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Premature differentiation and aberrant movement of pituitary cells lacking both *Hes1* and *Prop1*

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Abstract

In the pituitary, the transition from proliferating progenitor cell into differentiated hormone producing cell is carefully regulated in a time dependent and spatially restricted manner. We report that two targets of Notch signaling, *Hes1* and *Prop1*, are needed to maintain progenitors within Rathke's pouch and for the restriction of differentiated cells to the ventral pituitary. We observed ACTH and αGSU producing cells that had prematurely differentiated within Rathke's pouch along with correlated ectopic expression of *Mash1* only when both *Prop1* and *Hes1* were lost. We also discovered that downregulation of N-cadherin expression in cells as they transition from Rathke's pouch to the anterior lobe appears to be essential for their movement. In the *Prop1* mutant, cells are trapped in Rathke's pouch and N-cadherin expression remains high. Also, Slug, a marker of epithelial to mesenchymal transition, is absent in the dorsal anterior lobe. When *Hes1* is lost in the *Prop1* mutant, N-cadherin is downregulated and cells are able to exit Rathke's pouch but have lost their migrational cues and form ectopic foci surrounding Rathke's pouch. Our data reveal important overlapping functions of *Hes1* and *Prop1* in cell differentiation and movement that are critical for pituitary organogenesis.

Keywords

Pituitary; Notch; Hes1; Prop1; cadherin; Slug; Snai2; ACTH

Introduction

Proper development of the pituitary gland is very important because the pituitary is responsible for releasing hormones that affect growth, metabolism, and fertility, as well as the body's response to stress. In mice, the pituitary gland contains an anterior lobe, an intermediate lobe, and a posterior lobe. The anterior and intermediate lobes are derived from a structure called Rathke's pouch (RP) which is formed at embryonic day 8.5 (e8.5) from the invagination of the oral ectoderm (Burrows, et al. 1999). During embryonic development, Rathke's pouch is made up of undifferentiated, proliferating progenitor cells. Many of these cells leave the pouch and move ventrally to the anterior lobe where they differentiate into corticotropes, thyrotropes, somatotropes, lactotropes, and gonadotropes. It is from these cells that adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), growth hormone (GH), prolactin (PRL), and gonadotropins (FSH and LH) are secreted into the body. Toward the end of prenatal

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development, the cells that remain in Rathke's pouch differentiate into melanotropes which make up the intermediate lobe and secrete melanocyte stimulating hormone (MSH).

This transition of cells from the packed, columnar-like cells of Rathke's pouch to the more loosely distributed round cells of the anterior lobe is not well understood, but resembles epithelial-to-mesenchymal transition (EMT). An important process during embryonic development as well as oncogenesis, EMT is characterized by a loss of cell polarization as well as downregulation of adherens and tight junction markers such as E-cadherin. The purpose of EMT is to allow the cells to change shape and become motile, often accomplished in part by a rearrangement of various cytoskeleton proteins (Kalluri and Neilson, 2003). One gene that has been implicated in this process is *Slug (Snai2)*, a member of the Snail family of zinc finger transcription factors (Nieto, et al. 1994). SLUG has been shown to induce EMT by directly repressing *E-cadherin*, causing destabilization of cell-cell adhesion which in turn allows for cell migration (Cano, et al. 2000; Bolos, et al. 2003). Although the expression and function of *Slug* in the rodent pituitary has not been elucidated, E-cadherin and N-cadherin expression patterns have been characterized in the rat pituitary gland. It appears that both E- and N-cadherin are coexpressed at the beginning of pituitary development. Eventually the two become mutually exclusive as there is down-regulation of E-cadherin in the hormone producing cell types and N-cadherin in the marginal cells lining the residual lumen of Rathke's pouch (Kikuchi, et al. 2006; Kikuchi, et al. 2007). It is possible that SLUG may play a role in the downregulation of E-cadherin in the mouse pituitary which could allow the cells to move to the anterior lobe from Rathke's pouch.

Previous research into the mechanism by which the Rathke's pouch progenitor cells remain distinct from differentiating anterior lobe cells has uncovered pivotal roles for the Notch signaling pathway (Zhu, et al. 2006; Raetzman, et al. 2007). Interestingly, SLUG has also been linked to the Notch signaling pathway, through which Notch is able to regulate *E-cadherin* expression (Leong, et al. 2007). This link to Notch signaling may help illuminate potential roles of SLUG in pituitary cell movement and adhesion. Because Notch signaling has been shown to participate in EMT during development (Timmerman, et al. 2004), it is possible that Notch also plays an active role in the movement of cells from Rathke's pouch to the anterior lobe. A well characterized target of Notch signaling is HES1, which inhibits transcription of basic helix-loop-helix (bHLH) genes necessary for cell differentiation, such as *Mash1*. Therefore, it acts to maintain cells in a precursor state (Ishibashi, et al. 1994; Jarriault, et al. 1998). *Hes1* is strongly expressed in Rathke's pouch cells and its expression declines as cells transition to the anterior lobe (Raetzman, et al. 2007).

Another direct target of Notch signaling in the pituitary is PROP1, a pituitary-specific homeobox transcription factor (Zhu, et al. 2006). In humans combined pituitary hormone deficiency (CPHD) can result from a loss of *PROP1* (a gene homologous to *Prop1* in mice) resulting in hypothyroidism, dwarfism, and infertility (Wu, et al. 1998). Studies on the Ames dwarf mice, which lack functional *Prop1*, have revealed that *Prop1* is critical for pituitary cell differentiation and is responsible for activating the Pit1 lineage: thyrotropes, somatotropes, and lactotropes (Andersen, et al. 1995; Gage, et al. 1996b). Besides its role in *Pit1* activation, PROP1 also participates in movement of cells to the anterior lobe. In Ames dwarf mice, there are no noticeable differences in pituitary morphology between wild type and dwarf pituitaries at e12.5. However, by e14.5 Rathke's pouch is abnormally shaped and hypercellular in dwarfs, likely due to an inability of the cells to leave the pouch (Gage, et al. 1996a; Raetzman, et al. 2002; Ward, et al. 2005). Concurrently, *Prop1* deficient anterior lobes are hypocellular at e14.5; reduced to half the size of wildtype anterior lobes at this age (Gage, et al. 1996a). Although downstream targets of PROP1 are emerging (Douglas, et al. 2001; Brinkmeier, et al. 2003; Carninci, et al. 2003), little is definitively known about its role in transitioning cells from Rathke's pouch to the anterior lobe.

Based on their roles in Notch signaling and Rathke's pouch progenitor cell expression, we questioned whether *Prop1* and *Hes1* may interact or have redundant functions in pituitary development. Recent studies have shown that in *Prop1* mutants, no significant change is seen in levels of *Hes1* mRNA by *in situ* hybridization (Raetzman, et al. 2006) and *Prop1* expression remains in the *Hes1* mutant (Zhu, et al. 2006). In order to better determine the roles that *Hes1* and *Prop1* play in pituitary gland morphogenesis, specifically in cell differentiation and movement, we examined murine pituitaries from wild type, *Hes1* mutant, *Prop1* mutant, and double mutant embryos. We hypothesized that the two genes function together in the Notch signaling pathway and therefore are both necessary for pituitary organogenesis. Our findings demonstrate that both *Hes1* and *Prop1* are necessary for proper placement of the anterior lobe. We also show that loss of both of these genes results in severe mislocalization and premature differentiation of αGSU and ACTH producing cells which does not occur with the loss of *Hes1* or *Prop1* individually. These findings are likely due to a problem with the movement of the newly differentiated Rathke's pouch cells, some of which move to the wrong place while others do not migrate at all. These changes may be due in part to altered N-cadherin, E-cadherin, and SLUG expression that we observed. Taken together, these data suggest that not only are both *Hes1* and *Prop1* required for proper pituitary gland development but they also have redundant or overlapping functions within the Notch signaling pathway.

Materials and Methods

Mice

All mice were provided with chow and water ad libitum. A breeding colony of *Hes1* null heterozygotes was established from mice obtained from Dr. Ryoichiro Kageyama. A breeding colony of Ames dwarf *Prop1* loss of function mutants was established from a colony in Sally Camper's lab and backcrossed to C57BL6 mice obtained from Jackson Laboratory for at least ten generations. The heterozygous *Hes1* progeny were mated with heterozygous *Prop1* progeny to obtain mice heterozygous for both genes. These double heterozygote mice were then crossed and their embryos collected and genotyped. Three embryos of each genotype at each age were studied. All procedures involving mice were approved by the University of Illinois IACUC.

