Development 130, 221-232

doi:10.1242/dev.00190

DEVELOPMENT AND DISEASE

The zinc finger transcription factor *Gfi1*, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival

Deeann Wallis[1,2], Melanie Hamblen[3], Yi Zhou[1], Koen J. T. Venken[4], Armin Schumacher[1,4], H. Leighton Grimes[5], Huda Y. Zoghbi[1,2,4,6], Stuart H. Orkin[3] and Hugo J. Bellen[1,2,4,*]

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA
[2]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, USA
[3]Division of Hematology/Oncology, Howard Hughes Medical Institute, Children's Hospital and the Dana Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA
[4]Program in Developmental Biology, Baylor College of Medicine, Houston, TX 77030, USA
[5]Institute for Cellular Therapeutics and Department of Surgery, University of Louisville, Louisville, KY 40202, USA
[6]Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA
*Author for correspondence (e-mail: hbellen@bcm.tmc.edu)

Accepted 4 October 2002

SUMMARY

***Gfi1* was first identified as causing interleukin 2-independent growth in T cells and lymphomagenesis in mice. Much work has shown that *Gfi1* and *Gfi1b*, a second mouse homolog, play pivotal roles in blood cell lineage differentiation. However, neither *Gfi1* nor *Gfi1b* has been implicated in nervous system development, even though their invertebrate homologues, *senseless* in *Drosophila* and *pag-3* in *C. elegans* are expressed and required in the nervous system. We show that *Gfi1* mRNA is expressed in many areas that give rise to neuronal cells during embryonic development in mouse, and that Gfi1 protein has a more restricted expression pattern. By E12.5 *Gfi1* mRNA is expressed in both the CNS and PNS as well as in many sensory epithelia including the developing inner ear epithelia. At later developmental stages, *Gfi1* expression in the ear is refined to the hair cells and neurons throughout the inner ear. Gfi1 protein is expressed in a more restricted pattern in specialized sensory cells of the PNS, including the eye, presumptive Merkel cells, the lung and hair cells of the inner ear. *Gfi1* mutant mice display behavioral defects that are consistent with inner ear anomalies, as they are ataxic, circle, display head tilting behavior and do not respond to noise. They have a unique inner ear phenotype in that the vestibular and cochlear hair cells are differentially affected. Although *Gfi1*-deficient mice initially specify inner ear hair cells, these hair cells are disorganized in both the vestibule and cochlea. The outer hair cells of the cochlea are improperly innervated and express neuronal markers that are not normally expressed in these cells. Furthermore, *Gfi1* mutant mice lose all cochlear hair cells just prior to and soon after birth through apoptosis. Finally, by five months of age there is also a dramatic reduction in the number of cochlear neurons. Hence, *Gfi1* is expressed in the developing nervous system, is required for inner ear hair cell differentiation, and its loss causes programmed cell death.**

Key words: Gfi1, Gfi1b, Senseless, PAG-3, Inner ear hair cell, Basic helix-loop-helix (bHLH), Deafness, Mouse

INTRODUCTION

Function of *Gfi1* and its homologs

The *Gfi1* gene was first identified as causing interleukin-2 (IL2) independent growth in T cells (Gilks et al., 1993) by allowing them to escape G_1 arrest normally induced by IL2 withdrawal (Grimes et al., 1996a) – hence the name growth factor independent (Gilks et al., 1993). Proviral integration in *Gfi1* results in upregulated transcriptional activity of *Gfi1* and is associated with lymphomagenesis in mice (Gilks et al., 1993; Liao et al., 1997; Scheijen et al., 1997; Schmidt et al., 1996). Gfi1 and its homolog Gfi1b (Tong et al., 1998) are nuclear zinc-finger proteins. Gfi1 functions as a position- and orientation-independent transcriptional repressor through its 20 amino acid N-terminal repressor, or SNAG, domain (Grimes et al., 1996a). Gfi1b appears to function biochemically in a similar manner to Gfi1, as it binds the same DNA recognition site and represses transcription through its SNAG domain.

Recent publications suggest a variety of in vivo functions for *Gfi1* and *Gfi1b*. High levels of Gfi1 transgene result in a block of T-cell lymphopoiesis (Schmidt et al., 1998a; Schmidt et al., 1998b). Constitutive *Gfi1* expression accelerates entry of resting T cells into S phase of the cell cycle (Karsunky et al., 2002a); and causes decreased levels of apoptosis, increased

levels of cell proliferation and a decrease in the levels of negative cell cycle regulators $p27^{KIP1}$ and pRb. *Gfi1* may also regulate apoptosis through repression of multiple pro-apoptotic regulators (Grimes et al., 1996b). Outside the lymphoid system, *Gfi1* is expressed in granulocytes and activated macrophages (H. Hock, M. J. Hamblen, H. M. Rooke, D. Traver, R. T. Bronson, S. Cameron and S. H. Orkin, unpublished) (Karsunky et al., 2002b). Loss-of-function studies in mice mutant for *Gfi1* indicate that it is necessary during hematopoesis as it is required for the proper specification and differentiation of neutrophils and macrophages (H. Hock, M. J. Hamblen, H. M. Rooke, D. Traver, R. T. Bronson, S. Cameron and S. H. Orkin, unpublished; Karsunky et al., 2002b). Overexpression of *Gfi1b* results in inhibition of G1 arrest and differentiation by directly binding the $p21^{WAF1}$ promoter and repressing its activity (Tong et al., 1998). *Gfi1b* can also directly repress the activity of tumor suppressor genes *Socs1* and *Socs3* by binding their promoters (Jegalian and Wu, 2002). The Gfi1b zinc-finger domain may also act as a transcriptional activation domain (Osawa et al., 2002). Thus, Gfi1b may modulate transcription as a repressor or activator depending on promoter and cell type context. Loss of function studies in the mouse indicate that *Gfi1b* function is required for hematopoiesis as it is required for erythroid and megakaryocytic lineages. Mice deficient for *Gfi1b* are embryonic lethal and die by E15.5 because of a complete lack of erythrocytes (Saleque et al., 2002).

The Gfi proteins have invertebrate homologues, including *senseless* in *Drosophila* (Frankfort et al., 2001; Nolo et al., 2000; Nolo et al., 2001) and *pag-3* in *C. elegans* (Cameron et al., 2002; Jia et al., 1996; Jia et al., 1997). In *Drosophila*, *senseless* is required during the development of the embryonic and adult peripheral nervous system (PNS). Embryos that lack *senseless* differentiate the majority of PNS cells, but most cells die through apoptosis (Nolo et al., 2000; Salzberg et al., 1997). However, in adult sensory organs, *senseless* is both necessary and sufficient for their development. Mosaic analysis in imaginal discs shows that *senseless* mutant clones lack sensory organs. Expression of *senseless* is dependent on the proper expression of proneural genes, such as *atonal, scute, achaete* and *daughterless*. *senseless* in turn is required for the upregulation and maintenance of expression of the proneural genes in the sensory organ precursors (SOP), as loss-of-function mutations in *senseless* abolish the further upregulation and maintenance of proneural gene expression in the SOPs. Ectopic expression of *senseless* induces ectopic proneural gene expression and ectopic PNS organs. In addition, *senseless* has been shown to synergize with the proneural genes (Nolo et al., 2000).

PAG-3 is a *C. elegans* homolog of Senseless and Gfi1. *pag-3* is involved in touch neuron gene expression and coordinated movement (Jia et al., 1996; Jia et al., 1997). In addition, null mutations of *pag-3* can result in abnormal patterns of apoptosis in the ventral nerve cord as well as abnormal differentiation of certain interneurons and motoneurons. Hence, *pag-3* functions in diverse contexts within the developing nervous system. The finding that *pag-3* is expressed in many neuronal subtypes at different points in development suggests that it cooperates with different factors to regulate the expression of cell type- and developmental stage-specific sets of genes to generate the complex pattern of neuronal subtypes seen in *C. elegans* (Cameron et al., 2002).

The above data suggest that this small but specific gene family of Zn-finger transcription factors plays similar as well as different roles in different species and different tissues. These include suppression of apoptosis, suppression of cell cycle checkpoints, as well as promoting cell fate determination and cell differentiation. However, in some lineages it is not obvious what the precise role of these proteins is.

Inner ear hair cell development

Mammalian inner ear hair cells function as mechanoreceptors to transduce sound and proprioreception. The morphology and development of the mammalian inner ear are very complex in nature. The major structures of the internal ear consist of the utricle and saccule, three semicircular canals, the cochlea, and the endolymphatic duct and sac. The sensory neuroepithelia are innervated by the eighth cranial nerve which consists of two parts, the vestibular and cochlear nerves. Vestibular hair cells are located in the macula of the saccule and the utricle, as well as in the cristae located in the semicircular canals. These hair cells fall into two types, Type I and Type II, and are innervated by the vestibular nerve. The hair cells of the vestibule detect linear acceleration and head position with respect to gravity. They are responsible for the sense of balance and proprioreception. The auditory hair cells in the organ of Corti located in the cochlea also fall into two categories, inner hair cells and outer hair cells. They are innervated by the cochlear nerve. These hair cells are responsible for auditory sensation. The membranous labyrinth of the inner ear first begins to form from the otic cyst and is visible in mice at E10.75. By E17.5 the gross anatomy of the inner ear is mature (Cantos et al., 2000). Many hearing impairments are caused by loss of sensory neurons and inner ear hair cells (for a review, see Petit et al., 2001). Hence, a better understanding of the genetic mechanisms responsible for the development of these structures may help us dissect the mechanisms implicated in hearing impairment or deafness.

A homology between hair cells in vertebrates and chordotonal organs in flies has been recently revealed (Hassan and Bellen, 2000). The bHLH proneural gene *atonal* was shown to be required for the specification of chordotonal SOPs (Jarman et al., 1993). These chordonal organs function as proprioreceptive organs and hearing devices (Eberl, 1999; McIver, 1985; van Staaden and Römer, 1998), much like the hair cells of the balance organs and the auditory system. As mentioned previously, Atonal is required for *senseless* expression in the SOP, and ectopic *senseless* expression induces *atonal* expression (H. J. B., unpublished). One of the mouse homologues of *atonal*, *Math1* (*Atoh1* – Mouse Genome Informatics) is expressed in the inner ear hair cells during development. *Math1*-null mice die shortly after birth and lack hair cells in balance organs and cochleae (Bermingham et al., 1999). Interestingly, all the defects associated with loss of *Math1* can be rescued by the fly *ato* gene, suggesting that they are orthologs (Wang et al., 2002). In addition, *Math1* overexpression has been shown to induce hair cell growth in inner ear epithelia (Zheng and Gao, 2000). Hence, *Math1*, like *ato*, is necessary and sufficient for hair cell development in vertebrates.

