

# The Growth Regulators *warts/lats* and *melted* Interact in a Bistable Loop to Specify Opposite Fates in *Drosophila* R8 Photoreceptors

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## Summary

Color vision in *Drosophila* relies on the comparison between two color-sensitive photoreceptors, R7 and R8. Two types of ommatidia in which R7 and R8 contain different rhodopsins are distributed stochastically in the retina and appear to discriminate short (p-subset) or long wavelengths (y-subset). The choice between p and y fates is made in R7, which then instructs R8 to follow the corresponding fate, thus leading to a tight coupling between rhodopsins expressed in R7 and R8. Here, we show that *warts*, encoding large tumor suppressor (Lats) and *melted* encoding a PH-domain protein, play opposite roles in defining the yR8 or pR8 fates. By interacting antagonistically at the transcriptional level, they form a bistable loop that insures a robust commitment of R8 to a single fate, without allowing ambiguity. This represents an unexpected postmitotic role for genes controlling cell proliferation (*warts* and its partner *hippo* and *salvador*) and cell growth (*melted*).

## Introduction

The fly eye provides a powerful system to study cell-fate decisions: it develops from a flat epithelium into a complex three-dimensional structure of multiple cell types in less than a week (Wolff and Ready, 1993). The adult eye allows the fly to perform various visual tasks, ranging from motion detection and the discrimination of colors to measuring the orientation of polarized light for navigation.

In the fly compound eye, each of the 800 ommatidia is a single optical unit that contains 8 photoreceptor cells (PRs) (for review, see Wolff and Ready [1993]). The 8 PRs form widely expanded membrane structures, the rhabdomeres, which contain the photosensitive Rhodopsins (Rh). The rhabdomeres of the six outer PRs

(R1–R6) form a trapezoid (Figure 1A). R1–R6 all express the broad spectrum rhodopsin1 (*rh1* or *ninaE*; Figure 1B; Hardie, 1985; O'Tousa et al., 1985; Zuker et al., 1985) and are morphologically and functionally invariant in all ~800 ommatidia.

The center of the trapezoid is occupied by the two inner PRs, R7 and R8 (Figure 1A). The rhabdomeres of R7 are positioned on top of R8, so that they share the same optic path (Figure 1A). Inner PRs are involved in color vision and can be viewed as equivalent to vertebrate cones (for review see Cook and Desplan [2001]). Each R7 and R8 expresses only one of the four rhodopsins, *rh3*, *rh4*, *rh5*, or *rh6* in a highly regulated manner (Figure 1B; Fortini and Rubin, 1990; Huber et al., 1997; Montell et al., 1987; Zuker et al., 1987; Chou et al., 1996, 1999; Papatsenko et al., 1997), defining three different subtypes of ommatidia: “yellow” (y), “pale” (p) (for their appearance under UV illumination, Franceschini et al. [1981]; Kirschfeld et al. [1978]), and the “dorsal rim area” (DRA; Figure 1B). Ommatidia in the DRA express *rh3* in both R7 and R8 and are specified in a very restricted region by the gene *homothorax* (Wernet et al., 2003; Tomlinson, 2003). They are believed to function as polarized light detectors (Labhart and Meyer, 1999).

In contrast, color vision depends on the y and p ommatidial subtypes that are randomly distributed through the main part of the retina, with a bias of y (~70%) over p subtype (~30%) (Figure 2A; Fortini and Rubin, 1990; Montell et al., 1987). In the p subtype, R7 expresses the UV-sensitive Rh3 and R8 the blue-sensitive Rh5. In the y subtype, R7 expresses a distinct UV-sensitive Rh4 while R8 expresses the green-sensitive Rh6. (Figure 1B; Chou et al., 1996, 1999; Papatsenko et al., 1997). As in many other sensory systems, expression of a given Rhodopsin excludes all others to prevent sensory overlap (Mazzoni et al., 2004). While the p subtype is better suited to discriminate among shorter wavelengths, the y subtype should discriminate amongst longer wavelengths (S. Yamaguchi and C.D., unpublished data).

The choice between the p and y fate is first made in R7 (Chou et al., 1996, 1999; Papatsenko et al., 1997): once an R7 commits to the p fate and expresses *rh3*, it sends an instructive signal to the underlying R8, which then also commits to the p fate and expresses *rh5*. In the absence of the R7 signal (i.e., when R7 expresses *rh4* or in a *sevenless* mutant), R8 commits to the y fate and expresses *rh6*. The stochastic choice appears to be made by each R7 independently of its neighbors, resulting in the biased random distribution of p and y ommatidia throughout the main part of the retina (for review, Mikeladze-Dvali et al. [2005]).

Here, we report identification of four genes required in R8 cells for ensuring the correct choice of y versus p cell fate. We show that the *warts* (*wts*) gene, which encodes the *Drosophila* large tumor suppressor (also known as *lats*; Justice et al., 1995; Xu et al., 1995) and *melted* (*melt*; Salzberg et al., 1997) play a critical role in the specification of p and y R8 cells, without affecting the R7 choice. *wts* encodes a Ser/Thr kinase (Justice et al., 1995; Xu et al., 1995), while *melt* encodes a

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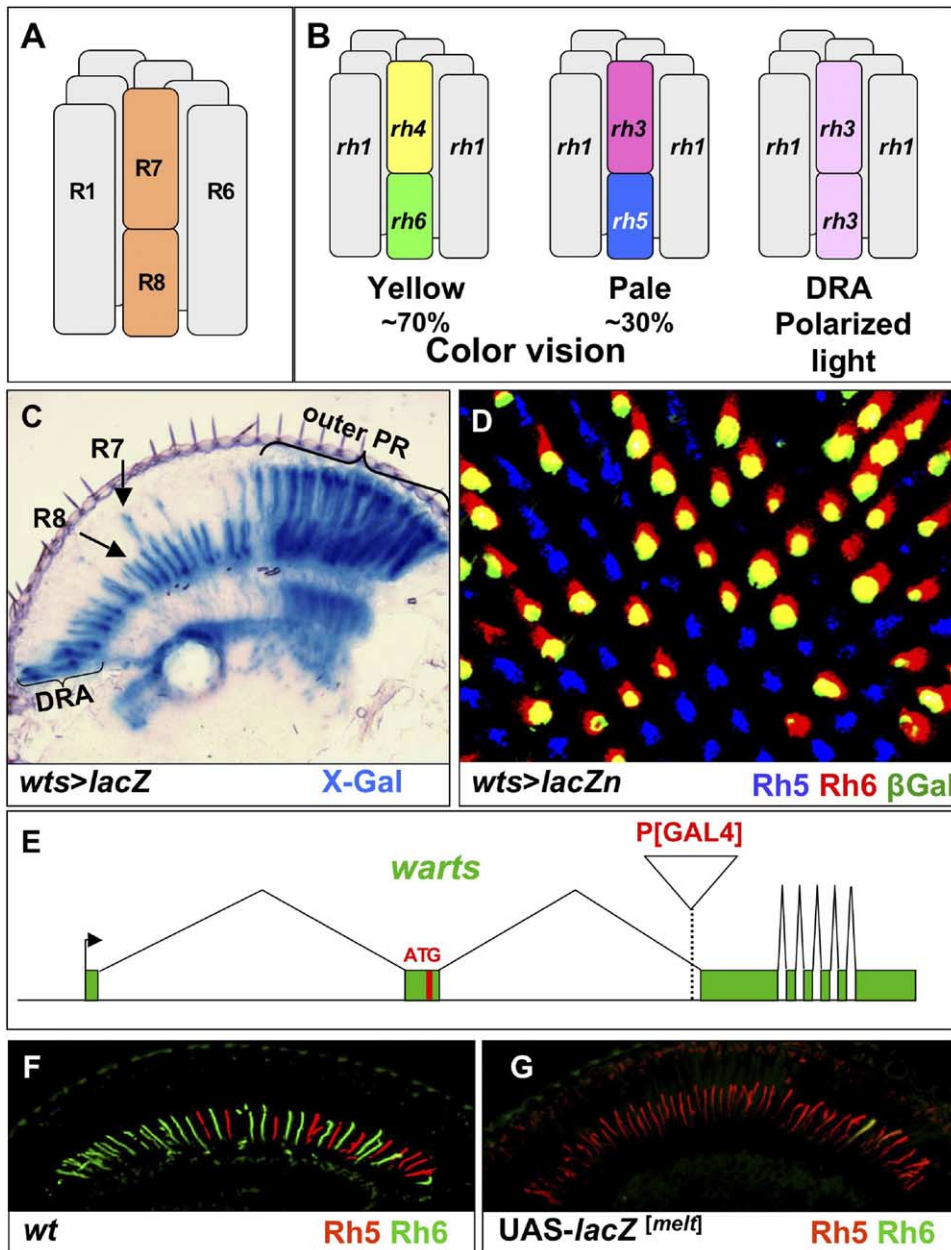


Figure 1. Expression of *wts*-Gal4 Is Specific to y R8

(A) Schematic diagram of one ommatidium. Rhabdomeres of the outer PR (R1–R6) span the whole retina. Inner PR (R7 and R8) are located in the center of the ommatidium, R7 being distal and R8 proximal.

(B) Ommatidia fall into 3 categories based on the morphology and Rhodopsin expression in the inner PR. The p and y ommatidia are involved in color vision. In the p subtype R7 expresses *rh3* and R8 *rh5*. y R7 and R8 express *rh4* and *rh6*, respectively. DRA ommatidia express *rh3* in R7 and R8.