To genotype the mice, DNA from tail biopsies was isolated using a DNA salt-out technique. The *Hes1* PCR reaction mixture contained 1.56mM of $MgCl_2$, 1 unit of Taq, and 12.5 pmol of each of four primers; two that amplified *Hes1*: 5' AGCCAGTGTCAACACGACACC 3' and 5' TGTTAAGTGCATCCAAAATCAGTG 3' ; and two that amplified the neomycin-resistant cassette used to knockout *Hes1*: 5' GTCTTGTCGATCAGGATGATCTG 3' and 5' CAATATCACGGGTAGCCAACGC 3'. The samples underwent 30 cycles of denaturing at 92°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds followed by a final elongation at 72°C for 10 minutes. The *Prop1* PCR reaction mixture contained $MgCl_2$, Taq, and 12.5 pmol of each of two primers that amplified *Prop1*: 5' GAGCTGGGGAGACCTAAGCTTTGCC 3' and 5' GCCCAGATGTCAGGATACTG 3'. The samples underwent 34 cycles of denaturing at 92°C for 30 seconds, annealing at 56°C for 30 seconds and elongation at 72°C for 30 seconds followed by a final elongation at 72°C for 10 minutes. The samples were then digested with HINF1 overnight before being loaded onto 2% agarose gels and undergoing gel electrophoresis.

Immunohistochemistry and *In situ* Hybridization

The timing of the pregnancies was monitored and the first day that the copulatory plug was detected was designated e0.5. The embryos were sacrificed at e12.5, e13.5, e14.5, and e16.5 and fixed in 3.7% formaldehyde in phosphate buffered saline (PBS, pH 7.2). They were then dehydrated in a graded series of ethanol before being embedded in paraffin and sectioned

sagittally at 6 micrometers. The sections were mounted onto charged slides and prepared for the staining procedures. Hemotoxylin and eosin was used to stain for morphology. For the hormone and PC2 staining procedures, the slides were deparaffinized in xylene and rehydrated in ethanol and PBS. They were then incubated in normal donkey serum (5% w/v) diluted in immunohistochemistry block (IHCB) which consists of PBS, BSA (3%), and Triton-X (0.5%), and followed by incubation overnight at 4°C with a primary antibody against the desired peptide: αGSU (1:1500; National Hormone and Pituitary Program-NHPP), ACTH (1:1000; DAKO), TSH (1:1000; NHPP), GH (1:1000; NHPP), or PC2 (1:100; Chemicon). The antibodies were diluted with IHCB. Next, an anti-rabbit secondary antibody conjugated to biotin (Jackson Immunoresearch) was added to the slides at a dilution of 1:200. Subsequent detection was carried out with a Vectastain kit (Vector Laboratories) diluted in PBS and Sigma Fast 3,3-Diaminobenzidine tablets (Sigma). The slides were counterstained with methyl green and mounted with Permount (Fisher). For colocalization experiments involving hormone antibodies, blocking and icubation of the primary antibody occurred as above, with ACTH being detected by an anti-rabbit secondary antibody conjugated to Cy2 (Jackson Immunoresearch) and αGSU with the TSA Kit #22 (Invitrogen). These slides were mounted with an aqueous mounting medium.

The slides for the LHX3, ISL1, PIT1, N-cadherin, E-cadherin, and SLUG staining procedures required boiling in 10mM citric acid, pH6, for ten minutes before incubation with 5% normal donkey serum diluted in IHCB. They were then incubated overnight at 4°C with a primary antibody against the desired marker: LHX3 (1:500; C651.6DbHn Developmental Studies Hybridoma Bank-DSHB, University of Iowa, Iowa City, IA), ISL1 (1:500; 40.2D6 DSHB), PIT1 (1:800; a gift from Dr. Simon Rhodes), N-cadherin (1:300; ZYMED), E-cadherin (1:100; Cell Signaling Technologies), or SLUG (1:100; Santa Cruz Biotechnology). The antibodies were diluted with IHCB. An anti-mouse secondary antibody conjugated to biotin was used with LHX3, ISL1, and N-cadherin while an anti-rabbit secondary antibody conjugated to biotin was used with PIT1 and E-cadherin. Anti-goat conjugated biotin was used with Slug. Either Strep-CY2 or Strep-CY3 for signal detection was used followed by mounting with an aqueous mounting medium. All secondary antibodies were obtained from Jackson Immunoresearch.

In situ hybridization was performed as previously described (Raetzman, et al. 2004). A *Mash1* clone was obtained from a RIKEN embryonic pituitary cDNA library and the probe made from it was labeled with digoxigenin (Carninci, et al. 2003).

After staining as described above, images of the slides were viewed at 200X magnification with a Leica DM2500 microscope, captured with the Retiga 2000R color camera (Q-imaging) attached to the microscope, and the pictures acquired in Q-Capture Pro (Q-imaging). An Axiovert 200M compound microscope, Axiocam HRm camera, and Axiovision software (Carl Zeiss) were used to visualize the slides at 640X magnification.

Results

Anterior lobe placement relies on both Hes1 and Prop1

As the cells in Rathke's pouch begin to differentiate they also move out along the rostral-caudal axis in a ventrally-restricted manner to form the growing anterior lobe. Generally the anterior lobe forms as a morphologically distinct structure around e12.5. The wildtype pituitary at e12.5 is composed of the Rathke's pouch (RP), an anterior lobe (AL), and a posterior lobe (PL) (Fig. 1A). The same is true for the *Prop1* mutant at this age (Fig. 1C). However, the *Hes1* mutant and double mutant both have an anterior lobe that is greatly reduced in size and is not visible as a morphologically distinct structure (Fig. 1B, D). Also, the posterior lobes are stunted in these genotypes.

By e13.5 the anterior lobe has continued to expand in the wildtype while the size of Rathke's pouch appears to be maintained (Fig. 1E). The anterior lobe of the *Hes1* mutant also has expanded greatly, and this expansion is restricted to the ventral part of the pituitary similar to the wildtype. However, Rathke's pouch is noticeably much smaller than in the wildtype (Fig. 1F). In the *Prop1* mutant, the anterior lobe has failed to expand as much as the wildtype, although the size of Rathke's pouch is maintained (Fig. 1G). The double mutant no longer closely resembles the *Hes1* mutant at this age. The anterior lobe appears to be forming ectopically in the dorsocaudal and dorsorostral Rathke's pouch (dcRP and drRP, respectively) in addition to the normal ventral expansion along the rostral-caudal axis (Fig. 1H).

At e14.5, the differences in morphology among the four genotypes are even more striking than at e13.5. The wildtype pituitary has maintained the size of Rathke's pouch while continuing to expand the anterior lobe (Fig. 1I). The anterior lobe of the *Hes1* mutant has continued to enlarge while the size of Rathke's pouch is only slightly larger (Fig. 1J), but is smaller than the wildtype pouch. In the *Prop1* mutant the anterior lobe remains nearly the same and, as a result, it is greatly reduced in size compared to the wildtype (Fig. 1K). However, the Rathke's pouch structure has grown, producing an elongated, often branched structure packed full of the columnar-shaped cells characteristic of Rathke's pouch. The double mutant is the most interesting at this age, with a morphology different than that of the wildtype or single mutants. The size of Rathke's pouch doesn't appear to be greatly altered, but the anterior lobe continues to form ectopically in the dcRP and drRP in addition to the normal ventral expansion (Fig. 1L).

Many of these same growth patterns are seen at e16.5. In the wildtype the anterior lobe and Rathke's pouch have both expanded (Fig. 1M), although more laterally than ventrally, which cannot be completely appreciated by viewing one sagittal section. The anterior lobe of the *Hes1* mutant has continued to enlarge without a noticeable change in Rathke's pouch structure (Fig. 1N). The *Prop1* mutant looks similar to e14.5 with an even more branched, elongated Rathke's pouch structure as well as an anterior lobe that does not look much larger than that of the e12.5 mutant (Fig. 1O). The morphology of the double mutant has continued to become more distinct with what seems to be a much smaller structure overall. It is difficult to distinguish the anterior lobe from Rathke's pouch by only viewing the morphology but it appears that the anterior lobe is located along the entire rostral axis in the dorsal and ventral areas.

It is clear from these morphological observations that the loss of either *Prop1* or *Hes1* has an effect on the size of both Rathke's pouch and the anterior lobe. It also appears that both of these genes may have redundant functions in restricting the formation of the anterior lobe to the ventral part of the pituitary. This is evidenced by the misplacement of the anterior lobe in the double mutant but not in either single mutant or wildtype.

Aberrant and premature expression of Mash1 correlates with misplacement of ACTH producing cells in the double mutant

To confirm the anterior pituitary identity of the misplaced cells in the double mutant, we examined hormone expression in wildtype, *Hes1* mutant, *Prop1* mutant, and double mutant pituitaries at e14.5 and e16.5. First, we examined the formation of the corticotrope lineage. *Mash1*, a bHLH transcription factor, is thought to promote the formation of ACTH producing cells and is known to be directly repressed by *Hes1* (Ohsako, et al. 1994; Van Doren, et al. 1994; Liu, et al. 2001). In the wildtype, *Hes1*, and *Prop1* mutants at e13.5 *Mash1* is expressed in the anterior lobe and the most dorsal part of Rathke's pouch (Fig. 2A-C).

