Inhibition of Sox2-dependent activation of Shh in the ventral diencephalon by Tbx3 is required for formation of the neurohypophysis

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SUMMARY

Tbx2 and Tbx3 are two highly related members of the T-box transcription factor gene family that regulate patterning and differentiation of a number of tissue rudiments in the mouse. Both genes are partially co-expressed in the ventral diencephalon and the infundibulum; however, a functional requirement in murine pituitary development has not been reported. Here, we show by genetic lineage tracing that Tbx2+ cells constitute the precursor population of the neurohypophysis. However, Tbx2 is dispensable for neurohypophysis development as revealed by normal formation of this organ in Tbx2-deficient mice. By contrast, loss of Tbx3 from the ventral diencephalon results in a failure to establish the Tbx2+ domain in this region, and a lack of evagination of the infundibulum and formation of the neurohypophysis. Rathke's pouch is severely hypoplastic, exhibits defects in dorsoventral patterning, and degenerates after E12.5. In Tbx3-deficient embryos, the ventral diencephalon is hyperproliferative and displays an abnormal cellular architecture, probably resulting from a failure to repress transcription of Shh. We further show that Tbx3 and Tbx2 repress Shh by sequestering the SRY box-containing transcription factor Sox2 away from a domain in this region, and a lack of evagination of the infundibulum and formation of the neurohypophysis. Rathke's pouch is severely hypoplastic, exhibits defects in dorsoventral patterning, and degenerates after E12.5. In Tbx3-deficient embryos, the ventral diencephalon is hyperproliferative and displays an abnormal cellular architecture, probably resulting from a failure to repress transcription of Shh. We further show that Tbx3 and Tbx2 repress Shh by sequestering the SRY box-containing transcription factor Sox2 away from a Shh forebrain enhancer (SBE2), thus preventing its activation. These data suggest that Tbx3 is required in the ventral diencephalon to establish a Shh+ domain to allow formation of the infundibulum.

KEY WORDS: Pituitary gland, Neurohypophysis, Infundibulum, Diencephalon

INTRODUCTION

The hypophysis or pituitary gland is a central regulator of the endocrine system in the control of growth, reproduction and homeostasis. It is composed of two anatomically and functionally distinct units: the neurohypophysis, also referred to as the posterior lobe, and the adenohypophysis, comprising the anterior and intermediate lobes. Adeno- and neurohypophysis derive from different germinal tissues, yet morphogenesis and differentiation of these organs is highly integrated.

In the mouse, morphological changes related to (anterior) pituitary development can first be seen at embryonic day (E) 8.5. At this stage, a subregion of the oral roof ectoderm that lies in close proximity to the ventral neuroectoderm of the forebrain thickens and starts to invaginate shortly afterwards to form Rathke’s pouch. Driven by continuous proliferation in its ventral aspect, the pouch continues to grow, and pinches off the oral roof ectoderm to form a closed vesicle by E11.5. Further proliferation of precursor cells within the primitive adenohypophysis and subsequent differentiation leads to the formation of anterior and intermediate lobes with six distinct endocrine cell types at E18.5. The posterior pituitary lobe develops from an evagination of the ventral midline of the forebrain adjacent to the dorsal aspect of Rathke’s pouch starting at E10.5. This infundibulum, or neurohypophyseal bud, elongates adjacent to the dorsal roof of the adenohypophysis and its neuroectodermal progenitors differentiate into specialized astroglial cells, collectively referred to as pituicytes. They envelop axonal projections of magnocellular neurosecretory cells of the paraventricular nucleus and the supraoptic nucleus of the hypothalamus, which release the hormones oxytocin (Oxt) and arginine vasopressin (Avp) (for reviews, see Amar and Weiss, 2003; Zhu et al., 2007).

Embryological and genetic experiments have shown that induction, growth and patterning of Rathke’s pouch depends on extrinsic cues from the adjacent neuroectoderm (Daikoku et al., 1982; Watanabe, 1982). Bmp4 from the ventral diencephalon induces the formation and initial growth of Rathke’s pouch, whereas expansion and maintenance of undifferentiated progenitors in the dorsal region of this tissue is mediated by members of the fibroblast growth factor family (Fgf8, Fgf10 and Fgf18) from the infundibulum (Ericson et al., 1998; Norlin et al., 2000; Treier et al., 1998; Treier et al., 2001). Infundibular Fgfs also counteract the activity of Bmp2 and Shh from the oral roof ectoderm, resulting in the patterning of Rathke’s pouch along its dorsoventral axis. An array of transcription factors subsequently orchestrates the position-dependent commitment and differentiation of progenitor cells to the endocrine cell types of the adenohypophysis (Ericson et al., 1998; Treier et al., 1998; for reviews, see Dasen and Rosenfeld, 2001; Zhu et al., 2007).

The molecular control of neurohypophysis development is much less clear. In mice mutant for Tcf4, Sox3 and Wnt5a, or conditionally mutant for Shh in the hypothalamus, evagination of the primary infundibulum is severely compromised, possibly owing to a failure to restrict expression of Bmp4 and Fgf8 in the ventral diencephalon.

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(Brinkmeier et al., 2007; Potok et al., 2008; Rizzotti et al., 2004; Zhao et al., 2012). In mice lacking the transcription factor genes *Hesx1, Lhx2, Nkx2-1* and *Rax*, as well as in *Hes1 Hes5* double mutants, the infundibulum does not form at all (Dattani et al., 1998; Kimura et al., 1996; Kita et al., 2007; Medina-Martínez et al., 2009; Takuma et al., 1998; Zhang et al., 2000; Zhao et al., 2010). How these and other factors are integrated to pattern the diencephalon and direct the localized formation of the infundibulum, and how subsequent differentiation of the neurohypophysis is controlled, are unresolved questions.

