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Morphogenesis of the mammalian tectorial membrane: unveiling the surface roles of a matrix organizer, alpha tectorin

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The tectorial membrane (TM) is an extracellular matrix (ECM) that lies over the organ of Corti. The TM exhibits complex ultrastructure and plays a critical role in auditory transduction. The TM is composed of collagens, non-collagenous glycoproteins, and proteoglycans that are produced by the cochlear supporting cells. Mutations in TM components result in TM malformation and hearing deficits. The expression of each TM component is tightly controlled in a time-dependent and cell type-specific manner. However, the mechanism by which TM components are organized outside of cells is poorly understood. We recently observed that the cell-ECM border plays a critical role in the organization of the matrix architecture. Surface-tethering of alpha-tectorin/TECTA via a glycosylphosphatidylinositol (GPI)-anchor is required to prevent the diffusion of secreted TM components into the luminal space and to form the matrix on the apical surface of the TM-producing cells. The release of TECTA is required for the growth of the TM layers. In contrast to the current extracellular-assembly model, which posits that the TM forms by a self-assembly process of its components in the luminal space, our findings support a novel three-dimensional (3D) printing model by which: (1) a surface-tethered structural organizer establishes a matrix layer on the cell surface and (2) as each successive layer is “printed”, the previously-established

layer is released along with the surface organizer. This repetitive process establishes the higher-order architecture of the TM. Based on the dynamic regulatory process of TECTA, we discuss potential morphogenesis mechanisms of the TM at different developmental stages.

Introduction

The mammalian cochlea is a fluid-filled, snail-like structure that is dedicated to auditory perception. The organ of Corti (OC), consisting of the inner and outer hair cells (IHC and OHC, respectively) and supporting cells (SC), functions as a receptor organ for hearing (*Figure 1*). Two extracellular matrices (ECMs), the basilar membrane (BM) and the tectorial membrane (TM), which sandwich the OC, play critical roles in transducing auditory stimuli [1-5]. The BM is the primary ECM structure that vibrates in response to auditory stimuli. Recent studies showed that the TM displays distinct vibration motions and plays pivotal roles in auditory perception, such as propagation, frequency tuning, and amplification of sound stimuli [4, 6, 7].

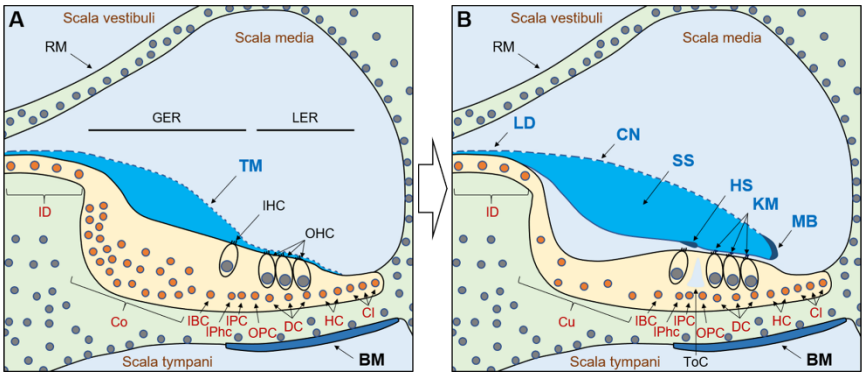


Figure 1. Schematics of a radial section of the developing and matured mammalian cochlea

The tectorial membrane (TM: light blue) and the basilar membrane (BM: dark blue) are extracellular matrix (ECM) structures essential for auditory perception. (A) The developing TM at postnatal day 2 (P2) in the scala media grows on the surface of auditory supporting cells, including interdental cells (ID), columnar cells (Co), inner border cells (IBC), inner phalangeal cells (IPhc), inner pillar cells (IPC), outer pillar cells (OPC), Deiter`s cells (DC), and Hensen`s cells (HC). So far, there is no report that the TM grows on the Claudius cells (Cl). (B) The mature TM exhibits characteristic ultrastructural features, which include the limbal domain (LD), top covernet (CN), striated sheet (SS), Hensen`s stripe (HS), Kimura`s membrane (KM), and marginal band (MB). Reissner`s membrane (RM) and the BM compartmentalize the scala vestibuli and scala tympani from the scala media, respectively. Rounds mark the position of the cell nucleus. Cu, cuboidal cells; GER, greater epithelial ridge; IHC, inner hair cells; LER, lesser epithelial ridge; OHC, outer hair cells; ToC, tunnel of Corti.

The TM is a gelatinous, poroelastic, and acellular structure that lies over the apical surface of the OC and is located within the luminal space that is filled with endolymph. The mature TM detaches from the OC, except at the spiral limbus and the tallest stereocilia of OHCs [1, 8, 9]. This unique configuration creates a shearing force on the tip of the stereocilia bundles of the OHC, which leads to the activation of OHC's electromobility (forward transduction) [5]. As a result, fluid dynamics within the narrow space between the bottom surface of the TM and the reticular lamina (apical cuticular structure of the cells in the OC) excite the IHC to transmit electrochemical signals to the brain [10, 11].

The cochlear SCs are aligned on the BM and are tightly connected to one another through gap junctions [12, 13]. The SCs play structural and functional roles in auditory perception. They provide mechanical support to the hair cells and regulate the balance of ions and small molecules between the endolymph and perilymph [13]. In addition, they are the primary cell types that produce the TM. They synthesize and release collagens and non-collagenous TM glycoproteins into the apical luminal space (scala media). There are multiple cell types of the SCs characterized by their distinct shape, position, and gene expression profiles. Notably, each type of SC shows the distinct spatial and temporal expression pattern of the TM's glycoproteins: Interdental cells in the spiral limbus are the primary cell type that expresses otoancorin/OTOA and carcinoembryonic antigen-related cell adhesion molecule 16/CEACAM16; Columnar cells in the Kölliker's organ generate the body domain of the TM and highly express otogelin/OTOG, and beta tectorin/TECTB; inner border cells express OTOA, otolin/OTOL; inner phalangeal cells express OTOL; inner and outer pillar cells express TECTB, OTOG, and OTOGL; Deiters` cells express TECTB, OTOG, and CEACAM16; Hensen`s cells express OTOG and otogelin-like/OTOGL; Claudius cells express OTOGL; All SCs are known to express alpha-tectorin/TECTA, except for Claudius cells [5, 8, 14-21] (Table 1). Moreover, each glycoprotein shows unique temporal expression profiles through developmental stages (Table 2). Mutations and/or dysregulated gene expressions of these glycoproteins result in structural defects of the TM and severe hearing loss [5, 10, 17, 22]. Thus, the proper assembly of these glycoproteins within the TM matrix in a spatiotemporal manner is significant in TM morphogenesis.

