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RESEARCH ARTICLE

Otx2 is a target of *N-myc* and acts as a suppressor of sensory development in the mammalian cochlea

Victor Vendrell[1], Iris López-Hernández[1], María Beatriz Durán Alonso[1], Ana Feijoo-Redondo[1], Gina Abello[2], Héctor Gálvez[2], Fernando Giráldez[2], Thomas Lamonerie[3] and Thomas Schimmang[1,*]

ABSTRACT

Transcriptional regulatory networks are essential during the formation and differentiation of organs. The transcription factor N-myc is required for proper morphogenesis of the cochlea and to control correct patterning of the organ of Corti. We show here that the *Otx2* gene, a mammalian ortholog of the *Drosophila orthodenticle* homeobox gene, is a crucial target of *N-myc* during inner ear development. *Otx2* expression is lost in *N-myc* mouse mutants, and *N-myc* misexpression in the chick inner ear leads to ectopic expression of *Otx2*. Furthermore, *Otx2* enhancer activity is increased by *N-myc* misexpression, indicating that *N-myc* may directly regulate *Otx2*. Inactivation of *Otx2* in the mouse inner ear leads to ectopic expression of prosensory markers in non-sensory regions of the cochlear duct. Upon further differentiation, these domains give rise to an ectopic organ of Corti, together with the re-specification of non-sensory areas into sensory epithelia, and the loss of Reissner's membrane. Therefore, the *Otx2*-positive domain of the cochlear duct shows a striking competence to develop into a mirror-image copy of the organ of Corti. Taken together, these data show that *Otx2* acts downstream of *N-myc* and is essential for patterning and spatial restriction of the sensory domain of the mammalian cochlea.

KEY WORDS: Inner ear, Cochlea, Otx, Myc, Organ of Corti, Mouse

INTRODUCTION

Otx2, a murine ortholog of the *Drosophila orthodenticle* (*otd*; *ocelliless* – FlyBase) gene, encodes a transcription factor required for early specification of the brain and for development of sensory organs, including the inner ear (Gat-Yablonski, 2011). In humans, heterozygous mutations of *OTX2* lead to eye and pituitary gland defects (Henderson et al., 2007, 2009) and have been also reported to cause hearing loss (Ragge et al., 2005). During mouse inner ear development, *Otx2* is initially expressed in the ventral portion of the otic vesicle, which gives rise to the cochlea. Later on, it is expressed in the non-sensory area of the roof of the cochlear duct corresponding to the future Reissner's membrane, which separates the *scala media* from the *scala vestibuli* (Morsli et al., 1999). Mouse mutants of the related *Otx1* gene display shortening of the cochlea, loss of the lateral semicircular canal and fusion of sensory epithelia (Morsli et al., 1999). Additional loss of one *Otx2* allele in an *Otx1* null background results in a more severe phenotype, especially in the cochlea. However, owing to the early lethality of *Otx2* mutant mice (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995), its role during inner ear development has not been addressed.

Inactivation of *N-myc* (*Mycn* – Mouse Genome Informatics) in the mouse inner ear affects proliferation, morphogenesis and differentiation of the inner ear (Dominguez-Frutos et al., 2011; Kopecky et al., 2011). Besides an overall size reduction of the inner ear, *N-myc* mouse mutants show more complex phenotypes affecting morphogenesis and patterning, including loss of the lateral semicircular canal and fusion of sensory epithelia. The cochlea is shortened and characterized by an increased number of hair cells in its apical part (Dominguez-Frutos et al., 2011; Kopecky et al., 2011).

In the present work, we show that in the course of a microarray-based screen designed to identify *N-myc* target genes in the cochlea, we found *Otx2* as a candidate to mediate some of the functions of *N-myc* during cochlear development. *Otx2* is one of the genes expressed in the roof of the cochlear duct that is most strongly downregulated in *N-myc* mutants. Conversely, *N-myc* misexpression in the chick inner ear leads to ectopic activation of *Otx2* expression, and we provide evidence that distal enhancer elements located within the 3′ genomic regulatory region of the *Otx2* gene are directly responsive to *N-myc*. To study the effects of Otx2 further, we generated conditional *Otx2* mutant mice and showed that *Otx2* inactivation in the inner ear results in re-patterning of the cochlea. *Otx2* ablation leads to the ectopic expression of several key genes involved in formation of the prosensory domain in the prospective non-sensory domain normally defined by *Otx2* expression and that gives rise to Reissner's membrane (Morsli et al., 1999). Accordingly, Reissner's membrane is absent in *Otx2* mutant cochleas, which exhibit a striking mirror-image duplication of the organ of Corti. Our results suggest that *Otx2* acts downstream of *N-myc* as a suppressor of sensory fate and that it is required for correct patterning of the non-sensory region that will give rise to Reissner's membrane.

RESULTS

A screen for *N-myc*-regulated genes reveals downregulation of genes in the non-sensory region of the roof of the cochlear duct

Loss of *N-myc* in the mouse cochlea leads to its shortening and to an increased number of differentiating sensory hair cells in the apex (Dominguez-Frutos et al., 2011; Kopecky et al., 2011). In order to identify potential target genes of *N-myc* in the mammalian cochlea, we performed a microarray-based screen for differential gene expression in *N-myc* mutant versus wild-type cochleas (for details, see Materials and Methods). We used whole cochleas at embryonic day (E) 15 when *N-myc* expression is detected in the floor of the cochlear duct, including differentiating hair cells (Dominguez-

[1]Instituto de Biología y Genética Molecular, Universidad de Valladolid y Consejo Superior de Investigaciones Científicas, C/Sanz y Forés 3, Valladolid E-47003, Spain. [2]CEXS, Universitat Pompeu Fabra, Parc de Recerca Biomédica de Barcelona, Barcelona E-08003, Spain. [3]Institut de Biologie Valrose, University of Nice Sophia Antipolis, UMR UNS/CNRS 7277/INSERM 1091, Nice F-06108, France.

*Author for correspondence (schimman@ibgm.uva.es)

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Frutos et al., 2011) (Fig. 1A). The results of the microarrays [summarized in supplementary material Table S1 and deposited in full in Gene Expression Omnibus (GEO) under accession number GSE61406] showed a broad downregulation of genes in the *N-myc* mutant cochleas. Among the genes predicted to be strongly downregulated in *N-myc* mutant cochleas, we found several that are expressed in the non-sensory portion of the roof of the cochlear duct, such as the low-density lipoprotein-receptor related protein 2 (Lrp2, also termed Megalin, MGI 95794, with 6.8-fold downregulation), endothelin-converting enzyme type 1 (Ece1, MGI 1343461, 5.3-fold downregulation) and Otx2 (4.4-fold downregulation).

Lrp2 is expressed at E15 in the roof of the cochlear duct (Fig. 1B), and later on in the *stria vascularis* and Reissner's membrane (Tauris et al., 2009) (Fig. 6K). *Ece1* and *Otx2* are both found in the medial (neural) portion of the roof of the cochlear duct, which gives rise to Reissner's membrane (Fig. 1E,H). Loss of expression of these genes in *N-myc* mutant cochleas was confirmed by either immunohistochemistry (Fig. 1C) or RNA *in situ* hybridization (Fig. 1D,F,G,I). Mouse *Lrp2* mutants suffer from progressive hearing loss associated with defects in the *stria vascularis* (Konig et al., 2008), but the reported malformations do not resemble the *N-myc* mutant phenotype. *Ece1* homozygous mouse mutants die at birth or soon after as a result of respiratory distress but no inner ear phenotype has so far been documented (Schweizer et al., 1999). Finally, *Otx2* mutants die during gastrulation, precluding the analysis of inner ear development (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995). However, the presence of an *Otx2* null allele in heterozygosity on an *Otx1* null background leads to enhancement of the *Otx1* mutant inner ear phenotype, consisting of loss of the lateral semicircular canal, fusion of sensory epithelia and shortening of the cochlea (Morsli et al., 1999). These alterations are very similar to those reported in *N-myc* mutant mice (Dominguez-Frutos et al., 2011; Kopecky et al., 2011), suggesting that *Otx2* may be instrumental for *N-myc* function in the ear. The onset of *Otx2* expression during inner ear development occurs in the otic vesicle and is lost in *N-myc* mutants (Fig. 1J,K). These results prompted us to analyze further the relationship between *Otx2* and *N-myc* during inner ear development.

