Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis

Xiaoyan Zhu,[1,7] Jie Zhang,[1] Jessica Tollkuhn,[1,2] Ryosuke Ohsawa,[3] Emery H. Bresnick,[4] François Guillemot,[5] Ryoichiro Kageyama,[3] and Michael G. Rosenfeld[1,6]

[1]Howard Hughes Medical Institute, Department and School of Medicine, University of California at San Diego, La Jolla, California 92093, USA; [2]Biomedical Sciences Graduate Program, University of California at San Diego, La Jolla, California 92093, USA; [3]Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto, Japan; [4]University of Wisconsin Medical School, Madison, Wisconsin 53706, USA; [5]Division of Molecular Neurobiology, National Institute for Medical Research, London NW7 1AA, United Kingdom

Mammalian organogenesis results from the concerted actions of signaling pathways in progenitor cells that induce a hierarchy of regulated transcription factors critical for organ and cell type determination. Here we demonstrate that sustained Notch activity is required for the temporal maintenance of specific cohorts of proliferating progenitors, which underlies the ability to specify late-arising cell lineages during pituitary organogenesis. Conditional deletion of *Rbp-J*, which encodes the major mediator of the Notch pathway, leads to premature differentiation of progenitor cells, a phenotype recapitulated by loss of the basic helix–loop–helix (bHLH) factor Hes1, as well as a conversion of the late (Pit1) lineage into the early (corticotrope) lineage. Notch signaling is required for maintaining expression of the tissue-specific paired-like homeodomain transcription factor, *Prop1*, which is required for generation of the Pit1 lineage. Attenuation of Notch signaling is necessary for terminal differentiation in post-mitotic Pit1$^+$ cells, and the Notch-repressed Pit1 target gene, *Math3*, is specifically required for maturation and proliferation of the GH-producing somatotrope. Thus, sustained Notch signaling in progenitor cells is required to prevent conversion of the late-arising cell lineages to early-born cell lineages, permitting specification of diverse cell types, a strategy likely to be widely used in mammalian organogenesis.

[*Keywords*: Notch; organogenesis; bHLH; Prop1; pituitary; precursor cells; differentiation]

Supplemental material is available at http://www.genesdev.org.

Received April 28, 2006; revised version accepted August 3, 2006.

Notch signaling is an evolutionarily conserved mechanism that regulates proliferation, apoptosis, cell fate determination, and morphogenesis in organisms ranging from nematodes to humans (for reviews, see Lewis 1998; Artavanis-Tsakonas et al. 1999). Notch signaling is mediated by the interaction between the Notch receptor and its ligands Delta and Serrate. Both receptor and ligand are cell-surface transmembrane proteins that contain extracellular arrays of epidermal growth factor (EGF) repeats. Specific EGF repeats mediate direct interaction between ligand and receptor. Mammals have four Notch receptors (Notch1, Notch2, Notch3, and Notch4) and five ligands (Delta-like1, Delta-like3, Delta-like4 [homologs of Delta], and Jagged1 and Jagged2 [homologs of Serrate]). Upon ligand binding, Notch receptors undergo successive proteolytic cleavages that lead to the release of the Notch intracellular domain (ICD) and subsequent nuclear translocation. Once in the nucleus, the ICD forms a complex with the Rbp-J DNA-binding protein, which is the primary mediator of Notch signaling, and the Mastermind coactivator; converts Rbp-J from a transcriptional repressor to a transcriptional activator; and induces transcription of target genes such as members of the Hairy enhancer of split (Hes) family of basic helix–loop–helix (bHLH) DNA-binding transcription factors Hes1 or Hes5 and the Hes-related protein (Herp) family (for review, see Iso et al. 2003). In the *Drosophila* nervous system, Notch regulates a process of lateral inhibition, whereby a single neuron differentiates within a field of similar precursor cells. During lateral inhibition, cells expressing high levels of Notch ligand commit to neural differentiation and activate Notch signaling in their neighbors, thereby preventing them from adopting the same fate. Though Notch is best known for its role in

Corresponding authors.
[6]E-MAIL mrosenfeld@ucsd.edu; FAX (858) 534-8180.
[7]E-MAIL xizhu@ucsd.edu; FAX (858) 534-8180.
Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.1444706.

lateral inhibition, activation of this pathway also promotes cell fate (Irvine 1999; Gaiano et al. 2000; Grandbarbe et al. 2003). Recent studies have shown that Notch signaling regulates a broad range of patterning processes during embryonic and postnatal development (Hitoshi et al. 2002; Krebs et al. 2003, Raya et al. 2003; Burns et al. 2005; Crosnier et al. 2005; Duncan et al. 2005; Fre et al. 2005; van Es et al. 2005; for reviews, see Lai 2004; Yoon and Gaiano 2005).

Coordination of signals from different pathways is essential for cell fate specification during animal development. The pituitary gland provides an excellent model system to study signaling events in organogenesis. The mature pituitary gland contains six hormone-producing cell types, including corticotropes secreting adrenocorticotrophic hormone (ACTH), a proteolytic product of proopiomelanocortin (POMC), thyrothopes secreting thyroid-stimulating hormone (TSH), somatotropes secreting growth hormone (GH), lactotropes secreting prolactin (PRL), gonadtropes secreting luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and melanotropes secreting melanocyte-stimulating hormone (MSH), a cleaved product of POMC. These cells derive from a common primordium, Rathke's pouch, and appear in a defined temporal and spatial fashion (Japon et al. 1994; for reviews, see Watkins-Chow and Camper 1998; Sheng and Westphal 1999; Dasen and Rosenfeld 2001; Rizzoti and Lovell-Badge 2005). Three of these cell types—thyrothopes, somatotropes, and lactotropes—differentiate from Pit1-expressing precursors and depend on the function of Pit1, a tissue-specific POU-class homeodomain transcription factor (Camper et al. 1990; Li et al. 1990). The expression of *Pit1* is positively regulated by the concerted efforts of the paired-like homeodomain transcription factor, Prophet of Pit1 (Prop1), and the Wnt/β-catenin signaling pathway (Gage et al. 1996; Sornson et al. 1996; Olson et al. 2006). Mutations in *Pit1* or *Prop1* result in a failure of Pit1 lineages differentiation, leading to a postnatal dwarf phenotype (Camper et al. 1990; Li et al. 1990; Ward et al. 2005). Differentiation of corticotropes is dependent on the T-box transcription factor, Tbx19, and regulated by other transcription factors and signaling events (Lamolet et al. 2001; Liu et al. 2001; Chesnokova and Melmed 2002; Pulichino et al. 2003).

Multiple signaling pathways converge to dictate molecular events underlying pituitary development. Loss-of-function and gain-of-function genetic studies together with ex vivo organ culture experiments have identified essential roles of FGF signaling emanating from the ventral diencephalon in proliferation and survival of progenitor cells comprising Rathke's pouch, as well as in establishing a dorsal to ventral gradient within the nascent pituitary gland (Ericson et al. 1998; Treier et al. 1998; Ohuchi et al. 2000; Revest et al. 2001). In addition, whereas BMP4 signaling is required for the pituitary organ commitment, BMP2 establishes a ventral to dorsal gradient necessary for cell type determination (Ericson et al. 1998; Takuma et al. 1998; Treier et al. 1998). Moreover, SHH signaling also exerts critical roles in supporting proliferation and terminal differentiation (Treier et al. 2001; Herzog et al. 2003; Sbrogna et al. 2003). Here, we report that the evolutionarily conserved Notch signaling pathway functions in the early phases of pituitary organogenesis to prevent premature differentiation of progenitors and drive specification of Pit1 precursors, which would otherwise emerge as the POMC-expressing corticotropes. Attenuation of Notch signaling at a later phase of pituitary development is required for the proper cell type terminal differentiation. These findings establish an additional role of the Notch signaling pathway in controlling the emergence of distinct precursor subtypes.

Results

Core components of the Notch signaling pathway during pituitary development

To explore the potential function of the Notch signaling pathway during pituitary development, in situ hybridization was carried out to examine the expression pattern of the known mammalian Notch ligands and receptors, *Dll1*, *Dll3*, *Dll4*, *Jag1*, *Jag2*, and *Notch*1–*Notch*4, respectively, as well as the direct downstream targets of Notch signaling, *Hes1*, *Hes5*, and *Hey1*. *Jag1*, *Dll1*, *Notch2*, and *Notch3*, as well as *Hes1* and *Hey1*, were expressed in the Rathke's pouch, indicating that Notch signaling is active during early pituitary development (Figs. 1, 2). At embryonic day 9.5 (E9.5), *Jag1*, *Notch2*, *Notch3*, and *Hes1* are expressed in the invaginating oral ectoderm. Between E10.5 and E12.5, *Jag1*, *Dll1*, *Notch2*, and *Notch3* are detected throughout Rathke's pouch, with *Hes1* demonstrating a restriction from the ventralmost region by E12.5. By the onset of *Pit1* expression at E13.5, *Dll1*, *Notch2*, *Notch3*, and *Hes1* expression have begun to be down-regulated in the perspective anterior pituitary, while persisting in the perilumenal cells. By contrast, *Jag1* expression appears largely restricted to mesenchymal cells lining the invaginated Rathke's pouch. At later stages of pituitary development for E14.5 and E17.5, *Dll1*, *Notch2*, *Notch3*, and *Hes1* were detectable only in cells adjacent to the lumen. These expression patterns reveal that both the ligands and receptors of the Notch pathway are expressed in early stages of pituitary development, and that subsequent down-regulation of expression correlates well with the onset of the pituitary gland maturation, consistent with an inhibitory role for Notch signaling in regulating cell terminal differentiation in the developing pituitary. While *Dll3* is not expressed in early stages of Rathke's pouch formation, its expression is detectable at E14.5 and E17.5 in the intermediate lobe as reported previously (Supplementary Fig. 1), although no defect in melanotrope differentiation was observed in *Dll3*$^{-/-}$ embryos (Raetzman et al. 2004).

