Chapter 4
Fast and Furious 800. The Retinal Determination Gene Network in *Drosophila*

Fernando Casares and Isabel Almudi

Abstract The *Drosophila* compound eye is formed by about 800 ommatidia or simple eyes, packed in an almost crystalline lattice. The precise ommatidial arrangement makes the fly eye especially sensitive to pattern aberrations. These properties, together with the fact that the eye is an external and largely dispensable organ, have made the *Drosophila* eye an excellent genetic model to investigate the mechanisms of cell proliferation, patterning and differentiation, as well as mechanisms of human disease, such as cancer, neurodegeneration or metabolic pathologies. Part of these studies have coalesced into the *Drosophila* eye (or retinal) gene regulatory network (GRN): a text-book example of an organ-specification gene network that has been used as a point-of-comparison in the study of the mechanisms of eye specification and evolution, as well as a paradigm of signaling integration. This paper reviews the gene network that covers the period from eye progenitor specification to the onset of retinal differentiation as marked by activation of the proneural gene *atonal,* while paying special attention to the dynamics of the network and its intimate relation to the control of eye size.

Keywords Eye disc · Compound eye · Visual systems · Drosophila development · Gene networks · Organ growth · Cell specification · Organ size

4.1 Introduction: Fast and Furious

The compound eyes of flies (Brachycerans or "higher diptera"), like *Drosophila,* have several important characteristics. First, they are large. The *Drosophila* eye has about 800 ommatidia, almost one order of magnitude larger than the eye of the flour beetle *Tribolium* (Coleoptera) which has close to 100 ommatidia. Moreover, some fly species, like the hoverfly *Episyrphus,* have eyes with over 3500 ommatidia, similar to

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J. Castelli-Gair Hombría and P. Bovolenta (eds.), *Organogenetic Gene Networks*,
DOI 10.1007/978-3-319-42767-6_4

grasshoppers (Orthoptera) and dragonflies (Odonata), which also have large eyes with thousands of ommatidia. However, eye development in these other insects takes significantly longer: while in *Drosophila* ommatidia differentiate at a rate of one row (starting with 7-8-cell rows at the onset of differentiation till several hundred of cells per row in most anterior regions of the disc) every 1.5 h, differentiating one row of ommatidia takes several hours in the grasshopper *Schistocerca americana* (Friedrich and Benzer 2000). Large compound eyes afford flies the wide field of view and high spatial resolution required for fast flying maneuverability, and for accurate detection of mates and food sources. In *Drosophila,* the embryonic eye rudiment comprises about 20 cells. Four days later, by the end of the third (and last) larval stage (L3), the eye primordium has grown 500 hundred times, reaching 15000 cells in size. Therefore, *Drosophila* eye development is fast. Despite this explosive growth, the final eye size in *Drosophila* adults of a given strain is almost constant (<5 % eye size difference between same sex individuals; (Hammerle and Ferrus 2003; Posnien et al. 2012), and robust in the face of environmental variation (Azevedo et al. 2002). Therefore, fast development, large size, and robustness are properties that need to be reflected in the gene regulatory network (GRN) for the *Drosophila* eye-specification. In this review, we will take this perspective and discuss what is currently known about this GRN.

4.2 The Eye Derives from the "Eye-Antennal" Imaginal Disc

The *Drosophila* adult eye has its origins in a broad region of the dorsal-anterior neuroectoderm of the embryo (Green et al. 1993; Younossi-Hartenstein et al. 1993), the visual anlage, that also gives rise to the larval eye (Bolwig's organ) and the optic lobes: the brain centers dedicated to the processing of eye-derived information. The visual anlage is characterized by the expression of *sine oculis (so),* a Six1, 2 type transcription factor (TF) that is required for the specification of all visual structures (Cheyette et al. 1994; Chang et al. 2001). Within the *so*-expressing region, the eye primordium cells fall within the domains of expression of two additional TFs: The Otx gene *orthodenticle (otd)* and *twin of eyeless (toy),* one of the two Pax6 paralogues in the *Drosophila* genome (Cohen and Jurgens 1990; Finkelstein and Perrimon 1990; Finkelstein et al. 1990; Czerny et al. 1999). By the end of embryogenesis, two symmetric elongated epithelial sacs invaginate from the neuroectoderm, forming the paired eye-antennal imaginal discs (EAD).[1] The EADs will remain attached to the mouthparts, anteriorly, and to the optic

[1]The origin of insect eyes from the cephalic neuroectoderm (Fernald 2000) resembles more the vertebrate sensory placodes (such as the lens, otic or olfactory placodes), which also derive from epithelial thickenings (Schlosser 2015), than the vertebrate retina, which forms as an evagination of the anterior neural tube. However, it is important to stress that the precursor cells *for both the eye and the optic lobes of the brain* originate from adjacent cell populations in the neuroectoderm. The difference being that the EAD invaginates as an epithelial sac, while the optic lobe neuroblasts internalize by delamination.

lobes, posteriorly, throughout development. The discs give rise to most structures of the adult head: the eyes, antennae, maxillary palps, ocelli and the head capsule (Fig. 4.1; Haynie and Bryant 1986). It is at the time of invagination that the EAD starts expressing the second Pax6 paralog, *eyeless (ey)* (Quiring et al. 1994), which is activated by *toy* (Czerny et al. 1999). During the first larval stage (L1) most or all EAD cells express *ey* and *toy*. However, it is during L2 that the first signs of regionalization within the EAD appear: a constriction of the disc results into two "lobes": the anterior lobe starts expressing the homeobox TF encoding gene *cut (ct)* while simultaneously loses *Pax6* expression (Kenyon et al. 2003; Figs. 4.1 and 4.2). The *ct*-expressing lobe will give rise to the antenna, the maxillary palp and associated head capsule, while the posterior lobe retains *ey* and *toy* and will give rise to the eye and the surrounding head capsule, which includes the small dorsal eyes called ocelli. This posterior lobe is usually called "eye disc" (the development of the ocelli will not be reviewed here) (Fig. 4.1). In what follows, we will focus on the gene network that operates from the establishment of the eye primordium, starting early in L2, through the transition of retina precursors into differentiating photoreceptor neurons, during L3, an event marked by the activity of the bHLH proneural TF gene *atonal (ato)* (Jarman et al. 1995). A number of excellent reviews have covered the processes following the initiation of *ato* expression and leading to the patterned differentiation of all retinal cell types (see for example (Charlton-Perkins and Cook 2010; Quan et al. 2012; Treisman 2013). In addition, recent efforts have successfully formalized the retinal differentiation and patterning network into a mathematical model that explains these two processes (Lubensky et al. 2011).

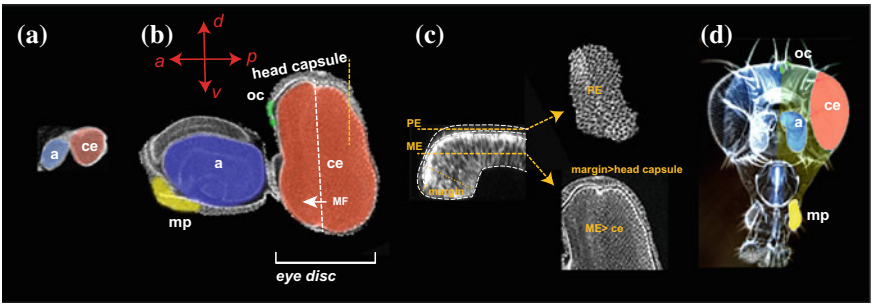


Fig. 4.1 The eye-antenna disc and its adult derivatives. Confocal images of phalloidin-stained L2 (**a**) and L3 (**b**) eye-antennal discs. In (**b**) the morphogenetic furrow (*MF*) has been marked by the *dashed line* and its direction of advancement indicated by the *arrow*. From L2, the eye antennal disc is bilobed. The posterior lobe is called "eye disc". **c** Z-plane optical section through the *orange line* in (**b**). The columnar (*ME*, main epithelium), cuboidal (margin) and squamous (*PE*, peripodial epithelium) epithelia are visible (outlined by the *dashed line*). Optical sections across the *ME* and the *PE* are shown in **c**. The margin gives rise to the head capsule; the *ME* differentiates into the eye. **d** The prospective regions of the adult head structures have been color-coded. *a* Antenna; *CE* compound eye; *oc* ocelli; *mp* maxillary palps. The *double-headed arrows* in (**b**) indicate the anterior (*a*), posterior (*p*), dorsal (*d*) and ventral (*v*) coordinates

