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The neuronal transcription factor Erect wing regulates specification and maintenance of *Drosophila* R8 photoreceptor subtypes

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Abstract

Signaling pathways are often re-used during development in surprisingly different ways. The Hippo tumor suppressor pathway is best understood for its role in the control of growth. The Hippo pathway is also used in a very different context, in the *Drosophila* eye for the robust specification of R8 photoreceptor neuron subtypes, which complete their terminal differentiation by expressing light-sensing Rhodopsin (Rh) proteins. A double negative feedback loop between the Warts kinase of the Hippo pathway and the PH-domain growth regulator Melted regulates the choice between `pale' R8 (pR8) fate defined by Rh5 expression and `yellow' R8 (yR8) fate characterized by Rh6 expression. Here, we show that the gene encoding the homologue of human Nuclear respiratory factor 1, *erect wing* (*ewg*), is autonomously required to repress *warts* and to promote *melted* expression to specify pR8 subtype fate and induce Rh5 expression. *ewg* mutants express Rh6 in most R8s due to ectopic *warts* expression. Further, *ewg* is continuously required to maintain repression of Rh6 in pR8s in aging flies. Our work shows that Ewg is a critical factor for the stable down-regulation of Hippo pathway activity to determine neuronal subtype fates. Neural-enriched factors, such as Ewg, may generally contribute to the contextual re-use of signaling pathways in post-mitotic neurons.

Introduction

The Hippo signaling pathway controls growth through the regulation of cell proliferation and apoptosis (Pan, 2010). Warts is the effector kinase of the Hippo tumor suppressor pathway and, along with Hippo, Salvador, and Mats, forms the core of the Hippo pathway that coordinates proliferation and apoptosis in developing tissues (Halder and Johnson, 2011; Pan, 2010; Zhao et al., 2011). Other than its function in growth, the Hippo pathway also regulates non-growth processes, such as follicle cell maturation in the fly oocyte (Polesello and Tapon, 2007) and the establishment of dendritic tiling in larva sensory neurons (Emoto et al., 2006). The core components of the Hippo signaling pathway are also re-used post-mitotically for a dramatically different purpose—to specify terminal photoreceptor fates in the *Drosophila* retina (Mikeladze-Dvali et al., 2005).

The *Drosophila* retina contains about 800 repeating unit eyes called ommatidia, each with 8 photoreceptors (Hardie, 1985). There are two main subtypes of ommatidia which are defined

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by the expression of Rhodopsin proteins in the color-detecting inner photoreceptors, R7 and R8 (Rister et al., 2013). In `pale' (**p**) ommatidia, **p**R7 expresses UV-sensitive Rh3 and **p**R8s expresses blue-sensitive Rh5, whereas in `yellow' (**y**) ommatidia, **y**R7 expresses UV-sensitive Rh4 and **y**R8 expresses green-sensitive Rh6 (Fig. 1A) (Chou et al., 1996; Chou et al., 1999; Papatsenko et al., 1997; Pichaud et al., 1999). The **y** and **p** ommatidia are distributed in a stochastic manner in the retina, with roughly 65% **y** and 35% **p** ommatidia (Fig. 1C and H) (Fortini and Rubin, 1990; Franceschini et al., 1981).

The ommatidial subtype decision is made randomly in R7s and then imposed onto R8s. Stochastic expression of Spineless (Ss), a PAS-bHLH transcription factor determines the random mosaic pattern of ommatidial subtypes. In one random subset of R7s, Ss is expressed and induces yR7 fate, including Rh4 expression. yR8 fate, including Rh6 expression is specified by default. In the complementary R7s that lack Ss, pR7 fate is induced including Rh3 expression. (Johnston et al., 2011; Thanawala et al., 2013; Wernet et al., 2006) (Fig 1B). An unknown signal from R7 is then transduced into a stable fate decision in R8s that become yR8 and express Rh6. This decision requires a feedback loop between the Hippo pathway and the Melted growth regulator (Fig.1B). The activity of the Hippo pathway during growth is regulated by multiple inputs to ensure correct proliferation and cell death (Halder and Johnson, 2011). However, only a subset of these upstream regulators of the Hippo pathway are involved in the control of R8 fate. Merlin, Kibra, and Lethal (2) giant larvae (Lgl), appear to constitutively activate the pathway to specify **y**R8 subtype (Jukam and Desplan, 2011). In **p**R8s, Warts is repressed while Melted is expressed, yielding expression of Rh5 and repression of Rh6. In **y**R8s, Warts is expressed and Melted is repressed, promoting expression of Rh6 and repression of Rh5. Mutual repression of Warts and Melted forms a bi-stable double-negative feedback loop for R8 subtype specification (Mikeladze-Dvali et al., 2005) (Fig. 1B).

Although the Hippo pathway has been extensively studied for its role in growth, the factors that determine its various roles in different developmental processes are not well known. Here, we show that *erect wing* (*ewg*), which encodes a neuronal transcription factor, is required to antagonize the Hippo pathway in the context of R8 subtype specification. *ewg* functions autonomously in R8 for terminal differentiation of R8 subtypes. It acts upstream of the Warts-Melted feedback loop to promote **p**R8 fate and Rh5 expression, and to prevent **y**R8 fate and Rh6 expression. Moreover, Ewg is required to maintain the repression of the **y**R8/Rh6 fate in adult R8 photoreceptor neurons. Thus, the input from *ewg* to down-regulate the Hippo pathway is required to specify and maintain **p**R8 fate. Such neuron-restricted regulation may help to repurpose the pathway for non-growth functions.