In the double mutant, *Mash1* appears to be expressed throughout much of Rathke's pouch (Fig. 2D). If *Mash1* activates the differentiation of ACTH producing cells, it would be expected that they too would be aberrantly located in the double mutant. In the wildtype pituitary at e14.5, ACTH producing cells are located exclusively in the anterior lobe (Fig. 2E). The same is true

for the *Hes1* mutant (Fig. 2F) as well as the *Prop1* mutant (Fig. 2G). However, in the double mutant the ACTH producing cells are located in the ventral part of the pouch, but they are additionally located ectopically within the dcRP and drRP (Fig. 2H). We also noticed that at e13.5 the double mutant started to have ACTH producing cells that were misplaced (all other phenotypes at this age were the same as at e14.5) (Supp. Fig. 1). At e12.5, we did not notice any difference among the wildtype, *Hes1* mutant, *Prop1* mutant, or wildtype pituitary concerning the placement of the ACTH producing cells (data not shown). Therefore, it appears that the cells begin to be misplaced in the double mutant at e13.5 and we begin to see a severely abnormal phenotype at e14.5.

By e16.5 there are two populations of POMC containing cells, the corticotropes within the anterior lobe (AL) and the melanotropes within Rathke's pouch, which is now referred to as the intermediate lobe (IL). The intermediate lobe cells can be distinguished by the presence of prohormone convertase 2 (PC2), which processes POMC into MSH. The wildtype pituitary has POMC immunoreactive cells in the AL that correspond to the corticotropes and cells in the IL that correspond to the melanotropes (Fig. 2I). PC2 expression marks the melanotropes in the IL (Fig. 2M). In the *Hes1* mutant, the corticotropes can be visualized in the AL (Fig. 2J). However, as previously reported by Raetzman et al. (2007), when *Hes1* is lost, very few, if any, cells in the IL differentiate into melanotropes and express PC2 (Fig. 2N). In the *Prop1* mutant at e16.5, ACTH expression appears similar to that of the wildtype pituitary (Fig. 2K), but we are able to see only a few PC2 immunoreactive cells in the IL (Fig. 2O). Ward et al. (2005) demonstrated expression of PC2 in the *Prop1* mutant at postnatal day 1 in the mouse, therefore we know that PC2 is expressed in the *Prop1* mutant. Low levels at e16.5 or delayed expression may help explain the reduction in PC2 expressing cells that we see. The double mutant contains ACTH positive cells aberrantly expressed throughout the entire pituitary (Fig. 2L) and is devoid of PC2 staining (Fig. 2P).

Clearly, both *Hes1* and *Prop1* are both required for proper restriction of *Mash1* expression to the anterior lobe and ventral part of Rathke's pouch. They also function redundantly to prohibit differentiation of cells within the pouch and restrict the movement of the ACTH producing cells ventrally from Rathke's pouch to the anterior lobe.

Hes1 and Prop1 are both required for proper restriction of αGSU positive cells to the anterior lobe

In addition to ACTH, the common alpha subunit of TSH, FSH, and LH (αGSU) is also expressed early in pituitary development. In the wildtype, *Hes1*, and *Prop1* mutant pituitaries at e13.5, αGSU expression is located exclusively in the developing anterior lobe (Supp. Fig. 1). However, we detected misexpression of the hormone subunit within the cells of Rathke's pouch of the double mutant beginning at this age (Supp. Fig. 1). This misexpression is more evident at e14.5 and e16.5 where the αGSU positive cells are clearly differentiated within the dcRP and drRP in addition to the normal expression in the ventral pituitary (Fig. 3D, H, arrows). In the wildtype, *Hes1*, and *Prop1* mutants at both ages, the αGSU positive cells are restricted to the anterior lobe in the ventral pituitary (Fig. 3A-C, E-G). This data indicates that *Hes1* and *Prop1* also are required for proper restriction of the αGSU positive cells, in addition to the ACTH producing cells, to the ventral area of the pituitary.

PIT1 and its lineages are lost in both the Prop1 and double mutants

After examining ACTH and αGSU producing cell types, we wanted to look at other hormones that are expressed in the pituitary to determine if the loss of both *Hes1* and *Prop1* affects those as well. By e16.5 TSH and GH are detected in the developing pituitary, in addition to ACTH and αGSU. It has been well established that *Prop1* is necessary for activation of PIT1 and its cell lineages: thyrotropes, somatotropes, and lactotropes (Gage, et al. 1996b; Sornson, et al.

1996). Although we hypothesized that the function of *Prop1* in PIT1 lineage specification is independent of *Hes1*, we explored this question by looking at hormone specification in double mutants at e16.5.

We first examined PIT1 and found that in the wildtype pituitary at e16.5, PIT1 is expressed throughout the anterior lobe (Fig. 4A). This is also true for the *Hes1* mutant, but PIT1 is also expressed in parts of the intermediate lobe (Fig. 4B). However, the *Prop1* mutant is completely devoid of PIT1 (Fig. 4C) as is the double mutant (Fig. 4D). We then examined TSH and GH, two of the cell lineages specified by PIT1. There are two distinct populations of cells expressing the TSHβ subunit during pituitary development: one that is PIT1-dependent and secretes functional TSH referred to as the thyrotropes, and one that is PIT1-independent and phenotypically disappears by birth referred to as the rostral tip thyrotropes (Lin, et al. 1994). Both of these cell lineages are visible at e16.5 in the wildtype (Fig. 4E) and *Hes1* mutant (Fig. 4F). The thyrotropes are located within the anterior lobe and are characterized by dark, round, distinct staining patterns (denoted with a bracket) while the rostral tip thyrotropes are typically located at the most rostral part of the anterior lobe and are characterized by a hazy staining pattern (denoted with asterisk). The *Prop1* mutant has no thyrotropes due to the lack of *Pit1* activation by PROP1 (Fig. 4G). However, the rostral tip thyrotropes are present in the rostral tip of the pituitary (asterisk). The double mutant appears to have a similar pattern to that of the *Prop1* mutant (Fig. 4H). The brown staining in the rostral part of the double mutant pituitary seems to have a hazy staining pattern similar to that of the rostral tip thyrotropes in the other genotypes. The absence of *Prop1*, location of this cell population, and staining pattern all indicate that this cell population (denoted with an asterisk) is likely the rostral tip thyrotropes and not the actual hormone-producing thyrotropes of the anterior lobe.

Another piece of evidence confirming that the PIT1 lineage is not rescued in the *Prop1* mutant when *Hes1* is lost is the lack of GH in the double mutant at e16.5 (Fig. 4L). The wildtype and *Hes1* mutant both contain GH within their anterior lobes (Fig. 4I, J). The *Prop1* mutant and double mutant both completely lack GH (Fig. 4K, L). These results indicate that the pathway of *Pit1* activation by PROP1 is independent of *Hes1*.

Early patterning molecules unaffected by the loss of Hes1 and Prop1

After confirming via hormone staining that the misplaced cells seen in the double mutant at e14.5 and e16.5 were differentiated anterior lobe cells, we examined early dorsal-ventral patterning in the pituitary by the expression of LHX3 and ISL1 at e12.5 (Thor, et al. 1991; Sheng, et al. 1997). Cells expressing LHX3 are found throughout both Rathke's pouch and the anterior lobe in the e12.5 wildtype (Fig. 5A), *Hes1* mutant (Fig. 5B), *Prop1* mutant (Fig. 5C), and double mutant pituitaries (Fig. 5D). Unlike LHX3 expression, ISL1 expression is restricted to the ventral anterior lobe in the e12.5 wildtype pituitary (Fig. 5E). The same is true for the *Hes1* mutant (Fig. 5F), *Prop1* mutant (Fig. 5G), and double mutant (Fig. 5H). Expression of LHX3 and ISL1 do not appear to be affected by the loss of *Hes1* or *Prop1* individually or by the loss of both at e12.5 or 14.5 (data not shown). Therefore, even though the double mutant has cells differentiating and moving improperly, the pituitary still retains some evidence of dorsal-ventral patterning, such as restricted ISL1 expression.

Morphology and movement of Rathke's Pouch cells correlates with E- and N-cadherin expression

Because the anterior lobe cells appear misplaced in the double mutants we wanted to explore possible causes of the improper cell movement. Cadherins are cell adhesion molecules that play important roles during morphogenesis and have been shown to be present in the rat pituitary (Gumbiner, 2005; Kikuchi, et al. 2006; Kikuchi, et al. 2007). A fine balance in adhesion is necessary for cell movement during development, especially during EMT (Kalluri

and Neilson, 2003). It is not yet understood if the pituitary cells of Rathke's pouch undergo EMT; however, the cells do appear to undergo similar changes: tightly packed columnar cells of Rathke's pouch transition to groups of round cells as they move to the anterior lobe. After staining for E- and N-cadherin at e13.5, the age when the improper cell movement appears to begin, subtle, yet noticeable differences in expression patterns were observed.

