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Lack of the ventral anterior homeodomain transcription factor VAX1 leads to induction of a second pituitary

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SUMMARY

The pituitary gland is an endocrine organ that is developmentally derived from a fold in the oral ectoderm and a juxtaposed fold in the neural ectoderm. Here, we show that the absence of *Vax1*, a homeodomain transcription factor known for its role in eye and optic chiasm development, causes the rostral oral ectoderm to form an ectopic fold that eventually develops into a separate second pituitary with all the pituitary cell types and neuronal fibers characteristic of the normal pituitary. The induction of the second pituitary is associated with a localized ectopic expression of *Fgf10*, a gene encoding a growth factor known to recruit oral ectodermal cells into the pituitary. Interestingly, there are rare cases of pituitary duplications in humans that are also associated with optic nerve dysplasia, suggesting that VAX1 might be involved in the pathogenesis of this disorder.

KEY WORDS: Midline defects, Septo-optic dysplasia, Adenohypophysis, Neurohypophysis, FGF signaling, Ectopic pituitary, Mouse

INTRODUCTION

The pituitary is a crucial hormone release site that is derived from an evagination of the oral ectoderm, termed Rathke's pouch, and a part of the diencephalic neuroectoderm, termed infundibulum. The developmental interactions between these tissues are regulated by many factors, including transcription factors such as *Pitx1* and *Pitx2* and their target, *Lhx3*, which are specifically expressed in the oral ectoderm/Rathke's pouch; others, such as *Six3/6*, *Sox2*, *Hesx1* and *Pax6*, which are found in both oral and neuroectoderm; and growth factors such as fibroblast growth factor 8 and 10 (FGF8, FGF10), bone morphogenic proteins 2 and 4 (BMP2/4), sonic hedgehog (SHH), NOTCH, and WNT, which are expressed in the neural or oral ectoderm, or in both tissues (Sheng et al., 1996; Treier et al., 1998; Suh et al., 2002; Charles et al., 2005; Zhu et al., 2007; Davis et al., 2009; Kelberman et al., 2009). Not surprisingly, transcriptional and signaling pathways intersect during pituitary development. For example, loss of TCF7L2, a high-mobility group transcription factor that serves as mediator of canonical WNT signaling, leads to a rostral expansion of FGF and BMP activities in the neuroectoderm, a rostral expansion of *Six6* expression in the oral ectoderm, and an increased recruitment of cells into the pituitary (Brinkmeier et al., 2007).

Here, we demonstrate a novel role for the ventral homeodomain protein VAX1 in pituitary development. In the mouse, *Vax1* is initially expressed in most of the ventral neuroectoderm and then is found in the telencephalon, the ventral diencephalon and the optic stalk (Hallonet et al., 1998; Bertuzzi et al., 1999). Mice with mutations in *Vax1* show prominent perturbations in the development of the optic chiasm and die at birth due to cleft palate (Bertuzzi et al., 1999). As shown here, they also display a second, more rostrally located, Rathke's pouch. This second pouch is completely separated from the primary pouch, matures into an ectopic adenohypophysis containing all pertinent cell lineages and exhibits posterior pituitary markers, indicating the presence of an ectopic neurohypophysis. Given that *Vax1* is not expressed in the oral ectoderm or the pituitary primordium, it probably exerts its effects indirectly. In fact, our results show that the major role of *Vax1* in pituitary development is to limit the zone in which FGF10 can act as a pituitary-inducing signal, thereby assuring that a single, correctly positioned pituitary is formed.

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MATERIALS AND METHODS

Mice

The *Vax1* knockout allele, *Vax1*^{tm1Grl}, here referred to as *Vax1*$^{-}$, has been described previously (Bertuzzi et al., 1999). The allele was kept on a mixed C57BL/6;129S1/Sv background.

Immunofluorescence, immunohistochemistry and in situ hybridization

All histological analyses were performed according to Bharti et al. (Bharti et al., 2008). Antibodies are shown in Table 1. The following in situ probes were used: *Vax1* (Bertuzzi et al., 1999); *Pit1*, *Pomc*, *Lhx3* (Sheng et al., 1996); *Six3* (Oliver et al., 1995); *Id3* (Jones et al., 2006); *Six6*, *Pitx2*, *Tcf7L2*, *Fgf10*, *Bmp4*, *Gli1*, *Shh* and *Ptch1* (all from Open Biosystems, Huntsville, Al). *Tcf7L2* encodes a high-mobility-group transcription factor that is also called *Tcf4* (T-cell specific factor 4; MGI:1202879), but the symbol *Tcf4* is officially assigned to another gene (transcription factor 4; MGI:98506), which encodes a bHLH protein.

