Functional analysis of embryonic brain development in *Tribolium castaneum*

Dissertation submitted in partial fulfillment of the requirements for the degree of
"doctor rerum naturalium (Dr. rer. nat.)"

of the Georg-August-University Göttingen

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from

Erlangen,
Germany

Göttingen, 2011

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Submission of the dissertation: June 2011

Date of the oral examination: August 22, 2011

Herewith I declare, that I prepared the dissertation

"Functional analysis of embryonic brain development in *Tribolium castaneum*"

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

_____ Göttingen, June 29, 2011

Nikolaus Koniszewski

Danksagung

Als erstes bedanke ich mich bei Herrn Professor Bucher, der mir sein Vertrauen entgegenbrachte, so dass ich diese Arbeit selbständig durchführen durfte. Besonders danke ich ihm für die hilfreichen Diskussionen, die das schnelle voranbringen der Projekte ermöglichte. Und schließlich danke ich ihm für die häufigen Möglichkeiten, meine Ergebnisse auf internationalen Tagungen präsentieren zu dürfen. Letztlich habe ich sehr viel bei Dir gelernt, wofür ich Dir sehr dankbar bin.
Als nächstes und nicht weniger Dank gebührt Herrn Professor Wimmer, der durch seine kritischen und hilfreichen Fragen einen erheblichen Einfluss auf den Verlauf und den Ausgang dieser Arbeit nahm. Auch dafür möchte ich mich bei ihm bedanken, dass er immer ein offenes Ohr und stets Ideen parat hatte um mögliche methodischen Probleme auszumerzen.

Herrn Professor Averof und Hendrikje Hein danke ich für die mir zur Verfügung gestellten Linien. Außerdem möchte ich Martin Kollmann und Herrn Professor Joachim Schachtner für die unkomplizierte Unterstützung und Kooperation danken. In diesem Kontext möchte ich auch Stefan danken.

Recht herzlich möchte ich allen Mitarbeitern des Instituts Entwicklungsbiologie danken, die mir alle immer hilfsbereit mit Rat und Tat zur Seite standen, wenn es nötig war. Besonderer Dank gilt Elke, die es immer wieder schaffte tot geglaubte Käfer wieder zu beleben, und Beate, die immer wieder versuchte mir die Welt der Computer näher zu bringen. Auch danke ich Birgit, Angelika, Helma, Barbara, Kathrin und Sabine, die immer zu ein kleinen netten Gespräch aufgelegt waren (oder auch zu andere Scherzchen), und damit ein wunderbare Arbeitsatmosphäre schafften.
Ein weiterer Dank gilt allen Bewohnern des Labor 3, auch jenen, die nur kurz vorbeikamen. Sie alle haben zu einem lebhaften, lustigen und turbulenten Arbeitsalltag beigetragen. Dabei möchte ich besonders Matthias, Georg, Inga, Sebastian und auch Monique danken.
Schließlich danke ich Jonas und Chris, die mich immer gut sicherten, wenn es darauf ankam.

Außerdem danke ich meinen Eltern und meiner Familie, die mich immer unterstützt haben und ohne die ich dies alles niemals erreicht hätte.

Aber der größte Dank gehört meiner Frau Sabine, einfach weil Du immer für mich da bist!

Vielen Dank!

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Summary

The brain is one of the most complex organs in animals. It allows the coordination of complex operations like orientation and directed movements. In insects, a structure in the brain has been identified that represents the superordinate center where such operations are processed. This center is called the Central Complex and encompasses five different subneuropilar structures in the adult insect brain. In *Schistocerca* embryos and *Drosophila* pupae, the respective progenitor cells, so called neuroblasts, were identified. However, only little is known about the molecular genetic background that enables the formation of the Central Complex. Respective embryonic genetic studies are not possible in *Drosophila,* since the Central Complex develops later during larval stages. In contrast, *Tribolium castaneum* larvae form a reduced Central Complex during embryogenesis which is easily accessible for genetic functional studies. Therefore, *Tribolium* was chosen to establish a new system to investigate embryonic insect brain development.

As a prerequisite for this aim, transgenic reporter lines were established, that allow studies of brain development in *Tribolium castaneum*. A transgenic line, where the regulatory regions of the *Tribolium* homologue to *reversed polarity* drives the reporter gene tGFP, was generated (reg. *Tc-repo*::tGFP). This line `reg. *Tc-repo*::tGFP´ appears to mark a subset of glial cells in the larval brain. Additionally, transgenic reporter lines, which should enable tracing of neuroblast development from the onset to the respective fully developed structure in the brain were generated. For this, various putative regulatory regions of the genes *Tc-six3* and *Tc-rx* were used to drive the reporter genes tGFP and DsRedExpress, respectively. The resulting transgenic lines where analyzed regarding embryonic expression as well as larval fluorescence patterns of the respective reporter genes. Thereby two lines were identified, which will be useful to study the development of a certain lineage. Furthermore, existing lines were characterized regarding their larval brain expression pattern. The promotor 6xP3 was shown to mark glial cells. Another line was shown to mark neural cells. Finally, a `Mushroom Body´ line derived from the GEKU screen marks the Mushroom Body neuropile. These lines were used in the second part of this work, where candidate genes were tested for a role in embryonic Central Complex formation in *Tribolium*. These analyses revealed that the genes *Tc-rx, Tc-chx,* and *Tc-six3* play important roles in different steps of the larval brain midline specification and Central Complex development in *Tribolium.*

Taken together, valuable tools, which allow investigations of the complex genetic network, which is needed for embryonic brain development in *Tribolium castaneum* were established in this work. Further, this system will allow identification of genes and studies of their function in embryonic

Central Complex formation in *Tribolium*. This is especially valuable, since the Central Complex develops only at later stages in *Drosophila*.

1. Introduction

1.1 Conservation of the bilaterian brain

The brain is the organ which filters, reacts to, and processes the stimuli of the environment as well as of the own body. Additionally, this organ enables complex activities like orientation, generalization, creation of strategies, and also communication.

The evolutionary origin of the central nervous system (CNS) in bilaterians has been controversially discussed. Its first appearance and its structure and function are not known. Even whether there is an independent evolutionary origin of vertebrates´ and invertebrates´ CNS has been debated (Arendt and Nübler-Jung, 1999; Holland, 2003; Lowe et al., 2003; Arendt et al., 2008). Recent data revealed a high degree of similarity of the molecular machinery building the brain of all bilaterian animals. This strongly indicates, that an Urbilaterian CNS precursor existed (Finkelstein and Booncinelli, 1994; Leuzinger et al., 1998; Loosli et al., 1998; Eggert et al., 1998; Acompora et al., 1999; Hirth and Reichert, 1999; Utting et al., 2000; Kammermeier and Reichert, 2001; Arendt et al., 2002; Sprecher and Reichert, 2003; Lichtneckert and Reichert, 2005; Tessmar-Raible et al., 2007; Denes et al., 2007; Arendt et al., 2008, Reichert, 2009; Hirth, 2010; Tomer et al., 2010; Steinmetz et al., 2010; Posnien et al., 2011).

The opinion that bilaterians CNS are homologous is based upon genetic patterning systems, which are conserved between vertebrates and invertebrates. There is the dorsoventral (dv) patterning mechanism, that specifies neurogenic and nonneurogenic tissue along the dv-axis. Vertebrates exhibit a dorsally located nerve cord, while invertebrates are characterized by a ventral nerve cord. Nevertheless, genes involved in dv-axis formation are homologues, like *short gastrulation* (*sog*, *Drosophila melanogaster*) and *Chordin* (vertebrates), *decapentaplegic* (*dpp*, *Drosphila melanogaster*) and *bone morphogenetic protein* 4 (*BMP4,* vertebrates), and feature conserved functions. The homologue genes *sog/Chordin* are expressed in the early embryonic neuroectoderm by inhibiting the antineural function of the *dpp/BMP4* signal. Thus, the functions of the homologue groups are conserved, albeit inverted regarding the dv-axis, which is thought to be the consequence of a body axis inversion in one of the two animal groups, Deuterostomia or Protostomia (Arendt and Nübler-Jung, 1994). Even the establishment of dorsal ventral polarity within the developing CNS is conserved. The *Drosophila* columnar genes *ventral nerve cord defective* (*vnd*), *intemediate neuroblast defective* (*ind*), and *muscle specific homeobox* (*msh*) are essential for the formation and specification of medial, intermedial, and lateral neuroblasts (NBs) along the anterior posterior axis (reviewed in Skeath and Thor, 2003). In vertebrates, the homologue genes *Nkx2*, *Gsh*, and *Msx* are involved in specifying the respective columns in the developing neural plate (reviewed in Arendt

and Nübler-Jung, 1999), which could be also shown for homologues in *Platynereis* (Denes et al., 2007).

Not only the developing nerve cord features conserved mechanisms, but also the anterior brain development of *Drosophila* and mouse. *Empty spiracles* (*ems*) is a cephalic gap gene in *Drosophila* and is broadly expressed in blastodermal stages during embryogenesis. Loss-of-function of the *ems* gene results in a gap-like phenotype in the brain (Hirth et al., 1995; Hartmann et al., 2000). Finally, this gene is required for the specification and formation of the anterior part of the embryonic brain in *Drosophila.* However, vertebrates have two orthologues *Emx1* and *Emx2*, which are expressed in the anterior region of the developing brain in the mouse. While a dysfunction of *Emx1* causes minor defects, a loss of *Emx2* leads to immediate death after birth (Bishop et al., 2002). In *Emx2* mutants, the anterior motor and sensory cortical areas are expanded caudally, whereas the posterior visual cortical areas are reduced in size (Hartmann et al., 2000; Bishop et al., 2002). Strikingly, rescue experiments with ubiquitous overexpression of *Emx2* in *ems* mutant flies demonstrated the conserved function of these genes among the phyla (Hartmann et al., 2000).

An additional functional conservation is demonstrated by the *otd/Otx* genes in embryonic brain development of *Drosophila* and mice. In *Drosophila, otd* is expressed anteriorily in the brain, while the anteriormost part of the neuraxis is devoid of *otd* expression (Younossi-Hartenstein, 1997). Mutation analyses revealed that most of the protocerebral and deuterocerbral neuroblasts are absent, which causes the absence of the entire anterior brain (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). In mice, two orthologues have been identified, *Otx1* and *Otx2*. Interestingly, these are expessed in the brain, but the most anterior part of the embryonic brain as well lacks *Otx2* expression (Simeone et al., 1992). A loss-of-function of *Otx1* leads to minor effects (Acampora et al., 1997), while *Otx2* mutant mice lack the rostral neurocetoderm (Acampora et al., 1995). Again, cross-phylum rescue experiments with the *Drosophila otd* cDNA sequence embedded by the 5´ and 3´ UTR of mouse *Otx2* restored the rostral brain in *Otx2* null mutant mice (Acampora et al., 2001). This supports the idea that *otd/Otx* function is conserved and derived from a common ancestor of vertebrates and insects (Lichtneckert and Reichert, 2005).

Apparently, many mechanisms important for the brain development are conserved between mice and insects. Thus it is very likely, that additional processes and molecular mechanisms in regionalization, specification, and formation of the embryonic brain are conserved. An additional argument for insect studies is the complexity of the vertebrate brain. The highly complex structure of the vertebrate brain complicates the research of genetic mechanisms of brain development. For example, the mammalian brain consists of billions neurons (reviewed in Luo et al., 2008), 1 mm^3

consists of 1 000 000 neurons, more than 4 km of axons, 500 m of dendrites, and more than 700 million synapses (Breitberg et al., 1998; Cardona et al., 2010). Compared to this, insect brains are not that large, for example *Drosophila melanogaster* has a small brain which develops from ~100 NBs per hemisphere. Finally, 200 000 neurons form the adult brain in *Drosophila melanogaster* (Younossi-Hartenstein et al., 1996; Urbach and Technau, 2003; Younossi-Hartenstein et al., 2006). Due to the lower complexity, it is much easier to study the general developmental processes of brain regionalization and specification in insect model organisms. Hence, in insect research it is easier to gain insight into the genetic network which is necessary for building such complex organs as the brain.

1.2. From neuroectoderm to neuropile

The development of the CNS in insects is a highly stereotyped process, starting with the sorting of neural and non-neural progenitors, which takes place during early embryogenesis. In *Drosophila,* the dorsoventral borders of the neuroectoderm get determined by the ventral to dorsal protein gradient of *Dorsal* (reviewed in Egger et al., 2008). On the one hand, the *Dorsal*-protein inhibits the expression of the gene *dpp* ventrally and on the other hand, low levels of this protein activate the expression of the gene *sog*, which outlines the presumptive neuroectoderm (reviewed in Egger et al., 2008).

The next step in CNS development is the determination of the neural stem cells which derive from neuroectodermal cells. Due to its relatively simple structure, the trunk neuroectoderm of *Drosophila* was chosen for studies of the processes and the underlying mechanisms regarding selection of single cells to become neuroblasts during the last decades. The neuroectoderm encompassing the ventral neurogenic region is structured by genetic patterning and segmentation cascades. I.e. the expression of pair rule, segment polarity, and hox genes determine the anterior posterior positions of NBs, while the dorsoventral axis is specified by the extracellular opponent protein gradients of *Dpp* and *Sog,* which together with EGFR signaling lead to the activation of the columnar genes (reviewed in Egger et al., 2008; Gilbert, 2003; Skeath and Thor, 2003, Stollewerk and Simpson, 2005). Eventually, the positioning of pronerual clusters and the specification of NB fate is finally based on the interaction of the pair rule, the segment polarity, and the columnar genes (reviewed in Stollewerk and Simpson, 2005).

The proneural clusters are groups of 5-7 equipotent cells, and are specified by the expression of the proneural genes *acheate* and *scute*. By this, all cells of such a cluster achieve neural competence (Skeath, 1999; Wheeler et al., 2003). However, only one cell of such a cluster is selected to become

a NB. This selection is enabled by juxtaposed cell-cell signaling and is called the lateral inhibition (e.g. reviewed in Skeath and Thor, 2003; Urbach and Technau, 2004; Pi and Chien, 2007). This is performed by the *Notch* pathway, which is involved in many other developmental processes, all of which have the decision between two different fates in common (reviewed in Beatus, 1998; Portin, 2002; Wheeler et al., 2008; Axelrod, 2010). The receptor *Notch* and its ligands *Delta* and *Serrat* are present at the cell surface of the equipotent proneural cluster cells. Stochastic changes in the representation of the levels of ligand and receptor among these cells enable the differentiation of the epidermal and neural fate. Meaning, high levels of *Notch* signaling inhibit the expression of the ligand and of the proneural genes in the surrounding and therefore lateral cells, which leads to an epidermal fate. At the same time, the selected proneural cell, which expresses high levels of the ligands of *Notch*, forms the neuroblast (e.g. Beatus and Lendahl, 1998; Pi and Chien, 2007; Axelrod, 2010).

In the ventral neurogenic region, after selection of neuronal fate, NBs delaminate in five temporal waves. Thus, 30 NBs derive per hemisegment, each forming a nearly invariant and unique cell lineage. The NB fate is specified by the position and the time of formation in the neuroectoderm (Urbach and Technau, 2004; Technau et al, 2005; Stollewerk and Simpson, 2006; Hartenstein et al, 2008). Shortly after delamination, these neuronal stem cells initiate asymmetrical and invariant cell division, thereby renewing themselves and creating a smaller daughter cell, the so called ganglion mother cell (GMC). The primary NBs divide 5-8 times in a stem-cell like manner, while the GMCs divide only once, 60-90 min after birth. By this final symmetrical division, the two daughter cells are differentiated postmitotically into glia and/or neuronal cells (Hartenstein et al., 1987; Hartenstein et al., 2008).

Similar to the trunk NBs, the formation of the brain NBs follows a reproducible pattern, where each NB is generated at a characteristic time (Urbach and Technau, 2004). For *Drosophila melanogaster,* it is known that 106 NBs are forming the brain. The protocerebrum takes the largest portion of NBs with 72, and gives rise to various neuropiles (see below). The deutocerebrum is associated with the antennal segment, where 21 NBs arise. Finally, the tritocerebrum encompasses 13 NBs in *Drosophila,* and represents the neuromere of the intercalary segment (reviewed in Urbach and Technau, 2003). While in the ventral neurogenic regions 30 NBs arise per hemisegment, ~100 NBs derive from the procephalic neurogenic region and form the brain (Younossi-Hartenstein et al., 1996; Urbach and Technau, 2003; Urbach et al., 2003). The ratio of cells with ectodermal and neuronal fate differs significantly between head and trunk neuroectoderm (Schmidt-Ott and Technau, 1994; Urbach and Technau, 2004). This is due to a less stringent lateral inhibition in the

procephalic neuroectoderm, where NBs, that derived from one pronerual cluster were identified (Urbach et al 2003; Urbach and Technau, 2003; Urbach and Technau, 2004;Technau et al., 2006). A further difference between ventral and proecephalic neurogenic region is that the brain NBs do not appear in waves, but rather continuously (Younossi-Hartenstein et al., 2006).

1.2.1. Specification of neuroblast identity

In *Drosophila,* the individual identity of the 30 NBs per hemisegment in the ventral neurogenic region is given by the spatial and temporal pattern of their delamination. The grid-like expression pattern of genes along the anterioposterior (segment polarity) and dorsoventral (columnar genes) axes subdivides the trunk hemisegment. Thus, NBs which arise in the respective quadrant of each hemisegment are serial homologues. Additionally, such serial homologues express the same combination of molecular markers and give rise to almost identical cell lineages (reviewed in Urbach and Technau, 2003; Urbach and Technau, 2004; Sprecher et al., 2007). Taken together, segment polarity and dorsoventral patterning genes in combination with temporal signals in the neuroectoderm explain how each arising truncal NB acquires an individual fate (Doe and Goodman, 1985; Technau et al., 2006)

However, the mechanisms of determining the different identities of the brain NBs are still unknown. Analogous expression of segment polarity genes and dorsoventral patterning genes occurs in specific domains in the procephalic neuroectoderm before NBs delaminate. Therefore it is discussed, that these genes are required for providing positional information and specification of brain NBs in analogy to the specification of trunk NBs (reviewed in Urbach and Technau, 2004). However, the segmental character, i.e. the expression of segment polarity genes and dorsoventral patterning genes, is less conserved for the procephalic neuromeres. Especially the protocerebrum comprises very little homology to truncal neuromeres compared to the deuterocerebrum and tritocerebrum. Due to this, the existence of serially homologous NBs in the anterior protocerebrum is less evident (Urbach and Technau, 2004; Technau et al., 2006). Nevertheless, the formation of NBs in the procephalic neuroectoderm follows a reproducible pattern. Urbach and Technau (2003) analyzed the expression pattern of more than 40 molecular markers, including proneural genes, segment polarity genes, dorsoventral patterning genes, and others, in the procephalic neuroectoderm of *Drosophila* (Figure 1). This study revealed that each of the ~100 brain NBs express a unique combination of transcription factors. This complex expression pattern is strongly suggested to support the machinery leading to the specification of individual NB fate, but the genetically relevant

network has not been identified yet (Urbach and Technau, 2003; Urbach and Technau 2004; Technau et al., 2006).

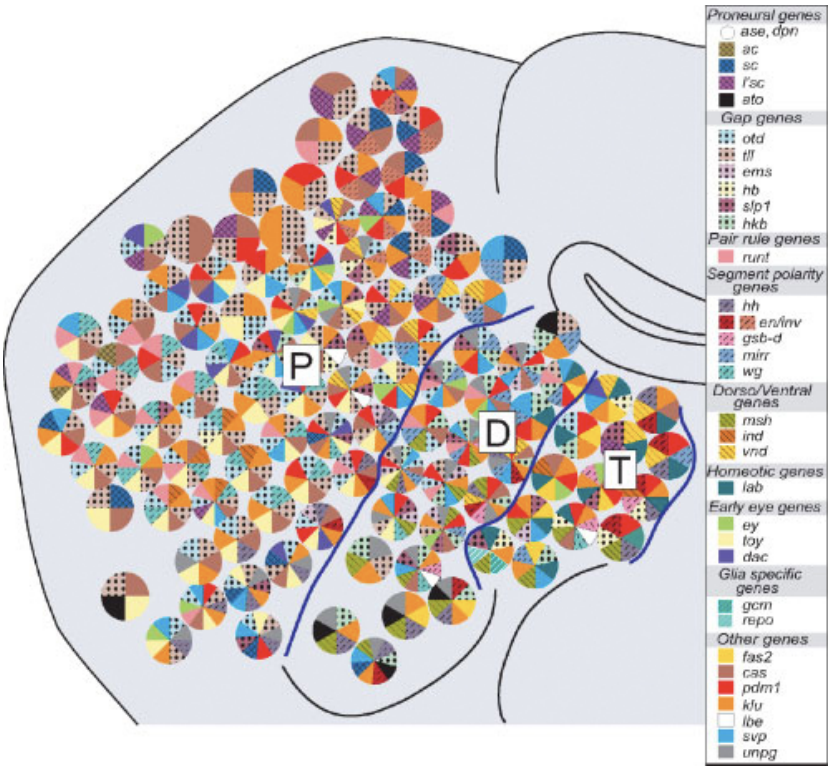

Figure 1: Combinations of marker gene expression reflect individual identities of brain NBs in *Drosophila*. Schematic presentation of about 100 NB populations of a *Drosophila* embryo. NBs are depicted as equal-sized circles at positions roughly corresponding to their positions *in situ*. More than 40 different molecular markers have been found to be specifically expressed in subsets of brain NBs. Depicted are 34 different genes, listed in the box on the right. Each brain NB reveals an individual combinatorial code of marker gene expression, which uniquely identifies each NB. Blue lines indicate the segmental boundaries between the tritocerebrum (T), deutocerebrum (D) and protocerebrum (P).(modified from Urbach and Technau, 2003; Urbach and Technau, 2004)

1.2.2. Neuroblast lineages

Tracing the development from NBs in the neuroectoderm of embryos to the respective neuropile in the adult brain was possible only in few cases so far. Younossi-Hartenstein and colleagues (2006) were able to establish a map of primary axon bundles of NBs and their progenies within the brain cortex of *Drosophila* embryos of stage 15 and 16. The progenies of NBs remain together in the cortex of the developing brain and form small cell clusters. Upon differentiation, they start axogenesis in a collective manner, therefore the neurons of one cone form a joint primary axon bundle (Younossi-Hartenstein et al., 2006). By mapping all bundles, it was possible to reveal which primary axon bundle contributed to which neuropile and partially from which NB in the cortex such bundles derived. This study enabled an investigation of the complex development of `macrocircuits´ in the developing embryonic brain, as well as the development from the larval to the adult brain of *Drosophila* (Younossi-Hartenstein et al., 2006; Hartenstein et al., 2008, Fung et al., 2009; Larsen et al., 2009; Spindler et al., 2009; Kumar et al., 2009; Cardona et al., 2009; Cardona et al., 2010). The first identified NBs which were localized in the neuroectoderm and functionally analyzed, were those of the Mushroom Body (MB). In previous studies, the NBs and their progenies could be

localized in the embryonic and larval brain, and it was shown that there were 4 NBs per hemisphere (e.g. Technau and Heisenberg, 1982; Ito and Hotta, 1992; Tettmani et al., 1997). Noveen and colleagues (2000) identified an important function of the genes *dachshund* and *eyeless* for the proper development of the Mushroom Body. Finally, they were able to trace the Mushroom Body NBs from the embryonic neuroectoderm to the adult Mushroom Body neuropile.

An additional NB lineage which could be traced from the embryo to the adult brain in *Drosophila,* was positive for *engrailed.* Kumar and colleagues (2009) could trace and analyze the *engrailed* positive serial homologue NBs within the procephalic neuromeres. By tracing these NBs to adulthood, they showed that *engrailed*-positive neurons of differing neuromeric origin do not project into neuromerically different compartments in the brain. Additionally, in embryonic, larval and adult stages, these neurons innervate the same neuropile compartments.

Thus, only few NBs could be traced from their origin within the procephalic neuroectoderm to the architectural structure within the adult brain so far. Especially the question of the genetic regulation of the protocererbral NB identity is still incompletely understood.

1.3. The insect brain

Already during the nineteenth century, the insect brain was studied and described based on investigations by bright field microscopy (e.g. Flögel 1876 and 1878; Weismann, 1884; Johansen, 1892; Wheeler, 1893; Kenyon, 1896). Here, the brain architecture was described morphologically. In the last decades, *Drosophila melanogaster* and *Schistocerca gregaria* were the most studied organisms regarding brain development and especially regarding the question which function can be attributed to the brain compartments (Olsen and Wilson, 2008; Hong et al., 2008; Johard et al., 2008; Pan et al., 2009; Heinze and Homberg, 2009; Young and Armstrong, 2010; Perenau et al., 2010; El Jundi et al., 2010; Zill, 2010; Ayali and Lange, 2010; Yu et al., 2010; Perenau et al., 2011; Träger and Homberg 2011).

The insect CNS derives from cells of the neuroectoderm, which consists of two major parts, the anterior procephalic neurogenic region and the ventral neurogenic region. While the ventral nerve cord derives from the ventral neurogenic region, the brain derives from the procephalic neurogenic region (reviewed in Technau et al., 2006). The procephalic neurogenic region comprises the pregnathal and gnathal segment part of the neuroectoderm. The brain is composed of two ganglions, the supraesophageal and the subesophageal ganglion, each consisting of three fused neuromeres. The subesophageal ganglion consists of the fused mandibular, maxillary and labial neuromeres. These neuromeres are structured similarly to those of the segmental ganglia of the ventral nerve

cord. The supraesophageal ganglion consists of the three neuromeres protocererbrum, deuterocerebrum, and tritocerebrum, which derive from the preocular and ocular region in case of the protocerebrum and from the antennal and intercalary segment for deutero- and tritocerebrum, respectively (Figure 1 and 2; Snodgrass, 1935; Bullock and Horridge, 1965; Burrows, 1996; Reichert and Boyan, 1997; Dettner et al., 2003). During head developmental processes in embryonic stages, the anterior most part of the neuroectoderm shifts to a dorsal position. As a consequence the anterior protocerebrum is located, relative to the body axis, dorsally in the head (reviewed in Reichert and Boyan, 1997), i.e. the neruaxis is bent with respect to the body axis (Figure 2; reviewed in Snodgras, 1935; Reichert and Boyan, 1997; Urbach and Technau, 2003).

As described above, the supraesophageal ganglion consists of three fused neuromeres, while the protocerebrum takes the largest portion of the brain. It includes the Optical Lobes, the Mushroom Bodies, and the Central Complex and various areas in the inferior, ventral, and superior protocerebrum (reviewed in Kurylas et al., 2008).

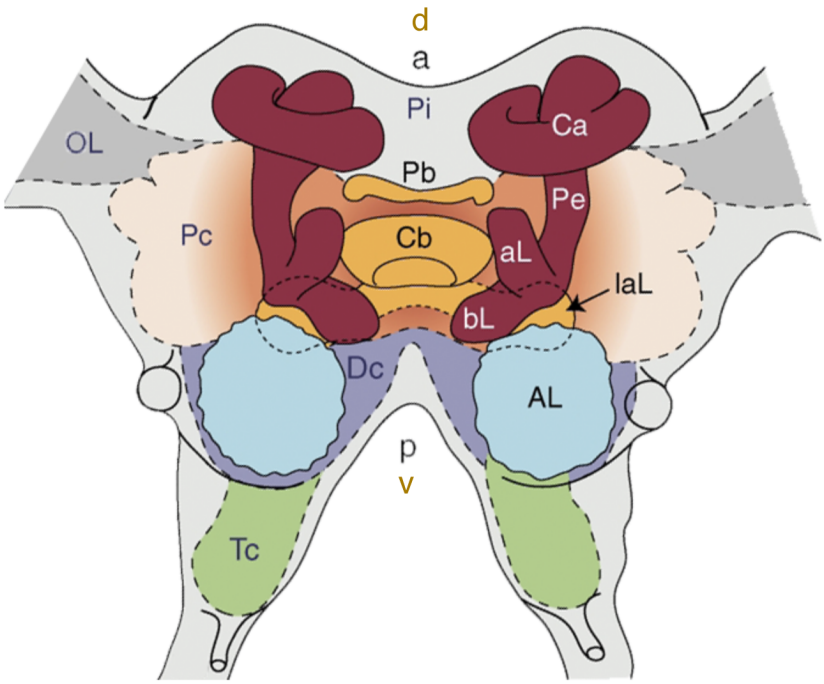

Figure 2: Scheme of an adult insect brain. The protocerebrum (Pc) encompasses the Mushroom Bodies (dark red) with the Calyx (Ca), Pedunculus (Pe), with median lobes ending in the beta lobe (bL) and the vertical lobe ending in the alpha lobus (aL). An additional unpaired neuropile in the Pc is the Central Complex (orange), which consists of the of the Protocerebral Bridge (Pb), the Central Body (Cb) and the lateral accessory lobes (laL). The Central Complex is mainly formed by neuroblasts located in the pars intercerebralis (Pi). The deutocerebrum (Dc) includes the Antennal Lobe (AL; light blue). The last neuromere is the tritocerebrum (Tc). The Optical Lobes (OL; dark grey) have been indicated, but are omitted in this scheme. Relative to the neuraxis anterior (a) is top and posterior (p) is bottom; relative to the body axis (depicted in brown) dorsal (d) is top and ventral (v) is bottom. (modified from Homberg, 1994; Urbach and Technau 2003).

The two Optical Lobes are the primary visual centers of the brain. Each Optical Lobe is divided into the lamina, medulla, and lobula complex. The lobula complex is subdivided into an anterior, a dorsal, an inner, and an outer lobe (Elphick et al. 1996). Visual projection areas in the median

protocerebrum include the anterior optic tubercles, consisting of upper and lower units. The lower unit is part of the polarization vision pathway (Homberg et al. 2003; Pfeiffer et al. 2005). The Mushroom Bodies represent a paired protocerebral brain structure which is found in all insects (Strausfeld et al., 2009). These neuropiles consist of axons of thousands of Kenyon cell fibers, which project through the peduncle (Pe) into the distinct lobes (median and vertical lobes). The calyces (Ca) of the Mushroom Bodies are mainly formed by dendrites of the Kenyon cells (Figure 2). The Mushroom Bodies are essential for olfactory learning and memory (reviewed in Fahrbach, 2006; Strausfeld 2009). The according NBs to this neuropile could be localized to the neuroectoderm as well as genetically characterized (see below).

The second prominent protocerebral neuropile is the Central Complex (CC; Figure 2). It is also the only neuropile complex within the insect brain, which spans the midline of the two hemispheres. The Central Complex comprises a group of neuropile modules: the Protocerebral Bridge (PB), the Central Body (CB) which consists of an upper and a lower unit, the noduli, and the lateral accessory lobes (laL) (reviewed in Homberg, 2008; Boyan and Reichert, 2011). The Central Complex is generated at different developmental stages in different arthropods. Hemimetabolous insects comprise a fully matured and developed Central Complex featuring all neuropilear modules at the end of embryogenesis (Boyan and Williams, 1997; Williams et al., 2005). Holometabolous insects, such as *Tenebrio molitor* possess a partially developed Central Complex at the end of embryogenesis, which successively develops postembryonically until the pupation (Wegerhoff and Breidbach, 1992). Only for the grasshopper *Schistocerca* and the fruit fly *Drosophila,* neuroblasts involved in the development of the Central Complex were identified (Boyan and Williams, 1997; Williams et al., 2005; Izergia et al., 2009). These NBs are located anterior median to the Mushroom Body, hence in the pars intercerebralis (Pi). In *Schistocerca,* 8 NBs, bilaterally in the brain, form clusters of progeny, which by their tracts contribute to the Central Complex (Boyan and Williams, 1997; Williams et al., 2005; Williams and Boyan, 2008; Boyan et al., 2008; Boyan and Reichert, 2011). Functionally, the Central Complex serves a role in sky compass orientation (Heinze and Homberg 2007), fine control of motoric behaviour (Martin et al., 1999; Loesel et al., 2002; Strauss, 2002; Homberg 2008, Poeck et al., 2008) and visual memory (Liu et al. 2006).

The neuromere of the antennal segment is the deutocerebrum. The Antennal Lobes are organized in globular structures, the so called glomeruli and serve as an olfactory integration center. The Antennal Lobes are connected to the calyces of the Mushroom Body and to the lateral horn of the protocerebrum. The tritocerebrum derives from the intercalary segment and is located posterior and ventral to the deutocerebrum. It connects the brain to the stomatogastric nervous system (reviewed

in Kurylas et al., 2008)

1.4. *Tribolium castaneum* as a model organism for studies of brain development

Currently, there are mainly two insect model organisms in which the development of the neural stem cells of the brain have been studied in detail, *Schistocerca gregaria* and *Drosophila melanogaster*. In *Schistocerca,* embryonic brain development was analyzed by morphological studies like immunohistochemistry, dye injection, BrdU incorporation, and Wigglesworth's osmium tetroxide-ethyl gallate histology (cell death; e.g. Boyan et al., 1995; Boyan and Williams 1997; Boyan et al., 2002; Ludwig et al., 2002; Wiliams et al., 2005; Boyan et al., 2010). Molecular and genetical investigations are difficult, since so far *Schistocerca* lack a sequenced genome. However, recently the transcriptome of the central nervous system in *Schistocerca gregaria* was analyzed and described (Badisco et al., 2011). Nevertheless, gene function analysis is still at the beginning and was first tested by RNAi in nymphal stages (Dong and Friedrich, 2005; Dong and Friedrich, 2010).

A very promising and highly developed morphological, molecular, and genetic model organism is *Drosophila melanogaster*. For this organism, many tools are available which allow studies of complex genetical specification and determination processes of the brain (e.g. Urbach and Technau, 2003; Edenfeld et al., 2005; Younossi-Hartenstein et al., 2006, Soustelle and Giangrande, 2006; Colonques et al., 2007; Berger et al., 2007; Spindler et al., 2009). However, the larval brain of *Drosophila* is reduced. For instance, this organism possess only a miniature Mushroom Body in the larval brain and lacks the Central Complex neuropile at this developmental stage (Boquet et al., 1999). Recent studies revealed that the onset of the development of the Central Complex in *Drosophila melanogaster* starts with the Protocerebral Bridge and the fan shaped body during the third larval instar. The Central Complex development in *Drosophila melanogaster* takes place during the larval to adult transition, most parts are formed and matured during puparium (Young and Armstrong, 2010). Thus, the genetic processes that specify cells forming a Central Complex are likely to be different to those during embryonic development. This means a different context and therefore, *Drosophila melanogaster* is unsuitable for investigating the embryonic signals required for Central Complex development.

However, a suited model organism to study this process is the red flour beetle *Tribolium castaneum*. Since its genome sequence is available (Richards et al., 2008) and the red flour beetle shows a robust and systemic RNAi response, functional analyses of genes by a knock down at any stage and in any tissue, is one of its largest potentials (e.g. Brown et al., 1999; Bucher et al., 2002; Tomoyasu et al., 2004; Posnien et al., 2009). The establishment of transposon mediated transgenesis in

Tribolium castaneum offers the development of tools to gain insight into gene functions, genetic control of genes, and the complex genetic network (Berghammer et al., 1999). The first large-scale insertional mutagenesis screen in an insect outside of *Drosophila* was performed by a consortium of different laboratories (GEKU), where various enhancer trap lines marked with eGFP were generated (see below; Trauner et al., 2009). In addition, imaging lines have been created and gene misexpression techniques have been established (Schinko et al., 2010). *Tribolium* has an insect typical head during larval stages (reviewed in Bucher and Wimmer, 2005; Posnien et al., 2010). Importantly, the larval brain of *Tribolium* posses parts of the Central Complex neuropiles, which are lacking in the larval brain of *Drosophila* (see below; Young and Armstrong, 2010). Therefore, *Tribolium castaneum* was chosen to study the genetic mechanisms of embryonic brain development, with the focus on the Central Body development.

1.5. The larval brain of *Tribolium castaneum*

Adult *Tribolium castaneum* beetles have an insect typical brain, featuring all neuropilear modules, which are described above (Dreyer et al., 2010). The first instar larval (L1) brain has not been investigated so far, due to technical limitations. However, after the generation of transgenic lines with distinct fluorescence signals in the brain, analyses regarding the architecture of the larval brain became easier. This was performed in parallel to this work in the laboratory of Joachim Schachtner (University of Marburg). Here, the first larval instar brain was studied regarding the morphology of the Mushroom Body, the Central Complex, the Optical Lobes, and the Antennal Lobes. Additionally, two transgenic lines were analyzed regarding the expression pattern and tissue specificity by colocalisation analyses. These lines were the GEKU screen derived `Mushroom Body´ line (Trauner et al., 2009; chapter 3.4) and the 6xP3::eCFP line (Hein, 2007; chapter 3.4).

In contrast to the adult brain, the composition of neuropiles in the larval brain differs among insect species. Hemimetabolous insects like *Schistocerca* have an insect typical brain already within the first larval instar, with all neuropiles described above (Bentley and Toroian-Raymond, 1981; Homberg, 2008). Holometabolous insect larvae often show reduced neuropilear structures, like in *Drosophila* where the Central Complex is absent in L1 brain, but gets formed during the last larval instar (Renn et al., 1999; Young and Armstrong, 2010). In others, neuropiles are reduced, like in *Tenebrio* where only the upper part of the Central Complex is present (Wegerhoff and Breidbach, 1992). The large differences in the presence of the Central Complex during larval or nymphal stages between holometabolous and hemimetabolous insects were discussed to be due to diverse efforts of locomotion in legless larvae (*Drosophila*) and larvae with legs (*Schistocerca, Tenebrio*). Kollmann

and colleagues showed, that the first larval instar brain of *Tribolium castaneum* mirrors the reduced situation. Furthermore, the larval brain is much more bent as the adult brain. The neuraxis in the larva is almost inversely parallel to the body axis, meaning the anterior part of the brain is localized posterior in the body, and vice versa the posterior part of the brain is localized anterior. Therefore, the descriptions of directions within the brain are given according to the directions of the body axis (Figure 3).

In the *Tribolium* larval brain, the Mushroom Body is already present with its typical projections of the Pedunculus (Pe) and its branching lobes (median, mL, and vertical, vL; Figure 3), while the Central Complex is only partially formed. As described above, the Central Complex is comprised of five modular neuropiles, the Protocerebral Bridge, the Central Body consisting of upper and lower unit, the noduli, and the lateral accessory lobes (reviewed in Boyan and Reichert, 2011). In the larval brain, the noduli and the lateral accessory lobes were not detected. The Protocerebral Bridge is not fused medially and is wedge shaped, whereas also a glial envelope was not detected (Figure 3). The Central Body is reduced, similar to that in *Tenebrio* larval satges. In *Tribolium,* it consists of one flattened structure, which by respective antibody staining was suggested to be the reduced upper unit (Figure 3; c.f. chapter 4.3.4). The Optical Lobes in larval stages are reduced, therefore they are called the anlagen of Optical Lobes (A-OL). The larval Antennal Lobes are organized in globular structures, like in the adult brain (Figure 3).

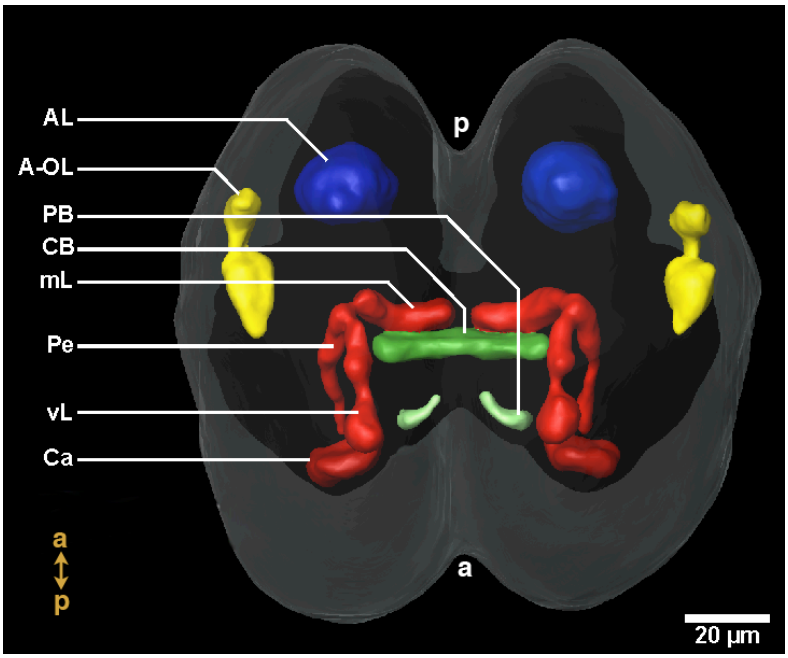

Figure 3: The larval brain of *Tribolium castaneum* (Martin Kollmann, University of Marburg).
The neuraxis (anterior: a; posterior: p; white letters) is inverted according to the body axis (brown letters; a and p). The larval brain comprises the Antennal Lobes (AL), the anlagen of the Optical Lobes (A-OL), the upper unit of the Central Body (CB), the paired Protocerebral Bridge (PB) and the Mushroom Bodies, with the calyx (Ca), the Pedunculus (Pe), and the branching median and vertical lobes (mL; vL).

The G 11410 enhancer trap line, which is derived from the GEKU screen, showed a prominent reporter gene expression in the larval Mushroom Bodies. The specificity of the tGFP expression pattern in the Mushroom Bodies was confirmed by a colocalisation assay with the antibody DcO (Farris and Strausfeld, 2003), therefore this line is further called `Mushroom Body´ line (chapter 3.4). To confirm the assumption, that the cells positive for the 6xP3::eCFP signal are glial cells, immunohistochemistry stainings were performed with an antibody against the glial marker *reversed polarity* (anti *Repo*; Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994; Lee & Jones 2005). These analyses revealed that 98,8% of the cells positive for the eCFP signal are colocalized with the glial marker *Repo*, which suggests that the 6xP3::eCFP signal marks glial cells (Kollmann and Schachtner, unpublished).

1.6. Candidate genes for NB identity specification in the insect brain

The genes which were selected for analysis of their function in the brain development of *Tribolium castaneum,* are known to be expressed in the embryonic head anlage or even to play an important role in head development. The expression pattern and function of *Tc-six3* and the homologues *Tc-otd1* and *Tc-otd2* were known from previous studies (Schröder, 2003; Posnien, 2006; Schinko et al., 2008; Posnien, 2009) and all these genes of interest are conserved and are required for proper brain development in other species.

Tc-six3 is a transcription factor, which encodes a homeobox and a Six domain and is the homologue to *SIX3* in vertebrates or to *Optix* in *Drosophila*. This gene is conserved in bilateria and marks the anterior-most region of the developing brain (Oliver et al., 1995; Steinmetz et al., 2010). It is known from vertebrates including humans, that a loss of function of this gene leads to severe phenotypes, the so called holoprosencephaly (HPE). Here, the median interhemispheric division and structures are affected (Pasquier et al., 2000; Dubourg et al., 2007; Domené et al., 2008; Salomon et al., 2009). In *Tribolium castaneum,* comparable phenotypes lacking the median head region and the labrum were generated by knock down of *Tc-six3* by parental RNAi (Posnien, 2006). An overexpression of the *six3* homologue in zebrafish lead to an enlargement of the rostral brain (Kobayashi et al., 1998). Analyses in *Xenopus* and zebrafish revealed that *Six3* itself is unable to induce neural tissue and overexpression promotes cell proliferation (Gestri et al, 2005). In *Drosophila,* an overexpression of *optix* leads to ectopic eye formation (Seimiya ad Gehring, 2000). Thus, *six3* homologues are involved in neural plate and brain specification, development of neuroendocrine systems, and eye development (e.g. Loosli et al., 1999; Seimiya and Gehring, 2000; Posnien, 2006; Steinmetz et al., 2010).

The gene *orthodenticle* belongs to the family of paired-like homeodomain transcription factors. Homologues were identified in various species like *Drosphila melanogaster, Ceratitis capitata, Anastrepha suspensa, Nasonia vitripennis, Parhyale hawaiensis, Euscorpius flavicaudis, Tegenaria saeva, Xenopus laevis, Mus musculus,* and others (Finkelstein et al., 1990; Simeone et al., 1993; Andreazzoli et al., 1997; Schröder 2003; Lynch et al., 2006; Browne et al., 2006; Schetelig et al., 2008). The *otd* homologues play a crucial role in head segmentation, brain regionalization and also photoreceptor development (Finkelstein et al., 1990; Simeone et al., 1993; Frantz et al., 1994; Hirth et al., 1995; Andreazzoli et al., 1997; Leuzinger et al, 1998; Blanco et al., 2009). In *Drosophila melanogaster, otd* belongs to the head gap genes and loss of function analyses revealed a loss of protocerebrum and anterior parts of the deuterocerebrum in brain development (Leuzinger et al., 1998). An additional putative function of *Drosophila otd* was the specification of progenies of mesectodermal neuroblasts in the ventral nerve cord (Finkelstein et al., 1990). In *Tribolium castaneum,* the two paralogue genes *Tc-otd1* and *Tc-otd2* were identified. *Tc-otd1* is required for the anterior embryonic patterning and it was suggested to substitute the anterior patterning system together with *Tc-hb* in short germ insects such as *Tribolium*, in parallel to the *bicoid* morphogene function in *Drosophila* (Schröder 2003; Schinko et al., 2008; Posnien, 2009). In additional studies, *Tc-otd1* function was revealed to be involved in dorsoventral axis formation in early embryonic stages of *Tribolium castaneum* (Kotkamp et al., 2010). A function in brain development in *Tribolium castaneum* was suggested, but could not be proven so far (Posnien, 2009).

Also *Tc-chx* is a transcription factor with a homeodomain and a homologue gene to *CHX/VSX,* which is conserved among the phyla. Furthermore in *Drosophila*, it is known to mark the pars intercerebralis (PI), the region which is known to give rise to neuroendocrine system and the Central Complex in the insect brain (Boyan and Williams, 1997; de Velassco et al., 2007). In *Tribolium,* it was suggested to be a target gene of *Tc-six3* (Posnien et al., unpublished). Dysfunction of *CHX10* in vertebrates is known to cause malformations in the visual system, in *Drosophila* a similar function was identified for the homologous *Vsx1* and *Vsx2* genes (Percin et al., 2000; Erclik et al., 2008; Gonzalez-Rodriguez et al., 2010). In *Tribolium castaneum,* one *Tc-chx* homologue was identified (Posnien, 2009) and a function in brain development has not been described so far.

The transcription factor *retinal homeobox* (*Rx/rax*) was originally discovered in vertebrates, where it plays a role in eye and brain development. The homeobox *rx* or *Rax* is conserved throughout various species like *Drosophila*, *Platynereis*, zebrafish, chicken, *Xenopus*, mouse, and humans (Eggert et al., 1998; Chuang et al., 1999; Ohuchi et al., 1999; Tucker et al., 2001; Tessmar-Raible et al., 2007). It encodes for a transcription factor featuring an N-terminal octapeptide domain, a

homeodomain, a *Rx* domain, and a C-terminal orthopedia-aristaless-*rx* domain (Eggert et al., 1998; Mathers et al., 1997, Davis et al., 2003). It is known to play major roles in eye development in vertebrates (Mathers et al., 1997; Voronina et al., 2004; Loosli et al., 2003; Rojas-Munoz et al., 2005). Homologues of *rx* were also discussed to be involved in forming the neuroendocrine system in *Drosophila, Platynereis, Zebrafish,* and other animals (Hartenstein, 2006; de Velasco et al., 2007; Tessmar-Raible et al, 2007).

