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Reciprocal Interactions of Pit1 and GATA2 Mediate Signaling Gradient–Induced Determination of Pituitary Cell Types

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Summary

The mechanisms by which transient gradients of signaling molecules lead to emergence of specific cell types remain a central question in mammalian organogenesis. Here, we demonstrate that the appearance of four ventral pituitary cell types is mediated via the reciprocal interactions of two transcription factors, Pit1 and GATA2, which are epistatic to the remainder of the cell type–specific transcription programs and serve as the molecular memory of the transient signaling events. Unexpectedly, this program includes a DNA binding–independent function of Pit1, suppressing the ventral GATA2-dependent gonadotrope program by inhibiting GATA2 binding to gonadotrope- but not thyrotrope-specific genes, indicating that both DNA binding–dependent and –independent actions of abundant determining factors contribute to generate distinct cell phenotypes.

Introduction

The development of specific cell types during mammalian organogenesis appears to involve molecular strategies analogous to those utilized for initial patterning in *Drosophila* development, involving gradients of signaling molecules that generate overlapping patterns of transcription factors (reviewed in Rusch and Levine, 1996). The pituitary gland provides an ideal model system for understanding the transcriptional programs that mediate the signaling gradient–induced positional determination events, as the cell phenotypes are well characterized and the critical morphogen gradients required for the emergence of cell types have been recently defined (Ericson et al., 1998; Takuma et al., 1998; Treier et al., 1998).

The mature pituitary gland contains six phenotypically distinct cell types that arise from a common primordium in a spatially and temporally specific fashion (Simmons et al., 1990; Japon et al., 1994) and serve critical homeostatic functions by regulating key endocrine organ targets in response to signals from brain and periphery. These six hormone-secreting cell types include corticotropes, secreting adrenocorticotropin hormone (ACTH); melanotropes, secreting melanocyte-stimulating hormone (MSH); thyrotropes, secreting thyroid-stimulating hormone (TSH); gonadotropes, secreting luteinizing hormone (LH) and follicle-stimulating hormone (FSH); somatotropes, secreting growth hormone (GH); and lactotropes, secreting prolactin (Prl). In addition, an embryonic cell type produces TSH (rostral tip cells). LH, FSH, and TSH are heterodimers sharing a common α-glycoprotein subunit (αGSU) and a specific β subunit (reviewed in Treier and Rosenfeld, 1996).

The pituitary gland is initially derived from the most anterior portion of the neural ridge and arises as an invagination of oral ectoderm, referred to as Rathke's pouch, that makes direct cell–cell contact with the overlying neural epithelium of the ventral diencephalon between embryonic days 8.5–9.0 (e8.5–e9.0) in mouse (Jacobson et al., 1979; Couly and Le Douarin, 1988; Eagleson and Harris, 1990). It has been demonstrated that direct contact of the ventral diencephalon with Rathke's pouch is required for pituitary organogenesis and that pituitary cell type determination occurs in response to a series of extrinsic and intrinsic signaling molecules (Ericson et al., 1998; Takuma et al., 1998; Treier et al., 1998). *BMP4*, *Wnt5a*, and *FGF8* are expressed in distinct, overlapping patterns in the ventral diencephalon, while *Shh*, uniformly expressed in the oral ectoderm, is excluded from the region that forms Rathke's pouch, creating a molecular boundary in the continuous ectoderm. Both in vivo (Treier et al., 1998) and organ culture experiments (Ericson et al., 1998; Treier et al., 1998) have demonstrated that BMP4 is required for the initial phase of organ commitment, with subsequent opposing ventral→dorsal BMP2 and dorsal→ventral FGF8 gradients serving to determine ventral/intermediate cell phenotypes (gonadotropes, thyrotropes, somatotropes, lactotropes) and dorsal cell phenotypes (melanotropes and corticotropes), respectively, with attenuation of the BMP2 signal ultimately required for terminal differentiation.

A consequence of these signaling gradients is apparently to establish overlapping expression patterns of several transcription factors in Rathke's pouch, allowing positional determination of pituitary cell types by e10.5–e12.5. Several factors exhibit spatially restricted domains of expression, including *Isl1* (Treier et al., 1998) and *Brn4* (Sornson et al., 1996) at the ventral boundary and *Prop1* (Sornson et al., 1996) and *Nkx3.1* (Bieberich et al., 1996) dorsally between e10.5–e12.5. Subsequently, prior to appearance of specific anterior pituitary cell types, the tissue-specific POU domain transcription factor Pit1, required for initial activation of growth hormone, prolactin, and thyroid-stimulating hormone in definitive thyrotropes, is expressed in an intermediate caudomedial field (Simmons et al., 1990; Li et al., 1990), while the orphan nuclear receptor SF1 is selectively expressed in the most ventrally arising pituitary cell type, the gonadotrope (Ingraham et al., 1994; Luo et al., 1994).

Thus, a central goal in pituitary development has been

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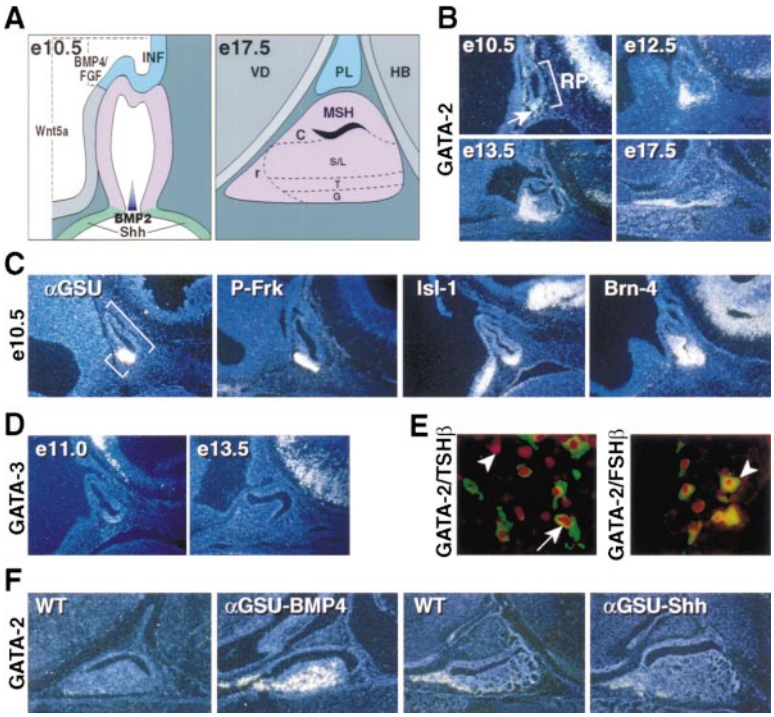

Figure 1. Ventral Induction of GATA2 in the Pituitary by BMP

(A) Schematic representations of the different stages of pituitary organogenesis. Pituitary cell types are positionally determined in response to signaling gradients between e10.5–e12.5, with definitive cell commitment occurring by e17.5. INF, infundibular component of the ventral diencephalon; r, rostral; C, corticotropes; S/L, somatotropes/lactotropes; T, thyrotropes; G, gonadotropes; VD, ventral diencephalon; HB, hindbrain; PL, posterior lobe.

(B) In situ hybridization demonstrating ontogeny of *GATA2* expression in the developing pituitary beginning at e10.5 at the ventral boundary of Rathke's pouch (RP) and remaining ventral throughout early embryonic development, with highest expression apparent in the most ventral region.

(C) Expression of ventrally expressed genes at e10.5; the common hormone subunit of thyrotropes and gonadotropes α*GSU* and the transcription factors *P-Frk*, *Isl1*, and *Brn4*.

(D) Transient expression of GATA3 is detected in the pituitary at e10.5 but is absent by e13.5.

