Supplemental Figures

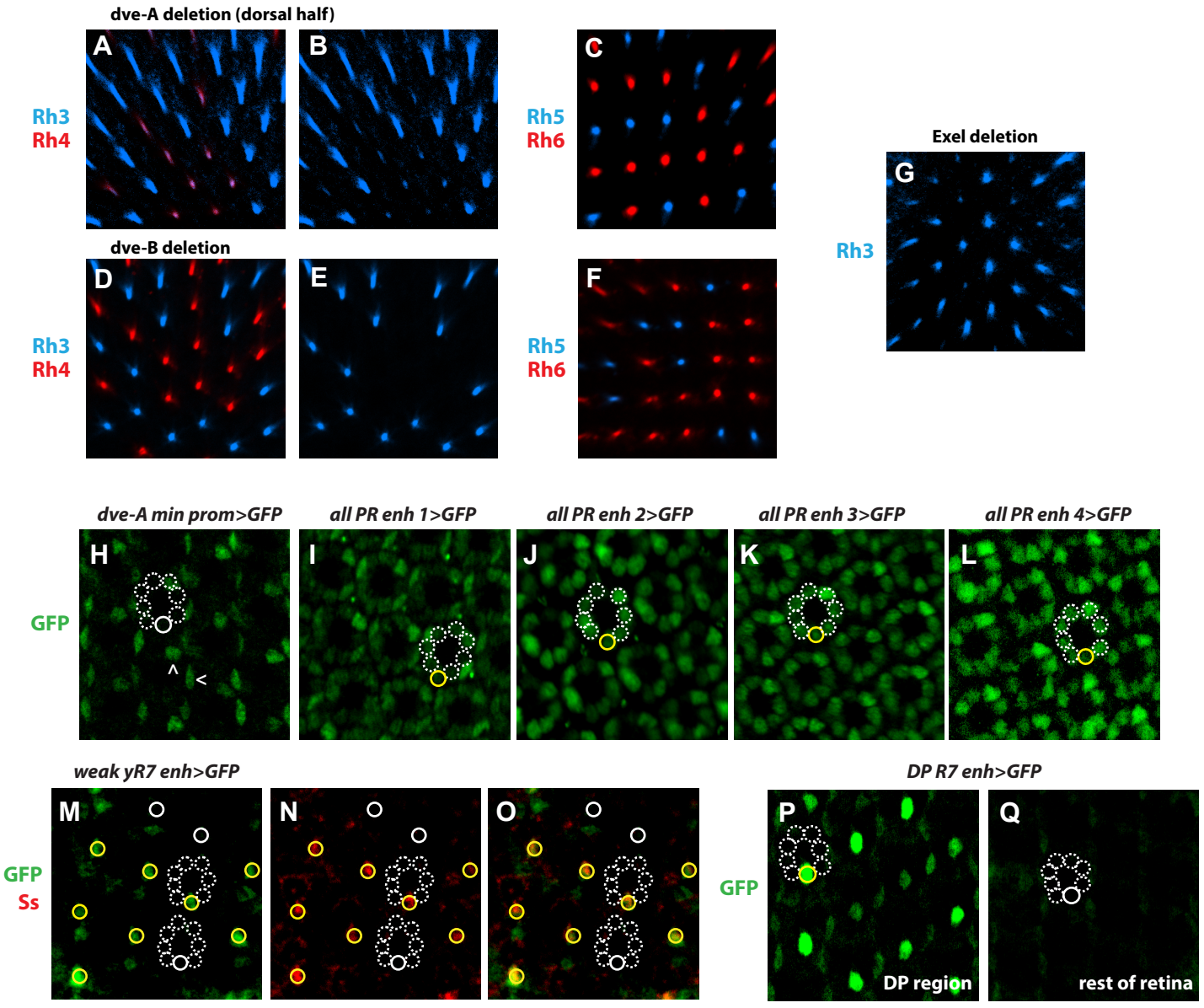

Figure S1. ***dve enh>GFP*** **constructs and** ***dve-A*** **and** ***dve-B*** **promoter deletions**

A-C. *dve-A* promoter deletion resulted in upregulation of Rh3 in all R7s in the dorsal half of the retina. Dve targets Rh5 and Rh6 displayed wild type expression.

