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Embryonic expression patterns of panarthropod Teneurin-m/odd Oz genes suggest a possible function in segmentation

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Abstract

Background A hallmark of arthropods is their segmented body, and the so-called *Drosophila* segmentation gene cascade that controls this process serves as one of the best-studied gene regulatory networks. An important group of segmentation genes is represented by the pair-rule genes (PRGs). One of these genes was thought to be the type-II transmembrane protein encoding gene *Tenascin-m* (*Ten-m* (aka *odd Oz*)). *Ten-m*, however, does not have a pair-rule function in *Drosophila*, despite its characteristic PRG-like expression pattern. A recent study in the beetle *Tribolium castaneum* showed that its *Ten-m* gene is not expressed like a segmentation gene, and hence is very unlikely to have a function in segmentation.

Results In this study, I present data from a range of arthropods covering the arthropod tree of life, and an onychophoran, representing a closely related group of segmented animals. At least one ortholog of *Ten-m/odz* in each of these species is expressed in the form of transverse segmental stripes in the ectoderm of forming and newly formed segments – a characteristic of genes involved in segmentation.

Conclusions The new expression data support the idea that *Ten-m* orthologs after all may be involved in panarthropod segmentation.

Introduction

63

64 Teneurins represent a highly-conserved family of type-II transmembrane-protein encoding

65 genes that possess a complex and invariant complement of functional domains (e.g. Tucker,

66 2018; DePew et al., 2019; Schöneberg and Prömel, 2019). Teneurins are present in at least all

67 of Bilateria (Tucker and Chiquet-Ehrismann, 2006), and although orthologs appear to be absent

68 in sponges, ctenophores and cnidarians, a possible ortholog of bilaterian teneurins has been

69 identified in a choanoflagellate (Tucker et al., 2012). The first teneurin genes were discovered

70 in the vinegar fly *Drosophila melanogaster*. Due to their structural similarity to the

71 extracellular matrix *glycoprotein* Tenascin, these were called *Tenascin-a* (*Ten-a*)

72 (Baumgartner and Chiquet-Ehrismann, 1993) and *Tenascin-m* (*Ten-m*) (Baumgartner et al.,

73 1994), the only two teneurins in *Drosophila*. Interestingly, *Ten-m* was independently identified

74 by another research group and named *odd Oz* (*odz*) after its expression in the brain and the

75 heart (the gifts bestowed by the wizard of Oz) (Levine et al., 1994). In recent years,

76 phylogenetic analyses revealed the presence of both orthologs, *Ten-a* and *Ten-m*, in all

77 arthropods, but also showed that in onychophorans, a closely related outgroup to arthropods,

78 and in another ecdysozoan, the nematode worm *Caenorhabditis elegans*, there is only one

79 teneurin gene (Minet and Chiquet-Ehrismann, 2000; Wides, 2019) (Figure 1A). This gene is

80 similar to both arthropod *Ten-a* and *Ten-m* and thus suggests that the latter are the result of a

81 gene duplication in the stem leading to Arthropoda.

82 Of the two *Drosophila* teneurin genes, *Ten-m* appeared to be the more interesting because it

83 was thought to represent a pair-rule gene (Baumgarnter et al., 1994; Levine et al., 1994), and

84 thus a component of the famous *Drosophila* segmentation gene cascade (StJohnston and

85 Nüsslein-Volhard, 1992). Critical reexamination, however, revealed that *odd paired* (*opa*) and

86 not *Ten-m* was responsible for the reported pair-rule phenotypes, and that *Ten-m* is involved in

87 motor neuron growth and guidance, and not segmentation (Zheng et al., 2011). At some point,

88   a pair-rule function was even attributed to *Ten-a*, but somewhat tragically, even this finding

89   was incorrect (Rakovitsky et al., 2007 (retracted in 2012)). However, since Ten-m protein is

90   expressed in a "typical" pair-rule like pattern in *Drosophila* (Baumgarner and Levine, 1994;

91   Levine et al., 1994), alternative functions in segmentation were discussed, and a possible

92   function as an oscillator in segmentation was suggested (Hunding and Baumgartner, 2017).

93   The very recent work of Jin et al. (2019) investigated the embryonic expression pattern of *Ten-*

94   *m* in the red flour beetle *Tribolium castaneum*. From their data, Jin and colleagues (2019)

95   concluded that *Ten-m* cannot be involved in segmentation, since it is not expressed in the

96   ectoderm and thus in tissue that is undergoing primary segmentation. The combined data from

97   *Drosophila* and *Tribolium* somewhat intuitively suggest that the pair-rule like expression of

98   *Ten-m* in the highly-derived dipteran fly *Drosophila* may represent a derived feature.

99   In this paper, however, I present the first comprehensive data on the embryonic expression

100 patterns of teneurin genes (*Ten-m* and *Ten-a* genes) in Panarthropoda, represented by the red

101 flour beetle *Tribolium castaneum* (Hexapoda: Coleoptera), the pill millipede *Glomeris*

102 *marginata* (Myriapoda: Diplopoda), the common house spider *Parasteatoda tepidariorum*

103 (Chelicerata: Arachnida), and the velvet worm *Euperipatoides kanangrensis* (Onychophora:

104 Peripatopsidae) (Figure 1B-E). The new data show unique expression profiles of each

105 investigated teneurin gene, but also reveal that at least one paralog of *Ten-m* in each species

106 (except for *Tribolium*) is indeed expressed in a pattern that is compatible with a possible

107 function in segmentation. The lack of such expression of *Ten-m* in *Tribolium* may thus

108 represent a derived rather than an ancestral feature of *Ten-m* function.

