Cubam receptor-mediated endocytosis in hindgut-derived pseudoplacenta of a viviparous teleost *Xenotoca eiseni*

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Abstract

Nutrient transfer from mother to the embryo is essential for reproduction in viviparous animals. Here, we focused on the molecular mechanism of nutrient absorption in hindgut-derived pseudoplacenta (trophotaenia) of the viviparous teleost *Xenotoca eiseni*. The candidate genes involved in the process were identified by RNA-Seq, and then protein distribution and functions were investigated using molecular biological methods. Our results suggested that Cubam (Cubilin-Amnionless) receptor-mediated endocytosis and cathepsin L-dependent proteolysis are involved in the maternal macromolecule absorption via the trophotaenia. Such a nutrient absorption mechanism involving endocytosis is not a specific trait to viviparous fish. Similar processes have been reported in the larval stage of oviparous fish or the suckling stage of viviparous mammals. Our findings suggest that the viviparous teleost acquired trophotaenia-based viviparity via a modification of the intestinal absorption system common in vertebrates. This is a fundamental study to understand the strategic variation in the reproductive system of vertebrates.

Introduction

August Krogh wrote, "*For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied*" [1]. This study aimed to investigate the molecular mechanism of maternal nutrient absorption in a species-specific pseudoplacenta of a viviparous teleost species belonging to the family Goodeidae.

Viviparity is a reproduction system, whereby the oocyte is fertilized in the female body, and subsequent embryo growth occurs with maternal component supply. Each viviparous animal has acquired processes specialized to the gestation in both the mother and embryo [2]. The placenta and umbilical cords in viviparous mammals are major components of the process for mother-to-embryo material transport [3,4]. Other viviparous components such as the extended yolk sac or pseudoplacenta are known in several viviparous vertebrates, except mammals [5].

The family Goodeidae is a freshwater small teleost distributed in Mexico, which includes approximately 40 viviparous species [6]. They possess trophotaenia, which is a hindgut-derived pseudoplacenta that absorbs the maternal component [7,8]. Trophotaenia is a ribbon-like structure consisting of a single epithelial layer, internal blood vessels, and connective tissues [9,10]. The epithelial cell is like an enterocyte in the intestine. Electron microscopy indicated that microvilli form in the apical side of the cell and intracellular vesicles in the cytoplasm [11]. Since the 1980s, these structures have been believed to be involved in maternal component absorption [12]. The nature of the maternal component was predicted to be proteins or other macromolecules secreted into the serum or ovarian fluids; however, no one has experimentally determined its distinct component [13,14]. Recently, we demonstrated that a yolk protein vitellogenin is secreted into the ovarian lumen of pregnant females, and the intraovarian embryo absorbs the nutrient protein via the trophotaenia using a goodeid species *Xenotoca eiseni* [15]. In that study, enterocyte-like microvilli and intracellular vesicles were also observed in the epithelial cells of the

68 trophotaenia. We hypothesized that the epithelial layer cell in the trophotaenia absorbs the maternal protein
69 and/or other components as macromolecules because the ovarian lumen lacks proteolysis activity like the
70 digestive intestine. However, the molecules responsible for the trophotaenia-mediated macromolecule
71 absorption have not been reported.

72 Vacuolated enterocytes involved in macromolecule absorption have also been reported in other vertebrate
73 species, including suckling mammals and stomachless fish [16-18]. Park et al. [19] reported that the
74 scavenger receptor complex Cubam (Cubilin-Amnionless) and Dab2 are required for macromolecule
75 uptake in zebrafish juveniles. Furthermore, the conditional knockout of *Dab2* in mice led to stunted growth
76 and severe protein malnutrition at the suckling stage [19]. Based on this report, we discuss the
77 commonality of molecular process for the macromolecule absorption between the intestinal enterocytes
78 and the trophotaenia [15]. However, the molecules responsible for macromolecule absorption in the
79 trophotaenia are still elusive.

80 Here, we report candidate molecules for nutrient uptake and subsequent proteolysis in the trophotaenia of
81 *X. eiseni*. An RNA-Seq for trophoteniae indicated candidate receptor molecules and
82 endocytosis-associated proteases expressed in the trophotaenia. Immunohistochemistry and biochemical
83 assays suggested the presence and functions of the candidate factors in the trophotaenia.