Chromatin immunoprecipitation (ChIP) and reporter assays

For ChIP assays, ventral forebrain tissue from six to eight embryos (E10.5-E11, wild-type or *Vax1*$^{-/-}$) was processed as described previously (Skuntz et al., 2009), using a rabbit anti-VAX1 antiserum (see Table 1) for immunoprecipitation. For sequences of primers used for ChIP assays and reporter construct cloning, see Table S1 in the supplementary material. Reporter assays were performed as described previously (Bharti et al., 2008) (see legend to Fig. S4 in the supplementary material).

RESULTS AND DISCUSSION

Loss of Vax1 expression leads to a second Rathke's pouch

As shown previously, in the mouse, *Vax1* expression starts at embryonic day (E) 8 (Hallonet et al., 1998). At both E11.5 and E13.5, *Vax1* is expressed in the diencephalic neuroepithelium but excluded from the infundibulum and the oral ectoderm or Rathke's pouch (Fig. 1A,B).

Table 1. Antibodies used

Number	Antibody	Species	Source	Dilution
1	Anti-phosphohistone H3	Rabbit	Upstate Biotechnology (Billerica, MA, USA)	1:100
2	Anti-phospho-Erk1/2	Rabbit	Cell Signaling Technologies (Danvers, MA, USA)	1:100
3	Anti-phospho-Smad1/5/8	Rabbit	Chemicon (Temecula, CA, USA)	1:200
4	Anti-GH	Rabbit	National Hormone Pituitary Program (NHPP) (NIDDK, NIH)	1:500
5	Anti-ACTH	Rabbit	NHPP (NIDDK, NIH)	1:500
6	Anti-TSH	Rabbit	NHPP (NIDDK, NIH)	1:500
7	Anti-LH	Rabbit	NHPP (NIDDK, NIH)	1:500
8	Anti-neurophysin (RN2) (Wray et al., 1988)	Rabbit	A gift from Dr Susan Wray (NINDS, NIH)	1:10,000
9	Anti-vasopressin (VA-4) (Alstein et al., 1988)	Rabbit	A gift from Dr Susan Wray (NINDS, NIH)	1:5000
10	Anti-TUJ1	Mouse	Convance (Princeton, NJ, USA)	1:1000
11	Anti-rabbit-alexafluor 488	Goat	Invitrogen (Eugene, OR, USA)	1:500
12	Anti-mouse-alexafluor 568	Goat	Invitrogen (Eugene, OR, USA)	1:1000
13	Anti-rabbit-AP	Goat	Vector labs (Burlingame, CA, USA)	1:1000
14	Anti-VAX1 (Bertuzzi et al., 1999)	Rabbit	A gift from Dr Greg Lemke (Salk Institute, La Jolla, CA, USA)	1:100

While analyzing *Vax1* mutant brain sections at E13.5, we noted the presence of an ectopic pouch-like structure underneath the diencephalic neuroectoderm rostral to Rathke's pouch (Fig. 1C,D; arrow in Fig. 1D). Interestingly, this pouch-like structure expressed the pituitary progenitor markers *Lhx3* and *Pitx2* (Fig. 1E-H; arrows in F,H), as did the normal Rathke's pouch in either wild type or *Vax1* mutants. It also expressed pro-opiomelanocortin (*Pomc*), which normally marks corticotropes, one of the first anterior pituitary cell types to differentiate at E13.5 (Japon et al., 1994; Pulichino et al., 2003) (Fig. 1I,J; arrow in J). These results suggest that the loss of *Vax1* causes the induction of a second, more rostrally located Rathke's pouch.

The second Rathke's pouch contains all anterior pituitary cell types and is associated with a second posterior pituitary