Misexpression of *N-myc* induces expression of *Otx2* in the otic vesicle

In order to study the regulation of *Otx2* by *N-myc*, we misexpressed *N-myc* in the chicken otic placode by electroporation of a cDNA encoding for *N-myc* together with a GFP reporter (see Dominguez-Frutos et al., 2011). The expression of *Otx2* in the otic vesicle was examined 1 day after electroporation using riboprobes specific for chicken *Otx2* (*cOtx2*). Like in the mouse, endogenous expression of *cOtx2* occurs in the ventral portion of the otic vesicle (Fig. 2A). However, after *N-myc* electroporation, ectopic *cOtx2* expression was detected in the anterior (Fig. 2B) and dorsal (Fig. 2C; supplementary material Fig. S1) portions of the otic vesicle (n=4/4; for details, see Materials and Methods). Interestingly, the ectopic *cOtx2* expression domain was always broader than GFP reporter activity, suggesting non-cell-autonomous interactions.

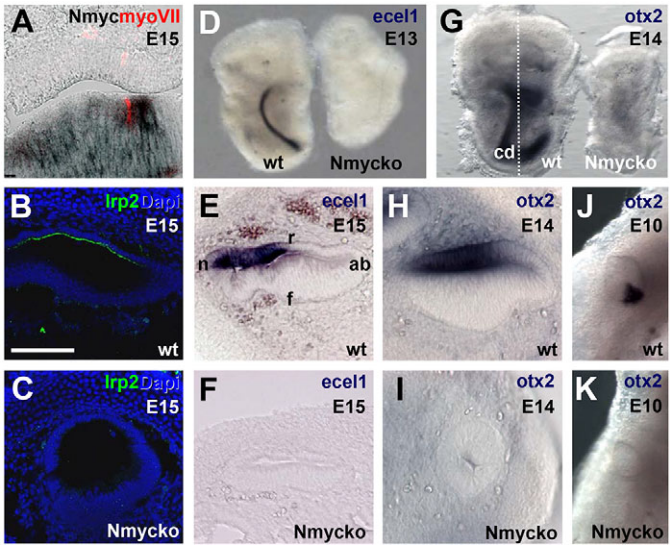

Fig. 1. Downregulation of genes expressed in the roof of the cochlear duct in *N-myc* mutants. (A) Section through the cochlear duct at E15 labeled with an RNA probe for *N-myc* and antibodies directed against myosin VIIA, which indicates differentiating hair cells. (B,C) Sections through the cochlear duct of wild-type (wt) and *N-myc* mutant mice labeled with antibodies against Lrp2 at E15. (D-I) Whole-mount *in situ* hybridized cochleas from wild-type and *N-myc* mutant mice labeled with RNA probes for *Ecel1* (D) and *Otx2* (G) and corresponding sections through the cochlear duct (E,F,H,I). Images shown in C, F and I correspond to representative sections derived from the apical, basal and midbasal portion of the cochlear duct of the *N-myc* mutant, respectively. The plane of the sections through the cochlear duct (cd) is indicated in G. The floor (f) and roof (r), and the neural (n) and abneural (ab) portion of the cochlear duct are indicated in E. (J,K) Wild-type and *N-myc* mutant embryos labeled with an RNA probe for *Otx2* at E10. Scale bar: in B, 100 µm for B,C,E,F,H,I; in A, 50 µm.

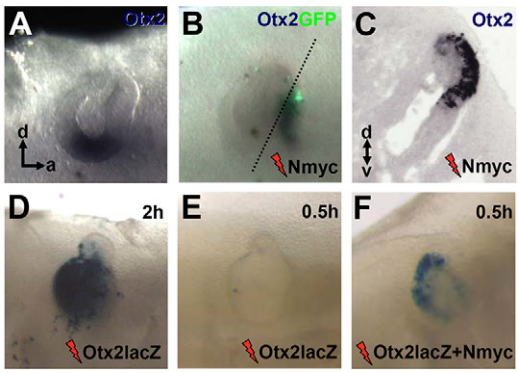

Fig. 2. Effects of *N-myc* on *Otx2* expression in the otic vesicle of chicken embryos. (A) Expression of *cOtx2* in the ventral portion of the otic vesicle detected by RNA *in situ* hybridization. (B,C) Detection of *cOtx2* expression upon electroporation of the otic vesicle with vectors containing *N-myc* and *GFP*. After a brief exposure to the chromogenic substrate, indicating the presence of the digoxigenin-labeled RNA probe, ectopic *cOtx2* expression is detected in the anterior (B) and dorsal (C; transversal section derived from B) portions of the otic vesicle. The plane of the section to produce C is indicated in B by a dotted line. (D-F) Otic vesicles electroporated with *Otx2lacZ* reporter, containing a 3′ enhancer of the murine *Otx2* gene, which drives *lacZ* expression. Note that in E and F, for better visualization of the effect of *N-myc* on the activity of the *Otx2lacZ* reporter, a short term exposure of the chromogenic substrate was used with respect to D (for details, see Materials and Methods). The exposure times for the chromogenic substrate are indicated. (D) Electroporation of *Otx2lacZ* reporter leads to expression of *lacZ* throughout the otic vesicle. (E,F) Upon co-electroporation of the *Otx2lacZ* reporter and *N-myc*, and following a short term exposure of the chromogenic substrate, the amount of *lacZ* expression detected in the otic vesicle is higher (F) compared with embryos electroporated only with the *Otx2lacZ* reporter (E). The orientation of the embryos and the section in C along the dorsal (d)-anterior (a) or dorsal-ventral (v) axis, respectively, are indicated.

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In the developing mouse embryo, *Otx2* expression is controlled by specific enhancers located 5′ and 3′ of its coding region (Kurokawa et al., 2004). A 1.2-kb fragment in the 3′ enhancer region of *Otx2* has been shown to drive expression of a *lacZ* reporter gene in the otic vesicle (Kurokawa et al., 2004). Although this *Otx2lacZ* reporter shows a broader expression than the endogenous *Otx2* gene, it nevertheless provides a tool to test potential transcriptional regulators that drive *Otx2* expression in the otic vesicle. Electroporation of the *Otx2lacZ* reporter construct confirmed the activity of this enhancer region in the otic vesicle of chicken embryos (Fig. 2D). Moreover, the co-electroporation of the *Otx2lacZ* reporter together with *N-myc* showed an increased activity of the reporter compared with the *Otx2lacZ* reporter alone (compare Fig. 2E and 2F). In summary, the data show that *N-myc* is sufficient to drive *Otx2* expression in the otic vesicle and increases the efficiency of transcription from the 3′ *Otx2* enhancer.

To explore whether *N-myc* and *Otx2* cross-regulate each other, we misexpressed *Otx2* in the otic placode of chicken embryos. After *Otx2* electroporation no ectopic expression of *N-myc* was observed and its endogenous pattern was unaffected (data not shown).

