rhomboid Mediates Specification of Blue- and Green-Sensitive R8 Photoreceptor Cells in *Drosophila*

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Color vision is based on the differential color sensitivity of retinal photoreceptors, however the developmental programs that control photoreceptor cell differentiation and specify color sensitivity are poorly understood. In *Drosophila* there is growing evidence that the color sensitivity of the R8 cell within an individual ommatidium is regulated by an inductive signal from the adjacent R7 cell. We previously examined the retinal patterning defect in *Scutoid* mutants, which results from a disruption of *rhomboid* expression. Here we show that loss of *rhomboid* blocks the induction of Rh5 expression and misexpression of *rhomboid* leads to the inappropriate induction of Rh5. These effects are specific to *rhomboid*, because its paralogue *roughoid* is neither required nor sufficient for the induction of Rh5 expression. We show that *rhomboid* is required cell-autonomously within the R8 photoreceptor cells and nonautonomously elsewhere in the eye for Rh5 induction. Interestingly, we found that the *Epidermal growth factor receptor* is also required for Rh5 induction, and its activation is sufficient to rescue the loss of Rh5 induction in a *rhomboid* mutant. This suggests that *rhomboid* may function in R8 cells to activate *Epidermal growth factor receptor* signaling in R7 cells and promote their differentiation to a signaling competent state.

Introduction

Drosophila melanogaster is capable of color vision and is a useful experimental system for examining the developmental programs that produce photoreceptor cells having different color sensitivities (Quinn et al., 1974; Spatz et al., 1974; Chou et al., 1996; Chou et al., 1999; Tang and Guo, 2001; Cook et al., 2003; Wernet et al., 2003; Mikeladze-Dvali et al., 2005). The compound eye is composed of ~750 ommatidia, each containing eight photoreceptor cells (R1–R8) (Hardie, 1985; Heberlein et al., 1993). These cells are distinguishable based on anatomical position, color sensitivity and visual pigment expression (Fig. 1A). The R1–R6 photoreceptor cells express the blue absorbing visual pigment Rhodopsin 1 (Rh1). Adjacent R7 and R8 cells in an individual ommatidium express visual pigments in a precisely coordinated manner (Fig. 1B–D). The two major types of ommatidia are referred to as yellow and pale, based on their appearance under blue illumination (Kirschfeld et al., 1978; Franceschini et al., 1981). Yellow ommatidia occupy ~65% of the eye and contain UV-sensitive Rh4-expressing R7yellow (R7y) cells and green-sensitive Rh6-expressing R8yellow (R8y) cells (Fig. 1D). Pale ommatidia

occupy ~35% of the eye and contain UV-sensitive Rh3-expressing R7pale (R7p) cells and blue-sensitive Rh5-expressing R8pale (R8p) cells (Fig. 1C). We have shown that the organization of the R7y and R7p cells is statistically random (Bell et al., 2007), consistent with the idea that R7y versus R7p cell fate is determined by a stochastic process that requires the gene *spineless* (Wernet et al., 2006). There is also a specialized class of ommatidia along the dorsal rim (DR) that are thought to be polarization sensitive; DR ommatidia express Rh3 in both the R7 and R8 cells, which are specified by *wingless*, the *iroquois* genes, and *homothorax* (Fortini and Rubin, 1991; Tomlinson, 2003; Wernet et al., 2003) (Fig. 1B).

The basis for specification of R7 and R8 pairs in yellow and pale ommatidia is unknown, however there is genetic evidence that the coordination of opsin expression in these cells is established by a switch from the default (Rh6, green sensitive) to induced state (Rh5, blue sensitive). This switch within the R8 cell is dependent on the phenotype of the adjacent R7 cell (Chou et al., 1996, 1999; Papatzenko et al., 1997) (Fig. 1C,D). The presumptive signal from R7 to R8 is thought to regulate the genes *melted* and *warts* that are required for proper Rh5 and Rh6 expression (Mikeladze-Dvali et al., 2005). Here we show that *rhomboid* (*rho*) mutants fail to induce the expression of Rh5 in R8 cells adjacent to Rh3-expressing R7 cells. *rho* is a component of the *Epidermal growth factor receptor* (EGFR) signaling pathway that is both required and sufficient for the induction of Rh5 expression in R8 cells. These results reveal an unexpected and highly specific role for *rho* and EGFR in generating photoreceptor cell diversity in the retinal mosaic.

Materials and Methods

Stocks and genetics. Stocks were maintained in humidified incubators on standard cornmeal/molasses/agar media. Unless otherwise specified, all

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Figure 1. Patterning of the R7 and R8 photoreceptor cells. **A**, Longitudinal view of a *Drosophila* ommatidium, composed of eight neuronal photoreceptor cells having rhabdomeres containing the visual pigments. The rhabdomeres of the outer photoreceptor cells (R1–R6) surround the apical and basal rhabdomeres of the inner R7 and R8 cells, respectively. **B**, Ommatidia along the dorsal margin of the eye contain Rh3 in both R7 and R8. The specification of ommatidia in the dorsal margin occurs from signaling events in the eye periphery. **C**, **D**, Tangential cross-sections of the two major types of ommatidia, yellow and pale, expressing Rh4 and Rh6 or Rh3 and Rh5 in adjacent R7 and R8 cells. Expression of Rh6 in R8/yellow cells (**D**) is not dependent on the presence of an adjacent R7 cell and likely reflects a default fate. Expression of Rh5 in R8/pale cells (**C**) is dependent on the presence of an adjacent Rh3-expressing R7 cell and likely reflects an inductive signal.

