Proneural gene requirement for hair cell differentiation in the zebrafish lateral line

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Abstract

The lateral line system comprises an array of mechanosensory organs, the neuromasts, distributed over the body surface. Each neuromast consists of a patch of mechanosensory hair cells surrounded by support cells. We show that, in the zebrafish, two proneural genes are essential for differentiation of the hair cells, *neuroD* (*nrd*) and *atonal homolog 1* (*ath1*). Gene knockdown experiments demonstrate that loss of function of either gene, but not of the related proneural gene *neurogenin1* (*ngn1*), abrogate the appearance of hair cell markers. This is in contrast to other sensory systems, such as the neurons of the lateral line ganglion, where *nrd* is regulated by *ngn1* and not by *ath1*. Overexpression of *ath1* can induce *nrd*, and the phenotype produced by loss of *ath1* function can be partially rescued by injection of *nrd* mRNA. This supports the conclusion that the activation of *nrd* probably requires *ath1* in the hair cell lineage, whereas in sensory neurons *nrd* activation requires *ngn1*. We propose that the emergence of two *atonal* homologs, *ath1* and *ngn1*, allowed the cellular segregation of mechanoreception and signal transmission that were originally performed by a single cell type as found in insects.

Keywords: Lateral line; Neuromasts; Hair cells; Proneural genes; bHLH; Atonal homolog; NeuroD

Introduction

The generation of neurons in vertebrates involves the sequential expression of genes that promote the acquisition of neuronal identity and terminal differentiation. Among the most well-known genes that participate in this process are the proneural genes (reviewed in Bertrand et al., 2002). Proneural genes were originally identified in *Drosophila* as providing groups of cells with the potential to become sense organ precursors (Ghysen and Dambly-Chaudière, 1989). They code for transcription factors of the basic helix–loop–helix (bHLH) class (Murre et al., 1989) that are generally involved in neurogenesis (Villares and Cabrera, 1987). Two major families of proneural genes are known in vertebrates, the *Achaete–Scute* family and the *Atonal* family which includes the *atonal homolog* (*ath*), *neurogenin* (*ngn*) and *neuroD* (*nrd*) classes. bHLH proneural genes belonging to the two major families have been identified subsequently in a wide range of multicellular animals.

The gene *nrd* is expressed in the central and peripheral nervous systems in vertebrates and has the capacity to induce neuronal differentiation in *Xenopus* (Lee et al., 1995; Lee, 1997). It is also expressed strongly in pancreatic cells where it regulates development of endocrine cells (Naya et al., 1997). In the mouse CNS, *nrd* is required for the formation of granule cells in the hippocampus and the cerebellum (Miyata et al., 1999) and for the formation of sensory neurons in the inner ear. One of the phenotypes of a mouse *nrd* null mutant is deafness (Kim et al., 2001).

Gain and loss of function studies have suggested that the neuronal expression of *nrd* is regulated by *ngn*, possibly directly (Ma et al., 1996, 1998; Fode et al., 1998; Perron et al., 1999; Andermann et al., 2002). *nrd* is expressed mostly in postmitotic cells (Cau et al., 1997; Korzh et al., 1998; Mueller and Wullimann, 2002; Cau and Wilson, 2003) and may be required for terminal differentiation rather than for neuronal precursor commitment.

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The *atonal* family of genes plays a major role in the development of the auditory system of vertebrates. Loss of function analysis of *neurogenin* has shown that it is required for formation of the inner ear ganglion in mammals (Ma et al., 1998, 2000), while the mouse *atonal homolog 1* gene *Math1* is required for the formation of hair cells (Bermingham et al., 1999; Ben-Arie et al., 2000). Overexpression of *Math1* is sufficient to induce the appearance of ectopic hair cells in the inner ear and can rescue hair-cell-deficient mice (Shou et al., 2003; Kawamoto et al., 2003).

We are interested in neuronal development in the fish lateral line, a mechanosensory organ involved in the detection of displacement waves in the water and which allows for schooling behaviors and predator or prey detection (Partridge and Pitcher, 1980; Coombs et al., 1989). The lateral line (LL) system arises from ectodermal placodes, as does the inner ear. Lateral line placodes develop anterior and posterior to the otic placode, generating, respectively, the anterior and posterior lateral line systems (ALL and PLL; Metcalfe et al., 1985; Andermann et al., 2002). The PLL placode gives rise to two cell populations: a stationary population which forms the PLL ganglion, and a migratory component which will deposit regularly spaced clusters of cells, the proneuromasts, along its way from the otic region to the tip of the tail (Metcalfe et al., 1985; Gompel et al., 2001). Deposited cells will differentiate as hair cells and accessory cells of two types: inner accessory (support) cells that surround the hair cells and a rim of outer accessory (mantle) cells.

In zebrafish, neurogenic and proneural genes are expressed in the lateral line components and some of these have been analyzed functionally. *neurogenin1* (*ngn1*) is essential for the development of the sensory neurons of most of the cranial ganglia, including those innervating the lateral line. In the PLL placode, *ngn1* precedes the expression of *nrd*, and *nrd* is strongly downregulated following injection of an antisense morpholino against *ngn1* (Andermann et al., 2002). This suggests that, as in other vertebrate systems, *nrd* acts downstream of *ngn1*. Interestingly, a residual expression of *nrd* is nevertheless observed after complete loss of function of *ngn1* (Andermann et al., 2002). The gene *atonal homolog 1* (*ath1*; Kim et al., 1997) is not detectably expressed in the PLL placode but can be observed in the PLL migrating primordium and in developing neuromasts (Itoh and Chitnis, 2001). In the *mindbomb* mutant, in which the Notch signaling pathway is disrupted (Itoh et al., 2003), there is ectopic expression of *ath1* and differentiation of supernumerary hair cells in PLL neuromasts (Itoh and Chitnis, 2001). Thus, genes of the *atonal* family seem to be involved in lateral line development, but their precise functions remain unclear.

In this work, we show that, in addition to *ath1*, *nrd* is also expressed in the lateral line neuromasts. We show that *nrd* is upregulated in the migrating PLL primordium in *mindbomb* mutants, much as is the case for *ath1*. We find that loss of function of either *nrd* or *ath1* results in a loss of hair cells, while the inactivation of two other proneural genes, *ngn1* and *achaete–scute homolog 1a* (*ash1a*), has no effect on hair cell determination or differentiation. Finally, rescue experiments lead us to propose that, in lateral line hair cell precursors, *nrd* is regulated by *ath1* rather than by *ngn1* as in all other systems studied so far.

Materials and methods

Fish and embryos

Zebrafish mutant and wild type embryos were collected from natural matings and kept at 28°C; stages are referred to as in hours post-fertilization, hpf. *mindbomb* mutants were a kind gift of Dr. Ajay Chitnis (NIH, Bethesda); transgenic lines ET4 and ET20 (Parinov et al., 2004) were kindly provided by Dr. Vladimir Korzh (IMCB, Singapore). All embryos were raised at 28°C and staged according to Kimmel et al. (1995). After 24 hpf, larvae were maintained in 0.03% phenylthiourea to prevent melanin pigment formation (Westerfield, 1994).

