Formation of the Clypeolabral Region During Embryonic Head Development of the Red Flour Beetle *Tribolium castaneum*

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Herewith I declare, that I prepared the Dissertation

"Formation of the Clypeolabral Region During Embryonic Head Development of the Red Flour Beetle *Tribolium castaneum*"

on my own and with no other sources and aids than quoted.

_____ Göttingen, April 30th, 2012

Sebastian Kittelmann

Für Maike

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1 Summary

Insects are segmented organisms but the number of segments contributing to the head is disputed. The most anterior head region is patterned differently from the rest of the body and, therefore, is considered to be non-segmental. During embryogenesis, the non-segmental region can be subdivided into an outer neurogenic region and an inner triangular part which is mostly free of neurogenic precursor cells. Due to the similarities with a structure known from embryonic development of the vinegar fly *Drosophila melanogaster* (MEIGEN, 1830), this median region will be referred to as the clypeolabral region (CLR). It gives rise to the larval clypeolabrum and comprises the anlagen of the anterior gut and the stomatogastric nervous system. The red flour beetle *Tribolium castaneum* (HERBST, 1797) has been established as an important model organism for insect head development due to its insect-typical head and its amenability to functional studies.

The formation of the CLR in *Tribolium* involves a previously unnoticed connection between mesodermal cells and the extraembryonic amnion as well as the formation of a fold in the anterior head. The anterior fold separates the ectoderm of both head sides during early embryogenesis and apparently accounts for the generation of the lateral head lobes found in insect embryos. It is probably also responsible for the relocation of anterior cells to a more posterior sub-terminal position. Moreover, it may be involved in the formation of the stomodaeum.

Seven genes were found to show a rather exclusive expression pattern in the CLR during embryogenesis. Surrounding head regions are marked by a distinct set of genes, as well. RNA interference-mediated knockdown of several genes expressed in the anterior head and the subsequent analysis of developing larvae and embryonic gene expression patterns led to the establishment of a model for a gene regulatory network of the CLR. While the late expression aspects of the analyzed genes show a high degree of evolutionary conservation throughout arthropods, the genetic interactions during CLR development appear to be only partly conserved between *Drosophila* and *Tribolium*.

Three genes, *Tc-six3*, *Tc-crocodile* (*Tc-croc*) and *Tc-cap'n'collar* (*Tc-cnc*) were identified as most important upstream regulators of CLR development. *Tc-six3* is necessary for all anterior ectodermal derivatives, *Tc-croc* for development of the foregut and *Tc-cnc* for clypeolabrum formation. I established transgenic animals for ubiquitous expression of *Tc-croc* and *Tc-six3* by using the φC31-based site specific integration system to further test the function of these genes during embryonic head development.

2 Introduction

2.1 Composition of the insect head

The arthropods are the largest phylum in the animal kingdom, with the insects alone comprising over one million described species (Grimaldi and Engel, 2005). One of the reasons for their evolutionary success is the subdivision of their body into single segments which allows specialization of parts of the body for distinct tasks (Tautz, 2004; Chipman, 2010).

In extant arthropods, segments in different body regions are closely grouped or even fused, thus forming functional units called tagmata. In the order of hexapods, to which the insects belong, the body is subdivided into three tagmata, head, thorax, and abdomen, each of which consists of a defined number of segments. It is widely accepted that the insect thorax consists of three and the abdomen ancestrally of eleven segments, while the number of the latter might be secondarily reduced (Snodgrass, 1935). However, the exact number of segments contributing to the insect head has been excessively disputed (Rempel, 1975; Jürgens et al., 1986; Diederich et al., 1991; Schmidt-Ott and Technau, 1992; Schmidt-Ott et al., 1994; Rogers and Kaufman, 1997; Haas et al., 2001; Budd, 2002; Scholtz and Edgecombe, 2006; Posnien et al., 2010).

The segmental origin of the clypeolabrum has been one of the main controversies among entomologists. The clypeolabrum consists of two cuticle structures, clypeus and labrum. The latter is often referred to as the insect upper lip (Rogers and Kaufman, 1997). It covers the mouthparts as well as the mouth opening, is involved in the feeding process and additionally protects the oral region. The clypeus forms the connection between the labrum and the head and bears the attachment sites for the dorsal mouth muscles (Snodgrass, 1935; Bitsch and Bitsch, 2010).

The labrum has been seen by different researchers either as the appendage of a particular anterior segment (e.g. Cohen and Jürgens, 1991; Finkelstein and Perrimon, 1991; Schmidt-Ott and Technau, 1992; Schmidt-Ott et al., 1994), as appendage of the intercalary segment (e.g. Haas et al., 2001) or as an appendage or a cuticular outgrowth of the non-segmental anterior part of the head (reviewed in Rempel, 1975; Rogers and Kaufman, 1997; Scholtz and Edgecombe, 2006; Posnien et al., 2010). This non-segmental anterior head region has historically been called acron.

The acron concept originates from the assumption that arthropods and annelids are sister taxa with a common ancestor. Annelids indeed possess an unsegmented anterior region termed prostomium (see e.g. Goodrich, 1897; Scholtz and Edgecombe, 2006). Therefore, a

homologous region was assumed for arthropods and termed acron. The acron was thought to give rise to the main brain structures and, according to some authors, also to the eyes and the antennae (Rempel, 1975). However, more recent works including molecular data (Eernisse et al., 1992; Aguinaldo et al., 1997) have shown that, in fact, arthropods and annelids are more distantly related, belonging to the Ecdysozoa and Lophotrochozoa, respectively (reviewed in Telford et al., 2008). As a result, the concept of an acron was obsolete, which reopened the discussion on the number of head segments and the segmental origin of the labrum. Studies by Posnien et al. (2009b) indicated that the labrum is the fused appendage pair of an anterior region that is not delimited by parasegment boundaries.

In this work, I will adapt the view from Posnien et al. (2010), according to whom the insect head can be subdivided into five segments and an anterior non-segmental, yet sectioned region (Fig. 1; see also Scholtz and Edgecombe, 2006). The posterior head (gnathocephalon) functions primarily in the feeding process and consists of the three segments carrying the mouthparts. From posterior to anterior, these are the labial, maxillary, and mandibular segments (Snodgrass, 1935). These segments are mostly patterned like the segments of the trunk (Pankratz and Jäckle, 1990; Rogers and Kaufman, 1997; Choe et al., 2006; Choe and Brown, 2007), whereas the mandibular segment is additionally influenced by anterior factors (Vincent et al., 1997).

The anterior procephalon is mainly involved in sensory perception and processing as it contains the brain, the eyes and the antennae. Embryonically, it consists of the intercalary and the antennal segments as well as the ocular region and also gives rise to the clypeolabrum and the mouth opening (Snodgrass, 1935; Posnien et al., 2010). The antennal and intercalary segments are still clearly serially homologous to the segments of the trunk, as inferred, for instance, by the expression of segment polarity genes. The ocular region is by some authors seen as a segment, as well (see e.g. Schmidt-Ott et al., 1994; Rogers and Kaufman, 1996; Rogers and Kaufman, 1997). As clear morphological boundaries to the anterior non-segmental region are lacking, I will use the phrase ocular region (see also Scholtz and Edgecombe, 2006; Posnien et al., 2010).

The extent of the non-segmental region of the head is unclear. The dotted line in Fig. 1B indicates a suggested boundary based on the deletion pattern after *Tc-six3* RNAi (see below; Posnien et al., 2011b) as well as the exclusive expression of genes in the anterior head like *Tc-six3*, *Tc-orthodenticle-1* (*Tc-otd-1*) or *Tc-tailless* (*Tc-tll*) (Li et al., 1996; Schröder et al., 2000; Steinmetz et al., 2010; see also Posnien et al., 2010).

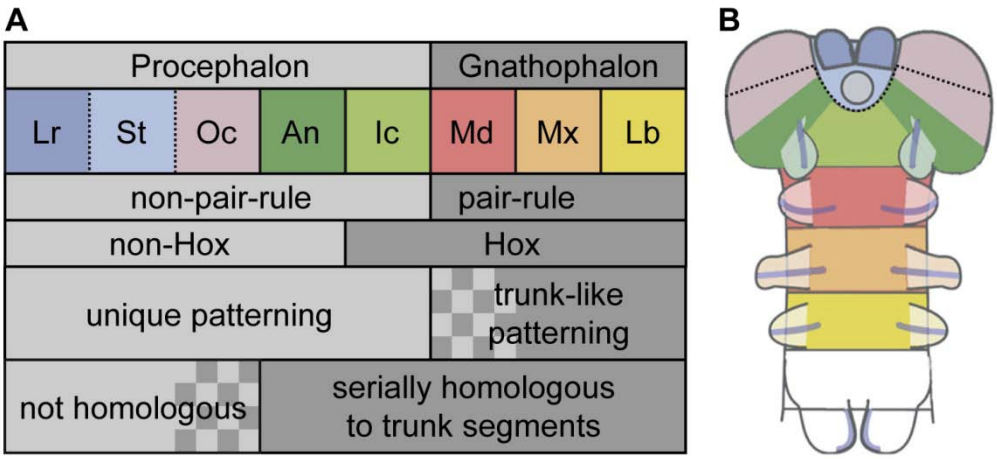


Fig. 1: Composition of the insect head. Modified after Posnien et al. (2010) and reprinted with permission from Elsevier. (A) Anterior to posterior organization of the non-segmental region and the segments constituting the insect head. Anterior is left. The procephalon consists of the labrum (Lr) and stomodaeum anlagen (St), the ocular/pre-ocular region (Oc), the antennal (An) and the intercalary segment (Ic). The gnathocephalon consists of the mandibular (Md), maxillary (Mx) and labial segments (Lb). Pair rule patterning is not involved in the formation of boundaries in the procephalon. Hox genes are only expressed in the intercalary and more posterior segments. The gnathocephalon is mostly patterned like the trunk segments, whereas the mandibular segment gets also input from the anterior system (indicated by shading). It is debated whether the ocular region is serially homologous to the trunk segments (indicated by shading). (B) Schematic of an embryo at a stage in which the appendages are elongating. The same color code as in (A) is used. Anterior is up. The dotted line indicates a suggested boundary between the non-segmental region and surrounding head regions/segments. This region can be subdivided into an outer neurogenic part (belonging to Oc in the scheme) and an inner region which is mostly free of neuronal precursors (Lr and St). The ocular region and the antennal segment are split by the non-segmental region.

The non-segmental region can be functionally subdivided into an outer neurogenic part and an inner area which is mostly free of cells giving rise to neurogenic tissue. Accordingly, neurogenic markers like *Tc-ventral nerve cord defective* (*Tc-vnd*) *Tc-asense* (*Tc-ase*) or *Tc-eyeless* are expressed along the midline and split in the anterior head (Wheeler et al., 2005; Posnien et al., 2010; Posnien et al., 2011b). Also other genes expressed along the midline like *Tc-spitz* (*Tc-spi*) show a Y-shaped expression pattern which frames the non-neurogenic part of the non-segmental region of the head (Grossmann, 2011; Kittelmann, 2008). Due to its similarities to the clypeolabral region of the vinegar fly *Drosophila melanogaster* (Rogers and Kaufman, 1997; see below), this non-neurogenic area in *Tribolium* will also be referred to as the clypeolabral region (CLR). The CLR does apparently not only split the ventral midline. Also, the antennal segment and the ocular region are pushed laterally by this median structure (Fig. 1B). The most prominent, i.e. clearly visible and easy to analyze, cuticle structures the CLR gives rise to, are the clypeolabrum and the foregut.

2.2 Genetic control of segmentation in insects

Much of our knowledge about arthropod development comes from studies in *Drosophila*. *Drosophila* is a great model organism which can be easily manipulated in various ways and is especially useful for the understanding of molecular and cellular interactions. It was, for example, the first arthropod in which the metamerization of a uniform embryo into a fully segmented one was studied molecularly. In *Drosophila*, this process is controlled by a cascade of genes which gradually subdivides the embryo into smaller compartments (Nüsslein-Volhard and Wieschaus, 1980). The gene products of maternal effect genes like *bicoid* (*bcd*) and *hunchback* are deposited in the egg during oogenesis and establish the primary body axes. In a concentration-dependent manner, they activate the expression of gap genes. These specify larger regions in the embryo. The more downstream pair rule genes subdivide the embryo into a series of segments. The pair rule genes activate expression of the segment polarity genes which define the parasegment boundaries. The unique identity of each segment is determined by the expression of Homeotic selector genes (Hox genes) of the Antennapedia Complex and the Bithorax Complex (reviewed in Akam, 1987; Ingham, 1988; Irish et al., 1989; St Johnston and Nüsslein-Volhard, 1992).

While several aspects of the cascade function by the same principle in other arthropods (Tautz and Sommer, 1995; Rogers and Kaufman, 1997; Davis and Patel, 2003; Damen, 2007), analysis of different species has also revealed that development of *Drosophila* is evolutionarily derived and cannot serve as an example for all insects (Tautz et al., 1994; Tautz and Sommer, 1995; Peel et al., 2005). Instead, the red flour beetle *Tribolium castaneum* shows a very different developmental mode that is believed to be more typical for insects (Klingler, 2004; Bucher and Wimmer, 2005; Schröder et al., 2008).

One important aspect is that *Tribolium* undergoes short germ embryogenesis, which means that the blastodermal fate map contains only the anlagen of head and thorax (germ rudiment) as well as a large extraembryonic portion (Handel et al., 2000; van der Zee et al., 2005; van der Zee et al., 2006). The abdominal segments are added successively to the posterior of the germ rudiment in a growth zone (Schoppmeier and Schröder, 2005; Choe et al., 2006; Sarrazin et al., 2012). In contrast to that, *Drosophila* forms all segments of the body at the same time in a process termed long germ embryogenesis. Short germ development is thought to be the ancestral mode of arthropod segmentation (Davidson, 1991; Tautz et al., 1994; Davis and Patel, 2002; Peel, 2008; Schröder et al., 2008).

Moreover, the larval head morphology of *Drosophila* is highly derived. In a process called head involution, the head segments become internalized into the trunk during late embryogen-

esis (Turner and Mahowald, 1979; Finkelstein and Perrimon, 1991). Genetic manipulation leading to defects in single segments often blocks head involution as well, thereby creating secondary defects that are not easy to interpret. Also, the head appendages are highly derived. The *Tribolium* larva, on the other hand, possesses an insect-typical head with all head appendages clearly visible on the outside (Bucher and Wimmer, 2005; Posnien et al., 2010).

Finally, *Tribolium* is easy to rear and genetic as well as molecular methods are well established (Bucher et al., 2002; Lorenzen et al., 2003; Pavlopoulos et al., 2004; Richards et al., 2008; Trauner et al., 2009; Kim et al., 2009; Schinko et al., 2010; Sarrazin et al., 2012). This makes *Tribolium* a very good model organism for insect development and especially for the analysis of head formation.

2.3 Formation of the anterior head

The segmentation cascade described for *Drosophila* has been shown to function in the segments of the trunk and the gnathocephalon. The situation is different in the procephalon, where the pair rule genes are not expressed. Also, no Hox-cluster genes are expressed anterior of the intercalary segment which is marked by expression of *labial* (Cohen and Jürgens, 1991; Diederich et al., 1991; Finkelstein and Perrimon, 1991; Brown et al., 1994; Nagy and Carroll, 1994; Rogers and Kaufman, 1997; Posnien et al., 2010; Choe et al., 2006; Choe and Brown, 2007; Farzana and Brown, 2008). Moreover, the most anterior parasegment boundary lies in the ocular region (Rogers and Kaufman, 1996; Rogers and Kaufman, 1997; but see Schmidt-Ott and Technau, 1992 and Schmidt-Ott et al., 1994 for a different view) and the segment polarity genes show unique interactions in each procephalic parasegment boundary (Gallitano-Mendel and Finkelstein, 1997).

As already mentioned, *Drosophila* is characterized by a derived developmental mode. This also applies for aspects of head formation. For example, the head gap genes account for the patterning of the segmented part of the anterior head in *Drosophila* (Cohen and Jürgens, 1991; Finkelstein and Perrimon, 1991; Rogers and Kaufman, 1997), but only *Tc-knirps* appears to be important in *Tribolium* (Cerny et al., 2008). Other genes do not show much functional conservation (Schinko et al., 2008). Also, the *bcd* gene encoding an important anterior determinant in *Drosophila* (St Johnston and Nüsslein-Volhard, 1992) has evolved only in the lineage of higher flies (Stauber et al., 1999), whereas different genes account for its function in other insects (Schröder, 2003; Lynch et al., 2006; van der Zee et al., 2006; Schoppmeier et al., 2009; Fu et al., in press).

These dissimilarities are at least partly due to the different germ anlagen (see above). The short germ rudiment of *Tribolium* does not extend to the anterior pole of the egg, complicating the determination of anterior head structures by a factor localized at the egg terminus like Bcd (Rosenberg et al., 2009). Instead, the posterior determinant *Tc-caudal* (*Tc-cad*) is repressed in the anterior germ rudiment (i.e. the central egg) by *Tc-Mex-3* (Schoppmeier et al., 2009). Also, repression of ubiquitous anterior (i.e. extraembryonic) determinants at the posterior pole by a localized factor has been proposed (Schröder, 2003). In order to understand how different modes of head development evolved in the various insect orders, head formation has to be studied detailed in different model organisms, one of which is *Tribolium*.

2.4 Genes involved in anterior head development

Patterning of the clypeolabral region in *Drosophila* (reviewed in Rogers and Kaufman, 1997) involves the maternal anterior, terminal and dorso-ventral systems. All three systems interact to activate the expression of the so-called terminal gap genes, *huckebein* (*hkb*) and *tailless* (*tll*) which regulate the expression of the gut patterning genes, *serpent* (*srp*) and *fork head* (*fkh*; Weigel et al., 1990; Brönner and Jäckle, 1991; Reuter and Leptin, 1994; Brönner et al., 1994; Brönner and Jäckle, 1996). *srp* is required for the formation of the endodermal anterior midgut (Reuter, 1994a), while *fkh* is necessary for proper development of the ectodermal foregut (Jürgens and Weigel, 1988; Weigel et al., 1989a; Weigel et al., 1989b). In combination with the maternal systems, *hkb* and *tll* also regulate expression of *cap'n'collar* (*cnc*; Mohler, 1993) and *crocodile* (*croc*; Häcker et al., 1995). These two genes are required for the formation of the clypeolabrum. In addition, *optix/six3* is necessary for proper labrum development (Coiffier et al., 2008). However, its position within the *Drosophila* network is uncertain. An overview of the patterning system in *Drosophila* is given in Fig. 2.

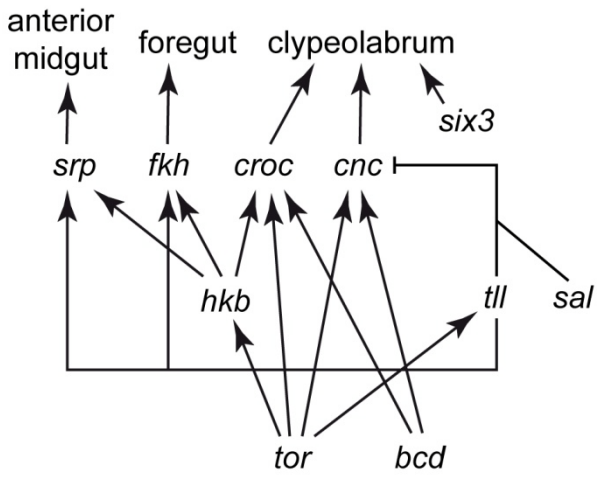


Fig. 2: Simplified scheme of the CLR patterning network in *Drosophila*. Modified after Rogers and Kaufman (1997) and reprinted with permission from Elsevier. The maternal systems (represented by *bcd* and *tor*) activate expression of the terminal gap genes, *hkb* and *tll*. Maternal systems and terminal gap genes act in concert to regulate expression of *srp*, *fkh*, *croc* and *cnc*. Also, *spalt* (*sal*) is involved in their regulation. The anterior midgut is dependent on *srp*, while the foregut is specified by *fkh*. The clypeolabrum is patterned by *cnc* and *croc* as well as *six3*.

Previous works indicated that the early determination of anterior head structures is not conserved between *Drosophila* and *Tribolium* because of the different extent of the germ anlagen (see above). Some of the orthologs of the factors acting further downstream in the CLR patterning network were identified in *Tribolium*, as well. Their early expression pattern is often not conserved because of the different embryonic fate maps. However, later expression aspects usually show a high degree of conservation (Schröder et al., 2000; Economou and Telford, 2009; Posnien et al., 2009b; Steinmetz et al., 2010). Functional data is only available for *Tc-six3* (Posnien et al., 2009b; Posnien et al., 2011b) and preliminary works were done for *Tc-crocodile* (*Tc-croc*) and *Tc-cap'n'collar* (*Tc-cnc*) (Kittelmann, 2008). A screen performed to identify further genes involved in *Tribolium* CLR patterning identified two more candidates, *Tc-chx* and *Tc-tailup* (*Tc-tup*) and led to the establishment of a hypothetical gene interaction scheme (Kittelmann, 2008). Due to its expression pattern and RNAi phenotype, *Tc-scarecrow* (*Tc-scro*) was expected to be involved in CLR development, as well (Posnien et al., 2011b).

2.4.1 *Tc-six3*

Optix/six3 has been recognized as the *Drosophila* ortholog to the vertebrate homeobox genes *Six3* and *Optx2* (Toy et al., 1998). Due to its expression pattern in the anterior head of *Drosophila* which is very similar to the vertebrate expression, a role in eye development had been suggested and was later confirmed (Seimiya and Gehring, 2000). It also plays a role in labrum development (Coiffier et al., 2008). Analysis of the expression pattern in other arthropods and two annelid species suggested that the most anterior head region is marked by *Six3* orthologs in apparently all bilaterian animals (Steinmetz et al., 2010). The *Tribolium* ortholog, *Tc-six3*, is necessary for proper development of the anterior median head region including the labrum (Posnien et al., 2009b) and anterior brain structures (Posnien et al., 2011b). Loss of its function leads to the deletion of a triangular region in the anterior *Tribolium* embryo that supposedly correlates to the CLR.

2.4.2 *Tc-crocodile*

The *fork head* transcription factor *crocodile* (*croc*; Häcker et al., 1992) functions in the formation of ectodermal and mesodermal derivatives of the *Drosophila* clypeolabrum (Häcker, 1995; Häcker et al., 1995). *croc* is also required for the formation of the stomatogastric nervous system (SNS; Schmidt-Ott et al., 1994). The expression pattern of the *Tribolium* ortholog, *Tc-croc*, is very similar to the later *Drosophila* pattern (Economou and Telford, 2009). It is also conserved in the more basal insect, *Oncopeltus fasciatus* (Dallas, 1852) (Birkan et al., 2011). Conserved expression is reported for the myriapod, *Glomeris marginata* (Villers,

1789), as well (Janssen et al., 2011). During later developmental stages, expression in a similar pattern is also observed in the crustacean *Parhyale hawaiensis* (DANA, 1853) (Schmid, 2011). Functional studies in *Oncopeltus* suggest a role in labrum patterning throughout insects (Birkan et al., 2011).

2.4.3 *Tc-cap'n'collar*

The leucine zipper transcription factor *cap'n'collar* (*cnc*) was named due to its expression in an anterior "cap" and a stripe in the anlagen of the mandibular and intercalary segments in *Drosophila* (Mohler et al., 1991). Loss of *cnc* function results in the lack of ectodermal labral structures as well as a transformation of mandible structures into maxillary identity (Mohler et al., 1995). Similar to *croc*, the expression pattern of *cnc* orthologs is conserved among insects and myriapods (Rogers et al., 2002; Economou and Telford, 2009; Birkan et al., 2011; Janssen et al., 2011). Knockdown of *Of-cnc* in *Oncopeltus* leads to a deletion of the labrum, but no effect on the mandibles is reported (Birkan et al., 2011).

2.4.4 *Tc-scarecrow*

In *Drosophila*, the homeobox gene *scarecrow* (*scro*) is expressed in the anlagen of the pharynx, in parts of the brain and in cells of the ventral nerve cord (Zaffran et al., 2000). The *Tribolium* ortholog, *Tc-scro*, is expressed surrounding the anlagen of the foregut and in the brain, as well. Expression is also detected at the base of the labrum, which is in accordance with its loss of function-phenotype: in *Tc-scro*-knockdown embryos, the labrum anlagen fail to fuse and L1 larvae exhibit a split labrum (Posnien et al., 2011b).

2.4.5 *Tc-fork head*

Expression of *fork head* (*fkh*) in *Drosophila* is detected in the ectodermal anlagen of the gut, i.e. in the anterior stomodaeum and the posterior proctodaeum (Weigel et al., 1989b). Loss of *fkh* function leads to a homeotic transformation of gut tissue to post-oral head structures (Jürgens and Weigel, 1988). The orthologous *Tc-fkh* is expressed in a comparable pattern (Schröder et al., 2000).

2.4.6 *Tc-chx*

The vertebrate gene *Chx10* is expressed during retina development (Levine et al., 1994; Liu et al., 1994). The *Drosophila* genome contains two orthologs, *Dchx1* and *Dchx2* (also *Visual system homeobox 1* and *2*, respectively) which play a role in eye development, as well (Erclik et al., 2008). Moreover, *Dchx1* has been established as a marker for the pars intercerebralis of the neuroendocrine system (de Velasco et al., 2007). Also for the *Tribolium* ortholog, *Tc-chx*,

expression in an anterior brain region probably corresponding to the pars intercerebralis as well as in the ocular region is reported. Additionally, expression is found in the labrum (Posnien et al., 2011b). Knockdown of *Tc-chx* leads to defects in anterior brain regions (Koniszewski, 2011).

2.4.7 *Tc-tailup*

The LIM-homeodomain transcription factor *tailup* (*tup*; also *islet*) plays a role in germ band retraction in *Drosophila* (Nüsslein-Volhard et al., 1984). Later studies showed that defects in amnioserosa development lead to the retraction defects (Frank and Rushlow, 1996). It is expressed in amnioserosa, mesoderm, pharynx and later also in the central nervous system (Thor and Thomas, 1997) where it plays a role in motor neuron pathway selection (Thor et al., 1999). In other dipterans, it is important for the development of the extraembryonic amnion (Rafiqi et al., 2010). The *Tribolium* ortholog, *Tc-tup*, is expressed in the extraembryonic membranes, the labrum anlagen, an ocular domain and in a bilateral segmental fashion (Kittelmann, 2008).

2.5 Analyzing gene function in *Tribolium*

2.5.1 Loss of function via RNA-interference

Gene function in *Tribolium* development is usually analyzed by reducing the gene expression via RNA-interference (RNAi; Meister and Tuschl, 2004; Mello and Conte, 2004; Hammond, 2005; Ketting, 2011). RNAi is a powerful tool in *Tribolium*, as it is easy to apply and causes systemic (i.e. not locally restricted) and strong effects (Brown et al., 1999; Bucher et al., 2002; Tomoyasu and Denell, 2004; Posnien et al., 2009a). Double-stranded RNA (dsRNA) of the gene to analyze is usually injected into female pupae which will pass the dsRNA on to their offspring by an unknown mechanism (Bucher et al., 2002). In cases in which the knockdown interferes with metamorphosis, oogenesis or the metabolism of the injected female, dsRNA is injected either into adult females or directly into eggs. RNAi-mediated defects in development are then studied in cuticles of L1 larvae as well as in developing embryos.

2.5.2 Gain of function via ectopic expression of genes

A second way to analyze gene function is via ectopic expression which is established in *Tribolium*, as well. The ectopic activation of genes via the GAL4/UAS system (Fischer et al., 1988; Ornitz et al., 1991; Brand and Perrimon, 1993) has been shown to be functional in *Tribolium* (Schinko et al., 2010). This system makes use of the expression of the transcription

factor GAL4 from yeast under control of endogenous regulatory sequences. GAL4 then activates expression of the desired transgene by binding to the upstream activating sequence (UAS). However, only few regulatory sequences that could be used for locally or temporally restricted expression of GAL4 are identified in *Tribolium*. Therefore, ectopic expression is at the moment facilitated via heat shock-induced ubiquitous overexpression (Schinko et al., 2010; Schinko et al., in preparation).

Transgenic *Tribolium* lines are usually generated by transposon-mediated mutagenesis, using either *piggyBac* (Lorenzen et al., 2003) or *Minos* transposable elements (Pavlopoulos et al., 2004). This results in nearly random insertion of transgenic sequences into the genome due to the short recognition sites for insertion (reviewed in Handler, 2001). Hence, the introduced gene can disrupt native genes and positional effects can lead to different expression patterns of identical transgenes. While being useful features for genetic screens (e.g. Trauner et al., 2009), studies using ectopic expression would benefit from site-specific integration (Wimmer, 2005).

The φC31-based site-specific integration system makes use of an integrase protein encoded by bacteriophage φC31 of *Streptomyces* (Kuhstoss and Rao, 1991). The integrase catalyzes recombination between the attachment sites in the genome of the phage (*attP*) and the bacteria (*attB*). The sites resulting from the recombination, called *attL* and *attR*, are no substrate for the integrase, making recombination unidirectional (Omer and Cohen, 1986; Omer et al., 1988; Boccard et al., 1989; reviewed in Groth and Calos, 2004 and Smith et al., 2010). The φC31 system is well established in different insects (Groth et al., 2004; Nimmo et al., 2006; Schetelig et al., 2009; Labbé et al., 2010), and is highly efficient (Groth et al., 2004). *Tribolium* as a model organism would benefit from the use of this site specific integration system.

Previous works to establish the φC31 system in *Tribolium* resulted in the generation of a line carrying a 228 bp *attP* site. Integration of the *attP* site into the genome was mediated by transposon-based mutagenesis carried out in an enhancer trap screen (Schinko and Bucher, unpublished). This line can be used for site-specific integration of the desired construct for ectopic gene expression. Therefore, the construct must include an *attB* site and has to be injected into the *attP*-carrying line together with the φC31 integrase. Fig. 3 gives an overview of the anticipated integration.

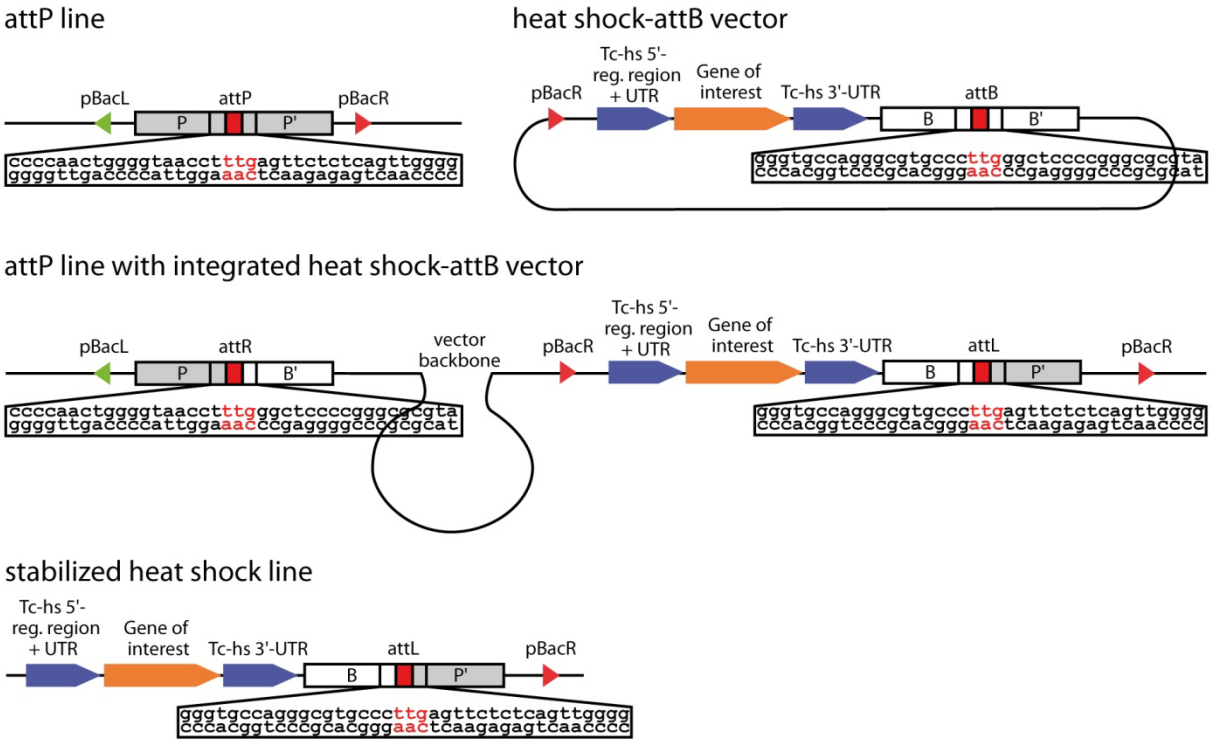


Fig. 3: Simplified schematic overview of the recombination reaction that was sought to be used for the generation of heat shock lines. Based on Schetelig et al. (2009) and Groth and Calos (2004). The *attP* line carries a *piggyBac* construct containing the *attP* site. *piggyBac* inverted repeats are indicated (pBacL, pBacR). The *attP* site consists of two arms, P and P'. The sequence of the core region is given, recombination sites are marked red. The heat shock vector contains the gene to be ectopically expressed under control of the regulatory region of *hsp68* as well as a *piggyBac* recognition site (pBacR) directly upstream of the heat shock construct and the *attB* site downstream of the heat shock construct. Similar to the *attP* site, the *attB* site consists of two arms, B and B', separated by the recombination sequence. Upon injection of the heat shock vector together with the φC31 integrase, the *attP* and *attB* sites recombine and the complete vector (ca. 8 kbp) is integrated into the genome of the line (indicated by the vector backbone). The resulting *attR* and *attL* sites consist of P and B' and B and P', respectively. It is possible to excise the vector backbone together with the *attR* site in case it interferes with ectopic expression due to its size or positional effects. Therefore, a *piggyBac* transposase would have to be supplied to the line. It would then excise the respective sequence due to the original *pBacL* site and the newly introduced *pBacR* site and leave the remaining parts of the construct in the stabilized heat shock line (see also Schetelig et al., 2009).

2.6 Aims

The aim of this work was to shed light on the formation of the CLR. I sought to use *in vivo* imaging as well as three dimensional reconstructions of staged fixed embryos to understand the morphogenesis of the anterior head. In order to test to which extent cell death and proliferation contribute to head formation, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and phosphorylated Histone H3 (PH3) immunostaining were to be performed, respectively.

I intended to identify genes involved in patterning of the CLR by studying their expression patterns and loss of function-phenotypes. Interactions between the single players were to be identified by analyzing the expression patterns of suggested downstream targets after loss of function of suggested upstream targets. Thereby, a model for a gene regulatory network was sought to be established.

To further test the hypothesized interactions between genes, I aimed to generate *Tribolium* lines for heat shock induced ectopic expression of assumed upstream genes. In order to do so, I intended to establish site-specific integration via the φC31 based integration system in *Tribolium*.

3 Materials and Methods

3.1 Animals

Animals were reared under standard conditions (Brown et al., 2009).

The wild type strain *San Bernadino* (*SB*) was used for RNAi experiments and staining.

The strain *171* was used for *in vivo* imaging. It expresses nuclear localized green fluorescent protein (GFP) in all cells (Sarrazin et al., 2012).

The *vermillion*^{white} (v^{w}) strain was used for crossings in the transgenesis experiments. A mutation of the *Tc-vermillion* gene leads to white eye color in this strain (Eddleman and Bell, 1963; Lorenzen et al., 2002).

The acceptor strain used for transgenesis was meant to be the *attP* line but might have been contaminated with the *SB* strain. The *attP* line has the genetic background of the v^{w} strain. Via *piggyBac* mutagenesis, a construct containing an *attP* site was introduced into this strain. The construct also contained a marker rescuing black eye color and a GAL4Δ construct to identify enhancer traps. Insertion apparently occurred into a region without positional effects, as black eye color was rescued but no expression of GFP after crossing to a UAS-GFP line was detectable in offspring (Bucher, personal communication).

3.2 Cloning of genes

Genes were cloned from complementary DNA (cDNA) of 0-48 h old embryos using specific primers. cDNA was prepared using the SMART™ cDNA Library Construction Kit (Clontech, Mountain View, CA, USA). Template messenger RNA (mRNA) was extracted from embryos using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA). Primers were designed on the basis of *Tribolium* sequences obtained from searching for orthologs of *Drosophila* proteins at BeetleBase (http://www.Beetlebase.org; Wang et al., 2007; Richards et al., 2008; Kim et al., 2009) using Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990). Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). A list of used primers is attached (p. 108). Gene fragments were amplified from cDNA by polymerase chain reaction (PCR) using Taq DNA Polymerase (Fermentas/Thermo Fisher Scientific, Waltham, MA, USA) or Advantage® 2 Polymerase (Clontech, Mountain View, CA, USA). Fragments were cloned into the pCR®II vector using the TA Cloning® Kit (Life Technologies, Carlsbad, CA, USA). The clone for *Tc-cnc* was provided by N. Schaeper (Developmental Biology, University Göttingen); the clones for *Tc-chx, Tc-scro* and *Tc-six3* were provided by N. Posnien (Developmental Biology, University Göttingen); the clone for *Tc-tll* was pro-

vided by R. Schröder (Institut für Biowissenschaften, Abt. Genetik, Universität Rostock); the clone for *Tc-hh* was provided by E. Ntini (Developmental Biology, University Göttingen).

For *Tc-cnc* and *Tc-croc*, rapid amplification of cDNA ends (RACE; Frohman et al., 1988) was performed using the SMART^{TM} RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). 5'-RACE was performed for *Tc-cnc* and 3'-RACE was performed for both, *Tc-cnc* and *Tc-croc*. The RACE-Ready cDNA pools used in the different experimental replicates had been prepared by N. Koniszewski, E. Ntini and M. Pechmann, respectively. Primer sequences are attached (p. 108).

3.3 RNAi

RNAi was performed according to established protocols (Posnien et al., 2009a). For dsRNA synthesis, the Ambion® MEGAscript® T7 kit (Life Technologies, Carlsbad, CA, USA) was used. The PCR product used as a template in the reaction was cleaned via gel electrophoresis and subsequent extraction from the gel using the NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany). Two non-overlapping fragments were injected for *Tc-cnc*, *Tc-croc*, *Tc-hh*, *Tc-scro*, *Tc-six3*, *Tc-tll* and *Tc-tup* to exclude off-target effects. Primer sequences for template synthesis are attached (p. 108). Pupal RNAi was performed in all cases. Additionally, adult RNAi was performed for *Tc-cnc*. Concentrations, GenBank entry and size of injected dsRNA fragments are given in Table 1.

Table 1: Injected dsRNA. Given are name of the gene as referenced in this work, concentration of injected dsRNA, GenBank entry and size of the injected fragments as indicated by first and last base pair (bp) of the respective sequence. More than one concentration indicates experimental replicates. Two fragment sizes indicate injection of non-overlapping fragments. For *Tc-homeobrain* (*Tc-hbn*), no GenBank entry was available. Fragment size is with respect to the cloned sequence. For *Tc-tup*, the annotation at NCBI is incorrect. Fragment sizes are with respect to the cloned sequence. For *Tc-cncB* and *Tc-cncC*, the fragment sizes are with respect to the cloned sequence.

gene	**dsRNA concentration (µg/µl)**	**GenBank entry**	**fragment size (ranging from bp X to bp Y)**
Tc-chx	3.86	FN295953.1	356-1142
Tc-cnc	0.75-4.00	NM_001170642.1	1279-1599; 1646-1975
Tc-cncB	2.00	n/a	1-582
Tc-cncC	2.00	n/a	1-217
Tc-croc	1.00-4.20	XM_001812646.1	8-435; 512-1010
Tc-hbn	6.5	n/a	1-735
Tc-hh	0.90-3.65	NM_001114365.1	9-552; 578-1163
Tc-scro	2.00-4.33	XM_968702.2	103-474; 551-852
Tc-six3	1.15-3.50	NM_001113467.1	1-642; 653-1377
Tc-tll	2.00-3.70	AF219117.1	53-702; 754-1521
Tc-tup	1.70-5.56	NM_001164807.1	1-374; 516-920

Developing L1 larvae after RNAi were embedded in 50 % Hoyer's medium 50 % lactic acid and were allowed to clear for 12 h at 65 °C. Head bristle patterns of cuticles were analyzed as described (Schinko et al., 2008) using a Zeiss Axioplan 2 microscope. Images were generated from stacks taken at a Zeiss LSM 510 using a 488 nm laser, a 505 nm longpass filter and Zeiss LSM image browser software (Carl Zeiss AG, Oberkochen, Germany).

3.4 Histology

3.4.1 Fixation

Embryos of an age of 0-72 h were used for FM 1-43® staining; embryos of an age of 0-24 h were used for all other experiments. Embryos were fixed using standard protocols (Schinko et al., 2009) with slight modifications: 2 ml fix buffer (1.3 x PBS and 67 mM EGTA, pH = 8.0) and 300 µl formaldehyde (37 %) were used.

3.4.2 Whole mount *in situ* hybridization

In situ hybridizations were performed as described (Schinko et al., 2009). Embryos were stained with Nitro blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP). In case of double *in situ* hybridizations, Fast Red (Roche Applied Science, Penzberg, Germany) or Vector® Red (Vector Laboratories, Burlingame, CA, USA) were used for staining of *Tc-wingless* (*Tc-wg*), and Iodonitrotetrazolium chloride (INT) was used for other genes. The yolk was removed from the embryos and they were embedded in 80-100 % glycerol. Analysis and imaging were performed using a Zeiss Axioplan 2 microscope with a DIC filter and Image-Pro® Plus software (Media Cybernetics, Bethesda, MD, USA).

3.4.3 Hoechst staining

Embryos were stained with 1 µg/µl Hoechst 33342 in 1X PBST for 1 h. The yolk was removed from the embryos and they were embedded in 80-100 % glycerol. Analysis and imaging were performed using a Zeiss Axioplan 2 microscope with a mercury-vapor lamp, a FITC filter and Image-Pro® Plus software (Media Cybernetics, Bethesda, MD, USA; when Hoechst staining was used to stage blastoderm stages after *in situ* hybridization) or a Zeiss LSM 510 using a 364 nm laser, a 385 nm longpass filter and Zeiss LSM image browser software (Carl Zeiss AG, Oberkochen, Germany; when Hoechst staining was performed in combination with FM® 1-43 and PH3 staining).

3.4.4 FM® 1-43 staining

Embryos were stained with 1 µg/ml *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl)pyridinium dibromide (FM® 1-43; Invitrogen/Life Technologies, Carlsbad, CA, USA).

The yolk was removed from the embryos and they were embedded in 70 % glycerol, 0.5 x PBST, 50 mM Tris-HCl (pH = 9.5), 10 mg/ml propyl gallate and 0.5 mg/ml *p*-phylodenediamine. Analysis and imaging were performed using a Zeiss LSM 510 using a 488 nm laser, a 505-550 nm bandpass filter and Zeiss LSM image browser software (Carl Zeiss AG, Oberkochen, Germany).

3.4.5 PH3 staining

Mitotic cells were stained using 0.5 µg/ml anti-phospho Histone H3 (Ser10) polyclonal antibody (Millipore, Temecula, CA, USA) as primary antibody and DyLight™488-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) as secondary antibody (see Hendzel et al., 1997). The yolk was removed from the embryos and they were embedded in 80-100 % glycerol. Analysis and imaging were performed using a Zeiss LSM 510 using a 488 nm laser, a 505-550 nm bandpass filter and Zeiss LSM image browser software (Carl Zeiss AG, Oberkochen, Germany). Proliferating cells were counted using Fiji (http://fiji.sc; Walter et al., 2010). Significance of differences between proliferating cells of untreated and *Tc-croc* RNAi-treated embryos was tested with a t-test.

3.4.6 TUNEL

Embryos were rehydrated, refixed and treated with proteinase K as described for whole mount *in situ* hybridizations (Schinko et al., 2009). TUNEL (Gavrieli et al., 1992) was performed as described for *Cupiennius salei* (KEYSERLING, 1877) (Prpic and Damen, 2005). The yolk was removed from the embryos and they were embedded in 80-100 % glycerol. Analysis and imaging were performed using a Zeiss Axioplan 2 microscope with a DIC filter and Image-Pro® Plus software (Media Cybernetics, Bethesda, MD, USA). Significance of differences between dying cells of untreated, *Tc-croc* RNAi-treated and *Tc-six3* RNAi-treated embryos was tested with a t-test.

3.5 Transgenesis

3.5.1 Constructs

All restriction enzymes used were provided by Fermentas/Thermo Fisher Scientific (Waltham, MA, USA). Vector maps are attached (p. 115) and were designed with ApE – A plasmid Editor v2.0.43 (M. Wayne Davis; http://biologylabs.utah.edu/jorgensen/wayned/ape/). Primer sequences for construct design and sequencing are attached (p. 108).

Plasmid pSLaf[3'pBac_Tc-hsp_Tc-hs5'3'UTR_attB_Pub_DsRed]af (#1) was generated as follows: The regulatory sequence of *hsp68* was amplified via PCR from vector pSLfa[Tc-

hsp5’3’UTR]fa (J.B. Schinko) using primers SK87 and SK88 (see p. 108). The PCR product and the acceptor vector pSL*af_3_pBac-attB_PUb-DsRed_af* (Schetelig et al., 2009) were cut with Bsp120I and subsequently ligated.

Plasmid pSLaf[3'pBac_Tc-hsp_Tc-hs'5'UTR_Tc-croc_Tc-hs3'UTR_attB_Pub_DsRed]af (#3) was generated as follows: The *Tc-croc* open reading frame (ORF) was amplified via PCR from cDNA of 0-48 h old embryos using primers SK78 and SK79 (see p. 108) and a 700 bp PCR product was sub-cloned into pCR®II (Invitrogen/Life Technologies, Carlsbad, CA, USA). The ORF was then amplified via PCR from pCRII[Tc-croc] with primers SK78 and SK99 (see p. 108). Plasmid #1 and the PCR product were cut with Bsp1470I and MluI and ligated.

Plasmid pSLaf[3'pBac_Hsp-p_Tc-hs5'3'UTR_attB_SV40_eCFP_Hsp-p_6xP3]af (#6) and pSLaf[3'pBac_Hsp-p_Tc-hs5'UTR_Tc-croc_Tc-hs3'UTR_attB_SV40_eCFP_Hsp-p_6xP3]af (#9) were generated as follows: The 6XP3-eCFP sequence was obtained from pSLfa[6xP3-Tc’hsp-ECFP-SV40] (Hein, 2007) by restriction with PauI. Pub-DsRed was removed from plasmids #1 and #3 by restriction with BglII and BspTI. Vector and insert were treated with T4 DNA Polymerase (Fermentas/Thermo Fisher Scientific, Waltham, MA, USA) to obtain blunt end DNA strands and were subsequently ligated.

