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Reversion of developmental mode in insects: evolution from long germband to short germband in the polyembrionic wasp *Macrocentrus cingulum* Brischke

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Abstract

Germband size in insects has played a central role in our understanding of insect patterning mechanisms and their evolution. The polarity of evolutionary change in insect patterning has been viewed so far as the unidirectional shift from the ancestral short germband patterning of primitive hemimetabolous insects to long germband patterning observed in most modern Holometabola. However, some Brachycera (higher insects) orders display both short and long germband development though the absence of clear phylogenetic context does not permit definite conclusions on the polarity of change. Derived hymenoptera, i.e. bees and wasps, represent a classical textbook example of long germband development. Yet, in some wasps putative short germband development has been described correlating with lifestyle changes, namely with evolution of endoparasitism and polyembryony. To address the potential reversion from long to short germband, we focused on the family Braconidae, which displays ancestral long germband development and examined embryonic development in the derived polyembryonic braconid *Macrocentrus cingulum*. Using SEM analysis of *Macrocentrus* embryogenesis coupled with analyses of embryonic patterning markers, we show that this wasp secondarily evolved short germband embryogenesis reminiscent of that observed in the beetle *Tribolium*. This work shows that the evolution of germband size in insects is a reversible process that may correlate with other life-history traits and suggests broader implications on the mechanisms and evolvability of insect and arthropod development.

Introduction:

One key question in evolutionary developmental biology is how variable morphology relates to variation in genetic circuitry (Wilkins, 2002). Moreover, independent and/or reverse evolution and how it relates to the underlying genetic architecture, is at the core of the evo-devo research agenda (Stern and Orgogozo, 2008)(Peel, 2008)(Gompel and Prud'homme, 2009)(Christin et al., 2010). Evolutionary changes in developmental programs manifest both at the cellular and molecular levels, and have been dissected in a number of processes such as axis formation (Lall and Patel, 2001)(Goltsev et al., 2007), cell fate specification and patterning (Carroll et al., 1994)(Sommer and Sternberg, 1996)(Wittkopp and Beldade, 2009), oogenesis (Lynch and Roth, 2011)(Vreede et al., 2013), gene expression (Kalinka et al., 2010)(Robinson et al., 2011), cleavage geometry and morphogenetic movements (Wray and Bely, 1994)(Green et al., 2010), amongst others. Reconstruction of the evolutionary history of development, by mapping changes in developmental programs onto solid phylogenies, can lead to the determination of the polarity of change (ancestral vs. derived) and is heuristic as it generates hypotheses about the association between developmental change and morphology (Wray and Bely, 1994)(Sucena and Stern, 2000) and the effects of environment and life-history (Peel, 2008)(Gilbert and Epel, 2009). One way to approach this question is to analyze development in species that independently evolved a particular morphological feature or mode of development (Sucena et al., 2003)(Jeffery, 2009)(Gompel and Prud'homme, 2009). Such an analysis may point to conserved parts of the genetic program and/or identify segments of the regulatory hierarchy that underwent

65 evolutionary change (Prud'homme et al., 2006)(Stern and Orgogozo, 2009)(Manceau et
66 al., 2010).

67 A major feature of insect development is the extreme variation in the
68 segmentation mode manifested in long germband and short germband insects (Sander,
69 1976)(Davis and Patel, 2002). Short germband embryos form a blastoderm that consists
70 of head lobes, the most anterior trunk segment and the terminus. Additional segments are
71 added progressively during the course of embryogenesis arising from a proliferative
72 posterior growth zone (Anderson, 1973). This form of development is displayed by
73 primitive insects such as the grasshopper in contrast with more derived insects such as
74 *Drosophila* which display predominantly long germband development (Peel et al., 2005).
75 In between these two extreme types of development some insects, such as crickets, reach
76 gastrulation with an intermediate number of segments (Mito, 2005). Short and
77 intermediate germband type of embryogenesis are predominant in primitive,
78 hemimetabolous, insects. More derived, holometabolous insects exhibit mostly long
79 germband development including the fly *Drosophila*, *Nasonia* wasps and the honeybee
80 (Davis and Patel, 2002).
81 The canonical short germband development of grasshoppers and the beetle *Tribolium* is
82 marked by formation of the cellular blastoderm that contains only anterior segments.
83 After the initiation of gastrulation, additional segments are added progressively from the
84 posterior growth zone in an anterior to posterior direction (Patel et al., 1992)(Patel et al.,
85 1994). The nature and mechanics of this growth zone is variable across organisms
86 arthropods (Peel et al., 2005) and, even within insects, is likely to obey very different
87 rules to those established in *Drosophila* (Pueyo et al., 2008).

These morphological differences are paralleled by the differential expression of the patterning genes and are diagnostic of the different types of germ band development. In *Drosophila*, interactions between gap genes expressed in broad domains along the anterior-posterior axis, are involved in the transition between the non-segmental and segmental organizations of the insect embryo (Jaeger, 2011). This organization is further refined through the double-segment periodic pattern of pair-rule gene expression. The expression of the pair-rule Even-skipped (Eve) protein at the time of gastrulation represents a reliable marker for germ band type (Davis and Patel, 2002). For example within Coleoptera, at the cellular blastoderm stage two Eve primary stripes are formed in the short germband *Tribolium* in contrast with the six Eve stripes displayed by its long germband counterpart *Calosobruchus* (Patel et al., 1994).

It has been established that the long germband mode of development is restricted to holometabolous insects and must have evolved from short germband development predominant in basal holometabolous and hemimetabolous insects (Sander, 1976)(Tautz et al., 1994). However, this division is not clear-cut, as illustrated above with the example of Coleoptera, composed of species displaying both short and long germband development (Patel et al., 1994). Moreover, it is likely that long germband development has evolved multiple times independently (Davis and Patel, 2002). Yet, no cases of reverse evolution from long germband to short germband have been reported thus far, suggesting a strict polarity in the transition between these two developmental modes. In many instances the difficulty lies in the absence of a strong phylogenetic framework that impedes definite conclusions on the polarity of this change. One putative exception lies in Braconidae wasps because of their phylogenetic framework (Dowton et al., 2002)(Shi et

al., 2005) and the distinct morphological characteristics of short germband development in the derived braconid *Aphidius* (Grbic and Strand, 1998). Yet, the lack of early molecular markers such as Eve precludes an unequivocal corroboration of reversibility in developmental mode.

Braconidae is a large family comprising close to 18,000 species and belonging to the Hymenoptera (the sister group of modern holometabolous insects) that groups sawflies, bees, wasps and ants (Savard et al., 2006)(Krauss et al., 2008). Derived parasitic wasps originated from an ancestor that displayed long germband development (reviewed in (Grbic, 2003)). Basal parasitic wasps from this family such as the Cyclostome braconid *Bracon hebetor*, display an ectoparasitic life style (Gauld, 1988). They oviposit their eggs on the surface of the host and the emerging parasitic larvae consume the host from the exterior. This species has large and yolky eggs and undergoes long germband development as determined by both morphological and molecular markers (Grbic and Strand, 1998). In contrast, derived braconids exhibit an endoparasitic lifestyle where females oviposit their egg in the host´s body cavity. The parasitic larva develops within the host body and consumes the host from within. Many different modifications of development are associated with endoparasitism including polyembryony (reviewed in (Zhurov et al., 2007)), a process whereby a single egg gives rise to multiple embryos. Phylogenetic analysis reveals that replicated shifts in life history strategy have occurred in the Hymenoptera such that free-living, ectoparasitic, endoparasitic and polyembryonic lifestyles have arisen independently multiple times within different monophyletic families (Whitfield, 1998)(Grbic, 2003). For example, polyembryony evolved four independent

times in parasitic insects, raising the question of how conserved are their respective programs of embryonic patterning (Grbic, 2000).