Given the similarities between *Math1* and *atonal* and the role of *senseless* in PNS development, we investigated the expression pattern and role of the *senseless* homolog, *Gfi1*, in

hair cell development. We find that *Gfi1* is expressed in many neuronal precursors as well as differentiating neurons during embryonic development. Consistent with these expression patterns, analysis of *Gfi1* function in inner ear development in a previously generated *Gfi1*-mutant line (H. Hock, M. J. Hamblen, H. M. Rooke, D. Traver, R. T. Bronson, S. Cameron and S. H. Orkin, unpublished) revealed that mutant hair cells are initially specified and express many hair cell markers, including *Math1*. However, *Gfi1* is required for proper differentiation and maintenance of inner ear hair cells. In *Gfi1* mutant mice, the vestibular and cochlear hair cells are morphologically abnormal, hair cell organization within the sensory epithelia is aberrant, the outer hair cells in the organ of Corti express a neuronal marker, and cochlear hair cells degenerate after separation. Thus, *Gfi1* is expressed in the developing nervous system and is required for the differentiation and survival of inner ear hair cells.

MATERIALS AND METHODS

Mice

Gfi1 mutant and *Math1* mutant mice were generated as previously described in 129/Sv × c57BL/6J backgrounds (Ben-Arie et al., 2000; H. Hock, M. J. Hamblen, H. M. Rooke, D. Traver, R. T. Bronson, S. Cameron and S. H. Orkin, unpublished). For some experiments, lines were crossed to yield mice heterozygous for *Math1*$^{\beta\text{-gal}}$ and *Gfi1* wild type or null.

Embryo staging and tissue preparation

Embryos were considered to be E0.5 days on the morning the vaginal plug was observed. To harvest the embryos, pregnant females were sacrificed by cervical dislocation and the embryos dissected out of the uterus. Regions of the yolk sac or tail were saved for genotyping. Embryos were fixed overnight in 4% paraformaldehyde, dehydrated in an ethanol series and embedded in paraffin wax for sectioning according to standard histological protocols. Sections (10 μm) were collected and analyzed by in situ hybridization or immunohistochemistry. Ear tissue for postnatal stages was collected by harvesting the temporal bones of the appropriately aged pup, fixing overnight in 4% paraformaldehyde, decalcifying in 1.35 N hydrochloric acid for at least an hour, dehydrating in an ethanol series and embedding in paraffin for sectioning. Sections (10 μm) were collected and analyzed by immunohistochemistry.

In situ analysis of *Gfi1*, *Math1* and *Brn3c*

The cDNA probe for *Brn3c* (*Pou4f3* – Mouse Genome Informatics) was kindly provided by Bill Klein. Probes for each of the genes were transcribed in the antisense direction and labeled with digoxigenin using the Dig RNA Labeling Kit from Roche. Probes were hybridized to paraffin sections and detected by anti-digoxigenin antibody coupled to alkaline phosphatase. Hybridization and stringent posthybridization wash steps were performed at 65°C.

Immunohistochemistry

Anti-Myosin VI/VIIa was kindly provided by Tama Hasson. Anti-TUJ1 was obtained from Babco. Anti-activated Caspase 3 was obtained from R&D Systems. An anti-Gfi1 antibody was generated in guinea pig. This antibody was raised against the domain of Gfi1 between the SNAG domain and the zinc fingers (amino acids 20-256), cloned into pET28a. This domain does not display homology to Gfi1b or other proteins. It is a specific nuclear antigen that is not present in *Gfi1* mutant mice and recognizes a specific band of the appropriate molecular weight on western blots of Gfi1-expressing yeast extracts and of mouse thymus protein extracts (data not shown). We used antibodies to Myosin, TUJ1 and Caspase 3 at a 1:1000 dilution, and Gfi1 at a 1:2000 dilution, and followed the ABC Vectastain directions with secondary anti-rabbit antibody (Myosin VI/VIIa and Activated Caspase 3), anti-mouse antibody (TUJ-1) or anti-guinea pig antibody (Gfi1) followed by DAB staining. Briefly, paraffin wax embedded sections were blocked in 1% H_2O_2 in methanol for 20 minutes at room temperature, rehydrated in a series of ethanols, boiled in citrate antigen retrieval solution in a microwave for 5-10 minutes, and blocked with horse serum (Vectastain) in PBS for 30 minutes at room temperature. Primary antibody was diluted in blocking solution and incubated on the section overnight at 4°C. Slides were rinsed in PBS and incubated in secondary for 30 minutes at room temperature. The slides were rinsed in PBS and incubated in Vectastain ABC solution for 30 minutes. The slides were again rinsed in PBS and the signal was detected with 2 mg/ml DAB, 0.02% H_2O_2 in PBS. Some slides were counterstained with Hematoxylin.

β-Gal staining

Mice heterozygous for the β-galactosidase cassette in the place of *Math1*-coding region were bred to *Gfi1* heterozygous mice to generate double heterozygotes, which in turn were crossed to obtain genotypes that were *Math1* heterozygous and either *Gfi1* wild type or *Gfi1* null. This allowed us to visualize hair cells in the *Gfi1* null mutants by staining the tissues for β-galactosidase. Appropriately staged mice were harvested, fixed briefly in 4% paraformaldehyde and stained overnight at 37°C for β-galactosidase according to established procedures (Ben-Arie et al., 2000). The tissue was then fixed overnight in 4% paraformaldehyde, and processed for paraffin wax embedding and sectioning or imaged immediately for whole mounts.

TEM

Staged cochleae were dissected and fixed in 0.1 M cacodylate buffer, 1% glutaraldehyde and 4% formaldehyde at 4°C for 2 hours. Specimens were then rinsed in 0.1 M cacodylate buffer and post fixed in 1% osmium tetroxide in cacodylate buffer at 4°C overnight. Samples were again washed in cacodylate buffer and rinsed with distilled water. Specimens were stained with 4% uranyl acetate for 3 hours and again washed in distilled water. Specimens were then dehydrated for 15 min each in a series of ethanols: 50%, 70%, 80%, 90%, 95% and 100% (twice), and then finally 100% overnight at room temperature. Samples were then rinsed in ethanol followed by rinsing in propylene oxide and embedded in scipoxy 812 resin with dodenyl succinate anhydride and nadric methyl anhydride. Semithin sections (0.5 μm) were obtained and then thin sections (50 nm) were obtained and grid stained with 4% uranyl acetate and 2.66% lead acetate and observed on an electron microscope.

RESULTS

Gfi1 is expressed in differentiating neurons and inner ear hair cells

As *Gfi1* and *Gfi1b* have not been reported to be expressed in neurons or neuronal precursors, we first searched for other *senseless* homologues that are expressed during embryonic mouse development. Two lines of evidence suggest that there may be no additional *senseless* homologs to *Gfi1* and *Gfi1b*. First, we did not identify another gene with similar sequence homology in BLAST searches of the human and mouse genomes. Second, RT-PCR with degenerate primers corresponding to the highly conserved zinc-finger sequences allowed us to identify transcripts in E10.5, E11.5, E12.5 and E13.5 embryos and adult thymus. Sixty-four clones were sequenced and all corresponded to *Gfi1* and *Gfi1b* (data not shown). These data show that *Gfi1* and *Gfi1b* are expressed in

embryos and that it is unlikely that other *senseless* homologues exist in mice.

To determine when and where *Gfi1* is expressed, we carried out in situ hybridization and immunohistochemistry in embryos. As shown in Fig. 1A, *Gfi1* mRNA is expressed in E12.5 embryos in the PNS, CNS and many sensory tissues. More specifically, *Gfi1* mRNA is expressed in the developing brain, optic epithelia, dorsal root ganglia, otic vesicle, trigeminal and vestibulo-cochlear ganglia, and gut epithelia (Fig. 1A). At later stages expression is also seen in many sensory organs such as the developing eye (Fig. 1B), presumptive Merkel cells (Fig. 1D,E), cells of the nasal epithelia (Fig. 1G), epithelia of the tongue (Fig. 1H), as well as in small clusters of neuroepithelial precursor cells in developing lung (Fig. 1I; D. W., unpublished), and many cells of the developing thymus (Fig. 1K). Hence, *Gfi1* mRNA is widely expressed in epithelia in which sensory cells are specified (tongue, nasal epithelia, gut, lung and eye), as well as in the developing brain and PNS ganglia. However, the Gfi1 protein has a more restricted expression pattern and localizes to several specialized sensory cells of the PNS. Gfi1 protein is present in the eye (Fig. 1C), the presumptive Merkel cells (Fig. 1F) and the lung (Fig. 1J). We did not detect Gfi1 protein expression in the brain or any of the ganglia, which is where we see *Gfi1* mRNA expression (see Discussion).

Whereas *Gfi1* is expressed in a variety of tissues, we chose to focus our analysis of the role of *Gfi1* in the developing ear. As shown in Fig. 2, *Gfi1* mRNA and protein are expressed in the ear throughout development. *Gfi1* mRNA is expressed in the developing otic vesicle and several ganglia, including the vestibulo-cochlear ganglia as early as E12.5 (Fig. 2A). Gfi1 protein can be detected at E12.5 in a more restricted pattern in the hair cell precursors in the otic vesicle, where Math1 is expressed (Bermingham et al., 1999). This timing is concomitant with the initial generation of hair cells during inner ear development as sensory hair cell precursors undergo their terminal mitosis between E11 and P2 (Ruben, 1967). Specification and differentiation of hair cells is initiated just after terminal mitosis between E12-P2 (Zheng and Gao, 1997), and hair cells then mature and grow sterociliary bundles during differentiation. By E14.5, we find *Gfi1* mRNA and protein in the developing vestibular organs in positions analogous to the newly developed hair cells (Fig. 2B,E). By E16.5 to E18.5, when the sensory structures are well defined, *Gfi1* mRNA and