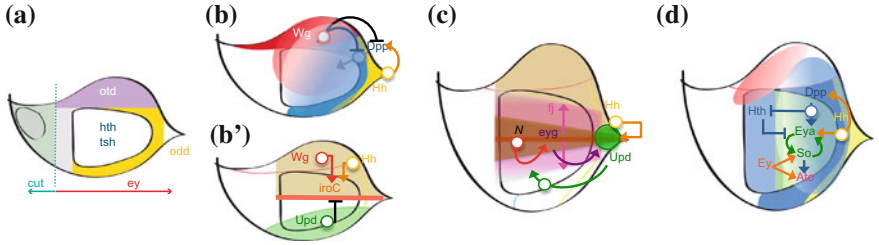


Fig. 4.2 Genetic organization of the early eye disc and major genetic interactions. Schematic representation of eye-antennal disc in L2 (**a**–**b**) and early L3 (**c**–**d**). In L2, the disc is subdivided in two major territories: the prospective antenna and eye lobes, marked by the exclusive expression of *cut* and *eyeless (ey),* that will give rise to the antenna and the eye, respectively, plus the associated head capsule. The eye region is subdivided in several major gene expression domains: *otd (*dorsal head); *odd* gene family (posterior/ventral head) and *tsh,* which marks the prospective eye proper. **b** In early discs, all eye disc cells are exposed to *Wg* and *Dpp* signals. *Wg* prevents the initiation of differentiation. **b′** The same *Wg* expression, restricted to the dorsal disc by the transient ventral expression of *Upd*, results in a genetic D/V subdivision that generates an iroC+/iroC− interphase. At this interface the *Notch* signaling pathway is activated (**c**). *Notch* signaling is translated into increased proliferation in the disc through two mechanisms: by generating a gradient of *ft,* which impacts the Hpo pathway, and by activating *Upd*, jointly with margin signals, which also increases proliferation. These two actions are intermediated by *eyg*. **d** The size increase frees the posterior disc region from *Wg*'s influence allowing the first steps towards eye differentiation. These involve the joint and partly redundant action of two signaling molecules: *Hh* and its target *Dpp*. Repression of *Hth* allows the recruitment of progenitors into precursor cells, where the retinal determination (RD) transcription factors Eya and So are simultaneously up-regulated. Signals and RD factors induce *atonal* as the first step towards retinal differentiation

4.3 The Phenomenon

First, we will describe briefly the structure and development of the disc from the start of L2 to the end of L3. This description will serve as framework to describe its molecular underpinnings (Fig. 4.1).

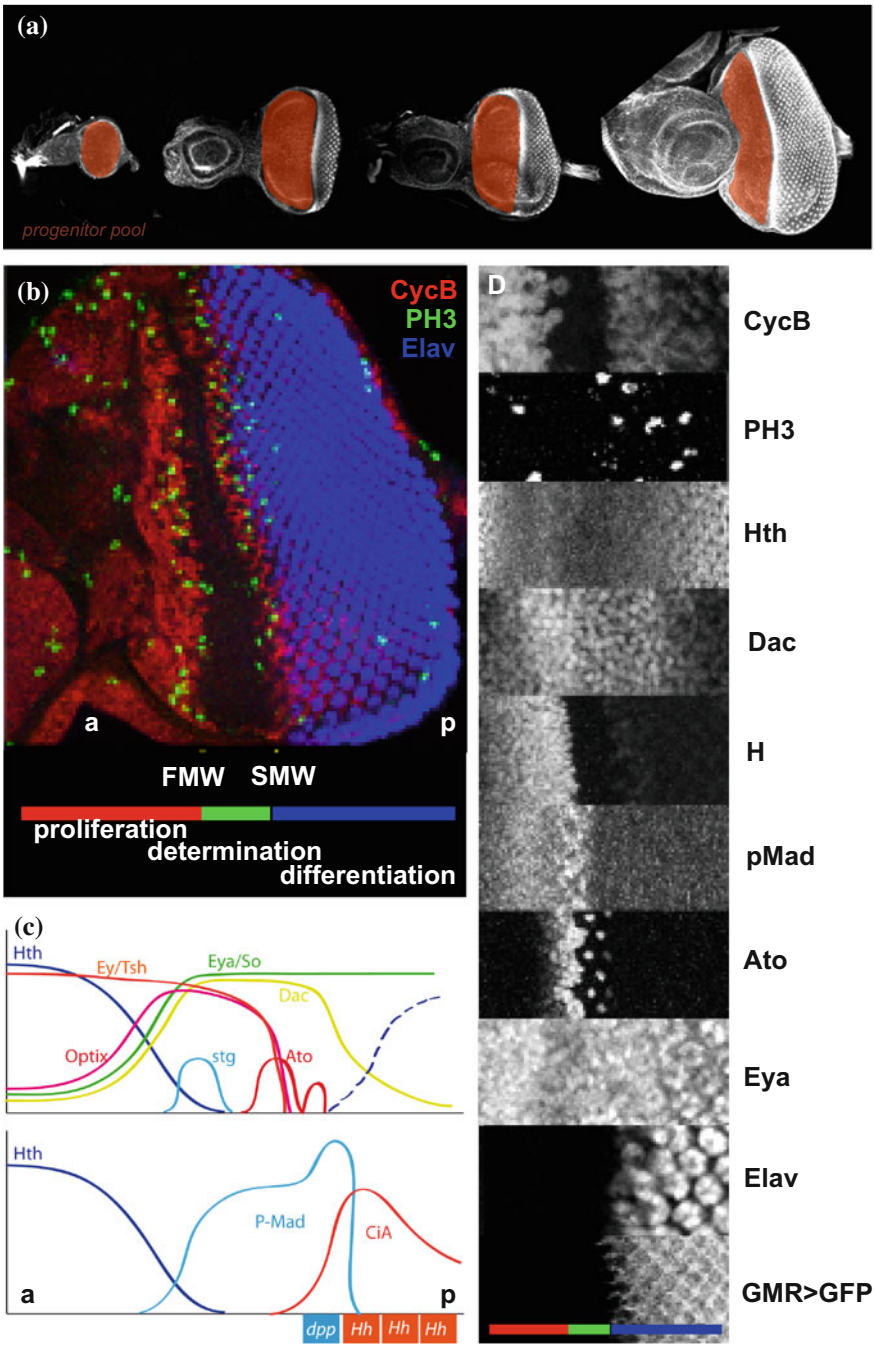
The eye disc is a flat sac. A cross-section through the disc reveals two closely apposed epithelial layers: one columnar, the other squamous. The columnar epithelium is called "disc proper" or "main epithelium" ("ME"). At the disc's margin, cell morphology changes from columnar to cuboidal (margin cells; "Ma") and then cells become squamous as they face the columnar layer. This squamous region is called peripodial epithelium ("PE"; Fig. 4.1; McClure and Schubiger 2005). Each of these regions develops into different structures that carry out distinct functions: the ME gives rise to the eye, and therefore constitutes the real eye primordium; the margin cells differentiate the head capsule that surrounds the eye and serves as a source of key patterning signals during development; and the PE participates in the fusion and final morphogenesis of the discs during metamorphosis.

