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Modulation of the Ras/MAPK Signalling Pathway by the Redox Function of Selenoproteins in *Drosophila melanogaster*

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Modulation of reactive oxygen species (ROS) plays a key role in signal transduction pathways. Selenoproteins act controlling the redox balance of the cell. We have studied how the alteration of the redox balance caused by *patufet* (*selD*^{ptuf}), a null mutation in the *Drosophila melanogaster selenophosphate synthetase 1* (*sps1*) gene, which codes for the SelD enzyme of the selenoprotein biosynthesis, affects the Ras/MAPK signalling pathway. The *selD*^{ptuf} mutation dominantly suppresses the phenotypes in the eye and the wing caused by hyperactivation of the Ras/MAPK cassette and the activated forms of the Drosophila EGF receptor (DER) and Sevenless (Sev) receptor tyrosine kinases (RTKs), which signal in the eye and wing, respectively. No dominant interaction is observed with sensitized conditions in the Wnt, Notch, Insulin-Pi3K, and DPP signalling pathways. Our current hypothesis is that selenoproteins selectively modulate the Ras/MAPK signalling pathway through their antioxidant function. This is further supported by the fact that a selenoprotein-independent increase in ROS caused by the *catalase* amorphic *Cat*^{n1} allele also reduces Ras/MAPK signalling. Here, we present the first evidence for the role of intracelular redox environment in signalling pathways in *Drosophila* as a whole organism. **© 2001 Academic Press**

Key Words: **selenophosphate synthetase; selenoproteins; reactive oxygen species; Ras/MAPK signalling pathway.**

INTRODUCTION

Selenoproteins are characterized by the presence of the amino acid selenocysteine. Incorporation of this amino acid into selenoproteins requires a translational step where UGA, which normally functions as a stop codon, specifies selenocysteine, and the presence of the selenocysteine insertion sequence (SECIS) in the 3′ untranslated region of the selenoprotein mRNA (for reviews see Low and Berry, 1996; Stadtman, 1996). Studies on mutant *Escherichia coli* strains exhibiting defects in selenium metabolism have identified four genes, *selA–selD*, encoding selenocysteine synthase (SelA), a selenocysteine-specific elongation factor (SelB), a selenocysteine-specific tRNA (tRNA^{Sec}, *selC* gene product), and selenophosphate synthetase (SelD), all of which are essential for bacterial selenoprotein synthesis (Bock *et al*., 1991; Bock, 2000). In eukaryotes, the selenocysteine tRNA, the selenocysteine-specific elongation factor (SelB), the SECIS binding protein (SBP2), and two selenophosphate synthetase genes (*sps1* and *sps2*) have so far been identified (Lee *et al*., 1990; Low *et al*., 1995; Guimaraes *et al*., 1996; Persson *et al*., 1997; Alsina *et al*., 1998; Zhou *et al*., 1999; Copeland *et al*., 2000; Fagegaltier *et al*., 2000; Hirosawa-Takamori *et al*., 2000; Tujebajeva *et al*., 2000). Sps1 and Sps2 differ in the presence of a cysteine codon in the predicted active site of the enzyme in the first while a selenocysteine codon occurs in the latter.

Disruption of the mouse *selenocysteine-tRNA* gene causes early lethality (Bosl *et al*., 1997). Similarly, the *selD*^{ptuf} mutation of the *Drosophila melanogaster sps1* gene (*selD*) results in larval lethality (Alsina *et al*., 1998). These results, together with the conservation of the selenoprotein synthesis machinery between different species, suggest an important role of selenoproteins in cell function. Several selenoproteins have been identified in different organisms, including the eukaryotic glutathione peroxidase and thioredoxin reductase families, iodothyronine deiodinases, or

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proteins of unknown function such as selenoprotein P or W (Stadtman, 1996; Burk and Hill, 1999). Most of them appear to have a role as antioxidants suppressing the formation or action of ROS, or catalyzing oxidation-reduction reactions (Stadtman, 1996). Although for many years ROS have been thought of as the unwanted and toxic by-products of living in an aerobic environment, recent evidence suggests that ROS such as superoxide anions and hydrogen peroxide function as intracellular second messengers. Emerging evidence suggests that ROS have an important role in signal transduction (for review see Finkel, 1998).

To explore the possible role of the redox cellular state driven by selenoproteins in the modulation of signalling pathways, we have used the null *selD^{ptuf}* mutation, which has been shown to block the biosynthesis of selenoproteins (Alsina *et al*., 1999). It is a recessive mutation causing lethality at third instar stage. Homozygous larvae have extremely reduced and abnormal imaginal discs, showing a completely disorganized epithelium, the cells of which accumulate free radicals and enter apoptosis (Alsina *et al*., 1998, 1999). In genetic mosaics, homozygous mutant *selD^{ptuf}* cells show similar phenotypes to those described for loss-of-function mutants of the DER signal transduction pathway. For example, clones are rounded and small, and suppress vein differentiation in both cases (Diaz-Benjumea and Hafen, 1994; Alsina *et al*., 1998). Although it is unlikely that an enzyme such as selenophosphate synthetase is an integral component of any growth factor signalling pathways, it may play an indirect role, modulating them. Thus, selenoproteins may be instrumental to maintain a certain redox state in the cell necessary for the different activities of the pathway.

We have performed a series of genetic interactions using the eye and wing of *Drosophila melanogaster* as model systems where the Ras/MAPK cassette is activated. The wild-type compound eye of the fly consists of a regular arrangement of ommatidia, each containing an invariant number of cells. Cell fate in the developing eye is determined by the activation of the Ras/MAPK cassette, which is triggered by two RTKs, the DER and Sev, required for the specification of the R1–R6 and R7 photoreceptor cell fate, respectively (Basler *et al*., 1991; Freeman, 1996). The *Drosophila* wing is also an important model system for analyzing pattern formation in a fully cellularized and proliferating epithelial sheet. Vein differentiation in the adult wing is achieved by the activation of the Ras/MAPK cassette through the DER (Diaz-Benjumea and Garcia-Bellido, 1990; Garcia-Bellido and de Celis, 1992; Sturtevant and Bier, 1995). Herein, we have analyzed gain-of-function mutations of members of the Ras/MAPK signalling pathway and RTKs that trigger their activation in combination with *selD^{ptuf}* loss-of-function mutation. Furthermore, we have altered the redox balance of the cell in a selenoprotein-independent way using a *catalase* amorphic allele instead of *selD^{ptuf}*. Altogether, the results obtained in this study suggest that the Ras/MAPK pathway is sensitive to the perturbation of the cellular redox balance caused either by the alteration of selenoprotein biosynthesis in the *selD^{ptuf}* mutant or by *catalase*.