(C) Gal4 enhancer trap in *wts* driving expression of *lacZ* (*wts>lacZ*) stained by X-gal shows expression in the R8, some R7, DRA R7, and R8 and in R8 and outer PRs in the ventral part of the eye.

(D) Whole-mounted retinas of *wts>lacZn* stained for Rh5 (blue), Rh6 (red), and  $\beta$ -Gal (green). The picture is taken at the level of the R8 nuclei. *wts>lacZn* is exclusively coexpressed with Rh6.

(E) Schematic representation of the *wts* locus showing that the *wts*-Gal4 enhancer trap is inserted in the second intron.

(F) Wild-type adult eye sections stained for the R8 Rhodopsins Rh5 (red) and Rh6 (green).

(G) Adult eye sections of flies homozygous for the *UAS-lacZ<sup>[melt]</sup>* insertion stained for Rh5 (red) and Rh6 (green). The pR8 subtype is expanded on expense of yR8.

Pleckstrin Homology (PH) domain protein (Teleman et al., 2005). *wts* is necessary and sufficient for R8 to adopt the y fate, while *melt* plays the opposite role and specifically induces the p fate in R8. *wts* and *melt* are

expressed in a complementary manner in the yR8 and pR8 subsets, respectively. We present evidence that the two genes repress each other's transcription to form a bistable loop. *melt* seems to respond to the R7

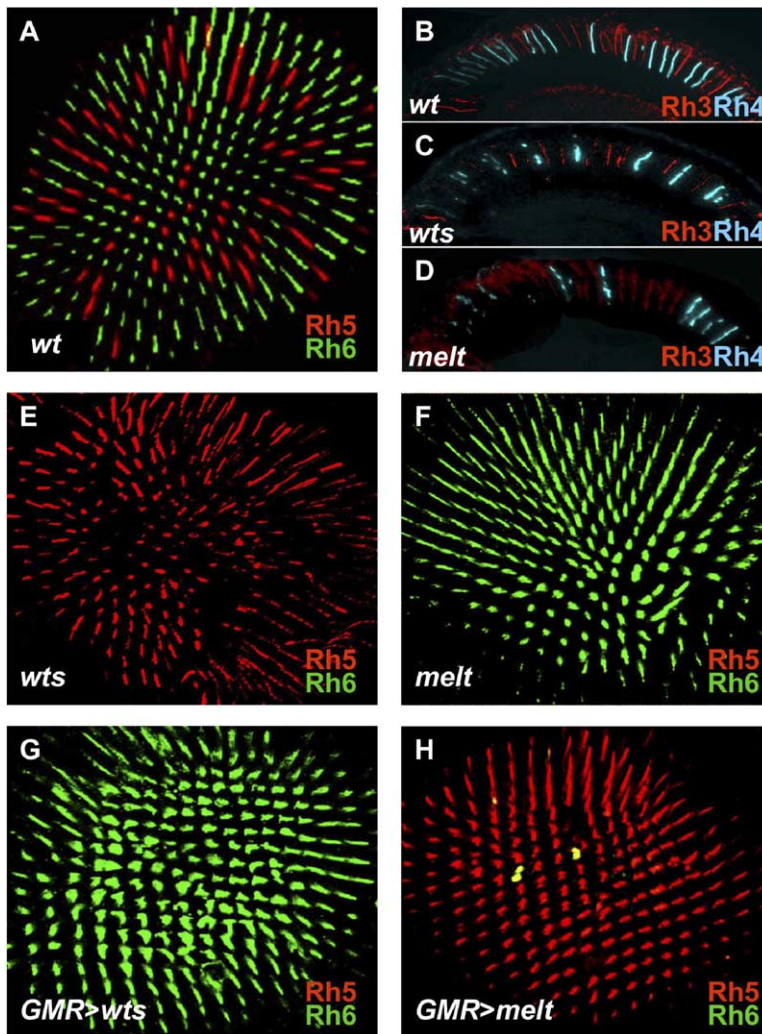


Figure 2. *wts* and *melt* Have Opposite Phenotypes in the R8

(A) Whole-mounted wild-type retina stained for Rh5 (red) and Rh6 (green), showing the distribution of the p (30%) and y (70%) subtypes.  
 (B) Adult eye section of a wild-type fly stained for the R7 Rhodopsins Rh3 (red) and Rh4 (blue).  
 (C) Section of whole clonal eyes for *wts<sup>latsP1</sup>* stained for Rh3 (red) and Rh4 (blue). R7 Rhodopsin expression is unaffected.  
 (D) Adult eye section of a fly homozygous for *melt<sup>-1</sup>* stained for Rh3 (red) and Rh4 (blue). R7 Rhodopsin expression is unaffected.  
 (E) Rh6 is lost and Rh5 expanded in R8 *wts<sup>latsP1</sup>* whole clonal eyes. Whole-mounted retina of *wts<sup>latsP1</sup>* eyes stained for R8 Rhodopsins with Rh5 (red) and Rh6 (green).  
 (F) In homozygous *melt<sup>-1</sup>* flies, all R8 express Rh6 instead of Rh5. Whole-mounted retina of *melt<sup>-1</sup>* homozygous flies stained for Rh5 (red) and Rh6 (green).  
 (G) When *wts* is misexpressed, Rh5 is replaced by Rh6 in all pR8. Whole-mounted retina of *GMR>wts* flies stained for Rh5 (red) and Rh6 (green).  
 (H) When *melt* is misexpressed, Rh5 is induced instead of Rh6 in all yR8; some ommatidia coexpress Rh5 and Rh6. Whole-mounted retina of *GMR>melt* flies stained for Rh5 (red) and Rh6 (green).

signal, while *wts* appears to regulate the output of the loop. Finally, we show that the tumor-suppressor genes *hippo* (*hpo*) and *salvador* (*sav*), which encode the two molecular partners of Wts/Lats, have phenotypes identical to *wts* (Harvey et al., 2003; Kango-Singh et al., 2002; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Pantalacci et al., 2003). Interestingly, *melt* has recently been reported to regulate growth and fat metabolism in *Drosophila* (Teleman et al., 2005). Thus, genes known to regulate both cell growth (*melt*) and proliferation (*wts*, *sav*, *hpo*) interact antagonistically during retinal patterning.

## Results

### *wts* Is Expressed in a Subset-Specific Manner in Postmitotic PRs

To identify genes involved in the differentiation of p or y PR subsets, we performed a Gal4 (pGawB) enhancer trap screen in adult flies using GFP expression as a reporter (Mollereau et al., 2001; Wernet et al., 2003). One insertion produced a strong GFP signal in inner PRs (data not shown). Staining of sectioned adult eyes for the UAS-*lacZ* reporter gene revealed Gal4 expres-

sion in a large subset of R8 cells. Additional expression was found in DRA R7 and R8, as well as in outer PRs in the ventral half of the eye (Figure 1C). Occasionally, weak expression was also found in some R7 cells, but not in any PR subset-specific pattern (Figure 1C and data not shown). Staining of the same enhancer trap (driving UAS-*lacZnuc* expression) with antibodies against  $\beta$ -Gal, Rh6 ( $\alpha$ -Rh6), and Rh5 ( $\alpha$ -Rh5) in whole-mounted retinas revealed that the reporter was specific to Rh6-positive R8 and was excluded from the Rh5-positive R8, indicating that the targeted gene is expressed in the yR8 subtype (Figure 1D).

We identified the genomic DNA flanking the pGawB transposon, which is inserted upstream of the third exon of the gene *warts* (*wts*). We stained an existing *wts* nuclear *lacZ* enhancer trap line P[*lacZ,w<sup>+</sup>*] (Justice et al., 1995; Xu et al., 1995). *lacZ* expression in this line (*wtsZn*) was also specific to the y subset of R8 cells as well as the DRA and some ventral outer PRs, confirming the restricted expression pattern of *wts* (data not shown).

*wts* encodes the ortholog of the human *Lats* genes (Figure 1E). The Ser/Thr kinase Lats plays a major role in the control of cell proliferation and cell death in flies

and vertebrates (Justice et al., 1995; Xu et al., 1995; St John et al., 1999; Hisaoka et al., 2002). Along with its partners *hippo* (*hpo*) and *salvador* (*sav*), *wts* has been reported to regulate the expression of Cyclin E and the *Drosophila* inhibitor of apoptosis 1 (DIAP1; Harvey et al., 2003; Kango-Singh et al., 2002; Tapon et al., 2002; Udan et al., 2003; Wu et al., 2003; Pantalacci et al., 2003). Cell proliferation is enhanced and apoptosis is repressed in *wts*, *sav*, or *hpo* mutants, hence the tumor-suppressor nature of these genes.

*wts*-Gal4 appears to be activated by a late eye-specific enhancer of *wts*, which first directs expression long after R8 has exited the cell cycle. *wts* therefore appears to play two distinct roles: a ubiquitous role in proliferating cells and a more restricted role in terminally differentiated PR.