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Results

111 *Complement and phylogenetic distribution of panarthropod teneurin genes*

112   Reciprocal BLAST searches revealed the presence of two teneurin genes in *Tribolium* and

113   *Glomeris*, five in the *Parasteatoda* and one in *Euperipatoides* (Figure 1A). The distribution of

114   teneurin genes reflects the relationship of the panarthropod species used in this study (e.g.

115   Campbell et al., 2011) (Figure 1 A). The phylogenetic distribution of these genes suggests that

116   a single teneurin gene was present in the last common ancestor of arthropods and

117   onychophorans, and that this gene was duplicated in the stem leading to the arthropods. In the

118   spider, these genes, *Ten-a* and *Ten-m*, underwent additional duplications. One of these

119   duplications is likely the result of a whole genome duplication that occurred in the last common

120   ancestor of Arachnopulmonata (Schwager et al., 2017) giving rise to *Ten-a1* and *Ten-a2* as

121   well as *Ten-m1/2* and *Ten-m3*. An additional duplication event then lead to the presence of

122   three paralogs of Ten-m in the spider (*Ten-m1/2* duplicating into *Ten-m1* and *Ten-m2*) (Figure

123   1A).

124

125   *Expression of arthropod Teneurin-m (Ten-m) orthologs*

126   *Glomeris Ten-m* is first expressed in the form of transverse segmental stripes in the *regio*

127   *germinalis* that originates from the early blastoderm (Figure 2A/B) (see Janssen et al., 2004).

128   *Ten-m* is also weakly expressed in the form of a stripe in the segment addition zone (SAZ)

129   (Figure 2A-E). The segmental stripes disappear as the segments mature and resolve into dots

130   of expression in the ventral tissue (likely associated with the developing ventral nervous

131   system), and solid blocks of expression in the dorsal segmental units (Figure 2C-H) (see

132   Janssen, 2011). Additional expression is in the ocular region that harbors the developing brain

133   (Figure 2A-H), the mesoderm of all appendages (Figure 2C-H and Supplementary Figure S1A)

134   and of the anal valves (Figure 2B-H). In late stages, *Ten-m* is also expressed in the developing

135   heart (Figure 2H).

136   *Parasteatoda Ten-m1* is only expressed in late developmental stages when it is in the head

137   lobes, the ventral nervous system, and in the form of a single dot dorsal in the base of the

138   pedipalps and the legs (Supplementary Figure S2).

139   *Parasteatoda Ten-m2* is expressed in transverse segmental stripes (Figure 3). First, a single

140   broad stripe appears anterior in the forming germ band; this stripe is likely associated with the

141   formation of the labrum and the cheliceral segment (Figure 3A). At subsequent developmental

142   stages, additional stripes form, each associated with a single segment (Figures 3B-H and 4C/D).

143   Later, it becomes clear that the positon of these stripes is located in the forming inter-segmental

144   grooves (Figures 3E, 4C and Supplementary Figure S3). *Parasteatoda Ten-m2* is also

145   expressed in the developing brain in the head lobes (Figures 3B-H and 4A/E/H), the ventral

146   nervous system (Figures 3F/G and 4A-C), and in a complex pattern in the appendages (Figure

147   4E/F and Supplementary Figure S5B).

148   *Parasteatoda Ten-m3* is expressed in dorsal most tissue corresponding to the head lobes

149   (Supplementary Figure S4A/B/E), the prosoma (Supplementary Figure S4A/C) and the

150   opisthosoma (Supplementary Figure S4A-E) with exception of the SAZ (Supplementary

151   Figure S4A/B/D). Later, expression in the opisthosoma likely relates to the development of the

152   alary muscles of the heart (Supplementary Figure S4H/I) (cf. Janssen and Damen, 2008). *Ten-*

153   *m3* is also expressed in the head lobes (Supplementary Figure S4E/G/I), the mesoderm of the

154   chelicerae and in the form of a distal mesodermal patch in the pedipalps and the legs

155   (Supplementary Figures S4F/J and S1C).

156

157   *Expression of the single onychophoran teneurin gene,* Teneurin-a/m *(*Ten-a/m*)*

158   *Euperipatoides Ten-a/m* is expressed in the frontal appendages (Figure 5), weakly in the mouth

159   and anus (Figure 5A-C), and in the mesoderm of newly formed posterior segments (Figure 5A-

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160   D). At later developmental stages, expression appears in the posterior part of the eyes (Figure

161   5D-F) and the mesoderm of all appendages and the anal valves.

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163   *Expression of arthropod Teneurin-a (Ten-a) orthologs*

164   *Tribolium Ten-a* is strongly expressed in the developing brain in the ocular region

165   (Supplementary Figure S5A-D), the ventral nervous system (Supplementary Figure S5B-D),

166   and in a subset of cells that likely correspond to the peripheral nervous system (Supplementary

167   Figure S5D). At later stages, it is also expressed in distal region of the developing hindgut

168   (Supplementary Figure S5D).

169   Like for *Tribolium*, *Glomeris Ten-a* is expressed in the brain and the ventral nervous system,

170   albeit much later and weaker than in *Tribolium* (Supplementary Figure S5E-G). Additional

171   expression is in the form of a small dot at the dorsal base of the antennae and in the anal valves

172   (Supplementary Figure 5E-G).

