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Genetic and molecular analyses of mutations involved in *Drosophila* raf signal transduction

Xiangyi Lu, Michael B.Melnick, Jui-Chou Hsu and Norbert Perrimon

Department of Genetics, Howard Hughes Medical Institute, 200 Longwood Avenue, Boston, MA 02115, USA

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We have identified dominant mutations that suppress the lethality associated with an R217 → L mutation in the GTP·Ras binding region (CR1) of the *Drosophila* raf (D-raf) serine/threonine kinase. Four intragenic and seven extragenic suppressors were recovered. Each of the four intragenic mutations contains one compensatory amino acid change located in either the CR1 or the kinase domain of D-raf. The seven extragenic suppressors represent at least four genetic loci whose effects strongly suggest that they participate in both the *sevenless* and *Drosophila* EGF receptor (*DER*) signaling pathways. One of these mutations, *Su(D-raf)*^{34B}, is an allele of *D-mek* which encodes the known signaling molecule MAPK kinase (MEK). A D83V mutation in D-MEK is identified and shown to be sufficient to confer the dominant activity of *Su(D-raf)*^{34B}.

Key words: genetic modifier/MAPK/MEK/Raf/Ras/receptor tyrosine kinase

Introduction

The Raf serine/threonine kinase was initially identified as an oncogene carried by transforming retroviruses (Rapp *et al.*, 1988). Since then, Raf has been shown to be a critical signal transducer used commonly by a number of receptor protein tyrosine kinases (RPTKs) in species as diverse as insects, worms and humans [reviewed by Perrimon (1993) and Egan and Weinberg (1993)]. Raf is a direct downstream target of p21^{ras} (Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Zhang *et al.*, 1993). Activated Raf phosphorylates and activates the tyrosine/threonine kinase MEK (Dent *et al.*, 1992; Kyriakis *et al.*, 1992), which in turn activates the serine/threonine kinase MAPK (Nakeilny *et al.*, 1992; Rossomando *et al.*, 1992). Sequential activations from Ras to Raf to MEK to MAPK seem obligatory in the pathways initiated by RPTKs. In contrast, yeast adopts various different MAPK activation pathways that are Ras/Raf-independent [reviewed by Levin and Errede (1993)]. For example, the yeast pheromone receptor triggers the activation of MEK via *Ste11* (*Saccharomyces cerevisiae*) or *Byr2* (*Schizosaccharomyces pombe*) which are kinases with no strong sequence homology to Raf (Rhodes *et al.*, 1990). Moreover, downstream of MEK, *S.cerevisiae* utilizes a combination of two related MAPKs, *FUS3* and *KSS1* (Courchesne *et al.*, 1989; Elion *et al.*, 1990), to mediate a full spectrum of effects in response to pheromone (Elion *et al.*, 1991). Recently, the mammalian homolog of *Ste11/Byr2* has been cloned and named MEKK (Lange-Carter *et al.*, 1993). Overexpression of MEKK leads to the activation of MEK in COS cells in the absence of the growth factor EGF. Addition of EGF to the same cells can activate MEK; however, this growth factor-induced MEK activation requires Raf activity. Thus, Raf represents an important MEK-activating mechanism responding primarily to growth factor RPTKs, whereas MEKK may respond primarily to G protein-coupled receptors (Lange-Carter *et al.*, 1993).

