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Uncovering the Secreted Signals and Transcription Factors Regulating the Development of Mammalian Middle Ear Ossicles

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Abstract

The mammalian middle ear comprises a chain of ossicles, the malleus, incus and stapes that act as an impedance matching device during the transmission of sound from the tympanic membrane to the inner ear. These ossicles are derived from cranial neural crest cells that undergo endochondral ossification and subsequently differentiate into their final functional forms. Defects that occur during middle ear development can result in conductive hearing loss. In this review, we summarize studies describing the crucial roles played by signaling molecules such as Sonic Hedgehog, Bone Morphogenetic Proteins, Fibroblast Growth Factors, Notch ligands and chemokines during the differentiation of neural crest into the middle ear ossicles. In addition to these cell-extrinsic signals, we also discuss studies on the function of transcription factor genes such as *Foxi3, Tbx1, Bapx1, Pou3f4*, and *Gsc* in regulating the development and morphology of the middle ear ossicles.

Keywords

Middle ear; Ossicle; Malleus; incus; stapes; columella; neural crest cells; growth factors; transcription factors

Introduction

The mammalian hearing apparatus consists of the outer, middle and inner ears, with all three parts being essential for hearing. Sound waves captured by the external ear (also known as the auricle or pinna) cause vibration of the tympanic membrane, which converts sound waves into mechanical vibrations that are conveyed to the chain of middle ear ossicles. These vibrations are applied to the inner ear through the oval window and travel along the cochlear duct, causing frequency-dependent vibrations of the basilar membrane of the cochlea at different positions along its length. These vibrations cause displacement of actin-rich hair bundles on the apical surface of cochlear hair cells, and the subsequent change in

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membrane potential of hair cells leads to synaptic vesicle release and transmission of nerve impulses along the auditory nerve to the brainstem [1]. The mammalian middle ear consists of a chain of three ossicles: the malleus, incus and stapes (Figure1A). One end of the malleus is connected to the tympanic membrane and the other end to the body of incus. The neck of the stapes is connected to the long process of the incus and the stapedial footplate is connected to the oval window of the inner ear [2].

Developmental defects in the middle ear ossicles can lead to conductive hearing loss. Some congenital diseases of the middle ear include absence or malformation of any of the middle ear ossicles, stapedial fixation at one or multiple fixed points, and fusion of malleus-incus elements [3-5]. Some hereditary syndromes exhibit middle ear malformations, including Branchio-oto-renal (BOR) syndrome, CHARGE syndrome, and DiGeorge syndrome [5-7]. Abnormal bone deposition or remodeling around the stapes (otosclerosis) is another cause of conductive hearing loss [8]. In children, conductive hearing impairment can also be caused by otitis media with effusion [9,10]. Although the genetic contribution to susceptibility to otitis media has been apparent for many years [11], it is only in the last 15 years that human and mouse studies have begun to identify genes associated with this disease [12-16]. Pathogenic variants in these genes can lead to altered innate immune responses in the middle ear, altered mucus production and abnormalities in the development of the middle ear cavity and Eustachian tube.

The three middle ear ossicles are largely derived from migrating cranial neural crest cells of the first and second branchial arches. An array of signaling molecules orchestrates the migration and condensation of neural crest into the three ossicle primordia. The production of these developmental signals, and the response of neural crest cells evoked by these signals are regulated by a large number of transcription factors. In this review, we summarize studies on the roles of these secreted signals and transcriptional regulators in regulating the development of the middle ear ossicles. We review Sonic Hedgehog (SHH), Bone Morphogenetic Protein 4 (BMP4), Fibroblast Growth Factors (FGFs), NOTCH and chemokine (CXCL12) signaling molecules, all of which play crucial roles during the early phases of middle ear development. Additionally, we summarize the roles of a number of transcription factors including Foxi3, Tbx1, Bapx1, Pou3f4 and Hoxa2 on middle ear ossicle development.

Evolution of the mammalian middle ear

As ancestral vertebrates began to exploit ecological niches on land, the differing densities and acoustic impedances of air and water presented physical constraints on the efficient transfer of sound wave energy from atmospheric air to the fluid of the inner ear labyrinth. The modern mammalian middle ear functions as an impedance matching device by reducing the loss of energy that occurs at the interface of air and liquid [17,18]. This impedance matching is dependent on the geometry and mechanics of the tympanic membrane and the middle ear ossicles. The relative surface areas of the tympanic membrane (large) and the stapedial footplate (small) also act to transfer acoustic energy so as to increase the force per unit area applied to the inner ear [19].

Mammals have a three-ossicle transmission apparatus, whereas most other land vertebrates have a single ossicle. In spite of this simple division, terrestrial middle ears are believed to have evolved independently many times [20,21]. Indeed, even the three ossicles of monotreme and therian mammals may have an independent origin [22]. It is believed that the hyomandibula of basal vertebrates that allowed the connection of the jaw to the brain case transformed into a sound-conducting bone, the columella, in amniotes (Figure1B) [20,23] and later, the stapes. The synapsid lineage incorporated the columella into the middle ear, but also developed the malleus and incus from the quadrate and articular bones [24], both of which are derived from condensations in the proximal region of Meckel's cartilage [25]. The loss of the original primary jaw articulation allowed the transformation of the proximal region of Meckel's cartilage into the middle ear ossicles. The exploitation of three fully ossified ear bones that emerged as a consequence of changes to the jaw to allow high frequency hearing has been discussed by Manley (2010). Interestingly, a recent study has shown that marsupials that begin feeding at a very early age still use their middle ear bones to articulate the jaw before the mature jaw articulation forms [26]. Although the precise sequence of events leading to the emergence of these two ossicles is still unclear, it is now believed that the miniaturization of the jaw that began in the Triassic-Jurassic period allowed the development of bones small and delicate enough to efficiently conduct high frequency sounds [27].