(E) Colocalization of GATA2 protein with the αGSU-expressing pituitary cell lineages: TSHβ in thyrotropes (arrow) and FSHβ in gonadotropes (arrowheads).

(F) Induction of *GATA2* expression in animals overexpressing *BMP2/4* but not *Shh* under control of α*GSU* regulatory sequences. *GATA2* expression is detected by in situ hybridization of e17.5 transgenic founder animals. Reduced sensitivity in detection of expression levels of *GATA2* in the wild-type littermate from the αGSU-BMP4 transgene is due to the smaller section width in the analysis of these animals (5 μm vs. 20 μm for all other sections in this figure).

to address the molecular mechanisms by which the transient signaling gradients establish the transcriptional programs that dictate cell type specification. In this paper, we demonstrate that two induced factors, Pit1 and GATA2, by reciprocal interactions and based on both DNA binding–dependent and –independent actions, provide the molecular memory in vivo that mediates the effects of transient signaling gradients in determining pituitary cell types.

Results

BMP2 Induces Ventral Expression of GATA2

To investigate the hypothesis that morphogen-induced transcription factors mediate the determination of pituitary cell types, we explored the transcription factor–encoding genes initially expressed at the ventral boundary of the developing Rathke's pouch. In vivo data have suggested that pituitary cell type positional determination can occur between e10.5–e12.5, long before terminally differentiated cell types appear between e15.5–e16.5 (Figure 1A). We observed that the gene encoding the zinc finger protein GATA2 exhibits ventral induction in the pituitary coincident with the closure of Rathke's pouch at e10.5 and is maintained with highest expression levels ventrally throughout early pituitary development (Figure 1B), later becoming expressed diffusely as the adult pituitary cell populations lose spatial restriction. *GATA2* therefore exhibits ventral inductive patterns similar to the genes encoding the transcription factors *Brn4*, *Isl1*, and *P-Frk* as well as α*GSU* between e10.5–e11 (Figure 1C). Transcripts for *GATA3* are also transiently detected in the pituitary, showing ventral expression similar to that of *GATA2* at e10.5, but become undetectable by e13.5, prior to the appearance of the initial markers of ventral cell determination (Figure 1D). Double-label immunohistochemistry colocalizes expression of GATA2 protein in the adult pituitary to the two most ventrally arising cell types, gonadotropes and Pit1-dependent thyrotropes (Figure 1E).

Based on the ventral induction of a series of transcription factors, including *GATA2*, we examined in vivo whether dorsally expanding the expression of the ventral signaling molecules *BMP2* or *Shh* under control of 15 kb α*GSU* 5′-flanking information, which targets expression to Rathke's pouch and later to thyrotropes and gonadotropes, would dorsally expand specific ventrally induced genes. *BMP2*, normally expressed at the ventral boundary of *Shh* restriction out of the nascent Rathke's pouch, is required for the appearance of four pituitary cell types (Ericson et al., 1998; Treier et al., 1998). We find that expression of BMP2/4 under control of α*GSU* results in a dramatic transcriptional induction as well as dorsal expansion of *GATA2* gene expression (Figure 1F), whereas other ventrally expressed genes are not directly induced. In contrast, overexpression of *Shh* in vivo under α*GSU* does not lead directly to transcriptional induction of *GATA2* (Figure 1F). These data are consistent with the hypothesis that expression of *GATA2* in the pituitary is selectively induced in response to the ventral BMP2 signal, with highest levels of GATA2 present in the most ventral cell type, the presumptive gonadotrope precursors.

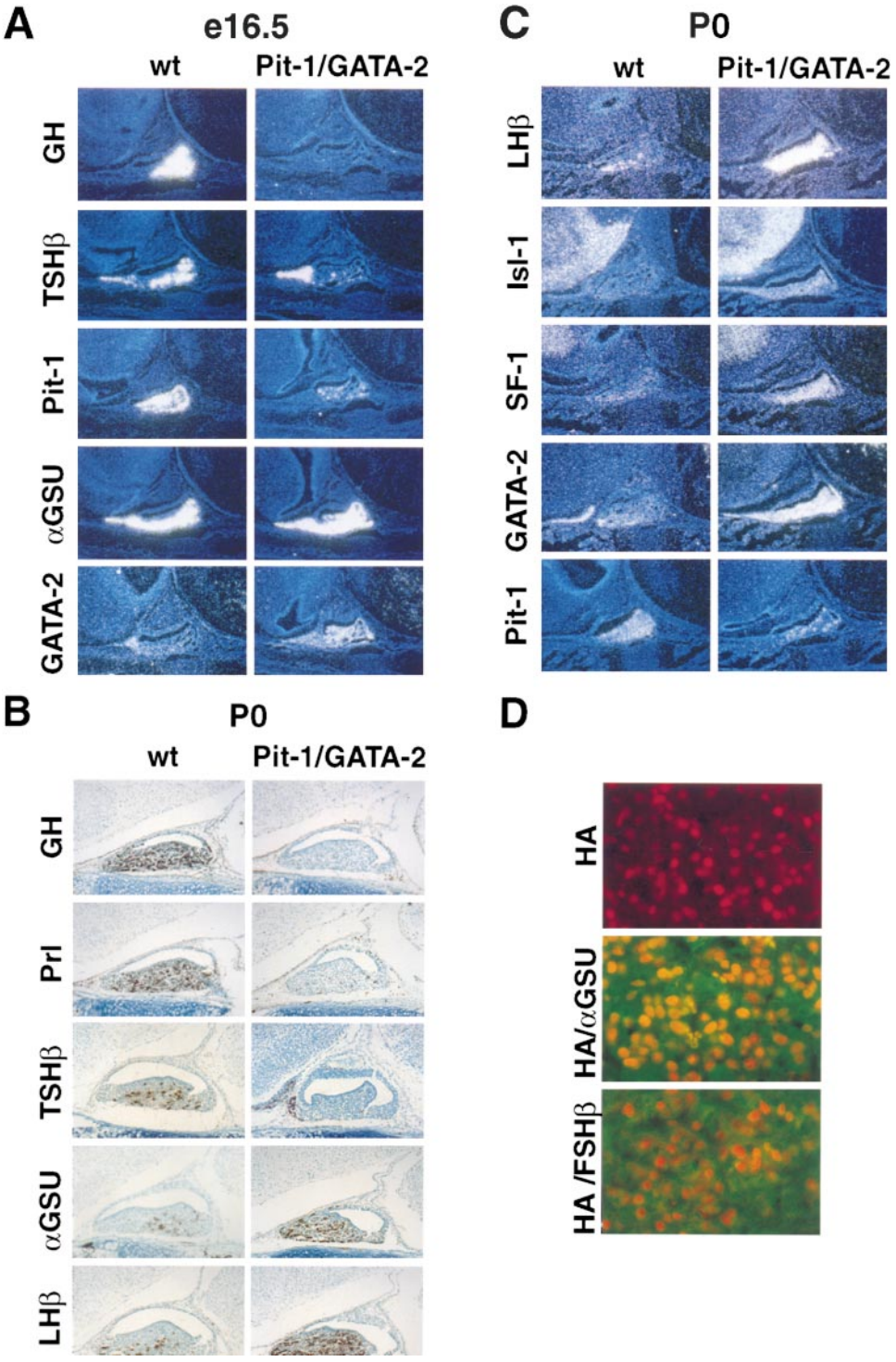

Figure 2. Conversion of Pituitary Cell Types by Dorsal Expression of GATA2

(A) Loss of Pit1-dependent cell lineages and *Pit1* expression in Pit1/GATA2 transgenic animals. In situ hybridization of e16.5 Pit1/GATA2 transgenic embryos demonstrated that expression of growth hormone (GH) and caudomedial thyroid-stimulating hormone (TSHβ) are absent with a severe inhibition of *Pit1* expression, whereas α*GSU* and *GATA2* expression expand dorsally. The Pit1-independent rostral tip thyrotrope population is unaffected in Pit1/GATA2 animals.

