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Patricia J. Polaczyk · Robert Gasperini · Greg Gibson

Naturally occurring genetic variation affects *Drosophila* photoreceptor determination

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Abstract The signal transduction pathway controlling determination of the identity of the R7 photoreceptor in the *Drosophila* eye is shown to harbor high levels of naturally occurring genetic variation. The number of ectopic R7 cells induced by the dosage-sensitive $Sev^{S11.1}$ transgene that encodes a mildly activated form of the Sevenless tyrosine kinase receptor is highly sensitive to the wild-type genetic background. Phenotypes range from complete suppression to massive overproduction of photoreceptors that exceeds reported effects of known single gene modifiers, and are to some extent sex-dependent. Signaling from the dominant gain-of-function *Drosophila Epidermal Growth Factor Receptor* (*DER-Ellipse*) mutations is also sensitive to the genetic backgrounds, but there is no correlation with the effects on $Sev^{S11.1}$. This implies that different genes and/or alleles modify the two activated receptor genotypes. The evolutionary significance of the existence of high levels of genetic variation in the absence of normal phenotypic variation is discussed.

Key words Genetic variation · Sevenless · EGF receptor · *Drosophila* · Photoreceptor

Introduction

Development of the *Drosophila* eye involves many genes, all of which probably harbor some polymorphism. Since genetic variation is usually assumed to lead to phenotypic variation, it is thus somewhat paradoxical that the ommatidial arrays of the eye are highly uniform. More generally, some of the most invariant structures in all of nature are produced by highly complex genetic pathways. The usual explanation for the absence of phenotypic variation is that selection for order and stability is so strong that it removes all new mutations that perturb the phenotype as soon as they arise. Circumstantial evidence might support this thesis, in that modifiers of major effect mutations accumulate quickly in laboratory stocks. This explanation makes the prediction that most wild-type genetic backgrounds should have similar effects when first crossed into mutant laboratory stocks, since there should be little genetic variation to modify the phenotype. Here we show that the situation is in fact the complete opposite for the *Drosophila* eye, as there is extensive genetic variation for photoreceptor determination that is hidden beneath the uniformity of wild-type development.

Though often regarded as a nuisance, since it hampers the molecular genetic dissection of particular processes, genetic variation is interesting to study from a number of perspectives. First, it provides the material basis for biological evolution. It is thus important to know the distribution of allelic effects in natural populations: how many genes contribute variation to each particular trait, how large are their effects, do they have pleiotropic functions, and how do they interact with one another? A related series of questions concern the maintenance of the variation – does it play some positive adaptive role, or is it more often the inevitable result of the balance between mutation and purifying selection? Second, genetic variation both contributes to the buffering of development and helps to ensure that every individual is a little bit different. Aside from bristle patterning in flies (Mackay 1995) and aspects of metabolism (Eanes 1994), not much is known about the molecular basis of either homeostasis or quantitative variation. Third, genetic variation is the basis of differential familial susceptibility to disease, including cancer. It is thus relevant to understand precisely which genes harbor genetic variation, and which types of polymorphism are functionally significant.

Eye development in *Drosophila* provides an excellent system with which to study genetic variation. The compound eye is a set of approximately 800 ommatidia, each of which consists of an invariant array of 8 photoreceptor

P. J. Polaczyk[1] · R. Gasperini · G. Gibson (✉)
Department of Biology, University of Michigan,
Natural Science Building, Ann Arbor, MI 48109-1048, USA

Current address:
[1] Laboratory of Molecular Biology, University of Wisconsin,
1525 Linden Drive, Madison, WI 53706-1514, USA

cells (R1–R8), 4 cone cells, and 3 types of pigment cell (Tomlinson 1985; Cagan and Ready 1989). Each of these cell types requires the activity of the *Drosophila Epidermal Growth Factor Receptor* protein (DER), which signals to the nucleus by way of Ras and the well defined MAP kinase pathway (Freeman 1996; Pawson 1994). One particular cell, R7, also requires a burst of activity from a second type of receptor tyrosine kinase protein encoded by the *sevenless* gene (Hafen et al. 1987; Banerjee et al. 1987). Modifier screens (Simon et al. 1991; Hafen et al. 1993) have shown that all of the common components of the Ras-MAP kinase pathway are also involved in Sevenless signaling (Wassarman et al. 1995). Furthermore, the observation that expression of an activated form of DER in R7 cells can functionally substitute for Sevenless protein has led to the model that these two receptors, though responding to different signals, act in the same way within cells (Freeman 1997; Tio and Moses 1997). This suggests that variation in the components of the signaling pathway should modify the two receptor genes to similar extents.

In order to test this prediction, as well as to begin to characterize the architecture of genetic variation for receptor tyrosine kinase activity in *Drosophila*, we have examined the effects of numerous wild-type genetic backgrounds on the activity of constitutively active forms of DER and Sevenless. For DER, we used the two *Ellipse* alleles, DER^{E1} and DER^{EB1}, which are now known to be gain-of-function mutations in the *DER* gene that result in ligand-independent signaling (Baker and Rubin 1992). Homozygotes have dramatically reduced eyes, possibly due to an early interference with lateral inhibition that prevents the proper maintenance of ommatidial preclusters (Baker and Rubin 1989; Freeman 1996). For Sevenless, we used a deliberately engineered transgene, $Sev^{S11.1}$, that consists of a C-terminal truncation of the gene expressed under the control of the normal *sevenless* enhancers (Basler et al. 1991), and shows ligand-independent signaling in the R7, cone and pigment cells. The severity of phenotypes caused by both types of mutation are greater in homozygotes than heterozygotes, indicating that signaling is dose-sensitive. We find that this sensitivity is heavily dependent on the genetic background, and interestingly that some backgrounds that enhance *Sevenless* activity suppress *DER* activity. The results are discussed with respect to the evolution of developmental stability and the sources of genetic variation.

