Tissue-specific roles of *Tbx1* in the development of the outer, middle and inner ear, defective in 22q11DS patients

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Abstract

Most 22q11.2 deletion syndrome (22q11DS) patients have middle and outer ear anomalies, whereas some have inner ear malformations. *Tbx1*, a gene hemizygously deleted in 22q11DS patients and required for ear development, is expressed in multiple tissues during embryogenesis. To determine the role of *Tbx1* in the first pharyngeal pouch (PPI) in forming outer and middle ears, we tissue-specifically inactivated the gene using Foxg1-Cre. In the conditional mutants, PPI failed to outgrow, preventing the middle ear bone condensations from forming. *Tbx1* was also inactivated in the otic vesicle (OV), resulting in the failure of inner ear sensory organ formation, and in duplication of the cochleovestibular ganglion (CVG). Consistent with the anatomical defects, the sensory genes, *Otx1* and *Bmp4* were downregulated, whereas the CVG genes, *Fgf3* and *NeuroD*, were upregulated. To delineate *Tbx1* cell-autonomous roles, a more selective ablation, exclusively in the OV, was performed using Pax2-Cre. In contrast to the Foxg1-Cre mutants, Pax2-Cre conditional mutant mice survived to adulthood and had normal outer and middle ears but had the same inner ear defects as the *Tbx1* null mice, with the same gene expression changes. These results demonstrate that *Tbx1* has non-cell autonomous roles in PPI in the formation of outer and middle ears and cell-autonomous roles in the OV. Periotic mesenchymal markers, *Prx2* and *Brn4* were normal in both conditional mutants, whereas they were diminished in *Tbx1−/−* embryos. Thus, *Tbx1* in the surrounding mesenchyme in both sets of conditional mutants cannot suppress the defects in the OV that occur in the null mutants.

INTRODUCTION

DiGeorge/Velo-cardio-facial/22q11 deletion syndrome (22q11DS) is one of the most common microdeletion disorders, characterized by severe malformations in the derivatives of the pharyngeal apparatus (1). The clinical phenotype includes ear defects, craniofacial abnormalities, thymus and parathyroid gland hypoplasia and outflow tract heart malformations (2,3). Most patients have external ear defects, chronic otitis media and hearing impairment

(3-5). The hearing loss is primarily conductive and correlates well with the presence of chronic otitis media, which is found in the majority of patients. However, 10% of the cases of hearing loss is of the sensorineural type, indicating that at least some patients have inner ear defects (6).

The vertebrate ear develops as a result of complex tissue interactions during embryogenesis (7). The outer (pinna) and middle ear develop as a result of interactions between cells in pharyngeal arches I (PA I) and II (PA II) and the migrating neural crest cells (NCCs) (7). The pinna forms as a result of fusion of PA I and II. The middle ear, consisting of the external auditory meatus and tubotympanic recess, and the middle ear ossicles (malleus, incus, stapes) derives from the first pharyngeal pouch (PPI) endoderm and NCC mesenchyme, respectively (8-11).

The inner ear, encompassing the sensory organs, including the cochlea and the vestibular system as well as the cochleovestibular ganglion (CVG), is derived from the otic vesicle (OV), which arises from the surface ectoderm (7). The inner ear sensory organs thus share a common embryonic origin with the primary sensory neurons that innervate the hair cells.

Despite the requirement for precise genetic control throughout ear development, mutations in only a handful of genes result in complete aplasia of inner, middle and outer ear structures (8,9). *Tbx1*, a member of the T-box gene family of transcription factors, is one of the few known essential genes (10,11). Mutations in T-box genes result in variety of human congenital malformations; *TBX1* haploinsufficiency has been implicated in the etiology of 22q11DS (12-15). As aforementioned, most 22q11DS patients have ear disorders including chronic otitis media with associated conductive hearing loss, while inner ear and cranial ganglion dysplasias have also been described in patients with this syndrome (6,8,9).

In mice, *Tbx1* homozygous mutations result in failure of middle and outer ear development and in early gestational arrest of OV morphogenesis, leading to hypoplasia of inner ear sensory organs (10,11). *Tbx1−/−* embryos lack the cochlea and the vestibular system, as the inner ear never develops past a rudimentary cystic structure. In contrast, the CVG primordium is expanded in *Tbx1−/−* mice, suggesting that *Tbx1* is required for sensory organ development and for suppression of neural cell fate determination in the OV (10). These studies propose that *Tbx1* acts to promote sensory organ fates through regulation of *Bmp4* and *Otx1* (10). In contrast, *Tbx1* negatively regulates *Ngn1* and *NeuroD*, two genes that specify neural fates, as well as *Fgf3* and *Lunatic fringe* (*Lfng*), markers of presumptive anterior otocyst neural competence (10).

During early ear development, *Tbx1* is expressed in the posterior half of the OV (10,11,16), in a complementary manner to the region from which neural precursors delaminate (10). *Tbx1* is also expressed in the periotic mesenchyme (PM) surrounding the OV, the pharyngeal pouch endoderm, pharyngeal arch mesenchyme, as well as additional sites of epithelial–mesenchymal interactions (17). However, it remains unclear whether the otic epithelial domain of *Tbx1* expression is sufficient to induce ear morphogenesis and whether the PM cells expressing *Tbx1* play a role in sensory organ formation and in suppression of neural cell fates.

In order to determine the precise role of *Tbx1* in middle ear and OV morphogenesis, we used the Cre/loxP strategy to conditionally inactivate the gene in the pharyngeal endoderm and OV, while leaving the mesenchymal domains of expression intact. Our results show that complete ablation of *Tbx1* in the OV and in the pharyngeal pouch endoderm causes an ear phenotype identical to that of *Tbx1* homozygous null mutants. This includes absence of outer and middle ears because of failed expansion of the PPI. It also includes failure to form inner ear sensory organs, coupled with an expansion of the CVG rudiment. Furthermore, OV-specific ablation of *Tbx1* with Pax2-Cre results in a similar inner ear phenotype, whereas outer and middle ear

formation remains unaffected. Thus, our experiments show that *Tbx1* in the OV has cell-autonomous roles in patterning the inner ear. Moreover, this study shows that *Tbx1* in the PM is not sufficient to suppress inner ear defects in the mutant embryos, suggesting that the gene has a distinct role in each tissue. Finally, these results suggest that *TBX1* may contribute to the etiology of middle and inner ear malformations observed in 22q11DS patients.

RESULTS

Conditional mutagenesis of *Tbx1*

Tbx1 is expressed in the OV and the PM, as well as in the pharyngeal pouch endoderm and the core mesoderm of the pharyngeal arches (10,11,14,17). It is also expressed transiently at E9.0 in the ectoderm of the distal pharyngeal apparatus (16,18). To determine the tissue-specific roles of *Tbx1* in ear development, we have used mice containing the previously described *Tbx1* conditional allele (*Tbx1 flox*) (19). When crossed to a tissue-specific Cre recombinase mouse strain, the floxed allele in *Tbx1 flox/flox* mice recombines to produce a *Tbx1 null* allele (Fig. 1A) (19). To assess the role of *Tbx1* in the epithelial tissues involved in ear development, we used two distinct Cre recombinase strains, Foxg1-Cre and Pax2-Cre (Fig. 1B) (20,21). In order to confirm the recombination pattern induced by the two Cre strains, we crossed Foxg1-Cre and Pax2-Cre mice with the ROSA26 reporter strain (22) and compared findings with endogenous *Tbx1* expression (Fig. 2A and B). As previously described (21), by E9.5 Foxg1-Cre-induced recombination is evident in the telencephalon, the otic and optic vesicles and in the pharyngeal pouch endoderm (Fig. 2C). The first pouch endoderm (PPI) forms the auditory tube and contributes, via tissue interactions, to the formation of the middle and outer ear. Stage-matched Pax2-Cre embryos show Cre expression in the OV (Fig. 2C). In contrast to the Foxg1-Cre mice (Fig. 2C), Cre is not expressed in PPI in Pax2-Cre mice (Fig. 2E and H).