Plasmid pSLaf[3'pBac_Hsp-p_Tc-hs5'UTR_Tc-six3-Tc-hs3'UTR_attB_SV40_eCFP_Hsp-p_6xP3]af (#10) was generated as follows: The *Tc-six3* ORF was amplified via PCR from pCRII[Tc-six3_full] (Posnien, 2009) with primers SK109 and SK110 (see p. 108). The PCR product and plasmid #6 were cut with XbaI and XhoI and subsequently ligated.

Plasmid pSLaf[3'pBac_Hsp-p_Tc-hs5'UTR_Tc-cncB_Tc-hs3'UTR_*attB*_SV40_eCFP_Hsp-p_6xP3]af (#11) was generated as follows: The *Tc-cncB* ORF was amplified via PCR from cDNA of 0-48 h old embryos using primers SK113 and SK114 (see p. 108). The PCR product and plasmid #6 were cut with MluI and XbaI and subsequently ligated. To circumvent point mutations that would lead to amino acid exchanges in the resulting Tc-Cnc protein, the 3’-end of the *Tc-cnc* ORF from clone B8 was excised by cutting with AdeI and replaced by the respectively cut fragment from clone hs2.

3.5.2 Injection

Injections were performed as described (Berghammer et al., 2009). The injected strain was probably the *SB* strain, which was mistaken for the *attP* line. Plasmids #9, #10 and #11, respectively, were injected together with φC31-integrase mRNA in the concentrations given in Table 2. Capped φC31-integrase mRNA was synthesized using the Ambion® mMESSAGE mMACHINE ® T7 Kit (Life Technologies, Carlsbad, CA, USA) as described (Schetelig et

al., 2009). DNA constructs and mRNA were mixed in desired ratio and filtered through a Millex-HV 0.45 µm Filter Unit (Millipore, Billerica, MA, USA). Injection was performed using a FemtoJet® (Eppendorf AG, Hamburg, Germany) and Bo-glass capillaries with filament (100 mm length; 1.0 mm outside diameter; 0.21 mm wall thickness; Hilgenberg GmbH, Malsfeld, Germany).

Table 2: Concentrations of injected phiC31 mRNA and plasmid for each experiment. Numbers of surviving offspring and names of established transgenic lines (where applicable) are indicated. *Tc-croc*_1 and *Tc-croc*_2, and accordingly, *Tc-six3*_1, *Tc-six3*_2 and *Tc-six3*_3 refer to separate experiments with different mRNA concentrations.

Gene	**Concentration mRNA (ng/µl)**	**Concentration plasmid (ng/µl)**	**Surviving offspring**	**Established transgenic lines**
Tc-cncB	500	300	31 (6.7 %)	-
*Tc-croc*_1	750	300	234 (35.7 %)	119_1
*Tc-croc*_2	600	400	228 (43.3 %)	124_1
*Tc-six3*_1	500	300	118 (31.9 %)	-
*Tc-six3*_2	400	400	254 (40.6 %)	-
*Tc-six3*_3	500	300	93 (17.8 %)	six3-1, six3-2

3.5.3 Generation of transgenic lines

Injected animals were crossed to not injected animals of the strain used for injection (in case of *Tc-six3*_1) or to animals of the v^w strain (in case of *Tc-croc*_1, *Tc-croc*_2 and *Tc-six3*_2). In case of *Tc-cncB* and *Tc-six3*_3, all surviving injected animals were kept together in one culture.

Offspring were screened for blue fluorescent eyes and crossed to animals of the v^w strain. Only animals with white eye background were used for generation of the *Tc-croc* ectopic expression lines 119_1 and 124_1.

3.5.4 Heat shock conditions

For proof of principle, eggs of strain 119_1 and 124_1 were collected for 24 h at 32 °C. Eggs were heat shocked in *Drosophila* vials (68 ml; 36 mm diameter; 82 mm height; Greiner Bio-One GmbH, Frickenhausen, Germany) in a water bath at 42 °C for 10 min. Eggs were fixed for *in situ* hybridization after developing for 4 h at 32 °C or were allowed to develop into L1 larvae and embedded and analyzed as described for RNAi.

For testing the optimal developmental time before heat shock, eggs were collected for 1 h at 32 °C and allowed to develop for 9, 10, 11, 12, 13, 14, 15, 16 or 17 h at 32 °C. Eggs were heat shocked as described above. Eggs were then allowed to develop into L1 larvae and were embedded and analyzed as described for RNAi.

For the heat shock experiment with subsequent staining of *Tc-wg* expression, eggs were collected for 2 h at 32 °C and allowed to develop for 16 h at 32 °C. Eggs were heat shocked as described above. In case of double heat shock, eggs were heat shocked again after developing for 3 h at 32 °C. Eggs were allowed to develop for another 4 h at 32 °C before fixation.

3.6 Digital Scanned Laser light sheet fluorescence Microscopy (DSLM)

In vivo imaging was performed using DSLM (Keller et al., 2008; Keller et al., 2010). Eggs of strain *171* were collected for 1 h and allowed to develop for another 8 h. The chorion was removed by washing two times for 30 sec in 50 % hypochlorous acid. The egg was embedded in 1.5 % Agarose Type VII (Sigma-Aldrich, St. Louis, MO, USA) in 1X PBS. Imaging was performed in 1X PBS at 30 °C.

The experimental setup for DSLM has been described elsewhere (Keller et al., 2008; Keller et al., 2010). Samples were imaged from four angles (0°/90°/180°/270°), with a z-spacing of 3.70 µm (experiments 1 and 2) and 2.96 µm (experiments 4 and 5). The objective used for detection was a Zeiss Plan Apochromat 20 x/1.0; i.e. the lateral voxel size was 0.37 µm (aspect ratio 10:1 for experiments 1 and 2, and 8:1 for experiments 4 and 5). In Experiment 1 we used structured illumination (SI30, see Keller et al., 2010), and a normal light sheet in the other three experiments. Time resolution of experiment 4 was 5 min during time points 0-4, 3 min during time points 5-25 and 5 min until the end of the experiment. Resolution of experiment 5 was 5 min. The z-range per angle lies between 450 and 460 µm, i.e. 155 planes per angle, thus 620 planes per time point. Every plane has 4 megapixels, i.e. 8 MB of data; thus, we produced roughly 5 GB data per time point.

4 Results

4.1 Morphogenesis of the CLR

4.1.1 *In vivo* imaging to visualize morphogenetic movements

Embryos undergo various morphogenetic movements during early development, and *Tribolium* is no exception. While many processes of early embryonic development have already been studied (Handel et al., 2000; Posnien et al., 2010), morphogenetic movements are easier to understand if they are directly looked at. In this case, a *Tribolium* line was used that expresses green fluorescent protein (GFP) with a nuclear localization signal under control of a constantly active basal promoter (Sarrazin et al., 2012). Tests showed that a fluorescent signal could not be detected until 6-8 h after egg lay (AEL; data not shown). DSLM was used to obtain good resolution in all three dimensions. The same technique had already been used to visualize early zebrafish development (Keller et al., 2008). Imaging was performed in collaboration with Dr. P.J. Keller in the Light Microscopy Group of Dr. E.H.K. Stelzer at the Cell Biology and Biophysics Unit, European Molecular Biology Laboratory, Heidelberg. Only experiment 5 gave satisfying results and allowed me to follow embryogenesis. The resulting video file is attached (p. 110). Fig. 4 shows selected frames of the video.

I started imaging of the embryo 8-9 h AEL (Fig. 4A). At this time, the embryo is already clearly separated into the anterior-dorsal serosa (extraembryonic membrane, em in Fig. 4A) and the posterior-ventral germ rudiment (gr in Fig. 4A). The serosal nuclei can be distinguished from the embryonic ones primarily by their size and spacing, as they are supposedly polyploid (Handel et al., 2000). The nuclei of the germ rudiment appear to be distributed in a salt and pepper pattern. This is due to the fact that the GFP is indeed nuclear localized but disperses over the cell when the nucleus breaks down during mitosis (arrowheads in Fig. 4A). Hence, cells are not marked during mitosis.

The embryo condenses during further development and starts to sink into the yolk (Handel et al., 2000). This process starts approximately after 1 h (9-10 h AEL) at the posterior end of the embryo and leads to the formation of the amniotic fold. The amniotic fold progresses to the anterior and forms the amniotic cavity, while the embryo sinks further into the yolk (arrow in Fig. 4D). At the same time, serosal nuclei are condensing at the left side of the imaged embryo (arrowhead in Fig. 4D). They are forming a cluster of cells that is visible throughout development. However, no such structure is reported in the literature and is also not visible in

the video published by Sarrazin et al. (2012). I therefore conclude that it is merely a developmental abnormality of the given embryo.

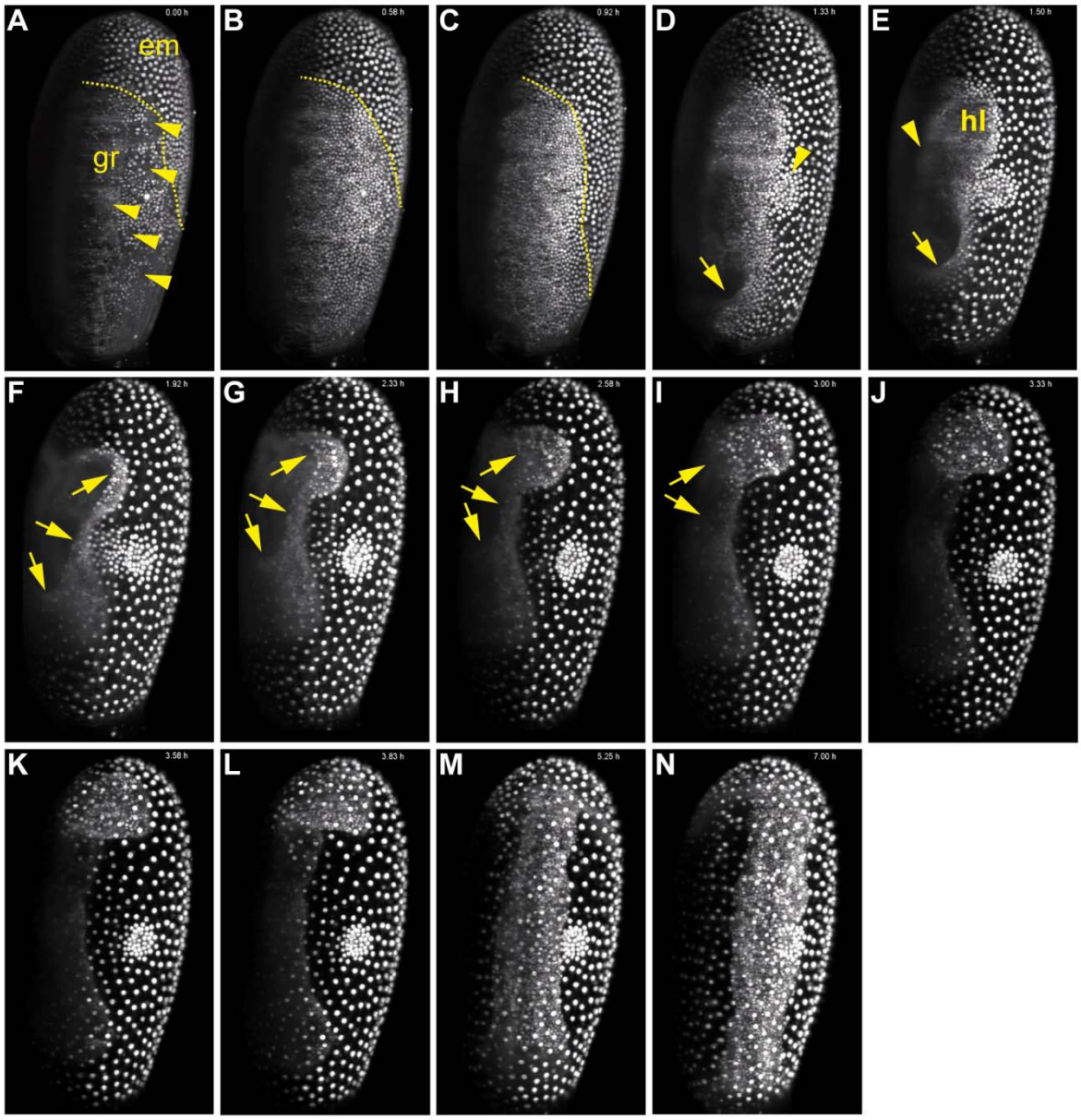


Fig. 4: Exemplary frames from the DSLM video. The egg is shown in a ventral left view; anterior is up. (A) At the beginning of imaging, the embryo was 8-9 h of age. The separation between extraembryonic membranes (em) and germ rudiment (gr) is already clearly visible. Nuclei in the extraembryonic membranes are larger than in the germ rudiment. Cells in the germ rudiment undergo a division cycle, leading to a reduction of the fluorescence signal due to nuclear breakdown (arrowheads). (B-C) The germ rudiment condenses at the ventral posterior side of the egg. (D) The embryo starts to sink into the yolk. The amniotic fold (arrow) is visible at the posterior of the germ rudiment at 9.3-10.3 h AEL. Cells appear to aggregate at the left side of the embryo (arrowhead). (E) At 9.5-10.5 h AEL, the ectodermal bulges closing over the invaginating mesoderm (arrowhead) and the head lobes (hl) are clearly distinguishable. The amniotic fold progresses towards the anterior (arrow). (F-I) As the embryo sinks further into the yolk, the amniotic fold starts to form also at the sides and the anterior of the embryo. During further development, the amnion grows to cover the elongating germ band (compare arrows). The area which is not yet covered by the serosa is termed the serosa window. (J) The serosa window is completely closed ca. 12.3-13.3 h AEL. (K-N) Starting at 11.6-12.6 h AEL, the embryo rotates in the egg for approximately 90° to the left. The rotation is completed 15-16 h AEL.

The head lobes become clearly distinguishable 9.5-10.5 h AEL (hl in Fig. 4E). Also, one of the ectodermal elevations which will fuse medially over the invaginating mesoderm is visible (arrowhead in Fig. 4E). The embryo now starts to elongate while the amniotic fold forms additionally at the sides and the anterior end of the germ band (arrows in Fig. 4F). The primary mechanism behind germ band elongation appears to be convergent extension (see also Sarrazin et al., 2012). The germ band gets narrower while it elongates and the head moves to a more anterior position. The amnion grows to cover the germ band completely. This leads to the formation of the serosa window, where the embryo proper is still uncovered by the extraembryonic membranes (arrows in Fig. 4F-I). The serosa window is completely closed 11.3-12.3 h AEL (Fig. 4J). The germ band now rotates in the egg for approximately 90° (Fig. 4K-N). Such a rotation has not been reported so far and it is unclear, whether just the imaged embryo behaved this way. The rotation takes ca. 3.5 h. The head eventually reaches its final position, at the anterior pole of the egg.

Three drawbacks eventually led me to decide that the use of DSLM in combination with the given *Tribolium* line was not suited to study the formation of the CLR. First, the nuclear localization of the GFP made it impossible to follow the fate of individual cells through mitosis. Hence, a fate map for single cells throughout development cannot be established. Second, the constitutive expression of the GFP in all embryonic tissues leads to a strong signal in the extraembryonic membranes overlying the embryo proper which impeded the tracing of embryonic cells. Third, the anterior head takes a ventral position only until ca. 12-13 h AEL (Fig. 4J) and a terminal position afterwards. Hence, for visualization of the anterior head throughout development, the egg has to be imaged in a tilted position, for which DSLM is unsuitable.

4.1.2 FM® 1-43 staining – snapshots of development

By investigating fixed embryos of many different developmental stages, I sought to get an insight into head morphogenesis. I stained embryos with the membrane marker FM® 1-43. This dye also stains the yolk cells, and thus the yolk had to be removed from the germ bands. To make sure that the embryos maintained their natural shape, they were embedded floating freely in the mounting medium. Besides the fact that the serosa is often peeled off the germ band during preparation, I did not detect any obvious preparation artifacts in the embryos shown in Fig. 5, Fig. 6 and Fig. 7.

In germ rudiments, the extraembryonic membranes (amnion and serosa) surround the embryo proper and are connected to its entire rim. Both, ectodermal (lateral) and mesodermal (median) embryonic anlagen are in contact with the extraembryonic membranes in the anterior embryo (not shown; Handel et al., 2000). The connection persists at least for some time

while the mesoderm invaginates. In early serosa window stages (Fig. 5A, A1), the ectoderm forms two elevations (ect in Fig. 5A1) and the invaginating mesoderm between them (arrowhead in Fig. 5A1) are clearly visible. The embryo has already started to sink into the yolk and the amniotic fold is beginning to expand over the germ rudiment (af in Fig. 5A1). A bridge between amnion and mesoderm is visible (arrow in Fig. 5A1) and splits the lateral ectodermal layer in the anterior head.

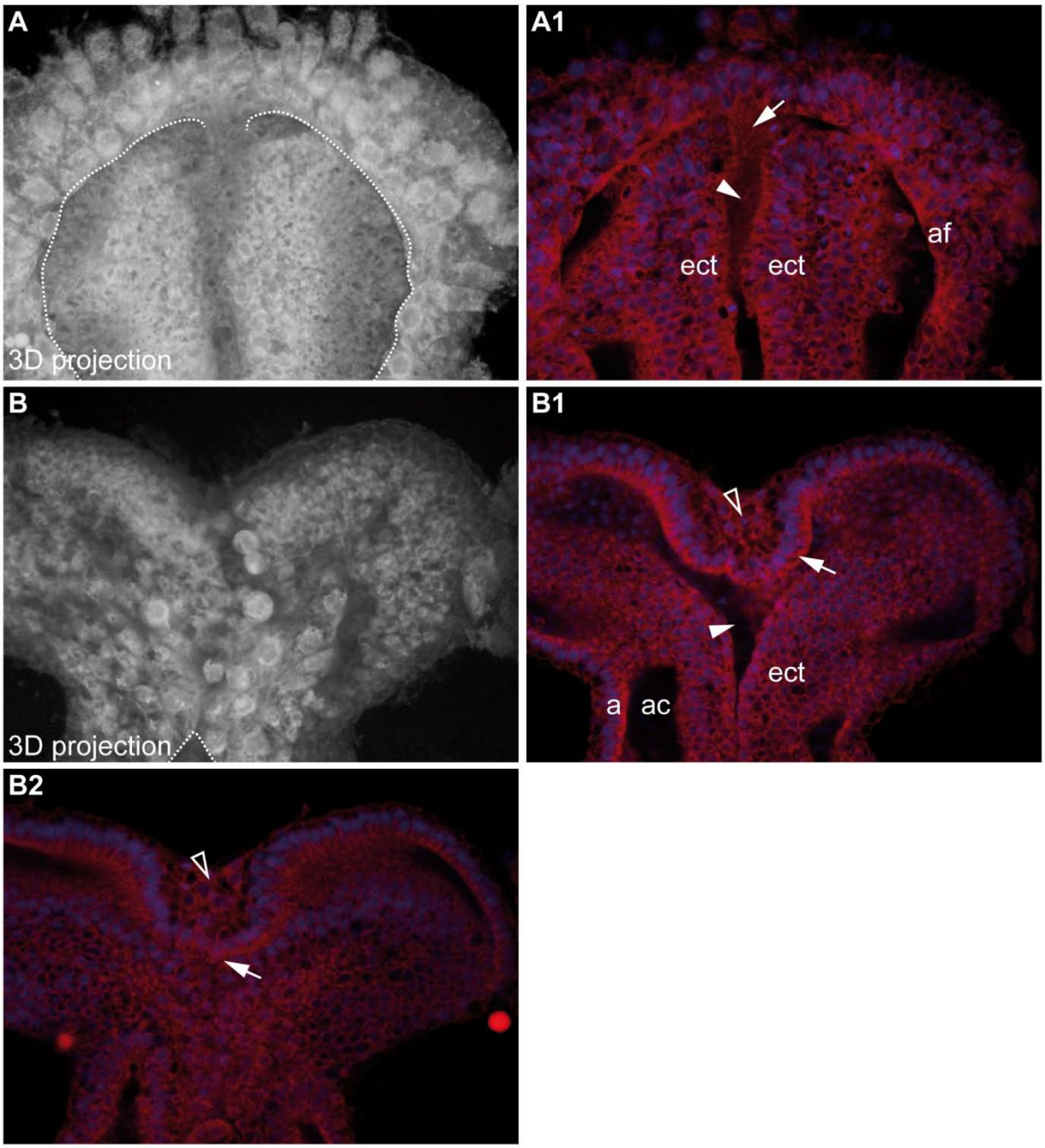


Fig. 5: FM® 1-43 staining reveals an involvement of the extraembryonic membranes in embryonic head morphogenesis. (A, B) 3D projections of confocal stacks of FM® 1-43 stained late germ rudiment and early elongating embryo. (A1, B1, B2) Single top-to-bottom planes of the respective stack; red: FM® 1-43; blue: Hoechst 33342. Anterior is up. (A, A1) The serosa window is still widely open in this germ rudiment stage embryo (dot-

ted line). The amniotic fold (af) is already present around the whole germ rudiment. The mesoderm is invaginating (arrowhead) between the bulged ectoderm (ect). The anterior invaginating mesoderm remains connected to the amniotic fold growing over the germ rudiment (arrow). (B-B2) Early elongating germ band stages still possess a small serosa window (dotted line). The amniotic cavity (ac) is clearly visible as the space between amnion (a) and embryo proper in optical sections. The ectodermal bulges fused over the mesoderm in the posterior head, while they remain separate in the anterior (white arrowhead). At the anterior tip of the germ band, a fold separating the head lobes is established (arrow in B1). Apparently, it is formed by amniotic cells. It is still connected to the mesoderm (arrow in B2). An apparently mesenchymal cell mass at the anterior tip of the embryo remains uncovered by the amnion (empty arrowhead).

As inferred from *in vivo* imaging, the position of the mesodermal-amniotic connection appears to be relatively static while the germ band starts to elongate (see attached video file on p. 110 and Sarrazin et al., 2012). An anterior movement of the ectodermal plates relative to the connection leads to the formation of a fold of the amnion between the ectodermal plates of both head sides (arrow in Fig. 5B1). This structure will be referred to as “anterior fold”. The ectoderm of the head lobes is separated by the anterior fold (Fig. 5B1, B2). The ectodermal bulges (ect in Fig. 5B1) fuse in the posterior head to cover the mesoderm completely, while they stay separated in the anterior by the anterior fold (white arrowhead in Fig. 5B1). Cells are visible within the anterior fold, which do not appear to be organized in an epithelium, but rather mesenchymal (empty arrowhead in Fig. 5B1 and B2). Thus, they probably belong to the mesoderm or are endodermal precursor cells.

The anterior fold separating the anterior ectoderm can still be recognized during further development (arrows in Fig. 6A1, A2). It still encloses the mesenchymal cell mass mentioned above (empty arrowhead in Fig. 6A3) and separates the ectoderm (ect in Fig. 6A2) of both head lobes. The amniotic cavity is clearly visible posterior to the anterior fold where the ectoderm apparently has not yet fused completely (arrowhead in Fig. 6A2).

The anterior fold is not detectable anymore when the labrum anlagen start to become clearly visible (Fig. 6B-B2). It has either merged with the embryo proper or both have become very closely attached. The ectodermal tissue of both head lobes appears to be continuous (Fig. 6B2). A sub-terminal connection between the germ band and the amnion persists (arrow in Fig. 6B1), which might be the remnants of the anterior fold. It appears to separate the paired labrum anlagen (lr in Fig. 6B1). The posterior end of this connection matches the position of the later forming stomodaeum.

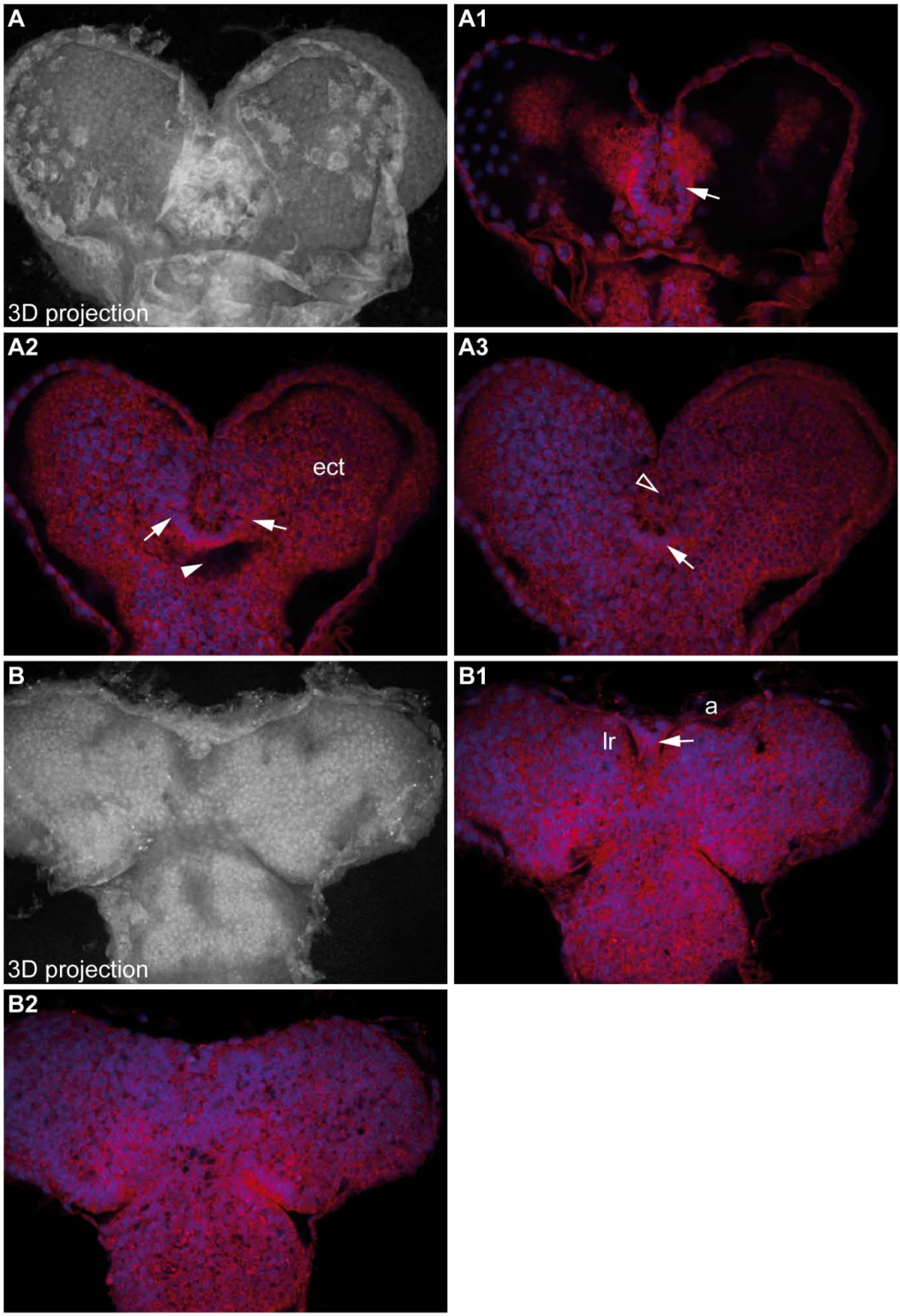


Fig. 6: The anterior fold separates the ectoderm of the head lobes. (A, B) 3D projections of confocal stacks of FM® 1-43 stained late germ rudiment and early elongating embryo. (A1-A3, B1, B2) Single top-to-bottom

planes of the respective stack; red: FM® 1-43; blue: Hoechst 33342. Anterior is up. (A1-A3) The anterior fold becomes narrower during following developmental stages and encloses the previously uncovered mesenchymal cell mass (empty arrowhead in A3). It still separates the ectoderm of both head lobes. The fold is connected to the ectoderm at the sides (arrows in A1). Posterior of the fold, a cavity is present in top planes (arrowhead in A2). Apparently, here the ectodermal bulges are still separated and the connection between anterior fold and mesoderm persists (arrow in A3). (B-B2) When the labrum (lr) starts to develop, the anterior fold is not visible anymore. The ectoderm of both head lobes has probably fused as it appears to be contiguous across the midline (B2). A connection between germ band and amnion (a) at a sub-terminal position might be the remnant of the anterior fold (arrow). It appears to separate the labrum anlagen.

The connection between germ band and amnion is partly still visible when the stomodaeum (st in Fig. 7) begins to invaginate (arrows in Fig. 7A1 and A2). It is located directly in front of the stomodaeal invagination site. As already mentioned, the stomodaeum invagination appears to match the posterior end of the sub-terminal connection between amnion and embryo proper. It remains to be confirmed by staining with appropriate markers (see below) whether the anterior fold forms the stomodaeum.

The connection cannot be recognized anymore during further development when major morphogenetic movements take place (Fig. 7B, B1): all head appendages are elongating and the stomodaeum invagination is progressing towards the interior and becomes enclosed by mesenchymal cells (mes in Fig. 7B1).

The apparent folding of the extraembryonic membranes and its role in the separation of the head lobes and the formation of the overall shape of the head were unexpected. I will come back to this finding when I discuss the development of gene expression patterns in the head.

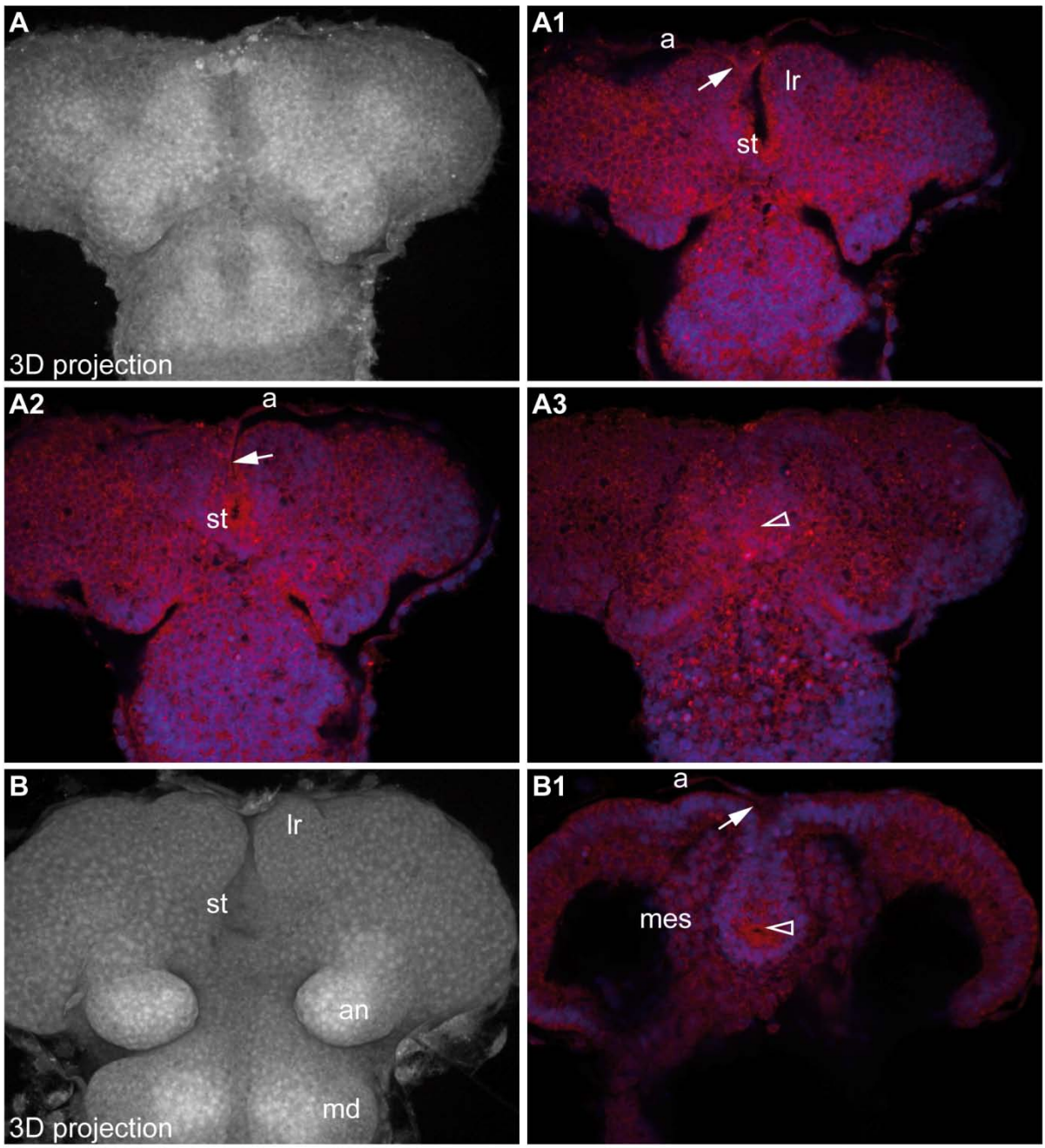


Fig. 7: The stomodaeum appears to form at the position of the anterior fold. (A, B) 3D projections of confocal stacks of FM® 1-43 stained late germ rudiment and early elongating embryo. (A1-A3, B1) Single top-to-bottom planes of the respective stack; red: FM® 1-43; blue: Hoechst 33342. Anterior is up. (A-A3) During the beginning invagination of the stomodaeum (st), the amnion (a) is still visible around the germ band. The apparent remnants of the anterior fold (arrows) still separate the labrum anlagen (lr). The stomodaeum appears to form at the position where the mesenchymal cell mass was enclosed by the anterior fold (compare its position to the empty arrowhead fold in Fig. 6). It is also visible in bottom planes (empty arrowhead in A3). (B, B1) When the anlagen of labrum, stomodaeum, antennae (an) and mandibles (md) are clearly visible, remnants of the amnion are still detectable at the anterior end of the embryo (arrow). The putative mesoderm (mes) surrounds the invaginating stomodaeum (empty arrowhead).

4.1.3 Contribution of cell death and proliferation to anterior head development

Proliferation and cell death are also important players when it comes to development of morphology. I analyzed to which extent they contribute to the morphogenesis of the anterior embryonic head. Embryos were subdivided into three developmental classes: (1) germ rudiments (Fig. 8A); (2) elongating germ bands (Fig. 8B-D, G, H); (3) retracting germ bands (Fig. 8E, F). Dying and proliferating cells were counted for each class. Cell death was visualized with the TUNEL method (Fig. 8A-F, I; see Gavrieli et al., 1992) and proliferating cells were immunolabeled with antibodies against phosphorylated Histone H3 (Fig. 8G, H, J, K; see Hendzel et al., 1997).

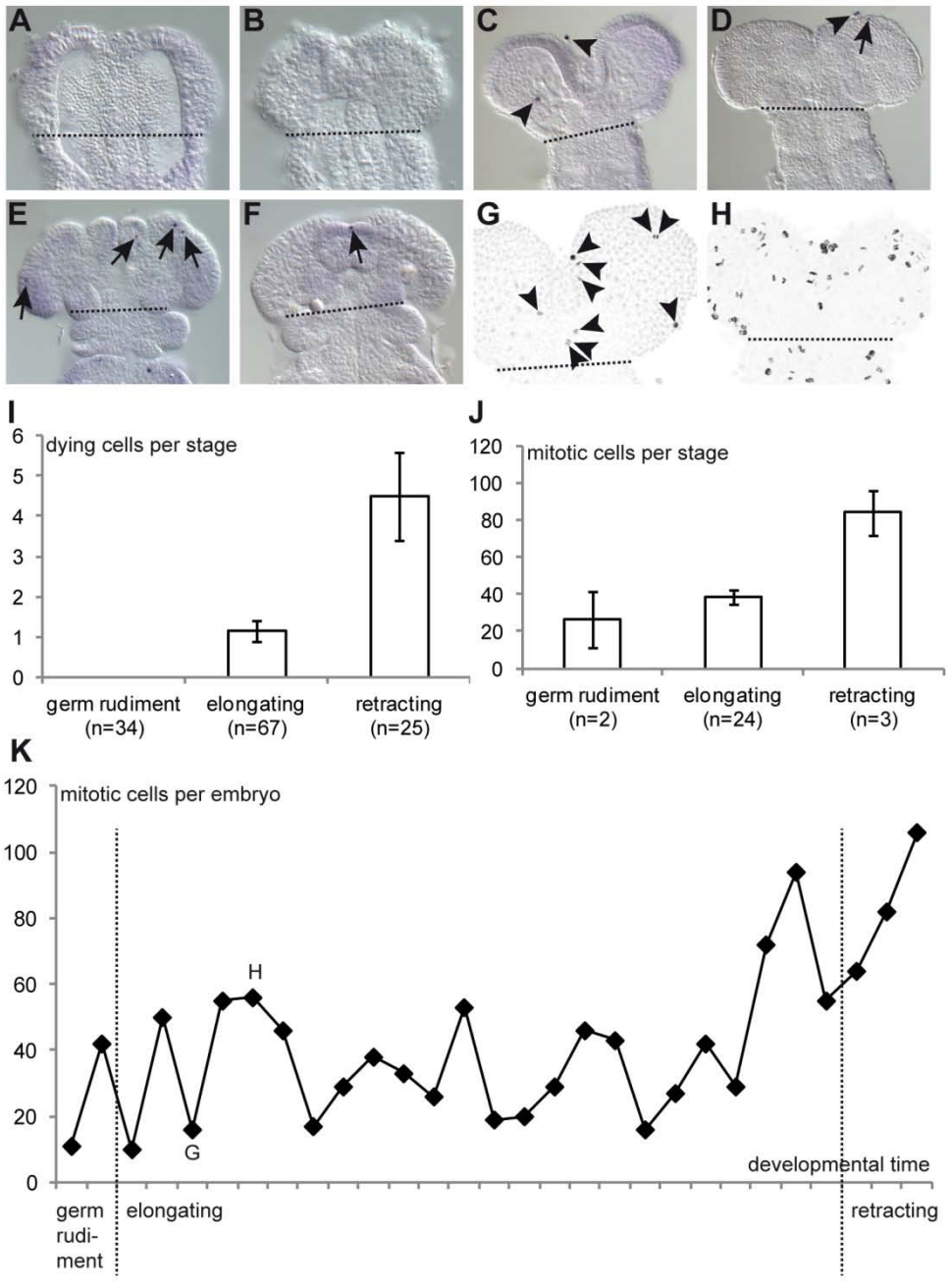


Fig. 8: Cell death and proliferation in the anterior head. (A-F) Successively older embryos after TUNEL. (G, H) Inverted maximum projections of the green channel of confocal stacks of embryos stained with anti-PH3 antibody. Dotted lines indicate the boundary between anterior and posterior head. Only cells anterior to this boundary were counted. Anterior is up. (I) Mean numbers of dying cells per stage. (J) Mean numbers of proliferating cells per stage. (K) Number of proliferating cells per embryo. (A) No cell death is detected in germ rudiments. (B-D) Few dying cells are visible during germ band extension. Cells in the serosa (arrowheads) as

well as in the embryo proper (arrows) undergo cell death. (E, F) More dying cells are found in retracting germ bands (arrows). All of them are cells of the embryo proper. (G, H) Two embryos of an age corresponding approximately to (C) show huge differences in the numbers of proliferating cells. 10 cells undergo mitosis in (G); 56 undergo mitosis in (H). I did not differentiate between cells of embryonic and extraembryonic tissue or between different germ layers. (I) Cell death levels rise during development. (J) Levels of proliferation appear to increase with age, but only two and three embryos were analyzed for germ rudiments and retracting germ bands, respectively. (K) Embryos were organized by age and proliferating cells were counted. Embryos that appear to be of similar age can vary largely in the number of proliferating cells. The embryos shown in (G) and (H) are indicated.

No dying cells were found in embryos of the germ rudiment stage (n = 34; Fig. 8A and I). During elongation, few dying cells can be found (1.2 dying cells per embryo; n = 67; Fig. 8B-D and I). Most of them are cells of the serosa, as inferred from their size and position (arrowheads in Fig. 8C and D). Retracting germ bands show highest levels of cell death with a mean value of 4.5 dying cells per embryo (n = 25; Fig. 8E, F and I). I find, however, embryos without dying cells as well as embryos with 19. Dying cells appear to be not localized to a specific region (e.g. Fig. 8E), which makes an important role in the shaping of specific head parts rather unlikely. Numbers of dying cells can also be found in the appendix (p. 112).

Proliferation appears to increase with age (Fig. 8J). However, due to time limitation, examination of proliferation in the anterior head was not done to a satisfying extent: only two germ rudiments and three retracting germ bands were analyzed. Numbers of proliferating cells per embryo range from 10 to 94 in elongating germ bands (n = 24; mean = 38.4) and no clear trend is observable (Fig. 8K). Numbers can also be found in the appendix (p. 111).

4.2 A set of genes is expressed in the CLR and is required for its proper development

4.2.1 Expression of genes in the anterior head

Previous works indicated that the anterior head can be subdivided into a median region which is mostly free of neurogenic cells (the CLR) and a lateral neurogenic region. Gene expression patterns in the head can be subdivided into three categories: (1) expression predominantly in the CLR; (2) expression excluding the CLR; (3) ubiquitous or broad expression (Kittelmann, 2008; Posnien, 2009; Posnien et al., 2010; Koniszewski, 2011; Posnien et al., 2011b). I wanted to test the hypothesis that the expression patterns recapitulate sharp boundaries between a median labral-stomodaeal and an outer neurogenic compartment.

First, I systematically searched for new genes expressed exclusively in the CLR (Kittelmann, 2008). Therefore, I screened the literature and the *Drosophila* databases BDGP

(Tomancak et al., 2002) and FlyExpress (Kumar et al., 2011; Konikoff et al., 2012) for genes that were expressed in clypeolabrum and foregut or in a similar pattern to *cnc*, respectively. Homologous genes were cloned from *Tribolium* cDNA and their expression pattern was determined by *in situ* hybridization (Schinko et al., 2009). Promising candidates were afterwards knocked down by parental RNAi (Posnien et al., 2009a) and defects were analyzed in developing L1 larvae as well as in embryos. I also did double *in situ* hybridizations with genes expressed in the CLR and in a surrounding fashion.

Most expression patterns of the candidate genes had not been described when I started my PhD thesis. In the meantime, however, all but *Tc-tup* have already been published. Hence, I am just briefly describing their expression patterns with focus on aspects required for subsequent discussion

Seven transcription factors are expressed specifically in the developing CLR

Most of the genes expressed specifically in the CLR (in the following: “CLR markers”) do not show highly dynamic changes of expression patterns. Rather, the pattern appears to change together with the already described morphogenetic movements in the head. This is especially true for the early expressed CLR markers. The major expression domain is shifted from a terminal to a sub-terminal location (*Tc-croc*, *Tc-scro*, median domain of *Tc-six3*, labral domains of *Tc-chx*) or the genes are expressed along the anterior fold and change with its appearance: they start to be expressed as two domains which fuse in the posterior and spread around the time when the anterior fold apparently merges with the ectoderm (*Tc-cnc*, *Tc-scro*). In most cases, expression in other head regions arises later than in the CLR.

Tc-six3 expression has been described in detail by Posnien et al., (2009b; 2011b). Expression starts in blastoderm stages (not shown), which makes *Tc-six3* the earliest CLR marker. Early germ rudiment stages (Fig. $9A_1$) show expression in a single domain, which is weaker in the anterior mesoderm (arrowhead in Fig. $9A_1$) and resolves into three separate domains during germ band elongation (Fig. $9A_2$-A_5). The outer domains (arrow in Fig. $9A_3$-A_7) mark neurogenic precursors (Posnien et al., 2011b) while the central domain marks clypeus, labrum and the roof of the stomodaeum. The central domain is expressed along the anterior fold during germ band extension.

Expression of *Tc-croc* has been described by Economou and Telford (2009). It starts only slightly later than *Tc-six3*. It is expressed in an anterior domain comprising the anterior mesoderm anlage in early germ rudiment stages (Fig. $9B_1$). Expression persists as a single domain until later elongating germ band stages (Fig. $9B_2$-B_3). Subsequently, expression decreases in the probably mesodermal center of the domain, while strong expression persists in the proba-

bly ectodermal lateral parts (Fig. 9B_4-B_5). The domain retracts from the anterior rim of the embryo, eventually leading to a half ring of expression around the posterior rim of the stomodaeum (Fig. 9B_5-B_7; compare to expression of *Tc-fkh* in the stomodaeum anlage, Fig. 9E_2-E_6). During germ band elongation, additional domains arise in the ocular region (arrow in Fig. 9B_5-B_7) and weak expression domains in a segmental pattern as well as a domain in the growth zone become visible (not shown). Weak expression can also be found in the labrum anlagen (arrowhead in Fig. 9B_6).

