

METHODS

Drosophila strains and crosses

Flies were raised on standard corn-meal-molasses-agar medium and grown at room temperature (RT) ($24\pm 1^\circ\text{C}$) unless otherwise noted. All genetic symbols not described in the text are in the *Drosophila* reference works. *y w* flies were used as control for *rh* expression. When a transgenic *white* was introduced in the genetic background, the color of the eyes were rendered white by using homozygous *cn br* flies or an RNAi construct against *white*⁴³. The *ss*^{D115.7} and UAS-*ss* were described in²⁴. *Long multimer glass reporter-Gal4* (LGMR-Gal4) is similar to the original GMR-Gal4 described in³¹ (here called sGMR for short), but each monomer is 38bp long (generous gift from B Mollereau). PanR7-Gal4 is a combination of *rh3* (-160/-56) and *rh4* (-63/+85) promoters, driving Gal4 (A. Tahayato). This driver is expressed in R7 cells and DRA R8 cells.

Antibodies

Antibodies and dilutions used were: mouse anti-Rh3 (1:100) (gift from S Britt, University of Colorado), rabbit-anti-Rh4 (1:400) (gift from C. Zucker, University of California at San Diego), mouse anti-Rh5 (1:50) (gift from S Britt, University of Colorado), rabbit anti-Rh6 (1:5000), rabbit anti- β Gal (1:5000) (Cappel), mouse anti- β Gal (1:500) (Promega), guinea pig anti-Sens (1:750) (H. Bellen, Baylor College of Medicine), mouse anti-pros (1:50) (DSHB), rabbit anti-Sal (1:100) (B. Mollereau, Rockefeller University), mouse anti-chaoptin, 24B10 (1:10) (DSHB) and sheep anti-GFP (1:800) (Biogenesis). All secondary antibodies were Alexa-conjugated (1:800) (Molecular Probes).

Generation of transgenic flies by germ line transformation

Transgenic lines were generated by injection of freshly purified plasmid DNA at a concentration of $0.3\ \mu\text{g}/\mu\text{l}$ into ~ 250 embryos of 0-10 minutes of age using standard procedures. Single G_0 flies were separately crossed with the balancer stock *wr135* (*w*¹¹¹⁸; *CyO* / *Sp* ; *TM2* / *MKRS*) and transgenic flies were

selected in F1 according to their eye color. Insertions were mapped and balanced again using balancer *wr135* flies by following the standard chromosome markers.

Constructs

The *ss* 'eye enhancer' / *ss*(E1.6) was identified as a ~1.6 kb *EcoRI* fragment that drives expression in the eye/antennal imaginal disc (D. Duncan and I. Duncan, in preparation). The eye enhancer was mapped initially using *ss-lacZ* reporters generated in the Duncan laboratory by Richard B. Emmons. This DNA fragment was ligated into the previously designed fly injection vector *pCasper[hs43-GAL4-SV40]* using *EcoRI* sites.

Antibody stainings on eye imaginal discs

Cerebral complexes of *Drosophila* late third instar larvae were dissected in PBS (1x) and fixed in PBS + 4% formaldehyde for 20 min at RT. After four washes in PBT (PBS + 0.3% Triton X-100), the first antibody was added overnight at 4°C. After four washes with PBT, the secondary antibody was then added for at least 2 h at RT. After another four washes in PBT, each eye imaginal disc was separated from the head skeleton by using two tungsten needles and then mounted in Aquamount (Lerner Laboratories).

Antibody stainings on mid-pupal retinas

Pupal cases were collected at 46, 48, 50 and 52 h after puparium formation at $25 \pm 1^\circ\text{C}$ and the head was dissected under ice cold PBS (1x). Several eye-brain complexes were extracted by gentle pipetting and collected in PBS (1x) on ice. After 20 min of fixation using PBS (1x) + 4% formaldehyde at RT, the samples were washed four times with PBT. The first antibody was added overnight at 4°C. After four washes with PBT, the secondary antibody was added for at least 2 h at RT. After another four washes in PBT, each retina was separated from the brain by using two tungsten needles and then mounted flat in

Vectashield (Vector Laboratories).

Antibody stainings on frozen fly head sections

10 μ m horizontal eye sections were performed using a cryostat (Zeiss) and deposited on Superfrost PLUS slides (Fisher). After drying at -20°C , the slides were then fixed 15 minutes in PBS (1x) + 4% formaldehyde at RT. Fixation was slightly different for anti-24B10 (chaoptin) stainings: Heads of anesthetized flies were cut, the proboscis was removed and the heads were fixed in PBS (1x) + 2% formaldehyde for 90 minutes at 4°C . The heads were washed and incubated in PBS (1x) + 12% sucrose overnight. After four washes with PBT, the first antibody was added overnight at 4°C . After four washes with PBT, the secondary antibody was added for at least 2 h at RT. After four washes with PBT, the slides were mounted in Aquamount.

Antibody stainings on adult whole-mounted retinas

Adult retinas were dissected out and after a rinse with PBS (1x) the retinas were fixed for 15 min with 4% formaldehyde at RT. After 3 washes in PBT the retinas were incubated with the primary antibodies diluted in BNT (PBS, 0.1% BSA, 0.1% Tween-20, 250 mM NaCl) overnight at 4°C . Following 2 rinses and a 30 min wash with PBT, the retinas were incubated with secondary antibodies for 2-4 h at RT. Two quick rinses with PBT were followed by an overnight wash at 4°C . Retinas were cleaned of any remaining cuticle and mounted in Vectashield.

In situ hybridization on pupal retinas

Pupal retinas were dissected as described for antibody stainings. Dissected retinas were mounted on Superfrost PLUS slides and dried for 2 h at 65°C . After fixation for 15 min with 4% paraformaldehyde, the slides were washed in PBS, Proteinase K treated for 5 min at 37°C and refixed for 10 min. Following a short PBS wash, the slides were treated with 0.2 M HCl for 10 min, washed in PBS and acetylated with 0.1M triethanolamine. Retinas were hybridized overnight at 65°C with 100 μ l hybridization buffer (50% formamide, 5x

SSC, 5x Denhardt's, 250 µg/ml yeast tRNA, 500 µg/ml herring sperm DNA, 50 µg/ml heparin, 2.5 mM EDTA, 0.1% Tween-20, 0.25% CHAPS) containing a fluorescein-labeled ss probe. After a series of washes in 5x SSC; 50% formamide, 2x SSC; 2x SSC; 0.2x SSC, and 0.1x SSC (5, 30, 20, 20, 20 min, respectively) the probe was detected using the TSA™ Biotin System (Perkin Elmer) and streptavidin-Alexa488 according to the manufacturers suggestions.

***In situ* hybridization and antibody staining of pupal retinas**

After *in situ* hybridization the sections were incubated with the appropriate antibodies overnight. Washes and detection with secondary antibody was as described for antibody stainings on frozen fly head sections.

MARCM

To generate single mutant R7 cells the MARCM system was used²⁹. After FRT mediated recombination, the transcriptional repressor Gal80 is removed from the *ss*^{D115.7} mutant cells, allowing expression of UAS-Syb:GFP under the control of PanR7-Gal4 as a positive clonal marker. Because GMR-flp was used as a source of Flipase³⁰, only cells originated after the second mitotic wave, R1, R6 and R7 can be homozygous for *ss*^{D115.7}. The activity of the PanR7-Gal4 is apparently higher than the *tub*-Gal80 situated in the FRT chromosome since flies older than one day express GFP in almost all R7 cells even in the presence of Gal80. Thus, we dissected very late pupa for our MARCM experiments.