Results

ewg regulates mutually exclusive R8 Rhodopsin expression

We identified a role for *ewg* in Rhodopsin regulation in an RNAi screen for transcription factors whose knockdown caused changes in Rhodopsin expression. RNAi knockdown of *ewg* led to ectopic *Rh1-GFP* expression in inner photoreceptors, a phenotype that will be described elsewhere (H-Y. H. & C.D., in preparation) (Suppl Fig. 1A). However, we also found that the proportion of R8s expressing Rh5 was dramatically lower in eyes expressing *ewg-RNAi* under the control of two strong eye-specific drivers, the *eyeless* (*ey*) and *lGMR-Gal4* driver (3% as compared to 17% in RNAi controls; $p \leq 0.001$) (Suppl Fig. S1B–D).

To further investigate the role played by *ewg* in the regulation of R8 *rhodopsin* expression, we used the Flp recombinase to excise an *elav>ewg-cDNA* rescue cassette in ewg^{11} null flies in order to generate and examine mutant tissue (Haussmann et al., In ewg^{11} whole mutant retinas, the proportion of Rh5 was dramatically lower than in the wild-type, with

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only 10% of R8s expressing Rh5 as compared to 35% in the control ($p \leq 0.0001$) (Fig. 1C, D and H). When the *elav>ewg-cDNA* cassette was not excised in ewg^{l1} homozygous mutant flies, we observed a wild-type Rh5:Rh6 ratio, showing that the R8 Rhodopsin phenotype was specifically due to loss of *ewg* (Fig. 1 E and H). Thus, *ewg* is required for the normal proportion of Rh5 and Rh6-expressing R8 photoreceptors.

Ewg acts autonomously for the regulation of Rhodopsin expression in R8

We next tested whether over-expression of Ewg was sufficient to affect Rh5 and Rh6 expression. We mis-expressed Ewg using *senseless (sens)-Gal4* that drives expression in all R8s from imaginal discs to adulthood (Pepple et al., 2008). The Rh5:Rh6 ratio in *sens>ewg* flies was not different from wild-type (Fig. 1F and H). We also over-expressed Ewg starting at late pupal stages and continuing through adulthood using *panR8-Gal4* (a combination of two drivers, *Rh5-Gal4* and *Rh6-Gal4*); the Rh5:Rh6 ratio in *panR8>ewg* retinas remained similar to wild type (Fig. 1G and H). Our data suggest that *ewg* functions permissively for **p**R8 subtype specification. However, we could not rule out the possibility that the level of overexpressed *ewg* via the Gal4-UAS system was only marginally higher than endogenous *ewg* expression levels, in which case overexpressed *ewg* might not be sufficient to force all R8s to adapt **p**R8 fate.

To investigate the cellular focus of Ewg function for R8 subtype specification, we examined Ewg expression. Using an anti-Ewg antibody (gift from M. Soller, University of Birmingham), we detected Ewg in all photoreceptor nuclei of the eye disc, starting at the 3rd instar larval stage and continuing throughout pupation and adulthood (Fig. 2A–E). Because Ewg was expressed in all photoreceptors, we tested whether loss of *ewg* caused defects in photoreceptors other than R8. However, in *ewg* whole mutant retinas (ewg^{l1}, *elav<ewg-cDNA>Gal4*; *ey-flp*), R7 Rhodopsins (Rh3 and Rh4) were expressed normally, suggesting that the *ewg* R8 Rhodopsin defect is not due to mis-specification of R7 subtypes and subsequent mis-regulation of R7 signaling to R8 (Suppl. Fig. S1E-E'). We recombined the ewg^{l1} allele on an FRT chromosome to generate mutant clones in which Ewg expression was lost, which allowed us to compare wild type and mutant tissue in the same eye (Suppl. Fig. S1F-F') and examine other genes important for photoreceptor specification. In ewg^{l1} mutant clones, expression of Spalt that specifies inner photoreceptors (Mollereau et al., 2001), and of Prospero that specifies R7 fate (Cook et al., 2003) were both normal (Suppl. Fig. S1G–H'), suggesting that *ewg* is not required for general inner photoreceptor specification or general R7 fate.

To directly test whether *ewg* functions autonomously in R8 to control Rh5 and Rh6, we removed *ewg* function in clones by flipping out the *elav>ewg-cDNA* rescue construct with *hs-flp* (see Methods) at 25–50% pupation, *i.e.* after the last cell division and before R8 subtypes are specified. Of 60 single cell R8 clones, only four expressed Rh5 (arrows in Fig. 2G-G'), whereas 56 expressed Rh6 (arrows in Fig. 2F-F'). The Rh5:Rh6 ratio of 7%:93% in R8 mutant clones is similar to the Rh5:Rh6 ratio of 10%:90% found in whole mutant retinas. This indicates that, although *ewg* is expressed in all photoreceptors, it functions autonomously in R8 to regulate Rhodopsin expression.

The transcriptional activation domain of Ewg is required for the regulation of R8 Rhodopsins

Different Ewg isoforms have context specific roles in their requirement for viability or synaptic growth (Haussmann and Soller, 2010). Specifically, the `D' exon that encodes a domain missing from the Ewg human homologue Nuclear respiratory factor 1, and the `J' exon that encodes a transcriptional activation domain conserved in humans (Haussmann and Soller, 2010), are included or excluded from different isoforms. To understand which Ewg

