1 **Immunological characterization of two types of ionocytes in the inner ear epithelium of**

2 **Pacific Chub Mackerel (*Scomber japonicus*)**

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8 **Abstract**:

9 The inner ear is essential for maintaining balance and hearing predator and prey in the

10 environment. Each inner ear contains three $CaCO_3$ otolith polycrystals, which are calcified

11 within an alkaline, K^+-rich endolymph secreted by the surrounding epithelium. However, the

12 underlying cellular mechanisms are poorly understood, especially in marine fish. Here, we

13 investigated the presence and cellular localization of several ion-transporting proteins within the

14 saccular epithelium of the Pacific Chub Mackerel (*Scomber japonicus*). Western blotting

15 revealed the presence of Na^+/K^+-ATPase (NKA), carbonic anhydrase (CA), Na^+-K^+-$2Cl^-$-co-

16 transporter (NKCC), vacuolar-type H^+-ATPase (VHA), plasma membrane Ca^{2+} ATPase

17 (PMCA), and soluble adenylyl cyclase (sAC). Immunohistochemistry analysis identified two

18 distinct ionocytes types in the saccular epithelium: Type-I ionocytes were mitochondrion-rich

19 and abundantly expressed NKA and NKCC in their basolateral membrane, indicating a role in

20 secreting K^+ into the endolymph. On the other hand, Type-II ionocytes were enriched in

21 cytoplasmic CA and VHA, suggesting they help transport HCO_3^- into the endolymph and remove

22 H^+. Additionally, both types of ionocytes expressed cytoplasmic PMCA, which is likely involved

23 in Ca^{2+} transport and homeostasis, as well as sAC, an evolutionary conserved acid-base sensing

24 enzyme that regulates epithelial ion transport. Furthermore, CA, VHA, and sAC were also

25 expressed within the capillaries that supply blood to the meshwork area, suggesting additional

26 mechanisms that contribute to otolith calcification. This information improves our knowledge

27 about the cellular mechanisms responsible for endolymph ion regulation and otolith formation,

28 and can help understand responses to environmental stressors such as ocean acidification.

29

32

Introduction

The inner ear senses gravity and sound waves, which is essential for maintaining balance and hearing predator and prey in the environment (Dijkgraaf, 1960; Furukawa and Ishii, 1967; reviewed in Ladich and Schulz-Mirbach, 2016). Enclosed within each inner ear are the sagittal, lapilli, and asterisci otoliths, which are composed of a protein matrix and calcium carbonate ($CaCO_3$). The higher density of the otolith compared to the inner ear fluid ("endolymph") results in differential inertia that stimulates the adjacent sensory hair cells, which the brain interprets as soundwaves or movement.

Being the largest of the three otoliths, the sagitta and its surrounding saccular epithelium have been most extensively studied. The saccular epithelium has been previously characterized as the macula, meshwork, patches, and intermediate areas (Mayer-Gostan et al., 1997; Pisam et al., 1998). The macula contains the sensory hair cells that detect otolith vibration and movement. This area is flanked by the meshwork area, which contains large ion-transporting cells ("ionocytes"). The patches area is positioned directly across from the macula and contains patches of smaller ionocytes. The intermediate area is largely devoid of ionocytes, but does contain some ionocytes in the area bordering the meshwork area and smaller ionocytes bordering the patches area. Each otolith is calcified within an alkaline, K^+-rich endolymph secreted by its respective saccule, utricle and lagena inner ear epithelium.

In the Rainbow Trout (*Oncorhynchus mykiss*), the endolymph has a pH of ~8, ~30 mmol of HCO_3^-, ~124 mmol of K^+, ~90 mmol of Na^+, and ~1.1 mmol of Ca^{2+} (Payan et al., 1997). When compared to its blood plasma, the endolymph is roughly 0.8 pH unit higher, has twice as much HCO_3^-, ~40-fold higher K^+, half as much Na^+, and twice as much Ca^{2+} (Payan et al., 1997). This dramatic differences between the endolymph and blood plasma are thought to be attributed to the surrounding ionocytes' activity. To date, two different types of ionocytes have been characterized: one is mitochondrion-rich (MR), has well-developed basolateral membrane infoldings (Mayer-Gostan et al., 1997), and abundantly expresses Na^+/K^+-ATPase (NKA) (Takagi, 1997), whereas the other one has abundant cytoplasmic carbonic anhydrase (CA) (Tohse et al., 2004, 2006). The NKA-rich ionocytes are proposed to be responsible for transporting K^+ (Payan et al., 1999), Ca^{2+} (Mugiya and Yoshida, 1995) and removing H^+ (Payan et al., 1997) from the endolymph, whereas the CA-rich ionocytes are thought to transport HCO_3^-

63 into the endolymph (Tohse and Mugiya, 2001; reviewed in Payan et al., 2004). These models

64 would imply the NKA-rich ionocytes should have different ion-transporting proteins than the

65 CA-rich ionocytes.

66   Moreover, the endolymph's composition is not homogeneous (Payan et al., 1999; Borelli

67 et al., 2003). The proximal endolymph, which is located between the otolith and the macula and

68 meshwork area, has lower [K_+] and total CO_2 compared to the distal endolymph, which is

69 located between the other side of the otolith and the intermediate and patches area (Payan et al.,

70 1999). Though [Ca_{2+}] does not differ between the proximal and distal endolymph (Payan et al.,

71 1999; Borelli et al., 2003), the proximal endolymph has a 3-fold higher concentration of

72 glycoprotein (Payan et al., 1999), which may chelate Ca_{2+} and catalyze aragonite crystallization

73 (Murayama et al., 2002; Ibsch et al., 2004). Correspondingly, the otolith's proximal surface

74 calcifies faster than the distal surface (Payan et al., 1999; Borelli et al., 2003; Beier et al., 2006).

75 And although it was not directly measured, it was further hypothesized that the pH in the

76 proximal endolymph is lower than the distal endolymph as increased otolith calcification would

77 locally increase [H_+] (Payan et al., 1999). This heterogeneity of the proximal and distal

78 endolymph was proposed to be the result of differential ion transporting activity of meshwork

79 and patches ionocytes. Under this model, the larger NKA-rich ionocytes in the meshwork area

80 remove K_+ from the proximal endolymph, whereas the smaller NKA-rich ionocytes in the

81 patches area secrete K_+ and absorb H_+ at the distal endolymph (Payan et al., 1999; Allemand et

82 al., 2008). Similarly, other studies speculated that the larger meshwork CA-rich ionocytes

83 remove H_+ from the proximal endolymph (Tohse et al., 2006). These models imply that NKA-

84 rich and CA-rich ionocytes in the meshwork area should express different proteins than their

85 counterparts in the patches area.