D-F. *dve-B* promoter deletion displayed wild type expression of Dve targets (Rh3, Rh5, and Rh6).

G. dve^{exel} deletion, covering ***y****R7 enh* and the *dve-A* promoter, resulted in Rh3 upregulation in R7s.

H. *dve-A min prom* drove weak expression in R4s (denoted by ^) and pigment cells (denoted by <). Dashed white circles represent outer PRs and R8s; solid white circle indicates R7.

I-L. Four enhancers (*all PR enh 1-4*) drove weak GFP expression in all PRs. Dashed white circles represent PRs. Yellow circles denote GFP-expressing R7s.

M-O. *weak* ***y****R7 enh* displayed weak GFP expression in **y**R7s; Ss is a marker for **y**R7s. Dashed white circles represent outer PRs and R8s, solid white circles denote **p**R7s, and solid yellow circles denote **y**R7s.

P-Q. *dorsal R7 enh* drove expression in R7s in the dorsal posterior (DP) region of the retina. Dashed white circles represent PRs. Yellow circles indicate GFP-expressing R7; solid white circle indicates non-expressing R7.

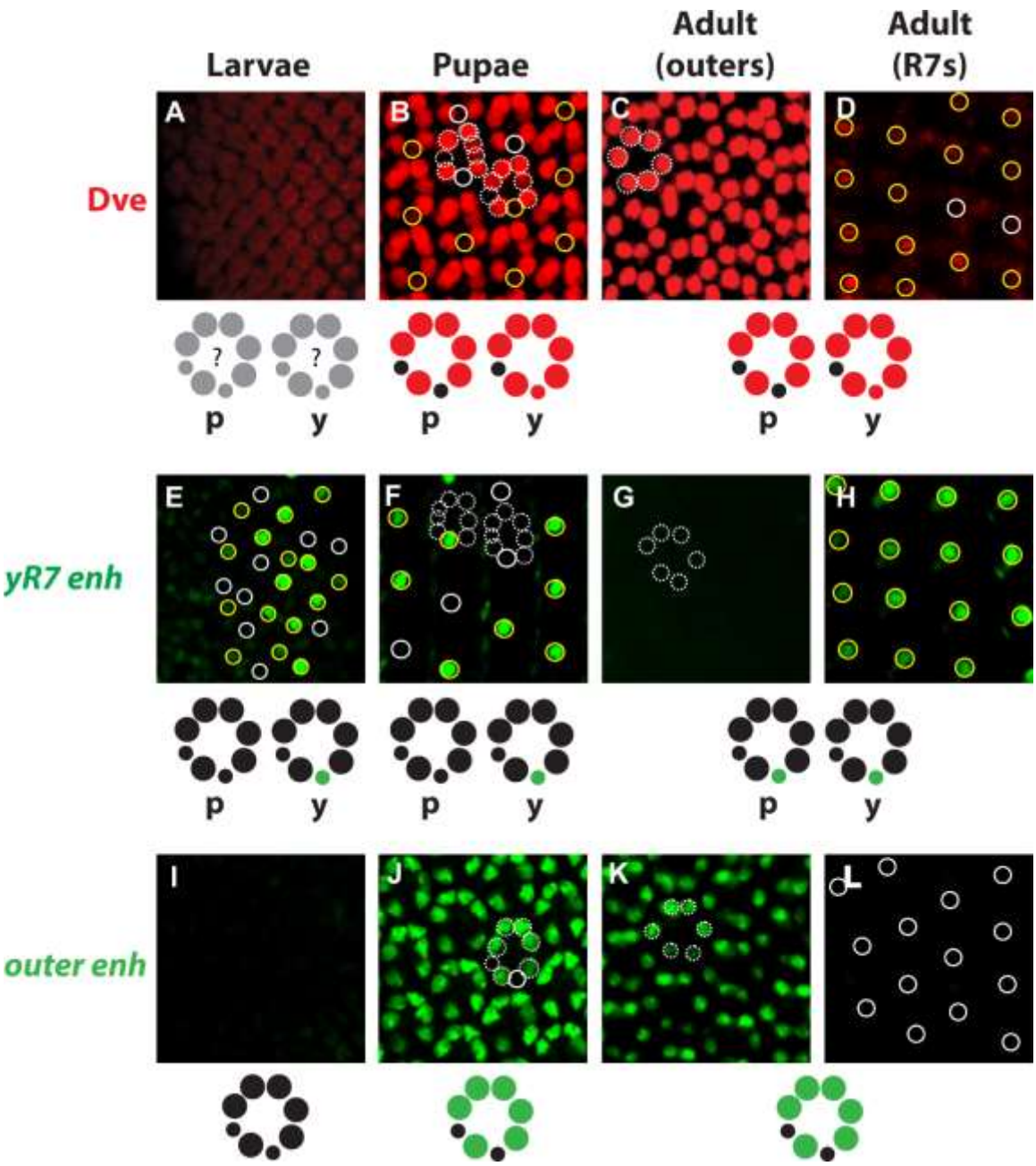

Figure S2. **Differential expression of Dve throughout development**

