The Role of *her4* in Inner Ear Development and Its Relationship with Proneural Genes and Notch Signalling

Marija Radosevic*, Laura Fargas*, Berta Alsina*

Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, Barcelona, Spain

### Abstract

The generation of sensory neurons and hair cells of the inner ear is under tight control. Different members of the *Hairy and Enhancer of Split* genes (HES) are expressed in the inner ear, their full array of functions still not being disclosed. We have previously shown that zebrafish *her9* acts as a patterning gene to restrict otic neurogenesis to an anterior domain. Here, we disclose the role of another her gene, *her4*, a zebrafish ortholog of *Hes5* that is expressed in the neurogenic and sensory domains of the inner ear. The expression of *her4* is highly dynamic and spatiotemporally regulated. We demonstrate by loss of function experiments that in the neurogenic domain *her4* expression is under the regulation of *neurogenin1* (*neurog1*) and the Notch pathway. Moreover, *her4* participates in lateral inhibition during otic neurogenesis since *her4* knockout results in overproduction of the number of *neurog1* and *deltaB*-positive otic neurons. In contrast, during sensorigenesis *her4* is initially Notch-independent and induced by *atoh1b* in a broad prosensory domain. At later stages *her4* expression becomes Notch-dependent in the future sensory domains but loss of *her4* does not result in hair cell overproduction, suggesting that there other her genes can compensate its function.

### Citation

Radosevic M, Fargas L, Alsina B (2014) The Role of *her4* in Inner Ear Development and Its Relationship with Proneural Genes and Notch Signalling. PLoS ONE 9(10): e109860. doi:10.1371/journal.pone.0109860

### Introduction

Distinct proneural genes from the atonal basic helix-loop-helix (bHLH) superfamily control cell fate specification in the inner ear. *Drosophila atonal* gene was first identified in 1993 [1], its mutation resulting in absence of PNS chordotonal organs and photoreceptors [2]. In vertebrate auditory system one of the *atonal* orthologs, *atoh1*, is initially expressed in the sensory epithelium to later restrict in nascent hair cells [3]. Either the loss or overexpression of *atoh1* results in complete absence or ectopic differentiation of hair cells [4–6]. In zebrafish, two *atoh1* genes are found in the inner ear, which act in two distinct phases of sensory development [7]. Initially, *atoh1b* appears in a broad prosensory territory to subsequently get restricted to smaller sensory domains. There, *atoh1a* is induced by *atoh1b* to direct the differentiation of hair cells [7]. In all organisms studied, the Notch pathway regulates the production of hair cells and supporting cells via lateral inhibition [8–10].

Another member of *atonal* proneural family, *neurog1*, is used to define the otic neurogenic domain where otic progenitors will acquire a neuronal lineage [11,12]. Again, Notch signalling is key to regulate the final number of otic sensory neurons [8,9,13,14]. The activities of Notch pathway and proneural genes are highly intertwined in many developmental contexts, generally conceived to work in concert. However, a detail mapping of Notch activity throughout inner ear development in correlation with proneural expression is still missing. The lack of good antibodies to detect the cleaved form of Notch Intracellular Domain (NICD) in other species than mouse hampers identifying Notch active cells, with only one report showing NICD localization during cochlear development [15].

Alternatively, expression of HES genes, considered direct targets of Notch pathway has been used to monitor Notch activity. Upon Notch activation *Hes5* is induced and suppresses hair cell fate while promoting supporting cell fate [16–18]. Similarly, *Hes5* is expressed complementary to *Delta1* expressing cells in the neurogenic domain [13]. *Her4* is one of the zebrafish orthologs of mammalian *Hes5* and it has been implicated in the control of primary neurogenesis, brain regeneration and neuronal target innervation [19–21]. In the CNS *her4* expression depends on Notch, while does not in the trigeminal sensory ganglia [22]. Nothing is known of *her4* in the inner ear, thus we aimed to study its function and regulation during neural and sensory specification. Here we show that zebrafish *her4* is expressed in the neurogenic and sensory domains and requires *neurog1* and *atoh1b* for its expression, respectively. Moreover, *her4* in the neurogenic domain is dependent on Notch, while initial broad induction of *her4* in the presumptive sensory domain does not require Notch but *atoh1b*. However, later on Notch restricts *her4* expression to the future sensory maculae. Furthermore, we show that loss of
her4 mediates lateral inhibition during neurogenesis, but not during sensorigenesis.

Materials and Methods

Fish maintenance and zebrafish stains
Zebrafish were maintained at the PRBB Animal Facility under standard conditions. The zebrafish protocols followed the guidelines and were approved by the IACUC, Comité Ético de Experimentación Animal-Parc de Recerca Biomédica de Barcelona (CEEA-PRBB); approved number JIB-08-1098P2. The zebrafish strains used were the following: wild-type (AB), mbt-AT1 [23] and neurog1/2 [24,25]. We also used the transgenic line Tg(her4:EGFP)85 [22]. A 3.4 kb 5’-flanking region of her4 DNA containing 22 bp of 5’ UTR sequence of her4 cDNA is controlling EGFP expression. Homozygous mutant mbt and neurog1 embryos were obtained by pairwise mating of heterozygous adult carriers. mbt descendant embryos were genotyped after in situ hybridization. Embryos were developed in an incubator at 28.5°C in system water containing methylene blue and staged after counting somite number. Embryonic stages are given as hours post-fertilization (hpf) at 28.5°C [26].

