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Atoh1 in sensory hair cell development: constraints and cofactors

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Abstract

The proneural gene, Atoh1, is necessary and in some contexts sufficient for early inner ear hair cell development. Its function is the subject of intensive research, not least because of the possibility that it could be used in therapeutic strategies to reverse hair cell loss in deafness. However, it is clear that Atoh1's function is highly context dependent. During inner ear development, Atoh1 is only able to promote hair cell differentiation at specific developmental stages. Outside the ear, Atoh1 is required for differentiation of a variety of other cell types, for example in the intestine and cerebellum. The reasons for this context dependence are poorly understood. So far, the pathways and key players that instruct Atoh1 to act as a mechanosensory cell fate determinant in the context of the inner ear are largely unknown. Here we review evidence that suggests that Atoh1 function in hair cell differentiation is modulated by interaction with other transcription factors. We particularly focus on the possible roles of Gfi1 and Pou4f3, drawing from studies in mouse, *Drosophila* and *C. elegans*.

1. Introduction

The conversion of mechanical stimuli into neuronal signals is achieved by mechanosensory cells. Senses like hearing, balance, proprioception and touch rely on these specialized mechanoreceptors, which display unique physiological characteristics not observed in any other cell type. Despite their specialized function (1), mechanosensory cells exhibit a great diversity in terms of shape, structure and cell type across animal phyla. The mechanoreceptors that have evolved to detect sound and proprioceptive movement in invertebrates and vertebrates are a good example of such diversity. In *Drosophila*, ciliated sensory neurons found in chordotonal organs (CHOs) such as the Johnston's organ (the fly's antennal hearing apparatus) detect sound and movement through a mechanotransduction unit located in its sensory cilium (2, 3). In contrast, the vertebrate's auditory function is mediated by an epithelial sensory receptor (known as the hair cell) that lacks an axon and is innervated by non-mechanosensory neurons. Additionally, the mechanotransduction machinery in vertebrate hair cells is located in actin-rich extensions (modified microvilli) called stereocilia rather than the true cilia of the fly chordotonal sensory neurons. Hair cells are located in the inner ear's vestibular system (for proprioception) and cochlear organ of Corti (for auditory function).

Regardless of their obvious anatomical differences, these fly and vertebrate mechanosensory cells share striking similarities in the pathways that regulate their development, particularly in the deployment of conserved transcriptional regulators (see Box 1). The *Drosophila* bHLH transcription factor Atonal and its vertebrate homolog Atoh1 play a critical role in the commitment of progenitors towards these mechanosensory fates. The role of Atonal/Atoh1 in mechanosensory cell development has been the subject of several excellent reviews (4-7) and we merely summarize here. Loss of Atonal and Atoh1 function leads to the absence of CHO neurons and hair cells, respectively, (8-10) while ectopic Atonal and Atoh1 expression causes ectopic CHO and hair cell formation (8, 11, 12). Importantly, the exchange of Atonal for its vertebrate homolog Atoh1 rescues fly CHOs development and the reverse experiment in the mouse shows that fly Atonal can also rescue hair cell development (13, 14). This remarkable functional conservation raises the possibility that Atonal and Atoh1 share conserved molecular interactions with common components of their gene regulatory networks as they drive mechanosensory differentiation.

Not all mechanosensory cells are, however, specified by the Atonal family. In *Drosophila*, the generation of external sensory organs (bristles) responsible for touch, requires the activity of bHLH transcription factors of the *achaete-scute* complex (15) (see Box 1). In vertebrates, Atoh1's function is confined to two different mechanosensory cell types: I) the above-mentioned hair cells, found both in the inner ear and in some animals also found in the lateral line and paratympanic organ (13, 16, 17); II) the epidermal Merkel cells necessary for encoding light touch responses (18). Thus, Atonal/Atoh1 govern the formation of only a subset of mechanosensory cells, but seem to have a particularly important conserved function in those cells that mediate hearing and balance.

The ability of Atoh1 to induce ectopic hair cell formation raises the hope that it can be used therapeutically to replace hair cells lost in sensorineural deafness. However, there are caveats to this approach. Firstly, the pro-differentiation activity of Atoh1 appears strictly limited to early stages of inner ear development. Secondly, Atoh1 genes are expressed in other tissues such as the cerebellum and the intestine where they instruct the specification and differentiation of neuronal and secretory cells respectively (Table 1). It is not well understood how this same transcription factor can play an instructive role in generating these different cell types. Here we review what is known of the context dependent activities of Atonal/Atoh1, and consider the implications for understanding the molecular mechanisms of hair cell differentiation.