Materials and methods

Fly stocks

The transgenic stock $Sev^{S11.1}$ [#P642: P($w^{-}sev^{d2};w^{+}$ sev^{pW8})50A] was constructed in the laboratory of Ernst Hafen (ETH, Zürich) and obtained from the Bloomington Stock Center in August, 1994. The P-element insertion was reported to be on the X chromosome (Basler et al. 1991), but genetic data placed it on the second chromosome and we subsequently mapped it to polytene interval 50A by in situ hybridization. In the course of clearing up this situation, we observed occasional loss of eye pigment due to excision of the transgene when crossed into wild-type backgrounds, but the transposition rate was too low to significantly bias the proportion of genotypes in F2 progeny in the crosses described here. Otherwise, the stock showed phenotypes exactly as previously described. Artificial selection for increased and decreased eye roughness was applied to this stock for 10 generations, taking the 20 most extreme individuals of 100 or so from a single vial. Five generations of inbreeding by single pair mating was then applied to generate the *rougher* and *smoother* lines reported here. The genetic variation may have been present in the original flies used to make the transgenic line, or may have arisen by mutation during the 5 years in culture. The two *Ellipse* alleles, $DER^{E1}$and DER^{EB1}, were obtained from Kevin Moses (University of Southern California) in April, 1997.

The wild-type isofemale lines were obtained from the Bowling Green Stock Center in February, 1996 (world wide wild type, wwwt: see Gibson and van Helden 1997 for list of source populations), or collected from the Kerrytown Fruit Market in Ann Arbor in July, 1996 by an undergraduate, Brian Haag (Ann Arbor wild type, aawt). The Ives laboratory strain was obtained from Brian Charlesworth (University of Chicago) in August, 1994. All stocks were maintained on 10 ml standard cornmeal supplemented with yeast powder at 25°C in glass vials.

The introgression lines listed in Table 2 were constructed by backcrossing the female hybrid progeny of a cross between $Sev^{S11.1}$*smoother* and the wild-type line, with males of that line, and repeating the procedure for ten generations. Since the transgene is only heterozygous in hybrids, in some cases it was not possible to pick these individuals, and so the introgression failed. At the tenth generation, hybrids were sib-mated to generate homozygotes that were distinguished by eye color (two copies of the $white^{+}$ marker gene produce more red pigment) and roughness, and again crossed to generate the homozygous lines.

Scoring of eye phenotypes

Sevenless

The $Sev^{S11.1}$transgene causes a roughening of the surface of the eye that was scored at ×20 magnification under a dissecting microscope with CO_2 anesthesia. The arbitrary scale used was as follows: 1 = wild type; 2 = very slight roughening; 3 = roughening in patches; 4 = clear roughening on patches of both eyes; 5 = roughening throughout both eyes; 6–8 = successively stronger roughening; 9 = some blistering on at least one side; 10 = strong blistering including loss of pigmentation in patches.

Ellipse

The phenotypes of viable homozygotes are qualitatively different from those of heterozygotes. Most homozygotes had eyes reduced to a small cluster of ommatidia in a field of bristles and pigmented tissue, but two grades of slightly reduced effect allowed scores of 8–10 to be distinguished. These eyes could not be sectioned easily. Most heterozygotes resembled $Sev^{S11.1}$eyes with a score of 4 or 5, but rougher examples could be discerned and were assigned scores of 6 or 7. Scores of 2 or 3 were given to eyes with slightly disordered portions, building up to successive roughening until reduction in the number of facets was observed starting at a score of 7.

Histology

Light microscopy

Fly heads were collected and immediately fixed in Carnoy's reagent (ethanol:chloroform:glacial acetic acid 6:3:1) for 3 h. After dehydration in absolute ethanol (3×1 h with agitation) they were cleared in methyl benzoate overnight. Tissue was infiltrated, embedded in Paraplast X-TRA (Oxford Scientific, St. Louis, Mo.)

and thin sections (3 μm) were cut on a rotary microtome. Sections were stained in dilute Giemsa solution (pH 6.5) and examined with a Zeiss Axioplan microscope.

Scanning electron microscopy

Fly heads were collected and fixed in acetone for 3 min followed by a graded series of ethanol in HMDS (hexamethyl-dichlorosilazane; Electron Microscopy Sciences, Washington, Pa.). Following three washes in 100% HMDS, heads were mounted on aluminum stubs with graphite adhesive and allowed to dry over a bed of desiccant overnight before being sputtered with gold and examined on an ISI DS-300 scanning electron microscope.

Cobalt nitrate staining

For staining of eye imaginal discs, whole heads were removed from wandering third instar larvae in phosphate-buffered saline (PBS), inverted, and placed in 2% glutaraldehyde fixative for 30 min. They were then treated with 2% cobalt nitrate for 30 min, washed in distilled water, stained with 1% ammonium sulfide for 5 min, and dehydrated in ethanol. Following further dissection, just the eye-antennal imaginal discs were cleared in methyl benzoate, and mounted in Permount (Fisher Scientific) for observation with a Zeiss Axioplan microscope.

Statistical analysis

All statistical analyses were performed with Statistica 4.1 software (StatSoft Inc 1996) for the Macintosh computer. The heritability estimate of the $Sev^{S11.1}$ phenotype given in the text was calculated from an analysis of variance on the eight homozygous introgression lines listed in Table 2 (see also Gibson and van Helden 1997). A normal probability plot was essentially linear, but the variance of intermediate scores was slightly greater than low or high scores. Heritability was estimated as the ratio of the between line genetic variance (V_G) to the total phenotypic variance ($V_P = V_G + V_E$). These quantitative genetic parameters were estimated from analyses of variance with V_G equal to $(1/2)\sigma^2_L$ where σ^2_L equals $(MS_L - \sigma^2)/n$, and the environmental variance, V_E equals the error mean square. The number of individuals scored for each line and sex, n, was 20.