1.7. Aims of the study

Before this work, functional studies of the larval brain were not feasible in *Tribolium castaneum* and therefore two aspects are encompassed: 1) Establishment of *in vivo* imaging systems for the analysis of embryonic brain development, in order to allow visualization of brain phenotypes and 2) functional analyses of several candidate genes with respect to brain development.

As a prerequisite for the analysis of NB lineages, transgenic lines marking a subset of NBs and their progenies should be established and characterized. Due to the exclusive anterior expression and their conservation in vertebrates, the NBs expressing the genes *Tc-rx* and *Tc-six3* were chosen. Various reporter lines, carrying upstream and downstream regions of the respective genes, were to be created. These lines should enable tracing of the development from the NB to the respective structure in the larval brain.

For analyzing the brain of animals in WT and knock down conditions, imaging lines, which mark neural, glial, and other specific structures of the brain were to be created and used. To this end, imaging lines driving various reporter genes under the control of the regulatory region of *Tc-embryonic lethal abnormal vision (elav)* and *Tc-reversed polarity (repo)* were to be created. Furthermore the existing lines `Mushroom Body´, derived from the GEKU screen, `6xP3::eCFP´, created from H. Hein (2007), and `EFII::DsRed´, created by M. Averof, were supposed to be characterized regarding their expression pattern in the larval brain. These lines allowed functional studies with genes, which by their anterior median expression were likely to contribute to central complex development. Those genes were *Tc-rx, Tc-otd1, Tc-six3,* and *Tc-chx.*

Taken together, this work aimed at providing a basis for the investigation of the complex genetic network of embryonic brain and Central Complex development in *Tribolium castaneum.*

2. Material and Methods

2.1. *Tribolium castaneum* strains used as source of genomic DNA and for embryonic transgenesis

For the amplification of regulatory regions, the isolated genomic DNA of animals of the San Bernardino (SB) strain (Richards et al., 2008) was used. For transgenesis, the embryos of the white-eyed Tc-vermilion line were used (Berghammer et al., 2009).

2.2. Amplification of the 5´UTR by RACE-PCR

For RACE experiment, the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) was used in combination with the Advantage 2 PCR Kit (Clontech Laboratories, Inc.). When generating first strand 5´-RACE Ready cDNA, the instructions of the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) were followed. The respective mRNA was isolated following the instructions of the mRNA Isolation Kit (Roche). For the amplification of the 5´UTRs the instructions of the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) were followed. The manufacturer´s instructions for the kits were followed regarding PCR-programs. In case of the *Tc-elav* 5´UTR, a semi nested PCR was performed, where 1µl of the PCR products of the first RACE-experiment were used as template for the consecutive. The PCR-program was for the gene specific primer (Table 1) under 70°C, additionally the gene specific primers for the second RACE-PCR were of the same primer batch. For *Tc-rx* and *Tc-six3* external and nested primers were designed. One set of primers allowed successful amplification of the 5´UTR of *Tc-rx* in two independent approaches (Table 1). With two sets of primers it was possible to amplify the 5´UTR of *Tc-six3* in independent approaches. Independent approaches here means the use of two different RACE 5´-cDNA pools.

Table 1: Primers used for 5´UTR amplification

gene/primername	RACE primer (5´-3´)	temperature
*Tc-elav/*RACE5_elav	CCATTCTGTTGTCCAACAACCCCCGAGT	69,0 °C
*Tc-rx/*rx_RACE3	CGAGTCCGAGTATGTTGTCGATGGTGTGCCTG	72,3 °C
*Tc-rx/*rx_RACE5	CGATGGTGTGCCTGGGTCCGGTTCC	72,7 °C
*Tc-six3/*Six3_RACE5b	GAAGTTGCCGCTGTGGAAGCAGA	66,5 °C
*Tc-six3/*Six3_RACE4	GCCACCGGCAGCGACCAGA	68,8 °C
*Tc-six/*Six3_RACE7	GCGGAAGTTGCCGCTGTGGAAGCAGACGATG	73,9 °C
*Tc-six3/*Six3_RACE6	GGTGGGCCACCGGCAGCGACCAGA	74,8 °C

2.3. Amplification and cloning of regulatory regions

Regulatory regions of the genes *Tc-repo, Tc-elav, Tc-rx,* and *Tc-six3* were amplified from gDNA isolated from animals of the San Bernardino strain. Isolation of gDNA was performed after Trauner and colleagues (2009). The primers included recognition sites for restriction enzymes cutting externally of their recognition sequence, thereby generating new compatible ends for cloning (small letters, Table 2). Amplified sequences were cloned into the Dual Promoter pCR®II vector by using the TA Cloning® Kit (Invitrogen). The final constructs were designed with the following sections from 5´ to 3´: 1) regulatory regions, 2) endogenous promoter, 3) reporter gene (Table 2). Care was taken to keep the linker sequence free of additional translation start sites (ATG). In case of the regulatory regions of *Tc-repo, Tc-elav,* `*Tc-rx* 5up´, and `*Tc-six3* 5up´, the amplification was performed including the endogenous promoter sequence. The respective reporter genes were amplified from other constructs by using according primers (Table 3). The constructs were designed and created in the vector pslfa1180fa. Afterwards, the cassette including the regulatory region, promoter, reporter gene, and SV40 was transferred into the *piggy*Bac[3xP3; Tc-ver; SV40]fa transformation vector by using the restriction enzymes AscI and/or FseI (New England BioLabs, Inc.). The 5´-ends of the regulatory regions and the promotor-reporter gene-region of achieved constructs were sequenced at Macrogen (Korea) using the primers listed in Table 4.

Table 2: Primers used for the amplification of regulatory regions including restriction sites

regulatory region	forward primer (5´-3´)	backward primer (5´-3´)	restriction enzyme	fragment size [bp]	reporter gene
Tc-repo	GTCAGTCCTTTGTGAAATC ACGG	*cgagctc*GATTGGCAATTGCTT AATGAACC	Sac I	4287	tGFP
Tc-elav	CGCTGAAAAATTAATAAGG ACCC	TCTAGAACTTCCTCAATGG TGGC		2551	mRFP
Tc-rx Prom	acggatccTCCTCTAATTATAGC TGAATACAGAGCCGA	atctacgctagcCTTCACAACGGT CCGATTCTATCGC	BamHI, NheI	625	
Tc-rx 10up	acctgaggACAGCAGCGACTC GTCACTCTGA	acggatccATTTGATAACAACG GCAACATACGTCGACA	Eco81I, BamHI	4757	DsRedEx
Tc-rx 5up	acggatccGGGATCAAGCGTAA ATGGGACGTCCCATACAATA	atctacgctagcCTTCACAACGGT CCGATTCTATCGC	BamHI, NheI	6090	DsRedEx
Tc-rx Intron	acctgaggCACCGTAATTACGC AAAGATAACAGACTCCA	acggatccCAGGGCGGTAATCA AGCCGACA	Eco81I, BamHI	7398	DsRedEx
Tc-six3 Prom	cacagatctGTGTACACCGGTCC TAAAGGTTTGAAATGA	cacggtaccATGGGTTGCCAGC TTGGGCTAGTGCACCATC	BglII,KpnI	657	
Tc-six3 10up	accgtctcggatccTCCATGCCCAT GGTGAATGGAGCACA	accgtctcagatctCTGACAAGTG CCGACAACGCTATTATCATA	Esp3I/BamHI, Esp3I/BglII	5416	tGFP

regulatory region	forward primer (5´-3´)	backward primer (5´-3´)	restriction enzyme	fragment size [bp]	reporter gene
Tc-six3 5up	cacagatctTTGGGCACCCGGA ACATTTCTCGAGCAATA	cacggtaccATGGGTTGCCAGC TTGGGCTAGTGCACCATC	BglII,KpnI	4133	tGFP
Tc-six3 5down	acgaagacaggatccGATGCGAGA TGGAGCAGATTAATCACTA	acgaagacgagatctCGCCACTGA CTTGCTCTCTGAG	BpiI/BamHI, BpiI/BglII	4373	tGFP
Tc-six3 10down	accgtctcggatccAAGGATTCGA CAAATTCGCTGTCAGAC	accgtctcagatctTTCTGAAGATT CACGATGACTTGCTCTGTA	BpiI/BamHI, BpiI/BglII	5246	tGFP

Table 3: Primers for the amplification of reporter genes

reportegene	froward primer (5´-3´)	backward primer (5´-3´)	size	annealing temperature
tGFP	ATGGAGAGCGACGAGAGC	ATTCTTCACCGGCATCTGCATC	697 bp	62 °C
DsRedExpress	ATGGCCTCCTCCGAGGA	CTACAGGAACAGGTGGTGG	678 bp	62 °C
mRFP	ATGGCCTCCTCCGAGGAC	ATTAGGCGCCGGTGGAGTG	679 bp	64 °C

Table 4: Sequencing primers of the pre-reporter gene regions

constructs	pre-reporter gene region (5´-3´)
for *Tc-six3* reporter lines	GGTGCTCTTCATCTTGTTGGTCA
for *Tc-rx* reporter lines	CTTGAAGCGCATGAACTCCTTGA
for *Tc-six3* overexpression	CGTACGGTATATATCACGTCAGTCT

2.4. Whole mount *in situ* hybridization

WT embryos (SB-strain) were used for Neuroblast (NB) quantification and for assembling the endogenous expression pattern of the genes *Tc-chx* and *Tc-repo*. For NB quantification, staged egg lays were used of slots of 12-18h, 18-24h, and 24-30h old embryos. Native expression pattern analyses were performed with staged egg lays of 0-24h, 24-48h, and 48-72h.

The generated reporter lines were analyzed by *in situ* hybridization with an antisense probe against the respective reporter gene, and staged egglays were used (0-24h, 24-48h, and 48-72h). In all cases, embryo rearing was performed at 32°C. Single (NBT/BCIP) and double *in situ* stainings (NBT/BCIP and INT/BCIP) were performed as described previously (Schinko et al., 2009). For the used probes of genes see Table 5.

Table 5: Antisense probes of genes used for *in situ* hybridization

Gene	Length [bp]	Labeling
Tc-ase	737	Dig/Flu
Tc-chx	787	Dig/Flu
DsRedEx	678	Dig
Tc-repo	920	Dig
Tc-rx	827	Dig/Flu
Tc-six3	1394	Dig/Flu
tGFP	697	Dig
Tc-wg	1100	Flu

2.5. Embryonic and pupal knock down of gene function by RNAi

Templates were prepared by PCR with T7-primers from plasmid templates of full length *Tc-six3* (1394 bp), *Tc-otd1* (1116 bp), *Tc-chx* (787 bp), and *Tc-rx* (827 bp) (Posnien et al., 2009). DsRNA was produced using the Megascript T7 Kit (Ambion). Final dsRNA concentrations between 2-5 µg/µl were diluted to the desired concentration between 100 ng/µl and 3,8 µg/µl, whereas an aberrance of 10 % was accepted (Table 6).

For embryonic RNAi, the protocol of Berghammer et al. (2009) was used as orientation (see variations in 2.6 embryonic injections). Embryos were collected for 1-2h, the respective beetle strains were kept on 32°C. Breeding for the various ages was performed at 32°C and embryos were finally injected with *Tc-six3* and *Tc-otd1* dsRNA (Table 6; *Tc-otd1*: 3.2.2; *Tc-six3*: 3.2.3). Pupal RNAi was performed according to Posnien et al. (2009) and for this, various dsRNA and concentrations were used to inject female pupae (see Table 6).

For analyzing RNAi efficiency, cuticles were scanned for malformations and defects. Cuticle clearing of the respective embryos was performed in a 1:1 Hoyer`s medium (lactic acid) at 65°C over night.

Table 6: Embryonic (emb.) and pupal (pup.) RNAi of different genes

dsRNA of gene	concentration	emb. RNAi	pup. RNAi
Tc-six3	100ng/µl		X
	1µg/µl	X	X
	2µg/µl		X
Tc-otd1	1µg/µl	X	

dsRNA of gene	concentration	emb. RNAi	pup. RNAi
Tc-chx	1,9µg/µl		X
	3,8µg/µl		X
Tc-rx	1µg/µl		X

2.6. Embryonic transgenesis

For transgenesis, embryos of the white-eyed Tc-vermilion-white strain were used. The embryos were collected at 25 °C for 1h and further reared for 1h at 25 °C. At the time of injections, embryos were aged 1-2h at 25°C. For injections, embryos were bleached for 1,5 min in 1% Danklorix (Colgate Palmolive GmbH). Constructs used for injections included the positive selection marker 3xP3::*Tc-vermillion*, rescuing black eyes in *Tc-vw* background, or 3xP3::eGFP, causing green fluorescent eyes (Table 7). To maintain a humid atmosphere, injected embryos were reared on apple agar plates at 32°C. Further steps and treatments were performed as described (Berghammer et al., 2009). The heat shock dependent *Tc-six3* over expression construct (Table 7) was designed and cloned by Schanda (2007), but was injected and analyzed in this work.

Table 7: Injected constructs and their positive selection marker

injected constructs	positive selection marker
pBac[3xp3,g-Tc`v,SV40; **Tc´repo**,tGFP,SV40]	3xP3::Tc-vermilion
pBac[3xp3,g-Tc`v,SV40; **Tc´elav**,mRFP,SV40]	3xP3::Tc-vermilion
pBac[3xp3,g-Tc`v,SV40; **Tc´rx-5up**,DsRed-Ex,SV40|	3xP3::Tc-vermilion
pBac[3xp3,g-Tc`v,SV40; **Tc´rx-10up**, rx-Prom; DsRed-Ex,SV40|	3xP3::Tc-vermilion
pBac[3xp3,g-Tc`v,SV40; **Tc´rx-Intron**, rx-Prom; DsRed-Ex,SV40|	3xP3::Tc-vermilion
pBac[3xp3,g-Tc`v,SV40; **Tc´six3 5 up**, tGFP, SV40]	3xP3::Tc-vermilion
pBac[3xp3,g-Tc`v,SV40; **Tc´six3 10 up**,Prom,tGFP,SV40]	3xP3::Tc-vermilion
pBac[3xp3,g-Tc`v,SV40; **Tc´six3 5 do**,Prom,tGFP,SV40]	3xP3::Tc-vermilion
pBac[3xp3,g-Tc`v,SV40; **Tc´six3 10 do**,Prom,tGFP,SV40]	3xP3::Tc-vermilion
pBac[3xp3, eGFP,SV40; hsp5´UTR, DsRedEx, 3´UTR; hsp5´UTR, **Tc´six3 ORF**, 3´UTR]	3xP3::eGFP

2.7. Larval brain *in vivo* imaging

For scanning larval brains of transgenic lines, offspring was collected for 1-2 d at 32°C. Breeding was allowed for 3,5-4d at 32°C. Embryos were bleached for 2,5 min in 1% Danklorix (Colgate Palmolive GmbH) and cleaned with water. To avoid dehydration during preparation, the mesh with embryos was kept on apple agar plates. In case of embryonic RNAi, animals were treated as described above (chapter 2.5).

Preparation of the larvae differed depending on the previous treatment. In case of transgenic lines, larvae hatched independently and only freshly hatched larvae were collected and prepared. In general, strongly affected RNAi treated animals did not hatch. By using the Leica MZ16FA fluorescence stereomicroscope, animals being close to or being hatching could be identified by two criteria: 1) a deformed egg shape, which was caused by the effort of hatching, and 2) embryos reacted with strong movements when touched. Animals not showing these characteristics had not concluded processes like dorsal closure or cuticle secretion. Embedding such animals in 80% glycerol (Sigma-Aldrich) immediately lead to dehydration and denaturation of tissues. For those close to hatching, the vitelline membrane was removed by using fine needles and eyelash. To facilitate preparation, embryos were transferred into a drop of 80% glycerol.

To avoid crushing the larval brain while scanning, two file reinforcement rings glued on top of each other were used as spacer between slide and coverslip. They were cut into quarters and fixed on the object slide (Dippel personal communication; Dreyer et al., 2010). The distance between the pieces was chosen according to the size of the coverslip (12x12 mm). The larva was arranged dorsal up in the center of an object slide prepared this way. After covering, the larva was fixed in this position by slight pressure on the cover slide during embedding with 80% glycerol from the side.

An alternative preparation was used for more fragile animals. Those were prepared within the vitelline membrane and standing on the abdomen. Forcing them into position, they were embedded by surrounding them with small pieces of apple agar, keeping only the head free in order to avoid signal disturbance during the scan. The corners of the coverslip were prepared with plasticine to avoid crushing the embryo or larva, respectively. Drops of 80% glycerol were given right on top of the head before covering.

In vivo brain scans were documented with a Zeiss LSM 510 and processed using the Zeiss LSM Image Browser (Version 4.2.0.121). Z-projections of substacks sized 10-40 slides were prepared using ImageJ (version 1.40 g) with the algorithm “average intensity“.

2.8. Immunohistochemistry on larval brains

Larval brains of the transgenic line `Brainy´ (see below, 3.1.4) were dissected using a Zeiss SteREO Lumar V12. When selecting embryos for brain dissections, the same criteria as in 2.7 were chosen. Brains of embryos almost hatching were considered to be fully developed like L1 brains.

`Brainy´-WT and -RNAi larvae were transferred into a drop of cold PBS on a cover slide. Here, the abdomen was cut between the thoracic segment T3 and the abdominal segment A1. Afterwards, the brain was pressed out of the head capsule by fixing the anteriormost tissue with one pair of forceps and slightly pushing the brain to the abdominal opening with another pair of forceps. Dissected brains were collected in cold 4% formaldehyde (Roth, Karlsruhe)/PBS up to 60 min, followed by fixation for 3h at room temperature (RT). For washing and rinsing, brains were transferred into the solutions using a 10µl pipette. They were rinsed three times at RT with PBS for 10 min, and afterwards the primary antibody incubation occurred at 4°C over night. The incubation solution included 5% normal goat serum (NGS; Jackson ImmunoResearch), PBS containing 0,3% Triton X-100 (PBT; Sigma-Aldrich), synapsin antibody, Alexa Fluor 488-coupled phalloidin (Molecular Probes®), and the according primary antibody (see below). The brains were rinsed three times in PBT for 10 min, followed by incubation with the secondary antibodies (see below) over night at 4°C. Afterwards, brains were washed three times in PBT for 10 min and finally embedded in 80% glycerol. To avoid compression of brains, reinforcing rings were used as spacers (see 2.7). Fixation of cover slide was achieved with nail polish (essence multi dimension, Cosnova GmbH).

Primary antibodies and concentrations used: 1:100 of monoclonal primary mouse antibody against synapsin (3C11, #151101 (13.12.06); Klagges et al., 1996; Utz et al., 2008; Dreyer et al., 2010). 1:10000 of polyclonal antiserum of rabbit against the biogen amin serotonin (5HT; DiaSorin; decribed in Dacks et al., 2006). 1:5000 of rabbit antiserum against neuropeptide *Periplaneta americana* myoinhibitory peptide (Pea-MIP, GWQDLQGGWamide, described by Predel et al., 2001). 1:200 rabbit antiserum against the catalytic subunit of the protein kinase A (PKAc) of *Drosophila melanogaster* for specific visualisation of Mushroom Body (Lane and Kalderon, 1993; Farris and Strausfeld, 2003).1:200 of Alexa Fluor 488-coupled phalloidin (Molecular Probes®).

Secondary antibodies and concentrations used: 1:300 of goat anti-mouse antibodies conjugated to either Cy5 (GAM Cy5) or Cy3 (GAM Cy3) (Jackson ImmunoResearch). 1:300 of goat anti-rabbit antibodies conjugated to either Cy5 (GAR Cy 5) or Cy3 (GAR Cy3) (Jackson ImmunoResearch). Antibodies were chosen to prevent overlapping emission, e.g. GAM Cy5 and GAR Cy3.

2.9. Heat shock in water bath

Embryos of the strain carrying the heat shock construct were reared before and after the heat shock (HS) at 25°C to avoid a latent over expression. Heat shock was performed as described by Hillebrand (2010), i.e. 10 minutes in a water basin prewarmed to 46°C. For this, embryos were transferred into a 15ml tube (120x17mm Sarstedt). Afterwards, breeding was allowed at different temperatures depending on the experiment. In case of *in situ* hybridization experiments, rearing was allowed after the HS at 25°C until the respective stage was reached and then the embryos were fixed (chapter 2.5; Posnien et al., 2009). In case of cuticle analysesrearing was allowed either at 25°C or at 32°C until the cuticle preparation (chapter 2.5 and chapter 3.1.1).

2.10. Documentation

In vivo brain scans were documented with a Zeiss LSM 510 and processed using the Zeiss LSM Image Browser (Version 4.2.0.121). Cuticles were documented using a Zeiss Axioplan microscope to record stacks of 10-20 plans. Deconvolution was performed by the “No Neighbour” method of the ImagePro software (Version 6.2; MediaCbernetics). Z-projections were made using the ImageJ (version 1.40 g) for cuticle images with the algorithm “maximum intensity“ and for *in vivo* brain projections either with "maximum intensity" or “average intensity“.

2.11. Used computer programs

NCBI/Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and "Blast against Baylor Data" (www.hgsc.bcm.tmc.edu/blast.hgsc) were used for obtaining and analyzing the regulatory regions of the genes described above. The Operon Oligo Analysis Tool (www.operon.com/technical/toolkit.aspx) and "OligoCalc: an online oligonucleotide properties calculator" (http://www.basic.northwestern.edu/biotools/oligocalc.html) were used for primer analysis. The APE-A plasmid Editor (v1.14), was used for sequence analysis and as a computational cloning tool. The MultAlin online tool was used for "Multiple sequence alignment with hierarchical clustering" F. Corpet, 1988 (http://multalin.toulouse.inra.fr/multalin/multalin.html). ImageJ (v 1.43u, 32bit and 64bit) was used for stack analyses and image preparations and Adobe Photoshop CS3 (Version 10.0.1) was used for image and panel preparation. Numbers´09 (Version 2.0.5) was used for stochastical calculations and bar graph illustrations and Pages´09 (Version 4.0.5) for scripting.

3. Results and Discussion Part I: Generation of transgenic reporter lines for the analysis of brain development in *Tribolium castaneum*

One part of this work was to establish a system to visualize the morphology of the larval brain in *Tribolium* and to adress questions regarding development. Some transgenic imaging lines already existed and showed distinct signals in the CNS or neural tissue. Those lines originated from the GEKU screen (`Mushroom Body´ line; chapter 3.1.4; Trauner et al., 2009), from collaborating laboratories (EFII::DsRedEx; chapter 3.1.4), or from work of former Diploma students in the group of G. Bucher (6xP3::eCFP, Hein; 2007; chapter 3.1.4) and were characterized here. Further lines were to be generated for the visualization of specific cell types of the CNS and brain tissue (chapter 3.1.2). Finally, reporter lines that enabled imaging of subsets of cells in the brain by the expression of reporter genes driven by the regulatory elements of the genes of interest could be generated (chapter 3.1.3). Additionally, transgenic animals for a *Tc-six3* overexpression system were generated and characterized.

3.1. The heat shock *Tc-six3* overexpression construct is not functional

By a former student, a construct for production of a ubiquitous *Tc-six3* overexpression (*piggyBac* [3xP3::eGFP; hsp5´UTR, DsRedEx, 3´UTR; hsp5´UTR, ORF *Tc-six3*, 3´UTR]) was designed. This construct included the *Tribolium* specific heat shock promoter *hsp68* (reviewed in Hillebrand, 2010) driving on the one hand the reporter gene DsRedEx and on the other hand *Tc-six3*. It was cloned by Julia Schanda and Johannes Schinko (unpublished) and was used here to generate transgenic animals (chapter 2.6). Six lines originating from one G_0 were characterized, meaning these lines are siblings.

3.1.1. Characterization of positive transgenic animals by *in situ* hybridization and cuticle phenotype

The functionality of the *Tc-six3* overexpression heat shock constructs in transgenic animals was tested by different heat shock (HS) experiments. Therefore, embryos of the transgenic lines were collected for 24h at 25°C and heat shocked for 10 min at 46°C. Heat shocked embryos were then kept on lower temperature to avoid further misexpression and hatched after approximately 5 days at 25°C. Additional heat shocked embryos were kept on 32°C to reduce potential self healing processes and larvae hatched after approximately 4d. In both cases, embryos were prepared in Hoyers Medium for cuticle analysis (chapter 2.5), and in both cases no cuticle defects or malformation caused by a gain of function of *Tc-six3* were detected.

To confirm the functionality of the construct, whole mount *in situ* hybridization (chapter 2.4) with heat shocked embryos of these lines was performed. Heat shocks had been done as described above, but embryos were reared for 1h, 2h, and 3h at 25°C after HS, followed by fixation. The experiments were repeated several times to collect enough embryos for staining. Due to the small number of eggs received by one collection, the patches were split and fixated. Thus, control embryos (no HS) and embryos fixed 2h after HS derived from one egg lay, 1h and 3h after HS respectively. All patches of heat shocked and non heat shocked embryos were stained simultaneously with the same antisense *Tc-six3* probe. Staining was stopped at the same time for all samples, when staining in the control animals first appeared (Figure 4, A).

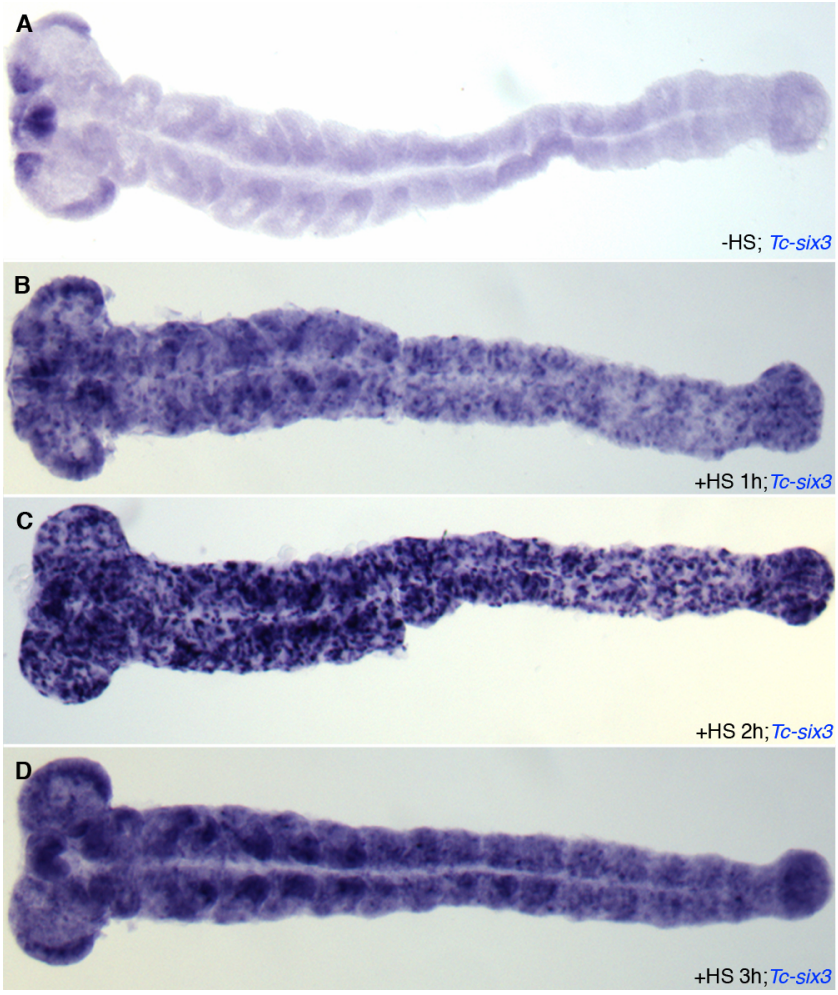

Figure 4: *In situ* hybridization for *Tc-six3* in the *Tc-six3* overexpression line after heat shock for 10 min at 46°C. (A) elongated germband without heat shock (HS). Expression pattern is WT-like. (B) Expression of *Tc-six3* one hour after HS. Staining shows a ubiquitous overexpression. (C) Ubiquitous overxpression 2h after HS. (D) Ubiquitous overexpression 3h after given HS.

All six lines showed the same expression pattern in all stages (not shown). Representative almost fully elongated embryos of the *Tc-six3* overexpression line 1 are shown in Figure 4. As a negative control, non heat shocked embryos of the transgenic lines were used. These embryos showed an expression pattern which was identical to the native *Tc-six3* expression in all stages (Figure 4, A). In general, in embryos with HS treatment a grainy expression pattern could be detected in all stages. In

embryos fixed 1h after HS, a strong ubiquitous expression pattern of *Tc-six3* could be detected (Figure 4, B). A slight increase of *Tc-six3* expression could be detected in embryos fixed 2h after HS (Figure 4, C), while embryos fixed 3h after HS showed a similar expression pattern compared to the embryos fixed 1h after HS (Figure 4, D). This confirmed that the construct was functional for expressing RNA of *Tc-six3* after HS treatment.

Nevertheless, a cuticle phenotype was not detected. This indicated either that overexpression of *Tc-six3* caused no cuticle phenotype or that no functional *Tc-six3* protein was produced. Since no antibody for *Tc-six3* protein detection was available, the construct sequence was analyzed for putative frame shifts or other disturbing properties.

3.1.2. Genomic DNA analysis reveals errors in construct sequence

The pre-reporter gene region of the overexpression construct was analyzed by sequencing the respective regions within the genomic DNA (gDNA; Figure 5), isolated from 3 to 5 individuals of each line (chapter 2.3). Sequencing of all *Tc-six3* overexpression lines confirmed the linker sequence between the open reading frame (ORF) of *Tc-six3* and the hsp5´UTR to be derived from the PCRII® vector (Figure 5; Appendix Figure 7.2).

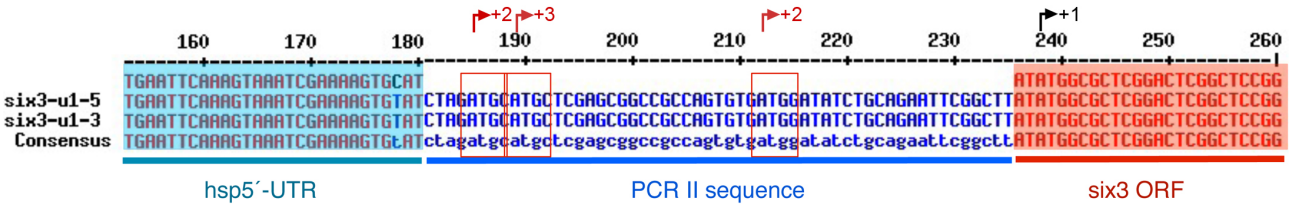

Figure 5: Sequence analysis of the linker between the hsp5`-UTR and the *Tc-six3* ORF reveals additional translation start sites.

The hsp5`-UTR is highlighted in turquoise, the *Tc-six3* ORF is highlighted in red. Sequences shown here are derived from lines 5 and 3. The linker sequence of the PCR II® vector is depicted in blue. Within the vector sequence there are three putative translation start sites (marked by red square), causing frame-shifts. The black arrow marks the original translation start site of *Tc-six3*.

In Figure 5, lines 3 and 5 are shown as representatives for all 6 hs-*Tc-six3* overexpression lines. The endogenous translation start site (TLS) of *Tc-six3* was present and unchanged (indicated by a black arrow, Figure 5). Within the linker sequence, several additional translation start sites (red squares, Figure 5) were found. The first TLS causing a frame shift of 1 base pair was coding for a short protein of 9 amino acids (AA) until the first stop codon. The second TLS caused a frame shift of 2

bp leading to a protein of 7 AA length. Both ORFs originating from the first TLSs stop before the *Tc-six3* ATG. The third TLS caused a frame shift of 1 bp and lead to a protein sequence of 17 AA. Here the coding sequence offerlapped with the ORF of *Tc-six3*.

Potentially, these additional TLSs disturbed the translation of *Tc-six3* mRNA. Therefore the total amount of functional *Tc-six3* protein after HS treatment was possibly too low to cause any cuticle phenotypes. To confirm the presence of ubiquitous *Tc-six3* protein after HS an immuno-histochemistry staining with a respective antibody against *Tc-six3* would be necessary.

3.2. Generation of reporter lines regulated by the native upstream regions of *Tc-elav* and *Tc-repo*

Marker lines showing reporter gene expression in specific tissues of the CNS were to be generated. Such lines can then be used for analysis of wildtype brains as well as for studies of loss or gain of function of various genes. The two genes *Tc-elav* and *Tc-repo,* were selected to generate such reporter lines. Homologues of *Tc-elav* were known to be expressed pan-neurally (Yannoni and White, 1997; Berger et al., 2007). Furthermore *Tc-elav* expression was also shown for *Tribolium* by whole mount *in situ* hybridization (Simonnet, personal communication). *Tc-repo* was chosen since homologous genes in other species are specifically expressed in glial cells arising from neuroectoderm (Yuasa et al., 2003). To establish the desired reporter lines, constructs including the putative regulatory regions of *Tc-elav* and *Tc-repo* in combination with the endogenous promoters driving various reporter genes were designed.

3.2.1. The transgenic line for the regulatory region of *Tc-elav* revealed a muscle expression pattern

Before cloning, RACE-PCR was performed to identify the 5´UTR of *Tc-elav* (chapter 2.2), which was also important for the identification of the endogenous promoter.

A 5´UTR of a total length of 409 bp could be identified. Interestingly, by comparison to gDNA, a 1586 bp long intron was found. This intron splits the 5´UTR into two fragments with 182 bp at the 5´end and 227 bp at the 3´end close to the translation start site ATG (Appendix Figure 7.1). For cloning the regulatory region, the upstream region of *Tc-elav* starting 28 bp and ending 2579 bp upstream of the translation start was cloned. Thus, the identified intron was included. The total length of this fragment was limited by a gene prediction for the Glean_4647, which lies on the negative strand and codes for a zinc carboxypeptidase domain. The 400 bp upstream of the gene prediction Glean_4647 were omitted to be included into the regulatory region of *Tc-elav* (reg. *Tc-*

elav). Thus, the total length of reg. *Tc-elav* was 2551 bp. This fragment was used for driving the reporter gene RFP with a membrane tag (mRFP). Transgenesis (chapter 2.6) using the construct `reg. *Tc-elav*::mRFP´ (Appendix Figure 7.3.1), yielded two transgenic lines from two independent G_0.

When analyzing the first larval instar (L1), no fluorescent signal was detected in *Tc-vw* larva (Figure 6, A), while the larvae of both `reg. *Tc-elav*::mRFP´ lines showed an identical expression pattern for mRFP. The expression was detected exclusively within the muscles (Figure 6, B). The brain is embedded by muscles positive for the mRFP signal (Figure 6, C). This was shown by crossing experiments with the 6xP3::eCFP line, which marks glial cells (chapter 1.4.2; for better contrast depicted in green). Due to the muscle expression pattern, no further investigations of the *Tc-elav* lines were performed.

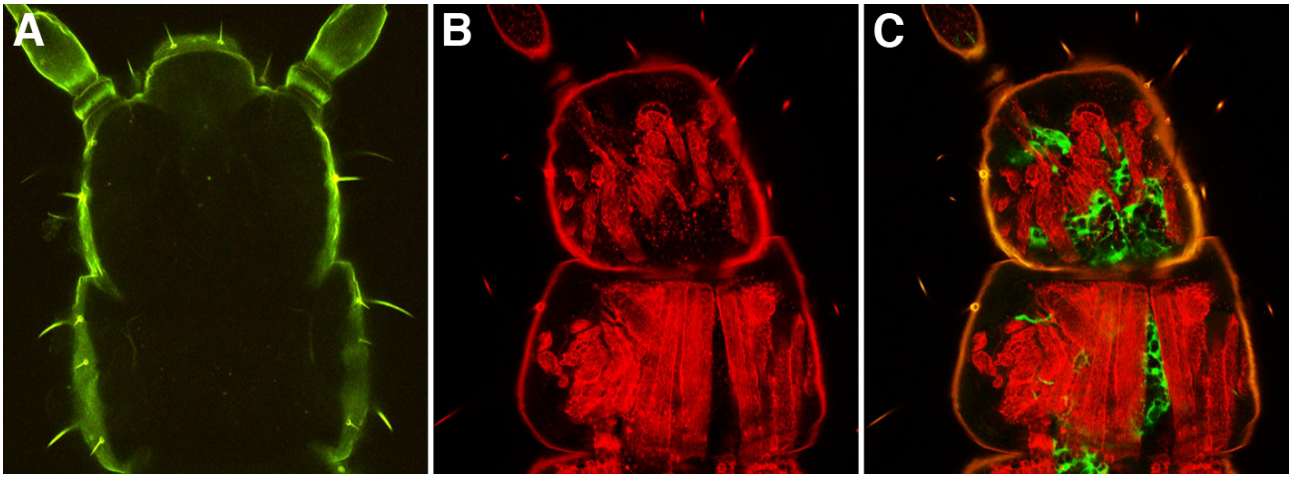

Figure 6: The line `reg. *Tc-elav*::mRFP´ drives reporter gene expression in muscles.
(A) The first instar larva (L1) of the vw strain shows an autofluorescence of the cuticle, shown as negative control. A larva derived from crossing of the lines 6xP3::eCFP and `reg. *Tc-elav*::mRFP´ is depicted in panel (B) and (C). (B) Muscular expression pattern of mRFP under the control of regulatory region of *Tc-elav,* only the red channel. (C) The eCFP signal is depicted in green. Brain tissue is visible under the muscles in the head capsule. Anterior is facing up.

Taken together, two independent lines whith similar expression patterns in the muscles were created. Due to the very similar expression pattern it is not very likely, that enhancer elements surrounding the insertion locus influence the expression pattern. However, to exclude a insertion locus dependent influence an inverse PCR on the gDNA of individuals of both lines could be performed. Due to the independent generation of such lines it is unlikely, that integration occured independently twice at the same genomic locus. Therefore it is suggested, that elements included within the cloned regulatory regions specifically cause this expression pattern.

For example, the `reg. of *Tc-elav´* could include sequences like so called insulators. It is known from other species like *Dorosphila,* that such elements are able to shield promoters from nearby enhancer or silencer elements (Brasset and Vaury, 2005; Bushey et al., 2008). The utilization of such elements to create transgenic reporter lines in *Tribolium castaneum* would facilitate the identification of enhancer elements including in putative regulatory sequences. That means, the expression pattern of reporter lines included such insulators would be due to the enhancers within the construct. Since locus specific integration was not feasible in *Tribolium* at that time, using insulators would display an alternative option to achieve specific expression.

3.2.2. The transgenic line for the regulatory region of *Tc-repo* shows a glial expression pattern

Genomic DNA of the SB strain was used to amplify a fragment upstream of *Tc-repo* with a total length of 4510 bp. *Tc-repo* possesses a 86 bp long 5´UTR with a TATA-box at -36 (relative to the transcription start site). These elements were included within the fragment. In order to select optimal primers, 44 bp of the 5´UTR were not included.

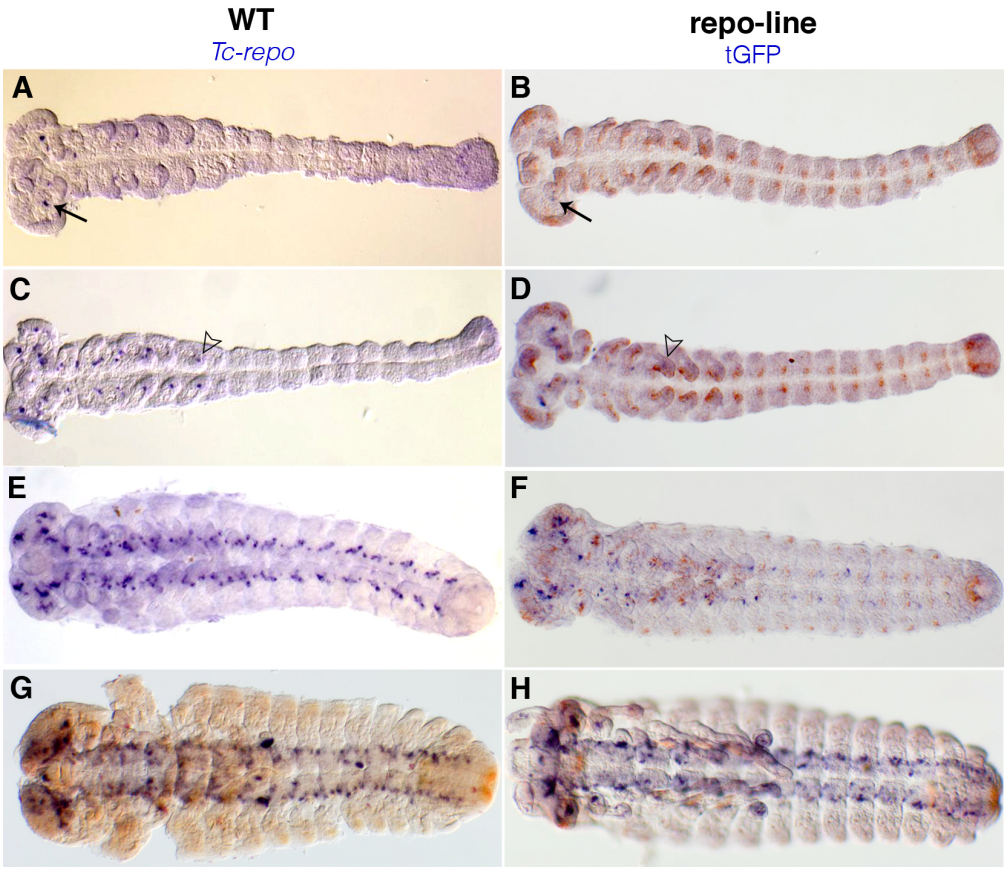

Figure 7: The reporter gene expression pattern of the line `reg. *Tc-repo*::tGFP´ mimics the endogenous *Tc-repo* expression in WT-embryos with delay. The expression pattern of *Tc-repo* is depicted in the WT-embryos (A, C, E, G) in blue. The tGFP expression pattern under the control of `reg. *Tc-repo´* (B, D, F, H) is also depicted in blue. Embryos in the panels (B, D, F, G, H) show a double staining with *Tc-wg,* shown in red. In embryos of the transgenic line `reg. *Tc-repo*::tGFP´, the reporter gene expression pattern is similar to the endogenous *Tc-repo* expression in WT-embryos. Arrows (A, B) and arrowheads (C, D) indicate similar expression domains in embryos of differing ages. Thus, tGFP expression in the transgenic line is delayed. Anterior is orientated to the left.

The regulatory region of *Tc-repo* (reg. *Tc-repo*) was used to drive the reporter gene turboGFP (tGFP; Appendix Figure 7.3.2). Transgenesis resulted in transgenic offspring of one G_0. This line was analyzed for tGFP expression. *In situ* hybridization experiments were performed to compare endogenous *Tc-repo* expression in WT embryos with tGFP expression of the reporter line `reg. *Tc-repo*::tGFP´. Transgenic embryos were stained with an antisense probe for tGFP and SB-embryos were incubated with an antisense probe for *Tc-repo*.

The endogenous expression of *Tc-repo* mRNA was detected first in two points in the head of an elongating embryo (black arrow; Figure 7, A). With ongoing development, a segmental expression pattern arose from anterior to posterior (Figure 7, C and E), lateral to the midline in parallel rows. The expression pattern in the head was very dynamic. In the labrum and the area around the stomodaeum no *Tc-repo* could be detected, initially(Figure 7, E), but was detectable in later stages (Figure 7, G). Testing the expression pattern of the reporter gene tGFP in the transgenic animals for the construct `reg. *Tc-repo*::tGFP´, a delayed expression pattern was detected, however very similar to the endogenous expression of *Tc-repo*. The two domains in the head were detectable at a fully elongated stage (black arrow; Figure 7, B). Segmental patterning arose in embryos being close to retracting movements (arrowhead; Figure 7, D and F). Taken together, even though delayed, the expression pattern of the reporter gene was similar to the endogenous *Tc-repo* expression.

First instar (L1) larvae of the transgenic line `reg. *Tc-repo*::tGFP´ were scanned for tGFP expression in the head. The tGFP signal was detectable in neural tissue. The expression within the soma of cells positive for the tGFP signal was visible as dots within the brain (open white arrowheads; Figure 8, C). Apparently, tGFP was located in the cell lumen, recognizable by faint lines spreading out from soma (Figure 8, C). A prominent expression domain (open white arrow; Figure 8, C) was present anterior lateral to the stomatogastric nervous system (STNS), which showed a tGFP signal as well (Figure 8, C). Starting from these lateral domains, two symmetric major projections were leading into the labrum and into the antenna. The tGFP positive cells projecting into the antenna were forming a tube (Figure 8, C`).

Comparing the signals of the line 6xP3::DsRedEx (Figure 8, B and B`) with the reporter line `reg. *Tc-repo*::tGFP´ in the antenna, it appeared that these signals are not overlapping. Rather, the cells positive for tGFP enclosed the bundles positive for DsRedEx (Figure 8, B` and C`). Since the line 6xP3::DsRedEx marks a subset of glial cells in the brain (chapter 1.4.2), it is very likely, that the transgenic line `reg. *Tc-repo*::tGFP´ marks an additional and non overlapping subset. It is known from *Drosophila,* that all glial cells derived from the neuroectoderm are positive for the transcription factor *repo* (reviewed in Edwards and Meinertzhagen, 2010). Furthermore the *repo*

positive cells are distinguishable into three types regarding their location, surface, neuropile, and cortex glia (Awasaki et al., 2008; Edwards and Meinertzhagen, 2010). Due to the position of the 6xP3::DsRedEx positive cells, it is suggested that these cells are cortex and neuropile glia. While cortex glia form a mesh in the cortex of the CNS, the neuropile glia enwrap the surface of neuropiles. Thus, these types of glia are embedded in the brain, as it is detectable in the case of the transgenic reporter line 6xP3::DsREdEx. In contrast the reporter gene signals of the `reg. *Tc-repo*::tGFP´ line seem to form a superficial ensheathing layer, which is once detectable in the antenna as well as in the case of the STNS (compare Figure 8, B`, C` and B, C). Thus it is suggested, that the line `reg. *Tc-repo*::tGFP´ marks a subset of surface glia.

