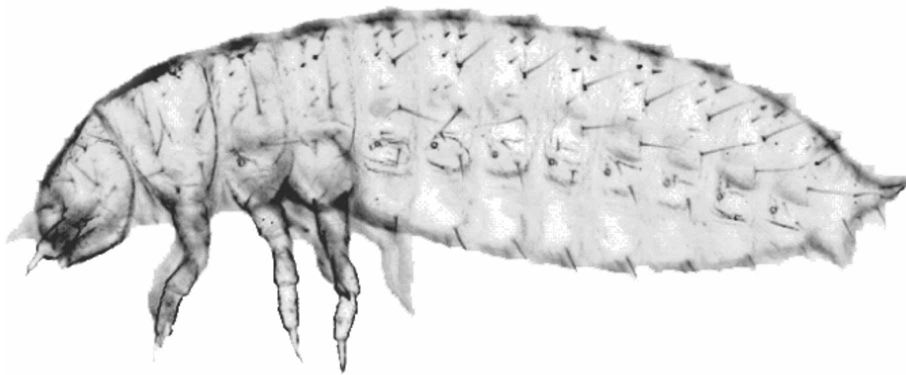
Functional analysis of a homolog of the pair-rule gene *hairy* in the short-germ beetle *Tribolium castaneum*



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Abbreviations:

aa amino acid

bp, kb base pairs, kilo base pairs

°C degrees Celsius

Dm'xxx Drosophila melanogaster gene xxx

DNA deoxyribonucleic acid

dsRNA double stranded RNA

h hours

mM milli molar

min minutes

mg milligram

ml milliliter

nl nanoliter

ng nanogram

mRNA messenger RNA

oligo oligonucleotide

PCR polymerase chain reaction

phenotype is due to a mutation in a gene

phenocopy is due to knocking down gene function by RNAi

pRNAi parental RNAi

primer oligonucleotide

RNA ribonucleic acid

RNAi dsRNA mediated interference

SB Tribolium wildtype strain *San Bernardino*

Tc'xxx Tribolium castaneum gene xxx

ul microliter

Summary

Drosophila melanogaster is by far the best-understood model for "long germ" developing insects. The most remarkable feature of its development is the specification of all segments during the syncitial blastoderm stage by a mechanism that relies on free diffusion of maternally provided and zygotic transcription factors between the uncellularized nuclei of the early blastoderm. In contrast to this, most insects undergo a short germ development, where only few anterior segments are specified at this stage. Although the ontogeny of several insects from different orders has been analysed so far, it still remains unclear if the principles discovered in *Drosophila* also apply for the generation of the post-blastoderm derived segments of short germ insects, and how this system might have evolved.

The functional analysis of the *Tribolium castaneum hairy* homolog by pRNAi provided in this thesis and the analysis of the knock down embryos by *in situ* hybridization using different molecular markers, strongly indicates that *Tc'h* functions as a pair-rule gene during the segmentation of blastoderm derived segments and suggests a conservation of this function between *Tribolium* and *Drosophila*. In contrast to this no such role could be observed for segments specified during the elongation process of the *Tribolium* germ-band. Interestingly, this function appears to be masked by an additional function of *Tc'h* in the further development of the embryonic head, which causes the loss of all segments anterior to the third thoracic segment and may indicate the presence of an anterior organizer which governs the proper maturation of this region during development.

Analysis of the regulation of *Tc'h*, in comparison to its *Drosophila* homolog, shows that the regulatory cascade governing its expression differs substantially between these species. Whereas the *Tribolium* gap gene orthologs *Tc'Kr* and likely also *Tc'hb* do not seem to participate in the regulation of the *Tribolium hairy* pair-rule pattern in a similar way as in *Drosophila*, a strong regulatory influence of *Tc'gt* could be observed in these experiments, reminiscent of the situation in *Drosophila*.

Analysis of the regulatory target gene *Tc'ftz* indicates that the regulatory interaction between these genes might be conserved, although the function of this interaction differs substantially between *Drosophila* and *Tribolium*.

Despite the differences found in the regulation of the *hairy* gene in *Tribolium* and *Drosophila*, a trans-species experiment using a *lacZ* reporter construct under the control of a *Tribolium hairy* enhancer, specifically regulating the expression of three central stripes #3-#5 in *Tribolium*, does indeed show expression of the corresponding stripes in transgenic *Drosophila* lines. Furthermore, crosses of these lines with *Drosophila* gap and pair-rule gene mutants strongly suggest a participation of these genes in the regulation of the observed pattern. Intriguingly, complementary experiments performed in *Tribolium*, using similar constructs harbouring stripe specific elements for the stripes #3-#4 from *Drosophila*, also exhibit stripe like expression at comparable positions in the *Tribolium* germ-band. However, pRNAi experiments performed with the *Tribolium* gap genes *Tc'hb* and *Tc'Kr* suggest a different regulation for the generation of these stripes.

The results obtained in this study reveal part of the regulation and function of the *Tribolium castaneum hairy* homolog and allow interesting speculations on the molecular events that may allow the transition from short- to long-germ development and the evolution of pair-rule patterning in higher insects.

1. Introduction

1.1 Evolution and Development

The history of life documented in the fossil record shows that the evolution of complex organisms such as animals and plants has involved marked changes in morphology, and the appearance of new features. However, evolutionary change occurs not by the direct transformation of adult ancestors into adult descendants, but rather when developmental processes produce the features of each generation in an evolving lineage. Therefore, evolution cannot be understood without understanding the evolution of development, and how the process of development itself biases or constrains evolution (Rudolf A. Raff, 2000).

One of the most astonishing processes in nature is the development of a complex organism from a single, fertilized cell. In this respect it may seem even more astonishing that some of the genes, networks and processes governing development are broadly shared over large evolutionary distances and organisms which are superficially as different as flies and man. According to evolutionary theories, changes in evolution are caused by hereditable changes in development. This implies that evolutionary changes, and thus phenotypic changes, can be tracked down to the genotypic level by comparison of developmental processes between different species, thus enabling us to understand the molecular mechanisms that drive evolution to shape species. Moreover it permits to infer the course of evolution between lineages and taxa, allowing the reconstruction of the tree of life, since innovations are structures based on genetic changes in descendent lineages that were not present in ancestral ones. The comparison of phenotypes on the genotypic and molecular level also allows the limitations of just morphological comparison to be overcome. Convergent evolution or the discrimination between real innovations and parallelism are some of the problems, which can only be solved and understood on the genetic level.
Ongoing development of new techniques to manipulate and analyse the processes governing ontogeny, as well as genome projects, allow insight into the genetic networks which direct the development of model organisms such as *Drosophila melanogaster* for insects or *Danio rerio* and *Mus musculus* for vertebrates. Inter specific comparison of the genetic basis of the regulation of these processes should therefore shed light on how evolution shapes the tremendous diversity of body plans using basically the same the molecular “tool box”.

1.2 Drosophila segmentation

The systematic analysis of the embryonic development of *Drosophila melanogaster* (*D.m.*) provided the basis for the understanding of the molecular processes that govern embryogenesis of this model organism. The *Drosophila* segmentation gene hierarchy consists of a cascade of transcription factor interactions, which define the anterior-posterior axis of the *Drosophila* embryo. It acts upon an array of nuclei that divide within a common cytoplasm termed syncitial blastoderm. After fertilization, ten fast synchronous nuclear divisions take place at a frequency of 8-10 min. followed by four additional, slower divisions prior to cellularization (Zalokar and Erk, 1976; Foe and Alberts, 1983), leading to a monolayer of approximately 6000 nuclei at the periphery of the egg on which the complex segmental blueprint is overlaid. Maternally provided factors, products of the so called maternal coordinate genes, which are deposited in form of mRNA at the poles of the embryo, begin to regulate their zygotic downstream targets after the tenth division when interphases lengthen and thus allow transcription of zygotic genes. With the onset of cellularization, during the fourteenth division, mitotic synchrony is lost. Free diffusion of the maternal factors within the syncitial blastoderm leads to the formation of long-range gradients that provide the initial positional information integrated by the zygotic segmentation genes, thus allowing the specification of all future segments before cellularization is complete. As a result, a “fate map” of the complete larval body can be mapped onto the blastoderm, which is the key feature of the “long germ mode” of development (Sander 1976).

1.2.1 Maternal coordinate genes

The *Drosophila* segmentation cascade is one of the most comprehensively studied gene networks to date. The cascade follows a hierarchical principle (Ingham, 1988; Tautz, 1992; Klingler and Tautz, 1999) beginning with the diffusion of maternal factors (*i.e.* bicoid, caudal, hunchback, etc.) from the anterior and posterior poles of the embryo which in turn control the spatial expression of the next hierarchical level, the so called gap genes (*e.g. hunchback*, *Krüppel*, *giant*, etc.), in a concentration and combination dependant manner. One of the most important maternal coordinate genes in this respect is *bicoid (bcd)*. *bcd* mRNA is localized in the anterior of the oocyte, but is not translated until fertilization after which it forms an anterior to posterior gradient (Driever and Nusslein-Volhard, 1988; St. Johnston *et al.*, 1989). BCD is a homeodomain containing transcription factor, but can also bind to RNA and regulate translation (Dubnau and Struhl, 1996; Rivera-Pomar *et al.*, 1996). One of its first

actions is to inhibit translation of the ubiquitously dispersed *caudal* (*cad*) transcripts, resulting in a complementary *CAD* gradient with lowest concentrations at the anterior pole of the embryo where *BCD* concentrations are highest (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987). Two other important gradients are formed by the gene products of maternal *hunchback* (mat-hb), a Cys2His2 class zink-finger transcription factor (Tautz *et al*., 1987), and *nanos* (*nos*) where *NOS* negatively regulates translation of the uniformly dispersed mat-hb, but also *bcd* transcripts, in a posterior to anterior fashion (Wang and Lehmann, 1991; Payre, 1994; Dahanukar and Wharton, 1996).

1.2.2 Gap genes

The gap genes identified in *Drosophila* belong to the "class" of transcription factors that have been defined by their mutant phenotype, since disruption of this class of genes leads to gaps in the developing larvae which span several segments, extending anterior and posterior of their respective expression domain (Pankratz und Jäckle, 1993). Gap genes are expressed in overlapping domains along the anterior posterior axis of the embryo. They are amongst the earliest expressed zygotic genes and are thought to act via the formation of short range gradients to sub-divide the embryo into broad domains, encompassing the progenitors of several contiguous segments (Hülskamp und Tautz, 1991) and to regulate the expression of the next set of genes in the hierarchy, the pair-rule genes. The borders of their expression domains are further refined by interactions amongst each other (Kraut und Levine, 1991a; Kraut und Levine, 1991b; Rivera-Pomar und Jäckle, 1996). As mentioned above, they are regulated by maternal factors in a concentration and combination dependent manner and sub-divide the embryo into anterior (head), central (thorax) and posterior (abdomen) regions. The concentration dependent activation of these genes has been well studied for the case of zygotic *hb* whose regulatory region contains several *Bcd* binding sites with different affinities, allowing transcriptional activation along the *Bcd* gradient in direct response to different protein concentrations (Driever and Nusslein-Volhard, 1989; Driever *et al*., 1989; Struhl *et al*., 1989). Another factor that determines where gap gene promoters can respond within the maternal gradients is how they interact with other transcription factors, since cooperative binding (Simpson-Brose *et al*., 1994) as well as cross-regulatory interactions among the gap genes have been shown to be involved in the refinement of their expression domains (Jäckle *et al*., 1986; Struhl *et al*., 1992). Well studied examples are the synergistic activation of *hb* by *BCD* and maternal *HB* (Simpson-Brose *et al*., 1994) as well as the mutual repression between

the posterior *Kr* and *gt* domains, which seems to be crucial for the proper refinement of their expression domains (Kraut and Levine, 1991b; Capovilla *et al.* 1992). Interestingly, it was found that some gap genes (*e.g. hb)* can serve both as repressor and activator depending on their concentration (Hülskamp *et al.*, 1990; Struhl *et al.*, 1992; Schulz and Tautz, 1994) an effect probably mediated by different binding sites and/or cofactors.

1.2.3 Pair-rule genes

The pair-rule genes form a diverse set of transcription factors, which further refine the broad domains, specified by the gap genes. They are the first genes to specify segmental regions, transforming the aperiodic pattern provided by the gap genes into a metameric pattern of double periodicity. Most pair-rule genes encode for transcription factors with predominantly repressive function, but also activators and factors, which can do both, are known. The majority of the pair-rule genes are initially expressed in broad gap gene like domains that refine into segmental stripes in every other segment during cellularization. In *D.m.* nine different pair-rule genes have been identified so far, including *even-skipped* (*eve*), *runt* (*run*), *hairy* (*h*) and *odd-skipped* (*odd*) which represent very different types of transcription factors.

The pair-rule genes have been initially divided into two groups, the primary and the secondary ones, where primary ones regulate the latter, but not vice versa. Newer studies though, suggest a more complex relation (Gutjahr *et al.*, 1993; Klingler and Gergen, 1993; Yu and Pick, 1995; Saulier-Le Drean *et al.*, 1998). The activity of the pair-rule genes is crucial for the definition of pre-segmental units called para-segments which are of the same size and width as the resulting segments, but shifted anteriorly by approximately a quarter segment. In addition they specify the polarity within the segments by regulating the segment polarity genes, which are the last level in the hierarchy of the segmentation cascade. Other functions involve for example the regulation of the *Hox* genes, which are responsible for the specification of segment identities.

The regulation of the pair-rule genes functions in a similar way as described for the gap genes. One of the best-characterized cases is the regulation of *eve*. Functional analysis, including expression analysis of reporter gene constructs harbouring mutated binding sites for certain factors and in vitro binding analysis, revealed that discrete stripe enhancer elements exist in the cis-regulatory region of at least some pair-rule genes. These enhancer elements are

comprised of clustered binding sites for suitable regulators of the segmentation cascade which, in concert, confine the expression of the respective stripes (Small *et al.*, 1991, 1992; Simpson-Brose *et al.*, 1994; Arnosti *et al.*, 1996a).

1.3 *Tribolium* segmentation

In contrast to *Drosophila*, *Tribolium* exhibits a more ancestral type of embryogenesis, the so-called "short germ mode" (Sander, 1976). Upon fertilization the nuclei divide synchronously under syncitial conditions and move to the periphery of the egg where additional asynchronous divisions take place and cellularization is believed to start. After the blastoderm stage most of the nuclei from the anterior dorsal site begin to move to the posterior ventral side of the egg to form the germ rudiment, whereas the few remaining nuclei dispersed along the anterior and dorsal region form the extra embryonic serosa. In contrast to *Drosophila* only the more anterior cephalic and the first thoracic segments are determined at this stage whereas the more posterior abdominal and likely the last thoracic segment are generated via a secondary growth process, where cells from a region in the most posterior part of the embryo, termed growth zone, are recruited to form segments in a sequential fashion under cellularized conditions by an yet unknown mechanism. The isolation and characterization of *Drosophila* segmentation gene orthologs spanning all hierarchical levels showed remarkable similarities in terms of expression and partially also function and regulation, but also fundamental differences (Schröder, 2003; Bucher *et al.*, 2004; Cerny *et al.* 2005). To date several genes from maternal coordinate to segment polarity genes have been identified and their expression as well as function has been analysed in *Tribolium*, but their regulatory interactions remain to be solved.

1.3.1 *Tribolium* maternal coordinate genes

Early studies of the *Tribolium* segmentation mechanisms involved the identification of orthologs of the maternal coordinate genes *cad* and *hb* to address the question whether anterior gradients of maternally provided factors, like *bcd* in higher dipterans, can indeed fulfill a similar function in short germ developing insects. Astonishingly it was found that these orthologs do not only share similar expression patterns (Wolff *et al.*, 1995), but are also regulated by maternal factors when transformed into *Drosophila,* suggesting a conservation of the anterior patterning system in *Tribolium* (Wolff *et al.*, 1998). Despite this initial finding no

bcd homolog could be isolated from *Tribolium castaneum*. Recent analyses though suggest a possible role for *otd* and *hb* in fulfilling the task of anterior patterning in *Tribolium* (Schröder, 2003).

1.3.2 Tribolium gap genes

As already described for *Drosophila,* gap gene products function as short-range gradients to sub-divide the embryo at the syncitial blastoderm stage into anterior, central and posterior regions, thus providing the positional information needed for the transition to the periodic pair-rule pattern, which in turn specifies all future segments at this stage, a prerequisite of long germ development. In contrast to this, the expression pattern of a *Tribolium gt* (*Tc'gt*) , for example, displays a similar expression pattern compared to its *Drosophila* ortholog, which is expressed in two primary domains, one in the head region and one in the trunk. While the anterior domain seems to be conserved between *Tribolium* and *Drosophila* in terms of position in the embryo, but also with respect to the expression domain of the *Kr* gene, which was found to negatively interact with both *gt* domains in *D.m.* (Capovilla *et al.*, 1992; Kraut and Levine, 1991a), the posterior one is shifted anteriorly and arises within the *Kr* domain. Despite the similarities and differences in expression the most prominent distinction becomes evident in the phenotype, which, upon depletion of *gt* RNA by RNAi, displays segmentation defects which are not restricted to a limited domain as in *Drosophila*, but affect all thoracic and abdominal segments (Bucher *et al.*, 2004). A similar and even more pronounced effect was described for the *Kr* ortholog of *Tribolium* (*Tc'Kr*) where pRNAi as well as an amorph mutant display segmentation defects, which are asymmetrically with respect to the *Tc'Kr* expression domain. Analysis of knock down embryos stained for the pair-rule gene *Tribolium eve* (*Tc'eve*) exhibit almost normal formation of the first five stripes, therefore suggesting no influence of *Kr* in defining specific stripe boundaries like in *D.m.*, since most of these stripes form within or adjacent to the *Kr* domain. Beside the formation of these five stripes no further stripes or segments are formed due to breakdown of segmentation at this point (Cerny *et al.*, 2005).

Despite the functional differences found between *Tribolium* and *Drosophila* in the use of these genes during the segmentation process, another role, namely the regulation of homeotic genes, appears to be conserved. The *Tribolium* gap gene orthologs analysed so far exhibit strong homeotic effects, a function which is also found for different gap genes in *Drosophila* (Harbecke and Janning 1989; Castelli-Gair 1998).

1.3.3 Tribolium pair-rule genes

The identification of pair-rule gene orthologs in *Tribolium,* which are expressed in double segmental periodicity (Sommer and Tautz, 1993; Brown *et al.*, 1994; Brown *et al.*, 1997), and mutants displaying phenotypes reminiscent of *Drosophila* pair-rule mutants (Sulston and Anderson, 1996; Maderspacher *et al.*, 1998) lead to the conclusion that the segmentation machinery of short and long germ development is conserved at the level of pair-rule genes. However, a recent analysis of the interactions of the *Tribolium* pair-rule genes *eve*, *runt (Tc'run)*, *h (Tc'h)*, *odd (Tc'odd)*, *paired* (*Tc'prd*) and *sloppy paired* (*Tc'slp*) revealed a strikingly different mode of action compared to *Drosophila* (Choe *et al.*, 2006). A circuit of three pair-rule orthologs, namely *Tc'eve*, *Tc'run* and *Tc'odd,* seem to regulate one another and their downstream targets *Tc'prd* and *Tc'slp* appear to define segments sequentially in double segment periodicity. Disruption of these genes by pRNAi produces truncated, almost asegmental phenotypes, while the latter display pair-rule phenotypes as known from *Drosophila,* where alternating segments are missing. Interestingly this mechanism seems to apply not only to the segments defined during the blastoderm stage, but also to the subsequently added posterior ones. In contrast to *D.m.* this main circuit is based on the pair-rule genes *eve* and *runt*, but not *h*, which has been defined as "primary pair-rule gene" in *D.m.* as mentioned above. Instead, the *D.m.* secondary pair-rule gene ortholog *odd* seems to play a more important role during the segmentation of the *Tribolium* embryo. The exact mode of regulation of this gene circuit remains unknown and could be based on a repetitive circuit or clock like mechanism as well as a combination of both.

1.4 The *hairy* gene

The pair-rule gene *hairy* encodes a transcription factor of the basic helix-loop-helix (bHLH) family (Sasai *et al.*, 1992). The bHLH domain mediates both dimerization as well as sequence specific DNA binding. In addition, an orange domain further specifies its dimerization capacities. Another conserved feature between *h* orthologes is the WRPW motif at the C-terminus of the protein, which is needed for the interaction with its main co-repressor *groucho* (GRO) (Paroush *et al.*, 1994; Fisher *et al.*, 1996; Jimenez *et al.*, 1997).

In Drosophila, *hairy* (*h*) is expressed in broad domains along the a-p axis of the embryo during blastoderm stage, which refine into a small anterior cap and seven stripes of double segmental periodicity during cellularization. Its function and regulation as well as its own regulatory capacity have been well studied. Amorphic mutations of *Dm`h* result in the

deletion of the posterior part of every odd-numbered segment in the resulting larvae, thus reflecting a classical pair-rule phenotype (Jurgens *et al.*, 1984) and analysis of transgenic embryos ectopically expressing *h* outside its natural boundaries suggests that segmental defects may be caused by the resulting misexpression of *fushi-tarazu* (*ftz*) and other pair-rule genes (Ish-Horowicz and Pinchin, 1987). Besides its function in embryonic development it has also been reported to be involved in processes during larval and adult development where it participates , for example, in the formation of sensilla (Blair *et al.,* 1992) and microchaete (Ingham *et al.*, 1985, Renaud and Simpson, 2002).

During segmentation *h* was found to negatively regulate the spatial expression of the pair-rule genes *runt* (Klingler and Gergen, 1993), *ftz* (Carroll *et al.*, 1988; Rushlow *et al.*, 1989; Tsai and Gergen, 1995 and *paired* (*prd*) (Baumgartner and Noll, 1990; Gutjahr *et al.*, 1993), hence it was classified as primary pair-rule gene.

The regulation of *hairy* during the segmentation of the *Drosophila* embryo has been systematically studied. Similar to the already described regulation of the pair-rule gene *eve*, stripe specific enhancer elements have been identified in the regulatory region of the *hairy* gene which respond to different concentration and combinations of maternal coordinate-, gap- and pair-rule gene products, resulting in the metameric seven stripe pattern (Pankratz *et al.*, 1990; Howard and Struhl, 1990). A well-characterized example for this type of regulation is the activation of the *h* stripe 6 element by *CAD* and *KNI* (Hader *et al., 1998) and* its repression by *KR*, *HB* and *TLL* (Pankratz *et al.*, 1990; Riddihough und Ish-Horowicz, 1991; Langeland *et al.*, 1994), which, in concert, define the expression boundaries of this stripe. Another example is the establishment of *h* stripe 5, which is defined by negative regulation through *Kr* and *gt*, although other factors are expected to be involved, too (Langeland *et al.,* 1994).

hairy orthologs are also common in vertebrates where they are involved in the generation of the so called somites, the vertebrate analogs to the segmental units of insects, although segmentation does not proceed in the ectoderm like in insects, but the mesoderm. Analysis of this process revealed a fundamentally different regulatory mode for the generation of somites. Reflecting segmentation in a cellular environment, the process is based on cell signalling factors of the Notch/Delta and other signalling pathways, which regulate oscillating waves of expression of several genes, including some of the vertebrate *hairy* orthologs, the *her* genes, in the so called *pre-somitic mesoderm* (psm) (reviewed in Rida *et al.*, 2004; Giudicelli and Lewis, 2004). Despite their role in segmentation, genes of this class participate in a multitude

of other developmental processes during the development of the vertebrate embryo, for example neurogenesis (Gratton et al., 2003; Ninkovic et al., 2005).

Intriguingly, a *hairy* ortholog is also expressed in a segmental fashion during segmentation in the spider *Cupiennius salei*. Moreover, functional analysis of the Notch/Delta pathway by RNAi shows strong disruption of segment formation and the pattern of the spider *hairy* homolog, an effect highly reminiscent of the situation found in vertebrates (Stollewerk *et al.*, 2003).

1.5 Aims of this project

All arthropod embryos pass through a segmented intermediate developmental stage that at the morphological level seems to be remarkably conserved. Therefore this phase was termed the 'phylotypic stage' (Sander, 1976). However, the developmental events before and after this stage are much less conserved.

The ontogeny of *Drosophila melanogaster* is by far the best-understood model for "long germ" developing insects. The most remarkable feature of its development is the specification of all segments during the syncitial blastoderm stage by a mechanism that relies on free diffusion of maternally provided and zygotic transcription factors between the uncellularized nuclei of the early blastoderm. In contrast to this, most insects undergo a short germ development, where only few anterior segments are specified at this stage. Although the ontogeny of several insects from different orders has been analysed so far, it still remains unclear if the principle of morphogenetic gradients, as discovered in *Drosophila*, also applies for the generation of the post-blastoderm derived segments of short germ insects and how this system might have evolved. One of the most promising model organisms to tackle this question is, undoubtedly, *Tribolium castaneum*. Beside its well-described ontogeny it offers a variety of genetic tools like RNAi, transformation systems, cDNA-, genomic-libraries and an almost complete genome sequence to analyse and compare the process of segmentation and the underlying regulator interactions on a molecular level. Another prerequisite for this approach is its more basal type of development, which is found in many insect orders of both holo- and hemimetabolous insects.

The regulation of pair-rule patterning is of particular importance for the understanding of the evolutionary changes in the regulatory cascade leading to the phylotypic stage in short and long germ developing insects, since these would most likely be reflected at this level. In this

thesis I analyse the function of the *Tribolium* pair-rule gene ortholog *Tc'h* using a pRNAi approach to knockdown gene function and compare the phenotype as well as the regulatory interactions discovered, to its *Drosophila* ortholog. Furthermore I analyse the regulatory conservation of these genes in trans-species experiments using reporter gene constructs in both species, in order to infer the degree of evolutionary conservation and divergence in function and regulation between these species. Based on the results obtained in this study I hypothesize possible evolutionary scenarios that can lead to the evolution of long germ development, but also to the high diversity of intermediate developmental modes observed in different insect orders.

2. Materials and methods

2.1 General methods and techniques

General methods, procedures and recipes are based on standard protocols (Sambrooks *et al.,* 1989) if not indicated otherwise.

2.2 Beetle handling and stock keeping

Beetle stocks were essentially kept as described by Berghammer, 1999a and information on development, biology and life cycle of *Tribolium* and was taken from Sokoloff (Sokoloff, 1974). All experiments were performed using the wild type strain "San Bernadino", provided by Dick Beeman, Kansas State University, if not indicated otherwise. Flour was kept at 65°C over night to prevent parasitic infections.