In this study we examined embryonic development in the derived polyembryonic braconid *Macrocentrus cingulum*. The morphological description of *Macrocentrus* development shows that this wasp undergoes a canonical short germband development. Further, we analysed the expression of maternal coordinate, gap, pair-rule and segment polarity genes and show that *Macrocentrus cingulum* utilizes the segmentation gene cascade with the hallmarks of short germband development. However, analysis of cell proliferation suggests that posterior growth is not associated with increased mitotic activity in this region. Mapping this mode of development onto the branch containing advanced wasps shows that long germband patterning can evolve to short germband development, and that the polarity of evolutionary change for this trait is reversible. Moreover, this work illustrates that reversion of germband type associates with the independent evolution of polyembryony observed also in this branch of the Braconidae, reinforcing the intimate relationship between life-history features and development.

Material and methods:

Colonies of *Macrocentrus cingulum* were cultured using european cornborer *Pyrausta nubilalis* as host at the insect rearing facilities of the Agriculture Canada at the Southern Crop Protection and Food Research Centre, London, Ontario. Host larvae were parasitized daily in acrylic cages by exposing 3-5 third instar host larvae placed onto cornmeal diet to 25-40 *M. cingulum* females. Following parasitisation, 2 hosts were

placed in 50 ml glass vials filled with cornmeal diet. Parasitized hosts were maintained at +27°C and 16:8 (L:D) photoperiod.

To analyse morphogenesis, *M. cingulum* embryos were dissected from the host body cavity in the PBS and fixed overnight at +4°C in the 3.7% formaldehyde in PBS. The following day, embryos were dissected from the extraembryonic membrane and dehydrated in ethanol (20-40-60-80-100% ethanol in PBS, 10 minutes each). Embryos were placed in Samdri-PVT-3B critical point dryer, mounted on stubs and gold coated in Hummer VI Sputter Coater. Processed embryos were examined on a Hitachi S-570 scanning electron microscope. Images were taken using a Quartz PCI digital imaging system and processed with Adobe Photoshop 5.5 software.

Cloning of *M. cingulum* Krüppel, *in situ* hybridization and immunocytochemistry

In order to isolate the *Kr* homolog of *M. cingulum,* we amplified by PCR a fragment of *M. cingulum* Krüppel using the conserved degenerate primers TAYAARCAYGTGYTRCARAAYCA and YTTYARYTGRTTRSWRTCRSWRAA taken from Sommer et al. (Sommer et al., 1992). The short PCR fragments were extended using the GenomeWalker System (BD Biosciences Clontech, USA). This sequence (Gene Bank accession number FJ685649) was used for in situ analysis using an *in situ* hybridization protocol described previously (Zhurov et al., 2004).

To characterize expression of Eve, Engrailed (En) and Vasa (Vas) proteins during *M. cingulum* morphogenesis, embryos were dissected from fifth instar hosts in PBS buffer. Dissected embryos were transferred to a nine-well dish and fixed for 40 minutes in 3.7% formaldehyde. Following fixation, embryos were dissected from the extraembryonic membrane in PBS using tungsten

needles. Antibody staining was performed as described by Grbic et al. (Grbic et al., 1996). The following concentrations of primary antibodies diluted in 60nl PBST and 1% BSA were used: 1:3 En (mAbEN4D9 (Patel et al., 1989)), 1:3 Eve (mAb2B8 (Patel et al., 1992)), 1:3 Vasa (formosa-1 (Chang et al., 2002)). Secondary antibodies (anti-mouse Cy5 and anti rabbit Cy2 Jackson) were diluted 1:200 in PBST. Analysis of mitoses was performed using rabbit anti-phosphoH3 in a 1:1000 concentration (Upstate, Inc., Lake Placid, NY, USA). Following antibody staining, embryos were counterstained for 40 minutes with rhodamine-phalloidin diluted 1:3 (Molecular probes). Embryos were mounted in glycerol and examined on a Zeiss 510 LSM Confocal microscope.

Results

Macrocentrus cingulum early morphogenesis

M. cingulum morphogenesis is initiated after a period of embryonic proliferation that generates up to 30 embryos from a single egg (Voukassovitch, 1927)(Parker, 1931). At the onset of embryogenesis, individual embryonic primordia are surrounded by a thick extraembryonic membrane (Fig 1A, asterisk). At an early stage, the embryonic primordium is radially symmetric, consisting of several hundred cuboidal cells that form a ball (Fig 1A). This ball-like embryonic primordium has a hollow blastocoel (Fig 1A, arrow). During the early embryonic primordium stage, embryonic cells are round and adhere loosely to each other (arrowhead). As development proceeds, the apical cell surfaces flatten to form the smooth surface of the primordium (Fig 1B). A cross section of the embryo shows that surface flattening is associated with changes in cell shape from cuboidal to columnar (Fig. 1C, arrowhead). These elongated cells become tightly inter-

digitated. This transformation of cell shape results in the formation of a unicellular epithelium that surrounds the blastocoel cavity. At this point in development it is not possible to determine the anterio-posterior embryonic axis in the radially symmetrical embryonic primordium. Following the change in cellular shape, the embryonic primordium increases in size (Fig. 1D, compare with B) and becomes ellipsoidal. This shortening of the vertical axis marks the initiation of dorso-ventral flattening. Dorso-ventral flattening proceeds, until the embryo becomes almost square-shaped (Fig. 1E) and opposing layers of cells come close to each other. At this point, an opening is formed on the dorsal side (Fig. 1F) initiating the formation of a flat epithelium. The longer axis of the dorsal opening runs perpendicularly to the presumptive anterio-posterior embryonic axis, separating future anterior and posterior regions (Fig. 1G, arrow). This results in a donut-shaped embryo. Further widening of the dorsal opening transforms it into a cup-shape (Fig. 1H). At this point, the wider side of the embryonic primordium will form the posterior region (Fig. 1H, arrow), while the narrower area will give rise to anterior structures (arrowhead). Both anterior and posterior folds form a symmetric furrow along the middle (pointed by arrow and arrowhead). Cells of the embryonic primordium extend cellular projections and filopodia consistent with active cellular movement during the reorganization of the embryo (Fig. 1H, inset).

Macrocentrus cingulum germband elongation and segmentation

The initiation of embryonic growth is marked by further flattening and elongation of the primordium and by the folding of the epithelial sheets at the presumptive anterior and posterior tips (Fig 2A). As a consequence, the hollow area on the future dorsal side of the

225 embryo widens. At the cup-stage, the embryo´s ventral ridge (Fig 2A, arrow) is seen
226 extending from the anterior and containing rounded cells, which appear to delaminate
227 from the tightly packed cells of the ectoderm. These ingressing cells represent the first
228 signs of gastrulation. Subsequently, the posterior region of the embryo starts to fold
229 forming a coiled structure (Fig. 2B). At this stage of development, the cup opening is
230 filled with extracellular matrix (arrows), which stains intensely with phalloidin (not
231 shown) indicating accumulation of actin in this area. In parallel with the onset of
232 gastrulation the embryo initiates germband extension. Initially, the embryo remains
233 tightly coiled with enlarged bilateral lobes formed at the posterior (Fig. 2C). As
234 morphogenesis advances, embryos become progressively more coiled as new regions of
235 the trunk are formed. The posterior region further elongates forming a transient furrow
236 separating the posterior tip from the newly formed trunk regions (Fig. 2D, arrow). In a
237 mechanically uncoiled embryo of a similar stage, we can observe the extension and
238 widening of the dorsal opening (Fig. 2E, arrowhead). The embryo continues to elongate
239 and the cephalic furrow appears at the anterior (Fig. 2F, arrowhead) demarcating the
240 future head. At this time, the gastrulation furrow extends along the ventral midline
241 (arrow). During germband extension, the embryo consists of a unicellular epithelial sheet
242 of elongated and tightly packed cells (Fig. 2G). Following the formation of the cephalic
243 furrow, the presumptive head region becomes elongated and the head lobes bulge out
244 (Fig. 2H). Subsequent to the enlargement of the head lobes, another furrow forms in the
245 future gnathal region (Fig. 2I, arrow). It is unclear whether this fold demarcates the entire
246 gnathal area or just the mandibular and maxillary segments. At a later stage, mandibular
247 and maxillary segments are refined (Fig. 2J) and a furrow forms in the cephalic region