### Identification of *melt* as a Regulator of the pR8 Subset

Flies with *wts*-Gal4 insertion were homozygous viable and did not exhibit any visible growth phenotype. However, we noticed that heterozygous *wts*-Gal4 flies always exhibited a strong *rh* phenotype when present in combination with one specific UAS-*lacZ* reporter construct (P{w[+mC] = UAS-*lacZ*.B}Bg4-2-4b, FlyBase #1777). The y/p R8 ratio was dramatically affected: most R8 expressed *rh5*, while *rh6* expression was almost completely lost, with *wts*-Gal4 expression reduced to the remaining *rh6* expressing R8 (data not shown). However, specification of R7 and of outer PRs was unaffected. This phenotype was only observed with this specific UAS-*lacZ* transgene, and not with UAS-*GFP* or other UAS-*lacZ* transgenes. When homozygous (in the absence of *wts*-Gal4), this UAS-*lacZ* line manifested an even more severe R8 opsin phenotype: about 90% of R8 expressed *rh5* at the expense of *rh6* (Figure 1G). This suggested that this particular insertion disrupted a gene affecting the p/y choice in R8.

We found that this UAS-*lacZ* P element was inserted 21 bp upstream of the transcriptional start site of the gene *meltd* (*melt*) (Figure 4A). The Melt protein has a C-terminal PH domain and is conserved from *C. elegans* to humans. Insertions in *melt* were initially identified in a screen for genes affecting peripheral nervous system development (Salzberg et al., 1997). We thus analyzed the role of *melt* in R8 subtype specification and its interaction with *wts*.

### *melt* and *wts* Are Necessary for the Differentiation of p and y R8 Subtypes

We examined PR subtype specification in mutants for *wts* and *melt*. Flies with whole mutant eyes for the null allele of *wts*<sup>*latsX1*</sup> exhibit severe tumorous overgrowth (Xu et al., 1995), preventing close examination of *rhodopsin* expression. We thus generated whole mutant eyes using the hypomorphic allele *wts*<sup>*latsP1*</sup> (Xu et al., 1995), which only mildly perturbed eye morphology. Staining these eyes with  $\alpha$ -Rh5 and  $\alpha$ -Rh6 antibodies revealed that all R8 cells expressed *rh5* and none *rh6* (Figure 2E), suggesting that the yR8 subset was lost, while the pR8 subset was expanded to all R8. Some R8 rhabdomeres were missing in *wts*<sup>*latsP1*</sup> retinas, but the number of degenerated R8 rhabdomeres could not ac-

count for the total absence of the yR8 subtype (89.3% of ommatidia expressed Rh5, while we detected neither Rh5 nor Rh6 expression in the remaining partially degenerated 10.7% [see Figure S1 in the Supplemental Data available with this article online]). We did not observe any change in the expression of *rh3* and *rh4* in R7 (35.6% Rh3:64.4% Rh4) or of *rh1* (Figure 2C and data not shown). As a consequence, we observed extensive miscoupling between *rh4* in R7 and *rh5* in R8 (Figure S2), a situation that is never observed in the wild-type.

The specific expression of *wts*-Gal4 in yR8 is consistent with its function in controlling *rh6* expression. The pR8 fate is normally induced by an overlying pR7 that expresses *rh3* and signals R8. In *wts* mutants, all R8 appeared as if they had received the inductive signal from R7 and had adopted the p fate, although the y/p fate decision remained normal in R7.

We then tested whether *melt* also had a role in R8 subtype specification. Immunostaining of eyes from viable *melt* null (*melt*<sup>*d1*</sup>) flies (Teleman et al., 2005) showed that *rh5* expression was completely lost. Instead, all R8 expressed *rh6* (Figure 2F). As in *wts* mutants, expression of *rh3* and *rh4* in R7 (34.7% Rh3:65.3% Rh4) and of *rh1* in outer PRs was not affected in *melt*<sup>*d1*</sup> (Figure 2D and data not shown). Thus, the loss of *melt* specifically affected pR8, resulting in the opposite phenotype to *wts*. An identical phenotype was observed with another null allele, *melt*<sup>*d3*</sup> (data not shown).

*melt*<sup>*d1*</sup> and *melt*<sup>*d3*</sup> also delete part of the adjacent gene *cornetto* (*corn*). However, neither a *corn* null allele nor misexpression of *corn* in all photoreceptors led to changes in R8 opsin expression (data not shown). This strongly suggested that the R8 opsin phenotype of *melt*<sup>*d1*</sup> was due to the absence of *melt*. Thus, *melt* appears to be necessary for *rh5* expression in the pR8 subtype, possibly by enabling these cells to receive or process the instructive signal from pR7.

### *melt* and *wts* Are Sufficient to Induce p or y R8 Subtypes

We then tested whether *wts* and *melt* were sufficient to induce a p or y fate. We misexpressed *wts* (UAS-*wts*) in all PRs using a PR-specific Gal4 driver (LongGMR-Gal4, referred to as GMR>*wts*). Retinas stained with  $\alpha$ -Rh5 and  $\alpha$ -Rh6 antibodies showed that ectopic expression of *wts* was sufficient to induce *rh6* expression in all R8, while *rh5* expression was completely lost (Figure 2G). Although GMR>*wts* is expressed in all PRs, only R8 PRs (except R8 of the DRA) responded to ectopic *wts* expression. *rh6* was not expanded to R7 or to outer PRs, and expression of *rh1*, *rh3*, and *rh4* was unaffected (Figure 2G and data not shown).

We also tested whether *melt* was sufficient to induce the pR8 fate: misexpression of *melt* using the same LongGMR-Gal4 driver (GMR>*melt*) induced *rh5* in all R8, while *rh6* was repressed in virtually all R8 (Figure 2H). Coexpression of *rh5* and *rh6* was observed in very few R8 cells (~2.7%). As with *wts* misexpression, only R8 outside of the DRA were transformed. Expression of *rh1*, *rh3*, and *rh4* was normal (Figure 2H and data not shown).

The fact that *wts*-Gal4 and UAS-*lacZ*<sup>[*melt*]</sup> interacted genetically in a positive manner and exhibited the *wts* phenotype suggested that UAS-*lacZ*<sup>[*melt*]</sup> was a gain-of-function allele. This might be caused by the promoter region of the *white* selector gene carried by the transposon acting on the *melt* gene (*white* is expressed in all photoreceptors during late pupation), leading to misexpression of *melt*. Indeed, homozygous UAS-*lacZ*<sup>[*melt*]</sup> exhibited the same phenotype as ectopic expression of *melt* (Figure 1G).

Therefore, *wts* and *melt* seem to have opposite roles for R8 specification. While *wts* is necessary and sufficient to induce the yR8 fate (*rh6*), *melt* is required for the pR8 subtype fate (*rh5*). Interestingly, manipulation of the two genes did not affect R7 fate, resulting in mis-coupling of *rhodopsin* expression between R7 and R8. Thus, R7 makes the initial stochastic choice between p and y fates. However, *melt* and *wts* consolidate the R7 decision in R8.

#### *wts* and *melt* Are Specifically Required in R8

The R8 *rh* phenotype in *wts* and *melt* mutants could be explained either by a cell nonautonomous role in R7, the signaling side of R7-R8 communication, or by a cell-autonomous role in R8, the receiving side. The expression of *wts*-Gal4 in yR8 suggested the latter. Indeed, flies doubly mutant for *wts* and *sevenless* (*sev*) phenocopied the *wts* phenotype (all R8 expressed *rh5*), indicating that *wts* is not required in R7 (Figure 3B).

To also test whether *melt* was required in R7, we selectively mutated *melt* in R7 and not in R8 using GMR-*flp* and the MARCM system: the medulla projections of *melt*<sup>Δ1</sup> R7 mutant cells, marked by GFP expression, could be found coupled with R8 projections expressing *rh5*>*lacZ*. Therefore, *melt* mutant R7 do not affect the R8 fate (Figure 3G).

We also tested whether misexpression of *melt* could rescue the loss of the pR8 subtype in *sev* mutants. In *sev*; GMR>*melt* flies, most R8 expressed *rh5* and only a small proportion still expressed *rh6* (Figure 3C). We also expressed *melt* with a pan-R8 (*rh5*-Gal4 + *rh6*-Gal4) or a panR7 driver. In panR8>*melt* flies, *rh5* expression was significantly increased, while coexpression of *rh5* and *rh6* was observed (62.8% Rh5 only, 16.8% Rh6 only, 20.4% Rh5 + Rh6; Figure 3F). In contrast, panR7>*melt* was unable to affect *rh5* or *rh6* expression (Figure 3E). The difference between GMR>*melt* and panR8>*melt* is likely due to the late onset of panR8 expression during late pupation compared to GMR expression that starts in larval discs.

Therefore, *melt* and *wts* are necessary in R8 rather than R7, suggesting that they are functioning downstream of the inductive signal from R7.

#### *melt* Is Specifically Expressed in the pR8 Subtype

Since *wts* and *melt* have opposite R8 opsin phenotypes and *wts* is expressed and required in the yR8 subtype, we tested whether *melt* was expressed in the complementary pR8 subset. We fused to Gal4 (or to *lacZ*:NLS) a 1.1kb promoter fragment (*melt*5'-Gal4) or a 4kb genomic fragment encompassing the first intron of *melt* (*meltZn*; Figure 4A). *melt*5'-Gal4 was not expressed in PRs, but *meltZn* was expressed in a subset of R8 in

late-pupal and young adult retinas, as shown by co-staining with the R8 specific nuclear marker Senseless (Sens; Figure 4B). *meltZn* was specifically coexpressed with *rh5* in pR8 and was always excluded from *rh6* expressing yR8 (Figures 4C and 4D). *meltZn* was also expressed in pigment cells. Thus, *melt* appears to be specifically expressed and required in *rh5*-expressing pR8.