173   *Parasteatoda Ten-a1* is expressed in the brain in late developmental stages, the ventral nervous

174   system and the mesoderm of the appendages (except for the labrum) (Supplementary Figures

175   S6A-F and S1D).

176   *Parasteatoda Ten-a2* is first expressed in the ventral nervous system associated with the

177   prosomal segments (Supplementary Figure S6G-L). In the pedipalps and the legs, but not the

178   other appendages, *Ten-a2* is expressed in the form of a sub-terminal domain (Supplementary

179   Figures S6G/K/L and S1E). At later developmental stages, *Ten-a2* is also expressed in the brain

180   and in the developing book lungs and tracheal lungs in the second and third opisthosomal

181   segment (Supplementary Figure S6L).

182

183   **Discussion**

184   *A possible function of Ten-m/odz in arthropod segmentation*

185   The *Drosophila* segmentation gene cascade (SGC) arguably is the most famous and one of the

186   best-investigated gene regulatory network. This hierarchic gene interaction is in control of

187   anterior-posterior body patterning of the fly, and thus the process of segment formation (e.g.

188   Nüsslein-Volhard and Wieschaus, 1980; Ingham, 1988; Cohen and Jürgens, 1991). *Drosophila*

189   development represents a derived form of segmentation in which most of its body is

190   patterned/segmented (almost) simultaneously from a uniform blastoderm (e.g. Pankratz and

191   Jäckle, 1993). Many of the genes involved in this process, however, likely play similar

192   function(s) in other arthropods and their closest relatives, the tardigrades and the

193   onychophorans, all of which add segments sequentially from a posterior segment-addition zone

194   (reviewed in e.g. Peel et al., 2005; Damen, 2007; Smith and Goldstein, 2017; Janssen, 2017;

195   Dunlop and Lamsdell, 2017). One important level of the SGC is represented by the pair-rule

196   genes (PRGs). PRGs are transcription factor-encoding genes that are typically expressed in the

197   form of a unique pattern of seven transverse stripes in the late blastoderm of the fly (e.g.

198   Harding et al., 1986; Carroll and Scott, 1986; Gergen and Butler, 1988; Carroll et al., 1988).

199   In arthropods with sequential addition and patterning of segments, the orthologs of these genes

200   are expressed in the form of transverse stripes or dynamic domains in the ectoderm of the

201   posterior segment addition zone (SAZ) or newly formed segments, or are expressed as

202   transverse stripes in the anterior segments that derive from the blastoderm (e.g. Damen et al.,

203   2005; Choe et al., 2006, 2017; Choe and Brown, 2007; Janssen et al., 2011, 2012; Eriksson et

204   al., 2013; Auman and Chipman, 2018).

205   In a recent study, Jin and colleagues (2019) demonstrated that in the beetle *Tribolium*, *Ten-m*

206   is not expressed like a segmentation gene, and thus is not involved in segmentation.

207   In *Glomeris* and *Parasteatoda*, however, at least one ortholog of Ten-m is expressed in the

208   form of transverse segmental stripes in newly forming segments, both in anterior segments that

209   originate from the early blastoderm (or the germ disc in the spider), and in segments that are

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210   added from the posterior SAZ (Figures 2-4 and S3). This suggests a possible function in

211   segment formation, or maintenance of segmental boundaries. The expression of *Glomeris Ten-*

212   *m* and *Parasteatoda Ten-m2* is very similar to that of other segmentation genes including the

213   pair-rule gene orthologs in these species (Damen et al., 2005; Janssen et al., 2008, 2011, 2012;

214   Janssen, 2012; Schönauer et al., 2016; Hemmi et al., 2018). In *Glomeris*, the primary PRGs

215   *runt* (*run*) and *even-skipped* (*eve*) both are expressed in the form of one (or more) circles around

216   the posterior pole (anus) of the developing embryo, representing a unique feature of PRG

217   expression (Janssen et al., 2011); and this detail of PRG expression is also present for *Ten-m*

218   (Figure 2G).

219   The phylogenetic distribution of segmentation-gene like expression of Teneurin-m genes in

220   Arthropoda thus suggests that the lack of such expression in *Tribolium* is a derived character.

221   The single teneurin gene of the onychophoran *Euperipatoides* is also expressed in transverse

222   segmental stripes in newly formed segments (Figure 5). In onychophorans, however, the PRG-

223   system appears to be little conserved (Janssen and Budd, 2013), and the segmentation-gene

224   like expression of *Ek-Ten-a/m* is not as obvious as in arthropods. Notably, however, the

225   expression of *Ten-a/m* is very similar to that of the only PRG ortholog that is possibly involved

226   in onychophoran segmentation (or at least posterior elongation), *even-skipped* (*eve*) (Janssen

227   and Budd, 2013). Thus, the expression of *Ten-a/m* could indicate that a possible segmentation-

228   gene (or related) function may date back to the last common ancestor of Panarthropoda, and

229   that such function was already established before the duplication of an ancestral panarthropod

230   teneurin gene (*Ten-a/m*) into *Ten-m* and *Ten-a*. It was therefore necessary to investigate

231   whether such function/expression could have been retained in the *Ten-a* orthologs of

232   arthropods including the beetle *Tribolium* that does not express *Ten-m* in a segmentation-gene

233   like pattern (Jin et al., 2019). Gene expression analysis of *Ten-a* orthologs in all investigated

234   arthropods, including *Tribolium*, however, revealed that neither of them is expressed like a

235   potential segmentation gene, revealing that the segmentation-gene like expression of arthropod

236   teneurin genes is a feature of *Ten-m*, and that the lack of such expression in *Tribolium* indeed

237   likely represents a derived character.