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isoform is responsible for regulating R8 Rhodopsin we tested the ability of four different Ewg isoforms to rescue ewg^{11} null mutants (Haussmann and Soller, 2010) (Fig. 3A). The full-length cDNA (SC3 isoform) that rescues viability and synaptic growth in *ewg* null alleles (Haussmann and Soller, 2010) also fully rescued the *ewg* R8 defects (Fig. 1E and H). The ΔDJ isoform (Fig. 3A) has the weakest ability to rescue viability and synaptic growth (Haussmann and Soller, 2010) and did not rescue R8 Rhodopsin expression defects (14% Rh5 and 86% Rh6 in R8) (Fig. 3B and E). The ΔD isoform (Fig. 3A) rescued the viability of *ewg* mutants as well as the Rh5 and Rh6 ratio (Fig. 3C and E). The ΔJ isoform, which can restore viability of *ewg* mutants, lacks the last exon. However, the ΔJ isoform did not rescue the Rh5:Rh6 phenotype of ewg^{11} mutants ($p<0.0001$) (Fig. 3D and E). Together, these data show that exon J, which contains the activation domain conserved to humans (Haussmann and Soller, 2010), is required for R8 terminal differentiation, whereas exon D is dispensable. As exon J is required for R8 fate regulation while both Exon D and J are required for synaptic growth. Ewg appears to have distinct protein domain requirements for different neuronal-specific functions.

ewg is required to maintain pR8 subtype fate

Young adult *ewg* mutant flies (0–7 days post-eclosion) exhibited a strong reduction in the proportion of R8s that expressed Rh5 (from 35% to 10%), but these remainging **p**R8s did not contain Rh6. However, in two-week old ewg^{11} mutant flies, Rh6 became de-repressed in the remaining **p**R8s as low levels of Rh6 protein could be observed with In 4 week old *ewg* mutant flies, Rh6 was expressed in all R8 cells (Fig. 4A-A"), leading to co-expression with Rh5 in all Rh5-expressing cells (10% of R8s), a phenotype never observed in aged wild type flies (Fig. 4B-B"). Restoring *ewg* function using *elav*-Gal4 driving *ewg-cDNA* in ewg^{11} mutants rescued the wild-type ratio in old adults (Fig. 4 C-C"). Therefore, *ewg* appears to have an adult function to specifically maintain repression of Rh6 in **p**R8s, in addition to its early role in the establishment of **p**R8 cells. Furthermore, as the Hippo pathway and Rh6 are required late to maintain **y**R8 fate (Jukam and Desplan, 2011; Vasiliauskas et al., 2011), this late requirement for Ewg in **p**R8s indicates that all R8s must actively maintain mutually exclusive Rh5 and Rh6 expression.

ewg determines pR8 fate by regulating melt and warts expression

p and **y** R8 fates are specified by the double-negative transcriptional feedback loop between Warts and Melted (Fig. 1B) (Mikeladze-Dvali et al., 2005). Therefore, *ewg* might control Rh5 and Rh6 by controlling the R8 subtype fate mechanism. We therefore analyzed expression of *warts* and *melted* using transcriptional reporters. In wild-type control retinas, *warts-lacZ* was perfectly co-expressed with Rh6 to specify **y**R8 (Fig. 5A). In *ewg* mutants, the frequency of R8s expressing *warts-lacZ* increased, but the *warts* reporter was always co-expressed with Rh6 (Fig. 5C-C'), suggesting that *ewg* is required to repress *warts* expression to establish **p**R8 fate and prevent **y**R8 fate. *melted-lacZ* is normally found in **p**R8s and is always co-expressed with Rh5 (Fig. 5B). The proportion of R8s expressing *melted-lacZ* was significantly decreased in *ewg* mutants and paralleled the decrease in the proportion of Rh5-expressing R8s, supporting the notion that ectopic Rh6-expressing R8s had adopted the complete **y**R8 fate. However, among the Rh5-expressing R8s in *ewg* mutants, only a subset expressed *melted-lacZ* (Fig. 5D-D'), suggesting that these Rh5-expressing **p**R8 cells were in the process of switching fate to becoming **y**R8. In this case, we would expect *melted-lacZ* to be completely lost in old flies in which Rh5 and Rh6 were co-expressed in pR8s. Indeed, in aged flies *melted-lacZ* was lost in R8s that still expressed Rh5, (Fig. 5E-E') whereas *warts-lacZ* was present in almost all R8s (Fig. 5F-F'). Since the expression of *warts* and *melted* is mutually exclusive, the progressive loss of *melted* and gain of *warts* is likely responsible for the co-expression of Rh5 and Rh6 in older *ewg* mutant flies, with Rh5 perduring in R8s that have switched fate late. This indicates that *ewg* is required to initiate and to maintain

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expression of *melted* and repression of *warts* in adults in order to promote **p**R8 fate and Rh5 expression.

ewg acts genetically upstream of warts and melted

We next performed genetic epistasis tests to determine whether the loss of *melted* or de-repression of *warts* caused the R8 Rhodopsin phenotype in *ewg* mutants. Mis-expression of *melted* in all photoreceptors with *lGMR-Gal4* represses *warts* transcription and induces Rh5 in all R8s (Mikeladze-Dvali et al., 2005). *melted* mis-expression (*lGMR-Gal4, UAS-melted*) suppressed the *ewg* mutant phenotype, and almost all R8s (>99 %) expressed Rh5 (Fig. 6A–B). Thus, *ewg* acts genetically upstream of *melted* to specify the **p**R8 subtype as *melted* induces **p**R8 fate and Rh5 expression in *ewg* mutants.

In *warts* mutants, all **y**R8s are converted into **p**R8s and express Rh5, a phenotype opposite to that of *ewg* mutants (Fig. 6C). *warts* mutants also suppressed the *ewg* mutant phenotype, as *ewg*; *warts* double mutants displayed expression of Rh5 in all R8s (Fig. Thus, *warts* is required to induce **y**R8 fate in *ewg* mutants, which suggests that *ewg* also functions upstream of *warts* to specify **p**R8 subtypes.

merlin encodes a FERM-domain containing protein that acts upstream of the Hippo pathway to constitutively promote Warts activity in R8 (Jukam and Desplan, 2011). Loss of *merlin* resulted in Rh5 expression in most R8s, similar to the *warts* mutant phenotype (Fig. 6E). When *merlin* was removed from *ewg* mutants, almost all R8s expressed Rh5 (Fig. 6F) suggesting that *merlin* is required to activate the Hippo pathway and Warts to induce **y**R8 fate in *ewg* mutants.

