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Hes1 is Required for Pituitary Growth and Melanotrope Specification

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Abstract

Rathke's pouch contains progenitor cells that differentiate into the endocrine cells of the pituitary gland. It gives rise to gonadotrope, thyrotrope, somatotrope, corticotrope and lactotrope cells in the anterior lobe and the intermediate lobe melanotropes. Pituitary precursor cells express many members of the Notch signaling pathway including the downstream effecter gene *Hes1*. We hypothesized that *Hes1* regulates the timing of precursor differentiation and cell fate determination. To test this idea, we expressed *Hes1* in differentiating pituitary cells and found that it can inhibit gonadotrope and thyrotrope differentiation. Pituitaries of *Hes1* deficient mice have anterior lobe hypoplasia. All cells in the anterior lobe are specified and differentiate, but an early period of increased cell death and reduced proliferation causes reduced growth, evident as early as e14.5. In addition, cells within the intermediate lobe differentiate into somatotropes instead of melanotropes. Thus, the *Hes1* repressor is essential for melanotrope specification. These results demonstrate that Notch signaling plays multiple roles in pituitary development, influencing precursor number, organ size, cell differentiation and ultimately cell fate.

Keywords

Pituitary; Notch; Hes1; melanotrope; proliferation; transgenic

Introduction

The pituitary gland controls the release of hormones that direct growth, metabolism, fertility and the body's response to stress. The anterior and intermediate lobes of this gland arise from a structure known as Rathke's pouch that invaginates from the oral ectoderm at embryonic day 8.5 (e8.5) in the mouse (Burrows et al., 1999;Zhu and Rosenfeld, 2004). Before birth, a cascade of signaling events activates transcription factors that serve to specify the five cell types of the anterior pituitary in a temporally discrete manner. The order of appearance of the fully differentiated cell types in rodents is corticotropes, thyrotropes, somatotropes, lactotropes and gonadotropes that secrete adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), growth hormone (GH), prolactin (PRL), and gonadotropins (FSH and LH). The

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intermediate lobe is composed of melanotropes that secrete melanocyte stimulating hormone (MSH).

We reported that many members of the Notch signaling pathway are expressed in the developing pituitary gland and hypothesized that Notch signaling regulates the transition from proliferation to differentiation (Raetzman et al., 2004). Notch is an evolutionarily conserved signaling system first identified in Drosophila. The transmembrane receptor Notch interacts with its ligand (Delta/Jagged) on a neighboring cell, resulting in the cleavage of the Notch intracellular domain (NICD), which then binds to Rbpsuh. This complex translocates to the nucleus and activates the expression of transcriptional repressor Hes and Hey genes. Hes genes classically function by inhibiting the transcription of bHLH genes that normally promote cellular differentiation. In the pituitary, there is a temporal and dorsal-ventral restriction of Notch receptors and ligands during development (Raetzman et al., 2004). The *Notch2* and *Notch3* receptors and *Hes1* are present in Rathke's Pouch progenitor cells, but are excluded from differentiating progenitors.

Proper expression of *Hes1* is essential for the development of many organs. In *Hes1* null mice, the pancreas is hypoplastic due to premature precursor differentiation (Jensen et al., 2000) and the biliary epithelium undergoes cell fate conversion to pancreatic tissue (Apelqvist et al., 1999;Sumazaki et al., 2004). In the intestine, loss of *Hes1* leads to increased *NeuroD* and *Math1* expression concomitant with premature hormone expression (Jensen et al., 2000). Muscle precursor differentiation is blocked by *Hes1* mediated repression of MyoD (Kuroda et al., 1999). Notch signaling through *Hes1* can also have a specific influence on endocrine cell selection. In lung development, neuroendocrine cells express Notch ligands and inhibit Clara cells from becoming neuroendocrine cells by activating Notch receptors and Hes genes (Ito et al., 2000). Intestinal lineage specification from crypt cells is also reliant on Notch signaling. Inhibition of Notch signaling by blocking receptor cleavage or by deleting *Hes1* causes Goblet and enteroendocrine cells to be formed preferentially, the other lineages are lost and there is overexpression of *Ngn3* (Jensen et al., 2000;Wong et al., 2004). *Ngn3* is necessary cell autonomously to promote enteroendocrine cell differentiation (Jenny et al., 2002). Taken together, these studies highlight a common theme in endocrine development, which is that Notch signaling represses endocrine cell development from undifferentiated precursors and/or from other differentiated cells.

To determine if Notch signaling mediates the transition from pituitary precursor proliferation to differentiation, or influences the specification hormone producing cell types, we undertook a gain and loss of function analysis of *Hes1* in pituitary organogenesis. We demonstrate that persistent expression of *Hes1* in pre-gonadotropes and pre-thyrotropes prevents their differentiation. *Hes1* deficient mice have abnormally small pituitary glands, due in part to increased cell death and decreased proliferation. Additionally, the intermediate lobe melanotropes exhibit a cell fate switch to somatotropes in the absence of *Hes1*. These data suggest that Notch signaling through *Hes1* both inhibits pituitary cell differentiation and promotes melanotrope cell fate.

Materials and Methods

Transgene Construction

To generate the *Cga-Hes1* construct, the *Hes1* open reading frame (Ishibashi et al., 1994) was inserted into a pBSK plasmid containing the mouse protamine intron, splice sites and polyadenylation sequences. A 4.6 kb fragment containing the mouse α subunit (*Cga*) promoter and enhancer (Brinkmeier et al., 1998;Kendall et al., 1994) was used to direct expression to the pre-gonadotropes and pre-thyrotropes of the anterior pituitary. Restriction enzyme mapping and partial sequencing were used to verify the identity of the construct. Prior to microinjection,

the construct was released from the vector by a restriction enzyme digest with *Kpn*1 and *Cla*1.

Generation and Genotyping of Transgenic Mice

The purified insert was microinjected into F2 zygotes from F1 (C57BL/6J X SJL/J) parents (JAX labs) by the University of Michigan Transgenic Animal Model Core. Embryos at the two-cell stage were implanted in pseudopregnant CD-1 females (Charles River) at embryonic day 0.5 (e0.5). For transgenic founder analysis, 106 embryos were collected at e18.5. Genotyping to identify the presence of the transgene was conducted using genomic DNA isolated from tail biopsy samples. Oligonucleotides were designed to amplify a region spanning the *Hes1* cDNA and the mouse protamine sequence: 5' TAACGCAGTGTCACCTTCCA 3' and 5' ATCTGCTCCTGCTTTTGCTG 3'. A standard reaction mixture was used containing the following concentrations of reagents per reaction; primers 12.5 pmol, BSA 5 μg, and Taq 5U. PCR amplification was conducted for 29 cycles of denaturing at 92°C for 30 seconds, annealing at 55°C for 30 seconds, and elongating at 72°C for 30 seconds with a final elongation step conducted at 72°C for 10 minutes.

Hes1 null mice

Hes1 null mice were previously generated by replacing the first 3 exons of *Hes1*, including the bHLH domain, with a neomycin-resistance cassette (Ishibashi et al., 1995). A breeding colony of *Hes1* null heterozygotes was established at University of Michigan by re-derivation. Embryos resulting from matings between heterozygote males obtained from Dr. Ryoichiro Kageyama and C56BL6/J females (Jackson Laboratory) were transferred to specific pathogen free surrogate mothers. The resulting heterozygote progeny were intercrossed for the experiments. Genotyping was performed as previously described (Jensen et al., 2000). Embryos at specific ages were obtained from *Hes1* heterozygote females mated with *Hes1* heterozygote males. All procedures involving the use of mice were approved by the University of Michigan Committee on the Use and Care of Animals. All experiments were conducted in accordance with the principles and procedures outlined in the NIH guidelines for the Care and Use of Experimental Animals.

