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Ancient genetic redundancy of *eyeless* and *twin of eyeless* in the arthropod ocular segment

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ABSTRACT

Pax6 transcription factors are essential upstream regulators in the developing anterior brain and peripheral visual system of most bilaterian animals. While a single homolog is in charge of these functions in vertebrates, two *Pax6* genes are in *Drosophila*: *eyeless* (*ey*) and *twin of eyeless* (*toy*). At first glance, their co-existence seems sufficiently explained by their differential involvement in the specification of two types of insect visual organs: the lateral compound eyes (*ey*) and the dorsal ocelli (*toy*). Less straightforward to understand, however, is their genetic redundancy in promoting defined early and late growth phases of the precursor tissue to these organs: the eye-antennal imaginal disc. Drawing on comparative sequence, expression, and gene function evidence, I here conclude that this gene regulatory network module dates back to the dawn of arthropod evolution, securing the embryonic development of the ocular head segment. Thus, *ey* and *toy* constitute a paradigm to explore the organization and functional significance of longterm conserved genetic redundancy of duplicated genes. Indeed, as first steps in this direction, recent studies uncovered the shared use of binding sites in shared enhancers of target genes that are under redundant (*string*) and, strikingly, even subfunctionalized control by *ey* and *toy* (*atonal*). Equally significant, the evolutionarily recent and paralog-specific function of *ey* to repress the transcription of the antenna fate regulator *Distal-less* offers a functionally and phylogenetically well-defined opportunity to study the reconciliation of shared, partitioned, and newly acquired functions in a duplicated developmental gene pair.

1. Introduction

The Pax gene family codes for genetic toolkit transcription factors with central roles in the development of complex animal body plans (Friedrich, 2015). Among them, members of the *Pax6* subfamily are required for the development of the visual system in a wide range of animal species (Gehring, 2014). This discovery culminated in one of the earliest examples of how developmental gene function conservation can unravel unexpected deep homology of diversified organs, in this case of the eyes in the lateral head of bilaterian animals (Gehring, 1996; Quiring et al., 1994).

In insects, the discovery of *Pax6* genes was pioneered by the molecular genetic characterization of the *Drosophila* mutants *eyeless* (*ey*) and *twin of eyeless* (*toy*) (Czerny et al., 1999; Kronhamn et al., 2002; Quiring et al., 1994). As a result of this work and complementing genetic studies on the *Pax6* transcription factor homologs in vertebrates, we now know that two *Pax6* genes, *ey* and *toy*, are essential for normal eye development in the fruit fly while a single *Pax6* gene is in charge of comparable functions in vertebrates. This raises the simple question of why there are two *Pax6* homologs active in the developing visual system of the insects while vertebrates get away with a single *Pax6* gene to execute the corresponding ancestral role in bilaterian brain and eye development? The question becomes even more puzzling if one considers the fact that the *Pax6* subfamily experienced a parallel expansion in vertebrates, spawning *Pax4* and *Pax10* and thus not only two but three paralogs together with *Pax6* (Feiner et al., 2014; Manousaki et al., 2011).

2. Paralog-specific functions of *ey* and *toy* in the developing visual system of *Drosophila*

An obvious answer to the above question is that *ey* and *toy* mediate, in part, paralog-specific functions in the developing visual system as well as components of the central nervous system (Daniel et al., 1999; Furukubo-Tokunaga et al., 2009; Glossop et al., 2014; Kammermeier et al., 2001; Noveen et al., 2000). As a well studied case in point, while the development of the most prominent visual organs of *Drosophila*, the compound eyes, is dependent on *ey* (Quiring et al., 1994), the

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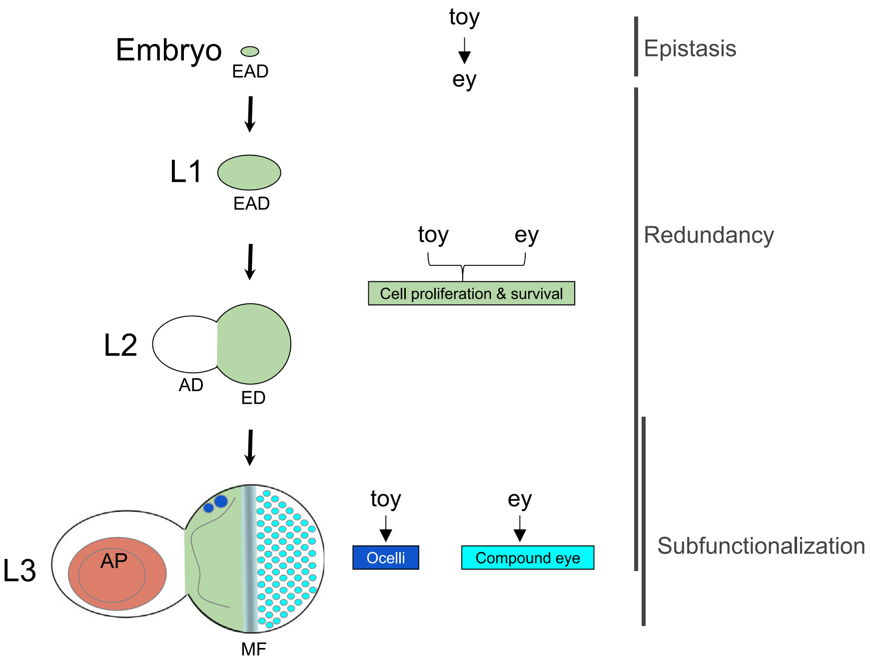