Cell labeling and microscopy

The neuromast hair cells were labeled in live embryos or larvae with 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (Di-Asp; Sigma D3418, USA) as described in Collazo et al. (1994). For live staining, 48–72-hpf larvae were incubated in 5 mM Di-Asp in embryo medium for 5 min and then rinsed with fresh medium and visualized under fluorescent light in a dissection microscope. We counted Di-Asp-stained neuromasts on both sides of the larvae when evaluating the effects of perturbing gene activity, and, thus, each side was counted as an independent result. The embryonic PLL usually comprises 5 lateral neuromasts (very rarely 4 or 6) aligned along the horizontal myoseptum and 2–3 terminal neuromasts (very rarely one) in a more ventral position at the tip of the tail. The total number of neuromasts is therefore 7–8 per side in more than 90% of the embryos. To take into account the rare occurrence of embryos with 5 neuromasts on one side (4 lateral and one terminal) in wild type populations, we classified as "normal" any side with 5–8 neuromasts, "subnormal" those with 1–4 neuromasts and "abnormal" sides where no PLL neuromasts have formed. For carrying out statistical tests, we used as data the number of neuromasts counted per side in all groups of embryos and compared them within experiments (see Table 1). To determine significance of differences, we used the Student's *t* test (SigmaStat 3.1).

To trace the PLL nerve, 30 hpf embryos were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) injection in fixed embryos was performed as described in David et al. (2002).

The posterior lateral line primordium and neuromasts were visualized in live or fixed animals under Nomarski optics using a Model III Zeiss Photomicroscope. In situ hybridized and antibody-stained embryos were post-fixed in 4% paraformaldehyde and transferred into glycerol for observation. Live embryos and larvae treated with Di-Asp were visualized in a Leica MZ12 fluorescence dissecting microscope in embryo medium.

Antibody staining and in situ hybridization

Embryos were staged and fixed overnight in 4% paraformaldehyde in PBS. Whole mount immunochemistry was performed as follows: embryos were rinsed three times for 5 min in PBS, washed one time for 1 h in distilled water, incubated for 7 min in acetone, washed one time in distilled water, two times for 5 min in PBS plus Tween-20 (polyoxyethylene-sorbitan monolaurate; Sigma, USA) and transferred to blocking solution (20% lamb serum, 1% dimethylsulfoxide, 0.1% Tween-20 in PBS) for 1 h. Embryos were then incubated overnight at 4°C with a mouse monoclonal anti-acetylated tubulin antibody (Sigma, USA) used at a 1:1000 dilution or with anti-HuC (Invitrogen) at 1:1000. They were then washed 4 times for 25 min in PBS plus Tween-20, incubated for 30 min in blocking solution and incubated overnight at 4°C in anti-mouse Ig, horseradish-peroxidase-linked whole antibody (Amersham Pharmacia Biotech, UK) diluted 1:200 in blocking solution. They were then rinsed four times for 20 min in PBS plus Tween 20, transferred to peroxidase staining solution (30 mg

Table 1
Loss of proneural gene function affects posterior lateral line hair cell development in zebrafish

	Normal (5–8 nm)	Subnormal (1–4 nm)	Abnormal (0 nm)
Controls ($P = 0.066$)			
No injection (58 embryos)	97%	3%	0%
*ash1a*MO [a] (75 embryos)	95%	5%	0%
Experiment 1 ($P < 0.001$)			
*nrd*MO (78 embryos)	22%	47%	31%
*nrd*MO co-injected with *nrd* (71 embryos)	68%	18%	14%
Experiment 2 ($P < 0.002$)			
ath1MO (53 embryos)	34%	55%	11%
ath1MO co-injected with *nrd* (46 embryos)	61%	37%	2%
Experiment 3 ($P = 0.051$)			
*nrd*MO (52 embryos)	50%	50%	0%
*nrd*MO co-injected with *ath1* (64 embryos)	52%	41%	7%

Embryos were injected with morpholino (MO) or with morpholino plus a DNA expression construct as indicated. They were raised to 72 hpf and stained with the vital dye Di-Asp. Labeled neuromasts (nm) were counted on each side for each larva. Larval sides were classified according to the severity of the effect: "normal" had between 5 and 8 neuromasts; "subnormal" had between 1 and 4 neuromasts; "abnormal" completely lacked neuromasts. Shown are the percentages for each category in the different experiments. Three different experiments were carried out; comparisons for rescue should be made within an experiment to eliminate differences in penetrance of the effect of the morpholinos that occur between experiments. To calculate significance values (P), we counted, for each side, the number of neuromasts present and compared the two conditions indicated in each experiment. Note that rescue is obtained by co-injecting the *nrdMO* with *nrd* ($P < 0.001$) and the *ath1MO* with *nrd* ($P < 0.002$), but not the *nrdMO* with *ath1* ($P = 0.051$).

[a] Injection of the *ash1a* morpholino does not affect formation of the posterior lateral line in zebrafish embryos and was used as an injection control.

diaminobenzidine in 100 mL water) for 30 min and then to peroxidase staining solution containing 0.003% H_2O_2. After the product of the peroxidase reaction was detected, the embryos were rinsed three times in PBS.

Whole mount in situ hybridization was performed essentially as described in Jowett and Lettice (1994). Riboprobes were synthesized from template plasmids including *ngn1* (Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998), *nrd* (Blader et al., 1997; Korzh et al., 1998), *ath1* (Kim et al., 1997), *eya1* (Sahly et al., 1999) and *claudinB* (Kollmar et al., 2001). For detection of *nrd* expression, we used a hybridization temperature of 58°C and we used a longer staining time (4–6 h at room temperature or overnight at 4°C). In all cases, control probes were negative for specific staining.

Constructs and antisense morpholinos

To generate an *nrd-myc* fusion construct, we used the full-length *nrd* cDNA (Blader et al., 1997) as a template for a PCR reaction. The primers contain a *Bam*HI site (upstream primer, contains the ATG, in bold below) and a *Cla*I site (downstream primer which mutates the stop codon). Their sequences are, respectively:

5′ CTAGGGATCCGAC**ATG**ACGAAGTCATAC 3′ and
5′ AGGATCGATAGTCGTGAAATATCGC 3′.

This PCR product was cloned into the pGEM vector (Promega, Madison, WI, USA) by TA cloning, digested with *Bam*HI and *Cla*I and subcloned into the pCS2MT expression vector, generating the plasmid construct pCS2*nrd*MT.

A similar approach was used to generate an *ath1* expression construct, also cloned by introducing *Bam*HI and *Cla*I sites and inserting into pCS2MT to generate plasmid pCS2*ath1*MT. Forward (sequence upstream of the ATG) and reverse primers were:

5′ CCGTCCCTGGATCCATAGCCAC 3′ and
5′ GTATCGATCGCACTTCAGTGAGG 3′.