For bromo-deoxyuridine (BrdU) experiments, pregnant mice were injected intra-peritoneally with 0.1mg/g body weight BrdU two hours prior to collecting the fetuses (Nowakowski et al., 1989).

Histology, Immunohistochemistry and *in situ* hybridization

Wild type, *Hes1* transgenic, and *Hes1* null embryos and adult pituitaries were fixed for 2-24 h in 10% formalin in phosphate buffered saline pH 7.2, dehydrated, and embedded in paraffin. Sagittal or coronal sections of 6 micrometers were then prepared for immunostaining or *in situ* hybridization. For transcription factor immunostaining, slides were boiled in 10mM citric acid, pH6, for 10 minutes and then incubated with the mouse monoclonal LHX3 antibody (1:1000, C651.6DbHN developmental Studies Hybridoma Bank, University of Iowa, Iowa city IA), mouse monoclonal ISL1 (1:600; 40.2D6 DSHB) or rabbit polyclonal T-PIT (1:600; gift of J. Drouin, Montreal, Canada) which were diluted using the M.O.M antibody kit (Vector Laboratories) for LHX3 and ISL1 or PBS containing BSA (3%), Tween-20 (0.5%), and normal donkey serum (5% w/v) for T-PIT as previously described (Raetzman et al., 2004;Raetzman et al., 2002). The Perkin-Elmer TSA kit was used for antibody detection. For immunostaining with Cyclin D2 (1:250, Santa Cruz) Prohormone convertase 2 (1:300; Chemicon) or bromo-deoxyuridine (BrdU, 1:100; Harlan Sera), the slides were boiled in citrate (see above) for 5 minutes. Antibodies were detected with anti-rabbit-cy2 (1:200, Jackson ImmunoResearch), anti-rabbit-cy3 (1:200, Jackson ImmunoResearch) or anti-rat TRITC (1:200, Jackson ImmunoResearch), respectively. All fluorescently stained slides were mounted in an aqueous

mounting media. Antibodies were also used to detect hormones: ACTH (also recognizes MSH; 1:1000; DAKO), MSH (1:500; Chemicon) LHβ (1:1500; National Hormone and Pituitary Program-NHPP), FSHβ (1:1800; NHPP), GH (1:1000; NHPP) and TSHβ (1:1000; NHPP). Vectastain kits (Vector Laboratories) were used for signal amplification and the Sigma Fast 3,3-Diaminobenzidine Tablet Sets (Sigma) were used for antibody detection. Before mounting in Permount (Fisher), slides were counterstained for 3 minutes with methyl green (Vector Laboratories).

Cell death was determined by the TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) method using the in situ cell death detection kit (Roche, Indianapolis IN) according to the manufacturer's protocol.

For *in situ* hybridization (ISH), embryos were collected and embedded in paraffin as they were for immunohistochemistry. Gene expression was detected using digoxigenin-labeled riboprobes as previously described (Raetzman et al., 2004). The in situ probes used were *Hes1* (Akazawa et al., 1992) and *Fgf10* (Bellusci et al., 1997) (a gift from Brigid Hogan, Durham, NC). The *Six6* ISH probe was derived from a sequence verified full length *Six6* clone from a RIKEN pituitary cDNA library (Carninci et al., 2003). The *Six6* clone in pFLCI was linearized with BamHI and transcribed with T7 polymerase to produce an antisense probe.

Results

Hes1 is expressed in the pituitary during development

Many members of the Notch signaling family are expressed in the pituitary at embryonic day 12.5 (e12.5) (Raetzman et al., 2004). To define the window of *Hes1* expression, we performed in situ hybridization on sections of mouse embryos from e10.5 through e18.5. At e10.5, *Hes1* expression is detected throughout most of Rathke's pouch and the adjacent ventral diencephalon (Fig. 1A). *Hes1* transcripts are localized in the dorsal and medial aspects of Rathke's pouch and are absent in the ventral region of the pouch at e11.5 (Fig. 1B). This pattern of *Hes1* expression corresponds to the area of the pouch that contains the proliferating, undifferentiated cells (Ikeda and Yoshimoto, 1991;Ward et al., 2006). *Hes1* is excluded from the ventral aspect of the pituitary that contains differentiating cells expressing αGSU, the alpha subunit common to the glycoprotein hormones TSH, LH, and FSH (Japon et al., 1994). By e13.5, *Hes1* expression is waning in Rathke's pouch and the ventral diencephalon (Fig. 1C) and is dramatically reduced by e14.5 (Fig. 1D). *Hes1* is no longer detectable in the pituitary or ventral diencephalon at e18.5, at which time terminal differentiation markers are detectable for all the hormone producing cell types (Fig. 2A and data not shown). The expression pattern of *Hes1* in the developing pituitary suggests that *Hes1* could have a role in undifferentiated precursor cells, and its expression is extinguished as pituitary precursors initiate their differentiation into hormone producing cells (Japon et al., 1994).

Hes1 expression must be silenced for pituitary cell differentiation to occur

Hes1 expression is spatially and temporally restricted during pituitary development. To test the hypothesis that *Hes1* maintains Rathke's pouch precursor cells in an undifferentiated state and that *Hes1* expression must be extinguished for differentiation to proceed, we created mice that express *Hes1* constitutively under the control of the αGSU promoter and enhancer sequences, *Cga* (Fig. 1F). This well characterized promoter confers cell specific and developmentally regulated expression of a variety of transgenes in pre-gonadotrope and pre-thyrotrope cells which persists in the corresponding fully differentiated cells (Charles et al., 2005;Cushman et al., 2001). It also directs reporter gene expression in Rathke's pouch and the rostral tip thyrotropes, but this expression is extinguished rapidly.

We examined pituitary development e18.5 in six independent *Hes1* transgenic founder mice. The transgenic mice with the highest levels of *Hes1* expression expressers were identified by *in situ* hybridization with a *Hes1*-specific probe (Fig. 2 B, C). Out of the six founder mice, four expressed detectable levels of the *Hes1* transgene, with the two shown being the highest expressers. *Hes1* transcripts are present in the anterior lobe of the transgenics but not in non-transgenics at this time because endogenous *Hes1* expression is already extinguished (Fig. 2A). The lumen of Rathke's pouch is dysmorphic in all of the transgenics, but there is no obvious difference in the overall size of these pituitary glands relative to those of non-transgenic mice.

The consequence of persistent *Hes1* expression on cell differentiation was examined by immunostaining for hormone expression. We assessed the presence of gonadotropes and thyrotropes by expression of the common alpha subunit (αGSU) and the distinct beta subunits luteinizing hormone (LH) and thyroid-stimulating hormone (TSH). In wild-type pituitaries, αGSU (Fig. 2D) positive cells are readily detectable in the ventral aspect of the anterior lobe. In contrast, the anterior lobes of the *Hes1* transgenics either lack αGSU positive cells (Fig. 2E) or only possess a few stained cells (Fig. 2F), indicating a failure to differentiate. The severity of the dysmorphology does not necessarily correlate with the degree to which differentiation is blocked. LHβ (Fig. 2G) and TSHβ (Fig. 2J) immunoreactive cells are abundant in the anterior lobe of wild-type pituitaries, however LHβ (Fig. 2H,I) and TSHβ (Fig. 2K,L) staining is severely reduced or absent in transgenic mice that express *Hes1* persistently. In contrast, immunostaining for POMC-derived proteins is identical in the intermediate and anterior lobes of wild-type (Fig. 2M) and transgenic (Fig. 2N, O) pituitaries. Taken together, these data demonstrate that *Hes1* can potently inhibit differentiation of gonadotropes and thyrotropes. A role of *Hes1* in pituitary development, then, may be to prevent Rathke's pouch precursor cells from differentiating into hormone producing cells.