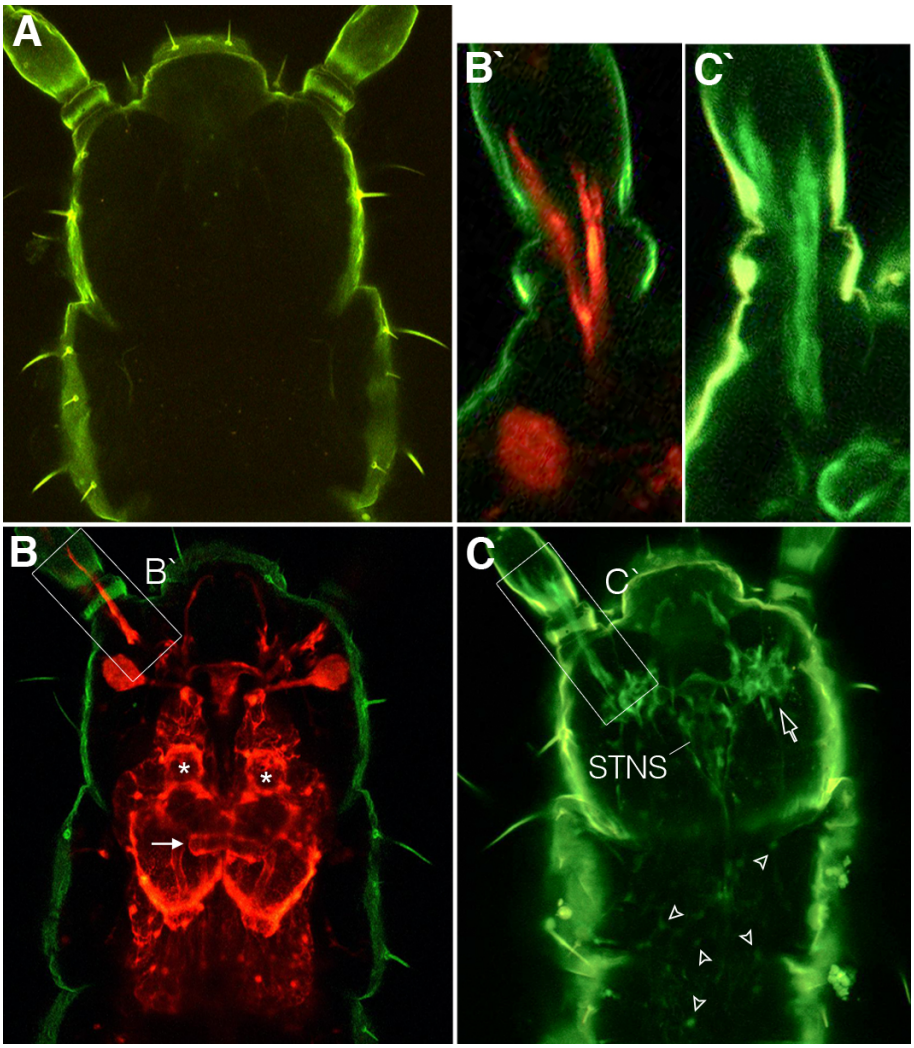

Figure 8: Expression analysis of tGFP in L1 larva of the line `reg. *Tc-repo*::tGFP´.
(A) The larva of the vw strain shows autofluorescence of the cuticle only. (B) Expression pattern in L1 larva transgenic for 6xP3::DsRedEx, which marks glial cells in red (see chapter 1.4.2 and 3.4 for details). Antennal Lobes are marked by white asterisks; the Central Body is pointed out by a white arrow. (C) L1 larva of the line `reg. *Tc-repo*::tGFP´, shown in green. tGFP positive cells in the brain are pointed out by white open arrowheads. The antennal nerve projects into a structure positive for tGFP expression, indicated by the open white arrow.The stomato-gastric nervous system (STNS) is also marked by tGFP expression. Enlarged antennal sections (B`, C`) are marked with a white square in the according image (B, C). The line `reg. *Tc-repo*::tGFP´ probably marks a subset of surface glia. Anterior is facing up.

To prove these hypotheses, colocalization experiments were performed by crossing the `reg. *Tc-repo*::tGFP´ line with the 6xP3::DsRedEx line. However, a full separation of signals was not possible with the available filter sets. For a detailed analysis of colocalization either antibodies against specific markers could be used, e.g. against *Tc-repo* as a glia marker (Halter et al., 1995) or anti DsRedEx and tGFP themselves in a crossed background as described above. Thereby it could be verified, if the transgenic lines mark additive subtypes of glia.

Taken together, the embryonal expression pattern of tGFP mRNA is similar to the endogenous expression pattern of *Tc-repo*. The tGFP signal present in cells of the neural tissue and its surroundings in larval brains, makes it very likely that the line `reg. *Tc-repo*::tGFP´ marks glial cells. In comparison to the glial marking line 6xP3::DsRedEx, it is very likely that both lines mark different subtypes of glia, while 6xP3::DsRedEx appears to mark cortex and neuropile glia, the line `reg. *Tc-repo*::tGFP´ appears to drive expression in surface glia. However, it also became apparent that the line 6xP3::DsRedEx provides a better marker for brain morphology. Therefore, further analyses of the line `reg. *Tc-repo*::tGFP´ were not performed.

3.3. Generation of *Tc-six3* and *Tc-rx* reporter lines

In order to analyze the regulation of the identity of neural lineages, lines where a reporter gene is driven under the control of the endogenous regulatory region and a promoter of a gene expressed in a very restricted set of cells in the brain were needed. Such lines should enable tracing the development starting from NBs to distinct regions or even neuropiles in the larval brain. For this, the two genes *Tc-six3* and *Tc-rx* were chosen. The reasons for selecting these genes are: 1) Both genes have an exclusive anterior most expression within the embryo, which stays restricted to the head during development. Hence, they are specific to the brain. 2) From other models it is known, that correlating regions positive for these genes are forming parts of the brain and in the case of *six3* of the Central Complex (Boyan and Williams 1997, de Velasco et al., 2007). 3) The expression domains are small and only a small number of NBs is positive for their expression, which alleviates the tracing from NB to neuropile in the larval brain.

3.3.1. Identification of *Tc-six3* and *Tc-rx* positive NBs in the anterior neuroectoderm

For analyzing the amount of NBs which were positive for *Tc-six3* or *Tc-rx* expression within the neuroectoderm, 6h staged egg lays were collected at 32°C: 12-18h, 18-24h, and 24-30h. This enabled estimation of the age of the according embryos. By double *in situ* hybridizations with the NB marker *Tc-asense* (*Tc-ase*; Wheeler et al., 2003) and *Tc-six3* or *Tc-rx,* the amount of NBs, which

arose in the respective gene expression region, was quantified. From *Drosophila,* it is known that *asense* remains expressed in delaminating NBs at least until the first division of the neural precursor (Brand et al., 1993).

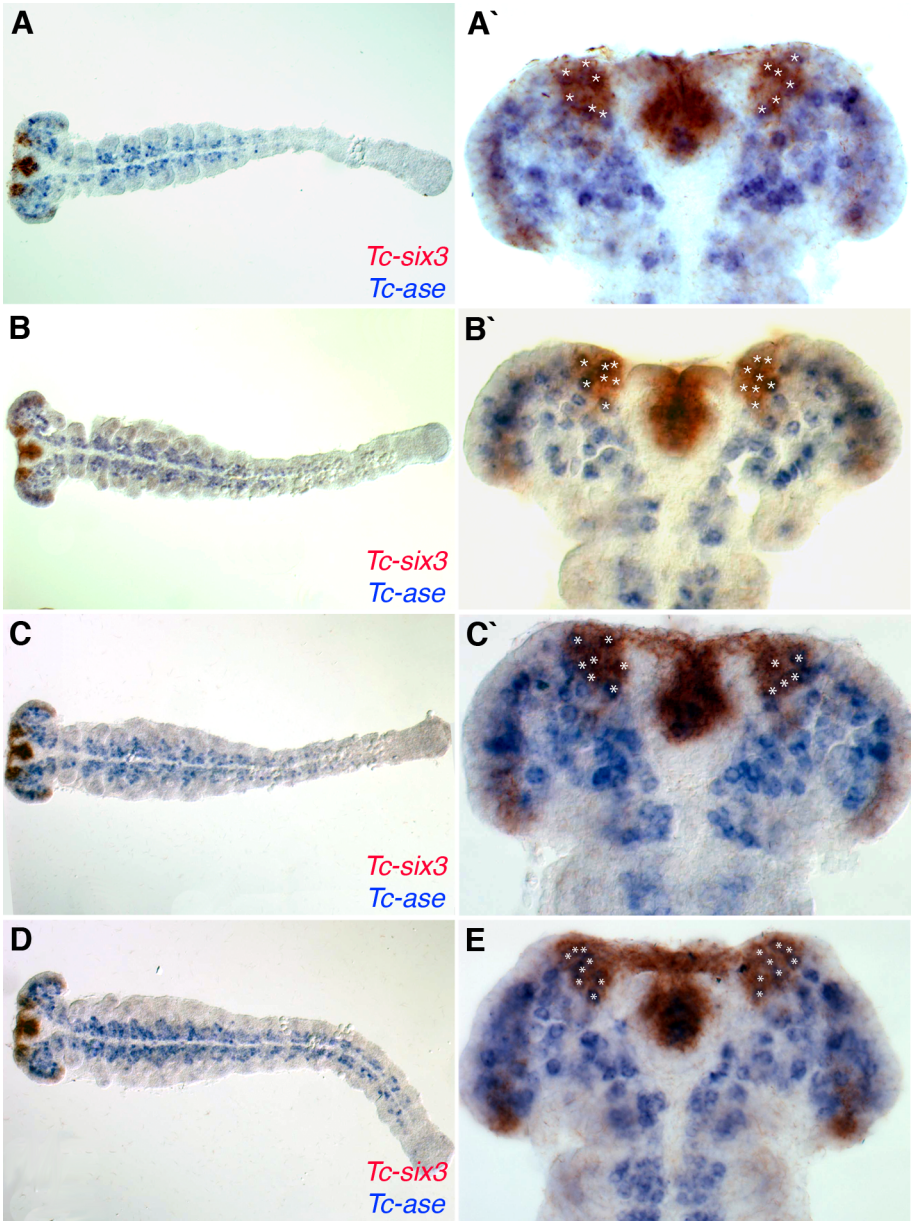

Figure 9: At least 8 NBs delaminate from the *Tc-six3* positive region.
(A-D) WT embryos at different developmental stages with double *in situ* hybridization for *Tc-six3 (*red) and the neuroblast marker *Tc-ase* (blue). (A) An embryo approximately 14h old. (B) An embryo approximately 16h old. (C) An embryo approximately 18h old. (D) An embryo approximately 24h old. (E) The head of an embryo of the approximately same age as the embryo depicted in D. Asterisks indicate NBs positive for *Tc-six3* expression. At an age of approximately 14h, 6 NBs in the region of *Tc-six3* arose up to a maximum amount of 8 NBs at 24h. Indicated ages correlate to the development at 32°C.

By staged egg lays, a clear number and temporal series of arising NBs from the *Tc-six3* region was to be elucidated. The expression pattern of *Tc-ase* started at approximately 12h at 32°C. The first NB positive for *Tc-six3* is the most posterior within the expression domain median lateral to the labrum. The numbers of NBs varied within same aged embryos and even per hemisphere (compare hemispheres in Figure 9, B` and C). In some cases, young embryos about 14h old at 32°C had more *Tc-ase* marked NBs within the *Tc-six3* region than older ones (compare Figure 9, A and C). Analysis of embryos older than 24h was not possible using this method, since technical limitation occurs

through the need to flatten the head without disturbing brain morphology and on the other hand because of advancing head morphology. In both cases the neural tissue separates from the ectoderm - meaning that the NBs are shifted relative to the ectoderm and cannot be assigned unequivocally. Taken together, up to 8 NBs positive for *Tc-six3* were detected in the neuroectoderm at an age of 24h at 32°C (Figure 9, D and E). However, it cannot be excluded that more NBs delaminate subsequently.

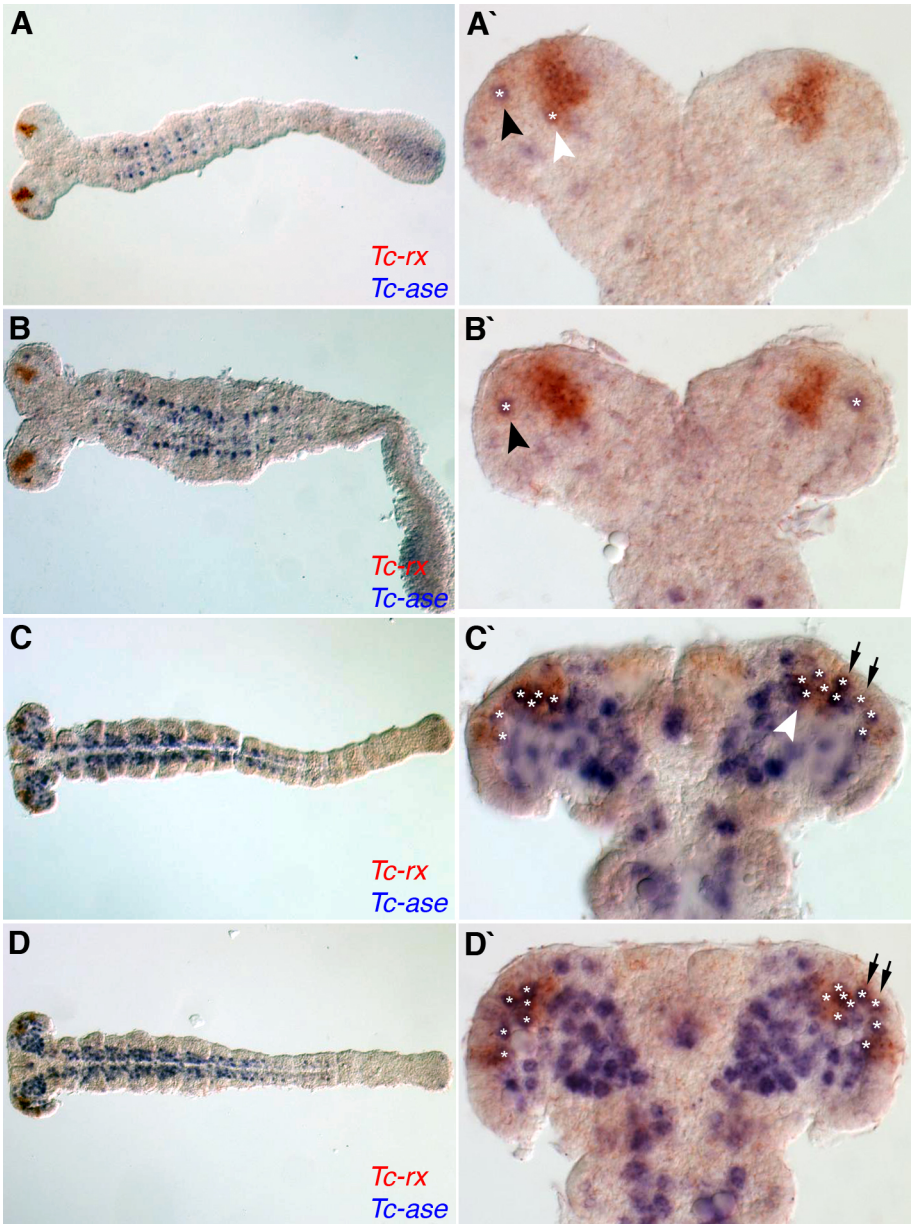

Figure 10: At least 9 NBs delaminate from the *Tc-rx* positive region.
(A-D) WT embryos at different developmental stages with double *in situ* hybridization for *Tc-rx* (red) and the neuroblast marker *Tc-ase* (blue). The *Tc-ase* expression pattern is detectable in elongating embryos, approximately 12h old (A). (C, D) The oldest embryo with an age of approximately 18h. Asterisks indicate NBs positive for *Tc-rx* expression. The first NBs arise in lateral part of *Tc-rx* region at approximately 12h (black arrowhead A`, B`). A group of NBs arise in the central domain, indicated by white a arrowhead (C`). In the lateral domain, 2 additional NBs arise in at an age of 18h, indicated by black arrows (D`). Thus, at least 9 NBs delaminate up to an age of 18h. Indicated ages correlate to the development at 32°C.

Double *in situ* analysis of *Tc-ase* and *Tc-rx* was performed to identify the amount of NBs in the *Tc-rx* positive region. The first NB positive for *Tc-rx* was detected in approximately 12h old embryos within the lateral domain at the rim of the head lobes (black arrowhead, Figure 10, A`, B`). The next group of NBs arose in the central domain with 2-3 NBs at the same time (white arrowhead, Figure

10, C`). In the lateral rim domain of the *Tc-rx* positive region, only two further NBs were detected in up to 18h old embryos (black arrows, Figure 10, C` and D`). Compared to the NB amount positive for *Tc-six3,* a variation of the NB number also occurred, both per hemisphere and in same aged embryos (Figure 10, C, D). Nevertheless, a maximum number of 9 NBs arising in the *Tc-rx* region could be detected up to an age of 18h at 32°C. Also here, it is unclear whether more NBs delaminate at later stages.

The variation of the NB amount among the different ages was remarkable. There are numerous possible reasons, for example that there is no distinct temporal pattern for arising NBs. However, the preparation technique could be another reason, meaning that NBs got out of place by flattening the embryo´s head. A difference in the NB amount between hemispheres and stages was also detected in embryos which had not been flattened, but left in natural shape for analysis. However, it is known that NBs delaminate from the neuroectoderm. This was also observed in older stages, where a correlation between the ectodermal surface layer and the neural tissue below was not given anymore. This indicates that in this case colocalization experiments by *in situ* hybridizations are only partially efficient to appoint the total amount of *Tc-six3* and *Tc-rx* positive NBs by light microscopy. In this case, antibody staining against the neuroblast marker *Tc-ASE* and either *Tc-Six3* or *Tc-Rx* should be performed. Simultaneous detection of these proteins within NBs would elucidate a definitive number. Nevertheless, at least 8 NBs positive for *Tc-six3* were detected up to an age of 24h, and 9 NB positive for *Tc-rx* up to an age of 18h.

3.3.2. *Tc-six3* reporter lines

Reporter lines expressing a reporter gene in the same pattern as a gene of interest were to be generated. The endogenous promoter and putative regulatory regions of *Tc-six3* were used to drive expression of the reporter gene. For this, the endogenous promoter of *Tc-six3* was analyzed first. By two independent RACE-PCR experiments (chapter 2.2) the 5´UTRs of *Tc-six3* were identified. Before sequences of regulatory regions were amplified by PCR of gDNA, the respective regions were analyzed for gene predictions or repetitive sequences (Figure 11, Panel B). Since the enhancer elements, which were driving the endogenous *Tc-six3* expression, were unknown, several regions with approximately 5kb length were cloned with an overlap of approximately 500 bp. These fragments were combined with the endogenous promoter of *Tc-six3* to drive the reporter gene tGFP (see Appendix 7.3.3. - 7.3.6.). The constructs were used to create transgenic lines, which were subsequently analyzed regarding their expression pattern.

3.3.2.1. Generation of *Tc-six3* reporter lines

In the case of *Tc-six3,* two variants of 5´UTRs with two different transcription start sites (TSS) were detected. The longer 5´UTR sequence was 96 bp long. This sequence starts with TSS 1 (highlighted pink, Figure 11, Panel A), which is embedded in a consensus sequence of a putative TFIIB Recognition Element (BRE, Lagrarange et al., 1998; Butler and Kadonaga, 2002). A BRE usually lies upstream of a TATA box, which was not given here. Therefore, it is probably not functional. However, this element had the expected distance of 30 bp to the second TSS. The latter led to a 5´UTR with a length of 66 bp. This TSS 2 (highlighted blue, Figure 11, Panel A) had a typical consensus sequence for TSS, which is also called Initiator element (reviewed in Butler and Kadonaga, 2002). No further core promoter elements were found.

The fragment called `*Tc-six3* 5up´ is shorter than 5 kb, it includes the entire 5´UTR starting from -1 and ending at -4133 upstream of the translation start site (TLS) of *Tc-six3* (purple line; Figure 11, Panel B). This construct already included the endogenous promoter sequence of the gene *Tc-six3*, while all other putative regulatory regions had to be fused to such a promoter.

The basal `*Tc-six3* promoter element´ was characterized carefully (short line under `*Tc-six3* 5up´ construct; Figure 11, Panel B), because it was used for all fragments. By analyzing the upstream region, 106 bp upstream of the TLS of *Tc-six3,* the gene prediction Glean_1196 was detected. This gene prediction has a total length of 225 bp and lies on the complementary strand. The`*Tc-six3* promoter element´ includes the entire 5´UTR and has a total size of 657 bp. Thus, also the glean prediction is included in the `*Tc-six3* promoter element´ (short line under `*Tc-six3* 5up´ construct; Figure 11, Panel B). The sequencing analyses of the finished constructs revealed two shortcomings. First, a deletion within the construct `*Tc-six3* promoter element´ was identified. 499 bp upstream of the TLS of the reporter gene tGFP, 9 bp (TGT GAT ACA) are deleted (Figure 11, Panel C). The construct `*Tc-six3* 5up´, which was amplified in one piece, has no deletion (Figure 11, Panel C). Second, the reverse primer for the endogenous promoter sequence as well as the `*Tc-six3* 5up´ construct include an additional translation start site of 18 bp upstream of the original ATG of the reporter gene tGFP. This does not lead to a frame shift, but to additional 9 amino acids at the N terminus of tGFP.

For the upstream and downstream sequences, primers were designed outside of repetitive sequences. The construct `*Tc-six3* 10 up´ starts at -8870 bp and ends -3454 bp upstream to ATG. It has a total size of 5416 bp and an overlap with the `*Tc-six3* 5up´ construct of 679 bp (blue line; Figure 11, Panel B).

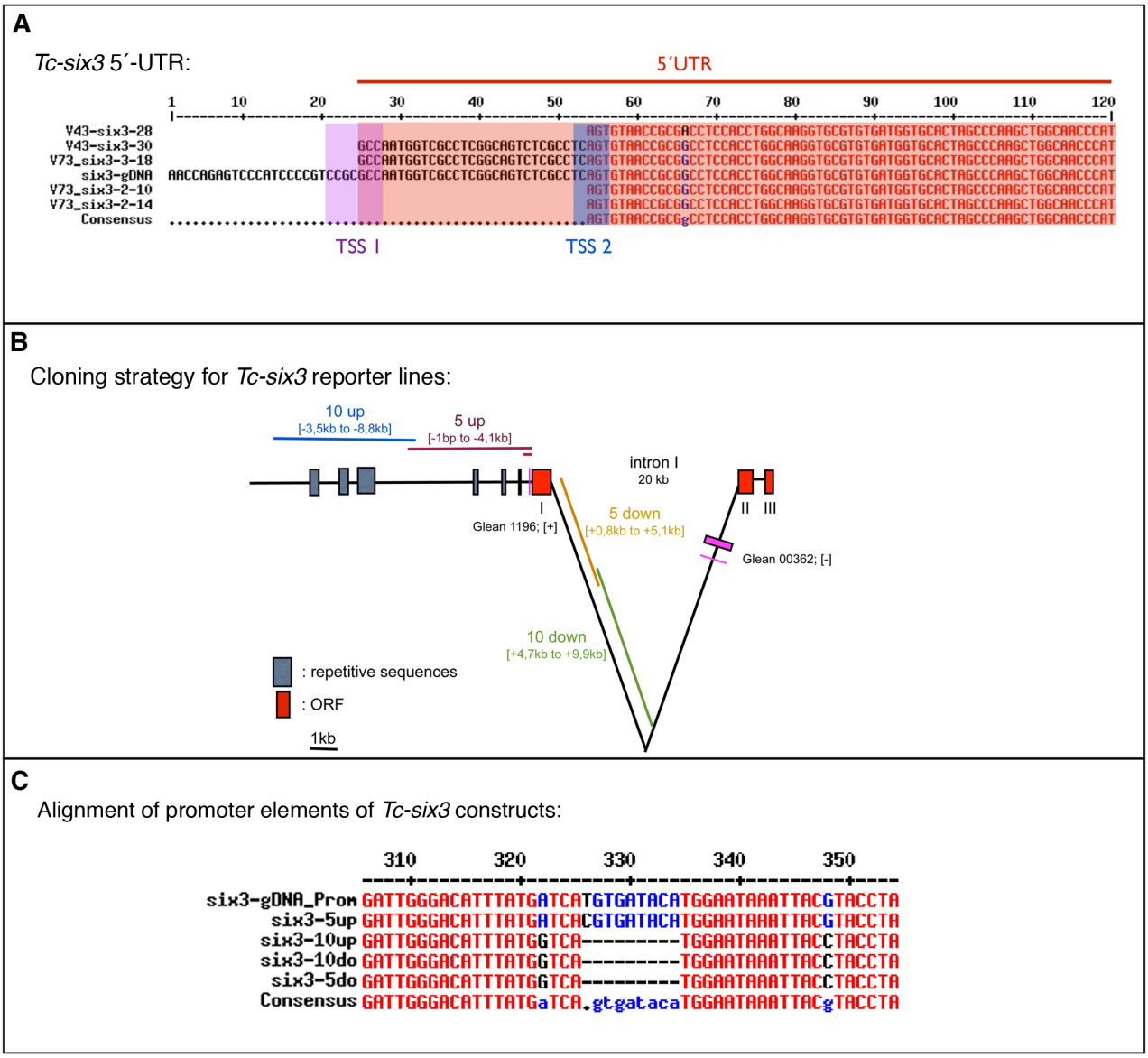

Figure 11: 5`UTR and cloning strategy for the transgenic reporter lines of *Tc-six3*

(A) Within the 5`UTR of *Tc-six3* (highlighted red) there are two different Transcription Start Sites (TSSs). TSS 1 (highlighted pink) was verified by two independent RACE PCR experiments and lead to a 5´UTR length of 96 bp. TSS 2 (highlighted blue) represents the typical consensus sequence with an 5´UTR length 66 of bp. (B) Schematic view of the genomic sequences, which were chosen for reporter gene constructs. A 657 bp long `*Tc-six3* promoter element´ was cloned, including the entire 5`UTR (depicted as a short line under the 5up construct). All sequences were designed to result in an overlap of at least 500 bp with adjacent regions. These regulatory regions were combined with the endogenous basal promoter driving tGFP, except for constructs 5up which already included the basal promoter. The downstream regulatory regions were cloned in front of the basal promoter. The generated lines with the construct `*Tc-six3* 10up´ (highlighted in blue) were positively tested for *in situ* and partially *in vivo* expression of tGFP. In lines carrying the constructs `*Tc-six3* 5up´ and `*Tc-six3* 5down´, the reporter gene expression was detected only in *in situ* hybridization experiments. No transgenic line was generated with the construct `*Tc-six3* 10down´. (C) Alignment of sequenced promoter regions of injected *Tc-six3* reporter constructs. High consensus sequences are depicted in red. Low consensus sequences are depicted in blue. Reporter constructs six3-10up, 10down, and 5down have a deletion of TGTGATACA, due to a deletion in the `*Tc-six3* promoter element´ construct (see chapter 2.3). Repetitive sequence regions are depicted as gray boxes; ORFs are depicted as red boxes; predicted gleans are depicted as pink boxes.

The constructs *Tc-six3* `down´ are located in the first intron of *Tc-six3,* which has a size of 19116 bp. At position +15254 bp downstream of the TLS of *Tc-six3,* the gene prediction Glean_362 starts on the sense strand. The sequences for reporter line constructs were chosen not to overlap with this gene prediction. Thus, the construct `*Tc-six3* 5down´ starts at +767 and ends +5140 bp downstream of the TLS of *Tc-six3* (yellow line; Figure 11, Panel B). It has a total size of 4373 bp. This construct overlapps with `*Tc-six3* 10down´ for 426 bp. `*Tc-six3* 10down´ starts +4714 bp and ends +9960 bp downstream of the TLS of *Tc-six3* with total size of 5246 bp (green line; Figure 11, Panel B).

All these amplified sequences were combined with the `*Tc-six3* promoter element´ and cloned in front of the reporter gene tGFP with adjacent SV40. Except the construct `*Tc-six3* 5up´, this was directly fused to tGFP with the adjacent SV40.

Taken together, four different fragments were amplified and cloned in front of tGFP and SV40. All of these have two TLS 18 bp in front of the reporter gene tGFP. Additionally, the `*Tc-six3* promotor element´, which was fused to all putative regulatory regions except to `*Tc-six3* 5up´, has a deletion of 9 bp. The assembled reporter constructs were used for transgenesis (chapter 2.6) and resulted in transgenic animals derived from two G_0 animals with the cassette for `*Tc-six3* 10up´, from two $G_0$ animals with the cassette for `*Tc-six3* 5up´, and from one G_0 animal with the cassette for `*Tc-six3* 5down´. No animal could be successfully transformed with the cassette for `*Tc-six3* 10down´.

3.3.2.2. Analysis of transgenic animals carrying for the Tc-six3 reporter line construct

To test the function of the constructs in transgenic animals, *in situ* hybridizations with an antisense probe for tGFP were performed in 0-24h and 24-48h old embryos. Furthermore, the lines were scanned and analyzed for tGFP expression in the first larval instar (L1) brain. Generally, lines were denominated according to the number of the respective G_0, from which they derived and the number of the sibling line. For example, the third sibling crossing originated from G_0 7 is further called line 7.3.

Transgenic lines carrying the construct `*Tc-six3* 10up´

For the cassette of `*Tc-six3* 10up´, two independent G_0 could be generated, line 6 and line 7. From these, regarding sibling lines, which were analyzed according their reporter gene expression pattern, were generated.

In the case of line 6, only two sibling lines could be generated, lines 6.1 and 6.2. Both lines were analyzed for *in situ* hybridization and fluorescence signal of tGFP in the respective developmental stages. These lines showed an identical expression pattern, hence only embryos of line 6.2 are

depicted in Figure 12. First, expression was detectable in embryos at late elongating stages (Figure 12, A). The construct drives tGFP expression distally in the appendages, except the mandibles (md; Figure 12). These distal expression domains remained present up to the oldest staged embryos analyzed here (Figure 12, C). In elongating embryos an expression domain was observed in the rim of the head lobes (Figure 12, A`). This domain decreased in size during ongoing development (Figure 12, B`). In fully elongated embryos, an additional expression domain arose within the labrum (Figure 12, B). After fusion of the labral segments, this expression pattern was still located in the anterior median part of the labrum (Figure 12, C`). The lateral expression domain within the rim of the head lobes was totally retracted to a small domain in the posterior part of the head, probably forming the larval eyes (Figure 12, C`).

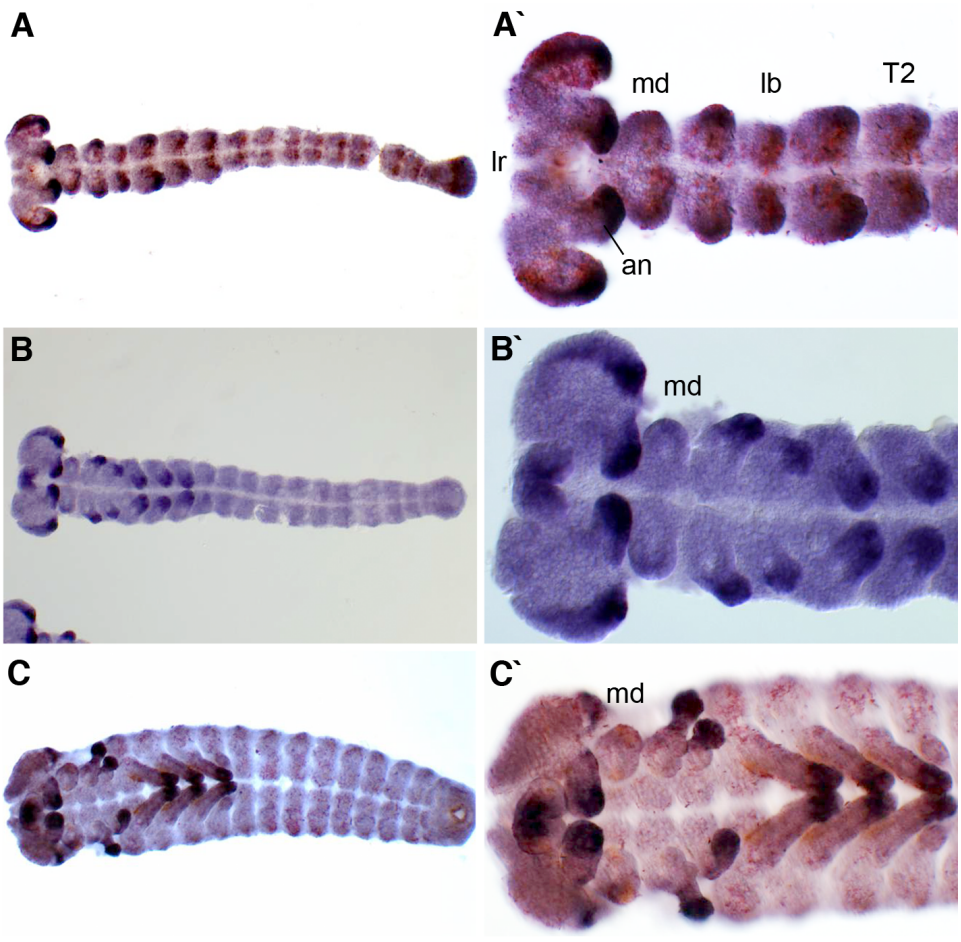

Figure 12: *In situ* hybridization with an antisense probe for tGFP in the transgenic line 6 containing the construct `*Tc-six3* 10up´.
(A) Elongating embryo, with expression in the rim of the head lobes and in the distal parts of budding appendages, except in the mandibles (md). (A`) Close up of the upper part of the body. (B) Fully elongated embryo with tGFP-expression in the labrum and all distal parts of appendages, except the md. The lateral expression in the head fades. (B`) Close up of the upper part of the body. (C) Fully retracted embryo with tGFP expression in the labrum and in the distal parts of appendages, except the md. (C`) Close up to the upper part of the body, with the lateral head expression restricted to lateral posterior. (A, A`, C, C`) show double *in situ* hybridizations with an antisense probe against *Tc-wg* depicted in red. lr: labrum; an: antenna; md: mandible; lb: labium; T2: thoracic segment 2. All embryos shown in total view are facing anterior to the left. All close ups to the head are facing anterior up.

The expression pattern of the reporter line `*Tc-six3* 10up´ 6.1 and 6.2 reflects only to a small portion that of the endogenous gene *Tc-six3*. Thus, in the reporter lines derived from G_0 6, the putative larval eyes domain (Figure 12, B and C) and the distal expression pattern in the labrum are the expression domains, which are overlapping with the endogenous expression pattern of the gene *Tc-six3*. The further expression domains in the appendages of `*Tc-six3* 10up´ line 6.1 and 6.2 indicate an enhancer trap situation.

The reporter gene expression pattern distal in the appendages resembled the expression of *Tc-dll* (Beermann et al., 2001). However, *Tc-dll* is also expressed in the pleuropodia, which was not true for embryos of line 6. Furthermore *Tc-dll* has a lateral head domain only during early elongating stages (Beermann et al., 2001; Prpic et al., 2001), while such a domain was present in line 6 also in almost fully elongated embryos (Figure 12, B). The distal expression domain suggests the influence of an enhancer which drives expression in the distal appendages. The determination of the integration site of the construct `*Tc-six3* 10up´ could reveal an enhancer trap dependent expression pattern. I.e. an integration close to a leg gene like *Tc-dll* could be the reason for the observed expression. However, fluorescence in larval brains was not detected in line 6.1 nor line 6.2.

Sibling lines of G_0 7 were also carrying the cassette for the `*Tc-six3* 10up´ construct. Three sister-lines were generated and analyzed for the expression pattern of tGFP.

The offspring of these lines was analyzed by *in situ* hybridization. All embryos showed the same expression pattern for the reporter gene tGFP, therefore only embryos of line 7.1 are depicted in Figure 13. The first expression pattern was detected in elongating embryos (Figure 13, A). Here, laterally in the head lobes, an elongated domain was strongly stained. In the median head region faint cellular expressions were detected (Figure 13, A`). During elongation, the lateral head domain as well as the median pattern were increased in intensity and spread out (black arrowhead, black arrow; Figure 13, B`). In the antennae and in the maxillae, faint expression domains arose *de novo* (open arrowhead, Figure 13, B` and Figure 13, B). In fully elongated embryos a faint distal expression pattern was detected in the appendages (Figure 13, C). This was located in the ectoderm and detectable distally in the labrum antennae, maxillae, labium and the legs, but not within the mandibles. Latter had two cellular expression domains on each side (open arrowhead, Figure 13, C`). The lateral head domain was condensed to a lateral posterior expression in a region suggested to form the larval eye. Surrounding this domain, several spots were detected (black arrowhead, Figure 13, C`). At later stages, dotted domains were spreading all over the body (Figure 13, C and C`). The distal superficial expression domains in the appendages could not be detected in older em-

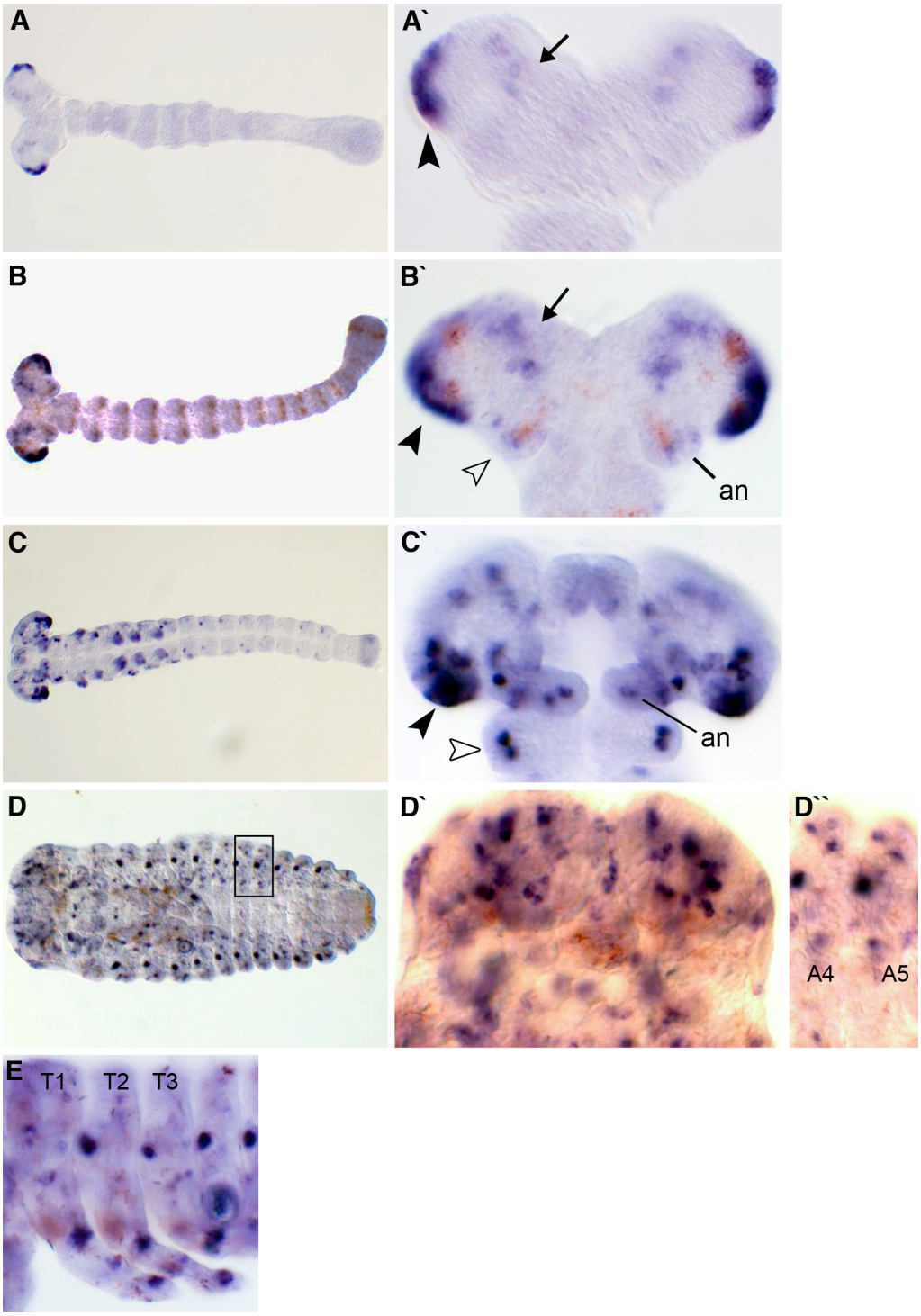

Figure 13: *In situ* hybridization with an antisense probe for tGFP in the transgenic line 7 containing the construct `*Tc-six3* 10up´.

(A) Elongating embryo with the tGFP expression pattern laterally in the head. (A`) Close up of the head, with the lateral expression domain (black arrowhead). The cellular median expression is pointed out by a black arrow. (B) Elongating embryo with exclusive expression in the head. (B`) The lateral head expression pattern is shifted laterally posterior (black arrowhead). The medial domain is diffuse and connected to the lateral domain (black arrow). Weak expression is detected at the antenna basis (open arrowhead). (C) Fully elongated embryo, predominant expression in the upper part of the body, with the signal covering the distal tips of appendages, except mandibles. (C`) Close up of the head with dotted expression domains within the head, especially in the lateral posterior region (black arrowhead). Segmental expression starting laterally in the mandibles (open arrowhead). (D) Approximately 48h old embryo, dotted expression over the entire body. (D`) Close up of the head, dotted domains scattered over the head. (D``) Close up of abdominal segments, dotted expression in lateral and dorsal tissue with a prominent medial expression domain. (E) Same aged embryo as depicted in F. Lateral view of the legs with posterior expression domains up to the tip. All embryos shown in total view are facing anterior to the left. All close ups to the head are facing anterior up.

bryos. Furthermore a complex spotty expression pattern covered the entire body (Figure 13, D). In the head, there were cellular expression domains (Figure 13, D`), which according to their location were possibly embedded in or part of the brain tissue. Interestingly, less expression was detectable ventrally in the embryo. The ventral abdomen was free of any expression domain, while a paired expression pattern was detected in the maxilla and first thoracic segment (Figure 13, D). However, the expression of tGFP was mainly located in lateral and dorsal tissues of the embryo. Here, many small spotty expressions could be detected, including one prominent, which in its location correlated to tracheal openings (Figure 13, D``). Such an expression domain was also visible in the third thoracic segment (T3), but this domain was smaller in comparison to the neighboring one. However, the first thoracic segment (T1) lacked such an expression domain. Additionally, dotted expression domains were detected posterior in the legs (Figure 13, E).

Thus, in comparison to the endogenous *Tc-six3* expression pattern, only few expression domains of the line 7 are correlative or overlapping. During elongation, the faint expression pattern of tGFP median in the head (black arrow; Figure 13, A` and B`) appears to be overlapping with the endogenous *Tc-six3* expression. Also in later stages, the cellular and spotty domains which are expressed in the labrum and in surroundings of the stomodeum, correlate to the endogenous *Tc-six3* expression (Figure 13, C` and D`). Interestingly, also the lines 7 appear to show a superficial and distal expression in the appendages, except the mandibles, only for a short period (Figure 13, C), like the line 6 (Figure 12). However, all the residual expression patterns of the line 7 do not show any domains correlating to the endogenous expression pattern of the gene *Tc-six3*. Rather, the pattern of line 7 is likely to have resulted from an enhancer trap.

The lines 7 were also analyzed for the fluorescence signal of tGFP in larval stages. Also here, the different sibling lines showed identical tGFP patterns. Therefore only line 7.3 will be described and is depicted in Figure 14. Single positive spots were detectable right below the cuticle and spread all over the head, which possibly indicated single cell bodies (open white arrowheads; Figure 14, B and C). Additional singular expression domains were detected around the oesophagus and in deeper sections tGFP positive cells could be detected laterally to the oesophagus (two anterior open white arrowheads; Figure 14, B). Also dorsally of the oesophagus, a group of single spots was present (open white square; Figure 14, C). In the anterior part of the head, a strong expression pattern projecting into the mandibles was detectable (white arrow; Figure 14, B).

Taken together, line 7 showed an embryonic expression pattern starting in the rim of the head. Also a distal expression pattern was detected in all appendages, except the mandibles. In later stages, the detected expression pattern became very complex with cellular as well as spotty domains spread

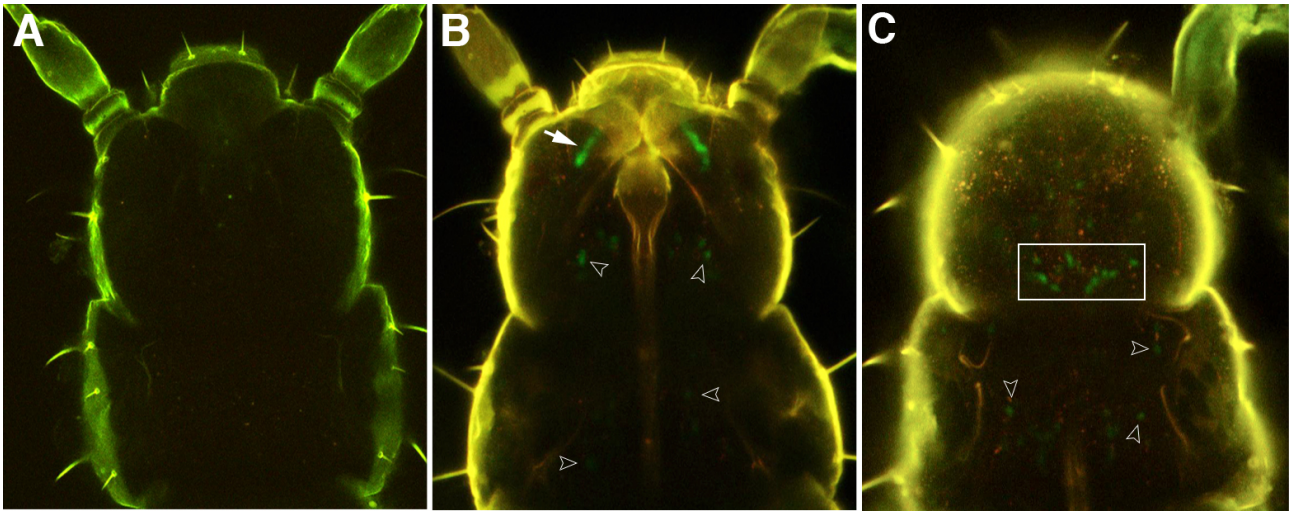

Figure 14: tGFP expression analysis in larvae of the transgenic line 7 carrying the construct `*Tc-six3* 10up´.
(A) As a control, a *Tc*-vermilion white larva in Green and Red was scanned. (B, C) Scans of line 7 transgenic for the construct `*Tc-six3* 10up´. (B) Single tGFP expressing cells within the brain region are indicated by open white arrowheads, strong expression projecting into mandibles (white arrow) (C) Scan close to the dorsal cuticle. Group of single cells positive for tGFP dorsal to the oesophagus (white square), additional single positive cells are spread all over the brain (open white arrowheads).

all over the body. In the larval brain, singular cells expressing tGFP were detected throughout the brain and especially dorsally of the oesophagus. Furthermore a bilateral strong expression was detected projecting into the mandibles.