"cornea neutralization" technique

The deep pseudopupil was neutralized by water immersion so that EGFP signals coming from individual ommatidia could be resolved. Living flies were anesthetized and immobilized on warm agarose (~50°C). After solidification, the flies were covered with a film of water. The eyes were then analyzed under blue illumination using an immersion microscope with a blue light source^{44,45}.

Plastic sections of adult fly heads

Eyes were fixed with 2% glutaraldehyde (sometimes plus 1% OsO₄) in 0.05 M Na-cacodylate buffer (pH 7.2) for 2 h at 4°C. Following post-fixation with 2% OsO₄ in 0.05 M Na-cacodylate buffer (pH 7.2) for 2 h at 4°C, the tissue was dehydrated with 2,2-dimethoxypropane and embedded in Epon 812. 1µm sections for light microscopy were stained with methylene blue.

Scanning electron microscopy of adult fly heads

Fly heads were dehydrated by a series of ethanol washes (12-24 h each): 25%, 50%, 75% and 100% (2x). Samples were then dried using a critical point drier. Finally, samples were mounted and sputter coated with platinum and analyzed using a standard scanning electron microscope.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1

(A) Three ommatidial subtypes can be distinguished in the *Drosophila* retinal mosaic. Outside the DRA (pink), two additional subtypes can be identified based on molecular markers (yellow and blue). Their stochastic distribution is simulated.

(B) Ommatidial subtypes in *Drosophila*. Left: yellow (y) ommatidia (~70%) express *rh4* in R7 (yellow) and *rh6* in R8 (green). Center: pale (p) ommatidia (~30%) express *rh3* in R7 (red) and *rh5* in R8 (blue). Right: DRA ommatidia (pink) have larger rhabdomere diameters, are monochromatic and express *rh3* in both R7 and R8. L = lamina; M = medulla.

(C) Current model for specification of **p** & **y** ommatidia can be divided into two steps: first, **p** and **y** subtypes are established in R7 (left) by promoting ~30% of the cells from a **yR7** 'default state' (*rh4*, yellow) into the **pR7** fate (*rh3*, red). In a second step, **pR7** cells (red) impose their subtype choice onto the underlying R8 cell, which subsequently adopt the **pR8** fate (*rh5*, blue). **yR8** cells (*rh6*, green) do not require instruction, as Rh6 is the R8 default opsin (does not require R7).

Supplementary Figure 2

(A) Adult whole mutant eyes lacking *ss* have normal eye morphology as documented by scanning electron microscopy (arrow = *aristapedia* phenotype).

(B) Plastic section through an adult retina with mitotic clones homozygous for *ss* (marked by the absence of eye pigment). No difference in PR morphology is detectable between homozygous (-/-) and heterozygous (+/-) tissue.

(C) PR cell fates get specified correctly in *ss* mutants. Left: Inner PRs (marked by Spalt, green) develop normally in *ss* mutant clones (marked by absence of Arm-lacZ, red) in a pupal retina (~55% pupation). Similarly, expression of the R7 marker Pros (right, green), the R8 marker Sens (left, green) and the DRA marker Hth (right, green) is unaffected in mitotic clones lacking *ss*.

Supplementary Figure 3

(A) With the exception of the DRA, expression of the **yR7** opsin reporter *rh4-lacZ* (green) is expanded into all PRs (labeled by 24B10, red) when *ss* is over-expressed. Within the DRA, inner PRs which project axons to the dorsal-most medulla (white arrow), do not express *rh4* (L = lamina; M = medulla).

(B) Expression of the **yR8** opsin reporter *rh6-lacZ* (green) is unaffected by *ss* over-expression (24B10, red; L = lamina; M = medulla).

(C) Ectopic *ss* does not affect specification of inner PRs (labeled by Spalt, red) and outer PRs (*svp-lacZ*, green) as visualized on pupal retinas (~55% pupation). Left: wild type; Right: over-expression of *ss* under GMR-control (IGMR > *ss*).

(D) Ectopic *ss* does not affect specification of R7 (labeled by Pros, red) and R8 cells (Sens, green) as visualized on pupal retinas. Left: wild type; Right: *ss* gain-of-function.

Supplementary Figure 4

(A) Late mis-expression of *ss* in all R7 cells (PanR7>*ss*) leads to re-specification of all R7 into the **yR7** fate, expressing Rh4 (cyan), while Rh3 (red) is lost as visualized on frozen sections.

(B) In the wild type, Rh4 (cyan) is always excluded from the DRA inner PRs as marked by Hth (red). Arrows mark the DRA.

(C) The DRA ommatidia of PanR7>*ss* flies express Rh4 (cyan) in both R7 and R8, as visualized by double labeling with Hth (red) on frozen sections (arrows).

