# Signaling circuitries in development: insights from the retinal determination gene network

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## Summary

**Context-specific integration of information received from the Notch, Transforming growth factor β, Wingless/Wnt, Hedgehog and Epidermal growth factor receptor signaling pathways sets the stage for deployment of the retinal determination gene network (RDGN), a group of transcription factors that collectively directs the formation of the eye and other tissues. Recent investigations have revealed how these transcription factors are regulated by their interactions with each other and with effectors of the above signaling pathways. Further study of the RDGN may provide insights into how common cues can generate context-specific responses, a key aspect of developmental regulation that remains poorly understood.**

## Introduction

Eye development in different organisms produces strikingly different structures: the primitive eye of planaria, the compound eye of insects, and the camera-like eye of vertebrates. Although these visual organs are morphologically distinct, the molecular mechanisms that underlie their development are remarkably conserved. The specification of the eye field in these diverse organisms requires the expression of homologous members of the retinal determination gene network (RDGN), a group of transcription factors and co-factors. Recent studies have explored a role for the RDGN as an interface for the integration of multiple signaling pathways, a function that is crucial for the proper development of many tissues, including the eye, gonad, muscle and ear. Together, these analyses indicate that this network affects, and is affected by, multiple signaling pathways in a context-specific manner. As such, studies that probe this specificity may provide a means to understanding the mechanisms that underlie specific responses to developmental regulatory circuits.

In this review, we discuss current knowledge of RDGN members and their functions, from studies predominantly carried out in *Drosophila*, beginning with an overview of the protein families that make up the RDGN (see Fig. 1). We then discuss their function in organ and tissue specification, focusing on the recently discovered links both in flies and vertebrates between network members and diverse signaling pathways. We highlight a few examples that illustrate the unifying concepts that have emerged from recent research. Specifically, the high degree of evolutionary conservation of the RDGN members encompasses not only the physical structure of the proteins, but also the functional interactions within the network and with exogenous signaling pathways. Superimposed on this strict conservation, context-specific adaptations reveal the enormous flexibility of genetic circuits, with respect to how they are deployed and how they respond to and integrate with other cellular signals.

## Key members of the RDGN

The proteins belonging to the PAX6, EYA (Eyes absent), SIX and DAC (Dachshund) families (see Fig. 1) make up the key members of the RDGN. Here, we briefly review what is currently known about their structure and function (for a more extensive description of RDGN members, see Pappu and Mardon (Pappu and Mardon, 2002).

### Eyeless/PAX6

*Drosophila eyeless* (*ey*) derives its name from the 'eyeless' phenotype that is caused by eye-specific, loss-of-function alleles of the *ey* gene (Bridges, 1935). The isolation of null alleles of *ey* highlighted its broader functions in the development of the fly embryo and brain (Table 1) (Kammermeier et al., 2001). The cloning of *ey* revealed its homology to the vertebrate *Pax6* transcription factors, which encode a subgroup of the large family of PAX proteins that each contain two DNA-binding motifs: a PAIRED box and a HOMEOBOX (Fig. 1) (Quiring et al., 1994). The *Drosophila* genome also contains a second closely linked *Pax6* homolog, *twin-of-eyeless* (*toy*), which probably arose by gene duplication during insect evolution (Czerny et al., 1999). TOY and EY are independently required for eye development (Kronhamn et al., 2002; Quiring et al., 1994).

Perhaps one of the most striking attributes of PAX6 family members is their ability to act as 'master regulators' of eye formation, as they can direct the formation of ectopic eyes upon overexpression (Halder et al., 1998). Consistent with this idea, TOY and EY function at the top of a transcriptional hierarchy, where they are required for the expression of

downstream members of the RDGN (Fig. 2) (Halder et al., 1998). Like *toy* and *ey*, members of the RDGN encode transcription factors, and include the *Pax6*-like gene *eyegone* (*eyg*), which acts in parallel to *ey* (Box 1) (Jang et al., 2003), and the downstream components of this network – *eya*, *sine oculis* (*so*) and *dac*. This transcriptional hierarchy is not absolute; ectopic expression of downstream members of the network, such as EYA or DAC, can also induce ectopic eye tissue and the expression of the upstream gene *eyeless* (Bonini et al., 1997; Shen and Mardon, 1997).

## EYA: a transcription factor and protein phosphatase

Studies of *Drosophila eya* and of its vertebrate homologs *Eya1-Eya4* have revealed important roles for these genes in cell survival and differentiation, particularly during tissue specification (Table 1) (Bonini et al., 1993; Bonini et al., 1998; Xu et al., 1999; Xu et al., 1997a; Xu et al., 1997b). The four mouse Eya genes have both discrete and overlapping expression patterns, suggesting that their functions may not be wholly redundant (Xu et al., 1997a; Zimmerman et al., 1997), although detailed studies of knockout combinations remain to be performed.

EYA family proteins are characterized by a conserved C-terminal domain called the EYA domain (ED), while the N terminus shows little conservation aside from the tyrosine rich EYA domain 2 (ED2), which is embedded within a proline/serine/threonine-rich region (Fig. 1) (Xu et al., 1997b; Zimmerman et al., 1997). These N-terminal domains are crucial for the transcriptional co-activator function of EYA (Ohto et al., 1999; Silver et al., 2003). The ED was initially characterized in flies as a protein-protein interaction domain that bound the other RDGN members SO (Pignoni et al., 1997) and DAC (Chen et al., 1997), an observation that was extended

to vertebrate EYA, SIX and DACH families (Heanue et al., 1999; Ohto et al., 1999).

EYA has been best characterized as a transcriptional co-activator that is recruited to the DNA of target genes via its interaction with SIX family members (Ohto et al., 1999; Silver et al., 2003). Recently, a second function has been described for EYA through the identification of the ED as a catalytic motif belonging to the haloacid dehalogenase enzyme family (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). Recombinant EYA can dephosphorylate tyrosyl phosphorylated peptides (Rayapureddi et al., 2003; Tootle et al., 2003) and serine/threonine phosphorylated peptides (Li et al., 2003), suggesting it may be a dual-specificity protein phosphatase. Thus far, only two substrates, RNA polymerase II (Li et al., 2003) and EYA itself (Tootle et al., 2003), have been shown to be dephosphorylated by EYA in vitro, although the in vivo relevance of both findings remains to be determined. However, the phosphatase function of EYA is required to rescue the phenotype of the eye-specific *eya²* allele (Rayapureddi et al., 2003; Tootle et al., 2003), indicating that this property of EYA is utilized in vivo during eye development in *Drosophila*.

