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Lmx1a is required for segregation of sensory epithelia and normal ear histogenesis and morphogenesis

David H. Nichols[1], **Sarah Pauley**[1], **Israt Jahan**[3], **Kirk W. Beisel**[1], **Kathleen J. Millen**[2], and **Bernd Fritzsch**[3,*]

1*Department of Biomedical Sciences, Creighton University, Omaha, Nebraska*

2*Departments of Human Genetics and Neurology, University of Chicago, Chicago, Illinois*

3*Department of Biology, University of Iowa, Iowa City, Iowa*

Abstract

At E8.5, the LIM-homeodomain factor *Lmx1a* is expressed throughout the otic placode but becomes developmentally restricted to non-sensory epithelia of the ear (endolymphatic duct, ductus reuniens, cochlea lateral wall). We confirm here that the ears of newborn dreher (*Lmx1a*^{dr}) mutants are dysmorphic. Hair cell markers such as *Atoh1* and Myo7 reveal for the first time that newborn *Lmx1a* mutants have only three sensory epithelia: two enlarged canal cristae and one fused epithelium comprising an amalgamation of the cochlea, saccule and utricle, a “cochlear-gravistatic” endorgan. The enlarged anterior canal crista develops by fusion of horizontal and anterior crista whereas the posterior crista fuses with an enlarged papilla neglecta that may extend into the cochlear lateral wall. In the fused endorgan the cochlear region is distinguished from the vestibular region by markers such as *Gata3*, the presence of a tectorial membrane and cochlea-specific innervation. The cochlea-like apex displays minor disorganization of the hair and supporting cells. This contrasts with the basal half of the cochlear region which shows a vestibular epithelium-like organization of hair cells and supporting cells. The dismorphic features of the cochlea are also reflected in altered gene expression patterns. *Fgf8* expression expands from inner hair cells in the apex to most hair cells in the base. Two supporting cell marker proteins, *Sox2* and Prox1, also differ in their cellular distribution between the base and the apex. *Sox2* expression expands in mutant canal cristae prior to their enlargement and fusion and displays a more diffuse and widespread expression in the base of the cochlear region whereas Prox1 is not detected in the base. These changes in *Sox2* and Prox1 expression suggest that *Lmx1a* expression restricts and sharpens *Sox2* expression thereby defining non-sensory and sensory epithelium. The adult *Lmx1a* mutant organ of Corti showed a loss of cochlear hair cells, suggesting that long term hair cell maintenance is also disrupted in these mutants.

Keywords

dreher; Lmx1a; ear; mouse; hair cell maintenance; sensory epithelium formation

INTRODUCTION

The vertebrate ear has 3 to 9 sensory epithelia consisting of hair cells and supporting cells (Lewis, et al., 1985). Mammals have three canal cristae, two gravistatic organs (utricle, saccule), an organ of Corti in the cochlea and a papilla neglecta that varies in size (Fritzsch

*Correspondence to: Bernd Fritzsch, Address: Department of Biology, University of Iowa, 143 Biology Building, Iowa city, Iowa. 52242, Phone: (319) 353-2969, FAX: (319) 335-1069, e-mail: bernd-fritzsch@uiowa.edu.

and Wake, 1988). The initially continuous sensory epithelia become separated as a result of unknown developmental mechanisms (Fritzsch, et al., 2002) by non-sensory epithelia that orient the sensory epithelia in space, channel fluid dynamics and maintain the endolymphatic environment (Lewis, et al., 1985). Sensory and non-sensory epithelia generate diffusible factors that govern the morphogenesis of nearby non-sensory epithelia (Chang, et al., 2004a, Chang, et al., 2008, Daudet, et al., 2002, Sienknecht and Fekete, 2008). Additional secreted factors originate from the hindbrain, ectoderm and mesenchyme (Chang, et al., 2004b, Fritzsch, et al., 2006b, Ohyama, et al., 2007, Pirvola, et al., 2004). Thus both global and local interactions of various diffusible factors regulate local transcription factors that govern the morphogenetic process of the non-sensory epithelium of the ear that ultimately channels physical stimuli to specific sensory epithelia. Likewise, differentiation of sensory epithelia into hair cells and supporting cells reflects temporal expression cascades of transcription factors (Fritzsch, et al., 2006a, Kelley, 2006, Kiernan, et al., 2005). However, no single factor has been described that is exclusively associated with the non-sensory epithelia during development and throughout the ear (Chang, et al., 2008, Kiernan, et al., 1997, Raft, et al., 2004) although the vast majority of Wnt transcripts are expressed in non-sensory domains (Sienknecht and Fekete, 2008). Thus a possible feedback loop between developing sensory and non-sensory areas of the ear could exist to fine tune the morphogenesis of the ear to the histogenesis of the sensory epithelia.

Lmx1a is one of four members of the Islet-Lim homeodomain transcription factor family (Hunter and Rhodes, 2005) that has three conserved members in triploblastic animals (*Isl1, Lmx1a, Lmx1b; Drosophila* orthologs: *tailup, CG32105, CG4328*, respectively). The Islet family belongs to a large family of Lim-homeodomain transcription factors that can bind to DNA in the form of monomers that form complexes with other transcription factors or in the form of heteromultimeres (Bhati, et al., 2008b, Hunter and Rhodes, 2005, Matthews and Visvader, 2003). GATA, bHLH and LMX factors interact with Lim-homeodomain factors during development. For example, such complexes are required during development of reticular formation in the hindbrain (Alenina, et al., 2006) and motoneuron formation in the spinal cord (Lee, et al., 2008Matthews and Visvader, 2003). Likewise, in insect mechanosensory development, *Isl* and *Gata/pannier* antagonize each other to regulate expression of bHLH genes necessary to develop sensory and non-sensory cells (Asmar, et al., 2008, Biryukova and Heitzler, 2005), presumably through competition for binding to another Lim-homeodomain factor. Consistent with the emerging concept of molecular conservation of essential neurosensory developmental modules across phyla (Adam, et al., 1998, Caldwell and Eberl, 2002, Fritzsch, et al., 2000, Fritzsch, et al., 2007, Pierce, et al., 2008), *Gata3* is necessary for neurosensory development of the vertebrate ear (Karis, et al., 2001, Lillevali, et al., 2006). Interestingly haploinsufficiency of *Gata3* causes hearing loss (Van Esch and Devriendt, 2001). In the ear, the expression of *Isl1* (Radde-Gallwitz, et al., 2004), *Lmx1a* (Failli, et al., 2002), Lhx3 (Hertzano, et al., 2007) and Lim only factors (LMOs) have been described (Deng, et al., 2006) but no functional analysis using LoF or Gof as yet exists.

The *dreherJ* (*Lmx1a*dr) point mutation is one of 13 known spontaneous mutations in the *Lmx1a* gene causing neurological, skeletal and otic abnormalities (Chizhikov, et al., 2006, Millonig, et al., 2000). The morphology of the *dreher*J mutant ear was initially described (Deol, 1964, Deol, 1983) and thought to be a consequence of malformations in the hindbrain (Manzanares, et al., 2000). More recent *in situ* expression show, however, a more robust and earlier expression of *Lmx1a* in the developing mouse (Failli, et al., 2002) and chicken ear compared to expression in the hindbrain (Giraldez, 1998). These *in situ* hybridization data raise the possibility that local, otic *Lmx1a* expression is required for ear development and its absence in the ear is causally linked to the ear defects. Since *Lmx1a* interacts with other Lim and LMO factors, it is an intriguing and likely possibility that *Lmx1a* and other *Isl* family members co-operate to regulate the sensory and non-sensory development of the ear. Their role in ear morphogenesis could thus parallel that of tailup/pannier in the fly mechanosensory

development (Biryukova and Heitzler, 2005) and could display a conserved interaction of bHLH, Gata and Lim transcription factors in the regulation of mechanosensory development across phyla (Fritzsch, et al., 2007). In agreement with this hypothesis, *Lmx1a* is predominantly expressed in the non-sensory otic epithelium and *Lmx1a*dr mutant mice show fusion and enlargement of sensory epithelia, dysmorphogenesis of the ear and disrupted histogenesis of sensory epithelia that eventually leads to the degeneration of hair cells. These data suggest that Lim domain factors indeed play a possibly conserved role in regulating the distinction between sensory and non-sensory epithelia in mechanosensory development across phyla. Further work is needed to unravel the details of the molecular interactions that are regulated by Lmx1a in the developing mouse ear.

Materials & Methods

Mice

*Atoh1*tm2Hzo mice were obtained from Dr. Huda Zoghbi (Bermingham, et al., 2001) and Lmx1adr/J mice from Jackson Labs and maintained in an AALAC approved facility under an IACUC approved protocol. Breeding and genotyping of the mice was as previously described (Bermingham, et al., 2001, Millonig, et al., 2000). Experimental animals were of mixed genetic stock. Timed breeding took place overnight, with midnight considered time 0.0; noon of the first day was considered as embryonic day 0.5 (E0.5). Postnatal day 0 (P0) was the equivalent of embryonic day 19 (E19) regardless of the actual birth date.

Detection of β-galactosidase activity

To detect β-gal activity, ears were dissected, briefly (30 min.) fixed in 4% paraformaldehyde/PBS, rinsed in phosphate buffer, and stained with X-gal. as previously described (Fritzsch, et al., 2005a). When required, we enhanced the X-gal. reaction using 2-photon photoactivation on whole mounts and sections (Matei, et al., 2006). Stained ears were mounted flat or alternatively, embedded in a soft epoxy resin, sectioned (3 μm) using a histology grade diamond knife (Dumont) , imaged using a compound light microscope (Nikon Eclipse 800) and captured using a Coolsnap camera and Metamorph software.

Immunohistochemistry & *In Situ* Hybridization

Primary antibodies were rat anti–mouse β-tubulin (Sigma #T6793, 1:800), Hoechst nuclear stain (Sigma), MyoVII (gift of T. Hasson, San Diego) and chicken anti BDNF (R&D Systems, 3AF248, 1:100). Whole mount *in situ* hybridization was carried out according to standard procedures (Pauley, et al., 2003) with digoxigenin-labeled riboprobes specific for *Sox2, Fgf8, Gata3* and *Fgf10*. Anti-dig-AP antibody and BM Purple (Roche) colorimetric signal detection was used. Some whole mount reacted ears were subsequently embedded in epoxy resin, cut at 5–10 μm thickness, counterstained with toluidine blue and viewed with a Nikon E800 microscope using DIC.

Secondary Alexa 488, 543, and 634–conjugated antibodies (Molecular Probes) were used predominantly on whole mounted, microdissected sensory epithelia (Matei, et al., 2005a). Sections and whole mounts were imaged using a confocal system (Biorad 2000 mounted on a Nikon E800 or Zeiss LSM 510). Images were assembled into plates using CorelDraw software.

Lipophilic dye tracing

PTI lipophilic tracers (NV red, NV Maroon) were used for afferent and efferent neuronal fibers (Fritzsch, et al., 2005b). Briefly, dyes were inserted into central targets and the fibers were filled with the diffusible dye, epithelia were microdissected and viewed with a confocal system (Zeiss LSM 510).

SEM imaging

Ears were microdissected, osmicated, dehydrated and critical point dried as previously described (Ma, et al., 2000). Ears were mounted on stubs and imaged with a Hitachi SEM.

