

RESEARCH ARTICLE

Visualizing Physiology-Using Novel Microscopy Methods to Investigate the Mechanisms Underlying Physiology and Pathophysiology

Immunohistochemical and ultrastructural characterization of the inner ear epithelial cells of splitnose rockfish (*Sebastes diploproa*)

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Abstract

The inner ear of teleost fish regulates the ionic and acid-base chemistry and secretes protein matrix into the endolymph to facilitate otolith biomineralization, which is used to maintain vestibular and auditory functions. The otolith is biomineralized in a concentric ring pattern corresponding to seasonal growth, and this calcium carbonate (CaCO_3) polycrystal has become a vital aging and life-history tool for fishery managers, ecologists, and conservation biologists. Moreover, biomineralization patterns are sensitive to environmental variability including climate change, thereby threatening the accuracy and relevance of otolith-reliant toolkits. However, the cellular biology of the inner ear is poorly characterized, which is a hurdle for a mechanistic understanding of the underlying processes. This study provides a systematic characterization of the cell types in the inner ear of splitnose rockfish (*Sebastes diploproa*). Scanning electron microscopy revealed the apical morphologies of six inner ear cell types. In addition, immunostaining and confocal microscopy characterized the expression and subcellular localization of the proteins Na^+/K^+ -ATPase, carbonic anhydrase, V-type H^+ -ATPase, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -cotransporter, otolith matrix protein 1, and otolin-1 in six inner ear cell types bordering the endolymph. This fundamental cytological characterization of the rockfish inner ear epithelium illustrates the intricate physiological processes involved in otolith biomineralization and highlights how greater mechanistic understanding is necessary to predict their multistressor responses to future climate change.

apical morphology; biomineralization; endolymph; otolith

INTRODUCTION

The inner ear of teleost fishes regulates the ionic and acid-base chemistry of the endolymph and secretes the proteins necessary for otolith biomineralization (1). Each inner ear contains three calcium carbonate (CaCO_3) otoliths called the sagitta, lapillus, and asteriscus, and each of these polycrystals serves as an inertial mass on adjacent sensory neurons to convey neuronal information to the brain to maintain proper vestibular and auditory functions. Otoliths grow continuously throughout the fish's life, and so must the inner ear expand to accommodate the larger polycrystals while maintaining proper homeostatic and sensory function. The inner ear's capacity to regulate endolymph chemistry is consistently robust, and the resulting otolith biomineralization patterns have been used to derive a myriad of chronological information for over half a century (1–3).

Otoliths are biomineralized in a concentric ring pattern that follows seasonal growth, thereby forming annual rings called “annuli” that serve as a proxy for fish age (4, 5). Fishery

managers incorporate this information into age-structured models to estimate growth rate, maturity age, recruitment, mortality, and overall population biomass (6, 7). These otolith-reliant age-structured models are used to make informed decisions on catch limits to sustainably regulate fish stocks around the world, thereby preserving both aquatic ecosystems and economies. In addition, otolith microchemistry has become an important tool to derive life history information. The analysis of trace elements and isotopes trapped within the otolith has been used to retroactively determine a variety of metabolic and environmental factors experienced by the fish such as metabolic rate (8, 9), temperature (10), diet (11), hypoxia (12), and salinity (13). As such, otolith-derived life-history information is particularly useful for agencies that manage endangered and migratory species as they explore methods to determine recruitment and dispersal (14), stock origin, migration pattern, and pollution exposure (15–17).

Of the three CaCO_3 otoliths, the saccule endorgan is the most commonly investigated due to its large size in most

teleost species. Past studies on the inner ear have generally described the endorgan in four regions: the macula, meshwork area, intermediate area, and patches area (18, 19), with the otolith resting on a gelatinous otolithic membrane that covers much of the macula (20–22) (Fig. 1). The otolithic membrane is a thin gelatinous membrane separating the macula from the otolith (Fig. 1), and each socket usually contains the hair bundles from a single hair cell (HC) (20, 21). As such, the otolithic membrane protects HCs from being crushed by the overlying otolith as well as in distributing the inertial movement across the HCs (20). The center of the macula is composed of HCs and supporting cells (SCs), and the periphery of the macula contains granular cells (GCs) (Fig. 1). The HCs are known to have basolateral $\text{Na}^+\text{-K}^+\text{-ATPase}$ (NKA) and cytoplasmic V-type $\text{H}^+\text{-ATPase}$ (VHA) in its basolateral soma (23, 24), tubulin-rich hair bundle (25), and rely on K^+ inflow as the driving force for its electrochemical potential similar to mammalian HCs (26, 27). The surrounding SCs are thought to aid in secreting the matrix proteins, and anchoring the filamentous material with cell boundary microvilli as proposed for inner ear systems of aves and amphibians (20, 28, 29). The GC has an extensive endoplasmic reticulum and numerous secretion granules (18) to synthesize and secrete proteins such as otolin-1 to chelate with Ca^{2+} ions to promote biomineralization (30–35). The meshwork area is known to have Type-I ionocytes (T1-Is) and Type-II ionocytes (T2-Is) (Fig. 1). The T1-Is are known to be mitochondrion-, NKA-, and $\text{Na}^+\text{-K}^+\text{-2Cl-cotransporter}$

(NKCC)-rich (24, 36–38), have short thick microvilli (18), and thought to be responsible for maintaining the ionic and acid-base parameters of the endolymph (19, 24, 37, 39, 40). T1-Is are interconnected and together form intracytoplasmic membrane systems, and their abundance decreases with distance away from the macula (18, 38). In contrast, the T2-Is are known to contain carbonic anhydrase (CA) (41, 42) and VHA (24, 37), and they become the more common with distance away from the macula. In fact, the intermediate area is dominated by T2-Is (Fig. 1). Finally, the patches area predominantly contains SQs (Fig. 1). The SQs have been called “small ionocytes,” and they are generally very “flattened” with highly infolded lateral plasma membranes and elongated mitochondria (18, 38). SQs also strongly express CA (41, 42) and otolith matrix protein-1 (OMP-1) (43), and their proposed role(s) include the secretion of ions and bicarbonate (41, 42) along with OMP-1 to facilitate biomineralization on the distal side of the otolith (19, 40, 44). Notably, the overwhelming majority of our current inner ear cell characterizations are derived from freshwater species such as the zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*); this greatly contrasts with the application of otolith-based tools, which are heavily used to regulate marine fisheries.

Recent research has suggested that near-future climate change scenarios could significantly alter otolith biomineralization patterns thereby threatening the many otolith-reliant analytical techniques that researchers have relied on for the past half century. In general, exposure to elevated CO_2 (45, 46) and warming (47, 48) accelerates otolith growth, whereas hypoxia hinders biomineralization (49–51) and can even generate false annual rings known as “checks” (49, 50). Indeed, inaccurate otolith-based aging and data misinterpretation *can* and *has* led to unsustainable fisheries, for example, those of the orange roughy (*Hoplostethus atlanticus*) in New Zealand (52) and the walleye pollock (*Gadus chalcogrammus*) in the Bering Sea (53). Despite the need to understand their cell-specific differences in morphology, protein localization and abundance, the characterization of inner ear cells, and their contribution to otolith biomineralization have received scant attention (1) and are recognized as a critical knowledge gap by the international sclerochronology community (3).

Since otoliths are formed inside the inner ear endolymph and are not in direct contact with the water, the altered biomineralization pattern and the accumulation of trace elements and isotopes within the polycrystal necessitate a degree of biological regulation by the inner ear epithelial cells. Indeed, the endolymph has higher average pH, $[\text{HCO}_3^-]$, $[\text{CO}_3^{2-}]$, total CO_2 , and $[\text{K}^+]$ than the blood plasma (24, 39, 40, 54, 55). Moreover, the endolymph in the proximal side of the saccule endorgan (facing the macula) has much lower $[\text{K}^+]$ and pH than the distal side (facing the patches area) (19, 40), a chemical heterogeneity that is most likely generated by the activity of the inner ear epithelial cells in each region. Further complicating matters is that the inner ear is known to contain at least six types of cells (18, 19) (Fig. 1), each with their own specific morphology and protein localization/abundance to fulfill their function(s).

Previous studies have examined the ultrastructure of the inner ear epithelial cells using transmission electron

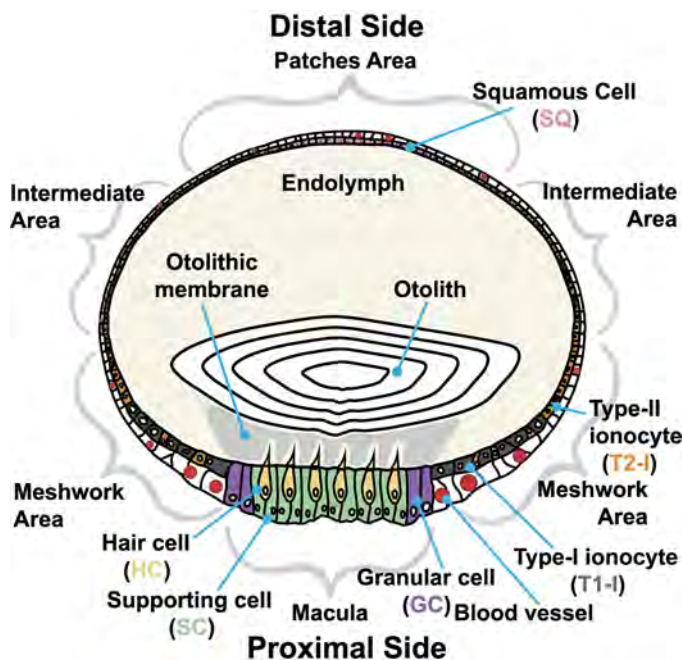


Figure 1. Schematic of an inner ear endorgan. The endorgan is generally categorized into four general areas: the macula, meshwork area, intermediate area, and patches area. The macula contains the hair cell (HC), supporting cell (SC), and granular cell (GC). The meshwork area contains the Type-I ionocyte (T1-I) and Type-II ionocyte (T2-I). The intermediate area only contains T2-I, and the patches area contains only squamous cell (SQ). The macula and meshwork area are considered to be on the proximal side of the inner ear, and the intermediate and distal areas are considered to be on the distal side of the inner ear. The endorgan is interwoven with blood vessels, most of which are on the proximal side of the inner ear.

microscopy (TEM), the expression of individual proteins in specific cell types using immunohistochemistry and fluorescence microscopy on tissue sections, and scanning electron microscopy (SEM), (18, 20, 21, 36–38, 41, 43, 56). Here, we combined multiplex immunohistochemistry and SEM on whole mounted inner ear sacculus to provide a comprehensive immunological and ultrastructural characterization of the six cell types lining the endolymph. This approach allowed us to generate unprecedented three-dimensional reconstructions of the expression patterns of NKA, CA, VHA, NKCC, OMP-1, and Otolin-1 in the six inner ear epithelial cell types that border the endolymph, together with matching visualizations of their apical morphology. Altogether, our results highlight the high cytological complexity of the fish's inner ear epithelium and provide critical foundational knowledge to help decode the mechanisms responsible for otolith biomineralization and their regulation.

METHODS

Tissue Sampling

Young-of-year splitnose rockfish (*Sebastes diploproa*) were caught from drifting kelp paddies off the shores of La Jolla and raised in the Hubbs Experimental Aquarium at the Scripps Institution of Oceanography, University of California

San Diego (La Jolla, CA). The animals were raised in a flow-through system with seawater continuously pumped from the Scripps Coastal Reserve for >2 yr and were fed EWOS food pellets (Cargill, Inc., Minneapolis, MN). In accordance with protocol S10320 of the University of California, San Diego Institutional of Animal Care and Use Committee, fish were euthanized with an overdose of anesthetic (tricaine mesylate 0.2 g/L buffered with NaOH) followed by spinal pithing before inner ear tissue sampling. In total, 24 splitnose rockfish (total length: 11.88 ± 0.29 cm; weight: 42.86 ± 2.79 g) were analyzed.