To test whether the second pouch would mature into an ectopic adenohypophysis and eventually lead to the formation of a full second pituitary, we performed additional histological and gene expression studies at later stages of development. With increasing developmental time, the second pouch grew to a larger structure that in some embryos showed a bipartite lumen (Fig. 2B, arrow and arrowhead). It continued to express *Lhx3* and *Pitx2*, as well as *Six3* (Fig. 2C-F, see Fig. S1A,B in the supplementary material). *Pit1*, a regulator of somatotropes, thyrotropes and lactotropes, that normally starts to be expressed at E13.5 (Dasen et al., 1999; Nica et al., 2004), was also found in the second pouch at E13.5 (not shown) and continued to be expressed at this site at E17.5 (Fig. 2G,H). The expression of *Pomc* continued through E15.5 (see Fig. S1C,D in the supplementary material) and beyond (not shown), and one of its products, adreno-corticotropic hormone (ACTH), started to be expressed at E13.5 (not shown) and continued through E15.5 and E17.5 (see Fig. S1E-G in the supplementary material; Fig. 2I,J). Moreover, additional markers were expressed in the second pouch in a temporal sequence reminiscent of that found in the primary pouch (Japon et al., 1994; Pulichino et al., 2003). They included thyroid-stimulating hormone (TSH), starting at E15.5 (not shown) and continuing through E17.5; growth hormone (GH) at E17.5; and luteinizing hormone (LH) at E17.5 (see Fig. S1H-L in the supplementary material; Fig. 2K,L). Interestingly, the temporal similarities in gene expression extended to the anatomical locations of the respective markers. For example, ACTH-positive cells, which are normally found at the periphery of the primary pouch, were also located at the periphery of the second pouch (Fig. 2J). Corresponding

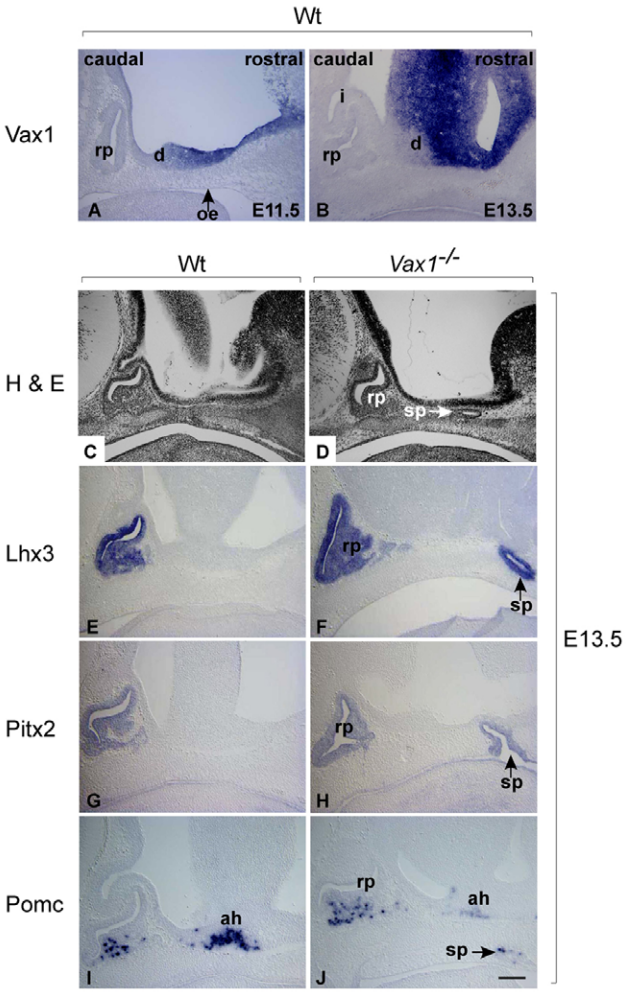

Fig. 1. Loss of *Vax1* leads to induction of a second pouch-like structure in the ventral diencephalon. For all figures, caudal is on the left and rostral is on the right. (**A**,**B**) *Vax1* in situ hybridization on wild-type sections at the indicated times. In wild type, *Vax1* is not seen in oral ectoderm, Rathke's pouch or infundibulum. (**C**,**D**) Hematoxylin and Eosin staining of E13.5 wild-type and *Vax1*$^{-/-}$ sections. Arrow in D shows a distinct, pouch-like structure. (**E-J**) In situ hybridization on E13.5 *Vax1*$^{-/-}$ sagittal sections shows ectopic signals (arrows) for the pituitary markers *Lhx3* (E,F), *Pitx2* (G,H) and *Pomc* (I,J). ah, anterior hypothalamus; d, diencephalon; i, infundibulum; oe, oral ectoderm; rp, Rathke's pouch; sp, second pouch. Scale bar: 113 μm for A,B,E-J; 141 μm for C,D.