Two independent G_0 were generated using the construct `*Tc-six3* 10up´. In case of line 6, the expression of the reporter gene tGFP was only found in *in situ* hybridization analysis. In case of line 7, tGFP expression was detected by *in situ* hybridizations and confocal scans. Comparing the two lines, only the distal superficial expression pattern in the appendages, except in the mandibles, and the expression in the lateral rim of the head lobes were common. This suggests, that these expression patterns are caused by an enhancer element included in the `*Tc-six3* 10up´ sequence.

Transgenic lines carrying the construct `*Tc-six3* 5up´

For the `*Tc-six3* 5up´ reporter construct (chapter 3.1.3.2), three lines from different G_0 with the serial numbers 8, 9, and 10 were generated. The offspring of the sibling lines originated by G_0 8 were negative for *in situ* hybridization and fluorescence analysis.

In case of G_0 9, only one line could be established. This line 9.1, showed a weak tGFP expression pattern in the labrum starting in elongating embryos (Figure 15, A and A`). After retraction, two expression domains could be detected in the labrum (Figure 15, B`) and furthermore a weak expression in the pleuropodia. In later stages, additional expression domains arose at the basis of the

labrum (open arrowhead; Figure 15, C`), while the other two expression domains in the labrum still remained (black arrowhead, Figure 15, C`). Furthermore, tGFP mRNA expression was detected in the proctodeum and in the pleuropodia (Figure 15, C). However, no fluorescence signal of tGFP was detected in larval offspring of line 9.1.

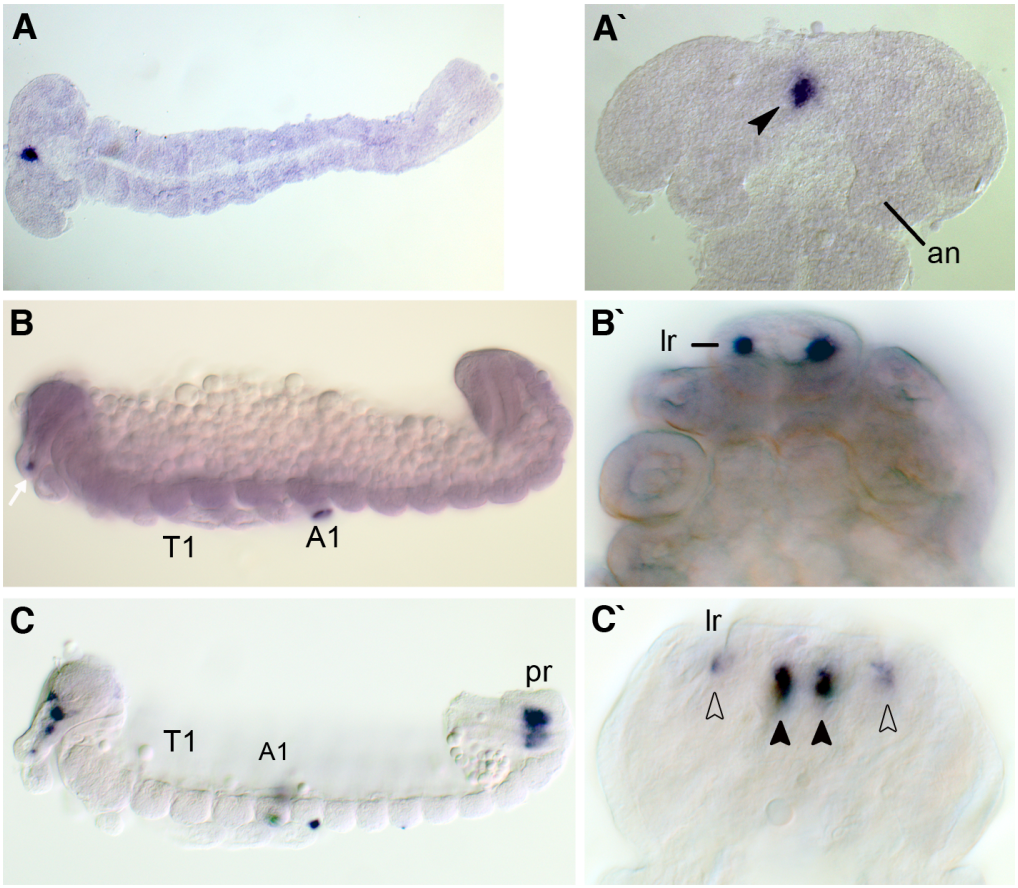

Figure 15: *In situ* hybridization with an antisense probe for tGFP in the transgenic line 9 containing the construct `*Tc-six3* 5up´.
(A) Ventral view of an elongating embryo with dotted expression in the anterior median head. (A`) Close up of the head, a black arrow points out the expression domain. (B) Lateral view on a fully retracted embryo. (B`) Ventral view of the labrum (lr) with two tGFP expression domains. (C) Lateral view of a approximately 30-35h old embryo with expression domains within the labrum and proctodeum (pr). (C`) Close up of the ventral view of the labrum with two stronger expression domains (black arrowhead) and weak expression domains (open arrowhead). All embryos shown in total view are facing anterior to the left. All close ups to the head are facing anterior up. Indicated ages correlate to the development at 32°C.

Two sibling lines were derived from G_0 10. Lines 10.2 and 10.3 showed identical expression patterns in *in situ* hybridization for tGFP. The expression pattern started in posterior pit stages, when the blastoderm was already differentiated and the germ rudiment started invagination. Here, the expression of tGFP was detectable in the ventral median of the germ rudiment (Figure 16, A) along the mesoderm. This expression pattern remained present in elongating embryos (Figure 16, B and C). This ventral domain fainted during elongation and a new expression domain arose in the developing labrum (Figure 16, C and C`). Eventually, the mesodermal expression pattern could not

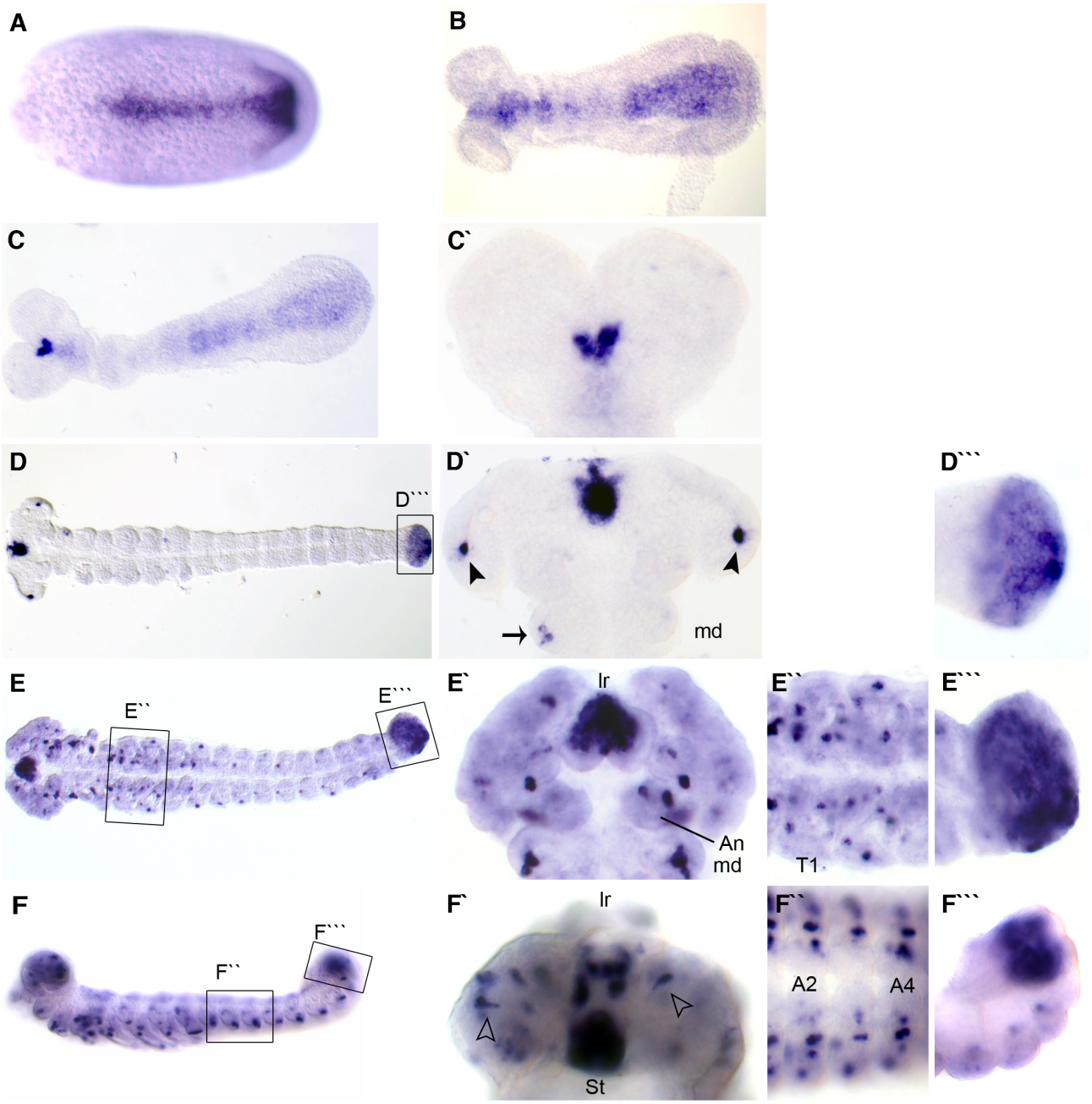

Figure 16: *In situ* hybridization with an antisense probe for tGFP in the transgenic line 10 containing the construct `*Tc-six3* 5up´.

In all embryos depicted in total view, anterior is facing to the left. (A-E) Embryos depicted ventrally, (F) depicted leterally. (C`-F`) Close up of embryonic heads oriented anterior upward. (A) Germ rudiment with a mesodermal expression pattern. (B) Early elongating embryo with a mesodermal expression pattern. (C) Fainting mesodermal expression pattern, arising of strong stomodeal expression. (C`) Close up of the head, with strong expression. (D) Almost fully elongated embryo, strong expression surrounding the labrum. (D`) Close up of the head, with strong expression surrounding the labrum basis. Point-shaped expression laterally in the head lobes (black arrowhead) and in the mandible (blak arrow) segment. (D```) Close up of the growth zone, with strong expression covering the posterior tip. (E) Fully elongated embryo before retraction, with a dotted segmental expression pattern and strong expression in the anterior and posterior tissue. (E`) Close up of the head, with strong expression in the labrum and dotted expression scattered over the head. (E``) Close up of the thorax T1 with a dotted expression pattern. (E```) Strong expression pattern covering the posterior tissue. (F) Lateral view of a retracted embryo, with a strong dotted expression pattern in the head and all segments. (F`) Strong expression at the interior stomodeal opening and in cells stretching out (open arrowheads).

(F``) Close up of the abdominal segments (A2-A4) with 4 dotted cell clusters lateral on each side. (F```) lateral view of stomodeum.

be detected in fully elongated embryos (Figure 16, D). The labral domain grew in size and in the lateral rims of the head lobes two point-shaped domains arose (black arrowhead, Figure 16, D`). The proctodeum was also positive for tGFP expression (Figure 16, D```). Only in the mandibles a faint spotty expression pattern was present (black arrow; Figure 16, D), while a complex expression pattern arose in later stages (Figure 16, E and F). In embryos close to retraction, a strong expression was present centrally in the labrum (Figure 16, E`) and scattered domains were arising in the head and spreading posterior. Within the thorax, each segment had a complex pattern (Figure 16, E``). Strong expression of tGFP mRNA was detected at the surface layer of the proctodeum (Figure 16, E```). In older embryos, the segmental dotted expression pattern was present in all segments (Figure 16, F). The labral domain was retracted to the interior part of the stomodeum, and in the labrum two point-shaped domains were present distally (out of focus). Cells positive for tGFP expression were bottle shaped and expanded interior (open arrowhead, Figure 16, F`). Within the appendages, a group of dotted domains was detected in the distal part and only single domains were detected proximally. In the proctodeum, tGFP mRNA was expressed strongly (Figure 16, F```).

Contrary to the strong expression pattern detected by *in situ* hybridization, no signal could be detected by confocal scans in the respective L1 larvae.

Taken together, lines 9 and 10, which were derived from two G_0, showed common tGFP expression patterns in the labrum and in the proctodeum. While the labral expression possibly reflects endogenous *Tc-six3* activity, the activity in the proctodeum is not seen in anti *Tc-six3 in situ* hybridization experiments.

Transgenic lines carrying the construct `*Tc-six3* 5down´

Two G_0 animals with the reporter construct `*Tc-six3* 5down´, numbered 2 and 3, could be created by transgenesis. From each G_0, three sibling lines were generated by crossings with Tc-vermillion. However, the sibling lines originated from the parental G_0 2 were negative for *in situ* hybridization and for fluorescence analysis of the reporter gene.

Interestingly, the same was found for the lines 3.1 and 3.2. Only the third crossing (3.3) was positive for *in situ* hybridization analysis for tGFP. Here, a pair rule gene like expression pattern was observed during elongation, starting in early germ bands close to the serosa and amnion fusion (Figure 17, A). This expression pattern remained present until the closure of the serosa window

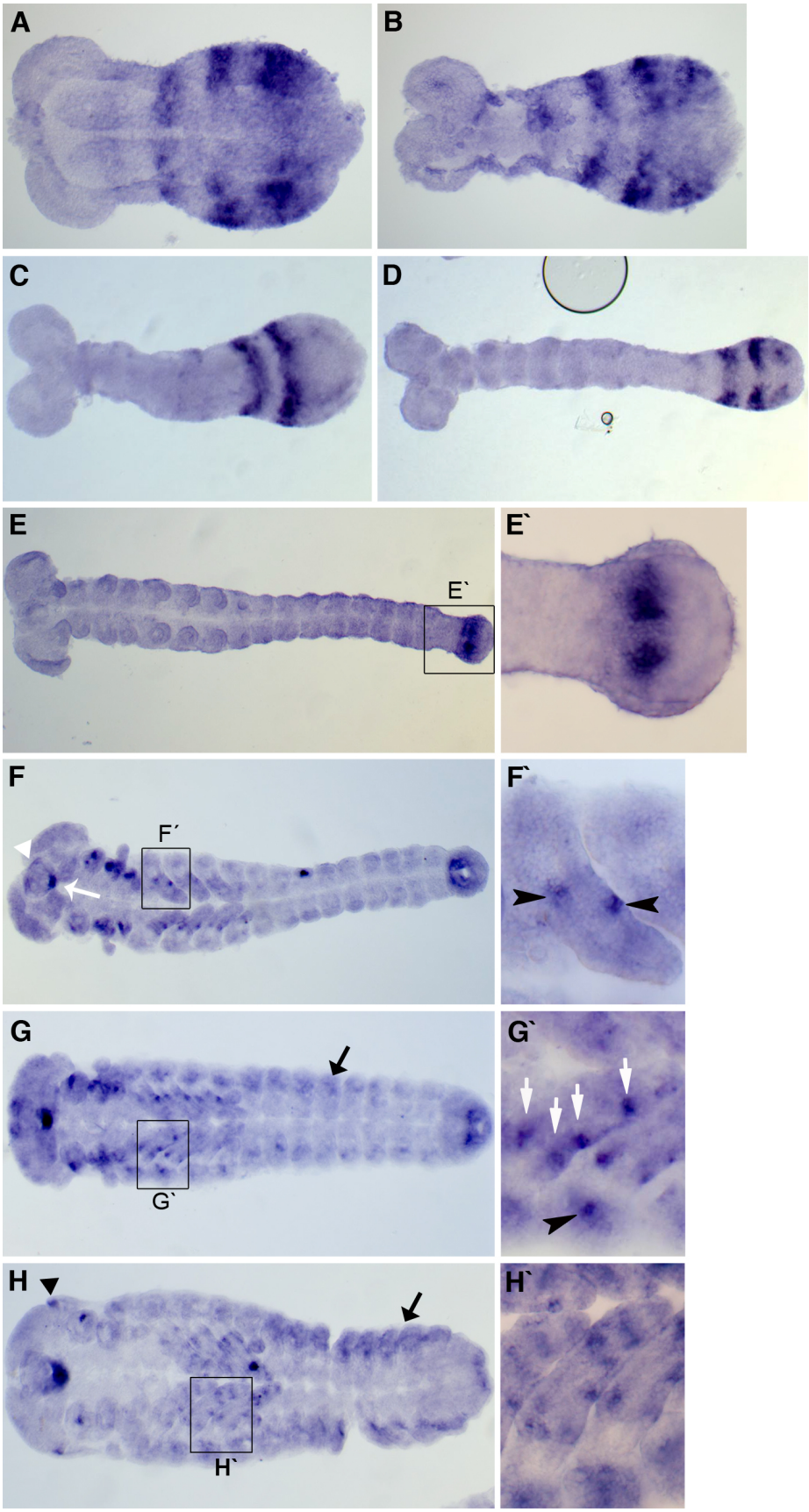

Figure 17: *In situ* hybridization with an antisense probe for tGFP in the transgenic line 3 containing the construct `*Tc-six3* 5down´.

(A) An approximately 10h old embryo with a pair rule gene like expression pattern. (B) An approximately 12h old embryo with a pair rule gene like expression pattern. (C, D) Approximately 14h and 16h old embryos with pair rule like expression patterns. (E) An approximately 18h old embryo with an expression domain in the putative proctodeum anlage. (E`) Close up of the putative proctodeum anlage. (F) An approximately 24h old embryo with an expression pattern anterior in the labrum (white arrowhead) and labrum basis (white arrow), as well as in the gnathal buds. Dotted expression pattern in the legs and roundish expression domain in the proctodeum. (F`) Close up of the leg. (G) 30-35h old embryo with a similar expression pattern compared to the embryo depicted in F; further domains arise segmentally in dorsal tissue (black arrow). (G`) Dotted expression pattern in the legs (white arrows) and in dorsal tissue of the thorax segment T2 (black arrowhead). (H) An approximately 45-50h old embryo with a similar expression pattern as the embryo depicted in G and in addition point-shaped expression laterally in the head (black arrowhead), stronger expression in dorsal tissue. (H`) Close up of legs with complex expression pattern. All embryos shown in total view with the anterior facing to the left, all close up images with the anterior facing to the left. Indicated ages correlate to the development at 32°C.

(Figure 17, B). During elongation, a striped expression pattern was detectable only in the posterior segmentanlagen close to the growth zone (Figure 17, C and D). This pattern could not be detected in fully elongated embryos. In these stages, the tGFP expression was observed only in the proctodeum (Figure 17, E and E`). A posterior expression pattern surrounding the proctodeum opening remained detectable throughout all developmental stages (Figure 17, F, G, and H). De novo domains arose in retracting embryos. Expression in the gnathal appendages (Figure 17, F) as well as a spotty expression pattern, which arose in the legs were detected (Figure 17, F`). Furthermore, a small expression domain appeared around the stomodeum (white arrow; Figure 17, F) and increased in size during development (Figure 17, G and H). In dorsal tissue a superficial expression arose and spread from anterior to posterior (black arrow; Figure 17, G). In the legs, the expression domains were more dotted (Figure 17, G`) and were more complex in older embryos (Figure 17, H`). The expression in dorsal tissue shifted posterior and the gnathal expression pattern could no longer be detected, except in the mandibles (Figure 17, H).

Considering the fact that only one line was generated, it is hard to distinguish wethter the expression pattern is influenced by enhancer elements of the construct `*Tc-six3* 5down´ or by local enhancer elements close to the insertion side. Nevertheless, this line does not show the expression pattern of *Tc-six3*.

3.3.3. *Tc-rx* reporter lines

Parallel to the *Tc-six3* reporter lines, transgenic lines for the gene *Tc-rx,* expressing the reporter gene DsRedExpress (DsRedEx) in the same pattern, were to be generated. To this end, the endogenous promoter was analyzed and the 5´UTR was identified by two independent RACE-PCR experiments (chapter 2.2.). The regulatory regions were amplified by PCR of gDNA of the SB strain. The respective sequences were analyzed for gene predictions and repetitive sequences. Finally, the generated transgenic lines were analyzed regarding their expression pattern of the reporter gene during embryonic stages as well as in the larval brain.

3.3.3.1. Generation of the *Tc-rx* reporter lines

The analysis of the 5´UTR of *Tc-rx* by RACE-PCR revealed a total length of 19 bp. *Tc-rx* has a typical insect TSS sequence (reviewed in Butler and Kadonaga, 2002; highlighted blue, Figure 18, Panel A). Further studies identified a downstream promoter element at position +28 relative to the TSS (DPE; highlighted blue, Figure 18, Panel A). This element was located within the first ORF of *Tc-rx.* To include the DPE within the endogenous promoter element, a primer, which also replaced

the endogenous translation start site ATG with ATA, was designed (black line, Figure 18, Panel A). Hence, an element of 625 bp was cloned as endogenous promoter and was fused with all *Tc-rx* reporter constructs (purple line under 5up construct; Figure 18, Panel B) except `*Tc-rx* 5up´, where this region was already included (purple line; Figure 18, Panel B).

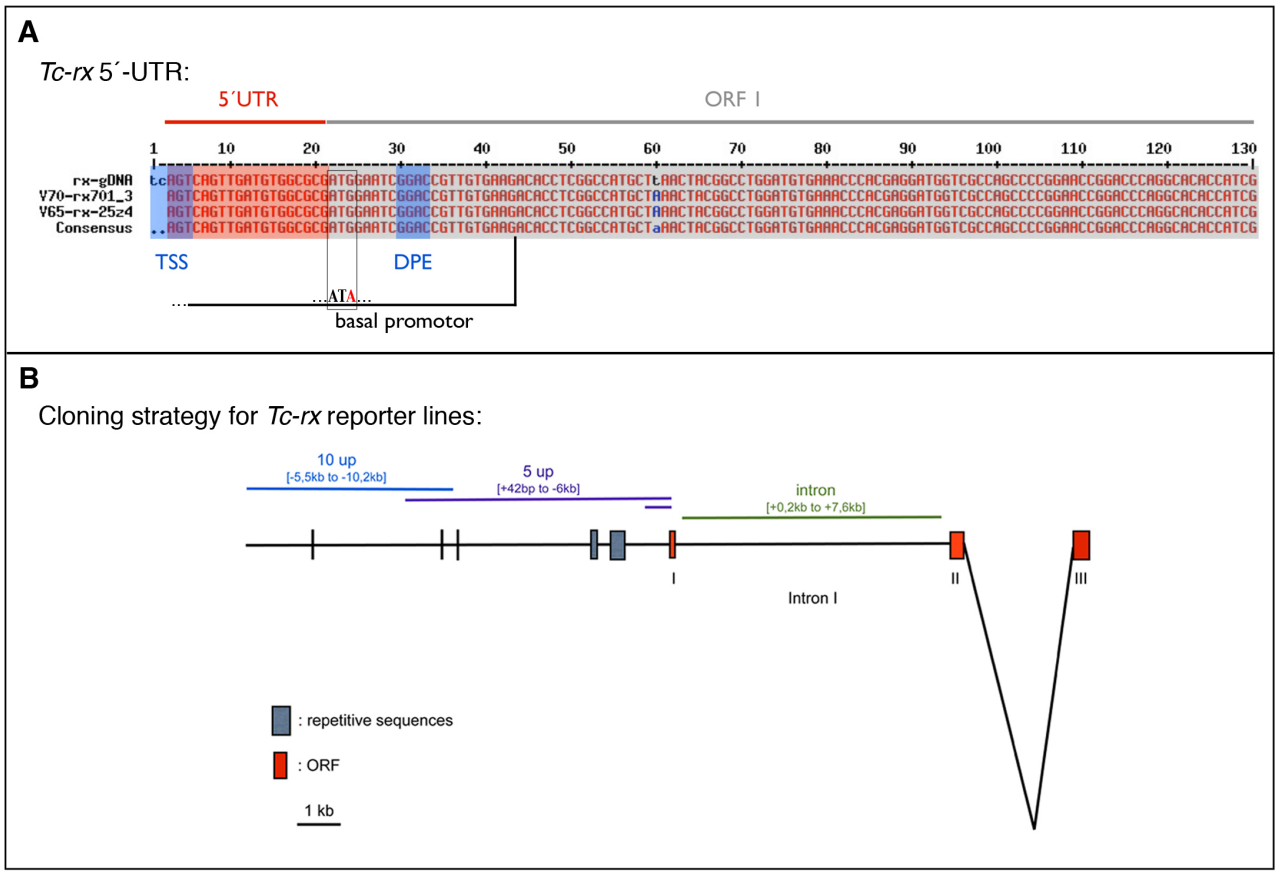

Figure 18: The 5`UTR and the cloning strategy for the transgenic reporter lines of *Tc-rx*.
(A) The 5`UTR of *Tc-rx* is 19 bp long. It has a transcription start site (TSS) with the typical consensus sequence. A downstream promoter element (DPE, highlighted blue) was found +28 bp of the TSS, within the ORF1 (highlighted gray). The DPE was included in the basal promoter (black line) and the native translation start site was disrupted by replacing G with A. (B) A basal promoter of 625 bp was cloned including the entire 5`UTR and the DPE (depicted as a shortened line under the 5up construct). The sequences 10up and 5up overlap by 577 bp. The regulatory regions were combined with the endogenous basal promoter driving DsRedExpress, except for the construct `*Tc-rx* 5up´, which already included the basal promoter. The `*Tc-rx* Intron´ was cloned in front of the basal promoter. Transgenic lines for various *Tc-rx* reporter gene constructs were generated: lines with the construct `*Tc-rx* 5up´ (highlighted in purple) and lines with the construct `*Tc-rx* Intron´ were generated and tested positively in *in situ* and *in vivo* experiments regarding DsRedEx expression. No transgenic line was generated with the construct `*Tc-rx* 10up´ (highlighted in blue). (A) 5`UTR is highlighted in red, Exon regions are highlighted in gray, and TSS elements highlighted in blue; (B) Repetitive sequence regions are depicted in gray boxes; ORFs are depicted in red boxes; predicted gleans are depicted in pink boxes.

The sequence for the construct `*Tc-rx* 10up´ started -10248 bp and ended -5491 bp upstream of the translation start site of *Tc-rx* (TLS; blue line; Figure 18, Panel B). It had a total size of 4757 bp and overlapped for 577 bp with the adjacent `*Tc-rx* 5up´ sequence. The sequence for the construct `*Tc-rx* 5up´ started -6068 bp upstream of the TLS of *Tc-rx* and ended +22 within the ORF 1 of *Tc-rx* (purple line; Figure 18, Panel B). The construct `*Tc-rx* Intron´ was located within the first intron of *Tc-rx* and was 7598 bp long. Nearly the complete first intron was included in the construct, starting at +213 bp and ending +7611 bp downstream of the TLS of *Tc-rx* (green line; Figure 18, Panel B), 174 bp upstream of ORF II.

Control sequencing revealed no disorders. Transgenic offspring from one G_0 animal for the cassette for `*Tc-rx* 5up::DsRedEx´ and one $G_0$ for the cassette for `*Tc-rx* Intron::DsRedEx´ could be generated. However, no animal was successfully transformed with the cassette for `*Tc-rx* 10up::DsRedEx´.

3.3.3.2. Analysis of transgenic animals carrying the *Tc-rx* reporter line constructs

For analysis of the functionality of the constructs in transgenic animals, *in situ* hybridization with an antisense probe for DsRedEx was performed. Therefore, egg lays were staged 0-24h, 24-48h, and 48-72h at 32°C. Additionally, the first larval instar brains (L1) of individuals of the transgenic lines were scanned for the fluorophor DsRedEx.

Transgenic lines carrying the construct `*Tc-rx* 5up´

In the offspring originating from the G_0 1, two animals were positive for the transfection marker carrying the cassette `*Tc-rx* 5up::DsRedEx´ and were established into sibling lines. Interestingly, only line 1.2 was positive for the reporter gene DsRedExpress, indicating independent transposition events.

The expression of DsRedEx mRNA started late in development. The first expression was detected in embryos with approximately 30-40h. The expression pattern was exclusively present in the head (Figure 19, A). Three paired expression domains were detected. One median domain was present close to the surface in the dorsal part of the head (open black arrowhead; Figure 19, A``). This domain expanded interior ventrally. At deeper levels, two lateral domains, which were located along the anterior-posterior axis, were observed (white arrowheads, Figure 19, A`). In later stages, these domains were shifted posterior in parallel to the lateral axis (white arrowheads; Figure 19, B`) and lateral to the median domain, where they showed a cellular expression (open black arrowhead; Figure 19, B`). A *de novo* expression domain arose at the ventral anterior segment boundary of the

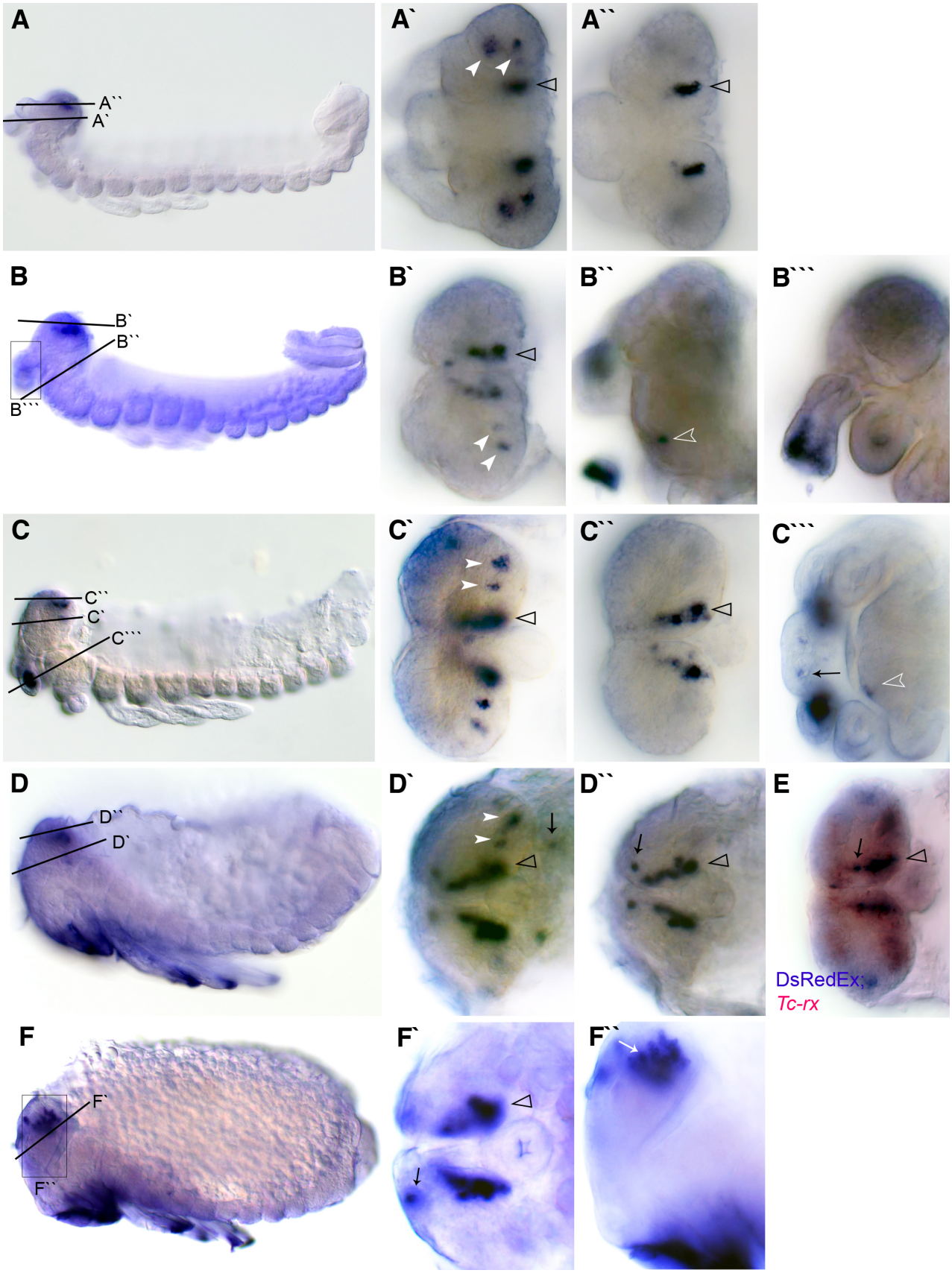

Figure 19: *In situ* hybridization with an antisense probe for DsRedEx in the transgenic line 1 containing the construct `*Tc-rx* 5up´.

(A-E) Lateral views. Horizontal cross sections are indicated by lines, lateral cross sections are indicated by squares. Lateral expression domains are indicated by white arrowheads. The median expression domain is indicated by an open black arrowhead. Arrows and arrowheads are only shown in one hemisphere. All indicated ages correlate to the

development at 32°C. (A) The expression pattern was first detectable in 30-40h old embryos. (A`) Horizontal cross section of the lateral expression domains. (A``) Horizontal cross section at the surface with a median expression domain. (B) 40-50h old embryo. (B`) Horizontal cross section with unchanged median and changed lateral expression domains. (B``) Symmetric expression domain at the anterior tip of the mandibular segment (open white arrowhead). (B```) Lateral cross section with an expression domain at the distal tip of the antenna. (C) 50-60h old embryo. (C`) Horizontal cross section with lateral expression domains. (C``) Horizontal cross section of the median expression domain at the surface. (C```) Ventral view with expression pattern at the tip of the mandibular segment (open white arrowhead). The distal tip of the antennal expression domain is indicated (out of focus). Two domains arise de novo in the labrum (black arrow). (D) Embryo older than 60h. (D`) Close up of the lateral expression domains, the mandibular expression domain indicated by a black arrow. (D``) Two domains arise *de novo* (black arrow) anterior of the median expression domain. (E) Double *in situ* hybridization with antisense probe for *Tc-rx* (red) and DsRedEx (blue). *Tc-rx* is expressed in the epidermis above the median expression domain. (F) Embryo older than 65h. (F`) Median expression domain with anterior expression domain (black arrowhead). (F``) Lateral cross section with the median expression domain and elongated cells (white arrow).

mandibular segment (open white arrowhead, Figure 19, B``). Furthermore, strong expression of the reporter gene was found in the distal part of the antennae (Figure 19, B```). The expression pattern did not change much in older stages (Figure 19, C). Here, the paired domains laterally in the head were detectable (white arrowheads; Figure 19, C`), the dotted median domains expanded over several z-sections (open black arrowhead; Figure 19, C``), the distal expression in the antennae got stronger (Figure 19, C and C``) and the dotted domain remained ventral anterior at the segment boundary of the mandibular segment (open white arrowhead, Figure 19, C```). Two cellular expression domains in the labrum were additionally found (black arrow, Figure 19, C```). The expression of DsRedEx mRNA remained restricted to the head and gnathal segments, even in embryos older than 60h (Figure 19, D). A small point-shaped expression arose anterior to the median expression pattern, which in the meanwhile had resolved into several cellular expression domains (open black arrowhead; Figure 19, D``). The stages that started with secretion of the cuticle developed background staining (Figure 19, D and F). Hence, in embryos older than 65h, the mandibular and labral expression was not detectable anymore, due to the background. The median expression domain was still located to brain tissue (open black arrowhead; Figure 19, F`) and consisted of cellular expression domains including anterior spots (black arrow; Figure 19, F`). Cells positive for DsRedEx expression within the brain tissue showed an elongated shape (white arrowhead; Figure 19, F``). DsRedEx mRNA was still detectable in embryos close to hatch.

Double *in situ* hybridizations with antisense probes for *Tc-rx* and the reporter gene DsRedEx were performed in embryos of the transgenic line `*Tc-rx* 5up:.DsRedEx´. Even though the obtained results were preliminary, the median expression domain (open black arrowhead; Figure 19; E) was

located right below the superficial expression pattern of *Tc-rx* (Figure 19, E). Also the anterior small roundish domain in front of the median domain overlapped with *Tc-rx* expression (black arrow; Figure 19, E). Due to the limitations of light microscopy in deeper layers, a colocalization analysis based on confocal scans needs to be performed. Here, fluorescently labeled antibodies could be used to stain the proteins of *Tc-Rx* and DsRedEx. Therby an analysis of putative colocalization of both proteins in the cells of the median domain in the `*Tc-rx* 5up´ line would be possible.

A fluorescent signal was detected in the L1 brains of individuals of the `*Tc-rx* 5up´ line. Posterior dorsal groups of cells sent projections towards anterior (white square, Figure 20, A). This projection crossed the interhemispheric brain fissure, thereby forming a chiasma (white arrowhead; Figure 20, A). Singular expression domains are detectable throughout the head, in most cases suggested to be neural tissue. Paired groups of cells positive for DsRedEx were detectable under the cuticle surface in the labrum (white arrow; Figure 20, C). A second, bigger and paired group of cells was located anterior of the cervix (white arrow; Figure 20, A and B) and an additional group of cells was found posterior (white arrow; Figure 20, A` and B`). A further signal was detectable within the brain, with no cellular, but thin projections (open white arrowhead; Figure 20, A and A`). A cellular expression pattern with thin elongations was observed within the antennae (Figure 20, A``). For a better allocation of the DsRedEx signaling caused by the `*Tc-rx* 5up´ reporter construct, crossings with other brain imaging lines were performed. For this, the line 6xP3::eCFP and the `Mushroom Body´ (`MB´) line, that originated from the GEKU-screen (chapter 3.1.4), were chosen.

The posterior group of cells forming the chiasma (white square; Figure 20, A) was embedded by cortex glial net like tissue (white square; Figure 20, B) and located median posterior to the endpoint of the vertical lobe of the Mushroom Body (white square; Figure 20, C). The position of the chiasma lay dorsally within the brain, above the median lobe (white arrowhead; Figure 20, C). This region, as well as the group of cells anterior to the cervix, was also covered by a net of cells which were glial cells (white arrow; Figure 20, B). This was also true for those cells embedded in the posterior glial tissue (white arrow; Figure 20, B`), surrounding the Kenyon cells of the Mushroom Body (Figure 20, C`). The thin projections within the brain described above were surrounding the Central Body neuropile. A stronger signal was detected anterior of the Central Body, expanding laterally in the protocerebrum (open white arrow; Figure 20, B` and C`), which could represent parts of the lateral accessory lobes. A DsRedEx signal could only be detected surrounding the Kenyon cells, but was not detected within the Mushroom Body (Figure 20, C`). Finally, the antennal cellular DsRedEx positive cells surrounded the axon bundles marked by 6xP3::eCFP (Figure 20, B``).

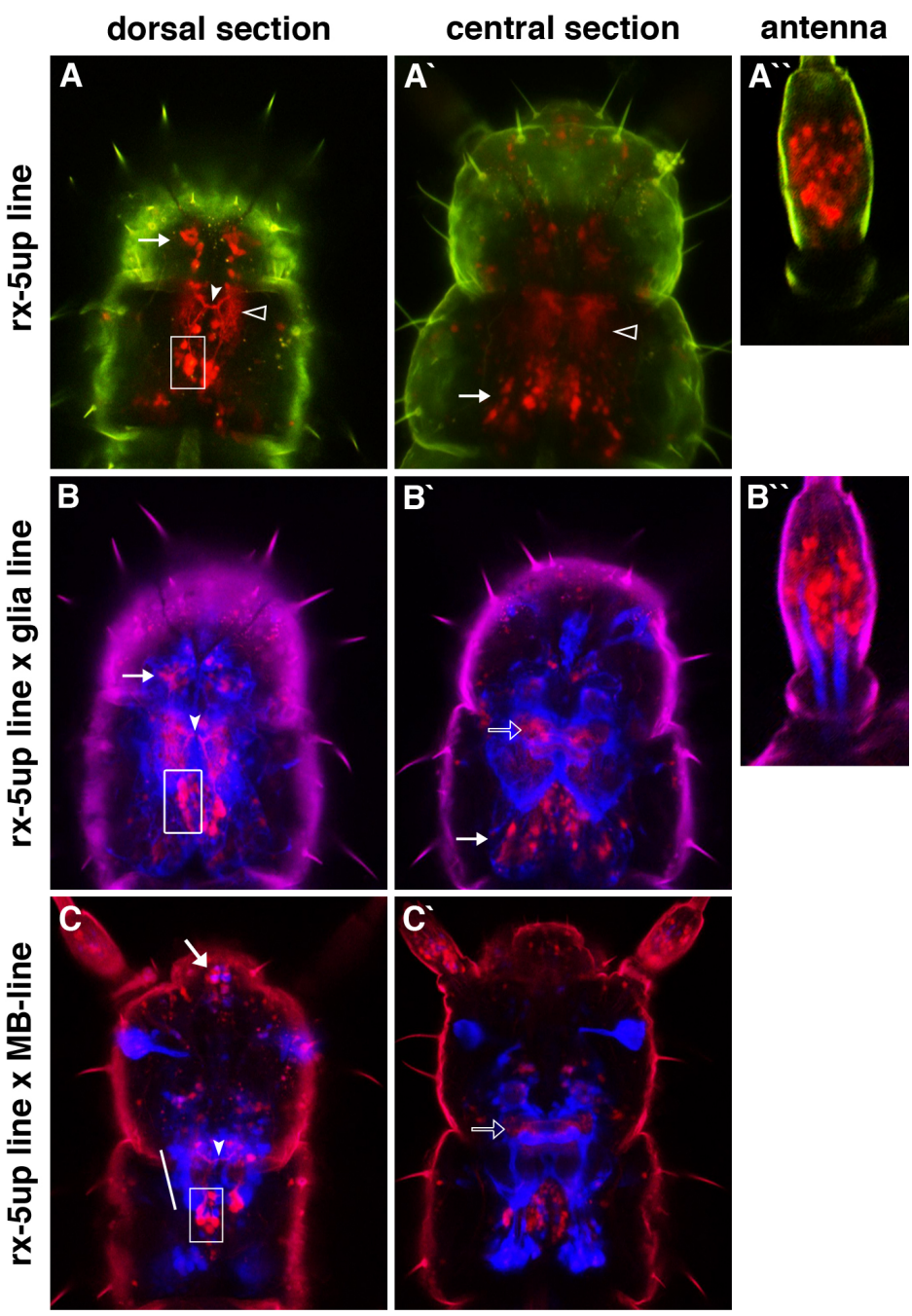

Figure 20: Analysis of reporter gene expression in the brain of a larva transgenic for `*Tc-rx* 5up´. (A-A``) The DsRedEx signal in the transgenic line `*Tc-rx* 5up´. (A) Dorsal sections of the brain; groups of cells projecting towards anterior (white square), forming a chiasma (white arrowhead); a net of thin projections is indicated by an open white arrowhead. (A`) Deeper section of the brain; groups of cells posterior in the brain (white arrow); net of thin projections indicated by open white arrowhead. (A``) Zoom into the antenna. (B-B``) Crossing of the lines `*Tc-rx* 5up::DsRedEx´ shown in red and 6xP3::eCFP shown in blue. (B) Dorsal sections of the brain; groups of cells forming the chiasma surrounded by glia (white square); chiasma (white arrowhead); anterior group of cells (white arrow). (B`) Deeper section of the brain; posterior group of cells surrounded by glia (white arrow); putative lateral accessory lobes (open white arrow) (B``) Zoom into the antenna, blue axon bundle within cell bodies positive for DsRedEx. (C-C`) Crossing of the lines `*Tc-rx* 5up::DsRedEx´ shown in red and the `MB´ line depicted in blue. (C) Dorsal section of the brain; the vertical lobe, parallel blue structure to white line; labral group of cells (white arrow); chiasma (white arrowhead) (C`) Deeper section of the brain; putative lateral accessory lobe (open white arrow). Anterior is facing up.

Taken together, DsRedEx was first detected in embryos of the line `*Tc-rx* 5up::DsRedEx´ at an age of approximately 30-40h by *in situ* hybridization eperiments. The exclusive expression pattern in the head and the gnathal segments is suggested to mark specific groups of cells throughout development and even so in L1 brains. The fluorescence signal of DsRedEx revealed a complex but

distinct expression pattern within the larval brain. The posterior group forming the chiasma could possibly be part of the pars intercerebralis and/or the pars lateralis. However, these cells probably derived from the median domain during embryonic stages, which were overlapped by *Tc-rx* expression. This specific pattern suggests the presence of a specific enhancer element within the reporter construct `*Tc-rx* 5up´. However, due to the lack of comparative lines, influences of the insertion locus on the expression pattern of DsRedEx cannot be excluded. Nevertheless, the line `*Tc-rx* 5up::DsRedEx´ represents a potentially valuable imaging line for further studies regarding the observed lineage positive for DsRedEx as well as additional aspects, e.g. axogenesis.

Transgenic lines carrying the construct `*Tc-rx* Intron´

For the construct `*Tc-rx* Intron´, it was possible to identify marked offspring from one G_0 11. From this G_0, 3 sibling lines were generated 11.1, 11.2 and 11.3. These showed DsRedEx mRNA expression in the embryo and a fluorescent signal in larval stages.

All sibling lines showed a range of diverse but in principle similar expression patterns. Therefore only line 11.1 and the most representative embryos are described here. The expression of DsRedEx mRNA was first detected in old embryos. In elongated embryos, a group of cells at the basis of the labrum was positive for DsRedEx mRNA (Figure 21, A`). A weak dotted expression started to be detectable laterally in the gnathal appendages (Figure 21, A``) as well as in the legs (Figure 21, A```). In additional embryos, single domains positive for DsRedEx mRNA were found in abdominal segments or even in the proctodeum (not shown).