In the wildtype pituitary, we observed E-cadherin (green) expression in an even pattern throughout Rathke's pouch and the anterior lobe while N-cadherin (red) appears in a strongly concentrated pattern along the lumen lining the dorsal part of Rathke's pouch. Along the ventral part of the lumen, the concentration of N-cadherin is lost (between the two arrowheads) and instead we observed a more disperse pattern (Fig. 6A-B). This is demonstrated more clearly in Fig. 6C where the cells within Rathke's pouch and the anterior lobe that are lining both the dorsal and ventral parts of the lumen, respectively, are shown at a higher magnification.

In the *Hes1* mutant we observed a similar pattern to that of the wildtype (Fig. 6D-F), which includes E-cadherin expression throughout the pituitary and concentrated N-cadherin in the cells lining the dorsal lumen of Rathke's pouch. However, it appears that N-cadherin may be concentrated in a smaller proportion of the luminal cells than in the wildtype (boundaries marked by arrowheads).

In the *Prop1* mutant we notice differences in the expression patterns of the two molecules (Fig. 6G-I). Specifically, we observe strong N-cadherin staining lining almost the entire lumen, except for a small area where anterior lobe cells appear to be leaving the pouch in the rostroventral part of the pituitary (boundaries marked by arrowheads). It also appears that E-cadherin staining is stronger in the ventral part of the pouch, particularly in a concentrated pattern along the lumen of Rathke's pouch. In the higher magnification image it is clear that there is a strong overlap of concentrated E- and N-cadherin (yellow) along the lumen of Rathke's pouch that is not seen in the wildtype or *Hes1* mutants (Fig. 6I).

We observe what appears to be an opposite staining pattern in the double mutant than that of the *Prop1* mutant. There is E-cadherin staining throughout Rathke's pouch in a pattern that appears to be dispersed throughout the cells (Fig. 6J). Also, a strong N-cadherin signal lines the lumen of the dorsal part of Rathke's pouch only, as opposed to nearly all of the lumen in the *Prop1* mutant (Fig. 6K). The lack of N-cadherin concentration along the ventral half of the lumen is better appreciated in the higher magnified merged image (Fig. 6L). These data point to a possible role of *Prop1* in permitting the down-regulation of N-cadherin in cells transitioning from proliferating progenitors to differentiated hormone cells. *Hes1* also likely plays an antagonistic role, perhaps by maintaining N-cadherin expression in cells along the lumen and therefore preventing differentiation or movement. We believe that loss of *Hes1* in the *Prop1* mutant may allow for the downregulation of N-cadherin, which in turn may allow for movement of the cells out of the pouch. However, even though the cells of the *Prop1* mutant are able to move with the loss of *Hes1*, they appear to have lost their directional cues and instead migrate aberrantly.

SLUG is present in the rostral tip but absent in the anterior lobe of the Prop1 and double mutants

After noticing changes in expression pattern of E- and N-cadherin as well as characteristics of EMT we examined the expression of SLUG, a zinc finger transcription factor known to directly repress *E-cadherin* (Nieto, et al. 1994). SLUG is upregulated during EMT, causing a decrease in E-cadherin expression which allows for cell movement (Bolos, et al. 2003). In the wildtype pituitary at e14.5, we observed dispersed SLUG expression throughout the entire anterior lobe as well as the rostral tip, with Rathke's pouch cells lacking expression (Fig. 7A). In the *Hes1* mutant we observed a similar pattern (Fig. 7B). Surprisingly, we discovered that the only place

SLUG is expressed in the *Prop1* mutant is in the rostral tip (Fig. 7C). We see the same expression pattern in the double mutant (Fig. 7D). Clearly, *Prop1* is necessary for activation of SLUG either directly or indirectly in the anterior lobe except in the cells comprising the rostral tip.

We then questioned whether SLUG is typically expressed in cells that have differentiated into hormone producing cells. We hypothesized that the loss of SLUG in αGSU and ACTH producing cells could result in the loss of directional cues seen in these cells in the double mutant. As described in Figs. 2 and 3, the wildtype, *Hes1*, and *Prop1* mutants at e14.5 all have αGSU and ACTH producing cells restricted to the anterior lobe in the ventral pituitary while the double mutant has ectopic ages and ACTH producing cells in the drRP and dcRP in addition to the ventrally located cells (Fig. 7E-H). When we examined the coexpression of SLUG and pituitary hormones, we observed that SLUG appears to colocalize with an occasional αGSU cell in the rostral tip, but not with any cells in the anterior lobe (Fig. 7I-L). Also, we did not observe any colocalization between Slug and ACTH in any of the genotypes (Fig. 7M-P). It is possible that SLUG may be expressed in cells during their transition from proliferating progenitor cell to differentiated hormone producing cell. Also, *Prop1* clearly appears to be involved in the activation of SLUG either directly or indirectly.

Discussion

Hes1 and *Prop1* are both involved in the Notch signaling pathway as direct targets of the Notch intracellular domain and RBPJ-κ transcriptional activator complex, (Jarriault, et al. 1998; Raetzman, et al. 2004). We demonstrate that *Prop1* and *Hes1* have overlapping and distinct functions during pituitary organogenesis (Fig. 8). Analysis of pituitary development in mice lacking both of these genes revealed their redundant role in the appropriate timing of pituitary precursor differentiation. We see ectopic ACTH and αGSU expression in the pituitary only when both genes are absent. Although the exact mechanism by which PROP1 and HES1 prevent premature differentiation is unclear, the ectopic corticotropes and αGSU positive cells correlate with the misexpression of *Mash1* in the double mutants. It is well established that HES1 represses *Mash1* transcription and often *Mash1* mRNA levels are upregulated in *Hes1* mutants (Ishibashi, et al. 1995; Kageyama and Nakanishi, 1997). For example, in the fetal lung, *Mash1* seems to be important for specification of one of the many cell types: the pulmonary neuroendocrine cells (PNEC). In the *Hes1* mutant there is increased expression of *Mash1* followed by an increase in PNEC in the fetal lung (Ito, et al. 2000). In the pituitary, we observe an increase in *Mash1* transcript levels only when both *Hes1* and *Prop1* are lost, indicating that PROP1 may also play a role in repressing *Mash1* expression.

Previous studies have seen a more subtle increase in ACTH immunoreactive cells and *Mash1* expression in the *Hes1* or *Rbpj-κ* conditional knockouts similar to our double mutant phenotype but unlike our *Hes1* mutant alone (Zhu, et al. 2006; Kita, et al. 2007). One explanation for the discrepancy in our findings is that genetic background plays a significant role in pituitary differentiation. In fact, the phenotype of the *Prop1* knockout is altered when moved to different strains of mice, although there is no change in its effect on cell specification (Nasonkin, et al. 2004). Our findings are unique in that neither previous study reports premature differentiation of αGSU containing cells. This strengthens our position that both *Hes1* and *Prop1* are critical for repressing pituitary precursor differentiation.

A major question in developmental biology is how signaling pathways coordinate complex morphological changes that occur as the organism grows. For the pituitary, the critical organ growth during development is the ventral expansion of the hormone producing cells of the anterior lobe. The placement of the anterior lobe is essential because it must be formed in close apposition with the vasculature to be able to receive signals from the hypothalamus and release

its hormone contents into the bloodstream (Treier and Rosenfeld, 1996). Our studies uncovered an interaction between *Prop1* and *Hes1* in coordinating formation of the anterior lobe in the ventral domain of the developing pituitary. In the *Prop1* mutant, few cells are able to leave the pouch to form the anterior lobe, resulting in a hypercellular, dysmorphic Rathke's pouch during embryonic development. However, after birth there is increased apoptosis in the remnant of Rathke's pouch which leads to a hypocellular pituitary (Ward, et al. 2005). We observe the opposite problem in the *Hes1* mutant where the Rathke's pouch is much smaller than normal likely due to a premature influx of cells to the anterior lobe around e13.5. When *Hes1* is eliminated from the *Prop1* mutant, cells are no longer trapped in Rathke's pouch but they do not migrate ventrally to form the anterior lobe. Instead, the double mutant cells appear to have lost their directional cues and are located both dorsally and ventrally on the rostral and caudal sides of Rathke's pouch. This phenotype has some similarity with the *Lhx3* knockout in which NOTCH2 is not expressed, resulting in abnormal dorsal-ventral patterning and cell movement (Ellsworth, Butts, Camper. 2008). By analyzing the double mutants, we were able to uncover the role of *Prop1* and *Hes1* in regulating corticotrope movement that was not appreciated in the *Rbpj-κ* knockout (Zhu, et al. 2006). In this model, *Hes1* and *Prop1* mRNA were significantly reduced, but not absent like they are in our double mutant system.