## SO/SIX family members

The SIX family comprises three subgroups, SO/SIX1/SIX2, SIX4/SIX4/SIX5 and OPTIX/SIX3/SIX6, each with one member in *Drosophila* (underlined) and two members in vertebrates (Kawakami et al., 2000; Seo et al., 1999). All family members are characterized by two conserved domains, the SIX domain, which mediates protein-protein interactions, and a homeobox DNA-binding domain (Fig. 1) (Kawakami et al., 2000; Seo et al., 1999). SIX family transcription factors are crucial for the development of many tissues and play an important part in regulating cell proliferation (Table 1) (Carl et al., 2002; Cheyette et al., 1994; Dozier et al., 2001; Li et al., 2002; Ozaki et al., 2004). For example, *Six1* is upregulated in a mouse model of metastatic skeletal muscle cancer, and increased levels of *Six1* are associated with a greater ability to form metastases (Yu et al., 2004). The role of SIX1 in malignancy-associated overproliferation may be to overcome mitotic checkpoints in G2 (Ford et al., 1998), as it can directly regulate the transcription of cyclin A1 to induce proliferation (Coletta et al., 2004).

The most divergent branch of the SIX family includes *Drosophila* OPTIX (Seimiya and Gehring, 2000) and the vertebrate counterparts SIX3 and SIX6, which, unlike the members of the SIX1/2 and SIX4/5 subfamilies, do not interact with EYA proteins (Kawakami et al., 2000). Instead, work in
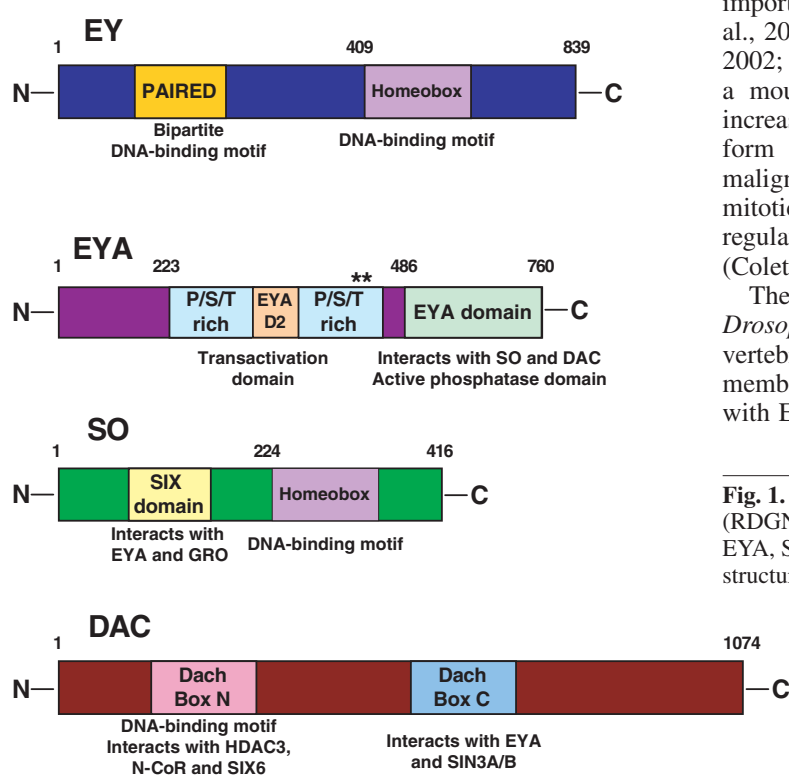


**Fig. 1.** Domain structures of the retinal determination gene network (RDGN) members. Representative members of the PAX6 (EY), EYA, SIX (SO) and DAC families from *Drosophila* show the domain structure of RDGN members and their functions. Numbers represent amino acid number; **, conserved MAPK phosphorylation sites in EYA. C, C terminus; DAC, Dachshund; EY, Eyeless; EYA, Eyes absent; EYA D2, EYA domain 2; GRO, Groucho; HDAC3, Histone deacetylase 3; N, N terminus; N-CoR, Nuclear co-repressor; P/S/T rich, proline, serine and threonine rich region; SO, Sine oculis.