Results

Gene expression in trophotaenia

Our previous findings and the hypothesis based on that are described in Figure 1A. In a viviparous teleost, *X. eiseni*, the embryo is raised in the ovary while receiving maternal nutrients. The trophotaenia is a pseudoplacenta that plays a role in the absorption of maternal nutrients consisting of proteins and other supplements. Based on previous studies, we hypothesized that several maternal proteins are absorbed by endocytosis-mediated proteolysis in the epithelial cells of the trophotaenia. To verify this hypothesis, we explored candidate genes for receptor, adaptor, vesicle, and protease proteins that are highly expressed in the trophotaenia. RNA-Seq analyses were performed using total RNA extracted from the trophotaeniae of the 3rd- or 4th-week embryos (Figure 1B). We selected candidate genes and compared their predicted expression level as transcription per million (TPM) values that were calculated using the known transcript sequence of *P. reticulata* as reference (Dataset S1). RNA-Seq suggested that *cubilin* (*cubn*) and *amnionless (amn*) were highly expressed in the trophotaeniae; however, other co-receptor genes (*lrp1aa*, *lrp2a*) were considerably lower (Figure 1C, Table S1). Adaptor protein-2 (AP2) subunit genes (*ap2a1*, *ap2b1*, *ap2m1a*, and *ap2s1*) were more highly expressed than the other family adaptor genes (*ldlrap1b* and *numb*) (Figure 1C, Table S2). Two families of the vesicle coating protein genes (*clta*, *cltbb*, *cltc*; *flot1b*, *flot2a*) were expressed higher than the vesicle proteins classified in other families (*cav2* and *cav3*) (Figure 1C, Table S3). The lysosomal endopeptidase enzyme gene (*ctsl.1*) exhibited a high TPM value (> 10,000) rather than that of not only protease family genes but also most of the genes expressed in the trophotaeniae (Figure 1C, Table S4). The trend of the predicted TPM values in each gene was not noticeably different between the 3rd- and 4th- week samples.

106 *Sequences for Cubam receptor genes*

107 A Cubam receptor is known to be a membrane-bound multi-ligand receptor consisting of Cubn and Amn
108 (Figure 2A). The secreted protein Cubn specifically binds to the transmembrane protein Amn; thus, the
109 CUB domain that associates with the ligands are localized on the apical surface of the plasma membrane.
110 We obtained the amino acid sequences of *X. eiseni* Cubn and Amn by *de novo* assembly of NGS reads
111 from the trophotaeniae. The sequences for Cubn and Amn were compared between four vertebrate
112 species, *Homo sapiens*, *Danio rerio*, *P. reticulata*, and *X. eiseni.* The binding motifs were conserved in the
113 species (Figure 2B and C). The intracellular domain of Amn included two conserved motifs to bind the
114 adaptor proteins, including AP2. The motifs were also conserved in the species (Figure 2D).

115

116 *Distribution of Cubilin and Amnionnless in trophotaenia*

117 To validate the expression of *cubn* and *amn* in the trophotaenia, semi-quantitative RT-PCR analyses were
118 performed using total RNAs extracted from the whole-embryo including the trophotaeniae, isolated
119 trophotaeniae, and adult skeletal muscle. The muscle sample was used as a control for tissue with low
120 endocytosis activity. The gene expression patterns did not conflict between the results of RT-PCR and
121 RNA-Seq. In the embryo and trophotaenia, both *cubn* and *amn* were more highly expressed than *lrp2a*
122 (Figure 3A). Conversely, no or few expressions of the target genes except *actb* as a positive control were
123 detected in the adult muscle. Next, to detect protein localization, immunohistochemistry was performed
124 using antibodies against Cubn or Amn. In both proteins, strong signals were observed in the epithelial
125 monolayer of the trophotaenia, while few background noises were detected in the control assay (Figure
126 3B-D, see also Figure S1 and S2). Confocal microscopy revealed the cellular distribution of the anti-Cubn
127 signals. The signals in the apical surface of the epithelial cell were detected as a homogeneous distribution,

128 while almost all signals in the cytoplasm were captured as a dot pattern (Figure 3E, Figure S3).
129 Immunoelectron microscopy revealed that the microvilli were distributed on the apical surface of the
130 trophotaenia epithelium, and intracellular vesicles were observed in the cytoplasm; furthermore, anti-Cubn
131 signals were distributed in the intracellular vesicles and overlapped with the microvilli on the apical surface
132 (Figure 3F).

133

134 *Proteolysis activity in trophotaenia*

135 Cathepsin L is a lysosomal cysteine proteinase characterized by three conserved protease regions and
136 active sites consisting of cysteine, histidine, or asparagine (Figure 4A). The functional regions were
137 conserved in the *X. eiseni* Cathepsin L protein translated from the coding sequence of *ctsl.1*. RT-PCR
138 analysis revealed a strong expression of *ctsl.1* in the trophotaenia (Figure 4B). To identify which type of
139 cells include proteolysis activity in the trophotaenia, acidic organelles including lysosomes and endosomes
140 were detected using a fluorescent probe. The LysoTracker® indicated the presence of acidic organelles in
141 the epithelial layer cells (Figure 4C). The signals were distributed in the cytoplasm and were not
142 components in the nuclei (Figure 4D). According to the RNA-Seq analysis, *ctsl.1* was presumed to be the
143 highest expressed *cathepsin* gene in the trophotaenia; thus, we calibrated the proteolysis activity of
144 cathepsin L in the trophotaenia using a fluorescent substrate-based measurement system. The
145 fluorescence indicating substrate digestion was significantly higher in the trophotaenia lysate than in the
146 control at one h after the reagent mixture. Furthermore, the intensity of the lysate condition continued to
147 increase for 7 h (Figure 4E, Table S5). Conversely, the increase in intensity was strongly suppressed by a
148 cathepsin L inhibitor.

