Cell Tissue Res (2008) 334:339–358
DOI 10.1007/s00441-008-0709-2

REGULAR ARTICLE

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

David H. Nichols · Sarah Pauley · Israt Jahan · Kirk W. Beisel · Kathleen J. Millen · Bernd Fritzsch

Received: 11 September 2008 / Accepted: 22 September 2008 / Published online: 5 November 2008

Abstract At embryonic day 8.5, 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 shows a loss of cochlear hair cells, suggesting that the long-term maintenance of hair cells is also disrupted in these mutants.

Keywords Dreher · Lmx1a · Ear · Hair cell maintenance · Sensory epithelium formation · Mouse

This work was supported by grants from the NCRR/COBRE (P20 RR 018788; D.H.N.) and NIH (RO1 DC 005590; B.F.). Parts of this investigation were conducted in a facility constructed with support of a Research Facilities Improvement Program Grant from the National Center for Research Resources, National Institutes of Health. We acknowledge the use of the confocal microscope facility of the NCCB, supported by EPSCoR EPS-0346476 (CFD 47.076), and of the University of Nebraska microarray facility, supported by NCRR/COBRE.

D. H. Nichols · S. Pauley · K. W. Beisel
Department of Biomedical Sciences, Creighton University, Omaha, NE, USA

K. J. Millen
Departments of Human Genetics and Neurology, University of Chicago, Chicago, IL, USA

I. Jahan · B. Fritzsch (✉)
Department of Biology, University of Iowa, 143 Biology Building, Iowa IA 52242, USA
e-mail: bernd-fritzsch@uiowa.edu

Introduction

The vertebrate ear has 3–9 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 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, 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, a process that ultimately channels physical stimuli to specific sensory epithelia. Likewise, the 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). Hence, 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), which 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 the development of reticular formation in the hindbrain (Alenina et al. 2006) and motoneuron formation in the spinal cord (Lee et al. 2008; Matthews and Visvader 2003). Likewise, in insect mechanosensory development, *Isl* and *Gata/pannier* antagonize each other to regulate the 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 the molecular conservation of essential neurosensory developmental modules across phyla (Adam et al. 1998; Caldwell and Eberl 2002; Fritzsch et al. 2000, 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 by Deol (1964, 1983) and was thought to be a consequence of malformations in the hindbrain (Manzanares et al. 2000). More recent in situ expression studies show, however, a more robust and earlier expression of *Lmx1a* in the developing mouse (Failli et al. 2002) and chicken ear compared with expression in the hindbrain (Giraldez 1998). These in situ hybridization (ISH) data raise the possibility that local otic *Lmx1a* expression is required for ear development, and that its absence in the ear is causally linked to the ear defects. Since *Lmx1a* interacts with other Lim and LMO factors, an intriguing and likely possibility is 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 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 eventually leading 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 and methods

Mice

Atoh1^{tm2Hzo} mice were obtained from Dr. Huda Zoghbi (Bermingham et al. 2001) and *Lmx1*$^{adr/J}$ mice from Jackson

Labs; they were 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 being 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/phosphate-buffered saline, rinsed in phosphate buffer, and stained with "X-gal." as previously described (Fritzsch et al. 2005a). When required, we enhanced the "X-gal." reaction by using two-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) with a histology-grade diamond knife (Dumont), imaged by using a compound light microscope (Nikon Eclipse 800), and captured by means of a Coolsnap camera and Metamorph software.

Immunohistochemistry and ISH

Primary antibodies were rat anti-mouse β-tubulin (Sigma; #T6793, 1:800), Hoechst nuclear stain (Sigma) and MyoVII (gift of T. Hasson, San Diego) and chicken anti-BDNF (anti-brain-derived neurotrophic factor; R&D Systems; 3AF248, 1:100). Whole-mount ISH 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 a thickness of 5–10 μm, counterstained with toluidine blue, and viewed with a Nikon E800 microscope by using differential interference contrast.

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

Imaging for scanning electron microscopy

Ears were microdissected, osmicated, dehydrated, and critical-point-dried as previously described by Ma et al. (2000). They were mounted on stubs and imaged with a Hitachi scanning electron microscope.

Results

Lmx1a expression is concentrated in certain non-sensory epithelia patches

Lmx1a expression was shown to be 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 by using ISH. 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 2 days, *Lmx1a* expression became focused in the developing endolymphatic duct (Fig. 1c,f,g) and the lateral margin of the cochlear duct (Fig. 1g). Strong expression also persisted in the saccular roof (Fig. 1h–j), in 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 $Lmxa1^{dr}$ 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 expression in the endolymphatic duct 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 the 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 whether 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 might indicate that *Lmx1b* expression indirectly affects ear development.

We next investigated the distribution of *Lmx1a* expression in near radial sections of epoxy-resin-embedded E18.5 ears analyzed by ISH for *Lmx1a* (Fig. 2). These data showed a clear medial to lateral organization of the cochlea in the mutant. However, the cellular organization of the organ of Corti did not show the single clear row of inner hair cells and the 3–4 rows of outer hair cells found in the wildtype (Fig. 2a-d). Consistent with our data on whole-mounted ears, we found a considerable expansion of the expression area of the mutated *Lmx1a* mRNA beyond the lateral wall area of the wildtype. Expression sometimes expanded to the stria vascularis and even to Reissner's membrane (Fig. 2a'-d'). In the absence of functional Lmx1a protein, mechanisms that help to focus *Lmx1a* expression onto the narrow part of the lateral wall between Claudius cells and the stria vascularis did not appear to work properly.

Mutation of *Lmx1a* gene produces unique inner ear phenotype

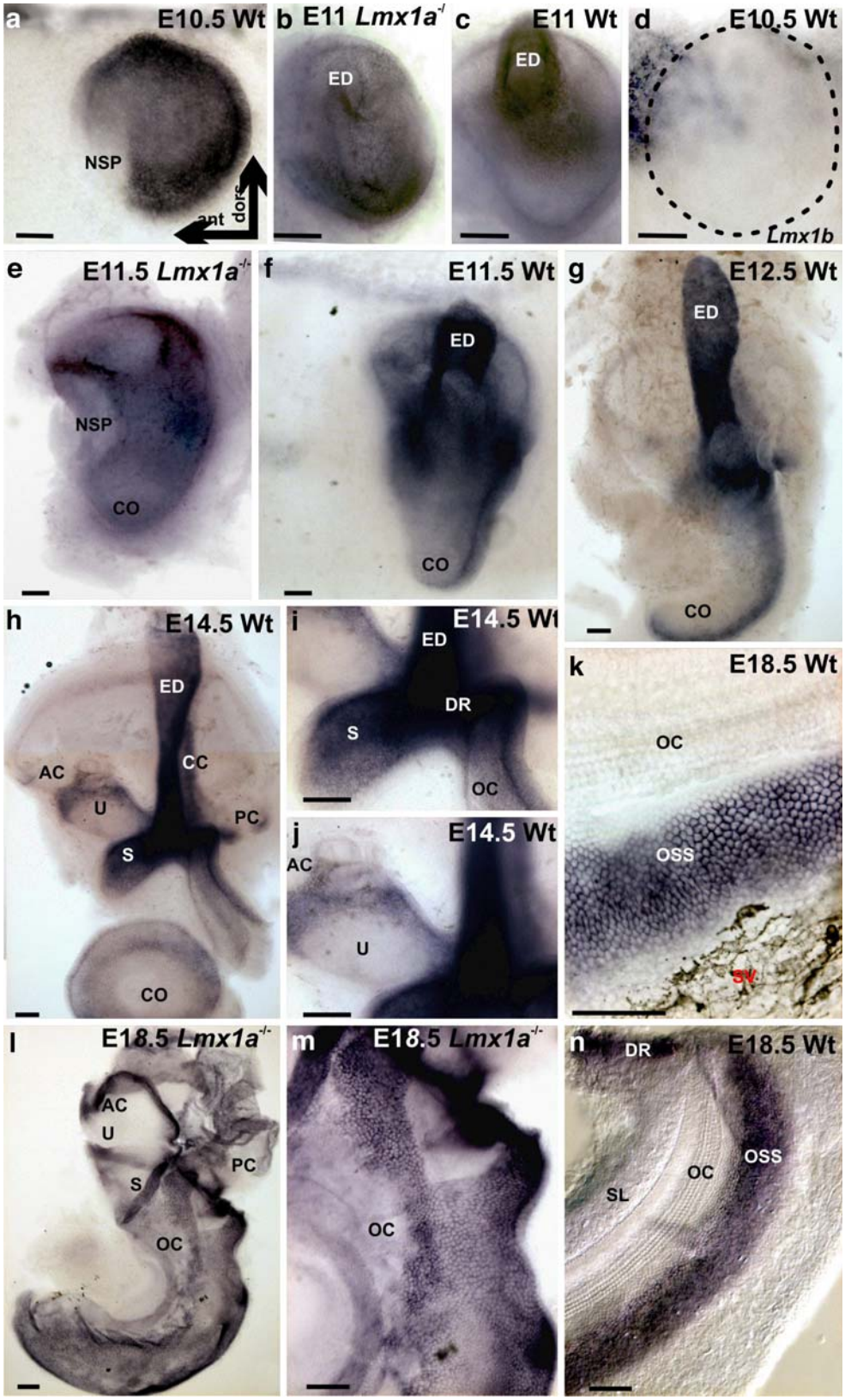
The altered *Lmx1a* expression pattern in *dr* mutants suggests a basis for the observed disruption of sensory epithelium segregation cues. Since the previous studies described only inner ear dysmorphogensis in the *Lmx1a* mutant lines (Deol 1964, 1983; http://www.informatics.jax.org/Lmx1a alleles), we extended these preliminary observations to define the extent of this interference in sensory and non-sensory epithelium formation. The *Atoh1*^{tm2Hzo} 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 1- and 2-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. Whereas each sensory epithelium of the wildtype ears resided within its own recess (three ampullae for the canal cristae, the utricular and saccular recesses being separated by the constricted utriculo-saccular foramen, and the cochlear duct being separated from the saccule by the ductus reuniens), none of these non-sensory constrictions was 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 (Figs. 1e,l, 3b).