Antibodies

NKA was immunodetected using the mouse monoclonal anti-NKA antibody $\alpha 5$ (57) purchased from the Developmental Studies Hybridoma Bank (DSHB, The University of Iowa, Iowa City, IA). VHA was immunodetected using custom-made rabbit polyclonal antibodies against a highly conserved epitope within subunit B (epitope: AREEVPGRRGFPGY; GenScript, Piscataway, NJ). Both NKA and VHA antibodies have been validated in the inner ear of splitnose rockfish (24) and Pacific chub mackerel (*Scomber japonicus*) (37). CA was immunodetected using rabbit polyclonal antibodies against human CA II (Rockland, Inc., Gilbertsville, PA), and this antibody has been validated in the inner ear of Masu Salmon (*Oncorhynchus masou*) (42) and Pacific chub mackerel (37). NKCC was immunodetected using the anti-NKCC antibody T4 (58) obtained

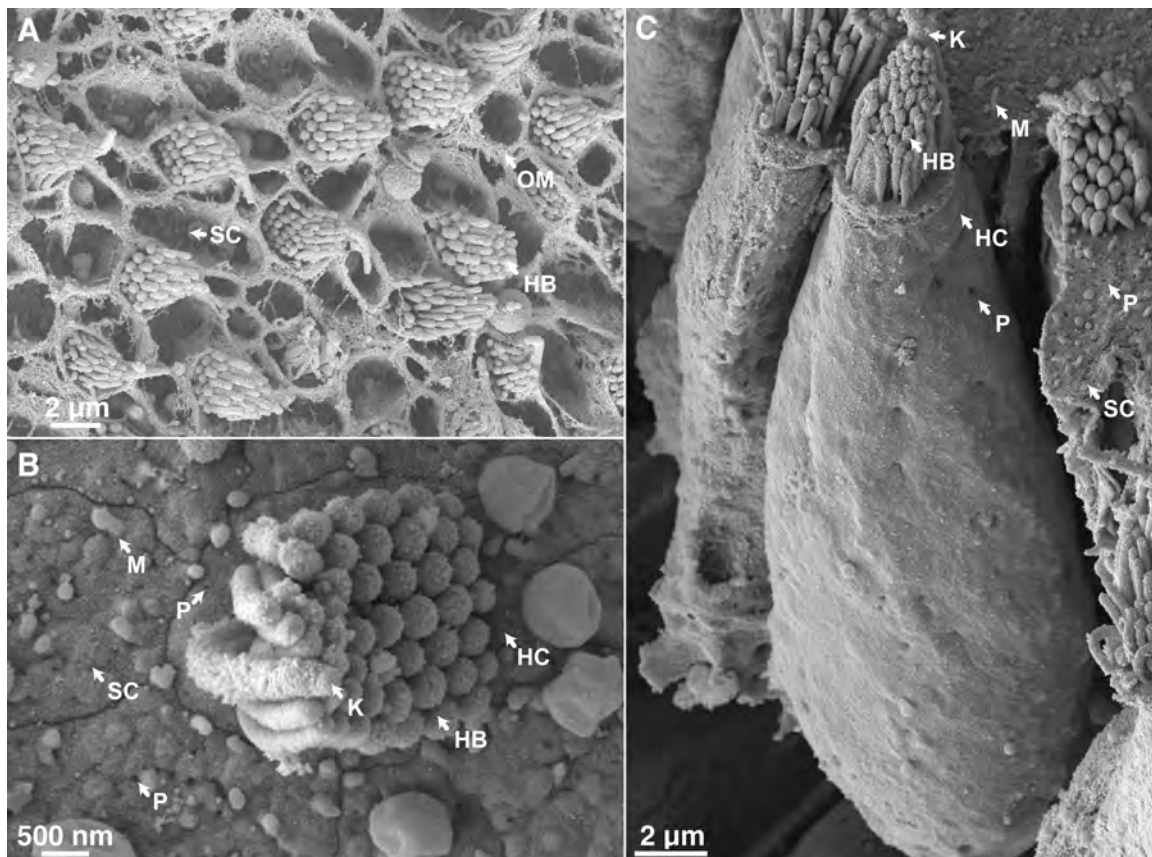


Figure 2. Scanning electron micrographs of inner ear hair cell (HC) and supporting cell (SC). **A:** the hair bundles (HB) of the HC protrudes through the otolith matrix (OM). **B:** when unobstructed, the pores (P) and microvilli (M) on the SC are visible. **C:** when viewed from the side, pores are also apparent on the HC's basolateral membrane. K, kinocilium.

from DSHB, and this antibody has been validated in the inner ear of zebrafish (59) and Pacific chub mackerel (37). OMP-1 and Otolin-1 were immunodetected using antibodies generously provided by Dr. Emi Murayama (Institut Pasteur, Paris, France), which were previously validated in rainbow trout inner ear (43). The secondary antibodies were goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP conjugate (Bio-Rad, Hercules, CA) for Western blotting, and goat anti-mouse Alexa Fluor 546, goat anti-rabbit Alexa Fluor 488, and/or goat anti-rabbit Alexa Fluor 555 (Invitrogen, Grand Island, NY) for immunohistochemistry.

The specificity of NKA, VHA, CA, NKCC, OMP-1, and Otolin-1 antibodies within splitnose rockfish inner ear tissues was verified using Western blotting. NKA, VHA, CA, and NKCC yielded bands matching their respective predicted size at ~100, ~55, ~40, ~210, ~80, and ~100 kDa (Supplemental Fig. S1).

Western Blotting

Frozen inner ear samples were immersed in liquid nitrogen, pulverized using a porcelain mortar and pestle, and submerged within an ice-cold, protease inhibiting buffer (250 mmol/L sucrose, 1 mmol/L EDTA, 30 mmol/L Tris, 10 mmol/L benzamidine hydrochloride hydrate, 1 mmol/L phenylmethanesulfonyl fluoride, 1 mmol/L dithiothreitol, pH 7.5). Samples were centrifuged at 3,000 g (10 min, 4°C) to remove debris, and the resulting supernatant was saved as the sample. Total protein concentration in the crude

homogenate was determined by the Bradford assay (60). On the day of SDS-electrophoresis, the samples were mixed with an equal volume of 2× Laemmli buffer containing 10% β-mercaptoethanol, heated (70°C for 5 min), and loaded (10 μg/lane) onto a 7.5% polyacrylamide mini gel (Bio-Rad, Hercules, CA). Proteins in the samples were separated by polyacrylamide gel electrophoresis at 200 V for 40 min and then transferred onto a polyvinylidene difluoride (PVDF) membrane using a wet transfer cell (Bio-Rad) at 100 mAmps (4°C overnight). On the following day, PVDF membranes were incubated in Tris-buffered saline with 1% tween (TBS-T) with milk powder (0.1 g/mL) for 1 h and then incubated with primary antibody (a5: 10.5 ng/mL; H300: 100 ng/mL; VHA *b*-subunit: 1.5 μg/mL; CA II antibody: 8 μg/mL; T4: 10.4 ng/mL; OMP-1: 1:1,000 dilution; Otolin-1: 1:1,000 dilution) at 4°C overnight. On the following day, PVDF membranes were washed in TBS-T (3 × 10 min), incubated in blocking buffer with the appropriate secondary antibodies (1:10,000 in blocking buffer) for 1 h, and washed again in TBS-T (3 × 10 min). Bands were made visible through the addition of ECL Prime Western Blotting Detection Reagent (GE Healthcare, Waukesha, WI) and imaged and quantified in the Universal III Hood (Bio-Rad) using ImageLab software (v. 6.0.1; Bio-Rad).

Immunohistochemistry

Inner ears were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 8 h, incubated in 50%

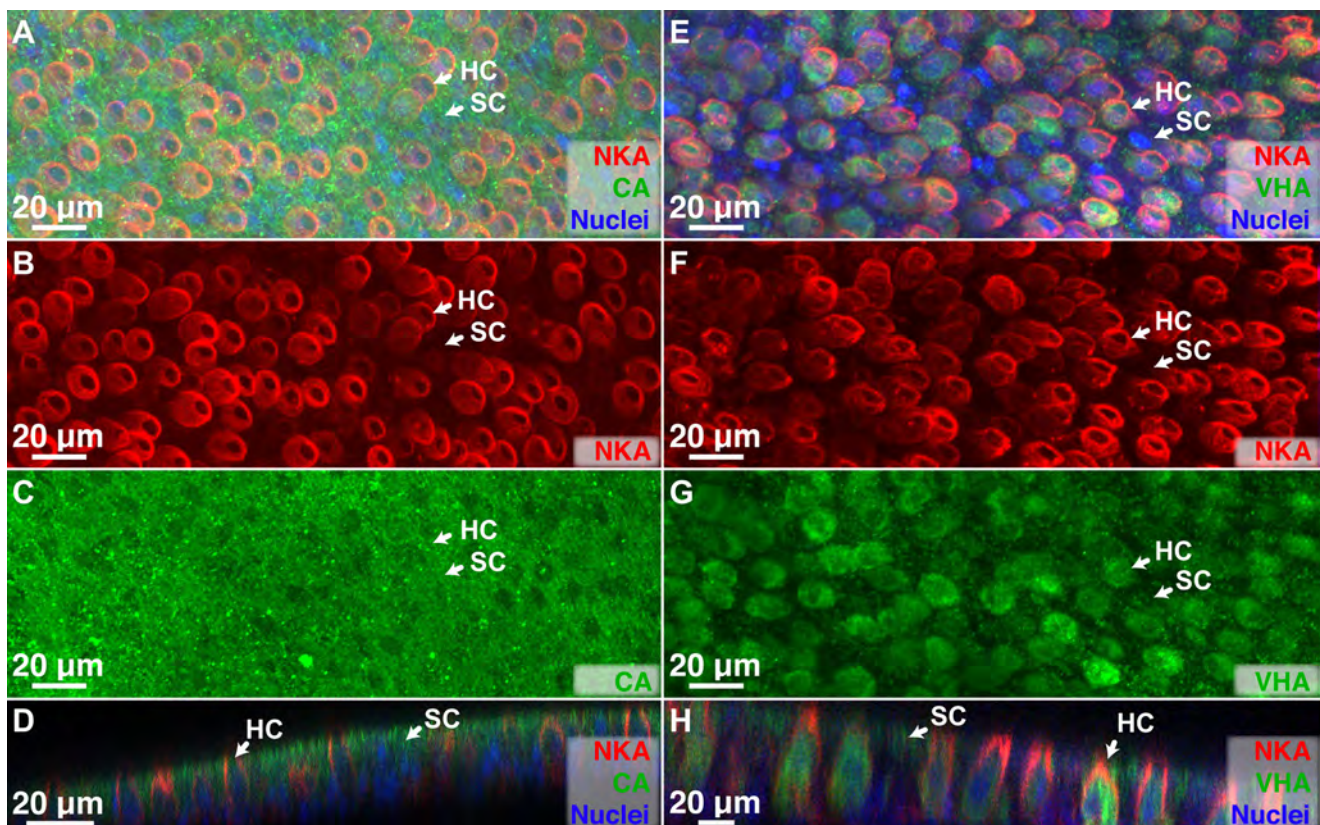


Figure 3. Immunolocalization of NKA, VHA, and CA in hair cell (HC) and supporting cell (SC). HC expresses NKA in the basolateral membrane (A and B), CA in their hair bundles (see Supplemental Fig. S2) (C and D), and VHA in their cytoplasm (E–H). A–D: adjacent SC does not express NKA but does express apical and subapical CA and VHA. CA, carbonic anhydrase; NKA, Na^+ - K^+ -ATPase; VHA, vacuolar H^+ -ATPase.

ethanol for 8 h, and stored in 70% ethanol. On the day of analysis, samples were rehydrated in PBS for 10 min. Next, the saccule was gently dissected from the rest of the inner ear under a dissecting microscope. Native autofluorescence was reduced using sodium borohydride (1 mg/mL) in ice-cold PBS (6 times; 10 min each) as demonstrated in an earlier study by Kwan et al. (61). Samples were then washed in PBS + 0.1% Tween (PBS-T) at RT for 5 min, incubated in blocking buffer (PBS-T, 0.02% normal goat serum, 0.0002% key-hole limpet hemocyanin) at RT for 1 h, and with the primary antibodies (a5: 42 ng/mL; VHA subunit B: 6 µg/mL; CA II antibody: 160 µg/mL; T4: 104 ng/mL; OMP-1: 1:500; Otolin-1: 1:500) in blocking buffer and kept in a humid chamber at RT overnight. On the following day, samples were washed in PBS-T (3 times; 10 min each) and incubated with the appropriate anti-rabbit or anti-mouse fluorescent secondary antibodies (1:1,000) and nuclear stain DAPI (1 µg/mL) at RT for 1 h. Samples were washed in PBS-T (three times; 10 min each), then whole mounted and flattened onto depressed glass slide fitted with a glass coverslip (No. 1.5, 0.17 mm). Samples

were Z-stacked imaged (Zeiss ZEN 2.6 blue edition software) using an inverted confocal microscope (Zeiss AxioObserver Z1 with LSM800; Oberkochen, Germany) at low ($\times 20$ objective: Plan-Apochromat $\times 20/0.8$ M27), high magnifications ($\times 40$ objective: Zeiss LD LCI Plan-Apochromat $\times 40/1.2$ ImmKorr DICM27 objective), or with Zeiss Airyscan super-resolution detector. Comparison of relative fluorescent signal across the different cell types was assessed in images visualized as maximum intensity projection or orthogonal cuts across the X-Z and Y-Z planes. Each of the six antibodies was immunotargeted in at least three rockfish.

