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Gene expression suggests double-segmental and single-segmental patterning mechanisms during posterior segment addition in the beetle *Tribolium castaneum*

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ABSTRACT In the model arthropod *Drosophila,* all segments are patterned simultaneously in the blastoderm. In most other arthropods, however, posterior segments are added sequentially from a posterior segment addition zone. Posterior addition of single segments likely represents the ancestral mode of arthropod segmentation, although in *Drosophila,* segments are patterned in pairs by the pair-rule genes. It has been shown that in the new model insect, the beetle *Tribolium*, a segmentation clock operates that apparently patterns all segments in pairs as well. Here, I report on the expression of the segment polarity gene *H15/midline* in *Tribolium*. In the anterior embryo, segmental stripes of *H15* appear in pairs, but in the posterior of the embryo stripes appear in a single-segmental periodicity. This implies that either two completely different segmentation-mechanisms may act in the germ band of *Tribolium*, that the segmentation clock changes its periodicity during development, or that the speed in which posterior segments are patterned changes. In any case, the data suggest the presence of another (or modified), yet undiscovered, mechanism of posterior segment addition in one of the best-understood arthropod models. The finding of a hitherto unrecognized segmentation mechanism in *Tribolium* may have major implications for the understanding of the origin of segmentation mechanisms, including the origin of pair rule patterning. It also calls for (re)-investigation of posterior segment addition in *Tribolium* and other previously studied arthropod models.

KEY WORDS: *segmentation, arthropod development, arthropod evolution, segment polarity*

Our understanding of arthropod segmentation comes primarily from studies on the model organism *Drosophila melanogaster*. Here, a hierarchic segmentation gene cascade operates to subdivide, in a stepwise fashion, a syncytial blastoderm that later develops without posterior segment addition into the complete adult body. Notably, one step of this segmentation mechanism comprises the temporal establishment of double-segmental units, as shown by the function (and expression) of the pair-rule genes. In most other arthropods, only anterior segments are formed from the blastoderm, and posterior segments are added from a posterior segment addition zone (Davis and Patel 2002). Posterior segment addition with a single-segmental periodicity likely represents the ancestral mechanism, as suggested by morphological observations and gene expression analysis (Schoppmeier and Damen 2005, Janssen 2011). Evidence for double-segmental patterning mechanisms in the blastoderm, superficially comparable to *Drosophila* pair-rule patterning, has, however, been found in distantly related arthropods (Dearden *et al.,* 2002, Janssen *et al.,* 2012). Double-segmental patterning has also been found in tissue that is generated from the posterior segment addition zone in the beetle *Tribolium castaneum* (Choe *et al.,* 2006) in addition to other insects (Davis *et al.,* 2001, Mito *et al.,* 2007, Erezyilmaz *et al.,* 2009) and a distantly related arthropod, the centipede *Strigamia maritima* (Chipman *et al.,* 2004). These findings support the idea that a double-segmental posterior patterning system may be a conserved component of arthropod (or at least mandibulate) segmentation. On the other hand, a vertebrate-like posterior segment addition mechanism was proposed for arthropods in which an oscillating clock mechanism would underlie posterior segment addition and patterning (Stollewerk *et al.,* 2003, Chesebro *et al.,* 2013). In vertebrates, posterior segments are strictly added and patterned as single segments (somites) (Gomez *et al.,* 2008). Recent studies have revealed the presence of an oscillating vertebrate-like pat-

Abbreviations used in this paper: HH, hedgehog; SPG, segment polarity gene; wg, wingless.

