Regional Modulation of a Stochastically Expressed Factor Determines Photoreceptor Subtypes in the *Drosophila* Retina

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SUMMARY

Stochastic mechanisms are sometimes utilized to diversify cell fates, especially in nervous systems. In the *Drosophila* retina, stochastic expression of the PAS-bHLH transcription factor Spineless (*Ss*) controls photoreceptor subtype choice. In one randomly distributed subset of R7 photoreceptors, *Ss* activates Rhodopsin4 (*Rh4*) and represses Rhodopsin3 (*Rh3*); counterparts lacking *Ss* express *Rh3* and repress *Rh4*. In the dorsal third region of the retina, the Iroquois Complex transcription factors induce *Rh3* in *Rh4*-expressing R7s. Here, we show that *Ss* levels are controlled in a binary on/off manner throughout the retina yet are attenuated in the dorsal third region to allow *Rh3* coexpression with *Rh4*. Whereas the sensitivity of *rh3* repression to differences in *Ss* levels generates stochastic and regionalized patterns, the robustness of *rh4* activation ensures its stochastic expression throughout the retina. Our findings show how stochastic and regional inputs are integrated to control photoreceptor subtype specification in the *Drosophila* retina.

INTRODUCTION

The *Drosophila* eye provides an excellent paradigm to study how stochastic and regionalized regulatory inputs intersect to affect cell fate specification. Underlying its uniform morphology, the fly eye contains two randomly distributed subtypes of ommatidia (unit eyes) defined by the mutually exclusive expression of specific Rhodopsin (Rh) proteins in the inner photoreceptors (R7 and R8). In the pale (*p*) subtype, *p*R7s express Rhodopsin3 (*Rh3*) and *p*R8s express Rhodopsin5 (*Rh5*), whereas in the yellow (*y*) subtype, *y*R7s express Rhodopsin4 (*Rh4*) and *y*R8s express Rhodopsin6 (*Rh6*). Although the *p* and *y* subtypes are randomly distributed, they consistently occur in a *p:y* ratio of ~35:65 (Bell et al., 2007; Johnston and Desplan, 2010) (Figures 1A, 1B, 1D, and 1F). Throughout the majority of the retina, the mutually exclusive expression of *Rhs* defines the *p* and *y* ommatidial subtypes. However, in the dorsal third region of the retina, *Rh3* is coexpressed with *Rh4* in *y*R7s. Thus, the dorsal third region consists of *p* ommatidia containing *p*R7s that express *Rh3* only and “dorsal third *y*” ommatidia containing *y*R7s that express both *Rh4* and *Rh3* (Figures 1C–1E) (Mazzoni et al., 2008).

Stochastic ommatidial subtype specification is controlled by the PAS-bHLH transcription factor Spineless (*Ss*) (Wernet et al., 2006). *Ss* is expressed in a random subset of R7s where it determines *y* subtype fate. In *y*R7s, *Ss* has three main functions: (1) activate *Rh4*, (2) repress *Rh3*, and (3) repress a signal from R7 to R8, leading to the default *y*R8 fate (*Rh6* expression) (Figure 1B). In the absence of *Ss*, *p*R7 fate (*Rh3* expression) and *p*R8 fate (*Rh5* expression) are induced (Figure 1A).

*y*R7-specific expression of *Rh4* appears to be simply activated by *Ss*. In contrast, *p*R7-specific expression of *Rh3* is regulated by complex interlocked feedforward loops of transcription factors (Johnston et al., 2011). The Defective Proventriculus (Dve) homeodomain protein, a repressor that directly binds the *rh3* promoter, is a critical node in this motif (Figures 1A and 1B). The Orthodenticle (Otd) homeodomain protein activates Dve expression in all PRs, whereas the Spalt zinc finger transcription factors (Salm and Salr, referred to collectively as “Sal”) repress Dve in R7s (Figures 1A and 1B). In *p*R7s, Sal and Otd together activate *Rh3* in the absence of Dve (Figure 1A). In *y*R7s, *Ss* reactivates Dve that represses *Rh3* despite the presence of Otd and Sal (Figure 1B) (Johnston et al., 2011; Sood et al., 2012).

The regionalized coexpression of *Rh3* in *Rh4*-expressing *y*R7s in the dorsal third of the retina is activated by the transcription factors of the Iroquois Complex (IroC) (Figures 1C–1E) (Mazzoni et al., 2008). Whereas *Ss* provides a stochastic input, IroC supplies a regionalized input into the regulation of *Rh* expression. Here, we show that, as in other biological contexts, Spineless acts with the ubiquitously expressed PAS-bHLH protein, Tango (Tgo) (Emmons et al., 1999), to regulate *Rh* expression. We show that the proper stochastic and regional control of *Rh* expression...
requires five mechanistic features: (1) Ss levels are high in yR7s in the main part of the retina to ensure repression of Rh3 and activation of Rh4, (2) Ss levels are reduced in dorsal third yR7s to allow Rh3 expression, (3) IroC activates Rh3 in dorsal third yR7s, (4) low Ss levels (as found in dorsal third yR7s) are sufficient to activate Rh4 expression, and (5) the absence of Ss expression produces pR7 fate including expression of Rh3 and absence of Rh4. The sensitivity of Rh3 to regional inputs is likely due to the presence of multiple IroC (activating) and Dve (repressing) binding sites in the rh3 promoter, whereas the robustness of Rh4 activation appears to be due to the presence of a single Ss (activating) binding site. Our data demonstrate how stochastic and regionalized regulatory inputs are integrated to determine ommatidial subtype specification throughout the retina.

