Molecular Endocrinology. First published ahead of print April 27, 2012 as doi:10.1210/me.2011-1319

ORIGINAL RESEARCH

# The HMG-Box Transcription Factor Sox4b Is Required for Pituitary Expression of *gata2a* and Specification of Thyrotrope and Gonadotrope Cells in Zebrafish

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The pituitary is a complex gland comprising different cell types each secreting specific hormones. The extensive network of signaling molecules and transcription factors required for determination and terminal differentiation of specific cell types is still not fully understood. The SRY-like HMG-box (SOX) transcription factor Sox4 plays important roles in many developmental processes and has two homologs in zebrafish, Sox4a and Sox4b. We show that the *sox4b* gene is expressed in the pituitary anlagen starting at 24 h after fertilization (hpf) and later in the entire head region including the pituitary. At 48 hpf, *sox4b* mRNA colocalizes with that for TSH (*tshβ*), glycoprotein subunit α (*gsuα*), and the Zn finger transcription factor Gata2a. Loss of Sox4b function, using morpholino knockdown or expression of a dominant-negative Sox4 mutant, leads to a drastic decrease in *tshβ* and *gsuα* expression and reduced levels of *gh*, whereas other anterior pituitary gland markers including *prl*, *slβ*, *pomc*, and *lim3* are not affected. Sox4b is also required for expression of *gata2a* in the pituitary. Knockdown of *gata2a* leads to decreased *tshβ* and *gsuα* expression at 48 hpf, similar to *sox4b* morphants. Injection of *gata2a* mRNA into *sox4b* morphants rescued *tshβ* and *gsuα* expression in thyrotrope cells. Finally, *sox4b* or *gata2a* knockdown causes a significant decrease of gonadotropin expression (*lhβ* and *fshβ*) at 4 d after fertilization. In summary, our results indicate that Sox4b is expressed in zebrafish during pituitary development and plays a crucial role in the differentiation of thyrotrope and gonadotrope cells through induction of *gata2a* expression in the developing pituitary. (***Molecular Endocrinology* 26: 1014–1027, 2012)**

In the last decades, knowledge concerning development of the pituitary gland has been gained from identification of genes involved in human congenital pituitary hormone deficiency, a pathology characterized by hypopituitarism (1). Also, spontaneous mutations and reverse genetics in murine models revealed that normal anterior pituitary formation is dependent upon a complex genetic cascade of signaling molecules and transcription factors (1–3). More recently, studies in mammals have been complemented by those in zebrafish that might prove useful to uncover thus far unidentified mechanisms and regulators of pituitary development and to better understand the evolution of this important gland.

The basic mechanisms of anterior pituitary development appear to be conserved between fish and mammals. The mature gland is formed of several cell types, each secreting a specific hormone (4): somatotropes producing GH, lactotropes secreting prolactin (Prl), thyrotropes synthesizing TSH, and gonadotropes producing LH and FSH. The glycoprotein α-subunit (Gsuα) is expressed in thyrotropes and gonadotropes, where it associates respectively with Tshβ or Lhβ and Fshβ. ACTH and MSH, both proteolytically cleaved from the same precursor proopiomelanocortin, are secreted by, respectively, corticotropes

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2011-1319 Received November 11, 2011. Accepted March 30, 2012.

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Abbreviations: BMP, Bone morphogenetic protein; coMO, control morpholino; dpf, days after fertilization; Gsuα, glycoprotein α-subunit; hpf, hours after fertilization; KO, knockout; MO, morpholino; PPE, preplacodal ectoderm; qRT-PCR, quantitative real-time RT-PCR; Prl, prolactin; SOX, SRY-related HMG box; WISH, whole-mount *in situ* hybridization; wt, wild type.

and melanotropes. An additional pituitary hormone is present in teleosts, somatolactin, closely related to Prl and Gh. Two *sl* genes have been identified in zebrafish that are expressed in specific domains of the adult gland (5). One of them, *slβ*, was shown to be expressed in wild-type (wt) zebrafish from 23 h after fertilization (hpf) onward (6).

Like in other vertebrates, the initial steps of zebrafish anterior pituitary formation start when gastrulation is completed at approximately 10 hpf. Specific transcription factors gradually restrict pituitary cell development and promote their differentiation into specific cell types. At this phase of development, the neural plate is bordered caudally by the neural crest and rostrally by the preplacodal ectoderm (PPE). Hedgehog signaling from the ventral forebrain specifies the median region of the PPE to form the pituitary placode (7). At this early stage, the PPE is characterized by the expression of multiple marker genes, such as eyes absent (*eya1*) and sine oculis (*six1*) (8), the bicoid-related paired-like homeobox gene *pitx3* or the *dlx3b* gene (7, 9). The first anterior pituitary-specific marker to come up during the mid-segmentation stage (18–20 hpf) is the Lim-domain homeobox gene *lim3*, which is expressed in all anterior pituitary precursor cells. Another early marker is the POU domain homeobox factor Pit1 (10, 11). In contrast to the pan-pituitary marker *lim3*, *pit1* is activated in just a subset of anterior pituitary cells. In mouse, loss of Pit1 function leads to absence of *gh*- and *prl*-expressing cells and a trans-fating of thyrotropes to gonadotropes (10, 11). Zebrafish *pit1* mutants appear to display a similar thyrotrope to gonadotrope trans-fating (11). Another factor specifically involved in thyrotrope and gonadotrope formation in the mouse is the Zn-finger transcription factor Gata2 (12). During normal thyrotrope development, Pit1 attenuates expression of the *lhβ* gene by binding and blocking the transcription factor Gata2 (12).

Transcription factors of the SRY-related HMG box (SOX) family are characterized by the presence of a highly conserved, DNA-binding HMG box domain. These factors are widely and dynamically expressed throughout embryogenesis and have been implicated in many developmental processes (13) ranging from early cell fate determination (14) to control of lens development (15–17) or chondrogenesis (18–20). Sox3 was the first member of this family to be associated with X-linked hypopituitarism in mice and humans (21, 22). It is expressed in the ventral diencephalon and in the infundibulum, adjacent to the Rathke's pouch, but not in the presumptive anterior pituitary (21–23). Sox2 was similarly found to be critical for the development of the hypothalamo-pituitary axis by maintaining a pool of undifferentiated cells in the pituitary (24, 25).

Sox4 is a member of the group C Sox proteins that contains a C-terminal transcription activation domain conserved within this group and has been involved in the development of various structures (26–30). Two Sox4 homologs are present in zebrafish, but only *sox4b* was shown to be expressed in pancreas and required for differentiation of glucagon-expressing cells (31), similar to the function of Sox4 in normal development of pancreatic islets in mouse (32). Recently, SOX4 was found to be one of the main transcription factors expressed in fetal pituitary in human (33). However, its function in the pituitary has not been investigated so far.

Here, we investigate the function of the zebrafish Sox4 homolog, Sox4b, in pituitary development. We show that *sox4b* is specifically expressed in the anterior pituitary primordium at 24 and 48 hpf, more specifically in *gsuα*-, *gata2a*- and *tshβ*-expressing cells. We show that *sox4b* is required for the differentiation of thyrotrope and gonadotrope cells, and finally, we demonstrate that *gata2a* expression is regulated by Sox4b in developing zebrafish pituitary.

## Materials and Methods

### Zebrafish maintenance, mutant lines, and microinjection

Zebrafish (*Danio rerio*) were maintained under standard conditions (34) in the GIGA-R zebrafish facility. Wild-type embryos from the AB strain were produced and staged according to Kimmel *et al.* (35). The *pit1* mutant was previously described (8, 11, 36).

Antisense morpholinos (MO) (Gene Tools, Philomath, OR) were used. Standard control MO (coMO) 5′-CCTCTTACCTCAGTTACAATTTATA-3′ is known to have no target and no significant biological activity in zebrafish embryos; *sox4b*MO 5′-GACTCAGTCTGATTGCACACAGTCC-3′ and *sox4b*MO2 5′-TGCTGCTGGATCTCTGGAGCAT-3′, targeting the 5′-untranslated region of *sox4b* or the first 25 bases of the coding sequence, respectively, to block translation, were previously described (31) and were injected at an optimal dose of 7 ng/egg or 2 ng/egg, respectively; and *gata2a*MO 5′-CATCTACTCACCAGTCTGCGCTTTG-3′ is a splice blocking MO targeting the third exon/intron boundary of the *gata2a* gene (30) and was used at a dose of 0.5 ng/egg.

The mRNA coding for the dominant-negative mutant Sox4bΔC (37) or enhanced green fluorescent protein was injected at 0.2 ng/egg. To perform phenotypic rescue experiments, *gata2a* mRNA was synthesized and then injected into *sox4b*- or *gata2a*MO-injected or wt embryos at concentrations of 100 and 75 pg, respectively. MO were diluted in Danieau's buffer containing 0.5% rhodamine-dextran for checking the injection.

Microinjection was performed at the one-cell stage; the injected embryos were fixed at different stages in 4% paraformaldehyde and stored in 100% methanol before analysis.

## Riboprobes and whole-mount *in situ* hybridization (WISH)

Single and double whole-mount and double-fluorescent *in situ* hybridizations were performed as previously described (37, 38) on wt and mutant embryos. Antisense RNA probes were prepared by transcribing linearized cDNA clones with SP6, T7, or T3 polymerase using digoxigenin labeling mix (Roche, Indianapolis, IN) or DNP-11-UTP ribonucleotides (TSAi Plus system; PerkinElmer, Norwalk, CT). The riboprobes used were *lim3*, *gata2a*, *pit1*, *prl*, *gsuα*, *tshβ*, *gh* and *slβ* (6, 11, 39–41) as described. The *lhβ* cDNA (438 bp) (accession no. HE608243) covering the partial coding region was obtained by performing RT-PCR on mRNA from 5-d-old [days after fertilization (dpf)] zebrafish larvae using the primers zfLHBfor3 (cagcctgctgagcaac) and zfLHBrev1 (cctctctctgggacatgcagaag) and contained three silent mutations relative to the reference *lhβ* sequence.

