Morphogenesis and Genetic Regulation of the Insect Head

Dissertation

for the award of the degree

“Doctor rerum naturalium”

Division of Mathematics and Natural Sciences

of the Georg-August-Universität Göttingen

within the doctoral program *Genes & Development*

of the Georg-August University School of Science (GAUSS)

submitted by

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Göttingen 2016

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Date of Oral Examination: 11.07.2016

DECLARATION

I hereby declare that the doctoral thesis entitled,
"*Morphogenesis and Genetic Regulation of the Insect Head*"
has been written independently and with no other sources and aids than quoted.

_____ Göttingen, May 30th , 2016
Peter Kitzmann

Für meine Eltern,

Sabine und Günter Kitzmann

Danksagung

An dieser Stelle möchte ich mich bei allen bedanken, die mich auf dem Weg zu dieser Arbeit begleitet und unterstützt haben. Ich bin froh und dankbar für eine sehr schöne Zeit, und glücklich, dass viele dieser Begleiter, falls sie es nicht schon vorher waren, Freunde geworden sind.

Zuallererst möchte ich mich bei Prof. Gregor Bucher bedanken, der es mir überhaupt erst ermöglicht hat an diesem spannenden Projekt zu arbeiten und diese Arbeit zu verfassen. Jedoch hat er mir die Arbeit an dem Projekt nicht nur ermöglicht, sondern mich vor allem auch in jeglicher Hinsicht, zu jeder Zeit aktiv unterstützt. Ich bin sehr dankbar für den wissenschaftlichen Austausch, motivierende Gespräche und unzählige hitzige Diskussionen über Gott und die Welt. Großen Dank und Respekt an einen der besten Betreuer -überhaupt-!

Vielen Dank gilt auch den Mitgliedern meines „Thesis Committees“ Prof. Jörg Großhans und Prof. Andreas Wodarz für das Opfern von Zeit, einen stets unkomplizierten Umgang, aber vor allem für konstruktive Diskussion die diese Arbeit mit geformt haben.

Für das fortwährende Feintuning des Projektes während unzähliger Seminare möchte ich mich auch gerne bei Prof. Ernst A. Wimmer, Dr. Nikola-Michael Prpic-Schäper und Dr. Gerd Vorbrüggen bedanken.

Danken möchte ich auch meinen Kollaborationspartnern in Lyon, Dr. Michalis Averof, Dr. Johannes B. Schinko und vor allem meiner großen Schwester Anna Gilles, für die stets unkomplizierte und konstruktive Zusammenarbeit. Auch meinen Kollaborationspartnern in Dresden, Dr. Pavel Tomancak und Dr. Stefan Münster, möchte ich für die Möglichkeit mit dem Lichtblatt-Mikroskop zu arbeiten danken und für die Aufnahme und das Prozessieren einiger live-imaging Videos.

Vom ganzen Herzen möchte ich mich auch bei Dr. Nico Posnien bedanken, welcher das Projekt in vielen Seminaren, aber auch jederzeit außerhalb dieser durch stetigen Austausch und Diskussionen mit hat reifen lassen. Vor allem möchte ich mich aber auch noch für viele kritische, lustige und ernste Gespräche -egal ob im Stehen, Sitzen oder Joggen- bedanken. Nicos „Koalafications“ haben es zu jeder Zeit und auch insbesondere zum Ende der Arbeit immer geschafft mich zu motivieren, nicht zuletzt durch feinste Musiktipps.

Sehr dankbar bin ich auch Dr. Sebastian Kittelmann, Dr. Bernhard G. M. Schmid und Dr. Georg Oberhofer, die alle stets Vorbild waren dazu beigetragen haben, dass ich zu dem Wissenschaftler geworden bin, der ich jetzt bin.

Großer Dank gilt aber vor allem auch Claudia Hinners, die mich jederzeit tatkräftig unterstützt hat und somit den Umfang dieser Arbeit erst mit ermöglicht hat. Danke, liebe Claudia!

Danken möchte ich auch Elke Küster, die mich nur allzu oft mit Käfern jeglicher Art versorgt hat und für die unermüdliche Pflege der Stocks. Beate Preitz bin ich dankbar für die Einweisung in alle Mikroskope und die Instandhaltung dieser, ohne die ein Großteil dieser Arbeit nicht möglich oder nur erschwert möglich gewesen wäre.

Für die Versorgung mit so einigen Plasmiden und guten Tipps, bin ich Stefan Dippel zu Dank verpflichtet.

Für das Korrekturlesen der Arbeit, möchte ich mich herzlich bei Max Farnsworth bedanken!

Weiterer Dank gilt auch allen HiWis und Studenten an denen ich mich probieren durfte und die mit mir an diversen Projekten gearbeitet haben. Danke für die Mühen und Geduld Julia, Felix und Magdalena! Ihr ward eine große Bereicherung für mich und das Projekt!

Special thanks goes to my best Lab-fellas Georg and Salim, I really enjoyed the time with you and I am happy to call you buddies!

Besonderer Dank gilt auch Hanna, die mich gerade in der heißen Phase der Arbeit, nicht zuletzt mit einem bestechenden Humor, immer wieder motiviert hat und es geschafft hat mich von allem Stress abzulenken! Danke Hanna!

Für eine unnormal gute Stimmung und große Hilfsbereitschaft, möchte ich mich bei allen jetzigen und ehemaligen Mitgliedern des Labors 3 bedanken. Danke, Julia, Janna, Sebastian, Felix, Magdalena, Yonggang, Jürgen, Daniela und Max!

Ich möchte mich bei der ganzen Abteilung für eine super Zeit und fortwährende Unterstützung und Hilfestellung bedanken. Hier geht ein besonders herzlicher Dank an: Christian, Jonas, Beni, Christoph, Natascha, Jana und Kolja.

Dankbar bin ich auch dem ganzen TA-Team, Claudia Hinners, Katrin Kanbach, Helma Griess, Angelika Löffers und Bettina Hucke, die den Laden die ganze Zeit im Hintergrund am Laufen gehalten haben und mir sehr viel Arbeit erspart haben. Ich möchte mich auch herzlich bei den Damen des Sekretariats, Birgit Rossi, Bettina Hucke, Merle Eggers, Selen Pfändner, Inga Schild und Constanze Gerhards bedanken, die es immer unkompliziert geschafft haben jeden komplizierten bürokratischen Kram zu erledigen und immer für ein netten Schnack zu haben waren.

Zu guter Letzt möchte ich mich bei all jenen bedanken, bei denen ich mich glücklich schätzen darf sie Freunde zu nennen. Euch namentlich zu erwähnen würde den Rahmen dieser Arbeit sprengen. Aber Ihr wisst von wem ich spreche. Ihr seid ja alle Füchse! Ich bin Euch allen mega dankbar, da Ihr mich alle stets auf Eure Art unterstützt, bespaßt und, wenn nötig, mit allen Mitteln abgelenkt habt.

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Summary

Natural selection and the struggle for ecological niches were the driving force for the origin of a tremendous number of animal groups with different body shapes. The segmental organization of insects enhanced their evolutionary specialization. Adaptations led to different morphologies, for instance with respect to the head. The head, which carries the feeding apparatus and the main sensory centers enabling interaction with the environment, is one prerequisite for evolutionary success. The segmental structure of the insect head facilitated evolution of adaptations with respect to morphology. However, although the head is of great importance for the evolutionary success, there is no comprehensive understanding of the gene network regulating anterior head development until now. Furthermore, the genetic basis for the different insect head morphologies is unknown. Moreover, there are open questions concerning the segmental structure of the head and how morphogenetic movements lead to the adult head.

During the last years the red flour beetle *Tribolium castaneum* was developed as a major model organism for studying insect head development. Recent studies were able to successively uncover the genetic interactions of anterior head development. However, so far the gene set and its interaction are not comprehensively unraveled. *Tc-foxq2* is a novel regulator of head development identified with a highly specific head phenotype in the iBeetle screen. Hence, I studied its potential function in the anterior head gene regulatory network. Another open question is the contribution of the different embryonic head regions to the adult head. To provide new insights regarding this question I wanted to provide new features for the *Tribolium* toolbox.

In this study I was able to show that *Tc-foxq2* is specifically expressed in the anterior pre-segmental head region, similar to the conserved expression pattern, which is located at the anterior pole in metazoan species. Further, I was able to show that *Tc-foxq2* is an upstream player within the anterior head gene regulatory network, forming a conserved patterning unit together with *Tc-six3*. *Tc-foxq2* knock-down results in an increased cell death rate within the clypeo-labral region, which consequently leads to reduced labral buds at embryonic stages. This

defect is also reflected in L1 larval cuticles showing a labrum that is strongly reduced or completely absent. Further, using neurogenic in vivo imaging reporter lines I was able to show that embryonic knock-down of *Tc-foxq2* function leads to central body and mushroom body defects. Moreover, I was able to generate new reporter lines that drive expression of the chimeric H2Av::EGFP nuclear marker protein under control of the *Tc-αTubulin1* promoter or the *Tc-ribosomal protein subunit3* promoter. I analyzed the lines with respect to localization of the marker protein, signal distribution within the embryo, signal intensity in different developmental stages and tissues, and viability of the different transgenic lines. I could show that the lines are functional and that the *Tc-αTubulin1* promoter line is best suited for being analyzed with light-sheet imaging. Finally, I was able to generate and to show functionality of new in vivo imaging lines for laser-induced cell marking and genetic cell marking.

2

Introduction

2.1 Patterning the insect body

2.1.1 Patterning the insect trunk

With more than a million described species, insects are the most diverse and species-rich animal class (Chapman et al., 2009; Grimaldi and Engel, 2005). The modular segmental body plan was the prerequisite for dramatic radiations and the evolutionary success (Chipman, 2010; Tautz et al., 1994). The genetic basis for the development of a segmented body plan was studied for decades in the model organism *Drosophila melanogaster* (*Drosophila*). Thus, anteroposterior (AP) patterning is well understood and best known in *Drosophila* (Pick, 1998). It has been shown that a multi level hierarchical gene cascade is responsible for building the segments along the AP-axis. This cascade involves in the first place maternal factors, which set up the anterior and posterior system by localized mRNAs like *bicoid* (*bcd*; anterior) and *nanos* (*nos*; posterior), as well as the terminal and dorso-ventral systems involving signaling pathways like *torso* (*tor*; terminal) and *decapentaplegic* (*dpp*; dorsal). These systems determine the primary body axes and the terminal regions of the embryo. The body is further subdivided in to smaller regions by the expression of gap genes, which are regulated by the maternal system. These regions are then subdivided further by the pair-rule gene expression regulated by gap genes. In turn, pair-rule genes regulate the expression of segment polarity genes, which determine segment boundaries and establish polarity in each segment. Eventually, all these levels are regulating the expression of homeotic selector (Hox) genes, which are required for segment identity specification (Akam, 1987; Cohen and Jürgens, 1991; Ingham, 1988; Johnston and Nüsslein-Volhard, 1992).

The insect head is of high importance due to its roles in sensory input integration, generation of adequate behavioral output, and feeding. But, in contrast to AP patterning, the genetic basis of head patterning is currently not understood comprehensively in any insect. However, due to the

fact that neither Hox genes nor pair-rule genes are expressed at the anteriormost region, this region has to rely on a completely different gene regulatory network (Bucher and Wimmer, 2005; Posnien et al., 2010).

2.1.2 Genetic regulation of insect head development

2.1.2.1 Head patterning in *Drosophila*

The subsequent description is mainly referring to (Rogers and Kaufman, 1997). In *Drosophila*, it has been shown that the posterior part of the head, built by the segments bearing the gnathal appendages, is patterned like the trunk segments via the classical patterning cascade (Akam, 1987; Cohen and Jürgens, 1991; Ingham, 1988; Johnston and Nüsslein-Volhard, 1992).

However, the segmental part of the anterior head composed of the intercalary segment and the antennal segment is patterned in a different way. The maternal contribution in anterior head development is the same. The anterior maternal morphogen *bcd*, terminal factors like *tor*, and dorso-ventral factors like *dorsal* (*dl*) are required for activating genes of the second level of hierarchy. The maternal factors are regulating head gap-like genes *orthodenticle*, *empty spiracles*, *buttonhead*, and *sloppy paired*, which are expressed in broad overlapping domains in the anterior head (Cohen and Jürgens, 1991, 1990; Dalton et al., 1989; Finkelstein et al., 1990; Grossniklaus et al., 1992; Mohler, 1995; Walldorf and Gehring, 1992; Wimmer et al., 1997, 1993). In contrast to the posterior head, there are no pair-rule genes expressed in the anterior head and consequently not involved in the development of this region. Instead, head gap-like genes are either directly acting on segment polarity genes, like engrailed, hedgehog, and wingless (Gallitano-Mendel and Finkelstein, 1998; Mohler, 1995; Rogers and Kaufman, 1997) or indirectly by regulation of second level regulators like *collier* (*col;* Crozatier et al., 1996) or segment identity genes like *cap'n'collar* (*cnc*; Crozatier et al., 1999).

2.1.2.2 Anteriormost head patterning in *Drosophila*

The subsequent description is mainly referring to Rogers and Kaufman, 1997. The anteriormost region of the head, which is composed of the clypeolabral, foregut, stomodeal, and endodermal anlagen (Rogers and Kaufman, 1997), depends on a very different gene regulatory network. The anteriormost region is patterned by the anterior, terminal, and dorsal maternal system

(Grossniklaus et al., 1994). In this region the maternal factors *bcd* and *torso* are required for regulation of the terminal gap genes *tailless* (*tll*) and *huckebein* (*hkb*). In turn, *tll* and *hkb* regulate the transcription factors and second order regulators *cnc* and *crocodile* (*croc*), which are involved in the formation of the clypeolabrum, *forkhead* (*fkh*), which is required for foregut formation, and *serpent* (*srp*), which is necessary for proper formation of the anterior midgut (**Figure 2.1**; Brönner et al., 1994; Bronner and Jackle, 1991; Brönner and Jäckle, 1996; Häcker et al., 1995; Mohler, 1993; Reuter and Leptin, 1994; Rogers and Kaufman, 1997; Weigel et al., 1989). The transcription factor *sine oculis homeobox homolog 3/optix* (*six3/optix*) (*six3*) is required for the formation of clypeolabrum as well, but its position in the gene regulatory network has not been studied so far (Coiffier et al., 2007). However, due to several limitations (**see next chapter**), there is no comprehensive understanding about patterning of the anteriormost head region in *Drosophila*. Therefore, in order to get a comprehensive knowledge of the anterior head gene regulatory network another organism has to be exploited. Only comprehensive understanding of the gene regulatory networks can provide information about the basis of evolution and diversification of head morphologies.

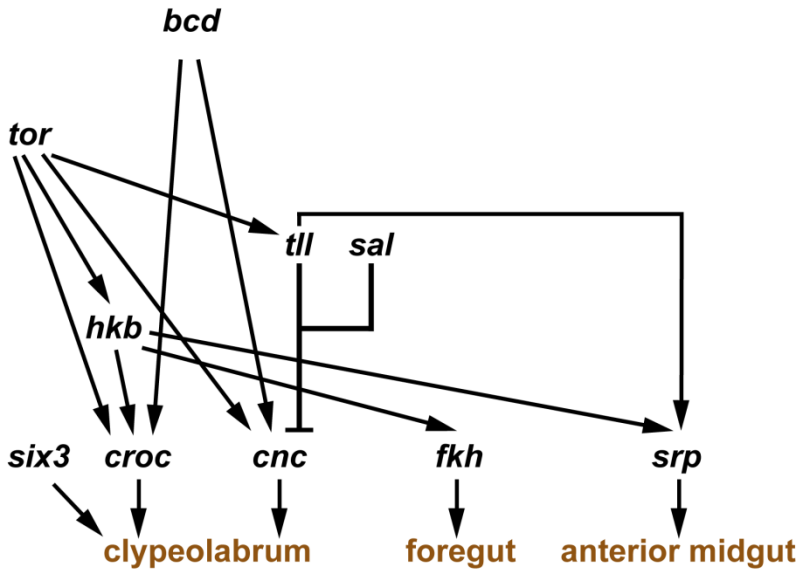


Figure 2.1 Gene regulatory network of the anteriormost region in *Drosophila*. Arrowheads indicate activation and cross-bars indicate gene repression. The terminal gap gene *tll* is already expressed at syncytial stages and represses *cnc*. At later stages *cnc* is additionally repressed by *spalt* (*sal*). However, loss of *sal* function leads only to a minor alteration of *cnc* expression. *six3* was shown to be required for clypeolabral patterning but interaction with other genes has not been studied. (Based on Coiffier et al., 2007; Rogers and Kaufman, 1997)

2.1.3 *Tribolium* - a model organism for insect head development

Drosophila is, for plenty of reasons, the prime model organism for studying insect development (Bolker, 2012; Kohler, 1994; St Johnston, 2002). However, the suitability for studying insect-typical head development is limited due to several reasons. First, *Drosophila* develops as a long-germ insect (Davis and Patel, 2002). Thus, the head anlagen are located at the anterior pole of the egg and depend on anterior, terminal, and dorsal signaling, while most insects show head anlagen located in the ventral median region at blastoderm stages (Sander 1976). Second, the *Drosophila* larval head is turned from outside to the inside during embryonic development, in a process called head involution (Grossniklaus et al., 1994). This leads to a highly derived head morphology and hampers the phenotypic analysis due to lack of morphological markers. Further, developmental defects due to mutations often interfere with head involution, thus causing additional secondary defects (Merrill et al., 1989; Posnien et al., 2010; Rogers and Kaufman, 1997).

I used the red flour beetle *Tribolium castaneum* (*Tribolium*; HERBST 1797) as model organism for insect-typical head development for several reasons (Bucher and Wimmer, 2005; Klingler, 2004). *Tribolium* is a representative of the most diverse and species-rich order across the tree of life, i.e. the coleopterans (beetles; Beutel, 2000; Grimaldi and Engel, 2005) and a cosmopolitan pest of stored grain (Klingler, 2004; Sokoloff, 1974; Zettler, 1991). *Tribolium* passes through embryonic development in the short germ-mode, in which posterior segments are progressively added from a posterior growth zone, reflecting a more insect-typical mode of development (Davis and Patel, 2002; Klingler, 2004; Lynch and Desplan, 2003; Tautz et al., 1994). At early embryonic stages the head is located at a ventral sub-terminal position, thus depending on ventral signaling as well as signaling from the more anterior extra-embryonic tissue (Posnien et al., 2010). Furthermore, larval stages show a fully everted head, carrying all typical appendages and a highly specific head bristle pattern, which provides landmarks for phenotypic analysis (Chapman, 1982; Posnien et al., 2010; Schinko et al., 2008). Moreover, a large versatile toolbox for studying insect development has been set up during the last two decades. The genome of *Tribolium* is fully annotated, serving as basis for many different questions and experiments (Richards et al., 2008). Further, the *Tribolium* toolbox provides several techniques for genetic manipulation, e.g. robust and systemic RNA interference (RNAi) feasible for all developmental stages (Brown et al., 1999; Bucher et al., 2002; Tomoyasu et al., 2008; Tomoyasu and Denell, 2004), spatio-temporal control of RNAi (J. Ulrich, unpublished), heat shock-based gene misexpression (Schinko et al., 2012), and GAL4/UAS-based misexpression (Schinko et al., 2010). Transposon-mediated transgenesis (Berghammer et al., 1999), as well as CRISPR (clustered

regularly interspaced short palindromic repeats) -mediated transgenesis has been established (Gilles et al., 2015; Gilles and Averof, 2014). Further, powerful in vivo imaging tools were generated, e.g. a nuclear reporter line (El-Sherif et al., 2012; Sarrazin et al., 2012), embryonic mRNA injection of reporter molecules (Benton et al., 2013), and protocols for light-sheet-based imaging and data processing (Strobl et al., 2015; Strobl and Stelzer, 2014). Moreover, the large-scale and unbiased 'iBeetle' RNAi screen can be used to find new genes required for a process apart from the classical candidate gene approach (Schmitt-Engel et al., 2015). Finally, the transposon-based mutagenesis screen 'GEKU' provided numerous transgenic enhancer trap and embryonic lethal mutant lines (Trauner et al., 2009).

2.1.3.1 *Drosophila* gene function is not conserved well in *Tribolium*

With respect to the genetic regulation of head development in *Tribolium* it appears that the mechanisms for patterning the gnathocephalic part of the head are similar to *Drosophila*, especially concerning downstream levels, like pair-rule genes and segment polarity genes (Brown et al., 1994; Choe et al., 2006; Choe and Brown, 2009, 2007; Farzana and Brown, 2008; Oppenheimer et al., 1999; Peel et al., 2013; Schaeper et al., 2010). However, there are some major differences considering the upstream regulators of head development in *Tribolium*, reflecting the more ancestral and insect-typical regulation of development (Bucher and Wimmer, 2005; Klingler, 2004; Schröder et al., 2008). It was predicted that early patterning of the anterior head region must be very different in *Tribolium* compared to *Drosophila*, because of the different positions of the head anlagen in *Drosophila* (Posnien et al., 2010). For instance, *tor* is present but plays no role in head development (Schoppmeier and Schröder, 2005), whereas *bcd* is not existent in *Tribolium* (Brown et al., 2001; Stauber et al., 1999), instead *Tc-axin* (Fu et al., 2012) and *Tc-mex3* (Schoppmeier et al., 2009) are required for specification of the head region. Regarding the anterior head it has been shown that the head gap-like genes diverged significantly in their function (Kittelmann et al., 2013; Schinko et al., 2008). The head patterning function of *Tc-orthodenticle* is conserved specifically during later stages. However, the early regionalization function is required for all segments formed at the blastoderm stage. Further, *Tc-sloppy-paired* affects only the head vertex in the procephalic region (Posnien et al., 2011b). *Tc-empty spiracles* shows a only partial loss of one segment upon deletion and *Tc-buttonhead* has no considerable function at all (Schinko et al., 2008). In turn, *Tc-knirps* (*Tc-kni*) is required for formation of the antennal and mandibular segments, but has no function in *Drosophila* head development (Cerny et al., 2008). Further, *Tc-kni* expression is regulated by the pair-rule gene

engrailed indicating an evolutionary difference in the classical gene hierarchy (Peel et al., 2013). However, more downstream acting genes that are involved in differentiation seem to be conserved, e.g. *Tc-cnc*, *Tc-croc*, and *Tc-fkh* (Economou and Telford, 2009; Kittelmann et al., 2013; Posnien et al., 2010).

2.1.3.2 Anteriormost head patterning in *Tribolium*

Recent studies tried to elucidate the gene regulatory network of the anteriormost pre-segmental head region and found out that this region is patterned by a unique gene regulatory network, which is independent of Hox genes and pair-rule patterning (**Figure 2.2B**, Kittelmann et al., 2013; Posnien et al., 2011b; Schaeper et al., 2010).

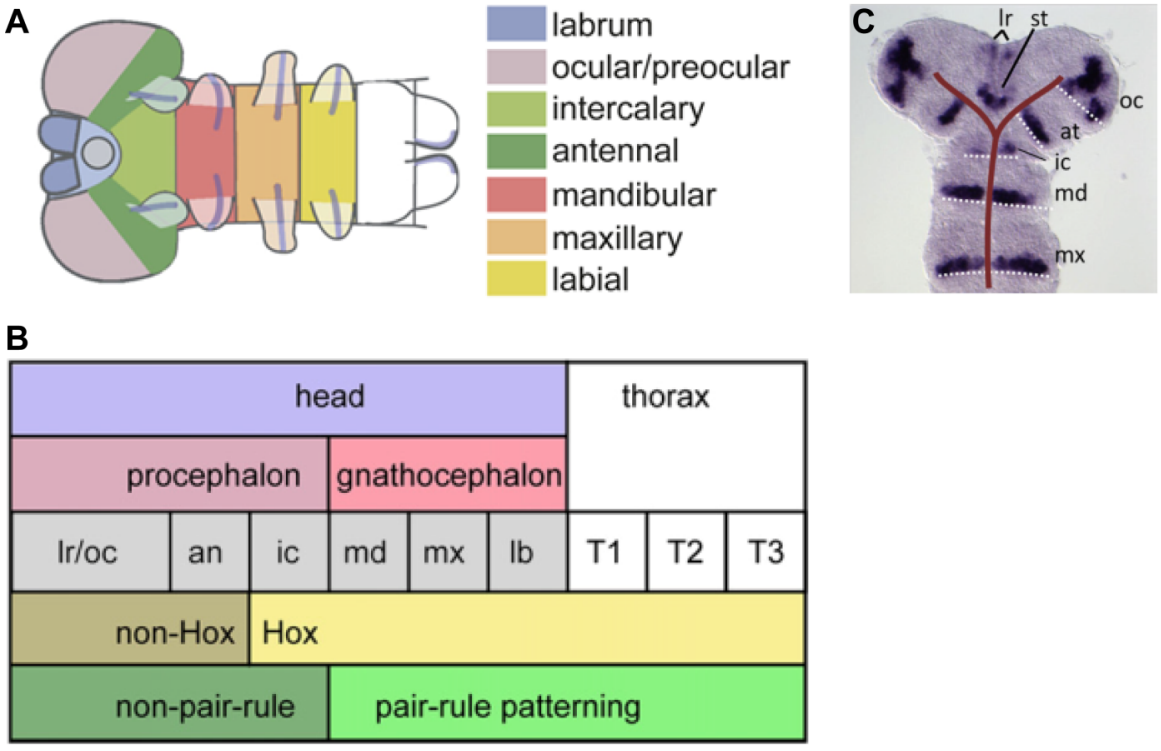


Figure 2.2 Composition and patterning of the *Tribolium* head. (**A**) The *Tribolium* head is composed of the posterior gnathocephalon and the anterior procephalon. The gnathocephalon comprises the gnathal segments, which give rise to the labium, maxillae, and mandibles, which are important for feeding. The procephalon is built by the intercalary segment, antennal segment, ocular/preocular region, and the labral region. This region gives rise to the antennae, compound eyes, ocelli, stomodeum, labrum, and the brain. The procephalon is mainly involved in sensing and subsequent integration of information. (**B**) The gnathocephalon is patterned like the trunk by Hox genes and pair-rule genes. However, the procephalon

shows no expression of these genes, except for the intercalary segment. (**C**) Using molecular markers, the procephalon could be subdivided further into a segmental region, with trunk-like parasegment boundaries (marked with dashed lines) and an anterior pre-segmental region, which show no trunk-like parasegment boundaries. The red line indicates a split of the Tc-*wg* expression in the anterior part of the head enclosing the non-neurogenic anterior median region (AMR), which comprise the labral and stomodeal region. (Posnien 2011b, modified): lr: labral region, oc: ocular region, an: antennal segment, ic: intercalary segment, md: mandibular segment, mx: maxillary segment, lb: labial segment, TS1-3: thoracic segments 1-3

The anterior pre-segmental region comprises the neurogenic ocular/preocular region and non-neurogenic 'anterior median region' (AMR, see **Figure 4.6.1**), which will give rise to the stomodeum and the labrum (Kittelmann et al., 2013, **Figure 2.2A**: blue region). This region was shown to rely on a highly conserved set of genes, which is also expressed in the vertebrate neural plate (Posnien et al., 2011b). Previous studies on the gene regulatory network of this region (**Figure 2.3**) were able to show that *Tc-six3* is an upstream regulator for this region (Kittelmann et al., 2013; Posnien et al., 2011b; Nico Posnien et al., 2009).

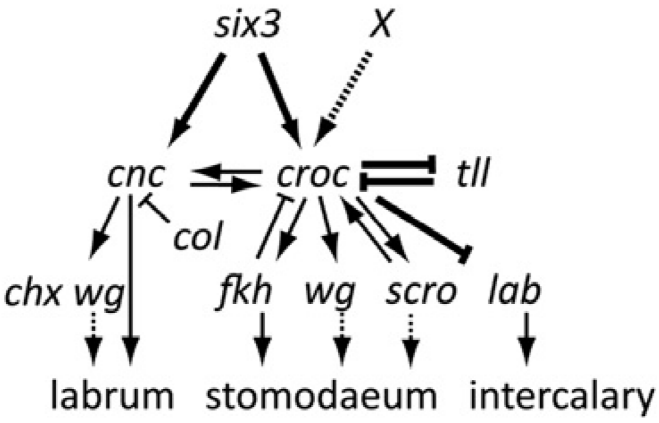


Figure 2.3 Gene regulatory network of the anteriormost head region in *Tribolium*. Arrowheads indicate activation and cross-bars indicate gene repression. Dashed lines indicate hypothetical effects. *Tc-six3* is a cardinal factor for pattering of the anteriormost region. *Tc-six3* is activating the anterior expression of *Tc-croc* within the AMR, while an unknown player '*X*' for the posterior portion is still unknown. (Taken from Kittelmann et al., 2013)

Tc-six3 activates *Tc-cnc*, which is responsible for formation of the anterior portion of the AMR, and *Tc-croc*, which is responsible for formation of the posterior portion of the AMR. Both transcription factors are required for formation of the labrum. *Tc-hkb* and *Tc-tll* show no

terminal gap gene function and are not involved in the formation of the anteriormost region of the head. Further, *Tc-croc* regulates *Tc-fkh* and thereby the stomodeum formation instead of *Tc-hkb,* as for *Drosophila* reported. Based on expression patterns it is also hypothesized that *Tc-wingless* (*Tc-wg*) is involved in labrum and stomodeum formation. *Tc-scarecrow* (*Tc-scro*) is hypothesized to play a role in the development of the stomodeum based on its expression pattern. However, there are still gaps concerning the knowledge of the gene regulatory network of the anterior head region, while the candidate gene approach seems to be exhausted. For example, an unknown player '*X*' that regulates the posterior part of the *Tc-croc* domain within the AMR is still missing. Further, it has not been reported, which factor activates *Tc-six3* (Kittelmann et al., 2013; Posnien et al., 2011b).

2.1.3.3 The 'iBeetle' screen - trying to find novel genetic regulators for head development

The knowledge about anterior head pattering gained so far is mainly based on analyzing candidate genes known from *Drosophila* head development and vertebrate neural plate (Economou and Telford, 2009; Kittelmann et al., 2013; Posnien et al., 2011a, 2011b). However, the candidate gene approach is biased towards conserved gene function (Schmitt-Engel et al., 2015). This approach appears to be exhausted, while it is obvious that important players patterning the anterior head are missing (**see above**; Kittelmann et al., 2013). To overcome the limitations of the candidate gene approach, a large-scale unbiased RNAi screen was started in 2011 for *Tribolium* with the aim to identify unknown function of genes, which are involved in essential processes, e.g. head development, muscle formation, and odoriferous gland formation. Several thousands of dsRNA fragments were injected to interfere with gene functions at embryonic as well as at postembryonic stages. The iBeetle screen is bipartite and composed of (1) a larval injection screen addressing genes, which are involved in post-embryonic developmental processes, and (2) a pupal injection screen, which addresses genes involved in embryonic development. The target genes were chosen randomly. In the next years the screen is planned to be finalized, thereby achieving genome-wide coverage (Bucher, pers. communication). The resulting developmental phenotypes are searchable in the online "iBeetle-Base" (http://ibeetle-base.uni-goettingen.de; Dönitz et al., 2015, 2013; Schmitt-Engel et al., 2015). In this database an interesting new candidate gene was annotated, which showed a labrum-specific cuticle phenotype, upon pRNAi. The labrum phenotype indicates a function in anterior head development. Thus, this was a promising candidate to further complement the

anterior head gene regulatory network. Information provided by the iBeetle genome browser (http://bioinf.uni-greifswald.de/gb2/gbrowse/tcas5/) suggested that the targeted gene is an ortholog of the *Drosophila fd102C* (*CG11152*), described as *foxq2*, a member of the *forkhead* gene family (Lee and Frasch, 2004; Mazet et al., 2003).

2.1.3.3.1 Forkhead box transcription factor family

The first *forkhead box* family member was identified 1989 in *Drosophila*. Mutant loss-of-function flies showed ectopic spike-shaped head structures, which led to the name of the gene and eventually to the name of the gene family '*fork head*' (Benayoun et al., 2011; Hannenhalli and Kaestner, 2009; Lam et al., 2013; Weigel et al., 1989). Forkhead proteins are known to function as transcription factors, which are required for initiation and regulation of transcription (**Figure 2.4**, Benayoun et al., 2011). All members of the Forkhead box family share the roughly 100 amino acid long Forkhead DNA-binding domain (Benayoun et al., 2011; Carlsson and Mahlapuu, 2002; Hannenhalli and Kaestner, 2009; Lai et al., 1990; Weigel and Jäckle, 1990).

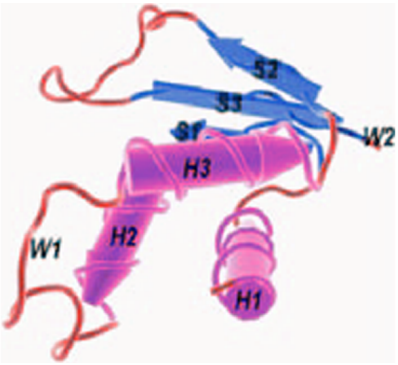


Figure 2.4 Structure of a classical Fox protein domain (FoxQ1). The classical FOX domain is composed of (N-terminal → C-terminal): three α-Helices (H1/H2/H3), three ß-strands (S1/S2/S3), and two loops (W1/W2). The conformation resembles butterfly wings and thus coined the nickname 'winged-helix'. (Benayoun et al., 2011, modified)

So far 19 *fox* subfamilies have been described (Benayoun et al., 2011; Hannenhalli and Kaestner, 2009; Kaestner et al., 2000; Larroux et al., 2008; Mazet et al., 2003; Shimeld et al., 2010), which are represented in more than hundred different species of animal and fungi with over 2000 members. Subfamilies are marked by an alphabetic character as suffix (*fox a-s*). However, the total number of *fox* genes and the number of represented families within each species is variable (Benayoun et al., 2011; Shimeld et al., 2010). Fox transcription factors tend to bind to DNA as monomers. Their function in biological processes is diverse ranging from insulin-signaling,

diabetes, ageing, cancer, vocal learning, chromatin remodeling, to nuclear receptor binding (Benayoun et al., 2011; Hannenhalli and Kaestner, 2009).

2.1.3.3.1.1 *foxq2*

Members of the *foxq2* gene family have been first described in 2003 in two different species. The *Caenorhabditis elegans* gene *C25A1.2*., although at the time not referred being member of the *foxq2* family, was shown to be expressed in nerve cells of the circumpharyngal nerve ring, but showed no phenotype, upon RNAi (Hope, 2003). Further, it has been shown that the *foxq2* gene in amphioxus is expressed at the anterior pole at embryonic and larval stages. This was the first study showing the apical expression pattern of the *foxq2* genes and, hence, suggesting an important role in AP patterning (Yu et al., 2003). *foxq2* subfamily members have been found in a large number of species across the metazoan kingdom (see taxa in **Figure 2.5**; Chapman et al., 2010; Chevalier et al., 2006; Fritzenwanker et al., 2014; Hope, 2003; Hunnekuhl and Akam, 2014; Koziol et al., 2016; Larroux et al., 2008; Lee and Frasch, 2004; Marlow et al., 2014; Martín-Durán et al., 2015; Martín-Durán and Hejnol, 2015; Mazet et al., 2003; Santagata et al., 2012; Shimeld et al., 2010; Sinigaglia et al., 2013; Tu et al., 2006; Yaklichkin et al., 2007; Yu et al., 2008, 2003; Zhang et al., 2014). Intriguingly, placental mammals lack a *foxq2* representative, whereas in other vertebrates, like *Danio rerio* and the monotreme *Ornithorhynchus anatinus*, representatives could be found (Shimeld et al., 2010; Yu et al., 2008).

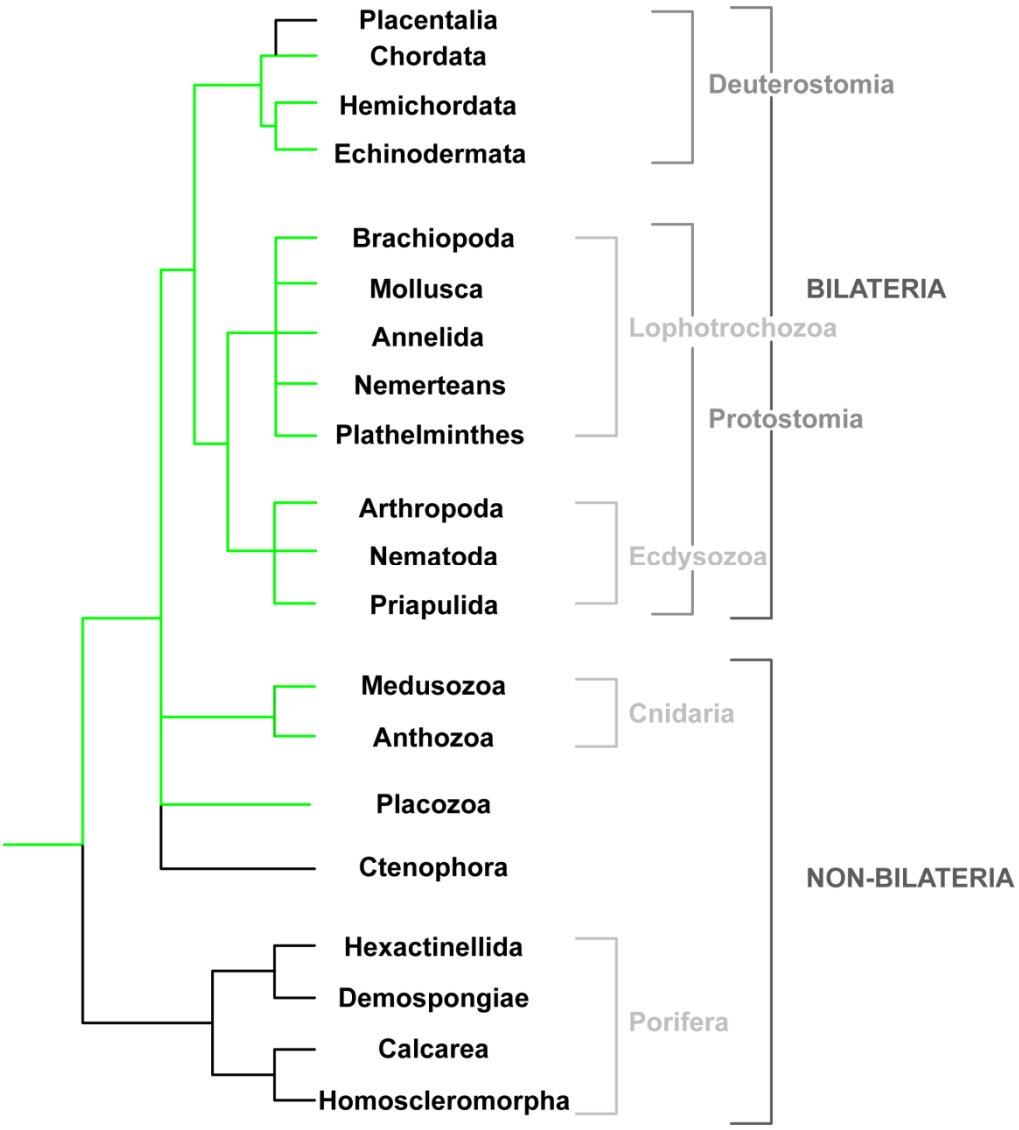


Figure 2.5 *foxq2* gene subfamily members are found in almost all phyla of the metazoan kingdom. Phyla showing *foxq2* gene subfamily members are marked in green. *foxq2* is not represented in the Placentalia and Ctenophora. Pre-metazoan clades like the Porifera lack members of the *foxq2* subfamily. (Tree is based on Dohrmann and Worheide, 2013; Prud'homme et al., 2003)

Most of the *foxq2* representatives in these different species appear to show comparable expression profiles, patterning a region hypothesized to be homologous (Fritzenwanker et al., 2014; Yaguchi et al., 2008). This homologous region is marked by a set of genes including *six3* and *rx* being conserved across protostome lophotrochozoans, deuterostomes, and cnidarians (**Figure 2.6**). It has been shown in *Nematostella* and *Platynereis dumerilii* (*Platynereis*) that *foxq2* is also part of this conserved pattering system (Marlow et al., 2014; Sinigaglia et al., 2013; Tosches and Arendt, 2013). Further, it has been shown that this conserved set of genes also

patterns an ancient neurogenic region of different species. In aquatic larval stages of *Nematostella*, *Strongylocentrotus*, *Terebratalia transversa*, and *Platynereis* these genes are patterning the sensory-neurosecretory apical tuft sometimes also referred to as apical organ (Howard-Ashby et al., 2006; Marlow et al., 2014; Santagata et al., 2012; Sinigaglia et al., 2013; Wei et al., 2009; Yaguchi et al., 2012, 2010, 2008). In the arthropod *Strigamia maritima* it has been shown that these genes are patterning a neurogenic region similar to the apical organ of other invertebrate marine larvae (Hunnekuhl and Akam, 2014). In *Tribolium*, the postulated pre-segmental region contributes to the central complex and the mushroom bodies, which are parts of the protocerebrum (Scholtz and Edgecombe, 2006). It was already shown for *Tc-six3* to play a major role in patterning and formation of the mushroom body and the central complex (Posnien et al., 2011b). However, data with respect to *foxq2* function is so far only provided for the cnidarian *Nematostella vectensis* (*Nematostella*; Sinigaglia et al., 2013) and the echinoderm deuterostome *Strongylocentrotus purpuratus* (*Strongylocentrotus*; Range and Wei, 2016; Yaguchi et al., 2012, 2010, 2008). In *Nematostella Nvfoxq2*a is involved in the development of the aboral region by regulating genes like *NvSix3/6* and *NvHoxF*. In knock-down experiments the overall larval morphology is unaffected but the apical organ size is reduced (Sinigaglia et al., 2013). In *Strongylocentrotus*, *foxq2* is involved in ectodermal patterning by regulating the oral-aboral axis specification, via repression by Wnt/ß-catenin signaling and repression of *nodal*. Knock-down of *foxq2* leads only to minor morphological defects, characterized by a slight thickening of the animal plate ectoderm. However, *foxq2* knock-down compromises the development of serotonergic neurons and the differentiation of long cilia in the apical organ/apical tuft (Yaguchi et al., 2010, 2008).

However, the expression and function of *foxq2* has so far not been characterized in insects. This study promises to provide information about the expression and function of *foxq2* in ectodermal and neural development in insects and thereby contribute to reconstruct the conserved anterior patterning gene regulatory network.

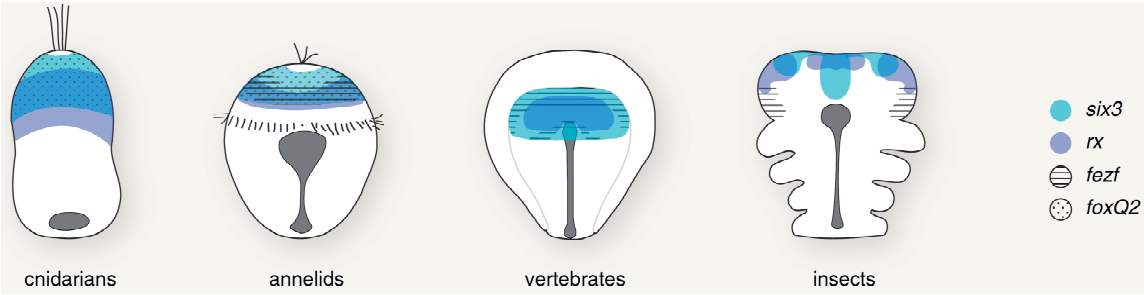


Figure 2.6 **Conservation of an apical patterning gene set in different species across metazoan species.** Schematic representation indicating expression of *six3*, *rx*, *fezf*, and *foxq2* at the apical pole of different metazoan species. *Nematostella* data represents cnidarians, *Platynereis* data represents annelids, *Xenopus laevis* data represents vertebrates, and *Tribolium* data represents the insects. (Taken from Tosches and Arendt, 2013)

2.2 The arthropod head problem

2.2.1 Origin and questions

The head and the brain were formed in a process called 'cephalization'. Starting point of this process was a common unsegmented bilaterian ancestor. Segments evolved separating the two non-segmental termini, forming the homonomously segmented ancestor of the arthropods. During arthropod cephalization, trunk segments were successively added to the anterior non-segmental part of the homonomously segmented ancestor to form an anterior tagma. Simultaneously, the ganglia of each segment fused at the anterior pole, building the brain. The different arthropods have varying numbers of segments building the head (Budd and Telford, 2009; Ou et al., 2012; Snodgrass, 1960). This composite structure of the head enabled a high diversification of the arthropod head shape, which allowed the different species to adapt to their environmental conditions and different ecological niches (Posnien et al., 2010). However, the composition of the arthropod head is disputed since decades, a discussion, which is called the 'arthropod head problem' (Budd, 2002; Haas et al., 2001; Jürgens et al., 1986; Posnien et al., 2010; Rempel, 1975; Rogers and Kaufman, 1997; Schmidt-Ott et al., 1994; Schmidt-Ott and Technau, 1992; Scholtz and Edgecombe, 2006). This problem comprises several open questions, but I will focus on the following subset: Is there evidence for the existence of (1) a labral segment, and/or (2) a non-segmental region called 'acron'? (3) Which of them will give rise to the labrum? (4) If there is a non-segmental region, what is its contribution to the adult head? And what is the contribution of the segmental parts to the adult head?

2.2.1.2 Morphogenesis of the insect head

In order to gain insights into the contribution of certain cells or tissues to a specific region were classically high-invasive experiments, e.g. hot needle cauterization or laser ablation, performed (Jürgens et al., 1986; Posnien et al., 2010; Sander, 1976; Wada, 1965). More recent studies used less invasive methods like analysis of mutants, RNAi phenotypes, marker gene in-situ hybridizations, fuchsin stainings, and membrane stainings to get insights into the *Tribolium* head fate map (Posnien et al., 2010; Posnien and Bucher, 2010). These analyses challenged the classical hypothesis about the head fate map. This hypothesis proposed that the gnathal segments of the embryonic head perform a dorsal closure similar to the trunk segments. Thus, the antennal, intercalary, and gnathal segments were suggested to contribute to lateral and dorsal parts of the adult head (Figure 2.7). However, the new findings led to the 'bend and zipper' model (Figure 2.8A-D), which predicts complex tissue movements resulting in a different prediction of the head fate map (Figure 2.8E). The new head fate map predicts that the maxillary and labial segment contribute to a minor extent to the dorsal capsule, whereas the other segments give only rise to lateral parts of the adult head capsule. Most of the dorsal head is built by the ocular/preocular region (Posnien et al., 2010).

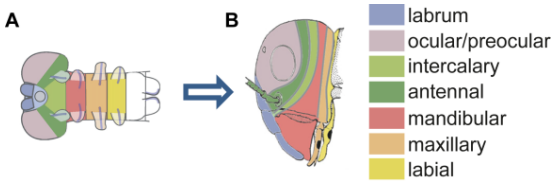


Figure 2.7 **Classical head fate map hypothesis.** (**A**) Embryonic segments posterior to the ocular/preocular region undergo trunk-like dorsal closure. (**B**) These segments each contribute to lateral and dorsal parts of the adult head capsule. The remaining portion of the dorsal head capsule is built by the ocular/preocular region. (Posnien et al., 2010, modified)

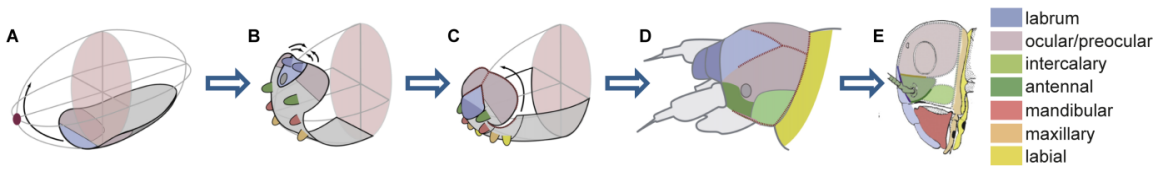


Figure 2.8 **The bend and zipper model predicts a new head fate map.** (**A**) The embryonic head anlagen are located at a ventral sub-terminal position within the egg. In the course of development, the germ band elongates towards the anterior pole of the egg. Thus, the head lobes will be bent upwards. (**B**) The lateral head lobes (ocular/preocular region) approach the embryos dorsal midline and start to fuse, in a zip-like

manner from anterior to posterior. (**C**) The labral buds fuse and form the labrum underneath the fused head lobes. The maxillary and labial segment will undergo trunk-like dorsal closure and fuse anteriorly with the head lobes. (**D**) Preliminary larval head fate map, resulting from the morphogenetic movements predicted by the bend and zipper model and mutant/RNAi phenotype analysis. (**E**) The data extrapolated to an adult head fate map. In this newly established head fate map, only the post-mandibular gnathal segments contribute to dorsal parts of the cuticle, whereas the more anterior segments only contribute to lateral parts of the head capsule. The dorsal head capsule is primarily built by the ocular/preocular region. The origin of some parts of the head capsule remains unclear (white region). (Posnien et al., 2010; Posnien and Bucher, 2010, modified)

However, also the new head fate map prediction is only preliminary. The bend and zipper model and the resulting new head fate map are based on invasive methods and fixed materials. Moreover, the fate map lacks cellular resolution and is thus limited by its accuracy. Therefore, it was one of my tasks to generate new transgenic lines for in vivo imaging experiments. These new tools should provide the basis to study the morphogenetic movements leading to the adult insect head in non-invasive imaging experiments. Further, cell marking lines should be generated and in the future used to mark the embryonic pre-segmental region and to look for its contribution to the larval head. The ultimate aim is to use these lines to establish an exact and comprehensive head fate map at cellular resolution.