Formation and patterning of the otic vesicle is unaffected in conditional *Otx2* mutant mice

The exact role of *Otx2* during inner ear development has not yet been addressed because of the early lethality of *Otx2* mutant mice (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995). To overcome this problem, we generated conditional *Otx2* mutant mice in which tissue-specific deletion of the essential *Otx2* exon 2 flanked by *loxP* sites (Fossat et al., 2006) was achieved using a Cre line driven by *Pax2* regulatory sequences. The *Pax2*Cre line has been used to inactivate floxed alleles during initial stages of inner ear development (Ohyama and Groves, 2004). Loss of *Otx2* in the otic vesicle of homozygous *Pax2*Cre-*Otx2* mutants, at the onset of *Otx2* expression, was confirmed by whole-mount RNA *in situ* hybridization (Fig. 3A). Additionally, loss of *Otx2* expression was observed in other *Pax2*-expressing tissues, such as the optic vesicle and the mesencephalon. As previously shown, loss of *Otx2* caused a severe reduction or absence of these structures (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995) (Fig. 3A) and homozygous *Pax2*Cre-*Otx2* mutants showed early postnatal lethality within the first week after birth.

The size of the otic vesicle in *Pax2*Cre-*Otx2* mutants was similar to that of control littermates (Fig. 3A), and the specification of its neurosensory region was unaffected (Fig. 3B-K). This region develops in an anterior-medial position flanking the *Otx2* expression domain and is characterized by the expression of *Sox2* (Pan et al., 2010), neurogenin 1 (*Ngn1*; *Neurog1* – Mouse Genome Informatics), *NeuroD* (Fritzsch et al., 2010), lunatic fringe (*LFng*) (Morsli et al., 1998) and *Fgf3* (Hatch et al., 2007). Whole-mount *in situ* hybridization of wild-type and *Pax2*Cre-*Otx2* mutant embryos revealed correct localization of mRNA for these genes in the otic vesicle (Fig. 3B-K). Moreover, *NeuroD* was expressed in the otic ganglion suggesting that neuroblast delamination was unaffected by the loss of *Otx2* (Fig. 3J,K). Finally, to investigate a potential cross-regulation between *Otx2* and *N-myc*, we examined *N-myc* expression in *Otx2* mutant otic vesicles. *N-myc* is initially expressed throughout the otic vesicle and, later on, becomes restricted to the prosensory regions (Dominguez-Frutos et al., 2011). Expression of *N-myc* was unaffected in the otic vesicle of *Otx2* mutants (Fig. 3L,M; data not shown), suggesting that *Otx2* expression is not required for the formation and early patterning of the otic vesicle.

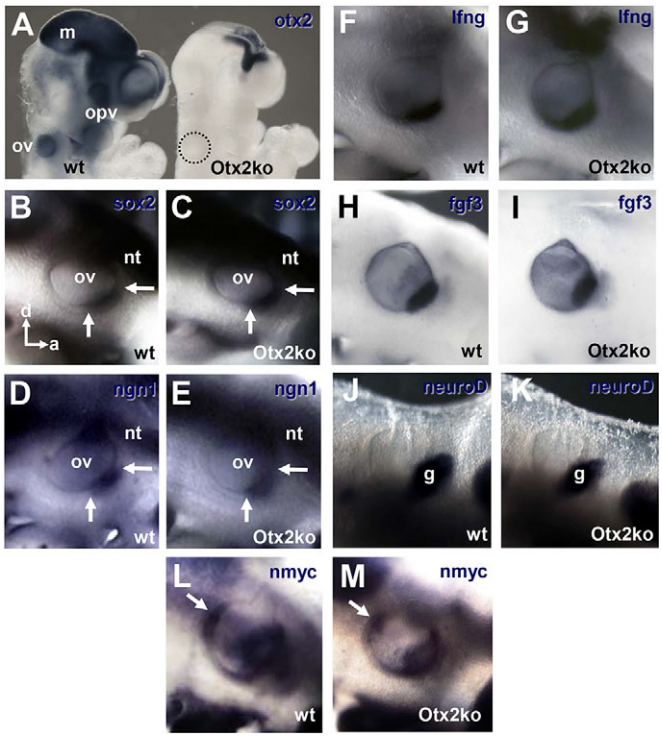

Fig. 3. Expression of otic genes upon conditional inactivation of the *Otx2* gene. (A) Detection of *Otx2* via whole-mount RNA *in situ* hybridization in the midbrain (m), optic vesicle (opv) and otic vesicle (ov) of E10 wild-type (wt) and an *Otx2* mutant embryo upon conditional inactivation with the *Pax2*Cre transgene. The circumference of the otic vesicle in the *Otx2* mutant is indicated by dotted lines. (B-M) Hybridization of E10 embryos with the indicated riboprobes reveals a correct localization of the indicated genes within the neurosensory domain in the otic vesicles of *Otx2* mutants compared with wild-type embryos. The borders of the expression domains of *Sox2* and *Ngn1* in the otic vesicle are indicated by arrows. (L,M) *N-myc* is broadly expressed in the otic vesicle. The arrow indicates the position of the future posterior prosensory region where *N-myc* expression has started to accumulate. Orientation of embryos shown in B-K along the dorsal (d)-anterior (a) axis are indicated in B. nt, neural tube; g, otic ganglion.

Loss of *Otx2* expression leads to ectopic expression of prosensory markers in the cochlear duct

The HMG-box transcription factor Sox2 and the Notch ligand Jag1 are necessary for the specification of sensory progenitors in the cochlear duct (Dabdoub et al., 2008; Kiernan et al., 2005, 2006) and both are initially expressed in the ventral part of the cochlea (Ohyama et al., 2012). Later on, Sox2 becomes restricted to the prosensory domain and to the region of the Kölliker's organ that gives rise to the inner sulcus (Fig. 4A), whereas Jag1 is progressively restricted to Kölliker's organ (Fig. 4C). The prosensory region is characterized by the expression of $p27^{kip1}$ (Cdkn1b – Mouse Genome Informatics) (Fig. 4E), which labels postmitotic hair and supporting cell progenitors (Lee et al., 2006). Expression of these markers was normal in the basal portion of the cochlea of *Pax2*Cre-*Otx2* mutants at E14 (data not shown). However, the midbasal and apical regions showed a dramatic expansion of the Sox2 expression domain towards the roof and the abneural portions of the cochlear duct (Fig. 4B). Sox2 expression was accompanied by an apparent thickening of the cochlear epithelium, a feature normally observed only in the floor of the cochlear duct but not in the non-sensory region of its roof. In parallel, and next to their normal regions of expression, Jag1 showed an additional ectopic expression domain in the neural portion of the

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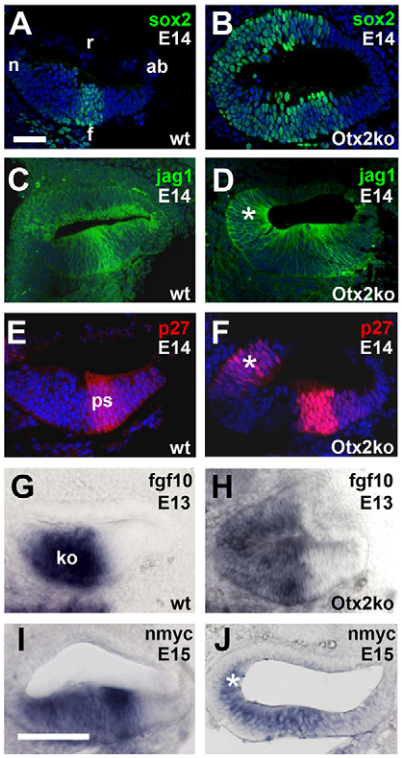

Fig. 4. Formation of the prosensory region in *Otx2* mutants. (A-F) Expression of the indicated proteins detected by immunohistochemistry in wild type (wt) and *Otx2* mutants at E14. Note the expression of Sox2 throughout the neural side and roof of the cochlear duct in *Otx2* mutants compared with the wild type (A,B). Asterisks label the ectopic domains of jagged (Jag1) in D and $p27^{kip1}$ expression in F, indicating the formation of an ectopic prosensory region (ps) in *Otx2* mutants. (G-J) Expression of *Fgf10* and *N-myc* detected by RNA probes at E13 and E15, respectively. *Fgf10* labels the Kölliker's organ (ko) whereas *N-myc* is present throughout most of the floor of the cochlear duct. Note the expanded domain of *Fgf10* and *N-myc* (indicated by an asterisk) extending to the roof of the cochlear duct in *Otx2* mutants. The floor (f) and roof (r), and the neural (n) and abneural (ab) portion of the cochlear duct are indicated in A. Scale bars: in A, 50 µm for A-H; in I, 100 µm for I,J.