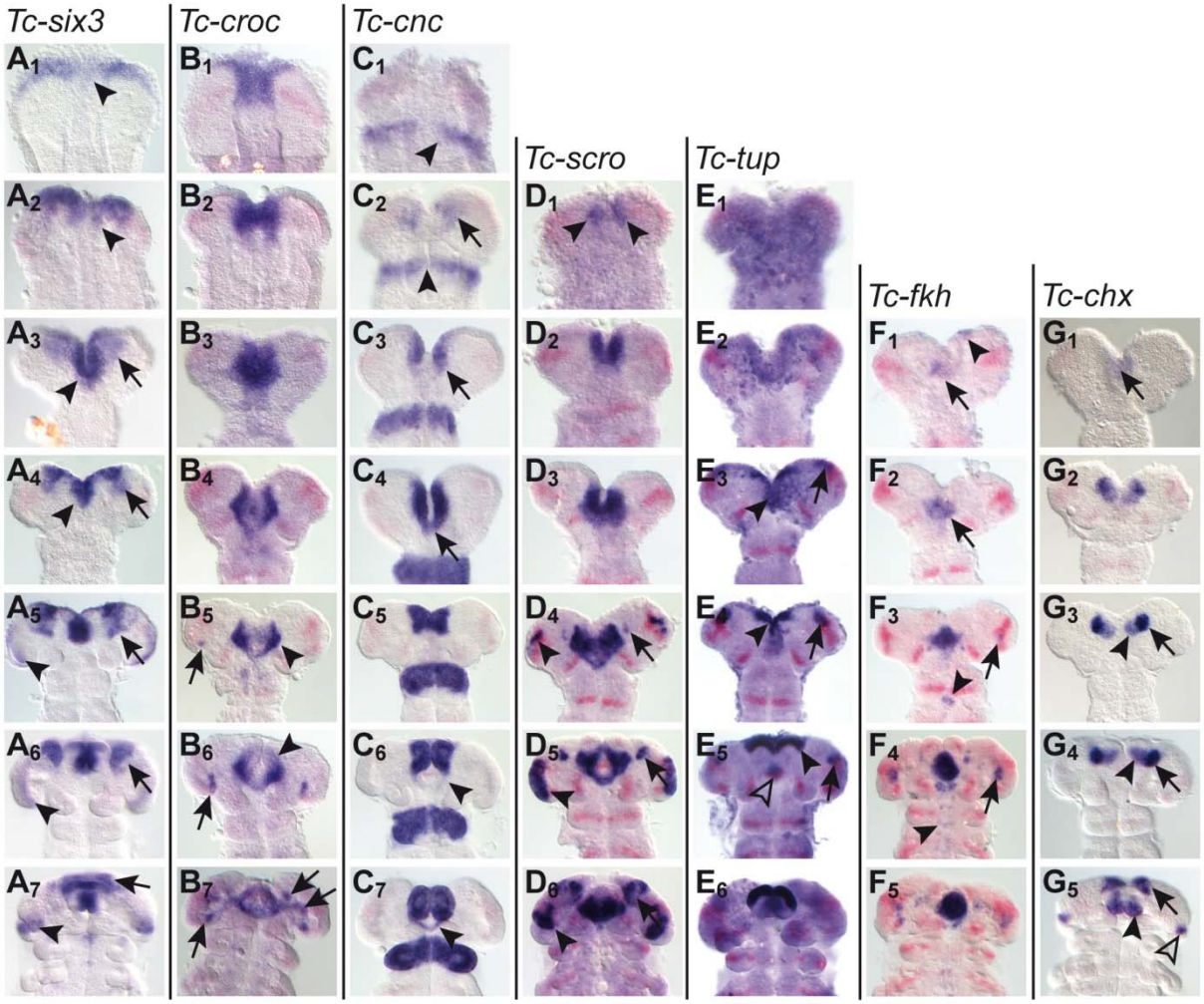


Fig. 9: Genes with a dominant expression in the CLR. Embryos in a row are of approximately the same age. Each column shows staining the same gene in successively older embryos; gene names are indicated. Red staining, where present, marks *Tc-wingless* (*Tc-wg*) expression. Anterior is up in all images. (A_1-A_7) Early germ rudiments show *Tc-six3* expression in a single domain at the anterior rim which is weaker in the center (arrowhead in A_1). Expression shifts towards the posterior in serosa window stages (arrowhead in A_2). During germ band elongation, expression splits into three separate domains, the two outer ones marking neurogenic tissue (arrow in A_3-A_7), the inner one marking the labrum and the stomodaeum roof (arrowhead in A_3 and A_4). The central domain is clearly marking the anterior fold (arrowhead in A_3). Additional domains arise in the ocular region during later germ band elongation (arrowheads in A_5-A_7). The *Tc-six3* expressing neurogenic tissue fuses dorsally from the labrum during later embryogenesis (arrow in A_7). (B_1-B_7) Expression of *Tc-croc* starts as a

single domain in germ rudiments, comprising the anterior mesoderm anlage (B_1). Expression condenses in serosa window stages (B_2) and is still detectable as a single domain comprising mesodermal cells during early germ band elongation (B_3). Expression ceases in the center during further development (B_4). The remaining expression surrounds the stomodaeum anlage posterior and is considerably stronger in two lateral spots (arrowhead in B_4). A secondary domain arises in the ocular region (arrow in B_5 and B_6). Weaker expression is detectable in the labrum anlagen during later developmental stages (arrowhead in B_6) leading to a ring of expression around the stomodaeum (B_7). Further expression domains connected to the central domain arise in the neurogenic region (arrows in B_7). (C_1-C_7) Expression of *Tc-cnc* starts in the ectoderm of the mandible segment anlage in older germ rudiments (arrowhead in C_1). The expression domains are separated by the mesoderm between them. The lateral plates fuse in the serosa window stage (arrowhead in C_2) and the mandible expression becomes a single domain. Two separate domains arise at the anterior of the embryo, which are expressed along the anterior fold in subsequent stages (arrow in C_2-C_4) and eventually fuse at the posterior (arrow in C_4). Slightly later, *Tc-cnc* is broadly expressed and marks the labrum anlagen (C_5-C_7). Weak expression stripes surround the stomodaeum in later stages (arrowheads in C_6 and C_7). (D_1-D_6) Expression of *Tc-scro* starts as two separate spots at the anterior (arrowheads in D_1). Like *Tc-cnc*, expression of *Tc-scro* marks the anterior fold in the anterior head, but at a slightly more posterior position (D_2, D_3) and has no contact to the anterior rim of the head in older embryos (D_4). Expression spreads out during germ band extension (D_3, D_4) and secondary domains arise in the ocular region (arrowhead in D_4-D_6) and in the neurogenic region (arrow in D_4-D_6). Expression ceases in the center of the median domain while the stomodaeum invaginates (D_5). Expression is thus restricted to the region around the stomodaeum and the proximal parts of the labrum anlagen, where it continues in older embryos (D_6). (E_1-E_6) *Tc-tup* is expressed in the extraembryonic membranes in early germ bands (E_1, E_2). Expression concentrates in the anterior fold (arrowhead in E_3 and E_4) and additional expression starts in the pre-ocular region (arrow in E_3-E_5). The anterior rim of the developing labrum shows strong expression in older embryos (black arrowhead in E_5), and weaker expression is detected in the stomodaeum (empty arrowhead in E_5). These domains persist during later stages (E_6). (F_1-F_5) Expression of *Tc-fkh* is first visible in yolk cells (arrowhead in F_1). It starts being expressed in the stomodaeum anlage during early germ band elongation (arrow in F_1 and F_2). Secondary domains arise in the ocular region (arrow in F_3 and F_4) and in the nervous system (arrowhead in F_3 and F_4) during later germ band extension. Expression is strongest in the stomodaeum (F_4, F_5). (G_1-G_5) Expression of *Tc-chx* is first detected as a single anterior domain during early germ band elongation (arrow in G1). The domain splits into two separate domains (G_2) with weaker expression medially (arrowhead in G_3 and G_4) and stronger expression laterally (arrow in G_3 and G_4). The stronger region marks the neurogenic tissue that fuses dorsally of the labrum during later development (arrow in G_5), whereas the weaker domain persists at the base of the labrum (arrowhead in G_5). An additional domain in the ocular region is detected in older embryos (empty arrowhead in G_5).

Also expression of *Tc-cnc* has been analyzed by Economou and Telford (2009). Expression is first seen in the mandibular segment (arrowhead in Fig. 9C_1 and C_2). Expression in the labrum starts as two separate domains (arrow in Fig. 9C_2) which expand towards the posterior along the anterior fold (arrow in Fig. 9C_3) and eventually fuse at the posterior tip of the fold (arrow in Fig. 9C_4). Expression spreads in the anterior median ectoderm during the time when the anterior fold merges with the ectoderm (Fig. 9C_5; see Fig. 6). The region *Tc-cnc* is ex-

pressed in will give rise to the labrum. Expression in the labrum anlagen persists throughout embryonic development. A half ring around the posterior rim of the stomodaeum arises in fully elongated germ bands (arrowheads in Fig. 9C_6 and C_7) and is expressed in the same region as *Tc-croc* (see Economou and Telford, 2009).

Similar to *Tc-cnc*, *Tc-scro* starts being expressed as two separate domains (arrowheads in Fig. 9D_1; Posnien et al., 2011b) which extend along the anterior fold and fuse during germ band elongation (Fig. 9D_2 and D_3). The expression domain retracts from the anterior rim of the embryonic head while spreading in the ectoderm during later elongating germ band stages, leaving the distal part of the labrum anlagen free of expression (Fig. 9D_4). At the same time, additional domains arise in the ocular region (arrowhead in Fig. 9D_4-D_6) and lateral of the central domain (arrow in Fig. 9D_4-D_6). Expression in the center of the major domain fades away in fully elongated germ bands, eventually leading to a ring shaped expression around the stomodaeum (Fig. 9D_5).

Expression of *Tc-tup* starts in the extraembryonic membranes (Fig. 9E_1 and E_2) and becomes stronger in the anterior fold (arrowhead in Fig. 9E_2 and E_3). It resolves into one domain at the anterior rim of the labrum anlagen (black arrowhead in Fig. 9E_5) and one in the stomodaeum (empty arrowhead in Fig. 9E_5). Expression domains in the ocular region (arrow in Fig. 9E_3-E_5) arise during germ band elongation.

Tc-fkh expression has already been characterized by Schröder et al. (2000). *Tc-fkh* is expressed in yolk nuclei from early germ rudiment stages (not shown) until early elongating germ band stages (arrowhead in Fig. 9F_1). It becomes expressed in the stomodaeum (arrow in Fig. 9F_1) and the proctodaeum (not shown) during germ band elongation. Here, expression persists throughout development. Additional expression domains arise along the ventral midline of the embryo (arrowhead in Fig. 9F_3 and F_4) and in the ocular region (arrow in Fig. 9F_3 and F_4).

In contrast to Posnien et al. (2011b), I find *Tc-chx* starting to be expressed in a single domain at the central anterior rim of the embryonic head (arrow in Fig. 9G_1). The exact assignment of the expressing cells to one of the germ layers is not possible. At this time, the anterior fold already starts to fuse with the ectoderm. Hence, cells in this location could be ectodermal. The domain subsequently splits into two separate domains with an inner part of weaker expression eventually marking the labrum (filled arrowhead in Fig. 9G_3-G_5) and an outer part of stronger expression eventually marking the anlagen of the pars intercerebralis (arrow in Fig. 9G_3-G_5; see de Velasco et al., 2007 and Posnien et al., 2011b). An additional domain arises in the ocular region of fully elongated germ bands (empty arrowhead in Fig. 9G_5).

A schematic overview of the CLR marker genes is given in Fig. 10. It was generated by analysis of single as well as double *in situ* hybridizations (see below).

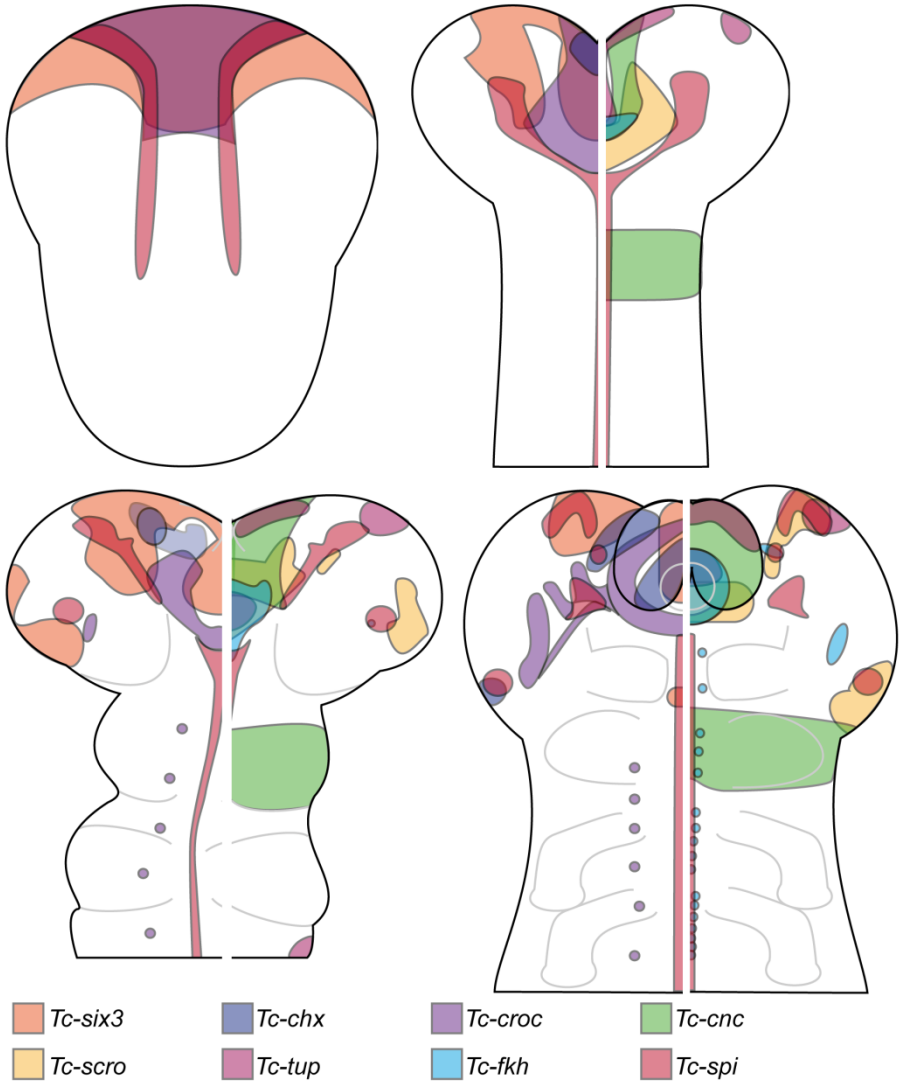


Fig. 10: Schematic overview of the CLR markers and *Tc-spi* (see below). Expression in germ rudiments (upper left), during germ band extension (upper right), in completely elongated (lower left), and in retracting germ bands (lower right) is shown. For each gene, expression in only one half of the embryo is given, except for germ rudiments, where only *Tc-croc* and *Tc-six3* are expressed. Only few markers show additional expression outside of the CLR during germ band extension. Secondary domains mostly arise during later germ band extension, but the most prominent expression domain persists in the CLR also in completely elongated germ bands. The expression patterns become more complex during germ band retraction. *Tc-spi* frames the CLR markers but overlaps with *Tc-croc* and probably also with *Tc-scro*. Outlines for the schematic embryos and for the expression patterns of *Tc-six3* and *Tc-scro* were provided by N. Posnien (see Posnien, 2009; Posnien et al., 2011b).

Another set of transcription factors frames the CLR

The CLR has been defined as the non-neurogenic part of the non-segmental anterior head. Clear boundaries between the neurogenic and the non-neurogenic region could indicate that both are in fact distinct developmental units. The genes described above mark the CLR specifically throughout embryonic development. In contrast to that, many genes expressed in the anterior head appear to exclude the CLR (in the following: “outside markers”). Double *in situ*

hybridizations for CLR markers and outside markers were used to test whether boundaries between the CLR and the neurogenic part of the anterior head could be defined.

Expression of the outside markers has already been described. I chose genes as markers which are expressed early in the anterior head in a pattern excluding the CLR. Taken together, all outside markers frame the CLR perfectly. The chosen outside markers are *Tc-otd-1* (Li et al., 1996; Schröder, 2003; Schinko et al., 2008), *Tc-tll* (Schröder et al., 2000; Posnien et al., 2011b), *Tc-empty spiracles* (*Tc-ems*; Schinko et al., 2008), *Tc-labial* (*Tc-lab*; Nie et al., 2001; Economou and Telford, 2009; Posnien and Bucher, 2010) and *Tc-spi* (Kittelmann, 2008; Grossmann, 2011). As a marker for neurogenic tissue, the neuroblast marker *Tc-ase* (Posnien et al., 2011b) was used.

The first CLR markers whose early expression patterns appear to be highly precise are *Tc-croc* and *Tc-cnc*. Expression of both genes together marks the complete CLR: *Tc-croc* marks the whole CLR during early development and the stomodaeum region during later stages, while *Tc-cnc* marks the anterior CLR with the labrum anlagen. They were therefore chosen as good CLR markers for counterstaining. The results of the double *in situ* hybridizations are shown in Fig. 11.

Tc-cnc is expressed mainly in the anterior CLR and only later in the posterior. Hence, co-expression was only tested for outside markers expressed in the anterior. *Tc-cnc* is not co-expressed with *Tc-tll*, *Tc-spi* or *Tc-ase* (Fig. 11A-D). Additionally to marking the lateral neuroectoderm, *Tc-ase* in also marks neuroblasts in the stomodaeum that will later probably contribute to the stomatogastric nervous system (arrow in Fig. 11G and I'; see Hartenstein et al., 1996; Posnien et al., 2011b). These neuroblasts are surrounded by *Tc-cnc*, but also this expression does probably not overlap.

In contrast to *Tc-cnc*, *Tc-croc* shows overlapping expression with *Tc-otd1* (Fig. 11E-F'), *Tc-ems* (Fig. 11G), *Tc-tll* (Fig. 11H, H'), *Tc-spi* (Fig. 11J, J') and *Tc-ase* (Fig. 11K, L) in the lateral part of its expression domain. The region I detect overlapping expression in appears to be the same for all genes (compare to arrowhead in Fig. 11M). It is the region with stronger *Tc-croc* expression lateral of the stomodaeum (compare to arrowhead in Fig. $9B_5$). In the posterior, expression of *Tc-croc* borders directly to that of *Tc-lab* (arrowhead in Fig. 11I; see Economou and Telford, 2009) and *Tc-spi* (arrow in Fig. 11J').

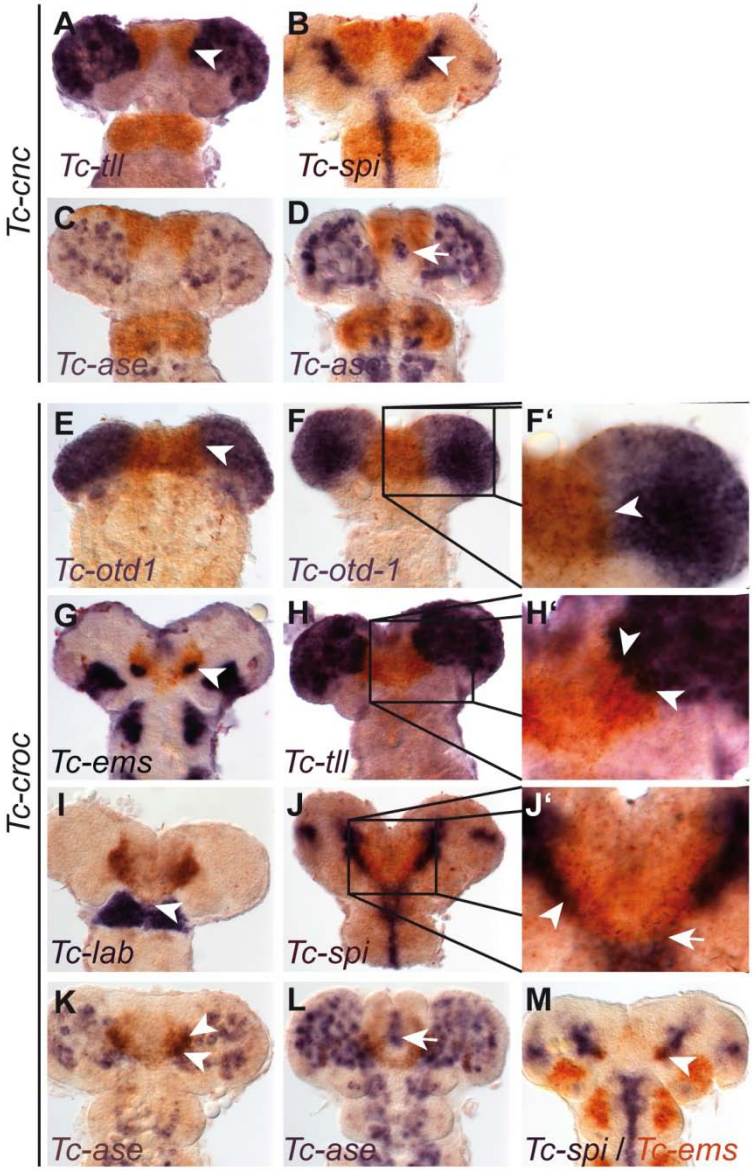


Fig. 11: Double *in situ* hybridizations of CLR markers and outside markers. Anterior is up in all images. (A-D) *Tc-cnc* expression is stained in orange; outside marker expression is stained in blue. (E-L) *Tc-croc* expression is stained in orange or brown; outside marker expression is stained in blue. (M) Double *in situ* hybridization of two outside markers. Gene names are indicated for each staining except for the magnifications. (A) *Tc-tll* is expressed in the neurogenic parts of the non-segmental region and in the ocular region (Schröder et al., 2000). *Tc-tll* and *Tc-cnc* are expressed directly adjacent (arrowhead in A), but expression does not overlap. (B) *Tc-spi* is expressed along the midline during germ band elongation and forms a Y-shaped expression pattern in the head (Kittelmann, 2008; Grossmann, 2011). Expression of *Tc-cnc* is directly adjacent to that of *Tc-spi* (arrowhead in E). (C, D) The neuroblast marker *Tc-ase* is expressed lateral of *Tc-cnc*. During later germ band elongation, *Tc-ase* expression can be detected in a small subset of neuroblasts delaminating from the stomodaeum and presumably forming the stomatogastric nervous system (arrow in D; Posnien et al., 2011b). Also these later forming neuroblasts develop in the *Tc-cnc*-free region. (E-F') *Tc-otd1* shows expression in the head lobes as well as along the midline (Li et al., 1996). The most lateral cells expressing *Tc-croc* also show expression of *Tc-otd-1* (arrowheads in E, F'). (G) *Tc-ems* is expressed in the antennal segment and in a segmental fashion. Secondary domains arise lateral from the stomodaeum anlage in elongating germ bands (Schinko et al., 2008). *Tc-ems* and *Tc-croc* are not co-expressed in early developmental stages (not shown). However, the secondary *Tc-ems* domains arise within the lateral tips of *Tc-croc* expression (arrowhead). (H, H') *Tc-tll* and *Tc-croc* show overlapping expression in the lateral region of the *Tc-croc* domain, as well (arrowheads in H'). (I) The intercalary marker *Tc-lab* (Nie et al., 2001) is expressed directly adjacent to the posterior tip of *Tc-croc* expression (arrowhead; see also Economou and Telford, 2009). (J, J') *Tc-spi* and *Tc-croc* expression overlap in the lateral part of the *Tc-croc* domain (arrowhead in F'). The overlapping region appears to be the same as for *Tc-tll* and *Tc-ems* (compare to arrowheads in B' and C). Expression in the posterior does not overlap (arrow in F'). (K, L) At least two cells show co-expression of *Tc-ase* and *Tc-croc* (arrowheads in K), whereas the presum-

able stomatogastric neuroblasts lie in the *Tc-croc* free region (arrow in K). (M) *Tc-spi* and *Tc-ems* are co-expressed in the same region, in which they overlap with *Tc-croc* expression (arrowhead).

In the posterior, *Tc-croc* and *Tc-lab* are expressed in adjacent cells. In the anterior, there is a clear boundary between expression of *Tc-cnc* and all lateral markers. Thus, the clypeolabrum and the stomodaeum anlagen are clearly separable from the surrounding neurogenic tissue and the posterior intercalary segment. However, *Tc-croc* marks in addition cells in the neurogenic head region, where its expression overlaps with the respective marker genes. Hence, it does not function as a marker for the lateral CLR boundary.

Fig. 12 shows a schematic of the outside markers.

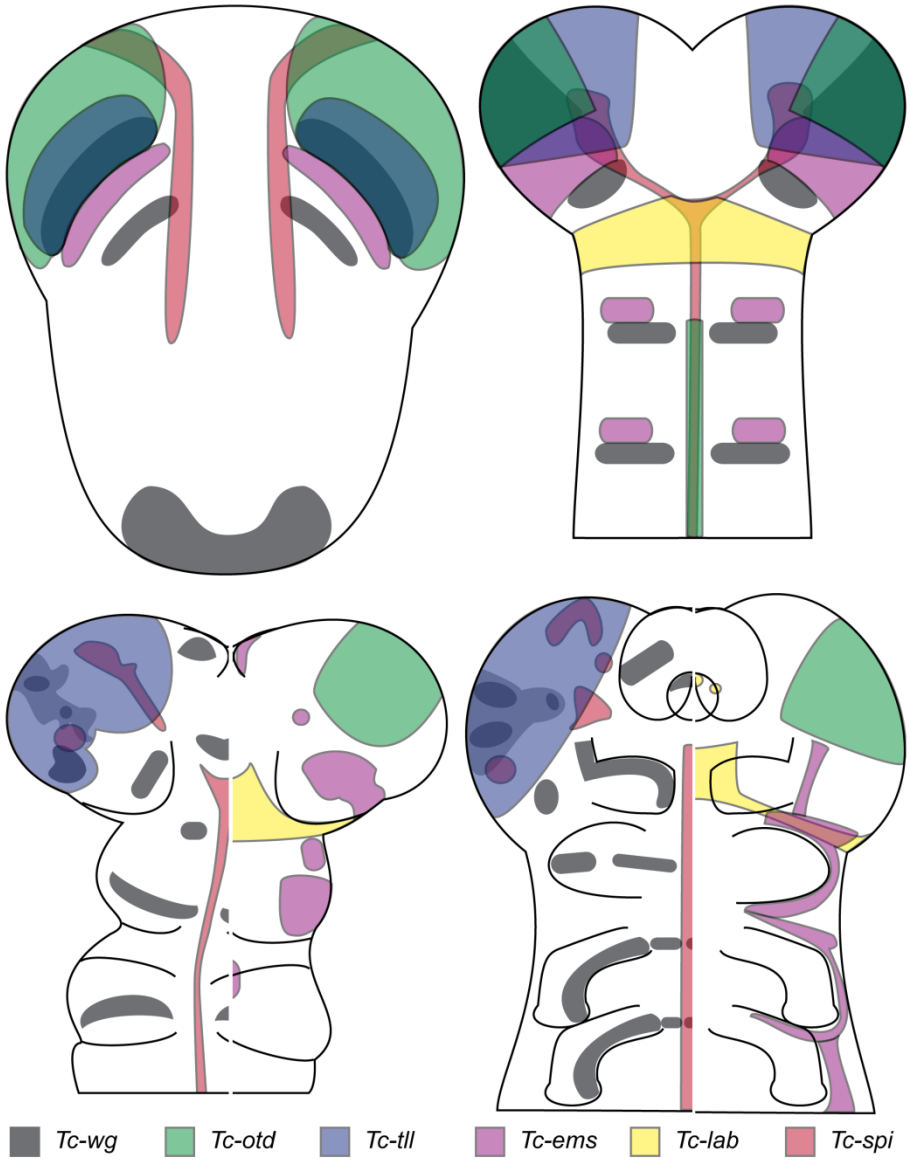


Fig. 12: Schematic overview of the outside markers. Expression in germ rudiments (upper left), during germ band extension (upper right), in completely elongated (lower left), and in retracting germ bands (lower right) is shown. For each gene, expression in only one half of the embryo is given for completely elongated and retracting germ bands. The outside markers show either no expression in the CLR and rather frame it or have transient and small expression domains apparently marking a subset of CLR cells. Outline for the schematic embryos and for the expression patterns of *Tc-wg*, *Tc-otd-1*, *Tc-tll* and *Tc-ems* provided by N. Posnien (see Posnien, 2009; Posnien et al., 2011b).

Expression of further genes tested as markers

Some of the genes I tested as CLR markers or outside markers due to their expression pattern in *Drosophila* turned out to be either expressed very differently than expected or in a pattern that was not specific enough to serve as good markers. Expression of *Tc-hbn* will be described for subsequent discussion; please refer to Kittelmann (2008) for details of the other genes.

Tc-hbn is not expressed in early blastoderm stages (not shown), but expression starts during blastoderm in an anterior cap (arrowhead in Fig. 13A). Early germ rudiment stages show expression in the anterior head, whereas the median region is free of expression (Fig. 13C). The expression becomes restricted to the outer anterior head lobes during germ band extension (Fig. 13D) and eventually the ocular region, where it is expressed as half circles (Fig. 13F).

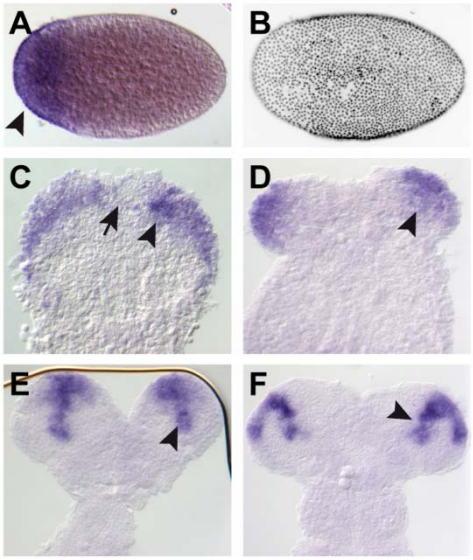


Fig. 13: Expression of *Tc-hbn*. Anterior is left in A and B; anterior is up in C-F. (A, B) Blastodermal stages start expressing *Tc-hbn* in an anterior cap (arrowhead). (C) Expression becomes restricted to the outer parts of the anterior head anlagen (arrowhead). The median region shows no expression (arrow). (D) Expression is found in the outer head lobes during early germ band extension (arrowhead). (E) Expression stripes extend towards the posterior in older elongating germ bands (arrowhead). (F) Eventually, the expression pattern becomes a half circle in the ocular region (arrowhead).

The terminal gap gene *hkb* is known as a marker for the endodermal anterior midgut primordium in *Drosophila* and has an important function in development of the anterior gut (Weigel et al., 1990; Brönner and Jäckle, 1991; Reuter and Leptin, 1994; Brönner et al., 1994; Brönner and Jäckle, 1996). *In situ* hybridization for *Tc-hkb* showed that the expression pattern is not conserved in *Tribolium*. Expression does not start before germ band elongation and is then found in a segmental fashion (Fig. 14).

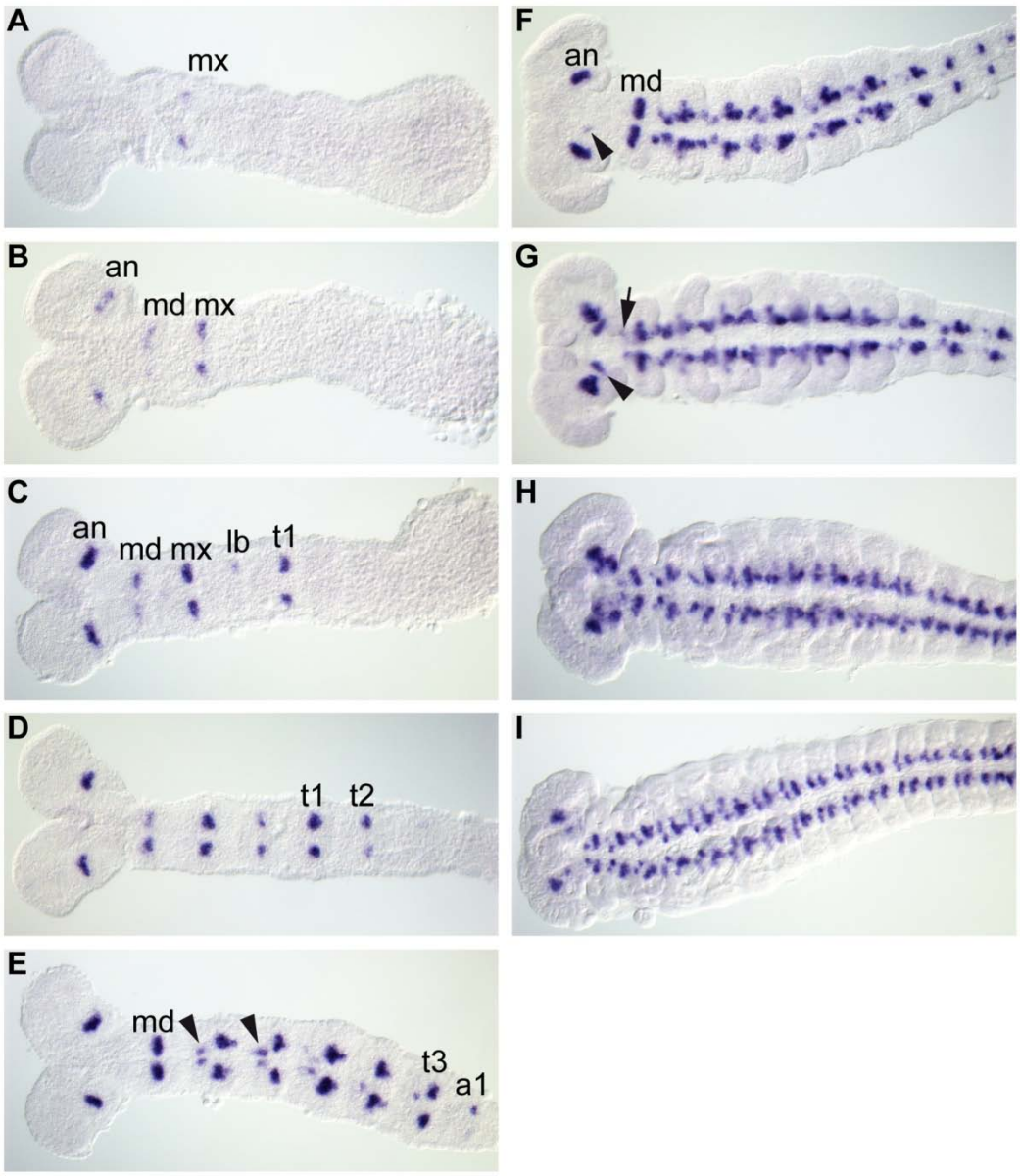


Fig. 14: Expression of *Tc-hkb* is shown in successively older embryos; anterior is left. (A) Expression starts as two spots in the maxillary segment (mx) during germ band elongation. (B) Bilateral expression is detected slightly later in the mandibular (md) and antennal segment (an). (C) More posterior segments start expressing *Tc-hkb*: labial segment (lb); first thoracic segment (t1). Expression becomes stronger in every second segment, starting with the antennal segment. (D, E) Segmental expression progresses towards the posterior (t1, t2: second and third thoracic segments; a1: first abdominal segment). Secondary expression domains are detected in the maxillary segment and more posterior segments (arrowheads). (F, G) Secondary domains in the antennal segment (arrowheads) are detected before secondary domains arise in the mandibular segment (arrow). (H) All expression domains are visible in completely elongated germ bands. (I) Expression of *Tc-hkb* decreases during germ band retraction.

4.2.2 Loss of function of the markers – drawing conclusions about their role in development and gene regulation

Tc-six3 is necessary for establishment of the anterior CLR

It has already been shown that *Tc-six3* RNAi leads to the loss of a triangular region in the anterior head of the *Tribolium* embryo. This triangular region contains the anlagen of the labrum as well as of parts of the brain. The remaining parts of the embryonic head often fuse along the midline, which leads to a characteristic change of gene expression patterns: domains outside of the deleted region come closer together due to the loss of tissue between them. L1 larvae are characterized by absence of the clypeolabrum, the central body of the brain and the foregut (Posnien et al., 2009b; Posnien et al., 2011b). I evaluated gene expression patterns of markers which had not been studied previously after *Tc-six3* loss of function.

Tc-croc is expressed almost like in untreated animals and only most anterior aspects are deleted. The deleted expression domains lie probably in ectodermal tissue, while the more posterior mesodermal expression aspects appear largely unchanged (Fig. 15A_1-B_4). Expression of *Tc-cnc* (Fig. 15C_1-D_4) and *Tc-fkh* (Fig. 15E_1-F_4) is lost, as both genes are expressed within the deleted area. Aspects that lie outside of the deleted region are expressed normally, but often appear misplaced due to the strongly altered shape of the head.

Expression of *Tc-tll* is not affected after *Tc-six3* RNAi (not shown; see Posnien et al., 2011b). Expression of the outside markers, *Tc-ems* (Fig. 16C_1-D_4) and *Tc-spi* (Fig. 16E_1-F_4), is mostly like in untreated animals as well, whereas the same alterations as for CLR markers can be found: expression domains which are usually separated come together at the midline after *Tc-six3* knockdown.

TUNEL was performed in *Tc-six3* RNAi embryos to test whether the tissue loss was generated by enhanced cell death. Like untreated embryos, germ rudiments show no cell death (Fig. 17G, J). However, cell death levels are increased compared to untreated embryos during germ band elongation ($p=0.0006$ after t-test; Fig. 17H, J) as well as during germ band retraction ($p=0.0329$ after t-test; Fig. 17I, J). Especially neurogenic and ocular regions are affected in embryos with high numbers of dying cells (arrows in Fig. 17I). However, I also found embryos with an obviously strong RNAi phenotype which showed no cell death in the anterior head (not shown). Numbers of dying cells can also be found in the appendix (p. 112).

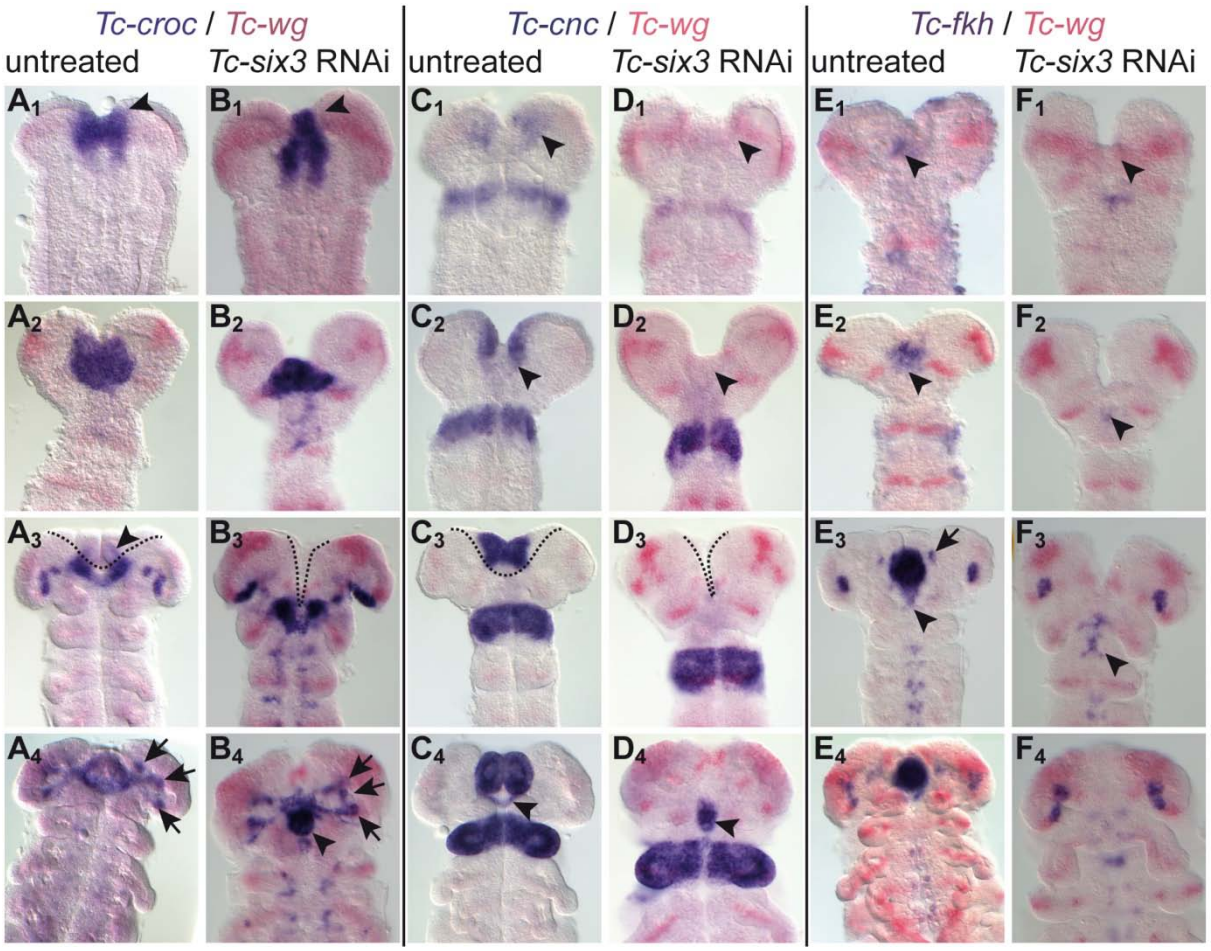


Fig. 15: *Tc-six3* RNAi leads to defects in anterior CLR marker expression. For each gene expression, untreated (left column) and *Tc-six3* RNAi embryos (right column) are displayed. Each column shows successively older embryos. Anterior is up in all images. Image (E_3) was provided by J. Ulrich. (A_1-B_4) Serosa window stage embryos show loss of *Tc-croc* expression in the anterior tips of the expression domain (arrowheads in A_1 and B_1). This region is characterized by overlapping expression of *Tc-six3* and *Tc-croc* (compare to Fig. 9A_1 and B_1). The loss of tissue in the anterior head due to *Tc-six3* RNAi becomes apparent during germ band extension. The *Tc-croc* expression domain has an irregular shape but is still present (compare A_2 and B_2). *Tc-croc* expression appears almost normal in fully elongated germ bands (compare A_3 and B_3) but the expression domains in the labrum anlagen (arrowhead in A_3) are lost, as they lie within the deleted area (compare dotted lines in A_3 and A_4). Expression in both head sides comes together at the midline due to the tissue loss. In retracting germ bands, the expression around the stomodaeum appears collapsed to a dense domain (arrowhead in B_4). Expression in the neurogenic region appears normal (compare arrows in A_4 and B_4). (C_1-D_4) Expression of *Tc-cnc* in the labrum is absent (compare arrowheads in C_1, C_2, D_1 and D_2 and dotted lines in C_3 and D_3), while it is normal in the mandibular segment. The expression domain around the stomodaeum is present, but appears collapsed (compare arrowheads in C_4 and D_4). (E_1-F_4) The stomodaeum expression domain of *Tc-fkh* does not develop after *Tc-six3* RNAi (compare arrowheads in E_1-F_2). Expression along the ventral midline is present (compare arrowheads in E_3 and F_3), while the domains directly lateral of the stomodaeum (arrow in E_3) are absent.

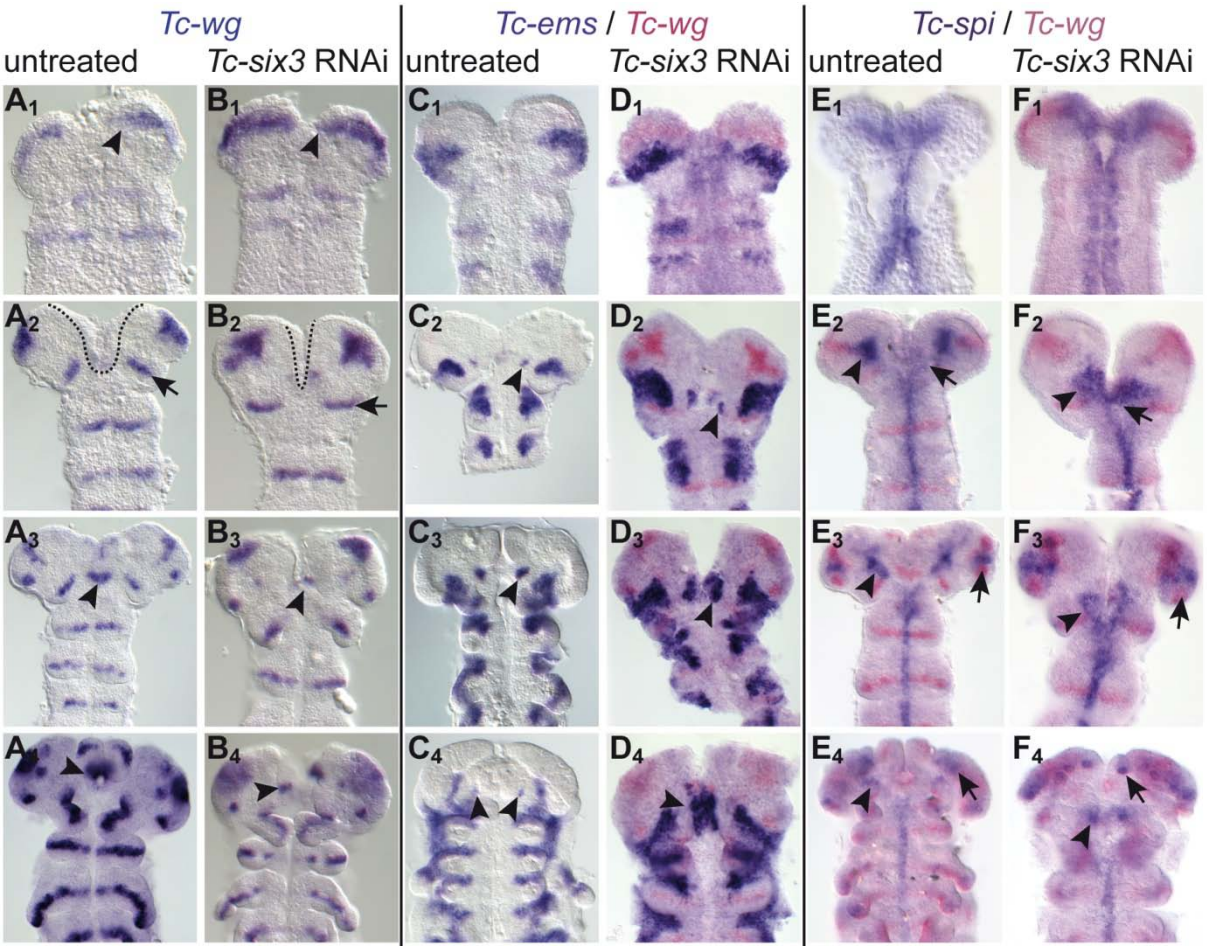


Fig. 16: Expression of outside markers after *Tc-six3* RNAi. For each gene expression, untreated (left column) and *Tc-six3* RNAi embryos (right column) are displayed. Each column shows successively older embryos. Anterior is up in all images. Image (A_4) was provided by J. Schwirz. (A_1-B_4) As already shown by Posnien et al. (2011b), the ocular expression domain of *Tc-wg* is expanded towards the midline of the embryo (arrowheads in A_1 and B_1). The antennal *Tc-wg* stripes are usually positioned in a 45° angle with respect to more posterior stripes (arrow in A_2). Due to the deleted region (compare dotted lines in A_2 and B_2), the head lobes move towards each other and the antennal *Tc-wg* stripes end up parallel to the posterior stripes (arrowhead in B_2). The stomodaeum domain of *Tc-wg* is often not deleted, but reduced (arrowheads in A_3, A_4, B_3 and B_4). (C_1-D_4) The segmental expression of *Tc-ems* is not altered after *Tc-six3* RNAi. In contrast to that, the secondary domains arising lateral of the stomodaeum during germ band extension come together along the midline and are often fused and expanded (compare arrowheads in C_2-C_4 and D_2-D_4). (E_1-F_4) Expression of *Tc-spi* appears normal in serosa window stage embryos (E_1, F_1). *Tc-spi* expression is weaker in the antennal region of untreated embryos during germ band elongation (arrow in E_2). The weaker expression domain is apparently absent after *Tc-six3* knockdown (compare arrows in E_2 and F_2) and the broad domain is fused to the midline expression (compare arrowheads in E_2-E_4 and F_2-F_4). Expression in the ocular region appears normal but is shifted (compare arrows in E_3, E_4, F_3 and F_4).

