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Maintenance of segment and appendage primordia by the *Tribolium* gene *knödel*

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Abstract

For homeotic and segment-polarity genes in *Drosophila*, a switch in gene regulation has been described that distinguishes patterning and maintenance phases. Maintenance of segment and organ primordia involves secondary patterning and differentiation steps, as well as survival factors regulating proliferation and organ size. In a screen for embryonic lethal mutations in the flour beetle *Tribolium castaneum*, we have recovered two alleles of the *knödel* gene, which result in short, bag-like embryos. These embryos have severely reduced appendages and differentiate a cuticle that lacks most overt signs of segmentation. In addition, they lack bristles and display defects in the nervous system. Early patterning in *knödel* mutant embryos is normal up to the extended germ band stage, as indicated by the formation of regular *even-skipped* (*Tc'eve*) and *wingless* (*Tc'wg*) stripes. Afterwards, however, these patterns degenerate. Similarly, proximo-distal growth and patterning of limbs are nearly normal initially, but limb primordia shrink, and proximo-distal patterns degenerate, during subsequent stages. *knödel* could be a segment polarity gene required for segment border maintenance in both trunk and appendages. Alternatively, it may have a more general role in tissue or organ maintenance.

Keywords: Tribolium; knodel; Naked cuticle; Segmentation; Segment polarity; Maintenance; leg development

1. Introduction

The principles underlying the formation of *Drosophila* body segments are well understood (Pankratz and Jackle, 1990; St Johnston and Nusslein-Volhard, 1992). Equally important, but less understood, is the maintenance of established segment and organ primordia. During pattern formation the embryo is subdivided in ever smaller units as for instance seen by the expression domains of gap and pair rule genes. These subdivisions are transient in nature and are not directly transformed into morphological traits. Only the last level of the segmentation hierarchy, the segment polarity pattern, provides direct cues for morphogenesis, i.e. compartment boundaries, cuticle differentiation or muscle attachment sites (Martinez Arias et al., 1988; Sanson, 2001; Larsen et al., 2003). Pattern maintenance, in contrast, requires stable cues that are able to maintain borders and regulate the size of morphological units. Only few of these cues are known. Mutual activation of *wingless* and *engrailed* expression is crucial for the maintenance of parasegmental boundaries during germ band extension. Afterwards, their expression domains become independent from each other (Heemskerk et al., 1991; Martinez Arias, 1993; DiNardo et al., 1994). *Engrailed* expression then comes under cell autonomous control involving the action of polycomb group genes (Moazed and O'Farrell, 1992). In the case of the segment polarity gene *gooseberry*, different enhancer elements are responsible for early and late functions (Li et al., 1993). Adjustment of parasegment size involves cell death and delamination of miss-specified cells (Hughes and Krause, 2001). In wing imaginal discs, maintenance of parasegmental borders involves *hedgehog* regulated cell sorting (Dahmann and Basler, 2000). It has been assumed that differential cell adhesion may be involved but the molecules responsible for the hypothetical differential cell adhesion remains elusive despite specific screening efforts (Dahmann and Basler, 1999).

All conclusions drawn from *Drosophila* research bear the drawback that this species develops through a highly derived

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mode of embryogenesis (long germ embryogenesis) that is not typical for insects (Tautz et al., 1994; Davis and Patel, 2002). Hence, statements on general properties of insect development have to be based on studies of insects with more typical features. We have chosen the red flour beetle *Tribolium castaneum* as model system, because it is readily manipulated by genetic, transgenic and RNAi approaches (Maderspacher et al., 1998; Berghammer et al., 1999; Brown et al., 1999; Bucher et al., 2002; Klingler, 2004). As in most insects, in *Tribolium* only the anteriormost segments are patterned in the blastoderm stage while all following segments are formed one by one from a posterior growth zone (short germ embryogenesis). In addition, *Tribolium* displays a fully developed larval head, larval appendages and extraembryonic tissue. The morphological conservation suggests that also the genetic control reflects the ancestral state more accurately than it does in *Drosophila*. Indeed, it has been shown that early patterning in *Tribolium* does not involve *bicoid* and that the abdominal gap gene orthologs have drastically changed their function (Brown et al., 2001; Bucher and Klingler, 2004; Cerny et al., 2005). In contrast, on the level of segment polarity genes studied so far, both expression and function appear to be conserved to great extent (Brown et al., 1994; Nagy and Carroll, 1994; Oppenheimer et al., 1999).

In a screen for embryonic lethal mutations in *Tribolium*, we have recovered *knödel* (*knö*), a mutant lacking cuticular segmentation. We show that segmentation itself is not compromised but the maintenance of the pattern is impeded. We speculate that *knö* may be a late acting segment polarity gene, or a gene required for adhesion. The phenotype of *knö* mutant embryos shows that also in short germ embryos, initial patterning of the growing germ band is followed by a distinct maintenance phase.