## Fluorescence imaging

For confocal analysis, images were acquired using a Leica TCS SP2 inverted confocal laser microscope (Leica Microsystems, Heidelberg, Germany) equipped with one argon and two helium-neon lasers. Digitized images were acquired using a ×10 (NA 0.4) or ×63 (NA 1.4) Plan-Apo water-immersion objectives at 1024- × 1024-pixel resolution. The diameter of the pinhole was set up equal to the Airy unit. Series of optical sections were carried out to analyze the spatial distribution of fluorescence, and for each embryo, they were recorded with a Z-step ranging set to 2.0 μm. Images were acquired under identical conditions, and we ensured that the maximal fluorescence signal was not saturating the photomultiplier tubes. For multicolor imaging, fluorescein isothiocyanate was visualized by using an excitation wavelength of 488 nm, and the emission light was dispersed and recorded at 500–535 nm. Cy3 was detected by using an excitation wavelength of 543 nm and the 488/543 dichroic mirror, and the emission light was dispersed and recorded at 595–650 nm. The acquisition was set up to avoid any cross talk of the two fluorescence emissions. Captured images were exported as TIFF format files and further processed using Adobe Photoshop.

## RNA extraction and reverse transcription

Total RNA was extracted from about 100 larvae per experiment using the RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After extraction, the quality and concentration of total RNA was evaluated by electrophoresis on capillary gel and the ratio of absorbance at 260/280 nm by spectrophotometer. Synthesis of cDNA was performed from 1 μg total RNA, which was reverse transcribed using the transcriptor iScript cDNA synthesis kit (Bio-Rad, Nazareth, Belgium) according to the manufacturer's instructions.

## Real-time PCR

Gene-specific oligonucleotide primers were developed using the Primer3 software and selected so as to span exon-exon junctions to avoid detection of genomic DNA (see Table 1 for primers used in quantitative RT-PCR) and synthesized by Eurogentec (Seraing, Belgium).

Real-time PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with SYBR green fluorescent label. Samples (25 μl final volume) contained the following: 1× SYBR green master mix (Diagenode, Liège, Belgium), 150 nmol of each primer, and 1 μl of the reverse transcription reaction (1/20 of the total cDNA). Samples were run in duplicate in optically clear 96-well plates (ABgene, Epsom, UK). Cycling parameters were 50 C for 2 min, 95 C for 10 min, and then 40 cycles of 95 C for 15 sec and 60 C for 1 min. A melting temperature-determining dissociation step was performed at 95 C for 15 sec, 60 C for 15 sec, and 95 C for·15 sec at the end of the amplification phase. For analysis by endpoint PCR, the final products of the quantitative real-time RT-PCR (qRT-PCR) obtained after 40 PCR cycles were loaded on agarose gel for electrophoresis.

For the test of gonadotropin expression (*lhβ* and *fshβ*), thermal cycling was performed on an Applied Biosystems 7900 HT sequences detection system. The cDNA was used for qRT-PCR with the SensiMix SYBR Kit (Bioline, London, UK), containing SYBR green. The standard conditions were used with a modification in the elongation step: 62 C for 20 sec for *lhβ* and 62 C for 30 sec for *fshβ* were used.

No-template controls were run for all reactions, and all RNA preparations were subjected to sham reverse transcription to check for the absence of genomic DNA amplification. The relative transcript level of each gene was obtained by the $2^{-\Delta\Delta Ct}$ method (42) and normalized relative to the housekeeping gene elongation factor1α (*ef1α*) with the program SDS version 2.2. Results are presented as percent expression in MO-injected embryos relative to clutchmate control embryos ± SD.

## Statistical analysis

Statistical analysis was performed on raw data using one-way ANOVA. Significance was set at $P < 0.01$. Data from biological replicates were averaged and shown as mean normalized gene expression ± SD.

**TABLE 1.** List of primers used for RT-PCR

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| *tshβ* | CTGTCAACACCACCATCTGC | GTGCATCCCCTCTGAACAAT |
| *gsuα* | TCACATCAGAAGCCACTTGC | GTGGCAGTCTGTGTGGTTGT |
| *lim3* | AGGAGGACGGCATGGACA | ATCGGACATGGGCGGC |
| *ef1α* | ACATGCTGGAGGCCAGCTC | TACCCTCCTTGCGCTCAATC |
| *pomc* | AGCTCAGTGTTGGGAAAACG | GGTAGACGGGGGTTTCATCT |
| *prl* | ATGACAAAGACCAAGCCATG | GTTCTGGATGTGCCAGACTG |
| *gh* | CCTCTGTCGTTCTGCAACTC | ACTCCCAGGATTCAATGAGG |
| *slβ* | GTATATTCCTGGAGGGGCTG | ATTCACCAATGGCTCAATCC |
| *lhβ* | GCAGAGACACTTACAACAGCC | AAAACCAAGCTCTGGAGCAGCC |
| *fshβ* | CTGCAGATGAGGATGCGTGTGC | CTATGCTGGACAATGGATCG |

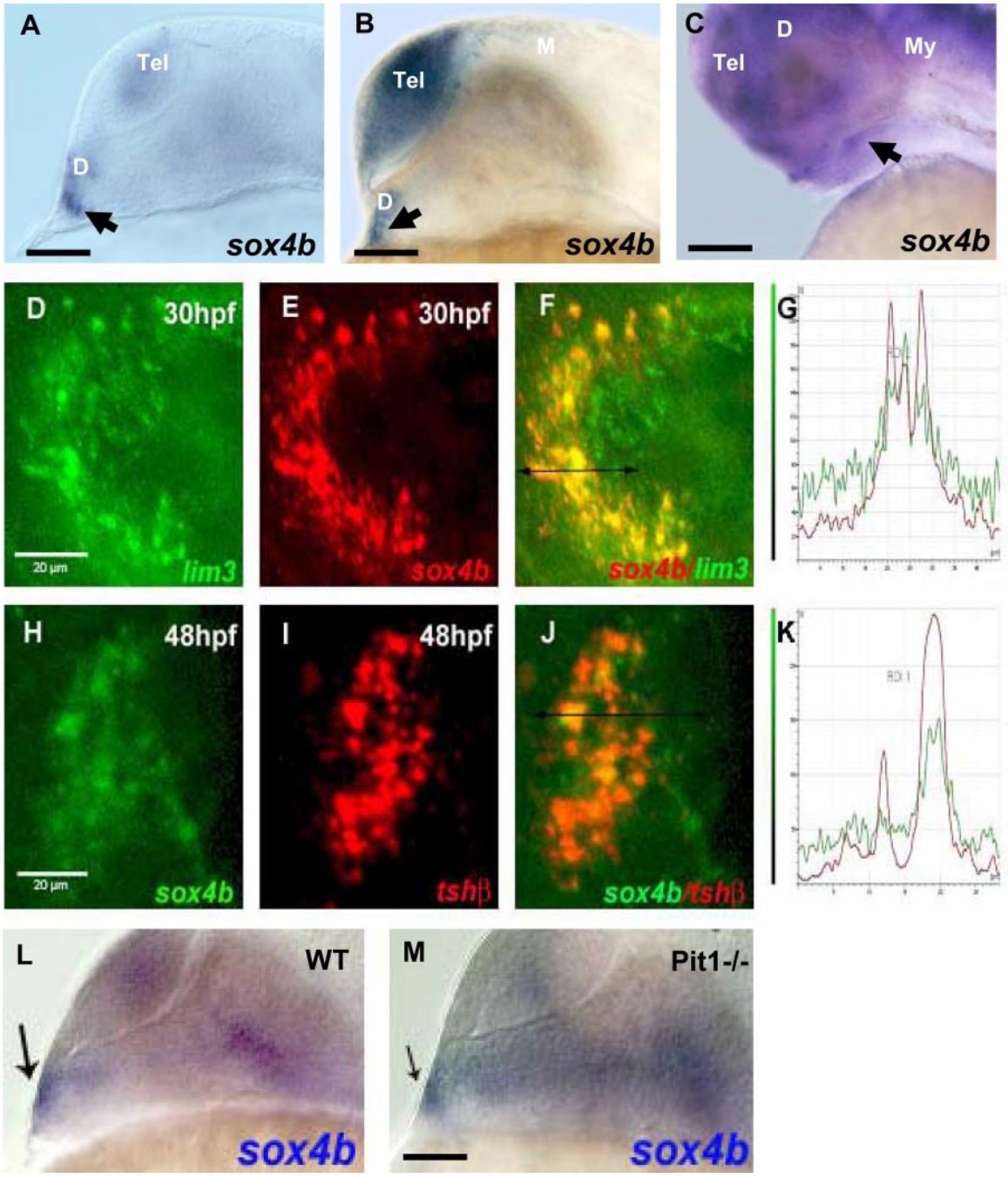


**FIG. 1.** *sox4b* is expressed in the pituitary during zebrafish organogenesis. WISH was performed on wt zebrafish embryos using the *sox4b* antisense probe. A–C, Lateral views (anterior to the *left*, dorsal to the *top*) of wt embryos labeled with the *sox4b* probe (*blue*) by WISH. The *arrowheads* indicate the pituitary placode at 24 (A), 26 (B), and 48 (C) hpf, respectively. D–F and H–J, Analysis by double-fluorescent WISH (anterior to the *left*), with one z-plane of the confocal images shown; D–F, expression of *sox4b* (*red*, E) and the pan-adenohypophyseal marker *lim3* (*green*, D) colocalize in the pituitary placode (F); G, fluorescence emission quantification in F; H–J, *sox4b* (*green*, H) and *tshβ* (*red*, I) are coexpressed in a subtype of pituitary cells (J); K, fluorescence emission quantification in J. In D–F and H–J, the ventral head regions were dissected before confocal microscopy. L and M, *sox4b* expression does not require *pit1* at 24 hpf. Analysis of *sox4b* expression by WISH in wt and homozygous *pit1* mutants. Lateral view, anterior to the left, the *arrow* indicates the label in the pituitary. *Scale bars*, 100 $\mu$m (A, B, C, L, and M) and 20 $\mu$m (D–J). D, Diencephalon; M, mesencephalon; My, myencephalon; Tel, telencephalon.