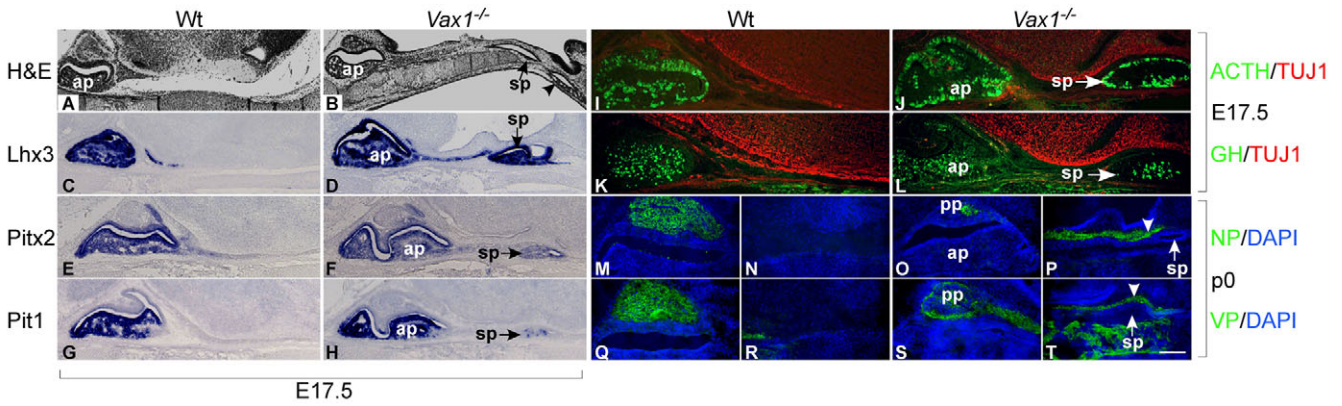

Fig. 2. The second pituitary expresses both anterior and posterior pituitary markers. (**A**,**B**) Hematoxylin and Eosin staining of E17.5 wild-type (A) and *Vax1*$^{-/-}$ (B) sagittal section, showing a large ectopic second pouch, marked with an arrow and arrowhead. (**C-H**) In situ hybridization for the indicated genes. (**I-T**) immunostaining for the indicated proteins. (I-L) Co-staining with TUJ1 antibody that labels neurons. For P,T, the second anterior pituitary is marked with arrows and the second posterior pituitary with arrowheads. M and N, O and P, Q and R, and S and T represent paired caudal and rostral parts of the ventral diencephalon. ap, anterior pituitary; pp, posterior pituitary; sp, second pituitary. Scale bar: 42 μm for A,B; 48 μm for C-H; 60 μm for I-L; 110 μm for M-T.

anatomical locations were also found for the centrally located GH- and TSH-positive cells (Fig. 2L, see Fig. S1I in the supplementary material) and the ventrally located LH-positive cells (see Fig. S1L in the supplementary material). Importantly, the primary pouch in *Vax1* mutants, though slightly dysmorphic, displayed temporal and spatial gene expression profiles and histological characteristics that are comparable with those in wild type.

To test whether the formation of an ectopic anterior pituitary was followed by development of a corresponding posterior pituitary, we then examined the expression of the carrier proteins neurophysin (NP) I and II and the hormone vasopressin (VP). In wild type, these markers label neurons extending caudally from the hypothalamus to the posterior pituitary lobe (Zhu et al., 2007). In *Vax1* mutants, we found these markers to extend caudally towards the primary pituitary and rostrally towards the second pituitary (Fig. 2M-T; see Fig. S1M,N in the supplementary material). The results suggest that in the absence of *Vax1*, the ectopically formed anterior pituitary is associated with the development of a second posterior pituitary.

The second Rathke's pouch originates from hyperproliferating rostral oral ectodermal cells

The above results show that a second pituitary develops in *Vax1* mutants but they do not address the initial steps, and hence the underlying molecular mechanisms, that lead to this ectopic pituitary. In fact, the first sign of an abnormality was seen at E10.5, when we observed a slight rostral shift of the rostral expression boundaries of *Lhx3* and *Pitx2* (Fig. 3A-D). The first anatomical alteration was not seen before E11.5, when the rostral oral ectoderm showed a thickening (Fig. 3E,F, compare insets showing magnifications) that now ectopically expressed the above two markers (Fig. 3G-J) as well as an additional Rathke's pouch marker, *Six6* (Fig. 3K,L). Furthermore, two targets and mediators of SHH signaling, *Gli1* (Fig. 3M,N) and *Ptch1* (see Fig. S2A,B in the supplementary material) (Treier et al., 2001), were also expressed at the ectopic rostral location, suggesting that SHH signaling was intact at this site. Moreover, *Shh*, which normally is excluded from the invaginating primary pouch, was also excluded from the second pouch (arrows in Fig. S2D,F in the supplementary material), but was present in the remainder of the rostral oral ectoderm (arrowheads in Fig. S2D,F in the supplementary material). The oral ectodermal thickening was probably due to increased cell proliferation as the number of phosphohistone H3-positive cells was increased in the oral ectoderm, but not in the primary Rathke's pouch of the mutants (Fig. 3O,P; oral ectoderm marked with white lines, rostral areas magnified in insets, Rathke's pouch marked with white stippled lines; see Fig. 3Q for quantitation). The increase in the number of phosphohistone H3-positive cells was consistent with an increase in the total number of oral ectodermal nuclei counted after DAPI staining (289±53 in wild type compared with 369±83 in the mutant; average of cell counts from three mid-sagittal sections of three different embryos each). Because no changes were found by TUNEL labeling (not shown), the oral ectodermal thickening was unlikely to be due to reduced rates in cell death. Furthermore, serial frontal sections labeled for *Lhx3* and *Pitx2* showed a label-free area of at least 160 μm between the labeled caudal and rostral sections, indicating a total separation of the two pouches even in the parasagittal area (see Fig. S2G-L in the supplementary material). The above observations confirmed that the formation of the second pouch was initiated from a separate region of the rostral oral ectoderm.