(B) Expansion of the gonadotrope lineage in Pit1/GATA2 animals. Immunohistochemistry of P_0 Pit1/GATA2 embryos revealed a loss of GH, TSHβ, and prolactin (Prl) protein and an expansion of gonadotrope cell types expressing glycoprotein subunit-α (αGSU) and luteinizing hormone (LHβ).

(C) Epistatic relationship of GATA2 and other ventral markers. Expression of the genes for the ventral markers *LH*β, *Isl1*, and *SF1* are dorsally expanded in P_0 animals. Expression of *P-Frk* is also dorsally expanded (data not shown).

(D) Immunohistochemistry of adult Pit1/GATA2 pituitaries reveals that gonadotrope cell types are derived from the transgene. The expression of the transgene (HA, rhodamine-labeled nuclei) colocalizes with gonadotrope markers (αGSU and FSHβ, fluorescein labeled). Nonlabeled nuclei represent the endogenous gonadotrope lineage. Wild-type pituitaries analyzed in parallel contained 5%–10% αGSU-expressing cells (data not shown). Identical results were obtained from two separate transgenic lines.

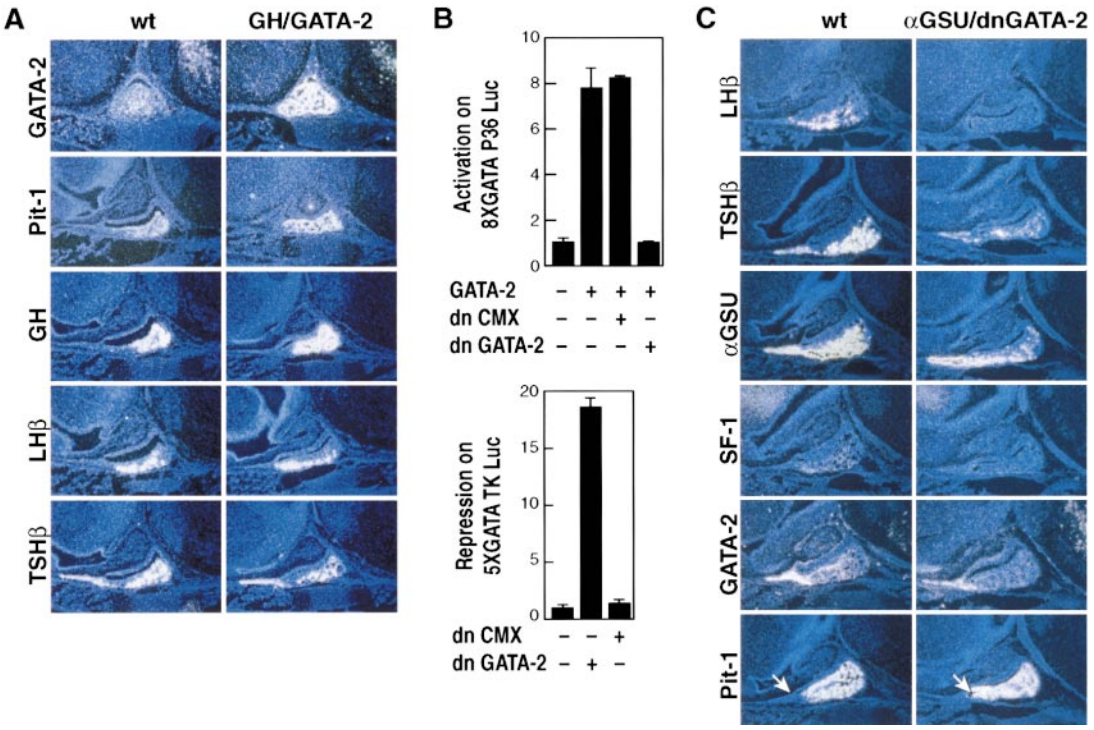

Figure 3. Roles of GATA2 in Ventral Pituitary Cell Type Determination

(A) Analysis of animals expressing *GATA2* under control of growth hormone regulatory sequences. Expression of *GATA2* is detected by in situ hybridization of e18.5 GH/GATA2 founder animals showing expression in the presumptive somatotrope region. No changes in *Pit1*, *GH*, *LH*β, or *TSH*β are detected.

(B) Dominant inhibition of GATA2 activation by an engrailed GATA2 fusion. "dnGATA2" refers to the dominant-negative GATA2 expression construct, whereas "dnCMX" contains the engrailed domain alone. These vectors are tested on promoters containing low-basal (P36; Lin et al., 1994) and high-basal (TK) activities and are presented as fold activation or repression over the reporter alone.

(C) In vivo expression of a dominant-negative GATA2 inhibits terminal differentiation of gonadotropes and thyrotropes. Analysis of e18.5 αGSU/dnGATA2 animals reveals the absence of *LH*β expression and a severe decrease in *TSH*β and α*GSU* expression. The gonadotrope-specific marker *SF1* is absent, and *GATA2* expression level is itself mildly reduced. *Pit1* expression also expands ventrally and rostrally. Two founders were analyzed for both transgenic constructs. Arrows indicate rostral shoulder region of anterior pituitary.

Dorsal Expression of GATA2 Converts Pituitary Cell Types to Gonadotropes

If GATA2 were to represent the critical factor that mediates the signal gradient–induced ventral determination program, then it should potentially be alone sufficient to impose this program on the more dorsally located Pit1 cell lineages. We therefore targeted expression of GATA2 in vivo using 15 kb of *Pit1* 5′-regulatory information (Rhodes et al., 1993) to test whether extending the expression of GATA2 more dorsally would be sufficient to alter the fate of the Pit1-dependent cell types that would normally generate somatotropes, lactotropes, and thyrotropes. Analysis of pituitary glands from animals expressing the Pit1/GATA2 transgene reveals that initial activation of growth hormone, prolactin, and Pit1-dependent thyroid-stimulating hormone β fails to occur at e16.5 (Figure 2A), remaining undetectable at P_0 (Figure 2B) and in the adult. In contrast, whereas the terminal differentiation marker for gonadotrope development, LHβ, is normally restricted to the most ventral portion of the gland at P_0 in wild-type animals, the pituitary glands of Pit1/GATA2 mice exhibit a dramatically expanded gonadotrope population, extending dorsally (Figures 2B and 2C).

In parallel, a series of markers normally confined to ventral expression also exhibit dorsal expansion in Pit1/GATA2 animals, including the ventral lineage marker *Isl1* and the gonadotrope marker *SF1* (Figure 2C). Conversely, there is marked inhibition of *Pit1* gene expression (Figures 2A and 2C). Double labeling with antibodies to define cells expressing the HA-tagged GATA2 transgene reveals that adult pituitaries contain predominantly gonadotrope cells (>90% of total cells) and that all GATA2-expressing cells derived from the transgene (HA positive) also express αGSU (Figure 2D). There is also a late appearance of some TSHβ-positive cells (data not shown). These results demonstrate that GATA2 is alone sufficient to induce the gonadotrope fate in the context of the more dorsally arising cell lineages and suggest an epistatic relationship between GATA2 and the remainder of the gonadotrope transcriptional program. In contrast, targeting of the ventrally induced homeodomain factor Isl1 under the identical promoter fails to convert the Pit1 lineages to gonadotropes (data not shown) or inhibit *Pit1* expression, demonstrating that the induction of the gonadotrope program is a specific effect of GATA2.