RESULTS

Tgo Is Required for Stochastic Rh Expression

In most biological contexts, the Tgo PAS-bHLH transcription factor is required as a heterodimeric partner for Ss function. Ss is expressed in specific cell types where it binds ubiquitously-expressed Tgo in the cytoplasm. The Ss/Tgo heterodimer then localizes to the nucleus to regulate target genes (Emmons et al., 1999; Ward et al., 1998). Although it has been suggested that Ss works independently of Tgo in some contexts, these analyses were conducted with available tgo alleles, which were all hypomorphic (Kim et al., 2006).

Because we could not detect staining in the eye using the available Tgo antibody (data not shown), we generated a tgo transcriptional reporter (tgo>nuGFP) that drove GFP expression in all cells in the retina, including all R7 cells, consistent with previous reports that Tgo is ubiquitously expressed (Figure 2O).

To clearly ascertain the role of Tgo in stochastic Rh regulation, we generated two tgo null mutant alleles, tgo
del16 and tgo
del25, using the hobo transposable element system (see Experimental Procedures). tgo
del16 removes the bHLH, PAS, and PAC domains required for dimerization and DNA binding, whereas tgo
del25 removes the entire tgo locus and part of the 3′ UTR region of the neighboring cg1986 gene (Figures 2A and 2B). Both tgo
del16 and tgo
del25 null mutant retinas displayed expression of Rh3
Tgo is required for Ss-mediated regulation of Rhs

Rh3 and Rh4 are expressed in mutually exclusive R7 subtypes in the main part of the retina. However, in the dorsal third region, IroC activates Rh3 coexpression in Rh4-expressing yR7s. We hypothesized that Ss could also play a role in this coexpression phenomenon and thus, we assessed the levels of Ss protein to determine regional differences across the retina. We defined four regions of the retina based on ommatidium position relative to the equator: dorsal third (DT), dorsal equatorial (DE), ventral equatorial (VE), and ventral third (VT). Ss was expressed in an on/off manner across the retina as indicated by the bimodal 

and loss of Rh4 in all R7s, similar to ss mutants (Figures 2D, 2E; Figure S1A available online). Retinas in which tgo was knocked down using RNAi as well as tgo null mutant clones displayed a similar phenotype (Figures 2F, 2J, and S1B). tgo null mutants faithfully phenocopied ss null mutants for all features of Rh regulation including derepression of the signal to R8s causing a dramatic increase in the frequency of Rh5-expressing R8s (Figures 2H and 2I), loss of Dve expression in yR7s (Figure 2L), and no effect on general cell fate markers (Figures S1C–S1G).

Consistent with the requirement of Ss/Tgo dimerization for efficient localization to the nucleus, Ss was not detectable in nuclei in tgo null mutant clones (Figure 2K). Further, although we could detect nuclear Ss in all photoreceptors when provided ectopically by a strong heterologous promoter (lGMR>Gal4), this localization was dramatically weaker in tgo mutant tissue compared to neighboring wild-type tissue (Figure 2N).

Tgo is cell autonomously required for regulation of Rh expression by Ss because ectopically-expressed Ss induced expression of Rh4 in all PRs and repression of Rh3 in all R7s in wild-type clones, but failed to do so in tgo null mutant clones, leading to Rh3 expression and loss of Rh4 in all R7s (Figure 2M).

Ss is also required for the elaboration of dendrites in “dendrite arborization” (da) sensory neurons. Although the hypomorphic tgo5 allele exhibited no dendritic arborization defects (Kim et al., 2006), tgo null mutant clones displayed decreases in the number of dendritic termini similar to ss null mutants, suggesting that Tgo is required for all Ss functions (Figures S1H–S1L).
distributions for each region (Figures 3A–3H). Interestingly, Ss levels were significantly lower for cells in the On state in the DT than in other regions of the eye (Figures 3A and 3B). Ss levels in IroC mutant clones were comparable to neighboring wild-type tissue (Figures 3I and 3J) and ectopic expression of IroC in all R7s did not significantly reduce Ss levels (data not shown), showing that this reduction of Ss levels in the DT is IroC-independent.

