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A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes

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ABSTRACT **Genetic screens in *Drosophila* have lead to the discovery of many genes important for patterning and signal transduction in diverse organisms. Traditionally, the phenotypic effects of loss-of-function mutations are analyzed. As an alternative way to link genes and function, I have developed a versatile misexpression screen in *Drosophila*, the first such screen in higher eukaryotes. The screen identifies genes that, when over- or misexpressed in a pattern of interest, give a specific phenotype or modulate an existing mutant phenotype. It is based on Gal4 transactivation of a mobile enhancer and promoter that "targets" random endogenous genes for expression. The modular design of the screen allows directed expression in any temporal or spatial pattern. When activated in the developing eye, 4% of target inserts gave dominant phenotypes. One insertion was in the gene encoding Ras GTPase-activating protein; its overexpression phenotype was strongly enhanced by a mutation in *Ras1*. Thus, biologically relevant phenotypes and genetic interactions are identified using this method. The screen is a powerful new tool for developmental genetics; similar approaches can also be developed for other organisms.**

Genetic screens are useful tools for identifying gene products involved in specific cellular or developmental processes. Traditionally, genes are characterized based on a loss-of-function phenotype: that is, based on which process is perturbed when the gene product is absent or functionally impaired. However, over- or misexpression phenotypes can be equally informative. For example, misexpression of homeotic genes cause striking transformations in *Drosophila* (1, 2), forced expression of MyoD converts fibroblasts to myoblasts (3), and overexpression of oncogenes causes tumors in mammals (4–6). In addition, it is estimated that over two/thirds of genes in flies, worms, and yeast (refs. 7–9; and perhaps even more in mammals) have no obvious loss-of-function mutant phenotype. For these genes, ectopic expression phenotypes can provide unique functional information.

If a mutation affecting a process of interest is already known, genetic interaction screens can be used to identify novel players in the same pathway. Interaction screens based on heterozygous loss-of-function mutations have been used successful in *Drosophila*, for example, to dissect the Sevenless-Ras signal transduction pathway (10, 11). But this type of screen requires exquisitely sensitized genetic background and phenotype. Controlled overexpression can also identify important genetic interactions; if increased expression of one gene enhances or suppresses the phenotype of a mutation in another gene, their products are likely to be involved in the same process. This notion is the basis of powerful genetic screens in yeast (12, 13), yet no equivalent tool is currently available in higher eukaryotes. Here, I describe the development of a tissue-specific conditional overexpression screen in *Drosophila*.

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MATERIALS AND METHODS

Construction of Target Elements. The fragments described below were all cloned into the polylinker of Casper4 P-element vector, which contains the mini-white marker gene and P-element ends (see Fig. 1*B*). Enhancer and promoter fragments were first cloned into pBluescript vectors (Stratagene) and sequenced. The enhancer contained a total of 14 Gal4 sites [three copies of the sequence (A) 5′-CAAGGCGGAGTACTGTCCTCCGGGCTGGCGGAGTACTGTCCTCCGG-3′ and four copies of the sequence (B) 5′-CAAGGTCGGAGTACTGTCCTCCGACACTAGAGGTCGGAGTACTGTCCTCCGACG-3′ in the order ABBBABA)] cloned into a *Sty*I site inserted in pBluescript. A few target elements contained seven copies of sequence A as Gal4 sites. The enhancer also contained GAGA sites (two copies of the sequence 5′-TCGAGAAAGAGAGAGAGAAGAGAAGAGAGAGAACAT-3′). The *hsp70* promoter (−45 to +33) was cloned by amplifying genomic fly DNA with the oligonucleotides 5′-GAAGGTACCGAGAGAGCGCCGGAGTAT-3′ and 5′-GAAGATCTCGACGTGTTCACTTTGCTTG-3′, and the cloning sites were subsequently changed to *Xho*I (5′ end) and *Pst*I (3′ end), using an artificial linker. The *Gypsy* Su(Hw) sites were on a 370-bp *Kpn*I–*Not*I fragment from the YES vector (kindly provided by Pamela Geyer). The plasmid rescue fragment (*Not*I–*Not*I) included a bacterial origin of replication and kanamycin resistance gene.