Primary sequences of Raf polypeptides from various species show three common structural motifs [reviewed by Heidecker *et al.* (1992)]. The kinase domain (CR3) constitutes the C-terminal half of the molecule. The N-terminal half of the molecule contains a cysteine motif (CR1) followed by a serine/threonine-rich region (CR2). The detailed mechanism by which Raf is activated is unknown. However, several lines of evidence suggest that the N-terminal half of Raf plays an important role in regulating its C-terminal kinase activity. First, mutations at the N-terminal half of Raf by either deletions or insertions generate constitutively active Raf kinases that are oncogenic in NIH 3T3 cells (Stanton *et al.*, 1989; Heidecker *et al.*, 1990). Presumably, Raf is folded such that its catalytic domain is prevented from interacting with substrates by the N-terminal domain. Upon removal or disruption of the N-terminal domain, the kinase becomes free to bind substrates for catalysis [reviewed in Heidecker *et al.* (1992)]. Second, the N-terminal half of Raf alone can act as a dominant negative protein when expressed in tissue culture cells, suggesting that this part of Raf protein may also play an active role, such as ligand binding, in the activation process of the kinase (Bruder *et al.*, 1992). Interestingly, the dominant negative activity maps to the CR1 region. Substitution of C168 by S in the cysteine finger motif within the CR1 region abolishes the dominant negative effect (Bruder *et al.*, 1992). Finally, recent studies show that the CR1 domain alone was sufficient to bind directly to the active GTP-bound form of Ras via the effector domain of Ras both *in vitro* and in the yeast 'two hybrid' assay (Vojtek *et al.*, 1993). Binding of CR1 to GTP·Ras may trigger conformational changes of Raf itself, leading to phosphorylation and activation of Raf kinase.

To understand the Raf signaling mechanisms we decided to isolate modulators of Raf activity using a genetic approach. In genetically manipulatable organisms, components of a given biological process can often be isolated as second site mutations which modify (enhance or suppress) the effect of a pre-existing mutation in the same pathway. In a sensitized genetic background it is possible to recover dominant genetic modifiers in one generation. This type of F1 screen has been extremely powerful in the identification of components of signal transduction pathways (Gertler *et al.*, 1989; Simon *et al.*, 1991; Doyle and Bishop, 1993). Many genes involved

in RPTK signaling pathways are essential genes required in multiple processes throughout development. In most situations, these types of genes can only be identified as dominant genetic modifiers.

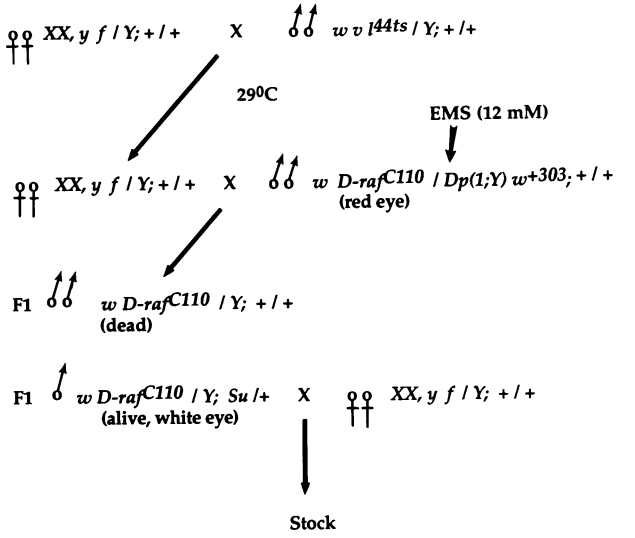

Fig. 1. Mutagenesis scheme to identify *Su(D-raf)*. Details of the mutageneses are described in Results and Materials and methods. *w D-raf^{C110}* hemizygote males die as pharate adults. The *w D-raf^{C110}* hemizygote parental males live and have red eyes because they carry the Y chromosome [*Dp (1;Y) w^{+303}*] which carries a fragment encompassing the *w^{+} D-raf^{+}* region from the X chromosome. If a suppressor mutation (*Su*) is present, *w D-raf^{C110}/Y* male progeny live and can be distinguished from the paternal males by their white eye color.