2. Context dependence of Atoh1

Studies in a variety of rodent genetic models have established that misexpression of Atoh1 can induce formation of supernumerary hair cells in the inner ear. However, this ability is confined to early developmental stages, with the competence of the inner ear epithelium to respond to Atoh1 strongly tapering off shortly after birth (19, 20). A second temporal aspect is that although Atoh1 is best described for its role in early hair cell specification, temporally controlled knock-out studies have suggested that it is also involved in later aspects of hair cell differentiation and survival (10, 21). These observations imply that Atoh1 has multiple roles at different stages of development, suggesting that its functions (and perhaps target genes) change over time.

Outside of the inner ear, genetic experiments in the mouse have revealed that Atoh1 is necessary, and in some cases sufficient, for the specification of particular neuronal lineages, including dorsal interneurons in the spinal cord and granule neurons in the cerebellum, plus some non-neural cell types including the secretory cells in the intestine (Table 1). Atoh1 is, therefore, a master regulator of distinct lineage-specific differentiation programs, further highlighting the fact that its activity and target genes are dependent on the developmental context.

The idea that Atoh1 regulates distinct targets in different cell types has been borne out in recent studies that have employed ChIP-seq to determine Atoh1 binding sites in cerebellum granule neurons (22) and intestinal secretory cells (23). Both studies identified a similar Atoh1 DNA binding motif (brain: (G/A)(C/A)CA(G/T)(C/A)TG(G/T)(C/T) and intestine: CA(G/C)CTG(G/T)(C/T)) indicating that differences in cellular context do not appear to strongly affect Atoh1 preference for its unique E-box motif (Fig. 1A). This context-independent binding to cognate E-boxes has also been shown for the *Drosophila* Atonal protein (24). Despite this, the Atoh1 binding sites found in intestinal secretory cells show only a small overlap with those found in cerebellum granule neurons (951 out of 8729 detected in the gut) (23). So far, there are no data on Atoh1 binding sites in hair cells or dorsal interneurons: Atoh1 ChIP-seq experiments are problematic due to the very small numbers of these cell types (25, 26). Nonetheless, the studies in

intestine and cerebellum provide a good example of how cellular context is able to induce dramatic changes in Atoh1 DNA-binding site occupancy. This leads us to an important question: what modulates Atoh1 DNA-binding preferences in these different contexts? We do not yet have an answer to this question, although there may be much to learn from our understanding of the context-dependent activity of related proneural bHLH transcription factors (27). Below, we review the possible mechanisms that could explain context-dependent activity of Atoh1.

3. Potential mechanisms underlying Atoh1's context dependence

3. 1 Chromatin landscape and epigenetic modifications

Some critical regulators of cell differentiation have the capacity to bind closed chromatin and to promote chromatin accessibility, and these are often referred to as 'pioneer factors'. The proneural bHLH transcription factor Ascl1 (closely related to Atoh1) is known to be a pioneer factor, capable of binding to nucleosomal-occluded DNA (28, 29) and promoting chromatin accessibility at the regulatory regions of its targets (29). However, pioneer activity remains to be determined for Atoh1. To identify active and poised enhancers as well as tissue-specific chromatin access, techniques for genome-wide chromatin profiling, such as DNase1 hypersensitivity, have been applied to intestinal crypt progenitors in the presence or absence of Atoh1 (23). Enhancers normally bound by Atoh1 showed the same chromatin access and histone activation pattern in Atoh1 depleted crypt progenitors (23), indicating that Atoh1 does not control the initiation and maintenance of chromatin accessibility and epigenetic modification changes in intestinal progenitors. It therefore seems likely that chromatin remodeling precedes Atoh1 binding, raising the possibility that the chromatin landscape may have a prominent role in controlling Atoh1 activity. If this also applies to the inner ear, a temporally changing chromatin environment may govern the competence of the ear to respond to Atoh1 expression. It is also tempting to speculate that the cell type-specific chromatin environment determines Atoh1´s target specificity. However, pioneer activity can also be context-specific (30) (31), and so it remains possible that Atoh1 is a pioneer factor in the inner ear.

