

Temporal regulation of a *paired*-like homeodomain repressor/TLE corepressor complex and a related activator is required for pituitary organogenesis

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Understanding the functional significance of the coordinate expression of specific corepressors and DNA-binding transcription factors remains a critical question in mammalian development. During the development of the pituitary gland, two highly related *paired*-like homeodomain factors, a repressor, *Hesx1/Rpx* and an activator, *Prop-1*, are expressed in sequential, overlapping temporal patterns. Here we show that while the repressive actions of *Hesx1/Rpx* may be required for initial pituitary organ commitment, progression beyond the appearance of the first pituitary (POMC) lineage requires both loss of *Hesx1* expression and the actions of *Prop-1*. Although *Hesx1* recruits both the Groucho-related corepressor TLE1 and the N-CoR/Sin3/HDAC complex on distinct domains, the repressor functions of *Hesx1* in vivo prove to require the specific recruitment of TLE1, which exhibits a spatial and temporal pattern of coexpression during pituitary organogenesis. Furthermore, *Hesx1*-mediated repression coordinates a negative feedback loop with FGF8/FGF10 signaling in the ventral diencephalon, required to prevent induction of multiple pituitary glands from oral ectoderm. Our data suggest that the opposing actions of two structurally-related DNA-binding *paired*-like homeodomain transcription factors, binding to similar cognate elements, coordinate pituitary organogenesis by reciprocally repressing and activating target genes in a temporally specific fashion, on the basis of the actions of a critical, coexpressed TLE corepressor.

[Key Words: Pituitary; transcriptional repression; *Hesx1/Rpx*; *Prop-1*; TLE1; N-CoR]

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Similar to developmental events in other organ systems, the transcriptional programs coordinating the commitment, patterning, and cell-type determination of the pituitary gland are associated intimately with the activities of localized signaling centers. The pituitary is derived from the midline of the anterior neural ridge (ANR), in which a series of transcription factors that are initially induced in the ANR remain present following the invagination of oral ectoderm to form Rathke's pouch, which will subsequently give rise to the pituitary gland (for review, see Sheng and Westphal 1999; Dasen and Rosenfeld 2001). Following Rathke's pouch formation, signaling events mediated by opposing dorsal FGF/

BMP4 and ventral Shh/BMP2 gradients impart positional and proliferative cues to the initially uniform pituitary progenitor field, acting in part through the induction of combinatorial patterns of transcription factor gene expression.

Genetic and biochemical studies have established the essential roles of multiple transcription factors in pituitary development, which appear to act in a sequential fashion to mediate the appearance of six hormone-producing cell types. These include homeodomain factors expressed in the anterior neural plate, including *Pitx1/2*, *Pax6*, and *Hesx1/Rpx*. Upon the invagination of oral ectoderm, the LIM homeodomain factor *Lhx3* is induced on embryonic day (E)9.5 in the nascent Rathke's pouch and is required for initial organ commitment and growth (Sheng et al. 1996). Subsequently, a second *paired*-like homeodomain factor, *Prophet of Pit-1 (Prop-1)* appears on E10.5, and is required for determination of four ventral cell types, including the Pit-1-dependent lineages

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(somatotropes, lactotropes, and thyrotropes) and gonadotropes (Sornson et al. 1996; Wu et al. 1998), with additional factors, including Pit-1, SF-1, and GATA-2 also required for cell-type specification within these four lineages (for review, see Dasen and Rosenfeld 2001).

The *paired*-like homeodomain factors Hesx1/Rpx and Prop-1 are expressed in spatially and temporally distinct and overlapping fashions throughout pituitary development. On the basis of the analysis of Ames dwarf (*df*) mice, which bear a hypomorphic mutation in the homeodomain of the *Prop-1* gene, and more severe human mutations, Prop-1 is required for the activation of gene programs required for the ventral proliferation and determination of four cell lineages. Hesx1, in contrast, is typical of developmentally critical transcriptional repressors and plays a broader role in the development of multiple placodally derived anterior structures including the eye, olfactory epithelium, forebrain, and pituitary (Dattani et al. 1998; Martinez-Barbera et al. 2000; Thomas et al. 2001). Attenuation of *Hesx1* expression in the developing pituitary coincides with the Prop-1-dependent progression of the pituitary, suggesting that the temporal regulation of *Hesx1* expression is essential for deployment of the Prop-1-dependent gene activation program (Gage et al. 1996; Hermes et al. 1996; Sornson et al. 1996).

The coregulatory apparatus that mediates Hesx1-dependent repression is unknown, although the nuclear receptor corepressor (N-CoR) has been linked to repression mediated by the homeodomain (Laherty et al. 1998; Xu et al. 1998). Another class of corepressors that have been linked to many homeodomain factors are the mammalian orthologs of the *Drosophila* protein Groucho (for review, see Chen and Courey 2000). Groucho is distantly related to the yeast corepressor Tup1, which binds several components of the core transcriptional apparatus, including Srb7, Srb10, Srb11, and Med6, as well as histone deacetylases (Kuchin and Carlson 1998; Gromoller and Lehming 2000; Watson et al. 2000; Wu et al. 2001). Both Groucho and Tup1 contain WD40 repeats, a well-characterized protein-protein interaction domain that has been linked to repression mediated by associations with DNA-binding proteins including transcription factors and histones (for review, see Chen and Courey 2000).

Here, we report that two related *paired*-like homeodomain factors, a Groucho-dependent repressor, Hesx1, and a transcriptional activator, Prop-1, exert temporally distinct reciprocal functions during pituitary development. Whereas Hesx1-dependent repression in a cell culture model system can involve the actions of either the N-CoR/mSin3/HDAC(1/2) and the Brg-1 complexes recruited to the homeodomain, recruitment of the coexpressed Groucho-like TLE1 corepressor by the Hesx1 N-terminal eh1 domain appears required and sufficient for the repressive actions of Hesx1 in vivo. A failure to extinguish expression of *Hesx1* and *TLE1* after E12.5 phenocopies the pituitary defects in *Prop-1*-defective mice, leading to pituitary dysmorphogenesis and loss of the Pit-1-dependent and gonadotrope lineages. Intriguingly,

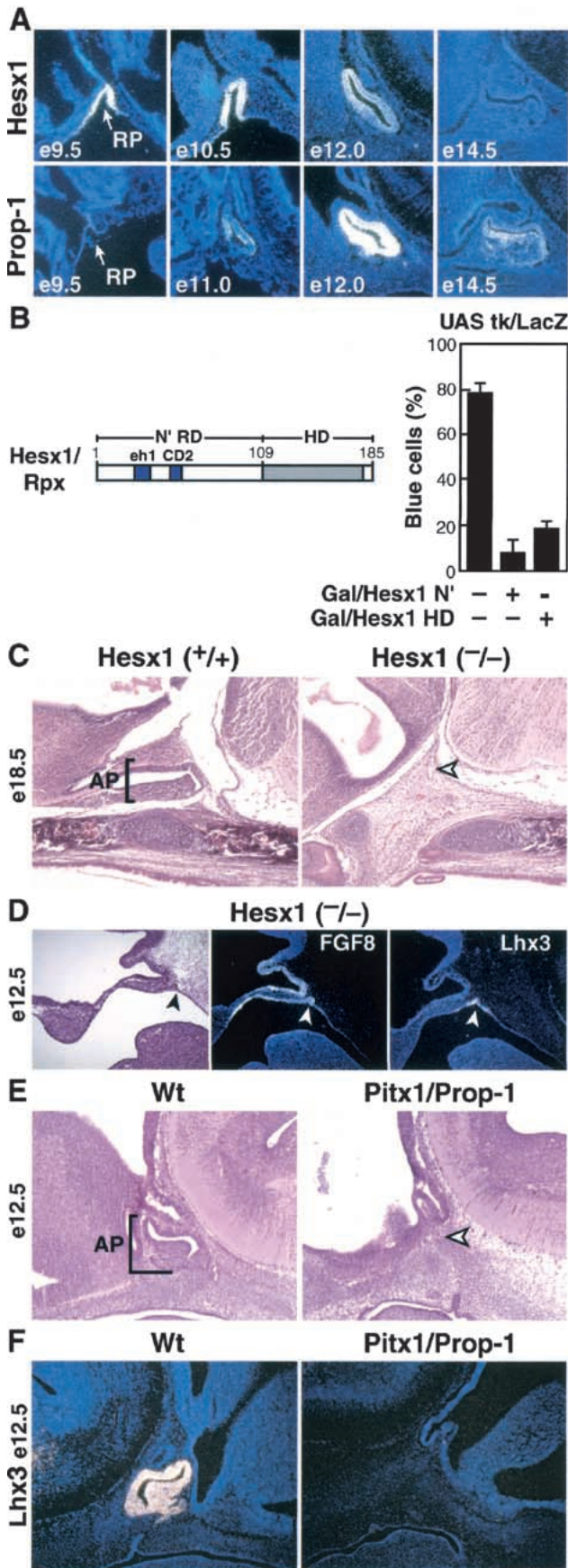
Hesx1 can also mediate cell nonautonomous effects on pituitary development by restricting *FGF8* and *FGF10* expression to discrete boundaries in the ventral diencephalon, without which multiple pituitary glands are ectopically induced in the oral ectoderm.