Scanning Electron Microscopy

Inner ears were dissected and immediately immersed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer at RT for 2 h. Samples were washed in the same buffer at RT (three times; 15 min each), postfixed in 1% osmium tetroxide (Electron Microscopy Science, Hatfield, PA) at RT for 1 h in dark, washed with ultrapure water (three

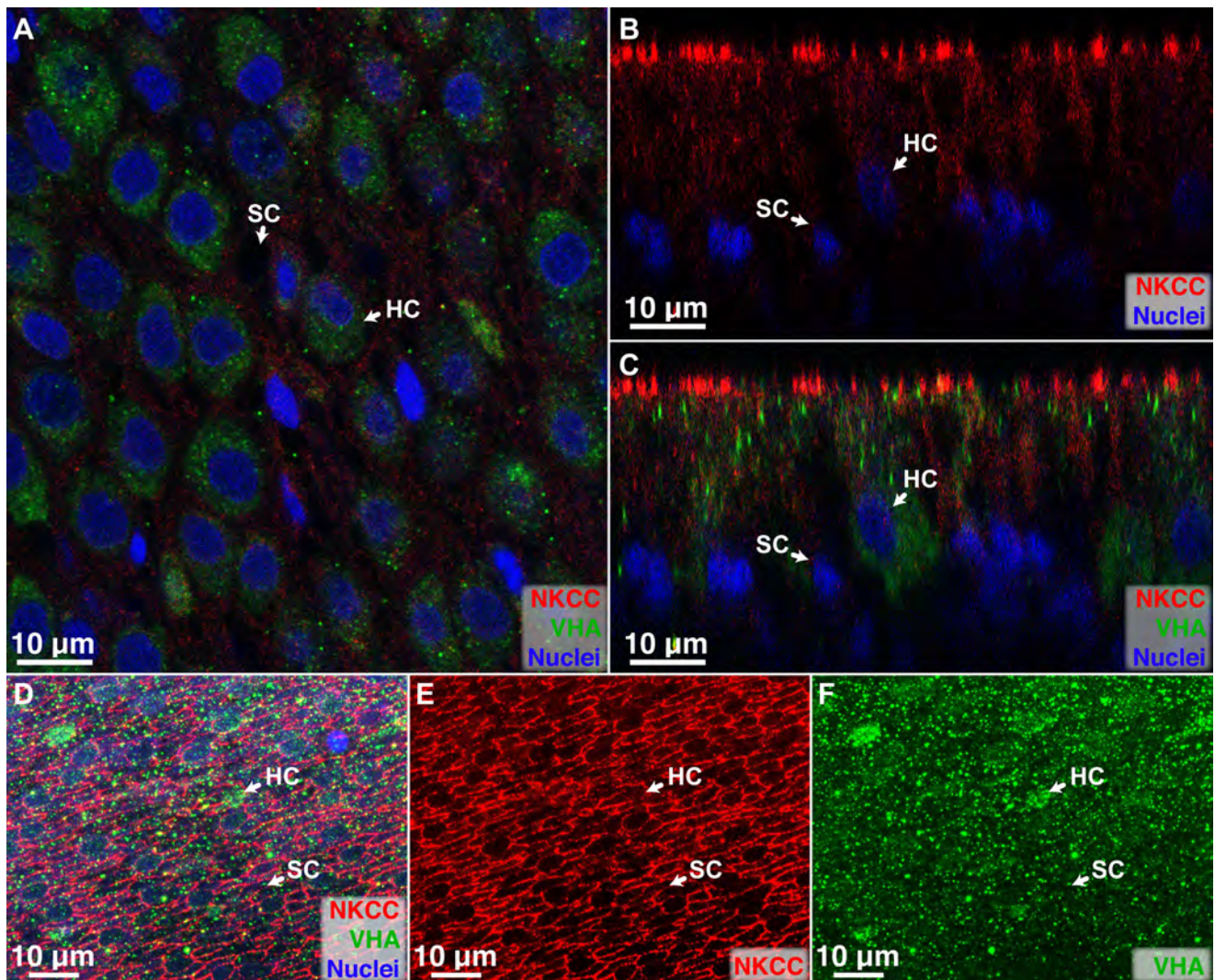


Figure 4. Immunolocalization of NKCC and VHA in hair cell (HC) and supporting cell (SC). A: The VHA-rich HC is surrounded by SC, which expresses basolateral NKCC (B and C) as well as apical NKCC in a honeycomb pattern that resembles the overlying protein matrix (D–F). NKCC, $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ -cotransporter; VHA, vacuolar H^+ -ATPase.

times; 15 min each), incubated with 1% tannic acid (Electron Microscopy Science) at RT for 1 h, washed again with ultrapure water (three times; 15 min each), and incubated a second time in 1% osmium tetroxide at RT for 1 h. After yet another round of ultrapure water washes (three times; 15 min each), samples were dehydrated in an ethanol series until absolute (three times), critical-point dried (Bal-Tec CPD-030), sputter-coated with 10 nm of gold-palladium (Leica EM SCD 500), and observed in Zeiss Sigma VP operated at 5 kV. Each cell type was examined in at least three rockfish.

Microvilli Measurements and Statistical Analysis

Microvilli length and width ($n = 20$ microvilli per cell type examined in three rockfish) on the apical surface of T1-Is and T2-Is were measured with FIJI (v. 2.1.0) and then analyzed with Student's t test using R (v. 4.0.3) (62). The assumption of normality was checked using Shapiro-Wilks test, and an α of 0.05 was applied.

RESULTS

Macula: Hair Cells and Supporting Cells

SEM imaging shows that the hair bundle of each HC protrudes through a single opening in the gelatinous otolithic membrane (Fig. 2A). Each HC hair bundle is composed of stereocilia arranged in a staircase pattern and one longer kinocilium at the top (Fig. 2, B and C). A circumstantial tear in the tissue revealed the soma of the HC, which has an ovoid shape (Fig. 2C). An unobstructed, higher magnification view shows the presence of numerous pores and budding vesicles (Fig. 2, B and C), especially in the SCs (Fig. 2B). Finally, microvilli are found on the SCs but not on the HCs (Fig. 2B).

Three-dimensional reconstruction images of whole mounted and immunostained inner ear tissue provide further insights into the inner ear epithelium structure and protein expression. The nucleus is located at the basal portion of the HC. The HC's basolateral membrane expresses abundant NKA that outlines

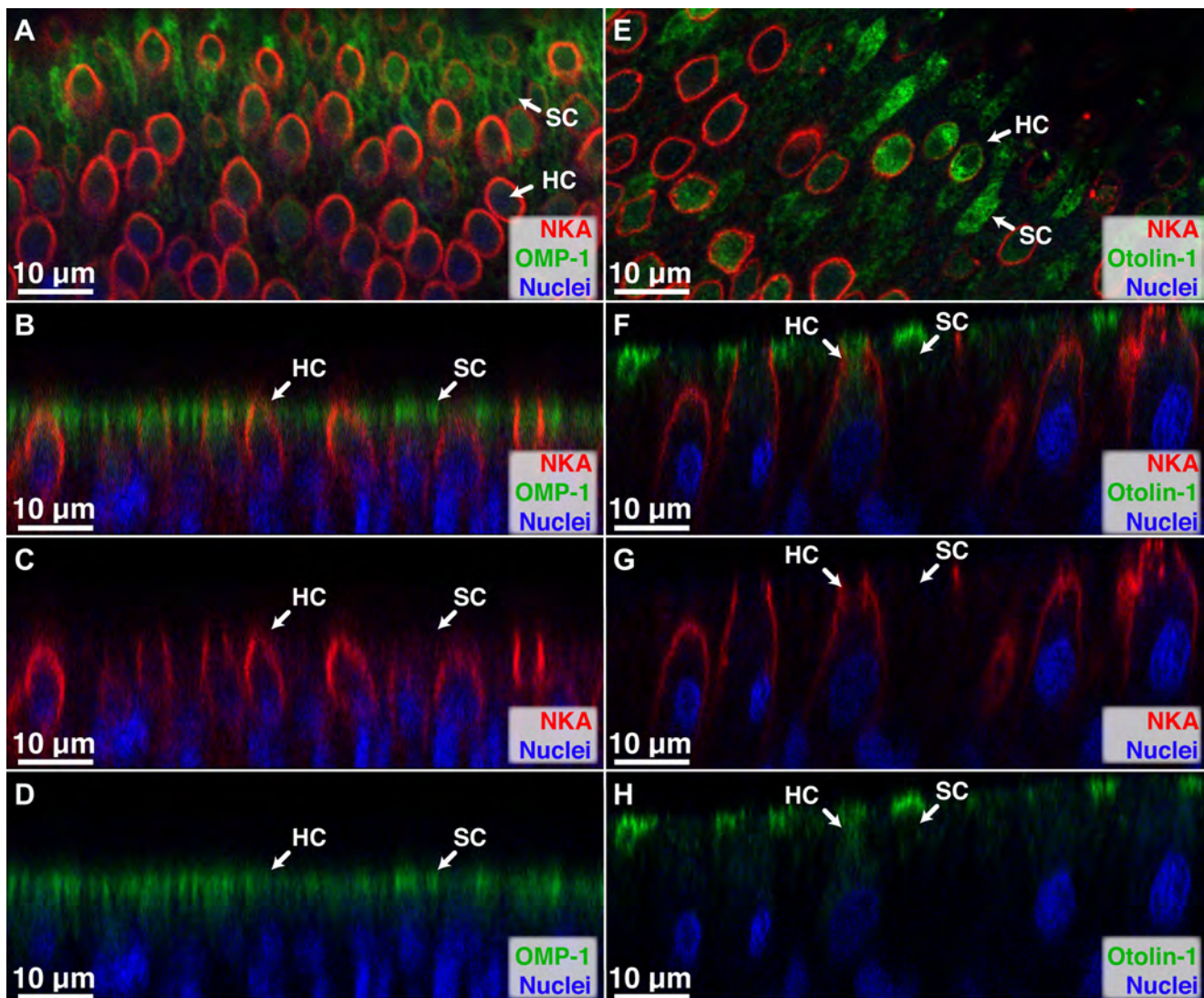


Figure 5. Immunolocalization of NKA, OMP-1, and Otolin-1 in hair cell (HC) and supporting cell (SC). The NKA-rich HC expresses some OMP-1 (A–D) and Otolin-1 in its subapical area (E–H). In contrast, the SC expresses OMP-1 (A–D) in a honeycomb pattern that resembles the overlying protein matrix, and otolin-1 (E–F) in its subapical area. NKA, Na^+/K^+ -ATPase; OMP-1, otolith matrix protein-1.

the ovoid shape of the soma (Fig. 3; Supplemental Fig. S2A). The HCs express CA at relatively low levels in their hair bundle (Fig. 3, A–D; Supplemental Fig. S2A), and they abundantly express VHA throughout the cytoplasm (Fig. 3, E–H) and in the kinocilium of their tubulin-rich hair bundle (Supplemental Fig. S2B). The HCs also have low expression of NKCC on its basolateral membrane (Fig. 4) and occasionally express OMP-1 and Otolin-1 within their subapical area (Fig. 5).