Results

Effects of $Sev^{S11.1}$ and *Ellipse* on eye development

Constitutively activated Sevenless and DER both cause roughening of the surface of the eye. The severity of this phenotype is dependent on the genetic background, and was quantitated simply by scoring individual flies under a dissecting microscope, using an arbitrary scale from 1 (wild type; Fig. 1A) to 10 (most severe phenotype). The scales developed for the two mutants, $Sev^{S11.1}$ and *Ellipse*, measure different cellular changes, so are only comparable in a relative sense. This is readily seen in flies with stronger phenotypes, as shown by the scanning electron micrographs in Fig. 1E ($Sev^{S11.1}$, score 9) and C (DER^{E1}, score 10). As Sevenless activity increases, the arrangement of the ommatidia becomes increasingly disordered and blistering of the retina occurs. As DER activity increases, the eye becomes smaller as fewer and fewer ommatidia form.

In order to confirm that the two activated receptor tyrosine kinases have different cellular effects in all genetic backgrounds, we cut thin sections across the retina and stained them to reveal the rhabdomeres of individual photoreceptors. As expected, the major effect of $Sev^{S11.1}$ is the production of ectopic R7 photoreceptors. Eyes with a score of 2 or 3 have occasional extra R7 cells (Fig. 1I), and this number gradually increases up to a maximum of four extra R7 cells per individual ommatid-

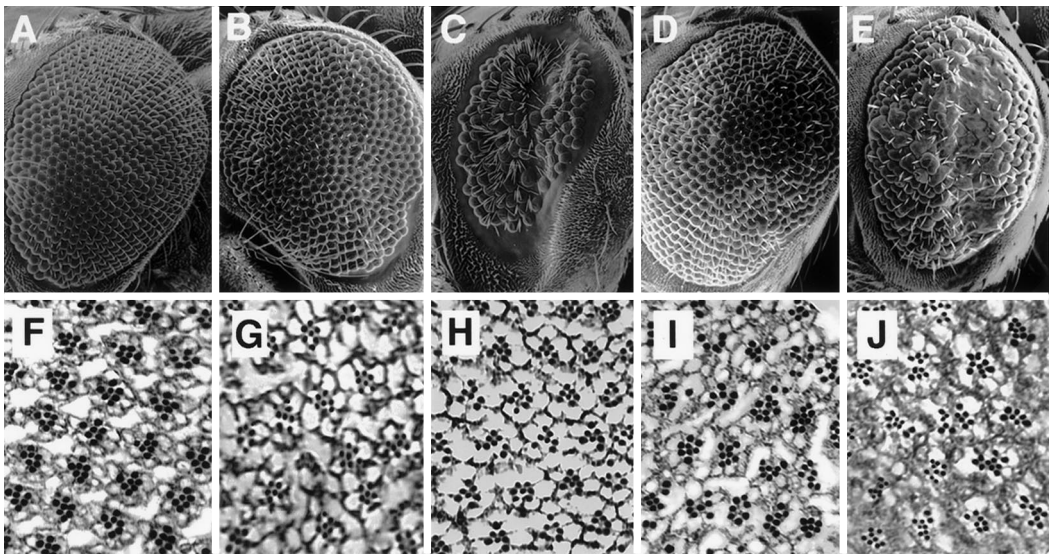

Fig 1A–J Variable eye phenotypes of *Sevenless* and *Ellipse* mutant flies. Scanning electron micrographs of wild type (**A**), Elp^1 heterozygote (**B**, score "4") and homozygote (**C**, "10"), and $Sev^{S11.1}$ homozygotes (**D**, "4" and **E**, "9") across the top row. The two eye roughness scales reflect different visible phenotypes, and these are actually easier to score in live flies under the light microscope. Sections of 3 μm across the bottom, stained with Giemsa to highlight the rhabdomeres of photoreceptors, show the normal pattern of seven photoreceptors per ommatidium in Elp^1 heterozygotes (**G**, compare with wild type in **F**), whereas increasing levels of eye roughness are associated with increased numbers of photoreceptors in $Sev^{S11.1}$ homozygotes (**H**, "1"; **I**, "4"; **J**, "9")

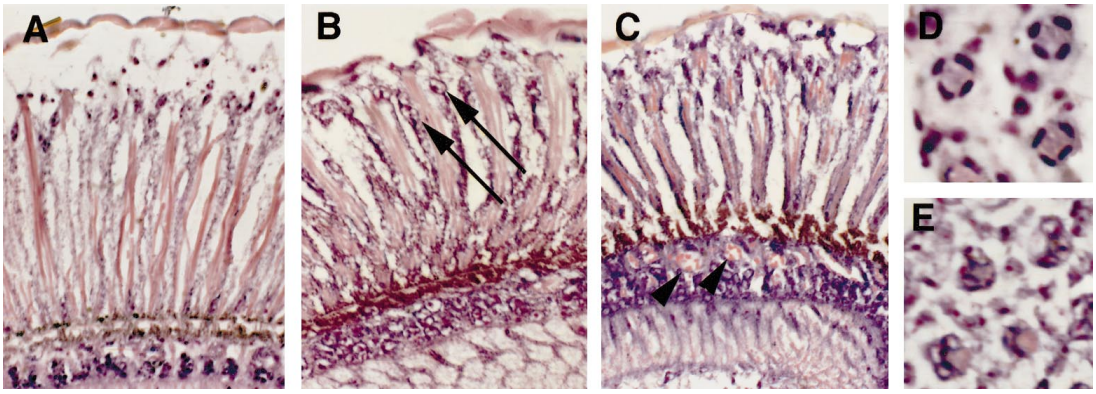

Fig 2A–E Aberrant cellular phenotypes in *Sevenless* and *Ellipse* mutant flies. Comparison of wild-type (**A**) with *Elp*^{1} heterozygote (**B**) and *Sev*$^{S11.1}$ homozygote (**C**) eyes in transverse sections shows that the two gain-of-function mutations have different effects. *Ellipse* animals have disordered rhabdomere assembly, with extra material at the apical ends of the ommatidia possibly indicating the presence of transformed ectopic cone cells. Sections across the level of the lens further show that the individual lens cells have large vauoles in *Ellipse* (**E**) but not wild-type (**D**) eyes. By contrast, extreme *Sevenless* signaling results in the migration of ectopic photoreceptors beneath the basal membrane – in even more extreme examples, these can take up a space similar in size to that of the normal array