Loss of *Hes1* prevents proper pituitary gland morphogenesis

To investigate the effect of *Hes1* deficiency on pituitary development, we utilized *Hes1* knockout mice (Ishibashi et al., 1995). This well-characterized null allele lacks the first 3 exons of the *Hes1* gene, which encodes the bHLH domain of HES1. *Hes1* null embryos die just before or at birth, allowing examination of the morphology of Rathke's Pouch and the anterior lobe up to that point in wild-type and *Hes1* knockout embryos. At e11.5, the wild-type infundibulum has formed by evagination of the neural ectoderm and Rathke's pouch has fully separated from the oral ectoderm (Fig. 3A). The infundibulum is smaller than normal and the process of Rathke's pouch separation is delayed in the *Hes1* mutants (arrow, Fig. 3E). By e14.5, the separation is complete in *Hes1* mutants. The cartilage that underlies the pouch has not formed a unified structure in mutants (arrow, Fig. 3F), but it has in wild-type embryos of the same age (Fig. 3B).

Normally, by e14.5 cells have migrated away from Rathke's pouch both ventrally and laterally to form the anterior lobe (Fig. 3B, C). The anterior lobe in *Hes1* mutants is smaller due to reduced expansion laterally (Fig. 3G). At e18.5, the anterior pituitary is still undersized in *Hes1* null embryos (Fig. 3H), and it appears to lack the posterior lobe that is prominent in wild-type pituitaries (Fig. 3E). These data demonstrate that the expression of *Hes1* in and around Rathke's pouch is necessary for generating the correct size of the anterior and posterior lobe.

Hes1 is necessary for appropriate cell survival and cell proliferation in Rathke's pouch

To investigate the mechanism of pituitary hypoplasia in *Hes1* mutant pituitaries, we compared proliferation and cell death during pituitary organogenesis in wild-type and *Hes1* mutant pituitaries. Cells in the dorsal and medial aspects of Rathke's pouch are normally highly proliferative at e11.5, as demonstrated by BrdU labeling and Cyclin D2 expression (Fig. 4A and data not shown). Although dying cells are detectable in the adjacent mesenchyme, no cell

death is occurring in Rathke's pouch at e11.5 in normal mice (Fig. 4B). In contrast, *Hes1* mutants have fewer cells labeled with the proliferation marker BrdU (Fig. 4C, white bracket) and numerous dying cells within Rathke's pouch (Fig. 4D, white bracket). By e13.5, Cyclin D2 expression in proliferating Rathke's pouch cells is similar in wild-type (Fig. 4E) and *Hes1* mutant (Fig. 4G) pituitaries. Additionally, cell death is no longer apparent in either wild-type (Fig. 4F) or *Hes1* mutant (Fig. 4H) Rathke's pouches at this time. It is likely that both an early decrease in progenitor cell proliferation and elevated progenitor cell death contributes to the smaller pituitary glands of *Hes1* mutants.

Pituitary cell death in *Hes1* mutants coincides with a loss of *Lhx3* expression

Cell survival during pituitary gland ontogeny is supported by FGF signaling from the diencephalon and the expression of the several transcription factors in Rathke's pouch including the LIM homeodomain factor LHX3 (Charles et al., 2005;Ericson et al., 1998;Norlin et al., 2000;Sheng et al., 1996;Takuma et al., 1998;Treier et al., 1998). It is possible that the cell death observed in *Hes1* mutant pituitaries at e11.5 is due to a decrease in one of these survival cues. At e10.5, the wild-type pituitary has detectable cell death along the oral ectoderm (Fig. 5A). This zone of death is expanded in the *Hes1* null mutant (Fig. 5B, white bracket) and extends into Rathke's pouch. *Lhx3* is normally expressed throughout Rathke's pouch at e10.5 (Fig. 5C), but expression is absent in *Hes1* mutant pouches in the area where increased cell death is observed (Fig. 5D, white bracket). The LIM homeodomain transcription factor, ISL1, exhibits indistinguishable expression patterns in wild-type (Fig. 5E) and *Hes1* mutant (Fig. 5F) Rathke's pouches. Likewise, the transcription factor SIX6, which promotes pituitary precursor proliferation (Li et al., 2002), is expressed similarly in *Hes1* mutants (Fig. 5H) and wild-type pouches (Fig. 5G) and the ventral diencephalon (Fig. 5 G, H, arrow). This indicates that *Hes1* deficiency alters LHX3 expression specifically.

FGFs released from the infundibulum are important for proliferation and survival of cells within Rathke's pouch (De Moerlooze et al., 2000;Ericson et al., 1998;Norlin et al., 2000;Ohuchi et al., 2000;Takuma et al., 1998). We examined the expression of *Fgf8* and *Fgf10* to determine whether the reduced cell proliferation and increased cell death in *Hes1* mutants resulted from deficiencies in these growth factors. *Fgf10* mRNA is detected in the infundibulum in wild-type mice at e11.5 (Fig. 5I, arrow). The levels and boundaries of *Fgf10* expression appear unchanged in *Hes1* mutants relative to wild-type (Fig. 5J, arrow). Similar results were obtained with *Fgf8* expression (data not shown). Because *Hes1* expression is not necessary for proper FGF expression in the infundibulum, but it is required for normal LHX3 expression in Rathke's pouch, the hypoplasia characteristic of *Hes1* mutants may be intrinsic to Rathke's pouch.

Intermediate lobe melanotropes are not specified in the absence of *Hes1*

Hes1 is necessary to specify enteroendocrine cells during intestinal organogenesis (Jensen et al., 2000). To determine if *Hes1* has a similar role the specification of endocrine cell types in the developing pituitary gland, we examined pituitary cell differentiation markers at e18.5. Wild-type and *Hes1* null embryos were immunostained with an antibody that recognizes the proteins produced from differential cleavage of pro-opiomelanocortin (POMC): MSH in the intermediate lobe (IL) and ACTH in the anterior lobe (AL). Wild-type pituitaries have POMC immunoreactive cells in the IL and AL (Fig. 6A), but *Hes1* null pituitaries only have immunoreactive cells in the anterior lobe, (Fig. 6B). The mutant intermediate lobe is completely devoid of POMC immunoreactive cells and POMC mRNA (data not shown), suggesting that *Hes1* is required for melanotropes but not corticotropes. The lack of melanotropes in *Hes1* null mice is confirmed in two ways. An antibody that recognizes αMSH reveals many stained cells in the IL of wild-type mice (Fig. 6C) but none in *Hes1* null mice (Fig. 6D). Pro-hormone

convertase 2 (PC2), the enzyme that processes POMC to produce αMSH, is clearly expressed in wild-type (Fig. 6E) but not mutant IL (Fig. 6F).