2.3 Embryo collection and fixation

Eggs were collected from 0-48h at 30°C to gather all developmental stages before dorsal closure. The embryos were rinsed with tapwater, mildly dechorionated for 1.5min. in 50% bleach and rinsed with tapwater afterwards to remove residual bleach. Fixation was performed in scintillation vials containing 3ml PEMS (0,1 M Pipes, 2mM MgSO4, 1mM EDTA, pH 6,9), 6ml heptane and 4% formaldehyd on a shaking platform for 25min.. The water phase was then substituted for 8ml methanol and the vial vigorously shaken for 30sec., resulting in devitellinization of the embryos by methanol shock. Undevitellinized embryos were mechanically devitellinized by squeezing them through a syringe using a 19G needle. Embryos were kept at –20°C in methanol for subsequent analysis.

2.4 Parental RNAi

Parental RNAi experiments were performed according to Bucher *et al.* (2002) with slight modifications. Approximatly 200 female pupae were fixed to microscope slides using double sided tape (Scotch 665). Pupae were taken of the slides after injection and transferred to "culture vials" containing full grain flour in order to facilitate eclosion. The first eggs were collected approx. 5 days after injection and incubated at 33°C for 4 days to allow full development in order to assess the amount and strength of phenocopies. Eggs were collected every 48h and fixed for subsequent analysis by in situ hybridization. Once a week a 24h collection was allowed to fully develop and cuticle preparations were performed in Hoyer´s

medium according to standard procedures (Berghammer *et al.* 1999a) in order to monitor the phenotype/phenocopies over time. Double stranded RNA was synthesized from PCR templates using the T7 MEGAscript RNAi Kit (Ambion) without additional annealing steps and injected at a concentration of 2µg/µl in H_2O with 10% Phenol red. The injection solution was thoroughly centrifuged at 13.000g before injection to pellet any particles and reduce clogging of the needle.

2.5 Histology:

2.5.1 *in situ* hybridization

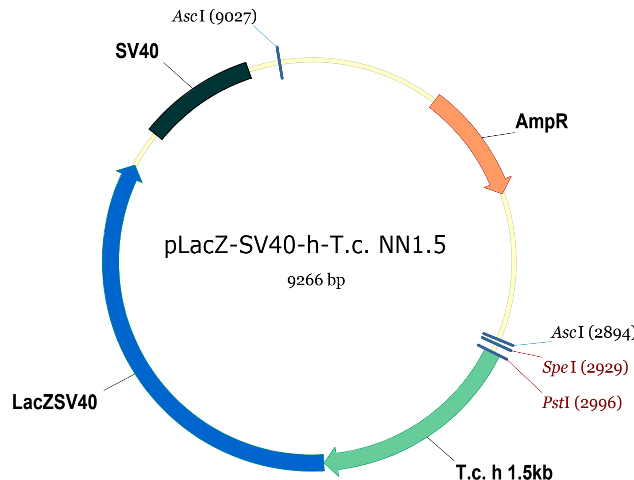
Whole mount in situ hybridizations were performed according to standard protocols (Tautz and Pfeifle, 1989; Klingler and Gergen, 1993) with slight modifications. For double stainings anti sense probes were generated labled with digoxigenin and fluorescein respectively using Roche chemicals and detected with appropriate antibodies from Roche. For double stainings with two alkaline phosphatase substrates (NBT/BCIP; INT/BCIP) detection was performed subsequently. In this case a heat inactivation step of 20 min. at 65°C was introduced after the first staining to inactivate the first antibody. To enhance sensitivity of alkaline phosphatase stainings polyvinyl alcohol was added to the staining solution at a final concentration of 5%. In cases of combined chromogenic and fluorescent stainings the alkaline phosphatase staining was performed first as described above to reduce artefacts. Fluorescent stainings were performed subsequently using the Alexa488 Tyramide Signal Amplification Kit from Molecular probes according to the manual provided by the manufacturer.

2.5.2 Immunological staining

Immunological staining was performed as described by MacDonald and Struhl (MacDonald and Struhl, 1986) with slight modifications. For the analysis of LacZ protein distribution in the transgenic lines, an additional signal amplification step, using a secondary biotinylated antibody and the Vectastatin ABC HRP KIT (Vector Labs), was introduced to the protocol. For apoptosis detection in the hairy pRNAi embryos using the anti cleaved caspase3 (Cell Signalling) amplifications steps were omitted and staining was performed using a secondary alkaline phosphatase coupled antibody.

2.5.3 plcZSV40- *Tc´h*-NcoINcoI1.5

The vector plcZSV40- *Tc´h*-NcoINcoI1.5 is a modified pEGFP vector (Clontech) and was used to build up the inserts of the transformation vectors. It contains a 1.5kb 5´ fragment from the *Tribolium castaneum hairy* gene, starting from the ATG, which was fused to a LacZ coding region and a SV40 terminator (see also Eckert *et al.* 2004). Regulatory elements were cloned upstream of the *Tribolium hairy* promotor using suitable restriction enzymes.

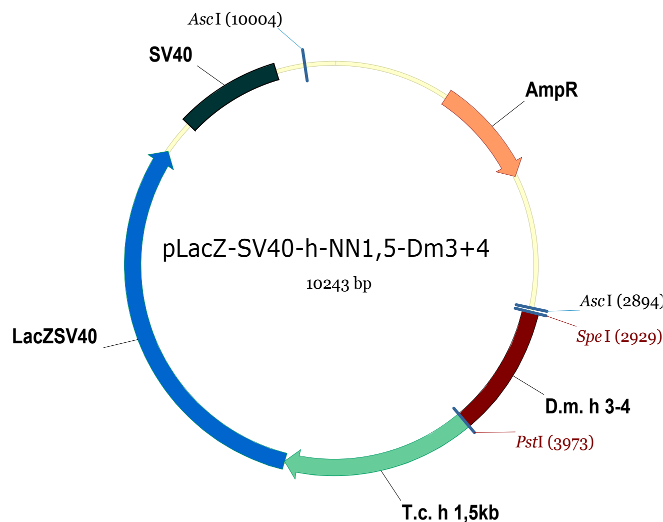


2.5.4 plcZSV40- *Tc´h*-NcoINcoI1.5 Dm3-4

A fragment of approximately 1kb containing the regulatory element of *Drosophila melanogaster hairy* stripe 3-4 was amplified via PCR using Roche High fidelity Taq polymerase to minimize amplification errors and cloned between the unique PstI/SpeI sites of plcZSV40- *Tc´h*-NcoINcoI1.5.

Primers used for PCR: Dm-*h*-3-4fwd: tactagtaggcctgtccttgagccactg

Dm-*h*-3-4rev: gtcctgcagcgtagccctcattatcacttc

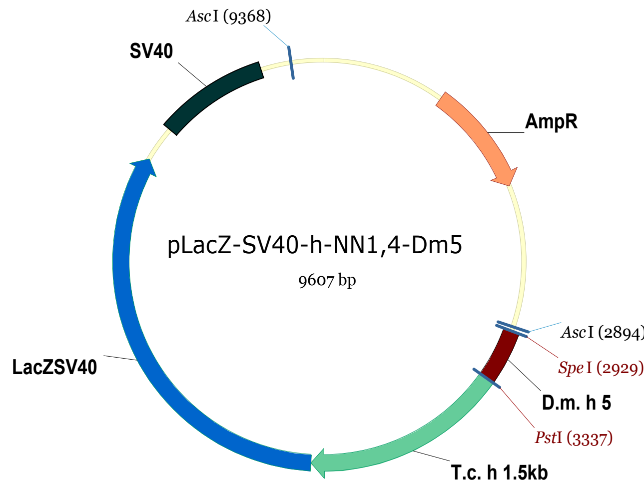


2.5.5 plcZSV40- *Tc´h*-NcoINcoI1.5 Dm5

A fragment of approximately 400bp containing the regulatory element of *Drosophila melanogaster hairy* stripe 5 was amplified via PCR (see above) and cloned between the unique SpeI/PstI sites of plcZSV40- *Tc´h*-NcoINcoI1.5.

Primers used for PCR: Dm-*h*-5fwd: atactagttaatcggccgaaagaggagag

Dm-*h*-5 rev: tactgcaggatcggggaatcgggaaaga



2.5.6 Transformation vectors

Reporter gene constructs were built based on the vector pB3xP3-EGFPafm (Berghammer *et al.*, 1999b; Horn and Wimmer, 2000). Inserts containing the reporter gene LacZ as well as 5`regulatory elements were inserted as a cassette behind the GFP coding region into the unique AscI restriction site.

2.5.7 Genetic transformation of *Drosophila*

Transgenic flies were essentially generated as described by Rubin and Spradling (Rubin and Spradling, 1982). *yw*-fly eggs were collected on apple juice agar plates for 30m. at 25°C, mildly dechorionated in 50% bleach for 30sec. and rinsed with tap water for 2 min.. The embryos were lined up on microscope slides with double sided tape (Scotch 665) with the posterior pole towards the edge of the slide and covered with Halo Carbon oil. The injection solution (10% phenol red, 500ng/µl respective *piggyBac*-vector and 300ng/µl helper plasmid pBachsp in H_2O) was applied into the posterior pole of the pre-blastodermal embryos using standard a microscope and an Eppendorf FemtoJet injection device set to manual in order to optimise the injection volume. The injection procedure was performed at 18°C whenever

possible to keep the developmental rate low. The injected embryos were kept in a closed plastic box with moist paper towels to prevent desiccation. Hatched larvae were collected for 2 days, reared to adulthood and backcrossed to *yw*-flies. The progeny of those flies were screened for GFP expression in the eyes.

2.5.8 Genetic transformation of *Tribolium*

Eggs from the eye mutant strain *Tribolium castaneum Pearl* were collected for 1h at 30°C and further incubated for 1h at 25°C to increase survival rate. The still pre-blastodermal eggs were rinsed with tap water and fixed to microscopic slides with the posterior pole towards the edge of the slide using a flour/water mixture. Injections, using the same solution as for the generation of transgenic flies, were performed at 25°C in the same fashion. Injected eggs were kept in a closed container with wet paper towels at 30°C for 2 days after which the lid was removed to allow desiccation. Hatched larvae were collected 3-4 days post injection and reared on full grain flour to pupal stage. Pupae were then sexed and backcrossed with *Pearl* beetles of the respective sex. Progeny of those beetles were screened for GFP fluorescence in the eyes to identify transgenic animals.

2.5.9 Crosses of transgenic and mutant lines

To test the effect of gap and pair-rule genes on the expression of the *Tc'h* constructs we crossed mutant lines with the transgenic lines T2M2 and T2M3 (generated by Ernst Wimmer) respectively, depending on the chromosomal localization of the mutant gene. In order to generate the respective lines, virgin females displaying the appropriate balancer phenotype were selected and mated with transgenic males of a suitable reporter line. Except for the crosses performed with the *Dm'gt* and *Dm'run* mutant lines, which could be analysed without subsequent crosses due to the localization of the mutation on the first chromosome, we selected virgin females and males of these crosses who lacked the balancer phenotype of both the transgenic and mutant line and crossed them with each other. The resulting progeny was thus expected to contain 25% homozygous mutants according to the experimental design.

Mutant strains used:

Hunchback:

BL-1755 hb^{12} st^{1} e^{1}/TM3, Sb^{1}

Giant:

BL-1529 y[1] sc[1] gt[X11]/FM6

Knirps:

BL-3332 kni[9] pum[13]/TM3, Ser[1]

Even-skipped:

BL-299 b[1] pr[1] eve[3]/CyO

Runt:

BL-4496 y[1] w[1] f[1] run[3]/FM7a/Dp(1;Y)y[+]mal[102]

Krüppel:

Kr^{2}/SM1 kind donation from Michael Hoch, University of Bonn

3. Results

3.1 Phylogenetic analysis of *hairy*

The *Tribolium hairy* ortholog isolated by Sommer and Tautz (Sommer and Tautz, 1993) shows high similarity to its *Drosophila* orthologs in terms of amino acid sequence and expression pattern. To test whether it is truly the closest ortholog of the *Drosophila hairy* gene and to analyse if duplications took place in the lineage towards *Tribolium*, as it is known from vertebrates (Umbhauer *et al.*, 2001; Gajewski and Voolstra, 2002), the recently available genome sequence was used to screen the *Tribolium* genome for *hairy* like genes, using the complete *Drosophila hairy* amino acid sequence. A total of four additional sequences with modest to high similarity have been revealed in this screen. Phylogenetic comparison of these sequences with orthologs known from arthropods and vertebrates confirms that the *hairy* gene identified first, indeed shares the highest similarity to other *hairy* orthologs isolated from insects and other arthropods. None of the other *hairy* like genes found during the screen showed expression during early segmentation except for Tc-h-3, which could be classified as a *Drosophila* deadpan ortholog.

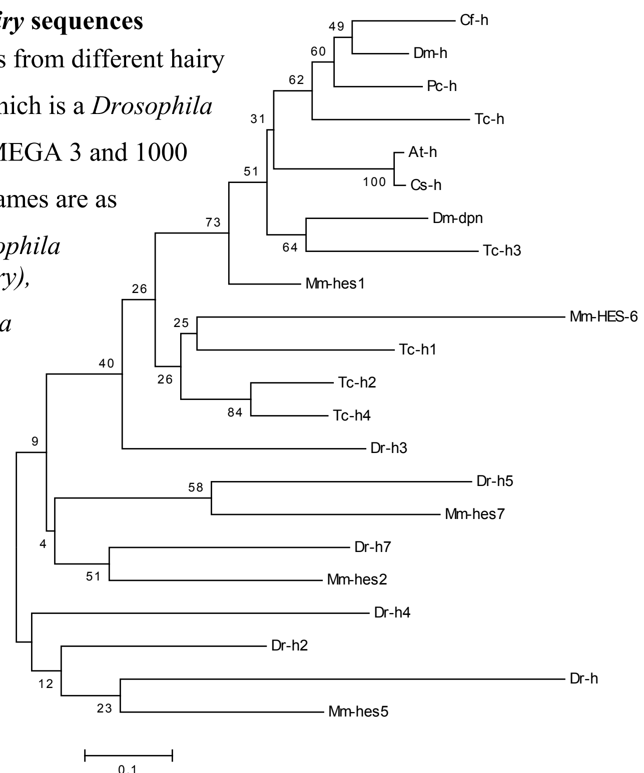


Figure 1 phylogenetic comparison of *Tribolium hairy* sequences
Neighbor-joining distance tree using full aa sequences from different hairy Orthologs from metazoan species, except Dm-dpn, which is a *Drosophila deadpan* homolog.. Sequences were analyzed using MEGA 3 and 1000 replicates were used for bootstrap analysis. Species names are as follows. Cf-h (*Coboldia fuscipes hairy*), Dm-h (*Drosophila melanogaster hairy*), Pc-h (*Platypeza consobrina hairy),* Tc-h (*Tribolium castaneum hairy*), At-h (*Achaearanea tepidariorum hairy)*, Cs-h (*Cupiennius salei hairy*), Dm-dpn (*Drosophila melanogaster deadpan*), Mm-hes1 *(Mus musculus hes1*), Mm-HES6 (*Mus musculus hes6*), Dr-h3 (*Danio rerio her3*), Dr-h5 (*Danio rerio her5*),Mm-hes7 (*Mus musculus* hes7), Dr-h7 (*Danio rerio her7*), Mm-hes2 (*Mus musculus hes2*), Dr-h4 (*Danio rerio her4*), Dr-h2 (*Danio rerio her2*),Dr-h (*Danio rerio her1*), Mm-hes5 (Mus musculus hes5). Accsesion numbers provided in supplemental material Figure 1

3.2 Expression of *Tribolium hairy*

Expression of *Tc'h* starts in a broad, circumferential stripe of expression positioned in the center of the early blastoderm (Figure 2A). In contrast to the direct appearance of the first stripe in the center of the blastoderm, expression of the second stripe starts as a posterior cap which slowly condenses at the anterior border and narrows down to become stripe #2, while expression retracts from the posterior pole at the same time. Simultaneously, the first stripe narrows down to about a third of its initial width. The third stripe forms shortly before the onset of posterior pit formation in a similar way as the second, starting as a cap in the posterior, but in contrast to the second stripe a condensation of the stripe at the anterior border of this cap cannot be observed. Instead, the stripe seems to form during the invagination of the posterior pole, *i.e.* the formation of the posterior pit and the beginning of germband elongation (not shown). At this stage expression can be detected in cells lining the posterior pit, but not in the center of the invagination. As soon as the germband rudiment is formed, expression of *Tc'h* is also detectable along the ventral midline and is maintained there throughout the whole segmentation process. The last five stripes also form sequentially, in a fashion comparable to the establishment of *Tc'h* stripe #2. In figure 2 (F-L) the formation of the stripes #3-#5 is depicted in more detail. The germband in figure 2 (F) shows the expression of stripe #1-#3, note that expression is not detected at the most posterior end of the embryo. In the following embryo the posterior border of stripe #3 is completely refined while expression in the growth zone covers the entire posterior part of the embryo. The following three pictures illustrate the formation of stripe #4 in more detail. In figure 2 (H-J) one can see how the expression retracts in the most posterior part of the growth zone while it condenses at the anterior border at the same time, thus forming stripe #4. Although expression at the posterior border seems to be highly dynamic the anterior border is always clearly refined. In figure 2 (K) the cycle starts all over, leading to the formation of stripe #5. The remaining three stripes are formed in an identical fashion. Shortly after the formation of the stripes in the growth zone, they split into segmental expression patterns, which persist for some time. Besides the expression of *Tc'h* in the double and later in the segmental fashion it is also expressed along the ventral midline throughout the segmentation process (Figure 2 F-P). During germband elongation *Tc'h* starts to be expressed in the developing nervous system in a dot like fashion in the lateral region of the embryo, headlobes and later in the growing appendages (Figure 2 M-R).

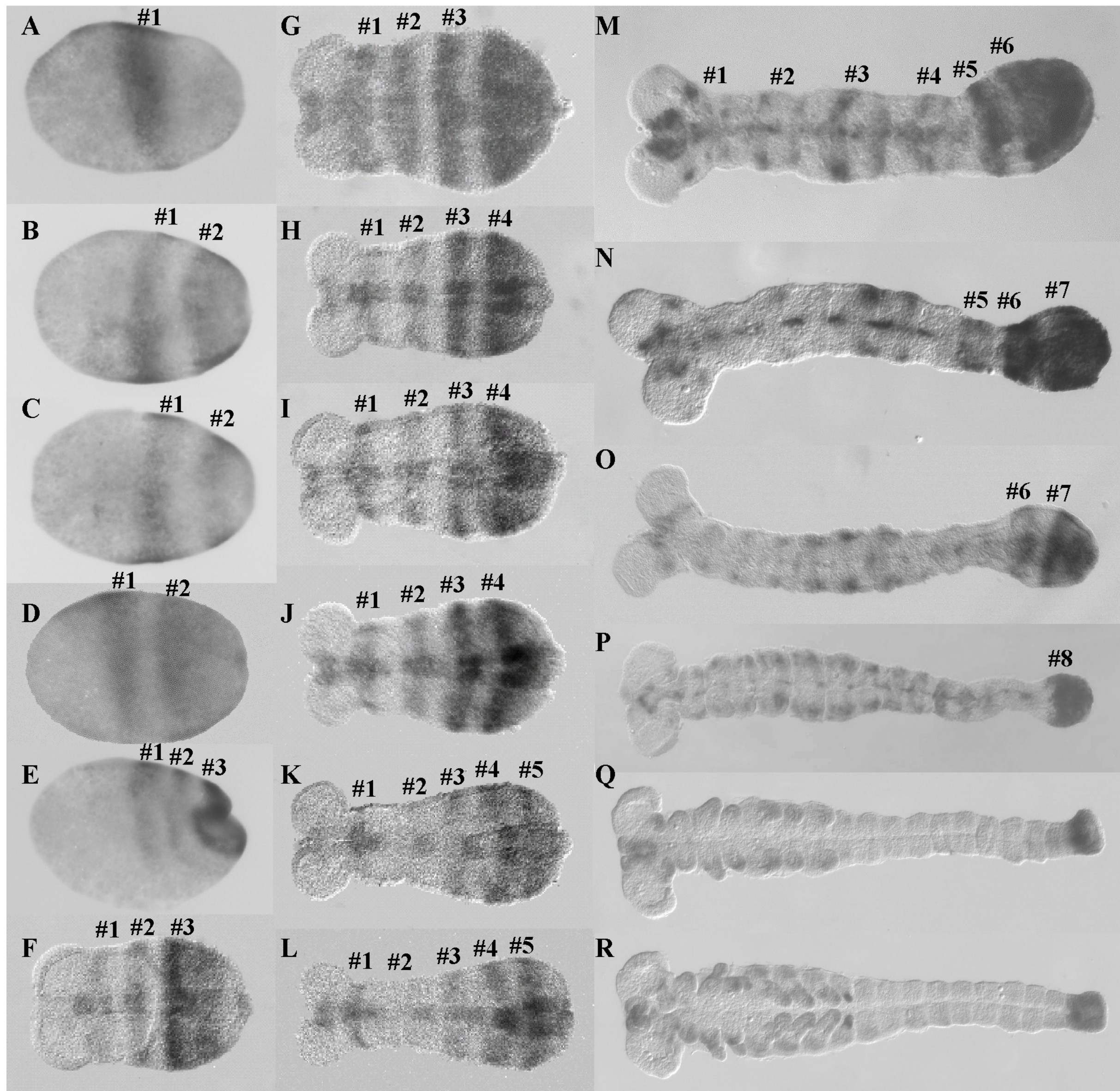


Figure 2

Expression of *Tc'h* in wild type. Blastoderm satges are aligned with the anterior to the left and the ventral side up, except for (B) which shows a ventral view. Germband stages are aligned with anterior to the left and numbers indicate the respective stripes.

(A-D) Expression during blastoderm stage. Expression of *Tc'h* starts in a broad, circumferential stripe of expression positioned in the centre of the early blastoderm (A). (B) Expression of the second stripe starts as a posterior cap which slowly condenses at the anterior border and narrows down to become stripe #2 while expression retracts from the posterior pole at the same time (C-D). In (E) gastrulation begins and all three blastodermal stripes are expressed. (F-L) Shows the formation of the stripes #3-#5 in detail. The germband in (F) shows the expression of stripe #1-#3, note that expression is not detected at the most posterior end of the embryo. In (G) the posterior border of stripe #3 is completely refined while expression in the growth zone covers the entire posterior part of the embryo. In (H-J) expression retracts in the most posterior part of the growth zone while condensing at the anterior border at the same time, forming stripe #4. Note that the expression at the posterior border seems to be highly dynamic while the anterior border is always clearly refined. In (K) the cycle starts all over leading to the formation of stripe #5. (L) The remaining three stripes are formed in an identical fashion. (M-R) In older stages expression is detectable in the presumptive nervous system in form of spots in the lateral regions of the embryo and in the headlobes.

3.3 Tc'*h* is not expressed in a wave

The way the stripes and in particular the posterior ones are formed, raises the question by which mechanism the observed retraction of expression in the posterior part of the growth zone could be achieved. One of the possibilities to achieve such a pattern would be a cell signalling pathway as it is known from vertebrate segmentation, where stripes are generated by an oscillatory mechanism based on cell signalling pathways, which regulate the cyclic expression of target genes that moves over the unsegmented region in form of an expression wave. Intuitively such a mechanism must imply fast degradation of specific transcripts in order to form stripes like seen in zebrafish (Gajewski *et al.*, 2003; Dill and Amacher, 2005). Another possibility to achieve such a dynamic pattern would be a mechanism based on cell movement where cells expressing *hairy* move towards the anterior leaving cells with no or low expression behind.

Based on the assumption that an expression wave, as it is known from vertebrate segmentation, should lead to signals in all cells which expressed the gene at some time point if no specific transcript degradation is included, as is seen in comparable experiments in the zebrafish embryo (Gajewski *et al.*, 2003), I used a *Tribolium* line carrying the reporter gene construct with the regulatory region necessary for the expression of all eight *Tc´h* stripes fused to the marker gene LacZ without endogenous UTRs (Eckert *et al.*, 2004) as a marker. Comparison of the expression of the construct, on both mRNA and protein level, with the endogenous expression pattern should allow to visualize a mechanism based on an expression wave due to the higher stability of the reporter gene transcript and protein. In case of an expression wave, a fusion of the stripes emanating from the growth zone is expected, caused by the remaining signal of the construct in the cells, which later reside in the interstripe region. In contrast to this, other mechanisms like cell movement should yield the same pattern as obtained in the staining of the endogenous transcripts. Comparison of the *in situ* staining for *Tc´h* and *lacZ* indeed show a higher stability of the reporter gene transcript. In figure 3 (L) the *Tc´h* stripe #2 is not detected anymore and stripe #3 is significantly weaker, in contrast to this both stripes are still well detectable in the lacZ staining (Figure 3 B and G). The immunological staining of LacZ protein resembles the picture obtained on mRNA level Although both, the reporter gene transcripts and the resulting protein, seem to be more stable than the endogenous *Tc`h* transcripts, no fusion of stripes could be detected as expected in the case of an expression wave. Instead the stripes, including the last formed one, appear well separated and refined on both mRNA and protein level (Figure 3 B-E and F-J), *i.e.* cells that lack staining have at no time expressed the reporter gene. The possibility of a higher turn over

rate of both mRNA and protein during segmentation seems highly unlikely since modified constructs using only different 5' sequences and no changes with respect to the reporter gene and UTR's do indeed show expression in the inter stripe region (see figure 18).