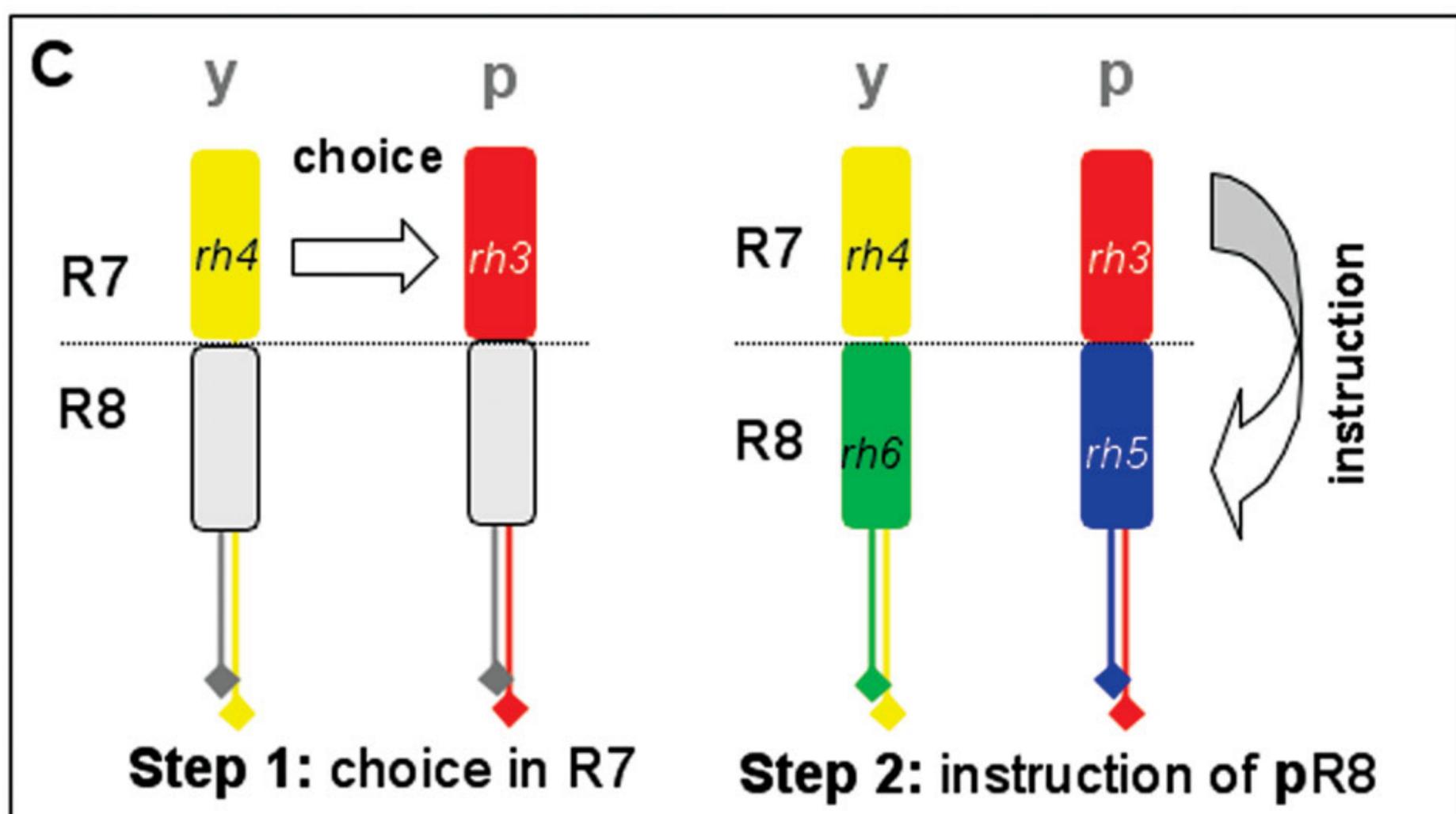
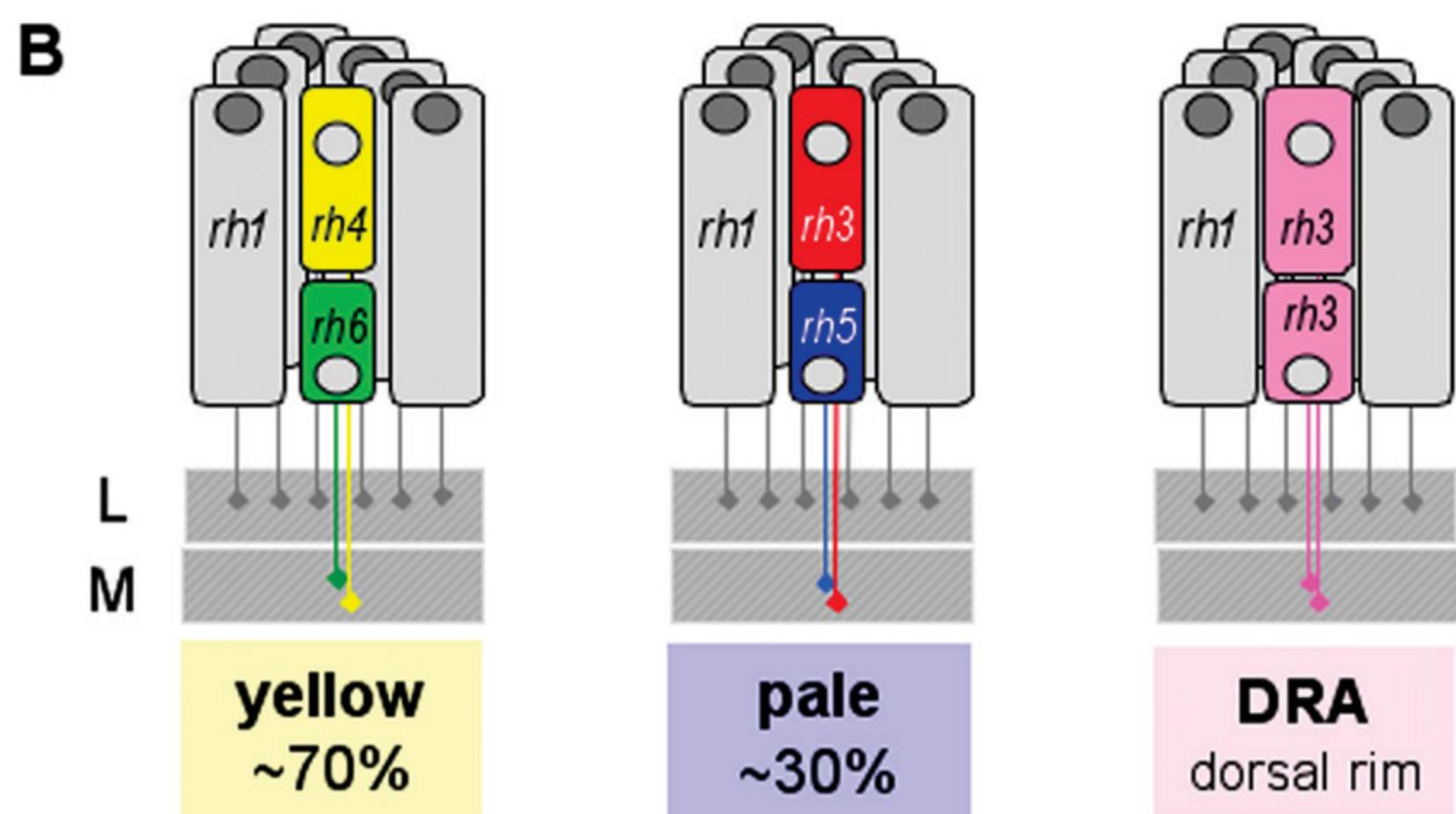
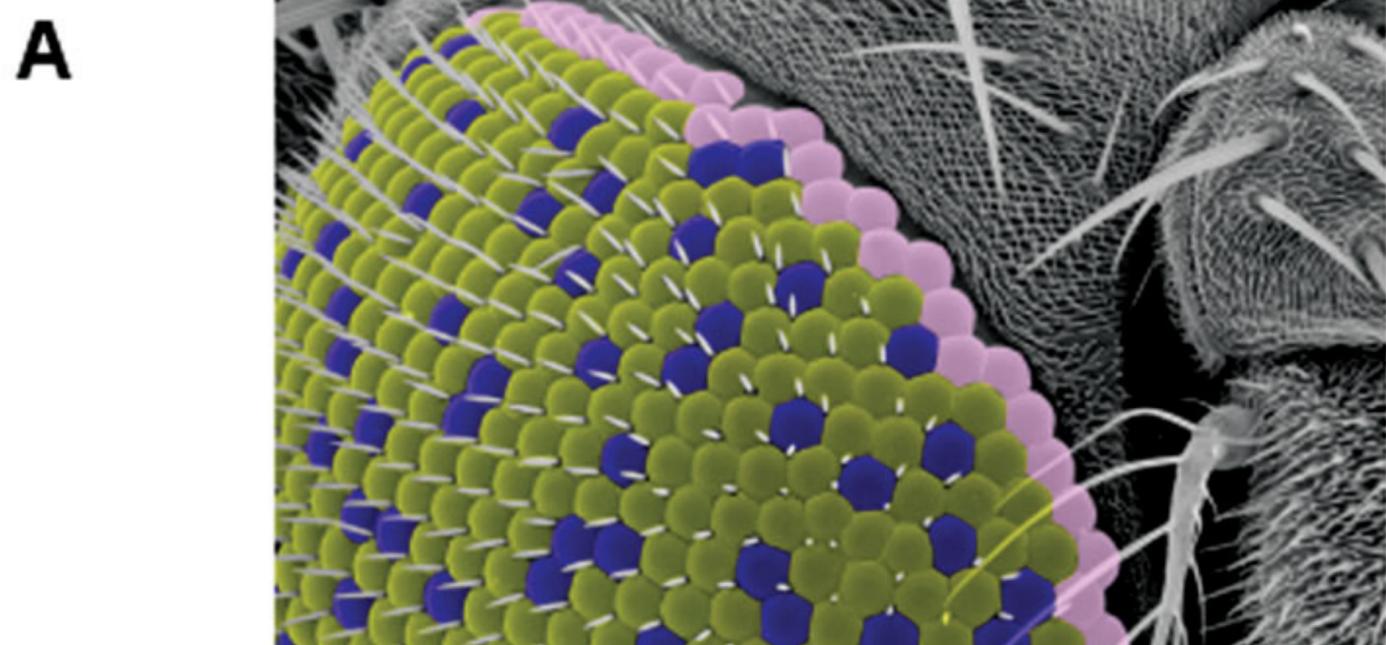
Supplementary Figure 5

