

A Misexpression Screen Identifies Genes That Can Modulate RAS1 Pathway Signaling in *Drosophila melanogaster*

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ABSTRACT

Differentiation of the R7 photoreceptor cell is dependent on the Sevenless receptor tyrosine kinase, which activates the RAS1/mitogen-activated protein kinase signaling cascade. Kinase suppressor of Ras (KSR) functions genetically downstream of RAS1 in this signal transduction cascade. Expression of dominant-negative KSR (KDN) in the developing eye blocks RAS pathway signaling, prevents R7 cell differentiation, and causes a rough eye phenotype. To identify genes that modulate RAS signaling, we screened for genes that alter RAS1/KSR signaling efficiency when misexpressed. In this screen, we recovered three known genes, *Lk6*, *misshapen*, and *Akap200*. We also identified seven previously undescribed genes; one encodes a novel rel domain member of the NFAT family, and six encode novel proteins. These genes may represent new components of the RAS pathway or components of other signaling pathways that can modulate signaling by RAS. We discuss the utility of gain-of-function screens in identifying new components of signaling pathways in *Drosophila*.

MULTICELLULAR organisms must coordinate growth and differentiation of many different cell types throughout the course of development. To do this, individual cells must be able to recognize environmental cues, integrate multiple signals, and produce the appropriate developmental response. Cells utilize several categories of signaling molecules: transmembrane receptors that recognize extracellular cues, intracellular proteins that relay and amplify the signals, and effector molecules that convert the signals to a developmental output. The molecular mechanisms responsible for regulating these signaling events appear to have been used repeatedly in different contexts throughout all developmental stages.

The RAS1/mitogen-activated protein kinase (MAPK) pathway is one of the many evolutionarily conserved signaling modules. This pathway can respond to a number of extracellular signals including growth factors, stress, and hormones. The signaling relay consists of three protein kinases, RAF, MAPK kinase (MEK), and MAPK, which together act downstream of the small GTP-binding protein, RAS1. Signals are transduced via a protein phosphorylation cascade leading to the activation of MAPK, which then activates both cytoplasmic and nuclear targets (reviewed in SEGER and KREBS 1995). Although the molecules involved in this signaling

cascade have been studied extensively, their interaction with other cellular targets and those effects on the course of development are not as clearly understood.

Numerous genetic screens have been conducted in *Drosophila* to identify novel components of the RAS1 pathway (SIMON *et al.* 1991; DICKSON *et al.* 1996; KARIM *et al.* 1996; NEUFELD *et al.* 1998; THERRIEN *et al.* 1998; REBAY *et al.* 2000, and accompanying article, THERRIEN *et al.* 2000, this issue). These screens were conducted in the adult eye because of the advantages it confers: it is not necessary for viability or fertility, and it is an easy tissue to screen. The eye is composed of a repeating array of ~800 ommatidial clusters, each of which comprises ~20 cells including 8 neuronal photoreceptor cells designated R1–R8. The eye arises from a monolayer of undifferentiated cells, the eye imaginal disc, and develops due to a concerted series of cell-cell interactions that utilize many intercellular signaling pathways.

One example is the specification of the presumptive R7 cell by the R8 photoreceptor cell (ZIPURSKY and RUBIN 1994). Proper differentiation of the R7 cell depends on local activation of the Sevenless receptor tyrosine kinase (RTK) by its ligand, BOSS, which is expressed on the adjacent R8 cell. The Sevenless RTK signals through the RAS1/MAPK pathway to activate downstream effectors required for specification of a neuronal cell fate. Genetic screens have identified many essential components of the RAS1/MAPK signaling cascade involved in R7 cell fate specification [SIMON *et al.* 1991; DICKSON *et al.* 1996; KARIM *et al.* 1996; NEUFELD *et al.* 1998; THERRIEN *et al.* 1998, 2000 (this issue); REBAY

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et al. 2000]. *Ras1*, *sos*, *gap1*, *drk*, *Dsor1*, *ksr*, *PTP-ER*, *cnk*, *yan*, and *sina* are all genes that were identified as essential downstream components of the Sevenless RTK pathway. The screens that led to the identification of these genes were all dominant modifier mutagenesis screens where loss-of-function mutations were identified by their ability to modify a RAS pathway-dependent rough eye phenotype.

Only one-quarter to one-third of all genes in the *Drosophila* genome can be mutated to an easily scored phenotype (MIKLOS and RUBIN 1996; ASHBURNER *et al.* 1999). For many genes, functional redundancy with related genes may prevent loss-of-function phenotypes from revealing gene function. Thus, classic loss-of-function genetic screens may miss many important components of regulatory pathways. Gain-of-function genetics have been widely used in multicopy screens in yeast (RINE *et al.* 1983; BENDER and PRINGLE 1989; RAMER *et al.* 1992), but have only recently come to fruition in *Drosophila* with the development of critical resources such as the EP and Gene Search collections (RORTH 1996; RORTH *et al.* 1998; TOBA *et al.* 1999). These are independent *P*-element lines where the *P* element contains a GAL4-inducible promoter that, in the presence of a tissue-specific source of GAL4, drives expression of the endogenous gene located proximal to the *P* element. Increasing gene dosage through overexpression may reveal gene functions that are not revealed through loss-of-function mutations.

To test the feasibility of using this gain-of-function misexpression approach as a means to identify nonessential genes that act in a signaling pathway, we took advantage of a RAS pathway-dependent rough eye phenotype caused by the expression of a dominant negative form of kinase suppressor of Ras (KSR). Many mutations in components of the RAS pathway can generate a rough eye; however, we chose to use this *ksr* phenotype because it is dosage sensitive and viable (see below). *ksr* was first identified in genetic screens in both *Drosophila* and *Caenorhabditis elegans* (KORNFELD *et al.* 1995; SUNDARAM and HAN 1995; THERRIEN *et al.* 1995). Genetic epistasis tests place it downstream of *Ras1* and either upstream of or parallel to *raf* (THERRIEN *et al.* 1995). KSR facilitates signaling between RAF, MEK, and MAPK (THERRIEN *et al.* 1996). In quiescent cells, KSR is bound to MEK in the cytoplasm (DENOUEL-GALY *et al.* 1998; YU *et al.* 1998). Upon RAS1 activation, KSR translocates to the membrane where it is found in a complex with RAF (THERRIEN *et al.* 1998). Although *ksr* encodes a putative protein kinase, substrates have not been identified. A genetic screen for dominant modifiers of a *ksr*-dependent phenotype identified genes known to play a role in RAS1/MAPK signaling as interactors of KSR as well as several new loci including a putative adaptor protein connector enhancer of KSR (CNK) [THERRIEN *et al.* 1998, 2000 (this issue)].

The KSR kinase domain functions as a dominant-nega-

tive molecule when separated from the noncatalytic N-terminal domain (THERRIEN *et al.* 1996). Overexpression of a KSR dominant-negative (KDN) protein in a subset of cells of the developing eye under the control of the *sevenless* enhancer/heat shock promoter (*sE-KDN*) blocks RAS1-dependent photoreceptor cell differentiation. In *sE-KDN* ommatidia, the R7 photoreceptor cell and one or two of the other photoreceptor cells are missing. Thus most ommatidia have five to seven photoreceptor cells instead of the normal eight, resulting in a visible rough eye phenotype, which is clearly distinguishable under a dissecting microscope [THERRIEN *et al.* 1996, 1998, 2000 (this issue)]. The rough eye phenotype generated in this manner is sensitive to the copy number of transgenes; that is, flies carrying two copies of the transgene have more severely disrupted eyes than flies carrying only one copy. The dose sensitivity of this phenotype has provided a good background for a genetic screen designed to identify loss-of-function mutations that dominantly modify this phenotype by disruption of one of two copies of the wild-type gene [THERRIEN *et al.* 1998, 2000 (this issue)].

