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The proneural gene *ascl1a* is required for endocrine differentiation and cell survival in the zebrafish adenohypophysis

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Mammalian basic helix-loop-helix proteins of the *achaete-scute* family are proneural factors that, in addition to the central nervous system, are required for the differentiation of peripheral neurons and sensory cells, derivatives of the neural crest and placodal ectoderm. Here, in identifying the molecular nature of the *pia* mutation, we investigate the role of the zebrafish *achaete-scute* homologue *ascl1a* during development of the adenohypophysis, an endocrine derivative of the placodal ectoderm. Similar to mutants deficient in Fgf3 signaling from the adjacent ventral diencepahalon, *pia* mutants display failure of endocrine differentiation of all adenohypophyseal cell types. Shortly after the failed first phase of cell differentiation, the adenohypophysis of *pia* mutants displays a transient phase of cell death, which affects most, but not all adenohypophyseal cells. Surviving cells form a smaller pituitary rudiment, lack expression of specific adenohypophyseal marker genes (*pit1*, *neurod*), while expressing others (*lim3*, *pitx3*), and display an ultrastructure reminiscent of precursor cells. During normal development, *ascl1a* is expressed in the adenohypophysis and the adjacent diencephalon, the source of Fgf3 signals. However, chimera analyses show that *ascl1a* is required cell-autonomously in adenohypophyseal cells themselves. In *fgf3* mutants, adenohypophyseal expression of *ascl1a* is absent, while implantation of Fgf3-soaked beads into *pia* mutants enhances *ascl1a*, but fails to rescue *pit1* expression. Together, this suggests that Ascl1a might act downstream of diencephalic Fgf3 signaling to mediate some of the effects of Fgf3 on the developing adenohypophysis.

KEY WORDS: Ascl1a (Zash1a), Pituitary, Adenohypophysis, Neurohypophysis, Zebrafish, Pia, Cell survival, Cell specification, Apoptosis

INTRODUCTION

The hypothalamic-hypophyseal axis constitutes a functional link between the nervous and the endocrine system. As a key regulator of hormone secretion, the hypophysis, also called pituitary gland, controls basic vital processes including growth, reproduction and overall body homeostasis. The mature pituitary consists of two functionally and anatomically distinct components: the neurohypophysis and the adenohypophysis, also named posterior and anterior pituitary lobe, respectively. In contrast to the neurohypophysis, which derives from ventral regions of the hypothalamus, the adenohypophysis originates from placodal ectoderm. At late gastrula and early segmentation stages, the placodal ectoderm is located at the interphase of the anterior neuroectoderm and the epidermal ectoderm, constituting a cell population that in several respects is the neural crest equivalent of the presumptive forebrain region. Anterior-most placodal ectoderm at the anterior neural ridge gives rise to the adenohypophysis, while more lateral and posterior regions of the placodal ectoderm give rise to sensory cells of nose, inner ear and lateral line, neurons of various cranial ganglia, and the lenses (for review, see Baker and Bronner-Fraser, 2001).

The adenohypophyseal primordium itself develops into multiple endocrine cell types, classified by the hormones they produce, namely lactotropes, somatotropes, thyrotropes, gonadotropes, corticotropes and melanotropes. Despite some crucial differences in morphogenesis and architecture of the adenohypophysis (Herzog et al., 2003), the molecular machinery that controls pituitary organogenesis appears largely conserved among different vertebrate species, following a program integrating extrinsic and intrinsic control elements. Extrinsic signaling molecules shown to be essential for pituitary development both in mouse and zebrafish are the fibroblast growth factors Fgf3/Fgf10 from the ventral diencephalon (Herzog et al., 2004a; Ohuchi et al., 2000), and Sonic hedgehog from the oral ectoderm and/or the hypothalamus (Herzog et al., 2003; Sbrogna et al., 2003; Treier et al., 2001). Together with other signals such as Fgf8, Bone morphogenetic proteins (Bmps) and Wnts (Ericson et al., 1998; Treier et al., 1998; Treier and Rosenfeld, 1996), they provide trophic and positional cues to the adenohypophyseal primordium, regulating the spatial and temporal expression patterns of transcription factor genes within the primordium (Burgess et al., 2002; Burrows et al., 1999; Scully and Rosenfeld, 2002; Zhu and Rosenfeld, 2004). Identified intrinsic transcription factors in control of adenohypohyseal cell proliferation and differentiation belong to different protein classes, including LIM homeodomain proteins (Lhx3, Lhx4, Isl1), Bicoid-like homeodomain proteins (Pitx1, Pitx2), Sine oculis-related homeodomain proteins (Six1, Six3, Six6), Paired-like homeodomain proteins (Hesx1, Prop1), POU domain homeodomain proteins (Pit1; Pou1f1 – Zebrafish Information Network), zinc-finger proteins (Gata2), T-box proteins (Tbx19) and STAT proteins (Stat3).

By contrast, basic helix-loop-helix (bHLH) transcription factors have been less well studied in the context of pituitary development. Two genes encoding bHLH proteins have been reported to be expressed in the zebrafish, mouse and/or human pituitary: *Mash1* and *Neurod1* (Allende and Weinberg, 1994; Ferretti et al., 2003;

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Guillemot and Joyner, 1993; Poulin et al., 2000; Wullimann and Mueller, 2002). They are homologues of proneural genes in *Drosophila*; Mash1 is a homologue of the *achaete-scute* (*asc*) complex genes, *Neurod1* a homologue of *atonal* (Kageyama et al., 1997). Both Mash1 and Neurod1 are known to be prominent regulators of mammalian neurogenesis (Casarosa et al., 1999; Farah et al., 2000; Guillemot et al., 1993; Olson et al., 2001; Sommer et al., 1995). In the mouse adenohypophysis, *Neurod1* expression is confined to corticotropes (Poulin et al., 2000; Poulin et al., 1997). Neurod1 has further been shown to bind to the proopiomelanocortin (*Pomc*) promoter, and to have a positive effect on *Pomc* transcription (Lamolet et al., 2004; Poulin et al., 2000; Poulin et al., 1997). Together, these findings suggested a requirement of Neurod1 for corticotroph differentiation. However, *Neurod1*-deficient mice just display delayed initiation of *Pomc* expression (Lamolet et al., 2004), indicating that Neurod1 is largely dispensable for corticotroph development, possibly because of functional redundancy with other transcription factors.

In contrast to *Neurod1*, the role of *Mash1* in pituitary development remained elusive. Remarkably, aside from controlling neurogenesis in the central and peripheral nervous system (CNS, PNS), mouse Mash1 is crucial for the development of cells of the so-called diffuse neuroendocrine system, including adrenal medullary chromaffin cells (Huber et al., 2002), thyroid parafollicular C-cells (Lanigan et al., 1998) and pulmonary neuroendocrine cells (Ball, 2004; Borges et al., 1997; Ito et al., 2000). In light of these data and the common expression of *Mash1* in the mammalian pituitary, it was speculated that Mash1 might play a similar role during the ontogeny of the adenohypophysis (Ferretti et al., 2003). However, genetic proof for such a function had been missing thus far.

Genetic screens in the zebrafish for mutations that disrupt adenohypophyseal *growth hormone* (*gh*) gene expression have led to the isolation of the *pituitary absent* (*pia*) mutant, characterized by a dearth of lactotrope-, corticotrope-, somatotrope- and thyrotrope-specific hormone expression during larval stages (Herzog et al., 2004b). Here, we report that the *pia* gene corresponds to zebrafish *achaete-scute-like 1a* (*ascl1a*; formerly called *zash1a*) (Allende and Weinberg, 1994), providing genetic evidence for an essential and cell-autonomous role of this proneural gene during endocrine specification of adenohypophyseal cells. In the absence of Ascl1a, all adenohypophyseal cell types fail to express their cognate hormone genes, although only some of them become apoptotic. This is in contrast to the situation in *fgf3* mutants, where failed specification leads to the loss of all adenohypophyseal cells. In view of this, the *ascl1a* mutation allows us to distinguish two different populations of adenohypophyseal cells in which cell specification and cell maintenance are either coupled or independent from each other.