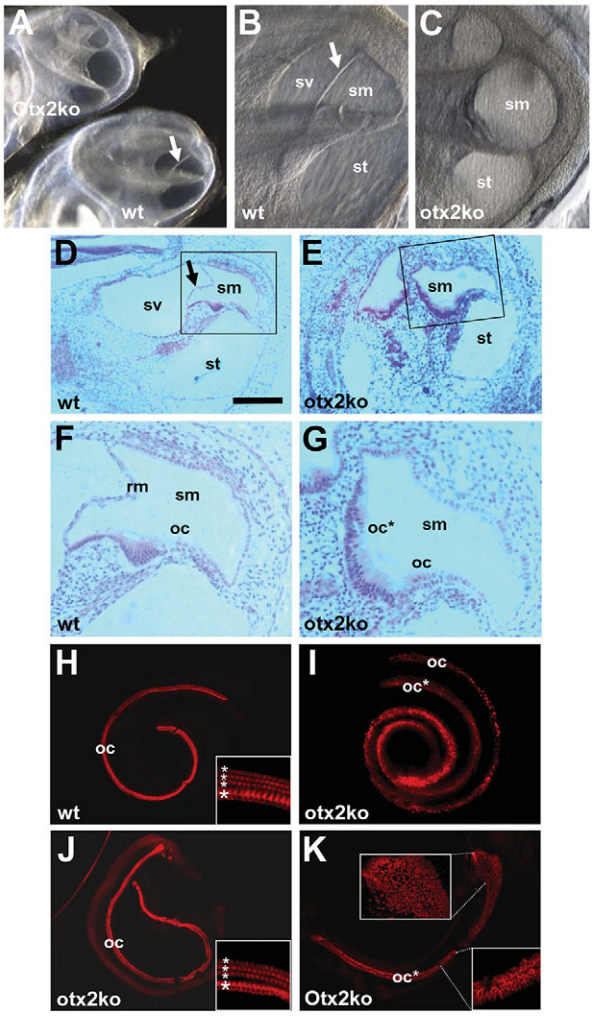

Fig. 5. Inner ear phenotype of postnatal *Otx2* mutants. (A-C) Clarification of inner ears at P0 reveals the absence of Reissner's menbrane in *Otx2* mutants (indicated by an arrow in the wild type, wt), which separates the *scala media* (sm) from the *scala vestibuli* (sv). (D-G) Histological sections through the cochlea confirm the absence of Reissner's membrane (rm) and indicates the presence of an ectopic organ of Corti (oc) in the *Otx2* mutant (oc*). F and G show higher magnifications of the boxed areas in D and E, respectively. (H-K) Whole-mount labeling of sensory epithelia with myosin VIIA antibodies confirms the presence of an additional stripe of hair cells (oc*) in *Otx2* mutants (I). (H,J) A normal staining patterning revealing the presence of one row of inner hair cells (large asterisk) and three rows of outer hair cells (small asterisks) is observed in a wild-type cochlea (inset in H) and the normotopic sensory epithelium (oc) of *Otx2* mutants (inset in J). (K) By contrast, the hair cells present in the ectopic sensory epithelium of *Otx2* mutants (oc*) show a highly disorganized pattern and an increase in their number leading to an expansion of the sensory region in its most apical portion (insets in K). st, *scala tympani*. Scale bar: 200 µm.

cochlear duct (Fig. 4D, asterisk) whereas $p27^{kip1}$ was present both in the neural region and the adjacent portion of the cochlear roof (Fig. 4F, asterisk). Finally, we examined *Fgf10*, a marker for the prospective Kölliker's organ (Ohyama et al., 2012) (Fig. 4G) and *N-myc*, which is restricted to the floor of the cochlear duct (Dominguez-Frutos et al., 2011) (Fig. 4I). The expression domains of both genes were expanded to the roof of the cochlear duct (Fig. 4H,J). Taken together, these results indicate that loss of *Otx2* in the non-sensory region of the cochlear duct leads to re-patterning of the mouse cochlea with ectopic expression of markers normally restricted to the prosensory domain and the neighboring Kölliker's organ.

Otx2 mutant mice lack Reissner's membrane and instead form an ectopic organ of Corti

In order to determine the consequences of the abnormal patterning of the cochlear duct in *Otx2* mutants, we next examined the early postnatal period when the organ of Corti fully develops. At postnatal day (P) 0, the different subcompartments of the cochlea can be distinguished in clarified wild-type inner ears: the *scala media* and *scala vestibuli* separated by Reissner's membrane, and the *scala tympani* (Fig. 5A,B). Strikingly, in *Pax2*Cre-*Otx2* mutants, no Reissner's membrane could be detected (Fig. 5A,C). Histological sections confirmed the absence of Reissner's membrane (Fig. 5D,E), and indicated the formation of ectopic hair cells on the neural side of the *scala media* (Fig. 5F,G). This was confirmed by whole-mount immunostaining with a myosin VIIA antibody (Fig. 5H-K). *Pax2*Cre-*Otx2* mutant cochleas revealed the presence of two parallel myosin VIIA-positive stripes (Fig. 5I). Further dissection of the sensory regions revealed that the stripe of hair cells present in the normotopic position of the cochlear duct of *Pax2*Cre-*Otx2* mutants was similar to that observed in the sensory epithelia from wild-type controls: one row of inner and three rows of outer hair cells (Fig. 5H,J). By contrast, the ectopic stripe of sensory

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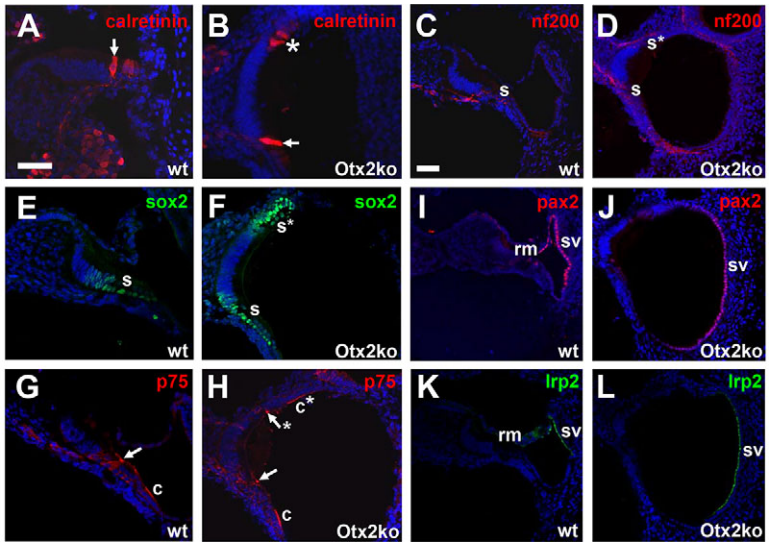