The SCs extend past and envelope the basolateral membrane of the HCs, and the nucleus of the SC is located at the basal portion of the cell and below the nucleus of adjacent HCs (Fig. 4, B and C). Thus, a lateral view of three-dimensional reconstructions shows two parallel rows of nuclei that clearly distinguish HCs from SCs (Supplemental Fig. S2C). The SCs do not show appreciable NKA signal (at least compared with the HCs); however, they express abundant CA throughout the cytoplasm and especially in the subapical area (Fig. 3). Notably, a view from the top shows that the CA signal follows a honeycomb pattern resembling that of the SCs perimeter (Fig. 3C). The SCs also express VHA in the subapical area; however, the signal is much dimmer than in the HCs (Fig. 3, G and H, Supplemental Fig. S2C). The NKCC signal is detected on both the basolateral (Fig. 4, A–C, Supplemental Fig. S2C) and apical side (Fig. 4, D and E; Supplemental Fig. S2C); interestingly, the latter forms a

honeycomb pattern resembling the extracellular otolithic membrane. The SCs express highly abundant OMP-1 and Otolin-1 in their subapical area (Fig. 5), although the Otolin-1 signal is somewhat patchy (Fig. 5H). Like CA and NKCC, the OMP-1 signal also forms a honeycomb pattern (Fig. 5A), whereas Otolin-1 appears throughout the apical surface of both HCs and SCs (Fig. 5E).

Macula: Granular Cells

The GCs line the periphery of the macula, separating the HCs and SCs from the T1-I and T2-I in the meshwork area. SEM imaging reveals that the apical surface of the GCs has a rhombic or pentagonal shape and contains numerous microvilli, pore-like structures, and budding vesicles (Fig. 6A). In addition, small hair bundles are occasionally seen protruding in between the GC (Fig. 6, B–D), which reminisce incipient HCs.

The GC nuclei are in the basal part of the cell and form a distinct row in lateral views of three-dimensional confocal reconstructions (Fig. 7, D and H and Fig. 8, C and F). The GCs express NKA within their basolateral membrane (Figs. 7 and 8) and abundant CA within its subapical region (Fig. 7, A–D). The GCs also contain cytoplasmic VHA and basolateral NKCC at levels similar to the SCs (Fig. 7, E–H). The most distinguishable feature of the GCs is their high abundance of

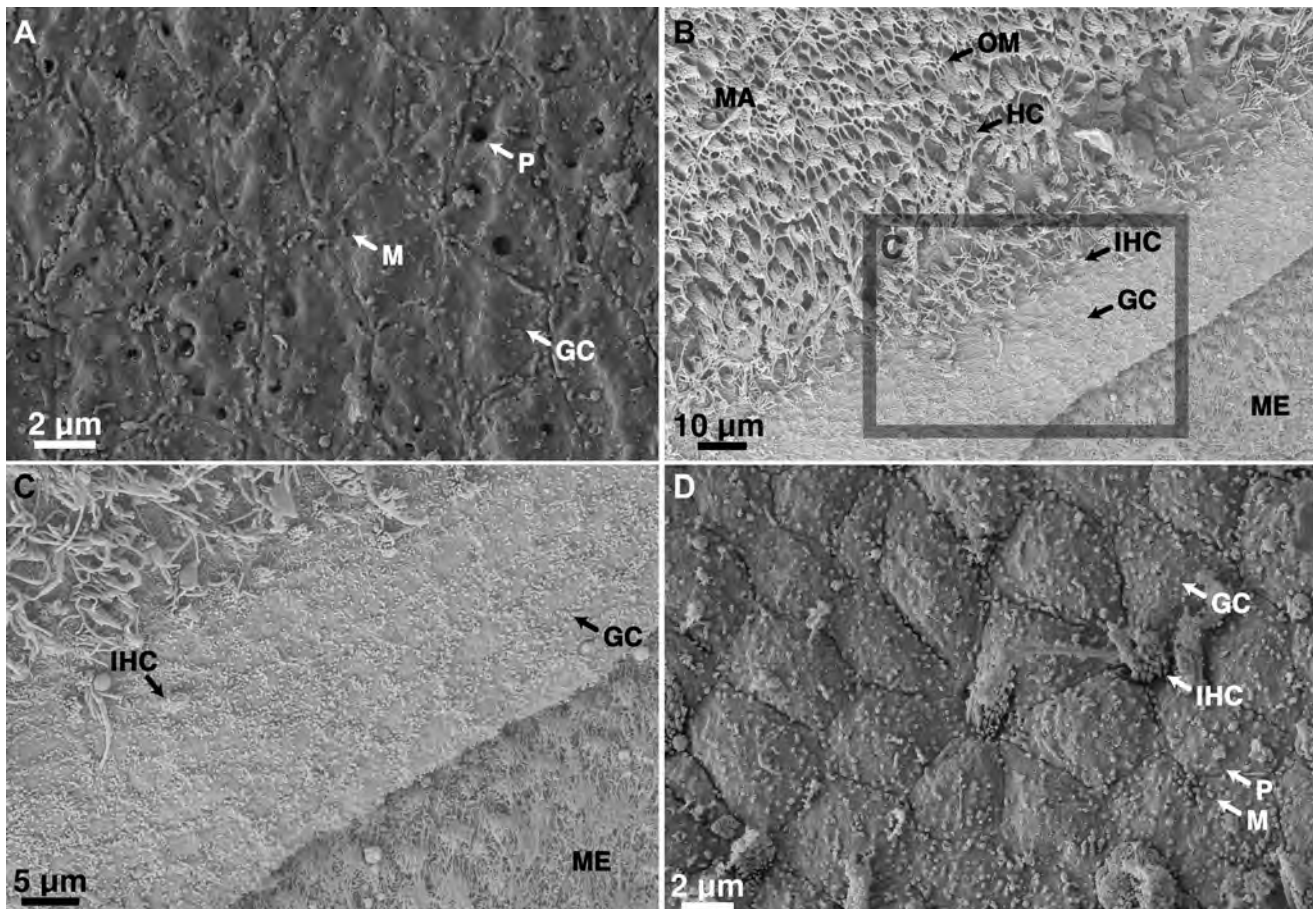


Figure 6. Scanning electron micrographs of inner ear granular cell (GC). A: apical surface of the GC exhibits numerous microvilli (M) and pores (P). B and C: GCs are located between the macula (MA) and meshwork area (ME), and hair bundles reminiscent of incipient hair cells (IHC) are occasionally observed. D: like the mature hair cell and supporting cell, IHC are never adjacent to one another and always surrounded by GC. OM, otolith matrix.

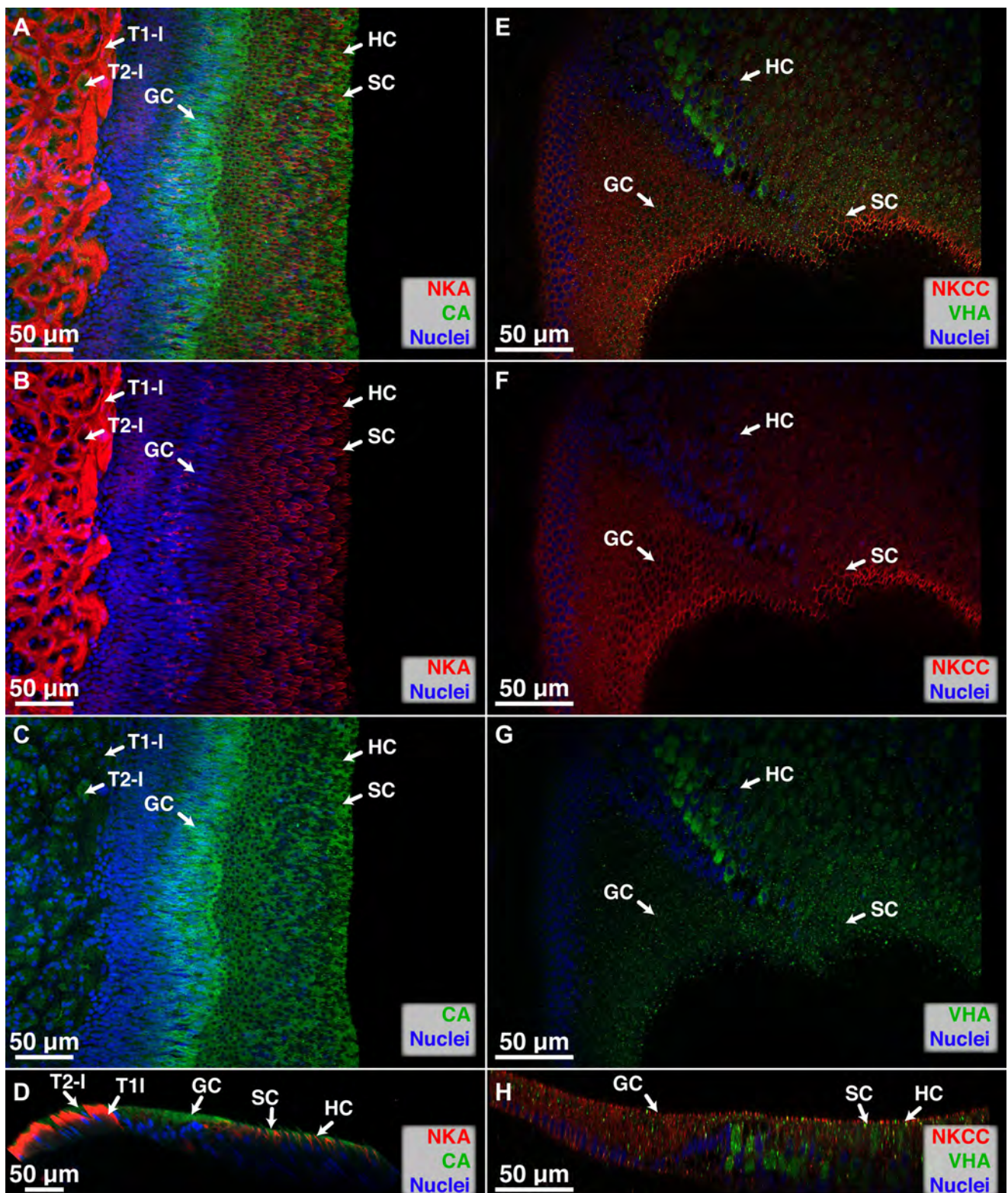


Figure 7. Immunolocalization of NKA, CA, VHA, and NKCC in the granular cell (GC). GC separates the hair cell (HC) and supporting cell (SC) from the Type-I (T1-I) and Type-II ionocytes (T2-I). A–D: GC expresses basolateral NKA—though not as intensely as the nearby T1-I and HC. GC also contains cytoplasmic and basolateral CA (A–D) and VHA (E–H) and basolateral NKCC. E and F: in particular, NKCC expression resembles the overlying protein matrix and links to that of the adjacent SC. CA, carbonic anhydrase; NKA, Na^+/K^+ -ATPase; NKCC, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ -cotransporter; VHA, vacuolar H^+ -ATPase.