stocks were obtained from the Bloomington Stock Center. *rho*^{P^{Δ5} FRT}, *rho*^{7M43}*ru*¹ FRT, *ru*^{PLLB} and *UAS-*rho** were provided by Mathew Freeman. *UAS-*rho** was provided by Ethan Bier. *UAS-EGFR^{DN}* was provided by Nicholas Baker. *sina* stocks were provided by Richard Carthew. Homozygous mutant clones in the eye of *rho*^{P^{Δ5}} and *rho*^{7M43}*ru*¹ were generated with the FLP/FRT system by standard techniques (Xu and Rubin, 1993) using the *eyeless* promoter-driven FLP recombinase (*ey-FLP*) (Newsome et al., 2000). For ectopic expression, flies containing the *UAS*-transgenes were crossed to *GMR-Gal4* flies at 28°C. *EGFR^{tsla}/CyO* and *EGFR²⁴/CyO* adults were mated and progeny were raised at 18°C. *EGFR^{tsla}/EGFR²⁴* white prepupae (P₀) were shifted to 29°C for 24 h and returned to 18°C until eclosion. As noted in Results, the *EGFR^{E1}/+* background yields a significant increase in the percentage of Rh3-expressing R7 cells. We found that this effect is very sensitive to genetic background and have therefore conducted experiments generating *EGFR^{E1}*, *sina*, and *FLP-FRT rho* mutant clones using sibling members of crosses as controls. This is noted in Results and in Table 1.

Scanning electron micrographs. Flies were dehydrated for 12 h each in a graded ethanol series (25%, 50%, 75% and 100%), followed by an incubation in hexamethyldisilazane. Samples were dried under house vacuum and sputter coated with a Gold Palladium Target (Electron Microscopy Sciences), mounted on 12-mm-diameter carbon adhesive tabs and placed on stubs. Samples were examined with a LEO 435VP scanning electron microscope (LEO Electron Microscopy Ltd.).

Immunohistochemistry. Cryosections (10 μm) were prepared and treated as previously described (Chou et al., 1999). Antibodies were used at the following dilutions: directly conjugated mouse monoclonal anti-Rh5 (Texas Red, 1:100), anti-Rh6 (FITC, 1:100), anti-Rh3 (clone 2B1, 1:20), anti-Rh4 (clone 11E6, 1:10), FITC-conjugated Fab fragment goat anti-mouse (1:400, Jackson ImmunoResearch Laboratories), AlexaFluor 568 goat anti-mouse IgG (H+L) (1:400, Invitrogen). Immunofluorescence images were acquired with an Axioskop plus/AxioCamHRC (Carl Zeiss) or by confocal microscopy using a Zeiss Pascal LSM (Carl Zeiss).

Comparisons of the proportions (percentages) of opsin expression in different genetic backgrounds were performed with a z-score and are shown in Tables 1 and 2 (Fleiss et al., 2003). The z-score was calculated using the following equation:

$$z = \frac{|p_2 - p_1| - \frac{1}{2}(1/n_1 + 1/n_2)}{\sqrt{\bar{p}\bar{q}(1/n_1 + 1/n_2)}},$$

where p_1 and p_2 are proportions of marker expression in each of the two different genotypes under comparison, n_1 and n_2 are the number of ommatidia counted for each genotype, $\bar{p}$ is the average proportion for both genotypes combined, and $\bar{q} = 1 - \bar{p}$. The significance of the difference between the two proportions was determined from the normal distribution as a two-tailed test.

Results

rhomboid is both necessary and sufficient for induction of Rh5 expression in R8 photoreceptor cells

We previously identified a retinal patterning defect in *scutoid* mutants that leads to a disruption in the normal paired expression of opsins in adjacent R7 and R8 cells (D. A. Birkholz, W.-H. Chou, M. M. Phistry, and S. G. Britt, unpublished observations). We found that this defect was due to the ectopic expression of the transcriptional repressor *snail* (Fuse et al., 1999), which causes *rho* to be misexpressed. To test whether *rho* regulates

specification of R8 photoreceptor cell fate, we generated *rho* mutant eye patches (Fig. 2A–D). Interestingly, we observed a dramatic decrease in the number of R8 cells that expressed Rh5, accompanied by a corresponding increase in Rh6 expression (Fig. 2C; Table 1). Loss of R7 cells, as occurs in the *sev* mutant, leads to a decrease in Rh5-expressing R8 cells and a corresponding increase in Rh6 (Chou et al., 1999). To verify that a loss of R7 cells did not increase Rh6 expression, we examined a *rho* mosaic and found that R7 cells are still present and express Rh3 and Rh4 at normal percentages (35% and 65% respectively, Table 1). This discrepancy between the normal proportion of Rh3 and Rh4 versus the decrease in Rh5 suggests that there must be a disruption in the coordination of adjacent R7/R8 cells. Indeed, we found this was the case. *rho* mutant animals have a large number of ommatidia with mispaired Rh3-expressing R7 cells and Rh6-expressing R8 cells; this effect is specific to the pale class of ommatidia and does not affect the dorsal rim (Fig. 2D). The loss of Rh5 expression in R8 cells adjacent to Rh3-expressing R7 cells demonstrates that *rho* is required for the induction of Rh5 expression, and is essential for establishing R7/R8 cell subtype adjacency (Fig. 1C,D).