Transformation and Fly Work. P-element germ-line transformation was done by injection into w^{1118};;Sb,P[ry$^+$,Δ2-3]/TM6,Ubx embryos. Additional insertion sites were obtained by exposing P-elements to P[Δ2-3]99B transposase source (14). The target elements mobilized with an efficiency similar to that of other P-elements. All target inserts were mapped to a chromosome and balanced, using standard fly stocks and procedures. For all target insert lines described in this paper, a single P-element was detected by Southern blot and by *in situ* hybridization to polytene chromosomes (using EP target element DNA as probe).

To test Gal4 pattern lines and the Gal4 responsive enhancer described above, the enhancer was cloned into HZ50 lacZ vector (15), and transgenic flies were made by germ-line transformation. These flies were crossed to Gal4 lines, and progeny were stained for β-galactosidase activity. The enhancer was highly responsive to Gal4 at 9 of 10 insertion sites and moderately responsive at one insertion site.

RNA Analysis. Brains, discs, and salivary glands were dissected from three instar larvae carrying the indicated target insertion and the "enhancertrap" T80, which expresses Gal4 in these tissues (16). Nucleic acids were isolated by standard proteinase K/SDS procedure and poly(A)$^+$ RNA was selected using the PolyATtract system (Promega). Approximately 1 μg of poly(A)$^+$ RNA and 100 fmol of 5′ end-labeled oligonucleotide was used for primer extension analysis, performed as described in ref. 17. A sequencing ladder was run alongside the extension reactions to measure the length of the products. The

Abbreviation: GAP, GTPase-activating protein.

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common primer EPp was: 5′-GCATGTCCGTGGGGTTT-GAATTAACTC-3′. For some target lines, Northern blot analysis was performed using plasmid rescued DNA as probe. It confirmed the primer extension data.

RESULTS AND DISCUSSION

The principle of the modular misexpression screen is outlined in Fig. 1*A*. First, a "pattern" line is chosen that expresses the yeast Gal4 transactivator in a tissue of interest. The pattern element can be a cloned regulatory sequence fused to Gal4 or an enhancertrap, which allows a genomic enhancer to control Gal4 expression (16). Gal4 acts as a sequence-specific transcription activator in *Drosophila* (16, 19). The pattern line is crossed to an extensive panel of "target" lines, each carrying a single target P-element inserted at a unique, random position in the genome. The target element contains a Gal4-responsive enhancer at one end. In progeny containing both elements, an endogenous gene adjacent to the target element should be induced in cells expressing Gal4 (determined by the pattern element). Phenotypes due to over- or misexpression of the gene adjacent to a target element can then be scored directly, or as suppression or enhancement of a preexisting mutation.

The screen takes advantage of two very useful techniques in *Drosophila*: single P-element mutagenesis (20, 21) and the modular Gal4 system (16). The modular design makes the screen flexible (genes can be induced in any spatial or temporal pattern) and conditional, such that dominant lethal and sterile mutations can be recovered. The mutagen is a (target) P-element, which greatly facilitates cloning of affected genes. Finally, the fact that P-elements often insert in 5′ regions of genes supports the feasibility of this design (21).

Several features of the target element were critical for the success of this screen. The enhancer should respond to Gal4 and activate transcription of an adjacent gene regardless of its position in the genome. Also, to generate a large number of target lines, the target element should transpose efficiently. An enhancer with 14 Gal4 binding sites and a set of GAGA elements (to prevent position effects; refs. 22–24) was constructed and included in two types of target elements: The EP element has a promoter immediately adjacent to the enhancer (Fig. 1*B*), so Gal4 activation should induce a transcript initiating within the element. The E element does not have this promoter, so the enhancer should act directly on a nearby genomic promoter. In target elements E^s and EP^s, Su(Hw) binding sites were inserted as "insulator" sequences (18) to prevent the enhancer from acting on the *white* and 5′ P promoter also present in the P-element. Target elements E, E^s, EP, and EP^s were introduced into the fly genome by P-element transformation and mobilized *in vivo* by exposure to a stable source of transposase (14). All elements transposed efficiently (data not shown).