Notch activity-dependent Hes1 *expression controls the timing of corticotropes differentiation*

The expression of multiple Notch receptors and ligands during early stages of pituitary development suggested

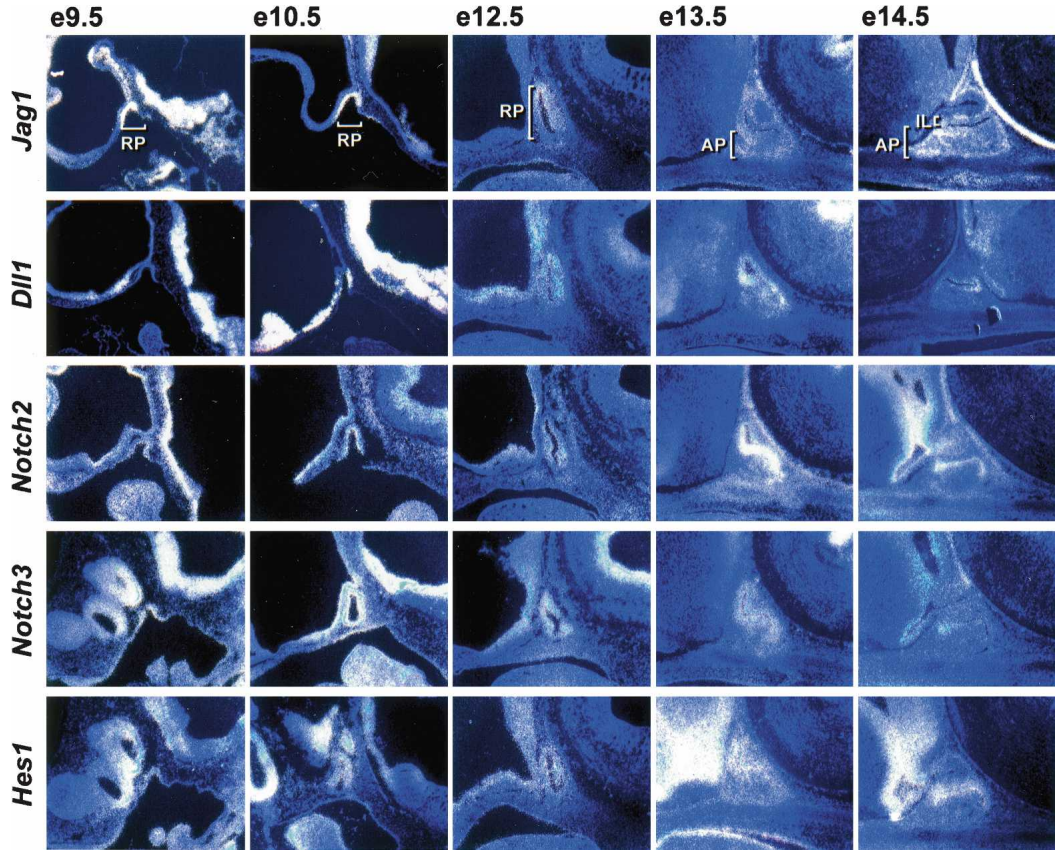

Figure 1. In situ analysis of expression pattern of core components of the Notch signaling pathway during pituitary development. Two ligands (*Dll1*, *Jag1*), two receptors (*Notch2*, *Notch3*), and the downstream target of Notch/Rbp-J signaling (*Hes1*) are expressed in the oral ectoderm and the Rathke's pouch (RP) by E12.5. At E13.5, *Dll1*, *Jag1*, *Notch2*, *Notch3*, and *Hes1* expression are down-regulated in the anterior pituitary (AP) and are further confined to the perilumenal cells. At E14.5, *Jag1* is expressed in the mesenchymal cells surrounding and within the pituitary gland. (IL) Intermediate lobe.

that analysis of the endogenous functions of Notch signaling would be most effective through manipulating the expression of the DNA-binding protein Rbp-J, the primary mediator of Notch signaling. We therefore deleted the *Rbp-J* gene in Rathke's pouch by crossing mice containing a floxed *Rbp-J* allele (Tanigaki et al. 2002) with transgenic mice expressing the Cre recombinase under control of the *Pitx1* promoter (*Pitx1*-Cre). This *Cre* allele exhibited efficient Cre-recombinase activity starting at E9.0 in all progenitors of Rathke's pouch (Olson et al. 2006). Quantitative RT–PCR of mRNA from microdissected E12.5 pituitaries revealed that floxed *Rpb-J* exons 6 and 7 were down-regulated fourfold in *Rbp-J*$^{f/f}$, *Pitx1*-Cre embryos in comparison with *Rbp-J*$^{f/+}$, *Pitx1*-Cre mice (data not shown). In situ hybridization readily detected a decrease in *Hes1* and *Hey1* expression at E11.5, suggesting that *Pitx1*-Cre can effectively mediate floxed *Rbp-J* recombination in pituitary premordium, and *Hes1* and *Hey1* are downstream targets of the Notch signaling in the pituitary (Fig. 2). Because *Hes1* is required in multiple tissues for proper development, we also probed the function of *Hes1* in pituitary organogenesis in order to compare the effects of loss of Notch activity and the loss of a downstream target of Notch signaling. Interestingly, *Rbp-J*$^{f/f}$, *Pitx1*-Cre embryos isolated at E12.5 exhibited similar phenotypes to those observed in *Hes1*$^{-/-}$ embryos (Fig. 2). In situ hybridization and immunofluorescence staining revealed premature differentiation of corticotropes at the most ventral region of the pouch, with concomitant up-regulation of *Tbx19*/*Tpit*, the function of which is necessary for corticotrope terminal differentiation (Pulichino et al. 2003). Expression of bHLH factors such as *Mash1*, *NeuroD1*, and *Math3* were also expanded or ectopically up-regulated, except that no apparent up-regulation of *Math3* was observed in *Hes1*$^{-/-}$. Interestingly, activation of these bHLH factors was restrained in the ventral-most region of the pouch where differentiated corticotropes were observed, suggesting that ablation of Notch activity, or relief from Hes1 repression, is insufficient to activate their expression and that additional components are required.

In the absence of either Notch activity or *Hes1*, there is a decreased number of proliferating pituitary progenitors and an increased number of cells exiting the cell cycle, as demonstrated by the proliferation marker Ki67 staining and bromodeoxyuridine (BrdU) labeling (Fig. 2; Supplementary Fig. 2). The cells in the vicinity of the

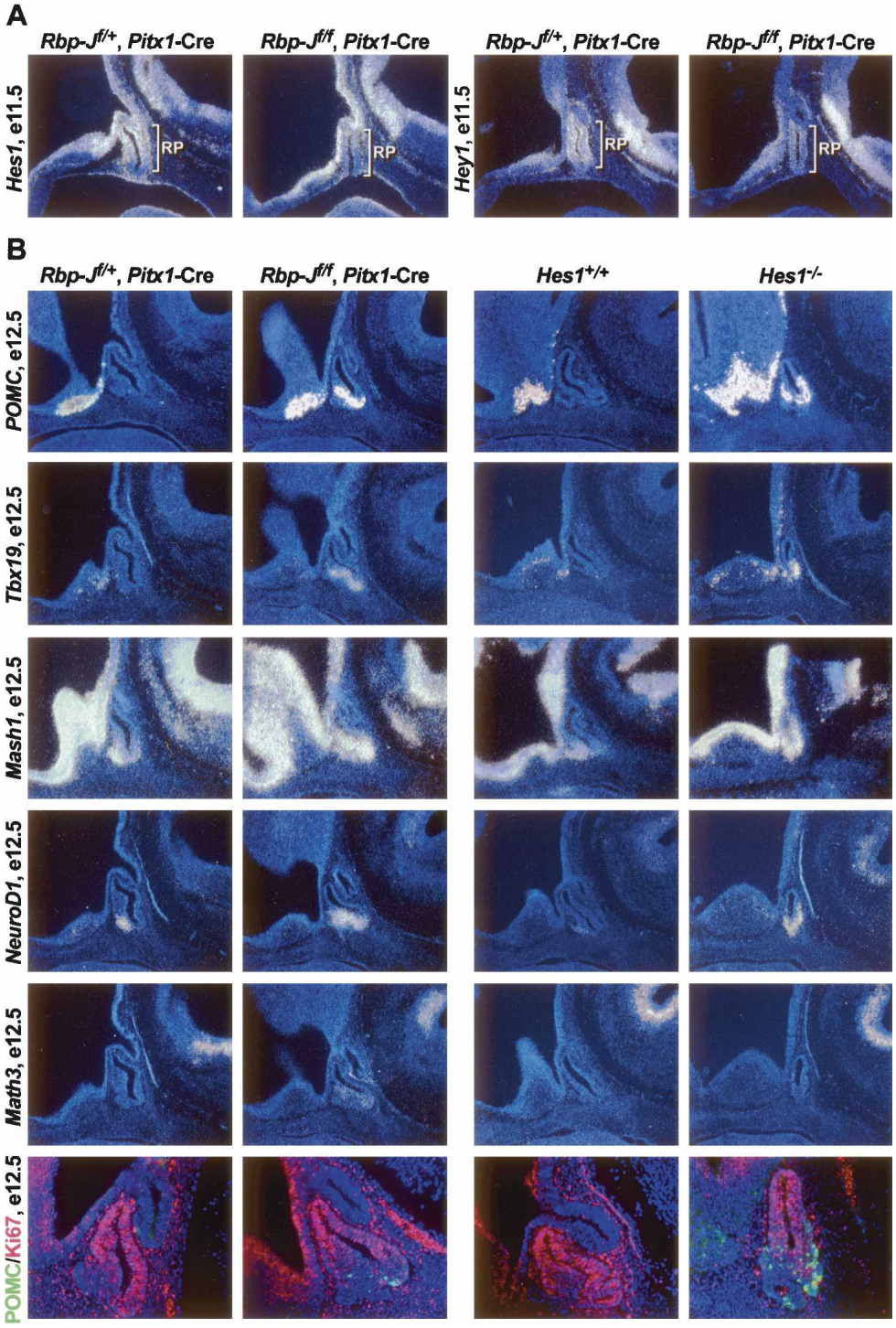

Figure 2. Notch signaling represses premature corticotrope differentiation via its downstream target *Hes1*. (*A*) *Hes1* and *Hey1* are down-regulated in *Rbp-J*$^{f/f}$, *Pitx1*-Cre mice at E11.5. (*B*) Premature corticotrope differentiation is indicated by *POMC* as well as *Tbx19* expression in *Rbp-J*$^{f/f}$, *Pitx1*-Cre mice at E12.5. Expression of bHLH genes, including *Mash1*, *NeuroD1*, and *Math3*, are up-regulated but restricted in the ventral region of Rathke's pouch. Corticotrope premature differentiation is evident in *Hes1*$^{-/-}$ mice, accompanied by up-regulation of *Tbx19*, *Mash1*, and *NeuroD1*. However, there is no pronounced ectopic expression of *Math3*. The posterior lobe of pituitary of *Hes1*$^{-/-}$ mice is absent. Double-immunofluorescence staining of POMC and Ki67 in E12.5 embryos showed increased POMC in *Rbp-J*$^{f/f}$, *Pitx1*-Cre and *Hes1*$^{-/-}$ embryos in comparison with their respective littermate controls. POMC^{+} cells are Ki67^{-}. Cells surrounding the lumen remain proliferative, while more cells at the ventral region of the pouch in mutant embryos exit the cell cycle and are negative for Ki67 staining.

lumen remained proliferative, whereas a higher proportion of cells in the caudomedial region ceased proliferation as compared with control littermates. These differences are not due to enhanced cell death as no significant differences in apoptosis were observed in both the *Rbp-J*$^{f/f}$/*Pitx1*-Cre and the *Hes1*$^{-/-}$ mice, demonstrated by staining for cleaved caspase-3 (data not shown). Double staining of Ki67 and POMC demonstrated that differentiated POMC$^+$ corticotropes are Ki67$^-$, therefore excluding the possibility that the increased number of corticotropes is a consequence of increased proliferation.

Taken together, these data demonstrate that Notch activation is required to prevent premature differentiation of corticotropes, and that *Hes1* is a primary target mediating the Notch pathway in the control of the timing of corticotrope differentiation. In addition, the presence of proliferating progenitors in the absence of Notch activity demonstrates that Notch signaling is not the sole determinant in maintaining the progenitor cell status during pituitary development.

