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**How to make a Teleost Adenohypophysis:
Molecular Pathways of Pituitary development
in Zebrafish**

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Abstract

The anterior pituitary gland, or adenohypophysis, represents the key component of the vertebrate hypothalamo-hypophyseal axis, where it functions at the interphase of the nervous and endocrine system to regulate basic body functions like growth, metabolism and reproduction. For developmental biologists, the adenohypophysis serves as an excellent model system for the studies of organogenesis and differential cell fate specification. Previous research, mainly done in mouse, identified numerous extrinsic signaling cues and intrinsic transcription factors that orchestrate the gland's developmental progression.

In the past years, the zebrafish has emerged as a powerful tool to elucidate the genetic networks controlling vertebrate development, behavior and disease. Based on mutants isolated in forward genetic screens and on gene knock-downs using morpholino oligonucleotide (oligo) antisense technology, our current understanding of the molecular machinery driving adenohypophyseal ontogeny could be considerably improved. In addition, comparative analyses have shed further light onto the evolution of this rather recently invented organ. The goal of this review is to summarize current knowledge of the genetic and molecular control of zebrafish pituitary development, with special focus on most recent findings, including some thus far unpublished data from our own laboratory on the transcription factor Six1. In addition, zebrafish data will be discussed in comparison with current understanding of adenohypophysis development in mouse.

Key words: Zebrafish, Adenohypophysis, Pituitary, Differential cell specification

Introduction

The vertebrate pituitary gland constitutes a functional link between the nervous and the endocrine system, acting as a relay between the hypothalamus of the diencephalon and downstream glands or tissues via the coordinated secretion of regulator and effector hormones. These hormones and their effectors, in turn, control an array of vital processes like growth, reproduction, metabolism and different aspects of body homeostasis.

Based on its ontogeny and anatomy the pituitary can be subdivided into two core compartments: the adenohypophysis (AH) or anterior pituitary gland and the neurohypophysis (NH), also known as posterior pituitary. The latter consists mainly of neurosecretory nerve endings of hypothalamic neurons that project downwards from the *nuclei supraopticus* and *paraventricularis*. Therefore, the neurohypophysis can be regarded as a ventral extension of the diencephalon, as also reflected by its alternative name *pars nervosa* (Norris, 1997). The two neurohypophyseal hormones are Vasopressin (Vasotocin in fish) and Oxytocin (Isotocin in fish) (Murphy et al., 1998).

In contrast, the associated AH is of non-neural origin and itself composed of two lobes, the *pars distalis* (PD) and the *pars intermedia* (PI). As the primary site of endocrine action the AH harbors at least six hormone secreting cell types: the PD contains corticotropes producing adrenocorticotropin (ACTH), lactotropes producing Prolactin (PRL), thyrotropes producing thyrotropin (TSH), somatotropes producing Growth Hormone (GH), and gonadotropes that secrete the gonadotropins (LH, FSH). The PI consists of corticotropes and melanotropes, which generate α -melanocyte stimulating hormone (α -MSH) (Liu et al., 2003). As a specific endocrine feature, teleosts share an additional adenohypophyseal cell type: the Somatolactin producing cells, which can be found in the PD of the embryonic and in the PD as well as PI of the adult AH (Zhu et al., 2004, Lopez et al., 2006).

In previous years, pituitary gland development has been a particular focus of biomedical research to unravel the genetic basis of hypopituitarism, congenital pathological conditions in humans characterized by the deficiency of pituitary hormones. Cloning of spontaneous mutations and reverse genetics in the mouse model led to the identification

of a set of transcription factors and paracrine signaling molecules that are crucial for pituitary organogenesis in mammals (Kelberman and Dattani, 2007, Mehta and Dattani, 2008, Zhu et al., 2005). More recently, the mouse work has been complemented by studies done in zebrafish, a vertebrate model organism amenable for forward genetics and especially suited for live imaging. This branch of pituitary research opened additional entry points towards the understanding of the molecular network regulating pituitary organogenesis and revealed further insights into the evolution of the gland.

Ontogeny of the zebrafish adenohypophysis

Like in other vertebrates, the initial steps of zebrafish AH formation can be traced back to early segmentation stages, which start shortly after the completion of gastrulation at approximately 10 hpf (hours post fertilization). At this phase of development, the neural plate is bordered by the neural crest caudally and the preplacodal ectoderm (ppe) rostrally (Fig.1). Similar to the neural crest, which develops into a variety of cell types like peripheral neurons, glia or pigment cells, the ppe harbors precursors of several tissues, including those of the various sensory ganglia and sensory organs of the head, the inner ear, lenses, olfactory epithelium and the AH (Schlosser, 2005). Within the ppe, these precursors are arranged in a specific spatial pattern, with the future AH cells located in the most anterior domain, the anterior neural ridge (ANR), in close vicinity to the more proximally positioned olfactory precursors and next to the prospective lens cells as their direct posterior neighbors (Dutta et al., 2005). Characteristic molecular markers of the zebrafish ppe are the *bicoid*-related *paired like* homeobox gene *pitx3* as well as *eyal* and *six1*, homologs of *Drosophila*'s *eyes absent* and *sine oculis*, respectively (Dutta et al., 2005, Nica et al., 2006, Zilinski et al., 2005). At these early stages the AH anlage is juxtaposed to the anterior tip of the neural plate, which will give rise to the telencephalon of the brain (Fig.1). However, during earliest steps of brain morphogenesis that are initiated at early segmentation stages (<16 hpf), the telencephalic anlage become positioned more dorsally, while from now on and during all further steps of development, the AH primordium is juxtaposed to the ventral diencephalon, which will later progress into the endocrine hypothalamus and the infundibulum. This close spatial relationship

allows the presumptive AH cells to be accessible for inductive signaling cues that emanate from the neighboring neuroectoderm (see e.g. (Herzog et al., 2004a, Pogoda and Hammerschmidt, 2007)). This is an important prerequisite for the proper formation of the gland, early, and its function, later (Gleiberman et al., 1999, Kawamura and Kikuyama, 1998, Pogoda and Hammerschmidt, 2007, Treier and Rosenfeld, 1996).

At about 18 hpf the zebrafish anterior pituitary becomes visible as a local thickening of the ANR. At the same time, the first AH-specific genes like the Lim-domain homeobox gene *lim3* and the Pou domain homeobox gene *pit1* start to be expressed (Glasgow et al., 1997, Herzog et al., 2003, Nica et al., 2004). In contrast to the pan-pituitary marker *lim3*, *pit1* is activated just in a subset of AH cells, indicating that adenohipophyseal patterning occurs right after placode formation, and before cells move inside the head. At 24 hpf the AH has formed a morphologically distinct structure, which is positioned ventro-rostrally to the hypothalamus and dorsal to the stomodeum, a thickening of the surface ectoderm that will give rise to the epithelium of the oral cavity (Fig. 1). Still in this position, in front of the head and before the onset of oral cavity formation and adenohipophysis internalization (23-24 hpf), the first endocrine adenohipophyseal cell lineages, lactotropes, Somatolactin producing cells, and corticotropes, differentiate, as indicated by *prolactin* (*prl*), *somatolactin β* (*sl β*), and *proopiomelanocortin* (*pomc*) expression, respectively (Herzog et al., 2003, Lopez et al., 2006). Careful *in vivo* microscopy, based on Nomarski optics, revealed the presence of an anterior and posterior lobe already at 24 hpf, presumably representing the future PD and PI, which are separated by a fine horizontal furrow within the placode (Guner et al., 2008). Defining the border on the molecular level, the homeobox gene *nkx2.2a* is expressed only in the anterior half, while transcripts of the paired box homeodomain gene *pax7* are confined to the posterior part of the adenohipophysis at 25 hpf (Guner et al., 2008).