Fig. 1 *Lmx1a* expression, as shown by in situ hybridization (ISH) undergoes dynamic changes in wildtype (*wt*) and mutants (*ant* anterior, *dors* dorsal, *AC* anterior crista, *PC* posterior crista, *S* saccule, *SL* spiral limbus, *U* utricle). **a** In the wildtype at embryonic day 10.5 (E10.5), 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 (labeling and be seen in and out of focus; *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 endolymphatic duct (*ED*), lateral cochlea, and non-sensory saccule. **h–j** By E14.5, expression centers on the ED and radiates to constricted spacer epithelia between sensory epithelial territories. Most conspicuous of these is the ductus reuniens (*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 (*OC*) is entirely free of *Lmx1a* expression (**n**). However, mutant Lmx1a mRNA is broadly expressed throughout the basal turn (**l**, **m**). All whole ears are oriented as in **a**. *Bars* 100 μm

Fusion of inner ear sensory endorgans

At P7/P14, only three distinct sensory epithelia instead of the usual six were found in *Lmx1a* mutants (Fig. 3a,b). Near the anterodorsal pole, a single crista consisting of two unequal sized hemicristae was 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), possibly representing a fusion of the posterior canal hemicrista with the papilla neglecta. The papilla neglecta generally consists only of a few hair cells in mammals but can be large in elasmobranchs and becomes the comparatively large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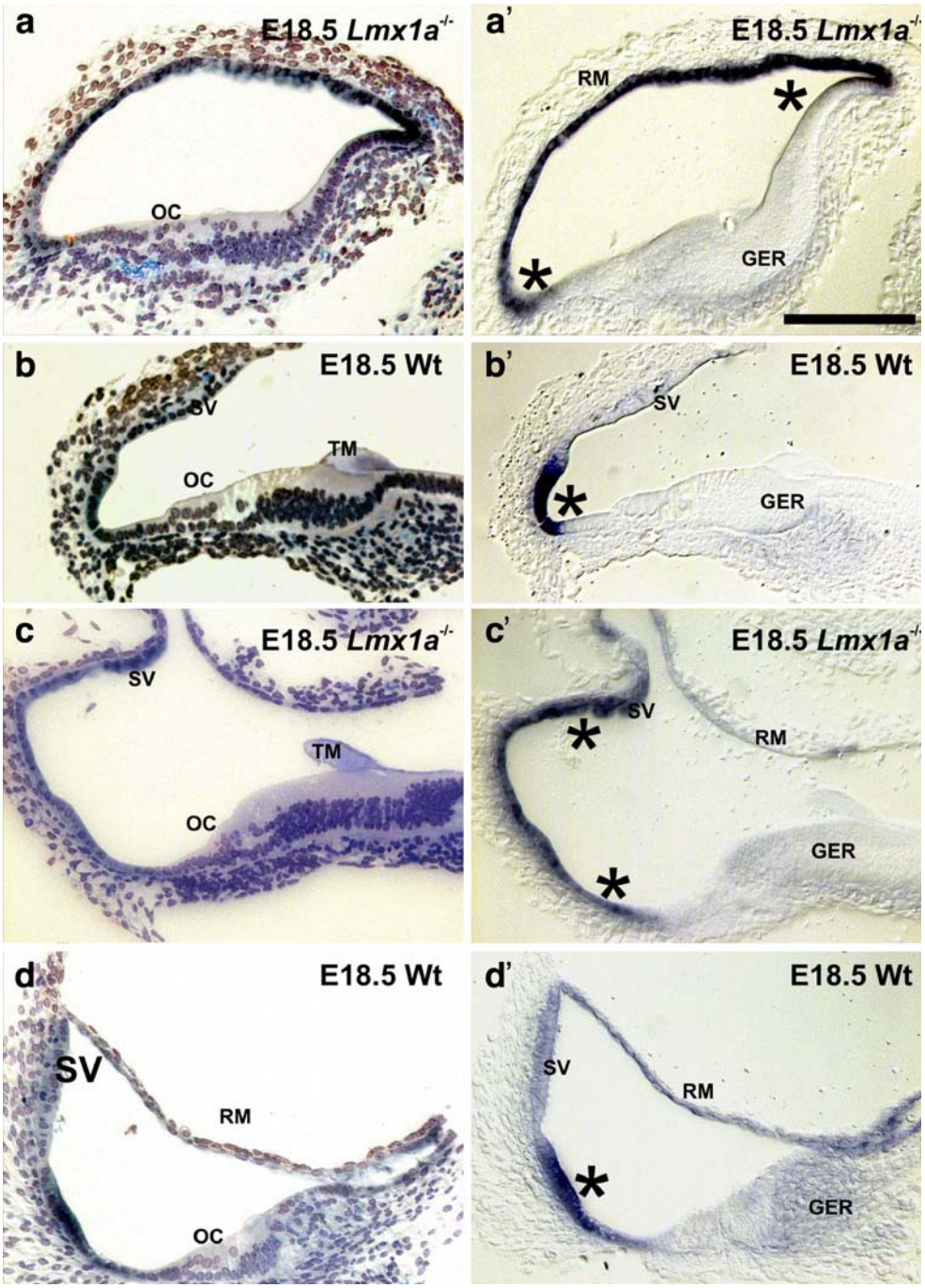


Fig. 2 *Lmx1a* expression is less restricted in mutants. The distribution of *Lmx1a* ISH reaction product is shown in wildtype (**b**, **b'**, **d**, **d'**) and *Lmx1a* mutant mice at embryonic day 18.5. Ears were reacted for whole-mount ISH (see Fig. 1), embedded in soft epoxy resin, and sectioned at 5–10 μm. These radial sections show a well-organized organ of Corti (*OC*) with 1 inner and 3–4 rows of outer hair cells in the wildtype (**b**, **b'**, **d**, **d'**). In contrast, hair cells are disorganized in the *Lmx1a* mutant mice (**a**, **a'**, **c**, **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 (*stars*) adjacent to the stria vascularis (*SV* in **b'**, **d'**). In contrast, in the *Lmx1a* mutant mice, the strong *Lmx1a* in situ signal expands to include the SV (*stars*) or even Reissner's membrane (*RM* in **a'**,**c'**). *Bar* 100 μm

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 an extensive patch that lay adjacent to the anterior crista, and that we tentatively identified as a utricle-like region based on its topology (Fig. 3b, see also below). This utricular area blended into a 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. Whereas the hair cells of this patch were continuous, the pattern of innervation indicated four distinct regions in this epithelium (Figs. 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 the wildtype (Fig. 4c). In contrast, the cochlea organ of Corti showed two distinct regions with