Results

Functional antagonism between Hesx1 and Prop-1 in pituitary organogenesis

The development of the pituitary gland appears to be coordinated by the sequential actions of a series of transcription factors, two of which, Prop-1 and Hesx1, belong to a family of *paired*-like homeodomain proteins sharing a conserved DNA-recognition site. The reciprocal expression and actions of Prop-1 and Hesx1 suggested that pituitary organogenesis might require their functional interactions. Subsequent to its induction in the anterior neural plate, *Hesx1* expression is maintained in the migrating oral ectoderm and invaginating Rathke's pouch, with selective expression in Rathke's pouch maintained until E13.5, but is excluded from the ventral rostral tip of the pituitary gland (Fig. 1A; data not shown). In contrast, *Prop-1* expression is undetectable until between E10.5 and E11, becomes maximal during the ventral migration of pituitary lineage precursors beginning at E12.5, and remains detectable between E14.5 and E15.5, after which its expression is maintained at low levels (Fig. 1A). Prop-1 and Hesx1 each bind to a well-described palindromic site (PrdQ; Wilson et al. 1993) as cooperative homodimers or heterodimers, with Prop-1 acting as an activator, but not as a repressor (Sornson et al. 1996; data not shown). Hesx1 acts only as a repressor in transient cotransfection assays and can inhibit Prop-1 activation function (Sornson et al. 1996; data not shown). Both the N-terminal and homeodomain (HD) regions of Hesx1 can act independently as repressors of the thymidine kinase (tk) promoter when evaluated as Gal4 fusions (Fig. 1B).

On the basis of the requirement for *Hesx1* in the development of anterior organs (Dattani et al. 1998; Martinez-Barbera et al. 2000; Thomas et al. 2001), we investigated defects in pituitary development in *Hesx1* gene-deleted mice. The most striking, although not frequent (~5% of *Hesx1*^{-/-} mice), phenotype was characterized by a complete lack of the pituitary gland. In this case, an initial thickening of oral ectoderm and minimal activation of *Lhx3* was observed at E12.5, but the pituitary gland was absent by E18.5 (Fig. 1C,D). In these mice, the ventral diencephalon appeared normal and expressed the normal FGF and BMP4 markers. Thus, it became of particular interest to investigate whether earlier expression of the related transcriptional activator, Prop-1, would, presumably by activation of Hesx1-repressed genes, be capable of phenocopying the pituitary arrest observed in *Hesx1*^{-/-} mice. Therefore, Prop-1 was targeted in transgenic mice under control of the 8-kb *Pitx1* regulatory sequences, which we have shown to be expressed only in oral ectoderm, Rathke's pouch, and first branchial arch



(Treier et al. 1998, 2001). Transgenic founders analyzed between E12.5 and E18.5 were found to exhibit entirely absent anterior pituitary glands, with no abnormalities of developing ventral diencephalon (Fig. 1E). Consistent with a loss of pituitary organ commitment, no initial induction of *Lhx3* expression was observed in Rathke's pouch at E12.5 (Fig. 1F). Thus, either absence of *Hesx1* or the premature presence of *Prop-1* serves to block pituitary organogenesis.

Multiple pituitary patterning and growth defects in Hesx1 mutant mice

In most cases of *Hesx1* gene deletion, pituitary development proceeds beyond formation of Rathke's pouch. In these less severe *Hesx1* mutants, the contact of the overlying neural epithelium of the ventral diencephalon with the dorsal part of the pouch proceeds normally from E8.5 to E11.5 (Fig. 2A). However, by E12.5, these mutants were characterized by the formation of multiple oral ectoderm invaginations, apparently reflecting pituitary glands, and were observed in most *Hesx1* embryos analyzed ($n > 30$) (Fig. 2A–C). Between E13.5 and E15.5, during the time in which pituitary lineages are sequentially generated, *Hesx1* mutants were also characterized by a dramatic cellular overproliferation, leading to a failure of the underlying mesenchyme to condense and form the sphenoid cartilage that separates the pituitary from the oral cavity. These two less severe phenotypes — the formation of multiple pouch invaginations and pituitary overproliferation — appeared to occur independently, as we have observed pituitary overgrowth in mice exhibiting a single pituitary invagination and delayed ventral proliferation in animals containing multiple Rathke's pouches (Fig. 2A,D; data not shown).

Figure 1. Opposing roles of *Hesx1* and *Prop-1* in initial phases of pituitary organogenesis. (A) *Hesx1* expression is detected in Rathke's pouch at mouse embryonic stage (e) 9.5 and is maintained until E12.5, after which its expression is rapidly extinguished. *Prop-1* expression, in contrast, initiates between E10.5 and E11 and is maintained throughout early pituitary development and attenuates beginning at E14.5. (B) The N terminus (N') and homeodomain (HD) of *Hesx1* act as separable repression domains when evaluated in nuclear microinjection experiments as Gal4 DNA-binding domain fusions on a UAS tk/*lacZ* reporter. Data represent the average of at least three independent experiments and error bars indicate the standard deviation from the mean. (C) Hematoxylin and eosin staining of midline sagittal sections showing that in the most severe class of *Hesx1* mutants, the anterior pituitary (AP) gland is absent at E18.5. Rostral is shown at left. (D) Earlier in development, there was a rudimentary pouch formed characterized by minimal *Lhx3* activation and ectopic *FGF8* expression in the oral ectoderm. (E) Analysis of *Pitx1/Prop-1* transgenic embryos showing that ectopic early activation by *Prop-1* phenocopies the loss of pituitary gland in the most severe class of *Hesx1* mutants. Embryonic founder mice analyzed at E12.5 are shown. Arrowheads indicate the region where the pituitary is normally found. No defects in the development of the infundibulum are observed. (F) *Pitx1/Prop1* mice also fail to induce *Lhx3* expression at E12.5.