86   Although many other proteins are known to be expressed in the fish inner ear, to our

87 knowledge NKA and CA are the only two ion-transporting proteins established to be specifically

88 present in ionocytes. Basolateral Na_+-K_+-$2Cl_-$-co-transporter (NKCC1; *slc12a2*), NKCC1) is

89 expressed in their developing inner ear of Zebrafish (*Danio renio*) larvae (Abbas and Whitfield,

90 2009). Although the lack of endolymph accumulation upon NKCC1 genetic disruption indicated

91 a role in K_+ and fluid secretion, the specific cell type where this protein is expressed was not

92 established. Another study detected abundant intracellular acidic compartments in a subset of

93 trout inner ear epithelial cells and hypothesized it indicated removal of H_+ from the endolymph

94 by V-type H+-ATPase (VHA) (Mayer-Gostan et al., 1997). However, a subsequent study did not

95 find VHA in Zebrafish inner ear ionocytes, and instead reported VHA expression within inner

96 ear sensory hair cells and proposed it acidified the proximal endolymph to retard otolith

97 calcification and maintain distance with the hair cells (Shiao et al., 2005). The plasma membrane

98 Ca2+-ATPase (PMCA; *atp2b1a*) was proposed to be expressed in MR-ionocytes and to transport

99 Ca2+ for otolith calcification (Mugiya and Yoshida, 1995; Payan et al., 2002). *In situ*

100 hybridization showed the presence of PMCA mRNA in some epithelial cells surrounding the

101 sensory macula of the developing inner ear of Zebrafish larvae; however, attempts to

102 immunolocalize the protein were unsuccessful in both larval and adult tissues and thus remain

103 unknown whether PMCA is expressed in ionocytes (Cruz et al., 2009). More recently, a

104 comprehensive transcriptomic and proteomic study concluded NKA, CA, VHA, and PMCA are

105 expressed in the inner ear of black bream (*Acanthopagrus butcheri*) (Thomas et al., 2019).

106 However, those analyses were conducted on samples that contained both inner ear and brain

107 tissue, and thus did not provide insights about protein expression in specific cells. In summary,

108 there are many excellent studies about the ion-transporting proteins involved in otolith

109 calcification, but their use of different fish species, life stages, and techniques greatly

110 complicates attempts to synthesize the available information into a single model describing the

111 ion transporting mechanisms that maintain the distinctive endolymph composition necessary for

112 proper inner ear function.

113   Although the cellular mechanisms underlying otolith calcification are not completely

114 understood, it is clear that they activities are sensitive to acid-base conditions (reviewed in

115 Allemand et al., 2008). Indeed, diurnal fluctuations in plasma [HCO3-] is one of the underlying

116 causes of the otolith's characteristic concentric rings (Tohse and Mugiya, 2008) used to estimate

117 age and growth in stock assessment studies (Pannella, 1971; Campana and Neilson, 1985). And

118 more recently, exposure to ocean acidification conditions has been reported to induce increased

119 otolith size and density in multiple fish species (Checkley et al., 2009; Bignami et al., 2013;

120 Maneja et al., 2013; Munday et al., 2011; Pimentel et al., 2014; Schade et al., 2014; Shen et al.,

121 2016), which has been linked to plasma [HCO3-] accumulation resulting from blood acid-base

122 regulation [c.f. (Esbaugh et al., 2012, 2016)]. One possibility is that otolith overgrowth is the

123 direct result of increased transport of plasma [HCO3-] into the endolymph. However, increased

124 otolith calcification rate also requires increased secretion of Ca2+ and glycoprotein into the

125 endolymph, and increased H+ removal. With this in mind, we explored whether the soluble

126 adenylyl cyclase (sAC, adcy10) is expressed within inner ear epithelial ionocytes. This

127 evolutionary conserved acid-base sensing enzyme is stimulated by HCO_3^- to produce cyclic

128 adenosine monophosphate (cAMP), a messenger molecule that can regulate multiple cellular

129 processes *via* protein kinase A mediated phosphorylation on target proteins (reviewed in

130 Tresguerres et al., 2010a; Tresguerres, 2014).

131   The goal of the current study was to determine how many types of ionocytes are present

132 in the inner ear epithelium of a single species, the Pacific Chub Mackerel (*Scomber japonicus*,

133 Houttuyn, 1782). To this end, we performed thorough immunohistochemical analyses using

134 specific antibodies against NKA, CA, NKCC, VHA, PMCA, and sAC. Unexpectedly, we also

135 detected high abundance of some of these proteins in the cells that form the arterioles that supply

136 blood to the meshwork area. The resulting model about the ion-transporting and regulatory

137 mechanisms underlying endolymph's unique composition improves our understanding about

138 how otoliths are calcified, and will inform subsequent experimental studies to determine if and

139 how they might be affected during environmental stress.

140

141 **Methods**:

142 *Tissue Sampling and Preparation*

143   Pacific Chub Mackerel were caught by hook and line off the Scripps pier in San Diego,

144 United States (standard length = 15.3 ± 0.3 cm; weight = 26.9 ± 2.2 g; n = 19). In accordance to

145 protocol S10320 of the University of California, San Diego Institutional of Animal Care and Use

146 Committee, fish were euthanized by spinal pithing and its inner ear tissue dissected. Tissue was

147 either flash frozen in liquid nitrogen and stored in -80°C, or fixed in 4% paraformaldehyde in

148 phosphate buffer saline (PBS) at 4°C for 8 hours, incubated in 50% ethanol for 8 hours, and

149 stored in 70% ethanol for immunohistochemistry. Protein integrity was prioritized; therefore, the

150 length and weight of the fish were recorded after dissection.