149

150 *Adaptors and vesicle coating proteins*

151 The expression of candidate genes for adaptors (*ap2a1*, *ap2b1*, *ap2m1a*, *ap2s1*, *ldlrap1b*, and *numb*) and
152 vesicle coating proteins (*clta* and *cltc*) were confirmed by RT-PCR (Figure S4). We determined an
153 incomplete transcript for *X. eiseni* Dab2 from the *de novo* assembly; however, it lacked internal sequences
154 in comparison with the proteins in other vertebrates (Figure S5). Furthermore, no amplicons were obtained
155 by RT-PCR using primers designed based on the Dab2-like sequence.

156

157 **Discussion**

158 RNA-Seq analyses revealed high expression of the genes for receptor-mediated endocytosis in the
159 trophotaenia of *X. eiseni* embryos. Cubn and Amn form a Cubam receptor complex that associates with
160 Vitamin-B12, albumin, transferrin, and other ligands [20,21]. The predicted amino acid sequences for *X.*
161 *eiseni* Cubn and Amn obtained from the *de novo* assembly both possessed a conserved motif which
162 allowed binding to each other, and Amn retained adaptor binding sites in the intracellular region [21, 22].
163 Immunohistochemical analysis indicated the presence of Cubn not only in the apical surface of the
164 epithelial layer but also in the intracellular vesicles in the cells, suggesting the incorporation and recycling
165 of endocytic receptors [23,24]. This evidence supports the idea that Cubam plays a role as a receptor for
166 the intraovarian nutrients and is involved in endocytosis. We also indicated the low presence of Lrp2 (also
167 known Megalin), which is a major co-receptor for Cubam [25]. This indicated that Cubam works
168 independently or in cooperation with other co-receptors, except Lrp2, in the trophotaenia. In zebrafish, a
169 previous study reported that Cubam-dependent endocytosis in the lysosome rich enterocytes (LREs) does
170 not require the presence of Lrp2 [19]. This supports our hypothesis; however, we do not exclude the
171 possibility that our sequencing and alignment processes could not catch other *lrp2* orthologous genes in *X.*
172 *eiseni*. Park et al. [19] also reported that Dab2 is an essential adaptor molecule not only in the zebrafish
173 larval intestine but also in the endocytic nutrient absorption in the ileum of suckling mice. However, we
174 obtained only an incomplete sequence for the *X. eiseni* Dab2-like protein without an internal region
175 compared with mammalian orthologs. This may be an alternative splice form with less endocytic activity
176 [26,27]. Furthermore, we did not detect Dab2-like expression by RT-PCR, and we could not exclude the
177 possibility that the predicted Dab2-like sequence is caused by an assembly error. Thus, the contribution of
178 Dab2 to endocytosis in the trophotaenia has not been confirmed in this study. The other adaptor or vesicle
179 coating proteins we detected were typical co-factors for receptor-mediated endocytosis. Conversely,

180 Caveolin is a vesicle coating protein involved in receptor-independent endocytosis [28]. Thus, a low
181 expression of *caveolin* genes (*cav2* and *cav3*) does not conflict with the activation of Cubam-mediated
182 endocytosis. Additionally, biochemical assays supported that cathepsin L is an active protease that
183 functions in the intracellular vesicles that is configured following membrane budding. This evidence
184 supports the idea that Cubam-mediated endocytosis and cathepsin L-dependent proteolysis is one of the
185 key mechanisms for the absorption of the maternal component in *X. eiseni* embryos.

186 Cubam is also known to be a scavenger receptor involved in nonspecific protein uptake [20]. In this study,
187 we did not determine the Cubam ligands in the trophotaenia. However, the candidates are limited to
188 intraovarian secreted proteins. In several goodeid species, previous studies indicated that the ovarian
189 fluids include various proteins in a pattern similar to that in the blood serum [13,29]. Therefore, several
190 serum proteins, albumen, transferrin, and others known as Cubam ligands, are potential targets in the case
191 of intraovarian nutrient absorption via the trophotaenia. Another possibility is that vitellogenin is also a
192 potential target because it is secreted into the ovarian fluids and is absorbed into the trophotaenia via
193 intracellular vesicles [15]. Another study suggested that the Cubn-Amn-Lrp2 receptor complex is related to
194 transport of yolk proteins, including vitellogenin, in endodermal epithelial cells during chicken yolk sac
195 growth [30]. These reports support the idea that Cubn and Amn are potent candidate molecules for a
196 receptor complex involved in maternal component uptake in the trophotaenia. However, functional
197 analyses such as gene knockout or transgenic technologies used in conventional model animals, such as
198 laboratory mice or small oviparous fish, have not been applied to goodeid fish. For a direct validation of our
199 hypothesis, these reverse genetic methods should also be developed in the viviparous teleost.

200 As described above, endocytosis-mediated nutrient absorption is not limited to viviparous fish; it has also
201 been reported in other animal species (Figure 5). In mammals, macromolecule absorption in the intestine is
202 limited to the suckling period [31]. In the stomachless fish, endocytosis-mediated absorption is considered

203 to continue in part of the intestine for life, because of low digestive activity in the enteric cavity [18]. In most
204 invertebrate species, because the extracellular digestive system is primitive, food particles are absorbed
205 into the intestinal cells by vesicle trafficking and degraded by intracellular digestion [32, 33]. Thus, we
206 hypothesize that endocytic nutrient absorption and intracellular digestion are ancestral traits common not
207 only in vertebrates but also in invertebrates, and their importance has decreased in certain vertebrates with
208 development of the extracellular digestive system in the enteric cavity. The ancestor of the goodeid species
209 may have applied the endocytic process to the reproductive system, and then configured the unique
210 hind-gut derived pseudoplacenta for matrotrophic nutrition during gestation [7,34]. To validate our
211 hypothesis, further exhaustive omics analyses using the goodeid species and comparative research using
212 the subfamily Empetrichthyinae, which is the oviparous species most closely related to the viviparous
213 goodeid fish, are required [6,35].