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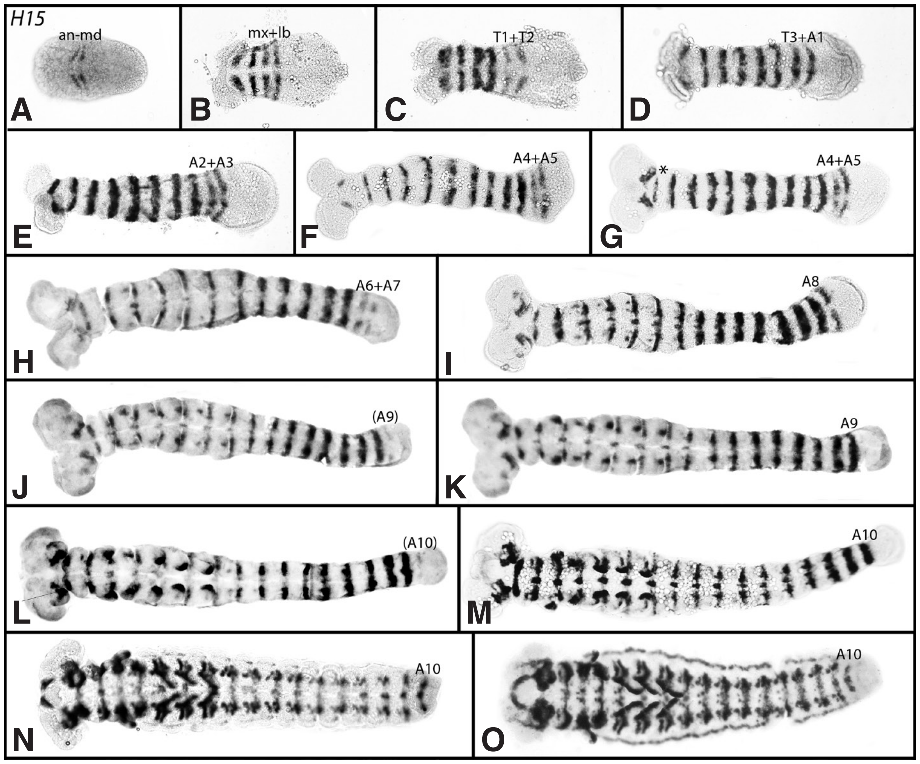

Fig. 1. Expression of *H15*: appearance of segmental stripes in pairs of two and as single stripes. *In all panels anterior is to the left. All embryos, except the embryo shown in (A), have been flat-mounted.* **(A)** *Two stripes of expression.* **(B)** *Four stripes of expression.* **(C)** *Six stripes of expression.* **(D)** *Eight stripes of expression.* **(E)** *10 stripes of expression.* **(F)** *12 Stripes of expression.* **(G)***The intercalary stripe (asterisk) forms; 13 stripes of expression.* **(H)** *15 stripes of expression.* **(I)***16 (!) stripes of expression.* **(J)***17th stripe of expression appears.* **(B)***17 stripes of expression.* **(L)***18th stripe of expression appears.* **(M)** *18 stripes of expression.* **(N)** *18 stripes of expression.* **(0)***18 stripes. Abbreviations: an, antennal segment; md, mandibular segment, mx, maxillary segment; lb, labial segment; T1-T3, first to third thoracic segment; A1-A11, first to eleventh abdominal segment. Segment abbreviations in brackets indicate nascent expression.*

terning mechanism in *Tribolium*, and at the same time show that this mechanism acts in a two-segment periodicity (Sarrazin *et al.,* 2013, El-Sherif *et al.,* 2013).

I analyzed the expression pattern of the segment-polarity gene *H15* (aka *midline*) in *Tribolium* and found that this gene is likely regulated in a double-segmental pattern in the blastoderm and most of the posterior segments. However, in the later-developed segments, *H15* is apparently regulated in a single-segmental fashion. Thus, my data reveal the presence of a single-segmental patterning system in *Tribolium*, different from the previously described double-segmental mechanism. This single mechanism, which is likely ancestral, may then have evolved into the double-segmental patterning present in the anterior germ band of *Tribolium*. Most importantly, however, the new data suggest that an additional mechanism of posterior segment addition may have escaped scrutiny in previous studies in this emerging model organism.