To test whether misexpression of *rho* in WT animals is sufficient for the induction of Rh5 expression and could potentially induce inappropriate induction of Rh5 expression, we used *GMR-Gal4* to drive *rho* expression (*UAS-*rho**) behind the morphogenetic furrow. These animals displayed two mutant phenotypes. First, misexpression of *rho* roughens the eye (Fig. 2E). As previously shown, ectopic *rho* expression hyperactivates EGFR signaling and produces excess cone and pigment cells giving the eye a roughened appearance (Wasserman et al., 2000). The second phenotype generated by misexpression of *rho* is an increase

Table 1. Opsin expression in different genetic backgrounds

Genotype	R8 cells expressing Rh5 [% (n)]	R7 cells expressing Rh3 [% (n)]	Mispairing	Figure
CS	33% (359)	38% (139)	NO	—
<i>w¹¹¹⁸</i>	29% (214)	34% (140)	NO	1B–D
<i>sev¹⁴</i>	12% (585)	—	—	—
	SDF <i>CS</i> $p < 10^{-13}$			
<i>rho^{Δ5} FRT</i>	6.0% (300)	35% (280)	YES (Rh3/Rh6)	2A–D
	SDF <i>CS</i> $p < 10^{-15}$			
<i>rho^{Δ5}/+</i>	40% (208)	ND	ND	—
<i>rho^{7M43} ru¹ FRT</i>	No eye	—	—	3E
<i>GMR-GAL4 → UAS-EGFR^{DN}</i>	No eye–scar	—	—	6C
<i>rho^{7M43} ru¹/++</i>	40% (218)	ND	ND	—
<i>rho^{7M43} ru¹/+ ru¹</i>	36% (222)	ND	ND	3D
<i>ru¹</i>	35% (154)	36% (191)	NO	3B
<i>ru^{PLlb}</i>	34% (339)	ND	ND	—
<i>GMR-GAL4 → UAS-rho</i>	51% (819)	ND	YES	2F
	SDF <i>CS</i> $p = 10^{-8}$		(Rh4/Rh5)	
<i>GMR-rho</i>	50% (250)	37% (590)	YES	6A
<i>y w; P{w, GMR-rho, Exel}3</i>	SDF <i>CS</i> $p < 10^{-5}$		(Rh4/Rh5)	
<i>sev; GMR-rho</i>	3.4% (583) SDF <i>GMR-rho</i> $p < 10^{-15}$	ND	ND	6B
<i>GMR-Gal4 → UAS-ru</i>	33% (231)	ND	NO	3F–H
<i>EGFR^{tsla}/EGFR^{CO}</i>	20% (182) SDF <i>CS</i> $p = 0.0016$	37% (139)	YES	5A–E
			(Rh3/Rh6)	
<i>EGFR^{E1}/+</i>	78% (177)	75% (236)	YES	6D–F
	SDF <i>CS</i> $p < 10^{-15}$	SDF <i>CS</i> $p < 10^{-11}$	(Rh4/Rh5)	
<i>sev; EGFR^{E1}/+</i>	32% (655)	Rh3-expressing R7 cells present	ND	6G
	SDF <i>EGFR^{E1}</i> $p < 10^{-15}$			
<i>EGFR^{E1}/+; sina²/sina³</i>	15% (143)	—	—	6H
	SDF sibling control (below) $p < 10^{-13}$			
<i>sibling control</i>	52% (443)	48% (96)	YES	—
<i>EGFR^{E1}/+; sina²/+</i>	SDF <i>CS</i> $p < 10^{-6}$		Rh3/6, 4/5	
<i>sina²/sina³</i>	3.7% (404)	—	—	—
	SDF <i>CS</i> $p < 10^{-15}$			
<i>EGFR^{E1}/+; rho^{Δ5} FRT</i>	42% (258)	51% (241)	YES	6I
	Not SDF sibling control (below)	Not SDF sibling control (below)	Rh3/6, 4/5	
<i>sibling control</i>	SDF <i>rho^{Δ5} FRT</i> $p < 10^{-15}$	SDF <i>CS</i> $p = 0.020$		
<i>EGFR^{E1}/+; rho^{Δ5}/+</i>	42% (366)	52% (236)	YES	—
	SDF <i>CS</i> $p = 0.011$	SDF <i>CS</i> $p = 0.015$	Rh3/6, 4/5	

Statistical comparisons of strains were carried out as described in Materials and Methods. Parentheses indicate the number of ommatidia counted. Unless indicated, the observed percentages are not significantly different from CS. Strains compared to another control are indicated. SDF, Significantly different from the strain indicated, at the *p* value shown; ND, not determined; —, not applicable.

in Rh5 expression (51%, Table 1) and a mispairing of Rh4-expressing R7 cells with Rh5-expressing R8 cells (Fig. 2*F*). This gain-of-function phenotype is the opposite of the *rho* loss-of-function phenotype, and demonstrates that *rho* is sufficient for the induction of Rh5 expression in R8 photoreceptor cells.

***rhomboid* is required for eye development but not for induction of Rh5 expression in R8 cells**

There is a large family of rhomboid-like genes in *Drosophila* and other organisms (Wasserman et al., 2000; Lee et al., 2001; Freeman, 2008). In addition to *rho*, *ru* is also expressed in the developing *Drosophila* eye (Wasserman et al., 2000). Previous studies have shown that *rho* and *ru* function redundantly in photoreceptor cell recruitment and loss of either gene has a mild phenotype (Figs. 2*A*, 3*A*). *rho* mutants show normal external eye morphology, whereas *ru* mutants show roughening

Table 2. Opsin expression in *rhomboid* mosaic ommatidia

Genotype	R8 cells (adjacent to Rh3-expressing R7 cells) that express Rh5 [% (n)]	Figure
CS	95% (98)	—
<i>R7⁺/R8⁺</i>		
<i>rho</i> mosaic <i>R7⁺/R8⁺</i>	64% (120) SDF <i>CS</i> <i>R7⁺/R8⁺</i> $p = 1.3 \times 10^{-7}$	4A, A'
<i>rho</i> mosaic <i>R7⁻/R8⁺</i>	50% (48) Not SDF <i>rho</i> mosaic <i>R7⁺/R8⁺</i>	4B, B'
<i>rho</i> mosaic <i>R7⁺/R8⁻</i>	9% (67) SDF <i>rho</i> mosaic <i>R7⁺/R8⁺</i> $p = 9.8 \times 10^{-13}$	4C, C'
<i>rho</i> mosaic <i>R7⁻/R8⁻</i>	20% (85) SDF <i>rho</i> mosaic <i>R7⁺/R8⁺</i> $p = 1.0 \times 10^{-9}$ Not SDF <i>rho</i> mosaic <i>R7⁺/R8⁻</i>	4D, D'

Statistical comparisons of strains were carried out as described in Materials and Methods. The genotype of the *rhomboid* mosaic animals was *y^{d2} w¹¹¹⁸ P[ey-FLP.N]2 P[GMR-lacZ.C(38.1)]TPN1 / w⁺; P[Ubi-GFP.D]61EF P[neoFRT]80B / rho^{Δ5} P[neoFRT]80B*. The numbers in parentheses indicate the number of ommatidia counted. The observed percentages were compared to the indicated controls and were significantly different from (SDF) or not SDF the strain indicated, at the *p* value shown. —, Not applicable.