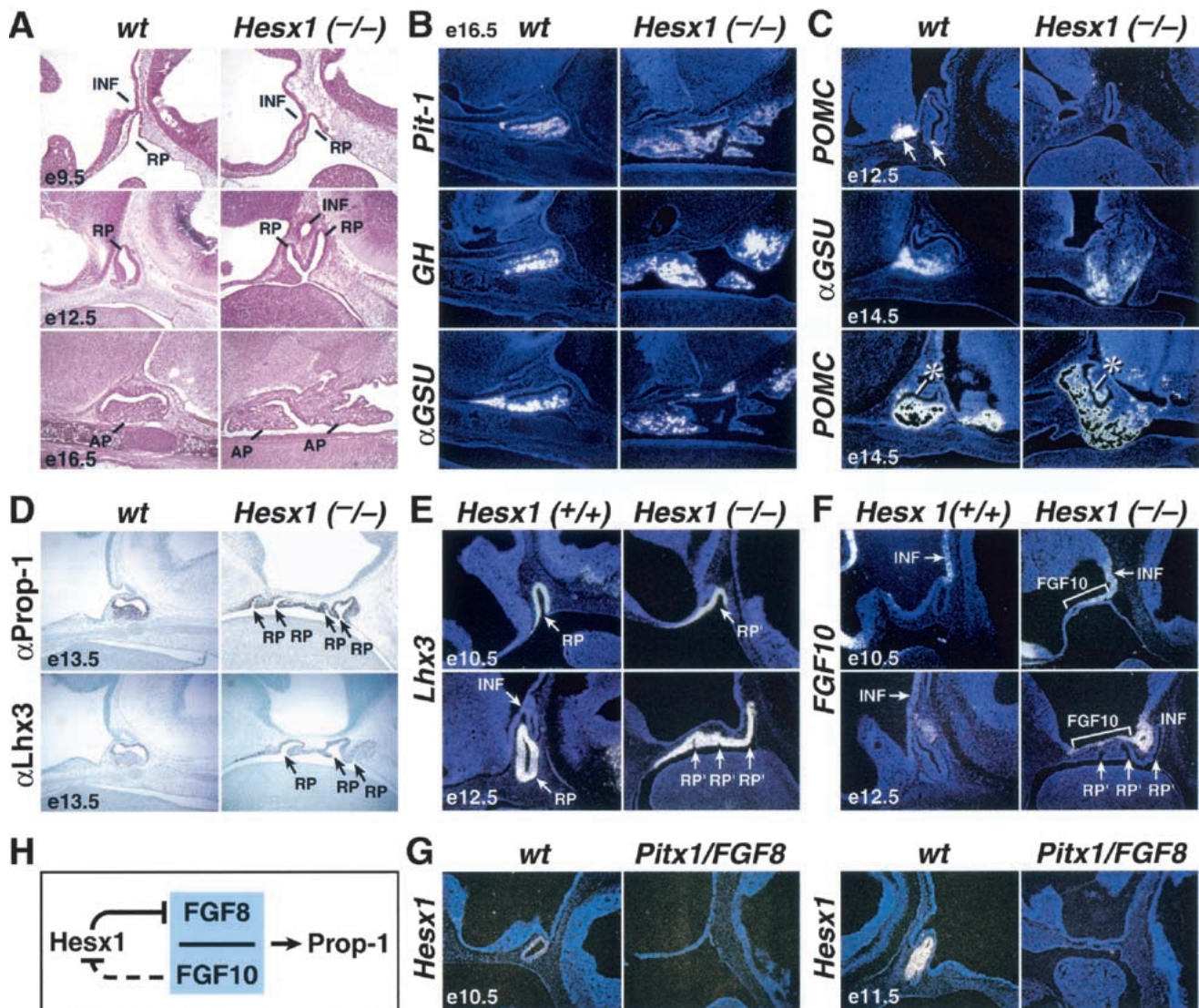


Figure 2. Pituitary dysmorphogenesis and patterning defects in other classes of *Hesx1* gene-deleted mice. (A) Hematoxylin and eosin staining of embryonic pituitary glands of *Hesx1* gene-deleted mice characterized by normal formation of Rathke's pouch (RP) at E9.5 and dysmorphogenesis beginning at E12.5. These classes of *Hesx1* mutant animals were characterized by formation of multiple pouch invaginations (E12.5 shown) or overproliferation of pituitary cells (E16.5 shown) into the sphenoid cartilage region. The morphology of the infundibular recess (INF) overlying Rathke's pouch was normal in these *Hesx1* mutants. (B) In situ hybridization showing that terminal differentiation of ventral pituitary cell lineages occurred normally in *Hesx1* mutant animals characterized by overproliferation as determined by normal expression of *Pit-1*, growth hormone (*GH*, expressed in somatotropes), and alpha glycoprotein subunit (α *GSU*, expressed in presumptive thyrotropes and gonadotropes) at E16.5. The relative proportion of cell types was greatly expanded in these *Hesx1* mutants. Asterisks, overexposed. (C) Expression of proopiomelanocortin (*POMC*, expressed in corticotropes and melanotropes) and α *GSU* in *Hesx1* mutants showing delayed appearance of *POMC* lineages at E12.5 and dual induction of α *GSU* and *POMC* (arrows) in the rostral and caudal regions of the ventral pituitary. (D) Immunohistochemistry of *Hesx1* mutant animals at E13.5, revealing that the additional invaginating oral ectoderm expressed the pituitary markers Prop-1 and Lhx3, indicating that the more rostral regions of oral ectoderm were being induced to form pituitary. (E) In situ hybridization ontogeny of *Lhx3* expression showing rostrally extended expression in most *Hesx1* mutant animals. (F) The extended expression domain of *Lhx3* correlated with extended expression domains in *FGF10* within the infundibulum. Similar results were obtained for *FGF8*. (G) Expression of *FGF8* inhibits *Hesx1* expression in *Pitx1/FGF8* transgenic mice. (H) Model for the reciprocal feedback regulation of FGF and *Hesx1* expression.

In the late stages of pituitary development, the terminal differentiation of the hormone-producing cell types appeared normal in most *Hesx1* mutants, with expression of α *GSU*, *TSH β* , *GH*, *POMC*, and *Pit-1* appearing by E16.5, indicating that cell lineages had differentiated

properly (Fig. 2B,C). The onset in the expression of most terminal markers appeared on a normal developmental schedule, but with consistently dramatic increases in numbers. Earlier in development, however, there was a delay in the onset of *POMC* expression both within

Rathke's pouch and the developing hypothalamus at E12.5, and there also appeared to be a dual induction of α GSU expression on both the rostral and caudal sides of Rathke's pouch (Fig. 2C). These data are consistent with Hesx1 also playing a general role in the early growth and patterning of the developing pituitary gland.

A negative feedback loop between Hesx1 and FGF8/10 establishes appropriate boundaries for pituitary organogenesis

The formation of multiple pouch invaginations in *Hesx1* mutants (Fig. 2A,D) suggested that additional oral ectoderm might be recruited to form pituitary. Consistent with this observation, analysis of *Hesx1* mutants revealed expanded expression domains of *Lhx3* and *Prop-1*, extending into more anterior regions of the oral ectoderm (Fig. 2D,E). Previous data have suggested pituitary organogenesis is initiated by inductive signaling originating from multiple signaling centers, including dorsal BMP4, FGF8, and FGF10 signals from the infundibular region of the ventral diencephalon, BMP2 from within the pouch and surrounding mesenchyme, and Shh from the ectodermal border between the pouch and oral ectoderm (Ericson et al. 1998, Treier et al. 1998, 2001). On the basis of previous studies showing that *Lhx3* expression can be regulated by FGF signaling (Ericson et al. 1998; Treier et al. 1998), we investigated potential defects in the expression domains of *FGF8* and *FGF10* in *Hesx1* mutants bearing multiple pouch invaginations. The expression domains of *FGF8* and *FGF10* in the infundibulum (Fig. 2F; data not shown), were expanded more rostrally, corresponding to the ectopic expression of *Lhx3* in the oral ectoderm (Fig. 2E), indicating that *Hesx1* is required for maintaining the proper domains of *FGF* expression. Furthermore, in the mutants characterized by severe pituitary defects, we observed *FGF* expression in the oral ectoderm (Fig. 1D), underscoring the requirement for Hesx1 function in defining *FGF* expression boundaries.

Because Hesx1 appears critical for maintaining boundaries of FGF expression and because increased infundibular FGF signaling correlates with *Hesx1* down-regulation, we were interested in exploring whether a negative feedback loop between *Hesx1* and *FGF8/10* expression might exist. Both *FGF8* and *FGF10* are initially expressed in the most dorsal aspect of the infundibulum, which makes contact with the dorsal tip of Rathke's pouch at E9.5. Between E12.5 and E14.5, as *Hesx1* expression attenuates, the domain of *FGF* expression begins to extend ventrally as the infundibulum makes contact with the dorsal wall of the pouch (Ericson et al. 1998; Treier et al. 1998). To investigate whether misexpression of a FGF would negatively regulate *Hesx1* expression earlier in development, we generated mice expressing *FGF8* under the control of the *Pitx1* regulatory sequences. In these mice, which express FGF8 in oral ectoderm (data not shown), *Hesx1* expression was completely absent in embryonic founders analyzed at E10.5 and E11.5 (Fig. 2G).

Thus, Hesx1 and FGF appear to exert reciprocal feedback regulation during pituitary development (Fig. 2H).

Hesx1 recruits the Groucho-related corepressor TLE1

The phenotypes of *Hesx1* mutants — the complete absence of the pituitary gland, the failure to restrict *FGF8/10* out of the rostral ventral diencephalon, and pituitary overgrowth — are consistent with Hesx1 acting in vivo as a transcriptional repressor. Attempts to delineate specific domains of Hesx1 required for transcription repression, provided that, under conditions of limiting plasmid concentrations, separation of the N terminus and homeodomain regions led to attenuated repression capacity, suggesting the presence of separable and cooperative repression domains (Fig. 1B). Comparison of Hesx1 sequences from several vertebrate species revealed the presence of two conserved motifs outside of the homeodomain, one of which showed similarity to the eh1 motif originally characterized in the *Drosophila* repressor engrailed (Smith and Jaynes 1996), whereas a second conserved motif showed similarity to the WRPW motif found in several bHLH proteins (Paroush et al. 1994). Because both of these motifs have been genetically and biochemically linked to the Groucho class of corepressors (Jimenez et al. 1997; Tolkunova et al. 1998), we determined whether TLE factors, mammalian counterparts of Groucho, mediate repression through the Hesx1 N terminus. We first investigated a physical association between Hesx1 and TLE proteins by combining HeLa cell nuclear extracts with Hesx1 affinity resin, finding a strong and specific interaction with TLE corepressors (Fig. 3A).

We performed in situ hybridization to analyze expression of the four mammalian Groucho homologs, TLE1–TLE4 (Stifani et al. 1992) between E9 and E16 of development, and found that expression of *TLE1* selectively overlaps with *Hesx1* during pituitary ontogeny (Fig. 3B). Thus, *Hesx1* and *TLE1* are coexpressed within Rathke's pouch between E9.5 and E12, but become rapidly extinguished beginning at E13.5, the time at which nascent anterior lobe pituitary cell types appear from Rathke's pouch. On the basis of the overlapping expression patterns of *TLE1* and *Hesx1* during pituitary ontogeny, we focused on TLE1 as the mediator of repression by the Hesx1 N terminus.

Coexpression of TLE1 and Hesx1 in heterologous 293T cells and immunoprecipitation against Hesx1 indicates a robust physical interaction in vivo (Fig. 3C). Furthermore, this interaction proved to be specific, as we did not detect interactions with other pituitary-restricted factors such as Pit-1, Prop-1, and GATA-2, or surprisingly, the eh1 domain-containing homeodomain repressor, Msx1 (Fig. 3D), for reasons that are not clear. In transfection-based assays, TLE1 supported transcriptional repression of the tk promoter by Hesx1 on the cognate PrdQ-binding site (Fig. 3E). Whereas Hesx1 alone could only partially abrogate the activity of Prop-1 on the PrdQ site, the presence of TLE1 completely abolished transcriptional activation mediated by Prop-1 (Fig. 3E). Furthermore, on