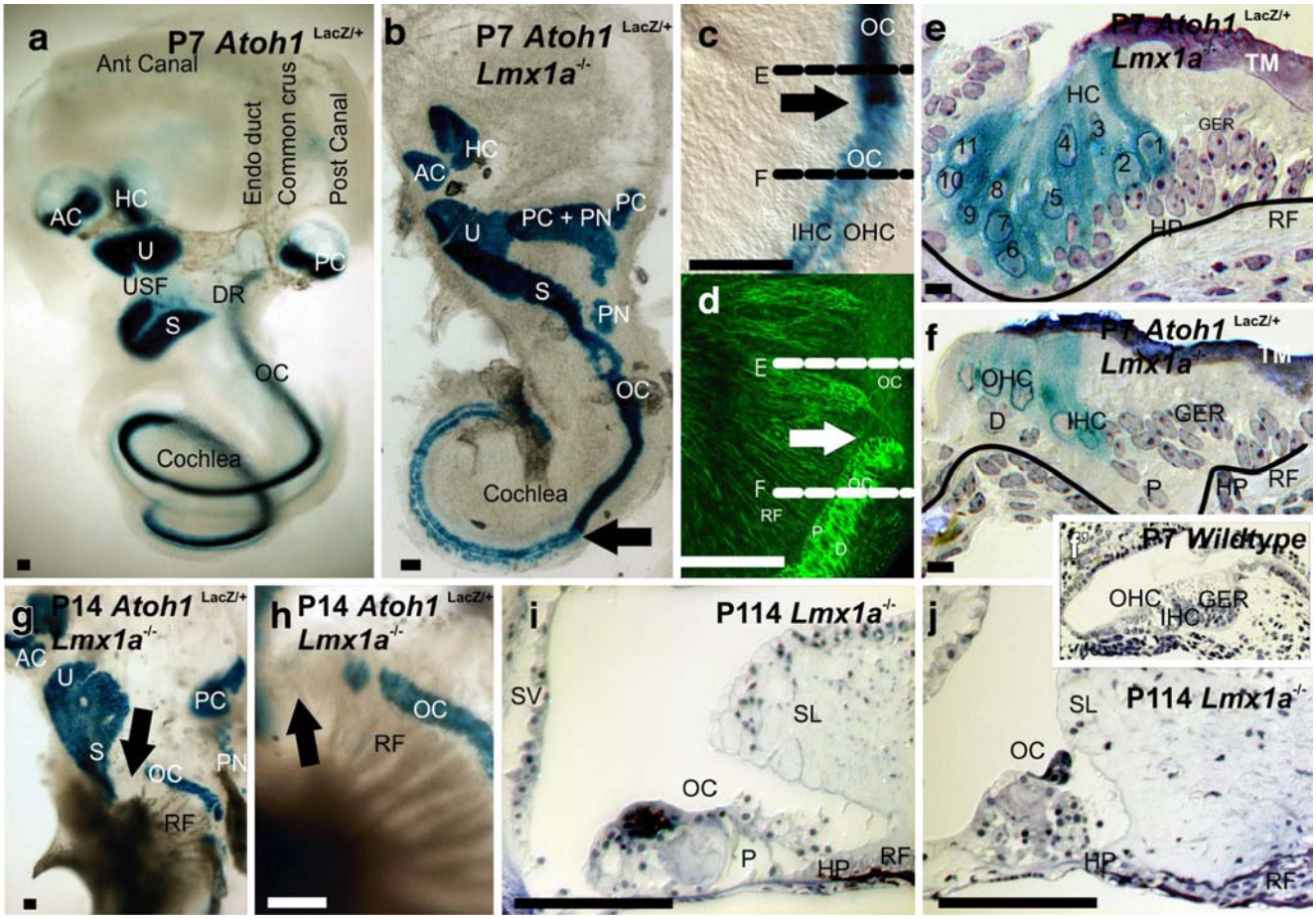


Fig. 3 Postnatal *Lmx1a* mutant ears reveal disorganized sensory epithelia. **a** In the wildtype, six discrete sensory epithelia are separated from one another by constricted non-sensory epithelial spacers. Two of three semicircular canals (*Ant Canal* anterior canal, *Post Canal* posterior canal) and an endolymphatic duct (*Endo duct*) can be identified as *shadows* (*Common cruc* common cruciate eminence, *DR* ductus reuniens, *OC* organ of Corti, *PC* posterior crista, *S* saccule, *U* utricle, *USF* utriculo-saccular foramen). **b** In the *Lmx1a* mutant, the anterior cristae (*AC*) and horizontal cristae (*HC*) are separated by a common cruciate eminence, whereas the posterior crista is grossly enlarged and extended by the presence of both embedded and detached papilla-neglecta-like sensory epithelia (*PN*). The utricle (*U*), saccule (*S*), and cochlear sensory epithelia seem to be continuous with one another. The basal turn of the organ of Corti (*OC*) 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 transition (*arrow*) in **b**. The densely packed hair cells of the basal cochlea lie *above* the *arrow*, with the apex (*IHC* inner hair cells, *OHC* outer hair cells) *below* it (*black lines* planes of section in **e**, **f**). **d** Same tissue as in **c** but stained for beta-tubulin to reveal nerve fibers (*RF* radial fibers), pillar cells (*P*), and Deiters' cells (*DC*). Note the absence of tubulin-containing pillar and Deiters' 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 (see *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 (*HP* habenula perforate, *HC* horizontal crista). **f** A recognizable organ of Corti with inner and outer hair cells is present in the apex (compare with wildtype, *inset*). **g**, **h** Beginning at ~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, although that its absence does not interfere with their initial formation. *Bars* 100 μm

respect to its innervation: the base received only a patchy and reduced density of nerve fibers, whereas the apex was densely innervated with radial fibers, like the wildtype (Figs. 3d, 4a–c). These fibers however arose from a typical spiral ganglion.

In addition to the differences in density of innervation, older *Lmx1a* mutant mice showed tubulin-positive pillar and Deiters' cells only in the apex (Figs. 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 (Figs. 3c-f, 4e-i). This was therefore identifiable as having an organ of Corti-like organization of cells. In

