Discovery of transcriptional regulators and signaling pathways in the developing pituitary gland by bioinformatic and genomic approaches

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Abstract

We report a catalog of the mouse embryonic pituitary gland transcriptome consisting of five cDNA libraries including wild type tissue from E12.5 and E14.5, *Prop1df/df* mutant at E14.5, and two cDNA subtractions: E14.5 WT-E14.5 *Prop1df/df* and E14.5 WT-E12.5 WT. DNA sequence information is assembled into a searchable database with gene ontology terms representing 12,009 expressed genes. We validated coverage of the libraries by detecting most known homeobox gene transcription factor cDNAs. A total of 45 homeobox genes were detected as part of the pituitary transcriptome, representing most expected ones, which validated library coverage, and many novel ones, underscoring the utility of this resource as a discovery tool. We took a similar approach for signaling-pathway members with novel pituitary expression and found 157 genes related to the BMP, FGF, WNT, SHH and NOTCH pathways. These genes are exciting candidates for regulators of pituitary development and function.

Keywords

Cap trapper; Homeobox gene; *Prop1*; Gene expression; Ames dwarf

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Introduction

The pituitary gland is a pivotal component of the endocrine system due to its role in controlling a wide range of fundamental bodily activities including growth, pubertal development, thyroid gland function, and the capacity to cope with stress. These critical functions are regulated by six different hormones secreted by five specialized cell types within the anterior pituitary gland, in response to local stimulatory and inhibitory peptides released by the hypothalamus.

Pituitary development in the mouse begins at embryonic day (E) 9.5 and continues after birth. All of the hormone-secreting cells in the anterior lobe derive from a single primordial tissue, Rathke's pouch, which forms from the oral ectoderm adjacent to the base of the developing brain. Formation of Rathke's pouch occurs in contact with a specialized region of the neuroectoderm, the infundibulum, which will later form the posterior lobe of the pituitary gland.

The molecular mechanisms underlying pituitary organ growth and cell differentiation are complex. Signaling molecules including FGF, BMP, NOTCH, WNT and SHH family members have roles in establishing regional identity within Rathke's pouch and contribute to its growth [1;2;3;4;5] The overall effect of these signaling molecules is to induce the regional expression of numerous critical transcription factors, which control the differentiation of the hormone secreting cells between E13.5 and birth.

Failure or incomplete differentiation of anterior pituitary cells during embryonic development can result in the genetically heterogeneous disorder, Multiple Pituitary Hormone Deficiency (MPHD). Molecular genetic analysis of spontaneous and genetically engineered mouse mutants with pituitary defects has revealed a functional role for many homeobox transcription factors in the etiology of MPHD in mouse and man. These include *Prop1*, *Pou1f1*, *Hesx1*, *Lhx3*, *Lhx4*, *Otx2*, *Pitx1*, and *Pitx2* [reviewed by[6;7;8]. Lesions in these genes only account for approximately half of the known genetic MPHD, suggesting that many of the key factors involved in the process of pituitary cell proliferation and differentiation remain unknown.

Mutations in *PROP1* are the most common known genetic cause of MPHD in humans [9]. Patients with *PROP1* mutations commonly exhibit deficiencies in Growth Hormone (GH), Prolactin (PRL), Thyroid Stimulating Hormone (TSH) and gonadotropins, with evolving adrenocorticotropin deficiency (ACTH). Similarly, the spontaneous Ames dwarf mouse model ($Prop1^{df/df}$), that harbors a homeodomain mutation in *Prop1* displays a phenotype that includes hypoplasia of the anterior pituitary accompanied by nearly complete loss of the hormone-secreting cell types that produce GH (somatotropes), PRL (lactotropes), and TSH (thyrotropes) [10;11;12;13]. Interestingly, genetic background has a more significant impact on the viability of *Prop1* deficient mice than any functional difference between the $Prop1^{df}$ and $Prop1^{null}$ alleles, suggesting that genetic modifiers may influence the clinical features of some MPHD patients [13].

Prop1 expression is first detectable in Rathke's pouch at E10 - 10.5. It reaches a peak at E12.5 after which time its expression declines rapidly [14]. A time of intense cell proliferation occurs in the developing pituitary from E12.5 - E14.5, which precedes the appearance of the differentiated pituitary cell types. The effects of mutations in *Prop1* and other critical transcription factors including *Pitx2*, *Lhx3* and *Hesx1* are evident at this time [15;16;17]. This suggests that potential *Prop1* targets and other important as yet unknown transcription factors may be expressed around E12.5 and/or E14.5.

At E12.5 the anterior pituitary is composed primarily of proliferating, undifferentiated cells. There is a very small group of non-proliferating cells that have begun to differentiate and express *Foxl2* and the alpha subunit of the gonadotropins and thyrotropin, *Cga* [18]. At this

developmental time *Prop1$^{df/df}$* and WT pituitaries are indistinguishable in size and morphology [13;19]. By E14.5 there are more cells in the anterior lobe of normal mice, and they appear loosely packed and glandular. In contrast, the pituitary glands of *Prop1$^{df/df}$* mutants are highly dysmorphic at E14.5 because cells apparently fail to migrate away from the dorsal aspect of the gland and colonize the anterior lobe. They exit the cell cycle but fail to differentiate [13; 19;20].

We exploited the *Prop1$^{df/df}$* model to gain insights into the embryonic pituitary transcriptome. We used cDNA subtractive hybridization between *Prop1$^{df/df}$* and wild type pituitary gland primordia at E14.5 to identify differentially expressed genes in mutant and normal embryonic pituitary [21]. We took a differential display approach to define an expression profile for the developing anterior pituitary at E12.5 and E14.5 by comparing Rathke's pouch tissue from wild type samples taken at these times [22]. Collectively, these studies represented the first expression profiles of the developing pituitary gland and also paved the way for demonstrating the role of the *Wnt* signaling pathway in regulating pituitary growth. More specifically, these studies identified *Wnt5a*, *Aes* and *Tcf7l2* in the developing pituitary. We have now demonstrated functional roles for each of these genes in pituitary development through the analysis of corresponding mutant mouse models [2;23;24;25].

Numerous gene expression profiling studies have been carried out using adult pituitary tissue and cell lines, but only a few studies have addressed developmental changes in pituitary gene expression aside from those with the *Prop1$^{df/df}$* model [26;27]. Alterations due to aging and caloric restriction have been investigated [28] and some studies with cell lines have implicated transcription factors as potential regulators of development and cell proliferation [29;30]. The expression studies with adult pituitary tissue and cell lines identified collections of potentially pituitary-specific genes [31;32], candidates for tumor biomarkers and mechanistic involvement in pituitary adenoma formation [reviewed in [33], and pathways induced by hormonal or growth factor treatment [34;35;36;37].

Our studies with the *Prop1$^{df/df}$* model have proven valuable for novel gene discovery and dissecting the genetic differences between the wild type and mutant pituitary; although, a major limitation for functional studies has been the lack of full-length cDNAs available. In addition, previous approaches have not addressed the depth and complexity of the developing pituitary transcriptome specifically. To overcome these limitations we generated a full-length cDNA encyclopedia of the mouse embryonic pituitary gland comprised of 5 transcriptome volumes including three samples and genotypes: E12.5 wild type pituitary (E12.5 WT), E14.5 wild type pituitary (E14.5 WT) and E14.5 *Prop1$^{df/df}$* pituitary [38], and two subtracted libraries: E14.5 WT - E14.5 *Prop1$^{df/df}$*, WT E14.5 – WT E12.5. We selected these times and genotypes because we expected E14.5 *Prop1$^{df/df}$* mutants to continue to express genes that are normally expressed at E12.5 and wane in expression by E14.5 such as *Hesx1* [12]. We also expected to find genes normally activated by *Prop1*, and expressed at E14.5 in WT mice, but absent in the E14.5 *Prop1$^{df/df}$* mutant, such as *Pou1f1* [10] and *Notch2* [3]. Using the Cap-trapper method to enrich for full-length clones [39;40;41;42;43], followed by aggressive subtraction and normalization, we produced full-length cDNA libraries and generated DNA sequence from 71,649 cDNA clones. The comparison and global analysis of the transcripts contained within each library reveals expression of 12,009 unique genes that we have integrated into a comprehensive searchable database. We demonstrate the integrity, validity, utility and novelty of the database by identifying 45 homeobox genes expressed in the developing anterior pituitary gland and 157 genes related to the BMP, FGF, WNT, NOTCH or SHH signaling pathways, many of which were not previously known to be part of the developing pituitary transcriptome.