MATERIALS AND METHODS

Genetic mapping, identification of mutation and genotyping

The *pia*^{t25215} mutation was mapped to LG4 via bulked segregation analysis, using a panel of simple sequence length polymorphism (SSLP) markers and standard techniques (Geisler, 2002). Linkage assignments were confirmed and further refined by genotyping single mutant and wild-type embryos, which placed *pia*^{t25215} close to SSLP marker z27201 (1 recombinant among 192 meiosis). In addition, we identified a new SSLP, amplifying a genomic fragment from contig BX511171.13 with primers 5′-GTACACTTGA-AGCTTGTGCG-3′ and 5′-GTTTTCTGCACCAGAACCTG-3′. For this marker, we found no recombination among 192 meioses.

The *acsl1a* open reading frame is encoded by a single exon. To search for lesions in *ascl1a*, we amplified overlapping *ascl1a* fragments by PCR from genomic mutant and wild-type DNA samples, followed by sequencing in forward and reverse directions.

The *pia*^{t25215} mutation generates a *Hind*III restriction site, which was used as a restriction fragment length polymorphism (RFLP) for genotyping. A 523 bp *ascl1a* fragment containing the polymorphic restriction site was amplified from genomic DNA of single embryos, using primers 5′-TCAGAGCATCCAACTCAGCC-3′ and 5′-CGAACGCTCAAAACCAG-TTG-3′, followed by *Hin*dIII digest. On wild-type DNA, this results in two fragments of 203 and 320 bp, whereas the mutant PCR product is cleaved into three fragments of 320, 139 and 64 bp. For genotyping of *pia* homozygotes injected with *ascl1a* BAC DNA, we identified an RFLP in the 3′-UTR of *ascl1a*, distinguishing the wild-type allele from the *pia*^{t25215} allele and the BAC-encoded gene. A 387 bp DNA fragment of BAC-injected embryos was amplified via PCR using primers 5′-AACAAGAGCT-CCTGGACTTC-3′and 5′-CACGGTGTCGTGGAAAGTCT-3′, followed by *Sty*I digest, which cleaved only the *pia*^{t25215} *ascl1a* allele and the BAC-derived fragment (301 bp and 86 bp), but not the endogenous wild-type allele.

Morpholinos, generation of constructs, mRNA synthesis and microinjection

The antisense MO for *ascl1a* (5′-CATCTTGGCGGTGATGTCCATTTCG-3′; corresponding to nucleotides –4 to +21 of *ascl1a* cDNA) was obtained from Gene Tools and diluted in 1× Danieu's buffer to a final concentration of 0.033 pmol/nl. The MO was injected in volumes of 1 to 1.5 nl into embryos at the one- to four-cell stage, as described (Nasevicius and Ekker, 2000).

To obtain an *ascl1a* expression construct, the *ascl1a* cDNA was cloned via *Sma*I/*Xho*I sites into pCS2+ (Rupp et al., 1994) to yield pCS2-*ascl1a*. To test the efficiency of the *ascl1a* MO (see below), a fusion construct encoding Ascl1a protein tagged with six C-terminal Myc epitopes was generated by amplifying the *ascl1a*-coding region from pCS2-*ascl1a* and cloning it into the *Bam*HI and *Spe*I sites of pCS2-3′MT (kind gift of U. Strähle). For *ascl1a*-VP16 fusion constructs, either the entire *ascl1a*-coding region without the termination codon (wild type), or bp 1-207 (mutant) were amplified via PCR, and cloned into the *Eco*RI site of pCS2+. The VP-16 activator sequence was excised from a pGMT-VP16 construct (Pogoda et al., 2000) and cloned 3′ to the *ascl1a* fragments. Capped mRNA was prepared after plasmid linearization, using the Message Machine kit (Ambion). Synthetic mRNA was injected into one- to four-cell stage embryos, as previously described (1.5 nl per embryo) (Hammerschmidt et al., 1999). For BAC injections, an *ascl1a* containing BAC was obtained from RZPD (ID HUKGB735K15264Q8), and injected at a concentration of 75 ng/μl into the cytoplasm of one-cell stage *pia*^{t25215} +/– intercross progeny.

Immunoblotting

Embryos were injected with 800 pg *ascl1a-myc* mRNA with or without *ascl1a* MO, and embryonic protein extraction were collected at 8 hpf as described (Westerfield, 1994). Protein samples were separated via SDS-PAGE on 12% Acrylamid/Bis-acrylamid gels, and blotted on Hybond P membranes (Amersham). Immunoblotting analyses were performed using either the anti-Myc antibody 9E10 (Roche Diagnostics) or an anti-pan cadherin antibody (Sigma) as a loading control.

In situ hybridization, sectioning, immunostaining and Acridine Orange staining

Single and double whole-mount in situ hybridization was performed and specimens were photographed as previously described (Hammerschmidt et al., 1996; Nica et al., 2004). Riboprobes of the following cDNAs were used as described previously: *ascl1a* and *ascl1b* (Allende and Weinberg, 1994), *elavl3* (Kim et al., 1997), *crybb1* (Chen et al., 2001), *omp* (Yoshida et al., 2002), *neurod* (Blader et al., 1997), *shh* (Krauss et al., 1993), *nkx2.1a* (Rohr and Concha, 2000), *fgf3* (Herzog et al., 2004a), *lim3* (Glasgow et al., 1997), *pitx3* (Dutta et al., 2005), *pit1* and *gsu* (Nica et al., 2004), and *gh*, *tsh*, *pomc* and *prl* (Herzog et al., 2003). Stained embryos were post-fixed, embedded in JB4 resin (Polysciences) and sectioned at 7 μm. Anti-Prl immunostaining was performed with rabbit anti-prolactin (Kawauchi et al., 1983; Sbrogna et al., 2003) and Cy3-labelled goat anti-rabbit antibodies (Jackson ImmunoResearch). Apoptotic cell corpses were visualized with the vital dye Acridine Orange, as previously described (Herzog et al., 2004a).

Electron microscopy

Wild-type and mutant larvae were fixed with 2.5% glutaraldehyde in PBS for 30 minutes each at ambient temperature and then on ice. After washing with PBS, the larvae were postfixed with 1% osmium tetroxide in 100 mM phosphate buffer pH 7.2 for 1 hour on ice, washed with H_20, stained with 1% aqueous uranylacetate for 1 hour, dehydrated in a graded series of ethanol and finally embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in Philips CM10 electron microscope. In parallel, Toluidine Blue stained Epon sections of 0.5 μm of the same specimen were prepared for light microscopy.

Cell transplantations, bead implantations and SU treatments

Donor embryos were injected at the one- to four-cell stage with biotin-dextran (Fig. 5A-C) or rhodamin-dextran (Fig. 5D-F) (Molecular Probes), and transplanted at the shield stage into recipient embryos from a cross of two *pia* heterozygous parents. Alternatively, *h2a*::*h2a-GFP* transgenics were used as donors (Fig. 5G-I) (Pauls et al., 2001). Chimeric embryos were analyzed either by in situ hybridization against *pit1* or *pomc*, followed by anti-biotin staining with the Vectastain Elite ABC kit (Vector Laboratories), or by Acridine Orange or anti-Prl immunostaining, followed by fluorescent evaluation in two different channels. Genotyping of recipients was performed with genomic DNA extracted from clipped tails lacking wild-type donor cells, as described above. Implantations of Fgf3-loaded beads into embryos of *pia*^{t25215} or *lia*^{t24149} intercrosses were carried out as described (Herzog et al., 2004a). Embryos were genotyped after photography as described above (*pia*) or by Herzog et al. (Herzog et al., 2004a) (*lia*). Treatments with the Fgfr inhibitor SU5402 (Calbiochem) were carried out as previously described (Herzog et al., 2004a).

RESULTS

The *pia* locus encodes *ascl1a*

During a large-scale screen for zebrafish mutants with altered *growth hormone* (*gh*) expression, we had isolated one ENU-induced *pituitary-absent* (*pia*) mutation (Herzog et al., 2004b). Segregation linkage analysis revealed that *pia* maps to a defined interval on linkage group 4 (Fig. 1A, upper panel; see Materials and methods). One of the genes that had been previously mapped to this genomic location (see http://zfin.org/cgi-bin/mapper_select.cgi) was the zebrafish *achaete-scute* homologue *ascl1a*, which appeared to be a reasonable candidate, as it is expressed in the pituitary (Allende and Weinberg, 1994; Wullimann and Mueller, 2002). Sequencing of the *ascl1a* gene from genomic DNA of *pia* mutant embryos uncovered a C to A mutation at coding nucleotide 210, changing a tyrosine residue to a premature stop codon. This results in a truncated Ascl1a protein that lacks all amino acid residues after position 69, including the entire basic DNA-binding domain and the HLH domain (Fig. 1A, lower panel). The found *ascl1* mutation generates an RFLP (see Materials and methods), which we used to genotype additional *pia* homozygotes, revealing no recombination between *ascl1* and *pia* in 215 tested embryos (430 meioses; <0.2 cM).