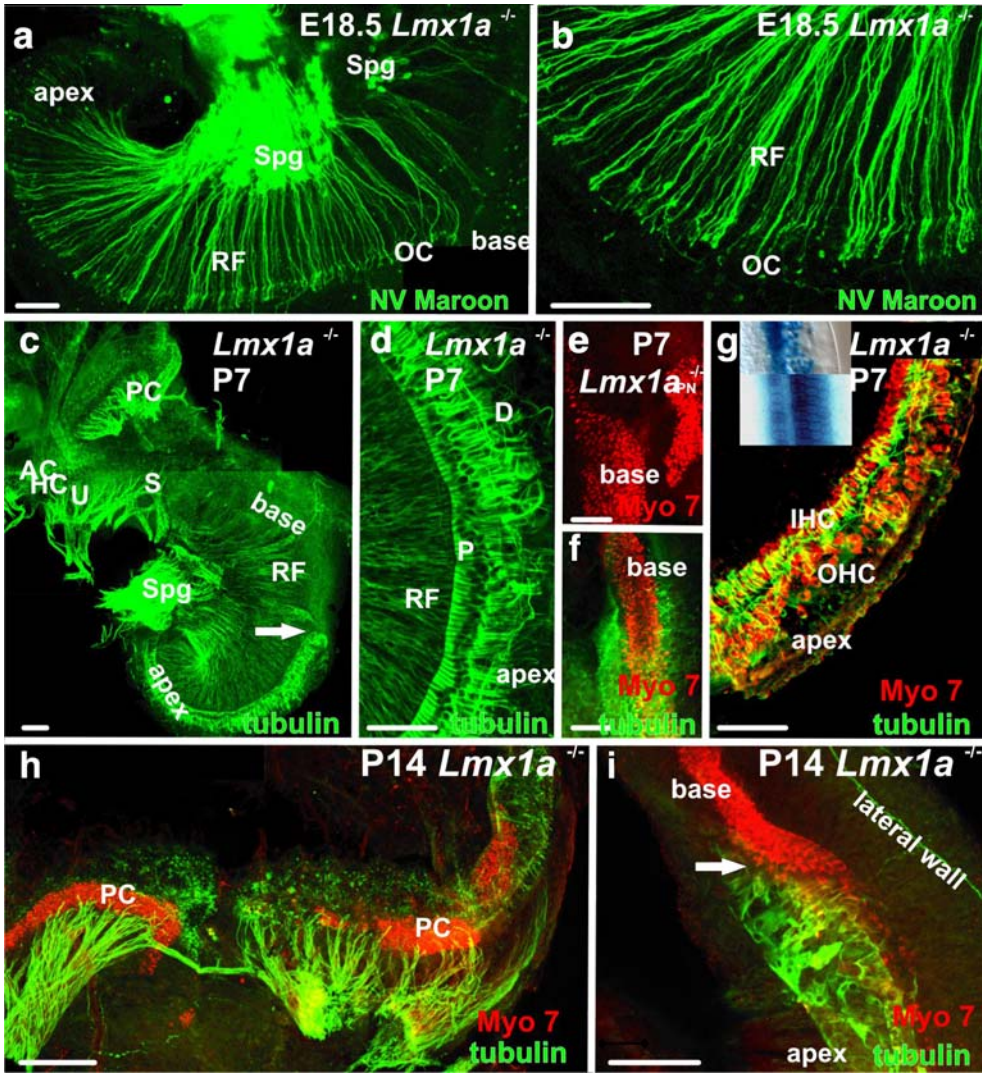


Fig. 4 Late innervation and sensory epithelia are disorganized in mutants. Afferent radial fibers to the base of mutant cochlea stained with the lipophylic dye, NV Maroon, at the E18.5 (**a**, **b**), and with anti-acetylated tubulin at P7/P14 (**c**–**i**). **a**, **b** Note that the fibers enter the organ of Corti(*OC*) but do not extend to the outer hair cells (*OHC*) as they would in a comparable wildtype ear. There is a notable difference between the packing density of radial fibers (*RF*) in the base and the apex consistent with the reduced presence of spiral ganglia (*Spg*) in the base. **c** There is a complete absence of pillar/Deiters' cells basal to the cochlear transition (*arrow*) and a clear distinction of innervation between the densely innervated saccule (*S*) and the poorly innervated basal turn of the cochlea (*AC* anterior crista, *HC* horizontal crista, *PC* posterior crista, *U* utricle). **d** Disorganized Deiters' cell processes (*D*) in the apex of the ear at P7 stained for β-tubulin and showing longitudinal extension along the cochlea (*P* pillar cells). **e** Myosin VIIa (*Myo7a*) staining revealing close proximity of the OC and hair cells of the papilla neglecta (*PN*) in the basal cochlea of a P7 mutant. **f** Basal/apical cochlear transition of the ear in **c**, with supporting cells stained for β-tubulin and hair cells stained for Myo7a. Note that the packing density of hair cells is inversely related to supporting cell labeling. This pattern is maintained for as long as hair cells are labeled by Myo7a antibodies (**i**). **g** Apical cochlea of the ear in **c**, **d**. Inner hair cells (*IHC*) and outer hair cells (*OHC*) can be recognized, but the organization is inferior to that of the wildtype (see *Atoh1*LacZ-stained hair cells in *inset*; *top* mutant apex, *bottom* wildtype). **h** Grossly enlarged and elongated posterior crista (*PC*) of a P14 mutant showing fibers targeted to the Myo7a-positive hair cells. *Bars* are 100 μm

contrast, in the base, hair cells formed multiple rows of uniform cells that defied any histological characterization as inner or outer hair cells (Figs. 3c,e, 4f,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 (Figs. 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

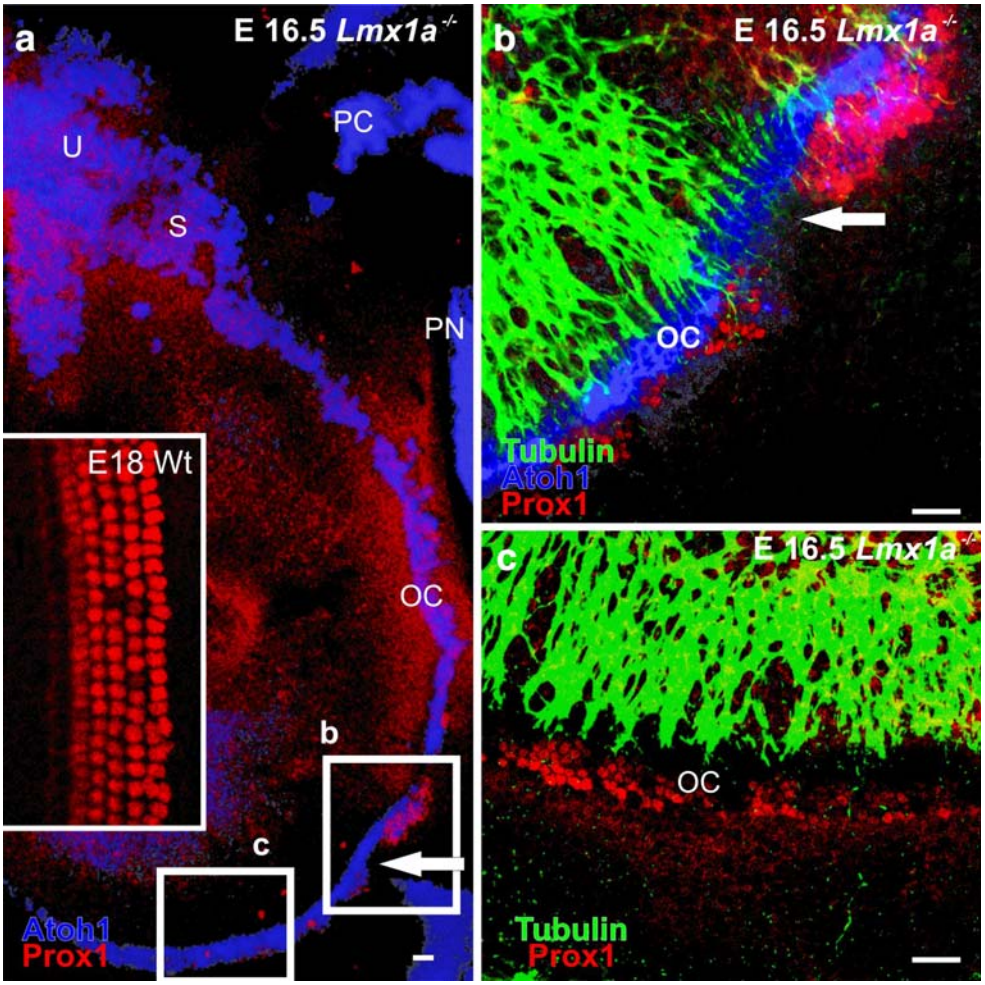


Fig. 5 *Atoh1*^{LacZ} staining and Prox1 immunodetection showing disorganization of hair and supporting cells. In these confocal micrographs of E16.5 *Lmx1a* null mutants, *Atoh1*LacZ is UV-activated and false-colored *blue*. Prox1 is immunostained *red*. **a** The fields shown in **b**, **c** are *boxed* (*OC* organ of Corti, *PC* posterior crista, *PN* papilla neglecta, *S* saccule, *U* utricle). *Inset*: Wildtype (*Wt*) Prox1 staining pattern of supporting cells at E18. Note that the supporting (pillar, Deiters') cells are precisely organized. **b** Note the basal-apical transition (*arrow*). The basal OC lies *upper right* of the *arrow*. Note that an unorganized mass of Prox1-positive cells is located just basal to the transition. *Blue* hair cells are located medial to the Prox1-positive cells. **c** Mutant E16.5 cochlear apex demonstrating a disorganized, but otherwise continuous band of supporting cells in the OC. *Bars* 100 μm

process during development and may have played the same role during ear evolution.

Absence of hair cells in adult *Lmx1a* mutant cochlea

Using *Atoh1*LacZ histochemistry, we found gaps in the distribution of cochlear hair cells as early as P14 (Fig. 1g,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). Investigation of 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 the spiral limbus, Reissner's membrane, and stria vascularis (Fig. 3i,j). A few hair cells remained in the vestibular organs (data not shown). These data suggest that *Lmx1a* not only plays a major role in early histogenesis and morphogenesis of the ear, but is also essential for the long-term maintenance of hair cells.