Progressive internalization of the AH into the head of the zebrafish embryo starts at 26 hpf. It is currently unclear to which extent this morphogenetic process is driven by active cell migration of placodal cells themselves, and to which extent by indirect tractions caused by the formation of the oral cavity and by the growth and folding of the fore- and midbrain. More live imaging analyses (see Liu et al., 2008) will be necessary to give

definitive answers. At the onset of AH internalization, the AH anlage has acquired a horseshoe-like shape, lining the anterior and lateral borders of the ventral diencephalon (Herzog et al., 2004a, Pogoda and Hammerschmidt, 2007). We previously hypothesized that the two lateral sides of this horseshoe might converge medially to fuse at the level of the midline, thereby generating the melanotropes- and corticotropes-harboring PI (Pogoda and Hammerschmidt, 2007). This model has recently been confirmed independently by two groups: using a *pomc:gfp* reporter transgene, Liu et al. showed via time-lapse studies that the GFP-positive *pomc*-positive cells of the intermediate lobe (PI) come from the bilateral arms of the horseshoe, from where they move towards the midline as the placode coalesces (Liu et al., 2008). In addition, these bilateral horseshoe domains express *pax7*, which also labels the PI of later stages (Guner et al., 2008) (Fig. 2). However, morphogenetic processes must be more complex, since the bilateral arms also contain *pit1*-positive cells (Nica et al., 2004), which will give rise to cells of the PD, rather the PI (see also below).

As the AH becomes progressively shifted posterior-wards, while being sandwiched between the ventral diencephalon and the dorsal roof of the mouth, the next endocrine cell lineages differentiate (Figs. 1 and 2). From 32 hpf onwards *gsu α* , marking thyrotropes and gonadotropes, can be detected in medial positions of the anlage. *thyroid stimulating hormone β* (*tsh β*) expressed by thyrotropes and *growth hormone* (*gh*) expressed by somatotropes come up at 42 hpf and 48 hpf, respectively (Herzog et al., 2003, Nica et al., 2004). By 60 to 72 hpf the zebrafish AH has reached its final position and becomes embedded in the fenestra of the developing cartilaginous neurocranium, while remaining attached to the ventral side of the endocrine hypothalamus. From these stages onwards, the distinct pituitary cell lineages display a specific pattern along the anterior-posterior axis of the gland: the anterior PD consists of lactotropes and corticotropes, while somato-, thyro-, somatolacto-, and also gonadotropes are located in the posterior PD. Finally, melanotropes of the PI constitute the posterior third of the AH (Fig. 2) (Pogoda and Hammerschmidt, 2007). This cellular anterior-posterior arrangement is maintained throughout the entire life of the fish. In addition, there is some, although less striking dorso-ventral polarity of the zebrafish adenohypophysis. For instance, gonadotropes are preferentially located ventral of thyrotropes and somatotropes

of the posterior PD (Glasgow et al., 1997, Herzog et al., 2003, Nica et al., 2004), consistent with the ventral location of gonadotropes in Rathke's pouch, the AH anlage of the mouse ((Treier et al., 1998, Zhu et al., 2005, Zhu et al., 2007). While the arrangement of PD in anterior and PI in posterior regions of the AH is conserved between zebrafish and mouse, the mouse PD *per se* does not display an apparent intrinsic anterior-posterior, but rather a dorso-ventral polarity (Treier et al., 1998, Zhu et al., 2005).

One reason for the prominent dorso-ventral patterning of the mammalian, versus the anterior-posterior patterning of the zebrafish PD, are differences in AH morphogenesis: the amniote AH anlage undergoes a further invagination, leading to Rathke's pouch formation, whereas the teleostean AH retains its linear subepithelial organization (see Fig. 2 for illustration). In this light, the posterior domain of the teleost PD corresponds to the dorsal domain of the mammalian PD, and the teleostean anterior domain to the mammalian ventral domain. Accordingly, anterior-posterior patterning of the zebrafish and dorso-ventral patterning of the mammalian anlage are driven by similar signals, such as Hedgehogs (anterior in fish, ventral in mouse) and Fibroblast growth factors (posterior in fish, dorsal in mouse) (see also below and Fig. 2) (Dutta et al., 2005, Dutta et al., 2008, Guner et al., 2008, Herzog et al., 2003, Liu et al., 2008, Sbrogna et al., 2003).

Molecules and genes with essential functions in zebrafish pituitary development

As aforementioned, pituitary development has already been extensively studied in mouse, where reverse genetics and transgenic-based overexpression analyses have provided crucial insights into the molecular network controlling AH organogenesis. However, all these approaches require a-priori knowledge of the gene or pathway to be studied. In contrast, forward genetics aim to identify genes based on the phenotype caused upon mutations, and without knowledge of their molecular nature. As one of its most powerful strengths, the zebrafish system allows such unbiased and systematic genetic approaches with reasonable investments in terms of time, costs, lab space and working load. Large-scale mutagenesis screens conducted during the early 1990s already led to the identification of hundreds of genes with essential functions in zebrafish development, physiology and behavior (Amsterdam et al., 1999, Driever et al., 1996, Haffter et al.,

1996). Since then, many additional screens using more specific or sophisticated screening assays greatly contributed to today's collection of available mutants (e.g. (Muto et al., 2005, Pogoda et al., 2006a, Sun et al., 2004)). At the same time, genomic tools have enormously improved, facilitating the cloning of randomly induced mutations (Dahm and Geisler, 2006, Ekker et al., 2007, Sivasubbu et al., 2007). In addition, methods of reverse genetics have been established, including gene knock-down via the injection of specific *antisense* morpholino oligos (MOs) (Nasevicius and Ekker, 2000, Doyon et al., 2008, Meng et al., 2008, Wienholds et al., 2003), target-selected ENU mutagenesis (also named TILLING; targeted induced local lesions in genomes) (Nasevicius and Ekker, 2000, Doyon et al., 2008, Meng et al., 2008, Wienholds et al., 2003), and, most recently, targeted introduction of small deletions via engineered zinc-finger-nucleases (Nasevicius and Ekker, 2000, Doyon et al., 2008, Meng et al., 2008, Wienholds et al., 2003).

Our laboratory participated in a large-scale forward genetics ENU mutagenesis screen under the leadership of the Max-Planck Institute of Developmental Biology in Tübingen, Germany, searching for mutations that disrupt *gh* expression in zebrafish larvae at 120 hpf. These efforts led to the identification of four essential loci / complementation groups: *lia*, *pia*, *pit1* and *aal*, which showed specific defects affecting the entire AH (*lia*, *pia*) or the formation of particular AH cell lineages (*pit1*, *aal*) (Herzog et al., 2004b). Subsequently, applying meiotic mapping and chromosomal walking, the affected genes of the four different mutants were identified as *fgf3* (Herzog et al., 2004a), *ascl1a* (Pogoda et al., 2006b), *pit1* (Herzog et al., 2003, Nica et al., 2004) and *eyal* (Nica et al., 2006), respectively. A smaller screen, recently conducted in our own laboratory and screening for altered *pomc* expression, added two antimorphic alleles of *six1* to the list (Pogoda HM, Slanchev K, Seibel SH, Hammerschmidt M, unpublished; see below). Additional data concerning the function of Pitx3, Dlx3/4, Hedgehog, Fgf and Notch/Delta were obtained, taking advantage of mutants isolated in other morphology-based screens, MO-based gene knock downs, or chemical in vivo blockage of certain signaling cascades (Dutta et al., 2005, Dutta et al., 2008, Guner et al., 2008, Herzog et al., 2003, Liu et al., 2008, Sbrogna et al., 2003). In this section, we will describe each of the identified

essential genes or pathways in the context of the particular processes of AH development they are involved in.