In L2, the main epithelium comprises uncommitted, proliferating progenitor cells. It is only at the L2/L3 transition that retinal differentiation begins. Retinal

differentiation proceeds like a wave from the posterior pole towards anterior. The differentiation wavefront is marked by a straight dorsoventral indentation in the epithelium, called the morphogenetic furrow (MF): Undifferentiated cells lie anterior to the MF while cells in its wake are differentiating. Therefore, as the MF moves anteriorly during L3, the eye disc shows an anterior-posterior "gradient of differentiation", with cells farthest anterior being the least differentiated while those at the posterior pole being the most differentiated (Fig. 4.3). Also, as the MF moves across the disc during L3, the uniform and asynchronous proliferation that characterized the eye primordium in L2 becomes patterned. The most anterior cells (progenitors) proliferate asynchronously; immediately anterior to the furrow, progenitor cells undergo 2–3 rounds of fast mitoses, called the first mitotic wave (FMW) to then become synchronized in G1 at the MF (Fig. 4.3). The G1-synchronized cells at the MF are genetically distinct from more anterior progenitors and are here referred as retinal "precursors". Posterior to the MF, a set of precursors exit the cell cycle permanently and begin to differentiate as photoreceptors R8 (the ommatidial founder cell), followed by R2 and R5 and R3 and R4 and R5 that exit the cell cycle permanently and differentiate. The other retinal cells (R1, R6 and R7, cone, pigment and interommatidial mechanosensory cells) are progressively recruited from the remaining pool of precursors posterior to the MF after having gone through one last mitotic round, the so-called second mitotic wave (SMW) (Baker 2001). Expansion of the progenitor pool occurs mostly during L2 and, anterior to the MF. During L3 until this pool is consumed as the MF advances, until the early pupal stage, when the MF reaches the anterior pole of the eye disc exhausting all progenitors. This expansion of the progenitor pool is critical in determining the final size of the eye as these progenitors are used as source of R8 cells: Since each R8 nucleates the formation of one ommatidium, the number of R8 generated during L3 (and early pupa) equals the number of ommatidia in the adult eye.

4.4 Specification of the Eye Progenitors

At the onset of L2, all eye disc cells (including margin and peripodial cells) express the two Pax6 genes, *toy* and *ey* (see above), which encode TFs with two DNA binding regions, a paired domain and a paired-type homeodomain (Quiring et al. 1994; Czerny et al. 1999); reviewed in Callaerts et al. (1997). Progenitors also express the Meis1 TALE-class TF homeodomain gene *homothorax (hth)* (Pai et al. 1998; Pichaud and Casares 2000; Bessa et al. 2002). However, only the main epithelium layer (where the eye primordium forms) expresses *teashirt (tsh)* and *tiptop (tio),* two paralogous genes encoding Zn-finger TFs (Fasano et al. 1991; Pan and Rubin 1998; Bessa et al. 2002, 2009; Singh et al. 2002; Tang and Sun 2002; Bessa and Casares 2005; Laugier et al. 2005; Datta et al. 2009). Expression of *tsh* coincides with the thickening of the eye primordium epithelium, and its ectopic


(a)
progenitor pool
(b)
CycB
PH3
Elav
a
p
FMW SMW
proliferation
determination
differentiation
(c)
Hth
Ey/Tsh
Eya/So
Dac
Optix
stg
Ato
Hth
P-Mad
CiA
a
p
dpp
Hh
Hh
Hh
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CycB
PH3
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H
pMad
Ato
Eya
Elav
GMR>GFP

◀ **Fig. 4.3** Transitions at the MF. **a** Phalloidin staining along L2–L3 stages. The pool of progenitor cells is highlighted in *red*. The number of progenitors first increases to then start decreasing over time until the pool is exhausted and the final number of ommatidia is attained. **b** L3 imaginal disc stained with Cyclin B (*CycB*, *red*), which marks cells in G2, Phospho-Histone H3 (*PH3*, *green*) a mitosis marker, and the pan-neural marker, *Elav* (*blue*). *CycB* positive cells indicate high levels of proliferation anterior to the MF. Flanking the MF, *PH3* positive cells accumulate at the First Mitotic Wave (*FMW*) anterior to the MF and the Second Mitotic Wave (*SMW*) posterior to the MF. Posterior to the MF, photoreceptor cells already specified are shown by *Elav* staining. **c** Schematic gene expression profiles in mid L3 (anterior region on the *left* and posterior on the *right*). These profiles are approximate, as they have never been quantified to date. **d** Expression patterns of key elements of the early eye GRN around the MF

expression in the PE converts the squamous cells into cuboidal/columnar cells. Despite *tsh* expression suffices to re-specify the PE into eye primordium, its removal is required later for morphogenesis of the neuronal array to proceed (Bessa and Casares 2005). What drives tsh/tio expression specifically to the ME is not known, but this should be related to the mechanisms that establish the distinction between ME and PE. This distinction requires yorkie (yki), the co-transcriptional activator of the Salvador/Warts/Hippo (SWH) pathway (Huang et al. 2005), in conjunction with the TEAD TF Scalloped (Sd). Thus, knocking down Yki or Sd results in the transformation of the PE into eye (Zhang et al. 2011), including the induction of *tsh* transcription. Hence, *tsh* expression (and presumably that of *tio* as well) is critical for assigning an eye fate to the eye disc cells. Little is known about the symmetry-breaking genetic step in the process—i.e. the mechanism that determines which of the two layers expresses *tsh.* Perhaps, the *odd-skipped (odd)* gene family contributes to this process, as *odd* family members *odd, drm* and *sob* are required for the specification of the margin/PE (Bras-Pereira et al. 2006).

Within the ME layer, eye progenitors are thus characterized by the combined expression of at least five TFs: Toy, Ey, Hth, Tsh and Tio. Arguably, *ey* is the most famous among them. The first *ey* mutation was reported one hundred years ago by Hoge (1915), and since then a number of hypomorphic and null *ey* alleles have been isolated. Homozygous *ey* flies show reduced or absent eyes, indicating a requirement for *ey* in eye development (Quiring et al. 1994; Clements et al. 2009). Even more impressive is Ey's capacity to trigger eye development when expressed ectopically in other imaginal discs, such as the antenna, legs or wings (Halder et al. 1995). A similar capacity of inducing ectopic eyes, even in the absence of *ey,* was demonstrated for *toy,* which suggested similar functional capacities, in accordance with their molecular similarity (Czerny et al. 1999). These results, together with the almost universal expression of *ey* in eyes from very different animal groups, led to the labeling of *ey/Pax6* as the "Eye Master" control gene (Gehring 1996). However, there are a number of unresolved issues about the precise role of *ey* and its mechanism of action. First, *ey* null mutants, are often not completely "eyeless", but exhibit reduced eyes. The residual eye was initially attributed to *toy,* which by being upstream of *ey* and functionally similar to it, could partially replace *ey* loss. However, although *toy-ey-* double mutant adults are often headless (Kronhamn et al. 2002), some *toy-ey-* pharate adults do form heads, and in these heads reduced eyes still develop (Gehring and

Seimiya 2010). Thus, eye specification appears to occur even in the absence of both Pax6 paralogues, which argues against Pax6 genes being indispensable for eye specification. In addition, the capacity of Ey to re-specify other tissues as eye is not unlimited. When Ey is ectopically expressed in other imaginal discs, only a limited number of areas are competent to be re-specified (Salzer and Kumar 2010), which has led to the concept that *ey,* rather than imposing an eye differentiation program, redirects development of cell populations of specially high developmental plasticity (Salzer and Kumar 2010). Furthermore, once the differentiation process has been initiated, the removal or the simultaneous attenuation of both *ey* and *toy* using RNAi causes only mild developmental defects (Lopes and Casares 2015). Even if *ey's* major role were not as an eye master, but instead as an eye "facilitator", it is unclear how Ey would play this role. An interesting notion is that Ey might be required to "maintain" an eye identity, instilled in eye progenitors by genes such as *so/Six2* and *Otd*, and fully expressed only during late L2.