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 was used to define the apical specializations in the area in which 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 the continuity of hair cells. Closer examination revealed distinct vestibular-type hair cells in the saccular and utricular regions (Fig. 6c–e). Near the tectorial membrane, we found that medial cells displayed vestibular-type long organ-pipe-like stereocilia. In contrast, more C-shaped, shorter stereocilia reminiscent of the inner hair cells of the organ of Corti prevailed more laterally, indicating an introgression zone of the vestibular and organ-of-Corti-type of hair cell differentiation. Ventral to

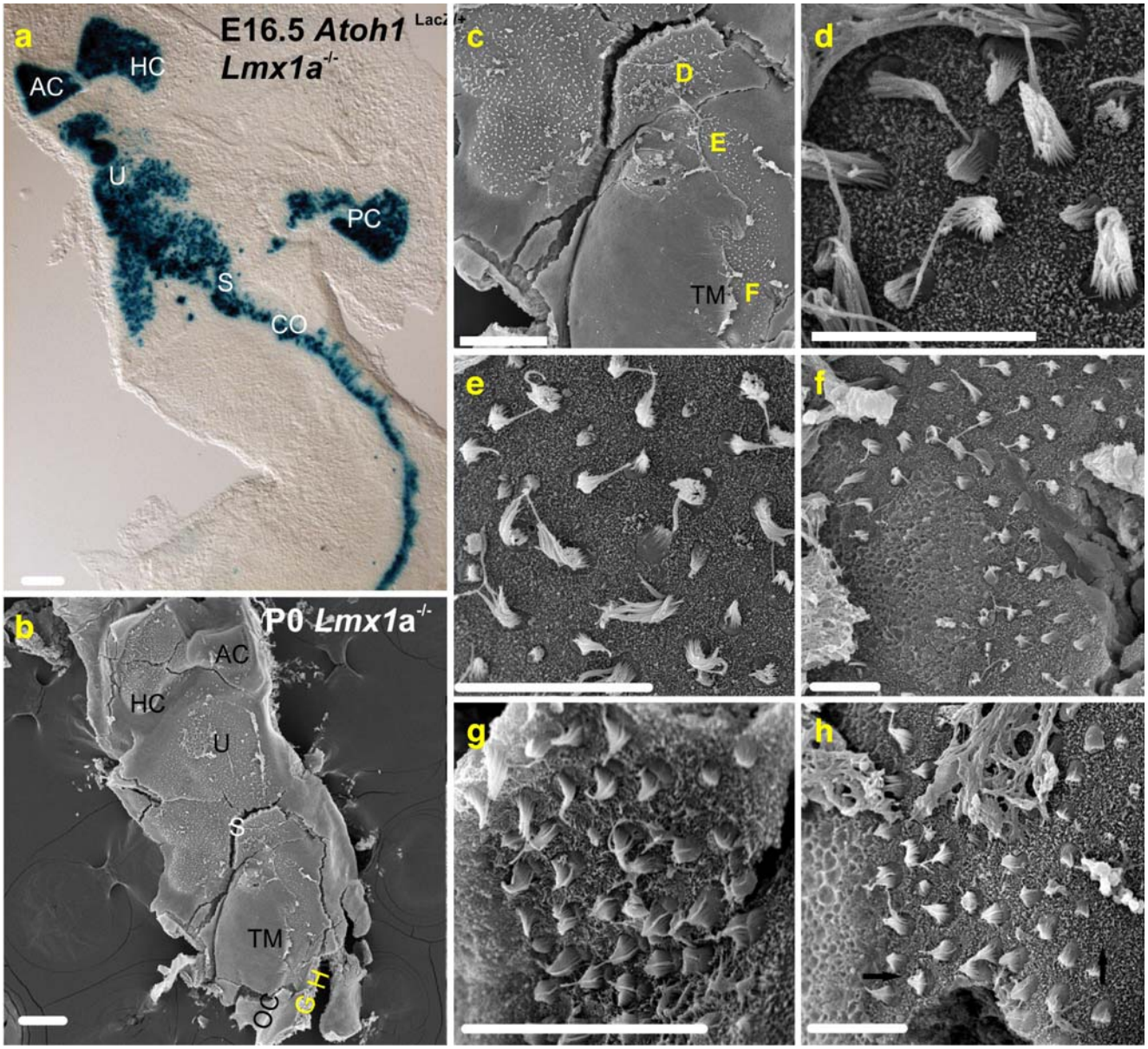


Fig. 6 Continuity of hair cells in late embryos; *Atoh1*^{LacZ} staining and scanning electron microscopy. **a** Sensory epithelia of the E16.5 *Lmx1a* mutant ear revealed by *Atoh1*^{LacZ} staining of hair cells. Note that the juxtaposition of sensory epithelia found at P7 in Fig. 1b is apparent even at E16.5 (*AC* anterior crista, *CO* cochlea, *HC* horizontal crista, *PC* posterior crista, *S* saccule, *U* utricle). **b** A P0 *Lmx1a* mutant ear oriented similar to that in **a** and viewed by scanning electron microscopy. The positions of the micrographs in **g** (*G*), **h** (*H*) are indicated *bottom* (*OC* organ of Corti, *TM* tectorial membrane). **c** Higher power micrograph centered on the saccule (*S*) in **b**. The positions of micrographs in **d–f** (*D-F*) are indicated, as is the smooth flat surface of the TM. **d** Saccular macula. The sterocilia appear as long and immature short variations of the pipe-organ arrangement characteristic of vestibular hair cells. **e** Region of the basal cochlea (note the adjacent TM 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, with cochear-like hair cells lying further laterally. **g** Still in the base, but further toward the apex, multiple "rows" of hair cells all possess short cochlea-like sterocilia bundles. **h** Variously oriented cochlear hair cells are present (*arrow* indicates polarity) adjacent to **g** in the base. Medial is *left* and lateral *right* in **d–h**. *Bars* 100 μm

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° 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, which was more typical in its supporting cell and hair cell organization (Figs. 5a-c, 7a-d). Instead of a regular pattern of supporting and hair cells, Prox1-positive cells in the apex of *Lmx1a* mutants showed an irregular distribution with an inconsistent number of rows. To obtain more information regarding the co-localization of hair cells and Prox1-positive supporting cells, hair cells were labeled by immunodetection for BDNF. In *Lmx1a* mutants, the innermost row of hair cells showed strong BDNF staining, much like that observed in wildtype mice. However, unlike in the wildtype, BDNF was just above background levels in the most lateral outer hair cells of the mutants (Fig. 7b"). The mutant distribution of Prox1 near the transition from the near normal apical organ of Corti to the more vestibular-like basal region was the most interesting. 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 that it occurred as early as E16.5 by using Prox1 immunodetection and *Atoh1*LacZ labeling of hair cells (Fig. 5). As in later stages, little to no Prox1 was expressed throughout the base, except at this transition zone (Figs. 5, 7). In wildtype mice, outer hair cells are regularly interspersed with Prox1-expressing Deiters' cells (Fig. 7a'"). 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. 7c'"). Combined, these data suggest that *Prox1*- and *Atoh1*-expressing cells partially segregate in the absence of *Lmx1a* expression further implying that *Lmx1a* functions in the setting up of the topology for these 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, 2b') where *Lmx1a* expression is most prominent.

Distinct basal cellular organization can be defined by gene expression

We next examined *Gata3*, *Fgf8*, and *Fgf10* transcript distribution to elucidate further the extent of the disorganization of the basal region at the level of gene expression.