AH pre-placode formation and AH induction

1. Pitx3

Zebrafish *pitx3* encodes a Pitx/Rieg homeodomain transcription factor that is related to Drosophila's morphogen Bicoid. During the final phase of gastrulation it starts to be expressed in cells bordering the anterior neural plate. In the course of early somitogenesis this expression domain expands and finally demarcates a field of preplacodal ectoderm (ppe) containing precursors of the AH, the nose, lenses and some cranial ganglia (Toro and Varga, 2007). Adenohypophyseal and lens progenitors maintain *pitx3* expression even beyond placode formation, up to 72 hpf (Dutta et al., 2005, Zilinski et al., 2005). Morpholino-based knock down of *pitx3* in zebrafish leads to failed pituitary placode initiation and AH cell specification, as demonstrated by the dearth of *lim3*, *nkx2.2a* and *pomc* expression indicating that this gene is crucial for initial steps in AH development (Dutta et al., 2005).

In mouse, instead of *Pitx3*, *Pitx1* and *Pitx2* are expressed in the embryonic anterior pituitary. While the single *Pitx1* knock out results in a rather mild AH phenotype (Szeto et al., 1999), *Pitx2* KO animals display reduced proliferation and increased apoptosis of AH precursors. This phenotype is further enhanced in the *Pitx1/2* double deficient mice (Charles et al., 2005), comparable to the defects described for zebrafish *pitx3* morphants (Dutta et al., 2005). This suggests that in fish *pitx3* has possibly subsumed the function of mammalian *Pitx1/2*. By trans-activating reporter assays it was shown that mouse Pitx1 can drive *Lhx3* (the mouse homolog of zebrafish *lim3*) as well as *prl* and *pomc* expression (Lamonerie et al., 1996, Szeto et al., 1999, Tremblay et al., 1998). Since Pitx binding sites have also been found in the zebrafish *pomc* promoter, and since lactotropes and corticotropes are the first differentiating AH cells of the zebrafish, one can speculate about a similar regulation of these genes by Pitx3 in teleosts (Liu et al., 2003).

2. Dlx3/Dlx4

Like *pitx3*, the *distal-less* related homeobox genes *dlx3b* and *dlx4b* are expressed in the ppe of early segmentation stage zebrafish embryos. However, their expression patterns only partially overlap with that of *pitx3*. Thus, *dlx* transcripts can be mainly detected in more medial regions of the anterior ppe. In addition, *dlx* expression within the ppe extends further posterior-wards than *pitx3* (Dutta et al., 2005). Fate mapping studies revealed that the *dlx* positive/*pitx3* negative part of the ppe preferentially gives rise to olfactory epithelium, whereas the *pitx3* positive/*dlx* negative domain develops either into lens or AH cells (Dutta et al., 2005, Toro and Varga, 2007). Concomitant knock down of *dlx3b* and *dlx4b* with morpholinos results in a loss of olfactory tissue, while at the same time the size of the AH placode increases significantly (Dutta et al., 2005, Solomon and Fritz, 2002, Whitlock and Westerfield, 2000). This suggests that Dlx3/Dlx4 restrict the area within the anterior ppe that is competent to respond to AH-inducing signals, thereby promoting olfactory fates. Thus, Pitx3 and Dlx3/Dlx4 seem to act in an antagonizing manner to pattern the anterior ppe along its proximo-distal (medio-lateral) axis. The *dlx3b*, *dlx4b*, *pitx3* triple knock down causes a phenotype similar to that of *pitx3* single morphants, indicating that *pitx3* is epistatic to the two *dlxes*, and that Dlx proteins promote nose at the expense of AH development by blocking *pitx3* (Dutta et al., 2005). In contrast to zebrafish, no role of Dlx factors in mouse pituitary development has been reported.

3. Hedgehog

Signaling by secreted glycoproteins of the Hedgehog (Hh) family play pivotal roles in many pattern formation events during animal development. This accounts also for early steps of AH formation in zebrafish. Mutations that severely block Hh signaling, either at the level of the Hh receptor complex protein Smoothened (the *slow-muscle-omitted* (*smu*) mutation), the Hh-responsive transcription factor Gli2 (the *you-too* (*yot*) mutation), or the Gli modulator Dzip (the *iguana* mutation), lead to a complete loss of anterior pituitary fates (Karlstrom et al., 1999, Sekimizu et al., 2004, Varga et al., 2001). The same is true for the double knock out of *sonic hedgehog* (*shh*) and *tiggy winkle hedgehog* (*twhh*),

which encode partially redundant Hh ligands (Herzog et al., 2003, Nasevicius and Ekker, 2000). Furthermore, defects like in these mutants or morphants can be obtained in wildtype backgrounds by chemical blockage of the Hh pathway using the drug Cyclopamine, which acts on the level of Smoothed (Sbrogna et al., 2003). Interestingly, and in contrast to *pitx3* morphants, loss of Hh signaling causes ectopic formation of lenses at the site of the AH placode, thus, the specification of more lateral-posterior ppe fates (Dutta et al., 2005, Kondoh et al., 2000, Sbrogna et al., 2003). In reverse, overexpression of *shh* causes induction of an excessive number of pituitary cells at the expense of lense fates, most likely due to an adoption of AH fates in lens precursor cells (Dutta et al., 2005, Herzog et al., 2003, Sbrogna et al., 2003). Based on these findings and in combination with careful fate mapping studies, a model was proposed according to which the entire *pitx3* positive domain of the ppe demarcates an equivalence field that will develop into lens cells unless AH fates are induced by Hh signals (Dutta et al., 2005, Toro and Varga, 2007). In line with this model, Cyclopamine studies have narrowed down the Hh-sensitive time window for AH induction to 10-15 hpf (Sbrogna et al., 2003), when *shh* and its paralog *twhh* are expressed in cells of the presumptive ventral diencephalon in the midline of the neural plate. This tissue extends along the anterior-posterior axis, while cells at the rostral tip of the plate become displaced dorsally, bringing the anterior *pitx3*-positive domain of the ppe in close proximity to the Hh domain (Toro and Varga, 2007, Pogoda and Hammerschmidt, 2007) (Fig. 1), while lens precursors of the more lateral *pitx3* expressing ppe region remain in some distance from that Hh source. It is a matter of debate whether these Hh signals induce AH fates directly, or whether they require a mediating signal of thus far unknown nature (Sbrogna et al., 2003, Dutta et al., 2005, Guner et al., 2008). Clearly, *gli* genes, encoding Hh-regulated transcription factors, are expressed in AH cells and required for proper pituitary development (Devine et al., 2008).

AH specification and survival

4. Fgf3

Like Shh and Twhh, Fibroblast growth factor 3 (Fgf3) is a secreted paracrine factor with essential functions in pituitary ontogeny, which is not expressed in the AH itself. At the onset of segmentation at 10 hpf, *fgf3* expression is restricted to cells of the presumptive telencephalon in the anterior neuroectoderm next to the anr, while at 18 hpf, when the future telencephalic cells have been displaced dorsally, *fgf3* transcripts are confined to the ventral posterior diencephalon, with the anterior tip of this expression domain touching the AH placode (see Fig. 1 for illustration). At mid-segmentation stages, when the AH anlage has acquired the aforementioned horseshoe-like shape with posteriorly extending bilateral arms, *fgf3* transcripts are present in the entire diencephalic region outlined by the horseshoe, in line with a role of Fgf3 signals on all AH cell types.