To further confirm that the defects observed in *pia* are due to a loss of Ascl1a function, we phenocopied the *pia* defects in wild-type embryos by knocking down *ascl1a* with antisense morpholino oligonucleotides (MO) (Nasevicius and Ekker, 2000). The used MO targeting a sequence covering the *ascl1a* start codon efficiently

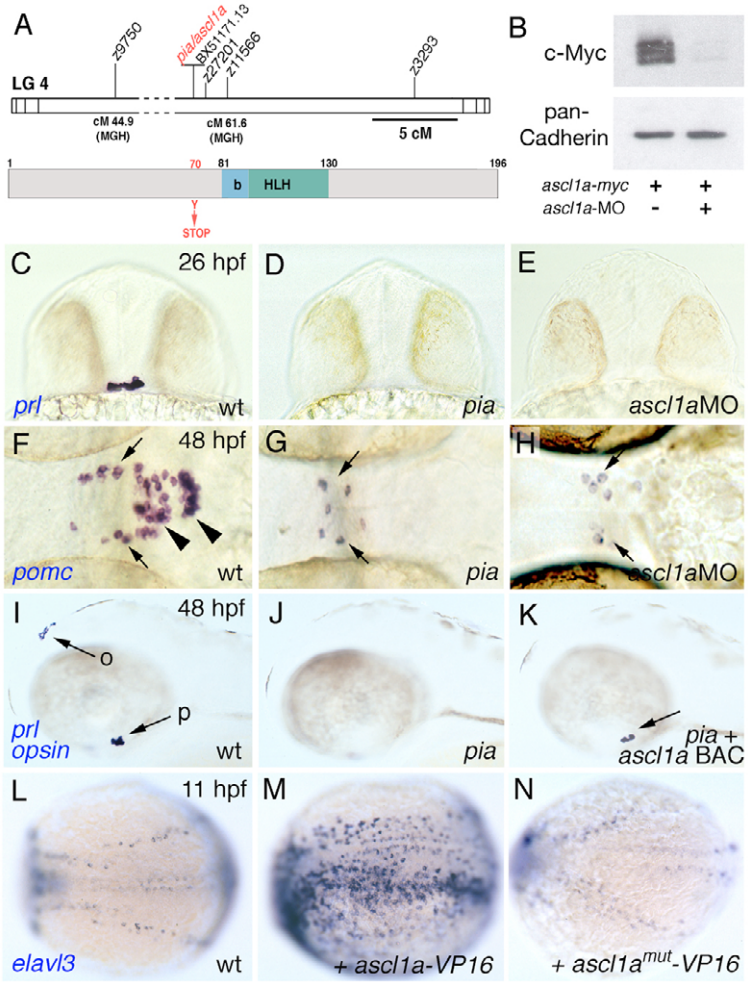

Fig. 1. *pia* encodes the *achaete-scute* homologue Ascl1a. (**A**) (Top) Genetic map of a region of linkage group 4 (LG4), showing positions of the *pia*^{t25215} mutation, the *ascl1a* gene and some markers used for mapping. Genetic distances of markers from the top of LG4 according to (http://zfin.org/cgi-bin/mapper_select.cgi) are indicated. (Bottom) Schematic representation of Ascl1a protein: the basic helix-loop-helix domain (blue and green) and the *pia*^{t25215} mutation (red) are indicated. (**B**) Anti-Myc western blot, showing that translation of chimeric *ascl1a-myc* mRNA is efficiently blocked in the presence of *ascl1a* MO (upper panel). The same blot was probed with an anti-pan-cadherin antibody for loading control (lower panel). (**C-H**) Whole-mount in situ hybridization detecting expression of *prl* (26 hpf; frontal view, dorsal up; C-E) and *pomc* (48 hpf; dorsal view, anterior towards the left; F-H) in wild-type (wt; C,F), *pia*^{t25215} mutant (*pia*; D,G) and *ascl1a* morphant embryos (*ascl1a*MO; E,H). (F-H) *pomc*-positive cells of the adenohypophysis are indicated with arrowheads, *pomc*-positive cells of the arcuate nucleus in the hypothalamus are indicated with arrows. (**I-K**) In situ hybridization at 48 hpf for the pituitary marker *prl* (p) and the epiphysis marker *opsin* (o). Injected BAC DNA usually does not distribute uniformly, but leads to chimeric embryos with only a subset of cells containing the injected DNA. Accordingly, the rescued *pia* embryo in K displays strong *prl* expression (arrow), but still lacks *opsin* expression in epiphysis, another phenotypic trait caused by loss of Ascl1a function (Cau and Wilson, 2003). The genotype of the embryo was further confirmed via PCR (see Materials and methods). (**L-N**) *elavl3* in situ hybridization at 11 hpf, dorsal views, anterior towards the left, demonstrating that wild-type Ascl1a fused to the VP16 transactivation domain can induce primary neurons in inter-proneural domains of the neural plate (M), whereas a fusion between VP16 and the truncated Ascl1a protein encoded by the *pia*^{t25215} allele is ineffective (N). hpf, hours post fertilization.

blocked *ascl1a* translation, as revealed by anti-Myc western blot analysis of protein extracts from embryos injected with mRNA encoding an Ascl1a-Myc fusion protein (Fig. 1B). Injection of the *acsl1a* MO into wild-type embryos led to a complete loss of adenohypophyseal *prolactin* (*prl*, 93% affected, *n*=58) and *pomc* (86% affected, *n*=48) expression, a phenotype indistinguishable from that of *pia* mutants (Fig. 1C-H). Furthermore, we were able to rescue *prl* and *pomc* expression in *pia* mutants upon injection of BAC DNA containing the *ascl1a* gene (Fig. 1I-K; *n*=7; and data not shown).

Finally, to investigate the severity of the pia^{t25215} mutation, we studied the effect of *ascl1a* overexpression on neurogenesis in the neural plate of zebrafish embryos. Although injection of mRNA encoding wild-type *ascl1a* had no effect (data not shown), injection of mRNA encoding a Ascl1a-VP16 fusion protein (0.6 ng/μl) led to a massive increase in the number of *elavl3*-positive neural precursor cells (Fig. 1M; 78/78) (compare with Bae et al., 2005; Kim et al., 1997). By contrast, the corresponding fusion between the VP16 transactivating domain and the truncated part of Ascl1a encoded by the pia^{t25215} allele was completely ineffective, even when injected at 60-fold higher molarity (12 ng/μl) (Fig. 1N; 0/42). In summary, these data indicate that the pituitary phenotype of *pia* mutants is caused by a null mutation in the zebrafish *achaete scute* homologue *acsl1a*.

ascl1a is expressed in the adenohypophysis and in adjacent domains of the diencephalon

To gain more detailed information about the spatiotemporal dynamics of adenohypophyseal *ascl1a* expression, we performed whole-mount in situ hybridization from early segmentation stages to 120 hpf. First expression of *ascl1a* in the pituitary placode could be detected at the 20-somite stage (18 hpf; data not shown), coincident with the onset of expression of other pituitary regulators such as *lim3* (*lhx3* – Zebrafish Information Network), *pitx3* and *pit1*. At 22 hpf and 24 hpf, *ascl1a* was co-expressed with *pitx3* in all adenohypophyseal precursor cells at the anterior neural ridge (Fig. 2A-D), a subset of which also displayed expression of *prl* (Fig. 2E,F). Consistently, at 26 hpf, *ascl1a* transcripts were uniformly distributed throughout the entire adenohypophyseal anlage, including anterior-most cells, which give rise to lactotropes and corticotropes, and more lateral-posterior cells, which most probably form somatotropes and thyrotropes (Fig. 2G) (cf. Herzog et al., 2004a; Nica et al., 2004). This uniform distribution of *ascl1a* transcripts persisted until 72 hpf (see Fig. 2H for 40 hpf). Then, expression became confined to anterior and posterior regions of the adenohypophysis, displaying a pattern resembling that of *pomc* expression (Herzog et al., 2004a), whereas the medial domain, characterized by the expression of *gh* and *tsh* (Herzog et al., 2004a), became devoid of *ascl1a* expression (Fig. 2I,J). This spatial shift from a uniform to a restricted distribution of transcripts suggests that *ascl1a* might initially be required in all pituitary cells, while later playing more restricted roles in particular cell types only. At all investigated stages (24 hpf –120 hpf), *ascl1a* in addition to the adenohypopyseal primodium itself was also expressed in adjacent cells of the diencephalon (Fig. 2C-J), such as the posterior ventral hypothalamus (pvh; Fig. 2I,J).