The induction of the second pouch correlates with ectopic Fgf10 expression in the neuroectoderm

It has been observed previously that embryos lacking *Tcf7L2* show a rostral expansion of the expression of *Bmp4* and *Fgf10*, both of which encoding pituitary inducing signals (Brinkmeier et al., 2007). *Tcf7L2* expression, however, was not changed in the neuroectoderm of *Vax1* mutants (Fig. 4A,B, arrowheads). Consistent with this finding, the expression of *Bmp4* and *Fgf10* remained largely unchanged in the caudal neuroectoderm, as did that of *Id3*, a *Bmp4* target, and phosphoSMAD (pSMAD), which serve as BMP effectors (see Fig. S3A-H in the supplementary material; Fig. 4C,D). Furthermore, *Bmp4* was also not induced in the neuroectoderm at the site of the second pouch (arrowhead in Fig. S3A,B in the supplementary material; Fig. 4C,D). Consistent with this observation, *Id3* and pSMAD were also not specifically expressed in the neuroectoderm or the underlying oral ectoderm at this site (arrowheads and arrows in Fig. S3C-F in the supplementary material), in contrast to *Fgf10*, which was strongly expressed at this location after E11.5 (see Fig. S3G,H in the supplementary material;

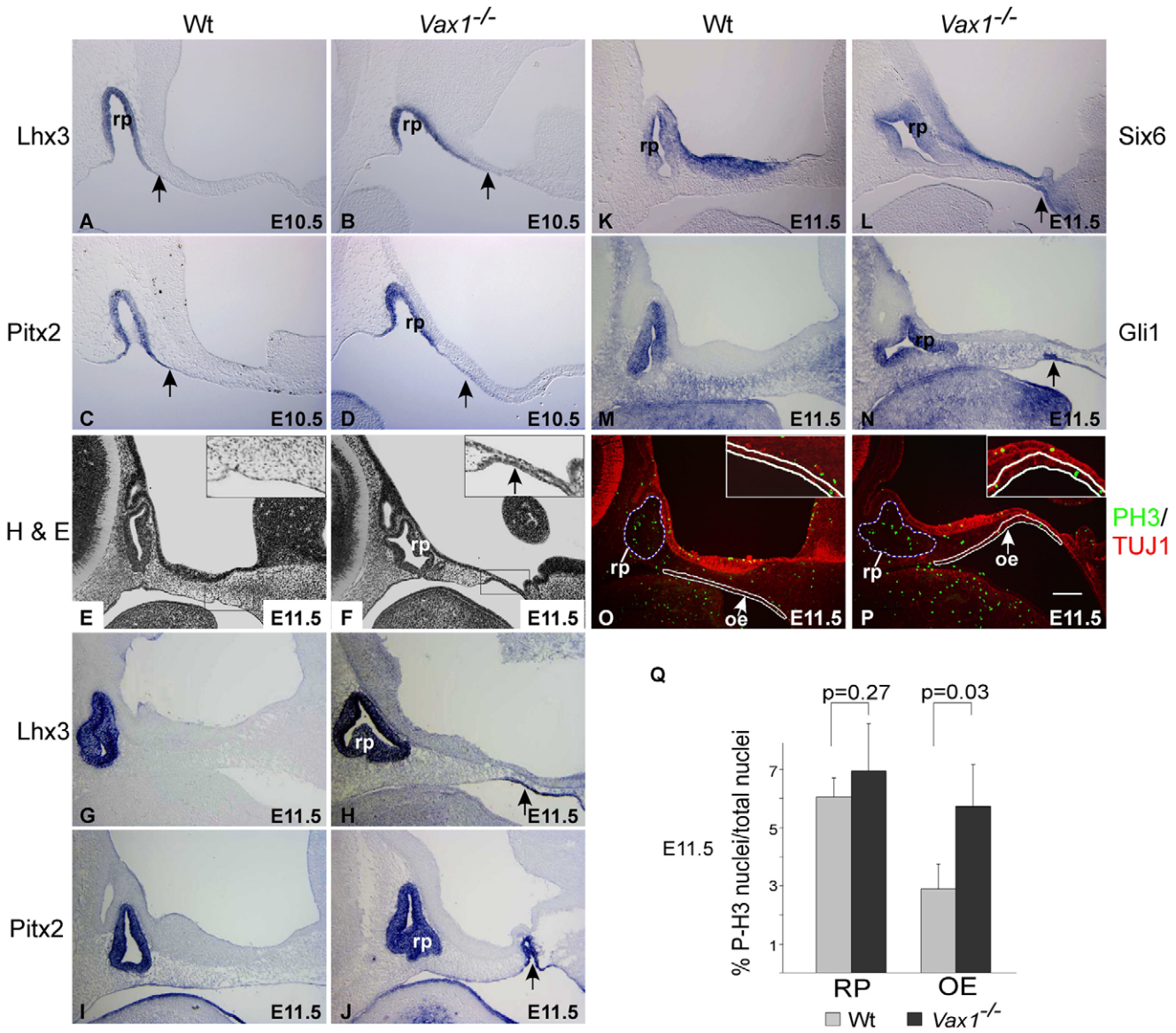

Fig. 3. The second pouch in *Vax1* mutants is induced from the rostral oral ectoderm. (**A-D**) In situ hybridization on E10.5 sections shows a slight rostral expansion of *Lhx3* (A,B) and *Pitx2* labeling (C,D) in *Vax1* mutants (arrows). (**E**,**F**) Hematoxylin and Eosin staining of E11.5 wild-type and *Vax1*$^{-/-}$ sections. Higher magnification in the inset marks thickening in *Vax1* mutant rostral oral ectoderm (arrow). (**G-N**) In situ hybridization performed on sections from E11.5 wild-type and *Vax1*$^{-/-}$ embryos shows rostral ectopic signals (arrows) for the indicated genes. (**O**,**P**) E11.5 embryo sections stained for phosphohistone H3 (green) and TUJ1 (red) show an increased number