Results

The embryonic pituitary encyclopedia is a rich source for gene discovery

We sought to investigate the depth and complexity of the developing pituitary transcriptome, while maximizing novel gene discovery and developing full-length clones that could be exploited for functional studies of the developing pituitary gland. 'Phase I' involved end sequencing of clones from three embryonic pituitary libraries (E12.5 WT, E14.5 WT and E14.5 *Prop1$^{df/df}$*) until less than 10% of the new sequences produced novel information [38]. Approximately 20% of the 32,476 sequences were obtained from the 5′ end and the remainders were sequenced from the 3′ end. Here, we present the identification and characterization of the unique cDNAs from these three libraries. We also present two additional libraries that were generated by a subtractive hybridization analysis between E14.5 WT and E14.5 *Prop1$^{df/df}$* (Sub1) and between E14.5 WT and E12.5 WT (Sub2).

We conducted single pass end-sequencing of 56,716 unique cDNA clones from these five libraries. The sequences were each queried using BLAST analysis against the UniGene unique subset of the NCBI *Mus musculus* database (Build #159, www.ncbi.nlm.nih.gov/UniGene/). The same sequences were also subjected to BLAST analysis against the mouse-specific Reference Sequence (RefSeq) (Release #20, www.ncbi.nlm.nih.gov/RefSeq/). Clones were identified based on the first BLAST identity recorded with an expect value of 1.0-e5 or lower. This analysis identified 51,352 clones present in the encyclopedia. 5,364 clones were not identified by BLAST analysis. While some of these clones have poor sequence quality through the polyA stretch at the 3′ end that interferes with BLAST analysis, we anticipate that some of these clones will be identified when compared to more recent UniGene and Refseq builds. Further analysis to determine the number of unique genes represented revealed 12,009 unique cDNA clones distributed between the five libraries.

Table 1 lists the total number of cDNA clones sequenced from each library, the number of library-specific genes represented, and the distribution of cDNA clones relative to each of the five libraries in different pair-wise combinations. In terms of library-specific transcripts, 1246 genes in the encyclopedia were unique to E12.5 WT, 642 to E14.5 WT, 1256 to E14.5 *Prop1$^{df/df}$*, 629 to the subtraction of E14.5 *Prop1$^{df/df}$* from E14.5 WT, and 1264 from the subtraction of E12.5 WT from E14.5 WT. 3029 genes were expressed universally in all libraries.

The majority of genes are represented by a small number of clones

The underlying principle of our approach was to develop a tool for novel gene discovery that addressed the depth and complexity of the embryonic pituitary transcriptome. Our sequencing effort was designed to maximize gene identification and enrich for those genes that were rare rather than abundant. The extremely high ranking of these libraries with regard to novelty in a pool consisting of 246 libraries generated from 71 different tissues is evidence of success [38]. The E14.5 *Prop1$^{df/df}$* library ranked 1/246, where 24% of the unique clones sequenced in this library were found to be novel, and the libraries from E14.5 WT and E12.5 WT also ranked highly, 8/246 and 16/246, respectively. Another measure of success is to ascertain the distribution of clone representation per unique gene with the expectation that the majority will have few replicates. This trend was observed in all five libraries. Figure 1 shows the results for the E14.5 WT library following analysis. The same trend was observed in all of the other libraries (data not shown).

All three non-subtracted cDNA libraries contribute unique genetic information

To gain greater insight into the unique cDNA distribution between the three non-subtracted libraries, we compared the relative distribution of 6,940 cDNA clones between the E12.5 WT,

E14.5 WT and E14.5 *Prop1$^{df/df}$* libraries (Figure 2a). Overall, there is similar overlap between transcripts detected in these three libraries, where any two of the three libraries considered share at least 11-12% clone representation. Each of the three libraries contains a significant proportion of apparently library-specific genes (14%), suggesting that each library contributes an important collection of unique genes to the encyclopedia that may be dependent on developmental stage and genotype.

The two subtracted libraries each add a dimension of genetic novelty to the encyclopedia

We compared the relative distribution of 5,765 cDNA clones between the E14.5 WT, E14.5 *Prop1$^{df/df}$* and Sub1 libraries (Figure 2b). The Sub1 library (E14.5 WT minus E14.5 *Prop1$^{df/df}$*) should theoretically represent those genes expressed at E14.5 in normal pituitaries but not in *Prop1$^{df/df}$* mutants. This trend is observed, suggesting that the subtraction was effective. Gene representation in the E14.5 WT and Sub1 libraries overlaps by 16% compared to a 13% overlap between E14.5 *Prop1$^{df/df}$* and Sub1. The percentage of genes expressed in both E14.5 WT and E14.5 *Prop1$^{df/df}$* is also 10%. This implies that the Sub1 and E14.5 *Prop1$^{df/df}$* libraries are as different as the E14.5 WT and E14.5 *Prop1$^{df/df}$* libraries in terms of the expressed genes being detected or sampled. In addition to those genes that were detected in both E14.5 WT and Sub1, there are also genes uniquely detected in one library. Uniquely represented genes constitute 10-14% of the total expressed genes, which equates to 2527 genes in the case of the Sub1 library, demonstrating that it adds a dimension of genetic novelty to the encyclopedia.

We evaluated the E12.5 WT, E14.5 WT and Sub2 libraries, comparing 7,590 clones (Figure 2c). The Sub2 and E14.5 WT libraries should share expression of a larger subset of genes compared to E14.5 WT and E12.5 WT, and this trend was observed. E14.5 WT and Sub2 unique transcript expression overlaps by 17%, compared to an overlap of 10% between E14.5 WT and E12.5 WT. This overlap is equivalent to that observed between Sub2 and E12.5 WT (13%) suggesting that this subtraction was also effective. The Sub2 library adds new information to the encyclopedia as 1,264 (16%) unique cDNA clones present in the Sub2 library were not sampled in the E14.5 WT or E12.5 WT libraries.

Gene Ontology (GO) terminology reveals novel library-specific biological processes

We wanted to determine the unique biological processes represented in each library as a means for identifying the genes that contribute to the morphological and functional differences in pituitary development [13;19]. Using Gene Ontology (GO) terms we probed the five sets of genes uniquely sampled from each library for enriched processes and also analyzed the set of genes that were detected universally. Twelve GO terms were significantly enriched (unadjusted p-value <0.01) in the universally expressed transcript set (Table 2). Enriched terms include 'translation', 'RNA splicing', 'protein folding' and 'protein transport', fundamental biological processes that are active in all tissues, irrespective of developmental stage or genotype.

The E14.5 WT library contains three significantly over-represented GO terms; 'spermidine metabolic process', 'hydrogen peroxide catabolic process', and 'defense response to bacterium' (Table 2). While these were unexpected, it is not difficult to envision how genes in these categories could be engaged in other known processes that occur predominantly at this time. For example, spermidine markedly increases DNA binding activity of certain transcription factors in the pituitary gland [44]. It may, therefore, play an important role in the changing transcription characteristic of the developing pituitary at this time. The 'defense response to bacterium' GO term is also significantly enriched in the *Prop1$^{df/df}$* library (Table 2) and is represented by more genes, compared to the E14.5 library (data not shown).

The E14.5 *Prop1^{df/df}* library sampling was enriched for six GO terms (Table 2), five of which are not over-represented in any of the other library samples, providing clues regarding the types of processes occurring in the *Prop1^{df/df}* pituitary that could differ from the normal state. For example, 'Response to oxygen radical' was enriched, a process that is involved in apoptosis, which is elevated in *Prop1^{df/df}* [19;45]. Two significantly over-represented GO terms emerged from the E12.5 WT library sample (Table 2). 'Positive regulation of calcium signaling' precedes the catabolism of spermidine in the pituitary, which was represented at E14.5, and the role for calcium signaling is well documented [46;47]. The Sub1 and Sub2 library samples collectively contain four over-represented GO terms, which do not appear in any of the other samples (Table 2). 'Exocytosis' and 'response to pH' may provide a platform on which to launch future functional studies for some of the genes represented.

Validation of the embryonic pituitary encyclopedia

To validate the five libraries that comprise the embryonic pituitary encyclopedia we queried the database for the presence of several 'knowns', consisting of some homeobox genes with well-characterized roles in the developing pituitary gland and related gene family members. Searches for gene name, UniGene ID, and DNA or peptide sequences identified nine homeobox genes in the database (Table 3). *Pou1f1*, a target of *Prop1* [14] was detected in the E14.5 WT library, and not in the E14.5 *Prop1^{df/df}* library, as expected. *Lhx3*, *Pitx1* and *Pitx2*, and two members of the *Six* gene family, were identified in several libraries [6;48;49;50;51]. Other expected genes detected included *Otx2, Prop1*, and *Pax6* [6;7;52]. Three of the expected homeobox genes, *Lhx4*, *Hesx1*, and *Pou3f4*, were not detected in the database following *in silico* searching. Absence from the database can be an artifact of the sampling strategy for sequencing. For example, *Hesx1* was detected by PCR at the expected developmental stages, suggesting that deeper sequencing would have revealed the presence of these genes (data not shown).