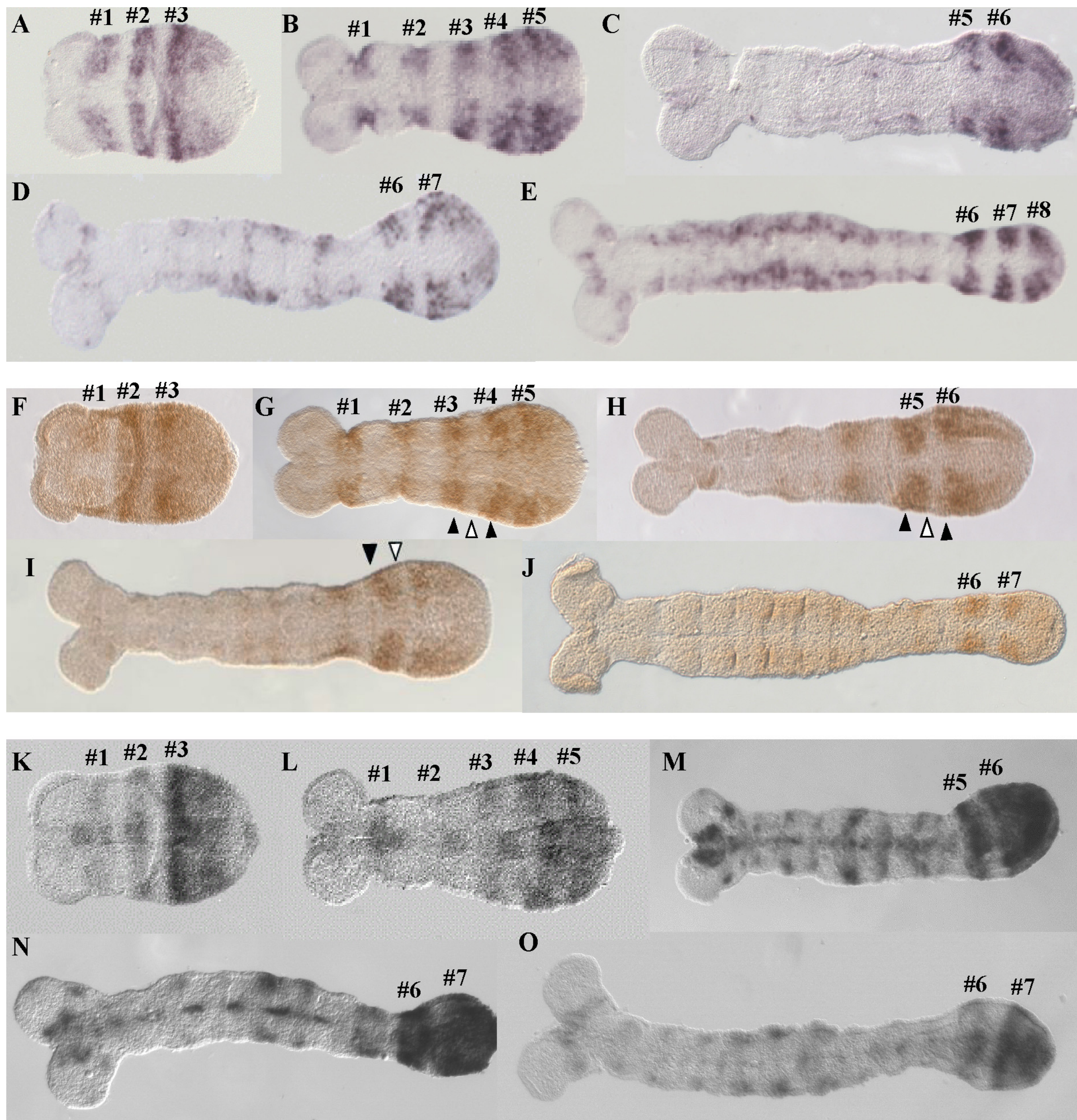


Figure 3

(A-E) Expression pattern of the *Tc'h-lacZ* reporter gene construct KN8.8 (Eckert *et al*., 2004). The construct drives the expression of eight stripes, faithfully mimicking the wild-type expression pattern. (F-J) shows an antibody staining for LacZ in the respective reporter line. Note, both the *lacZ* in situ (A-E) as well as the antibody staining (F-J) show formation of precise stripes (black arrows) with no staining in the inter stripe region (white arrows), i.e. no fusions are detected as expected in the case of an expression wave. (K-O) Wild-type *Tc'h* staining in comparable stages. Comparison of the expression in stripe #2 and #3 in (B, G and L) shows higher stability of the *lacZ* transcripts and protein compared to the endogenous hairy transcripts.

3.4 Functional analysis of the gene *Tc'hairy* by pRNAi

The classical pair-rule phenotype known from *Drosophila,* as previously described, is defined by a complete loss or defect of alternating segments, thus reflecting the double segmental expression pattern of this group of genes. In order to analyse if the expression pattern of *Tc´h* is correlated with a similar function I performed knock down experiments via parental RNAi (Bucher *et al.*, 2002) and analysed the phenotype of the resulting larvae. Injection of dsRNA, as high as 2 µg/µl, yielded the strongest phenotype on cuticle level. Cuticles of the progeny of such treated females exhibit segmentation defects in the anterior part of the larval body, ranging from loss of the mandibular and labial segments in weak up to deletion of all segments anterior to the third thoracic segment (Figure 4) in the strongest phenotype. Intermediate phenotypes display strong defects in gnathal segments including either complete or partial loss of gnathal appendages like labrum, mandible, maxilla and labium. Furthermore, left right asymmetry of this phenotype was observed frequently, where appendages were lost, malformed or strongly reduced on one side of the respective segment, but not the other without any side preference. Thus the phenotype observed in *Tc´h* knockdowns differs substantially from the phenotype observed in *Drosophila hairy* mutants.

Analysis of the phenotypes obtained by injection of lower and higher dsRNA concentrations did correlate well with the phenotype and no qualitative differences could be observed apart of a decrease or increase of stronger phenotypes and total amount of phenocopies.

Figure 4

(A) wild-type cuticle for comparison, lateral view. (B-E) Phenotypic series of cuticles obtained in the *Tc'h* pRNAi experiment, lateral view. In (B) the distance between the antennae and the maxillae is strongly reduced, suggesting that the mandibular segment is missing (see also magnification in I). (C-E) show successively stronger phenotypes were the entire head region is missing (C) and thoracic segments are affected (D-E). (F-G) show magnifications of the head region in wild-type (F) and intermediate *Tc'h* phenotypes (G) in a ventral view. In (G) no mandibular structures are visible and the labium is strongly reduced (white arrow). (H-K) Magnifications of the head region of a wild-type (H) and three *Tc'h* pRNAi cuticles (I-K) in a lateral view. (I) shows a magnification of the same phenotype as seen in (B), note the distance between the antennae and the maxilla which lay directly adjacent in contrast to the wild-type situation (H). In (J) the labrum and the mandibles are absent whereas in (K) the labrum is present, but an antennae is formed only on one side, the maxillas are both present. Frequencies of phenotypes obtained with 2 µg/µl *Tc'h* dsRNA (B) 45%; (I-K) 29%; (C) 7%; (D) 3%; (E) 2%; 16% wild-type, n=114.

3.5 Does *Tc'hairy* function as anterior organizer?

Due to the observed deletion of all cephalic and the first two thoracic segments in the strongest phenotype, I asked if *Tc'h* acts as a main organizer of anterior segment formation during the development of the *Tribolium* embryo. Furthermore the high number of larvae displaying specific deletion of the mandibular and labial segments, which correspond to the first and second *hairy* stripe, suggest a possible pair-rule function of *hairy* for blastoderm derived segments. To analyse these possibilities in more detail, the expression of the segment polarity gene *gooseberry* (*gsb*), which is expressed in the posterior region of the already determined segments, was examined in the respective embryos by *in situ* hybridisation (Figure 5). In weaker phenotypes the expression pattern of *Tc'gsb* shows significant changes in expression for the stripes corresponding to the mandibular and labial segments, which are either reduced, malformed or absent (Figure 5 F and G). In stronger phenotypes also the maxillary stripe is affected, but the effects on this stripe can only be observed at later stages i.e. after the establishment of the segment, suggesting a secondary loss of expression during germband elongation. Intriguingly in some cases expression of *Tc'gsb* can be detected in these segments although no respective appendage is formed (Figure 5 M). Compared to the wild type, embryos showing stronger defects display retarded head development (Figure 5 I and J), which leads to a very narrow connection of the head and trunk region with ongoing development (Figure 5M). Additionally, a deep groove can be detected along the ventral midline (Figure 6 I), where *Tc'h* is expressed in wild type embryos.

The loss of already initiated expression in these segments indicates that *Tc'h* may be crucial for the maintenance of *Tc'gsb* expression in the segments where it is not expressed. The observed retardation of development could thus be a consequence of a general "maintenance" function of *Tc'h* or a function in the establishment of a so far unknown organizer in this region. In no case did we detect a complete loss of all anterior segments before the end of segmentation, suggesting that the phenotype observed on cuticle level is caused in a secondary, post segmentation process. It is interesting to note that *Tc'delta*, one of the members of the Notch signalling pathway, is also strongly expressed in form of a stripe in the mandibular segment (Figure 7) and a regulatory connection of this pathway and *hairy* orthologues is found in a multitude of species and processes, including oogenesis (reviewed in Roth, 2001); neurogenesis and the segmentation of the vertebrate and spider embryo as mentioned before (Fisher and Caudy, 1998; Davis and Turner, 2001; Stollenwerk et al., 2003).

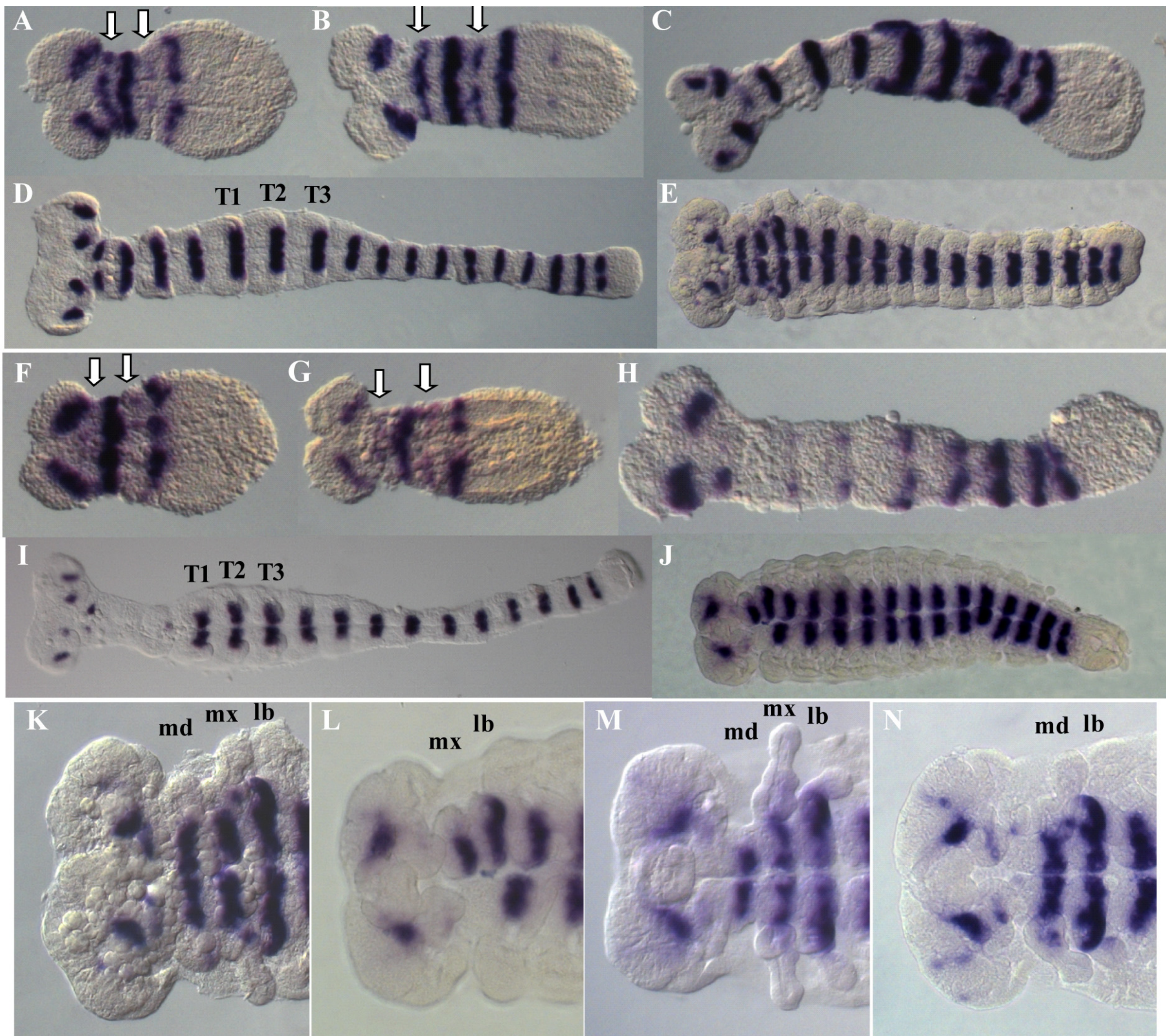


Figure 5

(A-E) *Tc'gsb* expression in wild-type. *Tc'gsb* is expressed in the posterior part of the specified segments and serves as segmental marker. The positions of the mandibular and labial segments are marked with white arrows in (A) and (B). (F-J) Expression of *Tc'gsb* in embryos depleted for *Tc'h* by pRNAi. The embryos depicted in (F and G) show disruption of *Tc'gsb* expression in the mandibular and labial segments (white arrows, compare to A and B). In older stages the stripe corresponding to the maxillary segment is also affected (H-J), suggesting a secondary loss of this stripe since a loss of this stripe at early stages was never observed. Furthermore, head development appears to be significantly retarded (compare I-J with D and E). (K-N) Blow up of the head region in (K) wild-type and (L-N) *Tc'h* pRNAi. In (L) the stripe in the mandibular segment is not detected whereas the stripes corresponding to the maxillary and labial segments are only formed on one side of the embryo. The embryo shown in (M) shows the formation of all gnathal *Tc'gsb* stripes, but no mandibular structures are detected whereas the maxilla and the labium are formed normally. (N) The embryo shows no stripe corresponding to the mandibular segment, but maxilla and labium are formed as in wild-type. Segment identities abbreviated as follows: (md), mandible; (mx), maxilla; (lb), labium; (T1-T3), thoracic segments.

3.6 Depletion of *Tc'h* leads to induction of apoptosis in anterior segments

The fact that no complete loss of cephalic segments could be observed before germband retraction, indicated an additional function for *Tc'h* during development. I therefore performed a staining for apoptosis in order to determine if segments and or gnathal structures are lost in a secondary process. Staining with an antibody directed against the activated form of caspase3 (see Materials and Methods for details), which allows the detection of apoptosis induction at early stages, shows indeed initiation of apoptosis in cells of the respective region (Figure 6 F-G). Interestingly no apoptosis was detected along the ventral midline, although this is clearly seen in wild type embryos (Figure 6 C and D). Instead, the ventral midline appears deeper as if cells were missing (Figure 6 I), suggesting a role for *Tc'h* in the specification of these cells. A comparable function is not known for *Drosophila hairy*, but interestingly a role for the specification of vertebrate midline structures, derived from the Spemann-Mangold organizer and its respective counterpart the dorsal shield, was found for different *hairy* orthologs in the zebrafish *Danio rerio* as well as the frog *Xenopus laevis* (Latimer *et al*., 2005; Murato *et al*., 2006) where the corresponding *hairy* orthologs function as mediators of the Notch signalling

Furthermore this pathway is also linked with the cell cycle via a *hairy* ortholog and can lead to apoptosis induction if components are altered (Sieger et al., 2003; Zweidler-McKay et al., 2005; Murata et al., 2005; Georgia et al., 2006; Fan et al., 2006). We therefore analysed the function of *Tc'delta* by parental as well as embryonic RNAi.

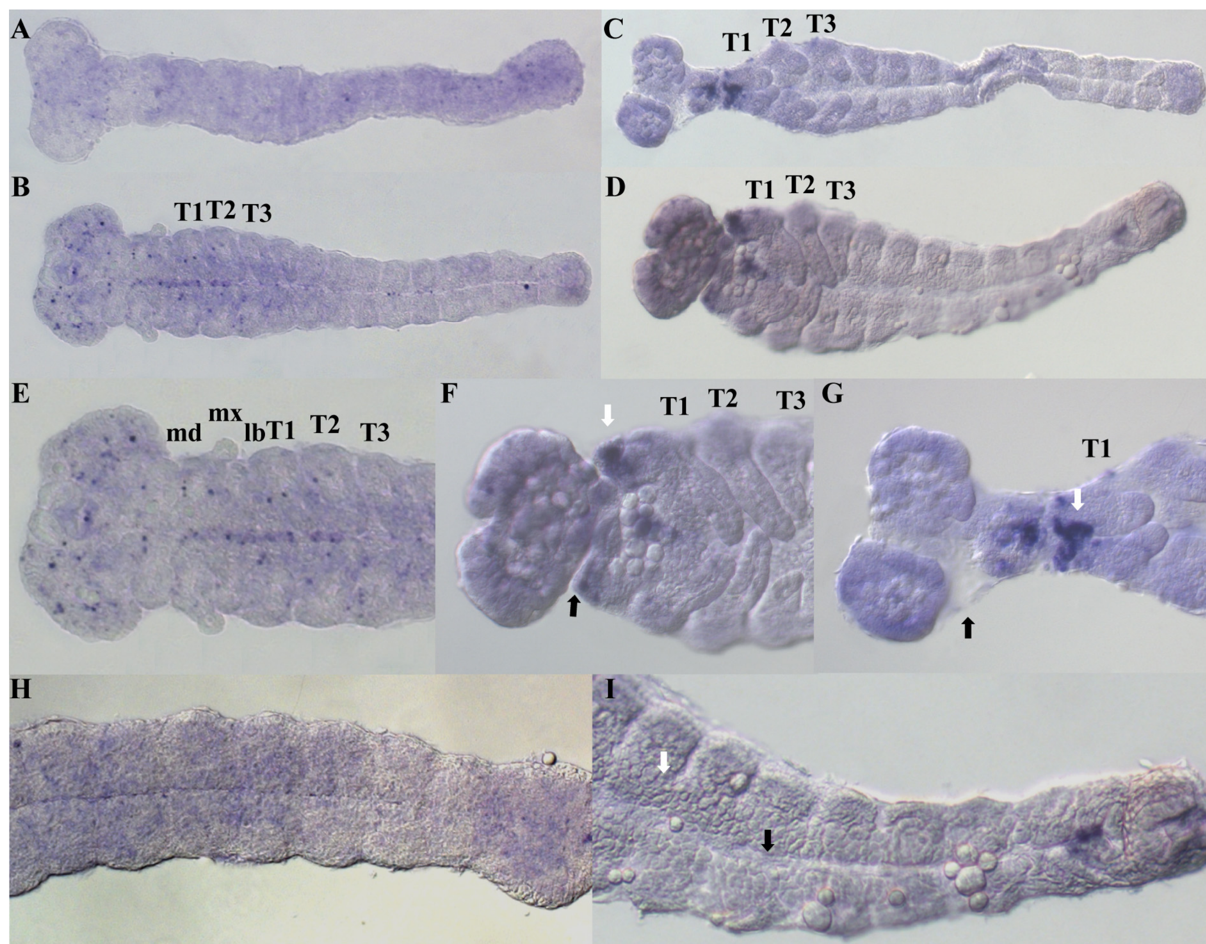


Figure 6

Antibody staining against cleaved caspase3, indicating induction of apoptosis, in wild-type (A and B, E and H) and in *Tc'h* pRNAi (C and D, F, G and I). In wild-type embryos apoptosis is only detected in a few cells in young stages (A). In older stages apoptotic cells are found in the headlobes, the ventral midline and a few dispersed cells (B). Tc'h depleted embryos in contrast show patches of strong staining in the gnathal region as well the first thoracic segment (C-D, F-G). Furthermore, strong disruption of the segments fusing the headlobes to the trunk region is seen, probably causing the detachment of all anterior segments during further development (F-G, black arrows). Note that no stained cells are detected along the ventral midline. Comparison of the morphology of the head region between the knockdown and the wild-type embryos show significant retardation of head development in the *Tc'h* knockdown (compare E and F). (H and I) Magnifications of the posterior segments of the embryos depicted in (B and D). The midline appears wider and deeper in *Tc'h* knockdown embryos (black arrow) and large cells are found lining the border to the lateral regions of the embryo (white arrow, compare H and I). Segment identities abbreviated as follows: mandible, (md); maxilla. (mx); labium, (lb); T1-T3, thoracic segments.

3.7 Functional analysis of *Tc'delta*

Injection of female pupae with double stranded *Tc'delta* RNA resulted in sterility and only few eggs were laid starting two weeks post injection, indicating a possible conservation of the role of this pathway during oogenesis (reviewd in Roth, 2001). Embryos injected with *Tc'delta* dsRNA indeed display severe defects in anterior segments, but the number of embryos developing a cuticle was very low in these experiments, suggesting high lethality for *Tc'delta* depletion. The few embryos that developed a cuticle displayed strong defects in the head region, where gnathal appendages were malformed or completely absent (Figure 7). In some cases appendages were only affected on one side of the respective segment, reminiscent of the phenotype observed in the *Tc'h* knockdowns.

In contrast to the pRNAi experiments with *Tc'h,* no specific loss of only the mandibular and labial segment was observed and comparison of the position of the antennae and the maxillas suggest that the mandibular segment is still present, even if the mandibles themselves are missing (compare figure 4 I and 7 O), suggesting no involvement of *Tc'delta* in the segmentation process of anterior segments. This is further supported by the fact that at least in some cases mandibular structures could also be observed in strong phenotypes.
Coincident with the function of this pathway during leg growth in *Drosophila* (Rauskolb and Irvine*,* 1999)*,* defects in the growth of the larval leg can also be observed in *Tc'delta* knockdowns, *i.e.* the legs are shorter compared to the wild type, although the corresponding segments themselves appear to be formed correctly.

Although the embryos examined in this experiments exhibit posterior segmentation defects, analysis of germbands depleted for *Tc'Su(H)* and stained for the segmental marker *Tc'gsb* suggest that the patterning process itself still seems to be functioning in these knockdowns (Aranda and Souza, unpublished). Even though a direct connection between the phenotypes observed in the experiment with *Tc'h* and *Tc'delta* can not be proven by these experiments, a regulatory connection appears possible, given that both *Tc'h* and *Tc'delta* are strongly expressed in the mandibular segment and that *hairy* orthologues are the main mediators of the Notch signalling pathway in a multitude of processes in both vertebrates and invertebrates (reviewed in Davis and Turner, 2001). Thus the secondary function of *Tc'h* evident in these experiments might be caused via the Notch/Delta pathway during post segmentation processes.

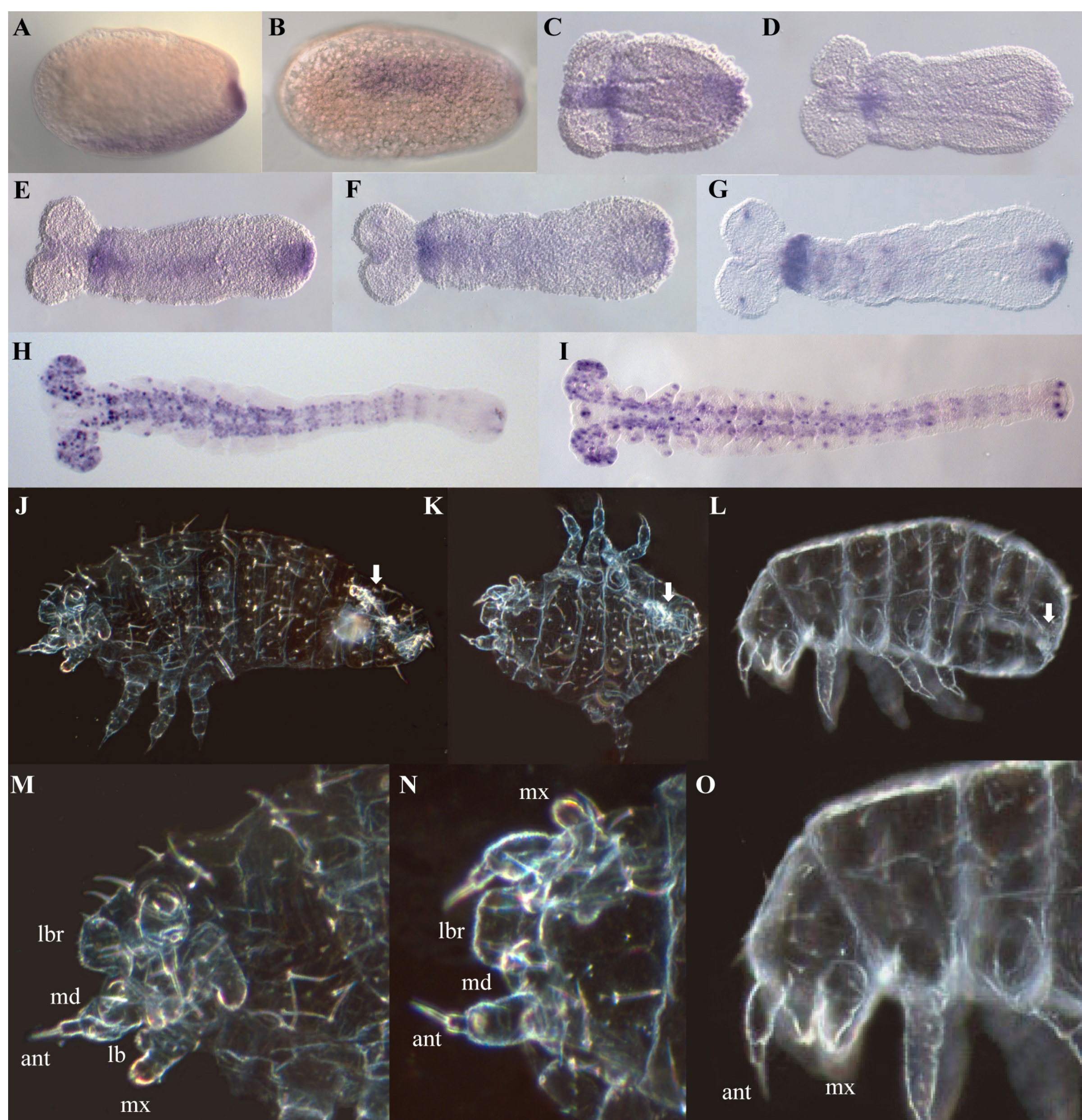


Figure 7

Expression of *Tc'delta* at different developmental stages (A-I). *Tc'delta* is first detected in a ventral stripe along the A-P axis of the blastoderm (A, lateral view; B, ventral view). In young germbands expression is detected in the mandibular segment in form of a stripe which persists until the end of segmentation (C-H). During growth of the germband *Tc'delta* is transiently expressed in form of a stripe along the A-P axis of the germband (E) and later in the developing nervous system (G-I). Embryonic injection of *Tc'delta* dsRNA at a concentration of 2 µg/µl leads to strong disruption of the gnathal region and to loss of abdominal segments (J-L). The cuticle depicted in (J) shows six abdominal segments and no terminal structures are visible, whereas in (K) only two to three abdominal segments are formed. (L) Shows a cuticle obtained from a pRNAi experiment. The larvae depicted shows similar defects in abdominal segments as in (K) and (L), whereas the formation of the gnathal structures appears less affected. (M-O) Show magnifications of the head region. In (M) all gnathal appendages are affected being either malformed or partially missing. In (N) the gnathal appendages are equally affected and the labium appears to be missing completely. The magnification of the cuticle from the pRNAi experiment (O) shows a less dramatic effect in the head region and the distance between the antennae and the maxilla appears to be normal compared to the results obtained in the pRNAi experiments with *Tc'h* (compare O and figure 4H). Note that all cuticles display internalized structures (white arrows). Of 78 embryos recovered 38% showed similar defects. The remaining 62% did not develop cuticles, in contrast to 72% developing a cuticle in the control experiment with GFP dsRNA. Segment identities abbreviated as follows: labrum, (lbr); antennae, (ant); mandible, (md); maxilla. (mx); labium, (lb).