of phosphohistone H3-positive nuclei in the rostral oral ectoderm (marked with white lines) but not in Rathke's pouch (marked with stippled white lines) of *Vax1* mutants. Insets show magnification of rostral oral ectoderm. (**Q**) Quantitation of number of nuclei positive for phosphohistone H3 labeling, from three mid-sagittal sections from three embryos each. Data are mean±s.d. oe, oral ectoderm; rp, Rathke's pouch. Scale bar: 141 μm for A-D,G-N; 113 μm for E,F,O,P.

Fig. 4E-H, arrowheads). At this time point, strong expression of the FGF effector proteins pERK1/2 extended caudally and rostrally in the neuroectoderm and the underlying oral ectoderm (Fig. 4I,J, arrows pointing to the oral ectoderm). *Bmp4*, however, was only seen at the site of the second pouch (see Fig. S3I,J in the supplementary material) after the *Fgf10* signal became prominent and the pouch was already induced. This suggests that *Bmp4* was not needed for inducing the second pouch, although it may be involved in its later development.

The above observations suggested that, in the neuroectoderm, *Vax1* might regulate *Fgf10* directly rather than through *Tcf7L2*. To analyze whether VAX1 protein binds putative regulatory elements in the *Fgf10* promoter, we performed chromatin immunoprecipitation (ChIP) assays using a VAX1 antiserum and chromatin extracts prepared from mouse E10.5 ventral forebrains, a stage at which the absence of *Vax1* does not yet cause major alterations in gene expression or anatomy. Sequences within ~5 kb of the *Fgf10* upstream region, containing potential conserved homeodomain-binding sites, were amplified (Odenwald et al., 2005; Mui et al., 2005) (see Fig. S4A,B in the supplementary material). As shown in Fig. 4K, ChIP assay showed amplification only of amplicons that contained highly conserved consensus homeodomain binding sites (amplicons 'b' and 'e' containing at least one ATTAA/TTAAT sequence), and not of amplicons that