To confirm the identity and integrity of the clones identified computationally from end sequence, at least one clone representing each homeobox gene identified in Table 3 was sequenced from both ends and examined to determine whether the libraries were enriched for full-length clones. In most cases the clones were full-length compared to the NCBI *Mus musculus* UniGene database (Build #159) validating the Cap-trapper technique for producing full-length clones. This validation strategy revealed that the high throughput first pass sequencing correctly identifies greater than 95% of the clones accurately. The misidentified clones tended to be ones with poor expect values from the initial BLAST analysis.

Identification of additional homeobox genes in the embryonic pituitary encyclopedia

Given the critical roles of numerous homeobox genes in the developing pituitary gland, we used the database as a tool for discovering additional homeobox genes expressed in the pituitary gland. The database is searchable using a combination of text searching with the terms 'homeo', 'homeobox' and 'homeodomain' or querying the database using the 60 amino acid homeodomain consensus sequence [53]. The most comprehensive method was to search using gene names that are included in a list of homeodomain containing proteins at Prosite (http://ca.expasy.org/prosite/). We recovered numerous additional homeobox genes expressed in the encyclopedia (Table 4).

Differential expression analysis confirms transcription of novel homeobox genes

We analyzed 23 of these genes with respect to expression in the developing pituitary gland at E12.5, E14.5, and E18.5 using a combination of in situ hybridization analysis, RT-PCR, and quantitative PCR. Figure 3 shows a subset of the in situ hybridization results. *Dlx1* expression was confirmed at E12.5 (Figure 3a) and *Dlx3*, *Rax1*, and *Zhx1* at E14.5 (Figure 3b-d). For PCR analyses cDNA was generated from E12.5, E14.5, and E18.5 WT, and E14.5 *Prop1^{df/df}*

pituitary primordia. The quality of these cDNAs was confirmed using a series of primers specific for *Prop1*, *Pou1f1*, *Hesx1*, and *Hprt* (Figure 4, data not shown). Using standard RT-PCR expression of 12 genes was confirmed during pituitary gland development. *Meis1*, *Adnp*, *Prrx2*, *Lhx2*, *Atbf1*, *Pknox2*, *Pbx2*, and *Mrg1* were expressed at similar levels at all time points analyzed in wild type and in *Prop1*^{df/df}Rathke's pouch cDNA. *Meox2*, *Emx2*, *Otx2*, and *Zeb2* appeared to exhibit developmentally regulated expression, with the peak expression of *Meox2* at E12.5, *Emx2* at E14.5, and *Zeb2* at E18.5 (Figure 4a). In addition, expression of *Meox2* and *Otx2* appeared elevated in *Prop1*^{df/df} mutant Rathke's pouch compared to wild type controls. Quantitative Real Time PCR was used to confirm the results suggested by standard RT-PCR (Figure 4b). cDNA was generated from additional E12.5, E14.5, E18.5 WT, and E14.5 *Prop1*^{df/df} pituitary primordia and the quality confirmed using primer sets for *Prop1* and *Pou1f1*. All samples were done in triplicate and normalized to *Hprt*. The fold activation was determined by comparison to E14.5 WT, which was set to 1. Both *Meox2* and *Otx2* peak in expression at E12.5 and are elevated 7.9 and 6 fold, respectively, relative to E14.5 WT. The peak expression of *Emx2* is between E12.5 and E14.5, while *Zeb2* peaks later in development at E18.5. *Meox2* and *Otx2* are slightly elevated in the E14.5 *Prop1*^{df/df}mutants relative to E14.5 WT by 1.59 fold and 2.06 fold, respectively. *Zeb2*, *Adnp*, and *Meis1* are decreased in the *Prop1*^{df/df}mutants relative to E14.5 WT by 2.09, 2.15, and 2.39 fold, respectively. *Prrx2* does not exhibit developmental regulation or differences between wild type and *Prop1*^{df/df}. Taken together these results suggest that *Meox2*, *Otx2*, *Zeb2*, *Adnp*, and *Meis1* could merit further evaluation as downstream targets of *Prop1*.

The identification of individual homeobox genes in the specific libraries does not necessarily correlate with the actual expression profile of that gene. For instance, *Meis1* was only identified in the E12.5 library; however, our RT-PCR data show that it is expressed at E12.5, E14.5, and E14.5 *Prop1*^{df/df} embryos. Therefore, it is misleading to assume that the identification of a gene in a specific library or even a subtracted library provides accurate differential expression information.

Database analysis implicates additional signaling molecules in pituitary development

Fundamental to the current understanding of the genetic mechanisms driving pituitary development has been the identification of numerous signaling pathways that exert their effects on the pituitary primordia. Previous studies have demonstrated the importance of individual members of the BMP, FGF, NOTCH, SHH and WNT signaling pathways in directing normal pituitary development [1;2;3;5;²³;24;25;54;55]. A continuing challenge in the field is to identify additional members of these pathways and to determine how these various signaling pathways interact with each other during pituitary organogenesis to promote development of the organ.

We used Genomatix software (www.genomatix.de/) to build functional genetic networks for signaling pathways known to affect pituitary development and queried the database for the expression of these genes. For example, in the case of the BMP pathway, a group of 78 *Bmp*-related genes emerged, and we identified 20 of them in the encyclopedia. This analysis was conducted for additional pathways, revealing a total of 61 genes expressed in the developing pituitary gland related to the BMP (20), FGF (13), NOTCH (2), SHH (1) or WNT (25) signaling pathways. A subset corresponding to the FGF pathway is shown in Table 5, and the complete list is available in Supplementary Table 1. 17 additional genes were identified that are involved in functional crosstalk between two or more signaling pathways (Table 5 and Supplementary Table 1).

As an alternative strategy we searched the library for GO terms related to signaling and for specific gene names for genes known to be involved in the BMP, FGF, NOTCH, SHH, and

WNT pathways. This search identified an additional 72 genes (Supplement Table 1). This list has not been verified by end sequencing yet.

Spatial expression of three *Bmp*-related genes in the developing pituitary

We searched the set of 61 signaling genes described above for those with previously demonstrated roles in the developing pituitary. Twelve "known" genes are in the set including *Aes* and *Tle4* [23], *Bmpr1a* and *Bmpr1b* [56], *Chrd* [55], *Fgf10* [1] *Titf1* [4], *Hey1* and *Notch2* [3], *Wnt5a* [2;25;55], *Axin2* [2;57] and *Fzd5* [21] (Supplementary Table 1). Previously we presented the expression pattern for the BMP signaling related genes *Nbl1* and *Fstl1*, found in our developmental library, at E12.5 and E14.5, and for *Id3* at E10.5 [54]. We expanded the analysis of *Id3* and examined the expression of *Id2* and *Tgfbi* (Figure 5). *Id2* expression was detected in the dorsal aspect of Rathke's pouch at E12.5 (Figure 5a). Expression persists at E14.5 in the expanding anterior pituitary (Figure 5b) but appears restricted to those cells closest to the lumen of the pouch. *Id2* does not appear to be expressed in the proliferating cells of the expanding Rathke's pouch. By E16.5 *Id2* expression is barely detectable in the pituitary (data not shown). *Id3* expression was detected in Rathke's pouch at E12.5, enriched in the ventral part of the pouch and in the intermediate lobe (Figure 5c). Similar to *Id2*, *Id3* expression does not appear in the proliferating cells of the expanding Rathke's pouch. Expression of *Id3* continues at E14.5 (Figure 5d) in similar pattern, restricted to the dorsal pouch and intermediate lobe and is barely detectable by E16.5 (data not shown). *Tgfbi* showed a striking expression pattern. While there are no transcripts in Rathke's pouch itself, the gene is expressed in the mesenchymal tissue immediately surrounding the pouch at E12.5 (Figure 5e). At E14.5, this expression continues more strongly and is also detectable surrounding the posterior pituitary (Figure 5f). Like *Id2* and *Id3*, *Tgfbi* expression wanes by E16.5. These studies confirm expression of these genes in the pituitary and support the encyclopedia as reflection of pituitary gene expression.

Interplay of signaling pathways in the developing pituitary

We surveyed the signaling genes represented in the encyclopedia using Genomatix software to identify those genes that displayed a functional overlap between two or more of the five pathways defined in Supplementary Table 1. This analysis revealed the presence of an additional 16 genes in our encyclopedia that are involved in functional cross talk during organ development, including pituitary (Supplementary Table 1). A subset of these (4/16) is shown in Table 5.