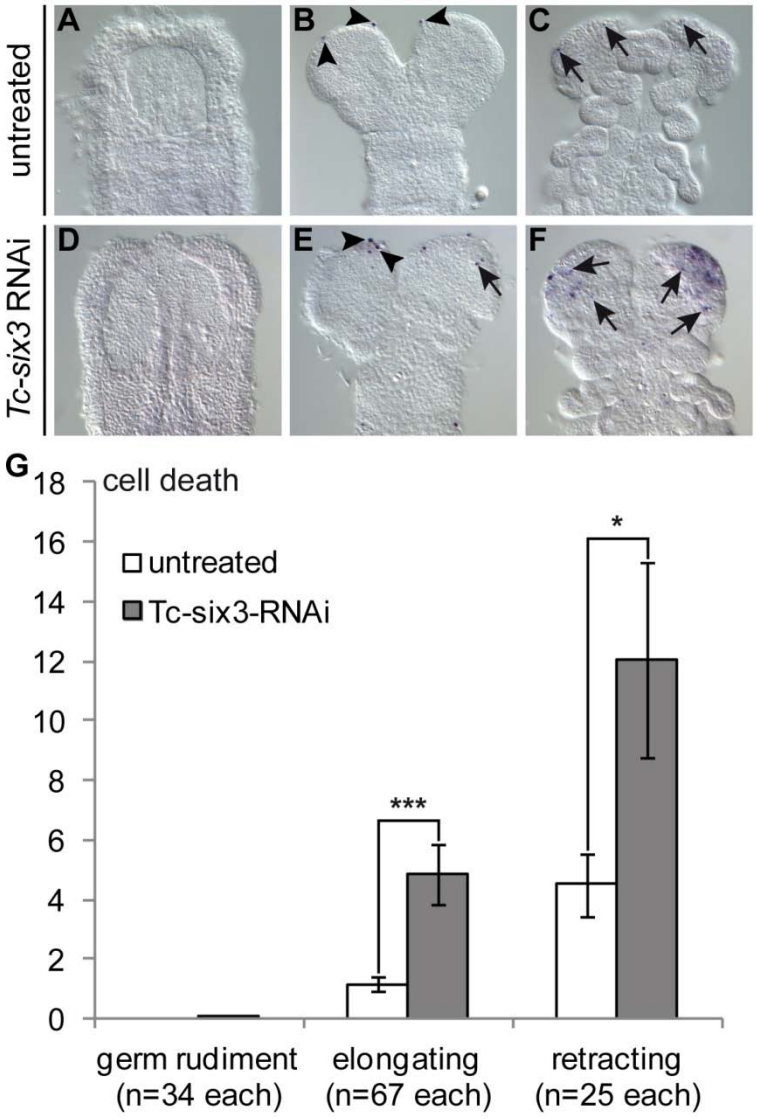


Fig. 17: Cell death increases after knockdown of *Tc-six3*. (A-F) Successively older embryos after TUNEL without RNAi treatment (first row) and after *Tc-six3* RNAi (second row). Each column shows embryos of comparable age. Anterior is up. (G) Mean numbers of dying cells per stage in untreated (white) and *Tc-six3* RNAi embryos (grey). Error bars indicate standard error of the mean. Asterisks indicate significance after t-test (one asterisk: $p \leq 0.05$; three asterisks: $p \leq 0.001$). (A, D, G) Untreated as well as *Tc-six3* RNAi embryos of germ rudiment stage show usually no cell death in the anterior head. (B, E, G) Few dying cells (1.2 per embryos; n = 67) are found in untreated embryos during germ band extension. Most of them can be assigned to extraembryonic tissues (arrowheads in B). Cell death is significantly higher in *Tc-six3* RNAi embryos (p=0.0006; 4.9 cells per embryo; n = 67). Dying cells can again be found in the serosa (arrowheads in E), but additionally, cells in the embryo proper undergo cell death (arrows). (C, F, G) Untreated retracting germ bands show higher levels of cell death than elongating ones (4.5 cells per embryo; n = 24). Cell death levels are again significantly higher after knockdown of *Tc-six3* (p=0.0329; 12.0 cells per embryo; n = 24). Apparently, all dying cells belong to the embryo proper (arrows). In *Tc-six3* RNAi embryos, more cells undergo cell death in the neurogenic and ocular regions than in the rest of the head.

Knockdown of *Tc-croc* leads to misplacement of the clypeolabrum and absence of the foregut

The most prominent phenotype after *Tc-croc* RNAi is a misplacement of the labrum from a position between the antennae to the dorsal side of the head (50 %, n=80; compare arrowheads in Fig. 18B with A). The labrum remains intact in many cases and can be identified by a typical set of four bristles, but it can also be reduced in size (arrowhead in Fig. 18C) or malformed. Analysis of the head bristle pattern (see Schinko et al., 2008; Posnien et al., 2011b) reveals that the anterior vertex seta is misplaced together with the labrum (arrow in Fig. 18B and A). The labrum is only slightly misplaced in more weakly affected larvae (21.25 %) and

in those cases appears to be bent upwards (arrowhead in Fig. 18E). Occasionally, antennae are bent upwards (15 %; arrow in Fig. 18E). The foregut is absent in most cuticles (97.5 %; for instance in the cuticles shown in Fig. 18B, C and E). It can still be shortened and misshapen in mildly affected cuticles in which a normal labrum is present (compare arrowhead in Fig. 18F with D). No developmental defects were detected in other body regions than the clypeolabrum and the foregut.

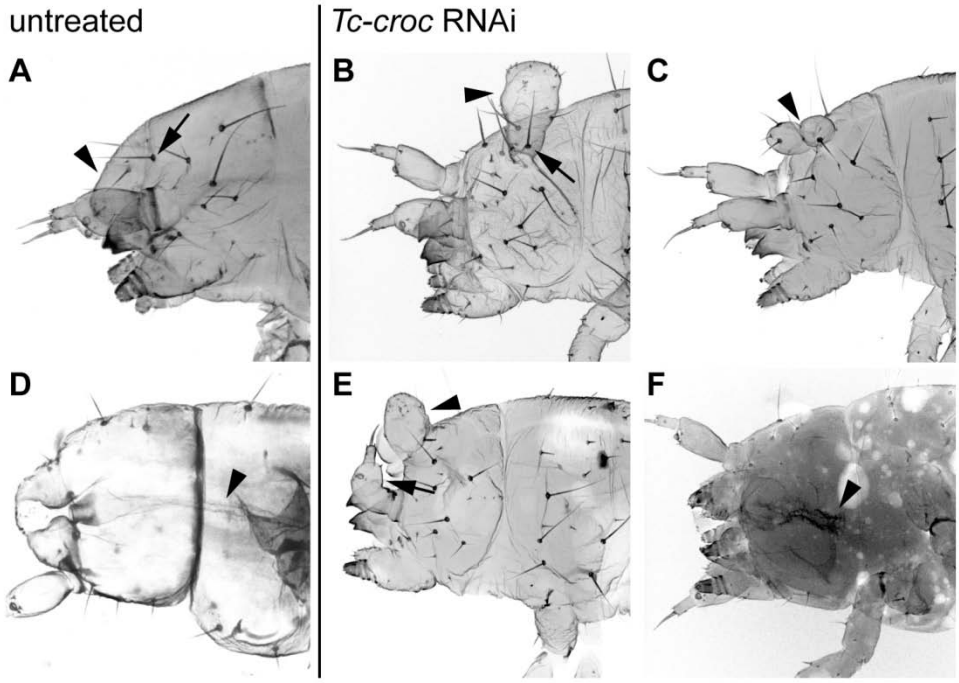


Fig. 18: L1 larval cuticles after *Tc-croc* RNAi. Anterior is left. (A, D) Untreated cuticles; lateral and ventral view. (B, C) Knockdown of *Tc-croc* leads to a misplacement of the labrum to the dorsal side of the head (compare arrowhead in A, B, C). Also the bristle marking the clypeus is misplaced (compare arrow in A, B). The misplaced clypeus and labrum are in many cases shaped like in untreated animals (B). Sometimes they are reduced in size or misshapen (C). (E) Dorsal movement of the labrum is less pronounced in more weakly affected animals and thus it appears to be bent upwards (arrowhead). Sometimes also one of the antennae is curved (arrow). (F) Most weakly affected larvae develop a normal labrum at the correct position but the foregut is shortened and misshapen (compare arrowheads in D and F). In B, C and E no foregut developed.

Embryonic defects after *Tc-croc* RNAi are restricted to the stomodaeal region

The genesis of the cuticle defects was investigated by analyzing the development of *Tc-croc* RNAi embryos. Despite the fact that *Tc-croc* is expressed from early germ rudiment stages on, developmental defects appear only when stomodaeum formation starts in elongating germ bands (ca. 10 *Tc-wg* stripes in the trunk). The stomodaeum fails to invaginate after *Tc-croc* knockdown and this is accompanied by loss or reduction of stomodaeal expression of several transcription factors: *Tc-wg* (Fig. 19A, B), *Tc-six3* (Fig. 19C, D), *Tc-scro* (Fig. 19E, F), *Tc-fkh* (Fig. 19G, H) and *Tc-cnc* (Fig. 19I, J). The earliest stages with clearly aberrant expression are shown, respectively. Other expression domains of these genes are not or only slightly affected (e.g. compare arrows in Fig. 19E and F). Expression of the intercalary marker *Tc-lab* is

found ectopically at a more anterior position after *Tc-croc* RNAi (Fig. 19K, L), indicating a switch from stomodaeal to intercalary identity of the respective cells. *Tc-tll* is usually expressed in neurogenic regions lateral from the labrum and stomodaeum. Knockdown of *Tc-croc* leads to an expansion into the stomodaeum region. The labral buds form normally in *Tc-croc* RNAi embryos apart from being twisted inwards (see bent arrows in Fig. 19B).

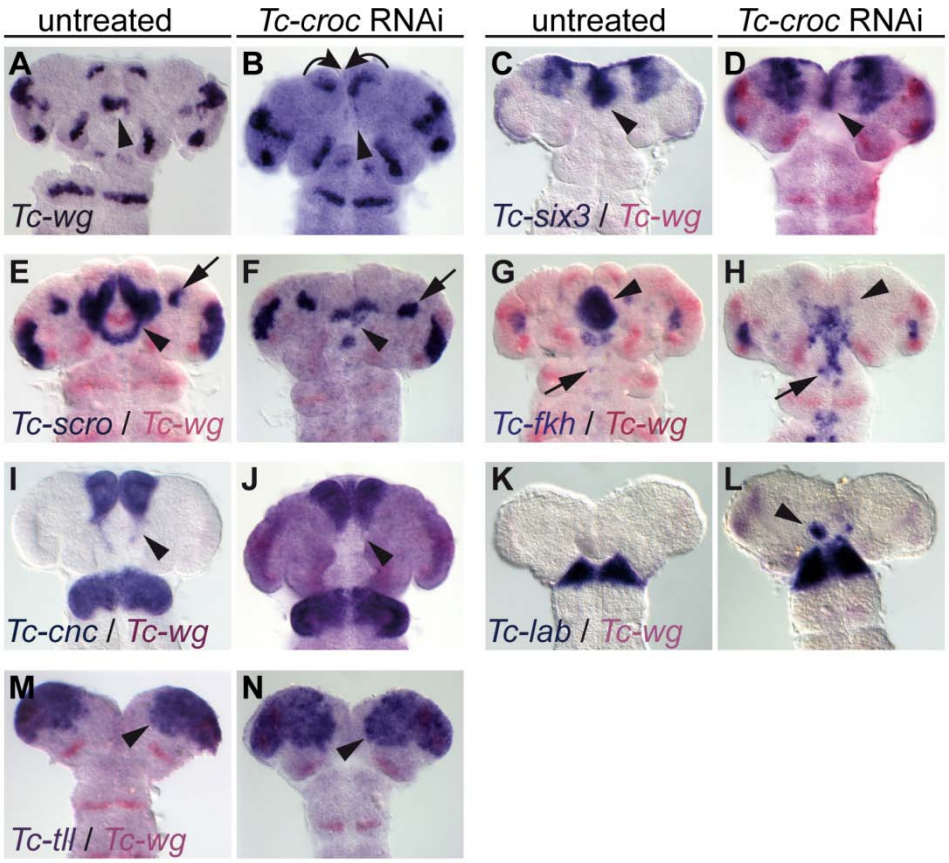


Fig. 19: *Tc-croc* RNAi leads to reduced expression of genes in the stomodaeum region. First and third column: untreated embryos; second and fourth column: *Tc-croc* RNAi embryos; anterior is up. The earliest developmental stage expression changes were detectable in is given for each staining. Both, one untreated and one *Tc-croc* RNAi embryo of the same stage are shown for each gene; gene names are indicated in the lower left corner of each pair. (A, B) Expression of *Tc-wg* is lost or severely reduced in the stomodaeum region (compare arrowheads). The labrum domains, facing outwards in untreated embryos are twisted inwards after *Tc-croc* RNAi (arrows in B). (C, D) The central expression domain of *Tc-six3* is reduced in size after *Tc-croc* RNAi (compare arrowheads). The lateral neurogenic domains are not affected. (E, F) The central expression domain of *Tc-scro* is severely reduced (compare arrowheads), whereas the lateral domains are unaffected (compare arrows). (G, H) The stomodaeum domain of *Tc-fkh* is reduced (compare arrowheads), while other expression domains are unaffected. Stronger staining in the RNAi embryo is due to overstaining (compare arrows). (I, J) The posterior expression stripes of *Tc-cnc* along the stomodaeum rim are lost after *Tc-croc* RNAi (compare arrowheads). The labrum anlagen are attached in a wrong angle. (K, L) The intercalary marker *Tc-lab* shows ectopic expression at a more anterior position after *Tc-croc* RNAi (arrowhead in L). These domains sometimes fuse with each other or, in older embryos, with the original expression domain (not shown). (M, N) Expression of *Tc-tll* is expanded towards the stomodaeum region after *Tc-croc* RNAi (compare arrowheads).

Aberrant morphogenesis leads to dorsal misplacement of the labrum after *Tc-croc* RNAi

The labrum is misplaced dorsally in *Tc-croc* RNAi cuticles although the labrum anlagen did not appear to be strongly affected. I wondered whether the primary defect around the stomodaeum would interfere with proper head morphogenesis, which would secondarily lead to the labrum phenotype. Indeed, aberrant morphogenesis was found in embryos stained with the membrane dye FM® 1-43. The earliest visible defect is the lack of stomodaeum formation (Fig. 20A, B, A', B'). In these embryos, the bases of the antennae are closer together (arrowheads in Fig. 20A, B) and the head lobes show unusual folds of tissues pointing towards the missing stomodaeum (arrows in Fig. 20 C, D). These bulges originate from lateral neurogenic head regions marked by *Tc-tll* (Fig. 19M, N) and the neuroectodermal expression of *Tc-six3* Fig. 20G, H). Hence, lateral tissues expand towards the missing stomodaeal tissue.

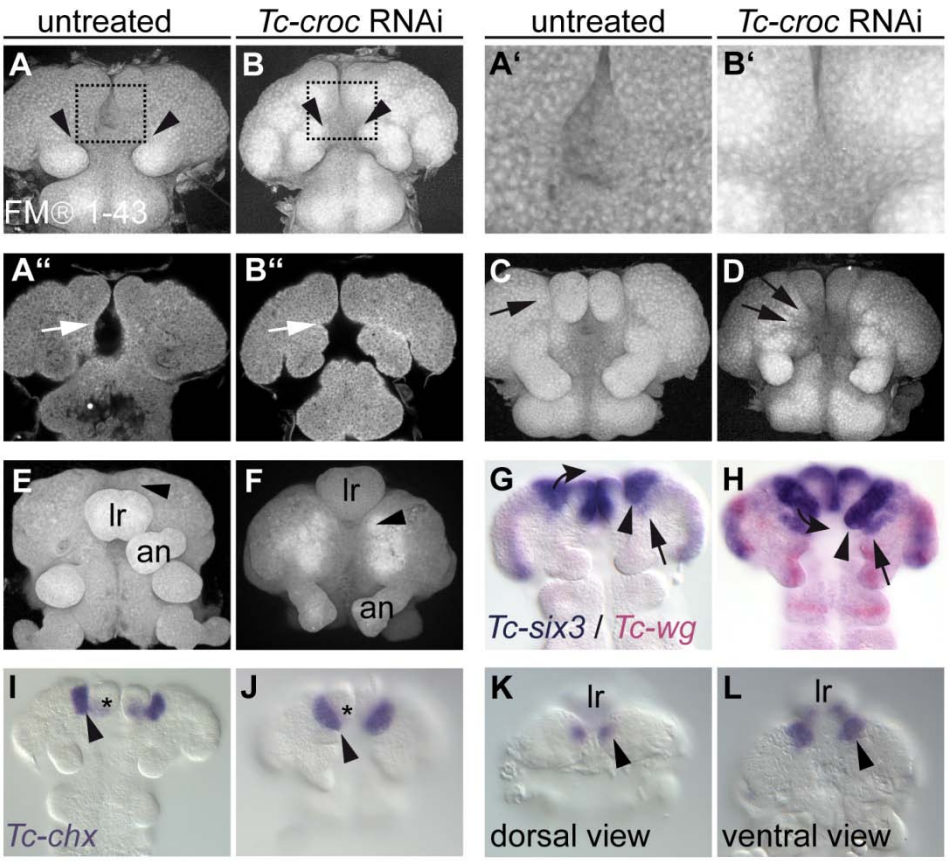


Fig. 20: Ectopic tissue growth instead of stomodaeum invagination leads to misplacement of the labrum. First and third column: untreated embryos; second and fourth column: *Tc-croc* RNAi embryos; anterior is up. (A, B and C-F) Projections of confocal stacks taken from FM 1-43® stained embryos. (A', B') Magnifications of the boxed region in the respective projection. (A'', B'') Single planes from the respective stacks. (G-L) *In situ* hybridizations. Both, one untreated and one *Tc-croc* RNAi embryo of the same stage are shown for each gene. Gene names are indicated in the lower left corner of each pair. All images show ventral views except for (K) which shows a dorsal view. (A, B, A', B') The stomodaeum invaginates in untreated embryos around the time when the labrum anlagen start budding. It fails to invaginate after *Tc-croc* RNAi. The antenna bases come closer to each other (compare arrowheads). (A'', B'') The rim of the invaginating stomodaeum forms the basis of the budding labrum anlagen in untreated embryos (compare arrows). The labrum grows out in a wrong angle due to the lack of the stomodaeum anlagen after *Tc-croc* RNAi. (C, D) Ectopic tissue growth takes place in *Tc-croc* RNAi embryos where the rim of the stomodae-

um should be. The ectopic tissue originates from the neurogenic region lateral of the stomodaeum. (E, F) The neurogenic tissue fuses dorsally of the labrum (lr) in untreated embryos. Instead, the ectopic tissue bulges in *Tc-croc* RNAi embryos come together ventrally of the labrum which leads to the formation of ectopic tissue between the mouthparts and the labrum (compare arrowheads). The distance between labrum and antennae (an) is unusually large. (G, H) *Tc-six3* expression in neurogenic regions expands after *Tc-croc* RNAi together with the ectopic tissue bulges towards the stomodaeal region (compare arrowheads and arrows). (I, J) The weak *Tc-chx* labrum expression domain is lost after *Tc-croc* RNAi (compare asterisks). The stronger neurogenic expression domain appears to be twisted inwards and expanded together with the ectopic tissue (compare arrowheads). (K, L) The *Tc-chx* expressing tissue fuses dorsally of the labrum in untreated embryos. In contrast, it comes together ventrally of the labrum after *Tc-croc* RNAi (compare arrowheads).

The most anterior median parts of head lobes have been identified as anlagen of the pars lateralis and pars intercerebralis marked by *Tc-six3* and *Tc-chx* in untreated embryos (Posnien et al., 2011b). While being separated at early stages, they eventually fuse dorsal of the labrum forming the dorsal head (arrowhead in Fig. 3E; see bend and zipper model in Posnien et al., 2010; Posnien et al., 2011a). In contrast, they appear to fuse ventral of the labrum after *Tc-croc* RNAi, thereby producing ectopic tissue between the labrum and the mouthparts (see arrowhead in Fig. 20F). This would explain why a largely normal labrum is misplaced dorsally in the cuticles. In order to test this idea, we stained *Tc-croc* RNAi embryos for *Tc-six3* and *Tc-chx* expression. Indeed, we find that the respective domains project towards the midline ventral of the labrum instead of dorsal (Fig. 20G-L) leading to an unusual large distance between the bases of the antennae and the labrum (compare Fig. 20E, F).

TUNEL was performed to test whether the defects in stomodaeum development were due to enhanced cell death in *Tc-croc* RNAi embryos. I did not find elevated levels of apoptotic cells in germ rudiment stages when comparing untreated and *Tc-croc* RNAi embryos (Fig. 21A, D and G). Cell death levels rise during germ band elongation in untreated embryos, but most dying cells can be identified as belonging to extraembryonic tissues (arrowheads in Fig. 21B). Corresponding dying cells are also found after *Tc-croc* RNAi, but in addition cell death is significantly increased in embryonic tissue ($p=0.0017$ after t-test; Fig. 21E and G). Most of the dying cells are located in the median portion of the head in cells underlying the ectodermal tissue while cell death in the ectoderm is scarce. Significantly elevated levels of cell death after *Tc-croc* knockdown are also found in retracting germ bands ($p=0.0011$ after t-test; Fig. 21C, F and G). Here, dying cells can be found all over the anterior head, but mostly in the ectopic bulges. Numbers of dying cells are attached (p. 112).

Proliferating cells were visualized by PH3 staining to analyze whether the ectopic bulges were due to aberrant proliferation. No significant changes in proliferation levels of elongating

embryos could be detected after *Tc-croc* RNAi (not shown; n=24; t-test; please find the numbers on p. 111). In summary, the ectopic bulges appear to arise by aberrant tissue movement rather than enhanced proliferation. Aberrant morphogenesis and loss of the median region comprising the stomodaeum anlagen are accompanied by elevated cell death.

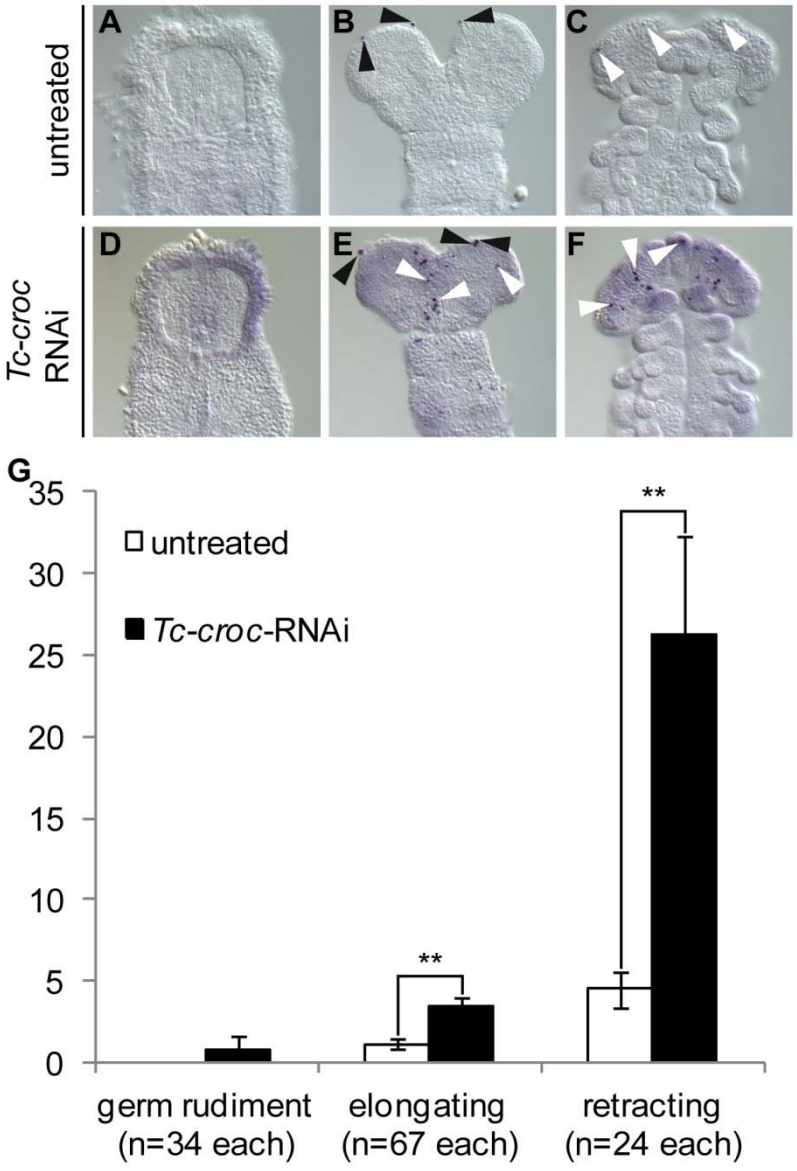


Fig. 21: Cell death and proliferation after *Tc-croc* RNAi. (A-C) Untreated embryos of representative stages in which dying cells were stained via TUNEL; (D-F) *Tc-croc* RNAi embryos of the same stages; anterior is up. The dotted line marks the posterior boundary of the analyzed region. (G) Average numbers of dying cells per stage. (A, D) No cell death was found in untreated germ rudiments. Cell death is not significantly higher after *Tc-croc* RNAi. (B, E) Cell death in untreated elongating germ bands is slightly higher than in germ rudiments (mean: 1.2 cells per embryo), whereas dying cells belong mostly to extraembryonic tissues as indicated by their size and position (black arrowheads). Cell death in the embryo proper (white arrowheads) is enhanced after *Tc-croc* RNAi leading to a significant enhancement in overall cell death (mean: 3.4 cells per embryo; p=0.0017; t-test). (C, F) Numbers of dying cells (arrowheads) are higher in untreated retracting germ bands than in earlier stages (mean: 4.5 cells per embryo). *Tc-croc* RNAi leads to a significant increase in cell death (arrowheads; mean: 26.3 cells per embryo; p=0.001; t-test).

Knockdown of *Tc-cnc* deletes the labrum anlagen

Tc-cnc RNAi leads to transformation of the mandibles to maxillae and to a loss of the labrum (Fig. 22A, B). Defects are also detected in the foregut which is sometimes deleted but in most cases shortened. While these phenotypes have at least partly also been reported for *Drosophila* (Mohler et al., 1995), furthermore, a strong reduction in the number of laid eggs after

the first egg collections is found. Starting around the third day of collection, egg numbers drop and collected eggs are smaller than usual and dissolve during the dechorionisation process. Moreover, females die after ca. two weeks. Dissection of RNAi treated females revealed a strong reduction of ovary tissue (not shown) typical for starved animals (Schmitt-Engel, personal communication). Probably, *Tc-cnc* is involved in metabolic processes, the dysfunction of which leads to the reduction of the reproductive organs (Bucher and Schmidt-Engel, personal communication). To overcome these problems and to obtain enough eggs for analysis of embryos, parental RNAi was performed in imagines instead of pupae.

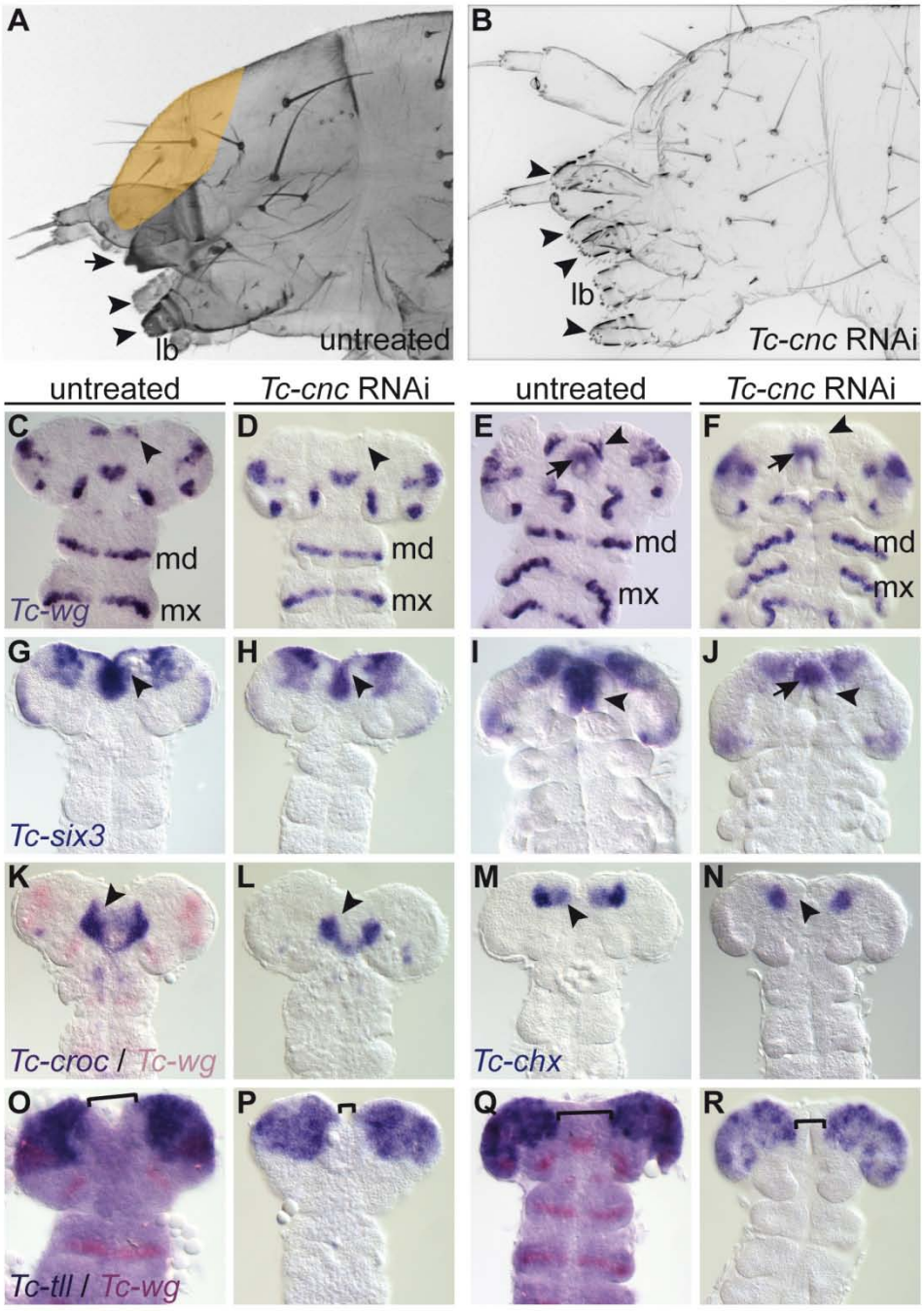


Fig. 22: Defects after *Tc-cnc* RNAi. (A, B) L1 larval cuticles. (C-R) *In situ* hybridizations. Two embryos of different age are shown for each staining except for *Tc-croc* and *Tc-chx*. Images (C and E) were provided by J. Schwirz. Anterior is left in (A, B) and up in (C-R). (A, B) The labrum (marked yellow in A) is deleted after *Tc-cnc* RNAi. The mandibles (arrow in A) are transformed into maxillae (arrowheads). The labium (lb) develops like in untreated animals. (C-F) *Tc-wg* expression reveals a lack of labrum anlagen, while the rest of the anterior head develops normally. The labrum expression domains are lost together with the labrum anlagen (compare arrowheads).

Expression in the stomodaeum roof is like in untreated embryos (compare arrows). No defects in the mandibular segment (md) are detectable in younger embryos (C, D). In older embryos (E, F), the appendages of the mandible segment have clearly transformed into those of the maxillary segment (mx). (G-J) The anterior part of the central *Tc-six3* expression domain is reduced in young embryos (compare arrowheads in G, H) while other expression domains appear normal. In older embryos, the labrum expression domain is lost along with the labrum anlagen (compare arrowheads in I, J). Expression in the stomodaeum roof is like in untreated animals (not visible in I; arrow in J). (K, L) Anterior aspects of *Tc-croc* expression are reduced in *Tc-cnc* RNAi embryos (compare arrowheads). The missing domains would resolve into the labrum expression domains during further development (not shown; compare to Fig. $9B_6$). (M, N) The labrum expression domains of *Tc-chx* are lost (compare arrowheads), while the neurogenic expression is normal. (O-R) Expression of *Tc-tll* appears to be shifted to a more median position (compare bars).

Analysis of the expression patterns of various genes revealed that the labrum anlagen and the respective expression domains are deleted. This is the case for *Tc-wg* (arrowheads in Fig. 22C-F), *Tc-six3* (arrowheads in Fig. 22G-J), *Tc-croc* (arrowheads in Fig. 22K, L) and *Tc-chx* (arrowheads in Fig. 22M, N). Other expression domains of these genes are not affected. Expression of *Tc-fkh* and *Tc-lab* is not altered (not shown). The expression domains of *Tc-tll* appear to be shifted to a more median position which might be a secondary effect due to loss of labrum tissue (compare bars in Fig. 22O-R).

Tc-scro RNAi leads to a split labrum

The effects of *Tc-scro* knockdown on larval cuticles have been described by Posnien et al. (2011b). Larvae are characterized by a split of the labrum due to the fact that the labrum anlagen do not fuse during embryonic development. The labrum anlagen can additionally be smaller than in untreated animals. I furthermore found that the foregut is absent or malformed. Especially posterior structures appear to be reduced (arrowheads in Fig. 23A, B). *Tc-scro* RNAi does not lead to complete depletion of mRNA. While younger RNAi embryos show no *Tc-scro* expression (not shown), weak expression is detectable in older embryos (Fig. 23C, D). I analyzed the expression of several markers in *Tc-scro* RNAi embryos. Expression changes are always rather subtle and localized.

The labrum expression domains of *Tc-wg* often take a more posterior position in older *Tc-scro* RNAi embryos (arrowheads in Fig. 23E, F). This might be a secondary effect caused by the size reduction of the labrum anlagen. Other expression domains appear normal. In younger embryos, expression of *Tc-croc* shows a slight reduction of the posterior expression stripe that will later surround the stomodaeum (arrowheads in Fig. 23G, H). This expression domain is also reduced in older embryos (black arrowheads in Fig. 23I, J). Additionally, expression in the labrum anlagen is lost (arrows in Fig. 23I, J). Expression of *Tc-cnc* is expanded in elongat-

ing germ bands (arrowheads in Fig. 23K, L). During germ band retraction, the expression domain around the stomodaeum is deleted (arrows in Fig. 23M, N), while other aspects appear normal. The expression patterns of *Tc-six3* and *Tc-fkh* appear like in untreated embryos (not shown) and no obvious change of *Tc-spi* or *Tc-tll* expression was detected (not shown).

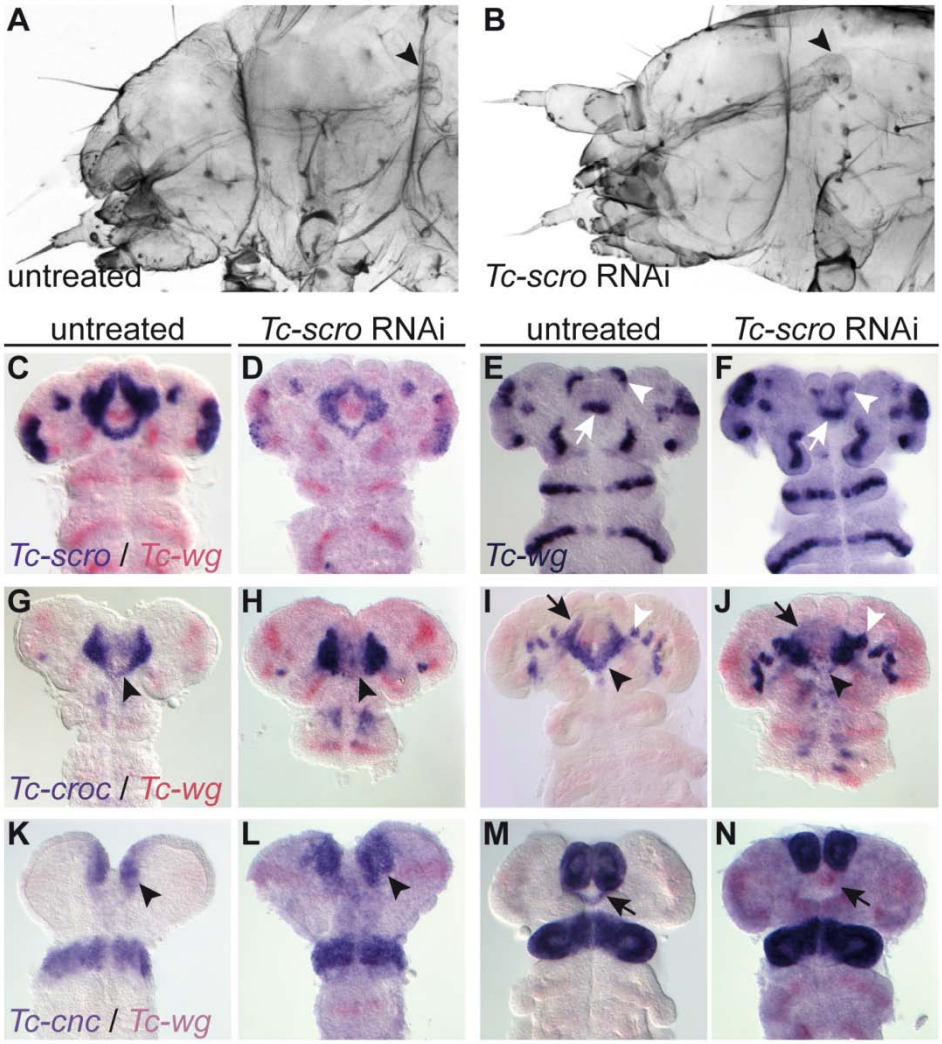


Fig. 23: Minor defects in marker expression patterns are detected after *Tc-scro* RNAi. (A, B) L1 larval cuticles; anterior is left. (C-N) *In situ* hybridizations in untreated (first and third column) and *Tc-scro* RNAi embryos (second and fourth column); anterior is up. (A, B) The anterior gut forms an open hook-like structure at its posterior end in untreated larvae. The foregut ends blind after *Tc-scro* RNAi (compare arrowheads). (C, D) Despite RNAi knockdown, *Tc-scro* expression is still detectable in older embryos. The expression pattern appears patchy, which is probably due to a high mRNA concentration in the nucleus and a lower one in the cytoplasm. (E, F) The labrum expression domain of *Tc-wg* is established at a more posterior position than in untreated embryos (compare arrowheads). Other expression domains appear normal. Also the stomodaeum domain looks unaffected (compare arrows). (G-J) *Tc-scro* RNAi embryos show a reduction of the most posterior aspects of *Tc-croc* expression (compare black arrowheads). The effect is first visible during later germ band elongation. The expression domains in the labrum anlagen are deleted (compare arrows). Expression in the neurogenic region appears expanded and fuses to the central expression domain (compare white arrowheads) which could, however, be due to stronger staining in the RNAi embryo. (K-N) Expression of *Tc-cnc* is expanded after *Tc-scro* RNAi in early elongating germ bands (compare arrowheads). Expression becomes normal during germ band elongation (not shown). However, the posterior expression stripes around the stomodaeum fail to be established (compare arrows).

Knockdown of *Tc-tup* affects labrum development and dorsal closure

Knockdown of *Tc-tup* leads to a wide range of cuticle phenotypes (Fig. 24). Most larvae are characterized by a loss of the clypeolabrum. The rest of the body appears unaffected in weakest phenotypes (Fig. 24A, A'). Additional defects in abdominal development are observed in stronger phenotypes. The abdomen appears narrower, and defects on its dorsal side (Fig. 24B) often lead to an upwards bending of the posterior abdomen (not shown). Wrinkles on the dorsal cuticle of thorax and abdomen indicate that dorsal closure does not occur correctly. The abdomen or parts of it can also be involuted into the body (Fig. 24C). The complete body is disarranged in stronger phenotypes, where still the abdomen is stronger affected than the rest of the body (Fig. 24D). Individuals with strongest defects show a typical inside-out phenotype in which external cuticle structures are developed towards the interior. This phenotype is indicative for defects in dorso-ventral patterning or dorsal closure (Bucher, personal communication). Head structures or legs (arrowheads in Fig. 24E) can sometimes be identified. Interestingly, gut structures can even be present if the cuticle is hardly recognizable (Fig. 24E).

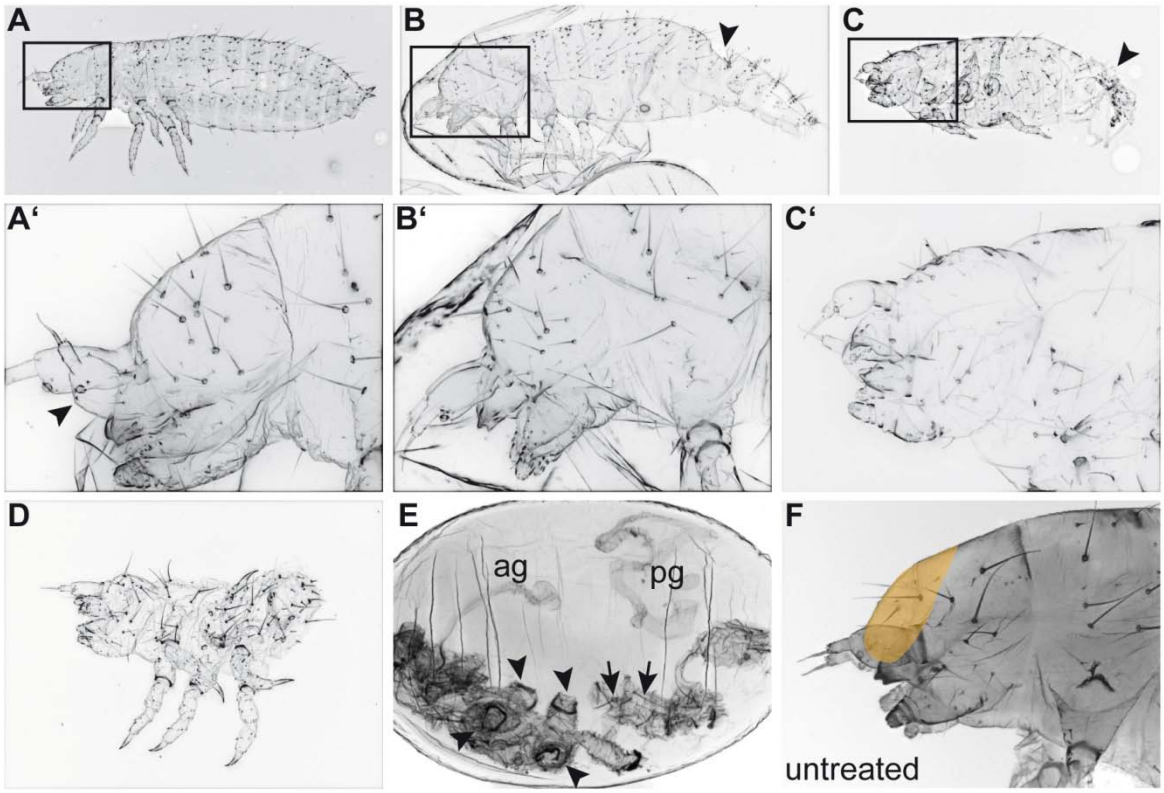


Fig. 24: L1 larval cuticles after *Tc-tup* RNAi. (A-E) *Tc-tup* RNAi cuticles; (A', B' and C') magnifications of the boxed area in the respective image; (F) untreated cuticle; anterior is left. (A, A') Weakly affected larvae are lacking the clypeolabrum (compare to yellow marked region in F). Sometimes, bent antennae are detected (arrowhead). Other than that, the body develops like in untreated animals. (B, B') Besides a lack of the clypeolabrum (compare to F), stronger phenotypes are characterized by defects in the abdomen. It is often bent upwards (not shown) due to an apparent fracture in its dorsal region (arrowhead). (C, C') The posterior abdomen

can also be internalized into the more anterior body. During preparation of the displayed cuticle, the abdomen was pushed out of the body again (arrowhead). The clypeolabrum is again missing (compare to F). (D) The whole body is affected in cuticles with even stronger phenotypes. Sometimes, thorax and head still develop, but are malformed. (E) The whole cuticle is inside-out in the strongest detectable phenotypes. Legs (arrowheads) and bristles (arrow) develop towards the interior of the individual. Anterior (ag) and posterior gut structures (pg) can be well identified.

Contribution of further genes to CLR development

As also revealed by Koniszewski (2011), *Tc-chx* RNAi has no detectable effect on cuticle development.

Tc-hbn was tested as a potential upstream regulator of anterior patterning, given its early expression in the anterior embryo. Its knockdown leads to a loss of the complete anterior and dorsal portions of the head up to the antennal segment. Posterior parts of the head (maxillae, mandibles and labium) develop normally (Fig. 25A, B). Analysis of embryos revealed that the anterior head is deleted (Fig. 25E, G) which also leads to the loss of expression of *Tc-six3* (not shown). This makes it difficult to draw conclusions about direct regulation of downstream targets.

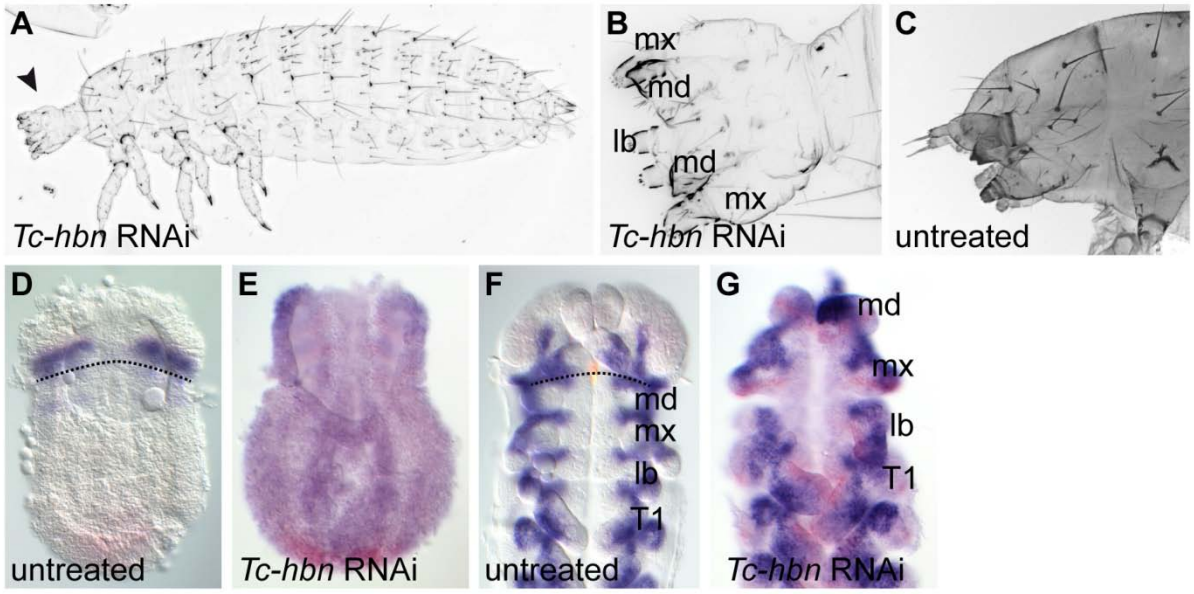


Fig. 25: *Tc-hbn* is required for the development of the whole procephalon. (A-C) L1 larval cuticles; anterior is left. (D-G) *In situ* hybridization of *Tc-ems*; anterior is up. The dotted line in D and F indicates the boundary between deleted and well established structures. (A) Defects in the cuticle are restricted to the head (arrowhead). (B) The mandibles (md), maxillae (mx) and labium (lb) develop like in untreated animals, while more anterior head structures are missing (compare to C). (D, E) The antennal *Tc-ems* stripe is not established in *Tc-hbn* RNAi germ rudiments. The anterior head anlagen are missing entirely. (F, G) The anlagen of the mandibular (md), maxillary (mx), labial (lb), first thoracic (T1) and more posterior segments (not shown) are well established after *Tc-hbn* knockdown. More anterior structures are deleted.