4.5 Maintaining Progenitors Undifferentiated and Proliferative

Of the five progenitor genes (Hth, Toy, Ey, Tsh and Tio), most research has focused so far on Hth, Ey and Tsh. These TFs are simultaneously involved in the control of the progenitor's eye identity as well as their proliferation—thereby providing a sufficiently large pool of progenitors for the development of the eye. Progenitors remain in an undifferentiated and proliferative state as long as they maintain *hth* expression. Thus, forced maintenance of *hth,* particularly in combination with *tsh,* causes tumor-like overgrowths of progenitor cells; whereas, loss of *hth* results in reduced cell proliferation and viability, and RNAi-*mediated hth* and *tsh* knock-downs result in a reduction of eye size (Pichaud and Casares 2000; Bessa et al. 2002; Bessa and Casares 2005; Peng et al. 2009; Lopes and Casares 2010). While we do not have a clear idea yet of what "undifferentiated" means in molecular terms (i.e. what genes are under direct Hth:Tsh:Ey control), Hth and Tsh are known to control proliferation via their interaction with Yki (Figs. 4.3 and 4.4). Hth (and its partner, the TF Exd), Tsh and Yki form a protein complex that regulates the transcription of *bantam (ban)*, a microRNA-encoding gene. The notion here, is that Hth:Tsh:Yki likely stimulate the proliferation and survival of progenitors through *ban* (Peng et al. 2009).

4.6 From Progenitors to Precursors: A Size-Balancing Act

The onset of retinal differentiation starts around the transition from L2 to L3. The onset of differentiation is presaged by the transition of progenitor cells into precursor cells. The precursor cell state is characterized by the loss of Hth expression

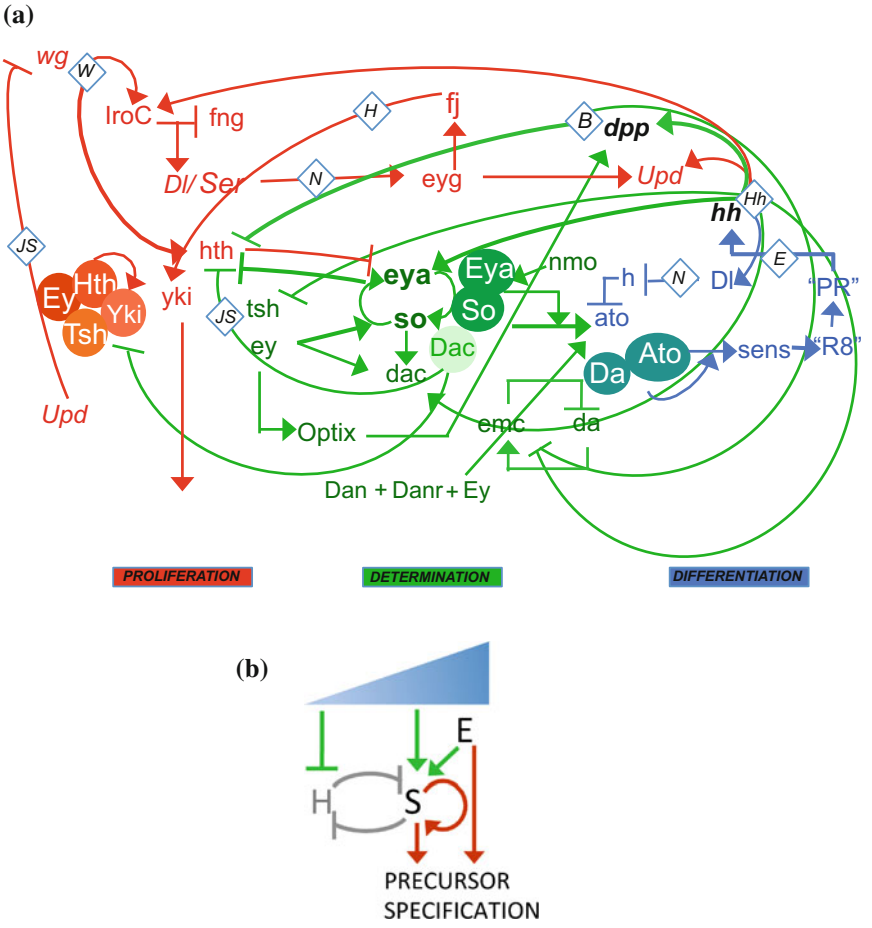

Fig. 4.4 Gene Network and network's logic. **a** Main elements of the early eye GRN. Genes (nodes) have been classified as involved in either proliferation (*red*), determination (*green*) or differentiation (*blue*), although this classification is not strict (as some factors are implicated in several of these processes). Key factors are: *Ey*, *Hth*, *Tsh*, *Yki* (proliferation), *Eya* and *So* (determination) and *Da* and *Ato* (differentiation). *Dpp* and *Hh* (*black*) contribute to all the stages. Main signaling pathways are represented by diamonds (*W* Wingless, *JS* JAK/STAT, *N* Notch, *H* Hippo, *B* BMP/Dpp, *Hh* Hedgehog, *E* EGFR). *Arrows* indicate activating links; T-ended links represent repression. Protein products are represented by *circles*. **b** General regulatory logic behind the specification of eye precursors. Signals (triangle: Hh + Dpp) contribute to specifying eye precursors in two ways: first, by cooperating with Pax6 genes (E: Ey + Toy) in activating So and Eya (S) genes and by clearing the repressor Hth (H), thus coordinating in time and space precursor specification. Next, S expression is locked-in through an autoregulatory loop. Precursor specification is further stabilized by E

(Bessa et al. 2002), the synchronous exit of the cell cycle (Escudero and Freeman 2007; Lopes and Casares 2010) through the FMW, and the upregulation of a number of transcription factors, including the retinal determination genes *eyes absent (eya), sine oculis (so), optix* and *dachshund (dac).*

The precise developmental time that triggers the onset of differentiation is linked to the action of two signaling centers within the eye disc that define the anterior/posterior (AP) and dorsal/ventral (DV) axes of the eye primordium. Both depend on the localized expression of *wingless (wg),* the *Drosophila* Wnt-1 homologue (Lee and Treisman 2001). In early L2 discs, the dorsal/anterior margin expresses *wg* (Baker 1988), while the posterior/ventral margin expresses *hedgehog* (*hh*) (Heberlein et al. 1993; Ma et al. 1993). *hh,* in turn, activates the transcription of *decapentaplegic (dpp),* a BMP2/4 molecule (Heberlein et al. 1993; Ma et al. 1993; Borod and Heberlein 1998). This subdivision depends on the disc's margin, marked by the differential expression of several transcription factors: *otd/ocelliless* in the anterior/dorsal margin (Royet and Finkelstein 1996) and the joint expression of the *odd-skipped* family Zinc-finger TFs (*odd, drm* and *sob* along the posterior/ventral margin (Bras-Pereira et al. 2006). *wg* and *dpp* play antagonistic roles, with *dpp* promoting and *wg* repressing retinal differentiation (Figs. 4.2 and 4.4; Ma and Moses 1995; Treisman and Rubin 1995; Chanut and Heberlein 1997; Royet and Finkelstein 1997). During early L2, the eye disc is small and the notion is that all eye progenitor cells receive enough Wg to counteract the pro-retinal action of Dpp (Lee and Treisman 2001; Kenyon et al. 2003). However, towards the end of L2, the disc has grown by Notch signaling-induced proliferation (see below, Kenyon et al. 2003), causing the separation of the anterior/dorsal Wg signaling center from the most posterior region producing Hh and Dpp. These posterior cells, now under the dominating influence of Dpp, would be the first ones to become retinal precursors and, thereby, the first to initiate differentiation.