4/16 genes of the pathway interaction genes identified belong to the helix-loop-helix transcription factor family (Table 5). These include *Ascl1* and *Id1*. The zebrafish *Ascl1* orthologue (*Ascl1a*) is a critical gene for pituitary cell differentiation and cell survival [58]. Zebrafish *Fgf3* mutants fail to express *Ascl1a*, suggesting that *Ascl1a* acts downstream of *Fgf*. Zebrafish *Fgf3* is orthologous to mammalian *Fgf10*, known for its function in mammalian pituitary development, implying a conserved mechanism for FGF signaling and *Ascl1* expression [1]. Additionally, *Id1* mediated expression of *Bmp2* leads to reduced *Ascl1* expression [59]. *Ascl1, Id1, Id2, Id3*, and *Id4* are all implicated as differentially expressed in pituitary tumors [28;60;61;62]. Together these studies suggest a functional link between FGF and BMP signaling and identify the *Id* gene family and *Ascl1* as interesting candidates in mediating cell differentiation in the developing pituitary. This example demonstrates the utility of the embryonic pituitary encyclopedia in providing a foundation for furthering our understanding of signaling in the developing pituitary.

Discussion

In previous studies we described a partial expression profile of the developing pituitary gland and paved the way for understanding the role of WNT signaling in pituitary development using a combination of differential display PCR [22] and subtractive hybridization [21]. The datasets generated in these studies were fairly small (<400 genes in each), but the gene discovery was significant, providing proof of principle that generation of a more comprehensive pituitary transcriptome profile would likely enable significant gene discovery and provide insight into the transcription factors and signaling molecules expressed in the developing pituitary.

In this study we generated a full-length cDNA encyclopedia of the developing pituitary at wild type E12.5 and E14.5 and in the *Prop1*$^{df/df}$ pituitary at E14.5 using an approach designed to enrich for full-length clones, maximize gene discovery, and permit high throughput gene recovery [38;40;41;42;43]. Using sequence data from these libraries we built a comprehensive database through which to access transcript information. It is searchable by Gene Ontology (GO) terminology; direct sequence query, including nucleic and amino acid sequence; gene name or text-string options; motifs for transcription factor binding; and UniGene ID and database-specific clone number. This encyclopedia contains 12,009 unique transcripts, represented by 56,716 cDNA clones, and accounts for over half the known genes in the mouse genome (22,723) (www.ensembl.org/Mus_musculus/index.html). Thus, the developing pituitary transcriptome has depth and complexity, and our study provides clones ideal for use in functional studies such as cell culture transfection and the generation of transgenic mice.

Each of the libraries contained within the encyclopedia includes a set of library-specific cDNA clones representing between 630 (Sub1) and 1328 (Sub2) unique transcripts. Exclusivity in any one library is not a true readout of the pituitary transcriptome, but rather, a reflection of the sequencing approach taken. Novelty and enrichment for rare transcripts were favored in place of the complete sequencing of any one library. Despite this limitation, the GO terminology analysis suggests that certain biological processes are significantly enriched in a library-specific manner. The genes represented by these processes could provide the basis for future novel mechanistic studies of embryonic pituitary development.

We identified a total of 45 homeobox genes that are expressed in the developing pituitary gland, expanding significantly on the subset of homeobox genes with well-characterized roles. Transcripts for seven of these newer genes have been detected in pituitary cell lines (*Lhx2, Mrg1, Pknox1, Pou6f1*), pituitary adenomas (*Pou4f1, Zeb2*), or other developing vertebrates (*Rax*) [37;60;63;64;65;66;67;68;69;70;71]. These genes correspond to over fifteen different homeodomain classes including the LIM, Paired (Prd), Paired-like (Prd-like) and POU homeobox families [reviewed by [72]]. Given the importance of known genes from these gene families in pituitary organogenesis such as *Prop1*, *Pou1f1*, *Pitx1*, and *Pitx2*, the identification of *Adnp* (POU-like), *Prrx2* (Prd), and *Prrxl1* (Prd), suggests that these genes may also play important roles in driving cell proliferation and/or differentiation during the formation of the pituitary gland. Mutations in some of these genes may underlie some cases of MPHD, where the current genetic cause is unknown.

Interestingly, *Meox2, Otx2, Emx2, and Zeb2* are differentially expressed in the developing Rathke's pouch between E12.5 and E18.5. No obvious differences in pituitary cell specification are noted in *Emx2* mutant mice (Potok, unpublished observation). *Meox2* and *Otx2* are elevated in the *Prop1*$^{df/df}$ E14.5 pituitary relative to E14.5 wild type, and *Prop1*$^{df/df}$ newborns have ectopic, elevated expression of *Otx2* compared to wild type littermates (Mortensen, unpublished observation). *Meis1* and *Adnp* are reduced in the *Prop1*$^{df/df}$ E14.5 pituitary relative to E14.5 WT. The dynamic expression patterns of these homeobox genes in the developing

pituitary raise the question of whether they are regulated by *Prop1* and whether they have functional roles in pituitary development.

Some of the remaining homeobox genes identified represent novel classes of homeobox genes, not previously found in the developing pituitary. For example, three zinc finger (ZF) homeobox genes were identified in the encyclopedia (Table 4). *Zeb2* expression was detected at low levels at E12.5, expression levels increased at both E14.5 and E18.5 in normal mice and expression was reduced in the *Prop1^{df/df}* E14.5 pituitary (Figure 4), and *Zhx1* has been detected at e12.5 by *in situ* hybridization (data not shown). Mutations in one of these genes, *ZEB2* or *ZFHX1B*, causes Mowat Wilson Syndrome, a human disorder characterized by a distinctive facial phenotype in association with mental retardation, microcephaly, and short stature [73]. Because this gene is expressed in human pituitary, it is intriguing to hypothesize that affected individuals with this syndrome have a pituitary defect [60].

Several genes belonging to the TALE family of homeobox genes were identified in the embryonic pituitary (*Pbx2*, *Meox2*, *Meis1*, *Mrg1*, *Pknox2*, *Tgif1*, *Tgif2*). Genes from this family are required to specify cell fate in numerous organs including the spleen, and they may play a similar role in the developing pituitary [74]. The TALE gene *Pbx1* regulates expression of the *Fshb* gene in pituitary gonadotrope-like cells and *Gnrh* in the hypothalamus [64;75]. MRG1 interacts with LHX2 to regulate *Cga* expression in pituitary gonadotrope-like cells [66]. Our expression analysis of *Meox2* suggests that *Prop1* may repress its expression during pituitary development.

One of the current challenges in the field is to determine how the various signaling pathways interact with each other during pituitary organogenesis. BMP and FGF signals influence anterior pituitary cell differentiation [1;55], and SHH signaling may participate in boundary formation with FGF and BMP signaling in the ventral diencephalon [54;55]. Cross talk between the WNT, BMP, FGF and SHH pathways is evident in *Tcf7l2, Wnt5a*, and *noggin* mutants, which exhibit pituitary developmental abnormalities [2;24;54]. Using the embryonic pituitary encyclopedia we identified 79 additional BMP, FGF, NOTCH, SHH or WNT pathway members with novel expression in the developing pituitary. One such example is *Fgf13*, which is critical for neural differentiation in *Xenopus* [76]. Additionally, three *Sprouty* genes were identified in the encyclopedia (*Spry1*, *Spry2* and *Spred2*). *Spry1*, *Spry2*, and *Spry4* expression was previously reported in E14.5 Rathke's pouch [77]. *Sprouty* genes have highly conserved roles as antagonists of FGF signaling, which can affect differentiation [78;79]. Thus, several members of the sprouty family may regulate FGF signaling during pituitary organogenesis.

An important application of our studies lies in the ability to utilize the full-length cDNA clones. Some examples are the full-length cDNA clones for the *Fstl1, Id3*, and *Six6* genes that were used for expression analysis by *in situ* hybridization in pituitary development in demonstrating the important role of *noggin* [54]. Full-length cDNA clones for the *forkhead* transcription factor *Foxl2* and numerous members of both the *Wnt* and *Frizzled* gene families, (*Fzd6*, *Axin2* and *Dvl2*, see Supplementary Table 1) have been invaluable [2;18]

In conclusion, the embryonic pituitary encyclopedia represents a valuable gene discovery tool that can be used to identify novel genes and pathways in the developing wild type and mutant pituitary (*Prop1^{df/df}*) and to initiate functional studies. We have just scratched the surface and anticipate that this full-length cDNA encyclopedia will act as an ongoing resource for studying pituitary development and disease.

Materials and Methods

Mice

Prop1^(df/+) mouse stocks were obtained from Dr. A. Bartke (Southern Illinois University, Carbondale, Ill.) in 1988 and maintained at the University of Michigan according to NIH guidelines. Heterozygote carriers were intercrossed to generate timed pregnancies and the morning after mating was designated as E0.5. Developing pituitary glands were dissected from E14.5 embryos with the aid of a dissecting microscope and were individually stored in RNA Later (Ambion, Austin, TX) at -20°C. Genomic DNA was extracted from the yolk sac, genotyping was performed as described [21], and pituitaries were pooled for RNA extraction and cDNA generation following genotyping. CD1 mice were purchased from Charles River Laboratories (Wilmington, MA) as timed pregnancies and pituitary glands were dissected from E12.5, E14.5, and E18.5 embryos. Pituitaries were pooled for RNA extraction and cDNA generation. The University of Michigan Committee on Use and Care of Animals approved all experiments.