RESULTS

Lmx1a expression is concentrated in certain non-sensory epithelia patches

Lmx1a expression was shown to be rather widespread throughout the ear between E8.5 and E10.5 (Failli, et al., 2002), but these expression analyses were limited to only those early embryonic ages. We therefore extended these investigations of *Lmx1a* expression using *in situ* hybridization. At E10.5 virtually the entire otocyst was positive for *Lmx1a* (Fig. 1A) except for a small anteroventral quadrant, the area of prosensory formation (Farinas, et al., 2001, Fekete and Wu, 2002, Ma, et al., 1998). Over the next two days, *Lmx1a* expression became focused in the developing endolymphatic duct (Fig. 1C, F, G) and the lateral margin of the cochlea duct (Fig. 1G). Strong expression also persisted in the saccular roof (Fig. 1H,I, J), the ductus reuniens (Fig. 1H,I) and near pigment cells in the utricular roof and the canal cristae (Fig. 1H,J). In the cochlea, *Lmx1a* was immediately lateral to the developing organ of Corti and medial to the pigment cells of the stria vascularis (Fig. 1K). These data suggest that *Lmx1a* outlines certain non-sensory epithelia of the ear and may be involved in specifying sharp boundaries between sensory and non-sensory epithelia.

Since *Lmxa1dr* is a nonsense mutation (Millonig et al., 2000), the presence of the mutated *Lmx1a* mRNA permits detection of alterations in *Lmx1a* expression patterns in these functional null mutants. Alteration of normal *Lmx1a* expression became evident at E11 in the mutant ears. Unlike the obvious concentrated endolymphatic duct expression in the wildtype, *Lmx1a* expression remained widespread in the mutant (Fig, 1B,C). The endolymphatic duct in *Lmx1a* mutants never developed beyond a rudimentary structure (Fig. 1E,F). The pattern of *Lmx1a* distribution in the mutants suggests a loss of segregation to non-sensory epithelia as evidenced by overlapping distribution of expression within the basal sensory region instead of clear segregation to the outer spiral sulcus (Fig. 1L–N). Although *Lmx1a* and *Lmx1b* diverged before the split of protostomia and deuterostomia, they both still share large areas of expression in the mammalian brain (Chizhikov et al., 2006). In order to determine if a similar overlapping expression pattern exists in the inner ear, we investigated the expression of *Lmx1b*. The well characterized expression of *Lmx1b* in the hindbrain and isthmus region was replicated, but no significant expression of *Lmx1b* was observed in the otocyst (Fig. 1D) or in later stages of the ear formation in wildtype or *Lmx1a* mutant mice. Based on these observations, *Lmx1b* could be eliminated as having a direct role in inner ear development. However, the strong and early expression in the adjacent hindbrain could indicate that *Lmx1b* expression might indirectly affect ear development.

We next investigated the distribution of *Lmx1a* expression in near radial sections of epoxy resin embedded, E18.5 *Lmx1a* ISH reacted ears (Fig. 2). These data show that in the mutant there is a clear medial to lateral organization of the cochlea. However, the cellular organization of the organ of Corti did not show the clear 1 row of inner and 3–4 rows of outer hair cells found in wildtype (Fig. 2 A–D). Consistent with our data on whole mounted ears, we find a considerable expansion of the expression area of the mutated *Lmx1a* mRNA beyond the lateral wall area of the wildtype. Expression could expand to the stria vascularis and even to Reissner's membrane (Fig. 2A'–D'). It appears that in the absence of functional Lmx1a protein mechanisms that help focus the *Lmx1a* expression onto the narrow part of the lateral wall between Claudius cells and the stria vascularis do not work properly.

Mutation of the *Lmx1a* gene produces a unique inner ear phenotype

The altered *Lmx1a* expression pattern in *dr* mutants suggests a basis for the observed disruption of sensory epithelium segregation cues. Since the previous studies described only an inner ear dysmorphogensis in the *Lmx1a* mutant lines (Deol, 1964, Deol, 1983); http://www.informatics.jax._org/Lmx1a_alleles), we extended these preliminary observations to define the extent of this interference in sensory and non-sensory epithelium formation. The *Atoh1tm2Hzo* allele carrying the targeted LacZ reporter (*Atoh1*LacZ) (Fritzsch, et al., 2005a) was used to identify both differentiated and undifferentiated hair cell precursors and in turn assisted in defining the regions of the developing sensory epithelium.

Postnatal one and two week old (P7 and P14) wildtype mice had six discrete *Atoh*LacZ positive sensory patches located in a complex three dimensional labyrinth of ducts and recesses (Fig. 3a). The papilla neglecta was barely detectable and consisted of 5–8 hair cells (data not shown). In contrast, P7 and P14 *Lmx1a*dr/dr mutants had only a single, undivided sac that was wide in the region of the canal cristae and continuously tapered toward the apical tip of the cochlea. While each sensory epithelium of the wildtype ears resided within its own recess (three ampullae for the canal cristae, the utricular and saccular recesses separated by the constricted utriculo-saccular foramen and the cochlear duct separated from the saccule by the ductus reuniens), none of these non-sensory constrictions were found in *Lmx1a* mutants. Nor was the constriction that normally separates the posterior ampulla from the cochlea. The data suggest that *Lmx1a* is, directly or indirectly, involved in the morphogenesis of the specific constrictions and ducts that separate the individual sensory epithelia. These mutant ears also lacked an endolymphatic duct/sac (Fig. 1E, L, 3B).

Fusion of inner ear sensory endorgans

At P7/14, only three distinct sensory epithelia instead of the usual six were found in *Lmx1a* mutants (Fig. 3A,B). Near the anterodorsal pole was a single crista consisting of two unequal sized hemicristae separated by a non-sensory cruciate eminence, suggesting that the horizontal crista had fused with one hemicrista of the anterior crista. Likewise, the posterior crista consisted of two asymmetric hemicrista separated by a cruciated eminence. The enlarged hemicrista sometimes had a distinct extension or patches extending into the cochlear duct (Fig. 3B), which might represent a fusion of the posterior canal hemicrista with the papilla neglecta. The papilla neglecta generally consists of only of a few hair cells in mammals but it can be very large in elasmobranches and becomes the comparatively large amphibian papilla in frogs (Fritzsch and Wake, 1988, Lewis, et al., 1985).

In addition to these two enlarged cristae, the *Lmx1a*dr ear contained one large continuous band of hair cells, with a very large patch adjacent to the anterior crista that we tentatively identified as a utricle-like region based on its topology (Fig. 3B; 5A). This utricular area blended into a somewhat smaller, elongated patch that we identified as a putative saccule. The saccular region continued into a progressively tapering band of hair cells that stretched to the apex of what appeared to be a shortened cochlear duct with the organ of Corti. While the hair cells of this patch were continuous, the pattern of innervation indicated four distinct regions in this epithelium (Fig. 3B,G; 4C,). The utricle region was innervated by fibers that were accompanied by others that continued on to the anterior crista, as in wildtype animals. Likewise, the saccular portion received fibers from both the superior and inferior vestibular ganglion as in wildtype (Fig. 4C). In contrast, the cochlea organ of Corti, showed two distinct regions with respect to its innervation: The base received only a patchy and somewhat reduced density of nerve fibers whereas the apex was densely innervated with radial fibers like wildtype (Fig. 3D; 4A,B,C). These fibers did, however, arise from a typical spiral ganglion.

Beyond the differences in density of innervation, older *Lmx1a* mutant mice showed tubulin-positive pillar and Deiter's cells only in the apex (Fig. 3D; 4D,F, G,I). The absence of these cells in the base correlated with a different distribution of hair cells. In the apex, both inner and outer hair cells could be identified and were separated from one another by unusually positioned but otherwise typical pillar cells (Fig. 3C–F, 4 E–I). It was therefore identifiable as an organ of Corti-like organization of cells. In contrast, in the base, hair cells formed multiple rows of uniform cells that defied any histological characterization as inner or outer hair cells (Fig. 3C,E; 4 F,I). Despite their unusual pattern of distribution, all were hair cells as revealed by both *Atoh1*^{LacZ} histochemistry (Fig. 3B,C,G) and immunofluorescent detection of the hair cell-specific marker, myosin VIIa (Myo7a) (Fig. 4D,F,H,I). The common utriculo-sacculo-cochlear sensory epithelium (Fig. 3B,5A) of *dr* mutant mice is thus reminiscent of the common macula of jawless vertebrates (Lewis, et al., 1985). This common macula is then the precursor of the several sensory epithelia that segregate from one another during the course of development and evolution (Fritzsch, 2003,Fritzsch, et al., 2002). Clearly, *Lmx1a* is a major molecular player in this process during development and may play the same role during ear evolution.

Hair cells are lost in the adult *Lmx1a* mutant cochlea

Using *Atoh1LacZ* histochemistry we found gaps in the distribution of cochlear hair cells as early as P14 (Fig. 1 G, H; arrow). Notably, these gaps tended to appear at the boundaries between the utricle, saccule and basal region of the cochlea. Likewise, the apex showed a patchy distribution of hair cells in the older epithelia (Fig. 3G). Investigating the cochlear histology at 2–3 months of age or older revealed a complete loss of all hair cells and severe dysmorphogenesis of the organ of Corti, including the adjacent areas such as spiral limbus, Reissner's membrane and stria vascularis (Fig. 3I,J). Few hair cells remained in the vestibular organs (data not shown). These data suggest that *Lmx1a* is not only playing a major role in early histogenesis and morphogenesis of the ear but is also essential for long term maintenance of hair cells.

The amalgamated cochlear-gravistatic endorgan boundary

In a series of experiments, we next characterized the unique morphological and histological phenotype of the fused cochlear-gravistatic endorgan. Scanning electron microscopy (SEM) was used to define the apical specializations in the area where the saccular and cochlear regions merged (Fig.6A,B). Despite our best efforts we could not mechanically remove the tectorial membrane near this transition site. Importantly, the presence of a tectorial membrane defines a molecular transition between the saccule (no tectorial membrane) and the cochlea (tectorial membrane) despite continuity of hair cells. Closer examination revealed distinct vestibular-type hair cells in the saccular and utricular regions (Fig. 6C,D,E). Near the tectorial membrane we found medial cells to display vestibular-type, long, organ pipe-like stereocilia. In contrast, more C-shaped, shorter stereocilia reminiscent of organ of Corti inner hair cells prevailed more laterally, indicating an introgression zone of vestibular and organ of Corti type of hair cell differentiation. Ventral to this zone, radial histological sections (Fig. 3E) showed that 8–11 rows of hair cells were present in the basal part the cochlear region. Interestingly, the polarity of these inner hair cell-like cells was normal in the more medial cells but the more lateral cells were rotated 90 degrees toward the base, much as in mice mutant for the *Foxg1* (Pauley, et al., 2006) and *Neurog1* (Ma, et al., 2000) genes.