**Rh3 and Rh4 Are Differentially Responsive to Ss/Tgo Activity Levels**

We hypothesized that the reduced levels of Ss in the DT lower repression and allow IroC-mediated activation of Rh3 in yR7s. rh4>ss generates a positive feedback loop to increase levels of Ss specifically in yR7s. These increased levels of Ss caused repression of Rh3 in yR7s in the DT (Figures 4A and 4G), showing that Ss must be maintained at low levels to allow for Rh3 expression.

Therefore, Rh3 and Rh4 appear to be differentially responsive to modulation of Ss levels in regions of the retina. Whereas Rh3 repression is sensitive to the reduction of Ss levels in the DT, Rh4 activation is robust. We characterized these differences further by evaluating a series of ss and tgo mutant alleles that cause premature termination and truncation of activation domains, leading to reduction in activity levels (Figures 2B and 5A). To determine the molecular lesions, we sequenced the ss116.4, tgo6 and other ss alleles (Figures 2B, 5A, and Experimental Procedures). Including the ectopic expression of Ss experiment, we observed six phenotypic classes:

1. Increased levels in yR7s (rh4>ss): Rh3 and Rh4 were expressed in stochastically distributed, complementary subsets of R7s throughout the retina, including the DT where Rh3 became excluded from yR7s (Figures 4A and 4G).
2. Wild-type (tgo3): Rh3 and Rh4 were expressed in stochastically distributed, mutually exclusive subsets of R7s in the majority of the retina (i.e., DE, VE, and VT). Rh3 was expressed in Rh4-expressing yR7s in the DT (Figures 4B and 4G).
3. Weak loss-of-function (tgo6, tgo7): Derepression of Rh3 occurred readily in yR7s of the DT and DE regions and sporadically in the VT and VE regions. Rh4 remained expressed in stochastically distributed yR7s (Figures 4D and 4G).
4. Medium loss-of-function (tgo5): Derepression of Rh3 occurred in nearly all R7s. Rh4 was still expressed in stochastically distributed yR7s (Figures 4C and 4G).
5. Strong loss-of-function (ss1116.4/def, ssn116.5/def): Derepression of Rh3 occurred in nearly all R7s. Rh4 was still expressed in stochastically distributed yR7s, but the frequency was subtly reduced in the DT region (Figures 4E and 4G).
6. Null (ssn1115.7, tgo6, tgo25): Rh3 was expressed and Rh4 was lost in all R7s (Figures 4F and 4G).

These data show that Rh3 and Rh4 respond differently to Ss/Tgo activity levels. Lowering Ss/Tgo activity allowed for derepression of Rh3 without affecting activation of Rh4 in...
yR7s. Rh3 was susceptible to an underlying dorsal/ventral gradient of regulation because the region in which Rh3 coexpresses with Rh4 in yR7s expanded ventrally as Ss/Tgo activity decreased. Whereas Rh3 was very sensitive to Ss/Tgo activity levels, Rh4 was robust, with only subtle changes in the frequency of Rh4 expression observed in the DT (where Ss levels are reduced) in the strongest loss-of-function alleles.

Our data suggest that Ss/Tgo activity occurs at specific levels to induce repression of Rh3 in yR7s throughout the majority of the retina while allowing coexpression with Rh4 in yR7s of the DT region.

**Rh Regulation Is Sensitive to the Activation Capacity of Ss**

The ss/tgo allelic series suggested that the C-terminal activation domains are important for the regulation of Rh expression in R7s. For the tgo alleles, the degree of activation domain truncation correlated with the loss of activation capacity and phenotypic severity (Figures 2B, 4C, 4D, 4F, and 4G) (Sonnenfeld et al., 2005).

To further characterize the differential response of Rh3 and Rh4 to Ss/Tgo activity levels, we used gain-of-function assays with Ss proteins with deletions of functional domains. The Ss bHLH domain binds DNA sequences in target genes upon

**Figure 4. Rh3 and Rh4 Are Differentially Responsive to Ss/Tgo Activity Levels**

Green lines mark the regional boundary of Rh3 expression in yR7s. Dotted yellow lines mark the regional boundary of the normal frequency of Rh4 expression in yR7s. Top, Rh3 and Rh4; middle, Rh3 alone; bottom, Rh4 alone. DT, dorsal third; DE, dorsal equatorial; VE, ventral equatorial; VT, ventral third.

(A) Ectopic expression of Ss (rh4>ss) represses Rh3 in dorsal third yR7s. Rh3 and Rh4 are expressed in exclusive subsets of R7s in all regions.

(b) In wild-type animals, Rh3 and Rh4 are expressed in exclusive subsets of R7s in the DE, VE, and VT regions. The DT region is composed of R7s that express Rh3 alone or coexpress Rh3 with Rh4.

(c) In tgo6 mutants, Rh3 expression in yR7s expands to the DE region. Rh3 and Rh4 are expressed in exclusive subsets of R7s in the VE and VT regions. The DT and DE regions are composed of R7s that express Rh3 alone or coexpress Rh3 with Rh4.

(d) In tgo5 mutants, Rh3 expression in yR7s expands to the entire retina. These retinas are composed of R7s that express Rh3 alone or coexpress Rh3 with Rh4.