For A-L, in schematics, red circles indicate endogenous Dve expression, green circles indicate GFP expression, black circles indicate no expression (endogenous Dve or GFP), and gray circles indicate indeterminate expression.

For B-D, yellow circles indicate **y**R7 cells. Solid white circles indicate **p**R7 cells. Dashed white circles are outer PRs and R8s.

For E-H, yellow circles indicate **y**R7 cells. Solid white circles indicate **p**R7 cells. Dashed white circles are outer PRs and R8s.

For J-L, dashed white circles represent outer PRs; solid white circles indicate R7s.

A. Antibody staining for Dve is nonspecific in larvae.

B-D. Dve is highly expressed in outer PRs and weakly expressed in **y**R7s in pupae and adults.

E-F. ***y****R7 enh* is expressed in **y**R7s in larvae and pupae.

G-H. ***y****R7 enh* is expressed in all R7 cells in the adult but is not expressed in outer photoreceptors.

I. *outer enh* is not expressed in larvae.

J-L. *outer enh* drives expression in outer PRs in pupae and adults, but is not expressed in R7s.

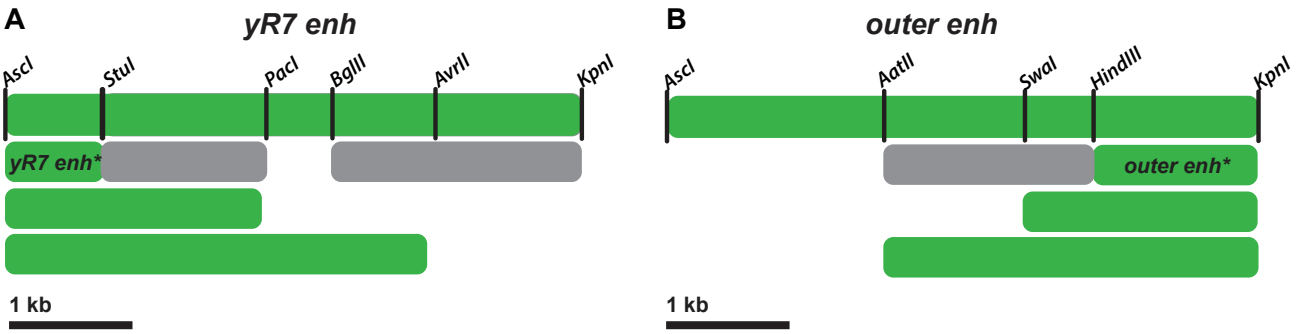

Figure S3. Restriction enzyme sites used to generate *dve enh* truncations

A. Schematic of the ***y****R7 enh* and the restriction enzyme sites used to generate the ***y****R7 enh** and other truncations.