Fig. 6. Differentiation of different cell types in the cochlear duct of *Otx2* mutants. (A-L) Detection of the indicated proteins in the cochlear duct of wild type (wt) and *Otx2* mutants at P0. (A,B) Calretinin antibodies preferentially label normotopic (arrows in A,B) and ectopic (asterisk in B) inner hair cells. (C,D) Nerve fibers labeled with antibodies against neurofilaments (nf200) are found underneath the normotopic (s) and ectopic sensory region (s* in F). (E,F) Sox2 expression is detected in supporting cells underlying the normotopic (s) and ectopic sensory epithelium (s* in F). (G,H) p75 immunoreactivity is found in the apical portions of normotopic pillar cells (arrows) and Claudius cells (c), and in the ectopic pillar and Claudius cells (arrow* and c* in H). (I-L) Pax2 and Lrp2 are expressed in Reissner's membrane (rm) and the *stria vascularis* (sv). Note the absence of Reissner's membrane and the expansion of the *stria vascularis* in *Otx2* mutants. Scale bars: in A, 50 µm for A, B,E-H; in C, 50 µm for C,D,I-L.

epithelium showed a poorly organized pattern that lacked the ordered rows of inner and outer hair cells (Fig. 5K). Moreover, the number of hair cells within the sensory epithelium of mutants was higher than that observed in the sensory epithelium of control ears. This phenotype was especially apparent in the apical region, which exhibited a club-shaped ending containing numerous myosin VIIA-positive cells (Fig. 5K, insets).

To characterize these changes further, we used a series of markers providing information on the differentiation of specific subtypes of cells, such as hair cells, nerve fibers, supporting cells and non-sensory cells of Reissner's membrane or the *stria vascularis*. The presence of inner hair cells was confirmed using calretinin antibodies (Dechesne et al., 1994) (Fig. 6A). Calretinin-positive cells were detected in the normotopic organ of Corti and also on the neural side of the ectopic patch of hair cells in *Pax2*Cre-*Otx2* mutants (Fig. 6B). Neurofilament antibodies labeled nerve fibers that reached both the normotopic organ of Corti and the ectopic hair cells present in *Pax2*Cre-*Otx2* mutant mice (Fig. 6C,D). We next examined the presence of supporting cells that underlie or flank hair cells within the organ of Corti. Upon differentiation of hair cells, Sox2, which is initially expressed throughout the developing organ of Corti, becomes restricted to supporting cells (Dabdoub et al., 2008) (Fig. 6E). Ectopic sensory regions of *Pax2*Cre-*Otx2* mice also showed a Sox2 staining pattern that was very similar to the normotopic organ of Corti, confirming the presence of supporting cells (Fig. 6F). Ectopic hair cells in *Pax2*Cre-*Otx2* mutants displayed a faint immunoreactivity for Sox2, probably indicating delayed differentiation. To specify further the supporting cell types present in the *Pax2*Cre-*Otx2* mutant, we examined the expression of the low-affinity neurotrophin receptor p75 (Ngfr – Mouse Genome Informatics), which shows a highly characteristic expression in the apical cell membranes of the inner pillar and Claudius cells during differentiation of the organ of Corti (Mueller et al., 2002; Shim et al., 2005) (Fig. 6G). As in control and in the normotopic sensory epithelium of *Pax2*Cre-*Otx2* mutants, p75 labeled the apical cell membrane of the 'inner pillar cell head' in ectopic hair cell clusters (Fig. 6G,H, arrows). In the vicinity of these cells, another group of cells also showed their apical sides strongly labeled with p75. These probably correspond to ectopic Claudius cells usually found on the abneural side of the organ of Corti (Fig. 6G,H).

Finally, we examined the expression of markers of non-sensory epithelia located in the roof and abneural portion of the cochlear duct. Pax2 and Lrp2 label the *stria vascularis* and are also expressed in Reissner's membrane (Burton et al., 2004; Tauris et al., 2009) (Fig. 6I,K). *Pax2*Cre-*Otx2* mutant mice had no Reissner's membrane and displayed an expansion of the abneural Pax2 and Lrp2 domains that correspond to the *stria vascularis* (Fig. 6J,L).

In summary, the results show that *Otx2* is required for the correct patterning of the cochlea (Fig. 7). *Otx2* directs the formation of Reissner's membrane, which in *Pax2*Cre-*Otx2* mutants is replaced by an ectopic cochlear sensory domain consisting of ectopic hair cells that are innervated and surrounded by supporting cells. The staining pattern of calretinin, together with the characteristic localization of the supporting cell markers Sox2 and p75 indicates that the loss of *Otx2* causes a mirror image duplication of the organ

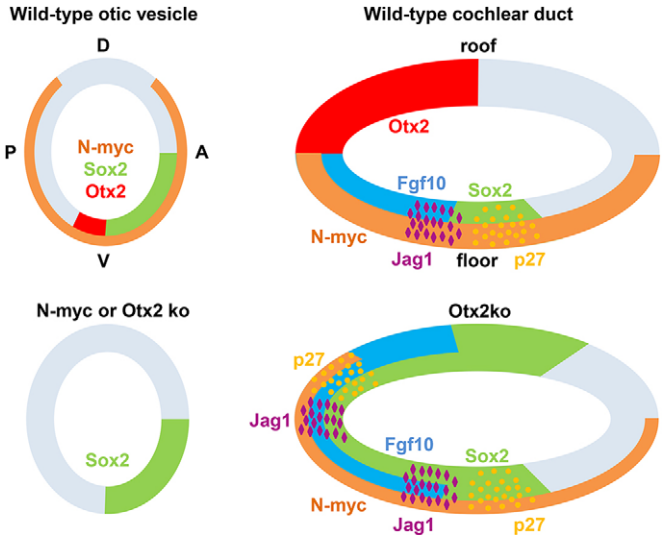

Fig. 7. Expression domains of genes involved in otic patterning during development upon loss of *Otx2* or *N-myc*. At the otic vesicle stage of wild-type embryos, *N-myc* is initially broadly expressed, whereas *Sox2* is found in the antero-ventral neurosensory region which abuts with the more posterior localized *Otx2* domain. In otic vesicles of *N-myc* mutants *Otx2* expression is absent. The loss of *Otx2* expression in the otic vesicle and/or the non-sensory regions of the roof of cochlear duct leads to the ectopic expression of Sox2, Jag1, $p27^{kip1}$, *Fgf10* and *N-myc*, and to the formation of an ectopic prosensory region and differentiation of hair cells. A, anterior; D, dorsal; P, posterior; V, ventral.

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of Corti across the neural-abneural axis of the cochlear duct. The ectopic expression of *Fgf10*, Sox2 and Jag1 during patterning of the prosensory region further suggests that this duplication also includes Kölliker's organ.

DISCUSSION

The relevance of the interaction between transcription factors has recently been underlined by the ENCODE project, which has revealed how analysis of transcriptional regulatory networks is crucial for understanding human biology and disease (Gerstein et al., 2012). Some of the regulatory interactions that occur during the formation of the inner ear are starting to be clarified (Raft and Groves, 2015; Schimmang, 2013). For instance, a transcriptional complex composed of Sox2, Six1 and Eya1 has been shown to bind directly to the *Atoh1* promoter, thereby promoting hair cell fate (Ahmed et al., 2012; Neves et al., 2012). Yet, the interactions between different transcription factors during inner ear formation remain largely unknown.