2.3 Aims

This study had two aims. On the one hand I wanted to gain more knowledge about the genetic regulation of insect head development, and on the other hand I wanted to shed more light on the embryonic morphogenetic movements, which lead to the larval head.

(I) In order to get new insights into the genetic regulation of the head development, I wanted to decipher the function of *Tc-foxq2* in the gene regulatory network of the anterior pre-segmental region using in-situ hybridization in wild-type, pRNAi and gain-of-function embryos. Moreover, I intended to find out the function of *Tc-foxq2* in ectodermal and neural development, via pRNAi and gain-of-function experiments. Finally, I aimed to correlate this *Tribolium* data with the *foxq2* data of other metazoan species in order to perhaps discover a conserved patterning system.

(II) In order to reveal the morphogenetic movements, which lead to the adult head, it was my aim to provide new transgenic lines expanding the *Tribolium* in vivo imaging tool kit. Simultaneously, I intended to find a promoter, which is ubiquitously active and drives strong expression at all developmental stages. Further, to provide the tools for the generation of a head fate map at better resolution it was my aim to generate lines, in which a small population of cells could be marked and subsequently tracked. To this end, I wanted to exploit photoactivatable fluorescent proteins for laser-induced cell marking, and the Cre/*loxP* system for a genetic cell marking system.

3

Material & Methods

3.1 Animals

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

The *San Bernadino* (*SB*) wild-type (wt) strain was used for cDNA synthesis, RNAi experiments, whole mount in situ hybridizations and antibody stainings.

The *black* (Sokoloff, 1974) and the *Pig-19* (based on the *pearl* strain;Lorenzen et al., 2003) strains were used for RNAi experiments only.

The Tc-*vermillion*^{white} (v_w) strain (Lorenzen et al., 2002) was used for transgenesis. The line is deficient for *Tc-vermillion*, which leads to white eyes.

The transgenic line *EFA-nGFP* drives expression of the green fluorescent protein (GFP) under the control of the *Tc-elongation factor1α* (*EF1α*) promoter (*EFA*) with nuclear localization due to a nuclear localization signal (nGFP; El-Sherif et al., 2012; Sarrazin et al., 2012). This line was used for comparison with my newly established nuclear reporter lines (see **section 3.10.1**).

The transgenic *MB-green* line (#176, enhancer trap line G11410 (http://www.geku.base.uni-goettingen.de)) was generated in a large-scale insertional mutagenesis screen (Trauner et al., 2009) and marks mushroom body tissue with enhanced GFP (EGFP; Binzer et al., 2014; Koniszewski et al., 2016; Posnien et al., 2011b).

The transgenic *brainy* line (#174) marks glial tissue (6xP3-ECFP) and neural cells (EFA-dsRed; Koniszewski et al., 2016; Posnien et al., 2011b). These lines were used for in vivo analysis of larval brain defects.

The Cre (*causes recombination*) recombinase driver line #16 (generated by J. Schinko) drives Cre expression under the control of the *Tc-heat shock protein68* promoter (*Tc-hsp68*). This line was used to drive the *loxP* (locus of crossing over (x), P1) responder line, upon heat shock treatment.

3.2 Phylogenetic analysis

Phylogenetic analysis of the Foxq2 proteins was done by using MEGA v.5 (Tamura et al., 2011). The multiple sequence alignment was conducted with the ClustalW algorithm with the preset parameters. Positions containing gaps were eliminated from the dataset. The phylogenetic tree was constructed using the Neighbor-Joining method with the Dayhoff matrix based substitution model (Schwartz and Dayhoff, 1979). Bootstrap tests (Felsenstein, 1985) were conducted using 1000 replicates to test the robustness of the phylogenetic tree.

3.3 RNAi

The templates for the non-overlapping double-stranded RNA (dsRNA) fragments were generated via standard PCR from a plasmid template using following primers (including T7 RNA polymerase promoter sequence): Fragment *Tc-foxq2*$^{RNAi\_a}$ (489 bp): 5'-GAATTGTAATACGACTCACTATAGGCTTACTTCAGGACCCGG-3' and 5'-GAATTGTAATACGACTCACTATAGGTCGCTTGTAACAATGCTTGA-3'; Fragment *Tc-foxq2*$^{RNAi\_b}$, (197 bp): 5'-GAATTGTAATACGACTCACTATAGGATGTGCAGTAACGAGACTCC -3' and 5'-GAATTGTAATACGACTCACTATAGGCTGGGGAAGAGCGGATAGC -3'.

The dsRNA was synthesized using the Ambion® T7 MEGAscript® kit (lifeTechnologies,Carlsbad,CA,USA). The transcribed dsRNA was extracted via isopropanol precipitation (*Tc-foxq2*$^{RNAi\_a}$) or phenol/chloroform extraction (*Tc-foxq2*$^{RNAi\_b}$) and dissolved in injection buffer (1.4 mM NaCl, 0.07 mM Na_2HPO_4, 0.03 mM KH_2PO_4, 4 mM KCl, pH 6.8). The injected dsRNA concentrations for parental RNAi with *Tc-foxq2*$^{RNAi\_a}$ and *Tc-foxq2*$^{RNAi\_b}$ were 1.0 µg/µl, 1.5 µg/µl and 3.1 µg/µl. If not stated differently, a dsRNA concentration of 1.5 µg/µl was used. Pupal injections were performed as previously described (Bucher et al., 2002; N. Posnien et al., 2009). The dsRNA was injected using the FemtoJet® express device (eppendorf, Germany). Cuticles of the L1 larval offspring were prepared as described (Wohlfrom et al., 2006). Head bristle patterns of cuticles were analyzed as described (Schinko et al., 2008).

3.4 Fixation

Embryos at an age of 6-26 h (32°C) AEL (for wt single whole mount in situ hybridization (ISH), wt double whole mount in situ hybridization (DISH) and loss-of-function ISH) or 14-18 h AEL (for

gain-of-function ISH) were fixed using standard protocols (Schinko et al., 2009) with slight modifications: 180 µm meshes were used and 2 ml PEMS buffer (0.1 M PIPES, 2mM MgCl, 5 mM EGTA, pH 6.9) in place of fixation buffer.

3.5 Immunostaining

3.5.1 Antibodies

Immunostaining was performed using the cleaved *Drosophila* Dcp-1 (Asp216) rabbit antibody (Cell Signaling Technology, Germany) with 1:100 dilution. Anti-rabbit coupled with Alexa Fluor 488 was used for detection with 1:1000 dilution.

3.5.2 Staining

The fixed embryos (see **section 3.4**) were successively rehydrated and freed from methanol, by washing with PBT. The embryos were blocked for one hour at room temperature with 3% BSA (Fraction V). Afterwards, the primary antibody (in 3% BSA) was added and incubated O/N at 4°C. After several washing steps with PBT the secondary antibody was added and incubated for 90 min at room temperature. The antibody was removed and the nuclei were afterwards stained with DAPI. The stained embryos were mounted in VECTASHIELD® (Vector Laboratories) to prohibit photobleaching.

3.5.3 Statistical analysis

The regions of interest (**Figure 4.5**: region1, 3 and **Figure 4.32**: region 2, 3; dashed lines) were all set on the basis of morphological traits. Cell counting was performed using the Fiji cell counter plug-in (Schindelin et al., 2012). The number of apoptotic cells is positively correlated with the age (data not shown). To circumvent potential staging errors, the apoptotic cell number in the posterior procephalon (**Figure 4.5** and **Figure 4.32**: region 3) was counted to normalize the data of interest (**Figure 4.5**: region 1 and **Figure 4.32**: region 2). This region was chosen, because it should be unaffected by RNAi experiments based on the expression data and the epidermal L1 larval phenotype. The correction value was calculated by dividing the mean number of apoptotic cells of RNAi embryos (region 3) by the mean number of apoptotic cells in wt embryos (region 3). For the normalization was each single data point for the region 1 (respectively region 2) divided by the correction value.

The normalized data was visualized in a box plot and statistically tested with R v.2.14.2 (http://www.R-project.org/). The dataset was tested for the homogeneity of the variances, via the box plot, and for normal distribution, via the Shapiro-Wilk test. To test for significance, three statistical tests were conducted: Welch t-test, two sample t-test and the Wilcoxon rank-sum test. All three tests show the same levels of significance. Stated *p*-values are based on the Wilcoxon rank-sum test results.

3.6 Whole mount in situ hybridization

3.6.1 Probes

ISH probes were synthesized with the DIG (Digoxegenin-UTP; ^{DIG}) RNA labeling kit (Roche, Germany) and the Fluorescin (Fluorescin-UTP; ^{FLUO}) labeling mix (Roche, Germany) using the T7 RNA polymerase.

3.6.2 Staining

ISH (alkaline phosphatase + NBT/BCIP) and DISH (alkaline phosphatase + NBT/BCIP & horseradish peroxidase mediated tyramide signal amplification (TSA) reaction: horseradish peroxidase + tyramide-Dylight550 conjugate) were performed as described previously (Oberhofer et al., 2014; Schinko et al., 2009; Siemanowski et al., 2015).

3.6.2 Co-expression analysis

The embryos, in the wt DISH assay (**Figure 4.8** and **Figure 4.9**), were staged based on morphological traits and *Tc-foxq2* expression pattern. The dashed lines indicating gene co-expression are based on comparisons of the *Tc-foxq2*^{DIG} signal (**Figure 4.8A**) with the signal of *Tc-foxq2*^{FLUO} in the other panels (**Figure 4.8B-E** and **Figure 4.9**).

3.7 Cloning of genes

Tc-foxq2 full coding sequence (1633 bp; Gen bank accession number: XM_008202469) was obtained from the *Tribolium* genome browser (http://bioinf.uni-greifswald.de/gb2/gbrowse/tcas5/). The following primers were used to amplify the full coding sequence from an embryonic cDNA pool (0-72 h AEL) via standard PCR: 5'-

ATGTGCAGTAACGAGACTCC-3' and 5'-TTAAGAGTCTGTGGTGTCGG-3'. The *Tc-foxq2* full coding sequence was cloned into the pJET1.2 vector (Thermo SCIENTIFIC™).

The *Tribolium* ortholog of *histone 2A variant* (*Tc-H2Av*, 387 bp, bank accession number: XM_970375) was amplified lacking the stop codon, via standard PCR, with the following primers: 5'-ATGGCTGGTGGCAAAGCAGG-3' and 5'-GACGGGCTGTGAGTGG-3'. The partial coding sequence was cloned into the pJET1.2 vector.

3.8 Generation of H2Av::EGFP and H2Av::C3PA-GFP chimeric reporter proteins

To generate the chimeric H2Av::EGFP and H2Av::C3PA-GFP nuclear marker proteins, overlap extension PCR was used as described previously (Yolov and Shabarova, 1990; Yon and Fried, 1989).

3.9 Cloning regulatory regions

The regulatory region of the gene *Tc-αTubulin1* (*Tc-αTub1*P) was already sub-cloned and described (Siebert et al., 2008). The regulatory region of *Tc-polyubiquitin* (*Tc-PUb*P; 948 bp fragment upstream of the start codon; Primer: 5´-TGTACTTTTCTTTGTCCCAAATGACC-3´and 5´-CTGCAACGACACAAAAAATTACTT-3´) and *Tc-ribosomal protein subunit3* (*Tc-rps3*P; 700 bp upstream of the start codon; Primer: 5'-TGTCAAACCACAAACATAAAAAATAG-3' and 5'-TTTGACGTTCTAAATGGAAAAGG-3') were obtained from the *Tribolium* genome browser (see **section 3.7**) and isolated from genomic DNA of *SB* adults. Amplified sequences were sub-cloned in the pSLfa1180fa shuttle vector. The regulatory regions were then cloned into the transformation vector, 5' upstream of the *H2Av::EGFP* chimeric marker protein and the *SV40* polyadenylation signal. For detailed sequences and vector maps: see **section 7.15-.20**.

3.10 Transgenesis

3.10.1 Constructs

All donor constructs were stably integrated into the genome using the *piggyBac* vector pBac[3xP3-gTc'v] (Lorenzen et al., 2003, 2002). Plasmids:
[3xP3-gTc'v;Tc'hsp68-Tc'foxq2], [3xP3-gTc'v;Tc'αTub1P- Tc'H2Av::EGFP], [3xP3-gTc'v;Tc'PUbP-Tc'H2Av::EGFP], [3xP3-gTc'v;Tc'rpS3P-Tc'H2Av::EGFP], [3xP3-gTc'v;Tc'αTub1P-C3PA-GFP], [3xP3-gTc'v;Tc'αTub1P- Tc'H2Av:: C3PA-GFP], [3xP3-gTc'v;Tc'αTub1P-*loxP*(mcherry)-Tc'H2Av::EGFP]. For detailed sequences and vector maps: see **section 7.15-.28**.

3.10.2 Germline transformation

Germline transformation was performed as described previously (Berghammer et al., 1999; Schinko et al., 2012), with slight modifications: the injection buffer was different (see **section 3.3**) and the TcasHylper (generated by S. Dippel) was used as helper construct. The TcasHylper helper construct carries the mammalian codon-optimized hyperactive transposase open reading frame (Yusa et al., 2011), which is flanked by the regulative sequence of the *Tribolium Tc-hsp68* promoter.

3.10.3 Transformation marker and marker detection

Tc-vermilion was used and detected as transformation marker as described previously (Lorenzen et al., 2002; Schinko et al., 2012).

3.11 Heat shock treatment

All lines for heat shock experiments were kept at 32°C. Heat shock treatments of embryos (for gain-of-function cuticle preparations: 0-24 h AEL, 9-13 h AEL, 14-20 h AEL, 20-25 h AEL; for gain-of-function ISHs: 9-13 h AEL) and pupae were performed as described previously (Schinko et al., 2012) for ten minutes at 48°C.

3.12 Photoactivation

Photoactivation was performed by using the LSM510 (ZEISS) with the corresponding ZEISS software (v.3.2 SP2). Larvae and pupae were fixed on a microscope slide with Fixogum (Marabu, Germany) and photoactivated using a 20x (air) objective. Embryos were mounted in Voltalef 10S Halocarbonoil and photoactivated using a 40x (oil) objective. For photoactivation the 351 nm and the 364 nm (both 100% transmission) UV-Laser line were used simultaneously. Using the software embedded 'Bleach control' tool were the region of interest (ROI), laser lines (351 nm, 364 nm), scan number (1), and the number of iterations (30x) set. The experiment was at the end conducted with single scans (scan speed 1) with a frame size of 512x512 pixels, resulting in a total excitation of 207.73 µs/pixel per activation cycle. The signal induced by photoactivation was observed using the 488 nm argon laser with a 505 nm long pass filter for detection.

3.13 Image documentation and processing

Cuticle preps were imaged as described (Wohlfrom et al., 2006). L1 larval brain imaging was performed as described previously (Posnien et al., 2011b). Immuno- and DAPI stainings were imaged using the LSM510 (ZEISS). Stacks of DAPI stained embryos, and stacks of in vivo imaged L1 larval brains were processed in Amira v.5.3.2 (FEI) and displayed as 'voltex' projections. ISHs were imaged using the Axioplan2 (ZEISS, Germany), 10x and 40x objectives, and recorded with the ImagePro v.6.2 software (Media Cybernetics). Individuals of the cell marking lines and the different nuclear reporter lines were imaged with the LSM510 (ZEISS). In vivo imaging videos were recorded using either the ZEISS Lightseet Z.1 (Strobl et al., 2015; Strobl and Stelzer, 2014) or the LSM780 (ZEISS) with the ZEN software (ZEISS). If not stated differently stacks were processed and visualized as average or maximum projections using Fiji (Schindelin et al., 2012). All images were level-adjusted and assembled in Photoshop CS (Adobe). All figures were imported into Illustrator CS5 (Adobe) for labeling and formatting.

4

Results

4.1 *Tc-foxq2* - a novel player in anterior head development of *Tribolium*

4.1.1 *iB_03837* targets the *Tribolium* ortholog of Foxq2

The iBeetle screen identified an RNAi-induced L1 larval cuticle phenotype which showed labrum specific defects at a high penetrance (Dönitz et al., 2015; Schmitt-Engel et al., 2015). This phenotype was caused by parental RNAi by injecting the iB_03837 dsRNA fragment into female pupae of the *pig19* strain, which were crossed with *black* males. The *iB_03837* dsRNA fragment targets a part of the coding sequence of the gene *TC004761* (Tcas_OGS 3.0; **GenBank accession number: XM_008202469**) In order to identify the orthologs of the targeted gene, a phylogenetic tree was built. Phylogenetic analysis revealed that TC004761 is the *Tribolium* ortholog of Foxq2 (Tc-Foxq2; **Figure 4.1**). As expected, Tc-foxq2 clustered together with the protostome orthologs. Interestingly, the *Tribolium* ortholog is more similar to the *Platynereis* ortholog, compared to the arthropod (*Drosophila* and *Strigamia*) orthologs. However, support for the respective branches is comparably low.

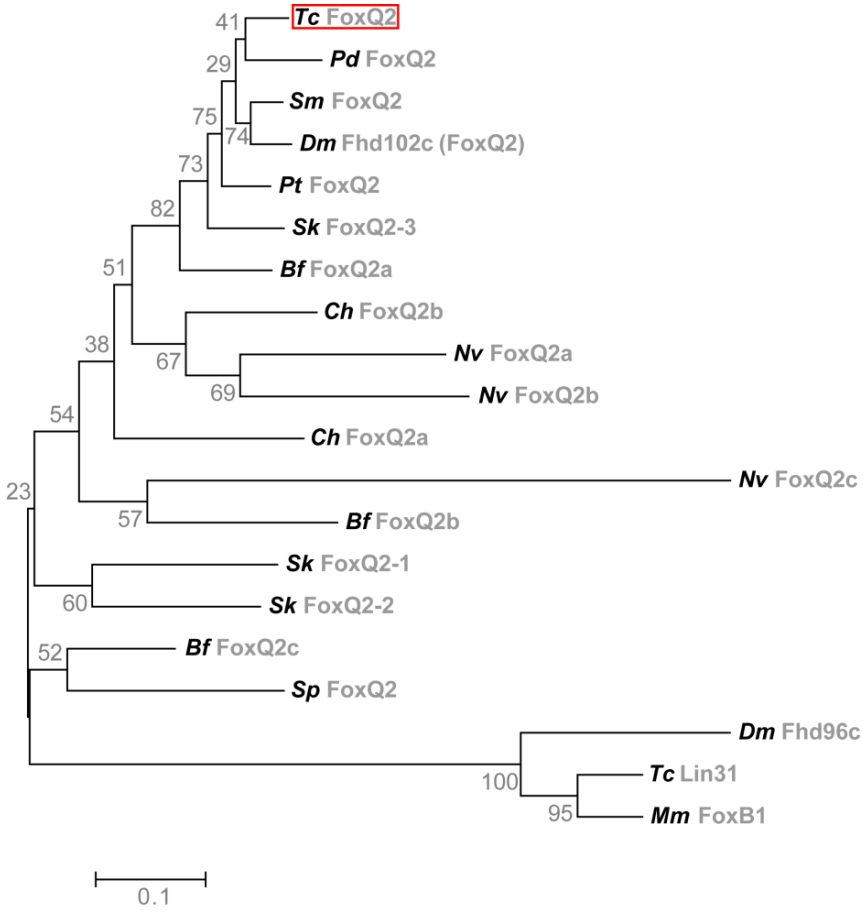


Figure 4.1 **Phylogenetic tree of Foxq2 proteins within the Metazoa.** *Tc-foxq2* encodes for a Foxq2 protein, which clusters together with the protostome orthologs. Interestingly, Tc-Foxq2 is related more closely to the annelid ortholog than to the orthologs of arthropods, which *Tribolium* is a member of. Shown is a Neighbor-Joining tree with bootstrap values (grey numbers). *Tc: Tribolium castaneum, Pd: Platynereis dumerilii, Sm: Strigamia maritima, Dm: Drosophila melanogaster, Pt: Parasteatoda tepidariorum, Sk: Saccoglossus kowalevskii, Nv: Nematostella vectensis, Ch: Clytia hemisphaerica, Bf: Branchiostoma floridae, Sp: Strongylocentrotus purpuratus, Mm: Mus musculus*.

4.1.2 *Tc-foxq2* knock-down phenotype in ectodermal tissue

4.1.2.1 Confirmation of the epidermal *Tc-foxq2* phenotype found in the iBeetle screen

I performed *Tc-foxq2* pRNAi using the *SB* strain to test the reproducibility of the labrum phenotype that was found under high-throughput conditions in the iBeetle screen. All of the analyzed L1 knock-down cuticles showed the same epidermal phenotype that had been previously described in the iBeetle screen. The cuticles showed either a size-reduced or

completely absent labrum (Figure 4.2B`, C`). Thus, the repetition of this experiment could confirm the *Tc-foxq2* pRNAi induced labrum phenotype that was annotated in the iBeetle screen. No other specific cuticle phenotypes were detected.

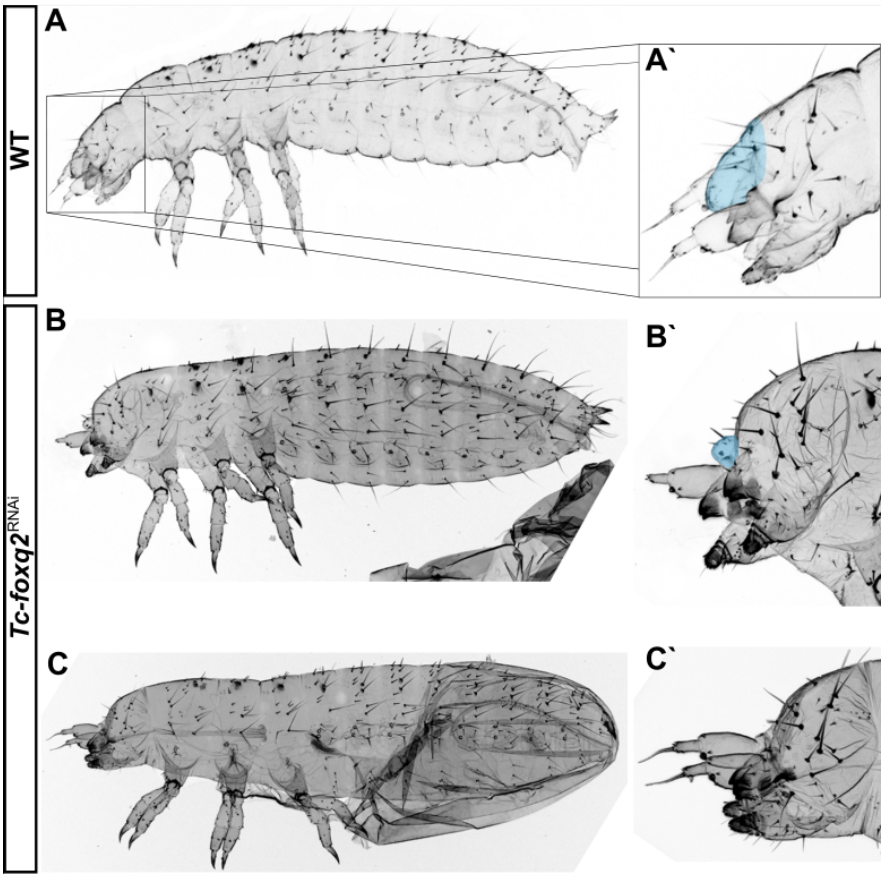


Figure 4.2 **Qualitative analysis of *Tc-foxq2* pRNAi reveals a labrum-specific phenotype in L1 larvae.** All larvae are depicted in lateral view. Anterior is left. (**A**, **A`**) Wild-type (wt) larval cuticle with the labrum marked in blue (**A`**). (**B**-**C`**) *Tc-foxq2*$^{pRNAi}$ cuticles show a labrum, which is either strongly reduced in size (**B**, **B`**) or completely absent (**C**, **C`**).

4.1.2.2 *Tc-foxq2* pRNAi affects the labrum and anteriormost setae

To get a precise and quantitative data set of the epidermal L1 phenotype, I performed detailed *Tc-foxq2* pRNAi experiments (raw counts and exact percentages are displayed in Table S7.1-.4). Further, I generated two new non-overlapping dsRNA fragments (*Tc-foxq2*$^{RNAi\_a}$ and *Tc-foxq2*$^{RNAi\_b}$) to exclude off-target effects (Qiu, 2005). Both are targeting parts of the *Tc-foxq2* coding sequence. Comprehensive analysis of L1 larval cuticles (Figure 4.3A: *Tc-foxq2*$^{RNAi\_a}$: n=838; B: *Tc-foxq2*$^{RNAi\_b}$: n=313) showed that most of the larvae have defects specific to the head

(Figure 4.3A, B). The proportion of eggs, which did not contain cuticles and eggs that only contained cuticle remnants were comparable (Figure 4.3A, B) and within in the range that is seen in RNAi experiments. Notably, the proportion of wt cuticles upon pRNAi using *Tc-foxq2*$^{RNAi\_b}$ was considerably higher (Figure 4.3B) compared to *Tc-foxq2*$^{RNAi\_a}$ (Figure 4.3A). This observation indicates that the *Tc-foxq2*$^{RNAi\_a}$ dsRNA fragment led to a more efficient knock-down of the endogenous *Tc-foxq2* mRNA. In cuticles showing the head-specific phenotype (Figure 4.3E, blue-marked region), the labrum setae (setae=long trichoid sensilla; Figure 4.3E, yellow dots), the clypeus setae (Figure 4.3E, orange dots), and the anterior setae of the vertex triplet were affected (Figure 4.3E, red dots). I used these setae as marker indicating the grade of the labrum phenotype and to evaluate the overall strength of the defects. I grouped the head defects into three different classes and quantified them (Figure 4.3C, D). Weak phenotypes showed a decreased labrum size, and one or both of the labral setae were missing (Figure 4.3F). Intermediate phenotypes were marked by a reduced labrum size and one or both of the anterior vertex triplet bristles were missing, indicating additionally more posterior defects (Figure 4.3G). Strong phenotypes ranged from cuticles with a strongly reduced labrum size, with deleted anterior vertex setae, and with maximal one labrum seta and one clypeus seta left to cuticles with a completely absent labrum (Figure 4.3H). The quantification indicates that the quality of head phenotypes was the same for both dsRNA fragments. Only the frequency of each of the head phenotypes was slightly different for both fragments, again indicating that the *Tc-foxq2*$^{RNAi\_a}$ dsRNA fragment leads to a stronger RNAi effect.

Asking whether the strongest phenotypes may have been missed, I also tested higher dsRNA concentrations (2 µg/µl and 3.1 µg/µl; data not shown) as well double RNAi using both dsRNA fragments together (*Tc-foxq2*$^{RNAi\_a}$ and *Tc-foxq2*$^{RNAi\_b}$, each 1.5 µg/µl; data not shown). None of these variations resulted in a stronger cuticle phenotype.

Taken together, these results showed that knock-down of *Tc-foxq2* results in a highly specific epidermal head phenotype, with defects restricted to the anteriormost region of the head. Furthermore, both non-overlapping dsRNA fragments lead to qualitatively equal phenotypes, indicating that the described phenotype is not due to off-target effects.

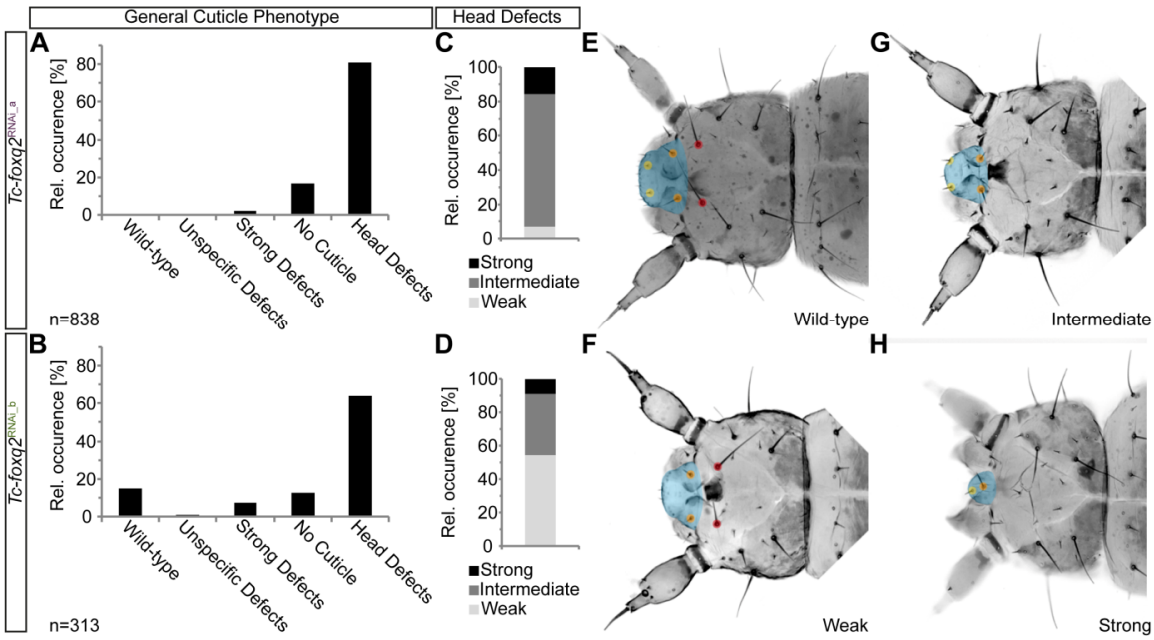


Figure 4.3 Quantitative analysis of the *Tc-foxq2*^{pRNAi} epidermal L1 defects confirms the phenotype and excludes off-target effects. (**A**, **B**) Knock-down of *Tc-foxq2* with two non-overlapping dsRNA fragments *Tc-foxq2*$^{RNAi\_a}$ (**A**) and *Tc-foxq2*$^{RNAi\_b}$ (**B**) leads to comparable portions of cuticle phenotypes. (**C**, **D**) Detailed analysis of head defects shows that the *Tc-foxq2*$^{RNAi\_a}$ fragment leads to a qualitatively comparable but quantitatively stronger phenotype, marked by more intermediate (**G**) and strong (**H**) head defects. (**E-H**) L1 cuticle heads, depicted in a dorsal view, are representing the different classes of head defects. Anterior is left. (**E**) Wt cuticle with the labrum marked in blue, two labrum setae (yellow dots), two clypeus setae (orange dots) and two anterior vertex setae (red dots). (**F**) Weak head defect with a reduced labrum and at least one deleted labrum seta. (**G**) Intermediate head defect is additionally lacking at least one of the anterior vertex seta. (**H**) Strong head defect with a strongly reduced labrum, one labrum seta and one clypeus seta. The strongest phenotypes show a completely absent labrum and deleted anterior vertex setae.

4.1.2.3 The *Tc-foxq2* pRNAi L1 epidermal phenotype is not strain specific

It has been previously described that RNAi-induced phenotypes could depend on the used strain, hence the given genetic background (Kitzmann et al., 2013). To test whether the observed phenotype is only specific to the used strain, I performed *Tc-foxq2* pRNAi in two different genetic backgrounds (raw counts and exact percentages are displayed in Table S7.1-.6). To this end, I injected the *Tc-foxq2*$^{RNAi\_b}$ dsRNA fragment into female pupae of the *pig19* strain (see section 3.1) and crossed them with *black* strain (see section 3.1) males (n=796; Figure 4.4), like

in the iBeetle screen. The repetition, within the changed genetic background, revealed a comparable general cuticle phenotype, showing high proportions of cuticles with head defects (**Figure 4.4B**). Furthermore, the quality of observed head defects was the same as in previous experiments. The *Tc-foxq2*$^{RNAi\_b}$ dsRNA fragment led to higher frequencies of intermediate and strong head defects in the *pig19*/*black* genetic background compared to the results in the *SB* genetic background (**Figure 4.4A**, compare **D** to **E**). However, compared to the pRNAi experiment using the *Tc-foxq2*$^{RNAi\_a}$ dsRNA fragment in *SB*, the expressivity of the head defect was very similar (**Figure 4.4** compare **E** to **F**). The observed defects in different genetic backgrounds are qualitatively equal and appeared in comparable proportions indicating that the epidermal phenotype after *Tc-foxq2* pRNAi is not strain specific.

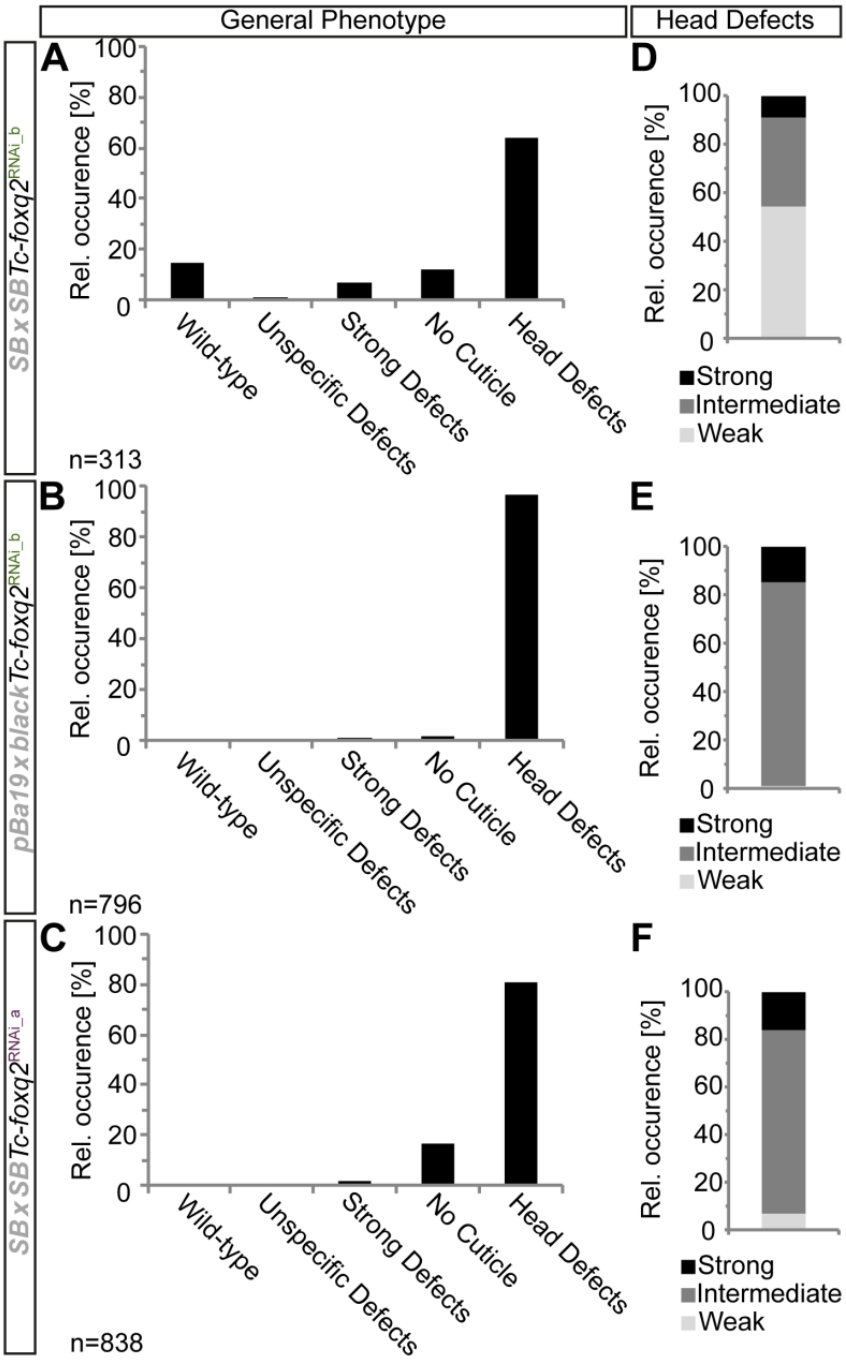
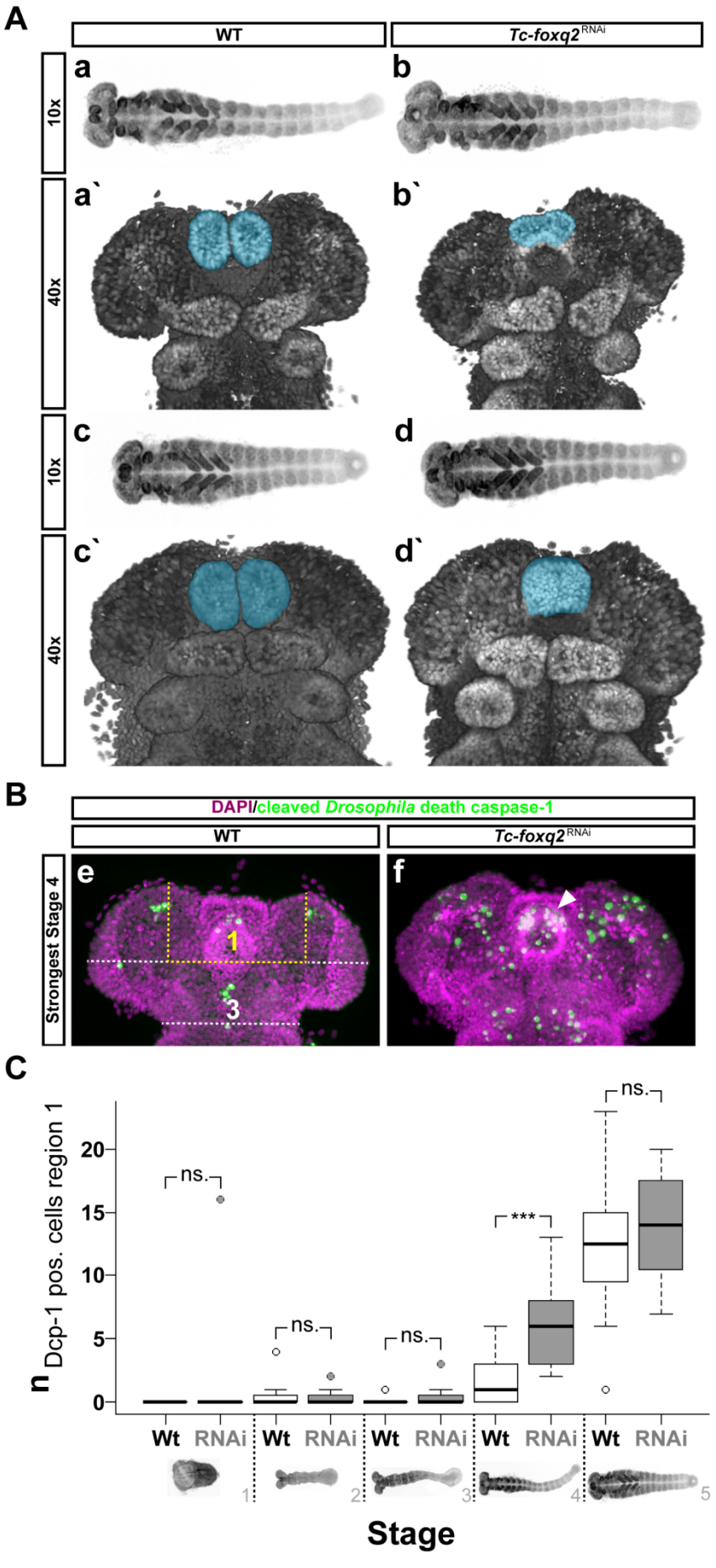


Figure 4.4 Quantitative analysis of the *Tc-foxq2*^{pRNAi} epidermal L1 phenotypes in two different strains shows no considerable strain specific effects. (**A**, **B**) Knock-down of *Tc-foxq2* using the *Tc-foxq2*$^{RNAi\_b}$ dsRNA fragment in the *SB* strain leads to a distribution of L1 larval general cuticle phenotypes (**A**), which is comparable to the distribution of phenotypes in L1 offspring, when the same dsRNA fragment is injected in pupal *pig19* females, which were crossed to *black* males (**B**). (**B**, **C**) The distribution of the general phenotype classes gets even more similar when compared to the RNAi experiment using the *Tc-foxq2*$^{RNAi\_a}$ dsRNA fragment in the *SB* strain (compare **C** and **B**). (**D**-**F**) Also the frequency and quality of head defects is comparable throughout the RNAi experiments in different genetic backgrounds. (Data from **A**, **D**, **C**, **F** taken from Figure 4.3)

4.1.2.4 *Tc-foxq2* pRNAi epidermal phenotype results presumably from an increased apoptotic cell death rate

In order to find out how *Tc-foxq2* pRNAi acts to interfere with labrum development, I asked when the labrum is decreased in size. To this end, I visualized the embryonic morphology by staining the nuclei with DAPI and compared labral bud sizes of *Tc-foxq2*^{pRNAi} embryos versus wt embryos (**Figure 4.5A**, blue marker). The comparison revealed that *Tc-foxq2*^{pRNAi} embryos showed a decreased labral bud size from fully elongated germ band stages onwards (**Figure 4.5A**$_{b', d'}$).


A
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Tc-foxq2RNAi
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40x
10x
40x
a
b
a`
b`
c
d
c`
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B
DAPI/cleaved Drosophila death caspase-1
WT
Tc-foxq2RNAi
Strongest Stage 4
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n Dcp-1 pos. cells region 1
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Figure 4.5 Qualitative analysis of the embryonic *Tc-foxq2*^{pRNAi} phenotype and its correlation with cell death rates. (**A**) Morphology of wt (**A_a**, **$A_{a`}$**, **$A_c$**, **$A_{c`}$**) and *Tc-foxq2*^{pRNAi} (**A_b**, **$A_{b`}$**, **$A_d$**, **$A_{d`}$**) embryos is visualized by nuclear staining (DAPI, grey). Anterior is left in 10x panels (**A_{a-d}**) and up in 40x panels (**$A_{a`}$**). The labrum is marked in blue (**$A_{a`-d`}$**). (**$A_{a`-d`}$**) *Tc-foxq2*$^{pRNAi}$ embryos (6-26 h after egg laying; AEL) show decreased labral buds, which appear to fuse prematurely (**$A_{b`}$**, **$A_{d`}$**). (**B**) For quantification of cell death rates, a region of interest and a control region were defined. The region of interest is the labral region (region 1, ROI 1, yellow dashed lines). The posterior procephalic region was used for data normalization (region 3, white dashed lines). Morphology of wt (**B_e**) and *Tc-foxq2*^{pRNAi} (**B_f**) embryos is visualized by nuclear staining (DAPI, magenta). Apoptotic cells are monitored by antibody staining (Cleaved *Drosophila* death caspase-1 (Dcp-1) - Alexa Fluor 488; green). (**B_e**, **B_f**) The fully elongated *Tc-foxq2*^{pRNAi} germ band with the most apoptotic cells within the labral region (ROI 1, marked with dashed lines) show apparently more marked cells (**B_f**) than the strongest representative of the wt embryos within this stage and region (**B_e**). (**B_f**) The *Tc-foxq2*^{pRNAi} embryo show an accumulation of apoptotic cells within the labral buds (arrowhead). (**C**) Box plot depicting the normalized number of apoptotic cells (y-axis) versus five different embryonic stages, subdivided in untreated wt and *Tc-foxq2*^{pRNAi} embryos. The ROI 1 values are normalized with the region 3 values (**Be**). Brackets display grade of significance. Germ rudiments (stage 1) to intermediate elongating germ bands (stage 3), as well as early retracting germ bands (stage 5) showed no significant increase in the number of apoptotic cells within the ROI 1 of *Tc-foxq2*^{pRNAi} embryos (stage 1: p=0.33 (wt: n=3, RNAi: n=7), stage 2: p=0.63 (wt: n=11, RNAi: n=12), stage 3: p=0.19 (wt: n=9, RNAi: n=19), stage 5: p=0.15 (wt: n=12, RNAi: n=11)). However, fully elongated germ bands (stage 4) showed significantly more apoptotic cells (p=0.00041) in *Tc-foxq2*^{pRNAi} embryos (n=15) compared to untreated ones (n=17). ns.: not significant

RNAi-induced labrum phenotypes could be due to altered embryonic cell proliferation or cell death rates (Kittelmann et al., 2013; Siemanowski et al., 2015). It has been shown that decreased cell proliferation rates, controlled by Notch signaling were the reason for a decreased labral bud size in one case (Siemanowski et al., 2015). To this end, I performed *Tc-serrate* (*Tc-ser*; marker for Notch signaling) whole mount in situ hybridization (ISH) in *Tc-foxq2*^{pRNAi} embryos (6-26 h after egg laying; AEL) and looked for an alteration of the expression profile. However, ISH experiments in *Tc-foxq2*^{pRNAi} embryos showed no considerable difference in *Tc-ser* expression compared to wt embryos (data not shown).

Thus, I asked whether altered embryonic cell death rates could be involved in the observed labrum phenotype. I performed cleaved *Drosophila* death caspase-1 (Dcp-1) antibody staining in embryos (6-26 h AEL) to visualize and quantify apoptotic cells (Florentin and Arama, 2012; **Figure 4.5B**, **C**). On the basis of morphological traits I defined two regions: Region 1 (**Figure 4.5B_e**: yellow dashed lines, 1) was the actual region of interest (ROI), encompassing the labral

region, while region 3 (Figure 4.5B$_e$: white dashed lines, 3) was the control region, which should show no RNAi-induced changes in cell death rates. The region 3 data set was used to normalize the actual data set in order to exclude unspecific changes of cell death rates (see section 3.5.3 for details). Furthermore, I set up five different groups, representing different embryonic stages (stage 1: germ rudiments, stage 2: early elongating germ bands, stage 3: intermediate elongating germ bands, stage 4: fully elongated germ bands, and stage 5: retracting germ bands (Figure 4.5C, x-axis)). The analysis revealed that fully elongated germ bands showed a significantly increased number of apoptotic cells, upon *Tc-foxq2* pRNAi (p=0.00041, n=15; Figure 4.5C: stage 4, B$_f$: arrowhead; for raw counts see Table S7.7-.8).

These data indicate that not an altered cell proliferation rate but an increased apoptotic cell death rate at early embryonic stages could be a reason for the reduced embryonic labral bud size and the L1 larval cuticle phenotype.

4.1.3 *Tc-foxq2* expression

4.1.3.1 *Tc-foxq2* is expressed in a highly dynamic pattern in the anterior head

It has been previously shown that *foxq2* shows a highly conserved expression pattern at the anterior pole of embryonic stages (Chevalier et al., 2006; Fritzenwanker et al., 2014; Hunnekuhl and Akam, 2014; Lee and Frasch, 2004; Martín-Durán and Hejnol, 2015; Santagata et al., 2012; Sinigaglia et al., 2013; Tu et al., 2006; Yu et al., 2003). To test whether this holds true for *Tribolium*, I conducted different ISH experiments to identify the onset and course of the *Tc-foxq2* expression pattern. To facilitate orientation during subsequent data description, a schematic representation of on embryonic head is shown in Figure 4.6.1.

The expression of *Tc-foxq2* starts at early embryonic stages at the anterior terminus (Figure 4.6A-C). At later stages it is expressed within the non-neural anterior median region (AMR: for orientation see Figure 4.6.1; Figure 4.6D, E). Subsequent stages show *Tc-foxq2* expression in the labral buds, enclosing parts of the stomodeum, and in the putative neuroectodermal region (Figure 4.6F-L).

These data show that *Tc-foxq2* is expressed within the AMR, in a highly dynamic expression pattern, and the adjacent neuroectodermal region. Its expression at the anterior pole of the

embryo confirms the conservation of the anterior function of *foxq2*. Further, the expression is in line with a direct function in the labrum and indicates a function in neural development.