## Results

### *Sox4b* is expressed in the developing anterior pituitary

The expression pattern of *sox4b* mRNA in developing pituitary was determined by WISH experiments in zebrafish embryos. Starting at 24 hpf, *sox4b* mRNA was detected in the anterior border of the neural plate, corresponding to the early pituitary placode (Fig. 1A). At 26 hpf, *sox4b* expression is increased in the adjacent ventral

diencephalon and predominant in the telencephalon (Fig. 1B), although at later stages (48 hpf), *sox4b* mRNA is detected in broad regions of the developing forebrain and midbrain and in a subpopulation of cells in the developing anterior pituitary (Fig. 1C). Double-fluorescent *in situ* hybridizations analyzed by confocal microscopy revealed that *sox4b*-positive cells in the ventral neural layer also express the pituitary precursor marker *lim3* at 30 hpf, confirming that they are located in the anterior pituitary primordium (Fig. 1, D–G). Finally, at 48 hpf, a clear colocalization of *sox4b* mRNA was observed with *tshβ* mRNA (Fig. 1, H–K), suggesting that *sox4b* is expressed in thyrotrope cells.

*Sox4b* expression was also assessed in the previously described pituitary mutant *pit1* (8, 11, 36). *Sox4b* mRNA was readily detected in pituitaries of *pit1* mutant embryos similar to their heterozygous or wt siblings at 24 hpf (Fig. 1, L and M).

### Knockdown of *Sox4b* eliminates expression of *tshβ* and *gsuα* in the pituitary

To investigate the function of Sox4b in pituitary development, we microinjected antisense MO oligonucleotides to ablate *sox4b* expression as previously described (31) and analyzed specific gene expression by WISH and by qRT-PCR in 48-hpf embryos. Control experiments checking pancreatic markers in the injected embryos confirmed the previously observed effect on glucagon expression (31) (Table 2). When the presence of the various pituitary hormone mRNA was investigated in these morphants (summarized in Table 2), we observed a drastic reduction of *tshβ* expression in *sox4b*MO-injected embryos at 48 hpf (Fig. 2, A and D). Similarly, the expression of *gsuα* was specifically reduced in *sox4b* morphants (Fig. 2, B and E), indicating that the two hormone subunits specific to thyrotrope cells are no longer expressed in these embryos. *gh* expression was reduced in 83% of the *sox4b*MO-injected embryos (Fig. 2, C and F), whereas *slβ*, *prl*, and *pomc* (Fig. 2, G–L) did not seem to be affected. Expression of *gh*, *tshβ*, and *gsuα* was also absent in 4-dpf *sox4b* morphants (Table 2) and in 48-hpf embryos previously injected with a different MO (*sox4b*MO2) against *sox4b* (Table 3). To confirm these results observed by *in situ* hybridization, qRT-PCR were performed using mRNA extracted from microinjected 48-hpf morphants and control embryos. The results confirmed the strong down-regulation of *tshβ*, *gsuα*, and *gh* expression (Fig. 3), whereas *prl*, the widely expressed *lim3* factor (see also below) or the control mRNA *ef1α* (elongation factor 1) were not affected.

To confirm the specificity of the effects observed by MO injection, we abolished Sox4b function by microinjecting an mRNA coding for a Sox4b dominant-negative mutant, lacking its transactivation domain (*sox4b*ΔC) (31). Injection of *sox4b*ΔC mRNA into wt embryos also resulted in a decrease of *gsuα*, *tshβ*, and *gh* expression, whereas *prl* is not affected (Fig. 4), thus confirming the specificity of the defects observed upon MO injection.

In conclusion, our results indicate that *sox4b* knockdown specifically affects expression and differentiation of thyrotrope and somatotrope cells.

**TABLE 2.** Sox4b regulates expression of *tshβ, gsuα*, and *lhβ* in zebrafish

| | NI normal | coMO normal | *sox4b*MO absence | *sox4b*MO decrease |
|---|---|---|---|---|
| **Embryo, 48 hpf** | | | | |
| ***tshβ*** | 10/10 | 89/100 | 12/122 | 110/122[a] |
| ***gsuα*** | 10/10 | 38/40 | 0/38 | 38/38[a] |
| ***glucagon*** | 10/10 | 70/78 | 0/78 | 78/78[a] |
| ***slβ*** | 8/10 | 38/40 | 5/40 | 0/40 |
| ***pomc*** | 10/10 | 40/40 | 0/40 | 0/40 |
| ***lim3*** | 10/10 | 38/38 | 2/40 | 0/40 |
| ***pit1*** | 10/10 | 27/27 | 0/35 | 0/35 |
| ***gata2a*** | 10/10 | 40/40 | 38/40[a] | 0/40 |
| ***gh*** | 9/10 | 45/45 | 0/58 | 48/58[a] |
| ***prl*** | 10/10 | 50/50 | 0/50 | 0/50 |
| **Larvae, 4 dpf** | | | | |
| ***lhβ*** | 10/10 | 50/50 | 0/50 | 28/50 |
| ***tshβ*** | 10/10 | 50/50 | 12/50[a] | 15/50[a] |
| ***gsuα*** | 10/10 | 50/50 | 0/50 | 30/50[a] |
| ***gh*** | 10/10 | 50/50 | 0/50 | 25/50[a] |
| ***prl*** | 10/10 | 50/50 | 0/50 | 0/50 |

Individual embryos, previously noninjected (NI) or injected with coMO or *sox4b*MO, were optically analyzed and compared for expression of different marker genes at 48 hpf and 4 dpf. The embryos were classified according to phenotype, and the number of each class is given relative to the total number of analyzed embryos for each condition. Absence indicates lost expression; decrease indicates that marker expression was reduced by more than 50%.

[a] Significant at $P < 0.001$.

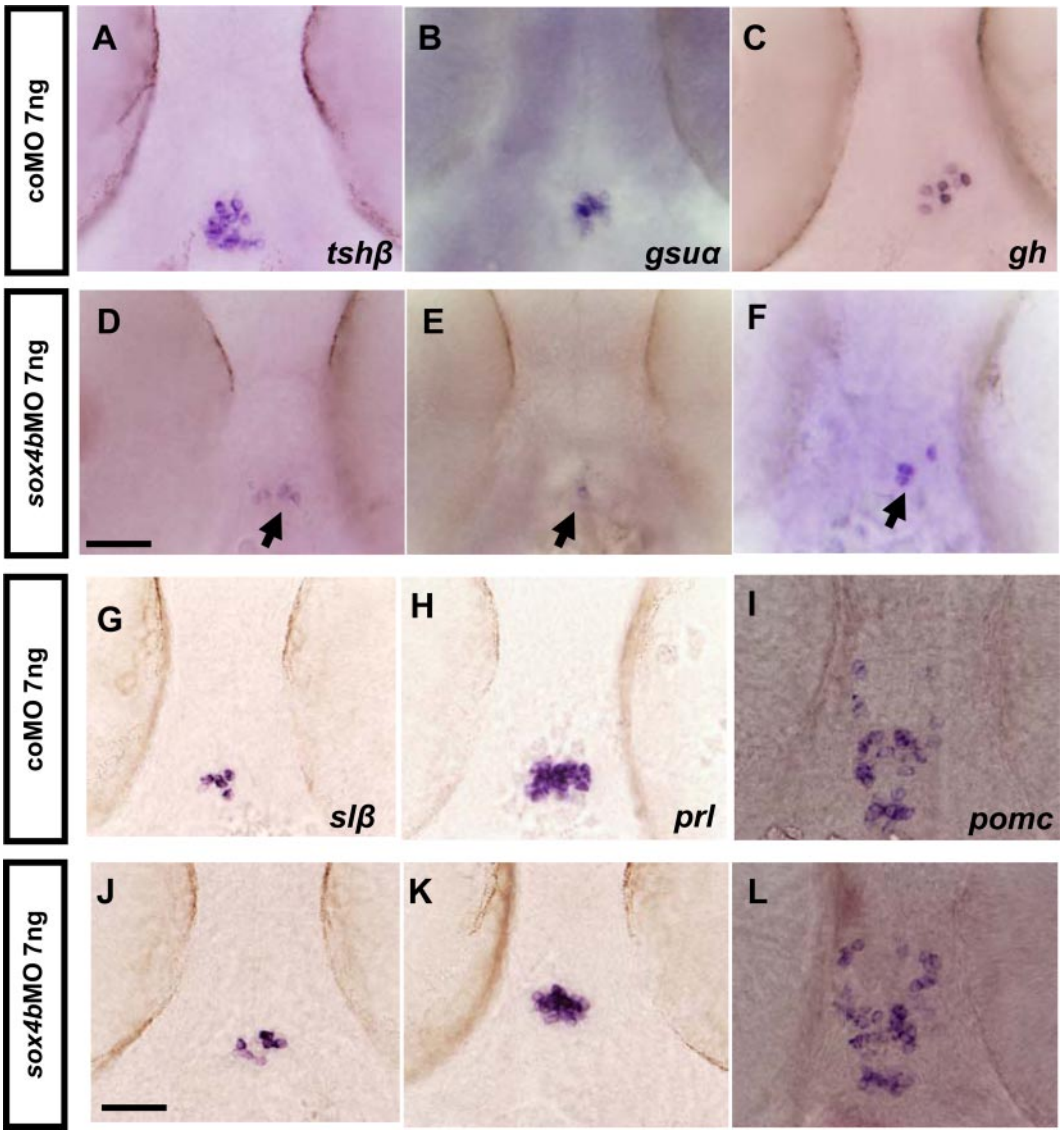


**FIG. 2.** *sox4b* knockdown decreases *tshβ*, *gsuα*, and *gh* expression in 48-hpf embryos. Fertilized eggs were microinjected with *sox4b*MO and analyzed by WISH for pituitary hormone expression. A–C and G–I, Control embryos; D–F and J–L, *sox4b* morphants, ventral views (anterior to the *top*); both *tshβ* (D) and *gsuα* (E) expression is almost completely lost, whereas *gh* expression is decreased (F) and *slβ*, *prl*, and *pomc* are unchanged (J–L). *Scale bars*, 50 $\mu$m (A–L).