To explore the molecular signature of the cells in the intermediate lobe of *Hes1* mutants further, we examined expression of the T box transcription factor T-PIT (*Tbx19*) and the bHLH transcription factor NeuroD1. Both transcription factors are normally expressed prior to *Pomc.* TPIT is necessary for IL cell specification (Pulichino et al., 2003), and *NeuroD1* deficient mice exhibit delayed corticotrope development (Lamolet et al., 2001). T-PIT expression is similar in the IL and AL of wild-type (Fig 6G) and *Hes1* mutant (Fig. 6H) pituitaries at e18.5. Both T-PIT and NeuroD1 expression are unchanged in *Hes1* mutants at e14.5 (data not shown). This indicates that the mechanism whereby *Hes1* controls melanotrope fate does not involve either T-PIT or NEUROD1.

Intermediate lobe cells adopt a somatotrope cell fate in *Hes1* mutants

The existing IL cells in *Hes1* mutants may be committed to the melanotrope fate, but not express the terminal differentiation markers MSH and PC2. Alternatively, the cells located in the *Hes1* null IL may have taken on another cell fate. To distinguish these possibilities, wild-type and *Hes1* mutant pituitaries were immunostained with an antibody to the homeodomain transcription factor PIT1. PIT1 is normally expressed in the anterior lobe and is necessary for TSH, GH, and PRL expression and lineage specific proliferation of thyrotropes, somatotropes, lactotropes (Lin et al., 1994;Ward et al., 2006). PIT1 expression is observed in the AL of wild-type pituitaries at e16.5, (Fig. 7A) and e18.5 (Fig. 7C). The *Hes1* null AL contains ample numbers of PIT1 positive cells, but PIT1 is aberrantly expressed in the IL at e16.5 (Fig. 7B, bracket) and at e18.5 (Fig. 7D, bracket). These data indicate that *Hes1* expression in Rathke's pouch is necessary to repress PIT1 expression in the IL.

PIT1 is thought to act in combination with other cell type restricted transcription factors to generate thyrotropes and lactotropes (Bradford et al., 1997;Charles et al., 2005;Gordon et al., 1997). Misexpression of PIT1 can be sufficient for GH expression, however (Dasen et al., 1999). Ectopic PIT1 expression in the IL of the *Hes1* null pituitaries, also leads to GH expression (Fig. F, bracket), while GH is only expressed in the anterior lobe of wild-type mice (Fig. E, bracket). No other ectopic hormone expression was detected in the IL of *Hes1* mutants (Fig. 7F, H, J, L). The absence of *Hes1*, therefore, results in a cell fate change from melanotropes to somatotropes.

Anterior lobe cell specification does not require *Hes1*

The anterior lobe is substantially smaller in *Hes1* mutants, but it contains all hormone producing cell types. POMC (Fig. 6B) and GH (Fig. 7F) are expressed in a normal pattern in the AL of *Hes1* mutants when compared to wild-type pituitaries at e18.5 (Fig. 6A, Fig. 7E), in spite of their altered expression within the IL. In addition, similar levels of TSHβ, LHβ and αGSU immunoreactivity are seen in wild-type (Fig. 7G, I, K, respectively) and *Hes1* mutant (Fig. 7H, J, L, respectively) pituitaries. To determine whether *Hes1* deficiency promotes premature differentiation of pituitary cells like it does in many other organ systems, we examined the appearance of hormones that mark corticotropes, somatotropes, thyrotropes, and gonadotropes during development. There was no evidence for consistent, precocious expression of hormone genes in *Hes1* mutants (Supp. Fig. 1 and 2).

Discussion

Notch signaling through *Hes1* is critically important in the normal development of many organs. A combination of gain of function and loss of function approaches has proven useful in deciphering the distinct role *Hes1* plays in cell fate selection. *Hes1* may participate in 2

distinct processes: directing a precursor to remain uncommitted while a daughter cell differentiates or biasing a precursor pool that usually generates diverse types of cells towards one particular differentiated cell fate. We have uncovered a key role for *Hes1* in pituitary cell fate specification (Fig. 8). *Hes1* is normally expressed in the progenitor cells of Rathke's pouch and its levels decrease as differentiation proceeds. Constitutive, ectopic expression of *Hes1* in developing gonadotropes and thyrotropes, results in dramatic reduction of LH and TSH production, indicating that *Hes1* expression is sufficient to inhibit differentiation. Besides its role in maintaining pituitary cells in an undifferentiated state, *Hes1* is also required for adoption of the melanotrope cell fate. The absence of *Hes1* results in ectopic somatotrope specification, at the expense of melanotropes. There are several possible mechanisms for this cell fate switch. *Hes1* may actively promote melanotrope differentiation and/or suppress *Pit1* expression. Alternatively, somatotropes may form in the intermediate lobe because *Hes1* deficiency permits cells to respond signaling molecules that stimulate differentiation at a time when *Hes1* should be inhibiting differentiation. By the time the cues are present to stimulate melanotrope differentiation, the precursor cells may have already assumed the somatotrope fate. The latter hypothesis is consistent with the requirement for *Hes1* to stimulate Rathke's pouch precursor cell proliferation. The undersized pituitary gland in *Hes1* mutants caused by a combination of reduced cell proliferation and increased cell death. These studies are the first to demonstrate a definitive role for Notch signaling in pituitary cell fate choices and organ growth.

Actively dividing progenitor cells line Rathke's pouch during the time *Hes1* is expressed (Ikeda and Yoshimoto, 1991). These cells cease dividing and migrate to the anterior lobe before expressing terminal markers of differentiation. The expression of *Hes1* in the same cells that express *Notch2* and *Notch3* suggests that *Hes1* is a major effecter of Notch signaling in Rathke's pouch. *Hey1* is expressed in the same cell population as *Hes1* (Raetzman et al., 2006), while *Hes6* appears to be in differentiating anterior lobe cells (Raetzman et al., 2004). It is possible that *Hes1* and *Hey1* have similar roles such that a significant amount of Notch signaling could occur in *Hes1* mutant pituitaries. Consistent with the idea that *Hes1* is only a part of Notch readout is that *Notch2* knockouts are embryonic lethal by e10.5 (Hamada et al., 1999), but *Hes1* knockouts can live until birth. In addition, loss of the pituitary specific transcription factor *Prop1* results in a profound diminution of *Notch2* expression, while *Hes1* levels are not appreciably altered. The consequence of *Prop1* loss in both humans and mice is failure of the *Pit1* lineage, which is composed of somatotropes, lactotropes and thyrotropes (Gage et al., 1996a;Gage et al., 1996b;Wu et al., 1998). The fact that these cell types are specified in the *Hes1* mutant, but melanotropes are absent, further indicates that the *Prop1* mutant phenotype is unlikely to involve *Hes1*, even though *Notch2* expression is dramatically reduced. Taken together, these data indicate that *Hes1* performs an important role in pituitary development, but that other downstream Notch targets also regulate cell proliferation and specification.

The function of *Hes1* in pituitary development is similar to its role during neurogenesis. In vertebrates, retinal progenitors rely on Notch signaling at two stages, first to block neuronal differentiation and next to direct precursors to produce glia. *Hes1* null retinas contain fewer neurons due to premature depletion of the precursors (Tomita et al., 1996). Reciprocally, misexpression of the Notch target *Hes1* in the retina inhibits neural differentiation and promotes production of Müller glial precursor cells (Bae et al., 2000;Furukawa et al., 2000). The Notch signal allows retinal progenitors to instructively generate glia despite the presence of neurogenic signals at the same time in development. In the pituitary it is likely that Notch signaling interacts with other signaling pathways present during the early stages of development as well.