ium in eyes with scores of 6 or 7 (Fig. 1J). The majority of genetic backgrounds produce phenotypes in this range, and they are presumably due to transformation of cone cells into photoreceptors. The reported phenotypes of a number of enhancers of activated *Sev* also fall in this range (Gaul et al. 1992; Hafen et al. 1993; Rebay and Rubin 1995).

Unexpectedly, we found that several genetic backgrounds can produce a much more severe phenotype in which it becomes difficult to distinguish individual ommatidia, and/or ommatidia with more than four extra R7-like rhabdomeres are observed. In flies with scores of 9 or 10, transverse sections through the optic tectum show that large clusters of mis-shapen ectopic ommatidia appear to lie underneath the basal cell layer close to the brain (Fig. 2C). This phenotype is most likely due to loss of structural support in regions of the eye disc where pigment cells are also transformed into ectopic photoreceptors, allowing ommatidia to drop through the basal lamina. It is possible that some interommatidial cells are also transformed. This interpretation is consistent with the notion that increasing eye roughness simply reflects an increase in signal transduction from the *Sev*$^{S11.1}$ transgene, resulting in more cells being transformed into photoreceptors. It is noteworthy that the extreme phenotype has not been described for enhancers of activated *Sev*transgenes that have been isolated in mutagenesis screens.

Ellipse mutant phenotypes are qualitatively distinct. The dramatic loss of ommatidia in viable homozygotes may be caused by aberrant recruitment of the R8 cells that found each precluster, or failure of the early photoreceptors to differentiate due to an excess of lateral inhibition immediately behind the morphogenetic furrow (Baker and Rubin 1989; Freeman 1997). This early ectopic DER function is undoubtedly responsible for much of the observed phenotypic variation. The disruption of ommatidial spacing in *Ellipse* heterozygotes is shown in eye imaginal discs stained with the cell-surface dye cobalt sulfide in Fig. 3B. By contrast, the spacing of ommatidial clusters in even extreme *sevenless* eye imaginal discs is relatively normal (Fig. 3C).

Since expression of a constitutively active DER protein can rescue R7 development in *sevenless* mutants, as well as cause transformations of pigment cells to extra cone cells (Freeman 1996) it is also possible that *Ellipse* heterozygote phenotypes result in part from altered differentiation of individual cells. We were unable to detect any ectopic photoreceptors in *Ellipse* mutant eyes with

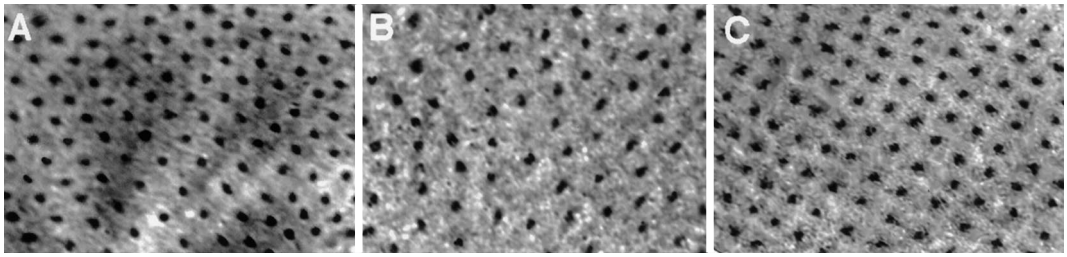

Fig 3A–C Abnormal patterning of ommatidia in *Ellipse* but not *Sevenless* eyes. Cobalt sulfide staining of third instar larval imaginal discs reveals the general pattern of ommatidia. This is clearly disordered in *Elp*^{1} heterozygotes (**B**) whereas the imaginal discs of *Sevenless* individuals (**C**) appear grossly normal compared with wild type (**A**). This indicates that *Drosophila* epidermal growth factor receptor (DER) is also involved in early aspects of ommatidial spacing, which presumably contributes to the overall roughness phenotype, whereas the Sev function is more restricted to R7 photoreceptor determination