GATA2 Restriction of Pit1 Initial Activation

The conversion of the Pit1 lineage by GATA2 to a gonadotrope fate, despite normal expression of GATA2 in both gonadotropes and thyrotropes, is potentially explained

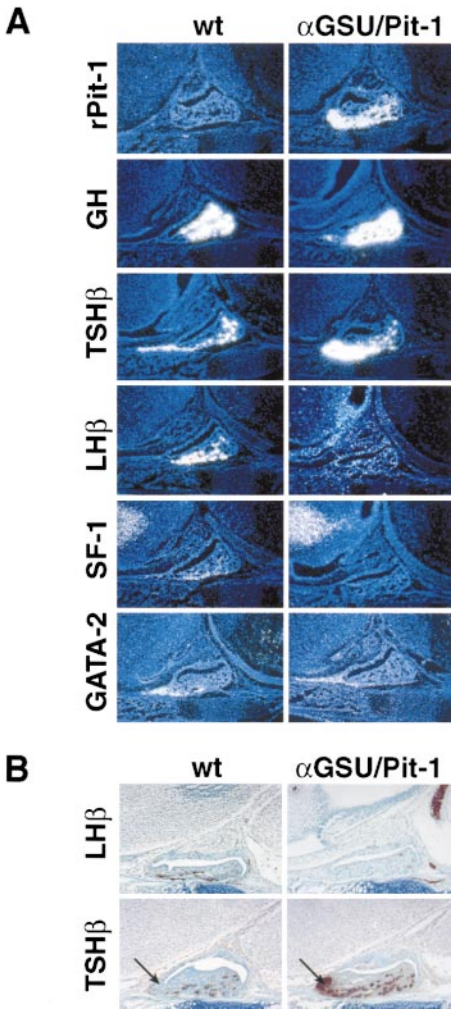

Figure 4. Ventral Expression of Pit1 Converts Gonadotropes to Thyrotropes

(A) In situ hybridization of e18.5 αGSU/Pit1 transgenic founder animals showing expression of the Pit1 transgene in the most ventral pituitary cell types (*rPit1*). Expression of *GH* is grossly normal, whereas the expression of *TSH*β expands into the presumptive gonadotrope region. Markers for gonadotrope determination (*LH*β and *SF1*) are lost. Ventral expression of Pit1 does not appear to affect *GATA2* levels. An occasional growth hormone–positive cell is also occasionally observed in the rostral tip region of the pituitary.

(B) Effects of ventral Pit1 expression analyzed by immunohistochemistry. Loss of LHβ-positive cells in the rostral/ventral region of the pituitary coincides with a reciprocal increase in the number of TSHβ-positive cells. Arrows indicate the expansion of the TSHβ$^+$ population in the rostral shoulder of the pituitary. Identical results were obtained from three separate founder animals.

by the marked inhibition of *Pit1* gene expression in Pit1/GATA2 animals (Figures 2A and 2B). *Pit1* gene expression is initially activated on e13.5 under control of a specific early gene enhancer but subsequently requires an autoregulated distal enhancer between e16.5–e17.5 (DiMattia et al., 1997). If GATA2 were to act by suppression of the Pit1 early—but not autoregulatory—enhancer, then GATA2 might fail to induce a cell type switch once *Pit1* autoregulation begins at the time of terminal differentiation (e16.5–e17.5). Indeed, targeting GATA2 expression under control of 1.7 kb of the rat growth hormone (*GH*) promoter, after *Pit1* autoregulation commences, results in transgenic embryos displaying high levels of *GATA2* expression in the presumptive somatotrope region but maintains normal expression of growth hormone and *Pit1* and no alterations from the normal expression of other cell type markers at e18.5 (Figure 3A). Thus, expression of GATA2 following terminal differentiation of a specific cell type (somatotropes) fails to alter the normal transcriptional programs or affect *Pit1* gene expression once it is driven by the late, autoregulatory gene enhancer.

Role of GATA2 in the Determination of Ventral Pituitary Cell Types

These actions of GATA2 in pituitary cell type determination are consistent with the critical developmental roles of members of the GATA family in hematopoietic, cardiovascular, and other organ systems (Pevny et al., 1991; Tsai et al., 1994; Pandolfi et al., 1995; Kuo et al., 1997; Molkentin et al., 1997). As mice homozygous for deletion of the GATA2 genomic locus die prior to critical events in pituitary organogenesis (Tsai et al., 1994), we wished to further investigate the requirement of GATA2 as a transcriptional activator in the determination of both gonadotropes and thyrotropes. We therefore generated transgenic mice expressing a dominant-negative GATA2 (dnGATA2) by replacing the N-terminal transcriptional activation domain of GATA2 with the repressor domain of the *Drosophila* Engrailed protein (Jaynes and O'Farrell, 1991; Han and Manley, 1993). In transient transfection assays, the dnGATA2 is capable of dominant inhibition of wild-type GATA2 function as well as repression of a GATA-TK reporter (Figure 3B). We targeted expression of the dnGATA2 in gonadotropes and thyrotropes under the control of 15 kb α*GSU* regulatory sequences and analyzed transgenic mice at e18.5. In these animals, both GATA2-expressing cell types are affected, as neither gonadotrope-specific (*LH*β, *SF1*) or thyrotrope-specific (*TSH*β) cell type markers are fully expressed, and α*GSU* expression is also diminished (Figure 3C). Consistent with the model that GATA2 restricts *Pit1* expression from presumptive gonadotropes, the field of *Pit1* expression in these pituitaries is now extended ventrally and rostrally to include the gonadotrope field (Figure 3C). Within the limitations inherent to in vivo dominant-negative experiments, these data are consistent with the hypothesis that GATA2 is required for both gonadotrope and thyrotrope cell type determination and further support the hypothesis that the high levels of GATA2 in the presumptive gonadotropes create the ventral exclusion boundary of the *Pit1* field.

Pit1 Can Convert Gonadotropes to Thyrotropes

Based on the requirement for Pit1 function in thyrotrope development (Li et al., 1990) and its absence in the gonadotrope, we next wished to test whether extending the expression domain of *Pit1* to include the field of GATA2$^+$ cells from which the gonadotropes are determined would "switch" their determination from a gonadotrope to a thyrotrope phenotype. Using 15 kb of α*GSU* regulatory sequences to target ventral expression of *Pit1*, we analyzed transgenic founder animals on e18.5, expressing *Pit1*, as determined using an in situ probe specific for the transgene (rPit1; Figure 4A). Pituitaries