B. Schematic of the outer enh and the restriction enzyme sites used to generate the *outer enh** and other truncations.

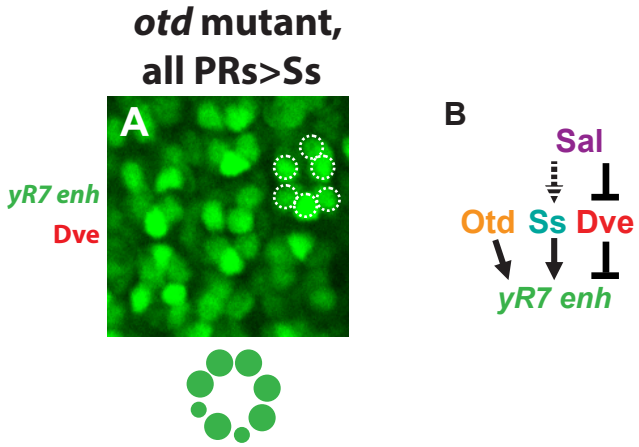

Figure S4. Sal activates yR7 enh by repressing Dve

A. In *otd* mutants, in which Dve is not expressed, ectopic Ss induces ***y****R7 enh* in all PRs.

B. The regulatory interactions governing ***y****R7 enh.* Otd and Ss activate ***y****R7 enh*, while Dve represses ***y****R7 enh*. Sal activates stochastic expression of Ss (denoted by dashed arrow) in **y**R7s and represses Dve.

Supplementary Materials and Methods

Generating *dve enh>GFP* constructs

3-6kb fragments (Fig. 2A) were amplified using DNA isolated from yw^{67} flies and ligated into the pGEM-T easy vector. The 699 bp *dve* minimal promoter was subcloned into a pJR16 nGFPcDNA reporter vector containing a w^{+} marker, generating the pGG14 vector. Other fragments of the *dve* locus were subcloned into pGG14 before microinjection into fly embryos. Constructs were then integrated into the genome via the attP/B system, and injected flies were crossed with a balancer stock with the genotype $y^{-}w^{-}$*; +/+; Tm2/Tm6b*. Red-eyed offspring were isolated, and transgenes were balanced over Tm6b. Primers and restriction enzymes used to generate each *dve* enhancer construct are shown in Table S1.

Enhancer constructs ***y****R7 enh>GFP* and *outer enh>GFP* were further truncated using restriction enzymes and blunt end ligation (Fig. S3A,B). Reporter vectors were microinjected into fly embryos, and transgenic lines were established using the same methods as above.

Drosophila strains

Flies were raised on standard cornmeal medium and grown at room temperature (25^{o}C). Transgenic lines used include *dve* enhancer gene constructs generated for this project, as well as the reagents in Table S2.

The GAL4-UAS system was used to ectopically express Sal, Ss, and Dve (Brand and Perrimon, 1993), while the FLP-FRT system was used to create *sal* and *dve* mutant phenotypes (O'Gorman et al., 1991). Shortened and complete genotypes of flies examined are found in Table S3.

Janelia and VDRC Stock Centers generated transgenic lines that express GAL4 driven by flanking non-coding or intronic regions of various genes. GAL4 lines

associated with *dve **y**R7* and *dve outer* enhancer elements were crossed with UAS-nlsGFP. See Table S4.

CRISPR-generated deletions

dve-B promoter, *outer enh*, and ***y**R7 enh* deletions were generated using CRISPR. We designed four gRNAs per deletion, two flanking either side of the deletion. Forward and reverse strands of gRNAs were designed and annealed together to have BbsI restriction site overhangs. gRNAs were then cloned into the pCDF3 cloning vector. Single stranded homologous bridges were generated with 80 bp homologous regions flanking each side of the deletion. An AscI restriction cut site was incorporated into the homologous bridge to facilitate screening. For every deletion, all four gRNAs were injected into *Drosophila* embryos at 125ng/ul each for a total of 500ng, along with 100ng/ul of homologous bridge oligos. Single adult males were then crossed with balancer stocks (yw ; if / cyo ; +), and the progeny were screened for the deletion via PCR. Homologous bridges, gRNAs, and PCR screening primers are shown in Table S5.

Otd/Dve binding site knockout

Otd/Dve binding site knockouts for *outer enh* and ***y**R7 enh* were generated using site-directed mutagenesis, where the K50 homeodomain consensus sites (TAATCC) were replaced with AAAAAA. Constructs were integrated into the genome and *Drosophila* strains were established using the same methods as described above.

Electrophoretic Mobility Shift Assay

Binding assays were performed as previously described (Johnston et al., 2011; Li-Kroeger et al., 2008).