Generation of the *Tbx1* conditional null mutant mice (*Tbx1nul/floxl;Foxg1-Cre/+* referred to as Foxg1-Cre KO) using the Foxg1-Cre strain has been previously described (Fig. 1) (19). To produce Pax2-Cre-driven conditional null mutants, termed Pax2-Cre KO, we crossed the *Tbx1 flox/flox* mice to mice heterozygous for *Tbx1* but also carrying the Pax2-Cre transgene (*Tbx1null/flox;Pax2-Cre tg*). The resulting conditional null offspring, generated using either Cre strain, are heterozygous for the *Tbx1* null allele in all tissues but homozygous for the null allele in tissues expressing Cre recombinase (Fig. 1). *Tbx1* heterozygosity is associated with chronic otitis media but not morphological defects, and does not interfere with the formation of the outer, middle and inner ear structures (23).

To verify that Foxg1-Cre and Pax2-Cre strains ablated *Tbx1* in the correct spatial and temporal manner, we analyzed *Tbx1* expression in control and conditional mutant embryos by *in situ* hybridization (ISH) and immunohistochemistry (IHC; Fig. 2). By E9.5, *Tbx1* expression in the OV is lost in the Foxg1-Cre and Pax2-Cre KO embryos, whereas the PM expression domain remains intact (Fig. 2D, F, H, J). In addition, in the Foxg1-Cre KO embryos, *Tbx1* is inactivated in PPI (19) (Fig. 2J). Control embryos of the same stage (*Tbx1 null/+; Cre*) show the previously described normal pattern of *Tbx1* expression (Fig. 2A, B, I). To further verify that the Pax2-Cre strain did not inactivate *Tbx1* in PPI, we performed IHC with a *Tbx1* polyclonal antibody (Fig. 2G and H). By E9.5, *Tbx1* expression is evident in the core mesoderm and in PPI of both control (Fig. 2G and I) and Pax2-Cre KO embryos (Fig. 2H). Furthermore, to ensure that the Pax2-Cre strain inactivated *Tbx1* in the OV prior to its onset of expression, we performed ISH on E9.0 control and Pax2-Cre embryos (Fig. 2K and L). By this stage, *Tbx1* is already expressed in the ventral part of the developing OV (Fig. 2K); however, Pax2-Cre KO mutants of the same stage show no *Tbx1* expression in the OV (Fig. 2L). Importantly, the pharyngeal pouch *Tbx1* expression is already evident in the same embryo (Fig. 2L), demonstrating that although both the Pax2-Cre and Foxg1-Cre strains inactivate *Tbx1* in the OV, only Foxg1-Cre mediates *Tbx1* ablation in the pharyngeal endoderm.

As previously reported, *Tbx1* conditional null embryos generated with the Foxg1-Cre strain do not survive embryogenesis and die in the perinatal period (E18.5–E20.5) with multiple malformations of the pharyngeal apparatus (19). To assess viability of the Pax2-Cre KO mice, we performed Mendelian ratio analysis of P10 progeny from *Tbx1 flox/flox* and *Tbx1 null/+*; *Pax2-Cre tg* crosses. Our results show that Pax2-Cre KO embryos survive in normal Mendelian ratios through adulthood (*n* > 100). Histological analysis of tissue sections revealed no anatomical defects besides the inner ear (data not shown; E17.5; *n* = 8).

Tbx1 in the pharyngeal endoderm is required to form the auditory tube, middle and outer ear

The Foxg1-Cre strain, congenic in the Swiss-Webster (SW) genetic background, inactivates *Tbx1* exclusively in PP endoderm, but not in the mesodermal tissues (Fig. 2C and D). We analyzed the morphogenesis of the outer and middle ear in Foxg1-Cre KO mutants. Foxg1-Cre KO mutants first show defects in middle ear formation at E10.5. At this stage, the pharyngeal endoderm of control embryos (*Tbx1 null/+*; *Cre*) starts to invaginate toward the surface endoderm to form the tubotympanic recess (Fig. 3A). However, the endodermal cells of the Foxg1-KO embryos fail to invaginate, resulting in a disruption of middle ear development (Fig. 3B). To assess later ear development, we analyzed E15.5–E17.5 mutant embryos. Neither the pinna nor the middle ear ossicles start the condensation process and thus fail to develop in Foxg1-Cre KOs or *Tbx1−/−* embryos, demonstrating that *Tbx1* in PPI is required for morphogenesis of the middle and outer ear structures (Fig. 3F and G).

In addition to the outer and middle ear defects, E17.5 Foxg1-KO embryos have a hypoplastic ear capsule, as well as severe malformations of craniofacial bone structures (Supplementary Material, Fig. S1C and D). As *Tbx1* is not expressed in NCCs, which give rise to craniofacial bones, these defects arise non-cell-autonomously as a consequence of *Tbx1* inactivation in the pharyngeal pouches.

In contrast to the Foxg1-Cre promoter, *Pax2* does not drive Cre expression in PPI and E10.5 Pax2-Cre KO embryos show normal development of the future tubotympanic recess (Fig. 3C). As *Tbx1* expression is normal in this tissue in Pax2-Cre KO mutants (Fig. 2L), normal middle and outer ear structures proceed to form in E17.5 Pax2-Cre KO embryos (Fig. 3H and I) and adult mice (Fig. 3J). The middle ear ossicles and pinnae are clearly evident in E17.5 Pax2-Cre KO (Fig. 3H and I), as compared with the Foxg1-Cre KO mice (Fig. 3F and G). Adult Pax2-Cre KO mice (Fig. 3J) also show normal development of the middle ear structures. Furthermore, formation of the ear capsule and the craniofacial bones is normal in E17.5 Pax2-Cre KO embryos, because of the presence of *Tbx1* expression in PPI (Supplementary Material, Fig. S1 E and F).

As compared with the Foxg1-Cre KOs, Pax2-Cre KO mice survive normally through adulthood (*n* > 100). To assess adult Pax2-Cre KO mutants for hearing defects, we performed auditory brainstem response (ABR) testing. As predicted, based on the loss of sensory structures, 3/5 mutants analyzed (*n* = 5) have no hearing on either the left or right side as compared with control littermates (Supplementary Material, Fig. S2). Normal hearing in two Pax2-Cre KO mutants is consistent with the incomplete penetrance of the inner ear phenotype observed at E17.5. We found that 12/16 ears show complete failure of inner ear development, whereas the rest appear completely normal. The sensorineural hearing loss in 10–15% of 22q11DS patients (6,19), may result from abnormal *TBX1* expression in the OV, as well.

Tbx1 expression in the OV is required for sensory organ formation

To assess embryonic development of the inner ear in Foxg1-Cre KO embryos, we analyzed E17.5 histological sections of mutant and control (*Tbx1 null/+*; *Cre*) embryos. By this stage, the control embryos have clear precursors of all major structures (Fig. 3D and E). The cochlea,

the utricle, saccule and the semicircular canals are all clearly evident (Fig. 3D and E). In contrast, the Foxg1-Cre KO embryos show severe hypoplasia of the inner ear, developing only a cystic OV and endolymphatic duct, with complete aplasia of the sensory organs (Fig. 3F and G, data not shown). The defects in the conditional mutants are identical to those in the *Tbx1−/− * embryos. However, it remains unclear whether inactivation of *Tbx1* in PPI plays a significant role in disrupting inner ear development.