Another possibility is that pioneer factor function in hair cell specification is provided by Sox2. This factor functions upstream of Atoh1 in prosensory progenitor specification (7, 32, 33). It is known to affect epigenetic priming in B cell development (34) and it has pioneer activity during reprogramming of fibroblasts to pluripotency (35). In hair cell specification, Sox2 may not only activate Atoh1 expression, but also prime chromatin to direct Atoh1 binding to hair cell target genes.

3.2 Protein levels and post-translational modifications

Posttranslational modifications have been shown to regulate proneural activity, DNA-binding specificity and protein stability of several bHLH proteins (27, 36, 37). In the case of Atoh1, recent studies of its

function in neurogenesis found that serine phosphorylations are responsible for the control of Atoh1 activity and protein stability: phosphorylations outside the bHLH domain in the C-terminus of the Atoh1 protein mediate interaction with HUWE1 (an E3 ubiquitin ligase), which targets Atoh1 for proteasomal degradation (38). In the bHLH domain, a highly conserved serine phosphorylation was recently revealed to be a binary switch for proneural activity by preventing DNA binding (39). While these modifications affect activity, at present there is no evidence that they affect target specificity. Neither is it known whether they are relevant to the inner ear.

3.3 Interactions between transcription factors

At any given time and place during development, diverse intrinsic and extrinsic signals are integrated to result in a specific combinatorial expression of transcription factors. These 'combinatorial codes' of transcription factors help to explain context-dependence of transcription factor binding in many different cell types (40, 41). Therefore, the cell-type-specific cooperation between transcription factors at enhancer regions could explain, at least in part, why Atoh1 exhibits a distinct DNA-binding profile in neurons compared with intestinal cells. For hair cells, analysis of motifs in the enhancers of Atoh1 target genes supports the possibility of it working in combination with other transcription factors to define target gene specificity (42). Heterodimerisation partners also play a role. Class II bHLH proteins such as Atoh1 typically bind to DNA as heterodimers with members of the class I family of bHLH factors known collectively as E proteins. For the generation of the hindbrain's pontine nucleus, it appears that Atoh1 must interact with a specific E protein, Tcf4 (43). The mechanism by which Atoh1/Tcf4 heterodimers promote this specific cell fate is currently unknown, although specific heterodimer interactions have been shown to cause differences in DNA-binding preferences in the context of *Drosophila* mesoderm formation (44).

Recently, Gfi1 and Pou4f3, two transcription factors of known importance in hair cell differentiation (45-47) have emerged as possible candidates for interaction with Atoh1 to promote hair cell development. In a mouse embryonic stem cell model, forced Atoh1 expression induces neuronal differentiation. In striking contrast, forced expression of Atoh1 in combination with Gfi1 and Pou4f3 instructs the cells to commit towards a hair cell fate (48). Atoh1 is vital to this programming process: Gfi1 and Pou4f3 are not alone able to drive differentiation (AC, unpublished data). The presence of Gfi1 and/or Pou4f3 appears able to switch Atoh1's activity from a neuronal cell fate determinant to a hair cell determinant. Thus, Atoh1, Gfi1 and Pou4f3 appear to be central players in the genetic network that drives hair cell formation. Since these three factors have not been discussed together before in the context of specificity, we review what is known about Gfi1 and Pou4f3 in mechanosensory cell development and in development of other cell types in order to ascertain how these factors might be connected to each other.

4. Gfi1 and the GPS family of transcription factors

Growth Factor Independence 1 (Gfi1) is the vertebrate member of the GPS (Gfi1/PAG-3/SENS) family of zinc finger transcription factors. GPS proteins are characterized by the presence of zinc finger domains frequently found at their C-terminus (Fig. 1B). The N-terminus of Gfi1 harbours a SNAG transcriptional repressor domain also fund in Snail/Slug zinc finger proteins (49).

In vertebrates, Gfi1 is best known as a major regulator of hematopoiesis, playing a prominent role in the development of the myeloid and lymphoid cell lineages (Table 1). Here it controls diverse developmental processes, such as cell fate determination, differentiation, proliferation and cell survival (for review, see (50-54)). Outside the hematopoietic system, Gfi1 mRNA has been detected in a wide range of other tissues, but expression of Gfi1 protein seems to be restricted to mechanoreceptors cells (hair cells and Merkel cells), neurons of the cerebellum and retina, and specialized epithelial cells in the gut and lung (Table 1). Gfi1 plays a key role in the differentiation or survival of these non-hematopoietic cell types.