We then tested whether *melt* expression was established in response to the instructive signal from pR7 by assessing *meltZn* expression in *sev* mutants. *meltZn* expression was lost in all R8 in *sev* mutants (Figure 4E), indicating that R7 is required for the induction of *meltZn* expression. Reciprocally, *wts*-Gal4>UAS-*lacZnuc* was expanded into all R8, suggesting that *wts* transcription is repressed by the inductive signal from pR7 (Figure 4G).

*meltZn* colocalizes with *rh5* and *wts*-Gal4 with *rh6*: cryosections from flies carrying *wts*-Gal4>GFP:NLS and *meltZn* transgenes revealed that *wts* and *melt* were expressed in two nonoverlapping and complementary R8 subsets (Figure 4H). Thus, besides the R8 opsin genes, *wts* and *melt* represent the only genes known to specifically mark the pR8 and yR8 cell fates.

#### *wts* and *melt* Act in a Negative Interaction Loop

*wts* and *melt* appear to repress each other transcriptionally. To test this, we assayed *meltZn* expression in *wts* loss- and gain-of-function backgrounds. Expression of *meltZn* was lost in all R8 when *wts* was misexpressed (GMR>*wts*; Figure 5B). Conversely, in *wts*<sup>*latsP1*</sup> mutant eyes, *meltZn* expression was expanded to all R8 (Figure 5C), but not to any other PR, indicating that *wts* represses *melt* in the yR8 subtype. *wts* also appears to be under negative control by *melt*: the *wtsZn* enhancer trap line was completely repressed in GMR>*melt* flies (Figure 5F). This effect was specific to the color-sensitive R8 since *wtsZn* expression in DRA R7 and R8 was unaffected (yellow arrows in Figure 5F). Since *wtsZn* and *wts*-Gal4 are weak alleles of *wts* and partially rescue the homozygous *melt* phenotype, it was not possible to examine *wtsZn* expression in a *melt* mutant.

The two genes should also be able to positively regulate their own expression. This is the case, as expression of *meltZn* was completely lost in *melt*<sup>Δ3</sup> mutants (Figure 5D). Furthermore, *meltZn* was expanded into most R8 in GMR>*melt* flies and expression levels were strongly increased (Figure 5H). This effect could be due to the repression of *wts* or to a positive autoregulation by *melt*. Similarly, *wtsZn* expression was expanded to all R8 in GMR>*wts* flies (Figure 5G).

#### *wts* Is the Output of the Negative Regulatory Loop

We tested the epistasis of *wts* and *melt* in double loss-of-function experiments: we recombined *melt*<sup>Δ3</sup> and *wts*<sup>*latsP1*</sup> on two arms of the same chromosome. Using *ey-flp*, we then generated eyes homozygous for both mutations. The double-mutant retina appeared disorganized with some missing rhabdomeres, but all remaining R8 expressed *rh5* while none expressed *rh6* (Figure 6A). We also assessed coupling between R7 and R8 rhodopsins in dissociated ommatidia stained with α-Rh5, α-Rh3, and α-Rh4 antibodies. Ommatidia expressed either Rh3 or Rh4 in R7, but always Rh5 in R8

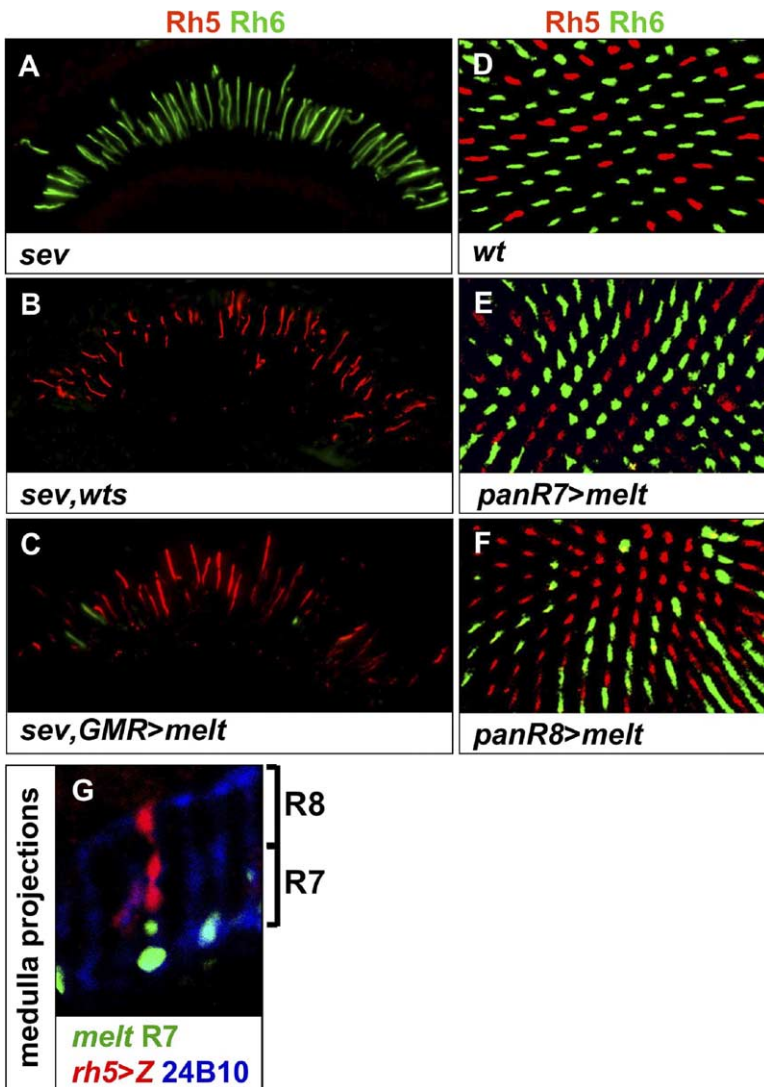


Figure 3. *wts* and *melt* Are Not Required in the R7

(A–C) Adult eye sections stained for Rh5 (red) and Rh6 (green). (A) In *sev* eyes, R7 are missing. R8 acquire the y fate and express Rh6. (B) In *sev; wts<sup>latSP1</sup>* double-mutant eyes, all R8 get specified as p and express Rh5. (C) Misexpression of *melt* reverts Rh6 expression to Rh5 in most ommatidia. In *sev; GMR>melt* eyes, Rh5 is expanded; some R8 coexpress Rh5 and Rh6. (D–F) Whole-mounted retinas stained for Rh5 (red) and Rh6 (green); (D) *wt*. (E) *panR7>melt* eyes have a *wt* Rh5:Rh6 ratio. (F) *panR8>melt* eyes show upregulated Rh5 expression. (G) *melt* is not required in the R7. Staining of medulla projections showing *melt<sup>Δ3</sup>* mutant R7 (marked by GFP staining in green) coupled with *rh5>lacZ* (red) positive R8. 24B10 marks the projections.

(Figure 6C). Thus, the yR8 (but not yR7) identity is lost in the double mutants.

To overcome the morphological disorganization of the double-mutant eyes, we generated mutant *wts<sup>latSP1</sup>* clones in the context of *melt<sup>Δ3</sup>* homozygous flies. In tissue mutant only for *melt<sup>Δ3</sup>*, all R8 expressed *rh6*, but double-mutant *melt<sup>Δ3</sup>; wts<sup>latSP1</sup>* clones had the opposite phenotype: all R8 expressed *rh5*, while *rh6* was lost (Figure 6D).

We thus concluded that *wts* acts downstream of *melt* and that it is absolutely necessary for yR8 specification, i.e., activation of *rh6* expression and repression of *rh5*. *melt* on the other hand, is sufficient but not necessary to specify the pR8 subtype that expresses *rh5*. The phenotype observed in *melt* mutant background is thus only due to the expansion of *wts* in the p subtype since removing *wts* in this background leads to generalized expression of *rh5* in R8.

Since *rh5* expression in the pR8 subtype depends on the instructive signal from pR7 and is repressed by *wts*, we tested whether expression of *rh5* in the *melt<sup>Δ3</sup>; wts<sup>latSP1</sup>* double-mutant background depended on the

R7 signal. We generated flies with eyes triply mutant for *sev*, *melt<sup>Δ3</sup>*, and *wts<sup>latSP1</sup>*, therefore eliminating the source of the R7 signal. *rh5* was still expressed in all R8 cells (Figure 6B). This again indicated that expression of *rh5* is a consequence of its derepression in the absence of *wts*, rather than the product of an independent signal coming from R7. This suggests that *wts* is not only required to induce the yR8 fate (*rh6*) but also to repress the pR8 (*rh5*) fate.

We also misexpressed both genes simultaneously in all PRs. The phenotype of *GMR>wts+melt* flies resembled that of *GMR>wts*, with all R8 expressing *rh6*. However, a few R8 also expressed *rh5*, a situation not observed in *GMR>wts* alone (Figure 6E). The double misexpression also resulted in a loss of *meltZn* expression and expansion of *wtsZn* in R8 (data not shown).