151

152 *Antibodies*

153   Mitochondria were labeled using a mouse monoclonal antibody against human

154 cytochrome *c* oxidase complex IV (MTC02, catalog #: MA5-12017, Invitrogen, Grand Island,

155 New York, USA); this antibody demonstrates specificity against a broad range of species

including coral (Barott et al., 2015b) and shark (Roa et al., 2014). The mouse monoclonal anti-NKA antibody α5 (Lebovitz et al., 1989) was purchased from the Developmental Studies Hybridoma Bank (DSHB, The University of Iowa, Iowa City, IA, USA). This antibody has been extensively validated in fish and is routinely used to detect NKA in multiple fish tissues (Wilson et al., 2000, 2002; Roa et al., 2014; Roa and Tresguerres, 2017; Kwan et al., 2019). Additionally, NKA was immunodetected using rabbit polyclonal antibodies against the mammalian NKA α-subunit (H300, catalog # SC-28800, Santa Cruz Biotechnology, Dallas, USA), which recognize NKA in gills from multiple fish (Roa et al., 2014; Michael et al., 2016; Allmon and Esbaugh, 2017). Rabbit polyclonal antibodies against human CA II were purchased from Rockland Inc., Gilbertsville, USA (catalog #: 100-401-136); these antibodies are routinely used to immunodetect CA from teleost fish [e.g. (Georgalis et al., 2006; Qin et al., 2010)], including in the saccular epithelium of Masu Salmon (*Oncorhynchus masou*) (Tohse et al., 2004). The mouse monoclonal anti-NKCC antibody T4 (Lytle et al., 1995) was obtained from DSHB; and has been widely used to detect NKCC in fish tissues (Tresguerres et al., 2010b; Esbaugh and Cutler, 2016), including Zebrafish saccular epithelium (Abbas and Whitfield, 2009). VHA was immunodetected using custom-made rabbit polyclonal antibodies against a peptide in the B subunit (epitope: AREEVPGRRGFPGY; GenScript, Piscataway, USA); this peptide is conserved from cnidarians to mammals (Barott et al., 2015), and has been successfully used to immunodetect VHA in elasmobranch tissues (Roa et al., 2014; Roa and Tresguerres, 2017). The mouse monoclonal anti-PMCA antibody 5F10 against human erythrocyte PMCA was purchased from ThermoFisher Scientific, Waltham, USA (catalog #: MA3-914). sAC was immunodetected using custom-made rabbit polyclonal antibodies against a peptide in the first catalytic domain of Rainbow Trout sAC (epitope: LSSKKGYGADELTR; GenScript). The secondary antibodies were goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP conjugate (Bio-Rad, Hercules, CA, USA) for western blot, and goat anti-mouse Alexa Fluor 546, goat anti-rabbit Alexa Fluor 488, and/or goat anti-rabbit Alexa Fluor 555 (Invitrogen, Grand Island, USA) for immunohistochemistry. Each antibody was tested in inner ear samples from at least three different fishes.

Western Blotting

186   Inner ear tissue was immersed in liquid nitrogen, pulverized in a porcelain grinder, and

187   submerged in an ice-cold, protease inhibiting buffer (250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA,

188   30 mmol l^{-1} Tris, 10 mmol l^{-1} benzamidine hydrochloride hydrate, 200 mmol l^{-1}

189   phenylmethanesulfonyl fluoride, 1 mol l^{-1} dithiothreitol, pH 7.5). Next, debris was removed by

190   low speed centrifugation (3000xg, 10 minutes, 4°C). Total protein concentration in the crude

191   homogenate was determined by the Bradford assay (Bradford, 1976). Samples were mixed with

192   an equal volume of 90% 2x Laemmli buffer and 10% β-mercaptoethanol, and heated at 70°C for

193   5 minutes. Protein (10 µg per lane) were loaded onto a 7.5% polyacrylamide mini gel (Bio-Rad,

194   Hercules, CA, USA) and ran at 200 volts for 40 minutes, then transferred to a polyvinylidene

195   difluoride (PVDF) membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). PVDF

196   membranes were then incubated in tris-buffered saline with 1% tween (TBS-T) with milk

197   powder (0.1 g/mL) at room temperature (RT) for 1 hour, then incubated with primary antibody

198   (a5: 10.5 ng/ml; H300: 100 ng/ml; CA II antibody: 8 µg/ml; T4: 10.4 ng/ml; VHA *b*-subunit: 1.5

199   µg/ml; Rainbow Trout sAC: 3 µg/ml; 5F10: diluted 1:10,000 from commercial stock) in

200   blocking buffer at 4°C overnight. On the following day, PVDF membranes were washed in TBS-

201   T (three times; 10 minutes each), incubated in the appropriate anti-rabbit or anti-mouse

202   secondary antibodies (1:10,000) at RT for 1 hour, and washed again in TBS-T (three times; 10

203   minutes each). Bands were made visible through addition of ECL Prime Western Blotting

204   Detection Reagent (GE Healthcare, Waukesha, WI) and imaged and analyzed in a BioRad

205   Universal III Hood using Image Lab software (version 6.0.1; BioRad). Peptide preabsorption

206   with excess peptide (1:5 antibody to peptide ratio; preabsorbed overnight at 4°C on shaker) was

207   performed to verify antibody specificity.

208

209   *Immunostaining*

210   After fixation, samples were immersed in decalcifying solution (NaCl 450 mM, KCL 10

211   mM, MgCl 58 mM, Hepes 100 mM, EDTA 0.5 M, pH 7.5, changed daily) for three days at 4°C

212   on a shake table to dissolve the otolith. Once the otolith dissolved, samples were incubated

213   overnight in 70% ethanol and dehydrated through a series of increasing ethanol steps (70%, 95%,

214   100%, 10 minutes each), SafeClear (3 times; 10 minutes each), warm paraffin (65°C; 3 times; 10

215   minutes each), before embedding tissue in a paraffin block on an ice pack overnight. The next

216   day, samples were sectioned using a microtome (~10 µm thickness) and mounted onto glass

217   slides. After drying overnight, paraffin was removed by incubation in SafeClear (3 times; 10

218   minutes each), and rehydrated in a series of decreasing ethanol steps (100%, 95%, 70%, 10

219   minutes each). To counter native autofluorescence, samples were immersed with sodium

220   borohydride (1 mg/mL) in ice cold PBS (6 times; 10 minutes each). Samples were then washed

221   in PBS + 0.1% tween (PBS-T) at RT for 5 minutes, incubated in blocking buffer (PBS-T, 0.02%

222   normal goat serum, 0.0002% keyhole limpet hemocyanin) at RT for one hour, and with the

223   primary antibodies (MTC02: 2 µg/ml; a5: 42 ng/ml; H300: 4 µg/ml; CA II antibody: 160 µg/ml;