Figure 2. *rhomboid* is both required and sufficient for induction of Rh5 expression in R8 cells. **A**, Loss of *rho* does not dramatically affect the structure of the eye. **B**, A $\rho^{\Delta 5}$ mosaic eye having a large mutant region (white) and smaller heterozygous region (red). **C**, A single longitudinal section of the eye in **B** with an entirely mutant retina labeled with antibodies against Rh5 and Rh6. Removal of *rho* dramatically increases the number of Rh6-expressing R8 cells compared with *w*. **D**, Section of an entirely *rho* mutant retina labeled with antibodies against Rh3, Rh5, and Rh6. Loss of Rh5 expression in *rho* mutants is accompanied by an increase in Rh6 expression and mispairing of Rh3 and Rh6 expression in individual ommatidia (arrows), consistent with a loss of induction of Rh5. R7 and R8 cells of the dorsal rim are unaffected and express Rh3 normally (arrowhead). **E**, Ectopic *rho* (*UAS-rho*) driven by *GMR-Gal4* results in a rough eye with both missing and double bristles. **F**, Ectopic expression of *rho* (as in **E**) results in an increase in the number of Rh5-expressing R7 cells and a mispairing of Rh4 and Rh5 (arrows).

of the eye and loss of cone cells, pigment cells and some photoreceptor cells (Freeman et al., 1992; Wasserman et al., 2000). However, loss of both genes causes major defects in photoreceptor cell recruitment and eye formation (Fig. 3E), similar to that observed on removal of *EGFR* (see Fig. 6C) (Wasserman et al., 2000; Urban et al., 2001). These previous studies showed that the functions of *rho* and *ru* overlap to such an extent that the presence of either gene is sufficient for eye formation, clearly demonstrating that their functions are redundant for eye formation.

To test whether *ru* may also be required for normal photoreceptor cell patterning, we examined the phenotype of *ru* loss-of-function mutants. Interestingly, although these animals have the roughened eye previously noted, the normal proportion of Rh5- and Rh6-expressing R8 cells are present (Fig. 3B; Table 1), as are Rh3- and Rh4-expressing R7 cells (Table 1). The *ru* allele shown (*ru¹*) is a strong loss-of-function allele. We also examined a viable null allele (*ru^{PLLb}*) and found the same phenotype (Table 1). Loss of a single copy of *rho* in a *ru* mutant background has no effect on R8 photoreceptor cell differentiation (Fig. 3D; Table 1), whereas animals mutant for *rho* alone have a very specific defect (Fig. 2C,D). Our studies clearly demonstrate that in the absence of *rho*, *ru* alone is not sufficient for R8 photoreceptor cell subtype specification. Furthermore, loss of *ru* does not sensitize the animal to the dosage of *rho* that is present. Although *rho* and *ru* have redun-

dant functions in photoreceptor cell recruitment, there is a specific requirement for *rho* and not *ru* in regulating the expression of Rh5 versus Rh6 in R8 photoreceptor cells.

rho and *ru* encode structurally related membrane proteases that function in the cleavage and secretion of the EGFR ligands. The requirement for *rho* but not *ru* in R8 cell differentiation may reflect a different spatial or temporal expression pattern rather than different biochemical functions. To test this hypothesis, we ectopically expressed *UAS-ru* using the *GMR-Gal4* driver described above. If *ru* has an identical function to *rho* then expression of *ru* behind the morphogenetic furrow should be sufficient to induce Rh5 expression in R8 photoreceptor cells. Interestingly, we found that this was not the case. Expression of *UAS-ru* caused the compound eye to be roughened (Fig. 3F), however opsin gene expression in the R7 and R8 photoreceptor cells was normal (Fig. 3G,H; Table 1). This demonstrates that in contrast to *rho*, *ru* is neither required nor sufficient for induction of Rh5 expression in R8 cells.

***rhomboid* is required in R8 cells and elsewhere in the eye for Rh5 induction**

To determine where *rho* is normally required for the induction of Rh5 expression, we generated *rho* mutant clones in a heterozygous background using the FLP-FRT system. Cells that are either wild type or heterozygous express GFP, whereas cells that are mutant for *rho* do not express GFP. We dissociated ommatidia from animals

constructed in this manner and counted the expression of Rh5 versus Rh6 in ommatidia in which the genotype of the R7 and R8 cells could be scored. It was essential to conduct this experiment in a rigorous statistical manner, because there are some R8 cells that express Rh5 in a completely *rho* mutant eye (e.g., Fig. 2C). Furthermore, in previous studies, we have shown that there is occasional mispairing of Rh3 and Rh6 in normal wild-type eyes (signal failure), as well as Rh5 expression in *sev* animals lacking R7 cells, in which there is no signal to induce Rh5 (Chou et al., 1999).