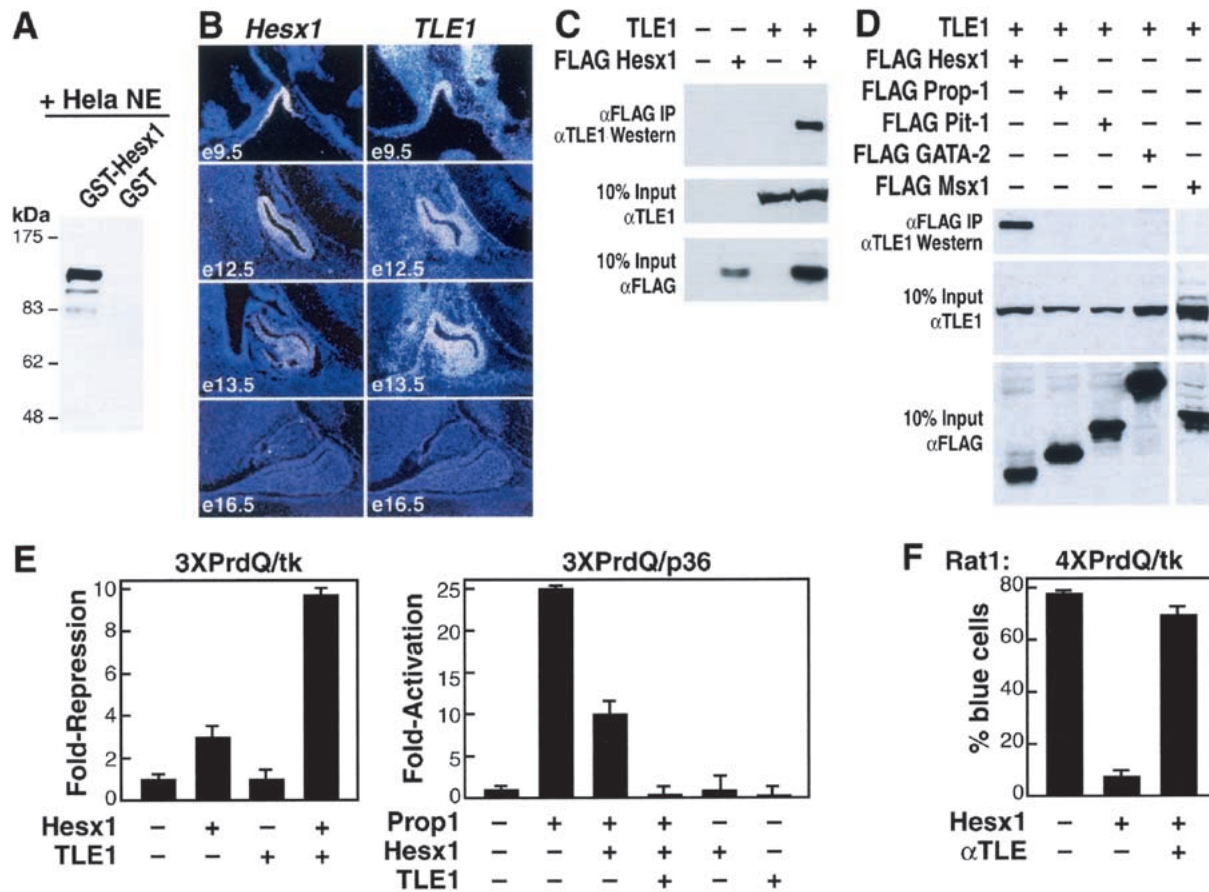


Figure 3. Expression and physical interactions of TLE1 and Hesx1 during pituitary development. (A) Hesx1 binds TLE family corepressors. GST-purified HeLa nuclear extracts (750 μ g) were combined with a full-length GST/Hesx1 fusion protein and tested for the presence of TLE using a pan-TLE antibody. (B) In situ hybridizations showing expression of *TLE1* overlaps with *Hesx1* during pituitary early ontogeny and is attenuated by E16.5. (C) Specific interactions between Hesx1 and TLE1 mediate transcriptional repression. FLAG epitope-tagged CMV-Hesx1 and CMV-TLE1 were expressed in 293T cells and coimmunoprecipitation was performed using anti-FLAG antibody. The presence of TLE1 was tested by use of a rabbit polyclonal TLE1 antibody. (D) No interactions with TLE1 were detected after immunoprecipitation with FLAG-tagged Prop-1, Pit-1, GATA2, or Msx1. (E) TLE1 mediates repression by Hesx1 and inhibition of Prop-1 function. TLE1-pcDNA3 (50 ng) supported repression by Hesx1-pCMX (50 ng) on the 4XPrdQ/tk reporter and also abolished Prop-1-dependent transcriptional activation of the 3XPrdQ/p36 reporter. (F) Nuclear microinjection in Rat1 cells showing the antibodies direct against TLEs can block repression mediated by Hesx1 on a 4XPrdQ/tk reporter.

the PrdQ/tk promoter, microinjection of α TLE IgG blocked repression mediated by Hesx1 (Fig. 3F). Together, these data suggest that Hesx1 can recruit TLE1 to specific promoters, leading to direct transcriptional repression as well as inhibition of the activity of other paired-like heterodimeric partners such as Prop-1.

An evolutionarily conserved interaction mediates TLE1-dependent repression by Hesx1

We further explored the mechanisms of TLE-dependent repression, focusing specifically on the mechanism by which TLE1 supported repression of Hesx1 and abrogated transcriptional activation mediated by Prop-1. Because recruitment of Groucho-class corepressors can be mediated through both the eh1 and WRPW motifs, we investigated the Hesx1 interaction domain. Using bacte-

rially expressed domains of Hesx1, we mapped the interaction interface to its N terminus, a region containing both of these motifs (Fig. 4A,B). Generation of point mutations within the two conserved motifs revealed the specific requirement for the eh1 domain for the Hesx1/TLE1 interaction in vivo (Fig. 4C). By generating fusions of Hesx1 fragments to the heterologous Gal4 DNA-binding, TLE-dependent repression of a UAS/tk promoter proved to be mediated through the N terminus of Hesx1, not the homeodomain (Fig. 4D). Furthermore, mutation in the eh1 domain, but not the second conserved motif, abrogated TLE1-mediated repression by the N terminus as well as repression by Gal4/hoHo-Hesx1 (Fig. 4E,F). Together, these data show that TLE1-dependent repression by Hesx1 is supported by a specific interaction with the eh1 domain.

We mapped the physical interaction on TLE1 in vivo and found that both the N-terminal tetramerization (Q

