Developmental Biology 333 (2009) 215–227

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

Probing the *Drosophila* retinal determination gene network in *Tribolium* (*II*): The *Pax6* genes *eyeless* and *twin of eyeless*

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ARTICLE INFO

Article history:
Received for publication 26 February 2009
Revised 18 May 2009
Accepted 7 June 2009
Available online 13 June 2009

Keywords:
Eyeless
Twin of eyeless
Dachshund
Tribolium
Evolution of development
Eye development
Redundancy
Retinal determination gene network
Drosophila

ABSTRACT

The *Pax6* genes *eyeless* (*ey*) and *twin of eyeless* (*toy*) are upstream regulators in the retinal determination gene network (RDGN), which instructs the formation of the adult eye primordium in *Drosophila*. Most animals possess a singleton *Pax6* ortholog, but the dependence of eye development on *Pax6* is widely conserved. A rare exception is given by the larval eyes of *Drosophila*, which develop independently of *ey* and *toy*. To obtain insight into the origin of differential larval and adult eye regulation, we studied the function of *toy* and *ey* in the red flour beetle *Tribolium castaneum*. We find that single and combinatorial knockdown of *toy* and *ey* affect larval eye development strongly but adult eye development only mildly in this primitive hemimetabolous species. Compound eye-loss, however, was provoked when *ey* and *toy* were RNAi-silenced in combination with the early retinal gene *dachshund* (*dac*). We propose that these data reflect a role of *Pax6* during regional specification in the developing head and that the subsequent maintenance and growth of the adult eye primordium is regulated partly by redundant and partly by specific functions of *toy*, *ey* and *dac* in *Tribolium*. The results from embryonic knockdown and comparative protein sequence analysis lead us further to conclude that *Tribolium* represents an ancestral state of redundant control by *ey* and *toy*.

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Introduction

Pax6 genes constitute a highly conserved orthology group of the Pax gene family of transcription factors, which are characterized by a paired-type DNA binding domain and a paired-class homeodomain (Chi and Epstein, 2002; Kozmik, 2005; Noll, 1993). *Pax6* has been found in all Bilaterians investigated so far including chordates, ascidians, sea urchin, nematodes, squid, annelids, insects, and planarians (Gehring, 2004; Kozmik, 2005). In most of these species, *Pax6* plays important roles in eye development. Examples are the severe forms of eye reduction in mouse *Small eye* mutants or in humans suffering from the congenital disorder Aniridia, which are caused by deleterious *Pax6* mutations (Hever et al., 2006). Similarly, mutations in either of the two closely related *Pax6* paralogs *ey* or *toy* cause loss or reduction of the adult compound eyes in *Drosophila* (Czerny et al., 1999; Quiring et al., 1994). Misexpression studies have shown that *Pax6* genes act high in the retinal determination gene network (RDGN), which controls the patterning of the eye primordium. Both in *Drosophila* and in vertebrates, *Pax6* is necessary and sufficient to trigger the RDGN. *Pax6* misexpression induces ectopic compound eye structures in *Drosophila* and differentiated ectopic lens eyes in the clawed frog *Xenopus laevis* (Halder et al., 1995; Chow et al., 1999). Intriguingly, ectopic eye induction in *Drosophila* can be put in motion by *Pax6* homologs from distantly related species (Halder et al., 1995; Kozmik et al., 2003). *Pax6* gene is therefore believed to represent an ancient master control gene in eye development (Gehring and Ikeo, 1999).

Much of how *Pax6* genes orchestrate basic steps of early eye development has been learned through the molecular genetic dissection of the RDGN in *Drosophila* (Fig. 1) (for review see Pappu

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0012-1606/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.ydbio.2009.06.013

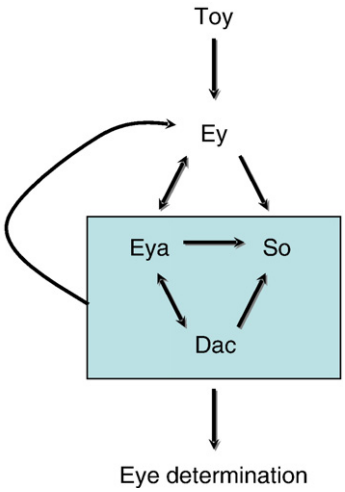


Fig. 1. Core interactions of the *Pax6* genes *toy* and *ey* in the *Drosophila* RDGN. Boxed genes also interact at the protein level. Information compiled from Ostrin et al. (2006) and Pappu and Mardon (2004).

and Mardon, 2004). In the case of *toy*, the earliest transcriptional activation takes place in the blastoderm embryo. *toy* is partially required for *ey* activation during embryonic development of the eye-antennal imaginal disc, which gives rise to the adult head including the compound eyes in the indirectly developing *Drosophila* (Czerny et al., 1999). In final instar *Drosophila* larvae, *toy* and *ey* are co-expressed in the region of the eye-antennal imaginal disc, which gives rise to the compound eye, the ocelli and a large part of the adult head cuticle, where these visual organs are embedded. In the prospective compound eye cells, this co-expression is maintained until the beginning of retinal differentiation, which is induced by a progressing front of neural induction, the morphogenetic furrow. Most transcription factor genes that have thus far been found to be required for patterning of the adult eye primordium in *Drosophila* are direct or indirect targets of *Pax6* activation (Fig. 1) (Ostrin et al., 2006; Pappu et al., 2005). This includes the *Six*-family homeobox genes *sine oculis* (*so*) and *optix*, the nuclear haloacid dehalogenase (HAD) group phosphatase *eyes absent* (*eya*) and the *ski*/*sno* gene family related transcriptional co-factor *dachshund* (*dac*) (Bonini et al., 1997; Cheyette et al., 1994; Mardon et al., 1994; Pappu and Mardon, 2004). In addition, biochemical evidence has indicated that Ey forms transcription factor complexes with the protein products encoded by *teashirt* (*tsh*) and *homothorax* (*hth*), which cooperate in maintaining the commitment state of retinal precursor cells in the eye disc (Bessa et al., 2002). This state is stabilized by positive feedback activation of *ey* by *eya* and *dac* (Bonini et al., 1997; Shen and Mardon, 1997). In addition to these interactions between RDGN-associated transcription factor genes themselves, extracellular signaling combinations play an important part in orchestrating the dynamic gene expression landscape of the developing *Drosophila* eye primordium (Firth and Baker, 2008).

Pax6 evolution and function has been intensely studied in *Drosophila*, but many aspects warrant further investigation. For instance, orthologs of *toy* and *ey* have been identified in other holometabolous insects including Lepidoptera, but only single *Pax6* genes were isolated from primitive directly developing insect species (Czerny et al., 1999). *Drosophila* is one of only two arthropod species in which two *Pax6* genes have been found. The second arthropod is the distantly related myriapod *Glomeris marginata* (Prpic, 2005). It is not clear if these insect and myriapod *Pax6* duplicates originated from the same or independent duplication events (Czerny et al., 1999; Prpic, 2005). Only one *Pax6* ortholog was found by degenerate PCR in the horseshoe crab *Limulus polyphemus*, which represents the arthropod subphylum Chelicerata (Blackburn et al., 2008).

The currently available genetic data indicate largely non-redundant and differential roles of *ey* and *toy* in *Drosophila* development including the eye-antennal imaginal disc (Benassayag et al., 2003; Clements et al., 2008; Kurusu et al., 2000; Punzo et al., 2004, 2002). This raises the question of how the diversification of *toy* and *ey* affected the architecture of the *Drosophila* RDGN in comparison to other species. Secondly, despite the critical role of *ey* and *toy* during the development of the adult eye in *Drosophila*, the larval eyes of *Drosophila*, the Bolwig organs, do develop normally in *toy* and *ey* double mutant embryos (Suzuki and Saigo, 2000). This situation is inconsistent with the fact that the *Drosophila* larval and adult eyes evolved from a shared ancestral compound eye and are therefore expected to share basic aspects of precursor cell determination (Friedrich, 2008).

In addition to its role in the eye, *Pax6* has also been found to be required for the development of other structures in the anterior head. The *Small eye* mutants of mouse, for instance, are characterized by lack of the nose, abnormal brain morphology and a ventralized pituitary gland (for review see Maekawa et al., 2005; Nomura et al., 2007). Consistent with this, mouse *Pax6* is activated in a broad domain in the neuroepithelium of the prosencephalon, including the region where the eye and the olfactory epithelium develop (Walther and Gruss, 1991). In the nematode *C. elegans*, the *Pax6* orthologue *vab-3* is expressed widely in the head region and *vab-3* mutants have gross abnormalities in head morphogenesis (Chisholm and Horvitz, 1995). In squid, the expression of the *Pax6* gene is not restricted to the embryonic eyes, but is also found in the olfactory organ and the brain (Tomarev, 1997). In *Drosophila*, *toy* null mutant animals display a "headless" phenotype, suggesting that the gene is required for all structures derived from the eye-antennal disc (Kronhamn et al., 2002). Also of note, *ey* is required for the development of the labial palps and the proper differentiation of insulin expressing neurons (Benassayag et al., 2003; Clements et al., 2008). In sum, the involvement of *Pax6* genes in many aspects of head development support the proposal that *Pax6* originated as a regional specification gene and was co-opted for eye development (Catmull et al., 1998; Harris, 1997).