(e) In ss116.5 mutants, Rh3 expression in yR7s expands to the entire retina. These retinas are composed of R7s that express Rh3 alone or coexpress Rh3 with Rh4. The frequency of Rh4 expression is slightly reduced in the DT.

(f) In tgo25 mutants, Rh4 is lost and Rh3 is expressed in all R7s throughout the retina.

(g) Quantification of the series of ss and tgo alleles. Data are presented in order of decreasing Ss/Tgo activity (i.e., increasing phenotypic severity). The six main phenotypic classes are separated by dashed lines.
dimerization with Tgo. The PAS domains mediate Ss/Tgo dimerization whereas the PAC motif contributes to folding of the PAS domain. The C-terminal region functions as an activation domain (Figure 5A) (Crews, 1998; Crews and Fan, 1999; Ponting and Aravind, 1997; Zhulin et al., 1997).

Considering our allelic series analysis, we predicted that Ss protein whose C-terminal activation domain had been truncated should activate Rh4 but not repress Rh3. We generated wild-type and modified UAS>Ss (Ssmodified) constructs that lacked one or more of the distinct functional domains (SsΔPAS/PAC, SsΔbHLH, SsΔPAS-N, SsΔPAS-C, SsΔPAC) or had truncations of the C-terminal activation region (SsΔC1, SsΔC2, SsΔC3) (Figures 5A and 5B). Upon ectopic Ss modified expression (panR7>Gal4; UAS>Ssmodified), we observed three classes of phenotypes that corroborated our ss/tgo allelic series:

1. Sswild-type, SsΔPAS-C, SsΔPAC, and SsΔC1 induced expression of Rh4 and repression of Rh3 in all R7s (Figures 5B, 5C, 5E, S2J, and S2L–S2N), suggesting that these constructs were fully functional. It was surprising that the second PAS domain and the PAC domain were not required to regulate Rh expression because they have important roles for PAS-bHLH protein function.

2. SsΔC2 and SsΔC3 induced Rh4 but did not repress Rh3 in pR7s (Figures 5B, 5D, 5F, and S2R, and S2S), consistent with the phenotype observed for ss and tgo mutants with truncated C-terminal domains.

3. SsΔbHLH, SsΔPAS-N and SsΔPAS/PAC caused no change in Rh expression (Figures 5B, 5G, and S2O–S2Q), consistent with critical roles for the bHLH and N-terminal PAS domains. We confirmed that these nonfunctional transgenes were indeed expressed using Ss antibody staining (Figures S2E–S2I).

Truncation of the C-terminal region reduces the transcriptional activity of the Ss protein. However, these changes in Ss could also impair heterodimerization with Tgo, prevent nuclear localization, or destabilize the protein in a nonspecific way. We therefore tested the Ssmodified proteins for their capacity to localize Tgo to the nucleus. Ectopic expression of Ss driven by the engrailed promoter (en>Gal4) caused Tgo nuclear localization in the ectodermal en stripes in the fly embryo (Figures 5B and 5F).
Stochastic and Regional Inputs Control PR Subtypes

Ss/Tgo does not regulate rh3 directly, but rather activates Dve to repress rh3 in yR7s (Figures 1A and 1B). However, Dve is expressed at levels that allow IroC to overcome repression and activate Rh3 in yR7s of the DT region (Johnston et al., 2011). We wondered whether Dve expression levels were affected in hypomorphic mutant situations that displayed Rh3 derepression. In tgo5 mutant clones, Dve expression was decreased but not lost (Figures 6A and 6B). Thus, the dramatic changes in Rh3 expression observed upon modulation of Ss/Tgo activity appear to be mediated by changes in Dve levels.

Rh3 regulation is controlled by repressing (Dve) and activating (Otd, Sal, IroC) inputs. We next tested whether the sensitivity of rh3 regulation extends to its promoter. The rh3 promoter contains three canonical binding sites (TAATCC) for the K50 homeodomain transcription factors, Dve and Otd (Figure 6C) (Johnston et al., 2011; Tahayato et al., 2003). In yR7s, these sites appear to mediate repression by Dve. A 194 bp rh3 promoter (rh3prom>GFP) induced expression in only pR7s in the main part of the retina and in both pR7s and yR7s in the DT region, similar to the Rh3 protein (Figure 6E). Mutation (K50 mut1) of the distal K50 site (K50–1) caused derepression in yR7s of the DE region (Figure 6F) whereas mutation (K50 mut2) of the proximal site (K50–2) led to derepression in yR7s of the DE and VE regions (Figure 6G). Mutation of both sites (K50 mut12) caused derepression in yR7s throughout the retina (Figure 6H).