The *neurogenin1* morpholino (*ngn1*MO) was kindly provided by Dr. Robert Cornell (Cornell and Eisen, 2002). *nrd*, *ath1* and *ash1a* antisense morpholino oligonucleotides (*nrd*MO, *ath1*MO and *ash1a*MO respectively) were synthesized by Gene Tools (Philomath, OR, USA). All morpholinos were directed against the 5′ region of the mRNA; triplets complementary to the ATG initiation codons are indicated in bold in the sequences (the *ath1*MO is complementary to the sequence just upstream of the ATG). The sequences of the antisense morpholino oligonucleotides used were:

5′ TGACTTCGT**CAT**GTCGGAACTCTAG 3′ (*nrd*MO)
5′ TCTGTTGGTTTGTGCTTTTGGGAGG 3′ (*ath1*MO)
5′ CCATCTTGGCGGTGATGTC**CAT**TTC 3′ (*ash1a*MO)
5′ TATACGATCTC**CAT**TGTTGATAACC 3′ (*ngn1*MO)

Note that the *nrd*MO should not bind to the RNA expressed from the pCS2*nrd*MT construct as there are 7 mismatches out of 25 bases (sequence upstream of the *Bam*HI site is lost after cloning in the pCS2 vector). For inhibiting gene function, 8.5 ng of morpholino oligonucleotide was injected into each embryo. In the rescue experiments, 4.25 ng of either the *nrd* or *ath1* morpholinos was co-injected with 125 pg of the pCS2*nrd*MT or pCS2*ath1*MT plasmids, per embryo.

Results

Expression of nrd in the zebrafish posterior lateral line

NeuroD (*nrd*) is a bHLH proneural gene implicated in neuronal differentiation. We investigated whether this gene is involved in the acquisition of neuronal identity and/or differentiation of PLL hair cells and sensory neurons. We examined the expression pattern of *nrd* in the different components of the PLL: placode, ganglion, primordium, proneuromasts and neuromasts, using in situ hybridization. We detected *nrd* transcripts beginning at 11 hpf in the brain and PLL placode and subsequently in the PLL ganglia, as described previously (Andermann et al., 2002). However, we were not able to detect expression in the lateral line primordium or neuromasts using our standard in situ hybridization protocol. As we had evidence from loss of function studies that *nrd* participates in hair cell development (see below), we forced the conditions of the in situ hybridization protocol to detect low levels of transcript. These became apparent only after modifying the hybridization and staining conditions (see Materials and methods) which also introduced high levels of background staining. Under these conditions, low levels of *nrd* expression were detected in deposited PLL neuromasts (Fig. 1A). Close inspection of neuromasts with higher magnification shows that nrd mRNA is localized to a group of 3–6 cells in the center of each neuromast (Figs. 1C–E), similar to what is observed with expression of the proneural gene *atonal homolog 1* (*ath1*) (Itoh and Chitnis, 2001). We were not able to detect expression of *nrd* in the migrating PLL primordium under these conditions.

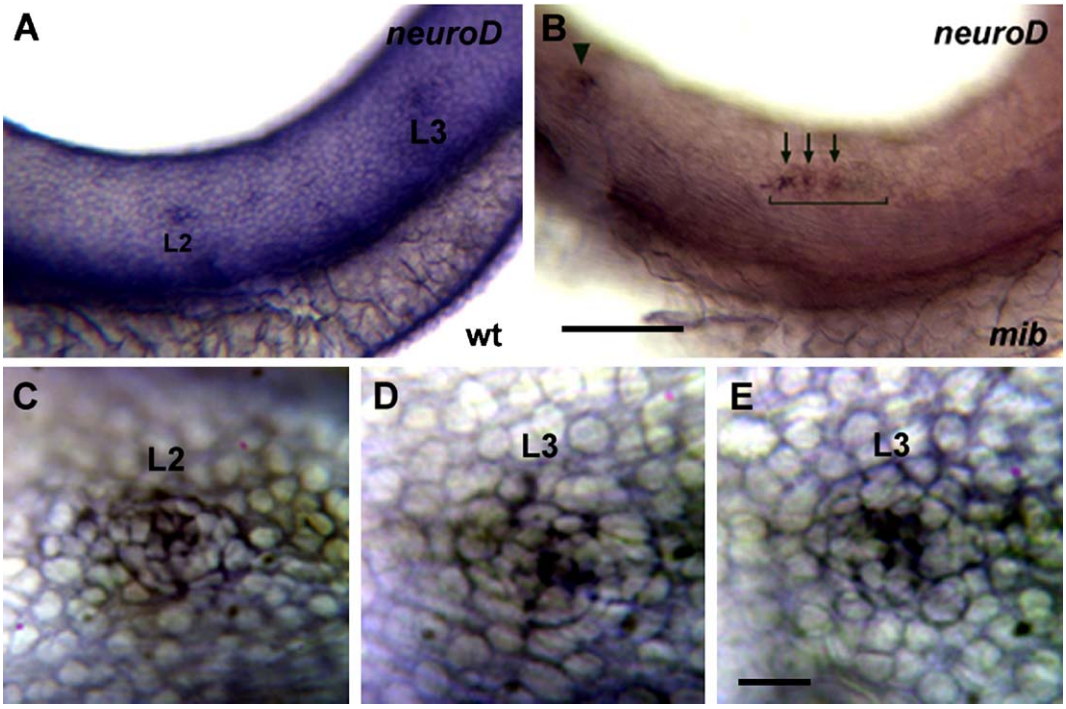

Fig. 1. Expression of *nrd* in the posterior lateral line system. *nrd* mRNA expression in wild type (A, C–E) or *mindbomb* mutant (B) embryos, detected by in situ hybridization. (A) *nrd* expression in the PLL neuromasts is detected when embryos are overstained. Indicated are the L2 and L3 neuromasts of a 42 hpf embryo. (B) *nrd* expression is observed in *mindbomb* 30 hpf embryos, in neuromasts (arrowhead) and often as clusters (arrows) in the migratory primordium (bracket) without the need for overstaining. (C–E) Close up images of neuromasts of wild type 42 hpf embryos labeled with the *nrd* riboprobe. All panels: lateral views, anterior to the left. Scale bar in B: 100 μM for A and B; scale bar in E: 10 μM for C–E.

In *mindbomb* (*mib*) mutants, there is an initial overproduction of hair cells in the ear and in LL neuromasts (Haddon et al., 1998), which is accompanied by increased expression of *ath1* in the primordium and neuromasts (Itoh and Chitnis, 2001). To determine whether this overproduction of hair cells is also preceded by increased expression of *nrd*, we performed in situ hybridization on homozygous *mib* embryos. *mib* embryos were fixed at 30 hpf, when the PLL migrating primordium has traveled about halfway to the tip of the tail and 2–3 proneuromasts have been deposited. We chose this stage because it is known that the hair cells produced in the ear and lateral line of *mindbomb* embryos die as they mature, around 48 hpf (Haddon et al., 1998; Itoh and Chitnis, 2001). We found that *mib* mutants show *nrd* label in the migrating PLL primordium (Fig. 1B, arrows) and expression appears increased in deposited proneuromasts (Fig. 1B, arrowhead). The increased expression is made evident by the fact that we did not have to use our modified in situ hybridization protocol to detect *nrd* expression in *mib* mutants (note the reduced background in Fig. 1B compared to Fig. 1A). Thus, the increase in *ath1* expression in *mib* embryos, which results in excess hair cells, is accompanied by elevated *nrd* expression, suggesting that *nrd* is also expressed in hair cell precursors. In addition, expression of *nrd* in the primordium in *mib* embryos suggests that there may be low levels of expression in these cells in wild type embryos, expression that is undetectable by in situ hybridization.