248 separating the labrum from the rest of the head (Fig. 2J, arrow). As embryonic elongation
249 proceeds, a progressive bulging of the gnathal segments can be observed (Fig. 2K)
250 followed by the appearance of a transient furrow in the future thoracic area (arrow). At
251 the complete extended germband stage (Fig. 2L), a furrow demarcating the next segment
252 of the thoracic area begins to form (arrow). Following germband extension, the embryo
253 undergoes germband condensation. In the condensed germband stage, all future segments
254 become visible (Fig. 2M) and bilateral protrusions along the ventral midline abdominal
255 segments show in the thoracic and the first two abdominal segments, representing proleg-
256 like primordia (arrowheads). The gnathal segments begin to involute (arrow) and the
257 labral segment extends anteriorly. Soon after, the gnathal segments ingress completely
258 into the oral cavity (Fig. 2N, arrow) and all future proleg-like primordia are formed (Fig.
259 2N, arrowheads). Finally, we can observe a completely segmented larva composed of
260 three thoracic and thirteen abdominal segments, each having proleg-like structures, and a
261 telson (Fig. 2O). The description of embryogenesis in *M. cingulum* indicates that this
262 species undergoes an extreme form of short germband development where only anterior
263 structures appear to be patterned at the blastoderm stage and that segments are
264 sequentially formed during the course of morphogenesis.
265
266 ***Expression of gap and maternal coordinate genes***
267 Previous description of another putatively short germband derived braconid, *Aphidius*
268 *ervi* (Grbic and Strand, 1998) failed to provide the early molecular markers necessary to
269 unequivocally classify its type of embryonic development. Thus, in *M. cingulum* we
270 examined the expression patterns of genes covering the main patterning cascade classes

271 defined in *Drosophila*: Krüppel mRNA (gap gene), and the proteins of the maternal
272 coordinate gene Vasa (Vas), the pair-rule gene *even-skipped* (*eve*) and the segment
273 polarity gene *Engrailed* (*en*).
274
275 *Vasa* is a *Drosophila* maternal coordinate gene and universal marker of the germ line in
276 metazoans (Saffman and Lasko, 1999). In the rounded stage *M. cingulum* embryo (Fig.
277 1E), a small population of the interior cells stains with anti-Vasa antibody (Fig. 3A).
278 These cells adopt a sub-terminal localization in the cup-shaped embryo, forming a
279 compact group of cells. Vasa-positive cells remain in a sub-terminal position (Fig 3 B, C)
280 even as the germband extends (marked by addition of En stripes Fig. 3 D-G, green).
281 Upon completion of germband extension, Vasa-positive cells localize between En stripes
282 13 and 14 in two bilaterally symmetrical cell patches corresponding to the gonadal
283 precursors (Fig. 3 H). Vasa protein localization suggests that the posterior terminus of the
284 *M. cingulum* embryo is specified early at the blastoderm stage and that additional
285 segments are added to a region anterior to Vasa-positive cells.
286
287 In *Drosophila*, *Krüppel* is a gap gene involved in the segmentation cascade and
288 demarcates the blastoderm between the mesothorax and the third abdominal segment.
289 However, in the short germband insect *Tribolium castaneum*, *Tc-Kr* marks a more
290 anterior region than in *Drosophila*, its expression domain encompassing only the thoracic
291 segments (Bucher and Klingler, 2004). Also, in intermediate germband insects such as
292 *Oncopeltus fasciatus* (Liu and Kaufman, 2004) and *Gryllus bimaculatus* (Mito et al.,
293 2006), the pattern of *Kr* demarcates the thoracic region suggesting that its expression

pattern can be used as a marker of thoracic structures. In *M. cingulum*, *Mc-Kr* is not expressed in the donut-shaped embryo (Fig. 4 A). Only later, at the cup-shaped embryo can *Mc-Kr* be detected as a sub-terminal band 10-12 cell diameters wide (Fig. 4B). The posterior terminus of the embryo does not express *Mc-Kr* mRNA. During the initiation of germband elongation, the *Mc-Kr* domain appears at a more anterior location (Fig. 4 C) but the posterior terminus continues to be devoid of *Mc-Kr* staining. Unfortunately, we were unable to perform simultaneous *in situ* hybridization and antibody staining to determine the boundaries of this early domain more precisely. However, our SEM sequence of developmental stages and carefully staged Eve and En stainings (see below) suggest that this early domain is posterior to the first two En stripes (compare Fig. 4 B and Fig. 6 A). Since the first En stripes to appear are mandibular and maxillary, it is likely that the early *Mc-Kr* expression domain covers roughly the future thoracic region, similarly to the short germband coleopteran *Tribolium castaneum*. As the germband elongates, *Mc-Kr* shows a sharp anterior boundary approximately at the level of the presumptive transition between thorax and gnathal segments (Fig. 4 D). This sharp expression limit is maintained midway through germband extension (compare Fig. 4 E and Fig 6 D). Following this stage, expression becomes more dynamic (Fig 4 F-G) culminating in the highly conserved neural expression observed across all insects studied thus far. In conclusion, the expression of *Mc-Kr* shows a pattern that parallels the domains and dynamics described in short and intermediate germband embryos.

Even-skipped expression

316 The expression of pair-rule genes at the onset of gastrulation represents the earliest
317 landmark of the periodic organization at the core of the metameric insect embryo (Davis
318 and Patel, 2002) and constitutes a canonical marker for germband type (Patel et al.,
319 1994). For example, at this stage the short germband *T. castaneum* expresses two Eve
320 primary stripes, the intermediate germband beetle *Dermestes* displays four primary
321 stripes and *Calosobruchus maculatus* exhibits six Eve stripes consistent with its long
322 germband mode of development (Patel et al., 1994).
323 To further sustain our classification of the type of development in *M. cingulum*, we
324 examined Eve expression pattern. At the embryonic primordium stage, Eve expression is
325 absent (corresponding to Fig. 2A-D, data not shown). We first detect Eve protein in the
326 flattened embryo stage (Fig. 5A), corresponding to the SEM stage in Fig. 1H. At this
327 stage Eve protein is observed at low intensity around the circumference of the embryo
328 starting from 70% of the embryo length to the posterior (Fig. 5A arrows). In subsequent
329 stages, corresponding to Fig. 1G-H, this pattern and sub-cellular localization are
330 maintained (Fig. 5B) in what recapitulates the well-established early broad domain of Eve
331 expression present in many insect embryos (Liu and Kaufman, 2005b). At the onset of
332 gastrulation (late cup stage, Fig. 2A), Eve expression disappears from the future inter-
333 stripe region defined by the first Eve stripe (Fig. 5C). This stripe (designated 1ab) is six
334 to seven nuclei-wide and is followed by a posterior domain of expression (arrowheads).
335 As the embryo starts to extend (as in Fig. 2B), the first wide Eve stripe starts to split into
336 the secondary (segmental) stripes. The split of the stripe is initiated by a fading of the
337 protein in the middle of the stripe (Fig. 5D, arrow), resulting in a 4-5 nuclei-wide strong
338 anterior stripe designated 1a, and a narrower 2 nuclei-wide posterior stripe named 1b.