Probes tested were as follows (bold/underline indicates K50 Otd/Dve site or mutated K50 Otd/Dve site):

***y**R7 enh* 1 5' –CGTGTTAGCCAAACC**TAATCC**AGGCTAAACGAGGG- 3'
***y**R7 enh* 2 5' –AAATACGCTTATGTC**GGATTA**TCCCATAATTTATG- 3'
***y**R7 enh* mutant 1 5' –CGTGTTAGCCAAACC**TGCGCC**AGGCTAAACGAGGG- 3'
***y**R7 enh* mutant 2 5' –AAATACGCTTATGTC**GGCGCA**TCCCATAATTTATG- 3'
outer enh 1 5' –AGCAAACAACAAAAA**GGATTA**AGTCCAAGACACAC- 3'
outer enh 2 5' –ATACTTATTTCATTA**GGATTA**TTTTTGACTAACAT - 3'
outer enh 3 5' –TCACGGCATTAATTA**TAATCC**GCTTAAAAGTTTCA - 3'
outer enh 4 5' –TCACACAAGGATTCG**TAATCC**TTGCGAGGGACCCA- 3'
outer enh mutant 1 5' –AGCAAACAACAAAAA**GGCGCA**AGTCCAAGACACAC- 3'
outer enh mutant 2 5' –ATACTTATTTCATTA**GGCGCA**TTTTTGACTAACAT- 3'
outer enh mutant 3 5' –TCACGGCATTAATTA**TGCGCC**GCTTAAAAGTTTCA- 3'
outer enh mutant 4 5' –TCACACAAGGATTCG**TGCGCC**TTGCGAGGGACCCA- 3'

Antibodies

Antibodies and dilutions used were as follows: mouse anti-prospero (1:10)(DSHB), rat anti-Elav (1:50)(DSHB), sheep anti-GFP (1:500), mouse anti-Rh3 (1:100)(gift from S. Britt, University of Colorado), rabbit anti-Rh4 (1:100)(gift from C. Zuker, Columbia University), mouse anti-Rh5 (1:2000)(Tahayato et al., 2003), rabbit anti-Rh6 (1:2000)(Tahayato et al., 2003), guinea pig anti-Ss (1:200)(Gift from Y.N. Jan, University of California, San Francisco), rabbit anti-Dve (1:500)(Nakagoshi et al., 1998). All secondary antibodies were Alexa-conjugated (1:400) (Molecular Probes).

Retina dissection and immunohistochemistry

Retinas were dissected and stained as described previously (Hsiao et al., 2012). Larvae were collected and dissected in ice cold PBS (1x), and retinas were isolated using forceps before fixing for 20 minutes in 4% formaldehyde at RT. Samples were washed three times with PBX and kept in primary antibodies diluted in PBX overnight at 4^{o}C. After three washes with PBX, secondary antibodies diluted in PBX were added, and samples were kept at RT for at least 2 hours. After three more washes, samples were kept in PBX at room temperature overnight, before being mounted flat in Vectashield (Vector Laboratories).

To facilitate pupae collection at the desired mid-pupae stage, flies were raised at 25^{o}C in a 12hr light/12hr dark cycle incubator. Pupae heads were dissected in ice cold PBS (1x) and eye-brain complexes were extracted via pipetting. Fixing, antibody staining and mounting procedures were consistent with those of larvae, but pupal retinas were not isolated from the brain until prior to mounting.

Adult flies were anesthetized on CO_2 pads before their heads were removed using forceps. Fly heads were dissected in ice cold PBS (1x), and retinas were isolated using forceps. Fixing and antibody staining procedures were consistent with those of larvae and pupae, although laminas were not removed until after fixing. Retinas were then mounted using SlowFade Gold Reagent (ThermoFisher Scientific).

For all stages of fly development, samples stained with antibodies were visualized under a Zeiss LSM 700 confocal microscope.

Quantification

Fluorescence intensity of nuclear GFP expression of single retinas was quantified using the ImageJ processing program. A small region in the center of each nucleus was selected for fluorescence intensity measurement. Images were taken under subsaturating conditions and comparisons of GFP intensity were drawn between cells of the same retina. Column scatterplots were generated using Graphpad Prism.