DISCUSSION

The complex expression pattern of Rhs in R7s requires the integration of stochastic and regional regulatory information. In the main part of the retina, high levels of Ss in yR7s ensure repression of Rh3 and activation of Rh4. In the DT, reduced Ss levels...
Figure 6. Regional rh3 Expression Is Sensitive to Activating and Repressing Inputs

(A) Dve levels in yR7s are decreased in tgo5 mutant clones. GFP− indicates tgo5 mutant tissue; GFP+ indicates wild-type tissue. Left, Dve and GFP; Right, Dve alone. White circles indicate high Dve expression in wild-type yR7s; light gray circles indicate low Dve expression in tgo5 mutant yR7s; dark gray circles indicate no Dve expression in wild-type and tgo5 mutant pR7s.

(B) Quantification of Dve levels in tgo5 mutant clones and wild-type tissue. Wild-type R7s (green) express Dve at higher levels than tgo5 mutant R7s (black).

(C) Schematic of rh3 promoter that recapitulates Rh3 protein expression. Orange, K50; purple, IroC site; green, RCSI; yellow, TATA box. The RCSI is a conserved element found in all rh promoters that is required for expression.

(D–H) When IroC sites are mutated, expression of rh3prom>GFP is lost in DT yR7s, similar to the loss of expression observed when Ss levels are ectopically high. As K50 sites are mutated, derepression of rh3prom>GFP expands ventrally similar to derepression of Rh3 protein in ss/tgo hypomorphic alleles. For all, rh3prom>GFP is expressed in pR7s in all regions of the retina. Dashed yellow lines mark regions where rh3prom>GFP is expressed in yR7s. DT, dorsal third; DE, dorsal equatorial; VE, ventral equatorial; VT, ventral third. For (D), gray circles indicate DT yR7s that have lost expression of rh3prom>GFP. For (E)–(H), white circles indicate ventral-most yR7s that express rh3prom>GFP.

(D) rh3prom IroC mut134>GFP is expressed in pR7s only. Expression in DT yR7s is lost.

(E) rh3prom wild-type>GFP is expressed in yR7s in the DT.

(F) rh3prom K50 mut1>GFP is expressed in yR7s in the DT and DE.

(G) rh3prom K50 mut2>GFP is expressed in yR7s in the DT, DE, and VE.

(H) rh3prom K50 mut12>GFP is expressed in yR7s throughout the retina (including DT, DE, VE, and VT).
Figure 7. Robust Rh4 Activation Requires a Canonical Ss Binding Site

(A–D) Colors indicate known cis-regulatory regions. Blue, RUS4A; red subset of RUS4A, XRE core site (Ss/Tgo binding site); green, RCSI (inverted in D. vir, D. moj, D. gri); yellow, TATA box.

(A) Schematic of rh4 promoter that recapitulates Rh4 protein expression. Sequence shows known critical cis-regulatory elements.

(B) rh4prom>GFP with wild-type XRE recapitulates Rh4 protein expression.

(C) Expression is lost with a point mutation in the XRE.

(D) Sequence alignment of the rh4 promoter for 12 Drosophila species highlighting the known cis-regulatory elements. The XRE core sequence is perfectly conserved in all 12 species. Sequence alignment was from the UCSC Genome Browser (http://genome.ucsc.edu/) (Fujita et al., 2011; Kent, 2002; Kent et al., 2002).

(E) rh4prom>GFP is expressed at similar levels in the DT.

(F) rh4prom>GFP is expressed at similar levels in the VT.

(G) rh4prom>GFP is expressed at similar levels in tgo5 and wild-type yR7s. Panel 1, GFP, Rh4, and Rh3; panel 2, GFP alone; panel 3, Rh4 alone; panel 4, Rh3 alone.

The white circle indicates a tgo5 mutant yR7 that expresses rh4prom>GFP with both Rh3 and Rh4. The solid gray circle indicates a wild-type yR7 that expresses rh4prom>GFP with Rh4 alone. The dotted gray circle indicates a ph7 that expresses Rh3 alone.

(H) Quantification of rh4prom>GFP levels in tgo5 and wild-type yR7s. tgo5 mutant yR7s (magenta) express rh4prom>GFP at similar levels to wild-type yR7s (red). Error bars are ±1 SD around the mean.
allow IroC-mediated activation of Rh3, yet are sufficient to activate Rh4.

IroC activates Rh3 in the DT where Ss levels are lower (Figure 8H). In IroC mutants, even the low levels of Ss in the DT are sufficient to induce repression of Rh3 (indirectly through Dve) (Figure 8G). Reciprocally, high levels of IroC are sufficient to induce Rh3 in the main part of the retina despite the presence of normal levels of Ss (Figure 8I).