Library generation

Pituitary tissue for library generation was prepared as described [38]. Briefly, starting from 50 micrograms of total RNA, first strand cDNA was prepared with an anchored oligo-dT primer adapter [80] and the cDNA was cap-selected [81] and subsequently cloned in a Lambda FLC I vector [41]. Libraries were excised into plasmids and sequenced. E14.5 WT was given the library ID K7, E14.5 *Prop1^(df/df)* was K8, and E12.5 WT was K9 [38]. Additionally, subtracted libraries were prepared from E14.5 WT minus E14.5 *Prop1^(df/df)* and E14.5 WT minus E12.5 WT, using established procedures [82].

Database generation

Mouse UniGene Build #159 (Mm.seq and Mm.seq.uniq) and Mouse RefSeq Release 20 were obtained from NCBI FTP servers, along with the 'blastall' program used to perform the BLAST identifications. Each cDNA sequence was first BLASTed against the Mm.seq.uniq dataset, and then against the full mouse UniGene set (Mm.seq). All cDNA sequences were also BLASTed against RefSeq to provide an alternative identification.

A database was constructed to contain the sequences themselves, along with Riken identification and a 'library' identification, indicative of the tissue source along with the UniGene and RefSeq identifications, where obtainable. Web-based search functions were developed to allow exploration of the contents of the various libraries by sequence, gene name, etc. From the GO Consortium (www.geneontology.org) we downloaded a database of GO identifiers in hierarchical format (10/31/06 release). This data, along with files from the NCBI 'Gene' database ('gene2go', 'gene2unigene') allowed us to query our UniGene identifications using GO terminology.

Gene Ontology (GO) terminology analysis

Over-represented Gene Ontology (GO) terms in the set of unique sequences from each library as well as the intersecting sequences for all libraries were detected using a modification of the standard Fisher's exact test [83]. Briefly, we selected those sequences that were unique to each library based on the UniGene ID and mapped these IDs to Entrez Gene IDs using the mouseLLMappings package of Bioconductor (http://www.bioconductor.org). We mapped the union of all sequences in each library to Entrez Gene to use as the 'universe' from which the unique sequences were selected. In other words, the usual heuristic explanation of a Fisher's exact test is the idea of selecting colored balls from an urn. In our case, the unique sequences from each library are the balls that were selected from the urn, and the union of all sequences

in each library is the 'universe' of balls in the urn. We computed over-representation of GO terms using the topGO package of Bioconductor, which uses the directed acyclic graph (DAG) structure of the GO ontologies to decorrelate significant subordinate terms from consideration when estimating the significance of higher-order terms.

In situ hybridization

RNA in situ hybridization was performed as described previously [84]. *Dlx1*, *Dlx3*, *Rax*, *Zhx1*, *Id2*, *Id3* and *Tgfbi* cDNAs were isolated from the embryonic pituitary encyclopedia [38]. *Id2*, *Id3* and *Tgfbi* were linearized with *NotI*, and *Dlx1*, *Dlx3*, *Rax* and *Zhx1* were linearized with SacII. T3 polymerase was used in all cases to generate antisense probes.

RT-PCR and PCR

RNA was extracted from pituitary tissue dissected from CD1 mouse embryos at E12.5, E14.5, and E18.5 and stored in RNAlater (Ambion, Foster City, CA) following the recommended commercial protocol. RNA was further purified via RNAqueous 4PCR (Ambion, Foster City, CA). cDNA was prepared from total RNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Briefly, 5μg total RNA was incubated at 42°C for 50 minutes in a 10μl reaction containing 2μl cDNA buffer, 4μl $MgCl_2$, 2μl 0.1μl DTT, and 1μl of Superscript II RT enzyme. Standard PCR reaction components at final concentrations were 1 X PCR Reaction buffer (Roche, Indianapolis, IN), 1.5mM $MgCl_2$ (Perkin Elmer, Waltham, MA), 200mM dNTP mix (Roche), and 0.4U GoTaq polymerase (Perkin Elmer) per 25μl reaction. Standard reactions contained 25-50ng template cDNA.

All PCR reactions were performed using the following conditions: 94°C; 4 minutes, followed by 30 cycles of 94°C; 30 seconds, PCR-specific annealing temperature; 30 seconds and 72°C; 30 seconds, followed by 1 cycle of 72°C; 10 minutes. PCR-specific annealing temperatures and primers are listed in Supplementary Table 2.