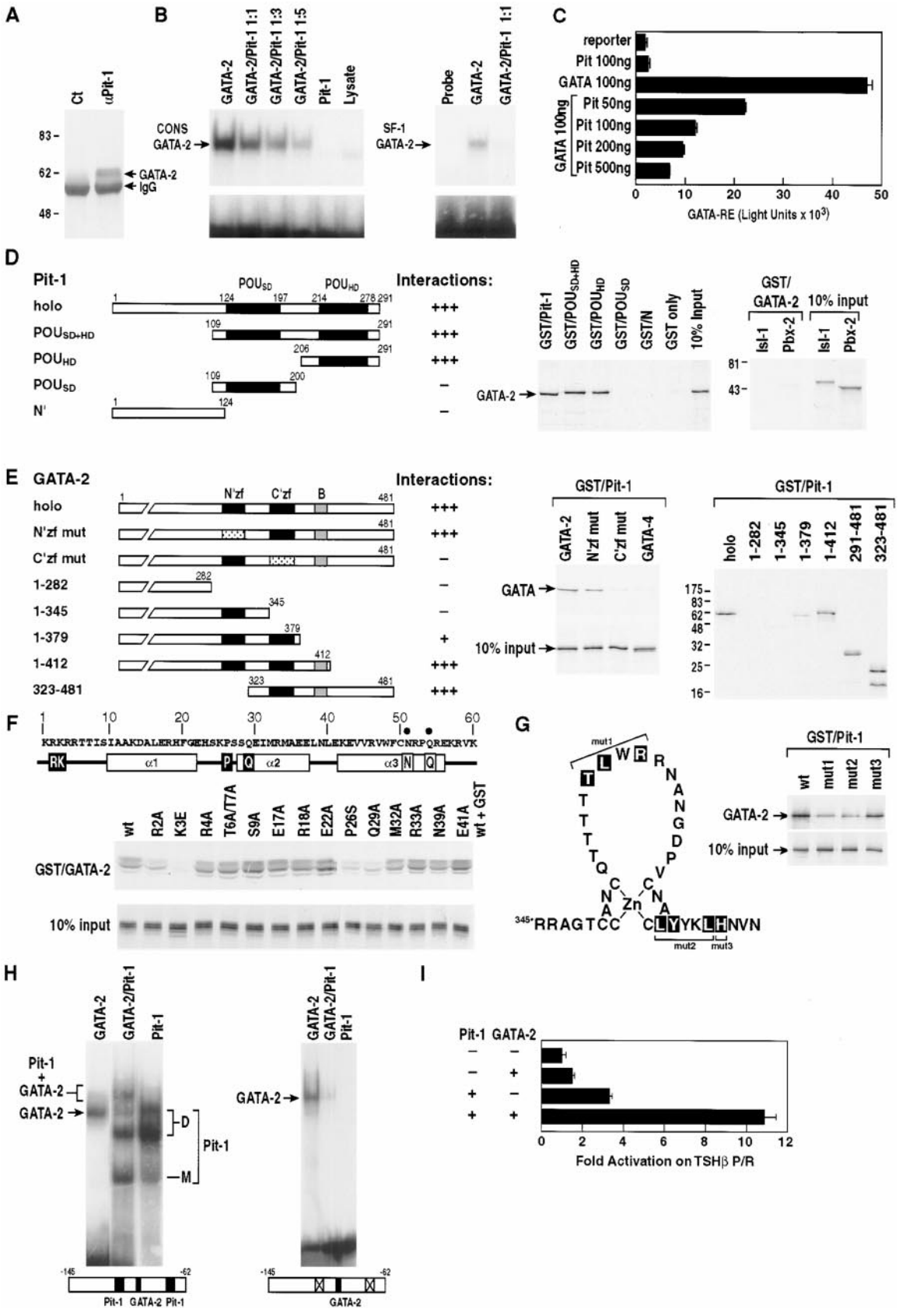

Figure 5. Physical and Functional Interaction between Pit1 and GATA2

(A) Immunoprecipitation of GATA2 from extracts of pituitary cells expressing Pit1 alone (lane 1) or Pit1 and GATA2 (lane 2) using αPit1 IgG, analyzed using a specific antibody to recognize GATA2. The secondary antibody also recognizes the IgG heavy chain (IgG). No coprecipitation is observed with nonspecific IgG (data not shown).

(B) Inhibition of GATA2 binding by Pit1. Electrophoresis gel mobility shift assays (EMSA) were performed using the indicated molar ratios of Pit1 and GATA2 protein generated by in vitro transcription and translation (TnT). GATA2 binding is inhibited by Pit1 on both a consensus "CONS" site and a site from the SF1 promoter.

(C) Similarly, Pit1 is capable of inhibition of GATA2-dependent transactivation of a reporter (8×GATA-RE P36) in transfection assays.

from αGSU/Pit1 animals are characterized by the near complete absence of the gonadotrope-specific markers *LH*β and *SF1* and a reciprocal, pronounced increase in the expression of both *TSH*β mRNA and protein (Figures 4A and 4B). The expansion of TSHβ-positive cells is particularly prominent at the rostral shoulder of the medial part of the gland (Figure 4B), presumably the location in which the gonadotrope lineage arises, as marked by the expression pattern of *SF1* during normal pituitary ontogeny. Extending the expression of Pit1 ventrally does not affect expression of *GATA2*, indicating that the loss of the gonadotrope cell type is not due to inhibitory actions of Pit1 on *GATA2* gene expression. Thus, within the context of the most ventral cell population, the presence or absence of Pit1 appears to be sufficient to determine differentiation to either a thyrotrope or a gonadotrope fate, respectively.

Functional Consequences of Pit1 and GATA2 Interactions

The ability of ventral expression of Pit1 to prevent the appearance of the gonadotrope phenotype without affecting *GATA2* levels has provided a model in which to explore the molecular mechanisms by which Pit1 may function as an inhibitor of the GATA2-dependent gonadotrope program in thyrotropes. Because the described interactions between homeodomain and GATA factors could result in either positive (Durocher et al., 1997) or negative transcriptional consequences, we evaluated the physical interaction between Pit1 and GATA2, initially by coimmunoprecipitation of the two proteins from pituitary-derived cell extracts (Figure 5A), finding Pit1–GATA2 interaction, as has been previously noted (Gordon et al., 1997). We find unexpectedly that Pit1, normally expressed at levels of up to 500,000 copies/cell, can effectively inhibit GATA2 binding to cognate DNA sites, including a putative GATA-binding site within the *SF1* promoter (Figure 5B; Woodson et al., 1997; Viger et al., 1998), and impair GATA2-dependent transactivation in cotransfection assays (Figure 5C). Conversely, GATA2 is incapable of inhibition of Pit1 binding to its cognate site (data not shown). The interaction interface maps to the homeodomain of Pit1 and to a region of GATA2 containing the C-terminal DNA-binding zinc finger and an adjacent cluster of basic residues (Figures 5D and 5E). Point mutations on the Pit1 interaction interface reveal a requirement for residues located in the N-terminal basic region (R2, K3) and the non-DNA-binding surface of the second helix of the homeodomain (P26, Q29) (Figure 5F). Further, mutation of residues defined by NMR structural analysis of GATA1 (Omichinski et al., 1993) to be the critical major groove DNA-binding residues in the zinc finger of GATA proteins appears to have no effect or to only partially weaken interactions with Pit1 (Figure 5G).

We find that this inhibition of GATA2 DNA binding by Pit1 does not occur on the *TSH*β promoter, which harbors both low-affinity Pit1 (Lin et al., 1994) and GATA2 DNA-binding sites (Figure 5H; Gordon et al., 1997). Binding of GATA2 to the *TSH*β promoter is, however, inhibited when the Pit1 sites have been deleted (Figure 5H). Similarly, in transient transfection assays, Pit1 and GATA2 exert synergistic effects on the *TSH*β promoter (Figure 5I), as has been previously reported (Gordon et al., 1997). These observations suggest a model in which Pit1 can inhibit transactivation by GATA2 on GATA2-dependent promoters that do not contain adjacent Pit1-binding sites, such as the gonadotrope-specific *SF1* promoter, and synergize with GATA2 on genes containing DNA-binding sites for both Pit1 and GATA2 as with the promoter for *TSH*β.

This model predicts that mutations of Pit1 that impair its interaction with GATA2 would consequently permit expression of gonadotrope-specific genes in the presumptive caudomedial thyrotrope population. The Pit1 point mutation (W48C) in the Snell genetic dwarf (*dw*) mouse (Li et al., 1990) disrupts the homeodomain structure (Jacobson et al., 1997) and inhibits both its interaction with GATA2 (Figure 6B) and DNA binding. As the *dw* mouse exhibits normal appearance of the $Pit1^+$ cell populations but fails to activate *TSH*β gene expression, we evaluated whether in this genetic model there might be an expansion in the gonadotrope cell types. Consistent with the hypothesis that Pit1 suppresses the gonadotrope program in thyrotropes, we find that in Snell mice the gonadotrope markers *LH*β and *SF1* (Figure 6A) are induced in the cells that presumably would have normally given rise to caudomedial thyrotropes. This mutation also provides independent evidence of the ability of a $GATA2^+$, $Pit1^-$ cell to assume a gonadotrope fate, complementing the gain-of-function transgenic experiments.