Activation of the Hippo pathway leads to the phosphorylation of Warts that negatively regulates the Yorkie (Yki) oncogene (Harvey and Tapon, 2007; Huang et al., 2005). As in growth, Yki is a co-transcriptional regulator that acts with the DNA binding factor Scalloped to activate Rh5 and repress Rh6 (D.J. et al., submitted). We tested whether *ewg* also acts upstream of *yki* to regulate Rhodopsin expression. Over-expression of *yki* caused all R8s to convert to **p**R8 with Rh5 expression (Fig. 6G). In *ewg* mutants, overexpression of *yki* in all PRs with *GMR-yki* led to Rh5 expression in all R8s (Fig. 6H), indicating that *ewg* acts upstream of *yki* to determine the **p**R8 fate.

These data suggest that *ewg* is required genetically upstream to activate *melted* and repress *warts* to induce **p**R8 fate (Fig. 6I).

Discussion

The proper specification of photoreceptor subtypes including Rhodopsin expression is critical for proper color-detection and related behavior (Yamaguchi et al., 2010). We found that the neural-specific transcription factor Ewg contributes to R8 subtype specification. In the absence of *ewg*, most **p**R8s are mis-specified as **y**R8s. Our analysis shows that *ewg* acts autonomously in R8 to regulate the Warts-Melted feedback loop controlling subtype fate. Ewg appears to regulate **p**R8 fate by promoting *melted* expression and *warts* repression, suggesting that *ewg* is necessary to promote the complete **p**R8 fate rather than directly regulating Rh5 expression. Furthermore, epistasis experiments with *merlin, warts* and *melted* place *ewg* genetically upstream of the Warts-Melted feedback loop.

In addition to its role in R8 subtype establishment, *ewg* also functions in subtype maintenance in adult flies, as Rh6 is de-repressed in **p**R8 and is co-expressed with Rh5 in 4-week old *ewg* mutant flies. Expression of Rh5 still remains in old **p**R8s. *ewg* mutants also progressively lose *melted* expression and gain *warts* expression in R8s. The gradual

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disappearance of *melted* in old *ewg* mutant flies likely allowsg expression of *warts* and reactivation of Rh6. This represents another genetic program required to maintain gene expression in differentiated sensory neuron subtypes of adult animals (Jukam and Desplan, 2011; Vasiliauskas et al., 2011). Previous studies have shown that the Hippo pathway is required both to specify and to maintain **y**R8 subtypes. Removing *merlin* after eclosion results in de-repression of Rh5 in all **y**R8s and co-expression with Rh6 (Jukam and Desplan, 2011), a phenotype opposite to that of old *ewg* mutant flies. Furthermore, an active Rh6 protein is required to repress *Rh5* to maintain its exclusive expression in **y**R8s, as loss of Rh6 results in the expansion of Rh5 to all R8s in old flies (Vasiliauskas et al., 2011). Our results are consistent with the model that establishment and maintenance programs are coupled by using the same genes, resulting in efficient long-term gene regulation.

How does the Ewg protein function in R8 subtype specification? Ewg has the same consensus DNA binding site as Nuclear respiratory factor 1 (Fazio et al., 2001). However, we could not find motifs matching the Ewg consensus sequence in the regulatory regions of *melted* and *warts*. The diverse transcriptional targets of Ewg in various organisms also prevent a clear assignment of a conserved Ewg protein function. For example, Nuclear respiratory factor 1 acts as a transcriptional activator in the regulation of expression of cytochrome C and mitochondrial genes (Efiok et al., 1994; Evans and Scarpulla, 1989). However, the sea urchin Ewg homolog, P3A2, limits expression of the cytoskeletal *cyIIIA actin* gene (Calzone et al., 1991). As human Nuclear respiratory factor 1 functions as an activator while sea urchin P3A2 negatively regulates *cyIIIA* (Hough-Evans et al., 1990), Ewg therefore appears to act either as an activator or as a repressor, consistent with the presence of a C-terminal activation domain and an N-terminal repression domain identified in *Drosophila* (Fazio et al., 2001). Although Ewg functions upstream of the *warts/melted* loop, neither *warts* nor *melted* contain canonical Ewg binding motifs, suggesting that Ewg likely regulates these genes indirectly.

Several other genes are expressed in all photoreceptors and act as permissive factors to regulate specific Rhodopsins and photoreceptor subtypes (Rister et al., 2013). For example, Orthodenticle (Otd), the fly homolog of vertebrate Crx and Otx proteins (Furukawa et al., 1997), is a K_{50} homeoprotein expressed in all photoreceptors. Loss of *otd* results in the loss of Rh3 and Rh5 in **p** ommatidia and de-repression of Rh6 in outer photoreceptors (Tahayato et al., 2003). However, like Ewg, Otd is not sufficient to activate these genes when mis-expressed. Otd is therefore a permissive factor that likely acts with co-factors to specify their activating or repressive functions in particular photoreceptors (Rister and Desplan, 2011; Tahayato et al., 2003). For Rh3, restricted Rh expression is achieved by repression by Dve, Senseless and Prospero (Johnston et al., 2011). The same principle might apply for Ewg: since Ewg is expressed in all photoreceptors, it might recruit co-factors specific to **p**R8 to promote the expression of *melted* or to negatively regulate the Hippo pathway. Recently, *ewg* was shown to be required for the recruitment of the cell specific Armadillo-TCF adaptor, Earthbound 1 (Ebd1), to specific chromatin sites to activate a subset of Wingless target genes (Xin et 2011). Ebd1 shares similar polytene chromatin binding sites with Ewg (Benchabane et 2011; Xin et al., 2011). It is possible that Ewg recruits a specific co-factor such as Ebd1 to function in **p**R8. However, we did not observe a decrease in Rh5 expression in *ebd1* mutant retinas, suggesting that Ewg acts differently in the retina. Nevertheless, it is likely that another subtype specific co-factor functions with Ewg to specify **p**R8 fate.