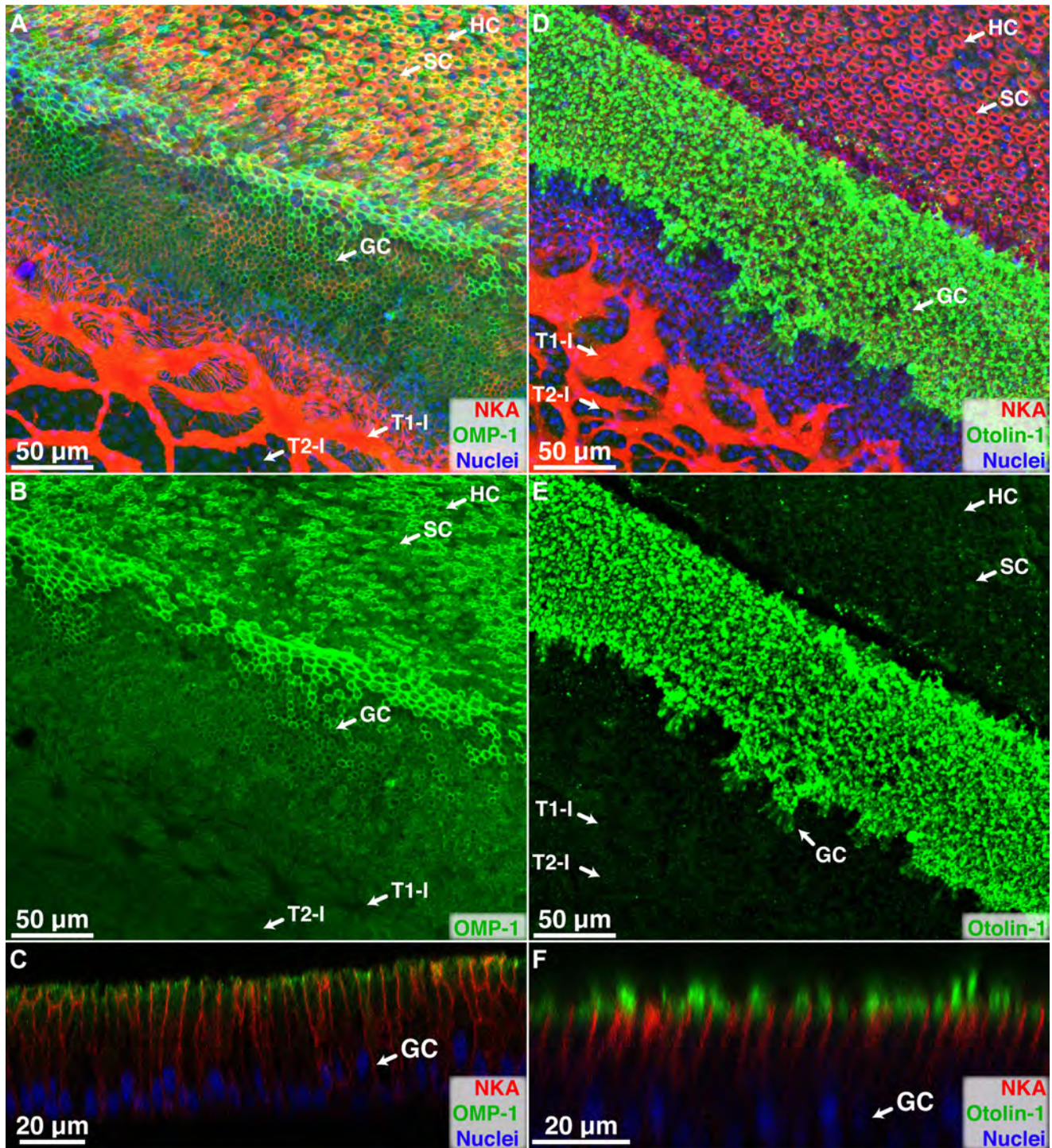
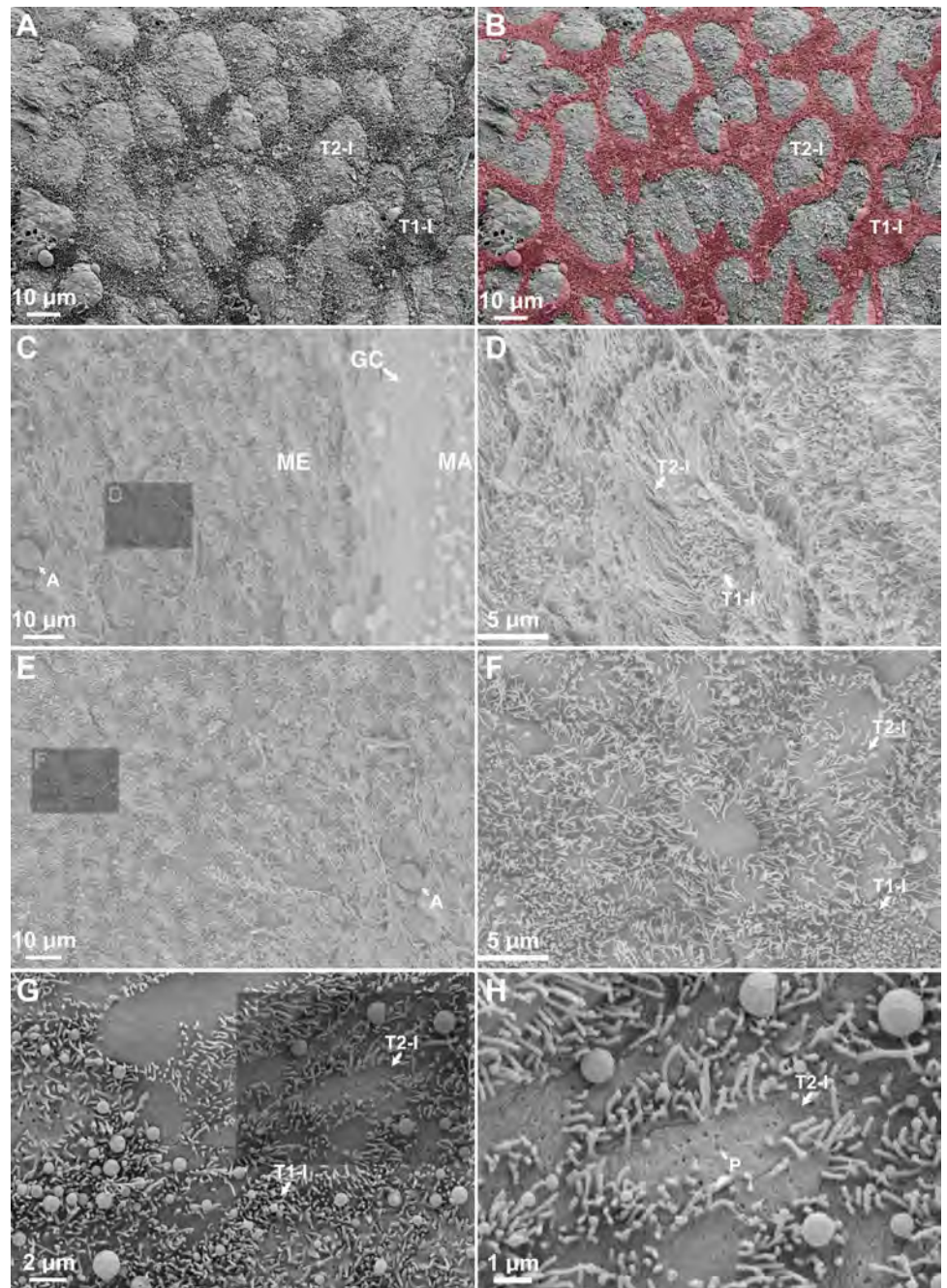


Figure 8. Immunolocalization of NKA, OMP-1, and Otolin-1 in the granular cell (GC). GC separates the hair cell (HC) and supporting cell (SC) from the Type-I (T1-I) and Type-II ionocytes (T2-I). The GC expresses basolateral NKA, and cytoplasmic and apical OMP-1 (A–C) and Otolin-1 (D–F). Apical OMP-1 expression resembles the overlying protein matrix and links to that of the adjacent SC. In contrast, Otolin-1 expression is in the central part of the apical membrane. NKA, Na^+/K^+ -ATPase; OMP-1, otolith matrix protein-1.

OMP-1 and Otolin-1 in their apical and subapical region (Fig. 8). As is the case in HC and SC, the GC's OMP-1 signal is present at the periphery of the apical membrane (Fig. 8B), whereas Otolin-1 signal is in the central part of the apical membrane (Fig. 8E). Notably, the OMP-1 signal is very intense in the GCs that are closest to the macula, but they

become much dimmer in cells closer to the meshwork area. In contrast, the Otolin-1 signal is uniformly very high across all GCs (Fig. 8, A and B). Finally, the expression patterns of CA (Fig. 7, A–D), NKCC (Fig. 7, E–H), and OMP-1 resemble that of the otolithic membrane, and this configuration is contiguous and adjoins with that of the SCs.

Figure 9. Scanning electron micrographs of inner ear Type-I (T1-I) and T2-I Ionocytes (T2-I). The apical surface of the T1-I has more numerous microvilli (M) than the T2-I (A) and matches the interconnected membrane system apparent in basolateral NKA and NKCC immunostaining (B). C and D: in contrast, the apical surface of the T2-I is relatively more barren, but its microvilli are longer than those of the T1-I. E and F: microvilli density and length decrease with distance from macula. Please note that C and E are contiguous, and the same artifact A is shown as a marker. G and H: numerous pores (P) can also be observed on the T2-I. A, artifact; GC, granular cell; MA, macula; ME, meshwork area; NKA, $\text{Na}^+\text{-K}^+\text{-ATPase}$; NKCC, $\text{Na}^+\text{-K}^+\text{-2Cl-cotransporter}$.



Meshwork and Intermediate Area: Type-I and Type-II Ionocytes

The macula is surrounded by the meshwork area, which is named after the interconnected network of T1-Is observed in past histological studies (Fig. 7, A and B). SEM imaging outlines a similar meshwork aspect resulting from the contrast in the apical membrane morphology between T1-Is and T2-Is (Fig. 9, A and B). Higher magnification SEM images (Fig. 9, C–F) reveal that the T1-Is have more abundant microvilli than the T2-Is, which contain numerous pores (Fig. 9, G and H). In addition, the microvilli in the T1-Is are significantly shorter ($0.568 \pm 0.023 \mu\text{m}$ vs. $1.467 \pm 0.095 \mu\text{m}$; $P < 0.0001$)

and wider ($0.184 \pm 0.006 \mu\text{m}$ vs. $0.105 \pm 0.004 \mu\text{m}$; $P < 0.0001$) than those in the T2-Is. As the distance from the macula increases, fewer T1-Is are observed, whereas T2-Is become increasingly abundant (Fig. 9, E–H). This pattern continues until the T1-Is are no longer present, and this signifies the transition from meshwork to the intermediate area (Supplemental Fig. S3).

Three-dimensional reconstructions of coimmunostaining combinations indicate that the T1-Is express abundant NKA throughout their basolateral membrane (Figs. 7, A and B, 8, A and D, Fig. 10, A–D, and Fig. 11), NKCC in the upper part of the basolateral membrane closer to the apical membrane (Fig. 10, E–H), and CA (Fig. 10, A–D) and VHA (Figs. 10, E–H