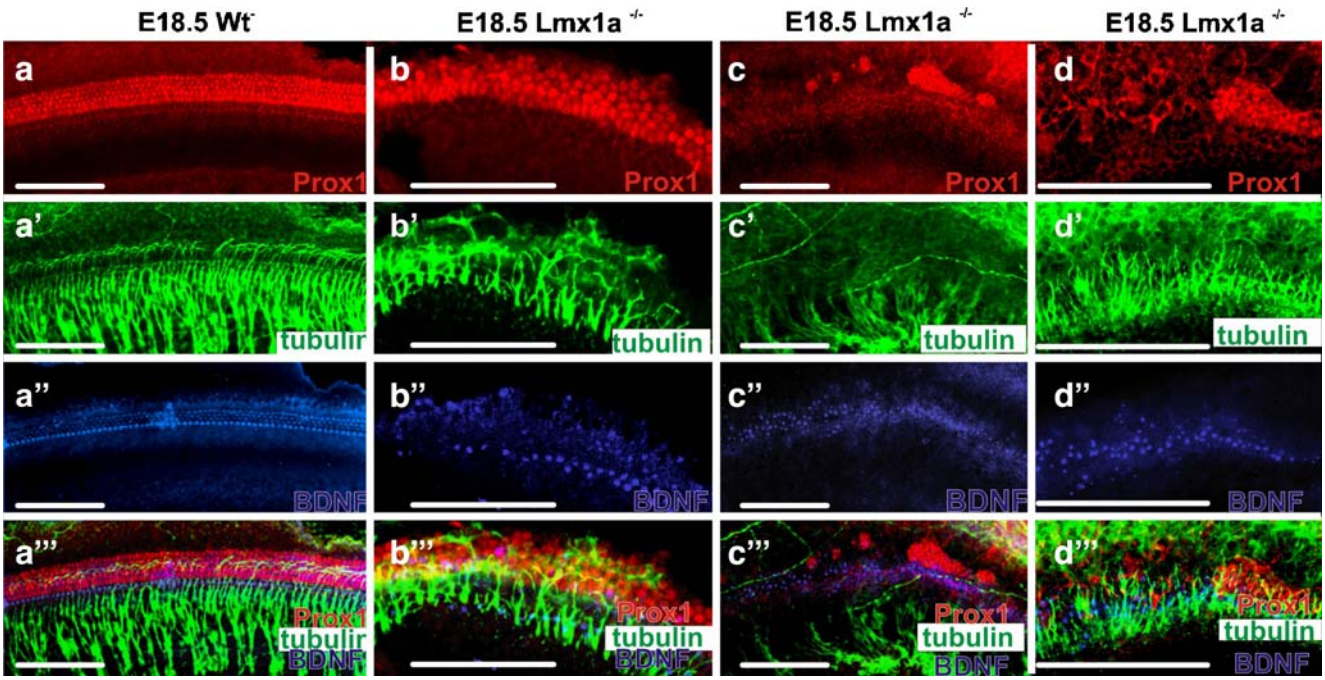


Fig. 7 Hair cells, supporting cells, and nerve fibers are disorganized in mutant mice. Organ of Corti at E18.5 stained immunohistochemically for Prox1, β-tubulin, brain-derived neurotrophic factor (*BDNF*), and all three combined (**a'"–d'"**), in the wildtype (**a–a'"**), mutant apex (**b–b'"**), mutant base (**c–c'"**), and high magnification of mutant base (**d–d'"**). **a–d** Immunohistochemistry for Prox1. Note the orderly expression in supporting cells in the wildtype, some organization in the apex in the mutant, but clumping of PROX1-positive supporting cells in the mutant base (see also **a'"-d'"**). **a'-d'** Immunohistochemistry for β-tubulin. Note that nerve fibers in the base stop short of the OC (see also **a'"-d'"**). **a"-d"** Immunohistochemistry for BDNF stains inner hair cells (IHC) strongly and outer hair cells (OHC) weakly in the wildtype. A row of IHCs 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 the wildtype, whereas hair cells and supporting cells do not overlap in mutants with disorganized fibers projecting between the labeled supporting cells (apex) or reaching the hair cells (base). *Bars* 100 μm

The distribution of the transcript for *Gata3*, the gene for a zinc finger transcription factor, is uniquely associated with the cochlea, but it is not observed in the vestibular (specifically, saccular) sensory epithelia (Karis et al. 2001). It is known to interact with the Lim-homeodomain and bHLH factors needed for cell-fate specification (Matthews and Visvader 2003). In addition, the two fibroblast growth factors (Fgf) have distinct expression patterns, *Fgf8* being associated with the inner hair cells and *Fgf10* being expressed in the greater epithelial ridge just adjacent to the 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 was distinct from the saccular region (Fig. 8a,b,e,g). There was also an expansion of *Gata3* expression across the cochlea in *Lmx1a* mutants compared with wildtype littermates (Fig. 8e,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. 8c,d,f). Most interestingly, *Lmx1b*, which shares 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. 8c), but no

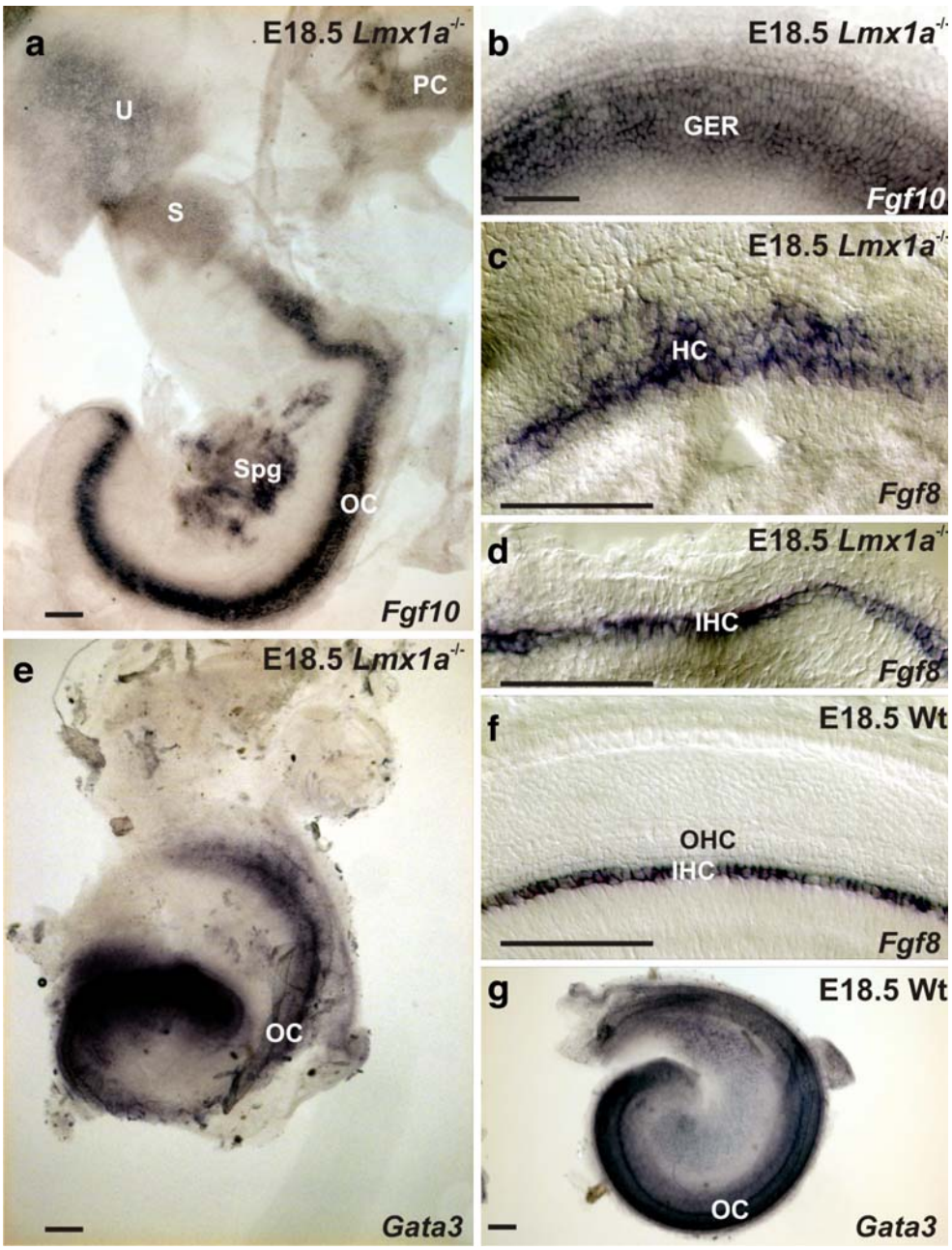


Fig. 8 *Fgf10*, *Fgf8*, and *Gata3* mRNA expression reveal disorganization of the organ of Corti in mutants. **a** In the E18.5 *Lmx1a* mutant, *Fgf10* is strongly expressed in the entire organ of Corti (*OC*), but more weakly in the vestibular sensory epithelia (*PC* posterior crista, *S* saccule, *Spg* spiral ganglion, *U* utricle). **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 and apex of the mutant, respectively. Note that expression is confined to the inner hair cells (*IHC*) in the apex but is scattered among the excess hair cells of the base (*HC* horizontal crista). **f** In the wildtype (*Wt*), *Fgf8* expression is neatly confined to the inner hair cells (*IHC*), with none in the outer hair cells (*OHC*). **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. *Bars* 100 μm

Lmx1b expression has been detected in the ear, this suggests that wildtype *Lmx1a* somehow restricts *Fgf8* expression in the basal cochlea to the 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 scanning electron-microscopic data of the Lmx1a mutant, viz., 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 and the near complete absence of Prox1-expressing cells in the base suggest that these too play roles in generating the altered phenotype of the 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 to 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 Deiters' 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 the early expression of the *Lmx1a* transcription factor appears to be necessary for the coordinated development of the organ of Corti.