### Sox4b is required for the expression of *gata2a* specifically in the pituitary

To shed some light on the molecular mechanisms involved in Sox4b function in the pituitary, we tested for the expression of known transcription factors in *sox4b* morphants. The pan-pituitary marker *lim3* was not affected upon *sox4b* knockdown (Fig. 5, A and D, and Table 2). One of the factors required for thyrotrope differentiation is the pituitary-specific, POU-homeodomain factor Pit1 (10). Zebrafish *pit1* mutants were shown to be deficient in *prl*, *gh*, *slβ*, and *tshβ* expression (6, 11). At 48 hpf, *sox4b*MO-injected em-

**TABLE 3.** Individual embryos, injected with coMO or *sox4b*MO2, were optically analyzed and compared as in Table 1 at 48 hpf

| Embryos, 48 hpf | coMo 2 ng normal | *sox4b*MO2 2 ng absence | *sox4b*MO2 2 ng decrease |
|---|---|---|---|
| *gsuα* | 25/25 | 5/25[a] | 20/25[a] |
| *tshβ* | 25/25 | 0/20 | 20/25[a] |
| *gh* | 25/25 | 15/25[a] | 10/25[a] |
| *prl* | 25/25 | 0/25 | 0/25 |

[a] Significant at $P < 0.001$.

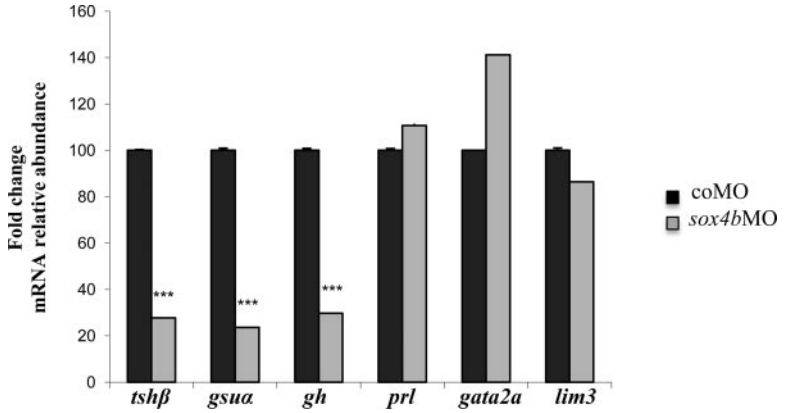


**FIG. 3.** qRT-PCR analysis of mRNA extracted from control and *sox4b* morphants. Total RNA was extracted from 48-hpf embryos injected with coMO or *sox4b*MO, and specific mRNA levels were determined by qRT-PCR. mRNA levels in control embryos were arbitrarily set to 100 for each primer pair and shown as mean relative levels ± SE. The results shown are representative of three independent experiments for each mRNA. ***, Significant at $P < 0.001$.

bryos displayed unaltered *pit1* expression in a frontal view (Fig. 5, B and E).

Another factor specifically involved in pituitary thyrotrope formation in mouse is the Zn-finger transcription factor Gata2 (12, 43). Although *gata2a* is widely expressed in the head region of the developing embryos at 48 hpf, we could clearly observe that its weak expression in the pituitary of coMO-injected embryos was completely absent in *sox4b* morphants (Fig. 5, C and F). Although qRT-PCR cannot directly assess the amount of *gata2a* or *lim3* expression in the pituitary due to their wider expression in the head region, it is noteworthy that the overall amount of *gata2a* and *lim3* mRNA was unchanged (or slightly increased) in the *sox4b* morphants (Fig. 3).

In conclusion, our data indicate that *sox4b* knockdown specifically affects the expression of *gata2a* in the developing pituitary and blocks differentiation of thyrotrope cells.

### *Sox4b* and *gata2a* are specifically coexpressed in the anterior pituitary

Expression of *gata2a* was clearly observed in the pituitary at 48 hpf (Fig. 6A). Relative to the *pomc*-expressing corticotropes (Fig. 6B) and the *prl*-expressing lactotropes (Fig. 6C) located at the anterior border of the pituitary, *gata2a*-expressing cells are detected in a posterior domain. Double-fluorescent *in situ* hybridization revealed that *gata2a* is clearly coexpressed with *gsuα* (Fig. 6, D–G). Similarly, *gata2a* mRNA is colocalized with *sox4b* mRNA (Fig. 6, H–K) and is also present in *tshβ*-expressing cells, whereas some *gata2a*-positive cells did not express *tshβ* (Fig. 6, L–N), probably representing gonadotrope precursor cells. No colocalization was observed between *gata2a* and *gh* mRNA (Fig. 6, O–Q).

Expression of *gata2a* was also tested by double *in situ* hybridization in the previously described *pit1* mutant (8, 11, 36). We observed that expression of *gata2a* was similar to wt or heterozygous siblings at 48 hpf (Fig. 6, R and S).

### Depletion of *gata2a* leads to loss of thyrotrope cells

To compare the effects of *gata2a* and *sox4b* depletion in developing embryos, we microinjected *gata2a* MO (37)

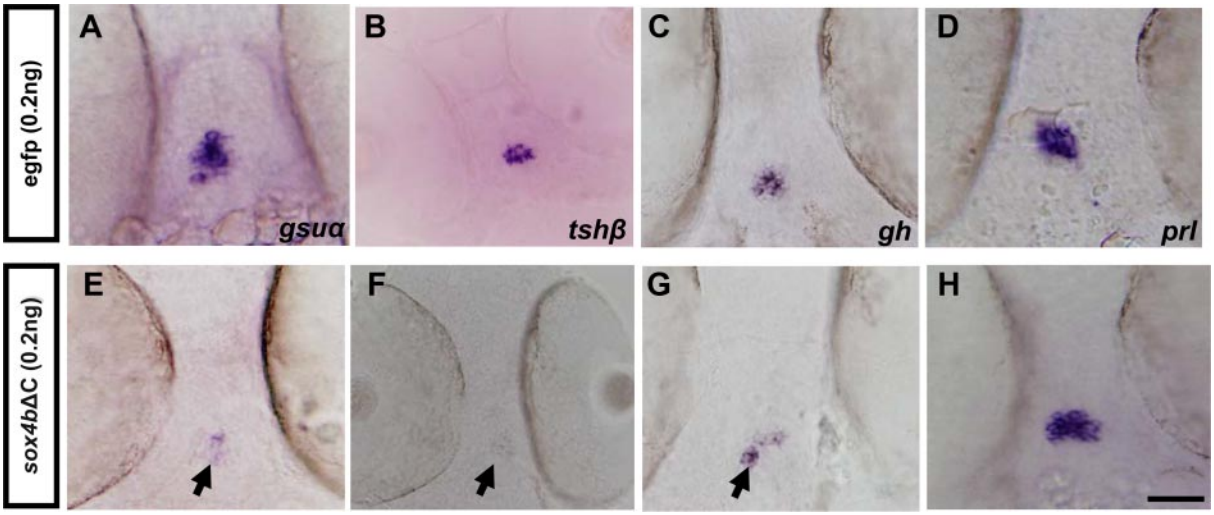


**FIG. 4.** Expression of the dominant-negative *Sox4bΔC* mutant decreases thyrotrope gene expression. *sox4bΔC* mRNA or *egfp* mRNA was microinjected into fertilized eggs, and WISH experiments were performed to detect *gsuα* (A and E), *tshβ* (B and F), *gh* (C and G), or *prl* (D and H) expression at 48 hpf. Ventral views (anterior to the *top*) are shown. *Scale bar*, 50 μm. *Arrowheads* in E–G point to the decreased expression in thyrotrope cells.

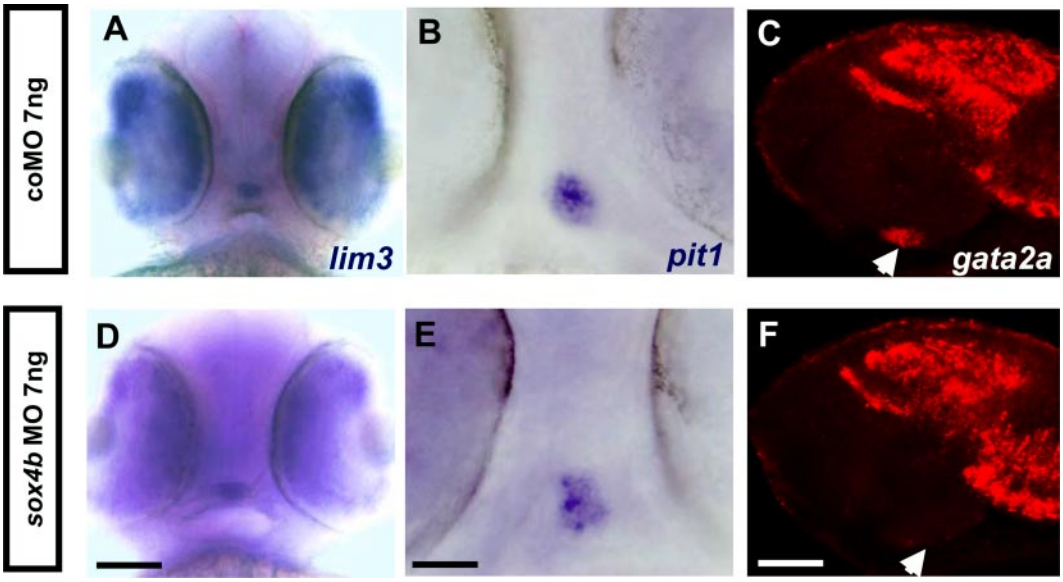


**FIG. 5.** *sox4b* knockdown leads to decreased expression of *gata2a*. Fertilized eggs were microinjected with *sox4b*MO and analyzed by WISH for expression of *lim3* and *pit1* or by single fluorescent whole-mount *in situ* hybridization for *gata2a* (*red*) in the embryos at 48 hpf. A–C, Control; D–F, *sox4b*MO-injected embryos; A and D, ventral view (anterior to the *top*) (*scale bar*, 100 $\mu$m); B and E, ventral view (anterior to the *top*) (*scale bar*, 50 $\mu$m); C and F, z-plane confocal image lateral view (anterior to the *left*) (*scale bar*, 60 $\mu$m). *Arrowheads* in C and F point at the position of *gata2a* expression in the pituitary.

into fertilized eggs and analyzed hormone expression in 48-hpf embryos. Expression of *tshβ* and *gsuα* were drastically reduced in *gata2a* morphants relative to control MO-injected embryos (Fig. 7, A, B, E, and F), whereas *prl* expression was not affected (Fig. 7, I and J) at 48 hpf. When we coinjected mRNA coding for *gata2a* together with *gata2a* MO, expression of both *tshβ* and *gsuα* was clearly rescued (Fig. 7, C and G), whereas injection of *gata2a* mRNA alone had no effect (Fig. 7, D and H).