This non-cochlear organization of hair cells and supporting cells in the cochlea-like basal region might result from mis-expression of transcription factors uniquely associated with the organ of Corti. One transcription factor associated with supporting cells is *Prox1* (Bermingham-McDonogh, et al., 2006, Fritzsch, et al., 2008, Puligilla, et al., 2007). *Prox1* distribution in *Lmx1a*dr mice showed a marked deviation from the wildtype, even in the apex

that was more typical in its supporting cell and hair cell organization (Fig. 5A–C, 7A–D). Instead of a regular pattern of supporting and hair cells, *Prox1* positive cells in apex of *Lmx1a* mutants showed irregular distribution with an inconsistent number of rows. To obtain more information about co-localization of hair cells and *Prox1* positive supporting cells, hair cells were labeled by BDNF immunodetection. In *Lmx1a* mutants, the innermost row of hair cells showed strong BDNF staining, much like that observed in wildtype mice. However, unlike the wildtypes, BDNF was just above background levels in the most lateral outer hair cells of the mutants (Fig. 7B"). Most interesting was the mutant distribution of *Prox1* near the transition from the near normal apical organ of Corti to the more vestibular-like basal region. *Prox1* could not be detected in the base, except for a large aggregate of labeled cells near the cochlear-saccular transition zone (Fig. 7C,D). We sought to determine how early this organization became apparent and found it in place as early as E16.5 using *Prox1* immunodetection and *Atoh1*LacZ labeled hair cells (Fig. 5). As in later stages there was little to no expression of *Prox1* throughout the base except at this transition zone (Fig. 5, 7). In wildtype mice, outer hair cells are regularly interspersed with *Prox1* expressing Deiter's cells (Fig. 7 A"'). However, in *Lmx1a* mutants at these stages, all hair cells were medial to the *Prox1* expression area, in both the apical and basal regions (Fig. 7 C"'). Combined these data suggest that *Prox1* and *Atoh1* expressing cells partially segregate in the absence of *Lmx1a* expression implying that *Lmx1a* functions in setting up the topology for those two cell types and their specific distribution along the cochlea. A possible candidate could be diffusible factors emanating from the lateral spiral sulcus (Fig. 1K; 2 B') where the *Lmx1a* expression is most prominent.

The distinct basal cellular organization can be defined by gene expression

We next examined *Gata3, Fgf8* and *Fgf10* transcript distribution to further elucidate the extent of the basal region disorganization at the level of gene expression. The distribution of *Gata3*, a zinc finger transcription factor, is uniquely associated with the cochlea, but not observed in the vestibular (specifically, saccular) sensory epithelia (Karis, et al., 2001). It is known to interact with Lim-homeodomain and bHLH factors needed for cell fate specification (Matthews and Visvader, 2003). In addition, the two fibroblast growth factors have distinct expression patterns where *Fgf8* is associated with the inner hair cells and *Fgf10* is expressed in the greater epithelial ridge just adjacent to inner hair cells (Pauley, et al., 2003, Pirvola, et al., 2002). Expression of both *Fgf10* and *Gata3* showed that the base region gradually merged into the apex with respect to these markers, but distinct from the saccular region (Fig 8 A,B,E,G). There was also an expansion of *Gata3* expression across the cochlea in *Lmx1a* mutants compared to wildtype littermates (Fig. 8 E,G). In *Lmx1a* mutants, *Fgf8* expression expanded across most of the several rows of hair cells in the basal organ of Corti, whereas only inner hair cells were labeled in the apical region, as in wildtype littermates (Fig. 8 C, D ,F). Most interestingly *Lmx1b*, which shares a high sequence homology with *Lmx1a*, is a known regulator of *Fgf8* in the isthmic region of mice and zebrafish (Alexandre, et al., 2006, Guo, et al., 2007, O'Hara, et al., 2005). Since our data show an expansion of *Fgf8* expression in the base (Fig. 8 C) but no *Lmx1b* expression was detected in the ear, this suggests that wildtype *Lmx1a* somehow restricts *Fgf8* expression in the basal cochlea to inner hair cells. This might result from, its early expression in this region during the otocyst stage, or from diffusible factors released as a result of its later expression in the outer spiral sulcus (Daudet, et al., 2002).

The Fgf8 data support the impression derived from the Lmx1a mutant SEM data that all hair cells in the basal portion of the cochlear region develop an inner hair cell phenotype. Furthermore, alterations in *Fgf8* and *Gata3* expression as well as the near complete absence of *Prox1* expressing cells in the base suggest that these too play roles in generating the altered phenotype of basal turn hair cells. Consistent with the *Lmx1a* expression pattern (Fig. 1N), *Lmx1a* is apparently necessary to define the lateral boundaries of the organ of Corti (which is more irregular in these mutants) and enhance the interaction of supporting cells and outer hair

cells. The absence of functional Lmx1a protein may underlie the disruption of outer hair cells and Deiter's cells observed in the *dr* mutant mice (Fig. 3), possibly through indirect effects on the integrity of sensory/ non-sensory boundaries. Proper cellular restriction of early expression of the *Lmx1a* transcription factor appears to be necessary for coordinated development of the organ of Corti.

The utricle, saccule and organ of Corti never segregate during development

Given that our *Lmx1a* expression analysis suggests a possible role in ear formation as early as E11 (Fig. 1B,C), the initial upregulation of *Atoh1*LacZ was compared in *Lmx1a* mutants and wildtype animals (Chen, et al., 2002, Matei, et al., 2005a). As early as E14.5 the hair cells of the six epithelia of the wildtype ear were distinctly labeled (Fig. 9A). In the mutant, and in contrast to later stages, three cristae could be recognized. However, a single cochlear-gravistatic endorgan was already in place, though distinct hair cell patches were observed within the common endorgan. Specifically, there was an area of constricted hair cell formation indicating the utricular/saccular regional transition Fig. 9B,C) and the cochlea showed little to no upregulation of *Atoh1-LacZ* in the base. In spite of this absence of staining, a basal cochlear prosensory precursor epithelium could be identified using differential interference microscopy (Fig. 9C). These data suggest a delay in hair cell maturation in the base of the organ of Corti that could contribute to the misexpression of the several of the factors we have already described above and contribute to the histological defects observed in the basal region of the organ of Corti in *Lmx1a* mutant mice.

In order to verify the presence of distinct subcompartments within the cochlear-gravistatic epithelium vestibular afferents were labeled from the brainstem and vestibule-cochlear efferents were labeled from rhombomere 4, where the olivocochlear bundle crosses (Bruce, et al., 1997, Fritzsch and Nichols, 1993). We had previously shown that with such double labeling, vestibular and cochlear fibers could be distinguished throughout development (Tessarollo, et al., 2004). As expected, brainstem vestibular projections labeled all afferents to vestibular organs and showed discrete innervation of a large utricular area and a smaller saccular portion (Fig. 9D, E). In contrast, the cochlear region received only efferent fibers and was thus identifiable based on this specific innervation. While anterior and horizontal cristae were normal in their innervation pattern, the posterior crista innervation was expanded by fibers targeting the papilla neglecta In summary, hair cell formation in the cristae and papilla neglecta of Lmx1a mutant mice starts as discrete patches, which only later fuse into composite structures. In contrast utricle, saccule and organ of Corti form as a common sensory epithelium. This common epithelium nevertheless shows differences in hair cell organization, maturation and innervation that warrant labeling them as run-on precursors of utricle, saccule and organ of Corti.

The unique phenotype of the *Lmx1a* mutant is foreshadowed by early alterations in *Sox2* expression

In the chick (Giraldez, 1998), as in mice (Faille et al., 2002), *Lmx1a* has been shown to be excluded from the neurogenic region of the otic placode. In addition, it was found that inhibiting the Notch signaling pathway allowed the spread of *Lmx1a* expression into the neurogenic region (Abello, et al., 2007). This was reminiscent of the 'non-neural' *Tbx1* gene of mice, that when mutated allowed neural genes, including *Neurog1*, to expand their expression domains, and when overexpressed caused the retreat of neural gene expression (Raft, et al., 2004). To obtain further insights into the molecular organization of supporting cells, the distribution of *Sox2* was next studied. *Sox2* is necessary for prosensory specification (Fritzsch, et al., 2006a, Holmberg, et al., 2008) and is later restricted to expression in supporting cells (Kiernan, et al., 2005). We therefore sought to determine whether prosensory gene expression would expand into non-neural territory in the *Lmx1a* null mice.

In E10.5 wildtype animals *Sox2* expression was in future sensory areas of the antero-ventral quadrant and in the anlage of the posterior crista (Fig. 10A). This general pattern was retained in the mutant, but the areas of expression were greatly expanded, with more diffuse margins. This was most apparent in the region of the posterior crista (Fig. 10B). By E11.5, however, while expression in the wildtype remained strong and neatly confined to future sensory areas, that in the mutant remained broader in the region of the posterior crista (Fig. 10C,D). It was noted that segregation of the anterior cristae from the utricle was completed in both wildtype and mutant mice. In contrast no horizontal crista prosensory patch appeared as a discrete entity in the *Lmx1a* mutants (Fig. 10C,D). Both wildtype and mutant showed a continuous expression band with focal increases in intensity of the utricle, saccule and organ of Corti (Fig. 10C,D). By E14.5, all six epithelia were distinct in the wildtype, but the mutant showed a combined anterior and horizontal crista, a combined posterior crista and papilla neglecta (including the extension along the cochlear lateral wall) and the fused cochlear-gravistatic endorgan. Most interesting were the pattern and intensity of *Sox2* expression in the cochlear region compared to the contiguous saccular portion. In both wildtype and mutant the cochlear expression was much reduced in intensity compared to the saccule. In addition, the cochlear expression was extremely broad in the base of the mutant and much less focused than in wildtype (Fig. 10E, F). This broader and more diffuse pattern of expression persisted in the mutant base through E18.5. The mutant apex developed a focused expression reminiscent of the entire wildtype cochlea (Fig. 10H,I,J,K).

In summary, the wildtype and *Lmx1a*dr mutant exhibited major differences in initial hair cell formation as revealed by *Atoh1*LacZ staining (Fig. 9B) and in prosensory patch formation as revealed by *Sox2* expression (Fig. 10G). Whereas prosensory patch formation maintained from its onset the fusion/combination of sensory epithelia, hair cell formation initially showed all six sensory epithelia as discrete entities embedded in the unsegregated prosensory patches specified by *Sox2*. These differences suggest that initial *Atoh1* upregulation as well as cell cycle exit of hair cells (Matei, et al., 2005b) and expression of *Sox2* might be under different regulatory control. However, *Atoh1* expression and initial cell cycle exit take place in the *Sox2* expression domains. These data also suggest that in the mutant, it is the initial expression changes in *Sox2* that foreshadow the later expansion and disorganization of hair cells in the mutant cochlea and the overgrowth of the papilla neglecta. It should also be noted that in the wildtype animal an Lmx1a expressing caudal constriction separates the posterior crista from the cochlea and thus contributes to the formation of the *Lmx1a* expressing ductus reuniens. This constriction remains rudimentary, and the ductus nonexistentin the *Lmx1a* mutant, while the papilla neglecta overgrows the site preserved by the failure to form the constriction. Thus, in the wildtype, an enlarged papilla neglecta does not form and fuse with the posterior crista both because *Lmx1a* expression confines *Sox2* expression and because it creates a non-sensory constriction at the site where the enlarged papilla would form in the mutant.

DISCUSSION

Lmx1a mutants show that non-sensory otic epithelium facilitates ear morphogenesis

Our *Lmx1a* expression data show a unique association with specific areas of non-sensory otic epithelium in older ears (>E10.5). These *Lmx1a* expressing non-sensory epithelia are involved in:

1. Separating and constricting the endolymphatic sac from the saccule by formation of the endolymphatic duct,
2. Separating and constricting the utricle from the saccule by forming the utriculo-saccular foramen, and

3. Separating and constricting the cochlea from the saccule and posterior crista by forming the ductus reuniens,

None of these non-sensory constrictions form in *Lmx1a* null mutants, implying that *Lmx1a* plays a direct or indirect role in all of these morphogenetic events and that these events are driven in part by the *Lmx1a* expression in the non-sensory epithelia. In addition *Lmx1a* is expressed in the outer spiral sulcus separating the organ of Corti from the stria vascularis (Fig. 1K; 2A–D). It is possible that a lack of Lmx1a expression adjacent to developing sensory epithelia is interfering with signaling from the sensory epithelia to govern coordinated morphogenesis of the non-sensory otic epithelia. Alternatively, *Lmx1a* expression might initiate the secretion of diffusible factors(s) from the non-sensory otic epithelium required for proper sensory epithelia maturation.