Transcriptional profiling of Myc-regulated processes has led to the discovery of a large number of genes controlled by the Myc gene family (Conacci-Sorrell et al., 2014). However, unlike c-myc (Myc – Mouse Genome Informatics), information on targets of N-myc remains scarce (Beltran, 2014). Our microarray-based screening has identified *Otx2* as one of the genes for which expression depends on *N-myc* during inner ear development. Somehow surprisingly, the downregulation of *Otx2* expression in the *N-myc* mutant cochlea was observed at E15, when the expression of both genes does not overlap. At this stage, *Otx2* expression is restricted to non-sensory regions of the inner ear, whereas *N-myc* is detected in the prosensory regions and cochlear hair cells (Dominguez-Frutos et al., 2011; Kopecky et al., 2011). However, the expression domains of both genes do coincide earlier in development, at the onset of *Otx2* expression in the most ventral part of the otic vesicle (Dominguez-Frutos et al., 2011; Kopecky et al., 2011; Morsli et al., 1999) (Fig. 1J; Fig. 7). Indeed, our results show that *N-myc* is required for *Otx2* expression at this stage (Fig. 1K). This suggests that the expression of *N-myc* at the otic vesicle stage is crucial for the initiation and maintenance of *Otx2* expression throughout later stages of inner ear development.

Experiments in the chick suggest that *N-myc* can activate *Otx2* directly. First, ectopic *Otx2* expression is observed following *N-myc* overexpression in the otic vesicle. Second, further experiments showed that, as in the mouse (Kurokawa et al., 2004), the 3′ *Otx2* enhancer is likely to be responsible for this activation. *In silico* analysis of the 3′ *Otx2* enhancer reveals the presence of several putative *N-myc* binding sites that may account for this activation.

The inner ear phenotype of *N-myc* mutants shows some interesting features in common with *Otx1* mutants (Dominguez-Frutos et al., 2011; Kopecky et al., 2011; Morsli et al., 1999). By contrast, unlike *N-myc* or *Otx1* knockout mice, the major defect of *Otx2* mutants consists of a duplication of the organ of Corti. Why then, if *N-myc* is upstream *Otx2*, does the loss of *N-myc* not result in a similar mutant phenotype? The loss of *N-myc* leads to a truncated cochlea caused by a lack of proliferation and an overall alteration of morphogenesis and patterning (Dominguez-Frutos et al., 2011; Kopecky et al., 2011). This suggests the possibility that *N-myc* knockout animals do not develop the *Otx2* mutant phenotype (duplication of the organ of Corti) because of the severity of the *N-myc* phenotype, which, besides *Otx2*, affects several other genes (supplementary material Table S1). Another surprising difference between *N-myc* and *Otx2* mutants is the fact that *N-myc* mutants maintain Reissner's membrane (Kopecky et al., 2011) whereas it is lost in *Otx2* mutants. In this case, we can only speculate that, although loss of Otx2 leads to the conversion of the non-sensory region, destined to give rise to Reissner's membrane, into an ectopic prosensory region, the dysregulation of additional genes, most likely acting upstream of *Otx2*, prevents this effect in the *N-myc* mutants and somehow rescues the formation of Reissner's membrane.

Interestingly, however, similarly to the *Otx2* mutant, the loss of *N-myc* also leads to the formation of ectopic hair cells in the apical portion of the cochlea (Dominguez-Frutos et al., 2011; Kopecky et al., 2011), which indicates that both *N-myc* and *Otx2* suppress the formation of hair cells. Further evidence for a suppressive activity of *Otx2* on sensory differentiation comes from mouse mutants for the *Gbx2* (Lin et al., 2005) and the *Kreisler* (*Mafb* – Mouse Genome Informatics) (Choo et al., 2006) genes. Loss of either of these genes leads to ectopic expression of *Otx2* and suppression of sensory development in the cochlear duct.

Otx2 expression is associated with non-sensory regions of the developing cochlea (Morsli et al., 1999) and, in the otic vesicle, it abuts the neurosensory region (Sánchez-Calderón et al., 2007). We have examined several markers for the neurosensory region, which might be altered by loss of the neighboring *Otx2* expression domain. However, at the otic vesicle stage, these markers are unchanged in the *Otx2*-deficient otic vesicles. The first signs of an alteration in gene expression are detected at E14, during the formation of the prosensory region. At this stage, an ectopic prosensory region is initiated, as indicated by the presence of $p27^{kip1}$ on the neural side of the roof of the cochlear duct, where *Otx2* is normally expressed (Morsli et al., 1999). Ectopic $p27^{kip1}$ expression is also accompanied by a broad expansion of Sox2 and by an ectopic patch of Jag1. Sox2 and Jag1-mediated Notch signaling are both required for sensory lineage formation during normal development (Kiernan et al., 2005, 2006), and their ectopic activation throughout the cochlear duct induces ectopic sensory patches (Hartman et al., 2010; Pan et al., 2013, 2010), which is similar to what we report on *Pax2*Cre-*Otx2* mutants. The most likely explanation for this effect is that Otx2 either suppresses Sox2, Notch signaling, or both, thereby preventing the development of sensory fate in the roof of the cochlear duct. However, misexpression of Otx2 during chicken inner ear development does not downregulate Sox2 or affect sensory development (G.A., H.G., A.F.-R. and F.G., unpublished), suggesting that Otx2 is necessary but not sufficient to prevent sensory fate. Therefore, rather than Otx2 and Sox2 mutually repressing each other, the initial widespread expression of Sox2 throughout the ventral part of the otic vesicle first appears to repress Otx2, but the subsequent downregulation of Sox2 then may allow Otx2 to appear in a ventro-lateral domain (supplementary material Fig. S2).

The suppressor activity of Otx2 has been previously documented in the cerebellum and the retina, and during myogenesis and neurogenesis (Bai et al., 2012; Nishida et al., 2003; Puelles et al., 2006, 2003). Sox2 and Otx2 are able to interact directly via their HMG and homeobox DNA-binding domains, respectively (Danno et al., 2008), and Otx2 has been shown to suppress the expression of Sox2 during retinal differentiation (Nishihara et al., 2012). The widespread ectopic expression of Sox2 in the cochlear duct of *Pax2*Cre-*Otx2* mutant mice may suggest that it acts as a permissive prosensory factor allowing ectopic expression of Jag1 and $p27^{kip1}$, which eventually generate an ectopic organ of Corti in the non-sensory region usually characterized by *Otx2* expression. However, ectopic activation of Notch in non-sensory regions of the cochlea has been shown to be more effective than Sox2 in promoting

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sensory fate (Pan et al., 2013). Therefore, it is possible that the combination of Sox2 and Notch activity underlies the generation of an ectopic organ of Corti in *Pax2*Cre-*Otx2* mutants.

So far only a few examples of mirror duplications of the organ of Corti have been reported. In the Jackson circler mouse mutant, loss of the transcriptional repressor Jxc1 (Sobp – Mouse Genome Informatics) leads to a partial mirror duplication of the organ of Corti and ectopic hair and pillar cells directly flanking the lateral side of the native sensory epithelium (Chen et al., 2008). More recently, a complete mirror image duplication of the organ of Corti has been shown to occur upon deletion of the transcriptional regulator *Lmo4* (Deng et al., 2014). Similar to the *Pax2*Cre-*Otx2* mutant, ectopic formation of the sensory epithelium in the *Lmo4* mutant is presaged by ectopic domains of Sox2, Jag1 and $p27^{kip1}$ in the cochlear duct. Until E14, we noted no ectopic expression of $p27^{kip1}$ in *Pax2*Cre-*Otx2* mutants. The onset of $p27^{kip1}$ expression in ectopic patches is therefore delayed with respect to the normotopic prosensory region (Lee et al., 2006). A similar delay of $p27^{kip1}$ expression was reported for the *Lmo4* mutants (Deng et al., 2014). However, eventually the differentiation of the ectopic sensory regions is accelerated because this delay disappears at later developmental stages in *Pax2*Cre-*Otx2* or *Lmo4* mutants (Deng et al., 2014). Further understanding of the basis of the unexpected plasticity of non-sensory regions of the cochlea may prove useful for defining pathways for the regeneration of hair cells by the manipulation of expression of transcriptional regulators and their associated networks.