The same conclusion has been drawn from the observation that the onset of retinal differentiation in *Drosophila* depends on differential levels of Decapentaplegic (Dpp), Wingless (Wg) and ecdysteroid signalling levels instead of *ey* itself (Niwa et al., 2004). However, other work has shown that *ey* does operate at the level of retinal development, including the direct activation of the proneural transcription factor *atonal* (*ato*) and rhodopsin genes like *Rh1* (Sheng et al., 1997; Zhang et al., 2006). Intriguingly, *Pax6* has also been found to directly regulate the expression of an *ato* homolog in mouse (Hufnagel et al., 2007; Riesenberg et al., 2009). This data speaks to the visual organ-specific functionality of *Pax6*. At the same time, the *Pax6*-independent induction of *ato* and development of larval eyes in *Drosophila* question the generality of this function. Interestingly, *Pax6* is not expressed in the developing photoreceptors but in the more posterior peripheral nervous system of the ancestral acoel species *Convolutribula longifissura* leading to the proposal that *Pax6* originated as a neuronal specification factor (Hejnol and Martindale, 2008).

Here we report the results from studying sequence conservation, expression and function of *ey* and *toy* in the red flour beetle *Tribolium castaneum*. A number of shared and diverged traits of *Tribolium* in comparison to *Drosophila* promised to shed light on ancestral patterning functions (for review see Friedrich, 2006b). Like *Drosophila*, *Tribolium* is an endopterygote insect that completes larval and pupal stages. The *Tribolium* larva is characterized by reduced visual organs, which are homologous to the *Drosophila* larval eyes. The *Tribolium* adult eyes develop in the lateral epidermis of the larval head capsule, which transforms into the adult head during pupal development. The specification of the adult head compartments, therefore,

takes place during embryogenesis in stark contrast to the postembryonic development of the adult head from the eye-antennal disc in *Drosophila*.

We used *Tribolium* to address three questions. Did *Pax6* duplicate before the diversification of endopterygote insects? Is the dispensability of *Pax6* for *Drosophila* larval eye development ancestral for endopterygote insects? Does *Pax6* affect wider aspects of head development in *Tribolium* besides the visual system? We find that *ey* and *toy* are conserved in *Tribolium*. Our investigations further demonstrate that larval eye development as well as global development of the larval head requires *Pax6* in *Tribolium*. Surprisingly, postembryonic knockdown reveals that adult eye development is comparatively mildly sensitive to *ey* and *toy* reduction. Eye-loss, however, is caused in triple knockdown experiments targeting *toy* and *ey* in combination with *dac*. We propose a model in which the embryonic specification of the peripheral visual primordia is redundantly regulated by *toy* and *ey*, while the subsequent maintenance of primordium commitment and expansion depends in part on redundant and in part on specific functions of *toy*, *ey* and *dac*.

Materials and methods

Tribolium stocks

Tribolium cultures were maintained as described (Liu and Friedrich, 2004). The *Tribolium toy* ortholog was isolated from the San Bernadino *Tribolium castaneum* strain (SB strain). The expressed sequence region of *Tribolium castaneum ey* was cloned from *Tribolium* strain Ga-1.

Molecular biology

The oligonucleotide primers used for nested RT-PCR amplification of part of *Tribolium toy* were the outer pair *Pax6-fw1* (CAY WSN GGN GTN AAY CAR YTN GGN GG) and *Pax6-bw1* (AA CCA NAC YTG DAT NCK NGC YTC NGG) and the inner pair *Pax6-fw2* (ACN MGN CAR AAR ATH GTN GAR YTN GC) and *Pax6-bw2* (TA RTG NGT NCK YTC RAA YTC YTT YTC). The oligonucleotide primers used for RT-PCR amplification of a part of *Tribolium ey* were Tc_*ey* A1 (5′-AGGTCATAGCGGTGTGAACCAG-3′) and Tc_*ey* B1 (5′-TGTTGTTGAAGCCCTGTAGC-3′). Terminal transcript regions of both genes were determined by 5′ and 3′ RACE with Marathon cDNA amplification kit reagents (CLONTECH). The *Tribolium toy* and *ey* transcript sequences are available under accession numbers TC_007490 and TC_008176 respectively.

Sequence analysis

Sequence reads were analyzed in FinchTV 1.4 (Geospiza) and MacVector 6.0.1 (Oxford Molecular Group). Multiple sequence alignments were generated with PRANK (Loytynoja and Goldman, 2008) and further edited in SeAl (Rambaut, 1996). Bayesian estimation of gene tree structure was carried out in MrBayes 3.1.2 applying a mixed discrete gamma (four rates) adjusted/invariant site JTT model of amino acid sequence evolution (Ronquist and Huelsenbeck, 2003). Two independent runs with four chains were performed over 5,000,000 generations and were sampled every 100 generations. Burn-in was initiated after 125,000 samples. See Supplementary data for accession numbers of sequences included in gene tree reconstruction and protein domain analysis.

In situ hybridization

Whole-mount *in situ* hybridization on embryos and postembryonic tissue was performed as described (Friedrich and Benzer, 2000).

RNA interference

Procedures for larval and parental RNAi in *Tribolium* were performed as described (Bucher et al., 2002; Tomoyasu and Denell, 2004). Templates for in vitro transcription were amplified by PCR reaction using primers that contain the promoter for the T7 RNA polymerase. dsRNA was produced by bidirectional in vitro transcription using Megascript T7 kit reagents (Ambion). Average size of injected foraging final stage larvae was 6 mm. Average size of early injected larvae was 3.2 mm. dsRNA fragments injected in *ey* knockdown experiments corresponded to regions 1–493 (*ey* 5′ dsRNA), 536–971 (*ey* RT-PCR dsRNA) and 1209–2108 (*ey* 3′ dsRNA) of the *Tribolium ey* sequence TC_008176. dsRNA fragments injected in *toy* knockdown experiments corresponded to regions 1–197 (*toy* 5′ dsRNA) and 1618–2210 (*toy* 3′ dsRNA) of the *Tribolium toy* sequence TC_007490. The *dac* dsRNA used corresponded to nucleotides 1–850 of the *Tribolium dac* gene sequence AJ307577 (Prpic et al., 2001). Primer sequences are available on request.

Imaging

To study larval head phenotypes, larvae were imbedded in immersion oil. Brightfield microscopy was carried out with differential interference contrast optics on a Zeiss axioplan. Autofluorescent cuticle structures were studied in larval heads after maceration in lactic acid using a Zeiss axiophot producing image stacks that were deconvoluted with AnalysisD software (Olympus). For scanning electron microscopy, head cases of adult *Tribolium* were cleaned with ultrasonic sound, dehydrated in an ethanol series, dried, and coated with gold (EmiTech K500 sputter coater). Images were taken on a Philips XL 30 ESEM and processed with Scandium 5 software.

Quantitative analysis of adult eye size

The number of ommatidia per eye was double-counted on stereoscope images. Data from left and right eyes were separately analyzed. *T*-test statistics was used to investigate significance of eye size differences between treatments. Consistent with results in a parallel study, no significant differences were detected between results from left and right eyes (Yang et al., 2009). Data are therefore shown for right eye only.

Results

toy and ey are conserved in Tribolium

The cloning of *toy* was initiated by nested RT-PCR reactions with degenerate primers targeting conserved coding regions. 17 clones were sequenced, all of which contained the same fragment. While these data suggested the presence of a single *Pax6* gene in *Tribolium*, BLAST searches of the *Tribolium* genome sequence draft version Tcas 2.0 at a later stage of the project revealed the presence of a second *Pax6* gene related to *ey* (Richards et al., 2008). The expression of this paralog during adult head development was confirmed by RT-PCR cloning. 5′ and 3′ terminal transcript regions were determined by RACE experiments for both genes.