The regional regulation of rh3 also requires reduction of Ss (and Dve) levels in the DT. In wild-type animals, Ss levels are high in yR7s in the main region to induce repression of Rh3 and low in the DT to allow Rh3 expression (Figure 8B). Increasing the levels of Ss in the DT causes Rh3 repression in yR7s (Figure 8A). As the activity of Ss/Tgo is lowered in mutant conditions, Rh3 expression expands ventrally in the DE (Figure 8C) and then throughout the retina (Figures 8D–8F). This intermediate expansion into the DE may be explained by the dynamic nature of IroC expression. IroC is initially expressed in all cells of the dorsal half (DT and DE) of the retina and then becomes restricted to the DT (Mazzoni et al., 2008; Sato and Tomlinson, 2007; Tomlinson, 2003). Perhaps, residual levels of IroC and/or chromatin changes induced at the rh3 promoter increase the activation capacity of rh3 in the DE (Figures 8B and 8C). At low levels of Ss activity or with complete ablation, the general activators Sal and Otd induce expression of Rh3 in all R7s, including those in the ventral half (VE and VT) (Figures 8D–8F). These observations support the requirement for the modulation of Ss levels to ensure proper regional Rh3 regulation. The presence of multiple K50 (repressing via Dve and activating via Otd) and IroC (activating) binding sites in the rh3 promoter is consistent with the nature of this regulatory mechanism.

In contrast, the control of Rh4 appears to be much simpler: Ss/Tgo, even at low levels, induces Rh4 expression (Figure 8B), likely by directly binding the lone canonical XRE (Ss/Tgo binding site) in the rh4 promoter. Perturbations of Ss/Tgo that yield derepression of Rh3, do not affect Rh4 expression (Figures 8C and 8D). A subtle decrease in the frequency of Rh4 expression occurs in the DT where Ss levels are reduced only when Ss/Tgo activity is strongly impaired, likely because Ss/Tgo activity levels are near the threshold for activation (Figure 8E). Therefore, rh4 is highly responsive to Ss ensuring that it is expressed in yR7s in the DT where Ss levels are low.
Stochastic and Regional Inputs Control PR Subtypes

Ss expression is controlled by two main inputs: stochastic on/off regulation and regional modulation of levels. The random, binary input determines pR7 (Ss Off) versus yR7 (Ss On) fate whereas the regional input determines main region (Ss high) versus DT (Ss low) yR7 fate. Although iroC is considered the critical factor determining dorsal identity in the retina, it does not appear to control regional modulation of Ss levels. Rather, another mechanism must work in parallel with iroC to control aspects of dorsal identity.

Despite dramatic changes in the activity of Ss and Tgo, Rh4 remains expressed in ~65% of R7s. If the mechanism controlling stochastic subtype specification was dependent on Ss levels (e.g., via a feedback mechanism), a decrease in Ss/Tgo activity levels should cause a decrease in the frequency of Rh4-expressing yR7s with a concomitant increase in the number of Rh3-expressing pR7s. Here, we have shown that this is not the case: Ss levels do not play a role in determining the frequency of stochastic expression but rather are modulated to allow Rh3 expression in Rh4-expressing yR7s in the DT. Thus, the stochastic mechanism controlling Ss expression requires regulation of the Ss promoter independent of feedback.

Proper Rhodopsin expression requires tight regulation of the levels of stochastically-expressed Ss. If Ss levels were highly variable, we would expect to see derepression of Rh3 in other R7s throughout the retina. Instead, we only observe expression of Rh3 in DT yR7s consistent with our findings that Ss levels are specifically lower there.

Our ongoing promoter dissection reveals that, not surprisingly, ss is controlled by a cis-regulatory logic (R.J.J., Jr. and C.D., unpublished data). It will be interesting to see how the ss gene integrates these two dramatically different types of inputs to produce its complex expression pattern.

**EXPERIMENTAL PROCEDURES**

*Drosophila Strains and Crosses*

Flies were raised on standard corn-meal-molasses-agar medium and grown at 25°C. In the Supplemental Experimental Procedures, we list all shortened genotypes, complete genotypes, figures in which they are examined, and original source for each reagent.

*Antibodies*

Antibodies and dilutions used were as follows: mouse anti-Rh3 (1:10) (gift from S. Britt, University of Colorado), rabbit anti-Rh4 (1:100) (gift from C. Zuker, Columbia University), mouse anti-Rh5 (1:200) (Chou et al., 1996), rabbit anti-Rh4 (1:100) (gift from C. Zuker, University of Colorado), rabbit anti-Rh4 (1:100) (gift from C. Zuker, University of Colorado), rabbit anti-Sens (1:100) (Xie et al., 2007), rat anti-Sal (1:100) (Kim et al., 2006), rabbit anti-Dve (1:500) (Nakagoshi et al., 1998), mouse anti-prospero (1:10) (DSHB), rabbit anti-Tgo (1:1, concentrated 10X) (DSHB), rat anti-Sal (1:100) (Kim et al., 2006), rabbit anti-Dve (1:500) (Nakagoshi et al., 1998), mouse anti-Rh4 (1:200) (Chou et al., 1996), rabbit anti-Ss (1:10) (San, 2004), mouse anti-Dve (1:500) (Nakagoshi et al., 1998), mouse anti-prospero (1:10) (DSHB), ms anti-Tgo (1:1, concentrated 10X) (DSHB), rat anti-Eia/V (1:50) (DSHB), rabbit anti-Sns (1:100) (Xie et al., 2007), rat anti-Sal (1:100) (Barrio et al., 1999), guinea pig anti-Od (1:750) (Vandendries et al., 1996), sheep anti-GFP (1:500), and Alexa488 Phalloidin (1:80) (Invitrogen). All secondary antibodies were Alexa-conjugated (1:400) (Molecular Probes).