We recently showed that the cell surface-localization of TECTA is required for preventing the diffusion of TM components and generating the TM matrix on the apical surface of the cochlear supporting cells. The release of TECTA is required for the development of the multi-layered architecture of the TM [23]. From these observations, we proposed that a novel three-dimensional (3D) printing model for TM morphogenesis, which is mediated by a repetitive process of surface-tethering and subsequent release of the matrix organizer, TECTA (*Figure 2*) [23]. In this chapter, we will discuss the mechanism of the TM's morphogenesis during cochlear development in mice, focusing on the surface roles of TECTA.

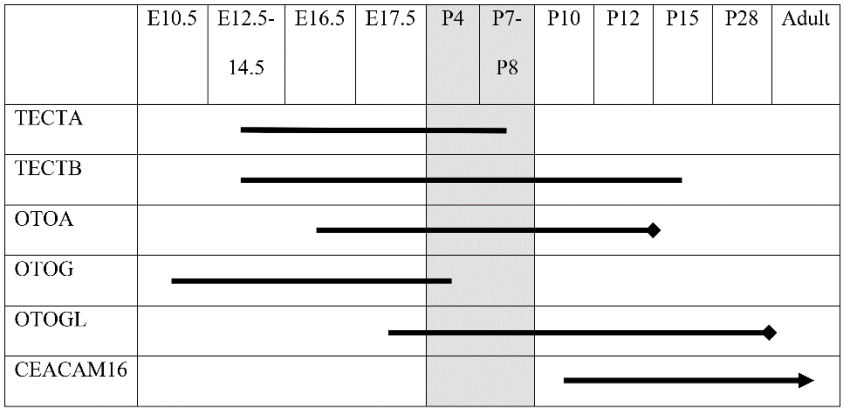
This chapter provides an example of the morphogenesis mechanism of a complex ECM architecture.

Table 1. Summary of the expression of TM glycoproteins in the cochlear supporting cells

	ID	Co	IBC	IPhc	PC	DC	HC	Cl
TECTA	+	+	+	+	+	+	+	-
TECTB	-	+	-	-	+	+	-	-
OTOA	+	-	+	-	-	-	-	-
OTOG	-	+	-	-	+	+	+	-
OTOGL	-	-	-	-	+	-	+	+
OTOL	-	-	+	+	-	-	-	-
CEACAM16	+	-	-	-	-	+	-	-

The TM glycoproteins show cell type-specific expression patterns in the developing cochlea. Cl, Claudius cells; Co, columnar cells; DC, Deiter`s cells; HC, Hensen`s cells; IBC, inner border cells; ID, interdental cells; IPhc, inner phalangeal cells; PC, inner and outer pillar cells. Expression profiles are collected from the following literatures: alpha tectorin/TECTA [19], beta tectorin/TECTB [19], otoancorin/OTOA [17], otogelin/OTOG [14], otogelin-like/OTOGL [21], otolin/OTOL [24], and carcinoembryonic antigen-related cell adhesion molecule 16/CEACAM16 [16]. TM glycoproteins expressed in a specific cell type are marked with +.

Table 2. Summary of the temporal expression patterns of the TM glycoproteins in the supporting cells during cochlear development



TM glycoproteins show a dynamic expression pattern during cochlear development. Lines indicate the period of gene expression. The uncertain termination of OTOA and OTOGL expression is depicted by diamonds. An arrow in CEACAM16 indicates persistent expression. The expression profiles are collected from the following literatures and are combined with *in situ* hybridization data shown in Fig. 4A: TECTA [19] (Fig. 5A), TECTB [19], OTOA [25], OTOG [26], OTOGL [21], CEACAM16 [16]. The initial expression of TECTA is controversial between E12.5 [19] and E14.5 [8, 27]. The temporal expression of OTOL is omitted in this table, due to a lack of data.

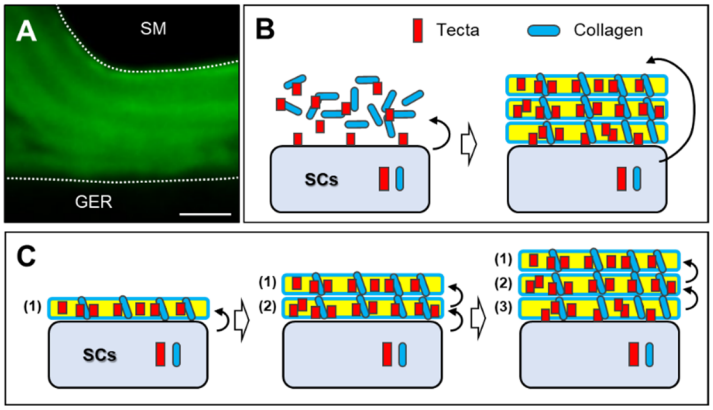


Figure 2. A multi-layered organization of the tectorial membrane (TM) and schematics of the TM morphogenesis mechanism

(A) *Pisum sativum* agglutinin (PSA)-lectin staining using radial sections of the mouse cochlea at postnatal day 2 (P2), analyzed by Airyscan high-resolution microscopy. The TM is specifically stained by PSA, which appears as multiple layers on the greater epithelial ridge (GER). The dashed line defines the TM. SM, scala media. Scale bar, 10 μm. (B) An extracellular-assembly model. Collagens and glycoproteins secreted or released from the cochlear supporting cells (SCs) may organize themselves in the luminal space to generate a multi-layered architecture. (C) A three-dimensional (3D) printing model. Printing of a new layer and simultaneous release of a pre-established layer may build a multi-layered TM structure. This model predicts the sequential generation of TM layers (depicted as a number next to each layer).