238   Further investigation including functional studies may reveal if arthropod *Ten-m* genes indeed

239   are involved in segmentation, and what their function(s) in this process may be.

240

241   **Experimental Procedures**

242   *Research organisms and embryos*

243   Research animals and their embryos were handled as described in Grossmann and Prpic (2012)

244   (*Tribolium castaneum*), Janssen et al. (2004) (*Glomeris marginata*), Prpic et al. (2008)

245   (*Parasteatoda tepidariorum*), and Hogvall et al. (2014) (*Euperipatoides kanangrensis*).

246   Developmental staging follows Strobl and Stelzer (2014) (*Tribolium*), Janssen et al. (2004)

247   (*Glomeris*), Mittmann and Wolff (2012) (*Parasteatoda*), and Janssen and Budd (2013)

248   (*Euperipatoides*).

249

250   *RNA extraction, gene cloning, whole mount* in-situ *hybridization, and nuclear counter staining*

251   Total RNA was extracted with TRIZOL (Invitrogen) from a mix of embryos representing all

252   stages from the blastoderm stage to hatching. Total RNA was then reverse transcribed into

253   cDNA using the SuperScript IV Reverse Transcriptase (Invitrogen). Gene fragments were

254   amplified by RT-PCR with gene-specific primers based on sequenced genomes (*Tribolium* and

255   *Parasteatoda*) and sequenced embryonic transcriptomes (*Glomeris* (SRA accession:

256   PRJNA525752) and *Euperipatoides* (SRA accession: PRJNA525753)). In all cases, a nested

257   PCRs was performed, using an initial PCR as template. Primer sequences are listed in

258   Supplementary Table 1. Gene fragments were cloned into the PCRII vector (Invitrogen) and

259   sequenced on an ABI3730XL automatic sequencer (Macrogen, Seoul, South Korea). Unique

260 gene identifiers are listed in Supplementary Table 2. *In-situ* hybridization was performed as

261 described in Janssen et al. (2018) using BM Purple (Roche) as staining substrate. For confocal

262 microscopy, embryos were stained with SIGMAFAST Fast Red TR/NaphtolAS-MX

263 (SIGMA). Morphology of the embryos was visualized with the nuclear dye SYBR Green in

264 phosphate buffered saline with 0.1% Tween-20 (PBST-0.1%).

265

266 *Phylogenetic analysis*

267 Reciprocal BLAST searches applying tblastn, blastp and blastv were run with the *Drosophila*

268 *melanogaster* sequences of Tenascin-m (aka Odd Oz (Odz)) and Tenascin-a to identify teneurin

269 genes. Amino acid sequences were aligned using T-Coffee with default parameters in

270 MacVector v12.6.0 (MacVector, Inc., Cary, NC), or Aliview 1.18.1 for Linux (Larsson, 2014).

271 The phylogenetic analysis was performed with MrBayes (Huelsenbeck and Ronquist, 2001)

272 with a fixed WAG amino acid substitution model with gamma-distributed rate variation across

273 sites (with four rate categories), unconstrained exponential prior probability distribution on

274 branch lengths, and exponential prior for the gamma shape parameters for among-site rate

275 variation. The phylogenetic tree was calculated applying 300000 cycles for the Metropolis-

276 Coupled Markov Chain Monte Carlo (MCMCMC) analysis (four chains; chain-heating

277 temperature of 0.2). Markov chains were sampled every 200 cycles. 25% of samples were

278 applied as burn-in. Clade support was determined with posterior probabilities in MrBayes.

279 Unique sequence identifiers for all sequences used in the phylogenetic analysis are listed in

280 Supplementary Table 2.

281

282 *Data documentation*

283 A Leica DC490 digital camera equipped with a UV light source mounted onto a MZ-FLIII

284 Leica dissection microscope was used for documentation of stained embryos. Confocal

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285 microscopy was performed using an inverted Leica TCS SP5 confocal microscope. For the

286 detection of Fast Red and DAPI signal. Emission wavelengths for Fast Red and DAPI were

287 561nm and 404nm, respectively. Collected wavelengths for Fast Red were between 600nm and

288 642nm, and for DAPI were between 430nm and 550nm. Whenever appropriate, contrast and

289 brightness were adjusted with the image-processing software Adobe Photoshop CC2018 for

290 Apple Macintosh (Adobe Systems Inc.).

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Acknowledgments

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439   adult brain. *Gene Expr Patterns*. 2003;3:397–405.

440

441   **Figure Legends**

442   Figure 1 – Phylogeny and research organisms

443   **A** Phylogenetic analysis of teneurin genes. Species abbreviations: Dm, *Drosophila*

444   *melanogaster* (Hexapoda: Diptera); Ek, *Euperipatoides kanangrensis* (Onychophora); Gm,

445   *Glomeris marginata* (Myriapoda: Diplopoda); Pt, *Parasteatoda tepidariorum* (Chelicerata:

446   Arachnida); Hs, Homo sapiens (Vertebrata); Tc, *Tribolium castaneum* (Hexapoda:

447   Coleoptera); Blue shade: Teneurin-a group. Purple shade: Teneurin-m (Odz) group. Node

448   support is given as posterior probabilities. **B** *Tribolium castaneum*. **C** *Glomeris marginata*. D

449   Adult female of *Parasteatoda tepidariorum*. **E** Adult female of *Euperipatoides kanangrensis*.

450

451   Figure 2 – Expression of *Glomeris Ten-m*

452   In all panels, except for panel H, anterior is to the left, ventral views. Panel H represents a

453   lateral view. Developmental stages are indicated. Arrows point to weak expression in the SAZ.