**Tbx2** and **Tbx3** encode a closely related pair of transcriptional repressors that direct differentiation and patterning processes in the development of numerous vertebrate organs (Naiche et al., 2005). Both genes have recently been shown to be expressed in the ventral diencephalon and the infundibulum (Pontecorvi et al., 2008), but a functional requirement in pituitary development has not been reported. Here, we show that **Tbx3**-deficient mice lack a neurohypophysis and have a degenerated adenohypophysis. We trace these defects to a failure to establish a Shh territory in the ventral diencephalon, and present the molecular mechanisms for repression of Shh by **Tbx3** in this domain.

**MATERIALS AND METHODS**

**Mice**

Mice carrying a null allele of **Tbx2** [**Tbx2**<sup>−/−</sup>;**R26mTmG**/+] or of **Tbx3** (Aanhaanen et al., 2009) or of **R26mTmG**/−/− and 2× **Rosa26mTmG**/−/− (Hoogars et al., 2007), and the **Tbx2**<sup>tm1.1(cre)Vmc**>**Tbx3**<sup>tm1.1(cre)Vmc**>**R26mTmG**/−/− (Muzumdar et al., 2007) were maintained on an NMRI outbred background.

**Tbx3**-deficient mice lack a functional requirement in pituitary development has not been reported. Here, we show that **Tbx3**-deficient mice lack a neurohypophysis and have a degenerated adenohypophysis. We trace these defects to a failure to establish a Shh territory in the ventral diencephalon, and present the molecular mechanisms for repression of Shh by **Tbx3** in this domain.

**Histological analysis, RNA in situ hybridization analysis and cellular assays**

Embryos were embedded in paraffin and sectioned at a thickness of 5 or 10 µm. For histological analysis, 5 µm paraffin sections were stained with Hematoxylin and Eosin. RNA *in situ* hybridization analysis on 10 µm paraffin sections was performed following a standard procedure with digoxigenin-labeled antisense riboprobes (Moorman et al., 2001). Cell proliferation rates were investigated by the detection of incorporated 5-bromo-2′-deoxyuridine (BrdU) on 5 µm paraffin sections according to published protocols (Bussen et al., 2004). For each specimen, five adjacent sections were assessed. The BrdU-labeling index was defined as the number of BrdU-positive nuclei relative to the total number of nuclei as detected by DAPI counterstaining. Apoptosis in tissues was investigated by the TUNEL assay using the ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon) on 5 µm paraffin sections. All sections were counterstained with DAPI.

**Immunofluorescence**

For the detection of antigens on 5 µm paraffin sections, the following primary antibodies and dilutions were used: polyclonal rabbit antisera against Otx (Millipore; 1:200) and Avp (Millipore; 1:200) and monoclonal antibodies from mouse against GFP (Roche; 1:200) and from rat against Emcn (a kind gift of D. Vestweber, Max Planck Institute for Molecular Biomedicine, Münster, Germany; 1:5). Labeling with primary antibodies was performed at 4°C overnight after antigen retrieval (Antigen Unmasking Solution, Vector Laboratories) and incubation in 2.5% normal goat serum in PBST (0.1% Tween-20 in PBS) or blocking solutions provided in the Mouse-on-Mouse Kit (Vector Laboratories). Primary antibodies were visualized with Alexa488- or Alexa555-conjugated secondary antibodies (Invitrogen; 1:200).

**Image analysis**

Sections were photographed using a Leica DM5000 microscope with Leica DFC300FX digital camera. Laser scanning microscopy was performed for immunofluorescence staining using a Leica TCS SP2 microscope. All images were processed in Adobe Photoshop CS4.

**Plasmid construction**

A cDNA encoding a Flag-tagged version of human SOX2 was cloned into the pcDNA3 expression vector (Invitrogen). The cDNA encoding wild-type human HA-tagged TBX2 was described previously (Habets et al., 2002). The HA-TBX2<sup>ΔRD</sup> deletion construct was generated by removing an internal BsrEI fragment encompassing the repression domain of TBX2 (Jacobs et al., 2000). Mutations of SOX2 and TBX2 were introduced into wild-type constructs by site-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene).

**Transient transfection and dual reporter assays**

CO1 cells were cultured under standard conditions in DMEM (GibcoBRL) supplemented with 10% fetal bovine serum, and transfected at 50–70% confluence using FuGENE 6 (Roche Applied Science). Wild-type or mutant SBE2-luciferase reporter construct (pGL3.23[flu2:2minP]) (1 µg; Promega) was mixed with 2 µg of Flag-SOX2-pcDNA3 and HA-TBX2-pcDNA3.1 plasmid in total, or empty vector for compensation, and 5 ng of pRL-TK vector (Promega) as an internal control, then applied to cells grown in a 3-cm dish. Cells were harvested 48 hours after transfection and analyzed for firefly and Renilla luciferase activities (Dual Luciferase Assay System, Promega). Enhancer activity was expressed as fold induction relative to that of cells transfected with the empty pcDNA3.1 vector. At least three independent experiments were performed for each construct in triplicate.

**Western blot and co-immunoprecipitation**

CO1 cells cultured in 15-cm dishes were transfected with Flag-SOX2-pcDNA3 or HA-TBX2<sup>wt/TBX2<sup>RD</sup>/ pcDNA3.1 or co-transfected with both in a 1:8 ratio. Forty-eight hours after transfection, cells were washed in cold PBS and transferred to cold lysis buffer (50 mM KCl, 20 mM HEPES, pH 7.9, 2 mM EDTA, 0.1% NP-40, 10% glycerol, 0.5% non-fat milk). The cell lysate was pre-cleared with protein A and G agarose beads (Upstate) by rotating at 4°C for 2 hours, then incubated with anti-Flag M2 beads or monoclonal anti-HA Agarose (Sigma) overnight. Beads were washed four times with lysis buffer by rotating for 4–5 minutes at 4°C followed by centrifugation at 3000 rpm (956 g) for 3 minutes. After the final wash, the protein beads were spun down at high speed, incubated in 2× sample buffer, boiled, and separated by SDS-PAGE. Western blots were performed as previously described (Zhao et al., 2009).