*Antibody Staining: Pupal and Adult Retinas*

Retinas were mounted in Slofade (adult retinas) or Vectashield (pupal retinas).

**Initial Screening of UAS>Ssmodified Lines with Water Immersion Microscopy**

We used the panR7->Gal4 (expressed in all R7a) and IGRMR->Gal4 (expressed in all PRs) drivers to induce UAS>Ssmodified expression in the eye and examined rhaprop-GFP expression. Flies were adhered to a Petri dish using nail polish and immersed in water. The retina was observed for GFP expression using a compound fluorescence microscope (40x lens) (Pichaud and Desplan, 2001). We tested a minimum of two independent lines and found consistent results among lines (Figures S2A–S2D). We selected a single line for each UAS>Ssmodified transgene for further analysis.

**Generating tgo Null Alleles**

The PwHy/tgogCas9/0 transposable element in tgo was used to generate null mutant alleles (Huet et al., 2002), yw; +; PwHy/tgogCas9/0T3A, TDM3 flies were crossed with yw; CyO, P(hsHT-2)/In(2L)Gla, wgG121, +; +. Parents were flipped after 1 day of egg laying. Progeny were heat shocked 30 min each day for 4 consecutive days. yw; cyo, P(hsHT-2)/+; PwHy/tgogCas9/0T3A, TDM3 progeny were crossed with yw; +; + and their progeny were screened for w y w y deletion stocks were established.

**Generating da Neuron Clones**

For MARCM, FRT +, FRT ssD178-7, FRT82b tgoD4, or FRT82b tgoD425 flies were mated to elav-GAL4, UAS->mCD8::GFP, hs->FPlP; FRT82b tubP-GAL80 flies (Lee and Luo, 2001). Embryos were collected for a 2 hr period and aged for 3 hr at 25°C. Embryos were then heat-shocked at 39°C for 50 min, allowed to recover for 30 min at 25°C, then heat-shocked again at 39°C for 45 min. Animals were reared at 18°C until the wandering larval stage, when GFP-positive clones were imaged. Morphology was analyzed in larval filet preparations (Ye et al., 2004) immunostained with 1:350 Alexa Fluor 488 rabbit anti-GFP (Invitrogen), mounted in 70% glycerol, and imaged on a Leica SP5 confocal microscope using a 40 x/1.25 NA oil objective. The total number of terminal branches was quantified in projections of individual ddaC neurons from the second through fifth abdominal segment as previously described (Lee et al., 2003). In Figure S1L, all error bars are ±1 SD around the mean.

**Transgenes tgoarm-GFP**

The tgo promoter was PCR amplified from wild-type flies (tgoprimer1: 5’-CTCAGATGATGGGATTGAGG-3’ and tgoprimer2: 5’-GAAACTACACCATGGAGG-3’) and cloned into the P-GEMT vector. The tgo promoter was subcloned into an attB vector containing nuclearGFP using PstI and BamHI. Constructs were inserted into the phi-C31 genome.

**Generating da Neuron Clones**

For MARCM, FRT +, FRT ssD178-7, FRT82b tgoD4, or FRT82b tgoD425 flies were mated to elav-GAL4, UAS->mCD8::GFP, hs->FPlP; FRT82b tubP-GAL80 flies (Lee and Luo, 2001). Embryos were collected for a 2 hr period and aged for 3 hr at 25°C. Embryos were then heat-shocked at 39°C for 50 min, allowed to recover for 30 min at 25°C, then heat-shocked again at 39°C for 45 min. Animals were reared at 18°C until the wandering larval stage, when GFP-positive clones were imaged. Morphology was analyzed in larval filet preparations (Ye et al., 2004) immunostained with 1:350 Alexa Fluor 488 rabbit anti-GFP (Invitrogen), mounted in 70% glycerol, and imaged on a Leica SP5 confocal microscope using a 40 x/1.25 NA oil objective. The total number of terminal branches was quantified in projections of individual ddaC neurons from the second through fifth abdominal segment as previously described (Lee et al., 2003). In Figure S1L, all error bars are ±1 SD around the mean.
transgenic (w*) lines were established. See the Supplemental Experimental Procedures for a list of primers used to generate UAS::ssmoded constructs.

\[ \text{rh3prom} \times \text{GFP} \]

The rh3 promoter was PCR amplified from yw flies and flanked by BglII and NotI sites (rh3promprimer1: 5'-AGATCTCGACTAATCCCTAGATGACG-3' and rh3promprimer2: 5'-GCGGCGCGTGTCGCGGCGCAAGAAGCTAATCGG-3') and cloned into the pGEM-T Easy vector.