Quantitative Real Time PCR was performed using TaqMan Gene Expression Assays On Demand (Applied Biosystems, Foster City, CA). Briefly 25-50ng of template cDNA was added to 10μl Taqman Universal PCR Master Mix (Applied Biosystems), 8 μl double distilled H_2O, and 1 μl TaqMan gene specific primer set. All samples were done in triplicate and standardized to *Hprt*. Real Time PCR was performed using the Prism 7000 Sequence Detection System (Applied Biosystems). Fold activation and standard deviations were determined as previously described [85].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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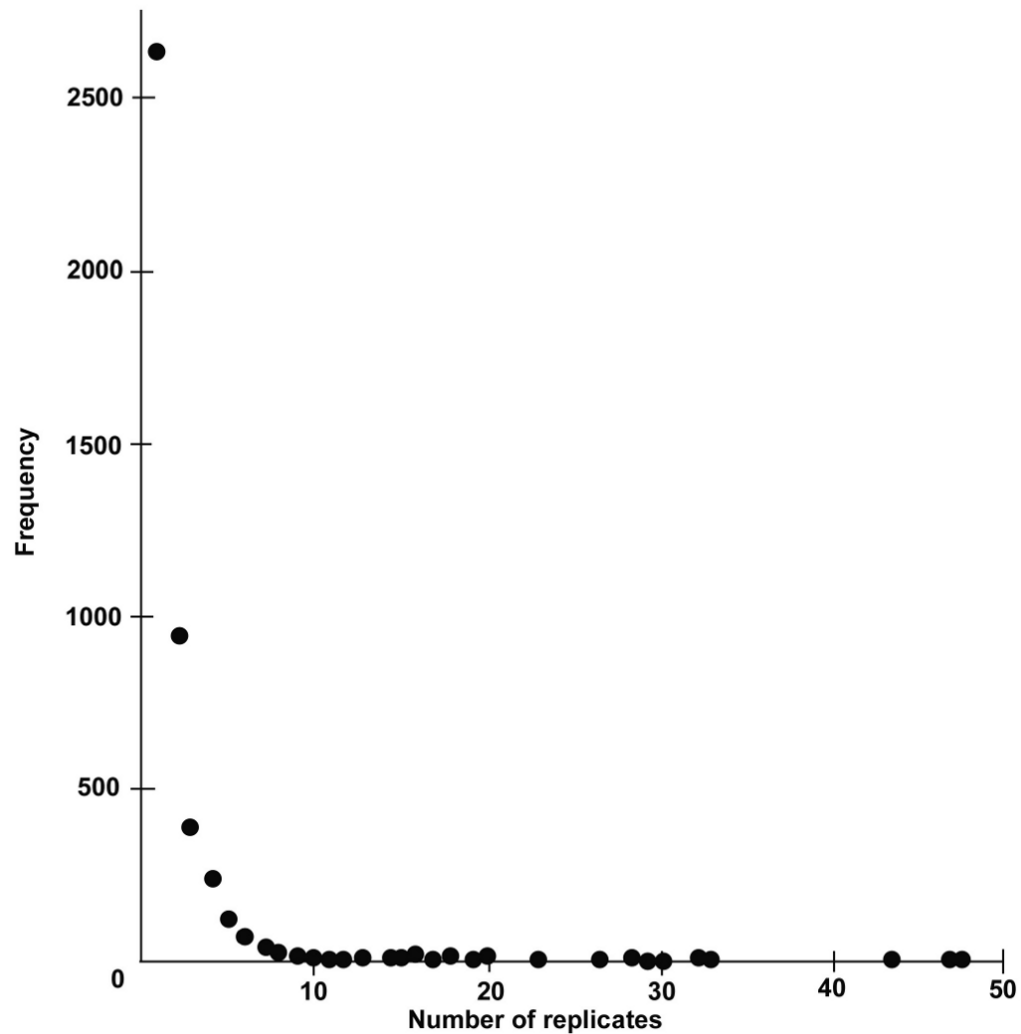
Figure 1. Distribution of clone representation per unique transcript for the E14.5 WT library
4339 unique UniGene entries are contained in the E14.5 WT library (Table 1). Over half of these (2644) are represented by a single cDNA clone (shown in the first column). A continually decreasing trend is observed, such that as the number of replicates or cDNA clones increases, the number of genes represented by these clones decreases. Number of Replicates (X axis) refers to the number of clones representing each unique UniGene entry. Frequency (Y axis) refers to the number of unique UniGene entries represented.
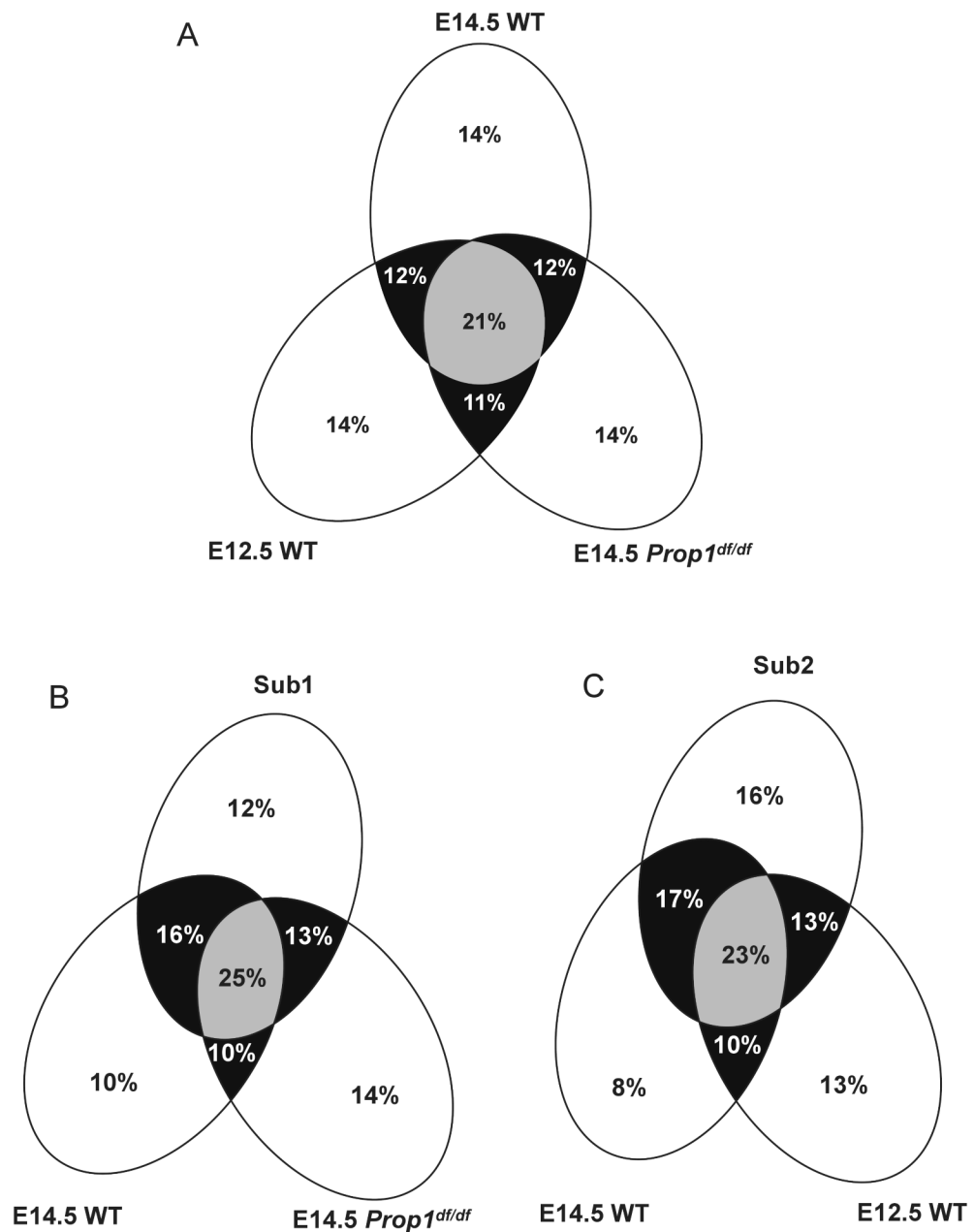
Figure 2. Venn diagrams illustrate the relationships between the five libraries in the encyclopedia
(A) Comparison of a total of 12,799 genes from the E12.5 WT, E14.5 WT and E14.5 *Prop1^{df/df}*. (B) Comparison of a total of 5,765 genes from the E14.5 WT, E14.5 *Prop1^{df/df}* and Sub1 libraries. (C) Comparison of a total of 7,590 genes from the E12.5 WT, E14.5 WT and Sub2 libraries.
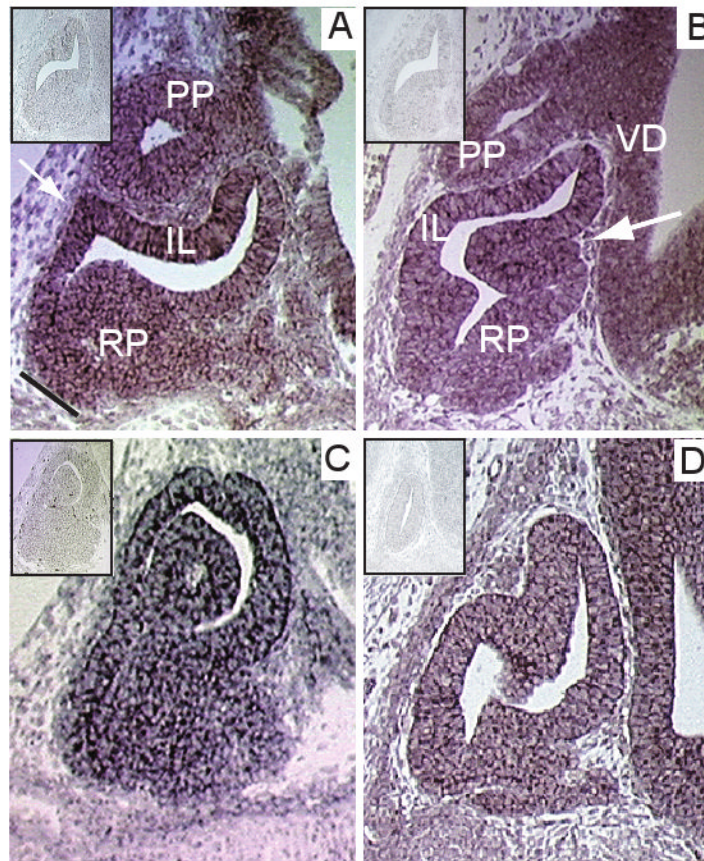
Figure 3. *In situ* hybridization of four homeobox genes with novel expression in the developing pituitary gland