454   Arrowheads point to transverse stripes of expression. Asterisks in panels A and B mark delayed

455   expression in some segments that originate from the *regio germinalis*. The asterisk in panel G

456 points to expression surrounding the posterior pole of the embryo. Filled circles mark

457 expression in the dorsal segmental units. Open circles in panel H mark lateral domains of

458 expression. Abbreviations: md, mandibular segment; h, heart; oc, ocular region (brain); SAZ,

459 segment addition zone; T1, first walking-leg bearing segment.

460

461 Figure 3 – Early expression of *Parasteatoda Ten-m2*

462 In all panels, anterior is to the left, ventral views (except for panel B, lateral view).

463 Developmental stages are indicated. Panels A´-H´ represent Cybr-Green counter-stained

464 embryos as seen in panels A-H. The arrows in panel H points to weak stripe of expression in

465 the SAZ. Arrowheads mark transverse segmental stripes of expression. Asterisks mark

466 expression in the anterior head (labral and cheliceral segment). Abbreviations: head lobe; L3,

467 third leg-bearing segment; SAZ, segment addition zone.

468

469 Figure 4 – Late expression of *Parasteatoda Ten-m2*

470 In all panels, anterior is to the left and ventral view (except panel D, lateral view and panel H,

471 dorsal view). Arrowheads point to transverse segmental stripes of expression. Arrows point to

472 expression in the brain. Asterisks in panels G/H mark expression in the alary muscles. Filled

473 circles mark expression in the ventral nervous system. Panels A´-G´ represent Cybr-Green

474 counter-stained embryos as seen in panels A-G. Abbreviations: br, brain; ch, chelicera; hl, head

475 lobe, L2, second walking-leg bearing segment; m, mouth; O2/4, second and fourth

476 opisthosomal segment respectively;

477

478 Figure 5 – Expression of the single *Euperipatoides* teneurin gene, *Ten-a/m*

479 In all panels, anterior is to the left. Panels A and B represent ventral views; panels B-F represent

480 lateral views. Arrowheads point to transverse stripes of expression in newly formed segments.

481   Abbreviations: a, anus; e, eye; fap, frontal appendage; m, mouth; saz, segment addition zone;

482   sp, slime papilla.

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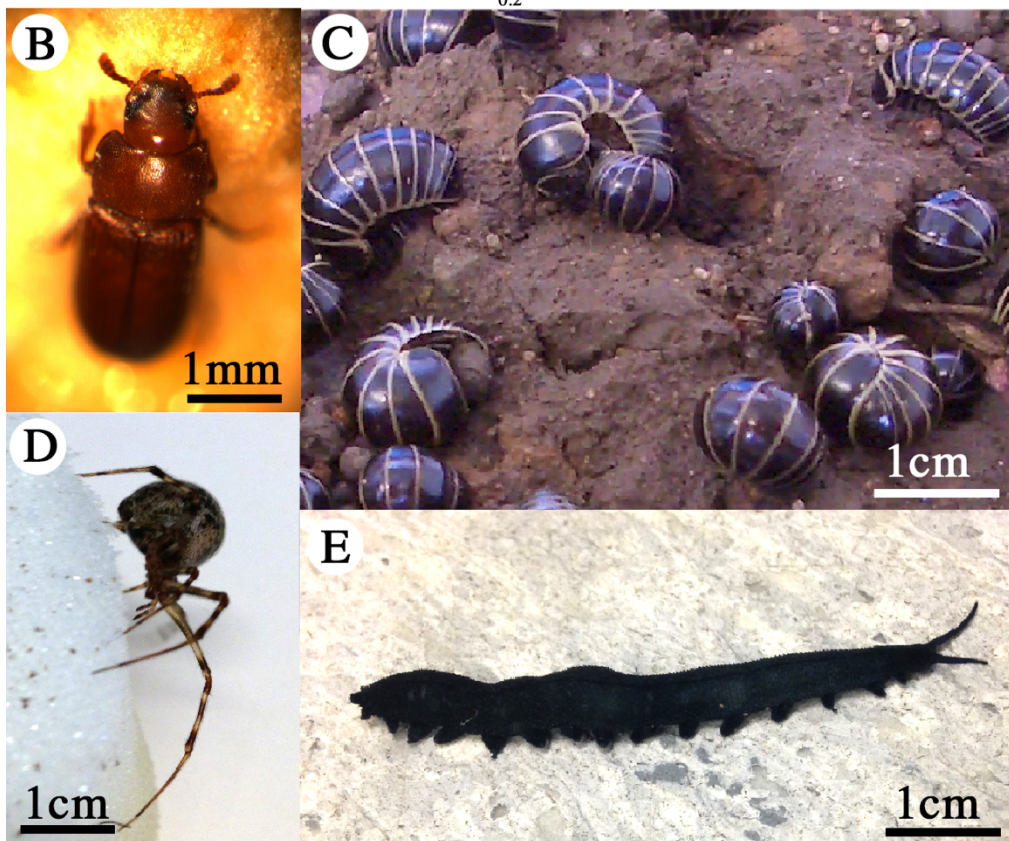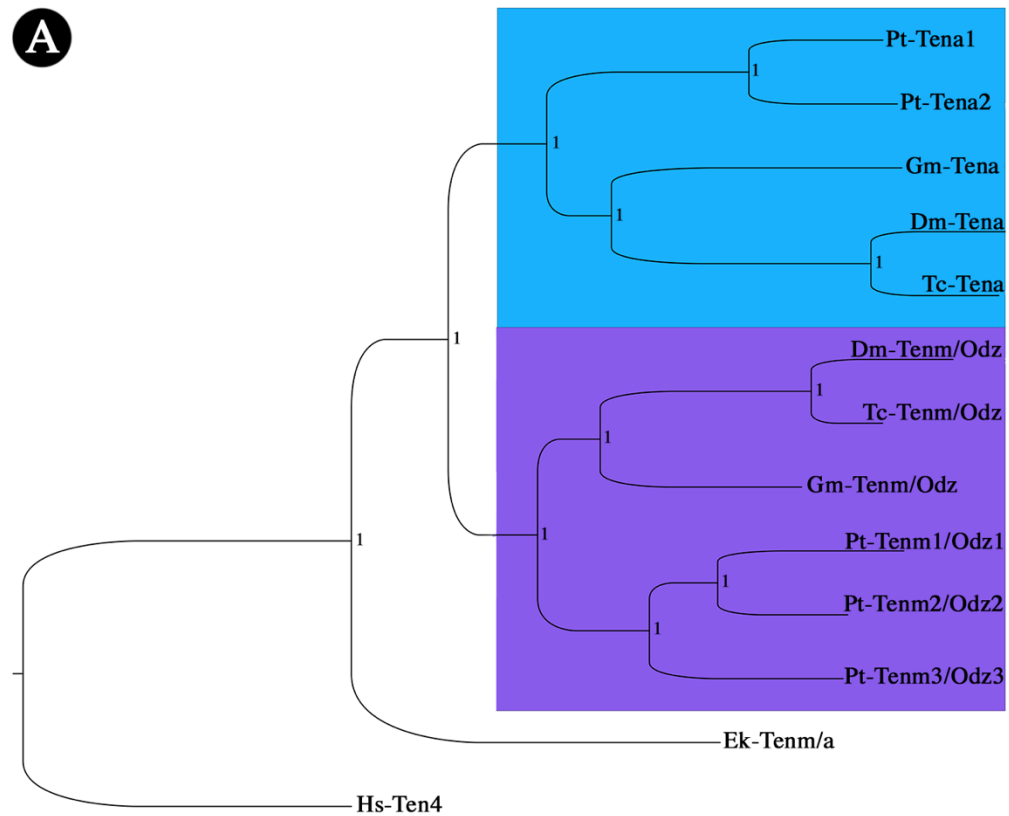490