Thus, *wts* seems to be regulating the output of the loop, inducing the y fate. *melt* might be induced in response to the instructive signal from *rh3* expressing pR7. We propose that the function of *melt* is to repress *wts*, allowing *rh5* expression and preventing *rh6* induction in pR8. In this model, expression of *melt* with the

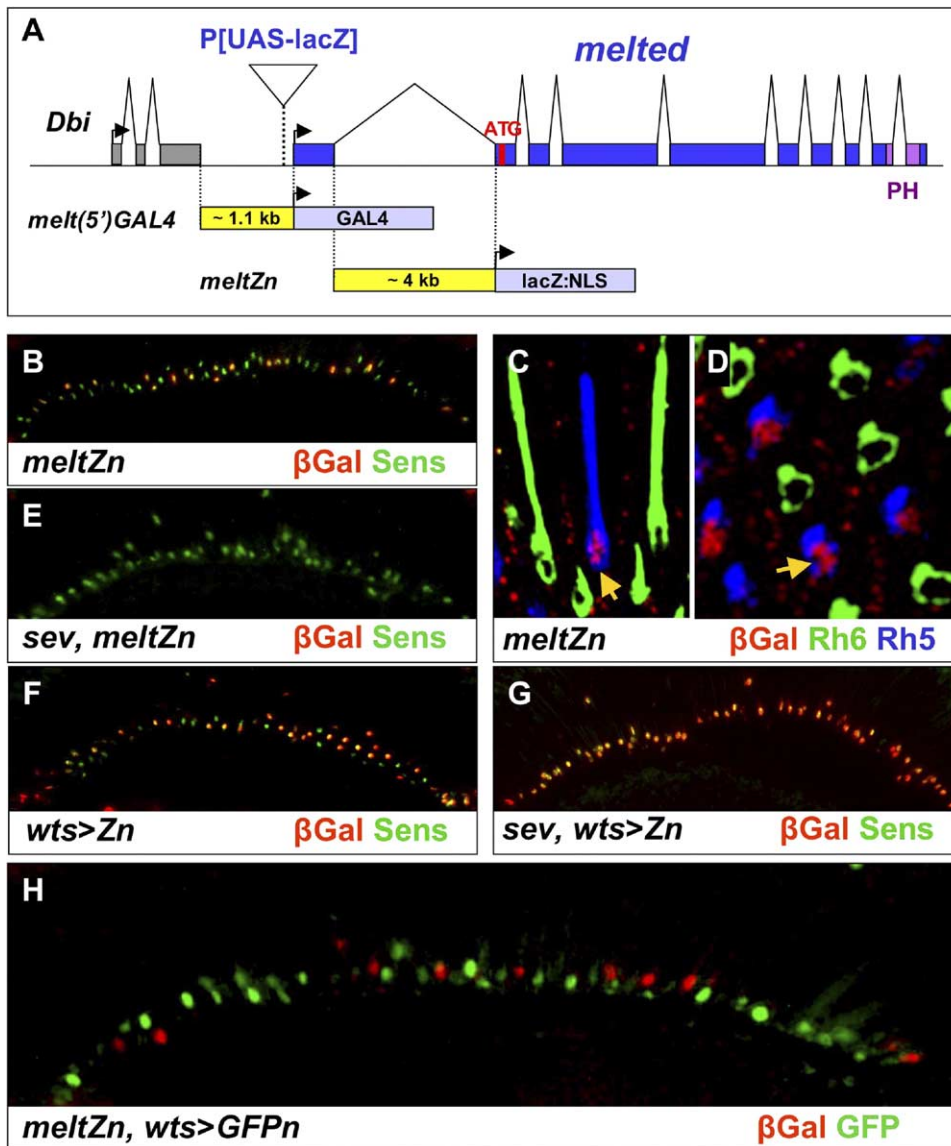


Figure 4. *meltZn* Is Expressed in the pR8

(A) Schematic diagram of the *melt* genomic region. P[UAS-*lacZ*<sup>(*melt*)</sup>] indicates the position of the UAS-*lacZ* insertion that was identified to genetically interact with *wts*-Gal4. *melt* expression constructs were built by fusing a 1.1 kb genomic fragment located 5' to the transcriptional start site (*melt*5'-Gal4) to Gal4 (data not shown for the ~4 kb first intron). *meltZn* was constructed by placing the first ~4kb intron in front of a *lacZ*:NLS reporter.

(B) Section of wild-type eyes carrying *meltZn* stained for  $\beta$ -Gal (red) and the R8 nuclear marker Sens (green). Only a subset of R8 nuclei expresses *meltZn*.

(C and D) In wild-type eyes, *meltZn* is exclusively expressed in the Rh5 expressing R8. Whole-mounted retina stained for Rh5 (blue), Rh6 (green), and  $\beta$ -Gal (red). Anti- $\beta$ -Gal staining is observed in the nuclei (the base of the rhabdomere) of the Rh5 expressing R8 (yellow arrow). The nuclei of Rh6 expressing R8 are empty.

(E) *meltZn* is induced by the R7 signal. Section of *sev* eyes carrying *meltZn* stained for  $\beta$ -Gal (red) and Sens (green). *meltZn* expression is lost in the absence of R7.

(F) Sections of wild-type eyes carrying *wts*>*lacZn* stained for  $\beta$ -Gal (red) and Sens (green). Only a subset of R8 nuclei expresses *wts*-Gal4.

(G) *wts*>*lacZn* is repressed by the R7 signal. Sections of *sev* eyes carrying *wts*>*lacZn* stained for  $\beta$ -Gal (red) and Sens (green). *wts*>*lacZn* expression is expanded to all R8 in the absence of R7.

(H) *meltZn* and *wts*-Gal4 are not coexpressed. Sections of eyes carrying *meltZn* and *wts*>GFP:NLS stained for  $\beta$ -Gal (red) and GFP (green).

*wts*-Gal4 driver should be sufficient to flip the loop into the pR8 state. Indeed, in *wts*-Gal4>*melt*, the pR8 fate (*rh5*) was highly expanded at the expense of yR8 (*rh6*) (Figure 6F).

#### The *hpo/sav/wts* Pathway Is Necessary for Yellow R8 Subtype Specification

The Ser/Thr kinase Wts/Lats is part of a signaling complex that involves the other Ser/Thr kinase Hippo (Hpo)

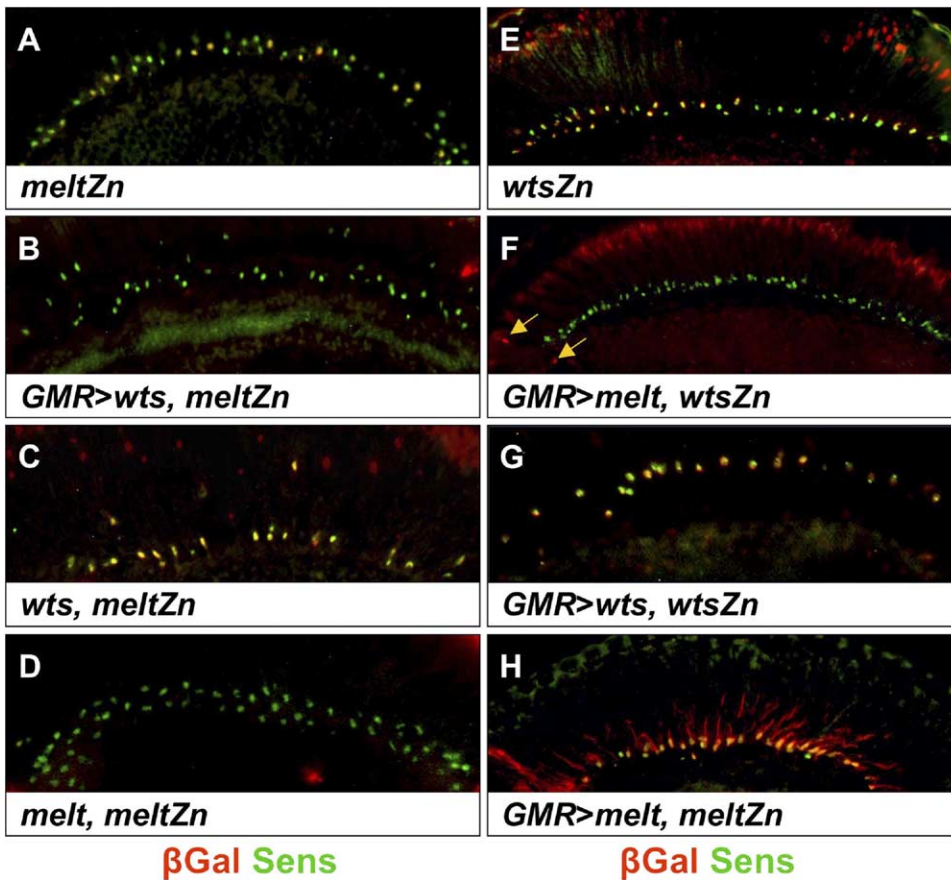


Figure 5. *melt* and *wts* Repress Each Other in a Bistable Loop

All figures are sections of adult eyes stained for β-Gal (red) and Sens (green).