In conclusion, *ewg* is autonomously required to specify the **p**R8 subtype and induce Rh5 expression. *ewg* appears to act upstream of the Hippo pathway, of Melted, and the feedback loops to determine **p**R8 fate. Therefore, a neuronal specific transcription factor, Ewg, contributes to the regulation of the Hippo pathway either directly or indirectly through regulation of *melted* to specify the fate of R8 photoreceptors.

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Material and Methods

Drosophila Stocks and Genetics

Mutant alleles and other fly stocks used in this study include: *ewg*^{l1} with *elav*-FRT-*ewg-cDNA*-FRT rescue cassette was a generous gift of the Soller lab (Haussmann et al., 2008) as were *elav*>EwgΔJ, *elav*>EwgΔJ, *elav*>EwgΔD flies (Haussmann and Soller, 2010). Other flies stocks include: *Rh1-GFP* (Pichaud and Desplan, 2001), *UAS-mer*^{DN} (LaJeunesse et al., 1998), *warts*^{P1}, *warts-lacZ* (Xu et al., 1995), *UAS-melt*, *melt-lacZ, panR8-Gal4* (Mikeladze-Dvali et al., 2005), *UAS-Dicer2*, *ey-Gal4+lGMR-Gal4* (Dietzl et al., 2007), *GMR-Yki* (Huang et al., 2005). *y*^{1}*w*^{67}, *lGMR-Gal4* (Wernet et al., 2003). *ey-FLP*, *ey3.5-FLP*, *UAS-CD8:GFP*, *UAS-GFP*, *UAS-FLP, hs-flp* were obtained from the Bloomington *Drosophila* Stock Center. The UAS-RNAi stocks used in the RNAi screen were obtained from the Vienna Drosophila RNAi Center (VDRC); *UAS-ewg-RNAi* was ID# 4559.

Flies were raised on cornmeal-agar-molasses-yeast medium at 25°C. *y*^{1}*w*^{67} flies were used as wild-type controls for *Rhodopsin* expression. The RNAi screen included ~1700 UAS-RNAi lines which targeted around ~900 transcription factors. The Gal4 driver line contained both *eyeless-Gal4* and *lGMR-Gal4* drivers recombined on chromosome 2. *eyeless-Gal4* is expressed early in the entire eye disc, whereas *lGMR-Gal4* is expressed only after the morphogenetic furrow and maintained in adults. Together, these two drivers induce RNAi expression in the whole eye from the time the eye is specified until adulthood. In addition, the RNAi driver stock carries *UAS-Dicer2* to enhance the efficiency of generating small interfering RNA. *Rh1-GFP*, which is expressed only in outer photoreceptors, was used as a readout in the screen. UAS-RNAi lines were crossed to the driver line (*eyeless>Gal4, lGMR>Gal4*; *UAS-Dicer2*; *Rh1-GFP*) at 25°C. The F1 progeny were analyzed under water immersion for a change in *rh1-GFP* reporter expression (Pichaud and Desplan, 2001).

The *ewg*^{l1} mutant allele containing the *elav<ewg-cDNA>Gal4* rescue cassette on the same chromosome was used to generate *ewg* mutant clones (Haussmann et al., 2008). The rescue constructs contains an *elav* promoter driving the *ewg-cDNA* flanked by FRTs at each side and followed by *Gal4*. *ey-flp* was used to remove *ewg* specifically in the eye in order to avoid embryonic lethality and generate whole mutant eyes. Mutant clones affecting R8 were generated by using *hs-flp* in *ewg*^{l1} flies containing the *ewg-cDNA* rescue cassette and *UAS-CD8:GFP*. These flies were raised at 25°C and were shifted to 37°C for 40 min at 0–25% pupation, when all the photoreceptors have been recruited, but *rhodopsins* are not yet expressed. After heat shock, the pupae were moved back to 25°C and raised to adulthood. Mutant clones were marked by GFP driven by Gal4 that was activated after removal of the *ewg-cDNA*. *ewg*^{l1} was also recombined with FRT19A to generate mutant clones.

In the *ewg* isoform rescue experiments, *ewg*^{l1}/FM7 females were crossed with males carrying *elav*-EwgΔDJ, *elav*-EwgΔJ or *elav*-EwgΔD provided by M. Soller, University of Birmingham.

Immunostaining and statistics

Dissection of adult retina was performed as described (Hsiao et al., 2012). Antibodies and dilutions were as follows: mouse anti-Rh1 (1:10, DSHB) mouse anti-Rh3 (1:100, gift form S. Britt, University of Colorado), rabbit anti-Rh4 (1:100, gift from C. Zuker, Columbia University), mouse anti-Rh5 (1:200, gift from S. Britt), rabbit anti-Rh6 (1:10,000), rabbit anti-Ewg (1:500, gift from M. Soller, University of Birmingham), goat anti-βgal (1:5000, Biogenesis), sheep anti-GFP (1:1000, AbD Serotec), mouse anti-Elav (1:40, DSHB), rabbit anti-Spalt (1:100)(Barrio et al., 1999), mouse anti-Prospero (1:10, DSHB). All secondary antibodies were Alexa Flour (488, 555, or 647)-conjugated made in donkey (1:800, Molecular Probes).