**Chromatin immunoprecipitation (ChIP)**

CO1 cells cultured in a 15-cm dish were co-transfected with an SBE2-luciferase reporter construct and one or more of the following: Flag-SOX2-pcDNA3, HA-TBX2<sup>wt/TBX2<sup>RD</sup>/ pcDNA3.1, or co-transfected with both in a 1:8 ratio. Forty-eight hours after transfection, cells were washed in cold PBS and transferred to cold lysis buffer (50 mM KCl, 20 mM HEPES, pH 7.9, 2 mM EDTA, 0.1% NP-40, 10% glycerol, 0.5% non-fat milk). The cell lysate was pre-cleared with protein A and G agarose beads (Upstate) by rotating at 4°C for 2 hours, then incubated with anti-Flag M2 beads or monoclonal anti-HA Agarose (Sigma) overnight. Beads were washed four times with lysis buffer by rotating for 4–5 minutes at 4°C followed by centrifugation at 3000 rpm (956 g) for 3 minutes. After the final wash, the protein beads were spun down at high speed, incubated in 2× sample buffer, boiled, and separated by SDS-PAGE. Western blots were performed as previously described (Zhao et al., 2012) using antibodies against Flag and HA (Sigma). PCR was performed as described (Jeong et al., 2008).

**Electromobility shift assay (EMSA)**

CO1 cells were transfected with HA-TBX2, TBX2<sup>R26mTmG</sup> or pcDNA3.1. Forty-eight hours after transfection, whole-cell lysates were prepared in a RIPA buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and 0.5% sodium deoxycholate and protease inhibitor cocktail. EMSA was performed as described (Jeong et al., 2008). The sequence of the sense strand of oligonucleotides probes is as follows: consensus T-box site: 5′-CTAGATTCTACCCCTAGGTGTTGAAATCTAG-3′, SBE2 T-box site (bold): 5′-ATGGGGGATTAACAGCTCAAGCTTAGCC-3′, SBE2 T-box site mutant (underlined): 5′-ATGGGGGATTAGCTCCAAGCTTGTCCCATAT-3′.

**Human Tbx2 and Tbx3** protein with C-terminally fused myc-tag were generated from pSP64 expression constructs using the TNT-Sp6 High-Yield
Wheat Germ Protein Expression System (Promega). HR-c and HR-c(ΔT-Site) DNA fragments were released as 88-bp NotI fragments from reporter plasmids RC9 and RC14 (Jeong and Epstein, 2003) and end-labeled with [α-32P]dCTP (Hartmann, Braunschweig, Germany). Binding reactions for EMSAs contained 1-1.5 µl of protein in a total volume of 11 µl of buffer (10 mM Tris, pH 7.2, 100 mM KCl, 1 mM DTT, 10% glycerol) with 1 µl complete protease inhibitor mixture (Roche Applied Science) and 1 µg of double-stranded poly(dI-dC) (Pharmacia). Reactions were pre-incubated for 15 minutes on ice before 4000 counts of probe were added. For supershift experiments, 1 µl of anti-myc antibody (9E10, Sigma) was added to the lysate. Complexes were allowed to form at room temperature for 15 minutes before the reactions were loaded on a native 4% polyacrylamide gel (0.5 M Tris-borate-EDTA). Gels were run at 10 V/cm at 4°C for 5 hours before they were dried and exposed to autoradiography film.

RESULTS

Tbx2 expression marks neurohypophysis progenitor cells within the Tbx3+ ventral midline of the diencephalon

Previous analyses demonstrated co-expression of Tbx2 and Tbx3 in the midline of the ventral diencephalon from E9.5 to E15.5. Tbx2 expression was primarily confined to cells within the ventral, whereas Tbx3 expression expanded into the posterior region of the ventral diencephalon. In the dorsoventral axis, Tbx2 was limited to cells within the ventral midline, whereas Tbx3 expression occurred in the mesenchymal cells as well. In addition, transient expression of Tbx2 was observed in mesenchymal cells surrounding the adenohypophysis, which contribute to the hypophyseal portal system, and of Tbx3 in the ventral aspects of the forming adenohypophysis (Pontecorvi et al., 2008). Our expression analysis by RNA in situ hybridization on whole embryos and mid sagittal sections of the head from E9.5 to E18.5 confirmed these findings and showed that expression of both genes was maintained in these domains at least until newborn stages (supplementary material Fig. S1).

To analyze whether Tbx2 expression in the ventral diencephalon is restricted to precursors of the neurohypophysis, we performed cre/loxP-based genetic lineage tracing using a cre knock-in allele of Tbx2 (Tbx2cre) in combination with the sensitive Rosa26ΔmTmG reporter line (Aanhaanen et al., 2009; Muzumdar et al., 2007). Reporter activity, as assessed by immunofluorescent detection of GFP in Tbx2cre/+:Rosa26ΔmTmG/G embryos was first observed at E10.5 in ventral midline cells of the diencephalon in a domain adjacent to Rathke’s pouch (Fig. 1A-C). In accordance with non-neural expression domains of Tbx2, GFP+ cells were also detected in the mesenchymal sheet between the anterior ventral diencephalon and the epithelium of the oral cavity. At E18.5, GFP+ cells were located in the neurohypophysis, including the infundibulum and the pars nervosa (Fig. 1D-F). Co-immunofluorescence with the endothelial marker endomucin (Emcn) (Morgan et al., 1999) demonstrated the contribution of GFP+ cells to the vasculature of the adenohypophysis (Fig. 1G). We conclude that Tbx2 expression within the ventral diencephalon marks progenitor cells of the neurohypophysis as early as E9.5. Tbx3 expression encompasses the domain of Tbx2 but extends more laterally and both anteriorly and posteriorly into the diencephalon.