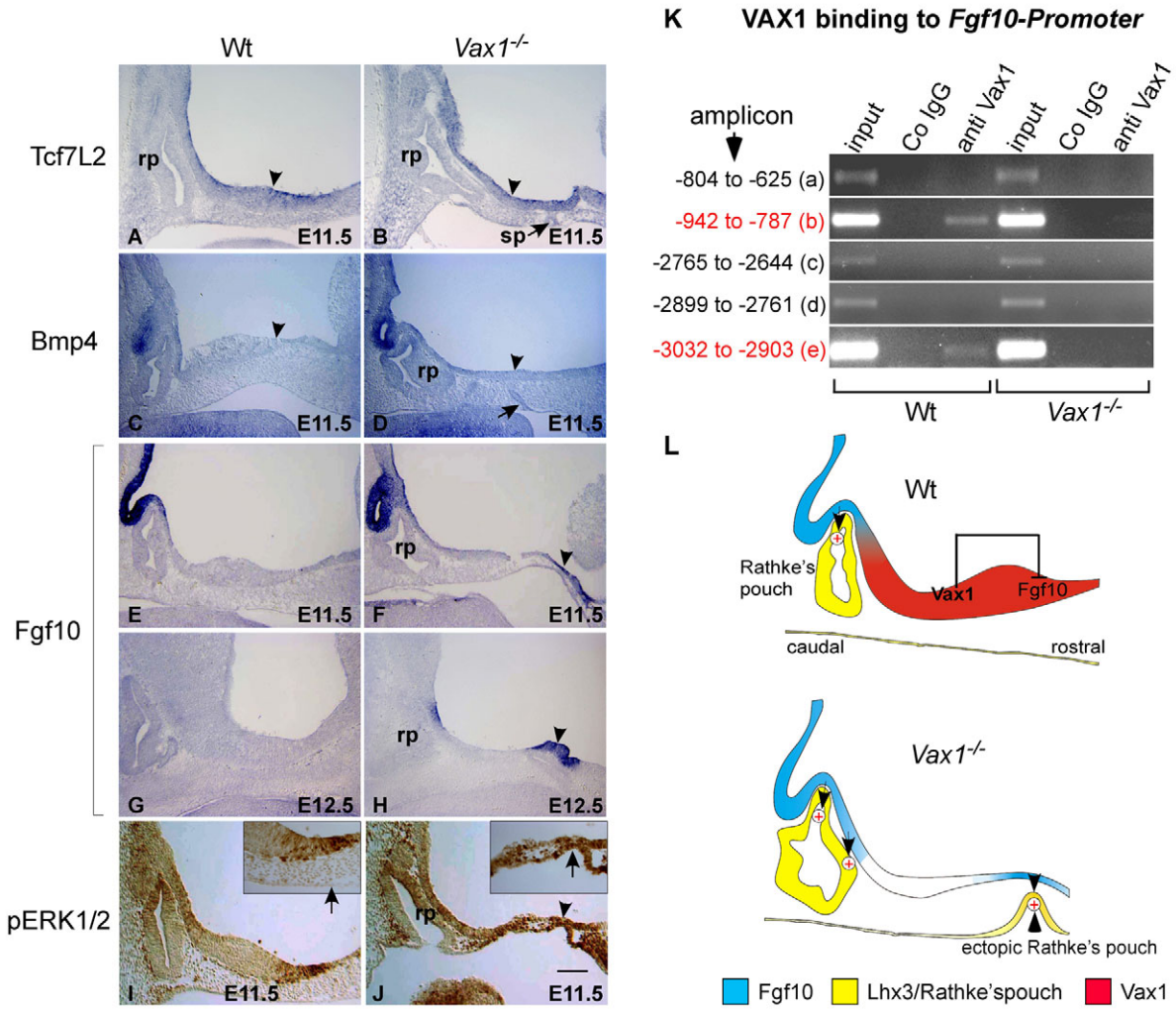

Fig. 4. Absence of *Vax1* induces *Fgf10* expression in the rostral neuroectoderm. (**A-D**) In situ hybridization for *Tcf7L2* (A,B) and *Bmp4* (C,D) indicates unchanged expression in the pituitary neuroectoderm (arrowheads). Arrow indicates the second pouch. (**E-H**) In situ hybridization for *Fgf10* shows ectopic expression in the rostral neuroectoderm (arrowhead) at E11.5 (E,F) and E12.5 (G,H). (**I**,**J**) Immunohistochemistry for pERK1/2, showing ectopic signal in rostral neuroectoderm (arrowhead) and rostral oral ectoderm (arrows; compare higher magnifications in insets). (**K**) VAX1 binds to conserved putative homeodomain-binding sites containing subregions of the *Fgf10* promoter in vivo. Amplicon positions are indicated relative to the translation start site (see also Fig. S4 in the supplementary material). Amplicons 'b' and 'e' show positive bands in wild type but not in *Vax1*$^{-/-}$ mutants. (**L**) A regulatory model highlighting the role of Vax1 in pituitary induction (for details, see text). Scale bar: 113 μm for A-J.