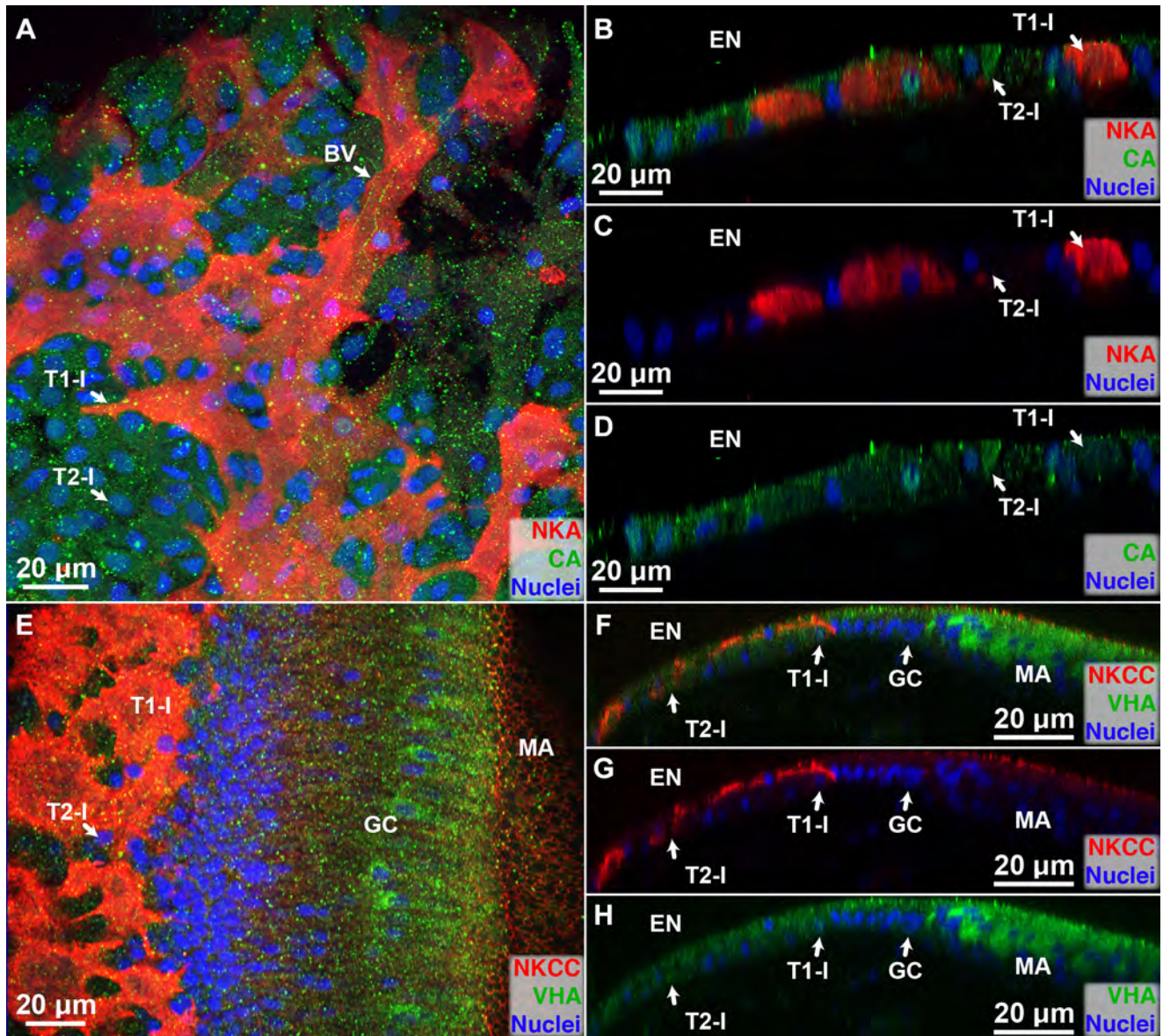


Figure 10. Immunolocalization of NKA, CA, VHA, and NKCC in the Type-I (T1-I) and T2-I Ionocytes (T2-I). The T1-I intensely expresses basolateral NKA (A–D) and NKCC (E–H), along with cytoplasmic CA and VHA. In contrast, T2-I does not contain NKA or NKCC but does express cytoplasmic CA and VHA. CA, carbonic anhydrase; EN, endolymph; GC, granular cell; MA, macula; NKA, Na^+/K^+ -ATPase; NKCC, Na^+/K^+ -2Cl-cotransporter; VHA, vacuolar H^+ -ATPase.

and 11) throughout the cytoplasm. The T1-Is also have some signal for OMP-1 (Fig. 8, A and B and Fig. 12, A–C) and Otolin-1 (Fig. 8, D and E and Fig. 12, D–F) in their subapical region; however, they were much fainter than in all other inner ear epithelial cell types. Notably, there is a lack of overlap in immunohistochemical signal between the cytoplasmic CA and VHA and the basolateral NKA (Figs. 10A and 11, A and D) and NKCC (Fig. 10E). Relative to the NKA signal, the NKCC signal in the T1-I appears to be concentrated closer to the apical side of the cell (Fig. 10, B and F). T1-Is appear to faintly express some OMP-1 (Fig. 8, A and B and Fig. 12, A–C) and Otolin-1 (Fig. 8, D and E and Fig. 12, D–F) in the subapical region of the T1-I.

In contrast, the T2-Is do not form a continuous membrane system, nor do they express NKA or NKCC (Figs. 10 and 11).

Instead, the T2-Is have abundant CA (Fig. 10, A–D) and VHA (Figs. 10, E–H and 11), and faintly express OMP-1 (Fig. 12, A–C) and Otolin-1 (Fig. 12, D–F).

Patches Area: Squamous Cell

The patches area is located on the opposite end of the macula and meshwork area and is entirely composed of squamous cells (SQs). SEM imaging reveals the border of the SQ contains abundant microvilli, whereas the rest of the cell appears to be relatively barren (Fig. 13).

The SQs express abundant NKA and can be mistaken as a T1-I when viewed laterally (Fig. 14, A and B) or at low magnification. Instead, the two cell types can be distinguished when viewed apically or at higher magnification settings: the edge of each SQ can be visualized using NKA immunostaining and

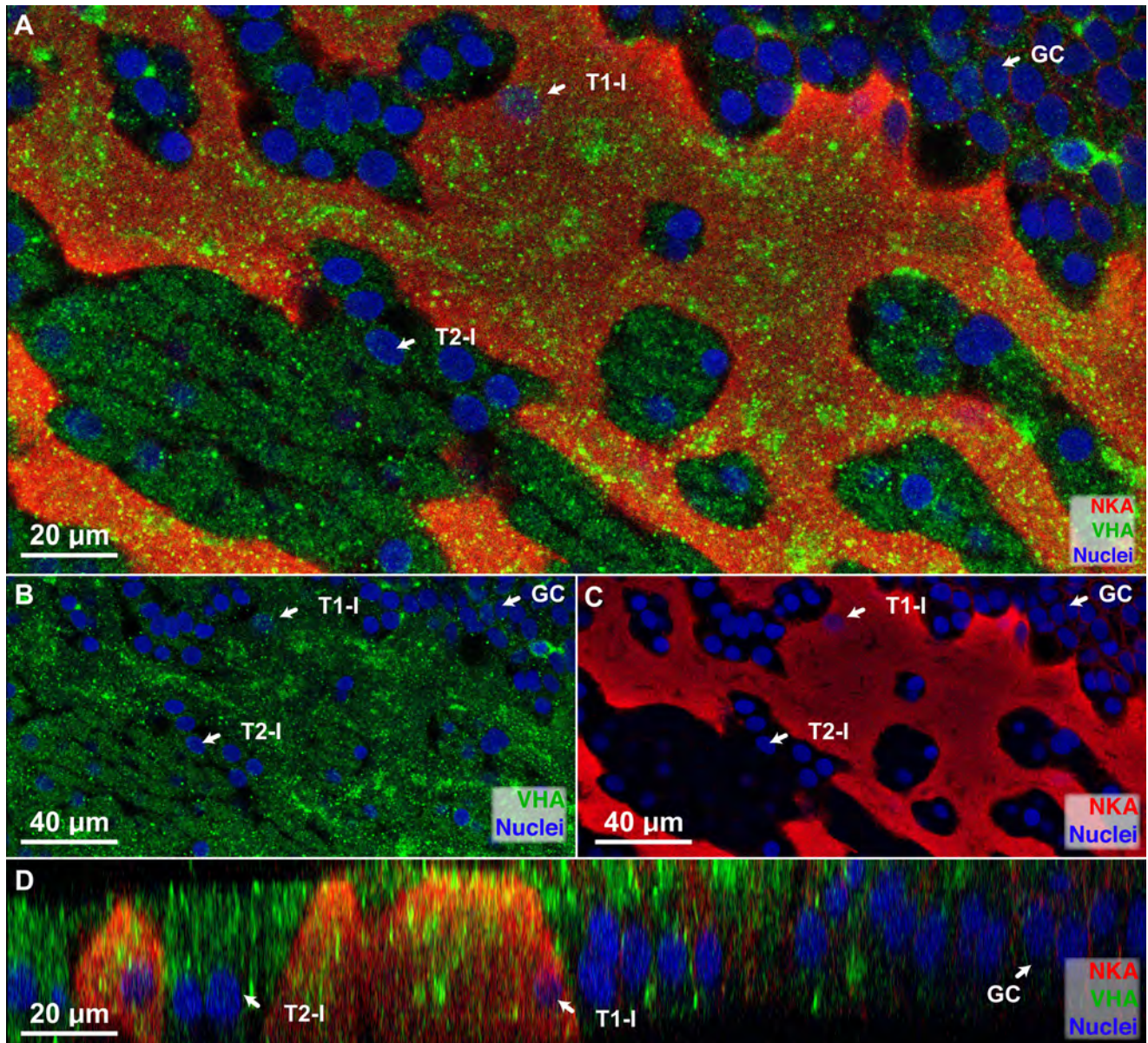


Figure 11. Immunolocalization of NKA and VHA in the Type-I (T1-I) and T2-I Ionocytes (T2-I). A–D: NKA-rich T1-I are interconnected with one another, forming a continuous membrane system. In contrast, T2-I do not form a continuous system. GC, granular cell. NKA, $\text{Na}^+/\text{K}^+/\text{ATPase}$; VHA, vacuolar H^+/ATPase .

the position of its nucleus is near the center of the cell (Fig. 14C), whereas the borders of the T1-I cannot be defined by NKA, and the location of its nucleus does not follow a specific pattern (Fig. 11, A and D). The SQs also contain abundant cytoplasmic and basolateral CA, with the latter appearing to clearly define the border of its cell membrane (Fig. 14C); this immunolocalization pattern sharply contrasts with basolateral NKA and NKCC (Fig. 14D) signal along the borders of the cell. VHA is expressed within the cytoplasm of the SQ. Finally, OMP-1 is expressed along the borders of the SQ (Fig. 14E), whereas Otolin-1 is more readily localized in the central portions of the SQ (Fig. 14F).

Altogether, the inner ear cell type-specific intracellular localization and relative protein abundance are summarized in Table 1.

DISCUSSION

The visualization of the six cell types lining the inner ear endolymph through immunohistochemical and ultrastructural techniques provides the foundational knowledge necessary to understanding the mechanism responsible for inner ear function and otolith biomineralization.

Hair Cell and Supporting Cell

The link between nominal inner ear sensory function and high endolymphatic $[\text{K}^+]$ appears to be evolutionarily conserved across teleost, amphibians, aves, and mammals (63). As the hair bundle is moved by the inertial force of the otolith/otoconia, endolymphatic K^+ and Ca^{2+} ions rush into the negatively charged HC and rapidly depolarize the cell (26, 63).

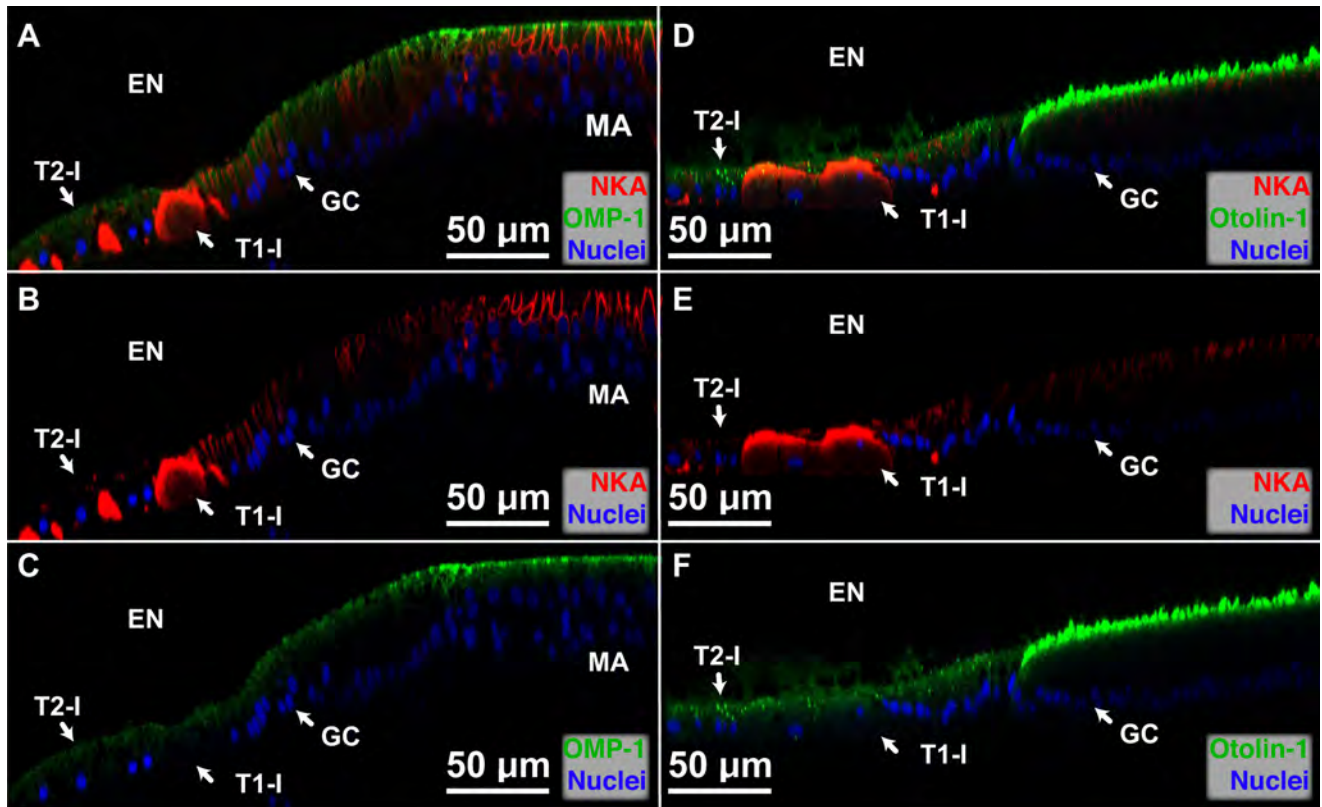


Figure 12. Immunolocalization of NKA, OMP-1, and Otolin-1 in the Type-I (T1-I) and Type-II (T2-I) Ionocytes (T2-I). A–C: OMP-1 is faintly expressed in both the NKA-rich T1-I, and in the T2-I. D–F: similarly, both T1-I and T2-I appears to express Otolin-1, with parts of it extruding into the endolymph (EN). GC, granular cell; MA, macula; NKA, Na^+/K^+ -ATPase; OMP-1, otolith matrix protein-1.