MATERIALS AND METHODS

Transgenic mice

Generation and genotyping of mice carrying the *Pax2*Cre transgene (Ohyama et al., 2012), and the floxed *N-myc* (Dominguez-Frutos et al., 2011) and *Otx2* alleles (Fossat et al., 2006) have been described previously. Experiments conformed to the institutional and national regulatory standards concerning animal welfare.

Screening for differentially regulated genes in *N-myc* mutants

RNA was isolated from E15 cochleas of wild type and *Pax2*Cre-*N-myc* mutants using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent). Labeling and hybridizations were performed according to protocols from Affymetrix. Briefly, 100-300 ng of total RNA were amplified and labeled using the WT Expression Kit (Ambion) and then hybridized to Mouse Gene 1.0 ST Arrays (Affymetrix) covering a total of 21,041 gene transcripts. Washing and scanning were performed using the Affymetrix GeneChip System (GeneChip Hybridization Oven 640, GeneChip Fluidics Station 450 and GeneChip Scanner 7G). The Robust microarray analysis algorithm was used for background correction, intra- and inter-microarray normalization, and expression signal calculation. The absolute expression signal for each gene was calculated in each microarray and significance analysis of microarrays was applied to calculate differential expression and find the gene probe sets that characterized the highly metastatic samples. The method uses permutations to provide robust statistical inference of the most significant genes and provides $>P$ values adjusted to multiple testing using false discovery rate. Probe synthesis, hybridizations and microarray data analysis were performed by the Genomics facility of the Centro de Investigación del Cancer (Salamanca, Spain). The microarray data from this screen have been deposited at GEO with accession number GSE61406. Genes downregulated twofold or greater were examined for their expression in the cochlea (Diez-Roux et al., 2011) and are listed in supplementary material Table S1.

Histology and RNA *in situ* hybridization

Preparation of histological sections stained with Hematoxylin and Eosin, β-galactosidase staining, sectioning of stained embryos and RNA whole-mount *in situ* hybridization have been described previously (Alvarez et al., 2003). Riboprobes were generated for detection of murine *N-myc* (Dominguez-Frutos et al., 2011), *Otx2* (Fossat et al., 2006; Morsli et al., 1999), *Sox2* (Pan et al., 2010), *Fgf3* (Alvarez et al., 2003), lunatic fringe (*LFng*), neurogenin 1 (*Ngn1*) and *NeuroD* (Vázquez-Echeverría et al., 2008), *Fgf10* (Ohyama et al., 2012) and chicken *cOtx2* (Hidalgo-Sánchez et al., 2000) and *N-myc* (Khudyakov and Bronner-Fraser, 2009). The *Ecel1* riboprobe was generated from its cDNA (Genbank reference BC057569.1) corresponding to nucleotides 760-1222.

Immunohistochemistry

For immunohistochemistry, cryostat sections were prepared and processed using standard protocols. The following primary antibodies were used: Pax2 (PRB-276P, Covance; 1:200), $p27^{kip1}$ (RB-9019-P0, Thermo Scientific; 1:1000), myosin VIIA (25-6790, Proteus; 1:50), calretinin (7699/3H, Swant; 1:1000), p75 (AB1554, Millipore; 1:200), NF 200 (N4142, Sigma; 1:500), Lrp2/megalin (sc-16478, Santa Cruz Biotechnology; 1:200), Sox2 (sc-17320, Santa Cruz Biotechnology; 1:50) and Jag1 (sc-6011, Santa Cruz Biotechnology; 1:50). An antigen retrieval step consisting of incubation in 1 mM sodium citrate and 0.005% Tween 20, pH 6.0, at 98°C for 20 min was required for $p27^{kip1}$, Jag1 and calretinin antibodies. The corresponding secondary antibodies used were Alexa 488-conjugated donkey anti-goat (1:400) and Alexa 568-conjugated goat anti-rabbit (1:1000) from Invitrogen. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Whole-mount immunolabeling with myosin VIIA antibody, dehydration and clearing of inner ears was performed as published (MacDonald and Rubel, 2008). Images of embryos, inner ears, sections and whole mounts of inner ear sensory epithelia were captured with a DFC 490 camera (Leica) on a Labophot-2 (Nikon) or MZ16FA fluorescence stereomicroscope (Leica). Immunofluorescence images of cochlear sections were taken with a Leica SP2 confocal microscope and processed using Adobe Photoshop.

N-myc and *Otx2* gain of function

For *in ovo* electroporation, fertilized chicken eggs were incubated until embryos reached stage HH 12-14 (Hamburger and Hamilton, 1992). An expression vector carrying either murine *N-myc* (2 µg/µl) under the control of the CMV promoter or a vector carrying a 1.2 Kb fragment of the 3′ enhancer of the *Otx2* gene (0.5 µg/µl, *Otx2lacZ*) or both, together with pEGFP-C1 (0.2 µg/µl, Clontech), were injected into the right otic cup by gentle air pressure through a micropipette. The platinum electrode was placed next to the otic cup and the anode electrode parallel to it on the other side of the embryo. Square pulses (eight pulses of 10 V, 50 Hz, 250 ms) were generated by a CUY-21 square wave electroporator (BEX, Tokiwasaiensu, Japan). The left otic vesicle was not injected and was always used as control. Electroporated embryos were collected 24 h post electroporation and selected for high GFP expression in the otic vesicle and further processed for whole-mount RNA *in situ* hybridization or detection of β-galactosidase activity. Ectopic expression of *Otx2* was detected after a short term exposure of 30 min to nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3′-indolyphosphate (BCIP), which serve as chromogenic substrates for alkaline phosphatase coupled to an RNA probe that hybridizes to chick *Otx2*. Longer exposures of 2 h lead to detection of the endogenous ventrally localized chick *Otx2* expression domain. Presence of β-galactosidase activity was detected using standard protocols (Alvarez et al., 2003). After a short term exposure of 30 min to the chromogenic substrates, embryos electroporated with *N-myc* and *Otx2lacZ* reporter (n=6) always showed a widespread blue precipitate within the electroporated vesicle whereas embryos electroporated with only the reporter showed no or few *lacZ*-positive cells (n=4). When the latter embryos were developed for 2 h, *Otx2lacZ* reporter activity was detected throughout the electroporated otic vesicle. Misexpression of Otx2 using an expression vector carrying murine *Otx2* (2 µg/µl) under the control of the CMV promoter in the otic cup was performed as described above. Expression of *N-myc* was monitored by RNA whole-mount *in situ* hybridization using a chicken *N-myc* probe (Khudyakov and Bronner-Fraser, 2009). Images were obtained by conventional fluorescence microscopy (Leica DMRB) with Leica CCD camera DC300F and images were processed with Adobe Photoshop. The images are representative of the original data.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
V.V., I.L.-H., M.B.D.A., A.F.-R., G.A., H.G., F.G. and T.S. performed research and analyzed data, T.L. contributed essential material, T.S. wrote the paper, and G.A., T.L. and F.G. edited the paper.