Development of middle ear and its associated structures

The middle ear ossicles derive largely from neural crest cells. However, a mouse lineage tracing study has shown the stapedial footplate to be derived from mesodermal cells, suggesting that the stapes has a dual origin [28]. Dye- and graft-based fate-mapping studies in chicken and mouse embryos show that neural crest migrates from rhombomeres 1-3 into the first pharyngeal arch (PA1) and from rhombomeres 3-5 into the second pharyngeal arch (PA2) [29-33]. In mice, genetic fate mapping confirms that Hoxb1- and Hoxa2-expressing crest from rhombomere 4 contribute to PA2 [34,35]. Some of the neural crest cells migrating into PA1 form the malleus-incus condensation [36], and cells migrating into PA2 form the stapedial condensation [23,37] and the external ear [35]. These neural crest cell condensations undergo endochondral ossification to form the middle ear ossicles [38]. In the mouse PA1, the malleus-incus forms as a single condensation attached to Meckel's cartilage at embryonic day (E) 10.5. Later, the malleus and incus separate from each other at E13.5, and the separation of Meckel's cartilage and malleus occurs postnatally in mice [3,25,37,39], requiring the action of chondroclasts [40]. The stapedial condensation forms separately in the PA2 mesenchyme at E10.5 and acquires its characteristic stirrup-like structure at E11.5, due to the stapedial artery passing through it [36]. By E15.5, the stapes is connected to the inner ear by the formation of annular ligaments on either side of the stapedial footplate [28,41]. The development of these middle ear ossicles is associated with the signals from the endoderm such as Shh and BMP4 [36,42], which we describe in more detail later in the review.

In addition to the middle ear ossicles, other elements associated with the definitive mammalian middle ear include the tympanic ring, gonial bone, styloid process, and middle ear cavity. The tympanic ring and gonial bone are neural crest-derived bony structures that anchor the middle ear ossicles to the skull and are generated by intramembranous ossification. They are homologous to the angular and prearticular structures seen in non-

mammalian amniote groups [43]. The styloid process is another mammalian-specific skeletal element derived from neural crest that forms by endochondral ossification in the PA2 region, and can act as an anchor for cranial muscles and ligaments [44-46].

The middle ear cavity also plays a very important role in sound conduction to the inner ear in addition to housing the middle ear ossicles. Whittmack proposed a developmental model of middle ear cavitation in which the first pharyngeal pouch endoderm that will ultimately form the auditory (Eustachian) tube extends into the middle ear, then expands and envelops the middle ear structures, resulting in endoderm lining the cavity caused by the evacuation of neural crest cells [47]. However, fate mapping studies with neural crest- and endoderm-specific Cre-expressing mice revealed that the epithelial cells derived from endoderm surround the eardrum and Eustachian tube, whereas epithelial cells derived from neural crest line more dorsal regions of the middle ear cavity, suggesting a dual origin for the epithelium surrounding the middle ear cavity [21,48].

Direct and indirect roles of secreted signaling molecules on middle ear development

In this section, we review studies on a number of signaling molecules (SHH, BMPs, FGFs, Notch and CXCL12) that play crucial roles in middle ear development, either directly or indirectly. Loss- or gain-of-function of these signals result in middle ear defects including an absence or reduction of middle ear structures, or alterations in the morphology of middle ear structures [23]. A summary of these phenotypes is shown in Figure 3 and Table 1.

SHH:

Hedgehog (HH) signaling plays very important roles in many tissues during embryogenesis and in the adult [49]. In brief, HH ligands bind to Patched1 (Ptch1), a transmembrane protein, which inhibits the binding of Ptch1 to Smoothened (Smo), another transmembrane protein. The resulting intracellular signaling cascade downstream from Smo allows proteolytic processing of Gli family transcription factors which translocate into the nucleus and regulate HH target genes [50]. There are three HH homologs in amniotes, SHH, Indian HH (IHH), and Desert HH (DHH). Loss of *Shh* in mice ($Shh^{-/-}$) causes cyclopia and defects in the outer, middle and inner ears [51]. At E10.5, *Shh* is expressed in the endoderm of the PA1, and its receptor *Ptch1* is expressed in the mesenchyme adjacent to the endoderm that will form the malleus-incus anlagen; however, *Shh* is not expressed in the endoderm beneath the stapes region in PA2, and *Ptch1* is also expressed at much lower levels in this region (Figure2B) [36]. Moreover, HH co-receptors such as *Cdon (Cell-adhesion molecules-related/down-regulated by oncogenes*) and *Gas1 (Growth arrest-specific 1*) are expressed between the middle ear joints and the annular ligaments in a similar pattern to *Gdf5*, a marker of joints. *Gas1* mouse mutants have a range of middle ear defects, including variable dysmorphology of the malleus and incus (with the stapes being less affected), as well as variable defects in the styloid process and tympanic ring [52]. *Biregional Cdon binding (Boc)* is another HH pathway protein expressed surrounding the joints and annular ligaments of the ossicles, similar to *Ptch1* expression [41].

Endodermal SHH signaling is essential for neural crest cells to initiate the malleus-incus condensation and is essential for neural crest survival in both the malleus-incus and stapes condensation regions [36]. Subsequently, endodermal deletion of *Shh* or neural crest-specific deletion of *Smo* results in the loss of middle ear ossicles (Figure 2B-D, 3B) [36,42,53]. Conversely, constitutive activation of SHH signaling in neural crest cells resulted in an enlargement of the middle ear condensations and altered the morphology and location of the middle ear ossicles at later stages (E15.5) [36]. These studies demonstrate that SHH signaling from pharyngeal endoderm directly regulates early stages of middle ear development.

BMPs:

BMP family members play a number of critical roles in craniofacial development, including neural crest induction and migration, and formation of the facial primordia, teeth, tongue and lips [54]. The binding of BMP ligands to BMP-type II receptors (BMPRII, ActIIR or ActRIIB), phosphorylates BMP-type I receptors (ALK1, ALK2, ALK3 and ALK6). These activated BMP-type I receptors phosphorylate receptor Smads (R-Smads), which in the case of Bmp signaling are Smad1, 5 and 8. The activated R-Smads form a complex together with the co-Smad Smad4, translocate into the nucleus, and activate Bmp target genes [55]. Noggin is a well-known secreted antagonist of BMP proteins. When BMP signaling was downregulated in cranial neural crest by ectopic expression of *Noggin*, embryos exhibited a loss of PA2 elements (including the stapes and styloid process) and more caudal arch elements [56]. Conversely, *Noggin* heterozygous ($Nog^{+/-}$) mutants exhibited ectopic bone formation in the ossicular chain due to incomplete separation of the stapes and styloid process. This ectopic bone led to a mild conductive hearing loss. The joints present between the three middle ear ossicles were also absent due to the fusion of all three middle ear ossicles [44]. Inactivation of BMP signaling in neural crest cells by deleting Smad4 with *Wnt1-Cre* mice, or by deleting *Bmp4* in the endoderm using *Foxg1-Cre* mice, the stapedial condensation failed to form in the PA2 as a result of the absence of neural crest cells in the prospective stapes region (Figure 2E-F) [36]. No evidence could be found of stapes formation even at later stages, confirming these defects were not simply due to a developmental delay. These observations suggest that endodermal BMP4 signaling plays a crucial role in early neural crest migration into the prospective stapes region, and normally acts to initiate stapes condensation.