Whole mount in situ hybridization

Synthesis of antisense RNA and whole-mount in situ hybridization were performed as previously described [27]. Probes were used as follows: atoh1b and atoh1a [7], neurog1 [28], her4 [29] and deltaB [9]. Embryos were isolated at desired developmental stages essentially as described [26]. Dechorionated zebrafish embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C and dehydrated in methanol series, rehydrated again and permeabilized with 10 mg/ml proteinase K (Sigma) at RT for 5–10 min depending on their stage. Digoxigenin-labeled probes were hybridized overnight at 70°C, detected using anti-digoxigenin-AP antibody at 1:2000 dilution (Roche) and developed with NBT/BCIP (Roche). Embryos were post-fixed overnight in 4% PFA and used for imaging either mounted in 100% glycerol or cryoprotected in 15% sucrose and embedded in 7.5%gelatine/15%sucrose. Blocks were fixed in 2-Methylbutan (Sigma) for tissue preservation and cryosectioned at 20 µm on a Leica CM 1510-1 cryostat.

Antisense morpholinos (MO)

MOS were obtained from Gene Tools. Embryos were injected at 1-cell stage. The her4-MO [21] was designed to block the translation of her4 mRNA transcript with sequence 5’-ATT GCT GTG TGT CTT GTG TTC AGT T-3’. her4-MO was injected at concentration 0.025 mM and its efficiency was assessed by the specific loss of GFP signal from the Tg(her4:EGFP)85 transgenic line [22]. The atoh1b-MO [7] was injected at concentration 5 µg/µl and its sequence is 5’-TCA TTG CTT GTG TAG AAA TGC ATA T-3’.

SU5402 inhibitor treatment

Dechorionated zebrafish embryos were incubated with 50 µM SU5402 (Calbiochem, 572630), a potent pharmacological inhibitor of Fgl signalling. Incubations were done at 28.5°C, starting at 10 hpf until the sacrifice of the animals at 16 hpf. The final solution was done in E3 medium from the 5 mM SU5402 stock solution (kept at −20°C in DMSO). For control treatments, embryos were incubated in an equivalent concentration of dimethyl sulfoxide (DMSO, Sigma).

Image acquisition

Pictures were acquired in a Leica DRM microscope or in Leica MZFLIII stereomicroscope using a Leica DFC300 FX camera and the Leica IM50 software. Adobe Photoshop 7.0.1 software was used for photograph editing.

Results

Spatiotemporal dynamics of her4 expression in relation to proneural genes

The family of her genes in zebrafish, orthologous to mammalian Hes genes, consists of 21 genes based on data published on Ensembl (Zv9). We analysed the expression of 10 her genes during otic development and only her6, her4 and her9 were found to be expressed in the otic vesicle from 12–14 hpf to 24 hpf (data not shown). We have previously demonstrated a role for her9 during otic patterning in restricting neurogenesis to the anterior domain, downstream of retinoic acid (RA) signalling [30] and here concentrated on her4 gene. Previous studies have analysed the development of the neurogenic and sensory territories separately, however since both domains develop simultaneously, a complete picture of the development of both territories should be taken into account. Moreover, the sequential phases of Notch activation is still not well defined. Consequently, we have generated a precise map of the expression of her4 at early stages of neuro- and sensorigenesis together with the expression of atoh1b, atoh1a and neurog1. her4 expression is first observed at 12 hpf throughout a broad band of cells just adjacent to the hindbrain. The pattern at this stage is strikingly similar to the one from atoh1b (compare Figure 1M with Figure 1A). By 13 hpf, both genes start to become down-regulated from the Central Medial Domain (CMD) of the otic placode and higher expression is observed in anterior and posterior patches (Figure 1N and Figure 1B, arrow pointing to CMD; [7]). At 16 hpf, her4 is only detected at the future anterior (utricular) and posterior (saccular) sensory maculae with higher levels of expression than atoh1b (compare Figure 1O with Figure 1C–D). atoh1a is expressed solely at the future anterior and posterior sensory domains from 13 hpf onwards (Figure 1E–H and [7,8]). At 20 hpf, the number of cells expressing atoh1a in the anterior macula increases, while only two cells remain expressing atoh1a at the posterior macula (Figure 1G and H) (as a result of the spherical shape of the otic vesicle, the posterior macula expressing atoh1a is not visible at dorsal planes but only in ventral planes).