A simple screen was performed to test the methodology and the different target elements. As a pattern element, I chose "sevenless-Gal4," which utilizes the sevenless enhancer to drive expression of Gal4 in differentiating photoreceptor and cone cells of the eye discs (25). This pattern line was crossed to 352 target lines (Fig. 1*C*), each carrying a single E, E^s, EP, or EP^s target element that had been mapped to a chromosome. Progeny were then scored for rough eyes, a phenotype indicating that eye development has been perturbed. Gal4 expression in six target lines resulted in mutant phenotypes; one was pupal lethal and five had rough eyes (Fig. 2). The mutant phenotypes were completely dependent on Gal4 was expression. Additional EP and EP^s target lines gave mutant phenotypes with pattern lines other than sevenless-Gal4 (data not shown). In conjunction with the molecular data presented below, this indicates that the screen uncovered specific effects.

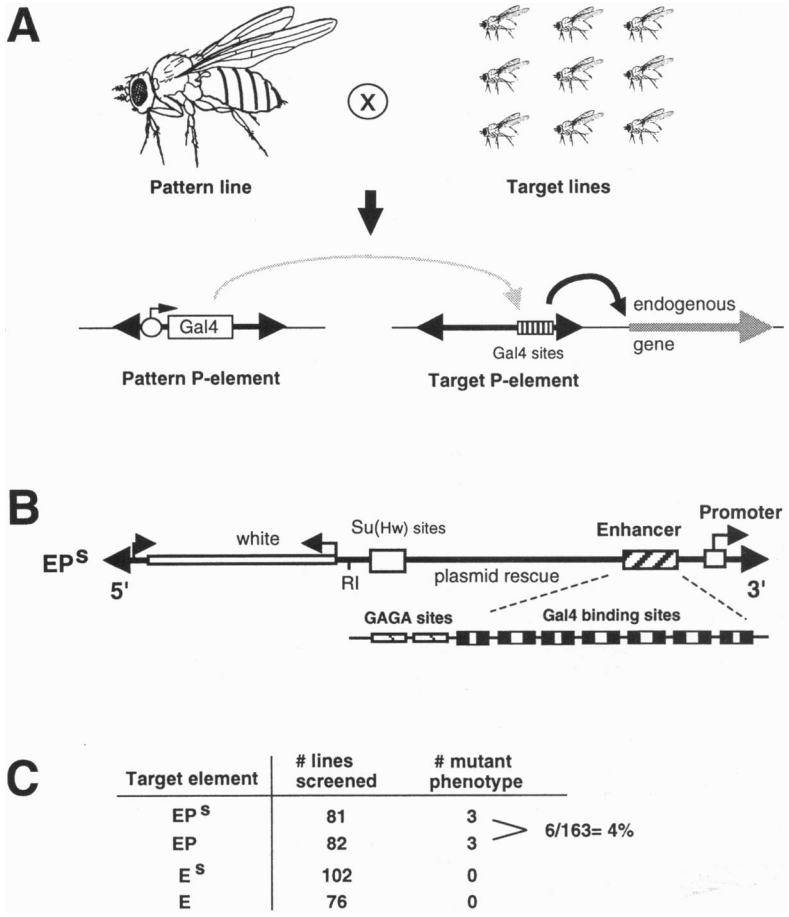


FIG. 1. The modular overexpression screen. (*A*) A specific pattern line is crossed to a large number of independent target lines. In progeny carrying both a pattern and a target element, Gal4 encoded by the pattern element should bind to Gal4 binding sites within the target element enhancer and activate an adjacent endogenous gene. Each independent target element insertion thus targets one endogenous gene for expression. (*B*) Structure of the EP^s target P-element. EP and E target elements do not contain Su(Hw) sites (18); E and E^s do not contain the promoter at the 3′ end. The plasmid rescue sequences and the unique *Eco*RI site allow rapid cloning of flanking genomic DNA. (*C*) Results of pilot screen using sevenless-Gal4 as pattern element; see text for details. The promoter was critical for target element function, but the Su(Hw) sites were not.