Meanwhile, the next Eve pair-rule stripe resolves from the posterior Eve expression domain (arrowhead, 2ab). As the embryo progresses through germband extension, the second stripe starts to split into segmental stripes (Fig. 5E, arrow). The split of this stripe results in the wider anterior (2a) and narrower posterior stripes (2b). Simultaneously, the first Eve stripe (1a and 1b) begins to fade and disappears at the stage shown in Fig. 2F, when second and third stripes form secondary a and b stripes and the fourth Eve stripe starts to split from the posterior zone (Fig. 5F, arrowhead). This dynamic logic is obeyed as development proceeds such that when the fourth and fifth stripes split into secondary stripes (a and b), the sixth and seventh Eve wide stripes appear almost simultaneously (Fig. 5G). Finally, at the completely extended germband stage almost all Eve stripes have faded and only the last pair of secondary stripes are visible (8a and 8b, Fig. 5H). At the condensed germband stage (Fig. 2 M), Eve protein is expressed in neurons and dorsal mesoderm (Fig. 5I). The morphogenetic movements of invaginating cells and the general morphology and stage of development shown in Fig. 2A demonstrate that these embryos are initiating gastrulation. Consequently, the presence of only one primary Eve pair-rule stripe at this stage (Fig 5C) classifies *M. cingulum* as a short germband insect where only a portion of the gnathal segments are likely to be specified at the time of gastrulation.

Engrailed expression

To extend our morphological and molecular analysis of *M. cingulum* morphogenesis we analysed expression of the segment-polarity protein En. Segment specification in arthropods is marked by the expression of En protein (Patel et al., 1989)(Fleig, 1990). In long germband insects, including *Drosophila* and honeybee, segments are established

362 nearly simultaneously with all En stripes forming almost at the same time (Patel et al.,
363 1989). In contrast, in short germband insects such as grasshopper and *T. castaneum* En
364 stripes are established one by one as segments are being formed sequentially from the
365 posterior growth zone (Peel et al., 2005).
366
367 In the early stages of the embryonic primordium, En protein was not detected (data not
368 shown). The first and second En stripes are detected in the cup stage embryo (Fig. 6A),
369 that corresponded to SEM stage described in Fig 3B. The first stripe (corresponding to
370 mandibular segment) is 3 cell diameters-wide (arrow) almost immediately followed by
371 the 1-cell diameter second stripe, corresponding to maxillary segment (arrowheads).
372 However, it increases in diameter during germband elongation. As the germband extends,
373 reaching the stage described in Fig. 2C, the third En stripe is formed (Fig. 6B, arrow)
374 corresponding to the labial segment. Formation of the third stripe is followed by the
375 initiation of the fourth En stripe that marks the first thoracic segment (Fig. 6B asterisk).
376 Again, this odd stripe is wider than even stripes. Upon cephalic furrow formation five En
377 stripes may be observed and formation of the sixth stripe has been triggered (Fig. 6C).
378 This stage is followed by a rapid germband extension such that, at the stage of formation
379 of gnathal furrows (see Fig. 3J), fifteen En stripes are observed (Fig. 6 D). The mature En
380 pattern, corresponding to 3 gnathal, 3 thoracic and 10 abdominal stripes is laid out at the
381 extended germband stage (Fig. 6E), corresponding to SEM stage described in Fig. 3K.
382 This pattern persists during the germband condensation stage with the addition of En
383 cephalic stripes (Fig. 3 F).

The sequential generation of En pattern further confirms that *M. cingulum* embryo exhibits short germband development containing only anterior structures at the cup-stage (blastoderm).

Posterior elongation in Macrocentrus cingulum

The budding of the posterior segments in short germband embryogenesis is not well characterized and it is yet unclear how body axis elongation is regulated by the putative growth zone (Peel et al., 2005). Two processes have been proposed to explain axial elongation: the higher mitotic activity of the growth zone and/or cell shape changes and convergent extension movements contributing to sequential segment formation (Heming, 2003). To characterize the elongation of the *M. cingulum* embryo, we examined expression of the segmental marker En together with expression of the phosphorylated histone that marks mitotically active cells. At the cup-stage embryo (prior to expression of the segmental markers) anti-histone H3 marks scarce groups of mitotically active cells interspersed with non-dividing cells (Fig. 7 A). At the onset of gastrulation, the posterior region of the embryo shows higher mitotic activity than the anterior (Fig. 7 B). However, a distinct mitotic domain was not observed and dividing cells appear to be spread uniformly throughout the entire posterior domain. During the process of germband elongation, mitotically active cells remain randomly distributed (Fig 7C) as well as during germband condensation (Figure 7 D). We failed to observe any distinct highly mitotic active domain. We also performed Z scans through multiple embryos at particular developmental stages (marked by En stripes, data not shown) but were not able to determine a specific region with higher mitotic activity.

Discussion

Macrocentrus cingulum is a short germband insect

The comprehensive SEM description of embryogenesis carried out in this work suggests that *M. cingulum* undergoes short germband morphogenesis. In addition, this mode of development is further confirmed through the use of classical molecular markers of segmentation, which show that at the time of gastrulation the embryo exhibits only anterior gnathal structures. Subsequent addition of segments at the posterior of the embryo is specified at the early blastoderm stage in a sequential manner. Cumulatively, both cellular and molecular aspects of *M. cingulum* development concur in that this wasp undergoes short germband development.

M. cingulum morphogenesis is initiated with formation of a hollow embryonic primordium, which quickly reorganizes into an embryonic blastoderm displaying the future head lobe and the posterior tip. At that time only one Eve stripe is present, suggesting that at this point only a portion of the gnathal segments are being specified and attesting that *M. cingulum* is the first *bona fide* example of short germband development in the Hymenoptera. Indeed, and despite the unpredictability across insects of the Eve expression pattern as pair-rule and/or segmental (Patel et al., 1994)(Liu and Kaufman, 2005a)(Mito et al., 2007), its dynamics constitute a solid diagnostic feature of developmental mode. In short germband insects such as *Tribolium castaneum*, the sequential growth of the germband is followed by a sequential expression of the subsequent Eve stripes in a pair-rule pattern (Patel et al., 1994)(Brown et al., 1997). In both species sequentially formed stripes are transient and disappear in an anterior to posterior progression. This sequential pattern of stripe formation is in sharp contrast with

the (near) simultaneous appearance of the complete Eve pair-rule pattern from an anterior domain in the honeybee (Wilson et al., 2010a) and *Bracon hebetor* (Grbic and Strand, 1998). In *Apis mellifera* pair-rule stripes split to form secondary, parasegmental, stripes in a brief anterior to posterior progression, while in *B. hebetor* they split simultaneously. Interestingly, *M. cingulum* also resolves a secondary, parasegmental Eve pattern by the split of the individual stripes. Spatially, Eve antigen disappears from the inter-stripe region in a manner reminiscent of that described for its long germband counterparts, the honeybee *Apis mellifera* and *B. hebetor*. However, temporally the split of the stripe happens soon after formation of the individual pair-rule stripe, in an anterior to posterior progression as described in the short germband insect *T. castaneum*. In contrast to the honeybee where Eve even stripes appear more intense than odd parasegmental stripes, in *M. cingulum* we find that after the split anterior (odd) stripes are more intense than the posterior (even) stripes. These aspects reinforce the notion that the role(s) of even-skipped is particularly labile in the evolution of insects and that short germband development as observed in *M. cingulum* may have an independent origin from that described in the canonical *Tribolium castaneum* system. Yet, our data suggests that other aspects of eve function, notably the (probably indirect) regulation of engrailed by eve, is a conserved feature of *M. cingulum* development. In *Drosophila*, Eve protein indirectly regulates expression of Engrailed (a segment polarity gene) that specifies the posterior segmental compartments (Fujioka et al., 1995). In all other examined insects except grasshopper (Patel et al., 1992) the expression patterns of Eve and En are consistent with this relationship. Due to technical difficulties we have not performed double staining of Eve and En proteins. However, based on morphological markers En expression appears

to be within Eve stripe domains and En stripes appear sequentially, following the appearance of Eve stripes. Also, Odd stripes of En expression are transiently larger than even stripes (see Fig 3A). Taken together, these observations suggest a regulation of *en* by *eve*.