Together with the activation of atoh1, another proneural gene, neurog1, is induced in the inner ear. In zebrafish, in contrast to what happens in other vertebrate species such as chick and mouse, sensory specification by atoh1 genes takes place before neuronal specification [7,9,31,32]. The expression of neurog1 is first detected at 15 hpf in the most anterolateral cells of the otic placode to progress overtime to more medial positions (Figure 1I and K, [30]). The expression of neurog1 lags the first appearance of atoh1b by three hours. At 17 hpf, her4 expression in the neurogenic domain appears, being the expression in the otic vesicle a sum of the expressions from the sensory and the neurogenic domains (Figure 1P, arrows pointing to the sensory and neurogenic expression of her4). Note that neurog1 expression begins in the neurogenic domain at 15 hpf but her4 expression is not initiated there until two hours later (compare Figure 1J, 1O and 1P). Later on, from 20 hpf her4 expression in the sensory domains remains only apparent in the anterior macula (Figure 1L). From the described expression patterns over time, we can infer that her4 is transcribed in both the neurogenic and sensory
Figure 1. Spatiotemporal expression of proneural genes at early stages of inner ear development. (A–D) In situ hybridization in wild-type embryos for atoh1b. atoh1b is expressed in a large medial domain adjacent to the hindbrain at 12 hpf (A) to then progressively restrict to two patches that correspond to the future anterior and posterior prosensory domains (B–D). Between 13 hpf and 14 hpf atoh1b is downregulated at the central medial domain (CMD) (arrow in B). (E–H) In situ hybridization in wild-type embryos for atoh1a. atoh1a is expressed in the anterior and posterior sensory domains by 13 hpf and 14 hpf (E, F). At 20 hpf the expression in the anterior domain increases (G) and just two positive cells remain in the posterior (H). (I–K) In situ hybridization in wild-type embryos for neurog1. Expression of neurog1 in the inner ear starts at 15 hpf (J) and it is extended from an anterolateral position to a postero medial domain (K). neurog1 progressively invades the CMD (arrow in K). (L–P) In situ hybridization in wild-type embryos for her4. her4 expression is found at 12 hpf (M) to become restricted to the future sensory maculae by 13 hpf (N). At 16 hpf her4 presents higher expression levels (O) and at 17 hpf, the expression of her4 appears in the neurogenic domain being a sum of the expressions from both domains (arrows in P). By 20 hpf the expression only remains at the anterior sensory domain (L). Dorsal views; anterior to the left, medial to the top. Dashed circles delineate otic vesicles. All images are at same magnification. Scale bar: 25 μm.

doi:10.1371/journal.pone.0109860.g001

Role and Regulation of her4 in the Inner Ear

Complex and dynamic regulation of her4 by Notch in the inner ear

In many cases, HES members are activated by Notch [19]. However, this is not a strict rule since in some cases BMP, Wnt or RA pathways but not Notch regulate her transcription [30,33,34]. Therefore, to test whether her4 expression is regulated by Notch pathway in the sensory and neurogenic domains, we have analysed the effect of Notch inhibition in mibta52b mutant embryos that lack the specific Notch ubiquitin ligase necessary for Delta endocytosis [23]. In mibta52b embryos, deltaB and deltaD are upregulated and lateral inhibition is impaired causing overproduction of hair cells at the expense of supporting cells [8]. When looking at what happens in the neurogenic domain, we found that her4 expression is abolished in mib mutant embryos of 20 hpf and 24 hpf (mib genotyped; n = 4/4) compared to heterozygous embryos that retain her4 expression (Figure 2A–D; n = 3/3). As mentioned, this phenotype highly resembles the phenotype of atoh1b expression in mibta52b embryos. Since her4 is induced in the CMD in mibta52b, instead of downregulated, her4 cannot be the atoh1b repressor in this region.

Since initial atoh1b expression has been shown to depend on Fgf signalling in the inner ear [7], next we decided to test whether blocking Fgf signalling also affects the induction of her4. Indeed, her4 expression is inhibited when embryos were treated pharmacologically with 50 μM SU5402 from 9 hpf until 14 hpf (n = 5/5) as well as atoh1b (n = 5/5) confirming that both have similar regulatory requirements (Figure 2G–J).

Taken together, Notch induces her4 in the neurogenic domain, while the regulation of her4 by Notch in the sensory domain is complex and changes over time. In a first phase her4 is set up by Fgf, but not by Notch signalling. Then, Notch down-regulates her4 expression in the CMD and finally maintains its expression in the future sensory maculae. These experiments indicate that her4 is not induced by Notch in the CMD to repress atoh1b.

atoh1b is upstream of her4 in the prosensory territory

Since her4 and atoh1b expression profiles over time are practically indistinguishable and also share the same pattern of regulation by Notch, one can formulate distinct reasoning. On one hand, her4 and atoh1b are under the same regulatory pathways or on the other hand, atoh1b and her4 belong to the same genetic pathway, either atoh1b regulating her4 or vice-versa.