Tbx2 is dispensable for pituitary development

To study the requirement of Tbx2 in pituitary development, we maintained a Tbx2-null allele (Tbx2−/−) on an NMRI outbred background that supported viability of homozygous mutant mice (Tbx2KO) until shortly after birth. Hematoxylin and eosin (HE) staining of mid sagittal sections of E18.5 Tbx2KO heads showed a pituitary gland that was indistinguishable from control embryos (supplementary material Fig. S2A,A’). To confirm the presence of a functional posterior lobe, we analyzed a set of region- and cell type-specific markers. In situ hybridization analysis showed that cre (from the mutant allele) was restricted to the posterior lobe of Tbx2KO mutants, similar to Tbx2 in wild-type embryos. Expression of Cacna1b (Wang et al., 2009) and Sox10 (Kuhlbrodt et al., 1998) was detected in pituicytes of both wild-type and Tbx2KO embryos. Finally, immunofluorescent detection of Otx and Ascl9 in Tbx2 mutants indicated a normal innervation of the neurohypophysis by projections from hypothalamic nuclei (supplementary material Fig. S2B-F’).

As the infundibulum provides essential cues for adenohypophysis development, we investigated the expression of a panel of molecular markers for adenohypophyseal cell lineages. Expression of Pcsk2 (melanotropes) (Marcinkiewicz et al., 1993), Pome (melanotropes and corticotropes), Gh (somatotropes), Prl (lactotropes), Tshb (thyrotropes) and Fshb (gonadotropes) was not changed upon loss of Tbx2 (supplementary material Fig. S2G-L’). Together, these data suggest that Tbx2 is dispensable for pituitary development.

Loss of Tbx3 disturbs pituitary morphogenesis

To investigate the role of Tbx3 in pituitary development, we performed histological analysis of embryos homozygous mutant for a previously generated Tbx3-null allele (Tbx3−−; synonym: Tbx3KO) (Hoogsaars et al., 2007) from the onset of pituitary formation at E9.5 to E14.5, i.e. shortly before these embryos die as a result of hepatic and cardiovascular defects (Lüdtke et al., 2009) (Fig. 2). At E9.5, a rudimentary pouch was present, and the ventral diencephalon formed a small kink at its most dorsal position both in wild-type and mutant embryos. In E10.5 wild-type embryos, the
pouch was significantly increased in size compared with a day earlier and featured an almost rectangular shape. The ventral diencephalon had evaginated and formed an infundibulum that extended along the dorsal surface of the pouch. In Tbx3KO embryos, the pouch was smaller and the dorsal tip was rounded. The ventral diencephalon adjacent to Rathke’s pouch was thickened and lacked any sign of evagination at this or subsequent stages of analysis. Irrespective of the genotype, Rathke’s pouch had pinched off the oral surface ectoderm to form the prospective adenohypophysis at E12.5. However, its size and morphological complexity was dramatically reduced at this stage and at E14.5 in Tbx3KO embryos (Fig. 2). Transverse sections of the ventral diencephalon at E12.5 confirmed increased stratification of the neuroectoderm in Tbx3KO embryos. The ventral midline in proximity to the adenohypophysis had a normal thickness but showed a reduced mediolateral expansion (supplementary material Fig. S3). We conclude that Tbx3 is crucial for formation of the infundibulum and the posterior pituitary lobe; hypodysplasia of the adenohypophysis might be secondary to changed signaling upon loss of Tbx3 in the ventral diencephalon.

Increased cell proliferation in the ventral diencephalon of Tbx3KO embryos

Abnormal thickening of the ventral diencephalon, loss of infundibulum formation and hypodysplasia of the adenohypophysis in Tbx3KO embryos might result from changed patterns of apoptosis and proliferation during early pituitary development. Apoptosis, as detected by the TUNEL assay, was invariably low in the ventral diencephalon and Rathke’s pouch of both wild-type and mutant embryos at E9.5 and E10.5 (Fig. 3A), but was strongly increased in both compartments at E11.5 and E12.5 (supplementary material Fig. S4), arguing against a causative role for the loss of the infundibulum. By contrast, proliferation, as detected by the BrdU incorporation assay, was significantly increased both at E9.5 (1.2-fold) and at E10.5 (3.4-fold) in the ventral diencephalon of Tbx3KO embryos. In Rathke’s pouch, proliferation was decreased by 15% at E9.5 and by 19% at E10.5 in Tbx3KO embryos (Fig. 3B,C).

As Tbx3 had been implicated in the control of cell cycle progression in previous studies (for a review, see Lu et al., 2010), we analyzed by in situ hybridization the expression of a number of genes encoding cell-cycle regulators at E10.5. We failed to detect expression of the genes of the anti-proliferative factors Cdkn1a (p21), Cdkn1b (p27), Cdkn2a (p16 and p19ARF), Cdkn2b (p15), Cdkn2c (p18) and Cdkn2d (p19) in the ventral diencephalon of both wild-type and Tbx3KO embryos at this stage (supplementary material Fig. S5). Cdkn1c (p57) was expressed in the posterior ventral diencephalon, including the infundibulum region, in wild-type embryos. In the diencephalon of Tbx3KO embryos, Cdkn1c expression was restricted to a smaller domain adjacent to the tip of Rathke’s pouch. Interestingly, we found strong ectopic expression of the pro-proliferative genes Mycn and Ccnd2 in the posterior ventral diencephalon of Tbx3KO embryos, whereas expression of these genes was restricted to small domains in the posterior diencephalon at the level of the mammillary recess in the wild type. Expression of Ccnd1 in the posterior ventral diencephalon was unchanged in the mutant (Fig. 3D). Hence, Tbx3 controls expression of cell-cycle regulators in the ventral diencephalon to restrict cell proliferation in this domain.