To identify new genes that can alter signaling through the RAS1 pathway in *Drosophila*, we present here the use of a misexpression screen to identify modifiers of a rough eye phenotype due to dominant-negative KSR. We screened the EP collection and identified several known signaling molecules as well as six novel loci that act as misexpression modifiers of this phenotype.

MATERIALS AND METHODS

EP screen: An isogenic driver stock homozygous for both the *sevenless* enhancer/heat shock promoter-GAL4 (*sE-GAL4*) and *sE-KDN* [THERRIEN *et al.* 1998, 2000 (this issue)] *P*-element insertions was first generated. Female *sE-KDN*; *sE-GAL4* flies were mated to male flies from each individual line from the EP collection (RORTH *et al.* 1998). F₁ progeny were scored under a dissecting scope for suppression or enhancement of the KDN rough eye phenotype. Potential modifiers were retested with a different isolate of the driver stock. Males from modifying lines were then crossed to *CyO*, *P[sev-RAS1^{V12}]/Sco*; *sE-GAL4* females and curly winged F₁ progeny were again scored for enhancement or suppression of the *sev-RAS1^{V12}* rough eye phenotype. Fly stocks were maintained at 25° on standard corn meal/molasses media.

The degree of enhancement or suppression by the misexpression lines was variable. For example, Figure 1D shows a strong suppressor of *sE-KDN*, yet the enhancement of *sev-RAS1^{V12}* in Figure 1E is mild. Figure 1F shows a weak enhancement of the *sE-KDN* phenotype, yet this line turns out to be a very strong suppressor of *sev-RAS1^{V12}*, as seen in Figure 1G. This was not due to a difference in genetic background of the driver lines for two reasons: (1) the driver lines were outcrossed and isogenized prior to screening, and (2) the strong interactions were seen with both driver lines. This eliminates the possibility that one line generally facilitated stronger interactions than the other.

Transgenic flies: cDNAs were cloned into the pUAST (BRAND and PERRIMON 1993) vector and constructs were introduced into the germline by injection in the presence of trans-

posase as previously described (RUBIN and SPRADLING 1982). *UAS-msn* flies were kindly provided by Jessica Treisman (SU *et al.* 1998).

Overexpression analysis: *dpp-GAL4* and *Act5C-GAL4* flies were obtained from the Bloomington Stock Center, and *vg-GAL4* flies were generated by Mike Brodsky.

Histology: Adult fly eyes were prepared for thin section analysis by fixing in 2% glutaraldehyde and 2% osmium tetroxide followed by dehydration through an ethanol series as described previously (TOMLINSON *et al.* 1987). Flies were prepared for scanning electron microscopy by dehydration through an ethanol series followed by a freon series.

Sequence analysis: DNA sequence tags flanking the *P*-element insertions were generated by the Berkeley Drosophila Genome Project (BDGP; <http://www.fruitfly.org/>) and used to BLAST search both the NCBI and BDGP databases to identify nearby genes. Expressed sequence tag (EST) clones identified by these means were sized by gel electrophoresis and the longest clones were then completely sequenced using the primer island transposon method (Applied Biosystems, Foster City, CA). Additional flanking sequence was obtained off of the other end of the *P* element by inverse PCR and, in some cases, plasmid rescue as described (HUANG *et al.* 2000). Full-length cDNA sequences are available in GenBank.

RESULTS

A misexpression screen for modifiers of KDN: The screen previously carried out was designed to isolate dominant modifiers of the *sE-KDN* phenotype [THERRIEN *et al.* 1998, 2000 (this issue)]. This was achieved by mutagenizing flies and identifying genes that, when mutated in one copy, could dominantly enhance or suppress the *sE-KDN*-dependent rough eye. These types of screens have been extremely successful and efficient in genetically dissecting signaling pathways (DICKSON *et al.* 1996; KARIM *et al.* 1996). These screens depend on a twofold difference in gene dosage to result in enhancement or suppression of a rough eye phenotype. However, since more than half of all genes in the genome do not mutate to any visible phenotype, it is probable that these previous screens have not identified all relevant interacting genes. In an effort to identify novel pathway interacting genes as well as test a new approach, we undertook a misexpression screen. The expectation is the same, that a change in dosage of an interacting molecule will affect the dosage-sensitive *sE-KDN* phenotype. In this study, we screened for genes that modify a rough eye phenotype when gene expression is increased rather than decreased.

The GAL4-UAS system from yeast has been used extensively to force ectopic expression of genes in flies (BRAND and PERRIMON 1993; BRAND *et al.* 1994). The EP collection (RORTH *et al.* 1998) is a collection of ~2300 independent *P*-element insertion lines. The EP *P* element contains a GAL4-inducible promoter at the 3' end of the transgene to drive expression of the closest downstream endogenous gene when combined with a transgene expressing GAL4 under the control of a tissue-specific promoter. In this screen we used a *sE-GAL4* line that expresses the yeast GAL4 activator in a subset

of cell types in the developing eye (including photoreceptor cells R3, R4, R7, and cone cells). By itself, the *sE-GAL4* transgene has no effect on normal eye development (Figure 1A). The *sE-KDN* transgene expresses the dominant-negative form of KSR in the same cell types (R3, R4, R7, and cone cells), which causes a loss of photoreceptor cells and leads to a roughened and disorganized eye (Figure 1B).

Since KDN blocks signaling efficiency through the RAS1/MAPK pathway, overexpression of genes that increase the overall output of the pathway will suppress the rough eye phenotype. Conversely, overexpression of genes that further reduce the signaling output will worsen the eye phenotype. Flies containing both the *sE-KDN* and *sE-GAL4* transgenes were crossed to 2254 individual lines from the EP collection and F₁ progeny from each cross were screened for alterations of the KDN-dependent rough eye phenotype due to the misexpression of genes downstream of the EP element. A total of 140 EP misexpression lines that suppress or enhance the rough eye phenotype were identified from the collection (Figure 1, D and F). Seventy-five of these lines generate a rough eye when crossed to the *sE-GAL4* driver alone in a wild-type background (A. M. BAILEY and G. M. RUBIN, unpublished results); these lines were not pursued any further because the enhancement of eye roughness likely reflects an additive effect of two unrelated phenotypes. Of the remaining 65, all exhibited wild-type eye morphology when misexpressed with *sE-GAL4* in a wild-type background. These were further characterized as potential genetic interactors.

Tests to identify pathway relevant lines: Although these lines showed no external phenotype when overexpressed in a wild-type background, there may be subtle phenotypes that were amplified by the presence of the *sE-KDN* transgene. Since it was already known that misexpression of 4% of the lines in this collection could generate rough eyes (A. M. BAILEY and G. M. RUBIN, unpublished results; RORTH 1996), we used additional criteria to determine which lines affected RAS1-dependent photoreceptor cell development. We examined the misexpression interactions in an activated RAS1 background as well as the effect on the R7 cell fate in particular. Examples of the RAS1 interaction are shown in Figure 1 and the results are summarized in Table 1.