Loss of function of nrd, but not of ngn1, affects posterior lateral line development

In order to test whether *nrd* is implicated in lateral line formation, we blocked mRNA translation of this gene by using an antisense morpholino oligonucleotide (*nrd*MO). Morpholino was injected into 1-cell-stage embryos that were raised and were analyzed for developmental defects. At the concentration used (8.5 ng), most of the embryos showed no obvious anatomical phenotypes after 1, 2 or 3 days post-fertilization. A small percentage (<5%) of embryos were severely deformed after 24 h; these were discarded from further analysis as we attribute the malformations to non-specific effects of the injection. Since *nrd* is strongly expressed in the cranial ganglia (Andermann et al., 2002), we analyzed the formation of these structures in *nrd*MO-injected embryos. Comparison of *nrd*MO and control embryos shows no difference in the development of the PLL ganglion or other ganglia as assayed with anti-acetylated tubulin and anti-HuC antibodies (not shown). Moreover, the PLL nerve can be labeled with DiI in the injected embryos, showing normal co-migration of the pioneering axons with the PLL primordium (Fig. 2).

We next analyzed *nrd*MO-injected larvae with Di-Asp, a vital stain for mature hair cells (Collazo et al., 1994). The assay consisted in classifying fish that showed either a normal number of Di-Asp-positive neuromasts (5–8 per side), less than normal (1–4), or absence of labeled neuromasts 48–72 h after injection (Table 1). At 72 hpf, the primary lateral line is completely

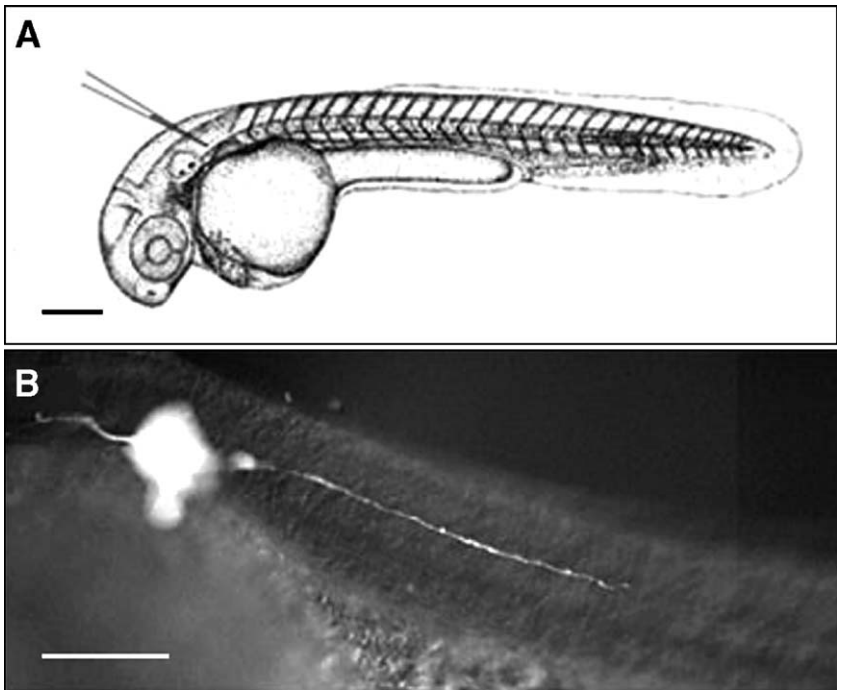

Fig. 2. The PLL nerve co-migrates with the PLL primordium in *NeuroD* (*nrd*) morphant embryos. (A) DiI injection in the posterior lateral line ganglion was performed at 30 hpf in *nrd* morphant embryos in order to mark the growing PLL nerve. (B) The PLL nerve is normally formed in a morphant embryo, can be labeled with DiI and is visualized under fluorescence microscopy; anterior to the left. Scale bar: 200 μM for A and 100 μM for B.

developed and neuromasts incorporate Di-Asp in wild type larvae (97% have between 5 and 8 neuromasts per side, see Table 1). In contrast, *nrd* morphants showed a marked decrease in the number of labeled neuromasts (Figs. 3B–D). Only 22% of the sides of *nrd*MO-injected larvae showed normal numbers of labeled neuromasts, 47% showing reduced numbers and 31% complete absence of labeling (Table 1). We attribute the heterogeneity among the injected embryo population to unequal delivery or distribution of the morpholino. Moreover, heterogeneity was observed within embryos as well since one side often showed more pronounced effects than the other (see Fig. 3C). As controls, we injected morpholinos directed against two other proneural genes, *zash1a* (Allende and Weinberg, 1994) and *ngn1* (Cornell and Eisen, 2002; Andermann et al., 2002). In the case of the *zash1a*MO, embryos injected with doses that produce loss of pineal neurons (Cau and Wilson, 2003) produce no defects in any of the lateral line components and neuromasts appear fully viable when assayed by Di-Asp staining (95% normal; Table 1). In the case of *ngn1*, morphants did not show a decrease in the number of labeled neuromasts when analyzed for Di-Asp staining and often showed an excess of neuromasts compared to the wild type larvae. This phenotype is due to the absence in *ngn1* morphant fish of the glia that normally accompany the migrating LL nerve (Lopez-Schier and Hudspeth, 2005; Grant et al., 2005).

We tested whether the inhibition in hair cell differentiation could be rescued by co-injecting the *nrd*MO together with a DNA construct encoding a NeuroD:Myc fusion protein, pCS*nrd*MT. The mRNA expressed from this construct should escape the translational block that inhibits the endogenous *nrd* gene as it has 7 mismatches out of 25 possible base pairings with the morpholino. The result shows that the effect is reverted when the morpholino is co-injected with the expression construct, as the number of normal sides increases to 68%, compared to 22% in fish injected with *nrd*MO alone (Table 1).

Atonal homolog 1 (ath1) is required for neuromast development

The lack of effect of the anti-*ngn1* morpholino on neuromast development suggests that *nrd* expression is not regulated by *ngn1* during the formation of posterior lateral line neuromasts, in contrast to what occurs in the lateral line placode and ganglia (Andermann et al., 2002) and in other neurons in the central nervous system (Ma et al., 1996, 1998; Perron et al., 1999). We therefore hypothesized that *nrd* is most likely controlled by another proneural gene in the primordium or in neuromasts. The *ath1* gene is a strong candidate for this function as it is important for development of hair cells in mammals (Bermingham et al., 1999; Chen et al., 2002; Kawamoto et al., 2003) and *ath1* expression has been observed in the zebrafish posterior lateral line primordium, in proneuromasts and mature neuromasts, in a progressively restricted way (Itoh and Chitnis, 2001). The narrowing down of *ath1* expression could be correlated with the selection of hair cell fate.