MATERIALS AND METHODS

Drosophila Strains

Canton-S was used as wild-type strain. The *PlacW* insertion lines *l(2)k11320/CyO* (*selD^{ptuf}* in the text) and *l(2)k07214/CyO* (Torok *et al*., 1993) were obtained from the Szeged Stock Center. The activated *sevenless* (*sev*) construct *w^{1118} sev^{d2};sevS11.5* (Basler *et al*., 1991), the activated *raf* construct *Raf^{torY9}/CyO* (Dickson *et al*., 1996), and the EMS gain-of-function mutation of *rolled* (*MAKP*): D-*raf^{C110}; rl^{Sem}* were used. *D-raf^{C110};rl^{Sem}* double mutant flies possess a normal complement of six outer photoreceptor cells and one R7 cell (Brunner *et al*., 1994). In order to eliminate the *D-raf^{C110}* allele, present to counteract the weak dominant sterility reported for *rl^{Sem}* heterozygous flies (Lim *et al*., 1999) and responsible for this wild-type eye phenotype, crosses were designed to score the male *+/Y;rl^{Sem}/+* progeny. The activated *ras* construct *e ftz ry/TM3 P(sev-rasV12)* (Fortini *et al*., 1992) was kindly provided by T. Laverty. The following stocks were also used: *2xsev hsp-rough/CyO* (Basler *et al*., 1991), *sev-wg*(III) (Brunner *et al*., 1997), *yw;GMR-GAL4, UAS-InRwt/CyO* (Huang *et al*., 1999), *GMR-GAL4, UAS-Dp110 CAAX* (II) (Leevers *et al*., 1996), and *sevGAL-4*. The *GMR-Rho1^{1} GMR-Rho1^{3}/TM6B* (Hariharan *et al*., 1995) stock was a gift from J. Settleman. J. Casanova kindly provided the activated *UAS-tkv* stock. For ectopic expression of *selD*, we used *UAS-selD 8.1* transgenic on the third chromosome made by B. Alsina. The *UAS-ΔD-Raf^{F20}* construct (Martin-Blanco *et al*., 1999) and *GAL-4^{604}* insertion line (Brand and Perrimon, 1993) were obtained from E. Martin Blanco. The *GAL-4^{604}* line is expressed in wing discs in late pupal stages (E. Martin Blanco, personal communication). J. Mahaffey kindly provided the *yw, Cat^{n1}/TM3* strain (Mackay and Bewley, 1989; Griswold *et al*., 1993). The *Elp^{B1}/CyO* stock and the *Ax^{28}* allele were obtained from the Bloomington Stock Center. Fly cultures were maintained at 25°C using standard medium.

Scanning Electron Microscopy and Histology

To prepare scanning electron microscopy (SEM) samples, flies were dehydrated in 25, 50, 75, 95, and 100% ethanol for 24 h each. To get rid of accumulated debris in the flies' eyes, they were sonicated for 30 s in an ultrasound bath followed by a final change of 100% ethanol. Flies were critical point dried and coated with gold to be examined in a Leica–360 scanning electron microscope.

Eyes to be sectioned were fixed and embedded in Spurr's medium as described previously (Basler and Hafen, 1988). Semithin sections were obtained and stained with methylene blue for light microscopy.

Statistical Analysis

For each genotypic combination, ommatidia from three different eyes were counted. As our data did not accomplish the minimal requirements to do a *t*-test, a chi-square test on contingency tables was performed. The aim was to see if there were significant differences between heterozygotes for the gain-of-function allele and transheterozygotes for the gain-of-function and the *selD^{ptuf}* mutation for the two parameters studied: normal versus abnormal ommatidia and number of R7 per ommatidium. The program used was STATGRAPHICS Statistical Graphics System Version 7.0.

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RESULTS

selD Modulates the Ras/MAPK Signalling Pathway in the Eye

One approach to identify new components of any given pathway is to search for mutations that dominantly modify the phenotype of another mutation in the same pathway. The rough eye phenotype caused by hyperactivation of components of the Ras pathway is dose sensitive and has been successfully used in dominant modifier screens that led to the identification of essential components in this signalling pathway (Simon *et al*., 1991; Olivier *et al*., 1993; Dickson *et al*., 1996; Karim *et al*., 1996). Given the similarities of the wing phenotypes caused by homozygous clones for $selD^{ptuf}$ and for loss-of-function mutations in genes coding for the components of the Ras/MAPK cascade (Diaz-Benjumea and Hafen, 1994; Alsina *et al*., 1998), we wanted to test whether the alteration of the cellular redox state generated by the loss-of-function mutation $selD^{ptuf}$ was interfering with the Ras/MAPK signalling pathway in the eye and the wing.

We analyzed whether the removal of one functional copy of the *selD* gene was sufficient to suppress the rough eye phenotype caused by gain-of-function mutations of several members of this signalling cassette: *sevenless* (*sev*), *ras*, *raf*, and *rolled* (*rl, MAPK*). Expression of transgenes encoding activated Sev, Ras, or Raf in a subpopulation of ommatidial precursor cells by the *sev* enhancer results in the recruitment of supernumerary R7 photoreceptor cells. The increased number of photoreceptor cells disrupts the regular hexagonal array of the ommatidial units thus leading to an irregular rough eye phenotype. The rl^{Sem} gain-of-function mutation in the *rolled* locus, the structural gene for MAPK, was generated in an EMS screen, and results in a prolonged activation of MAPK also leading to a rough eye phenotype and extra R7-like cells.

To assure that lowering *selD* gene function does not suppress the rough eye phenotype by reducing the expression of the *sev*-enhancer-*hsp*-promoter controlled transgenes, we tested whether the $selD^{ptuf}$ mutation suppressed an unrelated rough eye phenotype caused by the ectopic expression of the *rough* gene under the control of the same *sev-hsp* enhancer/promoter element (Basler *et al*., 1990). The ommatidial alteration caused by the overexpression of *rough* is independent of the Ras/MAPK pathway and transforms the presumptive R7 cell into R1–R6 cell fate. $selD^{ptuf}$ did not suppress the rough eye phenotype associated with the *sev-hsp-rough* (data not shown). We conclude from this that a reduction in functional *selD* product does not alter gene expression from the *sev-hsp* enhancer promoter.

The suppression of the rough eye phenotype caused by activating components of the Sev pathway was analyzed in two ways: the external arrangement of the ommatidial units and by the degree of eye roughness. Eyes were examined by scanning electron microscopy (SEM). To determine whether the degree of roughness of the eyes observed by SEM correlated with the number of extra R7 photoreceptor cells, we analyzed semithin sections and counted the number of normal and abnormal ommatidia per genotype and the number of R7 rhabdomeres per ommatidium.

sevenless. Constitutive activation of Sev in *sevS11.5/+* mutant flies showed a characteristic rough pattern of irregular ommatidia (Fig. 1B). In contrast, the regular arrangement of ommatidia was partially restored in $+/selD^{ptuf}$; *sevS11.5/+* individuals (Fig. 1C). Sections through the distal part of the eyes of flies carrying one copy of the Sev activated transgene confirmed the highly irregular ommatidial pattern (Fig. 1B). Less than 10% of ommatidia contained a normal set of photoreceptor cells. Many ommatidia contained up to six and seven small in addition to six or seven large rhabdomeres. Sections through haploinsuficient *selD* ($selD^{ptuf}/+$) eyes in the activated Sev background showed more than 50% wild-type ommatidia (Fig. 1C). The difference between the wild-type and haploinsuficient *selD* dosage in the activated Sev background in the proportion of normal and abnormal ommatidia is highly significant ($P < 0.005$). There was also a significant difference ($P < 0.005$) between both genotypes in the number of R7 rhabdomeres per ommatidium. While only 7.5% of the ommatidia in the activated Sev background contained one R7, 61.2% of the ommatidia haploinsuficient for *selD* in the Sev-activated background contained a single R7 cell (Fig. 2A).