Interaction with activated RAS1: The initial screen was performed in a background where RAS1 signaling is decreased due to the dominant-negative effect of the *sE-KDN* transgene resulting in fewer R7 and outer photoreceptor cells. If the RAS pathway is constitutively active in the R3, R4, R7, and cone cells, increased signaling activity results in the development of extra R7 photoreceptor cells. The *RAS1^{V12}* cDNA encodes a protein with a glycine-to-valine substitution at position 12. This mutation prevents the hydrolysis and release of the bound GTP, which maintains RAS1 in an active form (reviewed in BOLLAG and MCCORMICK 1991). Overexpression of

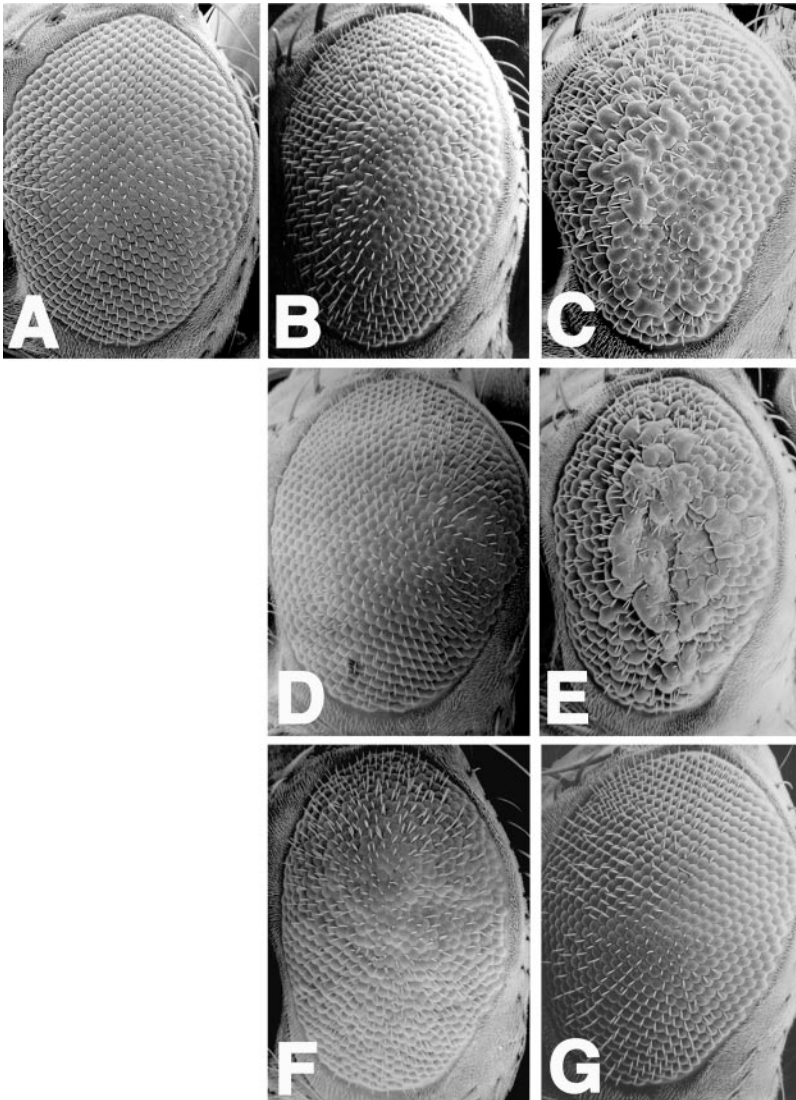


FIGURE 1.—Identification of misexpression enhancers and suppressors of *KDN*. Scanning electron micrographs of adult eyes of the following genotypes are shown: (A) $P[sE-GAL4]/+$; (B) $P[sE-KDN]/+$; (C) $P[sev-RASI^{V12}]/+$; (D) $P[sE-KDN]/P[EP(2)2347]; P[sE-GAL4]/+$; (E) $P[sev-RASI^{V12}]/P[EP(2)2347]; P[sE-GAL4]/+$; (F) $P[sE-KDN]/P[EP(2)2221]; P[sE-GAL4]/+$; and (G) $P[sev-RASI^{V12}]/P[EP(2)2347]; P[sE-GAL4]/+$. RAS pathway specific misexpression enhancers of *KDN* both make the *sE-KDN* eye rougher (F) as well as suppress the roughness of the *sev-RASI^{V12}* eye (G), whereas suppressors of *KDN* cause the *sE-KDN* eye to look more like wild type (D) yet cause the *sev-RASI^{V12}* phenotype to worsen (E).

this cDNA in the eye under the control of the *sevenless* enhancer/promoter (*sev-RASI^{V12}*) generates a dosage-sensitive rough eye phenotype (Figure 1C; FORTINI *et al.* 1992). The 65 putative interacting lines were crossed to a line expressing both the *sev-RASI^{V12}* and *sE-GAL4* transgenes. F₁ progeny were screened for enhancement or suppression of the *sev-RASI^{V12}* phenotype (Figure 1, E and G). The *KDN* and *RASI^{V12}* transgenes produce their respective rough eye phenotypes by affecting photoreceptor development in opposite directions and therefore should respond oppositely to overexpression of a given interacting gene. Misexpression of genes that specifically increase RAS pathway signaling by acting downstream of *RASI* should suppress the *sE-KDN* phenotype but enhance the *RASI^{V12}* phenotype. Conversely, misexpression of lines that decrease RAS pathway signaling by acting downstream of both *ksr* and *RASI* should enhance the *KDN* phenotype and suppress the *RASI^{V12}* phenotype. In contrast, enhancers that roughen the eye by the addition of two unrelated phenotypes should

enhance both lines. Of the 65 lines, 19 displayed the opposite interactions when misexpressed in the presence of the *sE-KDN* compared to the *sev-RASI^{V12}* transgene. The remaining 44 lines either had no visible effect on the *sev-RASI^{V12}* rough eye or exhibited the same phenotypic modification as with the *sE-KDN* transgene and were not pursued further.

The R7 photoreceptor cell fate: The RAS1/MAPK signaling cassette is involved in the cell fate specification of all eight neuronal photoreceptor cells as well as some of the nonneuronal cell types in the eye. However, this module is not always activated in a Sevenless RTK-dependent manner, which only occurs in the R7 photoreceptor. To quantify the degree of modification by each misexpression line, we focused on the R7 cell fate. We chose the R7 cell for two reasons: R7 appears most sensitive to changes in RAS1 signaling, and, whereas a rough eye may reflect changes in fates of many cell types, the number of R7 cells appears to directly reflect a change in the strength of RAS1 signaling.

TABLE 1
Summary of *sE-KDN* and *sev-RAS1^{V12}* interacting lines

Gene name, line no.	cDNA clone (GenBank accession no.)	Predicted protein size (amino acids)	Map position	KDN	Ras ^{V12}	No. of lines
<i>MESK1</i> , EP(2)0980	N/A	N/A	33B3–4	S+	E+	1
<i>MESK2</i> , EP(2)2347	LD23639 (AF195792)	486	57E6–7	S+++	E+	1
<i>MESK3</i> , EP(3)3728	N/A	N/A	77A4–B1	S+	E+	1
<i>MESK4</i> , EP(3)1015	LD15753 (AF195495)	281	89E5–7	S++	E+	1
<i>MESR1</i> ; Dm novel rel, EP(X)1353	LD28881 (AF195496)	1211	12A8–10	E++	S+	1
<i>MESR2</i> ; <i>DAKAP200</i> , EP(2)2254	LD27016	754	29C1–2	E+	S+	1
<i>MESR3</i> , EP(2)2221	LD08471 (AF195497)	281	36F6–7	E+	S+++	1
<i>MESR4</i> , EP(2)0386	LP07188 (AF195494)	1107	54C7–8	E++	S+	1
<i>MESR5</i> ; misshapen, EP(3)0609	LD34191	1658	62E5–7	E++	S+	1
<i>MESR6</i> , EP(3)3142	LD16107 (AF195499)	398	75F6–7	E+++	S+	1
<i>MESR7</i> , EP(3)3403	LD11488 (AF195498)	1492	78C1–2	E++	S+	1
<i>MESR8</i> ; <i>LK6</i> , EP(3)0886, EP(3)3094	LD17668	1143	86F6–7	E+++	S++	2

Genes isolated in this screen are listed along with the strength of interaction, map position of the *P* element, cDNA clone sequenced, GenBank accession number, and size of predicted protein. *MESK1* and *MESK3* have not been identified to date.