FGF:

FGFs play very important roles during craniofacial development through the regulation of cell proliferation, cell survival and migration [57,58]. FGF signaling is mediated by receptor tyrosine kinases (Fgf receptors, Fgfrs; Fgfr1-4) [59]. A number of human craniosynostosis syndromes (such as Apert, Pfeiffer, Crouzon, Beare-Stevenson, and Jackson-Weiss syndromes) that are caused by pathogenic variants in FGF receptors can also display inner and middle ear defects [60,61]. Among the FGF ligands, *Fgf8* is strongly expressed in arch surface ectoderm and the pharyngeal endoderm [62,63]. Ectodermal-specific deletion of *Fgf8* or hypomorphic *Fgf8* mutants exhibit similar phenotypes, including a severely reduced or absent malleus, incus and tympanic ring which are due to the failure in neural crest cell survival. However, the stapes remains unaffected in both of these *Fgf8* mutant strains (Figure 3C) [64,65]. Among the four FGF receptors, mice carrying a Neo cassette insertion in intron 7

(*Fgfr1*$^{n7/n7}$) exhibited a loss of middle ear ossicles [66]. In *Hush puppy* mutant mice, a W691R variant in the *Fgfr1* gene resulted in a severe loss of FGFR1 activity. Homozygotes for this variant die at E8.5, however, heterozygotes exhibited hearing loss due to the defects in outer, middle and inner ears. The middle ear phenotypes include an abnormal incus and a wide range of malformations in the stapes [67] including a thinning or absence of the posterior crus in the stapes, or anterior crus malformations [68].

Neural crest-specific deletion of *Fgfr2* causes conductive hearing loss due to structural defects in the middle ear ossicles. In these mutants, the measurements of the auditory bulla showed a significant reduction in the overall volume when compared to control littermates. The retrotympanic process and tympanic ring were dysmorphic and hypoplastic. Ectopic bone formations were observed on the manubrium of the malleus, the incudomalleal joint, the ligament insertion site on the anterior process, and the insertion site of the stapedial muscle on the stapes [69].

NOTCH Signaling:

Another important pathway involved in various processes of development is Notch signaling. Unlike secreted growth factors, Notch signaling occurs between two adjacent cells when they contact with each other. The transmembrane ligand present on the signal-sending cell activates transmembrane Notch receptors present on the adjacent signal-receiving cells [70]. Human studies showed that mutations in *JAGGED1 (JAG1*, loss-of-function) were found in 94% of Alagille Syndrome patients. Some of these patients were diagnosed with sensorineural, conductive or mixed types of hearing loss [71]. In zebrafish, a *jag1b* allele isolated in a mutagenesis screen, *jag1b*^{b1105} displayed a variety of defects in the hyomandibula, a result that was also seen in morpholino-mediated knockdown of the Notch2 receptor(Zuniga et al., 2010). *jag1b* is expressed in the most dorsal neural crest contributing to the hyoid and mandibular arches, a region analogous to the region that generates the stapes in mammals. The evolutionary relationship between the fish hyomandibula and the middle ear ossicles raised the possibility that Jag1-Notch2 signaling might be necessary for ossicle formation. Deleting either *Jagged1* or *Notch2* in neural crest caused significant defects in the stapes, which appeared as a monopodal structure lacking crural regions. The stapedial artery was found passing adjacent to this monopodal structure rather than passing through the stapes [72]. As expected, these mice exhibit significant conductive hearing loss. A more detailed examination of *Jag1* heterozygous mouse mutants revealed them to have a smaller stapes that lacked a foramen (Figure 3D) [72], suggesting that the primary defect in the stapes is due to its malformation, rather than the lack of the stapedial artery. These results suggest a surprising degree of conservation in pharyngeal arch patterning between mammals and fish.

Chemokine signaling:

The C-X-C motif chemokine ligand 12 (CXCL12), also known as stromal cell-derived factor 1 (SDF1), is a chemokine that mainly signals through two G-protein coupled chemokine receptors, CXCR4 and CXCR7. It plays critical roles in migration of primordial germ cells, gonadotropin-releasing hormone (GnRH) interneurons, and endothelial precursor cells [73]. CXCL12 can act also act as a chemoattractant for neural crest cells, as well as regulating the

patterning and morphogenesis of neural crest-derived tissues [74-77]. In our recent work, we observed that *Cxcl12* was expressed in a pattern partially overlapping with the malleus-incus condensation lateral to the pharyngeal endoderm in pharyngeal arch 1, and its expression overlapped with *Sox9* and *Tfap2a* in the stapes region of pharyngeal arch 2. Upon deletion of *Cxcl12*, the neural crest-derived stapedial condensation initially formed. The primitive stapedial foramen and stapedial artery initially formed at E11.5, but have degenerated by E12.5 due to the loss of CXCL12 signaling, resulting in the loss of the stirrup-shaped stapes (Figure 3D) [78]. However, the malleus and incus condensations remained morphologically unaffected in these mutant mice.

Functional roles of transcription factors implicated in middle ear development

In this section, we discuss the roles played by several transcription factors in middle ear ossicle development. Transcription factors including *Hoxa2, Dlx* genes, *Tbx1, Pou3f3*, and *Foxi3*, are known to have direct or indirect functional roles on ossicle development [79-85]. Some of these transcription factors also regulate the patterning of neural crest cells in the pharyngeal arch region along either the posterior-anterior or dorsal- ventral axes [81,84,86,87]. The middle ear defects observed in the absence of some of the transcription factors were summarized in Figure 3 and Table 1.