In order to distinguish between these possibilities, we have blocked atoh1b activity by injecting atoh1b-morpholino (atoh1b-MO) to

territories, with dynamics highly similar to those of atoh1b and neurog1.
assess the possible regulation of her4 by atoh1b. her4 expression precedes the onset of atoh1a in the otic placode, thus we reasoned that atoh1b instead of atoh1a was the best candidate for testing the upstream regulation of her4. Indeed, in atoh1b-MO injected embryos, expression of her4 is abrogated in the anterior and posterior sensory epithelia at 15 hpf (Figure 3B; n=5/5) and no her4 expression is present in the otic placode, as her4 is not yet induced in the neurogenic domain. Interestingly, neither precocious nor ectopic expression of her4 in the neurogenic domain is found. In control conditions, her4 expression is only present in the future sensory maculae (Figure 3A; n=5/5). At 24 hpf, again, expression of her4 is abolished from the sensory territory in atoh1b morphant embryos (compare arrow in Figure 3C and D; n=6/6) but her4 expression in the neurogenic domain remains intact (compare Figure 3C with Figure 3D). The expression of her4 in the neurogenic domain did not seem to change significantly after blocking atoh1b, having into account that there is some intrinsic otic vesicle to otic vesicle variability in the shape and extension of the neurogenic domain. To further confirm this result and analyze a possible cross-talk between atoh1b and neurog1, we checked for the extent of neurog1 expression in atoh1b-MO injected embryos. Again, no expansion of neurog1 in the sensory domain was observed in morphant embryos compared with controls (Figure 3E-F; n=4 for atoh1b-MO and n=4 for controls).

In summary, her4 is a target of atoh1b in the sensory domain and depletion of atoh1b function has no effect on her4 neither on neurog1 expression in the neurogenic domain.

Neurog1 regulates her4 expression in the neurogenic domain

We next assessed the genetic relationship of her4 with the proneural gene neurog1 by using the neurog1<sup>1<sup>hi1059</sup></sup> mutant line [24]. In neurog1<sup>1<sup>hi1059</sup></sup> mutants obtained from heterozygotic crosses of neurog1<sup>1<sup>hi1059</sup></sup> adults, her4 is not expressed in the neurogenic domain at 24 hpf (black arrow in Figure 4B' and Figure 4A'; n=7/21), whereas expression in the sensory domain is not abolished. Interestingly, the expression of her4 in wild-type embryos is only detected in the future anterior sensory macula (Figure 4A), whereas in neurog1<sup>1<sup>hi1059</sup></sup> mutant embryos it is also detectable in the posterior patch (arrowhead in Figure 4B and B'; n=5/7). This suggests that either down-regulation in the posterior patch does not take place in neurog1<sup>1<sup>hi1059</sup></sup> mutant embryos or alternatively reactivation of her4 in the future posterior patch occurs. Therefore, when neurogenesis is impaired in the inner ear from lack of neurog1, her4 expression in the posterior sensory patch is altered, suggesting a cross-talk in this territory between the different proneural genes. Recently it has been reported that absence of neurog1 activity expands the posterior sensory patch but not the anterior patch [35], which would be in agreement with our results showing increased her4 expression at the posterior patch in neurog1 mutants.

Altogether, our experiments show that her4 expression is dependent on atoh1b and neurog1 in the sensory and neurogenic domains respectively. Moreover, we corroborate previous studies indicating that neurog1 influences the development of the posterior macula and we show that atoh1b does not influence neurog1 expression.

Inhibition of her4 increases the number of neuronal fated cells but not the number of hair cells

In order to evaluate the role of her4 during inner ear development, we have blocked her4 function by injecting her4-MO at 1-cell stage embryos. The efficacy of her4-MO was assessed in the Tg(her4:EGFP) line [22]. In this line, a 3.4 kb region upstream of the her4 start site containing also 22 bp of the her4 5’ UTR is cloned together with EGFP. Her4-MO sequence binds specifically to the 5’ UTR affecting EGFP translation. In control embryos, strong EGFP is visible in the neural tube, while in 0.025 mM her4-MO injected embryos EGFP expression is completely abolished (Figure 5A-B). Next, we have analysed the effects of her4 on neurogenesis by analysing the expression of neurog1 and deltaB in 24 hpf and 27 hpf her4 morphant embryos, respectively. Increase of neurog1 expression is observed in
morphant embryos in dorsal and ventral planes compared to controls (Figure 5C–D'; n = 10/18 for her4-MO and n = 0/10 for controls). In cross-sections, an expansion of neurog1 expression is also observed (Figure 5G–H').

During lateral inhibition, cells adopting a primary fate inhibit their neighbours from acquiring the same fate. These cells express ligands of the delta/serrate family to activate the Notch pathway in their neighbours from acquiring the same fate. These cells express ligands of the delta/serrate family to activate the Notch pathway in their neighbours from acquiring the same fate. These cells express ligands of the delta/serrate family to activate the Notch pathway in their neighbours from acquiring the same fate. These cells express ligands of the delta/serrate family to activate the Notch pathway in their neighbours from acquiring the same fate. These cells express ligands of the delta/serrate family to activate the Notch pathway in their neighbours from acquiring the same fate. These cells express ligands of the delta/serrate family to activate the Notch pathway in their neighbours from acquiring the same fate. These cells express ligands of the delta/serrate family to activate the Notch pathway in their neighbours from acquiring the same fate. These cells express ligands of the delta/serrate family to activate the Notch pathway.