The neuronal photoreceptor cells of the eye are organized in a regular, repeated trapezoidal pattern as seen in apical cross section (Figure 2A). The six outer photoreceptor cells (R1–R6) outline the trapezoid and the R7 and R8 cells lie in the center with the R7 cell situated apical to the R8 cell (which is not visible in these sections; Figure 2). In *sE-KDN* animals, ~80% of ommatidia are missing R7 cells (Figure 2B; THERRIEN *et al.* 1996).

Suppressors of this phenotype should restore the R7 cell fate. However, this change in number of R7 cells did not provide a good means by which to quantitate an enhancement of the *KDN* phenotype. Instead, we took advantage of the previous requirement that misexpression enhancers of *sE-KDN* also act as suppressors of *sev-RAS1^{V12}*. In the *sev-RAS1^{V12}* background, 90% of ommatidia have supernumerary R7 cells (Figure 2C).

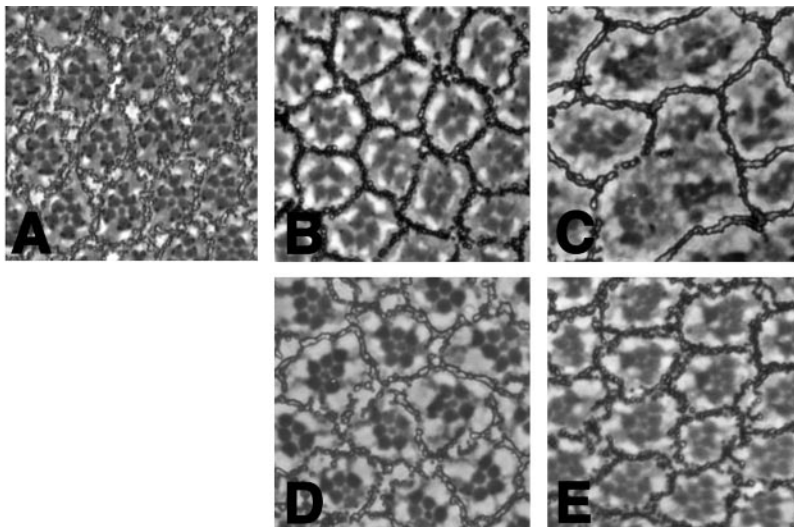


FIGURE 2.—Eye sections reveal suppression of R7 photoreceptor phenotype. Apical sections through eyes of the following genotypes are shown: (A) *P[sE-GAL4]/+*; (B) *P[sE-KDN]/+*; (C) *P[sev-RAS1^{V12}]/+*; (D) *P[sE-KDN]/+*; *P[sE-GAL4]/P[EP(3)1015]*; and (E) *P[sev-RAS1^{V12}]/P[EP(2)2221]; P[sE-GAL4]/+*. A suppressor of *sE-KDN* restores the R7 cell fate in most ommatidia (D); however, the suppression does not rescue the polarity defect. A suppressor of *sev-RAS1^{V12}* reduces the excess number of R7 cells to one per ommatidium (E).

There is also occasional loss of the outer photoreceptor cell types as well [KARIM *et al.* 1996; THERRIEN *et al.* 1998, 2000 (this issue)]. Suppressors of this phenotype restore the number of R7 cells to fewer than two per ommatidium in >10% of the ommatidia examined in each eye.

Misexpression lines that appeared to suppress the *sE-KDN* rough eye were crossed to the *sE-KDN*- and *sE-GAL4*-expressing line. F₁ progeny were fixed, the eyes sectioned, and ommatidia examined for presence of R7 cells. Of the six potential suppressor lines, only four restored the R7 cell fate and to varying degrees (Figure 2D and data not shown). The other two lines were assumed to affect other cell types in the eye and therefore possibly to act independently of the SEV/RAS1/MAPK pathway. The 13 misexpression lines that enhance *sE-KDN* and suppress *sev-RASI^{V12}* were examined for number of R7 cells. Of these 13 lines, 9 suppress the supernumerary R7 phenotype in the *sev-RASI^{V12}* background (Figure 2E and data not shown) and the other 4 do not show a significant change in R7 cell number over background. Thus, 13 of 19 lines that modified the rough eye phenotype also affected R7 cell fate specification. In the remaining 6 lines, the modification of eye roughness may reflect a change in the development of nonneuronal cell types in the eye. Having more carefully examined the effect of misexpressing these 19 lines in two different genetic backgrounds, we concluded that 13 show a clear effect on the R7 cell fate and therefore likely interact with the RAS1 signaling pathway when overexpressed.

Identification of misexpressed genes: All interacting EP lines were mapped cytologically; comparison of map positions and flanking sequences (see below; LIAO *et al.* 2000) with the genomic sequence revealed that the 13 interacting lines correspond to 12 independent genes (Table 1). The 4 genes that suppress the *sE-KDN* and enhance the *sev-RASI^{V12}* phenotypes are designated *misexpression suppressors of KDN* (*MESK 1–4*) and the 8 genes that enhance the *sE-KDN* and suppress the *sev-RASI^{V12}* phenotypes are designated *misexpression suppressors of RASI^{V12}* (*MESR 1–8*). This screen took advantage of *P*-element insertions in close proximity to the genes of interest. To identify the genes that were being misexpressed, genomic DNA sequence flanking the 13 *P* elements was obtained by inverse PCR and plasmid rescue (see MATERIALS AND METHODS) and used to search for similar sequences in the NCBI and BDGP databases. Database searches identified 3 previously described *Drosophila* genes and ESTs corresponding to 6 of the lines. In these six cases where only ESTs were identified, all EST clones corresponding to that particular locus were sized and the longest cDNA was sequenced completely. Flanking genomic sequence from 2 of the lines did not identify either known genes or ESTs and are not described any further in this report. One line, EP(3)3728, maps to cytological position 77A4–B1 and

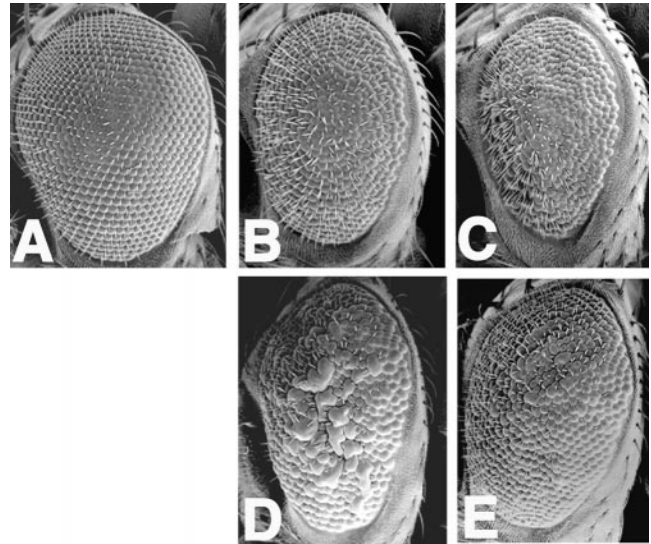


FIGURE 3.—Verification that EP(3)0886 is overexpressing the *Lk6* gene. Scanning electron micrographs of adult eyes of the following genotypes are shown: (A) *P[EP(3)0886]/P[sE-GAL4]*; (B) *P[sE-KDN]/+*; *P[sE-GAL4]/P[EP(3)0886]*; (C) *P[sE-KDN]/+*; *P[sE-GAL4]/P[UAS-Lk6]*; (D) *P[sev-RASI^{V12}]/+*; *P[sE-GAL4]/+*; and (E) *P[sev-RASI^{V12}]/+*; *P[sE-GAL4]/P[UAS-Lk6]*. Misexpression of the original *P*-element line in a wild-type background has no effect on normal eye development (A); however, it increases the severity of roughness of the *sE-KDN* rough eye phenotype (B). Misexpressing the *Lk6* cDNA shows a stronger enhancement than the original EP line on the *sE-KDN* rough eye (C) and somewhat suppresses the *sev-RASI^{V12}* rough eye (D and E).

is located ~300 nucleotides upstream of predicted gene CG13813. This 1.3-kb predicted mRNA contains one InterPro motif (PS00430), which is characteristic of the *Escherichia coli* tonB protein involved in shuttling substrates from the outer bacterial membrane to periplasmic space. The other line, EP(2)0980, maps to cytological position 33B3–4. There are no ESTs or predicted genes in the region of this *P*-element insertion. Further work is required to identify the genes misexpressed by these two insertion lines. The results are summarized in Table 1.