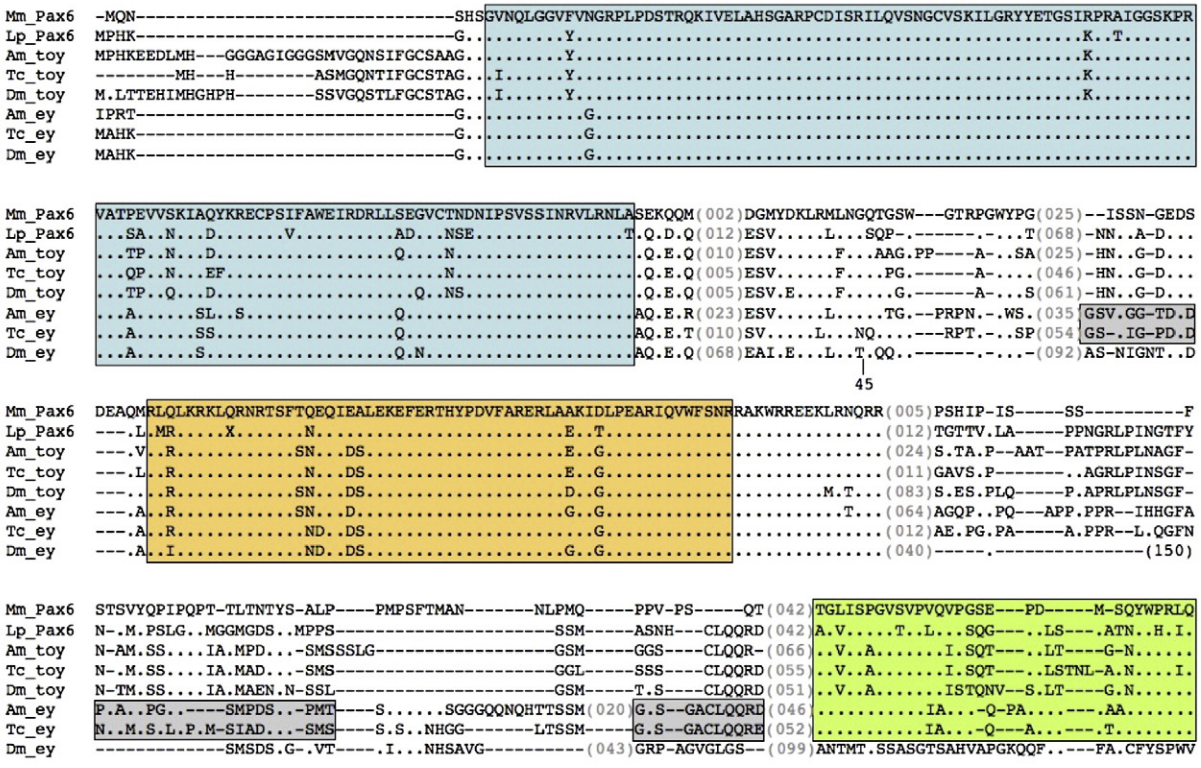
Fig. 2. Evolution and sequence conservation of arthopod *Pax6* genes. (a) Arthropod *Pax6* gene tree. Branches represent relative accumulation of sequence change. Branches with oblique separators have been shortened. Branch support numbers correspond to Bayesian support. Only branches with larger than 0.5 of Bayesian support are resolved. Scale corresponds to 0.2 substitutions per amino acid site. See Supplementary data for sequence alignment and accession numbers of sequences included. (b) Multiple sequence alignment. Dots indicate identity with top sequence. Dashes represent gaps. Length of omitted sequence indicated by numbers in parentheses. Light blue box identifies position of PD. Light orange box identifies HD and green box identifies the conserved C-terminus. *ey* domains conserved in *Tribolium* and honeybee but not *Drosophila* are boxed grey. Species abbreviations: Am = *Apis mellifera*, Dm = *Drosophila melanogaster*, Lp = *Limulus polyphemus*, Mm = *Mus musculus*, Tc = *Tribolium castaneum*. (c) JTT protein substitution distances between *ey* and *toy* in the PD and HD for *Drosophila* (Dm), *Tribolium* (Tc) and *Apis* (Am).

a

c

	PD	HD
Dm	0.1075	0.1018
Tc	0.0982	0.0421
Am	0.0810	0.0560

b



Analysis of the genome sequence drafts confirmed the conservation of *toy* and *eye* in *Tribolium* and the honeybee *Apis mellifera* (Honeybee Genome Sequencing Consortium, 2006). As expected, protein sequence comparison of these gene models revealed high sequence conservation in the paired domain (PD) and the homeodomain (HD) regions (Fig. 2b). Gene tree reconstruction with a multiple alignment of the PD, HD and a conserved undecapeptide motif in the spacer between PD and HD supported a monophyletic origin of all arthropod *Pax6* genes (Fig. 2A). Moreover, the insect *ey* and *toy* orthologs sorted into consistent subgroups. The tree further tentatively suggested that the duplication of *ey* and *toy* preceded the diversification of the arthropod subphyla.

Primary sequence conservation in insect toy and ey

Consistent with previous reports (Callaerts et al., 2006), we also found additional regions of primary sequence conservation outside the PD and HD such as at the C-terminus. The comparison of our more comprehensive sample of *toy* and *ey* sequences revealed that sequence conservation in the C-terminus is not restricted to the last eight amino acids but extends into an additional N-terminal adjacent stretch of 16 amino acids (Fig. 2b). This string is strongly conserved between vertebrate *Pax6* and all insect *ey* and *toy* sequences examined except for *Drosophila ey*. We further noted that a higher degree of sequence divergence was generally characteristic for *Drosophila ey* compared to the *toy* and *ey* orthologs in *Tribolium* and the honeybee. In the latter, small patches of sequence conservation existed both between PD and HD and between HD and the conserved C-terminus. Most of these motifs were less conserved or missing in *Drosophila ey*. The only strongly conserved additional domain in *Drosophila ey* was the previously noted undecapeptide between PD and HD (Callaerts et al., 2006) (Fig. 2b). Interestingly, we also found a consistently higher sequence divergence between *ey* and *toy* in the PD and HD of *Drosophila* compared to *Tribolium* and honeybee (Fig. 2c). This difference was particularly pronounced in the HD. In summary, these findings suggested that *ey* and *toy* have remained more similar at the primary sequence level in *Tribolium* and honeybee, while the *Drosophila ey* paralog is characterized by accelerated sequence divergence.

Embryonic expression of ey and toy

To obtain evidence whether *ey* and *toy* were involved in the development of the larval visual system of *Tribolium*, we studied embryonic expression by whole mount *in situ* hybridization. In contrast to the differential expression of *toy* in the *Drosophila* blastoderm embryo (Czerny et al., 1999), earliest expression of both genes was detected in blastoderm embryos of *Tribolium*. The spatial regulation of *toy* and *ey* was very similar suggesting co-expression in the prospective head region (Figs. 3a and e). This high degree of similarity persisted until early germband extension (Figs. 3d and h), when *toy* was also detected in clusters of cells in the ventral nervous system while the expression of *ey* remained restricted to the head region. By beginning of germ band retraction, the expression of *toy* and *ey* also diverged partially in the embryonic head (Figs. 3i and j). Both genes were widely expressed in the lateral head, but *ey* was also detected in large median cell clusters of the gnathal and procephalic

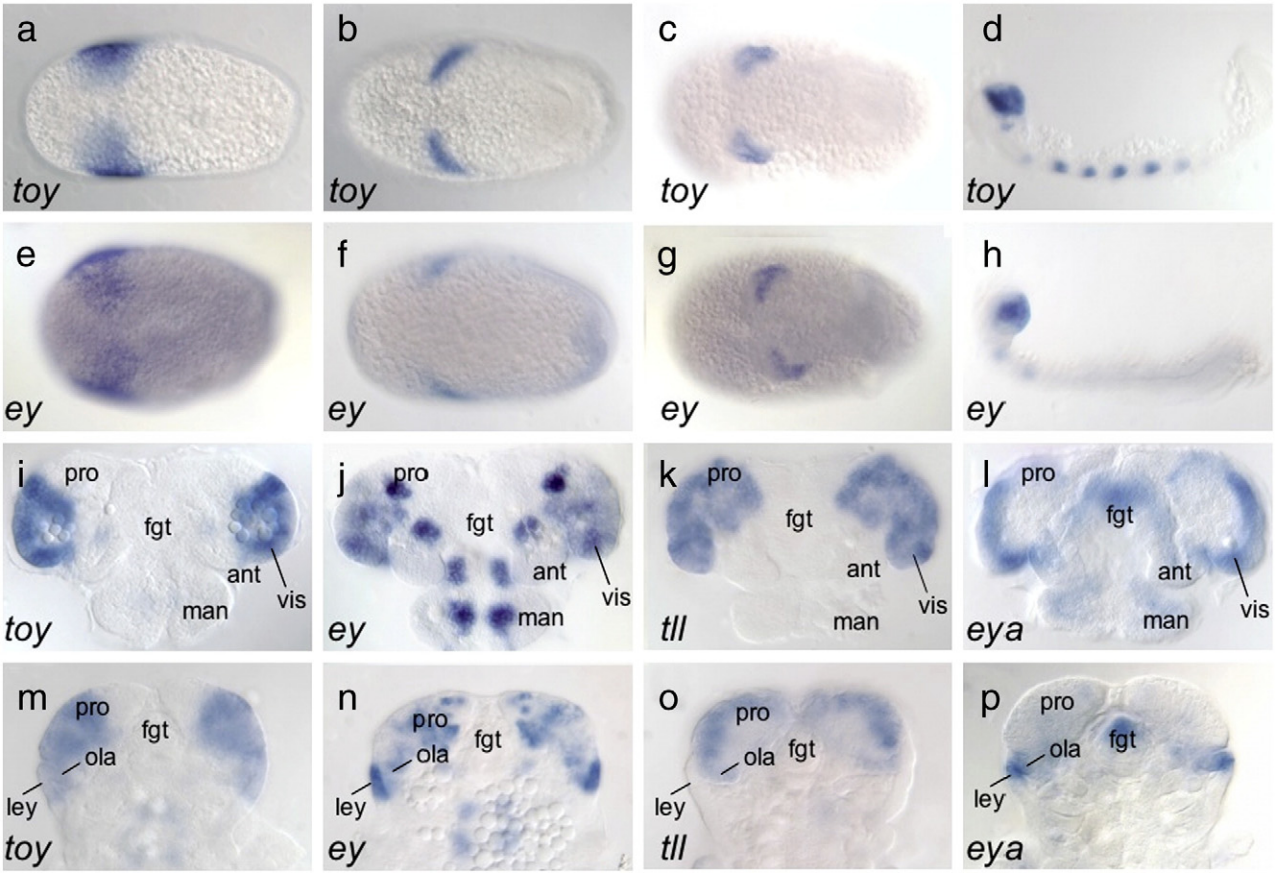


Fig. 3. Embryonic expression of *ey* and *toy* in *Tribolium*. (a–c and e–g) Ventral view of *Tribolium* embryo labeled by whole mount *in situ* hybridization for *toy* (a–c) and *ey* (e–g) expression. Anterior to the left. (a and e) early blastoderm stage, (b and f) later blastoderm/germ band formation stage, and (c and g) early germ band stage. (d and h) Lateral view of early germ band extension stage of (d) *toy* and (h) *ey* expression. Anterior to the left. (i–p) Frontal view of embryonic head labeled by whole mount *in situ* hybridization for *toy* (i + m), *ey* (j and n), *tll* (k and o), and *eya* expression (l and p). Anterior up. (i–l) Early germband retraction stage. (m–p) Late germband retraction stage. Ant = antenna, pro = protocerebrum, fgt = foregut, man = mandible, ley = larval eye primordium, ola = optic lobe anlage, vis = visual anlage.