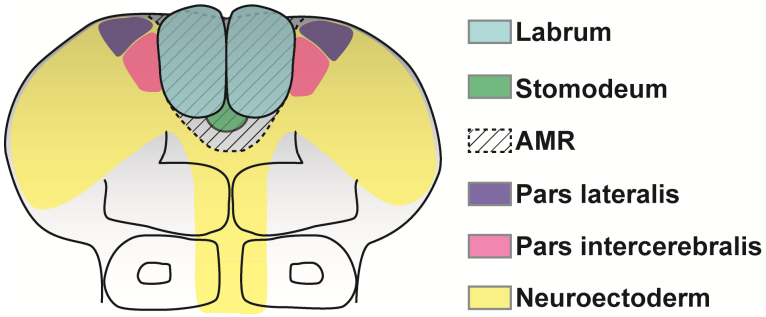
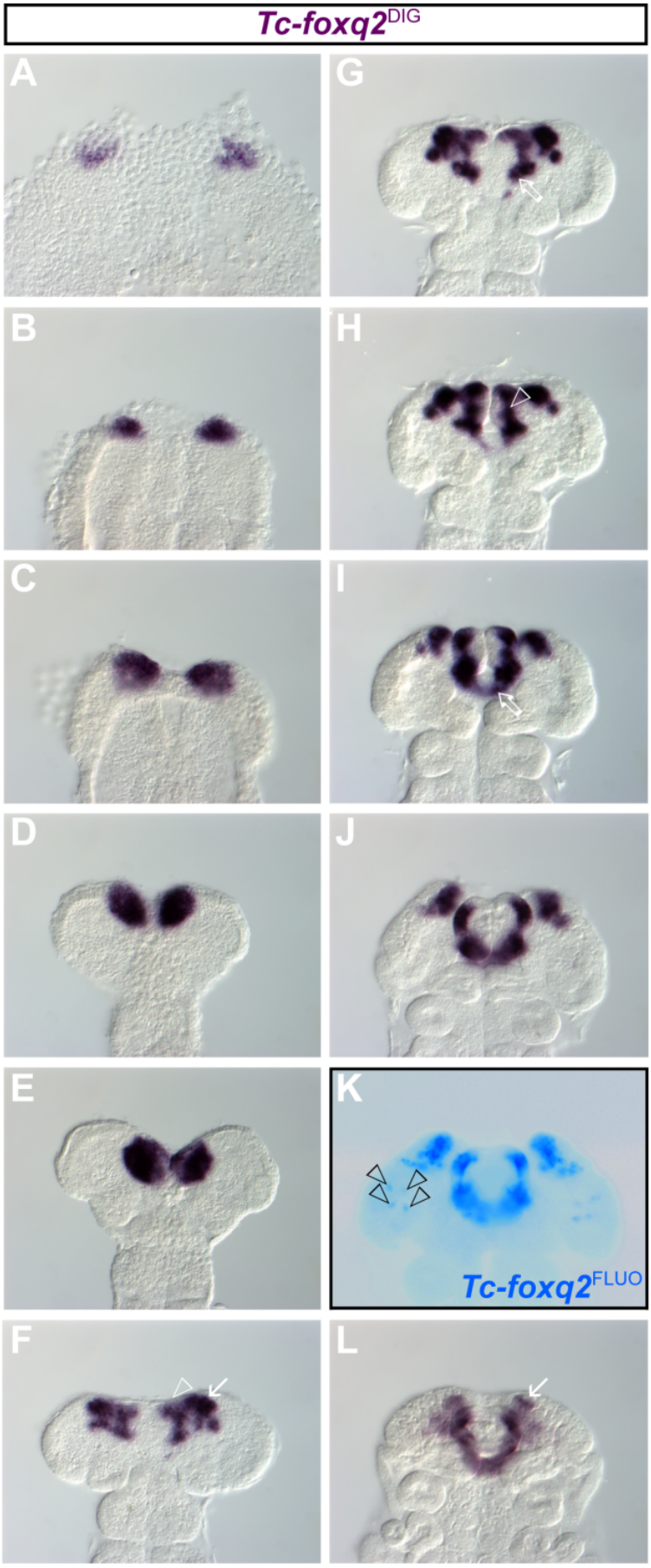


Figure 4.6.1 **Overview of the location of the most relevant embryonic head structures for this study.** Anterior is up. The non-neural anterior median region (AMR, striped region) harbors the labral buds (blue) and the stomodeum (mouth precursor, green). This region is enframed by the neuroectoderm (yellow), in which the central neuroendocrine centers, the pars lateralis (purple) and the pars intercerebralis (pink), are located (de Velasco et al., 2007). (Based on Kittelmann et al., 2013; Posnien et al., 2011b, 2010)


Tc-foxq2DIG
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G
B
H
C
I
D
J
E
K
Tc-foxq2FLUO
F
L

Figure 4.6 ***Tc-foxq2* is expressed in a highly dynamic pattern at the anterior pole.** Anterior is up. Expression of *Tc-foxq2* in wt embryos is monitored by whole mount in situ hybridization (ISH). (**A**) *Tc-foxq2* expression starts with formation of the germ rudiment. (**A**-**E**) Early *Tc-foxq2* expression is marked by two domains located at the anterior pole, which successively approach each other, probably due to morphogenetic movements, at the embryonic midline. (**F**) The expression pattern splits into several domains in late elongating germ bands, with expression domains in the putative neuroectoderm (white arrow, presumably including parts of the pars intercerebralis; Posnien et al., 2011b) and in the labral/stomodeal region (white empty arrowhead). (**G**) The expression domains flanking the prospective stomodeum become more prominent (empty arrow). (**H**) The anterior median expression domain frames the lateral parts of the labral buds (white empty arrowhead). (**I**, **J**) At fully elongated to early retracting germ band stages the two expression domains flanking the stomodeum are linked posteriorly to each other (empty arrow) and the expression domains within the labral buds are getting narrower. (**K**) Staining with the more sensitive TSA-Dylight550 (*Tc-foxq2*^{FLUO}) reveals four dot-like expression domains in the ocular region (black empty arrowheads). (**L**) At retracting germ band stages *Tc-foxq2* is expressed in a narrow U-shape pattern and the neuroectodermal expression domains are reduced in size (white arrow).

4.1.3.2 *Tc-foxq2* expression might involve a positive autoregulatory feedback loop

In order to get an impression of the knock-down efficiency upon *Tc-foxq2* pRNAi, I performed ISH experiments in *Tc-foxq2*^{pRNAi} embryos. ISH indicated that the signal intensity of *Tc-foxq2* expression seemed to be strongly reduced (Figure 4.7). However, increasing the exposure time revealed that there was an unequal efficiency of the endogenous *Tc-foxq2* mRNA knock-down. In early elongating embryos *Tc-foxq2* mRNA was almost completely abolished (Figure 4.7B). At later stages the anterior part of the *Tc-foxq2* expression domain within the neuroectoderm was highly reduced, whereas the posterior part of the neurogenic *Tc-foxq2* expression domain was still detectable (Figure 4.7F: white arrow). *Tc-foxq2* mRNA was still detectable around the stomodeum (Figure 4.7F: empty arrow) and in parts of the labrum (Figure 4.7H: empty arrowhead). Wt expression appeared to be rather equal in strength (Figure 4.7A, C, E, G). Therefore, the difference in knock-down efficiency might result from autoregulation in the respective domains.

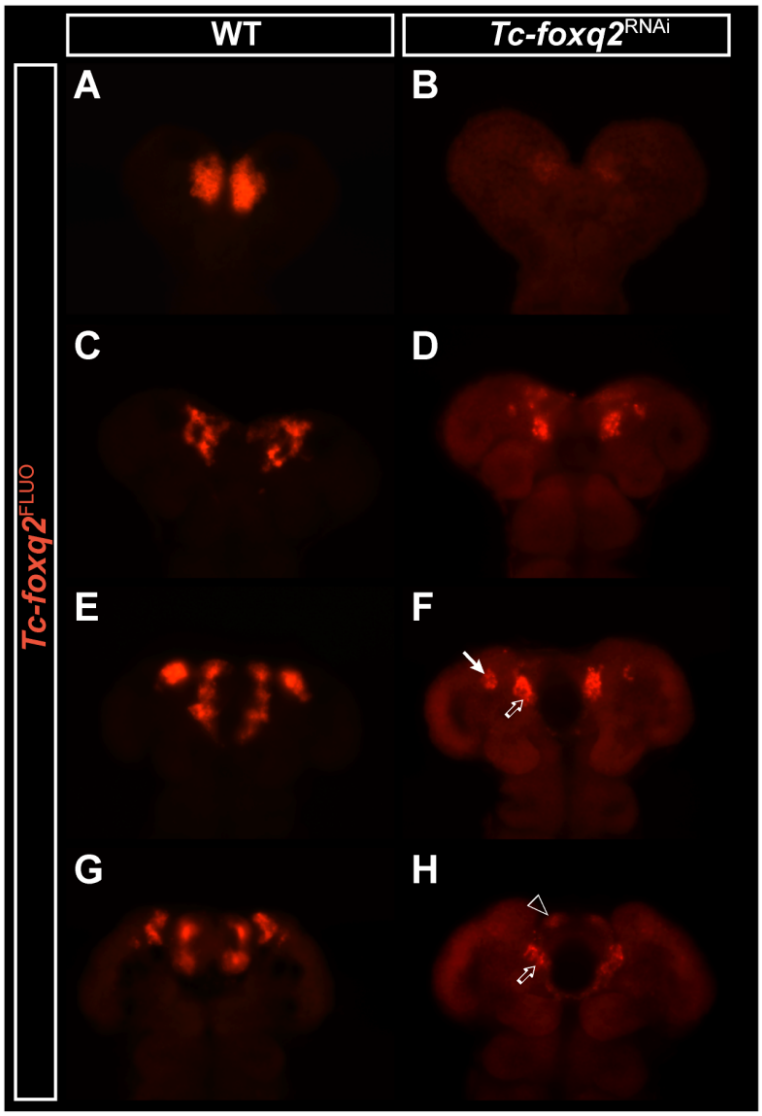


Figure 4.7 **Endogenous *Tc-foxq2* mRNA is not completely abolished after *Tc-foxq2* pRNAi.** Anterior is up. Expression of *Tc-foxq2* in wt (**A**, **C**, **E**, **G**) and *Tc-foxq2*^{pRNAi} (**B**, **D**, **F**, **H**) embryos is monitored by ISH. Shown are *Tc-foxq2*^{pRNAi} embryos with typical staining. Note that the exposure time had to be strongly increased in order to detect residual staining (see elevated background in **B**, **D**, **F**, **H**). (**B**) Upon RNAi treatment *Tc-foxq2* expression is almost completely deleted at early elongating germ band stages. (**D**) At *Tc-foxq2*^{pRNAi} late elongating germ band stages the posterior portion of the *Tc-foxq2* expression is still detectable. (**F**) Fully elongated germ band stages still show expression in the neurogenic region (arrow) and the stomodeal flanking region (empty arrow). (**H**) In early retracting *Tc-foxq2*^{pRNAi} embryos is *Tc-foxq2* expression in the anterior part of the labral buds (empty arrowhead) and the stomodeal region (empty arrow) detectable.

4.1.4 Uncovering the role of *Tc-foxq2* within the gene regulatory network of the anterior pre-segmental region

4.1.4.1 Co-expression of *Tc-foxq2* with other head patterning genes

To test for potential *Tc-foxq2* interaction partners within the gene regulatory network of the anterior pre-segmental head development, I looked for mutual co-expression. Genes co-expressed with *Tc-foxq2* are potentially regulating or are regulated by *Tc-foxq2*. To visualize co-expression, I performed double in situ hybridization (DISH) in wt embryos (6-26 h AEL). For better comparability I grouped these embryos according to their embryonic stage. Expression overlaps are marked with dashed lines.

To test whether *Tc-foxq2* is potentially interacting with the Wnt/ß-catenin signaling pathway, I analyzed *Tc-wingless/wnt1* (*Tc-wg*, Nagy and Carroll, 1994; **Figure 4.8A$_{0\text{-}6}$**) and *Tc-arrow* (*Tc-arr*, (Bolognesi et al., 2009); data not shown) for co-expression with *Tc-foxq2*.

Tc-wg is a segment polarity gene and ligand of the canonical Wnt signaling pathway. The co-expression analysis of *Tc-foxq2* and *Tc-wg* showed that there is only an expression overlap at retracting germ band stages in the anterior portion of the labral buds (**Figure 4.8A$_{5,\ 6}$**). *Tc-arr* is a co-receptor of *Tc-wg* and therefore also member of the segment polarity genes. In contrast to *Tc-wg*, *Tc-arr* is ubiquitously expressed throughout the embryonic development and therefore is covering the *Tc-foxq2* expression pattern entirely (data not shown).

Previous studies showed that *sine oculis homeobox homolog 3* (*six3*) is co-expressed with *foxq2* in various species (Fritzenwanker et al., 2014; Hunnekuhl and Akam, 2014; Marlow et al., 2014; Martín-Durán et al., 2015; Santagata et al., 2012; Sinigaglia et al., 2013; Tu et al., 2006; Wei et al., 2009). Thus, I tested whether *Tc-optix/six3* (*Tc-six3*), a transcription factor and major regulator of AMR and central complex development in *Tribolium* (Nico Posnien et al., 2009; Posnien et al., 2011b), shares also expression domains with *Tc-foxq2* (**Figure 4.8B$_{0\text{-}6}$**).

Tc-foxq2 and *Tc-six3* expression are largely overlapping from early stages on (**Figure 4.8B$_0$**). In intermediate elongating germ bands, *Tc-foxq2* shows no co-expression with *Tc-six3* (**Figure 4.8B$_2$**). During the course of development they are co-expressed in parts of the AMR, the labral

buds, and in the putative neurogenic region (**Figure 4.8B$_{0-6}$**). The previously described conserved co-expression of *foxq2* and *six3* is also represented at most of the analyzed *Tribolium* developmental stages. However, this holds not true for intermediate elongating germ bands, showing mutually exclusive expression of both factors.

I also tested *Tc-cap'n'collar* (*Tc-cnc*), a transcription factor involved in labrum development and proper mandible formation (Coulcher and Telford, 2012; Economou and Telford, 2009; Kittelmann et al., 2013), for co-expression with *Tc-foxq2* (**Figure 4.8C$_{0-6}$**). *Tc-cnc* and *Tc-foxq2* show an almost complete overlap in their expression profile at elongating germ band stages (**Figure 4.8C$_{0-2}$**). At later embryonic stages the expression overlap of these genes is restricted to the non-neural part the labral/stomodeal region (**Figure 4.8C$_{3-6}$**).

Tc-scarecrow (*Tc-scro* (*nk2.1* ortholog)), a transcription factor with roles in proper labrum formation and foregut development (Kittelmann et al., 2013; Posnien et al., 2011b), was also tested for overlapping expression with *Tc-foxq2* (**Figure 4.8D$_{0-6}$**). *Tc-foxq2* expression covers the complete *Tc-scro* expression domains at early elongating germ band stages (**Figure 4.8D$_{0}$**). In further elongated embryos *Tc-foxq2* expression is partially overlapping with the anterior parts of the *Tc-scro* domains (**Figure 4.8D$_{1-2}$**). At later stages *Tc-scro* expression shows overlaps with *Tc-foxq2* expression only in the lateral part of the AMR (**Figure 4.8D$_{3}$**) or additionally in small areas of the anterior neurogenic region (**Figure 4.8D$_{4-6}$**).

I tested *Tc-crocodile* (*Tc-croc*) for co-expression with *Tc-foxq2*, due to its role as a transcription factor in AMR formation, and formation of the stomodeum (Economou and Telford, 2009; Kittelmann et al., 2013; **Figure 4.8E$_{0-5}$**). At early germ band stages *Tc-croc* mRNA is co-localized with *Tc-foxq2* mRNA in latero-anterior parts of the AMR (**Figure 4.8E$_{0-3}$**). At later stages they are co-expressed in the posterior portion of the labral buds and around lateral and posterior parts of the stomodeum (**Figure 4.8E$_{4-5}$**).

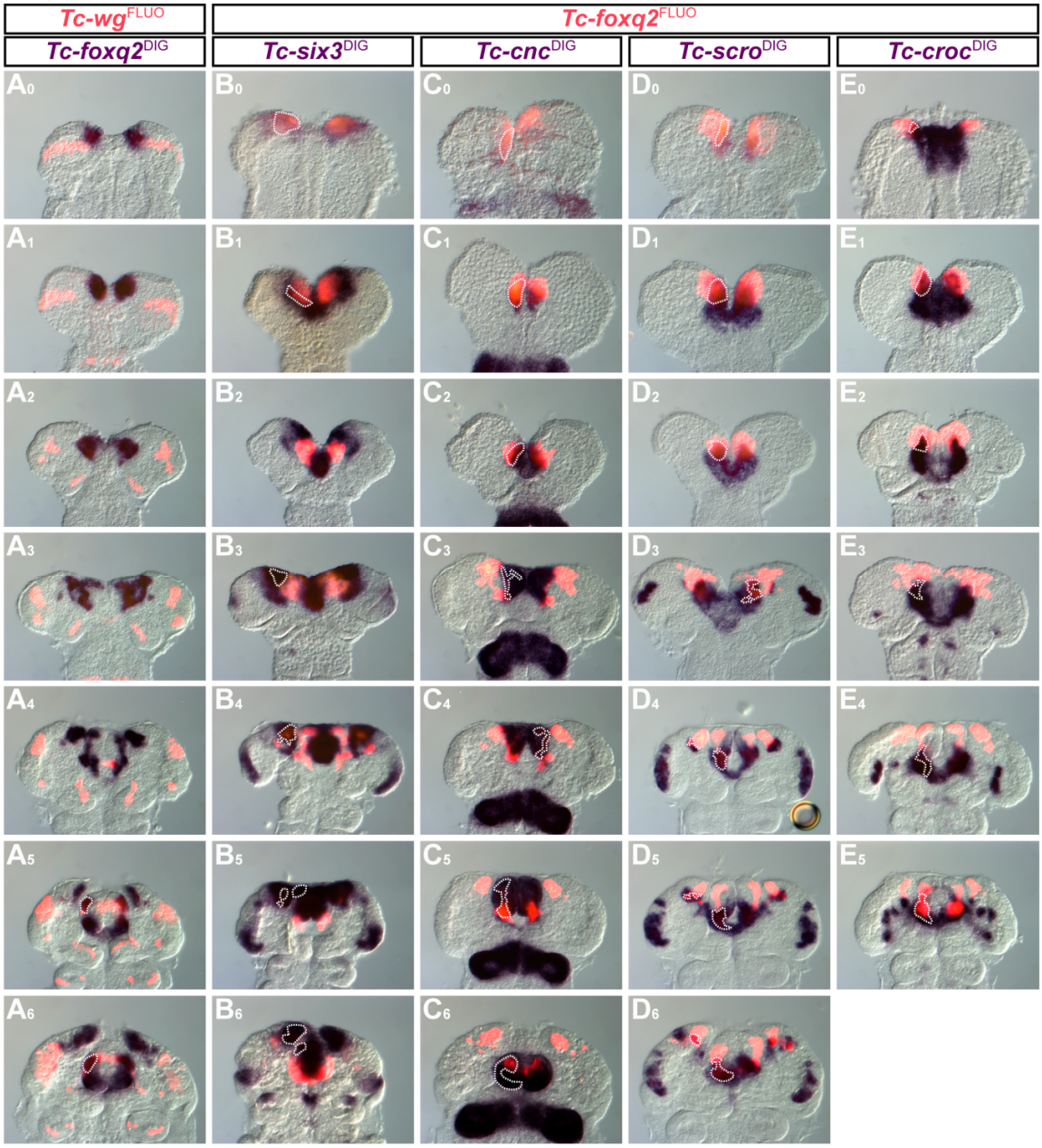


Figure 4.8 **Co-expression analyses of *Tc-foxq2* and anterior head patterning genes I.** Anterior is up. Expression is visualized by double-ISH (DISH), using NBT/BCIP (blue) and TSA-Dylight550 (red). For better comparison and potential TSA signal quenching effects by NBT/BCIP staining, *Tc-foxq2* was stained using NBT/BCIP in **A_{0-6}**. Co-expression is indicated with dashed lines. (**A_{0-6}**) Until fully elongated germ band stages no *Tc-foxq2*/*Tc-wg* co-expression is detectable (**A_{0-4}**). At later stages *Tc-foxq2* is co-expressed with *Tc-wg* in the anterior portion of the labral buds (**A_{5-6}**). (**B_{0-6}**) *Tc-foxq2* and *Tc-six3* are completely overlapping in their expression at germ rudiment stages (**B_0**). In early elongating germ bands the co-expression is limited to a narrow lateral stripe of the AMR (**B_1**). Intermediate germ bands show a mutually exclusive expression of *Tc-foxq2* and *Tc-six3*, within the AMR (**B_2**). At later stages *Tc-foxq2* and *Tc-six3* expression are overlapping within the neurogenic region (**B_{3-4}**). In early retracting germ bands and at later stages *Tc-foxq2* and *Tc-six3* expression additionally overlap in the anterior portion of the labral buds (**B_{5-6}**).

(**C_{0-6}**) *Tc-cnc* expression is almost completely covering the expression of *Tc-foxq2* at early embryonic stages (**C_{0-2}**). At later stages the co-expression is restricted to parts of the labral/stomodeal region (**C_{3-6}**). (**D_{0-6}**) *Tc-scro* is partially co-expressed with *Tc-foxq2* within the anterior part of the AMR at early embryonic stages (**D_{0-2}**). In late elongating germ bands the co-expression is restricted to a narrow lateral stripe of the AMR (**D_3**). Later stages show co-expression of *Tc-scro* and *Tc-foxq2* in the posterior portion of the labral buds, the stomodeum flanking region, and small areas of the neurogenic region (**D_{4-6}**). *Tc-croc* expression is partially overlapping with *Tc-foxq2* within antero-lateral parts of the AMR at early stages (**E_{0-2}**). At later stages of development the *Tc-foxq2*/*Tc-croc* co-expression is restricted to the stomodeum flanking region and the posterior portion of the labral buds (**E_{3-5}**).

I tested *Tc-retinal homeobox* (*Tc-rx*), a transcription factor involved in neuroectodermal development and labrum differentiation (Posnien et al., 2011b), for co-expression with *Tc-foxq2* (**Figure 4.9A$_{0-6}$**). Co-expression of *Tc-foxq2* and *Tc-rx* starts at late elongating germ band stages within parts of the neurogenic region (**Figure 4.9A$_3$**). Later stages additionally show expression overlaps in the anterior part of the labral buds (**Figure 4.9A$_{5-6}$**).

Tc-chx is known to be a transcription factor marking parts of the AMR, marking the neuroendocrine pars intercerebralis and being involved in anterior brain development (Koniszewski, 2011; Posnien et al., 2011b), therefore I tested co-expression with *Tc-foxq2* (**Figure 4.9B$_{0-5}$**). *Tc-chx*/*Tc-foxq2* co-expression starts at early stages within the AMR, and is later on located in the labral region/labral buds, and within the neuroectodermal region (**Figure 4.9B$_{0-5}$**). The partial expression overlap of *Tc-chx* and *Tc-foxq2* in the neuroectoderm is confirming that *Tc-foxq2* is also at least partially marking the pars intercerebralis.

Potential co-expression of *Tc-forkhead* (*Tc-fkh* (*foxa ortholog*)) with *Tc-foxq2* was analyzed (**Figure 4.9C$_{0-4}$**), because of its role as marker gene for the stomodeal part of the AMR, and its connection to the stomodeum formation (Kittelmann et al., 2013; Schoppmeier and Schröder, 2005). Only retracting germ bands show a small overlap of expression, flanking the stomodeum (**Figure 4.9C$_4$**).

Tc-six4 co-expression was analyzed (**Figure 4.9D$_{0-4}$**), because it is marking the insect head placode, and it is involved in proper formation of the anterior larval head (Posnien et al., 2011a). At the onset of *Tc-six4* expression, there is no overlap with the *Tc-foxq2* expression domains (**Figure 4.9D$_0$**). However, at later stages, *Tc-six4* activity is partially overlapping with *Tc-foxq2* activity at the anterior part of the neuroectoderm (**Figure 4.9D$_{1-4}$**).

In order to ask whether *Tc-foxq2* is interacting with the Notch signaling pathway, I analyzed co-expression of *Tc-foxq2* with *Tc-ser* (Figure 4.9E$_{0-4}$), and *Tc-mindbomb 1* (*Tc-mib1*; data not shown). *Tc-ser* is a ligand of *Tc-Notch* within the Notch signaling pathway and required for proper labrum formation (Siemanowski et al., 2015). The analysis revealed that there is a partial overlap of *Tc-ser* and *Tc-foxq2* within the AMR at early germ band stages (Figure 4.9E$_{0-1}$). At later stages *Tc-ser* is co-expressed with *Tc-foxq2* in the lateral part of the labral buds (Figure 4.9E$_{2-4}$).

Tc-mib1 is an E3 ubiquitin ligase of the Notch signaling pathway and required for proper labrum formation as well (Siemanowski et al., 2015). *Tc-mib1* is expressed ubiquitously throughout embryonic development (Siemanowski et al., 2015) and therefore at all embryonic stages of interest overlapping and potentially interacting with *Tc-foxq2* (data not shown).

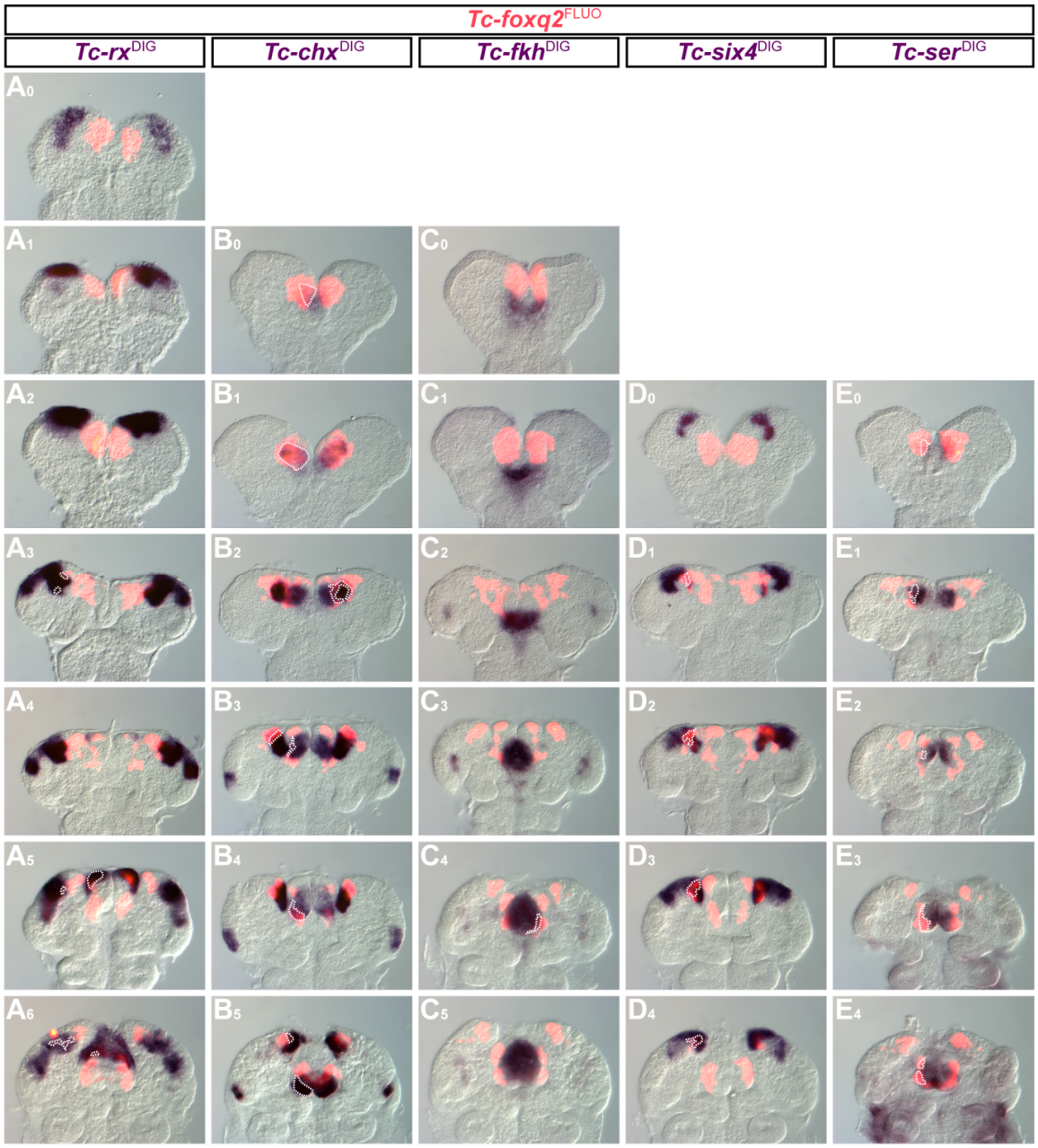


Figure 4.9 **Co-expression analyses of *Tc-foxq2* and anterior head patterning genes II.** Anterior is up. Expression is visualized by DISH, using TSA-Dylight550 (red) and NBT/BCIP (blue). Co-expression is indicated with dashed lines. (**A_{0-6}**) *Tc-rx* is not co-expressed with *Tc-foxq2* until fully elongated germ band stages (**A_{0-4}**), except for two little spots in the neurogenic region in late elongating germ bands (**A_3**). In retracting germ bands both genes are partially overlapping within the neurogenic region and in anterior parts of the labral buds (**A_{5-6}**). (**B_{0-5}**) *Tc-chx* expression is partially (**B_0**) and later almost completely (**B_1**) overlapping with *Tc-foxq2* expression. At later stages the co-expression is restricted to narrow stripes within the outer lateral labral and the neurogenic region (**B_{2-3}**). In early retracting germ bands *Tc-chx* expression shows only a little overlap within the posterior portion of the labral buds (**B_4**), and at later stages an additional overlap within the neurogenic region (**B_5**). (**C_{0-5}**) *Tc-fkh* shows almost no co-expression with *Tc-foxq2*, except for a small domain in the stomodeal region in early retracting germ bands (**C_5**). (**D_{0-4}**) *Tc-six4* is not co-expressed with *Tc-foxq2* in intermediate elongating germ bands (**D_0**). Co-expression starts in late elongating germ bands and is throughout the depicted stages restricted to a domain within the neurogenic region (**D_{1-4}**). (**E_{0-1}**) *Tc-ser* is partially co-expressed with *Tc-foxq2* within a small sub-region of the AMR at elongating germ band stages (**E_{0-1}**). (**E_{2-4}**) At later stages co-expression is restricted to outer lateral parts of the labral buds.

4.1.4.2 Knock-down of *Tc-foxq2* leads to changed expression profiles of several anterior head patterning genes

In order to gain more insights with respect to *Tc-foxq2* function within the gene regulatory network of the anterior head, I analyzed expression profiles of the previously described genes (see section 4.1.4.1) in *Tc-foxq2* knock-down embryos. Changes in their expression profile indicate a potential regulation by *Tc-foxq2*. To test this, I performed *Tc-foxq2* pRNAi and visualized the expression pattern of these genes in the embryonic offspring (6-26 h AEL), via ISH.

Only in the cnidarian *Nematostella* and the deuterostome *Strongylocentrotus* it has been described that *six3* and *foxq2* are interacting in the gene regulatory network of the apical pole (Range and Wei, 2016; Sinigaglia et al., 2013; Wei et al., 2009). In *Tribolium Tc-foxq2*^{pRNAi} germ rudiments *Tc-six3* expression was strongly reduced (Figure 4.10B, empty arrowhead). At later stages the labral/median expression domain was reduced in size (Figure 4.10D, F, H: empty arrowheads), but the neurogenic/lateral domains were affected even more (Figure 4.10D, F, H: arrows).

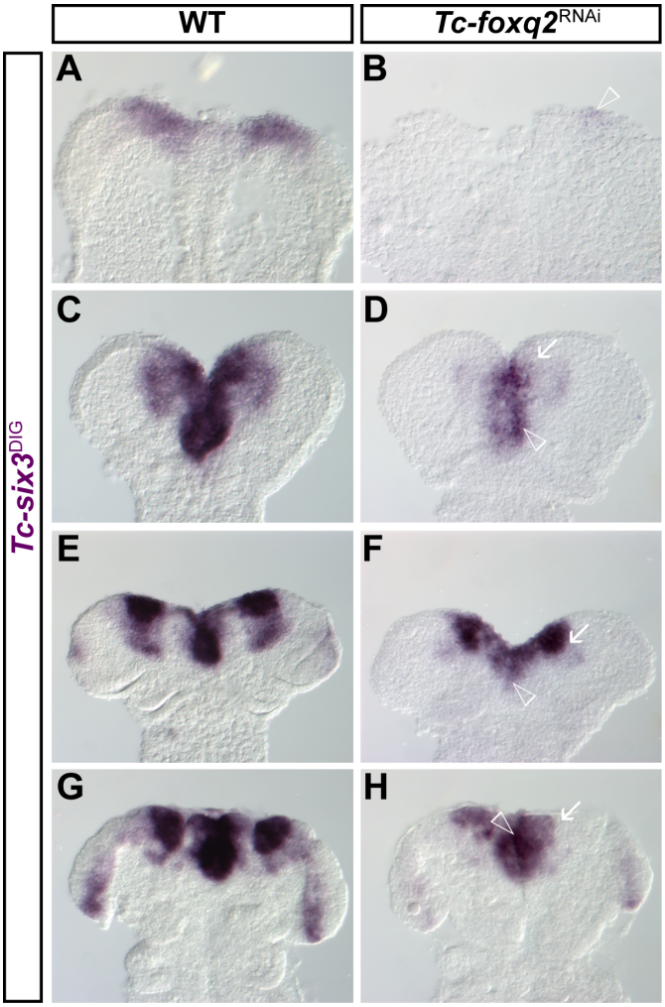


Figure 4.10 ***Tc-foxq2*^{pRNAi} embryos show reduced *Tc-six3* expression domains.** Anterior is up. Expression pattern of *Tc-six3* in wt (**A**, **C**, **E**, **G**) and *Tc-foxq2*^{pRNAi} (**B**, **D**, **F**, **H**) embryos is monitored by ISH. (**B**) *Tc-six3* expression is strongly reduced at *Tc-foxq2*^{pRNAi} germ rudiment stages (empty arrowhead). (**D**, **F**) At later stages the median (empty arrowhead) and neurogenic (arrow) *Tc-six3* expression domains are reduced in size. (**H**) In fully elongated germ bands the labral (empty arrowhead) and the neuroectodermal (arrow) aspects of expression are strongly reduced, while the posterior median domain persists. The ocular domain appears unchanged at late stages.

The anterior median expression domain of *Tc-cnc*, which marks the labrum, was strongly reduced in size after knock-down of *Tc-foxq2* (Figure 4.11B, D, F, H). Prior to early germ band stages no considerable changes in the expression pattern were observable. At early germ band stages the expression domain was almost completely vanished, except for an anterior remnant (Figure 4.11B, D: empty arrowhead). Subsequent stages showed predominantly reduced *Tc-cnc* expression within the labral buds (Figure 4.11D, F, H).

The anterior median expression domain of *Tc-croc* was reduced in size throughout the analyzed stages (Figure 4.11J, L, N, P: empty arrowhead). However, the rest of the *Tc-croc* expression profile was not considerably affected.

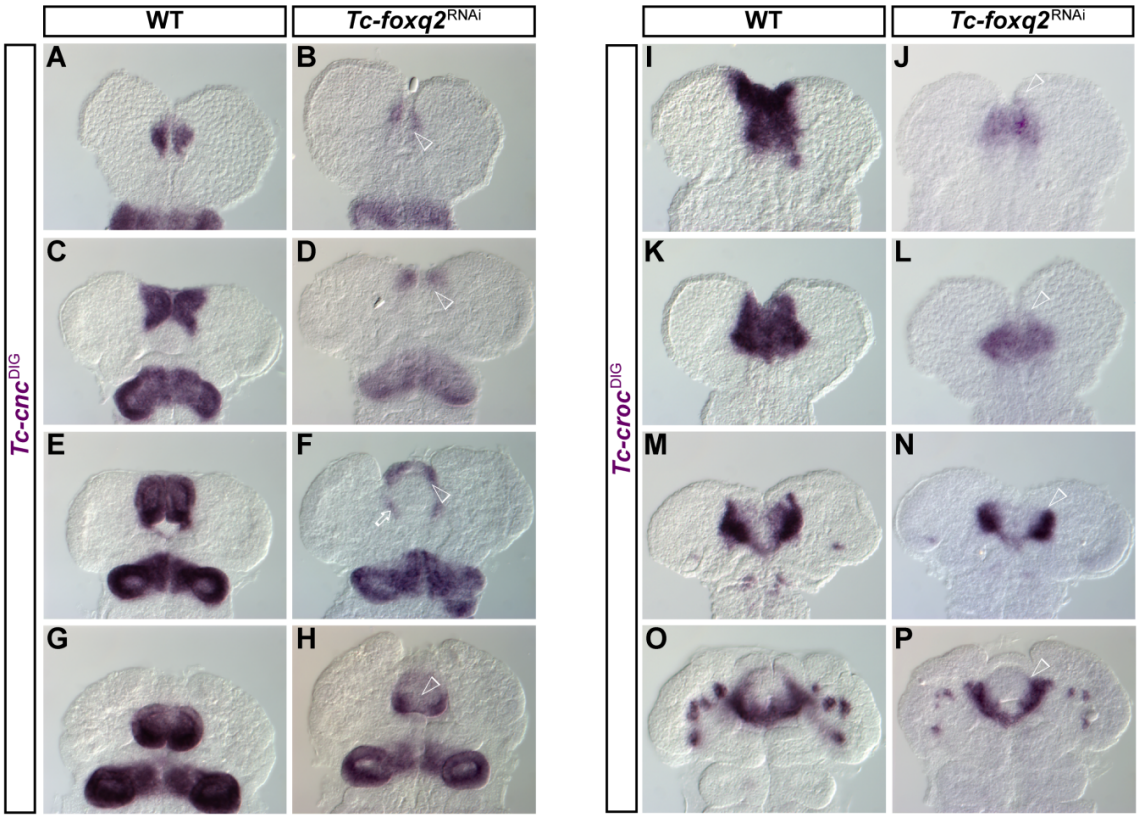


Figure 4.11 ***Tc-foxq2*^{pRNAi} embryos show reduced *Tc-cnc* and *Tc-croc* expression domains.** Anterior is up. Expression pattern of *Tc-cnc* in wt (**A**, **C**, **E**, **G**) and *Tc-foxq2*^{pRNAi} (**B**, **D**, **F**, **H**) embryos as well as expression of *Tc-croc* in wt (**I**, **K**, **M**, **O**) and *Tc-foxq2*^{pRNAi} (**J**, **L**, **N**, **P**) embryos monitored by ISH. (**B**, **D**) In *Tc-foxq2*^{pRNAi} embryos the AMR expression domain of *Tc-cnc* is reduced posteriorly during germ band elongation (empty arrowheads). (**F**) Fully elongated germ bands show reduction of expression in the labral buds as well, whereas the stomodeal expression domain appears to be only slightly decreased (empty arrow). (**H**) In retracting germ bands expression of *Tc-cnc* in the anterior and median region of the labral buds is strongly reduced (empty arrowhead). (**J**, **L**, **N**, **P**) Throughout the depicted stages, the *Tc-croc* expression pattern is lacking the anterior portion of its AMR domain (empty arrowhead).

Tc-scro expression was laterally reduced in *Tc-foxq2*^{pRNAi} early elongating germ bands (Figure 4.12B: empty arrowhead). In contrast to wt, *Tc-foxq2*^{pRNAi} embryos showed a stomodeal/labral expression domain that was connected to the expression domain in the

neurogenic tissue (Figure 4.12D, F, H: empty arrow). The ocular *Tc-scro* expression domains were not affected in *Tc-foxq2*^{pRNAi} embryos.

The expression of *Tc-fkh* was not considerably altered in *Tc-foxq2*^{pRNAi} embryos (Figure 4.12J, L, N, P).

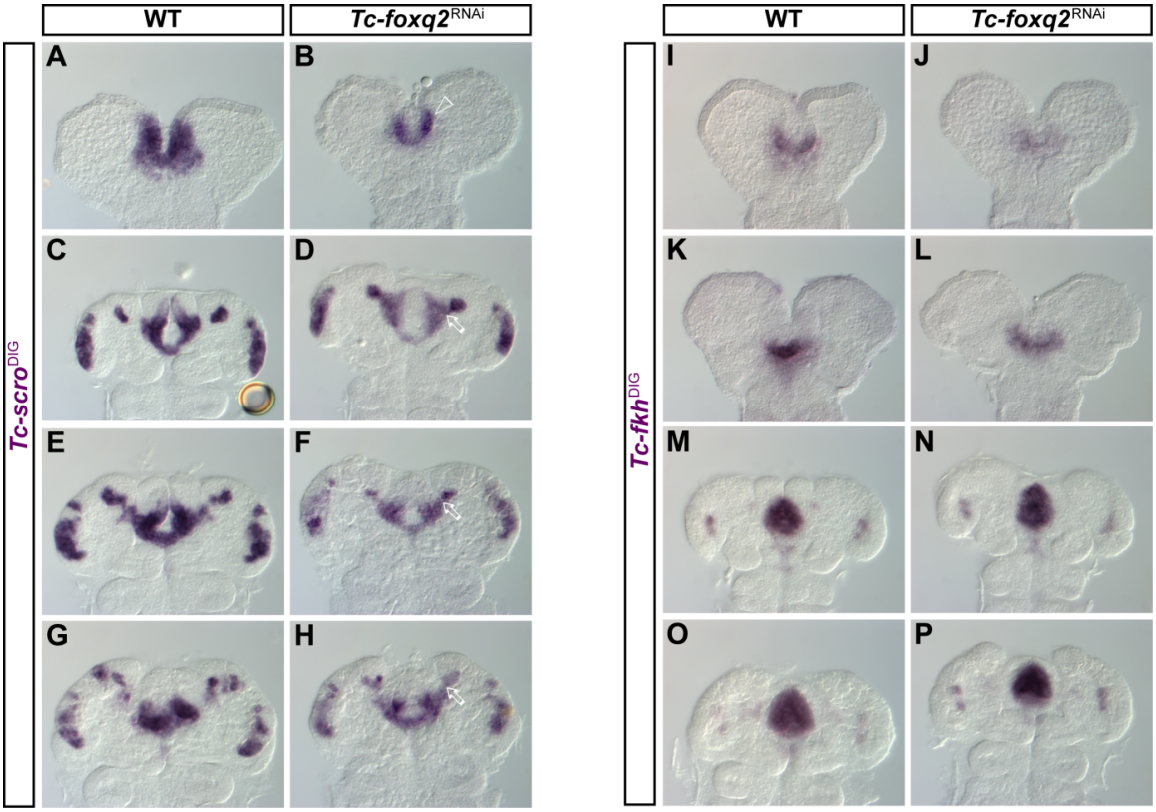


Figure 4.12 *Tc-foxq2*^{pRNAi} embryos show slightly altered *Tc-scro* and *Tc-fkh* expression profiles. Anterior is up. Expression pattern of *Tc-scro* in wt (**A**, **C**, **E**, **G**) and *Tc-foxq2*^{pRNAi} (**B**, **D**, **F**, **H**) embryos as well as expression of *Tc-fkh* in wt (**I**, **K**, **M**, **O**) and *Tc-foxq2*^{pRNAi} (**J**, **L**, **N**, **P**) embryos monitored by ISH. (**B**, **D**, **F**, **H**) Expression of *Tc-scro* is reduced to a narrow strip residing along the anterior fold (empty arrowhead) in *Tc-foxq2*^{pRNAi} embryos. Later stages show an atypical bridging between the labral/stomodeal and the neurogenic *Tc-scro* expression domains (empty arrows). (**J**, **L**, **N**, **P**) *Tc-fkh* expression is not considerably altered after *Tc-foxq2* pRNAi.

Tc-chx expression was completely lost in early elongating *Tc-foxq2*^{pRNAi} germ bands (Figure 4.13B: empty arrowhead). At later stages the expression domains in the labral buds (Figure 4.13D, F, H: empty arrowheads) and in the neurogenic region were reduced to a high degree (Figure 4.13D, F, H: arrow).

The *Tc-six4* neuroectodermal expression domain was highly reduced in early *Tc-foxq2*^{pRNAi} embryos, showing only small expression spots at the anterior rim (Figure 4.13J: arrow). Later embryonic stages showed a reduction of the inner lateral part of the *Tc-six4* expression domains (Figure 4.13L, N, P: arrows).

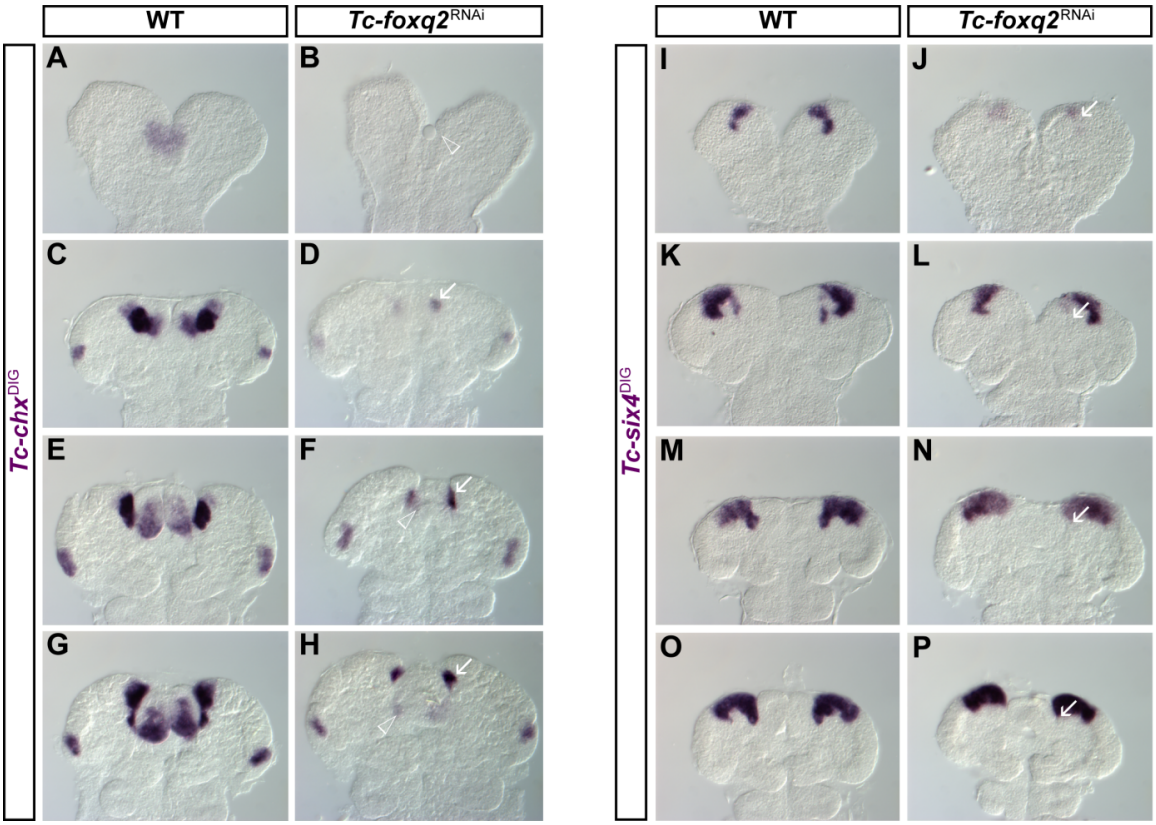


Figure 4.13 *Tc-foxq2*^{pRNAi} embryos show reduced *Tc-chx* and *Tc-six4* expression domains. Anterior is up. Expression pattern of *Tc-chx* in wt (**A**, **C**, **E**, **G**) and *Tc-foxq2*^{pRNAi} (**B**, **D**, **F**, **H**) embryos as well as expression of *Tc-six4* in wt (**I**, **K**, **M**, **O**) and *Tc-foxq2*^{pRNAi} (**J**, **L**, **N**, **P**) embryos monitored by ISH. (**B**) *Tc-chx* expression is completely absent in early elongating *Tc-foxq2*^{pRNAi} germ bands (empty arrowhead). (**D**-**H**) At later stages the labral *Tc-chx* expression domains are strongly reduced (empty arrowheads) as well as the anterior neurogenic expression domains, which show only a small leftover adjacent to the labral region (arrows). The ocular *Tc-chx* expression domains remain unaffected. (**J**) Expression of *Tc-six4* is strongly reduced in early elongating germ bands (arrow). (**L**, **N**, **P**) At later stages the inner postero-lateral parts of the neurogenic *Tc-six4* expression domains are reduced (arrows).

Tc-rx expression at early elongating germ band stages was either reduced or completely absent after *Tc-foxq2* pRNAi (Figure 4.14B). However, at later stages only the labral expression domain

of *Tc-rx* was reduced or lost (Figure 4.14D, F: empty arrowheads), but the neuroectodermal domains showed no considerable changes.

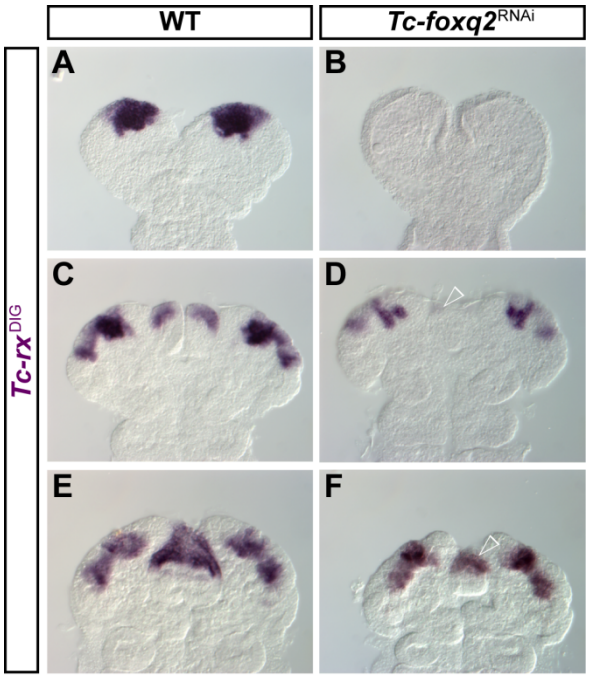


Figure 4.14 *Tc-foxq2*^{pRNAi} embryos show reduced *Tc-rx* expression domains. Anterior is up. Expression of *Tc-rx* in wt (**A**, **C**, **E**) and *Tc-foxq2*^{pRNAi} (**B**, **D**, **F**) embryos is monitored by ISH. (**B**) *Tc-rx* expression is strongly reduced or completely absent in early elongating *Tc-foxq2*^{pRNAi} germ bands. (**D**, **F**) At later stages the neurogenic *Tc-rx* expression pattern appears unaffected, but the labral expression domains are absent (**D**: empty arrowhead) or reduced in size (**F**: empty arrowhead).

Tc-foxq2^{pRNAi} embryos showed strongly reduced labral *Tc-wg* expression domains (Figure 4.15B, D: empty arrowheads). The other segmental *Tc-wg* expression domains were not considerably changed.

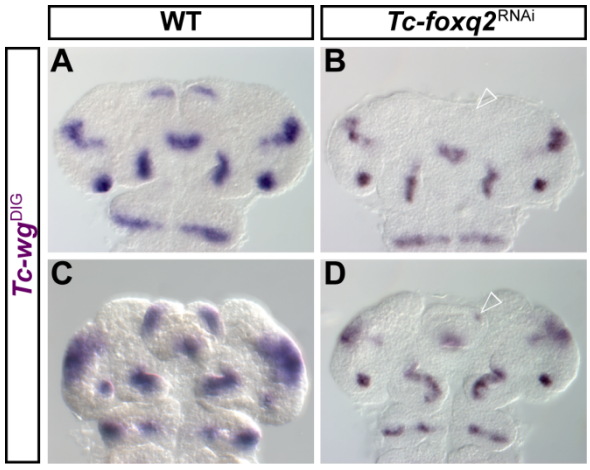


Figure 4.15 *Tc-foxq2*^{pRNAi} embryos show a reduction of the labral *Tc-wg* expression domains. Anterior is up. Expression of *Tc-wg* in wt (**A**, **C**) and *Tc-foxq2*^{pRNAi} (**B**, **C**) embryos is monitored by ISH. (**B**, **D**) *Tc-wg* expression within the labral region is completely absent (**B**: empty arrowhead) or strongly reduced (**D**: empty arrowhead), after *Tc-foxq2* pRNAi.