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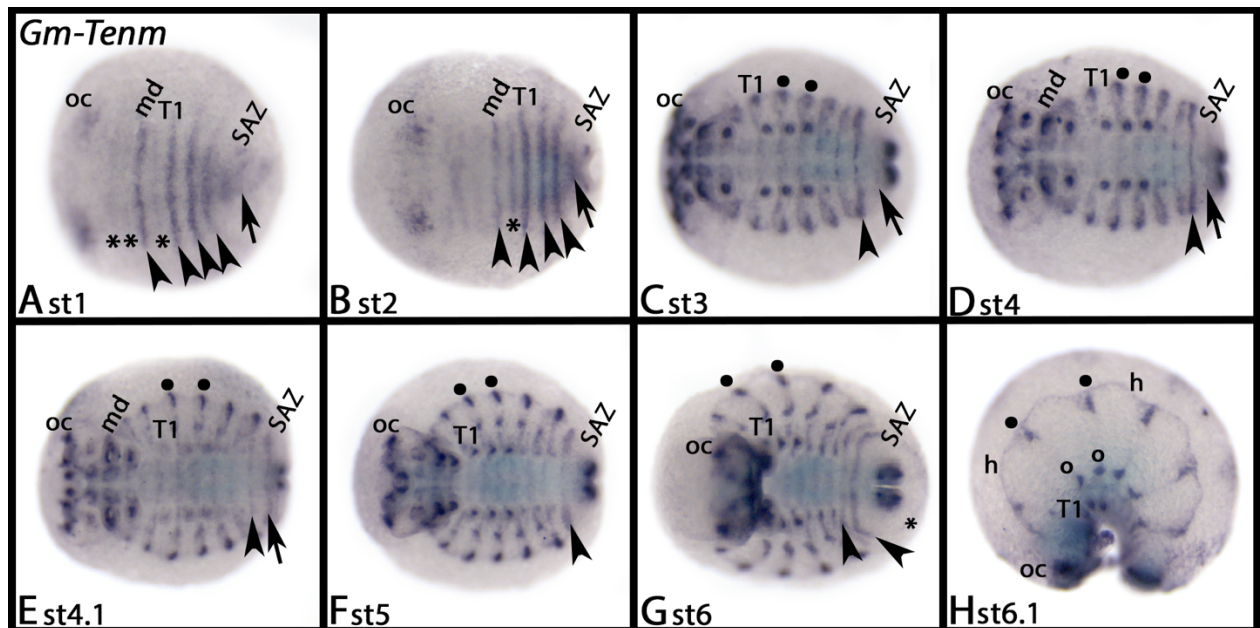496