The hypothesis that *Lmx1a* interferes with a sensory epithelium signal is in line with the emerging concept that crista epithelia express diffusible factors such as *Fgf10* and *BMP4* that regulate growth of the non-sensory part of the vertical canals (Chang, et al., 2004b, Chang, et al., 2008, Fritzsch, et al., 2006b, Pauley, et al., 2003). In contrast, the cochlea grows by intercalation of sensory and non-sensory epithelia (Wang, et al., 2006) and does so even when differentiating hair cells never form (Fritzsch, et al., 2005a) or when the prosensory anlage of the organ of Corti is disrupted (Kiernan, et al., 2005). The data support models that propose more sophisticated molecular interactions and possible feedback loops of sensory and non-sensory epithelia to complete morphogenesis (Chang et al., 2008). These hypothesis are in line with the known inner ear otic mesenchyme interactions that require bilateral signals, only some of which are presently understood (Pirvola, et al., 2004).

Overall, the *Lmx1a* mutant dysmorphogenesis is a more exaggerated form of the *Otx1* null dysmorphogenesis where utricle and saccule stay in communication via an open utriculo-saccular foramen and no ductus reuniens forms (Fritzsch, et al., 2001, Morsli, et al., 1999). The two phenotypes differ, however, in that the *Otx1* mutant organ of Corti remains distinct from the saccule in most cases and develops a normal histology. In contrast to *Lmx1a* expression, *Otx1* expression is found in both non-sensory and sensory compartments during ear morphogenesis (Morsli, et al., 1999) and thus does not permit the contention that its non-sensory expression that is uniquely involved in ear morphogenesis. Several other genes also affect ear morphogenesis (Chang, et al., 2004b, Fritzsch, et al., 2007). However, the limited characterization of their expression patterns does not allow making the distinctions we can make here for *Lmx1a* gene expression and function. In addition, the primary action of some of these genes is in the brain with the ear being secondarily affected. Others are overlappingly expressed with both sensory and non-sensory parts of the ear, as in the case with *Otx1* and *Foxg1* (Pauley, et al., 2006, Raft, et al., 2004). Based on the *Lmx1a* expression pattern and defects in null mutants, we show here for the first time that a gene expressing a non-diffusible factor exclusively in non-sensory areas of the differentiating ear is essential for aspects of ear morphogenesis and sensory organ histogenesis.

Lmx proteins are known to regulate *Fgfs*, *Wnts* and *Bmps* in parts of the CNS (Adams, et al., 2000, Alexandre, et al., 2006, Chizhikov and Millen, 2004, Guo, et al., 2007, Matsunaga, et al., 2002, O'Hara, et al., 2005). These secreted factors are major players for ear morphogenesis (Chang, et al., 2008, Pauley, et al., 2003, Riccomagno, et al., 2005, Wright and Mansour, 2003). Clearly, there is an altered expression of *Fgf8* in the basal organ of Corti of Lmx1a mutants, showing that indeed disregulation of at least one *Fgf* factor occurs in the ear and there is severe dysmorphogenesis that could be related to disregulation of both *Fgf*s and *Bmp*s. *Wnt*s have also been shown to be crucial for ear placode formation (Ohyama, et al., 2006) and to play a major role in ear morphogenesis (Riccomagno, et al., 2005). Moreover, both Wnt's and some Fgfs are secreted by the non-sensory part of the developing ear. It is therefore possible that a *Wnt* or *Fgf* factor such as *Fgf9* (Pirvola, et al., 2004) or Wnt4 (Daudet, et al., 2002) is

directly regulated by *Lmx1a* and that these and other factor(s) released from the *Lmx1a* expressing non-sensory areas of the developing ear regulate those crucial aspects of ear morphogenesis and provide a feed-back loop for sensory epithelium development. Absence of the endolymphatic duct in *Lmx1a* mutant could certainly relate to the similar absence of the endolymphatic duct in *Ffg3* null mice (Hatch, et al., 2007) and could explain the ultimate loss of all hair cells in *Lmx1a* null mice as a consequence of disturbed endolymphatic homeostasis, such as that reported in *Foxi1* and *Pendrin* mutant mice (Blomqvist, et al., 2006, Hulander, et al., 2003). The absence of an endolymphatic duct does in fact result in the absence of *Foxi1* expression (data not shown) and a likely lack of pendrin expression in the missing endolymphatic duct. Further studies are needed to test for ionic disregulation in the *Lmx1a* mutant mice that could also play a role in the adult hair cell loss.

In this context it is also important to note that no pigment cells reach the lateral wall of the cochlea but rather accumulate near the radial fibers in *Lmx1a* null mutant. WNTs, in interaction with Wnt signaling modulators, such as the Dickkopf (DKK) family of secreted factors, set up gradients along which cells migrate. For example, in the skin, DKK interacts with WNTs in a reactiondiffusion mechanism that sets up the spacing of hair follicles (Sick, et al., 2006). It also interacts with WNTs in head morphogensis (Lewis, et al., 2008). Similar issues of spacing between sensory epithelia are in part the cause of dysmorphogenesis in the *Lmx1a* mutant ear. The similarity of several aspects of the *Otx1* and *Lmx1a* phenotypes combined with the fact that *Dkk1* can rescue the *Otx2* phenotype (Kimura-Yoshida, et al., 2005, Lewis, et al., 2008) implies that *Lmx1a* may play an unspecified part in the *Wnt-Otx* mediated morphogenesis. It is similarly possible that the close proximity of pigment cells to *Lmx1a* expressing areas is suggests a modulated Wnt signaling. Such signaling may guide pigment epithelial cells to known areas of endolymph production (stria vascularis, dark cells of the utricle, and canal cristae) and resorption (endolymphatic duct). This possible involvement of Lmx1a protein in formation and resorption of endolymph, once verified in the *Lmx1a* mutant ear, could reflect a conserved function of this gene in the ear and hindbrain where *Lmx1a* is involved in the formation of CSF secreting choroid plexus (Chizhikov, et al., 2006, Elsen, et al., 2008). Wnts and their intracellular effector, β-catenin play important roles in ear formation (Ohyama, et al., 2006, Riccomagno, et al., 2005). However, more work is needed to elucidate the role of Lmx1a in local otic Wnt secretion (Daudet et al., 2002) and its modulation.

Histological defects of *Lmx1a* mutants relate to gene misexpression

Our data show that the enlarged anterior and posterior canal cristae of *Lmx1a* mutants result from the fusion of the horizontal crista and negelected papilla with the anterior and posterior crista, respectively. Interestingly, the initial upregulation of *Atoh1* to differentiate hair cells is discrete but embedded in an enlarged or fused *Sox2* expression domains (Fig. 10). It is only in late embryonic stages that the hair cell patches fuse into these enlarged epithelia (Fig. 3B, 9B). These data imply that two independent, but topographically related processes focus *Sox2* expression to the prosensory patches and *Atoh1* expression to the first differentiating hair cells inside the *Sox2* expression domains. Obviously, the effect of the absence of Lmx1a expression is more profound on prosensory patch formation which foreshadows the later phenotype that simply fills in the prosensory domain with differentiated hair cells. Neither the focal prosensory patch formation nor the focal *Atoh1* upregulation is understood at a molecular level (Kiernan, et al., 2005,Matei, et al., 2005b). Like the Lim domain factor tailup in insect sensory development (Biryukova and Heitzler, 2005), *Lmx1a* may counteract the bHLH gene upregulation mediated by *Gata3* (Karis, et al., 2001) and other factors. However, a complete inventory of Lim-homeodomain factors in ear development is necessary before such multimeric interactions (Bhati, et al., 2008a,Matthews and Visvader, 2003) can be understood.

We suggest that the common function of the Lim genes *Isl/tailup* (flies) and *Lmx1a* (mice) is to define non-sensory cells. This suggestion is in agreement with existing models comparing insect and vertebrate mechanosensory development (Caldwell and Eberl, 2002, Fritzsch, et al., 2000). However, whereas the sorting of insect sensory and non-sensory cells is reinforced by delta-notch signaling, we argue here that diffusible factor gradients may be altered in the multicellular non-sensory spacers that form between sensory epithelia of mice, complementing the delta-notch function in sensory epithelium segregation (Daudet and Lewis, 2005).

In contrast to canal cristae, the wildtype utricle, saccule and cochlea form as a single elongated epithelium that only segregates later in development (Farinas, et al., 2001, Fritzsch, et al., 2002, Morsli, et al., 1998). Obviously, neither prosensory epithelium formation nor hair cell differentiation demonstrate any such segregation in *Lmx1a* mutants. Segregation appears only during senescence of the adult ear when hair cells die. The blending of vestibular with cochlear hair cell types near the saccular-cochlear transition might be due to cell-type mingling across an undetected border. However, it might also suggest that proximity prevents specific factors associated with vestibular and cochlear hair cell differentiation or segregation from functioning normally. In addition, the absence of *Lmx1a* might change radial gradients of Wnts or other diffusible factors and cause the altered morphology. Certainly, the broad expression of *Fgf8* in *Lmx1a* mutant basal turn hair cells combined with the wider expression of *Sox2* and the absence of *Prox1* could result in sophisticated changes in signaling that would lead to the observed dismorphogenesis. Mutating Fgfr3 can clearly alter pillar and Deiter's cell differentiation (Puligilla et al., 2007). Obviously, changing the expression of *Fgf8* from a single to multiple rows can affect differentiation of pillar and Deiter's cells as shown for mutations of *Fgfr3* receptor (Puligilla, et al., 2007). Clearly, the next step is to define such factors that are disregulated by *Lmx1a* and cause dysmorphogenesis and/or altered histogenesis.

Evolutionary implications

The morphological and histological defects reported here suggest that in the absence of Lmx1a protein, the mouse ear reverts to a hagfish-like ear consisting of a simple torus with two canal cristae and a single common macula for the gravistatic organs (Fritzsch, et al., 2006b). Once DNA sequencing efforts in hagfish have been completed it will be possible to begin determining how *Lmx1a* expression differs across vertebrate phyla. It is possible that *Lmx1a* plays the same role for the segregation of the common macula into multiple end organs as *Otx1* (Fritzsch, et al., 2001) and *Foxg1* (Pauley, et al., 2006) play for the formation of the horizontal canal and horizontal canal crista , respectively, in development and evolution. Further work is needed to show whether the fly *Lmx1a* ortholog, *CG32105* is associated with chordotonal organ development. Alternatively, *Lmx1a* may play a conserved role in ionic homeostasis, which is critical for proper function of ear and mechanosensors alike (Fritzsch, et al., 2000, Fritzsch, et al., 2007, Todi, et al., 2004, Walker, et al., 2000). Irrespetive of the details, our results suggest local Lmx1a expression is a major player in ear morpho-and histogenesis and this function is likely conserved at the level of interacting modules of transcription factors.