In embryos that had finished the retraction, more domains, which in some cases spread all over the embryo, were detected. The labral expression pattern elongated laterally anterior (black arrow; Figure 21, B`). At the same level laterally in the head, two point-shaped, in most cases paired domains were often detected (black arrowhead; Figure 21, B`). Laterally in the gnathal appendages, single spots were detectable (Figure 21, B``), while similar spotty expression domains were localized anterior in the legs (Figure 21, B```). Single domains were spread all over the body in an irregular fashion (not shown). This was also observed for the expression pattern in the head. After retraction, the labral domains fused proximally (Figure 21, C`). The expression pattern in the appendages became more superficial but remained in the anterior part (Figure 21, C`` and C```). In old embryos (Figure 21, D), dotted expression domains were spread all over the body. Those in the appendages were most prominent and at the labrum basis a group of smaller expression domains formed a belt (black arrowhead; Figure 21, D`). Paired expression domains were found in the antennae (Figure 21, D`) and strong expression was also detected in the distal parts of the gnathal

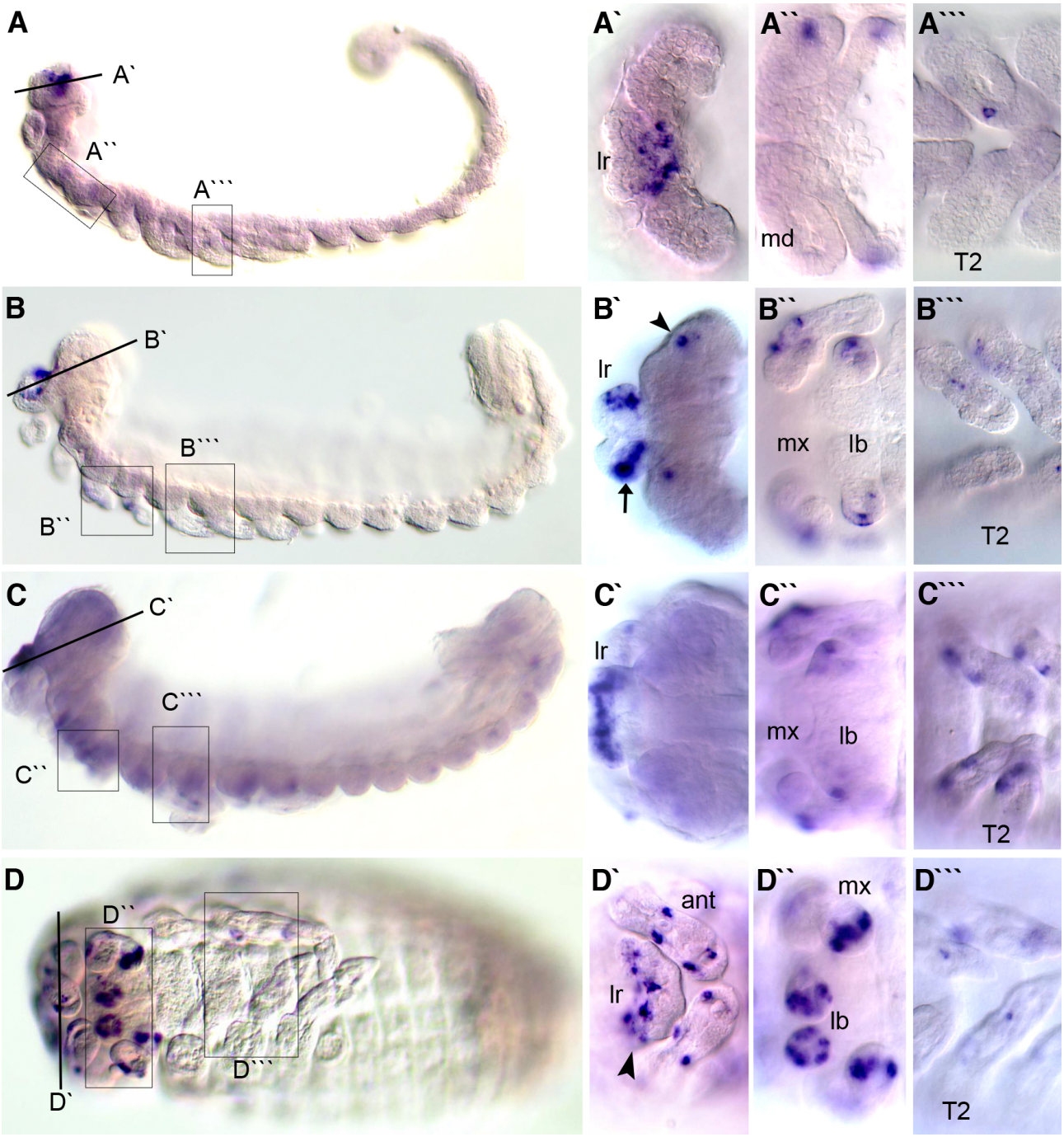

Figure 21: *In situ* hybridization with an antisense probe for DsRedEx in the transgenic line 11 containing the construct `*Tc-rx* Intron´.

(A-C) Lateral views of embryos of different age, except (D) which shows a ventral view. (A) An approximately 24h old embryo, where the first expression pattern was detectable in the head. (A`) Horizontal cross section: expression pattern in the basis of the labrum (lr). (A``) lateral expression domains within the gnathal segments mandibel (md) and maxilla (mx). (A```) The dotted expression domain anterior in the leg bud T2. (B) Embryo 25-30h old. (B`) Horizontal cross section; lateral expression in the lr (black arrow) and two point-shaped expression domains within the head (black arrowhead). (B``) Expression domain anterior in the gnathal segments mx and labium (lb). (B```) Small expression domain in the leg. (C) Embryo approximately 40-45h old. (C`) Horizontal cross section; fused labral expression pattern. (C``) Anterior expression pattern in the mx and lb. (C```) Split dotted expression pattern anterior in the legs. (D) Embryo approximately 55-60h old. (D`) Frontal view of the lr and antennae (ant), dotted expression patterns in the lr

basis (black arrowhead) and ant. (D``) Expression patterns distal in the tips of the mx and lb. (D```) Weak expression in the legs. In all pictures anterior are facing to the left. All indicated ages correlate to the development at 32°C.

appendages (Figure 21, D``). Interestingly, the expression pattern in the legs reflected the pattern detected in the antennae (Figure 21, D```). In some cases, a superficial expression was observed in the dorsal tissue. This was located one-sided along the anterior-posterior axis in the dorsal tissue in a different region in each embryo (not shown).

The expression patterns described here represent those which all lines derived from G_0 11 have in common. In comparison to the expression pattern of *Tc-rx,* none of the described expression patterns in the lines 11.1 - 11.3 showed a correlation, except the labral one. However, despite the difference of the embryonal expression patterns of the lines 11.1 - 11.3, the fluorescent signals within L1 heads were surprisingly similar. Therefore only one individual of line 11.1 will be described here. Cellular expression was scattered within the head capsule and probably within the brain (Figure 22, B). Some of these domains were connected with each other. Especially in the median region of the protocerebrum, very weak superficial domains were detected (open white arrowheads; Figur 22, B and C). Slightly anterior to the cervix, a group of DsRedEx positive cells formed a globular structure through their projections (white arrow, Figure 22, C). Cellular expression, possibly displaying the soma of sensory neurons, was found in the labrum (Figure 22; B`) and in the antennae (Figure 22; C`). From these cellular expressions, projections, which were detected in the tip of the antennae and in the head, arose (Figure 22, C`). The latter terminated in a network of DsRedEx positive tissue (white arrowhead; Figure 22, B). The most prominent expression domain was found within the labrum (white square; Figure 22, B). Putative sensory neurons were projecting to the distal tip of the labrum (Figure 22, B`). The projections into the head ended near those of the antennae, but not in the same structure (not shown). For further analysis, detailed localization by crossings with the glial line 6xP3 or with the `MB´ line are suggested.

Taken together, the sibling lines 11.1 - 11.3 showed a similar fluorescent pattern in the larval head, which only partially correlated with the DsRedEx mRNA expression found within the embryos. The variability of the embryonic expression pattern suggests that either more constructs were integrated in the genome or each sibling displays independent integrations. Both cases should be tested by inverse PCR. Further studies by crossings with the glial marker line and the `MB´ line could be performed, and by this the expression domains in the larval head could possibly be related to neuromeres or neuropiles. However, only one G_0 could be generated and therefore it is hard to

elucidate whether the described patterns are influenced by enhancer elements surrounding the integration locus or present in the `*Tc-rx* Intron´ construct.

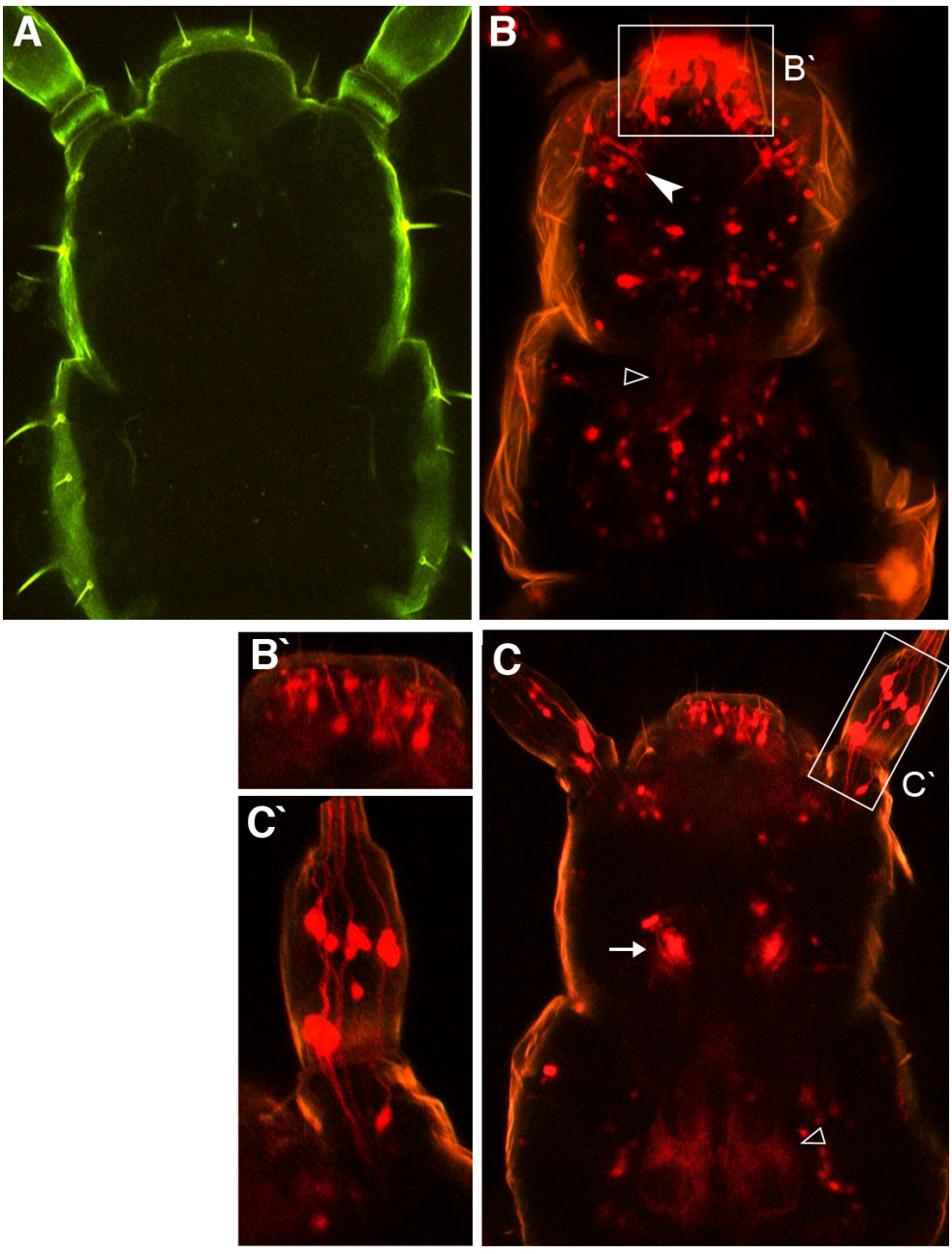

Figure 22: Analysis of reporter gene expression in the brain of a transgenic larva for `*Tc-rx* Intron´.
(A) As a control, a *Tc*-vermilion larva was scanned in green and red. (B - C) Scans of a `*Tc-rx* Intron´ positive larva. (B) Dorsal section of the head; scattered expression domains within the brain region and strong antennal projections (white arrowhead). Superficial domain, probably formed by thin projections (open arrowhead) (B`) Close up of the labrum with single cell bodies and projections. (C) Deeper section within the head; with cells form a globular domain (white arrow). Superficial domain, which is putatively formed by thin projections (open white arrowhead). (C`) Close up of the antenna, single positive cells with projections. Anterior facing up.

3.3.5. Verification of transgenic reporter lines by PCR and schematic summary of the characterization of the transgenic animals

At least 29 putative lines were analyzed regarding their reporter gene expression patterns in embryos by *in situ* hybridizations as well as in larvae by confocal scanning for fluorescent signals in the larval head. However, only 13 lines showed expression of the respective reporter gene in embryonic stages. From those, only 8 lines showed a fluorescent signal in the larval head (compare Figure 24). Thus, PCR experiments on genomic DNA of transgenic animals were performed (Figure 23) in order to clarify, whether the additional G1 with black eyes but without expression pattern in the embryo or in the larva, were false positive or whether no signal could be detected due to low expression of the reporter gene caused by the integration site.

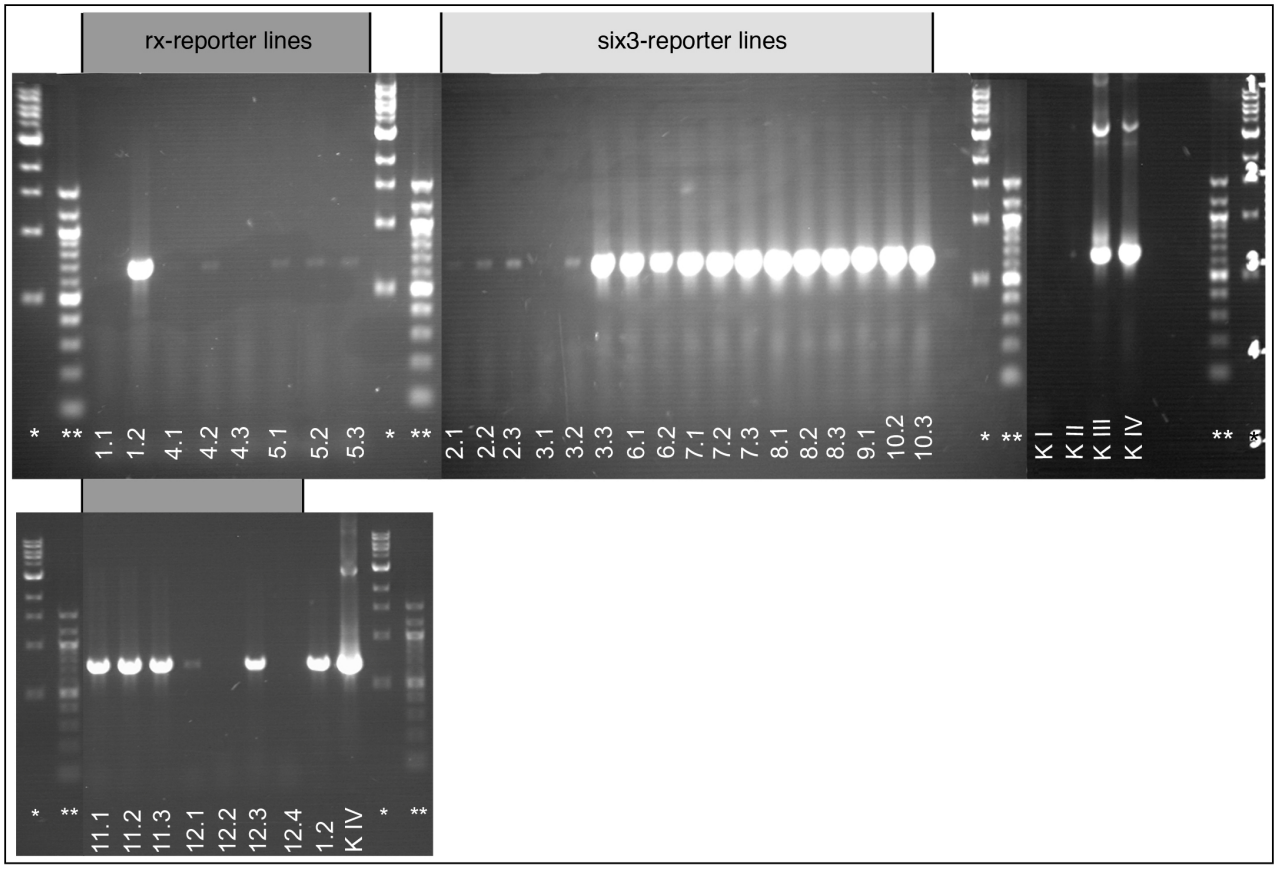

Figure 23: PCR analysis of *Tc-rx* and *Tc-six3* reporter lines by screening gDNA for reporter gene insertion.
Lines 1.1 and 1.2 carried the construct `*Tc-rx* 5up´; lines 4.1 - 4.3 and 5.1 - 5.3 should carry the construct `*Tc-rx* 10up´; lines 11.1 - 11.3 and 12.1 - 12.4 should carry the construct `*Tc-rx* Intron´. The reporter gene DsRedEx is not included in the gDNA of lines 4.1 - 4.3, 5.1 - 5.3, and 12.1, 12.2, 12.4. Lines 2.1 - 2.3 and 3.1 - 3.3 carried the construct `*Tc-six3* 5down´; lines 6.1 - 6.2 and 7.1 - 7.3 carried the construct `*Tc-six3*-10up´; lines 8.1 - 8.3, 9.1, 10.2 and 10.3 carried the construct `*Tc-six3* 5up´. The reporter gene tGFP is not included in the gDNA of lines 2.1 - 2.3, 3.1, and 3.2. KI, KII were negative controls: gDNA of *Tc-ver* strain with primer for DsRedEx and tGFP, respectively. KIII was a positive control: PCRII-tGFP plasmid with tGFP primer. KIV was a positive control: PCRII-DsRedEx plasmid with DsRedEx primer. *:1kb ladder; **: 100bp ladder

Sibling lines of G_0 2, 3, 6, 7, 8, 9, and 10 carrying the *Tc-six3* reporter constructs were analyzed by PCR for tGFP insertion in their genome. The animals positive for tGFP showed a distinct band of approximately 650 bp. Hence, the lines derived from G_0 6, 7, 8, 9, and 10 were positively tested by PCR (Figure 23). The residuals showed only a weak band, which was also detectable for gDNA of SB-wildtype animals and of Tc-vermilion animals, depending on the amount of gDNA used in the experiment (not shown). Therefore, these weak bands are regarded as negative results, which support the negative results in *in situ* hybridization and in confocal scanning experiments. Therefore, the offspring lines for G_0 2 and the lines 3.1 and 3.2 were negative for an insertion of the reporter constructs (Figure 23). In these lines no expression of the reporter gene could be identified by *in situ* hybridizations and confocal scanning experiments.

Interestingly, within lines derived from G_0 3, only line 3.3 was positive for *in situ* hybridization experiments. There are several possibilities why lines 3.1 and 3.2 were positive for the transfection marker *Tc-vermilion* (black eyes), but negative for the reporter construct. First, a contamination with animals of the SB-line could have occurred, since crossings were performed in pupal stages and young pupae have no pigmented eyes. Second, a possible contamination with animals of *Tc-pearl* strain could have occurred, which per se also have white eyes, but after crossings to animals of the *Tc-vermilion* line produce black-eyed offspring.

The PCR analysis was positive for the reporter gene tGFP in the genome of the offspring lines of G_0 8. Contrary to this, expression analyses by *in situ* hybridization and confocal scanning were negative, indicating that the `*Tc-six3* 5up´ reporter construct possibly inserted into a silencing genomic region.

For *Tc-rx* reporter constructs, the sibling lines of the G_0 animals 1, 4, 5, 11, and 12 were analyzed. Those animals positive for DsRedEx showed a distinct and strong band of approximately 650 bp. Hence offspring lines of G_0 1, 11, and some of 12 were positive.

Interestingly, line 1.1 was also positive for insertion analysis (not shown), but not for *in situ* hybridization or confocal scans. This could be explained by integration in a silenced or silencing locus in the genome, repressing any detectable expression. Additionally, to test the hypothesis of separate insertion loci for line 1.1 and 1.2 in detail, an inverse PCR could be performed.

Individuals of the line 12.3 were positive for DsRedExpress, but were not analyzed further, because confocal scans revealed a contamination with the `Brainy´ line (chapter 3.1.4).

The following scheme summarizes the results of the described characterization experiments for the transgenic animals carrying the *Tc-six3* and *Tc-rx* reporter gene constructs.

Table 1: six3-reporter lines

	six3-10up					six3-5up						six3-5do					
six3-line	6. 1	6. 2	7. 1	7. 2	7. 3	8. 1	8. 2	8. 3	9. 1	10. 2	10. 3	2. 1	2. 2	2. 3	3. 1	3. 2	3. 3
PCR analysis	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	×	×	×	×	×	✔
in situ stainings	✔	✔	✔	✔	✔	×	×	×	✔	✔	✔	×	×	×	×	×	✔
promotor deletions	yes	yes	yes	yes	yes	no	no	no	no	no	no	yes	yes	yes	yes	yes	yes
confocal scans	×	×	✔	✔	✔	×	×	×	×	×	×	×	×	×	×	×	×

Table 2: rx-reporter lines

	rx-10up						rx-5up		rx-Int			
rx- line	4. 1	4. 2	4. 3	5. 1	5. 2	5. 3	1. 1	1. 2	11. 1	11. 2	11. 3	12
PCR analysis	×	×	×	×	×	×	✔	✔	✔	✔	✔	×
in situ stainings	×	×	×	×	×	×	×	✔	✔	✔	✔	×
confocal scans	×	×	×	×	×	×	×	✔	✔	✔	✔	×

Figure 24: Summary of the analyses of transgenic reporter lines

Results are sorted by fragments from up- to downstream constructs. Constructs are depicted according to their color code (cf. Figures 11 and 18). Insertion analyses through amplification of the respective reporter gene with specific primers for either tGFP (six3-reporter lines) or DsRedEx (rx-reporter lines) are depicted in the row `PCR analysis´. Results from testing the reporter gene expression are summarized in the row `*in situ* stainings´. `Promoter deletions´ indicates absence for 9 bp within the promoter construct (see chapter 3.3.2.1). Results from larval brain scans analysis for the respective reporter gene are shown in `confocal scans´.

3.4. Analysis of the fluorescence signal of the `Mushroom Body´ line and the `Brainy´ line

For imaging the L1 brain, two different lines were used. One line is an enhancer trap was found in the GEKU screen and is called the `Mushroom Body´ (MB) line. A second line was created by crossing two lines, that mark glial cells of the central nervous system and neural tissue respectively, it is called the `Brainy´ line.

The `Mushroom Body´ line

The `MB´ line is an enhancer trap line with the number G 11410, in which the artificial promoter 3xP3 drives the fluorescent reporter gene eGFP. This construct inserted on the 4th chromosome near the Glean_07701-OG17474, which is the orthologue to *Dm-spite* (http://www.geku-base.uni-goettingen.de/Details.aspx?MainIDResult=G11410). The molecular function of *Dm-spite* is still unknown, but it is coding for a hydroxyacylglutathione hydrolase and in *Drosophila* it is expressed in the

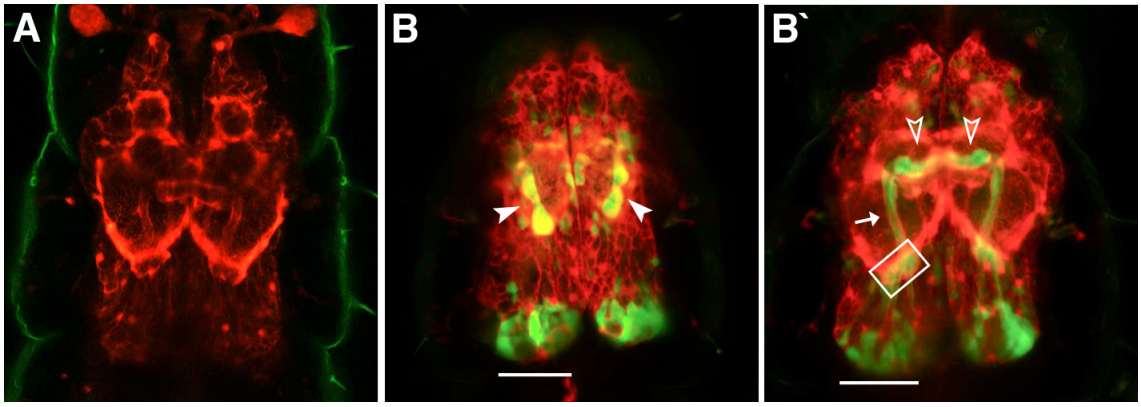

Figure 25: Characterization of the `Mushroom Body´ (`MB´) line by crossing with the glial marker line 6xP3::DsRedEx.

(A) Glial cells are marked by 6xP3::DsRedEx (see chapter 1.5), depicted in red. (B and B`) Crossing between the line 6xP3::DsRedEx and the `MB´ line. (B) Optical dorsal section. Vertical lobes (white arrowheads) are detected at the surface of the protocerebrum. Posterior lies a cluster of Kenyon cells (white line). (B`) Optical median section. The cluster of Kenyon cells is marked by a white line (one side indicated). The calyx is indicated by a white square. The median lobes (white open arrowheads) are located in a right angle to the Pedunclus (white arrow). Anterior is oriented to the top .

somatic mesoderm (Tomancak et al., 2002; Kwon et al., 2009).

To characterize the expression pattern of the `MB´ line, it was crossed with a line expressing DsRexEx under the control of 6xP3 (chapter 1.5; Figure 25, A). Some cells positive for eGFP were spread all over the brain, but these were not further characterized. The Kenyon cells of the Mushroom Body were the most prominently marked structure posterior in the brain (white dash; Figure 25, B and B`). These cells were embedded by putative cortex glial cells, that form a net like structure, which will be referred to as the `posterior glial tissue´. The putative Kenyon cells take up almost all of the posterior part of each brain hemisphere (white line; Figure 25, B and B`). They project to the anterior region through the posterior glial tissue into the protocerebrum. At the protocerebral neuropile border, elongated oval shapes, which represent the calyces could be detected (white square; Figure 25, B`). From here, marked axon bundles project anterior through the protocerebrum. Those bundles display the Pedunculus (white arrow; Figure 25, B`). At the end point, this structure splits into a horizontal projection forming the median lobes and into a vertical projection forming the vertical lobes. The median lobes (open arrowhead; Figure 25, B`) are parallel to the Central Body, which is located posterior to them. These lobes span almost completely across the interhemisphereic fissure. The vertical lobes (white arrowhead; Figure 25, B) are located at the surface of the protocerebrum. Thus, the larval brain comprises a Mushroom Body comparable to the adult one, which is visualized by the `MB´ line.

The `Brainy´ line

The `Brainy´ line was generated by crossing the 6xP3::eCFP glia reporter line (chapter 1.5) with a line, which was generated in the laboratory of Michalis Averof. This second line expresses DsRedEx under the control of the regulatory region of the elongation Factor II (EFII). Additionally, this line carries the positive transfection marker 3xP3::eGFP. After respective sibling crossings, all individuals of the `Brainy´ line showed the signals for EFII::DsRedEx and 3xP3::eGFP, and partially 6xP3::eCFP. For analysis, animals which were also positive for 6xP3::eCFP expression were chosen.

The `Brainy´ line allows to monitor the maturation of the larval brain *in vivo*. The developing embryonic structures and developing neuromeres were identified and named in their correlation to the morphology and position of neuropiles within the first larval instar brain.

The earliest expression of 6xP3::eCFP was detected in a 17h old embryo. Here, weak expression was detected in some cells, while expression of EFII::DsRedEx was not found at this time (data not shown). A similar pattern was visible in 24h old embryos. Here, faint expression of DsRedEx was detected (Figure 26, A). Both signals became stronger during ongoing development of the embryo. At an age of 30h an increasing number of cells expressed either eCFP or DsRedEx (Figure 26, B). Cells positive for the 6xP3::eCFP signal showed a strong branching in 40h old embryos. Cells positive for DsRedEx were detected within the branching tissue and are probably developing glial cells (Figure 26, C). An area free of signal appeared at an age of 45h, surrounded by cells positive for eCFP (white arrowhead; Figure 26, D) and increased to typical protocerebral shape and volume in later stages. A paired central structure was detected at an age of 55h, also surrounded by an eCFP signal (open arrowhead; Figure 26, F). Dorsally of this structure, the median lobes of the Mushroom Body and posterior to this structure, the Central Body was detected in later stages. The DsRedEx signal increased and was located in putative cell bodies of neural cells (Figure 26, F-I). In 60h old embryos, Antennal Lobes (AL, asterisks) were detected anterior to the protocerebral neuropile (Figure 26, G). Posterior to the paired central structure, the first faint and unpaired structure connecting the hemispheres was detected (white arrow; Figure 26, H), surrounded by cells positive for eCFP signal. At an age of 64h, cells expressing 6xP3::eCFP coated this structure, representing the developing Central Body. The latter could be clearly recognized at an age of 72h, where cells expressing a strong eCFP signal were covering this neuropile (Figure 26, I). At this stage, the first glial cells positive for the eCFP signal surrounded the Pedunculus of the Mushroom Bodies in the posterior dorsal part of the brain (not shown). Therefore, the `Brainy´ line represents a valuable tool to visualize the development and presence of neuropiles within brains of embryos to the first larval

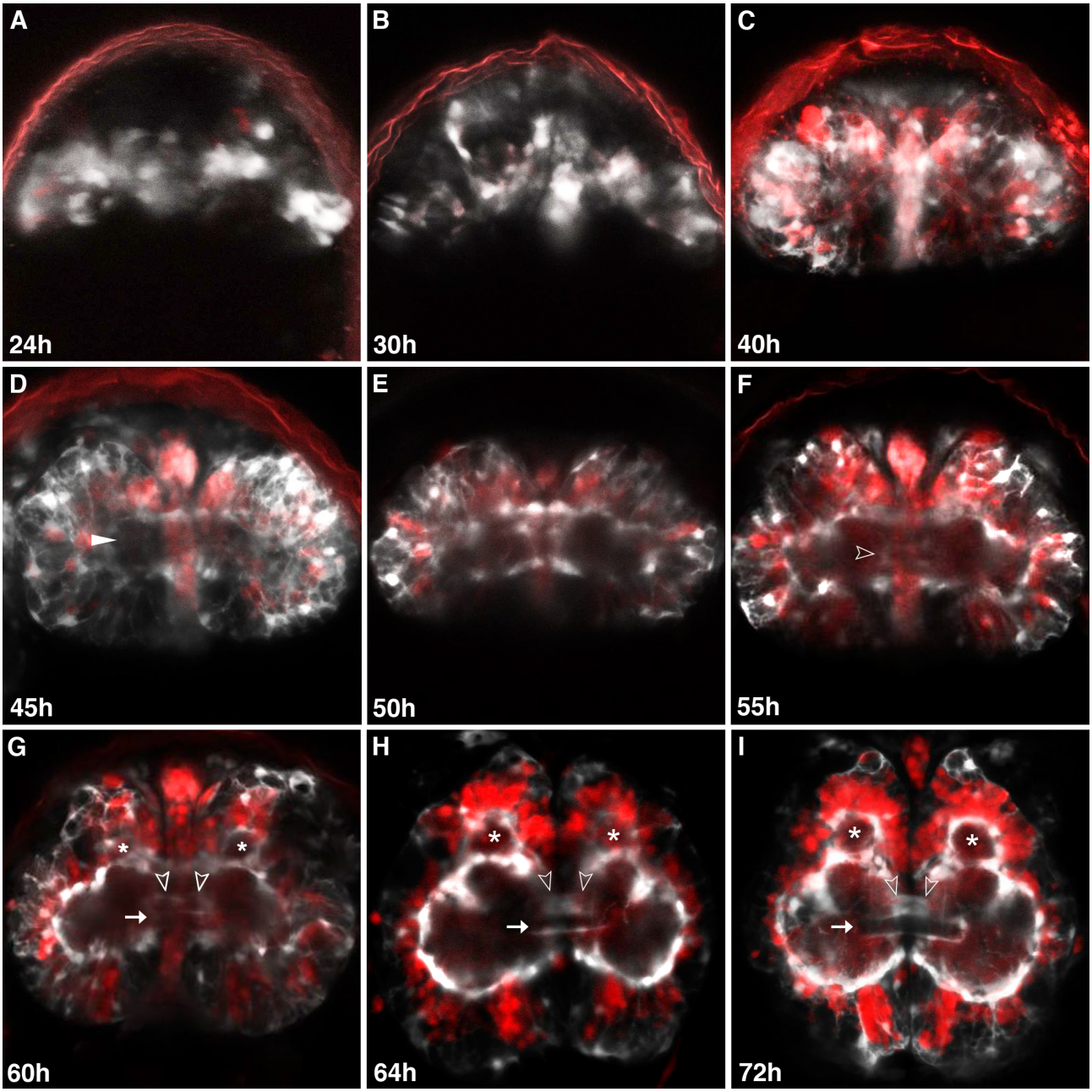

Figure 26: Embryonic developmental series of the fluorescence signal in the `Brainy´ (BA) line.
The 6xP3::eCFP (glial cells) signal is shown in white. EFII::DsRedExpress (neural cells) is depicted in red. Anterior is oriented to the top. The pace of embryonic development depends on the temperature, the indicated time relates to development at 32°C. (A) A 24h old embryo. The 6xP3::eCFP signal is weak in some cells. The EFII::DsRedExpress signal is beginning to appear in very few cells. (B) A 30h old embryo. (C) A 40h old embryo, where the 6xP3::eCFP signal is expressed in glial cells and starts to form a mesh like structure. (D) A 45h old embryo, where the putative Protocerebrum starts to form, marked by the absence of a 6xP3 signal (white arrowhead). (E) A 50h old embryo, where the shape of the protocerebrum is visible. (F) A 55h old embryo, where a paired structure is visible (open arrowhead). (G) A 60h old embryo. The Antennal Lobes (white asterisks) and the developing Central Body (white arrow) become visible (H) A 64h old embryo. (I) A 72h old embryo, where the Central Body is coated by eCFP positive cells. Asterisks mark the developing Antennal Lobes. Open arrowheads point to paired median structures, where finally the median lobes of the Mushroom Bodies appear. White arrows point to the forming Central Body.

instar brain.

Taken together, the `MB´ line as well as the `Brainy´ line are prerequisites for the following analyses of various genes in the context of embryonic brain development (second part of this work).

4. Results Part II: The genetic network of Central Body development

Little is known about the genes that give identity to the neural lineages, which in turn form and develop neuropiles in the brain. The same holds true for the Central Complex, which is a very prominent central structure within the insect brain. In *Schistocerca,* this structure is formed during embryonic stages, while in *Drosophila*, it develops partially in the last larval stage and is completed during the puparium. However, in both animals the corresponding NBs, which are involved in forming this neuropile, have been identified (Willams et al., 2005; Williams et al., 2008; Boyan et al., 2010; Itzergina et al., 2009). Yet, the underlying genetic mechanisms are still unknown. The regions of the brain, from which the Central Complex develops, are known in *Schistocerca* and *Drosophila.* On this basis, genes which are expressed in homologue regions in *Tribolium castaneum* were identified. These genes were analyzed with respect to their role in Central Body formation in *Tribolium castaneum.*

4.1. *Tc-rx* knock down leads to a split Central Body

The homeobox gene *Tc-rx* was chosen for functional analysis, because it is expressed close to the region, where the putative progenies of the Central Body originate. Furthermore, it is also expressed close to the region, where the putative Mushroom Body NBs arise (Hein, 2007). The onset of *Tc-rx* expression in embryos occurs during elongation, which correlates to embryos approximately 12h old at 32°C (Posnien, 2009; chapter 3.3.1). Additionally, its expression pattern is exclusively found in the head. Both the late onset and the exclusive head expression pattern indicate a brain specific differentiating function of *Tc-rx.* Especially, since knock down experiments in previous studies revealed only a weak cuticle phenotype. I.e. the labrum was reduced in size and bristles of the clypeus quartet were missing after pupal dsRNA injection of *Tc-rx* (Posnien, 2009), whereas the latter was not detected in this work. Taken together, all these findings regarding *Tc-rx* suggest a role in brain development.

In order to analyze the function of *Tc-rx,* pupal knock down was performed with different concentrations of dsRNA in the `Brainy´ line (1 or 4 µg/µl). The pupae injected with 4 µg/µl dsRNA died, probably due to the high dsRNA concentration. Offspring of the pupae injected with 1 µg/µl were analyzed by confocal laser microscopy scannings. Normally, the Central Body (white arrow; Figure 27, A) spans the interhemispheric fissure. At this stage, it is the only neuropile which connects the brain hemispheres. The median lobes of the Mushroom Body (white arrowhead; Figure 27, A) are located anterior to this prominent neuropile. This was also the case in the RNAi brains, where the median lobes were still found anterior to the Central Body (white arrowhead;

Figure 27, B). The Central Body, however, was split and had a short oval form (white arrow; Figure 27, B). Also, it did not span the interhemispheric fissure. This phenotype was detected in three of nine larvae. Interestingly, the non scanned siblings were able to hatch and to molt. Also, one second larval instar brain with split Central Body was scanned.

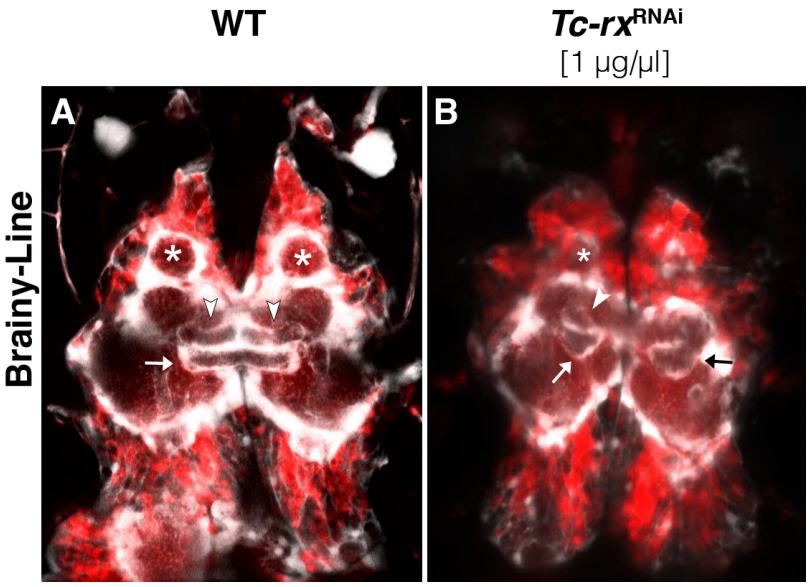

Figure 27: Knock down of *Tc-rx* leads to a split Central Body in first larval instar brains.
(A) The `Brainy´ line native situation. (B) Split Central Body (CB) after knock down of *Tc-rx*. Separation of the median (white arrowhead) lobe of the Mushroom Body (MB) and CB (white arrow) for the left hemisphere. The black arrow points out the same area, but a dsicrimination between MB and CB was not possible due to technical reasons. White asterisks: Antennal Lobes; white arrowhead: median lobes of the MB; white arrow: CB. For a better contrast the eCFP signal is depicted in white. Anterior is oriented to the top.

However, the described results are preliminary and need to be verified by a second independent knock down experiment for *Tc-rx*. Apparently, *Tc-rx* is required for a process mediating the crossing of the brain midline. I.e. *Tc-rx* is suggested to play an important role in establishing the resepective midline signaling for a proper Central Body formation (see chapter 5.4.2), but the split Central Body phenotype after *Tc-rx* RNAi is very likely to be due to secondary effects. Since the focus was to identify genes that influence the Central Body development in *Tribolium* as such, further analysis of *Tc-rx* regarding was not performed here, especially since the Central Body was missing after *Tc-six3* RNAi (chapter 4.3.3).

4.2. *Tc-otd1* is not the antagonist of *Tc-six3* in Central Body growth

In earlier studies, it was hypothesized that *Tc-six3* and *Tc-otd1* are antagonists in anterior head patterning during embryonic stages (Posnien; 2009). A knock down of *Tc-six3* led to absence of the larval Central Body (see chapter 4.3.3) and to an expansion of the *Tc-otd1* expression domain. This was visualized in whole mount *in situ* hybridization in respective embryos and suggested that *Tc-six3* represses *Tc-otd1* in the neuroectoderm. Based on the non overlapping expression patterns, a

negative feedback loop was suggested. Thus, it was hypothesized, that a knock down of *Tc-otd1* could enlarge the areas of *Tc-six3* expression, and vice versa. Due to the role of *Tc-six3* in Central Body development, it was hypothesized, that a knock down of *Tc-otd1* would lead to an enlargement of this neuropile.

It has previously been described, that a pupal *Tc-otd1* knock down leads to severe phenotypes and to high cellular lethality during blastodermal stages of the offspring (Kotkamp et al., 2010). This is congruent with the previously described "early regionalization" function of *Tc-otd1* in blastodermal stages (Schinko et al., 2008). Therefore, embryonic injections were performed to overcome these early regionalizing defects and to asses the late function determining the neurogenic tissue. By embryonic injections with 1µg/µl dsRNA into staged egg lays, a phenotype with weak to intermediate defects should be generated. Therefore, embryos aged 7-8h and 8-9h at 32 °C were injected. Larvae with weak phenotype showed a reduction in size of one hemisphere (indicated by white lines on different levels, Figure 28, B). Neuropiles like the Central Body, the Mushroom Body, and the Antennal Lobes showed no obvious defects. The protocerebrum and the posterior glial tissue were shortened along the anterioposterior axis. In case of intermediate phenotypes, both brain hemispheres were reduced in length. The Antennal Lobes, the Central Body and the Mushroom Body (out of focus in ; Figure 28, C) appeared to be unaffected (Figure 28, C).

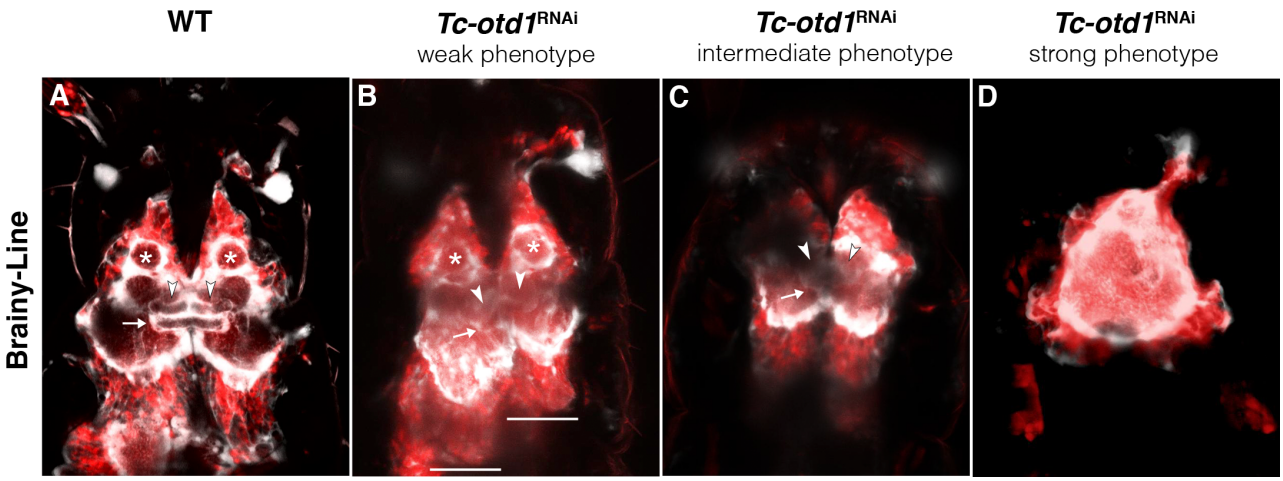

Figure 28: Knock down of *Tc-otd1* leads to a reduction of brain size.
Embryonal injections of 1µg/µl were performed in embryos 7-8h old at 32°C (A) `Brainy´ line native situation. (B) Weak phenotype of *Tc-otd1* knock down, where the right hemisphere is reduced; white lines indicate the end of posterior glial tissue. (C) Intermediate knock down phenotype, where both hemispheres are reduced. (D) Strong knock down phenotype, which leads to unstructured brain mass. White asterisks: Antennal Lobes; white arrowhead: median lobes of the Mushroom Body; white arrow: Central Body; white line: posterior end of the posterior glial tissue. Anterior is oriented to the top.

In larvae with strongly affected brains, the brain architecture was destroyed in a way that no neuropiles could be identified anymore (Figure 28, D).

The brain defects were correlated to the time slot of dsRNA injection, hence a knock down of *Tc-otd1* in young embryos caused stronger effects than a knock down in later stages (Figure 29). In the embryo batch injected at 7-8h at 32 °C, strong phenotypes occurred in 42,1 %, while weak and intermediate phenotypes, meaning those with reduced brain hemispheres, occurred in 21,1 % (Figure 29, 7-8h bar graphs). The strong brain phenotype occurred together with deletions of the dorsal head structures (compare Schinko et al., 2008). Injections into older embryos revealed no strong brain phenotypes, whereas weak brain phenotypes were predominant with 62,5% (Figure 29; 8-9h bar graphs).

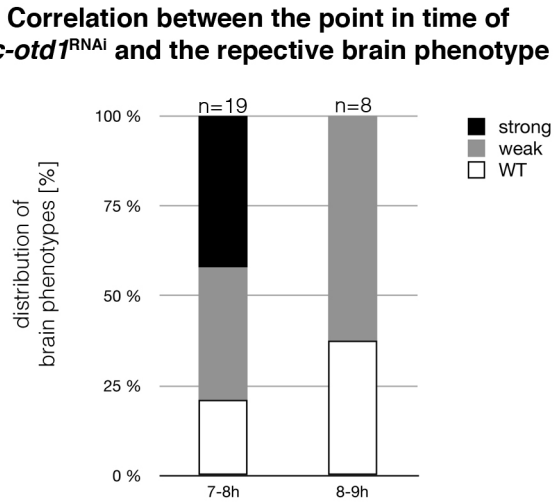

Figure 29: Correlation between the point in time of *Tc-otd1* RNAi and resulting brain phenotypes - early knock down leads to strong phenotypes.
The first bar shows the distribution of phenotypes detected in embryos injected with 1µg/µl *Tc-otd1* dsRNA at 7-8h old at 32°C; wild type (WT): 21,1%; weak phenotype (one or both hemispheres reduced): 36,8%; strong phenotype (brain unstructured tissue): 42,1%. The second bar shows distribution of detected phenotypes within embryos injected with 1µg/µl *Tc-otd1* dsRNA at 8-9h old at 32°C; WT: 37,5%; weak phenotype: 62,5%; strong phenotype: none.

In order to quantify the differences in neuropile size, 5 embryos of the injection performed after 8-9h at 32 °C with intermediate phenotypes (cf. Figure 28, C) were analyzed regardng their relative brain size in correlation to the size of the head capsule. Only embryos where brain and head capsule were almost parallel were selected. There, different tissues and neuropiles were measured in length and width. The resulting data were normalized to either the head capsule length or to the cervix width. For the head capsule length (line a; Figure 30) the distance between the antenna basis to the cervix fold (`neck´) was measured. For the head capsule width (line b; Figure 30), the distance from the most distant to the most lateral points of the cervix fold was measured (yellow lines in, Figure 30). For measuring length and width of brain tissues and neuropiles the most distant points were measured (Figure 30).

The bar graph in Figure 30 shows the arithmetical mean of all measured structures and neuropiles for WT and RNAi treated individuals. A students T-test was performed to verify statistical significance. Hence, the observed differences in brain length and width and also in protocerebrum

width were significant. No significant difference were found regarding the size of the Central Body. Thus, the hypothesis, that a knock down of *Tc-otd1* leads to an enlargement of the Central Body could not be confirmed here.