Zebrafish *fgf3* null mutants develop an intact ppe. Furthermore, at 25 hpf, the AH anlage is of normal size and shape. However, on the molecular level it lacks expression of all specification (e.g. *lim3*, *pitx3*) and differentiation (*prl*, *pomc*) markers, indicating a global failure of AH cell specification. From 28 to 32 hpf cells of the AH placode undergo massive apoptosis, resulting in a complete elimination of the pituitary (Herzog et al., 2004a). It remains to be elucidated, whether this cell death is a secondary consequence of failed cell specification, or whether Fgf3 promotes cell specification and survival in parallel. Studies of chimeric *fgf3* $-/-$ mutants with wild-type cells either in the tel- or diencephalon, and experiments with temporally controlled pharmacological blockage of FGF receptor function by the drug SU5402, further revealed that this specification- and survival-promoting effect is achieved by Fgf3 signals emanating from the ventral diencephalon between 18 and 22 hpf (Herzog et al., 2004a). This indicates that zebrafish hypophysis and diencephalon / hypothalamus not only form a functional axis during the regulation of adult body homeostasis, but also interact with each other during development, comparable to the situation in mouse (Treier and Rosenfeld, 1996). Accordingly, an AH phenotype comparable to that of *fgf3* mutant zebrafish is obtained in mice that are deficient for the FGF3 relative FGF10 or its cognate receptor Fgfr2IIIb (De Moerlooze et al., 2000, Ohuchi et al., 2000). Therefore, it appears that in the context of

AH ontogeny, FGF3 and FGF10, which signal both via the IIIb isoform of the Fgf receptor 2, have replaced each other during vertebrate evolution.

5. *Ascl1a*

The *pituitary-absent* mutant (*pia*) carries a potential null mutation in the *achaete-scute-like 1a* gene, which encodes a bHLH transcription factor homologous to *Drosophila*'s proneural *achaete-scute-complex* proteins. From 20 to 48 hpf *ascl1a* is uniformly expressed in the entire AH anlage, where during the third day of development, expression becomes confined to the anterior PD and the PI, resulting in a pattern very similar to that of *pomc* (Pogoda et al., 2006b). These dynamics in *ascl1a* expression point at a more global requirement for AH formation early, and cell type-specific functions later. In line with an initial global “pro-endocrine” function, *pia* mutants lack expression of all hormone genes. The pan-pituitary specification markers *lim3* and *pitx3* show reduced, but not completely abolished expression, while *pit1* expression is completely absent (Pogoda et al., 2006b). This failure of *pit1* activation is most likely the main reason for the later absence of *prl*, *gh*, and *tsh- β* expression (see paragraph 9), whereas the absence of *pomc* expression might point to a direct role of *Ascl1a* as a transcriptional activator of *pomc*, consistent with the presence of E-boxes, consensus binding sites for bHLH proteins, in the promoter of the zebrafish *pomc* gene (Liu et al., 2003).

Similar to the situation in *fgf3* mutants, failed cell specification of *ascl1a* mutant AH cells is followed by apoptosis in the AH around 30 hpf. However, in contrast to *fgf3* mutant embryos, it is just a fraction of AH cells that dies in *ascl1a* mutants. Survivors form a pituitary rudiment of smaller size that consists of *lim3* and *pitx3*-positive cells, which remain negative for hormone expression (Pogoda et al., 2006b). Given the very similar phenotypes of *fgf3* and *ascl1a* mutants, it is tempting to speculate that the Fgf3 signal fulfills at least parts of its function via *Ascl1a* (Fig. 2). Consistent with this hypothesis, *ascl1a* expression fails to be initiated in the AH anlage of *fgf3* mutants. Furthermore, implantation of FGF3-loaded beads next to the AH of *ascl1* mutants leads to enhanced *ascl1a* expression, while failing to rescue AH cell specification (Pogoda et al., 2006b).

Although the human homolog has been detected in ACTH and PRL adenomas, and although the mouse homologue *Mash1* is expressed in Rathke's pouch, no functions in AH formation have been assigned to the mammalian counterparts of zebrafish *Ascl1a* as yet (Ruebel et al., 2006, Zhu et al., 2006). However, *Mash1* is essential for the differentiation of neuroendocrine cells in the lung, the adrenal gland, the thyroid, and is thus an important factor regulating endocrine development in another context (Ball, 2004, Huber et al., 2002, Lanigan et al., 1998). Interestingly, differentiation and survival of the mouse olfactory epithelium depends on *Mash1* function comparable to that of *Ascl1a* in zebrafish pituitary development (Guillemot et al., 1993). Since olfactory and AH precursors originate from closely juxtaposed positions within the anterior preplacodal region (see above), the related pituitary and nose phenotype might point at a common evolutionary origin of the affected cell types (Gorbman, 1995).

AH patterning and differential cell specification

6. Shh

In mouse pituitary organogenesis, Shh secreted by the oral ectoderm and ventral cells of Rathke's pouch itself acts as a morphogen on the pouch where it promotes the differentiation of ventral cell fates and positively controls proliferation of pituitary precursors (Treier et al., 1998, Treier et al., 2001). A recent study by Guner and colleagues showed that Hh patterns the early zebrafish pituitary placode along its anterior-posterior axis, with highest signaling levels promoting anterior fates (Guner et al., 2008). This is already anticipated by the absence of *nkx2.2a* activity within the anterior AH lobe of mutants with moderately disrupted Hh signaling, while at the same time *pax7* expression of the posterior lobe remains unaltered. In contrast, overexpression of *shh* results in an expansion of the *nkx2.2a* domain at the expense of the *pax7* domain. Supporting a possible morphogenetic function of Hh in zebrafish pituitary patterning, Guner and colleagues further showed that the AH placode responds to Cyclopamine treatment in a concentration dependent manner: when zebrafish embryos were exposed to low doses of the drug from 10 hpf onwards, mainly *pomc* and *prl* expressing cells of the anterior PD were significantly reduced in number, whereas posterior PD and PI fates

were only suppressed after treatment with correspondingly higher Cyclopamine concentrations. Interestingly, however, weak Cyclopamine treatment did not lead to an increase in the number of AH cells with posterior fates. Rather, unspecified AH cells acquired lens fate, leading to the formation of one ectopic lens (instead of PD) after weak, and of two ectopic lenses (instead of PD and PI) after strong Hh inhibition (Guner et al., 2008). In this light, the morphogenetic effect of Hh signaling is a dose-dependent differential induction of anterior-posterior AH fates from a lens “ground” stage, rather than a patterning within an AH field. Most recent data further show that in addition to different concentrations of Hh signals, dynamic and differential presence of different Hh-regulated transcription factors (Gli1, Gli2a, Gli2b) in AH progenitor cells of the PD versus PI accounts for differential Hh-dependent cell fate specification (Devine et al., 2008). The supposed source of Hh signals regulating AH induction and patterning is the anterior ventral hypothalamus, which expresses *shh* and *twhh* in the vicinity of the presumptive anterior PD until the onset of AH internalization (Herzog et al., 2003, Sbrogna et al., 2003, Guner et al., 2008) (see Fig. 1). Later, *shh* is also in the epithelium of the oral cavity ventral of the AH primordium (Herzog et al., 2003). In mouse, this is the primary source of Shh patterning Rathke’s pouch (Treier et al., 2001), although also here, a contribution of diencephalic Shh cannot be ruled out. In zebrafish, Shh from the oral epithelium might account for some later patterning of the AH anlage, which, in contrast to the mouse primordium, does not invaginate to form a pouch, but remains in its initial subepithelial position and orientation (see also above). It could be involved in the specification of later differentiating and preferentially ventrally localized endocrine cell types. This, together with differential *gli* expression (Devine et al., 2008), could for instance explain why somatotropes of the posterior PD are even more sensitive to partial loss of Hh signaling than the lactotropes and corticotropes of the anterior PD (Herzog et al., 2003, Guner et al., 2008).