There are several other signaling pathways that are critical for early pituitary cell proliferation and specification. Many members of the Wnt signaling pathway are detected in embryonic

Rathke's pouches (Douglas et al., 2001). Wnt appears to act at several different stages of pituitary development, influencing proliferation (Kioussi et al., 2002), growth (Brinkmeier et al., 2003) and shape (Cha et al., 2004). Additionally, the Wnt target β-catenin interacts with *Prop1* to specify the PIT1 lineage: somatotropes, lactotropes and thyrotropes (Olson et al., 2006). Wnts or LEF1 can increase the levels of Notch ligands (Ayyanan et al., 2006;Galceran et al., 2004;Hofmann et al., 2004). Additionally, *Hes1* interacts with the groucho related genes known as transducin-like enhancer of split (*Tle*) to function as a co-repressor (Grbavec and Stifani, 1996;Ju et al., 2004). Notch and Wnt signals are also integrated to maintain hematopoetic stem cells. As in the pituitary, Notch is down-regulated as differentiation proceeds (Duncan et al., 2005). Notch signaling together with Wnt signaling maintains the cells in an undifferentiated state, while Wnt signaling alone promotes proliferation and survival. In the pituitary, it is likely that Wnt and Notch have overlapping roles in precursor maintenance while also performing distinct functions.

BMPs and FGFs provide important survival and differentiation cues during early pituitary organogenesis. We propose that these signaling pathways interact with the Notch pathway in the pituitary in a manner similar to that described for other organs. FGF8 and FGF10 are secreted from the diencephalon and are essential for proliferation and cell survival in Rathke's pouch (Ericson et al., 1998;Norlin et al., 2000;Ohuchi et al., 2000;Takuma et al., 1998). FGFs may enhance Notch signaling in the pituitary like they do in developing nervous system cells and pancreatic progenitor cells (Akai et al., 2005;Miralles et al., 2006;Norgaard et al., 2003;Yoon et al., 2004). BMP signaling appears to oppose FGF signaling and regulate the choice of Rathke's pouch precursors to differentiate into rostral tip thyrotropes or corticotropes (Ericson et al., 1998). BMPs may suppress corticotrope differentiation, in part, by augmenting the inhibitory effects of Notch signaling. This idea is supported by the observation that BMPs synergize with Notch signaling to increase the levels of *Hes* gene expression in developing osteoblasts (Nobta et al., 2005), myocytes (Dahlqvist et al., 2003) and endothelial cells (Itoh et al., 2004). BMP4 is expressed in the diencephalon dorsal to Rathke's pouch near the progenitors where it may suppress differentiation, and BMP2 is expressed in the ventral mesenchyme near the differentiating cells, where it may promote differentiation. Both of these processes may be linked with Notch signaling in the pituitary. This idea is supported by the observation that BMP4 acts with Notch to inhibit myogenic differentiation while BMP2 induced osteoblastic differentiation is promoted by Notch signaling.

A common role of *Hes1* is to prevent premature differentiation of precursor cells. This is evident in both neurogenesis and intestinal endocrine cell development (Jensen et al., 2000;Nakamura et al., 2000). *Hes1* functions to repress cell-patterning genes and when *Hes1* is absent, premature cell specification occurs. We predicted that we would also observe precocious hormone expression in the pituitary of *Hes1* deficient mice. Surprisingly, there were no consistent changes in the timing of pituitary hormone gene expression during development. It is difficult to eliminate the possibility that premature differentiation is occurring, however. The onset of hormone gene expression varied by one day among individual mice that were either wild type or *Hes1* mutants, possibly due to the mixed genetic background of this colony. Genetic background effects have been observed in analysis of *Pax6* and *Prop1* alleles, and this variation could obscure subtle changes in the timing of differentiation (Bentley et al., 1999;Cushman et al., 2001;Kioussi, 1999;Vesper et al., 2006). Premature differentiation could also be missed due to a paucity of markers for stages leading to terminal differentiation and hormone production.

Hes1 is necessary for the survival of cells in the caudal aspect of Rathke's pouch. The reason why cell death only occurs in the caudal region is not clear, but it could be due to interaction of Notch and other signaling pathways. Chordin and Noggin, potent inhibitors of BMP signaling, are expressed near the caudal and dorsal aspects of Rathke's pouch, respectively,

and affect FGF expression and pituitary cell survival (Treier et al., 1998)(Davis and Camper, unpublished observation). Reduced FGF signaling causes increased cell death in Rathke's Pouch (De Moerlooze et al., 2000). There may be a common mechanism whereby a combination of extracellular FGF signals and intrinsic Notch signaling establishes a milieu of transcription factors, all of which are essential for pituitary cell survival. This idea is supported by the observation that deficiencies in the transcription factors *Lhx3* or *Lhx4*, or the combination of both *Pitx1* and *Pitx2* causes increased cell death in the pituitary primordium (Charles et al., 2005;Raetzman et al., 2002)(Ellsworth, Butts, Camper, unpublished observation). Like *Hes1*, lack of *Lhx4* or the combination of *Pitx1* and *Pitx2* also decreases *Lhx3* expression levels. *Hes1* appears to play a role in cell survival in other organ systems. For example, *Hes1* null duodenal crypt cells exhibit similar increases in cell death (Jensen et al., 2000), and *Hes1* can repress the initiation of apoptosis in melanoblasts (Moriyama et al., 2006).

One of the most significant changes in observed in the *Hes1* mutant pituitaries is a cell fate change from melanotropes to somatotropes. The scenario is similar in foregut-derived cells in *Hes1* mutants in that biliary epithelial cells are absent, and pancreatic cells form in their place ((Fukuda et al., 2006;Sumazaki et al., 2004). Thus, *Hes1* either actively represses one cell fate or directs a multi-potential precursor to a specific fate in multiple organ systems. The cell fate change in *Hes1* deficient mice is another example in support of the idea that the cells in Rathke's pouch have the potential to differentiate into hormone producing cells of either the anterior or the intermediate lobe. Mice lacking the T box transcription factor T-PIT (TBX19) exhibit ectopic differentiation of gonadotropes in the intermediate lobe at the expense of POMC expression, presumably because T-PIT suppresses expression of the gonadotrope lineage-specific orphan nuclear receptor transcription factor SF1 (*Nur5a1*) (Pulichino et al., 2003). *Hes1* may be required to suppress *Pit1* expression in the cells normally fated to become melanotropes. This suggests that suppression of lineage specific transcription factor gene expression may be equally important as activation of gene expression to produce multiple types of hormone-producing cells from Rathke's pouch precursors. Cell fate choices in the pituitary may be more complicated than a series of binary choices (Fig. 8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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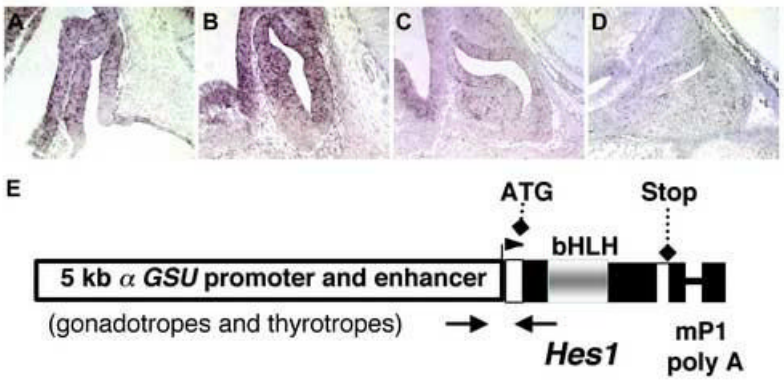

Fig 1.