Hox genes:

Hox genes play crucial roles in neural and craniofacial development, and also regulate the formation of some neural crest derived-pharyngeal arch elements. Neural crest cells contributing to pharyngeal arch 1 elements do not express *Hox* genes, but neural crest cells contributing to pharyngeal arch 2-6 elements, including the hyoid and other cartilages forming the neck region, all express *Hox* genes [88,89]. Lack of *Hoxa2* results in the homeotic transformation of pharyngeal arch 2 elements into pharyngeal arch 1 elements. As a result, *Hoxa2*$^{-/-}$ mutant embryos have no stapes, styloid process or hyoid cartilages; instead the malleus, incus, Meckel's cartilage, tympanic ring and gonial bone elements are duplicated (Figure 3E) [84,87,89,90]. Although they do not possess middle ear structures, the arch 1 duplication phenotype is conserved in amphibian and zebrafish models in which *Hoxa2* was downregulated, resulting in the homeotic transformation of pharyngeal arch 2 elements into pharyngeal arch 1 [91,92]. Temporal inactivation of *Hoxa2* at pre- or post-neural crest migration results in the duplication of some pharyngeal arch 1 elements in pharyngeal arch 2, suggesting that *Hoxa2* plays its role even after the completion of neural crest migration, and also exerts its temporal functions on specific individual neural crest derived elements [89]. Additionally, inactivation of *Hoxa2* resulted in external ear defects such as microtia [35,89], and pathogenic variants of Hoxa2 can also cause dominant microtia in humans [93].

When *Hoxa2* was ectopically expressed in neural crest cells (*NCC-Hoxa2*) [45], most of the *Hoxa2* over-expressing structures were affected. In these *NCC-Hoxa2* embryos, the distal parts of the dentary bone and Meckel's cartilage were normal or less affected, but the proximal parts of the dentary bone were hypoplastic and the proximal part of Meckel's cartilage was absent. Most of the pharyngeal arch 1 elements such as the malleus, incus,

tympanic ring, gonial bone, pterygoid and squamous bones were absent and instead were replaced with a duplicated stapes, styloid process, and the lesser horn of the hyoid cartilage. In neural crest cell-specific ectopic activation of *Hoxa2*, pharyngeal arch 1 elements underwent homeotic transformation to pharyngeal arch 2 elements [45]. Gain-of-function studies of *Hoxa2* in the first pharyngeal arch (Hox negative region) in chicken embryos, zebrafish or Xenopus also showed a conserved homeotic transformation of first pharyngeal elements into second pharyngeal elements [92,94,95]. In *NCC-Hoxa2*, the external ear auricle was duplicated, whereas the ear canal and tympanic membrane were absent [45]..

Dlx family:

Like *Hoxa2*, the six amniote *Dlx* family genes regulate pharyngeal patterning along the dorsal to ventral axis of each arch. Dlx genes arose from an ancestral duplication, and in modern amniotes, the *Dlx* genes occur in pairs (*Dlx1/2, Dlx3/4* and *Dlx5/6*), transcribed in opposite directions [96]. Expression studies of this family showed that *Dlx1/2 are* expressed throughout the proximo-distal pharyngeal arch mesenchyme, with *Dlx5/6* and *Dlx3/4* expression being progressively restricted to more distal regions of arch mesenchyme [86]. More than 50% of $Dlx1^{-/-}$ mutant mice exhibited an abnormal stapes and styloid process at postnatal day zero (P0) (Figure 3F) [97]. P0 $Dlx2^{-/-}$ homozygous mutant pups exhibited abnormal morphogenesis of the proximal parts of the first and second pharyngeal arches, which include an abnormal incus with ectopic palatoquadrate-like cartilage structures, a stapes lacking the central stapedial foramen, and a styloid process not connected to the inner ear (Figure 3G) [98]. The malleus in these mutant mice remains unaffected. In most $Dlx5^{-/-}$ homozygous mutants, all three middle ear cartilages were of normal shape, size and position. However, some of these mutants exhibiting exencephaly phenotypes were found to have their stapes missing [79,82]. Loss of *Dlx6* ($Dlx6^{-/-}$) gene alone did not affect any of the middle ear ossicles. However, together with the loss of other *Dlx* genes such as *Dlx1, Dlx2* or *Dlx5*, lack of *Dlx6* had abnormal middle ear defects [81,99]. Double mutant $Dlx5^{-/-};Dlx6^{-/-}$ embryos were severely affected and exhibited an exencephalic phenotype. These embryos lack external ear cartilages and their inner ear and middle ear cartilages were fused and severely dysmorphic. The incus was duplicated in these double knockout embryos. Also, the middle ear associated cartilages (Meckel's cartilage) and bones (tympanic ring and gonial bone) were absent in these double mutant embryos [86,100].

Tbx1:

Tbx1 is a member of the T-box transcription factor family, and is expressed in the ectoderm and endoderm of pharyngeal arches 1-3 at the time of neural crest migration (E9.5). Its expression shifts to the pharyngeal mesoderm (mesodermal core) at E10.5 [74,101]. Haploinsufficiency of *TBX1* in humans can result in DiGeorge Syndrome (also known as 22q11 deletion syndrome [101,102]. Some patients with DiGeorge syndrome are reported to present with congenital hearing loss, either conductive (majority of cases), or sensorineural (10%) [80,103]. $Tbx1^{-/-}$ neonatal mice exhibit a small and recognizable malleus and tympanic ring, a small cartilaginous nodule-shaped incus and no discernible stapes (Figure 3J). The gonial bone was normal in these mutant pups [103]. Although, *Tbx1* is not expressed in migrating neural crest, and the failure in the development of the neural crest-derived middle ear ossicles in embryos lacking endodermal *Tbx1* suggests that endodermal *Tbx1* acts

non-autonomously on neural crest cells during the development of the middle ear ossicles [80]. Consistent with this result, neural crest cell migration was not affected in *Tbx1* mutants and neural crest-specific deletion of *Tbx1* does not cause any craniofacial abnormalities [104].

Tbx1 acts indirectly to influence middle ear development through the regulation of a number of the signaling pathways discussed above. In the developing mandibular arch of $Tbx1^{-/-}$ embryos, the downstream targets of FGF8 signaling including *Lhx6, Lhx8, Pitx1, Spry1, Spry2* and *Erm2* were spatially shifted towards proximal and dorsal regions [104]. *Bmp4* and its downstream target gene, *Alx4* were also shifted towards the proximal side in the developing mandibular arch of $Tbx1^{-/-}$ embryos, and *Bmp4* expression was downregulated in the distal oral ectoderm [104]. Although *Shh* expression was not changed, *Ptch1* expression was expressed only in the proximal region of the mandibular arch, and diminished in the distal oral ectoderm [104].