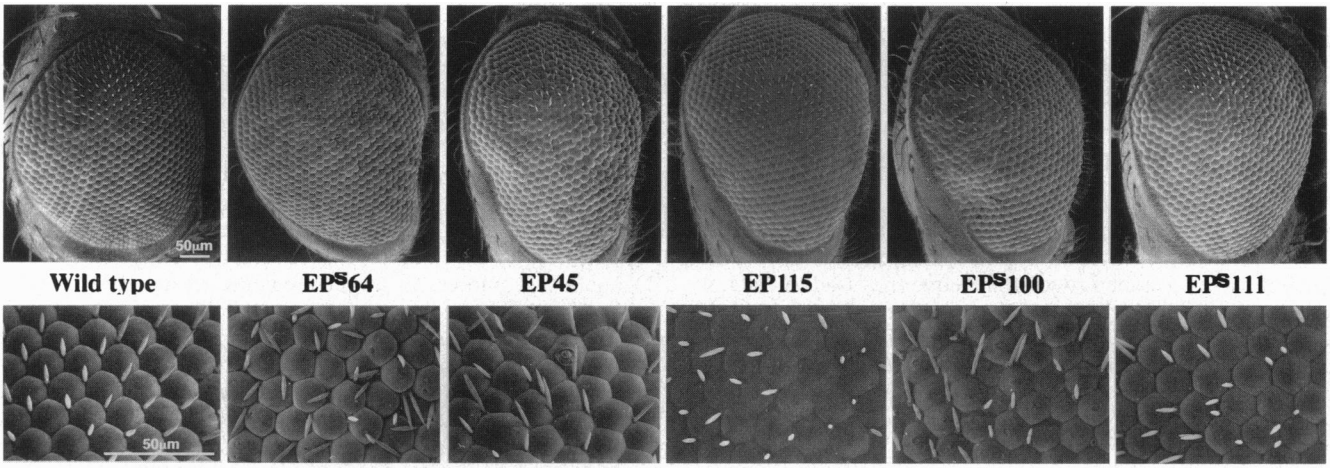


FIG. 2. Rough eye phenotypes. (*Upper*) Typical scanning electron microscopy images of whole eyes from flies carrying sevenless-Gal4 and the indicated target element (wild type, no target element). (*Lower*) Same eyes at higher magnification. (Bar = 50 mm.)

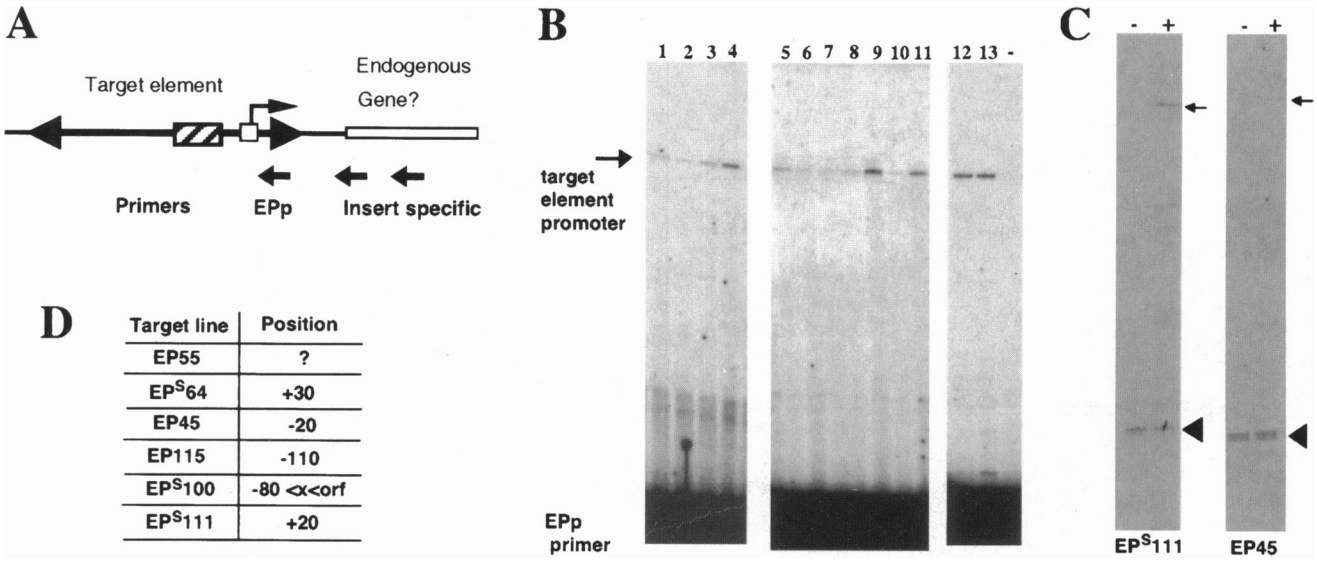
Thus the method worked as designed, and it worked efficiently: 4% of EP and EP^s target lines (6 out of 163) showed dominant mutant phenotypes in the eye (Fig. 1*C*).