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Abbreviations for all plates

AC, anterior crista
CO, cochlea
D, Deiter's cell
DR, ductus reuniens
GER, greater epithelial ridge
HP, habenula perforate
HC, horizontal crista
IHC, inner hair cell
OC, organ of Corti
OHC, outer hair cell
P, pillar cells
PC, posterior crista
PN, papilla neglecta
RF, radial fibers
S, saccule
SL, spiral limbus
Spg, spiral ganglion
SV, stria vascularis
U, utricle
USF, utriculo-saccular foramen

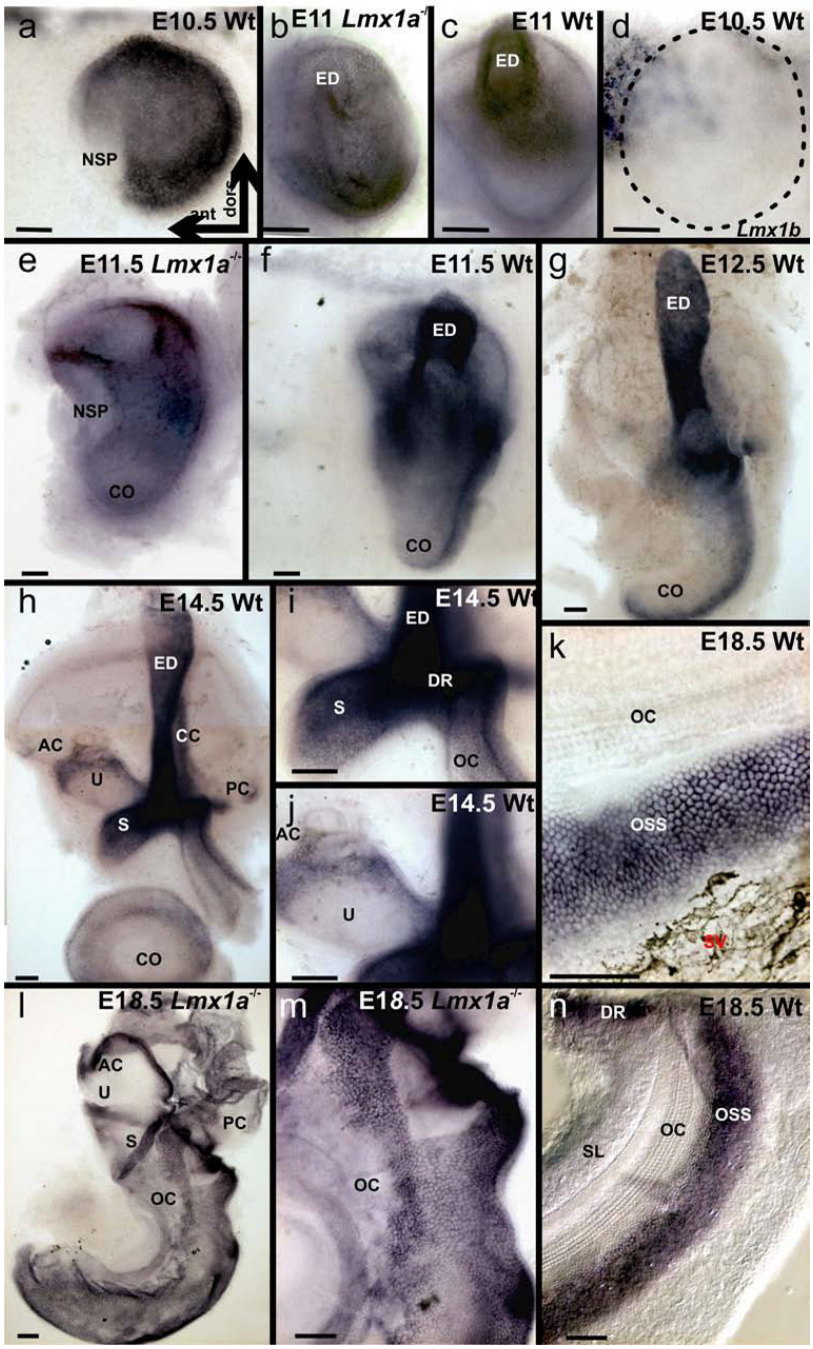

Fig. 1. *Lmx1a* expression undergoes dynamic changes in wildtype and mutants

A. In the E10.5 wildtype, expression is in all but the neurosensory precursor epithelium (NSP). B., C. Compare the wildtype endolymphatic duct (ED) with its vestigial mutant counterpart. D. *Lmx1b* is not expressed in the E10.5 ear (dotted line), but is strongly expressed in the hindbrain (in and out of focus labeling top left). E., F. In the E11.5 mutant, Lmx1a expression is incompletely segregated to the lateral/posterior cochlea (CO) and no trace of an endolymphatic duct is apparent. G. At E12.5, expression is becoming confined to the ED, lateral cochlea and the non-sensory saccule. H.-J. By E14.5, expression centers on the ED and radiates to more-or-less constricted spacer epithelia between sensory epithelial territories. Most conspicuous of these is the ductus reunions (DR). K. At E18.5, cochlear expression is localized

to the outer spiral sulcus (OSS). Melanocytes are conspicuous in the adjacent stria vascularis (SV). L.-N. In wildtype mice, the organ of Corti (OCin N.) is entirely free of *Lmx1a* expression. However, mutant Lmx1a mRNA is broadly expressed throughout the basal turn (L,M). All whole ears are oriented as in A. Scale bars are 100 μm.

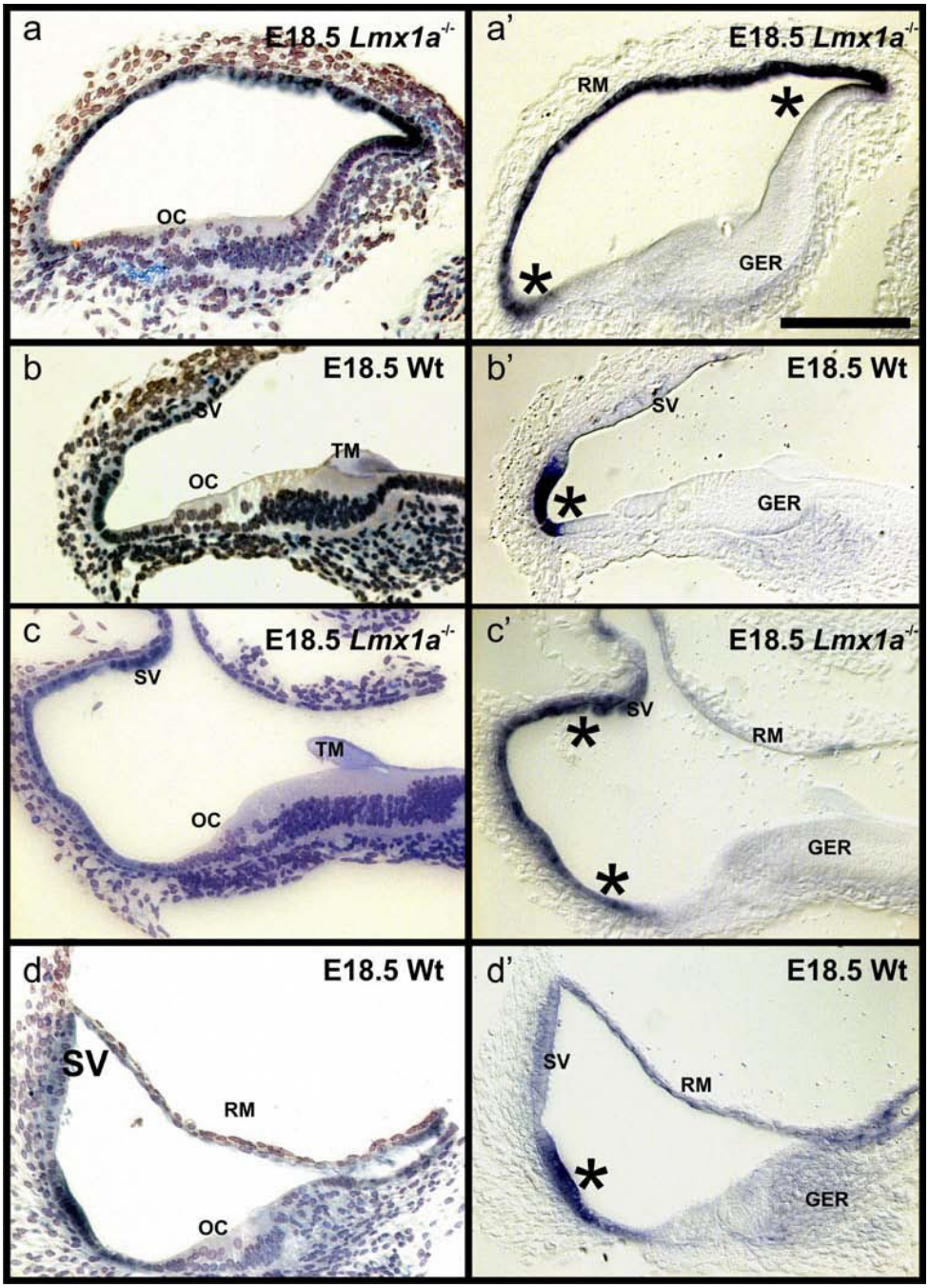

Fig. 2. *Lmx1a* expression is less restricted in mutants

These sections show the distribution of *Lmx1a* ISH reaction product in wildtype (B,D) and *Lmx1a* mutant mice of embryonic day 18.5. The ears were reacted for whole mount in situ hybridization (see Fig. 1), embedded in soft epoxy resin and sectioned at 5–10 μm. These radial sections show a well organized organ of Corti (OC) with one inner and 3–4 rows of outer hair cells in the wildtype (B,D). In contrast, hair cells are disorganized in the *Lmx1a* mutant mice (A,C). Nevertheless, the main medial-to-lateral areas of the cochlea such as greater epithelial ridge (GER) with a tectorial membrane (TM), OC and lateral wall are distinct. In wildtype mice the *Lmx1a in situ* signal is in the lateral wall (asterisk) adjacent to the stria vascularis (SV; B', D'). In contrast, in the *Lmx1a* mutant mice the strong *Lmx1a in situ* signal expands

to include the stria vascularis (asterisks) or even Reissner's membrane (RM; A',C'). Bar in A' indicates 100 μm for all images.