TM Morphogenesis

Development of the TM

An earlier significant study demonstrated that the mammalian TM is composed of collagens (type II, V, IX, and XI) and glycosylated polypeptides [28]. Subsequent studies using different types of lectins (carbohydrate-binding proteins) tracked the developmental changes in the distribution of specific sugar moieties within the TM during cochlear development [29, 30]. They observed that the covernet forms early when the TM is apparent on the apical surface of the greater epithelial ridge (GER). The distance between the covernet and cell border increases when the growing TM remains attached to the apical surface of the supporting cells. This macroscopic observation suggests that major layers of the

TM are sequentially generated in an order of covernet, central body, and then border layer [30]. Histological studies of the mouse TM using electron microscopy (EM), immunofluorescence, and Toluidine blue staining of semi-thin cochlear sections revealed that the rodent TM becomes identifiable around embryonic day (E) 14.5 forming the covernet (the first-born layer) on the apical surface of the GER [8, 27]. On E18, the TM also forms on the lesser epithelial ridge (LER). At this stage, the TM attains its area ranging from the spiral limbus to the outer hair cells and thickens. The thickening of the TM is initiated from columnar cells and extends laterally toward the outer supporting cells, while the limbal region of the TM remains thin and becomes condensed. Starting at postnatal day 4 (P4), the inner sulcus forms as the TM begins to detach from the columnar cells [8, 30, 31]. The TM vertically grows up to 50 microns at the apex, although the size of the TM varies along with the cochlear turn [32]. By P14, the body domain of the TM completely separates from the producing cells except in the spiral limbus and the tallest stereocilia of OHCs [8]. The detached TM no longer grows, and it maintains the same overall morphology throughout the lifespan that it has at this early developmental stage in the luminal space of the cochlea, known as the scala media.

Characteristic Ultrastructures

Cross-sectional views of the cochlea reveal characteristic ultrastructures: i) The covernet is a dense and reticularly organized structure located on the top of the TM. ii) The striated sheet is a thin filamentous structure and forms the central body together with parallel collagen fibers. iii) The marginal band is a rigid structure, located at the lateral end of the TM along the radial axis. iv) Kimura`s membrane is a compact, filamentous structure where the tallest stereocilia of the OHCs are embedded. v) Hensen`s stripe is a densely-packed structure located above the stereocilia of the IHCs. vi) The limbal domain (medial part) is a thin and compact structure and remains attached to the spiral limbus (*Figure 1B*) [9]. Mutations or knock-out (KO) studies of TM components revealed the essential components for these ultrastructures [5, 33-35]. These ultrastructural features are required for the proper functions of the TM: activation of the OHCs directly by TM displacement and the IHCs indirectly by endolymph flow in response to acoustic stimulation.

The major fiber of the covernet is organized longitudinally along the length of the cochlear turn [27]. The branches of the covernet display a meshwork densely packed with thin filaments and globular units. TECTA and TECTB are densely clustered in the covernet [36]. Since the covernet is the first-born layer of the TM, it has been speculated to play a role in confining the TM components of the central body layer. However, the mechanism by which these ultrastructures are precisely organized at their specific locations is still mysterious.

Reasonable clues for this question could be found in the spatiotemporal expression pattern of the TM glycoproteins: i) The SC consists of various types of cells that uniquely reside along the radial axis of the cochlea, where they express distinct TM glycoproteins (Table 1). ii) These glycoproteins are expressed in a time-dependent manner (e.g. TECTA, E12.5 or E14.5~P8; TECTB, E12.5~P15; OTOA, E16.5~P12 or later; OTOG, E10.5~P4 or later; OTOGL, E17.5~P28; CEACAM16, P12~) (Table 2) [16, 19, 21, 25-27]. iii) Specific ultrastructural features are lost when the expression of these glycoproteins is altered. For instance, the TM of *Tectb* or *Otoa* KO mice lacks the marginal band and Hensen`s stripe [17, 35]. *Otog* KO affects the limbal domain of the TM [37]. Loss of *CEACAM16* expression results in the absence of the striated sheet and Hensen`s stipe [33]. Overall, these observations suggest that the unique molecular compositions of each domain contribute to organizing their characteristic ultrastructures.

The Extracellular-assembly Model

Although the molecular composition of the TM has been extensively investigated, the mechanism by which this fine structure is organized outside of cells is poorly understood. One generally accepted model is that the TM is organized by the self-assembly process of the secreted and released TM components in the endolymph-filled, luminal space of the cochlea, the scala media (extracellular-assembly model) (*Figure 2B*) [36]. This model is supported by the following observations in the TM: i) The TM is an acellular structure from the beginning of its development, unlike matrices that form within the cells, such as crystallin fibers [38]. ii) Not only are secreted TM components such as collagens, OTOG, OTOGL, and CEACAM16 present away from the cell surface throughout the TM structure but so are the GPI-anchored TM components including TECTA and TECTB. Thus, TM components are either secreted or robustly released from the cell surface of the supporting cells (except for OTOA, which remains anchored to the surface membrane). iii) The TM is expanded on the cell surface during development, which may imply that the secreted molecules promptly assemble themselves upon their release. Nevertheless, this model hardly explains how the TM displays a defined morphological gradient along the length of the cochlea and the designated areas of characteristic ultrastructure. Moreover, the extracellular-assembly model cannot explain the characteristic anisotropy of the TM layers: the longitudinal arrangement of the major fibers of the covernet and radial alignment of collagen fibers in the central body [36]. Interestingly, a recent study showed that disruption of planar cell polarity (PCP) in the sensory epithelium impairs the characteristic anisotropy of collagen fibers [8, 27]. This observation raises the possibility that there exists a cell surface component that signals to the ECM compartment to direct the assembly of the TM matrix.