Absence of segregation of utricle, saccule, and organ of Corti during development in the *Lmx1a* mutant

Given that our *Lmx1a* expression analysis suggested 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 mice (Chen et al. 2002; Matei et al. 2005). 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 in place, even at this stage, although distinct hair cell patches were observed within the common endorgan. Specifically, an area of constricted hair cell formation indicated 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 by using differential interference microscopy (Fig. 9c). These data suggested a delay in hair cell maturation in the base of the organ of Corti that could contribute (1) to the misexpression of the several of the factors that we have described above and (2) 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 vestibulocochlear efferents were labeled from rhombomere 4, at which point 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 the 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. Whereas 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, the 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 warranting their labeling as run-on precursors of the utricle, saccule, and organ of Corti.

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

In the chick (Giraldez 1998), as in mice (Failli et al. 2002), *Lmx1a* has been shown to be excluded from the neurogenic region of the otic placode. In addition, inhibition of the Notch signaling pathway allows the spread of *Lmx1a* expression into the neurogenic region (Abello et al. 2007). This is reminiscent of the "non-neural" *Tbx1* gene of mice, which when mutated allows neural genes, including *Neurog1*, to expand their expression domains, and which when overexpressed causes the retreat of neural gene expression (Raft et al. 2004).

To obtain further insights into the molecular organization of supporting cells, we next studied the distribution of *Sox2*, which is first necessary for prosensory specification (Fritzsch et al. 2006a; Holmberg et al. 2008) and is only later restricted to 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 occurred in presumptive 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, whereas 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). We 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 in 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. The pattern and intensity of *Sox2* expression in the cochlear region compared to the contiguous saccular portion were most interesting. In both wildtype and mutant, the cochlear expression was much reduced in intensity compared with that of the saccule. In addition, the cochlear expression was extremely broad in the base of the mutant and much less focused than in the wildtype (Fig. 10e,f). This broader and more diffuse pattern of expression persisted in the mutant base through E18.5. The mutant apex developed focused expression reminiscent of the entire wildtype cochlea (Fig. 10h–k).

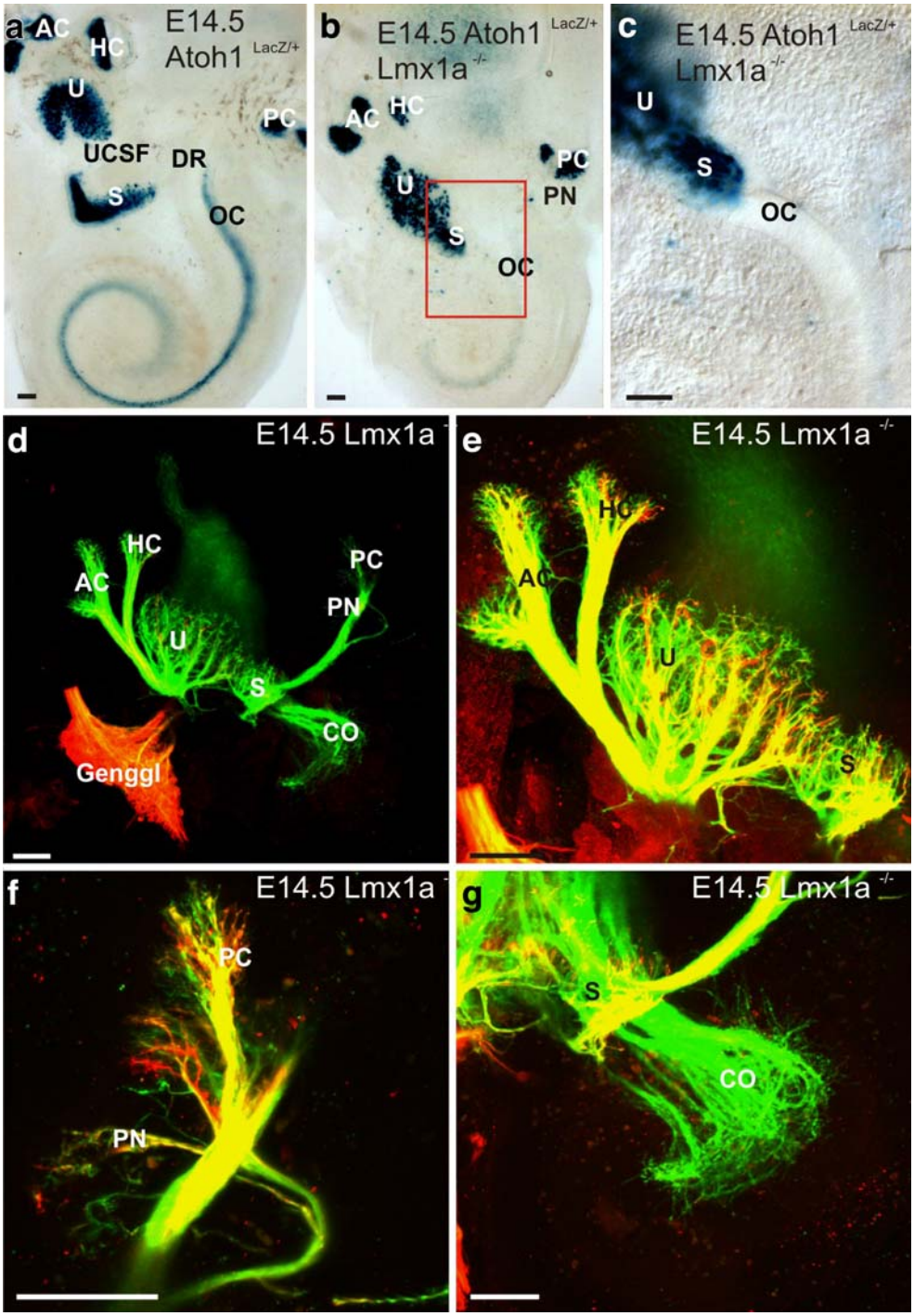
In summary, the wildtype and *Lmx1a*^{dr} mutant exhibited major differences in initial hair cell formation as revealed by *Atoh1*LacZ staining (Fig. 9b), and in prosensory patch formation as revealed by *Sox2* expression (Fig. 10g). Whereas prosensory patch formation maintained, from its onset, the 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 the initial *Atoh1* upregulation and withdrawal of the hair cells from the cell cycle (Matei et al. 2005) and the 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, the initial expression changes in *Sox2* foreshadow the later expansion and disorganization of the hair cells in the mutant cochlea and the overgrowth of the papilla neglecta. Notably, 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 is nonexistent in the *Lmx1a* mutant, whereas 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.

Fig. 9 *Atoh1*LacZ staining of hair cells and tracer dye studies showing initial segregation of sensory epithelia and their innervation. **a** Wildtype *Atoh1*LacZ-stained ear (*left* anterior, *up* dorsal, *AC* anterior crista, *DR* ductus reuniens, *HC* horizontal crista, *OC* organ of Corti, *PC* posterior crista, *S* saccule, *U* utricle, *UCSF* utriculo-saccular foramen). **b** An identically stained mutant ear. Note that the cristae are separated at this age and the posterior crista (*PC*) is not grossly enlarged, although a tiny papilla neglecta (*PN*) is present. The utricle (*U*) and saccule (*S*) are, however, joined. **c** Higher magnification of the *red boxed field* in **b**. The basal termination of the unstained though translucent organ of Corti (*OC*) overlaps the stained horizontal cristae of the saccule (*S*). **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 and 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 (*CO* cochlea). **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 (*AC*), horizontal crista (*HC*), utricle (*U*), and saccule (*S*) are almost normal, except for the limited segregation of the saccule from the utricle. **f** Innervation to the posterior crista (*PC*) is abnormally widespread and includes a large branch to the papilla neglecta (*PN*). **g** Innervation to the basal cochlea (*CO*) lacks a vestibular (*yellow*) component. *Bars* 100 μm

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 the 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 occur 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 *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 (Figs. 1k, 2a-d). A lack of *Lmx1a* expression adjacent to developing sensory epithelia might interfere 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 agreement with the emerging concept that crista epithelia express diffusible factors such as *Fgf10* and *Bmp4* (*bone morphogenetic protein 4*) that regulate the growth of the non-sensory part of the vertical canals (Chang et al. 2004b, 2008; Fritzsch et al. 2006b; Pauley et al. 2003). In contrast, the cochlea grows by the 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 hypotheses conform with the known interactions that occur in the inner ear otic