In order to determine if *ath1* participates in hair cell differentiation in the lateral line, we injected a morpholino directed against the *ath1* gene into one-cell stage zebrafish embryos. The injection of the *ath1*MO again results in a decrease in the normal number of Di-Asp-labeled neuromasts, with 34% of the sides displaying less than 5 neuromasts, instead of 97% in uninjected controls or 95% in embryos injected with a

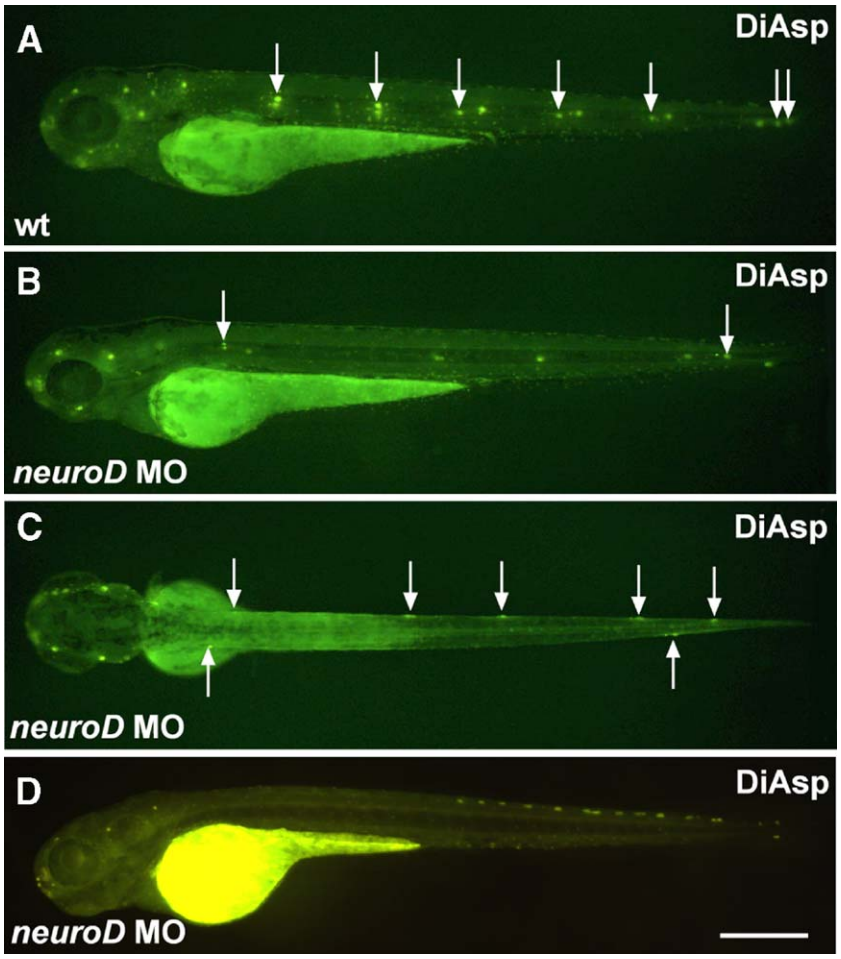

Fig. 3. *NeuroD* morpholino injection affects formation of the posterior lateral line. (A) Pattern of neuromasts (indicated by white arrows, only on the left side) in a 72-hpf larva stained with Di-Asp and visualized under fluorescence, revealing the presence of functional neuromasts. Lateral (B) and dorsal (C) view of a representative *nrd* morphant showing partial absence of neuromasts. Note that the *nrd* morpholino effect is different on each side. While the left side of the larva shows two neuromasts marked with Di-Asp, the right side is nearly normal in appearance. (D) Lateral view of a *nrd* morpholino-injected fish with complete absence of marked Di-Asp-labeled hair cells. All panels: anterior to the left. Scale bar: 100 μM.

morpholino against another bHLH gene (Table 1). These results were practically indistinguishable from those obtained by injection of *nrd*MO.

Though both *ath1* and *nrd* can be involved in the establishment of neural competence, *nrd* has been mostly implicated in neuronal differentiation (Lee et al., 1995; Korzh et al., 1998). It is tempting, therefore, to speculate that *ath1* acts upstream of *nrd* in the lateral line neuromasts. Obvious predictions are then that *ath1* and *nrd* are co-expressed and that, in the absence of *ath1*, *nrd* expression should be abolished in the primordium and neuromasts. Unfortunately, the expression of *nrd* is very weak, making double in situs impractical, and thus an effect of *ath1* inactivation on *nrd* expression cannot be reliably demonstrated. We tried to circumvent this problem by assessing whether the effect of loss of function of *ath1* can be reverted with *nrd* overexpression. We injected the Nrd:Myc expression construct together with the *ath1*MO into one-cell stage embryos. Embryos were raised and assayed for functional hair cells by Di-Asp labeling as before. We found that the mutant phenotype induced by the *ath1*MO alone is partially rescued by *nrd* co-injection (61% of larval sides had normal numbers of neuromasts in co-injected larvae versus 34% normal in *ath1*MO-injected larvae; Table 1). We then performed the opposite experiment, co-injecting the *nrd*MO with an *ath1* expression construct. In this case, we found no rescue (Table 1). Finally, we analyzed *nrd* expression after injecting the pCS*ath1*MT construct (see Materials and methods), which strongly drives the expression of *ath1* in wild type larvae. Larvae were injected at the one-cell stage and were analyzed at 72 hpf by in situ hybridization using an *nrd* probe. Expression of *nrd* was clearly induced, though most of the larvae showed a random punctate pattern, possibly reflecting mosaicism in the distribution of DNA (Fig. 4A). About 20% of the larvae ($n = 25$) showed strong induction of *nrd* in neuromasts, expression that is never seen in non-injected controls with the in situ hybridization procedure used in this experiment (Fig. 4B, compare background levels to Fig. 1A). Higher magnification images of individual neuromasts (Figs. 4C, D) show strong expression of *nrd* in the entire neuromast, in contrast to the more restricted, and substantially weaker expression in non-injected fish (compare Figs. 4C, D with Figs. 1C–E). The terminal neuromasts are also very strongly labeled in *ath1*-injected