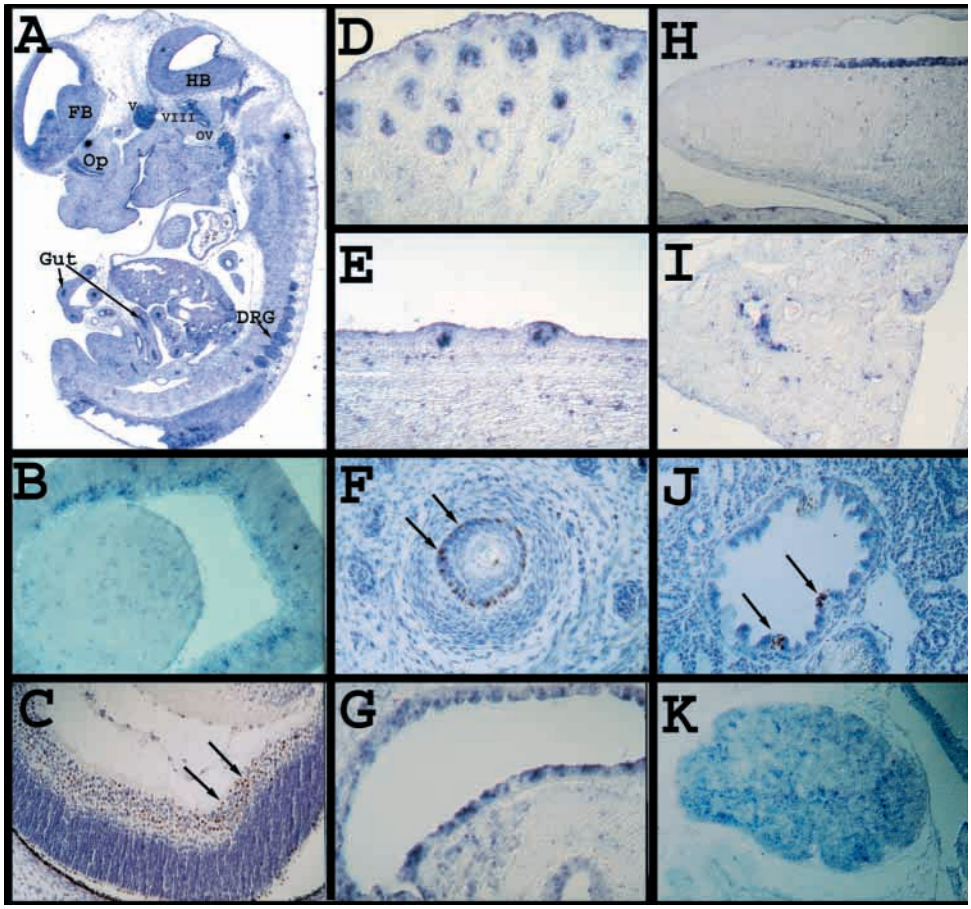

Fig. 1. *Gfi1* wild-type expression pattern. In situ hybridization using a specific antisense probe derived from the 3′ UTR of *Gfi1* and immunohistochemistry using a Gfi1-specific antibody. For in situ, positive cells are purple, and for immunohistochemistry positive cells are brown and counterstained with Hematoxylin (purple). (A) Sagittal section of E12.5 embryo. Areas of high expression of *Gfi1* mRNA are denoted and include the developing brain (FB, forebrain; HB, hindbrain), optic epithelia (Op), dorsal root ganglia (DRG) and gut epithelia (Gut). In addition, *Gfi1* mRNA is expressed in the developing otic vesicle (OV) and several ganglia including the trigeminal (V) and vestibulo-cochlear ganglia (VIII) (see Fig. 2A for enlarged view of ear and associated ganglia). (B) Sagittal section of E18.5 retina showing *Gfi1* expression primarily in the retinal ganglion cell layer. (C) Sagittal section of E16.5 retina showing immunohistochemistry of Gfi1 expression in specific cells denoted with arrows that are likely to be retinal ganglion cells. (D) *Gfi1* expression in E16.5 section through the upper lip and mouth area where the whiskers develop. Positive cells around the shaft of the whiskers are thought to be the Merkel cells. (E) *Gfi1* expression in E15.5 section through the skin. Positive cells are located under the touch domes and correspond in size and position to the Merkel cells. (F) E18.5 section through the upper lip and mouth area where the whiskers develop. Immunohistochemically positive cells (brown cells) around the shaft of the whiskers denoted by arrows are thought to be the Merkel cells. (G) *Gfi1* expression in E16.5 section of the olfactory epithelia. (H) *Gfi1* expression in E18.5 section of the tongue and its dorsal epithelium where taste papilla develop. (I) *Gfi1* expression in E15.5 section of the developing lung. Clusters of cells as well as individual cells express Gfi1. (J) Gfi1 protein expression in E16.5 section of the developing lung. Clusters of cells denoted by arrows express Gfi1. (K) *Gfi1* mRNA expression in E15.5 section of the developing thymus.

protein are clearly detected in the vestibular organs (Fig. 2C) as well as in the organ of Corti in the cochlea (Fig. 2D,F arrows), where it localizes to hair cells. *Gfi1* mRNA expression is also detected in the cochlear ganglia (Fig. 2D, asterisk); however, Gfi1 protein is not expressed in the cochlear ganglia at any time (data not shown). Hence, the temporal and spatial expression pattern of Gfi1 protein in the ear correlates with the specification of hair cells and is very similar if not identical to the expression pattern of *Math1* (Bermingham et al., 1999; Chen et al., 2002).

Gfi1 mutant mice display behavioral defects

We previously established a mouse line deficient for *Gfi1* (H. Hock, M. J. Hamblen, H. M. Rooke, D. Traver, R. T. Bronson, S. Cameron and S. H. Orkin, unpublished). This *Gfi1* allele has part of the 5′ UTR along with the entire first and second exon, as well as part of the third exon deleted. This deleted region contains the entire SNAG transcriptional repression domain but not the zinc fingers and creates a severe loss-of-function or null allele. We have found heterozygous mice to be phenotypically indistinguishable from wild-type littermates at all stages in our assays. The mutant mice are viable for 3-6 months. The mutants look similar to wild type and heterozygous littermates until about postnatal day 10 (P10). By P10, the mutants no longer continue to grow at the rate of their littermates (H. Hock, M. J. Hamblen, H. M. Rooke, D. Traver, R. T. Bronson, S. Cameron and S. H. Orkin, unpublished; Karsunky et al., 2002b) and ataxic behavior becomes apparent. This ataxia and the differences in size between mutant and heterozygous littermates increase in severity with age (see video at http://flypush.imgen.bcm.tmc.edu/lab/deeann/mouse-video1.avi). The mutant animals display several behavioral abnormalities suggestive of inner ear defects including hyperactive circling, head tilting, ataxia and lack of a proper startle response to loud noises. This phenotype coupled with *Gfi1* expression in the inner ear sensory epithelia suggests inner ear defects.

Gfi1 is required for proper differentiation of hair cells

As the overall gross morphology of the inner ear appeared normal at P0 in *Gfi1* mutant mice (data not shown), we immunocytochemically stained ear epithelia with several hair cell markers to identify differentiation defects. Myosin VI/VIIa is an early marker for hair cell differentiation and initiation of expression of this marker occurs properly at E13.5 in the mutant balance organs and cochlea (Fig. 3A,B) (Hasson et al., 1997). However, as shown in Fig. 3A,B, the mutant hair cells in the utricle are thinner and more elongated than the wild-type cells, and there are two to three layers of myosin VI/VIIa-positive cells in the mutant instead of the single layer observed in wild-type epithelia. Anti-myosin VI/VIIa staining of the utricle at E14.5 (Fig. 3C,D) also shows abnormal hair cell morphology and layering in the mutant. The vestibular organs normally show a straight line of hair cells at the edge of the lumen, but the mutant hair cells do not form this straight line as the hair cells are present in the support cell layer. Similarly, the organization of the auditory hair cells in the organ of Corti is also aberrant. The characteristic three rows of outer hair cells and single row of inner hair cells are not present in the mutant. Serial sections of the organ of Corti often show that one of the

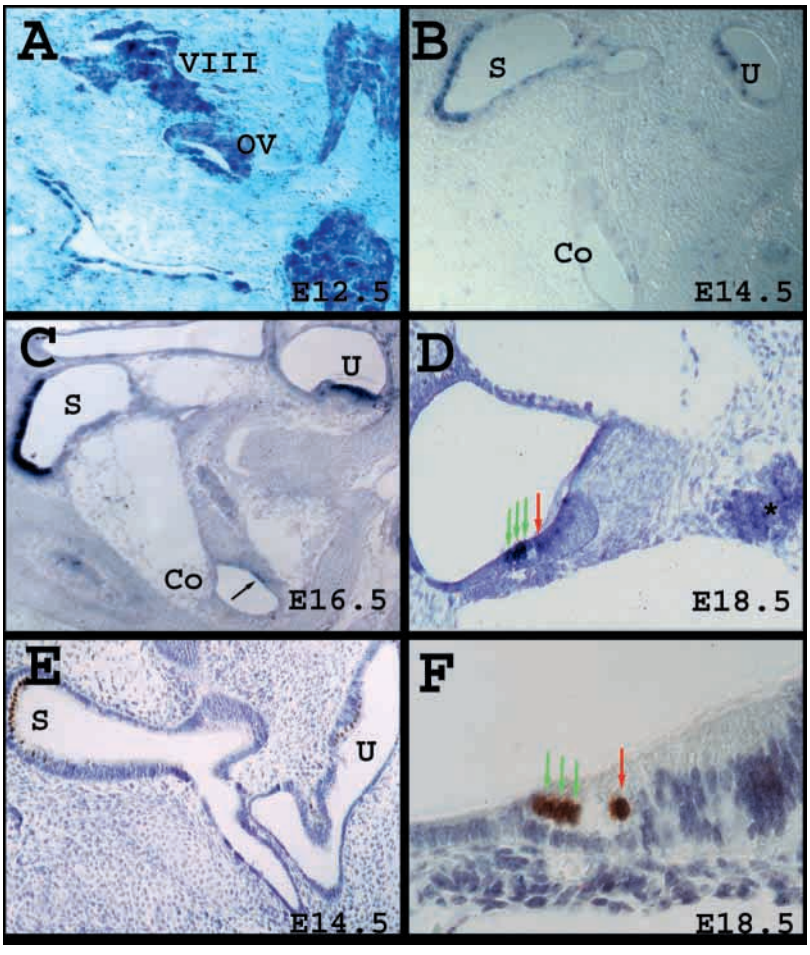

Fig. 2. *Gfi1* wild-type expression in the sensory epithelia of the inner ear. mRNA in situ hybridization using a specific antisense probe derived from the 3′ UTR of *Gfi1* and immunohistochemistry using a Gfi1-specific antibody. For in situ hybridization, positive cells are purple, and for immunohistochemistry, positive cells are brown and counterstained with Hematoxylin (purple). (A) Sagittal section of E12.5 embryo. *Gfi1* is expressed in the otic vesicle (OV) and several ganglia including the vestibulo-cochlear ganglia (VIII). (B) Sagittal section of E14.5 ear. *Gfi1* is expressed in the saccule (S) and utricle (U), but high levels of expression are not yet visible in the cochlea (Co). (C) Sagittal section of E16.5 ear. *Gfi1* is expressed in the saccule (S), utricle (U) and cochlea (Co). An arrow indicates the hair cells in the organ of Corti. (D) Sagittal section of E18.5 cochlea. High expression levels of *Gfi1* mRNA can be seen in the hair cells. The inner hair cell is indicated by a red arrow and the outer hair cells by green arrows. Lower levels of expression can be seen in the cochlear ganglia (asterisk). (E) Sagittal section of E14.5 ear. Gfi1 protein is expressed in the saccule (S) and utricle (U). (F) Sagittal section of E18.5 cochlea. High expression levels of Gfi1 protein can be seen in the hair cells. The inner hair cell is indicated by a red arrow and the outer hair cells by green arrows.