The expression profiles of *Tc-arr*, *Tc-ser* and *Tc-mib1* were not different comparing wt and *Tc-foxq2*^{pRNAi} embryos (data not shown).

Taken together, *Tc-foxq2* knock-down caused altered expression of several genes, which are involved in patterning of the AMR and the adjacent neurogenic region. Considering the unchanged cell death rate at these early stages and the co-expression analysis, most of the early effects of *Tc-foxq2* on these genes are presumably primary effects on gene regulation. However, the deleted expression of *Tc-rx* at early embryonic stages (**Figure 4.14B**) is an exceptional case, because of the lack of co-expression with *Tc-foxq2* at this stage (**Figure 4.9A$_{0-2}$**). This observation points to secondary effects, which could be responsible for the loss of the *Tc-rx* expression domain.

4.1.4.3 Knock-down of anterior head patterning genes altered *Tc-foxq2* expression profile

To get hints regarding the regulation of *Tc-foxq2* by other factors, within the anterior head gene regulatory network, I knocked-down the anterior head patterning genes and looked for altered *Tc-foxq2* expression. Alterations in the *Tc-foxq2* expression profile would point to a potential regulation of *Tc-foxq2* by these factors. To this end, I performed pRNAi of the head patterning genes and visualized the expression pattern of *Tc-foxq2* in embryonic offspring (6-26 h AEL) via ISH.

In *Tc-six3*^{pRNAi} embryos *Tc-foxq2* expression was completely vanished (**Figure 4.16B**, **D**, **F**, **H**), except for the posterior portion of the stomodeal *Tc-foxq2* expression domain in retracting germ bands (**Figure 4.16J**). The loss of *Tc-foxq2* expression was most likely due to a mixture of real genetic interaction and secondary effects, caused by a loss of neurogenic and anterior median tissue in *Tc-six3*^{pRNAi} embryos (Kittelmann, 2012; Posnien et al., 2011b). Based on the finding, that loss of tissue after *Tc-six3* pRNAi starts in elongating germ bands (Kittelmann, 2012), the altered *Tc-foxq2* expression was, at earlier stages, most likely caused by primary interactions.

In early elongating *Tc-croc*^{pRNAi} germ bands the bilaterally expressed *Tc-foxq2* domains were expanded towards the posterior pole and fused at the embryonic midline (**Figure 4.16L**). Earlier stages showed no considerable expression alteration. At late elongating germ band stages, the *Tc-foxq2* expression pattern was slightly misarranged and fused at the embryonic midline as well (**Figure 4.16N**). Following stages showed a deletion of the stomodeal domain (**Figure 4.16P**, **R**:

empty arrowheads) and an altered arrangement of the *Tc-foxq2* expression domains presumably caused by the previously described misplacement of the labrum (Kittelmann et al., 2013; **Figure 4.16P**, **R**: dashed arrows).

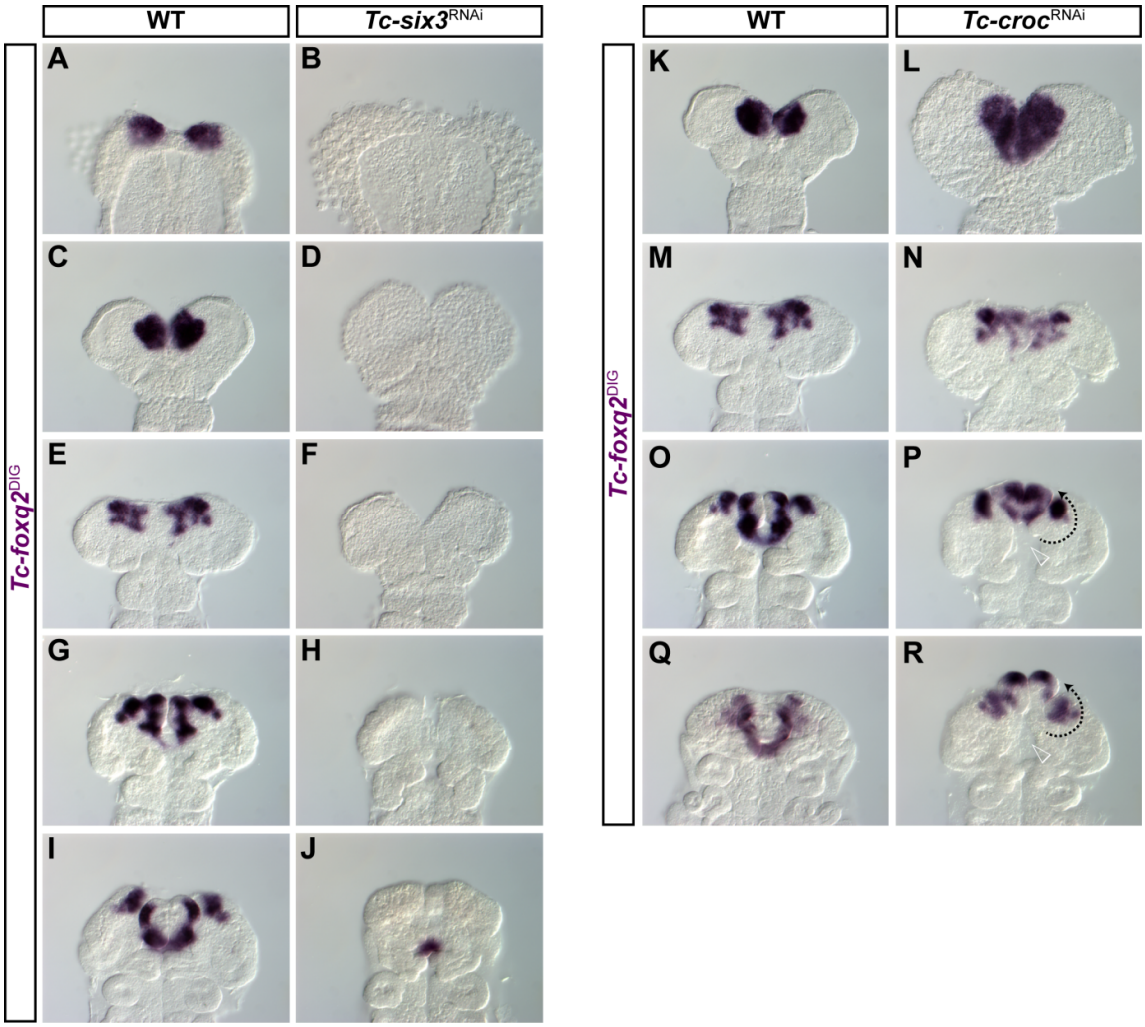


Figure 4.16 *Tc-six3*^{pRNAi} and *Tc-croc*^{pRNAi} embryos show altered *Tc-foxq2* expression profiles. Anterior is up. Expression of *Tc-foxq2* in wt (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**, **Q**), *Tc-six3*^{pRNAi} (**B**, **D**, **F**, **H**, **J**), and *Tc-croc*^{pRNAi} (**L**, **N**, **P**, **R**) embryos is monitored by ISH. (**B**, **D**, **F**, **H**) In *Tc-six3*^{pRNAi} embryos the *Tc-foxq2* expression is completely abolished. (**J**) Only the posterior part of the stomodeal *Tc-foxq2* domain remains in retracting germ bands after *Tc-six3* pRNAi. Loss of *Tc-foxq2* is most likely due to a combination of primary effects and loss of anterior tissue after *Tc-six3* pRNAi. (**L**) In early elongating germ bands *Tc-croc*^{pRNAi} embryos show a posterior expansion of *Tc-foxq2* expression. The embryo appears larger due to preparation. (**N**) Late elongating embryos show a reduced and disarranged expression pattern. (**P**, **R**) Later stages show a postero-medial deletion of *Tc-foxq2* expression (empty arrowheads). Additionally, the composition of the *Tc-foxq2* expression is altered due to a misplacement of the labral buds (indicated by dashed arrows).

The expression domains of *Tc-foxq2* at early embryonic stages were expanded laterally after *Tc-arr* pRNAi (**Figure 4.17B**, **D**, **F**). In late elongating and fully elongated *Tc-arr*^{pRNAi} embryos appear the neurogenic expression domains expanded towards posterior (**Figure 4.17H**, **J**: arrows), whereas the labral expression domains are reduced anteriorly (**Figure 4.17H**, **J**: empty arrowheads). I assume that the early effects are genetic effects due to an ectopic de-repression, because the head morphology showed no dramatic changes (**Figure 4.17B**, **D**). Also the later changes in expression pattern are presumably due to genetic effects may as well as secondary effects due to tissue loss (**Figure 4.17F**, **H**, **J**).

In early and intermediate elongating *Tc-mib1*^{pRNAi} embryos the *Tc-foxq2* expression domains appear laterally decreased in size (**Figure 4.17L**, **N**: empty arrowheads). Prior to this stage expression was not altered considerably. At following stages the neurogenic *Tc-foxq2* expression domains (**Figure 4.17P**, **T**: arrows) as well as the anterior portion of the labral bud expression domain were reduced (**Figure 4.17P**, **R**, **T**: empty arrowheads). Disturbance of *Tc-foxq2* expression caused by *Tc-mib1* pRNAi is most likely based on a combination of genetic interaction and secondary effects. The early defects are considered being primary effects. However, for these stages it is unknown whether head tissue is lost after *Tc-mib1* pRNAi treatment. Loss of the *Tc-foxq2* expression domain in the labral bud is due to the absence of the labral tissue caused by decreased cell proliferation rates (Siemanowski et al., 2015). The changes in the neuroectoderm are considered as genetic interaction of *Tc-mib1* on *Tc-foxq2*, because the tissue of this region is not affected upon *Tc-mib1* pRNAi (Siemanowski et al., 2015).

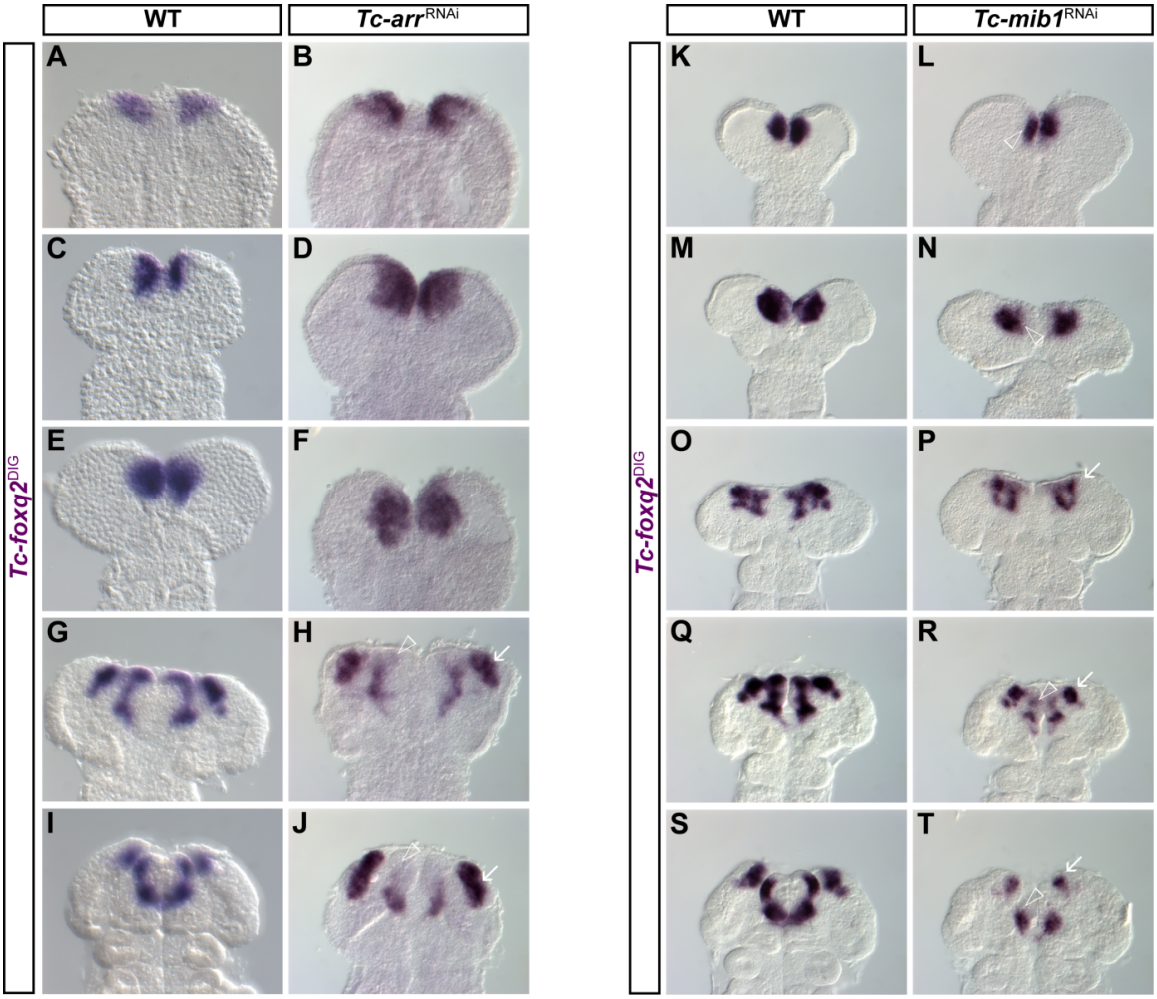


Figure 4.17 *Tc-arr*^{pRNAi} and *Tc-mib1*^{pRNAi} embryos show altered *Tc-foxq2* expression profiles. Anterior is up. Expression of *Tc-foxq2* in wt (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**, **Q**, **S**), *Tc-arr*^{pRNAi} (**B**, **D**, **F**, **H**, **J**), and *Tc-mib1*^{pRNAi} (**L**, **N**, **P**, **R**, **T**) embryos monitored by ISH. (**B**, **D**, **F**) Early elongating *Tc-arr*^{pRNAi} embryos show expanded *Tc-foxq2* expression domains. (**H**, **J**) At later stages, the neurogenic *Tc-foxq2* expression domains are posteriorly expanded (arrows), whereas the anterior portion of the labral bud expression domain is deleted (empty arrowhead). (**L**, **N**) Early elongating *Tc-mib1*^{pRNAi} embryos show medially reduced *Tc-foxq2* expression domains (empty arrowhead). (**P**) In late elongating *Tc-mib1*^{pRNAi} embryos is the neurogenic part of the *Tc-foxq2* expression reduced (arrow). (**R**, **T**) Later stages show a reduction of the neurogenic expression domains and a reduction of the anterior portion of the labral bud domains (empty arrowhead). The late effect, in the labral buds (**R**, **T**), is most likely due to a loss of tissue caused by *Tc-mib1* pRNAi.

Late *Tc-scro*^{pRNAi} germ band stages showed a reduced and misarranged *Tc-foxq2* expression pattern (Figure 4.18B, D: empty arrowheads). Earlier stages showed no considerable alteration of the expression profile. In retracting *Tc-scro*^{pRNAi} embryos the *Tc-foxq2* expression domain

within the labral buds seemed to be altered in its shape and in its composition, caused by malformation of the labrum (Posnien et al., 2011b; **Figure 4.18F**: white arrowhead).

Tc-cnc^{pRNAi} embryos showed a complete loss of the *Tc-foxq2* expression domains within the labral buds (**Figure 4.18H**, **J**, **L**: arrowheads). This loss of *Tc-foxq2* expression is due to the absence of labral tissue, caused by *Tc-cnc* pRNAi (Kittelmann et al., 2013). Prior to late elongating germ band stages expression was not altered considerably.

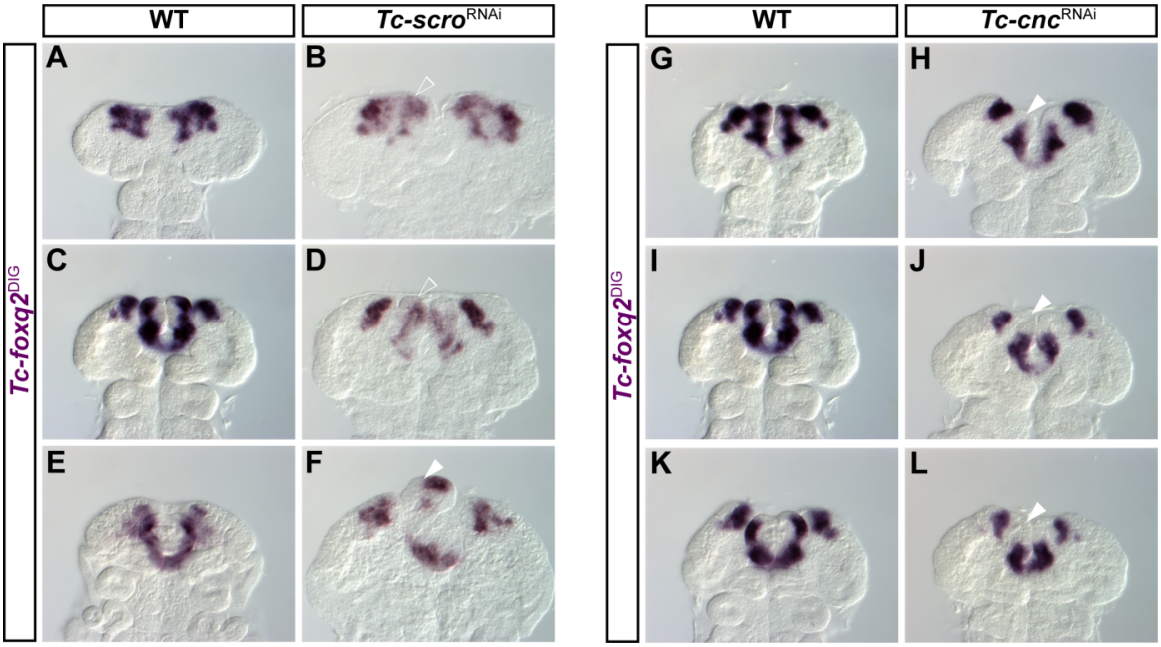


Figure 4.18 *Tc-scro*^{pRNAi} and *Tc-cnc*^{pRNAi} lead to a changed *Tc-foxq2* expression profile at late stages only. Anterior is up. Expression of *Tc-foxq2* in wt (**A**, **C**, **E**, **G**, **I**, **K**), *Tc-scro*^{pRNAi} (**B**, **D**, **F**), and *Tc-cnc*^{pRNAi} (**H**, **J**, **L**) embryos is monitored by ISH. (**B**) *Tc-scro*^{pRNAi} embryos show a slightly distorted *Tc-foxq2* expression pattern at late elongating germ band stages within the prospective labral/stomodeal region (empty arrowhead). (**D**) Fully elongated germ bands show a residual labral *Tc-foxq2* expression domain, which appears to be misplaced (empty arrowhead). (**F**) Retracting *Tc-scro*^{pRNAi} germ bands show a *Tc-foxq2* expression that appears to be altered mainly due to a disarrangement of the labral buds (arrowhead). (**H**, **J**, **L**) Late *Tc-cnc*^{pRNAi} embryos show no *Tc-foxq2* expression within the labral buds, caused by an RNAi-induced loss of tissue (arrowhead).

Embryos prior to late elongating germ band stages showed no considerable expression alteration. *Tc-six4*^{pRNAi} embryos appear to show a slight reduction of the *Tc-foxq2* expression domains within the neurogenic region (**Figure 4.19B**, **D**, **F**, **H**, **J**: arrows). It is known that

Tc-six4^{pRNAi} leads to slight malformations of the anterior L1 larval head (Posnien et al., 2011a), however the embryonic origin of this phenotype is unknown. Based on the overall intact head morphology, this effect is unlikely to be secondary.

Also *Tc-chx*^{pRNAi} had only an effect on the neurogenic *Tc-foxq2* expression domains, from late elongating germ band stages onwards. However, in this case the neurogenic expression domain was expanded, which resulted in a bridging between the neurogenic and the stomodeal *Tc-foxq2* expression domains (**Figure 4.19L**, **N**, **P**: arrows). The observed effect was most likely due to a de-repression of *Tc-foxq2* activity, after *Tc-chx*^{pRNAi}. Secondary effects are less likely due to the fact that *Tc-chx* pRNAi is not associated with epidermal defects and causes only a loss of the central body in the L1 larval brain (Koniszewski, 2011).

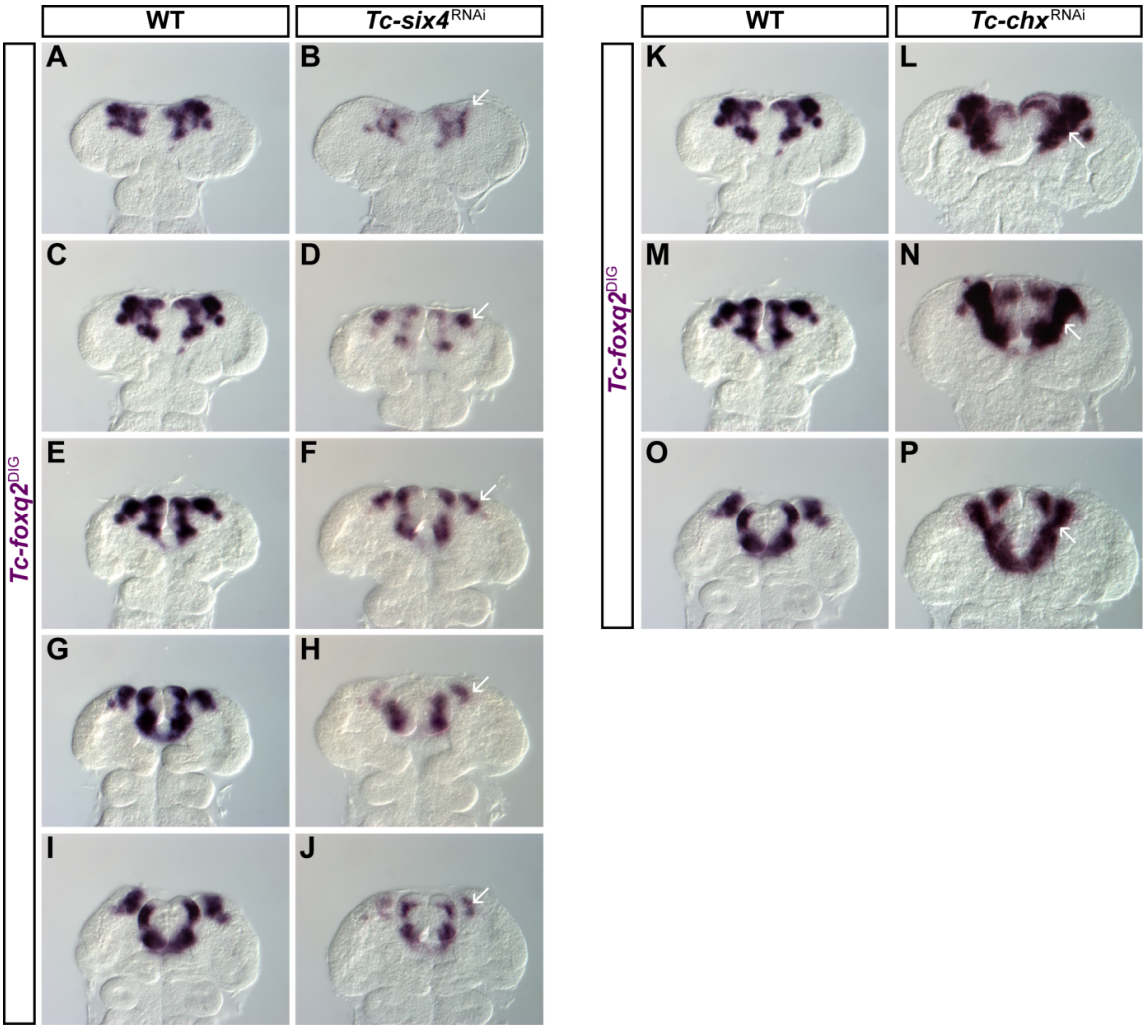


Figure 4.19 *Tc-six4*^{pRNAi} and *Tc-chx*^{pRNAi} embryos show an altered *Tc-foxq2* expression profile at late stages. Anterior is up. Expression of *Tc-foxq2* in wt (**A**, **C**, **E**, **G**, **I**, **K**, **M**, **O**), *Tc-six4*^{pRNAi} (**B**, **D**, **F**, **H**, **J**), and *Tc-chx*^{pRNAi} (**L**, **N**, **P**) embryos is monitored by ISH. (**B**, **D**, **F**, **H**, **J**) *Tc-six4*^{pRNAi} embryos show a reduction of the neurogenic *Tc-foxq2* expression domains (arrows). (**L**, **N**, **P**) Late elongating to retracting *Tc-chx*^{pRNAi} germ bands show an expansion of the neurogenic *Tc-foxq2* expression domains (arrows), which leads to a fusion with the stomodeal expression domain.

The expression pattern of *Tc-foxq2* at late elongating *Tc-fkh*^{pRNAi} germ band stages was misarranged, caused by a turning-in of the head lobes towards the midline (Figure 4.20B, C, E, F). The misarranged *Tc-foxq2* expression domains in *Tc-fkh*^{pRNAi} embryos were most likely caused by a malformation of the tissue. Although it has been reported for *Tc-fkh* to have only an embryonic function in hindgut development (Schoppmeier and Schröder, 2005) the embryos appeared to have also defects in the stomodeal tissue.

Tc-rx^{pRNAi} embryos showed no considerable change of the *Tc-foxq2* expression pattern (Figure 4.20H, J, L).

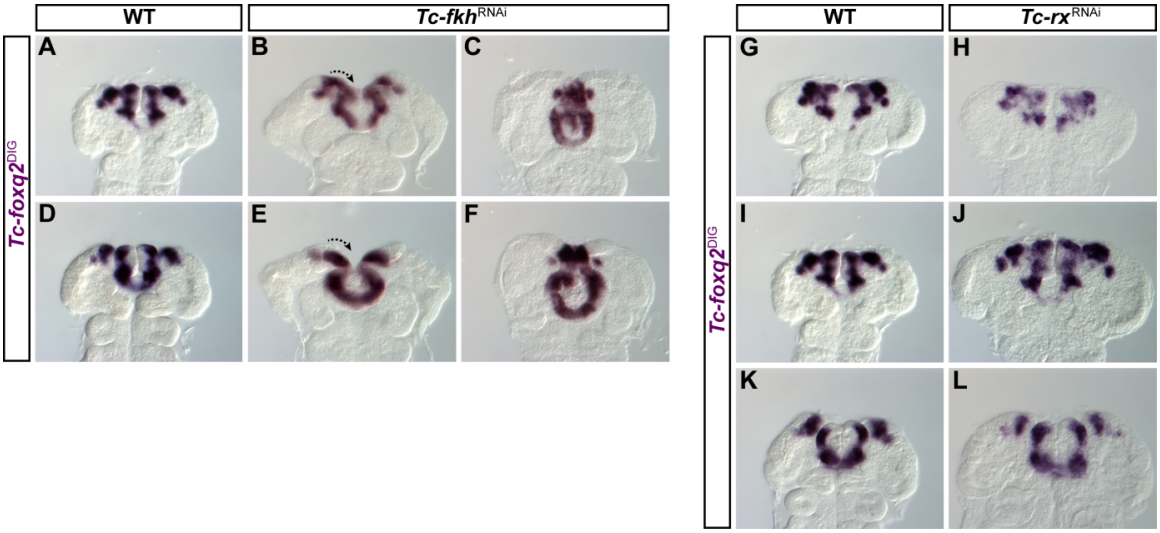


Figure 4.20 *Tc-fkh*^{pRNAi} embryos show a secondary alteration of *Tc-foxq2* expression, whereas *Tc-rx*^{pRNAi} embryos show no considerable change in *Tc-foxq2* expression profile. Anterior is up. Expression of *Tc-foxq2* in wt (**A**, **D**, **G**, **I**, **K**), *Tc-fkh*^{pRNAi} (**B**, **C**, **E**, **F**) and *Tc-rx*^{pRNAi} (**H**, **J**, **L**) embryos is monitored by ISH. (**B**, **C**, **E**, **F**) Fully elongated *Tc-fkh*^{pRNAi} embryos show an altered *Tc-foxq2* expression pattern. Change of the expression pattern is probably a secondary effect and caused by an intermediate (**B**, **E**) or strong (**C**, **F**) turning of the antero-lateral head tissue towards the embryonic midline (**B**, **E**: turning indicated by dashed arrows). (**H**, **J**, **L**) In *Tc-rx*^{pRNAi} embryos no considerable change of the *Tc-foxq2* expression pattern is detectable.

4.1.5 *Tc-foxq2* gain-of-function analysis

The knock-down experiments provided already some information about the function of *Tc-foxq2* and its role within the gene regulatory network of the anterior pre-segmental head. In order to substantiate these findings and to gain more information about *Tc-foxq2*, I exploited the heat shock system to drive ectopic *Tc-foxq2* expression (Lindquist, 1986; Schinko et al., 2012). To this end, I generated a construct, which ubiquitously drives the expression of *Tc-foxq2* (full coding sequence) under the control of the *Tc-heat shock protein 68* promoter (*hsp68*; Figure 4.21).

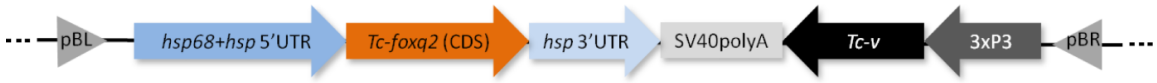


Figure 4.21 **Transgenesis construct for heat shock-inducible *Tc-foxq2* misexpression lines.** pBL and pBR: piggyBac sites for transposon based integration, *hsp68*+hsp 5'UTR: *heat shock protein 68* promoter + *heat shock protein* 5' untranslated region, *Tc-foxq2* (CDS): full coding sequence of *Tc-foxq2*, *hsp* 3'UTR: *heat shock protein* 3' untranslated region, SV40polyA: polyadenylation signal, *Tc-v*: *Tc-vermillion* (Transgenesis marker), 3xP3: eye-specific promoter.

4.1.5.1 Ectopic expression of *Tc-foxq2* - Proof of principle

In order to test whether the generated transgenic lines were functional, I performed a heat shock treatment with embryos (0-24 h AEL) of all independent transgenic lines, fixated them after 30 min, and visualized the *Tc-foxq2* expression via ISH. This control experiment showed that all independent transgenic lines (n=8) were functional, but the degree of ectopic expression was highly variable. Some lines showed a very patchy expression pattern, with only a few *Tc-foxq2* expressing cells (Figure 4.22B-B```). In contrast, other lines showed a spotty but an equally distributed *Tc-foxq2* expression throughout the embryo (Figure 4.22C-D```). It has to be noted that all lines showed ectopic expression, while the actual *Tc-foxq2* expression domains appeared not to be increased in their signal. For further analysis I used the transgenic line *hsp68-Tc-foxq2*$_{w5}$, which showed the most even signal distribution.

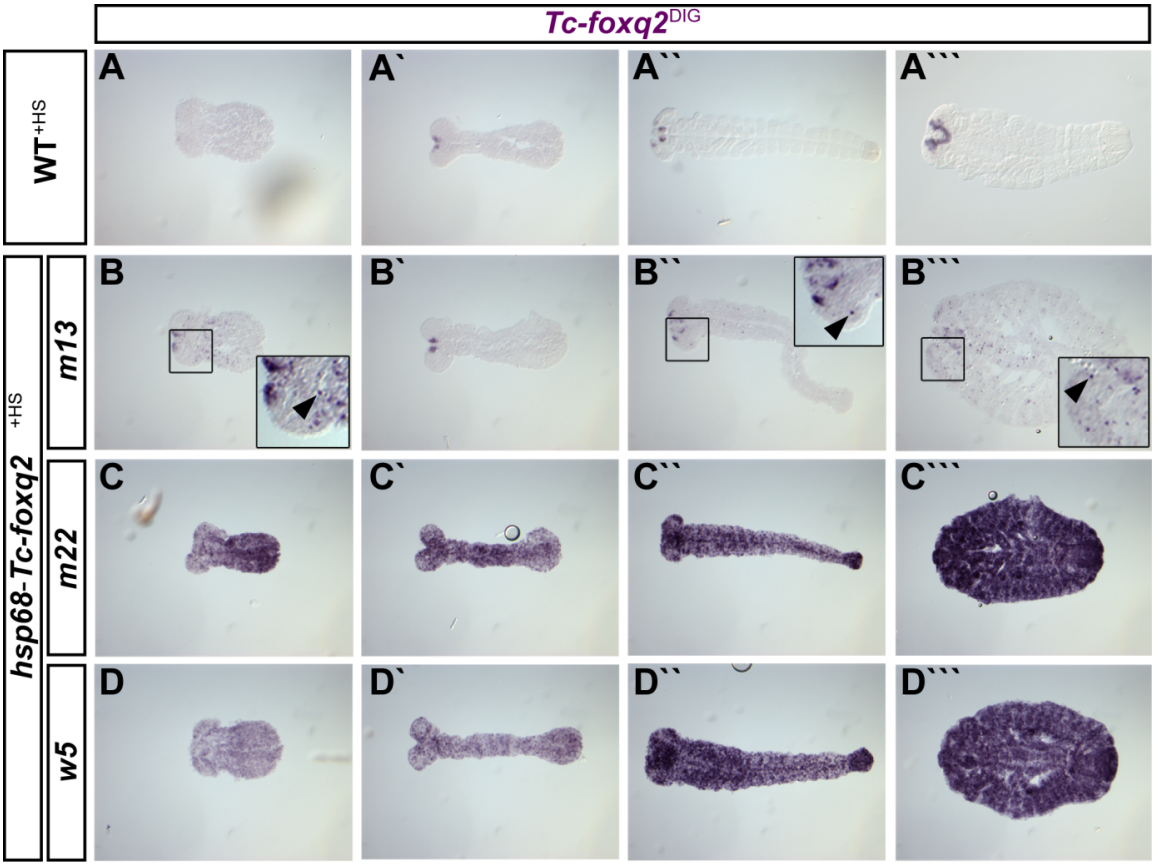


Figure 4.22 ***Tc-foxq2* gain-of-function lines show heat shock-induced ectopic *Tc-foxq2* expression.** Anterior is left. Expression of *Tc-foxq2* in heat shock-treated wt (**A**-**A**```), *hsp68-Tc-foxq2*$_{m13}$ (**B**-**B**```), *hsp68-Tc-foxq2*$_{m22}$ (**C**-**C**```), and *hsp68-Tc-foxq2*$_{w5}$ (**D**-**D**```) embryos is monitored by ISH. (**A**-**A**```) Wt embryos show no change in the *Tc-foxq2* expression pattern upon heat shock treatment. (**B**-**B**```) Individuals of the transgenic line *hsp68-Tc-foxq2*$_{m13}$ show ectopic *Tc-foxq2* expression in some cells that are scattered sparsely across embryo (boxes, arrowheads). (**C**-**D**```) Individuals of the transgenic lines *hsp68-Tc-foxq2*$_{m22}$ and *hsp68-Tc-foxq2*$_{w5}$ show a strong activation of ectopic *Tc-foxq2* expression throughout the embryo. For the following experiments the line *hsp68-Tc-foxq2*$_{w5}$ was used, because of the more even distribution of ectopic *Tc-foxq2* expression (**D**-**D**```).

4.1.5.2 Analysis of the epidermal *Tc-foxq2* gain-of-function phenotype

4.1.5.2.1 Ectopic *Tc-foxq2* expression resulted in a pleiotropic but specific epidermal L1 phenotype

To gain more insights about *Tc-foxq2* function, I was interested in the epidermal phenotype after ectopic expression of *Tc-foxq2* at early embryonic stages. For this gain-of-function experiment, I

collected and heat shocked embryos (0-24 h AEL) from the *hsp68-Tc-foxq2* line. After four days, I analyzed the L1 larval cuticles.

All of the analyzed cuticles showed various defects. However, two distinct classes of phenotypes were observed consistently. The first class showed defects in all three tagmata (**Figure 4.23B**, **B`**). The head capsule and the gnathal appendages appeared to be unaffected. However, the antennal flagellum was completely absent in almost all cuticles of this class and at times the head bristle pattern was partially disrupted (**Figure 4.23B`**: left panel). The legs showed a reduced number of podomeres, probably lacking the femur and tibia (**Figure 4.23B`**: mid panel). The abdomen lacked a variable number of segments and the remaining segments were dorsally fused (**Figure 4.23B`**: right panel). The urogomphi and pygopods were frequently affected (**Figure 4.23B`**: right panel). The second class of phenotypes showed defects that were restricted to the pre-abdominal region (**Figure 4.23D**, **D`**). Most of the cuticles showed defects in the head capsule, the head appendages, and very often defects in the head bristle pattern. (**Figure 4.23D-D``**: left panel). Notably, these defects were found in regions where *Tc-foxq2* pRNAi leads to loss of structures (e.g. clypeus bristle and anterior vertex setae). The legs showed the same defects as described above (**Figure 4.23D``**: right panel). Taken together, these results show that the phenotype is characterized by several defects affecting several epidermal structures, but that within this variation two different classes of phenotypes with specific sets of defects are recurring. The two different phenotype classes show a subdivision with the emphasis on more posterior (Class I) versus more anterior defects (Class II). This may reflect different phenotypic outcomes resulting from different timing of the ectopic *Tc-foxq2* expression.

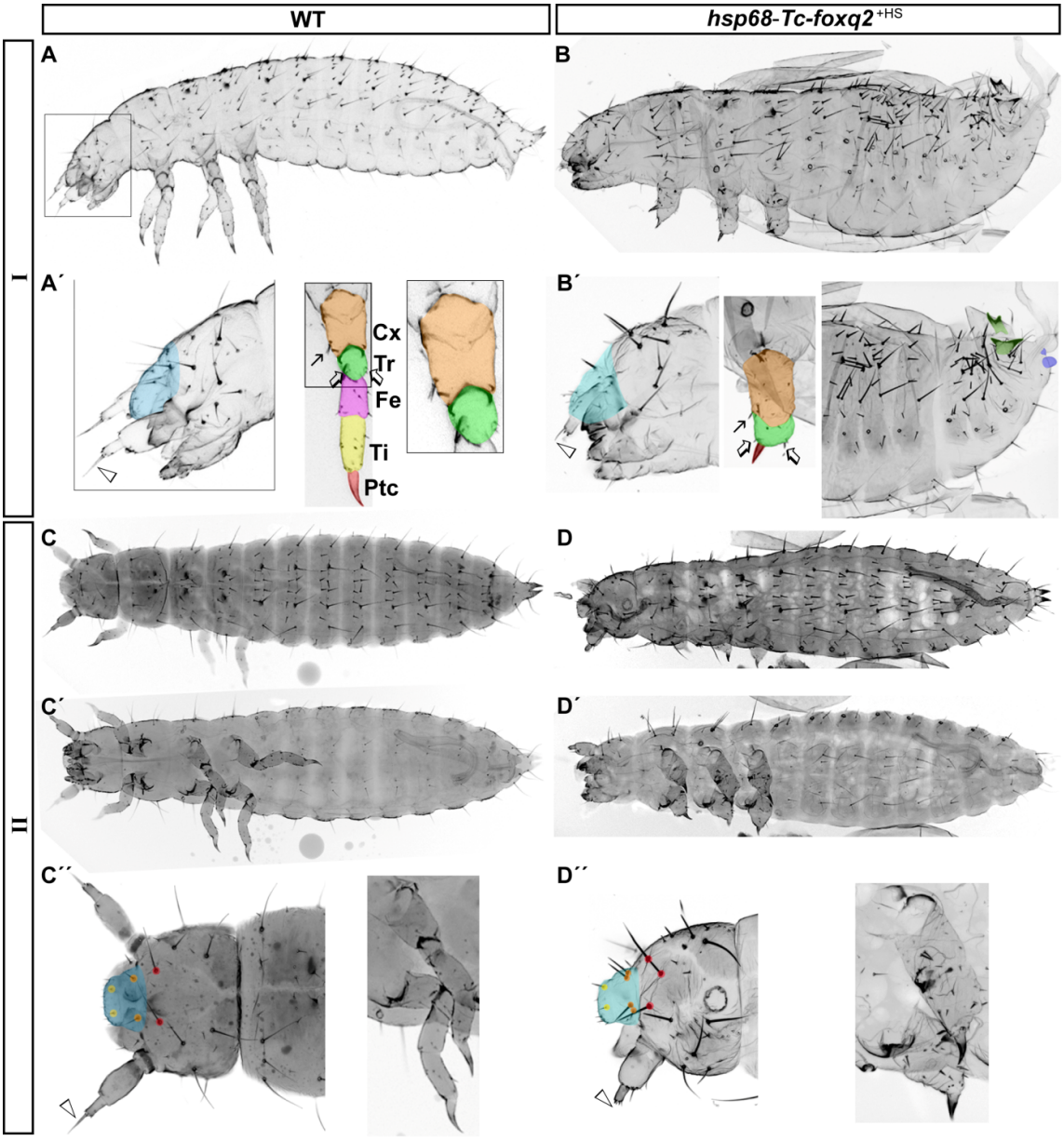


Figure 4.23 **Embryonic *Tc-foxq2* gain-of-function results in defects in L1 larval cuticles.** Anterior is left. Wt (**A**, **A`**, **C**-**C``**) and heat shock-treated *hsp68-Tc-foxq2* (**B**, **B`**, **D**-**D``**) L1 larval cuticles, grouped into two classes (I and II). (**B**, **B`**) L1 cuticles of the phenotype class I lack the antennal flagellum (**B`**, left panel: arrowhead) and a slightly disrupted bristle pattern. The legs (middle panel) have a reduced number of podomeres. Presumably only the coxa (orange), trochanter (green) and the pre-tarsal claw (red) are left (compare bristles marked by arrows in **A`** and **B`**). Abdominal segments (right panel) are reduced in number and remaining segments are dorsally fused. The urogomphi (green, duplicated in this specimen) and pygopods (blue, reduced in this specimen) are sometimes affected. (**D**-**D`**) L1 cuticles of the phenotype class II show defects restricted to the head and thoracic region. (**D``**) A larval head (left panel) of the phenotype class II lacking an antennal flagellum (arrowhead) and showing affected head appendages. The head bristle pattern is disrupted and frequently shows a duplication of the clypeus setae

(orange dots) and the anterior vertex setae (red dots). The legs (right panel) show comparable defects as the legs of the first phenotype class. Cx: coxa, Tr: trochanter, Fe: femur, Ti: tibia, Ptc: pre-tarsal claw

4.1.5.2.2 Epidermal phenotype - Separating late and early *Tc-foxq2* function

Based on the finding that the *Tc-foxq2* misexpression experiment results in distinct classes of epidermal L1 phenotypes, I tried to determine whether these differences are related to a late versus early *Tc-foxq2* function during development. Trying to separate these aspects, I collected embryos from the *hsp68-Tc-foxq2* line and grouped them in three different cohorts according to the timing of heat shock treatment (9-13 h AEL, 14-20 h AEL, and 20-25 h AEL). Subsequently, I collected and analyzed the L1 larval cuticles (exact percentages are listed in **Table S7.9-.14**).

The three different cohorts (**1**: 9-13 h AEL, germ rudiment stage in **Figure 4.5C:** stage 1; **2**: 14-20 h AEL, elongating germ band stages in **Figure 4.5C:** stage 2-3; **3**: 20-25 h AEL, fully elongated germ band stages in **Figure 4.5C:** stage 4-5) showed no drastic phenotypic differences (**Figure 4.24A-C**). Analyzing the cuticles with regard to their particular defects, it turned out that the first cohort (n=42, **Figure 4.24A`**) often had severe defects within the head region, showing a loss of gnathal appendages (Md: 63.1%, Mx: 64.3%, Lb: 76.2%) and a disrupted head bristle pattern. This cohort also showed a high penetrance in more posterior defects (leg pair 1-3: 82.2%, and abdominal segment 1-8: 48.1%). The second cohort (n=122, **Figure 4.24B`**) showed minor defects in the head region, especially the gnathal appendages were only rarely affected (Md: 0.8%, Mx: 0.8%, Lb: 1.6%). More posterior regions were frequently affected within this cohort (Leg pair 1-3: 65.6%, abdominal segment 1-8: 95.9%). The last cohort (n=132, **Figure 4.24B`**) showed comparable amounts of defects in the gnathocephalic region (Md: 2.9%, Mx: 4.9%, Lb: 5.0%), less frequent leg defects (Leg pair 1-3: 44.2%) and less severe and frequent abdominal defects (Abdominal segment 1-8: 89.7%). These data indicate that the timing of ectopic *Tc-foxq2* expression leads to different phenotypes. Due to my interest in the head phenotype, I decided to focus on the early cohort for subsequent experiments.

In order to get an impression of the basis of the cuticle phenotype, I tested whether the phenotype resulted from an increased cell death rate. To this end, I collected eggs of the *hsp68-Tc-foxq2* line and applied a heat shock treatment (9-13 h AEL), fixed the heat shocked embryos (14-18 h AEL), and performed a Dcp-1 antibody staining to mark apoptotic cells. Finally I investigated qualitative differences between wt and *Tc-foxq2* gain-of-function embryos. Heat shock-treated wt embryos showed a slight increase in the number of apoptotic cells compared

to untreated embryos (data not shown). *Tc-foxq2* gain-of-function embryos showed a dramatically increased number of apoptotic cells when compared to heat shock-treated wt embryos (Figure 4.25A-F`). The apoptotic cells were distributed all over the embryo independent of the analyzed stage. This result suggests that the increased cell death rate is one factor for the pleiotropic larval cuticle phenotype caused by ectopic *Tc-foxq2* expression.