ras. Like Sev, activation of Ras during eye development in *rasV12* flies causes a rough eye phenotype. Adult eye sections revealed that most ommatidia contained two or more supernumerary R7 cells (Fig. 1D). Eye SEM images and semithin sections of *selD* haploinsuficient eyes in the activated Ras background did not show any obvious suppression of the *rasV12* rough eye phenotype (Fig. 1E). The statistical analysis confirmed that the difference between both genotypes for the proportion of normal and abnormal ommatidia, and for the number of R7 rhabdomeres per ommatidium was not significant ($P > 0.05$) (Fig. 2B).

raf. Comparison of the eye SEM images of flies carrying the activated Raf transgene Raf^{torY9} and flies haploinsuficient for *selD* in the activated Raf background showed that the latter presented a more regular ommatidial arrangement (Figs. 1F and 1G). Distal sections through $Raf^{torY9}/+$ eyes revealed a highly irregular ommatidial pattern in which more than 70% of ommatidia had multiple R7-like cells (Figs. 1F and 2C). In distal sections through $Raf^{torY9}/selD^{ptuf}$ eyes more than 60% of ommatidia had the normal set of photoreceptors (Figs. 1G and 2C). Data from semithin sections showed that there was a significant difference ($P < 0.005$) in the proportion of normal and abnormal ommatidia between both genotypes. There was also a significant difference ($P < 0.005$) between the number of R7 rhabdomeres per ommatidium, which is reflected in the different distribution profiles of these genotypes (Fig. 2C).

rolled. Both $rl^{Sem}/+$ and $rl^{Sem}/selD^{ptuf}$ eyes displayed the same mild rough phenotype under the SEM (Figs. 1H and 1I). Analysis of sections revealed the presence of three or four R7-like cells in most ommatidia (Fig. 2D). However, an

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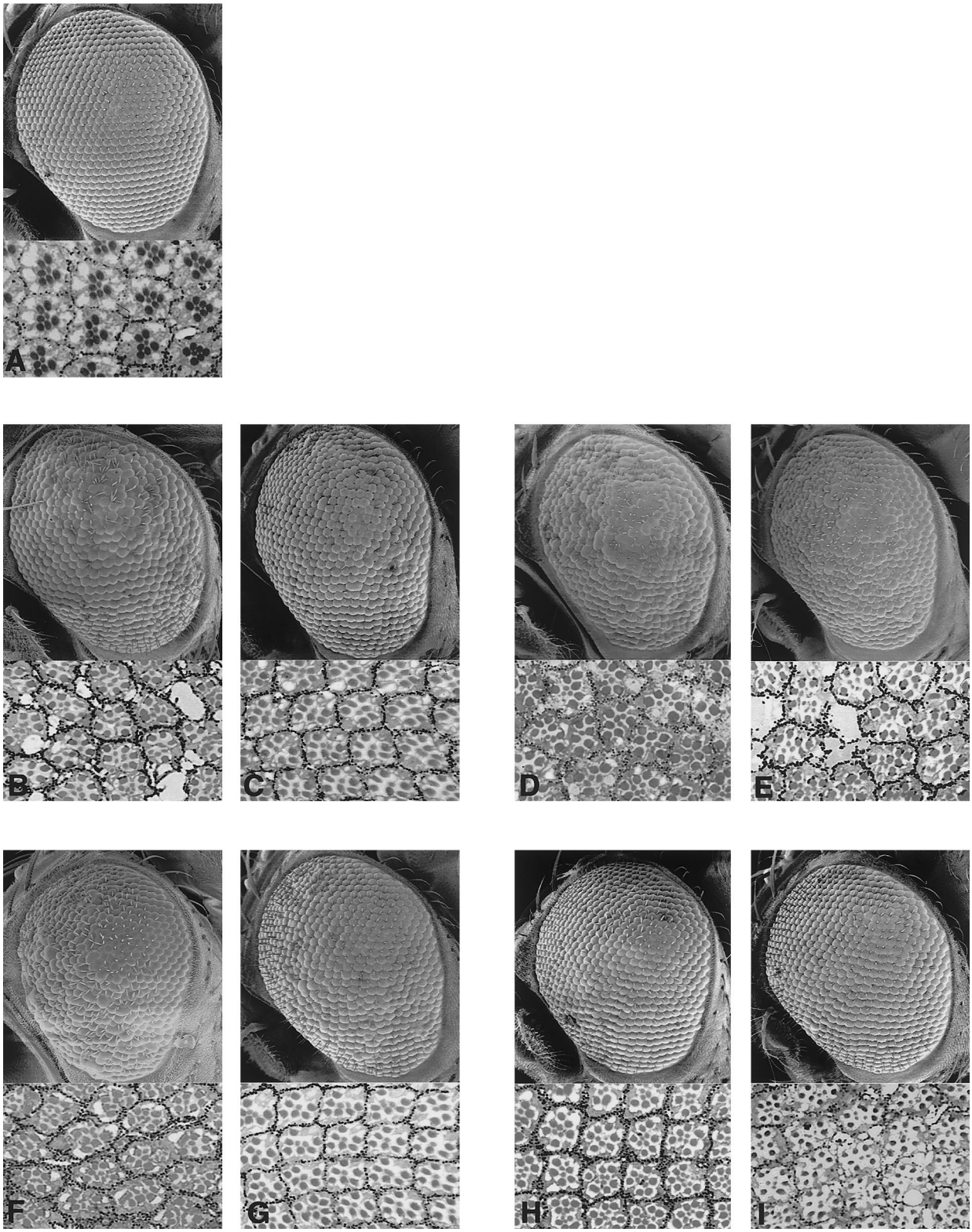

FIG. 1. Suppression of the rough eye phenotype of heterozygous eyes for gain-of-function alleles of the Sev/Ras/MAPK pathway by the *selD*^{ptuf} mutation. Scanning electron micrographs and semithin sections are shown as follows: (A) wild type, (B) *sevS11.5*/+, (C) +/*selD*^{ptuf}; *sevS11.5*/+, (D) +/+;+/*TM3 P(sev-rasV12)*, (E) +/*selD*^{ptuf} ;+/*TM3 P(sev-rasV12)*, (F) *Raf* ^{torY9}/+, (G) *Raf* ^{torY9}/*selD*^{ptuf} , (H) +/*Y;rl*^{Sem}/+, and