Results

Expression of Tribolium H15

Expression first appears in the form of two segmental stripes that are associated with the primordia of the antennal and the mandibular segments (Fig. 1A). Note that the rudimentary intercalary segment will subsequently form between those stripes and express *H15* at a later developmental stage. Individuals with a single stripe of expression were never found. At the subsequent stage, two additional stripes of expression appear (associated with the maxillary and labial segments) (Fig. 1B). Embryos with three stripes were never found. At the next stage, six stripes are present, of which the posterior most two bands (in the first two thoracic segments) are of the same weakened intensity (compared to the more anterior stripes) (Fig. 1C). Embryos with five stripes were never observed. This periodicity of two additional stripes (and no intermediates) is repeated in three further events, resulting in embryos with eight, 10, or 12 stripes (Fig. 1 D-F). The next change in the expression pattern concerns the delayed appearance of the intercalary stripe between the antennal and the mandibular stripe (Fig. 1G). In the next stage, embryos with two additional posterior stripes (sixth and seventh abdominal segment) can be found (Fig. 1H). Notably, the pattern of posterior stripe-addition now changes towards a single-segmental mode, in which abdominal stripes eight, nine and ten form (Fig. 1 I-N).

At later developmental stages *H15* is expressed along the ventral surface of the limbs (Fig. 1 K-O), the developing heart,

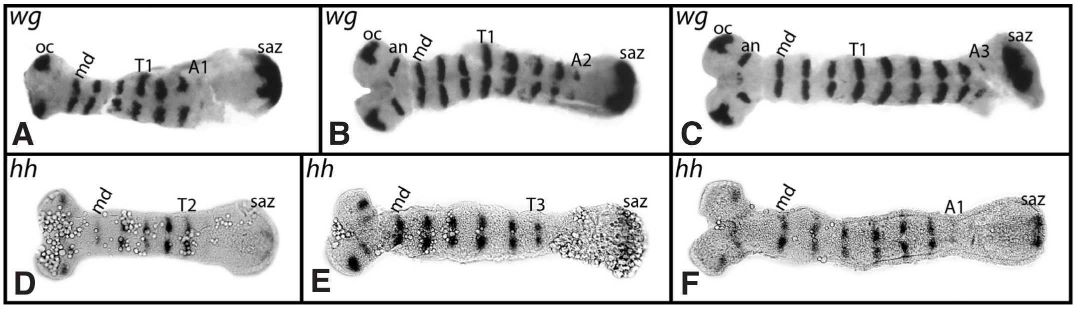

Fig. 2. Expression of *wingless* (*wg*) and *hedgehog* (*hh*): segmental stripes appear one by one. (A-C) *Expression of* wg. **(D-F)** *Expression of* hh. *In all panels anterior is to the left. Embryos have been flat-mounted.* **(A)** *Stripe of* wg *in the first abdominal segment forms.* **(B)** *Stripe in the second abdominal segment forms.* **(C)** *Stripe in the third abdominal segment forms.* **(D)** *Stripe of* hh *in the second thoracic segment forms.* **(E)** *Stripe in the third thoracic segment has appeared.* **(F)** *Stripe in the first abdominal segment forms. Abbreviations as in Fig. 1, oc, ocular region; saz, segment addition zone.*

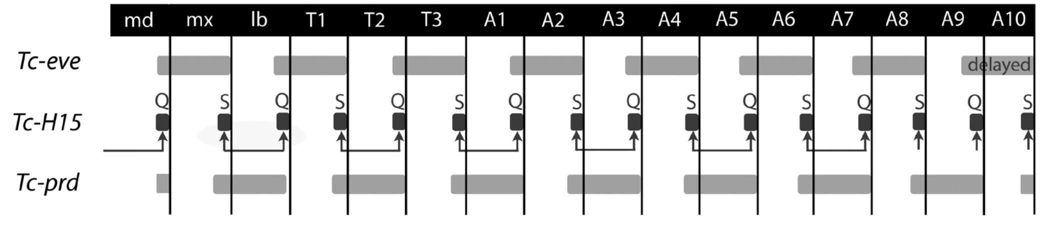

Fig. 3 Summary of *H15* expression and its theoretical activation by the pair rule genes *even-skipped* (*eve*) and *paired* (*prd*). *Light grey bars indicate double-segmental primary expression patterns of* eve *and* prd *respectively. Note that the last wave of* eve *is delayed. "Q" and "S" indicate potential quick and slow activation of* H15 *by* eve. *Dark grey bars indicate single segmental expression of* H15. *Stripes of* H15 *that appear simultaneously are connected by double-arrows. Stripes of* H15 *that appear one by one are indicated by simple arrows. Abbreviations: A1-A10, first to tenth abdominal segment; md, mandibular segment; mx, maxillary segment; lb, labial segment; T1-T3, first to third thoracic segment.*

dorsally in the labrum, and in the developing ventral nervous system (Fig. 1 N,O).