Figure 3. her4 expression requires atoh1b in the sensory domain. (A–D') In situ hybridization for her4 expression in wild-type and atoh1b-MO injected embryos. (A, B) In 15 hpf control embryos, her4 expression is detected in the future anterior and posterior sensory domains (A), while in morphant embryos her4 expression is lost (B). (C, D) At 24 hpf her4 expression is also lost in the sensory territory in morphant embryos (arrow in D) compared with controls (arrow in C). (C', D') The expression in the neurogenic domain is not affected in morphant embryos. (E, F) In situ hybridization for neurog1 expression in wild-type (E) and atoh1b-MO injected (F) embryos. neurog1 expression in atoh1b morphant embryos is not affected. Dorsal views; anterior to the left, medial to the top. Dashed circles delineate otic vesicles. All images are at same magnification. Scale bar: 25 μm.

doi:10.1371/journal.pone.0109860.g003

Figure 4. neurog1 is required to induce her4 expression in the neurogenic domain. (A, A') In situ hybridization for her4 expression in wild-type embryos. her4 expression is detected in both, the anterior sensory domain and the neurogenic domain (arrow A'), but not in the posterior sensory domain (arrowheads A, A'). (B–B') In situ hybridization for her4 expression in neurog1-MO embryos. Her4 is not expressed in the neurogenic domain (arrow B') and her4 expression in the posterior sensory domain is detected (arrowheads B, B'). Dashed circles delineate otic vesicles. All images are at same magnification. Scale bar: 25 μm.

doi:10.1371/journal.pone.0109860.g004

thence amplifying the differences between neighbouring cells (reviewed in [36,37]). Several delta genes are expressed in the zebrafish inner ear [8], many of them found in the sensory domains. Since deltaB is highly expressed in neuronal cells, we chose this ligand to study the effects of loss of her4 in neuronal differentiation as a possible mediator of Notch signalling during lateral inhibition. When analysing the expression of deltaB, increased number of deltaB-positive cells was found when analysed in flat-mount images (Figure 5F–F') and in transverse sections (Figure 5I). In morphant embryos the statoacoustic ganglion (SAG) is bigger, present in two consecutive transverse sections (Figure 5J), compared to one in control embryos (Figure 5I). At the level of the otic epithelium, increased number of neuroblasts was also visible (compare arrowheads in Figure 5J and J' in morphant embryos to Figure 5I in control embryos), accompanied with higher density of deltaB-positive cells in the neural tube. This is in accordance with a role of her4 in the suppression of a deltaB during the selection of neuronal cells upon Notch activation. The results suggest that her4 regulates deltaB, but does not feedback on neurog1.

We could expect a similar role of her4 in negatively regulating the number of differentiating hair cells in the prosensory domains. First, we have analysed the effects on atoh1b to know whether her4 can control its own main regulator. Interestingly, no effect on the expression of atoh1b in morphant embryos was observed at 14 hpf (Figure 6A–B; n = 9/9 for her4-MO and n = 4/4 for controls). As we have previously shown that atoh1b is upstream of her4, this result indicates that, as observed for neurog1, her4 does not feedback on atoh1b.

We have seen above that at later stages her4 becomes dependent of Notch, most probably participating in Notch-mediated lateral inhibition and repressing the differentiation of sensory progenitors to hair cells. If this hypothesis is true, we expected to find increased numbers of hair cells in her4-MO embryos. Intriguingly, the number of cells expressing atoh1a at 24 hpf and 28 hpf, as a readout of committed hair cells did not increase in her4-MO (Figure 6G–F) and was similar to controls. In few injected embryos, we found a reduction in the levels of atoh1a (n = 5/13).
Discussion

Here we have explored the role of her4 in inner ear development and its relationship with proneural genes and Notch signalling. In the neurogenic domain her4 and neurog1 expressions are correlated spatially, with a temporal delay between neurog1 induction and her4, suggesting an intermediate step. The fact that in the neurogenic domain her4 expression depends on Notch, positions Notch as the intermediary pathway that activates her4 downstream of neurog1. Moreover, depletion of her4 leads to an increase in the population of neurons. This is similar to what was previously reported for her4 role in primary neurogenesis [19]. Neither the loss of function of her4 nor Hes5 has already been analysed directly in inner ear neurogenesis (all studies being focused on hair cell development). The data demonstrate for the first time that in the inner ear, as in the CNS, her4 participates in Notch-mediated lateral inhibition to control the final number of neuronal cells.