A further evolutionary conserved function of Hh in mammalian and fish pituitary development is its mitogenic effect on the AH precursor cells. Consistent with data obtained with transgenic mice, in which ectopic expression of the Hh inhibitor Hip within Rathke’s pouch causes pituitary hypoplasia (Treier et al., 2001), zebrafish *syu* mutants display an anterior pituitary of significantly reduced size (Herzog et al., 2003).

7. Fgf

In mouse, the dorso-ventral axis of the AH anlage is patterned by opposing signaling gradients of paracrine factors. Shh, described above, acts together with Bone morphogenetic protein 2 (Bmp2) as a ventralizing factor, while Fgf8 secreted by the overlying ventral diencephalon promotes the specification of dorsal cell fates (Treier et al., 1998). As explained, the dorsoventral axis of the murine AH corresponds roughly to the anterior-posterior axis of the zebrafish, with Hh signals promoting anterior fates. Our studies with transgenic lines driving temporally controlled expression of Bmp2b or its inhibitor Noggin3 (Rentzsch et al., 2006) have failed to reveal a requirement of Bmp signaling during zebrafish pituitary development (unpublished own data). However, Guner et al. and Liu et al. have now identified Fgf3 as the posteriorizing opponent to Hh in AH patterning (Guner et al., 2008, Liu et al., 2008).

fgf3 expression in the posterior-ventral hypothalamus persists beyond the time window when it is required for global AH specification and survival (Herzog et al., 2004a). Fgf3 activity levels were gradually decreased, using the chemical SU5042, which blocks FGF receptors, or applying *fgf3* morpholinos at variable “hypomorphic” concentrations, which do not cause AH apoptosis. These treatments affect anterior-posterior patterning of the AH in reverse to the effects obtained by blocking Hh signaling: weak inhibition of Fgf3 signaling primarily results in a loss of melanotropes and corticotropes of the PI, while the numbers of thyro-, gonado-, lacto-, and corticotropes of the PD only decrease after intermediate or strong disruption of Fgf3 function (Guner et al., 2008, Liu et al., 2008). These effects are anticipated by a loss of *pax7* expression in the PI, complementary to the downregulation of the direct Hh target *nkx2.2a* in the PD after Cyclopamine treatment (Guner et al., 2008). An exception are again the somatotropes, which unlike the other PD cell types are already affected by low doses of SU5042 (Guner et al., 2008). Although being positioned most medially within the placode, it appears that this specific cell type depends particularly strongly on both Hh as well as Fgf3 signals.

8. Notch/Delta

Lateral inhibition is a mechanism by which short-range signals mediated by the ligand Delta and its cognate receptor Notch control binary cell fate decisions, allowing a cell to adopt a different developmental path than its direct neighbor (Schweisguth, 2004). From worms to humans, Delta-Notch signaling thereby controls patterning events in many different developmental contexts, with the segregation of neuroblasts from the neurogenic ectoderm of the *Drosophila* embryo as a classical example (Campos-Ortega, 1995). In each of the three compartments of the zebrafish AH (aPD, pPD, PI), at least two different AH cell types are present and largely organized in a salt-and-pepper-like spatial pattern (Fig. 2). Recently, Dutta and colleagues showed that Delta-Notch signaling is at play to regulate differential fate specification within the domains (Dutta et al., 2008). *dla*, *dlb* and *dld*, encoding different Delta ligands, as well as the receptor-encoding genes *notch1a*, *notch1b* and *notch5* are expressed in the zebrafish AH placode (24-28 hpf), initially in a salt-and-pepper distribution, while expression vanishes during the second and third day of development, when endocrine cells differentiate. Embryos treated with DAPT, a pharmacological γ -secretase inhibitor that blocks intracellular Notch activation, and mutants in the RING E3 ubiquitin ligase *mindbomb* (*mib*), which are devoid of Notch signaling (Itoh et al., 2003), display a significant increase in lactotropes at the expense of corticotropes in the aPD, an increase of thyrotropes and possibly gonadotropes at the expense of somatotropes in the pPD, and a loss of melanotropes in the PI (Dutta et al., 2008). In reverse, temporally controlled expression of a constitutively active version of the Notch1a receptor in transgenic zebrafish embryos results in a gain of corticotropes at the expense of lactotropes in the aPD, and a loss of thyrotropes in the pPD, while the number of melanotropes and the size of the PI are increased (Dutta et al., 2008). This suggests that lactotropes and thyrotropes are “primary” cell fates that develop unless they are actively suppressed by Notch signaling. Of the “secondary” fates (somatotropes, corticotropes, melanotropes) that require Notch signaling, at least somatotropes are not expanded upon constitutively active Notch signaling, demonstrating that Notch has a permissive, rather than instructive effect.

Studies with temporally controlled exposure of wild-type embryos to DAPT further showed that Notch function is required between 14 and 16 hpf. This is several hours before the first AH cells begin to differentiate, suggesting that cells acquire the ability of lateral inhibition long before their own endocrine differentiation. However, it cannot be excluded that the observed defects are at least to some extent indirect and caused by *delta-notch* expressing cells in neighboring tissue such as the ventral diencephalon. In any case, these studies show that Delta-Notch-mediated lateral inhibition controls distinct cell fate decisions within the three subdomains of the zebrafish AH, thereby directing subfunctionalization within subdomains (lacto- versus corticotropes in aPD, thyro/gonado- versus somatotropes in pPD) – as well as between subdomains (melano- versus thyrotropes) (Dutta et al., 2008).

An effect on differential cell fate specification has also been reported for *Rbp-J*, a major mediator of the Notch pathway, and for the Notch downstream effector and transcriptional repressor *Hes1* during pituitary development in mouse (Zhu et al., 2006, Raetzman et al., 2007). Like upon loss of Notch signaling in zebrafish, murine *Rbp-J* or *Hes1* mutants display reduced numbers of melanotropes in the PI. This, however, coincides with a gain of somatotropes, which is in striking contrast to the loss of somatotropes caused by loss of Notch signaling in zebrafish (Dutta et al., 2008, Raetzman et al., 2007). Thus, while the general concept of AH endocrine cell lineage segregation under the control of the Delta-Notch system might be evolutionary conserved, the dependence of particular cell types on Notch signaling seems to differ between fish and mouse. This might be due to differences in the temporal and spatial fine-tuning of Notch signaling and its integration into the global signaling system. Several lines of evidence indicate that in the mouse AH, *Rbp-1*, *Hes1* and its relative *Hes5* are primarily required for AH precursor proliferation, and to prevent the conversion of late-arising cells to early born cell lineages (Kita et al., 2007, Raetzman et al., 2007, Zhu et al., 2006). In this light, different dependences on Notch signaling could be due to differences in the time course with which the different cell types are born in mouse and fish (see also below and Fig. 1).