- (A) *meltZn* expression in wild-type eyes.
- (B) *meltZn* expression in *GMR>wts; meltZn* expression is lost in all R8.
- (C) *meltZn* expression in *wts<sup>latsP1</sup>* eyes; *meltZn* is expanded to all R8.
- (D) *meltZn* expression in *melt<sup>Δ3</sup>*; *meltZn* expression is lost in all R8.
- (E) *wtsZn* expression in wild-type eyes.
- (F) *wtsZn* expression in *GMR>melt; wtsZn* expression is lost in all color-sensitive R8; however, expression is unaffected in the DRA R8 nuclei (arrow points to the DRA R7 and R8; Sens does not mark the DRA R8).
- (G) *wtsZn* expression in *GMR>wts*; *wtsZn* expression is expanded to all R8.
- (H) *meltZn* expression in *GMR>melt; meltZn* is highly upregulated, note β-Gal staining in the cell body of R8.

and the adaptor protein Salvador (Sav) (Kango-Singh et al., 2002; Tapon et al., 2002; Harvey et al., 2003; Wu et al., 2003; Udan et al., 2003; Pantalacci et al., 2003). We asked whether *hpo* and *sav* were also involved in R8 subset specification. Flies with eyes homozygous for the hypomorphic *hpo<sup>KC203</sup>* allele exhibited an identical phenotype to *wts<sup>latsP1</sup>* mutants: all R8 adopted the p fate (*rh5*; Figure 6G).

Hpo is a Ste-20 family protein kinase and its function in cell growth depends on its kinase activity (Harvey et al., 2003; Udan et al., 2003; Wu et al., 2003). To test whether this was also true for PR differentiation, we misexpressed a kinase-dead version of *hpo*, *GMR>hpo<sup>K71R</sup>*. *hpo<sup>K71R</sup>* acted as a dominant-negative: most R8 expressed *rh5*, with only a few R8 expressing *rh6*, indicating that the Hpo kinase activity is required to define the yR8 fate (Figure 6H).

We also generated eye-clones mutant for the null al-

lele *sav<sup>shrp1</sup>* (Figure S3). Mutant clones expressed only *rh5*, although a few R8 coexpressed *rh5* and *rh6* (Figure S3, arrow). Thus, *wts*, *hpo*, and *sav* are all necessary for the yR8 fate and the activation of *rh6*.

#### The TOR and Insulin Pathways Are Not Involved in *melt* Function in R8

*melt* was recently shown to modulate tissue growth and fatty acid metabolism through the TOR and insulin receptor (DlnR) pathways (Teleman et al., 2005). The effect is mediated by interactions of Melt with TSC1 and FOXO. However, it appears that the TOR and insulin pathways (for review, see Goberdhan and Wilson [2003]; Kozma and Thomas, 2002) do not participate in PR specification by *melt*, as none of the members that we tested exhibited defects in opsin regulation (*GMR>TSC*, *TSC1<sup>R453X</sup>*, *PTEN<sup>DJ89</sup>*, *DlnR<sup>ex15</sup>*, *DlnR<sup>353</sup>*, *Dp110A*, or *GMR>Dp110<sup>D954A</sup>*, *GMR>Dp110-CAAX*,

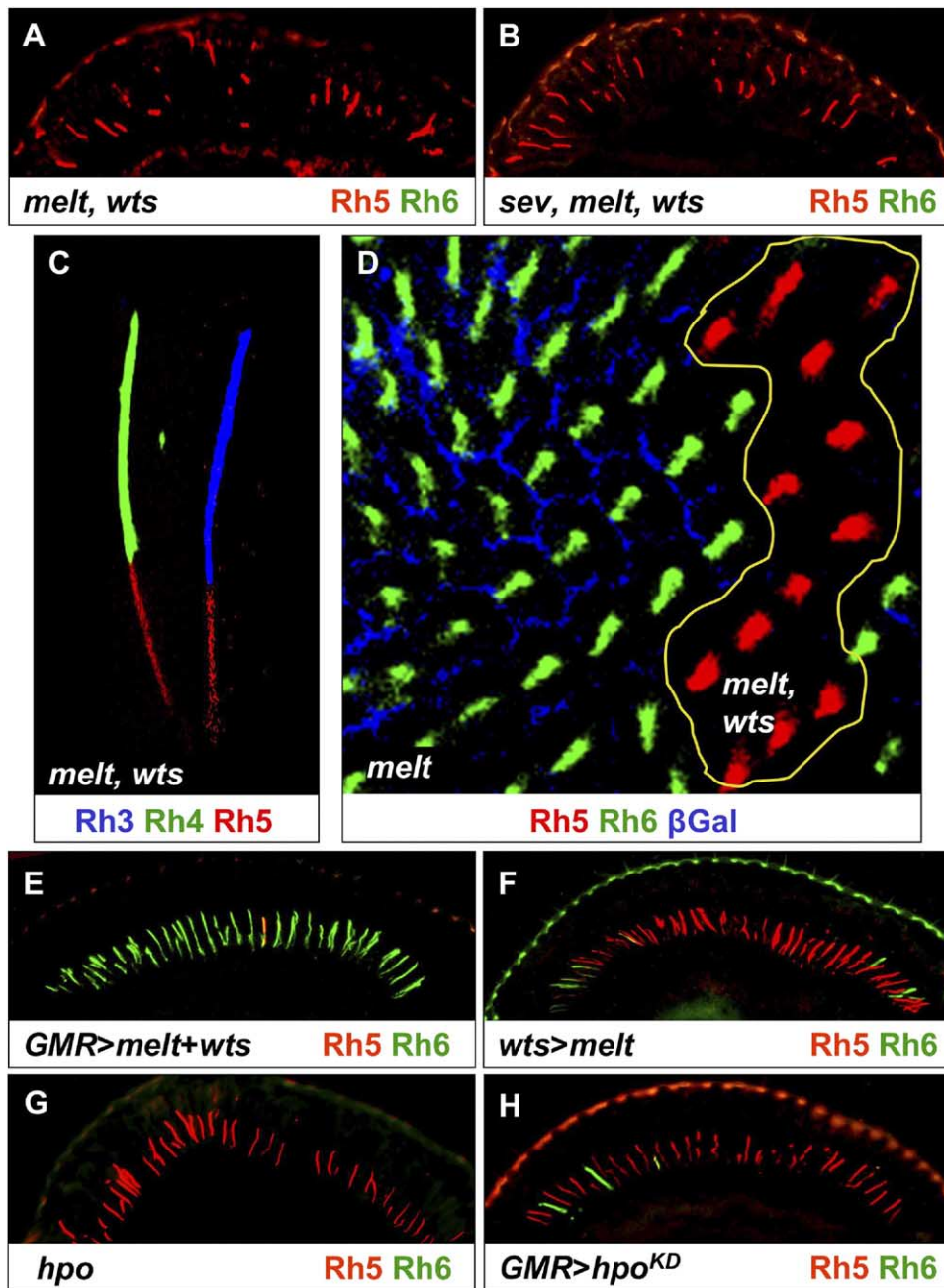


Figure 6. *wts* Regulates the Output of the Bistable Loop

(A and B) Section of eyes stained for Rh5 (red) and Rh6 (green).

(A) In *melt*<sup>*Δ3*</sup>, *wts*<sup>*latsP1*</sup> double-mutant eyes, Rh6 expression is lost and Rh5 expression is expanded. The retina is disorganized and some rhabdomeres are missing.

(B) Rh5 expression in the *melt*<sup>*Δ3*</sup>, *wts*<sup>*latsP1*</sup> double-mutant eyes is not induced by the R7 signal. Sections of *sev*; *melt*<sup>*Δ3*</sup>, *wts*<sup>*latsP1*</sup> triple-mutant eyes stained for Rh5 (red) and Rh6 (green). Rhodopsin expression is the same as in *melt*<sup>*Δ3*</sup>, *wts*<sup>*latsP1*</sup> double-mutant eyes.

(C) In *melt*<sup>*Δ3*</sup>, *wts*<sup>*latsP1*</sup> mutant eyes, the R8 fate is misspecified. Miscoupling of Rh4 and Rh5 in dissociated double-mutant ommatidia stained for Rh3 (blue), Rh4 (green), Rh5 (red).

(D) Reversion of the R8 fate in *melt*<sup>*Δ3*</sup>, *wts*<sup>*latsP1*</sup> double-mutant clones within *melt*<sup>*Δ3*</sup> whole-mutant eyes. A section of a whole-mounted retina stained for Rh5 (red), Rh6 (green), and  $\beta$ -Gal (blue). *melt*<sup>*Δ3*</sup>, *wts*<sup>*latsP1*</sup> clones are marked by the absence of *arm-lacZ* (blue). Rh5 expression is lost in the *melt*<sup>*Δ3*</sup> mutant eye, while Rh5 expression is restored in *melt*<sup>*Δ3*</sup>, *wts*<sup>*latsP1*</sup> clones.

(E–H) Section of eyes stained for Rh5 (red) and Rh6 (green).

(E) Section of eyes simultaneously misexpressing *GMR*>*melt* and *GMR*>*wts*; Rh6 is expanded and Rh5 expression is reduced.

(F) *wts*-Gal4>*melt* eyes. Misexpression of *melt* under the *wts* enhancer is sufficient to induce *rh5* expression in the y subtype.

(G) *hpo*<sup>*KC203*</sup> whole clonal eyes have the same phenotype as *wts*; Rh6 expression is lost and replaced by Rh5 in all R8.

(H) Misexpression of a kinase dead *hpo* has a dominant-negative phenotype. Rh6 expression is lost and replaced by Rh5 in majority of *GMR*>*hpo*<sup>*K71R3A*</sup> R8 eyes.

GMR>*Dp110*, GMR>*S6K<sup>KQ</sup>*, and GMR>*S6K<sup>STDETE</sup>*, data not shown).