Evidence of the Cell Surface Organizer

A Morphological Gradient of the TM along with the Cochlear Turn

The TM exhibits morphological and stiffness gradients along the tonotopic axis. The size of the TM gradually increases in width and thickness from the base (high-frequency hearing zone) to the apex (low-frequency hearing zone), while the stiffness decreases [32, 39, 40]. What determines the size and the stiffness of the matrix in each position, which is organized in the luminal space? Some clues can be found at the molecular and cellular levels. The cellular area of the sensory epithelium expands toward the apex (maximum up to ~250 microns in mouse) during early cochlear development and the size of the sensory epithelium matches that of the TM along with the cochlear length, suggesting that the radial size of the TM is confined to the length of the apical surface of the radial SC arrangements [27, 32, 41]. In addition, many genes show a gradient expression pattern along with the cochlear turn during development, which includes TM components such as TECTB [42]. The unique combination and assembly process of the TM's glycoproteins may give rise to the different ECM properties, such as stiffness, porousness, viscosity, and charges [43, 44]. These observations indicate that the assembly process of TM components takes place on or immediately above the SC surface.

A Layered and Anisotropic Structure of the TM

A recently advanced technique using high-resolution fluorescence microscopy allows the observation of the TM architecture in detail. When the TM is stained with *Pisum sativum* agglutinin (PSA), an alpha-linked mannose-binding lectin, the TM appears as a layered architecture (*Figure 2A*) [23]. Moreover, the ultrastructural analyses revealed that the TM matrics display unique anisotropic organizations [36]. The major fibers of the covernet are organized longitudinally. The collagen fibers in the underlying body layer are radially oriented with a tonotopy-specific, apically-directed slant [27].

Directional Organization by PCP Signals

The cochlear SCs are polarized cells [13]. TM components are specifically sorted to the apical surface of cells, thus the TM forms on the apical surface of the SCs. A recent study provided evidence that patterning of collagen type IX fibrils (a secreted TM molecule) on the cell surface during cochlea development is altered when PCP is disrupted [27]. Unlike wildtype mice that display collagen fibril

bundles with an apically-directed slant, *Vangl2* or *Ptk7* KO mice lose this slant. In addition, *Tecta* and *Tectb* double KO mice fail to form the proper orientation of collagen fibril bundles, indicating that tectorins play a role in the directional organization of the collagen fibers [27]. However, whether tectorins directly transduce the cellular signal or indirectly play a permissive role via collagen organization is unknown.

Possible Candidates

Secreted vs. membrane-tethered TM components

Since the late 1990s, mammalian TM glycoproteins have been identified and cloned: *Tecta* [45], *Tectb* [45], and *Otog* [14], *Otoa* [25], *Otol* [24], *CEACAM16* [46], and *Otogl* [21]. Several KO studies using these proteins demonstrated the importance of these proteins for the structure and function of the TM in relation to auditory transduction [17, 21, 33-35, 37, 47]. The TM components can be categorized into two groups: secreted proteins (collagens, OTOG, OTOL, and OTOGL) and membrane-tethered glycoproteins (TECTA, TECTB, and OTOA) via a glycosylphosphatidylinositol (GPI)-anchorage. TECTA and TECTB are the most abundant glycoproteins in the TM [34]. Intriguingly, immunofluorescence and immuno-electron microscopy analyses showed that both TECTA and TECTB are not only expressed on the cell surface but also broadly distributed throughout the TM away from the cell surface [15, 17, 23, 36]. In contrast, the expression of OTOA, another GPI-anchored protein, appears to be restricted to the apical surface of the interdental cells of the spiral limbus and border cells [25]. These findings suggest that TECTA and TECTB may play versatile roles in matrix organization depending on their localization: on the apical surface or in the extracellular space. Tectorins are expressed at E14.5 when the TM starts to form [27] and may function as a molecular glue by crosslinking ColII fibrils in the TM [36].

Domains of Tectorins

TECTB is small and contains only a zona pellucida (ZP) domain, which can mediate protein multimerization (*Figure 3A*). TECTA is large and contains multiple domains, which include an N-terminal entactin G1-like (NIDO) domain (G1LD), a von Willebrand factor type (vWF) C, multiple vWFD domains, and the C-terminal zona pellucida (ZP) domain (*Figure 3A*) [8, 45, 46, 48, 49]. The G1LD, vWFC, and vWFD domains are suggested to be for binding to collagens and ECM proteins. Mutations of *Tecta* resulted in severe malformation of the entire TM structure [5, 10, 34, 50], while deletion of *Tectb* leads to the loss of specific

ultrastructural features including the marginal band and Hensen's strip [35, 51]. Consistent with the histological observations, *Tecta* KO mice show more severe hearing deficits than *Tectb* KO mice [34, 35]. These observations suggest that TECTA plays a major role in the TM's organization during cochlear development.