Hes1 is expressed in the developing pituitary in a temporally and spatially restricted pattern. *Hes1* mRNA is detected by in situ hybridization in Rathke's Pouch, present in mid-sagittal sections of pituitaries, at e10.5 (A) and e11.5 (B). *Hes1* expression wanes at e14.5 (C) and is undetectable by e16.5 (D). To increase the length of time *Hes1* is expressed and to force its expression in differentiating cells, transgenic mice were generated with a construct (E) containing 4.6 kb of the mouse αGSU promoter and enhancer sequences, the *Hes1* open reading frame cDNA, and mouse protamine 1 intron and polyadenylation sequences.

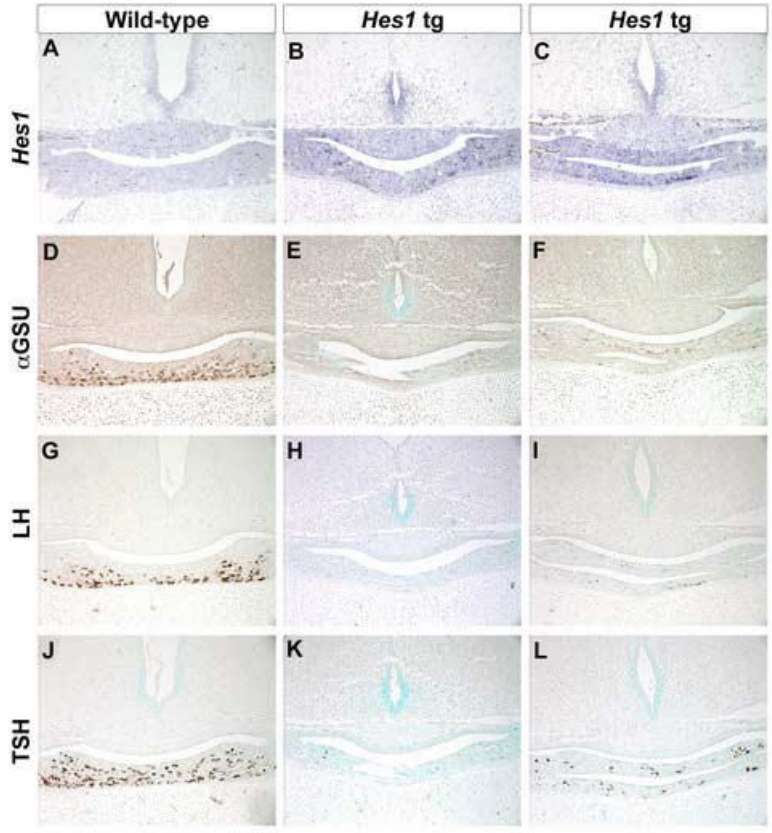

Fig 2.

Mis-expression of *Hes1* inhibits gonadotrope and thyrotrope differentiation. Coronal sections of pituitaries from e18.5 mice were probed for *Hes1* expression by in situ hybridization. *Hes1* in not detected in the anterior lobe of wild-type mice at this time (A), but transcripts are present in two independent, transient transgenic anterior pituitary lobes (B,C). The α subunit common to the dimeric hormones LH, FSH, and TSH is reduced in transgenic (E, F) pituitaries relative to wild-type (D). Both LHβ and TSHβ are also absent or substantially reduced in *Hes1* transgenics (LH H, I; TSH K, L) compared to wild-type (LH G; TSH J). POMC, a hormone marker unrelated to α subunit, is expressed similarly in wild-type (M) and transgenic (N,O) pituitaries.

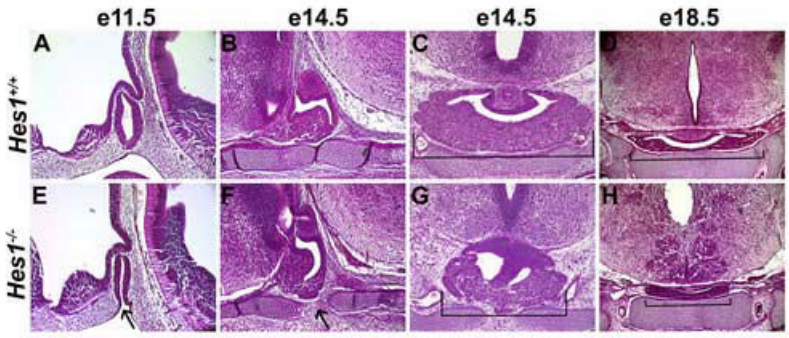

Fig 3.

Pituitary size is reduced in *Hes1* mutants. Hematoxylin and eosin (H&E) staining reveals the morphology of wild-type (A-D) and *Hes1* null (E-H) pituitaries during development. In e11.5 sagittal sections, the pouch of the *Hes1* mutant is formed, but has not completely separated from the underlying oral ectoderm (arrow, E) compared with the wild-type sagittal section (A). At e14.5, wild-type pituitaries have a nearly fused cartilage plate underneath Rathke's pouch (B), but *Hes1* mutants exhibit a large gap between the rostral and caudal aspects of the cartilage (F). Coronal sections at e14.5 demonstrate that the size of the wild-type anterior pituitary (C, bracket) is substantially greater than the *Hes1* mutant (G, bracket). This size difference between the wild-type (D, bracket) and *Hes1* mutant (H, bracket) is even more pronounced in coronal sections of e18.5 pituitaries.