Verification of overexpressed genes: The open reading frame located directly downstream of the UAS promoter-containing *P* element is most likely the target gene expressed. To confirm that the cDNAs isolated corresponded to the misexpressed gene that modifies RAS1 signaling, these cDNAs were directly tested for their ability to modify the eye phenotypes used in the initial screen. cDNAs for 9 of these genes were individually cloned downstream of a *GAL4*-inducible promoter. Two independent transgenic lines expressing each cDNA were crossed to the *sE-KDN*; *sE-GAL4* and *sev-RASI^{V12}*; *sE-GAL4* lines (with the exception of *misshapen*, for which only one line was tested). All nine cDNAs gave the same phenotypic modification as the corresponding original EP lines. An example is shown in Figure 3. The

strength of the interaction often varied from the original EP line; this difference can be explained most easily by a difference in expression levels (Figure 3, B and C). Of the 10 genes identified in this screen, 3 were previously characterized based on other functions: *Lk6* (KIDD and RAFF 1997; TREISMAN *et al.* 1997; LI *et al.* 1999), *misshapen* (*msn*), and *Drosophila A kinase anchor protein 200* (*Akap200*). Six genes are entirely novel in sequence and extensive database searches and sequence comparisons have not revealed any defined protein domains and/or motifs or homologs in other species. These novel genes do not map to any loci from previous RAS1 screens performed in the eye [DICKSON *et al.* 1996; KARIM *et al.* 1996; NEUFELD *et al.* 1998; THERRIEN *et al.* 1998, 2000 (this issue); REBAY *et al.* 2000]. Twelve of the 13 EP lines identified in this screen are homozygous viable and show no adult phenotypes. Only the EP insertion in the *msn* gene is recessive lethal and *msn* was previously determined to be an essential gene (TREISMAN *et al.* 1997). Other *P*-element alleles may exist for 3 of the 6 novel genes isolated. The GeneScene map viewer (<http://www.fruitfly.org>) identified the following *P*-element lines to map within 5 kb of the respective locus for each of the following genes: *MESK2* [1(2)k10317, 1(2)01862], *MESK4* [1(3)06713], and *MESR4* [1(2)k15815]. It is unknown whether these lethal *P*-element insertions affect the same genes as the EP insertions.

Overexpression phenotype analysis: The UAS-cDNA transgenic lines were also crossed to other tissue-specific sources of GAL4 to determine if misexpression of these genes affected the development of other tissue types. GAL4 drivers used included *dpp-GAL4* for expression in wing, leg, eye, and antenna; *vestigial-GAL4* for expression in the wing; and *actin-GAL4* for ubiquitous overexpression. Except for the examples mentioned below, misexpression of these cDNAs had no apparent effect on normal development.

When overexpressed under the control of *Act5C-GAL4*, the *MESR1* and *MESR6* crosses gave rise to 10 and 50% unclosed F₁ pupae, respectively. The adults that eclosed appeared wild type. When crossed to the other drivers, these two lines gave rise to completely wild-type-appearing F₁ progeny. When the *MESR2* cDNA was misexpressed using the *vestigial-GAL4* and *Actin5C-GAL4* drivers, adult wings of the progeny were reduced in size and very malformed (data not shown). This phenotype appears to be due to loss of intervein wing tissue whereas the amount of wing margin has remained constant or increased. Overexpression of *MESR2* in other tissue types did not seem to affect their development.

A new *Drosophila rel* domain protein: Sequence analysis of the longest EST clone corresponding to the *MESR1* gene reveals that it contains a region that shares high homology with the *rel* family of transcription factors (Figure 4). Known *Drosophila rel* proteins are Dorsal, Dif, and Relish (STEWART 1987; IP *et al.* 1993;

DUSHAY *et al.* 1996); however, the *MESR1* cDNA appears to encode a novel protein. The 4.2-kb cDNA clone contains a predicted open reading frame of 3633 bp in length encoding a predicted protein of 1211 residues. This protein contains a well-conserved *rel* homology domain (RHD) of 280 residues.

The RHD is loosely defined by a ~300-amino-acid region containing a DNA-binding domain, nuclear localization signal, and a conserved motif near the C terminus (JAIN *et al.* 1995). Alignment and sequence comparisons of the *MESR1* RHD with other *rel* proteins show that *MESR1* is most closely related to the mammalian nuclear factor of activated T-cells (NFAT) family of transcription factors. There are five distinct *NFAT* genes in human and most are expressed in multiple splice variants; however, the isoforms all contain the same RHD (RAO *et al.* 1997). *NFAT1–4* contain a Calcineurin binding domain found only in *NFAT* family members whereas *NFAT5* does not. *NFAT5* has two polyglutamine stretches in the C-terminal half of the protein that are not found in *NFAT1–4*. *MESR1* is most closely related to *NFAT5*; it is 51% identical and 63% similar to *NFAT5* in the RHD while it is only 37% identical and 52% similar to the RHD of *NFAT1*. *MESR1* shares even less sequence homology with other *Drosophila rel* proteins with only 38% identity to Relish, 26% identity to Dorsal, and 23% identity to Dif in the RHD. *MESR1* does not share any sequence homology with the Calcineurin binding domain of *NFAT1–4*. However, it does contain two polyglutamine stretches >20 residues in length, one on either side of the RHD.

The crystal structure of *NFAT1* interacting with AP-1 on DNA reveals 14 DNA contact residues in *NFAT1*, which are completely conserved among *NFAT1–4* (CHEN *et al.* 1998). *NFAT5* shares 11 of the 14 conserved residues (LOPEZ-RODRIGUEZ *et al.* 1999), and *MESR1* shares the same 11 residues (Figure 4). Structural analysis also implicates a histidine residue in the DNA recognition loop of *NFAT1–4* that confers binding specificity. However, *NFAT5* and *MESR1* contain an arginine instead of histidine at this site, which is also conserved in other members of the *rel* family including all other *Drosophila rel* proteins and human NF- κ B p50 (Figure 4 and CHEN *et al.* 1998; LOPEZ-RODRIGUEZ *et al.* 1999). Both the sequence similarity and structural similarities suggest that this novel *Drosophila rel* protein, *MESR1*, and human *NFAT5* form a new class of *rel* proteins.