Most probably owing to the additional round of genome duplication that occurred during teleost evolution (Postlethwait et al., 1998), the zebrafish has a second *ascl1* gene, named *ascl1b* (Allende and Weinberg, 1994). To study whether partial redundancy between Ascl1a and its paralog Ascl1b might account for the slightly weaker phenotype of *pia* mutants compared with *fgf3* mutants, we also analyzed the expression of *ascl1b* (Allende and Weinberg, 1994). However, at all stages examined (24-120 hpf), *ascl1b* expression was excluded from the adenohypophysis (Fig. 2K,L; and data not shown), indicating that the regulation of adenohypophysis development has been dedicated to the *ascl1a* paralog only.

ascl1a/*pia* is required for proper initiation of pituitary specification and terminal differentiation of all adenohypophyseal cell types

Thus far, the pituitary defects of *pia* mutants had been analyzed only briefly, revealing the absence of *gh*, *pomc*, *prl* and *thyroid-stimulating hormone* (*tsh*) transcripts at late larval stages (120 hpf) (Herzog et al., 2004b). We extended such expression analyses, looking at additional markers and at earlier stages of development. The placodal ectoderm, from which the zebrafish pituitary originates, specifies from late gastrulation through mid segmentation

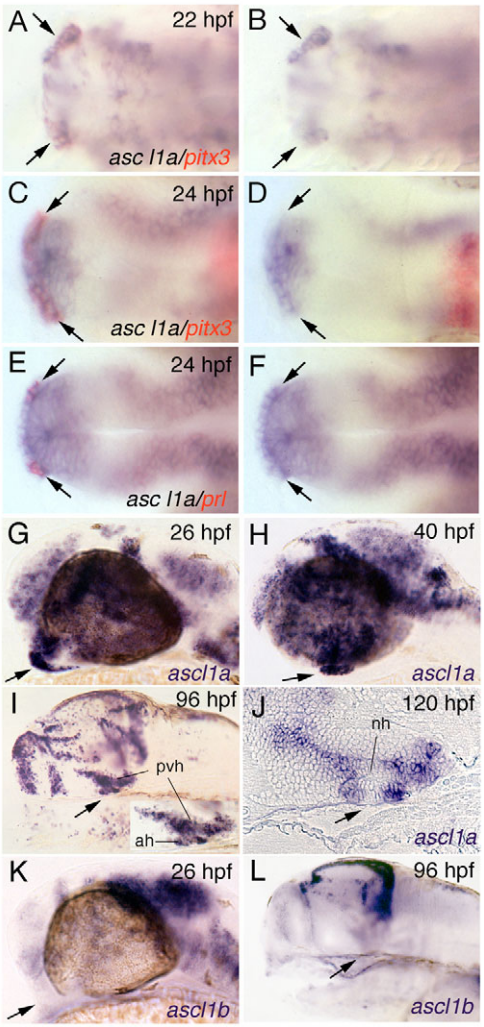

Fig. 2. *ascl1a* is expressed in the adenohypophysis and the adjacent diencephalon. All panels show double (**A-F**) or single (**G-L**) in situ hybridization with probes indicated in the lower right-hand corners and ages of specimen in the upper right-hand corners. (B,D,F) Same embryos as in (A,C,E), after the red staining has been washed out. (A-F) Dorsal views, anterior towards the left; (G-L) lateral views, anterior towards the left, dorsal upwards. Arrows indicate adenohypophysis (G-L) or its anlage at the anterior neural ridge (A-F). ah, adenohypophysis; nh, neurohypophysis; pvh, posterior-ventral hypothalamus.

stages, and is set up normally in *pia* mutants, as revealed by in situ hybridization against *eya1* transcripts (data not shown) (cf. Herzog et al., 2004a). Specification of the pituitary itself starts around 19 hpf, as indicated by expression initiation of adenohypophysis-specific marker genes, such as the pan-pituitary marker *lim3* (Glasgow et al., 1997). At 25 hpf, *lim3* expression was present in *pia* mutants, but the staining was very diffuse and strongly reduced compared with wild-type siblings (Fig. 3A). More moderately reduced expression was also observed for *pitx3* (Dutta et al., 2005; Zilinski et al., 2005), another marker of the entire adenohypophyseal anlage (Fig. 3C), and for *ascl1a* itself (Fig. 3E). Interestingly, expression levels of all three genes appeared to recover during later stages of development, when the pituitary of *pia* mutants was of reduced size (see also below); however, *lim3*, *pitx3* and *ascl1a* hybridization signals were of normal intensity (Fig. 3B,D,E, inset).

By contrast, expression of other regulator genes was completely lacking in *pia* mutants from earliest stages onwards. Thus, *neurod*, another bHLH gene that like *ascl1a* is expressed throughout the adenohypophysis of wild-type embryos (Blader et al., 1997; Mueller and Wullimann, 2002), was absent in the pituitary of *pia* mutants at all investigated stages (Fig. 3F; and data not shown). Similarly, expression of *pit1*, which encodes a POU domain transcription factor required for transcriptional activation of *prl*, *gh* and *tsh*, and for repression of *pomc* (Nica et al., 2004), was absent in *pia* mutants at 20 hpf, shortly after its expression initiation in wild-type embryos (data not shown) and at all investigated later stages (see Fig. 3G,H for 25 hpf and 72 hpf).

Early defects in pituitary formation were also visible at the morphological level. Using Nomarski optics, the adenohypophysis can normally be recognized as a separate organ from 25 hpf onwards. However, in *pia* mutants, adenohypophyseal borders appeared much less distinct than in their wild-type sibling embryos (Fig. 4A,B; 26 hpf). Together with the decreased intensity of early marker gene expression, this suggests that *ascl1a* is crucial for the proper onset of adenohypophysis specification and organ formation.

We also studied the expression of the different hormone genes at early time points of pituitary development. In zebrafish, terminal differentiation of the different adenohypophyseal cell types occurs in sequential steps between 22 hpf and 48 hpf (Herzog et al., 2003). The first cells that undergo terminal differentiation are the lactotropes, indicated by the expression of *prolactin* (*prl*), which is initiated at 22 hpf. The next lineages to differentiate are the corticotropes and melanotropes, which express *pomc* from 24 hpf onwards (Herzog et al., 2003). The somatotropes, marked by expression of *gh* (Herzog et al., 2003), the thyrotropes, marked by co-expression of *tsh* and *gsu* (Herzog et al., 2003; Nica et al., 2004), and the gonadotropes, marked by expression of *gsu* (Nica et al., 2004), start to differentiate between 36 and 42 hpf. In *pia* mutants,