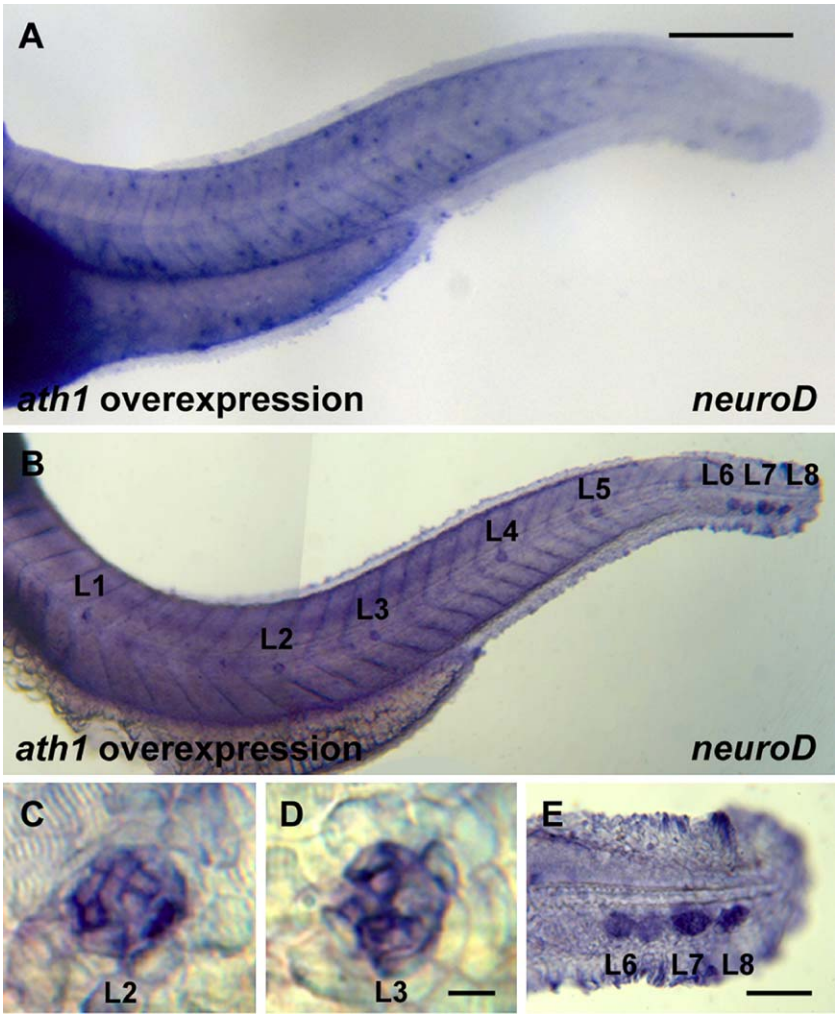

Fig. 4. *ath1* overexpression promotes ectopic *nrd* expression. (A–E) *nrd* mRNA expression in wild type embryos injected with the *ath1* cDNA, detected by in situ hybridization. (A) Punctate ectopic *nrd* expression can be observed along the trunk and tail of an injected embryo. (B) Increased *nrd* expression localized to neuromasts of an injected embryo; L1–L8 indicate positions of the individual PLL neuromasts. Close up views of the L2 (C), L3 (D) and terminal (E) neuromasts from the larvae shown in B. All panels: lateral views, anterior to the left. Scale bar in A: 200 μM for A and B; scale bar in D: 10 μM for C and D; scale bar in E: 50 μM.

larvae (Fig. 4E). Our results strongly suggest that *ath1* regulates *nrd* and that it might be the proneural gene that is primarily responsible for establishing hair cell fate in the lateral line system.

nrd and ath1 control hair cell development

The reduction in number or complete absence of Di-Asp-stained neuromasts found in *nrd* and *ath1* loss of function fish could have several causes. For instance, a failure in primordium migration, neuromast deposition or neuromast differentiation could give rise to this phenotype. We exclude the possibility that morpholino injections affect primordium migration based on two lines of evidence. First, even though morphant larvae show loss of Di-Asp staining in trunk and tail neuromasts, we often observed the presence of terminal neuromasts at the tip of the tail (Figs. 3B, C), indicating that the primordium had reached its final destination. Second, we carried out in situ hybridization using the *claudinB* probe, a gene that is expressed in the posterior lateral line primordium and in proneuromasts (Kollmar et al., 2001). *ClaudinB* expression in *nrd* or *ath1* morphant larvae does not exhibit significant differences compared to the controls (data not shown). Thus, primordium size and migration along the trunk and tail of the embryo do not depend on proneural gene function. Neuromast deposition appears to occur normally in morphant larvae as well since *claudinB* label is in most cases indistinguishable from that found in wild types, though we found a few instances where we could not detect *claudinB* expression at positions where neuromasts are normally deposited (see below).

Since most neuromasts are deposited correctly, but fail to become labeled by Di-Asp, we examined the morphology of the neuromasts by Nomarski/DIC optics in *nrd* morphant larvae and compared them to wild types. Wild type neuromasts have a circular shape. The centrally located hair cells form clearly distinguishable, radially patterned rosettes (Fig. 5A) and are easily visualized with Di-Asp stain (Fig. 5E). On the other hand, neuromasts in *nrd* morphants (Figs. 5B–D) show irregularly

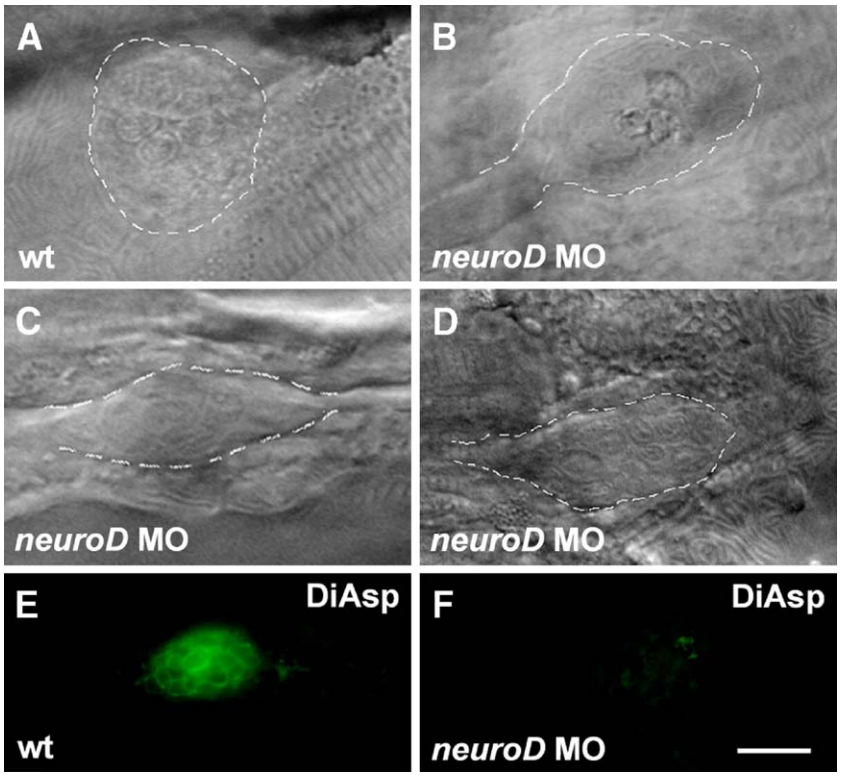

Fig. 5. Aberrant neuromasts form in *nrd* morphant larvae. (A–D) Neuromasts were localized in control and in *nrd* morphants and observed with Nomarski interference contrast microscopy. (A) A neuromast in a wild type larva showing a circular shape (delimited by a discontinuous white line) and a rosette pattern of hair cells concentrated in the center. (B–D) Three examples of Di-Asp-negative neuromasts found in *nrd* MO-injected larvae. Note the irregular shape of these neuromasts and the disorganized arrangement and size of the cells. (E) Wild type neuromast labeled with Di-Asp. (F) Aberrant neuromasts do not have Di-Asp-labeled cells. All panels: lateral views, anterior to the left. Scale bar: 10 μM.

placed cells of varying shapes and sizes. It is not possible to distinguish hair cells by morphology or by labeling with Di-Asp (Fig. 5F). Similar results were observed in *ath1* morphants (not shown).