DNA Binding–Independent Functions of Pit1

Because the interaction of GATA2 with Pit1 appears to involve a surface of the Pit1 homeodomain distinct from the DNA-binding domain, we wished to further evaluate whether the entire program of Pit1 action on cell type determination is DNA binding dependent or whether protein–protein interactions might account for critical aspects of cell type determination. We therefore generated a mutation in the homeodomain of Pit1 (N51A,

(D) Mapping of the Pit1 interaction interface with GATA2. Schematic representations of Pit1 constructs that were expressed as GST-fusion proteins for interaction assays with ^{35}S-labeled Met GATA2 are shown. Interactions with other classes of homeodomain proteins (e.g., Isl1 and Pbx2) are not detected.

(E) Mapping of the GATA2 interaction interface with Pit1. Interaction assays were performed using a GST fusion of the Pit1 homeodomain with various fragments and mutants of GATA2.

(F) Fine mapping of the Pit1 homeodomain interaction with GATA2 reveals a requirement for specific residues located in the N terminus of the homeodomain and near the second helix (reverse lettering).

(G) Alanine substitution of major groove–contacting residues (reverse lettering) in GATA2 only partially weaken interaction with Pit1.

(H) DNA binding of Pit1 and GATA2 on the *TSH*β promoter. GATA2 binding is inhibited when the Pit1 sites are removed.

(I) Synergistic activation of a multimerized Pit1/GATA2 site from the *TSH*β promoter (TSHβ P/R) by Pit1 and GATA2.

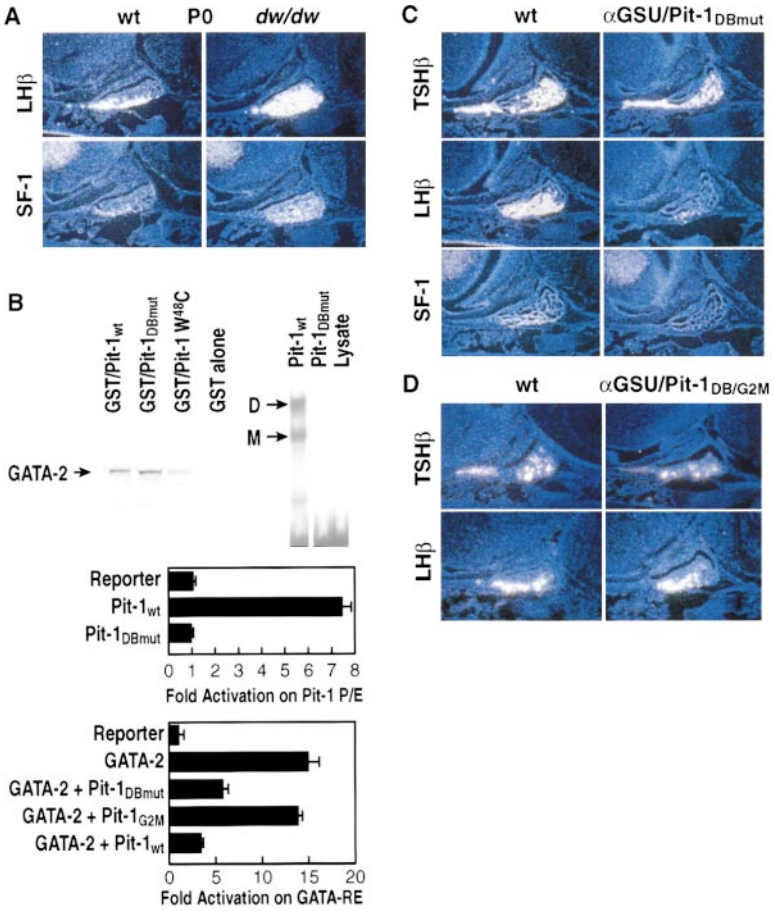

Figure 6. DNA Binding–Independent Inhibition of Gonadotrope Determination by Pit1

(A) Snell dwarf (*dw*) animals display an increased and dorsally expanded gonadotrope population. In situ hybridization was performed on P_0 animals and revealed a dorsal expansion of *LH*β and *SF1* expression. Similar results were obtained from analysis of five pairs of *dw/dw* and wild-type animals.

(B) Generation of a non-DNA-binding Pit1 mutant (N51A, Q54A) that retains GATA2 interaction. Interaction assays were performed using a GST fusion of Pit1 and ^{35}S-labeled GATA2 (upper left panel). EMSA and transfections with the Pit1 DNA-binding mutant ($Pit1_{DBmut}$) reveals a loss of DNA binding on a high-affinity Pit1-binding site from the Prl promoter (upper right panel) and activation of the autoregulatory Pit1 promoter/enhancer (P/E) (center panel). The $Pit1_{DBmut}$ is also capable of inhibition of GATA2-dependent activation of the GATA-RE reporter. With mutation of residues in Pit1 required for GATA2 interaction ("G2M"; K3E, P26S, Q29A as identified in Figure 5F), the ability to inhibit GATA2-dependent transactivation is lost (lower panel).

(C) In vivo expression of $Pit1_{DBmut}$ under control of αGSU regulatory sequences inhibits gonadotrope determination at e18.5. Whereas *TSH*β expression is normal, *LH*β and *SF1* levels are dramatically attenuated.

(D) Expression of $Pit1_{DBmut}$ containing the G2M mutations ($Pit1_{DB/G2M}$) under control of αGSU regulatory sequences does not inhibit appearance of either gonadotropes or thyrotropes, in contrast to gonadotrope-specific effects of the $Pit1_{DBmut}$. The $Pit1_{DB/G2M}$ transgene expresses at levels comparable to that of the Pit_{DBmut} (data not shown). Similar results were obtained in all transgenic mice analyzed.

Q54A) based on its DNA-bound crystal structure (Jacobson et al., 1997) that prevents DNA binding and DNA binding–dependent activation but has no effect on interaction with GATA2 (Figure 6B) and targeted its expression under control of α*GSU* regulatory sequences. As shown in Figure 6C, *TSH*β continues to be expressed normally in thyrotropes, indicating that the expression of the $Pit1_{DBmut}$ does not act to "squelch" the binding or transcriptional actions of the endogenous Pit1 protein. However, expression of the αGSU/$Pit1_{DBmut}$ protein effectively blocks the terminal differentiation program of gonadotropes, as indicated by the dramatic loss of *LH*β and *SF1* expression (Figure 6C). Thus, in contrast to the actions of the αGSU/wild-type Pit1 transgene, the αGSU/$Pit1_{DBmut}$ does not convert gonadotropes to thyrotropes but inhibits their terminal differentiation.

To more rigorously test whether this effect of the non–DNA binding form of Pit1 actually reflects its interaction with GATA2, we generated point mutations in the residues preceding helix 1 and within helix 2 of the Pit1 homeodomain ($Pit1_{G2M}$) that abolished both the interaction with GATA2 and the ability to inhibit GATA2-dependent transactivation (Figures 5F and 6B). In vivo expression of the Pit1-containing mutation of residues required for GATA2 interaction in the context of the $Pit1_{DBmut}$ reverts the inhibitory effects of Pit1 on gonadotrope determination (Figure 6D), consistent with this effect attributed to direct inhibition of GATA2 function. Thus, a critical function of the Pit1 homeodomain includes its ability to selectively alter components of gene activation programs required for cell type specification through protein–protein interactions, in this case interacting with GATA2, inhibiting activation of gonadotrope-specific genes in thyrotropes.