All target lines giving mutant phenotypes in this screen were generated with EP or EP^s elements, indicating that the promoter at the 3′ P-element end was critical for target element function. Several factors are likely to contribute to this: endogenous promoters may be selective and not interact with the artificial "enhancer"; the enhancer may only work at very short distance from the promoter; and finally, target elements often insert 3′ of transcription start sites (see Fig. 3*D*).

Two features required for proper and efficient function of the screen were tested by molecular analysis. (*i*) Gal4 should induce productive transcription from within the EP target element in most or all target lines. (*ii*) Mutant phenotypes should be due to over- or misexpression of endogenous genes (not truncated or antisense transcripts); thus, target elements should be inserted into the 5′ end of genes as depicted in Fig. 3*A*. Both requirements were fulfilled.

The first requirement was addressed using a primer specific to the common 5′ end of induced transcripts (EPp in Fig. 3*A*). The six mutant target lines identified above, as well as seven EP target lines without mutant phenotypes, were crossed to an active Gal4 pattern line and poly(A)$^+$ RNA was isolated from the progeny. Primer extension analysis revealed a mRNA initiating from the EP target promoter in all lines (Fig. 3*B*). This RNA was only detected in the presence of a Gal4 pattern element. Furthermore, the level of expression did not correlate with a mutant phenotype. Thus, the EP target element was activated by Gal4 in all lines tested.

To address the second requirement, induced and endogenous transcripts were compared for each mutant line. Genomic DNA adjacent to the target element was cloned by plasmid rescue and sequenced. This allowed the construction of insert-specific primers (Fig. 3*A*). Fig. 3*C* shows two examples of primer extension analysis on control RNA (−) and target line RNA (+). Endogenous transcripts (arrowheads) are detected in both − and + samples; induced transcripts (arrows) are



D

Target line	Position
EP55	?
EP^{S}64	+30
EP45	-20
EP115	-110
EP^{S}100	-80 <x<orf
EP^{S}111	+20

FIG. 3. Analysis of mRNAs produced upon Gal4 activation of EP target elements. (*A*) Relative positions of oligonucleotides used for primer extension analysis. (*B*) Primer extension with primer EPp. Lanes 1–6, target lines which had mutant phenotypes with sevenless-Gal4; lanes 7–13, target lines without mutant phenotypes; lane −, control RNA (no target insert). (*C*) Primer extension with insert-specific primers and RNA from larvae with (+) or without (−) the corresponding target insertion. The primer sites were 106 (EP^{s}111) and 139 (EP45) nucleotides 3′ of the target element insertion site. Induced (arrow) and endogenous (arrowhead) transcripts are indicated. (*D*) Approximate insertion site (in bp) of target elements relative to the corresponding endogenous transcription start site (in the same orientation). For EP^{s}100, the endogenous transcript was only detected by reverse transcription PCR. cDNA cloning and sequencing indicated that the target element was inserted between 80 bp 5′ of the endogenous transcription start site and the open reading frame. For EP55, a nearby endogenous transcript was not detected.