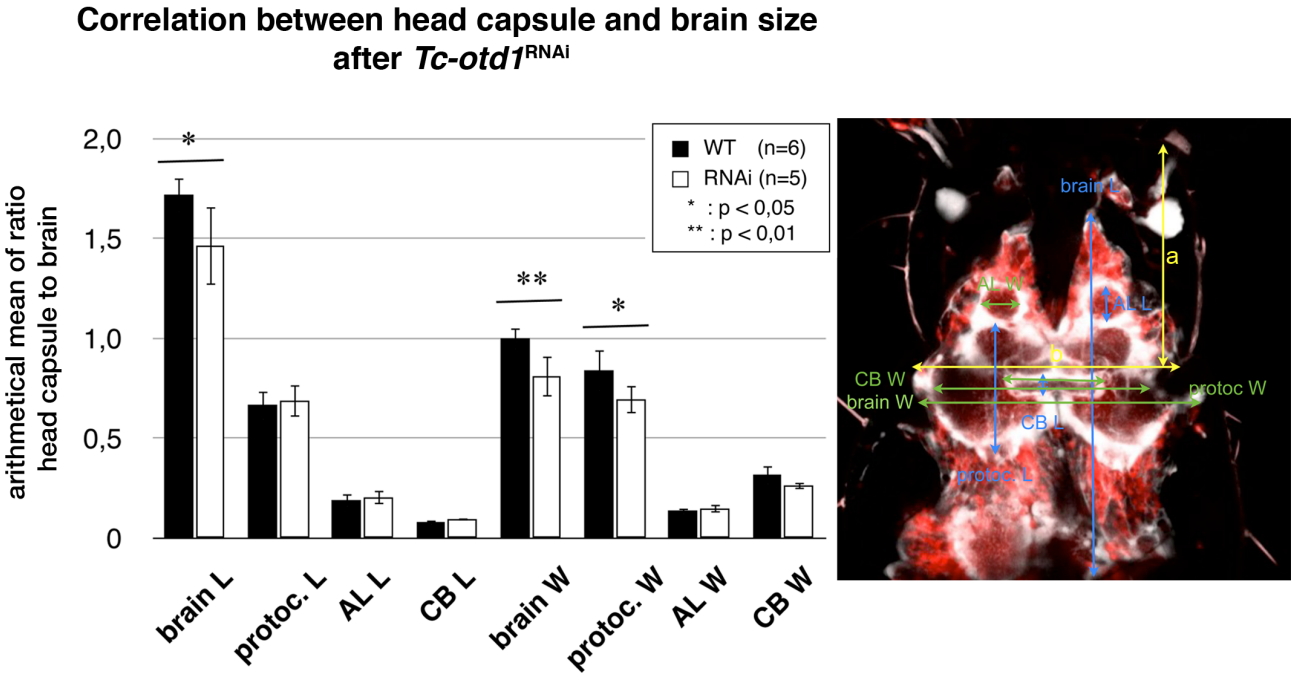

Figure 30: Correlation between head capsule and brain size after *Tc-otd1* knock down.
Embryonic knock down with 1µg/µl *Tc-otd1* dsRNA at 8-9h at 32°C was performed in the `Brainy´ (BA) line. Bar graph: the x-axis indicates different neuropiles which were measured in length (L) and width (W) for wild type (WT) and RNAi treated first instar larvae. The y-axis indicates the arithmetical mean of measured neuropiles in correlation to the head capsule. An unpaired and two-sided student´s T-test was performed testing the statistical significance. Significant results are indicated by one asterisk ($p < 0,05$); highly significant results are indicated by two asterisks ($p < 0,01$). The right panel shows the untreated BA situation (WT); green lines indicate start and end point of measured width; blue lines indicate start and end point of measured length; yellow lines indicate the measured width and length of the head capsule. (a) measurement for head capsule length starting at the antenna basis and ending at the cervix. (b) measurement for the head capsule width was performed at the farthest lateral points of the cervix. protoc.: protocerebrum; AL: Antennal Lobes; CB: Central Body; L: length; W: width.

4.3. *Tc-six3* and *Tc-chx* are required for Central Body formation

The NBs, which contribute to the Central Body formation, are located in anterior median regions of the brain in *Schistocerca* and *Drosphila* (Willams et al., 2005; Williams et al., 2008; Boyan et al., 2010; Itzergina et al., 2009). Thus, a screen for candidate genes, which are expressed in the respective regions in *Tribolium castaneum,* was performed. Thereby, the genes *Tc-six3* and *Tc-chx* were found (Posnien, 2009). From their expression pattern, it was hypothesized, that these genes play major roles in Central Body formation in *Tribolium castaneum.* First, in order to clarify this,

the expression patterns of *Tc-six3* and *Tc-chx* were analyzed for an overlap of their expression domains by double *in situ* hybridization experiments.

4.3.1 *Tc-chx* and *Tc-six3* coexpression analysis

Staged embryos of 0-24h and 24h-48h at 32°C were stained with probes for *Tc-six3* and *Tc-chx*. While *Tc-six3* expression started early in blastodermal stages (Posnien, 2009), *Tc-chx* was first expressed in embryos in elongating stages (Figure 31, A). Here, it was expressed in a small roundish pattern anterior lateral and partially overlapping with expression of *Tc-six3* (Figure 31, A`). The expression within this group of cells increased during ongoing elongation (Figure 31, B). A slightly longish and faint expression spread to the median tissue (black arrow; Figure 31, B`), later developing into basal expression within the labrum. In fully elongated embryos, this basal domain in the labrum was weaker than the expression lateral of the anterior median head tissue (black arrow; Figure 31, C`). In fully elongated embryos, an additional domain posterior in the rim of the head lobes, where the expression patterns of *Tc-six3* and *Tc-chx* were overlapping, was also detected (black arrowhead; Figure 31, C`). In embryos aged 24h at 32°C, the expression pattern of *Tc-chx* expanded slightly in all directions, especially the anterior domain (Figure 31, D). This domain almost completely covered the lateral expression domain of *Tc-six3* (open arrow; Figure 31, D`). The expression domains in the putative eye anlage were still overlapping, but *Tc-chx* overgrew *Tc-six3* (black arrowhead; Figure 31, D`). The posterior part of the *Tc-chx* expression domain lateral to the labrum basis still showed a slight expression of *Tc-six3* (black arrow; Figure 31, D`). However, in later stages the weak expression connecting the lateral expression domain and labrum basis could not be detected anymore (open arrowhead; Figure 31, E`). Furthermore, a small thin band of cells positive for only *Tc-chx* expression in the posterior part of the lateral expression domain of *Tc-chx* was detected (white arrow; Figure 31, E`). The expression patterns of *Tc-chx* and *Tc-six3* were still overlapping in the putative eye anlage (black arrowhead; Figure 31, E`). Until this stage, *Tc-chx* expression was located exclusively in the head (Figure 31, E), whereas additional domains positive for *Tc-chx* arose later, also within other regions of the body.

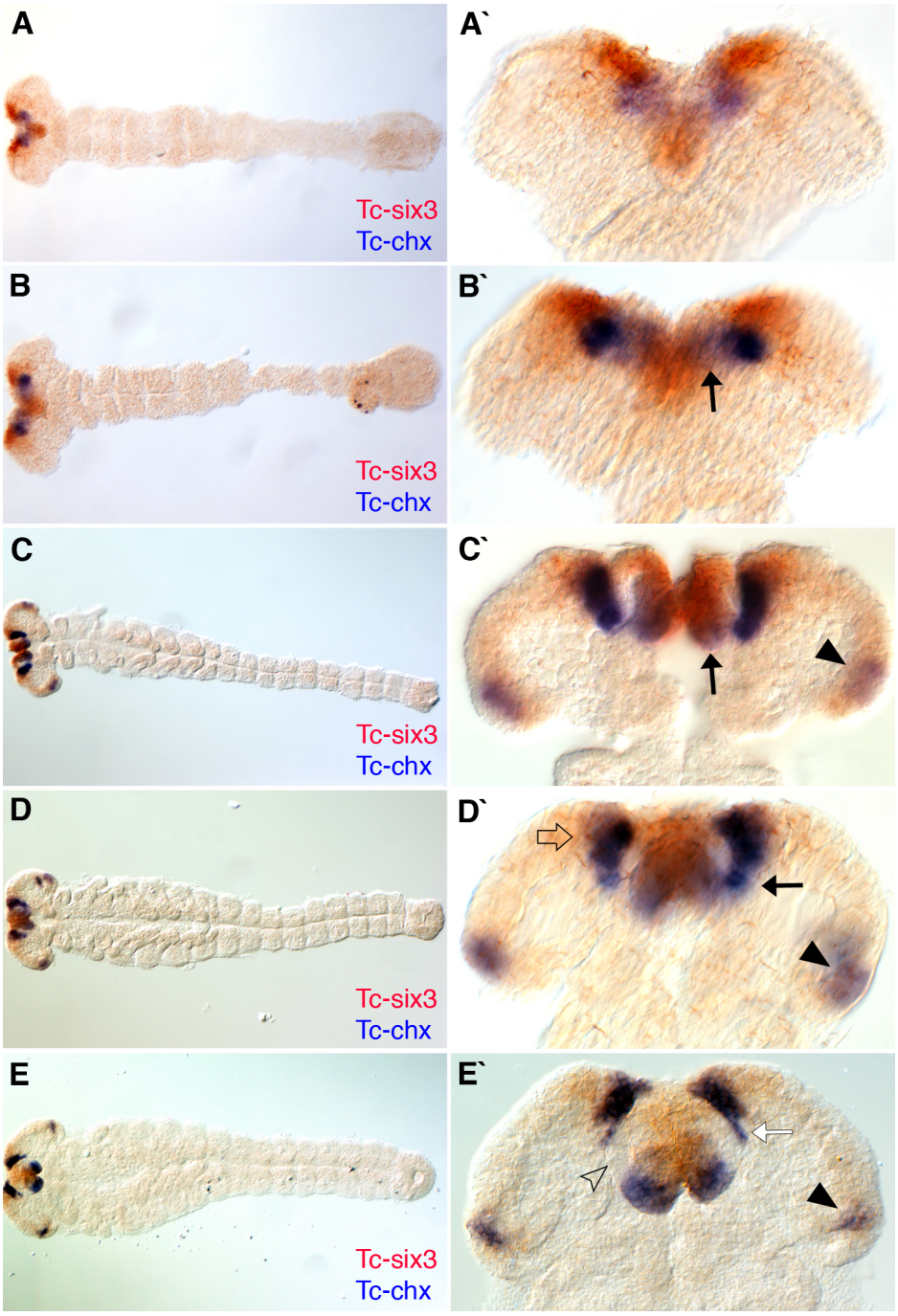

Figure 31: Double *in situ* hybridization of *Tc-chx* and *Tc-six3* demonstrates an overlapping expression pattern in up to 24h old embryos. (A-E) Ventral view of embryos, anterior facing to the left. (A`-E`) Close up of the head, anterior facing up. *Tc-chx* is depicted in blue and *Tc-six3* in red. (A and B) Approximately 13-14h old embryos. (A) shows a younger embryo than the embryo depicted in (B). (A`) *Tc-chx* expression is detectable in a dotted domain. (B`) A lateral domain with strong expression of *Tc-chx* and a longish domain developing into labral expression in later stages (black arrow). (C) 18h old embryo. (C`) The point-shaped domain had stretched out anterior, faint expression in the putative eye anlage (black arrowhead). The basal labral expression is indicated by a black arrow. (D) 24h old embryo. (D`) The domain lateral to the labrum has a strong expression (open arrrow), its posterior part shows still a coexpression with *Tc-six3* (black arrow). (E) 26h old embryo. (E`) The lateral domain and posterior expression of the labrum detach from each other (open arrowhead). *Tc-chx* shows a longish expression lateral to the labrum (white arrow). *Tc-six3* is only weakly expressed. The black arrowheads indicate the overlapping expression pattern in the putative eye anlage. All indicated ages correlate to the development at 32°C.

To analyze the expression pattern of *Tc-chx* at later stages, single *in situ* hybridization was performed in embryos staged 24-48h and 48-72h at 32 °C.

A segmental expression pattern arose *de novo* and spread out stepwise from the mandibular to the abdominal segments. More domains arose laterally in the head lobes, while the previous expression

remained. In embryos aged 40-55h, the segmental expression was finally detected in all segments (Figure 32, C). The head expression domains remained unchanged, i.e. the superficial expression median in the head (black arrow; Figure 32, B), the posterior domain in the rim of the head forming

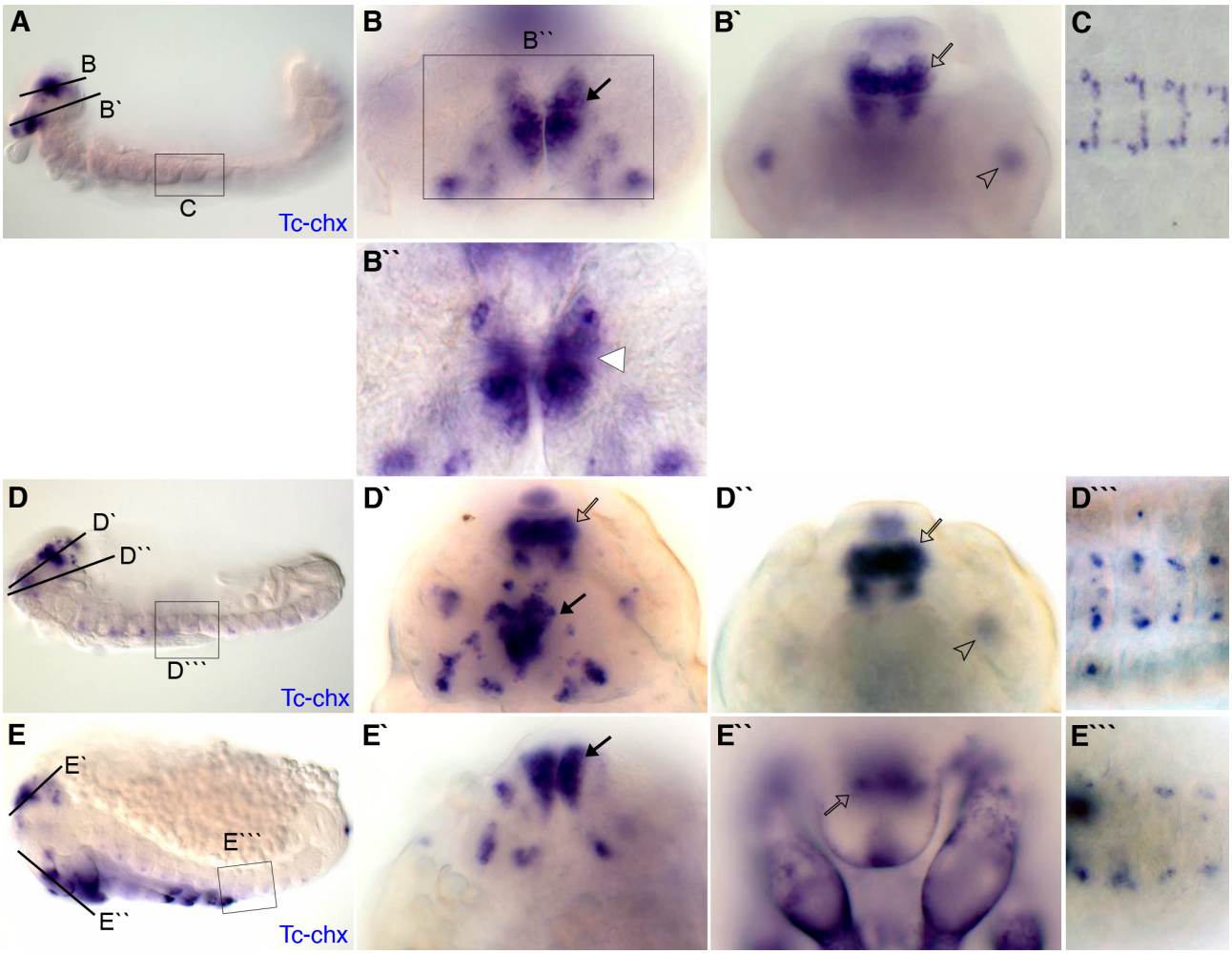

Figure 32: *Tc-chx* expression pattern.

(A, D, E) Lateral view, anterior facing left.(B, B`, D`, D``, E`, E``) Close ups of various optical sections in the head indicated by lines in A, D, and E respectively, anterior facing up. (C, D```, E```) Indicated by squares in A, D, and E respectively; ventral view of abdominal segments, anterior facing left. All images were prepared in bright field, except B``, which was prepared with a Nomarski-prism. The black arrow indicates the median expression domain, the open arrow indicates the labral expression domain, the open arrowhead indicates the putative larval eye expression domain in the respective images. (A) An embryo 40-55h old. (B) Close up of the dorsal expression domain of different embryos at similar stage. (B``) Same expression domain in Nomarski-optics, midline crossing structures are detectable (white arrowhead). (B`) The labral expression domain and faint lateral domains in the developing larval eyes. (C) Segmental expression pattern of different embryos at similar stages, here the abdominal segments A1-A4 are depicted. (D) An embryo 50-60h old. (D`) Complex expression pattern at the dorsal head. (D``) The labral expression from the ventral view with faint lateral domains. (D```) The segmental expression pattern decreases, abdominal segments A1-A4. (E) An embryo older than 65h. (E`) Expression domain in the dorsal head. (E``) Frontal view of the labral domain. (E```) Weak segmental expression pattern in abdominal segments A1-A4. All indicated ages correlate to the development at 32°C.

the larval eyes (open arrowhead; Figure 32, B`), as well as the labral expression (open arrow; Figure 32, B`). Through analysis with a DIC filter, interhemispherical projections could be detected on the level of the median strong expression domain of *Tc-chx* (white arrowhead; Figure 32, B``). The expression pattern in the head became more complex (Figure 32, D`). While the median expression domain remained constant (black arrow; Figure 32, D`), more cells and clusters in the surrounding tissue expressed *Tc-chx* (Figure 32, D`). The labral expression (open arrow; Figure 32, D``) as well as the eye expression domain of *Tc-chx* remained unchanged (open arrowhead; Figure 32, D``), whereas the segmental expression domain diminished with time (Figure 32, D```). Here, the lateral most domains remained constant in their expression level, while the median domain was hardly detectable (Figure 32, D```).

At an age of approximately 65h at 32°C, cuticle was secreted by epidermical cells. This leads to strong background stainings, especially in the head and appendages (Figure 32, E). However, the overall expression pattern in the head was found to remain unchanged with its median expression domain and single spots surrounding it (Figure 32, E`). The labral expression was still detectable, although in slightly changed shape (open arrow; Figure 32, E``). The segmental expression strongly decreased and only the lateral domains were detectable (Figure 32, E```).

Taken together, *Tc-chx* became expressed in the ventral tissue of the abdomen and also in a more complex manner in the head after an age of 24h. Since *Tc-six3* remains expressed only in the anterior part of the embryo, the coexpression pattern of *Tc-chx* and *Tc-six3* is suggested to be limited in time and location. However, during the 0-24h slot, a colocalization of *Tc-chx* and *Tc-six3* was found for a restricted area in the anterior head region. Therefore, the amount of NBs arising in the *Tc-chx* positive ectodermal region was analyzed next, especially since they are a subset of *Tc-six3* positive NBs.

4.3.2 At least 4 NBs delaminate from the *Tc-chx* positive anterior neuroectoderm

For the quantification of the NBs, that delaminate from the *Tc-chx* positive domain, double *in situ* hybridziations with staged egg lays of the SB-strain were performed at an age of 12-18h, 18-24h, and 24-30h. Analysis was performed with antisense probes for *Tc-chx* and *Tc-ase* as a NB marker.

The first NB arising in the *Tc-chx* expression domain was detected in elongating embryonic stages at approximately 13-14h of age (Figure 33, A`). Two additional NBs arose in fully elongated embryos (Figure 33, B`). In later stages, a maximum of four NBs was identified within the *Tc-chx* expression domain (Figure 33, D` and E`). At later stages, it was not possible to match NBs with the overlaying ectoderm, due to the morphological separation of neural from epidermal tissue.

Therefore, the detected number of NBs is a minimum number, as. it is very likely, that more NBs, which are *Tc-chx* positive, arise.

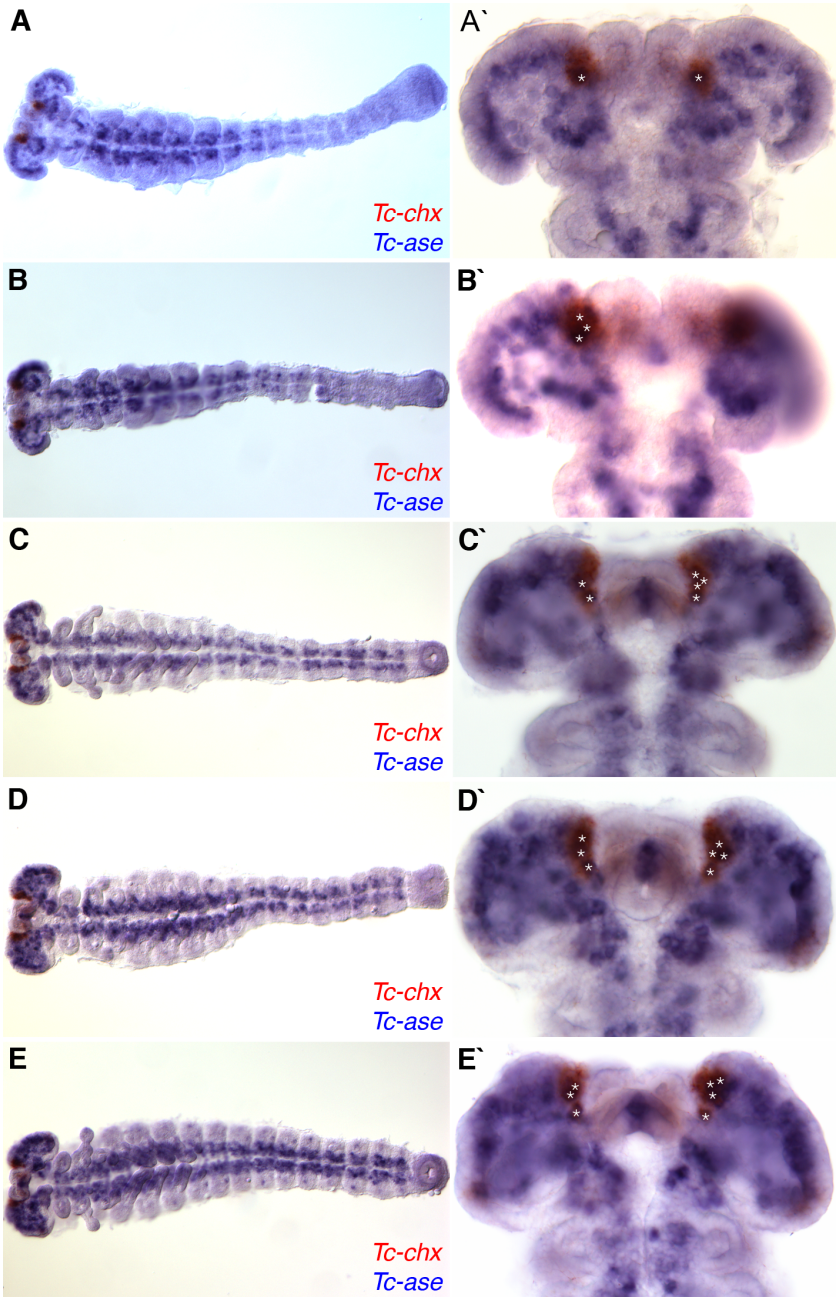

Figure 33: The number of NBs positive for *Tc-chx* varies in similarly aged embryos.
Staging of embryos was performed at 32°C. (A-E) Embryos are oriented facing anterior to the left. (A`-E`) Close up of the head, anterior facing up. NBs are marked by white asterisks. (A) Elongating embryo, approximately 13-14h old. (A`) One *Tc-chx* positive NB per hemisphere. (B) Almost fully elongated embryo, approximately 18h old. (B`) 3 NBs per hemisphere (right hemisphere out of focus). (C) Fully elongated embryo, approximately 20h old. (C`) 2 NBs in the left and 4 NBs in the right hemisphere. (D) Fully elongated embryo, approximately 24h old. (D`) 3 NBs in the left and 4 in the right hemisphere. (E) Embryo in retraction movement, approximately 26h old. (E`) 3 NBs in the left and 4 NBs in the right hemisphere.

The number of NBs within one developmental stage was found to be dynamic, rather than stereotypic. In some cases, older embryos had less NBs than younger ones (compare Figure 33, B` and C`). However, a maximum number of 4 NBs positive for *Tc-chx* expression could be detected until an age of approximately 26h at 32°C.

From the coexpression analysis of *Tc-chx* and *Tc-six3* (chapter 4.3.1) it is known that the identified NBs were positive for both genes, i.e. *Tc-chx* positive NBs are a subset of those positive for *Tc-six3*.

4.3.3 pRNAi of *Tc-six3* and *Tc-chx* in brain imaging lines led to loss of the Central Body

After the quantification of the number of the NBs positive for *Tc-six3* and *Tc-chx* within the neuroectoderm, the following questions arose: Which influence do these genes have on the development of the brain and which neural fate is determined by their expression in the NBs? To answer these questions, RNAi experiments starting with pupal injections of *Tc-six3* dsRNA were performed.

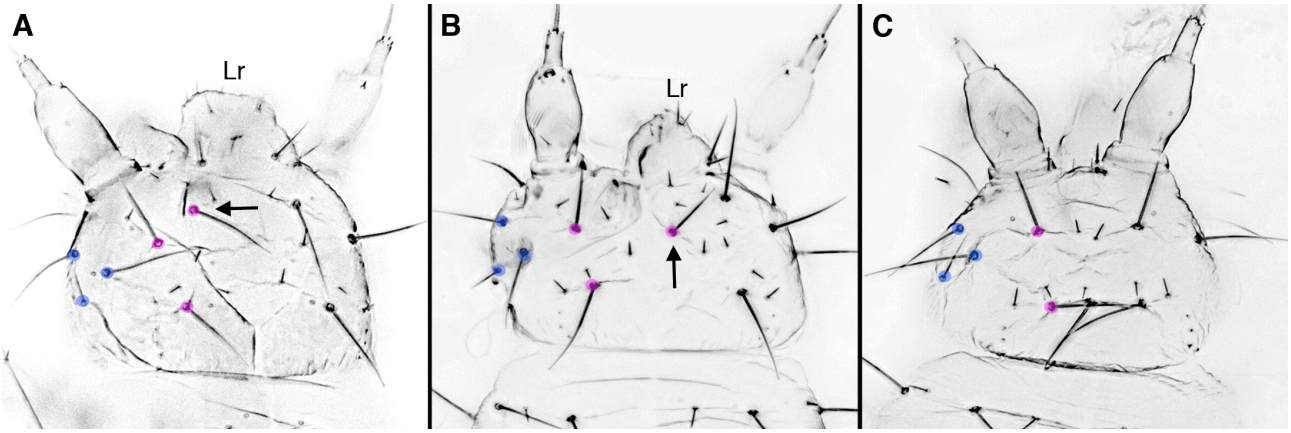

Figure 34: *Tc-six3* cuticle RNAi phenotypes
(A) WT cuticle. (B) Weak cuticle phenotype after injection of 100 ng/µl *Tc-six3* dsRNA. The labrum (Lr) is slightly affected and the anterior vertex seta is displaced (black arrow). (C) Strong cuticle phenotype after injection of 1 µg/µl *Tc-six3* dsRNA. The Lr and the median head tissue including the anterior vertex seta are missing. All heads are depicted by a dorsal lateral view, facing anterior up. Gena seta are marked blue. Vertex seta are marked pink. Black arrows point to anterior vertex seta.

It was known from prior studies, that *Tc-six3* RNAi causes severe cuticle phenotypes when injecting highly concentrated dsRNA, i.e. 1 µg/µl and higher. Therefore the efficiency of the RNAi treatment was monitored by analysis of the head cuticle. Indeed, the anterior median tissue of the head, including the labrum was deleted using 1 µg/µl of dsRNA (Figure 34, C). Less concentrated dsRNA, here 100 ng/µl, caused a malformation of the vertex seta and the bristle pattern on the dorsal head (Schinko et al., 2008; Posnien, 2009) as well as the labrum (Figure 34, B).

Functional analysis of *Tc-six3* regarding its role in brain development was initially performed with injections of 100 ng/µl dsRNA in female pupae of the `Brainy´ line. The brains of the RNAi treated offspring were similar to those of wildtype in size and shape. The posterior glial tissue was not affected. The Antennal Lobes (asteriks, Figure 35, B) and the median lobes of the Mushroom Bodies were present (white arrowhead, Figure 35, B). However, the position of the latter was

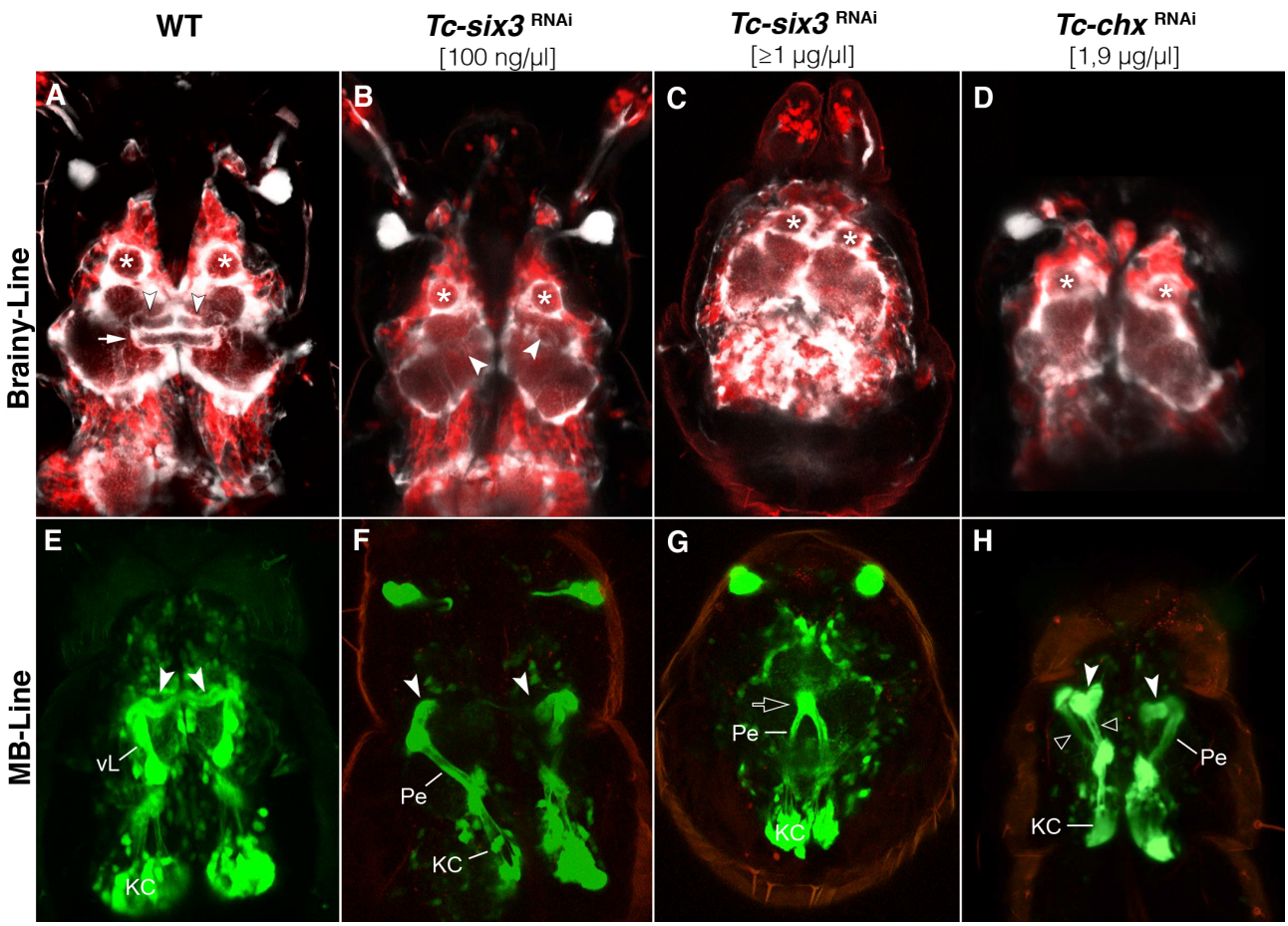

Figure 35: *Tc-six3* and *Tc-chx* knock down leads to a loss of the Central Body.
Larval offspring of animals treated with pRNAi for *Tc-six3* and *Tc-chx*. Larvae are presented with anterior to the top. (A-D) Larvae with various RNAi defects in the `Brainy´ (BA) line background, the 6xP3::eCFP signal is depicted in white. (E-H) Larvae with various RNAi defects in the `Mushroom Body´ (`MB´) line background. The Antennal Lobes are marked with white asterisks, the median lobes of the Mushroom Bodies (MB) are pointed out by white arrowheads. Kenyon cells are indicated by KC and the Pedunculus by Pe. WT with typical expression patterns of the BA line is shown in (A), and of the `MB´ line in (E) respectively. (B, F) Larvae with *Tc-six3* phenotype caused by pupal injection with a concentration of 100 ng/µl. The median lobes of the MB are disarranged (white arrowheads, B and F). (C) pRNAi with 2,1 µg/µl, and (G) 1 µg/µl. The MBs are severely affected and appear to touch each other (open arrow, G). (D, H) Larval phenotypes caused by pupal RNAi of *Tc-chx* with a concentration of 1,9 µg/µl dsRNA. (D) The Central Body is not detectable. (H) The MBs are disarranged within the protocerebrum and the median lobes are more flexed (white arrowheads). Two putative pedunculi are present in the left hemisphere (open arrowheads).

altered. The median lobes were not located between the brain hemispheres like in wildtypes, but more laterally (white arrowhead, Figure 35, A). Furthermore, the Central Body was not detectable in these animals.

Increasing the concentration of injected dsRNA to 1 µg/µl for pupal RNAi (pRNAi) resulted in severe brain phenotypes. The brains appeared to be smaller than the WT brains and the posterior glial tissue was not unfolded (Figure 35, C). Nevertheless, the protocerebrum and the Antennal

Lobes remained present (asterisks, Figure 35, C). The brain hemispheres were still separated, but the fissure between the hemispheres was strongly reduced. The Central Body and the median lobes of the Mushroom Bodies were not detectable (Figure 35, C). Further increase of dsRNA concentration to 2,1 µg/µl did not lead to any different or stronger phenotypes, neither on the brain nor on the cuticle level.

In order to test, whether Mushroom Bodies are deleted or absent, *Tc-six3* pRNAi experiments were repeated with the same concentrations as described above in female pupae of the `MB´ line. Following injection of 100 ng/µl dsRNA of *Tc-six3,* all typical structures for Mushroom Bodies were found, i.e. Kenyon cells (KC) in the posterior glial tissue, Pedunculus (Pe), vertical lobes (vL), and median lobes (n=6; white arrowheads; Figure 35, F). The median lobes were not located centrally between the brain hemispheres, but lay bent within the protocerebrum (white arrowheads, Figure 35, F).

The brain phenotypes caused by injection of 1 µg/µl *Tc-six3* dsRNA showed a strong reduction of the posterior glial tissue, whereas the Kenyon cells (KC) and the Pedunculus (Pe) were detectable (n=5; Figure 35, G). The latter partly touch each other at the midline, but appear not to be fused (open arrowhead; Figure 35, G). The vertical lobes and median lobes were not visible (Figure 35, G). Thus, the correct positioning of the Mushroom Body in the protocerebrum was affected by the weak *Tc-six3* knock down. Furthermore, pupal knock downs of the gene *Tc-six3* with higher concentrations caused severe defects in the brain as well. I.e. it affected the whole brain and also led to disarrangements of the Mushroom Body neuropile. Nevertheless, the most sensitive and most severely affected structure appears to be the Central Body. These results suggest, that *Tc-six3* plays an important role in the formation of the central brain and the Central Body of *Tribolium castaneum*.

Since *Tc-chx* marks a subset of *Tc-six3* positive NBs, this gene displayed a very promising candidate for influencing the Central Body development. Knock down experiments yielded no cuticle phenotype, i.e. the larval cuticle had no defects and resembled the WT situation, independent of injections with 1,9 µg/µl or 3,8 µg/µl *Tc-chx* dsRNA. After *Tc-chx* RNAi in the `Brainy´ line, the resulting brain phenotypes appeared almost identical to those of the weak *Tc-six3* RNAi phenotypes. The size of the brain hemispheres was the same as in WT situation, the Antennal Lobes were present and the posterior glial tissue showed no defects. Mushroom Bodies were present and only slightly malformed, i.e. the median lobes lay disarranged in the protocerebrum. The Central Body was not detectable (Figure 35, D).

Next to the `Brainy´ line, injections with the same concentration of *Tc-chx* dsRNA were repeated in

the `MB´ line to visualize possible effects on the Mushroom Body. Here, the Kenyon cells embedded by the posterior glial tissue were projecting to the calyx, from where the Pedunculus projected anterior ventrally into the protocerebrum, dividing into the vertical and the median lobe. The latter appeared to be slightly shortened and/or delocalized in the protocerebrum, while no abnormalities were detected for the position of the vertical lobe (Figure 35, H). However, in 50% (n=8/16) of the analyzed cases, malformed Mushroom Bodies were found. In these, the projections from the calyx were either split in two or more Pedunculi or even projections to the ventral lobes could be detected (open arrowheads; Figure 35; H). Furthermore, abnormal projections towards anterior arising, from the Kenyon cells and ending near the Antennal Lobes (not shown), were detected. However, such effects occurred in only one out of 13 *Tc-six3* knock down animals. Interestingly, an increase of phenotype was not achieved by increasing the concentration of injected *Tc-chx* dsRNA.

Since no cuticle phenotype was detected, these analyses suggest, that *Tc-chx* apparently exclusively influences the neural fate. Furthermore this gene was shown to play an important role in both Central Body formation and Mushroom Body development.

4.3.4 Immunohistochemistry analysis of the brain phenotypes resulting from *Tc-six3* and *Tc-chx* knock down

Possibly occurring subtle defects may not be detectable by using the `Brainy´ and the `MB´ line. Therefore, the pRNAi phenotypes were characterized in immunohistochemic analyses. To this end, different antibodies against neuropeptides expressed in the larval brain and the Central Body were used. Female `Brainy´ pupas were injected with 100 ng/µl and 1 µg/µl *Tc-six3* dsRNA to induce weak and strong phenotypes in the offspring. The brains of the larval offspring were dissected and stained with the respective antibodies (chapter 2.8).

Larvae with a weak RNAi impact showed separated brain hemispheres. Here, the anterior brain tissue surrounding the Antennal Lobes was separated like in the WT situation (weak RNAi phenotypes: Figure 36, B, F, K; WT situation: A, E, I). This was not the case in heavily affected RNAi phenotypes, where the median anterior tissue was fused (open arrows; Figure 36, C, G, L). Therefore, the brain was u-shaped here. Additionally, the whole brain appeared to be reduced in size (Figure 36, C, G, L). These morphological characteristics were used to classify the observed phenotypes.

For the analysis of the Mushroom Bod, the antibody DcO was used (Zhao et al., 2008). The brains of weakly affected *Tc-six3* pRNAi larvae had Pedunculi, vertical lobes, and median lobes (n=10),

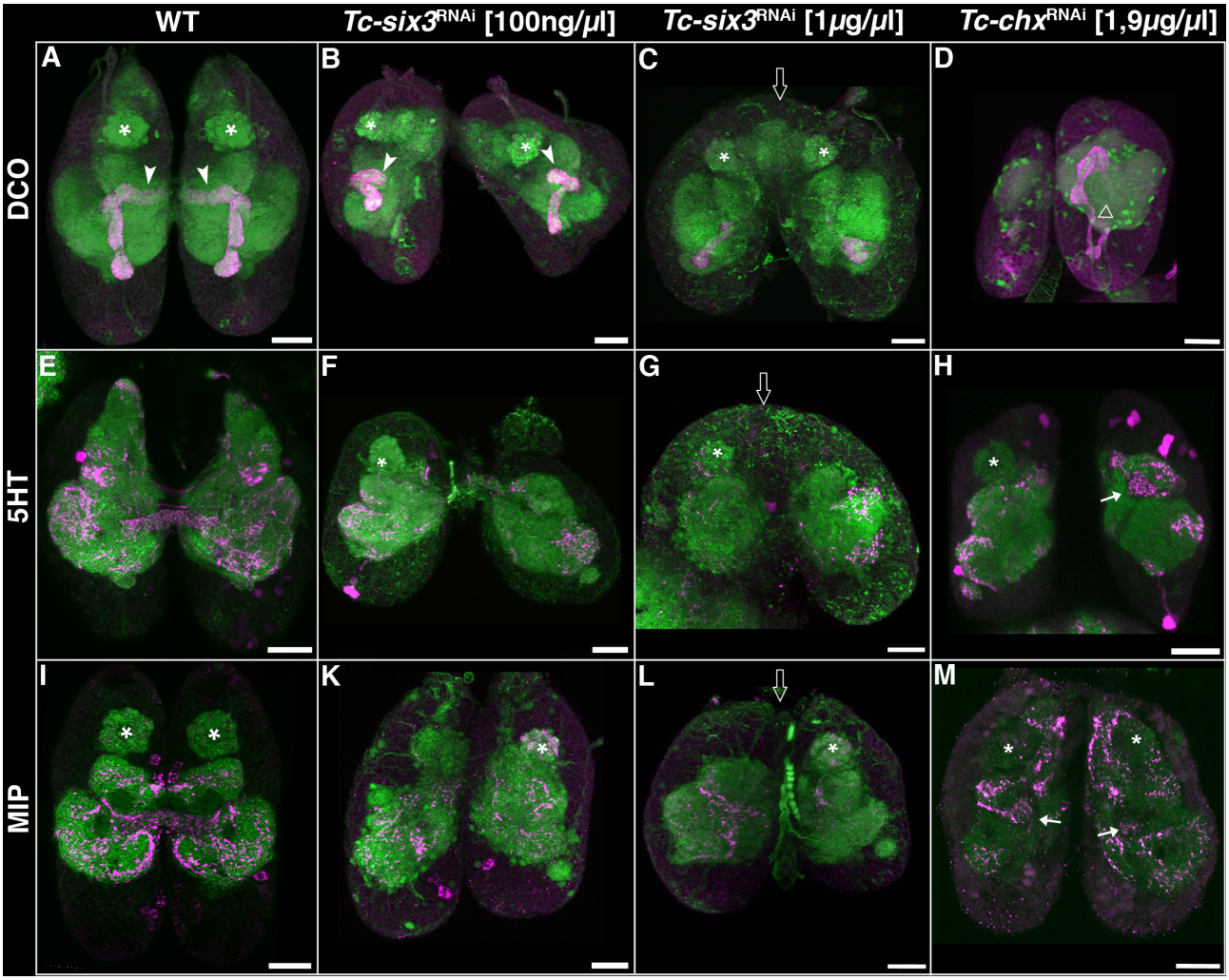

Figure 36: *Tc-six3* and *Tc-chx* knock down leads to loss or cleavage of the Central Body (CB).
Immunohistochemistry stainings on larval brains. Larvae are offsrpring of *Tc-six3* and *Tc-chx* RNAi treated female `Brainy´ pupae. Depicted are dissected brains of first larval instars (L1). (A, E, I) Wildtyp (WT) L1-brains with antibody stainings for (A) DcO, (E) 5HT/ Serotonin, (I) Myo inhibitoric Peptide (MIP). (B, F, K) *Tc-six3* pRNAi phenotypes caused by 100 ng/µl *Tc-six3* dsRNA injections. (C, G, L) *Tc-six3* pRNAi larval brain phenotypes caused by 1µg/µl *Tc-six3* dsRNA injections. (D, H, M) Tc-chx RNAi larval brain phenotypes caused by 1,9 µg/µl dsRNA injections. All brains were costained with phalloiden, shown in green. In the first row (A-D) the antibody against DcO was used to stain the larval Mushroom Body (magenta staining). In the second row (E-H), embryos were stained with the antibody against 5HT/ Serotonin (magenta staining). In the third row (I-M), larval brains were incubated with an antibody against MIP. All brain phenotypes caused by *Tc-six3* knock down show no CB. Phenotypes caused by *Tc-chx* knock down lead to a paired CB as indicated (white arrows; H, M), and further projections in the MB were detected (open arrowhed; D). Asterisks mark the larval Antennal Lobes. Anterior is facing up. All scale bars measure 20 µm. The preparation and staining of the WT and *Tc-chx* RNAi brains were performed by Martin Kollmann (University of Marburg) in collaboration.

even though these appeared to be reduced in size. The position within the protocerebrum was altered and especially the median lobes were heavily reduced (open arrowheads; Figure 36, B). The Antennal Lobes were present in all larvae. Strongly affected phenocopies of *Tc-six3* pRNAi, caused

by injections of 1 µg/µl dsRNA, showed the Pedunculi of the Mushroom Body in all larvae (n=6), whereas the vertical and the median lobes were strongly reduced and the latter sometimes even not detectable at all (Figure 36, C). Generally, the Mushroom Bodies of these animals appeared reduced in size like the rest of the brain. Also in the strong phenotypes, the Antennal Lobes were present (asterisks; Figure 36, C).

The antibodies against Serotonin and MIP were used to mark the Central Body (Dacks et al., 2006; Predel et al., 2001). By performing stainings on *Tc-six3* knock down brains, the absence of this neuropile could be confirmed in weakly affected larval RNAi brains. The neuropeptide serotonin was detected in the respective tissues of the brain (n=12, Figure 36, F), but a Central Body like structure could not be identified. In strong phenotypes this neuropile was also missing, while the neuropeptide was detectable in the rest of the brain (n=12, Figure 36, G). These results were confirmed by neuropeptide stainings for MIP, which marks the upper unit of the Central Body in adult animals (Martin Kollmann, personal communication; Predel et al, 2001). Here, an expression in the brain was detectable in both weak (n= 7; Figure 36, K) and strong (n=5; Figure 36, L) RNAi phenocopies of *Tc-six3*. However, the median pattern was lacking, indicating a loss of median brain tissue including the Central Body.

Through these experiments it could be shown, that *Tc-six3* essentially contributes to proper development of the *Tribolium castaneum* brain. Importantly, the Central Body neuropile appeared to be most sensitive and did not develop after a knock down of *Tc-six3*. Furthermore, these experiments indicated a correlation of the injected dsRNA concentration and the strength of the phenotypes on cuticle as well as neural tissue level.

Immunohistochemistry analysis was also performed for the *Tc-chx* RNAi larvae brain, similar to those described above. For this, *Tc-chx* pRNAi was performed by injecting female `Brainy´ pupae with a concentration of 1,9µg/µl dsRNA. Brains of the respective offspring were dissected and stained using the antibodies described above.

Tc-chx RNAi (1,9 µg/µl) did not affect the separation of the brain hemispheres and rather mirrored the situation shown for weak *Tc-six3* phenocopies. In *Tc-chx* phenocopies, no reduction of size and shape of the brain was detected, resembling the observations made in weak *Tc-six3* RNAi phenotypes. The antibody against DcO was used to visualize the Mushroom Body. In *Tc-chx* phenocopies, structures like Pedunculi, median and vertical lobes were present. The median lobes were slightly shortened (not shown). Axon bundles positive for DcO staining were detected, arising from Kenyon cells projecting into the vertical lobes (open arrowhead; Figure 36, D). Therefore, the observations from the *in vivo* images could be confirmed in these stainings.