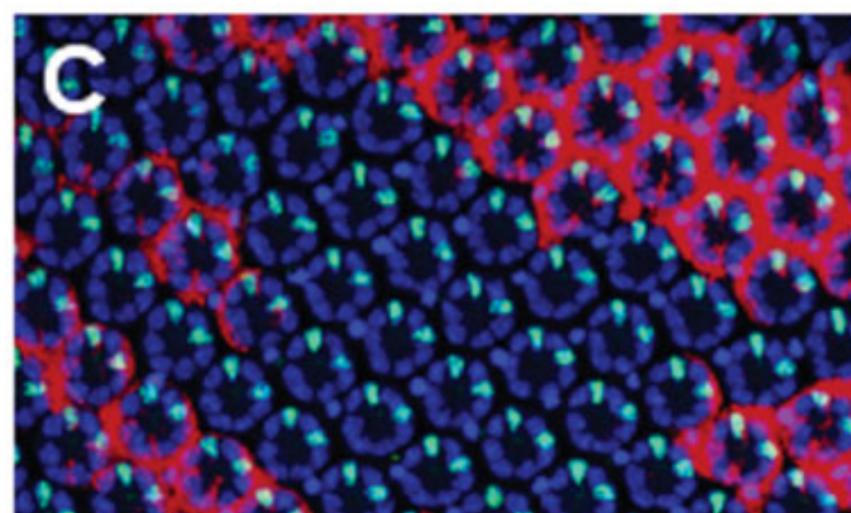
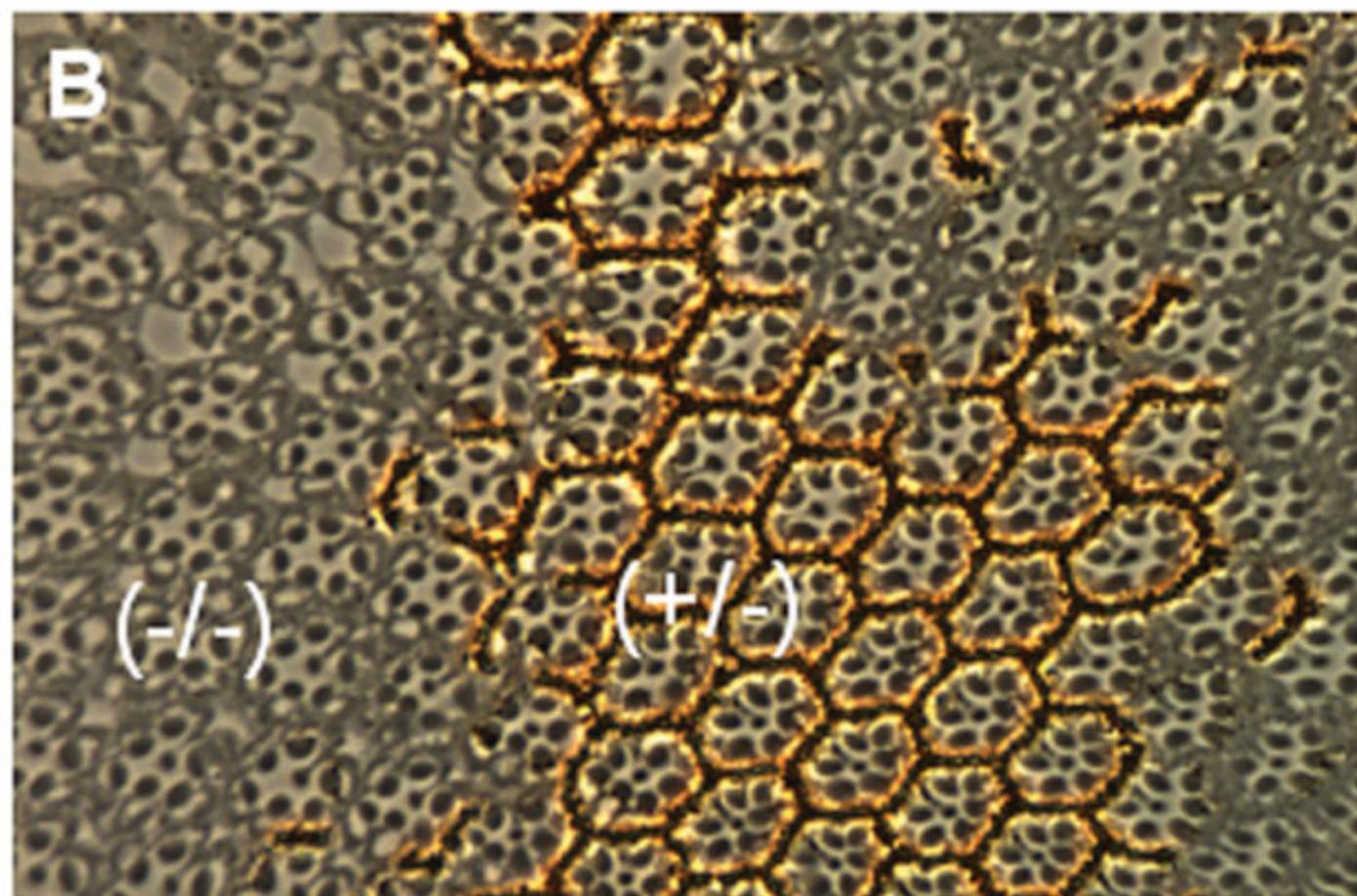
(A) Co-localization of *ss_{eye}*> $\eta\beta$ -Gal (red), ElaV (blue) and Pros (green). Note that Pros also labels cone cell feet located in the center of each ommatidial cluster.

(B) Expression of *ss_{eye}*> $\eta\beta$ -Gal (red) is never observed in Hth-positive (green) inner PRs of the DRA. Neurons are marked with ElaV (blue).

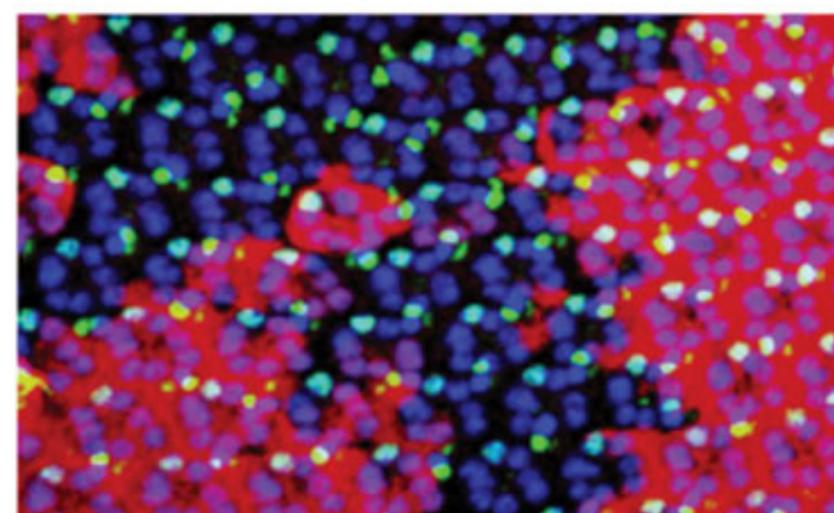
Additional References

43. Lee, Y. S. & Carthew, R. W. Making a better RNAi vector for *Drosophila*: use of intron spacers. *Methods* **30**, 322–329 (2003).
44. Mollereau, B. et al. A green fluorescent protein enhancer trap screen in *Drosophila* photoreceptor cells. *Mech Dev* **93**, 151-60 (2000).
45. Pichaud, F. & Desplan, C. A new visualization approach for identifying mutations that affect differentiation and organization of the *Drosophila* ommatidia. *Development* **128**, 815-26 (2001).