Collectively, our results show that the defects due to *gata2a* depletion are very similar to those in *sox4b* morphants, suggesting that the two factors are components of a common pathway.

## Exogenous Gata2a rescues Sox4b depletion in the pituitary

To understand the relationship between Gata2a and Sox4b in the regulatory pathways, we next asked whether ectopic expression of *gata2a* could restore expression in *sox4b*-depleted embryos.

Injection of *sox4b*MO resulted in a decrease of *tshβ* and *gsuα* expression, as expected, whereas coinjection of *gata2a* mRNA together with *sox4b*MO completely restored this expression at 48 hpf (Fig. 8).

Taken together, our results demonstrate that the presence of Sox4b is required for *gata2a* expression in the pituitary, which itself is required for thyrotrope differentiation.

## Sox4b and gata2a are both involved in the expression of gonadotropins (*fshβ* and *lhβ*) in the zebrafish developing pituitary

Gata2 is known in mouse to be required for expression of gonadotropins, whereas our own results show that both Gata2a and Sox4b control expression at 48 hpf of *gsuα*, the hormone subunit common to thyrotropes and gonadotropes. Consequently, we considered the possibility that both transcription factors might play a role also in gonadotropin expression.

Previous studies suggested that *fshβ* and *lhβ* transcript levels in the larval zebrafish pituitary are extremely low and detectable only via RT-PCR at 72 hpf (8). Therefore, we first tested the gonadotropin β-subunit mRNA levels by qRT-PCR in RNA extracted from 4-dpf larvae previously microinjected with control MO or MO directed against *sox4b* or *gata2a*. The results show that in *sox4b* morphants (Fig. 9A) and in *gata2a* morphants (Fig. 9B), both *lhβ* and *fshβ* mRNA levels are clearly decreased relative to control MO-injected larvae. When we analyzed *lhβ* expression by fluorescent *in situ* hybridization in 4-dpf larvae, we could clearly detect several *lhβ*-expressing cells in the controls, whereas no or only weakly labeled cells were detected in *sox4b* morphants (Fig. 9C). As an internal control for the efficiency of *in situ* hybridization and detection, we used in parallel a probe for the pancreatic marker insulin (*ins*), whose signal was not affected by *sox4b*MO injection.

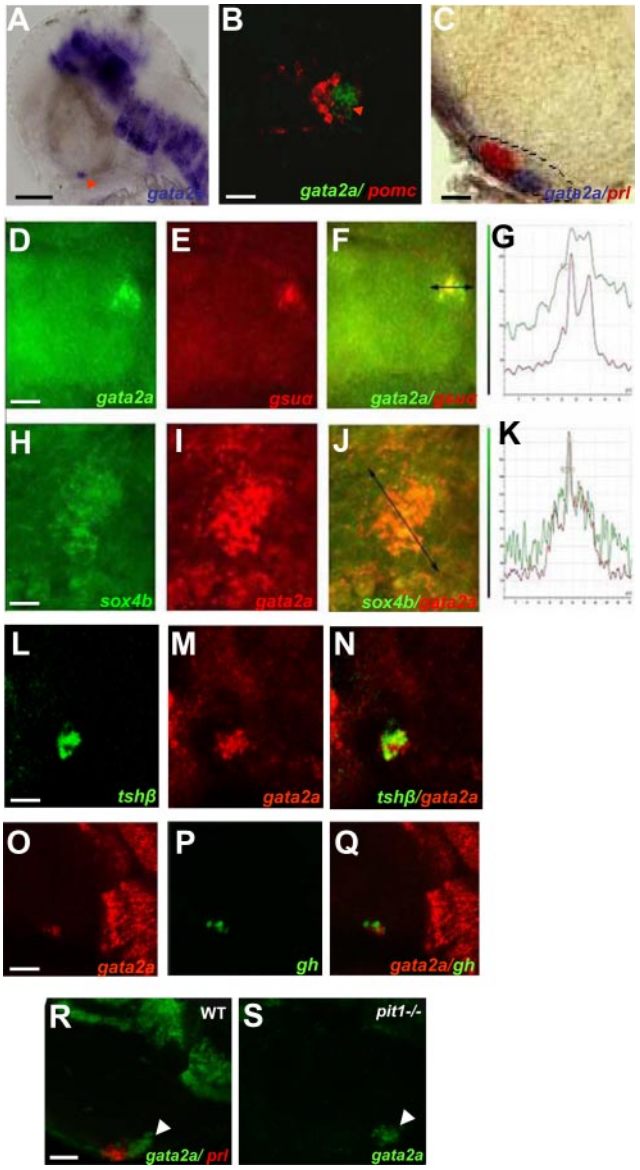


**FIG. 6.** *gata2a* mRNA is detected in *sox4b*- and *gsuα*-expressing cells in 48-hpf embryos. WISH was performed on wt zebrafish embryos using the *gata2a* antisense probe. A and C, Lateral view (anterior to the *left*); B, ventral view (anterior to the *top*). *Scale bars*, 100 μm (A), 45 μm (B), and 40 μm (C). *Arrowhead* in A points at *gata2a* expression in the pituitary. A, Single-probe *gata2a* (*blue*); B, double-fluorescent WISH showing *gata2a* (*green*) and *pomc* (*red*) mRNA (z-plane confocal image); C, double WISH showing *gata2a* (*blue*) and *prl* (*red*) mRNA; the ventral head regions were dissected before microscopy. D–N, Double-fluorescent WISH showing coexpression as follows: D-G, of *gata2a* (*green*, D) and *gsuα* (*red*, E); F, overlay of D and E; G, fluorescence emission quantification in F. H-K, *sox4b* (green, H) and *gata2a* (red, I); J, overlay of H and I; K, fluorescence emission quantification in J. L–N, *tshβ* (*green*, L) and *gata2a* (*red*, M); N, overlay of *tshβ* and *gata2a* expression; D–N, z-plane confocal image, ventral views (anterior to the *left*). O–Q, Z-plane of the confocal images, lateral view (anterior to the *left*); *gata2a* (*red*, O) and *gh* (*green*, P); Q, overlay of *gata2a* and *gh* expression. R and S, *gata2a* expression in wt and *pit1* mutants (*pit1-/-*) are shown in *upper right corner* in embryos at 48 hpf. Analysis by double-fluorescent WISH, lateral view (anterior to the *left*), one Z-plane of the confocal images is shown; *gata2a* (*green*, R and S) and *prl* (*red*, R) is shown, the *arrowhead* points to *gata2*a expression in the pituitary (R and S). *Scale bars*, 40 μm (D–F, L–N, R, and S), 20 μm (H–J), and 60 μm (O–Q).

Taken together, these results show that Sox4b and Gata2a are both required for gonadotropin expression in zebrafish pituitary.

## Discussion

The Sox4 transcription factor is highly conserved in vertebrates, encoded by a single exon, and expressed in different tissues during embryonic and adult life. In mammals, Sox4 is essential for heart, lymphocyte, and thymocyte development (44). Furthermore, Sox4 was shown to be required for normal endocrine pancreas development in mouse (32). Recently, SOX4 expression was found in the human fetal pituitary (33).

In zebrafish, two homologs for the mammalian *Sox4* gene exist, only one of them, *sox4b*, was shown to be required for glucagon-producing cell differentiation (31). In our study, we determined the expression and functional role of Sox4b in the specification of endocrine cell types during anterior pituitary development in zebrafish.

We observed *sox4b*-expressing cells in a domain located in the pituitary placode at early stages (24 hpf) and in the directly adjacent ventral diencephalon. At 30 hpf, *sox4b* expression covers most of the *lim3*-expressing domain (Fig. 1), confirming that *sox4b* is expressed in pituitary precursor cells. At later stages (48 hpf), *sox4b* mRNA is detected in the entire forebrain, and a clear signal is detected in the pituitary region expressing *tshβ* (Fig. 1), *gsuα*, and the transcription factor *gata2a*. All these observations confirm that Sox4b is expressed in pituitary cells in zebrafish, consistent with its recently described expression in human fetal pituitary (33).

Loss-of-function studies using MO knockdown or expression of a dominant-negative Sox4 mutant allowed us to uncover a key role for Sox4b in pituitary cell fate specification. Loss of Sox4b function results in dramatic reduction of *gsuα*, *tshβ*, *lhβ*, and *fshβ* expression and a moderate decrease in *gh* expression, whereas other pituitary markers are not significantly affected at 48 hpf, indicating that *sox4b* expression is mainly required for differentiation of the specific *gsuα*-expressing lineage giving rise to thyrotrope and gonadotrope cells during pituitary development.