During development, expansion of organs relies on differential cell sorting and movement. Changes in the actin cytoskeleton and adhesion molecules on the cell surface play pivotal roles in this process. We suspected that the loss of function of *Prop1* might interfere with important cell adhesion or movement cues, thereby preventing the cells from leaving Rathke's pouch. This would account for not only the branched, elongated structure of the Rathke's pouch in the *Prop1* mutant but also for the hypocellular anterior lobe. We discovered that *Prop1* is necessary for downregulating N-cadherin in the cells lining the lumen of Rathke's pouch. N-cadherin is a classical cadherin molecule known to affect cell-cell adhesion and migration during development. In pituitary tumor formation, reduced expression of N-cadherin can result in an increase in invasiveness of the tumor, likely due to the decrease in cell adhesion (Ezzat, et al. 2004; Gumbiner, 2005; Deramaudt, et al. 2006). However, there appears to be no canonical PROP1 binding sites within 10 kb upstream of N-cadherin, indicating that *N-cadherin* may not be a direct PROP1 target gene. One mechanism by which PROP1 could influence N-cadherin expression is through its interactions with the Wnt signaling pathway. Knocking out members of the Wnt signaling pathway such as *Wnt4* and *Wnt5a* has produced pituitaries with dysmorphology and/or altered cell specification, similar to the *Prop1* mutant (Cha, et al. 2004; Potok, et al. 2008). Additionally, PROP1 can directly interact with β-catenin, a central component of the Wnt signaling pathway (Olson, et al. 2006). N-cadherin is tethered to the cytoskeleton by direct interactions with β-catenin. It is possible that when PROP1 is lost, there is more β-catenin available to interact with N-cadherin, preventing its downregulation at the junction where cells need to exit Rathke's Pouch (Nagafuchi and Takeichi, 1988; Ozawa, et al. 1990; Douglas, et al. 2001; Gumbiner, 2005; Olson, et al. 2006). Alternatively, loss of *Prop1* may influence expression of other pathways that are known to regulate N-cadherin expression such as TGFβ, EGF, metalloproteases, or Rac1 (Grande, et al. 2002; Reiss, et al. 2005; Woods, et al. 2007).

Notch signaling also plays a role in cell movement and segregation. In Drosophila, the imaginal disc relies on Notch to maintain distinct populations of cells along the dorsal-ventral axis as it develops. Loss of Notch results in the absence of F-actin concentration which leads to the mixing of cells between the two compartments (Micchelli and Blair, 1999; Major and Irvine 2005). In *Hes1* mutants we observe a similar reduction in the boundary marker N-cadherin which correlates with a greater proportion of cells leaving Rathke's pouch. *Hes1* may have a direct effect on N-cadherin expression, or more likely, an indirect effect. This could include a role in cell sorting or actin cytoskeleton rearrangement, which can lead to changes in N-cadherin expression or function. Interestingly, in the double mutant only about half of the lumen

is lined with concentrated N-cadherin staining, despite the lack of *Prop1*. We speculate that the loss of *Hes1* in the *Prop1* mutant may permit the cells trapped in Rathke's pouch to be released by allowing for the downregulation of N-cadherin. It is possible that *Prop1* alone is imparts directional cues to Rathke's pouch cells. However, in the *Prop1* mutant, ACTH and αGSU positive cells move to the correct place. This leads us to hypothesize that both *Hes1* and *Prop1* work together to promote movement of cells ventrally from Rathke's pouch to the anterior lobe.

The presence of E- and N-cadherin in the pituitary suggests that the transition from the proliferating progenitor cells of Rathke's pouch to the differentiated cells of the anterior lobe may be an EMT. Classic characteristics of EMT that we are able to observe in the pituitary include presence of epithelial markers such as E-cadherin, loss of cell polarity, and change in cell shape allowing for cell movement or migration. However, we do not observe a downregulation of E-cadherin in the wildtype, which is a typical part of the process (Thiery, 2002; Kalluri and Neilson, 2003). Partial EMT (pEMT) is a similar process that differs from EMT in that often there is only a transient loss of polarity in the epithelial cells and the mesenchymal cells do not display all of their typical characteristics (Leroy and Mostov, 2007). It is possible that one of these processes is occurring in the pituitary.

Both EMT and pEMT involve a protein called SLUG, which is responsible for downregulating E-cadherin in EMT and maintaining cell survival in pEMT (Nieto, et al. 1994; Leroy and Mostov, 2007). We discovered SLUG expression throughout the dorsal area of the anterior lobe where cells appear to be transitioning from Rathke's pouch to the anterior lobe as well as in the rostral tip in the wildtype and *Hes1* mutant pituitaries. This specific expression of SLUG in the pituitary indicates that it could function to support migration like it does in the melanocyte cells (Jiang, et al. 1998). Surprisingly, in the *Prop1* and double mutants SLUG was detectable in the rostral tip, but not in the dorsal anterior lobe. This could explain why so many of the cells of Rathke's pouch fail to transition to the anterior lobe in the *Prop1* mutant. There appears to be no canonical PROP1 binding sites within 10 kb upstream of SLUG, therefore PROP1 likely regulates SLUG indirectly via Wnt or TGFβ signaling (Sakai, et al. 2005; Choi, et al. 2007). However, the double mutant also lacks SLUG, yet the cells are able to leave Rathke's pouch. This may be due to a combinatorial effect of the loss of *Hes1* in the *Prop1* mutant. Specifically, *Mash1* could be playing a role along with SLUG. In addition to regulation of the number and timing of differentiation, MASH1 and other proneural bHLH factors have been implicated in cell migration. A recent study by found that migration of neural precursor cells was enhanced in cell aggregate migration assays by exogenous MASH1 expression (Ge, et al. 2006). In total, our data provides insight into the way in which Notch signaling molecules and cell adhesion molecules can affect cell differentiation and movement (via N-cadherin, Slug, Mash1, etc.) and could be applied when studying Notch in the context of pituitary development and tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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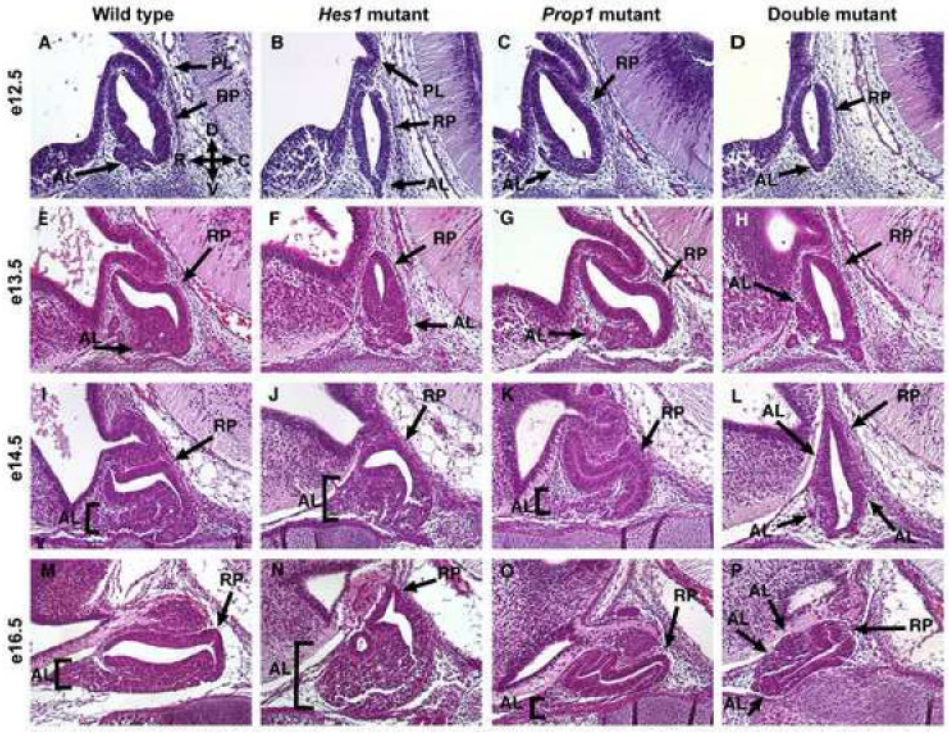

Fig. 1.