head segments (Fig. 3j). Importantly, *toy* and *ey* expression filled the ventral portion of the head lobes, which include the visual anlagen of *Tribolium* (Liu and Friedrich, 2004). The *Tribolium tailless* (*tll*) gene is expressed in the entire precursor cell population of the protocerebrum (Fig. 3k) (Daniel et al., 1999; Schroder et al., 2000). By comparison with *tll* expression we concluded that *toy* and *ey* were expressed in the protocerebral neuroectoderm (Fig. 3k). Further, comparison with the early retinal gene *eya* suggested that *ey* and *toy* were co-expressed with other eye selector genes in the embryonic visual system (Fig. 3l) (Yang et al., 2009).

During late germ band retraction, *toy* and *ey* continued to be expressed in the visual anlagen domains, where *tll*, however, was no longer detectable but *eya* was specifically expressed (Figs. 3m–p). This finding was reminiscent of the suppression of *tll* from the larval and adult eye anlagen during embryonic development of the visual system in *Drosophila* (Daniel et al., 1999). In combination, these observations suggested that *ey* and *toy* were co-expressed with *eya* in the visual anlage and, later, in the separating primordia of the larval eyes. This finding contrasted with *Drosophila*, where *ey* is not expressed in precursor tissue of the larval eye (Chang et al., 2001).

Larval eye development is sensitive to ey and toy knockdown

To test for a role of *toy* and *ey* in the developing visual system of *Tribolium* embryos, we generated *Tribolium toy* or *ey* single knockdown embryos using the parental RNAi protocol and injecting dsRNAs at concentrations of 1 μg/μl and 2 μg/μl (Bucher et al., 2002). Offspring from females injected with *ey* dsRNA contained a considerable fraction of larvae (>10%) with unilaterally or bilaterally reduced larval eyes (Figs. 4b, c, and j). No conspicuous difference in the percentage of phenotypic larvae was observed in offspring resulting from females injected at 1 μg/μl and 2 μg/μl concentrations. Knockdown of *toy* resulted in more dramatic larval eye phenotypes ranging from unilateral reduction to complete loss (Figs. 4d–f and j). In addition, the percentage of phenotypic larvae increased with the concentration of injected dsRNA. 85% phenotypic larvae were obtained at the 2 μg/μl concentration compared to over 58% at the 1 μg/μl concentration (Fig. 4j). Of note, no additional abnormalities of the larval head could be detected, suggesting specific sensitivity of larval eye development to *ey* and *toy* single knockdown. These results demonstrated that larval eye development is dependent on *ey* and *toy* in *Tribolium* in contrast to *Drosophila*.

Functional redundancy of toy and ey in the developing embryonic head

Considering the overlapping expression of *ey* and *toy* in the embryonic visual system, we further probed for genetic interaction by combinatorial knockdown of *ey* and *toy*. In these experiments, the fraction of larval eye-loss phenotypes increased to 90% (Fig. 4j). Similar penetrance of the larval eye-loss phenotype was obtained at concentrations of 1 μg/μl and 2 μg/μl concentration per injected dsRNA. This suggested that the difference in penetrance between double and single knockdown experiments was not primarily the consequence of dsRNA dosage increase. We therefore concluded that *toy* and *ey* cooperate in a redundant manner during embryonic head development in *Tribolium*.

ey and toy affect global development of the posterior embryonic head

Closer inspection of the *ey + toy* double knockdown larvae revealed isolated cases of asymmetric head deformities and posterior head reduction (~10%) (Figs. 4h and i). In some individuals, evidence of abnormally placed pigmented cells suggested extreme dislocation of larval photoreceptor cells (Fig. 4g). Further consistent with defects in global head patterning, the inspection of larval head cuticle preparations showed cases of loss of head bristles in *toy + ey* double knockdown animals (Fig. 4i). Quantitative analysis revealed deletion of the posterior vertex setae in all animals examined ($n = 6$) and

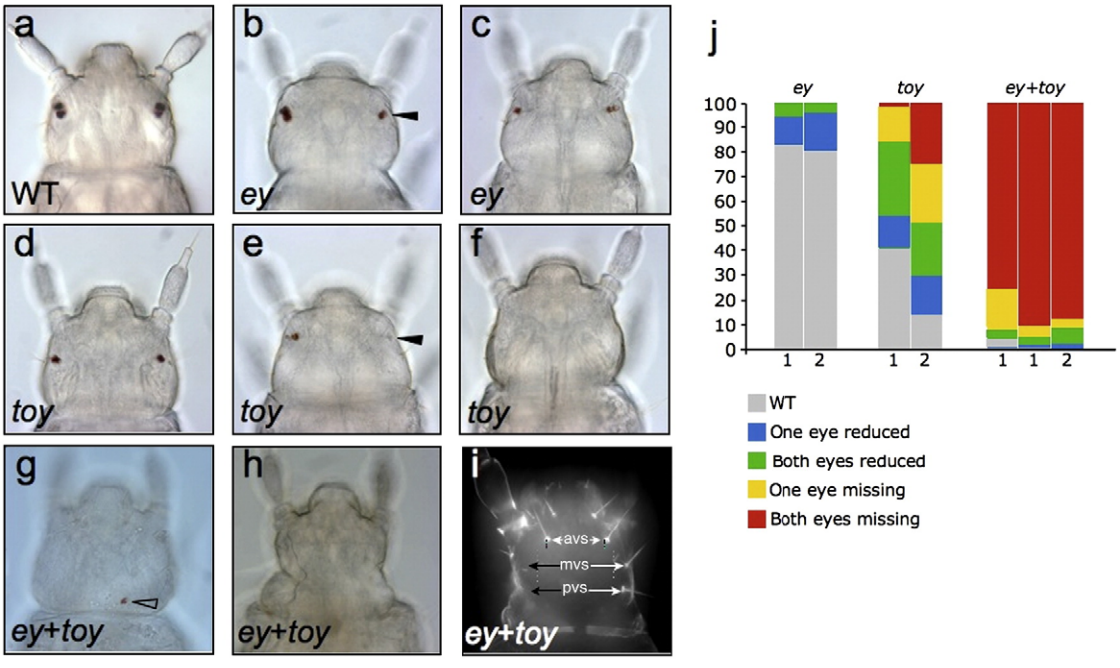


Fig. 4. Knockdown analysis of *Tribolium ey* and *toy* function during embryonic development. (a–i) Dorsal view of the head of freshly hatched first instar *Tribolium* larva. Anterior up. (a) Wild type (WT). (b) *ey* knockdown phenotype with one larval eye reduced (arrowhead). (c) *ey* knockdown phenotype with both larval eyes reduced. (d) *toy* knockdown phenotype with both larval eyes reduced. (e) *toy* knockdown phenotype with one larval eye missing (arrowhead). (f) *toy* knockdown phenotype with both larval eyes missing. (g) *ey + toy* larval eye-loss knockdown phenotype with abnormally positioned pigmented cells that may be displaced larval photoreceptors (arrowhead). (h) *ey + toy* knockdown larval eye-loss phenotype with reduced posterior larval head capsule. (i) Deconvoluted UV-autofluorescence image of *ey + toy* knockdown phenotype with posterior larval head patterning defects. Black arrows indicate positions of unilaterally missing bristles. White arrows point at anterior vertex seta (avs), median vertex seta (mvs), and posterior vertex seta (pvs). Nomenclature of setae based on Schinko et al. (2008). (j) Quantitative analysis of phenotype frequencies in *ey* and *toy* single and double knockdown experiments. Y-axis: phenotype fractions. X-axis: concentration of injected dsRNA in μg/μl.

deletion of the ventral vertex setae in 67% of animals examined. Taken together, these findings provided evidence that *toy* and *ey* were also involved in global patterning or growth control during embryonic head development in *Tribolium*.