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Fluorescent images were taken with a Leica SP5 confocal laser scanning microscope and processed with Leica AF-Lite software. The number of R8 cells that expressed Rh5, Rh6, or both, was counted in a single focal plane of confocal images. The statistical comparison measuring the Rh5% between different genotypes was performed with a two-tailed unpaired t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *erect wing* (*ewg*) controls the fate of photoreceptors in the *Drosophila* retina.
- *ewg* is expressed in all photoreceptors: It promotes Rh5 and represses Rh6.
- *ewg* promotes *melted* expression and represses *warts* to specify the pR8 subtype fate.
- *ewg* is continuously required to maintain repression of Rh6 in pR8s.
- *ewg* down-regulates Hippo pathway activity to determine neuronal subtype fates.

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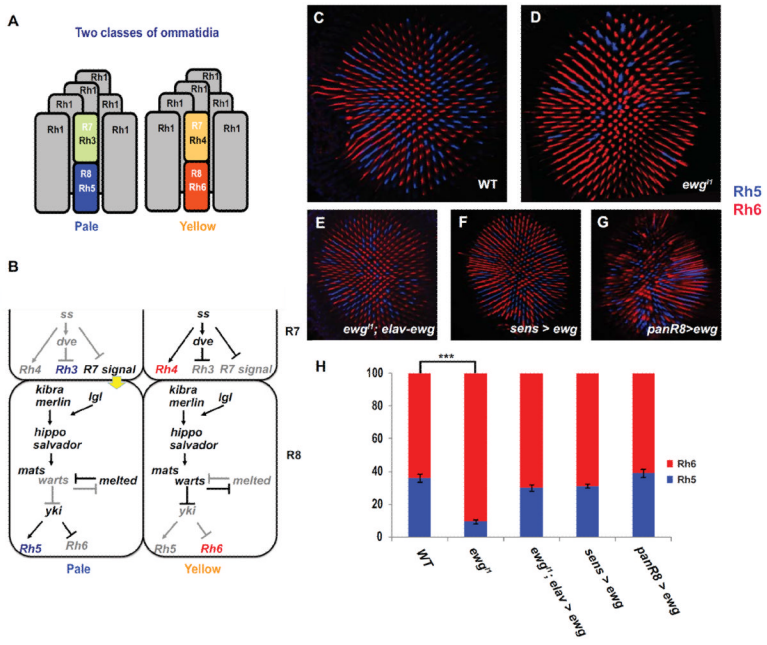

Fig. 1. Rh5 is expressed in fewer R8 cells in *ewg* mutants

(A) Two subtypes of ommatidia: **pale** ommatidia have Rh3 in R7 paired with Rh5 in R8 whereas **yellow** ommatidia contain Rh4 in R7 and Rh6 in R8. Outer photoreceptors (R1–R6) all express Rh1. **(B)** Model showing how R7 is specified into **pale** and **yellow** R7s, and how the Hippo pathway and Melted regulate R8 subtype specification. Model modified from (Jukam and Desplan, 2011). **(C–G)** Confocal images of adult retina showing antibody staining of Rh5 (blue) and Rh6 (red) to mark the two R8 subtypes: **(C)** $y^1 w^{67}$ retinas are used as wild-type controls with a ratio of Rh5 to Rh6 of 35:65. **(D)** ewg^{l1} mutant retina showing a lower number of Rh5 expressing R8. The ratio of Rh5:Rh6 is 10:90. **(E)** Using *elav-Gal4* to drive expression of the *ewg-cDNA* in ewg^{l1} mutant restores the ratio of Rh5:Rh6 to wild type. **(F)** Overexpression of Ewg using *senseless-Gal4* shows no significant changes in the Rh5:Rh6 ratio. **(G)** *panR8>ewg-cDNA* retinas have a wild-type Rh5:Rh6 ratio. **(H)** Quantification of Rh5 and Rh6 in *ewg* mutants. The graph shows the percentage of Rh5 (blue) and Rh6 (red) in R8. An unpaired t-test was performed to calculate the difference in mean % Rh5. Wild-type: n=10 retinas, N=3675 ommatidia; ewg^{l1} mutant: n=11 retinas, N=3214 ommatidia; ewg^{l1}, *elav>ewg-cDNA*: n=11, N=3321 ommatidia; all other genotypes throughout paper: n≥4, N≥ 800 ommatidia. *** $p<0.001$, error bars are mean ± one standard deviation (s.d.).

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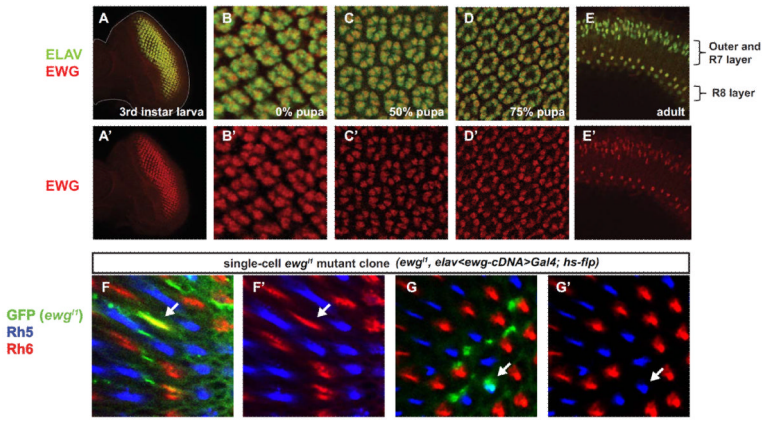

Fig. 2. *ewg* is expressed in all photoreceptors and functions autonomously to specify R8 subtypes