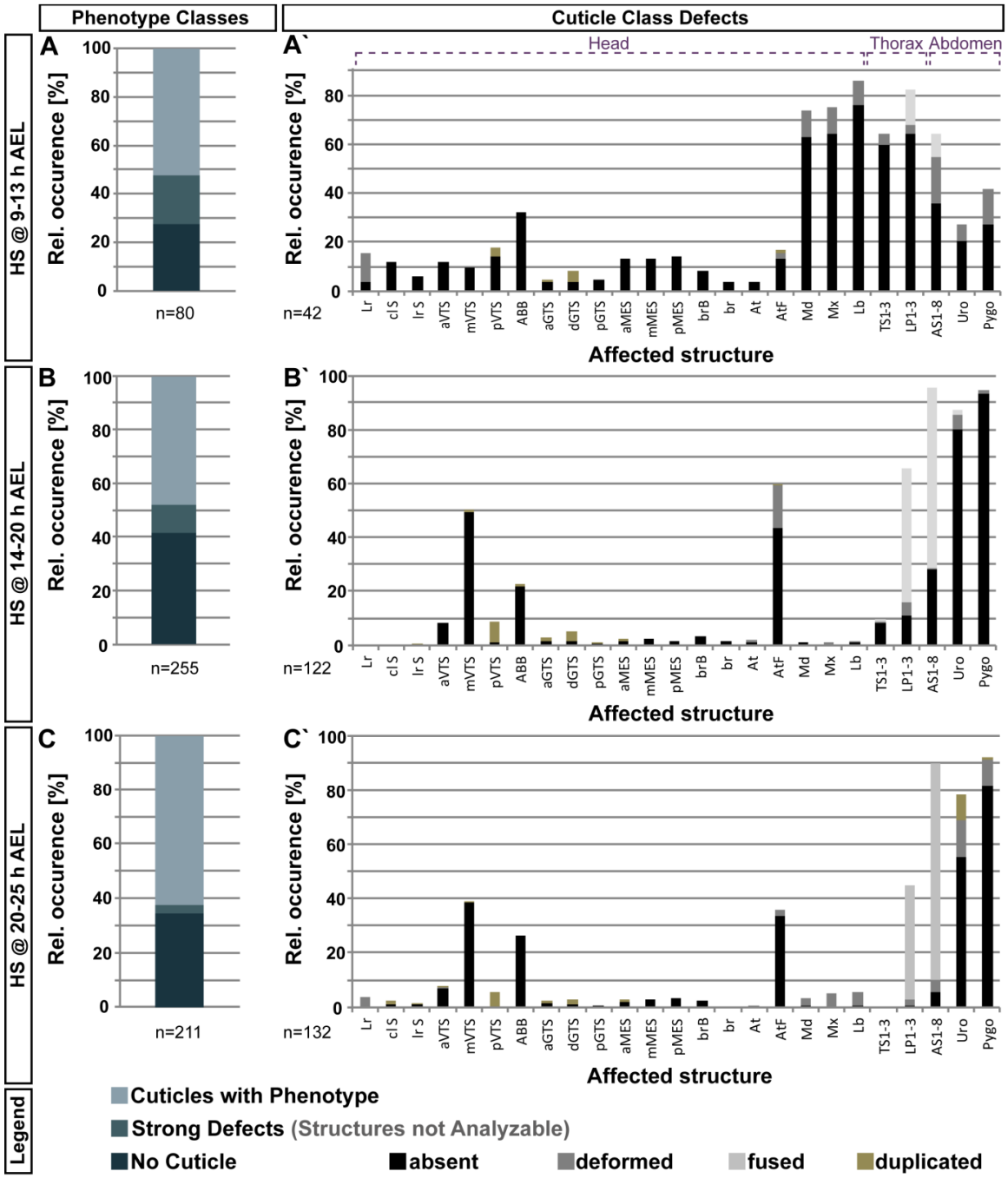


Figure 4.24 Larval epidermal defects after different onsets of embryonic *Tc-foxq2* gain-of-function. Relative occurrence of eggs without cuticle (dark blue), cuticles with strong defects (blue), and cuticles with an analyzable phenotype (light blue; **A**-**C**) and the defects present in the 'cuticle class' (**A`**-**C`**), resulting from heat shock treatment of *hsp68-Tc-foxq2* embryos at different stages. (**A**-**C**) The penetrance of eggs without cuticle (no cuticle) is highest within the second cohort, where the portion of analyzable cuticles is the lowest (**B**). (**A`**) Ectopic *Tc-foxq2* expression in 9-13 h AEL old embryos leads to cuticles that show defects in all three body parts frequently, with the strongest defects in the gnathal appendages. (**B`**) Heat shock-induced *Tc-foxq2* gain-of-function in 14-20 h AEL old embryos leads to minor head defects, showing a loss of the antennal flagellum or a disrupted bristle pattern. The thoracic structures are frequently but mostly slightly affected. Predominant are post-cephalic defects. (**C`**) Heat shock-induced *Tc-foxq2* gain-of-function in 20-25 h AEL old embryos leads to cuticles that show the fewest and weakest defects in all three body parts. Abdominal defects are predominant. Lr: labrum, cl S: clypeus setae, lr S: labrum setae, aVTS: anterior vertex setae, mVTS: median vertex setae, pVTS: posterior vertex setae, ABB: antenna basis bristles, aGTS: anterior gena triplet setae, dGTS: dorsal gena triplet setae, pGTS: posterior gena triplet setae, aMES: anterior maxilla escort setae, mMES: median maxilla escort setae, pMES: posterior maxilla escort setae, brB: bell row bristles, br: bell rows, At: antennae, AtF: antennal flagella, Md: mandibles, Mx: maxillae, Lb: labium, TS1-3: thoracic segments 1-3, LP1-3: leg pair 1-3, AS1-8: abdominal segments 1-8, Uro: urogomphi, Pygo: pygopods

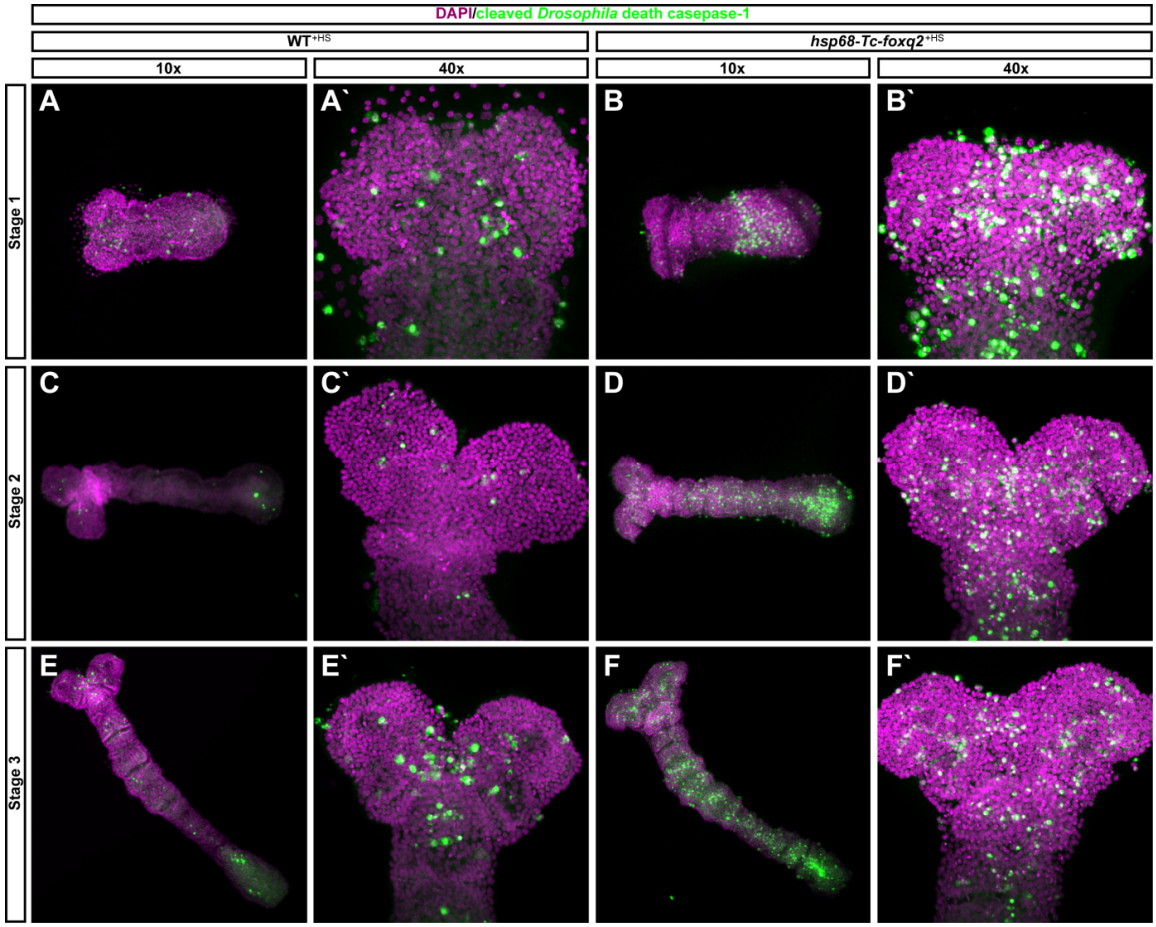


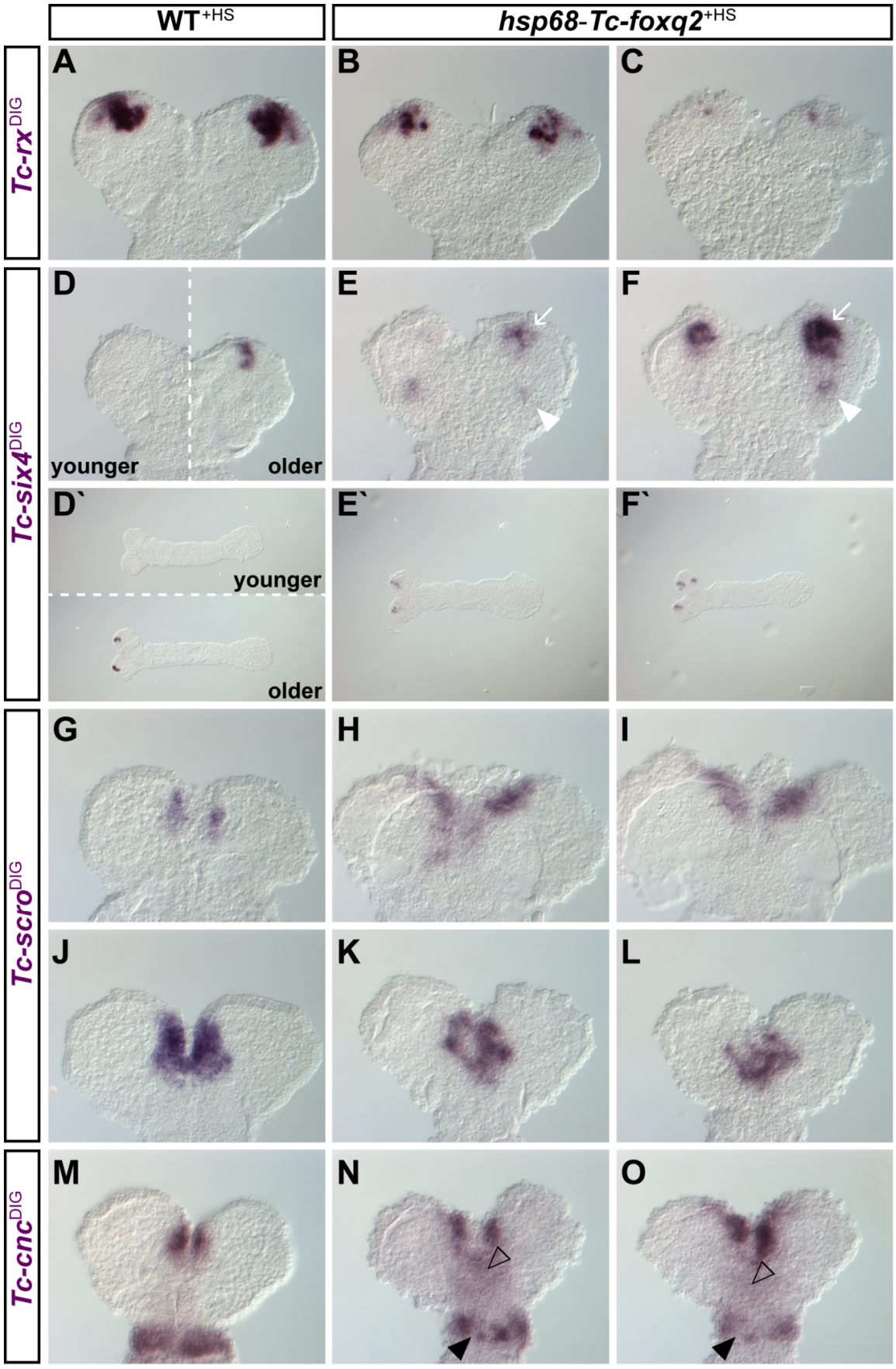
Figure 4.25 **Ectopic *Tc-foxq2* expression leads to an increased number of apoptotic cells.** Anterior is left in **A**-**F**. Apoptotic cells of heat shock-treated wt (**A**, **A`**, **C**, **C`**, **E**, **E`**) and *hsp68-Tc-foxq2* (**B**, **B`**, **D**, **D`**, **F**, **F`**) embryos are monitored by cleaved DCP-1 antibody staining (green). Nuclei are stained with DAPI (magenta) to visualize the embryonic morphology. Embryos are depicted as maximum projections. (**B**, **B`**, **D**, **D`**, **F**, **F`**) Ectopic *Tc-foxq2* expression in embryos (14-18 h AEL) leads to a strong increase in the number of apoptotic cells in all three body parts throughout the analyzed developmental stages compared to heat shocked wt embryos (**B**, **B`**, **D**, **D`**, **F**, **F`**).

4.1.5.3 *Tc-foxq2* gain-of-function and the impact on the anterior head gene regulatory network

With the aim to complement the picture of *Tc-foxq2* function in the gene regulatory network of the anteriormost head region, I analyzed *Tc-foxq2* gain-of-function embryos for changes in expression profiles of head patterning genes. To this end, I collected embryos from the *hsp68-Tc-foxq2* line, applied a heat shock treatment to the embryos (9-13 h AEL), fixed them

(14-18 h AEL) and performed ISH to visualize potential alteration of the expression profile. The same procedure was performed simultaneously using wt embryos as control.

Tc-rx expression was considerably reduced to a small or spotty domain upon ectopic *Tc-foxq2* expression (**Figure 4.26A-C**). Ectopic *Tc-foxq2* expression caused a premature onset of *Tc-six4* expression (compare **Figure 4.26D**, **D`**: younger and older with **E-F`**). Additionally, *Tc-six4* expression domains were enlarged (**Figure 4.26D**: right panel and **Figure 4.26E**, **F**: arrow). Further, *hsp68-Tc-foxq2*$^{+HS}$ embryos showed novel *Tc-six4* expression domains, which seem to be located within the antennal segment (**Figure 4.26E**, **F**: white arrowhead). The *Tc-scro* expression pattern was altered in two different ways depending on the developmental stage (**Figure 4.26G-L**). In *hsp68-Tc-foxq2*$^{+HS}$ germ rudiments the onset of expression *Tc-scro* was premature and the domains were uniformly enlarged and posteriorly elongated (**Figure 4.26G-I**). In contrast, elongating *hsp68-Tc-foxq2*$^{+HS}$ germ bands showed a spotty reduction of the *Tc-scro* expression domain compared to the wt expression domain (**Figure 4.26K**, **L**). The effect of ectopic *Tc-foxq2* expression at elongating germ band stages may be a modulated secondary effect elicited by apoptosis (**see Figure 4.25**). The anterior median domain of *Tc-cnc* expression appeared to be slightly expanded, after ectopic *Tc-foxq2* expression (**Figure 4.26O**: white empty arrowhead) and spread posteriorly (**Figure 4.26N**: black empty arrowhead). The *Tc-cnc* mandibular expression domain was reduced in a spotty manner (**Figure 4.26N**, **O**: black arrowheads).


WT+HS
hsp68-Tc-foxq2+HS
Tc-rxDIG
Tc-six4DIG
Tc-scroDIG
Tc-cncDIG
A
B
C
D
E
F
younger
older
D`
E`
F`
younger
older
G
H
I
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K
L
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Figure 4.26 **Ectopic *Tc-foxq2* expression impacts head patterning gene expression profiles (strong effects).** Anterior is up (left in **D`**-**F`**). Expression of head patterning genes in heat shock-treated wt (**A**, **D**, **D`**, **G**, **J**, **M**) and *hsp68-Tc-foxq2* (**B**, **C**, **E**, **E`**, **F**, **F`**, **H**, **I**, **K**, **L**, **N**, **O**) embryos (14-18 h AEL) is monitored by ISH. (**B**, **C**) Ectopic *Tc-foxq2* expression leads to reduced *Tc-rx* expression domains (**B**, **C**). (**E**, **E`**, **F**, **F`**) *Tc-six4* expression shows a premature onset (compare **E`**, **F`** with **D`**: younger and older) at the anterior tip (arrows). These premature expression domains are expanded (**F**: arrow) compared to the size of the wt domains. Further, *hsp68-Tc-foxq2*$^{+HS}$ embryos show an additional *Tc-six4* expression domain within the antennal segment (white arrowhead). (**H**, **I**) The *Tc-scro* expression domains are prematurely expressed and expanded in *hsp68-Tc-foxq2*$^{+HS}$ germ rudiments. (**K**, **L**) In contrast, early elongating germ bands show reduced and aberrant *Tc-scro* expression domains in *hsp68-Tc-foxq2*$^{+HS}$ embryos. (**N**, **O**) The anterior median *Tc-cnc* expression domains appear to be spread to the posterior (**N**, **O**: black empty arrowheads). The mandibular *Tc-cnc* expression domain is reduced in an irregular manner after ectopic *Tc-foxq2* expression (black arrowheads).

Tc-wg, *Tc-six3,* and *Tc-croc* showed comparably mild or less relevant alterations in expression profiles, after ectopic *Tc-foxq2* expression. *hsp68-Tc-foxq2*$^{+HS}$ embryos showed a slight reduction of the ocular *Tc-wg* domain (Figure 4.27B, C: empty arrowheads). The antennal *Tc-wg* stripes were heavily reduced or completely absent in almost all *hsp68-Tc-foxq2*$^{+HS}$ embryos (Figure 4.27B, C: white arrowheads). The subsequent segmental expressed *Tc-wg* domains were either reduced (Figure 4.27F: black empty arrowhead), absent (Figure 4.27F: white empty arrowhead) or collapsed (Figure 4.27E: white arrowhead). This abnormal expression of the segment polarity gene *Tc-wg* could relate to the cuticle phenotype, which showed deformed, absent, or fused segments (Figure 4.23). Neurogenic *Tc-six3* expression was anteriorly reduced in *hsp68-Tc-foxq2*$^{+HS}$ embryos (Figure 4.27H, I: white arrows). The expression pattern of *Tc-croc* appears to be enlarged (Figure 4.27K, L: empty arrowheads) and spread posteriorly (Figure 4.27K, L: white arrowheads) in *hsp68-Tc-foxq2*$^{+HS}$ embryos.

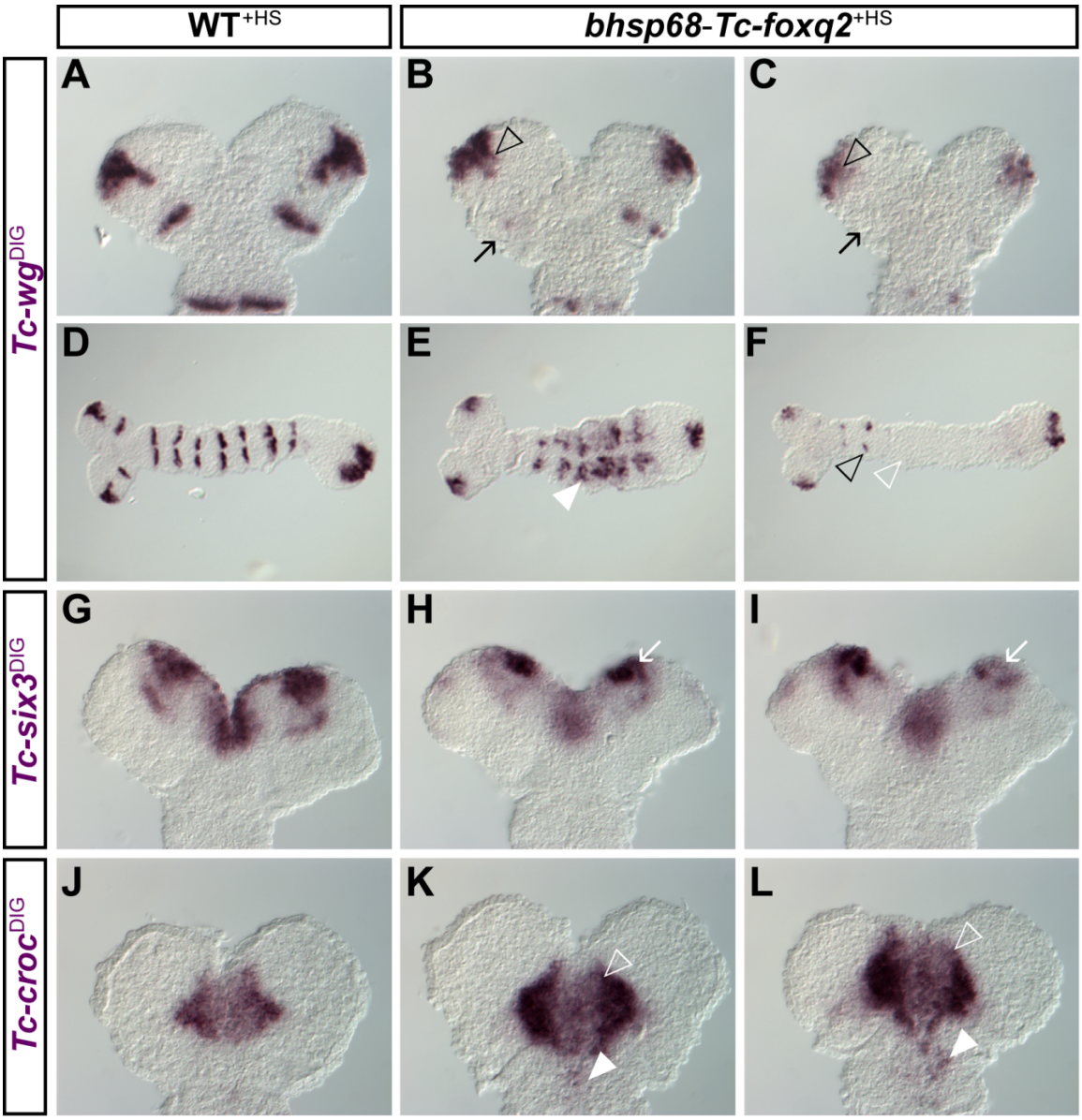

Figure 4.27 **Ectopic *Tc-foxq2* expression impacts head patterning gene expression profiles (mild effects).** Anterior is up (left in **D**-**F**)**.** Expression of head marker genes in heat shock-treated wt (**A**, **D**, **G**, **J**) and *hsp68-Tc-foxq2* (**B**, **C**, **E**, **F**, **H**, **I**, **K**, **L**) embryos (14-18 h AEL) is monitored by ISH. (**B**, **C**) The ocular *Tc-wg* expression domain is slightly (**B**) or heavily (**C**) reduced (empty arrowheads). The antennal expression domains are heavily reduced (**B**: arrow) or completely absent (**C**: arrow). (**E**, **F**) The *Tc-wg* stripes posterior to the procephalon are collapsed (**E**: white arrowhead), reduced (**F**: black empty arrow) or completely absent (**F**: white arrowhead), in *hsp68-Tc-foxq2*$^{+HS}$ embryos. (**H**, **I**) The *Tc-six3* expression domains within the neurogenic region are reduced (arrows). (**K**, **L**) The *Tc-croc* expression pattern appears to be slightly expanded (empty arrowheads) and posteriorly spread (arrowheads).

4.1.6 *Tc-foxq2* is required for proper brain formation

4.1.6.1 Embryonic *Tc-foxq2* knock-down causes neural phenotypes in L1 larvae

Tc-six3 is known to be required for the proper formation of the brain (Posnien et al., 2011b). Embryonic *Tc-six3* knock-down experiments resulted in defects of the central body, convergence of the brain hemispheres and reduction of the mushroom bodies in L1 larval stages (Posnien et al., 2011b).

Further, it has been shown for *Tc-chx*, a neuroendocrine marker of the pars intercerebralis (Posnien et al., 2011b), to be essential for the proper formation of the central body (Koniszewski, 2011). The findings that *Tc-foxq2* is co-expressed and interacting with *Tc-six3* and *Tc-chx* in neurogenic regions during embryogenesis raised the question whether *Tc-foxq2* has a function in neural development as well. In order to proof this hypothesis, I performed *Tc-foxq2* pRNAi within the *brainy* reporter line and analyzed L1 larvae regarding neural defects, via confocal in vivo imaging (Posnien et al., 2011b). The *brainy* line is a reporter line that visualizes the neuropils and a subset of glial tissue (Koniszewski et al., 2016; Posnien et al., 2011b).

The knock-down of embryonic *Tc-foxq2* function resulted in L1 larvae that showed different levels of neural defects (n=13). The weakest phenotypes showed mushroom bodies with shorter medial lobes (**Figure 4.28B`**, magenta marker) that appeared to be fused (61.5%; **Figure 4.28**: compare **A** and **B**: empty arrows). Further, the central complex appeared to be shortened (61.5%; **Figure 4.28B`**, yellow marker) and the brain hemispheres appeared to be more closely together or even fused (61.5%; **Figure 4.28A`**, **B`**: empty arrowheads). In stronger phenotypes of *Tc-foxq2* knock-down larvae the central complex was reduced in size as well, and the mushroom bodies were not detectable (23.1%; **Figure 4.28C`**). Also the brain hemispheres in this phenotype were fused at the midline (**Figure 4.28C**, **C`**). The strongest phenotype appeared to have no central complex, mushroom body, and antennal lobe at all (n=7.7%; **Figure 4.28D**, **D`**). The entire brain hemispheres seem to be heavily reduced in this phenotype. However, this class of phenotypes was hard to interpret as secondary effects may blur the primary phenotype. Interestingly, the strength of neural defects correlated with the strength of the epidermal defects. Weak neural defects also appeared with a size-reduced labrum, whereas strong defects lacked the complete labrum. Taken together, these data suggest that *Tc-foxq2* has indeed a neural function, presumably required for correct formation of the central body and the mushroom bodies. Further, the observed neural defects caused by knock-down of *Tc-foxq2*

function and the neural defects reported for *Tc-six3* knock-down larvae resemble each other (Posnien et al., 2011b).

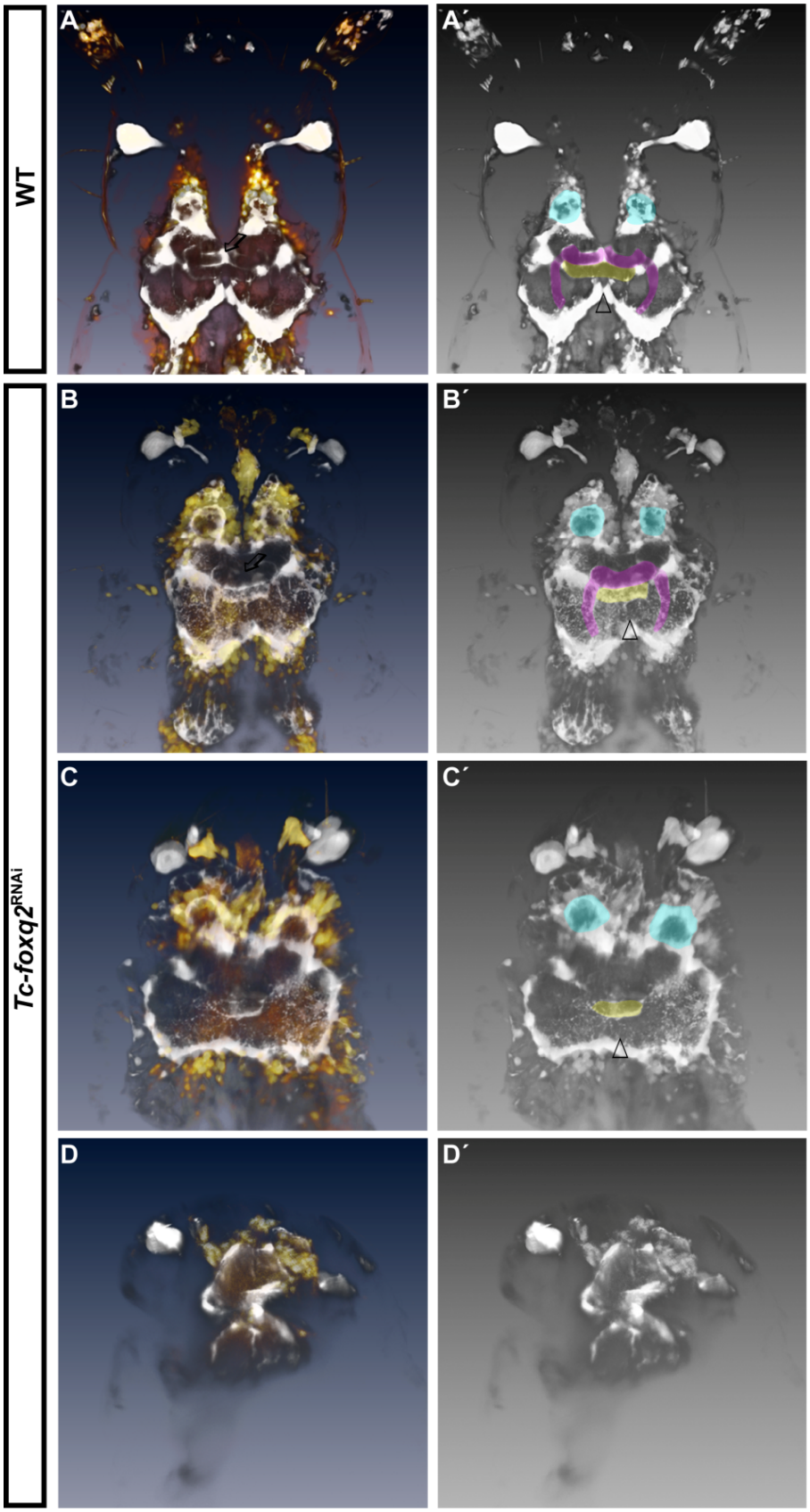

Figure 4.28 **Embryonic knock-down of *Tc-foxq2* function leads to defects in L1 larval brains.** L1 larval brains are shown with anterior up, visualized with the transgenic *brainy* reporter line. In **A** (wt) and **B**-**D** (*Tc-foxq2*^{pRNAi}) neural cells are shown in yellow and glial cells in white. In **A`**-**D`** glial cells are shown in white and neuropils are color-coded. (**A**, **A`**) Wt L1 larval brain with two brain hemispheres, each with a mushroom body (magenta), an antennal lobe (cyan), and the mid-line spanning central body (yellow). (**B**, **B`**) A weak *Tc-foxq2*^{pRNAi} larval brain phenotype showing the loss of the boundary between the medial lobes of the mushroom bodies (compare arrows in **B** and **A**). The central complex appears to be slightly reduced in size. (**C**, **C`**) Intermediate *Tc-foxq2*$^{pRNAi}$ larval brains appear to lack the complete mushroom bodies. The central body is reduced in size. (**D**, **D`**) Strong *Tc-foxq2*^{pRNAi} neural phenotypes show a completely disarranged and strongly reduced brain in L1 larvae. Furthermore the brain hemispheres appear to be more closely together or fused (**A`**-**C`**: empty arrowheads).

The *Tc-foxq2* knock-down experiment in the *brainy* reporter line showed that the most pronounced neural defects were found in the mushroom bodies. To get a better view on these defects, I performed *Tc-foxq2* pRNAi within the mushroom body reporter line ('*MB-green*'), which visualizes the overall structure of the mushroom body (Binzer et al., 2014; Koniszewski et al., 2016; Posnien et al., 2011b).

The experiment with the *MB-green* reporter line revealed different grades of phenotype strength. Only phenotypes that were observed at least twice are discussed. Knock-down of embryonic *Tc-foxq2* most frequently led to L1 larvae that lacked the border between the two medial lobes, indicating a fusion of the medial lobes (Figure 4.29B: empty arrowhead, compare with Figure 4.28B). Moreover, L1 larvae were frequently observed, which appeared to have intact but misarranged mushroom bodies, resulting in a loss of contact between the medial lobes from the two hemispheres (Figure 4.29C: arrowheads). In intermediate phenotypes, the mushroom bodies were twisted with each other at the midline (Figure 4.29D). In strong phenotypes appeared the mushroom bodies to be completely absent (Figure 4.29E). Intermediate and strong phenotypes were scarcely found. These findings substantiate the result observed within the *brainy* reporter line.

Taken together, the experiments using the neural reporter lines indicate that *Tc-foxq2* is required for the correct formation and arrangement of the mushroom bodies, for the proper formation of the central body and led upon knock-down to fused brain hemispheres. Interestingly, the neural defects observed in *Tc-foxq2* larvae are similar to the defects in *Tc-six3* knock-down larvae (Posnien et al., 2011b).

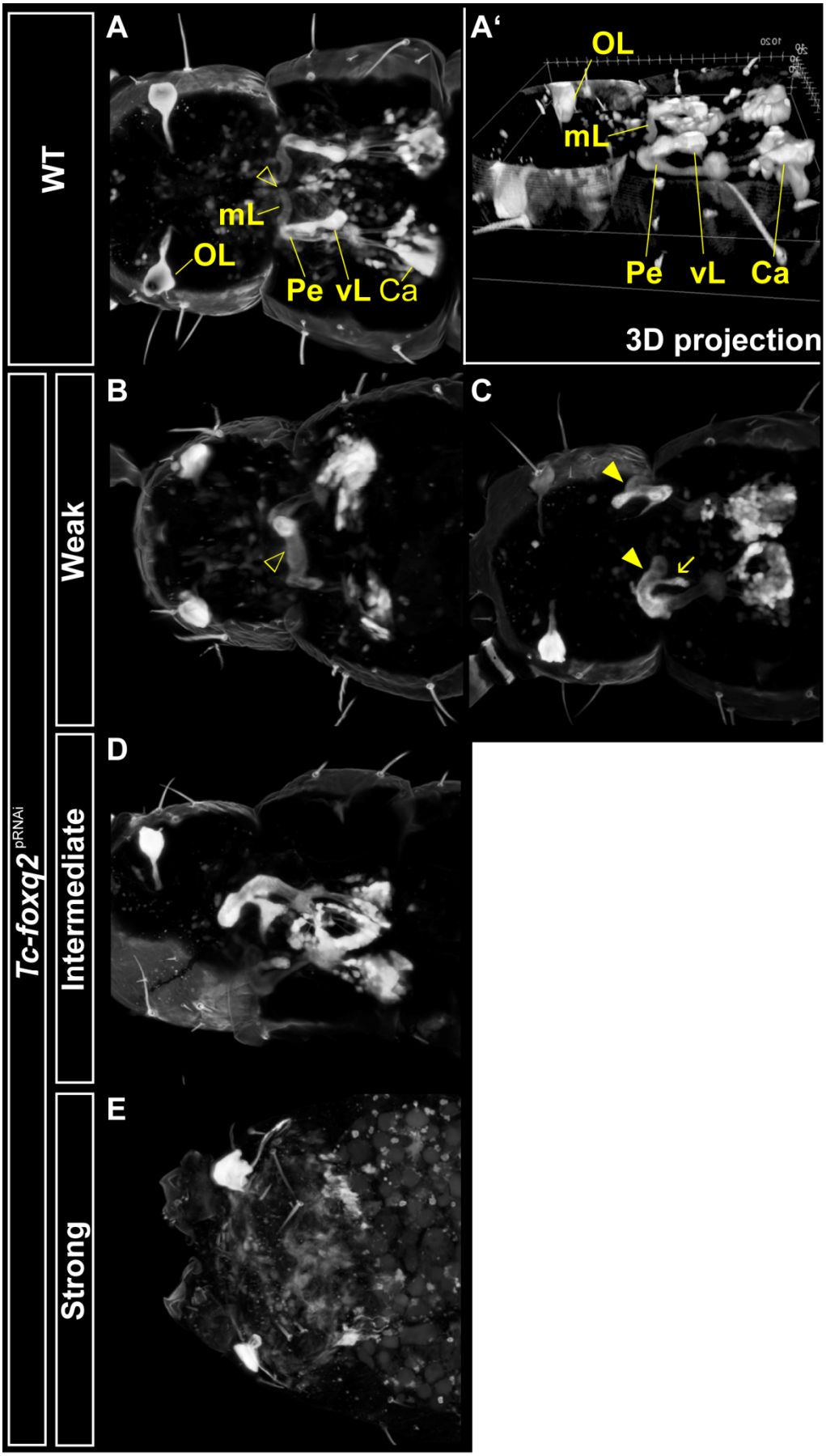
WT
A
mL
OL
Pe vL Ca
A'
OL
mL
Pe vL Ca
3D projection
Tc-foxq2 pRNAi
Weak
B
C
Intermediate
D
Strong
E

Figure 4.29 **Embryonic *Tc-foxq2* knock-down leads to mushroom body defects in L1 larvae.** Anterior is left. (**A**-**F**) Mushroom bodies are in L1 larvae visualized by using the transgenic *MB-green* reporter line. (**A**) Maximum projection of wt L1 larval mushroom bodies in a dorsal view. (**A`**) 3D projection of wt L1 larval mushroom body in a lateral view, providing a better overview of the organization of the structures. (**B**) A weak *Tc-foxq2* mushroom body phenotype, which lacks the border at the midline between the two medial lobes (compare empty arrowheads in **A** and **B**). (**C**) *Tc-foxq2*^{pRNAi} L1 larval mushroom bodies, which show distorted pedunculi, leading to a loss of contact between the two medial lobes (arrowheads), and slightly reduced vertical lobes (arrow). (**D**) Intermediate *Tc-foxq2*^{pRNAi} mushroom body phenotypes are either marked by an interdigitation of the two mushroom bodies (**D**). (**E**) In strong *Tc-foxq2*^{pRNAi} larval brain phenotypes the mushroom body structures are highly reduced or absent. OL: optical lobe, mL: medial lobe, Pe: pedunculus, vL: vertical lobe, Ca: calyx

4.1.6.2 Embryonic *Tc-foxq2* gain-of-function results in larvae showing neural phenotypes

I was also interested whether *Tc-foxq2* gain-of-function embryos result in larvae with neural defects. Thus, I crossed the ectopic *Tc-foxq2* expression line *hsp68-Tc-foxq2* with the *brainy* as well as with the *MB-green* line. Embryonic offspring (0-24 h AEL) of these hybrids was then heat shocked and the emerged L1 larvae were analyzed regarding neural deficiencies.

Heat shock treatment of both the *brainy* and the *MB-green* reporter lines alone did not lead to an increase of specific neural defects in larvae (data not shown). Heat shock-treated double heterozygous animals (reporter lines crossed with the *hsp68-Tc-foxq2* line) showed mild neural defects. The hemispheres looked normal (**Figure 4.30A-D`**), but the medial lobes of the mushroom bodies appeared to be slightly elongated and the pedunculi appeared to be slightly misarranged (**Figure 4.30B`**, magenta marker). In some cases the central body was elongated or its shape slightly altered (**Figure 4.30C`**, **D`**, yellow marker) and sometimes the medial lobes lost contact (**Figure 4.30C`**, magenta marker). The observed mushroom body phenotype was confirmed in the *MB-green* line. The mushroom bodies of these larvae appeared to have all units. However, the medial lobes of the mushroom bodies had lost contact to each other (**Figure 4.31**, compare **B** with **A**: yellow brackets) or were folded posteriorly (**Figure 4.31C**, empty arrowheads). In some cases he pedunculi appeared slightly dislocated (**Figure 4.31D**, arrowheads).

Taken together, the neural defects of the *Tc-foxq2* gain-of-function assay were less severe than the defects in the *Tc-foxq2* knock-down assay. However, both assays showed affected

mushroom bodies and central bodies, while the antennal lobes were not affected. Both assays showed dislocated medial lobes, which had lost contact, while only in the knock-down assay the mushroom bodies appeared to be fused. Further, loss of the central body and mushroom bodies were only found in the knock-down assay, while the gain-of-function assay showed enlarged medial lobes. Moreover, the midline fusion of the two brain hemispheres was only observed in the knock-down assay.

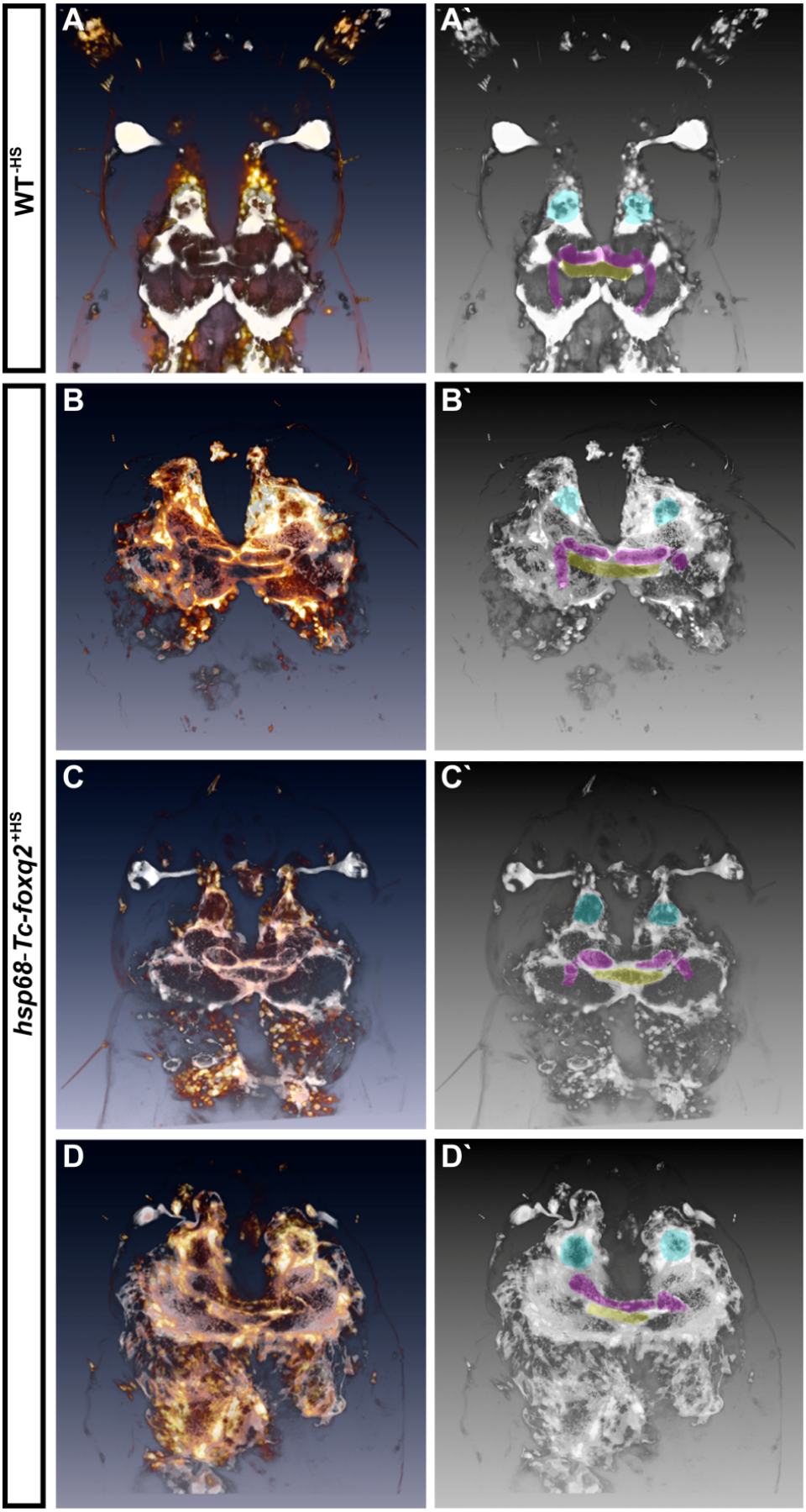
WT-HS
hsp68-Tc-foxq2+HS
A
A`
B
B`
C
C`
D
D`

Figure 4.30 **Embryonic gain of *Tc-foxq2* function leads to weak neural defects in L1 larvae.** L1 larval brains are shown with anterior up, visualized using the transgenic *brainy* reporter line. In **A** (wt) and **B**-**D** (*hsp68-Tc-foxq2*$^{+HS}$) neural cells are shown in yellow and glial cells in white. In **A`**-**D`** glial cells are shown in white and neuropils are color-coded. (**A**, **A`**) Wt L1 larval brain with two brain hemispheres, each with a mushroom body (magenta), an antennal lobe (cyan), and the mid-line spanning central body (yellow). (**B**, **B`**) L1 larval brain that shows enlarged medial lobes (magenta) and a slightly misarranged pedunculus after ectopic *Tc-foxq2* expression (magenta). (**C**, **C`**) L1 larval brain showing dislocated medial lobes (magenta) and a shape-altered central body (yellow). (**D**, **D`**) L1 larval brain with a slightly reduced central body (yellow) and dislocated pedunculi (magenta).

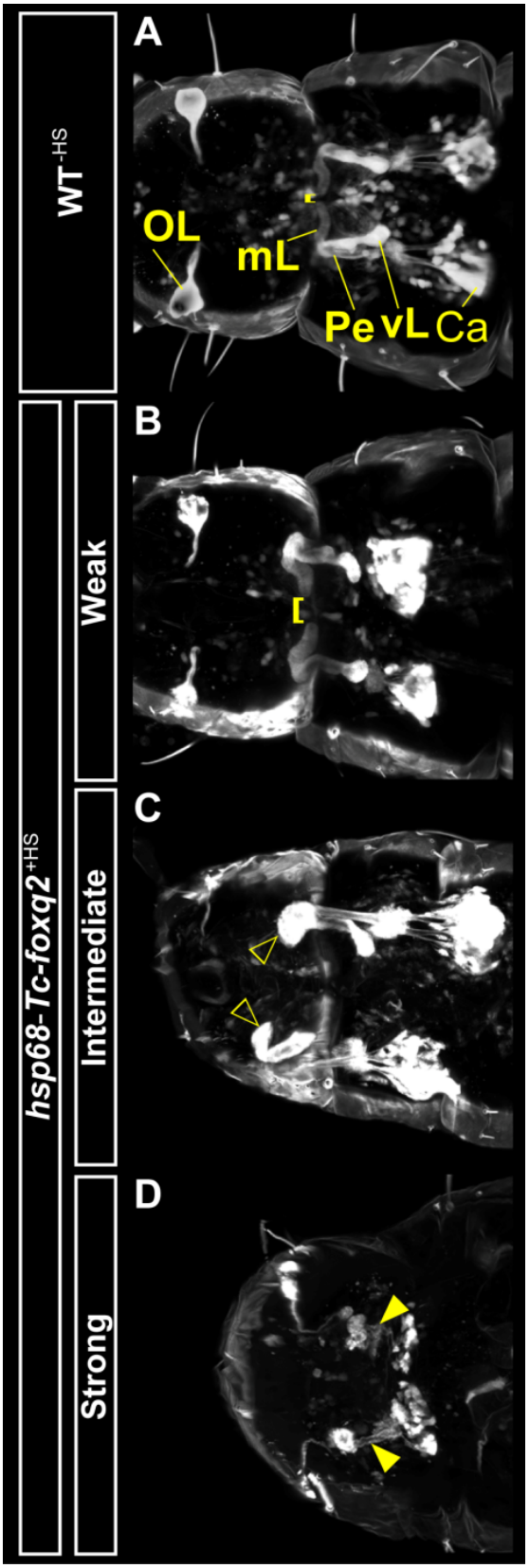

Figure 4.31 **Embryonic gain of *Tc-foxq2* function leads to affected mushroom bodies in L1 larvae.** Anterior is left. (**A**-**F**) L1 larval mushroom bodies are visualized by using the transgenic *MB-green* line. (**A**) Maximum projection of wt L1 larval mushroom bodies in a dorsal view. (**B**) Weak phenotypes are marked

by an enlarged gap between the medial lobes of the two hemispheres (compare brackets in **A** and **B**). (**C**) Intermediate phenotypes show strongly dislocated medial lobes (empty arrowheads). (**D**) The strongest *hsp68-Tc-foxq2*$^{+HS}$ phenotypes show reduced and misarranged mushroom bodies, in which the medial lobes appear to be absent. OL: optical lobe, mL: medial lobe, Pe: pedunculus, vL: vertical lobe, Ca: calyx

4.1.6.3 *Tc-foxq2* misregulation leads to increased cell death rates within the neurogenic head region

Trying to track down possible reasons for the neural phenotype in *Tc-foxq2* knock-down experiments, I analyzed the cell death rate in this particular region for several embryonic stages (<30 h AEL). To this end, I performed virtually the same experiment as in **4.1.2.2**, with the sole difference that the ROI was not the labral but the neurogenic head region (**Figure 4.32A$_a$**, region 2).

The first embryonic stages (germ rudiment to fully elongated stages) showed no differences in cell death rates in *Tc-foxq2*^{pRNAi} embryos compared to wt embryos (**Figure 4.32B**: stage 1-4). However, retracting germ bands showed a significant increase in the cell death rate within the neurogenic head region (**Figure 4.32B**: stage 5; p=0.023). Within the neurogenic region, the vast majority of apoptotic cells were located at the anterior rim of the head lobes (**Figure 4.32A$_b$**, empty arrowhead). This region is important for neural development. *Tc-six3* and *Tc-chx* are markers of the pars intercerebralis, both are expressed at this region, and lead to neural defects resembling the *Tc-foxq2* neural defects, upon knock-down (Posnien 2011). Further, it is known that neuroblasts, which are involved in central complex formation, form within this region in retracting embryos (Boyan and Reichert, 2011; Koniszewski, 2011). This indicates that increased cell death rates could be related to the observed *Tc-foxq2* knock-down neural phenotype.