Expression of ey and toy in the developing adult eye

We next investigated whether *toy* and *ey* were also involved in the development of the *Tribolium* adult eye. Specific expression of both genes was detected by whole mount *in situ* hybridization in the lateral head epidermis of resting stage larvae. In reference to the retracting larval eyes, the expression of *ey* and *toy* filled a wide area of the developing head epidermis in front of the eye primordium at this stage (Figs. 5a and b). The similarity of the expression domains suggested that *ey* and *toy* are co-expressed in a large area between the antenna and the compound eye placode. The posterior margin of the *toy* expression domain appeared sharply defined suggesting down-regulation in differentiating tissue or tissue close to retinal differentiation (Fig. 5a), while the expression of *ey* tapered off in a more central area of the eye primordium (Fig. 5b).

The similarity of *ey* and *toy* expression persisted into the pupal stage. In the 48 h old *Tribolium* pupa, in which the prospective retinal field has begun to differentiate, *toy* and *ey* were specifically expressed in cells surrounding the differentiating retina (Figs. 5c and d). This expression pattern was reminiscent of the situation in the *Drosophila* eye disc (Bessa et al., 2002) and suggested that *ey* and *toy* remain co-expressed in epidermal cells at the immediate vicinity of the retina but not in the differentiating retina.

Mild effect of ey and toy knockdown on adult eye size

The expression of *toy* and *ey* in the early adult eye primordium was consistent with the expectation that the development of the adult *Tribolium* eyes depended on *toy* and *ey*. To test this hypothesis, we knocked down *toy* and *ey* transcript levels by systemic RNAi in last instar *Tribolium* larvae (Tomoyasu and Denell, 2004). Strikingly, knockdown experiments varying transcript target regions and concentration resulted in only mild cases of detectable eye reduction (Supplementary data) (Figs. 6a–c). To quantify the impact of *toy* and *ey* knockdown we determined the average number of ommatidia in the right compound eye in experimental cohorts of treated and control animals. Based on this measure, an average eye size of 83 (+/−3) and 77 (+/−6) ommatidia was observed in *toy* and *ey* knockdown animals respectively compared to 93 (+/−2) ommatidia in untreated and 95 (+/−3) ommatidia in EGFP dsRNA-injected animals (Fig. 6g). While the differences between *ey* or *toy* knockdown animals and untreated or EGFP-injected animals were significant (*t*-test: $p<0.05$), the degree of eye reduction between *ey* and *toy* knockdown animals was not significantly different. Of note, no significant difference in eye size was detected between untreated and EGFP-injected animals suggesting insensitivity of eye development to injection injury and non-specific dsRNA (Fig. 6g).

Considering the functional redundancy of *ey* and *toy* in the *Tribolium* embryo, we further examined the consequence of knocking down *ey* and *toy* in combination during late larval development. The average eye size of 75 (+/−5) ommatidia in *toy*+*ey* knockdown animals was only significantly different from *toy* single knockdown animals (Fig. 6g). This suggested that *ey* and *toy* interacted in an epistatic manner during adult eye development in contrast to the situation in the embryo.

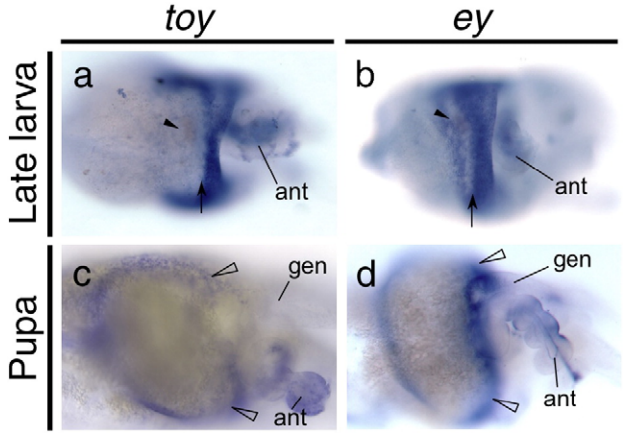


Fig. 5. *Tribolium toy* and *ey* expression during adult eye development. (a–d) Lateral view of larval or pupal head labeled for expression of *toy* (a, c) and *ey* (b, d). Anterior to the right and dorsal up. (a and b) Arrows indicate approximate position of the incipient morphogenetic furrow. Black arrowheads point at position of the retracting larval eyes. (c and d) Open arrowheads point at expression in the periphery of the differentiating eye field. Ant = antenna, gen = gena.

Distinct effects of ey knockdown on adult eye morphology

A detailed comparison of adult eye morphology revealed no conspicuous abnormalities in *toy* knockdown animals compared to untreated animals (compare Figs. 6a with b). *ey* knockdown animals, however, were characterized by widened separation of the compound eye facets (compare Figs. 6a′ with c′). Further, while the border between head cuticle and compound eye was indistinguishable between untreated and *toy* knockdown animals, the transition between compound eye surface and head cuticle was more pronounced in *ey* knockdown animals leading to a fold at the dorsal eye margin (Figs. 6a–c). Second, the gena of *ey* knockdown animals appeared shortened and formed a posterior-pointing tip in contrast to the rim-like gena of untreated or *toy* knockdown animals, which protruded deeply into the anterior eye margin (Figs. 6a′–c′). The compound eye morphology of *toy*+*ey* knockdown animals (not shown) was indistinguishable from *ey* single knockdown animals suggesting that *ey* was involved in processes that affect the patterning of the compound eye independently of *toy*.

Combinatorial knockdown of ey and toy with dac causes adult eye-loss

The failure to provoke adult eye-loss in *Tribolium* by *ey* and *toy* knockdown was remarkable considering the sensitivity of adult eye development to *Pax6* in *Drosophila*. The result was further notable in light of the fact that knockdown of other *Tribolium* RDGN genes such as *eya* and *so* can cause severe to complete adult eye reduction demonstrating that the RNAi protocol is sufficiently effective to cause eye-loss by RDGN gene transcript reduction (Yang et al., 2009). Wondering if *ey* and *toy* might cooperate with additional RDGN genes, we noted the similarity of *ey* and *toy* expression with *dac* expression during adult eye development in *Tribolium*. Like *ey* and *toy*, *dac* is expressed immediately in front of the developing *Tribolium* adult eye and, later, along the periphery of the differentiating retina (Yang et al., 2009). This similarity was intriguing because *dac* knockdown also failed to cause eye-loss in *Tribolium* (Yang et al., 2009). We therefore tested if *dac* cooperated with *ey* and *toy* in *Tribolium*.

Single injection with high concentration of *dac* dsRNA into last instar larvae led to an average reduction of eye size to 53 (+/−4) ommatidia consistent with results in independent experiments (Figs. 6d and g) (Yang et al., 2009). Four adult animals were obtained from larvae injected with a mix of *toy*, *ey* and *dac* dsRNA. Two of these represented eye-loss phenotypes (Fig. 6f). In the other two animals, eye size ranged from 26 to 57 ommatidia (Figs. 6e and g). Of further note, the retina of eye-reduced *toy*+*ey*+*dac* knockdown animals was partially interrupted by head cuticle, which was never observed in single knockdown animals (compare Figs. 6a′–d′ to e′). These results