Contribution of *Tc-tll* to CLR development was tested because it is expressed directly lateral of the CLR and because its expression expands after *Tc-croc* and *Tc-cnc* RNAi. The most prominent phenotype after *Tc-tll* RNAi is a shortening of the abdomen. Only 26.5 % of analyzed cuticles (n = 68) exhibit an abdomen with eight segments. 55.9 % have only four or less abdominal segments (not shown). Defects in the head are limited to the head bristle pattern. I can, however, not confirm the defects in posterior lateral head regions described by (Posnien et al., 2011b), but rather find them in the anterior lateral head (Fig. 26P, Q).

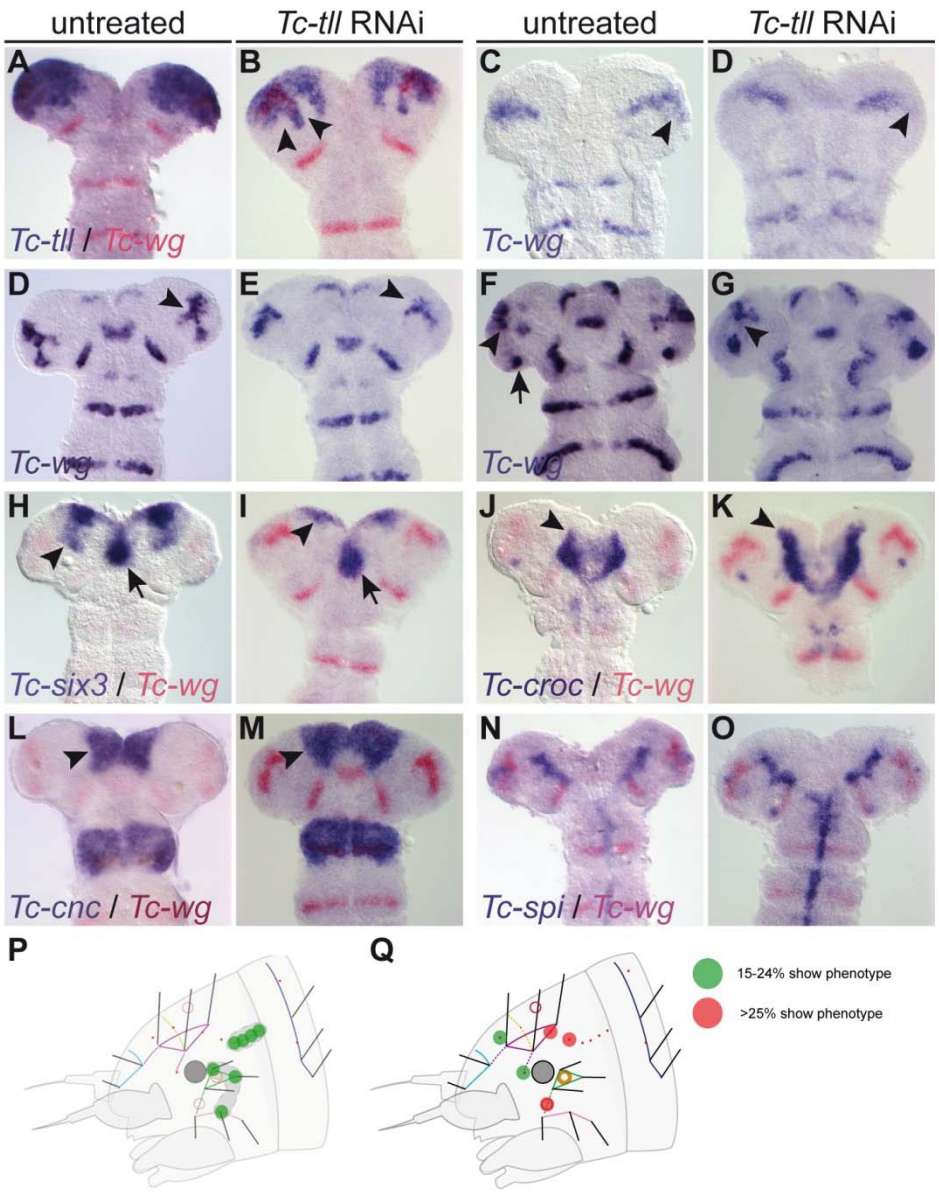


Fig. 26: Knockdown of *Tc-tll* results in an altered shape of the embryonic head. First and third column: untreated embryos; second and fourth column: *Tc-hh* RNAi embryos. Anterior is up in all images. All RNAi embryos show rather round head lobes compared to the untreated ones. (A, B) Expression of *Tc-tll* is reinitiated in RNAi embryos during germ band extension. The expression pattern differs from the untreated situation. Essentially, two areas are free of expression (arrowheads) and overall expression appears weaker. (C-G) The ocular expression domain of *Tc-wg* is reduced in its outer region in serosa window stage embryos. Also later developmental stages show defects in this area (compare arrowheads). It cannot be determined exactly which aspects of the expression domain are lost. Apparently, the most posterior expression spot (arrow in F) is deleted. (H, I) The lateral expression domains of *Tc-six3* are strongly reduced after *Tc-tll* RNAi (compare arrowheads). In contrast to that, expression in the central domain is normal (compare arrows). (J, K) Expression of *Tc-croc* is,

especially at its most anterior tip (compare arrowheads), slightly expanded after *Tc-tll* RNAi. (L, M) Also expression of *Tc-cnc* is expanded. The lateral expression boundary appears bulged outwards (compare arrowheads). (N, O) The anterior expression arms of *Tc-spi* appear to be shifted to an outer position, thereby widening the space between them. (P, Q) Schematics of L1 larval heads depicting the head bristle pattern. The schematics were provided by N. Posnien. (P) is reprinted from Posnien et al. (2011b). Defects are located in the posterior lateral head after Posnien et al. (2011b; P), whereas I detect defects in the more anterior lateral head (Q).

Young RNAi embryos lack *Tc-tll* expression completely (not shown), but expression is reinitiated in older ones. However, the expression pattern differs from that in untreated embryos (Fig. 26A, B). Embryos are characterized by a typical alteration of lateral head morphology. The head lobes are smaller and appear rounder than in untreated animals. This is accompanied by a reduction of ocular *Tc-wg* expression (Fig. 26C-G). The central domain of *Tc-six3* is expressed like in untreated embryos, but the lateral neurogenic domains are reduced (Fig. 26H, I). Expression of *Tc-croc* (Fig. 26J, K) and *Tc-cnc* (Fig. 26L, M) appears slightly expanded, which might, however, be due to the altered head shape. The same is true for *Tc-spi* where the expression arms in the head appear to be shifted to a more outer position, thereby widening the space between them (Fig. 26N, O). Expression of *Tc-fkh* is unaffected (not shown).

Posnien et al. (2009b) have shown that the labrum still develops when segment polarity gene function is knocked down by RNAi. Knockdown of *Tc-hh* leads to cuticle balls which do not show any signs of segmentation. However, a labrum is still formed (not shown). I tested some of the CLR marker genes in *Tc-hh* RNAi embryos and found that expression of *Tc-croc* (Fig. 27A-C) and *Tc-cnc* (Fig. 27E, F) is almost like in untreated embryos. In accordance with the lack of a foregut in RNAi cuticles and expression of *Tc-hh* in the developing gut of untreated embryos, expression of the stomodaeum marker *Tc-fkh* is severely reduced. No expression is found in the stomodaeum region (compare arrowheads in Fig. 27G, H), while expression along the ventral midline appears to be increased (compare arrows in Fig. 27G, H). Expression of the midline marker *Tc-spi* appears almost normal (Fig. 27J, K).

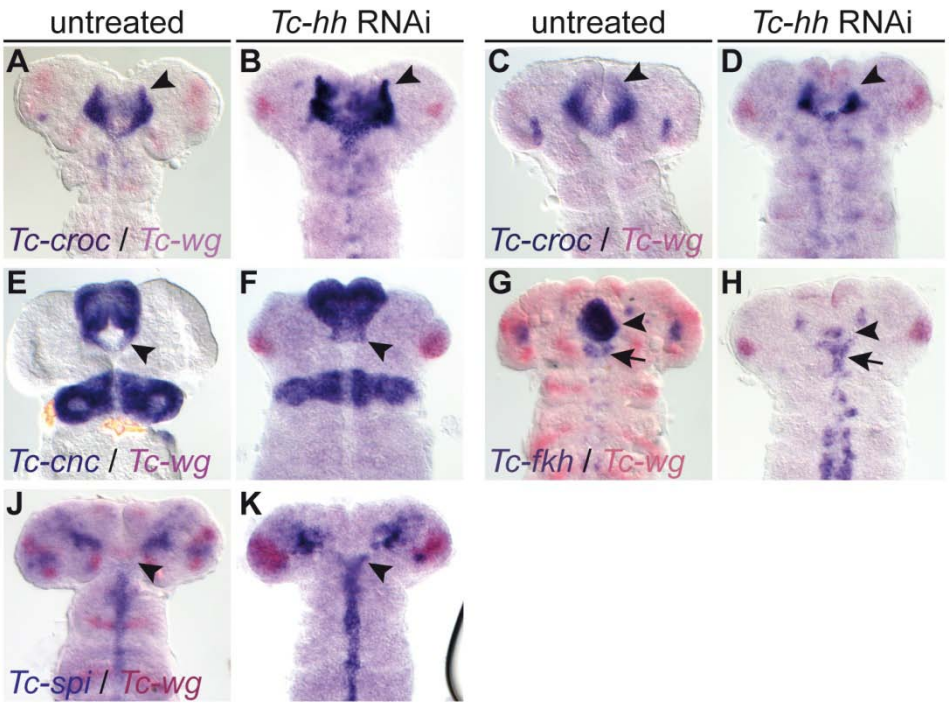


Fig. 27: *Tc-hh* RNAi leads to defects in stomodaeum but not labrum development. First and third column: untreated embryos; second and fourth column: *Tc-hh* RNAi embryos. Anterior is up in all images. (A-D) The *Tc-croc* expression pattern is still well recognized after *Tc-hh* RNAi. The expression appears to be slightly expanded during later germ band elongation, especially in the anterior part of the domain (compare arrowheads in A, B). The anterior domains become absent during germ band retraction (compare arrowheads in C, D) and the whole expression appears to be reduced. (E, F) *Tc-cnc* expression in the labrum anlagen appears like in untreated embryos, but the expression domain around the stomodaeum is lost after *Tc-hh* RNAi (compare arrowheads). (G, H) The stomodaeum expression domain of *Tc-fkh* is absent after *Tc-hh* knockdown (compare arrowheads), whereas other domains appear to be expressed in the correct region albeit stronger than usual (compare arrows). (J, K) The *Tc-spi* expression-free area found in untreated embryos appears to be absent after *Tc-hh* RNAi (compare arrowheads). Other than that, expression appears normal.

4.2.3 Gain of function of CLR genes to refine the interaction scheme

In order to further investigate the gene regulation network in the anterior head, I wanted to test the effect of ectopic expression of key genes on possible downstream targets. Ectopic expression via the Gal4-UAS system has been shown to be functional in *Tribolium* (Schinko et al., 2010). However, the current lack of enhancer lines makes ectopic expression in restricted body regions impossible. I therefore used a heat shock system to drive ectopic expression ubiquitously (Schinko et al., 2010; Schinko et al., in preparation).

The ectopically expressed genes should be high in the hierarchy of the genetic interactions in the CLR. Good candidates for upstream regulators should show an early expression and have a strong but distinct RNAi phenotype. On this basis, the CLR markers *Tc-six3*, *Tc-croc* and *Tc-cnc* were chosen as promising candidates. As *Tc-six3* was also studied by a colleague, I concentrated on *Tc-croc* and *Tc-cnc*.

Open reading frames of *Tc-croc* and *Tc-cnc*

The cloned gene fragments used in prior experiments as templates for synthesis of RNA probes and dsRNA were not whole open reading frames (ORFs) and were thus not sufficient for ectopic expression of a functional protein. The ORFs of *Tc-croc* and *Tc-cnc* were therefore confirmed via RACE-PCR.

The *Tc-croc* transcript contains a single exon which is identical to the GenBank entry XM_001812646.1 at the National Center for Biotechnology Information (NCBI).

The situation is more complicated for *Tc-cnc*. Three differentially expressed splice variants, *cncA*, *cncB*, and *cncC* are known in *Drosophila*. They show variation in their 5'-end: *cncA* contains three exons (A1-A3); *cncB* contains seven exons (B1-B5 plus A2 and A3); *cncC* contains eleven exons (C1-C5 plus B2-B5 plus A2 and A3). While *cncB* is expressed specifically in the anlagen of labrum and mandible segment, *cncA* and *cncC* show ubiquitous expression (McGinnis et al., 1998). For *Tribolium*, an annotation containing ten exons had been made by the group of Prof. M. Klingler, Erlangen (Fig. 28A; see http://www.Beetlebase.org). The prediction at NCBI (NM_001170642.1) has twelve exons (Fig. 28A; Richards et al., 2008). The nine 3'-exons are the same in both versions. They will be referred to as exons 1 through 9. The primer for 5'-RACE, SK0907152, was designed into exon 1. The NCBI prediction but not the BeetleBase annotation could be recovered. Also, a further variant with a unique exon of 217 bp was identified (Fig. 28A). Hypothetical protein sequences from both variants were aligned separately with the splice variants from *Drosophila* using CLUSTAL 2.0.12 (Larkin et al., 2007; Goujon et al., 2010). While the long splice variant clustered with *cncC*, the short one clustered with *cncB* (not shown). Hence, the shorter *Tribolium* splice variant will be referred to as *Tc-cncB* and the longer one as *Tc-cncC*. No splice variant corresponding to *cncA* could be identified using RACE primer SK109designed into exon 8.

In order to test whether both splice variants were differentially expressed, probes directed against the unique exons were synthesized. *Tc-cncB* is expressed in the same pattern as the original *Tc-cnc* fragment (compare Fig. 28B, C). In contrast, *in situ* hybridization with the longer probe (576 bp) for the unique exons of *Tc-cncC* results only in background staining (Fig. 28D).

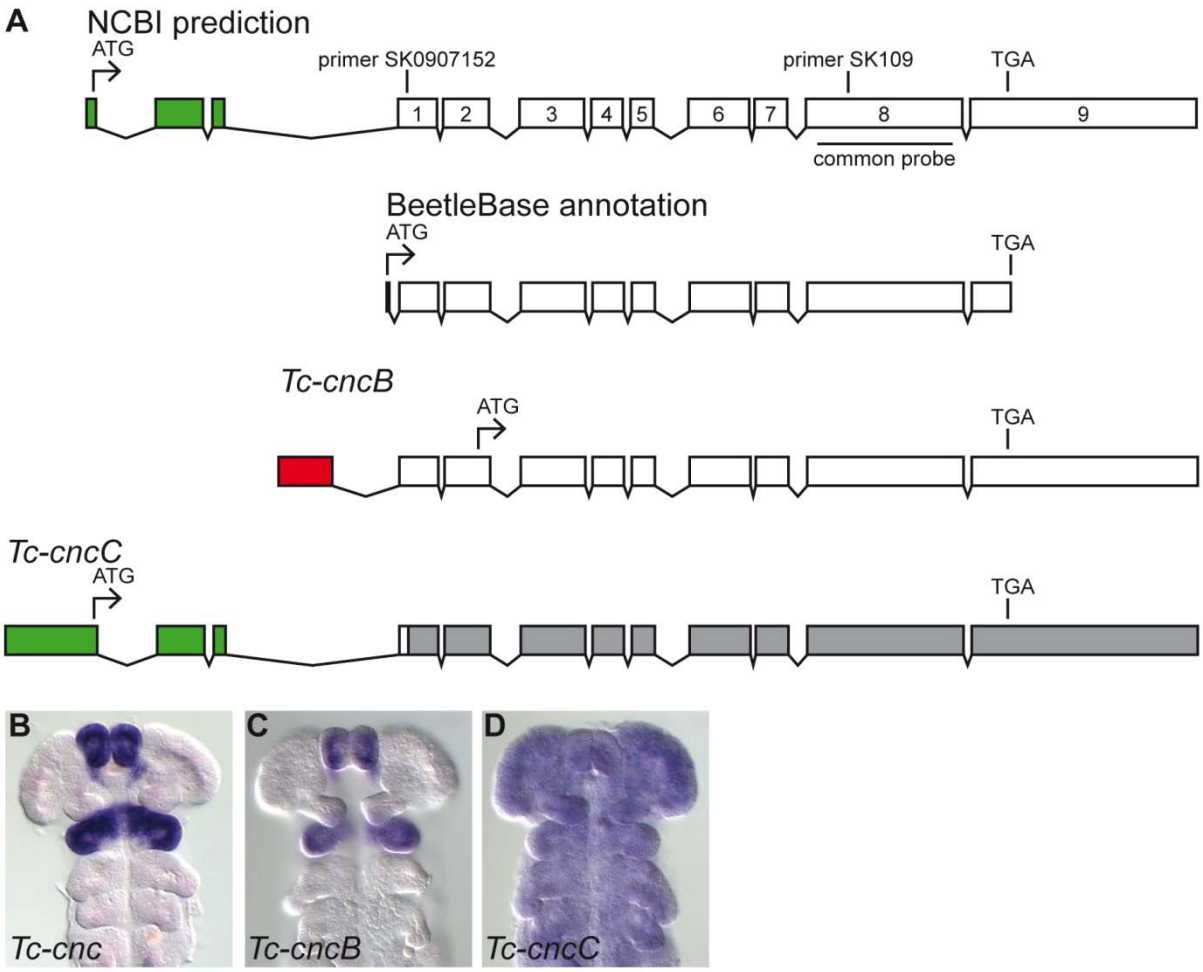


Fig. 28: *Tc-cnc* splice variants are differentially expressed in *Tribolium*. (A) Overview of the gene models for *Tc-cnc*. Exons are to scale, introns are not. The whole annotation spans more than 30 kbp. The NCBI prediction contains the nine exons common to all splice variants (white boxes) as well as three unique exons at the 5'-end (green boxes). The transcription start lies in the first unique exon, the stop codon in the ninth exon. RACE primer binding sites and the section the common RNA probe binds to are indicated. The BeetleBase annotation contains the nine common exons and a 10 bp 5'-exon starting with the ATG. The annotation only spans the coding sequence and ends with the stop codon. *Tc-cncB* has a unique 217 bp exon (red box) upstream of exon 1. RACE PCR with both primers confirmed *Tc-cncB*. *Tc-cncC* spans the same exons as the NCBI prediction, but the first unique exon has a length of 371 bp (compare green boxes). *Tc-cncC* was only detected after RACE with primer SK0907152. Hence, only the 5'-end of exon 1 is confirmed (white box). The region downstream of the RACE primer is assumed to be the same as for *Tc-cncB* (grey boxes). (B-D) *In situ* hybridizations for the different splice variants. Anterior is up. *In situ* hybridization for *Tc-cncB* (C) shows the same staining as the original fragment (B), whereas *Tc-cncC* gives just background (D).

I performed RNAi for both splice variants to analyze, whether knockdown of one of them would lead to the same developmental defects as the original fragment. However, none of the dsRNA fragments produced a phenotype of similar strength (not shown). Knockdown of *Tc-cncB* leads to minor defects in labrum and mandible patterning. The labrum appears smaller than in untreated larvae and is sometimes split in its distal portion. The antennae sometimes

appear to have a small outgrowth. The most common definite defect is a duplication of the clypeus bristle. *Tc-cncC* leads to even weaker defects in head appendage patterning. The clypeus bristle is not affected. However, the number of eggs laid by RNAi-treated females declines drastically after the first two egg collections. This phenotype was also observed in RNAi with the original *Tc-cnc* fragment and indicates a role in oogenesis or metabolic processes in the adult beetle.

Due to the specific expression pattern and the slightly more specific RNAi phenotype, I decided to clone the ORF of *Tc-cncB* for ectopic expression.

Establishment of strains for ectopic expression

The φC31 integrase based site specific integration system was used for transgenesis. The primary advantages of site specific integration over random integration are (1) avoidance of gene disruption by the integrated construct (2) reduction of positioning effects and (3) insertion of a single copy at a known location. The strain used for integration was reported to carry a transgenic construct containing an *attP* landing site (Bucher and Schinko, personal communication) and a black eye marker (rescuing the v^w mutation; Lorenzen et al., 2002). The exact position of the construct had not been determined.

The constructs for ectopic expression contained an *attB* site and a blue eye marker (ECFP under control of 6XP3; Horn and Wimmer, 2000; for the design of the constructs and vector maps, please refer to p. 27 and p. 115, respectively). Upon injection together with φC31 integrase, the *attB* site recombines with the *attP* site and the construct is thereby integrated into the genome (Fig. 3). Different concentrations of φC31 integrase mRNA and heat shock construct were injected into eggs of the *attP* line (see Table 2). One injection experiment was carried out for *Tc-cnc*, two for *Tc-croc* and three for *Tc-six3*. Table 3 gives an overview over the numbers of injected eggs, developing L1 larvae and developing adults in the single injection experiments.

Developing beetles were crossed to individuals of the v^w strain and offspring were screened for marker expression, i.e. blue fluorescent eyes. For *Tc-croc*, I was able to find 7 blue-eyed offspring in the G_0 crossings no. 119 of *Tc-croc_1* (total number of screened beetles: 204) and 22 in crossing no. 124 of *Tc-croc_2* (total number of screened beetles: 198).

Table 3: Establishment of transgenic lines for ectopic expression. Tc-croc_1 and Tc-croc_2, and accordingly, Tc-six3_1, Tc-six3_2 and Tc-six3_3 refer to separate experiments with different mRNA concentrations. Numbers of injected eggs, of developing larvae and of developing adults are given. For Tc-cncB and Tc-six3_3, developing adults were not quantified. Percentage is with respect to the previous column. Established transgenic lines are indicated.

Injection	**Number of injected eggs**	**Number of eggs developing to larvae**	**Number of larvae developing to adults**	**Established transgenic lines**
Tc-cncB	460	31 (6.7 %)	n/a	-
*Tc-croc*_1	655	234 (35.7 %)	138 (59 %)	119_1
*Tc-croc*_2	526	228 (43.3 %)	189 (82.9 %)	124_1
*Tc-six3*_1	370	118 (31.9 %)	95 (80.5 %)	-
*Tc-six3*_2	626	254 (40.6 %)	200 (78.7 %)	-
*Tc-six3*_3	523	93 (17.8 %)	n/a	six3-1, six3-2

One transgenic F_1 male from each crossing was used for establishment of transgenic lines 119_1 and 124_1 by crossing them to v^w females. Offspring of the F_2 generation of each strain were screened for marker expression. 50 % of the offspring were expected to have white eyes without fluorescence and another 50 % to have blue fluorescent black eyes because the *attB* construct (blue) and the original *attP* construct (black) should be linked after integration (Fig. 29A, B).

Surprisingly, beetles with four different eye marker expressions were found: (1) 30.3 % and 23.6 % white eyes, (2) 31.6 % and 29 % blue fluorescent eyes in a white background, (3) 18.7 % and 23.7 % black eyes, and (4) 19.4 % and 23.8 % blue fluorescent eyes with a black background (Fig. 29E). The black and the blue eye marker (i.e. the original pBac-*attP* construct and the newly introduced heat shock construct) must, hence, have been inherited separately from each other. This means, the insertion of the heat shock construct did not occur, as expected, into the *attP* site (see Fig. 3), but into a different genomic location. Most likely, the *attP* line was contaminated with a different black-eyed line (possibly *SB*) and injection was carried out into eggs of beetles without *attP* site (Fig. 29C, D).

I established different lines with each eye marker. PCR analysis on genomic DNA of the eight different strains revealed that the blue eyed lines in fact carry the heat shock construct. However, inverse PCR (performed by J. Ulrich) confirmed that the heat shock constructs integrated into a genomic region other than the *attP* site. In the following experiments, I worked with the lines established from animals with blue fluorescent eyes in a white eye background.

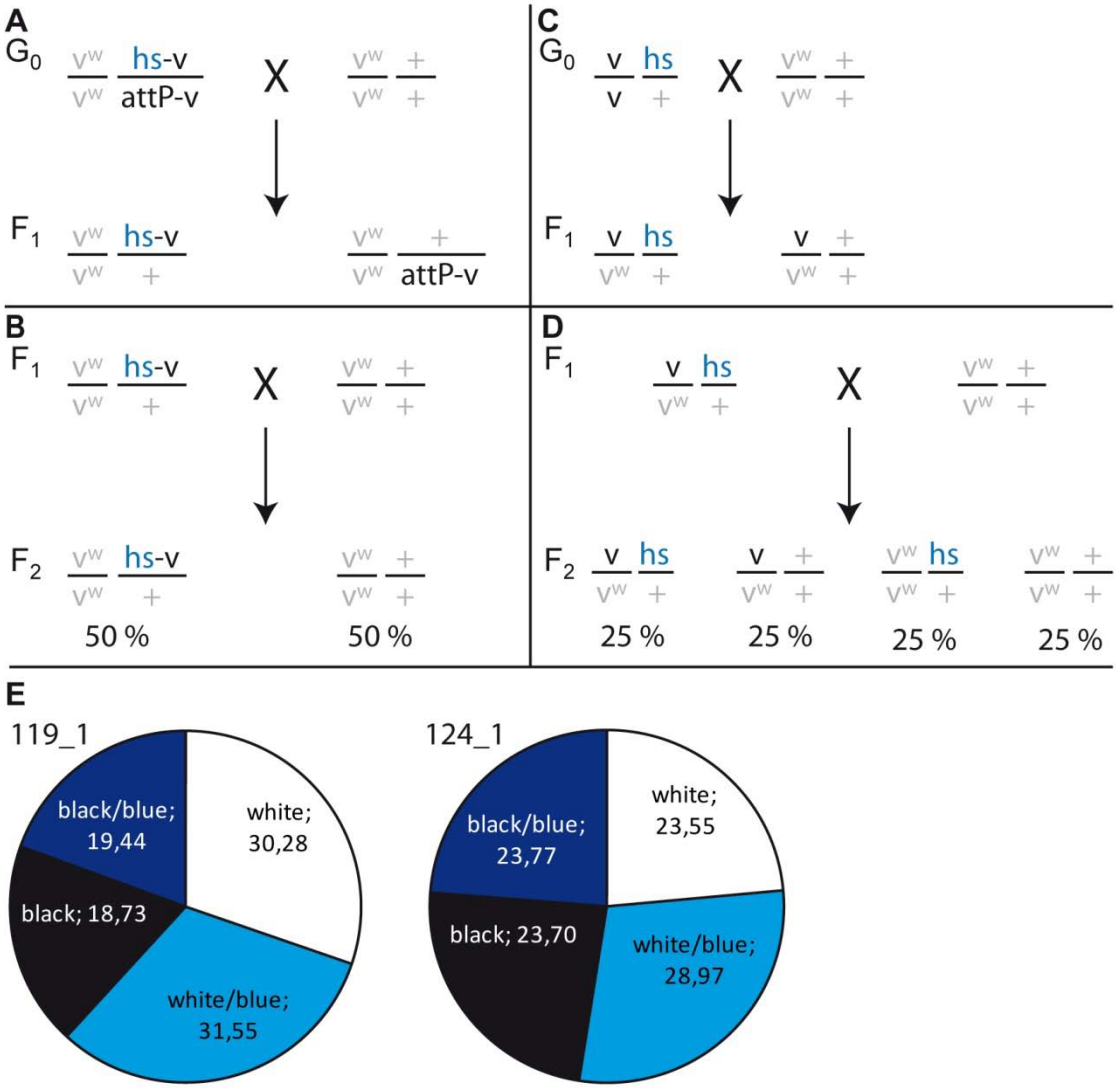


Fig. 29: Establishment of transgenic lines for ectopic expression of CLR markers. (A, B) Expected crossing scheme. (C, D) Hypothetical crossing scheme which would explain the findings. Color coding indicates marker expression (blue: blue fluorescent eyes due to ECFP expression; black: black eyes due to *vermillion* expression; grey: white eyes due to *vermillion*^{white} mutation). (A) The G_0 individuals should have a v^w background and should therefore be homozygous for the mutated version of the *vermillion* gene (v^w). The heat shock construct (hs) should have heterozygously integrated into the *attP* site, which is linked to the wild type *vermillion* gene (*v*) rescuing black eye color. The G_0 individuals are crossed to animals of the v^w strain. Offspring should be homozygous for v^w and carry either the original *attP* construct or the *attP* construct with the heat shock construct integration. (B) Blue-eyed F_1 males were then crossed to v^w females. Offspring should be homozygous for v^w and either heterozygously carry the heat shock construct linked to *v* or be homozygous for the respective wild type situation. (C) Probably, the heat shock construct integrated in the absence of *attP*. The resulting line appears to be homozygous for *v*, but might as well be heterozygous for v^w (not shown). Crossing to the v^w strain results in offspring which are heterozygous for v^w as well as for hs and offspring which are heterozygous for v^w as well as homozygous for the wild type situation. (D) Blue-eyed F_1 males were crossed to v^w females, resulting in four genetically different offspring each of which shows a unique eye marker combination. (E) Relative numbers of the different marker combination for offspring of the established lines.

In case of *Tc-six3_3*, the G_0 generation was crossed to other G_0 animals. Two beetles with blue fluorescent eyes were found. I established two transgenic lines for ectopic expression of *Tc-six3* by crossing the animals to v^w beetles. In both lines, all blue-eyed beetles have a black eye background. However, this does not necessarily indicate integration into the *attP* site but is expected when the injected line is the *SB* strain (Fig. 30).

No transgenic offspring were obtained after injection of the *Tc-cncB* heat shock construct or the first two injection rounds for *Tc-six3*.

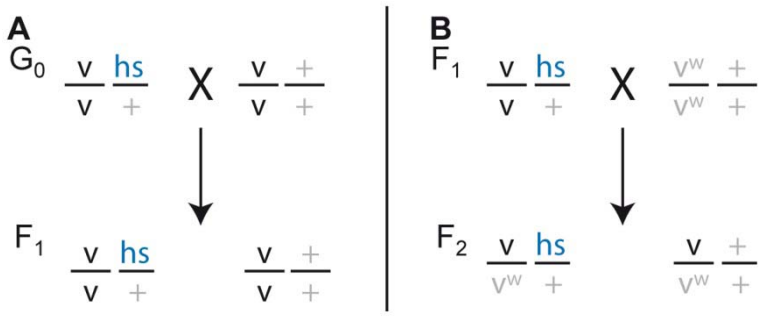


Fig. 30: Crossing scheme for *Tc-six3*. The crossing scheme is based on the assumption that injection was carried out with the *SB* strain instead of the *attP* line. Color coding indicates marker expression (blue: blue fluorescent eyes due to ECFP expression; black: black eyes due to *vermillion* expression; grey: white eyes due to *vermillion*^{white} mutation). (A) Injected animals were crossed to other injected animals of the G_0 generation which most likely did not carry a transgenic integration. The resulting F_1 animals had the same genetic background as their parents. (B) Transgenic (i.e. blue-eyed) beetles were crossed to animals of the v^w strain. 50 % of the offspring carried the transgene and all offspring were heterozygous for the black eye marker.

Ectopic expression of *Tc-croc* – proof of principle

Egg collections of 24 h from lines 119_1 and 124_1 were heat shocked, fixed and *in situ* hybridized with RNA probes for *Tc-croc* to test for ectopic expression. Staining revealed that *Tc-croc* was in fact expressed in the whole embryo. Expression looks, however, spotted and stronger along the midline (Fig. 31A-D). The batch of embryos also contained non-transgenic ones, due to the fact that the line was not homozygous. In these embryos, staining of endogenous *Tc-croc* expression was very weak (not shown), indicating that ectopic expression is very high in transgenic embryos. Longer staining would have probably covered the apparent patchy expression pattern. Expression appeared somewhat stronger in 119_1 than in 124_1.

I then analyzed whether ectopic expression had an influence on cuticle development. I collected eggs for 1 h and let each collection develop for 9-17 h before heat shock (see p. 29). Eggs were then allowed to develop into cuticles. I find high numbers of hatched larvae, indicating normal development (mean values: 60.9 % in 119_1; 86.6 % in 124_1). Many of the remaining eggs do not develop any cuticle structures (“empty egg phenotype”; mean values: 67.3 % for 119_1; 73.5 % for 124_1). L1 larvae which developed but did not hatch from heat shocked eggs show defects in the position and development of mouthparts (primarily 119_1;

23.2 % affected) and in the head bristle pattern (primarily 124_1; 46.1 % affected). However, the defects are rather diverse and also larvae allowed to develop without a heat shock displayed similar defects in 18.3 % (119_1) and 30.8 % (124_1).

I therefore analyzed, whether ectopic expression at a certain point during development led to clearer results. Timing of the heat shock (collection of 1 h; heat shock after 9-17 h) did not lead to a higher percentage of cuticles with defects in 119_1. In 124_1, a heat shock after 16-17 h or 17-18 h AEL leads to 58.1 % or 75 % of defective cuticles, respectively. These preliminary results are, however, based on low egg numbers. Experimental setup required egg collections of one hour each for a period of nine hours, but egg numbers dropped after the first two egg collections and only few larvae developed from the ones collected later (i.e. heat shocked during earlier development; Fig. 31E, F). Numbers can also be found in the appendix (p. 114).

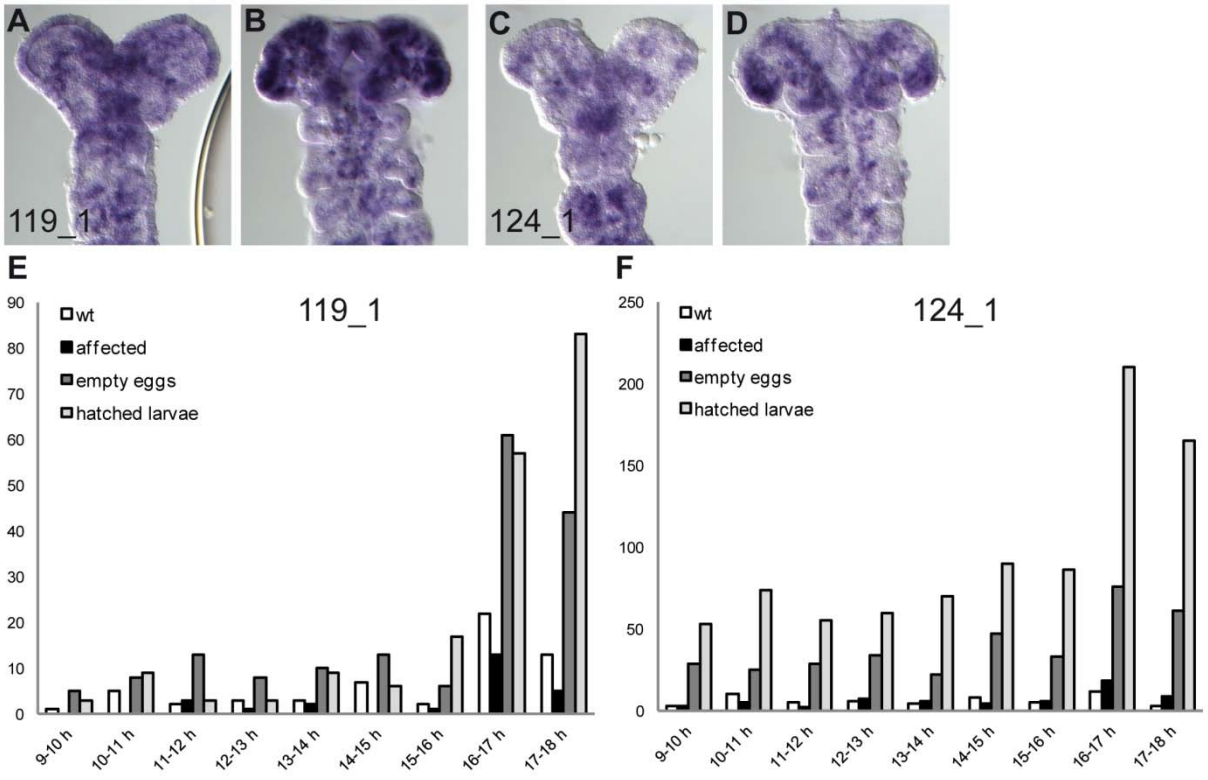


Fig. 31: Expression of *Tc-croc* and cuticle analysis after heat shock. (A-D) Expression of *Tc-croc* after heat shock in lines 119_1 (A, B) and 124_1 (C, D) is shown in two different developmental stages, respectively. Expression is ubiquitous, but looks patchy and stronger along the midline. Anterior is up. (E, F) Absolute numbers of wild type-like (wt; white bars) and affected cuticles (black bars) as well as empty eggs (dark grey bars) and hatched larvae (light grey bars) are given for each time point AEL eggs were heat shocked at. The first two egg collections were the oldest at the time of heat shock (16-17 h and 17-18 h). Egg numbers dropped notably in later collections.

In a last experiment, I analyzed whether a heat shock after 16-18 h leads to defects in the expression of the segment polarity gene *Tc-wg*. Preliminary analysis in both lines did not reveal strong alterations, but in some embryos *Tc-wg* staining in the stomodaeum appeared reduced (not shown).

I conclude that integration into the *Tribolium* genome using the φC31 system and subsequent ectopic expression of integrated genes works in principle. However, due to the fact that the injected line did not carry an *attP* site, integration occurred at a different position than expected. Preliminary results indicate that ectopic expression of *Tc-croc* does not lead to obvious defects.

5 Discussion

5.1 Morphogenetic movements during early anterior head development

5.1.1 The Amnion is involved in the formation of head structures

I showed that in *Tribolium*, the formation of the distinct shape of the anterior embryonic head depends on a previously unrecognized morphogenetic movement involving the germ band and the extraembryonic tissues. There are two extraembryonic membranes in insects, amnion and serosa. In higher flies including *Drosophila*, these two membranes are fused to one continuous dorsal structure, the amnioserosa, which does not cover the embryo (Campos-Ortega and Hartenstein, 1997). The formation of the amnioserosa in *Drosophila* depends on the expression of the *Hox3* ortholog *zerknüllt* (*zen*; Rushlow et al., 1987a; Rushlow et al., 1987b; Pultz et al., 1988). In other insects, *zen* orthologs are necessary for the development of the serosa, but not the amnion (van der Zee et al., 2005; Panfilio et al., 2006; Rafiqi et al., 2008). The serosa is probably not important for proper embryogenesis of the insect head. After RNAi knockdown of one of the two *Tribolium zen* orthologs, *Tc-zen1*, the serosa does not form but the head still develops normally albeit delayed (van der Zee et al., 2005).

The amnion, on the other hand, plays a role in insect head formation. Amnion development has been studied in the dipteran *Megaselia* and depends on the activity of the u-shaped (ush)-group of genes (Rafiqi et al., 2010). In *Drosophila*, these genes maintain the amnioserosa in later developmental stages when *zen* is not expressed anymore (Frank and Rushlow, 1996). Knockdown of u-shaped genes via RNAi in *Megaselia* leads to loss of the amnion and results in defects in dorsal closure as well as head formation (Rafiqi et al., 2010). One gene belonging to the ush-group is *tup*, the *Tribolium* ortholog of which I showed to be expressed in the extraembryonic membranes and to be necessary for labrum development. This strengthens the idea that formation of the clypeolabral region (CLR) is closely connected to amnion development which may depend on the ush-group of genes. Analysis of the other members of this group will be necessary to further test this hypothesis.

5.1.2 The anterior fold separates the ectoderm of the head lobes

The formation of the anterior fold during the transition of the germ rudiment to the serosa window stage appears to play an important role in the formation of the head lobes. Using FM® 1-43 staining, I showed that the anterior fold separates the ectoderm in the anterior head and leads to a relocation of anterior terminal cells to a more posterior sub-terminal position. Apparently, this leads to the outwards-directed shift of head lobe tissue. This movement has

been noticed before in *Tribolium* (Posnien et al., 2010) and is evident by the expression of genes along the anterior-posterior axis. Genes expressed along the midline (e.g. *Tc-mae, Tc-vnd, Tc-spi, Tc-gremlin, Tc-Sox21b*) are, in fact, often expressed as two parallel stripes along the ventral midline in early embryos (see Fig. 32A, B and e.g. Bucher and Klingler, 2005; Wheeler et al., 2005; Kittelmann, 2008; Grossmann, 2011). In the trunk and the posterior head, these stripes fuse medially during later development due to the fusion of the ectoderm covering the invaginating mesoderm (Fig. 32C, D). The fusion of the ectoderm is impeded in the anterior head by the anterior fold as shown by FM® 1-43 staining, eventually leading to a Y-shaped expression pattern (see Fig. 32E, F; Bucher and Klingler, 2005; Wheeler et al., 2005; Grossmann, 2011; Kittelmann, 2008). Also genes expressed in a segmental fashion, e.g. *Tc-wg*, follow the Y-shape. This leads to a displacement of the antennal expression stripe relative to more posterior stripes (Nagy and Carroll, 1994; see also expression of *Tc-ems* in Fig. 32A-F). Interestingly, the loss of *Tc-six3* function results in absence of the CLR and a fusion of the head lobes along the midline. In this derived state, the antennal *Tc-wg* stripes are again parallel to more posterior stripes (Posnien et al., 2011b; this work). It would be interesting to test, whether formation of the anterior fold is affected by *Tc-six3* RNAi or whether only ectodermal tissue is deleted.

Based on my findings as well as on previous results, I propose the following model for the morphogenesis of the anterior head (Fig. 32G-K; see Handel et al., 2000 for previously described morphogenetic movements): Prior to gastrulation, the anlagen of the amnion surround the complete anlagen of the embryo proper (Fig. 32G). During the sinking of the germ rudiment into the yolk and the formation of the amniotic fold, the amnion stays in contact with the mesoderm in the anterior (Fig. 32H, H'). The growth of the extraembryonic membranes over the germ band occurs simultaneously with germ band extension and fusion of the ectoderm over the invaginating mesoderm. In the anterior head, germ band extension appears to be directed outwards leading to the formation of the head lobes and the anterior fold between them (Fig. 32I, I'). The mechanical reason might be the static amnion-mesoderm connection which blocks the fusion of the ectoderm. The anterior fold separates the ectoderm of both head sides during early and mid germ band extension and encloses a mesenchymal cell population which is apparently uncovered by the amnion (Fig. 32J, J'). Subsequently, the anterior fold itself is laterally enclosed by the ectoderm and the mesenchymal cells become located where the stomodaeum develops later on (Fig. 32K, K'). Possibly, this cell mass or the anterior fold themselves form the stomodaeum. The anterior fold appears to vanish in later embryogenesis while the previously separated ectoderm of both head sides fuses.

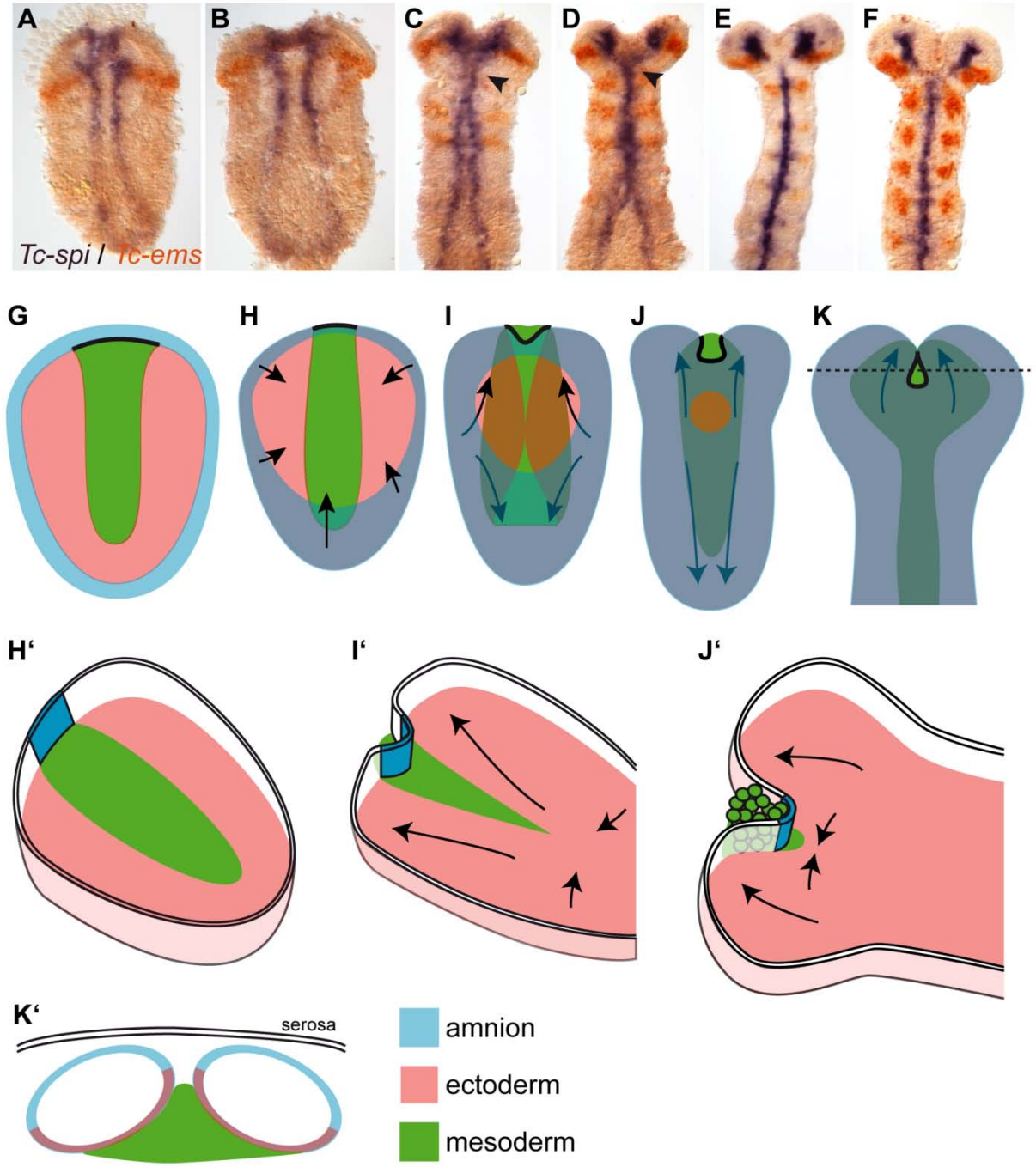


Fig. 32: A model for the formation of the anterior fold and early morphogenetic movements in the anterior head. (A-F) Expression of *Tc-spi* and *Tc-ems* recapitulates morphogenetic movements in the anterior head. In young embryos (A, B) *Tc-spi* is expressed in two parallel stripes along the midline. The stripes start to fuse posterior of the CLR during early germ band extension (arrowheads in C, D). The fusion progresses towards posterior. In the anterior head, the stripes branch (D-F) and become Y-shaped. The segmental expression of *Tc-ems* follows the Y-shape, as well. (G-K) Schematic explaining the model of anterior fold morphogenesis. (G) Prior to gastrulation, the anlagen of the embryo proper are surrounded by the anlagen of the amnion (blue). The mesoderm (green) is specified along the midline of the germ rudiment. It is connected to the amnion at the anterior (black line). The ectoderm (red) is situated lateral and posterior of the mesoderm. The posterior growth zone is not taken into account in the schematic. (H) The embryo starts to sink into the yolk and the amnion begins to grow over the embryo (indicated by arrows). The mesoderm stays connected to the amnion at its anterior tip (black

line; see also the three-dimensional scheme in H' where the amnion is white and only the connection between amnion and mesoderm is marked blue). (I) The amnion closes further over the germ band. In the anterior, the amnion grows partly over the mesoderm but stays connected to it (see also I'). The embryo starts to elongate through convergent extension (indicated by arrows). The ectoderm starts to fuse at the midline while the mesoderm invaginates. Fusion starts posterior of the CLR and progresses posteriorly. (J) The amnion is almost completely closed over the embryo. The germ band elongates further (indicated by arrows). The connection between mesoderm and amnion (black line) restrains the elongation of the ectoderm at the anterior tip, where the anterior fold is formed. This leads also to the formation of the head lobes. The anterior fold begins to enclose the mesenchymal cell mass at the anterior tip (see also J'). (K) The amnion is closed completely over the embryo except for the anterior where the anterior fold encloses the mesenchymal cell mass. The head lobes start to come together anterior of the anterior fold. (K') depicts a transverse cut along the dotted line. Each of the head lobes is covered by separate amniotic membranes. The ectodermal sheets of both head lobes are separated by the mesoderm between them.