Loss of Tbx3 affects growth and patterning of the developing adenohypophysis

To address further how the loss of Tbx3 affects the development of the adenohypophysis, we analyzed the expression of a panel of marker genes associated with growth, patterning and differentiation of this tissue by in situ hybridization at E10.5, E12.5 and E14.5 (Fig. 4).

Lhx3, SIX3 and Pax6 have been implicated in survival and proliferation of precursor cells and establishment of dorsal cell fates, respectively, in the developing adenohypophysis (Gaston-Massuet et al., 2008; Kioussi et al., 1999; Sheng et al., 1996; Zhao et al., 2006). At E10.5, the three genes were co-expressed in the dorsal-most two-thirds of Rathke’s pouch in wild-type embryos. In Tbx3KO embryos, Lhx3 expression was unchanged whereas expression of SIX3 and Pax6 was absent. In E12.5 wild-type embryos, Lhx3 was expressed in the entire adenohypophysis, Nkx3-1 was restricted to the most dorsal aspect, and Aldh1a2 (also known as Raldh2) was expanded ventrally. Pou3f4 (also known as Brn4) was found in an intermediate region overlapping with the ventral domain of Aldh1a2 but was excluded from the most ventral aspect of the adenohypophysis, which was positive for Gata2 and Foxl2. In Tbx3-deficient embryos, Lhx3 expression was preserved. Nkx-3-1 and Aldh1a2 expression was completely abolished whereas Pou3f4 expression was expanded to cover all cells of the rudimentary adenohypophysis excluding the most ventral aspect, which still maintained normal expression of Gata2 and Foxl2 (Fig. 4). At E14.5, expression of Tbx19 (also known as Tpit), Pou1f1 (also known as Pit1) and Cga marks common progenitor pools for the corticotrope/melanotrope, the lactotrope/somatotrope/thyrotrope and the thyrotrope/gonadotrope lineages, respectively, which are thought to be established from dorsal, intermediate and the most ventral precursor cells in the developing adenohypophysis (for reviews, see Dasen and Rosenfeld, 2001; Scully and Rosenfeld, 2002). In Tbx3KO embryos, expression of Tbx19 and Pou1f1 was abolished and strongly reduced, respectively, whereas expression of Cga was unchanged,
indicating the presence of ventral cell lineages only (Fig. 4). We conclude that Tbx3 and Tbx3-dependent signals from the ventral diencephalon are required to establish dorsal cell fates and lineages in the developing adenohypophysis.

**Disturbed anterior-posterior patterning of the ventral diencephalon in Tbx3KO embryos**

Lack of infundibulum formation might reflect the specific loss of primordial segments along the anterior-posterior axis of the ventral diencephalon. To investigate such patterning defects, we analyzed a panel of marker genes that show restricted expression in the ventral diencephalon and/or are required for normal development of the neurohypophysis. At E10.5, i.e. at a stage when the morphological changes in the ventral diencephalon were fully apparent, expression of the transcription factor genes Nkx2-1, Emx2, Otx2, Sox2, Sox3, Six3, Lhx2, Six6 and Rax (Brinkmeier et al., 2007; Furukawa et al., 1997; Jean et al., 1999; Kelberman et al., 2006; Mathers et al., 1997; Oliver et al., 1995; Rizzoti et al., 2004; Simeone et al., 1993; Simeone et al., 1992; Takuma et al., 1998; Zhao et al., 2010) was unchanged in Tbx3KO embryos (supplementary material Fig. S6). Cre expression from the mutant allele was established in Tbx3KO embryos, but appeared to be diminished compared with Tbx3<sup>lox/lox</sup> control embryos; Pou3f4 and Hes5 (Alvarez-Bolado et al., 1995; Hatakeyama et al., 2004; Kita et al., 2007) were ectopically expressed in this region (Fig. 5). By contrast, expression of Tbx2, which marks the prospective neurohypophysis in the wild type, was abolished in the ventral diencephalon of mutant embryos, indicating that Tbx3 is required for formation of the Tbx2<sup>+</sup> infundibulum subdomain.

We next analyzed expression of components of signaling pathways that may account for the observed patterning defects and cellular changes in both the ventral diencephalon and the adenohypophysis of Tbx3-deficient embryos. Fgf8, Fgf10 and BMP4 are expressed in the posterior ventral diencephalon, including the infundibulum, at E10.5 in the wild type (Ericson et al., 1998; Treier et al., 2001). In Tbx3KO embryos, Fgf8 and BMP4 expression was normal, but Fgf10 expression was clearly downregulated. Interestingly, Shh was ectopically expressed in the ventral midline of the anterior diencephalon (Echelard et al., 1993). Ectopic expression of Pch1, a transcriptional downstream target of the Shh pathway (Ägren et al., 2004; Vokes et al., 2007), confirmed that de-repression of Shh is associated with enhanced signaling activity of this pathway in the midline of the ventral diencephalon in Tbx3KO embryos.