Table 1 Summary of phenotypes

	ElpB1				*Elp1*				*SevS*				*SevR*			
	Male		Female		Male		Female		Male		Female		Male		Female	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
wwwt-1	1.4	0.6	1.5	0.7	1.1	0.3	1.3	0.6	5.9	1.6	6.0	1.6	4.9	2.1	4.0	1.8
wwwt-6	4.4	0.8	5.1	0.7	4.7	0.7	6.3	0.5	3.2	0.9	3.0	1.1	3.3	1.9	4.6	1.7
wwwt-8	4.7	0.6	4.9	0.6	5.7	0.6	5.7	0.6	3.9	1.5	2.9	0.8	3.5	0.8	3.9	1.3
wwwt-17	4.5	0.8	5.0	0.5	4.4	0.7	4.6	0.6	2.7	0.7	2.0	0.7	3.4	1.0	3.5	0.9
wwwt-21	4.0	0.9	4.4	0.8	4.5	0.5	5.0	0.5	7.3	2.1	8.0	0.6	7.1	1.1	5.9	1.6
wwwt-25	4.5	0.8	4.3	0.8	3.8	1.0	4.2	0.8	4.6	1.1	3.9	0.7	5.9	2.1	6.2	1.7
wwwt-26	4.1	0.7	4.6	0.9	4.0	0.5	4.2	0.6	3.1	1.0	3.3	0.9	5.2	0.6	4.5	1.0
wwwt-27	2.4	0.9	3.2	0.9	3.7	0.8	4.2	0.7	2.8	1.2	2.6	0.8				
wwwt-28	2.6	1.0	2.9	0.8	2.8	1.1	3.9	0.7					5.3	1.3	3.7	0.5
wwwt-32	4.8	0.9	4.5	0.9	3.4	0.7	3.4	0.8	3.9	0.8	2.4	1.2	5.8	1.5	4.7	1.4
aawt-1	6.8	1.6	6.6	1.2	5.2	0.6	5.2	0.5	6.7	1.7	5.9	1.7	4.9	1.6	4.0	1.1
aawt-3	5.1	0.8	4.9	0.7	4.0	0.6	4.0	0.5	5.6	1.1	4.7	1.0	5.4	1.5	5.9	1.2
aawt-4									7.5	1.4	6.5	1.7	7.0	2.0	6.9	1.9
aawt-7	4.0	0.8	4.2	0.5	3.6	0.7	3.3	0.9					5.5	1.4	5.8	1.8
aawt-10	4.6	0.8	4.3	1.0	4.4	0.7	4.3	0.5	4.1	1.4	3.3	1.3	5.3	0.6	4.8	2.4
aawt-15	5.0	0.9	5.0	0.6	4.6	0.7	4.2	0.4								
Ives	3.0	1.0	3.1	1.0	2.9	0.7	3.2	0.8	6.5	2.2	5.2	1.6	8.4	1.6	7.5	1.7
Mean	4.1	0.9	4.2	0.8	4.0	0.7	4.2	0.6	4.9	1.3	4.3	1.1	5.4	1.4	5.1	1.5

scores in the intermediate range, 4–5 (Fig. 1G), but extra cone cells do seem to form adjacent to the apical tips of rhabdomeres (Fig. 2B). These cells are aberrant in morphology, often appearing to contain large vacuoles (Fig. 2E) that are presumably full of excess unsecreted lens material. It is difficult to count the cone and pigment cells, since they are also displaced in the apical-basal axis and thus lie in different sections. Nevertheless, this abnormal non-photoreceptor development in *Ellipse* but not $Sev^{S11.1}$ eyes suggests that activated DER and Sev are not functionally interchangeable in cells that are not normally fated to become photoreceptors.

Distribution of genetic background effects on $Sev^{S11.1}$ and *Ellipse* activity

In order to estimate to what extent the wild-type genetic background can affect phenotypic variation, the two mutants were crossed to 17 lines from 3 different populations. Ten of these crosses were to isofemale lines from a world wide collection, "wwwt"; 6 were from isofemale lines recently established from an Ann Arbor fruit market, "aawt"; and several crosses were made to the long-term "Ives" laboratory population. Two different *Ellipse* alleles were tested, DER^{E1} and DER^{EB1}, and the $Sev^{S11.1}$ transgene was tested from 2 slightly different stocks ($Sev^{S11.1}$ *rougher* and $Sev^{S11.1}$ *smoother*) derived by 10 generations of artificial selection on the original stock. Since *Ellipse* heterozygotes are fully penetrant, phenotypes were scored in F1 progeny of crosses between 3 or 4 wild-type virgin females and an equal number of *Ellipse* males. An average of 30 flies were scored for each cross and sex, and most crosses were also replicated 6 months after the first experiment.

The $Sev^{S11.1}$ phenotype is only partially dominant, so in this case phenotypes were scored in F2 flies derived from crosses of several F1 siblings. These F2 populations generally contained Mendelian ratios of the three genotypic classes (no transgene, heterozygotes, and transgene homozygotes). The upper quarter of the distribution were assumed to be homozygotes, and the scores of only these individuals are considered here. In all cases, at least one quarter of the flies were wild-type in appearance, and hence homozygotes for absence of the transgene. In most cases, there was a distinct bimodal distribution, such that the cut-off for the top quarter was coincident with a natural trough in the distribution – in such cases, this trough was actually used to make the cut-off. The only cases where this was not possible involved strong suppression of the phenotype, in which case we conservatively estimated the cut-off, resulting in possible overestimation of the mean homozygote value in the lines with the lowest scores.

Mean eye roughness scores and standard deviations for each cross are listed in Table 1. The scores are plotted in Fig. 4, which allows direct comparison of the phenotypes obtained in each background. Standard errors for each cross were of the order of 1 eye roughness point, and the means of the two sexes of each cross differed by a maximum of 2 points, as did means of replicate crosses. Consequently, mean differences greater than 2 points generally indicate significant effects that most likely have a genetic basis. Both standard analysis of variance as well as non-parametric Kruskal-Wallace tests (not shown) confirmed that there are highly significant overall differences between lines for each mutant. They also indicated that the two sexes are more distinct for *Sevenless* than *Ellipse* mutants.