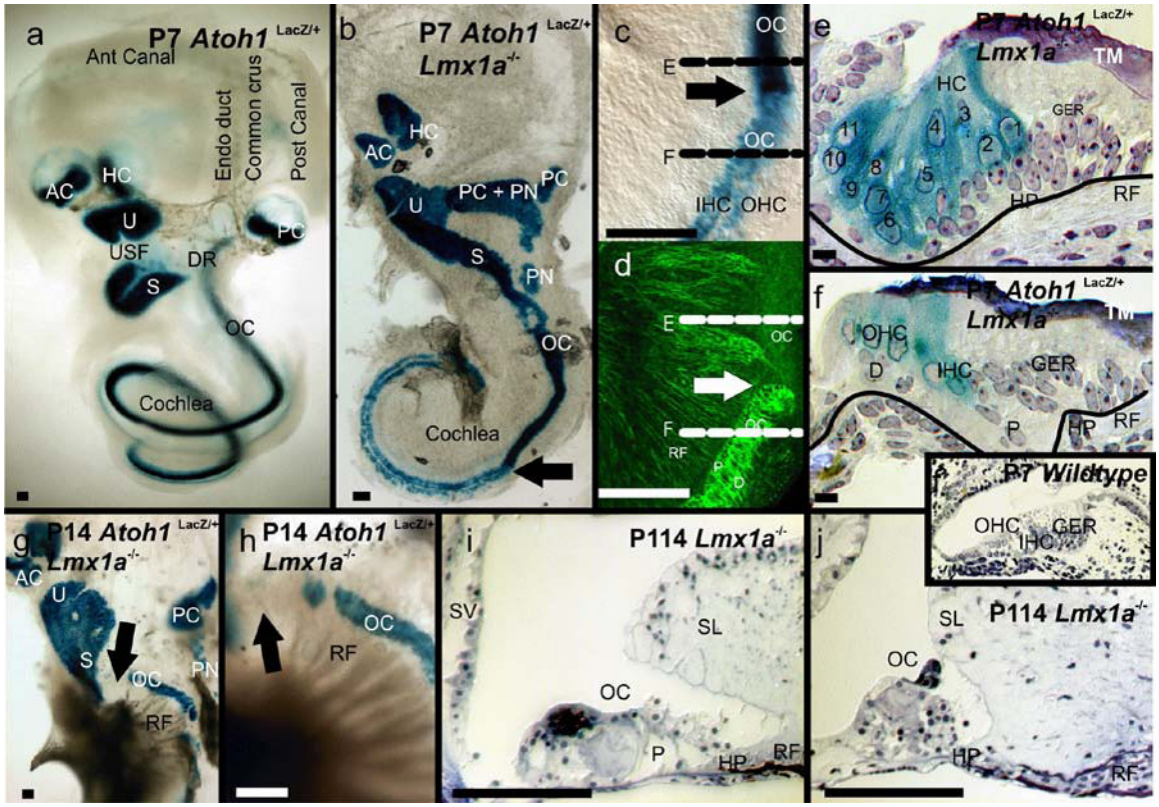

Fig. 3. Postnatal *Lmx1a* mutant ears reveal disorganized sensory epithelia

A. In the wildtype, six discrete sensory epithelia are separated from one another by constricted, non-sensory epithelial spacers. Two of three semicircular canals and an endolymphatic duct can be identified as shadows. B. In the *Lmx1a* mutant, the anterior and horizontal cristae are separated by a common cruciate eminence, while the posterior crista is grossly enlarged and extended by the presence of both embedded and detached papilla neglecta-like sensory epithelia (PN). The utricle, saccule and cochlear sensory epithelia appear continuous with one another. The basal turn of the organ of Corti appears as a uniform band of hair cells that is discretely separated (arrow) from an apex in which inner and outer hair cells can be identified. C, Higher magnification view of the arrowed transition in B. The densely packed hair cells of the basal cochlea are above the arrow and the apex below it. The black lines indicate the planes of section in E and F. D. Same tissue as C, but stained for beta-tubulin to reveal nerve fibers and pillar and Deiter's cells. Note the absence of tubulin-containing pillar and Deiter's cells in the base and their conspicuous appearance at the transition to the apex. E. A medio-lateral near radial section across the base of the mutant cochlear duct as indicated by the dotted line in C. Note the presence of a tectorial membrane (TM). Up to 11 rows of hair cells are marked by the blue Atoh1^{LacZ} reaction product, unlabelled supporting cells are present below the hair cells. F. A recognizable organ of Corti with inner and outer hair cells is present in the apex (compare with wildtype insert). G,H Beginning around P14, hair cells disappear, starting in the base (arrow). In contrast, nerve fibers continue to mature as indicated by the osmium tetroxide stained myelin (black fibers). I., J. By P114 the organ of Corti is grossly disorganized, lacks identifiable hair cells and shows massive aberrations in almost all associated epithelia such as the spiral limbus (SL) and the stria vascularis (SV). These data suggest that absence of *Lmx1a* is ultimately incompatible with hair cell maintenance though that its absence does not interfere with their initial formation. Scale bars are 100μm.

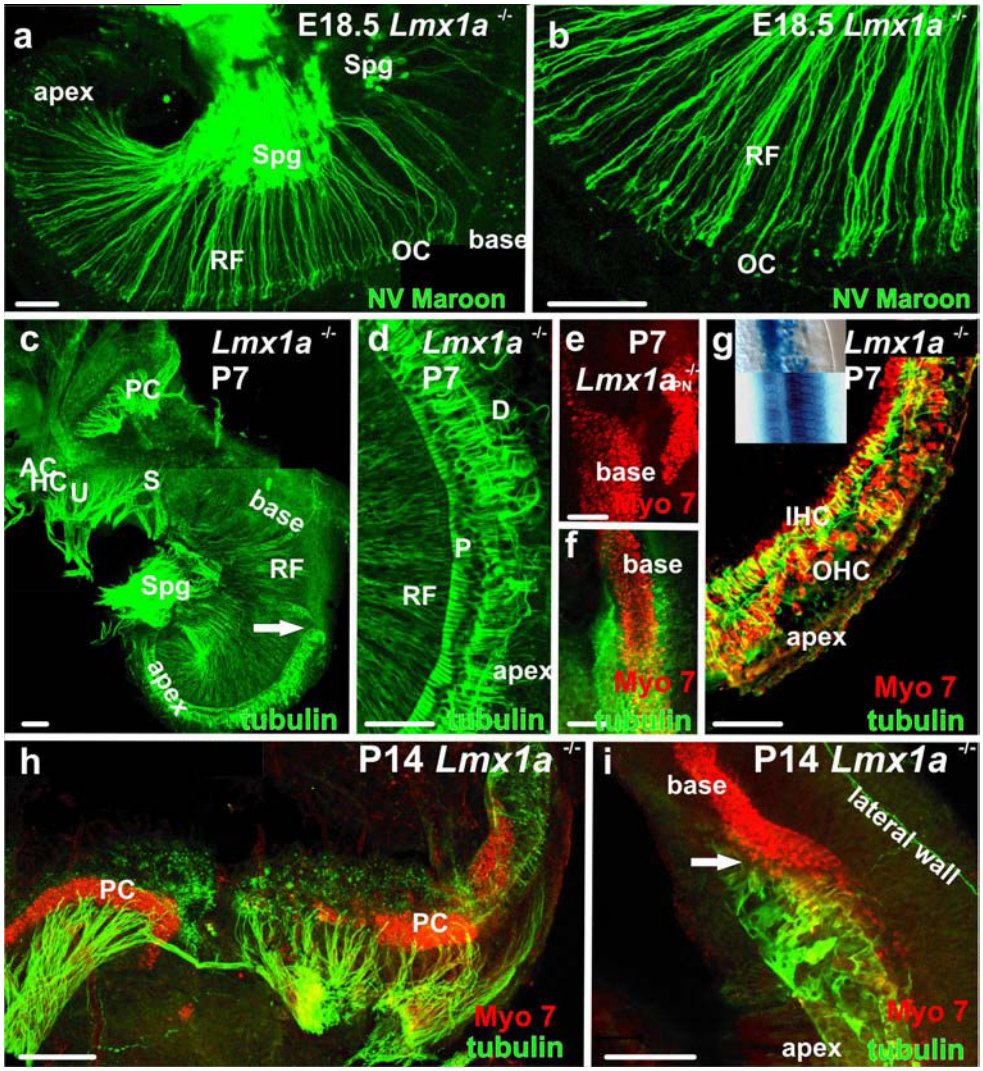

Fig. 4. Late innervation and sensory epithelia are disorganized

A, B Afferent radial fibers to the base of the E18.5 mutant cochlea stained with the lipophylic dye, NV Maroon (A,B) and anti-acetylated tubulin (C,D,F-I). Note that the fibers enter the organ of Corti but do not extend to the outer hair cells as they would in a comparable wildtype ear. There is a notable difference between the packing density of radial fibers in the base and the apex consistent with the reduced presence of spiral ganglia in the base. C There is a complete absence of pillar/Deiter's cells basal to the cochlear transition (arrow) and clear distinction of innervations between the densely innervated saccule and the poorly innervated basal turn of the cochlea. D. Disorganized Deiter's cell processes (D) in the apex of the ear in A (now stained for β-tubulin) show longitudinal extension along the cochlea. E. Myo7a staining shows close proximity of OC and papilla neglecta hair cells in the basal cochlea of a P7 mutant. F. The basal/apical cochlear transition of the ear in C., with β-tubulin stained supporting cells and Myo7a stained hair cells. Note that the packing density of hair cells is inversely related to supporting cell labeling. This pattern is maintained as long as hair cells can be labeled by Myo7a antibodies (I). G. Apical cochlea of the ear in C/D. IHC's, and OHC's can be recognized, but the organization is inferior to that of the wildtype (see *Atoh1*lacZ stained hair cells in the inset, mutant apex above, wildtype below). H. The grossly enlarged and elongated posterior crista of a P14 mutant shows fibers targeted to the Myo7a positive hair cells. Scale bars are 100 μm.

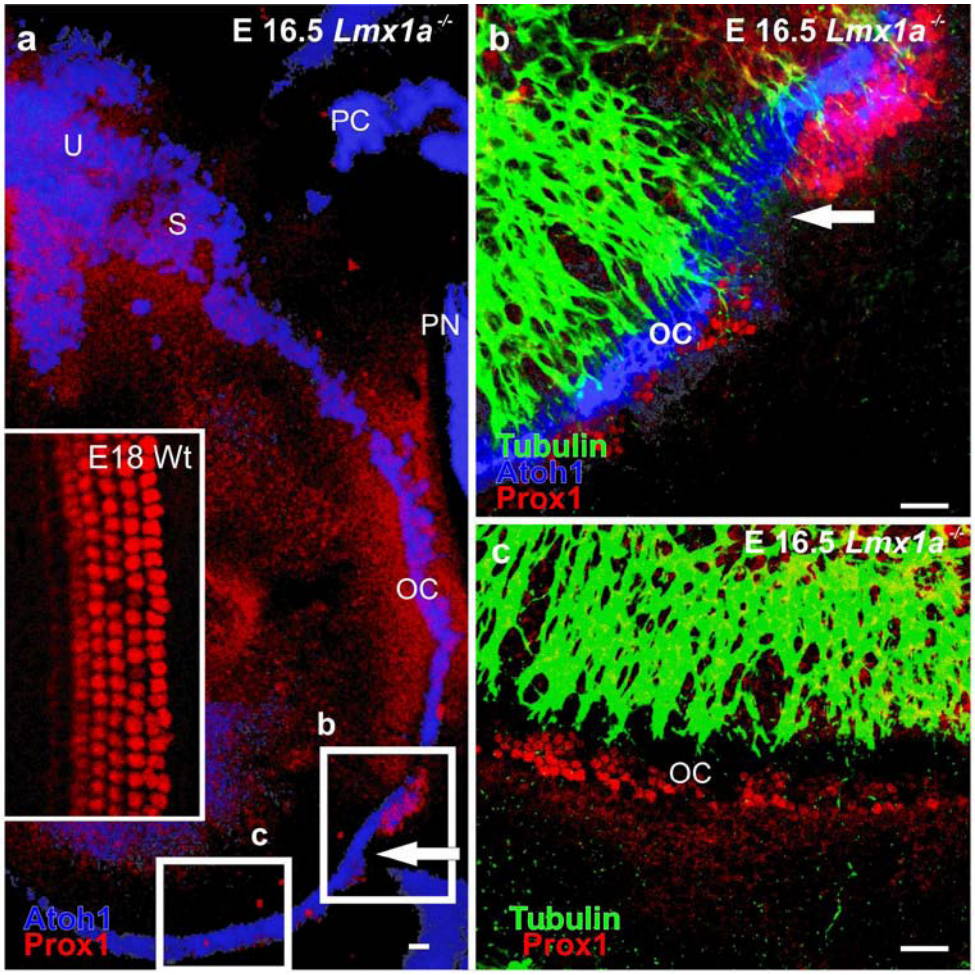

Fig. 5. *Atoh1*LacZ staining and Prox1 immunodetection show disorganization of hair and supporting cells

In these E16.5 *Lmx1a* null confocal micrographs, *Atoh1*lacZ is UV activated and false colored blue. Prox1 is immuno-stained red. A. The fields of B and C are boxed. The inset shows a wildtype Prox1 supporting cell staining pattern. Note that the supporting (pillar, Deiter's) cells are precisely organized. B. The basal-apical transition is arrowed. The basal OC is to the upper right of the arrow. Note that an unorganized mass of Prox1 stained cells is located just basal to the transition. Blue hair cells are located medial to the Prox1 positive cells. C. The mutant E16.5 cochlear apex demonstrates a disorganized, but otherwise continuous band of supporting cells in the OC. Scale bars are 100μm.