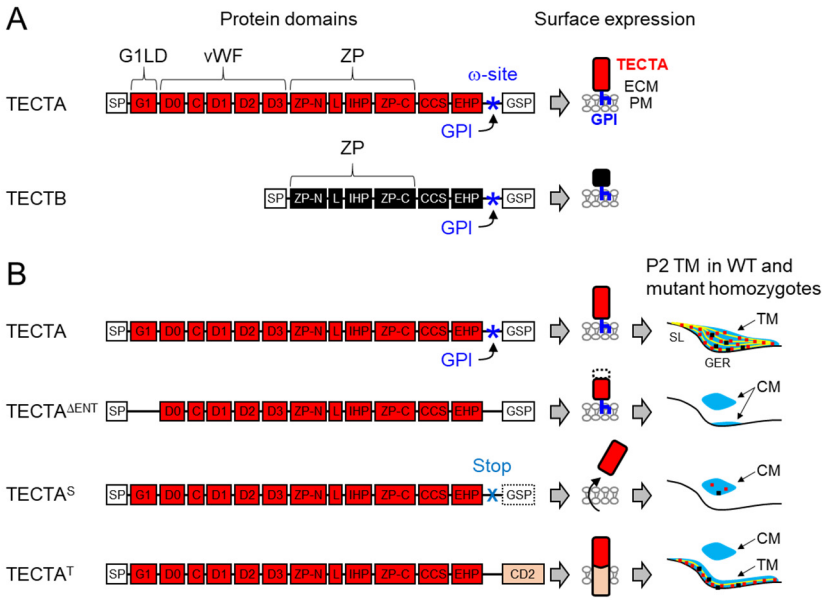


Figure 3. TECTA domains and the cellular localization of TECTA mutants

(A) TECTA contains the N-terminal ER targeting signal peptide (SP) followed by entactin G1-like (NIDO) (G1LD), von Willebrand factor type C (vWFC) and D (vWFD), and zona pellucida (ZP) domains, and C-terminal glycosylphosphatidylinositol (GPI)-anchorage signal peptides (GSP) [8]. TECTB is relatively short and contains the ZP domain in the mature form. The consensus cleavage site (CCS) and the external hydrophobic patch (EHP) domain, which blocks the polymerization of ZP domains, are located between the ZP domain and GSP. Both tectorins are localized on the cell surface via a GPI anchor. Open boxes indicate preprotein domains removed during posttranscriptional modification. *, GPI-attached site (ω-site); ECM, extracellular matrix compartment; IHP, internal hydrophobic patch; L, linker sequences; PM, plasma membrane; ZP-N, N-terminal of ZP; ZP-C, C-terminal of ZP. (B) Schematics of TECTA mutants and their corresponding TM morphologies in the P2 mouse cochlea [23, 34]. A dashed box indicates a domain not expressed due to the introduction of a premature stop codon at the ω-site. The developing TM of wildtype mice exhibits a multi-layered architecture. Cyan color indicates the collagen-based matrix (CM). The TM of the N-terminal G1LD deletion mutant mice (*TECTA*$^{\Delta ENT/\Delta ENT}$)

is severely disorganized. The disorganized collagen aggregates that are not associated with TECTA are found in the luminal space and on the cell surface at this time (P2), but the detailed structure of this immature matrix is unknown. The loss of GPI-anchorage of TECTA in *Tecta*$^{S/S}$ mice leads to similar disorganization of the TM as observed in *Tecta*$^{\Delta ENT/\Delta ENT}$ mice. A transmembrane form of TECTA (TECTA^{T}) has C-terminally extended amino acids with T-cell surface antigen CD2. *TECTA*$^{T/T}$ mice only form a thin TM layer on the cell surface, which does not mature into a multi-layered structure. Each TM layer, identified by PSA-positive glycoproteins, is depicted with a yellow line. Localization of TECTA and OTOG in the TM is present as red and black dots, respectively. The number of layers and protein, including TECTA in the schematics, does not represent the exact quantity.

TM Layer Formation by Surface-anchored TECTA

The TM of *Tecta*$^{\Delta ENT/\Delta ENT}$ mice lacking the G1LD domain of TECTA is disorganized and detached from the OC. The disorganized aggregate is associated with Reissner`s membrane in the luminal space (*Figure 3B*) [34]. The TM of *Tecta* and *Tectb* double KO mice displays similar disorganization of collagen fibrils, which are not associated with the microvilli of the auditory supporting cells during its development [27]. These observations indicate that TECTA plays a critical role in the organization of collagen fibrils and the TM matrix. However, the mechanism by which TECTA mediates the organization of collagen fibrils was unknown. Using mice in which the GPI-anchorage of TECTA is either lost (*Tecta*$^{S/S}$) or substituted with the transmembrane protein of T-cell surface antigen CD2 (*Tecta*$^{T/T}$), a recent study showed that the GPI-anchorage of TECTA plays critical roles in multiple steps of TM assembly (*Figure 3B*) [23]. In *Tecta*$^{S/S}$ mice, where TECTA is constitutively secreted by adding a premature stop codon before the C-terminal GPI-anchorage signal peptide (GSP), the TM is severely disorganized similarly to that of *Tecta*$^{\Delta ENT/\Delta ENT}$ mice, indicating that surface expression of TECTA plays a critical role in TECTA function. Collagen fibrils are not recruited to the cell surface and form an irregular aggregate in the luminal space (e.g. absence of the TM anisotropy and TM glycoprotein incorporations) (*Figure 3B*). Conversely, a transmembrane form of TECTA (TECTA^{T}) is localized on the apical surface of the supporting cells and is sufficient to recruit collagen fibrils to the microvillar surface of the supporting cells (*Figure 3B*) [23]. Interestingly, in *Tecta*$^{T/T}$ mice, the collagen network forms on the apical surface of the supporting cells and does not develop into a multi-layered structure (*Figure 3B*). Notably, TECTA^{T} is not released from the cell surface, indicating that GPI-dependent surface expression of TECTA plays an important role in TECTA release. The GPI-anchorage of TECTA may be a direct target of GPI-anchor lipases [52, 53]. Alternatively, the GPI-anchorage of TECTA may regulate substrate specificity or availability toward proteolytic sheddases. It is also possible that the

extended distance between the ZP domain and the plasma membrane introduced by the C-terminally fusion of CD2 in TECTA^{T} may affect accessibility toward surface sheddases. Collectively, these observations indicate that the release of TECTA plays a critical role in the growth of the TM matrix [23]. Besides, the collagen network, OTOG, a secreted TM component, is also deposited on the apical surface of the supporting cells in the *Tecta*$^{T/T}$ cochlea but not in the *Tecta*$^{S/S}$ cochlea (*Figure 3B*). These results indicate that surface-expression of TECTA is sufficient to induce the formation of both collagenous and non-collagenous matrices on the apical surface of the supporting cells. Overall, these observations suggest a possibility that interaction between surface-tethered TECTA and ECM components plays a critical role in the organization of the TM. So far, however, there is no biochemical evidence that shows the direct interaction between the TECTA domains and collagens. Moreover, which type of collagen among the TM collagens (e.g. fibril-forming collagens, II, V, XI; fibril-associated collagen, IX) is associated with TECTA remains unknown. Intriguingly, ColII, the major collagen type in the TM, is expressed early at E14.5 [54] when both tectorins start to be expressed. Other TM collagens, such as ColIX and ColXI are expressed later at E15.5 [27, 55]. Since type IX and XI collagens are known to be crosslinked or assembled with ColII [37, 56], it is possible that collagens expressed later than TECTA may associate with pre-assembled TECTA-ColII complexes. The temporal expression pattern of ColV (the smallest amount in the TM) in the cochlea has not yet been examined. Overall, *in silico* analysis, *in vivo* studies with TECTA mutations, and the temporal expression patterns of TECTA and collagens suggest that the interaction between TECTA and type II collagen via the N-terminus of TECTA may play a critical role in the matrix organization.