Pou3f4:

One of the most frequent causes of X-linked hearing impairments is X-linked mixed hearing loss, characterized by both conductive and progressive sensorineural hearing loss. This non-syndromic hearing loss is due to variants in the coding or regulatory regions of POU3F4, located on chromosome Xq13-q22 [105]. *Pou3f4* mRNA is expressed in the mesenchyme apposed to the stapes footplate, dorsal to the stapes within the developing S-V joint, but is not expressed in the developing malleus and incus. The occurrence of conductive hearing loss in patients with X-linked mixed hearing loss often results from stapedial fixation [106]. Replacement of the coding region of mouse *Pou3f4* with a *Cre* recombinase gene results in a functional null allele of *Pou3f4 (*$Pou3f4^{Y/Cre}$). These mice exhibit a hypoplastic stapes footplate and oval window dysplasia, contributing to disarticulation of the footplate from the oval window [85]. *Ephrin-B2 (Efnb2),* a type I transmembrane protein, acts as a cellcell interacting ligand for various Ephrin (Eph) receptor tyrosine kinases. *Efnb2* plays crucial roles during various developmental processes including epithelial, nervous and brain development. During embryogenesis, *Efnb2* regulates neural crest cell migration by acting as a repulsive guidance molecule [107-109]. In the inner ear spiral ganglion, *Pou3f4* expression in otic mesenchymal cells is essential to activate *Epha4*, a canonical receptor for *Efnb2*, and disruption of any of these genes can lead to fasciculation and guidance defects of the spiral ganglion neurons innervating the cochlea [110]. In middle ear development, both *Efnb2* and *Pou3f4* overlap in the stapes footplate, stapedio-vestibular joint, the region dorsal to stapes, and the pharyngeal arch mesenchyme dorsal to the stapedial artery at E12.5-E13.5. However, overlapping expression of *Efnb2* and *Pou3f4* was not observed in the malleus and incus regions [85]. Inactivation of *Efnb2* in the mesenchymal and epithelial ear components using *Sox9-IRES-Cre* driver (*Efnb-cko*) resulted in the joining of the stapes and styloid process and/or a reduced distance between the stapes and styloid process. This phenotype is similar to the $Pou3f4^{Y/Cre}$ mutant phenotype. The genetic interaction between Pou4f3 and Efnb2 is confirmed by the observation that $Pou3f4^{Y/Cre}$;$Efnb2^{flox/null}$ compound mutant mice exhibit more severe middle ear defects than $Pou3f4^{Y/Cre}$ mutant mice [85].

Emx2:

Emx2 is a homeobox transcription factor that is necessary for correct middle and inner ear development [111]. *Emx2* begins to be expressed in the incus primordium from E13.5, distinguishing it from the adjacent malleus condensation [25]. Homozygous *Emx2* mutants lack both the incus and the malleal structure that articulates with the incus (Figure 3K) [111]. This suggests that the malleal articulation requires the incus to form correctly, but is not itself dependent on *Emx2* function [111]. Although all the middle ear ossicles form in *Emx2* heterozygous mutants ($Emx2^{+/-}$), they also show articulation defects between the malleus and incus. In the same study, a missense mutation in *Emx2* identified in the ENU-induced mutant *Pardon* was shown to have defects in all three ossicles (Figure 3K) [111].

Msx1:

Msx1 is a homeobox-containing transcription factor, which plays a major role in the development of many craniofacial elements, including the palate, mandible and the middle ear ossicles [112]. Loss of *Msx1* results in deformation of the malleus; the malleus is slightly shorter and lacks a small prominence (Figure 3L) called the process brevis in humans and the orbicular hypophysis in mice [113]. However, no other middle ear ossicles were affected in these mice [112,114]. X-gal staining of $Dlx5^{lacZ}$ shows expression in the malleus and incus; and similar analysis of $Msx1^{nlacZ}$ mice shows expression in the malleus and cochlea. In $Dlx5^{-/-};Msx1^{-/-}$ compound mutants, mice show deformation of the malleus, with a lack of the processus brevis (a *Msx* mutant phenotype), and ectopic gonial bone formation (a $Dlx5^{-/-}$ mutant phenotype). However, no additional defects were observed in these double knockout mouse embryos compared to those observed in either mutant line. This suggests that *Msx1* and *Dlx5* have independent and non-overlapping functions in ossicular development [112,114].

Foxi3:

Foxi3 is a forkhead transcription factor that plays very important roles in pharyngeal arch development. *Foxi3* is expressed in the surface ectoderm and pharyngeal endoderm, but not in migrating neural crest cells that populate the arch [115]. It becomes progressively restricted to the cleft/pouch region of each arch, and disappears by E11 [115]. Mouse and chick *Foxi3* transcription factors are homologous to the zebrafish *foxi1* transcription factor [116]. The unique phenotype observed with the loss of *Foxi3* is that it lacks all three components of the ear (outer, middle and inner ear) (Figure 3I). Middle ear associated elements such as tympanic ring and gonial bone elements are absent in $Foxi3^{-/-}$ embryos [83]. In these phenotypes, neural crest migration is not affected. However, the pharyngeal arches are greatly reduced and dysmorphic. The cells present within these hypoplastic pharyngeal arches fail to survive, which result in the absence of most of the pharyngeal arch derivatives [83]. This phenotype is very similar to a previously characterized deletion of *Fgf8* in branchial arch 1 ectoderm [65]. Accordingly, $Foxi3^{-/-}$ embryos lack *Fgf8* in arch ectoderm, and FGF-responsive genes such *as Erm/Etv4* fail to be expressed in neural crest cells invading the first and second arches of *Foxi3* mutants, suggesting that the cell death observed in Foxi3 mutants may be due in part to a lack of FGF signaling from the ectoderm to neural crest [83]. Cell death of first and second arch neural crest cells and jaw defects is also observed in

zebrafish *foxi* loss of function embryos [117,118]. Heat shock activation of either *fgf3* or *fgf8* is able to rescue the cranial cell death phenotype in *foxi1* morphant embryos [83], although these animals were only analyzed at relatively short time points after rescue. It remains to be determined whether Foxi3 regulates other secreted signals in arch ectoderm or endoderm that regulate neural crest cell differentiation and survival in the branchial arches.

c-Fos:

c-Fos is a proto-oncogene which is a component of the AP-1 transcription factor complex. c-Fos acts as a key regulator in osteogenic-macrophage lineage determination [119]. During development, the malleus-incus initially form a single condensation together with Meckel's cartilage. Typically the malleus separates from the proximal part of Meckel's cartilage at postnatal day 3 in mice [40] and separates from the incus around E13.5 [3,36]. Chondroclast cells play an important role during the breakdown of the connection between the malleus and Meckel's cartilage. In *c-Fos* mutant pups, the malleus fails to separate from the proximal part of Meckel's cartilage due to a failure of chondroclast differentiation. Treatment of wild type P0 CD1 mouse pups with bisphosphate alderonate (a drug that inhibits clast cell activity) also shows a persistent connection between the malleus and Meckel's cartilage, similar to the *c-Fos* phenotype at P3. This phenotype in mice appears to phenocopy the morphology of early Mesozoic mammals [40], suggesting that full separation of the malleus from Meckel's cartilage was a later mammalian innovation.