3.8 Regulation of *Tc`h* by segmentation genes

In *Drosophila*, segmentation genes of different hierarchical levels, including the gap genes *Kr*, *gt* and *hb* as well as the pair-rule genes *eve* and *run*, regulate the expression of *h* during segmentation. To analyse whether *Tc`h* has a similar position in the regulatory network *i.e.* whether it is also regulated by orthologs of these genes in *Tribolium*, I performed pRNAi experiments with different *Tribolium* gap and pair-rule gene orthologs and examined the expression pattern of *Tc´h* in the respective knockdown embryos.

Regulation by Tc'gt

Tc`gt is expressed at different stages during segmentation, starting with putative maternal transcripts that are homogenously distributed throughout the syncitial blastoderm. With ongoing development expression retracts from both poles and intensifies in a circumferential stripe positioned in the later maxillary segment, comparable to the anterior domain in *Drosophila*, while expression in the remaining domain decreases. At the posterior pit stage a new domain arises in the posterior that later splits into two segmental stripes during germband elongation, corresponding to the third thoracic and second abdominal segment respectively (Bucher *et al.*, 2004), thus residing exactly between the *Tc´h* stripes #3-#4 and #4-#5, respectively.

In embryos of females injected with *gt* dsRNA, two stripes of expression appear at the position of the first and the third *Tc´h* stripe during blastoderm stage exactly as in wild type, while no expression at the position of the second stripe, which lies directly adjacent to the anterior *Tc'gt* stripe in the maxillary segment (Bucher *et al.,* 2004), can be detected (Figure 8 A). Although the anterior border of *Tc'h* stripe #3 seems well refined during the posterior pit stage and in young germbands, it becomes fuzzy and extends anteriorly during subsequent germband growth, suggesting a possible role for the anterior *Tc'gt* domain in the determination of the anterior border of this stripe. In addition, the third stripe does not bud off, but instead expression covers approximately 60% of the posterior region of the embryo (Figure 8 B-C). In weaker phenotypes this domain shows signs of splitting into stripes, but expression never ceases in the expected inter stripe regions, suggesting a fusion of the presumptive *Tc'h* stripes #3-#5 (Figure 8 C), which in turn speaks for a repressive function of *Tc'gt* on *Tc'h* either directly or indirectly. Interestingly *Dm'gt* was shown to repress *Dm'h* stripe #5 in the posterior region of the Drosophila embryo (Langeland *et al.,* 1994). Although the observed changes in expression do not fit the same region of the embryo due to the

anteriorly shifted expression of the posterior *Tc'gt* domain compared to its expression in *D.m.* (Bucher *et al.,* 2004), the observed derepression of *Tc'h* in *Tc'gt* knockdowns is highly reminiscent of the situation in *Drosophila*. Expression in the anterior part of this large domain decreases during further development, leaving a posterior cap of expression in the growth zone, which persists until the end or breakdown of segmentation (Figure 8 D-E). Formation of additional stripes was never observed, but the secondary expression in the presumptive nervous system at later stages appears unperturbed (Figure 8 F-G). It should be noted that the dynamic *Tc'h* expression observed in the growth zone of wild type embryos couldn't be detected in this remaining posterior cap, suggesting a breakdown of the pattern forming mechanism in the growth zone.

Regulation by Tc'Kr

The function of *Tc'Kr* was recently analysed in detail by Cerny *et al*. (Cerny *et al.*, 2005): Expression of *Tc'Kr* starts in the late blastoderm in form of a posterior cap with its anterior border residing between the labial and first thoracic segment. During growth of the germ rudiment expression retracts from the growth zone forming the posterior boundary of a domain that spans the three thoracic segments thus encompassing *Tc'h* stripe #3.

In *Tc'Kr* knockouts, the formation of the first five *Tc'h* stripes seems unperturbed although the fourth stripe does initially not bud of properly from the growth zone and refines somewhat later (Figure 8 J). No additional stripes are formed, but expression persists in the anterior part of the growth zone while the most posterior region stays free of expression. Furthermore, no secondary expression in the presumptive nervous system is detectable (Figure 8 M). Analysis in the *Tc'Kr* mutant *jaws* confirmed the results obtained in the pRNAi experiment (Alex Cerny, personal communication). It is interesting to note that the *Tc'eve* and *Tc'run* pattern in such knockdowns also shows normal formation of the first three stripes while the fourth is initially formed normally, but the segmental stripes after the split appear irregular. The fifth stripe is formed, but fails to split at all (Cerny *et al.*, 2005; Souza and Aranda, unpublished), a finding that further speaks against a direct involvement of *Tc'Kr* in pair-rule gene regulation since both these stripes lie outside the *Tc'Kr* domain and are therefore likely caused by a secondary process leading to the breakdown of segmentation.

Interestingly, it could be shown that expression of *Tc'gt* is altered in *Tc'Kr* pRNAi as well as in the mutant line "*jaws*" in a way that the posterior expression in A2 is missing whereas ectopic expression of *Tc'gt* is detectable in T1 (Cerny *et al.*, 2005). In case of a strict repressive function of *Tc'gt* on *Tc'h* expression, one would inevitably expect that the *Tc'h*

stripes #4-#5 are fused due to the lack of *Tc'gt* in the second abdominal segment. Instead we only see retardation in the formation of this stripe suggesting the involvement of additional factors in the refinement of this stripe. Although the central *Tc'Kr* domain encompasses *Tc'h* stripe #3 and is directly adjacent to *Tc'h* #2 in the anterior and *Tc'h* #4 in the posterior, no obvious effects can be detected during the formation of these stripes. This observation differs substantially from the effects of *Kr* mutations in *Drosophila* where the *h* stripes #2-#6 are strongly affected (Pankratz *et al*., 1990), including the stripes that are located within the primary *Tc'Kr* expression domain.

Regulation by Tc'hb

Tc'hb is provided maternally and distributed homogenously in the early blastoderm before it clears from the posterior pole and splits into an anterior cap, which coincides with the serosa, and a posterior domain that retracts posteriorly to form a broad band. This splits further into two stripes in the center of the embryo, the posterior of which coincides with *Tc'h* stripe #2, reminiscent of the situation in *Drosophila*. Apart from a secondary expression in faint segmental stripes, a posterior expression arises adjacent to the *Tc'h* stripe #6 and remains there until the end of segmentation, thus coinciding with Tc'h #7-#8.

In *Tc'hb* knockouts the first *Tc'h* stripe forms normally while the second seems broader than in wild type and does not split properly (Figure 8 O). The third stripe, which starts to be expressed as a posterior cap that refines to a stripe during germband growth, does not bud off. Instead expression in the growth zone is expanded towards anterior, forming a large posterior domain. In some cases a stripe is formed at the anterior border of this domain, but splitting is incomplete; *i.e.* the stripes appear to be fused (Figure 8 P). During further growth of the germband, expression fades in the anterior region of this domain while it remains in the growth zone until the breakdown of segmentation (Figure 8 R-U). The presumptive neurogenic expression seen in wild type embryos cannot be detected. Intriguingly, analysis of the gap gene interactions in *Tribolium* revealed that both the posterior domains of *Tc'gt* and the initial central domain of *Tc'Kr* are not present in *Tc'hb* knockouts (Souza and Aranda, unpublished). The observed fusion of *Tc'h* stripe #3-#5 could thus be a consequence of the missing *Tc'gt* expression since the effects observed in *Tc'gt* knockdowns at this stage are very similar. In *Drosophila hb* mutants the anterior *h* stripes are affected in a way that the first stripe appears significantly broader; whereas *Dm'h* stripe #2, which coincides with the second *Dm'hb* stripe, is missing (Figure 12 E). It should be noted that the effects of the gap genes on each other caused by their regulatory interactions and thus the resulting positional map in

such mutants or knockdowns differ substantially between *Tribolium* and *Drosophila*. It is therefore difficult to infer whether the effects observed are a direct consequence of depleted *Tc'hb* transcripts or indirectly mediated by other factors; for example like *Tc'gt*; since not all interactions are known in *Tribolium* and cannot be simply inferred from the *Drosophila* model.

Regulation by pair-rule genes

In contrast to *Drosophila eve* and *run* mutants, depletion of either *Tc'eve* or *Tc'run* transcripts by pRNAi produce truncated, almost asegmental embryos instead of the expected pair-rule phenotype. On cuticle level only few anterior segments and terminal structures are formed in these embryos (Choe *et al*., 2006).

Regulation by Tc'eve

In *Tc'eve* knockdowns *Tc'h* is first expressed in a broad posterior cap that covers approximately 70% of the early blastoderm, thus exhibiting an expansion towards both anterior and posterior compared to the initial circumferential stripe observed in wild type embryos. In some cases, which likely represent weaker phenotypes, establishment of an initial stripe at the anterior border of this domain can be observed, but the stripe appears broader and weakly refined apart from its anteriorly shifted position. Expression of *Tc'h* does not cease during the formation of the germ rudiment; resulting in strong expression of *Tc'h* in the entire embryo posterior to the head lobes (Figure 9 B), thus suggesting a general repressive function of *Tc'eve* on *Tc'h*. The fact that *Tc'eve* and *Tc'h* expression partially coincide (Brown et al., 1997) though implies the involvement of additional factors for this function. Due to the asegmental phenotype, which becomes evident at the level of the segment polarity gene *Tc'en* as it is only detectable in the antennal segment in these knockdowns *i.e.* no gnathal nor trunk *Tc'en* stripes are formed (Choe *et al*., 2006), no markers, neither molecular nor morphological, can be used to determine the exact position of the of the *Tc'h* domains. In contrast to this, the segment polarity gene *Tc'gsb*, which we found to be expressed earlier than *Tc'en*, shows additional expression in the intercalary segment as well as a broad central domain that follows the subsequent growth the embryo, thus again reflecting the asegmental phenotype of this region (Figure 20 A-F, supplemental material). In older stages a clearing of staining is observed in the central region of the germband, which starts as a broad domain that later exhibits a thin stripe of expression in its center (Figure 9 D). Intriguingly a posterior *Tc'gt* domain arises at a similar position at this stage in *Tc'eve* knockdowns, starting as a

broad central domain, which later splits into two stripes (Figure 20 A-C, supplemental material), highly reminiscent of the establishment of the posterior *Tc'gt* domains in wild type embryos. This observation further hints at a possible negative regulation of *Tc'h* by *Tc'gt*, although the exact position of these domains would have to be determined by double in situ. In addition it shows that the regulation of *Tc'gt* is indeed independent of the pair-rule cascade, as proposed by Choe *et al.*, even though the embryos appear almost completely asegmental, a finding that was also observed for the gap gene orthologs *Tc'Kr* and *Tc'hb* and *Tc'kni* (Choe *et al*., 2006, Souza and Aranda, unpublished). In late stages the anterior expression fades while the posterior domain becomes restricted to the posterior most region and eventually forms a stripe. At about the same time, expression arises in the lateral regions of the germband, probably correlated with the presumptive neurogenic expression observed in in wild type embryos.

Regulation by Tc'run

In *Tc'run* knockdowns the first *Tc'h* stripe is shifted anteriorly and appears wider than in wild type. Moreover the entire region posterior to this stripe exhibits low levels of expression (Figure 9 F). The formation of a second blastoderm stripe cannot be observed. In contrast to the pattern observed in *Tc'eve* knockdowns, stripes are still formed during further development, although they appear wider and unrefined at the beginning and narrow down somewhat later during the elongation process. This effect may be caused by the morphological phenotype of the embryos, which exhibit a condensed, bottle like morphology. In older stages expression is also detected in the lateral regions of the embryo as in *Tc'eve* knockdowns.

This result leads to the conclusions that the regulatory interaction between *Tc'run* and *Tc'h* during the segmentation of blastoderm derived segments differs from the interaction during the segmentation of the subsequently added segments during germband elongation, despite the finding of Choe and colleagues (Choe *et al*., 2006) that the pair-rule circuit is responsible for the patterning process in both phases of development.

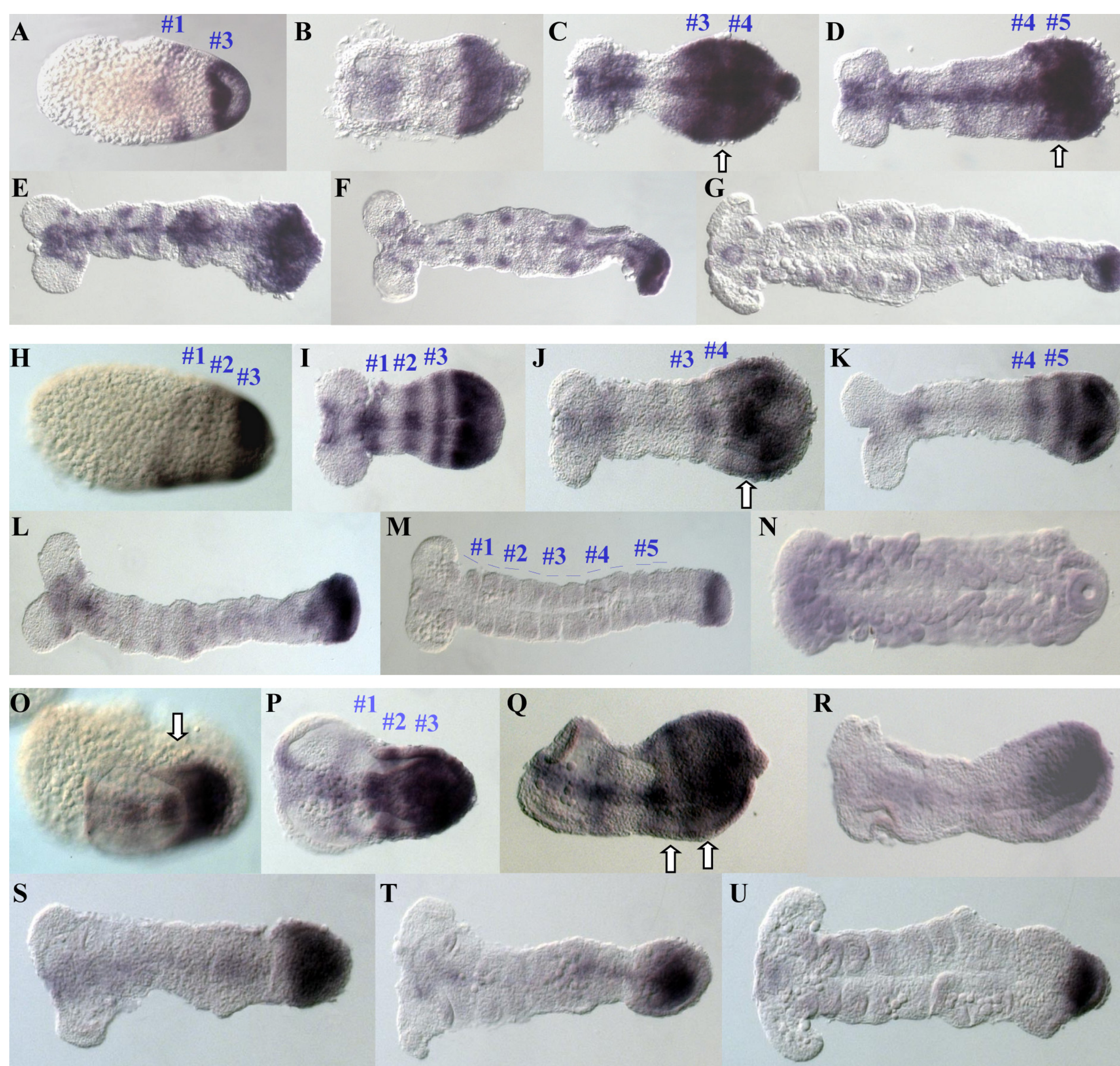


Figure 8

Tc'h expression in *Tc'gt* (A-G), *Tc'Kr* (H-N) and *Tc'hb* (O-U) pRNAi.

In *Tc'gt* knockdowns the formation of the first and third *Tc'h* stripe is not affected while the second stripe fails to form at blastoderm stage and is also absent in the germband (A-B). Although expression of *Tc'h* stripe #3 appears normal at blastoderm stage, the stripe never resolves during further growth and its anterior border appears fuzzy and poorly refined. Therefore a broad domain is formed, which shows a weak stripe of lower expression (C, white arrow), suggesting a possible fusion of the third and the fourth stripe, since *Tc'gt* is expressed in the segment in between. Similarly the fourth stripe also doesn't split properly (D, white arrow) and expression becomes restricted to the growth zone shortly after (E-G). Additional stripes are not formed and segmentation breaks down shortly after.

Depletion of Tc'Kr does not affect the formation of the first three *Tc'h* stripes (H-I). However, the fourth stripe does not split properly and refines somewhat later compared to the wild-type (J, white arrow). Thereafter a fifth stripe is formed before segmentation breaks down (L-N).

In *Tc'hb* pRNAi embryos the first *Tc'h* stripe is formed like in wild-type, while the posterior border of the second stripe is weakly defined (O-Q). The third *Tc'h* stripe never splits from the growth zone and a broad domain is formed in the posterior half of the germband which displays a faint stripe of weaker expression (Q, white arrow), suggesting a fusion of the third and fourth stripe. Expression in the anterior part of this broad domain ceases with ongoing development and expression is restricted to the growth zone until the breakdown of segmentation (R-U).

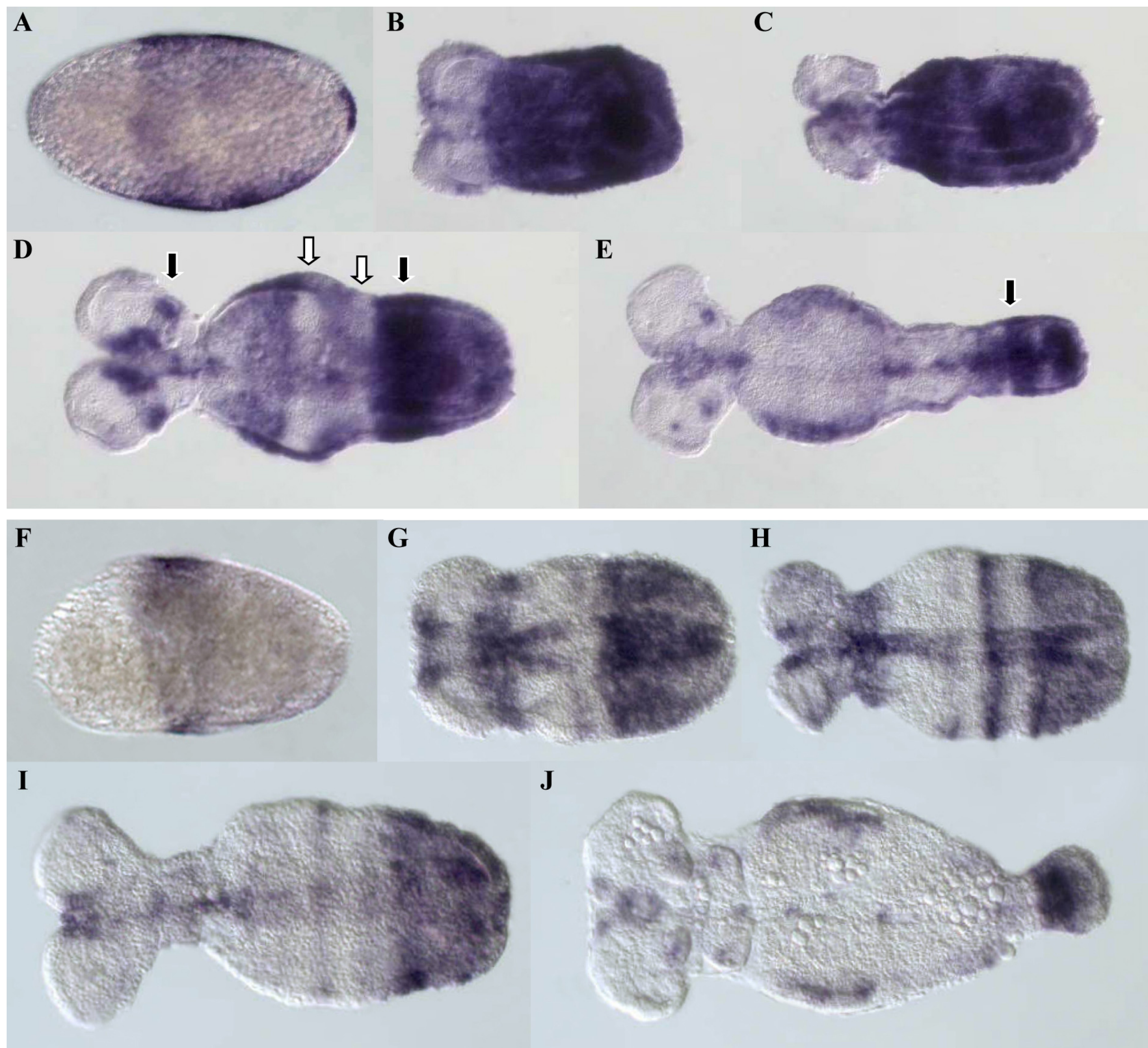


Figure 9

Expression of *Tc'h* in *Tc'eve* (A-E) and *Tc'run* (F-J) pRNAi. In *Tc'eve* pRNAi embryos *Tc'h* is severly affected. At blastoderm stage *Tc'h* is expressed in a broad domain covering approximately 70% of the blastoderm (A). Expression of *Tc'h* does not cease during the formation of the germband resulting in strong expression in the entire embryo posterior to the headlobes (B-C). During further growth expression ceases in the anterior part of the germband and a stripe of expression appears in the headlobes and in the posterior, which seems to move posteriorly during further growth (D-E, black arrows). Furthermore, two cleared regions are detected in the center of these germbands (D, white arrows). In *Tc'run* pRNAi embryos *Tc'h* expression is similarly affected at the blastoderm stage in that the first stripe is anteriorly shifted and poorly resolved. The posterior part of the blastoderm shows low levels of expression (F). During further growth of the germband several stripes split from the expanded growth zone, suggesting that the basic patterning mechanism regulating *Tc'h* expression is still functioning.

3.9 Conserved regulation of target genes

Drosophila hairy has been classified as primary pair-rule gene due to its regulation of secondary pair-rule genes like *Dm'prd* and *Dm'ftz*. To analyse if this regulation is conserved in *Tribolium*, the expression pattern of the pair-rule genes *paired* (*prd*) and *fushi tarazu* (*ftz*) were examined in *Tc`h* knockdown embryos generated by pRNAi.

The secondary pair-rule gene *ftz* plays an essential role during the segmentation of the *Drosophila* embryo and is negatively regulated by *h* (Ish-Horowicz and Pinchin, 1987), probably directly (Dearolf *et al.*, 1990). Although *Tc'ftz* is also expressed in a double segmental pattern, previous analyses have yielded no evidence for an involvement of this gene in the segmentation process of the *Tribolium* embryo (Stuart *et al.*, 1991). Since we found that *Tc'h* is not required in segmentation of posterior segments either, we asked if the regulatory interaction of *h* on *ftz* known from *Drosophila* is evolutionarily conserved. Therefore, we analysed the expression pattern of *Tc'ftz* in embryos depleted for *Tc'h* at different stages of development.

Regulation of Tc'ftz

Tc'ftz is expressed in a double segmental pattern which starts in the late blastoderm as a stripe in the presumptive maxillary segment, thus only after the expression of the first two or three *Tc'h* stripes and in a pattern complementary to *Tc'h*. Thereafter seven additional stripes appear sequentially *de novo* near the tip of the expanding germband, which initially appear wider than a para-segment before they refine (Figure 10 A-E).

In *Tc'h* pRNAi embryos, the anterior border of the first *Tc'ftz* stripe is clearly defined while the following seven stripes are initially formed, but appear to fuse with ongoing development, although expression in the presumptive interstripe region is slightly weaker. However, this effect could be caused by an incomplete depletion of *Tc'h* transcripts. Interestingly, the anterior expression does not vanish with subsequent elongation as seen for the stripes in wild type embryos, but instead appears stronger and persists until the end of segmentation (Figure 10 I-J). This result suggests a conservation of the regulatory interaction between *hairy* and *ftz* as found in *Drosophila*.

Regulation of Tc'prd

Like *Tc'ftz*, *Tc'prd* it is expressed in a pattern complementary to *Tc'h.* However, *Tc'prd* does act as a pair-rule gene during *Tribolium* segmentation (Choe *et al*., 2006). In wild type *Tc'prd* is first detected in a circumferential stripe corresponding to the maxillary segment at early blastoderm stage (Figure 11 A-B), which is followed by a second stripe, corresponding to the first thoracic segment, before the formation of the germ rudiment. The following stripes appear *de novo* in a similar fashion to the *Tc'ftz* stripes *i.e.* the most posterior tip of the elongating germband is free of expression and the stripes appear at a certain distance from the growth zone. Thus it contrasts the dynamic stripe formation observed for the pair-rule genes *Tc'eve, Tc'run* or *Tc'h*. Like *Tc'ftz,* the *Tc'prd* stripes seem broader and thus only poorly separated. Shortly after their appearance the stripes split resulting in a segmental expression pattern. At later stages *Tc'prd* is expressed in the mandibles as well as in two spots in the maxillae (Figure 11 M).

Regulation of Tc'prd

In *Tc'h* knockdown embryos the first *Tc'prd* stripe is initially formed like in wild type, while the second stripe appears directly adjacent to the first, suggesting a loss of the labial segment at the blastoderm stage (Figure 11 G-H). Furthermore the stripes do not split into the segmental pattern as seen in the wild type (Figure 11 I), instead a weak spotty pattern is observed in the region (Figure 11 C and I). The remaining stripes form virtually normally with respect to the wild type expression. In early germbands a premature loss of the first stripe can be detected in part of the embryos (Figure 11 J), comparable to the loss of *Tc'gsb* expression in this segment. At later stages no mandibular or labial appendages are seen in some of the embryos and the expression in the maxillae is reduced to one spot instead of the two seen in wild type embryos. Thus the results indicate a specific loss of the mandibular and labial segment, which fits with the observations made at the cuticle level. The missing spot in the maxilla may be a result of the secondary function of *Tc'h.* Thus, in contrast to the role of *h* in *Drosophila*, the results obtained in this experiment suggest no direct involvement of *Tc'h* in the regulation of *Tc'prd* and instead suggest the specific deletion of the labial segment.