9. Pit1

The Pou homeodomain protein Pit1 is the only known transcription factor to date with an expression and function that is strictly restricted to the AH. It was initially identified and cloned based on its ability to bind and transactivate the expression of the mammalian *prl* and *gh* genes. These findings were underscored by the identification of spontaneous mutations in the human and murine *pit1* gene, which cause an absence of lacto-, thyro-, and somatotrope differentiation, thereby defining the so-called Pit1 lineage (Li et al., 1990, Tatsumi et al., 1992, Pfaffle et al., 1992, Radovick et al., 1992). The more recently identified zebrafish *pit1* mutants lack exactly the same three AH cell types, indicating that the function of Pit1 during AH lineage specification is highly conserved among the different vertebrate classes (Nica et al., 2004).

pit1 is the earliest lineage-specific marker in the developing zebrafish AH, whose transcripts can be detected in two bilateral clusters of the placode already at 19 hpf, several hours before the transcriptional activation of the earliest hormone genes (Nica et al., 2004). This early onset of *pit1* expression in fish contrasts the situation in mouse, where *Pit1* comes up several days after AH specification, indicated by *lhx3* expression, and even a full day after the onset of corticotrope differentiation. This is most likely due to the adaption of fish to their aquatic habitat, which requires proper Prl-dependent osmoregulation already during embryonic stages. Disrupted salt-water-balance in zebrafish *pit1* mutants is probably also the reason why most of them die during larval stages (Nica et al., 2004), whereas their mammalian counterparts are fully viable, with an even prolonged lifespan (Hsieh et al., 2002).

Analysis of *Pit1* mutant mice revealed that this transcription factor does not only control specification of certain cell fates in a positive way, but also represses others, indicated by the transfating of thyro- to gonadotropes in mutant animals. As one of the underlying mechanisms, Pit1 was found to bind Gata2, thereby blocking the ability of this transcription factor to induce expression of the gonadotropin-specific hormone gene *Lh-β* (Dasen et al., 1999). Approving the conservation of such repressor properties, transfating of thyro- to gonadotropes also occurs in the zebrafish *pit1* mutant. But here, an even more prominent transfating affects presumptive lactotropes of the aPD, which in the

absence of Pit1 become *pomc*-positive (Nica et al., 2004). This indicates that Pit1 negatively regulates *pomc* expression under normal conditions. In line with that function, the *pomc* promoter harbors Pit1 binding sites (unpublished own data).

Based on these data, we previously proposed a model, according to which corticotropes and gonadotropes represent the “default”, and possibly evolutionary most ancient lineages of the AH, which have to be actively repressed by Pit1 to allow differentiation of the “secondary” fates, somato-, lacto-, and thyrotropes (Nica et al., 2004, Pogoda and Hammerschmidt, 2007). This classification, like the “default” concept in general, has of course to be taken with caution, and just refers to one particular regulatory system, rather than describing a general paradigm. For instance, in respect to Notch signaling instead of Pit1, lacto- and thyrotropes, rather than gonado- and corticotropes, appear as the primary cell fates that have to be suppressed by Notch signaling to enable cortico-, somato-, and melanotrope differentiation (see above) (Dutta et al., 2008).

Future studies have to reveal how *pit1* expression and its spatial confinement are regulated. In mouse, *Pit1* expression requires the concerted action of Wnt/ β -catenin signaling and the paired-like homeodomain transcription factor Prop1, which also affects the Notch pathway (Raetzman et al., 2004, Olson et al., 2006, Zhu et al., 2007). However, also here, it is not understood how these pathways might contribute to Pit1’s spatial restriction, while in zebrafish, neither Prop1 nor canonical Wnt genes have been described in the context of AH development at all. Interestingly, in mouse, the Notch effector Hes1 blocks Pit1 (Raetzman et al., 2007, Zhu et al., 2006), suggesting that cells of the Pit1 lineage employ the Delta-Notch system to attenuate further lineage expansion, thereby promoting the alternate lineage and ensuring lineage co-existence. In reverse, at least in zebrafish, there is a similar positive effect of genes promoting the “non-Pit1 lineage” on *pit1* expression and thereby on the other lineage.

10. The Eya1-Six1 complex

In zebrafish, cell types of the “non-Pit1” lineage, the cortico-, melano-, and gonadotropes, can also be positively defined by their particular dependence on the *eya1*

gene, which is affected in the zebrafish *aal* (*all-absent-but-lactotropes*) and *dog* (*dog-eared*) mutants (Nica et al., 2006). *eyal* is one of multiple homologs of *Drosophila*'s *eyes-absent* gene. They belong to the group of so-called Retinal Determination (RD) genes, which control eye formation in insects and mammals. Another member of this group is *sine oculis* (*so*) or its vertebrate homolog *six1*, which encode Six homeodomain transcription factors. Eya1 and Six1 are part of a multi-protein transcriptional complex. Eya1 protein carries an intrinsic tyrosine phosphatase activity, which converts the complex from a transcriptional repressor to an activator (Li et al., 2003). In the developing zebrafish AH, *six1* and *eyal* are coexpressed in all cells and from earliest ppe stages onwards (Nica et al., 2006). *eyal* mutants fail to initiate expression of *pomc* and the gonadotrope marker *gsu α /cga*, indicating that it is absolutely required for the specification of corticotropes, melanotropes and gonadotropes. Expression of *gh*, *tsh β* and *prl* and their upstream regulator *pit1*, although normally initiated, is secondarily lost, indicating that Eya1 is also required for the maintenance of *pit1* expression, contributing to the aforementioned mutual support of the two complementary lineages (Nica et al., 2006). Studies on the expression of *somatolactin- β* , a marker for a teleost-specific other AH cell type, the Somatolactin producing cells, further demonstrated that the Pit1 and Eya1 lineages are not completely complementary. Thus, *somatolactin- β* expression requires both factors, characterizing these cells as the intersection of both lineages (Lopez et al., 2006).

Unlike the Pit1 lineage, no trans-fating of the Eya1-dependent cell types can be observed in the absence of their specific regulator. Also, failed specification does not result in apoptosis, as in *fgf3* or *ascl1a* mutants. Rather, precursors of the Eya1 lineage pause in an immature status, thereby uncoupling differentiation-promoting and anti-apoptotic effects (Nica et al., 2006). This is different from the effect caused by loss of Eya1 function in sensory cells of the inner ear or lateral line organ, where failed specification coincides with apoptosis (Kozlowski et al., 2005). Future studies have to reveal the nature of Eya1-regulated target genes mediating cell specification. Furthermore, it will be interesting to study whether and up to which developmental stage unspecified AH cells of *eyal* mutants can be re-programmed to acquire their regular or other fates by introducing the corresponding downstream or alternative regulators.