Notch signaling is required for the commitment of Pit1$^+$ precursor cell fate by regulating Prop1 *expression*

During pituitary development, the next cell lineage commitment following the initial determination of corticotropes is characterized by the appearance of *Pit1* transcripts at E13.5. In the *Rbp-J*$^{f/f}$, *Pitx1*-Cre mice, *Pit1* expression fails to be initiated (Fig. 3A), and at E17.5, when the lateral region of the anterior pituitary would be normally populated with Pit1$^+$ cells, most of which would be GH$^+$ somatotropes, only a few Pit1$^+$ cells could be identified in the *Rbp-J*$^{f/f}$, *Pitx1*-Cre mice by either in situ hybridization or immunofluorescence labeling. Consequently, only a few GH$^+$ somatotropes, and almost no TSHβ$^+$ thyrotropes, could be detected (Fig. 3A). Instead, at E14.5 and E17.5, the anterior pituitary was composed predominately of POMC$^+$ corticotropes (Fig. 3C; Supplementary Fig. 3). Gonadotrope differentiation, which occurs independently of *Pit1* expression, was not significantly affected, as judged by expression of *SF1* and *LH*β (Fig. 3A). These results demonstrate that Notch signaling is necessary to induce the onset of Pit1 lineage commitment, and, in its absence, the progenitors take on a corticotrope fate at the expense of the Pit1 lineage. By contrast, analysis of the *Hes1*$^{-/-}$ mice revealed that the ontogeny of initial *Pit1* induction, terminal differentiation of Pit1 lineages, occurred normally (Fig. 3B; Supplementary Fig. 4). These data suggest that down-regulation of *Hes1* does not account for the defects in *Pit1* induction observed in *Rbp-J* mutant embryos, and that Notch signaling may control Pit1$^+$ lineage commitment by regulating downstream targets other than *Hes1*.

Because *Pit1* activation is regulated by the concerted actions of Prop1 and the Wnt/β-catenin signaling pathway, we sought to examine the expression of *Prop1* as well as *Axin2*, a direct downstream target of Wnt/β-catenin signaling and therefore serving as an indicator of signaling activity in this pathway (Olson et al. 2006). In situ hybridization analyses revealed no significant differences in *Axin2* expression in the *Rbp-J*$^{f/f}$, *Pitx1*-Cre mice as compared with heterozygous littermates, implying that the Wnt/β-catenin signaling remained intact in the absence of Notch activity (Fig. 4B). *Prop1* expression, conversely, was markedly diminished by E12.5, when levels of *Prop1* mRNA normally peak. We further noted that at E11.5, when *Prop1* is initially expressed at lower levels, there was no obvious difference in the *Prop1* expression profile exhibited by the mutant and the control (Fig. 4A), suggesting that Notch activity is required for the up-regulation of *Prop1* at E12.5, but not the initiation of *Prop1* expression. By comparison, *Prop1* transcripts in *Hes1*$^{-/-}$ mice at E12.5 were not significantly affected (Fig. 4B), consistent with the model that Rbp-J may modulate *Prop1* expression directly.

To further investigate the molecular mechanism underlying the genetic relationship between Notch activity and *Prop1* expression, an in silico search utilizing VISTA (http://rvista.dcode.org) was performed for conserved noncoding regions in the *Prop1* genes among six mammalian species: mouse, rat, dog, sheep, bovine, and human. Two highly evolutionarily conserved regions (>75%) were identified, in the promoter and the first intron, respectively. A search for conserved regulatory elements in these regions identified a consensus binding site for Rbp-J within the first intron (Fig. 4C). In order to examine whether this putative binding site might be recognized by Rbp-J, we performed electrophoretic mobility shift assays (EMSA) using synthetic oligonucleotides representing the putative binding site and flanking regions, and Rbp-J produced by in vitro transcription and translation. Rbp-J bound efficiently to the *Prop1* intron in vitro, and this binding could be competed with itself or a known Rbp-J-binding site, but not with oligonucleotides in which the putative recognition sites had been mutated (Fig. 4D).

In order to estimate whether Rbp-J is recruited to the *Prop1* gene in vivo, we performed chromatin immunoprecipitation (ChIP) analysis from E12.5 dissected pituitaries using antiserum specific for Rbp-J (Chu and Bresnick 2004). The results revealed recruitment of Rbp-J to the promoter of *Hes1*, as well as to the first intron of *Prop1*, suggesting that *Prop1* is a direct target of Notch signaling activity in the pituitary (Fig. 4E). Consistent with these in vivo findings, transient cotransfection reporter assays in the pituitary cell line GHFT1 employing either a 2-kb fragment including the promoter and first intron of *Prop1*, or the evolutionarily conserved intron region in conjunction with a heterologous minimal promoter, demonstrate that reporter activity was inhibited by small interfering RNA (siRNA) directed against Rbp-J (Fig. 4F,G). These data support a mechanistic role of Rbp-j in direct regulation of *Prop1*.

Notch activity is necessary to suppress melanotrope cell fate

While the ventromedial regions of Rathke's pouch differentiate into Pit1$^+$ precursors, cells originating from

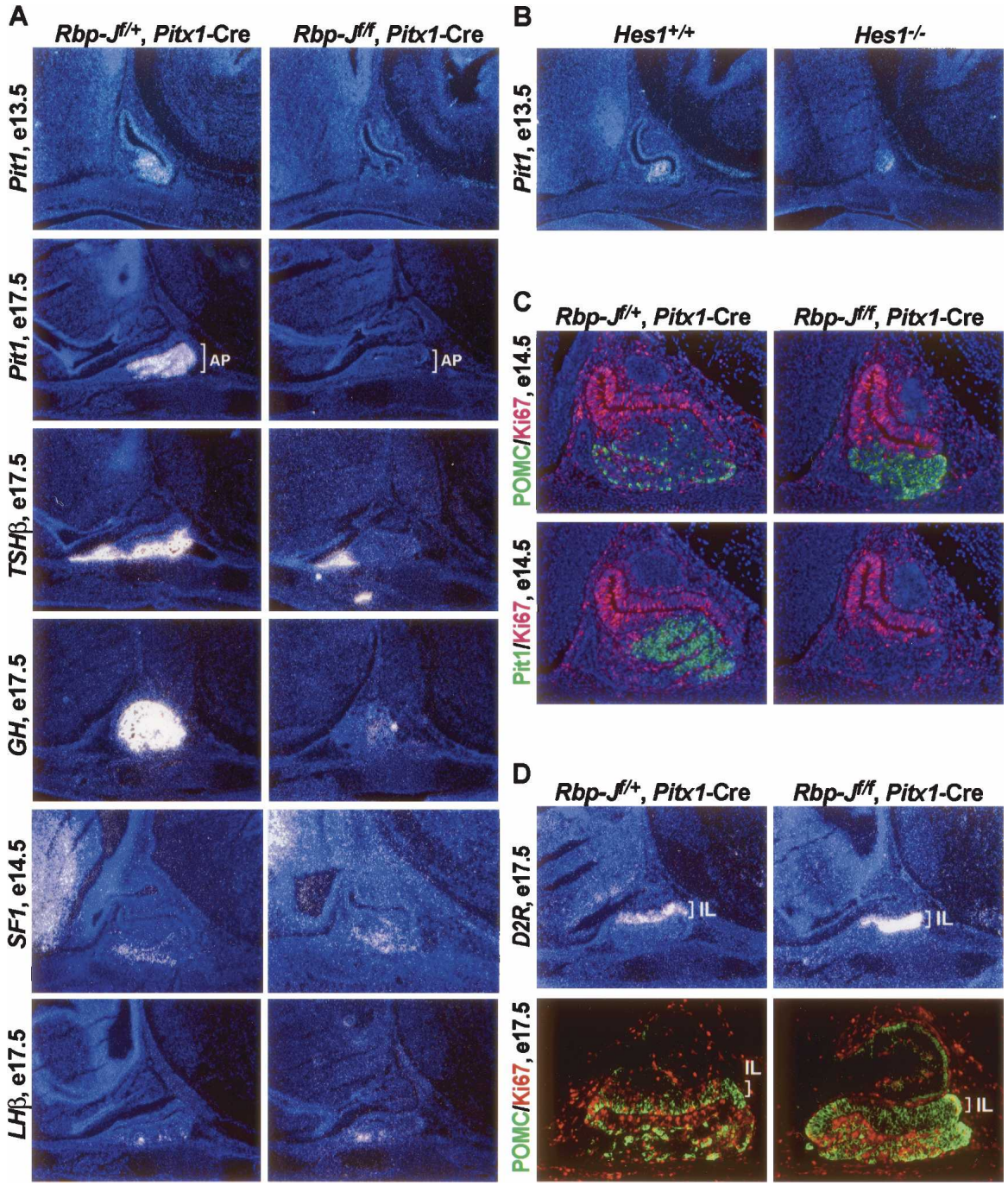

Figure 3. Notch signaling is required for Pit1 lineage commitment. (*A*) *Pit1* expression is absent in *Rbp-J*$^{f/f}$, *Pitx1*-Cre mutant embryos at E13.5. At E17.5, *Rbp-J*$^{f/f}$, *Pitx1*-Cre mutant embryos, *Pit1* expression, and differentiation of Pit1 lineages thyrotropes (*TSH*β) and somatotropes (*GH*) are impaired, while gonadotrope commitment, indicated by *SF1* expression at E14.5 and *LH* expression at E17.5, is not affected in *Rbp-J*$^{f/f}$, *Pitx1*-Cre mutant embryos. (*B*) *Pit1* induction occurs normally in E13.5 *Hes1*$^{-/-}$ embryos. (*C*) Double-immunofluorescence staining of POMC (green) and Ki67 (red), or Pit1 (green) and Ki67 (red) at E14.5 pituitaries shows that the anterior pituitary of the *Rbp-J*$^{f/f}$, *Pitx1*-Cre mutant is populated with an increased number of corticotropes and is devoid of Pit1^{+} cells. The differentiated cells are not proliferative, as indicated by Ki67 staining. Cells surrounding the lumen are Ki67^{+} in both *Rbp-J*$^{f/f}$, *Pitx*-Cre mutant embryos and littermate controls. (*D*) *D2R* expression at the intermediate lobe (IL) is increased in *Rbp-J*$^{f/f}$, *Pitx1*-Cre mutant embryos at E17.5. Dual-immunofluorescence labeling of POMC (green) and Ki67 (red) at E17.5 pituitaries shows almost all POMC^{+} cells in IL are Ki67^{-}.

the dorsal regions of the pouch continue to proliferate, eventually differentiating into Tbx19^{+}, Mash1^{+}, D2R^{+} (dopamine D2 receptor), POMC^{+} intermediate lobe melanotropes (Meador-Woodruff et al. 1989; Japon et al.