214 This study is an investigation of species-specific traits based on the transcriptome of a non-conventional
215 model species. The results revealed potential candidate molecules for nutrient absorption in the
216 pseudoplacenta of the viviparous teleost. As August Krogh wrote, this kind of study would be suitable for
217 investigation using the most appropriate species and is unsuitable for verification using alternative models
218 such as viviparous rodents or oviparous teleosts. We believe that this study is an important and
219 fundamental step in understanding the strategic variation of the reproductive system in vertebrates.
220

Methods

Animal experiments

This study was approved by the ethics review board for animal experiments at Nagoya University (Approval number: AGR2020028). We sacrificed live animals in minimal numbers under anesthesia according to the institutional guidelines.

Fish breeding

X. eiseni was purchased from Meito Suien Co., Ltd. (Nagoya, Japan). Adult fish were maintained in freshwater at 27 °C under a 14:10-h light: dark photoperiod cycle. Fish were bred in a mass-mating design, and approximately 30 adult fish were maintained for this study. The juveniles were fed live brine shrimp larvae, and the adults were fed Hikari Crest Micro Pellets and ultraviolet-sterilized frozen chironomid larvae (Kyorin Co., Ltd., Himeji, Japan). To accurately track the pregnancy period, the laboratory-born fish were crossed in a pair-mating design, and the mating behavior was recorded.

Sample collection

Fish samples were anesthetized using tricaine on ice before the surgical extraction of tissues or embryos. The obtained samples were stored on ice until subsequent manipulations. In this study, we dissected approximately 10 pregnant females and extracted 15–30 embryos in each operation.

240 *RNA-Seq*

241 Total RNA was extracted from trophotaenae of the 3rd or 4th week of the embryo extracted from the
242 pregnant female using the RNeasy Plus Mini kit (QIAGEN). A total of six samples were obtained from three
243 3rd week embryos and three 4th week embryos. Next-generation sequencing (NGS) was outsourced to
244 Macrogen Japan Corp. (Kyoto, Japan) using NovaSeq6000 (Illumina, Inc., San Diego, CA, USA).
245 Approximately 60 million 150-bp paired-end reads were obtained in each sample. The NGS data was
246 deposited to the DNA Data Bank of Japan (DDBJ, ID: DRA011209). *De novo* assembly and mapping to the
247 reference sequence were performed by CLC Genomics Workbench(Filgen, Inc., Nagoya, Japan). The
248 published transcript sequences of*Poecilia reticulata* (NCBI Genome, ID: 23338) was used as a reference.
249 The transcript sequences of *X. eiseni* were deposited into the DDBJ. The accession numbers were listed in
250 Table S6.

251

252 *Reverse transcription (RT) PCR*

253 Total RNA was extracted from tissues or whole embryos using the RNeasy Plus Mini kit and
254 reverse-transcribed using SuperScript IV reverse transcriptase (Thermo Fisher Scientific). PCR was
255 carried out using KOD-FX-Neo (Toyobo, Osaka, Japan) under the following conditions: 100 s at 94 °C,
256 followed by 32 cycles of 20 s at 94 °C, 20 s at 60 °C, 60 s at 72 °C, and 120 s at 72 °C. Primer sequences
257 are listed in the Resource List in Table S6.

258

259 *Antibodies and antiserums*

260 Antiserums against Cubn and Amn were generated in this study. The antigen sequences were 6×
261 His-tagged peptide of 151 amino acids (aas) corresponding to the intermediate region of *X. eiseni* Cubn
262 (Accession#, LC595284; aa 691–841) and 6× His-tagged peptide of 247 aas corresponding to the
263 C-terminal of *X. eiseni* Amn (Accession#, LC595285). The experimental procedure has been described in
264 our previous study [15]. All antibodies and antiserums used in this study are listed in Table S7.

265

266 *Immunohistochemistry*

267 Tissue samples were fixed in 4.0% paraformaldehyde/phosphate-buffered saline (PFA/PBS) at 4 °C
268 overnight. Samples were permeabilized using 0.5% TritonX-100/PBS at room temperature for 30 min.
269 Endogenous peroxidase was inactivated by 3.0 % hydrogen peroxide/PBS for 10 min. Then, the sample
270 was treated with Blocking-One solution (Nacalai Tesque, Kyoto, Japan) at room temperature for 1 h.
271 Primary antibody or antiserums were used at 1:500 dilution with Blocking-One solution. Samples were
272 incubated with primary antibody or antiserum at 4 °C overnight. Secondary antibodies were used at a 1:500
273 dilution in 0.1% Tween-20/PBS. Samples were treated with the secondary antibody solution at 4 °C for 2 h.
274 We performed a 3,3'-Diaminobenzidine Tetrahydrochloride (DAB) color development using the DAB
275 Peroxidase Substrate Kit, ImmPACT (Vector Laboratories, Inc., Burlingame, CA, USA). Microscopic
276 observation was performed using an Olympus BX53 microscope and photographed using a DP25 digital
277 camera (Olympus, Shinjuku, Japan).