The vital dye Di-Asp labels hair cells that are differentiated and mature (Collazo et al., 1994). It is possible, therefore, that hair cells were still present in the morphant neuromasts but had lost the ability to incorporate the dye. To evaluate this possibility, we used another hair cell differentiation marker, acetylated tubulin. In control neuromasts, immunolabeling with an anti-acetylated tubulin antibody marks hair cell bodies and cilia (Raible and Kruse, 2000; Figs. 6A and B). The underlying PLL nerve is labeled, as are the axons that innervate the neuromast (thick arrow in Fig. 6A). However, in *nrd* and *ath1* morphant larvae, labeling is limited to the PLL nerve (Fig. 6C, arrowhead). Neuromasts in morphants could reliably be found using Nomarski optics, allowing us to confirm the absence of acetylated tubulin in neuromast cells. Nerve branches were not observed under the neuromasts either. Finally, we tested both *nrd* and *ath1* morpholinos in the ET4 transgenic background; these fish express GFP in hair cells several hours prior to their terminal differentiation (Parinov et al., 2004). Both morpholinos produced loss of GFP expression in these fish, which was perfectly correlated with the loss of Di-Asp staining (data not shown). Therefore, hair cells do not develop in the absence of *nrd* or *ath1*.

Effect of nrd and ath1 on other cell types

To further characterize the effects of *nrd* or *ath1* loss of function, we analyzed other markers of neuromast cells in morphant embryos. First, we used the ET20 transgenic line, which expresses GFP in the outer rim of the accessory cells, the mantle cells (Parinov et al., 2004), as well as in the interneuromastic cells that will later form intercalary neuromasts (Grant et al., 2005; Lopez-Schier and Hudspeth, 2005). ET20 embryos were injected with morpholinos, were raised to 54 hpf, and were stained with Di-Asp to simultaneously detect hair cells and mantle cells (yellow and green label in Fig. 7, respectively). *nrd* and *ath1* morpholinos produced identical results in this experiment, though we show results only for *ath1* in Fig. 7. We observed that, in most cases (>75%), and despite losing the Di-Asp label as before, neuromasts showed normal GFP expression in morphants (compare Figs. 7A, B with C, D). This result indicates that neither neuromast deposition nor the formation of mantle cells or interneuromastic cells is altered in the absence of NeuroD or Ath1 protein (Figs. 7C, D). We found some cases in which GFP label was not detected at positions where neuromasts should normally lie. In these instances, we were not always able to find the aberrant neuromasts by Nomarski optics, leaving a doubt about whether the morpholinos occasionally affect neuromast deposition or whether they interfere with mantle cell determination or survival. We

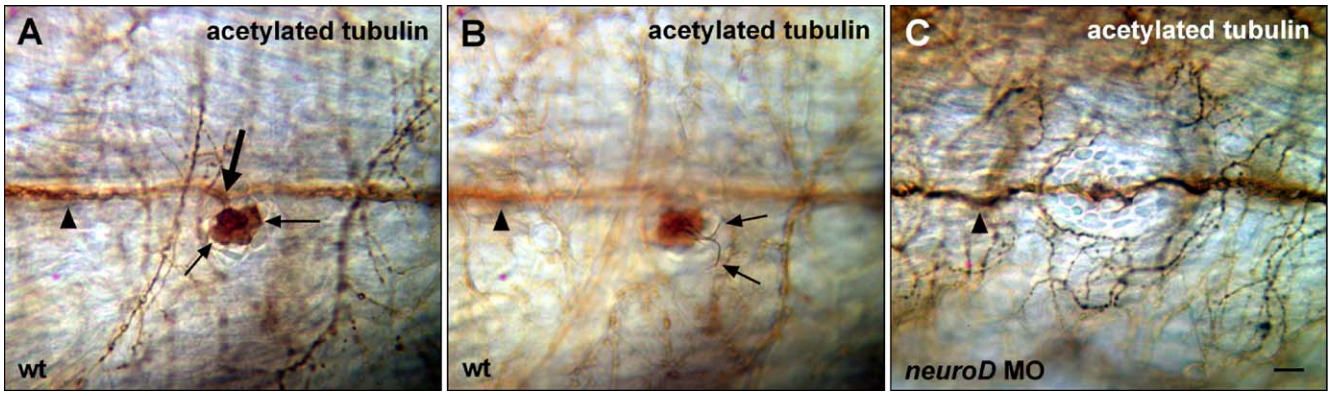

Fig. 6. Neuromasts formed in *nrd* morphant larvae lack hair cells. (A–C) Whole mount immunohistochemistry with anti-acetylated tubulin antibody. (A) In control larvae, anti-acetylated tubulin antibody marks the PLL nerve (arrowhead) and the two fibers that innervate the neuromast (thick arrow), as well as the hair cell somata (arrows). (B) Same image as in A, but the plane of focus was adjusted to visualize the hair cell kinociliae (arrows). (C) In neuromasts of *nrd* morphant larvae, there are no acetylated tubulin-labeled cells. The PLL nerve is unaffected (arrowhead). This particular neuromast looks somewhat larger than in the wild type, but this is not a consistent feature of morphant neuromasts. All panels: lateral views, anterior to the left. Scale bar: 10 μM.

therefore took single ET20 *ath1* morphant larvae at 54 hpf, recorded the pattern of GFP expression and processed them for in situ hybridization using the *claudinB* and *eya1* probes, genes that are expressed in all PLL neuromast cells (Lopez-Schier et al., 2004; Sahly et al., 1999). We found a strict correlation (n = 10 larvae, both sides analyzed) between GFP label in the mantle cells of the neuromast and expression of *claudinB* and *eya1* (Figs. 7E, F, and data not shown). Thus, *nrd* and *ath1* are critical for hair cell development, but not for other cell types in the neuromast. These genes could also play a minor role on neuromast deposition, possibly through their effect on hair cell determination in the migrating primordium. Alternatively, the loss of hair cells could indirectly affect the survival of accessory cells as has been observed previously (Erkman et al., 1996).

Discussion

Role of nrd and of ath1 in hair cell determination

In an attempt to define the proneural genes that are involved in promoting hair cell fate in the zebrafish lateral line system, we sought to determine whether *nrd* has a function in this process. While expression of *nrd* in the PLL placode is easily detected by in situ hybridization, expression is not detected in the migrating primordium and only weakly detected in recently deposited neuromasts. However, loss of function experiments using morpholino oligonucleotides designed against the *nrd* gene show that it is necessary for hair cell differentiation within neuromasts while it plays little or no role in lateral line ganglion specification, in primordium migration and in neuromast deposition. A previous study by Andermann et al. (2002) showed that *ngn1*, a proneural gene of the *Ath* family, is strongly expressed in the lateral line placode, regulates *nrd* and participates in ganglion development without affecting neuromast formation or hair cell differentiation. To reconcile both findings, it became necessary to search for an additional proneural gene responsible for the regulation of *nrd* in cells destined to become hair cells. A likely candidate was the *ath1* gene. Morpholino injections directed against *ath1* showed a phenotype indistinguishable to that observed with *nrd* morpholinos: migration of the primordium and deposition of neuromasts are not affected but hair cells do not differentiate. Moreover, we showed that the effect could be partially rescued by overexpression of *nrd* and that overexpression of *ath1* can induce *nrd*. These observations are consistent with the idea that *ath1* acts upstream of *nrd* in hair cell development.