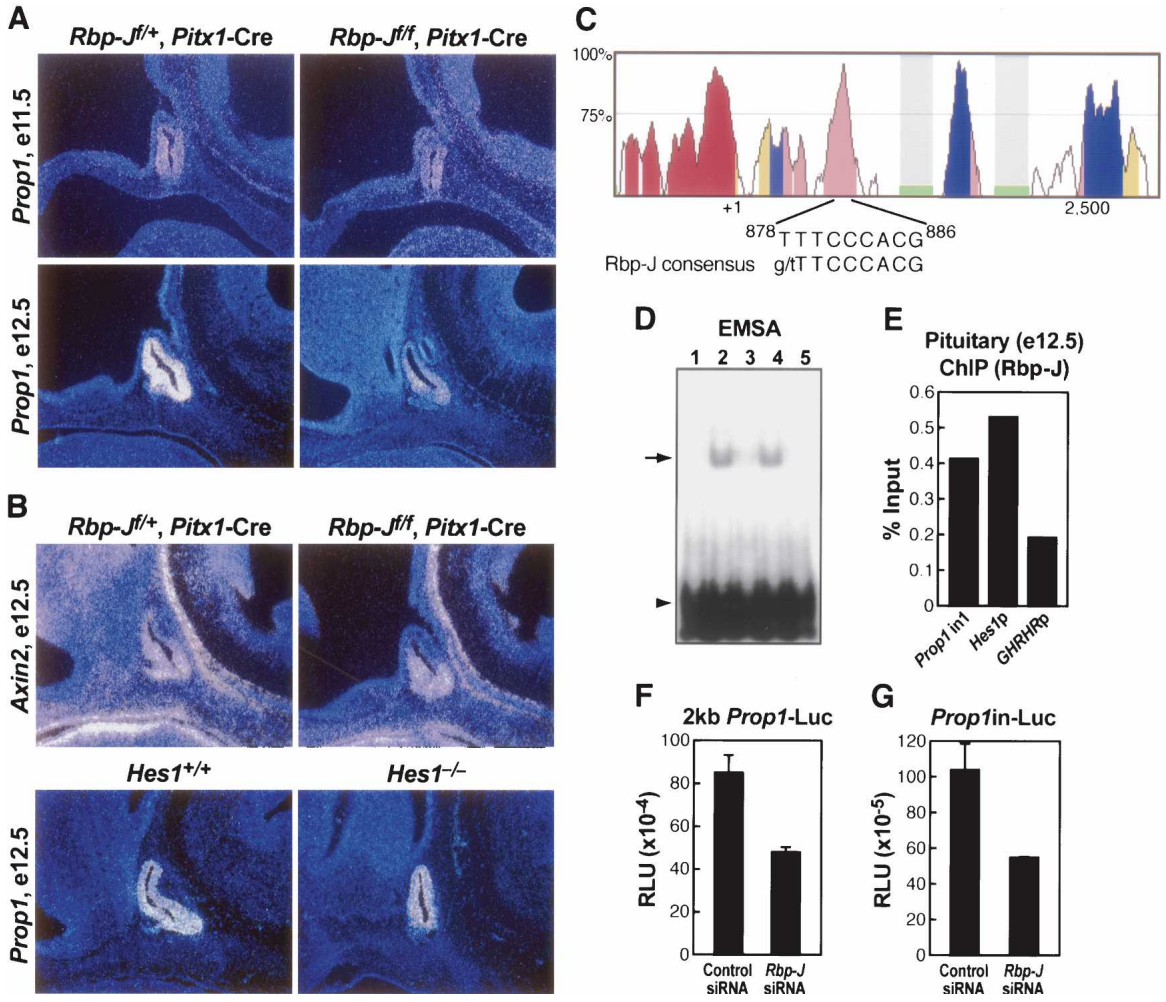

Figure 4. *Prop1* is a direct target of Notch signaling. (*A*) *Prop1* expression is significantly down-regulated in *Rbp-J*$^{f/f}$, *Pitx1*-Cre mutant embryos at E12.5 but is unchanged at E11.5. (*B*) *Axin2* expression in *Rbp-J*$^{f/f}$, *Pitx1*-Cre mutant embryos and *Prop1* expression in *Hes1*$^{-/-}$ at E12.5 are not significantly changed. (*C*) Genomic DNA sequences of mouse and human *Prop1* were compared using VISTA. (Red) Promoters, (yellow) UTRs, (blue) exons, (pink) introns. Two evolutionarily conserved regions, the promoter and the first intron, show >75% homology. A putative Rbp-J-binding site is identified in the first intron. (*D*) A ^{32}P-labeled 25-bp oligonucleotide encompassing the putative Rbp-J-binding site was incubated in the absence (lane *1*) or in the presence (lanes *2–5*) of in vitro translated Rbp-J and the competitors. Unlabeled oligonucleotides (lane *3*), equivalent oligonucleotides where the putative Rbp-J-binding site was mutated (lane *4*), or the oligonucleotides containing a Rbp-J-binding site from Epstein-Barr virus C promoter region (lane *5*) were used as competitors at 100× molar excess. The arrow indicates the shifted probe caused by Rbp-J binding, and the arrowhead indicates free probe. (*E*) Quantitative ChIP assay of E12.5 pituitaries using anti-Rbp-J showed recruitment of Rbp-J to the first intron of *Prop1*. The promoter regions of the *Hes1* and *GHRHR* were used as the positive and negative controls, respectively. (*F,G*) Transient transfection of 2-kb *Prop1*-Luc (*F*) or *Prop1*in-Luc (*G*) with control siRNA or siRNA specific to *Rbp-J*.

1994; Lamolet et al. 2001; Liu et al. 2001; Pulichino et al. 2003). In situ hybridization analyses of *D2R*, and immunostaining of POMC at E17.5, revealed an increase in the overall number of melanotropes in the intermediate lobe of the *Rbp-J*$^{f/f}$, *Pitx1*-Cre mice as compared with control littermates (Fig. 3D). Double-immunofluorescence staining of POMC and Ki67 revealed that intermediate lobe POMC$^+$ cells were negative for Ki67 staining, ruling out the possibility that expansion of the melanotrope population was caused by increased proliferation. By comparison, Ki67$^+$ cells in the intermediate lobe were significantly diminished in *Rbp-J*$^{f/f}$, *Pitx1*-Cre mice, suggesting that more progenitors were differentiated to melanotrope cell fate in the absence of Notch activity. Intriguingly, the intermediate lobe of the *Hes1*$^{-/-}$ embryo is virtually absent (Supplementary Fig. 4). This is likely due to a nonpituitary effect and is currently under investigation.

Ectopic Notch signaling prevents terminal cell differentiation

Notch activity, as indicated by *Hes1* expression, is down-regulated as cells undergo lineage commitment, suggesting that active Notch signaling may interfere with terminal differentiation. In order to test this hy-

pothesis directly, we employed a transgenic mouse model engineered to sustain Notch signaling in Pit1-expressing cells by expressing epitope-tagged Notch1 ICD (NICD) under the control of a 15-kb 5′ flanking region of the *Pit1* promoter, which has been shown to activate target gene expression in three distinct Pit1-dependent lineages. Transgenic mice exhibit a postnatal dwarf phenotype, weighing one-half of wild-type littermates at 1 mo of age; they remain smaller than wild-type littermates throughout life. Analyses of adult transgenic and wild-type littermates revealed hypoplasia of the anterior pituitary in transgenic mice, with prominent reduction of somatotrope, thyrotrope, and lactotrope populations (data not shown). These observations demonstrate that sustained expression of activated Notch in Pit1$^+$ precursors inhibited terminal differentiation of three Pit1-dependent lineages.

Examination of ectopic NICD expression by immunohistochemistry using an epitope-specific antibody demonstrated a spatial and temporal pattern of expression in the anterior pituitary almost identical to that of endogenous *Pit1* expression, initiated at E13.5, and sustained thereafter (Fig. 5A). Dual immunofluorescence labeling with anti-Pit1 and anti-HA revealed colocalization of Pit1 and NICD (Supplementary Fig. 3). Ectopic NICD expression in the Pit1 lineage did not affect apoptosis, as assessed by a TUNEL assay or cleaved caspase 3 immunostaining (data not shown), nor did it affect cell proliferation as assayed by BrdU labeling and Ki-67 staining at E17.5 (Fig. 5B; data not shown). In fact, at E13.5, when Pit1 is initially expressed, double-immunostaining analysis using anti-Pit1 and anti-Ki67 showed that almost all Pit1-expressing cell were Ki67-negative, suggesting that Pit1$^+$ cells were not proliferating at this embryonic stage. Double-immunofluorescence labeling of Pit1 and Ki67 in E14.5 and E17.5 transgenic mice and wild-type controls showed that there was no significant difference in the number of Pit1$^+$/Ki67$^+$ cells. Thus, NICD expression in Pit1$^+$ cells did not affect proliferation or survival of the Pit1 lineages (Fig. 5B). However, in situ hybridization analysis of E17.5 pituitaries from transgenic and wild-type littermates revealed a significant decrease of somatotropes and thyrotropes, as measured by *GH*, *GHRHR* (growth hormone-releasing hormone receptor), and *TSH*β expression (Fig. 5C), with only occasional GH-positive cells and TSHβ-positive cells observed at E17.5 pituitary in transgenic mice. To determine the origin of these cells, double-immunofluorescence labeling with antiserum specific for HA epitope and either GH or TSHβ revealed that the residual GH$^+$ or TSHβ$^+$ cells in transgenic pituitaries were HA-negative, indicating they were derived from Pit1$^+$ precursors that had failed to efficiently express the Pit1-NICD transgene (Fig. 5D). These results suggest that ectopic expression of NICD blocks terminal differentiation and that relief from the Notch repression is prerequisite for cells to undergo terminal differentiation. Pit1-independent corticotropes and gonadotrope lineages, expressing *POMC* and *LH*β, respectively, were not significantly affected by Pit1-NICD expression (Fig. 5C), suggesting that the effects of Notch signaling on inhibition of terminal differentiation occurred in a cell-autonomous manner.

In *Snell* mice, which harbor a point mutation in the *Pit1* gene that abolishes its transcriptional function, three Pit1 lineages are absent while gonadtropes are markedly increased (Dasen et al. 1999), and the latter phenotype was not observed in the NICD transgenic mice. Thus, our results implicated that NICD blocks lineage differentiation likely by interfering with pathways either parallel to or downstream from the Pit1 action.

Ectopic NICD expression down-regulates proneural bHLH expression

Because *Hes1* and *Hes5* are well established Notch targets in other tissues that in turn inhibit the expression or antagonize the functions of cells specifying bHLH proteins, we examined the expression of *Hes1* and *Hes5*, as well as *Mash1* and *NeuroD1*, which are expressed during pituitary development (Liu et al. 2001; Lamolet et al. 2004). In situ hybridization analysis demonstrated up-regulation of *Hes1* and ectopic expression of *Hes5* at E14.5 pituitaries of Pit1-NICD transgenic mice (Fig. 6A), whereas *Hes1* expression is almost undetectable in the anterior pituitary of wild-type embryos at this stage of development and *Hes5* is normally not expressed in pituitary (Figs. 1, 7A). These results indicate that the down-regulation of *Hes1* expression in the wild-type anterior pituitary is most likely due to down-regulation of Notch receptors and ligands, and that Rbp-J itself apparently functions as a transcription repressor at later stages of pituitary development, consistent with the observation that overexpression of a dominant-negative form of Rbp-J (Rbp-J + engrailed repressor domain) in Pit1$^+$ cells did not interfere with terminal differentiation of Pit1 lineages (X. Zhu, unpubl.).