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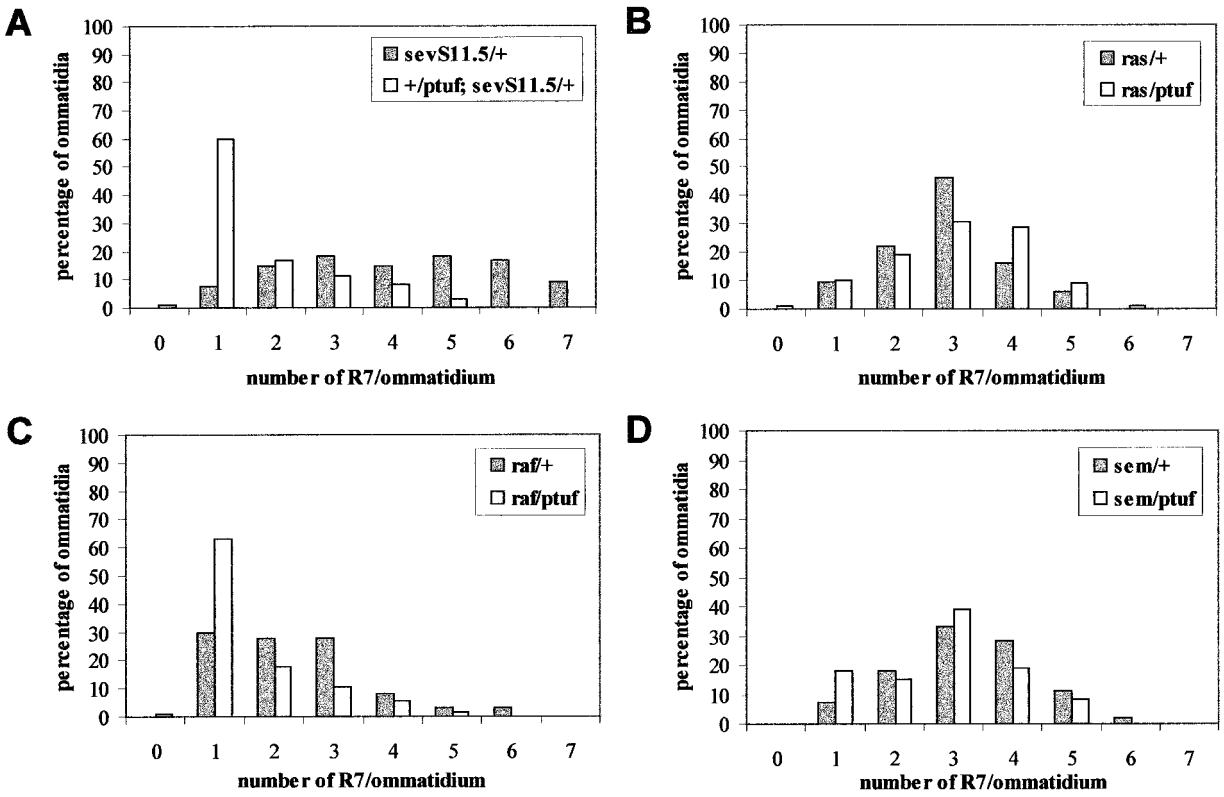

FIG. 2. Distribution profiles of the number of R7 rhabdomeres per ommatidium of heterozygous eyes for gain-of-function elements of *sev*, *ras*, *raf*, and *rl*, and for combinations of these elements with the $selD^{ptuf}$ mutation. Genotypes analyzed are as follows: (A) *sevS11.5/+* and $+/selD^{ptuf}$ *;sevS11.5/+*, (B) *+/+;+/TM3 P(sev-rasV12)* and $+/selD^{ptuf}$ *;+/TM3 P(sev-rasV12)*, (C) $Raf^{torY9}/+$ and $Raf^{torY9}/selD^{ptuf}$, and (D) $+/Y;rl^{Sem}/+$ and $+/Y;rl^{Sem}/selD^{ptuf}$. The X axis stands for the number of R7 rhabdomeres per ommatidium whereas the Y axis stands for percentage of ommatidia. The statistical analysis done on these data show that the number of R7 rhabdomeres per ommatidium distribution profile of *sevS11.5/+* and $+/selD^{ptuf}$*;sevS11.5/+* are significantly different (A) as well as they are for $Raf^{torY9}/+$ and $Raf^{torY9}/selD^{ptuf}$ genotypes (C) (in both analysis $P < 0.005$). A mild but significant difference ($P < 0.05$) has been detected between the number of R7 rhabdomeres per ommatidium distribution profiles of $+/Y;rl^{Sem}/+$ and $+/Y;rl^{Sem}/selD^{ptuf}$ (D). No difference has been detected in the case of *+/+;+/TM3 P(sev-rasV12)* and $+/selD^{ptuf}$ *;+/TM3 P(sev-rasV12)* distribution profiles (B). Note that in the one R7 rhabdomere per ommatidium category of (A), (C), and (D), the absolute difference between the gain-of-function mutant in a $selD^{ptuf}$ background and the gain-of-function alone decreases: a 52.6% in (A); a 33.3% in (C); a 10.7% in (D) (see Discussion)

increase of 8.4% was found in the percentage of $rl^{Sem}/selD^{ptuf}$ (15%) normal ommatidia when compared to the percentage of *the* $rl^{Sem}/+$ ones (6.6%). These numbers pointed to a slightly less severe phenotype of $rl^{Sem}/selD^{ptuf}$ compared to $rl^{Sem}/+$. Statistical analysis showed that there was a mild but significant difference ($P < 0.05$) between the two genotypes in the proportion of normal and abnormal ommatidia, as well as in the number of R7 rhabdomeres per ommatidium.

Summarizing, Sev, Raf, and MAPK, which are kinases, were modulated by *selD*, but Ras, which is a GTPase, was not. In order to discern whether the suppression of the

(I) $+/Y;rl^{Sem}/selD^{ptuf}$. In all scanning electron micrographs, anterior is to the left and dorsal is up. In the presence of one copy of the gain-of-function alleles the adult eye appears rough as compared to wild type (A); i.e., one can no longer see straight rows of ommatidia and the lenses over many of the ommatidia become fused (B, D, F, and H). The rough eye phenotype of the *sev* and *raf* gain-of-function alleles is suppressed by the $selD^{ptuf}$ mutation in heterozygosis and tangential sections display a closer wild type pattern (compare B with C and F with G). No suppression has been seen for the Ras gain-of-function construct (compare D with E). The statistical analysis of sections reveal that there is a mild suppression of the rough eye phenotype in $+/Y;rl^{Sem}/selD^{ptuf}$ though it is not obvious in SEM images and sections (compare H with I).

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TABLE 1
selD Transgene Partially Restores the Activated Sev Phenotype

Genotypes	Mean of R7 per ommatidium	No. of ommatidia counted
+/+;sevS11.5/+	4.10 ± s.d. 1.78	54
UAS-selD/selD^{ptuf};sevGAL-4/sevS11.5	2.50 ± s.d. 1.01	161
+/selD^{ptuf};sevS11.5/+.	1.71 ± s.d. 1.13	170

rough phenotype depends on the molecular nature of these elements of the Sev pathway, we checked how a different GTPase behaved in combination with *selD^{ptuf}* mutation. It has been published that flies carrying two independent copies of the wild-type *Rho1* gene, which encodes for the small-GTPase Rho1, under the expression of the GMR driver display an intermediate rough eye phenotype, which allows for the detection of either enhancement or suppression of eye roughness (Hariharan *et al.*, 1995). No suppression of the rough eye phenotype was observed in *selD^{ptuf}/+;GMR-Rho1^{1} GMR-Rho1^{3}/+* flies (data not shown).