278

279 *Fluorescent Immunohistochemistry*

280 Tissue samples were fixed in 4.0% PFA/PBS at 4 °C overnight. Samples were permeabilized using 0.5%

281 TritonX-100/PBS at room temperature for 30 min. Endogenous peroxidase was inactivated by 3.0 %
282 hydrogen peroxide/PBS for 10 min. Then, the sample was treated with Blocking-One solution (Nacalai
283 Tesque, Kyoto, Japan) at room temperature for 1 h. Primary antibody (anti-Cubn) was used at a 1:500
284 dilution with Blocking-One solution. Samples were incubated with primary antibody at 4 °C overnight.
285 Secondary antibody was used at a 1:500 dilution in 0.1% Tween-20/PBS with
286 4',6-diamidino-2-phenylindole (DAPI). Samples were treated with the secondary antibody solution at 4 °C
287 overnight. Microscopic observation was performed using a Leica TCS SP8 microscope (Leica
288 Microsystems, Wetzlar, Germany).

289

290 *Immunoelectron microscopy*

291 Embryo samples were fixed in 4.0% PFA/PBS. Fixed samples were washed in PBS, then reacted with a
292 primary antibody (anti-Cubn) at 4 °C overnight, and then reacted with biotinylated anti-rabbit IgG (Vector,
293 Burlingame, CA, USA) at room temperature for 2 h. Samples were performed with the
294 avidin–biotin–peroxidase complex kit (Vector), and visualized with 0.05% DAB (Dojindo Laboratories,
295 Kumamoto, Japan) and 0.01% hydrogen peroxide in 50 mM Tris buffer (pH 7.2) at room temperature for 10
296 min. The procedure for electron microscopy is described in our previous study [15].

297

298 *Labeling acidic organelles*

299 The live embryos immediately after extraction from the pregnant female at the 4th-week post-mating were
300 incubated in PBS with a 1:1000 dilution of LysoTracker® Red (Thermo Fisher Scientific) for 1 h at room
301 temperature. The samples were fixed with 4.0 % PFA/PBS and stained with DAPI. Microscopic observation

302 was performed using a Leica DM5000 B microscope (Leica Microsystems, Wetzlar, Germany).

303

304 *Measurement of Cathepsin L activity*

305 The trophotaenia lysate was prepared from six littermate embryos obtained from the pregnant females at
306 the 4th-week post-mating. The trophotaeniae were manually extracted from the embryo under a microscope.
307 Proteolysis detection was performed using the Cathepsin L Activity Fluorometric Assay Kit (BioVision, Inc.,
308 Milpitas, CA, USA). The fluorescence intensities were measured using a Qubit 4 Fluorometer (Thermo
309 Fisher Scientific).

310

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Data accessibility

The data that support the findings have been provided with the manuscript.

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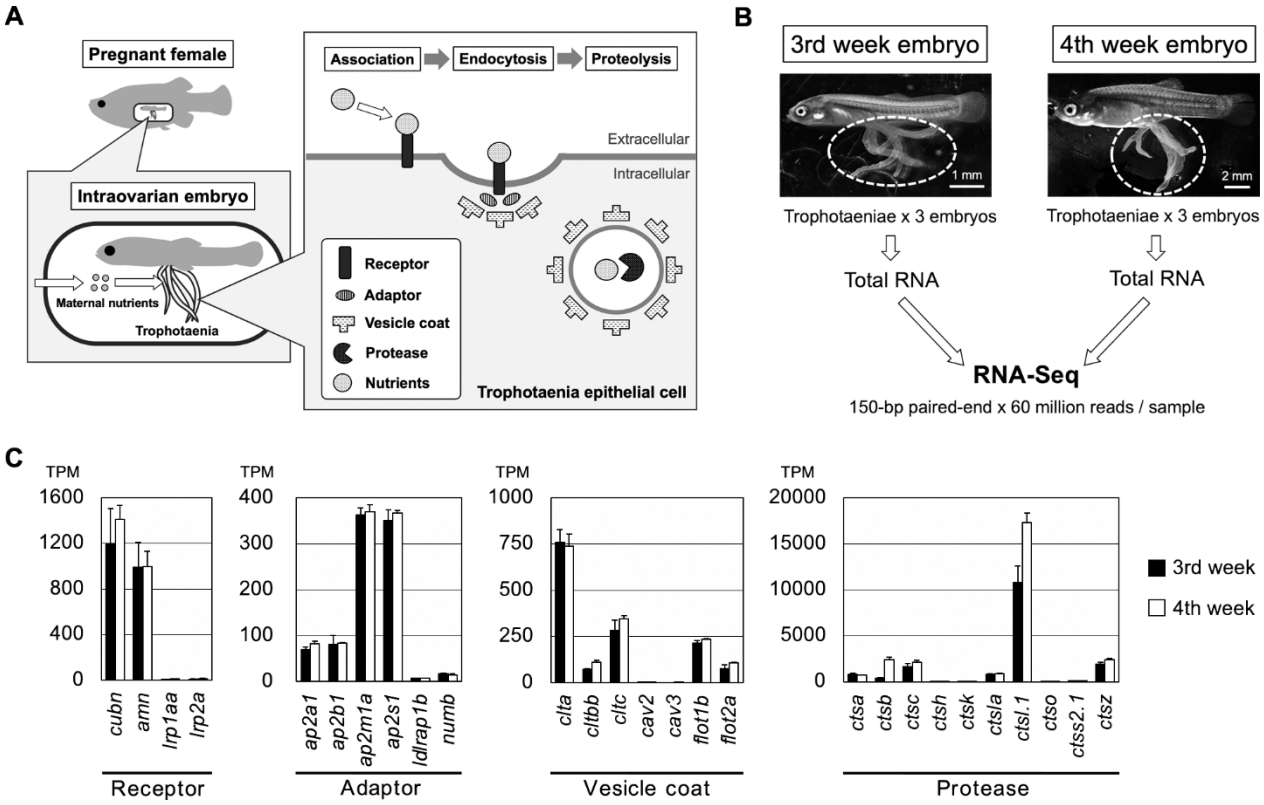