**Table 1. Phenotypes of RD gene network members in *Drosophila* and vertebrates**

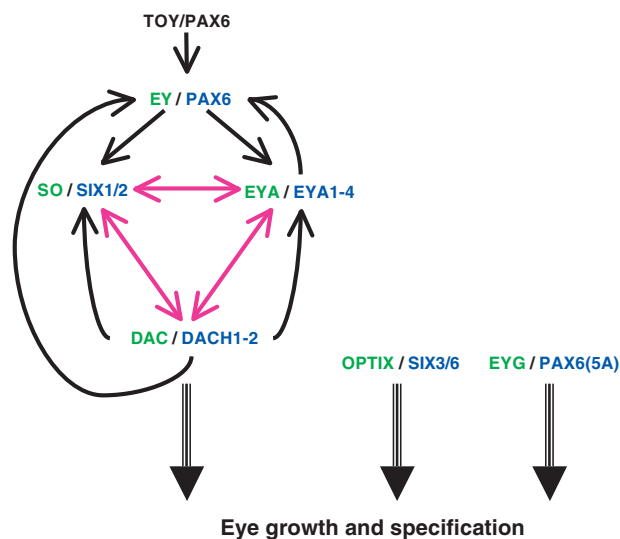| *Drosophila* gene | Mutant phenotype | Mammalian gene (human/mouse) | Mutant phenotype | References |
|---|---|---|---|---|
| *eyeless (ey)* | Loss of head structures; embryonic lethal; eye-specific alleles cause loss of eyes; essential for adult brain function. | *PAX6/Pax6* | In humans, dominant aniridia and Peters anomaly; recessive severe CNS defects. Similar phenotypes observed in *Pax6* knockout mice. | Callaerts et al., 2001; Czerny et al., 1999; Hanson et al., 1994; Kronhamn et al., 2002; Quiring et al., 1994 |
| *twin of eyeless (toy)* | Loss of head structures; embryonic lethal. | | | |
| *eyes absent (eya)* | Embryonic lethal with anterior defects; hypomorphs can have loss of eye tissue, as well as male and female sterility. | *EYA1/Eya1* *EYA2/Eya2* *EYA3/Eya3* *EYA4/Eya4* | Mutations in *EYA1* linked to Branchio-oto-renal (BOR) syndrome, which is characterized by lung, kidney and ear defects; similar defects seen in *Eya1* knockout mice. Mutations in human *EYA4* linked to deafness. | Abdelhak et al., 1997; Bonini et al., 1993; Bonini et al., 1998; Wayne et al., 2001; Xu et al., 1999; Xu et al., 2002; Zimmerman et al., 1997 |
| *sine oculis (so)* | Defects in eye, brain and gonad development; embryonic lethal; eye-specific alleles cause loss of eye tissue. | *SIX1/Six1* *SIX2/Six2* | Mutations in *SIX1* also associated with BOR syndrome. *Six1* mutant mice display defects in ear, kidney, thymus, skeletal muscle and nose. | Cheyette et al., 1994; Fabrizio et al., 2003; Laclef et al., 2003; Ozaki et al., 2004; Ruf et al., 2004; Xu et al., 2003; Zheng et al., 2003 |
| *six4* | Defects in muscle and gonad development. | *SIX4/Six4* *SIX5/Six5* | *SIX5* mutations associated with myotonic dystrophy (DM1); *Six5* knockout mice develop cataracts similar to individuals with DM1. Mouse knockouts of *Six4* are viable and have no gross defects. | Kirby et al., 2001; Klesert et al., 2000; Ozaki et al., 2001; Wansink and Wieringa, 2003 |
| *optix* | No reported mutants. | *SIX3/Six3* *SIX6/Six6* | *SIX3* mutations are associated with holoprosencephaly; loss of *SIX6* is associated with bilateral anophthalmia and pituitary defects. Similar to the human phenotype, *Six6*-null mice survive but have retinal and pituitary hypoplasia. | Carl et al., 2002; Gallardo et al., 1999; Li et al., 2002; Pasquier et al., 2000; Seimiya and Gehring, 2000; Wallis et al., 1999 |
| *dachshund (dac)* | Lack eye tissue; leg defects; those that survive to adulthood die within days. | *DACH1/Dach1* *DACH2/Dach2* | *Dach1* knockout mice die soon after birth with no obvious defects; may reflect partial redundancy of *Dach1* and *Dach2*, which have overlapping expression. | Backman et al., 2003; Davis et al., 2001a; Davis et al., 2001b; Mardon et al., 1994 |

vertebrates suggests that SIX3/SIX6 act as transcriptional repressors that are important for proper eye and brain formation, through their interactions with the GROUCHO (GRO) family of co-repressors (Box 2) (Kobayashi et al., 2001; Lopez-Rios et al., 2003; Zhu et al., 2002).

Transcriptional repression may not be limited to the SIX3/6 subfamily, as these proteins interact with GRO co-repressors through an Engrailed homology 1 (eh1) motif that is present in the SIX domain of all SIX proteins (Kobayashi et al., 2001). In fact, *Drosophila* GRO can also interact with SO, and can repress SO-mediated transcription of a reporter gene, probably by competing with EYA for binding to SO (Silver et al., 2003). Studies in mice have revealed that SIX1 also has a transcriptional repressor function (Li et al., 2003), suggesting that SIX family members might generally operate as both activators and repressors, depending upon their specific co-factors and context.

### DAC: a novel DNA-binding protein

*dachshund* (*dac*) in *Drosophila*, and its vertebrate homologs, *Dach1* and *Dach2*, encode novel nuclear proteins characterized by two conserved domains, the DachBox-N and the DachBox-

TOY/PAX6

EY / PAX6

SO / SIX1/2 ⟷ EYA / EYA1-4

DAC / DACH1-2

OPTIX / SIX3/6    EYG / PAX6(5A)

**Eye growth and specification**

**Fig. 2.** Retinal determination gene network (RDGN). The RDGN is expressed in a transcriptional hierarchy (black arrows), in which Twin of eyeless (TOY) leads to Eyeless (EY) expression, which leads to the expression of Sine oculis (SO), Eyes absent (EYA) and Dachshund (DAC). However, the hierarchy is not only linear, as the lower tier members EYA, SO and DAC contribute to positive feedback loops that ensure the continued expression of EY, and also physically interact with each other (pink arrows). Other RDGN members Eyegone (EYG) and OPTIX are required independently for proper eye development (see Boxes 1 and 2). *Drosophila* proteins are shown in green and their vertebrate homologs in blue. PAX6(5A), 5A splice isoform of PAX6.

C (Fig. 1) (Davis et al., 2001b; Kozmik et al., 1999), although recent studies in *Drosophila* have suggested that only the DachBox-N is essential for function (Tavsanli et al., 2004). The crystallization of the human DachBox-N has revealed its structural resemblance to the winged helix/forkhead subgroup of the helix-turn-helix family of DNA-binding proteins, a finding that had not been predicted by amino acid sequence homology (Kim et al., 2002). Although no specific DNA-binding sites for DAC have been identified, it has been shown to bind naked DNA (Ikeda et al., 2002). The DachBox-C is thought to be a protein-protein interaction motif that interacts with the ED of EYA family members (Chen et al., 1997). DAC synergizes with EYA to increase both the size and frequency of ectopic eyes when the two are expressed together (Chen et al., 1997), supporting the model that these two proteins act in a complex to direct fly eye development. Thus, like SO, DAC (with its DNA-binding ability) may recruit the transcriptional activator and/or phosphatase activity of EYA to the promoter of target genes.

Of the RDGN members, DAC remains perhaps the least well mechanistically understood; the fact that *Dach1*-null mice die postnatally with no obvious defects has provided little additional insight into its function (Table 1) (Backman et al., 2003; Davis et al., 2001a). With respect to transcription, DAC is a novel nuclear factor with the potential to promote (Ikeda et al., 2002) and to repress gene expression (Box 2) (Li et al., 2002). A repressor complex between SIX1 and DAC has also been described that might switch from being a repressor to an activator through the function of the EYA protein phosphatase (Li et al., 2003). All of the reported experiments that observe

a DAC-dependent effect on transcription use endogenous promoter sequences of several hundred bases or more, raising the intriguing possibility that the DachBox-N must bind directly to DNA to co-regulate its target genes. Furthermore, as discussed later in this review in the context of vertebrate ear development, DAC may, at times, function independently of, or even antagonistically to, the rest of the RDGN (Heanue et al., 1999; Ozaki et al., 2004).