Sox4b does not affect expression of the pituitary transcription factors Pit1 and Lim3; however, it is required for expression of the zinc finger transcription factor Gata2a specifically in the zebrafish developing pituitary. The function of Gata2 was intensely studied in the hematopoietic tissues for proliferation and survival of hematopoietic stem cells (45). A function in urogenital development (46) and in adipocyte (47) and endothelial cell (48)

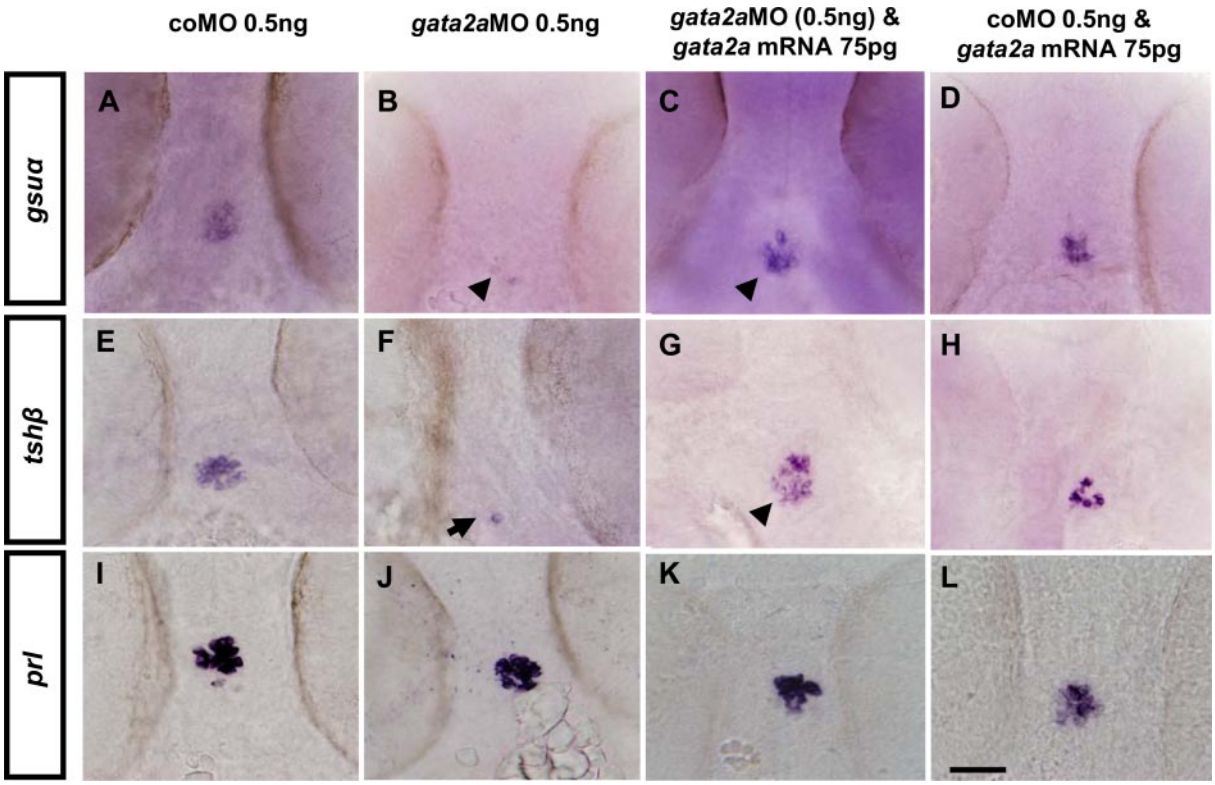


**FIG. 7.** Thyrotrope expression depends on *gata2a* expression. WISH analysis for expression of *tshβ*, *gsuα*, and *prl* in 48-hpf embryos previously microinjected with (A, E, and I) coMO; (B, F, and J) *gata2a*MO, (C, G, and K) *gata2a*MO and *gata2a* mRNA or (D, H, and L) coMO and *gata2a* mRNA. A–L, Ventral views (anterior to the *top*). *Scale bar*, 50 $\mu$m.

differentiation was also described. In mouse, Gata2 expression was shown in thyrotropes and gonadotropes starting at embryonic day 10.5 (12) and transfection studies in cell cultures revealed that Gata2 activates the *Tshβ* promoter through a synergistic action with Pit1 in thyrotropes (49). Gata factors also stimulate the *Gsuα* promotor in mouse gonadotrope cells (50). Recently, a mouse Cre-Lox knockout (KO) model was generated where *Gata2* gene disruption specifically in *Gsuα*-expressing cells decreased the number of thyrotropes and gonadotropes at birth and severely reduced *Tsh*, *Lh*, and *Fsh* expression in the adult (43).

In zebrafish, *gata2a* transcripts are detected in the ventral ectoderm at 75% epiboly and in the yolk syncytial

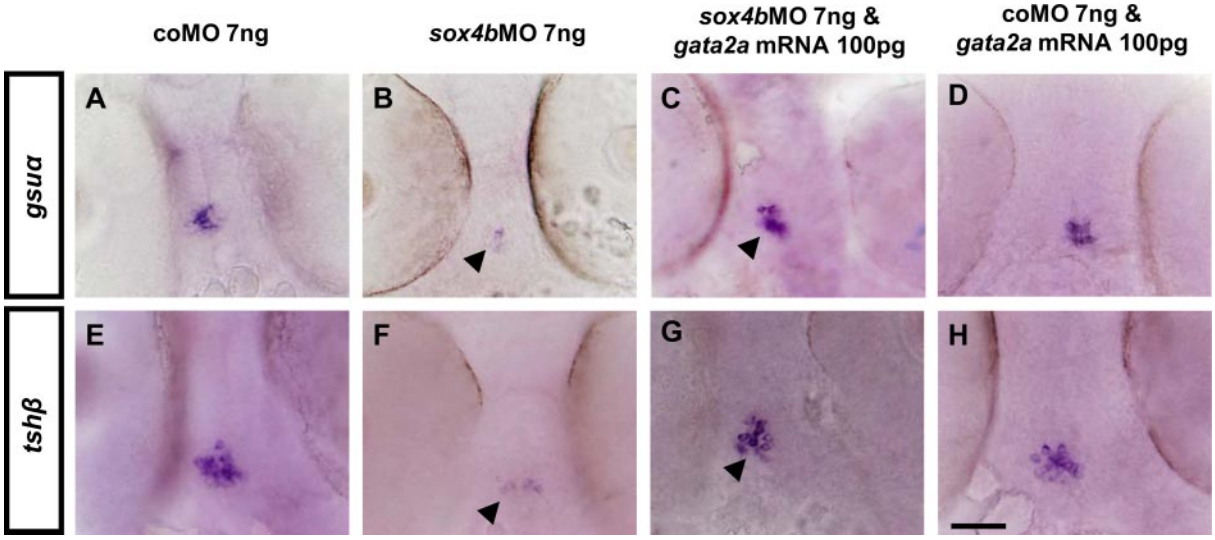


**FIG. 8.** Expression of exogenous Gata2a rescues the pituitary defects in *sox4b* morphants. WISH analysis was performed for expression of *tshβ* and *gsuα* in 48-hpf embryos previously microinjected with coMO (A and E), *sox4b*MO (B and F), *sox4b*MO and *gata2a* mRNA (C and G), or coMO and *gata2a* mRNA (D and H). A–H, Ventral views (anterior to the *top*). *Scale bar*, 50 $\mu$m.

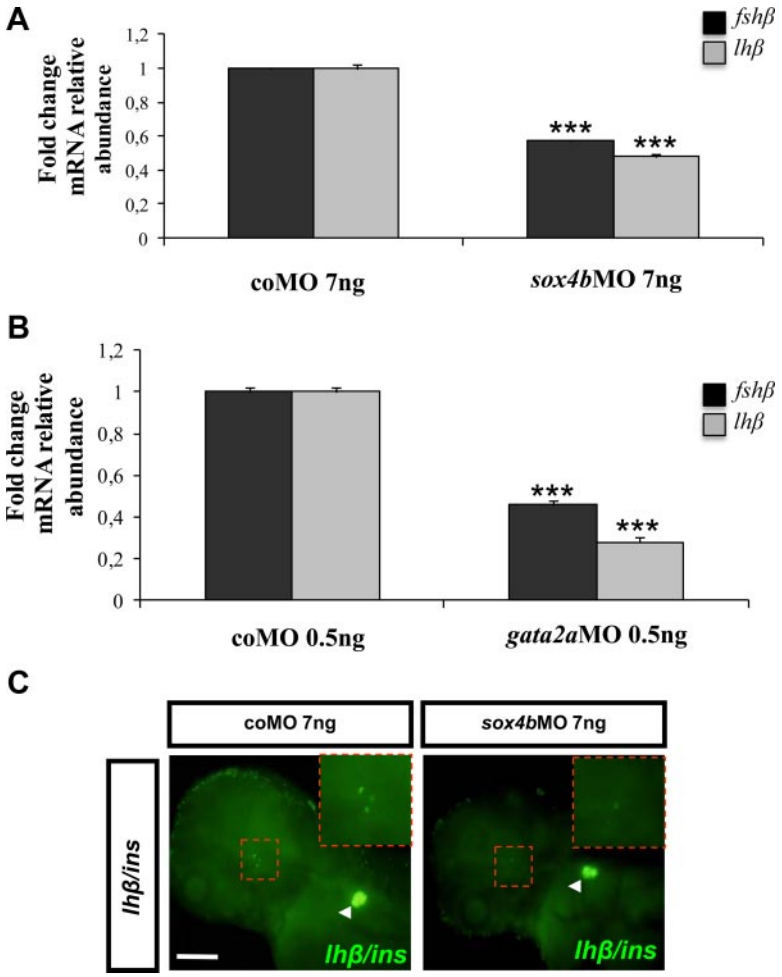


**FIG. 9.** Both Sox4b and Gata2a are required for gonadotropin expression. Expression of *lhβ* and *fshβ* was measured by qRT-PCR in RNA extracted from *sox4b* morphant (A) and *gata2a* morphant (B) larvae of 4 dpf. The data were normalized relative to the *ef1α* transcript, and the levels in coMO-injected embryos were arbitrarily set to 1. Mean fold change relative to control morphants is shown with the corresponding SD (n = 3). ***, Significant at $P < 0{,}001$. The results shown are representative of three independent experiments. C, Analysis by double-fluorescent WISH for expression of *lhβ* and *ins* at 4 dpf previously microinjected with coMO or *sox4b*MO; pancreatic *ins* (*arrowhead*) expression was assessed as invariant control. C, Ventral view (anterior to the *left*). *Scale bar*, 200 μm.

layer at 90% epiboly (39); later, it is expressed in hematopoietic tissues and the central nervous system (51). Similar to mouse, Gata2a can modify the expression of specific genes in erythrocytes (37). In the developing pituitary, we found *gata2a* expression to be highly overlapping with that of *sox4b* and *gsuα*, indicating a close relationship between *gata2a* and the *gsuα* cell lineage. We also found that *gata2a* is expressed in all *tshβ*-expressing cells at 48 hpf. When we investigated hormone expression in *gata2a* morphants, we indeed observed a drastic decrease of *tshβ* and *gsuα* expression at 48 hpf and of gonadotropins at 4 dpf. These results are consistent with those previously described in the conditional KO mouse model (43). However, the defects observed in zebrafish appear to be much stronger than those reported in mouse; the number of thyrotrope and gonadotrope cells was lower at birth but recovered in the adult, whereas *Tsh*, *Lh*, and *Fsh* expression remained severely reduced in the adult. Thus, in the mouse pituitary, Gata2 appears to be dispensable for initial gonadotrope and thyrotrope cell fate determination but important for expansion of the cell lineage during embryogenesis and for optimal gonadotrope and thyrotrope function in the adult. Unfortunately, no data are available concerning *Tshβ*, *Lhβ*, and *Fshβ* expression at early stages of the conditional KO mouse pituitary development; thus, it remains unclear whether the *Gata2* depletion in mouse leads to a drastic delay of thyrotrope and gonadotrope differentiation or whether the recovery in cell number results from a compensatory mechanism, such as the reported upregulation of *Gata3* expression in *Gata2*-deficient mice (43).