The Notch-driven proliferative thrust happening during L2 starts also with *wg.* Dorsal expression of *wg* initiates, together with *hh,* the expression of the TALE-homeodomain TFs of the *Iroquois* complex (Iro-C): *araucan (ara), caupolican (caup)* and *mirror (mirr)* (Heberlein et al. 1998; Cavodeassi et al. 1999; Yang et al. 1999). The expression of the Iro-C genes is restricted to the dorsal region by the repressive action of the JAK/STAT signaling pathway, activated by the transient, ventral-specific expression of its ligand Unpaired (Upd; the *upd* gene is also known as *outstretched*, *os*) (Gutierrez-Aviño et al. 2009). The ventral repression of iroC is maintained after the early ventral expression of Upd has disappeared by epigenetic silencing (Netter et al. 1998). Then, the dorsal-specific iroC TFs repress *fringe (fng),* a glycosyl-transferase that modifies Notch affinity for its ligands *Delta (Dl)* and *Serrate (Ser).* This, together with the asymmetric distribution of Dl and Ser along the DV axis, results in *Notch* signaling activation *only* across the DV *fng-/fng* + border, called "equator" (Cho and Choi 1998; Dominguez and de Celis 1998; Papayannopoulos et al. 1998; Yang et al. 1999). Modulation of Notch signaling through the regulation of its ligands is further exerted by *Lobe* (*Lb*) (Singh and Choi 2003) and the fork-head TF paralogues Slp1 and Slp2 (Sato and Tomlinson 2007). In turn, Notch activates the transcription of the Pax6(5a) type

gene *eyegone* (*eyg*) (and presumably of its paralogue *twin of eyegone* (*toe*) too) in a wedge straddling the DV boundary (Jang et al. 2003; Dominguez et al. 2004; Yao et al. 2008). This *Notch/eyg* interaction is translated into progenitor proliferation through, at least, two mechanisms. First, *Notch/eyg* would act through the transcriptional activation of the Golgi transmembrane type II glycoprotein *four-jointed* (*fj*). Thereby, *fj* would be expressed in a gradient, with its maximum straddling the equator (where Notch signaling is activated and *eyg* expression driven) and decreasing toward the dorsal and ventral poles of the disc (Gutierrez-Aviño et al. 2009). The proto-cadherin *dachsous* (*ds*) is expressed in an opposing expression gradient to *fj* (i.e. with *increasing* expression towards the poles) (Yang et al. 2002). Interestingly, in the wing primordium, the juxtaposition of cells with different levels of *fj* and *ds* leads to the activation of another proto-cadherin, *fat* and the regulation of the Hippo growth control pathway (Rogulja et al. 2008), suggesting that a similar mechanism of growth control could be operating during eye development. *Notch* signaling activity is modulated by the apical determinant *crumbs* (*crb*) and so is the proliferation rate of progenitors. In *crb* mutant cells, there is increased endocytosis of *Notch* and its ligand *Dl* and a concomitant enhancement of *Notch* signaling. As a consequence, *crb* mutant eyes are larger than normal (Richardson and Pichaud 2010). The second mechanism by which the *Notch* → *eyg* link regulates proliferation is through the ligand of the JAK/STAT pathway, Upd. After its early ventral phase of expression, *upd* is induced by the end of L2 specifically at the intersection of the *eyg* domain with the posterior margin, expressing *hh,* in a small region (Bach et al. 2003, 2007; Chao et al. 2004; Tsai and Sun 2004; Reynolds-Kenneally and Mlodzik 2005). This "singularity" is called *the firing point*, as it represents the origin of the retinal differentiation process (Fig. 4.2c). Upd produced at the firing point increases the proliferation of progenitors (Bach et al. 2003; Tsai and Sun 2004; Flaherty et al. 2009, 2010). The expression of *upd* at the firing point is transient: as soon as retinal differentiation starts, Upd fades, so that the effect of *upd* expression at the firing point may be to cause a proliferation burst. Upd levels are instrumental in controlling the final size of the eye. In os^{1} mutants, which lack the transient Upd pulse, the eyes are smaller than wild type, while increasing Upd levels cause overgrown eyes. Still the differently sized eyes produced by modifying Upd levels are normally patterned (Bach et al. 2003). Interestingly, Upd and the JAK/STAT signaling feeds back on *wg* repressing its expression also at these late stages (Tsai and Sun 2004; Ekas et al. 2006) to favor initiation of retinal differentiation, closing a complicated circle of regulatory interactions (Fig. 4.2).

Mechanistically, the two key processes—Wg/Dpp antagonism and Notch-induced proliferation—are known to different degrees. Wg acts by repressing *dpp* transcription but also Dpp signaling (Wiersdorff et al. 1996; Hazelett et al. 1998). Part of Wg's action might be mediated by Hth, a *wg's* target (Pichaud and Casares 2000). Forced maintenance of Hth delays differentiation (Pai et al. 1998; Pichaud and Casares 2000), while loss of Hth in progenitors results in their premature differentiation (Pai et al. 1998; Pichaud and Casares 2000; Bessa et al. 2002). Interestingly, Dpp is a major Hth repressor (Bessa et al. 2002; Firth and Baker 2009; Lopes and Casares 2010). Hence, the eye primordium has to grow

beyond a critical size to permit Dpp to repress *hth*, thus allowing the transit from hth + progenitors to hth- precursors (Fig. 4.2d). In addition, *wg* limits dorsally the extent of the eye disc margin with capacity to trigger retinal differentiation, by repressing *hh* and *dpp* transcription along this margin. *wg* might be doing this indirectly, through the repression of *drm/odd/sob,* which are necessary for *hh* expression along the margin (Bras-Pereira et al. 2006). Thus, reduction of *wg* function in *wg* hypomorphic mutants results in an anterior/dorsal extension of retinal differentiation, premature exhaustion of progenitors and, globally, smaller eyes (Treisman and Rubin 1995). However, as the head capsule also depends on *wg* function, loss of *wg* also compromises the development of the head capsule surrounding the eye.

4.7 Transiting from Progenitors to Precursors and the Onset of MF Movement

By the end of L2, the separation of the Wg and Dpp sources would allow Dpp to repress *hth* in the posterior half of the eye primordium, recruiting the first precursor cells out of their proliferative, undifferentiated progenitor state. Concomitant with this repression, there is a simultaneous increase in levels of the retinal determination (RD) genes *eya, so, dac* and *optix* and of the cdc25 phosphatase *string* (*stg*). *stg* expression forces cells to undergo mitosis as they lose *hth*, resulting in a synchronized entry into G1 (Mozer and Easwarachandran 1999; Escudero and Freeman 2007; Lopes and Casares 2010, 2015). Therefore, precursor cells maintain *toy, ey* and *tsh* expression, gain Eya, So, Dac and Optix and enter G1 in preparation for their further differentiation. Activation of Eya and So is particularly important. So is a Six1/2 type homeodomain TF. Eya is a transcriptional activator without any known DNA binding domain. So and Eya form a protein complex, in which So provides the DNA binding domain and Eya acts as a transactivator (Pignoni et al. 1997). Mutants lacking either *eya* or *so* function in the developing eye are eye-less (see review by Silver and Rebay (2005)). The Eya/So activity is, in addition, modulated. The Nemo (Nmo) Ser/Thr-kinase directly phosphorylates Eya, stimulating its transactivating action on So which enhances the eye-specifying function of the complex (Braid and Verheyen 2008; Morillo et al. 2012). The antagonistic regulatory interactions between Hth and Eya, So and Dac (Bessa et al. 2002; Lopes and Casares 2010), together with the positive feedback between Eya, So and Dac (Chen et al. 1997; Pignoni et al. 1997) explains why, once Hth is repressed, the precursor program sets in irreversibly. Precursor cells are primed to differentiate, but do not do so immediately, as they also express high levels of Hairy (H) (Brown et al. 1995) a transcriptional repressor of the bHLH (basic Helix-Loop-Helix) proneural gene *ato*. Like *eya* or *dac,* the expression of H is activated by Dpp (Greenwood and Struhl 1999) and limited anteriorly by Hth (Bessa et al. 2002). Closer to the Hh source, Hh induces Dl to activate Notch signaling which, in turn,

represses H allowing initiation of *ato* transcription (Baonza and Freeman 2001; Fu and Baker 2003). Transcriptional activation of *ato* is carried out by Ey, So and the Dpp pathway (Sun et al. 1998; Niwa et al. 2004; Zhang et al. 2006; Tanaka-Matakatsu and Du 2008). Ato function is further regulated through dimerization with E and Id-type proteins. Thus, Ato is activated by binding to the E protein Daughterless (Da), while Da itself is sequestered by the Id protein Extramacrochaete (Emc). While Da activates Emc expression, Emc represses Da, with the net result of no Da availability. It is again the Hh signaling center, providing Hh and its relay signal Dpp, that represses Emc locally, allowing the upregulation of Da and functional activation of Ato (Bhattacharya and Baker 2011). At this point, cells expressing Ato/Da dimers close to the Hh/Dpp signaling cells initiate a cascade of events that results in the specification of spaced R8 cells, followed by the further induction and recruitment of the remaining retinal cell types.