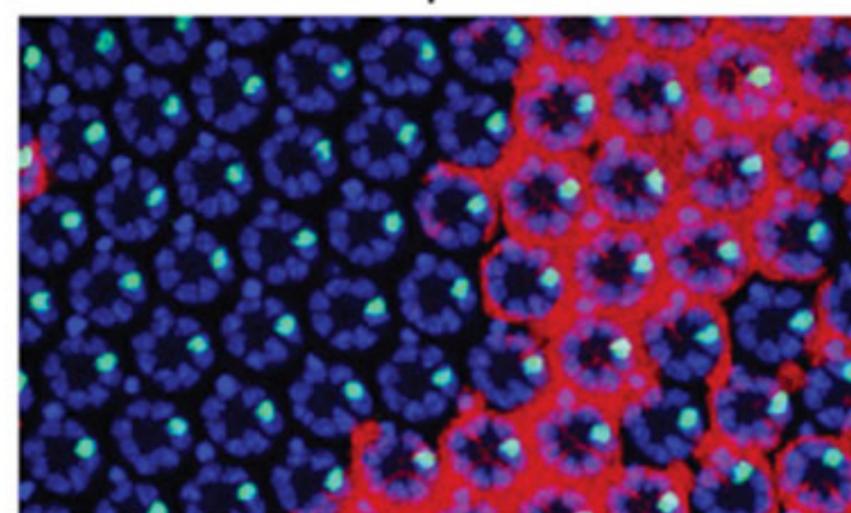




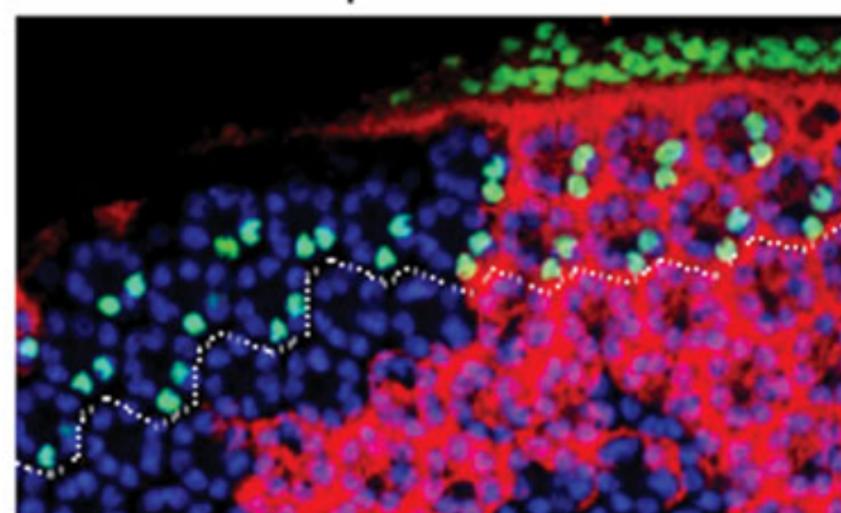
Sal Elav Arm-Z
Inner PR specification



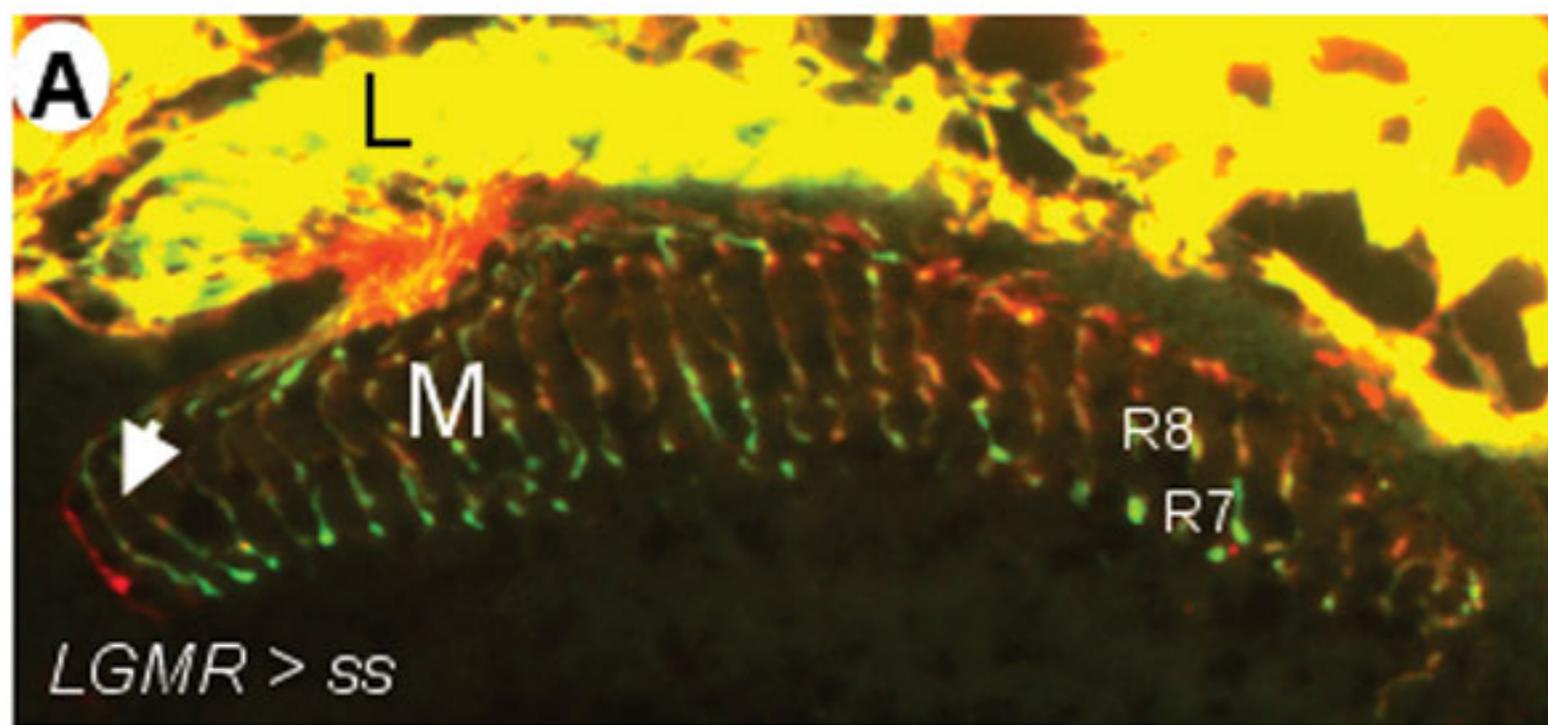
Pros Elav Arm-Z
R7 specification