2. Experimental procedures

2.1. Identification of the knödel mutant strain

knö was identified independently in two genetic backgrounds. In a screen for embryonic lethal mutations (Maderspacher et al., 1998), the allele 13h8 was isolated in an EMS-treated SB wild-type background. Independently, we found that our copy of a Tiw-1 wild-type strain harbored a large number of chromosomes carrying a *knö* allele: 39 of approximately 400 inter se backcrosses segregated embryos indistinguishable from 13h8. Complementation analysis showed that the two mutants ($knö^{13h8}$ and $knö^{Tiw1}$) are allelic. knödel (dumpling) is a dish typical for southern Germany and consists of amorphous balls made of potato flour or bread that resembles the knödel phenotype.

2.2. Complementation study

One $knö^{Tiw1}$ male was crossed to three $knö^{13h8}$ females and the offspring checked for mutant offspring ($n=8$) and vice versa ($n=10$). Mutant offspring was detected in 3/8 and 7/10 of the crosses, respectively. Therefore, the mutations do not complement and are alleles of the same locus.

2.3. Histology

Whole-mount in situ hybridizations were performed according to established protocols (Tautz and Pfeifle, 1989). For initial double stainings, fluorescein- and digoxigenin-labeled probes were detected using alkaline phosphatase and beta-galaktosidase, the latter after signal enhancement via biotin deposition (Prpic et al., 2001). For the remaining double in situs both stainings were detected successively by alkaline phosphatase using NBT/BCip and the fluorescent FastRed reaction (Sigma), respectively. The false color pictures were produced by pasting the inversed bright field image into the red and blue channels of the image of the fluorescent staining. Detailed protocols are available from the authors. The stained embryos were dissected free from the yolk and embedded in 50% glycerol. Cuticles were embedded in Hoyer's medium mixed with lactic acid (1:1) and analyzed using standard dark field illumination. In addition, confocal microscopy of the auto fluorescent cuticle was used (excitation: argon laser, 488 nm; emission filter: long pass 505 nm)

2.4. Embedding in low refraction medium

In glycerol, the yolk granules disturb optical observation. Therefore, embryos usually have to be dissected from the yolk. In order to observe embryos within the egg, we have developed an embedding method for making yolk transparent for normal bright field microscopy. Embryos are dehydrated in alcohol (ethanol or methanol, 25, 50, 75, 100%). They are then embedded in a solution of high refractory index, 'benz-mix', a 4.3:1 mixture of benzyl benzoate: benzyl alcohol for embryos dehydrated in ethanol (3:1 for embryos dehydrated in methanol). This technique was applied to the whole-mount *Tc'even-skipped* staining.

2.5. Scanning electron microscopy

Embryos were dehydrated by 10 min incubations in raising acetone concentrations (70%, 80%, 2×98%, 2×100%). They were then immediately transferred to HMDS (1,1,1,3,3,3 hexadimethyldisilazane; Merck-Suchardt, Darmstadt) and incubated for 30 min. HMDS was then removed and embryos transferred to an exsiccator loaded with silica gel balls (Merck-Suchardt, Darmstadt). The exsiccator was evacuated in order to avoid contamination with water (which causes shrinkage), and evaporation was performed over night. Embryos were then transferred to an aluminium carrier (Plano, Wetzlar) that had been prepared with a double-sided adherent disc (Plano, Wetzlar). The embryo was then sputtered with gold (BIO-RAD SC 510, München) at 200 V for 120 s. Scans were performed with a Philips XS-20 scanning electron microscope at 20 kV.

3. Results

3.1. knödel embryos lack segment boundaries and bristles

knö mutant larvae differentiate as nearly amorphous cuticular sacs that lack most bristles, proper appendages and any sign of abdominal segmentation (Fig. 1A–C). The cuticle of *knö* embryos appears to be shaped by the egg shell because the cuticle closely lines the vitelline membrane. Accordingly, the proportions of *knö* embryos differ strongly from wild-type: they are about half as long, but approximately 30% more wide than wild-type 1st instar (L1) larvae. The anterior posterior axis is well established, however, as in all larvae the anterior pole can be recognized. In the head, several rudimentary appendages are usually formed, although it is often difficult to assign their identity unequivocally. The labrum is commonly discernable as are shortened antennae that lack the flagellum. Mandibles, maxillae and labium are strongly reduced to cuticular humps that sometimes carry distal structures like reduced palps or sclerotized tips (mandible). In most cases, remnants of most but not all gnathal segments can be found. Legs are either missing or discernable as