Figure 1. Exploring candidate genes for nutrient absorption

A. A working model and hypothesis of this study. In the goodeid viviparous fish (*X. eiseni*), intraovarian embryo absorbs maternal nutrients via the trophotaenia. We hypothesized that endocytosis-mediated proteolysis is related to nutrient absorption. Based on the scenario, potential target genes are for endocytic receptors, adaptors, vesicle coating proteins, and proteases. **B**. An experimental scheme for the RNA-Seq analysis. RNA samples were obtained from the trophotaeniae (white dotted line) of the single intraovarian embryos extracted from the pregnant females of the 3rd- or 4th-week post-mating. The RNA-Seq was performed using three samples at every stage. **C**. The graphs indicate the TPM values of the genes selected from the RNA-Seq data that are involved in the endocytosis-mediated proteolysis pathway.

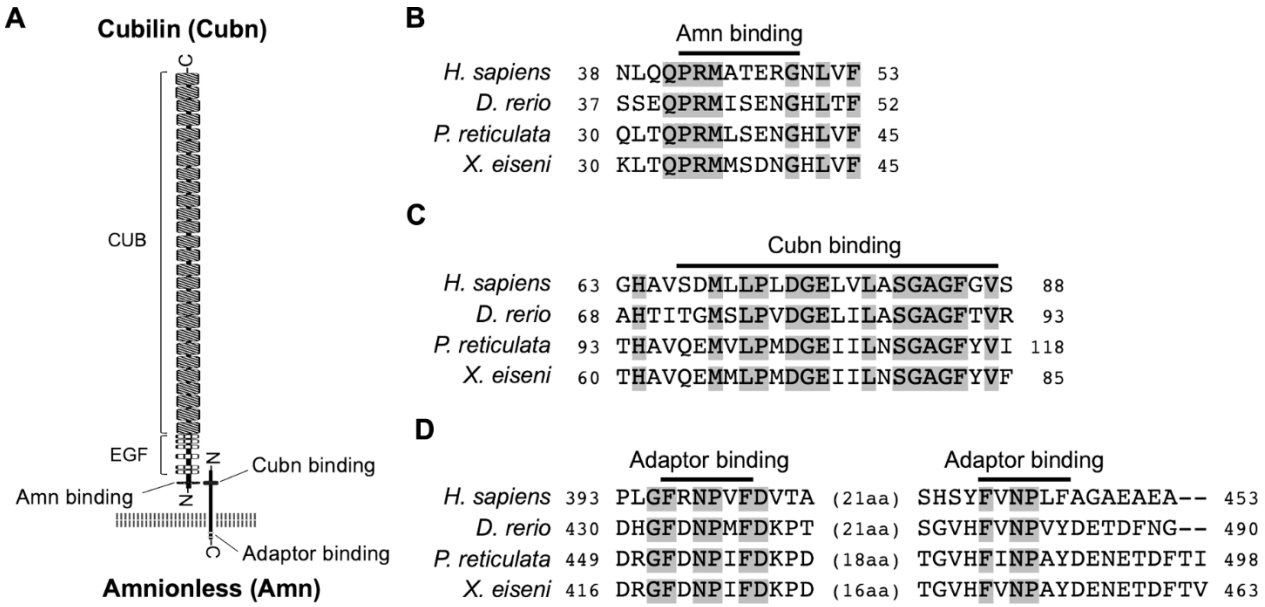

Figure 2. Structures and amino acid sequences for *X. eiseni* Cubn and Amn

A. The illustration indicates that a typical structure of Cubam receptor complex consists of Cubn and Amn. Both proteins possess a conserved motif to bind each other in the N-terminal regions, and the Amn possesses two adaptor binding motifs in the C-terminal intracellular domain. **B-D**. A comparison of amino acid sequence around the Amn-binding motif in Cubn (**B**), Cubn-binding motif in Amn (**C**), and adaptor binding motifs in Amn (**D**) between *X. eiseni* and three other vertebrate species. The gray filled texts are conserved sequences among the four species.