To compare the effectiveness of Rh5 induction resulting from the R7 to R8 signal, we compared the percentage of Rh5 expression in R8 cells in ommatidia expressing Rh3 in the R7 cell and having readily scoreable R7 and R8 cell genotypes. Comparisons of the percentage of opsin expression in ommatidia having R7 and R8 cells of different genotypes were performed with a z-score (Fig. 4; Table 2) (Fleiss et al., 2003). In each panel we show a montage of ommatidia labeled against Rh3, Rh5 and GFP. Wild-type or heterozygous R7 and R8 cells are labeled with GFP, indicated with arrows in each panel, and the GFP image alone is shown as a separate panel (Fig. 4A' for example). Cells that are mutant for *rho* do not express GFP. We found that in ommatidia having genetically mutant R7 cells and genetically wild-type R8 cells (Fig. 4B,B'), the percentage of ommatidia expressing Rh5

Figure 3. *roughoid* is neither required nor sufficient for induction of Rh5 expression in R8 cells. **A**, Loss of *ru* results in a rough eye phenotype. **B**, Rh5 (35%) and Rh6 (65%) expression are normal in *ru*¹, a strong hypomorph. We examined a viable null allele, *ru*^{PLB}, which also has normal Rh5 expression (34%, Table 1). **C**, Loss of one copy of *rho* does not alter the *ru* rough eye phenotype. **D**, Loss of both copies of *ru* does not sensitize the eye to the dosage of *rho*. Animals heterozygous for *rho* and homozygous mutant for *ru* (*rho*^{7M43} *ru*¹/+, *ru*¹) are normal and display WT percentages of Rh5 and Rh6. **E**, Removal of both *rho* (*rho*^{7M43}) and *ru* (*ru*¹) results in the loss of all ommatidia in the eye, similar to that found with the loss of *EGFR* function (Fig. 6C). **F**, Expression of ectopic *ru* (*UAS-ru*) driven by *GMR-Gal4* results in a very rough eye. **G**, **H**, Ectopic expression of *ru* does not affect the percentage or pairing of opsin gene expression in the R7 and R8 photoreceptor cells. Normal pairing of Rh4 and Rh6 as well as Rh3 and Rh5 is observed, demonstrating that *ru* is not sufficient for induction of Rh5 expression in R8 cells (33%, Table 1).

was not statistically different from ommatidia having genetically wild-type R7 and R8 cells (Fig. 4A,A') (50% vs 64%, respectively, Table 2). Conversely, there was a dramatic decrease in Rh5 expression in ommatidia having a genetically mutant R8 cell and a genetically wild-type R7 cell (Fig. 4C,C') (9%, Table 2). The percentage of Rh5 expression in ommatidia having both genetically mutant R7 and R8 cells (Fig. 4D,D', 20%; Table 2), was statistically different from that of ommatidia having genetically wild-type R7 and R8 cells, whereas it was not significantly different from ommatidia having a genetically mutant R8 cell alone (Fig. 4C,C'). These results demonstrate that signaling is impaired in ommatidia in which the R8 cell is genetically mutant for *rho*. Impaired signaling is not enhanced when the R7 cell is also genetically mutant. When the R7 cell is genetically mutant and adjacent to a genetically wild-type R8 cell, signaling and induction of Rh5 expression is the same as when both cells are genetically wild type. One caveat to these results is that we were unable to genotype every cell in the ommatidium. It does appear that there is a nonautonomous requirement for *rho* elsewhere in the ommatidia or developing eye. We believe this is responsible for the reduction in Rh5 when both R7 and R8 are genetically wild type (64% vs 95% for C5, Table 2). These findings demonstrate that *rho* function in the R7 cell is not required; however, loss of *rho* in the R8 cell or elsewhere in the ommatidium or eye compromises induction of Rh5 expression.

Epidermal growth factor receptor is required for induction of Rh5 expression in R8 photoreceptor cells

rho is an activator of the *EGFR* pathway throughout most of *Drosophila* development. *EGFR* is a transmembrane receptor tyrosine kinase (RTK) that acts reiteratively throughout eye development to regulate cell fate specification, control ommatidial spacing, and inhibit programmed cell death (Freeman, 1996; Domínguez et al., 1998; Kumar et al., 1998). Having demonstrated the requirement for *rho* in photoreceptor cell subtype specification, we wanted to determine whether *EGFR* was also required for this process. We used the temperature-sensitive *EGFR* allele, *EGFR*^{tsla}, to study the consequences of *EGFR* loss during eye development. Removal of *EGFR* for 24 h during late larval/early pupal development (L3/P0) produced a very rough compound eye (Fig. 5A,B). Loss of *EGFR* in the anterior eye, before the passage of the morphogenetic furrow, leads to missing ommatidia. Loss of *EGFR* in the posterior eye, after the passage of the morphogenetic furrow, roughens the eye due to the loss of cone and pigment cells (Kumar et al., 1998). Loss of *EGFR* also impairs the induction of Rh5 expression and causes an increase in the expression of Rh6 (Fig. 5C; Table 1). Similar to the loss of *rho*, loss of *EGFR* does not alter the proportions of Rh3- and Rh4-expressing R7 cells (Fig. 5D; Table 1), however there is a dramatic mispairing of Rh3-expressing R7 cells with Rh6-expressing R8 cells (Fig. 5E). These findings demonstrate that *EGFR* is required

Figure 4. *rhomboid* is required in R8 photoreceptor cells and elsewhere in the eye for induction of Rh5 expression. *rho* mutant mosaic animals were constructed having GFP-marked wild-type and *rho*-heterozygous cells. *rho*^{Δ5} mutant cells are unlabeled. Each genotype is shown in paired panels, labeled against Rh3, Rh5, and GFP on the left (**A**, **B**, **C**, **D**) and against GFP alone on the right (**A'**, **B'**, **C'**, **D'**). Arrows indicate GFP-marked cells. Statistical comparisons between results are detailed in Table 2. **A**, **A'**, Ommatidia from *rho*^{Δ5} mosaic animals with GFP-marked R7 and R8 cells show 64% pairing between Rh3- and Rh5-expressing cells. This is significantly less than in completely wild-type CS animals, indicating that *rho* is required elsewhere in the eye (see Results and Table 2). **B**, **B'**, Ommatidia having mutant R7 cells and GFP-marked R8 cells show 50% pairing between R7 and R8 cells that is not statistically different from the ommatidia in Figure 7A, **A'**. **C**, **C'**, Ommatidia having GFP-marked R7 cells and mutant R8 cells show a dramatic decrease in pairing between R7 and R8 cells (9%). **D**, **D'**, Ommatidia having mutant R7 and R8 cells have reduced pairing (20%) that is similar to that seen in Figure 7C, **C'**.

for regulating the induction of Rh5 expression in R8 cells and establishing R7/R8 photoreceptor cell adjacency.