Table S1. *dve enh>GFP* Constructs and Primers

Construct	Primers	Restriction sites
***y**R7 enh*	agtcggcgcgcccacaaccatttcactcctgc agtcggtacccttctcccagtcttcgaatg	*AscI* *KpnI*
outer enh	agtcggcgcgcctcatcctcatccctacctac agtcggtaccacaactgcctttgccttgtg	*AscI* *KpnI*
***y**R7 enh extended to right*	agtcggcgcgccgcctagctaccgtgatcaac agtcggtaccgtttagctcgattacgcttc	*AscI* *KpnI*
dve min promotor	agtcagatcttgatctggctctctggactc agtcggatccgtgggaaagtgttggtaagc	*BglII* *BamHI*
*weak **y**R7 enh*	agtcggcgcgcccggtcagcaggtgagttgag agtcggtacctacgatgacaccgataagcg	*AscI* *KpnI*
dorsal R7 enh	agtcggcgcgcccataatcacaacacgagtcgg agtcggtaccgatggtggcttaactcaatc	*AscI* *KpnI*
all PR enh 1	agtcggcgcgccgcttatctgcggctttgtgg agtcggtaccctcgtcttgtcccgattcca	*AscI* *KpnI*
all PR enh 2	agtcggcgcgccgctagcgcatagagcatagatg agtcggtaccgttgctggcaccaatacacg	*AscI* *KpnI*
all PR enh 3	agtcggcgcgccgtgctgcctacaagtttgga agtcggtaccgccttctgaagactagcac	*AscI* *KpnI*
all PR enh 4	agtcggcgcgcccgaaactcctcgactcacac agtcggtaccccaattcgctgattg	*AscI* *KpnI*
dve enh 1	agtcggcgcgcccactgacatcaattcaccgtc agtcggtaccaggagaaaggagtgagttcg	*AscI* *KpnI*
dve enh 2	agtcggcgcgccccatcccttagagagctttg agtcggtacctgtatctggggaatcggatg	*AscI* *KpnI*
dve enh 3	agtcggcgcgccgccacaatgtcaagcatcaaag agtcggtacccactttcccacagtatcatcttg	*AscI* *KpnI*
dve enh 4	agtcggcgcgcccagagctgaactgaacaatc agtcggtacccgtgtctctgcgctttgtga	*AscI* *KpnI*
dve enh 5	agtcggcgcgccgtcttagtgcagctactgtt agtcggtaccgaaggcttacgaaactaatg	*AscI* *KpnI*
dve enh 6	agtcggcgcgcccagctcgttaagcataagca agtcggtacccgttcccaaattaccccatc	*AscI* *KpnI*
dve enh 7	agtcggcgcgcctggtggtggcgattcatttg agtcggtaccctaccacaaaactagagcaccc	*AscI* *KpnI*

Table S2. *Drosophila* reagent descriptions

Name	Description	Source
otd^{uvi}	hypomorphic allele, fails to produce protein product in the eye	(Vandendries et al., 1996)
FRT40 sal$^{Df(2L)32FP5}$	a deficiency that removes the *sal* gene	(Barrio et al., 1999)
FRT42d dve^{186}	Dve protein null mutant	(Terriente et al., 2008)
FRT40 GMR-hid, cL	eye-specific enhancer driving hid, an activator of apoptosis	Bloomington
FRT42d GMR-RFP	eye-specific enhancer driving RFP	Bloomington
ey-FLP	eye-specific enhancer driving flippase	Bloomington
ss$^{d115.7}$	Ss protein null mutant	(Duncan et al., 1998)
ss$^{Df(3R)Exel7330}$	Deficiency covering the *ss* locus	Bloomington
UAS-Sal	UAS enhancer driving Sal	(Kuhnlein and Schuh, 1996), (Wernet et al., 2003)
UAS-Dve	UAS enhancer driving Dve	(Nakagoshi et al., 1998)
UAS-Ss	UAS enhancer driving Ss	(Duncan et al., 1998)
lGMR-Gal4	eye-specific enhancer driving Gal4	Bloomington
dve^{Exel}	deletion that removes the first exon of *dve, dve-A* promoter, and *dve* ***y****R7* enhancer element	See footnote*
dve^{E181}	deletion allele for the *dve-A* promoter	(Nakagawa et al., 2011)
UAS-nlsGFP	UAS driving nuclear GFP	Bloomington

*We generated the dve^{exel} deletion by using hsFLP-mediated recombination between two FRT sites, inserted by P-elements P(XP)d05100 and P(XP)d08355.