During depolarization, the intracellular VHA within the HC may be forming acidic vesicles for neurotransmitter transport for their exocytosis (64) and appears to look similar to the intracellular VHA pattern in nearby neurons. This also explains why nearby SCs have considerably less VHA expression. As the hair bundle reverts to its original position, intracellular $[\text{K}^+]$ within the HC is removed from the cell via basolateral K^+ channels (e.g., *KCNB1*; *KCNQ1*) (65). Simultaneously, basolateral NKA repolarizes the HC. The removed K^+ ions are transported away from the HC by the SC and eventually recycled back into the endolymph. Given that SCs completely envelope the HC's basolateral membrane, the role of the SC is likely to assist the HC in repolarization by removing surplus K^+ as well as resupplying Na^+ into their interstitial space. The exact mechanism(s) is still being debated, and multiple pathways have been proposed for mammalian and avian models (26, 63). However, the mechanistic pathway for K^+ recycling in fish likely differs, as mammalian Deiters' cells (analogous to the SCs) have neither NKA nor NKCC, whereas avian Deiters' cells have both basolateral NKA and NKCC (66), whereas our results suggest teleost SCs do not express NKA but do have apical and basolateral NKCC signal. It is unlikely for a cell to possess both apical and basolateral NKCC. Instead, the apical NKCC signal could instead be a part of the otolithic membrane given that it extends past the soma of the HCs (Fig. 4, B and C) and is shaped in a pattern resembling that of the otolithic membrane (Fig. 2A, Fig. 4, D and E, and Fig. 7, E and F). Alternatively, the NKCC antibody could simply be labeling an off-target protein. Future research should

focus on identifying the K^+ channel within teleost HC and SC and verifying the presence of NKCC in the SC.

In addition to NKCC, SCs also have CA and OMP-1 signals matching the otolithic membrane pattern observed via SEM. This suggests that the SCs play a critical role in secreting the otolithic membrane that protects the adjacent HC from being crushed by the overlying otolith, as well as anchoring the matrix structure to the macula (20, 28, 67). This otolithic membrane must expand outward to support the perennial growth of the otolith, yet remain porous to allow endolymph K^+ ions to reach the HCs at the center of the macula. Some SC also appears to express Otolin-1 in the middle of its apical membrane, which suggests that it may be contributing to the biomineralization of the otolith near the central region of the macula (30–32). Interestingly, previous Otolin-1 immunostaining on the chum salmon (*Oncorhynchus keta*) and the rainbow trout (*Oncorhynchus mykiss*) was immunonegative (31, 43). Perhaps the prevalence of Otolin-1 within the inner ear is linked to the relative otolith size: rockfishes (*Sebastes* spp.) are notorious for having large otoliths, whereas salmonids generally have relatively smaller otoliths. Alternatively, differences in Otolin-1 presence could be related to phylogenetic trends and would require further examination across salmonids and various percomorpha fishes.

Granular Cell

Similar to previous studies (31, 43), Otolin-1 presence is significantly greater within the granular cell (GC) than in any

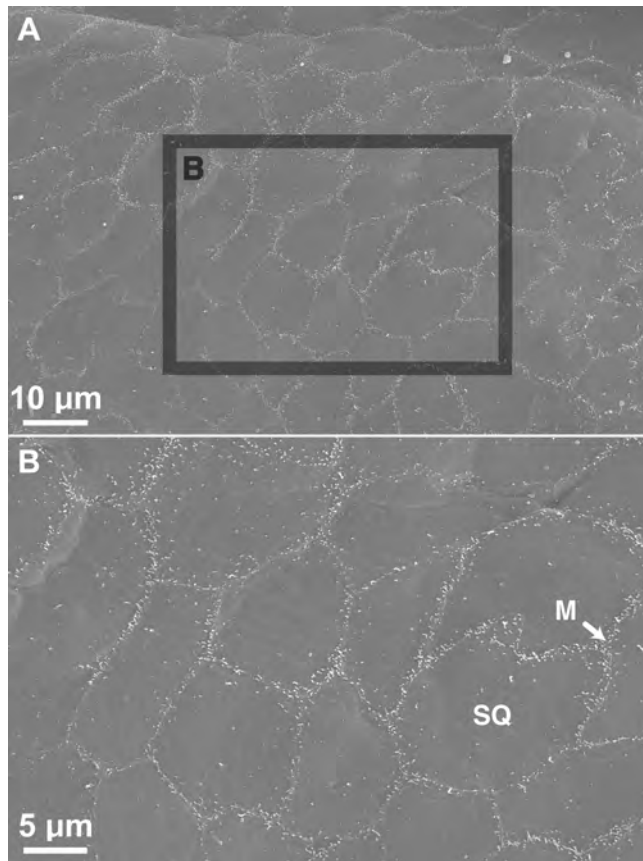


Figure 13. Scanning electron micrographs of inner ear squamous cell (SQ). Most of the apical surface on the SQ is relatively barren (A), but its periphery is marked with abundant microvilli (M) (B).

other inner ear cell type. This suggests that the GC is not only critical to otolith biomineralization but also necessary for proper CaCO_3 arrangement: calcite has a rhombohedral crystal structure and is formed in the presence of exclusively Otolin-1, whereas aragonite has an orthorhombic crystal structure formation that requires the presence of both Otolin-1 and otolith matrix molecule 64 (OMM-64) during *in vitro* trials (30, 68). The abundant presence of Otolin-1 in the GC around the macula also matches the natural biomineralization patterns: the proximal side (facing the macula) is known to grow faster and have wider increments than the distal side (facing the patches area) of the otolith (40).

In contrast, OMP-1 expression is not uniform. OMP-1 is more abundantly expressed in the GCs closest to the macula, and this spatial pattern suggests their role in supporting and accommodating the growing otolith. As the otolith grows, so must new HC and SC be synthesized and expand outward—thereby displacing the adjacent GC. In this study, incipient HCs are observed amid GCs at a significant distance away from the rest of the macula (Fig. 6). As such, do GCs gradually morph into SCs? This may also explain why the SC and GC share similar CA, VHA, and basolateral NKCC expression (both in intracellular localization and relative abundance). Other proteins critical to otolith biomineralization that the GCs may be secreting include *starmaker* (69, 70) and OMM-64 (68), though species-specific variation exists [c.f. *Oreochromis mossambicus* lacks OMM-64 and has calcitic otoliths (71)]. There is

much that is not known about GC, and further research is necessary to confirm whether GCs are displaced or transformed into SCs as the otolith enlarges, and to verify whether they secrete other otolith-relevant proteins.

Type-I and Type-II Ionocytes

The primary role of the T1-I has long been proposed to be responsible for maintaining the ionic and acid-base balance of the inner ear endolymph given its ample expression of mitochondria (18, 38), NKA (24, 37, 54), NKCC (37, 59), and the potassium channel KCNQ1 (59). In fact, the proposed mechanism for K^+ secretion within the inner ear appears to be evolutionary conserved (63, 66): the teleost T1-I (37) seems to function very similarly to their analogous counterparts, the mammalian marginal cells (72, 73), and the avian vestibular dark cells (74). However, unlike their mammalian and avian counterparts, the teleost inner ear is unique in that their otoliths biomineralizes continuously throughout the fish's life.

In contrast, the role of T2-I does not express NKA nor NKCC, and it likely does not play a role in K^+ secretion that the T1-I specializes in. Given this, the T2-I may simply play a role in maintaining the ionic gradients generated by the T1-I. This likely requires the expression of tight junctions via a claudin-like protein to prevent ion leakage and to maintain the endolymphatic ionic K^+ gradient similar to the inner ears of mice (18, 75, 76).

Continuous H^+ removal is necessary to promote perennial otolith biomineralization, and both T1-I and T2-I express CA and VHA, two proteins often involved in $\text{H}^+/\text{HCO}_3^-$ transport. The role of H^+ removal is often linked to VHA since it has been shown to translocate from the cytoplasm to the cell membrane during acid-base disturbance (77–79). However, VHA does not appear to play a role in mitigating an environmentally relevant acid-base disturbance (24, 37). Instead, VHA may be forming acidic vesicles to sequester H^+ within the T1-I to promote the formation of amorphous CaCO_3 ; a similar process has been proposed in the calcifying primary mesenchyme cells of the sea urchin (80). This may explain the presence of CA, which would catalyze local CO_2 into H^+ and HCO_3^- to the formation of amorphous CaCO_3 within the T1-I. The use of acidic vesicles to promote amorphous CaCO_3 has long been proposed for teleost otolith biomineralization (81–83), and there is evidence of this mechanism in calcifying corals (84–86). Further research is necessary to confirm whether the VHA expression within the T1-I is indeed intracellular vesicles involved in sequestering and removing H^+ to promote biomineralization.