Possible Release and Multimerization Mechanisms of TECTA for the Growth of the TM

Although TECTA is GPI-anchored, TECTA is released from the producing cells and is present throughout the TM structure. The release of TECTA plays a critical role in matrix growth [23]. However, the release mechanism of TECTA is unknown. *In silico* analysis of TECTA shows that C-terminus of mature TECTA protein (located proxy to the membrane anchorage site) may contain three potential cleavage sites (two proteolytic consensus cleavage sites (CCSs) and a GPI-anchor by GPI-lipases) and two external hydrophobic patch (EHP) domains, which can block the polymerization of the ZP domains (*Figure 4A*) [48, 49]. The polymerization of other ZP domain-containing proteins such as ZP3 and Uromodulin (UMOD) requires surface expression and subsequent removal of the EHP domain [48, 57, 58]. Thus, the release process of TECTA may play a role in the multimerization of TECTA and the organization of other TM components.

Indeed, western blot analysis using the P0 mouse cochlea shows that TECTA appears as multiple bands with high molecular weight (*Figure 4B*), indicating that TECTA forms multimers (either homomeric or heteromeric) in the TM.

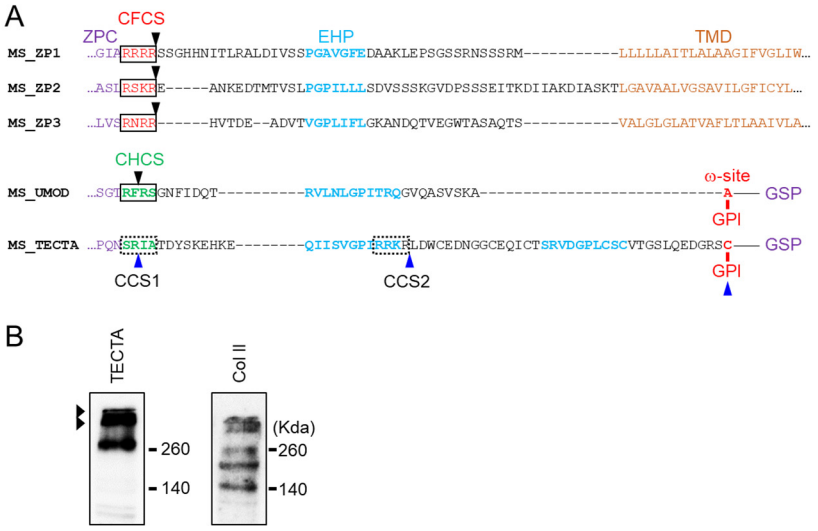


Figure 4. Sequence alignments of the C-terminus of the ZP-domain and following regulatory domains that regulate the multimerizatin of the ZP domains.

(A) ZP1, ZP2, and ZP3 are transmembrane proteins, while TECTA and uromodulin (UMOD) are GPI-anchored proteins. ZP1-3 are cleaved by furin at the consensus furin cleavage site (CFCS) [59] and UMOD is cleaved by a serine protease, hepsin/TMPRSS1 at the consensus hepsin cleavage site (CHCS) [48]. Black arrowheads indicate the cleavage site. The external hydrophobic patch (EHP), which prevent the multimerization of the ZP domain is located downstream of the cleavage site. Surface expression and subsequent removal of the EHP domain by an endopeptidase facilitates the multimerization of the released proteins. Sequence alignment indicates that TECTA may have three cleavage sites (blue arrowheads) recognized by serine proteases-consensus cleavage site 1 (CCS1) [48], furin-CCS2 [49], and GPI-anchor lipases [52, 53] flanking two EHP domains (cyan). The ω-site indicates an amino acid where a GPI-anchor is attached in the mature form [23]. Boxes and dashed boxes indicate the known and predicted consensus cleavage sequence, respectively. EHP, external hydrophobic patch; GSP, glycosylphosphatidylinositol (GPI)-anchorage signal peptide; Ms, mouse form; TMD, transmembrane domain; ZPC, C-terminal ZP domain. (B) Western blots of the P0 wildtype mouse cochlea. TECTA is detected as multiple bands with a high molecular weight (arrows). Collagen type II (Col II) is expressed at this developmental stage. The numbers indicate the size marker (kDa).

There are three potential release sites within the C-terminus of mature TECTA that are processed by distinct release mechanisms (*Figure 4A*).

(1) CCS1 (upstream of both EHP domains). UMOD, another GPI-anchored ZP domain-containing protein is cleaved by hepsin/TMPRSS1 (a type II transmembrane serine protease) at a consensus hepsin cleavage site (CHCS), Rφ ↓ RS (where φ is hydrophobic amino acids, F, Y, W, H) (*Figure 4A*) [58, 60]. The sequence alignment of TECTA and UMOD suggested that TECTA may also be cleaved by serine proteases at the corresponding site (CCS1), (RI ↓ AT in human TECTA and SR ↓ IA in mouse TECTA) [48]. Indeed, mice lacking hepsin showed a malformed TM structure and profound hearing loss [61]. Cleavage of TECTA at CCS1 would generate and release a truncated form of TECTA that lacks both EHP domains.

(2) CCS2 (between the two EHP domains). Transmembrane-anchored ZP domain-containing proteins (type I transmembrane proteins), such as ZP1, ZP2, and ZP3 share a consensus furin cleavage site (CFCS, RX(R/K)R ↓) [57, 59]. TECTA contains the conserved tetrabasic amino acids (RRKR ↓) between two potential EHP domains [49]. Cleavage of TECTA at CCS2 would generate and release a truncated form of TECT that contains one EHP domain.