4.8 Making the Wave Move: Again a Role for Hh and Dpp

The mechanisms described up to now would give rise to a very small eye: if the Hh/Dpp signaling center were static, only the cells closest to the posterior margin (where Hh and Dpp are initially produced) would undergo this whole cascade of regulatory events to start differentiation. This is not the case because the signaling center becomes motile due to differentiating PRs (except R8) expressing Hh (Rogers et al. 2005). By doing so, the PRs induce Dpp which, acting at a longer range, recruits progenitor cells into new precursors to differentiate into Hh-producing PRs. This process establishes a feedforward loop (Hh → Dpp → → PR → Hh…) that spreads the differentiation process as a forward-moving wave. In hh^{bar3} mutants, which lack the PR-specific *hh* enhancer, eyes are comprised of only 6–10 ommatidial rows, as compared to the 28–30 rows of normal eyes (Rogers et al. 2005). Therefore, a large eye requires a moving differentiation wave. In addition to Hh and Dpp, MF movement requires the action of the EGFR pathway as the MF is constantly reinitiated along the eye disc margins as it travels across the disc (Kumar and Moses 2001). The epithelial cells at the wave front experience an apical constriction, contract in the apico-basal axis and their nuclei move basally, so that the morphogenetic furrow, MF, forms. Since the final eye size depends on how fast this differentiation wave progresses (all other things being equal, the faster the wave, the smaller the eye), the mechanism controlling MF formation need also to be integrated in the gene network. Again, the process is driven jointly by Dpp and Hh. The loss of either Dpp or Hh signaling alone results in delayed MF, and only when *both* signaling pathways are blocked the MF stalls (Greenwood and Struhl 1999; Fu and Baker 2003; Vrailas and Moses 2006). As mentioned above, precursors express the Six3-type TF *optix*. While ectopic Optix expression in the antenna and wing discs induces ectopic eyes (Seimiya and Gehring 2000; Salzer and Kumar 2010), possibly in an Ey-independent manner (Seimiya and Gehring 2000), its role during

normal eye development seems more related to progression of differentiation than to specification (Li et al. 2013). Thus, *optix* mutant cells lose *dpp* expression at the moving MF, thereby delaying differentiation progression. *optix* is not expressed in the embryonic primordium of the eye disc (Seimiya and Gehring 2000; Dominguez-Cejudo and Casares 2015) but is activated anew during eye disc development by Eya, So (Li et al. 2013) and probaly Ey (Ostrin et al. 2006). According to their distinct function, the two Six proteins, So and Optix, partner up with specific cofactors, including the exclusive use of Eya by So as partner (Seimiya and Gehring 2000; Kenyon et al. 2005a, b; Anderson et al. 2012).

As part of the mechanism that makes the differentiation wave move, Dpp and Hh also control the tissue changes that cause the furrowing of the disc's epithelium by promoting the localized accumulation of non-muscle Myosin II (Corrigall et al. 2007; Escudero et al. 2007). This "furrowed" state is transient, though, and once the furrow has passed, Hh signaling is attenuated. This signaling attenuation is caused by the regulated degradation of the activator form of Ci (Ci155), the nuclear transducer of the Hh pathway. This is carried out by the BTB protein *roadkill (Rdx)* which is induced in differentiating PRs by their production of Hh and EGF ligands. Rdx couples Ci to Cullin-3 to mediate Ci's proteasomal degradation, thus extinguishing Hh signal posterior to the MF (Baker et al. 2009). The reason why the differentiation process is linked to tissue morphological changes is not totally clear. However, abrogating MF formation by altering the actin cytoskeleton causes abnormal differentiation (Benlali et al. 2000). In any case, one of the RD genes, *dac,* seems to have a major role in MF movement. When Dac function is removed from posterior margin cells, MF initiation does not occur. Once the MF is moving, it can traverse a patch of *dac*-mutant cells but does so more slowly. Still *dac*-mutant cells differentiate (Mardon et al. 1994). These results link the RD genes (*dac* is activated by Eya and So, see below) and tissue morphogenesis. However, the mechanism by which Dac controls MF movement is unknown. In addition, MF movement is coupled to the ecdysone pathway, the hormonal system that regulates developmental timing and metamorphosis, although the exact cellular mechanisms through which the ecdysone pathway affects MF dynamics are not clear yet (Brennan et al. 1998, 2001).

As the MF moves, not only PRs differentiate in its wake, but the expression of Ey and Tsh is turned off by MF signals (Firth and Baker 2009; Atkins et al. 2013). Otherwise, the persistence of Ey (or Tsh) impairs retinal differentiation (Atkins et al. 2013). In contrast, the expression of Eya and So continues in differentiating PRs and other cells behind the MF (Bonini et al. 1993; Cheyette et al. 1994), whereas that of *dac* continues in the region just posterior to the MF but eventually fades away completely in more differentiated cells (Mardon et al. 1994; Bras-Pereira et al. 2015). Eya expression in differentiating retinal cells is required for the normal differentiation of cone and pigment cell development, perhaps also associated to So (Karandikar et al. 2014). In this work, Karandikar make another interesting observation: *eya*'s expression anterior and posterior to the MF is controlled by two different enhancers (called IAM and PSE, respectively). Therefore, what appears as seamless continuous expression across the MF, at mRNA or

protein levels, masks, in fact, a regulatory switch, reflecting two distinct states hinging around the MF: the precursor state, anterior, and the differentiating state, posterior. Interestingly, a similar CRE organization has been described for *ato* (Sun et al. 1998; Niwa et al. 2004; Zhang et al. 2006; Tanaka-Matakatsu and Du 2008) and for *stg* (Lopes and Casares 2015), together strengthening the idea of an abrupt regulatory state switch driven by the passing MF.

4.9 Controlling Proliferation During the Differentiation Phase

Retinal differentiation progresses in the wake of the MF at the expense of proliferating progenitors. The cell cycle of these progenitors is characterized by a long G2 phase, relative to G1 and S/mitosis (Fig. 4.3; Lopes and Casares 2010). As we mentioned before, progenitor's proliferation requires Yki, the *Drosophila* YAP/TAZ homologue and co-transcriptional activator of the Hippo signaling pathway. Yki, which lacks a DNA binding domain, depends on partner TFs to regulate transcription. In the developing eye, these partners are Hth, which is specifically expressed and required in progenitors, and Tsh (Peng et al. 2009). The complex also includes the TALE-homeodomain PBX-type protein Extradenticle (Exd), which is an obligatory partner for Hth (Rieckhof et al. 1997), and very likely Ey as well, as Ey, Hth and Tsh have been shown to be able to form a protein complex in vivo (Bessa et al. 2002). Of the known targets of the Hippo/Yki pathway, the microRNA *ban* seems to mediate the proliferative (and anti-apoptotic) action of the Yki-Hth-Tsh complex (Peng et al. 2009). As the MF advances, Dpp produced at the MF reaches anteriorly and represses Hth. This repression is progressive and during the transition period two events participate in the control of the cell cycle. The first one is the sharp upregulation of *stg* expression. As described above, this burst of the *Drosophila* cdc25 phosphatase drives all cells in G2 into mitosis and G1. As most progenitor cells spend most of their cell cycle in G2, *stg*-driven mitoses occur almost synchronously and are visualized as the FMW. Therefore, the G1 zone that results is the product of a synchronization, rather than an arrest. Still, the G1 state is maintained closer to the MF by *dacapo,* the p21/p27 homologue, induced by Hh (de Nooij et al. 1996; Lane et al. 1996; Duman-Scheel et al. 2002) and the cyclin-dependent kinase inhibitor (CKI) *roughex* (Thomas et al. 1994, 1997). The second event related to proliferation is the upregulation of *dac* transcription as Hth expression decays. Dac-mutant clones proliferate faster than wild type ones, and this is a consequence of Dac repressing the Hth-Yki-mediated proliferation. In addition, Dac and Hth repress each other's transcription. These interactions likely occur in the transition domain between progenitors and precursors, where low levels of both Hth and Dac transiently coexist. This mutual antagonism ensures a clear separation between the proliferation regimes of progenitors and precursors, with progenitors engaged in active proliferation and

precursors securely synchronized in G1 (Bras-Pereira et al. 2015). This G1 synchronization is necessary for normal retinogenesis. In the *string* mutant allele *stgHwy,* in which the burst of *stg* at the FMW is lost, precursor cells keep cycling. The resulting *stgHwy* eyes show patterning defects (Mozer and Easwarachandran 1999). All these intrinsic mechanisms of growth control are also coupled with the global regulation of the animal's growth, ensuring that the growth of organs and that of the whole individual are in synchrony. In insects, the levels of the steroid hormone ecdysone regulate the major developmental transitions of the individual, including the larval molts and metamorphosis. Recent work shows that the ecdysone pathway is a global regulator of disc growth during L3. Ecdysone would increase the activity of the insulin/insulin-like signaling pathway (which is a major growth regulator (Mirth and Shingleton 2012) by repressing Thor/4E-BP, a growth repressor downstream of the insulin and Tor pathways (Herboso et al. 2015). Specifically in the eye, additional effects of the ecdysone pathway on MF progression (described above) maybe necessary to coordinate differentiation speed and growth rates.