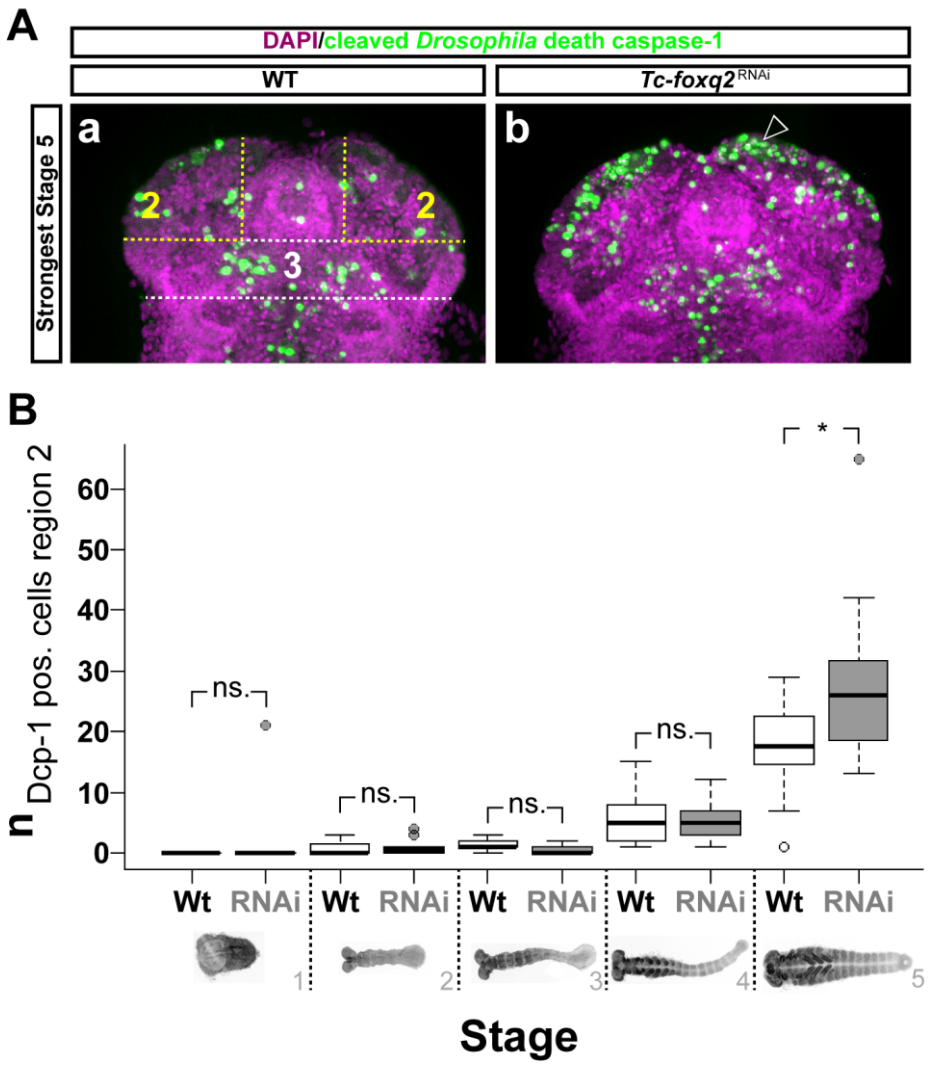

Figure 4.32 **Analysis of cell death rates within the neurogenic head region in *Tc-foxq2*^{pRNAi} embryos.** Anterior is up (**A$_a$**, **A$_b$**). Apoptotic cells, in wt (**A$_a$**) and in *Tc-foxq2*^{pRNAi} (**A$_b$**) embryos, are monitored by antibody staining (Dcp-1 – Alexa Fluor 488, green). Nuclei are stained (DAPI, magenta) to visualize embryonic morphology. (**A$_a$**, **A$_b$**) Retracting germ bands with the highest number of apoptotic cells in wt (**A$_a$**) and *Tc-foxq2*^{pRNAi} (**A$_b$**) embryos. Indicated are the neurogenic region (ROI 2, yellow dashed lines) and the region, which was used for normalization of the data set (region 3, white dashed lines). The *Tc-foxq2*^{pRNAi} retracting germ band shows a strong accumulation of apoptotic cells within the neurogenic region (**A$_b$**). (**B**) Box plot depicting the number of apoptotic cells (y-axis) versus five different embryonic stages. The ROI 2 values are normalized with the region 3 values. Brackets display grade of significance. Germ rudiments (stage 1) to fully elongated germ bands (stage 4) show no significant increase of apoptotic cells (stage 1: p=0.33 (wt: n=3, RNAi: n=7), stage 2: p=0.35 (wt: n=11, RNAi: n=12), stage 3: p=0.99 (wt: n=9, RNAi: n=19), stage 4: p=0.23 (wt: n=17, RNAi: n=15)). However, retracting germ bands showed in the ROI 2 significantly more apoptotic cells (p=0.023) in RNAi embryos (n=11) compared to untreated embryos (n=12). ns.: not significant

4.2 Expanding the *Tribolium* toolbox

4.2.1 Generating transgenic lines driving strong and ubiquitous expression of a nuclear localized EGFP

Nuclear reporter lines are well-suited for in vivo imaging experiments (Clarkson and Saint, 1999; El-Sherif et al., 2012; Pauls et al., 2001; Sarrazin et al., 2012; Strobl et al., 2015). However, for embryonic in vivo imaging experiments the signal of the reporter line needs to (1) be strong enough for imaging, (2) mark all nuclei, and (3) be always localized to the nucleus. The first reported transgenic *Tribolium* nuclear reporter line used for in vivo imaging was the *EFA*-nGFP line (El-Sherif et al., 2012; Sarrazin et al., 2012). This line encodes the green fluorescent protein (GFP) with a nuclear localization signal (nls or n; nGFP) under the control of the *Tc-elongation factor 1α* (*Tc-EFA*) promoter, which should drive ubiquitous expression. This line was well-suited for some questions, but it has also two major drawbacks. First, the nGFP signal gets blurry during the Prophase of mitosis (**Figure 4.33F**: box), because of the breakdown of the nuclear envelope (Smoyer and Jaspersen, 2014) and the efflux of the nGFP into the cytoplasm. Furthermore, the *Tc-EFA* promoter does not drive complete ubiquitous expression, showing an uneven signal distribution throughout the embryo (**Figure 4.33O**: box). On the basis of these findings, I tried to generate transgenic lines, which effectively drive ubiquitous and strong expression of a fluorescent protein, which is DNA-bound, and does therefore not show a blurry signal during mitotic stages. To this end, I generated a chimeric protein, which consists of the histone Tc-H2A variant (Tc-H2Av) and the fluorescent protein enhanced GFP (EGFP; H2Av::EFGP). Afterwards I cloned three different constructs each with a different promoter (*Tc-alpha tubulin 1* promoter (*αTub1*P; Siebert et al., 2008), *Tc-polyubiquitin promoter* (*PUb*P) and *Tc-ribosomal protein subunit 3* promoter (*rps3*P)), which should drive strong and ubiquitous expression of the chimeric H2Av::EGFP protein. I cloned these reporter cassettes into a transgenesis vector and generated three different imaging lines. Subsequently I analyzed them concerning their expression profile and compared them to the *EFA-nGFP* imaging line.

4.2.1.2 Qualitative promoter comparisons

I compared the three generated imaging lines (*rps3*P-*H2Av::EGFP*, *αTub1*P-*H2Av::EGFP,* and *PUb*P-*H2Av::EGFP*) with each other and with the already published *EFA-nGFP* line. To this end, I

imaged all lines with the same microscope settings and processed them all in same the way. Afterwards, I analyzed these lines regarding the signal localization, intensity, and distribution as well as with regard to their viability. However, the *PUb*P-*H2Av::EGFP* imaging line died after some weeks. Thus, the detailed description is restricted to the lines, which survived. The new lines were a pool of hetero- and homozygous animals, while the *EFA-nGFP* line was completely homozygous. For the comparison only individuals of each line with the strongest signal intensity were used.

At first I compared the different lines regarding the promoter-based activation intensity and the signal localization at embryonic stages (**Figure 4.33A-O**). The comparison revealed that the *αTub1*P-*H2Av::EGFP* line showed the strongest signal intensity at blastoderm stages (**Figure 4.33B**). However, in the course of development the signal intensity of the *αTub1*P-*H2Av::EGFP* line got weaker (**Figure 4.33B**, **E**, **H**, **K**, **N**) and showed signal intensities comparable to the signal of the *rps3*P-*H2Av::EGFP* line (**Figure 4.33M**). In contrast, the signal intensity of the *EFA-nGFP* line became stronger during these stages (**Figure 4.33C**, **F**, **I**, **L**, **O**). As a consequence, retracting germ band stages showed the strongest signal intensity in the *EFA-nGFP* line (**Figure 4.33O**). The *αTub1*P-*H2Av::EGFP* and the *rps3*P-*H2Av::EGFP* line showed an ubiquitous signal with no obvious expression gaps. In contrast, the *EFA-nGFP* line showed regions where the signal was not detectable (**Figure 4.33** compare **M**: box with **O**: box). The signal of the fluorescent chimeric protein of both of the new lines was always sharp and tightly localized to the DNA (**Figure 4.33D**: box), whereas the signal of the *EFA-nGFP* line was blurry and distributed over the whole cell during mitosis (**Figure 4.33F**: box). The blurry, equally distributed signal is due to the breakdown of the nuclear envelope in the Prophase (Smoyer and Jaspersen, 2014), leading to an efflux of the unbound nuclear localized nGFP.

I also compared signal intensities of the different lines at larval (**Figure 4.34A-C**), pupal (**Figure 4.34D-F**), and adult stages (data not shown). At larval as well as at pupal stages the *αTub1*P-*H2Av::EGFP* line showed the strongest signal intensities (**Figure 4.34B**, **E**). Signal intensities of the *rps3*P-*H2Av::EGFP* (**Figure 4.34A**, **D**), and the *EFA-nGFP* (**Figure 4.34A**, **D** and **C**, **F**) line had comparable signal intensities. In adult stages the *αTub1*P-*H2Av::EGFP* and the *EFA-nGFP* line showed the best signal intensities (data not shown).

Comparison of the distribution, localization, and intensities of the signal showed no considerable differences in ovaries among the different lines. In all imaging lines a signal was found within the follicle cells, the nurse cells as well as in the pro-nucleus in germ cells (**Figure 4.35A-C`**).

However, in all lines the signal of the pro-nucleus was not always detectable (Figure 4.35B-C`). The basis of this phenomenon was not further analyzed.

Taken together, the *PUb*P-*H2Av::EGFP* line showed the strongest signal intensities at all developmental stages (Figure 4.36), but the line was not viable. Comparing the *αTub1*P-*H2Av::EGFP* line with the *rpS3*P-*H2Av::EGFP* line, the *αTub1*P-*H2Av::EGFP* line showed comparable or even better signal intensities at the analyzed developmental stages (Figure 4.36). Both lines showed similar viabilities (Figure 4.36). In contrast, the *EFA-nGFP* line showed the best viability and the strongest signal intensities at post-elongation germ band stages, with the drawback of signal gaps in the embryonic tissue and blurry signals in dividing cells (Figure 4.36). It is possible that the strong expression of histone-tagged EGFP is interfering with viability (see section 5.2.3), which could restrict the maximal signal intensity.

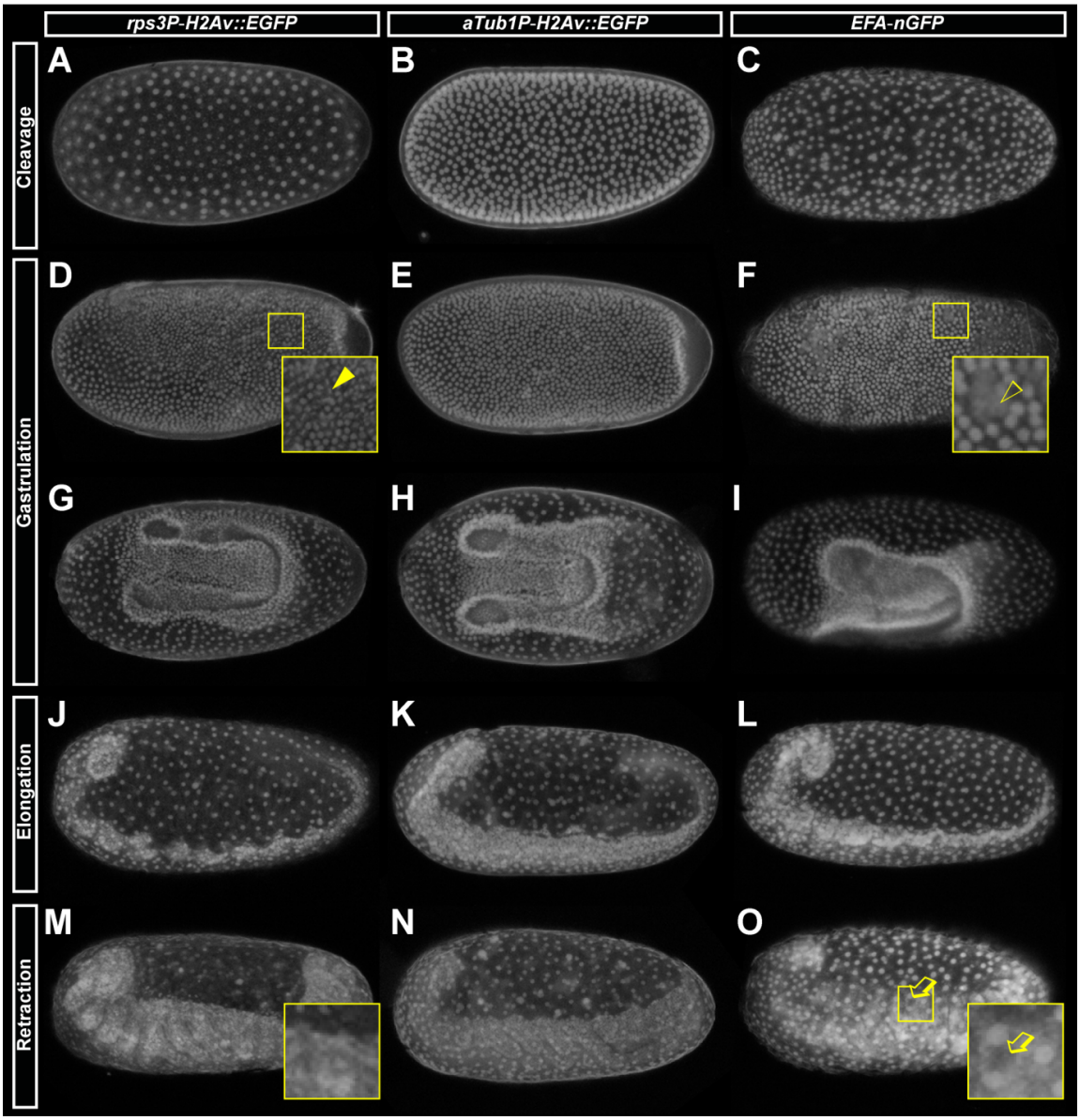

Figure 4.33 **Qualitative comparison of signal intensities and localizations of three different ubiquitous nuclear reporter lines at early embryonic stages.** Anterior is left. Nuclei are visualized by using the transgenic imaging lines *rps3*P-*H2Av::EGFP* (**A**, **D**, **G**, **J**, **M**), *αTub1*P-*H2Av::EGFP* (**B**, **E**, **H**, **K**, **N**), and *EFA-nGFP* (**C**, **F**, **I**, **L**, **O,** El-Sherif et al., 2012). The embryos are depicted as average projections. (**A**-**C**) Blastoderm stages of the *αTub1*P-*H2Av::EGFP* line (**B**) show the strongest and blastoderm stages of the *rps3*P-*H2Av::EGFP* line (**A**) the weakest signal intensity. (**D**-**F**) With the onset of the amniotic fold all three lines show comparable signal intensities. (**G**-**O**) At later stages the signal of the *EFA-nGFP* line becomes successively stronger and shows the strongest signal intensity in all subsequent stages (**I**, **L**, **O**). In contrast, the *αTub1*P-*H2Av::EGFP* line shows a successive decrease of the signal intensity (**H**, **K**, **N**), and consequently shows the weakest signal intensity in retracting germ bands (**N**). However, the advantages of the two new nuclear reporter lines, *rps3*P-*H2Av::EGFP* and *αTub1*P-*H2Av::EGFP*, are that they show a crisp and DNA-associated signal also during mitosis (compare **D**: box, yellow arrowhead with **F**: box, yellow

empty arrowhead) and that the signal is ubiquitously detectable, without signal gaps in the embryonic tissue (compare **M**: box with **O**: box, empty arrow).

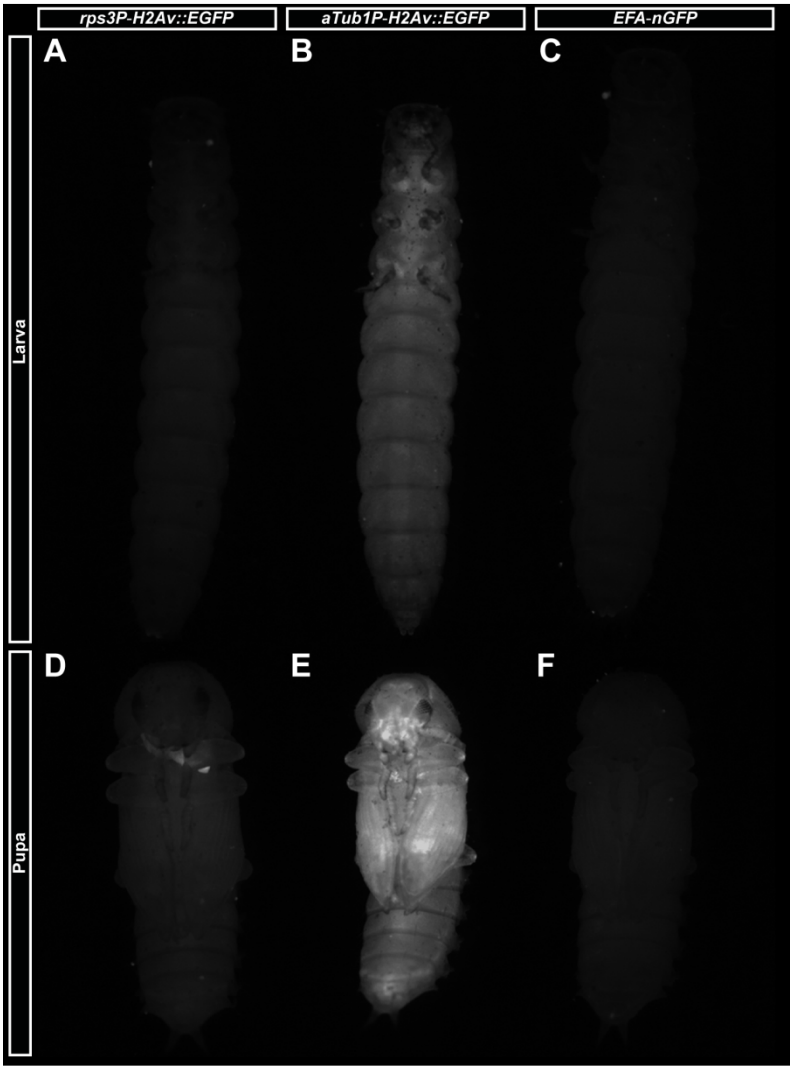

Figure 4.34 **Qualitative comparisons of signal intensities of three different ubiquitous nuclear reporter lines at larval and pupal stages.** Anterior is up. Larvae and pupae are monitored by using the transgenic imaging lines *rps3*P-*H2Av::EGFP* (**A**, **D**), *αTub1*P-*H2Av::EGFP* (**B**, **E**) and *EFA-nGFP* (**C**, **F**, El-Sherif et al., 2012). (**A**-**F**) The *αTub1*P-*H2Av::EGFP* line shows the strongest signal intensity at larval (**B**) as well as at pupal stages (**E**). The other lines show weak signals, which are comparable with respect to intensity.

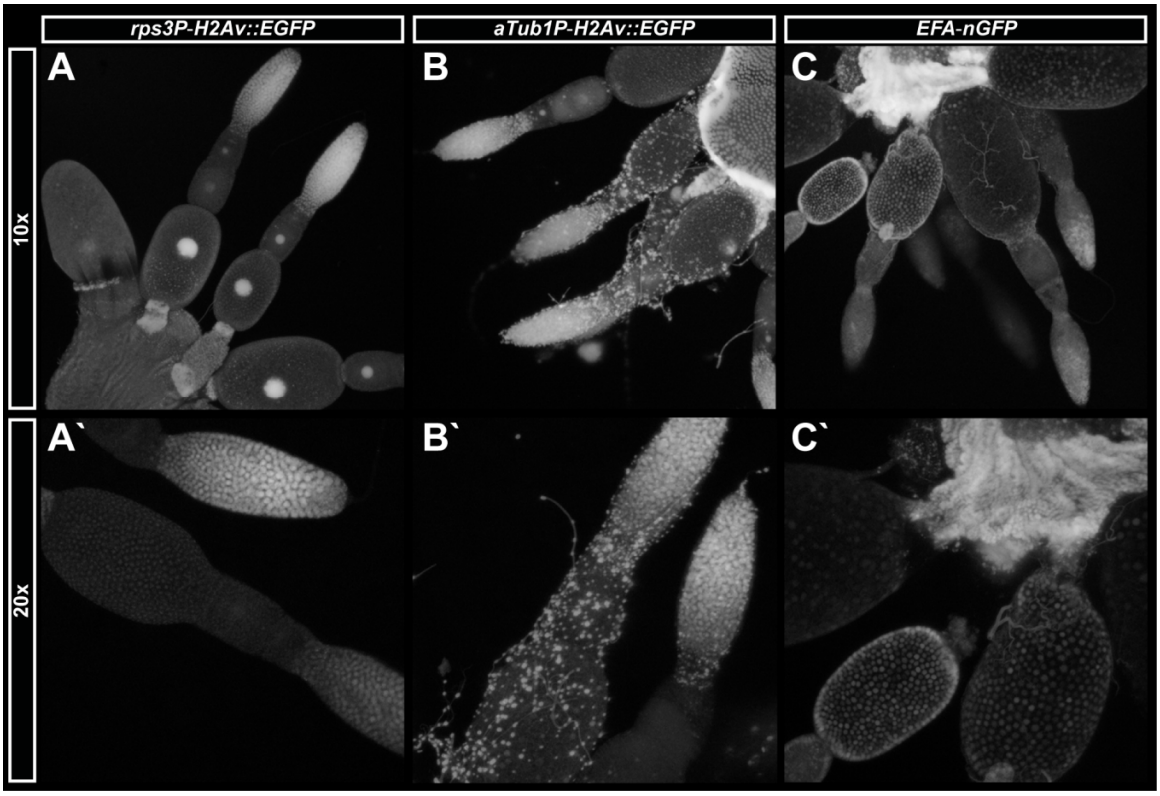

Figure 4.35 **Qualitative comparison of signal intensity and localization of three different ubiquitous nuclear reporter lines in ovaries.** Nuclei are visualized using the transgenic imaging lines *rps3*P-*H2Av::EGFP* (**A**, **A`**), *αTub1*P-*H2Av::EGFP* (**B**, **B`**) and *EFA-nGFP* (**C**, **C`**; El-Sherif et al., 2012). The dissected ovaries are depicted as maximum projections. (**A**-**C`**) All three transgenic lines show comparable signal intensities in all structures of the ovaries. Although, only in the ovary of the *rps3*P-*H2Av::EGFP* line depicted (**A**), could be in all three lines ovaries observed that show a signal in the pro-nucleus of germ cells.

Trait / Promoter	Signal			Viability
	Embryonic	Larval/Pupal	Adult	
*αTub1*P-*H2Av::EGFP*	→	↗	↗	↘
*rps3*P-*H2Av::EGFP*	→	→	→	↘
*PUb*P-*H2Av::EGFP*	↑	↑	↑	↓
EFA-nGFP	↑*	→*	↗*	↑

Figure 4.36 **Summary of the qualitative analysis of four different ubiquitous nuclear reporter lines.** The strongest signal was detectable in the transgenic *PUb*P-*H2Av::EGFP* nuclear reporter line. However, due to low viability the line died out before a detailed analysis could be started. The *αTubulin1*P-*H2Av::EGFP* line showed the best quality, due to stronger signals especially at post-embryonic stages. The already published *EFA-nGFP* line (El-Sherif et al., 2012) was the best, considering the signal at early embryonic stages and the viability. However, a patchy expression pattern and the blurry signal during mitosis are drawbacks (asterisks).

4.2.1.1 Ubiquitous H2Av::EGFP expression - Proof of principle

In order to test whether the new imaging lines are suitable for future in vivo imaging experiments, I performed a first proof of principle experiment using the *αTub1*P-*H2Av::EGFP* imaging line. Collaborating with Dr. Sven Poppelreuther (Carl Zeiss Microscopy GmbH), I had the opportunity to image the line with the Lightsheet Z.1 microscope (ZEISS) for several hours (18 h, 3-21 h AEL). We imaged simultaneously from a dorsal and ventral view in time intervals of three minutes. The video (see section **S7.29** and the representative time frames: **Figure 4.37**) shows that the *αTub1*P-*H2Av::EGFP* imaging line is functional, showing a signal, which is localized to the DNA and is equally distributed over the embryo throughout the stages (**Figure 4.37A-J**). Each nucleus appears to have a strong signal, which reveals the overall morphology of the embryo and key steps of early embryonic development like gastrulation (**Figure 4.37B**), the amniotic fold (**Figure 4.37C**), germ band elongation (**Figure 4.37D-J**) and limb bud growth (**Figure 4.37J**). Based on this result, I also collaborated with Dr. Stefan Münster (Tomancak Lab; MPI-MCBG in Dresden), trying to further exhaust imaging using the Lightsheet Z.1 microscope, and specifically in order to record videos, which are well suited for cell tracking and fate mapping. To this end, we tried to record several in vivo imaging videos of developing embryos. However, most of them died during the process only one embryo survived the treatment. The video was taken from three different views (with 180° spacing each) and a time interval of three minutes. Afterwards, the video was completely processed by Dr. Stefan Münster, including registration and rendering (see section **S7.30** and representative time frames: **Figure 4.38**). The video depicts a rendered 3D projection of an embryo (1.5-24 h AEL) from a lateral view. The video shows that light-sheet-based fluorescence microscopy (LSFM) is in combination with the *αTub1*P-*H2Av::EGFP* imaging line a powerful tool, but the recorded video is so far not well suited for fate mapping and especially not for automatic cell tracking, due to a bad signal to noise ratio, resolution and the signal of the extra-embryonic tissue, which partially covers the embryonic signal.

More interested in the morphogenetic movements of the head, I also tried to image only the head in order to perform cell tracking and fate mapping experiments. To this end, I imaged the anterior cap using the *αTub1*P-*H2Av::EGFP* line with the LSM 780 microscope (ZEISS) and Dr. Stefan Münster did the same with the Lightsheet Z.1 microscope. These two videos (see **section S7.31** and **S7.32**, representative frames: **Figure 4.39**) give a short impression about the morphogenetic movement of the head. Both microscopes are suitable for this purpose in principle, but the LSFM technique allows following up complete morphogenesis of the head with its potential to image from different angles. With head development starting at a mid-ventral region of the egg (**Figure 4.37C**) and ending dorsally at the anterior pole (**Figure 4.37J**), it is only

possible to record certain time frames of the development by using the LSM. Thus, the LSFM technique is more suitable for long-term cell tracking of the embryonic head development, whereas conventional confocal imaging could be used to image certain aspects of development.

Taken together, these proof of principle experiments indicate that the *αTub1*P-*H2Av::EGFP* imaging line is suitable for in vivo imaging experiments as well as in combination with LSFM.

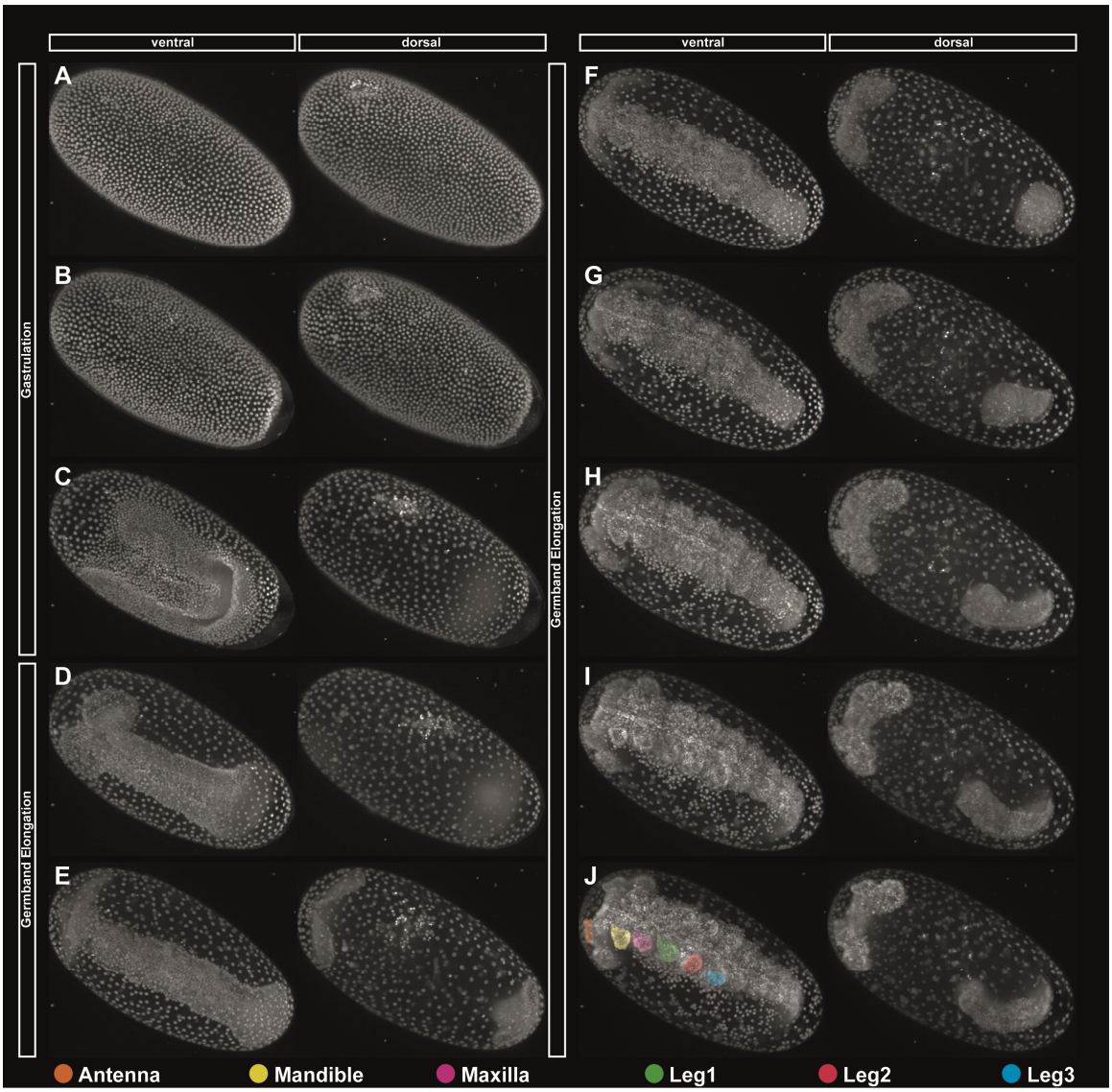

Figure 4.37 **Early embryonic development imaged using the transgenic *αTubulin1*P-*H2Av::EGFP* line in combination with LSFM technique I.** Anterior is left. Nuclei are visualized by using the transgenic *αTub1*P-*H2Av::EGFP* nuclear reporter line. Ventral and dorsal views were simultaneously imaged and are depicted as maximum projections. (**A**) Blastoderm stage, which has finished the last round of cell division. (**B**) Start of amniotic fold. (**C**) Formation of the germ rudiment. (**D**-**J**) Germ band elongation. Note that the anterior head is first localized at a ventral sub-terminal position within the egg (**C**) and ends up at the anterior cap of the egg (**J**). Limb buds start to grow out (**G**) and became later clearly recognizable (**J**).

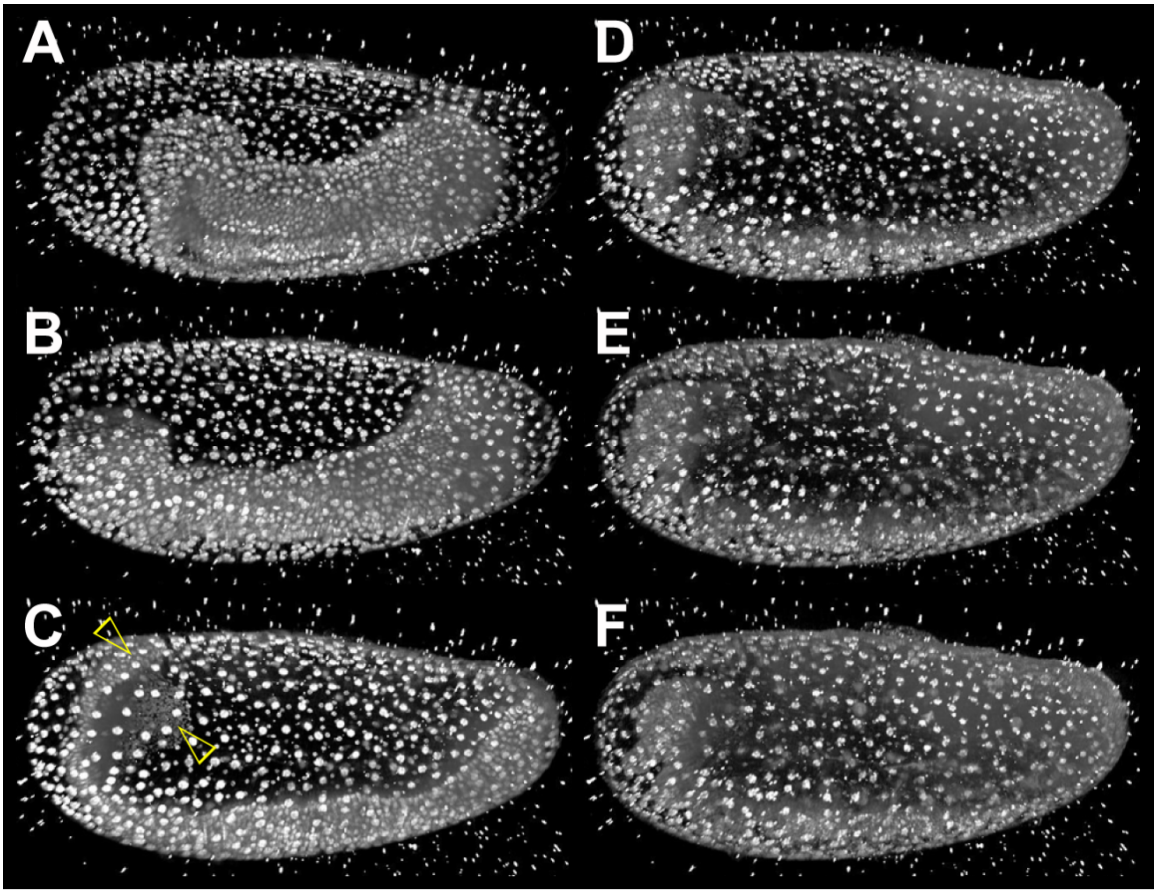

Figure 4.38 **Early embryonic development imaged with the transgenic *αTubulin1*P-*H2Av::EGFP* line in combination LSFM technique II.** Anterior is left. The embryo was simultaneously imaged from three different angles. The embryo is depicted as rendered 3D projection. Spotty signals, outside of the embryo, are resulting from fluorescent beads, which are added to the mounting medium. These beads are used as landmarks to reconstruct the embryo by registration and fusion of the three separately imaged angles into one. (**A**-**F**) The imaging of three different angles and the subsequent reconstruction of the data by bead-based registration and rendering allows having a look through the entire embryo (**C**: empty arrowheads).

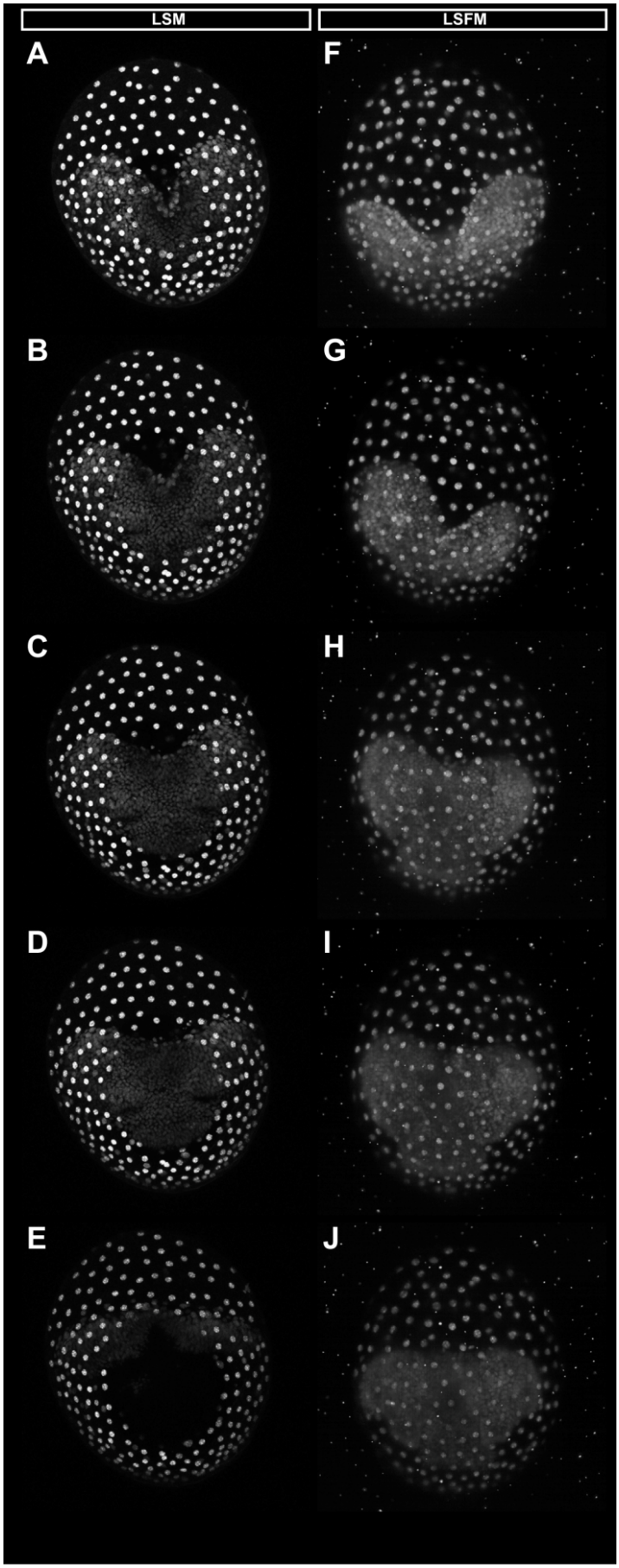

Figure 4.39 Comparison between conventional LSM and LSFM imaging of the anterior embryonic cap. Anterior is up. Nuclei are visualized by using the transgenic *αTub1*P-*H2Av::EGFP* line and imaged either by laser scanning microscopy (**A**-**E**, LSM) or light-sheet-based fluorescence microscopy (**F**-**J**, LSFM) imaging techniques. The embryos are depicted as maximum projections. (**A**-**E**) The movement of the anterior head from the ventral sub-terminal position of the egg to the anterior cap impedes tracing of the complete embryonic head development until larval stages via conventional LSM microscopy. (**F**-**J**) A proof of principle experiment shows that the possibility of different imaging views could be also exploited trying to trace the complete embryonic head development. The embryo was imaged from three different frontal views (1. ventro-frontal, 2. frontal, and 3. dorso-frontal).

4.2.2 Generation of cell marking lines for tracking experiments to assemble an exact head fate map

In order to get a better understanding of the morphogenetic movements of the head, I wanted to generate stable transgenic lines, which allow non-invasive cell marking and tracking. With this new tool it would be possible to precisely track down the movement of cells from early embryonic to L1 larval stages. This information would allow to assemble an exact head fate map and to get information about the cellular basis of head defects in loss-of-function or gain-of-function experiments. I tried to generate two different cell marking systems. (**1**) I wanted to generate a targeted laser-induced cell marking system on the basis of a photoactivatable fluorescent protein. This system allows transient but precise marking of single cells or small cell groups. Laser-induced cell marking in *Tribolium*, has so far only been shown by injecting mRNA of the actin-binding peptide fused to the photoconvertible Eos fluorescent protein (Benton et al., 2013; Izeddin et al., 2011) into embryos. Drawbacks of this transient system are that (I) the mRNA synthesis is expensive in the long-term, (II) embryonic injection is time-consuming, and (III) the method is invasive and could interfere with normal development. The generation of stably transgenic lines that allow laser-inducible cell marking would help to overcome these obstacles. (**2**) In addition, I wanted to generate a cell marking system on the basis of genetically marked cell clones, which lead to a random but permanent marking of small cell groups, which can be then traced throughout development. To this end, the Cre/*loxP* based *brainbow* cell marking system (Livet, 2007) for permanently genetic cell marking was already established in *Tribolium* (Averof, pers. communication). However, the system was so far suffering from low numbers of marked cells, and weak signal intensity of these marked cells (Bucher pers. communication). On this basis, I also wanted to exploit the Cre/*loxP* system (Metzger, 1999; Sternberg and Hamilton, 1981), with the following modifications concerning the responder line: (I) Use of another, more active ubiquitous promoter (instead of *EFA*); (II) Use of a stronger fluorescent marker protein (instead of m-RFP, m-YFP, and m-CFP); (III) Use of only one *loxP* flanked marker cassette (instead of *loxP*, *loxN*, and *lox2272*); (IV) Use of a fluorescent body marker expressed in unmarked cells (instead of no body marker).

4.2.2.1.1 Photoactivatable GFP lines for precise cell marking

For the transient laser-induced cell marking system, I decided to use the photoactivatable fluorescent protein 'Cycle 3' mutant of GFP (C3PA-GFP; Ruta et al., 2010). The C3PA-GFP is an advancement of the first generation of photoactivatable-GFP (PA-GFP; Patterson, 2002) and shows a better and more stable signal after photoactivation (Ruta et al., 2010). These photoactivatable GFPs are mutants of the wt GFP (Tsien, 1998), marked by several amino acid substitutions. These substitutions lead to an altered, non-fluorescent confirmation of the GFP molecule. Upon laser treatment (appr. 400 nm) the molecule undergoes a conformational change leading to strong fluorescence after excitation (appr. 488 nm; Patterson, 2002). I generated two different transgenic lines with C3PA-GFP as fluorescent marker. Both lines drive ubiquitous expression under the control of the *Tc-αTub1* promoter. However, for one line I generated a chimeric C3PA-GFP, which is histone-tagged (H2Av::C3PA-GFP) and in consequence nuclear localized, whereas the other line carries the untagged cytosolic C3PA-GFP version.

4.2.2.1.2 Photoactivatable GFP lines - Proof of principle

I was able to generate several independent lines for both variants of the transgenic lines (*αTub1*P-*C3PA-GFP* (n=6) and *αTub1*P-*H2Av::C3PA-GFP* (n=7)). Afterwards, I tested all lines for functionality at embryonic stages. I collected eggs (0-24 h AEL) of all lines (pool of hetero- and homozygotes), and performed photoactivation via LSM for several times in an arbitrarily chosen region of the embryo. Before photoactivation and after each activation cycle I imaged the embryos to analyze changes regarding the fluorescent signal. The results of these lines showing the most efficient photoactivation capacity are shown in **Figure 4.40A-D```**. Embryos of the two independent *αTub1*P-*C3PA-GFP* lines (*αTub1*P-*C3PA-GFP*$_{1\_1}$ and *αTub1*P-*C3PA-GFP*$_{2\_3}$) showed a considerable activation of the cytosolic fluorescent signal (**Figure 4.40A-B```**) already after the first cycle of photoactivation within the activated region (**Figure 4.40A**, **B**: dashed circles). However, the *αTub1*P-*C3PA-GFP*$_{2\_3}$ line showed a better signal-to-noise ratio after each photoactivation cycle (**Figure 4.40**, compare **A`** and **B`**). The two independent transgenic lines carrying the histone-tagged C3PA-GFP (*αTub1*P-*H2Av::C3PA-GFP*$_{w7}$ and *αTub1*P-*H2Av::C3PA-GFP*$_{w5}$*)* showed a weaker photoactivation capacity (**Figure 4.40C-D```**) compared to the lines with the untagged C3PA-GFP (**Figure 4.40A-B````**). Furthermore, the experiment showed -with the used laser lines (351/361 nm) and settings (**see section 3.12**) - that the signal of the *αTub1*P-*H2Av::C3PA-GFP* lines unexpectedly was localized in the cytosol but not

in the nucleus (Figure 4.40C````: yellow box). However, Anna Gilles (Averof lab, IGFL in Lyon (France)) was able to reveal, with a different laser line (405 nm), that the lines show a nuclear localized fluorescent signal upon photoactivation (Figure 4.40E).

Additionally, I was asking whether photoactivation is also feasible at larval and pupal stages. To this end, I performed a proof of principle experiment using all independent lines of *αTub1*P-*C3PA-GFP* and *αTub1*P-*H2Av::C3PA-GFP* (data not shown). The analysis revealed that only the lines expressing the cytosolic C3PA-GFP showed a detectable photoactivation-dependent fluorescent signal (data not shown). The line *αTub1*P-*C3PA-GFP*$_{2\_3}$ showed the best results, with a clear photoactivation-dependent fluorescent signal at larval and pupal stages (Figure 4.41A-B`). However, the signal was not uniformly distributed indicating an uneven distribution of cells or uneven expression (Figure 4.41A`, B`).

Taken together, these proof of principle experiments show that the transgenic laser-inducible cell marking lines are functional concerning their photoactivation capacity as well as the localization of the activated fluorescent signal. However, first long-term experiments revealed that the embryos were arrested in development upon photoactivation at 350-400 nm.

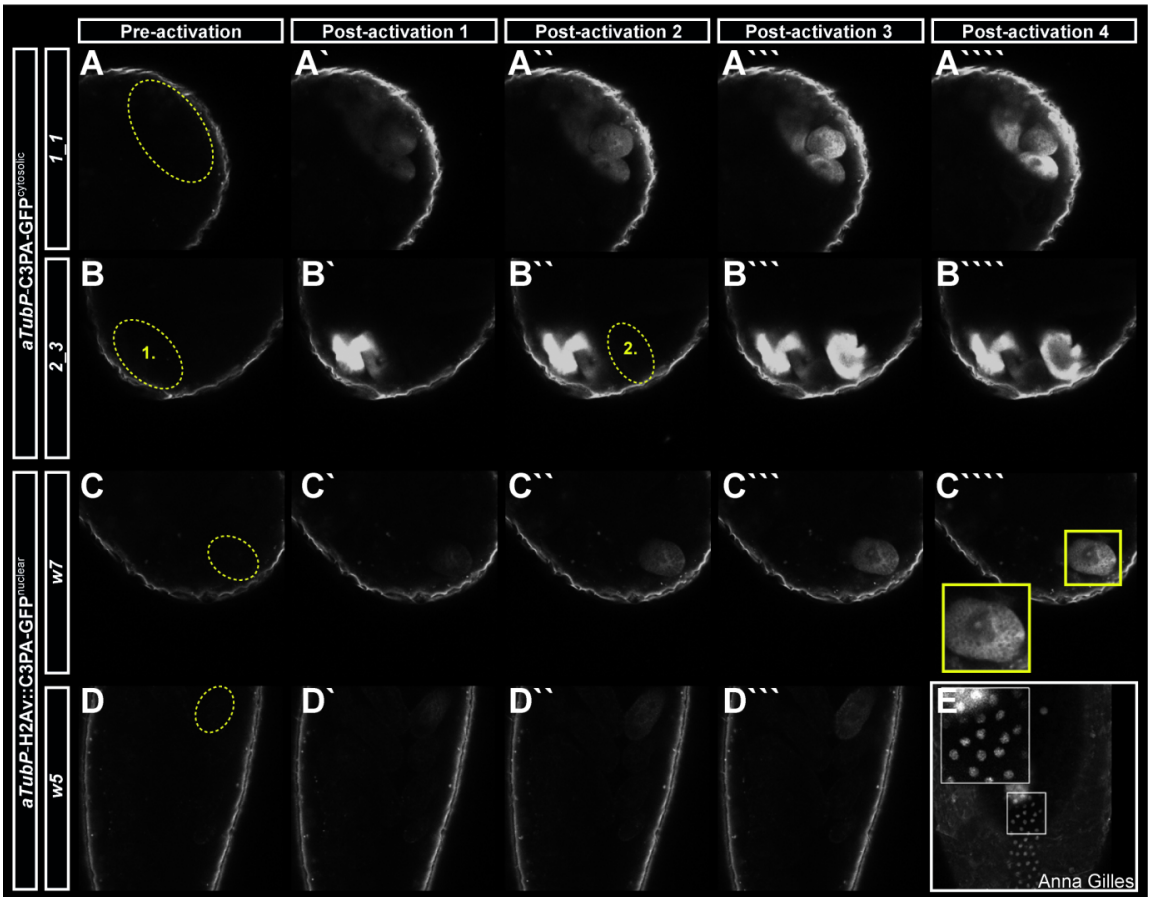

Figure 4.40 **Test of C3PA photoactivation in different transgenic lines.** Depicted are embryos from two independent lines, carrying the *αTub1*P-*C3PA-GFP* construct (**A**-**B````**), and two independent lines carrying the *αTub1*P-*H2Av::C3PA-GFP* construct (**C**-**E**), before (**A**, **B**, **C**, **D**) and after photoactivation. Regions of photoactivation are marked by dashed circles. (**A**, **B**, **C**, **D**) Before photoactivation no C3PA-GFP signal is detectable. (**A`**-**A````**) In embryos of the *αTub1*P-*C3PA-GFP*$_{1\_1}$ line the signal intensity is successively increases after each cycle of photoactivation. (**B`**-**B````**) Embryos of the *αTub1*P-*C3PA-GFP*$_{2\_3}$ line show already strong fluorescent signal intensity after one cycle of photoactivation (**B`**: ROI 1 and **B```**: ROI 2). The signal of both lines is localized within the cytoplasm (**B````**). (**C**-**C````**) Embryos of the *αTub1*P-*H2Av::C3PA-GFP*$_{w7}$ line show a weak, but increasing, signal intensity after each cycle of photoactivation. (**D**-**D```**) Embryos of the *αTub1*P- *H2Av::C3PA-GFP*$_{w5}$ line show the weakest photoactivation capacity. With the used laser line (351/364 nm) and settings nuclear localization of the signal could not be obtained (**C````**: box). However, Anna Gilles was able with the same transgenic H2Av::C3PA-GFP line, but a different laser line (405 nm), to get a nuclear localized signal upon photoactivation (**E**: box).

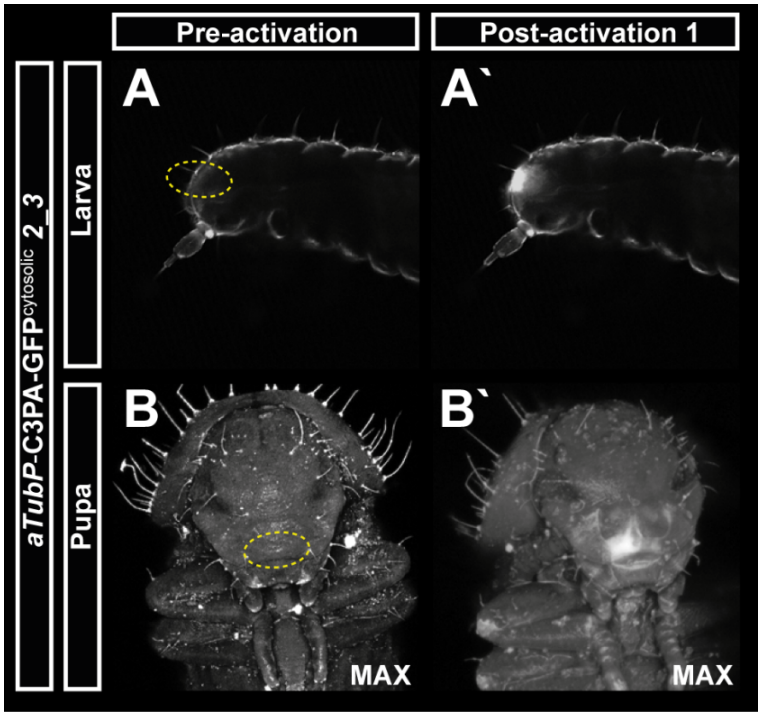

Figure 4.41 **Test of C3PA photoactivation capacities at larval and pupal stages.** Depicted is a larva, in single plane section (**A**, **A`**) and a pupa, as maximum projections (**B**, **B`**) from the line *αTub1*P-*C3PA-GFP*$_{2\_3}$ before (**A**, **B**) and after photoactivation (**A`**, **B`**). Regions of photoactivation are marked by dashed circles. (**A**, **B**) Before photoactivation no C3PA-GFP signal is detectable. (**A**-**B`**) Photoactivation of the C3PA-GFP in larvae and pupae of the transgenic line *αTub1*P-*C3PA-GFP*$_{2\_3}$ is feasible, but shows an uneven signal distribution.