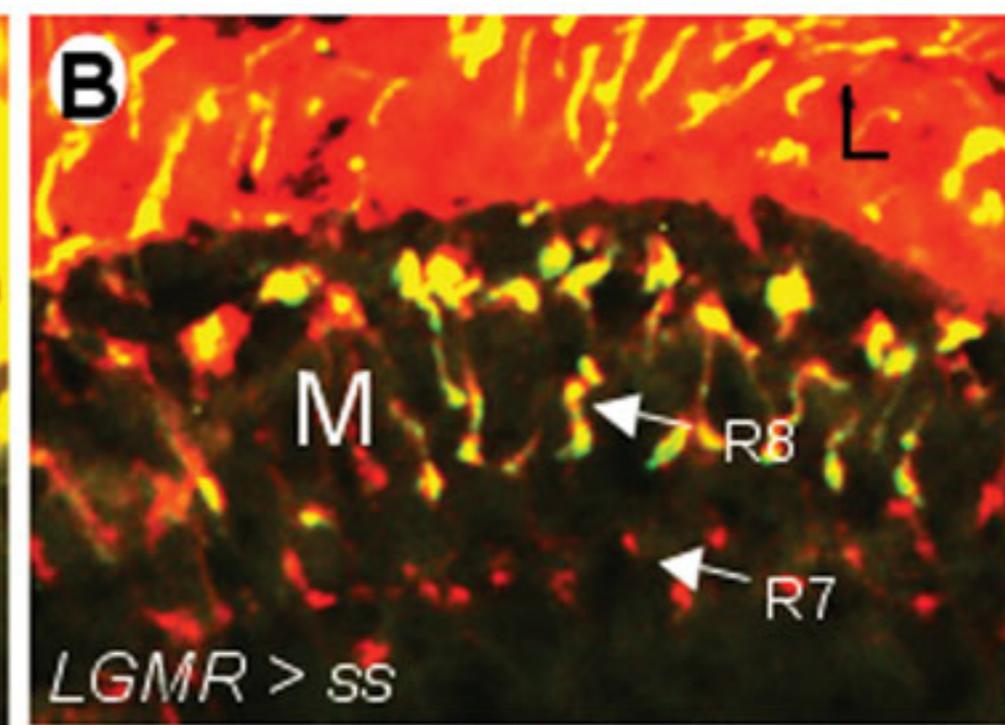
Sens Elav Arm-Z
R8 specification



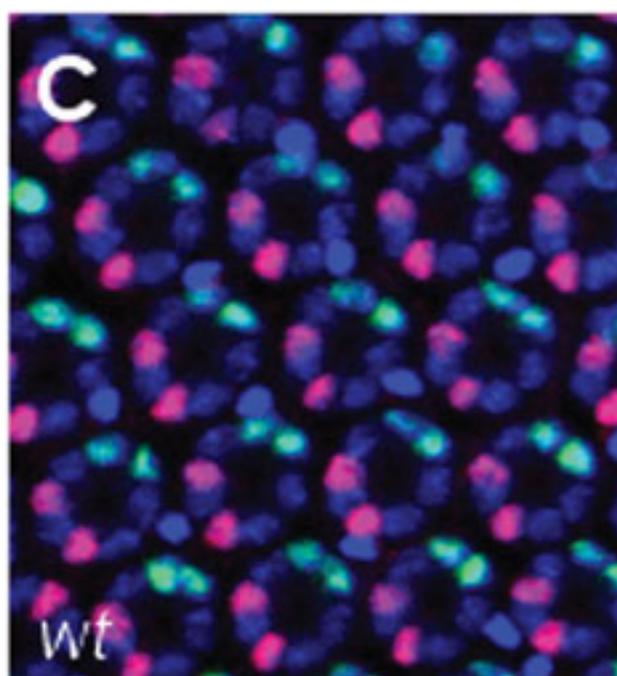
Hth Elav Arm-Z
DRA specification



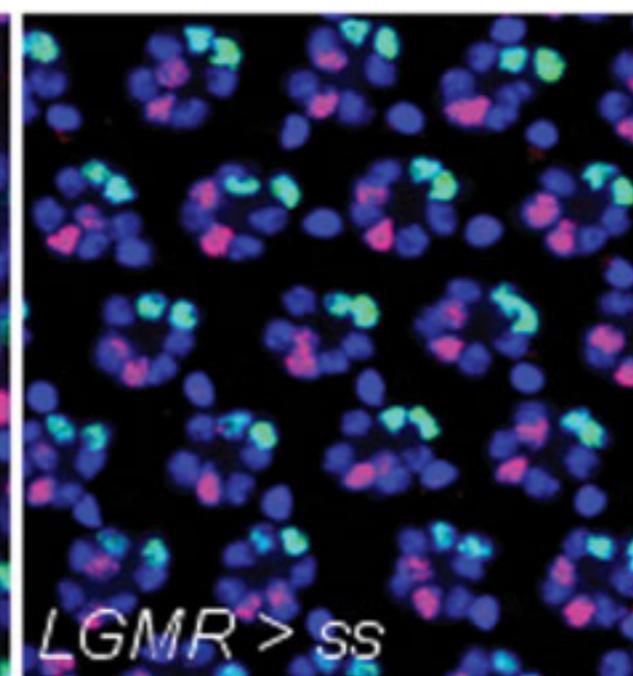
24B10 *rh4-lacZ*



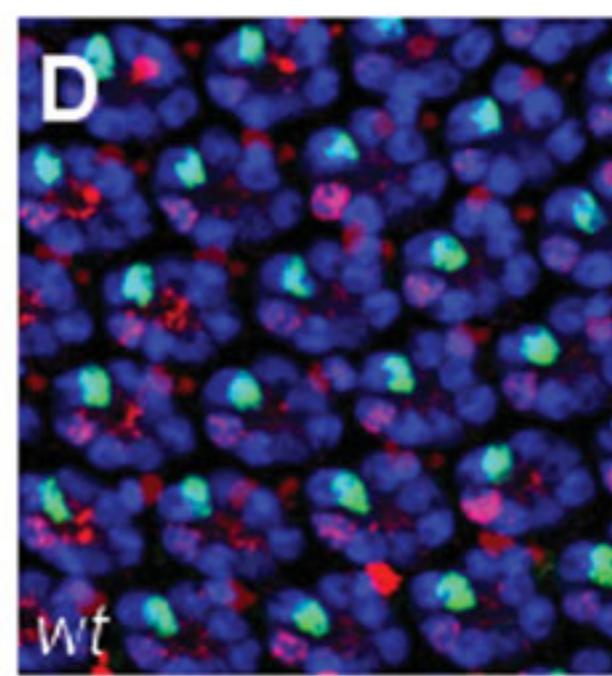