But what happens in the sensory domain? There, her4 is regulated differently. In the presumptive sensory territory her4 expression is highly dynamic and identical to atoh1b. It initially encompasses a broad medial territory of the otic placode to progressively restrict to the future anterior and posterior maculae. Initial her4 expression requires atoh1b and Fgf signalling but not Notch, indicating that it cannot be assumed that her4 is always regulated by Notch. Nonetheless, in an intermediate development period, in the CMD Notch regulates negatively but not positively her4 in the CMD. Our work thus shows that her4 is not the downstream target of Notch to repress atoh1b expression. her6, another member of her repressors, cannot perform this role since it is not expressed in the CMD at 12.5–13 hpf (Figure S1). Thus, it still remains elusive how Notch represses atoh1b in the CMD to obtain two segregated the sensory patches.

Notch, in addition to her4, also down-regulates atoh1b in the CMD [7]. Since her4 expression depends on atoh1b, we propose that the effect of Notch on her4 is most probably mediated by atoh1b. However, we cannot exclude that Notch inhibits her4 directly in the CMD in parallel to atoh1b. At later stages, her4 persists at the sensory maculae requiring Notch-activity. Interestingly, by 16 hpf, her4 expression levels appear higher than atoh1b.
Figure 6. Blockade of her4 has no increase on atoh1b and atoh1a expression. (A–B) Dorsal views of 14 hpf control (A) and her4-MO injected (B) embryos stained by in situ hybridization for atoh1b. atoh1b expression is not affected in the prosensory domains in her4-MO injected embryos. (C–D) Dorsal views of 24 hpf control (C) and her4-MO injected (D) embryos stained by in situ hybridization for atoh1a. atoh1a expression decreases in her4-MO injected embryos. (E–F’) Transversal sections anterior (E, F) and posterior sections (E’, F’) of 28 hpf control (E, E’) and her4-MO injected (F, F’) embryos stained by in situ hybridization for atoh1a. atoh1a expression is as wild-type in her4-MO injected embryos. Dashed circles delineate otic vesicles. All images are at same magnification. Scale bar: 25 μm.

doi:10.1371/journal.pone.0109860.g006

suggesting that from this period onwards, her4 expression can be maintained independently of atoh1b. This coincides with the period of atoh1a activation and probably also Notch pathway. Thus, we propose that by 16 hpf, her4 regulation changes from a direct regulation by the proneural atoh1a to a regulation by atoh1a and Notch.

The complex spatiotemporal regulation of her4 expression suggests multiple cis-regulatory regions controlling her4 transcription. Yet no data exists on the regulatory regions of this locus. The promoter of mouse Hes5 has been characterized [38] and, in chick, labels cells responding to Notch and undergoing lateral inhibition during hair cell formation [39]. Here we show evidences for a Notch independent regulation of her4, not been described yet in chick and mouse. This might be due to a lack of data at early developmental times or to a divergence in the regulatory mechanisms from amniote to anamniote animals.

The role of Notch in hair cell development has been widely studied. The best known role of Notch in hair cell development is in the process of lateral inhibition, in which cells activated by Notch activate target genes and suppress hair cell fate versus supporting cell fate [8,10,40–42]. In agreement with a putative role of her4 downstream of Notch activity during sensorigenesis, we expected to observe an increase in the number of hair cells after her4 knockdown. However, we failed to observe increased numbers of atoh1a expressing cells. This is not due to inefficacy of her4-MO since it was tested in Tg(her4:EGFP)y83 embryos and also it resulted in expansion of deltaB expression. One of the most plausible explanations is that contrary to what happens in the neurogenic domain, other her genes compensate for her4 loss in the sensory domain. Her6, a Hes1 ortholog, is expressed exclusively in the sensory domain already from 12.5 hpf (Fig. S1) could act redundantly with her4. The presence of her6 in the sensory patches but not in neurogenic domain could explain why depletion of her4 has a stronger effect in the latter. This is in agreement with data in mouse, where Hes and Hey genes cooperate in hair cell development and a graded increase in hair cell number was related to a reduction in Hes/Hey dosage, being the highest increase in compound mutants for Hes1, Hes5 and Hey1 [18].

In chick, the transcription of atoh1 lags by almost 2 days the expression of neurog1 in the anteroventral domain of the otic placode [31,32]. Therefore, the specification of otic neurons as judged by the induction of neurog1 proceeds hair cell specification. Moreover, hair cells from the utricle and saccule derive from a neurog1-positive domain in mice [43]. In chick, a clonal relation between sensory neurons and utricular epithelial cells was also

Figure 7. Sequential steps in her4 activation and its regulation during sensori- and neurogenesis. (A) In the sensory domain her4 expression is detected at 12 hpf in a broad medial territory of the otic placode and, at this early stage, it requires atoh1b and FGF signalling. (B) By 13 hpf her4 gets restricted to the future anterior and posterior sensory domains being down-regulated in the CMD by Notch activity. (C) At later stages, her4 expression requires Notch in order to be maintained in the future maculae. (D) In the neurogenic domain neurog1 activates Notch, which in turn activates her4 during lateral inhibition. Notch*: Notch active. her4 expression in dark (sensory domain) and light (neurogenic domain) violet.