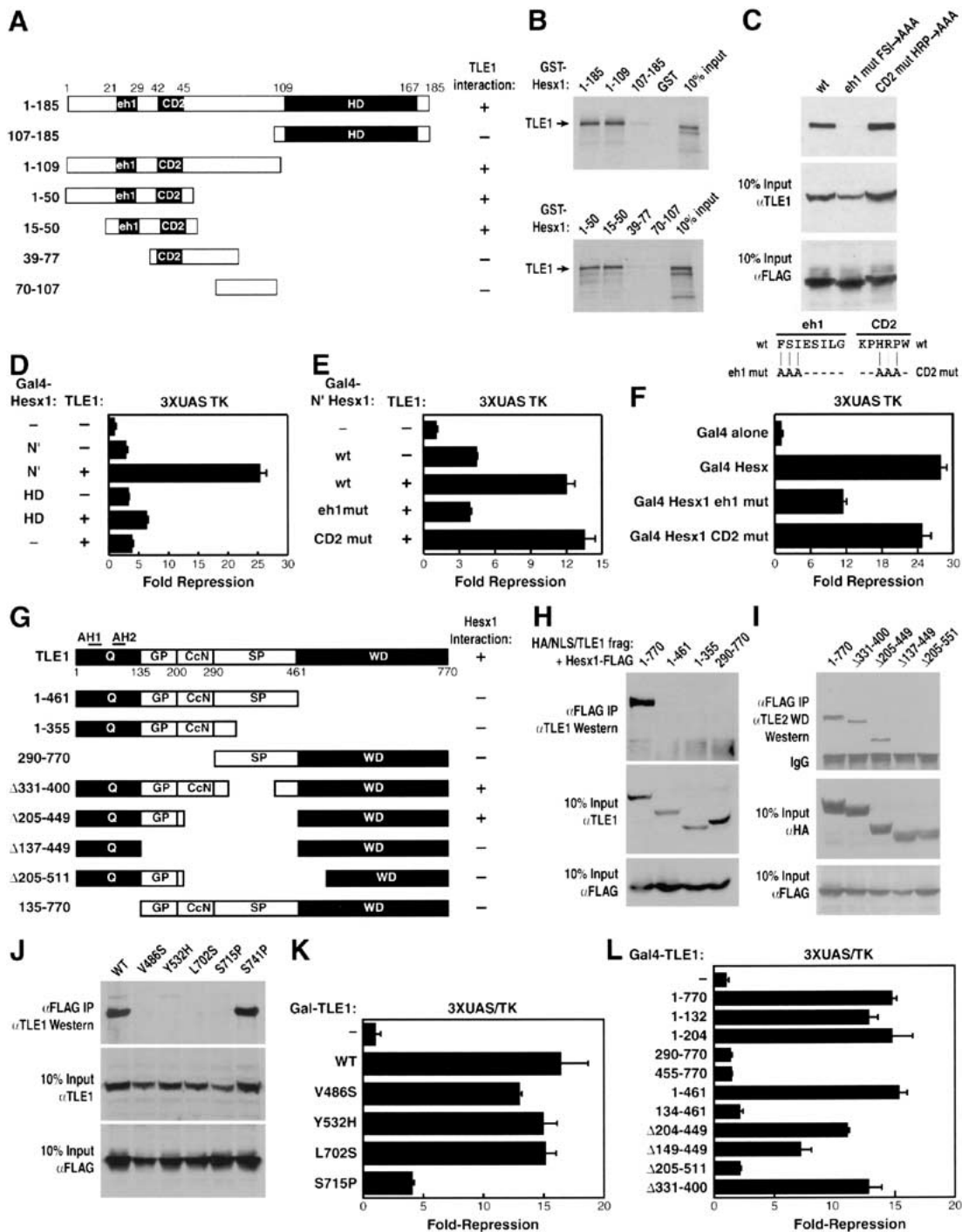


Figure 4. Conserved functions of eh1 domain/WD40 domain interactions in mediating transcriptional repression. (A) Schematic representations of Hesx1 fragments expressed as GST-fusion proteins. (B) ^{35}S -labeled full-length TLE1 was combined with bacterially expressed GST-fusions of Hesx1 and analyzed for interaction after SDS-PAGE and film exposure. TLE1 bound to an N-terminal region containing the conserved N-terminal amino acid residues. (C) Point mutation in the eh1 domain but not the second conserved domain (CD2) of Hesx1 were tested for TLE1 interaction by immunoprecipitation and revealed a specific requirement for the eh1 domain. (D) Repression by TLE1 is mediated through the Hesx1 N terminus. Gal4 fusions of Hesx1 domains were tested for repression on a 3XUAS/tk luciferase reporter in the presence or absence of TLE1. (E-F). Mutation in the eh1 domain of Hesx1 abrogated repression by TLE1 by both the N terminus and repression of full-length Hesx1-Gal4 fusion. (G) Schematic representations of TLE1 domains. (H-I) Coexpression of FLAG-Hesx1 and TLE1 subdomain expressed as HA tagged/nuclear localization signal (NLS) fusion proteins were tested for interaction by coimmunoprecipitation assays. (Right) An antibody directed against the conserved WD40 domain of TLE2 was used. (J) Point mutations in the WD40-repeat region of TLE1 revealed that residues conserved in Tup1 are required for Hesx1 interaction. (K) Nonstructural point mutations in TLE1 had no effect on the ability of Gal-TLE1 to repress. (L) Gal4 fusions of TLE1 subdomains revealed a requirement for the GP and WD40 domain in transcriptional repression.

and GP) domain and WD-repeat region were required for the interaction (Fig. 4G–I). On the basis of analogy to interactions between Tup1 and the MAT α 2 homeodomain, we focused on the analysis of the WD40 region as potentially forming the interaction surface. We generated point mutations in the WD40 domain within conserved or analogous residues between TLE1 and Tup1, which, in Tup1, have been shown to be required for the interaction of the MAT α terminal peptide and the channel surface formed by the propeller blades of the WD40 domain (Sprague et al. 2000). Mutation in four of the five mutated residues tested in these assays abolished Hesx1/TLE1 interactions (Fig. 4J). Three mutations (V486S, Y532H, and L702S) are predicted to maintain the overall structural integrity of the WD40 repeat, whereas the S715P mutation is predicted to disrupt the overall structure (Komachi et al. 1997; Sprague et al. 2000).

To determine which domains in TLE1 were required for transcriptional repression, we generated Gal4 fusions of the WD40 point mutations as well as subdomains of TLE1. Fusions of the nonstructural point mutations in the WD40 repeat had no effect on the ability of TLE1 to repress a UAS/tk reporter, whereas the structural S715P mutation consistently attenuated TLE1-mediated repression (Fig. 4K). The Q tetramerization domain was sufficient for repression, likely due to recruitment of endogenous TLE proteins (Chen et al. 1998; Ren et al. 1999). Gal4 fusions of TLE1 subdomains demonstrated that, within the context of the TLE tetramerization motif, full transcriptional repression required the GP and WD40 domains (Fig. 4L). Thus, as in the case Tup1, the repression and interaction domains appear to be separable, although the WD40 repeat appears to be required for aspects of both functions.

Regulation of Hesx1 by distinct corepressor complexes

On the basis of our previous observations that many homeodomain proteins, including Hesx1, require N-CoR and associated corepressors for transcriptional repression (Xu et al. 1998), we explored a potential interface between the N-CoR complex and the Groucho class of corepressors. We initially investigated whether N-CoR might, at least under certain cellular conditions, be required for Hesx1-mediated repression in vivo. In quiescent Rat-1 cells, both N-CoR and TLE1 were required for repression by Gal4/Hesx1, as microinjection of antibodies against at either factor significantly relieved repression of a UAS/tk reporter (Fig. 5A).

Both N-CoR and Groucho have been reported to bind class I histone deacetylases (HDACs), but because Groucho has been shown to bind the HDAC dRpd3 through its GP domain (Chen et al. 1999), an interaction domain poorly conserved in vertebrate TLEs, and because we were unable to find detectable HDAC/TLE interactions (data not shown), we investigated the possibility that any HDAC interaction with Hesx1 might involve indirect recruitment. HeLa nuclear extracts were combined with Hesx1 affinity resin consisting of either

the N terminus, homeodomain, or full-length protein, and following washing and elution, tested for the presence of components of known repressor complexes. As expected, we found that the N terminus was effective in recruitment of TLE corepressors in HeLa cells (Fig. 5B). The Hesx1 homeodomain was required and sufficient to recruit components of N-CoR associated corepressors, including Sin3A/B and HDAC1 and HDAC2, but did not interact with TLE proteins (Fig. 5B). Although the homeodomain was sufficient to mediate N-CoR/HDAC interactions, the presence of the N terminus significantly enhanced binding of N-CoR and HDAC1, and to a lesser extent, Sin3A/B (Fig. 5B). Furthermore, we also found that whereas Mi2, MTA1, and HDAC3–HDAC6 were not recruited, components of the mammalian SWI/SNF complex (Brg1) and RbAp46, were found in the homeodomain-purified fraction (Fig. 5B). Consistent with these data, the eluates from the Hesx1 homeodomain column contained TSA-sensitive HDAC activity (data not shown).

On the basis of these data, we wished to evaluate the functional importance of these components in actions of the Hesx1 N' and HD repression domains. Repression by the N-terminal domain of Hesx1 was not inhibited by α HDAC1–HDAC6, α Mi2, α Sin3, α Brg1, α BAF57, α BAF55, or α BAF110 IgGs, but was inhibited by α TLE-1 IgG, indicating that it alone could serve as a component required for Hesx1-dependent repression (Fig. 5C; data not shown). To further evaluate this issue, we examined repression by the Hesx1 holoprotein, or the Hesx1 eh1 mutant form that is incapable of interacting with TLEs. With wild-type protein, TLE was required for repression, whereas HDAC1–HDAC6, N-CoR, Brg1, and BAF activities were not required (Fig. 5D). In contrast, in the absence of the TLE interaction domain (Hesx1 eh1 mutant), repression was still achieved, but now required the actions of HDAC2, HDAC5, and Brg-1, as well as BAF57, BAF155, and BAF110 (Fig. 5E; data not shown). On the basis of these data, we conclude that, whereas the TLE-dependent complex may recruit substoichiometric amounts of deacetylases, HDAC1–HDAC6 are not required for TLE-mediated repression. In contrast, repression can be mediated by the Hesx1 homeodomain, requiring both specific HDACs and the actions of the Brg1 complex.

Attenuation of Hesx1 and TLE1 is required for Prop-1-dependent specification of four pituitary cell lineages

The recruitment of N-CoR, Sin3A/B, HDAC1, and HDAC2, as well as TLE to Hesx1 raised the question of whether either complex is critical for the biological functions of Hesx1 within the pituitary. The ability of Hesx1/TLE1 interactions to inhibit the activity of Prop-1, and the requirement for Prop-1 in the determination of four anterior pituitary cell types, initially suggested that the spatially and temporally restricted coexpression patterns of *Hesx1* and *TLE1* expression might be a critical component of normal pituitary organogenesis. In an at-