Bapx1:

Bapx1 is a *bagpipe*-related homeobox gene belonging to the NK family of transcription factors. Initially, this family was identified in *Drosophila* and is considered one of the earliest marker of prechondrogenic cells in vertebrates. Among the three vertebrate *Bapx1* genes, two are found in mammals: *Nkx3.1* and *Bapx1* (*Nkx3.2*) [120]. In humans, the homologue for *Bapx1* maps to chromosome 4p16.1, and this region is associated with many skeletal diseases [120]. In mice, loss of *Bapx1* results in a failure to form many skeletal elements due to early defects in cartilage development. Several genes associated with cartilage differentiation including *Sox9, Col2a1* and *Indian hedgehog* were downregulated in sclerotomal precursors, resulting in cartilage development defects [121]. *Bapx1* is expressed in the first pharyngeal arch (mandibular region) at E10.5, and its expression is later observed in the region medial to malleus-incus condensations at E11.5, and in the region surrounding the malleus at E13.5. At E12.5, its expression domain is closely associated with Meckel's cartilage, and in the condensing regions which become the tympanic ring and gonial bones [36,43]. In zebrafish, loss of *bapx1* resulted in the loss of the jaw joint, whereas its homologous structure in mice (the malleus-incus joint) remains unaffected in the absence of *Bapx1* [43,122]. Middle ear defects were observed in *Bapx1* mutant embryos: the width of the malleus, and middle ear-associated elements such as the gonial bone were absent and tympanic ring was hypoplastic (Figure 3M) [43]. The gonial bone was also hypoplastic in *Bapx1* heterozygous pups [43].

Goosecoid (Gsc):

Goosecoid is a homeobox-containing gene which is conserved among different vertebrates including zebrafish, *Xenopus*, chicken and mouse. *Gsc* is initially expressed in the mouse

primitive streak during gastrulation at E6, and later during craniofacial development in the mandibular mesenchyme, Eustachian tube, external auditory meatus, and malleus between E10.5 to E14.5 [123-125]. In *Gsc* null mutant embryos, the tympanic ring and external auditory meatus are absent and the manubrium and processus brevis of the malleus are reduced compared to controls. The gonial bones in *Gsc* null mutant mice embryos are either hypoplastic or reduced to small vestiges [125-127]. At E10.5, *Gsc* and *Bapx1* expression domains overlap in a small region of first arch mandibular mesenchyme. At later stages (E15.5), *Bapx1* was expressed within and surrounding the malleus region and coincides with *Gsc* expression in the anterior part of the tympanic ring. In the developing external acoustic membrane, *Bapx1* and *Gsc* overlap with each other. The similar expression patterns of *Bapx1* and *Gsc*, and the superficially similar mutant phenotypes suggests that the two transcription factors may be acting in a regulatory network. However, *Gsc* expression in *Bapx1* mutant embryos and *Bapx1* expression in *Gsc* mutant embryos are both unchanged, suggesting these genes act independently regulating tympanic ring development [43].

Tshz1:

TSHZ family members are zinc finger transcription factors which play crucial roles during embryogenesis. Of the three *Tshz* genes, only *Tshz1* is expressed in neural crest derived mesenchymal cells in the first and second pharyngeal arches [128]. The mesenchymal expression of *Tshz1* in the pharyngeal arches is controlled by the opposing actions of two epithelial-derived signaling molecules FGF8 (which induces *Tshz1* expression) and BMP4 (which blocks *Tshz1* expression) [129]. *Tshz1* mutant mice displayed middle ear defects, including an absence of the orbicular apophysis in the malleus (Figure 3N, black asterisk), a shortened and thickened tympanic ring, and an abnormal gonial ring. The incus and stapes developed normally and the stapes inserted normally into the oval window of the inner ear. Analysis of the manubrial area in $Tshz1^{-/-}$ mice showed a small rounded cartilaginous structure between the external acoustic meatus and the tubo-tympanic recess. Although *Tshz1* is expressed in pharyngeal arches 1 and 2, it not essential for early patterning of the arches, rather it specifically regulates the development of the malleus and middle ear-associated structures. As discussed above, genes such as *Msx1, Gsc* or *Bapx1* are essential for malleus, tympanic ring and gonial bone development, but expression of these genes in the developing middle ear structures of *Tshz1* mutant were unaffected. However, markers for developing bones (*Cbfa1/Rux2*) were greatly downregulated in the prospective tympanic ring and gonial bone regions of *Tshz1* mutants. suggesting that the tympanic ring and gonial bone defects are due to mis-regulation of *Cbfa1* [130] and placing Tszh1 function either downstream or acting in parallel to *Msx1, Gsc* and *Bapx1* action.

Future directions and unanswered questions in middle ear development research

The work above describes many of the transcriptional regulators and secreted signals that regulate neural crest cell migration into the prospective middle ear region and initiate the condensation of middle ear ossicle primordia. However, many questions remain unanswered. It is currently not clear when neural crest cells migrating into the first and second pharyngeal arches receive patterning information that directs them to form a particular component or

structure in the ossicular chain. At one extreme, neural crest cells could start to become distinct from each other as the migrate through the arches, while at the other extreme, they could remain as a relatively homogenous population until they cease migration and begin to condense into cartilage. The advent of single cell RNA sequencing (scRNA-seq) may help to shed light on this question [131]. scRNA-seq has already shown that crest cells in different parts of the migratory stream may be transcriptionally different from each other [132], and the ability to analyze single cell transcriptomes to greater read depths may reveal further heterogeneity during the different stages of ossicle formation. Similarly, although the expression patterns of growth factors, including those described in this review, have been described broadly in pharyngeal endoderm and ectoderm, it has been difficult in the past to map the spatial and temporal relationship of different signaling factors at high resolution. Once again, the ability to interrogate the transcriptomes of arch endoderm and ectoderm at the singe cell level may help identify unique populations of cells whose combined signaling output may help shape ossicle formation in their immediate vicinity.