doi:10.1371/journal.pone.0109860.g007
found [44]. Together with a clonal relationship between neurons and hair cells, mutual antagonism between atoh1 and neurog1 has been shown [43,45,46]. In Neurog1^-/- mouse embryos expansion of hair cells in the future utricle was observed, conversely increased number of neuronal cells was detected in Atoh1 mutants [43]. We show that zebrafish atoh1b and atoh1a are induced before neurog1. Whether this discrepancy has any functional relevance is still not known. Disruption of neurog1 by MO injection caused an expansion of hair cells from the posterior macula [35]. This was further confirmed in the present study, since neurog1 mutants also display an increase on the expression of her4 only in the posterior macula.

However, we also explored neurog1 expression after blockade of atoh1b, as the first proneural gene defining the prosensory domain. Our data suggests that loss of atoh1b proneural activity does not modify neurog1 expression, suggesting that the definition of the neurogenic domain is not influenced by atoh1b proneural gene. However, since two atoh1 genes are present in zebrafish, further work deleting both atoh1b and atoh1a genes should provide better insights into proneural cross-regulation between sensory and neurogenic fates.

In conclusion, we show here that both, neurog1 and atoh1b proneural genes, are main regulators of her4 in the neurogenic and sensory domains. In the first, her4 is involved in lateral inhibition to regulate the balance between neuronal precursors and differentiating neuronal cells, whereas in the latter, her4 alone cannot regulate the number of hair cells during sensorogenesis, most probably helped by her6. Furthermore, in the sensory domain, Notch only acts upstream of her4 at late stages but initial her4 expression is downstream of atoh1b. Instead, at the neurogenic domain, the genetic cascade differs and neurog1 first activates Notch, which in turn activates her4 (Figure 7).

### Supporting Information

**Figure S1** Her6 is expressed in similar domains than her4. Her6 expression is restricted to the anterior and posterior sensory domains from its onset and is not induced at the CMD. (TIF)

### Acknowledgments

We want to thank Dora Saépe and laboratory members for helpful comments on the paper. We also thank colleagues for providing zebrafish strains and Morpholinos used here: Laure Bally-Cuif for her probes and the Tg(her1:EGFP), Patrick Blader for the neurog1^TIG1059, Hernán López-Schier for the mβ^257b and Bruce Riley for atoh1b morpholino. Marta Linares and Mi quel Sax for technical assistance. The authors declare no competing financial interests.

### Author Contributions

Conceived and designed the experiments: MR LF BA. Performed the experiments: MR LF. Analyzed the data: MR LF BA. Contributed reagents/materials/analysis tools: BA. Wrote the paper: BA LF MR.

### References

1. Jarman AP, Grau Y, Jan LY, Jan YN (1993) atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. Cell 73: 1307–1321.

2. Jarman AP, Sun Y, Jan LY, Jan YN (1995) Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors. Development 121: 2019–2030.

3. Shaham L, Lanford PJ, Dolinsky CM, Norton CR, Gridley T, et al. (1999) Expression of proneural and neurogenic genes in the embryonic mammalian vestibular system. J Neurosci 28: 809–819.

4. Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, et al. (1999) Contributions of neurog1 proneural gene. Dev Biol 215: 291–306. doi:10.1006/dbio.1999.9304.

5. Zheng JL, Gao WQ (2000) Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nat Neurosci 3: 500–506. doi:10.1038/75755.

6. Woodh C, Monteconquio M, Kelley MW (2004) Math1 regulates development of the sensory epithelium in the mammalian cochlea. Nat Neurosci 7: 1130–1138. doi:10.1038/nrn1499.

7. Millenikki BB, Sweet EM, Dhasil MS, Riley BB (2007) Zebrafish atoh1 genes: classic proneural activity in the inner ear and regulation by Fgf and Notch. Development 124: 595–595. doi:10.1242/dev.02734.

8. Haddon AP, Sun Y, Jan LY, Jan YN (1995) Role of the proneural gene, atonal, in the development of the Drosophila peripheral nervous system. Development 125: 359–370.

9. Adam J, Myat A, Le Roux I, Edmondson M, Henrique D, et al. (1998) Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with Drosophila sensoneurogenic development. Development 124: 4645–4646.

10. Lanford PJ, Lan Y, Jiang R, Lindell C, Weimaster G, et al. (1999) Notch signalling pathway mediates hair cell development in mammalian cochlea. Nat Genet 21: 209–292. doi:10.1038/60894.

11. Mia Q, Chen Z, del Barco Barrantes I, de la Pompa JL, Anderson DJ (1998) Neurogenin1 is Essential for the Determination of Neuronal Precursors for Proximal Cranial Sensory Ganglia. Neuron 20: 469–482.

12. Andermann P, Ungrats J, Raphael DW (2002) Neurogenin1 defines zebrafish cranial sensory ganglia precursors. Dev Biol 253: 1–5.