(A–E) Ewg (red) is co-expressed with Elav (green), a neuronal marker, in all photoreceptors starting from 3rd instar larval stage and remains in pupal and adult retinas. **(A'–E')** Ewg (red) channel only. **(A)** $y^1 w^{67}$eye imaginal disc at the 3rd instar stage. Ewg is co-expressed with Elav only in differentiated photoreceptors. Expression of Ewg remains at 0% **(B)**, 50% **(C),** 75% pupation **(D)** and throughout adult stages **(E)**. Ewg is visible both in nuclei of outer and R7 photoreceptors (outer layer) as well as in R8 in the lower nuclear layer. **(F)** Most *ewg* R8 mutant cells (arrow) in clones marked by GFP (green) express Rh6 (red). **(F')** Red (Rh6) and blue (Rh5) channel only. Among 60 mutant R8s, 56 expressed Rh6. **(G)** One *ewg* mutant R8 (arrow) marked by GFP (green) expresses Rh5 (blue). **(G')** Red (Rh6) and blue (Rh5) channel only.

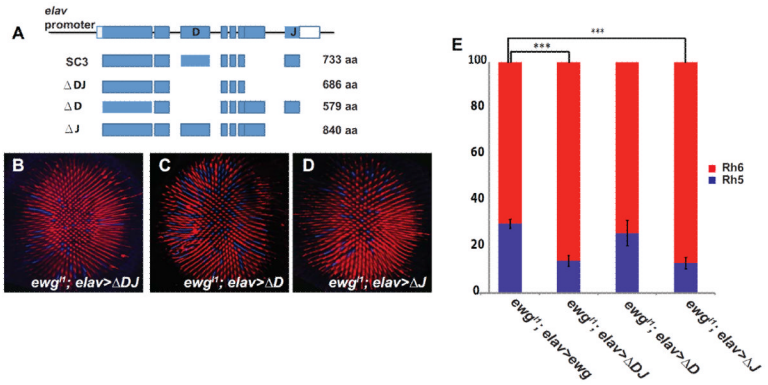

Fig. 3. The Ewg activation domain is required for R8 subtype specification

(A) Schematic of *ewg* genomic organization and four isoforms of Ewg. Exons are indicated in blue. Modified from (Haussmann and Soller, 2010). **(B)** Expression of the ΔDJ isoform fails to restore the normal ratio of Rh5 (blue) to Rh6 (red). **(C)** Expression of the ΔD isoform restores a wild type Rh5:Rh6 ratio. **(D)** Expression of the ΔJ isoform is not able to rescue the *ewg* mutant phenotype. **(E)** Quantification of Rh5 and Rh6 rescue with different Ewg isoforms. An unpaired t-test was performed to calculate the difference in mean % Rh5. ewg^{l1}; *elav-ewg-cDNA*, n=11 retinas, N=3321 ommatidia; ewg^{l1}; *elav*-ΔDJ, n=5 retinas, N=1656 ommatidia; ewg^{l1}; *elav*-ΔD, n=6 retinas, N=2096 ommatidia; ewg^{l1}; *elav*-ΔJ, n=6 retinas, N= 2103 ommatidia. *** $p<0.001$, error bars are mean ± one standard deviation (s.d.)

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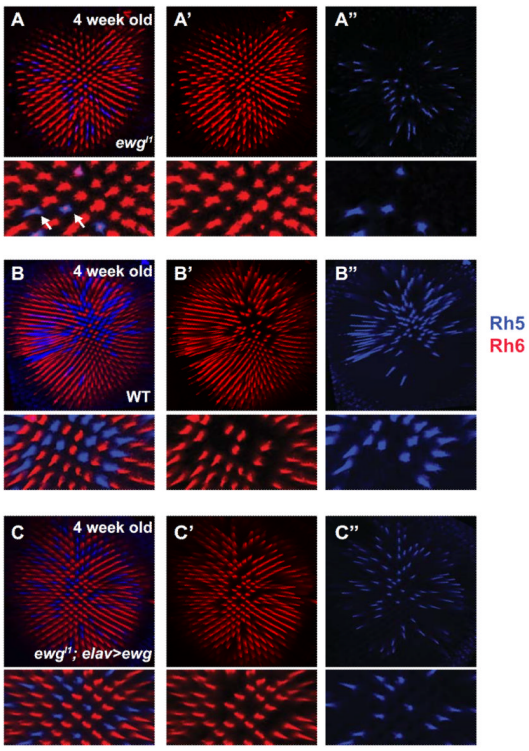

Fig. 4. *ewg* functions in R8 subtype specification and is required to maintain repression of Rh6 in pR8 photoreceptors

(A–C) Confocal images of 4 weeks old adult retina stained with antibodies against Rh5 (blue) and Rh6 (red) with zoomed-in images. **(A)** ewg^{l1} mutant retina shows expanded expression of Rh6 in all R8. Arrows point R8 cells co-expressing Rh5 and Rh6. **(A')** Rh6 channel only. **(A")** Rh5 channel only. Note the co-expression of Rh5 and Rh6 in the bottom panels of A and A'. **(B)** A four week old wild-type retina shows no expansion of Rh6 expression, and maintains mutually exclusive Rh5 and Rh6 expression. **(B')** Rh6 channel only. **(B")** Rh5 channel only. **(C)** ewg^{l1} mutant rescued with *ewg cDNA* shows no expansion of Rh6 in 4 weeks old flies. **(C')** Rh6 channel only. **(C")** Rh5 channel only.