Interestingly, the *sox4b* and *gata2a* genes in zebrafish display similar characteristics; both are coexpressed in the same pituitary precursor cells also expressing *gsuα*, and both are involved in thyrotrope and gonadotrope differentiation. We further showed that *sox4b* knockdown led to decreased expression of *gata2a* in the pituitary, suggesting that Sox4b activates *gata2a* expression. We confirmed this hypothesis by demonstrating that exogenous Gata2a expression can compensate for the lack of Sox4b function in *tshβ* and *gsuα* cell differentiation in *sox4b* morphants. Our *in situ* experiments show that *sox4b* is expressed at later stages (48 hpf) in the hypothalamus of the developing zebrafish; thus, an indirect effect of hypothalamic signaling on pituitary *gata2* expression can be envisaged. However, although we cannot completely rule out a role of hypothalamic Sox4b, the perfect coexpression of *sox4b* and *gata2a* in *gsuα*-expressing cells argues in favor of a cell-autonomous regulation of *gata2a* expression by the Sox4b transcription factor.

We show that the expression of the pan-pituitary marker *lim3* is unchanged in *sox4b* knockdown embryos, indicating that the number of pituitary-specified cells remains unaltered, whereas their capacity to terminally differentiate is affected. Furthermore, thyrotrope cells de-

pend on the transcription factor Pit1 for their differentiation. Our results clearly show that *pit1* expression is not affected in *sox4b* morphants and, conversely, that *sox4b* and *gata2a* expression is not affected in *pit1* mutants. Thus, the *sox4b*/*gata2a* cascade acts independently of Pit1 to promote *gsuα* and *tshβ* expression in the *pit1*-expressing thyrotropes, consistent with the synergistic activation of the *Tshβ* promoter that was described in mouse (49).

Another cell lineage that is affected in the *sox4b* morphants is the somatotropes expressing *gh*. Although less affected than the thyrotrope and gonadotrope cells, a clear decrease in *gh* expression was observed in the *sox4b* morphants in zebrafish. In the conditional *Gata2* KO mouse model, *Gh* expression was normal at birth, whereas a transient growth retardation was observed in males between wk 3 and 9 (43). In this model, *Gata2* gene depletion is obtained by expressing the CRE recombinase in *Gsuα*-expressing cells; thus, the somatotrope cells are presumably *Gata2* positive. However, no Gata2 expression was observed in mouse somatotropes, and we show also here no colocalization of *gata2a* and *gh* mRNA in zebrafish, indicating that the effect of Sox4b or Gata2a depletion on *gh* expression might be cell nonautonomous, through altering the numbers or properties of other cell types. The precise significance and mechanism of the Sox4b/Gata2a regulatory cascade on GH expression will require further investigation.

Numerous reports have been published on *Fsh* and *Lh* in teleosts. Both hormones are synthesized in gonadotrope cells in two separate populations exhibiting distinct patterns of distribution in the zebrafish pituitary (52). Their expression is low in zebrafish larvae and was only detectable by RT-PCR at 72 hpf (8), whereas sexual differentiation of the gonads was shown to start in 3-wk-old juveniles (53). Using the more sensitive fluorescent *in situ* hybridization, we were able to observe several *lhβ*-positive cells at 72 hpf in control embryos and observed a clear decrease in the number of these cells in *sox4b* morphants. qRT-PCR analysis further confirmed the decreased expression of both *lhβ* and *fshβ*, consistent with previous studies in mammalian cell culture revealing the activation of the gonadotropin subunit genes *Gsuα* (50) and *Lhβ* (54) by Gata factors. Our results clearly show that the Sox4b and Gata2a factors are required for formation of *lhβ*- and *fshβ*-expressing cells in developing zebrafish; thus, they represent to date the only factors specifically involved in gonadotrope cell differentiation in zebrafish.

Recently, induction of *SOX4* expression by TGFβ1 was shown in the human HP75 cell line derived from a silent gonadotropinoma (55), suggesting that *Sox4* genes could be targets for TGFβ/bone morphogenetic protein (BMP) signaling pathways. The TGFβ family member activin is known to activate gonadotropin expression in the adult pituitary, whereas inhibin represses this effect (56). A function for activin in maintaining cellular homeostasis within the pituitary was also proposed. During pituitary development in the mouse, a crucial role was demonstrated for BMP, also belonging to the TGFβ family. BMP4 is required early for formation of the Rathke's pouch and thus for development of the entire pituitary, whereas BMP2 exerts a ventralizing effect within the developing Rathke's pouch, thus favoring the formation of, *e.g.* gonadotropes (57). These observations are consistent with a role for Sox4b in the transduction of TGFβ signaling at least in gonadotropes; however, in zebrafish, manipulation of BMP signaling failed to cause any defect in pituitary development (58). Thus, at present, the relationship between Sox4 factors and TGFβ signaling remains an open question.

Taken together, our results show that the Sry-related HMG-box transcription factor Sox4b is involved in the differentiation of thyrotrope and gonadotrope cells and that this function is mediated by specifically activating *gata2a* expression in *gsuα*-expressing pituitary precursor cells.

## Acknowledgments

We thank the GIGA-R zebrafish facility for providing zebrafish adults and fertilized eggs and the GIGA-R Transcriptomics platform for DNA sequencing and RNA quality control.

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This work was supported by the Fonds National pour la Recherche Scientifique (FNRS) 2.4542.00/2.4555.99/2.4561.10/2.4631.11/2.4531.09, the "Belgian Science Policy" agency: Pôle d'attraction Interuniversitaire P5/35, the University of Liège: "GAME" project to M.M. and the Special Fund for Research to P.M. M.L. held a fellowship from the Walloon Region and the "Belgian Science Policy" agency. Y.Q. was supported by the European Commission and the Walloon Region (Alma-in-Silico project). M.M. is a Chercheur Qualifié du FNRS.

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Disclosure Summary: The authors have nothing to disclose.