24B10 *rh6-lacZ*



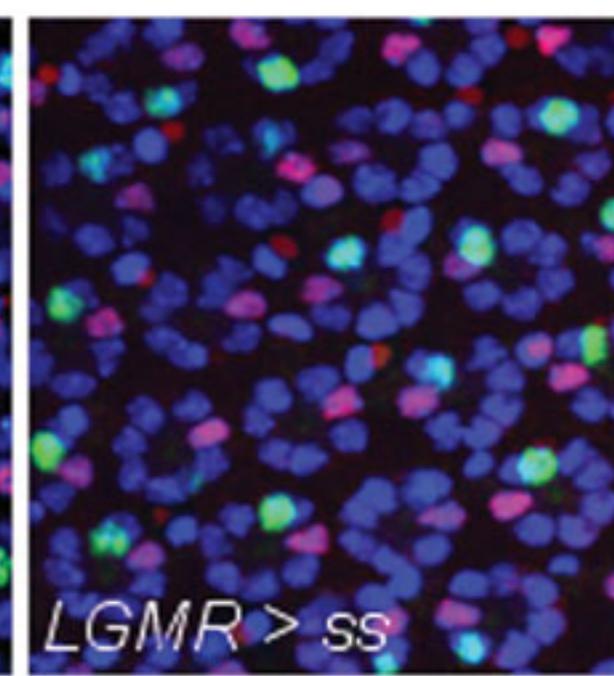
Sal ElaV *svp*



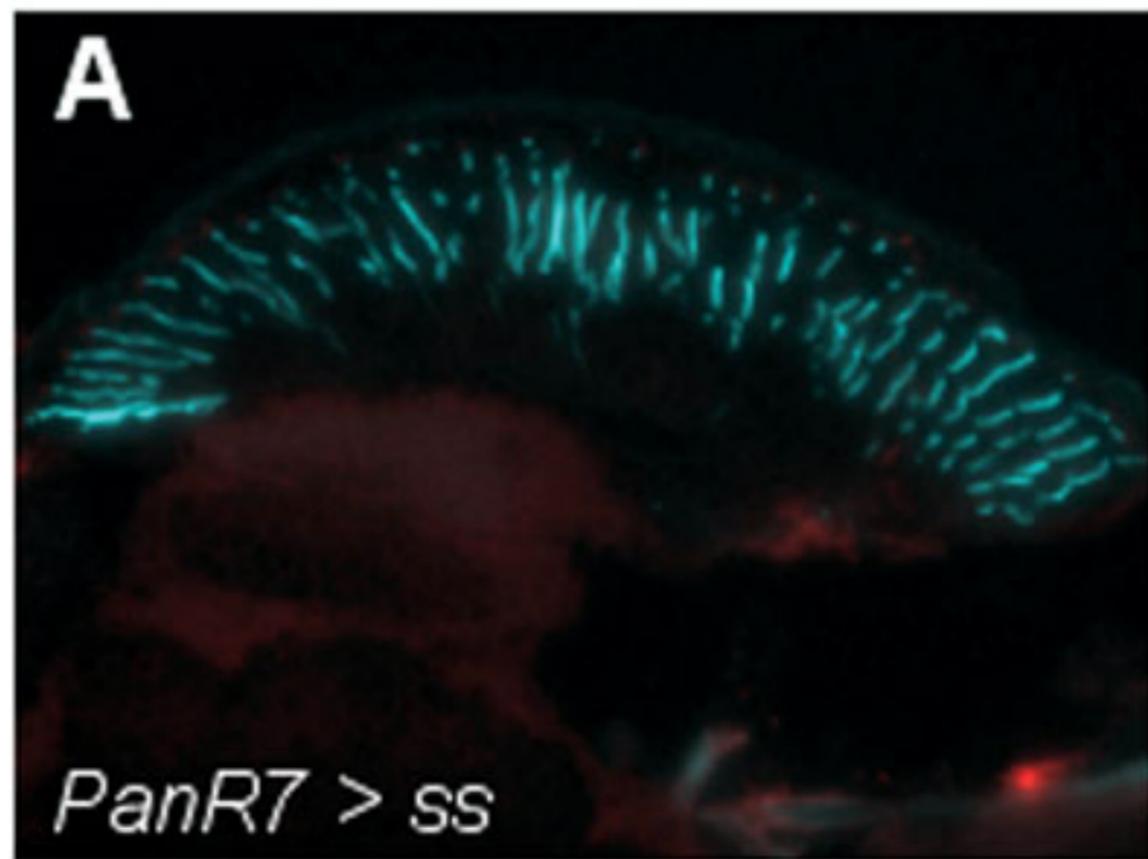
Sal ElaV *svp*



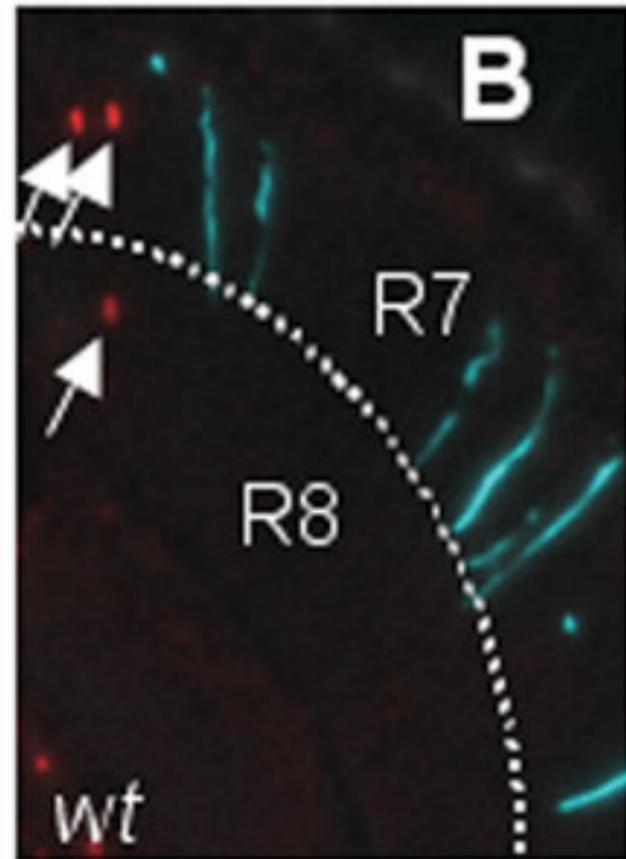
Pros ElaV Sens