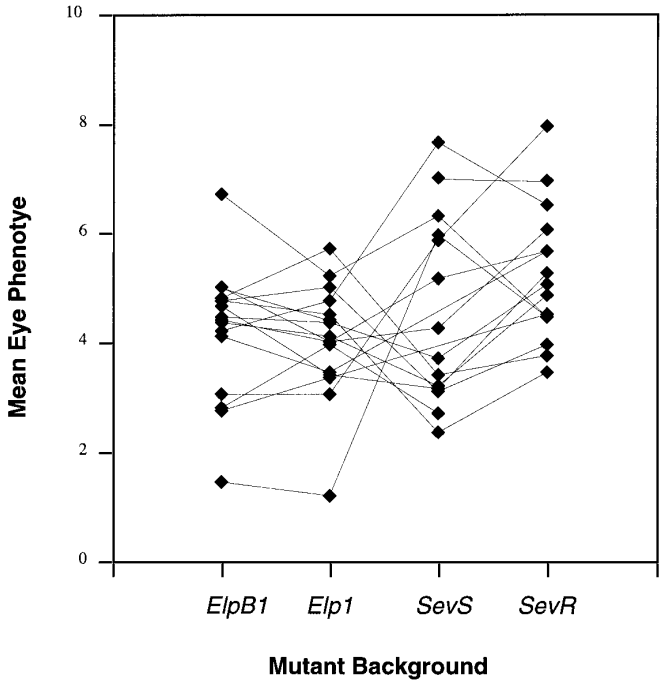

Fig 4 Comparison of mean eye roughness scores in 17 different genetic backgrounds of two alleles of *Ellipse* (*Elp*^{1} and *Elp*^{B1})and the *Sev*$^{S11.1}$ transgene in two different artificially selected lines (*smoother, SevS* and *rougher, SevR*). Crosses to individual isofemale lines are joined on the figure by thin black lines, which show the high level of crossing of line means between the two mutant types. Eye score represents two arbitrary scales from 1 (wild type) to 10 (most extreme) for each mutation, as described in the text

For *Ellipse*, most wild-type genetic backgrounds modified the eye phenotype to similar extents, producing roughness scores in the range of 3–5 points. Two lines were outliers, one (wwwt-1) strongly suppressing the mutant, the other (aawt-1) enhancing just the *DER*^{EB1} allele. Overall, the effects of the two alleles were highly correlated (R^2 = 0.69, P < 0.001), but interestingly *DER*^{E1} homozygote females were lethal in most lines despite having similar heterozygote mean phenotypes as *DER*^{EB1} relatives, which were usually homozygous viable. This may indicate that viability is modified by different genes than is photoreceptor determination.

For *Sev*$^{S11.1}$, there was much greater variance between lines with a spread of mean roughness scores from 2.4 to 8.4, and the effects on the two artificially selected (*rougher* and *smoother*) mutant backgrounds were only weakly correlated (R^2 = 0.43, P = 0.04). This indicates both that there is substantial naturally occurring genetic variation affecting Sevenless signaling, and that there are non-additive interactions among modifiers. Strikingly, two of the lines with the strongest suppression when crossed to the *rougher* background showed above average enhancement in the *smoother* background. These were the same two lines that were extreme modifiers of the *Ellipse* phenotype. Three further lines (aawt-4 and wwwt-21, and each of the Ives crosses) consistently enhanced *Sev*$^{S11.1}$more strongly than has been reported for loss-of-function mutations in the *Gap1* gene identified in a mutagenesis screen for enhancers of a different activated *Sev* transgene (Gaul 1992). All of the extreme effects were confirmed in replicate crosses.

There was no significant correlation between the effects of the wild-type lines on the phenotypes of *Ellipse* and *Sevenless*. This can be seen by the extensive crossing of line means between *DER*^{EB1} and *Sev-smoother* in the center of Fig. 4, and is supported by the non-significance of correlation coefficients for all pairwise comparisons between the two backgrounds of each mutant or the averaged mutant effects (R^2 = 0.06, P = 0.86). Since the two receptor tyrosine kinases are thought to signal through the same pathway, variation in components of this pathway was expected to modify the phenotypes to similar extents. It was even more surprising to observe negative correlations between some lines. Thus, Ives crosses consistently enhance *Sev*$^{S11.1}$ but suppress *Ellipse*, while lines wwwt-17 and aawt-10 suppress *Sev*$^{S11.1}$ *smoother* but enhance *Ellipse*. Taken together with the observation of sex-specific differences within crosses, we conclude that signal transduction in the eye is regulated in a complex manner by modifiers that need not be restricted to components of the Ras-Raf pathway.

Heritability and the magnitude of allelic effects

It was not possible with our design to make accurate estimates of the heritability of the eye phenotypes, since the various lines used were not isogenic. Nevertheless, the mean square difference between line means is clearly greater than the within-line variance, suggesting that the contributions of fixed genotype differences are greater than the environmental variance. For *Sev*$^{S11.1}$ we also constructed a series of introgression lines by repeated backcrossing of isofemale lines to *Sev*$^{S11.1}$ heterozygotes for ten generations. Eight lines survived this procedure and were further inbred to homogenize the *Sev*$^{S11.1}$ allele, with phenotypes ranging again from almost complete suppression to very strong enhancement (Table 2). Heri-

Table 2 Summary of *sevenless* Introgression Lines. Means and standard deviations of eye roughness scores for homozygous introgression lines derived by ten generations of backcrossing of *Sev*$^{S11.1}$ to aawt (AS1, 3, 9) or wwwt (WS1, 8, 11, 15, 25) stocks. The overall line mean square from an ANOVA of this data set was 230.6, with sex by line and residual mean squares of 1.6 and 1.0 respectively. This yields a negligible sex by line intereaction term, and provides estimates for the genetic variance, V_G = 2.9 units, and the environmental variance, V_E = 1.0 units, whence a heritability estimate of 0.74 (*aawt* Ann Arbor wild type, *wwwt* world wide wild type)

Line	Mean	σ
AS1	6.8	1.2
AS3	7.7	0.7
AS9	8.5	1.1
WS1	7.4	1.2
WS8	3.5	0.9
WS11	4.2	1.3
WS15	2.2	0.8
WS25	3.3	0.8