## Integrating signaling pathways with RDGN components

Studies of the RDGN have revealed new paradigms for transcriptional regulation, and the network has provided a valuable model for studying tissue specification, as discussed later in this review. However, these nuclear factors do not act alone, but are employed coordinately by, and with, components of conserved signaling pathways to achieve the specificity of transcriptional response that is necessary for appropriate development. This section focuses on the regulation of the RDGN genes by different signaling pathways and on examples where the RDGN signals back to these pathways in the context of *Drosophila* eye development.

### DPP and HH signaling regulate RDGN member expression

The *Drosophila* eye develops in a wave of differentiation that moves from the posterior to the anterior of the eye disc, which can be visualized by the progression of the morphogenetic furrow (Fig. 3A) (for a review, see Treisman and Heberlein, 1998; Voas and Rebay, 2004). The Decapentaplegic (DPP) and Hedgehog (HH) signaling pathways, which are required for the establishment and progression of the furrow, influence eye specification by regulating the expression of the RDGN members.

In flies transheterozygous for *dpp*, the expression of RDGN members *eya*, *so* and *dac* is lost in the eye disc (Chen et al., 1999b), although *ey* expression is retained (Kenyon et al., 2003). This suggests that DPP acts downstream of or in parallel to EY to activate the expression of RDGN members. More detailed work using somatic mosaic analysis has revealed that DPP signaling is required for EYA, SO and DAC protein expression only prior to morphogenetic furrow initiation (Curtiss and Mlodzik, 2000), and that once they are turned on, their expression can be maintained independently of DPP signaling.

High levels of HH protein are expressed just posterior to the DPP signal in the morphogenetic furrow, and are required for proper furrow progression (Fig. 3A) (Pappu et al., 2003). HH signaling inside the cell is affected by changes in the transcription factor Cubitus interruptus (CI); in the absence of signal, CI is cleaved to a shorter repressor form (CI$^r$), which enters the nucleus and downregulates target genes, whereas in the presence of HH, the phosphorylation of CI is blocked, preventing its cleavage and allowing the transcription of target genes (Chen et al., 1999a). HH regulates *eya* expression by eliminating a transcriptional block, rather than by directly activating it; removal of CI$^r$ is sufficient to promote *eya* expression, while the active full-length form of CI (CI$^{act}$) is not necessary for eye formation (Pappu et al., 2003).

The eye field undergoes a wave of differentiation that is directed by the expression of DPP and HH at the

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**Box 1. EYEGONE and EYELESS: insights into PAX6-mediated coordination of growth and differentiation**

Coordinated regulation of cell proliferation and differentiation is crucial for the proper development all tissues and organs. In the *Drosophila* eye, the PAX6 members of the retinal determination gene network (RDGN) reveal a 'divide and conquer' approach to solving this problem.

In addition to the PAX6 orthologs Twin of eyeless (TOY) and Eyeless (EY), two *Drosophila* *Pax6*-like genes not found in vertebrates [*eyegone* (*eyg*) and *twin-of-eyegone* (*toe*)] may act in parallel to *ey* during eye formation (Jang et al., 2003). Recent results suggest EY and EYG play discrete, but coordinated, roles, with EY providing important eye-specification cues and EYG regulating eye growth (Dominguez et al., 2004) (reviewed by Rodrigues and Moses, 2004).

How then does vertebrate PAX6 independently control growth and differentiation when two different genes are required in flies? The answer may lie in the structural differences that distinguish EYG from EY. *eyg* (and *toe*) encode PAX proteins that contain a truncated PAIRED domain (termed RED) and a complete HOMEOBOX domain (Jang et al., 2003; Jun et al., 1998), similar to the protein encoded by the 5A splice isoform of vertebrate *Pax6* (Jun et al., 1998). PAX6(5A) and EYG may be functionally homologous, as the RED domain of EYG and the RED domain of PAX6(5A) can bind to similar sequences in vitro (Jun et al., 1998), and overexpression of either EYG or PAX6(5A) in *Drosophila* produces the same overgrowth effects (Dominguez et al., 2004). Thus, two different strategies, gene duplication and alternative splicing, create structurally and functionally homologous proteins designed to coordinate growth and differentiation.
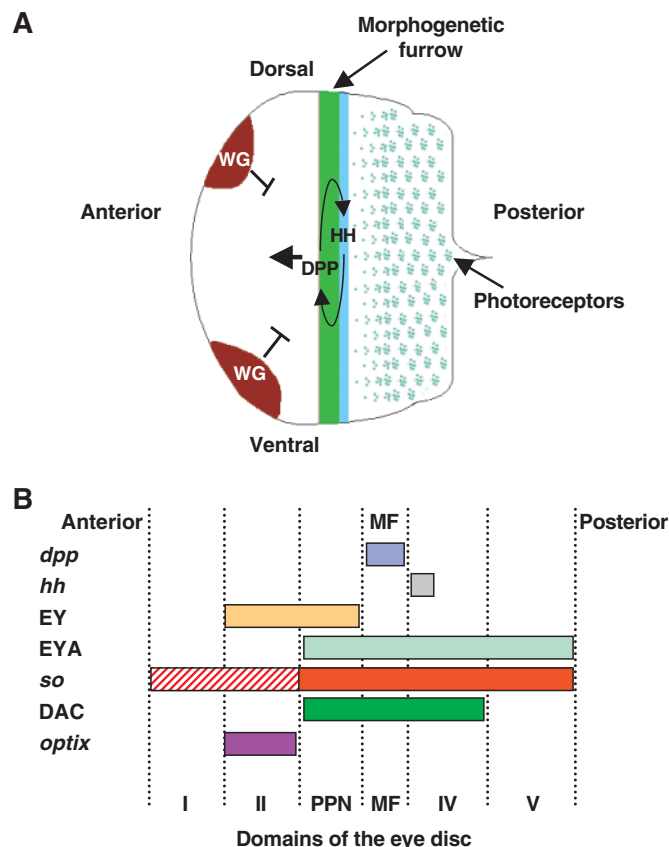
**Box 2. SIX3/6 and transcriptional repression**

Like other SIX proteins, SIX3/6 family members play an important role in cell proliferation (Carl et al., 2002; Li et al., 2002), but in contrast to other SIX family members, SIX3/6 have thus far been characterized solely as transcriptional repressors. One mechanism for this repression is through their interactions with the Groucho family of co-repressors (GRG or TLE in vertebrates). Groucho proteins are broadly expressed co-repressors that are recruited to specific target promoters through their interaction with DNA-binding proteins (Fisher and Caudy, 1998). In overexpression studies, the GRG family member TLE synergizes with SIX3 and SIX6 to expand the eye field (Lopez-Rios et al., 2003), suggesting that this transcriptional repressor complex positively regulates cell proliferation. Another co-factor of SIX6-mediated repression is Dachshund (DACH), which recruits the co-repressors Histone deacetylase 3 (HDAC3), a chromatin remodeling enzyme, and nuclear receptor co-repressor (N-CoR), another transcriptional repressor, to the cyclin-dependent kinase inhibitor p27Kip1 (Li et al., 2002).