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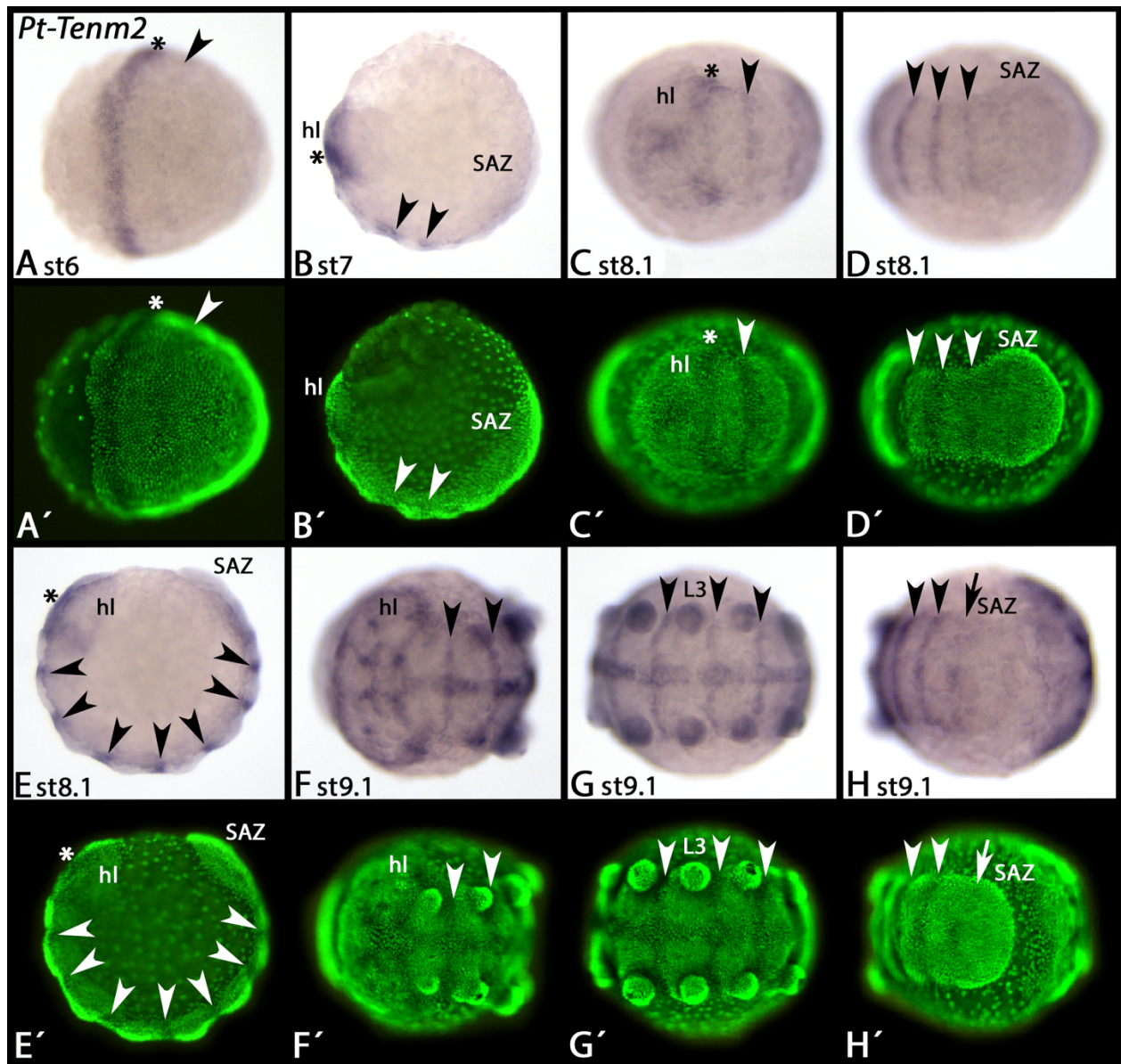506 **Figures**

508 Fig. 1 Teneurin Phylogeny and research organisms
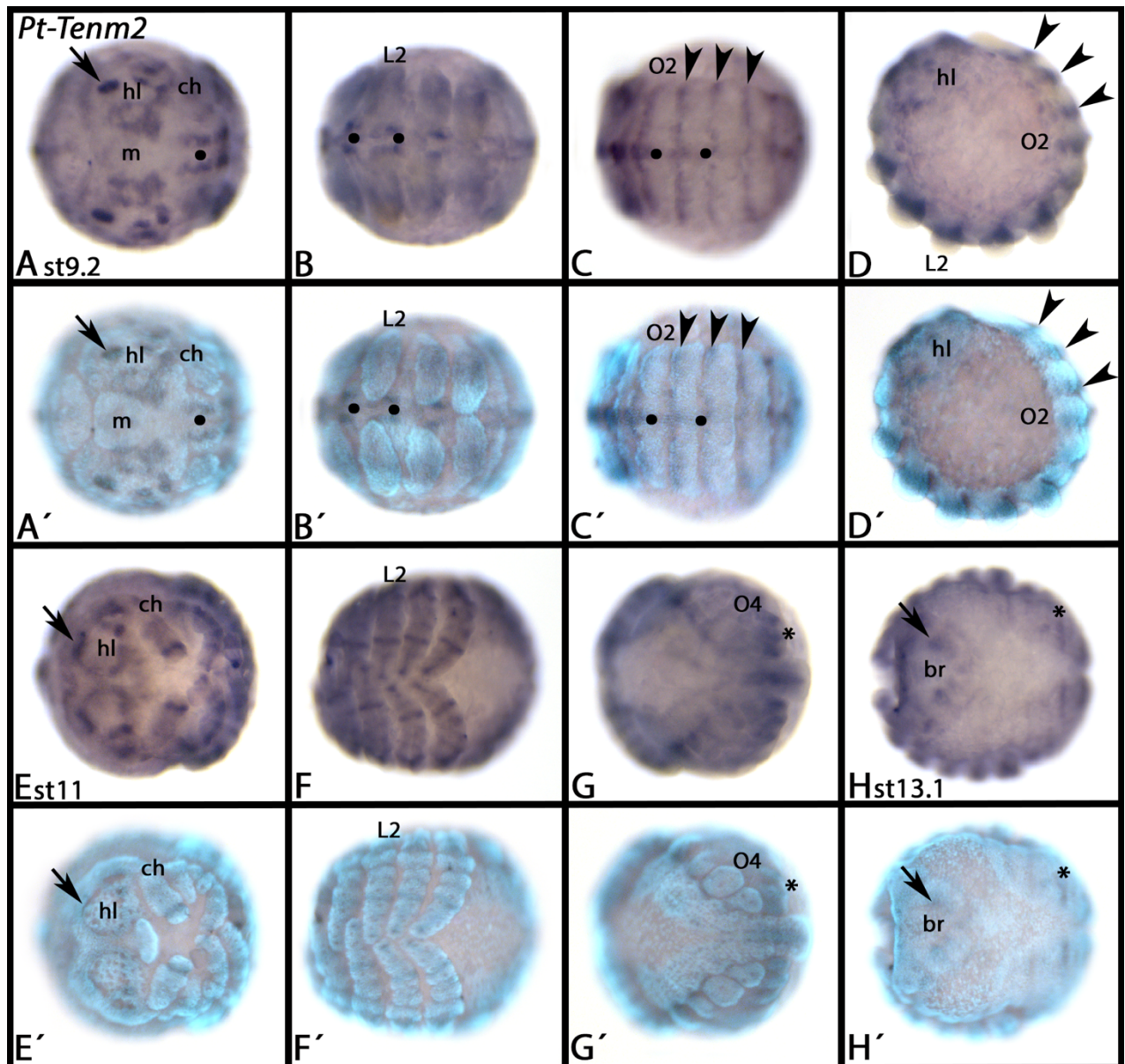
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511   Fig. 2 Expression of *Glomeris Ten-m*