Reverse evolution to short germband development

The phylogeny of Hymenoptera suggests that the suborder Apocrita (parasitic wasps, ants and bees) originated from a dryinid-like ancestor that underwent long germband development. The canonical representative of the basal Apocrita is the long germband *Apis mellifera* (reviewed in Grbic 2000). The long germband development of cyclostome braconids illustrated by *Bracon hebetor* suggests that long germband development also represents the ancestral type of development in braconidae (Grbic and Strand 1998). In contrast, the phylogenetic position of the non-cyclostome braconid *Macrocentrus cingulum* (Shi et al., 2005) suggests that its short germband development constitutes a secondarily derived trait. This notion is reinforced by the strong suspicion that *Aphidius ervi*, which occupies the sister clade to *M. cingulum*, undergoes short germband development (Grbic, 2003).

This direction of evolutionary trajectories demonstrates that evolutionary processes can drive not only evolution from the short germband to long germband development, but also that the directionality of evolutionary change can be reversed. Our data suggest that short germband embryogenesis evolved multiple times complementing the proposal that long germband development may have evolved on multiple occasions (Dawes and Patel 2002).

It is firmly established that at the base of the insect lineage lies some version of short germband development (Sander, 1976)(Davis and Patel, 2002). Though originally defined for insects by Krause (Sander, 1976), the short and long germband contrasting modes of development (and all intermediate forms) may be extended to arthropods as a whole, since this dichotomy has been reported in chelicerates, myriapods and crustaceans (Scholtz and Wolff, 2013). Also, in recent years it has suggested that this ancestral mode of segmentation could be shared by vertebrates and invertebrates and date back to the Urbilateria (Peel, 2004)(De Robertis, 2008). One particularly interesting model points to the generic involvement in this mechanism of the Wnt and Notch pathways, coordinately controlling proliferation and segmentation, respectively (McGregor et al., 2009). Indeed, Wnt signalling appears to play a role in axis elongation in all short/intermediate germband arthropods analysed thus far, including spiders (McGregor et al., 2008), *Gryllus bimaculatus* (Miyawaki et al., 2004) and *Tribolium* (Bolognesi et al., 2009)(Beermann et al., 2011). Also, central to this idea is evidence showing that Notch signalling participates in short germband embryo segmentation in arthropods such as spiders (Stollewerk et al., 2003) and the cockroach *Periplaneta americana* (Pueyo et al., 2008), in which the presence of a clock-like mechanism was established recently (Sarrazin et al., 2012). Yet, conflicting evidence gathered in *G. bimaculatus* (Mito et al., 2011)(Kainz et al., 2011), as well as parsimony arguments speak against the common ancestry scenario and point to an independent co-option of this gene regulatory network (GRN) in the parallel evolution of the short germband mode of segmentation in vertebrates and arthropods (Couso, 2009)(Chipman, 2010). Interestingly, strong evidence

supports the idea that germband growth and segmentation may be decoupled in crustaceans (Williams et al., 2012), chelicerates (Stollewerk et al., 2003) as well as insects, namely *Gryllus bimaculatus* (Kainz et al., 2011) and *Apis mellifera* (Wilson et al., 2010b).

It has been proposed that long germband development may have evolved through a heterochronic shift in the ancestral short germband development through a relative delay in segmentation and/or an acceleration of embryo growth (Scholtz, 1992).

Thus, the evolution of short germband development in *M. cingulum* could be explained as a reversion of this heterochronic change in growth dynamics or a temporal extension of the patterning mechanisms from the ancestor typified by *Bracon hebetor*. In any case, a burning question raised by this independently evolved short germband type of development, concerns the involvement and importance of the Wnt (and caudal) and Notch pathways in this process. Confirming a role of these pathways would strengthen the notion that the decoupling of elongation and segmentation is plastic and probably a result of the intrinsic properties of the underlying GRN. This would reinforce the hypothesis that segmentation has evolved independently through co-option of an ancient Notch/Wnt-based interaction module devoted to balance cell fate decisions in a wide range of animal development contexts (Hayward et al., 2008). Moreover, the proposed pivotal role of the Notch/Wnt regulatory module in the shift between germband modes may help resolving the difficulties raised by the extreme difference in signalling contexts of *Bracon hebetor* (long) and *M. cingulum* (short). Indeed, *B. hebetor*, the honeybee and *Drosophila* develop in a syncytium where nuclear divisions are not followed by cytokinesis and depend on a diffusion-based patterning system. In contrast, the *M.*

cingulum embryo, as other short and intermediate germband arthropods, consists of individualized cells from very early on and must rely on a cell-cell communication patterning system. Future work on the role of this pathway in *M. cingulum* and other insects and arthropods will clarify the mapping of germband development modes and associated mechanisms onto a high-resolution phylogenetic context. This will permit more robust conclusions on the polarity of change and the mechanistic bases of germband type and segmentation evolution.

Comparing the patterning of *Macrocentrus cingulum* and *Tribolium castaneum*, as representative of the putative ancestral state of short germband development, reveals striking similarities. Yet, the extent to which this reversion from long germband development back to short germband may parallel the ancestral situation remains to be determined. One putatively interesting departure from the canonical process of segmentation and growth described for short germband organisms, specifically *Tribolium castaneum*, is the apparent absence of a growth zone at the posterior tip of the embryo. Indeed, we have failed to confirm a higher density of proliferating cells across the extending abdominal region of the developing embryo. This observation suggests a putative change in the mechanisms operating in these independently evolved short germband organisms. At this point, our analysis is too broad to ascertain conservation or divergence in the players that read the early (maternal) determinants and in those, which regulate the balance between growth and segmentation. We have hypothesized that the Wnt/Notch module may be at the core of this reversion. To test this hypothesis will be highly informative in this respect (the biological system permitting) as the

characterization of a larger regulatory network will help to ascertain the similarities and differences of this GRN to that of *Tribolium* and provide a putative mechanistic basis for this homoplasy. Moreover, the comparison of the GRN operating in *M. cingulum* to that of other Braconidae or Hymenoptera would reduce the timescale of the comparison and possibly provide a comprehensive map of GRN topology evolution. The role of constraints and the evolvability of fundamental developmental processes such as segmentation may be best studied and properly quantified at short time scales (Peter and Davidson, 2011) such as those provided by the Braconidae. Indeed, it is becoming more and more evident that the topology of well-established GRNs (typically defined in *D. melanogaster*) is evolutionarily more plastic than expected (Hinman and Davidson, 2007)(Vreede et al., 2013) despite the undisputed weight of historical contingency (Payne and Wagner, 2013).

Future work on the genetic architecture of germband development modes in *M. cingulum* and other insects and arthropods, where solid phylogenies for relatively short timescales containing multiple examples of developmental programme (reverse) change, constitute a fertile ground for a proper understanding of the intimate relationship between development and evolution.

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Figure legends:

Figure 1

Early morphogenesis of *Macrocentrus cingulum*

(A) Early embryonic primordium. The arrow points to the hollow cavity. Asterisk marks extraembryonic membrane and arrowhead points to rounded cells with specific "cobblestone" appearance. (B) Radially symmetric early embryonic primordium dissected from the extraembryonic membrane. Apical cell surface flattened (compare to A). (C) Broken embryonic primordium showing the blastocoel cavity (arrow) and tightly organized columnar cells (arrowhead). (D) Initiation of dorso-ventral flattening of the embryonic primordium. (E) Flattened embryonic primordium. (F) Onset of dorsal opening formation (arrow). (G) Expansion of dorsal opening (arrow) and formation of donut-shaped embryo. (H) Folding of the embryonic epithelium at anterior (arrowhead) and posterior (arrow). Embryo forms a cup-shape. Inset: high magnification of posterior area marked by rectangle. Anterior is up.

