch signaling has a role in maintaining progenitor number during the postnatal period, which is not surprising given the large body of work showing that Notch signaling is an important progenitor maintenance factor (Ehm, et al. 2010; Aujla, et al. 2013). It is proposed that these progenitor cells give rise to intermediate progenitors, which are responsible for the majority of the proliferation in the pituitary. These intermediate progenitors are then thought to differentiate into endocrine cells. We observe a reduction in proliferation and which indicates there may be a deficit in number or proliferation of this putative intermediate progenitor population. Defects in different progenitor populations may also contribute to the differences in endocrine cell number that is observed. Future studies aimed at

identifying a marker of these intermediate progenitor populations and determining how they contribute to postnatal pituitary expansion will be essential to our understanding of postnatal pituitary development and the pathways that are important during this developmental window.

An important finding of this study is that *Notch2* is dispensable for embryonic proliferation. Previous studies have shown that global loss of the Notch effector *Hes1* results in a decrease in pituitary proliferation (Monahan, et al. 2009). However, it is unclear from this study whether the defect in proliferation is intrinsic to the pituitary or caused by loss of *Hes1* in the surrounding tissues, namely the hypothalamus which releases signals that are important for pituitary development and proliferation (Treier, et al. 1998; Ericson, et al. 1998). Our findings suggest that the latter may be true. In agreement with this conclusion, mice with pituitary-specific loss of the Notch cofactor *Rbpj* also show no alterations in proliferation at e14.5(Zhu, et al. 2006). Further studies examining mice with pituitary-specific and hypothalamic-specific loss of *Hes1* will necessary to determine how this factor regulates pituitary proliferation.

We also show that *Notch2* helps to regulate the balance of differentiated cells in the developing pituitary. Specifically, inhibition of Notch leads to an increase in corticotropes and a decrease in cells of the Pit1 lineage. When Notch is chemically inhibited in postnatal mice, similar results are found in *Pomc* and *Pit1* mRNA levels, suggesting this role is maintained throughout postnatal development. These findings are in agreement with studies showing that mice with pituitary specific loss of *Rpbj* have decreased *Pit1* expression (Zhu, et al.

2006). It is also in agreement with the observation, described in Chapter 2, that persistent Notch signaling inhibits the differentiation of corticotropes. This study indicates that Notch singling is necessary to inhibit corticotrope differentiation and promote Pit1 lineage expansion, acting as a choice point between these two lineages. These effects may be direct, with Notch signaling acting in differentiating cells to influence their specification. Additionally, Notch may also act in progenitor cells to bias them towards distinct intermediate progenitor populations.

Identification of Potential Notch Targets

In chapter 3, we show that Notch signaling is necessary for postnatal pituitary proliferation and progenitor maintenance. In chapters 2 and 3, we also highlight a role for Notch signaling in inhibiting the corticotrope lineage and promoting the Pit1 lineage. Previous studies have shown that Notch signaling may transcriptionally regulate *Prop1*, which is necessary for transcription of *Pit1*, and thus for GH, TSH and Prl hormone synthesis. We developed a DAPT washout assay and used it to validate *Prop1* as a bona fide Notch target. This finding is important, as human mutations in the first intron of *PROP1*, where an RBPJ consensus sequence is found, can lead to CPHD (Godi, et al. 2012). The confirmation that Notch signaling likely regulates *Prop1* expression provides clues as to how a pathway, like Notch, that is necessary for many aspects of embryonic development, can have a tissue specific effect.

In order to extend the findings in Chapters 2 and 3 and to identify novel Notch targets in the pituitary, we performed a microarray comparing control and

Notch2 cKO pituitaries. Many of the genes identified by this array are involved in various aspects of cell proliferation. This includes factors that are involved in mitosis, cell cycle progression and replication. We also identified changes in genes involved in pituitary cell differentiation. Of these we identified changes in factors that may regulate corticotrope number. These genes are of specific interest since we show Notch is important to inhibit corticotrope differentiation. Specifically, our array revealed a significant reduction in *Hsd11b1* when *Notch2* is lost the pituitary. The *Hsd11b1* gene encodes for the protein 11β-HSD1, which is necessary for the conversion of inactive corticosteroids to their active forms. In late prenatal and early postnatal development, the pituitary is thought to begin to respond to external cues from the hypothalamus and its target organs. Reductions in *Hsd11b1* would decease effective glucocorticoid signaling in the pituitary, as there would be a reduction in active corticosteroids, which bind to and activate the glucocorticoid receptor (GR). Loss of the GR in the pituitary results in an increase in corticotrope number, whereas an increased GR leads to deceased ACTH production (Reichardt et al, 2000, Schmidt et al, 2009). We hypothesize that reductions in *Hsd11b1* levels in *Notch2* cKO mice lead to deceased glucocorticoid signaling and increased corticotrope number.