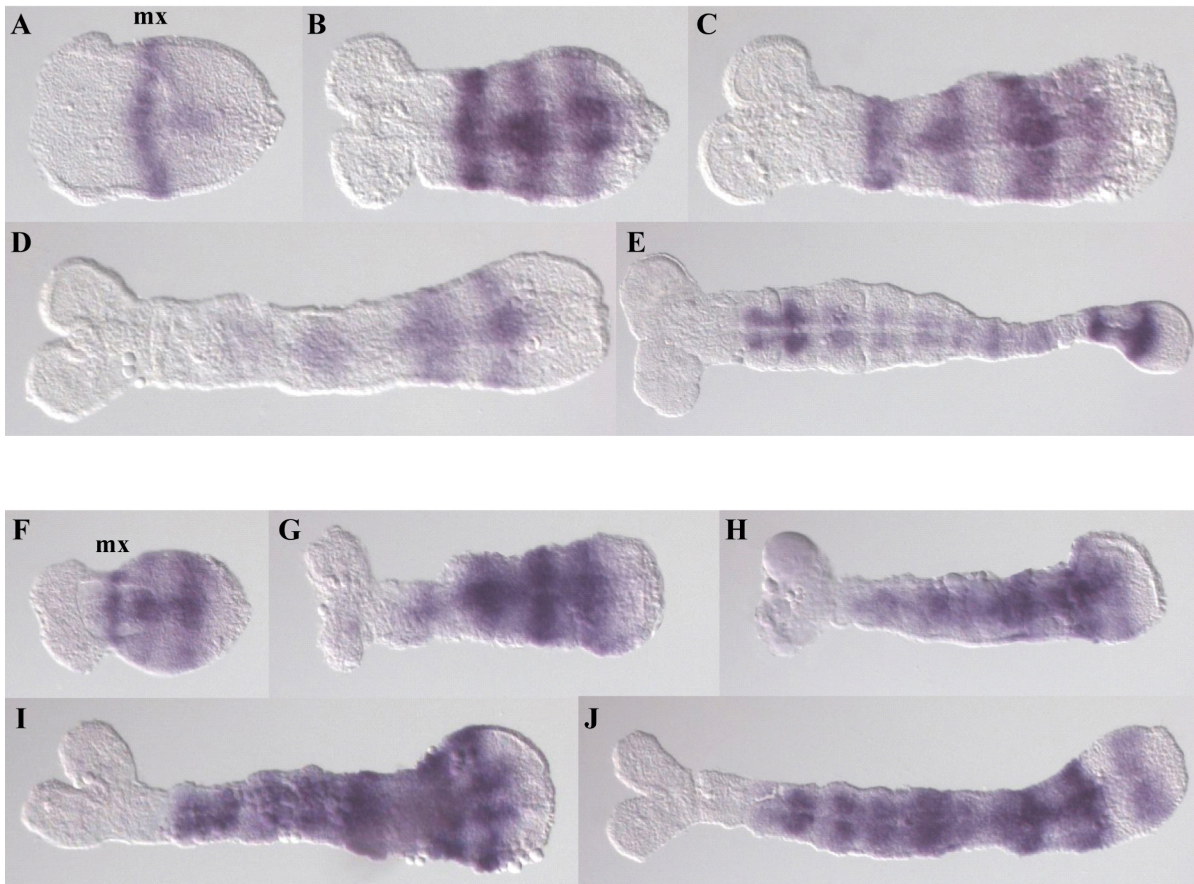


Figure 10

Wild-type expression of *Tc'ftz* (A-E) and in *Tc'h* pRNAi (F-J). *Tc'ftz* is expressed in a pair-rule like fashion complementary to *Tc'h*. Expression is detected in the maxillary segment of young germbands (A). Thereafter seven additional stripes appear sequentially, *de novo* near the tip of the expanding germband (B-E). In later stages a second expression appears in the developing nervous system (E). In *Tc'h* knockouts the anterior border of the first stripe seems unaffected (D) while the remaining stripes are formed, but appear to fuse with ongoing development (G-J). Interestingly expression in the anterior region does not cease, as seen for the wild-type expression. Instead the expression persists throughout the segmentation process.

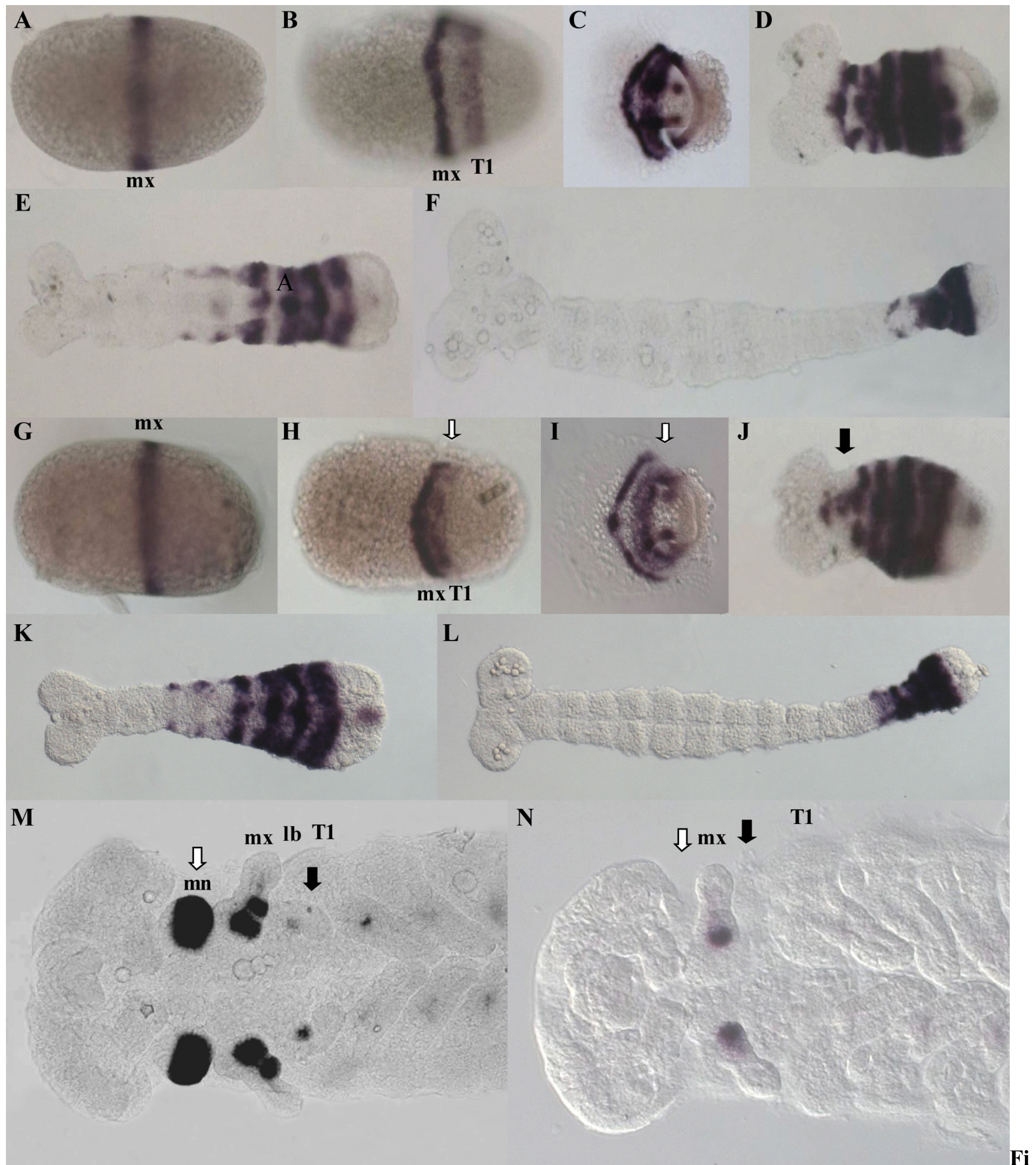
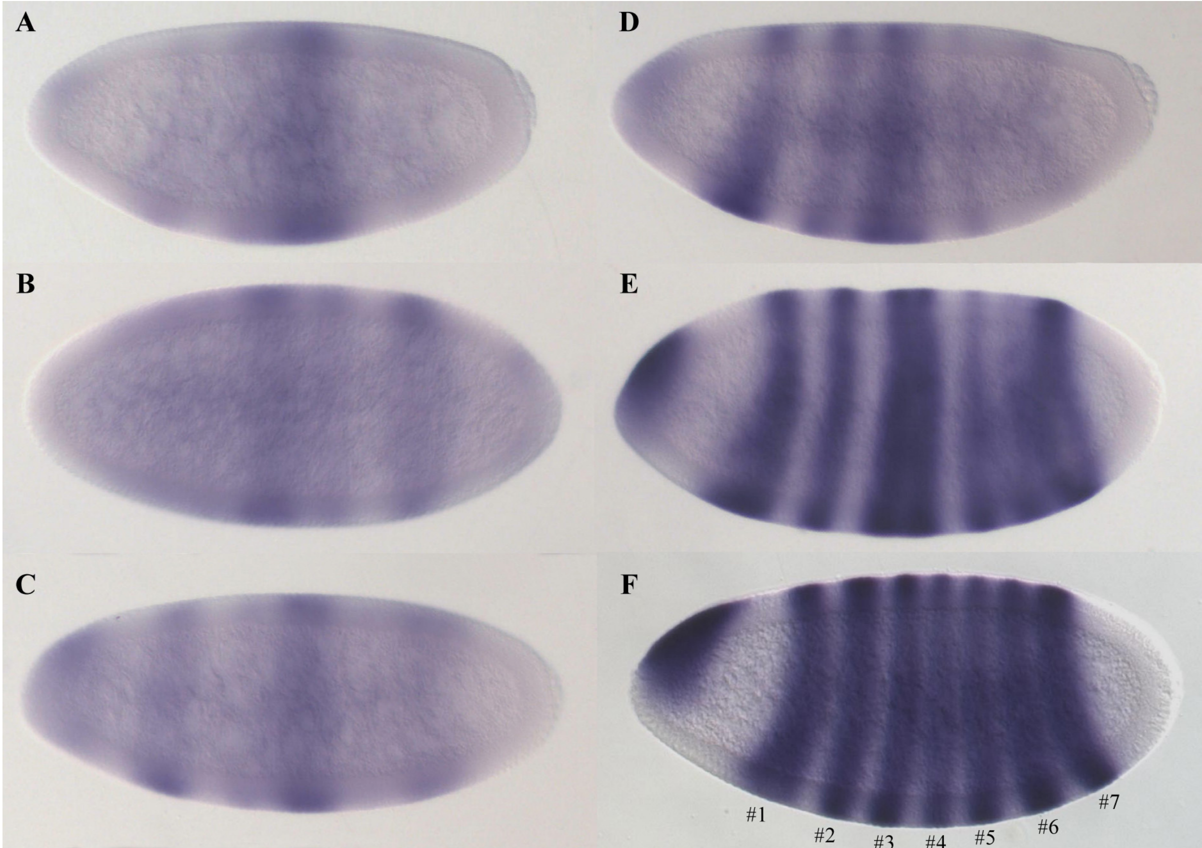


Figure 11

Expression of *Tc'prd* in wild-type (A-F) and Tc'h pRNAi embryos (G-L). Expression of *Tc'prd* starts as a circumferential stripe in the blastoderm in the maxillary segment, which is followed by a second sripe of expression in the segment corresponding to T1 before the formation of the germ rudiment (A-B). The following stripes appear *de novo* at the anterior border of the growth zone (C-F). Shortly after the appearance of the stripes they split into a segmental expression pattern. At the end of segmentation *Tc'prd* is strongly expressed in the mandible and the maxilla (M). In *Tc'h* knockdown embryos the first *Tc'prd* stripe appears normally in the blastoderm (G), whereas the second stripe is formed directly adjacent to the first (H), suggesting a loss of the intermediate labial segment. During further development these stripes do not split as seen in wild-type instead, a weak, spotty pattern is detected in the region of the presumptive labial segment (compare I and B, white arrow). In some cases a loss of expression in the maxillary segment is observed during further germband growth (J, black arrow). The remaining stripes appear unaffected (K-L). (M-N) show magnifications of the head region in wild-type (M) and *Tc'h* depleted embryo (N). Expression in the mandible is not detected and no mandibular nor labial structures were formed (compare N and M).

3.10 Expression of *Tc'h* constructs in Drosophila

To test whether the *Tribolium* regulatory regions identified in a previous study (Eckert *et al.*, 2004) are also functional in *Drosophila*, I have used genetically transformed Drosophila lines carrying identical *Tc'h* constructs (transgenic lines provided by Ernst Wimmer). The 3.1 kb construct BN3.1, which includes the regulatory elements for the expression of three central *Tc'h* stripes corresponding to the stripes #3/#5 in *Tribolium* (Eckert *et al*., 2004), is expressed in a broad anterior domain encompassing about 70% of the early blastoderm, which subsequently refines to a broad anterior and central domain. Thereafter, an additional domain arises in the posterior part in some, but not all embryos, and the central domain eventually resolves into three distinct stripes (Figure 12 G-L). Double staining with the endogenous *h* gene shows that these stripes are slightly out of phase but overlap partially with the endogenous stripes #3-#5 (Figure 13B). The anterior and posterior domains, however, do not resolve into stripes. While the anterior domain is not located in the region of stripes #1 or #2, the posterior domain posterior of the endogenous stripe #7 (Figure 13 A-D). The origin and significance of these domains can currently not be assessed, but expression of a domain reminiscent of the anterior domain driven by this construct seems to be associated with the first 1.5 kb upstream of the reporter gene start codon consistent with other constructs containing this region. In addition, a larger construct, which includes the regulatory elements for all eight *Tc'h* stripes (Eckert *et al*., 2004), yields the same pattern as the two shorter constructs (Eckert, 2004). The fact that the stripes are not expressed at exactly the same position as the endogenous stripes is not surprising, since this is even observed for reporter gene constructs harbouring endogenous *Drosophila* enhancer elements (Pankratz, et al., 1990) and may be partially associated with the lack of endogenous UTR regions in these constructs and a resulting retardation in transcript degradation.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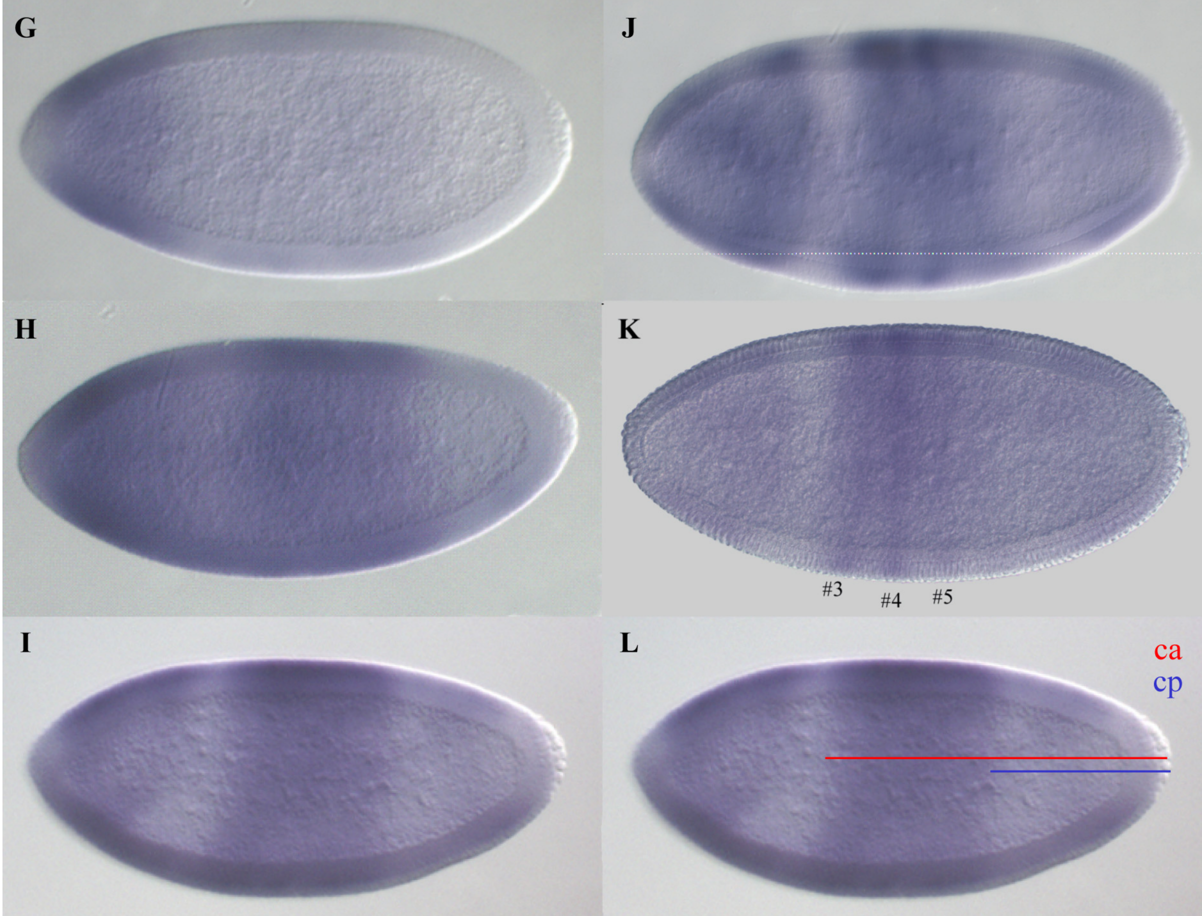
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Figure 12

Expression of *Dm'h* (A-F) and BN3.1 (G-L) in wild-type embryos. Expression of Dm'h starts as a broad stripe in the center of the blastoderm, which is flanked by domains of weaker expression (A). With ongoing development further stripes appear anteriorly and posteriorly of this central domain and a cap of expression is detected in the anterior most region of the blastoderm (B-C). The broad domains start to split into the double segmental pattern and expression intensifies (D-E). Finally the seven stripe pattern is fully resolved (F).

Expression of the *Tc'h* construct BN3.1 in the transgenic line T2M3 starts as a large anterior domain encompassing approximately 80% of the blastoderm (G). During further development, expression intensifies in the central and anterior region, thus forming an anterior cap and a broad central domain (H-I). In some, but not all embryos, an additional posterior domain appears (I-K). Around the end of the cellularization process the central domain splits into three distinct stripes at the position of the endogenous stripes #3-#5(K). Embryos of the stage depicted in (L) have been chosen for the single staining analysis in the mutant experiment, since they present a distinct pattern but are still young enough to exclude major influences of pair-rule genes. The lines ca and cp mark the distances measured for the statistical analysis.
The numbers indicate the endogenous and reporter driven stripes, respectively.

3.11 *Tc'h* is regulated by gap genes in Drosophila

We used the genetically transformed *Drosophila* lines carrying the BN3.1 construct to analyse the regulation of the reporter gene by different *Drosophila* gap- and pair-rule genes. Therefore two different transgenic lines were crossed into *Drosophila* mutants of *hunchback*, *Krüppel*, *knirps*, *giant*, *even-skipped* and *runt* respectively and the resulting embryos were stained both for endogenous *hairy* expression by fluorescence in situ hybridisation, as well as for *lacZ* RNA expression by chromogenic in situ hybridisation. Due to the highly dynamic expression of *Dm'h* during the early blastoderm stage, I first performed a single staining analysis to determine all possible wild type patterns in order to prevent an analysis of false positive mutants. An overview of the different patterns observed is provided in figure 12 (A-H) for comparison. Apart from the double staining analysis I performed single staining of the reporter gene expression in the different mutants and compared the position of the domain boundaries at earlier stages with the wild type expression. The data sets obtained in this experiment were analysed for significant changes compared to the wild type pattern using the non-parametric Mann-WhitneyU test and Bonferroni corrected.

The regulation by Dm'hb

In *hunchback* mutants the second *h* stripe is missing in *Drosophila* while the stripes #3 and #4 are fused, resulting in a broad domain. The central stripes driven by the *Tc'h* construct also form a broad domain, which shows a stripe of weaker expression, thus separating it weakly into a posterior and anterior part, the latter likely formed by fusion of the first two stripes (Figure 13 E-G). The statistical analysis of single staining places the anterior border of this central domain at a position around 60% egg length in wild type, whereas it resides at about 64% e.l. in *hunchback* mutants, thus being slightly, but significantly extended towards the anterior. The posterior margin of this domain resides directly adjacent to the endogenous *h* stripe #6 on the dorsal side while it is some cells apart on the ventral site and exhibits a slight posterior expansion (Figure 17 D and L). Interestingly, a similar effect of *Tc`hb* on the endogenous *Tribolium* stripes can be seen, but it is difficult to assess if the fused stripes observed in the pRNAi experiment are really the expected stripes #3 and #4 and if this effect is indeed caused by *Tc`hb* directly as mentioned above. A similar indirect effect as cause for the observation appears unlikely due to the different effects of *hb* on *gt* expression in these species.

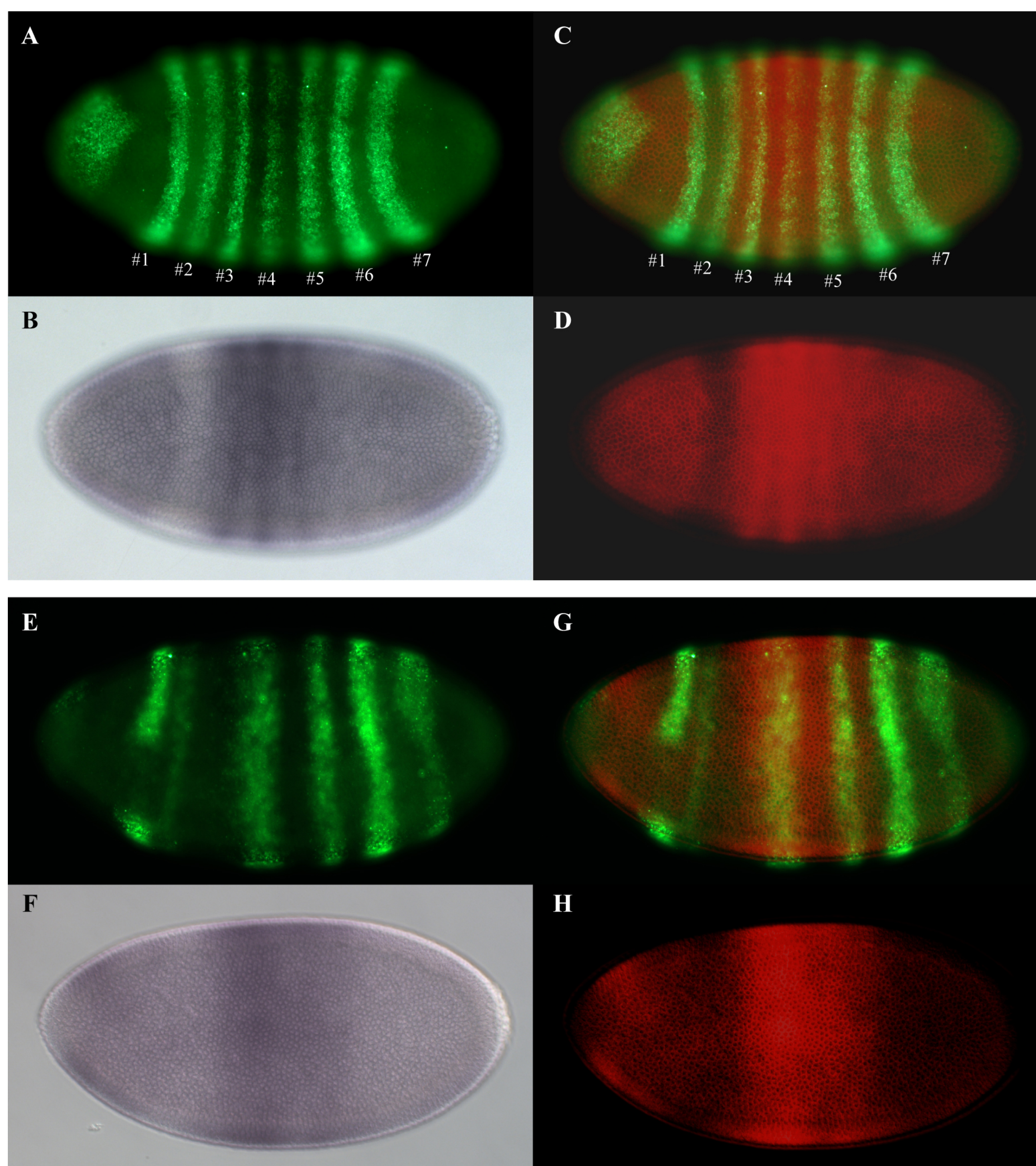


Figure 13

Double staining of *Dm'h* expression (in green) and BN3.1 (blue and red, respectively) in wild-type (A-D) and a *Dm'hb* mutant (E-H) embryo. Embryos are positioned with anterior to the left and the dorsal side up. (A) shows the seven Dm'h stripes in a wild-type embryo. In (B and D) expression of the BN3.1 construct in the same embryo as (A) stained with NBT/BCIP in (B) and artificially colored in red in (D). (C) The overlay of (A) and (E) shows that the three stripes driven by the BN3.1 reporter gene construct overlap with the endogenous stripes #3-#5. The stripes appear wider though and are slightly shifted anterior by about 2-3 cells. The anterior domain covers the first *Dm'h* stripe and seems to coincide at the posterior border. (E-H) shows a *Dm'hb* mutant embryo. Expression of *Dm'h* is severly affected in that stripe #2 is missing whereas stripes #3-#4 appear fused. The remaining stripes are slightly wider than in wild-type (compare A and F). In (F and H) expression of the BN3.1 construct in the same embryo as (E) stained with NBT/BCIP in (F) and artificially colored in red in (G). Expression of the BN3.1 construct is expressed in a broad central domain, suggesting a fusion of the three stripes driven by the construct in wild-type embryos (F and H). The broad domain is directly adjacent to the presumptive *Dm'h* stripe #6, while the anterior domain appears unaffected (G). The numbers indicate the endogenous Dm'h stripes.

The regulation by Dm'kni

In *Dm'kni* mutant embryos, the endogenous *h* stripes #4-7 are fused and form a broad domain in the posterior region of the embryo. *Tc'h* stripe #3 appears unaffected in this mutant while stripes #4-#5 do not resolve, but form a broad domain that extends towards the posterior by approximately 7% e.l. compared to the expression in wild type at this stage (Figure 14 A-D). The posterior border of this domain overlaps with the posterior *Dm'h* domain formed by the fused endogenous stripes #4-#7. Analysis of the regulatory region of *Tc`h* revealed several clusters of putative *Dm'kni* binding sites and band shift experiments performed in collaboration with Paolo Struffi confirmed that *Dm'kni* is indeed able to bind fragments of approximatly 200bp containing a minimum of three putative binding sites *in vitro* (Paolo Struffi, personal communication), but the regions tested in the binding experiments lay further upstream than the 3.1kb used in this construct. No predicted clusters of *Dm'kni* binding sites are found within the construct used in the transgenic experiment.