To further determine the precise role of *Tbx1* solely in the OV, we used the Pax2-Cre strain to inactivate the gene in this tissue. In order to assess embryonic development of the ear, we examined Foxg1-Cre and Pax2-Cre KO embryos as well as controls (Tbx1+/−; Cre) at E10.5–E17.5. The OV was present in both sets of mutant embryos, however, in both, it is smaller and surrounded by an expanded CVG, similar to the phenotype of *Tbx1−/−* embryos (Fig. 3L-N and Fig. 4). A total of 6/8 Pax2-Cre mutant embryos analyzed had a severely hypoplastic inner ear, identical to the Foxg1-Cre KOs (Fig. 3H and I). The severe hypoplasia was bilateral and present in 12/16 ears. In these mutants, the cochlea and the vestibular system never develop and the inner ear persists in a rudimentary OV stage. These results demonstrate that inner ear sensory organ formation requires *Tbx1* expression within cells of the OV and that inner ear dysplasia is independent of *Tbx1* activity in PPI.

Reciprocal functions of *Tbx1* in the OV

In addition to hypoplasia of the inner ear sensory structures, homozygous null *Tbx1* mutants (*Tbx1−/−*) have an expanded CVG rudiment (10). To determine if inactivation of *Tbx1* exclusively in the OV is sufficient to reproduce CVG defects, we examined the expression pattern of *NeuroD*, a marker of committed neural precursors, and found that it was expanded in both the Foxg1-Cre (Fig. 4C) and Pax2-Cre (Fig. 4E) KO mutant embryos at E10.5. To assess later stages of CVG development, we used the anti-neurofilament monoclonal 2H3 antibody (m2H3) (Fig. 4B, D, F). As in the *Tbx1−/−* mutants, the CVG at E11.5 is duplicated around the OV anterior–posterior midline in both Foxg1-Cre and Pax2-Cre conditional mutants (Fig. 4E and F). These results suggest that *Tbx1* expressed by cells of the OV represses neurogenesis either locally through cell–cell interactions or cell-autonomously, and that this activity is separable from *Tbx1*'s role in middle and outer ear morphogenesis. These findings are also consistent with the role of *Tbx1* in the etiology of 22q11DS, as VIIIth cranial ganglion dysplasia has been inferred in human patients with the syndrome on the basis of hearing loss (24-26).

Tbx1 has been implicated as the determinant of anterior–posterior patterning in the OV (10). In that study, *Tbx1* loss of function mutation was shown to cause anterior (neurogenic) transformation of posterior OV regions that normally contributes to sensory organ development. In *Tbx1−/−* embryos *Fgf3*, which normally co-localizes with neural *bHLH* gene expression in the anterior OV (Fig. 5A), is ectopically expressed in the posterior OV. In contrast, expression of *Otx1*, a gene essential for sensory organ development and expressed in the posterior OV (Fig. 5D) is absent in *Tbx1−/−* embryos (10). However, it remains unclear if the PM domain of expression of *Tbx1* plays a role in establishing the anterior–posterior boundary in the OV.

To determine if the PM plays a role in anterior–posterior patterning of the OV, we examined expression of several molecular markers in Foxg1-Cre or Pax2-Cre KO embryos (Fig. 5). As in the *Tbx1−/−* embryos, at E10.5 *Fgf3* is expressed ectopically in the posterior OV in both mutants (Fig. 5B and C), as compared with controls (Fig. 5A, *Tbx1 null*/+ Cre). Conversely, expression of posterior OV marker *Otx1* is lost in E10.5 mutant embryos (Fig. 5E and F). These results suggest that *Tbx1* in the OV regulates sensory organ formation by the activation of posterior OV genes and by suppression of anterior, neurogenic gene expression. Moreover, our results suggest that *Tbx1* in the PM is not sufficient to compensate for the loss of the gene

in the OV, suggesting that it plays a permissive rather than an inductive role in early patterning of the OV.

To further analyze the role of OV-specific *Tbx1* activity on sensory organ patterning, we analyzed *Bmp4* expression in both Foxg1-Cre KO and Pax2-Cre KO embryos. *Bmp4* has been localized to a site of juxtaposition of *Tbx1* and neural *bHLH* (*Ngn1/NeuroD*) gene expression. Furthermore, the precise pattern of *Bmp4* expression was lost in OVs of *Tbx1−/−* embryos (10). As expected, in control embryos (*Tbx1 null/+ Cre*), *Bmp4* is present in an anterior stripe (E10.5), marking the presumptive anterior and lateral cristae (Fig. 5G) (10). However, in Foxg1-Cre and Pax2-Cre KO embryos, the *Bmp4* signal is downregulated, indicating that OV-specific *Tbx1* activity is responsible for regulating *Bmp4* patterning.

Tbx1 has independent functions in the PM

Inactivation of *Tbx1* in the OV leads to severe malformations of sensory organs derived from the OV. However, *Tbx1* mutation in the OV could also disrupt essential epithelial–mesenchymal interactions, interrupting cross-talk with the PM. Throughout mid-gestation (E10.5–E12.5), *Tbx1* continues to be expressed in the PM, surrounding the OV. *Tbx1−/−* embryos have defects in the otic capsule, directly derived from these cells. Early indication of developmental defects in the PM of the *Tbx1−/−* mutants has been detected by the disappearance of *Prx2* expression, an early homeodomain containing gene required for the condensation of mesenchyme to form cartilage (11,27). The loss of *Prx2* expression has been hypothesized to be due to the reduction in the number of cells in the lateral PM (11). To determine if the loss of *Tbx1* in the OV affects the expression of PM specification, we assessed expression of *Prx2* in Foxg1-Cre KO and Pax2-Cre KO mutant embryos (Fig. 6A-C). In contrast to the reported loss of *Prx2* expression in the *Tbx1* null mutants (*Tbx1−/−*), when compared with controls (Fig. 6A, *Tbx1 null /+ Cre*), expression of the gene was intact in both Foxg1-Cre KO (Fig. 6B) and Pax2-Cre KO (Fig. 6C) embryos at E10.5.

These findings suggested that PM development was unaffected in Foxg1-Cre and Pax2-Cre KO mutants. To further confirm normal specification of mesenchymal cells surrounding the OV, we assessed expression of marker genes in both Foxg1-Cre KO and Pax2-Cre KO mutants. *Brn4* expression is normally restricted to the condensing mesenchyme surrounding the ear capsule and mutations in the gene lead to mesenchymal defects in auditory bones (10,28). In addition, *Brn4* homozygous null mutant mice have defects in OV-derived structures, including a reduction in the number of cochlear turns, suggesting that *Brn4* plays a role in epithelial–mesenchymal interactions necessary for inner ear development (28,29).

By E11.5, *Brn4* is strongly expressed in the mesenchymal tissue surrounding the OV of control embryos (Fig. 6D and E, *Tbx1 null/+ Cre*). As expected, *Brn4* expression is severely downregulated in the PM of *Tbx1−/−* embryos, indicating mesenchymal specification defects or loss of cells (Fig. 6J and K). Consistent with the *Prx2* findings, expression of Brn4 was not downregulated in the PM of either Foxg1-Cre KO (Fig. 6F and G) or Pax2-Cre KO embryos (Fig. 6H and I) at E11.5. These results suggest that *Brn4* expression is not regulated by signaling from the OV and may be induced cell autonomously by *Tbx1* in the PM. Furthermore, the data suggests that PM specification is uninterrupted in both Foxg1-Cre KO and Pax2-Cre KO mutant embryos.