Once neural crest cells cease migration in the first two pharyngeal arches, they undergo endochondral ossification to form cartilages including Meckel's cartilage, the malleus, incus, stapes, and styloid process. The signaling mechanisms and cell-intrinsic transcriptional codes that distinguish the ossicle cartilage precursors from each other and from other cartilages or bones in the head is not currently clear. Similarly, mouse mutants have given us only a rudimentary understanding of the signals that produce the unique shape and position of each ossicle. To give one example, previous studies showed that the malleus and incus initially develop from a common condensation and eventually separate from each other by forming the malleo-incudal joint. This cells within the prospective malleo-incudal joint down-regulate early condensing markers (*Sox9, Col2a1*) and up-regulate joint markers (*Gdf5*) [3,25,36]. However, it is still not clear that how the joint between incus and stapes (incudo-stapedial joint) is formed.

A final area of research concerns the large number of genetic disorders that affect the formation of the auditory conduction apparatus. Although the effects of many disorders and syndromes on the middle ear have been characterized, such as DiGeorge or Treacher-Collins syndromes, and a large number of deafness genes affecting inner ear function have been found, it is likely that many new gene variants may contribute to more subtle defects in the development of the middle and external ears. With the advent of large-scale genome and exome sequencing, more potentially pathogenic gene variants associated with conductive hearing loss may be identified. Moreover, the ability to rapidly create mouse models of such variants using CRISPR offers the potential for a new approach to understanding the genetics of middle ear-associated hearing loss.

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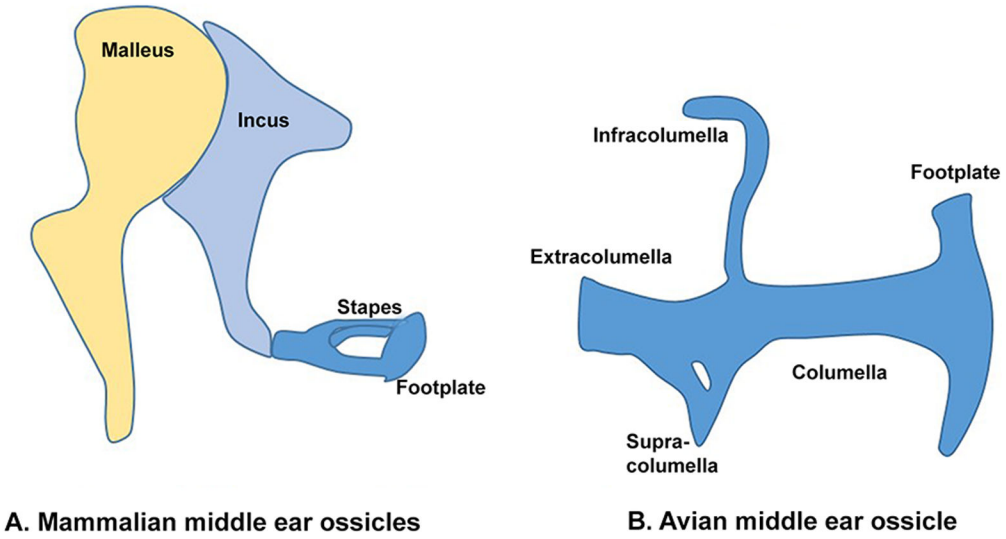

Figure 1: Comparison of the mammalian middle ear ossicles and avian columella.

(A) The mammalian middle ear ossicles consists of the malleus, incus and stapes. The stapes is connected to the oval window of the inner ear through the stapedial footplate. (B) The avian columella is homologous to the mammalian stapes. It consists of the extracolumella, infracolumella, supracolumella, columella and footplate.

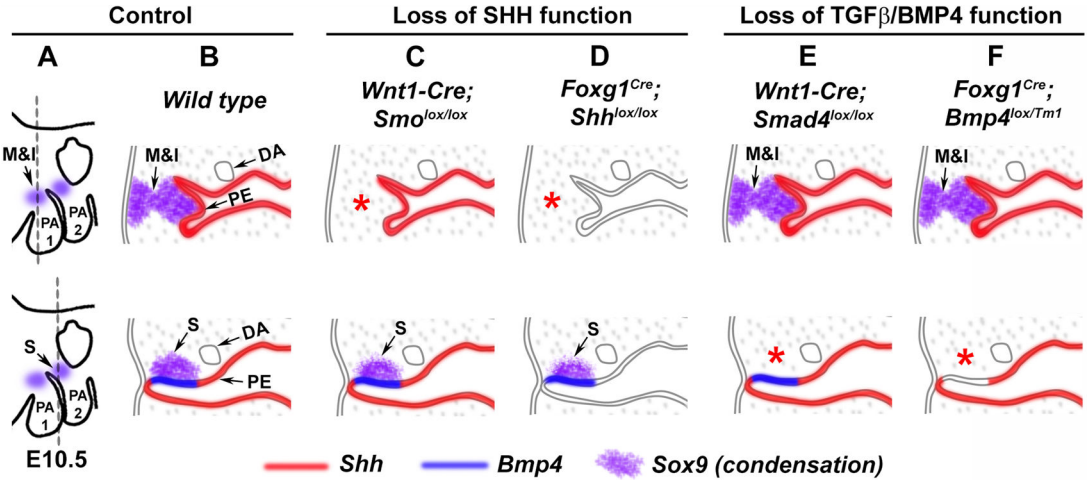

Figure 2: Endoderm acts as a signaling center for developing neural crest derived middle ear ossicles.

Loss of endodermal *Shh* (*Foxg1*^{Cre};*Shh*$^{lox/lox}$) function, or inactivation of SHH signaling by deleting *Smo* in neural crest cells (*Wnt1-Cre;Smo*$^{lox/lox}$) lead to loss of the malleus and incus condensation, but not the stapes (C, D). Similarly, loss of endodermal *Bmp4* (*Foxg1*^{Cre};*Bmp4*$^{lox/Tm1}$) function, or inactivation of TGF-β/BMP signaling in neural crest cells by deleting *Smad4* in neural crest cells resulted in the loss of the stapes condensation, but not the malleus or incus condensations (E,F). M, malleus; I, incus; S, stapes; DA, Dorsal aorta; PE, pharyngeal endoderm. This figure is adopted from Ankamreddy et al., Development (2019) with permission.