224   T4: 104 ng/ml; VHA *b*-subunit: 6 µg/ml; Rainbow Trout sAC: 6 µg/ml; 5F10: diluted 1:500

225   from commercial stock) in blocking buffer and kept in a humid chamber at RT overnight. On the

226   following day, samples were washed in PBS-T (3 times; 10 minutes each) and incubated with the

227   appropriate anti-rabbit or anti-mouse fluorescent secondary antibodies (1:1,000) and nuclear

228   stain Hoechst 33342 (5 µg/mL; Invitrogen) at RT for 1 hour. Samples were washed in PBS-T

229   (three times; 10 minutes each), then mounted in Fluoro-gel with Tris (Electron Microscopy

230   Sciences). Samples were examined and imaged on an epifluorescence microscope (Zeiss

231   AxioObserver Z1). Digital images were adjusted, for brightness and contrast, using Zeiss

232   Axiovision software. Some low magnification images were stitched together to provide pictures

233   of the entire saccular epithelium using Helicon Focus 6 (Helicon Soft Ltd., Kharkov, Ukraine).

234   Peptide preabsorption with excess peptide (1:10 antibody to peptide ratio; preabsorbed overnight

235   at 4°C on shaker) was performed to verify antibody specificity against VHA and sAC.

236

237   **Results**:

238   Western blotting revealed high abundance of NKA, CA, NKCC, VHA, sAC, and PMCA

239   protein in Pacific Chub Mackerel inner ears (Fig. 1). The immunoreactive bands matched the

240   predicted size of each target protein (NKA-α subunit: ~100 kDa with both mono- and polyclonal

241   antibodies; CA: ~30 kDa; NKCC: ~200 kDa; VHA-b subunit: ~55 kDa; PMCA: ~140 kDa; sAC:

242   ~180, 110, and 50 kDa), were sharp and distinct, and were absent in control blots in which the

243   primary antibody was omitted. No bands were detected in anti-VHA and anti-sAC antibodies'

244   pre-immune and peptide pre-absorption controls.

245   Next, we examined the expression of these proteins within specific saccular epithelial

246   cells using immunohistochemistry. NKA was abundantly expressed within cells adjacent to the

247   endolymph (Fig. 2a). Higher magnification images revealed NKA immunostaining produced a

248   dense intracellular speckled pattern (Fig. 2b), which indicates NKA is present in the highly

249   infolded basolateral membrane. Double immunolabeling with anti-complex IV antibodies

250   revealed the NKA-rich ionocytes are MR (Fig. 2c) and contain abundant NKCC (Fig. 2d).

251   Furthermore, the resulting "yellow" signal from dual NKA and NKCC immunolabeling indicated

252   a strong overlap in the basolateral membrane. CA was also highly expressed in specific saccular

253   epithelial cells; however, double immunolabeling revealed CA was present in cells that were not

254   labeled for NKA (Fig. 3a) or NKCC1 (Fig. 3b). Similarly, double immunolabeling of NKA and

255   VHA (Fig. 4a, b, c) revealed that these two proteins were expressed in different cells. By default,

256   this indicates the CA and VHA were expressed in the same cell type. Overall, these results

257   indicate the presence of two types of ionocytes in the saccular epithelium. "Type-I" ionocytes

258   abundantly express NKA and NKCC1 and are MR, and "Type-I" ionocytes abundantly express

259   CA and VHA.

260   PMCA was also abundantly expressed in saccular epithelial cells adjacent to the

261   endolymph. The pattern observed following dual immunostaining with NKA indicates PMCA is

262   present in Type-I and Type-II ionocytes (Fig. 5a, b). Unlike NKA and NKCC1 (Fig. 2d), NKA

263   and PMCA immunofluorescent signals did not overlap significantly (Fig. 5c), suggesting PMCA

264   is predominantly present in cytoplasmic vesicles and not in the basolateral membrane.

265   Additionally, abundant sAC immunolabeling was detected throughout the saccular

266   epithelium (Fig. 5d). Dual immunostaining of sAC and NKA (Fig. 5e, f) and sAC and PMCA

267   (Fig. 5g, h, i) revealed sAC was abundantly expressed in both Type-I and Type-II ionocytes.

268   Type-I and Type-II ionocytes in the meshwork area were larger than in the patches area

269   ($\sim$40 μm vs. $\sim$10 μm wide, respectively; Fig. 4b, c). However, the protein expression profile in

270   each ionocyte type was identical regardless of size. In addition to the previously reported

271   presence of PMCA (Cruz et al., 2009) and VHA (Shiao et al., 2005), we detected NKA (Fig. 2a)

272   and sAC (Fig. 5d) within the sensory hair cells. Unexpectedly, we also observed intense CA

273   (Fig. 6a, b), VHA (Fig. 6c, d), and sAC (Fig. 6e, f) immunoreactivity within the endothelial cells

274   that form the abundant capillaries surrounding the meshwork area.

275

276   **Discussion**:

277   Here, we characterized two types of ionocytes within the Pacific Chub Mackerel's

278   saccular epithelium: Type-I ionocytes are MR and express abundant NKA, NKCC1, PMCA, and

279   sAC whereas Type-II ionocytes express abundant CA, VHA, PMCA, and sAC (Fig. 8). Ionocyte

280   distribution and size patterns were similar to those reported in most previous studies (Mayer-

281   Gostan et al., 1997; Pisam et al., 1998): larger ionocytes bordered the meshwork area while

282   smaller ionocytes were found in the patches area. However, there were no differences in protein

283   expression between the larger Type-I and Type-II meshwork ionocytes and the smaller Type-I

284   and Type-II patches ionocytes, further supporting the idea that only two types of ionocytes exist

285   within the saccular epithelium. This suggests that the differences in ionic composition between

286   the proximal and distal endolymph are the result of different ion transporting rates in these two

287   regions and not due to the presence of different ion transporting mechanisms. Additional factors

288   that surely contribute to the heterogeneous endolymph ionic composition and otolith calcification

289   rates include the activity of hair cells and the secretion of glycoproteins that promote carbonate

290   precipitation, both taking place in the meshwork area and proximal endolymph (reviewed in

291   Payan et al., 2004; Allemand et al., 2008).

292   Our results on the marine Pacific Chub Mackerel generally agree with the literature about

293   ion transporting mechanisms in fish inner ear epithelia, which is largely based on research on

294   freshwater fishes. The main differences were the localization of VHA and PMCA. The former

295   was reported to be exclusively expressed in sensory hair cells in the inner ear of Zebrafish

296   embryos (Shiao et al., 2005), and the latter was only studied at the mRNA level and

297   predominantly found in hair cells as well (Cruz et al., 2009). Future experiments should confirm

298   whether the differences between Pacific Chub Mackerel and Zebrafish are species or life stage-

299   specific, environmentally based (i.e. freshwater vs seawater), or due to different immunostaining

300   techniques and antibodies.