DISCUSSION

The majority of 22q11DS patients have ear disorders consisting of outer ear anomalies and chronic otitis media with associated conductive hearing loss, whereas 10% have sensor-ineural hearing loss (6). In addition, several reports have described patients with balance problems, although it is not known whether these are due to vestibular defects or to hypotonia (6). The

transcription factor, *TBX1* is hemizygously deleted in 22q11DS patients and mutated in a subset of patients without the deletion, implicating the gene in the etiology of the disorder (15,30). Furthermore, its pattern of expression during embryogenesis is consistent with the defects in the 22q11DS patients and in *Tbx1 null* (*Tbx1−/−*) mutant embryos (11,13,14,17,31). *Tbx1* is expressed in multiple tissues forming the outer, middle and inner ear. The molecular pathogenesis of *Tbx1* in the ear has now been delineated by tissue-specific inactivation using Foxg1-Cre and Pax2-Cre. Analysis with molecular markers makes it possible to form a model of *Tbx1* function in ear development.

Genetic pathway of *Tbx1* in ear development

We showed that ablation of *Tbx1* in the foregut endoderm disrupts the formation of the PPI (double-sided arrow; Fig. 7). When the endodermal pouch does not extend towards the overlying ectoderm, the cleft and pouch cannot become juxtaposed. This juxtaposition is necessary for the morphogenesis of the outer ear and for neural crest-derived mesenchymal condensations forming the middle ear ossicles (9). Epithelial–mesenchymal interactions between the mesenchyme of the arches and the epithelia of the endodermal pouches and ectodermal clefts are essential for formation of the outer and middle ear structures (7,32). Our data are consistent with non-cell autonomous roles of *Tbx1* in mediating epithelial–mesenchymal interactions.

Inactivation of *Tbx1* in the OV leads to severe hypoplasia of the sensory organs and expanded neurogenesis, similar to the *Tbx1−/−* phenotype (10). *Tbx1* may act cell-autonomously to induce *Otx1* and *Bmp4*, required to form sensory organs, and to suppress *Ngn1* and *NeuroD*, required to form the ganglion, in the same cells (Fig. 7). In parallel, *Tbx1* may serve important but distinct cell-autonomous functions in the PM via positive regulation of genes such as *Prx2* and *Brn4* or via mesenchymal cell proliferation. Overall, the data presented in this study defines distinct, tissue-specific roles of *Tbx1* in the endoderm, PM and the OV, leading to a better understanding of the gene's function in ear development.

Tbx1 in the OV is required for sensory organ formation

The mechanism behind the failure in sensory organ development in *Tbx1* mutants remains elusive. A previous study addressed this question using chimeric embryos to analyze developmental potential of *Tbx1−/−* cells (11). The analysis showed that homozygous *Tbx1* mutant cells rapidly disappear from the OV in chimeras, suggesting that they have a proliferation/survival disadvantage that prevents them from populating the OV. Together with the inner ear defects observed in *Tbx1−/−* embryos, these experiments suggested that *Tbx1* is required for an expansion/differentiation of a subpopulation of cells necessary for cochlear and vestibular development in the OV. Alternatively, or in addition, it was proposed that patterning defect in *Tbx1−/−* embryos might occur as a result of interrupted signaling from the abnormal PM, because of the inactivation of *Tbx1* in this tissue, as suggested by down-regulation of a transcription factor, *Prx2* in *Tbx1−/−* PM (11). Our results show that OV-specific inactivation of *Tbx1* phenocopies inner ear defects of the null mutant (*Tbx1−/−*), suggesting that *Tbx1* in the PM does not have a major role in OV patterning. Instead, the results of this study further argue for a cell-autonomous role of *Tbx1* in the OV. This does not rule out the possibility that *Tbx1* in the mesenchyme may be required for later aspects of cochlea and vestibular system morphogenesis.

Severe inner ear phenotypes, involving developmental arrest of both the OV and the CVG, were also found in mice mutant for *Eya1* or *Six1*, two genetically linked genes that lie upstream of *Tbx1* (8,9,33,34). In contrast to the *Tbx1* mutants, increased apoptosis and abnormal cell proliferation are responsible for the OV-derived defects in *Eya1−/−* and *Six1−/−* mice (8,9, 33,35). Furthermore, the ear defects in these mutants correlate with the expression domains of

the genes, whereas global inner ear sensory malformations in *Tbx1* mutants are not directly obvious from the *Tbx1* expression pattern (10,11). Although expression of *Tbx1* in the OV correlates with the prospective vestibular area, it is transcribed only in a small area overlapping with the *Pax2* marked prospective cochlear domain (10,11,36). *Tbx1* inactivation, either globally, or tissue specifically in the OV results in failure of cochlear development, suggesting that *Tbx1* in the ventral OV plays a role in inductive cellular signaling events with the cells of the dorsal OV and that these events are required for morphogenesis of the cochlea.

One possible explanation for this discrepancy comes from observations of altered spatial expressions of several marker genes (10,36). In *Tbx1−/−* and in conditional mutant embryos, *NeuroD* and *Lfng* expression domains, which specify neurogenic cell fates, are extended posteriorly into the region that normally contributes to sensory organ development (10,36). Furthermore, other cochlear markers, such as *Bmp4* and *Otx1*, normally found in the posterior OV are downregulated in *Tbx1−/−* and conditional null embryos (Foxg1-Cre and Pax2-Cre KO) (10). It has therefore been suggested that *Tbx1* specifies a midline boundary between the anterior (neurogenic and sensory) and posterior (sensory) regions of the OV (10). In addition, other reports have suggested that *Tbx1* in the OV might be required to specify global gene expression patterns, required for formation of the cochlea and the vestibular system (36).

Role of *Tbx1* in the PM

Tbx1 is also expressed in the PM surrounding the OV; the role of the gene in this tissue remains unknown (11). Previous studies have shown that *Tbx1* loss of function leads to PM defects, as demonstrated by downregulation of several markers, including *Prx2* (11). It has therefore been suggested that abnormal cochlear marker specification and the ensuing defects observed in *Tbx1−/−* embryos arise in a non-cell-autonomous manner, as a result of *Tbx1* absence in the PM. Alternatively, it is possible that inactivation of *Tbx1* in the OV disrupts tissue interactions between the OV and the PM, leading to abnormal development of the inner ear. Thus it was proposed that *Tbx1* in the mesenchyme plays an inductive role in patterning the OV (11).

Our study instead argues that epithelial *Tbx1* activity is required for OV patterning, which is disrupted when *Tbx1* is inactivated. First, *Tbx1* expressing cells in the PM are present in Foxg1-Cre KO and Pax2-Cre KO mutants. Second, although *Brn4*, a marker of these cells, is downregulated in *Tbx1−/−* embryos, its expression domain is unaltered in the PM of the conditional null mutants. This suggests that PM development in the conditional mutants is normal; the presence of *Tbx1* in this tissue cannot rescue the defects caused by inactivation in the OV, suggesting that the gene plays a non-inductive role in the mesenchyme.