A recently described transcription-independent role of SIX3/6 in controlling cell proliferation in vertebrate eye development involves interactions with the DNA replication inhibitor Geminin (Del Bene et al., 2004). Geminin inhibits cell proliferation by sequestering CDT1, a component of the replication machinery. SIX3 can compete with CDT1 for Geminin, thus releasing CDT1 and with it cell cycle inhibition (Del Bene et al., 2004). This, combined with the direct transcriptional repression of cell cycle antagonists, such as p27Kip1, illustrates how cell proliferation is coordinated with organ development by the SIX3/6 family. Molecular studies of *Drosophila Optix* should determine whether this function of SIX3/6 proteins is conserved in invertebrates.



**Fig. 3.** Expression of signaling molecules and retinal determination gene network (RDGN) members during *Drosophila* eye development. (A) A schematic of the eye disc that shows morphogenetic furrow progression. The eye disc undergoes waves of differentiation as the morphogenetic furrow, which is driven by the cooperative actions of the Hedgehog (HH, blue) and Decapentaplegic (DPP, green) signaling pathways, moves from the posterior to the anterior of the eye disc. The most posterior cells have differentiated into the photoreceptor cells, while anterior cells are still proliferating. Wingless (WG) expression in the dorsal- and ventral-most anterior regions of the disc prevents eye tissue formation in that region, leading to head cuticle formation. (B) A schematic of the expression pattern of RDGN members, where the eye disc is broken up into six regions based on the level of differentiation, as described by Bessa et al. (Bessa et al., 2002). There are discrepancies in the literature regarding the expression pattern of Sine oculis (SO). All reports agree on the expression shown in solid red. The red hatching indicates expression reported by antibody staining (Cheyette et al., 1994) and in situ hybridization (Seimiya and Gehring, 2000) that is not seen with antibody staining by Halder et al. (Halder et al., 1998) or in the commonly used *so-lacZ* enhancer trap line, which reflects expression via a specific enhancer (Punzo et al., 2002). Eyegone is not shown, as it is expressed in a stripe along the dorsoventral boundary. EY, Eyeless; EYA, Eyes absent; SO, Sine oculis; DAC, Dachshund; MF, morphogenetic furrow; PPN, prepreneural region.

morphogenetic furrow, together activating or allowing transcription of the second tier of the RDGN, *eya*, *so* and *dac* (Curtiss and Mlodzik, 2000; Pappu et al., 2003). These three gene products are coincident only at and surrounding the morphogenetic furrow (Fig. 3B) (Bessa et al., 2002; Cheyette et al., 1994), where they and the DPP signal come together to direct terminal differentiation of the eye. Recently, a mechanism has been proposed that defines the anterior limits of *eya* and *dac* expression (Bessa et al., 2002). Homothorax (HTH), a homeodomain protein, and Teashirt (TSH), a zinc-finger transcription factor, interact with EY anterior to the furrow in Domain II to form a transcriptional repressor that prevents *eya* and *dac* expression in this region. Expression of *hth* is itself downregulated through DPP and EYA, providing a strict boundary of expression between the undifferentiated and prepreneural regions of the eye disc. Further studies of these diverse transcription factors, alone and in combination, should yield insights into how this precise program of eye development is orchestrated.

## Connections between EGFR signaling and the RDGN

The Epidermal growth factor receptor (EGFR)/RAS/mitogen-activated protein kinase (MAPK) pathways play conserved roles in growth and differentiation in many organisms (Widmann et al., 1999). In the *Drosophila* eye, EGFR signaling is required in all cells to prevent apoptosis (Bergmann et al., 2002), but is also used selectively to specify many of the cell types of the eye (for a review, see Voas and Rebay, 2004). As discussed below, the EGFR pathway provides one of the few direct links between a signaling pathway and the RDGN, where part of the molecular mechanism linking signaling to the network is understood.

Multiple genetic interactions have suggested that a complex interface exists between EGFR signaling and the RDGN. For example, mutations in *dac* were initially isolated as suppressors of the dominant-active EGFR allele *Ellipse* (*Elp*;

*Egfr* – FlyBase) (Mardon et al., 1994), suggesting that DAC plays a positive role in the transduction of the EGFR signal in the eye. Another RDGN member that is genetically implicated as a positive transducer of EGFR signaling is EYA (Rebay et al., 2000), which is phosphorylated by MAPK in response to RAS activation (Hsiao et al., 2001). Recent work has demonstrated that MAPK-mediated phosphorylation of EYA increases the activity of the EYA-SO transcription factor, although it is not absolutely required for transcription factor function (Silver et al., 2003). Thus, MAPK-mediated phosphorylation of EYA may represent a context specific mechanism for increasing transcriptional output. Whether MAPK-mediated phosphorylation regulates other aspects of EYA function, such as its phosphatase activity, or also phosphorylates DAC and SO, is not known.

The connection between the RDGN and EGFR signaling extends beyond *Drosophila*, as molecular data have also linked PAX6 family members to this pathway in vertebrates. Studies of zebrafish Pax6 have revealed a conserved MAPK phosphorylation site, serine 413 (Ser413), which is phosphorylated in vitro and in vivo by MAPK family members (Mikkola et al., 1999). Ser413 lies within the transactivation domain of Pax6, and, as in *Drosophila* EYA, may provide a context-specific mechanism by which zebrafish Pax6 targets are modulated.

In conclusion, these examples illustrate a conserved link between the RDGN and the EGFR signaling pathway in flies and vertebrates. Whether the underlying mechanisms of this link are identical or distinct remains to be determined. To address this issue it will be informative to explore whether *Drosophila* EY and vertebrate EYA proteins are regulated by MAPK-mediated phosphorylation, as has been shown for vertebrate PAX6 and *Drosophila* EYA. If they are, this would indicate extensive conservation of this particular mechanism of signal integration; if regulation appears distinct, this would suggest that the EGFR signaling pathway interfaces with the RDGN using different points of crosstalk in flies and vertebrates.