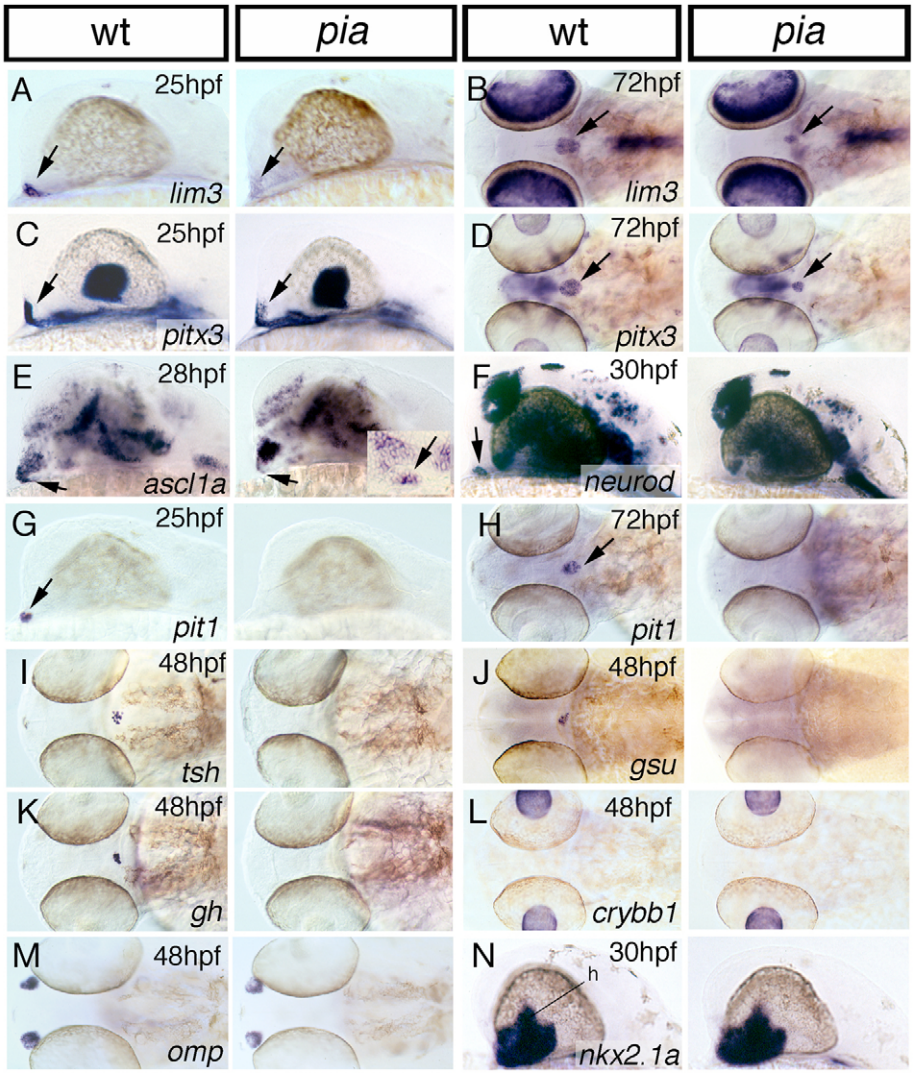

Fig. 3. *ascl1a* mutants show decreased expression of the *lim3*, *pitx3* and *ascl1a*, and fail to initiate expression of *neurod*, *pit1* and all adenohypophyseal hormone genes, whereas the hypothalamus appears unaffected. Columns 1 and 3 show wild-type siblings (wt); columns 2 and 4 show *pia* mutants. Probes used for whole-mount in situ hybridization are indicated in lower right-hand corners, ages of embryos in the upper right-hand corners of wild types. (**A**,**C**,**E**,**F**,**G**,**N**) Lateral views, anterior towards the right, dorsal upwards. (**B**,**D**,**H-M**) Dorsal views, anterior towards the right. Arrows in A-H indicate expression in the adenohypophysis. Inset in E shows section through pituitary of *pia* mutant at 120 hpf (see Fig. 2J for wild-type control). h, hypothalamus.

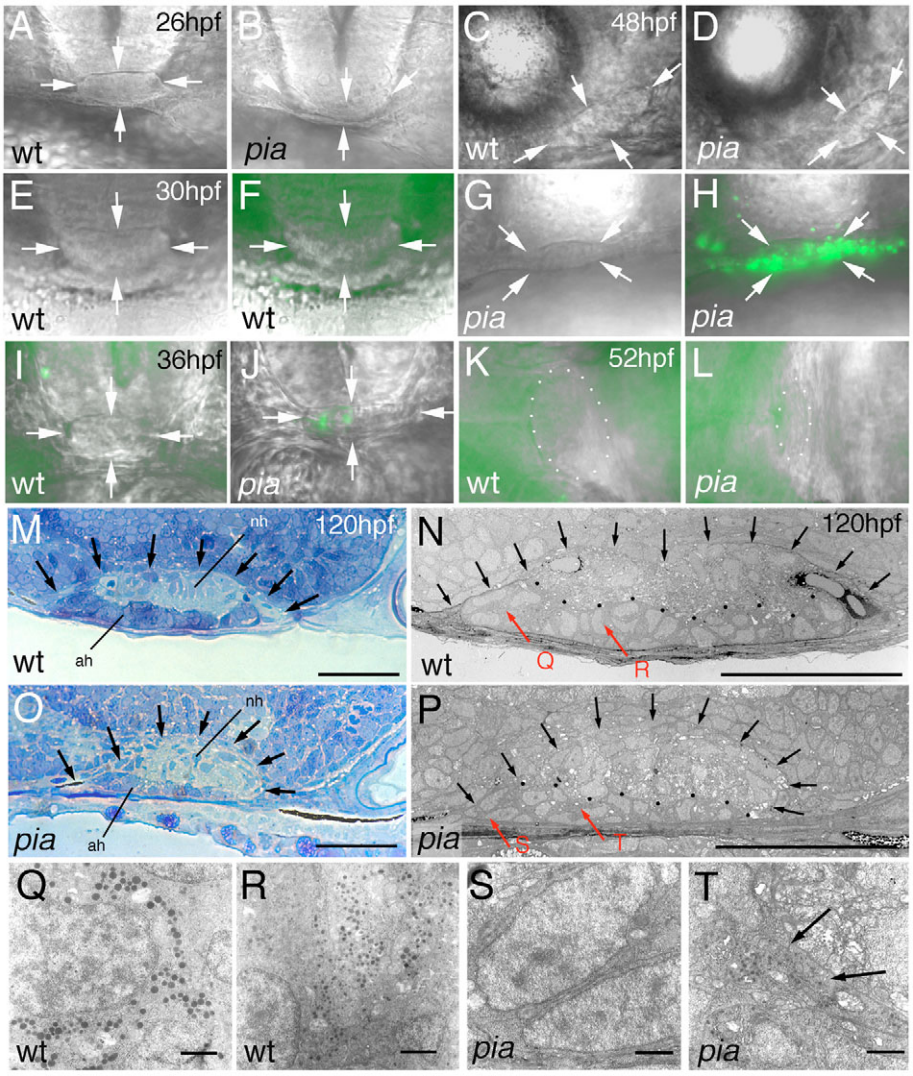

Fig. 4. *pia* mutants display indistinct early pituitary morphology, followed by a transient phase of adenohypophyseal cell death and the formation of a smaller, but distinct, pituitary gland with cells of rather primitive ultrastructure. (**A-L**) Nomarski images of live *pia* mutant embryos (pia) and their corresponding wild-type siblings (wt). (F,H,I-L) Images are superimposed by Acridine Orange staining for apoptosis. (A,B,E-J) Frontal views, dorsal upwards; (C,D) lateral views, anterior towards the left, dorsal upwards; (K,L) ventral views, anterior towards the right. Ages of embryos are indicated in upper right-hand corners of wild types. Genotypes were determined via PCR after photography. Arrowheads in A-J indicate borders of the pituitary gland; in K,L, pituitary borders are outlined by dots. (**M-T**) Pituitary ultrastructure at 120 hpf; longitudinal sections, anterior towards the left, dorsal towards the top. (M,O) Toluidine Blue staining; (N,P-T) electron micrographs. (M-P) The border of the pituitary is indicated by arrows; (N,P) the border between adenohypophysis (ah) and neurohypophysis (nh) is outlined by black dots. (Q-T) Higher magnifications of regions indicated by red arrows in N,P. Vesicles (as in T, indicated by arrows) were seen in three out of ~20 adenohypophyseal cells present in the section of the *pia* pituitary (P). They could contain matrix proteins and hormone-binding proteins, which can be made even in the absence of hormone production (compare with Norris, 1997). Scale bars: 30 μm in M-P; 1 μm in Q-T.

no *prl* transcripts could be detected at 26 hpf (Fig. 1C,D), and no *gh* or *tsh* transcripts at 48 hpf (Fig. 3I,K). In addition, transcripts of *pomc* and *gsu* were absent from *pia* mutant pituitaries at 26 hpf (data not shown) and at 48 hpf (Fig. 1F,G; Fig. 3J). In summary, this indicates that Ascl1a is required for the initiation of cognate hormone genes of all adenohypophyseal cell types.