DISCUSSION

We have performed a gain-of-function modifier screen for enhancers and suppressors of the rough eye phenotype associated with overexpression of a dominant-negative form of KSR in the developing eye; this screen identifies genes that can alter signaling through the RAS1 pathway when misexpressed. Genetic tests suggest that 12 genes from the EP collection can alter

A

MLLKFTKTSAGPSAVPLLTAPATVTSASMQATTTSYAARGTGMRMTMSTNSTMSARI
 HRKGFRIPSKRQPGKGLPGKLHTITRTGPGKMVPGKRIPQRPHPPPCDNDSDGLGFDQH
 TELRSSAGAGGVDPAANGGSGSNGQRSSLLVNSALNTVAAAAAASNTLQQH
 QQHH**QQQQQQQQQQQQQQQQQQQQQQQQ**HSPQQLIRAI PVSRFISARNVTRVANKRQP
 TTPLNSVASSNDGQVQLEILSQPEQQHRARYQTEGSRGAVKDRSGNGFPVRLTGYDKVA
 VLQVFIGTDIGRVAPHMFYQACKVAGKNSTQCNEKVDGTMVIEIDFKPETDMTITCDCV
 GILKERNVDVEHRFPEHLAQKNNKSTRCRMVFRQLTRDDGTTETLQVCSNPIICTQPP
 GVP EICKLSNSCPVDGGLLEFII GKNFLKDT HVVFQET YDSVNGDDPAT EIAVRQQLIG
 GTAALWEQSVLPDKEYLHQTHLICTVPPYLHQNILKPVQVQVSVSVSGKKSEPHFTFYTA
 KGQYTTLAAASTLSNTIHAQDVS GFMDTTSASNASGSSGWSAAGSGNNTSTGDNVEAKH
 EIDSGMPPFITTPMGVRRSSLSVTPMITDQQLVHLNAVAASAEALKTELDDSNHS
 PLTGESTPDPNAALQYHRFARKPSLDTIMYDQSNLPGFPVVPATAAAVAAVDIDP
 TAVAVAVELAVKNEIVKHVVQQHQMQEQMH**QQQQQQQQQQQQQQQQQQQQQQQQQV**
 HKFIDELTKSTSVSSNGTTEPALFTTS AVIDHALDILQAKVGIAPPNVVLERLSLSS
 TNSSSSMSGSESSPNSPLTQDII LNSEPAALAGAAALGGPAPVDVTGGLSTDIIMNPA
 VSPSTILCSANGAATAVVPNILAPHQVTMANSILNDIAMQPEPTQQDAVAALALSNIMM
 SPPTAASGVGVDLTPPTATMQPEVAATATSTAVSNMIKAAADFITTQEQEHYHHH
 GRIHSHSPQAAVGVSGNPAETASDPLVDLLNHSNTPETAAAAAAVAVEAANFQSMNHS
 HSHHGHGHSHGHGHSRVTHGVS GHVTSHHRGHHLVPPVPTPQESLIVALASENALQKS
 VATAAVTTNGAVMTQQASAPNTAGSILPAAVGAVAAAAAVAVQPIPELTTMSDQDLIS
 YINPSTFDQL

B

R A R Y L T E G
 * * * * *

Dm novel rel Q P T T P L N S V A S S N D **G Q V Q L E I L S Q P E Q Q** - H **R A R Y Q T E G** - - S R G A V K - - D R S G N G - - - F P I
 Hs NFAT5 K K S P M L C G Q Y P V K S E G K E L K I I V V Q P E T Q - H R A R Y L T E G - - S R G S V K - - D R T Q Q G - - - F P T
 Hs NFAT1b A S L P P L E W P L S S Q S G S Y E L R I E V Q P K P H - H R A H Y E T E G - - S R G A V K - - - A P T G G - - - H P V
 Hs NFkB - - - - - - - - - - - A D G P - - Y L Q I L E Q P K Q R G F R F R Y V C E G P - S H G G L P G A S S E K N K - K S Y P Q
 Dm Relish - - - - - - - - - - - K H V P - Q L R I V E Q P V E K - F R F R Y K S E M H G T H G S L N G A N S K R T P - K T F P E
 Dm dorsal - - - - - - - - - - - R K K P - Y V K I T E Q P A G K A L R F R Y E C E G R - S A G S I P G V N S T P E N - K T Y P T
 Dm Dif - - - - - - - - - - - R S G P - H L R I V E E P T S N I I R F R Y K C E G R - T A G S I P G M N S S S E T G K T F P T
 R F R Y X C E G

Dm novel rel **V R L T G Y D K** - - V A V L Q V F I G T - D I - G R V A P H M F Y Q A - - - - - C K V A G K N S T Q C N E - - K K V D
 Hs NFAT5 **V K L E G H N E** - - P V V L Q V F V G N - D S - G R V K P H G F Y Q A - - - - - C R V T G R N T T P C K E - - V D I E
 Hs NFAT1b **V Q L H G Y M E N K** P L G L Q I F I G T A D E - R I L K P H A F Y Q V H - - - - - R I T G K T V T T S Y - E K I V G
 Hs NFkB **V K I C N Y V G** - - - P A K V I V Q L V T N G K N I H L H A H S L V G K H - - - - - C E D G I C T V T A G P K D M V V G F
 Dm Relish **V T L C N Y D G** - - P A V I R C S L F Q T N L - D S P H S H Q L V V R K D D R D V C D P H D L H V S K E R G - Y V A Q F
 Dm dorsal **I E I V G Y K G** - - R A V V V V S C V T S T K D T P Y R F P H N L V G K E - - - - - G C K K G V C T L E I N S E T M R A V F
 Dm Dif **I E V C N Y D G** - - P V I I V V S C V T S D E P F R Q H P H W L V S K E E A - D A C K S G I Y Q K K L P P E E R R L V F

Dm novel rel G T M V **I E I D F K P E T D M T I T C D C V G** - - - - - I L K E R N V D V E H R - - - - -
 Hs NFAT5 G T T V **I E V G L D P S N N M T L A V D C V G** - - - - - I L K L R N A D V E A R - - - - -
 Hs NFAT1b N T K V L E I P L E P K N N M R A T I D C A G - - - - - I L K L R N A D I E L R - - - - -
 Hs NFkB A N L G I L H V T **K K K V F E T L E A R M T E A C I R G Y N P G L L V H P D L A Y L Q A E G G G D R Q L G D R E K E L I**
 Dm Relish I N M G I I H T A K K Y I F E E L C K K K Q D R - - - - - L V F Q M N R R E L S H K - - - - - Q L Q E L
 Dm dorsal S N L G I Q C V K K K D I E A A L K A R E E I - - - - - R V D P F K T G F S H R - - - - -
 Dm Dif Q K V G I Q C A K K L E M R D S L V E R E R R - - - - - N I D P F N A K F D H K - - - - -

Dm novel rel F P E H L A **Q K N K K S T R C R M V F R T Q L T R D D G** - - - T T E T L Q V C S N P I I C T Q P - - P G V P E I C K K
 Hs NFAT5 I G I A G S - - - K K K S T R A R L V F R V N I M R K D G - - - S T L T L Q T P S S P I L C T Q P - - A G V P E I L K K
 Hs NFAT1b - - - K G E T D I G R K N T R V R L V F R V H I P E S S G - - - R I V S L Q T A S N P I E C S Q R S A H E L P M V E R Q
 Hs NFkB R Q A A L Q **Q T K E M D L S V V R L M F T A F L P D S T G** - S F T R R L E P V V S D A I Y D S K A P N A S N L K I V R M
 Dm Relish H Q E T E R E A K D M N L N Q V R L C F E A F K I E D N G - A W V P L A P P V Y S N A I N N R K S A Q T G E L R I V R L
 Dm dorsal - - - - - F Q P S S I D L N S V R L C F Q V F M E S E Q K G R F T S P L P P V V S E P I F D K K A - - M S D L V I C R L
 Dm Dif - - - - - D Q I D K I N R Y E L R L C Y Q A F I T V G N S - - - K V P L D P E V S S P I Y G K - - - S S E L T I T R L