Development of the anterior pituitary requires both *Hes1* and *Prop1*. Embryos were sectioned sagittally at e12.5, e13.5, e14.5, and e16.5 and stained for morphology with hematoxylin and eosin. At e12.5 the wild type consists of an anterior lobe (AL), posterior lobe (PL), and Rathke's pouch (RP) (A, arrows). In panel A, arrows point to D, V, R, and C which correspond to the directions of dorsal, ventral, rostral, and caudal, respectively. The anterior lobe at e13.5 has grown and Rathke's pouch has begun to elongate (E). This trend continues with the e14.5 (I) and e16.5 (M) wildtype pituitaries. At e12.5 the *Hes1* mutant lacks a distinct anterior lobe and the posterior lobe is noticeably smaller (B). The anterior lobe gets larger at e13.5 (F), e14.5 (J), and e16.5 (N) while Rathke's pouch gets smaller. At e12.5 the *Prop1* mutant (C) is nearly indistinguishable from the wild type, however the anterior lobe doesn't enlarge much by e13.5 (G). By e14.5 (K) and e16.5 (O) of the *Prop1* mutant, Rathke's pouch is abnormally branched and elongated while the anterior lobe is much smaller than the wildtype anterior lobe. The double mutant at e12.5 (D) and e13.5 (H) has a morphology very similar to the *Hes1* mutant at those ages. However, at e14.5 the anterior lobe is forming along the sides of Rathke's pouch (L, arrows). By e16.5 the Rathke's pouch is difficult to distinguish from the anterior lobe however, some anterior lobe cells do appear to be located more dorsally than ventrally (P). n=3 Magnification: 200X

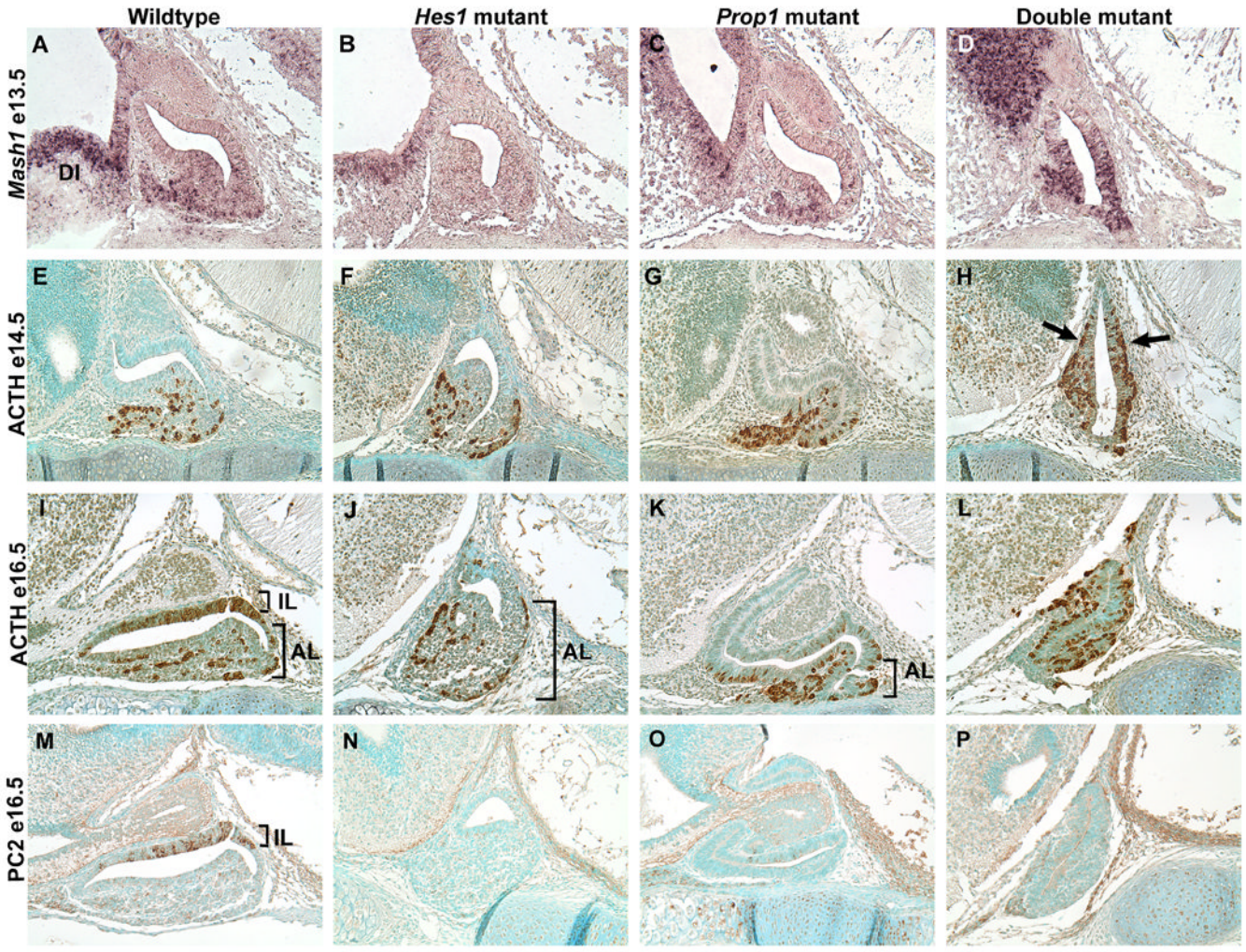

Fig. 2.

Mash1 and ACTH containing cells are misplaced in double mutants. *In situ* hybridization on sagittal sections reveals *Mash1* mRNA located in the anterior lobe of the wildtype (A), *Hes1* mutant (B), *Prop1* mutant (C), and double mutant (D) at e13.5 as well as the adjacent diencephalon tissue (DI). However, the double mutant also contains *Mash1* within Rathke's Pouch and the cells that are ectopically located (D). At e14.5 we observe a similar pattern in ACTH with immunohistochemistry on sagittal sections. In the wildtype (E), *Hes1* mutant (F), and *Prop1* mutant ACTH expression is restricted to the anterior lobe. In the double mutant ACTH is expressed throughout Rathke's pouch (H, arrows). By e16.5 the melanotropes have differentiated and are detected with the POMC antibody in the intermediate lobe (IL) while the corticotropes are detected in the anterior lobe (AL) in the wildtype (I). The *Hes1* mutant lacks melanotropes but contains corticotropes in the anterior lobe (J, bracket). In the *Prop1* mutant corticotropes are evident in the anterior lobe (K, bracket). Dark staining likely corresponding to ACTH producing cells is observed throughout an indistinguishable Rathke's pouch and anterior lobe in the double mutant (L). PC2, marking the melanotropes, is present throughout the intermediate lobe (IL) of the wildtype (M) and is absent in the *Hes1* mutant (N). An occasional PC2 positive cell can be observed in the *Prop1* mutant (O). Similar to the *Hes1* mutant, the double mutant lacks PC2 staining (P). n=3 Magnification: 200X

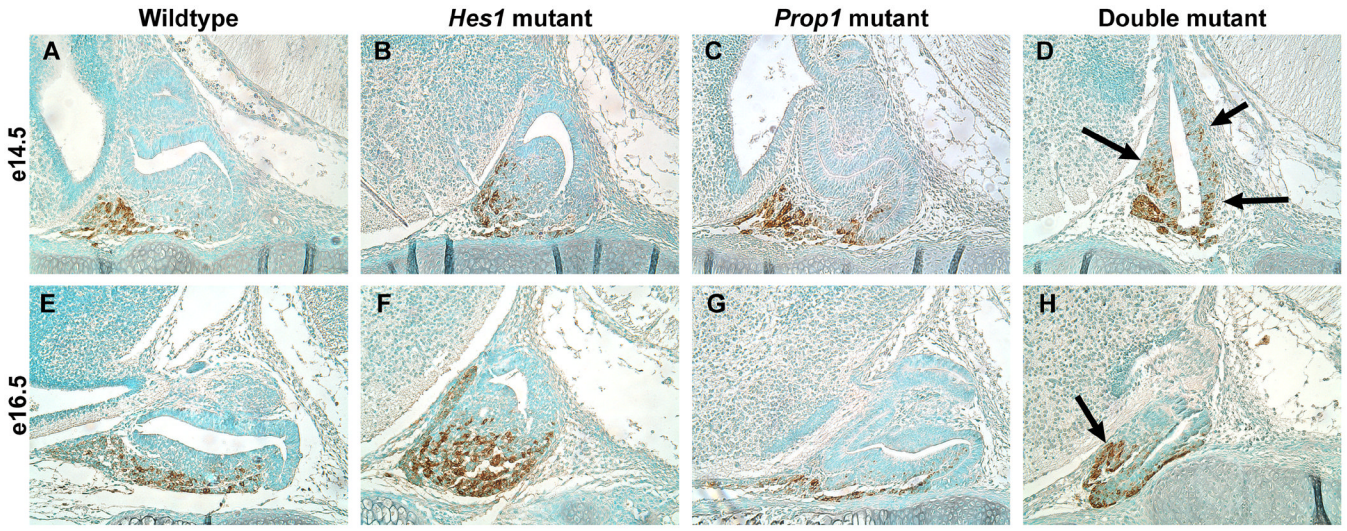

Fig. 3.