301

302   *Putative functions of fish inner ear epithelial ionocyte function.*

303   Based on the presence of NKA and NKCC1, the Type-I ionocytes are likely responsible

304   for secreting K_+ into the endolymph, where it can reach concentrations >40 higher than in blood

305   plasma (Payan et al., 1997, 1999; Ghanem et al., 2008). Given that NKCC1 knockout results in

306   inner ear collapses due to lack of fluid in Zebrafish larvae (Abbas and Whitfield, 2009), one of

307   the roles of NKCC1-driven K_+ secretion is to osmotically drive fluid transport. Additionally, the

308   K_+-rich endolymph is essential for mechanoreception by the sensory hair cells (Zdebik et al.,

309   2009). This model would imply that Type-I ionocytes express K_+ channels in their apical

membrane, and should be further investigated in future studies. The outwardly conducting

KCNQ1/KCNE1 K+ channels found on the apical membrane of the analogous "dark" cells of

mammalian inner ear are promising candidates (Nicolas et al., 2001).

In contrast, the high abundance of CA and VHA in Type-II ionocytes suggests these cells

are involved in promoting otolith calcification by secreting HCO_3^- into the endolymph and

removing H^+. The CA-catalyzed hydration of CO_2 (for example from the abundant mitochondria

from the adjacent Type-I ionocytes) would provide HCO_3^- to be secreted into the endolymph by

yet unidentified apical anion exchangers. The H^+ that is simultaneously produced might be

removed by VHA, either into intracellular vesicles as proposed by Mayer-Gostan *et al* (1997) or

upon VHA insertion into the basolateral membrane as reported in the base-secreting cells of

elasmobranch gills (Tresguerres et al., 2005; Roa and Tresguerres, 2016).

Both Type-I and Type-II ionocytes also expressed PMCA, which has been previously

shown to be important for otolith calcification based on the effects of genetic knockdown (Cruz

et al., 2009) and pharmacological inhibition of calmodulin-antagonist of PMCA activity (Mugiya

and Yoshida, 1995). The presence of PMCA throughout the cytoplasm suggests Ca^{2+}

sequestration in vesicles, which may be transported to the apical membrane and its contents

exocytosed into the calcifying fluid as proposed in coral calcifying cells (Barott et al., 2015b;

Barron et al., 2018). Other proposed transcellular pathways for Ca^{2+} transport include Ca^{2+}

channels and Na^+/Ca^{2+} exchangers (Mugiya and Yoshida, 1995; Thomas et al., 2019), and the

identification of their cellular and subcellular localizations would contribute greatly to the

mechanistic model of otolith calcification.

A potential regulatory mechanism of otolith calcification.

Both Type-I and Type-II ionocytes contained sAC, an evolutionary conserved acid-base

sensing enzyme that produces the messenger molecule cAMP (Chen et al., 2000; Tresguerres,

2014). The effects of plasma and endolymph acid-base status on otolith calcification are well

established (Takagi, 2002; Payan et al., 2004; Allemand et al., 2008), and sAC may be one of the

underlying signaling mechanisms that senses and regulates the activity of calcification-relevant

ion transporting proteins. Supporting this possibility, some of the same ion-transporting proteins

found in Type-I and Type-II ionocytes have been shown to be under sAC regulation in many

other epithelia. In the intestine of marine teleosts, sAC senses elevations in $[HCO_3^-]$ and

341 regulates NKA and NKCC activity to promote luminal carbonate precipitation and fluid transport

342 (Tresguerres et al., 2010b; Carvalho et al., 2012). In marine elasmobranchs gills, sAC senses

343 blood alkalosis and activates VHA -and possibly the apical anion exchanger pendrin- to mediate

344 compensatory HCO_3^- secretion and H_+ absorption (Tresguerres et al., 2010c; Roa et al., 2014;

345 Roa and Tresguerres, 2016). In addition to being directly stimulated by HCO_3^-, sAC is stimulated

346 by Ca_{2+}(Litvin et al., 2003), providing another potential regulatory mechanism for otolith

347 calcification. Interestingly, sAC is also abundantly expressed in coral calcifying cells (Barott et

348 al., 2017) and in oyster mantle (Barron et al., 2012), suggesting a conserved role in regulating

349 transepithelial ion transport for calcification.

350

351 *A novel regulatory role of capillaries in regulating otolith calcification?*

352   The connective tissues surrounding the inner ear contain numerous capillaries, which are

353 especially abundant near the meshwork area (Saitoh, 1990; Mayer-Gostan et al., 1997).

354 Unexpectedly, we found the endothelial cells that form such capillaries to abundantly express

355 CA, VHA, and sAC. This is consistent with previous reports of CA within the cytoplasm of

356 capillaries in the analogous mammalian inner ear (Watanabe and Ogawa, 1984). We tentatively

357 propose that the activities of these proteins are relevant for otolith calcification by mediating the

358 transport of CO_2/HCO_3^- from the blood to the endolymph, and by facilitating the removal of

359 excess H_+ generated as a result of $CaCO_3$ precipitation. In addition, the local acidification of the

360 capillary lumen could trigger the Root effect in circulating red blood cells, thus promoting O_2

361 offloading to sustain aerobic metabolism of ionocytes and sensory hair cells within the saccular

362 epithelium. Such a mechanism was originally described in fish swim bladder and eye (reviewed

363 in Pelster, 2001), and more recently proposed to apply more broadly to other highly aerobic fish

364 tissues including the eye (Fairbanks et al., 1969), muscle (Rummer et al., 2013), and intestine

365 (Cooper et al., 2014).