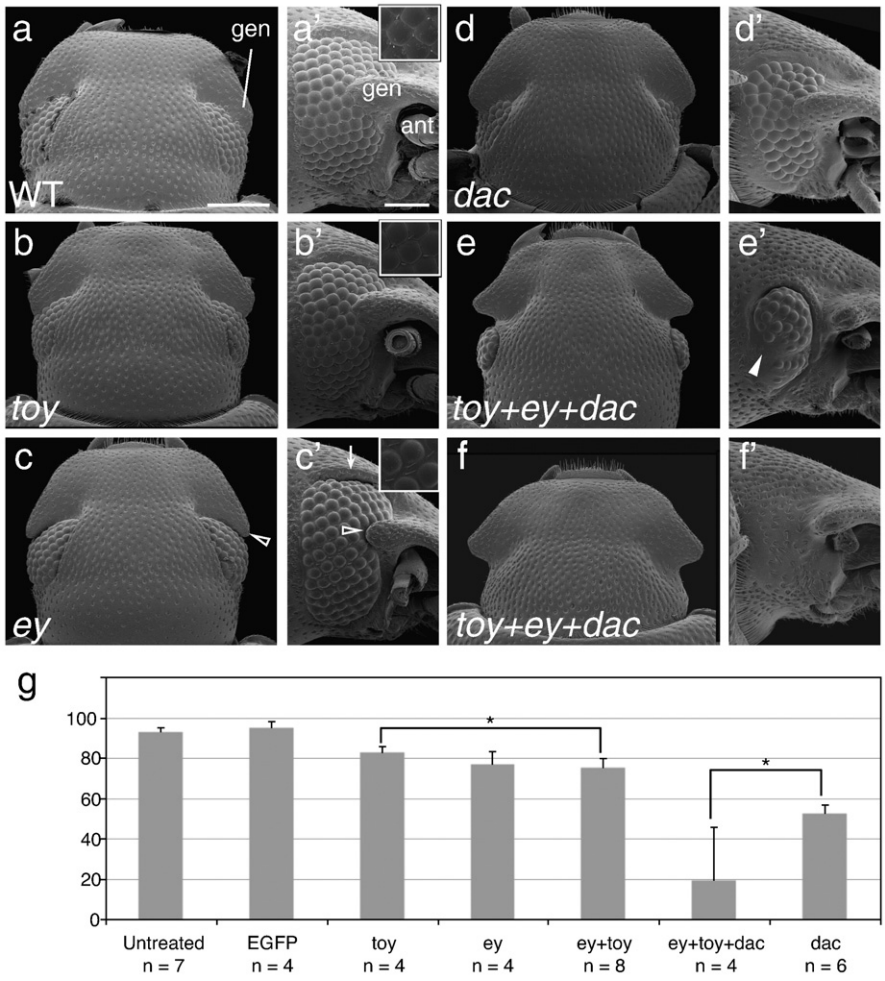


Fig. 6. Effect of single and combinatorial *toy*, *ey* and *dac* knockdown on *Tribolium* adult eye development. Results obtained from injecting dsRNA at 2 μg/μl concentration per target gene into final instar larvae. (a–f) Dorsal view scanning electron microscopy image. Scale bar in panel a represents 200 μm for all dorsal perspective panels. Antennae removed. Anterior up. (a′–f′) Lateral view scanning electron microscopy image. Scale bar in panel a′ represents 100 μm for all lateral perspective panels. Antenna removed. Anterior right. Dorsal up. Insets show compound eye surface at high magnification. (a and a′) Untreated specimen. (b and b′) *toy* knockdown specimen. (c and c′) *ey* knockdown specimen. Open arrow indicates abnormally shaped gena. Arrow indicate dorsal cuticle fold. (d and d′) *dac* knockdown specimen. (e and e′) Combinatorial *toy* + *ey* + *dac* knockdown eye reduction phenotype. Arrow points at compound eye area where facets are replaced by head cuticle. (f and f′) Combinatorial *toy* + *ey* + *dac* knockdown eye-loss phenotype. (g) Quantitative analysis of eye reduction. Bar graph describes average number of ommatidia in right eye in untreated and knockdown animals. Y-axis represents number of ommatidia. Error bars represent standard deviation. Select significant differences are indicated by asterisk representing $p<0.05$ in *t*-test. All average eye sizes in the *toy*, *ey* or *dac* dsRNA-injected animals are significantly reduced compared to control animal data (Untreated and EGFP). Ant = antenna, gen = gena.

suggested that *toy* and *ey* cooperate with *dac* and that the combination of these three genes was essential for normal eye specification or eye primordium growth in *Tribolium*.

Evidence of partial functional redundancy of Pax6 and dac during late adult eye primordium development

The triple knockdown of *dac*, *ey* and *toy* provoked stronger average eye reduction (80%) than the sum of eye reduction observed in *ey* + *toy* and *dac* knockdown animals (20% + 45%) (Fig. 6g). This result suggested that *dac* interacted synergistically with *toy* and *ey*. To test this and explore further how *dac*, *ey* and *toy* interacted during adult eye development, we compared the effects of knocking down *dac*, *ey* and *toy* in all possible combinations. To maximize the consistency of knockdown efficiency, injections were carried out with single preparations of *ey*, *toy* and *dac* dsRNA each at a concentration of 1 μg/μl (Fig. 7).

Indicating dosage sensitivity, average eye reduction in animals injected with dsRNA at a concentration of 1 μg/μl was significantly milder than in experiments with higher dsRNA concentration (*t*-test: $p<0.05$) (compare Figs. 6g and 7). Relative differences in average eye

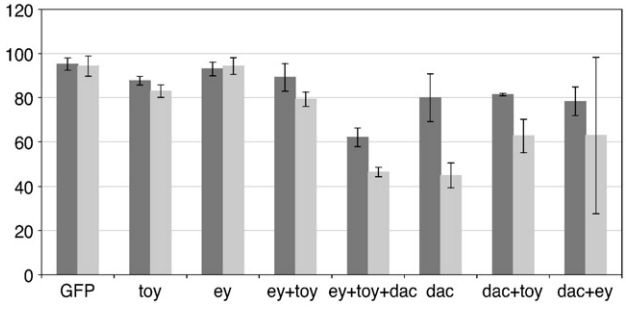


Fig. 7. Quantitative analysis of eye reduction in postembryonic *toy*, *ey* and *dac* knockdown experiments. Bar graphs describe average number of ommatidia in right eye in untreated and knockdown animals injected with dsRNA at 1 μg/μl concentration per target gene. Y-axis represents number of ommatidia. Results obtained from early and late injection experiments are given for each injected dsRNA or dsRNA combination. Right-positioned dark grey bars describe results obtained from injecting into final instar larvae. Left-positioned light grey bars describe results obtained from injecting into early instar larvae. Error bars represent standard deviation. Sample sizes, i.e. eyes counted, per late injection experiment: GFP = 4, *toy* = 10, *ey* = 14, *toy* + *ey* = 17, *ey* + *toy* + *dac* = 2, *dac* = 18, *toy* + *dac* = 3, *toy* + *ey* = 3. Sample sizes per early injection experiment: GFP = 4, *toy* = 2, *ey* = 6, *toy* + *ey* = 3, *ey* + *toy* + *dac* = 2, *dac* = 2, *toy* + *dac* = 3, *toy* + *ey* = 2.

reduction resulting from single and combinatorial dsRNA injections in last instar larvae, however, were largely consistent with those obtained from injection of high concentration dsRNAs. One exception was the milder eye reduction in *ey* knockdown animals than in *toy* knockdown animals. The average eye size among *ey* knockdown animals (93+/−3 ommatidia) was not significantly different from EGFP dsRNA control injected animals (95+/−3 ommatidia), while average eye size among *toy* knockdown animals (88+/−2 ommatidia) was. Further, average eye size in *toy*+*ey* double knockdown animals (89+/−6 ommatidia) was similar to *toy* single knockdown animals. Eye reduction tested statistically significant compared to untreated or EGFP-injected animals in the *toy* knockdown animals but not in the *toy*+*ey* double knockdown animals due to higher variation in the latter. Notwithstanding, these results were compatible with the evidence for epistatic interaction between *ey* and *toy* in the high concentration knockdown experiments.

Likewise consistent with the results in the high concentration knockdown experiments, targeting *dac* yielded the strongest eye size reduction in single knockdown experiments (80+/−10 ommatidia) (compare Figs. 6g and 7). Of note, single and combinatorial knockdown experiments involving *dac* persistently caused higher larval lethality, reducing the number of adult phenotypes available for analysis (Supplementary data). Nonetheless, the combinatorial knockdown experiments involving *dac* revealed clear trends that were consistent with the results in knockdown experiments with higher dsRNA concentrations (compare Fig. 6g with Fig. 7). Most importantly, combinatorial knockdown of *toy*, *ey* and *dac* resulted in a 34% reduction of ommatidia (62+/−4) compared to 7%, 1% and 15% in the *toy*, *ey* and *dac* single knockdown experiments, respectively. This data supported the conclusion from high concentration injection experiments that *toy* and *ey* synergize with *dac* in processes that affect adult eye size during the final stages of retinal primordium patterning. The equivalent impact of *dac* single knockdown and *toy*+*dac* or *ey*+*dac* double knockdown further suggested that *dac* interacts with *ey* and *toy* in part epistatically and in part synergistically. Moreover, the fact that synergistic enhancement of eye reduction was only observed when *ey* and *toy* together were silenced in combination with *dac* suggested redundant roles of the *Pax6* paralogs in the synergistic interaction with dac.

Evidence of a Pax6-independent role of dac on early eye primordium development

Because *toy* and *ey* affect adult eye primordium development at very early stages of larval development in *Drosophila*, we also compared the effect of initiating gene knockdown in early instar and last instar *Tribolium* larvae (Fig. 7). No significant differences between early and late injection were found in experiments that targeted only *ey*. A mild but significant difference, however, existed between the early and late *toy*-injected animals. The average number of 88 (+/−3) ommatidia the in late injected *toy* knockdown animals compared to 83 (+/−3) ommatidia in the early injected *toy* knockdown animals. Conspicuously more dramatic phenotype enhancement was observed in early *dac* knockdown experiments. In this case, an average eye size of 80 (+/−10) ommatidia in late injection experiments compared to 45 (+/−6) ommatidia in early injection experiments (Fig. 7). Taken together, these results suggested that *ey* and *toy* affected adult eye size specifically in the final larval instar in contrast to *dac*, which seemed to execute an additional role during early larval eye development that affects adult eye size.

In all early injection experiments that targeted *dac* in combination with *ey* or *toy*, eye size was further reduced compared to the *toy* and *ey* single knockdown treatment experiments. The eye reduction in *dac* single knockdown experiments, however, was not exceeded. The equivalent eye size reduction in *dac* single knockdown and *dac*+*toy*+*ey* triple knockdown animals (46.5 +/−2) suggested that *dac* influenced early eye size in a process that is upstream of that which is also influenced by *toy* and *ey* in the late larva (Fig. 7). However, in the double knockdown experiments that targeted *dac* in combination with either *toy* or *ey* only, eye size reduction was of intermediate strength compared to single *toy*, *ey* and *dac* knockdown experiments, inconsistent with a role of *dac* being epistatic to *ey* and *toy*. Considering the limited statistical power of early injection experiments, due to the low number of surviving animals (<5), we concluded that the exact nature of *dac* interaction with *toy* and *ey* during early adult eye development requires a larger sample of experimental animals.