Research on the mechanism of transcription regulation mediated by Gfi1 has been largely restricted to hematopoietic development. These studies revealed that Gfi1 acts mainly as a transcriptional repressor by recruiting chromatin regulatory complexes such as histone demethylase complex (LSD1/CoRest), histone deacetylases (HDACs 1-3) and histone methyltransferase (G9a) (55-57). At Gfi1 DNA target sites, these corepressors and enzymes remove active histone modifications and apply repressive marks to prevent transcription and potentiate long-term changes in chromatin structure. The SNAG domain is necessary for the recruitment of the LSD1/CoRest complex whilst interactions with other histone-modifying enzymes (HDACs and G9a) are mediated via the intermediary and zinc-finger regions of the Gfi1 protein (56, 57). Despite these varied interactions, a single mutation in the SNAG domain, which disrupts the interactions with the LSD1/CoRest complex, leads to a phenotype apparently identical to that observed in *Gfi1* null mice in both hematopoietic and non-hematopoietic systems (57, 58). Further support for this mode of Gfi1 function comes from a recent study showing that LSD1 deficiency phenocopies the developmental arrest of the haematopoietic stem cells observed in Gfi1 null mice (59). In summary, the suppression of gene expression through LSD1/CoRest appears to be vital for Gfi1 function regardless of the tissue and developmental context.

4.1 Gfi1 in the vertebrate inner ear

Mouse genetic studies have shown that Gfi1 is required for hair cell development. The loss of *Gfi1* seems not to disrupt hair cell specification grossly as hair cells are formed and express early hair cell markers, such as Myo7a and Myo6 (47, 60, 61). However, these hair cells have defects that become increasingly apparent as differentiation progresses. In the cochlea, hair cells at E16.5 are already disorganized, and by E18.5 the outer hair cells show signs of apoptosis. In addition, inner and outer hair cells show stereociliary bundle defects, being shortened and poorly organized. Eventually, first outer hair cells then inner hair

cells are lost by apoptosis. By P14, all cochlear hair cells are lost (47, 60). In the vestibular apparatus, hair cells are morphologically abnormal from the earliest stages (E14.5) onwards, but are not lost by apoptosis.

It will be important to explore whether Gfi1 functions in the inner ear by recruiting LSD1/CoRest, as in the hematopoietic system. In addition, studies on a second family member, Gfi1b, indirectly suggest that Gfi1's intermediary domain may be important for its function in the context of the inner ear. Gfi1b is important in hematopoiesis but is not required in hair cell development. Interestingly, when the Gfi1b coding region is knocked-in to the mutated *Gfi1* locus, it can completely restore the defects in hematopoiesis, but not the defects in hair cell differentiation (58). Gfi1b shares high identity with Gfi1 in its SNAG and zinc finger domains and they are able to recognize the same DNA motif (62). This suggests that the functional difference arises from recruitment of proteins mediated by the intermediary region of the Gfi1 protein in the context of the inner ear.

4.2 How might Atoh1 and Gfi1 interact?

It is notable that Gfi1 and Atoh1 both function in hair cells, Merkel cells, and gut neurosecretory cells (Table 1). In hair cells, Gfi1 is a downstream target gene of Atoh1 (47). It could be that their interaction is simply one in which Atoh1 activates Gfi1 transcription, with Gfi1 subsequently functioning independently of Atoh1. Another possibility is that once activated Gfi1 enhances Atoh1 function by antagonising HUWE1-dependent degradation. In addition to these possibilities, work in flies suggests the intriguing possibility that the two proteins work together directly during hair cell development.

The *Drosophila* orthologue of Gfi1 – Senseless (Sens) – is expressed in several cell types, including all sensory neurons. In the peripheral nervous system the mutant phenotype of Sens resembles that of Gfi1 in the vertebrate inner ear in that sensory cells are specified, but fail to differentiate properly and eventually die (63). Like Gfi1, Sens acts as a DNA-binding-dependent transcriptional repressor (63, 64), but there is also strong evidence that Sens can activate transcription by promoting the activity of proneural bHLH transcription factors (Fig. 2). Sens directly binds to proneural proteins, including Atonal, via its Zn-finger domains (24, 64-66). This interaction enhances the activity of the bHLH factor at its target genes. One of the affected targets appears to be bHLH gene autoregulation itself. Sens is therefore thought to be an important regulatory switch for proneural bHLH expression and function during sensory precursor specification, on the one hand acting as a repressor, but also able to act as a co-activator. Conversely, the Atonal-Sens interaction also modulates the transcriptional repressor activity of Sens: during sensory precursor development, Atonal binding to Sens prevents it from repressing its target gene *rhomboid* (67).