As might be expected, sustained induction of *Hes1* and *Hes5* in Pit1-NICD transgenic mice results in significant down-regulation of *Mash1* and *NeuroD1* in the anterior pituitaries of transgenic mice (Fig. 6A). It has been reported recently that *NeuroD1* is required for the early corticotrope differentiation but not lineage commitment (Lamolet et al. 2004), and in related studies, we have found that *Mash1* exerts roles in terminal differentiation of thyrotropes, gonadtropes, and corticotropes (our unpublished data). Intrigued by these findings, we sought to explore if other proneural bHLH factors are expressed in the pituitary and, if so, whether they are also down-regulated by Pit1-NICD expression. A semiquantitative RT–PCR comparing E14.5 wild-type and transgenic pituitaries was performed to characterize the potential expression of a panel of proneural bHLH factors and *Math3* was identified, expression of which was markedly inhibited in transgenic embryos in comparison with wild-type littermates (Fig. 6A).

Math3 *is required for somatotrope maturation and function*

Targeted disruption of *Math3* leads to cerebellar defects, with increased apoptosis in the external granular layer as

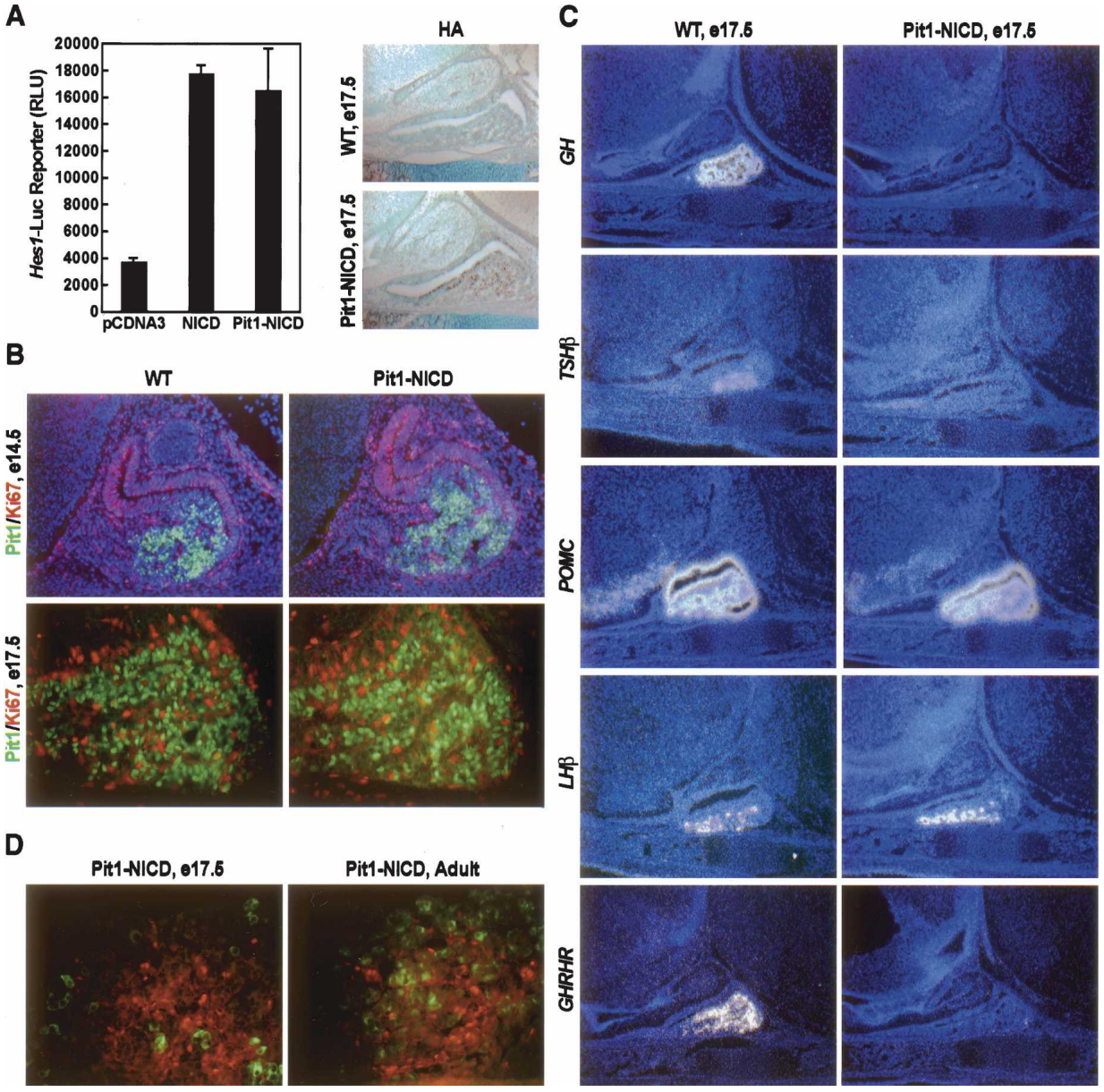

Figure 5. (*A*) Transient transfection in the pituitary GC cell line shows that the Pit1-NICD transgene construct can stimulate *Hes1*-Luc activity. Immunohistochemical staining with anti-HA antibody to detect transgene expression in E17.5 transgenic pituitary. Transgene expression is detectable as early as E13.5 when Pit1 expression begins. (*B*) Double-immunofluorescence staining of Pit1 (green) and Ki-67 (red) in wild-type and transgenic pituitary at E14.5 and E17.5 showed almost all proliferating cells are Pit1-negative at these stages and ectopic NICD expression in Pit1 lineage did not induce proliferation. (*C*) Prolonged activation of Notch signaling inhibits terminal cell differentiation of Pit1 lineages. In situ analysis of the transgenic pituitaries was performed with the *GH*, *TSH*β, *POMC*, *LH*β, and *GHRHR*. Differentiation of corticotropes and gonadotropes is less affected. (*D*) Double-immunofluorescence staining of HA (red) and terminal differentiation markers GH (*left*, green) and TSHβ (*right*, green) in pituitaries of transgenic mice showed that differentiated cells did not express the transgene.

well as postnatal growth retardation (Tomita et al. 2000). However, the underlying molecular mechanism(s) regulated by *Math3* during postnatal growth remains largely unknown. Because *Math3* is expressed in the pituitary, and the postnatal dwarfism phenotype in *Math3*$^{-/-}$ mice resembles those of the Pit1-NICD and *Snell* mice, we probed *Math3* functions in pituitary development. In situ hybridization analysis revealed that *Math3* expression in the anterior pituitary begins at E13.5 and persists throughout adulthood (Fig. 6B). The spatio-temporal expression profile of *Math3* is reminiscent of that of *Pit1*. In silico comparison of the promoter regions of *Math3* from mouse and human using VISTA identified a consensus Pit1-binding site within a highly conserved region (>75%) in the promoter (Fig. 6D), suggesting that *Math3* is likely a direct downstream target of Pit1. ChIP

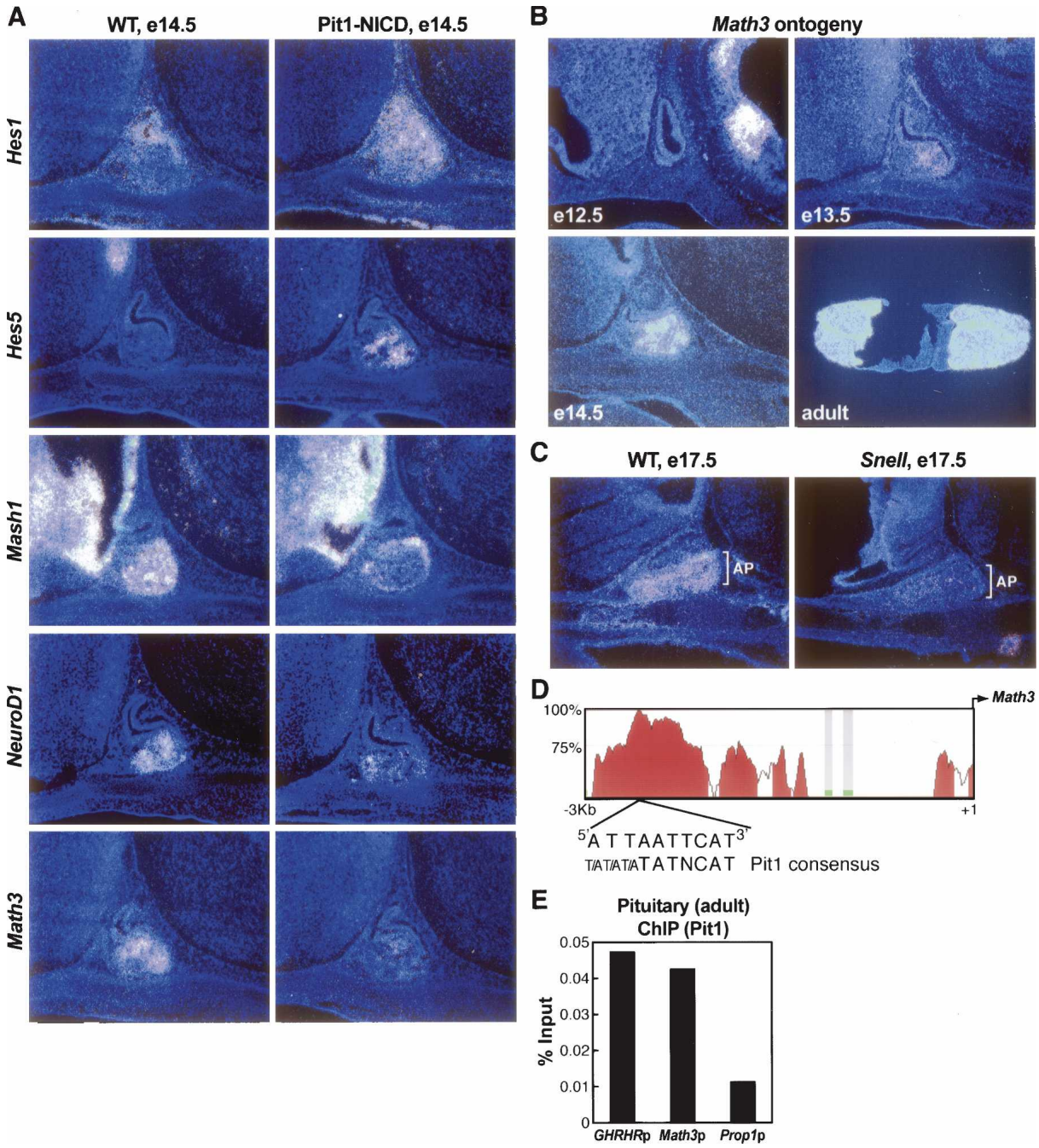

Figure 6. (*A*) In situ analysis of bHLHs expression in E14.5 pituitaries of transgenic mice and wild-type control. Both *Hes1* and *Hes5* were induced by ectopic NICD expression while *Mash1*, *NeuroD1*, and *Math3* were significantly repressed. (*B*) *Math3* expression ontogeny during pituitary development. Expression of *Math3* begins at E13.5 in the caudomedial region of the anterior pituitary and persists in the anterior lobe of the adult pituitary. (*C*) Expression of *Math3* is down-regulated in *Snell* mice at E17.5. (*D*) Alignment of the promoter regions of human and mouse *Math3* using VISTA identified an evolutionarily conserved region in the promoter with >75% homology. A putative binding site for Pit1 lies in this region. (*E*) ChIP from adult pituitaries using anti-Pit1 shows Pit1 is recruited to the *Math3* as well as *GHRHR* promoter regions.