Discussion

toy and ey predate the diversification of holometabolous insects

In this paper we describe conservation, expression and function of the *Pax6* gene orthologs *toy* and *ey*, which were first described in *Drosophila* (Czerny et al., 1999; Quiring et al., 1994), in the model coleopteran *Tribolium castaneum*. Previous efforts identified duplicated *Pax6* forms in other dipteran and lepidopteran species but not in more distantly related insects including *Tribolium* or grasshopper (Czerny et al., 1999). Assisted by completion of the honeybee and flour beetle genome sequence projects, we have been able to identify orthologs of both *toy* and *ey* in these species. This finding implies that *toy* and *ey* derived from duplication prior to the diversification of endopterygote insects (Czerny et al. 1999).

It has previously been argued that the linkage of *ey* and *toy* on chromosome 4 in *Drosophila* (separated by 286.6 kb) reflects the recentness of the duplication event that generated *ey* and *toy* (Czerny et al., 1999, Gehring and Itoh 1999). Given that the diversification of *ey* and *toy* dates back deeper in time, the genetic linkage of *Drosophila ey* and *toy* is more likely to reflect gene regulatory constraints. Consistent with this, *ey* and *toy* in *Tribolium* are also located on the same chromosome, separated by 1118 kb.

Our molecular phylogenetic analysis tentatively suggests that the duplication generating *ey* and *toy* may have occurred before the diversification of the major arthropod subgroups Pancrustacea (Hexapoda+Crustaea), myriapods and chelicerates. The support for some of the critical internal nodes, however, is low, which is undoubtedly in part due to the limited number and high conservation of alignment sites that can be extracted from Pax genes for gene tree estimation. Similar limitations were encountered in previous attempts to resolve arthopod *Pax6* gene evolution (Blackburn et al., 2008). The forthcoming genome sequence information for a wider range of arthropod species may yield more definitive insights into the diversification of *Pax6* in this phylum.

Diverged Pax6 expression and function in the embryonic visual system

Our analyses unravel major differences in expression and function of *toy* and *ey* during embryonic visual system development between *Tribolium* and *Drosophila*. Development of the larval eye is highly sensitive to reduction of *ey* and *toy* in *Tribolium*, while these genes are dispensable for larval eye development in *Drosophila* (Suzuki and Saigo, 2000). This difference has corollaries at the level of gene expression. In *Drosophila*, only *toy* is transcriptionally activated during early development of the cephalic region in the blastoderm embryo, while *ey* is not activated in the visual system before the segregation of the eye-antennal imaginal disc (Chang et al., 2001; Czerny et al., 1999; Sheng et al., 1997). In *Tribolium*, *toy* and *ey* are both expressed in a region of the blastoderm embryo that is reminiscent of the cephalic expression of *toy* in *Drosophila* and can be assumed to include the embryonic visual anlage. Moreover, the co-expression of *Tribolium ey* and *toy* persists into the differentiating larval eye primordium in

Tribolium, which may be the case for *ey* but can be excluded for *toy* in *Drosophila* (Chang et al., 2001; Sheng et al., 1997).

The larval eyes of endopterygote insects share evolutionary ancestry with the adult eye (Friedrich, 2008). This leads to the conclusion that the *Pax6* dependence of larval eye development in *Tribolium* likely represents an ancestral state, while the situation in the *Drosophila* embryo is derived. It has previously been speculated that larval eye development became independent from *Pax6* in *Drosophila* because the extreme reorganization of the larval head in the higher Diptera rendered ancestral growth and patterning processes obsolete (Friedrich, 2006a). Consistent with this hypothesis, the *Tribolium* larva develops a typical insect head capsule, and combinatorial knockdown of *ey* and *toy* revealed wider roles in *Tribolium* head development, implying that *toy* and *ey* function as broader patterning genes instead of visual organ-specific determination genes. Interestingly, this model is also supported by combined interpretation of the larval and adult eye knockdown data in *Tribolium* (see below).

Ancestral redundancy of toy and ey in Tribolium

The strong effect of combinatorial *ey* and *toy* knockdown on larval eye development compared to single knockdown experiments in *Tribolium* is further noteworthy because it suggests that *ey* and *toy* can complement each other in the regulation of shared targets. The milder effect of *ey* compared to *toy* single knockdown further suggests that *toy* contributes a somewhat larger fraction of this overlapping functionality. In addition, *toy* may partially be activating *ey* consistent with the situation in the embryonic eye disc in *Drosophila* (Chang et al., 2001).

The evidence for redundant regulation of embryonic head development by *toy* and *ey* in *Tribolium* is significant considering the lack of genetic evidence of similar redundancy in *Drosophila*, although it has been suspected that the variability of eye reduction in *ey* null mutant *Drosophila* could reflect complementing activity of *toy* (Punzo et al., 2004). During embryonic development, *ey* and *toy* seem to engage in independent functions in *Drosophila* (Benassayag et al., 2003; Clements et al., 2008; Kurusu et al., 2000; Punzo et al., 2004, 2002). Moreover, the activation of *ey* in the early eye-antennal imaginal disc is strongly dependent on *toy*, implying epistasis (Kronhamn et al., 2002). During adult eye development, *Drosophila toy* acts largely, although not exclusively, through *ey* (Punzo et al., 2004). However, the difficulties with generating *toy* mutant *Drosophila* and the dramatic effects of *Pax6* reduction on early eye disc development may obscure more subtle interactions between *toy* and *ey* in *Drosophila*. Indeed, the direct activation of the early retinal gene *so* by both *toy* and *ey* in *Drosophila* is consistent with synergistic interaction models (Punzo et al., 2002).

Interestingly, the evidence for a higher degree of functional divergence between *toy* and *ey* in *Drosophila* correlates with a higher level of sequence divergence between *toy* and *ey* in *Drosophila* compared to *Tribolium* and honeybee. The striking loss of the conserved *Pax6* C-terminus in *Drosophila ey* has been previously discussed (Callaerts et al., 2006). Our data reveal that this protein sequence modification is specific for *Drosophila ey*, because the *Pax6* C-terminus is strongly conserved in *Tribolium* and honeybee *ey*. We also discovered additional examples of *Drosophila ey*-specific loss of conserved protein domains. Taken together, these findings indicate evolutionary changes of Ey protein interaction in the *Drosophila* lineage. This is significant in light of the multiple protein–protein interactions, which *Drosophila* Ey has been found to engage in (Bessa et al., 2002).

Also noteworthy in this context is the repression of *Distal-less* (*Dll*) expression by *ey* in *Drosophila* (Punzo et al., 2004). This interaction is essential for the instruction of eye versus antenna primordium commitment in the *Drosophila* eye-antennal imaginal disc and cannot be fulfilled by *toy*. Comparing the embryonic expression of *toy* and *ey* with the previously published expression and function of *Dll* in *Tribolium* leads to the conclusion that *Dll* transcription initiates in the embryonic head lobes despite co-expression with *toy* and *ey* (Beermann et al., 2001). This suggests that neither *toy* nor *ey* is a negative regulator of *Dll* expression during antenna and visual primordium specification in the *Tribolium* embryo. The relationship between these genes needs to be studied in additional species to determine which condition is ancestral. Notwithstanding, it is reasonable to speculate that the repression of *Dll* by Ey evolved during the origin of the *Drosophila* specific eye-antennal imaginal disc.

Point-mutated variants of the Ey HD fail to repress *Dll* in the *Drosophila* eye disc (Punzo et al., 2004). This implies the HD as critical facilitator for this *Drosophila* specific function although the *ey* HD is less extensively diverged than adjacent sequence region and the C-terminus. The functional significance of the most dramatic sequence changes in the *Drosophila ey* therefore needs further study.

Genetic redundancy is the instantaneous consequence of gene duplication (Force et al., 1999). A growing number of studies suggest that this type of regulatory redundancy is advantageous and under purifying selection in development (Wagner, 2000). This leads to the conclusion that the higher level of functional redundancy of *toy* and *ey* in *Tribolium* represents a conserved ancestral state.

Diverged Pax6 functionality during adult eye development

In contrast to the results in the embryo, *ey* and *toy* single or double knockdown affects adult eye size and morphology only mildly in *Tribolium*. Notwithstanding the caveat that RNAi knockdown may fall short of genetic null mutant phenotypes, the *ey* and *toy* knockdown phenotypes are milder than expected based on the *Drosophila* paradigm. However, by exploring alternative models of *Pax6* function in *Tribolium* we have also found that the combinatorial knockdown of *ey* and *toy* with *dac* can enforce eye-loss. The sum of our larval knockdown results leads to the conclusion that *Pax6* and *dac* contribute in part independently and in part redundantly to eye primordium development in *Tribolium* (Fig. 8). Independent functions of *ey* and *toy* as well as *dac* are implied by the *ey*-specific facet defects. Further, independent functions of the *Pax6* paralogs and *dac* are implied by the significantly stronger eye reduction in *dac* single knockdown animals compared to *ey* or *toy* knockdown animals. Redundant overlap of *Pax6* and *dac* functions is indicated by the non-additive enhancement of eye reduction in $toy + ey + dac$ triple knockdown animals, which suggests the existence of shared regulatory targets.