To distinguish primary from secondary changes, we analyzed expression of the genes altered at E10.5 in the ventral diencephalon at E9.5, i.e. before infundibulum formation occurs. Expression of Fgf8, Fgf10 and BMP4 was unchanged at this stage whereas ectopic expression of Pou3f4, Hes5, Shh and Pch1 and loss of Tbx2 expression preceded the morphological and histological changes in the Tbx3-deficient ventral diencephalon. As repression of Tbx2 and induction of Pou3f4 by Shh has been suggested in other developmental contexts (Riccomagno et al., 2002; Zhao et al., 2012), we assume that these changes are subordinate to altered activity of the Shh pathway in the ventral diencephalon of Tbx3KO embryos.

**TBX3 inhibits SOX2 from activating SBE2**

Shh brain enhancer-2 (SBE2) is a long-range acting regulatory element that controls the expression of Shh in the rostral diencephalon, including its repression from the ventral midline at the level of the infundibulum (Jeong et al., 2006). Shh transcription in the rostral diencephalon is dependent on several transcription factors, including Sox2 and Sox3, which directly bind to and activate SBE2 (Zhao et al., 2012). To determine how Tbx3 mediates its repressive effects on Shh transcription, we performed a series of co-transfection experiments in COS-1 cells with wild-type and mutant forms of human SOX2 and TBX2/3 (Fig. 6A). As previously demonstrated, SOX2 is a potent activator of SBE2-luciferase expression (Zhao et
al., 2012; Fig. 6B). By contrast, TBX3 (or TBX2) had no effect on SBE2 when transfected on its own (Fig. 6B). However, in the presence of both SOX2 and TBX3 (or TBX2), there was a significant reduction in SBE2-luciferase activity, compared with SOX2 alone (Fig. 6B) suggesting that repression of Shh transcription by Tbx3 results from inhibition of SBE2 activation by Sox2.

Tbx and Sox proteins are known to interact with a variety of protein partners, including each other (Boogerd et al., 2011; Habets et al., 2002; Reményi et al., 2003). To determine whether SOX2 and TBX2/3 can physically interact, we performed co-immunoprecipitation (co-IP) experiments. Given the high degree of homology between TBX2 and TBX3, the availability of already existing TBX2 variants, and the finding that Tbx2 can repress Shh in chick embryos, all subsequent experiments were performed with TBX2 (Lingbeek et al., 2002; Manning et al., 2006). COS-1 cells were transfected with constructs encoding Flag-tagged SOX2, Hemagglutinin (HA)-tagged TBX2 and/or empty vector (Fig. 6A,C). Western blot analysis demonstrated that the anti-HA antibody was able to immunoprecipitate SOX2 and the anti-Flag antibody was able to immunoprecipitate TBX2, suggesting a physical association between SOX2 and TBX2 (Fig. 6C).

Additional co-IP experiments were performed to identify the domains within each protein that are required for their interaction. Three residues at the C-terminus of the SOX2-HMG domain were previously shown to be involved in ternary complex formation with other co-factors (Reményi et al., 2003) (Fig. 6A). To determine whether the same residues are needed for binding with TBX2, constructs carrying mutations in each of them were used in co-IP experiments. Although SOX2R100E/M104E and SOX2R115E maintained their ability to interact with TBX2, a construct containing mutations in all three residues, SOX2R100E/M104E/R115E, did not associate with TBX2 (Fig. 6D).

Key residues in the T-box domain of TBX2 (Arg122/Arg123) are crucial for its DNA-binding activity (Habets et al., 2002; Sinha et al., 2000). Interestingly, SOX2 did not pull down TBX2R122E/R123E, suggesting that these residues constitute a previously unappreciated protein-interaction domain (Fig. 6E, lane 4). In addition to the T-box domain, the C-terminal repression domain of TBX2 was also required for interaction with SOX2 (Fig. 6E, lane 6).

The domains of TBX2 that interact with SOX2 are also needed to repress SBE2 activation by SOX2 (Fig. 6F). Whereas wild-type TBX2 repressed the SOX2-dependent activation of SBE2 in a dose-dependent manner, neither TBX2R122E/R123E nor TBX2RD impeded SOX2 from activating SBE2, even when co-transfected at a much higher ratio (Fig. 6F). Taken together, our results indicate that TBX2/3 can repress SBE2 activation by SOX2 and that this repression might depend on physical interactions between the two proteins.
TBX2/3 disrupts the binding of SOX2 to SBE2

In order to clarify the mechanism by which TBX2/3 prevents SOX2 from activating SBE2, it was important to know whether TBX2/3 binds directly to SBE2 and, if so, what impact this might have on SOX2 activator function. We first addressed this question by performing chromatin immunoprecipitation (ChIP) followed by quantitative PCR (QPCR) to evaluate the extent of SOX2 and TBX2 recruitment to SBE2.

COS-1 cells transfected with SOX2 and SBE2 showed significant enrichment of SBE2 DNA overlapping the previously characterized SOX2-binding site, in SOX2- versus IgG-bound chromatin (Fig. 7A) (Zhao et al., 2012). A similar region of SBE2 DNA was also significantly enriched in TBX2-bound chromatin in COS-1 cells transfected with TBX2 and SBE2 (Fig. 7A). Remarkably, the recruitment of SOX2 and TBX2 to SBE2 was completely abolished in COS-1 cells co-transfected with SOX2 and TBX2 (Fig. 7A). Mutations in TBX2 that blocked its interaction with SOX2 did not compromise the binding of SOX2 to SBE2 (Fig. 7A). Thus, Tbx2/3 might repress Shh transcription by interfering with SOX2 binding to its target recognition sequence in SBE2.

As the TBX2 mutations that disrupt contact with SOX2 also compromise other important aspects of TBX2 function, including DNA binding (R122E/R123E) and co-repressor recruitment (∆RD), it remained uncertain whether ternary complex formation (TBX2-SOX2-SBE2) was a necessary prerequisite for TBX2/3-mediated repression of Shh. Based on the ChIP data, TBX2 is recruited to SBE2. We next set out to determine whether TBX2 binds directly to SBE2.