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Supplementary material
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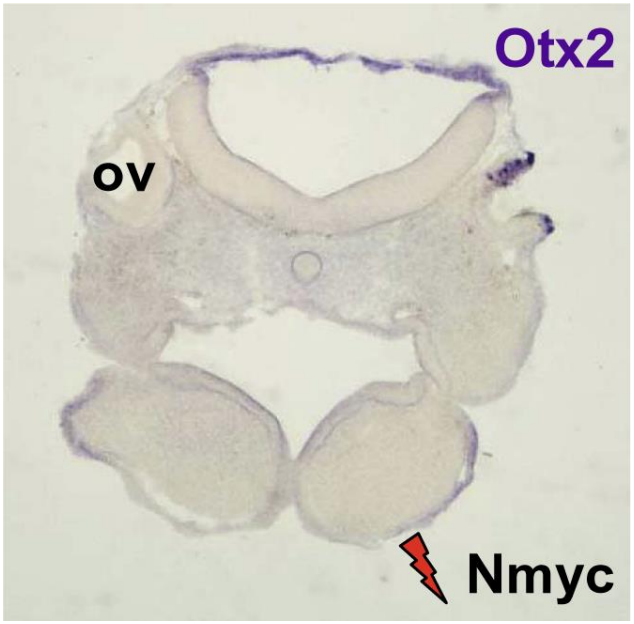
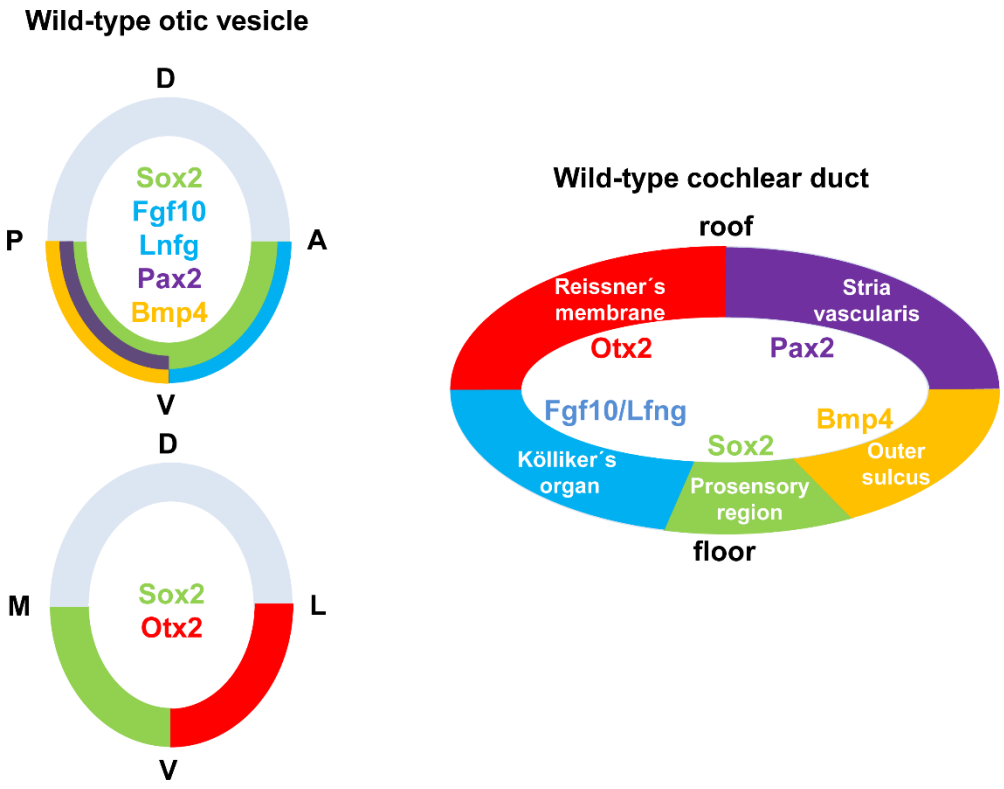


Supplementary Fig. 1. Misexpression of N-myc leads to ectopic Otx2 expression in the otic vesicle of chicken embryos.

Transversal section of a chicken embryo upon electroporation of the otic vesicle (ov) with vectors containing N-myc and GFP. Ectopic cOtx2 expression is detected in the dorsal portion of the electroporated otic vesicle (right) after a brief exposure to the chromogenic substrate, indicating the presence of the digoxigenin-labelled RNA probe.



Supplementary Fig. 2. Expression domains of genes involved in otic patterning in the otic vesicle and the cochlear duct.

Sox2 is initially broadly expressed throughout the ventral portion of the otic vesicle (top, left) but is then downregulated in the ventro-lateral domain at the level where Otx2 expression is now found (bottom, left). Results from the present work and previous publications (Burton et al., 2004; Kiernan et al., 2005; Mak et al., 2009; Morsli et al., 1998; Morsli et al., 1999; Ohyama et al., 2012; Pauley et al., 2003) indicate that the future cochlear duct may be mapped onto the ventral portion of the otic vesicle. A molecular map of otic patterning genes in the cochlear duct is shown on the right. The expression of these patterning genes in the ventro-medial (Sox2) and ventro-lateral (Otx2) (bottom, left), and antero-ventral (Lfng, Fgf10) and postero-ventral domains (Pax2, Bmp4) (top, left) of the otic vesicle are indicated. Note that in the case of Pax2 and Bmp4 only the postero-ventral domains of expression which are likely to be relevant for the formation of the cochlear duct are shown. Abbreviations: a, anterior; d, dorsal; l, lateral; m, medial; p, posterior; v, ventral.

Table S1. Summary of genes downregulated in the cochlea of *N-myc* mutants

Gene (MGI reference)	Fold Downregulation	Expression Pattern
low density lipoprotein receptor-related protein 2 (95794)	6.8 x	roof of cochlear duct
endothelin converting enzyme-like 1 (1343461)	5.3 x	roof of cochlear duct
solute carrier family 6, member 15 (2143484)	4.7 x	cochlear ganglion
calbindin 1 (88248)	4.6 x	cochlear ganglion
orthodenticle homolog 2 (MGI:97451)	4.4 x	roof of cochlear duct
otoancorin (2149209)	3.2 x	roof of cochlear duct
protein kinase C, theta (97601)	3.0 x	cochlear duct
mucolipin 3 (1890500)	2.7 x	cochlear duct
solute carrier family 27, member 2 (1347099)	2.6 x	roof of cochlear duct
EF hand domain containing 1 (1921607)	2.6 x	neural portion of cochlear duct
family with sequence similarity 20, member A (2388266)	2,5 x	cochlea
hepatocyte growth factor (96079)	2.3 x	roof of cochlear duct
ectonucleoside triphosphate diphosphohydrolase 3 (1321386)	2.3 x	roof of cochlear duct, cochlear ganglion

arginase type II (1330806)	2.2 x	cochlear duct
Ca2+-dependent activator protein for secretion 2 (2443963)	2.2 x	roof of cochlear duct
tubulin, beta 4 (107848)	2.2 x	cochlear ganglion
TOX high mobility group box family member 3 (3039593)	2.1 x	cochlear duct
major facilitator superfamily domain containing 2A (1923824)	2.1 x	neural portion of cochlear duct
adaptor protein complex AP-1, mu 2 subunit (1336974)	2.1 x	cochlear duct
upstream transcription factor 1 (99542)	2.0 x	cochlear ganglion
family with sequence similarity 107, member A (3041256)	2.0 x	neural portion of cochlear duct
myosin VB (106598)	2.0 x	cochlear ganglion
chloride channel Ka (1329026)	2.0 x	roof of cochlear duct
claudin 9 (1913100)	2.0 x	cochlear duct
glutamyl aminopeptidase (106645)	2.0 x	cochlear duct
G protein-coupled receptor 56 (1340051)	2.0 x	cochlear ganglion
RIKEN cDNA 1190002H23 gene (1913464)	2.0 x	neural portion of cochlear duct
tumor necrosis factor receptor superfamily, member (1352474)	2.0 x	cochlear duct, surrounding mesenchyme
melanoregulin (2151839)	2.0 x	cochlear ganglion

The localization of the genes within the cochlea was predicted according to Diez-Roux et al. (2011) who show expression of the listed genes at E14.5 using RNA in situ hybridization. The table shows a list of genes which are expressed in the cochlea and downregulated by twofold or higher in *N-myc* mutant cochleas according to the results obtained by the microarrays.