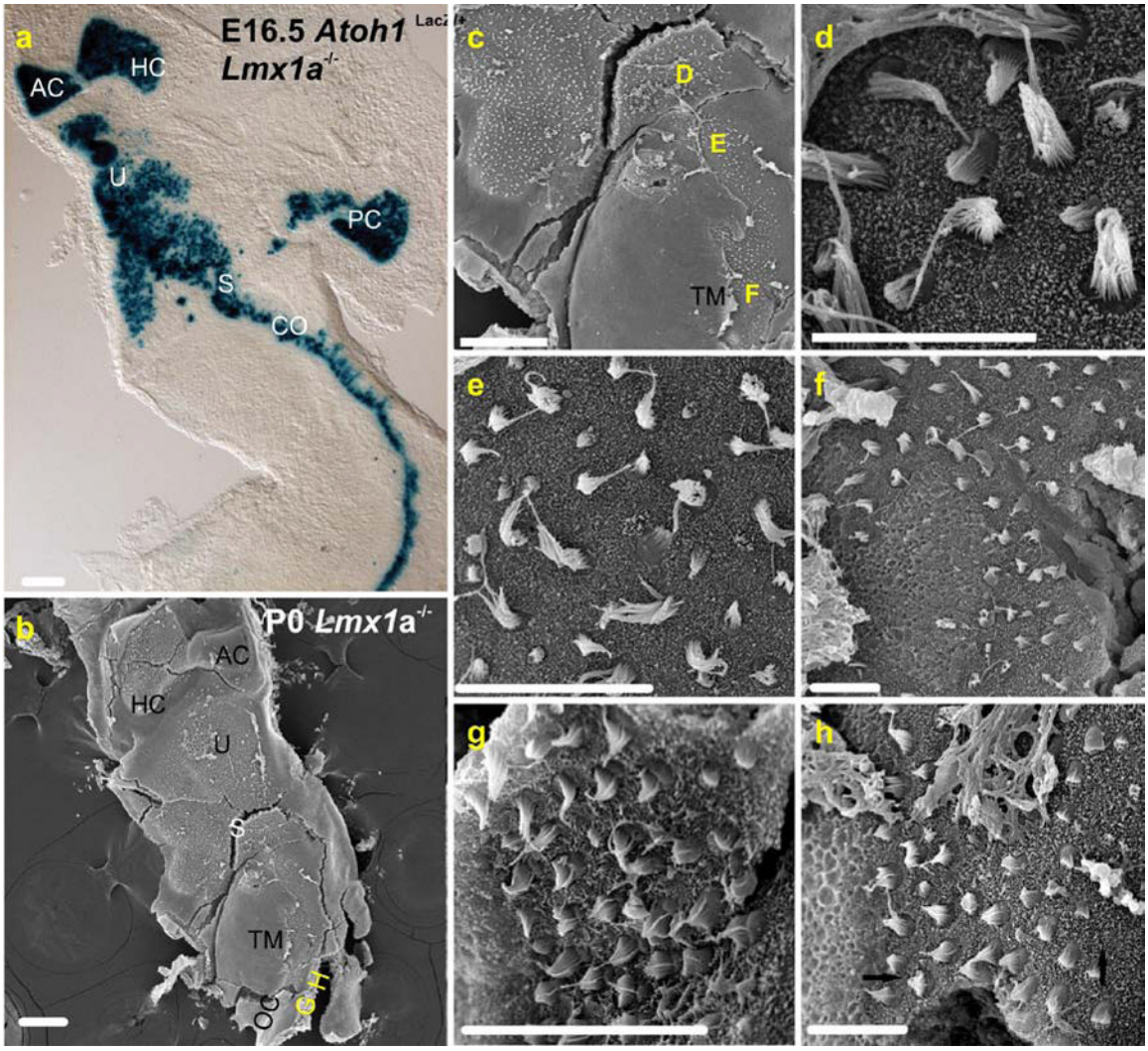

Fig. 6. Continuity of hair cells in late embryos as revealed by *Atoh1*LacZ staining and SEM

A The sensory epithelia of the E16.5 *Lmx1a* mutant ear revealed by *Atoh1*lacZ staining of hair cells. Note that the juxtaposition of sensory epithelia found at P7 in Fig. 1B is already apparent at E16.5. B. A P0 *Lmx1a* mutant ear oriented similar to that in A and viewed in the scanning electron microscope. The positions of the micrographs in G and H are indicated at the bottom of the micrograph. C. A higher power micrograph centered on the "S" (saccule) of B. The positions of micrographs in D–F are indicated, as is the smooth, flat surface of the tectorial membrane (TM). D. Micrograph of the saccular macula. The sterocilia come in long and immature short variations of the pipe-organ arrangement characteristic of vestibular hair cells. E. A region of the basal cochlea (note the adjacent tectorial membrane in C) close to the saccule. A mix of hair cells with long, vestibular-like and short, C-shaped cochlear inner hair cell-like sterocilia are present. F. Further toward the apex, but still in the basal cochlea, vestibular type hair cells are found adjacent to the tectorial membrane, and cochear-like hair cells further lateral. G. Still in the base, but further toward the apex, multiple "rows" of hair cells all possess short, cochlea-like sterocilia bundles. H. Adjacent to G. in the base, variously oriented cochlear hair cells are present (arrows indicate polarity). For abbreviations, see Fig.1. Medial is left and lateral right in Fig's. 3 D–H. Scale bars are 100μm.

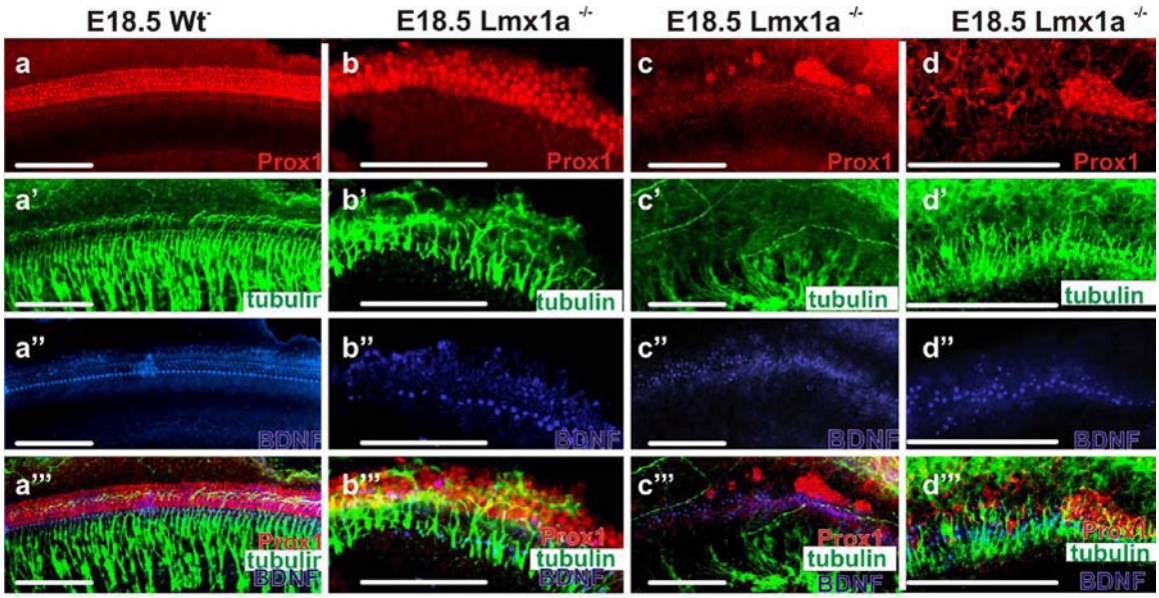

Fig. 7. Hair cells, supporting cells and nerve fibers are disorganized

E18.5 organs of Corti immunohistochemically (ImHC) stained for Prox1, β-tubulin, BDNF, and all three combined (rows A-A’’’), in the wildtype, mutant apex, mutant base, and high mag. mutant base (columns A–D). A–D Prox1 ImHC. there is an orderly expression in supporting cells in wildtype, some organization in the apex in the mutant but clumping of PROX1 stained supporting cells in the mutant base (see also A’’’–D’’’). A’–D’ β-tubulin ImHC. Note that nerve fibers in the base stop short of the OC (see also A’’’ – D’’’). A’’–D’’’ BDNF ImHC stains IHC’s strongly and OHC’s weakly in the wildtype. A row of IHC’s can be recognized in the mutant apex, but strongly stained cells are scattered in the base. A’’’ – D’’’ The superimposed images show that hair cells and supporting cells overlap and orderly nerve fibers project between the supporting cells in wildtype whereas hair cells and supporting cells do not overlap in mutants with disorganized fibers projecting between the labeled supporting cells (apex) or reaching hair cells (base) Scale bars are 100μm.

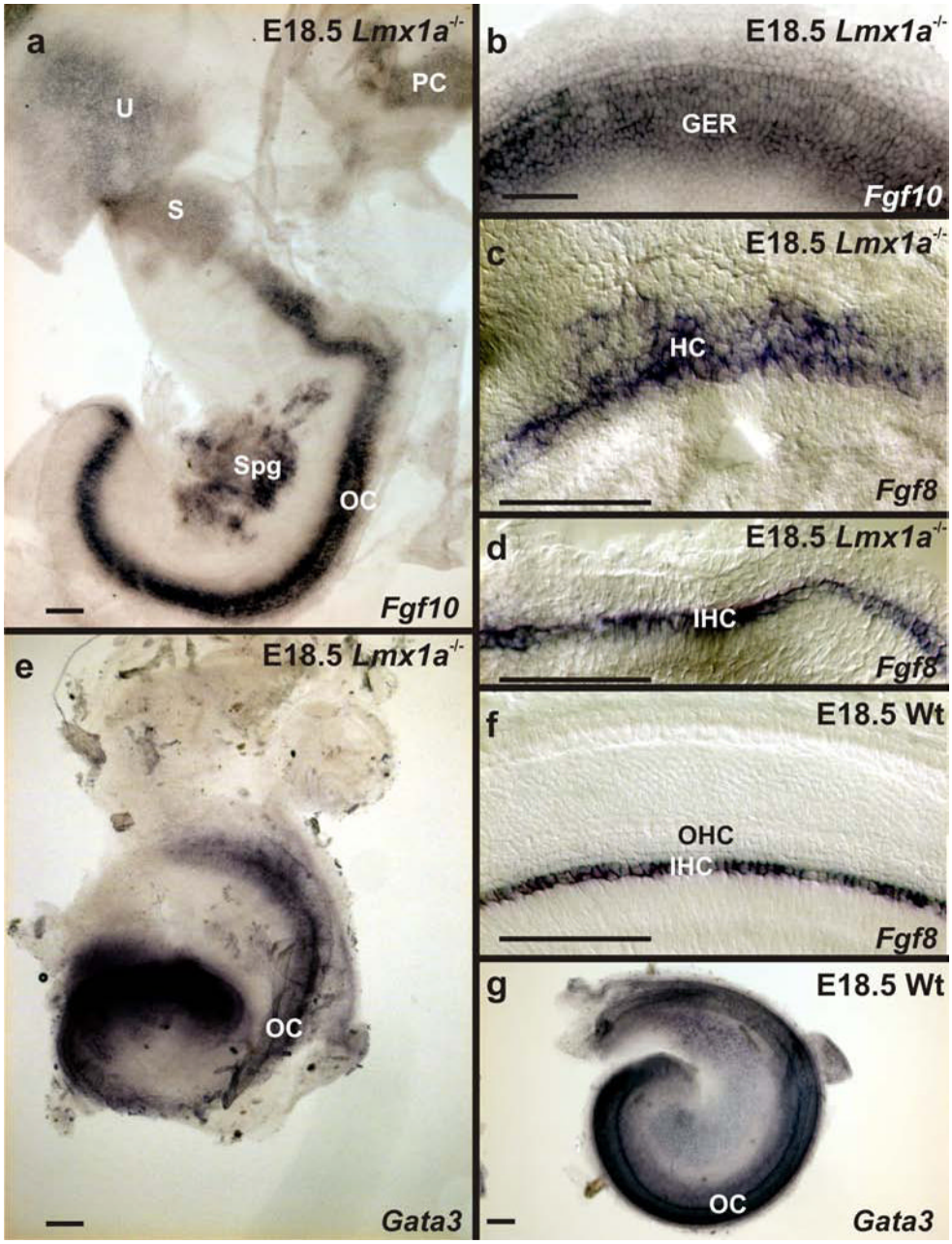

Fig. 8. *Fgf10, Fgf8* and *Gata3* mRNA expression reveal disorganization of the organ of Corti