4.10 Finishing Up: Attaining a Final Size

Retinal precursor cell recruitment ends when the MF having reached the anterior-most edge of the eye primordium exhausts the progenitor pool. This is suggested by the correspondingly smaller and larger size of eyes from undergrown or overgrown eye discs. Although this fact—finishing the recruitment of progenitors—may seem trivial, it requires precise coordination of a number of processes. For example, an imbalance in proliferation and differentiation (were progenitor proliferation too fast or MF advancement too slow) would cause a failure to arrest with presumably catastrophic consequences for head formation. It would be basically impossible for the morphogenetic furrow to differentiate all progenitors. Also, the shape of the primordium might have a critical role in determining the time to differentiation termination. Imagine two primordia of identical size, but one circular, the other very oblong and elongated along the DV axis. For the same progenitor proliferation rate and same MF speed, the primordium with the very elongated shape would complete differentiation earlier, resulting in an eye with fewer ommatidia. A comprehensive study of the potential factors affecting final eye size through the morphogenetic process is lacking, but work by Wartlick et al. (2014) suggests that dedicated mechanisms may be in place to control it. Studying the dynamics of growth and differentiation of the eye, they observed that the progenitor proliferation rate decreases exponentially with developmental time (something that may be required for consistent differentiation termination). A number of experiments had indicated that Dpp has a role in proliferation control in the eye (Penton et al. 1997; Horsfield et al. 1998; Firth and Baker 2005). Wartlick et al. (2014) found that the dynamic changes in the Dpp signaling gradient, as the MF moves, could explain the slowing down of progenitor proliferation if progenitor cells underwent division only

after "sensing" a fixed relative increase in Dpp signaling. This model was supported by previous work indicating that the same mechanism might be controlling the proliferation rates of wing disc cells (Wartlick et al. 2011). Nonetheless, proliferation, though affected in Dpp pathway mutants, is not halted completely and the proliferation profiles are still maintained to some extent. These results indicate that sensing Dpp signaling dynamics cannot be the only mechanism regulating the cells' proliferation slowdown. In addition, as we have reviewed above, the effects of Dpp signaling may not be direct, but mediated by a number of regulated events (e.g. Hth repression, *stg* upregulation) with complicated feedbacks whose effects may obscure the relation between Dpp and proliferation control. Ultimately, the eye reaches a final size that shows little variation within and between individuals. Whatever the mechanisms that explains the termination of neurogenesis, they must also explain the robustness of the process.

4.11 Molecular Regulatory Logic Through the Eyes of Some Enhancer Regions

Up to this point we have reviewed the regulatory interactions from genetic and phenomenological points of view. To gain a deeper molecular insight, a number of works have investigated the regulatory interactions happening at the *cis*-regulatory elements (CREs; basically enhancers) of relevant genes, as these CREs act as integrating nodes in regulatory networks. It is somehow surprising that, despite the dense network of regulatory interactions knitting the eye network, the characterization of these nodes is sparse. Until recently, the identification of these CREs had been generally guided by the prior mapping of regulatory mutations affecting eye development. Eye-specific CREs have been molecularly characterized to different degrees for *ey* (Hauck et al. 1999), *eya* (Bui et al. 2000; Karandikar et al. 2014), *so* (Niimi et al. 1999; Punzo et al. 2002), *dac* (Pappu et al. 2005), *optix* (Ostrin et al. 2006), *ato* (Sun et al. 1998; Zhang et al. 2006; Tanaka-Matakatsu and Du 2008; Zhou et al. 2014), *hh* (Pauli et al. 2005; Rogers et al. 2005), *dpp* (Blackman et al. 1991), *wg* (Pereira et al. 2006), *da* (Bhattacharya and Baker 2011), *eyg* (Wang et al. 2008) and *stg* (Lopes and Casares 2015). Figure 4.3c represents the common positive feed-forward regulatory logic governing precursor gene activation, extracted from the regulatory interactions controlling the activation of *dac, stg* and the first phase of *ato* expression, as examples of this logic. Still, the molecular structure of the CREs involved varies: from the single enhancer of *stg,* through the bipartite enhancer that activates *ato* to two distinct and separate enhancers for *dac.*

A comprehensive diagrammatic representation of the GRN is shown in Fig. 4.4a. At the core of this network lay the partner genes So and Eya. Not only these transcription factors seem to be in charge of retinal specification, but they also simultaneously stabilize eye fate by avoiding the spurious activation within the eye field of antennal and head capsule specification (Roignant et al. 2010; Weasner and Kumar 2013).

4.12 Looking Inside: Molecular Characterization of the Process and Its Network Extensions

It is likely that the transcription factors and signaling molecules *driving* the transit from eye progenitors to *ato*-expressing precursor have been identified. The genetic (and sometimes, molecular) linkages between some of them, defined as control of enhancer activity by direct TF binding to CREs, have also been defined. However, there are still two important gaps between the general phenomenon and the molecular and cellular details. First, CRE sequence conservation beyond the few BS motifs known to date strongly suggests that there must be other sequence-specific DNA-binding proteins involved, in addition to the characterized retinal determination TFs. Second, what is downstream the GRN backbone? Answering this question requires a description of the global expression changes the cells experience along their differentiation journey. Thus, it is important to define their biological properties at each developmental time-point, to identify the links connecting these targets to the backbone, to find how these properties (target gene functions and connectivity) constrain the dynamics of the network and which are the mechanisms that confer robustness to the process. Two initial attempts to identify *ey* targets were carried out by Michaut and coworkers and by Ostrin and collaborators, using gene expression profiling (Michaut et al. 2003; Ostrin et al. 2006). Although both experiments yielded transcriptional profiles of limited overlap, they included genes with varied functions, suggesting that Ey would control many aspects of the cell's biology. New computational methods combine transcriptomics, motif discovery and epigenomic profiling to knit much more complete GRNs, capable to predict direct links between TFs and cognate CREs with ever increasing predictive power (Aerts et al. 2010; Naval-Sanchez et al. 2013; Potier et al. 2014). The massive identification of CREs was initially based on histone profiling using chromatin immunoprecipitation followed by sequencing (ChIP-seq). More recently, FAIRE-seq (Giresi et al. 2007) and especially ATAC-seq (Buenrostro et al. 2013) are making affordable the profiling of open chromatin (a good correlate of active CREs) with fast protocols that require modest amounts of *Drosophila* tissue (Davie et al. 2015). These methods have been applied to derive gene networks involved mostly in retinal differentiation but similar studies need to be carried out on earlier stages. Furthermore, methods are still to be developed to determine to what extent the network models not only highlight gene targets and molecular and biological functions, but also the dynamics of the network—that is, whether feeding the model an initial state, the network will progress through successive intermediate states until reaching the target state. One major stepping-stone towards this goal will be to generate genome-wide DNA-binding maps for most key TFs in the network to identify bona-fide, in vivo bound CREs.