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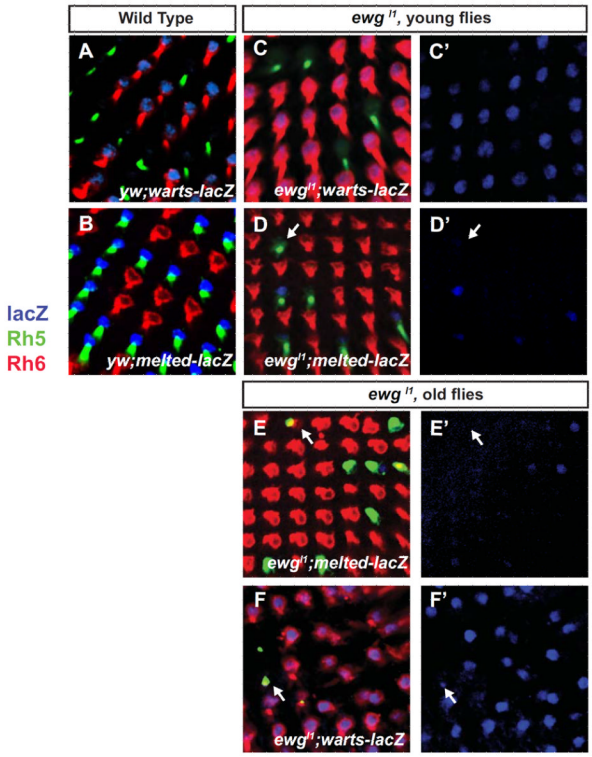

Fig. 5. *ewg* is required to specify the pR8 subtype fate

In controls **(A)** *warts-lacZ* (antibody to β–galactosidase, blue) is expressed only in **y**R8s, marked by Rh6 (red) while **p**R8s are marked by Rh5 (green) while **(B)** *melted-lacZ* expression (blue) in **p**R8s (labeled with Rh5) is mutually exclusive with *warts-lacZ* expression in **y**R8s (marked by Rh6). **(C–F)** ewg^{11} mutant retinas. **(C)** R8s co-express Rh6 and *warts-lacZ*. **(C')** *warts-lacZ* channel only. **(D)** Not all R8s expressing Rh5 contain *melted-lacZ*. The white arrow marks a photoreceptor expressing Rh5 that lacks *melted-lacZ* expression. **(D')** *melted-lacZ* channel only. **(E–F)** Two weeks old ewg^{11} mutant flies. **(E)** Small amounts of Rh6 are co-expressed in R8s that express Rh5. In those R8s (white arrow), *melted-lacZ* expression is lost **(E')** *melted-lacZ* channel only. **(F)** *warts-lacZ* expands in R8s that are co-expressed with Rh5 and Rh6 (arrows). **(F')** *warts-lacZ* channel only.

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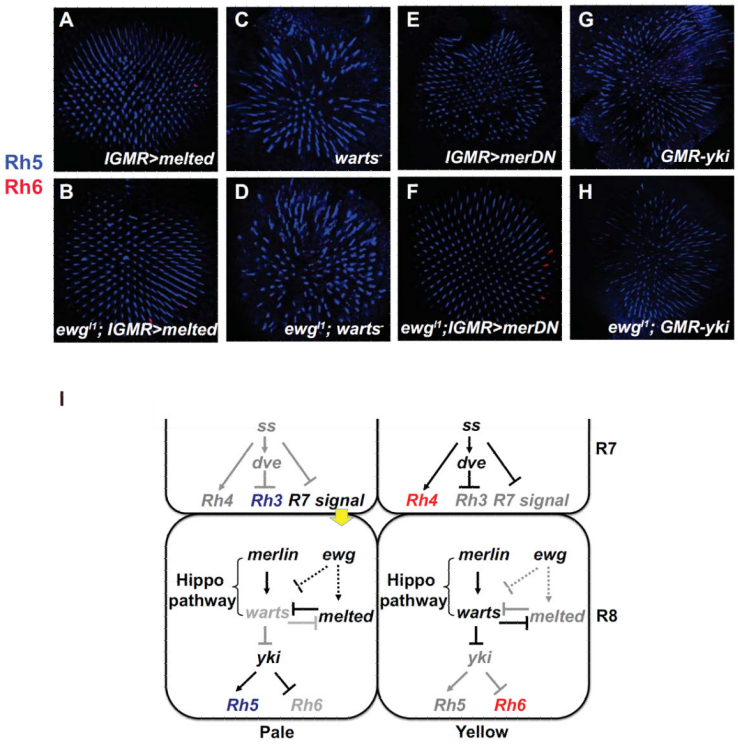

Fig. 6. *ewg* acts genetically upstream of *warts* and *melted*

(A–H) Confocal images of adult retinas stained with antibodies for Rh5 (blue) and Rh6 (red). **(A)** Over-expression of *melted* driven by *IGMR-Gal4* results in Rh5 expressed in all R8s. **(B)** In ewg^{l1}; *IGMR>melted* flies, overexpression of *melted* suppresses the ewg^{l1} phenotype, leading to Rh5 expression in all R8s. **(C)** In a *warts* mutant, all R8s are converted to **p**R8 expressing Rh5. **(D)** ewg^{l1}; $warts^{-}$ double mutants show Rh5 expression in all R8s. **(E)** Overexpression of a dominant negative form of Merlin (mer^{DN}) in all PRs leads to expression of Rh5 in all R8s. **(F)** A mer^{DN};*ewg* double mutants show Rh5 expression in all R8s. **(G)** Over-expression of *yki* driven by *GMR* causes Rh5 expression in all R8s. **(H)** Over-expression of *yki* in ewg^{l1}mutants suppresses the *ewg* phenotype and leads to Rh5 expression in all R8s. **(I)** Model of *ewg* interaction with the Warts-Melted feedback loop. *ewg* acts upstream of *melted* to promote its expression, allowing expression of Rh5 in **p**R8s. *ewg* might also be required to repress the Hippo pathway, leading to Rh5 expression.