Figure 2

Germband extension and segmentation of *Macrocentrus cingulum*

(A) Initiation of the embryonic growth and gastrulation. Arrow marks the gastrulating cells. (B) Posterior coiling of the embryo. Arrow points to extracellular matrix in dorsal area. (C) Embryo undergoing the germband extension. (D) Progressive elongation of the embryo. Posterior transient furrow is marked by the arrow. (E) Embryo at the similar stage as D but uncoiled, showing enlarged dorsal opening (arrow). (F) Initiation of cephalic furrow formation (arrowhead). At this time, the gastrulation furrow extends along the ventral midline (arrow). (G) Broken embryo showing tightly interdigitated columnar epithelium. (H) Bulging of the head lobes. (I) Formation

of the gnathal furrow (arrow). (J) Separation of mandibular (m), maxillary (ml) and labial (l) segments. An arrow marks labral furrow. (K) Bulging of gnathal segments and formation of the thoracic furrow (arrow). (L) Formation of another furrow in the thoracic region. (M) Germband condensation and initiation of gnathal involution (arrow). All segmental furrows are visible. Arrow marks primordia of the proleg-like structures. (N) Completely condensed embryo. Involution of gnathal segments is marked by arrow and proleg-like structures by arrowheads. (O) Completely formed larva comprising of 3 thoracic (T1-3) 10 abdominal segments (A1-10) and telson (T). Anterior is to the left and dorsal is up.

Figure 3

Expression of Vasa and Engrailed proteins in *Macrocentrus cingulum*

(A-D) The initial Vasa protein localization to the posterior terminus of the embryo is consistent with a conserved role in specifying posterior in *M cingulum.* (C-E) At later stages, the number of Engrailed stripes increases through sub-terminal growth of the embryo, anterior to Vasa-positive cells. (F-H) As the embryo grows, Vasa-positive cells exhibit the canonical migration to a more medial-posterior bilateral localization, consistent with germ cell placement at the differentiating gonad primordium.

Figure 4

In situ hybridization analysis of *Macrocentrus cingulum Krüppel*

Krüppel expression in *M. cingulum* is reminiscent in its patterns and dynamics to other described short and intermediate germband embryos. (A) Very early embryos do not

813 show *Kr expression.* (B-E) Early *Mc-Kr* expression exhibits a typical gap-like pattern and
814 covers the presumptive thoracic region. (F-H) As in all insects studied thus far, later
815 expression in CNS can be observed.
816
817
818 Figure 5
819 Expression of Eve in *Macrocentrus cingulum*
820 (A) Expression of Eve in the flattened embryo primordium. The arrow marks nuclear
821 antigen localization in the anterior region. (B) Expression of Eve in the cup-stage
822 embryo. The arrow marks the posterior group of cells that do not express Eve and
823 arrowheads the anterior Eve boundary. (C) Formation of the first Eve stripe (marked
824 1ab). Arrowheads mark the anterior boundary of the posterior Eve domain, which is
825 visible as a continuous expression only on edges of the embryo due to embryo curvature
826 (D) Split of the first stripe (arrow) where anterior stripe is marked 1a and posterior 1b. A
827 second stripe has been formed (arrowhead). (E) The split of the second Eve stripe (2a and
828 2b). Note that the first Eve stripe already started to fade. Arrowheads mark the anterior
829 boundary of the posterior Eve domain. (F) Formation of third secondary (3a and 3b) and
830 fourth pair-rule (4ab) Eve stripes. The arrowhead demarcates the posterior zone
831 expressing Eve. (G) Split of fourth and fifth Eve stripe and formation of sixth and seventh
832 pair-rule stripe. Stripe 5b is partly obscured by the embryo head. (H) Completely
833 extended germband showing expression of Eve in stripe 8a and 8b. Note that all Eve
834 anterior stripes have faded. (I) Expression of Eve in the nervous system and dorsal

mesoderm. The arrow marks Eve-positive neurons and the arrowhead expression in dorsal mesoderm that has faded in the anterior region.

Figure 6

Expression of En in *Macrocentrus cingulum*

(A) Formation of first (arrow) and second (arrowheads) En stripes (red) in the cup-stage embryo. (B) Formed third (arrow) and initiation of the fourth En stripe (asterisk marks this stripe in B-F) during germband extension. (C) Sequential formation of fifth and sixth En stripe. Asterisk marks the first thoracic stripe and arrowhead shows the initiated sixth En stripe. (D) Embryo at the extended germband stage displaying fifteen En stripes. (E) Completely extended germband with sixteen En stripes. (F) Embryo undergoing the germband condensation expressing the mature pattern on En. Embryos are counterstained with phalloidin (green) and oriented with anterior to the left and dorsal up except A and C, which display a ventral view.

Figure 7

Cell proliferation profile throughout *Macrocentrus cingulum* development

(A-D) Simultaneous anti-En and anti-Histone H3 antibody staining in the segmenting embryo show no clear preferential mitotic domain associated to embryo elongation. mb - mandibular; mx-maxillary.