Brn4 and *Prx2* are robustly expressed at E10.5 in the condensing mesenchyme surrounding the ear in wild-type (WT) embryos (28,37). Normal *Brn4* and *Prx2* expression in conditional mutant embryos also diminishes the possibility that *Tbx1* in the OV mediates signaling events necessary for proper specification of the PM. Instead, the results of this study suggest a distinct local role for *Tbx1* in the PM. It is possible that *Tbx1* in the PM, although not required for early OV patterning, may be required for later development of the sensory organs or otic capsule. Conditional ablation in this tissue will be required to address the later developmental roles of *Tbx1* in the PM. It has been shown that *Sonic hedgehog (Shh)* acts upstream of *Tbx1* in the mesenchyme surrounding the OV (38). This process is independent from OV signaling pathways and may be regulated via *Shh* signaling from the notochord and the floor plate (Fig. 7).

Summary

Conditional inactivation of *Tbx1* in the OV points to an essential and cell-autonomous role for the gene in this tissue. Ablation of *Tbx1* in the OV using both the Foxg1-Cre and Pax2-Cre

strains leads to severe disruption in inner ear development. In addition to the OV-specific requirement of *Tbx1* for inner ear morphogenesis, tissue-specific ablation of the gene in the OV and in the pharyngeal endoderm demonstrates the non-cell-autonomous role for endodermal *Tbx1* in outer and middle ear development. Analysis of Foxg1-Cre and Pax2-Cre conditional mutants thus provides crucial evidence for tissue-specific roles of *Tbx1* in the formation of outer, middle and inner ear structures. Finally, this study contributes to the general understanding of embryonic and molecular processing governing the development of the auditory and vestibular systems, disrupted in 22q11DS and possibly in other human congenital malformation disorders.

MATERIALS AND METHODS

Experimental animals

The generation and maintenance of the *Tbx1 null/ +* and *Tbx1 flox/flox* mice has been previously described (19). The Foxg1-Cre strain was maintained in the congenic SW background. This strain mediates inactivation of the floxed allele in the OV and in the pharyngeal endoderm but not in the PM (19). The Pax2-Cre strain, which induces recombination in the OV, was maintained in the mixed N1 C57B/6 background (20). *Foxg1-Cre/ +* and *Pax2-Cre tg* mice were crossed to *Tbx1* null/ + mice kept in the N5 SW and N4 C57Bl/6 backgrounds, respectively. To generate conditional null embryos, *Tbx1 null*/+; *Foxg1-Cre*/+ and *Tbx1 null/ + ; Pax2-Cre tg* mice were subsequently crossed with the *Tbx1 flox/flox* mice. The *flox/flox* mice were kept in the N2 C57Bl/6 background to minimize ectopic recombination. Both the Foxg1-Cre and Pax2-Cre strains were genotyped as previously described (20,21). WT morphology and marker expression was analyzed in both SW and *C57Bl/6* background. All animals were held on a 24-h dark/light cycle; the day of the plug was recorded as E0.5. Embryos were staged according to the number of somites (39).

Histology

Mouse embryos were dissected in phosphate-buffered saline (PBS), and fixed in 10% neutral buffered formalin solution (Sigma) overnight. Following fixation, the embryos were dehydrated through graded ethanol, embedded in paraffin and sectioned (5–7 μM). All sections were stained with hematoxylin and eosin. Skeletal staining of E17.5 embryos was performed using Alcian blue and Alizarin red as previously described (12). For histopathological analysis of adult mice, animals were sacrificed and their ears perfused with 10% formalin and 1% acetic acid, decalcified, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

ABR testing

Hearing status of adult mice 2–3 months of age was assessed by obtaining ABR thresholds. ABR signals were recorded from needle electrodes inserted through the skin (vertex to ipsilateral tragus). Computer-assisted evoked potential systems were used to obtain responses to tone pips at 5, 8, 11, 16, 22, 32 and 45 kHz (tone pip duration 5 ms; repetition rate 30/s) and averaged responses to 512 pips of alternating polarity.

Whole-mount in situ hybridization

Digoxigenin-labeled RNA probes for *Tbx1* (40), *Fgf3* (cloned PCR fragment), *NeuroD*, *Bmp4* and *Otx1* were prepared by standard methods (10). Whole-mount and section ISH were performed as previously described (10,41). Some *Tbx1*-hybridized embryos were embedded in paraffin and microtome-sectioned at a thickness of 10–12 μM after the reporter reaction.

Immunohistochemistry

Tissue was prepared by 10% formaldehyde fixation of whole embryos, which were paraffin wax embedded and sectioned at a thickness of 10 μM. Affinity-purified rabbit anti-*Tbx1* (Zymed) was diluted 1:100 in TBS/0.1% Triton X-100/5% goat serum/2%BSA, incubated for 1 h at room temperature and detected with a biotinylated horse anti-mouse IgG conjugate (1:200; Vectalab), avidin–biotin complex formation (Vectalab) and DAB reaction (Research Genetics). Monoclonal 2H3 antibody was used as previously described (10). The polyclonal anti-Brn4 antibody was generously provided by Dr E. Bryan Crenshaw and used in a 1:400 dilution as described earlier (10).

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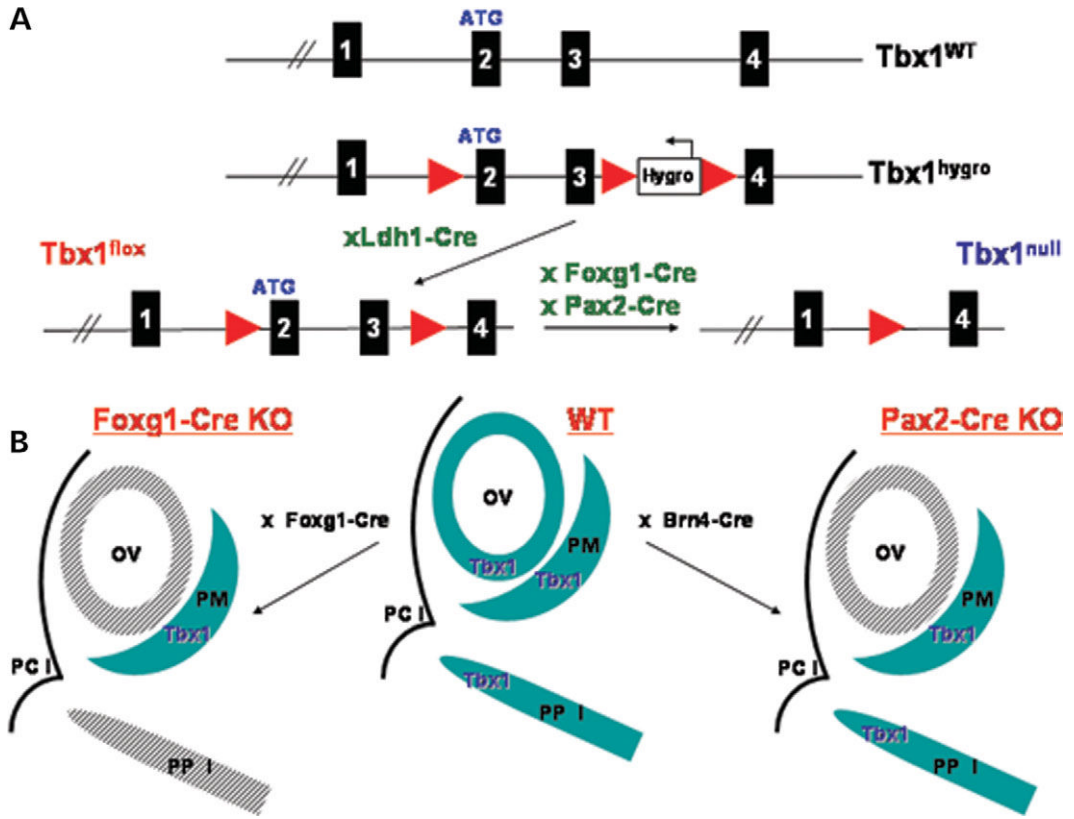41. Epstein JA. Pax3 and vertebrate development. Meth. Mol. Biol 2000;137:459–470.