ascl1a/pia mutants display a transient phase of apoptosis affecting a subset of non-differentiated adenohypophyseal cells

In a previous study, we have shown that in *fgf3* mutant zebrafish embryos, failed pituitary specification is followed by destruction of the entire organ, driven by apoptosis of non-differentiated adenohypophyseal cells between 28 and 32 hpf (Herzog et al., 2004a). As early adenohypophyseal specification seems similarly disrupted in *pia* mutants, we determined apoptosis rates in *pia* mutant pituitaries, performing Acridine Orange staining at various stages of development. At 24 hpf, no Acridine Orange-positive cells could be detected at the anterior neural ridge, where the adenohypophyseal primordium is located (data not shown; two genotyped mutants). However, at 27 hpf (data not shown; four genotyped mutants) and more dramatically at 30 hpf (Fig. 3E-H; three genotyped mutants), mutant embryos displayed significantly increased numbers of apoptotic cells within the adenohypophyseal anlage. At this stage, the mutant organ was morphologically much more distinct than at 26 hpf (compare Fig. 4G with 4B), although smaller and flatter compared with the pituitary of 30 hpf wild-type siblings (compare Fig. 4G with 4E). In addition to the adenohypophysis itself, Acridine Orange-positive signals were found in regions adjacent to the organ (Fig. 4H). These signals most probably represent debris of apoptotic cells that have been extruded from the adenohypophysis, as previously shown in *fgf3* mutants by cell-tracing experiments (Herzog et al., 2004a). At 36 hpf, the number of dying cells in *pia* mutants was significantly decreased compared with the situation at 30 hpf (three genotyped mutants; Fig. 4I,J), while no apoptotic adenohypophyseal cells at all could be detected at 48 hpf (data not shown; three genotyped mutants), and at 52 hpf (two genotyped mutants; Fig. 4K,L). In contrast to *fgf3* mutants (Herzog et al., 2004a), however, *pia* mutants of these late stages had a morphologically distinct adenohypophysis of smaller size (Fig. 4C,D), indicating that a subset of non-differentiated adenohypophyseal cells must have survived the transient phase of cell death. Together, these results indicate that in addition to, or as a consequence of, its role during cell differentiation Ascl1a is also required for the survival of most, but not all adenohypophyseal cells.

Surviving pituitary cells of *pia* mutants display molecular and ultrastructural features of adenohypophyseal precursors

As mentioned above, surviving cells in the pituitary rudiment of later stage *pia* mutants displayed rather normal levels of *lim3*, *pitx3* and *ascl1a* transcripts (Fig. 3B,D,E). In addition, they lacked expression of genes marking other derivatives of the placodal ectoderm such as *crystallin bb1* (lens tissue; Fig. 3L) or *omp* (olfactory epithelium; Fig. 3M), ruling out trans-differentiation to these other placodal cell types, and strongly suggesting that pituitary identity was maintained to some extent. To gain further insight into the nature of cells in the pituitary rudiment, we carried out transmission electron microscopy. At 120 hpf, the pituitary of *pia* mutants showed a neurohypophyseal compartment of rather normal size and morphology (compare Fig. 4M,N for wild type with Fig. 4O,P for *pia*). However, striking differences were apparent in the adenohypophysis. In wild-type siblings, most, if not all, adenohypophyseal cells contained secretory vesicles. By morphology, at least two types of secretory vesicles could be distinguished (Fig. 4Q,R), most probably reflecting different hormone-producing cell types (Nica et al., 2006). By contrast, the adenohypophysis of *pia* mutants not only contained fewer cells (Fig. 4O,P); ~90% of the cells also displayed a rather primitive ultrastructure. Thus, their cytoplasm lacked an elaborated endoplasmic reticulum and secretory vesicles, whereas the chromatin of the nucleus was very homogeneous (Fig. 4S). However, a few adenohypohyseal cells of *pia* mutants did contain a particular type of secretory vesicles (Fig. 4T), although in smaller numbers than in wild-type siblings, and despite the absence of hormone synthesis. This suggests that *pia* mutant adenohypophyseal cells in their entirety are not fully differentiated, although they differ in their exact specification state.

Ascl1a is required cell-autonomously in adenohypophyseal cells

As described above, *ascl1a* is expressed not only in adenohypophysis itself but also in adjacent tissues, such as the ventral diencephalon, the source of signaling proteins such as Shh or Fgf3, both of which are required for proper pituitary development (Herzog et al., 2004a; Herzog et al., 2003; Sbrogna et al., 2003). Thus, Ascl1a might either have an essential intrinsic role in adenohypophyseal cells themselves, or might influence adenophypophysis indirectly via the diencephalon. In order to distinguish between these possibilities, we generated chimeric embryos. For chimeras with donor cells in the pituitary, labeled cells were transplanted at early gastrula stages into regions of host embryos ventral to the animal pole, whereas for telencephalic or diencephalic clones, cells were transplanted right at the animal pole, or slightly animal of the dorsal shield, respectively (compare with Herzog et al., 2004a). Chimeric embryos were raised to 26-48 hpf, and stained either for *pit1* transcripts to evaluate early adenohypophyseal specification, with Acridine Orange to evaluate cell survival, or for Prl protein to evaluate terminal endocrine differentiation. When wild-type cells ended up in the telencephalon, which is located adjacent to the adenohypophyseal anlage early, or in the ventral diencephalon, which is in close proximity to the adenohypophysis later (Herzog et al., 2004a), no induction of adenohypophyseal *pit1* expression was obtained (16/16 embryos; Fig. 5B). By contrast, when wild-type donor cells were located within the adenohypophysis of 26 hpf *pia* mutant hosts, most of them were *pit1* positive (4/5 embryos; Fig. 5C; *pit1*-negative wild-type cells most probably represent corticotropes, melanotropes or gonadotropes). Similarly, at 32 hpf, transplanted wild-type cells within the adenohypophysis of *pia* mutant hosts all were Acridine Orange negative, while many of the host pituitary cells were apoptotic (nine embryos; Fig. 5D,E). In reverse, some of the *ascl1a* morphant cells transplanted into wild-type pituitaries became apoptotic, while the host tissue was completely Acridine Orange negative (11 embryos; Fig. 5F). Finally, many of the wild-type cells transplanted into *pia* mutant pituitaries contained *pomc* transcripts (four embryos; data not shown) or Prl protein at 48 hpf (eight embryos; Fig. 5I), while *pia* mutant cells were Pomc- and Prl-negative (Fig. 5H,I; and data not shown). In summary, this strongly suggests that Ascl1a is required in a cell-autonomous fashion in the adenophypophysis itself to promote early steps of specification, cell