In *Drosophila*, *D-raf* plays multiple roles throughout development in the *torso*, *sevenless* and *DER* signaling pathways (Ambrosio *et al.*, 1989; Dickson *et al.*, 1992; Melnick *et al.*, 1993; Brand and Perrimon, 1994). We have isolated mutations which suppress a partial loss of function mutation in *D-raf*. Both intragenic and extragenic suppressors were recovered. The extragenic mutations define at least four genetic loci. We have shown that these mutations affect the development of ommatidia in the eye and the *DER*-mediated dorsal/ventral patterning of egg chambers during oogenesis. One of the extragenic mutations is allelic to *D-mek* which encodes *Drosophila* MEK, D-MEK (Tsuda *et al.*, 1993).

Results

Isolation of D-raf suppressors

To identify new genes involved in *D-raf* signaling, we decided to design an F1 interactive screen to isolate suppressors of a hypomorphic *D-raf* allele, *D-raf^{C110}*. *D-raf^{C110}* contains a single amino acid change, R217 → L (Melnick *et al.*, 1993), within the CR1 domain which has been shown recently to mediate binding to GTP·Ras. It is probable that *D-raf^{C110}* decreases the effectiveness of the interaction between *D-raf* and *Ras1* (Vojtek *et al.*, 1993). The residual activity of *D-raf^{C110}* is just slightly below what is required for survival. *D-raf^{C110}*-associated lethality occurs at late pupal stages and is fully penetrant since all *D-raf^{C110}* hemizygous males die either in the pupal case or a few hours following emergence (Perrimon *et al.*, 1985). These features allow us to isolate genetic suppressors that rescue *D-raf^{C110}* lethality presumably by up-regulating the

Table I. Characteristics of the *Su(D-raf)*

Mutation	Location	Viability			Oogenesis	Eye development	
		C110; Su/ C110	C110; Su/ EA75	Su/Su	(Percentage with two appendages)	Regularity	Percentage with R7
Su(D-raf)3	X - 0.9 ± 0.4 cM	98%	V	V	ND	WT	ND
Su(D-raf)8	X - 0.9 ± 0.4 cM	96%	V	V	ND	WT	ND
Su(D-raf)34B	X - 21.6 cM	61%	L	V	0.40	WT	88
Su(D-raf)5	X - 0.9 ± 0.4 cM	30%	L	V	ND	WT	ND
Su(D-raf)2	X - 0.9 ± 0.4 cM	18%	L	V	ND	WT	ND
Su(D-raf)6	III - 42 ± 2 cM	V	L	V	23	– –	ND
Su(D-raf)43B	III - 48 ± 2 cM	V	L	V	13	–	ND
Su(D-raf)9	II	V	L	ND	0.16	– –	ND
Su(D-raf)1	II - 55 ± 1 cM	V	L	L	62	–	64
Su(D-raf)7	II	V	L	ND	17.5	– –	ND
Su(D-raf)4	II or III	V	L	ND	0.6	– – – –	ND
D-raf^{C110}	X - 0.9 ± 0.4 cM					– – – – – –	22

