Minireview

Targeting *Drosophila* eye development

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Abstract

In order to understand the role of transcription factors in particular developmental processes it is necessary to know their target genes. A combination of bioinformatics, comparative expression profiling and microarray-based epistasis experiments has recently identified new targets of Eyeless, a key transcription factor in *Drosophila* retinal determination.

The *Drosophila* retinal determination gene network (RDGN) consists of seven transcription factors, conserved from flies to humans, that cooperate to regulate cell specification and determination during eye development. At the top of this network are the master regulators Twin of Eyeless (Toy) and Eyeless (Ey), homologs of vertebrate Pax6, which activate expression of the genes for the conserved downstream transcription factors Eyes Absent (Eya), Sine Oculis (So), Optix and Dachshund (Dac) (Figure 1). The transcription factor Eyegone (Eyg) is also a retinal determination protein, but has not yet been positioned within the pathway. At present only a small number of direct targets of any of these transcription factors have been identified (see Figure 1). An elegant strategy integrating bioinformatics and microarray-based expression profiling with *in vivo* genetics has recently been used by Ostrin *et al.* [1] to isolate and validate transcriptional targets of Ey.

Bringing genetics to genomics: microarray epistasis

The strength of a large-scale screen lies in its design and in the implementation of effective secondary tests to distinguish the desired output from the inevitable background. Standard microarray analysis results in long lists of genes whose expression changes under different experimental conditions, but does not reveal which genes reflect direct transcriptional targets. For example, a previous study by Michaut *et al.* [2] compared expression profiles of wild-type eye and leg imaginal discs (the larval tissues from which the adult structures develop) from third-instar larvae, as well as leg discs ectopically expressing *eyeless* (*ey*), and identified 371 genes relevant to eye specification and development; which of these genes actually represent direct transcriptional targets of Ey remained an open question, however [2]. Ostrin *et al.* [1] have taken a novel approach, utilizing microarray-based epistasis analysis to identify genes expressed in a spatial and temporal pattern consistent with that of direct Ey targets. Epistasis refers to an interaction between two genes such that mutation in one gene masks the phenotype of mutation in the second; epistasis analysis is commonly used to determine the relative order of gene action within a linear signaling pathway - for example, a transcriptional target acts downstream of, or is 'epistatic to', the transcription factor that regulates its expression.

First, Ostrin *et al.* [1] compiled a set of 300 potential Ey targets from comparative expression profiling of different types of discs: wing, antennal and leg imaginal discs engineered to express *ey* ectopically; their wild-type counterparts that normally lack *ey* expression; and wild-type eye discs that normally express *ey*. Only genes whose expression was consistently enriched upon *ey* induction in all three non-eye tissues and that were also expressed in wild-type eye discs were considered for subsequent analysis. Thus, by sampling four different imaginal disc types, the authors

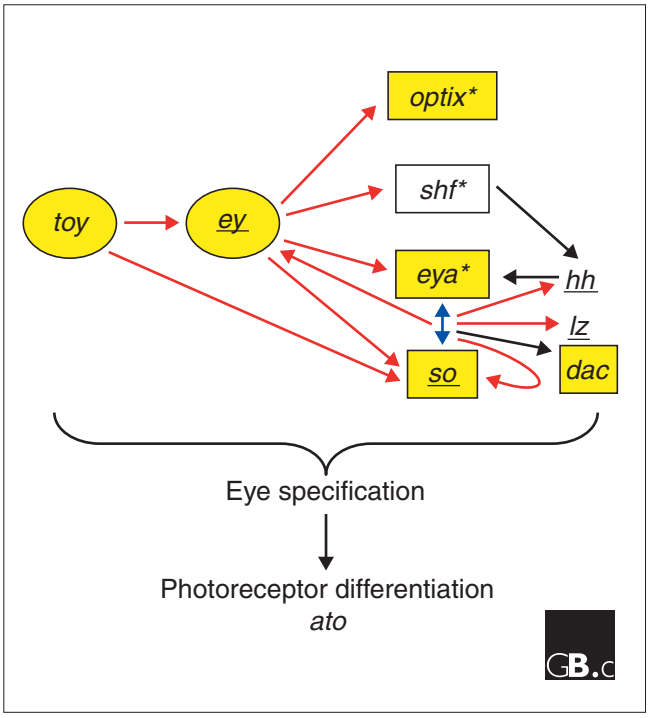

Figure 1
The retinal determination gene network (RDGN). Members of the RDGN are indicated on a yellow background. Toy activates expression of *ey* as well as *so* (reviewed in [3]). Ey activates expression of *so, eya*, optix** and *shf** [10] (boxed genes are direct Ey targets; asterisked genes were isolated by Ostrin *et al.* [1]). Eya and So interact to directly activate expression of their targets: *lz, hh, ey* and *so* (direct targets are underlined), as well as indirectly activating expression of the downstream gene *dac* [14,16,17]. Evidence suggests that Shf regulates the activity of Hh, and that Hh regulates *eya* expression [8,9,15]. Members of the RDGN are required for eye specification, upstream of photoreceptor specification and differentiation, with *ato* functioning to regulate specification of the first photoreceptor cell, R8 [3,4]. Protein-protein interactions are depicted by blue arrows. Red arrows indicate direct transcriptional regulation. Black arrows indicate an undetermined level of regulation. The position of the retinal determination gene *eyg* in the network is not yet clear, and so it is not included in this figure.

increased confidence in the data and presumably minimized the false-positive rate relative to studies based on the analysis of fewer tissue types.