analysis of adult mouse pituitaries revealed recruitment of Pit1 to the *Math3* promoter, as well as to the promoter of *GHRHR*, a known target of Pit1 (Fig. 6E). Moreover, *Math3* expression in the pituitary was significantly reduced in *Snell* mice (Fig. 6C). Together, these data suggest that *Math3* is a direct downstream target of Pit1 and uncover a developmentally regulated program whereby *Math3* is repressed by Notch signaling and activated by

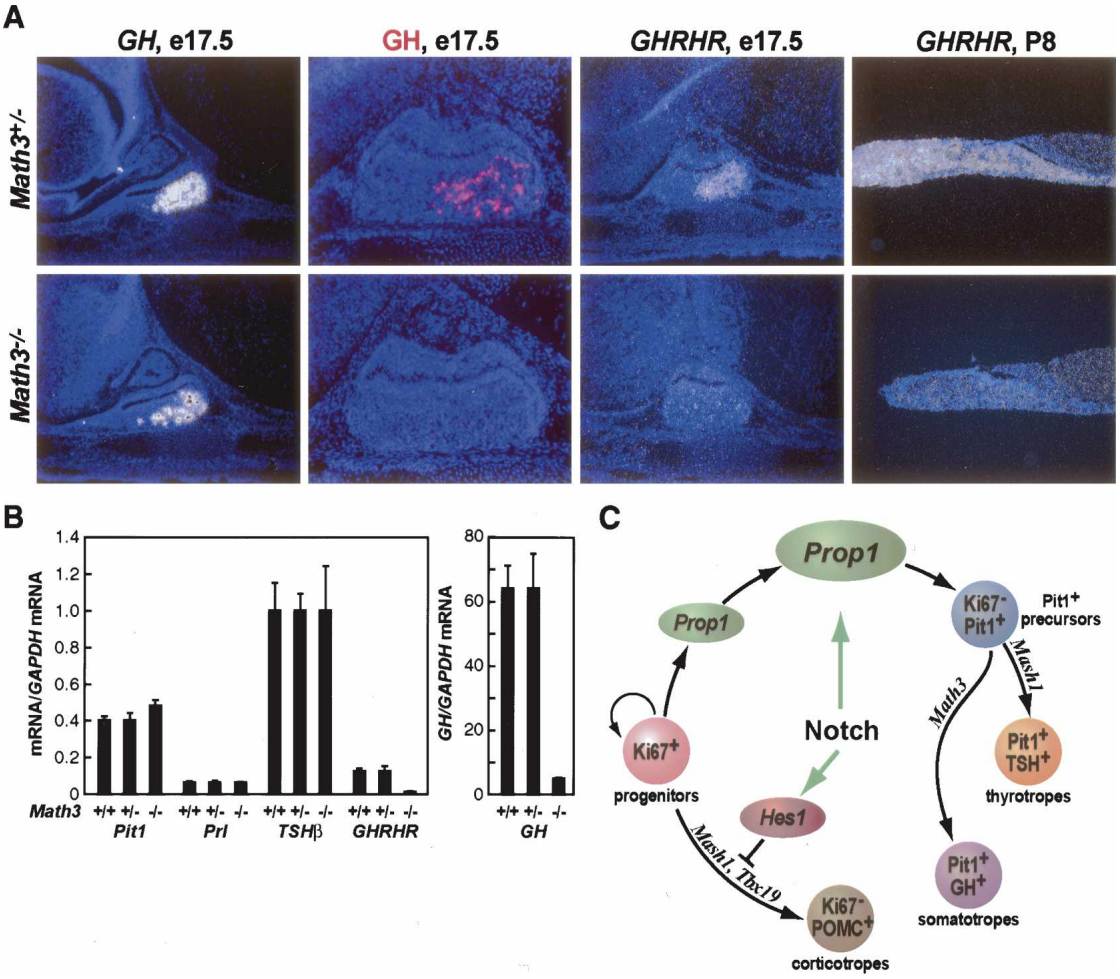

Figure 7. (*A*) *Math3* is required for somatotrope maturation. In situ analysis in E17.5 pituitaries of *Math3*$^{-/-}$ and littermate controls showed that somatotrope markers *GH* and *GHRHR* are down-regulated in *Math3*$^{-/-}$. Immunofluorescence staining showed GH protein is undetectable at E17.5 in *Math3*$^{-/-}$ embryos. *GHRHR* expression remained undetectable in postnatal *Math3*$^{-/-}$ pituitaries. (*B*) Quantitative RT–PCR of *Pit1*, *Prl*, *TSH*β, *GHRHR*, and *GH* at E17.5 pituitaries showed significant and specific down-regulation of *GH* and *GHRHR* mRNA. (*C*) Model of Notch signaling in pituitary development. Notch-regulated *Hes1* expression maintains self-renewal of the Ki67^{+} progenitor and prevents precocious corticotrope differentiation. Notch activity promotes *Prop1* up-regulation at E12.5 and drives progenitors to adopt the fate of Pit1^{+} precursors. *Mash1* and *Math3* are required for proper development of thyrotropes and somatotropes, respectively.

the tissue-specific POU domain transcription factor Pit1. To our knowledge, in addition to *Pit1* itself, *Math3* is the first identified transcription factor regulated by Pit1.

Examination of pituitaries from P8 and P15 *Math3*$^{-/-}$ and wild-type littermates revealed a hypoplastic anterior pituitary with decreased numbers of somatotropes in *Math3*$^{-/-}$ mice (data not shown). At E17.5, expression of *POMC*, *LH*β, *Pit1*, α*GSU*, and *TSH*β were not affected in *Math3*$^{-/-}$ mice (Fig. 7B; data not shown). However, expression of *GHRHR* was almost completely abolished. The number of somatotropes expressing *GH* was markedly decreased and, more strikingly, even fewer GH^{+} cells could be detected by immunostaining (data not shown), suggesting that *Math3* is required in somatotropes for expression of *GHRHR* and *GH* at embryonic stages. Quantitative RT–PCR assay confirmed reduced levels of total *GH* mRNA in E17.5 *Math3*$^{-/-}$ pituitaries, and further studies revealed no aberrant mRNA splicing of *GH* mRNA that might lead to defective GH protein synthesis (data not shown). The molecular mechanisms of the apparent discrepancy between the levels of *GH* mRNA and protein have clear implications that will be the subject of future investigation. However, the block of somatotrope maturation in *Math3*$^{-/-}$ mice is transient and recovers postnatally, because in P8 and P15 *Math3*$^{-/-}$ pituitaries, GH^{+} somatotropes, although obviously present in reduced cell number, were readily detectable by immunostaining, suggesting that *Math3* is necessary for the proper onset of somatotrope specification. Expression of *GHRHR*, in contrast, remained minimal (Fig. 7A). The postnatal dwarf phenotype in *Math3*$^{-/-}$ mice is likely attributable to the reduced number of somatotropes, as is observed in *little* mice, which harbor a point mutation in the *GHRHR* gene, rendering

somatotropes incapable of responding to the hypothalamic trophic factor GHRH (Lin et al. 1993).

Discussion

The distinct cell types in the pituitary gland are generated in a temporal and spatial fashion. We have shown here that Notch activity operates in a precise temporal window during pituitary development. In early developmental stages, Notch signaling prevents premature differentiation by regulating *Hes1* expression. Perhaps more importantly, Notch signaling plays an essential role in controlling the lineage commitment of Pit1$^+$ precursors, without which they would "switch" to an alternative cell fate. It does so, at least in large part, by directly regulating the expression of *Prop1*, a gene that is necessary for the genesis of Pit1$^+$ precursors. In the late phases of pituitary development, however, Notch activity is dramatically attenuated in Pit1$^+$ cells, in part because expression of a subset of bHLH factors that are otherwise negatively regulated by active Notch signaling is required for terminal differentiation of Pit1 lineages. One of these bHLH factors, *Math3*, is itself a downstream transcription target of Pit1, and is crucial for maturation and expansion of somatotropes through regulation of *GHRHR* expression.

Notch activation controls formation of Pit1 precursors

We have shown that both ligands and receptors of the Notch signaling pathway are expressed in proliferating progenitors in Rathke's pouch during early pituitary development. These cells transduce Notch signaling, as indicated by the loss of the Notch downstream effectors *Hes1* and *Hey1* when Notch activity is impeded by deletion of the *Rbp-J* gene. As cells within the Rathke's pouch exit the cell cycle, migrate ventrally and laterally out of the proliferative zone, and undergo lineage commitment and subsequent terminal differentiation, Notch signaling is turned off, and remaining Notch activity can be detected in only periluminal cells. Thus, the proliferative zone in pituitary development to a certain extent is similar to the ventricular zone in cerebral cortex development. In that system, Notch signaling is required to maintain the progenitor pools and inhibit neuronal differentiation (for review, see Yoon and Gaiano 2005). Due to the great inherent redundancy within the Delta/Notch pathway and the pleiotropic defects associated with deletion of some of the Notch pathway components, blocking the Notch signaling results in premature differentiation and depletion of progenitor pools, precluding the analysis of Notch function in later-born cell lineage determination.

We have observed that in Rathke's pouch when *Rbp-J* is conditionally removed in progenitor cells, there is no immediate complete conversion of progenitor cells into post-mitotic corticotropes. Instead, the Ki67$^+$ proliferative zone remains, which is capable of supporting the differentiation of gonadotropes and intermediate lobe melanotropes, implying that the progenitor cells retain a certain degree of pluripotency. However, Pit1$^+$ precursors are almost completely missing, and instead are converted into corticotropes. Our data suggest a model in which Notch signaling controls the formation of diverse precursor subtypes from a progenitor pool. In this model, progenitors receiving no or only a short pulse of Notch signaling progress to the first-born cell lineage corticotropes, while those receiving longer durations of Notch signaling are suggested to undergo irreversible changes in gene expression and/or epigenetic status, such that they are competent to assume a later-born cell fate such as the Pit1 lineage precursor. Consistent with this model is our finding that Notch signaling is essential for the maintenance of *Prop1* expression, providing a molecular mechanism underlying Notch signaling-dependent commitment of Pit1 precursors. This proposed model is likely generally employed in other developmental contexts. It has been shown that in the ventricular zone during mammalian cerebral cortex development, radial glial cells that have been temporarily subjected to Notch activation skip the early-born cell fate and differentiate into later-born upper-layer neurons (Mizutani and Saito 2005). In a detailed analysis of the kinetics and mechanisms of T-lineage differentiation in an in vitro culture system, continuous Notch signaling is obligatory to maintain development along the T-cell pathway (Taghon et al. 2005).