Fig. 1. Expression and function of *ey* and *toy* in the developing eye-antennal imaginal disc of *Drosophila*. The two components of the peripheral *Drosophila* visual system, the compound eyes and ocelli, originate in a specialized type of postembryonic precursor tissue, the eye-antennal imaginal disc. This tissue is generated in the late embryo and constitutes a small pocket of developmentally and morphologically equivalent cells, which coexpress the *Pax6* transcription factor paralog duo *ey* and *toy*, as indicated by green shading. While the onset of *ey* expression depends partially on *toy*, maintenance of the expression of the two factors is mutually independent in the eye-antennal imaginal discs of first instar larvae and promotes the cell proliferation and survival in a genetically redundant manner. The coexpression domain of *ey* and *toy* recedes from disc areas that eventually acquire antennal precursor fate in the second instar larva or enter differentiation such as the ocelli and compound eye in the third instar larva. Although *ey* and *toy* continue to be coexpressed even at that late stage, the two factors facilitate the specification of ocelli and compound eye precursor cells in a paralog-specific manner. L1-3 = Larval instars 1–3, EAD = Eye-antennal imaginal disc, AD = Antennal disc, ED = Eye disc, AP = Antenna primordium, MF = Morphogenetic furrow.

development of a second set of visual organs of *Drosophila*, the ocelli, is specifically dependent on *toy* but not *ey* (Fig. 1) (Blanco et al., 2010; Brockmann et al., 2011; Punzo et al., 2004, 2002).

To experts in the field of gene duplication, these data suggest subfunctionalization following gene duplication (Hahn, 2009). In the framework of this model, *ey* and *toy* are the descendants of a singleton precursor *Pax6* gene that was essential for both compound eye and ocellus development. Following the duplication of this ancestral pleiotropic factor, *ey* and *toy* acquired their individual rights of existence through the differential adoption of the compound eye- and ocellus-specific regulatory functions. While the actual course of events was likely different and more complicated as will become apparent later below, at this point, it is sufficient to note that the acquisition of paralog-specific functions by *ey* and *toy* provides one explanation for their evolutionary persistence as a paralog pair.

A second explanation is that the genetic data in *Drosophila* imply that *ey* has become partly dependent on *toy* during early visual development. Before executing their ocellus- and compound eye-specific functions, *ey* and *toy* are already both active during the early development of a pair of specialized postembryonic precursor tissues, which produce the precursor cell populations of the ocelli and compound eyes: the eye-antennal imaginal discs (EADs) (Fig. 1). While both genes are coexpressed in the EADs from the very beginning of their formation in the late *Drosophila* embryo, the activation of *ey* is, partly, contingent on the direct input by Toy (Czerny et al., 1999; Kronhamn et al., 2002). Partly contingent, because *ey* is detectable in the EADs of *toy*-depleted flies (Zhu et al., 2017), although at reduced levels and in a temperature-sensitive manner (Kronhamn et al., 2002). Thus, while not completely, *ey* is critically dependent on *toy* for the reliable onset of its expression in the EAD. Consistent with this, *toy* begins to be expressed at a much earlier stage in the founding cells of the EAD (Czerny et al., 1999).

Following this epistatic interaction, however, the maintenance of *ey* expression becomes independent of *toy* (Czerny et al., 1999; Hauck et al., 1999; Zhu et al., 2017). Thus, by the time the two paralogs are coexpressed in the early EAD of 1st instar *Drosophila* larvae, *ey* and *toy* are using independent regulatory devices. And yet, the two genes continue to be coexpressed in indistinguishable domains (Fig. 1). There is, however, evidence of expression level differences. *toy*, for instance, is more strongly expressed in the ocellus precursor cell population (Brockmann et al., 2011). The close spatial coexpression of *ey* and *toy,* however, is even maintained into the wide undifferentiated region of the anterior eye disc after its visible and genetic separation from the antennal disc region (Fig. 1). This is significant by ruling out the spatial separation of expression domains as the mechanism that led to the subfunctionalization of *ey* and *toy* with respect to ocelli vs compound eye development.

Thus, the overall persistent coexpression of *ey* and *toy* during postembryonic visual system development in *Drosophila* raises a question of its own regarding its possible functional significance. In the early EAD, this status is now mechanistically well explained by yet another form of genetic interaction between the two sister paralogs. And although this interaction has been recently reexamined in depth, it still begs for an evolutionary explanation: Genetic redundancy.

3. Genetic redundancy of *ey* and *toy* in promoting tissue growth in the early *Drosophila* eye-antennal imaginal disc

Most components of the peripheral adult *Drosophila* head such as the antennae develop from the EADs, in addition to the compound eyes and ocelli (Fig. 1) (Haynie and Bryant, 1986). In order to supply the required cell material, the EADs have to gain dramatically in size from the time point they have been generated in the final embryonic stages. This happens while the EADs remain tucked away inside the larva, which itself grows to final size by undergoing three molts. Thus, invisible from outside, the development of the adult peripheral visual system in *Drosophila* begins with the setting aside and, next, early growth of the EADs (Fig. 1). It is this critical process where new work