(3) A GPI-anchor, a GPI-anchor lipase. GPI-anchored proteins can be released by GPI-anchor cleaving enzymes such as GPI-PLD and glycerophosphodiesterase (GDEs) family (*Figure 4A*) [52, 53, 62, 63]. We observed that expression of GDE3 and GDE6, but not GDE2, facilitate the release of TECTA from HEK293T cells into the medium (unpublished data), suggesting that the GPI-anchorage of TECTA is a substrate of a GPI-anchor cleaving enzyme. An enzymatically dead mutant, GDE3 H230A, fails to release TECTA, indicating that the catalytic activity is required for the release of TECTA. Cleavage of TECTA within the GPI-anchor would generate and release full-length TECTA that contains both EHP domains.

Depending on the release mechanisms (CCS1 cleavage, which removes both EHP domains from N-terminal fragment; CCS2 cleavage, which removes only the 2nd EHP; and GPI-anchor cleavage, which releases a full length protein), different forms of TECTA can be released, which may play roles in organizing the specific shape of the matrix architecture. The released TECTA lacking one or two EHPs may interact with itself (homo-multimer) or with the ZP domain of TECTB (hetero-multimer, TECTA-TECTB). Further study is required to identify the releasing enzymes and their target sequences within TECTA and to characterize the *in vivo* role of each release mode of TECTA for matrix organization.

Detachment and Maturation of the TM

Although the developing TM grows on the cell surface of auditory supporting cells, its body domain detaches from the producing cells, except at the spiral limbus and stereocilia of the OHCs. In mice, this event occurs between P4~P8, starting from the base of the cochlea to the apex [8]. Along the radial axis, the detachment starts from the medial columnar cells and proceeds laterally forming the inner sulcus. How is this large acellular ECM separated from the producing cells without structural damages? During cochlear development, the majority of columnar cells in the GER undergo programmed cell death or the transition into the cuboidal shape [64, 65]. It might be possible that the TM's detachment is the consequence of columnar to cuboidal transition of the Kölliker's organ (a GER replacement model). However, a recent timecourse study showed that apoptosis of the columnar cells occurs later than the TM's detachment [31]. Activated caspase 3-positive cells begin to appear at P7 when the TM's detachment is already observed in those cells. Besides, this model does not explain the TM's detachment from the surface of Deiter`s cells and Hensen`s cells originated from the LER, which maintain their shape before and after the TM's detachment. Interestingly, the expression of *Tecta* mRNA is reduced after P0 and is not detected at P14 (*Figure 5A*) [19], which is correlated with a period of TM detachment. Thus, the ratio of the incorporation of newly-synthesized TECTA into the surface membrane to the release of TECTA from the surface may regulate the timing of TM detachment. Whether the release rate of TECTA remains constant or is regulated at the detachment site along the radial axis and/or during the maturation gradient of the cochlear turn is to be determined (*Figure 5B*). Consequently, the depletion of the surface-localized TECTA may allow for the detachment of the TM from the cell surface before the onset of hearing.

Although the detached TM maintains its overall architecture throughout the lifespan, the ultrastructure of the TM may further mature after its detachment into the extracellular space. For example, the striated sheet in the central body and Hensen`s stripe become apparent between P8~P14 (after the detachment of the TM) [33]. This process requires CEACAM16, which maintains its expression after the completion of TM detachment. In addition, TECTB expresses until P15, and the TM in *Tectb* KO mice does not properly form the marginal band and Hensens` stripe [35]. Likewise, OTOGL continues to be expressed after the TM's detachment (till P28). This protein may also be involved in the TM's maturation. Collectively, these observations suggest that the cell-ECM border determines the pattern of the matrix architecture, which is further matured by the incorporation of secreted components into the TM.

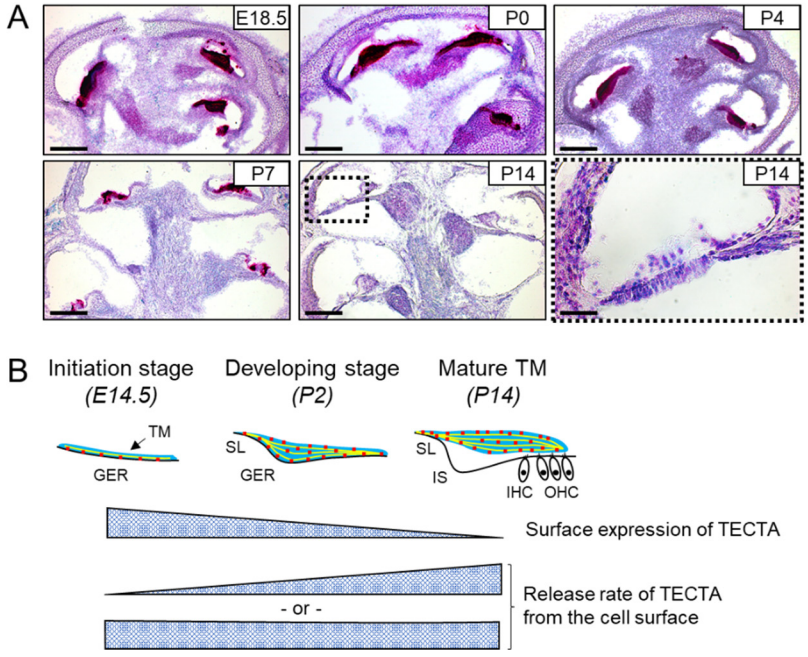


Figure 5. TECTA expression and TM morphogenesis

(A) *in situ* hybridization of *Tecta* mRNA during cochlear development. The expression of *Tecta* mRNA (red signal) ceases between P7~P14 when the TM is detached [23]. Scale bars, 200 μm; inset 50 μm. (B) Schematic of TM morphogenesis. In mice, the primordial TM (cyan) forms on the apical surface of the supporting cells in the greater epithelial ridge (GER) at E14.5 when TECTA (red dots) starts to be expressed. TECTA on the cell surface formed a thin layer (yellow line) consists of collagenous and non-collagenous matrices. The TM grows on the cell surface, as seen in the increased number of layers in the TM. The mature TM detaches from the cell surface except at the spiral limbus (SL) and the stereocilia of the outer hair cells (OHC). Inner sulcus (IS) is formed by the transition of the columnar cells to the cuboidal cells. The detachment of the TM from the cell surface may be tightly regulated by a balance between the incorporation of newly-synthesized TECTA to the cell surface and its release from the cell surface. Hypothetically, the reduced expression level of TECTA may lead to the TM detachment which occurs between P4-P8 (Table 2).