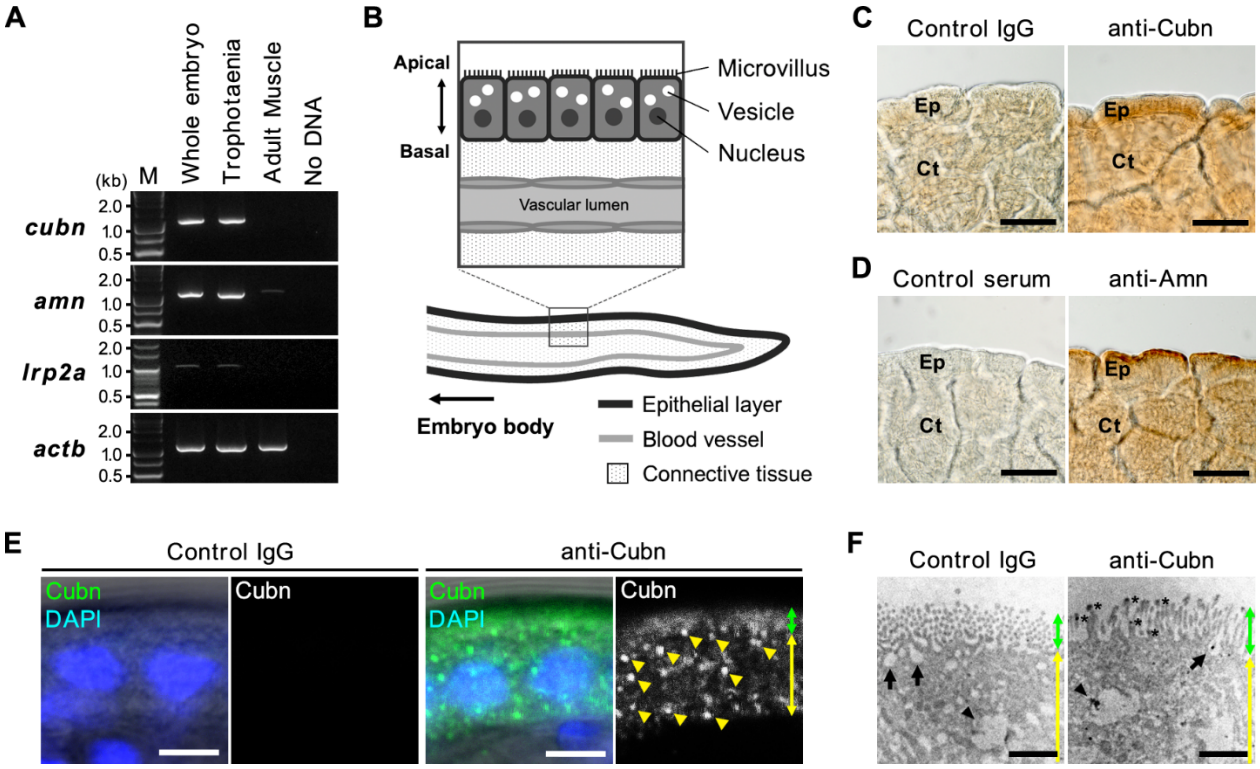

Figure 3. Distribution of receptors involved in endocytosis

A. RT-PCR for the candidate genes for the receptors involved in the endocytosis. All amplicons were detected as single band on the expected sizes based on the transcript sequences obtained from the *de novo* assembly. **B**. The illustration indicates an internal structure of the trophotaenia. An epithelium cell layer configures an outermost structure of the trophotaenia, which contacts the ovarian luminal fluids. The layer consists of an enterocyte-like cell with microvilli on the apical surface and vesicles in the cytoplasm. **C-D**. Immunohistochemistry using the Cubn antibody or Amn antiserum in the trophotaenia. In both samples, DAB color development was observed in the epithelial layer. Ep, epithelial layer. Ct, connective tissue. Scale bar, 50 μm. **E**. Confocal microscopy images of fluorescent immunohistochemistry using the Cubn antibody in the epithelial layer cells. Yellow triangles indicate the dotted signals in the epithelial cells of the trophotaenia. Green double-headed arrow indicates the apical surface defined by the flat signal.

467 Yellow double-headed arrow indicates the cytoplasmic region including the dotted signals. Scale bar, 5 μm.

468 **F.** Immunoelectron microscopy using the Cubn antibody in the epithelial layer cells. Green double-headed

469 arrow indicates the microvilli on the apical surface. Yellow double-headed arrow indicates the cytoplasmic

470 region including the dotted signals. Asterisks indicate anti-Cubn signals in the microvilli. Arrows indicate

471 endocytic vesicles in the invagination phase. Arrowheads indicate intracellular vesicles after endocytosis.