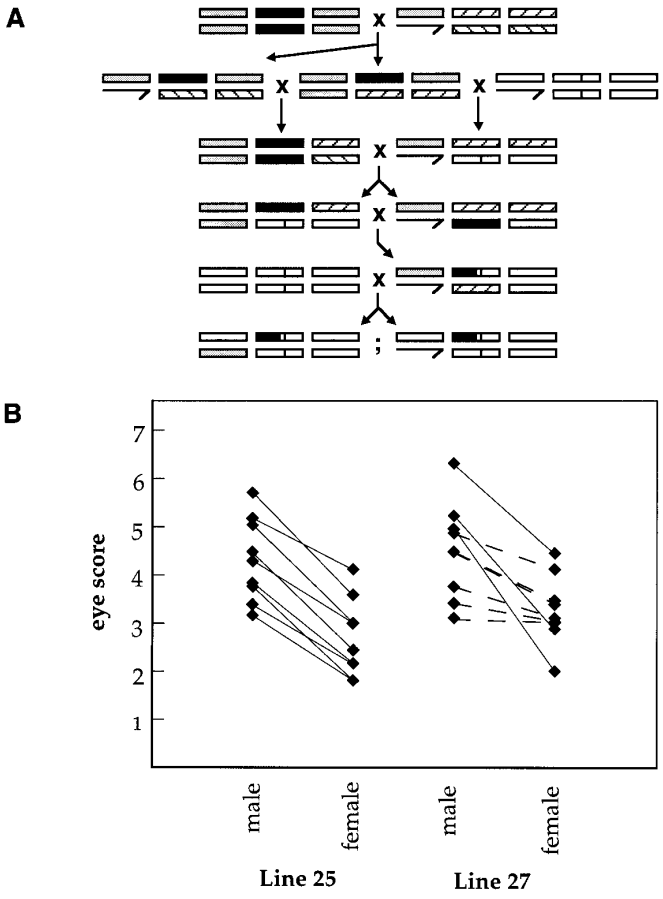

Fig 5A, B Genetic variation affecting sex-dependence of *Sevenless* activity maps to the second chromosome. **A** The crossing scheme used to compare the mean phenotypes of recombinant chromosomes between the *Sev-smoother* and isogenic second chromosomes from lines 25 and 27. Individual second chromosomes (*black*) were first isogenized in the Harwich genetic background (*light shading*) using standard balancer crosses, and then tested as shown. *Cross-hatching up to the right* indicates a balancer (CyO for Chr 2, TM6 for Chr 3), and *down to the right* indicates a dominantly marked chromosome (*Pm, Sp* or *Sb*). The *Sev-smoother* background is shown as *unshaded boxes*, with a *vertical bar* to indicate the location of the transgene on chromosome 2. Y chromosomes in males are shown as a *one-sided arrow*, and the small fourth chromosome is disregarded. Each heterozygous male used to set up the last cross will have a different break point between the line 25 or 27 chromosome and the Sev chromosome, but all of its non-Curly progeny will be genetically identical. For both of these lines, heterozygotes (the *curly* progeny) had almost wild-type eyes, so only the (non-*curly*) homozygotes were scored. **B** The relationship between males and females in nine different recombinant genetic backgrounds. There is a consistent difference between the sexes for all crosses involving line 25 recombinants, whereas several of the crosses involving line 27 show no difference between the sexes (*dotted lines on right hand side*)

tability for both sexes treated separately was estimated from analysis of variance as 0.74, in good agreement with the F2 data and confirming that there is widespread genetic variation affecting Sevenless signaling in natural populations. This may be an underestimate since the error term includes a component due to within-line genetic variation, but on the other hand, the sample may have been biased toward lines that gave a strong phenotype as some without a dominant phenotype were lost by genetic drift during the introgression.

To determine whether it might be possible to apply quantitative genetic methods to the mapping of loci that affect Sevenless signaling, two enhancing second chromosomes were extracted into an isogenic background, and recombined with the $Sev^{S11.1}$*smoother* background. Crosses were designed (Fig. 5A) to allow measurement of an average of 20 flies of the same genetic constitution. The results are plotted in Fig. 5B, which compares male and female scores for nine recombinant *wwwt25*, and nine recombinant *wwwt27* second chromosomes. Each of these chromosomes were found to produce significantly different mean scores among recombinants ($P < 0.0001$, ANOVAs for the two lines considered separately). However, the mean difference between chromosomes was similar to the environmental deviation, implying that interval mapping of quantitative trait loci (for example, Zeng 1994) would be difficult. It is also apparent from Fig. 5 that there is genetic variation on chromosome 2 that affects the differences between the two sexes. All nine line 25 recombinants have sex-specific differences of about 1 roughness score, whereas the line 27 recombinants vary significantly ($P < 0.05$; ANOVA) in the difference between males and females, and might be used to map the locus or loci responsible for this effect.

Our data allows a weak estimate of the magnitude of effects attributable to individual loci. From the increase in phenotypic variance of F2 flies from crosses between the $Sev^{S11.1}$ *rougher* and $Sev^{S11.1}$ *smoother* lines, which differ by 3 scale units, we used the Wright-Castle approximation (Castle 1921) to estimate that one or two genes might be responsible for this difference (not shown). Such effects would not be greatly different from those uncovered in mutagenesis screens for modifiers of activated *Sevenless* alleles, given that the most extreme reported phenotypes have a value of 6 or 7 on our scale. The magnitude of between-line effects is also much larger relative to the trait mean than is observed for most continuously variable morphological traits (Houle 1992). The data are at least consistent with the segregation in natural populations of major-effect alleles that affect *Sevenless* signaling. Tests of association between particular alleles of candidate genes and eye roughness might be used to test this hypothesis.