αGSU producing cells are misplaced in the double mutant. In the wildtype (A), *Hes1* mutant (B), and *Prop1* mutant (C) at e14.5 the αGSU immunoreactive cells are located exclusively in the anterior lobe as detected by immunohistochemistry on sagittal sections. However, in the double mutant, αGSU producing cells are detected within Rathke's pouch (D, arrows). At e16.5 we continue to observe αGSU producing cells strictly within the anterior lobes of wildtype (E), *Hes1* mutant (F), and *Prop1* mutant (G) pituitaries while in the double mutant, αGSU producing cells appear to be located within Rathke's pouch (H, arrow) in addition to the anterior lobe. n=3 Magnification: 200X

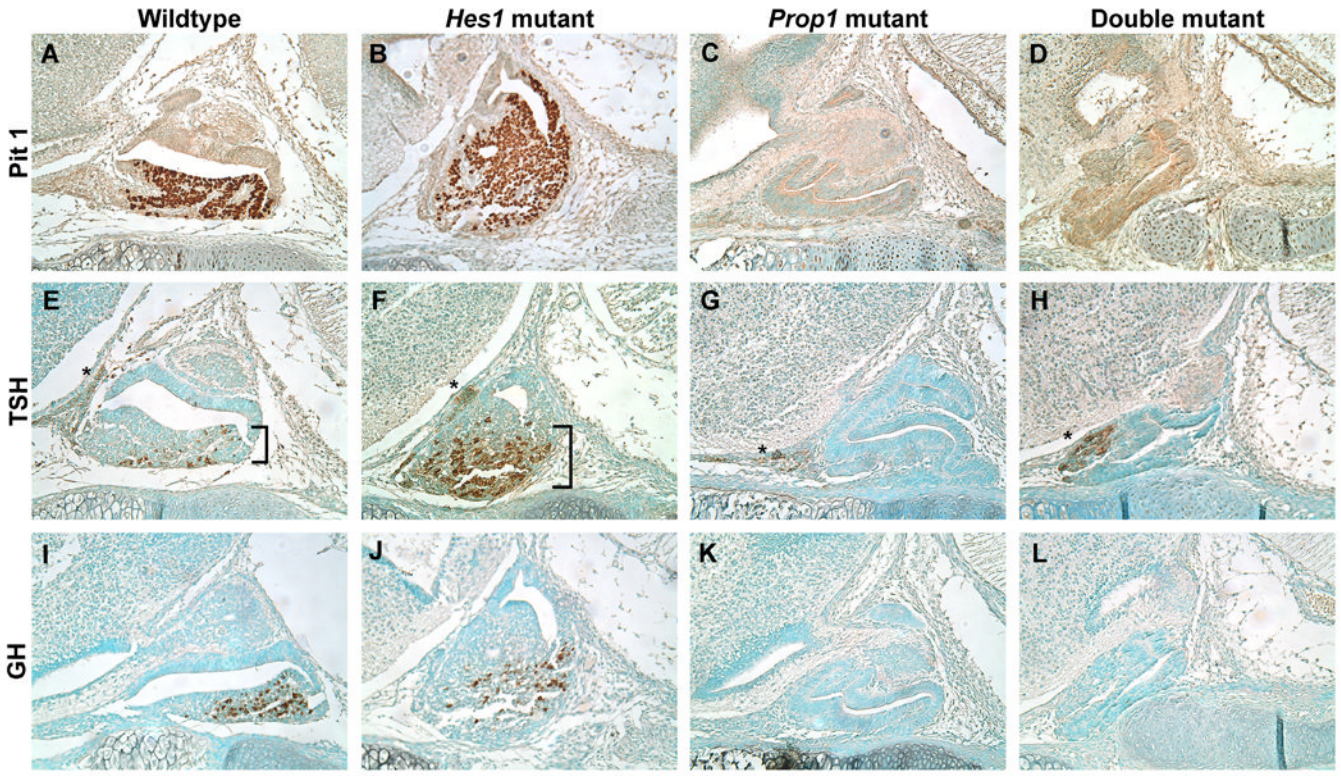

Fig. 4.

Prop1 and double mutant pituitaries lack PIT1 and its lineages. Sagittal sections at e16.5 stained via immunohistochemistry for PIT1 (A-D), TSHβ (E-H), and GH (I-L). PIT1 is present in the anterior lobes of the wildtype (A) and *Hes1* mutant (B) but entirely absent from the *Prop1* (C) and double mutant (D) pituitaries. The TSH antibody detects two distinct cell populations, the hormone producing thyrotropes of the anterior lobe (brackets) and the rostral tip thyrotropes (asterisks). In the wildtype (E) and *Hes1* mutant (F) the thyrotrope cell population can be seen as darkly stained round cells within the anterior lobe while the rostral tip thyrotropes can be seen as a hazy brown staining in the most rostral part of the pituitary. In the *Prop1* mutant (G) and double mutant (H) only the rostral tip thyrotropes appear to be present. These same patterns hold true for GH. In the wildtype (I) and *Hes1* mutant (J) GH is located within the anterior lobe while in the *Prop1* mutant (K) and double mutant (L) no GH is detected. n=3 Magnification: 200X

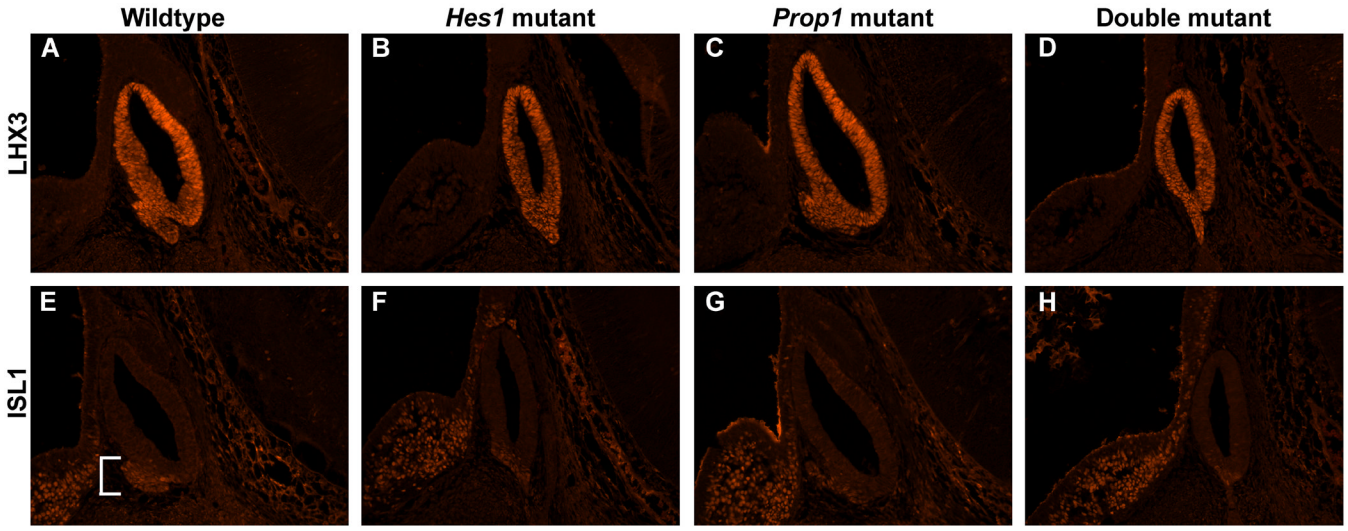

Fig. 5.

Loss of *Hes1* and *Prop1* does not affect initial stages of pituitary patterning. Sagittal sections of embryos at e12.5 were stained for expression of LHX3 (A-D) or ISL1 (E-H). LHX3 is detected throughout Rathke's pouch and the anterior lobe in the wildtype (A), *Hes11* mutant (B), *Prop1* mutant (C), and double mutant (D). ISL1 expression is restricted to the anterior lobe in the ventral part of the pituitary in the wildtype (E, bracket), *Hes1* mutant (F), *Prop1* mutant (G), and double mutant (H). n=3 Magnification: 200X

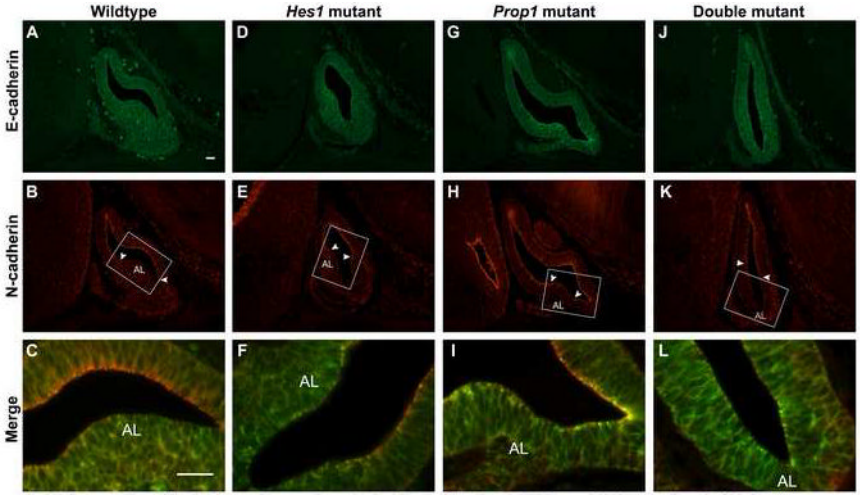

Fig. 6.