5.1.3 Gene expression changes follow the morphogenetic movements

It has been enigmatic for some time why some genes start being expressed at the anterior tip of the *Tribolium* germ band during early embryogenesis but end up at a more posterior position during later stages. This is, for instance, the case for *Tc-six3* (Posnien et al., 2009b; Steinmetz et al., 2010; Posnien et al., 2011b), *Tc-croc* (Economou and Telford, 2009), *Tc-scro* (Posnien et al., 2011b) or *Tc-hh* (Farzana and Brown, 2008). The movement of the expressing cells to a more posterior position would provide an elegant mechanism explaining the expression shift. As already mentioned above, also the development of Y-shaped expression patterns of ventrally expressed genes can now be explained by tissue movements.

Other genes are expressed along the anterior fold, e.g. *Tc-cnc*. Its expression spreads over the anterior ectoderm around the time when the anterior fold becomes indistinguishable from the ectoderm. According to my model, this is the point when the ectodermal sheets of both head lobes merge. Again, the drastic change of an expression pattern can be explained with the help of these morphogenetic movements. It will be helpful to analyze the expression of *Tc-cnc* in the context of morphogenesis to test this. Staining with NBT/BCIP in FM® 1-43-stained embryos results in quenching of the fluorescence signal (see Posnien et al., 2009b), making it possible to determine the point of *Tc-cnc* spreading. This technique will also help to determine whether the posterior end of the anterior fold coincides with the stomodaeal expression domain of *Tc-fkh* and, hence, with the site of stomodaeum invagination.

Two significant findings based on the analysis of morphogenetic movements can be summarized as follows: (1) the formation of the *Tribolium* head involves a previously not described structure, the anterior fold; (2) expression changes of many genes can be explained by the morphogenetic movements caused by the anterior fold.

5.1.4 *In vivo* imaging approaches to further study head morphogenesis

To further test the hypothesis that early head morphogenesis involves the amnion and the anterior fold, *in vivo* imaging experiments should be carried out. In a recent publication by Sarrazin et al. (2012), the same *Tribolium* line as in my experiments was used. Early development is well traceable but as soon as the amnion closes over the germ band, embryonic cells become difficult to follow. The same was observed in my DSLM experiments. In order to circumvent these difficulties, *in vivo* imaging of only germ rudiment tissue is desirable. One approach is the establishment of a *Tribolium in vivo* imaging line that expresses GFP under control of a germ rudiment enhancer. A gene which is expressed only in germ rudiment tissue throughout development is, however, yet to be discovered. Alternatively, GFP expression in only parts of the germ rudiment is possible. This could be facilitated by the regulatory sequences of *Tc-tll* marking the anterior head lobes and *Tc-cnc* or *Tc-six3* marking the CLR.

Similarly, a fluorescent protein other than GFP could be expressed under control of the regulatory sequence of an early CLR marker gene. In combination with ubiquitous GFP expression, this approach would allow to trace CLR precursor cells while overall development could still be followed. Koniszewski (2011) tried to identify the regulatory sequence of *Tc-six3* but was unsuccessful. In this work, I identified a non-coding 5'-exon of the *Tc-cncB* splice variant. Possibly, this 5' untranslated region or the introns 3' of it provide cis regulatory elements for the specific expression of *Tc-cncB* in the CLR. Also the region upstream of the *Tc-croc* coding region could provide useful regulatory elements.

Moreover, the regulatory sequence of a gene expressed specifically in the amnion could be used to control the expression of an inverted repeat of part of the GFP coding sequence. This would lead to the production of double stranded RNA and subsequent RNAi-mediated degradation of the GFP mRNA in the amnion. The respective line would then have to be crossed to the line previously used for *in vivo* imaging. The resulting offspring would express GFP in all cells but degrade the GFP mRNA specifically in the amnion via RNAi. It has been shown in mice that ubiquitous expression of GFP can be reduced by expression of small interfering RNA directed against GFP (Hasuwa et al., 2002). The u-shaped group of genes might hold some promising candidates for genes expressed specifically throughout the amnion. Hence, their regulatory sequences could be used in this context.

A further and very elegant approach would be the use of photoconvertible proteins (reviewed in Lukyanov et al., 2005; Müller-Taubenberger and Anderson, 2007; Shaner et al., 2007). A *Tribolium* line expressing mEos2 (McKinney et al., 2009) under control of an α-tubulin promoter has recently been established (Dippel, unpublished results). All cells of indi-

viduals from this line express green fluorescent mEos2 which switches to a red fluorescent form upon activation with ultraviolet light. Activation of the red fluorescence in only a subset of cells (e.g. the anterior median tip of the germ rudiment) would allow the tracing of these cells while overall embryogenesis can still be followed.

As already mentioned, another drawback of the GFP line established by Sarrazin et al. (2012) was the nuclear localization of the GFP which disperses throughout the cell upon mitosis and therefore hampers the tracing of single cells throughout development. In their reconstruction of zebrafish development, Keller et al. (2008) used a fusion protein of human Histone 2B and GFP (Kanada et al., 1998) staying localized to chromatin through mitosis. A *Tribolium* line expressing a similar fusion protein with the *Tribolium* ortholog of Histone 2B is currently being established (Kitzmann and Bucher, unpublished results). Besides allowing the tracing of cells throughout development, resolution of single nuclei might be higher. Moreover, this line will allow assessing mitotic activity *in vivo*. As the immunostaining of phosphorylated Histone H3 in this work did not result in a clear picture, this provides an alternative opportunity to test to what extent proliferation is an important player in morphogenesis of the head as well as overall development.

5.2 The boundaries of the CLR as inferred from gene expression and morphological markers

Tc-croc marks the posterior boundary of the CLR, as it is expressed directly adjacent to the intercalary segment (Economou and Telford, 2009). Without its expression, the intercalary segment marker *Tc-lab* is detected ectopically at a more anterior position, indicating a repression by *Tc-croc*. It could not be clarified, whether this repressive function is mutual but data from Schaeper et al. (2010) indicate that *Tc-lab* is not needed for the establishment of the posterior CLR boundary. RNAi for *Tc-collier* (*Tc-col*), a gene usually repressed by *Tc-lab* leads to a loss of segmental markers in the intercalary segment. *Tc-lab* is still expressed normally, indicating the further presence of the respective cells. In this background, the stomodaeal expression stripes of *Tc-cnc* which are overlapping with *Tc-croc* expression (Economou and Telford, 2009) are shifted towards the posterior and are now in contact with the mandibular *Tc-cnc* expression domain. This indicates a shift of the posterior CLR boundary on expense of the intercalary segment, despite normal *Tc-lab* expression. Hence, another factor, possibly *Tc-col* or one of its targets must act in concert with *Tc-croc* to establish the posterior CLR boundary. In *Drosophila*, expression of *croc* is not affected in *col* mutants and vice versa (Crozatier et al., 1999). It will be interesting to test the interaction of both genes in *Tribolium*.

In contrast to the definite boundary at the posterior, expression of *Tc-croc* in the anterior CLR overlaps with virtually all tested outside markers. As these genes are expressed in neurogenic tissue, it is likely that *Tc-croc*, besides its function in stomodaeum development, is also involved in the patterning of distinct parts of the nervous system. In *Drosophila*, *croc* is necessary for the development of the stomatogastric nervous system (SNS; Schmidt-Ott et al., 1994). I did not test the presence of the SNS in *Tribolium* after *Tc-croc* knockdown. However, the SNS forms from invaginations of the stomodaeum roof (Hartenstein et al., 1994), and due to the fact that the stomodaeum is absent, loss of the SNS appears likely. In this regard, *Tc-croc* would at least be indirectly necessary for the development of the SNS. The overlapping expression of *Tc-croc* and neurogenic markers is, however, not found in the stomodaeum roof, but lateral of the stomodaeum. It will be interesting to analyze, which brain parts develop from this region. Therefore, RNAi could be carried out in a *Tribolium* brain imaging line which was established by Koniszewski (2011; see also Posnien et al., 2011b).

The anterior CLR boundary appears to be set at least partly by *Tc-cnc*, as it restricts the expression of *Tc-tll* from the labrum region. However, after *Tc-cnc* RNAi, a median portion of the CLR is still free of *Tc-tll* expression. This indicates a partial redundancy of *Tc-tll* repression by a second factor. An incomplete expansion of *Tc-tll* expression was also detected after *Tc-croc* RNAi. Thus, both *Tc-cnc* and *Tc-croc* and maybe also additional factors could be necessary for the repression of *Tc-tll* expression in the whole CLR. This can be tested by RNAi for both genes simultanously.

Tc-tll on the other hand, is apparently not involved in establishing the CLR boundary, as loss of its function does not affect expression of *Tc-cnc* or *Tc-croc*. The discrepancies of head bristle defects after *Tc-tll* RNAi found in this work and by Posnien et al. (2011b) might be due to the fact that after loss of head lobe tissue, much compensation by the epidermis might take place during dorsal closure where the smaller head lobes have to fuse (see bend and zipper model in Posnien et al., 2010). A similar effect has been observed after knockdown of *Tc-abrupt* (*Tc-ab*). *Tc-ab* RNAi leads to smaller head lobes and random defects in the head bristle pattern. In an attempt to discriminate between weak and strong defects, RNAi was performed with lower dsRNA concentrations. However, this did not lead to a more distinct phenotype but just less affected bristles which still occurred randomly. Moreover, dorsal closure was delayed (Bucher, personal communication).

It remains to be tested which gene expressed in the neurogenic region of the non-segmental head establishes the CLR boundary in concert with the CLR factors.

5.3 Establishing a model for a gene regulatory network of the anterior head

One of the purposes of this work was to identify the genetic interactions that play a role during the formation of the CLR and its various structures. The genes were therefore analyzed under three aspects: (1) when does expression of the gene start, (2) which defects are observed after loss of function, and (3) loss of which gene function leads to loss of expression aspects of other genes. An overview of the affected areas in the head after RNAi can be found in Fig. 33A. Based on my results as well as on previous data, a model for a CLR gene regulatory network can be established revealing some major differences to *Drosophila* (Fig. 33B; compare to Fig. 2).

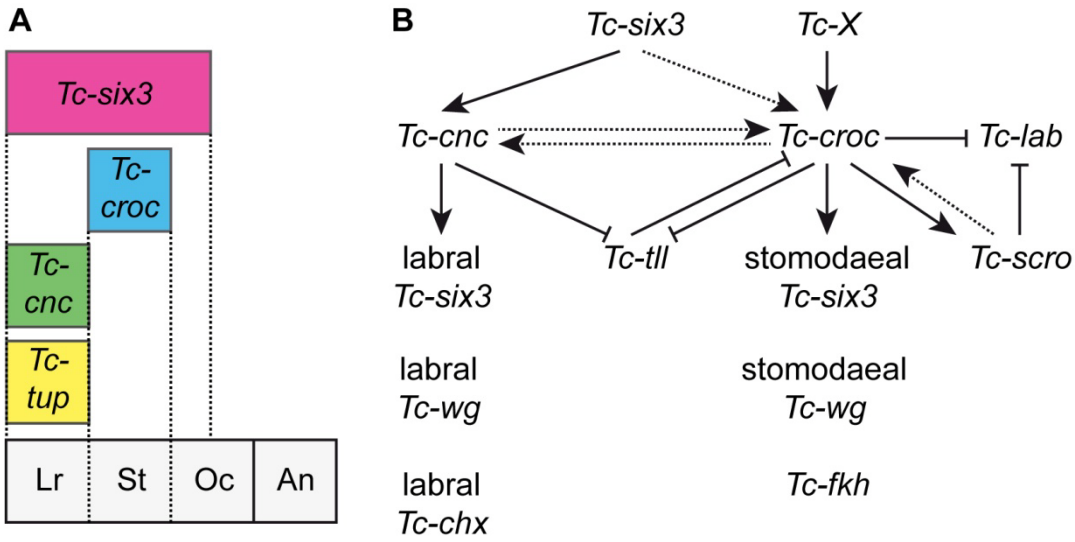


Fig. 33: Deletion areas of the CLR markers and genetic interactions of *Tc-six3*. (A) The different CLR markers are necessary for the development of different parts of the procephalon. Loss of *Tc-six3* function leads to deletion of a triangular region comprising the labrum and stomodaeum anlagen as well as part of the neurogenic tissue in the pre-ocular region. *Tc-croc* is needed for the establishment of the stomodaeal invagination. Knockdown of *Tc-cnc* or *Tc-tup* abolishes development of labral structures. An: antennal segment; Ic: intercalary segment; Lr: labral region; Oc: ocular region; St: stomodaeal region. (B) Arrows indicate activation; bars indicate repression; dotted arrows indicate activation of only certain aspects of the target. *Tc-six3* is the earliest expressed CLR marker and necessary for formation of the ectodermal aspects of the CLR. After loss of its function, the ectodermal CLR is deleted; hence, a direct regulation of *Tc-cnc* and the ectodermal *Tc-croc* expression is likely. As the posterior aspects of *Tc-croc* are still expressed normally after *Tc-six3* RNAi, it needs at least one additional factor to be activated, indicated by *Tc-X*. *Tc-croc* represses *Tc-lab* and *Tc-tll* expression in the CLR and is itself repressed by *Tc-tll*. *Tc-croc* is needed for expression of *Tc-six3*, *Tc-wg* and *Tc-cnc* in the stomodaeum. It also activates *Tc-fkh* and *Tc-scro* expression. In turn, *Tc-scro* activates the most posterior expression aspect of *Tc-croc*. *Tc-cnc* is necessary for the labral expression domains of *Tc-six3*, *Tc-wg*, *Tc-chx* and *Tc-croc* and represses expression of *Tc-tll*. The mutual activation of *Tc-cnc* and *Tc-croc* is only in the labrum and stomodaeum region, respectively.

5.3.1 *Tc-six3* is an early upstream activator of CLR development

The earliest gene expressed in the CLR is *Tc-six3*, which also correlates to its drastic RNAi effect deleting most of the CLR (Posnien et al., 2009b; Posnien et al., 2011b; this work). In this work, I showed that it is also required for the expression of *Tc-cnc* and anterior aspects of *Tc-croc*. The tissue loss is first morphologically detectable during germ band extension, but already in germ rudiments anterior ectodermal expression of *Tc-croc* is reduced. The fact that mesodermal/endodermal expression of *Tc-croc* is more or less normal leads me to suggest, that *Tc-six3* is only necessary for the formation of the ectodermal aspects of *Tc-croc* expression. In contrast to that, expression of *Tc-cnc* is lost entirely after *Tc-six3* RNAi.

Possible reasons for and timing of the tissue loss

It is unclear, whether the loss of tissue after *Tc-six3* RNAi is mediated by a deletion of cells or whether the tissue is not established at all. Loss of *Six3* function leads to defects in eye and forebrain development after higher apoptosis levels in the ricefish *Oryzias latipes* (Carl et al., 2002). On the other hand, It is reported for *Xenopus* and zebrafish that Six3 promotes proliferation by antagonizing the inhibitive function of Geminin on DNA replication (Del Bene et al., 2004) and by transcriptional activation of cell cycle regulators (Gestri et al., 2005). Both scenarios are therefore possible in *Tribolium*.

I tried to solve this issue by testing in different embryonic stages whether the tissue loss was due to cell death. I did not find an enhancement of cell death in germ rudiments. The numbers of dying cells during germ band extension are higher compared to untreated animals, but still appear to be too low to account for the tissue loss, as the numbers are much higher in retracting germ bands where no further tissue loss is detectable. This might be indicative for further defects in eye and brain development after *Tc-six3* loss of function as it is the case for *Oryzias*. It remains to be tested whether apoptosis levels are higher during blastodermal stages and therefore account for the drastic tissue loss in the anterior head.

It is possible that not cell death but reduced proliferation leads to the loss of tissue after *Tc-six3* RNAi. To further investigate this, *in vivo* imaging with the described Histone-GFP line should be carried out before and after *Tc-six3* RNAi. Also, further phospho-Histone immunostaining might give a clearer insight into the generation of the *Tc-six3* RNAi phenotype.

Alternatively, *Tc-six3* could be necessary for proper formation of the anterior fold and subsequent development of the CLR. After loss of its function, the fold might fail to be established and in turn the head lobes never become separated from each other. FM® 1-43 staining of *Tc-six3* RNAi embryos would help to solve this issue.

Regulation of *Tc-six3* expression

Tc-six3 is very high in the hierarchy of anterior head patterning but is not provided maternally (Posnien et al., 2009b). Therefore, other genes must activate its expression during blastodermal stages. Only one candidate for activation of *Tc-six3*, *Tc-hbn* was analyzed in this work and loss of the entire procephalic head was found. An important role in early head patterning appeared unlikely for *Tc-hbn* because of the location of the head anlagen in the *Tribolium* egg. In short germ insects, the germ rudiment forms in the posterior ventral region of the egg, while the anterior dorsal part contains the anlagen of the extraembryonic membranes (Sokoloff, 1972; Tautz et al., 1994; Handel et al., 2000; van der Zee et al., 2006). Hence, the the head does not develop at the anterior tip of the egg during early stages and a gene expressed here is unlikely to be involved directly in head development. The *Tribolium* expression pattern of *Tc-hbn* in an anterior cap during the blastoderm stage does not speak for a direct interaction with *Tc-six3* during blastodermal stages. Nevertheless, *Tc-hbn* is necessary for the establishment of the procephalon.

In *Drosophila*, the loss of function-phenotype of the orthologous *hbn* is reminiscent of the one in *Tribolium* but less drastic. Only the labrum and anterior brain parts are deleted (Kaspar, 2008). *hbn* and *optix/six3* appear to be expressed in the same region in *Drosophila* (compare Walldorf et al., 2000 and Seo et al., 1999), offering the possibility of direct activation or interaction. Also in *Tribolium*, both genes are likely to be co-expressed in young elongating germ bands and *Tc-hbn* could influence expression of *Tc-six3*. Otherwise, it could be involved in the establishment of axial polarity, which in turn might be necessary for proper *Tc-six3* expression. It remains to be tested by double *in situ* hybridization, whether the two genes are truly co-expressed and could, hence, interact in head development.

Other genes that might be involved in the control of *Tc-six3* expression are genes of the anterior patterning system. In *Drosophila*, the anterior system depends on activity of the anterior localized determinant, Bcd, which functions as a transcriptional activator for anterior target genes and represses translation of the posterior factor, Cad (Frohnhöfer and Nüsslein-Volhard, 1986; Berleth et al., 1988; Struhl et al., 1989; Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). The *bcd* gene has, however, evolved rather recently only in higher dipterans by a duplication of the *Hox3* ortholog *zen* and is not present in other insects (Sommer and Tautz, 1991; Schröder and Sander, 1993; Stauber et al., 1999; Brown et al., 2001). In *Tribolium*, the translational repression of *Tc-cad* mRNA is carried out by Tc-Mex-3 (Schoppmeier et al., 2009). The genes *Tc-otd-1* and *Tc-hunchback* (*Tc-hb*) have been suggested to substitute for the lacking *bcd* in terms of transcriptional activation due to the head defects found after loss

of their function (Schröder, 2003). However, more recent studies have shown that these defects are, in fact, resulting from incorrect dorso-ventral patterning and a homeotic transformation, respectively (Kotkamp et al., 2010; Marques-Souza et al., 2008). The role of *Tc-otd-1* in head formation indicates that proper dorso-ventral patterning is crucial for development of the *Tribolium* head. This is due to the fact that the head in *Tribolium* forms in the ventral blastoderm. Also Bone morphogenic protein (BMP) signaling is involved in determination of the dorso-ventral axis and head formation in *Tribolium*. Knockdown of *Tc-short gastrulation* (*Tc-sog*) which is the ortholog of the vertebrate BMP antagonist, Chordin, results in an enlargement of the dorsal serosa on cost of ventral head tissue. On the other hand, RNAi against the BMP ortholog, *Tc-decapentaplegic* (*Tc-dpp*), leads to a loss of serosal tissue and to an expansion of the head lobes (van der Zee et al., 2006). Due to the fact that the dorso-ventral system is involved in head formation, activation of the early CLR patterning factor *Tc-six3* is likely to involve the dorso-ventral system, as well. Based on its early expression in the anterior head anlagen and the head defects after RNAi, *Tc-otd-1* is a good candidate for activation of *Tc-six3* expression.

Moreover, canonical Wnt signaling has proven to function in *Tribolium* head patterning. Similar to vertebrates, canonical Wnt signaling is repressed in the anterior. The anterior localized factor, Tc-Axin, is involved in the β-catenin destruction complex and its knockdown results in translocation of β-catenin to the nucleus. Here, it activates expression of targets genes involved in the development of posterior structures leading to the loss of head structures (Fu et al., in press). Hence, canonical Wnt signaling might be involved at least indirectly in *Tc-six3* activation by the repression of posterior factors.

Possible downstream targets of *Tc-six3*

Tc-six3 is necessary for proper activation of several genes in the anterior CLR (Fig. 34). More posterior expression aspects are, however, not dependent on *Tc-six3*. *Tc-cnc* expression around the stomodaeum anlagen and the stomodaeal expression domain of *Tc-wg* are established regardless of a loss of *Tc-six3* activity. The expression domains of both genes are, however, malformed due to the lack of anterior ectodermal tissue in *Tc-six3* RNAi embryos. Also, the stomodaeum does not invaginate as indicated by the lack of a foregut in developing L1 larvae. Hence, a second factor besides *Tc-six3* is necessary for a normal establishment of stomodaeal gene expression. Most likely, *Tc-croc* is this factor, as its stomodaeal expression domain is not affected by *Tc-six3*.

It is difficult to determine whether the loss of expression of various genes after *Tc-six3* knockdown indicates direct activation or whether it is merely a secondary loss due to the

missing tissue (see Fig. 33). Ectopic expression of *Tc-six3* in the established heat shock lines might help to answer this question. Furthermore, *Tc-six3* can be knocked down by embryonic RNAi at different stages. Expression of *Tc-croc* starts in early germ rudiments, expression of *Tc-cnc* slightly later. These developmental stages correspond approximately to 9.5 and 10 h AEL at 32 °C, respectively. Therefore, injection of *Tc-six3* dsRNA at 8-9 h AEL should not interfere with its possible early function in cell death inhibition or proliferation activation but knock down its transcriptional activity at the given time. This procedure has proven helpful to discriminate between early and late function of *Tc-otd-1* (Schinko et al., 2008) and in the case of leg development, where staged knockdown of *Tc-wg*, *Tc-dpp* and the Epidermal growth factor receptor pathway reduced defects in early development of other structures and made the analysis of specific limb patterning defects possible (Grossmann et al., 2009; Grossmann, 2011).

The assumed interactions between *Tc-six3* and other genes in the anterior head are depicted in Fig. 34.

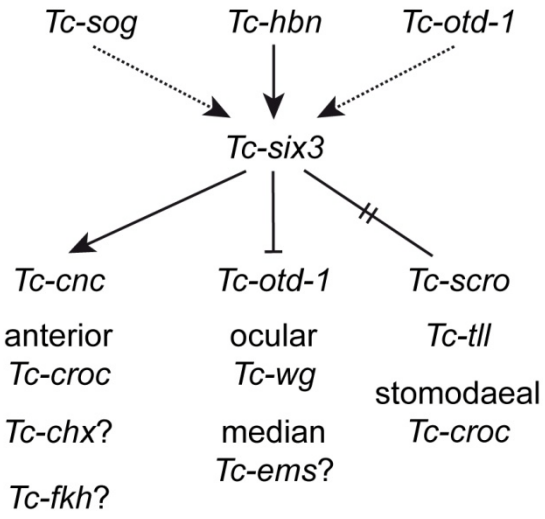


Fig. 34: *Tc-six3* is the earliest CLR patterning factor. Arrows indicate activation; the bar indicates repression; the crossed bar indicates no effect; question marks indicate uncertainty; dotted arrows indicate possible activation that has not been tested. The interaction scheme is based on findings published by Posnien et al. (2011b) as well as own observations. The assumed activation of *Tc-six3* by *Tc-otd-1* and/or *Tc-sog* is based on findings by Schröder (2003), Kotkamp et al. (2010) and van der Zee et al. (2006) indicating early anterior determining functions of these genes. Due to the fact that the complete anterior head including *Tc-six3* expression is lost after *Tc-hbn* RNAi, a direct activation of *Tc-six3* by *Tc-hbn* is an open question and the expression might be lost secondarily. Early regulation of *Tc-croc* and *Tc-cnc* by *Tc-six3* is likely to be direct because of co-expression. The lack of the later expression of *Tc-chx* and *Tc-fkh* after *Tc-six3* RNAi could be a secondary effect due to the loss of the CLR tissue. *Tc-otd-1* and ocular *Tc-wg* expression expand after *Tc-six3* RNAi. Hence they are repressed by *Tc-six3* (Posnien et al., 2011b). However, the expansion of the late median *Tc-ems* domains could be a secondary effect. No interaction of *Tc-six3* and *Tc-scro*, *Tc-tll* or the stomodaeal expression domain of *Tc-croc* was found.

5.3.2 *Tc-croc* and *Tc-cnc* pattern the posterior and the anterior part of the CLR, respectively

While loss of *Tc-cnc* function deletes the labrum anlagen, i.e. the anterior CLR, *Tc-croc* is needed for the formation of the stomodaeum and proper patterning of the posterior CLR.

Hence, they further subdivide the ectodermal region that is set up by the action of *Tc-six3* and possibly other early anterior determinants. In turn, *Tc-cnc* and *Tc-croc* probably activate the expression of further patterning factors. The genetic interactions are summarized in Fig. 33B.

Tc-cnc is necessary for labrum development and proper mandible patterning

In *Tribolium*, knockdown of *Tc-cnc* leads to a loss of the labrum anlagen. The orthologs of *Tc-cnc* in other insects functions in labrum development, as well. Studies are available for *Drosophila* (Mohler et al., 1995) and the milkweed bug *Oncopeltus fasciatus* (Birkan et al., 2011). The expression pattern is also conserved in the millipede *Glomeris marginata* indicating functional conservation, but functional data is not available (Janssen et al., 2011). The exact mechanism of *cnc* in labrum development is not known in any species. Its early expression suggests that it acts as an important upstream factor. It will be interesting to test whether loss of *Tc-cnc* function results in enhanced cell death in the CLR. Due to its expression along the anterior fold, *Tc-cnc* might also be involved in its development and, therefore, embryonic morphology should be studied by FM® 1-43 staining of *Tc-cnc* RNAi embryos.

While *cnc* is a determining factor for labrum development throughout insects, only in *Drosophila* and *Tribolium* its loss of function has a homeotic effect in the mandibular segment (Harding et al., 1995; Mohler et al., 1995; this work). It has been proposed that Cnc antagonizes the activating function of Deformed (Dfd) on downstream targets in the mandibular segment. In the absence of Cnc, Dfd promotes the development of maxillary as opposed to mandibular structures, while ectopic expression of *cnc* in the maxillary segment leads to reduced maxillary structures (McGinnis et al., 1998; Veraksa et al., 2000). In *Oncopeltus*, no function in mandible patterning has been detected (Birkan et al., 2011). This could be due to a secondary loss of the repressive function of *cnc* in the Hemiptera or to a newly evolved gain of this function in the Holometabola. Studies in further insects and arthropods might clarify this issue. It would also be interesting to test whether ectopic expression of *Tc-cnc* leads to defects in maxillary development of *Tribolium*, as well.

Activation of _Tc-cnc_ expression is probably not evolutionarily conserved

In *Drosophila*, the clypeolabral expression domain of *cnc* is established by the anterior and terminal maternal systems. Namely, *bcd* and *torso* are necessary for the establishment of the primary domain which is subsequently shaped by the gene products of *tll*, *spalt* (*sal*) and *giant* (*gt*). Expression in the mesoderm anlage is prevented by Snail (Sna). Also *hkb* and *otd* are involved in establishing the expression pattern, but the effects of these genes have been considered to be secondary (Mohler, 1993).

As already pointed out, the anterior patterning system in *Tribolium* and also more basal insects is different from the *Drosophila* situation because *bcd* is lacking. Hence, other genes must be involved in *Tc-cnc* activation. The *torso* gene is conserved in *Tribolium* and like in *Drosophila* necessary for the establishment of terminal structures. However, as described above, the *Tribolium* head does not develop at the eggs terminus and head development is not affected by *Tc-torso* knockdown (Schoppmeier and Schröder, 2005). Hence, also *Tc-torso* is unlikely to be involved in regulation of *Tc-cnc*. Nevertheless, expression control of *Tc-cnc* in the labral region is probably mediated by early anterior factors, given the relatively early start of expression. Obviously, *Tc-six3* is necessary for its activation and ectopic expression of *Tc-six3* might reveal whether it is sufficient, as well.

As already mentioned, a loss of labrum development is also found after *Tc-tup* RNAi. *Tc-tup* could act upstream of *Tc-cnc* by being necessary for the formation of the anterior fold which is in turn needed for proper patterning of the anterior head and expression of *Tc-cnc*. Also other genes of the ush-group might be necessary for *Tc-cnc* activation. It remains to be tested whether *Tc-cnc* expression is affected by loss of *Tc-tup* function.

Repression of *Tc-cnc* expression by Tc-Tll, as it is found in *Drosophila*, cannot be confirmed for *Tribolium*. While expression of *Tc-cnc* appears to expand slightly in later stages after *Tc-tll* RNAi, this is probably a secondary effect due to the altered head morphology. In contrast to that, *Tc-tll* expression expands towards the midline after *Tc-cnc* knockdown. *Tc-tll* is important for the establishment of the neurogenic non-segmental region of the head (see also Posnien et al., 2011b) and its median expression boundary is set up by *Tc-cnc* but apparently not vice versa.

In *Drosophila*, *gt* is required for is the retraction of the cap of *cnc* expression from the anterior tip of the embryo during cellularization (Mohler, 1993). This is unnecessary in *Tribolium*, where the anterior *Tc-cnc* domain is established long after cellularization in the later germ rudiment (Economou and Telford, 2009; this work). Likewise, *Of-cnc* is never expressed in a cap at the anterior egg pole in *Oncopeltus* (Birkan et al., 2011) arguing in favor of a derived patterning mode in *Drosophila*. Moreover, the expression pattern of *Tc-gt* in the whole anterior head (Bucher and Klingler, 2004) makes a repression of *Tc-cnc* rather unlikely, and the phenotype after knockdown of *Tc-gt* does not show any alteration of labrum tissue. Defects in the head are detected more posteriorly in the maxillary and labial segments, which are homeotically transformed to trunk identity (Bucher and Klingler, 2004). However, the homeotic function of *gt* has not been confirmed in other insects, where *gt* always acts as a gap gene (Petschek et al., 1987; Petschek and Mahowald, 1990; Eldon and Pirrotta, 1991; Brent et al.,

2007; Liu and Patel, 2010). Therefore also other functions of *gt* like a repression of *cnc* might have been lost during *Tribolium* evolution.

Also, repression of *Tc-cnc* expression in mesodermal cells by Tc-Sna or another mesodermal factor appears not necessary. As already mentioned, *Tc-cnc* is never expressed like *Drosophila cnc* in an anterior cap in the blastoderm which withdraws from the mesoderm primordium (Mohler et al., 1991; Mohler, 1993; Economou and Telford, 2009).

No data on the expression of *Tc-sal* in the *Tribolium* head is available. Hence, a conservation of the repressive effect on *Tc-cnc* cannot be excluded.

The later *Tc-cnc* expression aspects around the stomodaeum are dependent on *Tc-croc*, possibly via *Tc-scro* as a mediator. After RNAi for these genes, the *Tc-cnc* expression around the stomodaeum is lost. Vice versa, *Tc-cnc* is necessary for the establishment of the labral expression stripes of *Tc-croc*. It is, however, uncertain whether this apparently mutual activation is not only a secondary effect due to the loss of the labrum anlagen after *Tc-cnc* RNAi and, respectively, the stomodaeum identity after *Tc-croc* RNAi. An early mutual interaction was not detected.

The expansion of early *Tc-cnc* expression after *Tc-scro* RNAi indicates that the posterior boundary of *Tc-cnc* could be set up by *Tc-scro*. During early embryogenesis, *Tc-scro* expression is apparently posterior to *Tc-cnc*, suggesting a negative regulation. However, both genes are clearly co-expressed during later stages and apparently no repression occurs. This could indicate that a second factor cooperates with *Tc-scro* to suppress *Tc-cnc* expression during early stages. Probably, knockdown of *Tc-scro* was not complete, as indicated by the fact that weak *Tc-scro* expression could be detected in older RNAi germ bands.

Tc-croc expression is activated by unknown factors

Expression of *croc* in *Drosophila* starts as an anterior cap which is established by the interaction of Bcd, Dorsal (Dl) and the terminal system. Also the later withdrawal of the expression domain from the anterior pole is dependent on the terminal system. The target genes of the maternal systems turned out to have no effect on *croc* expression, placing *croc* at the same level as the zygotic gap genes (Häcker et al., 1992; Häcker, 1995; Häcker et al., 1995).

As already pointed out, *bcd* is not conserved among insects and the terminal system is not involved in *Tribolium* head patterning. Nevertheless, anterior factors (e.g. Tc-Otd-1 or Tc-Hbn) are most likely involved in the onset of *Tc-croc* expression. In contrast to *Tc-cnc*, only the anterior ectodermal aspects of *Tc-croc* expression are affected by a *Tc-six3* knockdown. The posterior expression is established normally without *Tc-six3* function. The *dorso-ventral* system is almost certainly involved in *Tc-croc* regulation, as a large portion of the *Tc-croc*

expression domain lies in anterior ventral tissue. The actual factors activating *Tc-croc* expression are yet to be identified.

The withdrawal of *Tc-croc* from the anterior pole of the germ band is probably not due to changes in expression regulation but to a morphogenetic movement of the tissue *Tc-croc* is expressed in.

Downstream targets of *Tc-croc* are expressed in the stomodaeal region

The posterior *Tc-cnc* expression stripes around the stomodaeum are lost after *Tc-croc* RNAi. However, this is likely to be a secondary effect (see above). Also, the reduction of *Tc-six3* expression in the stomodaeum after *Tc-croc* RNAi might be a secondary effect, as early *Tc-six3* expression was not found to be affected. In contrast to that, the loss of the expression domains of other genes in the stomodaeum region (e.g. *Tc-fkh*, *Tc-wg*) after *Tc-croc* RNAi is likely to result from direct interaction. This makes *Tc-croc* an important upstream factor for stomodaeum formation and gut development in *Tribolium*.

Tc-croc function is also necessary for proper establishment of the *Tc-scro* expression domain. However, *Tc-croc* does not account for the complete domain. *Tc-scro* expression in the labrum base appears to be narmal despite *Tc-croc* RNAi. This is also indicated by the fact that the fusion of the labrum anlagen still takes place after *Tc-croc* RNAi while it is abolished after *Tc-scro* RNAi. During later stages, *Tc-scro* even appears to be necessary for *Tc-croc* expression posterior of the stomodaeum but not in other areas. The *Tc-scro* RNAi phenotype includes a loss of the most posterior hook-like structure of the foregut. Instead, the foregut ends blind. I therefore suggest that *Tc-scro* is necessary for the establishment of the most posterior ectodermal expression aspect of *Tc-croc*, which in turn is necessary for the proper formation of the posterior end of the foregut.

***Tc-croc* patterns the stomodaeal region and might replace *Tc-hkb* in gut formation**

The developmental function of Croc in *Drosophila* is the formation of labral derivatives and the posterior pharynx wall (Häcker, 1995; Häcker et al., 1995) as well as the SNS (Schmidt-Ott et al., 1994; see above). In *Tribolium*, the labrum defects detected after *Tc-croc* knockdown are of secondary nature and result from the replacement of the mouth opening by ectopic tissue and the subsequent dorsal misplacement of the labrum. The complete loss of a stomodaeum invagination and the reprogramming of the tissue fate have not been described in other organisms than *Tribolium*. The defect found in *Oncopeltus* after *Of-croc* RNAi is a disruption of labrum patterning (Birkan et al., 2011). The nature of these defects is not further stated and might be secondary. No information is available about stomodaeum or foregut de-

velopment after *Of-croc* RNAi in *Oncopeltus*. It will be interesting to test whether *croc* has a function in foregut patterning in other arthropods, as well.

The molecular genetics of gut formation in arthropods have been studied only in *Drosophila* (see Murakami et al., 1999, for review). The insect gut consists of three major parts, anterior foregut, midgut and posterior hindgut. The anlagen of the gut develop at both embryonic poles and elongate during embryogenesis to eventually fuse. Foregut and hindgut develop from ectodermal invaginations, stomodaeum and proctodaeum, respectively. The midgut is of endodermal origin, the anlagen of which invaginate at the same time as the mesoderm (Reuter and Leptin, 1994; Campos-Ortega and Hartenstein, 1997).

In *Drosophila*, the anterior gut primordia are specified by the action of the anterior and terminal maternal systems. They activate expression of the terminal gap genes, *tll* and *hkb*. Hkb controls the formation of the anterior endoderm (i.e. anterior midgut) in concert with Twist (Twi) and Sna. While the gene products of *twi* and *sna* alone specify the mesodermal part of the ventral furrow, *hkb* is expressed at its anterior tip and is necessary for specification of the endoderm. One downstream target of *hkb* is *serpent* (*srp*). The loss of function of either *hkb* or *srp* leads to a homeotic transformation of endodermal to ecto- and mesodermal tissue (Brönner et al., 1994; Reuter, 1994b; Reuter and Leptin, 1994; Brönner and Jäckle, 1996).

hkb and *tll* also activate the expression of another *Drosophila* gut determinant, *fkh* (Weigel et al., 1990), which specifies the ectodermal foregut. Loss of its function leads to a homeotic transformation of the stomodaeal tissue into post-oral head structures (Jürgens and Weigel, 1988). Later studies showed that also midgut and hindgut are affected after loss of *fkh* function (Weigel et al., 1989a; Weigel et al., 1989b).

The *Drosophila* pattern of gut formation is apparently not conserved in *Tribolium*. *Tc-tll* RNAi does not interfere with *Tc-fkh* expression or gut formation (Posnien et al., 2011b; this work). The expression pattern of *Tc-hkb* makes a functional role in foregut development unlikely, but functional tests remain to be performed. In contrast, *Tc-croc* is necessary for proper expression of *Tc-fkh* and loss of its function leads to an abolishment of foregut formation. In the absence of *Tc-croc*, the stomodaeal tissue is replaced by ectodermal tissue originating from surrounding head regions. This phenotype is reminiscent of a homeotic transformation as reported for loss of function of *fkh* in *Drosophila*. In *Tribolium*, however, early expression of genes in the stomodaeal region appears not affected and only later, ectopic tissue is found. This tissue is then marked by the expression of genes expressed in surrounding head regions. It is, therefore, uncertain whether this is a true homeotic transformation.

Also, the expression pattern of *Tc-croc* in *Tribolium* is reminiscent of *hkb* expression in *Drosophila*. In *Tribolium*, *Tc-croc* instead of *Tc-hkb* is detected at the anterior tip of the invaginating mesoderm in germ rudiments (see Economou and Telford, 2009). Also during early elongating stages, the *Tc-croc* expression pattern has the same shape as mesenchymal cells in the anterior head (compare expression of *Tc-croc* in Fig. $9B_3$ to expression of *Tc-twi* in Fig. 4B and C in Handel et al., 2004) and might therefore specify the endodermal anlagen. It remains to be tested whether this is a derived state or whether more basal arthropods also use *croc* instead of *hkb* to specify the anterior gut. Interesting data comes from the basal dipteran *Clogmia albipunctata* (WILLISTON, 1893). In *Clogmia*, expression of *Calb-hkb* starts rather late in development and is like in *Tribolium* probably not involved in early gut specification (García-Solache et al., 2010). Hence, it is likely that in the lineage leading to *Drosophila, hkb* gained a function as an important anterior patterning factor and replaced *croc* in gut formation. *croc* only retained part of its function in gut development in *Drosophila*, as the posterior pharynx wall and the SNS are still dependent on *croc* expression (Häcker et al., 1995; Schmidt-Ott et al., 1994).

Further support for the theory that *Tc-croc* functionally replaces *Tc-hkb* comes from yet another similarity between *Tc-croc* in *Tribolium* and *hkb* in *Drosophila*: *Tc-croc* is not sufficient to activate *Tc-fkh* expression. In the *Tc-six3* loss of function background, *Tc-croc* is still expressed, but *Tc-fkh* expression fails to be established. Like in *Drosophila*, at least one more factor is therefore needed to activate *Tc-fkh*. *Tc-six3* could be this factor, which remains to be tested by staged embryonic RNAi or ectopic expression of *Tc-six3*.

To further investigate the role of *Tc-croc* in gut formation, the functions of its hypothesized downstream targets remain to be analyzed. It appears likely that the defects in gut development after *Tc-croc* knockdown are actually due to the loss of *Tc-fkh* expression. This remains to be tested by *Tc-fkh* RNAi. Also, no data is currently available on the expression, function or regulation of *Tc-srp*. If *Tc-croc* in *Tribolium* has, in fact, the role of *hkb* in *Drosophila*, it should not only specify ectodermal but also endodermal anlagen by activation of *Tc-srp*. Interestingly, *Tc-croc* loss of function leads to higher cell death in mesenchymal cells during later embryogenesis. Possibly, the respective cells are not properly specified as endoderm without *Tc-croc* function and die subsequently. It will be interesting to determine the role of *Tc-srp* in gut development and to elucidate whether its expression is activated by *Tc-croc*, as well. The regulation of the gut patterning factors as suggested here is depicted in Fig. 35.

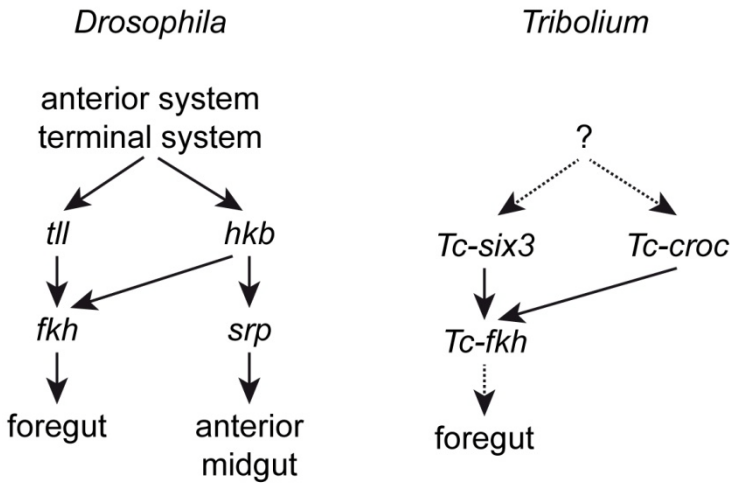


Fig. 35: Differences and similarities in gut patterning of *Drosophila* and *Tribolium*. In *Drosophila*, the anterior and terminal systems activate expression of the terminal gap genes, *tll* and *hkb*. *hkb* activates expression of *srp* which is needed for mesodermal anterior midgut formation. Together with *tll*, *hkb* also activates expression of *fkh* which in turn leads to stomodaeum and foregut development. In *Tribolium*, *Tc-tll* and *Tc-hkb* have probably no function in gut formation. Instead, both *Tc-six3* and *Tc-croc* are needed for the development of the stomodaeum and the expression of *Tc-fkh*. The role of *Tc-fkh* in foregut formation is likely to be conserved.

5.4 Ectopic expression of CLR patterning factors

For further analysis of the function of the CLR factors, I established *Tribolium* lines for their ectopic expression. Two lines were established for *Tc-croc* and *Tc-six3*, respectively. The φC31 system I used for site specific integration of the transgenic construct into the genome worked in principle, as several transgenic lines were generated. However, transformation efficiency was very low (ca. 0.2 %) and the eye markers of the *attP* construct and the insert were not linked. I assume that the injected *attP* line was contaminated with beetles of the *SB* strain and hence, that no *attP* site was present in the genome. Nevertheless, the construct integrated which might be due to putative pseudo *attP* sites in the *Tribolium* genome. It has been demonstrated in *Drosophila*, that recombination between the *attB* site and endogenous pseudo *attP* sites can occur (Groth et al., 2004). Inverse PCR of the transgenic *Tribolium* lines revealed that, in fact, integration occurred in a different region than the *attP* site (Ulrich, personal communication). Further analysis of this region might lead to the finding of pseudo *attP* sequences in *Tribolium*, as well. Moreover, the model organism *Tribolium* would benefit from another attempt to use of the φC31 system for site specific integration. This could be carried out in the context of creating a strain for ectopic expression of *Tc-cncB* with a newly established stock of the *attP* line.