366

367 *Conclusions, future directions and significance.*

368   Our proposed model is consistent with previous functional studies conducted on isolated

369 fish inner ear organ that suggested the involvement of NKA, CA, and PMCA (as well as

370 Na_+/Ca_{2+} exchanger, Ca_{2+} channels, and Na_+/H_+ exchanger) based on acid-base titration and

371 $_{45}Ca_{2+}$ incorporation experiments in combination with pharmacological inhibitors (Mugiya and

372 Yoshida, 1995; Payan et al., 1997). Furthermore, functional evidence for the roles of NKCC1

373 (Abbas and Whitfield, 2009) and PMCA (Cruz et al., 2009) is available through the genetic

374 downregulation experiments on Zebrafish larvae mentioned above. More recently, the presence

375 of many of those proteins as well as VHA has been confirmed through an extensive proteomic

376 and transcriptomic survey (Thomas et al., 2019) (with the caveat that analyses were conducted

377 on samples that contain both inner ear and brain tissue). Our results expand and complement

378 those previous studies by establishing the transporter's cellular and subcellular localization,

379 ultimately leading to the identification of two types of ionocytes. In addition, our results revealed

380 sAC is present in both types of ionocytes, providing a potential mechanism that can regulate

381 otolith calcification in response to acid-base variations. Ongoing efforts in our laboratory are

382 attempting to functionally characterize the putative regulatory role of sAC on inner ear function;

383 however, sAC's presence within both types of ionocytes, sensory hair cells, and capillaries is a

384 significant hurdle for studies at the organ and whole organism level. For example, putative

385 changes in protein or mRNA abundance in ionocytes in response to experimental manipulations

386 would be confounded by the background provided by all the other cell types in the tissue, which

387 are the majority. Thus, detailed functional studies on the underlying ion transport mechanism

388 would require the development of ionocyte primary cultures. Similar considerations apply to

389 efforts to elucidating the functional roles of CA, VHA, and sAC in the capillaries near the

390 meshwork area.

391   The inner ear organ allows fish to sense and respond to its environment and therefore is

392 essential for survival. In addition, analyses on otolith rings provide valuable information

393 regarding daily and seasonal growth bands, trace element signatures (Swearer et al., 1999),

394 exposure to environmental salinity and temperature (Campana, 1999; Elsdon and Gillanders,

395 2002), and diet (Radtke et al., 1996; Nelson et al., 2011; von Biela et al., 2015). Thus, in addition

396 to its intrinsic value from physiological and evolutionary perspectives, information about the

397 cellular mechanisms underlying otolith calcification can improve current fisheries assessment

398 tools and help predict the effects of environmental stressors, and in particular ocean acidification,

399 on otolith growth and function from a mechanistic perspective.

400

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406

407

408 **Figure Captions:**

409 Fig. 1: Western blot analysis of inner ear homogenates. Antibodies against monoclonal Na+/K+-

410   ATPase (mNKA), polyclonal Na+/K+-ATPase (pNKA), carbonic anhydrase (CA), Na+-

411   K+-Cl-co-transporter (NKCC), V-type H+ ATPase (VHA), plasma membrane calcium

412   ATPase (PMCA), and soluble adenylyl cyclase (sAC) reveal bands matching the

413   predicted size of respective proteins. Molecular marker is shown on the left of each

414   respective blot.

415 Fig. 2: Characterization of Type-I ionocytes within the saccular epithelium. Histological saggital

416   section immunostained with (**a**) Na+/K+-ATPase (NKA, green). (**b**) Magnified view of

417   the NKA-rich ionocytes revealed abundant staining in a dense, speckled pattern

418   resembling a developed basolateral infolding. Dual-immunostaining revealed the NKA-

419   rich (green) ionocyte is also (**c**) mitochondrion-rich (red) and contain abundant (**d**) Na+-

420   K+-2Cl-co-transporter (NKCC, red). Nuclei are stained blue. EN = endolymph. SO =

421   sagittal otolith protein. SHC = sensory hair cell.

422 Fig. 3: Evidence for two types of ionocytes within the saccular epithelium. Dual-immunostaining

423   of ionocytes within the saccular epithelium revealed carbonic anhydrase (CA; red) is

424   expressed in cells that are different from the (**a**) Na+/K+-ATPase (NKA; green) and (**b**)

425   Na+-K+-2Cl-co-transporter (NKCC, green)-rich Type-I ionocyte. Nuclei are stained blue.

426   EN = endolymph.

427 Fig. 4: Characterization of Type-II ionocytes within the saccular epithelium. (**a**) Histological

428   saggital section immunostained with Na+/K+-ATPase (NKA, green) and V-type H+-

429   ATPase (VHA, red). (**b**) Higher magnification image of saccular ionocytes of the larger

430   meshwork ionocytes and (**c**) the smaller patches ionocytes indicate NKA-rich and VHA-

431   rich cells are different cells. Nuclei are stained blue. EN = endolymph; SO = sagittal

432   otolith protein. SHC = sensory hair cell.

433 Fig. 5: Presence of plasma membrane Ca$_{2+}$ ATPase and soluble adenylyl cyclase in Type-I and
434   Type-II ionocytes. (**a, b, c**) Dual immunostaining of plasma membrane Ca$_{2+}$-ATPase
435   (PMCA, green) with Na$_+$/K$_+$-ATPase (NKA, red). Notice that PMCA is present in all
436   NKA-rich cells (Type-I ionocyte), as well as in adjacent cells without NKA signal (Type-
437   II ionocytes). (**d**) Histological saggital section immunostained with soluble adenylyl
438   cyclase (sAC, red) and Na$_+$/K$_+$-ATPase (NKA, green). (**e,f**) higher magnification images
439   reveal sAC is present in both the NKA-rich Type-I ionocytes (green) and Type-II
440   ionocytes (indicated by ionocytes lacking NKA signal). (**g, h, i**) The presence of PMCA
441   (green) and sAC (red) in both Type-I and Type-II ionocytes was further conformed by
442   dual-staining. Nuclei are stained blue. EN = endolymph; SO = sagittal otolith protein.
443   SHC = sensory hair cell.

444 Fig. 6: Inner ear saccular epithelium capillaries express CA, VHA, and sAC. Histological section
445   dual-stained with (**a, b**) carbonic anhydrase (CA, red) and Na$_+$-K$_+$-2Cl$_-$-co-transporter
446   (NKCC, green), (**c, d**) V-type H$_+$-ATPase-rich (VHA, green) and Na$_+$/K$_+$-ATPase (NKA,
447   green), and (**e, f**) soluble adenylyl cyclase (sAC, red) and NKA (green). Nuclei are
448   stained blue. EN = endolymph; CAP = capillary.