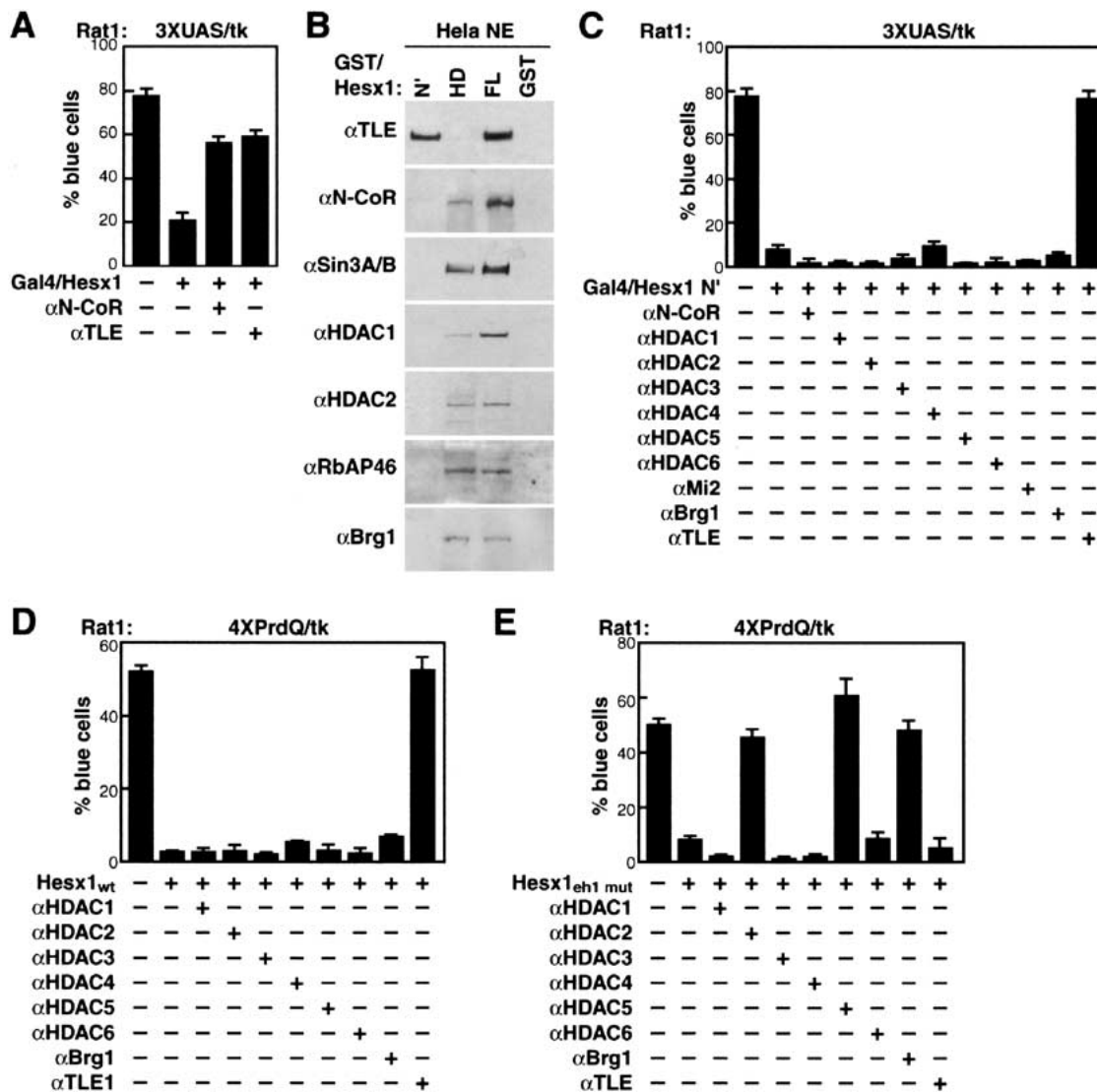


Figure 5. Cooperative interactions of TLE1 and N-CoR and recruitment of Brg1 mediate repression by Hesx1. (A) In Rat 1 cells, on a UAS/tk promoter using Gal4/Hesx1, both N-CoR and TLE1 are required components of Hesx1-mediated repression. Antibodies against TLE1 and N-CoR were microinjected into the nuclei of Rat-1 cells and tested for the ability of Gal4/Hesx1 to repress a UAS/tk *lacZ* reporter. (B) Coordinate recruitment of distinct corepressor proteins by the Hesx1 homeodomain and N' terminus. Affinity chromatography using Hesx1 N', homeodomain, or holoprotein was performed with HeLa nuclear extract, after washing and elution, equivalent aliquots were fractionated by SDS-PAGE and used for Western blot analysis. (C) TLE is required for repression by Gal4/Hesx1 N' on a UAS/tk reporter. (D) TLE is required for repression by the Hesx1 wild-type protein on a 4× response element-dependent reporter. (E) Repression by the Hesx1 eh1 mutant on the identical reporter was TLE independent, but was blocked by αBrg1, αHDAC2, and αHDAC5. In single-cell nuclear microinjection experiments, ~300 cells were injected for each point; M ± SEM; results were repeated in these independent experiments.

tempt to address the functional significance of TLE1 and Hesx1 actions in pituitary development, we generated transgenic mice expressing *Hesx1* under the control of regulatory sequences the *Pitx1* gene, which would target misexpression to all ventral pituitary cell types, or the α*GSU* gene, which would maintain *Hesx1* expression in specific cell lineages through later stages of development. In these transgenic animals we observed only minimal phenotypes characterized by a modest reduction in some cell lineages (Fig. 6A,B). In light of the temporally restricted patterns of *TLE1* expression, we next

evaluated the effects of *TLE1* expression alone, finding all cell types present (data not shown).

On the basis of these results, we next addressed whether maintained coexpression of both *TLE1* and *Hesx1* would be sufficient to inhibit the Prop-1-dependent determination program. We therefore generated transgenic animals, temporally maintaining expression of both *Hesx1* and *TLE1* under control of regulatory sequences of the *Pitx1* gene, which targets expression in the oral ectoderm, and then to progenitor cell types in developing Rathke's pouch. These DNAs were coin-

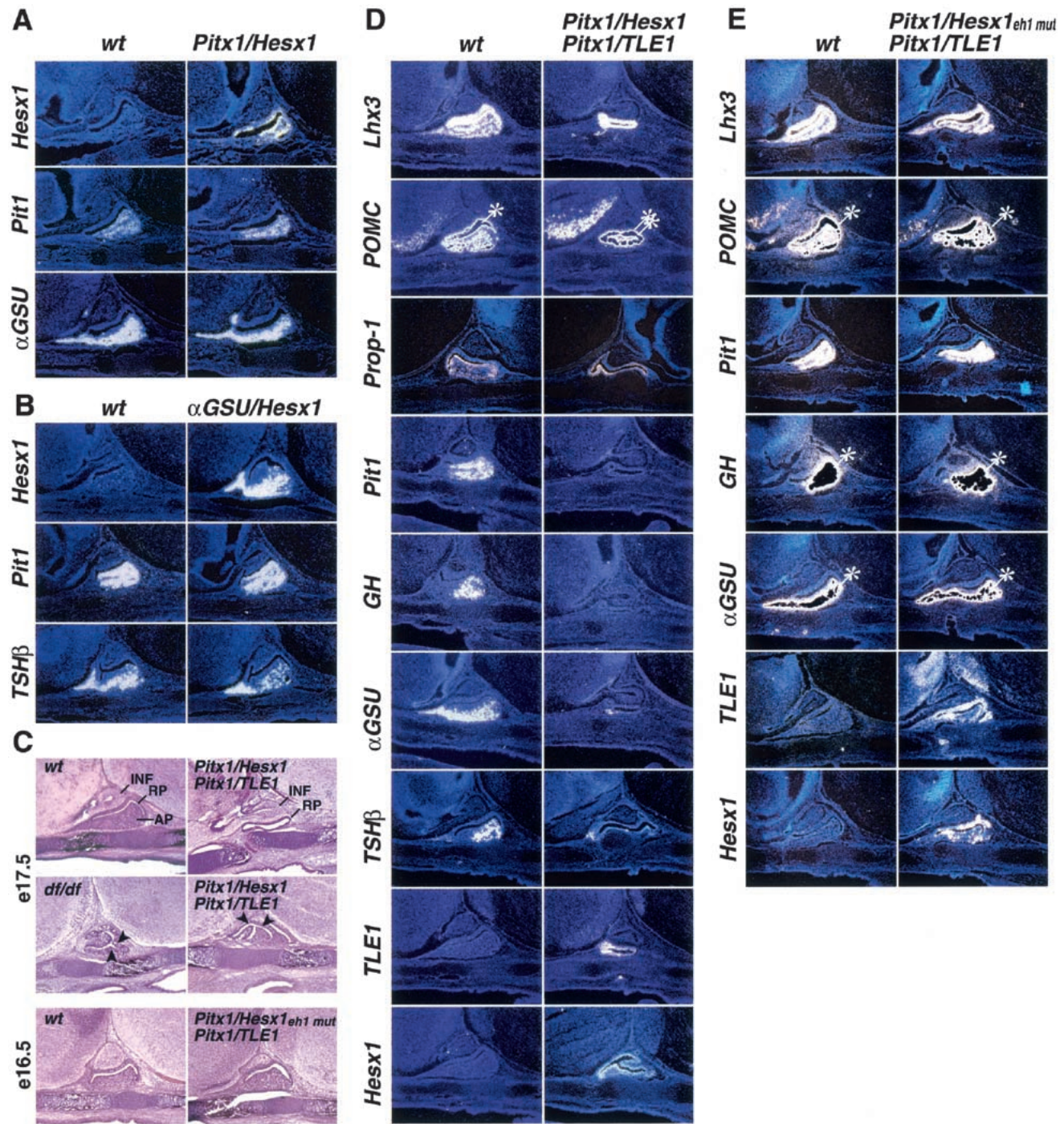


Figure 6. Temporal misexpression of *Hesx1* and *TLE1* abrogates the *Prop-1*-dependent ventral proliferation and determination of four pituitary cell types. (A,B) Expression of *Hesx1* under the control of regulatory sequences of the *Pitx1* and α *GSU* genes has no adverse effects on the appearance of pituitary cell types analyzed in E16.5 founder animals. (C) Hematoxylin and eosin staining showing the loss of ventral pituitary cell types of *Pitx1/Hesx1:Pitx1/TLE1* double-transgenic embryonic founders and similarity to pituitary defects in Ames *df/df* mice. (D) In situ hybridization analysis of *Pitx1/Hesx1:Pitx1/TLE1* double-transgenic founder mice at E16.5 showing loss of *GH*, *Pit-1*, *Prop-1*, α *GSU*, and *TSH β* expression, although expression of *POMC* appears to be unaffected near the lumen of Rathke's pouch. *Lhx3* continues to be expressed in the pouch ectoderm, whereas expression of *Prop-1* and *FGF10* were unaffected (data not shown). (E) Mutation in the *eh1* domain of *Hesx1* reverts the phenotypes of *TLE1* coexpression as determined by normal expression of pituitary markers in *Pitx1/Hesx1^{eh1 mut}/Pitx1/TLE1* transgenic mice.

jected into embryos and allowed to concatenate in vivo. *Pitx1/Hesx1:Pitx1/TLE1* double-transgenic embryos were characterized by the near-complete absence of all ventral anterior pituitary cell types (Fig. 6C,D). Rathke's pouch appeared to have formed normally in these animals and continued to express early determination markers such as *Lhx3* (Fig. 6D). In some transgenic animals (~30%), a dysmorphogenesis of Rathke's pouch was observed, characterized by bifurcation of Rathke's pouch ectoderm and dysmorphogenesis, remarkably similar to the pituitary dysmorphogenesis (Gage et al. 1996; Sornson et al. 1996) of *Prop-1*-defective *Ames* dwarf mice (Fig. 6C).