Sens differs from Gfi1 in several aspects: it lacks a SNAG domain and only has four Zn-fingers (Fig. 1B). Nevertheless, it is possible that the protein interactions demonstrated in *Drosophila* sensory neuron

development are conserved in hair cell development. The ability of Atonal and Atoh1 to replace each other has already been mentioned. Moreover, mouse Atoh1 misexpressed in *Drosophila* can interact genetically with Sens, whereas a related vertebrate bHLH family transcription factor Neurogenin cannot, indicating some specificity for this interaction (68). Our unpublished experiments also suggest that Gfi1 can enhance Atoh1 transcriptional activity in a cell culture system (LP, unpublished).

More generally, mouse Gfi1 is known to interact with other transcription factors in a variety of ways. It can synergistically associate with transcription factors through binding to adjacent DNA sites (69) or bind directly to particular transcription factors thereby inhibiting their activity (70, 71). Whilst these mechanisms are most commonly associated with repression, some reports have shown that such interactions can lead to transcriptional activation (72-74), and thus Gfi1 might be a context-dependent repressor or activator like Sens. Understanding whether transcription activation is an important aspect of Gfi1 function in its different contexts and what mechanisms control the switch between activation and repression will constitute the basis for inner ear future studies. Similarly to Sens, there is *in vitro* evidence that transcriptional activation by Gfi1 could be dependent on promoter sequence, concentration of Gfi1 protein and cellular context (75).

What are the opportunities for direct interaction between Atoh1 and Gfi1 in the inner ear? In one report that examines both proteins, Atoh1 and Gfi1 were found to be co-expressed in cochlear inner hair cells as early as E15.0, and in outer hair cells by E15.5 (76). Transcriptome analyses also support their coexpression during later stages of differentiation (77). The earliest expression of Atoh1 in the cochlea is around E13.5, a time when Gfi1 is not yet detectable (10). There is thus scope for Gfi1 to alter Atoh1's later activity and/or specificity, providing temporal context. In particular, Atoh1 activity between E13.5 and E15.5 is crucial for hair cell survival (10, 78). Given that Gfi1 is also required for hair cell survival at these stages (see above), one might speculate that Atoh1 prevents cell death by regulating Gfi1 expression at these stages, or alternatively that Atoh1 and Gfi1 cooperate in regulating survival. The time course of Atoh1/Gfi1's co-expression in hair cell development remains to be determined completely. Finally, it should be noted that in addition to differentiation and survival defects, the *Gfi1* mutant mouse seemed to develop fewer outer hair cells (47), suggesting that perhaps an early interaction between Atoh1 and Gfi1 in hair cell specification should not be ruled out.

5. The vertebrate POU-IV transcription factors

Pou4f3 is required for development of functional hair cells (45, 46, 79). Pou4f3 protein is a member of the POU-IV class of transcription factors. The POU-domain is essential for DNA-binding and is characterized by two distinct sub-domains separated by a non-conserved variable linker (Fig. 1C). All three members of the mammalian POU-IV class (Pou4f1, Pou4f2 and Pou4f3) share a high degree of sequence similarity between their POU domains and are able to bind to the same consensus DNA motif (80-82). The POU-IV

factors are expressed in overlapping patterns predominantly confined to sensory systems and the central nervous system (79, 80, 83, 84). Pou4f3 itself is expressed in several neuronal types, including dorsal spinal cord, dorsal root ganglia, retinal ganglion neurons, and it is the only POU-IV factor to be present in hair cells (85) (Table 1).