### Antagonistic signaling: determining eye from cuticle

In the formation of the *Drosophila* eye, two major decisions must be made by the developing imaginal disc that gives rise to the eye, antenna and head: one is to distinguish between eye region and antennal region (as discussed in Box 3); the other is to distinguish, within the eye region, tissue destined to become eye from that which destined become head cuticle, using the opposing signals of the DPP/Transforming growth factor β (TGFβ) and Wingless (WG) pathways. High levels of DPP at the most posterior region of the eye disc repress the WG signal and allow the morphogenetic furrow to form, while high levels of WG at the most dorsal and ventral boundaries of the disc inhibit eye formation (see Fig. 3A) (Hazelett et al., 1998). The RDGN members play important roles in the specification and maintenance of these expression patterns and may themselves be regulated directly by DPP and WG signaling.

Genetic manipulations of the WG pathway in the fly have revealed its role in regulating the genes of the RDGN; for example, loss of WG signaling results in ectopic morphogenetic furrows and in the ectopic protein expression of EYA and DAC, while ectopic WG signaling leads to inappropriate cell proliferation and to the formation of ectopic

head cuticle at the expense of eye tissue (Baonza and Freeman, 2002; Ma and Moses, 1995; Royet and Finkelstein, 1997; Treisman and Rubin, 1995). Although eye specification is prevented by ectopic WG signaling, the expression of EY remains unchanged (Baonza and Freeman, 2002), suggesting that this block occurs either at the level of EY protein function, or via downstream components of the network. Consistent with either of these mechanisms, EYA, SO and DAC protein synthesis is reduced by ectopic WG pathway activation (Baonza and Freeman, 2002). The downregulation of EYA and DAC is likely to be important for formation of head cuticle, as in EYA or DAC mutant tissue, head cuticle can form in place of the eye (I.R., unpublished) (Mardon et al., 1994). Could repression of DPP signaling be an indirect mechanism by which WG downregulates RDGN members? Epistasis experiments indicate that this is unlikely, as WG-mediated repression cannot be overcome by activation of DPP/TGFβ signaling (Hazelett et al., 1998).

However, blocking the transcription of RDGN members is not the only mechanism that underlies the WG-mediated repression of eye formation, as ectopic expression of EYA, which leads to elevated levels of SO and DAC proteins, cannot rescue the loss of eye tissue (Baonza and Freeman, 2002). Thus, in tissue with high levels of WG signal, increasing the levels of EYA, SO and DAC is not sufficient to direct eye formation. This suggests that WG signaling may have post-translational effects on RDGN function, or may affect unknown factors that act in parallel to the RDGN. The response of RGDN genes to multiple signaling pathways makes them key nodes of signal integration, as seen in the *Drosophila* eye, for the DPP and WG morphogens.

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**Box 3. Eye versus antenna: EGFR, Notch and the RDGN**

During *Drosophila* development, epidermal sacs known as imaginal discs give rise to adult tissues. One of the earliest decisions the eye-antennal imaginal disc makes is its subdivision into two discrete primordia: the eye region and the antennal region. Initial experiments using overexpression of dominant-active or dominant-negative pathway components suggested this restriction derives from a balanced antagonism between Epidermal growth factor receptor (EGFR) and Notch signaling, where EGFR induces antennal fate by repressing *eyeless* (*ey*), and Notch induces eye fate by repressing the antennal gene *Distal-less* (*Dll*) and by activating *ey* (Kumar and Moses, 2001). However, recent work has revealed that Notch is not required for expression of RDGN members *ey, eyes absent* (*eya*), *sine oculis* (*so*) and *dachshund* (*dac*) (Kenyon et al., 2003). Thus, how these primordia are restricted remains unclear. Notch might indirectly activate *eya* expression as the delayed onset of EYA protein expression that is associated with the absence of Notch signal can be restored independently of Notch signaling by increasing cell division (Kenyon et al., 2003). However, EYA protein expression does not correlate with proliferation in the developing eye disc, suggesting that the overall size of the eye field, rather than proliferation signals, induces *eya* expression (Kenyon et al., 2003). Altering the field size affects the local concentrations of signals received by cells from the opposing Decapentaplegic (DPP) and Wingless (WG) morphogens, and consistent with field size in the induction of *eya*, reducing the WG-mediated 'anti-eye' signal (Ma and Moses, 1995) is sufficient to restore the early onset of EYA in a smaller field (Kenyon et al., 2003).

## The RDGN and tissue specification

Although the RDGN has been best characterized for its role in eye development, insight into its function and into its interactions with developmentally important signaling pathways has been gained from the comparative study of different organs and tissues. We discuss three examples outside the eye in which the RDGN has been particularly well studied. First, studies of *Drosophila* gonad development, in which multiple members of the network play crucial and context-specific roles, have revealed new modes of regulation and potential combinatorial codes of action of the RDGN members that may provide insight into the regulation and function of the RDGN in vertebrate tissues (Bai and Montell, 2002; Bonini et al., 1998; Fabrizio et al., 2003; Keisman and Baker, 2001). Second, we describe the role that the RDGN plays in vertebrate muscle development (Heanue et al., 1999), and how studies of this tissue first highlighted the evolutionary conservation not only of the individual RDGN genes, but also of the complex meshwork of interactions that links them into a functional network. Finally, recent analysis of the interplay between PAX, EYA, SIX and DAC family members in the vertebrate ear placode highlights the additional regulatory potential that context-specific variations in the use of the RDGN components provides (Ozaki et al., 2004; Zheng et al., 2003). Because signal integration and context specificity are important components of the genetic circuits that are used throughout development, the principles elucidated from studies of the RDGN are likely to apply to diverse biological processes.

### Sex-specific regulation of RDGN members

Many patterning genes are expressed in homologous regions in the male and female genital discs of *Drosophila*. For example, in both sexes, *wg* is expressed in a stripe along the anterior-posterior border, and is flanked by broad stripes of *dpp* expression (Keisman and Baker, 2001). Other genes, including *dac* (Keisman and Baker, 2001; Sanchez et al., 2001), are expressed in a sex-specific manner.

In males, DAC protein expression overlaps the *dpp* stripes, whereas in females DAC expression overlaps the central *wg* expression domain (Keisman and Baker, 2001; Sanchez et al., 2001). DAC function is important for development of both male and female genitalia, as males that lack *dac* have reduced claspers, which are structures of the external male genitalia, and females that lack *dac* have defects in ovarian duct formation (Keisman and Baker, 2001).