In most investigated developmental processes, loss of Eya1 and loss of Six1 cause very similar, if not identical, defects (Zou et al., 2004). However, this is not the case for the zebrafish AH. Here, injection of *six1* morpholinos, although efficiently knocking down *six1* translation and although pheno-copying the lateral line defects of *eyal* mutants, failed to give comparable AH phenotypes, most likely due to functional redundancies with other Six protein of thus far unknown nature (Nica et al., 2006). However, in a recent screen for mutations that disrupt *pomc* expression in the zebrafish hypothalamo-hypophyseal system, we isolated two mutants that displayed a strikingly similar, but slightly weaker phenotype compared to *eyal* mutants. By 96 hpf, these mutants with the allele numbers *fr15* and *fr16* show slightly reduced numbers of lactotropes, but strongly affected thyro- and somatotropes; most strikingly, the *pomc* expressing cortico- and melanotropes are absent (in the case of *fr15*) or severely diminished (in the case of *fr16*) down to one to three cells (Fig. 3; Suppl. Fig. 1). Complementation crosses revealed that *fr15* and *fr16* are allelic, whereas they did complement *dog/aal*, ruling out that they are mutations in *eyal*. Initial bulked segregation mapping placed the *fr15/fr16* locus on chromosome 20 in an interval between the SSLP markers z10056 (8 recombinations in 50 meioses) and z35375 (2/80), close to the marker z22659 (0/42) and an SSLP marker found in *psmc6* (0/72). Among other genes, the interval contained the *six1* gene that we had previously knocked down with morpholinos (see above). However, sequencing of the *six1* gene from mutant embryos revealed homozygous mutations in both alleles.

fr15 mutants harbor an A to C exchange of the second base of the termination codon, transforming it into a serine-encoding codon and elongating the protein by 28 amino acid residues. This mutation induces a *TspRI* restriction site, which could be used as a Restriction Fragment Length Polymorphism (RFLP). Analysis of 214 *fr15* embryos revealed co-segregation of the RFLP with the mutant phenotype (< 2.3 cM). In the case of Bmp2b, a similar C-terminal protein extension caused by stop codon mutations have been shown to result in a fully penetrant null phenotype (Kishimoto et al., 1997), while for Six1^{fr15}, the mutation seems to be of neomorphic or antimorphic nature (see below).

fr16 mutants display a G to A exchange at nucleotide position 47 of the *six1* coding region, resulting in a tyrosine to cysteine exchange of amino acid residue 16. This residue

is part of a highly conserved stretch of the Six-domain, which is involved in protein-protein interactions of the transcription factor (Fig. 3). Meiotic mapping with an *fr16*-induced RFLP generated by the dCAPS method (Neff et al., 1998) again revealed full co-segregation of mutation and phenotype (0 recombination / 112 meioses). In humans, mutations in *SIX1* have been found to be linked to the Branchio Oto Renal Syndrome (BOR, OMIM 113650), a genetic disorder that includes malformations of the ear and cysts in the neck, hearing loss, and malformations of the kidney. According to the autosomal dominant mode of BOR inheritance, the corresponding mutations in human *SIX1* appears to confer dominant-negative properties to the transcription factor (Kochhar et al., 2008, Ruf et al., 2004). One of the antimorphic human mutations disrupts the conserved valine at position 17, right next to the cysteine mutated in the zebrafish *fr16* allele (Fig. 3F). However, in contrast to the human mutations, neither zebrafish *fr15* nor *fr16* heterozygosity caused apparent larval defects. Nevertheless, disrupted *pomc* expression in homozygous mutants could be restored close to wild-type condition by injection of a translation-inhibiting ATG-morpholino, while a cognate control morpholino containing five mismatches had no effect (Fig.3 C-E). A similar scenario (very mild dominant effects, but alleviation of strong defects of homozygotes by morpholino injection) has for instance been described for gain-of-function mutations in the cell adhesion molecule N-cadherin (Warga and Kane, 2007). We can only speculate about the molecular basis of the antimorphic effect of the two mutant Six1 alleles. The Six domain has been implicated in protein-protein interaction. In this light, Six1^{fr16} might interfere with transcriptional complex formation, for instance by trapping Eya1 or other cofactors in inactive complexes. While further studies are necessary to better understand these alleles, they demonstrate the usefulness of ENU mutagenesis and forward genetics to identify potential disease genes that might have been missed via loss-of-function approaches.

Conclusions

By now, isolation and characterization of zebrafish mutants with disrupted AH ontogeny have lead to the identification of 14 essential genes. Six of them encode components of

the Hh pathway. Hh, together with Fgf3, appears to establish the main extrinsic signaling cues regulating AH induction, growth, patterning and survival in teleosts. Recent data have added intrinsic short-range signaling by Delta-Notch to the list of secreted stimuli that control pituitary patterning. One challenge of the future will be to elucidate how the different signaling pathways and the identified essential transcription factors are interconnected. For instance, it will be interesting to learn more about the genes that act up- and downstream of the Notch input, since its time point of action appears to be far earlier than pituitary cell fate commitment was thought to happen. In this respect, ongoing screening might help to identify additional genes with crucial functions in AH formation to progressively fill the remaining gaps in our current understanding of the process.

Admittedly, thus far, the forward genetics approach did not identify as many new players of pituitary development as we had hoped. Rather, most identified regulators or pathways had already been known based on the wonderful work carried out in mouse (Zhu et al., 2007). Just in one case (*ascl1a*), zebrafish genetics provided first functional information on a transcription factor family that had not been studied much in this context thus far, since in mouse, the corresponding mutants did not show a pituitary phenotype, most likely due to functional redundancies. However, there also is an academic value of the zebrafish studies beyond the identification of novel regulators: given that the AH is a relatively recent advance of the chordate phylum, it is very interesting to understand its development in a vertebrate system other than mouse. Comparing the genetic control systems in different vertebrate classes, and ideally with non-vertebrate chordates (Christiaen et al., 2002), should allow fruitful insights into the evolution of this important gland. In fact, we have already learned both about class-specific adaptations of pituitary development, as well as evolutionary conserved traits (e.g. for the Pit1 lineage).

In the future, existing and new zebrafish pituitary mutants will hopefully also serve as valuable tools for studies of the physiological processes controlled by the hypothalamo-hypophyseal-system (Liu et al., 2006, To et al., 2007). Here, one major advantage of the zebrafish system will be the high accessibility of the animals for both experimental manipulations and live imaging. The latter is further potentiated by the generation of

mutant strains that are even largely transparent during adulthood (White et al., 2008), and by the employment of transgenic reporter lines for live imaging on the cellular, subcellular and possibly even the molecular level.

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Figure legends:**Figure 1: Ontogeny of the zebrafish adenohypophysis, temporal expression profiles of the different AH hormones and some intrinsic regulators, and spatial expression patterns and temporal requirements of Hedgehog, Fgf3 and Notch/Delta signals**

The three top rows show simplified cartoons of zebrafish pituitary development at 10, 26 and 60 hpf, and in relation to surrounding neural and non-neural tissues; row 1, lateral views on the head regions of embryos and larvae; rows 2,3, dorsal views; row 3 shows magnified views of AH region. The AH is in light blue, the neural ectoderm (ne) in yellow, and the pre-placodal ectoderm (ppe) in purple. In the first and third row, the expression domains of *shh* and *fgf3* are marked in red and dark blue, respectively. In all panels, anterior is to the left. Black arrows point at the AH, white arrows in the second panel of row 3 indicate the postulated medial movement of posterior AH cells. Other abbreviations: anr, anterior neural ridge; e, eye; h, hypothalamus; le, lens; oc, oral cavity; sto, stomodeal ectoderm.

At the end of gastrulation (10 hpf, left column), AH precursors are located in the anr, the most anterior part of the ppe. The ventral neuroectoderm expresses *hedgehog* genes (*shh* and *twhh*), with the rostral tip of their expression domains extending anteriorly up to the anr (indicated in red in rows 1 and 3). At these early stages, Hedgehog signals are essential for the general induction of AH specificity. During further development, ppe cells at the anr aggregate to form the AH placode, which becomes visible as a distinct structure at the anterior edge of the head by 19 hpf. At this and during the following stages, the hypothalamic *hedgehog* expression domain is located at the anterior aspect of the AH anlage. At the same time, *fgf3* expression comes up in the ventral diencephalon, in close dorsal proximity of AH anlage. The *fgf3* expression domain progressively shifts towards posterior regions of the gland. Diencephalic Fgf3 signals are required for AH specification and survival between 18 and 22 hpf (indicated by blue bar in bottom part). In addition, opposing Hedgehog and Fgf3 signaling gradients pattern the pituitary placode along its anterior-posterior axis during mid-segmentation stages, with Hh specifying anterior and Fgf3 specifying posterior fates (see also Fig. 3). Beginning at 26 hpf, the AH placode gets internalized into the head, coinciding with the formation of the

oral cavity, and reaches its final position between 48 and 72 hpf. Endocrine differentiation of the distinct AH lineages starts around 24 hpf, as indicated by the onset of cognate hormone gene expression. In contrast to mouse, where the Pit1 lineage is established rather late, *pit1* and *prl* of the Pit1 lineage are the first lineage- and cell type-specific genes to be expressed in the zebrafish AH (see black bars in middle part for progressive onset of other hormone genes).