FIGURE 1

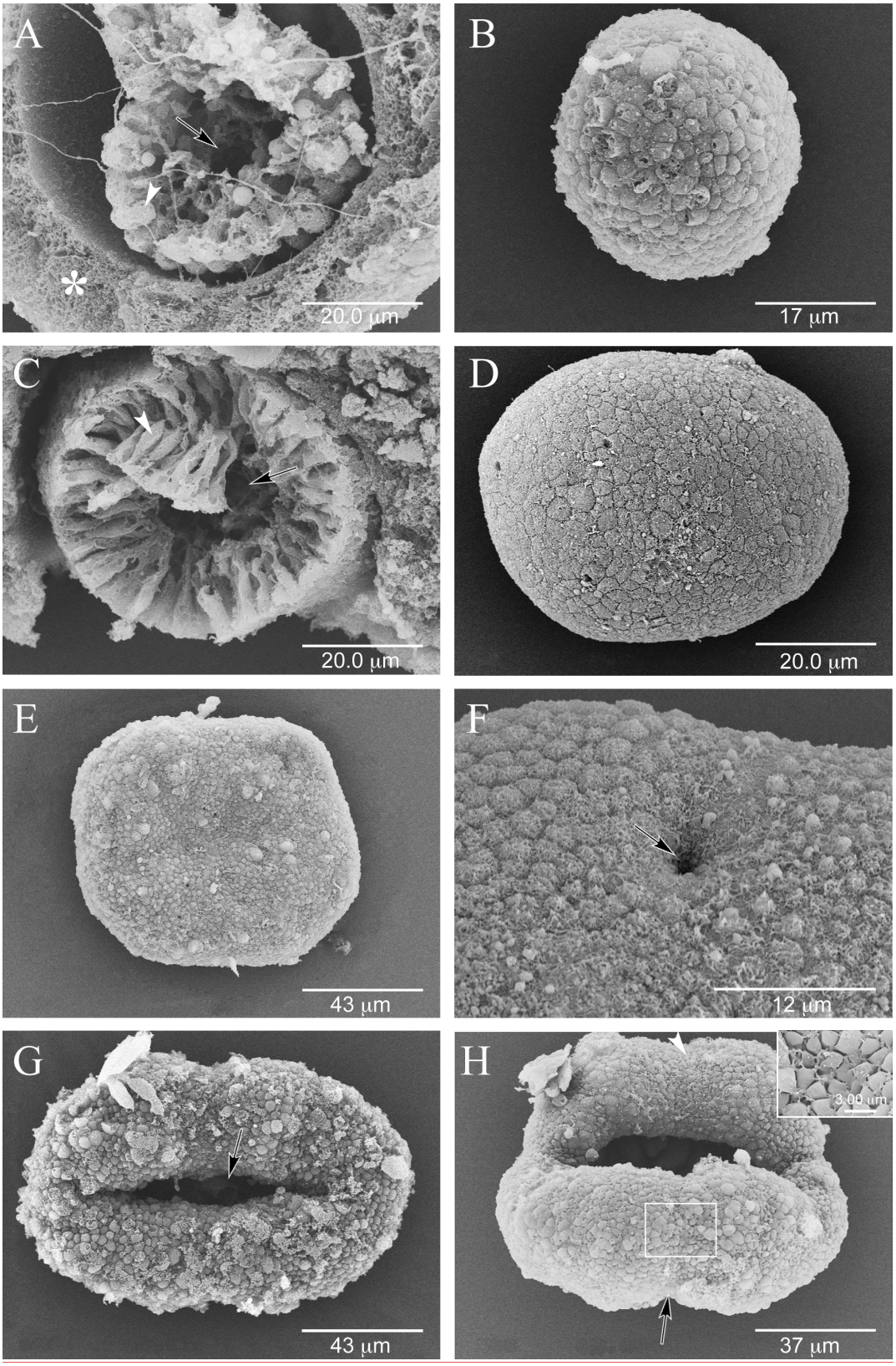

FIGURE 2

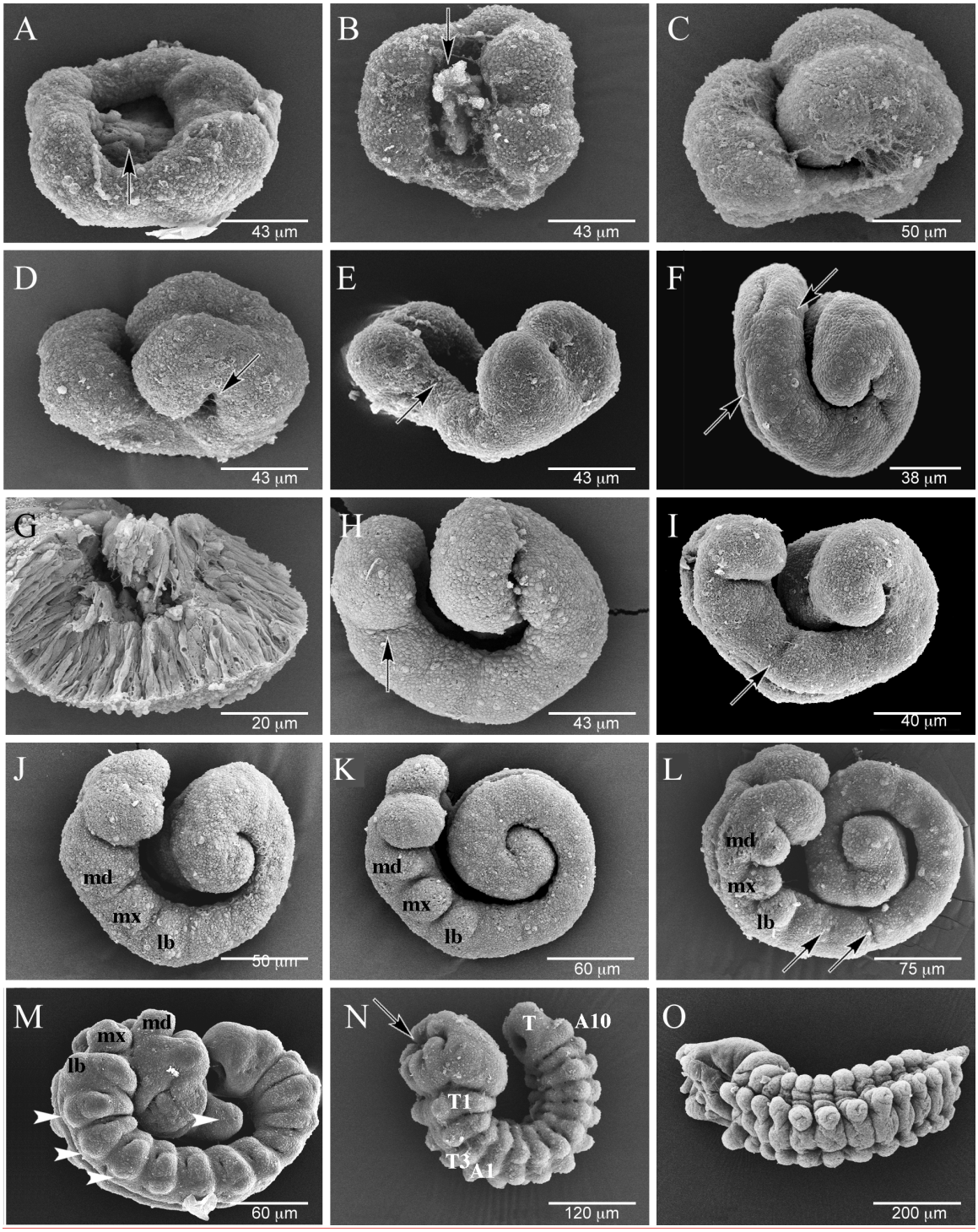
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FIGURE 3

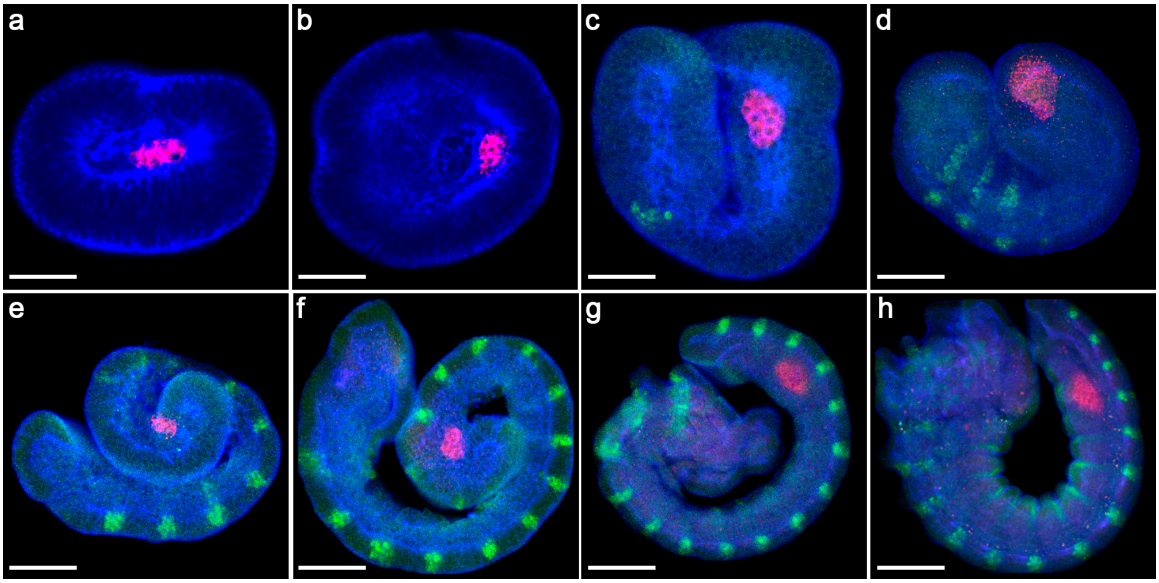
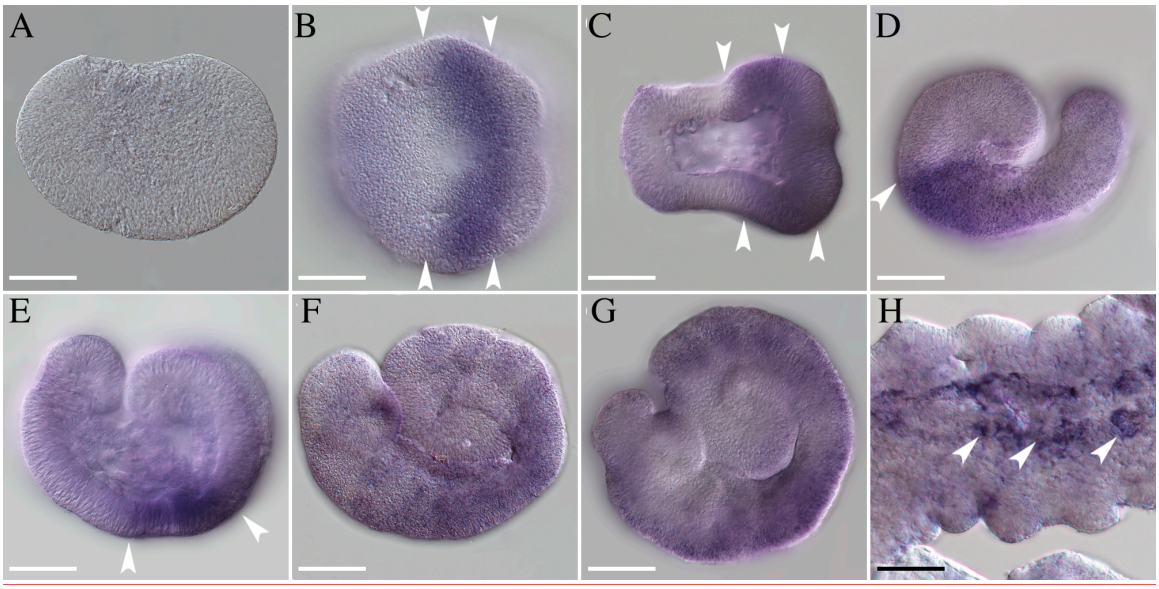