In addition to the well-characterized Notch targets *Hes1* and *Hey1*, we have identified *Prop1* as a Notch downstream target. However, their expression exhibits different dynamics, with *Hes1* and *Hey1* detectable at E9.5 in Rathke's pouch—indicative of active Notch signaling (Fig. 1; data not shown)—whereas *Prop1* is not expressed until E11.5, suggesting that Notch activity is not sufficient to induce *Prop1* expression (Sornson et al. 1996) and that initiation of *Prop1* expression is independent of the Notch activity. However, the sustained expression of *Prop1* is Notch-dependent. These data are consistent with the view that expression of Notch targets is dependent on the developmental status and appears to be target gene specific (Umesono et al. 2002; Anthony et al. 2005; Taghon et al. 2005). Premature expression of *Prop1* in Rathke's pouch proves to be deleterious, leading to agenesis of the anterior pituitary gland, probably by inhibiting the endogenous function of *Hesx1* (Dasen et al. 2001). Prolonged expression of *Prop1* interferes with anterior pituitary cell differentiation (Cushman et al. 2001). Therefore, identifying the critical factors initiating *Prop1* gene expression will provide further insights into the integration of the Notch pathway with other developmental programs. It has been reported recently that *Prop1* is required for Notch2 protein expression in the pituitary (Raetzman et al. 2004). However, our in situ hybridization analyses of *Prop1*$^{-/-}$ embryos showed that expression of *Notch2* and *Hes1*, which is dependent on Notch signaling, is not significantly affected (Raetzman et al. 2004; our unpublished data), suggesting that deletion of *Prop1* is not sufficient to downregulate Notch signaling during pituitary development.

Down-regulation of Notch activity at a later stage is necessary for terminal differentiation

Notch signaling is down-regulated as cells exit the cell cycle and undergo terminal differentiation. We show that forced expression of NICD in Pit1$^+$ post-mitotic cells is sufficient to prevent them from differentiating into hormone-producing cells, consistent with the role of Notch signaling in maintaining progenitor fate. These data highlight the importance of properly controlled Notch signaling during development and raise a fundamental question as to the regulation of Notch activity. Our expression data show that Notch activity in the pituitary is closely correlated with the expression of Notch receptors and ligands, implying a key component of this regulation would be their own expression, although other cellular mechanisms may also be involved (for review, see Schweisguth 2004). A recent study in Zebrafish retinal neurogenesis has demonstrated that a mutation in histone deacetylase 1 (Hdac 1) results in a defect in the transition from proliferating progenitor cells to post-mitotic neurons due to a failure to suppress Notch and Wnt pathways. Although this mechanism may be cell type specific, it suggests that transcription corepressors of Rbp-J may feedback to regulate Notch activity (Yamaguchi et al. 2005).

NICD expression in Pit1$^+$ post-mitotic cells leads to induction of *Hes1*, *Hes5*, and *Hey1* and repression of *Mash1*, *NeuroD1*, and *Math3*, implicating their functions in Pit1 lineage terminal differentiation. Indeed, *Mash1* executes roles in differentiation of thyrotrope as well as corticotrope, melanotrope, and gonadotrope (our unpublished data). In this study, we demonstrate that *Math3* is critical for maturation and expansion of somatotropes by regulating the expression of *GHRHR*. Thus, using the Pit1-NICD transgenic mouse as a genetic approach, we have uncovered bHLH factors as new components involved in Pit1 lineage differentiation. Deletion of neither *Mash1* nor *Math3* alone account for the full spectrum of defects in the Pit1-NICD transgenic mouse and, therefore, whether they exert overlapping roles during pituitary development, as has been demonstrated in neurogenesis (Tomita et al. 2000), is the subject of ongoing investigation.

Materials and methods

Mice

A DNA-encoded mouse Notch1 intracellular domain, amino acids 1744–2183, was generously provided by Dr. R. Kopan (Washington University, St. Louis, MO) (Schroeter et al. 1998). A hemagglutinin (HA) tag was added at the C terminus of NICD cDNA. The Notch1-ICD-HA ORF was inserted between a rabbit 0.65-kb β-globin intron and a 0.63-kb poly(A) fragment of the human growth hormone gene at the 3′ end. The 15-kb *Pit1* promoter was inserted 5′ of this cassette. Transgenic mice were generated as described in Treier et al. (1998), and transgenic animals were genotyped by PCR using primers 5′-GCAACGT GCTGGTTATTGTGC-3′ and 5′-CGGTCTGTCTGGTTGTG CAAGCTG-3′. The transgenic line was maintained on a CB6F1 background as a heterozygote. The male transgenic animals are dwarf and fertile while the females are dwarf and sterile. Other mice used in this study—floxed *Rbp-J*, *Hes1* knockout, and *Math3* knockout—have been described previously (Ishibashi et al. 1995; Tomita et al. 2000; Tanigaki et al. 2002). The *Rbp-J*$^{floxed/floxed}$, *Pitx1*-Cre mutant embryos were obtained by crossing *Rbp-J*$^{floxed/floxed}$ mice with mice heterozygeous for floxed *Rbp-J* and *Pitx1*-Cre (Olson et al. 2006).

In situ hybridization and immunofluorescence

In situ hybridization and immunofluorescence were carried out as previously described (Simmons et al. 1990). Mouse embryos from E9.5 to E17.5 were fixed in 10% neutral formalin, penetrated with 20% sucrose in PBS, and embedded in OCT compound. Serial 16-µm sections were hybridized with ^{35}S-labeled antisense RNA probes. The probes used in this study were either purchased as EST clones from ATCC or generated by RT–PCR from various tissues and verified by sequencing. For immunofluorescence staining, the sections were boiled for 10 min in 10 mM citrate buffer (pH 6.0) to retrieve antigens and stained with mouse mAbs against the HA tag (Babco, 1:400), Ki-67 (Pharmingen, 1:50), BrdU (ICN Biomedicals, 1:20), rabbit polyclonal antibodies against GH (DAKO, 1:200), TSHβ (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, rabbit, 1:400), ACTH (Sigma, 1:100), and Pit-1 (1:200). Secondary peroxidase, Alexa Fluor 488-, and Alexa Fluor 594-conjugated antibodies were from Jackson ImmunoResearch and Molecular Probes. Slides were coverslipped in Vectashield Mounting Medium with DAPI (Vector Laboratories). The results were analyzed on a Zeiss Axioplan2 microscope with a Hamamatsu camera, and pictures were superimposed in Adobe Photoshop.

EMSA, transfection, ChIP, and quantitative PCR

EMSA experiments were performed as previously described (Reizis and Leder 2002). Double-stranded oligonucleotides were labeled with γ-^{32}P-ATP. Rbp-J was transcribed and translated using the TNT Quick Coupled Transcription/Translation Systems (Promega). In vitro translated proteins were incubated with 1× binding buffer (25 mM Tris at pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol), DNA competitors, and 1 µg of poly-dIdC for 15 min on ice prior to adding probe. Probe was added to the reaction and allowed to bind for 20 min at room temperature, and then protein–DNA complexes were resolved by electrophoresis. Oligonucleotides used for EMSA are 5′-CTTGAGCTCGTGGGAAAGGCTTGCC-3′, 5′-GGCAAG CCTTTCCCACGAGCTCAAG-3′ (*Prop1* intron); 5′-CTTGA GCTCGTGaacAAGGCTTGCC-3′, 5′-GGCAAGCCTTgttCA CGAGCTCAAG-3′, (*Prop1* intron with mutations); and 5′-AAACACGCCGTGGGAAAAAATTTGG-3′, 5′-CCAAATT TTTTCCCACGGCGTGTTT-3′ (Rbp-J-binding site from Epstein-Barr virus C promoter region). Transient transfection of pituitary cell lines GC and GHFT1 using SuperFect (Qiagen) or Lipofectamine 2000 (Invitrogen) was performed according to the manufacturers′ instructions. ChIPs were performed as previously described (Wang et al. 2006) with some modifications. The pituitaries were dissected from wild-type E12.5 embryos or adult mice and cross-linked with 2% formaldehyde for 20 min at room temperature. Aliquots (1 µL) of 30 µL of purified DNA fragments were analyzed by quantitative real-time PCR. For RT–PCR analysis, total RNA was isolated from dissected pituitary using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using SuperScript II (Invitrogen). Quantitative RT–

PCR using Sybr Green was performed on an Mx3000P QPCR System (Stratagene). Primers sequences are available on request.

Acknowledgments

We thank T. Honjo for the floxed *Rbp-J* mice, R. Kopan for the NICD cDNA and *Hes1*-Luciferase reporter constructs, and R.J. McEvilly and C.R. Lin for critical reading of the manuscript and suggestions. We thank the UCSD transgenic core facility for assistance in generating transgenic mice, H. Taylor for animal husbandry, C. Nelson for cell culture assistance, and J. Hightower and M. Fisher for figure and manuscript preparation. X.Z. was supported by National Institutes of Health Individual National Research Service Award 5F32NS10819. M.G.R. is an investigator with the Howard Hughes Medical Institute. This research was supported by a grant from the NIDDK to M.G.R.

References

Anthony, T.E., Mason, H.A., Gridley, T., Fishell, G., and Heintz, N. 2005. Brain lipid-binding protein is a direct target of Notch signaling in radial glial cells. *Genes & Dev.* **19:** 1028–1033.

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. 1999. Notch signaling: Cell fate control and signal integration in development. *Science* **284:** 770–776.

Burns, C.E., Traver, D., Mayhall, E., Shepard, J.L., and Zon, L.I. 2005. Hematopoietic stem cell fate is established by the Notch–Runx pathway. *Genes & Dev.* **19:** 2331–2342.

Camper, S.A., Saunders, T.L., Katz, R.W., and Reeves, R.H. 1990. The Pit-1 transcription factor gene is a candidate for the murine Snell dwarf mutation. *Genomics* **8:** 586–590.

Chesnokova, V. and Melmed, S. 2002. Minireview: Neuro–immuno–endocrine modulation of the hypothalamic–pituitary–adrenal (HPA) axis by gp130 signaling molecules. *Endocrinology* **143:** 1571–1574.

Chu, J. and Bresnick, E.H. 2004. Evidence that C promoter-binding factor 1 binding is required for Notch-1-mediated repression of activator protein-1. *J. Biol. Chem.* **279:** 12337–12345.

Crosnier, C., Vargesson, N., Gschmeissner, S., Ariza-McNaughton, L., Morrison, A., and Lewis, J. 2005. Delta–Notch signalling controls commitment to a secretory fate in the zebrafish intestine. *Development* **132:** 1093–1104.

Cushman, L.J., Watkins-Chow, D.E., Brinkmeier, M.L., Raetzman, L.T., Radak, A.L., Lloyd, R.V., and Camper, S.A. 2001. Persistent Prop1 expression delays gonadotrope differentiation and enhances pituitary tumor susceptibility. *Hum. Mol. Genet.* **10:** 1141–1153.

Dasen, J.S. and Rosenfeld, M.G. 2001. Signaling and transcriptional mechanisms in pituitary development. *Annu. Rev. Neurosci.* **24:** 327–355.