Although Notch has been most studied for its roles in progenitor maintenance and cell fate choice, there is precedent to believe that Notch signaling is crucial for the regulation of metabolic processes, including insulin sensitivity and glycolysis (Landor et al, 2011, Valenti et al, 2013). Understanding the extent to which Notch controls *Hsd11b1* and how this factor is important for

pituitary development and function are important new areas of research in our laboratory. Furthermore, we are investigating the possibility that of whether Notch influences expression of *Hsd11b1* in other important metabolic organs, such as the liver. These studies will be another area of focus in the Raetzman laboratory and will contribute to a more global understanidng of *Hsd11b1* regulation and function.

Overall Conclusions

The studies presented in Chapters 2-4 have greatly expanded our understanding of the role of Notch signaling throughout pituitary development. Specifically, this work is the first to define a role for this pathway in postnatal pituitary expansion. In doing so, we have identified the first signaling pathway to be active during this period, which is an emerging area of research in the pituitary development field. Each of the mouse models presented throughout this work represent valuable tools to further explore remaining questions. These questions include, but are not limited to, how isolated ACTH deficiency affects development of other cell types in the pituitary, how progressive loss of pituitary progenitors can lead to hypopituitarism and how glucocorticoid signaling alterations can affect corticotrope cell number. These areas of research are potential avenues that can be explored in the future.

REFERENCES

Aujla, P.K., Naratadam, G.T., Xu, L., Raetzman, L.T., 2013. Notch/Rbpjkappa Signaling Regulates Progenitor Maintenance and Differentiation of Hypothalamic Arcuate Neurons. Development .

Budry, L., Balsalobre, A., Gauthier, Y., Khetchoumian, K., L'honore, A., Vallette, S., Brue, T., Figarella-Branger, D., Meij, B., Drouin, J., 2012. The Selector Gene Pax7 Dictates Alternate Pituitary Cell Fates through its Pioneer Action on Chromatin Remodeling. Genes Dev 26, 2299-2310.

Carbajo-Pérez, E., Watanabe, Y.G., 1990. Cellular Proliferation in the Anterior Pituitary of the Rat during the Postnatal Period. Cell and tissue research 261, 333-8.

Castinetti, F., Reynaud, R., Saveanu, A., Barlier, A., Brue, T., 2012. Genetic Causes of Combined Pituitary Hormone Deficiencies in Humans. Ann Endocrinol (Paris) 73, 53-55.

Daly, A.F., Rixhon, M., Adam, C., Dempegioti, A., Tichomirowa, M.A., Beckers, A., 2006. High Prevalence of Pituitary Adenomas: A Cross-Sectional Study in the Province of Liege, Belgium. The Journal of clinical endocrinology and metabolism 91, 4769-75.

Drouin, J., Bilodeau, S., Vallette, S., 2007. Of Old and New Diseases: Genetics of Pituitary ACTH Excess (Cushing) and Deficiency. Clinical genetics 72, 175-82.

Ehm, O., Göritz, C., Covic, M., Schäffner, I., Schwarz, T.J., Karaca, E., Kempkes, B., Kremmer, E., Pfrieger, F.W., Espinosa, L., Bigas, A., Giachino, C., Taylor, V., Frisén, J., Lie, D.C., 2010. RBPJkappa-Dependent Signaling is Essential for Long-Term Maintenance of Neural Stem Cells in the Adult Hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 30, 13794-807.

Ericson, J., Norlin, S., Jessell, T.M., Edlund, T., 1998. Integrated FGF and BMP Signaling Controls the Progression of Progenitor Cell Differentiation and the Emergence of Pattern in the Embryonic Anterior Pituitary. Development (Cambridge, England) 125, 1005-15.

Gaston-Massuet, C., Andoniadou, C.L., Signore, M., Jayakody, S.A., Charolidi, N., Kyeyune, R., Vernay, B., Jacques, T.S., Taketo, M.M., Le Tissier, P., Dattani, M.T., Martinez-Barbera, J.P., 2011. Increased Wingless (Wnt) Signaling in Pituitary Progenitor/Stem Cells Gives Rise to Pituitary Tumors in Mice and Humans. Proc Natl Acad Sci U S A 108, 11482-11487.

Ge, W., Martinowich, K., Wu, X., He, F., Miyamoto, A., Fan, G., Weinmaster, G., Sun, Y.E., 2002. Notch Signaling Promotes Astrogliogenesis Via Direct CSL-Mediated Glial Gene Activation. Journal of neuroscience research 69, 848-60.