Discussion

Unique regulation of H15 in Tribolium

In *Drosophila*, *H15* acts as a segment-polarity gene (SPG) and its function is required to break symmetry of the otherwise bi-directional Hedgehog (Hh) signaling (Buescher *et al.,* 2004). Since the overall expression pattern of *H15* is conserved in all hitherto studied arthropods (Prpic *et al.,* 2003, Buescher *et al.,* 2004, Janssen *et al.,* 2008a, b, Svendsen *et al.,* 2009), and since the SPG-network itself is also highly conserved in arthropods including *Tribolium* (Farzana and Brown 2008, Janssen *et al.,* 2004, 2008a), this implies also that the function of *H15* in the beetle is likely conserved. Notably, however, *H15* appears to be the only SPG that is regulated in a double-segmental fashion. Other SPGs such as *wingless* (*wg*) (Nagy and Carroll 1994) (Fig. 2 A-C), *engrailed* (*en*) (Brown *et al.,*1994) and *hedgehog* (*hh*) (Farzana and Brown 2008) (Fig. 2 D-F) appear to be regulated in a single-segmental fashion. It has been shown, however, that the genetic interaction that leads to the activation of *wg* in odd and even numbered parasegments (i.e. in adjacent segments) differs considerably (Choe and Brown 2009). It may then be the case that the single-segmental (and strictly anterior to posterior) appearance of *wg* is merely the result of differences in the upstream regulatory network. This could lead to a temporal delay of *wg* expression in the posterior of two simultaneously established segmental units.

What is the cause of the double vs. single-segmental appearance of H15?

It is obvious that the regulation of *H15* in the posterior abdomen in *Tribolium* is different when compared to the patterning of the more anterior segments. It is either the case that: two generally different patterning mechanisms function in *Tribolium* (like in the myriapod *Strigamia* (discussed in the following section) (Brena and Akam 2013)); or that the apparent regulation of *H15* in pairs is just the result of upstream clock dynamics. This could be the case if the anterior stripe of *H15* that is regulated by a dynamic wave of pair-rule gene expression comes up quickly, while the posterior stripe regulated by the previous wave of pair-rule gene expression comes up slowly. In that way both stripes may appear at the same time. This would be in line with the shifted appearance of *H15* in adjacent segments compared to the waves of *eve*n-skipped (*eve*) expression (Choe and Brown 2007) and the fact that the last wave of *eve*-expression is delayed (El-Sherif *et al.,* 2012). Slowing down of the first 'tick' of the clock towards the end of embryogenesis would then lead to the appearance of single stripes of *H15* in the last formed segments (summarized in Fig. 3). An alternative scenario with *paired* (*prd*) being in control of *H15* would not require slow and quick activation of *H15* in adjacent segments because the double-segmental domains of *prd* are in register with the appearance of *H15*. It would, however, not explain the delayed appearance of *H15* in A8 and A9 without further modification of *H15*-regulation.

A drastic alternative is that the segmentation-clock may change its periodicity from double- towards single-segmental in nature. If this is the case, then the question is what causes this *switch*? This may be a matter of available space. Firstly, it is known that the vertebrate segmentation clock "ticks" for as long as presomitic mesoderm is present (the amount of this tissue is consumed during the process of segment addition) (Gomez *et al.,* 2008). It is therefore not unlikely that the arthropod segmentation clock requires comparable tissue (the segment addition zone), independent of whether vertebrate and arthropod segmentation clocks are homologous or analogous. Secondly, we find that in the centipede *Strigamia* (Geophilomorpha) a double-segmental patterning mechanism exists (likely clock-based as suggested by dynamic gene expression patterns in the saz) (Chipman *et al.,* 2004, Brena and Akam 2013). In this species the saz is expansive. In other myriapods such as the centipede *Lithobius forficatus* (Lithobiomorpha) the saz is much reduced and no evidence of a double-segmental patterning mechanism has been found. Although it is not unlikely that the double-segmental mechanism in *Strigamia* is the result of convergent evolution, the large saz may have provided the morphological prerequisite for the evolution of this patterning mechanism. For *Tribolium*, this could mean that the *switch* from double- to single-segmental patterning is caused by the shrinking of the saz towards the end of ontogenesis. In order to test this hypothesis it would be interesting to study gene expression of *H15* (and other SPGs) in arthropod species with small, intermediate and large segment addition zones.