One caveat in the interpretation of the combinatorial knockdown data is that the enhancement of eye reduction in the $toy + ey + dac$ triple knockdown animals may reflect dosage-dependent regulation. In this case, however, enhancement of the *dac* phenotype should also have been observed in the double knockdown experiments that paired *dac* with *ey* or *toy* only, which was not the case. Moreover, the results from our parallel study of *dac* suggest that the *dac* knockdown experiments achieve maximum lack-of-function impact on adult eye development (Yang et al., 2009). Of note, the same study has shown that appendage truncation can serve as marker to identify experimental animals in which the knockdown of *dac* expression was effective. Eye size was nevertheless averaged across all animals in the analysis of *dac* interaction with *ey* and *toy* in this study due to the lack of comparable markers for the latter genes. However, the *dac* data demonstrate that knockdown efficiency differs between individuals in the same dsRNA injection series (Yang et al., 2009). An important improvement of future studies will therefore be an assay that quantifies knockdown efficiency for every gene, such as qPCR.

Another caveat in the interpretation of the larval knockdown results is that stronger *ey* or *toy* phenotypes may not be uncovered

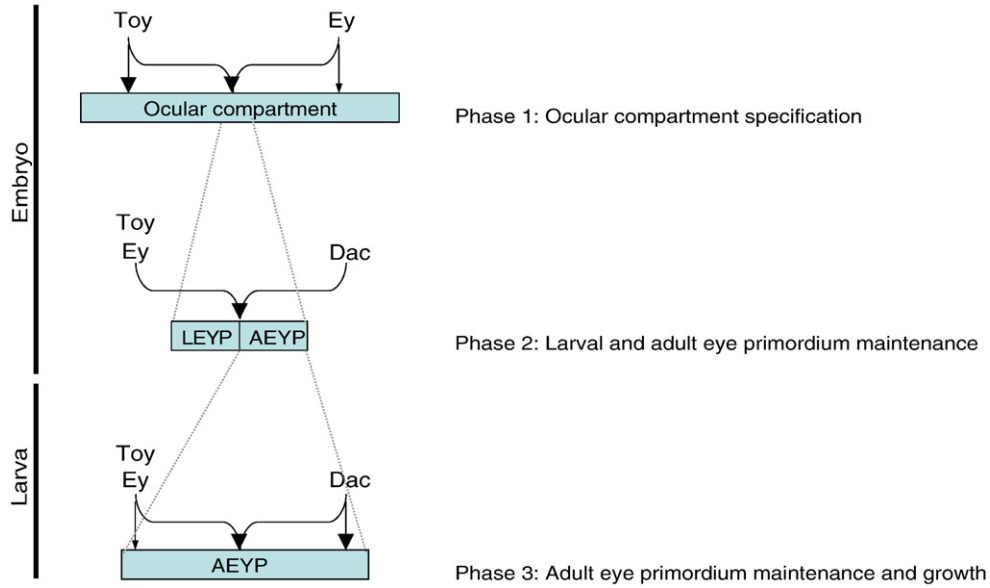


Fig. 8. A model of *toy*, *ey* and *dac* interaction in the *Tribolium* visual system. The model is based on the integrated analysis of gene knockdown data in this study and the parallel study on *dac* (Yang et al., 2009). Phase 1: *toy* and *ey* cooperate in a partly redundant manner during the specification of the head region which gives rise to the peripheral visual system, which includes both the larval and adult eyes. Phase 2: The commitment state of the larval and adult eye primordia is redundantly upheld by *toy*, *ey*, and *dac* during embryonic head development. Phase 3: Adult eye primordium commitment and expansion is regulated by overlapping and specific function of *toy*, *ey*, and *dac*. Arrow tip size indicates relative importance of gene regulatory input. LEYP = larval eye primordium, AEYP = adult eye primordium.

because highly efficient transcript reduction causes larval lethality. However, the survival rates in the late larvae injected with *ey* or *toy* dsRNA were generally in the same range as in control injections (>50%) suggesting that *ey* and *toy* knockdown did not affect viability at this stage (Supplementary data). Injections involving *dac* were associated with lower survival rates (<20%) compared to control injections (37%). Moreover, larval survival was further reduced in experiments, which combined *dac* with *ey* and *toy* knockdown (<15%). These results raise the possibility of stronger *dac* effects on eye development hidden by larval lethality. The persistent effect of *dac* on eye development in moderate and strongly appendage-truncated animals, however, suggests that this is not the case.

A model of Pax6 and dac function in the Tribolium peripheral visual system

Even in light of the limitations of the evidence obtained in our knockdown experiments, it is difficult to avoid the conclusion that major architectural differences exist between the *Tribolium* RDGN and the hierarchical organization in the *Drosophila* RDGN. It is therefore tempting to develop a working model of RDGN architecture in *Tribolium*, which integrates the embryonic and postembryonic data. Such a model is meant to serve as a guide for hypothesis testing in follow-up studies that need to explore the effect of *toy*, *ey* and *dac* reduction on target gene expression, primordium specification and head morphogenesis.

Taken together, our postembryonic knockdown data lead to a model in which adult eye size in *Tribolium* depends in part on regulatory interactions between *dac*, *toy* and *ey*, and in part on redundant regulation as well as unique functions by some of these genes. Approximately 50% of the formation of the *Tribolium* adult eye size reduction can be accounted for by mechanisms in which *dac* acts partially through *toy* and *ey*, or in which the latter act through *dac*, which, however, depends on additional activating factors (Fig. 8). Both scenarios are compatible with the equivalent reduction of eye size in the *dac* double and single knockdown experiments. The deletion of an additional 50% of the eye in the *toy* + *ey* + *dac* triple knockdown experiment implies the redundant regulation of the development of part of the eye by *dac* and *Pax6*.

In the embryo, larval eye development is highly sensitive to *Pax6* reduction. One interpretation of this finding is that *toy* and *ey* are essential for final photoreceptor determination. This hypothesis, however, conflicts with the lack of detectable effects of *toy* and *ey* knockdown on photoreceptor development in the adult retina. An alternate model is that the loss of larval eyes is the indirect consequence of perturbed specification of the head region, from where the elements of the peripheral visual system (larval and adult eyes) originate. Because the organization of the anterior insect head remains debated (Urbach and Technau, 2003), we will preliminarily refer to the *Pax6*-dependent region of the *Tribolium* larval head as the ocular compartment. Consistent with a role of *toy* and *ey* in specification or patterning of the ocular compartment is the effect on additional structures such as the vertex bristles. Another attractive aspect of this model is that it also explains the mild effect of *Pax6* knockdown on the adult eyes. That is, the insensitivity of adult eye development to *Pax6* reduction is due to redundant regulation of eye primordium commitment maintenance through the *Pax6* paralogs and *dac*.

When integrating the role of *dac*, it is further important to note that *dac* is not co-expressed with *ey* and *toy* before formation of the embryonic headlobes. The expression data therefore suggest that *dac* does not interact with *toy* and *ey* during early ocular compartment patterning. The subsequent co-expression of *dac* with *toy* and *ey* in the visual primordium could be related to redundant maintenance of visual primordium commitment. This is consistent with both the lack of *dac* knockdown effect on larval eye development documented in our parallel study (Yang et al., 2009) and the lack of *Pax6* knockdown effect on adult eye development, where the redundant regulation by *dac* and *Pax6* genes is uncovered by the eye-loss in the *toy* + *ey* + *dac* triple knockdown situation. A testable prediction of this model is that the double knockdown of *dac* with *ey* or *toy* is expected to lead to enhancement of larval eye phenotypes compared to the mild consequence of *ey* single knockdown.

The high level of redundant regulation by *Pax6* and *dac* in *Tribolium* contrasts with the more hierarchically structured RDGN model in

Drosophila. However, both *dac* and *ey* null mutant *Drosophila* exhibit a range of eye reduction (Mardon et al., 1994; Quiring et al., 1994). Exploring the regulatory redundancy, which has previously been speculated to explain the high level of phenotypic variability in *ey* mutant *Drosophila*, may reveal more similarities between the *Drosophila* and *Tribolium* RDGN. Interestingly, there is also precedent of redundant regulation by *ey* and *dac* during larval mushroom body formation in *Drosophila* (Kurusu et al., 2000). Moreover, *Tribolium* is not the first system in which *Pax6* genes have been found associated with redundantly organized gene regulatory network architecture. In the mouse eye, *Pax6* and *Pax2* cooperate in specification of the retinal pigment epithelium (Baumer et al., 2003). Even more strikingly, the development of anterior structures in *C. elegans* is in part redundantly controlled by orthologs of *Pax6* and *Eya* genes (Furuya et al., 2005). Taken together, it is tempting to speculate that redundancy-fueled evolutionary turnover of RDGN architecture explains the mix of conserved and diverged aspects in the control of eye development in distantly related species.

Acknowledgments

We are grateful to Diethard Tautz for support in an initial phase of the project, William Branford and the anonymous reviewers for numerous important comments on the manuscript, Ashisna Khadka for invaluable help with larval knockdown experiments and eye size analyses, and Jason Caravas for help with molecular gene tree reconstruction. The work of W.D. was supported by a Marie Curie Fellowship of the European Commission (Brussels) and a DFG grant (Da526/1-2), the work of M.K. by DFG (KL656/3-1, 3-2) and HFSP grants (RG 0303/1999), the work of R.B and R.F. by the DFG (grant BE1789/4-12 to RGB).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.06.013.

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