4.2.2.2 Responder line for a genetic cell marking system

Embryonic development of *Tribolium* takes several days (at 32°C about three days; (Brown et al., 2009)). It is questionable whether the amount and photo-stability of the photoactivated molecules will be high enough to image the development repeatedly for a longer period of time. Trying to overcome this potential drawback of the photoactivation based cell marking system, I also generated transgenic responder lines, which are able, with the adequate driver line, to genetically and therefore permanently mark cells. For this purpose I exploited the Cre/*loxP* system (Metzger, 1999; Sternberg and Hamilton, 1981). The driver line was already established in my lab (unpublished). This transgenic driver line is expressing the Cre (*causes recombination*) recombinase under the control of the *Tc-hsp68* promoter. Upon heat shock treatment the Cre recombinase is ubiquitously expressed. However, stochastic distribution of Cre expressing cells and level-dependent activity of the Cre recombinase lead only in a small subset of cells to excision events of *loxP* (locus of crossing over (x), P1) flanked sequences (Bucher, pers. communication). My aim was to generate a transgenic *loxP* responder line (**Figure 4.42**). In the off-state, the *loxP* responder line should drive ubiquitous expression of cytosolic monomeric Cherry (mCherry, red flourescent protein; **Figure 4.42A**) as body marker. After crossbreading with the driver line and heat shock treatment cells should express the Cre recombinase (**Figure 4.42B**, **C**). In consequence, cells expressing the Cre recombinase should stop the mCherry expression and start the expression of H2Av::EGFP, due to a cyclization and excision event of the *loxP*-flanked cassette (**Figure 4.42C**, **D**, **E**). Thus, a small cell population should be marked by a green nuclear localized signal (**Figure 4.42E**), whereas the unmarked cells should still express mCherry marked by a red cytosolic signal (**Figure 4.42A**).

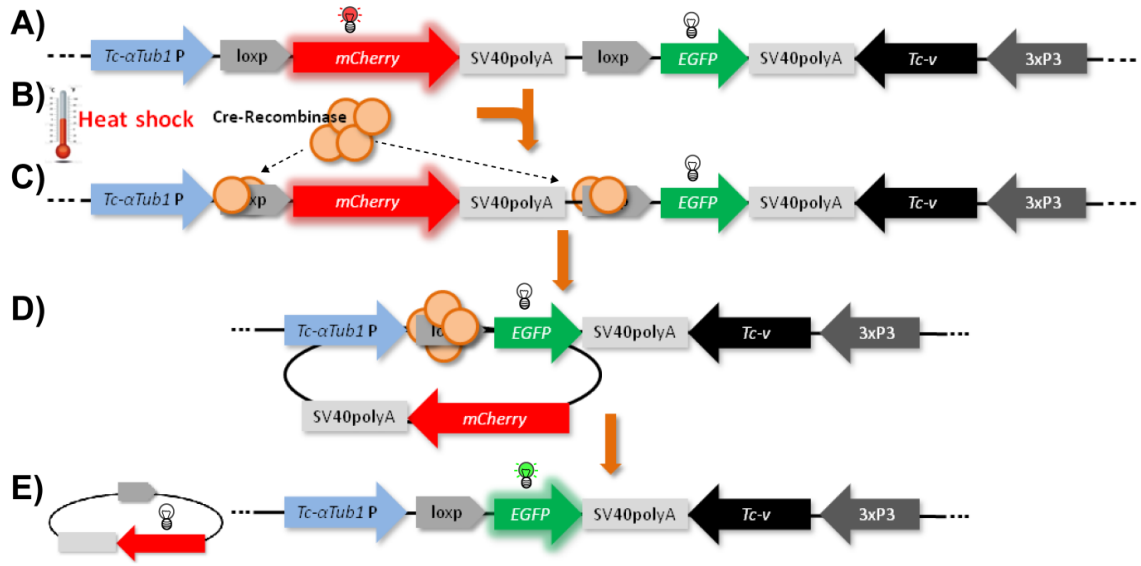

Figure 4.42 **Scheme of the genetic cell marking system exploiting the Cre/*loxP* system.** (**A**) In the off state, the *loxP* responder line carrying the ubiquitous promoter (*Tc-αTub1*P), which drives ubiquitous expression of the red body marker (mCherry, cytosolic). The body marker is flanked by two *loxP* sites, which are oriented in the same direction. The body marker cassette is followed by the green cell clone marker (EGFP, histone-tagged for a nuclear localization), which is not expressed in the off state. The transgenesis marker (*Tc-v*), which is driven by an eye-specific promoter, rescues the eye color in transgenic individuals within the *Tc-v*^{w} strain (white eyes switch to black eyes). The Cre driver line drives expression of the Cre recombinase, upon heat shock treatment (not depicted here). (**B**) Crossing of the *loxP* responder line with the Cre driver line and the subsequent heat shock treatment of the offspring leads to random expression of Cre recombinase. (**C**) The Cre recombinase enzyme binds to the *loxP* sites. (**D**) Due to the orientation of the *loxP* sites, the Cre recombinase starts the cyclization and excision of the *loxP*-flanked sequence. (**E**) The excision abolishes expression of the body marker, but induces expression of the green cell clone marker. Thus, cells will be marked by a green signal within the nuclei.

4.2.2.2.1 Genetic cell marking system - Proof of principle

In order to test whether the generated *loxP* responder lines are functional, I performed two proof of principle experiments. The first experiment should reveal whether the uncrossed *loxP* responder line shows only the red body marker signal for unmarked cells. The second experiment should show whether, in offspring of the crossbred driver and the responder lines, a small population of cells is marked by a green signal upon heat shock treatment. To this end, I collected eggs (0-48 h AEL) from the *loxP* responder line and from crossbreds of the independent *loxP* responder lines and the Cre driver line. Offspring were either untreated (neg. control) or treated with one heat shock. The individuals were analyzed the following day.

Analysis of the offspring of the uncrossed independent *loxP* responder lines revealed that most of the lines showed the red body marker (n=4/6) but no green signal (data not shown) as expected. Heat shock treatment of the crossbred offspring (Cre driver x *loxP* responder lines) led to marked cells showing a green signal in all four lines (result of the independent line *αTub1*P-*Lox(mcherry)*;*H2Av::EGFP*$_7$ as representative: **Figure 4.43A-B``**). Further, the signals were correctly localized. The red signal was, although weak and granular, localized to the cytosol (**Figure 4.43A**, **B**) and the green signal was nuclear localized (**Figure 4.43A`**, **B`**: white box). Both signals could be traced in embryos (**Figure 4.43A``**, **C``**) as well as in L1 larvae (**Figure 4.43B``**, **D``**). However, the number of genetically marked cells was very high (**Figure 4.43A``**, **B``**). Asking whether those embryos that received no heat shock treatment, showed a lower number of marked cells, I analyzed them regarding the number of cells expressing H2Av::EGFP. The analysis revealed that also the untreated individuals showed a very high number of marked cell clones (**Figure 4.43C-D``**).

In summary, the generated *loxP* responder lines are functional and are able to respond to the Cre expression of the driver line. However, the number of marked cells was very high, even without heat shock treatment, and therefore not suitable for cell tracking and fate-mapping experiments.

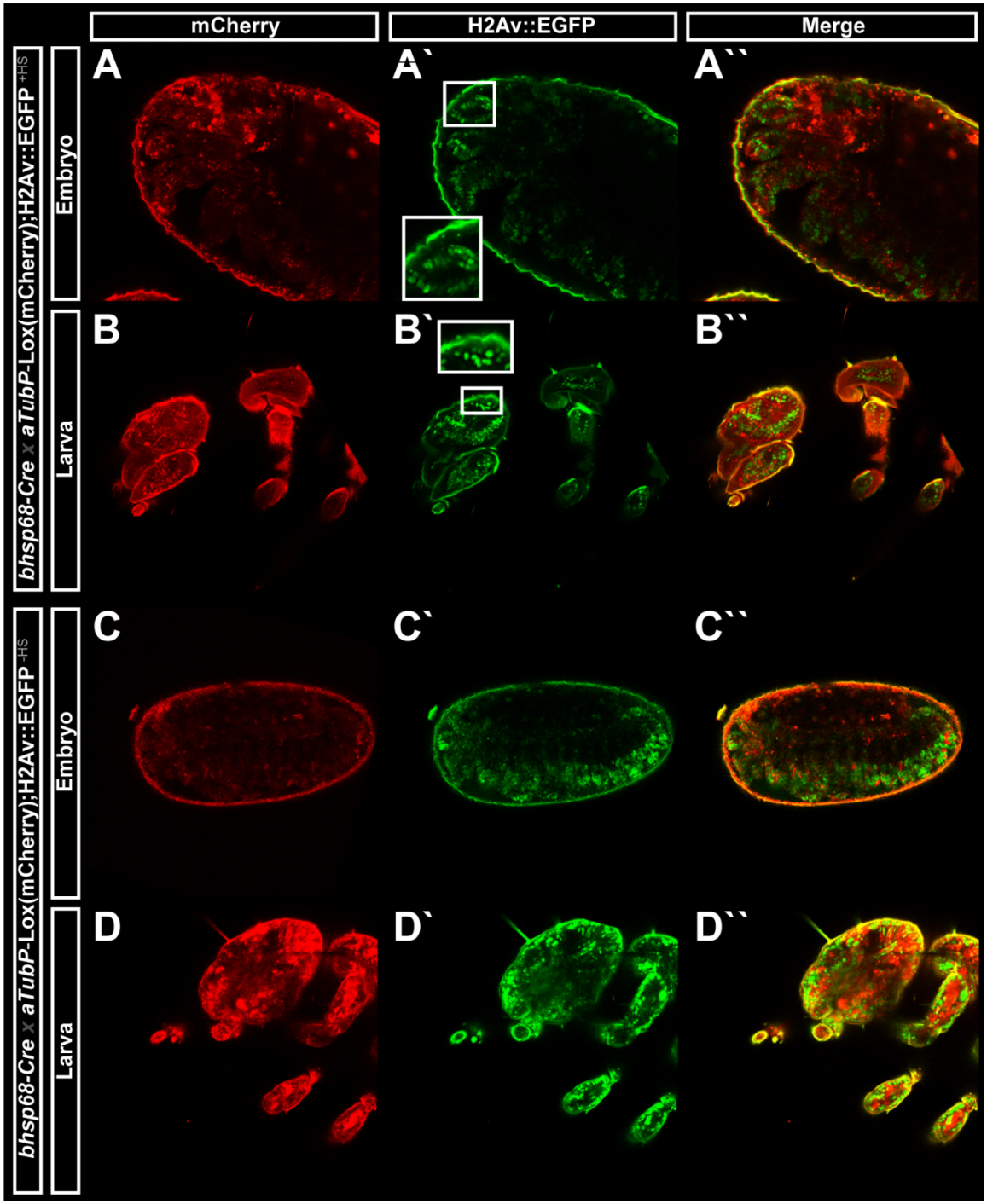

Figure 4.43 **Test of the genetic cell marking system.** Depicted are embryos and larvae from the crossbreeding of the *hsp68-Cre* driver and the *αTub1*P-*Lox(mcherry)*;*H2Av::EGFP*$_7$ responder line, which were either heat shocked during embryonic stages (0-48 h AEL; **A**-**B``**) or remained untreated (**C**-**D``**). (**A**-**A``**) Embryos of the crossbred show a weak but ubiquitous mCherry expression (**A**). Further, a subset of cell clones is marked by the expression of H2Av::EGFP (**B**) which is nuclear localized (white box). These findings suggest functionality of the *loxP* responder line and the cell marking system. However, the number of marked cell clones is very high. (**B**-**B``**) The body marker and marked cell clones are detectable

also at larval stages. (**C**-**D**``) The number of marked cell clones (**C**`, **D**`) is also very high in crossbred offspring, which were not heat shocked.

5

Discussion

5.1 *Tc-foxq2* is required for head and brain development

5.1.1 Significance of the study

foxq2 was shown to be expressed at the anterior pole in many metazoan species. Within arthropods, *foxq2* expression was so far only reported for *Drosophila* and *Strigamia,* being located at the anterior pole, as well. Furthermore, *foxq2* was suggested to form together with *six3* a conserved core regulatory module, which is required for anterior patterning. So far functional data was only reported for the deuterostome *Strongylocentrotus* with having a function in ectodermal and neural development, and for the cnidarian *Nematostella* with having a function in neural development as well. However, data on *foxq2* function in protostomes was completely missing. In this work, I was able to show that *foxq2* function is required for proper epidermal and neural development. Further, I was able to reveal a comprehensive gene regulatory network that shows that *foxq2* is acting -together with *six3*- as a core regulatory module for anterior head development.

5.1.1.1 Gene regulatory network of anterior head development

One of the main aims of this study was to identify the function of *Tc-foxq2* in the gene regulatory network responsible for pattering the anterior head. This study revealed that *Tc-foxq2* expression is localized at the apical pole (**Figure 5.3D**), and is antagonized by Wnt/ß-catenin signaling (**Figure 5.1**), as expected from previous data. Its function is essential for the correct formation of the anterior ectoderm. On the basis of the expression pattern of *Tc-foxq2*, co-expression studies with head patterning genes as well as knock-down and gain-of-function studies I integrated *Tc-foxq2* into the already established interaction network (Based on Kittelmann et al., 2013; Posnien et al., 2011b; Siemanowski et al., 2015). The hierarchal order of the genes and their interactions shown in **Figure 5.1** were based on: 1. Expression onset,

2. time/region of co-expression, 3. expression data gathered from knock-down/gain-of-function experiments, and 4. extent of defects in L1 larvae after knock-down. It is noteworthy that both cell death rate and cell proliferation showed no significant differences during early patterning of the anterior head in *Tc-foxq2*^{pRNAi} embryos compared to wt embryos. Therefore, the early effects of *Tc-foxq2* on the head patterning genes are very likely primary regulatory interactions and not secondary effects due to tissue loss. My data revealed that *Tc-foxq2* is a key upstream factor in pattering the anterior ectoderm and neuroectoderm (Figure 5.1).

I was able to show that *Tc-foxq2* and *Tc-six3* are forming a core regulatory unit responsible for patterning the anterior head. This finding is based on the fact that both show similar epidermal and neural phenotypes, in knock-down experiments. Further, *Tc-foxq2* is co-expressed with *Tc-six3* at the anterior pole, at most developmental stages (Figure 5.4). Both together share an upstream position in the anterior head gene regulatory network and show a highly similar regulation of several downstream targets. Moreover, *Tc-foxq2* and *Tc-six3* are regulating each other mutually from early stages onwards. This is similar to the eye network where mutual activation by *eyeless*, *eyes absent*, *dachshund*, *twin of eyeless*, and *sine oculis* is required for eye development and where mutations in either of those genes lead to loss of eyes (Wagner, 2007).

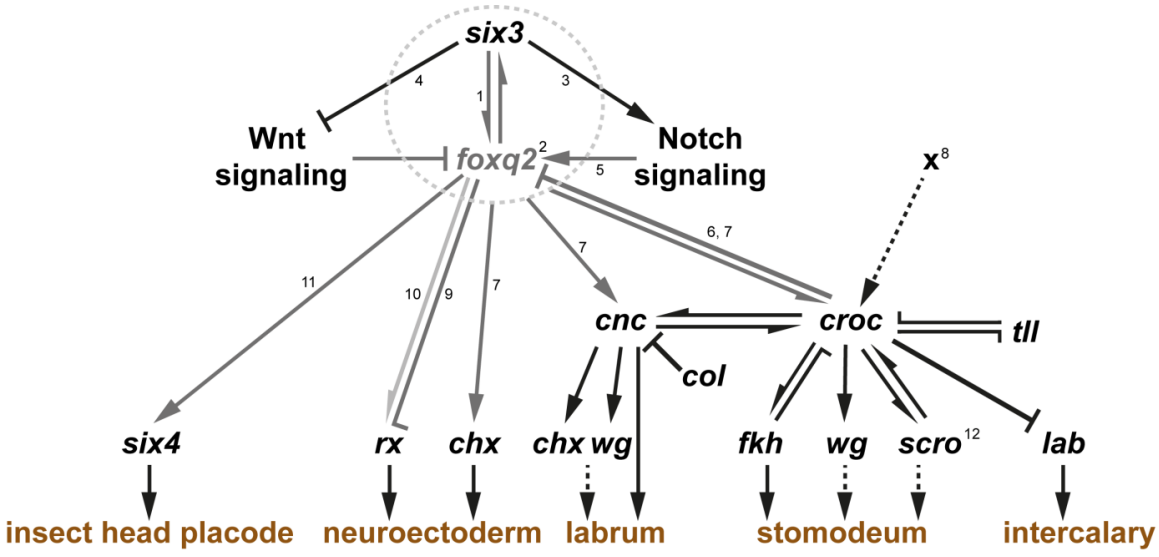

Figure 5.1 *Tc-foxq2* is an upstream player within the gene regulatory network of the anterior *Tribolium* head. Black lines indicate previously reported parts of the network (Based on Kittelmann et al., 2013; Posnien et al., 2011b; Siemanowski et al., 2015). Grey lines represent new data based on *Tc-foxq2* experiments. Arrows represent gene activation, and cross-bars gene repression. Dashed lines indicate hypothetical effects. (**1**) *Tc-six3* is the major factor for patterning the anterior head due to the early onset of expression and the strongest knock-down cuticle phenotypes with epidermal defects spanning the

labral to the ocular regions and neural defects marked by a loss of the central body, defective mushroom bodies and fused brain hemispheres. (**2**) *Tc-foxq2*, like *Tc-six3*, is a key player in anterior head development with a somewhat later onset of expression than *Tc-six3*, but similar cuticle phenotypes (epidermal & neural), and comparable activities in patterning of the anterior head. Mutual activation and similar phenotypes suggest that they form a regulatory module (indicated by a dashed circle). (**3**) *Tc-six3* acts on Notch signaling via *Tc-ser*. However, it is not clear whether the effect is primary or secondary. (**4**) *Tc-six3* prevents the ocular *Tc-wg* domain from expansion into the AMR, but is not acting on other *Tc-wg* domains. (**5**) Notch signaling-dependent activation of *Tc-foxq2* is only partial and restricted to lateral parts of the anterior median *Tc-foxq2* domains (*Tc-mib1* data). (**6**) *Tc-foxq2* like *Tc-six3*, only regulates the anterior (ectodermal) part of the *Tc-croc* expression domain. (**7**) Regulative activity on several downstream targets is essentially indistinguishable between *Tc-foxq2* and *Tc-six3* knock-down experiments. (**8**) An unknown factor '*X*' is predicted to regulate the posterior (mesodermal) part of the *Tc-croc* expression, because *Tc-six3* and *Tc-foxq2* regulate only the anterior (ectodermal) portion of the *Tc-croc* AMR domain (6). (**9**, **10**) Results of *Tc-foxq2* gain-of-function and knock-down experiments are conflictive. Since, *Tc-rx* is repressed when *Tc-foxq2* is ectopically expressed (9), simultaneously *Tc-rx* expression vanishes in *Tc-foxq2* knock-down experiments (10), indicating activation by *Tc-foxq2*. However, in the relevant stages both genes are mutually exclusively expressed, which argues against direct activation (10, indicated by light grey arrow) and points to interaction via diffusible signaling molecules. (**11**) Data for interaction of *Tc-six3* with *Tc-six4* is missing, and thus it is impossible to judge whether it is regulated by the *Tc-foxq2*/*Tc-six3* regulatory module so far (dashed line). (**12**) The late effect of *Tc-foxq2* on *Tc-scro*, observed in gain-of-function experiments, is most likely secondary and is, hence, not considered.

5.1.1.2 Late *Tc-foxq2*-associated effects

The genetic interaction studies on *Tc-foxq2* do not only reveal alterations in the expression pattern and morphology at early embryonic stages, but also at stages from limb formation onwards.

Figure 5.2 gives an overview of the later effects of *Tc-foxq2* knock-down. However, most of these interactions could not be clearly determined as being a primary or secondary effect. This is due to the fact that in knock-down experiments most of the genes at these stages show an altered cell death rate, cell proliferation rate or tissue malformation (**Figure 4.5**, Kittelmann et al., 2013; Posnien et al., 2011b; Siemanowski et al., 2015).

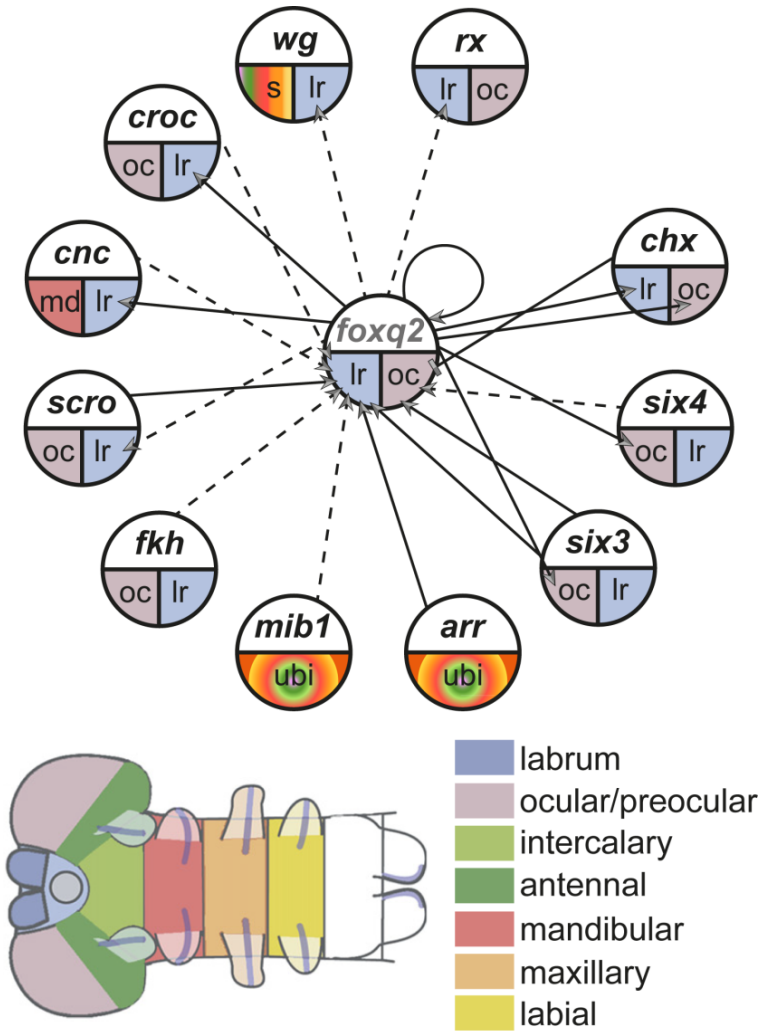

Figure 5.2 **Late effects of and on *Tc-foxq2*.** The upper part shows the non-hierarchical interaction network at late embryonic stages. Each circle shows in the top part the gene name and in the bottom part the regions, in which the respective gene shows expression. The continuous lines indicate interactions, which are most probably primary. Dashed lines indicate interaction, which could be primary or secondary. Ends of the lines indicate activation (arrowheads) or repression (crossbar) and point to the affected expression domain. The lower part of the figure serves as legend demonstrating the location of the affected region (Embryonic scheme taken from Posnien and Bucher, 2010). lr: labrum, md: mandibular, oc: ocular/preocular, s: segmental, ubi: ubiquitous

5.1.1.4 *foxq2* and *six3* - a highly conserved signaling center for pattering the apical pole

Representatives of the *foxq2* subfamily were found in various species distributed over the metazoan kingdom (Chapman et al., 2010; Chevalier et al., 2006; Darras et al., 2011; Fritzenwanker et al., 2014; Hope, 2003; Hunnekuhl and Akam, 2014; Koziol et al., 2016; Larroux

et al., 2008; Lee and Frasch, 2004; Marlow et al., 2014; Martín-Durán et al., 2015; Martín-Durán and Hejnol, 2015; Mazet et al., 2003; Santagata et al., 2012; Shimeld et al., 2010; Sinigaglia et al., 2013; Tu et al., 2006; Yaklichkin et al., 2007; Yu et al., 2008, 2003; Zhang et al., 2014). The majority of these species show a well-conserved expression of *foxq2* at the anterior/apical pole (**Figure 5.3**; Chevalier et al., 2006; Darras et al., 2011; Fritzenwanker et al., 2014; Hunnekuhl and Akam, 2014; Lee and Frasch, 2004; Martín-Durán et al., 2015; Santagata et al., 2012; Sinigaglia et al., 2013; Tu et al., 2006; Yu et al., 2003). Furthermore, some recent studies showed co-expression of *foxq2* and *six3* at the apical pole (**Figure 5.4**; Fritzenwanker et al., 2014; Hunnekuhl and Akam, 2014; Marlow et al., 2014; Martín-Durán et al., 2015; Santagata et al., 2012; Sinigaglia et al., 2013; Tu et al., 2006; Wei et al., 2009). So far mutual co-regulation for *foxq2* and *six3* was only shown in *Strongylocentrotus*. In *Strongylocentrotus*, *six3* activates *foxq2*, while *foxq2* is repressing *six3* (Range and Wei, 2016; Wei et al., 2009; Yaguchi et al., 2010). Further, it was shown that both together form a conserved regulatory unit for pattering the anterior ectoderm, antagonized by Wnt/ß-catenin signaling (Darras et al., 2011; Fritzenwanker et al., 2014; Marlow et al., 2014, 2013; Range and Wei, 2016; Sinigaglia et al., 2013; Wei et al., 2009; Yaguchi et al., 2008).

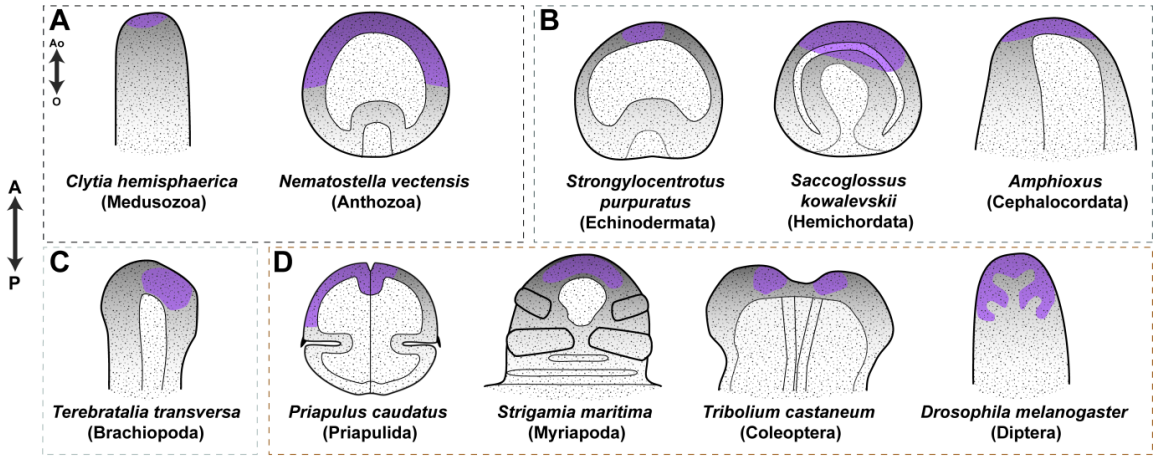

Figure 5.3 Conserved expression pattern of *foxq2* in metazoan kingdom-spanning species. Schematic representation of *foxq2* expression (purple) in cnidarians (**A**), deuterostomes (**B**), lophotrochozoans (**C**), and ecdysozoans (**D**). All species are oriented with the anterior/aboral pole to the top. Ectodermal expression of *foxq2* at the most anterior part is highly conserved in different members distributed across the metazoan kingdom. (Based on Chevalier et al., 2006; Fritzenwanker et al., 2014; Hunnekuhl and Akam, 2014; Lee and Frasch, 2004; Martín-Durán and Hejnol, 2015; Santagata et al., 2012; Sinigaglia et al., 2013; Tu et al., 2006; Yu et al., 2003); A: anterior, P: posterior, Ao: aboral, O: oral

I showed that *foxq2* and *six3* in *Tribolium* show the previously described co-expression pattern, characterized by largely overlapping expression domains of both factors (**Figure 5.4**, left column). However, in *Tribolium* elongating germ bands the expression of both factors becomes mutually exclusive to each other (**Figure 5.4H``**), which so far has only been reported for late gastrulae of *Strongylocentrotus* (**Figure 5.4B`**; Range and Wei, 2016; Tu et al., 2006). Also other species show diversification of the *foxq2*/*six3* expression/co-expression pattern at later stages, ranging from a complete *six3*/*foxq2* co-expression with a *six3*-free region at the most apical part (**Figure 5.4A`**, **E**) to an absence of both factors in the most apical part, being only co-expressed in more posterior regions (**Figure 5.4D`**, **F**). At later *Tribolium* embryonic stages co-expression of *Tc-foxq2* and *Tc-six3* starts again, but with a quite complex expression profile compared to other species at later stages (**Figure 5.4H```**, **H````**). Taken together, at least at early stages the co-expression of *foxq2* and *six3* appear to be the ancestral condition. From the distribution of co-expression it is even unclear what the ancestral condition was for later stages. In *Nematostella* and *Platynereis* both factors are co-expressed at later stages, while *Strongylocentrotus*, *Terebratalia*, *Lineus*, and *Tribolium* show only minor expression overlaps at later stages.

With respect to the co-regulation of both factors it is known that *six3* is repressed by *foxq2* in *Strongylocentrotus* (Range and Wei, 2016). In contrast, in *Tribolium foxq2* and *six3* both regulate each other positively forming an upstream regulatory module (see **Figure 5.1**). Due to the lack of functional data in other taxa statements about the ancestral condition remain impossible.

On the one hand, these data support the notion that the set of factors acting on the regulation of anterior tissue is well-conserved (Range and Wei, 2016; Sinigaglia et al., 2013). On the other hand, the exact expression dynamics, co-expression, and interactions of these conserved factors appear to have evolved substantially depending on the evolutionary situation within the metazoan kingdom, culminating in a complete loss of *foxq2* representatives in the Placentalia. This differential regulation reflects a high degree of plasticity of these factors in evolutionary processes (Sinigaglia et al., 2013). This suggests that the core apical patterning gene set evolved to a significant degree and being involved in head shape diversification. This is similar to trunk patterning by Hox genes. The highly conserved Hox genes of the conserved Hox gene cluster play a crucial rule in AP patterning and building the body. However, evolutionary changes of these genes influence development and in consequence trunk morphology (Carroll, 1995; Lemons, 2006; Pearson et al., 2005).

The importance of Hox genes is due to the fact that these genes are involved in many processes of development e.g. cell cycle control, cell adhesion, cell division rates, cell death, and cell movement (Pearson et al., 2005; Weatherbee et al., 1998).

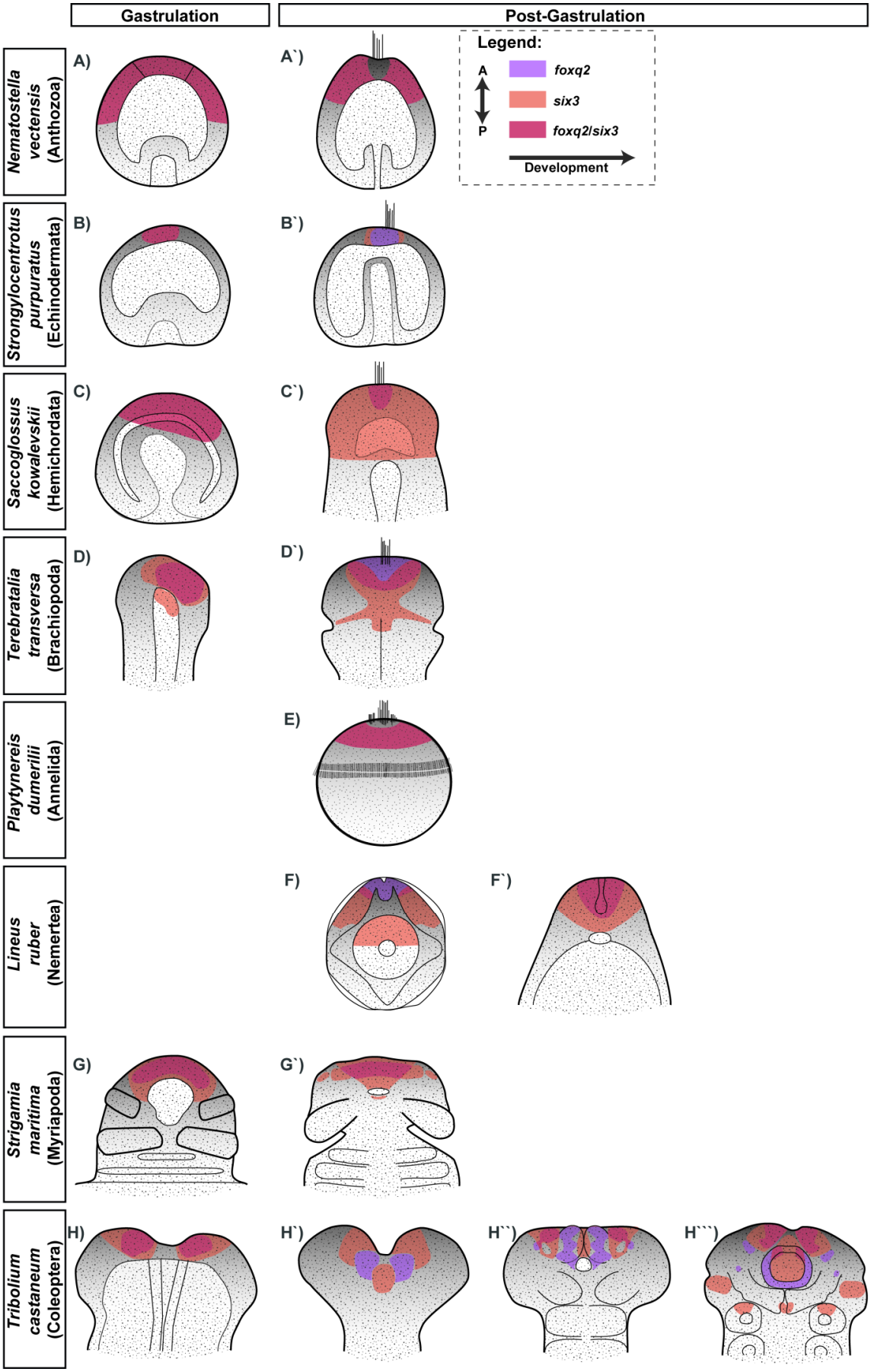
Gastrulation
Post-Gastrulation
Legend:
A
P
foxq2
six3
foxq2/six3
Development
Nematostella vectensis (Anthozoa)
Strongylocentrotus purpuratus (Echinodermata)
Saccoglossus kowalevskii (Hemichordata)
Terebratalia transversa (Brachiopoda)
Platynereis dumerilii (Annelida)
Lineus ruber (Nemertea)
Strigamia maritima (Myriapoda)
Tribolium castaneum (Coleoptera)
A)
A`)
B)
B`)
C)
C`)
D)
D`)
E)
F)
F`)
G)
G`)
H)
H`)
H``)
H```)

Figure 5.4 **Co-expression of *foxq2*/*six3* at different developmental stages of different Metazoa.** Schematic representation of *foxq2* (purple) and *six3* (orange) expression in metazoan species at different developmental stages. Co-expression is marked in red. The anterior/apical pole is oriented to the top. Expression/co-expression of *foxq2* and *six3* at early stages of different metazoan species is highly conserved marked by largely overlapping domains at the anterior/apical pole (left column). At later stages the expression/co-expression of both factors diversifies. (**A`**, **E**) *Nematostella* and *Platynereis* larvae show a *foxq2*/*six3* co-expression during early stages like the other species, with the exception that the most apical region is free of *foxq2*/*six3* expression. (**C`**, **F`**, **G`**) Late embryonic stages of *Saccoglossus* and *Strigamia* as well as early *Lineus* juveniles show a *foxq2* expression at the anterior/apical pole, which is completely covered by *six3* expression. (**B`**, **H`**) *Strongylocentrotus* late gastrulae and *Tribolium* elongating germ bands show mutually exclusive expression of *foxq2* and *six3*. (**D`**, **F`**) Early tri-lobed *Terebratalia* larvae and *Lineus* Schmidt's larvae show *foxq2* expression at the anterior/apical pole overlapping only posteriorly with *six3*. (**H``**, **H```**) Fully elongated and retracting *Tribolium* germ bands show a complex expression pattern of *foxq2* and *six3* with partial overlaps in the neuroectoderm (**H``**, **H```**) and in the anterior labral buds (**H```**). (Based on Fritzenwanker et al., 2014; Hunnekuhl and Akam, 2014; Marlow et al., 2014; Martín-Durán et al., 2015; Santagata et al., 2012; Sinigaglia et al., 2013; Tu et al., 2006; Wei et al., 2009); A: anterior, P: posterior

5.1.1.6 Ectopic expression of *Tc-foxq2*

Heat shock-based ubiquitous ectopic expression is a powerful tool for studying gene function due to the adjustable time point and strength of ectopic expression (Brand et al., 1994; Schinko et al., 2012). This method has been established previously but has not been applied so far. In this study, I generated a functional transgenic line for ubiquitous ectopic expression of *Tc-foxq2*. The transgenic line in combination with expression studies provided a good complementary view on *Tc-foxq2* function in patterning of the anterior head region. Expression analyses showed that the majority of the head pattering genes are responding, if at all, only in certain regions to ectopic *Tc-foxq2* expression. This indicates that gene activation/repression of *Tc-foxq2* target genes is also dependent on a permissive environment, which is created by other activators or repressors acting on the particular gene. The pleiotropic effect of ectopic expression of the transcription factor *Tc-foxq2* indicates that there are the same or further target genes, which respond to the presence of *Tc-foxq2* in tissue posterior to the anterior part of the head. This is also confirmed by the disrupted expression pattern of the segment polarity gene *Tc-wg*. While the effect on the trunk and the appendages are reproducible it does not reflect biologically meaningful interactions because this occurs outside the *Tc-foxq2* expression domains. The results of the

expression pattern analysis of head patterning genes were partially ambiguous in *Tc-foxq2* gain-of-function experiments. In some cases the effect was not very pronounced or could not be clearly assigned to be a primary effect.

However, although heat shock-induced ubiquitous expression is a well-suited method for studying gene function it also has some drawbacks: the ectopic expression is ubiquitous and cannot be restricted to a subset of cells, and basal expression levels of the heat shock promoter are to be expected (Brand et al., 1994). In order to circumvent these disadvantages it would be helpful to generate and exploit specific GAL4 enhancer lines (Brand et al., 1994; Prelich, 2012; Schinko et al., 2010). It would be interesting to drive overexpression of head patterning genes in the *Tc-foxq2* region and look for alterations in *Tc-foxq2* expression. Moreover, reciprocal *Tc-foxq2* ectopic expression in the pattern of other head patterning genes and subsequent analysis of altered expression patterns of the head patterning gene set could be also useful to understand genetic dependencies and phenotypic causalities.

5.1.1.7 *Tc-foxq2* misregulation leads to neural phenotypes

foxq2 was already shown to have a neural function in the cnidarian *Nematostella* and in the deuterostome *Strongylocentrotus* with an alteration in the neuro-secretory apical organ, in knock-down experiments (Bisgrove and Burke, 1987; Page, 2002; Sinigaglia et al., 2013; Yaguchi et al., 2012, 2010, 2008). A neural *foxq2* function was also proposed for protostomes based on the expression pattern (Hunnekuhl and Akam, 2014; Marlow et al., 2014). Here I show that *foxq2* has indeed neural functions in protostomes and that *Tc-foxq2* is involved in brain formation, resulting in defects in the central body and the mushroom bodies upon knock-down and ectopic expression. It appears as if the phenotypes of the knock-down and the gain-of-function experiments were to some degree complementary. In both experiments the antennal lobes remained unaffected, whereas only knock-down experiments showed a fusion of the midline of the two brain hemispheres. In the *Tc-foxq2* knock-down phenotype the central body was decreased in size, while in the gain-of-function experiment the central body appeared to be elongated. Further, the medial lobes of the mushroom bodies were fused in knock-down experiments, whereas ectopic *Tc-foxq2* expression resulted in an increased spacing between the medial lobes. The neural phenotype in *Tc-foxq2* knock-down larvae shared some features with the neural phenotype observed upon *Tc-six3* knock-down confirming a mutual regulation of these genes. It has been shown that the neural phenotype correlates with *Tc-six3* function in

early neuroectodermal patterning events (Posnien et al., 2011b). These findings indicate an early *Tc-foxq2* function in neural development as well.

5.1.2 Outlook

5.1.2.1 Potential extensions of work on *Tc-foxq2* function

This study could be expanded in the future. New tools of the *Tribolium* toolbox could be exploited to get a better understanding about *Tc-foxq2* function and thus a better understanding about genetic and morphogenetic processes happening in the embryonic head.

This work showed that *Tc-foxq2* knock-down results only in a small amount of cuticles showing the strongest phenotype, which is characterized by the loss of the labrum. Thus, the question arises whether this is due to a low penetrance of RNAi treatment or whether this is a compensatory trait of the system.

In order to clarify this issue, one of the first additional experiments, which should be done is the generation of a transgenic *Tc-foxq2*-null mutant by using the CRISPR/Cas9 system (Gilles et al., 2015; Gilles and Averof, 2014; Hsu et al., 2014; Jinek et al., 2012). This could be achieved by a knock-in of a transformation marker into the coding sequence of *Tc-foxq2*. In consequence, this would lead to a loss of *Tc-foxq2* function and would simultaneously provide a marker. These mutants could then be analyzed with respect to their epidermal defects in L1 larvae. It would be interesting whether the penetrance of L1 larvae with a completely absent labrum would be increased and thereby the expressivity of the phenotype strengthened. Further, it should be analyzed whether *Tc-foxq2*-null mutants show additional defects. Indeed, recent studies in zebrafish showed that phenotypes emanating from transient knock-down experiments (morphants) could be very different from phenotypes resulting from stable transgenic loss-of-function lines (mutants) due to compensatory effects in these mutants (Rossi et al., 2015; Stainier et al., 2015). Hence, a *Tc-foxq2*-null mutant line could be an opportunity to test whether the epidermal loss-of-function phenotype was complete.

Further, if the mutant shows a more expressive phenotype -including a high penetrance of expressive defects- it would be worth to cross the *Tc-foxq2*-null mutant line with the *αTub1P-H2Av::EGFP* nuclear reporter line. Mutant offspring could be used for in vivo imaging experiments with the LSFM to get new insights into the emergence of the epidermal phenotype. This could give reciprocal new insights into the morphogenesis of the *Tribolium* head.

Subsequently, the same should be done using the neural reporter lines to substantiate and refine the findings described in this study (this study, Binzer et al., 2014; Koniszewski et al., 2016; Posnien et al., 2011b).

Exploiting the CRISPR technique it would be also interesting to establish a *Tc-foxq2* enhancer trap line. For this purpose the construct has to be brought into the proximity of the endogenous *Tc-foxq2* promoter and under the control of its enhancers. With this line one could perfectly mark *Tc-foxq2* expressing tissue e.g. the AMR, labrum, and neuroectoderm for in vivo imaging in combination with RNAi to analyze head patterning genes and their effects on embryonic structures. Further it could help to understand head morphogenesis, and it could be used for multiple antibody stainings, when using an anti-EGFP antibody.

5.1.2.2 Outlook on neural function

To track down a potential neural function of *Tc-foxq2* it would be helpful to gather more information about the *Tc-foxq2* wt expression in the brain. To this end, a *Tc-foxq2*-specific antibody should be generated. This could be used in immunostainings to get more precise insights about the expression pattern. It would be interesting to analyze whether *Tc-foxq2* is expressed in the brain at later embryonic and postembryonic stages (>40 h AEL). In addition to the early neuroectodermal patterning function this would indicate a late neural function. Furthermore, to test whether *Tc-foxq2* is expressed in neuroblasts it would be interesting to investigate co-localization with the marker for neuroblasts, *Tc-asense* (*Tc-ase*, Brand et al., 1993; Wheeler, 2003). To this end the previously published '*ase-Gal4*' line (Koniszewski et al., 2016) could be used in combination with a GAL4-specific antibody together with the *Tc-foxq2* antibody in immunostainings. These immunostainings could provide exact data about co-localization and number of *Tc-foxq2* positive neuroblasts.

To get a better understanding about the neural phenotype in *Tc-foxq2* knock-down experiments it could be tested whether the phenotype is due to *Tc-foxq2* function in early neuroectodermal patterning or in neural cells at later stages. This could be tested via *Tc-foxq2* embryonic RNAi at different stages (Posnien et al., 2011b) or via RNAi within the *hsCrPVi1A* RNAi inhibitor line, which allows temporal control of RNAi inhibition via heat shock application (J. Ulrich, in prep.). Further, it would be worthwhile to perform *Tc-asense* (*Tc-ase*) ISH in *Tc-foxq2*^{pRNAi} embryos. *Tc-ase*, marking the neuroblasts, could provide information in *Tc-foxq2* knock-down experiments

whether neuroblasts show an alteration in number or onset of delamination. Another approach to track down whether *Tc-foxq2* has a neural-specific function would be to knock-down *Tc-foxq2* function in the pattern of *Tc-ase*, by using the *ase-Gal4* line driving a hairpin construct. This line allows interfering specifically with the potential neural aspects and not the ectodermal aspects of *Tc-foxq2* function. The resulting offspring could then be analyzed regarding altered number and composition of neuroblasts, as well as regarding altered expression patterns of neuroblast-specific transcription factors. The neurogenic reporter lines (*brainy* and *MB-green* line) could be used to analyze the overall brain defects in *Tc-ase* driven *Tc-foxq2* RNAi offspring.

Moreover, the heat shock-based ectopic *Tc-foxq2* expression line could be used for further studies. The described neural phenotype resulted from using embryos at an age of 0-24 h AEL. In order to look for a late neural function of *Tc-foxq2* it would be interesting to perform the heat shock treatment at later stages and more narrow time frames. This could help to get more prominent or more specific defects, which could be related easier to *Tc-foxq2* function in neural development.

To get a more precise view on the neural defects in *Tc-foxq2* knock-down larvae, it would be interesting to dissect L1 larval brains and perform immunostainings with several neural markers, like anti-synapsin, anti-5HT (serotonin), anti-periviscerokinin (PVK), and anti-myoinhibitory protein. Immunostaining against these neuromodulators help to specifically mark parts of the mushroom body and the central body (Koniszewski et al., 2016), which allows more detailed analyses of the defects in larval brains. Especially in combination with high resolution LSM imaging and 3D reconstruction it would be perfect to get an impression of the three-dimensionality of the defects (Dreyer, 2010). However, due to the pleiotropic defects of the brain phenotype it will be difficult to assign the observed effects to certain structures unless more specific imaging lines are available.

5.1.2.3 Behavioral assays in *Tc-foxq2* knock-down post-embryonic individuals

This study showed that some *Tc-foxq2* knock-down embryos are able to reach L1 larval stages and that these larvae are able, even with the described neural defects, to hatch and to move. Based on these observations it would be interesting to conduct simple behavioral tests with knock-down larvae or if possible with adult individuals. Mushroom bodies are involved in olfactory learning and memory (Akalal et al., 2006; Davis, 2011; Heisenberg, 1998) and the central body, as subunit of the central complex, is involved in locomotor activity, courtship, sky compass orientation, and memory amongst others (Homberg, 2008; Pfeiffer and Homberg,

2014). Thus, I would propose four different simple behavioral tests: (**1**) the open field test (Gould et al., 2009; Tremmel and Muller, 2013), (**2**) light-dark exploration tests (Bourin and Hascoët, 2003; Tremmel and Muller, 2013), (**3**) odorant recognition tests (Loschiavo, 1965), and (**4**) the food recognition tests (Campbell and Hagstrum, 2002).

(**1**) The most promising and easiest test to conduct is the open field test. In this test a group of individuals are put in an empty box and their movement is recorded and tracked with a camera from above. With this test different question could be addressed. (I) Do individuals with neural defects show the same complexity in movement, i.e. the same exploratory behavior than wt individuals? (II) Is the locomotor activity changed? (III) Is the courtship behavior altered?

(**2**) Light-dark exploration tests are easy to conduct and can give information whether the visual sensing is disrupted. Usually *Tribolium* individuals tend to choose dark places (C. Schmitt-Engel, personal communication). Loss of preference would point into the direction of processing visual inputs.

(**3**) With odorant recognition tests it could be analyzed whether they are still responding correctly to different attractive or aversive odors. However, some trials to establish this test within *Tribolium* already failed because it is hard to get statistically robust results (A. Metzger, personal communication).

(**4**) The food recognition test could be also very interesting and may give more clear and valid data, because with this test several senses are tested simultaneously. However, the results will give only vague insights of the real causality of the erratic behavior, because it could be based on deficiencies in processing visual, olfactory or tactile clues.