In order to check the specificity of our observations, we tried to restore the activated Sev phenotype in a *+/selD^{ptuf}; sevS11.5/+* background, driving the expression of a *selD* transgene (*UAS-selD*) with a *sevGAL-4* construct. The mean of R7 per ommatidium for each genotype is shown in Table 1. The *selD* transgene was able to partially restore the activated Sev phenotype. The statistical analysis performed on the number of R7 rhabdomeres per ommatidium of these three genotypes showed that their distribution profiles (Fig. 3) were significantly different ($P < 0.005$). This suggests that the ectopic expression of *selD* in an activated Sev and *selD^{ptuf}* heterozygous background restores the abnormal Sev phenotype. The fact that just a partial rescue was observed is probably due to the delay in the activation of the *selD* transgene inherent to the GAL4-UAS system. To assure that we were not scoring effects of the overexpression of the *selD* transgene in a *selD^{ptuf}* background, we checked if the eyes of these flies presented any alteration that could interfere with the restoration of the characteristic activated Sev rough eye phenotype. Both eye SEM images and sections proved to be wild type (data not shown).

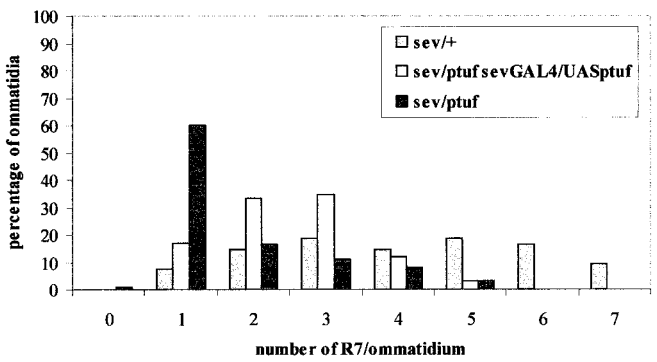

FIG. 3. Distribution profiles of the number of R7 rhabdomeres per ommatidium of the following genotypes: *+/+;sevS11.5/+*, *UAS-selD/selD^{ptuf};sevGAL-4/sevS11.5*, and *+/selD^{ptuf};sevS11.5/+*. The X axis stands for the number of R7 rhabdomeres per ommatidium whereas the Y axis stands for percentage of ommatidia. The comparison between the distribution profiles has shown that there is a significant difference ($P < 0.005$) between them.

Altogether, these results show that lower amounts of functional *selD* gene product suppress the rough eye phenotype caused by the activated forms of Sev and Raf. A mild but statistically significant suppression has also been observed for the *rl^{Sem}* eye phenotype. In contrast, no suppression of the *ras* rough eye phenotype has been detected. Moreover, no suppression of eye roughness caused by overexpression of *Rho1*, another GTPase, has been observed. A different *selD* allele, the *PlacW* insertion line *l(2)k07214*, which has the same lethality phase and imaginal disc's morphology as *selD^{ptuf}*, behaves in the same way (data not shown).

selD Modulates the Ras/MAPK Signalling Pathway in the Wing

During *Drosophila* development a number of RTKs besides Sevenless activate the highly conserved Ras/MAPK cassette, but each RTK elicits a distinct response. One of the processes in which the DER signalling pathway has been extensively studied is cell fate determination in the *Drosophila* wing. Flies carrying viable combinations of *DER* loss-of-function alleles exhibit a partial loss of wing veins (Clifford and Schupbach, 1989). Overexpression of downstream effectors of DER or gain-of-function alleles results in ectopic wing veins (Brunner *et al.*, 1994; Martin-Blanco, 1998). It has recently been found that the level of DER pathway activity is regulated in time and space during wing development. An early activation of DER signalling is necessary for the acquisition of "vein competence" whereas a later downregulation is necessary to implement vein differentiation. Hence, overexpression of activated Raf in late pupal stages results in vein loss instead of ectopic vein tissue (Martin-Blanco *et al.*, 1999).

To test whether lowering *selD* gene function had an effect on the Ras/MAPK signalling cassette independently of tissue and RTK through which it was activated, we used the wing as a model system. Following the same rationale

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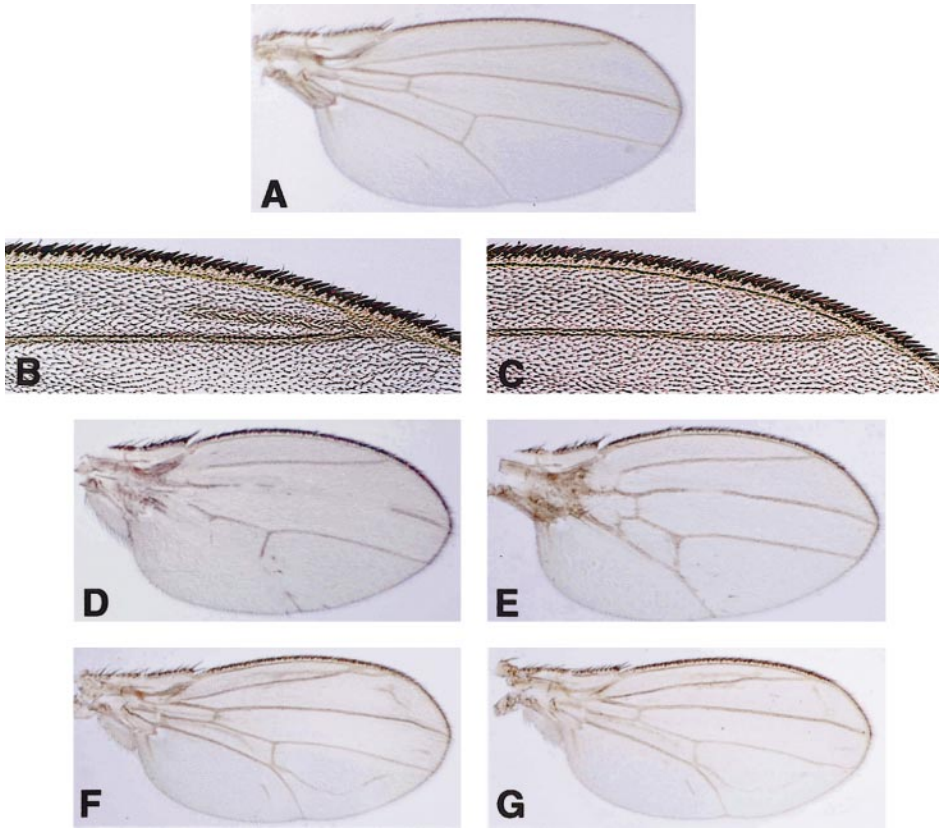