## References


1. **Mehta A, Dattani MT** 2008 Developmental disorders of the hypothalamus and pituitary gland associated with congenital hypopituitarism. Best Pract Res Clin Endocrinol Metab 22:191–206
2. **Zhu X, Lin CR, Prefontaine GG, Tollkuhn J, Rosenfeld MG** 2005 Genetic control of pituitary development and hypopituitarism. Curr Opin Genet Dev 15:332–340
3. **Kelberman D, Dattani MT** 2007 Hypothalamic and pituitary development: novel insights into the aetiology. Eur J Endocrinol 157(Suppl 1):S3–S14
4. **Rhodes SJ, DiMattia GE, Rosenfeld MG** 1994 Transcriptional mechanisms in anterior pituitary cell differentiation. Curr Opin Genet Dev 4:709–717
5. **Zhu Y, Stiller JW, Shaner MP, Baldini A, Scemama JL, Capehart AA** 2004 Cloning of somatolactin alpha and beta cDNAs in zebrafish and phylogenetic analysis of two distinct somatolactin subtypes in fish. J Endocrinol 182:509–518
6. **Lopez M, Nica G, Motte P, Martial JA, Hammerschmidt M, Muller M** 2006 Expression of the somatolactin $\beta$ gene during zebrafish embryonic development. Gene Expr Patterns 6:156–161
7. **Dutta S, Dietrich JE, Aspöck G, Burdine RD, Schier A, Westerfield M, Varga ZM** 2005 *pitx3* defines an equivalence domain for lens and anterior pituitary placode. Development 132:1579–1590
8. **Nica G, Herzog W, Sonntag C, Nowak M, Schwarz H, Zapata AG, Hammerschmidt M** 2006 Eya1 is required for lineage-specific differentiation, but not for cell survival in the zebrafish adenohypophysis. Dev Biol 292:189–204
9. **Zilinski CA, Shah R, Lane ME, Jamrich M** 2005 Modulation of zebrafish pitx3 expression in the primordia of the pituitary, lens, olfactory epithelium and cranial ganglia by hedgehog and nodal signaling. Genesis 41:33–40
10. **Li S, Crenshaw 3rd EB, Rawson EJ, Simmons DM, Swanson LW, Rosenfeld MG** 1990 Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. Nature 347:528–533
11. **Nica G, Herzog W, Sonntag C, Hammerschmidt M** 2004 Zebrafish *pit1* mutants lack three pituitary cell types and develop severe dwarfism. Mol Endocrinol 18:1196–1209
12. **Dasen JS, O'Connell SM, Flynn SE, Treier M, Gleiberman AS, Szeto DP, Hooshmand F, Aggarwal AK, Rosenfeld MG** 1999 Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. Cell 97:587–598
13. **Wegner M** 1999 From head to toes: the multiple facets of Sox proteins. Nucleic Acids Res 27:1409–1420
14. **Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R** 2003 Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 17:126–140
15. **Kondoh H, Uchikawa M, Kamachi Y** 2004 Interplay of Pax6 and SOX2 in lens development as a paradigm of genetic switch mechanisms for cell differentiation. Int J Dev Biol 48:819–827
16. **Kamachi Y, Uchikawa M, Collignon J, Lovell-Badge R, Kondoh H** 1998 Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. Development 125:2521–2532
17. **Lang RA** 2004 Pathways regulating lens induction in the mouse. Int J Dev Biol 48:783–791
18. **Ikeda T, Kawaguchi H, Kamekura S, Ogata N, Mori Y, Nakamura K, Ikegawa S, Chung UI** 2005 Distinct roles of Sox5, Sox6, and Sox9 in different stages of chondrogenic differentiation. J Bone Miner Metab 23:337–340
19. **Smits P, Dy P, Mitra S, Lefebvre V** 2004 Sox5 and Sox6 are needed to develop and maintain source, columnar, and hypertrophic chondrocytes in the cartilage growth plate. J Cell Biol 164:747–758
20. **Pevny L, Placzek M** 2005 SOX genes and neural progenitor identity. Curr Opin Neurobiol 15:7–13
21. **Solomon NM, Ross SA, Morgan T, Belsky JL, Hol FA, Karnes PS, Hopwood NJ, Myers SE, Tan AS, Warne GL, Forrest SM, Thomas PQ** 2004 Array comparative genomic hybridisation analysis of boys with X linked hypopituitarism identifies a 3.9 Mb duplicated critical region at Xq27 containing SOX3. J Med Genet 41:669–678
22. **Woods KS, Cundall M, Turton J, Rizotti K, Mehta A, Palmer R, Wong J, Chong WK, Al-Zyoud M, El-Ali M, Otonkoski T, Martinez-Barbera JP, Thomas PQ, Robinson IC, Lovell-Badge R, Woodward KJ, Dattani MT** 2005 Over- and underdosage of SOX3 is associated with infundibular hypoplasia and hypopituitarism. Am J Hum Genet 76:833–849
23. **Laumonnier F, Ronce N, Hamel BC, Thomas P, Lespinasse J, Raynaud M, Paringaux C, Van Bokhoven H, Kalscheuer V, Fryns JP, Chelly J, Moraine C, Briault S** 2002 Transcription factor SOX3 is involved in X-linked mental retardation with growth hormone deficiency. Am J Hum Genet 71:1450–1455
24. **Kelberman D, de Castro SC, Huang S, Crolla JA, Palmer R, Gregory JW, Taylor D, Cavallo L, Faienza MF, Fischetto R, Achermann JC, Martinez-Barbera JP, Rizzoti K, Lovell-Badge R, Robinson IC, Gerrelli D, Dattani MT** 2008 SOX2 plays a critical role in the pituitary, forebrain, and eye during human embryonic development. J Clin Endocrinol Metab 93:1865–1873
25. **Kelberman D, Rizzoti K, Avilion A, Bitner-Glindzicz M, Cianfarani S, Collins J, Chong WK, Kirk JM, Achermann JC, Ross R, Carmignac D, Lovell-Badge R, Robinson IC, Dattani MT** 2006 Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. J Clin Invest 116:2442–2455
26. **Busslinger M** 2004 Transcriptional control of early B cell development. Annu Rev Immunol 22:55–79
27. **Ya J, Schilham MW, de Boer PA, Moorman AF, Clevers H, Lamers WH** 1998 Sox4-deficiency syndrome in mice is an animal model for common trunk. Circ Res 83:986–994
28. **Cheung M, Abu-Elmagd M, Clevers H, Scotting PJ** 2000 Roles of Sox4 in central nervous system development. Brain Res Mol Brain Res 79:180–191
29. **Hunt SM, Clarke CL** 1999 Expression and hormonal regulation of the Sox4 gene in mouse female reproductive tissues. Biol Reprod 61:476–481
30. **Friedman RS, Bangur CS, Zasloff EJ, Fan L, Wang T, Watanabe Y, Kalos M** 2004 Molecular and immunological evaluation of the transcription factor SOX-4 as a lung tumor vaccine antigen. J Immunol 172:3319–3327
31. **Mavropoulos A, Devos N, Biemar F, Zecchin E, Argenton F, Edlund H, Motte P, Martial JA, Peers B** 2005 *sox4b* is a key player of pancreatic $\alpha$-cell differentiation in zebrafish. Dev Biol 285:211–223
32. **Wilson ME, Yang KY, Kalousova A, Lau J, Kosaka Y, Lynn FC, Wang J, Mrejen C, Episkopou V, Clevers HC, German MS** 2005 The HMG box transcription factor Sox4 contributes to the development of the endocrine pancreas. Diabetes 54:3402–3409
33. **Ma Y, Qi X, Du J, Song S, Feng D, Qi J, Zhu Z, Zhang X, Xiao H, Han Z, Hao X** 2009 Identification of candidate genes for human pituitary development by EST analysis. BMC Genomics 10:109
34. **Westerfield M** 1995 The zebrafish book: guide for the laboratory use of zebrafish (*Danio rerio*). 3rd ed. Eugene, OR: University of Oregon Press
35. **Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF** 1995 Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310
36. **Herzog W, Sonntag C, Walderich B, Odenthal J, Maischein HM, Hammerschmidt M** 2004 Genetic analysis of adenohypophysis formation in zebrafish. Mol Endocrinol 18:1185–1195
37. **Galloway JL, Wingert RA, Thisse C, Thisse B, Zon LI** 2005 Loss of *gata1* but not *gata2* converts erythropoiesis to myelopoiesis in zebrafish embryos. Dev Cell 8:109–116
38. **Hauptmann G, Gerster T** 1994 Two-color whole-mount in situ hybridization to vertebrate and *Drosophila* embryos. Trends Genet 10:266
39. **Detrich 3rd HW, Kieran MW, Chan FY, Barone LM, Yee K, Rund-**

**stadler JA, Pratt S, Ransom D, Zon LI** 1995 Intraembryonic hematopoietic cell migration during vertebrate development. Proc Natl Acad Sci USA 92:10713–10717

40. **Glasgow E, Karavanov AA, Dawid IB** 1997 Neuronal and neuroendocrine expression of lim3, a LIM class homeobox gene, is altered in mutant zebrafish with axial signaling defects. Dev Biol 192:405–419
41. **Herzog W, Zeng X, Lele Z, Sonntag C, Ting JW, Chang CY, Hammerschmidt M** 2003 Adenohypophysis formation in the zebrafish and its dependence on sonic hedgehog. Dev Biol 254:36–49
42. **Pfaffl MW** 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45
43. **Charles MA, Saunders TL, Wood WM, Owens K, Parlow AF, Camper SA, Ridgway EC, Gordon DF** 2006 Pituitary-specific *Gata2* knockout: effects on gonadotrope and thyrotrope function. Mol Endocrinol 20:1366–1377
44. **Schilham MW, Oosterwegel MA, Moerer P, Ya J, de Boer PA, van de Wetering M, Verbeek S, Lamers WH, Kruisbeek AM, Cumano A, Clevers H** 1996 Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking Sox-4. Nature 380:711–714
45. **Shimizu R, Yamamoto M** 2005 Gene expression regulation and domain function of hematopoietic GATA factors. Semin Cell Dev Biol 16:129–136
46. **Zhou Y, Lim KC, Onodera K, Takahashi S, Ohta J, Minegishi N, Tsai FY, Orkin SH, Yamamoto M, Engel JD** 1998 Rescue of the embryonic lethal hematopoietic defect reveals a critical role for GATA-2 in urogenital development. Embo J 17:6689–6700
47. **Tong Q, Tsai J, Tan G, Dalgin G, Hotamisligil GS** 2005 Interaction between GATA and the C/EBP family of transcription factors is critical in GATA-mediated suppression of adipocyte differentiation. Mol Cell Biol 25:706–715
48. **Minami T, Murakami T, Horiuchi K, Miura M, Noguchi T, Miyazaki J, Hamakubo T, Aird WC, Kodama T** 2004 Interaction between hex and GATA transcription factors in vascular endothelial cells inhibits flk-1/KDR-mediated vascular endothelial growth factor signaling. J Biol Chem 279:20626–20635
49. **Gordon DF, Lewis SR, Haugen BR, James RA, McDermott MT, Wood WM, Ridgway EC** 1997 Pit-1 and GATA-2 interact and functionally cooperate to activate the thyrotropin $\beta$-subunit promoter. J Biol Chem 272:24339–24347
50. **Steger DJ, Hecht JH, Mellon PL** 1994 GATA-binding proteins regulate the human gonadotropin $\alpha$-subunit gene in the placenta and pituitary gland. Mol Cell Biol 14:5592–5602
51. **Meng A, Tang H, Ong BA, Farrell MJ, Lin S** 1997 Promoter analysis in living zebrafish embryos identifies a cis-acting motif required for neuronal expression of GATA-2. Proc Natl Acad Sci USA 94:6267–6272
52. **So WK, Kwok HF, Ge W** 2005 Zebrafish gonadotropins and their receptors. II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits: their spatial-temporal expression patterns and receptor specificity. Biol Reprod 72:1382–1396
53. **Uchida D, Yamashita M, Kitano T, Iguchi T** 2002 Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J Exp Biol 205:711–718
54. **Lo A, Zheng W, Gong Y, Crochet JR, Halvorson LM** 2011 GATA transcription factors regulate LH$\beta$ gene expression. J Mol Endocrinol 47:45–58
55. **Ruebel KH, Leontovich AA, Tanizaki Y, Jin L, Stilling GA, Zhang S, Coonse K, Scheithauer BW, Lombardero M, Kovacs K, Lloyd RV** 2008 Effects of TGF$\beta$1 on gene expression in the HP75 human pituitary tumor cell line identified by gene expression profiling. Endocrine 33:62–76
56. **Bilezikjian LM, Justice NJ, Blackler AN, Wiater E, Vale WW** 4 February 2012 Cell-type specific modulation of pituitary cells by activin, inhibin and follistatin. Mol Cell Endocrinol 10.1016/j.mce.2012.01.025
57. **Zhu X, Gleiberman AS, Rosenfeld MG** 2007 Molecular physiology of pituitary development: signaling and transcriptional networks. Physiol Rev 87:933–963
58. **Pogoda HM, Hammerschmidt M** 2009 How to make a teleost adenohypophysis: molecular pathways of pituitary development in zebrafish. Mol Cell Endocrinol 312:2–13