A. In the E18.5 *Lmx1a* mutant, *Fgf10* is strongly expressed in the entire organ of Corti, but more weakly in the vestibular sensory epithelia. B. Mutant *Fgf10* expression extends from the greater epithelial ridge (GER) laterally to the inner hair cells in a pattern similar to that of the wildtype. This pattern is the same in the base and the apex. C., D. *Fgf8* expression in the base (C) and apex (D) of the mutant. Note expression is confined to the inner hair cells (IHC) in the apex, but scatters among the excess hair cells of the base. F. In the wildtype, *Fgf8* expression is neatly confined to the inner hair cells. E. In the E18.5 mutant, *Gata3* expression is present in the basal cochlea, but not the saccule. G. Similar *Gata3* expression in the wildtype cochlea. Sgl, spiral ganglion; OHC, outer hair cells. Scale bars are 100μm.

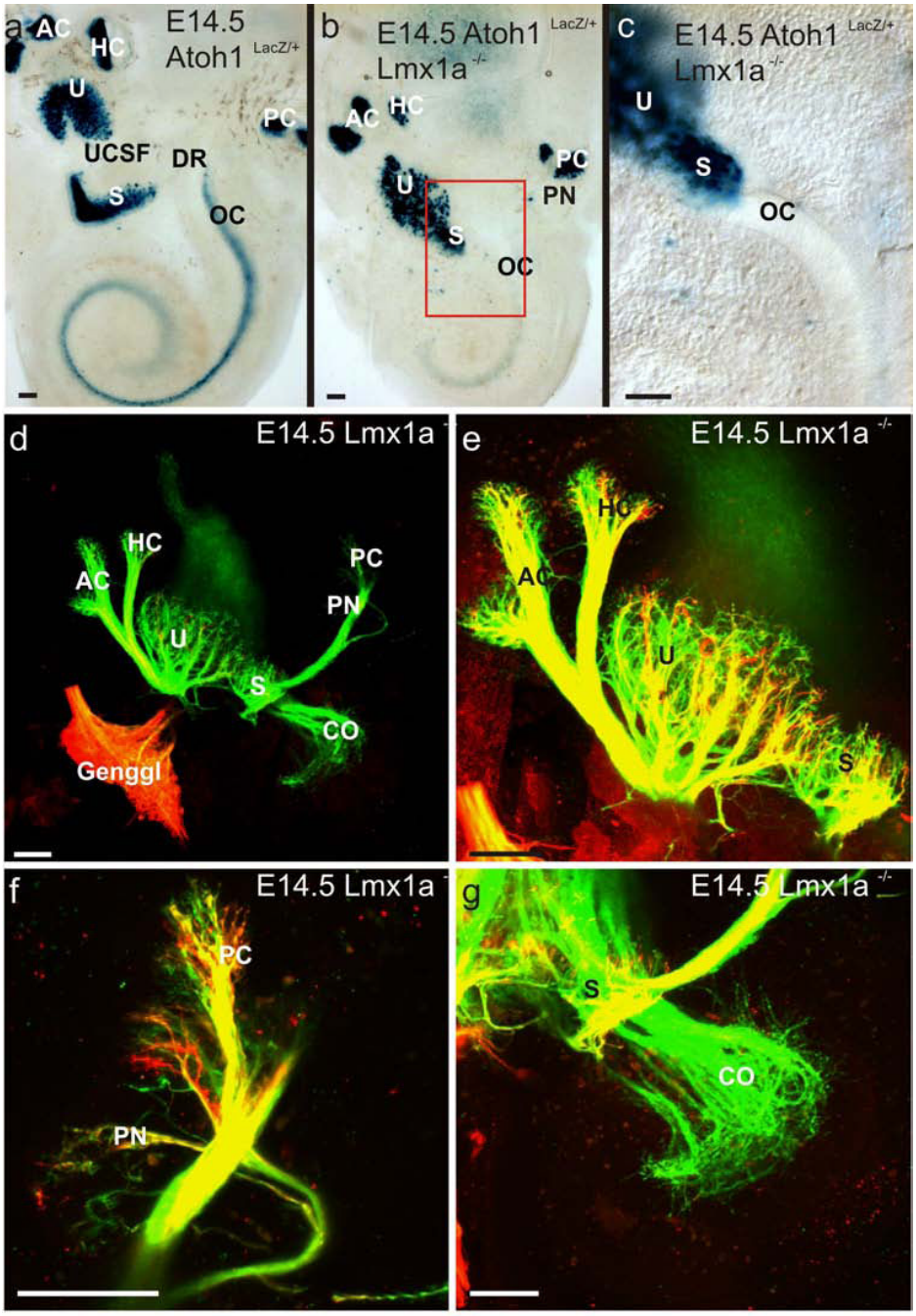

Fig. 9. *Atoh1-lacZ* staining for hair cells and tracer dye studies show initial segregation of sensory epithelia and their innervation

A. Wildtype *Atoh1*lacZ stained ear. Anterior left; dorsal up. B. An identically stained mutant ear. Note that the cristae are separated at this age and the posterior crista is not grossly enlarged (though a tiny papilla neglecta (PN) is present). The utricle and saccule are, however, joined. C. Higher magnification of the red, boxed field in B. The basal termination of the unstained though translucent organ of Corti (CO) overlaps the stained HC's of the saccule. D-G. Lipophylic dye tracings in E14.5 mutant ears. The red dye was placed in the solitary nucleus and descending vestibular nucleus. The dye then backfilled collaterals to the vestibular sensory epithelia as well as sensory neurons of the 7th cranial nerve in the geniculate ganglion (Genggl).

The green dye was placed in the (otic) efferent nerve fibers near the floor of the fourth ventricle to both the vestibular and cochlear sensory epithelia. D. Overview in which the green channel dominates. All sensory epithelia receive efferents. E. Vestibular afferent (red) and efferent innervation (green) to the anterior crista, horizontal crista, utricle and saccula are almost normal except for the limited segregation of the saccule form the utricle. F. Innervation to the posterior crista is abnormally widespread and includes a large branch to the papilla neglecta. G. The innervation to the basal cochlea lacks a vestibular (yellow) component. Scale bars are 100 μm.

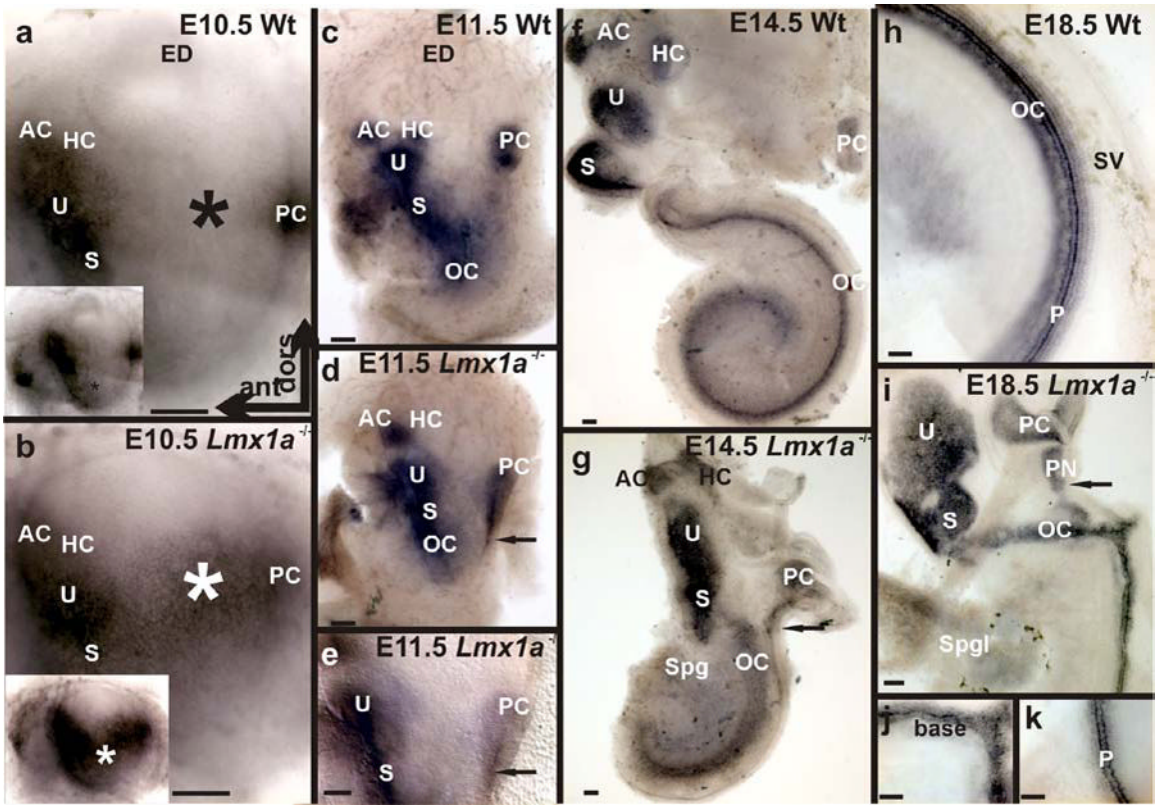

Fig. 10. *Sox2* mRNA expression shows *Lmx1a* defines early prosensory patch development

A., B. *Sox2* expression is similar, but broader in the mutant without a distinct gap between posterior and anterior prosensory patch expression (asterisk in A,B and lower mag. insets). C.-E. At E11.5 wildtype expression is localizing to individual sensory epithelia (Note separate AC and HC), while in the mutant the AC and HC are joined. Note also the PC/PN extending ventrally toward the cochlea (arrows in D, E, G, I). F,G. By E14.5 the wildtype sensory epithelia are clearly segregated (by *Lmx1a* expressing epithelia – compare Fig. 8H.), while continuities and near continuities are apparent in the mutant. H. In the E18.5 wildtype cochlea, *Sox2* expression is most intense in the inner pillar cells. I. Mutant *Sox2* expression identifies the fused U-S-C and enlarged PC-PN. J., K. At E18.5, *Sox2* expression in the mutant cochlear base is broad and diffuse, while that in the apex (K.) is, like the wildtype, focused on the inner pillar cells (P). All whole ears oriented as in A. Scale bars are 100 μm in A–G and 50 μm in H–K.