Dm novel rel S L N S C P V D G G L E L F I I G K N F L K - D T H V V F Q E T Y D S V N G D D P A T E I A V R Q Q L I G G T A A L W E
 Hs NFAT5 S L H S C S V K G E E E V F L I G K N F L K - G T K V I F Q E N V S D E N S - - - - - W K
 Hs NFAT1b D T D S C L V Y G G Q Q M I L T G Q H F T S - E S K V V F T E K T - - T D G Q - - - - - Q I W E
 Hs NFkB D R T A G C V T G G E E I Y L L C D K V Q K D I Q I R F Y E E E - E N G - - - - - G V W E
 Dm Relish S K P T G G V M G N D E L I L L V E K V S K K N I K V R F F E E D - - E D G E - - - - - T W E
 Dm dorsal C S C S A T V F G N T Q I I L L C E K V A K E D I S V R F F E E - - K N G Q - - - - - S V W E
 Dm Dif C S C A A T A N G G D E I I M L C E K I A K D I E V R F Y E T D - - K D G R - - - - - E T W F

Dm novel rel Q S V L P D K E Y L H - Q T H L I C T V P P Y L H Q N I L K P V Q V Q V S I V S - - - S G K K S E P H T F T Y T A
 Hs NFAT5 S E A E I D M E L F H - Q N H L I V K V P P Y H D Q H I T L P V S V G I Y V V T N - - A G R S H D V Q P F T Y T P
 Hs NFAT1b M E A T V D K D K S Q - P N M L F V E I P E Y R N K H I R T P V K V N F Y V I N G - - K R K R S Q P Q H F T Y H P
 Hs NFkB G F G D F S P T D V H R Q F A I V F K T P K Y K D I N I T K P A S V F V Q L R R K S - D L E T S E P K P F L Y Y P
 Dm Relish A Y A K F R E S D V H H Q Y A I V C Q T P P Y K D K D V D R E V N V Y I E L I R P S D D E R S P A L P F R Y K P
 Dm dorsal A F G D F Q H T D V H K Q T A I T F K T P R Y H T L D I T E P A K V F I Q L R R P S - D G V T S E A L P F E Y V P
 Dm Dif A N A E F Q P T D V F K Q M A I A F K T P R Y R N T E I T O S V N V E L K L V R P S - D G A T S A P L P F E Y Y P

signaling efficiency through the RAS1 pathway when misexpressed in the eye. Of these 12 genes, 3 are previously cloned *Drosophila* genes, 1 is a new member of the rel family of proteins, 6 are completely novel, and 2 have yet to be molecularly characterized. Below we discuss the possible roles of 4 of these genes in the RAS pathway.

Drosophila Akap200: One of the misexpression interactors, *MESR2*, was an insertion upstream of the *Akap200* locus. DAKAP200 is *Drosophila A kinase anchor protein* of molecular weight 200 kd and binds the regulatory II (rII) subunit of cyclic AMP-dependent protein kinase (PKA; LI *et al.* 1999). The *Akap200* gene produces two different transcripts, one that contains the binding site for RII and one where the exon encoding for the RII binding site is spliced out to generate a protein that does not interact directly with PKA (LI *et al.* 1999). Both isoforms of AKAP200 are expressed at relatively similar levels throughout development as well as in adult heads (LI *et al.* 1999).

PKA is the principal mediator of signals that activate adenylate cyclase. cAMP signals are often targeted to effectors that accumulate to discrete intracellular locations. This targeting is due to a nonuniform distribution of PKA molecules within cells. In *Drosophila*, PKA has been implicated in normal developmental events in all imaginal tissues through the Hedgehog signaling pathway (JIANG and STRUHL 1995; LI *et al.* 1995; PAN and RUBIN 1995) and is involved in signaling pathways that generate cell polarity, which requires it to be localized to distinct intracellular locations.

Subcellular localization of PKA occurs through association with AKAPs. AKAPs are a functionally related family of proteins that are defined by their ability to associate with PKA (RUBIN 1994; COLLEDGE and SCOTT 1999). Each AKAP contains a unique targeting domain that directs the complex to a defined intracellular location where PKA is placed proximal to both a signal generator (adenylate cyclase) as well as to potential PKA effector molecules. Coordinate binding of specific combinations of enzymes can allow such complexes to respond to distinct second messenger-mediated signals.

Studies in mammalian cells have suggested that PKA signaling via Rap1, another small molecular weight GTP-binding protein, antagonizes RAS1 signaling by competing for RAS pathway components such as B-Raf and MAPK (KITAYAMA *et al.* 1989; VOSSLER *et al.* 1997). How-

ever, more recent studies suggest no genetic interaction between *Drosophila Rap1* and *RAS1* (ASHA *et al.* 1999). In *Drosophila*, overexpression of *Rap1* in a heterozygous *RAS1* mutant background had no effect on photoreceptor determination, suggesting no interaction between the two gene functions. A heterozygous *Rap1* mutation did not reduce the number of R7 cells in a *sev-RAS1^{V12}* rough eye, also suggesting that the two pathways do not interact (ASHA *et al.* 1999). Although there is no direct evidence linking PKA activation to MAPK activation via Rap1, there may be a still unknown pathway by which these molecules can signal.

Akap200 was isolated as a misexpression enhancer of *KDN* and suppressor of *RAS1^{V12}* in our screen. This suggests that overexpression of this AKAP decreases signaling through RAS1. Overexpression of an AKAP might cause mislocalization of PKA molecules to the plasma membrane. This could activate a signaling pathway that normally is not utilized in this cell or at this time in development. If PKA and Rap1 are involved in RAS signaling, why were they not uncovered in previous loss-of-function screens? One possibility is that mutations in either gene may not be dose sensitive and therefore be unable to dominantly modify a rough eye phenotype. Another is the possibility that overexpression of an AKAP causes abnormal targets of PKA to become activated. Whether PKA signals through Rap1 is still unclear; however, the reported effects of attenuating RAS1/MAPK signaling are supported by this study. The enhancement of the *KDN* rough eye phenotype could be due to the additive effects of inefficient signaling due to *KDN* as well as the attenuation of MAPK by mislocalized PKA. In the activated *RAS1^{V12}* background, the attenuating effects of activated PKA due to mislocalization to the plasma membrane might reduce the amount of signaling through the pathway to suppress the RAS1-dependent rough eye phenotype.

misshapen: Overexpression of *msn* in a *sE-KDN* background enhances the rough eye phenotype. *msn* encodes the *Drosophila* homolog of Nck interacting kinase (NIK), a member of the mammalian SPS1 subfamily of the STE20 kinase family (TREISMAN *et al.* 1997; SU *et al.* 1998). *msn* is an essential gene involved in dorsal closure during embryogenesis in *Drosophila* and mutant clones result in misshapen rhabdomeres (due to defects in polarity, malformed and missing photoreceptors) in the adult eye (TREISMAN *et al.* 1997). STE20, the founding

FIGURE 4.—A novel *Drosophila* rel domain-containing protein. (A) The 1211-residue predicted amino acid sequence of a novel rel protein. rel homology domain (RHD) and polyglutamine stretches are underlined and boxed, respectively. (B) Sequence alignment of the RHD from seven different rel proteins (and GenBank accession numbers): Dm, MESR1 identified in this screen (AF195496); human NFAT5 (AF200687); human NFAT1b (U43341); human NF- κ B p50 (M55643); *Drosophila* Relish (U62005); *Drosophila* dorsal (M23702); and *Drosophila* Dif (L29015). Identities shared in at least four of seven proteins are highlighted in black and similarities in light gray. The 14 residues involved in DNA contact in NFAT1b are marked above with a dot. The invariant histidine in the NFAT1 DNA recognition loop is marked above with an asterisk. The DNA recognition motif is underlined, the NFAT consensus sequence is above, and the rel consensus sequence is below.