Epidermal growth factor receptor activation is sufficient to induce Rh5 expression and rescue the loss of *rhomboid*

We found that *rho* is required and sufficient for the induction of Rh5 expression. *rho* is required in the R8 cell, and the induction of Rh5 expression is also dependent on *EGFR*. Because of the well described pathway in which *EGFR* is activated by *Spi*, after its transport by *Star* and cleavage by *rho*, we thought it was likely that a similar process may occur in the regulation of Rh5 expression and photoreceptor cell patterning. Specifically, we believe that *rho* may function in the R8 cell to cleave an *EGF* ligand, that activates *EGFR* in the R7 cell. Because this signal is directed from the R8 cell to the R7 cell, it seems unlikely to be directly responsible for the induction of Rh5 expression. More likely, this *rho*/*EGFR* signal from R8 to R7 may play a role in conferring signaling competency to the R7 cell. This model predicts that (1) induction of Rh5 expression through misexpression of *rho* is dependent on

the presence of the R7 cell, (2) activation of *EGFR* is sufficient to induce the expression of Rh5, (3) this effect is dependent on the presence of the R7 cell, and (4) activation of *EGFR* is sufficient to rescue the defect in Rh5 expression found in *rho* mutant patches.

To test the first prediction, we used a transgene in which *GMR* directly drives the expression of *rho*, rather than using the binary *GAL4-UAS* system. We found that the phenotype of these animals is identical to flies carrying *GMR-GAL4; UAS-rho*. *GMR-rho* expressing flies show a dramatic increase in the proportion of Rh5-expressing R8 cells compared with CS (50.4%, Table 1), a normal proportion of Rh3-expressing R7 cells (37%, Fig. 6A; Table 1) and mispairing between Rh4-expressing R7 cells and Rh5-expressing R8 cells (Table 1). The *GMR-rho* transgene was crossed into a *sev* mutant background that lacks R7 cells, and we found that the increase in Rh5 expression was completely suppressed (Fig. 6B; Table 1), to levels equivalent to that found in animals mutant for *sev* alone. This finding confirms that induction of Rh5 expression by misexpression of *rho* is dependent on the presence of the R7 cell and suggests that *rho* may act on the R7 cell as described in the model above.

Figure 5. Epidermal growth factor receptor is required for induction of Rh5 expression, and establishment of R7/R8 cell adjacency. **A**, SEM of an *EGFR^{tsla}/EGFR²⁴* animal that was heat shocked for 24 h beginning at P0 (~240 h AED). **B**, Bright-field view of animal treated as in (**A**) shows similar anterior ommatidial loss and roughening throughout. **C**, Longitudinal section of heat-shocked *EGFR* animal shown in **B** displays an increase in Rh6 expression (80%). **D**, **E**, In adjacent sections (**D**) R7 cells are present and expression of Rh3 (37%) and Rh4 (63%) is normal, and (**E**) loss of *EGFR* results in a mispairing of Rh3-expressing R7 cells with Rh6-expressing R8 cells (white arrows/white asterisks), consistent with a loss of induction of Rh5 expression in R8 cells.

To test the second prediction that activation of *EGFR* is sufficient to induce Rh5 expression, we examined the phenotype of animals carrying the activated *Ellipse* allele of *EGFR* (*EGFR^{E1}*). This mutation causes a single amino acid substitution in the kinase domain of the protein, which activates tyrosine kinase activity. The mutation behaves as a hypermorph, and is effectively a dominant active *EGFR* (Lesokhin et al., 1999). We found that animals carrying *EGFR^{E1}/+* show a dramatic increase in the number of R8 cells that express Rh5 (77%, Fig. 6D; Table 1); however, this is accompanied by an increase in the number of R7 cells that express Rh3 (75%, Fig. 6E; Table 1). There are nonetheless instances of mispairing between Rh4-expressing R7 cells and Rh5-expressing R8 cells (Fig. 6F). This finding demonstrates that activation of *EGFR* is sufficient to induce Rh5 expression, including inappropriate Rh5 expression in R8 cells adjacent to Rh4-expressing R7 cells.

To test the third prediction that the increase in Rh5 expression is dependent on the presence of R7 cells, as opposed to the possibility that *EGFR* activation could be acting on R8 cells to induce Rh5 expression, we examined *EGFR^{E1}* in a *sev* mutant background. In *sev; EGFR^{E1}/+* mutants, we found that Rh5 induction was dramatically reduced (32%) but not eliminated (Fig. 6G; Table 1). Interestingly, some R7 cells remain in this genotype (data not shown) consistent with the ability of *EGFR* activation to partially rescue loss of the *sev* receptor tyrosine kinase. To definitively determine whether the *EGFR^{E1}* effect on R8 cells is dependent on the presence of R7 cells, we crossed the *EGFR^{E1}* allele into *seven in absentia (sina)*, which functions downstream of *Ras* activation in R7 cell recruitment (Carthew and Rubin, 1990; Fortini et al., 1992). Sibling controls of the genotype *EGFR^{E1}/+; sina/+* showed an increase in Rh5-expressing R8 cells compared with CS (51.7% that was similar to that of *EGFR^{E1}/+*, Table 1). Furthermore, *EGFR^{E1}; sina* double mutants that lack R7 cells have a dramatic reduction in the expression of Rh5 (15%, Fig. 6H; Table 1). These animals are red eyed and demonstrate significant fluorescence background, nonetheless only 3 R8 cells within the montage in Figure 6H show specific staining for Rh5 (arrowheads). This level of Rh5 expression is comparable although somewhat higher than that found in *sina* mutants (3.7%, Table 1), and demonstrates that the induction of Rh5 expression on *EGFR* activation is dependent on the presence of R7 cells.