Analysis of the expression of pituitary cell type markers in *Pitx1/Hesx1:Pitx1/TLE1* mice revealed the complete absence of Prop-1-dependent cell lineages as determined by loss of expression of the *Pit-1*, *GH*, *TSH β* , and α *GSU* genes (Fig. 6D). Expression of *Prop-1* in the pituitary and *FGF10* in the ventral diencephalon was unaffected, consistent with this phenotype occurring as a result of the cell-autonomous inhibition of Prop-1 activation function (Fig. 6D; data not shown). Expression of *POMC*, a marker for the Prop-1-independent melanotrope lineage, as well as the corticotrope lineage, appeared normal in the dorsal, melanotrope cell field. Remarkably, the ventral aspect of Rathke's pouch now expressed a uniform field of *POMC* in a manner similar to that in the intermediate lobe of the pituitary gland.

To further test whether the effects of maintained *TLE1* and *Hesx1* expression on pituitary development required a specific interaction between these proteins, we generated a mutation in the eh1 domain that abolished TLE1 interaction within the context of the *Pitx1/Hesx1* transgene and then coinjected it with the *Pitx1/TLE1* expression cassette. These animals robustly expressed both transgenes, but exhibited no defects in the appearance of pituitary cell types nor altered expression of pituitary determination markers (Fig. 6E). This is consistent with the requirement of specific interactions between TLE1 and Hesx1 as the critical repressor complex serving to prevent the appearance of the Pit-1 and gonadotrope lineages.

Discussion

The sequential actions of transcriptional repressors and activators on overlapping sets of gene targets, in concert with requisite coregulatory machinery, is likely to be a central strategy in mammalian organogenesis. Here, we explored the opposing roles of two highly-related *paired*-like homeodomain factors, Hesx1/Rpx and Prop-1, that exhibit temporally distinct, but overlapping patterns of expression over the entire period of pituitary organ commitment, patterning, and cell-type determination. Our data suggest that Hesx1, although clearly modified by the actions of linked modifier genes on the basis of genetic background, is required for early organ commitment and cell determination events. These actions extend temporally to include the appearance of the dorsal POMC lineage, which then, with a specific coexpressed

corepressor, Hesx1, serves to prevent Prop-1 from initiating the program required for asymmetric division and proliferation of the Pit-1 and gonadotrope lineages (Fig. 7). Premature expression of Prop-1 can block pituitary organogenesis, phenocopying the effects of *Hesx1*-gene deletion, suggesting that the switch of binding of a *paired*-homeodomain repressor for a *paired*-homeodomain activator, with resultant alteration in the expression of key target genes, now prevents organogenesis. Conversely, expression of Hesx1 with the obligate corepressor TLE1 can block the activation of Prop-1-dependent genes required for the appearance of four anterior pituitary cell types. This provides a striking example of a potent strategy in mammalian organogenesis, in which opposing actions of related repressors and activators, putatively binding to overlapping sets of gene targets, provide critical temporal control of organ development. Interestingly, later persistent expression of *Prop-1* under control of the α GSU promoter caused decreased gonadotrope differentiation and caused increased adenomatous hyperplasia (Cushman et al. 2001).

A Hesx1/FGF regulatory loop in boundary formation

In addition to its early and later roles in pituitary organogenesis and cell type determination, analysis of *Hesx1*^{-/-} mice have also revealed an intriguing regulatory loop. Early in development, *Hesx1* is expressed in a broad region of the anterior neural plate that will later give rise to the ventral diencephalon and pituitary. Deletion of the *Hesx1* gene causes a rostral extension of *FGF8* and *FGF10* expression in the ventral diencephalon, into an area that transiently expresses *Hesx1*, leading to ectopic *Lhx3* induction and formation of supernumerary pituitary glands, confirming that FGF8/FGF10 signaling is required and sufficient to signal pituitary commitment from oral ectoderm (Treier et al. 2001). Further, our data showing that FGF8 suppresses *Hesx1* gene expression indicates a negative regulatory loop with *Hesx1* acting early to repress *FGF8/FGF10*, which in turn, directly or indirectly, represses *Hesx1* gene expression at the time of the emergence of pituitary cell types from Rathke's pouch. Thus, a *paired*-like homeodomain repressor serves to establish boundaries of *FGF8/10* gene expression in the ventral diencephalon and thus restricts the spatial domains at which pituitary organogenesis can occur.

Together, our data suggest that Hesx1 can exert both cell-autonomous and noncell-autonomous roles in pituitary development. Early in development, Hesx1 is required for restricting and maintaining the proper expression domains *FGF8* and *FGF10*, consistent with its putative role as a repressor in the anterior neural plate, which establishes boundaries of morphogen expression. Later in development, after its expression becomes restricted to Rathke's pouch between E9 and E12, Hesx1 is required for regulating the appropriate ventral proliferation patterns of pituitary progenitor lineages. These observations are based on the analysis of *Hesx1* mutants, in which the pituitary did not exhibit defects in the ventral

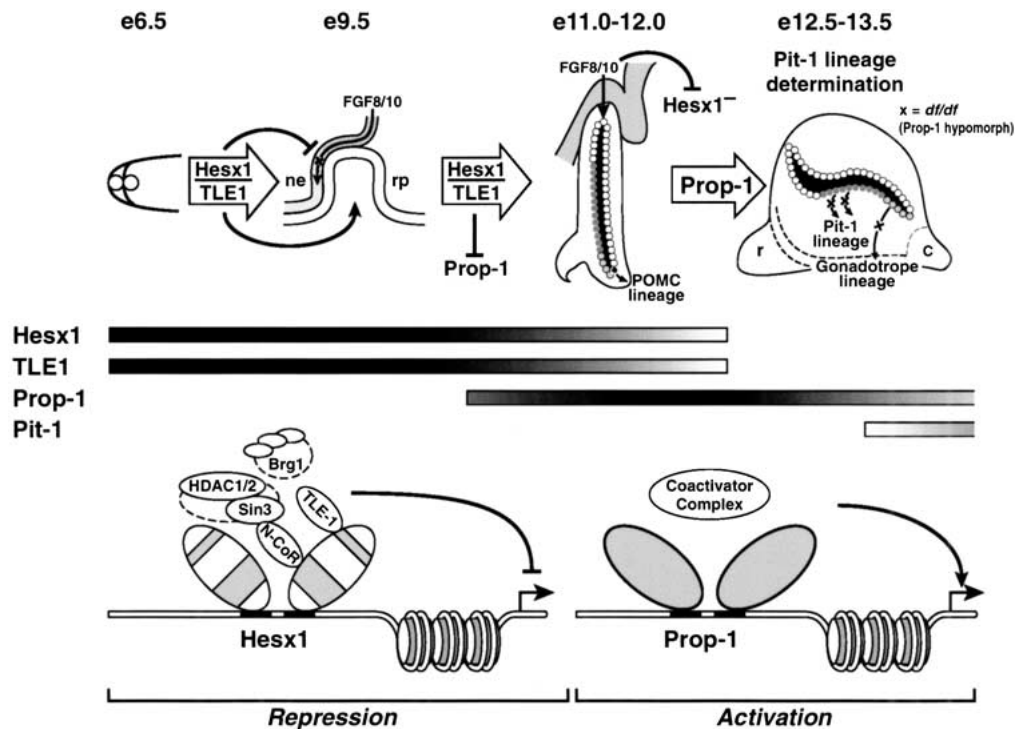


Figure 7. Model for the temporal switch of a homeodomain repressor for an activator, mediated by the coordinate recruitment of distinct corepressor complexes in pituitary organogenesis. In the anterior neural plate, *Hesx1* is required for restricting the domain of *FGF8* and *FGF10* expression to distinct regions of the ventral diencephalon and is later required for the inhibition of *Prop-1* function to temporally control the proliferation and determination of four pituitary cell lineages. Conversely, FGF signaling down-regulates *Hesx1* expression, necessary for the deployment of the *Prop-1* gene program. *Hesx1* requires the recruitment of coordinately expressed *TLE1*, but also recruitment of N-CoR, and Brg1 complexes, to inhibit *in vivo* activation events mediated by *Prop-1*.

diencephalon, but continued to proliferate, and are further supported by the *in vivo* effects of maintained *TLE1* and *Hesx1* expression.