FIG. 4. Suppression of the wing phenotypes of gain-of-function elements of the DER pathway by the $selD^{ptuf}$ mutation. Genotypes are shown as follows: (A) wild type, (B) $Elp^{B1}/+$, (C) $Elp^{B1}/selD^{ptuf}$, (D) $UAS\text{-}\Delta D\text{-}Raf^{F20}/GAL\text{-}4^{604}$, (E) $+/selD^{ptuf};UAS\text{-}\Delta D\text{-}Raf^{F20}/GAL\text{-}4^{604}$, (F) $+/Y;rl^{Sem}/+$, and (G) $+/Y;rl^{Sem}/selD^{ptuf}$. The $selD^{ptuf}$ mutation suppresses the extra vein tissue produced by the gain-of-function allele Elp^{B1} (compare B with C). It restores as well the altered venation pattern caused by the overexpression of $UAS\text{-}\Delta D\text{-}Raf^{F20}$ driven by the $GAL\text{-}4^{604}$ stock (compare D with E), although the reduced size of these wings is not rescued. No suppression of the wing rl^{Sem} phenotype is seen in $+/Y;rl^{Sem}/selD^{ptuf}$ wings (compare F with G).

as in the eye, we have analyzed components of this pathway in the wing in combination with $selD^{ptuf}$ mutation.

Drosophila EGF receptor (DER). We used a gain-of-function allele of *DER*: the $Elp^{B1}/+$, which gives rise to ectopic vein tissue in all wings (Fig. 4B). In 66% of $Elp^{B1}/selD^{ptuf}$ wings, the extra vein phenotype was completely reverted and in the remaining 34% the suppression was partial (Fig. 4C).

raf. We used a $UAS\text{-}\Delta D\text{-}Raf^{F20}$ transgene, which produces a constitutively activated form of Raf, in presence of the $GAL\text{-}4^{604}$ insertion line. As previously described (Martin-Blanco *et al.*, 1999), flies expressing this transgene had reduced viability (50%) and displayed the following wing phenotypes at 25°C: deletion of the central region of vein L3, total or partial loss of vein L5 and a reduction of the overall wing size (compare Fig. 4D to Fig. 4A). An almost wild-type vein pattern was recovered in $selD^{ptuf}/+;UAS\text{-}\Delta D\text{-}Raf^{F20}/GAL\text{-}4^{604}$ wings (Fig. 4E).

rolled. Besides the rough eye phenotype, the rl^{Sem} gain-of-function mutation produces extra vein tissue, which is seen in $rl^{Sem}/+$ wings (Fig. 4F). The $rl^{Sem}/selD^{ptuf}$ combination seemed unable to suppress the phenotype displayed by the gain-of-function (Fig. 4G).

Consistent with the results in the eye, *selD* haploinsufiency suppresses the phenotype of the Elp^{B1} gain-of-function mutation of *DER* and the activated Raf construct. In contrast, no suppression of the rl^{Sem} wing phenotype has been detected.

The Catalase Allele Cat^{n1} Mimics the $selD^{ptuf}$ Mutation Effects on the Ras/MAPK Signalling Pathway

Our current hypothesis is that a reduction in SelD product due to $selD^{ptuf}$ null mutation causes an alteration to the redox balance of the cell, to which the Ras/MAPK signalling pathway is sensitive. To test our hypothesis further, we sought to alter the redox balance by reducing the copy number of another gene whose product is known to participate in its control. Using the same genetic approach out-

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lined above, the *catalase* amorphic allele Cat^{n1} (Mackay and Bewley, 1989; Griswold *et al*., 1993) was used. Indeed, lowering the amount of catalase suppressed the rough eye phenotype of activated Sev and Raf. Both SEM images of $Cat^{n1}/sevS11.5$ and $Raf^{torY9}/+;Cat^{n1}/+$ eyes showed a rescue of the rough eye phenotype to the same extent as $selD^{ptuf}$ (compare Fig. 1C to Fig. 5B and Fig. 1G to Fig. 5D). Furthermore, 17% of $Elp^{B1}/+;Cat^{n1}/+$ wings displayed a complete wild-type phenotype and the rest a partial suppression (data not shown). The activated Ras construct was as well tested against the Cat^{n1} allele for a reduction of rough eye phenotype. Again, as with $selD^{ptuf}$ mutation, the result was no suppression (data not shown). These results show that an independent increase in ROS caused by the *catalase* allele Cat^{n1} has the same effects on the Ras/MAPK signalling pathway as $selD^{ptuf}$ mutation.

Other Pathways Are Not Sensitive to the Change of the Redox Balance Caused by the $selD^{ptuf}$ Mutation

The above results suggest that the Ras/MAPK signalling pathway is able to sense changes in the redox balance of the cell. To test whether this sensitivity to the alteration of the redox potential is specific of the Ras/MAPK cassette or a shared feature with other pathways, we analyzed if some of them could be modulated by *selD*.

In order to test whether $selD^{ptuf}$ would have the same effect on another RTK not directly involved in the activation of the Ras/MAPK cassette, we used the *Drosophila* insulin receptor (InR) of the insulin pathway, shown by several laboratories to regulate growth in *Drosophila* (Bohni *et al*., 1999). Flies with transheterozygous combinations of hypomorphic mutations in *InR* are smaller than wild type (Chen *et al*., 1996). In contrast, overexpression of *InR* by the *GMR-GAL4* driver causes enlarged eyes due to an increase in cell number and cell size. This phenotype was not suppressed in a heterozygous $selD^{ptuf}$ background (Figs. 6B and 6C).

Another element of this pathway is the 1-phosphatidylinositol 3-kinase (Pi3K), which like Raf is also a Ras effector. Although there is ample evidence for the cooperation of Ras-effector pathways in mammalian cells, the genetic characterization of the corresponding pathways in *Drosophila* has not yet provided genetic support for such interactions. Whereas Ras affects cell fate, Pi3K (Dp110) appears to control cell growth during development (for review see Rommel and Hafen, 1998). However, as Pi3K and Raf/MAPK are parallel pathways, and the latter seemingly modulated by *selD*, we attempted to uncover if their possible interaction was based on this modulation. It has been reported that the expression of a constitutively active membrane targeted *Dp110* construct (*Dp110-CAAX*) driven by the GMR-GAL4 insertion line generates enlarged eyes (Leevers *et al*., 1996). Eye SEM images from flies expressing the *Dp110-CAAX* construct in a heterozygous $selD^{ptuf}$ background displayed the same phenotype as the control (data not shown).