449 Fig. 7: Proposed model for otolith calcification by the two types of ionocytes within the inner ear
450   saccular epithelium. Abbreviations: Na$_+$/K$_+$-ATPase (NKA), Na$_+$-K$_+$-Cl$_-$-co-transporter
451   (NKCC), mitochondria (mito), carbonic anhydrase (CA), V-type H$_+$ ATPase (VHA),
452   plasma membrane calcium ATPase (PMCA), soluble adenylyl cyclase (sAC), anion
453   exchanger (AE), and K$_+$ channel (KC). Capillaries that supply O$_2$ (and potentially HCO$_{3-}$)
454   are not shown for simplicity, though they are especially important in the meshwork area.
455   Ion transport is indicated by a solid line, and gas diffusion is indicated by a dashed,
456   squiggly line.

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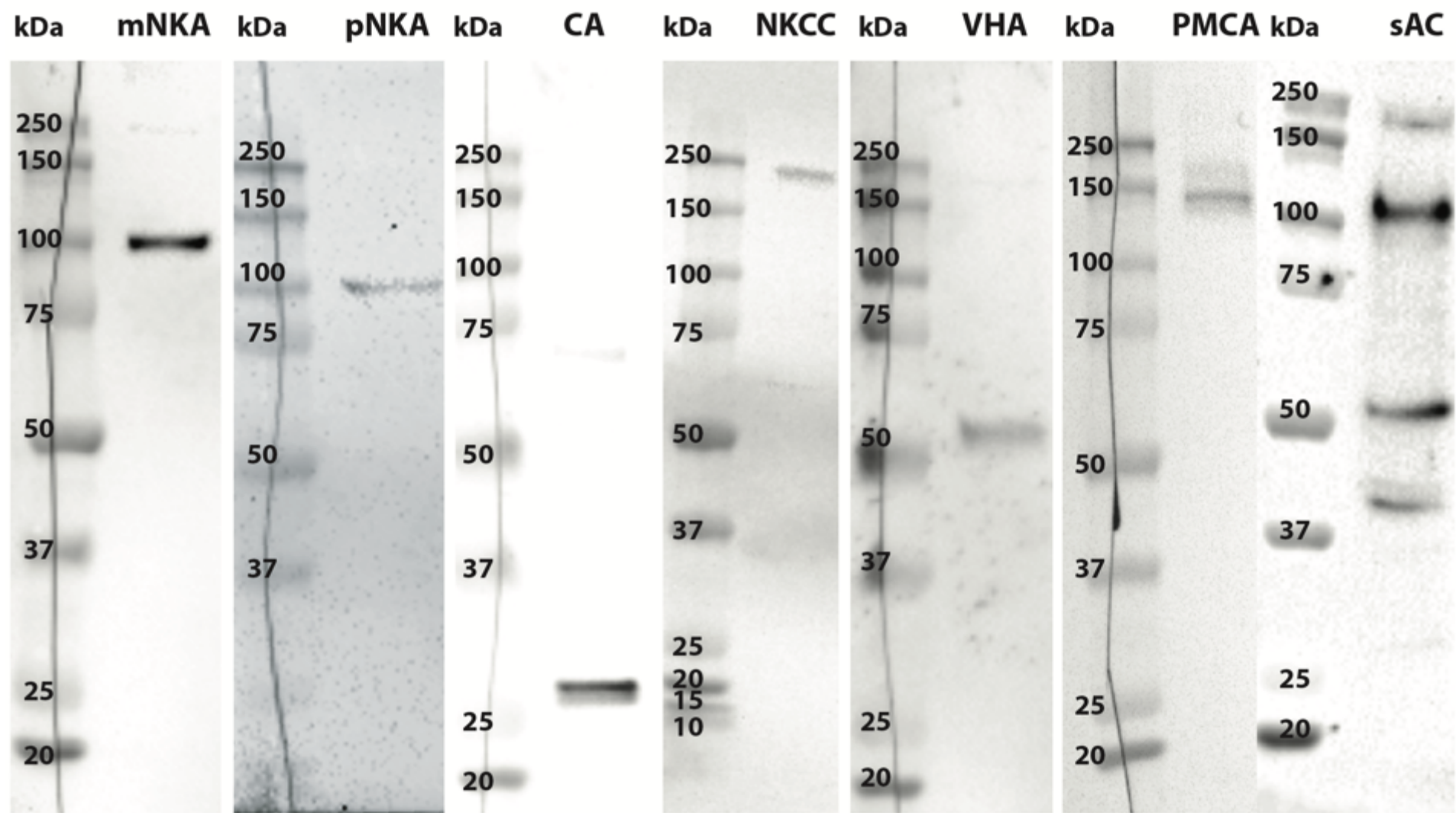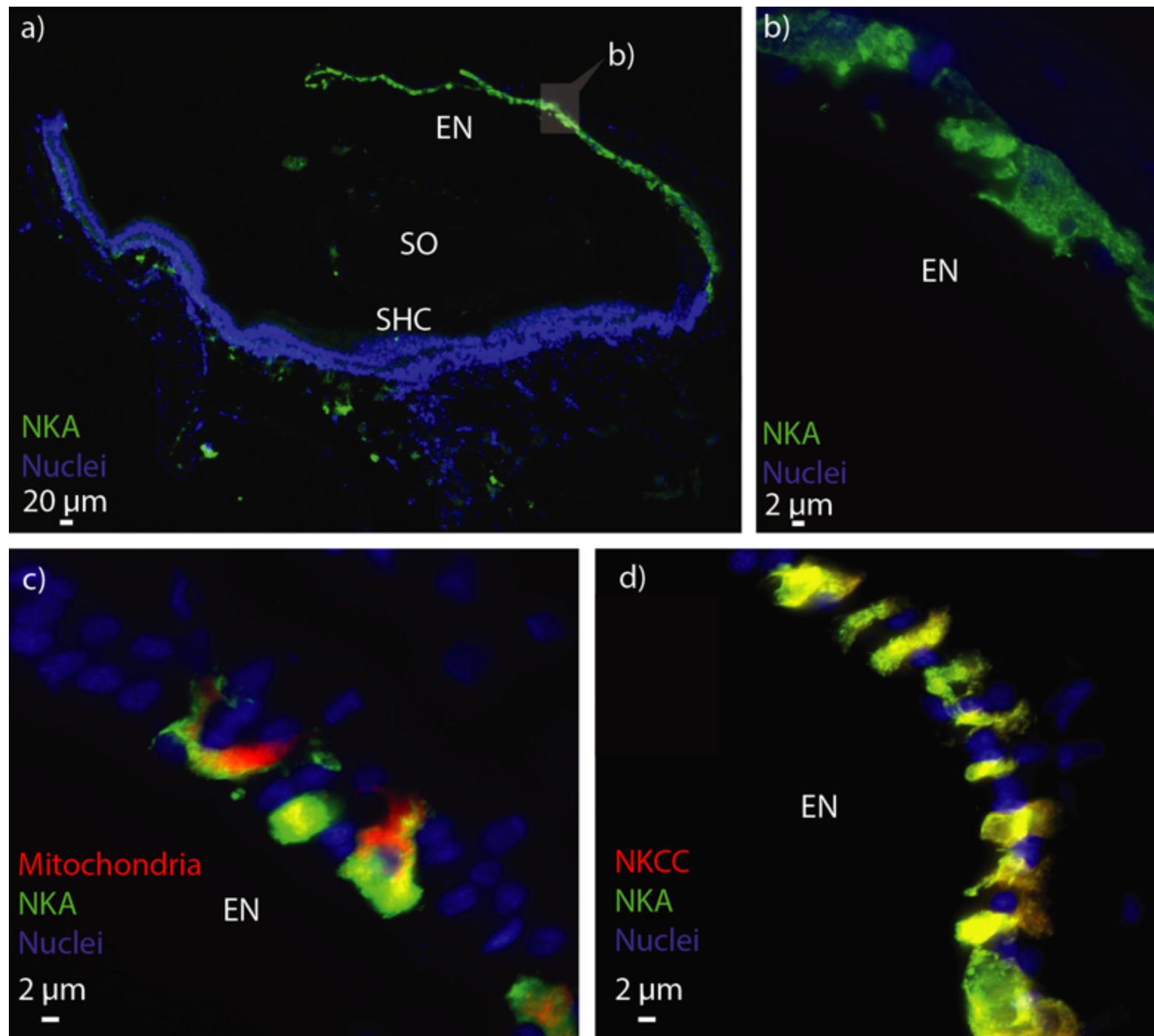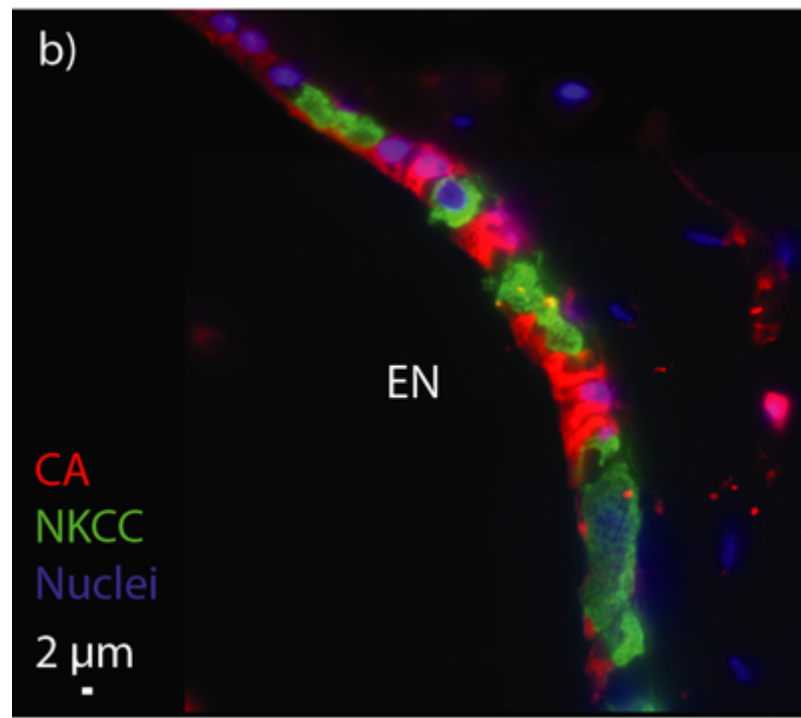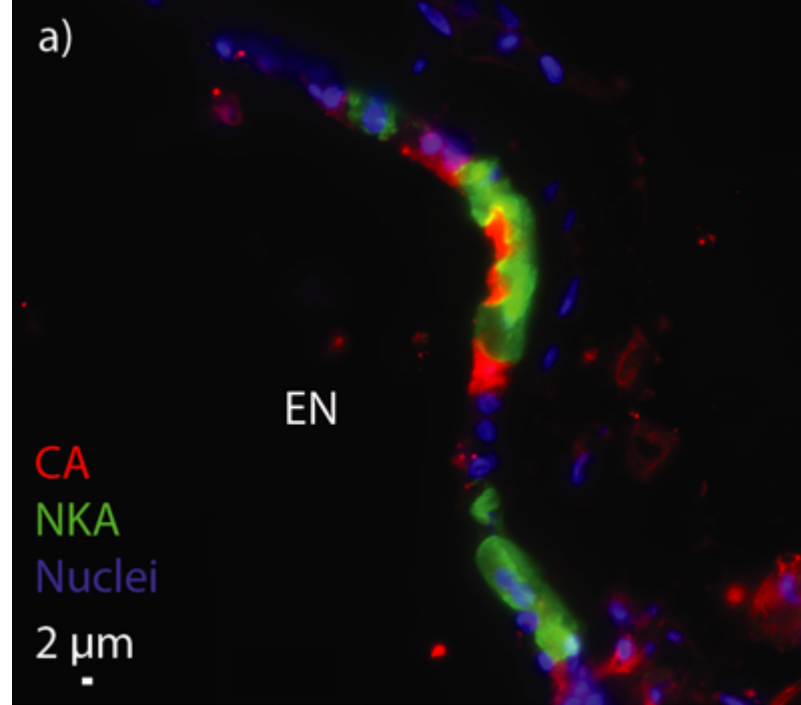
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Fig 1

Fig 2

Fig 3

a)

EN

CA
NKA
Nuclei

2 μm

b)

EN

CA
NKCC
Nuclei

2 μm

Fig 4

Fig 5

Fig 6

Fig 7