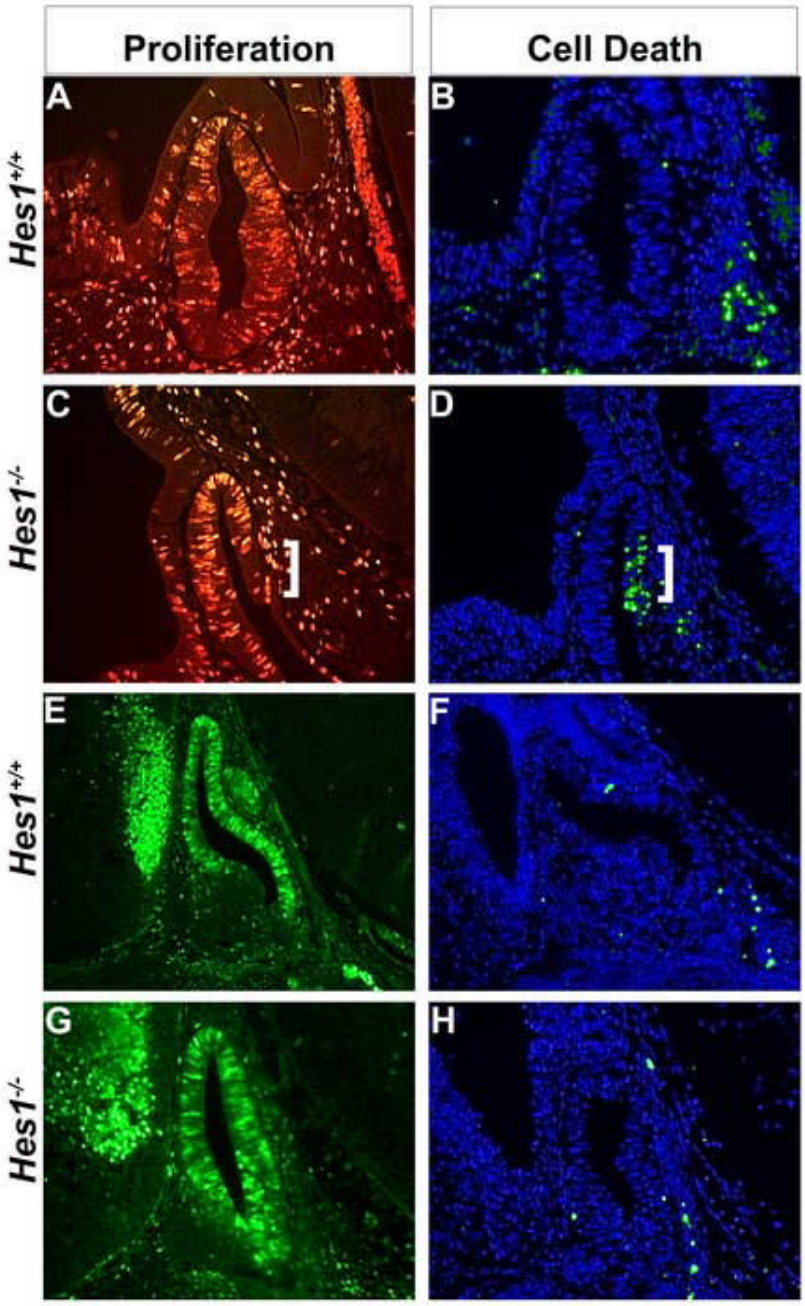

Fig 4.

Decreased cell proliferation and increased cell death in Rathke's pouch of *Hes1* mutants.

Proliferation was assessed by BrdU immunohistochemistry at e11.5 (A, C) and Cyclin D2 immunohistochemistry at e12.5 (E,G). There is an area of decreased density of proliferating cells in the *Hes1* mutants at e11.5 (C, white bracket), compared to the wild-type Rathke's pouch, where proliferating cells are evenly spacing throughout (A). At e12.5 proliferating cells are located throughout Rathke's pouch of both wild-type (E) and *Hes1* mutants (G). Cell death was detected by the TUNEL method in sagittal sections of wild-type (B, F) and *Hes1* mutant (D, H) pituitaries. Dying cells are labeled green and all nuclei are marked blue with DAPI. At e11.5, wild-type pituitaries have no dying cells within Rathke's pouch, but *Hes1* mutants have

many dying cells (D, white bracket). At e12.5, no cell death is detected in Rathke's pouch of wild-type (F) or *Hes1* mutants (H).

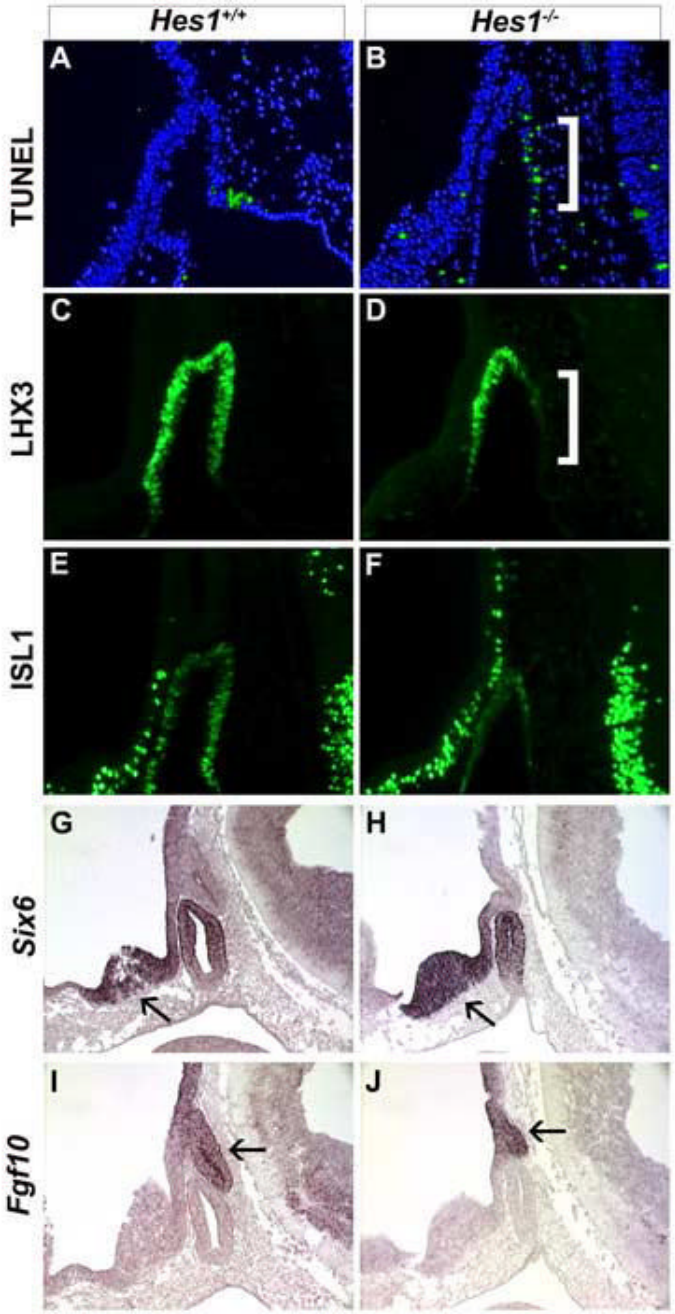

Fig 5.

Hes1 is necessary for cell survival and LHX3 expression. Cell death (A, B), LHX3 (C, D) and ISL1 (E, F) expression were examined in wild-type and *Hes1* mutant embryos collected at e10.5. *Six6* (G, H) and *Fgf10* (I, J) mRNA expression was examined by in situ hybridization on sagittal sections of e11.5 embryos. At e10.5, wild-type embryos exhibit cell death at the junction between Rathke's pouch and the attached oral ectoderm (A, green stained cells). *Hes1* mutants have ectopic dying cells in the caudal aspect of Rathke's pouch (B, white bracket) that corresponds with lack of LHX3 expression (D, white bracket). *Six6* expression in the diencephalon (arrow) and Rathke's pouch is equivalent in wild-type (G) and *Hes1* mutants (H). *Fgf10* expression in the infundibulum (arrow) is similar in wild-type (I) and *Hes1* mutants (J).