Table 1. Phenotypic effects of induced expression in different tissues

Target line	Cytological position of element	No Gal4	Gal4 line: 2xsev (eye disc posterior)	T80 [(all) discs salivary gland]	69B [embryo epidermis discs (wing)]	24B (embryo mesoderm)	32B (embryo epidermis discs)	h[1J3] (*hairy* pattern)
EP55	$4C_{5-6}$	WT	Lethal	Lethal	Semilethal	Lethal	Lethal	Lethal
EP^{s}64	$50A_{1-2}$	WT	Rough eyes	WT	Black spots on eyes	WT	WT	WT
EP45	$67D_{2-3}$	WT	Rough eyes	WT	WT	WT	WT	WT
EP115	$98F_{10-14}$	WT	Rough eyes	Lethal	Semilethal	Lethal	Lethal	Lethal
EP^{s}100	$42E_{4-7}$	WT	Rough eyes	WT	WT	WT	WT	WT
EP^{s}111	$92D_{1-2}$	WT	Rough eyes	Tiny Salivary glands/ other defects	Altered wing vein	Lethal	Semilethal	Lethal

Mutant phenotypes were completely penetrant (except semilethality) and dependent on Gal4 expression (see No Gal4). Five of the six target lines were also homozygous viable and normal; EP^{s}100 was inserted on a balancer chromosome and therefore could not be made homozygous. All but 2xsev (sevenless-Gal4) are Gal4 enhancertrap lines described in ref. 16. The cytological position of the target element in each line is listed. wt, Wild type; sal., salivary; emb., embryo; epid., epidermis.

detected in + samples only and are longer, due to the added P-element sequences. For all "mutant" target lines, primer extension analysis was performed using two or three insert specific primers and RNA from different tissues, giving consistent results (data not shown). This analysis as well as DNA sequence information (discussed below), showed that five of the six mutagenic target elements had inserted close to the transcription start site of endogenous genes (Fig. 3*D*). Thus over- or misexpression of essentially normal transcripts was the predominant mechanism of generating the observed phenotypes.

More detailed phenotypic analysis was carried to examine the specificity of the dominant phenotypes. Scanning electron microscopy showed that sevenless-Gal4 induced distinctive rough eye phenotypes in each target line (Fig. 2). For example, in EP45, the ommatidia appeared to be variable in size, and many bristles were missing or mispositioned. In EP115, lenses were flat and nondistinct, whereas, in EP^{s}100, the lenses had structural defects, such as holes and bulges. Different phenotypes were also observed when these target lines were crossed to enhancertrap lines that expressed Gal4 in other tissues (Table 1). For three lines (EP45, EP^{s}100, and EP^{s}64), defects were only observed in the eye. For two lines (EP55 and EP115) induced expression either in embryos or in larvae was lethal. Finally, Gal4 induction in EP^{s}111 resulted in specific defects, such as underdeveloped salivary glands, missing scutellar bristles, and minor disruptions of wing veins. Thus, the six targeted genes had distinct effects on development.

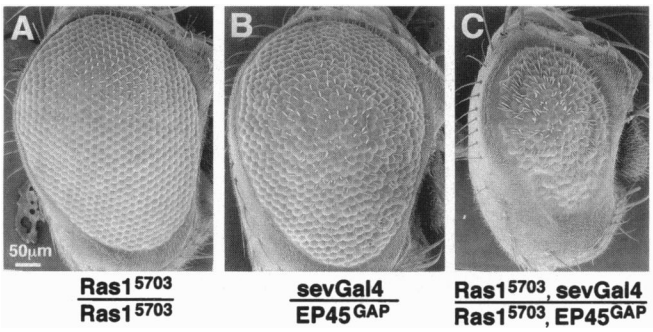


FIG. 4. Decreased Ras1 activity enhances the EP45^{GAP} overexpression phenotype. Typical scanning electron microscopy of eyes from flies of the indicated genotypes. Wild type is shown in Fig. 2. The rough eye phenotypes (*B* and *C*) were only observed in the presence of sevenless-Gal4.