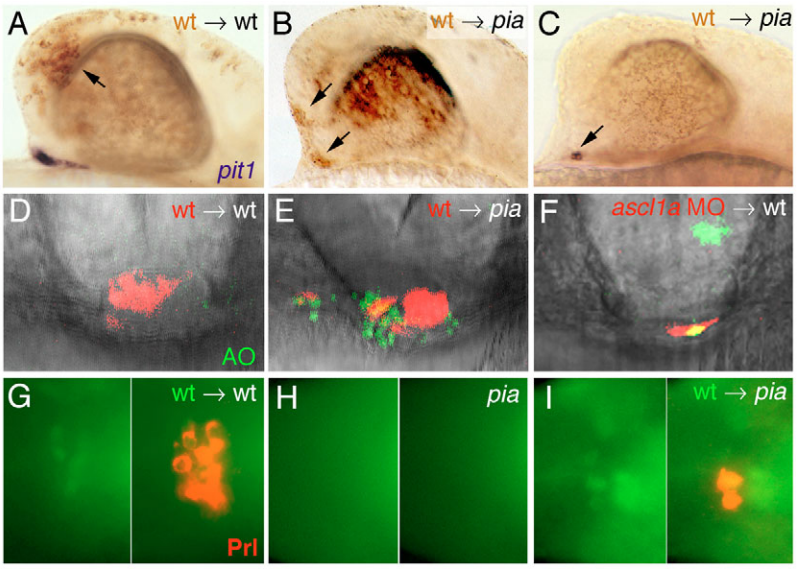

Fig. 5. Ascl1 is required cell autonomously to promote *pit1* expression, cell survival and Prl production. (**A-C**) Lateral views on chimeric embryos at 26 hpf embryos, in situ hybridized for *pit1* transcripts in blue and with transplanted wild-type cells in brown (indicated with arrows). (A) Wild-type control recipient with wild-type donor cells in the telencephalon and normal *pit1* expression. (B) *pia* recipient with wild-type cells in eyes, telencephalon and ventral regions of diencephalon, lacking adenohypophyseal *pit1* expression. (C) *pia* embryo with few wild-type cells within adenohypophyseal domain, showing *pit1* expression in these wild-type cells only. (**D-F**) Frontal views of pituitaries at 32 hpf. Nomarski images superimposed with red fluorescence revealing transplanted cells; green fluorescence revealing Acridine Orange (AO)-positive cells. (D) Wild-type control recipient with AO-negative wild-type donor cells in the pituitary. (E) *pia* recipient, with AO-negative transplanted wild-type cells (red), but many AO-positive host cells (green) in pituitary. (F) Wild-type recipient, with one AO-positive (yellow) and two AO-negative *ascl1a* morphant donor cells in pituitary. (**G-I**) Ventral views of pituitaries at 48 hpf, anterior towards the left. Left panels show transplanted *h2a::h2a-GFP* transgenic cells weakly labeled in green, right panels an overlay of the same images with anti-Prl immunostaining in red. (G) Wild-type host; (H) *pia* host without transplanted cells in pituitary; (I) *pia* host with three wild-type cells in pituitary, two of which produce Prl protein.

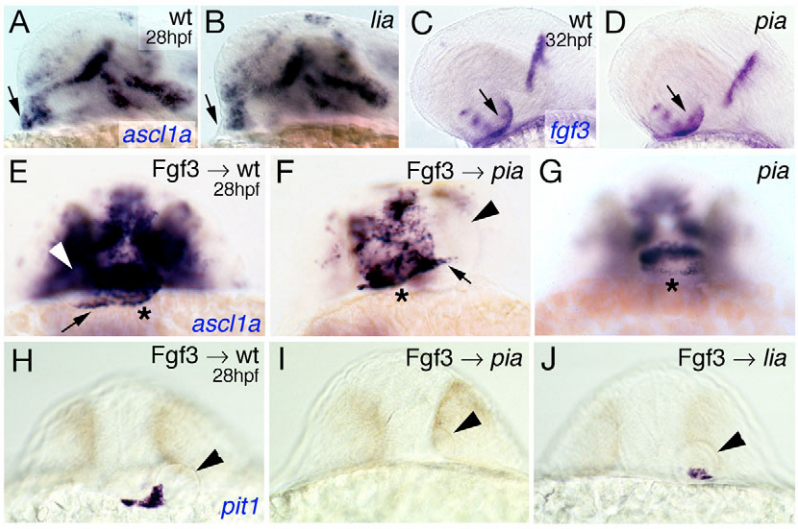

Fig. 6. Fgf3 is required for adenohypophyseal *ascl1a* expression and requires Ascl1a to induce *pit1* expression. (**A-D**) Whole-mount in situ hybridization (lateral views with anterior towards the right) showing that adenohypophyseal *ascl1* expression (indicated by arrows in A,B) is lacking in *fgf3/lia* mutants at 28 hpf, while diencephalic *fgf3* expression (indicated by arrows in C,D) is normal in *ascl1a/pia* mutants at 32 hpf. (**E-J**) Frontal views on embryos at 28 hpf, after implantation of Fgf3-soaked beads in embryos of indicated genotype, and in situ hybridization for *ascl1a* (E-G) or *pit1* (H-J). Beads in E,F,H-J are marked with arrowheads. Arrows in E,F mark laterally expanded *ascl1a* expression; asterisks in E-G indicate medial adenohypophyseal *ascl1a* expression, which is much stronger in the *pia* mutant after Fgf3 bead implantation (F) compared with untreated *pia* sibling (G).

survival and terminal endocrine differentiation. By contrast, *ascl1a* expression in telencephalon and diencephalon appears to be dispensable for proper adenohypophysis development, consistent with the normal expression of all investigated marker genes in the forebrain of mutant embryos (*nkx2.1a*, Fig. 3N; *fgf3*, Fig. 6C,D; *shh*, data not shown).

Implanted Fgf3 beads enhance adenohypophyseal *ascl1a* expression, but fail to rescue disrupted adenohypophysis development of *pia* mutants

The findings that *fgf3* and *ascl1a* mutants display similar pituitary phenotypes, and that Fgf3 acts as an extrinsic signal (Herzog et al., 2004a) and Ascl1a as an intrinsic transcription factor, suggests that Ascl1a might act downstream of Fgf3 signaling to mediate at least some of its effects. This notion is further supported by the lack of adenohypophyseal *ascl1a* expression in *fgf3/lia* mutants at 28 hpf (Fig. 6A,B), before the appearance of massive cell death (Herzog et al., 2004a), although in reverse, diencephalic *fgf3* expression is normal in *ascl1a/pia* mutants (Fig. 6C,D). Similarly, adenohypophyseal *ascl1a* expression was absent in 28 hpf wild-type embryos after treatment with the Fgf receptor inhibitor SU5402 from 18-22 hpf (data not shown). To investigate more directly whether Ascl1a is required to mediate diencephalic Fgf3 signaling, we implanted Fgf3-loaded beads into the diencephalon of embryos obtained from $pia^{+/-}$ intercrosses at 18 hpf, briefly before first adenohypophyseal defects in *pia* mutants become visible. Such beads caused a significant normalization of adenohypophyseal *ascl1a* expression in *pia* mutants at 28 hpf (4/6; Fig. 6F). However, they failed to rescue *pit1* expression in *pia* mutants of the same age (5/5; Fig. 6I), in contrast to the striking rescue obtained in control implantations into *fgf3/lia* fish (6/6; Fig. 6J). This indicates that in the absence of Ascl1a, Fgf3 signaling can activate the expression of *ascl1a* itself, but not the expression of downstream genes, consistent with the notion that Fgf3 requires Ascl1a to mediate at least some of its effects on the adenohypophysis.

DISCUSSION

Zebrafish Ascl1a is required for endocrine differentiation in the adenohypophysis

In *Drosophila*, bHLH proteins encoded by the so-called proneural genes of the *achaete-scute* complex (AS-C; *achaete*, *scute*, *asense* and *lethal of scute*) and *atonal* are not only required for the development of neurons and sensory cells (for reviews, see Calleja et al., 2002; Jan and Jan, 1994; Skeath and Carroll, 1994), but also in other cell types that arise by singling out from clusters of progenitor cells, such as mesodermal and endodermal precursors (Carmena et al., 1995; Tepass and Hartenstein, 1995).

Also in vertebrates, Asc-like (Ascl) proteins and members of the Atonal superfamily, such as Math/Neurod1/neurogenin (Ledent et al., 2002), have not only been described in the context of neural differentiation (Westerman et al., 2003). Thus, mouse Mash1 is not only required for the specification of neurons and sensory cells of the CNS or PNS, but also for the differentiation of neuroendocrine cells in the lung (Ball, 2004; Borges et al., 1997; Ito et al., 2000), the thyroid (Lanigan et al., 1998) and the adrenal gland (Huber et al., 2002). Furthermore, Mash2 is required for trophoblast formation in the placenta (Guillemot et al., 1994; Tanaka et al., 1997), Mash3/Sgn1 for salivary gland duct cells (Yoshida et al., 2001), and neurogenin 3, Neurod1 and Math1 for diverse endocrine cells of the gastrointestinal system (Jenny et al., 2002; Naya et al., 1997; Yang et al., 2001) and the pancreas islets (Gradwohl et al., 2000; Naya et al., 1997). Although some of these cell types represent specialized neurons, deriving from the neural crest (Lanigan et al., 1998), others lack any sign of neuronal specification, and are supposed to derive from non-ectodermal tissues, such as the endoderm in the case of the endocrine pancreatic cells (Percival and Slack, 1999).