With the onset of oral cavity formation, *sonic hedgehog (shh)* also starts to be expressed in the oral ectoderm, establishing a second source of Hh signals with probable impact on AH patterning along its dorsal-ventral axis. *notch* and *delta* genes are transiently expressed within the zebrafish AH placode between 14 and 48 hpf. They are required for differential cell fate specification between 14 and 16 hpf, thus several hours before the onset of endocrine differentiation, and before the first lineage-specific regulators like *pit1* come up (indicated by yellow bar in bottom part).

Figure 2: Molecular network regulating adenohypophysis organogenesis in zebrafish.

Diagrams showing the interaction and impact of the identified regulators of zebrafish AH formation. Arrowheads indicate stimulating activities, crossbars inhibitory functions. The grey ellipse in the center represents the AH placode, with anterior to the left and posterior to the right. It consists of two main entities: the *pars distalis* (PD, dark grey), characterized by *nkx2.2a* expression, and the *pars intermedia* (PI, light grey), defined by *pax7* expression. The PD can be further subdivided into the anterior PD (aPD) and the posterior PD (pPD). Thus, the zebrafish AH is made of three (sub)domains that are arranged along the anterior-posterior (a-p) axis of the organ. Each domain harbors a specific set of endocrine cell types. The aPD consists of lactotropes (L) and corticotropes (C), the pPD of somatotropes (S), thyrotropes (T) and gonadotropes (G), and the PI of melanotropes (M) - due to space constraints corticotropes of the PI and Somatolactin producing cells of the pPD and the PI are not indicated). The open arrow at the border between pPD and PI indicates the corresponding position of the mouse AH anlage. In mouse, it becomes the leading edge / dorsal tip of Rathke's Pouch, whereas in fish, a

corresponding invagination does not occur, and the AH anlage remains in its subepithelial position.

The Bicoid related transcription factor Pitx3 defines a pre-placodal equivalence field, which is specified to develop either into (lateral) lenses or (more medial) pituitary cells (left panel). Hh signals emanating from the ventral midline of the neuroectoderm induce Pitx3-positive ppe cells at the anterior neural ridge (anr) to acquire AH fates (red arrow), while more lateral cells of the equivalence domain that do not receive Hh give rise to lens tissue. Subsequently, Hh further acts as a morphogen on the AH anlage, promoting anterior fates in a dose-dependent fashion. An opposing signaling gradient that promotes posterior fates is provided by Fgf3 from the posterior-ventral hypothalamus. An initial hypothalamic Fgf3 pulse is also required for general AH survival and AH specification (by transcriptional induction of intrinsic transcription factors like Lim3 and Ascl1a, or transcriptional maintenance of Eya1 and Six1) (blue arrows). Pit1 drives the differentiation of lacto-, somato-, and thyrotropes, while it inhibits gonado-, cortico-, and melanotrope formation (pink arrows). In contrast, the Eya1-Six1 complex positively regulates the development of the three latter lineages. It further promotes maintenance of *pit1* expression, thereby also supporting somato- and thyrotropes in the alternate lineage (black arrows). The bHLH transcription factor Ascl1a acts as a global “pro-endocrine” player of AH development, which is crucial for the differentiation of all endocrine cell types. Its positive effect on lacto-, somato-, and thyrotropes might be mediated by transcriptional activation of the *pit1* gene, while its positive effect on corticotropes is most likely achieved by direct transcriptional activation of the *pomc* gene itself, as suggested by the presence of E-boxes in the *pomc* promoter element driving corticotrope-specific expression. Ascl1a also controls cell survival of a subset of AH cells (light turquoise arrow). Lateral inhibition mediated by intrinsic Delta/Notch short-range signaling controls the segregation of distinct cell lineages within or across the AH subdomains (yellow). For details see text. Abbreviations: encircled I, transcriptional induction; encircled M, transcriptional maintenance (also indicated by dashed lines); L, lactotropes, C, corticotropes, S, somatotropes; T, thyrotropes; G, gonadotropes; M, melanotropes.

Figure 3: Isolation of two anti- or neomorphic *six1* alleles that disrupt *pomc* expression in the pituitary.

(A-B, D-E) *pomc* expression in wild-type (A), *six1^{fr16}* mutants (B), *six1^{fr15}* mutants injected with a *six1* 5-mismatch control MO (D), and in a *six1^{fr15}* mutant rescued by *six1* MO injection (E). The black arrowhead marks *pomc* expression of the aPD, the grey arrowhead marks *pomc* expression in the PI. The unaffected bilateral *pomc* expression domains represent cells of the hypothalamic *arcuate nucleus*, which in mammals is involved in the regulation of feeding behavior and energy homeostasis. Images show dorsal views on the head region between the eyes, anterior is to the left. Frequencies of obtained phenotypes for the different injection experiments were as follows: *fr15*: uninjected control = 15 embryos with mutant phenotype / 65 evaluated embryos (expected ration ¼); injection of *six1* MO = 3/71; injection of *six1* 5mm MO 11/48; *fr16*: uninjected control = 16/60; plus *six1* MO = 4/74; plus *six1* 5mm MO 13/56.

(C) Schematics of wild-type (top) and mutant (bottom) versions of Six1. Note the C-terminal extension (grey) of the Six1^{fr15} allele. The *six1^{fr16}* mutation converts a cysteine to a tyrosine within the Six domain at amino acid position 16. (F) Sequence alignment of the N-terminal 21 amino acid residues of the human, mouse, frog, zebrafish and *Drosophila* Six1/So proteins. The highly conserved cysteine residue, which is replaced by a tyrosine in *six1^{fr16}*, is highlighted in grey. Note the adjacent highlighted valine residue (in salmon), which is affected in an antimorphic allele of human Six1. Abbreviations: SD, six domain (in red); HD, homeodomain (in blue); h, human; m, mouse; X, *Xenopus laevis*; z, zebrafish; Dm, *Drosophila melanogaster*.

Supplementary Figure 1: *six1^{fr15}* mutants show a similar, but slightly weaker AH phenotype than *eyal^{t22744}* embryos, while *six1^{fr15} / eyal^{t22744}* double mutants are indistinguishable from *eyal^{t22744}* single mutants.

Expression of *prolactin* (*prl*) (A-D), *gh* (E-H), *thyroid-stimulating hormone* (*tshβ*) (I-L) and *pomc* in larvae at 120 hpf and of the indicated genetic backgrounds. All images are

dorsal views with anterior to the left. The double mutant analysis shown in (D,H,L,P) is in line with previous results obtained by *six1* MO injection in *eyal* mutants, indicating that the *eyal* mutant phenotype results from the loss of the transcription-activating activity of the Eya1-Six1 complex, rather than an aberrant activity of the complex as a transcriptional repressor (Nica et al., 2006). In the latter case, the defects should be alleviated in the double mutant.