In situ hybridization analysis of *Dlx3* at E12.5 (A) and *Dlx1* (B), *Rax* (C) and *Zhx1* (D) at E14.5 in the developing pituitary indicates robust expression. (A) At E12.5 *Dlx3* is expressed strongly in the intermediate lobe (IL), shown by the white arrow, as well as in the posterior pituitary (PP) and throughout the expanding Rathke's pouch (RP), though predominantly in the dorsal aspect. The dorsal aspect is indicated by the black diagonal line. (B) At E14.5 *Dlx1* is expressed throughout Rathke's pouch and the intermediate lobe. Expression appears concentrated in the ventral aspect of the pouch, as shown by the white arrow. *Dlx1* expression is also present in the ventral diencephalon (VD). *Rax* (C) and *Zhx1* (D) are expressed throughout the developing Rathke's pouch (RP). Sense controls corresponding to each gene are shown in the top left hand corner of each panel.
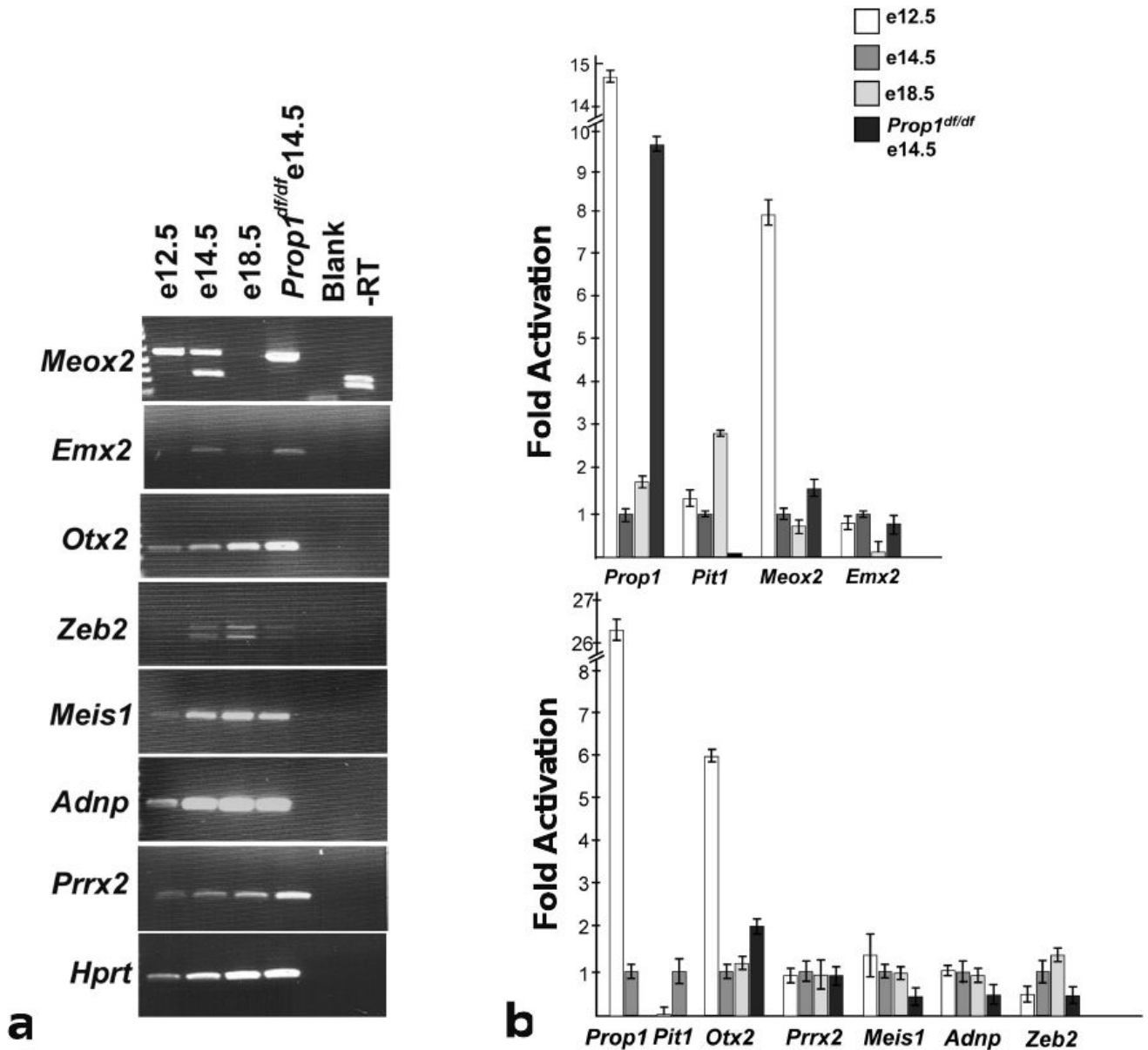
Figure 4. Expression of homeobox genes in the developing wild type and *Prop1^{df/df}* pituitary
(A) Pituitary expression of a set of homeobox genes identified in the embryonic pituitary
encyclopedia was validated by RT-PCR using cDNA generated from pituitary primordia at
E12.5, E14.5, E18.5, and *Prop1^{df/df}* E14.5. The homeobox genes *Meox2*, *Emx2, Otx2*, and
Zeb2 show differential expression between E12.5-E18.5 and in the *Prop1^{df/df}* E14.5 pituitary,
while *Meis1, Adnp*, and *Prrx2* showed similar expression in all samples. *Hprt* is a control for
RNA and cDNA quality. Differential expression was quantitated using Real Time PCR (B).
The quality of the cDNA samples was confirmed by demonstrating the expected pattern of
Prop1 and *Pit1* expression. *Meox2, Emx2, Otx2, Prrx2, Meis1* and *Adnp* expression levels were
validated using pituitary cDNA from E12.5 WT (white bars), E14.5 WT (dark gray bars), E18.5
WT (light gray bars). Different E12.5 cDNA preparations were used in the top and bottom
panels of B. All samples were done in triplicate and standardized to *Hprt*. Fold activation and

standard deviations were calculated relative to E14.5 WT based on the ΔCt values of each sample compared to the ΔCt of *Hprt*.
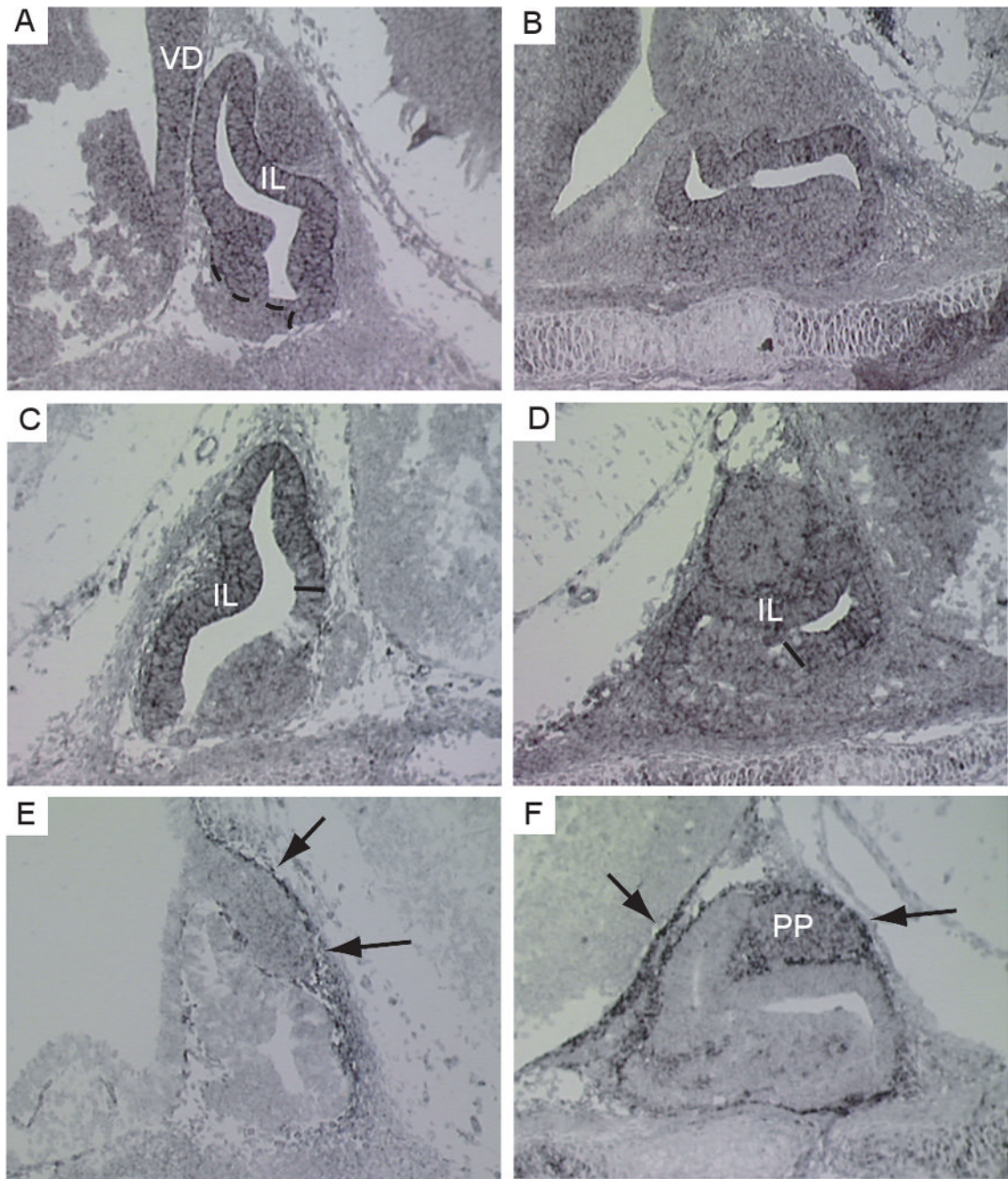
Figure 5. Developmental expression of 3 novel *Bmp*-related genes in the embryonic pituitary gland
(A) Expression of *Id2* is predominantly in the intermediate lobe (IL) and around the lumen of the pituitary. Expression appears restricted to the cells that are not in the expanding proliferating Rathke's pouch (delineated by the black dashed line). Expression is also detected in the ventral diencephalon (VD). This expression pattern persists at E14.5 (B). (C) At E12.5 *Id3* is expressed in the IL and dorsal part of the cells surrounding the pituitary lumen (indicated by the horizontal black line) and this expression pattern continues at E14.5 (D). (E) At E12.5 *Tgfbi* was detected in the mesenchymal tissue immediately surrounding the developing pituitary (shown by the black arrows). By E14.5 (F), *Tgfbi* expression becomes stronger in the mesenchyme and is also detected surrounding the posterior pituitary (PP) (shown by the black arrows).