Here, we extend this list of Ascl-dependent cell types, showing that zebrafish Ascl1 is required for endocrine differentiation of adenohypophyseal cells. The adenohypophysis derives from the placodal ectoderm, in close proximity to the olfactory epithelium (Dubois and ElAmraoui, 1995). In mouse, the *ascl1a* homologue *Mash1* is required for early olfactory development (Guillemot et al., 1993), while in zebrafish *ascl1a* mutants, the olfactory epithelium develops normally, indicated by unaffected expression of *omp* (Fig. 3). This is most probably due to the presence of the *ascl1a* paralog *ascl1b* in this tissue (Fig. 2) (Allende and Weinberg, 1994). Alternatively, it might be due to functional redundancy between Ascl1a and other proneural bHLH proteins, similar to the situation described for zebrafish Ascl1a and neurogenin 1 during epiphysial neurogenesis (Cau and Wilson, 2003).

It is unclear why, in vertebrates, Ascl proteins in addition to neurons are particularly required for the specification of endocrine fates. In the case of the adenohypophysis and the olfactory epithelium, it might result from their common developmental and

evolutionary origin. Thus, both tissues derive from the placodal ectoderm, and according to a current model, they have evolved from a common chemoreceptive structure already present in Bilaterian ancestors (De Velasco et al., 2004; Gorbman, 1995).

Different roles of Ascl1a during zebrafish adenohypophysis development

In line with the proposed common origin of adenohypophysis and neural structures, we found striking similarities in the modes of Ascl1 action during endocrine differentiation in the zebrafish adenohypophysis and neurogenesis in *Drosophila* and mouse. In *Drosophila*, the function of *asc* members is divided into distinct developmental steps (Brunet and Ghysen, 1999; Jan and Jan, 1994; Westerman et al., 2003). The first is the establishment of neural precursors out of a population of progenitors (proneural function), the second is the specification of specific neural fates (subtype specification step). The same is true for Mash1 during neural development in the mouse. Thus, in the subventricular zone of the ventral telencephalon, Mash1 is required for the formation of a pool of neural precursor cells (Casarosa et al., 1999), whereas in autonomic ganglia, the olfactory epithelium and some regions of the brain, it activates the expression of specific neuronal differentiation genes and initiates neuronal differentiation (Cau et al., 1997; Guillemot et al., 1993; Hirsch et al., 1998; Parras et al., 2002).

A similar biphasic proneural-like and subtype-specification function also appears to apply for Ascl1a in the zebrafish adenohypophysis. Thus, in the absence of Ascl1a function, all adenohypophyseal cell types fail to differentiate, in line with a general role of Ascl1a during the specification of pituitary precursor cells. Actually, Ascl1a is the first transcription factor shown to be required for the proper differentiation of all adenohypophyseal cell types, whereas mutants in all other previously described transcription factors only lack particular adenohypophyseal lineages (for a review, see Zhu and Rosenfeld, 2004).

In somatotropes and thyrotropes, *ascl1a* is switched off after endocrine differentiation and the initiation of cognate hormone gene expression (Fig. 2). This indicates that, in these cell types, *ascl1a* is exclusively involved in earlier specification steps, such as the activation of *pit1*, the expression of which fails to be initiated in *ascl1a* mutants (Fig. 3). By contrast, *ascl1a* expression persists in the Pomc lineage (Fig. 2), suggesting that in these cell types, Ascl1a might be involved in later, subtype-specific differentiation processes. Interestingly, the zebrafish *pomc* promoter contains E-boxes, consensus binding sites for bHLH transcription factors (Liu et al., 2003), suggesting that Ascl1a might be involved in the transcriptional activation of the *pomc* gene. In summary, it appears that Ascl1a regulates adenohypophysis development at multiple levels, acting during both early and terminal steps of the transcriptional control cascade.

Differential apoptosis within the *ascl1a* mutant pituitary and the nature of surviving cells

In addition to cell specification processes, *ascl1a* is required for the survival of some, but not all adenohypophyseal cells, allowing us to distinguish two cell populations: one in which cell differentiation and cell survival are strictly coupled; and another in which cells can survive in an undifferentiated state (Fig. 4). In mutant embryos, cell death occurs during a rather narrow time window (2-3 hours), several hours after the failed differentiation of lactotropes and corticotropes, but before somatotropes and thyrotropes would differentiate under wild-type conditions (Herzog et al., 2003). These surviving cells of *ascl1a*-deficient pituitaries have adenohypophyseal character, as revealed by the presence of *lim3*, *pitx3* and *ascl1a* transcripts at 72 hpf (Fig. 3), and their ultrastructural morphology at 120 hpf, which resembles that of undifferentiated adenohypophyseal precursor cells (Fig. 4). In the mouse olfactory epithelium, where Mash1 plays a subtype-specific role to drive final steps of neural differentiation (see above), loss of Mash1 leads to massive cell death; however, apoptosis only starts after the initiation of the differentiation arrest (Cau et al., 1997). Assuming that the same is true for the zebrafish adenohypophysis, this would mean that apoptosis (between 30 and 32 hpf) occurs only in the early differentiating lineages (corticotropes, melanotropes and lactotropes; 24-28 hpf). Along the same lines, surviving cells would represent somatotrope and thyrotrope precursors, which normally differentiate later (42-48 hpf). However, their differentiation in the pituitary rudiment of *ascl1a* mutants would be blocked because of the absence of Pit1 (Fig. 3), which is absolutely required for *tsh* and *gh* hormone gene expression (Nica et al., 2004). Clearly, this is only one of several possible interpretations, and further experiments, such as transgene-driven re-introduction of *pit1* gene products into the pituitary rudiment of *pia* mutants will be necessary to prove this notion. In addition, it appears that even within this population of surviving cells, Ascl1a might have differential effects, as suggested by the different ultrastructure of adenohypophyseal cells in older mutants (Fig. 4).

Does Ascl1a act downstream of Fgf3 and upstream of Neurod?

In mouse, out of all bHLH proteins, only Neurod1 has been described in the context of pituitary development. In particular, Neurod1 has been shown to bind and activate the *Pomc* promoter, acting in concert with a Pitx homeodomain protein and another bHLH factor (Horton et al., 1999; Poulin et al., 2000; Poulin et al., 1997; Westerman et al., 2003). To search for bHLH proteins that might act in parallel or downstream of Ascl1a, we studied the expression pattern of other zebrafish *achaete scute* and *atonal* homologues. However, we only found *neurod* (Fig. 3) to be expressed in the adenohypophysis, whereas *ascl1b*, *neurod2, ngn1*, *atho1.1*, *atho1.2*, *atho2a/ndr1a*, *atho2b/ndr1b*, *ath3/neurod4*, *atho4/ngn3* and *ath5* were not (H.M.P. and M.H., unpublished). Interestingly, in *ascl1a* mutants, *neurod* expression was absent at all investigated stages, suggesting that it acts downstream of Ascl1a. However, in contrast to *Neurod1*-deficient mice, which show moderate defects during corticotroph differentiation (Lamolet et al., 2004), we failed to observe any abnormalities during zebrafish pituitary development when knocking down zebrafish Neurod with specific antisense morpholino oligonucleotides (H.M.P. and M.H., unpublished). This indicates that Neurod is neither an essential partner, nor an essential downstream mediator of Ascl1a during zebrafish pituitary development. To address whether Neurod might nevertheless mediate Ascl1a function, e.g. in redundancy with another as yet unidentified factor, it will be necessary to generate transgenic lines to drive Ascl1a-independent adenohypophyseal *neurod* expression, investigating whether forced *neurod* expression is sufficient to rescue the adenohypophyseal defects of *ascl1a* mutants.

A similar transgenic approach will be necessary to provide ultimate proof for a role of Ascl1a downstream of Fgf3 signaling from the ventral diencephalon. For several reasons, such a role seems quite likely. First, *fgf3* mutants show a similar combination of failed specification and apoptosis of adenohypophyseal cells (Herzog et al., 2004a), as described here for *ascl1a* mutants. Second, *fgf3* mutants lack adenohypophyseal *ascl1a* expression, and third,

implantation of Fgf3-loaded beads into *ascl1a* mutants can enhance adenohypophyseal *ascl1a* expression, while genes downstream of Ascl1a such as *pit1* do not respond. Clearly, without the aforementioned rescue of *fgf3* mutants by forced *ascl1a* expression, these data do not rule out that Ascl1a acts in parallel to, rather than downstream of, Fgf3. In any case, Fgf3 must have other mediators in addition to Ascl1a, given that the pituitary in *fgf3* mutants is more severely affected than in *ascl1a* mutants, with apoptosis of all rather than a subset of adenohypophyseal cells.

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