4.13 Perspectives

The study of *Drosophila* eye development is yielding one of the most complete pictures of an organogenetic GRN. Already equipped with a very powerful technical toolbox, *Drosophila* research is ever adapting to the latest technology often serving to benchmark them—so this research will be quickly furthering our understanding of this network. What are the next frontiers?

Perhaps surprisingly, one of the most interesting questions still standing is the exact roles played by the Pax6 gene *ey*. Neither *ey* nor *toy,* alone or jointly, seem absolutely required for eye specification and differentiation. Although the association between Pax6 and eyes is widespread, it is not universal. In *Drosophila,* the larval eye, the small Bolwig's organ, does not express nor requires the fly Pax6 genes, *Toy* or *ey* (Daniel et al. 1999; Suzuki and Saigo 2000), and studies in representative species of chelicerates (Schomburg et al. 2015), planarians (Martin-Duran et al. 2012), polychaete annelids (Arendt et al. 2002) or scyphozoan cnidarians (Nakanishi et al. 2015) show that Pax6 genes are not expressed during the development of their eyes. Still, in *Drosophila, ey* is the most powerful retinal determination gene, in inducing ectopic eyes, both in terms of size as well as in the number of locations. *ey*-induced eyes are large, while *ey* mutant eyes are reduced in size, albeit this reduction is variable. Therefore, large size and Ey seem related, but it is not clear how. One possibility is that the Ey expression domain defines the eye-competence territory, by inducing the expression of Eya/So. Thereby, the larger the domain, the larger the eye. This is certainly not the only thing that Ey does, as Eya plus So generate smaller eyes than Ey does in ectopic expression assays (Halder et al. 1995; Bonini et al. 1997; Pignoni et al. 1997; Weasner et al. 2007). The ectodermal locations susceptible to *ey*-induced transformation are very specific (Niwa et al. 2004; Salzer and Kumar 2008)—called "transformation hotspots" (Salzer and Kumar 2008). These hotspots coincide geographically with the so-called "transdetermination weak point", locations in the discs prone to switch their organ identity when disc fragments are transplanted for long periods into the abdomen of host females, or when exposed to Wg during development (Schubiger 1971; Sustar and Schubiger 2005; Schubiger et al. 2010). The cells at these weak points may be especially plastic. In a "Waddingtonian landscape" view (Waddington 1957), these cells might have several developmental trajectories (or "creodes") almost equally accessible, at least transiently, with Wg signaling increasing their indeterminacy. In this context, Ey might render more accessible the eye trajectory—perhaps repressing the non-eye creodes (see also Salzer and Kumar 2010), rather than activating the eye program. In fact, expression of antennal determinants is occasionally derepressed in *ey* mutant cells (Punzo et al. 2004). Larger eye sizes can also be achieved by stimulating progenitor proliferation and by delaying the onset of eye differentiation (thus providing for an extended proliferative period). In any case, the developmental window for Ey's action seems to be early, because the simultaneous attenuation of Ey and Toy (with RNAi) to undetectable levels during L2 in cell clones does not result in severe eye developmental

defects (Lopes and Casares 2010). In any case, a better understanding of the function of this conserved family of TFs will require the characterization of the full set of its direct targets and their further functional characterization along eye development.

Related to the ability of Ey to facilitate the development of large eyes, understanding the regulation of Tsh and its function, in molecular detail, is key. The definition of the eye field depends on differential gene expression of Tsh in one of the two disc layers, the one becoming the columnar main epithelium. The mechanism regulating Tsh is thus involved in establishing/limiting eye competence. The capacity of Tsh to respecify the squamous peripodial epithelium and to change cell morphology into cuboidal hints at a relationship between cell morphology and fate specification. The fact that there is a very limited knowledge on the function of Tsh and the identity of its targets hinders progress in this direction.

If Pax6 genes favor eye competence and help producing large eyes, the partners Eya and So seem to be the actual eye selectors. If this is indeed the case, again, to translate "eye" in molecular terms, the full complement of Eya + So targets needs to be identified. This collection of target genes may contain the minimal set of genes required to specify a "generalized eye". Testing this hypothesis is becoming increasingly feasible by extending the application of new technologies to a larger range of organisms at key phylogenetic positions.

Another aspect of the network that is poorly understood molecularly is the integration of Dpp and Hh pathways. Both pathways are partially redundant in *hth* regulation and cell cycle control as well as in triggering the epithelial changes that generate the morphogenetic furrow. Yet the Dpp and Hh pathways are very little connected—if at all. How come that their functions are redundant?

The network's backbone is a positive feed-forward loop with an autoregulation (between Eya and So), a motif that generally ensures a consistent output (Guantes and Poyatos 2008). This, on its own, justifies the very consistent final output of the developmental system: the tight activation of *ato*. However, up to date, all the analyses have been generally carried out over the *average* of the cells, as if there were no intercellular variation (either mean profiles of a single gene's expression or average transcriptomic profiles). However, biological processes are intrinsically variable. What the degree of variability is, to what extent mechanisms to minimize this intrinsic noise are built-in within the network (and which are their components), or whether noise is also fueling some of the transitions, are questions that can only be addressed through single-cell level of analysis. With such descriptions, a given cell "state" will no longer be a vector comprising mean gene/protein expressions, but rather vectors of probability distributions. The challenge for GRNs will be to take a leap from describing linkages and defining simple regulatory motifs to become predictive and analytic tools for some sort of "biological statistical mechanics" (Garcia-Ojalvo and Martinez Arias 2012).

In addition to gene regulatory motifs, gene expression is stabilized through epigenetic modifications. In fact, mutations that affect components of the

chromatin-modifying Polycomb and Trithorax complexes derail early eye development (Janody et al. 2004). However, in the case of the eye, while on the one hand the transitional states must be stable to ensure robust eye development, they ought to be also flexible to allow fast transitions. The specific role of chromatin modifiers has still to be integrated with the action of more "conventional" TFs.

In addition, the eye GRN is highly dynamic and contingent—i.e. each step is dependent upon the previous ones. We have presented here just a window through this dynamics. However, the challenge is to knit the GRN starting at the inception of the eye primordium in the embryo through to the differentiation of PRs and other cell types. The early larval stages are poorly characterized and it is a working assumption that L1/L2 cells are very much like the anterior progenitors in L3, but it may be a mistake to assume that the logic in L3 (in the progenitor field) faithfully reflects the earlier stages. Recent efforts at defining the GRN downstream of *ato* are seeing great progress. However, there is a bridge to be built between the events happening anterior to the MF (reviewed here) and posterior to it.

The eye determination GRN works in a growing tissue with precisely defined shape, that includes a constriction of the whole disc marking the separation between antenna and eye, different cellular morphotypes, furrows and folds, all potential causes or consequences of differential tensions. Whether physical forces are to be included in models regulating the growth and differentiation of the eye, and how these mechanical parameters should eventually be integrated in the gene network are questions that need to be studied.

The *Drosophila* eye is an organ of exemplar constancy. However, the size (and shape) of eyes across diptera is remarkably variable. It is very likely that these changes have occurred by introducing developmental variations, which in one way or another, must be connected with the early eye gene network—e.g. by varying the speed at which the MF travels, or altering proliferation rates of progenitors. Finding out these changes and their genetic, cellular, molecular and/or physical bases may throw light on the understudied problem of how organ size varies during evolution. Looking beyond diptera, comparative studies based on *Drosophila* research should identify genetic kernels, common to most insects (and beyond), as well as evolutionary variations generating morphologically and functionally diverse eyes.

The works reviewed in this chapter set strong foundations for continuing efforts in *Drosophila* to tackle all these fascinating questions, and more.

Acknowledgments Recent work in the Casares lab related to the subject of this review has been partly funded through grants BFU2009-07044 and BFU2012-34324 from the Spanish Ministry of Science and Innovation/MINECO. We specially thank S. Aerts (KU, Leuven), M. Friedrich (Wayne State Univ., Detroit), F. Pichaud (UCL, London) and F. Pignoni (Upstate Medical Univ., Syracuse) for their critical comments. IA has been supported through "Programa de Fortalecimiento" of Pablo de Olavide University and a MSC postdoctoral contract from the EU H2020 Program.

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