To analyze the Central Body, antibodies against serotonin/5HT and MIP were used. The staining of knock down *Tc-chx* RNAi brains indicated that the Central Body was present but split. Anti 5HT staining was detected in the brain like in the WT situation. In addition, two out of four brains were found to have separated Central Bodies positive for anti 5HT (white arrow; Figure 36, H). The situation of the Central Body in the residual two brains could not be analyzed due to optical resolution. Similar results were obtained by the analysis of anti MIP stainings. All scanned brains showed separated Central Bodies (n=8; white arrows; Figure 36, M). In two cases a Central Body was detected in only one hemisphere, while the other hemispheres could not be analyzed due to optical resolution.

Tc-chx function is suggested to act exclusively on neural fate, since no cuticle phenotype was detected. Taken together, this gene plays an important role in the medial fusion of the Central Body and in the orientation of the developing Mushroom Body.

4.3.5 Temporal separation of the epidermal and neural functions of *Tc-six3*

Neurogenesis is a spatiotemporal process within the neuroectoderm (Urbach and Technau, 2003). In *Tribolium,* the first NBs delaminate at approximately 12h at 32°C (chapter 3.3.1), while epidermal patterning starts much earlier. Now the question was, whether it is possible to separate the influences of *Tc-six3* in epidermic and neural fate by injecting embryos at different developmental stages. To address this, eggs of the `Brainy´ line were collected for 2 h and bred until the desired age at a temperature of 32°C. Injections were performed with *Tc-six3* dsRNA concentrated to 1 µg/ µl.

Approximately one-third of the injected embryos survived the treatment and after breeding for 3-4 days on 32°C developed to larvae. The older the embryos were at the time of injection, the more embryos survived. Surviving larvae were randomly picked for preparation, scanning and analysis of the brain phenotype. This batch of larvae will further be called `scanned larvae´. Afterwards, these larvae were embedded in Hoyers Lactic Acid to analyze the cuticle phenotype. Those embryos or larvae which were not selected, were used to prepare cuticles, in order to confirm that the picked embryos were showing a representative ratio of the respective phenotype. This batch will be called `residual larvae´.

The earliest embryos were injected when developed 0-2h hours at 32°C (Figure 37, 0-2h bar graphs). These embryos were in a very early blastodermal stage, where only a few energids exist. The `scanned larvae´ showed severe cuticle phenotypes. In 45,5% (5 out of 11 analyzed cases), the anterior median tissue was deleted (compare Figure 34, C; Figure 37, E, 0-2h cuticle bar graphs).

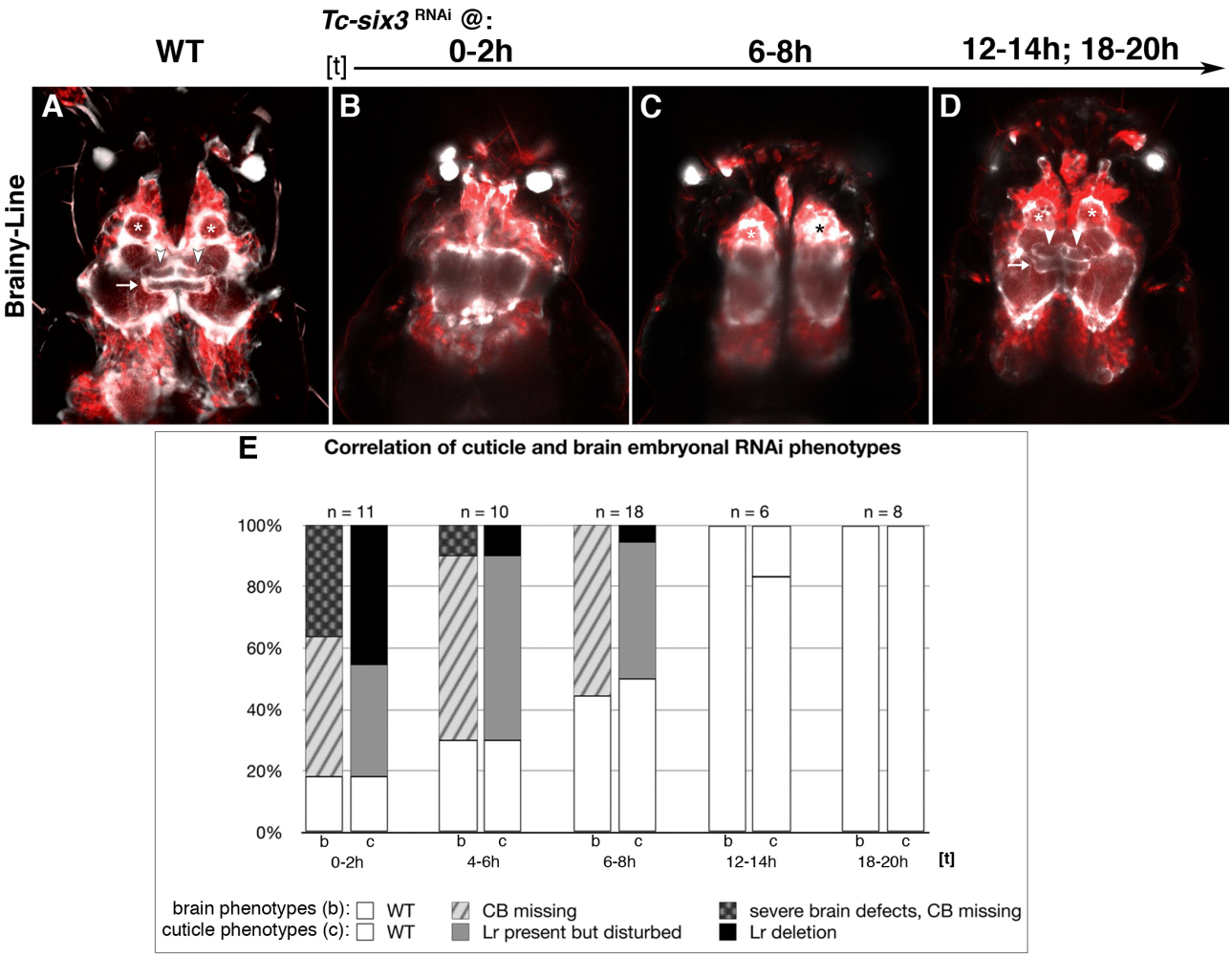

Figure 37: The epidermal and neural function of *Tc-six3* is not separable by late embryonic RNAi.
Embryos of the `Brainy´ (BA) line were injected with 1µg/µl dsRNA of *Tc-six3* at 0-2h, 4-6h, 6-8h, 12-14h, and 18-20h age. (A) WT situation in untreated BA line larva. (B) Strong brain phenotype induced by *Tc-six3* dsRNA injection at 0-2h. (C) Weak brain phenotype induced by *Tc-six3* dsRNA injection at 6-8h, where the Central Body (CB) is not detectable and the brain hemispheres are separated. (D) Injections performed at 12-14h and 18-20h, where no defects were detectable. The larva depicted here originated from 18-20h injections series. The larvae are oriented to face anterior to the top. White arrowheads indicate the median lobes of the Mushroom Body. Asterisks indicate the Antennal Lobes, the arrow indicates the CB. (E) The graph depicts the distribution of larval brain and cuticle phenotypes caused by embryonic RNAi against *Tc-six3* in percent depending on the time of injection. The left bars within each group reflect the distribution of larval brain phenotypes (b), while the right bar reflects the distribution of cuticle phenotypes in the respective embryos (c). The detached area in the cuticle bar [14-16h] represents a defect cuticle not reflecting the typical *Tc-six3* RNAi phenotype. The defect was probably caused by an injection artefact. n = the number of individuals which were analyzed for brain and cuticle defects. `Lr present but disturbed´ represents the weak cuticle phenotype. `Lr deletion´ represents the strong cuticle phenotype.

This result correlated with the `residual larvae´, where 70% (7 out of 10) showed a strong phenotype. Among the `scanned larvae´ the proportion of the weak cuticle phenotypes was 36,4% (4 out of 11; compare Figure 34, B; Figure 37, E, 0-2h cuticle bar graphs), which correlated with 30% (3 out of 10) in the `residual larvae´. No WT-like cuticle was detected among the `residual

larvae´, while 18,2% WT-like phenotype were detected among the `scanned larvae´ (2 out of 11; Figure 37, E, 0-2h cuticle bar graphs). Thus, in both cuticle analyses the strong phenotypes were dominant.

Following a knock down of *Tc-six3* at later stages (4-6h), strong cuticle phenotypes were drastically reduced to 10% in the `scanned larvae´(1 out of 10, Figure 37, E, 4-6h cuticle bar graphs). This also correlated with the analysis of the `residual larvae´, where the contribution of the strong phenotype decreased to 35,1% (13 out of 37). Larvae with weak cuticle phenotypes were represented with 60% among the `scanned larvae´ (6 out of 10; Figure 37, E, 4-6h cuticle bar graphs), while these represented 54,1% (20 out of 37) in the `residual larvae´. The portion of WT-like cuticles rose to 30% (3 out of 10; Figure 37, E, 4-6h cuticle bar graphs) among the `scanned larvae´ and to 10,8% (4 out of 37) in the `residual larvae´.

In even later injections (6-8h), strong cuticle phenotypes were again drastically reduced to 5,6% (1 out of 18; Figure 37, E, 6-8h cuticle bar graphs) in the `scanned larvae´ and within the `residual larvae´ no cuticle was found to show this phenotype. Even the batch of larvae with weak cuticle defects were decreased in both the `scanned larvae´ with 44,4% (8 out of 18; Figure 37, E, 6-8h cuticle bar graphs) and the `residual larvae´ with 30,9% (17 out of 55). The portion of larvae without any phenotypes rose up to 50% (n=9/18) among the `scanned larvae´ and to 69,1% (38 out of 55) among the `residual larvae´. Embryonic knock down of *Tc-six3* at later stages than 12h at 32°C age caused no detectable cuticle phenotype (Figure 37, E, 12-14h and 18-20h cuticle bar graphs). Thus, the severity of the *Tc-six3* phenotypes decreased with the age of the embryos at the time of RNAi treatment.

For grouping the brain defects into strong or weak phenotypes, the same parameters as mentioned above were used. Strong phenotypes also had severe brain defects. Here, the median lobes of the Mushroom Body were not detectable, the posterior glial tissue appeared to be strongly reduced and the brain hemispheres were close to each other (Figure 37, B). Weak phenotypes showed malformed Mushroom Bodies and clearly separable brain hemispheres, but no Central Body (Figure 37, C). Strong brain malformations decreased from 36,4% at an injection age of 0-2h (4 out of 11; Figure 37, E, 0-2h brain bar graphs), to 10% at an injection age of 4-6h (n=1/10; Figure 37, E, 4-6h brain bar graphs), and no strong phenotypes could be detected in injections in older embryos. Weak brain phenotypes were represented in 45,5% of the 0-2h old RNAi treated embryos (5 out of 11; Figure 37, E, 0-2h brain bar graphs), in 60% of the 4-6h old embryos (6 out of 10; Figure 37, E, 4-6h brain bar graphs), and finally in 55,6% of the 6-8h old embryos (10 out of 18; Figure 37, E, 6-8h brain bar graphs).

A similar distribution of phenotypes became apparent when comparing brain and cuticle phenotypes (Figure 37, E). However, the brain tissue seems to be sensitive for RNAi with *Tc-six3* for a longer time (Figure 37, E, 6-8h bar graphs). Apparently, there are two separate functions for *Tc-six3*. These can be divided into an early function leading, to strong phenotypes when performing RNAi, while a later function leads to impairment of the Central Body development. However, the *Tc-six3* function essential for brain development cannot be separated from the epidermal function. Generally, *Tc-six3* appears to be required for a proper brain development even at very early stages(6-8h still is an undifferentiated blastoderm).

5. Discussion

5.1. Reporter systems in *Tribolium castaneum*

One part of this work was to establish transgenic reporter lines for visualization of the brain in *Tribolium castaneum*. The expression pattern of most of the lines that were generated differed from the expectations. This depends on many factors, e.g. the choice of the cloned regulatory regions, cloning and amplification artifacts, and also positional effects. In this paragraph, some features that could generally improve the reporter systems like the specific ones established here, will be discussed.

5.1.1 Improving the *Tc-repo* and *Tc-elav* reporter lines

In case of the `reg.-*Tc-repo*::tGFP´ reporter line, the expression pattern of the reporter gene appears to mirror that of the endogenous *Tc-repo* in early glia precursor cells. However, double *in situ* hybridization in `reg.-*Tc-repo*::tGFP´ embryos would further prove, if the mRNA expression patterns of *Tc-repo* and tGFP overlap exactly. In larval stages, only a weak fluorescent tGFP signal was detected. Both the delay in the tGFP mRNA expression pattern and the weak fluorescent signal in the larval stages indicated only a low expression level of the reporter gene. The resulting questions are, why the expression pattern is that light and how this could be improved?

One possibility to enhance tGFP expression, is to clone the total 5´UTR of *Tc-repo*. As described, half of the 5´UTR was not cloned due to the selection of optimal primers (chapter 3.2.2). The sequence of these primers was selected for a small number of hairpins and a high amount of GC bases, which was the case only 44 bp upstream of the translation start site. This deletion could have resulted in a reduction the transcription of the reporter gene. This could be the reason that the levels of tGFP mRNA, which were detectable by whole mount *in situ* hybridization, were reached with a slight delay and that the fluorescent signal in the larval brain was only weak.

An alternative possibility to increase the reporter gene signal would be to duplicate the regulatory region. In many other reporter lines, a duplication of the regulatory region led to enhanced expression of the reporter gene. For example in the line 6xP3::eCFP, the promoter sequence `3xP3´ was cloned twice in tandem (Hein, 2007). In case of the `reg.-*Tc-repo*::tGFP´ line, the regulatory region has a length of 4510 bp. Therefore, an enhancer analysis would make sense in order to minimize the size of the sequence that needs to be duplicated. After duplication of this enhancer, the reporter gene signal in the embryonic stages could possibly be detectable earlier and closer resemble the endogenous *Tc-repo* expression. Furthermore, the signal in the larval brain would be enhanced, which would facilitate detailed colocalization analyses regarding the glial cells which are

marked in the `reg.-*Tc-repo*::tGFP´ line. In any case, the expression of the reporter gene of the `*Tc-repo*::tGFP´ reporter line is congruent to the endogenous *Tc-repo* expression. This indicates that no positional effects of the insertion locus are present.

The reporter lines of `*Tc-elav*::mRFP´ showed an unexpected pattern in two lines derived from independent G_0. Both showed an identical larval expression pattern in the muscles. An independent integration at the same genomic locus, resulting in the same enhancer trap, is very unlikely. This could only be proven by an identification of the respective integration locus of both transgenic lines. Interestingly, a search for orthologues of the gene *Tc-elav* revealed two genes in *Drosophila, found in neurons* (*fne*) and *elav*. A blast of the *Drosophila* genes within the *Tribolium* genome revealed in contrast only the single gene prediction Glean_4718, called *Tc-elav* (Simonnet, personal communication). Despite this unclear situation for the *Tribolium* homologues, the *Drosophila* transcripts and proteins of the genes *Dm-fne* and *Dm-elav* are restricted to neurons of the CNS during embryogenesis (Yannoni and White, 1997; Samson and Chalvet, 2003; Berger et al., 2007). *In situ* stainings for *Tc-elav* also showed an expression in the CNS during embryogenesis in *Tribolium* (Simonnet, personal communication).

One reason for the muscle expression pattern could be enhancer elements surrounding the gene locus of *Tc-elav*. The glean prediction Glean_4647, coding for a zinc carboxypeptidase, was identified in the upstream region of *Tc-elav* (chapter 3.2.1). This gene and its function are still unknown, so possibly it is expressed in muscles. Since access to alternative lines with specific CNS expression was possible (`MB´ line and `Brainy´ line, chapter 3.4), the `*Tc-elav*::mRFP´ reporter line was not analyzed further.

5.1.2. Low efficiency of the *Tc-six3* reporter constructs

The ability of promoters to be controlled by surrounding enhancer elements of the insertion locus was often proven and is used for large enhancer trap screens in various species (Klämbt and Goodman, 1991; Yang et al, 1995; Wurst et al., 1995; Horn et al., 2000; Balciunas et al., 2005; Trauner et al., 2009). This possibly explains, why the different lines carrying the same *Tc-six3* reporter construct show different expression patterns. I.e. the offspring derived from two independent G_0 carrying either the construct `*Tc-six3* 10up´ or `*Tc-six3* 5up´ did not show similar expression patterns. This suggests influences of surrounding enhancer and silencer elements of the insertion locus and low regulatory activity of the cloned fragments. To improve reporter lines, influences of surrounding enhancers could be avoided by either using insulator sequences or by site specific integration, for example by attP and attB integration (reviewed in Groth and Palos, 2004;

Smith et al., 2010) which has recently become possible also for *Tribolium castaneum* (Kittelmann, personal communication).

Generally, the fluorescent reporter signal was very weak or absent in the lines carrying the constructs derived from *Tc-six3*. The construct of the `*Tc-six3* promoter element´ which was used as core promoter for all *Tc-six3* constructs except for `*Tc-six3* 5up´, had a deletion of 9 bp, 484 bp upstream of the endogenous translation start site. This deletion, which occurred due to a PCR artifact, possibly caused a decrease in the transcription of the reporter gene tGFP. Thus, to exclude such a possible influence, new constructs which are including the entire core promotor could be created.

Another potential problem does not appear to play a role: The core promoter element of *Tc-six3* included an additional ATG upstream of the reporter gene tGFP. These additional 18 bp are in frame, and cause additional 6 amino acids at the N-terminus of the tGFP protein. However, a fluorescent signal was succesfully detected for example in the line 7 carrying the reporter construct `*Tc-six3* 10up´. Additionally, a fluorescent signal of tGFP was detected *in vivo* in the embryos of the reporter lines 6 and 3, which are carrying the construct `*Tc-six3* 10up´ and `*Tc-six3* 5down´ (data not shown). Therefore these constructs in principle express detectable tGFP levels.

5.2. The `*Tc-rx* 5up´ line for studies of axon guidance and brain midline cues

The transgenic line `*Tc-rx* 5up´ shows a complex expression pattern of DsRedExpress in the larval brain. This appears to correspond to the endogenous *Tc-rx* expression (chapter 3.3.3.2). Therefore, this line is very promising for future studies of the development of the Central Complex and axon guidance in *Tribolium*.

The DsRedEx positive cell cluster is located in the posterior median part of the larval brain. This region correlates to the pars intercerebralis in insects, which is defined by its localization in between the brain hemispheres anterior mediolateral to the Mushroom Body and dorsally to the Central Body (white square, Figure 20; De Velasco et al., 2007; Steinmetz et al., 2010; Boyan and Williams, 2011; Boyan and Reichert, 2011). For *Drosophila,* it is known that the protein *Chx1* is expressed in the pars intercerebralis, while the homologue of *Rx* could be detected in the surrounding protocerebral tissue (De Velasco et al., 2007; Steinmetz et al., 2010). A definite verification and localization within the brain of the DsRedEx positive cells could possibly be performed by using antibodies specific for neuropeptides, which are produced by pars intercerebralis cells or specific for the *Tc-Rx* protein.

In *Schistocerca* and *Drosophila,* 4 NBs, which are playing an important role in formation of the Central Complex and which are located in the pars intercerebralis could be identified in the embryo and pupal brain. The neuronal progenies of such NBs are projecting anterior and crossing the brain midline. The orientation for axonal growth is given by an axonal scaffold forming commissures and connectives within the embryonic brain. At the onset of the Central Complex development, the commissures of the scaffold are already spanning the brain midline (Boyan and Williams, 1997; Williams et al, 2005; Boyan et al., 2008; Pereanu et al., 2011). In the transgenic line *`Tc-rx*-5up´ the DsRed positive cell cluster forms an axon bundle, which is projecting anterior and forms a chiasma by crossing the brain midline (white arrowhead, Figure 20). This axon bundle is located dorsally in the brain, but ventrally in relation to the neuraxis. Crossing experiments with the 6xP3 line revealed that this projection lies outside of the Central Body (chapter 3.3.3.2). Therefore, this axon bundle clearly does not belong to the Central Body and it thus did not derive of progenies of the 4 NBs known from *Schsitocerca* and *Drosophila*. However, the DsRedEx positive contralateral projections could belong to the commissures which form the scaffold and therefore give orientation to further axon growth. Immunohistochemistry stainings for horse radish peroxidase as a general neuronal marker (Jan and Jan, 1982; Kurosaka ate al., 1991), and for the reporter gene DsRedExpress should be performed in order to localize the respective axon bundle within the brain. It is known from several other species that the primary commissure pioneer (PCP) axons present the antigen Term-1, which is a glyocprotein secreted on the growth cones of neurons during axogenesis. At the same time, these axons are stainable with anti HRP (Meier et al., 1993; Ludwig et al., 2002). This is suggested to hold true for PCPs in *Tribolium castaneum* as well and colocalizaton should be tested with the respective antibodies in the *`Tc-rx* 5up´ line.

In this line, an additional DsRedEx positive structure could be detected anterior to the larval Central Body (chapter 3.3.3.2). By its shape and position within the brain, it possibly correlates to the *Schistocerca* lateral accessory lobes. These protocerebral functional units are tightly connected to the Central Complex (e.g. Boyan and Williams, 1997; Kurylas et al., 2008). In *Schictocerca,* a partial origin of the lateral accessory lobes lies in the pars intercerebralis (e.g. Boyan and Williams, 1997). However, in *Tribolium,* a connection between such anterior structure and the DsRedEx positive cells posterior in the brain surrounding the Kenyon cells is not detectable in the line *`Tc-rx* 5up´. Still, a detailed analysis should be performed in dissected brains for better optical resolution.

Taken together, the *`Tc-rx* 5up´ line marks an interesting axon bundle in the *Tribolium castaneum* brain as well as possibly the putative lateral accessory lobes (LAL). To address the embryonic axogenesis of the commissure and the putative LAL development, *in vivo* imaging could be

performed, especially in combination with a 6xP3::eCFP line. This would reveal, which of these parts arises first. If the mentioned axon bundle is a part of the scaffold surrounding and guiding the Central Body development, these axon bundles should be detectable before the Central Body is formed.

Furthermore, the `*Tc-rx* 5up´ line enables functional analyses regarding axon guidance. For example, a knock down of the genes *Tc-robo* and *Tc-split* could deliver insight into the ability of axons to cross the brain midline. The respective orthologue genes are known to act as receptor and ligand and to be involved in correct commissure formation in the CNS of vertebrates and invertebrates (Kidd et al., 1999; Brose et al., 1999; Jen et al., 2004; Dickson et al., 2006; Furrer et al., 2007; Coleman et al., 2010). Performing these functional analyses by using the `*Tc-rx* 5up´ line, defects on axon projection could be easily studied.

5.3. The `Brainy´ reporter line allows analyses of embryonic brain development regarding the function of *Tribolium* homologues to vertebrate neural plate genes

The `Brainy´ line is a crossing between two different lines that mark neuronal cells and glial cells (chapter 3.4). It is unknown, how much of the neuronal tissue is marked by the `Brainy´ line, and whether this line also marks neuronal cells derived from the mesectoderm. However, for the glial marking part (6xP3::eCFP) of this line, a coexpression analysis with an antibody for *Repo* was performed. From other species it is known that the *repo* homologue is expressed in all glial cells derived form the neuroectoderm, whereas glial cells derived from mesectoderm do not express this gene (Yuasa et al., 2003; Lee & Jones 2005; reviewed in Parker et al., 2006). The coexpression analyses of the 6xP3::eCFP and the anti *Repo* signal in *Tribolium* were performed in colaboration with Martin Kollmann and Joachim Schachtner (University of Marburg). They revealed that 98,8 % of the eCFP positive cells at the same time also express the protein *Repo*, whereas 84,2% of the *Repo* positive cells are also positive for the eCFP signal (Kollmann et al., unpublished). This suggests, that the eCFP positive cells of the `Brainy´ line are glial cells, but that not all glial tissue is marked by this line, as approximately 15% of the *Repo* positive glial cells are not detectable in the `Brainy´ line.

Although not all cells may be marked, this reporter line enables visualization of embryonic brain development in *Tribolium castaneum* (chapter 3.1.4). Surface glia marked by the expression of eCFP enable visualization of the borders of most neuropiles like the Antennal Lobes, the Central Body and the Mushroom Body in the larval brain. Nevertheless, the neuropiles themselves are not detectable in this line. Also the paired Protocerebral Bridge in the posterior part next to the calyces

(chapter 1.5), appears not to be enveloped by glial cells yet. Additionally, in the *Tc-chx* RNAi phenotypes, the split Central Body was not detectable by *in vivo* imaging but required antibody staining. This was probably due to the lacking glial surface. These two examples demonstrate the limitations of this line in investigating gene functions in neuropilear organization. Despite these limitations however, the `Brainy´ line offers the opportunity to investigate the overall larval brain architecture. It offers the first step in the identification of genes, which have a strong influence on neural architecture and/or glial tissue. Thus, the `Brainy´ line established in this work is a very promising tool for first gene function analyses regarding embryonic brain development in *Tribolium castaneum*.

In *Schistocerca,* it is known that the protocerebral commissure develops when 31% of embryonic development is completed and when the protocerebral lobes lie next to each other. By dissolving of the glial bound borders, primary commissure pioneer axons derived from the mesectoderm cross the brain midline and serve as a scaffold for further midline crossing axons (Boyan et al., 1995; Ludwig et al., 2002). In the case of *Tribolium castaneum*, the development of the protocerebral commissure has not been studied yet. Here, the `Brainy´ line gives first clues to the point in time of its development. After a development of 30h at 32°C (35%), the protocerebral lobes of the embryo become detectable and then grow to strong branches in up to 40h (48%) old embryos. The hemispheres lie close to each other, separated by a border consisting of glial cells. In 45h (53%) old embryos, the commissure is already visible and could also be confirmed by using the DIC-filter in case of *Tc-chx in situ* hybridizations in an embryo aged 40h - 55h (chapter 4.3.1). This indicates, that in the time slot of 30h to 40h which correlates to approximately 35% to 48% of the embryonic development, the primary commissural pioneers axon should have passed the brain midline. Apparently, the timing is very conserved between the mentioned insects. A more detailed analysis of axon scaffold and commissure formation would be possible by anti HRP and anti-Term1 stainings.

Various aspects could be addressed in future studies using this line. How are the brain midline features established, i.e. are genes like *netrin*, *roundabout*, *slit*, and *Dscam* also involved in midline passing controls in the brain of *Tribolium castaneum* (reviewed in Evans and Bashaw, 2002; Edwards and Meinertzhagen, 2010)? Which genes expressed in the neuroectoderm influence the development of neuropiles or parts of the brain? For example, what is the role of the identified 18 genes in *Tribolium castaneum* which are homologues to genes expressed in the vertebrate neural plate genes and which are also expressed in the head during embryonic stages (Posnien, 2009)? Until now, only effects on the epidermis could be studied by RNAi technique in *Tribolium*

castaneum, but using the `Brainy´ line now also neurogenic aspects can also be addressed. This will be especially interesting for the genes, which showed only minor defects on the larval head cuticle after the respective knock down, e.g. *Tc-ey, Tc-toy, Tc-so/six1, Tc-dbx, Tc-ptx/pitx, Tc-mirr/irx, Tc-mun/arx, Tc-fez, Tc-gsc, Tc-tll* and *Tc-Dll* (Posnien, 2009). The `Brainy´ line allows the rapid identification of genes with strong effects on brain morphology upon RNAi. This would be especially interesting for the embryonic Central Body development in *Tribolium castaneum,* because *Drosophila melanogaster* does not develop this structure in the embryo.

5.4. Results regarding the embryonic brain formation in *Tribolium castaneum*

In this work, various genes were analyzed regarding their function in embryonic brain development in *Tribolium castaneum*. The major focus lay on those genes, which had a putative influence on the Central Body formation, since *Drosophila* lacks such a neuropile in larval stages. The different aspects and gene functions according their loss-of-function phenotypes will be discussed and finally summarized in a model.

5.4.1. *Tc-otd1* has a conserved function in specifying the anterior part of the brain

In previous analyses, a knock down of *Tc-six3* by parental RNAi led to the expansion of the *Tc-otd1* domain in the neuroectoderm. Vice versa, a repressing function for *Tc-otd1* on the expression of *Tc-six3* was hypothesized, forming a negative feedback loop to retain the boundaries of the expression domains (Posnien, 2009). During embryonic development, two major functions were suggested to be carried out by *Tc-otd1*: First of all in axis formation and regionalizing of blastodermal fate during the early blastodermal stages and secondly in head patterning in embryonic later stages (Schinko et al., 2008; Kottkamp et al., 2010).

To gain insight into the *Tc-otd1* function on brain patterning, embryonic knock down was performed in different stages in this work (chapter 4.2). Here, in weak phenotypes a reduction of the posterior glial tissue was observed, whereas in strong phenotypes the entire brain architecture was affected (chapter 4.2). Intermediate phenotypes, which reflect the transition from weak to strong effects were not detected. Thus, a true intermediate phenotype as it was expected to be caused by time dependent embryonic injections was missing.

In previous studies, embryonic *Tc-otd1* RNAi experiments were performed and revealed cuticle based phenotypical series. The various outcomes of time dependent injections suggested different functions in development: Once a regionalizing function in blastodermal stages and secondly a head patterning function in embryonic later stages (Schinko, et al., 2008). In the case of head patterning,

Tc-otd1 function has to take place in embryonic stages where the head anlage is already existing. Hence it influences the ectodermal as well as the neural fate. Interestingly, in this work, the cuticle phenotype and the brain phenotype correlate with each other. Whenever a strong cuticle phenotype occurred, the brain was strongly affected as well. The same correlation was observed in weakly affected RNAi embryos. Focusing on the brain phenotypes, in embryos injected at an age of 7-8h old at 32°C, the strong phenotypes were dominant while only weak phenotypes occurred in embryos injected later (chapter 4.2). This indicates a temporal specification and patterning function of *Tc-otd1* in the anterior brain. The obtained results led to the question, when this stage of transition between strong and weak phenotype occurs? Apparently, this time is between 7-8h at 32°C when the embryos are still in a blastodermal stage and *Tc-otd1* is already strongly expressed (Schröder, 2003; Schinko etal., 2008; Kotkamp et al., 2010). Therefore, a knock down of *Tc-otd1* mRNA does not immediately affect the *Tc-Otd1* protein level which is dependent of the protein turn over. Antibody stainings for *Tc-Otd1* could reveal how fast levels of this protein are reduced after RNAi. Thereby, it would be possible to better identify the transition between the strong and the weak phenotype.

The function of *otd* homologues in regionalizing the anterior part of the brain was suggested to be conserved for invertebrates and vertebrates (Hirth and Reichert, 1999; Lichtneckert and Hirth, 2005; Lichtneckert and Reichert, 2008; Hirth, 2010). In *Drosophila,* a loss of *otd* results in the loss of the entire anterior brain, probably due to the absence of most protocerebral and partially also the deuterocerebral neuroblasts in such mutants (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). In mouse, *Otx2* null mutants the entire forebrain and midbrain are absent (Acampora et al., 1995). Therefore, the strong phenotypes in *Tc-otd1* RNAi probably reflect an anterior brain deletion. However, in some scans of strong *Tc-otd1* knock down brain phenotypes, structures which possibly are parts of the Mushroom Bodies could be detected (not shown). To verify this situation, *Tc-otd1* knock down experiments together with immunohistochemic analyses using the Mushroom Body marker DcO could be performed. In *Drosophila,* the NBs which form the Mushroom Body neuropile arise in the protocerebrum (Noveen et al., 2000). Studies of correlating expression patterns of homologue genes like *dachshund* and *eyeless* also identified a correlating region in the protocerebrum that gives rise to Mushroom Body NBs in *Tribolium* (Hein, 2007; diploma thesis). This suggests, that the Mushroom Bodies are protocerebral structures and therefore part of the anterior brain. Therefore, if the hypothesis of anterior brain deletion holds true, the Mushroom Body should not be detectable after a knock down of *Tc-otd1* (Acampora et al., 1995; Hirth et al., 1995; Younossi-Hartenstein et al., 1997).

In an alternative hypothesis, *Tc-otd1* is required for cell survival and therefore a knock down leads to a reduction of the brain. Even though mostly unknown, one of the putative target genes of *Tc-Otd1* during blastodermal stages was suggested to be a housekeeping gene (Kotkamp et al., 2010). Thus, a disturbance induced by knock down of *Tc-otd1* could lead either to a loss of cell fate in the developing brain, followed by programmed cell death, or to the loss of the housekeeping gene, which in turn also leads to cell death. Both situations could explain the drastically reduced brains with only partially present neuropiles. To identify, whether a gap like deletion occurs after a knock down of *Tc-otd1,* specific antibody stainings against proteins like *Tc-Six3, Tc-Rx, Tc-Chx* or DcO expressed in the protocerebrum could be performed. If these proteins are still detectable, a complete deletion of the anterior brain can be excluded. To investigate the possibility of cell death, a TUNEL (terminal deoxyribonucleotidyl transferase [TDT]-mediated dUTP-digoxigenin nick end labeling) assay of the larval RNAi brain could be performed (Gavrieli et al., 1992; Prpic and Damen, 2005). By this it could be estimated, if a knock down of *Tc-otd1* leads to an increase in cell death in the developing embryonic brain.

Nevertheless, the function of the anterior regionalization of the anterior brain is conserved among the species (reviewed in Lichteneckert and Reichert, 2005). Therefore it is likely, that the knock down phenotype of *Tc-otd1* in *Tribolium* mirrored this conserved function and lead to the deletion of the anterior parts of the brain. For this function, *Tc-otd1* appears to be required rather early in development. Alternative options for this drastical brain phenotype would be that genes involved in cell fate specification require *Tc-otd1* input, or that the expression of a housekeeping gene could be affected after a knock down of *Tc-otd1* in embryonic stages (Kotkamp et al., 2010).

5.4.2. The role of *Tc-rx* in Central Body formation

In vertebrates, *Rx* homologues play an important role in eye and brain development. An overexpression can induce ectopic retinal pigmented epithelium, duplications of the retina and the neural tube (Chuang and Raymond, 2001; Bailey at al., 2004). In contrast, the *Drosophila* homologue *drx* was found not to be required for establishing the visual system (Davis et al, 2003) and it was not even found to be expressed in embryonic and larval eye primordia (Eggert et al., 1998). *Tc-rx* is not expressed within the developing eye field (Posnien, 2009; chapter 3.3.1). This seems to be confirmed by *Tc-rx* RNAi phenotypes, where no detectable malformation was observed in the larval eyes. This aspect is however preliminary and has to be explicitly studied by immunohistochemistry. However, *rx* homologues of insects and other invertebrates appear not to have functions in eye development (Arendt et al., 2002; Davis et al., 2003; Arendt et al., 2004).

Yet, *Tc-rx* RNAi leads to a split Central Body which is located at the midline in the larval brain. The split parts are surrounded by glial cells and indicate a loss in midline passing signals (chapter 4.1). *Tc-rx* expression starts at the point in time when 5-8 *wingless* stripes occur, which can be correlated to an approximate age of 12-15h at 32°C. At the onset of expression, it is bilaterally expressed at the anterior median rim of the head lobes. In later stages, the expression pattern shifts to a more lateral position at the rim of the head lobes. After head morphogenesis, the *Tc-rx* expression domains are positioned in the middle of the head laterally to the midline (Posnien, 2009; chapter 3.3.1). A quantification of the amount of NBs which arise from *Tc-rx* positive regions revealed 11 up to an age of 18h at 32°C (see chapter 3.3.1). Furthermore it is suggested by the double stainings, that the *Tc-rx* positive cells in the brain may derive from such NBs.

The role of *Tc-rx* in formation of the neuroendocrine system was suggested to be conserved among bilaterians. Homologues of *rx* were discussed to be involved in forming the neuroendocrine system in *Drosophila, Platynereis,* zebrafish and other animals (Hartenstein, 2006; de Velasco et al., 2007; Tessmar-Raible et al, 2007). Although the specific role of *drx*-positive neurons in the pars lateralis of *Drosophila* are still obscure, they possible form extra neurosecretory cells which are not connected with the ring glands (de Velasco et al., 2007). Steinmetz and colleagues (2010) assume that the neurosecretory system in bilaterians derives from cells in the anterior part of the neuroectoderm/brain precursor which are *six3* positive. The Central Body forming neuroblasts were identified to be located in the pars intercerebralis in the embryonic brain of *Schistocera*, as well as in the pupal brain of *Drosophila* (reviewed in Boyan and Reichert, 2011). There is no obvious connection between *rx* positive regions and these NBs which contribute the Central Complex. Interestingly, in *drx* mutant *Drosophila* flies, the development of the ellipsoid body which is part of the Central Complex and the homologue structure to the lower unit in other insects, is malformed or even unfused (Davis et al., 2003). Davis and colleagues (2003) discussed a mechanism where neurons in *drx* mutant flies either change the ability of their growth cones to respond to midline cues or that pioneer tracts or glia for proper ellipsoid body placement and formation are missing. In the larval head of *Tribolium,* only the lower part of the upper unit of the Central Body exists (chapter 1.5). However, a knock down of *Tc-rx* leads to an unfused Central Body, similar to that observed in *Drosophila* mutants. Despite the different stages, *rx* appears to play a similar role in embryonic and larval Central Body formation in *Tribolium* and *Drosophila*, respectively.

Eventually, the question how a split Central Body can arise, remains. In *Drosophila,* genes, which play important roles in axon pathfinding and especially in midline crossing, could be identified (reviewed in Evans and Bashaw, 2010). The receptor proteins *Round about* (*Robo*) have been

analyzed with respect to their contralateral axon guidance ability in the *Drosophila* brain. Here, a loss-of-function study in *Robo2* and *Robo3* mutants discovered malformations of the Central Complex, especially the fan-shaped body and the ellipsoid body. The axons which are crossing the brain midline in the WT-situation, are concentrated at the brain midline here but do not cross (Nicholas and Preat, 2005). When comparing this to the *Tribolium Tc-rx* knock down phenotype, certain similarities appear, as this leads to a split Central Body (chapter 4.1). Similar to the *Drosophila Robo* mutants, the split parts of the Central Body are at the correct positions within the protocerebrum in *Tribolium*. Additionally, the split compartments are enclosed by glial cells like in the WT-situation in *Tribolium*. The presence of surface glia in *Tc-rx* knock down phenotypes makes a specific glial function of *Tc-rx* unlikely, but nevertheless it cannot be excluded. In contrast, the described split Central Body phenotype in *Tc-rx* knock down *Tribolium* larvae suggests a lack of cues for midline crossing. Possibly the growth cones of the processing Central Body neurons are affected, which would lead to a loss of their capability to cross the brain midline. Also extrinsic cues, for example the proper formation of a primary axon scaffold, or even the presence of ligands or other factors, which influence the axon guidance at the brain midline, could be missing. From the expression pattern of *Tc-rx* in embryonic stages (chapter 3.3.1 and 3.3.3.2) and from the `*Tc-rx* 5up´ reporter line (chapter 3.3.3.2) no evidence that *Tc-rx* could be expressed in Central Body forming neurons is provided. It is more likely that *Tc-rx* is required for formation of the axon scaffold which is necessary for the midline crossing of the Central Body axons.

To prove the axon scaffold building hypothesis, an additional *Tc-rx* RNAi experiment could be performed in the `*Tc-rx* 5up´ reporter line. An absence of such a scaffold would lead to a general breakdown of the midline spanning projections in larval stages, meaning the DsRedEx positive axon bundles would not cross the brain midline. An anti HRP staining in *Tc-rx* RNAi animals would confirm this and address this question in an embryonic stage. Also, antibodies specific for *Tc-rx* would offer the possibility to identify putative targets of *Tc-rx* by chromatin immunoprecipitation (e.g. Orlando, 2000). The findings from such analyses would possibly help to elucidate the function of *Tc-rx* concerning the cell specification and formation of the Central Body in the embryonic and larval brain of *Tribolium castaneum*.

5.4.3. *Tc-six3* and *Tc-chx* are required for Central Body formation

The results of this work showed that *Tc-six3* is important for the proper development of the larval Central Body during embryogenesis in *Tribolium castaneum*. What role does *Tc-six3* play during the embryonal development and the development of the Central Body? Taken together, *Tc-six3* is

exclusively expressed in the head during later stages, i.e. in the anterior most part of the embryo tissue, the labrum and surrounding tissue. Within the *Tc-six3* positive tissue, at least 8 NBs arise. RNAi experiments revealed that in strong phenotypes the median head epidermal as well neural tissue is deleted, while in weak phenotypes mainly the brain is affected through the loss of the Central Body.

Due to these results, two functional phases for *Tc-six3* during embryogenesis are suggested, once a proliferative and second a neural fate specifying function. The strong phenotype suggests a proliferative function of *Tc-six3* and is characterized by the loss of the median head including the median brain. Especially the immunohistochemistry experiment revealed the absence of MIP positive cells in the median brain region after a knock down of *Tc-six3* (chapter 4.3.4). The absence of these cells in knock down larval brains indicates their origin in tissue, which depends on *Tc-six3* function. In vertebrates, a cell proliferative function for *SIX3* was detected and a misfunction of *SIX3* leads to apoptosis in the anterior neural ectoderm and finally to holoprosencephaly (e.g. Loosli et al., 1999; Gestri et al., 2005; Geng et al., 2008). In mice, mutants with hypomorphic alleles of *SIX3* and *SHH* revealed higher apoptosis rates in distinct regions of the lateral dorsal telencephalon, which was suggested to cause a microcephaly phenotype and in addition showed a slight reduction of proliferating cells (Geng et al., 2008). Due to this, *Tc-six3* is suggested to play an important role in cell proliferation in the anterior median tissue. To test this, apoptosis and proliferation changes should be quantified by TUNEL (Gavrieli et al., 1992) assay and phosphohiston-3 staining (Hendzel et al., 1997). An enhanced cell death and/or a reduced proliferation in *Tc-six3* RNAi could explain the loss of the anterior neuroectoderm, from which cells positive for MIP and serotonin and cells required for Central Body formation normally arise.

While the strong phenotype indicates an early function of *Tc-six3* in cell proliferation and therefore in forming the correct amount of cells, the weak phenotype indicates a more specific neural determining function. Weak phenotypes revealed a total loss of the Central Body, while the cuticle was weakly or not at all affected (chapter 4.3.3, 4.3.4, and 4.3.5). Neither in RNAi *in vivo* scans nor in the respective immunohistochemistry stainings, were this neuropile or parts of it detectable, while the remaining brain appeared to be physiologically and morphologically unaffected (chapter 4.3.3 and 4.3.4). Due to the expression pattern of *Tc-six3,* which overlaps with the pars intercerebralis marker *Tc-chx* (chapter 4.3.1; de Velasco et al., 2007; Steinmetz et al., 2010), it is suggested that the pars intercerebralis tissue is affected by a knock down of this gene. From *Schistocerca* it is known that some NBs of the pars intercerebralis contribute to the Central Complex and to neurosecretoric cells, and to the primary commissure pioneer cells, which are

expressing Term-1 and are positive for anti HRP ((Boyan and Williams, 1997; Williams et al., 2005; Williams et al., 2008; Boyan et al., 2010, Herbert et al., 2010; Ludwig et al., 2002). Thus it is suggested that a knock down of *Tc-six3* leads to the loss of large portions or even to the loss of the entire pars intercerebralis in *Tribolium.* Taking into account that the primary commissure pioneers arise in the pars intercerebralis of *Tribolium* as well, no commissures would be detectable in knock down brains. This could be tested by respective antibody stainings, e.g. anti HRP.

Most evidence for such separable functions of *Tc-six3* during the embryonic development is delivered by the embryonic knock down experiment (chapter 4.3.5), where RNAi was performed at various times of embryonic development. The early knock down of *Tc-six3* reveals strong phenotypes and indicates the proliferative function of *Tc-six3*, as described above. I.e. the knock down of *Tc-six3* at early stages finally leads to the loss of the accurate cell number, hence to the loss of the median tissue, epidermally as well as neurally. However, a knock down of *Tc-six3* at later stages, revealed the weak phenotype (see above), which much more represents a neural function. Here, *Tc-six3* seems to be required to specify neural cells, which form the Central Body (chapter 4.3.5). Thus, the function of *Tc-six3* seems to switch from an early proliferative to a late neural specifying function during the embryonic development in *Tribolium castaneum.*

Intriguingly, these functions appears to be dose dependent. Pupal knock down experiments with differing *Tc-six3* dsRNA concentrations (1µg/µl and 100ng/µl) revealed that injections of low concentrations cause mainly Central Body defects, thereby reflecting the weak phenotype, while injection of highly concentrated *Tc-six3* dsRNA lead to a additional loss of median brain marking MIP expression, thereby reflecting the strong phenotype (chapter 4.3.4). In medaka, loss and gain of function experiments of *Six3* revealed a dose dependency in proliferation and differentiation in retinogenesis (Del Bene et al., 2004; Singh and Tsonis, 2010). High levels of *Six3* allowed proliferation and inhibited both the antagonist *Germinin* and premature neuronal differentiation (Del Bene et al., 2004; Singh and Tsonis, 2010). In correlation to pupal RNAi in *Tribolium*, the injection of a higher concentration of *Tc-six3* dsRNA appears to lead to the loss of the regarding threshold to enable cell proliferation at all, i.e. those cells either acquire a different fate, undergo cell death or start differentiation. Nevertheless, the anterior median region is lost. In contrast, the phenotypes caused by injections of lower concentrations of dsRNA could be explained by either a partial loss of cells, due to a reduced proliferative phase, or by premature differentiation, which results in the situation of too little cell numbers, at the end. Furthermore, it became clear by these experiments, that the first and early function of cell proliferation is highly regulative, while the second and later function of neural cell specification is not at all or not strongly regulative. I.e. to

achieve that the cell proliferative function of *Tc-six3* is interrupted, high doses of *Tc-six3* dsRNA are needed, while injections of lower dsRNA doses are still affecting the neural cell specification, especially the Central Body progenitor cells. Thus, *Tc-six3* function in embryonic development has two phases which are additionally dose dependent.