Godi, M., Mellone, S., Tiradani, L., Marabese, R., Bardelli, C., Salerno, M., Prodam, F., Bellone, S., Petri, A., Momigliano-Richiardi, P., Bona, G., Giordano, M., 2012. Functional SNPs within the Intron 1 of the PROP1 Gene Contribute to Combined Growth Hormone Deficiency (CPHD). J Clin Endocrinol Metab 97, E1791-7.

Goldberg, L.B., Aujla, P.K., Raetzman, L.T., 2011. Persistent Expression of Activated Notch Inhibits Corticotrope and Melanotrope Differentiation and Results in Dysfunction of the HPA Axis. Dev Biol 358, 23-32.

Gremeaux, L., Fu, Q., Chen, J., Vankelecom, H., 2012. Activated Phenotype of the Pituitary Stem/Progenitor Cell Compartment during the Early-Postnatal Maturation Phase of the Gland. Stem cells and development 21, 801-13.

Himes, A.D., Raetzman, L.T., 2009. Premature Differentiation and Aberrant Movement of Pituitary Cells Lacking both Hes1 and Prop1. Dev Biol 325, 151-161.

Kopan, R., Ilagan, M.X.G., 2009. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. Cell 137, 216-33.

Landor, S.K., Mutvei, A.P., Mamaeva, V., Jin, S., Busk, M., Borra, R., Gronroos, T.J., Kronqvist, P., Lendahl, U., Sahlgren, C.M., 2011. Hypo- and Hyperactivated Notch Signaling Induce a Glycolytic Switch through Distinct Mechanisms. Proc Natl Acad Sci U S A 108, 18814-18819.

Langlais, D., Couture, C., Kmita, M., Drouin, J., 2013. Adult Pituitary Cell Maintenance: Lineage-Specific Contribution of Self-Duplication. Mol Endocrinol 27, 1103-1112.

Monahan, P., Rybak, S., Raetzman, L.T., 2009. The Notch Target Gene HES1 Regulates Cell Cycle Inhibitor Expression in the Developing Pituitary. Endocrinology 150, 4386-94.

Patten, B.A., Peyrin, J.M., Weinmaster, G., Corfas, G., 2003. Sequential Signaling through Notch1 and erbB Receptors Mediates Radial Glia Differentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 23, 6132-40.

Pulichino, A., Vallette-Kasic, S., Couture, C., Gauthier, Y., Brue, T., David, M., Malpuech, G., Deal, C., Van Vliet, G., De Vroede, M., Riepe, F.G., Partsch, C., Sippell, W.G., Berberoglu, M., Atasay, B., Drouin, J., 2003. Human and Mouse TPIT Gene Mutations Cause Early Onset Pituitary ACTH Deficiency. Genes & development 17, 711-6.

Raetzman, L.T., Ross, S.A., Cook, S., Dunwoodie, S.L., Camper, S.A., Thomas, P.Q., 2004. Developmental Regulation of Notch Signaling Genes in the Embryonic Pituitary: Prop1 Deficiency Affects Notch2 Expression. Developmental biology 265, 329-40.

Raetzman, L.T., Cai, J.X., Camper, S.A., 2007. Hes1 is Required for Pituitary Growth and Melanotrope Specification. Developmental biology 304, 455-66.

Raetzman, L.T., Wheeler, B.S., Ross, S.A., Thomas, P.Q., Camper, S.A., 2006. Persistent Expression of Notch2 Delays Gonadotrope Differentiation. Molecular endocrinology (Baltimore, Md) 20, 2898-908.

Reichardt, H.M., Umland, T., Bauer, A., Kretz, O., Schutz, G., 2000. Mice with an Increased Glucocorticoid Receptor Gene Dosage show Enhanced Resistance to Stress and Endotoxic Shock. Mol Cell Biol 20, 9009-9017.

Schmidt, M.V., Sterlemann, V., Wagner, K., Niederleitner, B., Ganea, K., Liebl, C., Deussing, J.M., Berger, S., Schutz, G., Holsboer, F., Muller, M.B., 2009. Postnatal Glucocorticoid Excess due to Pituitary Glucocorticoid Receptor Deficiency: Differential Short- and Long-Term Consequences. Endocrinology 150, 2709-2716.

Treier, M., Gleiberman, A.S., O'Connell, S.M., Szeto, D.P., McMahon, J.A., McMahon, A.P., Rosenfeld, M.G., 1998. Multistep Signaling Requirements for Pituitary Organogenesis in Vivo. Genes & development 12, 1691-704.

Valenti, L., Mendoza, R.M., Rametta, R., Maggioni, M., Kitajewski, C., Shawber, C.J., Pajvani, U.B., 2013. Hepatic Notch Signaling Correlates with Insulin Resistance and Nonalcoholic Fatty Liver Disease. Diabetes 62, 4052-4062.