WG signaling activates DAC protein expression in the female genital discs (Keisman and Baker, 2001; Sanchez et al., 2001). Strikingly, the opposite effect is observed in male genital discs, where WG appears to restrict DAC expression (Keisman and Baker, 2001; Sanchez et al., 2001), as in the eye (Fig. 4). The converse is true for DPP signaling, which activates DAC expression in the eye and in male genital discs, but represses DAC expression in female genital discs (Fig. 4) (Keisman and Baker, 2001; Sanchez et al., 2001).

This sex-specific regulation of *dac* is likely to be mediated by components of the sex-determination pathway *doublesex* (*dsx*) and *transformer* (*tra*) (Keisman and Baker, 2001; Sanchez et al., 2001), although there is some disagreement as to whether both male and female forms of DSX play active roles in *dac* regulation (reviewed by Estrada et al., 2003). Although the sex-specific forms of DSX and TRA participate

in determining tissue response to WG and DPP, it is not clear how this response then regulates the expression of downstream genes such as *dac*.
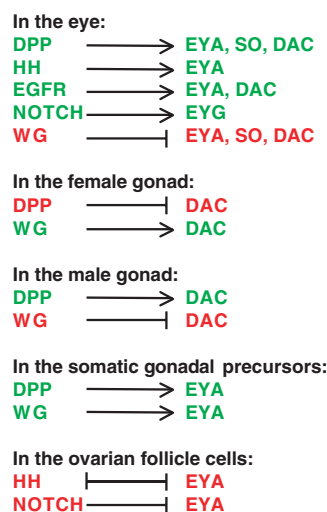
### EYA is crucial for gonad development in *Drosophila*

EYA also plays a role in both female and male fertility in *Drosophila* (Table 1) (Boyle et al., 1997; Fabrizio et al., 2003). It is expressed in the somatic gonadal precursor (SGP) cells that associate with the germ cells and ensure their incorporation into the gonad, and is required for the maintenance of SGP cell fate (Boyle et al., 1997). In this context, EYA may function downstream of WG signaling, as ectopic activation of the WG pathway induces ectopic EYA and the recruitment of extra SGPs (Boyle et al., 1997). Here, WG plays a positive role in EYA regulation, whereas in the eye, WG and EYA act antagonistically (Fig. 4).

By contrast, DPP and EYA have a positive relationship in the developing gonad, as in the eye; DPP signaling is crucial for EYA expression and for the formation of SGPs (Boyle et al., 1997). Thus, context, once again, determines the direction of interaction between signaling pathways and the RDGN; as in the gonad WG and DPP together activate EYA in contrast to their opposing functions in the eye (Fig. 4).

In addition to its roles in patterning the early gonad, EYA also functions during oogenesis in flies. Three types of somatic



**Interactions between *Drosophila* retinal determination genes and signaling pathways**

In the eye:

DPP ⟶ EYA, SO, DAC
HH ⟶ EYA
EGFR ⟶ EYA, DAC
NOTCH ⟶ EYG
WG ⊣ EYA, SO, DAC

In the female gonad:

DPP ⊣ DAC
WG ⟶ DAC

In the male gonad:

DPP ⟶ DAC
WG ⊣ DAC

In the somatic gonadal precursors:

DPP ⟶ EYA
WG ⟶ EYA

In the ovarian follicle cells:

HH ⊣ EYA
NOTCH ⊣ EYA

**Interactions between vertebrate retinal determination genes and signaling pathways**

Mouse muscle

WNT3A ⟶ SIX1
WNT3A ⟶ PAX3
SHH ⟶ PAX3

**Fig. 4.** Context-dependent relationships between signaling pathways and the retinal determination gene network (RDGN). Positive interactions are shown in green, negative interactions in red. Arrows represent activation or induction; blunt-end lines represent transcriptional and/or post-translational repression. DAC, DACHSHUND; DPP, Decapentaplegic; EGFR, Epidermal growth factor receptor; EYA, Eyes absent; HH, Hedgehog; SHH, Sonic hedgehog; SO, Sine oculis; WG, Wingless.

follicle cells surround the developing oocyte and are crucial for proper germ cell development and function: polar cells, stalk cells and main body epithelial cells (Spradling, 1993). Ovaries mutant for *eya* have extra polar cells, whereas ectopic expression of *eya* prevents polar cell specification (Bai and Montell, 2002). Unlike the eye, where Notch and HH signaling exert positive, or at least permissive, effects on EYA, in the ovary both Notch and HH function antagonistically to EYA, again illustrating the importance of context on signal integration. For example, ectopic Notch or HH signaling can reduce EYA protein expression in the follicle cells and induce the formation of ectopic polar cells (Bai and Montell, 2002). Although the molecular mechanisms underlying Notch- and HH-mediated repression of EYA have not been elucidated, *eya* mutant cells exhibit higher levels of $CI^{act}$, the transcriptional effector of HH signaling (Bai and Montell, 2002), indicating that a mutually repressive relationship exists between HH signaling and EYA during the differentiation of ovarian follicle cells.

### RDGN members direct muscle specification

In early mouse skeletal muscle development, the expression of *Pax3*, a gene related to *Pax6* but not orthologous to *ey*, overlaps with that of *Dach2*, and their expression is mutually regulated through positive feedback loops (Heanue et al., 1999), similar to those observed between EY and DAC during *Drosophila* eye development (Shen and Mardon, 1997). *Pax3* is required for skeletal myogenesis and, when overexpressed, can induce *Eya2* and *Six1* expression (Ridgeway and Skerjanc, 2001). *Six1* mutant mice also display defects in myogenesis (Laclef et al., 2003), but *Eya2* knockout mice have not yet been reported. Strikingly, when *Eya2* and *Six1*, as well as *Eya2* and *Dach2*, are misexpressed in combination, they can synergize to direct the expression of muscle markers that are indicative of myogenic differentiation, including PAX3 (Heanue et al., 1999; Kardon et al., 2002). This is similar to the synergism observed between EYA and SO, and EYA and DAC in ectopic eye induction in *Drosophila* (Chen et al., 1997; Pignoni et al., 1997), indicating that analogous patterns of interactions between these proteins play conserved roles in the development of multiple tissues and organ types in flies and vertebrates.