Figure 1.
(**A**) Generation of the *Tbx1* conditional mutant mice. Genomic structure of the *Tbx1* WT locus from exon 1 to 4 is shown on top. Standard gene targeting techniques were used to generate the *Tbx1 hygro* allele by inserting a single loxP site between exons 1 and 2 and a Hygromycin resistance cassette between exons 3 and 4. The *Tbx1hygro/ +* mice were mated with Ldh1-Cre mice to delete the Hygromycin resistance cassette and generate the *Tbx1 flox/+* mice. *Tbx1 floxed* mice were crossed to Foxg1-Cre and Pax2-Cre mice to produce the *Tbx1* conditional mutant mice, containing the *Tbx1 null* allele shown on the right. (**B**) Strategy to generate tissue-specific *Tbx1* mutants. In WT embryos *Tbx1* is expressed in both the OV and the surrounding PM, as well as in the PPI. *Tbx1* expression is shown in blue. The Foxg1-Cre (left) and Pax2-Cre (right) strains mediate *Tbx1* inactivation in the OV (shaded grey) while the PM expression domain remains intact. The Foxg1-Cre strain also mediates inactivation of *Tbx1* in the PPI (shown in shaded grey). PCI, first pharyngeal cleft; Foxg1-Cre KO, Tbx1 null/flox;Foxg1-Cre/ +, Pax2-Cre KO, Tbx1 null/flox; Pax2-Cre tg.
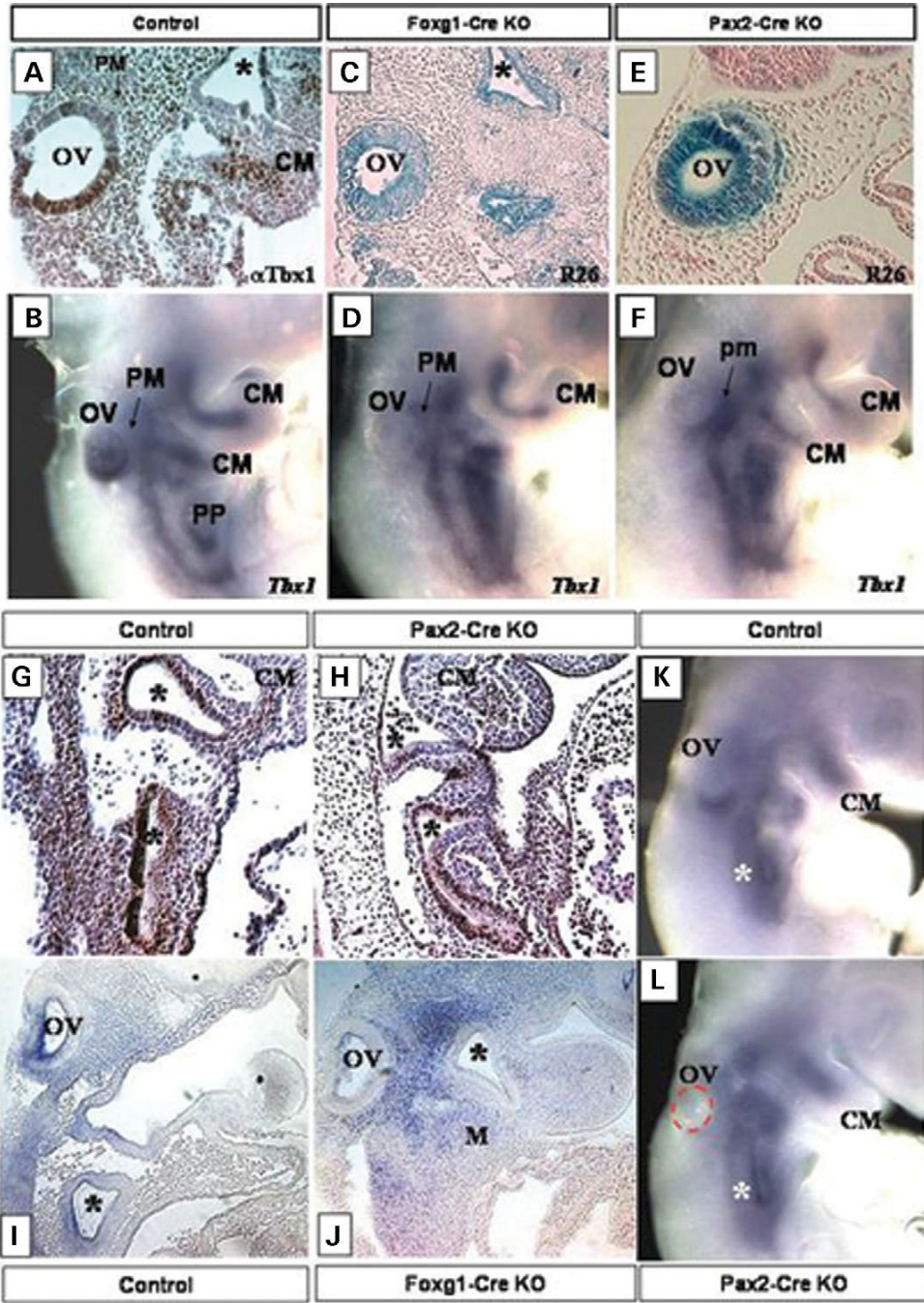
Figure 2.
Tbx1 expression in E9.5 control embryos shown by IHC on sagittal sections with a Tbx1 polyclonal antibody (**A**) and by whole mount ISH with *Tbx1* (**B**). Note Tbx1 expression in the OV, the pharyngeal pouches (PP, asterisks) and in the periotic mesenchyme (PM) and the core mesoderm of the pharyngeal arches (CM) in (A and B). Foxg1-Cre (**C**) and Pax2-Cre (**E**) activity shown by x-gal staining (lacZ) on E9.5 sagittal sections of Foxg1-Cre/ROSA26 (R26) and Pax2-Cre/ROSA26 (R26) progeny. Both Cre strains are active in the OV, while the Foxg1-Cre strain is also active in the pharyngeal pouches (stars in C). (**D** and **F**) *Tbx1* expression by ISH in E9.5 *Tbx1* conditional null mutant embryos, generated with the Foxg1-Cre strain (Foxg1-Cre KO) (D) and with the Pax2-Cre strain (Pax2-Cre KO) (F). *Tbx1* is inactivated in