Downregulation of N-cadherin expression correlates with the ability of cells to leave Rathke's pouch. Sagittal sections of e13.5 embryos were stained via immunohistochemistry for E- and N-cadherin and then the images were merged. In the wildtype pituitary, E-cadherin is dispersed throughout Rathke's pouch and the anterior lobe (A) while N-cadherin is concentrated along the lumen of Rathke's pouch except for the ventral area where the anterior lobe (AL) is forming (B, arrowheads mark boundaries of N-cadherin concentration along lumen). The boxes in BE, H, and K correspond to magnified images C, F, I, and L. These images were merged and viewed at higher magnification to show the dorsal aspect of the pouch with N-cadherin concentration and the ventral aspect with only E-cadherin (C). In the *Hes1* mutant E-cadherin is dispersed throughout the pouch and anterior lobe (D) while N-cadherin is concentrated along the lumen of the dorsal half of the pouch (E, arrowheads). In the merged image of the *Hes1* mutant it is clear that the ventral half of the pouch is void of N-cadherin concentration. The *Prop1* mutant contains E-cadherin dispersed throughout the pituitary with areas of concentration along the lumen in the ventral part of the pouch (G). Nearly all of the lumen of Rathke's Pouch is lined with N-cadherin except for the small area where cells are exiting the pouch for the anterior lobe (H, arrowheads). E-cadherin is dispersed throughout the pouch in the double mutant (J). N-cadherin lines the dorsal half of the lumen of Rathke's pouch and is absent along the ventral half where the anterior lobe is forming (K, arrowheads). In the merged image of J and K it is clear that there is no N-cadherin concentration along the ventral half of the double mutant Rathke's pouch (L). n=3 Magnification: 200X (A-H) 640X (I-L), scale bars are 25 μm

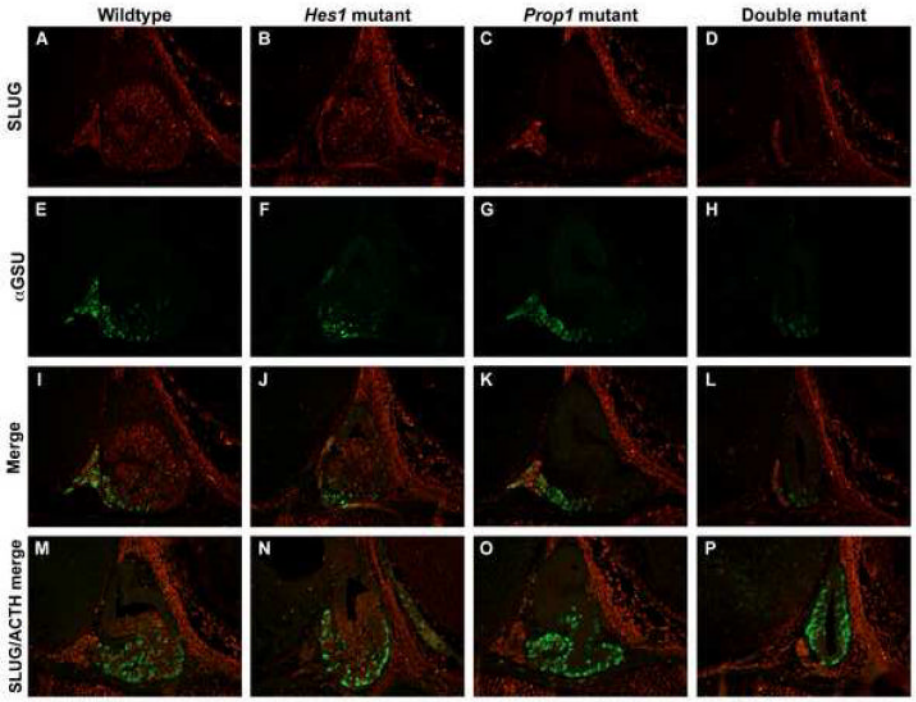

Fig. 7.

SLUG is absent in *Prop1* and double mutant anterior lobe cells. Immunohistochemical staining at e14.5 on sagittal sections revealed that SLUG is present in the anterior lobes of the wildtype (A) and *Hes1* (B) mutant pituitaries. In the *Prop1* (C) and double mutants (D) SLUG is found only in the most rostral tip of the pituitary. In the wildtype (E), *Hes1* mutant (F), and *Prop1* mutant (G) pituitaries αGSU is found exclusively in the anterior lobe while in the double mutant (H), αGSU is also expressed in the ventral part of Rathke's pouch. When these two stainings were merged we saw an overlap of SLUG and αGSU in an occasional cell in the rostral tip in the wildtype (I), *Hes1* mutant (J), *Prop1* mutant (K), and double mutant (L). We saw no overlap when SLUG and ACTH stainings were merged in the wildtype (M), *Hes1* mutant (N), *Prop1* mutant (O) and double mutant (P). n=3 Magnification: 200X

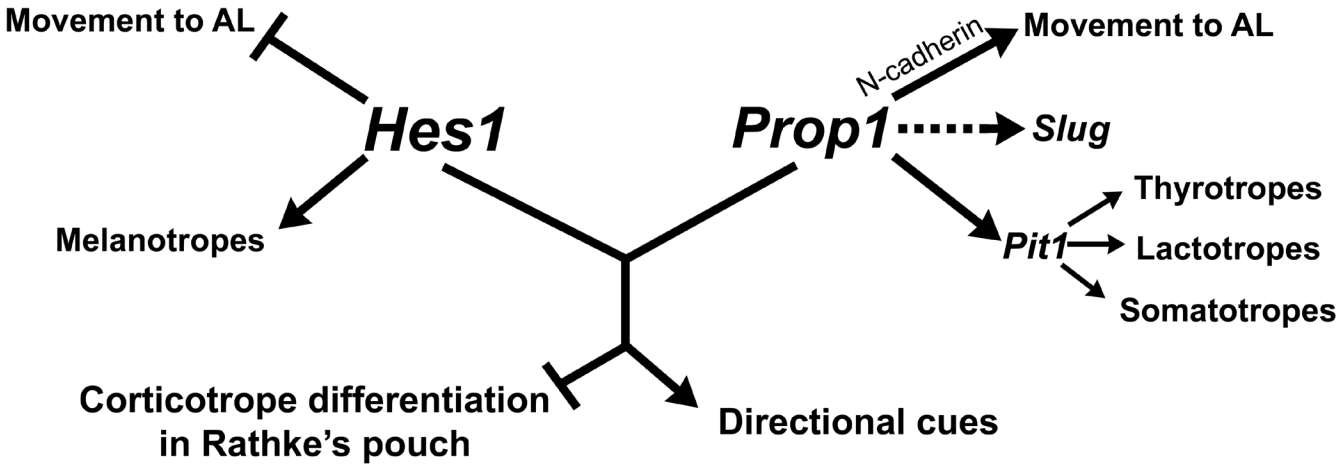

Fig. 8.

The roles of *Hes1* and *Prop1* during pituitary organogenesis. Alone, *Hes1* is likely necessary for preventing movement of cells from Rathke's pouch to the anterior lobe. Also, it has been shown to be necessary for the differentiation of the melanotrope cell lineage. *Prop1* alone is likely necessary for promoting the movement of cells from Rathke's pouch to the anterior lobe. This may be done through the downregulation of N-cadherin. *Prop1* may also be responsible for activating *Slug*, evidenced by the lack of SLUG in the *Prop1* and double mutants. It is also well established that *Prop1* is necessary for activation of *Pit1*, a transcription factor that promotes the thyrotrope, lactotrope, and somatotrope lineages. Together, *Hes1* and *Prop1* appear to prevent differentiation of the corticotropes within Rathke's pouch as well as promote factors that provide directional cues to the cells as they transition from Rathke's pouch to the anterior lobe.