To test the fourth prediction that activation of *EGFR* is sufficient to rescue the *rho* mutant phenotype, we generated flies car-

rying the *EGFR^{E1}* allele in a background in which *rho^{PΔ5}* mutant clones were formed. Sibling controls carrying *EGFR^{E1}/+*, the *FRT-FLP* components and heterozygous for *rho^{PΔ5}* show an increased proportion of Rh5-expressing R8 cells compared with CS (42.4%, Table 1) and numerous examples of Rh3/Rh6, and Rh4/Rh5 mispaired ommatidia. In *EGFR^{E1}* animals having *rho^{PΔ5}* mutant patches, the percentage of Rh5-expressing R8 cells is unchanged (41.9%, Fig. 6I; Table 1). This demonstrates that in the absence of *rho*, activation of *EGFR* is sufficient to induce the expression of Rh5.

Discussion

The precise pairing of adjacent R7 and R8 photoreceptor cells establishes three very specific classes of ommatidia that have unique sensitivities to different colors and polarized light (Kirschfeld et al., 1978; Hardie, 1979, 1985). We have shown that *rho* is required to coordinate the differentiation of adjacent R7 and R8 cells over the majority of the retina. Furthermore, we have shown that expression of *rho* posterior to the morphogenetic furrow is sufficient for the induction of Rh5 expression in R8 cells. Interestingly, although *rho* and *ru* are redundant for early stages of eye formation and retina development, *ru* is neither required nor sufficient for this process.

Photoreceptor cell subtype specification

The compound eye of *Drosophila* is a classic model system in developmental biology, however only recently have the mechanisms that regulate photoreceptor cell subtype specification been investigated. Genes that participate in this process include *orthodenticle*, *prospero*, *spalt*, *wingless*, the *iroquois* complex, *homothorax*, *warts*, *melted*, and *spineless* (Mollereau et al., 2001; Cook et al., 2003; Tahayato et al., 2003; Tomlinson, 2003; Wernet et al., 2003, 2006; Mikeladze-Dvali et al., 2005; Mazzoni et al., 2008). These studies have shown that specific genes are required to (1) activate and repress the transcription of the visual pigment genes, (2) distinguish the R7 and R8 cells from each other, (3) distinguish the R7 cells from each other, (4) distinguish the R7 and R8 cells from the R1–6 cells, (5) distinguish the R7 and R8 cells of the dorsal margin from those of the rest of the retina, and (6) regulate the induced versus default switch in R8. The present study demonstrates the requirement for *rho* and *EGFR* in this process and suggests that they may establish the competency of R7 cells to induce Rh5 expression in adjacent R8 photoreceptor cells.

Figure 6. Epidermal growth factor receptor activation is sufficient for induction of Rh5 expression in R8 cells and can rescue the *rhomboid* mutant phenotype. **A**, Misexpression of *rho* under the direct control of *GMR* does not alter the percentage of Rh3-expressing R7 cells (37%). **B**, Removal of R7 cells in *sev; GMR-rho* mutant flies results in a dramatic decrease in the percentage of Rh5-expressing R8 cells (3.4%). **C**, Downregulation of *EGFR* throughout the eye with a dominant negative transgene severely reduces the eye similar to the phenotype of *rho ru* double mutants (Fig. 3E). **D**, Activation of *EGFR* in the *Ellipse* allele leads to a dramatic increase in Rh5 expression (77%). **E**, **F**, *EGFR^{E1}* flies show increased expression of Rh3 (75%) (**E**) and mispairing between Rh4-expressing R7 cells and Rh5-expressing R8 cells (**F**). **G**, Removal of most R7 cells with the *sev* mutation suppresses the effect of *EGFR^{E1}* on the induction of Rh5 expression (32%). **H**, Removal of all R7 cells with the *sina* mutation leads to further dramatic

In addition to *Drosophila*, photoreceptor cell subtype adjacency has been observed in the retinal mosaic of teleost fish. The fish retina consists of a crystalline-like array in which rows of red- and green-sensitive double cones alternate with rows of UV- and blue-sensitive cones so that each cone type is regularly positioned with respect to other cone types (Stenkamp and Cameron, 2002). It seems likely that one functional consequence of this organization is to ensure adequate spectral sampling throughout the retina, thus preserving high spatial acuity at different wavelengths. Although the basis for photoreceptor cell subtype adjacency in this system is not known, it seems likely that it is established by local inductive signals similar to those we believe regulate this process in *Drosophila*.

A novel *rhomboid* function in eye development

The recruitment of the R7 photoreceptor cells is a complex process that requires input from at least three signals (Fig. 7). These include contributions from *Notch* (Cagan and Ready, 1989; Cooper and Bray, 2000; Tomlinson and Struhl, 2001), *EGFR* (Freeman, 1996; Yang and Baker, 2001), and the *boss/sev* signaling pathways (Zipursky and Rubin, 1994). Our identification of a requirement for *rho* in the induction of Rh5 expression in R8 cells, suggests that there is an additional role for *EGFR* signaling in eye development.