member of the family in yeast, acts in the pheromone signaling pathway and activates the yeast MAPK module (HERSKOWITZ 1995). However, the upstream activators of the STE20 pathway are not known. *msn*, the STE20 homolog in *Drosophila*, acts upstream of the c-Jun amino-terminal kinase (JNK) MAPK cascade required for dorsal closure (SU *et al.* 1998) and has recently been implicated to act downstream of the Frizzled receptor in the epithelial planar polarity pathway (PARICIO *et al.* 1999). Previously published results suggest that *msn*, when overexpressed in the eye in an otherwise wild-type background, can generate a rough eye (PARICIO *et al.* 1999). However, in our experiments we found no effect on eye morphology using *sE-GAL4* to drive either *UAS-msn* or EP(3)0609 and EP(3)0549, the two EP lines upstream of the *msn* gene (data not shown). *Drosophila* Jun has been implicated as a downstream target of both the JNK MAPK and RAS1/MAPK signal transduction pathways by overexpression analysis of dominant-negative mutations (KOCKEL *et al.* 1997); however, no other components of either pathway are shared. If the JNK pathway can partially compensate for the RAS1 pathway in eye development, one would expect that *msn* overexpression would suppress *sE-KDN*. The overexpression results from our screen suggest that as a misexpression suppressor of RAS1, *msn* decreases signaling in the pathway. *msn* may independently inhibit neuronal cell fate, although there is no previous evidence for this. It is possible that the JNK signaling pathway may compete with the RAS pathway for common components. Alternatively, this interaction may be tissue specific and only uncovered in the eye with overexpression. There is also the possibility that misexpression of *msn* causes promiscuous signaling through an independent pathway that also affects eye morphology. Although we do not see a phenotype when *msn* is overexpressed in the eye, this situation may sensitize the eye and nonspecifically enhance the *sE-KDN* phenotype.

Lk6: Another misexpression interactor, *MESR8*, corresponded to two independent insertions upstream of the *Lk6* gene. The *Lk6* gene was originally identified biochemically as a microtubule-binding protein (KIDD and RAFF 1997). LK6 localizes to centrosomes in the early blastoderm embryo and appears to be expressed ubiquitously throughout development. Loss-of-function mutations in *Lk6* are viable and have no visible adult phenotype (A. M. HUANG and G. M. RUBIN, unpublished results). Constitutive overexpression of *Lk6* under the *ubiquitin* promoter causes microtubule defects in both eggs and embryos; these defects include fragmented mitotic spindles, abnormal asters, and abnormal metaphases, suggesting that LK6 may stabilize microtubules *in vivo* (KIDD and RAFF 1997). Overexpression of phenotypes in the embryo suggests the same. Overexpression of *Lk6* does not appear to affect imaginal tissues (KIDD and RAFF 1997; A. M. HUANG and G. M. RUBIN, unpublished results).

The identification of a cytoskeleton-associated kinase that can genetically interact with the RAS pathway is consistent with studies in mammalian cells that indicate that the activation of the RAS/MAPK pathway can lead to spindle instability (SAAVEDRA *et al.* 1999). Activated RAS induces chromosome aberrations such as dicentrics, acentrics, and double minutes (DENKO *et al.* 1994). Genomic instability as measured by micronucleus formation increases tenfold in cells expressing activated RAS (SAAVEDRA *et al.* 1999). Micronucleus formation induced by activated MAPK was due largely to disruption of the mitotic spindle rather than double-strand chromosome breaks.

In our experiments the overexpression of *Lk6* in the developing eye has no visible phenotype. However, when overexpressed in the *sev-RAS^{V12}* background, *Lk6* is able to partially suppress the associated rough eye by reducing the number of supernumerary R7 cells. Since the supernumerary R7 cells in the *sev-RAS^{V12}* background are generated postmitotically by cone cell to R7 transformations, LK6 may be playing a role in differentiation rather than proliferation in this instance. LK6 may be redundant with other kinases expressed in the eye, normally act at other stages of development, or activate normally inactive signaling pathways when overexpressed. It is possible that expressing LK6 in the *sev-RAS^{V12}* background could have a deleterious effect on cell differentiation and cause the improperly fated photoreceptors to die.

A new rel family member in flies: The *MESR1* interactor identified in this screen is an insertion upstream of a novel gene that encodes a protein containing a rel domain. Sequence comparison of the rel domain with other known proteins of this class identifies it as a distant member of the NFAT family of transcription factors with mammalian NFAT5 its closest relative. NFAT1–4 are highly regulatable transcription factors that are sequestered in the cytoplasm of resting cells of the mammalian immune system. Upon activation of Calcineurin by intracellular calcium release, NFAT1–4 translocate to the nucleus where they bind cooperatively to DNA with the AP-1 heterodimer, FOS and JUN (RAO *et al.* 1997). The mammalian NFAT5 protein is distantly related to the others in sequence homology as well as function. NFAT5 appears to be constitutively nuclear, does not seem to interact with FOS and JUN, and does not transactivate well-characterized NFAT1–4 target promoters; its function and specificity remain unclear (LOPEZ-RODRIGUEZ *et al.* 1999).

Overexpression of this gene in the eye suppresses an activated *RAS1* rough eye, implying that it acts to decrease RAS signaling efficiency. This protein may act to promote a nonneuronal cell fate or actively repress neuronal differentiation. There are four putative MAPK phosphorylation sites in the *Drosophila* NFAT5 homolog but it is unknown whether any of these sites are phosphorylated *in vivo*. No loss-of-function mutations

have been identified to date. This protein may bind to RAS-dependent promoters; identification of target genes will help elucidate the wild-type function of this gene in flies.

We have identified two kinases and one kinase anchor protein that can modulate RAS signaling efficiency when overexpressed. It is possible that increased levels of these signaling proteins lead to the activation of targets, of which the novel *rel* protein may be one, not normally active in the developing eye. The genetic interaction we see may be the result of activation of signaling pathways not used in eye development, or represent processes found in other tissues or other stages of development.

The gain-of-function screens in animals: Gain-of-function genetics has been a successful approach in identifying regulatory pathway components in many systems. Multicopy screens in yeast revealed drug-resistant loci (RINE *et al.* 1983), overexpression in mammalian cell culture identified MyoD as a crucial cell fate determinant (DAVIS *et al.* 1987), and overexpression in *Xenopus laevis* identified Noggin as a potent neural inducer (SMITH and HARLAND 1992). In *Drosophila*, overexpression has identified critical genes involved in development and oogenesis (MILAN *et al.* 1998; RORTH *et al.* 1998).

The genetic screens performed to date looking for genetic interactors with RAS pathway components have been dominant modifier screens [SIMON *et al.* 1991; DICKSON *et al.* 1996; KARIM *et al.* 1996; MAIXNER *et al.* 1998; NEUFELD *et al.* 1998; THERRIEN *et al.* 1998, 2000 (this issue); REBAY *et al.* 2000]. These screens required that a twofold reduction in gene dosage be sufficient to alter the phenotype of a genetically "sensitized" background. In some cases, these mutageneses were done to near saturation and have uncovered many RAS pathway components. However, the observation that two-thirds of all genes are not mutable to any visible phenotype suggests that the function of many genes may not be revealed by loss-of-function mutations. In addition, one concern with dominant modifier screens is that many genes that normally interact with the RAS1/MAPK pathway were not recovered because the reduction in dosage of one gene is compensated by other genes in the genome.

To identify additional genes that can modulate RAS signaling, we screened through a collection of *P*-element insertions that drive overexpression of otherwise wild-type genes. Six of the genes isolated in this screen have never been previously characterized. These six insertion lines are homozygous viable and show no adult phenotype (A. M. HUANG and G. M. RUBIN, unpublished observations). To exclude lines that modify the RAS-dependent rough eye due to the additive effect of two causally unrelated phenotypes, we screened for lines that have opposite effects on phenotypes due to decreased (*sE-KDN*) or increased (*sev-RAS1^{V12}*) signaling

through the RAS pathway. Further work is required to determine the specificity of these interactions, the functions of these molecules in development, and the roles they may play in RAS signaling events.

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