The molecular basis for the eye-specific phenotypes in target lines EP^{s}100 and EP45 was explored by sequencing flanking DNA and identifying the targeted genes.

The target element in EP^{s}100 was inserted in the 5′ end of a gene with the potential to encode a DnaJ-like protein. A cDNA fragment derived from the 5′ end of the induced RNA in EP^{s}100 was sequenced and revealed a long open reading frame with good *Drosophila* codon usage. It was ≈45% identical over 70 aa to the N terminus of DnaJ homologs from different species, typical of eukaryotic DnaJ-like proteins (26). DnaJ-like proteins may assist Hsp70-like proteins in processes such as protein folding, translocation, and complex assembly (26). The mRNA was also detected in wild-type embryos, but at very low levels. Overexpression of this gene in the developing eye resulted in abnormal lenses (Fig. 2), perhaps by perturbing folding or secretion of lens proteins by the cone cells.

The target element in EP45 was inserted immediately upstream of the *Gap1* gene, encoding Ras GTPase-activating protein (GAP; ref. 27). Sequencing of EP45 plasmid-rescued DNA revealed a 100% match with the 5′ end of a Gap1 cDNA sequence, from 65 nucleotides 3′ of the target element insertion site. Since the expression pattern of an enhancertrap inserted in *Gap1* (27) is similar to the sevenless expression pattern (25), the phenotype described here is probably due to overexpression rather than misexpression of Gap1. This result fits very well with previous analyses of eye development. Loss-of-function mutations in *Gap1* cause rough eyes and, based on dose-dependent genetic interactions with other mutants in the Sevenless-Ras pathway, appear to increase Ras activity (27). These genetic interactions agree with biochemical data showing that Ras GAP converts active, GTP-bound Ras into inactive GDP-bound Ras by stimulating its intrinsic GTPase activity. Decreased *Ras1* gene dosage, which would be be similar to overexpression of Gap1, also disrupts eye development (11).

To confirm that induction of EP45^{GAP} acted by decreasing Ras activity, I examined the effect of further reducing Ras activity in the eye. *Ras1*^{5703} is a hypomorphic loss-of-function mutation caused by a P insertion 28 bp 5′ of the transcription start site of *Ras 1* (28). This recessive mutation causes semilethality and female sterility, but no detectable eye defects

(Fig. 4*A*). Overexpression of Gap1 in this mutant background resulted in severely disorganized eyes (Fig. 4*C*), a striking enhancement of the original EP45^{GAP} overexpression phenotype (Fig. 4*B*). *Ras1*^{5703} did not affect the induced expression phenotypes of EP115, EP^{s}64, EP^{s}111 or EP^{s}100 (data not shown). Thus increased Gap1 expression synergized with partial loss of Ras activity, and the effect was specific. This strong and reproducible interaction shows that the modular misexpression screen can be used to identify biologically relevant genetic interactions.

In conclusion, I have developed a conditional overexpression screen in *Drosophila* that can be applied to many developmental processes. This is a novel genetic approach to link genes and function in higher eukaryotes: identifying genes that, when over- or misexpressed in a pattern of interest, give a specific phenotype or modulate an existing mutant phenotype. The modular design makes the screen simple to use for the following reasons: many useful Gal4 pattern lines are already available; target lines are easy to generate and can be used repeatedly; and induced, mutated genes are easily identified. I have shown that meaningful dominant effects are uncovered using this approach, exemplified by the overexpression phenotype of Ras GAP, which is consistent with traditional genetic analyses. The synergism of Ras and Ras GAP mutations furthermore indicated that the method can identify important genetic interactions. This screen is likely to be very useful for dissecting signal transduction pathways and other processes in *Drosophila*. Finally, similar approaches may also be developed for other organisms, using transposable elements or retroviruses as mutagens.

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