outer rows of cells is lacking as shown with anti-myosin VI/VIIa staining at E16.5 (Fig. 3E,F). Similarly, *Math1* in situ hybridization at E17.5 (Fig. 3G,H), and *Brn3c* in situ hybridization at E18.5 (Fig. 3I,J) each show one inner hair cell and two outer hair cells. Thus, though some hair cells appear to be missing, hair cell-specific markers are expressed and maintained throughout inner ear hair cell development in *Gfi1* mutant mice. In addition, we find that the remaining mutant hair cells in the organ of Corti are not properly innervated. As shown in Fig. 3K,L, staining with anti-TUJ1, a marker for β-tubulin in neurons, reveals a very different staining pattern in mutant animals. In wild-type hair cells (Fig. 3K), the cochlear neurons synapse with the base of the three outer hair cells forming a cup-like staining pattern at the base of each outer hair cell (indicated by green arrows). In the mutant outer hair cells, the anti-TUJ1 labels the entire cell body including the cytoplasm. However, the base of the cells where synaptic sites are normally seen as cup-like structures in wild-type embryos are not, or are barely visible (indicated by green arrows in Fig. 3L). This aberrant pattern is not observed for the inner hair cells. Hence, as the cytoplasm of the outer hair cells in *Gfi1* mutants stain with TUJ1 antibody, the outer hair cells express a neuronal marker that is not normally expressed in these cells. We conclude that based on aberrant morphology of vestibular hair cells and ectopic expression of the neuronal marker TUJ1 in outer hair cells, hair cell differentiation is affected in *Gfi1* mutants.

Is *Gfi1* expression dependent on *Math1* expression and vice-versa?

In flies, proneural gene expression is required for initiation of *senseless* expression, and *senseless* expression is required for maintenance of proneural gene expression (Nolo et al., 2000; Frankfort et al., 2001). To determine if a similar relationship exists between *Math1* and *Gfi1*, we investigated the expression of *Gfi1* in *Math1* mutants and *Math1* in *Gfi1* mutants. As *Math1* and *Gfi1* expression overlap temporally and spatially in the inner ear epithelia, *Math1* may be required for *Gfi1* expression. We therefore tried to assess *Gfi1* expression at the earliest stage of development, just prior to hair cell formation when hair cell precursors are specified (E12.5). At this stage, *Gfi1* mRNA expression in the *Math1* mutant is present in the entire otic epithelia similar to wild-type controls (Fig. 4A,B). However, Gfi1 protein expression is drastically reduced or absent (Fig. 4C,D). These observations indicate that initiation of *Gfi1* mRNA expression is not dependent on *Math1*, but that Gfi1 protein expression is *Math1* dependant. At later stages in development (E14.5, E16.5 and E18.5), *Gfi1* mRNA and protein are both drastically reduced or absent in *Math1* mutants (Fig. 4E,F and data not shown). This may imply that *Math1* is required for *Gfi1* expression, or, alternatively, that the cells in which *Gfi1* is expressed are not specified (Chen et al., 2002). It also indicates that *Gfi1* mRNA and protein expression is confined to hair cells at later developmental stages.

Is *Gfi1* required to maintain *Math1* expression? To monitor *Math1* expression in *Gfi1* mice, we used a mouse containing the β-galactosidase-coding region that replaced the entire *Math1*-coding region. β-galactosidase staining of heterozygous *Math1* animals faithfully mimics *Math1* expression, whereas hair cell specification appears normal (Ben-Arie et al., 2000). Mice that were *Math1* heterozygous ($Math1^{+/\beta Gal}$) and *Gfi1* wild-type or null mutant were derived. In the *Gfi1* mutants, $Math1^{\beta Gal}$ expression is present in all inner ear sensory epithelia (Fig. 4G-J, Fig. 5A-L). However, sections of P0 saccules stained with β-galactosidase show

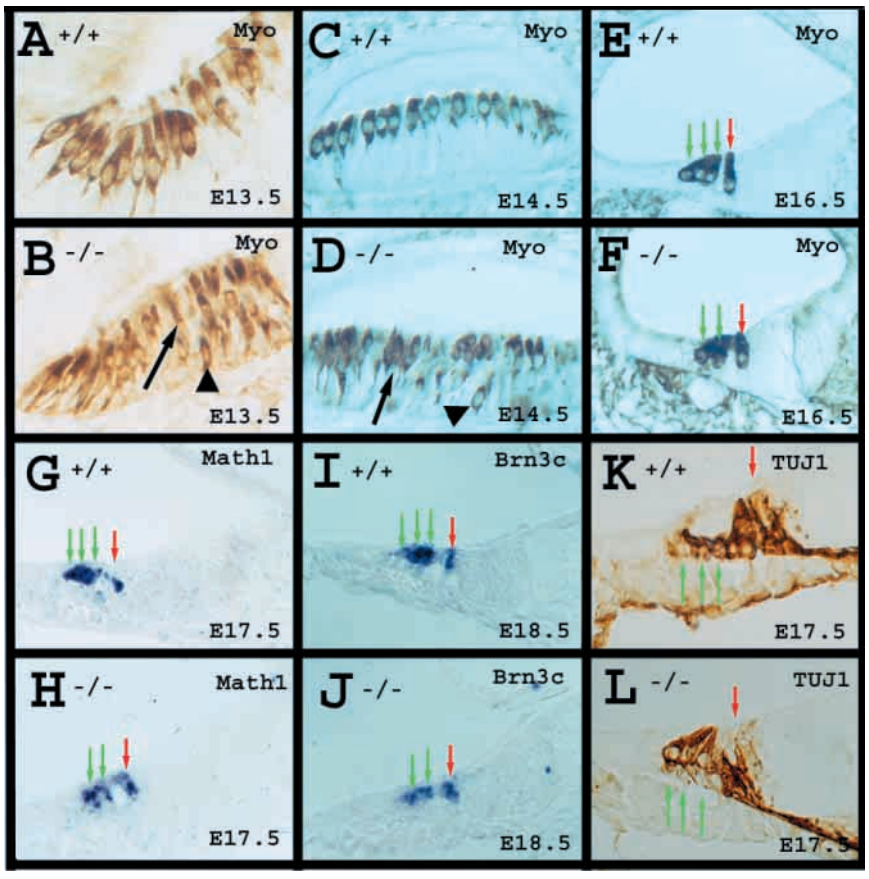

Fig. 3. Expression profile of markers in *Gfi1* mutant ears. (A,C,E,G,I,K) *Gfi1* wild type (+/+); (B,D,F,H,J,L) *Gfi1* null (–/–) mice. The inner hair cell is denoted by a red arrow and the outer hair cells by green arrows. (A-F) Anti-myosin VI/VIIa (Myo) staining of inner ear hair cells showing abnormal cell shape (arrow) and organization (arrowhead) at E13.5 (A,B) and E14.5 (C,D) in the developing vestibular organs. Note that some mutant hair cells are maintained in the support cell layer (arrowhead). (E,F) Hair cells in the mutant organ of Corti are disorganized. (G,H) *Math1* in situ showing that mRNA expression in the organ of Corti at E17.5 is unaffected in the mutants, but hair cells are disorganized. (I,J) *Brn3c* in situ showing *Brn3c* mRNA expression in the organ of Corti at E18.5 is unaffected in the mutant, but the hair cells are disorganized. (K,L) Anti-TUJ1 staining marking the neurons at E17.5 showing abnormal TUJ1 staining in the outer hair cells of the mutant. Note that in wild type, innervation stops at the base of the outer hair cells, but the TUJ1 staining of the mutant outer hair cells appears to be all over the cell body. The inner hair cell is indicated by a red arrow and the outer hair cells by green arrows.

some disorganization and some cells appear to be present in the supporting cell layer (Fig. 4G,H). The mutant hair cells also seem to have less well organized stereocilli than do wild-type cells. Note, however, that the cristae appear to be less affected or unaffected as they have a very similar morphological appearance to wild-type cristae (Fig. 4I,J). Similar data were also observed with *Math1* in situ hybridization in *Gfi1* mutant organ of Corti (Fig. 3G,H). In summary, we found no obvious changes in *Math1* expression pattern in *Gfi1* mutants. These data suggest that there is no dependence of *Math1* on *Gfi1* in mouse. This is in contrast to what we observed in fruit fly between *atonal* and *senseless* (Frankfort et al., 2001; Nolo et al., 2000).

Gfi1 is required for cochlear hair cell survival

The *Math1*$^{\beta Gal}$/+;*Gfi1* mice provided us with a convenient tool to follow hair cell development in wholemounts of organ of Corti and asses differences in apical and basal areas of the cochlea. Normally, by E15.5 *Math1*/β-galactosidase expression is visible in the developing hair cells of the organ of Corti and rows of hair cells are beginning to differentiate in a basal-to-apical gradient. As shown in Fig. 5A,B, by E15.5, *Math1*/β-galactosidase positive cells are present in wild-type and mutant embryos. However, the rows are not as clearly defined in the mutant as in the wild type. By E17.5, the wild-type hair cells have formed the characteristic one row of inner hair cells and three parallel rows of outer hair cells (Fig. 5C). At E17.5, the mutant hair cells are disorganized and less numerous in the basal cochlea (Fig. 5D). As shown in Fig. 5E-H, by P0 the loss of hair cells has progressed in a basal to apical gradient. Fig. 5E shows the orderly arrangement of wild-type hair cells at P0, whereas the basal cochlea of the *Gfi1*-null mice has lost the majority of its hair cells as gauged by *Math1* expression (Fig. 5F). The medial cochlea is also severely affected, but the inner hair cells are still present (Fig. 5G). At P0 the apical cochlea shows little to no loss of hair cells, but does exhibit a disorganization similar to that seen in basal cochlea as early as E15.5 (Fig. 5H, compare with Fig. 5B). By P3 the loss of hair cells in the basal to apical gradient is more severe in the mutants (Fig. 5I-L). The basal cochlea has few hair cells by P3 (Fig. 5J). The medial cochlea still retains the majority of inner hair cells but has lost almost all outer hair cells (Fig. 5K). Even the apical cochlea is beginning to show drastic reduction in the number of hair cells by P3, though again it appears as though the outer hair cells degenerate first. Hence, it appears that the inner and outer hair cells are initially specified by E15.5 but are subsequently lost in a basal-to-apical gradient. In all cases, the outer hair cells in a given region degenerate prior to the inner hair cells.