## Discussion

### *wts* and *melt* Have Opposite Functions in R8 Subtype Specification

As R7 and R8 in a given ommatidium share the same optic path, their fates must be tightly regulated. The decision of a given ommatidium to become y or p is initially made by R7. Once R7 has chosen its fate, it imposes it onto the underlying R8. To coordinate opsin expression between R7 and R8, R8 has to respond to the R7 signal with high fidelity.

Here, we have shown that *wts* and *melt* act in R8 to prevent an ambiguous response to the instructive R7 signal. *wts* and *melt* play opposite roles in the specification of R8 subtypes. In the absence of *wts*, the yR8 subtype is completely misspecified into pR8. By contrast, in *melt* mutants, the pR8 subtype is lost with expansion of yR8. Overexpression of *wts* or *melt* leads to the transformation of all R8 into the y or p fate, respectively. The complementary expression patterns of the two genes in y or p R8 subtypes are set up in response to the pR7 signal. Therefore, *wts* and *melt* appear to interpret the signal from R7, and mutations in *wts* and *melt* render R8 insensitive to this signal without influencing R7 or outer PR.

### *wts* and *melt* Form a Bistable Loop Controlling the Robust Choice between R8 Fates

The decision to express *wts* or *melt* in R8 is determined by R7, but the two genes repress each other's transcription. Thus, *wts* and *melt* act in a loop of negative crossregulation. However, if R7 imposes its fate upon R8, what then is the role of this crossregulation? We suggest that the bistable loop allows only an unambiguous readout while R7 provides an asymmetric bias of this choice.

In a negative bistable crossregulatory loop, the input signal biasing cell-fate choice might act at any level. Similarly, any member of the loop can serve as the output. For instance, *wts* could positively regulate *rh6* expression (yR8 fate), while *melt* could activate *rh5* (pR8 fate). Our double misexpression and double loss-of-function experiments suggest that *wts* is the output regulator of the loop. When both *wts* and *melt* are ectopically expressed, all R8 acquire the y fate, i.e., the fate imposed by *wts*. In *melt*, *wts* double mutants, all R8 acquire the p fate. These phenotypes resemble the single gain- or loss-of-function phenotypes of *wts*, which appears to be necessary and sufficient for *rh6* expression. In contrast, while *melt* is sufficient to induce *rh5* in yR8, *rh5* remains expressed in the absence of *melt* in the double mutant. This argues that *melt* is not necessary for the pR8 fate (*rh5*). In *melt*, *wts* double-mutant eyes, *rh5* does not depend on instruction from pR7, which confirms that *rh5* expression is a consequence of the absence of *wts* (a derepression rather than activation by the pR7 signal).

We propose the following model (Figure 7): in the absence of an instructive pR7 signal, i.e., in y ommatidia, the loop is biased in favor of *wts* expression, which represses *melt*. In p ommatidia, the R7 signal either induces *melt* expression in R8 (as shown in Figure 7) or represses expression of *wts* in R8. In either case, the balance of the loop is shifted, leading to upregulation of *melt* and complete suppression of *wts* expression. This system is able to amplify a weak or transient signal to ensure that the cell-fate decision is made unambiguously.

There are clearly a number of examples of bistable loop that often reinforce stochastic decisions or transient differentiation stimuli (Ferrell, 2002). Bistable systems require positive feedback loops as proposed for the BMP signaling during dorso-ventral patterning in *Drosophila* (Wang and Ferguson, 2005) or double-negative feedback loops as in the case of the *wts-melt* loop. The left-right choice by chemosensory ASE neurons in *C. elegans* is a similar example where a negative bistable loop is involved in making an unambiguous cell-fate decision. This loop includes two transcription factors and two microRNAs. In the left ASE, this loop is strongly biased toward Na<sup>+</sup>-sensitive fate and in the right ASE, toward Cl<sup>-</sup> sensitivity (Johnston et al., 2005). This strong bias is likely imposed by a factor outside of the loop. In R8 cells, the *wts-melt* loop is inherently biased toward y fate. The signal from R7 in p ommatidia biases the choice toward the pR8 fate. The transcription loop described here is clearly incomplete since neither Wts nor Melt is a transcription factor. We have recently identified a mutation, *daltonien* (*don*), which genetically interacts with *melt*, activates the expression of *melt* (D.P. and C.D., unpublished data), and appears to encode a component of this loop. Another potential member of the loop is the newly identified transcriptional coactivator Yorkie (Yki), a direct target of the Wts kinase (Huang et al., 2005).

The bistable loop is specific to those R8 that are involved in color vision: in DRA ommatidia, *melt* misexpression does not lead to *wts* downregulation. This is not surprising since R7 and R8 in DRA are specified independently by positional information and do not appear to communicate (Wernet et al., 2003).

### Transcriptional Regulation of Signaling Molecules

The transcriptional regulation of *wts* and *melt* expression is surprising, since kinases and PH domain proteins are usually regulated by changes in their activity or subcellular localization. For instance, Wts/Lats kinase activity is regulated through phosphorylation by Hpo in the presence of Sav (Harvey et al., 2003; Wu et al., 2003). However, the nature of the signal that triggers activation of the Wts/Hpo/Sav proliferation control pathway has remained elusive. Thus, identification of the signal from pR7 to R8 could provide important insights into the mechanism by which this tumor-suppressor complex is regulated to control proliferation and cell death.

The ability of *wts* to indirectly regulate transcription of other genes (here *melt*) is less surprising. *wts*, *sav*, and *hpo* have been reported to negatively regulate the transcription of Cyclin E and DIAP1, leading to a de-

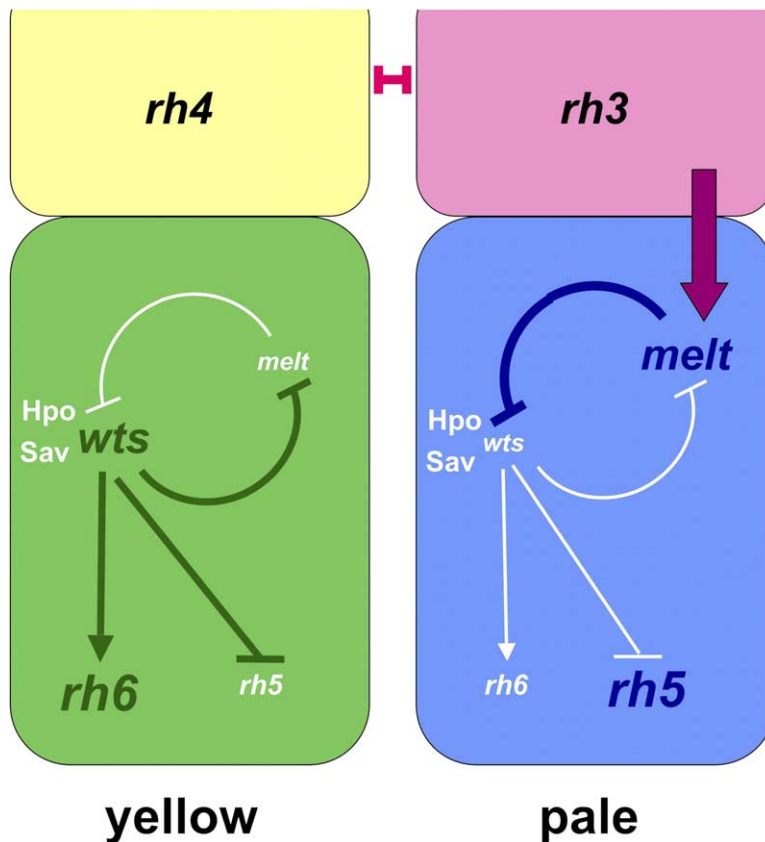


Figure 7. Model: *wts* and *melt* Interact in a Bistable Loop to Specify the y and p R8 Fate, Respectively

A loop involving transcriptional repression of *wts* and *melt* lies in the center of the R8 fate decision. The loop is able to swing the system into one of the two fates, depending on the presence or absence of the inductive R7 signal. In the absence of an instructive signal from the yR7, the underlying R8 expresses *wts*. *wts* in turn represses *melt* and *rh5*, allowing the activation of the yR8 rhodopsin *rh6*. Upon induction from the pR7, the loop swings the system into the p fate. We suggest that the R7 signal relieves *melt* from the repression by *wts*. *melt* is able to repress *wts*, and without *wts* *rh6* is repressed and the pR8 rhodopsin *rh5* becomes expressed. *hpo* and *sav* are necessary to allow *wts* activity, presumably through phosphorylation by Hpo.

crease in cell cycle progression and to an increase in cell death (Justice et al., 1995; Xu et al., 1995; Tapon et al., 2002; Kango-Singh et al., 2002; Wu et al., 2003; Harvey et al., 2003; Udan et al., 2003; Pantalacci et al., 2003). The same (unknown) transcription factor required downstream of *wts* could therefore also play a role in repressing *melt* and *rh5*, and possibly in activating *rh6*.

Cbk1, the Lats/Wts homolog in *S. cerevisiae* has been shown to regulate a broad range of daughter specific genes during budding (Colman-Lerner et al., 2001). The asymmetric gene expression between mother and daughter cells is due to Cbk1-dependent activation and nuclear localization of the transcription factor Ace2 in daughter cells. Cbk1 kinase activity requires another gene, Mob2. Recently, a member of the Mob family in *Drosophila*, Mats, has been shown to bind and synergistically interact with Wts/Lats to control proliferation and apoptosis (Lai et al., 2005). Although Melt is not known to regulate the transcription of other target genes, it can affect subcellular localization of FOXO and the TSC1/TSC2 complex to regulate fat metabolism. However, in our hands, the members of the TOR or InR do not play a role in the specification of R8 subtypes.