On the origin of pair rule-like patterning mechanisms

The current study revealed the possible involvement of a single and a double-segmental patterning system in *Tribolium*. This is strikingly similar to what a very recent study has demonstrated to be the case for the centipede *Strigamia* (Brena and Akam 2013). However, in *Strigamia* the change from double- to single-segmental patterning apparently comes with a general change of genetic regulation, and is not the result of a slower-ticking clock mechanism (Brena and Akam 2013). With our current knowledge we cannot decide whether the similarities found in *Tribolium* and *Strigamia* are the result of convergent evolution or, alternatively, the evolutionary remnant of an ancestral mandibulate patterning system that involved single- and double-segmental patterning mechanisms.

This is because the unique patterning of the posterior-most abdominal segments has not been recognized until now, except for the statement of El-Sherif *et al.,* (2012) that the appearance of the last stripe of *even-skipped* expression is significantly delayed.

Future perspectives

As a consequence of the current study, it will now be necessary to further investigate posterior (single) segment addition in *Tribolium* in order to find out if it underlies different regulatory mechanisms than double-segmental patterning, and if those are potentially similar to the mechanisms of single segment addition in *Strigamia*. We also will have to investigate posterior segment addition in other insects that pattern segments in pairs. The question is whether they pattern all segments by the same double-segmental mechanism, and if this is not the case, if single segmental posterior segment patterning underlies the same (or similar) genetic regulation system as in *Strigamia* and/or *Tribolium*. A first step must be to study the expression of known posterior segmentation genes, such as the pair-rule genes, in relation to the expression of *H15*, and to study functional aspects of *H15* during anterior and posterior segmentation in *Tribolium*. The aim of this paper is to demonstrate that differences in anterior and posterior segmentation exist in the model arthropod *Tribolium*, and to highlight the urgent need for further detailed investigation of *Tribolium* segmentation mechanism(s). If both, single and double-segmental patterning mechanisms were present in the last common ancestor of arthropods (or at least mandibulates), this would explain the widespread appearance of pair rule-like expression patterns throughout Arthropoda.

Materials and Methods

Gene cloning and expression of *Tribolium H15* in the developing heart has been described before (Janssen and Damen 2008). Fragments of *wingless* (*wg*) and *hedgehog* (*hh*) were amplified with the degenerate primers described by Damen (2002) and Janssen *et al.,* (2004). Expression of *wg* was described by Nagy and Carroll (1994) and expression of *hh* has been described by Farzana and Brown (2008). *In-situ* hybridization of embryos was performed as described by Tautz and Pfeifle (1989). Flat-mounted embryos were analyzed under a Leica MZFLIII dissection microscope equipped with a Leica DFC490 digital camera, or under a Nikon ECLIPSE E400 microscope equipped with a Nikon D70 portable digital camera. Brightness, contrast and color values were adjusted in all images using the image processing software Adobe Photoshop CS2 (Version 9.0.1 for Apple Macintosh).

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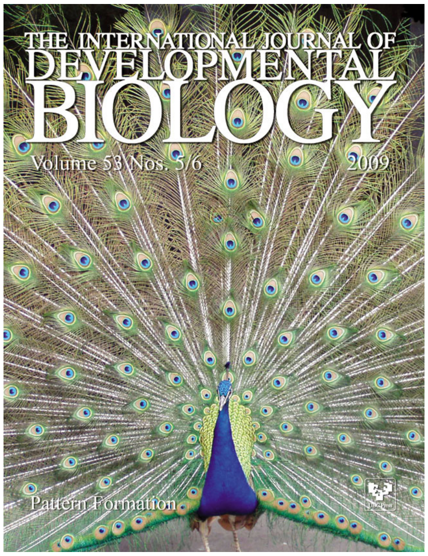

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