TLE1-mediated recruitment of repressor function to Hesx1 in pituitary development

We have provided evidence *in vivo* that *Hesx1* contains two repressor domains, located in the N-terminal and homeodomain regions of the protein, each recruiting a distinct corepressor complex. The component recruited by the N terminus includes one mammalian Groucho ortholog, *TLE1*, that appears to be required for *Hesx1*-dependent repression, both in cell culture models, in which anti-*TLE1* IgG blocks *Hesx1* repression activity, and *in vivo*, in which coexpression of both *Hesx1* and *TLE1* are sufficient to phenocopy the effects of *Prop-1* gene deletion. The strong association between *TLE1* and *Hesx1* is mediated by a highly conserved helical motif (FXLXXIL), which is present in the *Hesx1* N terminus and selectively recruited by purification from the nuclear extracts, as well as by coimmunoprecipitation assay in cells, analogous to interactions of *Nkx*, *Six*, and certain *Pax* homeodomain factors with other *TLE* family members (Eberhard et al. 2000; Muhr et al. 2001). The inability of the eh-1 domain-containing repressor *Msx-1*

to bind *TLE1* suggests specificity codes between homeodomain repressors and specific *TLE* corepressors may exist.

In the case of *Hesx1*, our data suggest that the N-CoR/Sin3/HDAC(1/2) complex can also be recruited to the *Hesx1* homeodomain. However, the component required for effective repression function appears to be dependent on cellular and promoter context. The homeodomain region is alone capable of mediating repression, requiring a corepressor complex that includes mSin3A/B, HDAC1, and HDAC2, suggesting recruitment of the mSin3 complex, but not of the high-affinity N-CoR/TBL1/HDAC3 complex (Guenther et al. 2000; Underhill et al. 2000) to the *Hesx1* homeodomain. Intriguingly, the *Hesx1* N-terminal domain-binding *TLE1* permits cooperative binding of N-CoR, HDAC1, and Sin3A/B to the homeodomain, a strategy that might be quite common with respect to many homeodomain repressors, and could provide a linkage between one class of corepressors — the *TLEs* — and the N-CoR/mSin3 complexes in the actions of a strong homeodomain repressor.

The molecular basis of actions of the mammalian *TLE* orthologs of *Tup1* and *Groucho* are still not fully clarified. In yeast, the *Tup1/SSN6* complex has been suggested to contact components of core machinery including *Srb7*, *Srb10*, *Srb11*, and *Med6*. However, we did not

find comparable direct associations between TLE1 and the mammalian counterparts of these highly conserved RNA polymerase-associated proteins (J. Dasen and M. Rosenfeld, unpubl.). Genetic approaches actively link some Tup1 repression events to the yeast homologs of class I (Rpd3) and class II (HDA1) HDACs, and *Drosophila* Groucho can interact with dRpd3 (Kuchin and Carlson 1998; Gromoller and Lehming 2000; Watson et al. 2000; Wu et al. 2001), however, we also do not find high-affinity interactions between TLE1 and mammalian HDAC1–HDAC6. Although HDACs may be recruited to TLE1 in a substoichiometric fashion, we suggest that cooperative recruitment of HDAC complexes to the homeodomain may be mediated through a TLE1-dependent mechanism. TLE1 contains the well-characterized WD40 repeat, which we have documented to be required for interactions with Hesx1, including conserved residues used for protein–protein interactions. The domains N-terminal to the WD40 repeats are critical for repression by TLE1; therefore, it is possible that a complex containing several components is required for TLE1 actions.

Whereas the SWI/SNF complex was initially identified as a critical component of HO gene activation events, subsequent genetic studies have revealed that activation of components of this complex have effects on both gene activation and repression (for review, see Sudarsanam and Winston 2000); one initial suggestion was that at least some repression events reflected activation of gene-specific repressors. Our data concerning Hesx1 supports the idea that components of the mammalian SWI/SNF complex (including Brg-1, BAF 51, BAF155, and BAF170) can be recruited and might serve as a required component of repression in cases in which the HDAC-dependent machinery recruited by the homeodomain is required. Recent biochemical purification of the Brg1 complex revealed the presence of Sin3A/B and HDAC1 (Sif et al. 2001). This is consistent with a model in which chromatin remodeling is an important component of both repression and activation, and we suggest that, as was found to be also required for other classes of repressors including nuclear receptors, this complex exerts its role in repression by recruiting many classes of DNA-binding factors. Formal documentation in the case of Hesx1 will require the identification of the critical target genes.

In summary, we have provided evidence for the importance of coordinated regulation of a repressor (Hesx1) and corepressor (TLE1) as a determinant of organ determination and the temporal control in the generation of pituitary cell lineages. The synergy between TLE1 and N-CoR complexes, recruited by separate domains, may underlie the strong repressor actions of Hesx1. Further, our data suggest that sequential repression and activation of a common set of regulatory genes may prove to be an underlying strategy in the temporal code of pituitary organ development, with initial repression required for organ commitment and proliferation, and subsequent activation for commitment of specific cell lineages.

Material and methods

Generation and analysis of transgenic animals

Genotype analysis of *Hesx1*^{-/-} and *Prop-1*^{df} mice and generation of transgenic have been described previously (Sornson et al. 1996; Dattani et al. 1998; Treier et al. 1998). Founder animals were analyzed for integration by PCR and Southern blot analysis. All transgenic animals used in this study were determined to have integrated ~3–10 copies of the transgene and were obtained from embryonic founders. The following number of positive founders were analyzed for each construct: α GSU/*Hesx1*, *n* = 5; *Pitx1/Hesx1*, *n* = 5; *Pitx1/Prop-1*, *n* = 4; *Pitx1/FGF8*, *n* = 4; *Pitx1/Hesx1:Pitx1/TLE1*, *n* = 5; *Pitx1/Hesx1^{eh1mut}:Pitx1/TLE1*, *n* = 3. For generation of double-transgenic embryos, DNAs were mixed at a 1:1 molar ratio prior to injection.

In situ hybridization and immunohistochemistry

In situ hybridization and immunohistochemistry was performed as described previously (Simmons et al. 1990; Dasen et al. 1999).

Transfection and nuclear microinjection assays

Cotransfection experiments were performed as described previously (Rhodes et al. 1993) in 293T and COS-7 cells, using 1 μ g of luciferase reporter, 50–500 ng of pCMX expression plasmids, and 500 ng of pRSV β Gal as an internal control for differences in transfection efficiencies. The 4XPrdQ/tk luciferase reporter was generated by multimerization of annealed oligonucleotides containing a *paired*-like binding site (5'-TAATTGAATTA-3') and cloned upstream of the thymidine kinase promoter in luciferase and *lacZ* reporter plasmids. Microinjection of reporter plasmids and IgG-purified antibodies staining for β -galactosidase activity and fluorescence microscopy analysis was performed essentially as described (Jepsen et al. 2000).

Characterization of Hesx1 interacting proteins from HeLa nuclear extracts

Frozen pellets of HeLa cells, corresponding to 20 L of spinner cultures were obtained from the National Cell Culture Center. HeLa nuclear extracts were prepared by standard methods (Dignam et al. 1983). For interaction studies, 0.75–1 mg of HeLa nuclear extracts were precleared twice with 50 μ L of GST-saturated glutathione agarose beads, each at 4°C for 1 h and then combined with 10–50 μ g of immobilized GST–Hesx1 fragments at 4°C for 3 h. The beads were washed four times with binding buffer (20 mM Tris at pH 7.8, 150 mM NaCl, 0.1% NP40, 10% glycerol, 0.5 mM EDTA) and the protein complexes were then eluted twice at 4°C in binding buffer containing 0.3% Sarkosyl for 1 h each. The eluates were pooled and complexes separated on a 5%–15% gradient SDS–polyacrylamide gel and transferred to nitrocellulose membranes for Western blotting. All antibodies used in these studies were obtained from Santa Cruz Biotechnology and used at a 1:2000–1:5000 dilution. HDAC assays were performed as described (Hoffmann et al. 1999), using material purified from 500 μ g of HeLa nuclear extracts.

Coimmunoprecipitations and protein-interaction assays

Coimmunoprecipitation and other protein-interaction studies were performed as described previously (Dasen et al. 1999). Whole-cell lysates were incubated with 1 μ L of anti-FLAG M2 monoclonal antibody (Sigma) for 30 min on ice, precipitated with Protein A/G plus agarose at 4°C for 2 h, and washed four

times in binding buffer. Complexes were resolved by SDS-PAGE and transferred to nitrocellulose membranes. TLE1 was detected by use of a polyclonal antibody (SantaCruz Biotechnology) at a 1:3000 dilution, followed by anti-rabbit HRP-coupled secondary antibodies (Jackson Immuno Research) at 1:5000, and developed by ECL (Amersham).

Fragments encoding subdomains of Hesx1 and TLE1 were generated by PCR amplification and cloned in frame into the GST expression plasmid pGEX2TK (Pharmacia) or into the plasmid pCDNA3-KATG for in vitro transcription and translation from rabbit reticulocyte lysates using ³⁵S-labeled methionine (Promega). Point mutations in Hesx1 and TLE1 were generated using the Quick Change Mutagenesis kit (Stratagene) and were confirmed by DNA sequencing.

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