5.1 Pou4f3 expression and function in the vertebrate inner ear

Pou4f3 expression in the vertebrate inner ear appears to be mainly confined to hair cells. In the cochlea, the onset of Pou4f3 expression occurs at E14.5, prior to Gfi1 but in a similar basal-to-apical gradient (21, 46, 76). Moreover, *Gfi1* expression is affected in *Pou4f3* mutant mice (60). Therefore, some effects observed upon deletion of *Pou4f3* could result directly from the consequent loss of Gfi1 expression. There are indeed similarities between the *Pou4f3* and *Gfi1* mouse phenotypes. In both cases, hair cells show morphological defects, aberrant or absent stereocilia-like structures, abnormal localization of vestibular hair cells in the supporting cell layer, and hair cell degeneration (46, 47, 60). The severity of the phenotype caused by the loss of *Pou4f3*, however, is more pronounced: unlike *Gfi1*, deletion of *Pou4f3* leads to apoptosis not only of cochlear hair cells but also of vestibular hair cells (45, 46). Moreover, signs of apoptosis appear earlier, being more prominent already at embryonic stages, and those stereocilia-like structures that do form have more pronounced morphological defects than those in the Gfi1 knockout mouse (60).

In the vestibular sensory epithelium (the earliest site of hair cell formation in the inner ear) Pou4f3 and Gfi1 are both present in incipient hair cells as early as E12.5, which precedes the expression of other early hair cells markers (46, 47). Given this early co-expression in vestibular hair cell precursors one might speculate that Pou4f3 (and Gfi1) cooperates with Atoh1 to promote hair cell formation in addition to later roles in hair cell differentiation and maintenance. However, just as in the *Gfi1* knockout mice, hair cells do undergo initial differentiation upon *Pou4f3* deletion (45, 46, 79), suggesting that any early hair cell function must be subject to compensatory mechanisms.

It seems plausible that Pou4f3 may also interact with Atoh1. Of note is the observation that the related POU-III class factor, Pou3f2 cooperates with the bHLH factor Ascl1 to activate transcription of the Notch ligand Dll1 during neurogenesis (86). Moreover, evidence from *C. elegans* shows a regulatory interaction between Atonal-like and Pou4f3-like factors in mechanosensory cells, although a physical interaction has not been demonstrated. The Atonal-like bHLH protein, Lin-32, is necessary to activate expression of the Pou-IV factor, Unc-86, in most touch neuroblasts, which is consistent with the function of its vertebrate homologue in hair cells (Fig. 1C) (87). In addition Unc-86 collaborates (indirectly) with the Gfi1 homologue, Pag-3, in the differentiation of BDU neurons (88). In contrast to *C. elegans*, the *Drosophila* Pou-IV factor, Acj6 is exclusively expressed in the sensory and nervous systems but it appears not to be required during mechanosensory development (89-91).

In addition to these interactions, Unc-86 interacts with the Lim homeodomain (Lim-HD) transcription factor Mec-3 to synergistically co-regulate downstream targets necessary for touch receptor differentiation (92, 93). In vertebrates, cooperative interactions between POU-IV (Pou4f1) and Lim-HD (Islet1) factors have been shown to be important for differentiation of retinal ganglion, trigeminal, and dorsal root ganglion neurons (94, 95). For hair cells, the Lim-HD factor Lhx3 is specifically expressed at their early stages of differentiation, leading to the possibility that it interacts with Pou4f3 in this context. *Lhx3* null mice show no phenotype in the inner ear (61), but there is the possibility of redundancy with other Lim-HD factors that are known to be present in hair cells, although their function during inner ear development has never been studied (96).

6. Conclusions

Atoh1, Gfi1 and Pou4f3 play critical roles in hair cell development, but the mechanisms by which they cooperate remain to be explored. The expression pattern and the loss-of-function phenotype of Atoh1 suggest that it initially acts upstream of Gfi1 and Pou4f3 in the genetic network of hair cell differentiation. However, once all three factors are expressed together, there is abundant opportunity for them to interact and modulate each other's function, perhaps generating context specificity.

It is attractive to suggest that cooperation with Gfi1 and Pou4f3 at later stages of hair cell differentiation might explain how Atoh1 can be required for temporally distinct functions in hair cell specification and differentiation. Moreover, despite differences in their mouse mutant phenotypes, it should perhaps not be ruled out that the factors interact during hair cell specification. This idea is strongly supported by the remarkable observation that Gfi1 and Pou4f3 appear to confer specificity on Atoh1 for driving hair cell differentiation from pluripotent cells (48). It will be of great interest to explore how this relates to the interactions between these three factors during development *in vivo*. It is notable that in the *Gfi1* knock out mouse, neuronal markers were aberrantly expressed in the outer hair cells (47). This is strongly reminiscent of the observation that in ES cells Atoh1 in the absence of Gfi1/Pou4f3 induces neuronal differentiation, supporting the idea that at least Gfi1 may influence Atoh1 specificity.