Discussion

GATA2 Mediates the Effects of BMP2 in Determination of Pituitary Cell Types

The mediation of the effects of transient signaling gradients on positionally determining the appearance of specific cell types in mammalian organogenesis has been hypothesized to be achieved by the induction of overlapping, spatially distinct patterns of transcription factor expression. In pituitary development, opposing BMP2 and FGF8 gradients are associated with induction of distinct combinations of transcription factors and positional determination of the component pituitary cell types in a distinct spatial fashion by e12.5. Our data suggest that the cell-autonomous programs established by the transient morphogen gradients are, surprisingly, ultimately mediated by the actions of two induced transcription factors, GATA2 and Pit1, which are both required and sufficient to establish the remaining transcriptional programs that dictate terminal differentiation

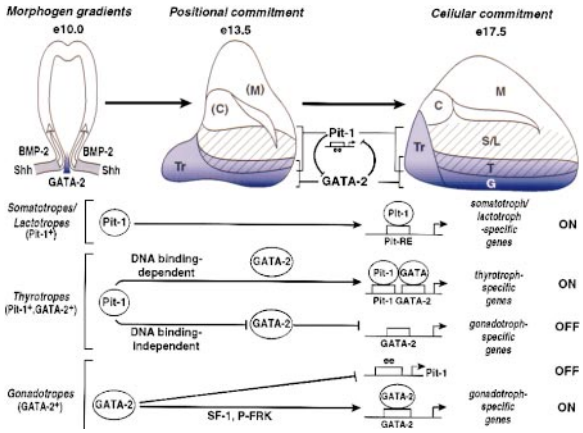

Figure 7. Model for Pituitary Cell Type Determination by Pit1 and GATA2

The reciprocal interactions of Pit1 and GATA2 and the DNA binding–independent actions of Pit1 serve as the cell-autonomous "molecular memory" that mediates the generation of four pituitary cell phenotypes in response to transient signaling gradients. The complement of Pit1 and GATA2 *cis*-active elements dictates gonadotrope- and thyrotrope-specific gene expression. This model provides a molecular mechanism by which the specific interaction of two factors can simultaneously generate an "activator" of a cohort of genes expressed in a specific type and a "repressor" of a distinct second set of genes that are excluded.

of four anterior pituitary cell types. In part, this cell type specification program is achieved by reciprocal, mechanistically distinct inhibitory and synergistic interactions between these two induced factors (Figure 7).

Our data suggest that the ventral→dorsal BMP2 gradient induces GATA2 in a corresponding gradient in presumptive gonadotropes and thyrotropes and that the high levels of GATA2 in the most ventral aspect of the gland directly or indirectly restricts *Pit1* gene expression out of the presumptive gonadotropes, creating the critical delineation of the gonadotrope and Pit1 cell lineages. In the absence of Pit1, GATA2 expression appears sufficient to induce the entire set of transcription factors that are typical of the gonadotrope cell type, including the transcription factors SF1, P-Frk, and Isl1. Conversely, the absence of GATA2 dorsally is critical for differentiation of Pit1$^+$ cells to somatotrope/lactotrope fates, as the targeting of GATA2 more dorsally inhibits initial *Pit1* expression and converts these cells to gonadotropes. Similarly, targeting overexpression of BMP2/4 also inhibits *Pit1* expression, although the cell types fail to terminally differentiate (Treier et al., 1998). We hypothesize that the level of GATA2 expression in the thyrotropes, however, is below the threshold required to inhibit activation of the *Pit1* gene early enhancer, permitting the emergence of a Pit1$^+$, GATA2$^+$ cell, dictating a pattern of gene expression that defines a thyrotrope. This interpretation is supported by the ability to convert a gonadotrope to a thyrotrope cell fate by ventral extension of Pit1 expression to the Pit1$^-$, GATA2$^+$ field.

Thus, between e13.5–e17.5, Pit1$^+$, GATA2$^-$ cells will terminally differentiate to somatotropes/lactotropes; Pit$^+$, GATA2$^+$ cells to thyrotropes; and GATA2$^+$, Pit$^-$ cells to gonadotropes. These results conceptually extend observations suggesting that a single transcription factor can induce cell determination events by triggering activation of downstream regulators as occurs in *Drosophila* eye development (Halder et al., 1995), in the actions of bHLH factors in myogenesis (Weintraub, 1993; Firulli and Olson, 1997), and by a homeodomain protein in the emergence of specific motor neurons in the neural tube (Tanabe et al., 1998). Thus, GATA2, which earlier in development is required for the expansion of hematopoietic precursors (Tsai et al., 1994) and the development of the placental trophoblast (Ma et al., 1997) and urogenital system (Zhou et al., 1998), also plays a critical role in pituitary cell type determination.

DNA Binding–Independent Roles of Pit1 in Cell Type Determination

Pit1 is prototypic of developmentally important transcription factors that are expressed at unusually high levels, in this case up to 500,000 copies of Pit1/cell, making it of particular interest to determine whether actions in addition to its role as a DNA-binding transcription factor are critical for its function. In this paper, we have provided evidence that Pit1 can modify the actions of a second factor, GATA2, based on protein–protein interactions that contribute specifically to cell type determination. We demonstrate that the interaction of the Pit1 homeodomain with the C-terminal zinc finger of GATA2 inhibits its binding to a subset of cognate DNA sites, unless there is an adjacent Pit1 DNA-binding site. This appears in vivo to account for the ability of Pit1 to inhibit gonadotrope-specific aspects of the ventral cell type differentiation program.

The residues of the Pit1 homeodomain required for interaction with GATA2 suggest a bipartite interaction surface, including the basic cluster of amino acids preceding the first helix of the homeodomain and the N-terminal portion of the second helix, consistent with the ability of Pit1 to bind cognate DNA sites even in the presence of excess GATA2. In contrast, the inhibition of GATA2 binding in the presence of Pit1 reflects an interaction interface of GATA2 that is its DNA-binding surface. However, a subset of Pit1- and GATA2-dependent genes, such as *TSH*β, which have adjacent binding sites for both proteins, escape this inhibiting effect. This is apparently due to allosteric effects imparted by the binding of Pit1 to DNA sites that permit a Pit1–GATA2 conformation that no longer inhibits specific DNA binding by GATA2. Thus, Pit1–GATA2 protein–protein interactions can lead to gene-specific actions that have come to assume cell-specific roles.

We therefore suggest that a critical component of the cell type determination program is achieved through the inhibition by Pit1 of GATA2-dependent gonadotrope-specific genes while simultaneously permitting GATA2-dependent gene activation critical for establishing the thyrotrope phenotype. Two types of in vivo data support a DNA binding–independent role of Pit1. The first is that targeted expression of a non-DNA-binding form of Pit1, still capable of interaction with GATA2, inhibits the gonadotrope-specific terminal differentiation program, while point mutations that abolish the Pit1–GATA2 interaction revert this inhibitory effect. Second, this hypothesis receives genetic confirmation based on developmental events in the Snell *dw* dwarf mouse in which the W48C mutation in Pit1 disrupts the homeodomain structure and impairs interaction with GATA2 and in

which the presumptive thyrotropes now express the GATA2-dependent gonadotrope gene activation program. This provides direct evidence that Pit1-dependent inhibition of GATA2-dependent activation of gonadotrope-specific genes is a critical component by which Pit1 controls the thyrotrope-specific program. Thus, DNA binding–independent inhibitory protein–protein interactions by the highly abundant Pit1 transcription factor, in addition to its DNA-dependent transcriptional activation roles, is a critical component of the cell type specification and suggests similar functions for other POU domain or other classes of homeodomain factors.