472 Anti-Cubilin signals were observed in both vesicles. Scale bar, 1 μm.

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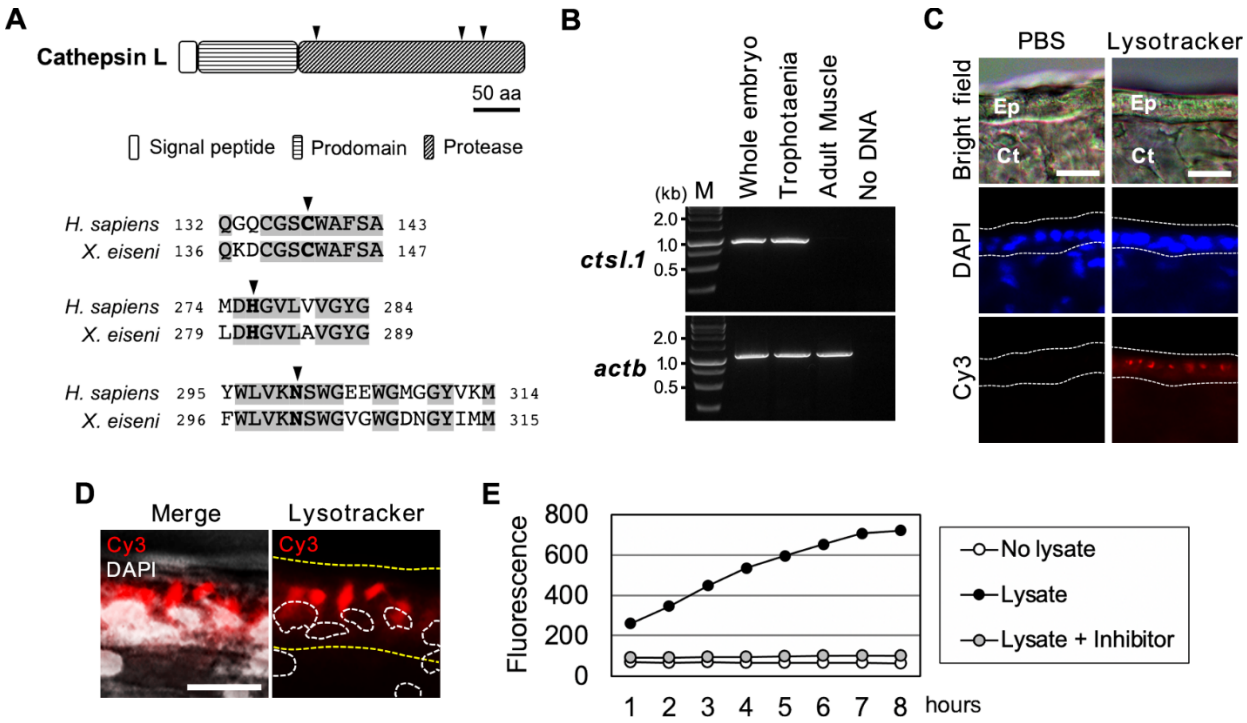

Figure 4. Proteolysis activities in trophotaenia

A. The illustrations indicate a typical structure of cathepsin L and a comparison of the protease domains of cathepsin L between *H. sapiens* and *X. eiseni*. The gray filled texts are conserved sequences among the species. The black triangles indicate protease active sites. **B**. RT-PCR for *cstl.1* exhibited the highest TPM value in the RNA-Seq analysis. The amplicons were detected as a single band on the expected size based on the transcript sequences obtained from the *de novo* assembly. **C**. Labeling of acidic organelles including lysosomal vesicle in the trophotaenia. The lysotracker treatment exhibited red fluorescence in the epithelial layer (white dotted line). Ep, epithelial layer. Ct, connective tissue. Scale bar, 20 μm. **D**. High magnification image of the lysotracker-treated epithelial cell layer of the trophotaenia. Yellow dotted line indicates the epithelial layer and white dotted circles are the nuclei of the epithelial cells. The lysotracker fluorescence was observed in the cytoplasm. Scale bar, 10 μm. **E**. Measurement of cathepsin L activity based on fluorescent substrate degradation. The vertical line indicates the accumulation of cleaved fluorescent substrates, which means cathepsin L activity in the sample solution. The fluorescent value of the

488 trophotaenia lysate sample was increased by 8 h after the reaction started.

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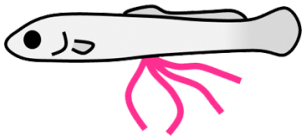
	Viviparous mammal (*Mus musculus*)	Stomachless fish (*Danio rerio*)	Viviparous fish (*Xenotoca eiseni*)
			
Stage	Suckling	Larva ~ Adult	Intraovarian embryo
Tissue	Intestine (Ileum)	Intestine (LREs)	**Trophotaenia**
Factor	*Dab2*	*cubn*, *dab2*	*cubn*, *amn*, *ctsl.1*
Target	Oral food (Milk)	Oral food (Normal diet)	**Maternal supplement**

Figure 5. Comparison of endocytosis-mediated nutrient intake in vertebrates

Endocytosis-mediated nutrient intake has also been reported in a viviparous mammal and stomachless fish. In these species, oral ingestion macromolecules are absorbed from a region of the intestine via endocytosis. In contrast, intraovarian embryos of the viviparous fish (family Goodeidae) absorbs the maternal component in the ovarian fluids from the trophotaeniae via endocytosis. Endocytosis in each species is predicted to be driven by similar molecular process in the enterocytes of the intestine or the enterocyte-like cells of trophotaenia; however, the biological ontology would be divergent between the viviparous fish (intraovarian growth) and the others (feeding after birth).