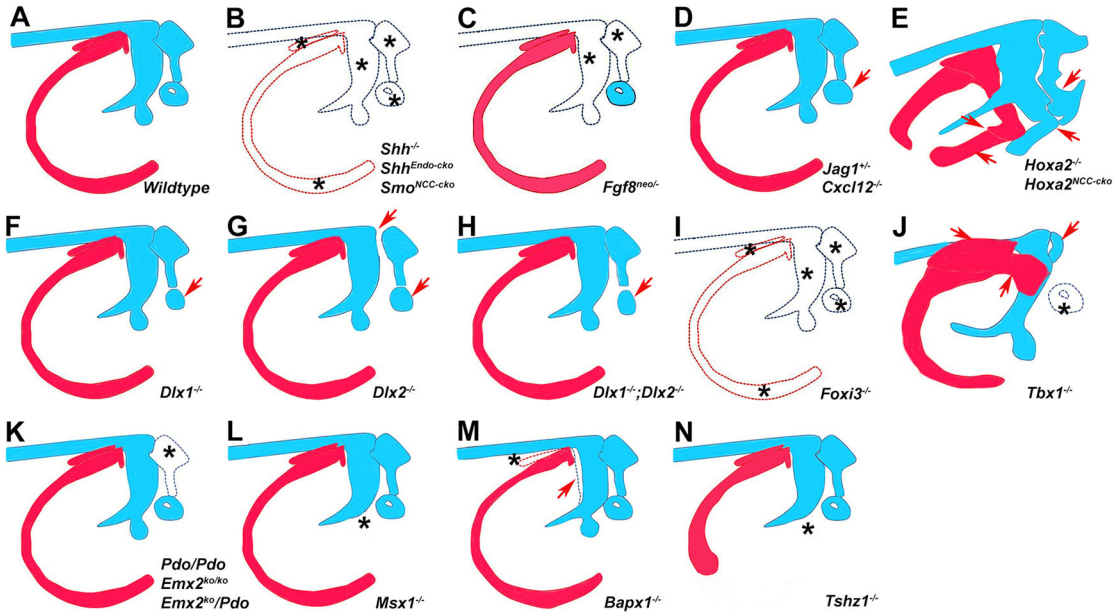

Figure 3: Summary of middle ear defects observed in different lines of mutant mice.

The middle ear ossicle defects observed in various mouse mutants are summarized in comparison to the wild type ossicles (A). Deletion of genes result in the loss of all three middle ear ossicles including their associated parts such as the tympanic ring and gonial bone (B, I, M), or a subset of the ossicles such as loss of the malleus and incus (C), incus (K) or stapes (E, J). Deletion of some genes can result in malformation of middle ear ossicles, such as a malformed malleus (J, L, M, N), malformed incus (G), malformed stapes (D, F, G, H) or malformed tympanic ring and gonial bone (J, N). Inactivation of Hoxa2 resulted in a mirror image duplication of the malleus, incus, tympanic ring and gonial bone in place of the stapes and other second pharyngeal arch derivatives (E).

These phenotypes are summarized in Table 1. Solid red and blue colors indicate normal bone and cartilages, respectively. Dotted red and blue colors indicate the absence of bone and cartilages, respectively.

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Table 1:

Middle ear defects observed in various mutant mouse embryos.

A summary of the middle ear defects observed in various mouse embryos shown in Figure 3

Panel in Fig.3	Mouse Mutant	Malleus	Incus	Stapes	Tympanic Ring	Gonial Bone	Stapedial Artery	Stapedial Foramen	References
A	Wildtype	Normal	Normal	Normal	Normal	Normal	Normal	Normal	
B	*Shh* $^{-/-}$	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Chiang et al., 1996
	Nkx2.5Cre;Shh $^{lox/-}$	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Billmyre and Klingensmith, 2015
	Wnt1-Cre;Smo $^{lox/lox}$	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Jeong et al., 2004; Ankamreddy et al., 2019
C	*Fgf8* $^{neo/-}$	Absent	Absent	Normal	Normal	Normal	Normal	Normal	Abu-Issa et al., 2002
D	*Jag1* $^{+/-}$	Normal	Normal	Malformed	Normal	Normal	Present	Absent	Teng et al., 2017
	Cxcl12 $^{-/-}$	Normal	Normal	Malformed	N/A	N/A	Absent	Absent	Ankamreddy et al., 2020
E	*Hoxa2* $^{-/-}$	Normal (duplicated)	Normal (duplicated)	Absent	Normal (duplicated)	Normal (duplicated)	Absent	Absent	Kanzler et al., 1998
	Wnt1-Cre;Hoxa2 $^{lox/lox}$	Normal (duplicated)	Normal (duplicated)	Absent	Normal (duplicated)	Normal (duplicated)	Absent	Absent	Santagati et al., 2005
F	*Dlx1* $^{-/-}$	Normal	Normal	Malformed	Normal	Normal	N/A	N/A	Qiu et al., 1997
G	*Dlx2* $^{-/-}$	Normal	Malformed (Not connected with	Malformed	Normal	Normal	N/A	N/A	Qiu et al., 1995
H	*Dlx1* $^{-/-}$*; Dlx2* $^{-/-}$	Normal	Normal	Malformed	Normal	Normal	N/A	N/A	Qiu et al., 1997
I	*Foxi3* $^{-/-}$	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Edlund et al., 2014
J	*Tbx1* $^{-/-}$	Normal	Malformed	Absent	Malformed	Malformed	N/A	N/A	Moraes et al., 2005
K	*Pdo/Pdo*	Normal	Absent	Normal	Normal	Normal	Normal	Normal	Rhodes et al., 2003
	Emx2 $^{KO/KO}$	Normal	Absent	Normal	Normal	Normal	Normal	Normal	Rhodes et al., 2003
	Emx2 $^{KO/Pdo}$	Normal	Absent	Normal	Normal	Normal	Normal	Normal	Rhodes et al., 2003
L	*Msx1* $^{-/-}$	Malformed process bravis	Normal	Normal	Normal	Normal	Normal	Normal	Satokata and Maas, 1994