EGFR signaling plays multiple roles in development, both within the eye as well as throughout embryogenesis, imaginal disc differentiation, spermatogenesis, oogenesis and other developmental processes (Shilo, 2003). The principal *EGFR* ligand in the *Drosophila* eye is thought to be Spitz (Spi), a TGF- α related, membrane-tethered molecule that must be activated for complete functioning (Rutledge et al., 1992; Freeman, 1994, 2008; Tio et al., 1994; Schweitzer et al., 1995; Tio and Moses, 1997). Spi first interacts with Star in the endoplasmic reticulum (ER) for transport to the Golgi complex, where it is cleaved by *rho* and secreted from the signal-sending cell (Freeman, 1994; Bang and Kintner, 2000; Wasserman et al., 2000;

← suppression in the effect (Rh5 expression, 15%). Arrowheads indicate the few remaining Rh5-expressing R8 cells. **I**, In contrast, loss of *rho* does not significantly suppress the *EGFR^{E1}* induction of Rh5 expression (42%), compared with sibling controls (*EGFR^{E1/+}; rho/+*, 42%). This demonstrates that loss of Rh5 expression in *rho* mutant patches can be rescued by *EGFR* activation.

Figure 7. Model for signaling events regulating recruitment and patterning of R7 and R8 photoreceptor cells. There are three distinct requirements for *Notch*, the *Epidermal growth factor receptor*, and *bride of sevenless/sevenless* signaling in the recruitment and induction of R7 cells (left side). The current work demonstrates a specific requirement for *rhomboid* function in R8 cells and elsewhere in the eye that may confer signaling competency to R7 cells, and allow induction of Rh5 expression and the establishment of precise cell–cell adjacency (right side).

Lee et al., 2001; Urban et al., 2001; Tsruya et al., 2002). *rho* has also been shown to cleave *Star* and may thus participate in a negative feedback loop to restrict the amount of *Spz* that is trafficked from the ER to the Golgi (Tsruya et al., 2007). It is currently thought that three waves of *spz/EGFR* signaling occur during eye development. In the first wave, *rho* expression in R8, R5 and R2 cells leads to *Spz* activation and recruitment of the remaining photoreceptors (R1, R3, R4, R6 and R7). These cells then initiate a second wave of signaling to recruit the cone cells, which then initiate a third wave to recruit the pigment cells (Freeman, 1996). There is additional evidence that *EGFR* is required in the developing eye for cell proliferation and survival (Domínguez et al., 1998).

These and other recent studies indicate that both the R7 and R8 cells express *rho* during the period when the cells are recruited and specified, and when Rh5 expression is likely to be induced (Yogev et al., 2008). The precise time at which Rh5 expression is induced in R8 cells is unknown, however it must follow the recruitment and specification of the R7 cells during late third instar and early pupal development, and precede the expression of the opsin genes beginning at 79% of pupal development (Earl and Britt, 2006).

Because *rho* is required in the R8 cell (and elsewhere in the eye) and not in the R7 cell, it is unlikely that a *rho/EGFR* mediated signal is directly responsible for the induction of Rh5 expression in R8 cells. Nonetheless, our observation that induction of Rh5 expression by *GMR-rho* misexpression is blocked in *sev* mutants suggests that *rho* is sufficient to induce signaling inappropriately in Rh4-expressing cells, but that this is dependent on the presence of the R7 cell. Furthermore, our observation that activation of *EGFR* can rescue the *rho* mutant phenotype suggests that *rho* functions upstream of *EGFR* activation in the induction of Rh5 expression. This suggests that *rho* and *EGFR* may function to establish a signaling competent R7 cell that can then induce Rh5 expression in the R8 cell (Fig. 7). This *rho* function could be a component of the requirement for *EGFR* signaling in R7 cell recruitment mentioned above, or could be an additional *rho*-specific requirement that regulates R7 cell signaling competency. Given that loss of *rho* or *EGFR* does not affect the proportion of Rh3- and Rh4-expressing R7 cells, our results suggest that they may act after or independently of *spineless* (Wernet et al., 2006).

In the context of *rho* specificity, *rho* and *ru* belong to a conserved family of rhomboid proteins that are intracellular membrane proteases (Wasserman et al., 2000; Lee et al., 2001; Urban et al., 2002). The similarity of their enzymatic functions *in vitro* and

their genetic redundancy has led to the proposal that they have limited specificity with regard to substrate cleavage and function within the *EGFR* signaling pathway (Wasserman et al., 2000; Lee et al., 2001; Urban et al., 2002). Obviously, the fact that *ru* is not required for regulating R8 cell color sensitivity may simply reflect that it is not expressed at the appropriate location or time. Similarly, it is possible that the inability of misexpressed *ru* to induce Rh5 expression may result from inadequate expression under the *GAL4/UAS* system. However, the observation that *ru* is not sufficient for induction of Rh5 expression suggests that despite the similar biochemical activity of *rho* and *ru in vitro*, their functions *in vivo* are quite specific. This raises the intriguing possibility that *rho* and *ru* may act on different substrates or ligands, require different cofactors, or they may generate ligands that are processed or modulated differently, producing qualitatively different signals. In addition, it was recently shown that *rho* may function distinctly from *ru*, based on differences in intracellular localization, cleavage of the chaperone *Star*, and the temporal and quantitative regulation of *Spitz* trafficking (Yogev et al., 2008). Each of these raises potential mechanisms by which *rho* and *ru* may function in a distinct manner and may underly the specific requirement for *rho* and not *ru* in the induction of Rh5 expression.

Thus in addition to demonstrating the requirement for *rho* and *EGFR* in the induction of Rh5 expression, our work raises many questions concerning their mechanism of action. Perhaps the two most important issues to be resolved in future studies are (1) what are the functions of the traditional components of *EGFR* signaling, *Spz* and *Star*, in the induction of Rh5 expression, and (2) what are the temporal and spatial requirements for *rho* and *EGFR* with respect to the functions of genes known to establish R7 and R8 photoreceptor cell subtype identity (*spineless*, *warts*, and *melted*)? Answering these questions will help place the function of *rho* and *EGFR* in R8 cell subtype specification into context with the activities of other genes and *EGFR* signaling processes more broadly and better define the role of inductive signaling in retinal development overall.

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