Vallette-Kasic, S., Pulichino, A.M., Gueydan, M., Barlier, A., David, M., Malpuech, G., Deal, C., Van Vliet, G., de Vroede, M., Riepe, F., Partsch, C.J., Sippell, W., Berberoglu, M., Atasay, B., de Zegher, F., Kyllo, J., Donohoue, P., Dechelotte, P., Fassnacht, M., Noordam, K., Dunkel, L., Pigeon, B., Weill, J., Yigit, S., Brauner, R., Leger, J., Heinrich, J.J., Enjalbert, A., Brue, T., Drouin, J., 2004. A Neonatal Form of Isolated ACTH Deficiency Frequently Associated with Tpit Gene Mutations. Endocrine research 30, 943-4.

Zhu, X., Zhang, J., Tollkuhn, J., Ohsawa, R., Bresnick, E.H., Guillemot, F., Kageyama, R., Rosenfeld, M.G., 2006. Sustained Notch Signaling in Progenitors is Required for Sequential Emergence of Distinct Cell Lineages during Organogenesis. Genes & development 20, 2739-53.

FIGURE

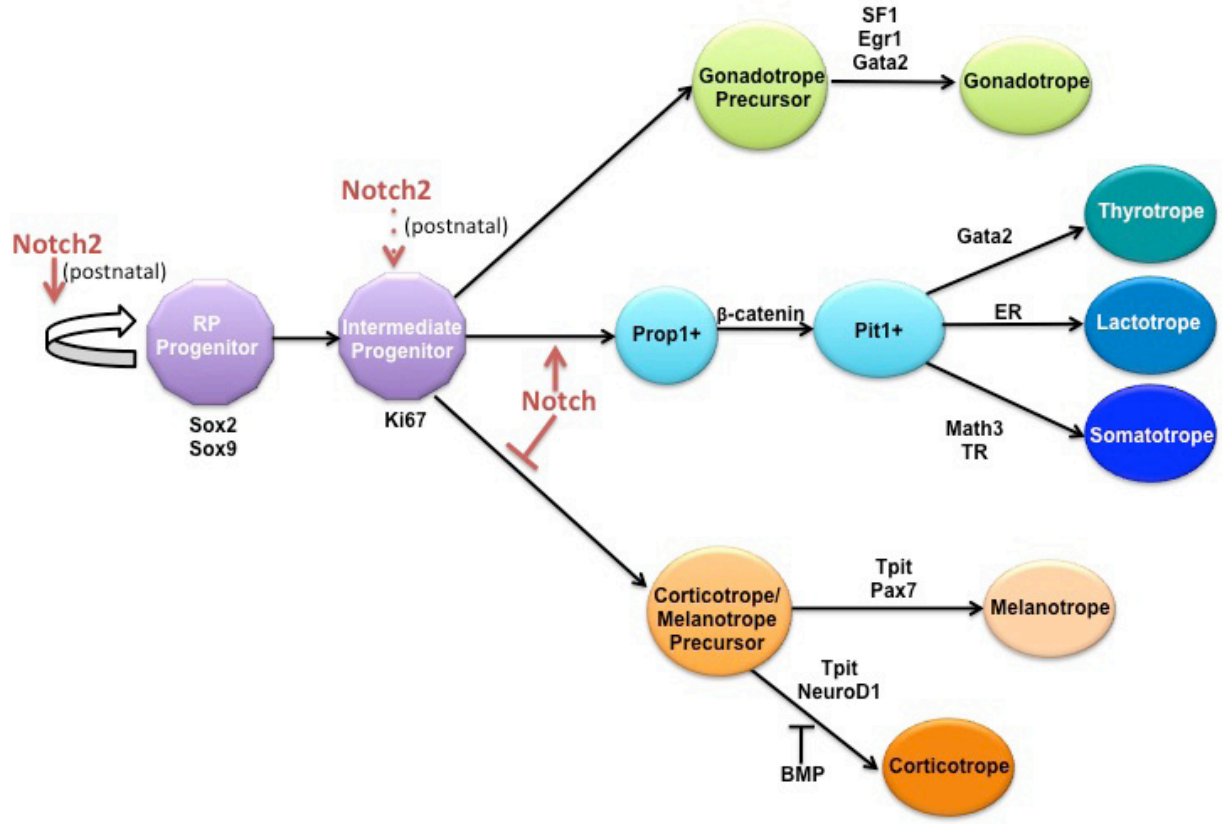

Figure 5.1 Summary of key findings. Our studies show that *Notch2* is necessary for postnatal progenitor maintenance and proliferation. Additionally, gain and loss of function models reveal that Notch signaling promotes the Prop1/Pit1 lineage and inhibits the corticotrope/melanotrope lineage, thus regulating differentiated cell number in the developing pituitary.