The regulation by Dm'Kr

In *Dm'Kr* mutants all central *h* stripes are affected, resulting in two broad domains in the central region of the embryo, which are flanked by a single stripe on each side. Expression of the *Tc'h* stripes in these mutants shows a similar effect, since the central domain never resolves into stripes (Figure 14 E-H). In addition the domain appears narrower than in wild type. Single staining analysis places the posterior boundary of this domain at around 40% e.l. compared to the observed 34% in wild type, but the statistical analysis shows no significance for this effect. A possible reason for the non-significance of the observed retraction of the central domain in the statistical analysis lies in the presence of wild type embryos in this experiment. Based on the crosses performed between the transgenic and mutant lines only 25% of the embryos analysed are homozygous mutants, whereas the remaining 75% of the embryos are heterozygous and homozygous wild type. For this reason, the non-parametric Mann-WhitneyU test was chosen, which by itself is a highly conservative test. With respect to the *h* expression in these mutants the domain covers the posterior region of the fused domain formed by the endogenous stripes #2-#4, extending posteriorly by approximately 3 cells and could thus resemble part of this domain. According to Langeland *et al.*, *Dm'Kr* is essential for the establishment of the anterior borders of the *Dm'h* stripes #5 and #6, since expression of reporter gene constructs harbouring minimal enhancer elements for these stripes show anterior expansion of these stripes in *Dm'Kr* mutants (Langeland *et al.*, 1994). In contrast, *Tc'h* expression appears to be posteriorly restricted in these mutants. A possible explanation

for the different effect observed for *Kr* on the endogenous and the reporter gene expression driven by the *Tc`h* construct may lie in the gap gene interactions. In previous analyses it was shown that mutation of *Kr* in *Drosophila* leads to an expansion of the posterior *gt* domain towards the anterior (Kraut and Levine, 1991; Capovilla and Eldon, 1992). Hence the anteriorly expanding *Dm'gt* domain in these mutants could cause the observed effects on the reporter gene expression, which is further supported by the finding that *Tc'gt* is also involved in the regulation of these stripes in *Tribolium*. These results also contrast with the results from *Tribolium* in which no comparable effect of *Tc`Kr* was detected on the presumptive *Tc'h* stripes #3-#5.

The regulation by Dm'gt

Dm'gt is reported to repress the posterior *h* stripes in *Drosophila,* where it refines the borders of the endogenous stripe #5, resulting in a fusion of the endogenous stripes #5-#7 (Figure 15 A-E) in the respective mutant. Similarly, the *Tc`h* pattern exhibits an additional stripe in the posterior that appears to be slightly separated from the first three stripes. Intriguingly the stripe appears in the region where *Dm'gt* is expressed in wild type embryos at this time, again suggesting a possible repressive function of *Dm'gt* on the *Tc`h* construct, as seen for the wild type *h* expression. Compared to the effect of *Tc`gt* on *Tc`h* it is interesting to note that knockdown of *Tc`gt* transcripts also leads to a broad domain reminiscent of a fusion of the presumptive stripes #3-#5 (see section 3.6) in *Tribolium*. The anteriorly shifted position of this resulting domain, with respect to *Drosophila* reflects the different positions of the posterior *Dm'gt* domains in these species. In *Tribolium* the posterior *Tc'gt* domains reside between the third, fourth and fifth *Tc`h* stripe respectively whereas the broad posterior *Dm'gt* domain covers the region of the *Dm'h* stripes #6 and #7, which corresponds to a shift of five segments (Bucher *et al*., 2004).

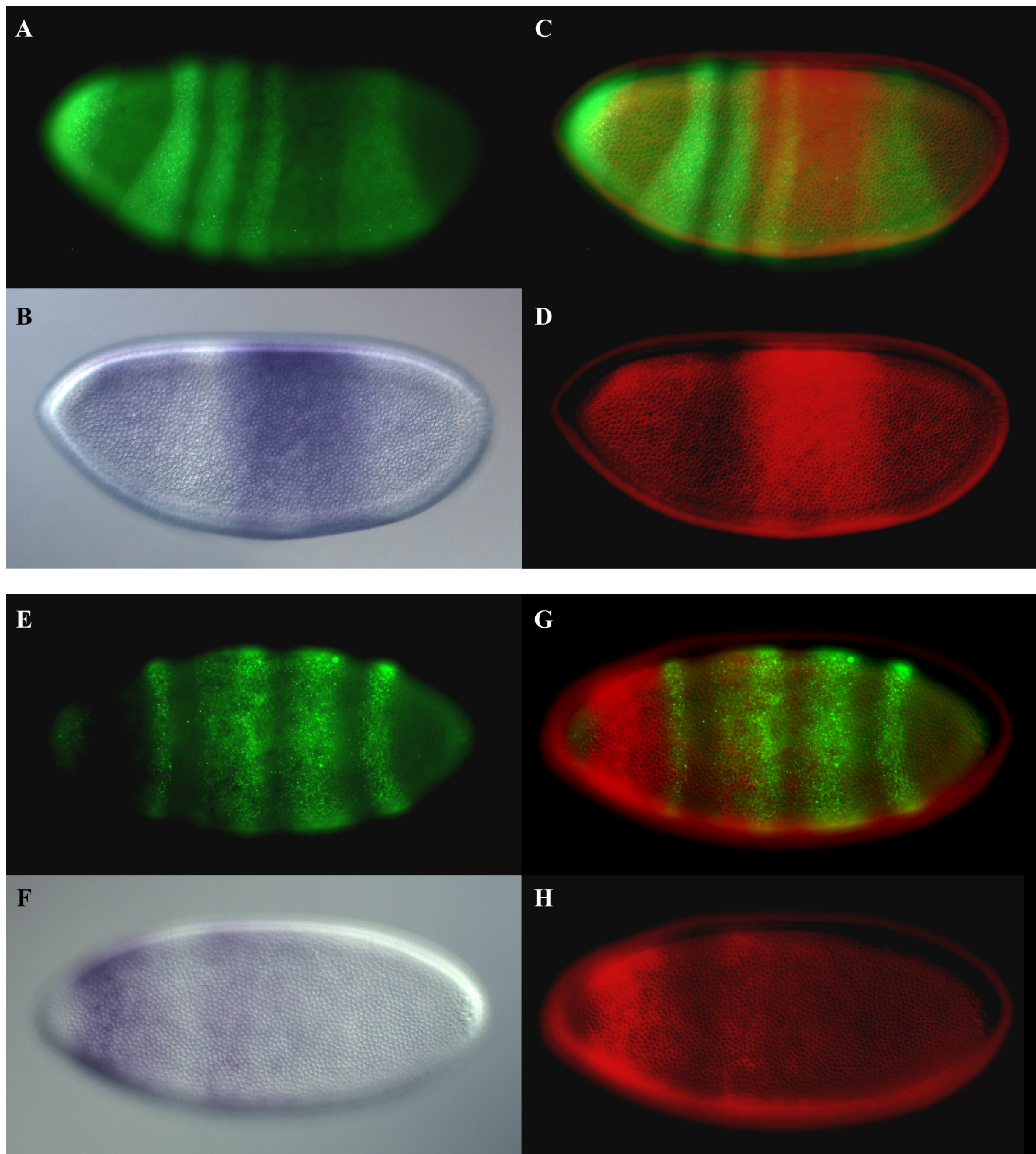


Figure 14

Double staining of *Dm'h* expression (in green) and BN3.1 (blue and red, respectively) in a *Dm'kni* mutant (A-D) and a *Dm'Kr* mutant (E-H) embryo. Embryos are positioned with anterior to the left and the dorsal side up.The endogenous *Dm'h* stripes #4-#7 are fused in *Dm'kni* mutant embryos while the anterior three stripes are broader than in wild-type (A). In (B and D) expression of the BN3.1 construct in the same embryo as (A) stained with NBT/BCIP in (B) and artificially colored in red in (D). The central domain driven by the BN3.1 at younger stages does not fully resolve into three stripes as seen in wild-type (B and D). Whereas *Tc'h* stripe appears unaffected and partially coincides with the endogenous stripe #3 (C) the *Tc'h* stripe #4-#5 form a broad domain, which extends posteriorly compared to the expression in wild-type embryos and overlaps with the broad posterior domain of the fused *Dm'h* stripes #4-#7.
In the *Dm'Kr* mutant embryo all central *Dm'h* stripes are affected, forming two broad domains in the central region of the blastoderm (E). In (F and H) expression of the BN3.1 construct in the same embryo as (E) stained with NBT/BCIP in (F) and artificially colored in red in (G). The BN3.1 construct is expressed in a central domain that appears to be narrower compared to the expression in the wild type (F and H) and fails to resolve into stripes. The anterior border of this domain coincides with the anterior border of the fused endogenous stripes #2-#4.

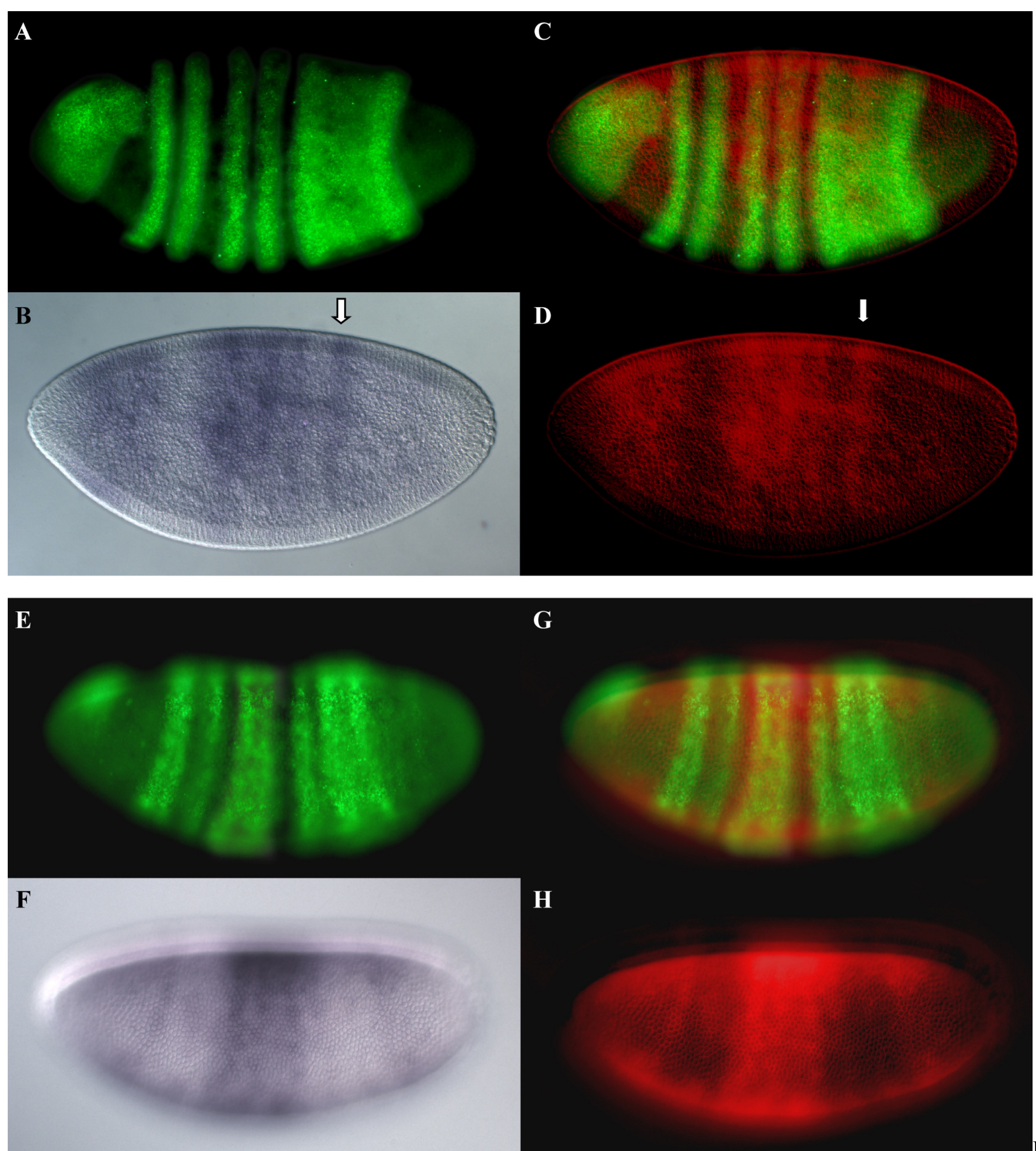


Figure 15

Double staining of *Dm'h* expression (in green) and BN3.1 (blue and red, respectively) in a *Dm'gt* mutant (A-D) and a *Dm'run* mutant (E-H) embryo. Embryos are positioned with anterior to the left and the dorsal side up. Mutation of *Dm'gt* affects the posterior *Dm'h* stripes #5-#7 resulting in a broad fused domain on the posterior region of the blastoderm (A), whereas the *Dm'h* stripes #1-#4 are almost unaffected. In (B and D) expression of the BN3.1 construct in the same embryo as (A) stained with NBT/BCIP in (B) and artificially colored in red in (D). Expression of the three central stripes driven by the BN3.1 construct is not affected in that the stripes are formed and overlap with the endogenous *Dm'h* stripes #3-#5. However, an additional stripe of expression appears in the posterior region (B and D, white arrow) which resides within the broad domain formed by the endogenous *Dm'h* stripes #5-#7 (C). Note that *Dm'gt* is expressed in this region in wild-type embryos.

All *Dm'h* stripes are affected in *Dm'run* mutant embryos, as they fail to resolve properly (E). In (F and H) expression of the BN3.1 construct in the same embryo as (E) stained with NBT/BCIP in (F) and artificially colored in red (G). Expression of the BN3.1 construct is less affected in that the stripes are still formed, but appear fuzzy and poorly resolved. In some cases faint expression, reminiscent of additional stripes is seen (F and H). Interestingly, the stripes driven by the construct still seem to overlap with the endogenous *Dm'h* stripes (G).

The regulation by Dm'run

All *Drosophila* stripes are affected in runt mutant embryos, as they fail to resolve properly. The effect of runt on the *Tc`h* stripes is slightly weaker though, since they are still formed but remain fuzzy and poorly resolved. It is interesting to note that a similar effect is also seen in *Tc`run* knockdowns on the expression of *Tc'h*, as shown above*,* in that stripes are still formed, but do not resolve properly. As expected, a significant shift of the boundaries of the central domain with respect to the expression in wild type background at earlier stages could not be observed, thus arguing against an earlier function of *Dm'run* on the reporter gene expression (Figure 15 E-H) and excluding an influence of *Dm'run* on the observed effects in the gap gene mutant experiments.

The regulation by Dm'eve

The opposite effect is seen for *Dm'eve* where most of the endogenous *h* stripes are only weakly affected while the *Tc`h* stripes fail to resolve. In some cases a split of the central domain into two stripes positioned at the anterior and posterior margins of the central domain can be observed, but the stripes appear poorly resolved. The anterior stripe is shifted anteriorly with respect to the endogenous stripe #3 while the posterior one lies anteriorly adjacent to the endogenous stripe #5 (Figure 16 A-D). In *Tc`eve* knockdowns *Tc`h* is expressed in almost the entire germband with only a small anterior and central region exhibiting no expression, an effect comparable to the unresolved stripes observed in this experiment.

Gap and pair-rule genes genes regulate the Tc'h construct

One of the biggest difficulties for the precise determination of the factors which cause the observed effects is the cross regulatory interaction between gap and pair-rule genes. As already mentioned before, the cross regulatory interactions between the gap and the pair-rule genes lead to shifts of the expression domains of several factors, if components of the network are altered. Some of these interactions and the resulting effects on factors of the *Drosophila* segmentation cascade, such as the mutual inhibition of the *Dm'Kr* and *Dm'gt* domains, have been well characterized and are likely to contribute to the observed changes in reporter gene expression. Other interactions characterized so far include the observation of lower levels of *Dm'gt* in its posterior domain in *Dm'kni* mutants (Kraut and Levine, 1991), which may contribute to the posterior expansion of the central domain detected in *Dm'kni* mutants. However, the results obtained in these experiments show that the regulation of the expression

driven by the *Tc'h* reporter gene construct depends on the input of the gap and primary pair-rule genes, although the exact mode of interaction is evidently not identical. The finding that gap gene mutations already affect the pattern at an early stage, as seen in the statistical analysis, makes it unlikely that cell-cell signalling is involved in the generation of the patterns detected. Thus the interactions between the *Drosophila* gap genes and the *Tribolium* enhancers within the construct are likely to be direct.

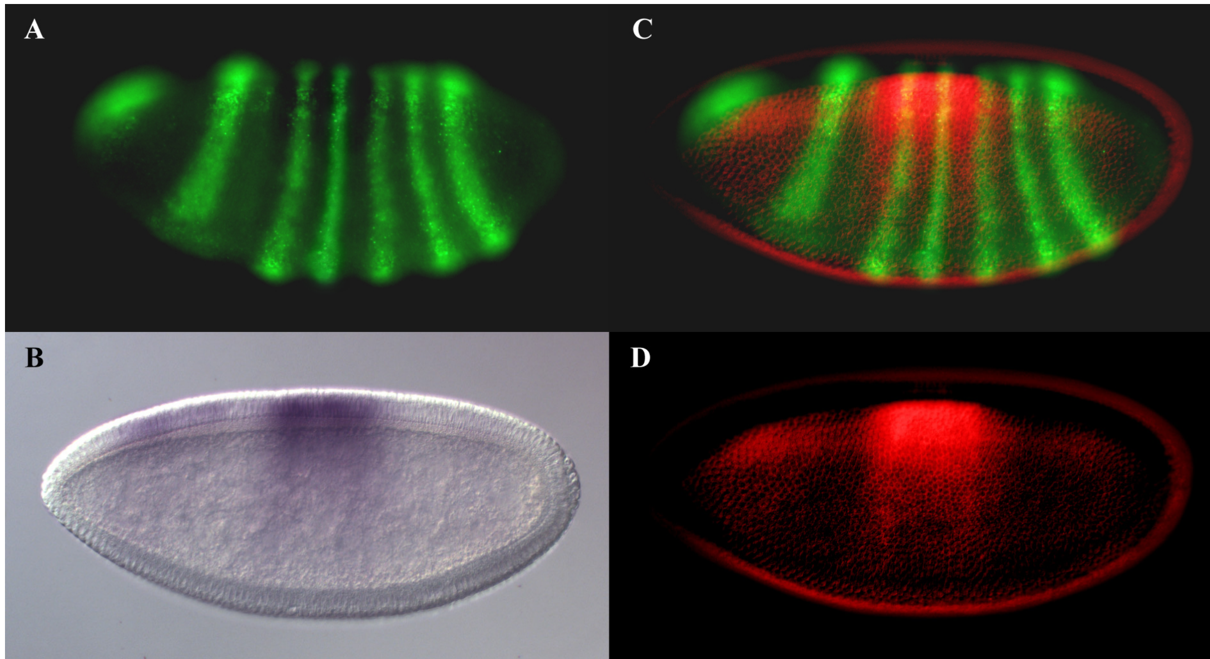
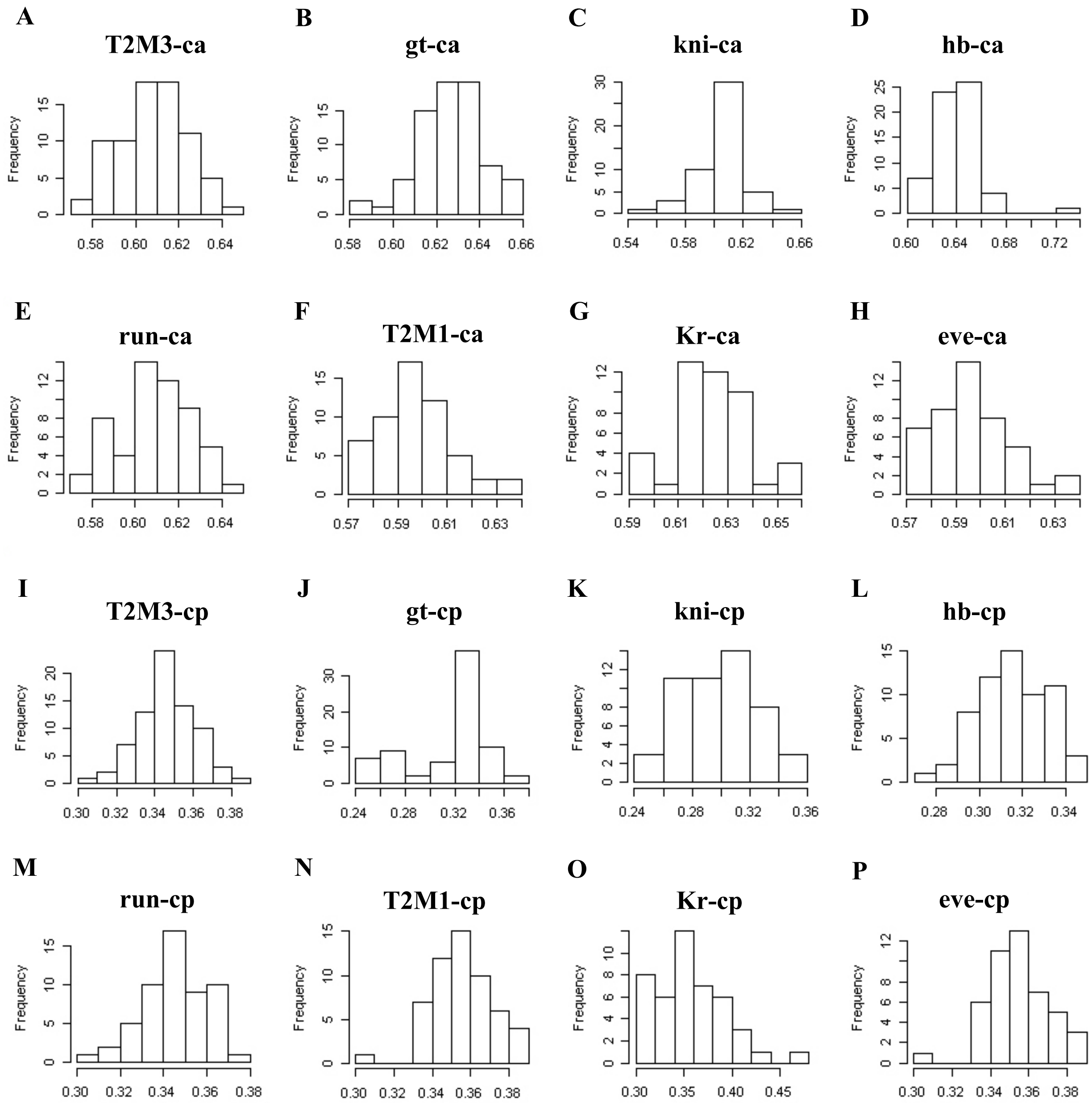


Figure 16

Double staining of *Dm'h* expression (in green) and BN3.1 (blue and red, respectively) in a *Dm'eve* mutant embryo (A-D). Embryos are positioned with anterior to the left and the dorsal side up. In *Dm'eve* mutant embryos *Dm'h* expression is only weakly affected besides the missing stripe #2 (A), whereas the expression of the BN3.1 construct fails to resolve into stripes (B and D), instead they form a broad domain in the center of the blastoderm which exhibits stronger staining at the anterior and posterior margins. The posterior border of this domain coincides with the endogenous *Dm'h* sripe #5. In (B and D) expression of the BN3.1 construct in the same embryo as (A) stained with NBT/BCIP in (B) and artificially colored in red in (D).

Figure 17

Distribution of the values measured for the margins of the central expression domain in the BN3.1 transgenic lines in wild-type and mutant background. (A and F) shows the distribution of the values obtained for the anterior border of the central domain (ca, see figure 12 L) in the transgenic lines T2M3 and T2M1 which were used for the crosses with *Dm'gt*, *Dm'kni*, *Dm'hb, Dm'run* (crossed with T2M3) and *Dm'Kr, Dm'eve* (crossed with T2M1) mutant lines. Note, the values display normal distribution. The datasets were Bonferroni corrected for the subsequent statistical analysis. The values obtained for the mutant lines were compared to the wild-type data sets using the non parametric Mann-WhitneyU Test. Frequency is depicted on the y-axis and the distance measured in percentage with respect to egg length on the x-axis. ***Comparison of the transgenic lines in wild-type background:*** Comparison of the values obtained for the two transgenic lines showed comparably weak, but significant changes (ca, W=2854, p-value = 0.0001934; cp, W=1377, p-value = 0.001247 n=56). Note that the embryos measured were composed of 25% mutants, 25% wild-type and 50% heterozygous. ***Changes in the position of the anterior border (ca:)*** Distribution of the values obtained for the position of the anterior border discovered significant anterior expansion of the central domain for the mutants *Dm'gt* (W=1002.5, p-value = 2.883e-11, n=74 in B), *Dm'hb* (W=288, p-value < 2.2e-16, n=63 in D) and *Dm'Kr* (W=256, p-value = 1.889e-11, n=45 in G). No significant changes in expression were found for the crosses with *Dm'kni*, *Dm'run* and *Dm'eve* mutant lines. ***Changes in the position of the anterior border (ca):*** (I-P) shows the same as (A-H), but for the posterior margins of the central domain (cp, see also figure 12 L). The distribution of values obtained for the transgenic lines are depicted in (I) and (N). Significant changes were found for the position of the posterior boundary of the central domain for the mutants *Dm'gt* (W=4635.5, p-value = 3.397e-13, n=74 in J), *Dm'kni* (W=3541, p-value < 2.2e-16, n=51 in K) and *Dm'hb* (W=4319, p-value < 2.2e-16, n=63 in L), which showed a posterior expansion. ***Notes:*** No influence has been found for the pair-rule genes *Dm'eve* and *Dm'run* as expected for the early stage analyzed in this experiment. Furthermore, no significant changes have been found for the position of the posterior domain in *Dm'Kr* mutants although the double staining clearly shows a retraction of this border towards anterior (see figure 14 B). This may be caused by the presence of wild-type embryos in the cross and weaker heterozygous effects, which are likely to contribute to the changes observed in other mutant lines. Note the bimodal distribution for *Dm'gt* (J), which is caused by the additional stripe in the posterior region (see also figure 15 B, white arrow).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eve-ca
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T2M3-cp
J
gt-cp
K
kni-cp
L
hb-cp
M
run-cp
N
T2M1-cp
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eve-cp
Frequency

3.12 Expression of *Drosophila* constructs in *Tribolium*

The result that at least some of the enhancers driving expression of individual pair-rule stripes in *Tribolium* show sings of conservation between and *Tribolium* and *Drosophila* lead us to analyse if this conservation is also evident in the complementary experiment. We therefore transformed *Tribolium* using a reporter gene construct harbouring the previously identified elements driving expression of *Drosophila h* stripes #3-#4 and #5 respectively (Hartmann *et al*., 1994; Langeland *et al*., 1994). To test whether these constructs are indeed functional, we first transformed *Drosophila* and analysed the expression pattern in several lines generated.