As the mutants age, the organ of Corti becomes unrecognizable. Analysis of serial sections stained with Hematoxylin and Eosin indicates that by P14 all cochlear hair cells and most support cells have

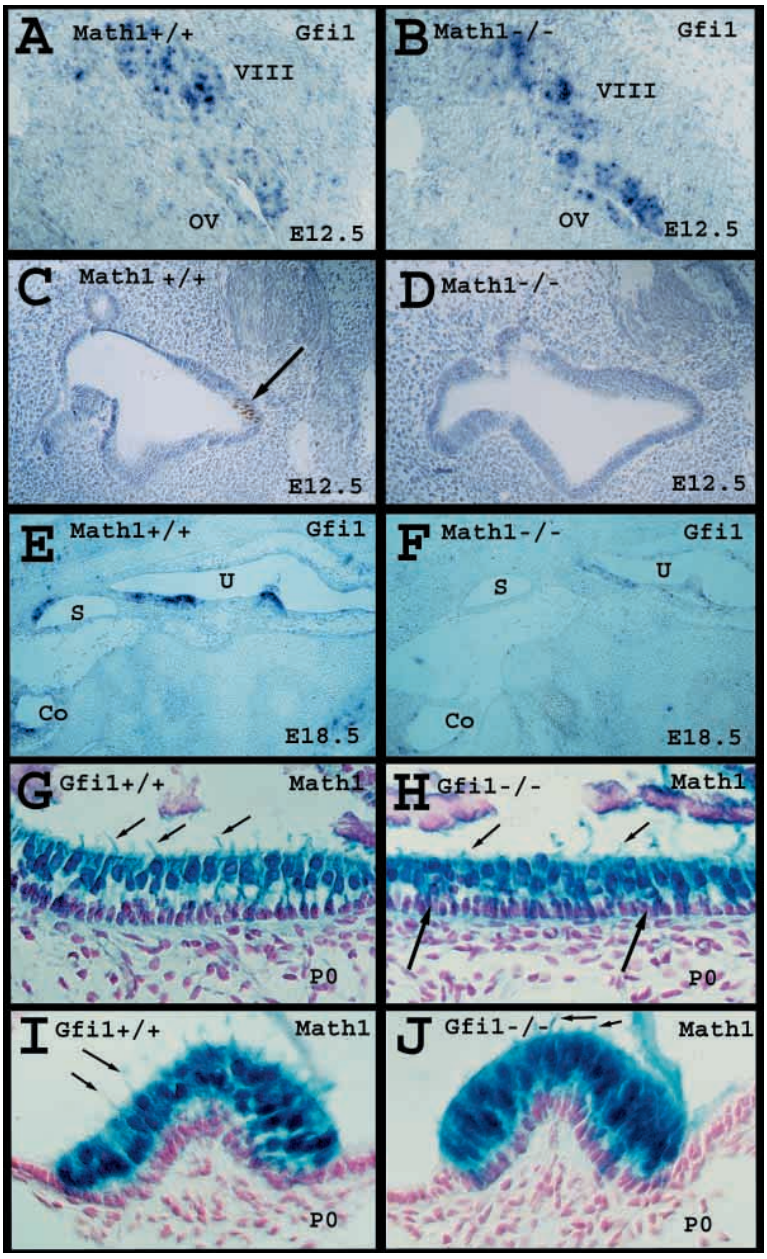

Fig. 4. *Gfi1* and *Math1* expression in hair cells of *Math1* and *Gfi1* mutants. (A-F) *Gfi1* expression in wild-type and *Math1* mutant mice.(G-J) *Math1*/β-Gal expression in *Gfi1* mice. (A,C,E,G,I) Wild-type littermates (+/+). (B,D,F) *Math1* null mutants (–/–). (H,J) *Gfi1*-null mutants (–/–). (A,B) *Gfi1* mRNA expression in both wild-type and *Math1* null otic vesicle (OV) at E12.5. (C,D) Gfi1 protein expression in wild-type but not *Math1* null otic vesicle (OV) at E12.5. Arrow indicates position of Gfi1-positive cells (E,F) Loss of *Gfi1* mRNA expression at E18.5 in the *Math1* null mutant utricle (U), saccule (S) and cochlea (Co), presumably due to loss of hair cells. (G-J) *Math1*/β-Gal expression (blue) and hair cell placement are visualized by the β-galactosidase cassette driven by the *Math1* promoter in P0 coronal sections of the ear. Sections are counterstained with Nuclear Fast Red (pink). Some of the stereocilli are visible (small arrows). (G) *Gfi1* wild-type saccule showing a single layer of blue stained hair cells and a single layer of pink stained support cells. (H) *Gfi1*-null saccule showing the disorganization of the hair cell 'layer'. Note that some hair cells are placed completely beneath others in the layer (large arrows) (see Fig. 3A-D for comparison). (I) *Gfi1* wild-type cristae showing normal development of hair cells and expression of *Math1*. (J) *Gfi1*-null cristae showing normal development of hair cells and expression of *Math1*.

disappeared in mutant animals (Fig. 6A,B). However, despite the rapid degeneration of cochlear hair cells, the hair cells in the vestibular organs do not degenerate, but remain unorganized (Fig. 6C,D). Note the separation of hair cells and support cells in the saccule of the wild-type mouse (Fig. 6C). This layering is again not as clearly defined in the mutant when compared with wild type (Fig. 6D).

As shown in Fig. 2D (asterisk), *Gfi1* mRNA is also expressed in the cochlear ganglion neurons, although we did not observe Gfi1 protein expression. We therefore examined number and morphology of the cochlear ganglion neurons. As shown in Fig. 6E,F, at P7, both wild-type (Fig. 6E) and mutant (Fig. 6F) cochlear ganglion neurons show similar cell densities and a low level of apoptosis as indicated by anti-activated-caspase 3 (C3) staining. Low levels of apoptosis at P7 in wild-type cochlear ganglion neurons has been previously observed (Kamiya et al., 2001). At P21, the cochlear ganglion neurons in the wild-type (Fig. 6G) and mutant mice (Fig. 6H) show slightly different cell densities and quite a few mutant cells express activated-caspase 3, suggesting that these cells undergo cell death by apoptosis. We did not observe apoptosis in the wild-type mouse at P21. By 5 months of age there is a dramatic reduction of neurons in the cochlear ganglion, as seen by Hematoxylin and Eosin staining (Fig. 6I,J). Hence, cochlear ganglion neuron degeneration occurs after hair cell loss and is progressive.

Ultrastructural analysis of the organ of Corti

To determine the ultrastructural defects in the cells of the organ of Corti, we carried out transmission electron microscopy (TEM). TEM shows abnormal hair cell morphology at E18.5 in the mutant mice and confirms the disorganization of hair cells in the organ of Corti. The stereocilli are nicely preserved and easily identifiable in the wild type outer hair cells (Fig. 7A,C), but the stereocilli of the mutant hair cells are not well preserved and are barely visible in some cells. Some of the mutant outer hair cells also display typical morphological signs of apoptosis, such as shrinkage of the cell body, extensive blebbing and vacuolization (Fig. 7B,D). Also, consistent with an apoptotic mechanism of cell death, the mutant mitochondria appear indistinguishable from the wild-type mitochondria, in contrast to what happens when cells die by necrosis. These data suggest that the outer and inner hair cells die by apoptosis.

DISCUSSION

Gfi1 and the development of the nervous system

As both *senseless* and *pag-3* play a role in the development of the nervous system, we wished to determine if *Gfi1* and *Gfi1b* play a role in nervous system development in vertebrates. However, expression of Gfi genes in nervous system development had not been documented. We therefore determined the developmental expression patterns for both *Gfi1* and *Gfi1b*. We found *Gfi1* mRNA to be expressed in a variety of CNS, PNS and sensory epithelia, whereas *Gfi1b* seems to be predominately expressed in the fetal liver with

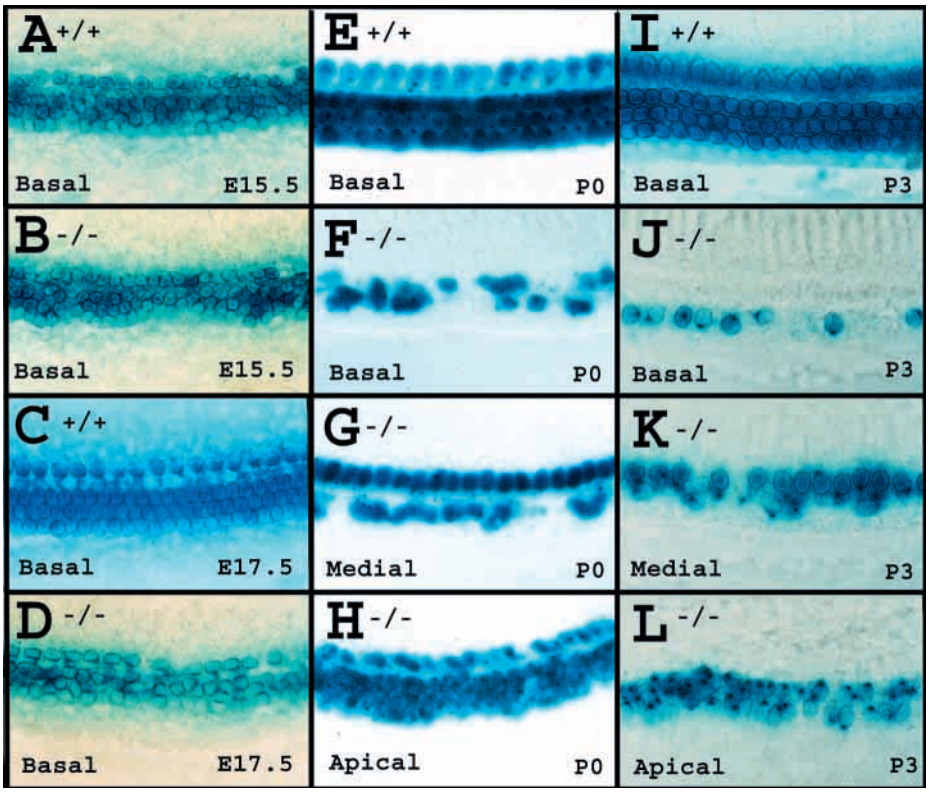

Fig. 5. Cochlear hair cells degenerate rapidly in *Gfi1* mutants. *Math1*/β-Gal expression (blue) and hair cell placement are visualized by the β-galactosidase cassette driven by the *Math1* promoter in whole mounts of the cochlea. (A,C,E,I) *Gfi1* wild type (+/+). (B,D,F-H,J-L) are *Gfi1* null (–/–). (A,B) *Math1*/β-galactosidase at E15.5. (A) *Gfi1* wild-type (+/+) region of the basal cochlea, showing the development of the orderly arrangement of three outer and one inner row of hair cells. (B) *Gfi1* null (–/–) basal cochlea showing *Math1* expression and hence specification of hair cells is unaffected in the null cochlea. However, the characteristic rows of inner and outer hair cells are not as clearly defined as in wild type. (C,D) *Math1*/β-Gal expression at E17.5. (C) *Gfi1* wild-type region of the basal cochlea showing the orderly arrangement of three outer and one inner row of hair cells. In the wild-type mouse, these orderly rows are continuous along the entire length of the cochlea. (D) *Gfi1*-null basal cochlea showing persistent disorganization of hair cells and decreased hair cell numbers. (E-H) *Math1*/β-galactosidase at P0. (E) *Gfi1* wild-type region of the basal cochlea showing the orderly arrangement of three outer and one inner row of hair cells. (F) *Gfi1* null basal cochlea, (G) *Gfi1* null medial cochlea, (H) *Gfi1* null apical cochlea. Note the degeneration of the cochlea in a basal to apical gradient. (I-L) *Math1*/β-galactosidase at P3. (I) *Gfi1* wild-type region of the basal cochlea showing the orderly arrangement of three outer and one inner row of hair cells. (J) *Gfi1* null basal cochlea; (K) *Gfi1* null medial cochlea;(L) *Gfi1* null apical cochlea. Note the degeneration of the cochlea in a basal to apical gradient.

minimal or no expression in the nervous system (data not shown). Hence, we chose to focus our analysis on *Gfi1*.