Initial tests for ectopic *Tc-croc* expression showed ubiquitous expression throughout embryogenesis, but no developmental defects were observed. This could indicate that *Tc-croc* is merely a permissive factor. It might only function in the local environment of the CLR and co-factors might be necessary for its proper effect on downstream targets. On the other hand, it might be that *Tribolium* has a high regulatory potential rescuing early defects resulting from ectopic *Tc-croc* expression. It could also be that defects were not detectable using *Tc-wg* as a

marker. Due to its role in stomodaeum development, gut formation after ectopic *Tc-croc* expression should be analyzed. As described above, *Tc-croc* might act in the specification of endoderm instead of mesoderm. Hence, the complete mesodermal tissue might be transformed into endodermal tissue after ectopic *Tc-croc* expression. To test this, the muscle pattern of larvae developing from heat-shocked eggs or the expression of mesodermal markers in embryos should be analyzed.

The high numbers of hatched i.e. not affected larvae after the heat shock experiments are probably due to the fact that the strains are not homozygous. Thus, 25 % of laid eggs have a wild type-like genetic background and develop normally. There were also a relatively high number of empty eggs indicating severe developmental defects. Further staged heat shock experiments might give better results.

5.5 Implications for the arthropod head problem

The “endless dispute” about the arthropod head problem as formulated by Rempel (1975) has become less controversially disputed in recent years. It is now, for instance, widely accepted that there is indeed an intercalary segment in hexapods which corresponds to the second antennal segment in crustaceans and the pedipalp segment in the chelicerata (see e.g. Scholtz and Edgecombe, 2006). It remains unclear whether the ocular region is a true segment, while most authors refuse this idea and define the ocular region as part of the anterior non-segmental head (e.g. Budd, 2002; Angelini and Kaufman, 2005; Scholtz and Edgecombe, 2005; Scholtz and Edgecombe, 2006; Posnien et al., 2010; but see Rogers and Kaufman, 1997 for an opposing view). There is little doubt that the labrum evolved from fused appendages which are supposedly homologous to the onychophoran antennae (Eriksson et al., 2003; Kimm and Prpic, 2006; Posnien et al., 2009b; Eriksson et al., 2010; but see Bitsch and Bitsch, 2010 for an opposing view). The fact that development of the labrum functions independently from *hedgehog* signaling or canonical Wnt signaling and, hence, segmentation (Farzana and Brown, 2008; Bolognesi et al., 2009; Posnien et al., 2009b) further supports the hypothesis that these appendages belong to a non-segmental region.

Within this work, I provide evidence that the anterior non-segmental region can ontogenetically be further subdivided into an outer neurogenic region giving rise to the procephalon and an inner non-neurogenic region, the CLR, forming the clypeolabrum and anterior gut structures as well as probably the SNS. Two of the earliest markers for the CLR and the procephalic region are *Tc-six3* and *Tc-otd-1*, respectively. The expression patterns of these genes appear to be conserved in all bilaterians (Steinmetz et al., 2010; Posnien et al., 2010). The fact

that also other genes expressed in the anterior head show highly conserved expression patterns throughout arthropods or even bilaterians (Arendt et al., 2001; Hirth et al., 2003; Angelini and Kaufman, 2005; Reichert, 2005; Arendt et al., 2008; Eriksson et al., 2010; Birkan et al., 2011; Janssen et al., 2011; Posnien et al., 2011a) might indicate that also the CLR is an evolutionary conserved structure.

The relocation of the mouth opening from an anterior to a more posterior and ventral position is suggested to have taken place during arthropod evolution (see e.g. Budd, 2002; Kimm and Prpic, 2006). My suggested model for the morphogenesis of the anterior head provides evidence for the ontogenetic movement of anterior tissue to a posterior position. This tissue appears to comprise the stomodaeum anlagen. The model also offers an explanation for the problem that the position of the stomodaeum appears to vary within the arthropods (see e.g. Figure 2 in Budd, 2002 or Fig. 2 in Eriksson et al., 2010). The posterior boundary of the CLR is defined by the extent of the anterior fold which could vary in different species. Further investigation of early developmental processes and analysis of the conservation state of CLR marker expression in other arthropods will give insights into the evolution of the anterior head.

5.6 Concluding remarks

I showed that the CLR and the resulting split of the antennal segment and the ocular region develop primarily by morphogenetic movements and that proliferation apparently plays no major role. Instead, a newly identified structure formed by interaction of the germ band and the amnion, the anterior fold, is largely involved in anterior head formation. In order to further analyze the formation of the CLR and its derivatives, several experiments should be carried out. Investigation of the u-shaped group of genes in *Tribolium* is necessary to strengthen the hypothesis of an involvement of the amnion in early head formation. In addition, it will be helpful to analyze early head formation more detailed *in vivo*. The GFP expressing *Tribolium* line as well as other strains currently being established are valuable prerequisites.

Different genes are expressed during CLR development and their expression pattern changes with the morphogenetic movements. I provided further evidence that *Tc-six3* is important for the establishment of the complete ectodermal CLR. *Tc-cnc* and *Tc-croc* act downstream of *Tc-six3* and are necessary for the development of clypeolabrum and anterior gut structures, respectively. Additionally, *Tc-croc* appears to have taken over the function of *hkb* as gut-determining factor. In order to better understand the emergence of the defects after loss of function of the different genes, analysis of RNAi phenotypes *in vivo* will be helpful. Investi-

gation of FM® 1-43-stained embryos after *Tc-six3* and *Tc-cnc* knockdown might also provide interesting findings. Finally, the effect of loss of *Tc-croc* function on brain development needs to be analyzed.

Also, a better understanding of gut development in *Tribolium* is desirable. Currently, functional analysis of *Tc-fkh* and *Tc-hkb* is performed. Also other factors known to be involved in *Drosophila* gut patterning should be examined in *Tribolium*. The conservation state of expression and function of *Tc-srp* will give further insight into the evolution of endoderm formation. Moreover, the genes involved in *Tribolium* and *Drosophila* gut development should be investigated in other arthropods.

In an attempt to further elucidate the genetic interactions in the anterior head, I developed lines for ectopic expression of *Tc-croc* and *Tc-six3*. These lines will help to understand the regulatory interactions of these CLR patterning factors. A line for ectopic expression of *Tc-cncB* using the φC31 site specific integration system will give further insights into the gene regulatory network of the CLR and establish this system in *Tribolium*. A more exact characterization of the established lines is desirable. In the future, the effects of ectopic expression on downstream targets as well as on development can be analyzed.

Closer investigation of early morphogenetic processes, the formation of the *Tribolium* gut and the conservation of the CLR patterning network in other arthropods will teach us more about the nature and composition of the anterior head. The use of new techniques like *in vivo* imaging and site specific integration will further strengthen *Tribolium* as an important arthropod model organism.

6 Literature

Aguinaldo, A. M. A., Turbeville, J. M., Linford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A. and Lake, J. A. (1997). Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* **387**, 489–493.

Akam, M. (1987). The Molecular Basis for Metameric Pattern in the Drosophila Embryo. *Development* **101**, 1–22.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Angelini, D. R. and Kaufman, T. C. (2005). Comparative developmental genetics and the evolution of arthropod body plans. *Annu Rev Genet* **39**, 95–119.

Arendt, D., Technau, U. and Wittbrodt, J. (2001). Evolution of the bilaterian larval foregut. *Nature* **409**, 81–85.

Arendt, D., Denes, A. S., Jékely, G. and Tessmar-Raible, K. (2008). The Evolution of Nervous System Centralization. *Philos Trans R Soc Lond B Biol Sci* **363**, 1523–1528.

Berghammer, A. J., Weber, M., Trauner, J. and Klingler, M. (2009). Red Flour Beetle (Tribolium) Germline Transformation and Insertional Mutagenesis. *Cold Spring Harb Protoc* **2009**, pdb.prot5259.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nüsslein-Volhard, C. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo. *EMBO J* **7**, 1749–1756.

Birkan, M., Schaeper, N. D. and Chipman, A. D. (2011). Early patterning and blastodermal fate map of the head in the milkweed bug Oncopeltus fasciatus. *Evolution & Development* **13**, 436–447.

Bitsch, J. and Bitsch, C. (2010). The tritocerebrum and the clypeolabrum in mandibulate arthropods: segmental interpretations. *Acta Zoologica* **91**, 249–266.

Boccard, F., Smokvina, T., Pernodet, J.-L., Friedmann, A. and Guérineau, M. (1989). Structural analysis of loci involved in pSAM2 site-specific integration in Streptomyces. *Plasmid* **21**, 59–70.

Bolognesi, R., Fischer, T. and Brown, S. (2009). Loss of Tc-arrow and canonical Wnt signaling alters posterior morphology and pair-rule gene expression in the short-germ insect, Tribolium castaneum. *Dev Genes Evol* **219**, 369–375.

Brand, A. H. and Perrimon, N. (1993). Targeted Gene Expression as a Means of Altering Cell Fates and Generating Dominant Phenotypes. *Development* **118**, 401–415.

Brent, A. E., Yucel, G., Small, S. and Desplan, C. (2007). Permissive and Instructive Anterior Patterning Rely on mRNA Localization in the Wasp Embryo. *Science* **315**, 1841–1843.

Brönner, G. and Jäckle, H. (1991). Control and function of terminal gap gene activity in the posterior pole region of the Drosophila embryo. *Mech Dev* **35**, 205–211.

Brönner, G. and Jäckle, H. (1996). Regulation and function of the terminal gap gene huckebein in the Drosophila blastoderm. *Int J Dev Biol* **40**, 157–165.

Brönner, G., Chu-LaGraff, Q., Doe, C. Q., Cohen, B., Weigel, D., Taubert, H. and Jäckle, H. (1994). Sp1/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in Drosophila. *Nature* **369**, 664–668.

Brown, S. J., Patel, N. H. and Denell, R. E. (1994). Embryonic expression of the single Tribolium engrailed homolog. *Dev Genet* **15**, 7–18.

Brown, S. J., Mahaffey, J. P., Lorenzen, M. D., Denell, R. E. and Mahaffey, J. W. (1999). Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. *Evolution & Development* **1**, 11–15.

Brown, S., Fellers, J., Shippy, T., Denell, R., Stauber, M. and Schmidt-Ott, U. (2001). A strategy for mapping bicoid on the phylogenetic tree. *Curr Biol* **11**, R43–R44.

Brown, S. J., Shippy, T. D., Miller, S., Bolognesi, R., Beeman, R. W., Lorenzen, M. D., Bucher, G., Wimmer, E. A. and Klingler, M. (2009). The red flour beetle, Tribolium castaneum (Coleoptera): a model for studies of development and pest biology. *Cold Spring Harb Protoc* **2009**, pdb.emo126.

Bucher, G. and Klingler, M. (2004). Divergent segmentation mechanism in the short germ insect Tribolium revealed by giant expression and function. *Development* **131**, 1729–1740.

Bucher, G. and Klingler, M. (2005). Tribolium mae expression suggests roles in terminal and midline patterning and in the specification of mesoderm. *Dev Genes Evol* **215**, 478–481.

Bucher, G. and Wimmer, E. A. (2005). Beetle a-head. *BIF Futura* **20**, 164–169.
Bucher, G., Scholten, J. and Klingler, M. (2002). Parental RNAi in Tribolium (Coleoptera). *Curr Biol* **12**, R85–R86.
Budd, G. E. (2002). A palaeontological solution to the arthropod head problem. *Nature* **417**, 271–275.
Campos-Ortega, J. A. and Hartenstein, V. (1997). *The embryonic development of Drosophila melanogaster*. Berlin; New York: Springer.
Carl, M., Loosli, F. and Wittbrodt, J. (2002). Six3 Inactivation Reveals Its Essential Role for the Formation and Patterning of the Vertebrate Eye. *Development* **129**, 4057–4063.
Cerny, A. C., Grossmann, D., Bucher, G. and Klingler, M. (2008). The Tribolium ortholog of knirps and knirps-related is crucial for head segmentation but plays a minor role during abdominal patterning. *Dev Biol* **321**, 284–294.
Chipman, A. D. (2010). Parallel evolution of segmentation by co-option of ancestral gene regulatory networks. *Bioessays* **32**, 60–70.
Choe, C. P. and Brown, S. J. (2007). Evolutionary flexibility of pair-rule patterning revealed by functional analysis of secondary pair-rule genes, paired and sloppy-paired in the short-germ insect, Tribolium castaneum. *Dev Biol* **302**, 281–294.
Choe, C. P., Miller, S. C. and Brown, S. J. (2006). A Pair-Rule Gene Circuit Defines Segments Sequentially in the Short-Germ Insect Tribolium Castaneum. *Proc Natl Acad Sci U S A* **103**, 6560–6564.
Cohen, S. and Jürgens, G. (1991). Drosophila headlines. *Trends Genet* **7**, 267–272.
Coiffier, D., Charroux, B. and Kerridge, S. (2008). Common Functions of Central and Posterior Hox Genes for the Repression of Head in the Trunk of Drosophila. *Development* **135**, 291–300.
Crozatier, M., Valle, D., Dubois, L., Ibnsouda, S. and Vincent, A. (1999). Head versus trunk patterning in the Drosophila embryo; collier requirement for formation of the intercalary segment. *Development* **126**, 4385–4394.
Damen, W. G. M. (2007). Evolutionary conservation and divergence of the segmentation process in arthropods. *Dev Dyn* **236**, 1379–1391.
Davidson, E. H. (1991). Spatial Mechanisms of Gene Regulation in Metazoan Embryos. *Development* **113**, 1–26.
Davis, G. K. and Patel, N. H. (2002). Short, Long, and Beyond: Molecular and Embryological Approaches to Insect Segmentation. *Annu Rev Entomol* **47**, 669–699.
Davis, G. K. and Patel, N. H. (2003). Playing by pair-rules? *Bioessays* **25**, 425–429.
de Velasco, B., Erclik, T., Shy, D., Sclafani, J., Lipshitz, H., McInnes, R. and Hartenstein, V. (2007). Specification and development of the pars intercerebralis and pars lateralis, neuroendocrine command centers in the Drosophila brain. *Dev Biol* **302**, 309–323.
Del Bene, F., Tessmar-Raible, K. and Wittbrodt, J. (2004). Direct interaction of geminin and Six3 in eye development. *Nature* **427**, 745–749.
Diederich, R. J., Pattatucci, A. M. and Kaufman, T. C. (1991). Developmental and evolutionary implications of labial, Deformed and engrailed expression in the Drosophila head. *Development* **113**, 273–281.
Dubnau, J. and Struhl, G. (1996). RNA recognition and translational regulation by a homeodomain protein. *Nature* **379**, 694–699.
Economou, A. D. and Telford, M. J. (2009). Comparative gene expression in the heads of Drosophila melanogaster and Tribolium castaneum and the segmental affinity of the Drosophila hypopharyngeal lobes. *Evolution & Development* **11**, 88–96.
Eddleman, H. L. and Bell, A. E. (1963). Four new eye-color mutants in Tribolium castaneum. *Genetics* **48**, 888.
Eernisse, D. J., Albert, J. S. and Anderson, F. E. (1992). Annelida and Arthropoda Are Not Sister Taxa: A Phylogenetic Analysis of Spiralian Metazoan Morphology. *Syst Biol* **41**, 305–330.
Eldon, E. D. and Pirrotta, V. (1991). Interactions of the Drosophila Gap Gene Giant with Maternal and Zygotic Pattern-Forming Genes. *Development* **111**, 367–378.
Erclik, T., Hartenstein, V., Lipshitz, H. D. and McInnes, R. R. (2008). Conserved Role of the Vsx Genes Supports a Monophyletic Origin for Bilaterian Visual Systems. *Curr Biol* **18**, 1278–1287.
Eriksson, B. J., Tait, N. N. and Budd, G. E. (2003). Head development in the onychophoran Euperipatoides kanangrensis with particular reference to the central nervous system. *J Morphol* **255**,

1–23.

Eriksson, B., Tait, N., Budd, G., Janssen, R. and Akam, M. (2010). Head patterning and Hox gene expression in an onychophoran and its implications for the arthropod head problem. *Dev Genes Evol* **220**, 117–122.

Farzana, L. and Brown, S. J. (2008). Hedgehog signaling pathway function conserved in Tribolium segmentation. *Dev Genes Evol* **218**, 181–192.

Finkelstein, R. and Perrimon, N. (1991). The Molecular Genetics of Head Development in Drosophila Melanogaster. *Development* **112**, 899–912.

Fischer, J. A., Giniger, E., Maniatis, T. and Ptashne, M. (1988). GAL4 activates transcription in Drosophila. *Nature* **332**, 853–856.

Frank, L. H. and Rushlow, C. (1996). A Group of Genes Required for Maintenance of the Amnioserosa Tissue in Drosophila. *Development* **122**, 1343–1352.

Frohman, M. A., Dush, M. K. and Martin, G. R. (1988). Rapid Production of Full-Length cDNAs from Rare Transcripts: Amplification Using a Single Gene-Specific Oligonucleotide Primer. *Proc Natl Acad Sci U S A* **85**, 8998–9002.

Frohnhöfer, H. G. and Nüsslein-Volhard, C. (1986). Organization of anterior pattern in the Drosophila embryo by the maternal gene bicoid. *Nature* **324**, 120–125.

Fu, J., Posnien, N., Bolognesi, R., Fischer, T. D., Rayl, P., Oberhofer, G., Kitzmann, P., Brown, S. J. and Bucher, G. (2012) Asymmetrically Expressed axin Required for Anterior Development in Tribolium. *Proc Natl Acad Sci U S A*. (accepted)

Gallitano-Mendel, A. and Finkelstein, R. (1997). Novel Segment Polarity Gene Interactions during Embryonic Head Development in Drosophila. *Dev Biol* **192**, 599–613.

García-Solache, M., Jaeger, J. and Akam, M. (2010). A systematic analysis of the gap gene system in the moth midge Clogmia albipunctata. *Dev Biol* **344**, 306–318.

Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). Identification of Programmed Cell Death in Situ Via Specific Labeling of Nuclear DNA Fragmentation. *J Cell Biol* **119**, 493–501.

Gestri, G., Carl, M., Appolloni, I., Wilson, S. W., Barsacchi, G. and Andreazzoli, M. (2005). Six3 functions in anterior neural plate specification by promoting cell proliferation and inhibiting Bmp4 expression. *Development* **132**, 2401–2413.

Goodrich, E. S. (1897). On the Relation of the Arthropod Head to the Annelid Prostomium. *Quart J Micr Sci* 247–268.

Goujon, M., McWilliam, H., Li, W., Valentin, F., Squizzato, S., Paern, J. and Lopez, R. (2010). A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res* **38**, W695–W699.

Grimaldi, D. A. and Engel, M. S. (2005). *Evolution of the Insects*. Cambridge University Press.

Grossmann, D. (2011). Comparative studies on the role of Egfr, Wingless and Decapentaplegic signalling in leg development in the red flour beetle Tribolium castaneum. *PhD Thesis; Dpt. for Developmental Biology* **Georg-August-University Goettingen**.

Grossmann, D., Scholten, J. and Prpic, N.-M. (2009). Separable functions of wingless in distal and ventral patterning of the Tribolium leg. *Dev Genes Evol* **219**, 469–479.

Groth, A. C. and Calos, M. P. (2004). Phage Integrases: Biology and Applications. *J Mol Biol* **335**, 667–678.

Groth, A. C., Fish, M., Nusse, R. and Calos, M. P. (2004). Construction of Transgenic Drosophila by Using the Site-Specific Integrase From Phage φC31. *Genetics* **166**, 1775–1782.

Haas, M. S., Brown, S. J. and Beeman, R. W. (2001). Pondering the procephalon: the segmental origin of the labrum. *Dev Genes Evol* **211**, 89–95.

Häcker, U. W. (1995). *Isolierung und Charakterisierung einer Familie fork-head-verwandter Gene in Drosophila melanogaster und Studien zur Struktur und Funktion des Lätzchen-Gens, eines Vertreters dieser Genfamilie*. Göttingen: Cuvillier.

Häcker, U., Grossniklaus, U., Gehring, W. J. and Jäckle, H. (1992). Developmentally regulated Drosophila gene family encoding the fork head domain. *Proc Natl Acad Sci U S A* **89**, 8754.

Häcker, U., Kaufmann, E., Hartmann, C., Jürgens, G., Knöchel, W. and Jäckle, H. (1995). The Drosophila fork head domain protein crocodile is required for the establishment of head structures. *EMBO J* **14**, 5306.

Hammond, S. M. (2005). Dicing and slicing: The core machinery of the RNA interference pathway. *FEBS Lett* **579**, 5822–5829.

Handel, K., Grünfelder, C. G., Roth, S. and Sander, K. (2000). Tribolium embryogenesis: a SEM study of cell shapes and movements from blastoderm to serosal closure. *Dev Genes Evol* **210**, 167–179.

Handel, K., Basal, A., Fan, X. and Roth, S. (2004). Tribolium castaneum twist: gastrulation and mesoderm formation in a short-germ beetle. *Dev Genes Evol* **215**, 13–31.

Handler, A. M. (2001). A current perspective on insect gene transformation. *Insect Biochem Mol Biol* **31**, 111–128.

Harding, K. W., Gellon, G., McGinnis, N. and McGinnis, W. (1995). A screen for modifiers of Deformed function in Drosophila. *Genetics* **140**, 1339.

Hartenstein, V., Tepass, U. and Gruszynski-Defeo, E. (1994). Embryonic development of the stomatogastric nervous system in Drosophila. *J Comp Neurol* **350**, 367–381.

Hartenstein, V., Tepass, U. and Gruszynski-deFeo, E. (1996). Proneural and Neurogenic Genes Control Specification and Morphogenesis of Stomatogastric Nerve Cell Precursors in Drosophila. *Dev Biol* **173**, 213–227.

Hasuwa, H., Kaseda, K., Einarsdottir, T. and Okabe, M. (2002). Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Lett* **532**, 227–230.

Hein, H. (2007). Identifizierung von Neuroblasten als Modell für die Regulierung ihrer Spezifizierung in Tribolium castaneum. *Diploma Thesis; Dpt. for Developmental Biology* **Georg-August-University Goettingen**.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348–360.

Hirth, F., Kammermeier, L., Frei, E., Walldorf, U., Noll, M. and Reichert, H. (2003). An Urbilaterian Origin of the Tripartite Brain: Developmental Genetic Insights from Drosophila. *Development* **130**, 2365–2373.

Horn, C. and Wimmer, E. A. (2000). A versatile vector set for animal transgenesis. *Dev Genes Evol* **210**, 630–637.

Ingham, P. W. (1988). The molecular genetics of embryonic pattern formation in Drosophila. *Nature* **335**, 25–34.

Irish, V. F., Martinez-Arias, A. and Akam, M. (1989). Spatial regulation of the Antennapedia and Ultrabithorax homeotic genes during Drosophila early development. *EMBO J* **8**, 1527–1537.

Janssen, R., Budd, G. E. and Damen, W. G. M. (2011). Gene expression suggests conserved mechanisms patterning the heads of insects and myriapods. *Dev Biol* **357**, 64–72.

Jürgens, G. and Weigel, D. (1988). Terminal versus segmental development in the Drosophila embryo: the role of the homeotic gene fork head. *Wilhelm Roux's Arch Dev Biol* **197**, 345–354.

Jürgens, G., Lehmann, R., Schardin, M. and Nüsslein-Volhard, C. (1986). Segmental organisation of the head in the embryo of Drosophila melanogaster. *Dev Genes Evol* **195**, 359–377.

Kanada, T., Sullivan, K. F. and Wahl, G. M. (1998). Histone–GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr Biol* **8**, 377–385.

Kaspar, P. (2008). Die Bedeutung des Homeoboxgens homeobrain für die embryonale Gehirnentwicklung von Drosophila melanogaster. *PhD Thesis; Entwicklungsbiologie, Medizinische Fakultät* **Universität des Saarlandes, Homburg/Saar**.

Keller, P. J., Schmidt, A. D., Wittbrodt, J. and Stelzer, E. H. K. (2008). Reconstruction of Zebrafish Early Embryonic Development by Scanned Light Sheet Microscopy. *Science* **322**, 1065–1069.

Keller, P. J., Schmidt, A. D., Santella, A., Khairy, K., Bao, Z., Wittbrodt, J. and Stelzer, E. H. K. (2010). Fast, high-contrast imaging of animal development with scanned light sheet-based structured-illumination microscopy. *Nat Methods* **7**, 637–642.

Ketting, R. F. (2011). The Many Faces of RNAi. *Dev Cell* **20**, 148–161.

Kim, H. S., Murphy, T., Xia, J., Caragea, D., Park, Y., Beeman, R. W., Lorenzen, M. D., Butcher, S., Manak, J. R. and Brown, S. J. (2009). BeetleBase in 2010: revisions to provide comprehensive genomic information for Tribolium castaneum. *Nucleic Acids Res* **38**, D437–D442.

Kimm, M. and Prpic, N.-M. (2006). Formation of the arthropod labrum by fusion of paired and rotated limb-bud-like primordia. *Zoomorphology* **125**, 147–155.

Kittelmann, S. (2008). Das anterior-mediane Gewebe des Insektenkopfes. *Diploma Thesis; Dpt. for*

Developmental Biology **Georg-August-University Goettingen**.

Klingler, M. (2004). Tribolium. *Curr Biol* **14**, R639–640.

Konikoff, C. E., Karr, T. L., McCutchan, M., Newfeld, S. J. and Kumar, S. (2012). Comparison of embryonic expression within multigene families using the flyexpress discovery platform reveals more spatial than temporal divergence. *Dev Dyn* **241**, 150–160.

Koniszewski, N. D. B. (2011). Functional analysis of embryonic brain development in Tribolium castaneum. *PhD Thesis; Dpt. for Developmental Biology* **Georg-August-University Goettingen**.

Kotkamp, K., Klingler, M. and Schoppmeier, M. (2010). Apparent role of Tribolium orthodenticle in anteroposterior blastoderm patterning largely reflects novel functions in dorsoventral axis formation and cell survival. *Development* **137**, 1853–1862.

Kuhstoss, S. and Rao, R. N. (1991). Analysis of the integration function of the streptomycete bacteriophage φC31. *J Mol Biol* **222**, 897–908.

Kumar, S., Konikoff, C., Van Emden, B., Busick, C., Davis, K. T., Ji, S., Wu, L.-W., Ramos, H., Brody, T., Panchanathan, S., et al. (2011). FlyExpress: visual mining of spatiotemporal patterns for genes and publications in Drosophila embryogenesis. *Bioinformatics* **27**, 3319–3320.

Labbé, G. M. C., Nimmo, D. D. and Alphey, L. (2010). piggybac- and PhiC31-Mediated Genetic Transformation of the Asian Tiger Mosquito, Aedes albopictus (Skuse). *PLoS Negl Trop Dis* **4**, e788.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947–2948.

Levine, E. M., Hitchcock, P. F., Glasgow, E. and Schechter, N. (1994). Restricted expression of a new paired-class homeobox gene in normal and regenerating adult goldfish retina. *J Comp Neurol* **348**, 596–606.

Li, Y., Brown, S. J., Hausdorf, B., Tautz, D., Denell, R. E. and Finkelstein, R. (1996). Two orthodenticle -related genes in the short-germ beetle Tribolium castaneum. *Dev Genes Evol* **206**, 35–45.

Liu, P. Z. and Patel, N. H. (2010). Giant Is a Bona Fide Gap Gene in the Intermediate Germband Insect, Oncopeltus Fasciatus. *Development* **137**, 835–844.

Liu, I. S. C., Chen, J., Ploder, L., Vidgen, D., van der Kooy, D., Kalnins, V. I. and Mclnnes, R. R. (1994). Developmental expression of a novel murine homeobox gene (Chx10): Evidence for roles in determination of the neuroretina and inner nuclear layer. *Neuron* **13**, 377–393.

Lorenzen, M. D., Brown, S. J., Denell, R. E. and Beeman, R. W. (2002). Cloning and Characterization of the Tribolium castaneum Eye-Color Genes Encoding Tryptophan Oxygenase and Kynurenine 3-Monooxygenase. *Genetics* **160**, 225–234.

Lorenzen, M. D., Berghammer, A. J., Brown, S. J., Denell, R. E., Klingler, M. and Beeman, R. W. (2003). piggyBac-mediated germline transformation in the beetle Tribolium castaneum. *Insect Mol Biol* **12**, 433–440.

Lukyanov, K. A., Chudakov, D. M., Lukyanov, S. and Verkhusha, V. V. (2005). Innovation: Photoactivatable fluorescent proteins. *Nat Rev Mol Cell Biol* **6**, 885–890.

Lynch, J. A., Brent, A. E., Leaf, D. S., Pultz, M. A. and Desplan, C. (2006). Localized maternal orthodenticle patterns anterior and posterior in the long germ wasp Nasonia. *Nature* **439**, 728–732.

Marques-Souza, H., Aranda, M. and Tautz, D. (2008). Delimiting the Conserved Features of Hunchback Function for the Trunk Organization of Insects. *Development* **135**, 881–888.

McGinnis, N., Ragnhildstveit, E., Veraksa, A. and McGinnis, W. (1998). A cap “n” collar protein isoform contains a selective Hox repressor function. *Development* **125**, 4553 –4564.

McKinney, S. A., Murphy, C. S., Hazelwood, K. L., Davidson, M. W. and Looger, L. L. (2009). A bright and photostable photoconvertible fluorescent protein. *Nat Methods* **6**, 131–133.

Meister, G. and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343–349.

Mello, C. C. and Conte, D. (2004). Revealing the world of RNA interference. *Nature* **431**, 338–342.

Mohler, J. (1993). Genetic regulation of CNC expression in the pharnygeal primordia of Drosophila blastoderm embryos. *Dev Genes Evol* **202**, 214–223.

Mohler, J., Vani, K., Leung, S. and Epstein, A. (1991). Segmentally restricted, cephalic expression of a leucine zipper gene during Drosophila embryogenesis. *Mech Dev* **34**, 3–9.

Mohler, J., Mahaffey, J. W., Deutsch, E. and Vani, K. (1995). Control of Drosophila head segment

identity by the bZIP homeotic gene cnc. *Development* **121**, 237–247.
Müller-Taubenberger, A. and Anderson, K. (2007). Recent advances using green and red fluorescent protein variants. *Appl Microbiol Biotechnol* **77**, 1–12.
Murakami, R., Takashima, S. and Hamaguchi, T. (1999). Developmental genetics of the Drosophila gut: specification of primordia, subdivision and overt-differentiation. *Cell Mol Biol (Noisy-le-grand)* **45**, 661–676.
Nagy, L. M. and Carroll, S. (1994). Conservation of wingless patterning functions in the short-germ embryos of Tribolium castaneum. *Nature* **367**, 460–463.
Nie, W., Stronach, B., Panganiban, G., Shippy, T., Brown, S. and Denell, R. (2001). Molecular characterization of Tclabial and the 3' end of the Tribolium homeotic complex. *Dev Genes Evol* **211**, 244–251.
Nimmo, D. D., Alphey, L., Meredith, J. M. and Eggleston, P. (2006). High efficiency site-specific genetic engineering of the mosquito genome. *Insect Mol Biol* **15**, 129–136.
Nüsslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature* **287**, 795–801.
Nüsslein-Volhard, C., Wieschaus, E. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster I. *Wilhelm Roux's Arch Dev Biol* **193**, 267–282.
Omer, C. A. and Cohen, S. N. (1986). Structural analysis of plasmid and chromosomal loci involved in site-specific excision and integration of the SLP1 element of Streptomyces coelicolor. *J Bacteriol* **166**, 999–1006.
Omer, C. A., Stein, D. and Cohen, S. N. (1988). Site-specific insertion of biologically functional adventitious genes into the Streptomyces lividans chromosome. *J Bacteriol* **170**, 2174–2184.
Ornitz, D. M., Moreadith, R. W. and Leder, P. (1991). Binary System for Regulating Transgene Expression in Mice: Targeting Int-2 Gene Expression with Yeast GAL4/UAS Control Elements. *Proc Natl Acad Sci U S A* **88**, 698–702.
Panfilio, K. A., Liu, P. Z., Akam, M. and Kaufman, T. C. (2006). Oncopeltus fasciatus zen is essential for serosal tissue function in katatrepsis. *Dev Biol* **292**, 226–243.
Pankratz, M. J. and Jäckle, H. (1990). Making stripes in the Drosophila embryo. *Trends Genet* **6**, 287–292.
Pavlopoulos, A., Berghammer, A. J., Averof, M. and Klingler, M. (2004). Efficient Transformation of the Beetle Tribolium Castaneum Using the Minos Transposable Element Quantitative and Qualitative Analysis of Genomic Integration Events. *Genetics* **167**, 737–746.
Peel, A. D. (2008). The Evolution of Developmental Gene Networks: Lessons from Comparative Studies on Holometabolous Insects. *Philos Trans R Soc Lond B Biol Sci* **363**, 1539–1547.
Peel, A. D., Chipman, A. D. and Akam, M. (2005). Arthropod Segmentation: beyond the Drosophila paradigm. *Nat Rev Genet* **6**, 905–916.
Petschek, J. P. and Mahowald, A. P. (1990). Different requirements for l(1)giant in two embryonic domains of Drosophila melanogaster. *Dev Genet* **11**, 88–96.
Petschek, J. P., Perrimon, N. and Mahowald, A. P. (1987). Region-specific defects in l(1)giant embryos of Drosophila melanogaster. *Dev Biol* **119**, 175–189.
Posnien, N. (2009). Function and evolution of highly conserved head genes in the red flour beetle Tribolium castaneum. *PhD Thesis; Dpt. for Developmental Biology* **Georg-August-University Goettingen**.
Posnien, N. and Bucher, G. (2010). Formation of the insect head involves lateral contribution of the intercalary segment, which depends on Tc-labial function. *Dev Biol* **338**, 107–116.
Posnien, N., Schinko, J., Grossmann, D., Shippy, T. D., Konopova, B. and Bucher, G. (2009a). RNAi in the red flour beetle (Tribolium). *Cold Spring Harb Protoc* **2009**, pdb.prot5256.
Posnien, N., Bashasab, F. and Bucher, G. (2009b). The insect upper lip (labrum) is a nonsegmental appendage-like structure. *Evolution & Development* **11**, 480–488.
Posnien, N., Schinko, J. B., Kittelmann, S. and Bucher, G. (2010). Genetics, development and composition of the insect head--a beetle's view. *Arthropod Struct Dev* **39**, 399–410.
Posnien, N., Koniszewski, N. and Bucher, G. (2011a). Insect Tc-six4 marks a unit with similarity to vertebrate placodes. *Dev Biol* **350**, 208–216.
Posnien, N., Koniszewski, N. D. B., Hein, H. J. and Bucher, G. (2011b). Candidate Gene Screen in the Red Flour Beetle Tribolium Reveals Six3 as Ancient Regulator of Anterior Median Head and

Central Complex Development. *PLoS Genet* **7**, e1002416.

Prpic, N.-M. and Damen, W. G. M. (2005). Cell death during germ band inversion, dorsal closure, and nervous system development in the spider Cupiennius salei. *Dev Dyn* **234**, 222–228.

Pultz, M. A., Diederich, R. J., Cribbs, D. L. and Kaufman, T. C. (1988). The Proboscipedia Locus of the Antennapedia Complex: A Molecular and Genetic Analysis. *Genes Dev* **2**, 901–920.

Rafiqi, A. M., Lemke, S., Ferguson, S., Stauber, M. and Schmidt-Ott, U. (2008). Evolutionary Origin of the Amnioserosa in Cyclorrhaphan Flies Correlates with Spatial and Temporal Expression Changes of Zen. *Proc Natl Acad Sci U S A* **105**, 234–239.

Rafiqi, A. M., Lemke, S. and Schmidt-Ott, U. (2010). Postgastrular zen expression is required to develop distinct amniotic and serosal epithelia in the scuttle fly Megaselia. *Dev Biol* **341**, 282–290.

Reichert, H. (2005). A tripartite organization of the urbilaterian brain: Developmental genetic evidence from Drosophila. *Brain Res Bull* **66**, 491–494.

Rempel, J. G. (1975). The evolution of the insect head: the endless dispute. *Quaest Entomol* **11**, 7–25.

Reuter, R. (1994a). The gene serpent has homeotic properties and specifies endoderm versus ectoderm within the Drosophila gut. *Development* **120**, 1123–1135.

Reuter, R. (1994b). The Gene Serpent Has Homeotic Properties and Specifies Endoderm Versus Ectoderm Within the Drosophila Gut. *Development* **120**, 1123–1135.

Reuter, R. and Leptin, M. (1994). Interacting functions of snail, twist and huckebein during the early development of germ layers in Drosophila. *Development* **120**, 1137–1150.

Richards, S., Gibbs, R. A., Weinstock, G. M., Brown, S. J., Denell, R., Beeman, R. W., Gibbs, R., Beeman, R. W., Brown, S. J., Bucher, G., et al. (2008). The genome of the model beetle and pest Tribolium castaneum. *Nature* **452**, 949–955.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J. and Jacklë, H. (1996). RNA binding and translational suppression by bicoid. *Nature* **379**, 746–749.

Rogers, B. T. and Kaufman, T. C. (1996). Structure of the Insect Head as Revealed by the EN Protein Pattern in Developing Embryos. *Development* **122**, 3419–3432.

Rogers, B. T. and Kaufman, T. C. (1997). Structure of the Insect Head in Ontogeny and Phylogeny: A View from Drosophila. *Int Rev Cytol* **174**, 1–84.

Rogers, B. T., Peterson, M. D. and Kaufman, T. C. (2002). The development and evolution of insect mouthparts as revealed by the expression patterns of gnathocephalic genes. *Evolution & Development* **4**, 96–110.

Rosenberg, M. I., Lynch, J. A. and Desplan, C. (2009). Heads and tails: Evolution of antero-posterior patterning in insects. *Biochim Biophys Acta* **1789**, 333–342.

Rushlow, C., Doyle, H., Hoey, T. and Levine, M. (1987a). Molecular Characterization of the Zerknüllt Region of the Antennapedia Gene Complex in Drosophila. *Genes Dev* **1**, 1268–1279.

Rushlow, C., Frasch, M., Doyle, H. and Levine, M. (1987b). Maternal regulation of zerknüllt: a homoeobox gene controlling differentiation of dorsal tissues in Drosophila. *Nature* **330**, 583–586.

Sarrazin, A. F., Peel, A. D. and Averof, M. (2012). A Segmentation Clock with Two-Segment Periodicity in Insects. *Science* **336**, 338–341.

Schaeper, N. D., Pechmann, M., Damen, W. G. M., Prpic, N.-M. and Wimmer, E. A. (2010). Evolutionary plasticity of collier function in head development of diverse arthropods. *Dev Biol* **344**, 363–376.

Schetelig, M. F., Scolari, F., Handler, A. M., Kittelmann, S., Gasperi, G. and Wimmer, E. A. (2009). Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly Ceratitis capitata. *Proc Natl Acad Sci U S A* **106**, 18171–18176.

Schinko, J. B., Kreuzer, N., Offen, N., Posnien, N., Wimmer, E. A. and Bucher, G. (2008). Divergent functions of orthodenticle, empty spiracles and buttonhead in early head patterning of the beetle Tribolium castaneum (Coleoptera). *Dev Biol* **317**, 600–613.

Schinko, J. B., Posnien, N., Kittelmann, S., Koniszewski, N. and Bucher, G. (2009). Single and double whole-mount in situ hybridization in red flour beetle (Tribolium) embryos. *Cold Spring Harb Protoc* **2009**, pdb.prot5258.

Schinko, J. B., Weber, M., Viktorinova, I., Kiupakis, A., Averof, M., Klingler, M., Wimmer, E. A. and Bucher, G. (2010). Functionality of the GAL4/UAS system in Tribolium requires the use of endogenous core promoters. *BMC Dev Biol* **10**, 53.

Schmid, B. G. M. (2011). Molecular Studies on Head Development of the Amphipod Crustacean

Parhyale hawaiensis. *PhD Thesis; Dpt. for Developmental Biology* **Georg-August-University Goettingen**.

Schmidt-Ott, U. and Technau, G. M. (1992). Expression of en and wg in the embryonic head and brain of Drosophila indicates a refolded band of seven segment remnants. *Development* **116**, 111–125.

Schmidt-Ott, U., González-Gaitán, M., Jäckle, H. and Technau, G. M. (1994). Number, identity, and sequence of the Drosophila head segments as revealed by neural elements and their deletion patterns in mutants. *Proc Natl Acad Sci U S A* **91**, 8363–8367.

Scholtz, G. and Edgecombe, G. (2005). Heads, Hox and the phylogenetic position of trilobites. In *Crustacea and Arthropod Relationships* (ed. Koenemann, S. and Jenner, R.), pp. 139–165. CRC Press.

Scholtz, G. and Edgecombe, G. D. (2006). The evolution of arthropod heads: reconciling morphological, developmental and palaeontological evidence. *Dev Genes Evol* **216**, 395–415.

Schoppmeier, M. and Schröder, R. (2005). Maternal torso signaling controls body axis elongation in a short germ insect. *Curr Biol* **15**, 2131–2136.

Schoppmeier, M., Fischer, S., Schmitt-Engel, C., Löhr, U. and Klingler, M. (2009). An ancient anterior patterning system promotes caudal repression and head formation in ecdysozoa. *Curr Biol* **19**, 1811–1815.

Schröder, R. (2003). The genes orthodenticle and hunchback substitute for bicoid in the beetle Tribolium. *Nature* **422**, 621–625.

Schröder, R. and Sander, K. (1993). A comparison of transplantable bicoid activity and partial bicoid homeobox sequences in several Drosophila and blowfly species (Calliphoridae). *Dev Genes Evol* **203**, 34–43.

Schröder, R., Eckert, C., Wolff, C. and Tautz, D. (2000). Conserved and divergent aspects of terminal patterning in the beetle Tribolium castaneum. *Proc Natl Acad Sci U S A* **97**, 6591.

Schröder, R., Beermann, A., Wittkopp, N. and Lutz, R. (2008). From development to biodiversity—Tribolium castaneum, an insect model organism for short germband development. *Dev Genes Evol* **218**, 119–126.

Seimiya, M. and Gehring, W. J. (2000). The Drosophila Homeobox Gene Optix Is Capable of Inducing Ectopic Eyes by an Eyeless-Independent Mechanism. *Development* **127**, 1879–1886.

Seo, H.-C., Curtiss, J., Mlodzik, M. and Fjose, A. (1999). Six class homeobox genes in Drosophila belong to three distinct families and are involved in head development. *Mech Dev* **83**, 127–139.

Shaner, N. C., Patterson, G. H. and Davidson, M. W. (2007). Advances in Fluorescent Protein Technology. *J Cell Sci* **120**, 4247–4260.

Smith, M. C. M., Brown, W. R. A., McEwan, A. R. and Rowley, P. A. (2010). Site-specific recombination by φC31 integrase and other large serine recombinases. *Biochem Soc Trans* **38**, 388.

Snodgrass, R. E. (1935). *Principles of Insect Morphology*. Ithaca, NY, USA: Cornell University Press.

Sokoloff, A. (1972). *The biology of Tribolium with special emphasis on genetic aspects*. Oxford: Oxford University Press.

Sommer, R. and Tautz, D. (1991). Segmentation gene expression in the housefly Musca domestica. *Development* **113**, 419–430.

St Johnston, D. and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. *Cell* **68**, 201–219.

Stauber, M., Jäckle, H. and Schmidt-Ott, U. (1999). The anterior determinant bicoid of Drosophila is a derived Hox class 3 gene. *Proc Natl Acad Sci U S A* **96**, 3786–3789.

Steinmetz, P. R. H., Urbach, R., Posnien, N., Eriksson, J., Kostyuchenko, R. P., Brena, C., Guy, K., Akam, M., Bucher, G. and Arendt, D. (2010). Six3 demarcates the anterior-most developing brain region in bilaterian animals. *Evodevo* **1**, 1–9.

Struhl, G., Struhl, K. and Macdonald, P. M. (1989). The gradient morphogen bicoid is a concentration-dependent transcriptional activator. *Cell* **57**, 1259–1273.

Tautz, D. (2004). Segmentation. *Dev Cell* **7**, 301–312.

Tautz, D. and Sommer, R. J. (1995). Evolution of segmentation genes in insects. *Trends Genet* **11**, 23–27.

Tautz, D., Friedrich, M. and Schröder, R. (1994). Insect embryogenesis – what is ancestral and what is derived? *Development* **1994**, 193–199.

Telford, M. J., Bourlat, S. J., Economou, A., Papillon, D. and Rota-Stabelli, O. (2008). The Evolution of the Ecdysozoa. *Philos Trans R Soc Lond B Biol Sci* **363**, 1529–1537.

Thor, S. and Thomas, J. B. (1997). The Drosophila islet Gene Governs Axon Pathfinding and Neurotransmitter Identity. *Neuron* **18**, 397–409.

Thor, S., Andersson, S. G. E., Tomlinson, A. and Thomas, J. B. (1999). A LIM-homeodomain combinatorial code for motor-neuron pathway selection. *Nature* **397**, 76–80.

Tomancak, P., Beaton, A., Weiszmann, R., Kwan, E., Shu, S., Lewis, S. E., Richards, S., Ashburner, M., Hartenstein, V., Celniker, S. E., et al. (2002). Systematic determination of patterns of gene expression during Drosophila embryogenesis. *Genome Biol* **3**, R88.

Tomoyasu, Y. and Denell, R. E. (2004). Larval RNAi in Tribolium (Coleoptera) for analyzing adult development. *Dev Genes Evol* **214**, 575–578.

Toy, J., Yang, J.-M., Leppert, G. S. and Sundin, O. H. (1998). The Optx2 Homeobox Gene Is Expressed in Early Precursors of the Eye and Activates Retina-Specific Genes. *Proc Natl Acad Sci U S A* **95**, 10643–10648.

Trauner, J., Schinko, J., Lorenzen, M. D., Shippy, T. D., Wimmer, E. A., Beeman, R. W., Klingler, M., Bucher, G. and Brown, S. J. (2009). Large-scale insertional mutagenesis of a coleopteran stored grain pest, the red flour beetle Tribolium castaneum, identifies embryonic lethal mutations and enhancer traps. *BMC Biol* **7**, 73.

Turner, F. R. and Mahowald, A. P. (1979). Scanning electron microscopy of Drosophila melanogaster embryogenesis: III. Formation of the head and caudal segments. *Dev Biol* **68**, 96–109.

van der Zee, M., Berns, N. and Roth, S. (2005). Distinct Functions of the Tribolium zerknüllt Genes in Serosa Specification and Dorsal Closure. *Curr Biol* **15**, 624–636.

van der Zee, M., Stockhammer, O., Levetzow, C., Fonseca, R. N. and Roth, S. (2006). Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. *Proc Natl Acad Sci U S A* **103**, 16307.

Veraksa, A., McGinnis, N., Li, X., Mohler, J. and McGinnis, W. (2000). Cap "n" collar B cooperates with a small Maf subunit to specify pharyngeal development and suppress deformed homeotic function in the Drosophila head. *Development* **127**, 4023–4037.

Vincent, A., Blankenship, J. T. and Wieschaus, E. (1997). Integration of the Head and Trunk Segmentation Systems Controls Cephalic Furrow Formation in Drosophila. *Development* **124**, 3747–3754.