Expression of Dm3-4 and Dm5 in Drosophila

In figure 18 (A-C) embryos carrying the Dm3-4 constructs indeed show expression of two central stripes although the stripes resolve somewhat later compared to the wild type, an effect possibly caused by the lack of the endogenous UTRs, which may result in retarded transcript degradation. The same effect is seen in figure 18 (D-F) for the lines carrying the Dm5 construct, which is expressed in a broad stripe in the posterior region of the embryo that refines only after cellularization is completed at the presumptive position of the endogenous h stripe #5.

Dm3-4 is expressed in Tribolium

Expression of the Dm3-4 constructs starts in the antennal segment at early germband stage and as a faint ‘salt and pepper’ staining in the head lobes (Figure 19 A-G). In addition, a domain of stronger expression arises in the posterior region of the embryo with its anterior border lying around the first thoracic segment, although patches of expression are also detected in the labial and maxillary segment. During germband elongation, expression starts to fade at the anterior limits of the posterior domain, which still extends posteriorly and covers the growth zone. Intriguingly, two stripes of stronger expression can be detected in this domain, the first of which corresponds to the segment T2 where the endogenous *Tc’h* stripe #3 is expressed. The position of the posterior stripe is difficult to assess, since expression fades quickly and becomes restricted to the growth zone at the time of the appearance of the *Tc’gsb* stripe corresponding to the segment A2, in which *Tc’h* stripe 4 is actually expressed. Taking into account that these stripe-like regions are slightly broader than a wild type *Tc’h* stripe, it is possible that the posterior stripe of expression covers the region of the second abdominal segment and thus *Tc’h* stripe #4.

Dm5 is expressed in Tribolium

Analysis of different *Tribolium* lines, carrying the Dm5 construct, showed only a faint expression of in the growth zone around the time point of the establishment of the *Tc'gsb* stripe corresponding to the segment T3, which fades away shortly after its appearance (Figure 19 H-L). However, the time point of appearance of this domain in the posterior correlates well with the onset of expression in the growth zone during the establishment of the endogenous stripe #5. Due to the lack of proper expression domains we excluded these lines from the following pRNAi experiments.

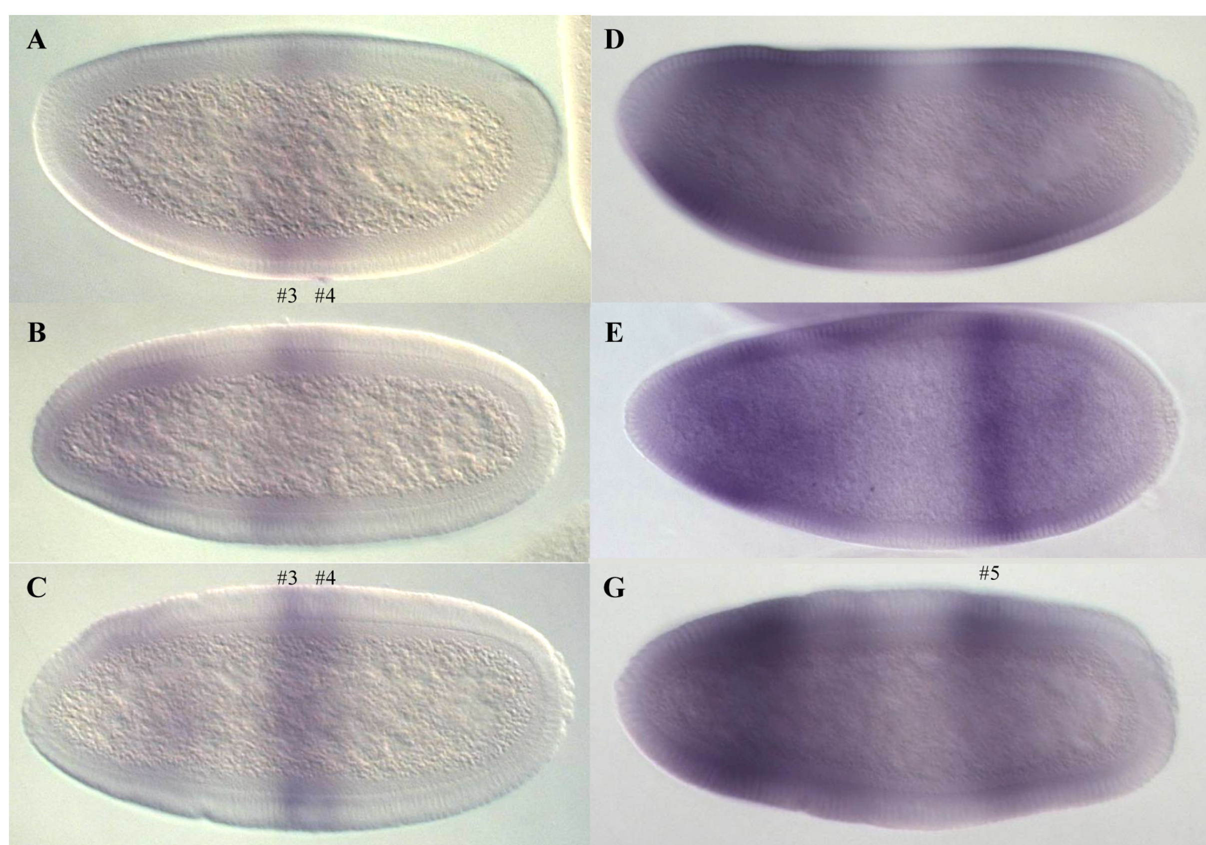

Figure 18

Expression of the DM3-4 (A-C) and Dm5 (D-F) constructs in *Drosophila melanogaster.* Embryos are positioned with anterior to the left and the dorsal side up.

Expression of the Dm3-4 construct, which harbours the regulatory element for the expression of the endogenous Dm'h stripes #3 and #4, starts in an anterior cap and a broad domain in the center of the blastoderm (A) that splits into two stripes residing at the presumptive position of the endogenous Dm'h stripes #3 and #4 during further development (B-C).

The Dm5 construct is also expressed in an anterior cap and a broad posterior domain is seen (D) which refines to a stripe at the presumptive position of the Dm' stripe #5 during further development (E-F).

Note that the anterior domain is also detected for the BN3.1 construct in the previous experiment.

3.13 Regulation of *Drosophila* constructs in *Tribolium*

To test whether gap genes regulate the observed expression in the Tc-Dm3-4 lines, I performed pRNAi experiments with *Tc'Kr* and *Tc'hb* with one of the lines, which showed a typical pattern.

Regulation by Tc'hb

Expression of the Dm3-4 construct in *Tc'hb* knockdowns appears essentially normal in early germbands. With ongoing development the central domain retracts to the segment corresponding to T1 earlier than in wild-type and fails to display the stripes of stronger expression seen in the wild type transgenic lines (Figure 19 M-S), reminiscent of the fusion of the endogenous Tc'h stripes #3-#4 in these knockdowns. It should be noted that a similar effect of *Dm'hb* mutation on the expression of the *Tc'h* construct can be seen in *Drosophila* in that the stripes do not resolve (described above), which also implies similarities with the *Tc'h* expression. However, this is not necessarily a sign of homology because the effect observed in this experiment could also be caused by the fusion of the segments T2 and T3 as well as A2 and A3 in these knockdowns as mentioned above. Furthermore, it is difficult to assess the exact margins of the central domain at a given developmental time point, due to the different morphology of the pRNAi embryos, which also display a slightly retarded development.

Regulation by Tc'Kr

Tc'Kr knockdown embryos display expression of the construct in the entire anterior region with darker staining in the antennal segment and the posterior domain, starting at the position of the presumptive maxillary segment, which then retracts to the labial segment somewhat later. In contrast to the expression in wild type embryos, the expression within the labial segment does not cease and the entire central domain, now ranging from the labial up to the presumptive third thoracic segment, resolves into four stripes. This effect is in stark contrast to the results obtained in the *Dm'Kr* mutant experiments, where the central stripes driven by the *Tc'h* construct as well as the endogenous *Dm'h* stripes #2-#4 are actually fused and with the effects observed on the endogenous stripes in *Tribolium,* which are not affected by *Kr* RNAi. The finding that *Tc'Kr* depletion does not affect the formation of the endogenous *Tc'h* stripes must not imply that it doesn't affect expression of the Dm3-4 construct, since Kr evidently regulates these stripes in *Drosophila*.

The effects observed in these experiments are less pronounced compared to the effects observed for the *Tc'h* construct in *Drosophila* mutants and do actually not show signs a of a conservation of regulation of the central stripes. The similarities observed with respect to the expression pattern in both species appear therefore even more enigmatic since the regulation must be apparently different. However, the fact that the construct drives the expression at the right time and in the right region of the embryo, despite the differences in regulation may hint to further regulatory inputs for the generation of the pattern observed in both species.

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#3
R
S
T
U
V
W
#1
#2
#3
X
Y
Z

Figure 19

Expression of the Dm3-4-lacZ (A-G) and Dm5-lacZ (H-L) construct in *Tribolium* wild-type embryos and expression of Dm'3-4 in *Tc'hb* (M-S) and *Tc'Kr* pRNAi (T-Z) embryos. Embryos stained for lacZ (blue/black) and Tc'gsb (red). The numbers indicate the presumptive position of the endogenous *Tc'h* stripes.

Expression of the Dm3-4 construct in wild-type embryos (A-G)

Expression of the construct is detected at early germband stage in a 'salt and pepper' pattern in the headlobes, with stronger staining in the antennal segment, and as broad domain in the region posterior to the first thoracic segment (A), with patches of expression in the maxillary and labial segments. Expression retracts to the first thoracic segment with ongoing development (B-C) although faint expression is still detected in the labial segment. Intruigingly, two stripes of stronger expression appear in this domain, the first of which resides in the segment T2 where *Tc'h* stripe #3 is expressed in the wild-type (D white arrows). At later stages expression is restricted to the growth zone (E-G).

Expression of the Dm 5 construct in wild-type embryos (H-L)

The construct Dm5 is only expressed transiently during the time point of the establishment of the *Tc'gsb* stripe corresponding to the third thoracic segment (J, black arrow)

Expression of the Dm3-4 construct in Tc'hb pRNAi embryos (M-S)

Expression of the Dm3-4 construct in *Tc'hb* depleted embryos starts essentially normal (M-N). However, with ongoing development expression of the broad domain retracts rapidly to the segment T2 (O-P), although faint expression is also detected in the segment T1 in a few embryos (Q). In contrast to the expression in wild-type embryos the domain does not show signs of stripe like expression (compare D and Q). Expression in the growth zone persists until the breakdown of segmentation (R-S). Note that four *Tc'gsb* stripes are formed before the first segmental fusion (black arrow).

Expression of the Dm3-4 construct in Tc'Kr pRNAi embryos (T-Z)

In *Tc'Kr* depleted embryos early expression of the Dm3-4 construct seems unaffected in the anterior region (T). With ongoing development expression in the central domain resolves transiently into four stripes (U), the first of which resides in the labial segment (white arrow, black arrows mark the presumptive position of the endogenous *Tc'h* stripe #2 and #3 in U and #3, #4 in V). Expression is restricted to the growth zone until the breakdown of segmentation (W-Z).

4. Discussion

4.1 *Tc`hairy* appears to function like a pair-rule gene at blastoderm stage

Dm'h is classified as pair-rule gene based on its amorphic mutant phenotype in which the posterior borders of every second segment are lost. The phenotype of *Tc'h* observed in these experiments differs substantially from the findings in *Drosophila*. *Tc'h* only affects segmentation in more anterior, namely blastoderm specified segments in a possibly pair-rule like fashion. This effect appears to be masked by a secondary function that leads to induction of apoptosis and to the loss of the anterior segments in strong phenotypes. It is therefore difficult to asses whether the observed phenotype is truly due to a failure of establishment of the mandibular and labial segment, which seem to be the most susceptible ones to *Tc'h* transcript depletion, or if *Tc'h* has only a maintenance function as some of the germbands do exhibit expression in the respective segments without inducing growth of gnathal appendages (Figure 5 M) although this effect could also result from incomplete depletion of *Tc'h* transcripts. Nevertheless the observed phenotype could be caused by a developmental arrest of the anterior segments. However, several independent lines of evidence do suggest a pair-rule function of *Tc'h* at blastoderm stage. First, the most prominent phenotype in the pRNAi experiment displays the specific loss of the mandibular and labial segment and staining of pRNAi embryos with the segmental marker *Tc'gsb* shows specific loss of the stripes corresponding to the mandibular and labial segments at an earlier stage and at higher frequencies, which is in agreement with the cuticular phenotypes scored in this experiment. Moreover, the first and second *Tc'prd* stripe lie directly adjacent to each other, further indicating a real loss of the labial segment, which becomes evident at later stages. And finally we find a specific loss of *Tc'h* stripe #2 and the corresponding segment in *Tc'gt* knockdowns. According to Bucher *et al.*, analysis of *Tc'gt* pRNAi embryos using the segmental marker *Tc'en* show normal formation of the three *Tc'en* stripes corresponding to the gnathal segments mandible, maxilla and labium, followed by a disturbance of the formation of the stripe in T1, which was interpreted as fusion of the segments T1 and T2 (Bucher *et al.*, 2004). However, expression of *Tc'gt* in the posterior region of the embryo is detected in the segments corresponding to T3 and A2. Thus the effects, manifested in the observed fusion of the corresponding *Tc'en* stripes, lay one segment anterior to the *Tc'gt* domain in T3. As mentioned before, knockdown of *Tc'hb* leads to a loss of both posterior *Tc'gt* domains, but not the anterior one. Analysis with the segmental marker *Tc'gsb* displays normal formation of

four segmental stripes prior to the first detectable segmental fusion (compare figure 20 G-I and J-L, supplemental material), thus exhibiting one additional segment. It should be noted that these segmental fusions observed in *Tc'gt* knockdowns are both also seen in *Tc'hb* knockdowns, but are shifted posteriorly by one segment. It seems therefore safe to conclude that the segmental fusions observed in *Tc'gt* knockdowns are actually comprised of the segments T2 and T3, which would be intuitively expected. The missing labial segment would thus cause the positional shift of these fusions as observed in the comparison with the *Tc'hb* knockdowns. Another implication of this finding is that the missing anterior *Tc'gt* domain likely causes the loss of *Tc'h* stripe #2, although other factors could contribute. A possible involvement of the Notch signaling pathway as cause for the specific deletion of these segments appears highly unlikely, since *Tc'delta* is only expressed in the mandibular segment after the segment has formed, whereas the lack of the *Tc'gsb* stripe as well as the fusion of the first and second *Tc'prd* stripe in *Tc'h* knockdowns is already evident at blastoderm stage. Furthermore, the specific loss of the labial or both the mandibular and labial segments was never observed in *Tc'delta* knockouts. The fact that the loss of the labial segment does not lead to a complete loss of the anterior segments, as seen in *Tc`h* knockdowns, allows the assumption that this effect is indeed associated with the expression in the mandibular segment where *Tc'h* appears to be coexpressed with *Tc'delta*. This is further supported by the unusually strong expression of *Tc'h* in this segment, which in addition persists for a longer time than in any other *Tc'h* stripe.

Besides the main effect on segment formation and maintanance, knockdown embryos display a deep groove at the position of the ventral midline where *Tc'h* is expressed, a feature not observed with *Drosophila hairy*. The lack of apoptosis induction in this region, as seen in the wild type is likely caused by a lack of the respective cells in this region since the midline appears deeper than in wild type. Interestingly *hairy* “orthologs” are known to play a crucial role during the formation of midline structures in vertebrate development, where they participate in cell fate decisions during the establishment of these structures by the so called Spemann-Mangold organizer and its respective counterpart, the dorsal shield (Latimer *et al*., 2005; Murato *et al*., 2006). However, this coincidence may not be a true homology, since members of the *hairy* family have been independently recruited to several processes of cell fate decisions (Latimer *et al*., 2005, Winkler *et al*., 2003), but may indicate the presence of a so far undetected organizer in this region. Another indication for the possible presence of an anterior developmental “organizer” in this species comes from the secondary function

observed for *Tc'h* during development. The developmental arrest and the later loss of anterior segments due to apoptosis induction, which is likely related to the possible interaction between *Tc'h* and *Tc'delta,* may hint to the presence of such an organizer given the early stages at which these effects become evident. The fact that no comparable effect is known from *Drosophila* development might be correlated with the highly specialized head development in this species.

4.2 The evolutionary conservation of regulation

The regulation of the secondary pair-rule gene *Dm'ftz* and others has been suggested to be a main cause of the segmental defects observed in *Dm'h* mutants (Ish-Horowicz and Pinchin, 1987) implying a more indirect function of *Dm'h* in segmentation. The results obtained in these experiments show that this regulatory interaction is evolutionary conserved between *Tribolium* and *Drosophila*, although the function is not. One implication of this finding is that *Tc'ftz* has been recruited to the process of segment formation in the line leading towards *Drosophila* and that this regulatory interaction might have been already present in the common ancestor. The regulation of *ftz* by *hairy* could thus have facilitated the evolutionary recruitment of this gene into the segmentation cascade of *Drosophila* due to its resulting pair-rule like expression. The fact that *ftz* is expressed in a A-P modulated pattern similar to its Hox complex neighbors in primitive mites, millipedes and onychophorans (Telford, 2000), but not crustaceans (Mouchel-Vielh, 2002, Janßen and Damen; 2006) may indicate that the expression and regulatory interaction observed in *Tribolium* evolved the insect clade, which is in line with the finding that the ftz homolog of the primitive insect *Thermobia* is expressed in a segmental pattern (Hughes *et al*., 2004), and thus facilitated the recruitment of *ftz* to the segmentation process in the line towards *Drosophila* . However, segmental expression of *ftz* homologs is also found in more basal arthropods like the centipede *Lithobius* (Hughes and Kaufman, 2002b) and a segmental expression pattern appears during the growth of the millipede *Glomeris marginata* embryo (Janssen and Damen, 2006). An explanation for the variation found in the expression pattern of *ftz* within and between different arthropod clades, based on the given interpretation, may be that a functional interaction between *ftz* and *h* is evolutionary conserved and facilitated the evolution of the segmental *ftz* expression several times independently. Functional analysis of this interaction in other insect species would help to pinpoint the evolutionary branching point at which this interaction was established and recruited to the segmentation process.

An obvious reason for the recruitment of additional factors to the process of segmentation could be the need of a denser positional map for the segmentation of the entire embryo at the syncitial blastoderm stage vs. the segmentation in a progressive fashion like seen in *Tribolium* and most other insect species. Such an additional recruitment of factors to the segmentation process in the line towards Drosophila seems not to be restricted to these two factors, since other *Tribolium* pair-rule orthologs have been shown not to function as such during segmentation (Choe *et al*., 2006). However, not all factors involved in the segmentation process of *Tribolium* and likely also *Drosophila*, are known. In a recent publication we presented a novel gap gene termed *Tc'mlpt* (millespattes), which is involved in the segmentation process of the *Tribolium* embryo (Savard *et al*., 2006) and which is also present in *Drosophila*. Yet its function during the segmentation of the *Drosophila* embryo, if it has any, has still to be proven.

4.3 The regulation of *Tc'hairy*

The regulation of *Dm'h* strongly reflects the derived mode of segmentation in a syncitial environment. The presence of stripe specific cis-regulatory elements that drive the expression of single stripes can be seen as a hallmark, and maybe even a prerequisite of long germ development and is found for several *Drosophila* pair-rule genes (*e.g. eve*, *prd*, *run*). The finding in our previous analysis that such enhancers do also exist in the short germ developing beetle *Tribolium* (Eckert *et al*., 2004) is a strong indication for at least a partial conservation of the pattern forming mechanism, and inevitably raises the question whether the underlying genetic networks regulating the homologous expression of the so called pair-rule pattern are also conserved between these species, despite the striking differences in the mode of segment formation. Comparison of the effects of gap gene mutations in *Drosophila* vs. knockdown by pRNAi in *Tribolium* show striking differences in the way they affect *hairy* expression in these species, but also similarities. Whereas, the *Kr* mutation has a strong influence on the formation of the *h* pattern in *Drosophila*, no comparable changes in expression could be found for the expression of the *Tc'h* stripes formed before the breakdown of segmentation in *Tc'Kr* knockdowns. Furthermore, a similar effect of *Tc'Kr* was also seen for the expression of the pair-rule genes *eve* and *runt* (Cerny *et al.*, 2005, Souza Aranda, unpublished) suggesting no major involvement of *Tc'Kr* in the establishment of the pair-rule pattern. It is difficult though to assess the true identity of the stripes formed, since segmentation breaks down prematurely, but analysis of the phenotype using different markers currently argues against a

loss of intermediate segments and instead suggests a deletion of segments posterior to the *Tc'Kr* domain, thus defining *Tc'Kr* as non-canonical gap gene (Cerny *et al.,* 2005). Interestingly *Kr* homologs were also studied in the more basal species *Gryllus bimaculatus* and *Oncopletus fasciatus*, were they have been found to act as canonical gap genes and in the case of *Gryllus* even to affect the pair-rule pattern of *eve* (Mito *et al*., 2006). It is therefore difficult to assess whether the function of *Kr* in the segmentation is ancestral or if this factor has been recruited to this process several times independently.

A similar situation is seen for *Tc'hb*, although the effects on all stripes posterior to the second *Tc'h* stripe are evidently strong. The early breakdown of segmentation after the third thoracic segment in these embryos does not allow analysis of the influence on *Tc'h* expression in the posterior regions.

The effect of *Tc'gt* depletion shows the highest similarity with respect to effects observed in the corresponding *Drosophila* mutant. The lack of *Tc'h* stripe #2 at blastoderm stage resembles a classical phenotype of gap gene mutation although this effect differs from the observations in *Drosophila* were stripe #2 is not affected by the *gt* mutation. The latter fusion of the presumptive stripes #3-#5 though are highly reminiscent as there are also fusions of the presumptive *Tc'h* stripes #3-#5 in *Tc'gt* knockdowns, despite the different position of the posterior *Tc'gt* domains. In both *Drosophila* and *Tribolium*, *gt* seems to have a repressive function on *hairy,* although the mode of action in *Tribolium*, being either direct or indirect via the failure in the establishment of these segments, can currently not be assessed. Furthermore the effects are seen both in blastoderm-derived segments (stripe #2) as well as in post-blastodermal stripes (stripes #3-#5), thus *Tc'gt* affects stripes that are specified during and after the syncitial stage in contrast to *Dm'gt*. A possible implication of this result is that even though the mode of segment formation has undergone dramatic changes on the morphological level during the evolution of long germ development, the changes on the genetic level with respect to the regulation of pattern formation may have been less dramatic such that the "read out" is still similar. Therefore the regulatory position of *Tc'gt* in the process of segmentation in regulating the expression of *Tc'h* at both the blastoderm and post-blastodermal stages could be interpreted as an "intermediate state" in the transition from short to long germband development in which a gap gene regulates the expression of the blastodermal stripes in a similar fashion as known from *Drosophila* and in the same time serves as a "landmark" during the generation of post-blastodermal stripes. The patterning function during the generation of these post-blastodermal stripes implies the presence of a regulatory element,

which responds to the signal provided by the gap gene. With ongoing evolution this regulatory element and thus the secondary function of this gap gene as a “landmark” could evolve to a stripe specific enhancer and allow the gap gene to pattern these segments in a syncitial environment.

Taken together the results obtained in this study indicate that, with respect to the regulation in *Tribolium*, the gap genes gained regulatory influence on the expression of *hairy* in the line towards *Drosophila* compared to *Tribolium*. The recent study on pair-rule gene regulation in *Tribolium* show that a variety of the pair-rule genes of *Drosophila* like *ftz*, *tna*, *odd* and *paired* do not acts as pair-rule genes during *Tribolium* development (Choe *et al*., 2006). Based on the main differences in the mode of development it is conceivable that more factors were needed to provide a sufficiently dense and precise positional map to pattern the entire embryo in a syncitial environment as mentioned above. The recruitment of factors and mechanisms to developmental processes based on their expression pattern has already been suggested before, and examples for convergent recruitment of pathways and mechanisms to such processes have been found in other model organisms (Harrison *et al*., 2005). This assumption is further nourished by the finding that several factors of different hierarchical levels, based on the knowledge of *Drosophila* development, seem to be interchangeable per se during the evolution of the regulatory network gouverning insect segmentation. In *Oncopeltus fasciatus* for example, the pair-rule gene *eve* does not act as a pair-rule gene during segmentation, but inherits a function in regulating the expression of the gap genes *Kr* and *hb* besides an “unusual” segmental expression (Liu and Kaufman, 2005), whereas analysis of the expression of these genes in *Tc’eve* knockdowns does not show any influence on the appearance of the respective gap gene domains in *Tribolium* (Choe *et al.*, 2006, Souza and Aranda, unpublished). Thus the pair-rule gene *eve* inherits different developmental functions in different insect lineages. A similar diversity in function is seen for the gap gene *Kr*, which acts as canonical gap gene in species like *Gryllus*, *Oncopeltus* and *Drosophila* (Mito *et al.*, 2006; Liu and Kaufman, 2004), but not *Tribolium* (Cerny *et al*., 2005), as mentioned before. One of the most clear examples for the de novo recruitment of factors to specific developmental processes is the anterior maternal coordinate gene *bcd*. It is assumed that *bcd* is a divergent duplication of the *Hox3* gene *zerknüllt* (Stauber *et al*., 1999; Stauber *et al*., 2002) and became the main anterior “organizer” in higher dipteran (*Cyclorrhapha*) development, thus switching its role from specifying non embryonic tissue to being a main regulator of segmentation.

4.4 Evolutionary Conservation of Regulation

Despite the different mode of segment formation, which occurs in a progressive fashion from a growth zone in *Tribolium* compared to the blastodermal patterning observed in *Drosophila* we find strong indications for at least partial conservation of the underlying regulatory network. Both in *Drosophila* and *Tribolium* the BN3.1 construct is expressed in a three-stripe pattern in the center of the embryo covering stripes #3-#5, thus in a homologous region. Furthermore this expression seems to be regulated by the same genes, although it is still unclear how the gap genes, which work in a syncitial environment, can act under cellularized conditions in the *Tribolium* embryo. Therefore it remains to be shown if these gap genes interact directly with the *hairy* enhancers in *Tribolium* or a if still unknown cell signaling pathway is required to mediate their function. The fact that the expression of the *Tribolium* constructs occurs at the same stage as the endogenous *hairy* expression, which is evidently regulated by these genes, makes it unlikely that another tier of regulation is involved in transmitting the gap gene signal. Additionally, no cell signaling pathways are known to act so early during *Drosophila* development.