Gfi1 mRNA is present in a variety of tissues during development. It is expressed in the CNS, a variety of ganglia and many specialized sensory cells of the PNS. However, the Gfi1 protein expression pattern is more restricted. We detect Gfi1 protein primarily in specialized sensory cells of the PNS. However, we did not detect Gfi1 protein in the CNS or any ganglia. The difference in *Gfi1* mRNA and protein expression may have several possible explanations. First, *senseless* mRNA is also more widespread than its protein expression pattern in the fly (Nolo et al., 2000). Thus, it is likely that this is a real phenomenon and not just an artifact of in situ or immunohistochemical analysis. Alternatively, the RNA and/or the protein stability may vary from cell to cell type. Third, it is possible that Gfi1 is only translated under specific conditions, i.e. in the presence of Math1.

The temporal and spatial distribution of *Gfi1* transcripts in many cells and epithelia overlaps with that of many bHLH gene expression patterns. In most tissues in which *Gfi1* is expressed, there is a corresponding bHLH gene that may regulate/control *Gfi1* expression. For example, *Mash1* (*Ascl1* – Mouse Genome Informatics) is expressed in the developing olfactory epithelia (Cau et al., 1997), the neuroendocrine cells of the lung (Borges et al., 1997; Ito et al., 2000) and the tongue (Seta et al., 1999), where we observe *Gfi1* mRNA expression. *Math1* is expressed in the developing ear epithelia (Bermingham et al., 1999), gut epithelia (Yang et al., 2001) and Merkel cells (Ben-Arie et al., 2000) where we observe *Gfi1* mRNA and protein expression. *Math5* is expressed in the developing eye and retinal ganglion cells (Wang et al., 2001). *Neurod1* is expressed in the ear epithelia, as well as the ganglia that innervate the ear (Liu et al., 2000). These bHLH genes are homologous to the *Drosophila* bHLH proteins Achaete, Scute, Atonal or Amos and are required for the specification of subtypes of cells. Similarly, other bHLH proteins such as the neurogenins have been shown to be expressed and required in some of the PNS ganglia where *Gfi1* mRNA is expressed (Ma et al., 1997; Ma et al., 2000b). These observations suggest that, similar to fruit flies, *Gfi1* expression may be regulated by bHLH genes.

Because we found *Gfi1* to be expressed in the developing ear, we chose to focus our analysis on the developing ear to test potential interactions of *Gfi1* with the bHLH gene *Math1* (Bermingham et al., 1999). We assessed the expression pattern of *Gfi1* in *Math1* mutants and *Math1* expression in *Gfi1* mutants. Interestingly, *Math1* expression is unaffected in *Gfi1* mutants. As atonal positively regulates its own expression in the fly (Sun et al., 1998), it is possible that *Math1* may also regulate its own expression in the mouse. This provides a potential explanation as to how *Math1* expression may be maintained in the *Gfi1*-deficient mouse. This data may also suggest that *Gfi1* is not required for maintenance of *Math1* expression. However, *Math1* is required for Gfi1 protein expression, but not required for initial *Gfi1* mRNA expression. Hence, it remains to be established how *Gfi1* mRNA and protein expression is precisely controlled.

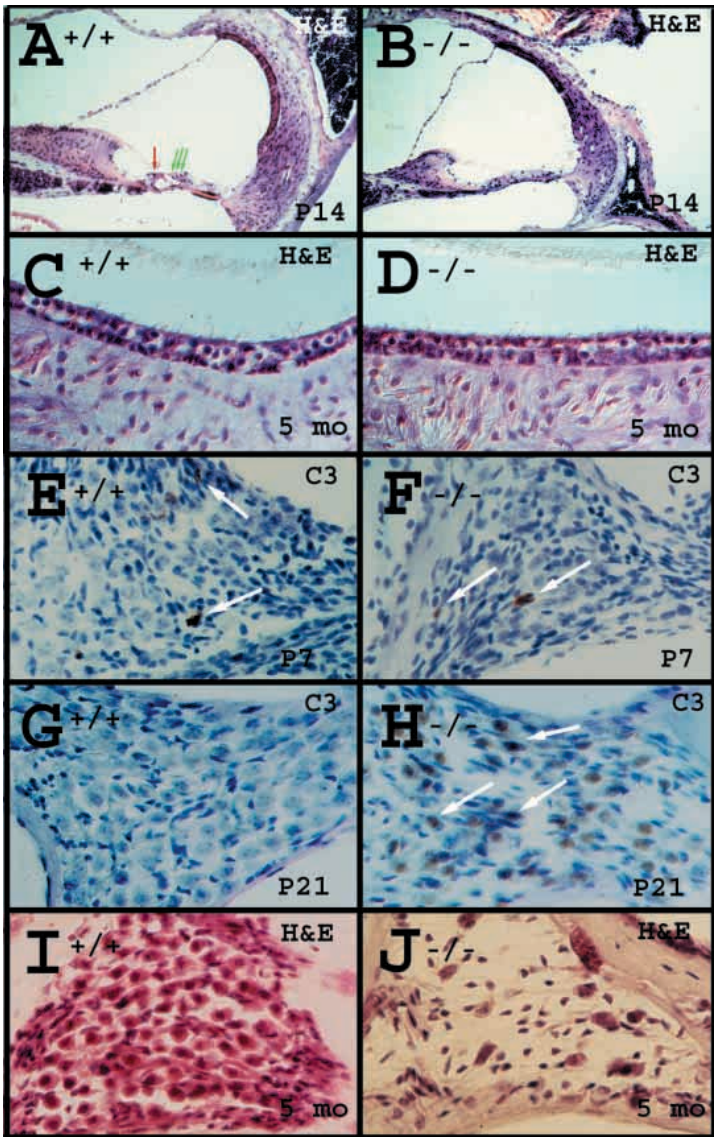

Fig. 6. The inner ear phenotype in *Gfi1* mutant mice. (A,C,E,G,I) are *Gfi1* wild-type (+/+) mice and (B,D,F,H,J) are *Gfi1* null (–/–) mice. (A,B) Hematoxylin and Eosin (H&E) morphological stains of the organ of Corti at P14. Note the development of the organ of Corti with one inner (red arrow) and three outer (green arrows) hair cells in the wild type, but its complete degeneration in the mutant section. (C,D) Hematoxylin and Eosin morphological stains of the saccule at 5 months of age. Note the presence of hair cells with stereocilli in both the wild-type and mutant. However, the mutant saccule has disorganized layering of the hair and support cells. (E-J) The progressive degeneration of the cochlear ganglion neurons in the mutant mice. (E-H) Sections stained with anti-activated-caspase-3 (C3) as a marker of apoptosis (brown cells) and counterstained with Hematoxylin. Arrows in E, F and H indicate activated-caspase-3-positive cells. (E,F) P7 mice. Note similar cell densities and levels of apoptosis in both samples. (G,H) P21 mice. Note that by P21 we see a slightly lower cell density in the mutant as well as a high level of apoptosis in the mutant that is not present in the wild-type mouse. (I,J) 5-month-old mice. Sections stained with Hematoxylin and Eosin.

Our data support a model where Gfi1 is downstream of *Math1*, but may not support a model in which *Gfi1* functions in a positive feedback loop with *Math1*. However, it is possible that another bHLH gene expressed in the vertebrate ear

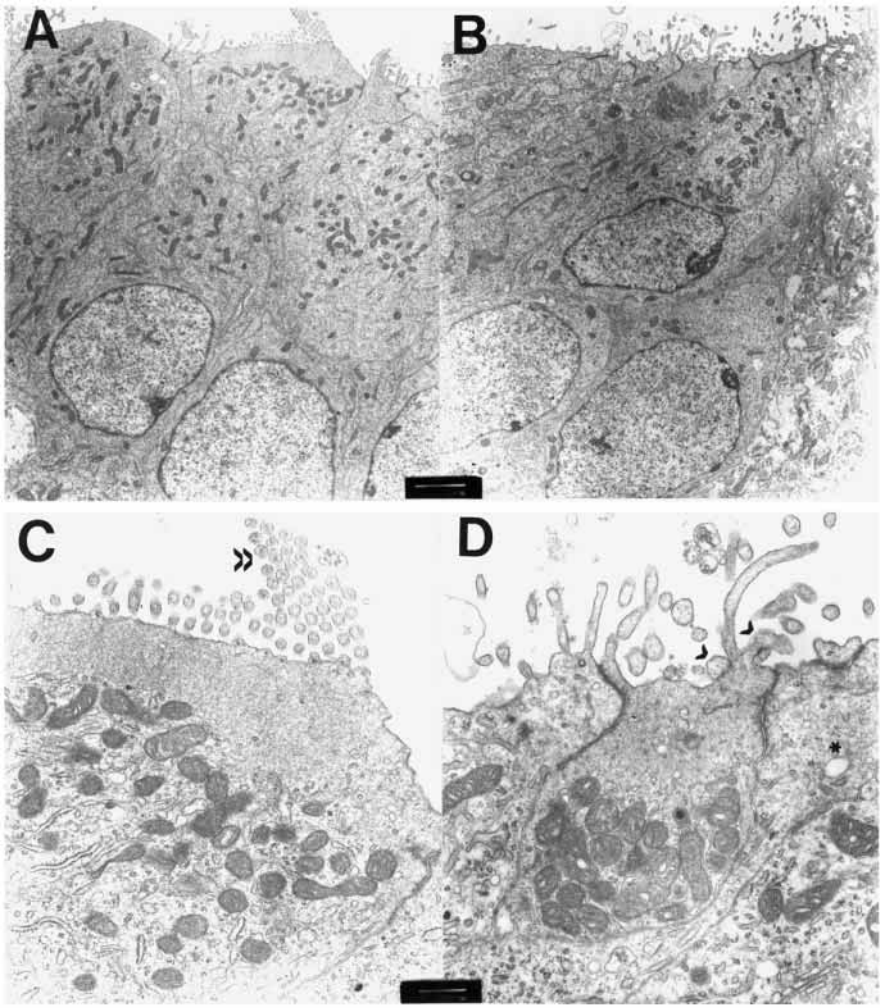

Fig. 7. Ultrastructural analysis of the organ of Corti at E 18.5 using transmission electron microscopy (TEM). (A,C) *Gfi1* wild-type mice. (B,D) *Gfi1*-null mice. (A,B) An overview of the outer hair cell. (C,D) The same outer hair cells as in A,B at a higher magnification. Double arrowheads indicate stereocilli of the wild-type outer hair cell, while single arrowheads indicate the blebbing seen in the mutant hair cell. Note the shrinkage of the cell body in the mutant outer hair cell. The mutant hair cell also contains vacuoles (asterisk). There is no detectable difference between wild-type and mutant hair cell mitochondria.