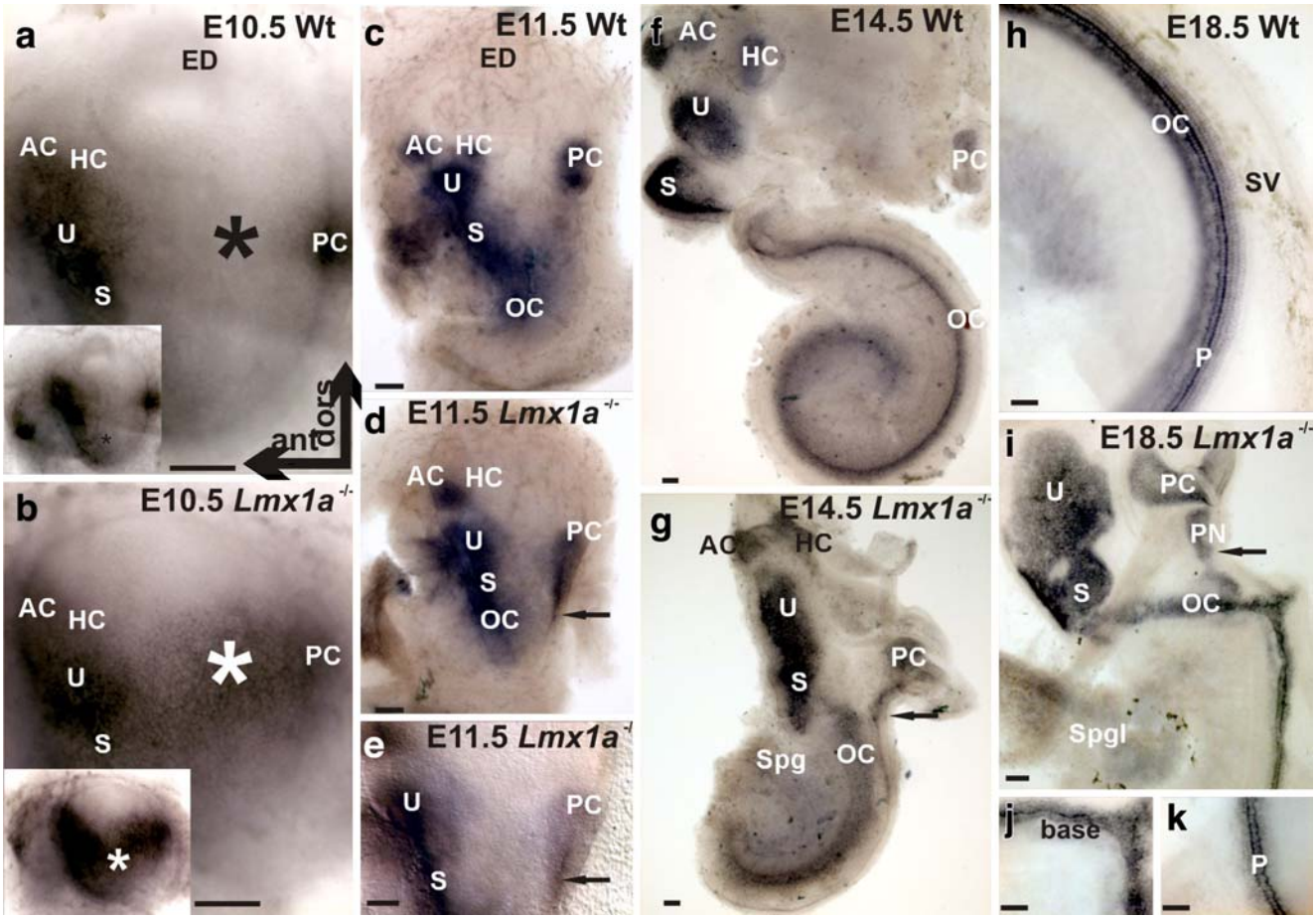


Fig. 10 *Sox2* mRNA expression shows that *Lmx1a* defines early prosensory patch development (*ant* anterior, *dors* dorsal, *AC* anterior crista, ED HC horizontal crista, OC organ of Corti, PC posterior crista, PN papilla neglecta, S saccule, Spg spiral ganglion, SV stria vascularis, U utricle) **a**, **b** *Sox2* expression is similar, but broader in the mutant without a distinct gap between posterior and anterior prosensory patch expression (*star* in **a**, **b**; see also lower magnification *insets*). **c–e** At E11.5, wildtype expression is localizing to individual sensory epithelia (note the separate AC and HC), whereas 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; cf. Fig. 8h), whereas 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 utricle-saccule-cochlear and enlarged posterior crista/papilla neglecta. **j**, **k** At E18.5, *Sox2* expression in the mutant cochlear base (**j**) is broad and diffuse, whereas that in the apex (**k**) is, as in the wildtype, focused on the inner pillar cells (*P*). All whole ears oriented as in **a**. *Bars* 100 μm (**a-g**), 50 μm (**h**, **k**)

mesenchyme, and that require bilateral signals, only some of which are presently understood (Pirvola et al. 2004).

Overall, *Lmx1a* mutant dysmorphogenesis is a more exaggerated form of *Otx1* null dysmorphogenesis in which the utricle and saccule remain 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 organ of Corti in the *Otx1* mutant 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 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 the distinctions that 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. Other genes are overlappingly expressed in 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 central nervous system (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, the expression of *Fgf8* is altered in the basal organ of Corti of *Lmx1a* mutants, showing that disregulation of at least one *Fgf* indeed occurs in the ear and that the severe dysmorphogenesis might be related to disregulation of both *Fgfs* and *Bmps*. *Wnts* 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 Wnts and some Fgfs are secreted by the non-sensory part of the developing ear. Therefore, a *Wnt* or *Fgf* agent such as Fgf9 (Pirvola et al. 2004) or Wnt4 (Daudet et al. 2002) might be directly regulated by *Lmx1a*, and these and other factor(s) released from the *Lmx1a*-expressing non-sensory areas of the developing ear might regulate crucial aspects of ear morphogenesis and provide a feed-back loop for sensory epithelium development. The absence of the endolymphatic duct in the *Lmx1a* mutant might certainly be related 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 indeed 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 *Lmx1a* mutant mice as this might also play a role in adult hair cell loss.

In this context, we should stress 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 reaction-diffusion 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 finding that *Dkk1* can rescue the *Otx2* phenotype (Kimura-Yoshida et al. 2005; Lewis et al. 2008) implies that *Lmx1a* plays an unspecified part in *Wnt-Otx* mediated morphogenesis. Similarly, the close proximity of pigment cells to *Lmx1a*-expressing areas suggests 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 the Lmx1a protein in the 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 the cerebrospinal-fluid-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 papilla neglecta with the anterior and posterior crista, respectively. Interestingly, the initial upregulation of *Atoh1* for the differentiation of hair cells is discrete but is embedded in enlarged or fused *Sox2* expression domains (Fig. 10). The hair cell patches fuse into these enlarged epithelia only in late embryonic stages (Figs. 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 focal prosensory patch formation nor focal *Atoh1* upregulation is understood at the molecular level (Kiernan et al. 2005; Matei et al. 2005). 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). 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 attributable to cell-type mingling across an undetected border. However, this 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. Obviously, changing the expression of *Fgf8* from a single to multiple rows can affect the differentiation of pillar and Deiters' cells, as shown for mutations of the *Fgfr3* receptor (Puligilla et al. 2007). Hence, the next step is to define those 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, we should be able to begin a determination of the way that *Lmx1a* expression differs across vertebrate phyla. *Lmx1a* might play the same role for the segregation of the common macula into multiple end organs that *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* might be involved in ionic homeostasis, which is critical for the proper function of ear and mechanosensors alike (Fritzsch et al. 2000, 2007; Todi et al. 2004; Walker et al. 2000). Irrespetive of the details, our results suggest that local *Lmx1a* expression is a major player in ear morpho- and histogenesis, and that this function is probably conserved at the level of interacting modules of transcription factors.

Acknowledgement We are grateful to Jennifer Kersigo, Anne Lindgren, Yuriko Mishima, and Amanda Branch for technical assistance and collection of mouse embryos, to Dr. Garret Soukup, Marsha Pierce, and Jason Pecka for help in designing and preparing primers, to Drs. Engel, Ornitz, Hogan and Chea for providing plasmids with probes, and to Dr. Huda Zoghbi for mice.

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