Walldorf, U., Kiewe, A., Wickert, M., Ronshaugen, M. and McGinnis, W. (2000). Homeobrain, a novel paired-like homeobox gene is expressed in the Drosophila brain. *Mech Dev* **96**, 141–144.

Walter, T., Shattuck, D. W., Baldock, R., Bastin, M. E., Carpenter, A. E., Duce, S., Ellenberg, J., Fraser, A., Hamilton, N., Pieper, S., et al. (2010). Visualization of image data from cells to organisms. *Nat Methods* **7**, S26–S41.

Wang, L., Wang, S., Li, Y., Paradesi, M. S. R. and Brown, S. J. (2007). BeetleBase: the model organism database for Tribolium castaneum. *Nucleic Acids Res* **35**, D476–D479.

Weigel, D., Bellen, H. J., Jürgens, G. and Jäckle, H. (1989a). Primordium specific requirement of the homeotic gene fork head in the developing gut of the Drosophila embryo. *Dev Genes Evol* **198**, 201–210.

Weigel, D., Jürgens, G., Küttner, F., Seifert, E. and Jäckle, H. (1989b). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell* **57**, 645–658.

Weigel, D., Jürgens, G., Klingler, M. and Jäckle, H. (1990). Two gap genes mediate maternal terminal pattern information in Drosophila. *Science* **248**, 495–498.

Wheeler, S. R., Carrico, M. L., Wilson, B. A. and Skeath, J. B. (2005). The Tribolium columnar genes reveal conservation and plasticity in neural precursor patterning along the embryonic dorsal–ventral axis. *Dev Biol* **279**, 491–500.

Wimmer, E. A. (2005). Insect transgenesis by site-specific recombination. *Nat Methods* **2**, 580–582.

Zaffran, S., Das, G. and Frasch, M. (2000). The NK-2 homeobox gene scarecrow (scro) is expressed in pharynx, ventral nerve cord and brain of Drosophila embryos. *Mech Dev* **94**, 237–241.

7 Appendix

7.1 Abbreviations

AEL	after egg lay
bp	base pair
cDNA	complementary DNA
CLR	clypeolabral region
DSLM	Digital Scanned Laser light sheet fluorescence Microscopy
dsRNA	double stranded RNA
ECFP	enhanced cyan fluorescent protein
GFP	green fluorescent protein
kbp	kilo base pair
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
ORF	open reading frame
PCR	polymerase chain reaction
PH3	phosphorylated Histone H3
RACE	rapid amplification of cDNA ends
RNAi	RNA interference
SB	*San Bernardino*; a *Tribolium* wild type strain
SNS	stomatogastric nervous system
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
v^{w}	*vermillion*^{white}; a white-eyed *Tribolium* strain

7.2 Gene abbreviations

ase	*asense*	*bcd*	*bicoid*
cad	*caudal*	*cnc*	*cap'n'collar*
croc	*crocodile*	*dl*	*dorsal*
dpp	*decapentaplegic*	*ems*	*empty spiracles*
fkh	*fork head*	*hbn*	*homeobrain*
hh	*hedgehog*	*hkb*	*huckebein*
lab	*labial*	*otd*	*orthodenticle*
scro	*scarecrow*	*sna*	*snail*
spi	*spitz*	*srp*	*serpent*
tll	*tailless*	*tor*	*torso*
tup	*tailup*	*twi*	*twist*
vnd	*ventral nerve cord defective*	*wg*	*wingless*
zen	*zerknüllt*	*Tc-*	prefix, if the gene is a *Tribolium* ortholog

7.3 Primers used in this work

Table 4: Primers for cloning of gene fragments. Name of the primer, sequence and cloned gene are indicated.

Name	Sequence	Gene
SK_Tc_croc_fwd	CGATTTTCACCGAACATCAGAATGC	*Tc-croc* (forward, bp 8 - 32)
SK_Tc_croc_rv	ACAGTCCTGTTGGTAGTAAGCCGCC	*Tc-croc* (reverse, bp 987-1011)
SK_croc_fw2	ATTCCTACATCGCGCTCATCG	*Tc-croc* (Nested forward, bp 191-211)
SK_croc_rv2	GGGCTGTACCAGGGGCTGTA	*Tc-croc* (Nested reverse, bp 843-862)
SK_Tc_hbn_fwd	GAAGAAATGCGTCCGAGGGCC	*Tc-hbn* (forward, bp 16-36)
SK_Tc_hbn_rv	GGCACTAAAATTTCTTGTGTCCGTAACC	*Tc-hbn* (reverse, bp 722-749)
SK_hbn_fw2	AGTTAACAGCTCGTCCACCTCTGC	*Tc-hbn* (Nested forward, bp 63-68)
SK_hbn_rv2	CGACTTCGCGTGCAGTTTCTG	*Tc-hbn* (Nested reverse, bp 763-783)
SK85	GCCCTTCCTGCAATAACGAAAC	*Tc-hkb* (forward)
SK86	GGCACGTAGGGGTAGGTCGC	*Tc-hkb* (reverse)
SK107	GCGTCGAACGGGAGCATAAC	*Tc-hkb* (Nested reverse)
SK108	CCTGCAATAACGAAACCAACAC	*Tc-hkb* (Nested forward)
SK_tup_fwd	AACGAGCGATAGCGAAAAAGCCC	*Tc-tup* (forward, bp 38-60)
SK_tup_rv	TGACGTCAGACGGCGGCGC	*Tc-tup* (reverse, bp 1144-1162)
SK_tup_fw2	CCAGATCCACGACCAATACATCC	*Tc-tup* (Nested forward, bp 93-115)
SK_tup_rv2	TGACGAGCTGCTGGAAGGCC	*Tc-tup* (Nested reverse, bp 1044-1063)

Table 5: Primers for RACE. Name of the primer, sequence and purpose are indicated.

Name	Sequence	Purpose
croc-3RACE	CGGCTTACTACCAACAGGACTG	3'-RACE of *Tc-croc*
SK0906221	GCGGCGGCTTACTACCAACAGGACTG	3'-RACE of *Tc-croc* (Nested)
SK0906222	CTGTCAATTGGCGGGGTTTAGGCACTC	3'-RACE of *Tc-croc*
SK0907151	CAATCCGCTCCAGTCAAAGACTCTGATAATTCC	5'-RACE of *Tc-cnc*
SK0907152	CTGATAATTCCTCGGCCTTTACTACATACTCCC	5'-RACE of *Tc-cnc* (Nested)
SK0907153	GAATCCAGAACACAAGGAGCCACCACCTCAAG	3'-RACE of *Tc-cnc* (Nested)
SK0907154	CAGTCTTCAGACGTCTGCAGATGGGTCGATATTGG	3'-RACE of *Tc-cnc*
SK73	GGTGTCCGGTTTTCGTATCCATCTC	Cloning of unique sequence of *Tc-cncC* (forward)
SK74	CGGTGTCTATGGTGTAGTTGAAGCC	Cloning of unique sequence of *Tc-cncC* (reverse)
SK75	CTGTTGGGCTGGGTCCTGATTG	Cloning of unique sequence of *Tc-cncB* (forward)
SK76	CTTGGAACAGAAATCACCGAAGCAC	Cloning of unique sequence of *Tc-cncB* (reverse)
SK109	TGGTTGTGCTGGATGTGGTCGAGGGAGTTTC	5'RACE of *Tc-cncA*
SK112	GGTCGAGGGAGTTTCTCACCAAGTGGCTG	5'RACE of *Tc-cncA* (Nested)

Table 6: Primers for tests for off target effects after RNAi. Name of the primer, sequence and gene are indicated.

Name	Sequence	Gene
SK20090902 1	TAATACGACTCACTATAGGGCGCCAACAGCTCGACTCC	*Tc-croc* (5'-fragment, reverse)
SK20090902 2	TAATACGACTCACTATAGGCGAACATGTTGTAGGAGTCTGGG	*Tc-croc* (3'-fragment, reverse)
SK20090902 3	TAATACGACTCACTATAGGGTGTTGTGCTTGTTCACGACCG	*Tc-tup* (5'-fragment, reverse)
SK20090902 4	TAATACGACTCACTATAGGGGCAAGAACTCTGTCAGGACGTG	*Tc-tup* (3'-fragment, reverse)
SK20090902 5	TAATACGACTCACTATAGGCCATGCCCGACTCCAAGC	*Tc-scro* (5'-fragment, reverse)
SK20090902 6	TAATACGACTCACTATAGGGCGGGAAATTGCAAATCGG	*Tc-scro* (5'-fragment, reverse)
SK91	TAATACGACTCACTATAGGCCGGCTGGGAGCTGTTGTAGG	*Tc-cnc* (5'-fragment, reverse)
SK92	TAATACGACTCACTATAGGGATGGAGTTCGGGAGACACAGCC	*Tc-cnc* (3'-fragment, forward)
SK93	TAATACGACTCACTATAGGGACCGAGCAGTGTATGTGGGC	*Tc-hh* (5'-fragment, reverse)
SK94	TAATACGACTCACTATAGGAATACGGCGGTTGTTTCTCGG	*Tc-hh* (3'-fragment, forward)
SK95	TAATACGACTCACTATAGGCACTTGCGTCGGCGTCAGTC	*Tc-six3* (5'-fragment, reverse)
SK96	TAATACGACTCACTATAGGTCAAGAACCGGAGGCAGCG	*Tc-six3* (3'-fragment, forward)
SK97	TAATACGACTCACTATAGGGGATGCTTCGGACCCATTGG	*Tc-tll* (5'-fragment, reverse)
SK98	TAATACGACTCACTATAGGGCTGGCTAGACCTATTCGTCCTG	*Tc-tll* (3'-fragment, forward)

Table 7: Primers for design of constructs for ectopic expression. Name of the primer, sequence and purpose are indicated.

Name	Sequence	Purpose
SK78	GCTGTACATCAGTATTTCTGTCCCACACAGTCCTG	Cloning of *Tc-croc*-ORF with 3'-Bsp1407I-overhang (reverse)
SK79	ATACCGGTAAACATGCATACGATTTTCACCGAACA	Cloning of *Tc-croc*-ORF with 5'-BshTI-overhang (forward)
SK87	ATAGGGCCCATATATGGATCCCAACCTTCAAT	PCR-amplification of Tc-hsp-5'-3'-UTR with Bsp120I-overhang (forward)
SK88	ATAGGGCCCCCACTAGTGAGCAGCCAGTTGTT	PCR-amplification of Tc-hsp-5'-3'-UTR with Bsp120I-overhang (reverse)
SK99	GATACGCGTTCTGTAAACATGCATACGATTTTCACCGAACATCAG	Cloning of *Tc-croc* into pSLaf[3pBac-Tc'hsp5'3'UTR-attB-PUb-DsRed]af with MluI-overhang (forward)
SK100	AGTAGGCATTCTGATGTTCGGTG	Sequencing Tc-hsp5'UTR in #1245+croc
SK103	GGCGATTAAGTTGGGTAACG	Sequencing 6xP3-eCFP in hs-overexpression vectors
SK104	GGGTGTTCTGCTGGTAGTGG	Sequencing 6xP3-eCFP in hs-overexpression vectors
SK105	GCGTACTCCACCTCACAGATC	Sequencing 6xP3-eCFP in hs-overexpression vectors
SK106	CCACTACCAGCAGAACACCC	Sequencing 6xP3-eCFP in hs-overexpression vectors
SK110	ACATCTAGAATGGCGCTCGGACTCGGCTC	Cloning of *Tc-six3*-ORF in hs-attB-vector with XbaI-overhang (forward)
SK111	ATACTCGAGTCACGTCAGTCTCGGCGGCGA	Cloning of *Tc-six3*-ORF in hs-attB-vector with XhoI-overhang (reverse)
SK113	GCATCTAGAATGATACAGACGTCGCAATTCC	Cloning of *Tc-cncB*-ORF in hs-attB-vector with XbaI-overhang (forward)
SK114	TCTACGCGTTCAATCTTTGTGACCTTGAGGTG	Cloning of *Tc-cncB*-ORF in hs-attB-vector with MluI-overhang (reverse)
SK115	CTGTTGGGCTGGGTCCTGATTG	*Tc-cncB* (forward)
SK116	TTTGTCTGGTTTCAGCTCCGCAGTC	*Tc-cncB* (Nested forward)
SK119	AATTAGCTCCACTATCTCCCAGTTCC	*Tc-cncB* (reverse)
SK120	TCCTAGCACCAATAACGTCAGTCAC	*Tc-cncB* (Nested reverse)
SK121	GCACAACCAGTTTCCACTATTCTCC	Sequencing primer for ORFs in hs-attB-vector
SK122	AGGCCACGACTAATGCGGTAAG	Sequencing primer for cncB-ORF in hs-attB-Vector
SK123	CCCTGCACAATTACAGCCACC	Sequencing primer for cncB-ORF in hs-attB-vector
SK124	CTATCAGATGGCGAGTGGTGCGACG	Sequencing primer for ORFs in hs-attB-vector
SK125	AGCTCAACAAGAACGAGGCCGTGC	Sequencing primer for six3-ORF in hs-attB-vector
SK126	ATTCCGCTTCGGCAATCACTCC	Sequencing primer for six3-ORF in hs-attB-vector
SK127	GTCGGCAAATAGCACCAATATC	Test for attP-attB recombination (forward in Gal4delta-Mutator)
SK128	CACTGTCACCTGGTTGGACG	Test for attP-attB recombination (reverse in Gal4delta-Mutator)
SK129	CCGCATTAGTCGTGGCCTTG	Test for attP-attB recombination (reverse in hs-croc- attB-Vektor)

7.4 Video file of DSLM *in vivo* imaging

7.5 Proliferating cells in untreated embryos and after *Tc-croc* RNAi

Table 8: Numbers of proliferating cells per embryo. Cells were counted in the anterior head (see Fig. 8).

	untreated		***Tc-croc* RNAi**	
	ID of the embryo image	**proliferating cells**	**ID of the embryo image**	**proliferating cells**
germ rudiment	2011_12_06-1_2000-08	11	MAX_C1-2011_12_20-04	86
	2011_12_06-1_1000-01-ab_slide_9	42	MAX_C1-2011_12_20-10	87
elongating	2011_12_01-1_1000-03	10	MAX_C1-2011_12_20-23	16
	2011_12_06-1_2000-04	50	MAX_C1-2011_12_20-06	12
	2011_12_06-1_5000-02	16	MAX_C1-2011_12_20-02	30
	2011_12_06-1_1000-04-ab_slide_11	55	MAX_C1-2011_12_20-15	38
	2011_08_19-20	56	MAX_C1-2011_12_20-07	16
	2011_12_06-1_2000-02	46	MAX_C1-2011_12_20-08	81
	2011_08_19-14	17	MAX_C1-2011_12_20-13	43
	2011_12_06-1_2000-01	29	MAX_C1-2011_12_20-25	49
	2011_12_01-1_1000-04	38	MAX_C1-2011_12_20-18	50
	2011_12_06-1_2000-06	33	MAX_C1-2011_12_20-17	25
	2011_12_06-1_2000-05	26	MAX_C1-2011_12_20-19	30
	2011_12_01-1_1000-02	53	MAX_C1-2011_12_19-01	32
	2011_12_06-1_1000-02-ab_slide_11	19	MAX_C1-2011_12_20-16	47
	2011_12_06-1_1000-05-ab_slide_9	20	MAX_C1-2011_12_20-11	38
	2011_12_06-1_1000-03-ab_slide_10	29	MAX_C1-2011_12_20-22	64
	2011_08_19-08	46	MAX_C1-2011_12_20-12	46
	2011_12_06-1_2000-07	43	MAX_C1-2011_12_20-24	20
	2011_12_06-1_5000-02	16	MAX_C1-2011_12_20-14	56
	2011_12_06-1_2000-03	27	MAX_C1-2011_12_20-01	54
	2011_12_01-1_1000-01	42	MAX_C1-2011_12_20-21	61
	2011_08_19-12	29	MAX_C1-2011_12_20-26	54
	2011_08_19-07	72	MAX_C1-2011_12_20-05	44
	2011_08_19-31	94	MAX_C1-2011_12_20-20	72
	2011_08_19-18	55	MAX_C1-2011_12_21-01	57
retracting	2011_08_19-16	64	MAX_C1-2011_12_20-03	84
	2011_08_19-22	82	MAX_C1-2011_12_20-09	75
	2011_08_19-01	106	MAX_C1-2011_12_20-27	46

Table 9: Test for significance of differences between cell proliferation in untreated embryos and after RNAi.

		untreated	***Tc-croc* RNAi**	**t-test**
germ rudiment	mean	26,5	86,5	0,061
	n	2	2	
	standard deviation	21,920	0,707	
	standard error of the mean	15,5	0,5	
elongating	mean	38,375	43,125	0,392
	n	24	24	
	standard deviation	19,934	18,116	
	standard error of the mean	4,069	3,698	
retracting	mean	84	68,333	0,402
	n	3	3	
	standard deviation	21,071	19,858	
	standard error of the mean	12,166	11,465	

7.6 Cell death in untreated embryos, after *Tc-croc* RNAi and after *Tc-six3* RNAi

Table 10: Dying cells in the anterior head of germ rudiments.

	untreated		***Tc-croc*-RNAi**		***Tc-six3*-RNAi**	
	ID	dying cells	ID	dying cells	ID	dying cells
	02.08.2011_1	0	02.08.2011_2	0	02.08.2011_3	0
	02.08.2011_2	0	02.08.2011_11	0	02.08.2011_7	0
	02.08.2011_3	0	02.08.2011_12	0	02.08.2011_9	0
	02.08.2011_4	0	02.08.2011_14	0	02.08.2011_15	0
	02.08.2011_5	0	02.08.2011_15	0	02.08.2011_19	0
	02.08.2011_6	0	02.08.2011_17	0	02.08.2011_22	0
	02.08.2011_7	0	02.08.2011_19	0	02.08.2011_25	0
	02.08.2011_8	0	02.08.2011_24	0	02.08.2011_26	0
	02.08.2011_9	0	02.08.2011_25	0	02.08.2011_27	0
	02.08.2011_10	0	02.08.2011_26	0	02.08.2011_33	0
	02.08.2011_11	0	02.08.2011_27	0	02.08.2011_36	0
	02.08.2011_12	0	02.08.2011_30	0	02.08.2011_37	0
	09.11.2011_5	0	02.08.2011_35	0	02.08.2011_38	0
	09.11.2011_16	0	02.08.2011_37	0	02.08.2011_41	0
	09.11.2011_21	0	09.11.2011_45	0	02.08.2011_42	2
	09.11.2011_28	0	09.11.2011_48	0	02.08.2011_43	0
	09.11.2011_30	0	09.11.2011_84	0	09.11.2011_15	0
	09.11.2011_34	0	09.11.2011_85	0	09.11.2011_23	0
	09.11.2011_35	0	09.11.2011_90	0	09.11.2011_37	0
	09.11.2011_36	0	09.11.2011_93	0	09.11.2011_38	0
	09.11.2011_37	0	09.11.2011_96	0	09.11.2011_41	0
	09.11.2011_39	0	09.11.2011_109	1	09.11.2011_48	0
	09.11.2011_45	0	09.11.2011_121	0	09.11.2011_50	0
	09.11.2011_46	0	09.11.2011_142	23	09.11.2011_53	0
	09.11.2011_51	0	09.11.2011_145	0	09.11.2011_54	0
	09.11.2011_53	0	09.11.2011_156	0	09.11.2011_57	0
	09.11.2011_58	0				
	09.11.2011_60	0				
	09.11.2011_65	0				
	09.11.2011_69	0				
	09.11.2011_70	0				
	09.11.2011_75	0				
mean		0		0,8571		0,0714
n		34		28		28
standard deviation		0		4,3437		0,3780
standard error of the mean		0		0,8209		0,0714
t-test				0,3011		0,3218

Table 11:Dying cells in the anterior head of extending germ bands.

	untreated		***Tc-croc*-RNAi**		***Tc-six3*-RNAi**	
	ID	dying cells	ID	dying cells	ID	dying cells
	02.08.2011_14	0	02.08.2011_1	0	02.08.2011_1	0
	02.08.2011_15	0	02.08.2011_3	0	02.08.2011_2	0
	02.08.2011_16	0	02.08.2011_4	0	02.08.2011_4	5
	02.08.2011_17	0	02.08.2011_5	0	02.08.2011_5	0
	02.08.2011_18	0	02.08.2011_6	0	02.08.2011_6	0
	02.08.2011_19	0	02.08.2011_7	0	02.08.2011_8	1
	02.08.2011_20	0	09.11.2011_2	6	02.08.2011_10	2
	02.08.2011_21	0	09.11.2011_3	1	02.08.2011_12	2
	02.08.2011_22	0	09.11.2011_5	4	02.08.2011_13	0
	02.08.2011_23	0	09.11.2011_6	1	02.08.2011_14	0
	02.08.2011_24	0	09.11.2011_8	0	02.08.2011_16	0
	02.08.2011_25	0	09.11.2011_10	2	02.08.2011_17	0
	02.08.2011_26	1	09.11.2011_12	22	02.08.2011_18	0

	02.08.2011_27	1	09.11.2011_14	16	02.08.2011_21	0
	02.08.2011_28	0	09.11.2011_15	22	02.08.2011_23	19
	02.08.2011_29	0	09.11.2011_22	1	02.08.2011_24	31
	02.08.2011_30	0	09.11.2011_23	1	09.11.2011_1	1
	02.08.2011_31	1	09.11.2011_25	4	09.11.2011_3	22
	02.08.2011_32	0	09.11.2011_27	0	09.11.2011_4	1
	02.08.2011_33	0	09.11.2011_35	1	09.11.2011_5	0
	02.08.2011_34	0	09.11.2011_38	0	09.11.2011_7	0
	02.08.2011_35	0	09.11.2011_40	7	09.11.2011_8	0
	02.08.2011_36	0	09.11.2011_41	0	09.11.2011_9	2
	02.08.2011_37	0	09.11.2011_46	0	09.11.2011_11	0
	02.08.2011_38	0	09.11.2011_49	0	09.11.2011_12	19
	02.08.2011_39	5	09.11.2011_51	6	09.11.2011_13	1
	02.08.2011_40	0	09.11.2011_60	2	09.11.2011_16	3
	02.08.2011_41	7	09.11.2011_64	10	09.11.2011_17	5
	09.11.2011_3	0	09.11.2011_74	0	09.11.2011_19	0
	09.11.2011_4	2	09.11.2011_79	6	09.11.2011_21	14
	09.11.2011_6	4	09.11.2011_83	0	09.11.2011_22	0
	09.11.2011_10	1	09.11.2011_88	0	09.11.2011_26	1
	09.11.2011_14	0	09.11.2011_91	0	09.11.2011_27	5
	09.11.2011_15	0	09.11.2011_92	10	09.11.2011_28	1
	09.11.2011_19	0	09.11.2011_7	4	09.11.2011_29	1
	09.11.2011_25	0	09.11.2011_13	2	09.11.2011_30	1
	09.11.2011_27	2	09.11.2011_33	9	09.11.2011_31	0
	09.11.2011_31	3	09.11.2011_47	0	09.11.2011_33	0
	09.11.2011_38	1	09.11.2011_59	3	09.11.2011_34	0
	09.11.2011_40	5	09.11.2011_62	6	09.11.2011_36	1
	09.11.2011_41	0	09.11.2011_70	5	09.11.2011_43	4
	09.11.2011_42	0	09.11.2011_76	15	09.11.2011_44	0
	09.11.2011_43	0	09.11.2011_80	6	09.11.2011_45	3
	09.11.2011_44	0	09.11.2011_97	1	09.11.2011_46	1
	09.11.2011_49	0	09.11.2011_99	2	09.11.2011_47	0
	09.11.2011_50	1	09.11.2011_94	3	09.11.2011_52	1
	09.11.2011_55	0	09.11.2011_95	12	09.11.2011_55	5
	09.11.2011_56	0	09.11.2011_102	0	09.11.2011_58	22
	09.11.2011_57	1	09.11.2011_103	0	09.11.2011_59	14
	09.11.2011_63	1	09.11.2011_104	1	09.11.2011_60	1
	09.11.2011_68	1	09.11.2011_110	0	09.11.2011_66	25
	09.11.2011_71	0	09.11.2011_111	0	09.11.2011_69	13
	09.11.2011_77	0	09.11.2011_112	3	09.11.2011_72	1
	09.11.2011_79	0	09.11.2011_113	0	09.11.2011_73	6
	09.11.2011_81	0	09.11.2011_115	3	09.11.2011_74	0
	09.11.2011_85	2	09.11.2011_117	20	09.11.2011_75	24
	09.11.2011_1	3	09.11.2011_125	1	09.11.2011_81	9
	09.11.2011_12	1	09.11.2011_126	1	09.11.2011_86	11
	09.11.2011_20	0	09.11.2011_128	0	09.11.2011_88	36
	09.11.2011_24	6	09.11.2011_133	3	09.11.2011_89	0
	09.11.2011_32	0	09.11.2011_137	0	09.11.2011_91	1
	09.11.2011_48	3	09.11.2011_143	0	09.11.2011_92	0
	09.11.2011_53	6	09.11.2011_147	0	09.11.2011_94	0
	09.11.2011_54	5	09.11.2011_151	1	09.11.2011_97	0
	09.11.2011_59	2	09.11.2011_152	6	09.11.2011_103	4
	09.11.2011_74	11	09.11.2011_154	0	09.11.2011_107	7
	09.11.2011_83	2	09.11.2011_158	0	09.11.2011_108	0
mean		1,1642		3,4179		4,8657
n		67		67		67
standard deviation		2,1291		2,1291		2,1291
standard error of the mean		0,2601		0,2601		0,2601
t-test				0,0017		0,0006

Table 12: Dying cells in the anterior head of retracting germ bands.

	untreated		***Tc-croc*-RNAi**		***Tc-six3*-RNAi**	
	ID	dying cells	ID	dying cells	ID	dying cells
	02.08.2011_42	0	09.11.2011_1	29	09.11.2011_2	19
	02.08.2011_43	0	09.11.2011_4	19	09.11.2011_6	9
	09.11.2011_7	1	09.11.2011_9	2	09.11.2011_10	8
	09.11.2011_8	19	09.11.2011_11	0	09.11.2011_14	0
	09.11.2011_9	5	09.11.2011_16	37	09.11.2011_18	6
	09.11.2011_11	1	09.11.2011_17	46	09.11.2011_20	2
	09.11.2011_13	10	09.11.2011_18	4	09.11.2011_24	0
	09.11.2011_17	2	09.11.2011_19	10	09.11.2011_25	0
	09.11.2011_18	2	09.11.2011_20	12	09.11.2011_32	19
	09.11.2011_22	0	09.11.2011_21	11	09.11.2011_35	0
	09.11.2011_23	16	09.11.2011_24	1	09.11.2011_39	0
	09.11.2011_26	0	09.11.2011_26	31	09.11.2011_40	0
	09.11.2011_29	8	09.11.2011_28	5	09.11.2011_42	17
	09.11.2011_33	1	09.11.2011_29	7	09.11.2011_49	11
	09.11.2011_47	3	09.11.2011_30	23	09.11.2011_51	60
	09.11.2011_61	14	09.11.2011_31	33	09.11.2011_56	14
	09.11.2011_62	3	09.11.2011_32	16	09.11.2011_61	14
	09.11.2011_64	1	09.11.2011_34	24	09.11.2011_62	0
	09.11.2011_66	5	09.11.2011_36	35	09.11.2011_64	31
	09.11.2011_67	3	09.11.2011_37	28	09.11.2011_65	51
	09.11.2011_72	7	09.11.2011_39	149	09.11.2011_67	7
	09.11.2011_73	1	09.11.2011_42	54	09.11.2011_68	0
	09.11.2011_78	3	09.11.2011_43	37	09.11.2011_70	1
	09.11.2011_82	3	09.11.2011_44	17	09.11.2011_71	20
mean		4,5		26,25		12,0417
n		24		24		24
standard deviation		5,3079		30,0756		15,9360
standard error of the mean		1,0835		6,1392		3,2529
t-test				0,0011		0,0329

7.7 Staged heat shock

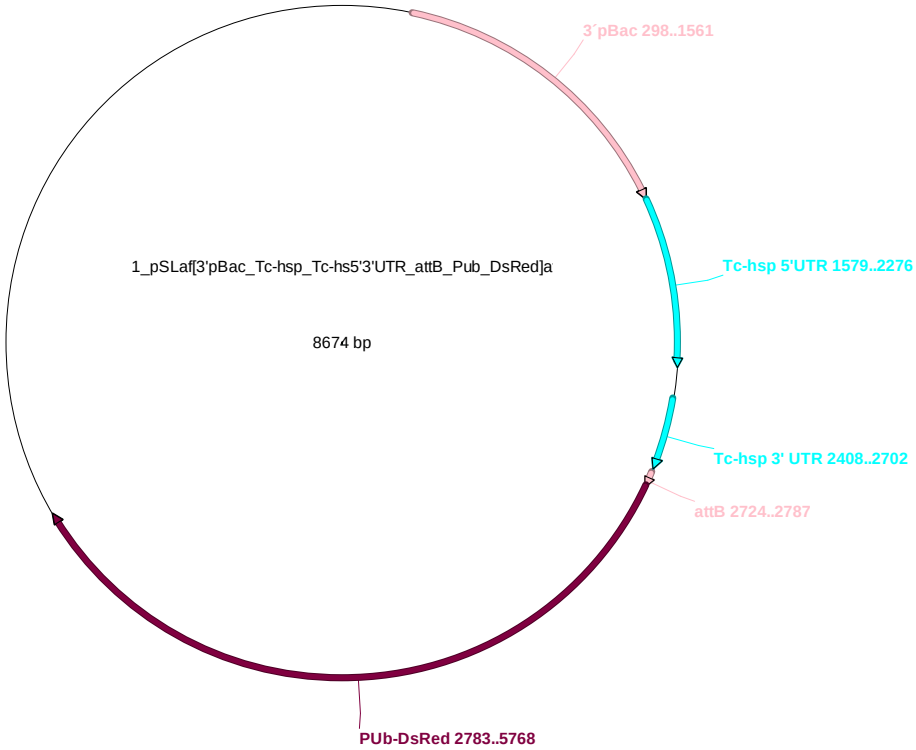
Table 13: Numbers of wild type-like larvae, affected larvae, empty eggs and hatched larvae of strain 119_1 after different developmental times before heat shock. Hatched larvae were not quantified when no heat shock was applied.

	no heat shock	**9-10 h**	**10-11 h**	**11-12 h**	**12-13 h**	**13-14 h**	**14-15 h**	**15-16 h**	**16-17 h**	**17-18 h**
wild type-like larvae	129	1	5	2	3	3	7	2	22	13
affected larvae	30	0	0	3	1	2	0	1	13	5
empty eggs	229	5	8	13	8	10	13	6	61	44
hatched larvae	n/a	3	9	3	3	9	6	17	57	83

Table 14: Numbers of wild type-like larvae, affected larvae, empty eggs and hatched larvae of strain 124_1 after different developmental times before heat shock. Hatched larvae were not quantified when no heat shock was applied.

	no heat shock	**9-10 h**	**10-11 h**	**11-12 h**	**12-13 h**	**13-14 h**	**14-15 h**	**15-16 h**	**16-17 h**	**17-18 h**
wild type-like larvae	59	3	10	5	6	4	8	5	12	3
affected larvae	28	3	5	2	7	6	4	6	18	9
empty eggs	219	29	25	29	34	22	47	33	76	61
hatched larvae	n/a	53	74	55	60	70	90	86	210	165

7.8 Vector maps



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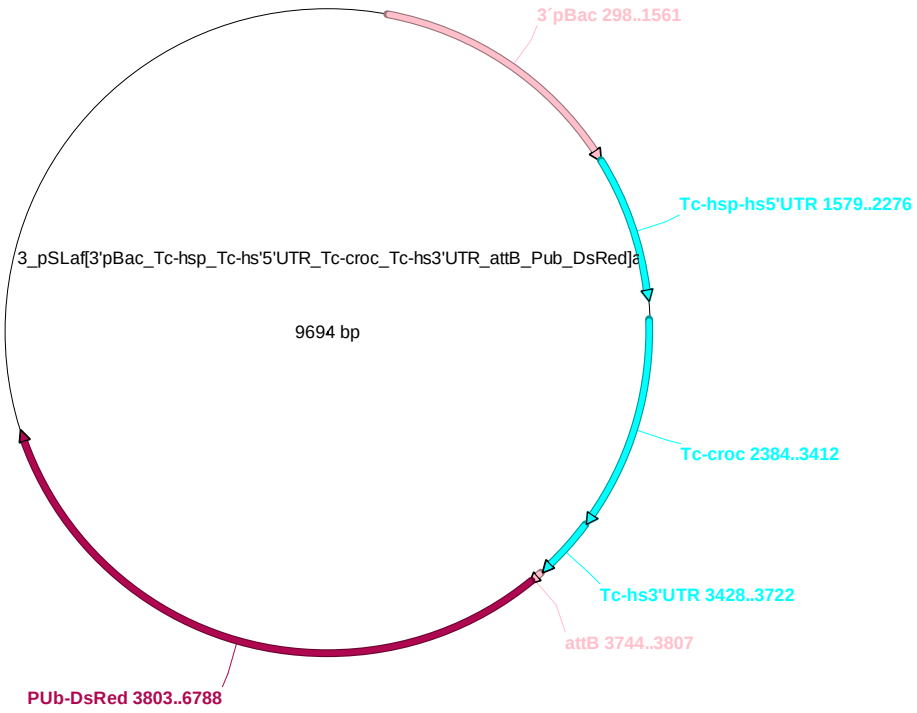

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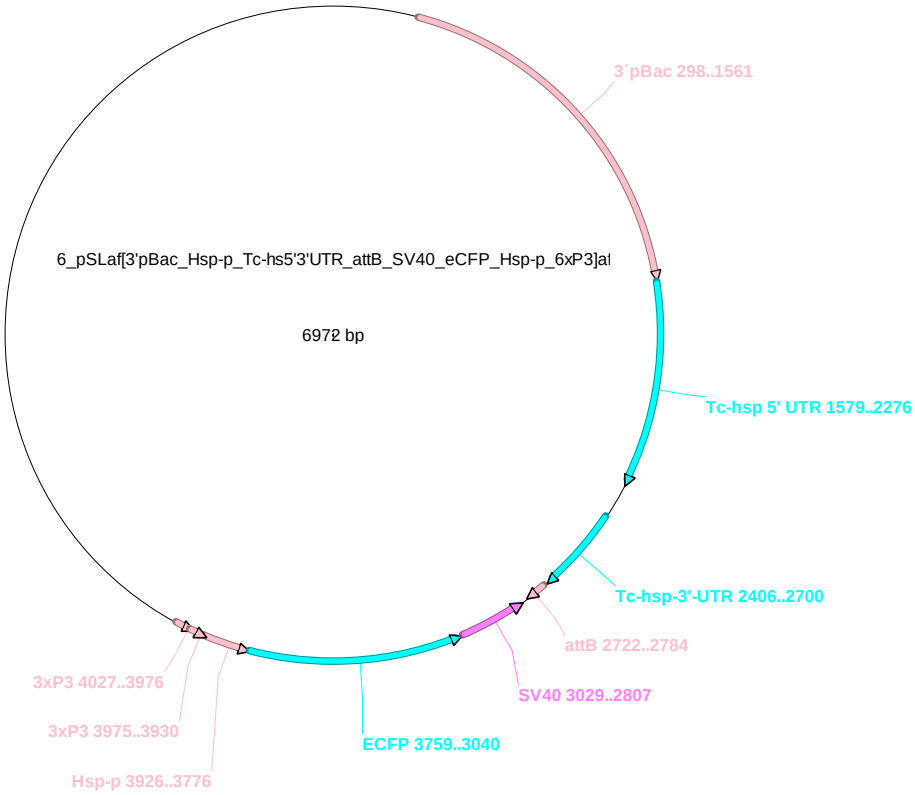

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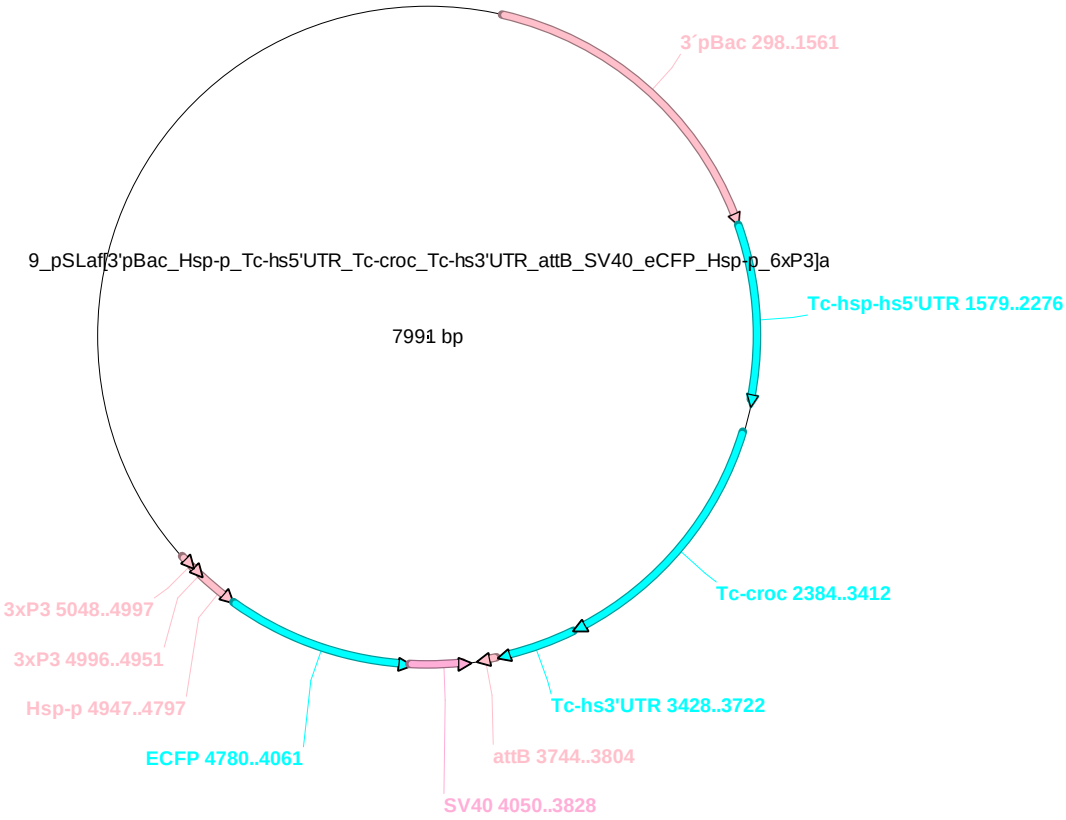

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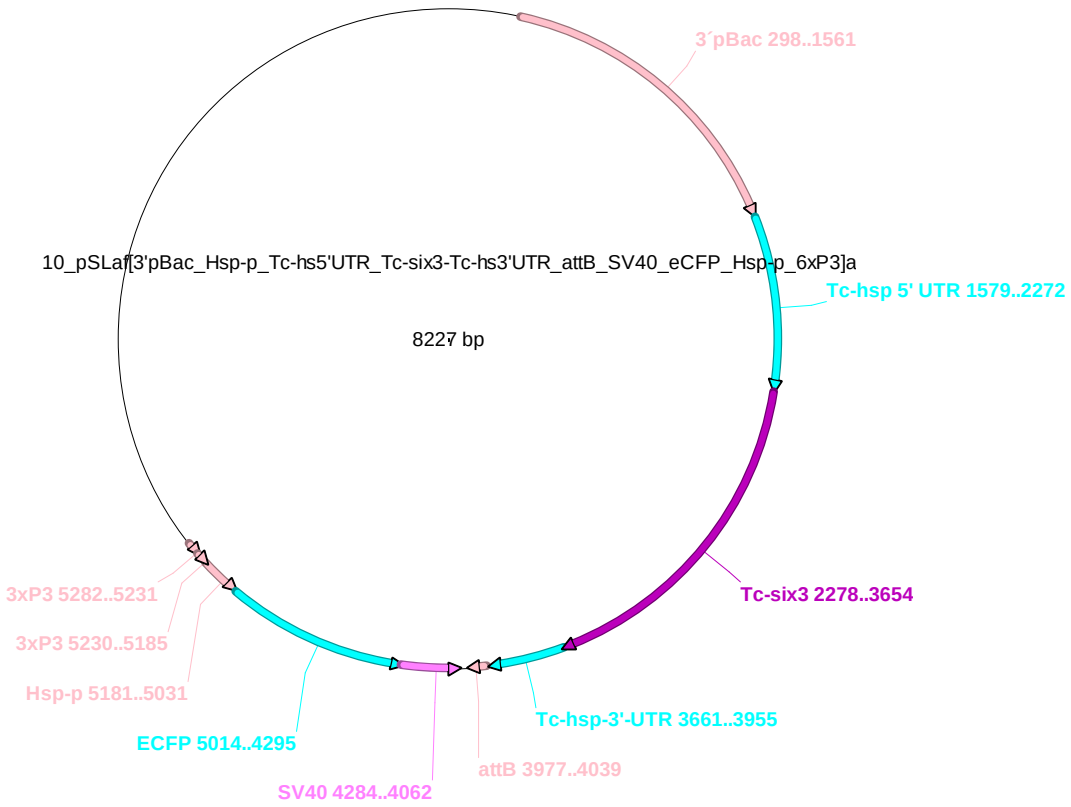

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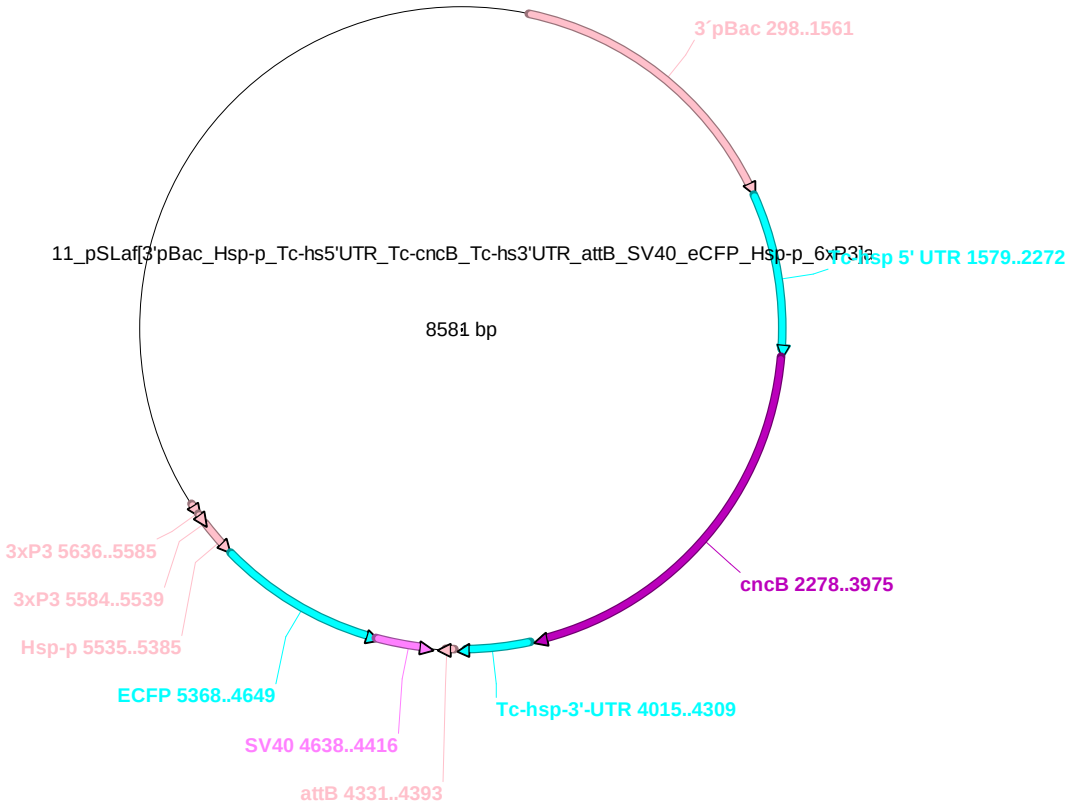

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8 *Curriculum vitae*

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University studies

since 11/08	PhD thesis "Formation of the Clypeolabral Region During Embryonic Head Development of the Red Flour Beetle *Tribolium castaneum*" at the Göttingen Graduate School for Neurosciences and Molecular Biosciences (GGNB) in the group of Gregor Bucher, Department for Developmental Biology, University of Göttingen. Partially funded by GGNB Junior Group Stipend.
02/08-10/08	Diploma thesis "The anterior median tissue of the insect head" in the group of Gregor Bucher. Final grade: sehr gut (A)
2003- 2008	Undergraduate studies of biology at the Georg-August-University of Göttingen with main focus on developmental biology, zoology and biochemistry.

Teaching experience

02/09	Supervision of practical course in developmental and cell biology
06/09-08/09	Supervision of Bachelor Thesis of Peter Kitzmann "Molecular and phenotypical analysis of insertion mutants of the red flour beetle *Tribolium castaneum*"

02/10-11/10 Supervision of Diploma Thesis of Julia Ulrich “Evolution and regulation of the anterior-median region”

04/11-10/11 Supervision of Master Thesis of Peter Kitzmann “Analysis of RNAi phenotypes”

Publications (published and in preparation)

Schetelig, Scolari, Handler, **Kittelmann**, Gasperi, Wimmer. Site-specific recombination for the modification of transgenic strains of the Mediterranean fruit fly *Ceratitis capitata*. *Proc Natl Acad Sci U S A*. 2009 Oct 27;106(43):18171-6.

Posnien, Schinko, **Kittelmann**, Bucher. Genetics, development and composition of the insect head--a beetle's view. *Arthropod Struct Dev*. 2010 Nov;39(6):399-410.

Schinko, Posnien, **Kittelmann**, Koniszewski, Bucher. Single and double whole-mount *in situ* hybridization in red flour beetle (*Tribolium*) embryos. *Cold Spring Harb Protoc*. 2009 Aug;2009(8):pdb.prot5258.

Kittelmann, Ulrich, Bucher. Development of the median non-segmental head of the red flour beetle *Tribolium castaneum*. In preparation.

Oral presentations

The anterior median tissue of the insect head. 101st annual meeting of the “Deutsche Zoologische Gesellschaft e.V.” (German zoological society), Jena, Germany, September 2008

Development of the Anterior Median Tissue of the *Tribolium* head. Colonia Coleoptera, Bi-annual Regional *Tribolium* Meeting, Cologne, Germany, July 2009

Patterning of the anterior median non-segmental region (AMR). International *Tribolium* meeting, Kansas City, USA, June 2011

Patterning of the anterior median non-segmental region (AMR) during *Tribolium* head development. Invited talk in the seminar of the Laboratory for Development and Evolution, Department of Zoology, University of Cambridge, UK, October 2011