The selection of hair cell fate has been proposed to be under the control of the *Notch* and *Delta* families of neurogenic genes (Henrique et al., 1995), the latter being under the control of proneural genes both in flies and in vertebrates. In the lateral line, *delta* is expressed in the migrating primordium in a pattern that is consistent with a role in hair cell selection (Itoh and Chitnis, 2001) and that corresponds well to the pattern of expression of *ath1*. In the *mindbomb* mutant, supernumerary hair cells are produced in neuromasts concomitantly with overexpression of *ath1* (Itoh and Chitnis, 2001). Thus, *ath1* is a prime candidate for being the proneural gene responsible for the determination of presumptive hair cells in the zebrafish lateral line through activation of *nrd* expression.

We observed an occasional absence of all neuromast cell types after inactivation of either *ath1* or *nrd*. A simple explanation for the loss of support of cell markers or of neuromasts is that the integrity of the entire organ is dependent on the presence of hair cells. Indeed, it has been shown that ablation of hair cells can induce cell death in support cells (Erkman et al., 1996). We cannot rule out, however, involvement of *ath1* or *nrd* in the determination of other cell fates and/or in the process of neuromast deposition, though we clearly favor a specific role for *ath1* and *nrd* in hair cell development.

Evolutionary origin of hair cells and afferent neurons

What may be the origin of the dual control of cell determination by *ngn1* for the afferent neurons and by *ath1* for hair cells? The fish lateral line (and ear) shares impressive similarities with fly mechanosensory organs, suggestive of a common ancestry (Adam et al., 1998; Fritzsch et al., 2000). One

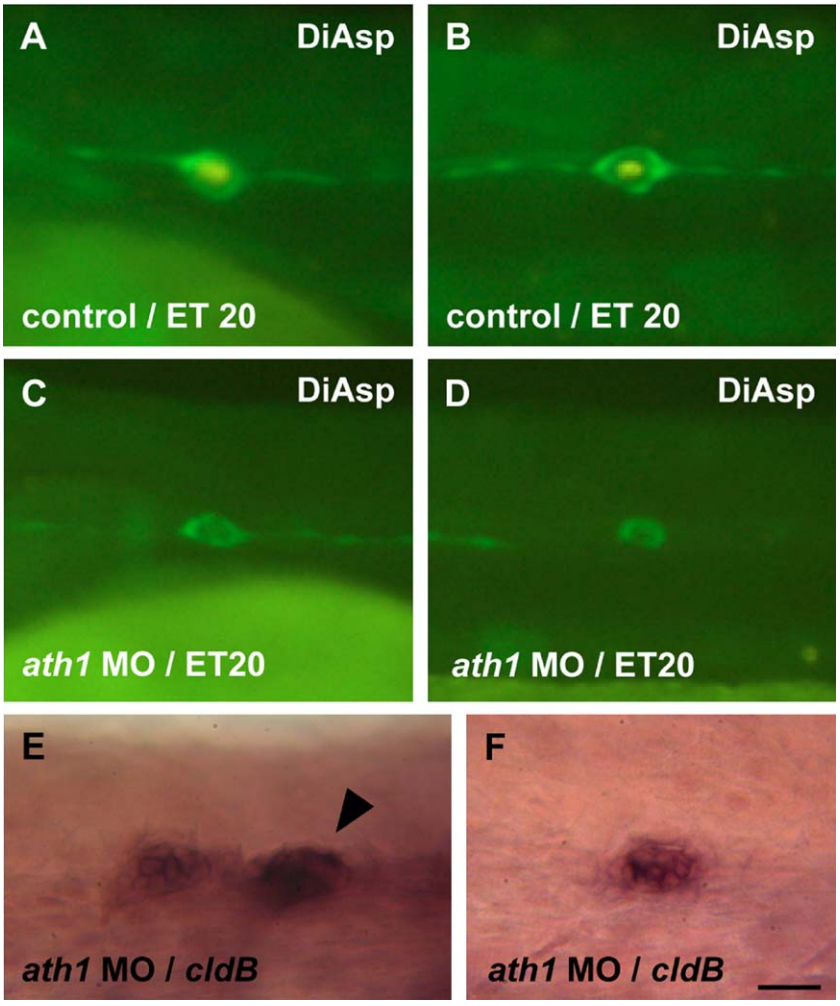

Fig. 7. Role of *ath1* in neuromast differentiation. (A–D) Control or *ath1* morphant ET20 transgenic larvae were incubated with Di-Asp and visualized under fluorescent illumination, allowing simultaneous visualization of GFP in mantle cells (green) and Di-Asp label in hair cells (yellow). Close up views of the L1 and L2 neuromasts from a control larva (A, B) and from an *ath1*MO-injected larva (C, D). Hair cells, but not mantle cells, are absent in *ath1* morphants. (E–F) The same morphant larva was processed for in situ hybridization using the *claudinB* (*cldB*) riboprobe, and the same neuromasts (L1 and L2) were analyzed. Both neuromasts are labeled with the *cldB* probe. Arrowhead in E corresponds to the secondary PLL primordium, which is also labeled by *cldB*. All views are lateral, anterior to the left. Scale bar: 20 μM.

major difference exists between the two types of organs, however: the function of the mechanosensory neuron present in arthropods has been subdivided in two cell types in vertebrates, the mechanosensory hair cell on the one hand and the afferent neuron on the other. It seems plausible that this subdivision reflects evolutionary history and that a single precursor cell type present in a common ancestor to flies and vertebrates subsequently diversified in the vertebrate lineage into two descendant cell types (Ghysen, 2003). Such cell types related by common evolutionary origin have been called "sister cell types" by Arendt (2003).

Assuming that hair cell and afferent neuron are indeed derived from an ancestral mechanosensory neuron (through sub- and neofunctionalization), this required some way to uncouple the developmental program of the two divergent cell types. It has been argued that divergence of bHLH genes has precisely the effect of uncoupling subsets of neurons from each other, thus making it possible to introduce gradual changes in each set without perturbing the function of the other set (Brunet and Ghysen, 1999). The diversification of the two *atonal* homologs, *ngn1* and *ath1*, from each other may therefore have been followed by the diversification of the two sister cell types, hair cells and afferent neurons, from a single mechanosensory cell type precursor still found in arthropods.

Advantages of this diversification include the possibility to increase the number of receptors relative to the number of afferent neurons, leading to an improved sensitivity and signal-to-noise ratio. Neuromasts comprise two populations of hair cells with opposite polarities and are innervated by two afferent neurons (Fig. 6A), each of which corresponds to one polarity. Increasing the number of hair cells innervated by a given afferent will obviously increase the sensitivity of the system. A similar trend is observed in the vertebrate retina, where sister cell types (rhabdomeric and ciliary photoreceptors) have also been combined to build a highly efficient sensory organ (Arendt, 2003). In the case of the mechanosensory organs, however, we need to know much more about the combination of transcription factors and effector genes

present in hair cells and afferent neurons before we can decide whether hair cells and afferent neurons are truly sister cell types.

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