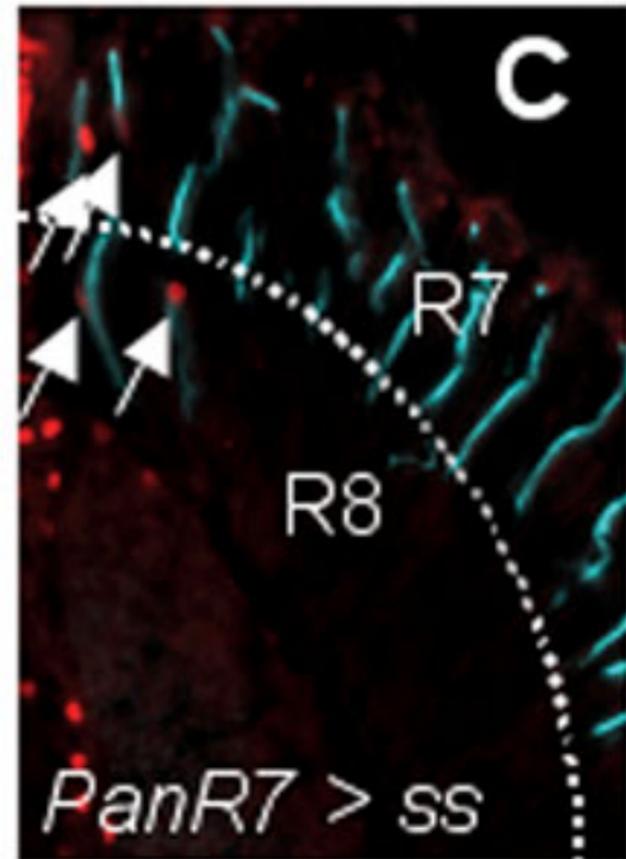
Pros ElaV Sens



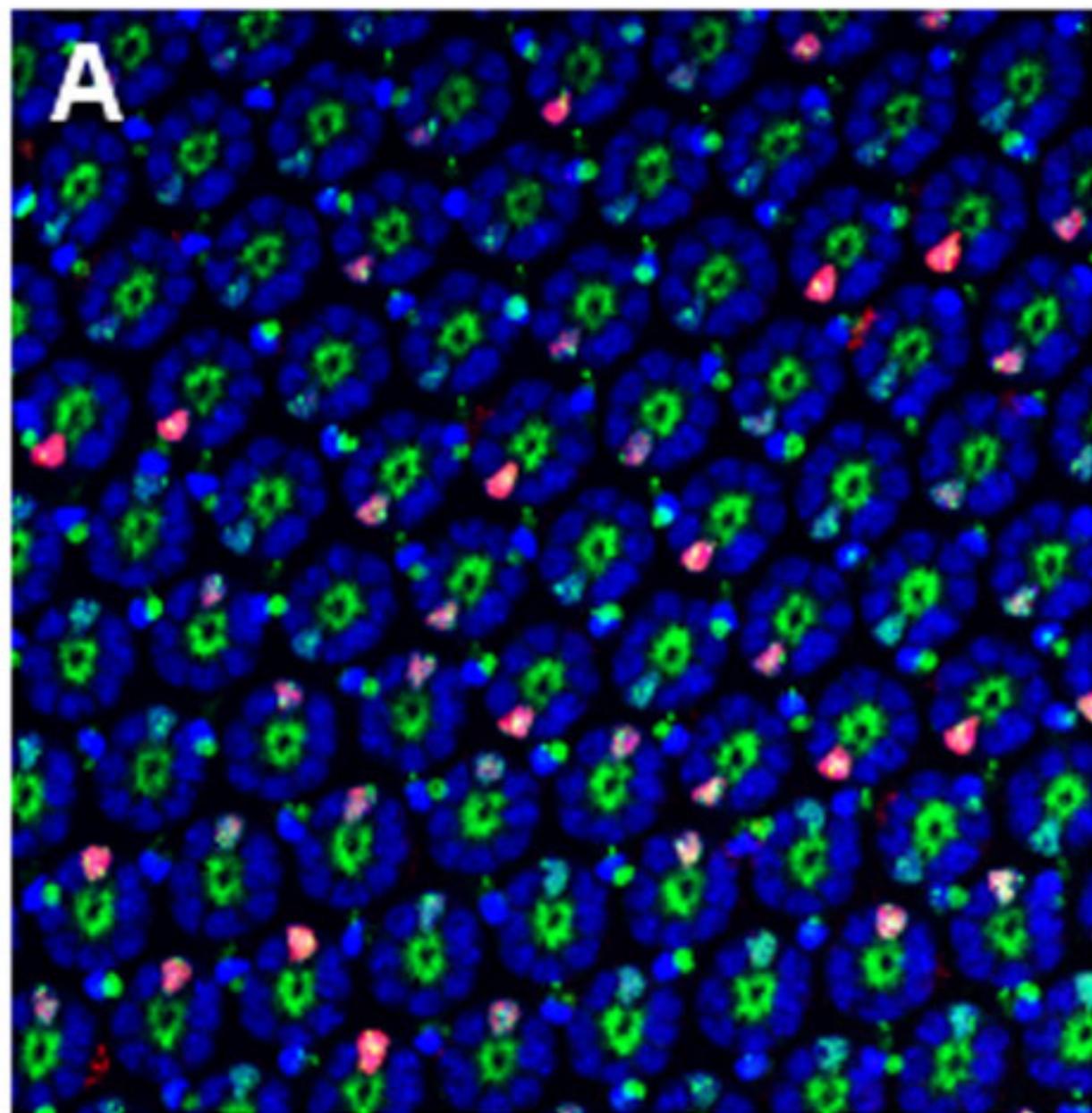
Rh3 Rh4



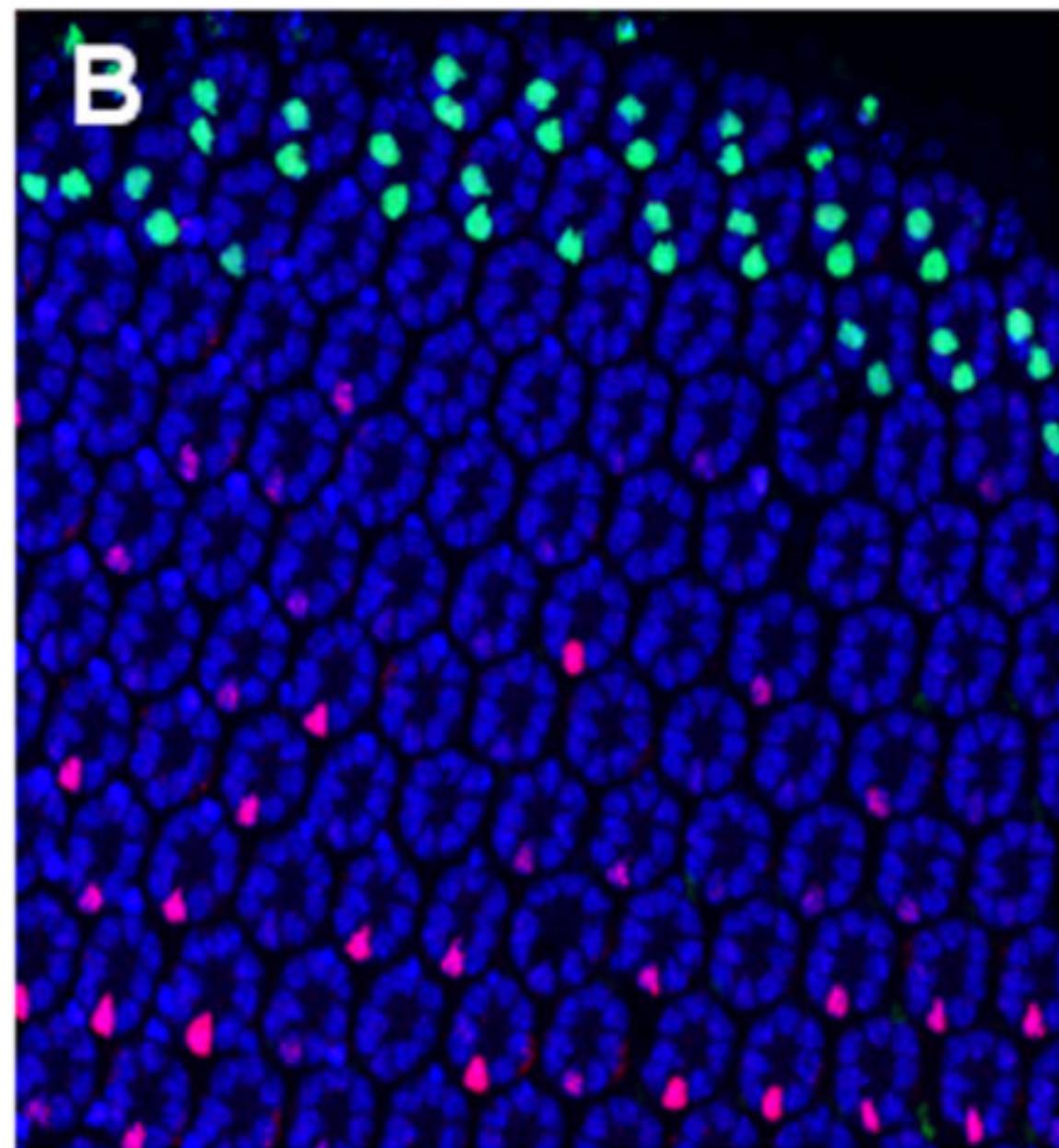
Hth Rh4



Hth Rh4



nβ-Gal **ElaV** **Pros**



nβ-Gal **ElaV** **Hth**