contained only the core part (ATTA,TAAT) of this sequence (amplicons 'a', 'c', 'd') (see Fig. S4A,B in the supplementary material). The fact that extracts from *Vax1* mutant embryos gave no signals indicates that the ChIP assays were specific. To confirm that these sites were functional, we co-transfected luciferase reporter constructs containing *Fgf10* promoter fragments with or without 131 bp representing amplicon 'e' (HD6-9 in Fig. S4B in the supplementary material) into HEK293 cells. These cells were chosen because they share neuroepithelial/neuronal characteristics (Shaw et al., 2002). As shown in Fig. S4C in the supplementary material, the HD6-9 deletion construct gave a higher signal compared with that containing HD6-9, as it did in a fibroblastic (NIH3T3) and a retinal pigment epithelial (ARPE19) cell line (not shown). This indicated that this region has repressive functions. As expected, *Vax1* co-transfection repressed the full-length construct but interestingly also the deletion construct, conceivably because this deletion construct still contained a consensus HD-binding site (see Fig. S4A,B in the supplementary material).

As schematically depicted in Fig. 4L, our findings lead to a model of pituitary biogenesis in which *Vax1* plays an important role as a repressor of *Fgf10* expression in the neuroectoderm rostral to the infundibulum, thereby restricting its expression to the infundibulum. When *Vax1* is absent, *Fgf10* is prominently expressed at the site of second pouch induction. Nevertheless, this *Fgf10* expression is not contiguous between primary and second pouch, conceivably because of the continuous expression of *Tcf7L2,* the absence of which, as mentioned, leads to an expansion of *Fgf10* expression (Brinkmeier et al., 2007). This interpretation is consistent with the fact that expression of constitutively active β-catenin, which is known to act in conjunction with Tcf factors, suppresses the *Fgf10* promoter (not shown). Finally, it is worth mentioning that the phenotype of the *Vax1* mutation in mice shares similarities with septo-optic dysplasia in humans, which is characterized by malformed optic discs, a missing septum pellucidum and pituitary defects (Mutlu et al., 2004; Kelberman and Dattani, 2006; Mazzanti et al., 2009; Kelberman et al., 2009).

This disorder is genetically heterogeneous and in a subset of individuals has previously been associated with mutations in *HESX1*. Although *Hesx1* expression was unchanged in *Vax1* mutant mice (not shown), the human homolog *VAX1* remains a candidate gene for pituitary duplications in humans in which no *HESX1* mutations have been found.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Table S1. Primers

Number		
	ChIP primers – *Fgf10* promoter	**Amplicon position**
1	For: 5′-acctccttggaagctggcagg-3′ Rev: 5′-atgtgtttctgccttccagatcc-3′	–804 to –625
2	For: 5′-ttcatttgcgccaagagagagg-3′ Rev: 5′-ccagcttccaaggaggtctcg-3′	–942 to –788
3	For: 5′-ggctttctggggctaagctaagagg-3′ Rev: 5′-ccaaaggaaaagctgacatagctttttag-3′	–2765 to –2644
4	For: 5′-gaagtaactgagaatgattcagg-3′ Rev: 5′-agcctgagaaaagttccacagg-3′	–2899 to –2761
5	For: 5′-gggtaccattctaatctgaagc-3′ Rev: 5′-gactgaaattctacctacctaccttcc-3′	–3032 to –2889
	Cloning primers – WT *Fgf10* promoter	**Cloning site**
	For: 5′-atctg<u>gaagctc</u>attctgccctttcaatcttaacag-3′ Rev: 5′-ctgct<u>aagctt</u>tcttgggcgagaggagtggc-3′	*Sac*I *Hin*dIII
	Cloning strategy – del (HD6-9) *Fgf10* promoter	
	Wild-type promoter was cut at *Sac*I and *Stu*I restriction sites, which flank homeodomain-binding sites sites 6-9. The cut construct was end filled by T4 DNA polymerase for blunt-end cloning, resulting in a construct that contains a small 131 bp deletion and lacks HD sites 6-9.	*Sac*I *Stu*I T4 DNA polymerase filled in and blunt end ligated