The 750-bp SBE2 sequence was scanned for T-box binding sites using the rVista tool, but no sequences perfectly matching the consensus were found. After relaxing the stringency to include one or two nucleotide mismatches, two imperfect T-box sites were identified 8 and 30 nucleotides upstream of the Sox2-binding site (Fig. 7B). We determined binding of TBX2 to either of these putative sites by EMSA. A specific protein-DNA complex was observed when a radiolabeled probe overlapping T-box site1, but not site2, was incubated with COS-1 cell lysates transfected with HA-TBX2wt (Fig. 7B; data not shown). This complex showed a similar mobility shift to that formed between TBX2 and a probe matching a consensus T-box binding site (Fig. 7B). No such complex formed in the presence of the DNA binding-defective TBX2R122E/R123E mutant (Fig. 7B). The specificity of this interaction was further confirmed by super-shifting the TBX2-SBE2 complex, albeit partially, with an anti-HA antibody (Fig. 7B).
...of SBE2 T-box site1 did not impede TBX2 from repressing SOX2-dependent SBE2-luciferase activity (Fig. 7D). These results indicate that although TBX2/3 binds SBE2 directly, this binding is not required for TBX2/3 to sequester SOX2 from SBE2.

In summary, Shh expression in the ventral diencephalon is regulated, in part, by SoxB1 (Sox2/3) transcription factors, which directly bind and activate SBE2, a long-range Shh forebrain enhancer (Fig. 7E). However, for the pituitary to develop properly, Shh transcription must also be extinguished in the ventral midline at the level of the infundibulum. Tbx3 mediates this repression of Shh by physically associating with Sox2 and blocking its recruitment to SBE2 (Fig. 7E).

DISCUSSION

Repression of Shh by Tbx2/3 is crucial for infundibulum formation

The infundibulum forms in a highly localized manner from a subregion of the ventral diencephalon. The molecular mechanisms that pattern the ventral diencephalon to drive this dynamic morphogenetic process have been poorly defined. Our analysis has shown that the neurohypophysis derives from a Tbx2+/Shh– subdomain within the ventral diencephalon. Although expression of Tbx3 (and hence cre from the Tbx3cre allele) in the inner cell mass prevented us from using a conditional approach to test directly the functional significance of ectopic Shh signaling in the Tbx3-
deficient ventral diencephalon, a number of findings suggest that repression of Shh by Tbx2/3 is crucial for the formation of the infundibulum.

First, upon conditional deletion of Shh in the diencephalon of SBE2-cre,Shh\textsuperscript{fl/fl} mice, the infundibulum exhibits an ectopic, anteriorly shifted position coinciding with an anterior expansion of Fgf10, Bmp4 and Tbx2 and a reduction of the Shh’ Six6’ domain in the ventral diencephalon (Zhao et al., 2012). Therefore, loss of Tbx2 in Tbx3KO embryos is a likely consequence of ectopic Shh signaling. We cannot exclude a direct regulation, i.e. activation, of Tbx2 by Tbx3. However, as Tbx3 is described as a transcriptional repressor (Brummelkamp et al., 2001), we deem it unlikely.

Second, Shh has been implicated in mitogenic responses in many developmental contexts (Ishibashi and McMahon, 2002; Wang et al., 2010), in some of which increased proliferation was promoted by upregulation of the proto-oncogene Mycn and the G1-cyclin gene Ccnd2 (Kenney et al., 2003; Mill et al., 2005). Previous \textit{in vitro} and \textit{in vivo} analyses showed that Tbx2 and Tbx3 mediate cell cycle progression through repression of cell-cycle dependent kinase inhibitor genes of the Cdkn1 and Cdkn2 families (Lingbeek et al., 2002; Lüdtke et al., 2013). However, we did not detect upregulation of any of these family members in the mutant diencephalon but rather found repression of Cdkn1c and upregulation of the Shh target genes Mycn and Ccnd2. The correlation of increased proliferation and lack of infundibulum formation in Tbx3-deficient embryos does not support a concept that this morphogenetic process is solely driven by the localized increase of cell proliferation. We suggest that coordinated changes of cell shapes could be a crucial factor in initiation of infundibulum formation, and that these tissue changes are disturbed in Tbx3KO embryos, possibly owing to Shh-induced overproliferation.

A crucial requirement for a T-box function to repress Shh in the ventral diencephalon was also reported in the chick. However, prolonged activation of Shh signaling in this domain in the chick resulted in growth arrest and prevented progression of floor plate cells to proliferative Ems2’ hypothalamus progenitor cells but a pituitary defect was not reported (Manning et al., 2006). This suggests that the cellular and molecular programs regulated by Tbx2, Tbx3 and Shh in the ventral diencephalon may have diverged in birds and mammals.

Previous studies showed that mice mutant for Hesx1, Lhx2, Nkx2-1 and Rax, as well as mice double mutant for Hes1 and Hes5 do not form the infundibulum, whereas loss of Tcf4, Sox3 or Wnt5a is associated with impaired infundibulum evagination (Dattani et al., 1998; Kimura et al., 1996; Kita et al., 2007; Medina-Martínez et al., 2009; Takuma et al., 1998; Zhang et al., 2000; Zhao et al., 2010). As we did not detect changed expression of Lhx2, Nkx2-1, Rax, Tcf4 or Sox3 in Tbx3KO embryos, Tbx3 probably acts downstream of these genes or in a parallel pathway to regulate infundibulum morphogenesis.