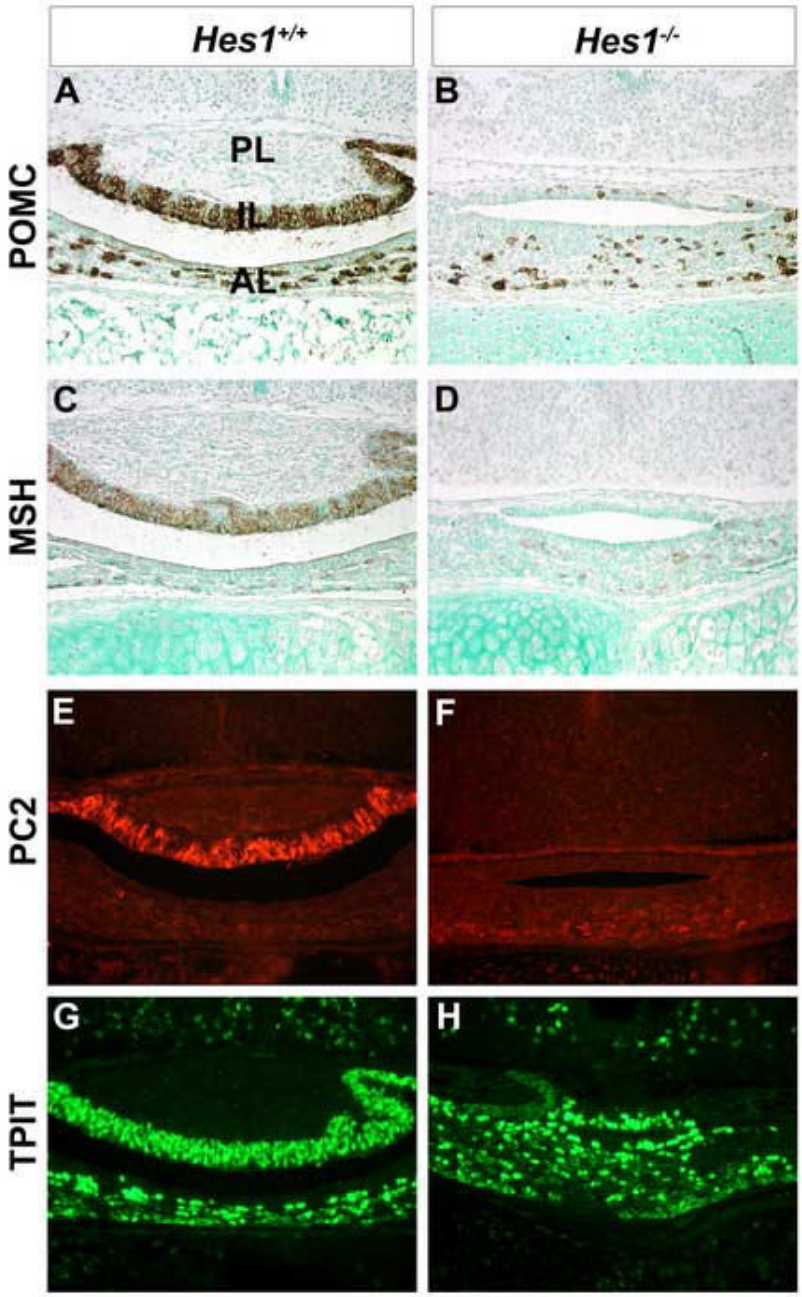

Fig 6.

Intermediate lobe melanotropes are lost in the absence of *Hes1*. Coronal sections of wild-type (A, C, E, G) and *Hes1* null (B, D, F, H) pituitaries were examined at e18.5. An antibody that recognizes POMC derivatives in the intermediate lobe (IL) and anterior lobe (AL) shows that in the wild-type pituitary, the posterior lobe (PL) is devoid of staining whereas the IL and AL contain many stained cells. In *Hes1* mutants, the AL contains POMC stained cells, but the IL has a dramatic reduction in staining. MSH and PC2 immunoreactivity is apparent in the intermediate lobe of wild-type pituitaries (C, E, respectively) but not in *Hes1* mutants (D, F, respectively). T-PIT immunoreactivity is similar in wild-type (G) and *Hes1* mutant anterior and intermediate lobes (H).

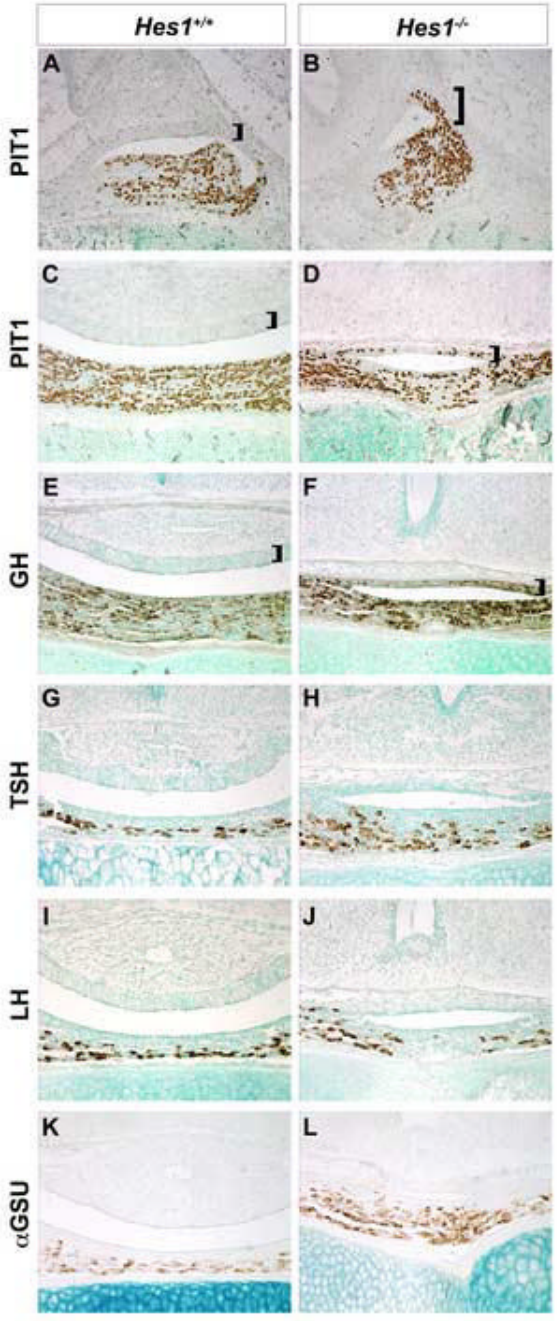

Fig 7.

Hes1 deficiency causes intermediate lobe cell fate switch to somatotrope. PIT1 immunoreactive cells are normally detected only in the anterior lobe of wild-type mice at e16.5 (A) and e18.5 (C). The PIT1 negative intermediate lobe is denoted with a bracket. *Hes1*mutant mice express PIT1 becomes expressed in the caudal part of the intermediate lobe at e16.5 (B, bracket), and PIT1 is expressed throughout by e18.5 (D, bracket). Additionally, GH is expressed in the intermediate and anterior lobes of *Hes1* mutants (F, bracket), but only in the anterior lobe of wild-type mice (E, bracket). There is no change in TSHβ, LHβ or αGSU immunostaining between wild-type (G, I, K, respectively) and *Hes1* mutant (H, J, L, respectively) pituitaries at e18.5.

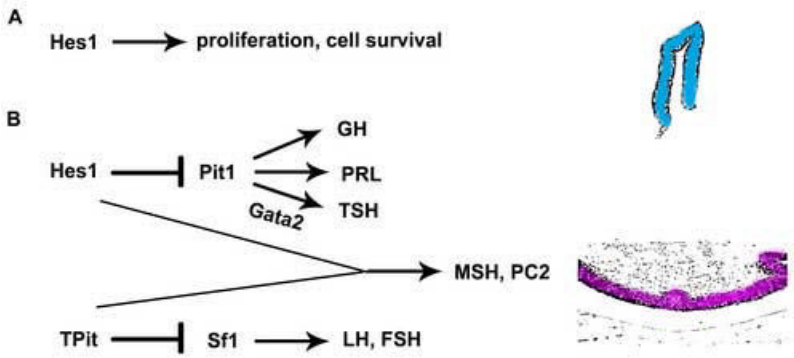

Figure 8.

Genetic model of pituitary development and dependence on *Hes1*. During initial organogenesis, *Hes1* is necessary for survival and proliferation of Rathke's pouch precursors (A). *Hes1* is also necessary for intermediate lobe cells to become melanotropes and not PIT1-containing somatotropes (B).