\[ \text{rh3prom} \times \text{oc4mut} \]

The QuikChange mutagenesis kit (Stratagene) was used to mutate the IroC motif (rh3mutprimer1For: 5'-CCCAAACGGGTAATC-3' and rh3mutprimer2Rev: 5'-GCCAGCGAAAATGTCAGCAAGGGGCGAGGCCAATC -3') and cloned into the P-GEMT Easy vector. The QuikChange mutagenesis kit was used to mutate the IroC motif 2 in rh3prom IroC1234>GFP, and rh3prom IroC134>GFP were subcloned into an attB vector containing GFP and miniwhite as selectable marker using BglII construct.

\[ \text{oc4mut} \]

InoC motifs 1 and 2 were mutated by amplifying the wild-type promoter using modified oligos with mutations at the 5' and 3' end (J36attP vector containing GFP and miniwhite as selectable marker using BglII construct).

\[ \text{rh3prom} \times \text{NotI} \]

Constructs were inserted into the J36 attP site on the third chromosome using NotI sites (5'-5GATCTCGACTAATCCCTAGATGACG-3').

\[ \text{rh3prom} \times \text{GFP} \]

The QuikChange mutagenesis kit was used to mutate the IroC motif 2 in the rh3 promoter and cloned into the P-GEMT Easy vector. The rh3 promoter and the mutated rh3 promoters were subcloned into an attB vector containing GFP and miniwhite as selectable marker using BglII and NotI. Constructs were inserted into the J36 attP site on the third chromosome using phi-C31-mediated transgenesis (Bischof et al., 2007).

\[ \text{rh3prom} \times \text{K5mut} > \text{GFP} \]

The QuikChange mutagenesis kit was used to mutate the K50-1 (rh3mutprimer1For: 5'-GCGGCGCGTGTCGCGGCGCAAGAAGCTAATCGG-3' and rh3mutprimer1Rev: 5'-GATTACCGGCTTGGAATTTGCGCCCTTGCATTCTTGCGG-3') and/or K50-2 (rh3mutprimer2For: 5'-AGGCCCAATCCAAGGCGTGCCCGCGCGGCAAATC -3' and rh3mutprimer2Rev: 5'-TAGAATTTGCGGAGGCGGCCGCTTGGAATTTGCGG-3') to generate the rh3prom K5mut>GFP construct.

\[ \text{rh3prom} \times \text{rh3prom} \]

The rh3 promoter was PCR amplified from transgenic flies (rh3promprimer1: 5'-CTTGGTGAGTAGCAAATGTCGTCG -3' and rh3promprimer2: 5'-GTCGAAGCTCGACGAGAATGACG-3') and cloned into the P-GEMT Easy vector. The rh4 promoter was subcloned into PBS using BamHI and EcoRI. rh4promprimerFor (5'-CAATTACGTTGTGCTTGCGCGCGAGAAGCTATGTCGC -3') and rh4promprimerRev (5'-GAAAATCTGTGCGCGCCGAACGAAACAAAGCTAACT -3') were used to induce the point mutation. The rh4 promoter and the rh4 promoter (point mutation) were subcloned into an attB vector containing GFP using BamHI and EcoRI. Constructs were inserted into the J36 attP site on the third chromosome using phi-C31-mediated transgenesis (Bischof et al., 2007).

Quantification of Expression

Using cell-specific markers, antibody staining frequency was assessed for five or more retinas. Greater than 25 cells were scored per retina/region.

Identification of Molecular Lesion in ss and tgo Alleles

\[ \text{ss}^{AT16.4} \]

We PCR amplified and sequenced the coding regions of the ss gene. We identified a deletion causing missense mutations and early termination (Figure 5A).

\[ \text{tgo}^o \]

We PCR amplified and sequenced the coding regions of the tgo gene. We identified a missense mutation causing early termination (Figure 5B).

Dp(3;2)P10

We used next-gen whole genome DNA sequencing to identify the breakpoint of this duplication in the ss locus (3R: 12,201,754).

\[ \text{Tf}(1;3)\text{ssD114.3} \]

We used next-gen whole genome DNA sequencing to identify the breakpoints of this reciprocal translocation in the ss locus (3R: ~12,237,800) and X chromosome (XHet: ~192,100).

We generated imprecise P-element excision lines from the P(Switch2) GSG2553 P-element. We used next-gen whole genome DNA sequencing to determine that the allele contains an inversion (breakpoint in ss at ~12,230,000) and a deletion (3R: ~12,230,000–12,235,000).

\[ \text{ss}^{RS627} \text{shw}^{44} \]

We generated imprecise P-element excision lines from the P(RS3)CB-6279-3 P-element. We used next-gen whole genome DNA sequencing to identify the endpoints of the deficiency that lie in the region upstream of the ss coding region (3R: 12,237,765) and in the second intron of the Pak3 gene (3R: 12,274,176).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.02.016.

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