#### A New Postmitotic Function for the *hpo/sav/wts* Pathway

Wts, together with the Ser/Thr kinase Hpo and the adaptor protein Sav, acts as a potent tumor suppressor

(Justice et al., 1995; Xu et al., 1995; Kango-Singh et al., 2002; Tapon et al., 2002; Harvey et al., 2003; Wu et al., 2003; Udan et al., 2003; Pantalacci et al., 2003). Here, we have shown that all three genes play a critical role for the establishment of the R8 subtypes. The function described here for *hpo/sav/wts* represents an unexpected new role unrelated to their tumor-suppression function: R8 PRs have exited the cell cycle for at least 4 days when they choose to express a particular rhodopsin, and these cells are not prone to die (PRs are particularly difficult to kill through induction of the cell death pathway). Furthermore, there is no detectable difference in cell size or shape between y and p R8, which specifically express or exclude *wts* or *melt* expression. However, it is interesting to note that p and y inner photoreceptors are morphologically distinguishable in *Calliphora* blowflies (Wunderer and Smola, 1982). Perhaps Wts and Melt represent an evolutionary remnant of a system in large flies where subtypes required different morphologies. Therefore, specification of the correct R8 fate utilizes two signaling cassettes used for different purposes earlier in development, after these cassettes are no longer in use in these highly differentiated PR cells.

Lats1, the human ortholog of Wts, is able to rescue the lethality of *wts* in flies (Tao et al., 1999). Recently, it was shown that a canine Lats1 splice variant is specifically expressed in the retina (Akhmedov et al., 2005). Moreover, a gene responsible for an autosomal dominant cone dystrophy (involving impaired color vision,

sensitivity to light, and gradual loss of visual activity) has been mapped close to the *Lats1* locus (Akhmedov et al., 2005). Thus, we might expect that the *hpo/sav/wts* pathway functions in the human retina as well. Although, *melt* knockout mice are viable and fertile (Muto et al., 2004), it will be interesting to test whether they are defective in cone differentiation or vision.

## Experimental Procedures

### Fly Stocks

The following lines were used for the study: *melt<sup>Δ1</sup>* is described in Teleman et al. (2005); *melt<sup>Δ3</sup>* is a deletion that covers *melt* and the adjacent genes CG32390 and *corn*; *melt<sup>Δ1</sup>* deletes ~300 bp more of *corn* than *melt<sup>Δ3</sup>*; *FRT82wts<sup>latsP1</sup>*, *FRT82wts<sup>latsX1</sup>*, *FRT82sav<sup>shrp1</sup>*, *FRT82P[arm-lacZ,w<sup>+</sup>]*, *FRT42hpo<sup>KC203</sup>*, *FRT82TSC1<sup>R453X</sup>*, *FRT40PTEN<sup>DJ89</sup>*, *FRT82DlnR<sup>ex15</sup>*, *FRT82DlnR<sup>353</sup>*, *FRT82Dp110<sup>A</sup>*, *sev<sup>14</sup>*, *corn<sup>Δ84</sup>*, *UAS-lats*, *UAS-melt*, *UAS-lacZ<sup>[melt]</sup>*, *UAS-hpo<sup>K71R</sup>*, *UAS-TSC1*, *UAS-TSC2*, *UAS-corn*, *UAS-Dp110<sup>D954A</sup>*, *UAS-Dp110-CAAx*, *UAS-Dp110*, *UAS-S6K<sup>KO</sup>*, *UAS-S6K<sup>STDETE</sup>*, *P[lacZ,w<sup>+</sup>]*, *aktP[lacZ:NLS]*, *rh5-lacZ:NLS* (T. Cook and C.D., unpublished data), *FRT82P[GMR:hid,w<sup>+</sup>]*, *UAS-lacZ*, *UAS-GFP:NLS*, *UAS-lacZ:NLS*. The “long GMR” driver was previously described (Wernet et al., 2003). All flies were raised at 24(+1)°C.

The panR7 driver is a combination of the *rh3* (−206 to −44) and *rh4* (−44 to +76) promoters driving Gal4 in all R7 cells (A. Tahayato and C.D., unpublished data). The panR8 is a combination of the *rh5-Gal4* and *rh6-Gal4* and is expressed in all R8. To generate *melt* clones in R7 cells only, we used *GMR>flp;rh5>lacZ* (or *rh6>lacZ*)/*panR7-Gal4>syb-GFP*; *FRT80melt<sup>Δ1</sup>*/ *FRT80tub-Gal80* 0- to 6-hr-old flies (later the strength of panR7-Gal4 overcomes the repression by tub-Gal80).

Recombined lines were as follows: *melt<sup>Δ3</sup>*, *FRT82wts<sup>latsP1</sup>*, *melt<sup>Δ3</sup>*, *FRT82GMR-hid*, and *melt<sup>Δ3</sup>*, *FRT82arm-lacZ* were generated by recombining the *melt<sup>Δ3</sup>* to *FRT82wts<sup>latsP1</sup>*, *FRT82GMR:hid*, and *FRT82arm-lacZ*, respectively. To generate *melt<sup>Δ3</sup>*, *wts<sup>latsP1</sup>* double-mutant clones in *melt<sup>Δ3</sup>* mutant eyes, *melt<sup>Δ3</sup>*, *FRT82wts<sup>latsP1</sup>* flies were crossed to *ey-flp*; *melt<sup>Δ3</sup>*, *FRT82arm-lacZ* flies.

### The *melt*-Gal4 and *melt*-lacZ:NLS Reporter Constructs

The *melt* enhancers (~1.1 kb 5' and ~4 kb first intron) were amplified by PCR from a genomic fragment flanking the *P[lacZ, w<sup>+</sup>]<sup>S1441/14</sup>* in *melt* (Salzberg et al., 1997). The ~1.1 kb 5' (−1054 to +60) fragment was PCR amplified, introducing EcoRI and BamHI sites. The EcoRI-BamHI fragment was ligated into the fly injection vector pCasper[hs43-Gal4-SV40] (T. Cook and C.D., unpublished data). The ~4 kb (+628 to +4636) first intron was PCR amplified and cut using EcoRI. It was ligated into the fly injection vectors pCasper[hs43-Gal4-SV40] and pCasper[hs43-lacZ:NLS-SV40] (T. Cook and C.D., unpublished data). Transgenic lines were generated by injection of purified plasmid DNA at a concentration of 0.3 μg/μl into ~250 embryos of 0–30 min of age using standard procedures.

### Microscopy and Immunohistochemistry

GFP expression in photoreceptors of living flies was assessed by neutralizing the cornea using water immersion (Pichaud and Desplan, 2001).

Cryosection and whole-mounted retina staining of adult heads were performed as previously described (Cook et al., 2003; Tahayato et al., 2003). Dissociated ommatidia were as follows: retinas were dissected as for whole-mounted staining (see above). The retina was scooped out of the cornea using two Tungsten needles in a drop of PBS (1×) on a microscope slide and gently dissociated using the two needles. The slides were air dried for 15–20 min and treated as for cryostat sections (see above). All transgenic constructs were crossed into a *cn bw* or *p[WIZ]* (Lee and Carthew, 2003) background to eliminate eye pigmentation.

Primary antibodies used were as follows: anti-β-Gal rabbit 1:5000 (Cappel), anti-β-Gal chicken 1:800 (Ab-cam), anti-GFP rabbit 1:500 (Molecular Probes), anti-GFP sheep 1:1000 (Biogenesis), anti-Sens guinea pig 1:10 (a gift from H. Bellen, Baylor College of Medicine), anti-Rh3 chicken 1:20 (Cook et al., 2003), anti-Rh4 rabbit 1:300 (a

gift from C. Zuker, University of California, San Diego), anti-Rh5 mouse monoclonal 1:100 (Chou et al., 1996), anti-Rh6 rabbit 1:1000 (Tahayato et al., 2003), anti-Rh5-TxR 1:500 and anti-Rh6-FITC 1:500 (a gift from S. Britt, University of Colorado), Phalloidin-Alexa-Fluor546 1:50 (Molecular Probes), anti-24B10 mouse monoclonal 1:50 (Developmental Studies Hybridoma Bank).

Secondary antibodies used were as follows: AlexaFluor488 coupled made in goat or donkey anti-rabbit, -mouse, -guinea pig, -sheep; AlexaFluor544 coupled made in donkey anti-rabbit, -chicken, -mouse; AlexaFluor645 coupled made in donkey anti-rabbit and -mouse (Molecular Probes); Cy3 coupled made in goat or donkey anti-rabbit, -chicken; and Cy5 coupled made in donkey anti-mouse (Jackson Immunochemicals).

All fluorescent microscopy was performed using a Nikon Microphot-Sa and super high-pressure mercury lamps (Hg 100 watts, Ushio Electric). Confocal microscopy was performed using the Leica TCS S2 system. Digital images were produced using SPOT software.

### Supplemental Data

Supplemental Data include three figures and can be found with this article online at <http://www.cell.com/cgi/content/full/122/5/775/DC1>.

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