Second, thinking as geneticists accustomed to using epistasis analysis to position genes within a pathway, Ostrin *et al.* [1] cleverly exploited the genetic hierarchy governing eye development to develop a microarray-based epistasis approach to identify the subset of genes most likely to be direct Ey targets. Previous work showed that Ey and other RDGN members function to specify the eye field, upstream of the genes involved in directing the differentiation of the retinal photoreceptor cells [3]. The gene *atonal* (*ato*) is required for the recruitment of R8, the first photoreceptor to be specified, and therefore acts downstream of the RDGN [4]. Thus, Ostrin *et al.* [1] reasoned that a direct Ey target gene should operate upstream of *ato*, should not exhibit altered expression in an *ato* mutant eye disc and should be equally well induced upon ectopic *ey* expression whether the downstream effector *ato* is present or not. By comparing the expression of their potential Ey targets in wild-type and *ato*-mutant eye discs, and in leg discs overexpressing *ey* in wild-type or *ato*-mutant backgrounds, Ostrin *et al.* [1] refined their list to include 188 genes whose spatial and temporal presence was consistent with that of direct Ey transcriptional targets.

Computational prediction of direct transcriptional targets

In order to distinguish valid targets from background, secondary screens are vital. To narrow down the list of 188 potential direct Ey targets to a smaller set worthy of *in vivo* validation, a bioinformatics approach was used that incorporated DNA binding-site data into the analysis of the potential targets. For transcription factors with clusters of binding sites in the *cis*-regulatory regions of their known target genes, position-weight matrices (PWMs) can be generated to search for such conserved clustered binding sites in other genes using web-based tools such as CIS-ANALYST [5]. While such approaches are useful, genes with few identified targets pose a challenge. Ostrin *et al.* [1] used the three known Ey-binding sites in the enhancer that directs *so* expression in the eye disc [6], in combination with binding sites in mammalian Pax6 target genes, to create a PWM to identify genes with potential Ey-binding sites, and assessed the conservation of these sites by aligning the genome sequences of seven *Drosophila* species [7]. This analysis resulted in 20 predicted targets, including *so,* the only Ey target identified before this study [6]. Ey-mediated regulation of the expression of three of these potential targets was confirmed by *in situ* hybridization and reporter analysis, while electrophoretic mobility-shift assays confirmed direct binding of Ey to predicted sites. Investigation of the remaining 17 shortlisted genes is likely to yield additional targets of Ey.

Biological implications of the identified Ey target genes

The identification of three new Ey targets by Ostrin *et al.* [1] answers some questions about *Drosophila* eye development, but raises others. Out of the three newly identified Ey targets, two - *eyes absent* (*eya*) and *optix* - had previously been described as retinal determination genes. The third target, *shifted* (*shf*), however, encodes a secreted protein known as the Wnt-1 inhibitory factor, which is required for the extracellular transport of the signaling protein Hedgehog (Hh) [8,9] - we shall return to this later.

Previous work has shown that the initiation of expression of both *eya* and *so* requires Ey, and that the induction of ectopic eyes by Ey also requires the expression of these two genes [10]. Interestingly, the converse is also true; Eya and So cannot induce ectopic eyes in the absence of Ey, suggesting

that Ey is required to induce the expression of additional genes needed for eye specification [11]. Further exploration of other putative targets identified by Ostrin *et al.* [1] may lead to the identification of these missing links.

In addition to *eya* and *so,* the Six-family gene *optix* was identified as a target of Ey [1]. Optix is a member of the RDGN, but its position within the network is unclear. For example, unlike Eya and So, Optix can induce ectopic eye formation independent of Ey [12]. Furthermore, whereas mammalian homologs of So operate as transcriptional activators through their association with Eya, Optix and its homologs do not exhibit this interaction, suggesting a novel mechanism for their function [13]. Surprisingly, it was previously reported that *ey* is not required for the expression of *optix* [12]. This discrepancy could reflect a caveat to using overexpression to identify transcriptional targets, but given the overlapping expression patterns of *ey* and *optix*, a more likely explanation is that in addition to being regulated by Ey, *optix* may be the target of another retinal determination gene or signaling pathway during eye development. One candidate for this regulation could be the retinal determination transcription factor Toy; Toy and Ey have been shown to co-regulate the expression of *so* [6], suggesting that they might converge to redundantly regulate other target genes.

The signaling protein Hh is important for the progression of the morphogenetic furrow, a wave of cell differentiation that moves across the eye disc leaving specified retinal cell clusters in its wake. The gene for Hh has recently been identified as a transcriptional target of the RDGN members Eya and So [14], and Hh acts as a regulator of *eya* expression posterior to the furrow [15]; thus, it is interesting that Ostrin *et al*. [1] identify *shf,* a positive regulator of Hh transport, as a target of Ey [8,9]. As Shf is predicted to regulate Hh localization and accumulation in coordination with heparin-sulfate proteoglycans, it seems likely that Ey, through its regulation of *shf*, contributes to Hh localization [8,9]. These results give insight into the multiple levels at which a network of factors contributes to the activation or repression of a targeted signaling pathway. It seems likely that identification of additional targets of the RDGN will further complicate this story, by introducing additional regulatory inputs that ensure proper eye development.

In summary, Ostrin *et al.* [1] have used a multifaceted temporal and spatial approach combining microarray-based expression profiling, computational analysis of binding sites and *in vivo* expression analysis to identify downstream targets of retinal determination genes. In particular, the incorporation of epistasis analysis into a microarray approach provides a powerful new strategy for exploring the dynamic transcriptional circuitries that regulate development. Its usefulness will extend well beyond the exploration of the RDGN.

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