In this review we have drawn from the broader literature to suggest a number of possible biochemical and transcriptional mechanisms by which Gfi1, Pou4f3, and Atoh1 might cooperate during the development of mechanosensory cells. The paucity of hair cells in the inner ear has until now hampered progress in this area because it is difficult to perform genome-wide studies and biochemical analysis on such small numbers of cells. The recently developed transcriptional programming strategy (48) provides a much-needed opportunity to access and interrogate this important developmental transition in a more experimentally tractable system. This may bring some clarity to the intriguing question of how

Atonal/Atoh1 conserves the ability to drive mechanosensory cell differentiation across invertebrate and vertebrate species, yet is able to direct differentiation of several distinct cell types in different tissues within the same organism.

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Box 1: Development of mechanosensory cells in flies and mouse

A) At the onset of *Drosophila* sense organ development, clusters of ectodermal cells are marked by the expression of proneural genes, such as those of the *achaete–scute* complex (AS-C) or *atonal* (Ato). The Sensory Organ Precursor (SOP) is subsequently selected from the cluster by accumulating high levels of proneural protein, which activates the transcription program for sense organ development. The SOP divides and the progeny differentiate into the ciliated mechanosensory neuron (N) and supporting cells (S) of bristle and chordotonal organs (the fly's mechanotransduction organs). The upregulation of proneural genes in the SOP and their downregulation in neighboring cells is mediated by the Notch signaling pathway. Notch activation caused by Delta binding, results in a downregulation of proneural genes in Notch expressing cells which prevents their differentiation into a SOP. Subsequently, Notch signaling is also involved in cell fate determination of the different cell types of the sense organs.

B) The auditory sensory epithelium in vertebrates arises from the otic placode, an ectodermal thickening that lies on either side of the posterior hindbrain. Expression of Sox2 marks sensory domains in the otic placode where a neurogenic event occurs prior to differentiation of hair cells and supporting cells. In mouse, upregulation of the bHLH proneural gene Neurogenin1 (Ngn1) is required for determination of neuronal fate. These neural committed cells prevent their neighbours from developing into neuroblasts via Notch signaling. Next, several prosensory patches are specified (regions where cochlear and vestibular epithelia will develop). Sox2 is expressed and plays an important role in the specifications of these domains. Subsequently, the expression of the bHLH Atoh1 in the prosensory patch initiates the hair cell differentiation program. This causes the upregulation of Notch ligands (Dll1, Jag2), and consequently, Notch activation in neighouring cells, which ultimately differentiate into supporting cells. Similar the fly mechanosensory organs, proneural genes and Notch pathway regulate cell fate decisions that are necessary for the development hair cell, supporting cells and neurons.

[Figure.Box to be inserted here]