Signaling by sonic hedgehog (SHH) and WNT family members induce many muscle-specific factors, and these signals are balanced by negative regulation through bone morphogenetic proteins (BMPs) and NOTCH (Parker et al., 2003). For example *Pax3* and *Six1* can be induced in a cell culture model of myogenesis by WNT3A and β-catenin (Petropoulos and Skerjanc, 2002), and *Pax3* may also be upregulated by GLI2, suggesting a positive role for SHH in RDGN regulation (Petropoulos et al., 2004). Furthermore, SIX family members can directly regulate muscle specification genes, such as *myogenin* (Spitz et al., 1998), making them the possible link between the RDGN, the signals it interacts with and the muscle-specific genes that control skeletal muscle development. Further experiments examining the expression and function of RDGN genes in vivo in response to perturbation of the signaling pathways that function in mouse skeletal muscle are required to test this hypothesis and the extent to which the signaling circuitries that operate in flies and vertebrates are conserved.

### Otic development reveals new relationships within the RDGN

Comparisons of RDGN function and regulation in the *Drosophila* eye versus vertebrate muscle highlight the striking conservation of relationships both within the network and with exogenous pathways. Here, we describe how recent investigations of the RDGN in vertebrate ear development have illustrated how distinct combinations of regulatory relationships may operate in different developmental programs (Fig. 5).

The vertebrate ear develops from a region termed the otic placode, which is initially a thickened part of the surface ectoderm adjacent to the hindbrain (Fig. 5A). *Pax8* and *Pax2* are among the earliest markers of otic placode fate (Riley and Phillips, 2003), although *Pax2* knockout mice have relatively mild otic defects, perhaps owing to redundancy with *Pax8* (Burton et al., 2004; Torres et al., 1996). *Six1* and *Eya1* are co-expressed ventrally in the otic placode, and mice mutant for
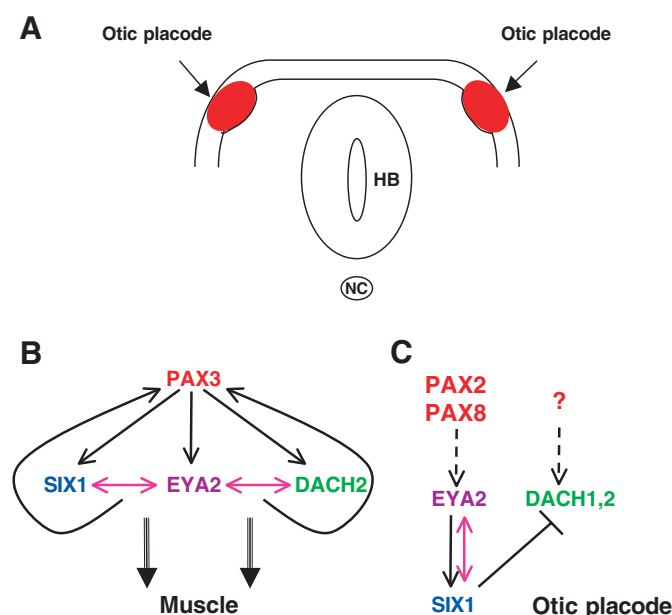


**Fig. 5.** Differential use of the retinal determination gene network (RDGN) in muscle versus ear development. (A) A schematic of the vertebrate otic placode, which can be identified at the 4- to 13-somite stage of mouse development, shown as a cross-section through the developing embryo. HB, hindbrain; NC, notochord. The placode invaginates at embryonic day 9 (E9) to form the otic vesicle, which will close to form the otocyst. (B) The RDGN hierarchy in vertebrate muscle development is analogous to that operating in the *Drosophila* eye (see Fig. 1), with respect to transcriptional regulation, protein-protein interactions and positive feedback loops. However, the PAX protein that functions in muscle development is PAX3, as opposed to PAX6, which functions in the eye. (C) The functions of the RDGN during otic placode development are distinct from those operating in the muscle. Most strikingly, DAC/DACH proteins appear to function in a parallel pathway to Eyes absent (EYA)/SIX that is negatively regulated by SIX1. Feedback loops in which downstream members influence the expression of upstream components are also not apparent. Black arrows indicate transcriptional regulation, either positive or negative. Broken black arrows reflect the uncertainty in the positioning of PAX2 and PAX8 upstream of EYA2 and SIX1. Double-headed pink arrows indicate the synergistic interactions that reflect possible direct protein-protein associations.

Development

*Six1* or *Eya1* have defects in ear development (Ozaki et al., 2004; Xu et al., 1999; Xu et al., 2003; Zheng et al., 2003). Similarly, mutations in human *EYA1* and *SIX1* have been implicated in branchio-oto-renal syndrome, an autosomal dominant disorder that is partly characterized by abnormal ear development (Abdelhak et al., 1997; Ruf et al., 2004). The likely redundancy between *Pax2* and *Pax8* has made it difficult to place these PAX proteins definitively upstream of *Eya1* and *Six1* (Burton et al., 2004; Torres et al., 1996), although the fact that *Pax2* expression is unchanged in *Six1* mutants suggests it does not act downstream of *Six1* (Ozaki et al., 2004; Xu et al., 1999; Xu et al., 2003; Zheng et al., 2003).

In contrast to vertebrate skeletal muscle (Heanue et al., 1999) and the *Drosophila* eye (Bessa et al., 2002), *Dach* is not co-expressed with other members of the RDGN in the otic placode (Ozaki et al., 2004), and its expression appears to be independent of either *Pax2* or *Eya1* (Heanue et al., 1999). Furthermore, expression of *Dach1* and *Dach2*, which are both normally restricted to the dorsal region of the otic vesicle, expands ventrally in *Six1* mutant mice, suggesting that *Six1* represses *Dach* (Ozaki et al., 2004). Thus, in the ear, *Six1* and *Dach* provide opposing differentiation cues, while in the skeletal muscle and fly eye, they work cooperatively in tissue specification. Further analysis of the molecular function of EYA, DACH and SIX in these tissues, including the identification of their protein interactions and target genes, should provide insight into the mechanism that underlies the sometimes synergistic, sometimes opposing and sometimes independent, relationships between RDGN members.

## Concluding remarks

The RDGN provides a model of signal integration, the study of which may provide us with insights into the broader issue of how signal specificity is brought about in different developmental contexts. Crosstalk between the RDGN members and signaling pathways provides a mechanism for coordinately linking the interdependent processes of differentiation and cell division that are likely to be conserved from flies to humans. Future work aimed at elucidating the conserved roles of these genes and the molecular mechanisms that underlie the context specificity of developmental signaling will benefit greatly from continued comparative investigations of RDGN function in flies and vertebrates.

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