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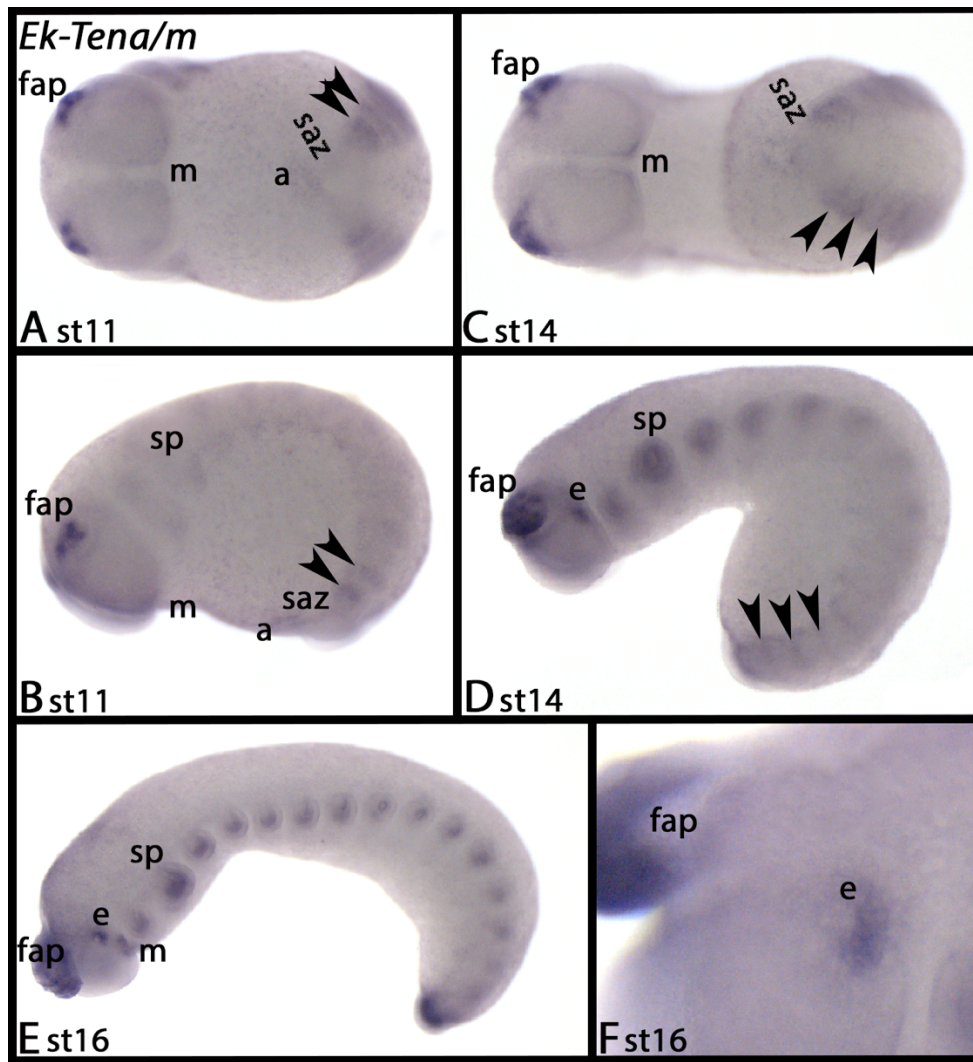Fig. 3 Expression of *Parasteatoda Ten-m2*, early stages

Fig. 4 Expression of *Parasteatoda Ten-m2*; late stages

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520   Fig. 5 Expression of *Euperipatoides Ten-a/m*