The ability to overcome the inhibitory effects of Pit1 on GATA2 on promoters not containing adjacent Pit1 and GATA2 DNA-binding sites is analogous to events in the well-documented transrepression of AP1 by glucocorticoid receptor (Jonat et al., 1990; Kamei et al., 1996). Indeed, the biological importance of the non-DNA binding component of glucocorticoid receptor actions has been shown in vivo by mutation of the glucocorticoid receptor DNA-binding domain (Reichardt et al., 1998). The data presented here show that this type of transrepression has been functionally adapted in cell type determination events. Thus, while the role of protein–protein interactions in the function of transcription factors such as Hox and Pbx factors (reviewed by Mann and Chan, 1996), Bob1/OCA-B/Obf1 for Oct1 (Luo et al., 1992; Gstaiger et al., 1995; Strubin et al., 1995), bHLH and MADS box factors (Molkentin et al., 1995), and FOG for GATA1 (Tsang et al., 1997) has been established, our study reveals that a critical component of the cell type–specific actions of developmentally regulated DNA-binding transcription factors is to modulate, by DNA-independent interactions, the function of other DNA-dependent transcription factors on a subset of their target genes.

We suspect that these DNA binding–dependent and –independent actions of Pit1 will prove to be prototypic of the actions of many highly expressed transcriptional regulatory factors in the development of other mammalian organs.

Experimental Procedures

Plasmid Constructions for Transgenic Animals

Plasmid DNA for generation of transgenic mice were constructed by insertion of cDNAs into a vector cassette containing a 5′-β-globin intron and a 3′-polyadenylation signal from the human growth hormone gene as previously described (Treier et al., 1998). The murine *GATA2* cDNA was obtained by PCR amplification of e14.5 pituitary cDNA and cloned in frame with the HA epitope at the N terminus. The dnGATA2 transgene was constructed by fusion of the Engrailed repressor domain (aa 2–229) to the DNA-binding region and C terminus of GATA2 (aa 260–481). For the Pit1 transgenic constructs, the full-length 1.6 kb rat cDNA was used, and point mutations were generated using the Quick Change Mutagenesis kit (Stratagene).

Generation of Transgenic Animals

Plasmid DNA for transgenic constructs was prepared by standard procedures, purified by gel electrophoresis, dialyzed against 10 mM Tris (pH 7.5), 1 mM EDTA, and microinjected as described (Crenshaw et al., 1989). Founder animals were analyzed for integration by PCR and Southern blot analysis. All transgenic animals used in this study were determined to have integrated approximately three to ten copies of the transgene. Data for the Pit1/GATA2 transgene were obtained from two independent transgenic lines, showing similar levels of expression and identical phenotypes. All other transgenic animals were obtained from embryonic founders. Tissues were fixed in either 10% formalin for analysis by in situ hybridization or in 10% formaldehyde, 60% EtOH for 2–3 hr for immunohistochemistry.

In Situ Hybridization and Immunohistochemistry

In situ hybridization was performed as previously described (Simmons et al., 1990) on 20 μm cryosections using ^{35}S-labeled antisense RNA probes. For immunohistochemistry, embryos or adult pituitaries were dehydrated in ethanol, embedded in paraffin, sectioned at 8–10 μm, and mounted on Superfrost Plus slides (Fischer). Sections were incubated with primary antibody at a 1:200 dilution in PBS, 0.3% Triton X-100, 5% normal goat serum overnight at 4°C. After washing three times in PBS, slides were incubated with HRP-coupled secondary antibodies (Chemicon) at a 1:200 dilution for 3 hr at room temperature. Staining was visualized using a DAB metal conjugate (Pierce). Sections were counterstained with methyl green and mounted on Permount (Fischer). For analysis of adult pituitaries by immunofluorescence, pituitaries were incubated with primary antibody overnight at 4°C, followed by incubation with TRITC- or FITC-coupled secondary antibodies (Chemicon) at a 1:200 dilution. Sections were then mounted using the Slow Fade antifade kit (Molecular Probes).

Primary antibodies used were obtained as follows; αGH, αPrl, αTSHβ, DAKO, Carpinteria, CA; α-αGSU, αLHβ, National Hormone and Pituitary Program, NIDDK, Rockville, MD; αHA, Babco; and αGATA2, provided by S. Orkin, Harvard, Cambridge, MA.

Transfections and DNA Binding Assays

Cotransfection experiments were performed as previously described (Rhodes et al., 1993) in HeLa and COS7 cells, using 1 μg of luciferase reporter, 50–500 ng pCMX expression plasmids, and 500 ng of pRSVβGal as an internal control for differences in transfection efficiencies. The GATA-RE luciferase reporter was generated by multimerization of annealed oligonucleotides containing two GATA-binding sites (5′-CACTTGATAACACAAAGTGATAACTCT-3′) and cloned upstream of a minimal promoter (P36) derived from the prolactin gene (Lin et al., 1994) or the thymidine kinase (TK) promoter. The TSHβ reporter was generated by PCR amplification of the murine promoter region (nt −145 to −62) and similarly multimerized. The GATA site in the *SF1* gene was derived from nt −189 to −157 of the murine promoter (5′-GGGCCCCCATAAAGATAGGGATATTTT TTTTTC-3′). DNA binding assays were performed using γ-^{32}P-labeled ATP double-stranded and gel-purified oligonucleotides as previously described (Rhodes et al., 1993). Other reporter constructs and DNA sites used in this study have been described previously (Rhodes et al., 1993)

Immunoprecipitations and Protein Interaction Assays

For coimmunoprecipitation studies, 100 mm plates of pituitary GC cells were transfected with 5 μg of a CMV-HA-GATA2 expression vector using the Lipofectamine Plus reagent (GIBCO–BRL). After 48 hr, cells were harvested and lysed in 600 μl binding buffer containing 20 mM Tris (pH 7.8), 150 mM NaCl, 0.1% NP-40, 10% glycerol, 0.5 mM EDTA, and 1× Complete Protease Inhibitor Cocktail (Boehringer Mannheim). Lysates were incubated with 3 μl of rabbit polyclonal α-Pit1 antiserum for 8 hr at 4°C, precipitated with protein A/G plus agarose, and washed four times in binding buffer. Complexes were resolved by 8% SDS-PAGE and transferred to nitrocellulose membranes. GATA2 was detected using an α-HA monoclonal antibody (Babco) at a 1:3000 dilution, followed by anti-mouse HRP-coupled secondary antibodies (1:3000), and developed by ECL (Amersham).

Protein preparation and interaction studies were performed as described (Kamei et al., 1996). Fragments encoding the full-length Pit1 (aa 1–291), the homeodomain of Pit1 (aa 206–291), and the zinc finger region plus the C terminus of GATA2 (aa 291–481) were generated by PCR amplification and cloned in frame into the GST expression plasmid pGEX2TK (Pharmacia). Fragments of Pit1 and GATA2 for mapping interaction domains were also generated by PCR and cloned into the plasmid pCDNA3-KATG for in vitro transcription and translation from rabbit reticulocyte lysates using ^{35}S-labeled methionine (Promega). Point mutations in Pit1 and GATA2 were generated using the Quick Change Mutagenesis kit

(Stratagene) and were confirmed by DNA sequencing. For the interaction assays, 0.1–0.5 μg of GST proteins were combined with ^{35}S-labeled proteins in a binding/washing buffer containing 150 mM NaCl, 20 mM Tris (pH 7.8), 10% glycerol, 0.02% NP-40, and 0.5 mM DTT. GST-coupled and ^{35}S-labeled proteins were bound for 1 hr at 4°C, washed four times, and interactions visualized after SDS-PAGE and autoradiography.

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