Dasen, J.S., O'Connell, S.M., Flynn, S.E., Treier, M., Gleiberman, A.S., Szeto, D.P., Hooshmand, F., Aggarwal, A.K., and Rosenfeld, M.G. 1999. Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. *Cell* **97:** 587–598.

Dasen, J.S., Barbera, J.P., Herman, T.S., Connell, S.O., Olson, L., Ju, B., Tollkuhn, J., Baek, S.H., Rose, D.W., and Rosenfeld, M.G. 2001. Temporal regulation of a paired-like homeodomain repressor/TLE corepressor complex and a related activator is required for pituitary organogenesis. *Genes & Dev.* **15:** 3193–3207.

Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N., et al. 2005. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat. Immunol.* **6:** 314–322.

Ericson, J., Norlin, S., Jessell, T.M., and 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* **125:** 1005–1015.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., and Artavanis-Tsakonas, S. 2005. Notch signals control the fate of immature progenitor cells in the intestine. *Nature* **435:** 964–968.

Gage, P.J., Brinkmeier, M.L., Scarlett, L.M., Knapp, L.T., Camper, S.A., and 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. *Mol. Endocrinol.* **10:** 1570–1581.

Gaiano, N., Nye, J.S., and Fishell, G. 2000. Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* **26:** 395–404.

Grandbarbe, L., Bouissac, J., Rand, M., Hrabe de Angelis, M., Artavanis-Tsakonas, S., and Mohier, E. 2003. Delta–Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. *Development* **130:** 1391–1402.

Herzog, W., Zeng, X., Lele, Z., Sonntag, C., Ting, J.W., Chang, C.Y., and Hammerschmidt, M. 2003. Adenohypophysis formation in the zebrafish and its dependence on sonic hedgehog. *Dev. Biol.* **254:** 36–49.

Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A.J., Nye, J.S., Conlon, R.A., Mak, T.W., Bernstein, A., and van der Kooy, D. 2002. Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes & Dev.* **16:** 846–858.

Irvine, K.D. 1999. Fringe, Notch, and making developmental boundaries. *Curr. Opin. Genet. Dev.* **9:** 434–441.

Ishibashi, M., Ang, S.L., Shiota, K., Nakanishi, S., Kageyama, R., and Guillemot, F. 1995. Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix–loop–helix factors, premature neurogenesis, and severe neural tube defects. *Genes & Dev.* **9:** 3136–3148.

Iso, T., Kedes, L., and Hamamori, Y. 2003. HES and HERP families: Multiple effectors of the Notch signaling pathway. *J. Cell. Physiol.* **194:** 237–255.

Japon, M.A., Rubinstein, M., and Low, M.J. 1994. In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. *J. Histochem. Cytochem.* **42:** 1117–1125.

Krebs, L.T., Iwai, N., Nonaka, S., Welsh, I.C., Lan, Y., Jiang, R., Saijoh, Y., O'Brien, T.P., Hamada, H., and Gridley, T. 2003. Notch signaling regulates left–right asymmetry determination by inducing Nodal expression. *Genes & Dev.* **17:** 1207–1212.

Lai, E.C. 2004. Notch signaling: Control of cell communication and cell fate. *Development* **131:** 965–973.

Lamolet, B., Pulichino, A.M., Lamonerie, T., Gauthier, Y., Brue, T., Enjalbert, A., and Drouin, J. 2001. A pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. *Cell* **104:** 849–859.

Lamolet, B., Poulin, G., Chu, K., Guillemot, F., Tsai, M.J., and Drouin, J. 2004. Tpit-independent function of Neurod1(β2) in pituitary corticotroph dfferentiation. *Mol. Endocrinol.* **18:** 995–1003.

Lewis, J. 1998. Notch signalling and the control of cell fate

choices in vertebrates. *Semin. Cell Dev. Biol.* **9:** 583–589.
Li, S., Crenshaw III, E.B., Rawson, E.J., Simmons, D.M., Swanson, L.W., and Rosenfeld, M.G. 1990. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature* **347:** 528–533.
Lin, S.C., Lin, C.R., Gukovsky, I., Lusis, A.J., Sawchenko, P.E., and Rosenfeld, M.G. 1993. Molecular basis of the little mouse phenotype and implications for cell type-specific growth. *Nature* **364:** 208–213.
Liu, J., Lin, C., Gleiberman, A., Ohgi, K.A., Herman, T., Huang, H.P., Tsai, M.J., and Rosenfeld, M.G. 2001. Tbx19, a tissue-selective regulator of POMC gene expression. *Proc. Natl. Acad. Sci.* **98:** 8674–8679.
Meador-Woodruff, J.H., Mansour, A., Bunzow, J.R., Van Tol, H.H., Watson Jr., S.J., and Civelli, O. 1989. Distribution of D2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci.* **86:** 7625–7628.
Mizutani, K. and Saito, T. 2005. Progenitors resume generating neurons after temporary inhibition of neurogenesis by Notch activation in the mammalian cerebral cortex. *Development* **132:** 1295–1304.
Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S., and Itoh, N. 2000. FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochem. Biophys. Res. Commun.* **277:** 643–649.
Olson, L.E., Tollkuhn, J., Scafoglio, C., Krones, A., Zhang, J., Ohgi, K.A., Wu, W., Taketo, M.M., Kemler, R., Grosschedl, R., et al. 2006. A homeodomain-mediated mechanism for distinct β-catenin-dependent switching events dictating cell lineage determination. *Cell* **125:** 593–605.
Pulichino, A.M., Vallette-Kasic, S., Tsai, J.P., Couture, C., Gauthier, Y., and Drouin, J. 2003. Tpit determines alternate fates during pituitary cell differentiation. *Genes & Dev.* **17:** 738–747.
Raetzman, L.T., Ross, S.A., Cook, S., Dunwoodie, S.L., Camper, S.A., and Thomas, P.Q. 2004. Developmental regulation of Notch signaling genes in the embryonic pituitary: Prop1 deficiency affects Notch2 expression. *Dev. Biol.* **265:** 329–340.
Raya, A., Kawakami, Y., Rodriguez-Esteban, C., Buscher, D., Koth, C.M., Itoh, T., Morita, M., Raya, R.M., Dubova, I., Bessa, J.G., et al. 2003. Notch activity induces Nodal expression and mediates the establishment of left–right asymmetry in vertebrate embryos. *Genes & Dev.* **17:** 1213–1218.
Reizis, B. and Leder, P. 2002. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. *Genes & Dev.* **16:** 295–300.
Revest, J.M., Spencer-Dene, B., Kerr, K., De Moerlooze, L., Rosewell, I., and Dickson, C. 2001. Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. *Dev. Biol.* **231:** 47–62.
Rizzoti, K. and Lovell-Badge, R. 2005. Early development of the pituitary gland: Induction and shaping of Rathke's pouch. *Rev. Endocr. Metab. Disord.* **6:** 161–172.
Sbrogna, J.L., Barresi, M.J., and Karlstrom, R.O. 2003. Multiple roles for Hedgehog signaling in zebrafish pituitary development. *Dev. Biol.* **254:** 19–35.
Schroeter, E.H., Kisslinger, J.A., and Kopan, R. 1998. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393:** 382–386.
Schweisguth, F. 2004. Notch signaling activity. *Curr. Biol.* **14:** R129–R138.
Sheng, H.Z. and Westphal, H. 1999. Early steps in pituitary organogenesis. *Trends Genet.* **15:** 236–240.
Simmons, D.M., Voss, J.W., Ingraham, H.A., Holloway, J.M., Broide, R.S., Rosenfeld, M.G., and Swanson, L.W. 1990. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. *Genes & Dev.* **4:** 695–711.
Sornson, M.W., Wu, W., Dasen, J.S., Flynn, S.E., Norman, D.J., O'Connell, S.M., Gukovsky, I., Carriere, C., Ryan, A.K., Miller, A.P., et al. 1996. Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. *Nature* **384:** 327–333.
Taghon, T.N., David, E.S., Zuniga-Pflucker, J.C., and Rothenberg, E.V. 2005. Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling. *Genes & Dev.* **19:** 965–978.
Takuma, N., Sheng, H.Z., Furuta, Y., Ward, J.M., Sharma, K., Hogan, B.L., Pfaff, S.L., Westphal, H., Kimura, S., and Mahon, K.A. 1998. Formation of Rathke's pouch requires dual induction from the diencephalon. *Development* **125:** 4835–4840.
Tanigaki, K., Han, H., Yamamoto, N., Tashiro, K., Ikegawa, M., Kuroda, K., Suzuki, A., Nakano, T., and Honjo, T. 2002. Notch–RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat. Immunol.* **3:** 443–450.
Tomita, K., Moriyoshi, K., Nakanishi, S., Guillemot, F., and Kageyama, R. 2000. Mammalian achaete-scute and atonal homologs regulate neuronal versus glial fate determination in the central nervous system. *EMBO J.* **19:** 5460–5472.
Treier, M., Gleiberman, A.S., O'Connell, S.M., Szeto, D.P., McMahon, J.A., McMahon, A.P., and Rosenfeld, M.G. 1998. Multistep signaling requirements for pituitary organogenesis in vivo. *Genes & Dev.* **12:** 1691–1704.
Treier, M., O'Connell, S., Gleiberman, A., Price, J., Szeto, D.P., Burgess, R., Chuang, P.T., McMahon, A.P., and Rosenfeld, M.G. 2001. Hedgehog signaling is required for pituitary gland development. *Development* **128:** 377–386.
Umesono, Y., Hiromi, Y., and Hotta, Y. 2002. Context-dependent utilization of Notch activity in *Drosophila* glial determination. *Development* **129:** 2391–2399.
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., et al. 2005. Notch/γ-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435:** 959–963.
Wang, Z., Qi, C., Krones, A., Woodring, P., Zhu, X., Reddy, J.K., Evans, R.M., Rosenfeld, M.G., and Hunter, T. 2006. Critical roles of the p160 transcriptional coactivators p/CIP and SRC-1 in energy balance. *Cell Metab.* **3:** 111–122.
Ward, R.D., Raetzman, L.T., Suh, H., Stone, B.M., Nasonkin, I.O., and Camper, S.A. 2005. Role of PROP1 in pituitary gland growth. *Mol. Endocrinol.* **19:** 698–710.
Watkins-Chow, D.E. and Camper, S.A. 1998. How many homeobox genes does it take to make a pituitary gland? *Trends Genet.* **14:** 284–290.
Yamaguchi, M., Tonou-Fujimori, N., Komori, A., Maeda, R., Nojima, Y., Li, H., Okamoto, H., and Masai, I. 2005. Histone deacetylase 1 regulates retinal neurogenesis in zebrafish by suppressing Wnt and Notch signaling pathways. *Development* **132:** 3027–3043.
Yoon, K. and Gaiano, N. 2005. Notch signaling in the mammalian central nervous system: Insights from mouse mutants. *Nat. Neurosci.* **8:** 709–715.

Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis

Xiaoyan Zhu, Jie Zhang, Jessica Tollkuhn, et al.

Genes Dev. 2006 20: 2739-2753
Access the most recent version at doi:10.1101/gad.1444706