13. Abello G, Khatri S, Giraldez F, Alsina B (2007) Early regionalization of the otic placode and its regulation by the Notch signaling pathway. Mech Dev 124: 631–645. doi:10.1016/j.mod.2007.04.002.

14. Daudet N, Ariza-McNaughton L, Lewis J (2007) Notch signalling is needed to maintain, but not to initiate, the formation of pro-neuronal patches in the chick inner ear. Development 134: 2369–2376. doi:10.1242/dev.001842.

15. Murata J, Tokunaga A, Okano H, Kubo T (2006) Mapping of atoh1 gene activation during cochlear development in mice: implications for determination of
of oscillation in medaka. Dev Genes Evol 216: 315–332. doi:10.1007/s00427-006-0059-6.

30. Radosevic M, Robert-Moreno A, Coolsen M, Bally-Cuif L, Alsina B (2011) Her9 represses neurogenic fate downstream of Tbx1 and retinoic acid signaling in the inner ear. Development 138: 397–408. doi:10.1242/dev.056093.

31. Pujades C, Kamaid A, Abello G, Ulhoa E, Henrique D, Pujades C, et al. (2006) BMP-signaling regulates the generation of hair-cells. Dev Biol 292: 53–67. doi:10.1016/j.ydbio.2006.01.001.

32. Alsina B, Abello G, Ulhoa E, Henrique D, Pujades C, et al. (2004) FGF signaling is required for determination of otic neuroblasts in the chick embryo. Dev Biol 267: 119–134. doi:10.1016/j.ydbio.2003.11.012.

33. Bae YK, Shinmin T, Hibi M (2005) Patterning of proneuronal and inter-proneuronal domains by hairy- and enhancer of split-related genes in zebrafish neuroectoderm. Development 132: 1373–1385. doi:10.1242/dev.01710.

34. Scholpp S, Delogu A, Gilthorpe J, Peukert D, Schindler S, et al. (2009) Her6 regulates the neurogenic gradient and neuronal identity in the thalamus. Proc Natl Acad Sci U S A 106: 16424–16434. doi:10.1073/pnas.0906994106.

35. Supp B, Dbyalla S, Pujades C (2002) Cell lineage analysis reveals three different progenitor pools for neurosensory elements in the otic vesicle. J Neurosci 32: 16424–16434. doi:10.1523/JNEUROSCI.3685-12.2012.

36. Chitnis AB (1995) The role of Notch in lateral inhibition and cell fate specification. Mol Cell Neurosci 6: 311–321.

37. Lewis J (1998) Notch signalling and the control of cell fate choices in vertebrates. Semin Cell Dev Biol 9: 583–589. doi:10.1006/scdb.1998.0266.

38. Takebayashi K, Akazawa C, Nakamichi S, Kogaysama R (1995) Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-5. Identification of the neural precursor cell-specific promoter element. J Biol Chem 270: 1342–1349.

39. Chrysostomou E, Gale JE, Daudet N (2012) Delta-like 1 and lateral inhibition during hair cell formation in the chicken inner ear: evidence against cis-inhibition. Development 139: 3764–3774. doi:10.1242/dev.074476.

40. Kiernan AE, Cordes R, Kopan R, Gessler A, Gridley T (2005) The Notch ligands Dll1 and Jag2 act synergistically to regulate hair cell development in the mammalian inner ear. Development 132: 4353–4362. doi:10.1242/dev.02002.

41. Brooker R, Honnmi K, Lewis J (2006) Notch ligands with contrasting functions: Jagged1 and Deltal in the mouse inner ear. Development 133: 1277–1286. doi:10.1242/dev.02204.

42. Takebayashi S, Yamamoto N, Yabe D, Fukuda H, Kojima K, et al. (2007) Multiple roles of Notch signaling in cochlear development. Dev Biol 307: 165–176. doi:10.1016/j.ydbio.2007.04.035.

43. Rall S, Koundakjian EJ, Quinones H, Javasena CS, Goodrich LV, et al. (2007) Cross-regulation of Ngn1 and Math1 coordinates the production of neurons and sensory hair cells in the inner ear development. Development 134: 4405–4415. doi:10.1242/dev.009118.

44. Satoh T, Tottori DM (2005) Clonal analysis of the relationships between mechanosensory cells and the neurons that innervate them in the chicken ear. Development 132: 1687–1697. doi:10.1242/dev.01730.

45. Matei V, Pauley S, Kiiing S, Rowitch D, Beisel KW, et al. (2005) Smaller inner ear sensory epithelia in Neurog 1 null mice are related to earlier hair cell cycle exit. Dev Dyn 234: 623–630. doi:10.1002/dvdy.20551.

46. Jahan I, Pan N, Kerstogi J, Calisto LE, Morriss KA, et al. (2012) Expression of Neurog1 instead of Atoh1 can partially rescue organ of Corti cell survival. PLoS One 7: e30853. doi:10.1371/journal.pone.0030853.