epithelium prior to *Math1* expression is required for *Gfi1* mRNA expression. The identity of this putative bHLH protein is unknown. However, the existence of such factor is suggested because in *Math1*-null mutants, hair cell precursors form a zone of non-proliferating cells that delineate the sensory primordium within the cochlear anlage, and a significant subpopulation of these precursors die because of apoptosis in a basal-to-apical gradient (Chen et al., 2002). The fact that these cells die instead of becoming support cells indicates that these cells have a different fate than their surrounding cells in the absence of or prior to *Math1* expression. We surmise that this difference is induced by the presumptive factor. Our data are consistent with the idea that this factor is upstream of both *Math1* and *Gfi1*. Such a factor could function similar to a proneural gene as it might be initially expressed in a cluster of cells rendering them competent to become neural cells and then refine to a specific cell that also expresses *Math1* and *Gfi1* to become a hair cell (Chen et al., 2002; Hassan and Bellen, 2000). This factor could be responsible for the initial expression of *Gfi1* and explain why in a *Math1* mutant we observe *Gfi1* mRNA expression early on. Candidate bHLH transcription factors expressed prior to *Math1* in ear development include *Neurod1* (Liu et al., 2000) and neurogenin 1 (Ma et al., 2000a). Both are required for proper development of the inner ear, but *Neurod1* and neurogenin 1 mutant mice display very different phenotypes from the ones we observe in *Gfi1* mutants (Liu et al., 2000; Ma et al., 1998), suggesting that neither *Neurod1* nor neurogenin 1 corresponds to the proposed factor.

Gfi1 is required for hair cell development in the vestibule and hair cell differentiation and viability in the organ of Corti

The hair cells of the inner ear seem to be specified properly as they express many of the typical hair cell markers such as myosinVI/VIIa, *Math1* and *Brn3c*. Thus, *Gfi1* is not required for the specification of hair cells as they are formed in both the vestibule and the cochlea. However, the loss of *Gfi1* seems to affect the vestibular and cochlear hair cells differently. In the vestibule, the hair cells are morphologically abnormal at the earliest stages of hair cell differentiation and at all subsequent stages. In addition, hair cells are not specifically localized to a lumenal monolayer, and are more variable in size and shape. This disorganization of hair cells in the vestibule may account for the ataxic behavior of the mice. In the cochlea, *Gfi1* is required for the organization and maintenance of both inner and outer hair cells. Although the mutant hair cells seem to be specified in the developing organ of Corti as early as E15.5 and express typical hair cell markers, they are disorganized. In addition, the outer hair cells express the neuronal marker TUJ1 at E17.5. This abnormal/ectopic TUJ1 expression may indicate a partial transformation of outer hair cells into neurons, or the de-repression of a single neuronal marker. It is thus possible that these cells are part hair cell and part neuron, and this ambiguity could trigger apoptosis. In fact, the outer cochlear hair cells are the first to disappear starting at E17.5. Based on TEM analysis, we see some of the classical morphological signs of apoptosis in the mutant hair cells at E18.5, including shrinkage of the cell body, blebbing and vacuolization. Whole-mount analysis of the cochlea indicates that this loss of hair cells occurs in a basal to apical gradient and affects outer hair cells prior to inner hair cells in any given region of the cochlea. The hair cells and support cells of the organ of Corti continue to disappear until the entire organ of Corti has been destroyed by P14. Because wild type mice do not perceive sound until

after P12 (Kamiya et al., 2001), and because *Gfi1* null mice have no hair cells by P14, we assume that these mice are deaf, which is in agreement with the lack of a startle response to loud noises.

Upon degeneration of the organ of Corti, the cochlear ganglion neurons also degenerate. This degeneration is progressive, beginning after P7 but fairly extensive by five months of age. As *Gfi1* mRNA is expressed at low levels in the neurons, it may be directly required for neuronal survival. However, *Gfi1* may not be directly responsible for neuronal loss because Gfi1 protein is not expressed in the cochlear ganglion, and cochlear neurons normally die after degeneration of hair cells, presumably because of the withdrawal of trophic support (Dodson, 1997; Lefebvre et al., 1992). Thus, it seems likely that the loss of cochlear ganglion neurons is secondary to the loss of hair cells in the organ of Corti.

There are other mutant mice with similar, yet distinct phenotypes. *Brn3c*-deficient mice have a similar vestibular phenotype with a small number of hair cells retained in the support cell layer in the vestibular sensory epithelia (Xiang et al., 1998). The hair cells in *Brn3c*-null mice are also initially specified, but fail to mature and form stereocilli. The *Brn3c*-deficient hair cells then rapidly degenerate by apoptosis (Xiang et al., 1997). The loss of hair cells occurs in the organ of Corti as early as E17.5 with nearly complete loss by P5. This is similar to the *Gfi1* mutant, but unlike in the *Gfi1* mutants, this degeneration is also detected in the vestibule as early as E18.5. In *Brn3c* mutants, the loss of hair cells is then followed by a loss of the cochleo-vestibular neurons with a substantial loss as early as P4, earlier than in the *Gfi1* mutants. Note that *Brn3c* expression is maintained in all inner ear sensory epithelia of *Gfi1* mutant mice (Fig. 3I,J; data not shown). *Barhl1*-deficient mice also show a progressive degeneration of cochlear hair cells (Li et al., 2002). This degeneration is much slower than in *Gfi1* mutants, occurring roughly from P6 to 2 months of age for outer hair cells and between six months and ten months for inner hair cells. Interestingly, the outer hair cells degenerate first in an apical-to-basal gradient, and the inner hair cells degenerate second in a reverse basal-to-apical gradient.

Gfi1 has a novel phenotype with respect to its effect on the inner ear. The fact that different types of hair cells expressing *Gfi1* in the different sensory organs are affected differently is mirrored in *Drosophila senseless* mutants and *C. elegans pag*-3 mutants. In the *Drosophila* embryonic PNS, some mutant neuronal subtypes undergo apoptosis like the auditory hair cells in *Gfi1* mutants (Nolo et al., 2000). In *C. elegans*, other neuronal subtypes are improperly differentiated or abnormal such as the BDU interneurons, the Pn.aa neuroblasts, and the VA and VB motoneurons similar to the vestibular hair cells in the *Gfi1* mutants (Cameron et al., 2002).

Hence, it is possible that the function of *Gfi1* and its homologs is dependent on the tissue in which it is expressed as it may have a variety of functions depending on its environment. It is most likely that *Gfi1* plays a variety of different roles as a transcriptional repressor or activator. Thus, different genes are repressed or activated in different tissues resulting in a variety of functions. A more precise explanation as to the function of *Gfi1* will have to await the further analysis of *Gfi1* function in other tissues and the identification of direct *Gfi1* target genes and interaction partners.

We thank Fred Pereira, Vincent Wang, Qi (Melissa) Yang, Bill Klein, Tama Hasson, Ruth Anne Eatock, Ming-Jer Tsai, Loretta Doan, Hamed Jafar-Nejad, Mahira Zaheer and Suzanne Paylor for discussions, reagents and technical assistance. Funding was provided by NASA grants NAG2-1496 and NAG2-1355 to H. J. B., and NIH NRSA grant F32 DC 5109 to D. W. Support was provided by the cores of the Baylor Mental Retardation Research Centers HD 24064 to H. Y. Z., a Centers of Excellence in Hematology Award to S. H. O., and by a Cancer Research Foundation of America Hope Street Kids grant to H. L. G. H. Y. Z., H. J. B. and S. H. O. are investigators of the HHMI.

REFERENCES

Ben-Arie, N., Hassan, B. A., Bermingham, N. A., Malicki, D. M., Armstrong, D., Matzuk, M., Bellen, H. J. and Zoghbi, H. Y. (2000). Functional conservation of atonal and Math1 in the CNS and PNS. *Development* **127**, 1039-1048.

Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A. and Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837-1841.

Borges, M., Linnoila, R. I., van de Velde, H. J., Chen, H., Nelkin, B. D., Mabry, M., Baylin, S. B. and Ball, D. W. (1997). An acheate-scute homolog essential for neuroendocrine differentiation in the lung. *Nature* **386**, 852-855.

Cameron, S., Clark, S. G., McDermott, J. B., Aamodt, E. and Horvitz, H. R. (2002). PAG-3, a Zn-finger transcription factor, determines neuroblast fate in C. elegans. *Development* **129**, 1763-1774.

Cantos, R., Cole, L. K., Acampora, D., Simeone, A. and Wu, D. K. (2000). Patterning of the mammalian cochlea. *Proc. Natl. Acad. Sci. USA* **97**, 11707-11713.

Cau, E., Gradwohl, G., Fode, C. and Guillemot, F. (1997). Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* **124**, 1611-1621.

Chen, P., Johnson, J. E., Zoghbi, H. Y. and Segil, N. (2002). The role of Math1 in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell fate determination. *Development* **129**, 2495-2505.

Dodson, H. C. (1997). Loss and survival of spiral ganglion neurons in the guinea pig after intracochlear perfusion with aminoglycosides. *J. Neurocytol.* **26**, 541-556.

Eberl, D. F. (1999). Feeling the vibes: chordotonal mechanisms in insect hearing. *Curr. Opin. Neurobiol.* **9**, 389-393.

Frankfort, B. J., Nolo, R., Zhang, Z., Bellen, H. J. and Mardon, G. (2001). senseless repression of rough is required for R8 photoreceptor differentiation in the developing Drosophila eye. *Neuron* **32**, 403-414.