5.1.2.4 Uncovering the gene regulatory network of the head & trying to find novel players for head development

Recent studies showed that there are still unknown players, which are active in the anterior head gene regulatory network (Kittelmann et al., 2013; Siemanowski et al., 2015). However, the candidate gene approach appears to be exhausted (Economou and Telford, 2009; Kittelmann et al., 2013; Posnien et al., 2011b; Schinko et al., 2008; Schmitt-Engel et al., 2015). The *Tribolium* iBeetle screen (Schmitt-Engel 2015) is a powerful approach to find novel players acting in the anterior head, even more when the screen achieves the genome-wide level. To this end the

iBeetle database (Dönitz et al., 2015) can be scanned for annotated labrum specific phenotypes and subsequently analyzed regarding their function in the anterior head gene regulatory network.

In order to identify additional *Tc-foxq2* target genes and to uncover all genetic interactions of the gene regulatory network of the anterior head region, it would be worth exploiting transcriptomic approaches in parallel. A promising approach in *Tribolium* was recently published using RNAi together with RNA sequencing (RNAi-seq; Oberhofer et al., 2014). *Tc-foxq2* would be an interesting candidate gene for this approach, due to its upstream position within the gene regulatory network of the anterior head and because of the lack of secondary effects in early head patterning stages upon knock-down. To this end, *Tc-foxq2*^{pRNAi} embryos have to be generated and afterwards the RNA of early embryonic stages (10-16 h AEL) has to be isolated and sequenced. The *Tc-foxq2* RNAi-seq results could substantiate and refine the findings of this study, by quantitatively measuring the effects. A potential drawback of the published RNAi-seq screen (Oberhofer et al., 2014), was that whole embryos were used for the RNA sequencing. This could weaken or blur minor changes in expression level of head players. Thus interesting candidates could be overseen. To overcome this issue I propose to enhance the accuracy of this approach by combining it with the laser capture microdissection (Emmert-Buck et al., 1996) or the laser cutting microdissection (Böhm et al., 1997) technique. With this method the procephalic region could be dissected from the rest of the embryo and afterwards used for RNAi-seq on the basis of morphological traits.

Another approach to get information about the direct genetic interactions of *Tc-foxq2* with the head patterning genes could be the ChIPSeq (chromatin immunoprecipitation combined with DNA sequencing) technique (Johnson et al., 2007). To this end, a *Tc-foxq2* antibody has to be generated first. ChIPSeq using a *Tc-foxq2* antibody could help to identify the DNA binding sites of the transcription factor *Tc-foxq2*. Thus, this technique could provide both new interaction partners and information about direct genetic interaction with other head patterning genes.

5.1.2.5 Outlook on the evolutionary aspects of *foxq2*

It would be interesting to look at *foxq2* function in other arthropods and investigate evolutionary differences. For *Drosophila* it is known to be expressed at the anterior tip including the clypeo-labral region, pharyngeal structures and the brain (Lee and Frasch, 2004). Also the spider *Parasteatoda tepidariorum* shows an expression at the anterior pole, including the labral region

(M. Schacht, personal communication). This confirms that the highly conserved expression pattern is also reflected in different arthropod species. *Parasteatoda* shows a complex expression pattern resembling the expression pattern in *Tribolium*. Although, co-expression data is lacking so far, it appears that *Pt-foxq2* and *Pt-six3.1/ Pt-six3. 2* are partially co-expressed, similar to the situation in *Tribolium* (M. Schacht pers. communication, Schomburg et al., 2015). It would be interesting to determine co-expression of *foxq2* and *six3* in wt and in *six3/ foxq2* knock-down individuals of these species to uncover potential similarities and changes in function. This would help to get deeper insights into the evolution of the gene regulatory network and, hence, the bases for morphological evolution.

5.2 New tools to study morphogenetic movements

5.2.1 The transgenic C3PA-GFP photoactivation lines are a powerful tool for cell marking and fate mapping

I was able to generate the first stable transgenic photoactivation lines in *Tribolium*. These lines are well-suited for marking cells in a targeted manner and for subsequent cell tracing and tracking. However, a potential drawback of this principle could be that the activated signal is reduced over time and with each imaging cycle. This disadvantage hampers imaging over a long time with many imaging intervals. Both lines (*αTub1P-C3PA-GFP* and *αTub1P-H2Av::C3PA-GFP*), described in this study, show a strong fluorescent signal after activation using the conventional LSM in combination with UV-light (350-360 nm). However, the first proof of principle experiments revealed some potential obstacles. Photoactivation with the described settings (**see section 3.12**) could result in cell cycle arrest, cell death, or changed signal localizations (own observations, A. Gilles personal communication). These observed side-effects are most likely due to phototoxic effects evoked by irradiation with high intensities of UV-light. Since decades it is known that UV-light leads to pyrimidine dimerization, DNA-protein cross-links, and DNA strand break (Kornhauser, 1980; Marrot and Meunier, 2008). These effects interfere with DNA replication, mitosis, and DNA transcription, which eventually result in cytotoxicity, mutagenicity, and the induction of apoptosis leading to cell death and absorption (Marrot and Meunier, 2008; Post et al., 2005). The described effects would explain the observed cell cycle arrest and cell death. Absence of the nuclear signal when activating the H2Av::C3PA-GFP via UV-light could be

due to cross-linking of these molecules with the DNA, which inhibits the correct folding of the protein, thus resulting in the inability to emit a fluorescent signal. To avoid these side-effects caused by photoactivation, it should first be tested whether the settings of activation could be optimized. To this end, it should be tried to lower the laser intensity or exposure time of the photoactivation. Another possibility could be to use different laser-lines or microscope techniques. First, it could be tested to use a conventional LSM with a 405 nm laser-line (instead of UV light at 351 nm and 364 nm) for photoactivation, which was already successfully used in *Drosophila* (Mavrakis et al., 2009). Wavelengths about 400 nm have less energy and thereby show massively lower rates of side-effects like pyrimidine dimerization and freeing of cytotoxic reactive oxygen species (Marrot and Meunier, 2008). Second, instead of using a conventional LSM, a two-photon microscope could be used. Photoactivation with a two-photon microscope, using light in the infra-red spectrum (780-840 nm), is shown to be efficient and non-toxic (Post et al., 2005; Ruta et al., 2010). If possible, it would be best suited to use a wavelength of 820 nm for photoactivation for whole mounted embryos (Post et al., 2005).

Another approach could be to use photoactivatable fluorescent proteins, which are activated at a different wavelength. One promising and versatile candidate would be the kindling fluorescent protein-1 (KFP1) based on the chromotprotein asulCP extracted from the sea anemone *Anemonia sulcata* (Chudakov et al., 2005; Lukyanov et al., 2005). KFP1 shows a red fluorescent signal (572/595 nm) upon photoactivation with intense green light (590 nm). This signal could be reversibly or irreversibly photoactivated, depending on the activation intensity and duration. Photoactivation could be reversed by using blue light (450 nm). One advantage of this versatile protein is that e.g. at first the overall morphology can be visualized via a first activation cycle. Afterwards, when the ROI is selected the overall signal could be switched off and only single cells or cell groups within the ROI could be then reactivated. Thus, KFP1 is perfectly-suited for cell-tracking experiments.

5.2.2 The genetic Cre/*loxP* cell marking system is a powerful tool, but has to be improved

I was able to generate a stable transgenic *loxP* responder line, which could be used, upon crossing with adequate Cre driver lines to mark cells genetically. In this study the *loxP* responder line was crossed to a heat shock-inducible Cre line, leading to random marking of cells upon heat shock treatment. The *loxP* responder line, described in this study, is functional regarding the

fluorescent body marker (mCherry) to visualize embryonic morphology and also regarding the marking of a subset of cells with a different fluorescent marker protein (EGFP). However, the *loxP* responder line and the used heat shock-inducible driver line have two problems. First, the fluorescent signal of the mCherry body-marker is weak. This could be explained by the lack of a Kozak consensus sequence (Kozak, 1984) in front of the start codon. The Kozak sequence is involved in ribosomal binding and thereby important for translation initiation. An altered or missing Kozak consensus sequence could decrease translation efficiency and thereby the protein level (Kozak, 1986; Nakagawa et al., 2007). Second, the number of marked cells was far too high for cell tracking experiments both in heat shock-treated as well as in untreated embryos. This suggests some constitutive expression of the Cre recombinase, which is most likely due to the leakiness of the *hsp* promoter at the integration locus of the pB[hsp68-Cre] construct. This problem could be overcome by remobilization of the integrated pB[hsp68-Cre] construct into a less active locus. To achieve this result the Cre driver line has to be crossed to a line that expresses the piggyBac transposase and, which enables the jump of the construct into another locus (Trauner et al., 2009). Afterwards, the resulting offspring has to be tested regarding the green signal indicating the number of marked cells. This should be done in heat shock-treated and in untreated offspring individuals. Offspring that shows no signal when untreated, but an intermediate or strong signal after heat shock treatment should be raised to a larger population and studied in detail.

5.2.3 The *αTub1/rps3/PUb* promoters are ubiquitously active at all embryonic stages

I was able to generate different functional transgenic ubiquitous nuclear reporter lines by synthesizing an H2Av::EGFP chimeric reporter protein that was driven either by the *Tc-αTub1*, the *Tc-rps3*, or the *Tc-Pub* promoter. However, all these lines showed a strongly decreased viability or led even to an extinction of the complete population after a few generations, as described for the *Tc-PUbP-H2Av::EGFP* line. The reason for this decreased viability is probably not due to an interference with the overall chromatin structure and cell cycle progression, caused by the 27 kDa EGFP tag (Kanda et al., 1998). It is more likely that the oversupply of the histone H2A variant could be the reason for the decreased viability. The transgenic lines carried on the one hand a second copy of this gene and on the other hand this was additionally driven by a highly active promoter. It was already described in several publications that the level of

histones is crucial for normal development and that excessive histone amounts could lead to RNAi effects, replication errors, altered gene expression, chromosome loss, and death (Groth et al., 2007; Gunjan et al., 2006; Li et al., 2014; Singh et al., 2010). Possible solutions to overcome this problem could be implemented by the use of the CRISPR/Cas9 system (Literature see above). The system could be used to directly fuse a fluorescent protein to the endogenous histone, which is then under the control of the respective histone promoter. Another possibility could be to perform a knock-out of a particular histone and to bring in a histone::fluorescent protein cassette into the first intron of a strongly expressed gene. Thus, the reporter protein will be under control of the desired endogenous promoter.

The newly generated ubiquitous nuclear reporter lines indeed show ubiquitous expression. However, the comparison with the *EFA-nGFP* (El-Sherif et al., 2012) showed that the expression level is higher only at some developmental stages than in the *EFA-nGFP* line. At most embryonic stages the *EFA-nGFP* line showed the best results with respect to signal intensity. However, the new lines and the *EFA-nGFP* line are not completely comparable due to different reporter proteins. Thus, the search for a good ubiquitous promoter, that drives strong expression, should be continued. The *Tc-PUbP* was already tested and it showed a strong signal in embryonic stages, but it showed an expression pattern with gaps like the *EFA* (A. Gilles, personal communication). Another promoter, which could be tested is the *Tc-glyceraldehyde-3-phosphate dehydrogenase* (TC006170, Tcas_OGS 3.0) promoter, a housekeeping gene, which is active in glycolysis. The *Tc-actin5C* (TCO06296, Tcas_OGS 3.0) promoter could also be an interesting candidate, which is involved in cytoskeletal protein formation. Both show strong expression at embryonic stages of *Drosophila* (Fisher et al., 2012).

5.2.4 Utilization of the new imaging lines

The newly generated imaging lines serve as an expansion for the already versatile *Tribolium* toolbox. These lines could be used to gain deeper insights into the morphogenesis of the *Tribolium* head and the complete embryo. These insights could be used to get a better understanding on the cellular processes and tissue movements, which build the final head. Better knowledge of these processes in wt and developmental phenotypes would help to get more precise and detailed information about gene function in morphogenetic processes.

The *αTubP1-H2Av::EGFP* line could be used to get more insights on large tissue movements during embryogenesis. By following the established protocols (Strobl et al., 2015) the LSFM technique could be exploited to image the embryo from different angles and hence generate a comprehensive data set (Goldberg et al., 2005). This data set could be processed (Strobl et al., 2015) to finally generate a 4D embryo. Using the Fiji implemented BigDataViewer plug-in (Schindelin et al., 2012) the embryo can be visualized and analyzed from any desired view. Even more, the cell movements could be annotated and tracked in a 4D environment, by using the MaMuT plug-in, which is a combination of the BigDataViewer and TrackMate (Schindelin et al., 2012). However, cell tracking using the ubiquitous nuclear reporter lines is impeded by the fact that the nuclei are tightly packed, permanently in motion and at times covered by the extra-embryonic tissue. Thus, automated cell tracking using these lines is impossible and manual cell tracking is at least very difficult and time-consuming. Nevertheless, the combination of the 4D data set and different Fiji plug-ins could be well-suited to test features of the bend & zipper model (Posnien et al., 2010) or to set up new hypotheses.

The newly established transgenic cell marker line could overcome the drawback of the ubiquitous nuclear reporter lines. These lines could be used to either mark transiently and targeted or permanently and randomly a subset of cells. Marking only small cell populations or single cells facilitates manual cell tracking and could be the first step into the direction of automated cell tracking. This could provide new insights in cell movement and organization, which are needed to set up tissues and to finally build up the larval head. However, this approach is not limited to the head capsule. With these cell marking lines it would be possible to get new insights into the generation of segments from the posterior growth zone. Due to the fact that *Drosophila* develops as a long-germ embryo, there is no comprehensive data in any insect about embryonic growth involving a posterior growth zone (Benton et al., 2013; Nakamoto et al., 2015; Oberhofer et al., 2014; Sarrazin et al., 2012; Schröder et al., 2008). In contrast to *Drosophila*, *Tribolium* develops as short-germ insect, in which segments are progressively added from a posterior growth zone (Tautz et al., 1994). The new cell marking lines could be used to analyze cell behavior in the posterior growth zone and how cells, originating from the posterior growth zone, will contribute to form new segments.

With the *loxP* responder line cells could be marked genetically and in a stable manner. The advantage of this method is that marked cells could be traced and tracked in short imaging intervals over a long time of development. The drawback of this tool is that, due to the random marking of cells, a lot of embryos have to be imaged in order to trace the desired cell

population. However, this tool enables to image the cells of interest throughout embryonic and postembryonic development, which allows the generation of an exact, non-invasive fate map at a cellular resolution. The ultimate goal would be to track down the movement and the final destination for every single cell. Imaging of wild-type embryos and embryos with induced developmental defects would allow new insights into morphogenesis and would facilitate and precise to describe developmental defects and to relate these to gene function.

5.3 The arthropod head problem

5.3.1 *Tc-foxq2* - Implementations for the arthropod head problem

Tc-foxq2 is expressed in the anterior head but not expressed segmental reiterated. Hence, *Tc-foxq2* is another marker for the pre-segmental region. Thus, *Tc-foxq2* is substantiating that this region is very different from the rest of the posterior head and from the trunk and relying on a very different set of genes, which is required for its patterning.

The notion that this region is not only a “special segment”, but indeed a non-segmental region is even more substantiated by evolutionary data. As already mentioned before, the pre-bilaterian sea anemone *Nematostella* shows a conserved gene set (*foxq2*, *six3*, and *rx*) required for patterning the aboral (presumably homologous to the anterior) pole (Marlow et al., 2013). These data indicate that these genes were involved in patterning the anterior pole of a pre-bilaterian ancestor, which was unsegmented. The conservation of this gene set, throughout the Metazoa, points to a homology of the anteriormost region among bilateria and its non-segmental structure.

5.3.2 Imaging lines and implementations for the arthropod head problem

One aspect of the arthropod head problem is dealing with the question, which part of the adult head capsule is built by the embryonic anterior pre-segmental region. The *αTubP1-H2Av::C3PA-*

GFP and the *αTubP1-C3PA-GFP* cell marking lines could be used to answer this question. The transgenic lines allow testing for the contribution of the embryonic anterior pre-segmental region to the larval head capsule as well as the exact contribution of the other segments. To this end, an embryo at a stage where the limbs start to grow out could be mounted. This stage would be well-suited, because the antenna, labral buds and the stomodeum become visible and could be used as morphological landmarks. The anterior pre-segmental region is defined by the expression of marker genes (e.g. *Tc-foxq2*, *Tc-six3*, *Tc-cnc*, *Tc-tll*, and *Tc-orthodentilce1*). This defined region could be photoactivated and the marked tissue analyzed regarding its proportion on the L1 larval head. This could shed light onto the acron, i.e. pre-segmental region, contribution to the arthropod head, by revealing its location in larval respectively adult heads. Subsequently, this could be done for every head segment, thereby revealing the contribution to the head capsule in larvae for each head segment. With this fate map it would be easier to understand the origin of head defects, observed in L1 larval cuticle preparations.

6

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7

Appendix

7.1 General abbreviations

2A	self cleaving peptide allowing polycistronic expression
AEL	after egg laying
AMR	anterior median region
BCIP	5-bromo-4-chloro-3'-indolyl phosphate
bhsp68	regulatory region of *heat shock protein 68*
bp	base pair
C3PA-GFP	'Cycle 3'mutant of photoactivatable GFP
Cas9	CRISPR-associated DNA nuclease9
CFP	cyan fluorescent protein
Cre	'*causes recombination*' - recombinase
CRISPR	clustered regularly interspaced short palindromic repeats
CrPVi1A	*Cricket Paralysis virus* extracted RNAi suppressor protein
DAPI	4',6-diamidino-2-phenylindole (DNA intercalating fluorescent stain)
Dcp-1	Cleaved *Drosophila* death-caspase-1
DIG	digoxigenin labeled RNA (for ISH)
DISH	double-ISH
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EFA	*Tc-elongation factor1α* promoter
EGFP	enhanced GFP
FLUO	fluorescin labeled RNA (for ISH)
GFP	green fluorescent protein
H2Av	histone H2A variant
HS	heat shock
hsp68	*heat shock protein68*
ISH	in situ hybridization
KFP	kindling fluorescent protein-1 (based on chromotprotein asulCP)
L1	first larval instar
loxP	locus of crossing over (x), P1
LSFM	light-sheet-based fluorescence microscopy
LSM	laser scanning microscope
m	prefix, means monomeric (in front of fluorescent proteins)
mRNA	messenger RNA
n	prefix, indicating a tag with a nuclear localization signal
NBT	nitro-blue tetrazolium
P	suffix, indicating that 5' regulatory (promoter) region of a gene was used
PA	prefix, photoactivatable (in front of fluorescent proteins)

pB	*piggyBac* (transformation vector)
pRNAi	parental RNAi
PUb	*polyubiquitin*
RFP	red fluorescent protein
RNA	ribonucleic acid
RNAi	RNA interference
ROI	region of interest
rps3	*ribosomal protein subunit 3*
SB	*San Bernadino* (*Tribolium* wt strain)
SV40PolyA	stop/poly adenylation-signal (extracted from *Herpes simplex virus*)
TSA	tyramide signal amplification
v^{w}	*vermillion*^{white} *(Tribolium*, eye color deficient strain)
wt	wild-type
YFP	yellow fluorescent protein
αTub1	*αTubulin1*

7.2 Gene abbreviations

arr	*arrow*
ase	*asense*
cnc	*cap'n'collar*
col	*collier*
croc	*crocodile*
fkh	*forkhead*
foxa	*forkhead box a*
foxq2	*forkhead box q2*
lab	*labial*
mib1	*mindbomb 1*
nk2.1	*nk2 homeobox 1 (thyroid transcription factor1)*
rx	*retinal homeobox*
scro	*scarecrow*
ser	*serrate*
six3	*sine oculis homeobox homolog 3*
six4	*sine oculis homeobox homolog 4*
Tc	*prefix, if the gene is a Tribolium ortholog*
tll	*tailless*
wg	*wingless*
wnt1	*int1 (wingless-related1)*

7.3 Species

***C**lytia **h**emisphaerica*	(Cnidaria, Medusozoa)
***N**ematostella **v**ectensis*	(Cnidaria, Anthozoa)
***L**ineus **r**uber*	(Protostomia; Nemertea)
***P**latynereis **d**umerilii*	(Protostomia; Annelida)
***T**erebratalia **t**ransversa*	(Protostomia; Brachiopoda)
***P**riapulus **c**audatus*	(Protostomia; Priapulida)

***C**aenorhabditis **e**legans*	(Protostomia; Nematoda)
***S**trigamia **m**aritima*	(Protostomia; Arthropoda, Myriapoda)
***P**arasteatoda **t**epidariorum*	(Protostomia; Arthropoda, Arachnida)
***T**ribolium **c**astaneum*	(Protostomia; Arthropoda, Insecta, Coleoptera)
***D**rosophila **m**elanogaster*	(Protostomia; Arthropoda, Insecta, Diptera)
***S**trongylocentrotus **p**urpuratus*	(Deuterostomia; Echinodermata)
***S**accoglossus **k**owalevskii*	(Deuterostomia; Hemichordata)
***B**ranchiostoma **f**loridae*	(Deuterostomia; Chordata, Cephalochordata (Amphioxus))
***D**anio **r**erio*	(Deuterostomia; Chordata, Actinopterygii)
***X**enopus **l**aevis*	(Deuterostomia; Chordata, Amphibia)
***O**rnithorhynchus **a**natinus*	(Deuterostomia; Chordata, Mammalia (Monotremata)
***M**us **m**usculus*	(Deuterostomia; Chordata, Mammalia (Placentalia))

7.4 Supplementary tables, figures, sequences, and videos

***Tc-foxq2*^{pRNAi} cuticle phenotype**

Table S7.1 *Tc-foxq2*$^{RNAi\_a}$ general cuticle phenotype using 1 µg/µl dsRNA in *SB*.

Pt:	Wildtype	Unspecific defects	Strong defects	No cuticle	Head defects	Total n
∑	4	0	18	140	676	838
%	0.5	0.0	2.1	16.7	80.7	

Table S7.2 *Tc-foxq2*$^{RNAi\_a}$ head defects using 1 µg/µl dsRNA in *SB*.

Phenotype:	Weak	Intermediate	Strong
∑	47	522	107
%	7.0	77.2	15.8

Table S7.3 *Tc-foxq2*$^{RNAi\_b}$ general cuticle phenotype using 1 µg/µl dsRNA in *SB*.

Pt:	Wildtype	Unspecific defects	Strong defects	No cuticle	Head defects	Total n
∑	47	4	23	39	200	313
%	15.0	1.3	7.3	12.5	63.9	

Table S7.4 *Tc-foxq2*$^{RNAi\_b}$ head defects using 1 µg/µl dsRNA in *SB*.

Phenotype:	Weak	Intermediate	Strong
∑	109	73	18
%	54.5	36.5	9.0

Table S7.5 *Tc-foxq2*$^{RNAi\_b}$ general cuticle phenotype using 1 µg/µl dsRNA in *pBa19* x *black*.

Pt:	Wildtype	Unspecific defects	Strong defects	No cuticle	Head defects	Total n
∑	0	4	8	12	772	796
%	0.0	0.5	1.0	1.5	97.0	

Table S7.6 *Tc-foxq2*$^{RNAi\_b}$ head defects using 1 µg/µl dsRNA in *pBa19* x *black*.

Phenotype:	Weak	Intermediate	Strong
∑	8	652	112
%	1.0	84.5	14.5

Quantification of cell death rates in wt and *Tc-foxq2*^{pRNAi} embryos

Table S7.7 Number (pre-normalization) of apoptotic cells per untreated *SB* embryo.

Stage/Region	Region 1	Region2	Region3
Stage1_1	0	0	0
Stage1_2	0	0	0
Stage1_3	0	0	0
Stage2_1	4	3	8
Stage2_2	0	0	0
Stage2_3	4	0	0

Stage2_4	1	2	0
Stage2_5	0	0	0
Stage2_6	0	0	0
Stage2_7	0	0	1
Stage2_8	0	1	0
Stage2_9	0	0	0
Stage2_10	0	0	0
Stage2_11	0	3	2
Stage3_1	1	1	3
Stage3_2	0	1	2
Stage3_3	0	1	1
Stage3_4	0	0	0
Stage3_5	0	2	0
Stage3_6	0	2	1
Stage3_7	0	1	0
Stage3_8	0	1	0
Stage3_9	0	3	3
Stage4_1	0	2	5
Stage4_2	2	2	3
Stage4_3	0	5	8
Stage4_4	3	4	1
Stage4_5	1	9	1
Stage4_6	5	2	2
Stage4_7	1	5	6
Stage4_8	2	5	0
Stage4_9	6	15	4
Stage4_10	0	5	2

Stage4_11	5	8	9
Stage4_12	0	4	1
Stage4_13	0	1	5
Stage4_14	4	8	4
Stage4_15	3	2	0
Stage4_16	0	8	0
Stage4_17	1	1	3
Stage5_1	6	23	22
Stage5_2	13	7	18
Stage5_3	14	17	19
Stage5_4	9	25	17
Stage5_5	13	29	23
Stage5_6	10	15	7
Stage5_7	17	18	21
Stage5_8	16	15	12
Stage5_9	23	22	12
Stage5_10	12	18	16
Stage5_11	11	14	15
Stage5_12	1	1	2

Table S7.8 **Number (pre-normalization) of apoptotic cells per *Tc-foxq2*^{pRNAi} embryo.**

Stage/Region	Region 1	Region2	Region3
Stage1_1	0	0	1
Stage1_2	16	21	36
Stage1_3	0	0	0
Stage1_4	0	0	0
Stage1_5	0	0	0

Stage1_6	0	0	0
Stage1_7	0	0	0
Stage2_1	0	4	0
Stage2_2	6	4	13
Stage2_3	5	3	6
Stage2_4	0	0	1
Stage2_5	1	1	1
Stage2_6	1	8	2
Stage2_7	0	1	0
Stage2_8	1	0	0
Stage2_9	0	1	1
Stage2_10	4	11	9
Stage2_11	0	3	1
Stage2_12	0	0	1
Stage3_1	1	0	1
Stage3_2	4	0	4
Stage3_3	2	8	25
Stage3_4	0	6	12
Stage3_5	3	4	3
Stage3_6	1	2	10
Stage3_7	0	5	6
Stage3_8	5	3	1
Stage3_9	8	10	11
Stage3_10	9	14	8
Stage3_11	0	1	5
Stage3_12	2	0	0
Stage3_13	25	11	39

Stage3_14	0	0	15
Stage3_15	2	2	7
Stage3_16	0	0	4
Stage3_17	0	0	12
Stage3_18	3	2	4
Stage3_19	8	4	5
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Stage4_2	7	18	15
Stage4_3	23	17	9
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Stage5_2	14	36	14
Stage5_3	10	18	5
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Stage5_5	19	57	46
Stage5_6	23	38	14

Stage5_7	15	26	22
Stage5_8	24	20	7
Stage5_9	18	42	35
Stage5_10	23	44	31
Stage5_11	24	29	4

Cuticle phenotype after embryonic *Tc-foxq2* gain-of-function at differet stages

Table S7.9 General cuticle phenotype after embryonic *Tc-foxq2* gain-of-function (HS: 9-13 h AEL).

Phenotype:	Cuticles	Strong	No cuticles	Total n
∑	42	16	22	80
%	52.5	20.0	27.5	

Table S7.10 Cuticle defects (%) after embryonic *Tc-foxq2* gain-of-function (HS: 9-13 h AEL).

%/Structure	Lr	clS	lrS	aVTS	mVTS	pVTS	ABB	aGTS	dGTS	pGTS	aMES	mMES	pMES	brB	br	At	AtF	Md	Mx	Lb	TS1-3	LP1-3	AS1-8	Uro	Pygo
Deformed	11.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	10.7	10.7	9.5	4.8	3.6	19.0	7.1	14.3
Absent	3.6	11.9	6.0	11.9	9.5	14.3	32.1	3.6	3.6	4.8	13.1	13.1	14.3	8.3	3.6	3.6	13.1	63.1	64.3	76.2	59.5	64.3	35.7	20.2	27.4
Fused	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.3	9.5	0.0	0.0
Duplicated	0.0	0.0	0.0	0.0	0.0	3.6	0.0	1.2	4.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table S7.11 General cuticle phenotype after embryonic *Tc-foxq2* gain-of-function (HS: 14-20 h AEL).

Phenotype:	Cuticles	Strong	No cuticles	Total n
∑	122	27	106	255
%	47.8	10.6	41.6	

Table S7.12 Cuticle defects (%) after embryonic *Tc-foxq2* gain-of-function (HS: 14-20 h AEL).

%/Structure	Lr	clS	lrS	aVTS	mVTS	pVTS	ABB	aGTS	dGTS	pGTS	aMES	mMES	pMES	brB	br	At	AtF	Md	Mx	Lb	TS1-3	LP1-3	AS1-8	Uro	Pygo
Deformed	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	16.0	0.0	0.8	0.8	0.4	4.9	0.8	5.7	1.6
Absent	0.0	0.0	0.0	8.2	49.2	0.8	21.7	1.6	1.2	0.4	1.6	2.5	1.6	3.3	1.2	0.8	43.4	0.8	0.0	0.8	8.2	11.1	27.9	79.9	93.4
Fused	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8	49.6	67.2	1.6	0.0
Duplicated	0.0	0.0	0.4	0.0	0.8	7.8	0.8	1.2	3.7	0.4	0.8	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table S7.13 General cuticle phenotype after embryonic *Tc-foxq2* gain-of-function (HS: 20-25 h AEL).

Phenotype:	Cuticles	Strong	No cuticles	Total n
∑	132	6	73	211
%	34.6	2.8	62.6	

Table S7.14 **Cuticle defects (%) after embryonic *Tc-foxq2* gain-of-function (HS: 20-25 h AEL).**

%/Structure	Deformed	Absent	Fused	Duplicated
Lr	3.8	0.0	0.0	0.0
clS	0.0	1.1	0.0	1.1
lrS	0.0	1.1	0.0	0.4
aVTS	0.4	6.8	0.0	0.4
mVTS	0.0	38.3	0.0	0.4
pVTS	0.0	0.0	0.0	5.3
ABB	0.0	26.1	0.0	0.0
aGTS	0.0	1.5	0.0	0.8
dGTS	0.0	1.1	0.0	1.5
pGTS	0.0	0.8	0.0	0.0
aMES	0.0	1.9	0.0	0.8
mMES	0.0	2.7	0.0	0.0
pMES	0.0	3.0	0.0	0.0
brB	0.0	2.3	0.0	0.0
br	0.0	0.0	0.0	0.0
At	0.8	0.0	0.0	0.0
AtF	2.7	33.3	0.0	0.0
Md	2.7	0.4	0.0	0.0
Mx	4.9	0.0	0.0	0.0
Lb	4.5	0.8	0.0	0.0
TS1-3	0.0	0.0	0.0	0.0
LP1-3	1.9	0.8	42.0	0.0
AS1-8	4.5	5.3	79.9	0.0
Uro	13.6	55.3	0.0	9.1
Pygo	9.8	81.4	0.0	0.8

Vector maps and sequences

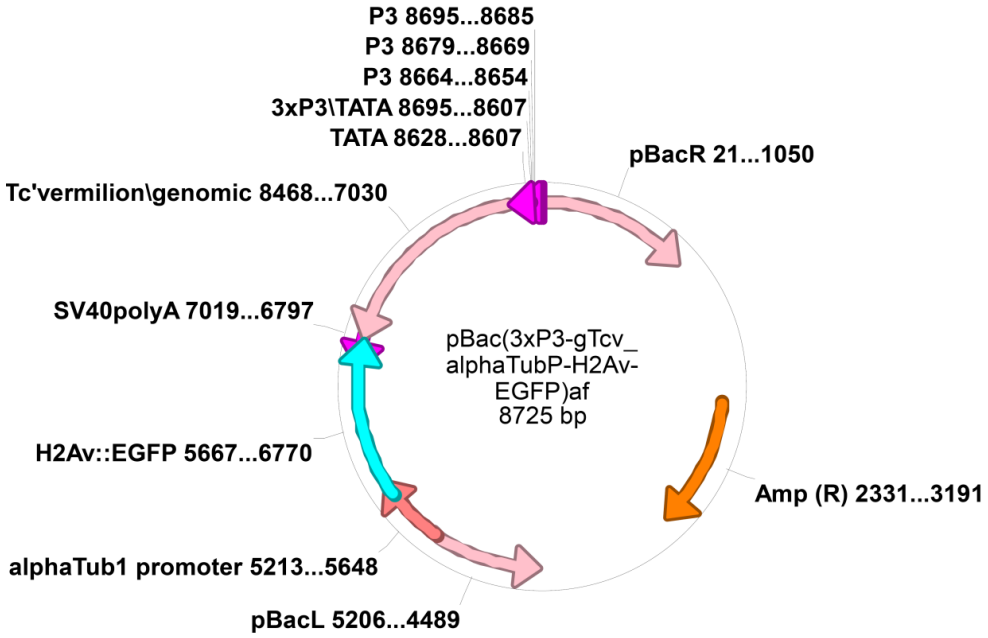

Figure S7.15 **pB[3xP3-gTc'v;Tc'αTub1P- Tc'H2Av::EGFP].**

Sequence S7.16 **pB[3xP3-gTc'v;Tc'αTub1P- Tc'H2Av::EGFP], GenBank formatted.**

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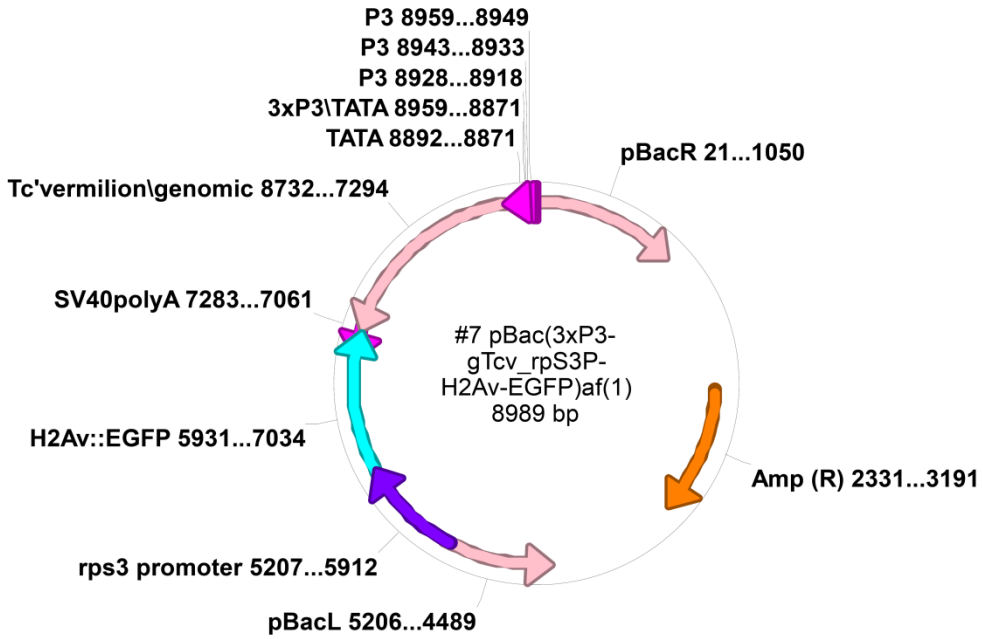

Figure S7.17 **pB[3xP3-gTc'v;Tc'rpS3P-Tc'H2Av::EGFP].**

Sequence S7.18 **pB[3xP3-gTc'v;Tc'rpS3P-Tc'H2Av::EGFP], GenBank formatted.**

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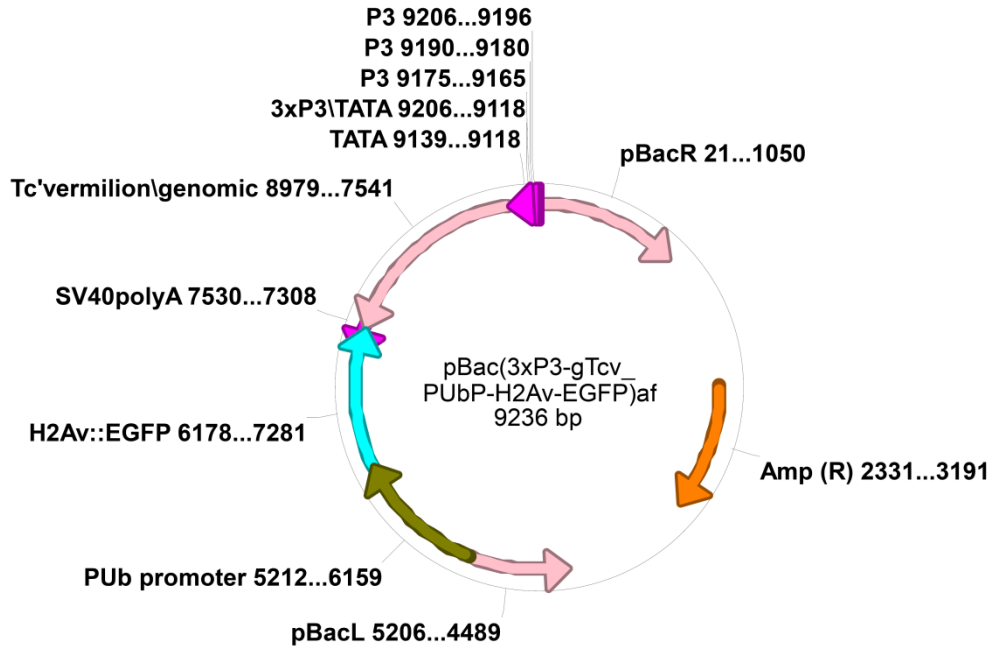

Figure S7.19 **pB[3xP3-gTc'v;Tc'PUbP- Tc'H2Av::EGFP].**

Sequence S7.20 **pB3[3xP3-gTc'v;Tc'PUbP- Tc'H2Av::EGFP], GenBank formatted.**

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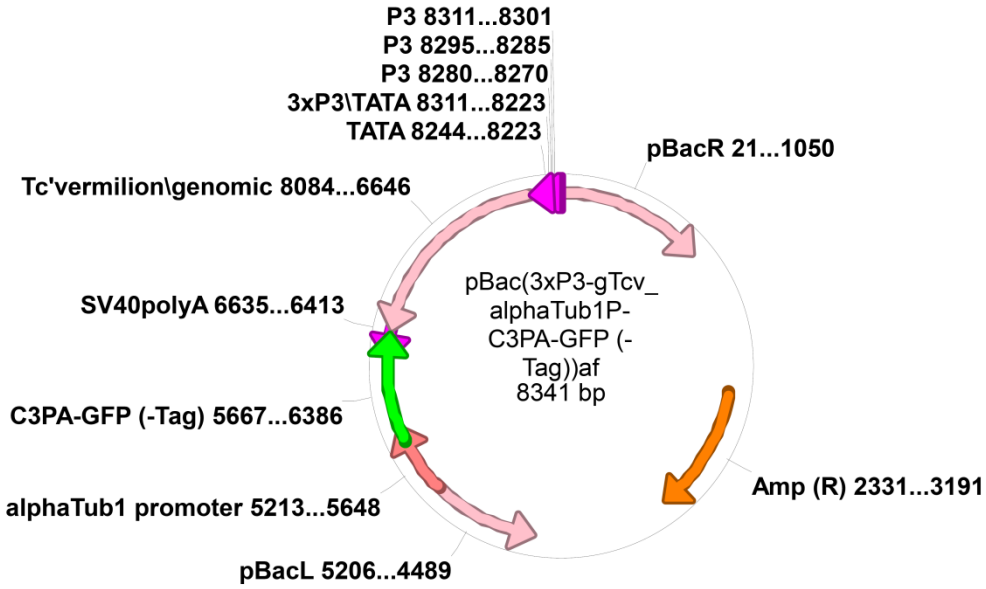

Figure S7.21 **pB[3xP3-gTc'v;Tc'αTub1P-C3PA-GFP].**

Sequence S7.22 **pB[3xP3-gTc'v;Tc'αTub1P-C3PA-GFP], GenBank formatted.**

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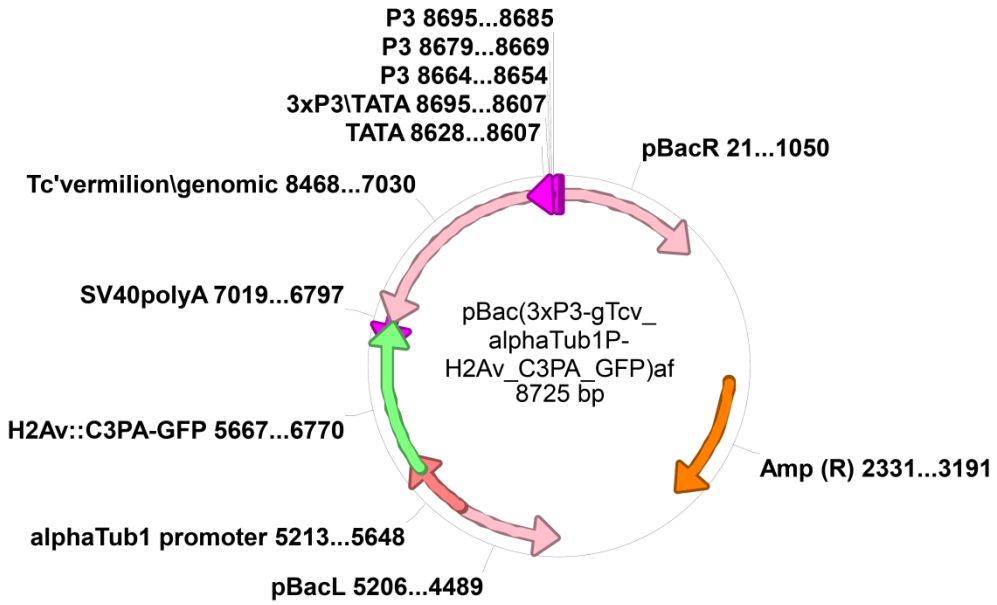

Figure S7.23 **pB[3xP3-gTc'v;Tc'αTub1P- Tc'H2Av:: C3PA-GFP].**

Sequence S7.24 **pB[3xP3-gTc'v;Tc'αTub1P- Tc'H2Av:: C3PA-GFP], GenBank formatted.**

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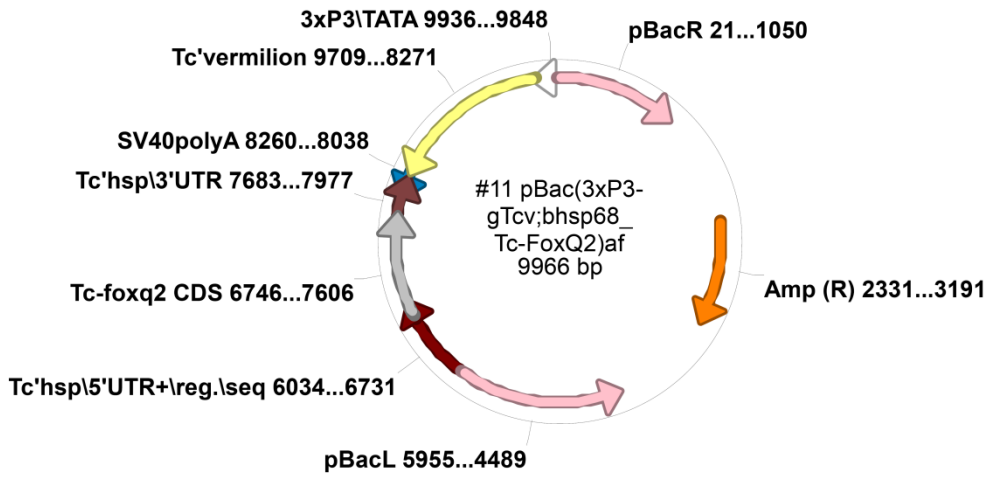

Figure S7.25 **pB[3xP3-gTc'v;Tc'hsp68-Tc'foxq2].**

Sequence S7.26 **pB[3xP3-gTc'v;Tc'hsp68-Tc'foxq2], GenBank formatted.**

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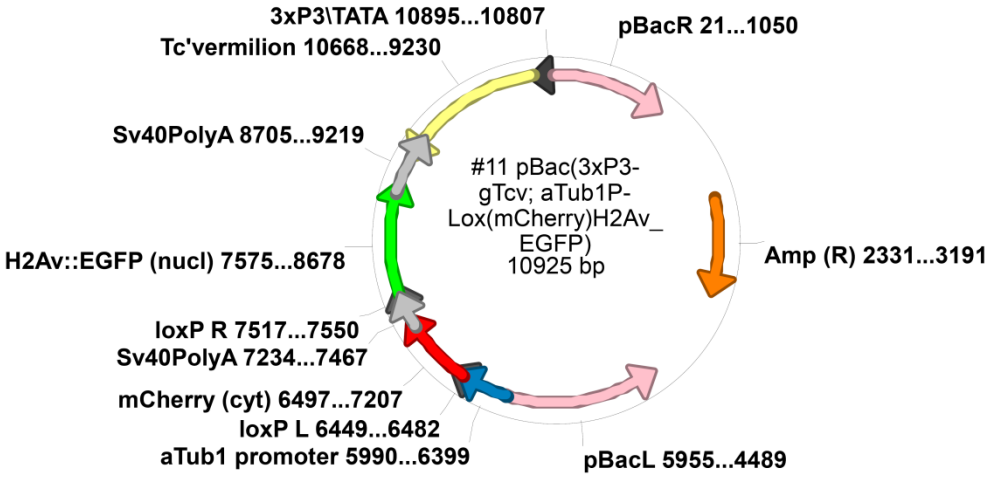

Figure S7.27 **pB[3xP3-gTc'v;Tc'αTub1P-*loxP*(mcherry)-Tc'H2Av::EGFP].**

Sequence S7.28 **pB[3xP3-gTc'v;Tc'αTub1P-*loxP*(mcherry)-Tc'H2Av::EGFP], GenBank formatted.**

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Videofiles

Videofile S7.29 Compacted LSFM video, ventral and dorsal view, maximum projections.

Videofile S7.30 Compacted LSFM video, lateral view, rendered 3D projection.

Videofile S7.31 Compacted LSM video, antero-frontal view, maximum projections.

Videofile S7.32 Compacted LSFM video, antero-frontal view, maximum projections

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Curriculum Vitae

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UNIVERSITY STUDIES

SINCE 08/12	**Ph.D. Thesis** "Morphogenesis and Genetic Regulation of the Insect Head" at the Göttingen Graduate School for Neurosciences and Molecular Biosciences (GGNB) in the group of Gregor Bucher, Department of Developmental Evolutionary Genetics, Georg-August-Universität Göttingen.
02/11-10/11	**Master Thesis** "Analysis of RNAi Phenotypes" in the group of Gregor Bucher, Department of Developmental Biology, Georg-August-Universität Göttingen

10/09-10/11 **M.Sc. Studies** in the "Developmental, Neural and Behavioral Biology" program with emphasis on Cell and Developmental Biology, Georg-August-Universität Göttingen

04/09-08-09 **Bachelor Thesis** "Molekulare und phänotypische Analyse von Insertionsmutanten des rotbraunen Reismehlkäfers *Tribolium castaneum*" in the group of Gregor Bucher, Department of Developmental Biology, Georg-August-Universität Göttingen

10/06-08/09 **B.Sc. Studies** in the "Biology" program, Georg-August-Universität Göttingen

SUPERVISION

07/15-01/16 **Master Thesis** of Felix Kaufholz "CRISPR-mediated Transgene Knock-in to Disrupt and Investigate Function of *FoxQ2* in the Beetle *Tribolium castaneum*"

08/14-11/14 **Lab Rotation** of Felix Kaufholz "Molecular Cloning of Two Fluorescent α-Tubulin Fusions and Evaluation of Heat shock induced Cell Marking for Fate-mapping in *Tribolium castaneum* (Coleoptera)"

08/12-10/12 **Lab Rotation** of Julia Meyer "Improvement of the Visualizing Techniques to Study Insect Head Development in *Tribolium castaneum*"

INTERNSHIPS

01/11-03/11 **Lab Rotation**: "Characterization of the Annotated Lipase CG9186 in *Drosophila melanogaste*r", in the group of Mathias Beller, Department of Molecular Developmental Biology, Max-Planck-Institute for Biophysical Chemistry.

09/10-11/10 **Lab Rotation**: "Molecular and Phenotypic Analysis of the Transgenic Line G11721 in *Tribolium castaneum*", in the group of Gregor Bucher, Department of Developmental Biology, Georg-August-Universität Göttingen.

CONTRIBUTION TO CONFERENCES

TALK

03/13 "RNAi Phenotypes are Influenced by the Genetic Background of the Injected Strain", iBeetle symposium "New Horizons in Molecular Zoology", Göttingen, Germany

09/12 "RNAi Phenotypes are Influenced by the Genetic Background of the Injected Strain", 9th GfE Summer School, Günzburg, Germany

POSTER

03/15 "Morphogenesis and Genetic Regulation of the Insect Head", Joint Meeting of the German and French Societies of Developmental Biologists, Nuremberg, Germany

07/14 "Morphogenesis and Genetic Regulation of the Insect Head", 5th Meeting of the European Society for Evolutionary Developmental Biology, Vienna, Austria

12/13 "Morphogenesis and Genetic Regulation of the Insect Head", GGNB Science Day, Göttingen, Germany

PUBLICATIONS

Kitzmann P, Schwirz J, Schmitt-Engel C and Bucher G. 2013, RNAi phenotypes are influenced by the genetic background of the injected strain. BMC Genomics 2013, 14:5

Fu J, Posnien N, Bolognesi R, Fischer TD, Rayl P, Oberhofer G, **Kitzmann P**, Brown SJ, Bucher G. 2012, Asymmetrically expressed *axin* required for anterior development in *Tribolium*. PNAS 7782–7786 May 15, 2012