The utilization of CA and VHA to generate H^+ necessitates the concurrent expression of an HCO_3^- transporter, and like other models, pendrin is the likely culprit. Pendrin (*slc26a4*) is an anion exchanger that has been demonstrated to be critical to hearing in mammalian models, and knock-out mice have more acidic endolymph pH and higher Ca^{2+} (87, 88). This suggests that an anion exchanger could be present on the apical membrane to transport HCO_3^- into the endolymph to buffer H^+ accumulation, or on the basolateral membrane to amass HCO_3^- in the endolymph to promote biomineralization. However, the presence, cellular and subcellular localization of an anion exchanger in the fish inner ear remains to be established, and further research is

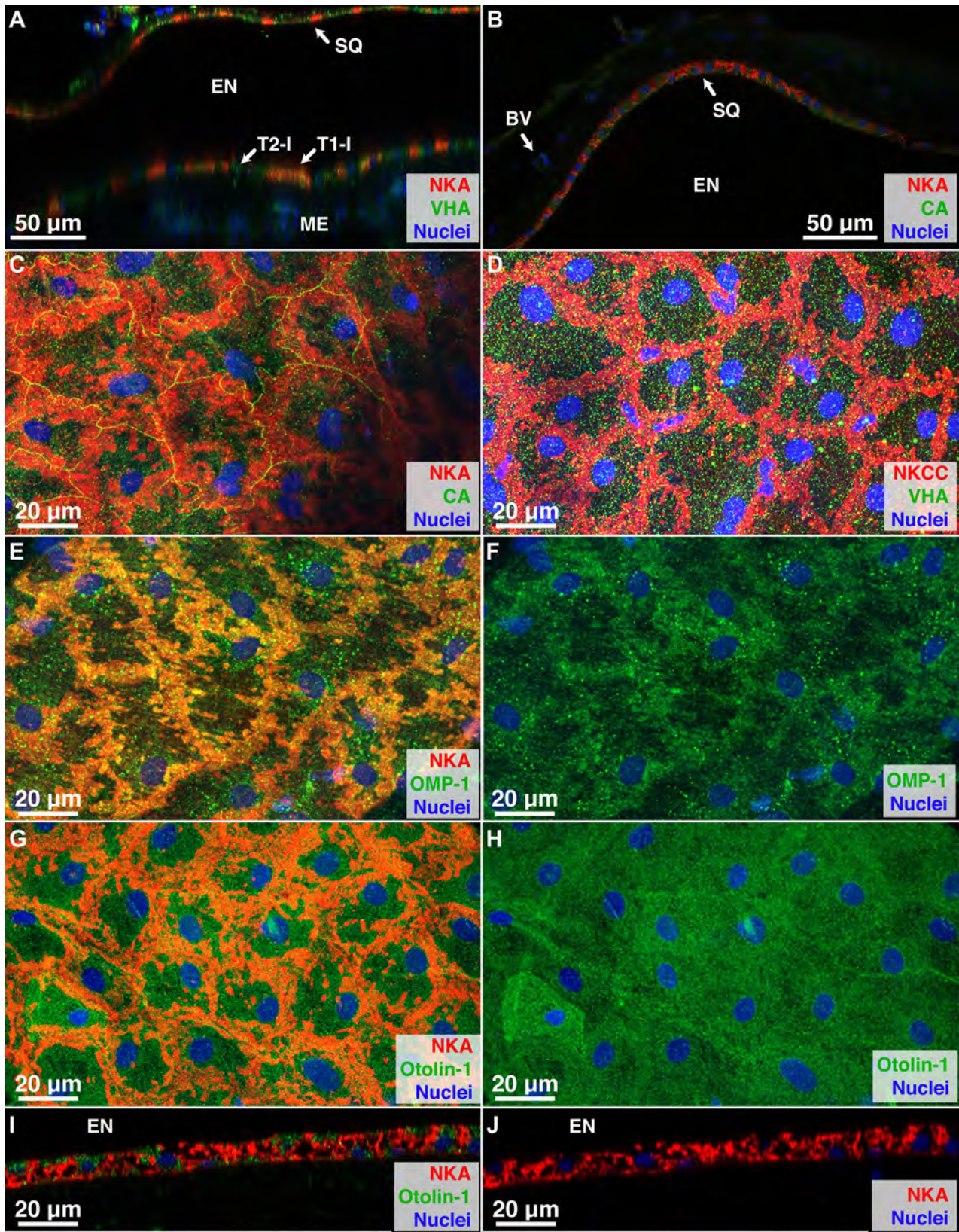


Figure 14. Immunolocalization of NKA, VHA, CA, NKCC, OMP-1, and Otolin-1 in the squamous cell (SQ). A–D: the SQ expresses basolateral NKA and NKCC, and cytoplasmic CA and VHA. The SQ also expresses OMP-1 at the periphery portions of the cell (E and F) but Otolin-1 near the central part of the cell (G–J). BV, blood vessel; CA, carbonic anhydrase; EN, endolymph; ME, meshwork area; NKA, $\text{Na}^+ - \text{K}^+ - \text{ATPase}$; NKCC, $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ -cotransporter; OMP-1, otolith matrix protein-1; T1-I, Type-I Ionocyte; T2-I, Type-II Ionocyte; VHA, vacuolar $\text{H}^+ - \text{ATPase}$.

Table 1. Summary of the six proteins and their relative expression within the inner ear cells adjacent to the endolymph

	NKA	CA	VHA	NKCC	OMP-1	Otolin-1
Sensory hair cell (HC)	++ (b)	+(hb)	+++ (c, hb, sa)	+(b)	+(sa [†])	+(sa [†])
Supporting cell (SC)	–	+++ (a, om, sa)	+(c)	++ (om, b)	+++ (a [†] , sa [†] , om [†])	+(a [†] , sa [†])
Granular cell (GC)	+(b)	++ (a, om, sa, b)	+(c)	++ (om, b)	++ (a, om [†] , sa)	+++ (a, sa)
Type-I ionocyte (T1-I)	+++ (b)	+(c, sa)	+(c, sa)	+++ (b)	+(sa)	+(sa)
Type-II ionocyte (T2-I)	–	++ (c, sa)	+(c, sa)	–	+(a, sa)	+(a, sa)
Squamous epithelial cell (SQ)	++ (b)	++ (b, c)	+(c)	++ (b)	++ (b)	++ (c)

A, apical; b, basolateral; c, cytoplasmic; hb, hair bundle; om, otolithic membrane; sa, subapical; [†]not uniformly expressed in all cells.

necessary to determine its intracellular expression within the inner ear cell type(s).

Both T1-I and T2-I appear to faintly express OMP-1 and/or Otolin-1 near the subapical and apical portions of the cells, and the two proteins are likely involved with the intracellular synthesis of amorphous CaCO_3 as proposed in sea urchins (80). OMP-1 and Otolin-1 staining appears to be extruded from the T1-I and T2-I into the endolymph (Fig. 12) in a manner similar to past TEM observations (89). Furthermore, SEM analysis revealed numerous pores suggesting exocytosis. However, the initial reporting in rainbow trout T1-I and T2-I failed to immunolocalize OMP-1 or Otolin-1 (43), and this could be due to differences in the sensitivity of the microscope and technique. Even so, additional studies are needed to determine whether OMP-1, Otolin-1, or another similar matrix protein is present within the T1-I and T2-I, and whether it is involved in synthesizing amorphous CaCO_3 .

Squamous Cell

SQ is the primary cell type facing the distal portion of the otolith, and its roles include ion transport (42) and otolithic protein secretion (31). The colocalized expression of NKA and NKCC and the extended microvilli along the perimeter of the cell resembles that of the T1-I, and thus SQ is likely also generating an electrochemical gradient for ion transport. Given the basolateral NKA and NKCC and the endolymph's elevated $[\text{K}^+]$, the potassium channel KCNQ1 is likely expressed on the apical membrane to facilitate K^+ secretion (59). Unlike the macula and meshwork area, the patches area is known to be particularly thin and has relatively few capillaries (18). Thus, the SQs could be sourcing their ions from the perilymph (rather than from blood capillaries). Yet unlike the T1-I, the perimeter of the SQ also expresses CA. Moreover, the presence of Otolin-1 and OMP-1 has been observed (43). Although the exact mechanism remains unknown, SQs are likely integral in maintaining the nominal endolymph chemistry and generating the necessary substrates for otolith biomineralization on the distal side of the otolith (40).

Proximal-Distal Gradient and Growth-Influenced Alteration

Altogether, the spatial position, cell specialization, and endolymph chemistry align with past observations on proximal-distal ionic gradient (19, 40) and relative number of amorphous CaCO_3 (34, 35). The network of mitochondrion-rich T1-I and the abundant GC located on the proximal side of the inner ear are likely able to significantly transport greater ions and synthesize more proteins compared with the SQ on the distal side of the inner ear. To elaborate, the relatively lower $[\text{K}^+]$ in the proximal endolymph (19, 40) likely stems

from its utilization by the HCs and removal by SCs. Next, the relatively lower pH in the proximal endolymph (19) suggests greater biomineralization (which produces H^+) and cellular respiration (which produces CO_2 that hydrates into HCO_3^- and H^+). Furthermore, the relatively greater protein concentration within the proximal endolymph (40) suggests the outsized production by the GC. Finally, there are more amorphous CaCO_3 both in the endolymph and bound to the edge of the otolith (34, 35), which ultimately leads to greater biomineralization on the proximal face of the otolith (33). Conversely, the distal endolymph is bordered by the SQ, which has significantly less mitochondria (38) and NKA, and must fulfill the role of multiple cell types. As such, the distal endolymph has relatively higher $[\text{K}^+]$ and pH (19, 40), lower protein concentration (40), less amorphous CaCO_3 (34, 35), and slower otolith biomineralization (33).

The otolith grows continuously throughout the fish's life, and thus the inner ear organ must also remodel itself to meet the demand. As the otolith expands, the need for additional matrix protein to protect the underlying HC is necessary. This means that the HC and SC in the macula must expand outward, and this study provides evidence that incipient HC is synthesized in the area occupied by GC. However, it is unclear how new SCs are formed: do GCs become SCs, or would new SCs be synthesized? Nevertheless, as the GC becomes displaced, new GCs must be created in the area where the T1-I and T2-I are located. This results in the displacement of T1-I toward the Intermediate Area, and could explain why the T1-I at the edge of the macula has a "scrunched up" appearance.

The capacity for the inner ear to remodel itself suggests that it could also balance and control the demands for ion transport and otolith biomineralization. First, the presence of extended microvilli across most of the described cell types suggests a degree of control of activities: increased ion transport could be generated with greater extension, whereas slowing of this process is possible with microvilli retraction. Moreover, as the inner ear grows, there is a potential for adjusting the number of cell types: the synthesis of more GC could indicate the need to boost otolith growth, whereas increasing T1-I may suggest the need for additional $[\text{K}^+]$ to meet HC demands. Altogether, this suggests the inner ear is capable of remodeling and could potentially alter its protein abundance, and apical morphology, and adjust the number of cell types in response to chronic stressors much like the gill ionocytes to various environmental stressors (c.f., 90–92). As a result, it remains possible that the inner ear's long-term response to future ocean acidification, warming, and hypoxia may not be predictable using short-term studies. Future research focused on understanding the dynamics of inner ear control over otolith biomineralization and the

interplay between cell types could potentially explain why otolith size varies across species.

Implications for Otolith-Reliant Tools under Future Climate Change Scenarios

This study showcases the intricate complexities of the inner ear, highlights numerous unanswered questions, and demonstrates the urgent need to understand the mechanism underpinning otolith biomineralization. Already, otolith biomineralization has already been shown to be impacted by future climate change scenarios including ocean warming (47, 48), acidification (45, 46), and hypoxia (49–51). Moreover, studies on otolith responses to multiple stressors are scarce: ocean acidification and warming impacts are synergistic and led to significantly greater otolith size (48), but the effects on ocean acidification and hypoxia remain undocumented in fish. However, in embryonic market squid (*Doryteuthis opalescens*) statolith (which is analogous to fish otolith), combined ocean acidification and hypoxia exposure resulted in a significantly smaller statolith (93). In summary, future climate change threatens to upend the decades of work spent to build the otolith-reliant toolkit that fishery managers, ecologists, and conservation biologists rely upon. This body of work showcases the need to consider inner ear cell responses to more accurately anticipate and predict otolith biomineralization patterns under future climate change scenarios.

Emerging technology such as using Fourier Transformed Near-Infrared Spectroscopy (FT-NIRS) to fish age by estimating its protein absorbance (94–97) is also at risk. Although FT-NIRS has the potential to greatly increase cost efficiency and reduce human error, climate change could still introduce unanticipated errors to the FT-NIRS by altering the ratio of otolithic proteins (e.g., OMP-1, Otolin-1) and CaCO_3 , as biomineralization rate increases due to ocean warming and acidification, or as biomineralization is stymied by hypoxia. Moreover, the remodeling of the inner ear (similar to that of fish gills) could also alter otolith biomineralization patterns, elemental incorporation, and protein production/abundance. As such, there remains an immense need for long-term studies that examine the future impacts of ocean warming, acidification, and hypoxia on the otolith metrics (e.g., annulus, elemental and isotopic signature, protein abundance) that fishery managers, ecologists, and conservation biologists rely on.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Figs. S1–S3: <https://doi.org/10.5281/zenodo.10231676>.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

G.T.K. and M.T. conceived and designed research; G.T.K., L.R.A., and K.J.P. performed experiments; G.T.K., L.R.A., and M.T. analyzed data; G.T.K., L.R.A., and M.T. interpreted results of experiments; G.T.K. prepared figures; G.T.K. and M.T. drafted manuscript; G.T.K., L.R.A., and M.T. edited and revised manuscript; G.T.K., L.R.A., K.J.P., and M.T. approved final version of manuscript.

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