Conclusions and Prospects

Since the late 1980s, molecular compositions of the TM have been extensively explored. Several inner ear-specific genes that encode the TM glycoproteins were cloned and subjected to the loss or gain of function studies to uncover the function of those molecules. Since 1992 when TECTA was identified in the avian inner ear, the role of TECTA in TM organization has been extensively studied. TECTA is necessary for TM matrix organization and functions as a molecular glue for the assembly of the TM components. A recent study shows that the dynamic process of surface-localization and the release of TECTA plays a critical role in TM matrix organization. Future studies are needed for determining the release mechanisms of TECTA, its interacting partners, and the roles of surface-tethered vs. released TECTA in the organization of the characteristic ultrastructure in a time-dependent and location-specific manner. The present chapter provides new insights into the morphogenesis mechanism of complex ECM regulated by a versatile cell-surface organizer.

Materials and Methods

Animals

All experiments and mouse (C57Bl/6J) care were carried out according to the NIH guidelines and approved by IACUC (18-02004). Mice were maintained in a standard a 12:12 h light/dark cycles and freely accessible to food and water. Only wild type mice were used in this study.

Cochlea Sample Preparations

All mice used in the present study were sacrificed after deep anesthesia with either ice or isoflurane. For immunohistochemistry, mice at P2 were used for *Pisum sativum* agglutinin (PSA)-lectin staining. Mice at E18.5, P0, P4, P7, and P14 were used for *in situ* hybridization (RNAscope). Small holes were made at the apex and two windows (oval and round) of the cochlea, through which 20 µl of a fixative (4% paraformaldehyde in PBS) was injected. The cochlea was further fixed with the fixative at RT for 1 h. After washing three times with PBS, the cochlea was incubated with the cryoprotectant solution (30% sucrose in PBS (w/v)) for 1 h. To reduce the viscosity of OCT (Ted Pella, 27050) for better penetration into the cochlear duct, the OCT solution was mixed with 30% sucrose

solution and applied to the cochlea samples for 1 h. The cochlea block was made in the embedding mold on a dry-ice and sectioned radially with a cryostat with a 14 μm-thickness. Sections were collected on a glass slide (Fisher Scientific, 12-544-2) and kept at -20 ℃ till use. For western blot, eight cochleae from four P0 pups were pooled and homogenized in 400 μl RIPA buffer (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton™ X-100, protease inhibitors in PBS). Samples were sonicated (50% duty cycle, 2 output, 6 strokes) and the homogenates were subjected to centrifugation with 13,000 rpm. The supernatant was collected for protein quantification. Samples were diluted, reduced with a gel loading buffer, and kept at -20 ℃ till use.

SDS-PAGE and Western Blot

Samples were loaded into the well of SDS-PAGE gels (12 μg/well, 7% running gel. Proteins were separated in electrophoresis buffer (2.5 mM Tris-base, 19.2 mM Glycine and 0.1% SDS) with 120 V for 2-3 h and transferred to the PVDF membrane in transfer buffer (2.5 mM Tris-base, 19.2 mM Glycine and 20% MeOH) with 100 V for 1-1.5 h. After blocking with TBST buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% skim milk, membranes were incubated at 4 ℃ overnight with primary antibodies, including an anti-TECTA antibody (a gift from Dr. Guy P. Richardson, 1:1,000, rabbit) or an anti-collagen type II (ColII) antibody (Abcam, ab34712, 1:5,000, rabbit). Membranes were washed three times with TBST and incubated with peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Jackson Immuno Research, 711-035-152, 1:5,000) at RT for 1 h. After washing three times with TBST, membranes were treated with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, PI34580) or SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, PI34095) for 5 min and imaged under a ChemiDoc™ XRS+ system (Bio-Rad).

Immunohistochemistry

Cochlea sections were rinsed with PBS three times and blocked with heat-inactivated normal goat serum (10%) in PBST (PBS containing 0.2% Triton™ X-100) for 1 h. Sections were incubated with FITC-PSA (Sigma-Aldrich, L0770, 20 μg/ml) with Hoechst 33342 (1:20,000) at RT for 1 h. Hoechst was used to identify the greater epithelial ridge (GER). After washing three times with PBS, sections were coverslipped with cover glass after treatment with mounting media, fluromount-G® (ThermoFisher scientific, 00-4958-02). Images were acquired by a Zeiss 880 Airyscan confocal microscope with a Zen Black software at the

Fluorescence Microscopy Core Facility of the University of Utah and processed with a FIJI software (https://fiji.sc/).

In Situ Hybridization

Tecta mRNA sequences were retrieved from the National Center for Biotechnology Information (NCBI, NM_001324548.1). The complementary RNA was designed for targeting a 900 bp *Tecta* mRNA that covers the exon 9 to 11 (ACDBio, 494191). The spatial expression of mRNA was developed by RNAscope® 2.5 HD Duplex Detection Reagents (ACDBio, 322500). Briefly, sections on the glass slide were oxidized by hydrogen peroxide and epitopes were exposed by the target retrieval procedure. After dehydrating with ethyl alcohol, sections were treated with proteases for 5 min. Samples were then briefly rinsed with sterilized water, and subjected to the probe hybridization step at 40 ℃ for 2 h. The localization of *Tecta* mRNA in the cochlea was detected by chromogenic development, according to the manufacturer`s instruction. After counterstained with 50% hematoxylin, samples were covered with the cover glass after applying fluromount-G®. Images were obtained using a Leica DM2500 optical microscope with Leica Las software V3.8.

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