the OV of both sets of mutants (OV in D and F), while the core mesoderm (CM) expression domain is still present. *Tbx1* expression by IHC with a polyclonal Tbx1 antibody on E9.5 control (**G**) and Pax2-Cre KO (**H**). Note *Tbx1* expression in the pharyngeal pouches (asterisks), and in the core mesoderm (CM). G and H are sagittal sections. ISH with *Tbx1* on E9.5 sagittal sections of control (**I**) and Foxg1-Cre KO embryos (**J**). Note that *Tbx1* expression is gone from the OV, and pharyngeal pouches (asterisks) of the Foxg1-Cre KO embryo (J). ISH with *Tbx1* on E9.0 control (**K**) and Pax2-Cre KO mutant (**L**). *Tbx1* expression is evident in the ventral OV in the control (K), but is absent in the Pax2-Cre KO mutant (L). The OV in the Pax2-Cre KO mutant (L) has been outlined with a red dashed line. *Tbx1* is still present in the pharyngeal pouches (asterisks) of the mutant (L). Foxg1-Cre KO, Tbx1 null/flox; Foxg1-Cre/+, Pax2-Cre KO, Tbx1 null/flox; Pax2-Cre tg.
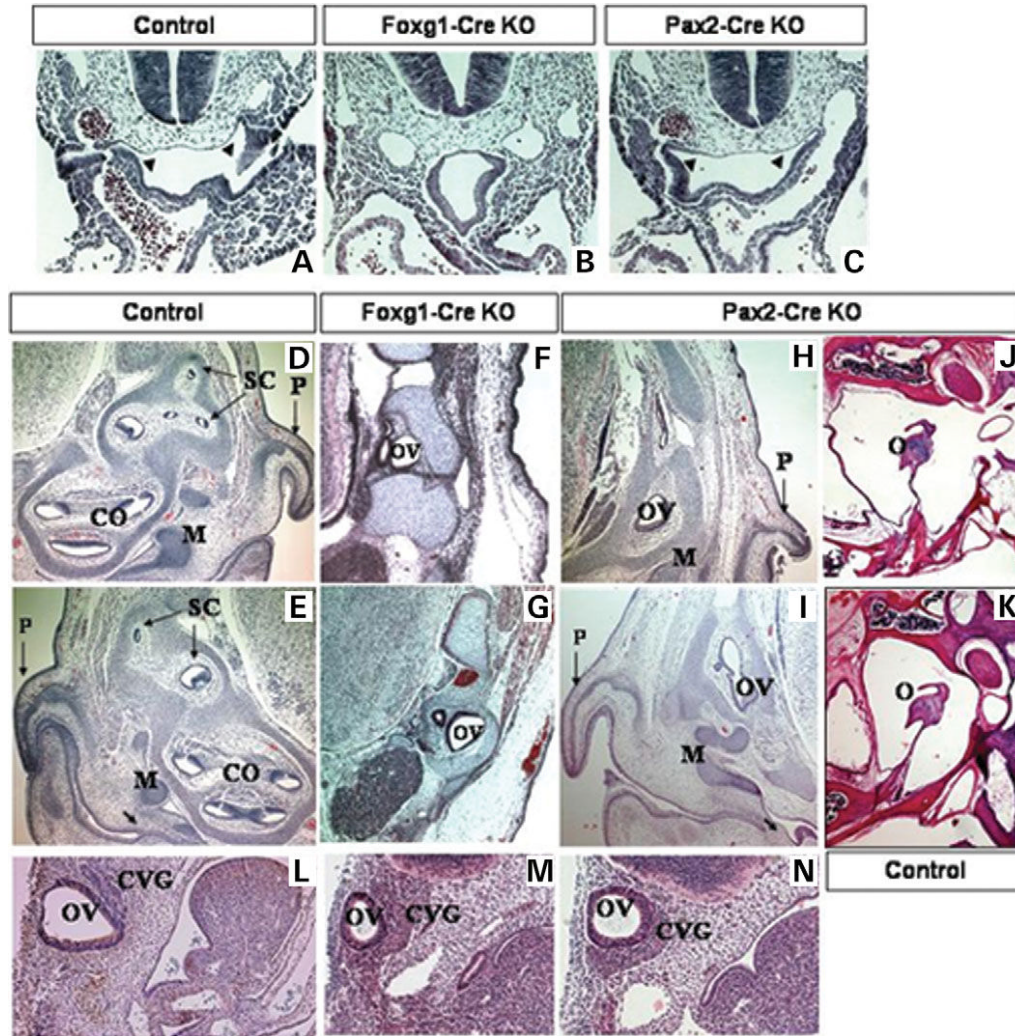
Figure 3.
E10.5 transverse histological sections of control (**A**), Foxg1-KO (**B**) and Pax2-Cre KO (**C**) embryos. The invaginating pharyngeal endoderm in control (A) and Pax2-Cre KO (C) embryos is denoted with arrowheads. E17.5 transverse histological sections of control (**D** and **E**), Foxg1-Cre KO conditional mutants (**F** and **G**) and Pax2-Cre KO (**H** and **I**) embryos. Note the presence of the nascent tympanum in controls (E) and Pax2-Cre KOs (I), denoted by an arrow, and the middle ear bones (M), as opposed to Foxg1-Cre KOs (F,G). Horizontal histological sections of adult conditional mutant Pax2-Cre KO (**J**) and control (**K**) mice show the presence of normal middle ear structures in mutants (J). Sagittal histological sections of E10.5 control (**L**), Foxg1-Cre (**M**) and Pax2-Cre (**N**) mutant embryos. Early OV development is normal in both sets of mutant embryos, but the structure is slightly hypoplastic by E10.5 (M and N). The CVG is also enlarged in both Foxg1-Cre (M) and Pax2-Cre (N) mutants, when compared with a control embryo (L). CO, cochlea, SC, semicircular canals, P, pinna, O, ossicles (middle ear), Control, Tbx1+/−, Foxg1-Cre KO, Tbx1 null/flox; Foxg1-Cre/+, Pax2-Cre KO, Tbx1 null/flox; Pax2-Cre tg.
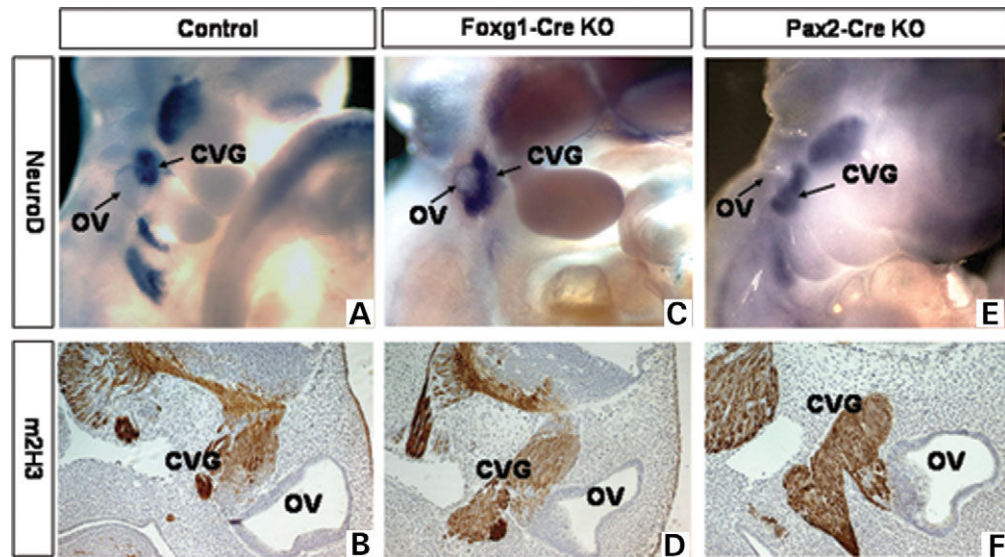
Figure 4.