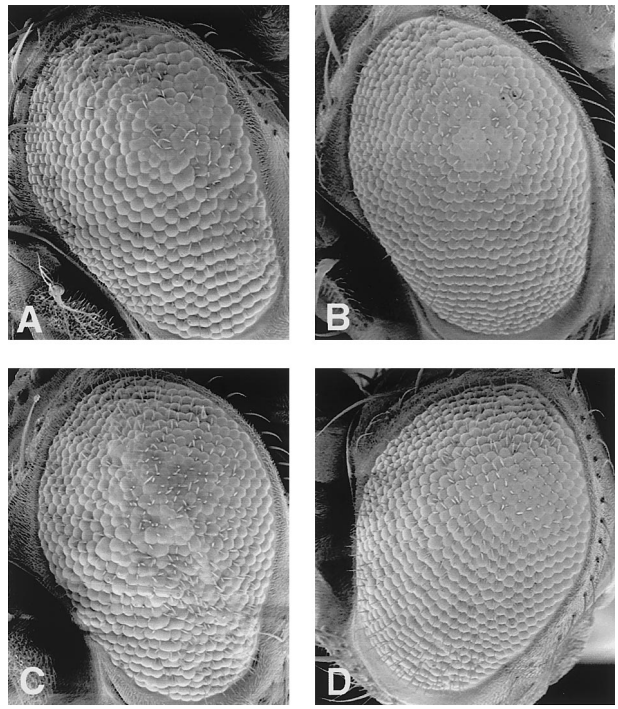

FIG. 5. *Catalase* suppresses the rough eye phenotype caused by the *sev* and *raf* gain-of-function alleles. Scanning electron micrographs are shown as follows: (A) $sevS11.5/+$, (B) $sevS11.5/Cat^{n1}$, (C) $Raf^{torY9}/+$, and (D) $Raf^{torY9}/+;+/Cat^{n1}$. In all pictures, anterior is to the left and dorsal is up. Both $sevS11.5/Cat^{n1}$ (B) and $Raf^{torY9}/+;+/Cat^{n1}$ (D) lack most of the characteristic fused ommatidia seen in their respective controls (A and C).

In addition to the Insulin pathway, we also tested genetic interactions between $selD^{ptuf}$ and activated components of the Wnt (Wg), the Dpp and the Notch pathways. In the case of the Wnt pathway, it has been described that the eyes of *sev-wg* flies appear normal, except that the interommatidial bristles, normally found at alternating vertices in the eye's hexagonal array, are almost completely missing (Cadigan and Nusse, 1996; Brunner *et al*., 1997). Though *sev-wg/+* eyes were completely devoid of interommatidial bristles, we also found that ommatidia along the anterior and ventral posterior margin of the eye had lost their array organization. In $selD^{ptuf}/+;sev\text{-}wg/+$ eyes, we found the same phenotype as in our controls (data not shown). To test the Dpp pathway, a gain-of-function allele of the *Drosophila* type I TGF-β receptor family: *thickveins* (*tkv*) was driven under the control of the $GAL\text{-}4^{604}$ driver. The $UAS\text{-}tkv/GAL\text{-}4^{604}$ wings present extravein tissue surrounding vein L5 severely distorting and thickening it. This phenotype was not reverted in a $selD^{ptuf}$ heterozygous background (Figs. 6D and 6E). To test the Notch pathway the *Notch* gain-of-function allele Ax^{28}, which in hemizygous condi-

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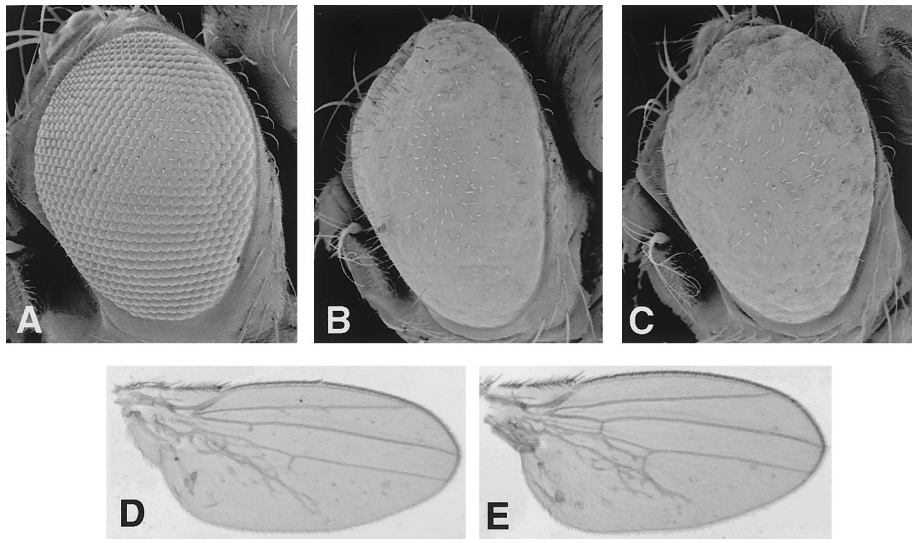

FIG. 6. The Insulin and the Dpp pathways are not sensitive to the alteration of the redox balance caused by the $selD^{ptuf}$ mutation. Genotypes are as follows: (A) wild type, (B) *GMR-GAL4, UAS-InR/+*, (C) *GMR-GAL4, UAS-InR/*$selD^{ptuf}$, (D) *UAS-tkv/GAL-*4^{604}, and (E) +/$selD^{ptuf}$*;UAS-tkv/GAL-*4^{604}. In all scanning electron micrographs, anterior is to the left and dorsal is up. No suppression of the eye and wing phenotype caused by the overexpression of *InR* (compare B with C) and an activated form of *tkv* (compare D with E), respectively, has been observed in the presence of $selD^{ptuf}$.

tions lacks the distal most parts of vein L5 and L4 when cultured at 25°C, was used. $Ax^{28}/Y;selD^{ptuf}/+$ wings showed the same phenotype as the Ax^{28}/Y control ones (data not shown).

In summary, none of the tests performed with elements of other characterized pathways has revealed a role of the *selD* in their modulation.

DISCUSSION

The results presented in this work indicate that the Ras/MAPK signalling cassette is modulated by the redox state of the cell during *Drosophila* development. This is supported by the following evidence: (1) dominant suppression of gain-of-function phenotypes of several elements of the Ras/MAPK signalling payhway by $selD^{ptuf}$ mutation in heterozygosis in both the eye and wing; (2) reversion of $selD^{ptuf}$ suppression by the expression of a *selD* transgene; (3) suppression by the *catalase* amorphic allele Cat^{n1} which is equivalent to a $selD^{ptuf}$, but selenoprotein independent, alteration of the redox balance; and (4) nonreversion of overexpression or gain-of-function phenotypes of elements of other pathways, such as Wnt, Dpp, Notch and InR/Pi3K, suggesting a highly specific suppression on the Ras/MAPK signalling pathway by $selD^{ptuf}$.

The most striking result is that *selD* function modulates the activity of Sev and DER RTKs, in the eye and wing respectively**,** suppressing in transheterozygous combination the phenotype caused by the gain-of-function mutations of these RTKs. In contrast, InR, another well-known RTK belonging to the insulin pathway, shows no modulation by $selD^{ptuf}$ mutation neither at the InR RTK level nor at the level of the Pi3K. Therefore, *selD* somehow modulates RTKs involved in the activation of the Ras/MAPK cassette but not other RTKs. The suppression of the Raf gain-of-function phenotype has been detected both in the eye and wing. A mild but statistically significant suppression of the rl^{Sem} phenotype by $selD^{ptuf}$ has been found in the eye but not in the wing. This is probably due to the different sensitivity of both systems. The mild suppression of rl^{Sem} phenotype could be due to the fact that the MAPK is the last step of the pathway prior to the transcription of target genes. At this point of the pathway, it may be difficult to block or dilute the amplified signal triggered by the rl^{Sem} gain-of-function mutation.