Gilks, C. B., Bear, S. E., Grimes, H. L. and Tsichlis, P. N. (1993). Progression of interleukin-2 (IL-2)-dependent rat T cell lymphoma lines to IL-2-independent growth following activation of a gene (Gfi-1) encoding a novel zinc finger protein. *Mol. Cell. Biol.* **13**, 1759-1768.

Grimes, H. L., Chan, T. O., Zweidler-McKay, P. A., Tong, B. and Tsichlis, P. A. (1996a). The Gfi-1 proto-oncoprotein contains a novel transcriptional repressor domain, SNAG, and inhibits G1 arrest induced by interleukin-2 withdrawl. *Mol. Cell. Biol.* **16**, 6263-6272.

Grimes, H. L., Gilks, C. B., Chan, T. O., Porter, S. and Tsichlis, P. N. (1996b). The Gfi-1 protooncoprotein represses Bax expression and inhibits T-cell death. *Proc. Natl. Acad. Sci. USA* **93**, 14569-14573.

Hassan, B. A. and Bellen, H. J. (2000). Doing the MATH: is the mouse a good model for fly development. *Genes Dev.* **14**, 1852-1865.

Hasson, T., Gillespie, P. G., Garcia, J. A., MacDonald, R. B., Zhao, Y., Yee, A. G., Mooseker, M. S. and Corey, D. P. (1997). Unconventional myosins in inner-ear sensory epithelia. *J. Cell Biol.* **137**, 1287-1307.

Ito, T., Udaka, N., Yazawa, T., Okudela, K., Hayashi, H., Sudo, T., Guillemot, F., Kageyama, R. and Kitamura, H. (2000). Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. *Development* **127**, 3913-3921.

Jarman, A. P., Grau, Y., Jan, L. Y. and Jan, Y. N. (1993). atonal is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. *Cell* **73**, 1307-1321.

Jegalian, A. G. and Wu, H. (2002). Regulation of Socs gene expression by the proto-oncoprotein GFI-1B: two routes for STAT5 target gene induction by erythropoietin. *J. Biol. Chem.* **277**, 2345-2352.

Jia, Y., Xie, G. and Aamodt, E. (1996). pag-3, a Caenorhabditis elegans gene involved in touch neuron gene expression and coordinated movement. *Genetics* **142**, 141-147.

Jia, Y., Xie, G., McDermott, J. and Aamodt, E. (1997). The C. elegans gene pag-3 is homologous to the zinc finger proto-oncogene gfi-1. *Development* **124**, 2063-2073.

Kamiya, K., Takahashi, K., Kitamura, K., Momoi, T. and Yoshikawa, Y. (2001). Mitosis and apoptosis in postnatal auditory system of the C3H/He strain. *Brain Res.* **901**, 296-302.

Karsunky, H., Mende, I., Schmidt, T. and Moroy, T. (2002a). High levels of the onco-protein Gfi-1 accelerate T-cell proliferation and inhibit activation induced T-cell death in Jurkat T-cells. *Oncogene* **21**, 1571-1579.

Karsunky, H., Zeng, H., Schmidt, T., Zevnik, B., Kluge, R., Schmid, K. W., Dührsen, U. and Möröy, T. (2002b). Inflammatory reactions and severe neutropenia in mice lacking transcriptional repressor Gfi1. *Nat. Genet.* **30**, 295-300.

Lefebvre, P. P., Weber, T., Rigo, J. M., Staecker, H., Moonen, G. and van de Water, T. R. (1992). Peripheral and central target-derived trophic factor(s) effects on auditory neurons. *Hear. Res.* **58**, 185-192.

Li, S., Price, S. M., Cahill, H., Ryugo, D. K., Shen, M. M. and Xiang, M. (2002). Hearing loss caused by progressive degeneration of cochlear hair cells in mice deficient for the Barhl1 homeobox gene. *Development* **129**, 3523-3532.

Liao, X., Tang, Y., Chattopadhyay, S. K., Hartley, J. W. and Morse, H. C. R. (1997). Upregulation of Gfi-1, a gene involved in IL-2-independent growth of T cells, in a murine retrovirus-induced immunodeficiency syndrome. *In Vivo* **11**, 9-12.

Liu, M., Pereira, F. A., Price, S. D., Chu, M. J., Shope, C., Himes, D., Eatock, R. A., Brownell, W. E., Lysakowski, A. and Tsai, M. J. (2000). Essential role of BETA2/NeuroD1 in development of the vestibular and auditory systems. *Genes Dev.* **14**, 2839-2854.

Ma, Q., Sommer, L., Cserjesi, P. and Anderson, D. J. (1997). Mash1 and neurogenin1 expression patterns define complementary domains of neuroepithelium in the developing CNS and are correlated with regions expressing notch ligands. *J. Neurosci.* **17**, 3644-3652.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J. L. and Anderson, D. J. (1998). neurogenin1 is essential for the determination of neural precursors for proximal cranial sensory ganglia. *Neuron* **20**, 469-482.

Ma, Q., Anderson, D. J. and Fritzsch, B. (2000a). Neurogenin 1 null mutant ears develop fewer, morphologically normal hair cells in smaller sensory epithelia devoid of innervation. *J. Assoc. Res. Otolaryngol.* **1**, 129-143.

Ma, Y., Certel, K., Gao, Y., Niemitz, E., Mosher, J., Mukherjee, A., Mutsuddi, M., Huseinovic, N., Crews, S. T., Johnson, W. A. et al. (2000b). Functional interactions between Drosophila bHLH/PAS, Sox, and POU transcription factors regulate CNS midline expression of the slit gene. *J Neurosci* **20**, 4596-4605.

McIver, S. (1985). Mechanoreception. In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology* (ed. G. Kerkut and L. Gilbert), pp. 71-132. Oxford: Pergamon Press.

Nolo, R., Abbott, L. A. and Bellen, H. J. (2000). Senseless, a Zn Finger transcription factor, is necessary and sufficient for sensory organ development in Drosophila. *Cell* **102**, 349-362.

Nolo, R., Abbott, L. A. and Bellen, H. J. (2001). Drosophila Lyra mutations are gain-of-function mutations of senseless. *Genetics* **157**, 307-315.

Osawa, M., Yamaguchi, T., Nakamura, Y., Kaneko, S., Onodera, M., Sawada, K., Jegalian, A., Wu, H., Nakauchi, H. and Iwama, A. (2002). Erythroid expansion mediated by the Gfi1b zinc finger protein: role in normal hematopoiesis. *Blood* **100**, 2769-2777.

Petit, C., Levilliers, J. and Hardelin, J.-P. (2001). Molecular genetics of hearing loss. In *Annual Review of Genetics*, Vol. 35 (ed. A. Campbell, W. Anderson and E. Jones), pp. 589-646. Palo Alto, CA: Annual Reviews.

Ruben, R. (1967). Development of the inner ear of the mouse: a radioautographic study of terminal mitosis. *Acta Otolaryngol.* **220**, 4-44.

Saleque, S., Cameron, S. and Orkin, S. H. (2002). The zinc-finger proto-oncogene Gfi1b is essential for development of the erythroid and megakaryocytic lineages. *Genes Dev.* **16**, 301-306.

Salzberg, A., Prokopenko, S. N., He, Y., Tsai, P., Pal, M., Maroy, P., Glover, D. M., Deak, P. and Bellen, H. J. (1997). P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: mutations affecting embryonic PNS development. *Genetics* **147**, 1723-1741.

Scheijen, B., Jonkers, J., Acton, D. and Berns, A. (1997). Characterization of pal-1, a common proviral insertion site in murine leukemia virus-induced lymphomas of c-myc and Pim-1 transgenic mice. *J. Virol.* **71**, 9-16.

Schmidt, T., Zornig, M., Beneke, R. and Moroy, T. (1996). MoMuLV proviral integrations identified by Sup-F selection in tumours from infected myc/pim bitransgenic mice correlate with activation of the Gfi-1 gene. *Nucleic Acids Res.* **24**, 2528-2534.

Schmidt, T., Karsunky, H., Gau, E., Zevnik, B., Elsasser, H. P. and Moroy, T. (1998a). Zinc finger protein GFI-1 has low oncogenic potential but cooperates strongly with pim and myc genes in T-cell lymphomagenesis. *Oncogene* **17**, 2661-2667.

Schmidt, T., Karsunky, H., Rodel, B., Zevnik, B., Elasser, H. P. and Moroy, T. (1998b). Evidence implicting Gfi-1 and Pim-1 in pre-Tcell differentiation steps associated with β-selection. *EMBO J.* **17**, 5349-5359.

Seta, Y., Toyono, T., Takeda, S. and Toyoshima, K. (1999). Expression of Mash1 in basal cells of rat circumvallate taste buds is dependent upon gustatory innervation. *FEBS Lett.* **444**, 43-46.

Sun, Y., Jan, L. and Jan, Y. (1998). Transcriptional regulation of atonal during development of the Drosophila peripheral nervous system. *Development* **125**, 3731-3740.

Tong, B., Grimes, H. L., Yang, T. Y., Bear, S. E., Qin, Z., Du, K., El-Diery, W. S. and Tsichlis, P. N. (1998). The Gfi-1B proto-oncoprotein represses $p21^{WAF1}$ and inhibits myeloid cell differentiation. *Mol. Cell. Biol.* **18**, 2462-2473.

van Staaden, M. J. and Römer, H. (1998). Evolutionary transition from stretch to hearing organs in ancient grasshoppers. *Nature* **384**, 773-776.

Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H. and Gan, L. (2001). Requirement for math5 in the development of retinal ganglion cells. *Genes Dev.* **15**, 24-29.

Wang, V. Y., Hassan, B. A., Bellen, H. J. and Zoghbi, H. Y. (2002). Drosophila atonal fully rescues the phenotype of Math1 null mice: New functions evolve in new cellular contexts. *Curr. Biol.* **12**, 1611-1616.

Xiang, M., Gan, L., Li, D., Chen, Z. Y., Zhou, L., O'Malley, B. W., Klein, W. and Nathans, J. (1997). Essential role of POU-domain factor Brn-3c in auditory and vestibular hair cell development. *Proc. Natl. Acad. Sci. USA* **94**, 9445-9450.

Xiang, M., Gao, W. Q., Hasson, T. and Shin, J. J. (1998). Requirement for Brn-3c in maturation and survival, but not in fate determination of inner ear hair cells. *Development* **125**, 3935-3946.

Yang, Q., Bermingham, N. A., Finegold, M. J. and Zoghbi, H. Y. (2001). Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* **294**, 2115-2118.

Zheng, J. L. and Gao, W. Q. (1997). Analysis of rat vestibular hair cell development and regeneration using calretinin as an early marker. *J. Neurosci.* **17**, 8270-8282.

Zheng, J. L. and Gao, W. Q. (2000). Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. *Nat. Neurosci.* **3**, 580-586.