Discussion

Hidden genetic variation affecting eye development

The major results of this study are that (i) there is widespread genetic variation affecting signal transduction in the *Drosophila* eye, (ii) wild-type genetic backgrounds can cause phenotypes in excess of those detected in mutagenesis screens, (iii) the variation is expressed to different degrees in the two sexes, with this difference itself being genetically variable, and (iv) at least some of the variation seems to affect the *Sevenless* and *DER* path-

ways antagonistically. All of these observations are paradoxical in light of simple evolutionary genetic theory which predicts that invariant phenotypes are invariant because of strong purifying selection that removes all genetic variation. Since ommatidial development is constant and uniform, despite pools of underlying hidden genetic diversity, there must be very strong buffering mechanisms operating. Understanding how this buffering works will require knowledge of the precise molecular genetic makeup of variation in the relevant pathways.

The situation where phenotypic variance increases in a mutant background relative to the wild type is referred to as "canalization" (Waddington 1942; Wagner and Gibson 1998). Canalization is usually regarded as an evolved state, in the sense that natural selection ought to favor the evolution of genetic systems that prevent the expression of sub-optimal phenotypes. Aberrant ommatidial development is undoubtedly suboptimal, and there is likely to be strong selection for developmental stability. Under such circumstances, modifiers that prevent the phenotypic effects of new mutations and random environmental noise might accumulate, with the result that the genetic pathway can actually hide increased levels of underlying variation. However, Wagner et al. (1997) have recently analyzed this problem mathematically, and shown that the conditions under which canalization can evolve are quite restricted. It is not clear that canalizing selection can completely suppress all phenotypic variation as observed here. An alternative "explanation" is that the observed canalization is an intrinsic property of the genetic pathways, so that the interactions among polymorphic alleles somehow ensure developmental buffering. That is to say, it seems that photoreceptor determination is threshold-dependent, but this is not very satisfying either; it may well be, but that does not explain either how the threshold works, how it came to be, or why there remain such high levels of underlying variation.

Part of the key to resolving the paradox will be to understand the molecular basis of both the dominance and sex-specificity of allelic effects. $Sev^{S11.1}$ is a perfect example of a 'mutation' whose degree of dominance is a function of the genetic background – in some lines it is purely recessive, in some it is recessive in males and dominant in females, and in some purely dominant. It is unclear why the degree of buffering should be sex-specific, unless the variation serves some adaptive pleiotropic purpose for which there is differential selection in the two sexes. In any case, the sex-specificity provides yet another example of the emerging notion that the two sexes provide remarkably different 'environments' in which context the maintenance of genetic variation should be considered (Mackay 1995).

Interpretation of the negative correlation between $Sev^{S11.1}$ and *Ellipse* activity

If Sev and DER proteins really are functionally interchangeable (Freeman 1997; Tio and Moses 1997), and simply initiate the Ras-Raf kinase pathway, genetic variation present in the activity of the *raf*, *D-sor* and *rolled* kinases in the wild-type lines would be expected to modify both types of constitutively active protein to similar extents (Wassarman et al. 1996). This may be the case, but the negative relationship observed between $Sev^{S11.1}$ and *Ellipse* phenotypes in the Ives and wwwt-1 lines is the opposite of this prediction. There are two other potential sources of variation that could more readily explain the fact that some enhancers of $Sev^{S11.1}$ suppress *Ellipse* and vice versa. One is that modifiers of expression level have opposite effects on the two genes, so that polymorphisms that, for example, increase *sevenless* transcription decrease *DER* transcription. A more intriguing possibility is that there is polymorphism in one or more of the components that physically interact with the receptor molecules and link them to Ras and the MAP kinase pathway. Thus, a protein variant that bound more tightly to Sev protein might naturally have a lower affinity for DER protein, or vice versa. One obvious candidate, Drk, has an alibi since the gene is tightly linked to the site of insertion of the $Sev^{S11.1}$ transgene at cytological interval 50B, and would be homogeneous in all of the lines (Simon et al. 1993). Nevertheless, this hypothesis makes the testable prediction that naturally occurring protein polymorphism in proximal components of RTK signaling, such as the Gap1 and SOS proteins, could be associated with variation for eye phenotypes in the mutant backgrounds.

Other sources of variation include the transcription factors and target genes that mediate the outputs of the signaling pathways. For example, loss-of-function mutations in *yan*, which encodes a repressor of the *phyllopod* target gene in R7 cells, enhance the $Sev^{S11.1}$ phenotype (Rebay and Rubin 1995), and naturally occurring variation in this gene could have a similar quantitative effect. It is also possible that some of the modifiers of $Sev^{S11.1}$ are unrelated to normal signaling, and act instead on genomic regulatory sequences adjacent to the site of insertion of the transgene. An argument against this possibility is that there was no correlation between eye roughening and eye color produced by the $white^{+}$ marker included in the P-element construct carrying the transgene (data not shown). It is also difficult to see how position effects could result in a negative correlation between $Sev^{S11.1}$ and *Ellipse* phenotypes. It should be recognized as well that some, or perhaps most, of the variation might be attributed to genes unrelated to signal transduction, including factors that affect dosage compensation and the determination of photoreceptor sub-type identity, to name just two possibilities. Genetic interactions need not imply biochemical interactions.

Implications

Finally, we emphasize two implications of our results. First, the interpretation of even fully penetrant phenotypes may easily be affected by the genetic background.

This has clearly been shown for knock-out mutations of the mouse epidermal growth factor (EGF)-receptor, the lethality of which is peri-implantation in some genetic backgrounds and perinatal in others (Threadgill et al. 1995). Particular caution should be applied to the interpretation of allelic series where genetic backgrounds are different, and it may not be valid to conclude that a particular amino acid change is responsible for a particular novel aspect of a phenotype. Second, though usually ignored or regarded as a nuisance, polygenic modifiers can have genetic effects at least as great as mutations isolated in screens. It is astonishing that viable combinations of wild-type alleles that have no obvious effect on normal development, interact with particular mutations to produce stronger phenotypes than certain lethal point mutations. As methods are developed for mapping quantitative trait loci, advantage might be taken of the fact that nature provides a vast supply of mutations that have important but subtle effects that elude classical Mendelian analysis.

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