Table 1

Number and distribution of cDNA clones in the embryonic pituitary encyclopedia

	E12.5 WT	E14.5 WT	E14.5 *Prop1$^{df/df}$*	Sub1 E14.5 WT-E14.5 *Prop1$^{df/df}$*	Sub2 E14.5 WT-E12.5 WT
Total UniGene entries/library	4192	4339	4268	4710	5191
E12.5 WT^a	1246^b	544	837	610	821
E14.5 WT	544	642	517	539	854
E14.5 *Prop1$^{df/df}$*	837	517	1256	563	847
Sub1	610	539	563	629	1160
Sub2	821	854	847	1160	1264
Genes represented in all five libraries			3029		

^{a}Example interpretation: Clones sequenced from the E12.5 WT library matched 4192 different UniGene entries, 1246 were unique to this library, 3029 were found in all libraries.

^{b}Unique library-specific transcripts are on the diagonal, shaded in gray.

Table 2

Enriched gene ontology (GO) terms

GO Term[a]	Description	P value[b]
Universally expressed gene set		
GO:0006412	Translation	0.0000012
GO:0006457	Protein folding	0.0000027
GO:0015671	Oxygen transport	0.000043
GO:0008380	RNA splicing	0.00013
GO:0006397	mRNA processing	0.00029
GO:0009408	Response to heat	0.00143
GO:0006986	Response to unfolded protein	0.00235
GO:0015031	Protein transport	0.00242
GO:0046907	Intracellular transport	0.00419
GO:0006334	Nucleosome assembly	0.00629
GO:0000398	Nuclear mRNA splicing, via spliceosome	0.00739
GO:0030198	Extracellular matrix organization and biogenesis	0.00907
E12.5 WT		
GO:0019068	Virus assembly	0.0007
GO:0050850	Positive regulation of calcium-mediated signaling	0.0077
E14.5 WT		
GO:0008216	Spermidine metabolic process	0.002
GO:0042744	Hydrogen peroxide catabolic process	0.002
GO:0042742	Defense response to bacterium	0.005
E14.5 *Prop1df/df*		
GO:0042742	Defense response to bacterium	0.0003
GO:0006952	Defense response	0.0012
GO:0048015	Phosphoinositide-mediated signaling	0.0062
GO:0051046	Regulation of secretion	0.0062
GO:0000305	Response to oxygen radical	0.0083
GO:0006111	Regulation of gluconeogenesis	0.0083
Sub1 (E14.5 WT - E14.5 *Prop1df/df*)		
GO:0030174	Regulation of DNA replication initiation	0.002
GO:0007264	Small GTPase mediated signal transduction	0.005
Sub2 (E14.5 WT - E12.5 WT)		
GO:0009268	Response to pH	0.002
GO:0006887	Exocytosis	0.004

[a] www.geneontology.org/

[b] GO terms with an unadjusted p-value < 0.01 are listed

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Table 3

Libraries contain homeobox genes implicated in pituitary development

Gene Symbol	Gene Name	Unigene ID[a]	NUMBER OF CLONES IDENTIFIED[b] Pituitary cDNA Source				
			E12.5 WT	E14.5 WT	E14.5 *Prop1*[df/df]	Sub1[c]	Sub2[d]
Lhx3	LIM homeobox protein 3	Mm.386765	5	3	2	5	
Otx2	Orthodenticle homolog 2	Mm.134516					1
Pax6	Paired box gene 6	Mm.3608			1		
Pitx1	Paired-like homeodomain transcription factor 1	Mm.135195	1	1		2	3
Pitx2	Paired-like homeodomain transcription factor 2	Mm.246804		2	1	2	5
Pou1f1	POU domain, class 1, transcription factor 1	Mm.457971		3			
Prop1	Paired like homeodomain factor 1	Mm.1386	2	2			4
Six3	Sine oculis-related homeobox 3 homolog	Mm.370208	1	1	1	3	
Six6	Sine oculis-related homeobox 6 homolog	Mm.57138	4	8	5	1	2

[a] NCBI UniGene Accession Number

[b] number of cDNA clones identified in each library corresponding to indicated gene. Blank indicates none.

[c] E14.5 WT - E14.5 *Prop1*[df/df]

[d] E14.5 WT - E12.5 WT

Table 4

Identification of additional homeobox genes in the embryonic pituitary encyclopedia

Gene Symbol	Gene Name	NUMBER OF CLONES IDENTIFIED[a] Pituitary cDNA Source					Homeobox Class[b]
		E12.5 WT	E14.5 WT	E14.5 Prop1[df/df]	Sub1	Sub2	
Adnp	Activity-dependent neuroprotective protein			1	3	3	POU-like
Dlx1	Distal-less homeobox 1	1			3		Dll
Dlx3	Distal-less homeobox 3	1					Dll
Emx2	Empty spiracles homolog 2			3	1		Ems
Hlx1	H2.0-like homeo box					1	Hlx
Homez	Homeodomain leucine zipper-encoding	2				1	ZF
Isl1	Islet 1 Tanscription factor	2	1				LIM
Lass2	LAG1 homolog, ceramide synthase 2	3	2				
Lass3	LAG1 homolog, ceramide synthase 3		1				
Lass4	LAG1 homolog, ceramide synthase 4	3	3				
Lass5	LAG1 homolog, ceramide synthase 5		1		1	2	
Lass6	LAG1 homolog, ceramide synthase 6		3			2	
Llx2	LIM homeobox protein 2 [69]					1	LIM
Meis1	Myeloid ecotropic viral integration site 1	1					TALE
Meox2	Mesenchyme homeobox 2	1					TALE
Mrg1	Myeloid ecotropic viral integration site-related gene 1 [66;71]			2	1		TALE
Msx1	Homeo box, msh-like 1 [67;86]		1	1		1	MSH
Nkx2-4	NK2 transcription factor related, locus 4					2	NK
Nkx3-1	NK-3 transcription factor, locus 1					3	NK
Obox3	Oocyte specific homeobox 3	2					OBOX
Pax1	Paired box gene 1				2	2	Prd
Pbx2	Pre B-cell leukemia transcription factor 2		1		3		TALE
Pknox1	Pbx/knotted 1 homeobox 1 [64]			1			TALE
Pknox2	Pbx/knotted 1 homeobox 2		1				TALE
Pou4f1	POU domain class 4, transcription factor 1 [37]				1	1	POU

Gene Symbol	Gene Name	NUMBER OF CLONES IDENTIFIED[a] Pituitary cDNA Source					Homeobox Class[b]
		E12.5 WT	E14.5 WT	E14.5 Prop1$^{df/df}$	Sub1	Sub2	
Pou6f1	POU domain class 4 transcription factor 1 [70]				1		POU
Prrx2	Paired related homeobox 2					1	Prd
Prrxl1	Paired related homeobox protein-like 1					2	Prd
Rax	Retina and anterior neural fold homeobox [63]	2			2	1	Prd
Satb1	Special AT-rich sequence binding protein 1	2	1		3	1	Cut
Six1	Sine oculis-related homeobox 1 homolog	5	2	4	10	6	Six
Six4	Sine oculis-related homeobox 4 homolog	1	3		2		Six
Tgif1	TGFB-induced factor homeobox 1		1		1		TALE
Tgif2	TGFB-induced factor homeobox 2		2				TALE
Zeb2	Zinc finger E-box binding homeobox 2 [60]			1			ZF
Zfhx3	Zinc finger homeobox 3	1				2	ZF
Zhx1	Zinc fingers and homeoboxes protein 1	2					ZF

[a]Number of cDNA clones identified in each library corresponding to indicated gene.

[b]Homeobox class is indicated where known. None known for *Lass3*.

Table 5

Identification of signaling-related genes in the embryonic pituitary encyclopedia[a]

Gene Symbol	Gene Name
Fgf-related[b]	
Fgf10	Fibroblast growth factor 10
Fgf13	Fibroblast growth factor 13
Fgf14	Fibroblast growth factor 14
Fgf17	Fibroblast growth factor 17
Fgfr1	Fibroblast growth factor receptor 1
Fgfr2	Fibroblast growth factor receptor 2
Fibp	Fibroblast growth factor intracellular binding protein
Frs3	Fibroblast growth factor receptor substrate 3
Spry1	Sprouty homolog 1 (Drosophila)
Spry2	Sprouty homolog 2 (Drosophila)
Spred2	Sprouty-related, EVH1 domain containing 2
Sp3	Trans-acting transcription factor 3
Tbx3	T-box 3
BMP-related[c]	
Id2	Inhibitor of DNA binding 2
Id3	Inhibitor of DNA binding 3
Tgfβi	Transforming growth factor, beta induced
Functional Cross Talk[d]	
Ascl1[*]	Achaete-scute complex homolog-like 1 (Drosophila)
Id1[*]	Inhibitor of DNA binding 1
Jun[*]	Jun oncogene
Neurod1[*]	Neurogenic differentiation 1

[a] A subset of the 63 genes identified is shown. The complete list is available in Supplementary Figure 1.

[b] Of the *Fgf* genes detected, only *Fgf10* was previously known to be expressed in the pituitary gland.

[c] The expression patterns of these were studied by in situ hybridization.

[d] The asterisk

[*] indicates genes in the basic helix-loop-helix family.