Development of the CVG in E10.5–E11.5 control (**A** and **B**), Foxg1-Cre KO (**C** and **D**) and Pax2-Cre KO (**E** and F) embryos. Whole-mount ISH with a *NeuroD* probe on E10.5 embryos (A, C and E). IHC with a monoclonal m2H3 anti-neurofilament antibody on E11.5 sagittal sections (B, D and F). Control, Tbx1+/−, Foxg1-Cre KO, Tbx1 null/flox; Foxg1-Cre, Pax2-Cre KO, Tbx1 null/flox; Pax2-Cre tg.
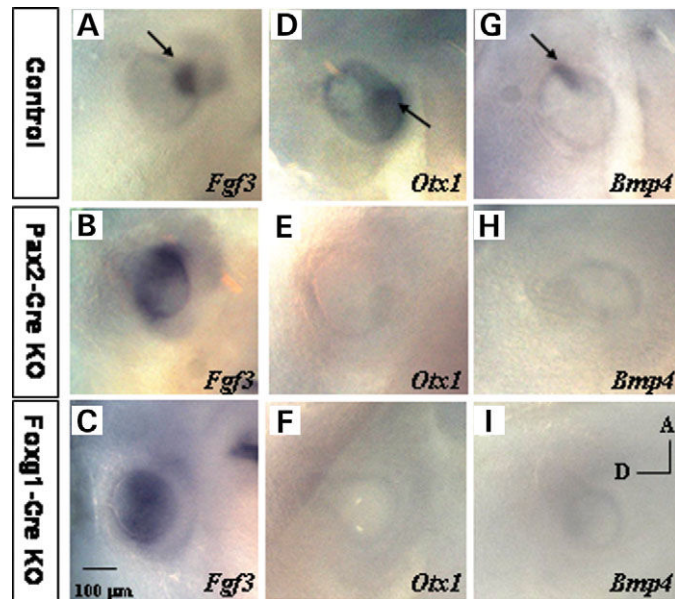
Figure 5.
Molecular marker analysis by ISH. *Fgf3* expression in control (**A**), Foxg1-Cre (**B**) and Pax2-Cre (**C**) mutant embryos at E10.5. Expression of *Otx1* in E10.5 control (**D**), Foxg1-Cre (**E**) and Pax2-Cre (**F**) mutant embryos. *Bmp4* expression in control (**G**), Foxg1-Cre (**H**) and Pax2-Cre mutant embryos (**I**). Control, Tbx1+/−, Foxg1-Cre KO, Tbx1 null/flox; Foxg1-Cre/+, Pax2-Cre KO, Tbx1 null/flox; Pax2-Cre tg.
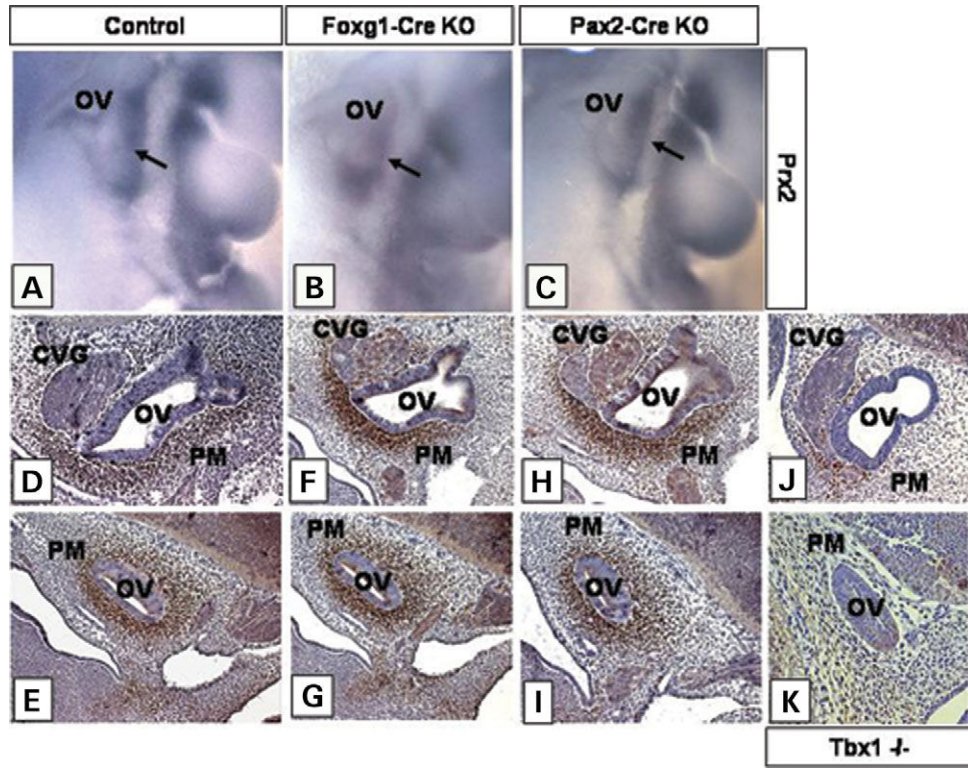
Figure 6.
PM specification shown by expression of marker genes. *Prx2* expression is shown by ISH on
E10.5 control (**A**), Foxg1-Cre KO (**B**) and Pax2-Cre KO (**C**) embryos. The arrow points to the
PM expression domain. Brn4 expression by IHC with a polyclonal Brn4 antibody on control
(**D** and **E**), Tbx1 null/flox; Foxg1-Cre/+ (**F** and **G**), Tbx1 null/flox; Pax2-Cre tg (**H** and **I**) and
Tbx1−/− (**J** and **K**) sagittal sections of E11.5 embryos. Control, Tbx1+/−, Foxg1-Cre KO, Tbx1
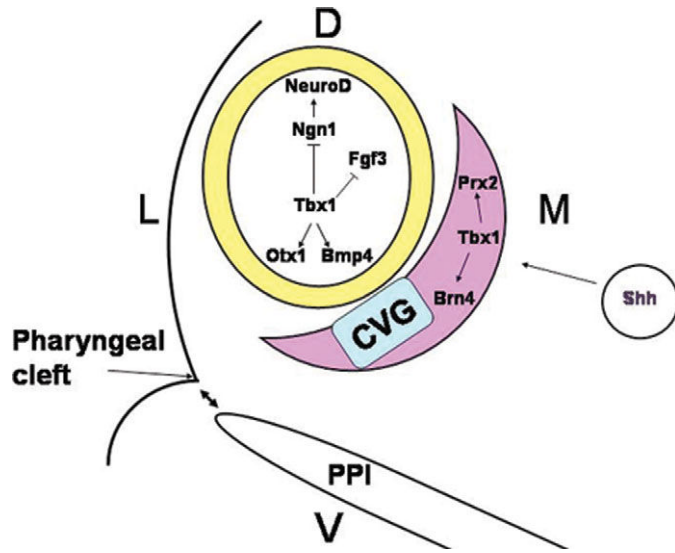null/flox; Foxg1-Cre/+, Pax2-Cre KO, Tbx1 null/flox; Pax2-Cre tg.

Figure 7.
Model of *Tbx1* function in ear development. The inner ear develops from the ectodermally derived OV, shown in yellow. Adjacent to the OV is the PM (pink). *Tbx1* in the OV plays a dual role in inner ear development by inducing sensory organ formation in the posterior OV through induction of *Otx1* and *Bmp4* and by suppressing neurogenesis in the anterior OV through negative regulation of *Ngn1* and *NeuroD*. The suppression of neurogenesis leads to the presence of an expanded CVG rudiment, shown in blue. In parallel, *Tbx1* in the PM plays a role in mesenchymal tissue specification through induction of *Brn4* and *Prx2*. This process might be mediated by *Shh* signaling from the notochord (circle) and the floor plate. *Tbx1* in the PPI mediates tissue interactions between the endoderm of the pouches and the ectoderm of the first pharyngeal cleft to promote middle and outer ear development (double-headed arrows).