FIGURE 4



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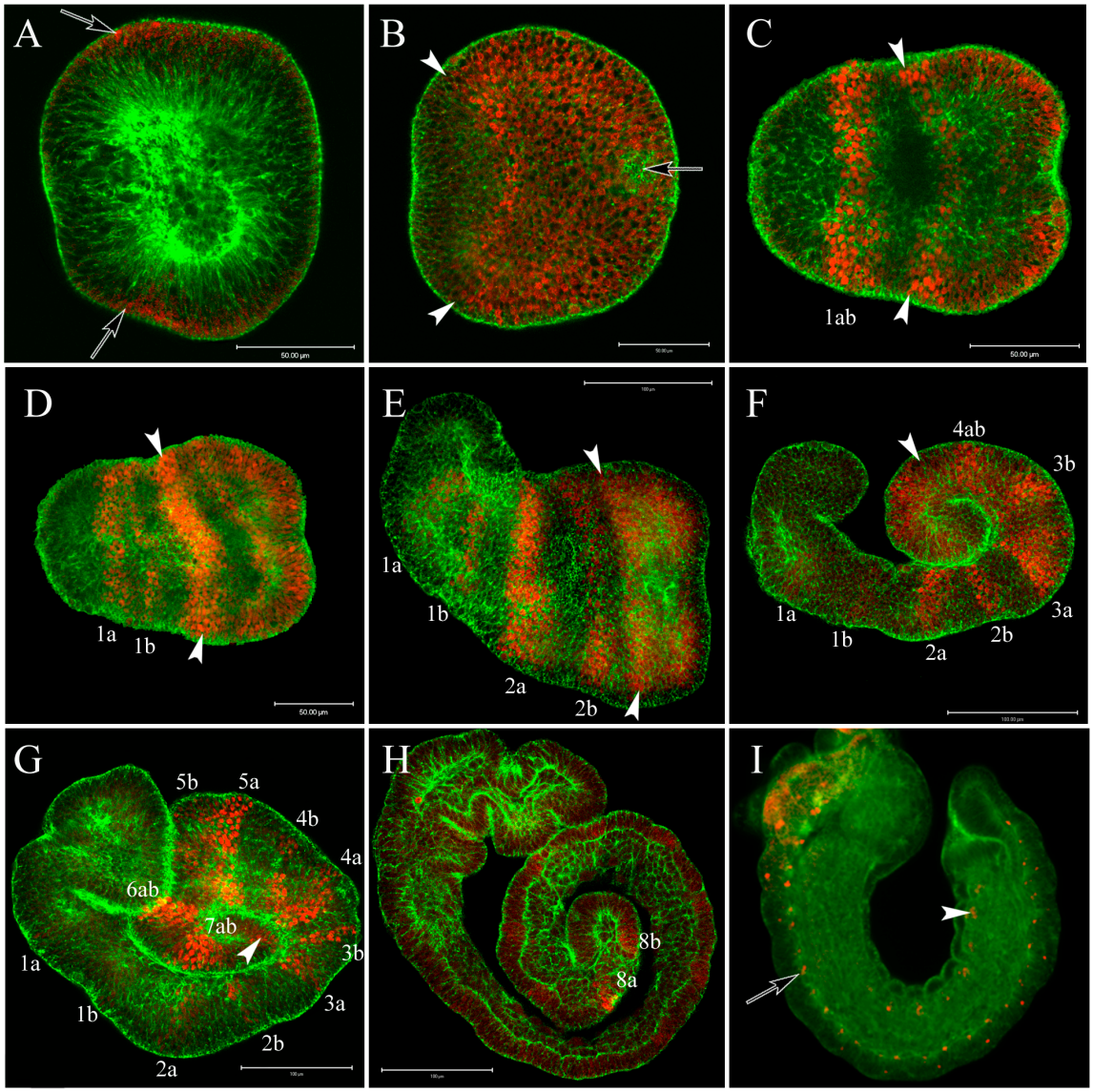
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FIGURE 6

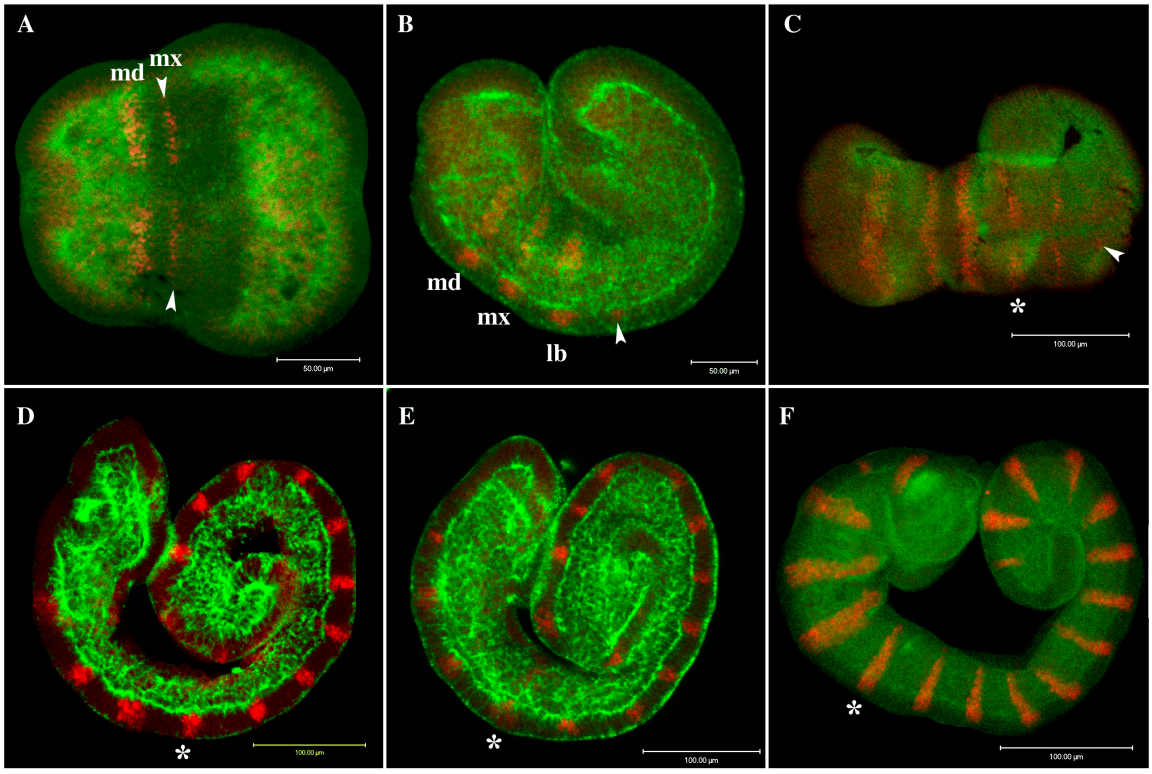

FIGURE 7