Surprisingly, no modulation of *ras* by $selD^{ptuf}$ mutation has been detected. There are two main explanations for this result: (1) *ras* is not modulated by *selD* at all. The Sev, DER, Raf and MAPK molecules, which are modulated by an alteration of the redox balance, either by $selD^{ptuf}$ or Cat^{n1} mutations, are kinases, whereas Ras is a GTPase. It is possible that GPTases are not sensitive to changes in the redox balance. This is supported by the fact that *rasV12* rough eye phenotype was not suppressed neither by $selD^{ptuf}$ or Cat^{n1} mutations. Similarly, the Rho1 Small-GTPase rough eye phenotype was not suppressed by $selD^{ptuf}$ either; (2) *ras* is actually modulated by *selD* but beyond our detection level, and therefore, no supression can be scored. It could also be that this modulation is towards an enhance-

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ment. It has been reported that 3T3 fibroblasts stably transformed with *rasV12*, produce large amounts of ROS superoxide (O_2-) by the activation of the NADPH oxidase enzyme, which is triggered by the Rac signalling pathway (Irani *et al*., 1997). There is evidence that in certain cells MAPK activation in response to growth factors is dependent on the production of ROS (Lo and Cruz, 1995; Sundaresan *et al*., 1995). Reduction of 50% of *selD* dose might be not enough to alter the initial *ras* rough eye phenotype neither to detect a suppression nor an enhancement. Besides, we have to bear in mind that Ras is a crosstalk point for other signalling pathways (such as Pi3K) and that the Raf/MAPK pathway is also activated in a Ras independent way (Hue *et al*., 1995).

With the exception of *ras*, we have observed a gradient in the strength of the suppression of phenotype from *sev* being the strongest to rl^{Sem} the weakest (Fig. 2, comment in legend). This could be attributed to the differential sensitivity of the particular gain-of-function alleles or to the position in the pathway of the triggering gain-of-function element.

Taking together the eye and wing results, it appears that the *selD* modulation of the Ras/MAPK pathway is independent of tissue and RTK through which this highly conserved signalling cassette is activated. Moreover, the genetic approach used in our experiments has not uncovered any modulation of other signalling pathways by *selD*. Though we can not discard that these modulations may exist, the fact that modulation of the Ras/MAPK pathway has been detected indicates that this pathway is sensitive to the change in the redox balance caused by one-dose reduction of *selD*.

We have previously shown that homozygous $selD^{ptuf}$ mutants lack selenoproteins and accumulate free radicals (Alsina *et al*., 1999). Heterozygous $selD^{ptuf}$ individuals show no sign of impaired Ras/MAPK signalling due to the loss of one dose of *selD*. However, when using activated elements of the Ras/MAPK pathway, the effects of the lack of one dose of *selD* are evident. Therefore, it is tempting to speculate that $selD^{ptuf}$ in heterozygosis results in an accumulation of ROS due to lower activity of the selenoproteins biosynthesis. This accumulation would be sufficient to impair the Ras/MAPK signalling, suppressing the gain-of-function phenotypes of elements of the pathway. However, our findings are in contrast with the results obtained in tissue culture experiments. It has been observed that ligand stimulation with peptide growth factors acting through RTKs results in an increase in intracellular ROS (Krieger-Brauer and Kather, 1992; Sundaresan *et al*., 1995; Bae *et al*., 1997). Moreover, ligand-stimulated ROS generation appears to have a role mediating tyrosine phosphorylation. In addition to that, it has also been shown that extracellular administration of non-lethal concentrations of H_2O_2 activates MAPK (Stevenson *et al*., 1994; Guyton *et al*., 1996; Kamata *et al*., 2000). Altogether, these results in tissue culture systems point to activation of the pathway by ROS. In our case, elimination or reduction of selenoprotein function could result in prolonged activity of the signalling pathway (due to the ROS increase caused by $selD^{ptuf}$) and consequently induce apoptosis. Therefore, one could conclude that the suppression observed in our experiments could be due to cell death of the extra R7s. Loss of any cell of the *sev* equivalence group by apoptosis will result in rough eye phenotype. However, we have instead demonstrated a rescue of the normal ommatidium organisation, which can only be achieved if the number of cells of the ommatidium is not altered. For this reason, we think that apoptosis does not explain the strong suppression observed in our experiments. Rather, our results point to a downregulation of the pathway. The discrepancies found between cell culture and *Drosophila* as a whole organism (i.e., activation versus downregulation of the pathway by ROS respectively) may be due to the inherent differences among the two systems used. A more important difference, however, is that experiments performed in tissue culture study the effect of transient low concentration increases of ROS whereas in our system we are dealing with a gene-dosage dependent constant increase in ROS. To reconcile these discrepancies, we propose that transient increases in ROS could have a physiological function on activation of phosphorylation and thus triggering the Ras/MAPK signalling pathway, whereas a constitutive pathological change in redox potential could activate a defense mechanism blocking the pathway.

We have been using a genetic approach to address a problem that has mainly been tackled biochemically. The heterozygous combinations used in our experiments are viable; therefore, increases in ROS would be very subtle and difficult to detect using conventional methods. What we actually present here is a biological response due to the loss of one dose of either *selD* or *catalase*. Although we did not measure changes in ROS, the read out of our experiments strongly supports that there must be differences in ROS accumulation betweeen the heterozygous and the wild type.

The converse experiments to overexpress SelD and study its effects when in combination with loss-of-function mutations of members of the Ras/MAPK pathway are not feasible due to the nature of SelD. SelD is an enzyme of the biosynthesis pathway of selenocysteine. Overexpressing one component of the pathway does not imply an increase in selenocysteine as other enzymes in the pathway may be limitant; therefore, overexpression of SelD does not imply an increase in selenoproteins. In fact, overexpression experiments done in our laboratory using different GAL-4 drivers gave no visible phenotype.

This is the first example of the role of intracelular redox environment on the Ras/MAPK signalling pathway in a whole organism. The high specificity of our results (i.e., no interaction with other signalling pathways and results confirmed in two different developing tissues: wing and eye) gives strong support to the notion that signalling through the Ras/MAPK pathway is modulated by ROS. Our current hypothesis is that selenoproteins, through an un-

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disclosed subset of ROS, modulate the Ras/MAPK signalling pathway. This is consistent with the finding that this pathway is also modulated by *catalase*. A selenoprotein-independent increase of a subset of ROS (i.e., H_2O_2) is able to modulate this pathway as well. This observation favors a scenario in which selenoprotein modulation of the Ras/MAPK pathway would be achieved by their control of the redox balance rather than one in which selenoproteins would exert their role directly interacting with one or more elements of this signalling cassette. These results may help to shed light on the role of redox on signalling events under physiological conditions in multicellular organisms.

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