**Secondary changes in adenohypophysis development**

In Tbx3KO embryos, Rathke’s pouch formed, grew and detached normally from the oral roof ectoderm, compatible with our finding that expression of Bmp4, the crucial factor for induction of Rathke’s pouch (Takuma et al., 1998), and of Fgf8 and Fgf10, which mediate expansion of precursors cells in the dorsal pouch (Ericson et al., 1998), were established normally at E9.5. However, we observed reduced Fgf10 expression at E10.5, and ectopic expression of Ptc1, the target of Shh signaling in the dorsal aspect of Rathke’s pouch at E9.5 and E10.5 (Fig. 5). Subsequent changes in growth (reduced proliferation, increased apoptosis), patterning and differentiation of the developing adenohypophysis (loss of dorsal cell lineages and expansion of ventral intermediate fates) might therefore result from the lack of an infundibular source of Fgf signals as well as from ectopic Shh signals from the ventral diencephalon. In fact, loss of Fgf signaling results in increased apoptosis (De Moerloose et al., 2000; Ohuchi et al., 2000), whereas overexpression of Shh in the developing adenohypophysis causes an expansion of ventral cell lineages and overexpression of the Shh antagonist Hhip in the oral ectoderm in transgenic mice results in loss of the ventral Gata2 and Pou3f4’ cell lineages of the adenohypophysis (Treier et al., 2001). Although mostly associated with pro-proliferative effects (Treier et al., 2001; Wang et al., 2010), abundant Shh signaling may also elicit apoptosis in some developmental contexts (Oppenheim et al., 1999).

As Tbx3 expression is not restricted to the ventral diencephalon but also occurs in the ventral aspects of Rathke’s pouch and the developing adenohypophysis, the observed changes might derive (at least partly) from a cell- and/or tissue-autonomous requirement for Tbx3 in the adenohypophysis. However, the selective loss of dorsal cell lineages and the maintenance of the ventral most cell fates argues against this possibility.

**Repression of Shh expression in the ventral diencephalon by DNA-dependent and DNA-independent activities of Tbx2 and Tbx3**

Between E8.5 and E9.0, Shh is expressed in a continuous band of ventral midline cells of the CNS reaching from the rostral diencephalon to the spinal cord. Expression intensifies and extends into the more rostral aspects of the forebrain until E9.5, but then becomes specifically excluded from the ventral midline and confined to two adjacent ventrolateral stripes in the posterior diencephalon (Echelard et al., 1993). Transgenic reporter assays showed that this complex and dynamic expression pattern results from the combinatorial activity of multiple distinct cis-regulatory elements spread in and around the Shh locus. Shh floor plate enhancer 1 and 2 (SFPE1, SFPE2) were found to mediate expression in the hindbrain and the spinal cord, Shh brain enhancer 1 (SBE1) in the midbrain and caudal diencephalon, SBE2 and SBE4 in the ventral diencephalon and SBE3 and SBE4 in the ventral telencephalon. Members of several transcription factor families, including forkhead (Foxa1, Foxa2), homeobox (Nkx2-1, Six3) and Sox-type HMG-domain proteins (Sox2, Sox3) have been identified as positive regulators of Shh expression in the CNS via binding to these elements (Epstein et al., 1999; Jeong et al., 2006; Jeong and Epstein, 2003; Jeong et al., 2008; Zhao et al., 2012). The identification of an evolutionary conserved T-box transcription factor binding site (T-site) within SFPE2, deletion of which leads to ectopic reporter activation in the ventral midline of the diencephalon, indicated the contribution of T-box transcriptional repressors to the Shh expression pattern as well (Jeong and Epstein, 2003).

Our molecular characterization of Tbx2- and Tbx3-deficient mice identified Tbx3, and not Tbx2 as in the chick, as the crucial factor for Shh repression in this domain (Manning et al., 2006). However, Tbx2 and Tbx3 might actually act redundantly in this context in the mouse as Tbx2 is lost in the Tbx3-deficient ventral diencephalon. Further support for this notion derives from the finding that Tbx2 and Tbx3 behave in an equivalent fashion with respect to their DNA-binding and protein-interaction properties (for reviews, see Lu et al., 2010; Plageman and Yutzey, 2005).

Our experiments identified a novel biochemical mechanism for transcriptional repression by Tbx2 and Tbx3 that is independent
from direct binding to DNA via the T-box but relies on protein interaction by the same domain to sequester the transcriptional activators Sox2 and Sox3 from their binding site in the SBE2 element. Such a DNA-independent function of T-box proteins is not without precedence. In fact, we and others have recently shown that Tbx1 and Tbx20, two related members of the Tbx1 subfamily, attenuate Bmp signaling by sequestering Smad1, Smad5 and Smad8 (Smad – Mouse Genome Informatics) from binding to Smad4 (Fulco et al., 2009; Singh et al., 2009).

Repression of Shh expression in the midline of the ventral diencephalon may not only rely on protein interaction but may additionally utilize the conserved DNA-binding activity of Tbx2 and Tbx3. In fact, in an EMSA with *in vitro* translated myc-tagged Tbx2 and Tbx3 protein and an 88-bp DNA fragment of SFP2E (also termed homology region c, *HR-c*) that harbors the identified T-site (Jeong and Epstein, 2003) as a probe, we found binding of both proteins to this fragment but not to one with a mutated T-site, indicating that Tbx2 and Tbx3 might regulate Shh expression in this region by direct transcriptional repression (supplementary material Fig. S7).

To this point, we cannot formally distinguish whether Shh repression in *in vivo* relies on DNA-dependent (via SBE2) and/or on DNA-independent (via SFP2E) mechanisms. An answer to this question may derive from analysis of SFP2E and SBE2 reporter expression in a Tbx3-deficient background. However, given the severe consequences of a failure in downregulating Shh in the midline of the ventral diencephalon, we assume that *in vivo* both mechanisms cooperate to ascertain Shh repression.

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