The function of *six3* homologues have been discussed to be conserved among the bilaterians. In mice it is expressed in the most anterior region of the neural plate, from which neural derivates like hypothalamus, optic vesicle, ventral forebrain and neurohypohysis derive (Oliver et al., 1995). Steinmetz and colleagues (2010) showed that *six3* homologues are expressed in the anterior tip during embryonic stages in *Platynereis, Pristina, Euperipatoides, Strigamia,* and *Tribolium* and thereby show a conserved expression pattern among bilaterians. Furthermore the authors showed that the *Drosophila Six3* homologue protein is detectable in a large region in the embryonic brain, which gives rise to the neuroendocrine system. However, the expression domain overlaps with cells of the pars intercerebralis, which are marked by the expression of *Drosophila chx1* (Steinmetz et al., 2010). In *Schistocerca,* a lot is known about neuroarchitecture and its development of the Central Complex during embryogenesis (Boyan and Williams, 1997; Williams et al., 2005; Williams et al., 2008; Boyan et al., 2010, Herbert et al., 2010). Here, eight bilateral NBs are located at the brain midline in the pars intercerebralis and contribute to the formation of the embryonic Central Body. Four per hemisphere play the key role in Central Body development and their progenies establish the basic columnar morphology of this neuropile (Boyan and Reichert, 2011). In *Drosophila,* recent studies identified 4 NBs to be involved in establishing the basic morphology of the Central Complex during late larval stages and the puparium (Itzergina et al., 2009). The expression pattern of *Tc-six3* had already been shown to be conserved (Steinmetz et al., 2010; chapter 3.3.1). Additionally, the respective *Tc-six3* positive region correlates to the location of the arising NBs, which form the Central Body in *Schistocerca* and in *Drosophila*. *Tc-six3* plays an important role in specifying the NBs to form the Central Body, which is demonstrated by the fact that the Central Body is lost after *Tc-six3* RNAi in *Tribolium* larvae (chapter 4.3.3). Thus in *Tribolium*, the Central Body forming NBs arise in the *Tc-six3* positive region (chapter 3.3.1). Therefore, the suggested role for *Tc-six3* in establishing parts of the neuroendocrine system in *Tribolium* could be confirmed by this work. The first indication was delivered by the overlapping expression pattern with the pars intercerebralis marker *Tc-chx*, but a clear evidence is the loss of the expression of the neurotransmitter serotonin and the neuropeptide MIP in the central brain after *Tc-six3* RNAi (chapter 4.3.4).

Due to the facts, that in *Drosophila chx1* was identified to specifically mark the pars intercerebralis

(de Velasco et al., 2007) and the correlation of NBs derived from the pars intercerebralis forming the Central Complex in *Schistocerca* (Boyan and Williams, 1997), the *Tc-chx* orthologue was an interesting candidate gene for the elucidation of the NBs in *Tribolium*. Additionally, it was identified to be overlapping in its expression with the gene *Tc-six3* in *Tribolium* (chapter 4.3.1) and this was also observed for the orthologue genes in *Drosophila* (Steinmetz et al., 2010). This combination of observations lead to the hypothesis that *Tc-chx* could specify the neural lineage responsible for the pars intercerebralis and NBs, which form the Central Body in *Tribolium*. After RNAi for *Tc-chx* in the `Brainy´ line, no structure ensheathed by glia resembling the Central Body in the larval brain could be detected. However, in immunohistochemistry analyses, structures positive for MIP and serotonin located laterally in the larval brain were detected. These findings support the hypothesis of displaying remnants of the Central Body (chapter 4.3.4). However, what could be the reason for the `split Central Body phenotype´, which is positive for MIP and serotonin staining, after a knock down of *Tc-chx*? Are these lateral structures really Central Body remnants?

The position of the MIP and serotonin positive structures do not support the theory of a split Central Body. In WT, the Central Body is located in the center of the brain, while in *Tc-chx* RNAi embryos, the position of the MIP and serotonin positive structures is lateral within the protocerebrum and they appear to be shifted to a ventral position. In comparison to the *Tc-rx* phenotype, which shows a split Central Body (chapter 4.1), the positioning of such remnants was WT-like. Additionally, the remaining parts of the Central Body were surrounded by glia cells in *Tc-rx* RNAi phenotypes, which was not the case in *Tc-chx* RNAi. Also, since the regarding glial cells are missing after *Tc-chx* RNAi, it is suggested that those derive from the *Tc-chx* lineage.

In *Tenebrio molitor,* immunohistochemistry analyses for serotonin positive neurons revealed that such neurons innervate the fan-shaped body in later larval stages (Wegerhoff and Breidbach, 1992). I.e. serotonergic neurons enter an existing Central Complex. Transferred to the situation in *Tribolium*, the Central Body is possibly absent after a knock down of *Tc-chx*, but that MIP positive and serotonergic neurons innervate any structure in the protocerebrum, but not the Central Body. This `non Central Body structure´ could be what was finally detected in immunohistochemistry analyses of *Tc-chx* knock down brains (chapter 4.3.4). Thus, these facts argue against the theory that the remnant structures after *Tc-chx* RNAi are parts of the Central Body.

However, it is more likely that in *Tc-chx* RNAi the Central Body is split and misplaced. In WT larva no similar structures positive for MIP and serotonin were detected. Furthermore, the shape is rectangular and longish, exactly like the split Central Body situation after *Tc-rx* RNAi. That such ectopic structures appeared by chance in *Tc-chx* RNAi is very unlikely.

Due to the assumption that *chx* homologues mark and specify the pars intercerebralis and that a knock down of *Tc-chx* leads to the loss of the regarding region, additional NBs, which are involved in Central Body formation, could be existing. Those NBs are not the putative homologue w, x, y, and z NBs known from *Schistocerca* and *Drosophila* (reviewed in Boyan and Reichert, 2011), but also do support the Central Body formation. The progenies of these additional NBs would not be affected by a knock down of *Tc-chx* and form the parts of Central Body, which are detectable in immunohistochemistry analyses for MIP and serotonin (chapter 4.3.4).

An alternative to the presence of such additional NBs is suggested by the fact that in *Schistocerca,* serotonergic neurons arise from the y and z NBs. These NB lineages form a part of the Central Complex. Boyan and colleagues (2010) identified serotonergic neurons, which occurred at stereotypic locations in their respective y and z lineages. In the context of the results of this work, a knock down of the respective gene did not lead to a total loss of Central Body forming NBs in the immunohistological analysis of *Tc-chx* RNAi brains (chapter 4.3.4). Therfore, the NB lineages forming the MIP and serotonin positive structure are not affected by *Tc-chx* RNAi. Eventually, to verify whether these MIP and serotonin positive structure in *Tc-chx* RNAi larval brains are parts of the Central Body, stainings with additional markers for the Central Body should be performed. One promising candidate could be *AP-2,* which is expressed in the Central Complex of adult *Drosophila* (Monge et al., 2001).

Assuming that the remnant structures occurring after *Tc-chx* RNAi are parts of the Central Body, the question why the Central Body is split after *Tc-chx* RNAi arises. One possibility is that *Tc-chx* is involved in forming the primary axon scaffold in the brain. Whole mount *in situ* hybridization with an antisense probe for *Tc-chx* revealed a strong expression pattern ventrally of a bundle of contra lateral projections (capter 4.3.1). These projections are suggested to be the onset of the protocerebral commissure (chapter 4.2). In conclusion of the expression pattern and RNAi phenotype, *Tc-chx* is possibly important for establishing the primary axon scaffold, which enables neuronal structures to cross the brain midline. From *Schistocerc,* it is known that the primary axon scaffold is important for the projecting axon bundles of the Central Body NBs for orientation. The progenies, which form this axon scaffold, are located in the pars intercerebralis of *Schistocerca* (Boyan and Williams, 1997; Williams and Boyan, 2008). Since *Tc-chx* is assumed to be a pars intercerebralis marker, it is likely that the progenies forming such an axon scaffold are affected after RNAi, which finally leads to a disturbed or lacking scaffold. Therefore, the midline crossing properties are lost, which could in turn have led to the observed split Central Body phenotype in the *Tc-chx* RNAi animals. Whether the primary axon scaffold progenies are affected by *Tc-chx* RNAi,

could be tested by anti HRP staining and in this case no protocerebral commissures should be detectable.

An additional hint for *Tc-chx* to be important for primary scaffold formation is the observed Mushroom Body phenotype after *Tc-chx* RNAi. This phenotype shows additional and misguided projections arising from the calyces and ending anywhere anterior in the brain. In other cases, additional projections are forming additional Pedunculi or innervate the vertical lobe of the Mushroom Body. By studying the expression pattern of the `Brainy´ line, it was revealed that the Mushroom Bodies develop late in the embryo (chapter 3.1.4). Therefore it is suggested that the Mushroom Body defects, which occur after a knock down of *Tc-chx,* are due to a secondary effect. Known from *Drosophila*, the developing projecting Mushroom Body tracts use the already existing neuronal scaffold as orientation (Kurusu et al., 2002; Grillenzoni et al., 2007). In this context, the Mushroom Body phenotype after *Tc-chx* RNAi could be caused due to scaffold defects. Therefore, additional projections as well as misguided projections arise from the Kenyon cells or calyces, because of wrong cues, which are due to a damaged or absent scaffold after *Tc-chx* RNAi. A direct interaction of *Tc-chx* and the Mushroom Body NBs is unlikely, because of the distance between the *Tc-chx* expression domain and the position of the arising Mushroom Body NBs in the neuroectoderm. Thus it is likely, that *Tc-chx* is involved in primary axon scaffold formation in the *Tribolium* brain, but it is still obscure how this occurs. Assuming that the progenies of the primary axon scaffold forming neurons are located in the pars intercerebralis like in *Schistocerca* (Williams and Boyan, 2008; Boyan and Williams, 2008), it is possible that these cells are affected by *Tc-chx* RNAi, which leads to the loss of the primary axon scaffold. On the other hand, it is also known from *Schistocerca* and *Drosophila* that glia are important for the axon guidance to establish the primary axon scaffold (Boyan et al., 1995; Lemke, 2001). Glial cells surrounding the remnants of the Central Body are missing after *Tc-chx* RNAi (chapter 4.3.3). Possibly, this lack of glia is actually necessary to enable the formation of the primary axon scaffold and for following midline crossing properties for projecting axon bundles of the Central Body. To what extent and what kind of glia are affected by *Tc-chx* RNAi could be analyzed by anti *Repo* stainings.

Finally, this work indicates that *Tc-six3* acts upstream of *Tc-chx.* This suggestion is based on the facts, that (1) *Tc-six3* is expressed earlier than *Tc-chx* (chapter 4.3.1 and Posnien, 2009), (2) a knock down of *Tc-six3* causes a severe cuticle RNAi phenotype, while in *Tc-chx* knock down no cuticle defects can be detected, that (3) in both cases a knock down lead to a loss of a properly developed Central Body, and that (4) the *Tc-chx* expression is lost after a knock down of *Tc-six3* (Posnien, personal communication).

Taken together, *Tc-chx* was shown to be essential for proper development of the Central Body and is likely to be involved in establishing the primary axon scaffold, which gives orientation to axon tracts of the developing Central Body as well as axon tracts of the developing Mushroom Body. In addition, it was discussed to act downstream of *Tc-six3*.

In conclusion, *Tc-six3* and *Tc-chx* are very important players in specifying the cells in the median brain region, including the pars intercerebralis, in *Tribolium*. Additionally, these genes are important to establish the respective cues in the center of the brain, which are needed for the proper development of the Central Body as well as the Mushroom Body. Thus, this work identified two novel genes, *Tc-six3* and *Tc-chx,* to be essential for Central Body development in *Tribolium*.

5.4.4. Personal assumption of a hierarchical function of the studied genes in the context of Central Body development in *Tribolium castaneum*

The here assumed antagonistic function of *Tc-six3* and *Tc-otd1* was shown not to hold true. Instead, both genes seem to act on the same hierarchical level. Both genes play a crucial role in early embryonic regionalization of the anterior head in the neuroectoderm. *Tc-otd1* is important for the specification of the anterior part of the developing brain, which is consistent with the conserved function that was suggested for vertebrates and insvertebrates (Hirth and Reichert, 1999; Lichtneckert and Hirth, 2005; Lichtneckert and Reichert, 2008; Hirth, 2010).

Tc-six3 is important for cell proliferation and differentiation in the median anterior neuroectoderm region. During early embryonic stages, *Tc-six3* maintains cell proliferation, while later on, *Tc-six3* is needed for the specification of NBs that form the Central Body. Additionally, it activates the expression of *Tc-chx*. *Tc-chx* is suggested to be involved in establishing a functional central brain glia. These glia are important to guide the outgrowing axons of the neurons, which form the primary axon scaffold. Finally, this scaffold enables the development of the Central Body.

In contrast to *Tc-chx, Tc-rx* is suggested to be required in axogenesis at the brain midline, while its target genes are unknown. *Tc-rx* is assumed to act on the growth cones, either by initiating the midline spanning through triggering extrinsic cues, which influence the axonal growth, or even by enabling the putative *Tc-rx* positive axons to react on extrinsic factors. As mentioned above, *Tc-chx* and *Tc-rx* possibly act in a synergistic fashion in order to establish the essential properties in the central midbrain, on the glial and on the axonal level.

5.5. Holoprosencephaly and *Tribolium castaneum* as model organism

In the context of conserved processes and mechanisms among brain formation in bilaterians, the findings of this work regarding the function of *Tc-six3* and *Tc-chx* in brain formation will be discussed with respect to their relevance for the vertebrate brain. Interestingly, there are many similarities in the phenotypes in vertebrates and invertebrates caused by dysfunctions of the genes studied here during embryonic insect brain development. The vertebrate expression pattern of *SIX3* starts early and marks the anterior neural plate (Oliver et al., 1995), which is similar in *Tribolium* (Posnien, 2009). During further development, *SIX3* is expressed in the developing retina, lens, hypothalamus, and pituitary. It has been shown that hypomorphic mutations leads to a severe phenotype in mammals including humans, the holoprosencephaly. This phenotype is caused by a dysfunction in the specification of the interhemispherical region within the developing brain. In severe cases, this leads to fatal malformations where the interehemispherical fisura (fisura longitudinalis cerebri, Sobotta and Becher, 1964) is absent and no hemispherical division has taken place. Even the corpus callosum connecting the brain hemispheres is affected (reviewed in Dubourg et al., 2007). In *Tribolium,* a knock down of *Tc-six3* leads to a loss of median tissue in the brain and finally to dramatic effects on the median head capsule, which is comparable to the phenotype of holoprosencephaly in vertebrates.

An additional gene identified in this this work was *Tc-chx.* This gene is homologous to *CHX/VSX* in vertebrates. Here, a gene dysfunction leads to microphthalmia (Percin et al., 2000). In *Tribolium,* a function of *Tc-chx* in the larval eye development was not detected. Possibly this effect would occur in adult beetles, but a knock down of *Tc-chx* does not allow embryos to hatch. Nevertheless, a severe phenotype regarding the central brain in *Tribolium castaneum* was observed. In vertebrates, *CHX*-10 is expressed in the thalamus, hindbrain ventral spinal cord, and lip, but no other malformation besides that of the eyes was detected (Liu et al, 1994; Burmeister et al., 1996). Apparently, there is a redundant function, which prevents a dramatic brain phenotype. In *Tribolium castaneum,* there is only one homolog gene of *CHX*, which is why a knock down of this gene leads to dramatic effects within the brain.

Thus, two genes were presented and studied, both of which are conserved in bilaterians. In one case, the function is still conserved and a knock down leads to phenotypes with many similarities. In the other case, the gene expression is conserved, but the functions differ.

Finally it will be interesting, which of the 18 *Tribolium* homologue genes to the vertebrate neural plate genes also possess a conserved function in brain development among insects and vertebrates. With the brain visualizing system for *Tribolium castaneum* established in this work, this question

could be answered. Therefore ,with this work the first steps to understand the genetic processes and mechanisms of brain organogenesis for *Tribolium castaneum* were taken. Possibly, a genetic machinery can be identified, which gives hints to the reasons underlying malformations like holoprosencephaly. Therefore, *Tribolium castaneum* is suggested as an additional model organism to study homologous genes involved in brain formation and to gain insight into processes essential also for vertebrate brain development.

5.6. Outlook

In this work, the `Brainy´- and `MB´-lines were characterized regarding their expression pattern in the larval brain and the colocalisation of their signals with antibodies for glia and Mushroom Body, respectively, which had been done in cooperation with the laboratory of Schachtner (University of Marburg). These lines offer a promising tool to screen for genes which are involved in brain formation in general. Some candidate genes, which could play important roles in brain development in *Tribolium,* have already been found by Posnien (2009). He identified 18 genes, which are orthologues to the vertebrate neural plate genes and feature expression domains in the head neuroectoderm in *Tribolium*. Some of these genes showed only minor cuticle defects after RNAi, as for example *Tc-six4,* and possibly their function is more specific to neural development.

However, Posnien could also show (2009), that *Tc-six4* expression overlaps with *Tc-six3.* Thus, this gene could also be involved in Central Body formation. The opportunity to study embryonic Central Body formation in *Tribolium* is what distinguish this model from *Drosophila,* since *Drosophila*, one of the best established model organism for genetical studies, forms such structure only during puparium. With the `Brainy´- and the `MB´-line, a promising set of tools to screen for additional genes, which could be involved in or necessary for proper Central Body formation in *Tribolium,* was created.

The generated *Tc-rx* reporter lines show a defined expression pattern, but still it is not clear whether the detected cells positive for the reportergene expression arose from *Tc-rx* positive NBs. One option to answer this question, could be a colocalisation experiment with the reportergene and *Tc-rx*. Assuming, that the reporter lines `*Tc-rx* 5up::DsRedEx´ and `*Tc-rx* Intron::DsRedEx´ are marking *Tc-rx* NB lineages, these lines offer the opportunity to study the selection of NB identity by RNAi with candidate genes. Parallel to that, additional NB lineage marking lines could be generated. One reporter gene construct for *Tc-rx* is still missing. In addition, new constructs for *Tc-chx* could be designed and used to generate NB reporter lines.

In this work, it could be shown that *Tc-rx, Tc-chx,* and *Tc-six3* are important for proper Central Body development. In the regarding paragraph, different opportunities and putative roles in Central Body formation were discussed. The target genes of *Tc-rx, Tc-chx,* and *Tc-six3* are still unknown, but these could be revealed by chromatin immunoprecipitation (Chip; Orlando et al., 1997; Orlando, 2000) with regarding antibodies. By this, further candidate genes for creating additional lines could be identified. Furthermore, the identification of the target genes of *Tc-rx, Tc-chx* and *Tc-six3* could deliver additional valuable details regarding Central Body development.

6. References

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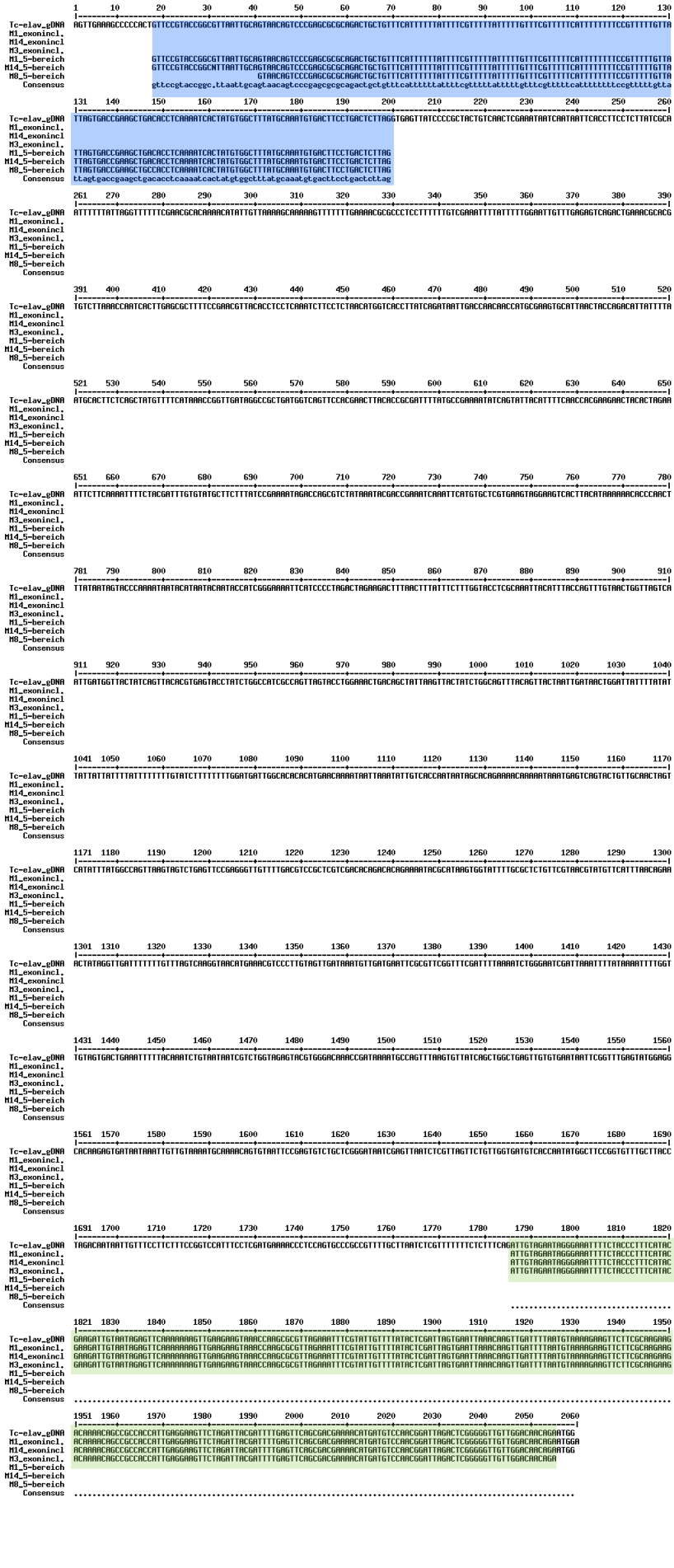
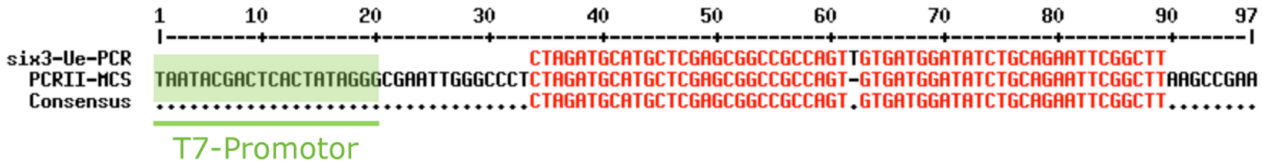
7. Appendix

7.1. 5´UTR alignment of *Tc-elav* with gDNA



Appendix Figure 7.1: 5´UTR aligment of the RACE PCR of *Tc-elav* and gDNA.
Different RACE products were aligned. 5´end of the UTR at the top (blue background) and 3´end of the UTR at the bottom (green background). Translation start site highlighted in red.

7.2. Identification of the linker sequence of the *Tc-six3* overexpression construct

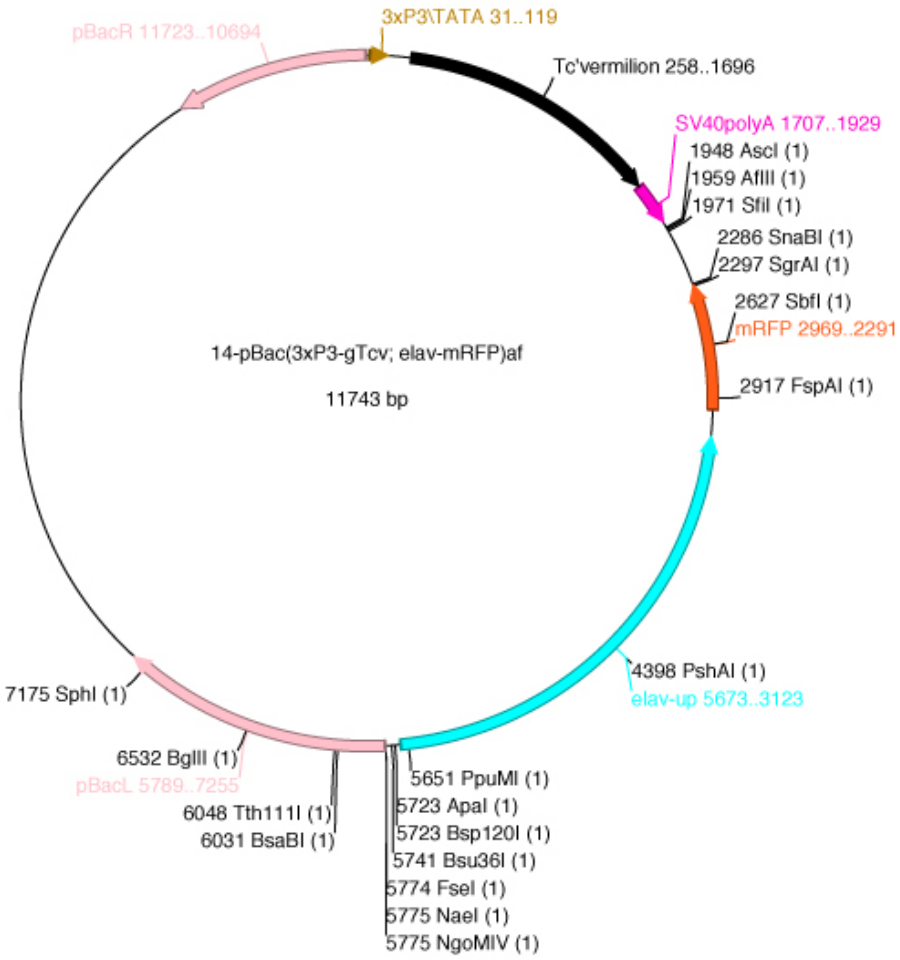


Appendix Figure 7.2: Linker of the HS construct and PCR II Multiple Cloning Site alignment. The upper sequence reflects the linker sequence between the HS-promoter and the ORF of *Tc-six3* of the HS-six3-Overexpression construct. Lower sequence reflects part of PCR II-vector, green highlights the T7-Promoter. The alignments demonstrate, that the linker sequence originates from the PCRII vector (highlighted in red).This part of the PCRII-MCS includes three additional ATGs, which are included also in the HS-six3-overexpression construct (see chapter 3.1.2).

7.3. Vector maps of the constructs used for embryonic transgenesis

The following depicts the maps of the constructs, which were used for transgenesis. The backbone vector was pBac[3xP3;*Tc-vermillion*;SV40polyA]af. Only the sequences which were inserted into the respective backbone at the position of AscI and/or FseI are presented. The color code highlights the respective elements in the vector maps and the sequence. SV40 poly A is depicted in purple. Regulatory regions and the respective promoters are depicted in turquoise. The reporter genes mRFP and DsRedExpress are highlighted in orange, tGFP in green.

7.3.1. Vector map of the *Tc-elav* reporter construct



> reg.-*Tc-elav*::mRFP

cgcgccaagcttaaggtgcacggcccacgtggccactagtacttctcgagctctgtacatgtccgcggtcgcgacgtacgcgtatcgatggcgccagctgcaggcggccgc
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7.4.2. Vector map of the *Tc-repo* reporter construct

> reg.-*Tc-repo*::tGFP

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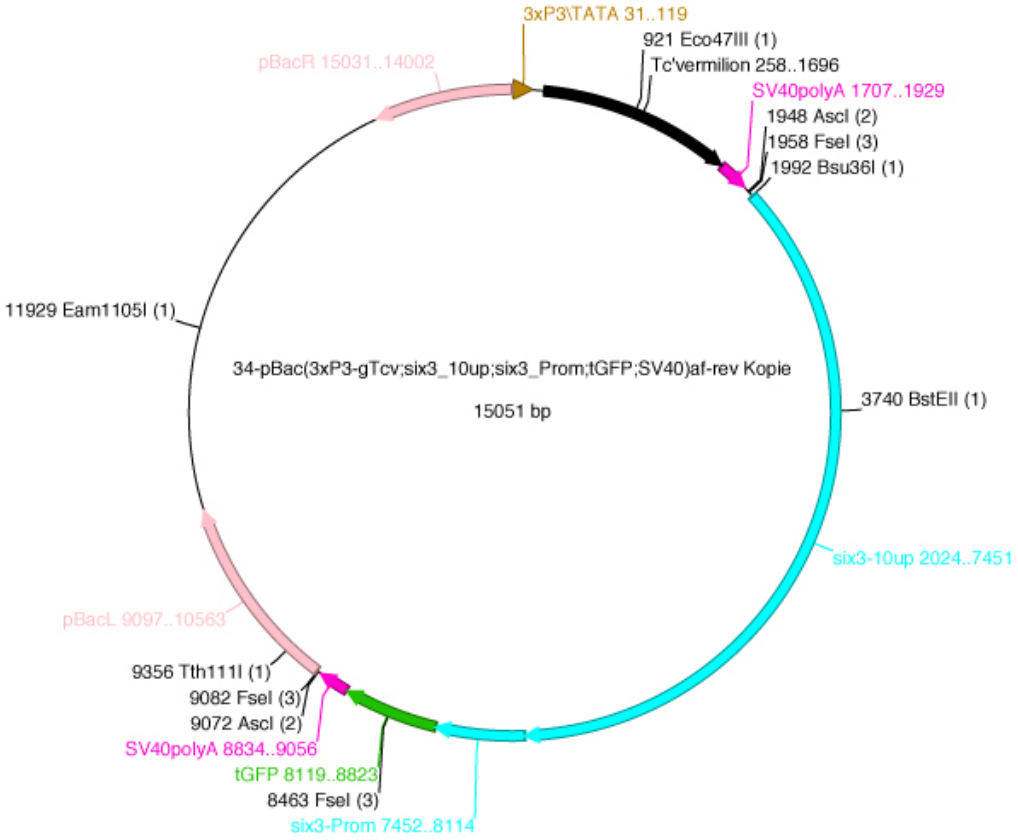
7.3.3. Vector map of the `*Tc-six3* 10up´ construct



> *Tc-six3* 10up::tGFP

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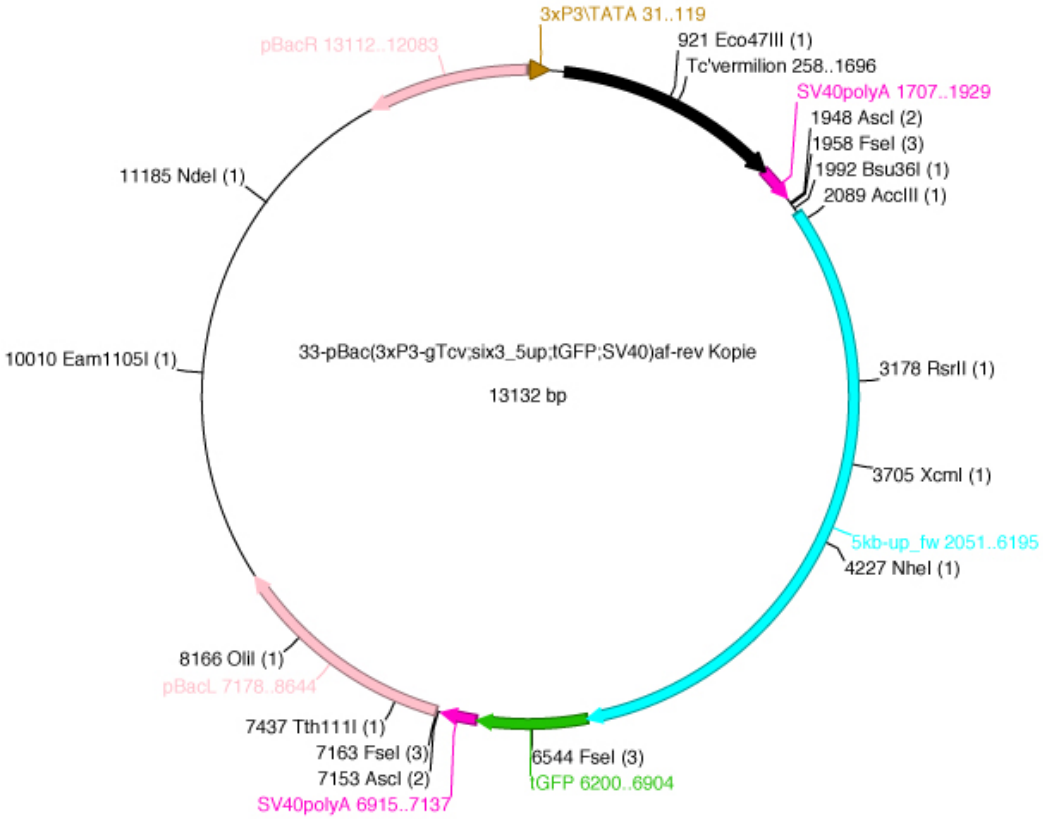
7.3.4. Vector map of the `*Tc-six3* 5up´ construct



> *Tc-six3* 5up::tGFP

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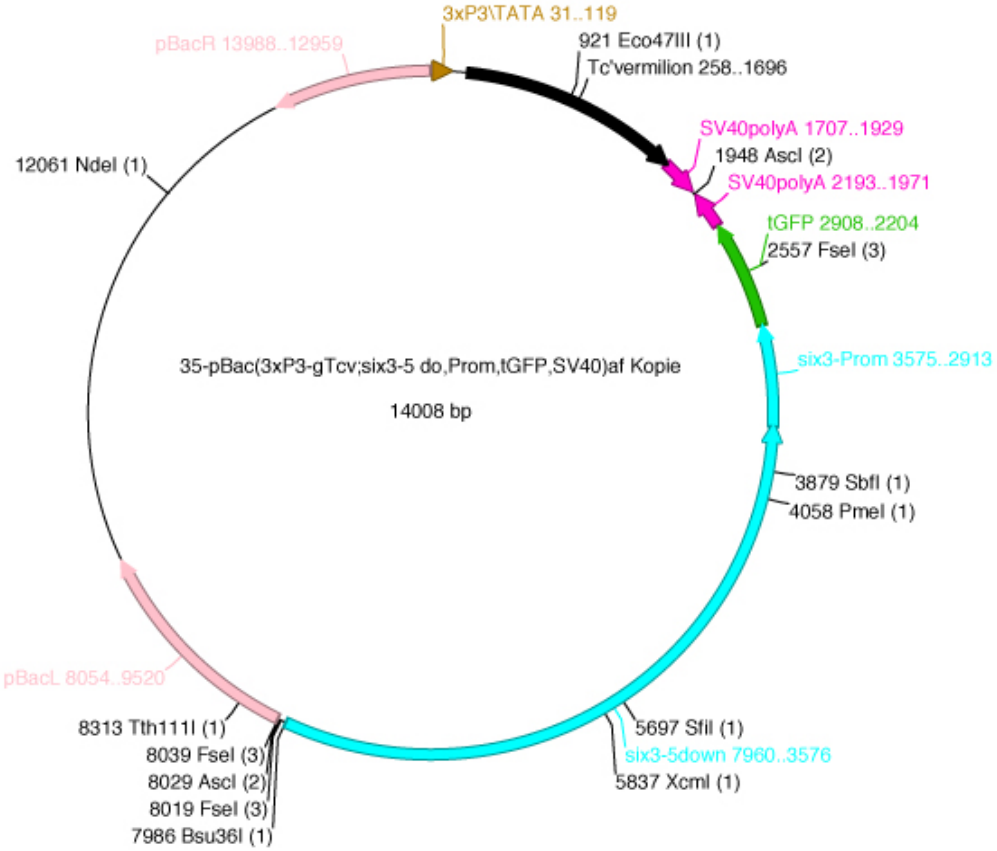
7.3.5. Vector map of the `*Tc-six3* 5down´ construct



> *Tc-six3* 5down::tGFP

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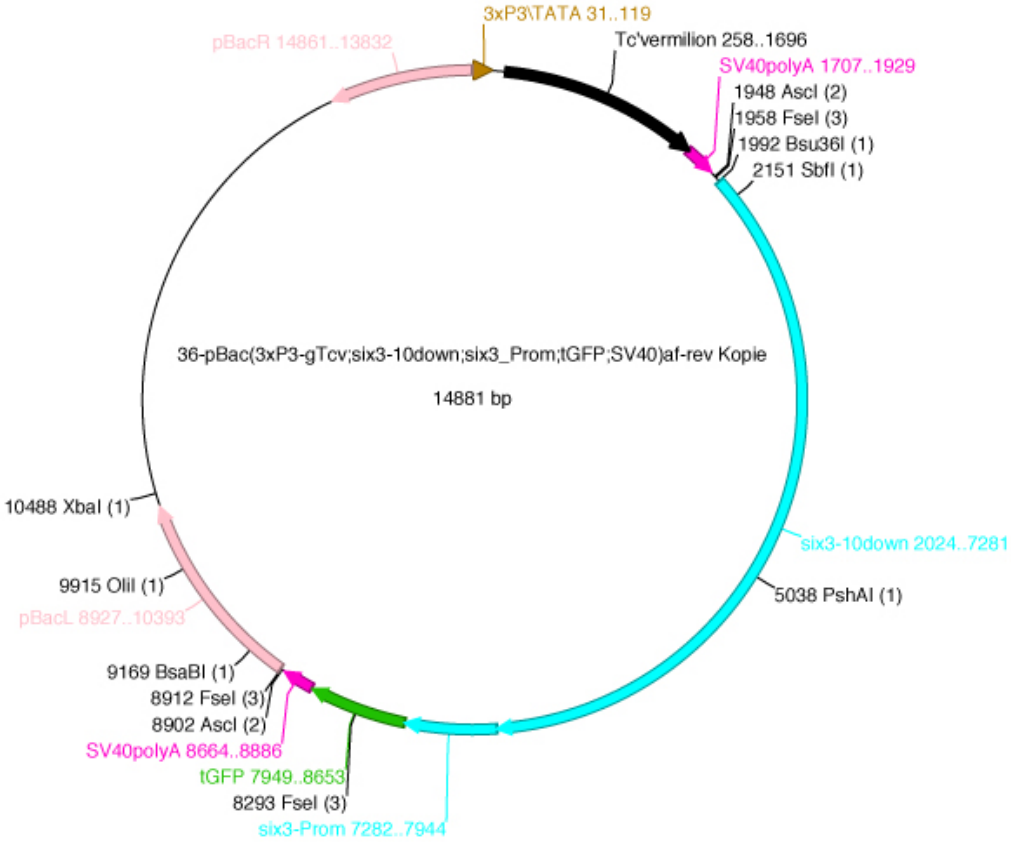
7.3.6. Vector map of the `*Tc-six3* 10down´ construct



> *Tc-six3* 10 down::tGFP

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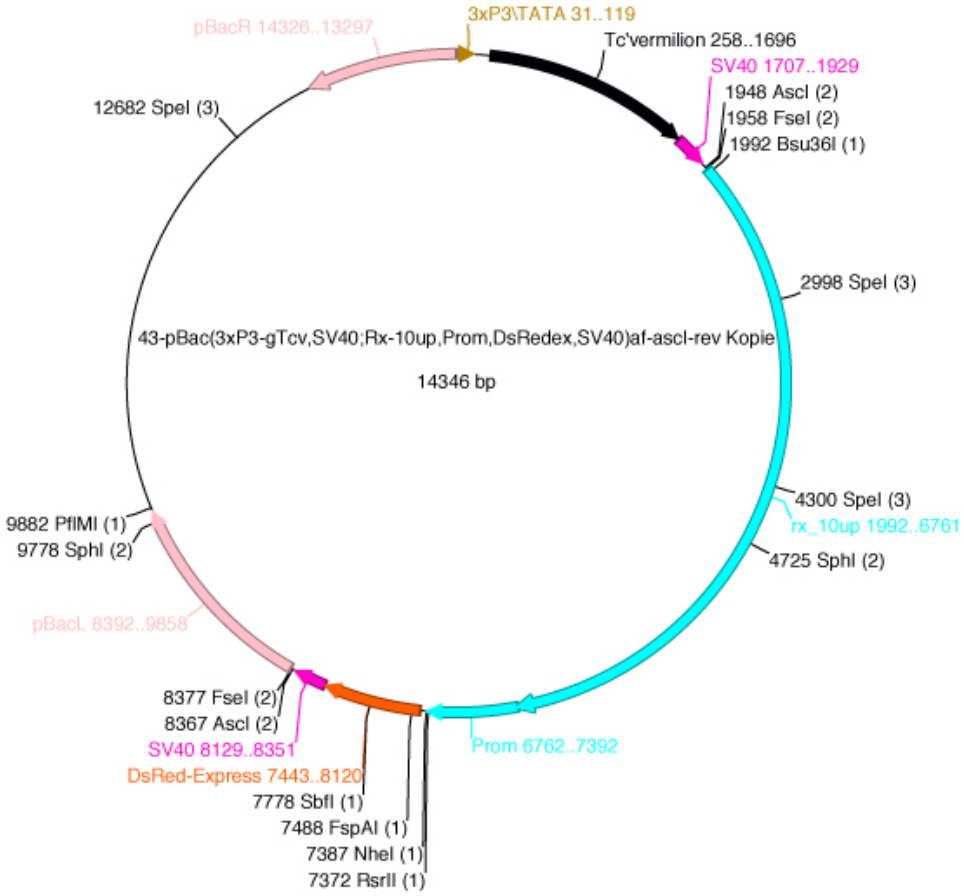
7.3.7. Vector map of the `*Tc-rx* 10up´ construct



> *Tc-rx* 10up::DsRedEx

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7.3.8. Vector map of the `*Tc-rx* 5up´ construct

> *Tc-rx* 5up::DsRedEx

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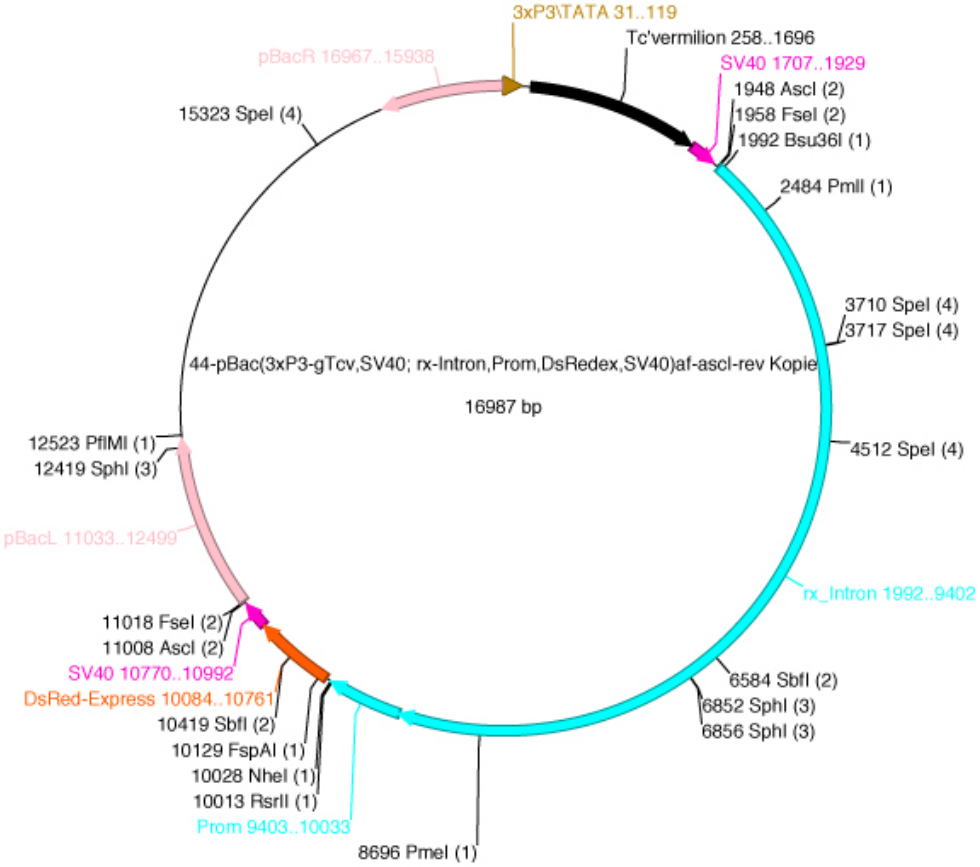
7.3.9. Vector map of the `*Tc-rx* Intron´ construct



> *Tc-rx* Intron::DsRedEx

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8. Abbreviations

AL	Antennal Lobe
CB	Central Body
CC	Central Complex
CNS	central nervous system
D	deuterocerebrum
DsRedEx	*Discosoma sp.* red fluorescent protein Express
dsRNA	double-stranded RNA
gDNA	genomic DNA
HS	heat shock
MB	Mushroom Body
NB	Neuroblast
P	protocerebrum
PB	Protocerebral Bridge
Pe	Pedunculus
PI	pars intercerebralis
RNAi	RNA interference
SB	San Bernardino
StNS	stomatogastric nervous system
T	tritocerebrum
tGFP	turbo green fluorescent protein
TLS	translation start site
TSS	transcription start site
UTR	untranslated region
vw	vermillion white
WT	wild type

9. Curriculum vitae

Name:	Nikolaus Dieter Bernhard Koniszewski
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Phd studies

since 2007: Georg-August-University Göttingen
PhD student under the supervision of
Juniorprofessor Dr. Gregor Bucher at the
Department of Developmental Biology
Title of thesis: Functional analysis of embryonic brain development in *Tribolium castaneum*

University studies

2007: Diploma in Biology of the Friedrich-Alexander-University Erlangen/Nürnberg

2006-2007: Friedrich-Alexander-University Erlangen/Nürnberg
Diploma thesis under the supervision of
Professor Dr. Martin Klingler at the
Department of Developmental Biology
Title of the thesis: Das Terminale System in *Tribolium castaneum*

2004-2006: Main course in Biology at the Friedrich-Alexander University Erlangen/Nürnberg

2002-2004: Basic studies in Biology at the Friedrich-Alexander University Erlangen/Nürnberg

Civil service:

2001-2002: Civil service at the "Klinikum Forchheim der Vereinigten Pfründnerstiftung"

High School:

2001: Abitur at the Ehrenbürg Gymansium Forchheim, Germany

Scientific memberships

- Member of the Göttingen Center for Molecular Biosciences (GZMB)
- Member of the Göttingen Center for Molecular Physiology of the Brain (CMPB)

Publications

- Schinko, J., 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
- Posnien, N., Koniszewski, N., and Bucher, G. (2011). Insect Tc-six4 marks a unit with similarity to vertebrate placodes. Dev Biol 350, 208-216

Public presentations

- Koniszewski N., Bucher G.
  What makes neuroblasts different from each other? **(Poster and Oral presentation)**
  3. International *Tribolium* Meeting, Göttingen (Germany), September 6, 2007
- Koniszewski N., Hein H., Bucher G.
  Tools for analyzing neural stem cell identity in the larval brain of *Tribolium castaneum* **(Poster)**
  From Neurogenesis to Synaptogenesis, Göttingen (Germany); September 25 - 26, 2008
- Koniszewski N., Hein H., Bucher G.
  Tools for analyzing neural stem cell identity in the larval brain of *Tribolium castaneum* **(Poster)**
  The 8th Meeting of the German Neuroscience Society, Göttingen (Germany), July 25 - 29, 2009
- Koniszewski N., Bucher G.
  Transgenic imaging lines to analyze *Tribolium* brain patterning **(Poster and Oral presentation)**
  Cologne's Regional Tribolium Meeting, Cologne (Germany), July 27 - 29, 2009
- Koniszewski N., Bucher G.
  Transgenic imaging lines to analyze *Tribolium* brain patterning **(Oral presentation)**
  20th Neuro Do-Wo Würzburg ´09, Würzburg (Germany), July 30 - 31, 2009
- Koniszewski N., Bucher G.
  Tc-six3 is required for central complex development **(Oral presentation)**
  International Tribolium Meeting Paris, Paris (France), July 5 - 6, 2010