Table S3. *Drosophila* shortened and complete genotypes

Shortened	Complete genotype	Figure
dve enh>GFP	*yw ; + ; dve enh>GFP*	2B-E, 3A, 5A, S1H-Q, S2E-L
all PRs>Ss	*yw ; lGMR>Gal4, UAS>Ss ; dve enh>GFP*	3E, 5D
all PRs>Sal	*yw, UAS>sal ; lGMR>Gal4 ; dve enh>GFP*	3G
all PRs>Dve	*yw ; lGMR>Gal4 ; dve enh>GFP/ UAS>dve*	4A, 6A
sal mutant	*yw ;sal$^{Df(2L)32FP5}$FRT40 GMR>hid FRT40 ; dve enh>GFP/ey>Flp*	3F, 5C
otd mutant	*otd^{uvi} ; + ; dve enh>GFP*	3B, 5B
dve mutant	*ey>FLP ;FRT42d dve^{186}/ FRT42d GMR>RFP ; dve enh>GFP/ +*	4B-E, 6B-C
ss mutant	*yw ; + ; dve enh>GFP, ss^{BL7985def}/ ss$^{d115.7}$*	3C
all PRs>Ss and Sal	*yw, UAS>sal ; lGMR>GAL4, UAS>ss ; dve enh>GFP*	3H
otd mutant, all PRs>Ss	*otd^{uvi} ; lGMR>GAL4, UAS>ss ; dve enh>GFP*	S4A
dve-A del	*yw ; FRT42d* dve^{186} / dve^{E181} *; +*	S1A-C

Table S4. Janelia and VDRC Gal4 stock numbers

Janelia GMR			
49373	49927	48655	46230
45702	50133	45682	46238
45284	46241	50066	48150
41238			
VDRC Stock			
020724	020725	020737	020739

Table S5. Primers for CRISPR

dve-B del	
Homologous bridge	ttttatggatcgcttggcattataatgaacagcggcgtcgccggctggccatgggcgcatggcgc gcccatgggagcaagttggagctgggcaagcccccacatcccatccgcccactgacctaagc c
dveBgRNA1 F	gtcgctggccatgggcgcataat
dveBgRNA1 R	aaacattatgcgcccatggccagc
dveBgRNA2 F	gtcgggataagtacggtgcatgg
dveBgRNA2 R	aaacccatgcaccgtacttatccc
dveBgRNA3 F	gtcgtcatccttccagtgcccat
dveBgRNA3 R	aaacatgggcactggaaggatgac
dveBgRNA4 F	gtcggtgtctgccactgttgaac
dveBgRNA4 R	aaacgttcaacagtggcagacacc
DveBscr F	gctgttgggagattaagttt
DveBscr R	tgccttctgaagactagcac
outer enh del	
Homologous bridge	gctgcctgggcgtccttttctcgggcacttgatagaatttgacaaattgaaaatccttttggcgcgcc gaagcctacttaagtcccttgaaatccttgagatttttgcactggtcaagcaatgataa
outergRNA1 F	gtcggacaaccgctcgccacaaa
outergRNA1 R	aaactttgtggcgagcggttgtcc
outergRNA2 F	gtcgttcaagagtccaggcgacc
outergRNA2 R	aaacggtcgcctggactcttgaac
outergRNA3 F	gtcgaaattaagcaatagtctta
outergRNA3 R	aaactaagactattgcttaatttc
outergRNA4 F	gtcggacttaagtaggcttccca
outergRNA4 R	aaactgggaagcctacttaagtcc
outer-scr F	ccagtgattatgtatggttc
outer-scr R	gagtgatttgggtatttagg
yR7 enh del	
Homologous bridge	acttgctccccgtccgtcgatcgattcaaattaccagcgatttattggcgatcgccagccggcgcg ccgctatggcaatgcaaacaggtgagggtgaattacttgtcctagacaactttgcagtcagc
yR7gRNA1 F	gtcgttgatcacggtagctaggc
yR7gRNA1 R	aaacgcctagctaccgtgatcaac
yR7gRNA2 F	gtcgtgttcgcataacgctggtc
yR7gRNA2 R	aaacgaccagcgttatgcgaacac
yR7gRNA3 F	gtcgtttagctcgattacgcttc
yR7gRNA3 R	aaacgaagcgtaatcgagctaaac
yR7gRNA4 F	gtcgtttgcattgccatagctac
yR7gRNA4 R	aaacgtagctatggcaatgcaaac
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