The evolutionary conservation of the enhancers driving the expression of the central stripes is further supported by the expression of the Dm3-4 construct in *Tribolium*. Although its regulation by *Tribolium* gap genes could not be fully elucidated, the mere fact that it is expressed in the right region and that it resolves into two stripes coinciding with the endogenous stripe #3 and likely also stripe #4 is a separate line of evidence for a conservation of the underlying regulatory network involved in the observed expression.

Analysis of the region driving the observed expression indeed contains predicted binding sites for at least some of the factors tested in these experiments. Although prediction of binding sites does not imply their functionality, we could show for *Dm'kni* that binding to these sites in vitro is indeed possible. The experiments performed by Paolo Struffi on selected sequences prooved that *Dm'kni* is indeed able to bind predicted binding sites in the *Tc'h* regulatory region in vitro, but the regions tested lie further upstream than the ones used in this construct (Paolo Struffi, personal communication), indicating hat the posterior expansion of the central domain driven by the *Tribolium* construct may indeed be indirect. Furthermore the *Tc'kni* ortholog identified so far does not seem to be involved in segmentation at all (Alex Cerny, personal communication).

One astonishing aspect of these results is that we find partial conservation of regulation for the central stripes #3-#5. Comparison of the mode of development obviously shows the

highest similarity for the formation of anterior segments, which are generated in a syncitial environment in both species, whereas at least stripe #4-#5 are generated in a cellular context in *Tribolium* vs. syncitial conditions in *Drosophila*. However, the exact mode of regulation of the central stripes #3-#5 is not fully understood in *Drosophila* (Hartmann *et al*., 1994; Langeland *et al*., 1994). The positioning of stripes #3-#4 in particular does not only depend on the input by gap genes, but also requires cross regulation through the pair-rule gene *runt* (Hartmann *et al*., 1994), whereas the *Tc'h* stripes seem to depend on *Tc'gt* for proper expression. Therefore additional studies on these stripe enhancers will be required to clarify if and to which extend cell-cell signaling is involved in their regulation.

The fact that we do not find a completely identical regulation of these patterns, despite the high similarities in the trans-species experiments in the *Drosophila* mutant experiments, hints to a conservation of the phenotypic character despite a diverged regulation and implies that major regulatory changes can occur without changing the phenotypic character.

Despite the conservation found in the expression of the central stripes we do not find conservation of the stripes #1 and #2 elements as expected from the similarities of the embryonic enviroment in which these stripes are generated. However, this finding correlates well with an evolutionary innovation in the line towards *Drosophila*, namely the appearance of *bcd* as provider for anterior positional information. Evolutionary, *bcd* inherited this function during the evolution of the higher dipterans (*Cyclorrhapha)* (Stauber *et al*., 2002) and hence also the regulation of the anterior stripes #1 and #2 (Riddihough and Ish-Horowicz, 1991). It remains to be analyzed if this evolutionary change is also evident in the regulation of these stripes in *Tribolium*, *i.e.* if *Tc'otd*, which is believed to fulfill a similar function in concert with *hb* in *Tribolium* (Schröder, 2003) and in the wasp *Nasonia* (Lynch *et al*., 2006), also acts in the regulation of *Tc'h* stripe #1 and #2 since the binding sites of *BCD* and *OTD* are highly similar (Lynch and Desplan, 2003). Similar to the evolutionary changes of the anterior patterning system we find strong divergence of the terminal patterning system between these species. Besides the involvement of *Dm'bcd* in the regulation of *Dm'h* stripe #7 (La Rosee *et al*., 1997) the expression of posterior acting gap genes differ substantially. *Tc'gt* for example is expressed in more anterior segments compared to its *Drosophila* orthologue and *Tc'tll* is not expressed at all during the formation of the posterior segments (Schröder *et al*., 2000).

Interestingly analysis of enhancer elements between *Drosophila* species show indeed that major changes in the composition and organization, including gain and loss as well as

changes in spacing, of transcription factor binding sites within these elements can occur in relatively short evolutionary timescales without changing the overall output, thus implying an evolutionary constraint on the phenotypic character rather than the underlying regulation (Ludwig *et al*., 2000; Ludwig *et al*., 2005). As a consequence enhancer elements have a high architectural flexibility allowing the loss or appearance and even rearrangements of transcription factor binding sites, which is compensated by evolutionary changes in other parts of these elements, *e.g.* the evolution of new binding sites. It is therefore conceivable that within even larger evolutionary time scales binding sites for new factors, previously not involved in the regulation of a given gene, may evolve and take over its regulation. Comparison of such an evolved regulation between distantly related species might thus show no obvious regulatory conservation despite a maintained evolutionary continuum, and would serve as explanation for the flexibility in the usage of factors like *Kr* or *eve* and other factors in developmental processes observed in the functional comparison of related insect species, as seen in *Anopheles*, where homologous pair-rule stripes are regulated by different combinations of gap repressors (Goltsev *et al*., 2004).

4.5 General conclusions

The results obtained in this study indicate a possible evolutionary intermediate state of *Tc'h* in the evolution of long germ development compared to *Drosophila*. Whereas *Dm'h* is involved in the segmentation of the entire *Drosophila* embryo we only find conservation of this function during the blastoderm stage of *Tribolium*. Most interestingly this functional conservation seems not to be correlated with the regulatory conservation observed in the trans-species experiments, as we do not find conservation of the regulation of the stripes generated during blastoderm stage, but for stripes generated in the subsequent segmentation process likely for the reasons mentioned above. Furthermore, we find conservation in the regulation of the target gene *ftz*, which is not directly involved in the segmentation of the *Tribolium* embryo, but it is in *Drosphila*. Based on theses results one might formulate a possible evolutionary scenario in which the function of *hairy* in segmenting blastoderm-derived segments was expanded in the line towards *Drosophila* to pattern the entire embryo. Such a functional expansion may also be reflected in the partially conserved regulation and even more in the conserved phenotypic character observed in the trans-species experiment, since expression at the right place and time is a prerequisite to inherit a function in a given process. One of the reasons for such an expansion could be the need of additional factors to

provide a sufficiently dense map for the segmentation of the entire embryo at blastoderm stage as seen in *Drosophila*. Functional analyses of several *Tribolium* pair-rule orthologs show no involvement in the segmentation, even if they are expressed in a pair-rule like fashion like *Tc'ftz*. Thus the function and evolutionary conservation found for *Tc'h* could be interpreted as an "intermediate state" in the recruitment of such a factor to the segmentation process in the evolution of long germ development. Given the evolutionary distance of more than 260 myr, one might speculate that the observed conservations evident in the expression patterns in the trans-species experiments were already present in the common ancestor of *Tribolium* and *Drosophila,* although their regulation is evidently not conserved in all aspects. The expression in a pair-rule like fashion thus might have facilitated the recruitment of *h* and factors like *ftz* into the segmentation process of the entire embryo in the line towards *Drosophila* and maybe also other long germ developing insects. However, there is no direct evidence for a selective force driving such a transition in *Tribolium castaneum*, but nebertheless it could still reflect some of the mechanisms which allow and facilitate such a transition.

Interestingly, the computational simulation of the evolution of expression patterns performed by Ciudad-Salazar and colleagues makes several intriguing predictions for the evolution of pattern forming networks and the evolutionary transition from short to long germ development, although the complexity of such a simulation does not reflect the intricacy found in the networks governing pattern formation in insect embryos (Ciudad-Salazar *et al*., 2001a; Ciudad-Salazar *et al*, 2001b). One of the predictions of this model is the need of more factors for a hierarchical system to generate a pattern with more than four stripes compared to a so called "emergent" system in which a self organizing system generates a periodic pattern, as known from vertebrate segmentation. Analysis of the segmentation cascade in *Tribolium* currently suggests the initiation and regulation of pair-rule gene expression by a circuit comprised of three pair-rule genes, namely *Tc'eve*, *Tc'run* and *Tc'odd*, which are connected in a regulatory circuit building the "core" mechanism of the pair-rule patterning system, in contrast to the regulation by gap genes as found in *Drosophila* (Pankratz and Jackle, 1990). Moreover the authors conclude that a smaller number of factors may comprise the core pair-rule mechanism compared to the hierarchical system known from *Drosophila*, although it is likely that not all factors involved in the segmentation process of *Tribolium* have been found. Furthermore, the sequential function of this circuit provides evidence for the presence of a regulation by some kind of periodic mechanism (Choe *et al*., 2006). Such a circuit appears

apparently more closely related to an ‘emergent’ system as seen in vertebrates, and raises the question how such a system can evolve to the hierarchical segmentation cascade found in *Drosophila*. Intriguingly, the computational simulation presented by Salazar-Ciudad *et al*., shows the tendency for a replacement of emergent systems with hierarchical ones during further evolution. Moreover they find that intermediate systems are generated, which are by themselves adaptive, a finding that also serves as explanation for the fact that different germ types are distributed throughout the insect orders and that germ types do not sort into individual monophyletic groups (Patel *et al*., 1994). Furthermore the authors found that many of the evolved networks exhibiting temporally oscillatory patterns can produce stripe patterns when they are allowed to function in a syncitium.

A main conclusion from the interpretation of the results mentioned above would be that segmentation is ancestrally driven by an emergent system comparable to the vertebrate segmentation clock or the pair-rule circuit found in *Tribolium*, and that the ‘state’ present in *Tribolium* may represent such an adaptive intermediate system in which the gap genes started to take over the regulatory network governing pair-rule gene expression, and that long germ development evolved several times independently. With respect to the underlying mechanism generating these stripes it might also explain the presence of stripe specific enhancer elements in a system not based on morphogenetic gradients as previously explained for the case of *Tc’gt* (see 4.3).

Although *Tc’h* seems not to participate in the core mechanism regulating the pair-rule pattern in *Tribolium* presented by Choe and colleagues (Choe *et al*. 2006), it could still serve as model for how a gene can get under the control of gap genes in an emergent system and be integrated into a hierarchical system. Furthermore it is evident that gap genes do participate in the segmentation process of *Tribolium* as we see fusion of stripes in *Tc’gt* knockdowns for several pair-rule genes including *Tc’eve* and *Tc’run*, and the breakdown of the pair-rule circuit, evident in the premature termination of segmentation as observed in the respective knockdowns. Nevertheless it cannot be excluded that the pair-rule function of *Tc’h* in patterning post-blastodermal segments was lost in the line towards *Tribolium*.

As mentioned earlier, homologs of the vertebrate segmentation clock, such as the Notch-Delta cell signaling system are indeed required for the proper segmentation of the spider *Cupiennius salei* (Stollenwerk *et al.*, 2003), and members of this cell-cell signaling system may be involved in the segmentation of the cockroach *Periplaneta americana* (Juan Pablo Couso, personal comunication). However, functional analysis of members of this signalling pathway,

like *Tc'delta* and *Tc'Su(H),* did not provide convincing evidence for an involvement in the segmentation process of the *Tribolium* embryo.

Intriguingly, we found that the polycistronic transcript coding for the novel gap gene *Tc'mlpt* (Savard *et al.,* 2006) mentioned above, likely encodes for four small peptides, the last of which contains a stretch of four arginine residues. Since arginine-rich peptides have also been found in investigations of cell-penetrating peptides (Melikov and Chernomordik, 2005), the involvement of morphogenetic gradients in the regulation of the segmentation process in the cellularized enviroment of the nascent germband can currently not be excluded. However, further analyses are needed to elucidate the function of these peptides on the molecular level.

Analysis of the molecular mechanism of segmentation in other insects, and in particular long germ developing beetles like *Callosobruchus maculates*, but also more basal hemimetabolous insects, may shed light onto the mechanism regulating the segmentation process in these species and allow to test the hypothesis presented in this study and finally help to understand the basic mechanisms that allow evolution to shape this tremendous diversity of body plans using the same molecular ‘tool box’.

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Supplemental Material

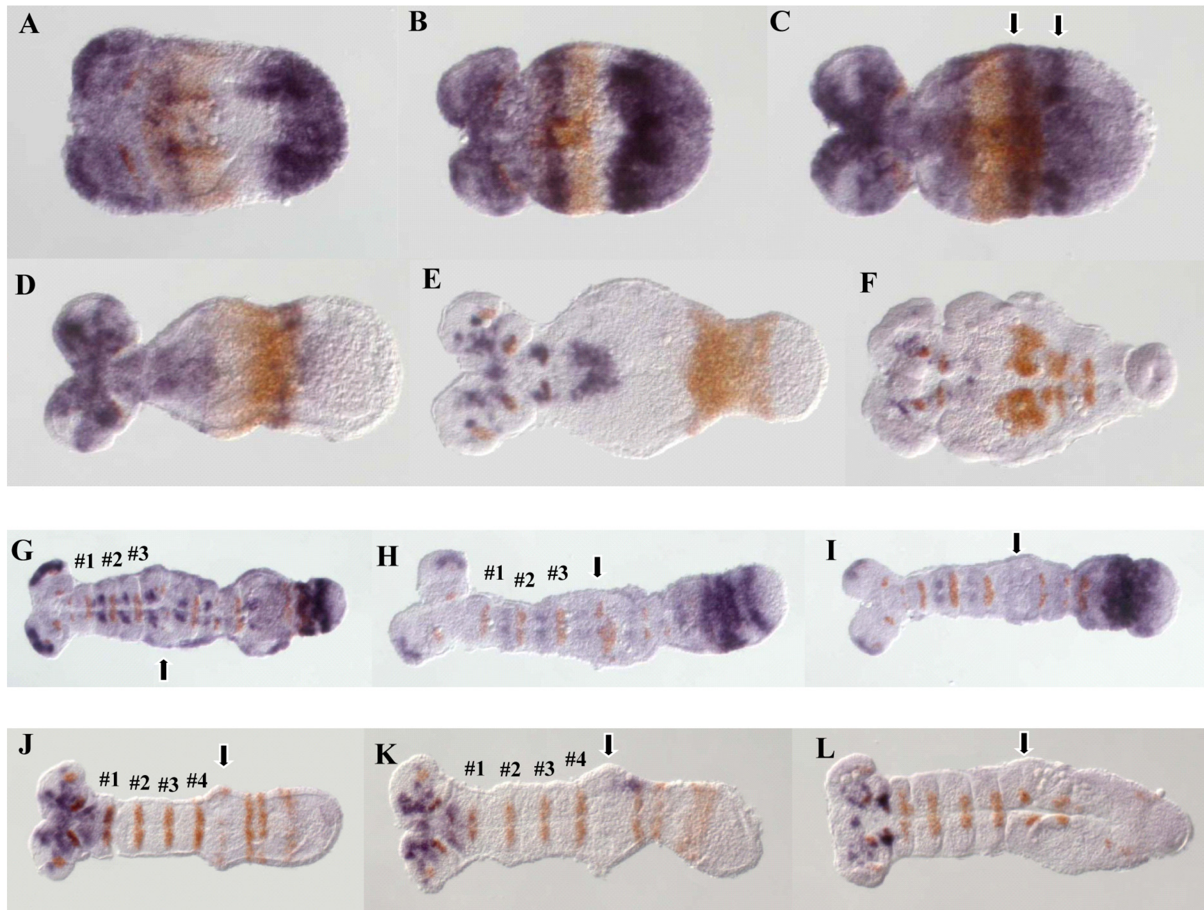

Figure 20

Expression of *Tc'gt* (blue) and *Tc'gsb* (red) in *Tc'eve* knockdown embryos (A-H); expression of *Tc'run* (blue) and *Tc'gsb* in *Tc'gt* knockdowns (G-I); expression of *Tc'gt* (blue) and *Tc'gsb* (red) in *Tc'hb* knockdowns (J-L). Numbers indicate the *Tc'gsb* stripes starting from the mandibular segment.

(A-F) *Tc'gt* is expressed in *Tc'eve* knockdown embryos in a similar fashion as in wild type. At early stage *Tc'gt* is expressed in an anterior domain and a posterior cap (A and B), which later splits into two stripes of expression similar as in wild type (C, black arrows). In contrast to this, *Tc'gsb* is not segmentally expressed as in wild type but shows expression in a broad domain around the center of the germband, which appears to move along the embryo during the elongation process.

(G-I) *Tc'gsb* expression in *Tc'gt* knockdown embryos shows the expression of three stripes posterior to the intercalary segment before the first segmental fusion is detected.

(J-L) Tc'gsb (red) and *Tc'gt* (blue) in *Tc'hb* knockdowns. Note that no posterior *Tc'gt* expression can be detected in the segments T3 and A2, instead segmental fusions are seen at these positions (black arrows). In contrast to the result obtained in *Tc'gt* knockdowns, four virtually normal *Tc'gsb* stripes are formed before the first segmental fusion (compare to G-I)

Figure 1 detailed sequence information

Abbreviation	Species name	Gene name	Accsession number
At-h	*Achaearanea tepidariorum*	*hairy*	BAD01491
Cf-h	*Coboldia fuscipes*	*hairy*	gbAAT92565
Cs-h	*Cupiennius salei*	*hairy*	embCAB89491
Dm-dpn	*Drosophila melanogaster*	*deadpan*	Q26263
Dm-h	*Drosophila melanogaster*	*hairy*	gbCAA34018
Dr-h	*Danio rerio*	her1	gbAAU10470
Dr-h2	*Danio rerio*	her2	NM_131090
Dr-h3	*Danio rerio*	her3	NM_131080
Dr-h4	*Danio rerio*	her4	NM_131090
Dr-h5	*Danio rerio*	her5	NP_571152
Dr-h7	*Danio rerio*	her7	AAG24398
Mm-hes1	*Mus musculus*	*hes1*	NP_032261
Mm-hes2	*Mus musculus*	*hes2*	NP_032262
Mm-hes5	*Mus musculus*	*hes5*	spP70120
Mm-HES6	*Mus musculus*	*hes6*	Q9JHE6
Mm-hes7	*Mus musculus*	*hes7*	NP_149030
Pc-h	*Platypeza consobrina*	*hairy1*	gbAAT92575
Tc-h	*Tribolium castaneum*	*hairy*	AJ457831
Tc-h1	*Tribolium castaneum*	*hairy1*	see below
Tc-h2	*Tribolium castaneum*	*hairy2*	see below
Tc-h3	*Tribolium castaneum*	*hairy3*	see below
Tc-h4	*Tribolium castaneum*	*hair4*	see below

Sequence Tc-h1:

atgcactcgatcctcgctaccgcacgcgccaccccccgtcactgccaccgccgcctggcagctcgcactcgcccactcgtcggctc
gtgctgcagcagttcgcctcgtcctctcgccggcatcgccgccgatgcgcctctagtatgaccgccaagagcaagcgagcctccgag
ccacgccgcgccaacaagcccctcatggagaagcgccgtcgggctcgcatcaaccagagcctggccgccctcaagaccctcatcct
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caccgcagtctggacatcaagggagtgcaccagtacaaggccggctacgcggactgcgtgcgcgaggtgcagcgctacctggaca
cgccggacgcccagacgatgaccgtcgtcgacgctggcgtgcgccagcggctgctccggcacctggacaactgcgtcgccgaggt
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acgctctgggggtgaatttgaggcacagtgagggtggagccgattccggggcctcctcgggggagagtagcgatagttcgaaacagt
caagtggggaaaaaccgctcgattttagcaaaagtaacgacaattgtgatatgtggaggccgtgg

Sequence Tc-h2:

atgggtgtcaggctagcggagcaaaggacgggagatcgtcgtcgtccatccacgcacccaatgctttccgtcgaagagccccagcc
gatctcccggacctaccagtaccggaaggtgatgaagccgatgttggagcgcaaacggcgcgcccgcatcaaccgctgcctcgacg
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tctactccatgcctctgacgccggcctcctcccaggcgccgtcgcccaaccagcccatggactgctccaccgctggccttctcaaagtc
gcccacaagaccgacgatgtgtggaggccgtgg

Sequence Tc-h3:

atgcccataagtgaagacgagtacgagattcgaccttcccaagaatcagtcacaatgtcgaaagccgaactacgaaaagtatgtccttt
gatttatcaaaatataataatttcaacacacattttccagacgcacaaacccataatggagaagcgcagaagagccagaattaaccactg
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atccgaagttgacgacggcctcaaagaccggatgcgatcccacttgcaaaaatgcatcaacgggatcgaccatgttgcccatttcaact
tccccaactttccaaatttaccgttcacttcaacttcaaacgtaccttcttcaagtatcggtgaccaaaacaataacgcaagagtgcaaata
ccacaaagcatccaattgataccaagccgacttccaagcggggaaatcgccttgcttttacccaactcgagtaacttaccatttttgcaac
aaagagaacgaccaagtgctttcgtaactgtgataccgtcatcttcatcaacttcggtaagtcctccggcaagccccaaggggttccgtc
cagtgcaaccaacaacctaccacgaacaaccccaagtgccccaagtgtcttccacttcgattcctccagcaatggaagtcaaaagtatg
aaattcccgattcatcaacgcattatcagtccgaaaaaaacgatagagcctttgtgtattataacaaatcagtcggagcgcttcaaacagg

cgcaaactcgcgaagatggcgccgattttgaggagaatcgcactcaaggagtgaagagaaaatatgccgaaatgactcaaggtttgct
cactgtggccgaatatccggctactaaaataattaaaacggaatcagcgacgagtacgacagaggccacagcgtccacttctcggga
gtcgaatcctccaggggatgggaacagtgatatgtggaggccgtgg

Sequence Tc-h4:

atggcgccgaccccgtatatggatgaaccgattagccgcacctatcagtatcgcaaagtgatgaaaccgatgctggaacgcaaacgc
cgcgcgcgcattaaccgctgcctggatgaactgaaagaactgatggtgaccgcgctgcagagcgaaggcgaaaacgtgagcaaact
ggaaaaagcggatattctggaactgaccgtgcgccatctgcatggcctgaaacgccagcatcagctggtgattccgccggaaggctat
gcggatcgctttcgcgcgggctttacccagtgcgcgcaggaagtgagccagtttctgaccaccccgaccgaaaccgtggatgcggtg
gcgggccgcaaactgctgcagcatctgggcgcgtgcgtgcgccagctggaatgcgcgcaggtgtataccccgccggcgagcccgc
aggtgaaagcgagcatgtggcgcccgtgg

Zusammenfassung

Drosophila melanogaster ist der bestuntersuchte Modellorganismus der Langkeim- Insekten. Eines der außergewöhnlichsten Merkmale seiner Entwicklung ist die Spezifikation aller Segmente während des syncitialen Blastodermstadiums mithilfe eines Mechanismus, der auf freie Diffusion von maternalen und zygotischen Transkriptionsfaktoren zwischen den unzellularisierten Zellkernen des jungen Blastoderms basiert. Obwohl die Entwicklung verschiedener Insekten aus verschiedenen Ordnungen bereits untersucht wurde, ist es immer noch unklar, ob die Mechanismen, die man in *Drosophila* entdeckt hat, auch auf die Entwicklung der nichtsyncitial angelegten Segmente von Kurzkeim- Insekten zutreffen und wie dieses System evolutiv entstand.
Die funktionelle Analyse des *Tribolium castaneum hairy* Homologs mithilfe der parentalen RNAi und die Analyse verschiedener molekularer Marker mittels in situ Hybridisierung in knockdown Embryos weisen darauf hin, dass die Segmentierungsfunktion von *Tc'h* als Paarregel-Gen während des Blastodermstadiums konserviert ist, wohingegen keine solche Funktionen für die Segmentierung während des weiteren Wachstums des Tribolium-Keimstreifens beobachtet werden konnte. Interessanterweise scheint diese Paar-Regel Funktion jedoch durch die Beteiligung von *Tc'h* an einem anderen Entwicklungsschritt, nämlich der Entwicklung des embryonalen Kopfes, überlagert zu werden, welche in knockdown Embyonen zum Verlust der anterioren Segmente bis zum 3. thorakalen Segment führt. Dies weist auf die mögliche Präsenz eines anterioren Organisers hin, der für die weitere Entwicklung dieser Region notwendig ist.
Analysen der Regulation von *Tc'h* zeigen im Vergleich zu seinem *Drosophila* Homolog, dass sich die regulatorische Kaskade, durch die sie beide gesteuert werden, erheblich unterscheidet. Während die *Tribolium* gap- Gen orthologe *Tc'kr* und möglicherweise auch *Tc'hb* nicht an der Regulation des *Tribolium hairy* Paarregelmusters beteiligt zu sein scheinen, wie sie es in *Drophila* tun, konnte ein starker regulatorischer Einfluss von *Tc'gt* in diesen Experimenten beobachtet werden, was Ähnlichkeiten zu *Drosophila* aufweist.
Analysen des regulierten Zielgens *Tc'ftz* weisen darauf hin, dass die regulatorische Interaktion zwischen diesen Genen konserviert sein könnte, obwohl die Nutzung dieser Interaktion bei *Drosophila* und *Tribolium* unterschiedlich ist.
Trotz der Unterschiede, die für die Regulation des *hairy* Gens zwischen *Drosophila* und *Tribolium* entdeckt wurden, zeigen Experimente mit transgenen Fliegen, die ein lacZ Reportergen- Konstrukt tragen, welches unter der Kontrolle eines *Tribolium* Enhancers steht, der die Expression der Steifen 3 bis 5 reguliert, tatsächlich die Expression von 3 Streifen an ähnlicher Position. Des weiteren zeigen Kreuzungen dieser transgenen Linien mit *Drosophila* gap- und Paarregelgen- Mutanten eine Beteiligung dieser Gene an der Regulation der beobachteten Muster. Interessanterweise zeigen Experimente mit ähnlichen Konstrukten, die die Streifen- spezifischen Elemente für die Streifen #3-#4 aus *Drosophila* enthalten, eine Streifen- ähnliche Expression an vergleichbarer Position im *Tribolium* Keimstreifen. Jedoch lassen pRNAi- Experimente mit den *Tribolium* gap- Genen *Tc'kr* und *Tc'hb* vermuten, dass die Regulation dieser Streifen unterschiedlich ist.

Die Ergebnisse dieser Studie enthüllen einen Teil der Funktion und Regulation des *Tribolium castaneum hairy* Homologs und erlauben einige interessante Spekulationen über die molekularen Ereignisse, die eine Transition von der Kurz- zur Langkeim- Entwicklung und der Evolution der Paarregelmuster in höheren Insekten ermöglicht haben könnten.

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Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Diethard Tautz betreut worden.

Köln den, 25.09.2006 Manuel Aranda