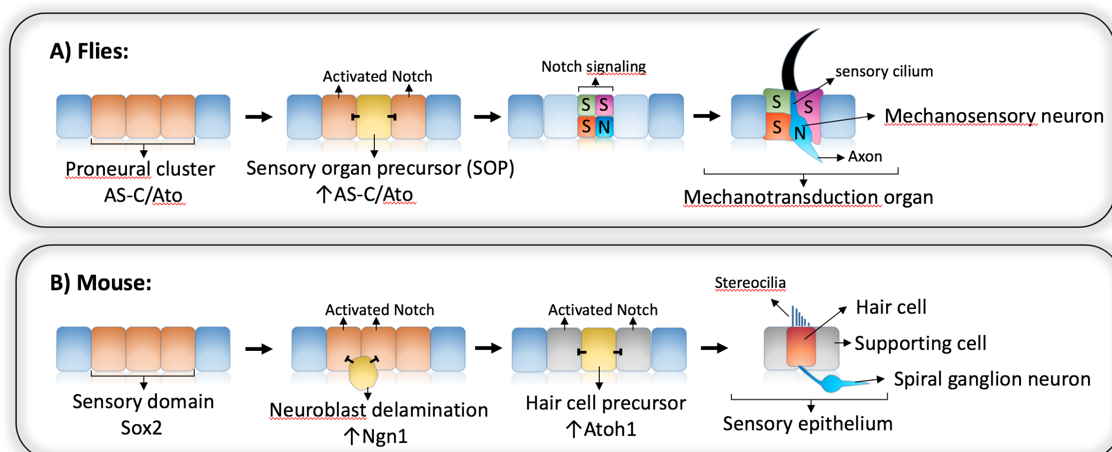
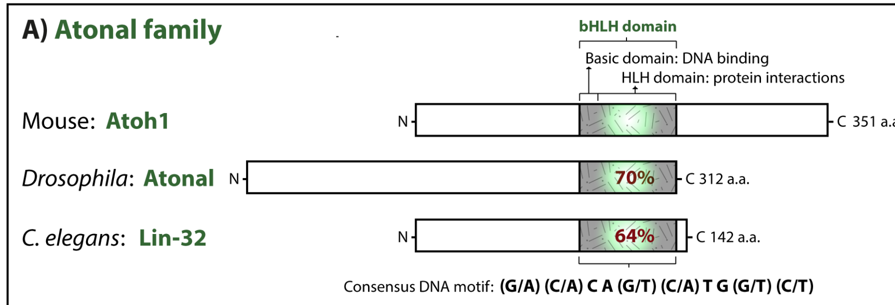
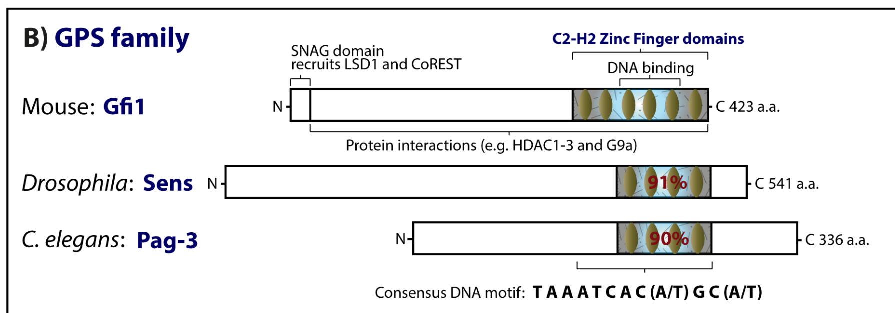
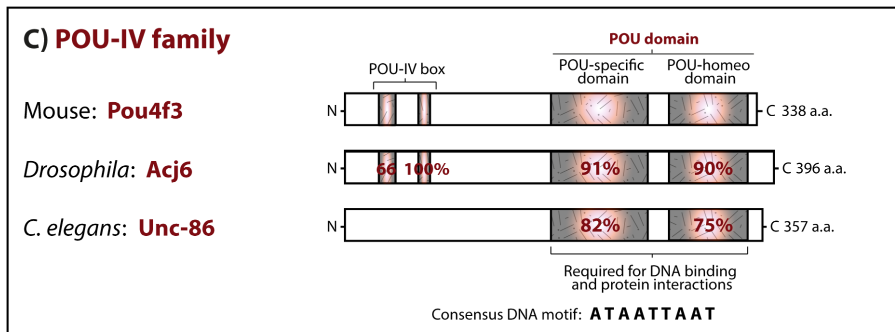


Figure Legends

Figure 1: Schematic representations of the Atonal (A), GPS (B) and POU-IV (C) transcriptional factor proteins found in mouse, *Drosophila* and *C. elegans*.







Conserved region

Non-conserved region

% of identity relative to mouse a.a. sequence

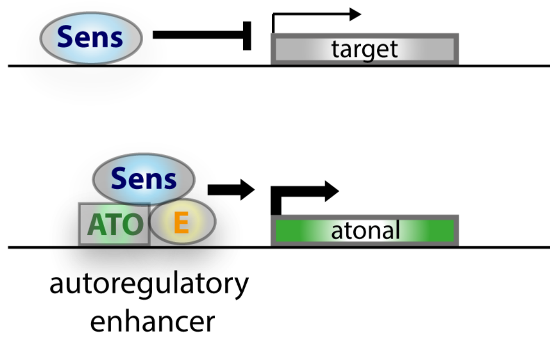


Figure 2: Schematic summary of the ability of *Drosophila* Sens to act as a transcriptional repressor when bound to DNA, and as a transcriptional co-activator when bound to Atonal or other bHLH proneural factors. When acting as a co-activator, Sens is proposed to stimulate proneural gene autoregulation, thereby promoting sensory precursor specification. E is the bHLH E protein dimerisation partner.