The locations of *Su(D-raf)* mutations were determined as described in Materials and methods. To determine the strength of each X-linked suppressor, progeny from the cross between *FM7/D-raf^{C110}* females with *D-raf^{C110} w Su(D-raf)/Y* males were scored. The percentage (%) viability is calculated by dividing the number of *D-raf^{C110}/D-raf^{C110} Su(D-raf)* female progeny by the number of *FM7/D-raf^{C110} Su(D-raf)* females. The activities during oogenesis were measured as the percentage of eggs with two dorsal appendages among eggs laid by *D-raf^{C110}/D-raf^{C110}* females that carry one copy of the suppressor. The activities in eye development were determined by the appearance of ommatidia in *D-raf^{C110}* males carrying one copy of each suppressor. The number of '–' indicates the relative degree of disorganization of the eye. The percentages of ommatidia that contain R7 photoreceptor cells are shown. ND, not determined. Two suppressors, *Su(D-raf)^{3}* and *Su(D-raf)^{8}*, are strong enough to allow *D-raf^{C110}/D-raf^{EA75}* females to survive (*D-raf^{EA75}* is a complete loss of function *D-raf* allele; Ambrosio *et al.*, 1989). Because the mutagenized male parent of genotype *D-raf^{C110}/Dp(1;Y)w^{+303}* carries a duplication of *D-raf^{+}* on the Y chromosome, we tested whether these suppressors may be generated by a translocation of the *D-raf^{+}* gene from *Dp(1;Y)w^{+303}*. *In situ* hybridization to polytene chromosome with a *D-raf* cDNA probe showed that *Su(D-raf)^{3}* only had a single band of hybridization located at 2F6 where *D-raf* maps (data not shown). All *Su(D-raf)/+* heterozygotes show no dominant visible phenotypes other than the suppression of *D-raf^{C110}* lethality. Homozygous *Su(D-raf)^{6}*, *Su(D-raf)^{34B}* and *Su(D-raf)^{43B}* flies have no visible phenotypes. *Su(D-raf)^{1}* is homozygous embryonic lethal (the lethality maps to the same site as the suppressing activity). *Su(D-raf)^{1}* dead embryos show collapsed head skeleton and deletion of a discrete part of the posterior filzkorper (data not shown) suggesting that *Su(D-raf)^{1}* may be involved in terminal development. *tll* and *ftz*, two of the target genes regulated by *torso*, are expressed normally in these embryos (data not shown).

level of signal transmission. Using the scheme shown in Figure 1, we have isolated 11 independent suppressors from ~400 000 progeny that carry mutagenized chromosomes. Five of these suppressors are X-linked and six are autosomal mutations. The genetic properties of these mutations are summarized in Table I.

Molecular nature of D-raf intragenic suppressors

Four of the five X-linked suppressors map near the *D-raf* locus and they show differential suppressing activities in the order of *Su(D-raf)*^{3} ≥ *Su(D-raf)*^{8} > *Su(D-raf)*^{5} > *Su(D-raf)*^{2} (Table I). To provide evidence that these four suppressors indeed contain compensatory mutations located within *D-raf*^{C110}, we sequenced *D-raf* genomic DNA isolated from *D-raf*^{C110} males that carried the individual suppressor mutations. In each case, the *D-raf* open reading frame acquired one additional nucleotide mutation that resulted in an amino acid change besides the original *D-raf*^{C110} mutation (R217L). Interestingly, these compensatory mutations are of two groups (Figure 2). One group of mutations [*Su(D-raf)*^{3} and *Su(D-raf)*^{2}] were localized, like the *D-raf*^{C110} mutation itself, to the CR1 domain. *Su(D-raf)*^{3} contains the amino acid change F290 → I [amino acid position refers to the *D-raf* sequence described in Melnick *et al.* (1993)]. Both R217 and F290 are conserved residues found in Raf polypeptides from other species, including *Caenorhabditis elegans* Raf encoded by *lin-45* (Han *et al.*, 1993) and vertebrate C-raf-1 and A-raf-1 [reviewed in Heidecker *et al.* (1992)]. *Su(D-raf)*^{2} also reveals a change in a conserved residue in the CR1 domain, P308 → L. The second group of compensatory mutations [*Su(D-raf)*^{8} and *Su(D-raf)*^{5}] are located in the CR3 or kinase domain. *Su(D-raf)*^{8} has G621 changed to S. G621 is located between kinase subdomains 7 and 8 and is a conserved residue among Raf kinases, but not among other serine/threonine kinases. *Su(D-raf)*^{5} has mutated L733 → Q. L733 is located at the end of subdomain 11 at the very C-terminus of the kinase domain. This residue is not conserved among Raf proteins from different species. Our results indicate that compensatory mutations of *D-raf*^{C110} appear clustered in either the CR1 or the kinase domains, although the strength of a suppressor does not correlate with a specific domain of the D-Raf protein.

Su(D-raf)^{34B} *encodes D-MEK*

The other X-linked suppressor, *Su(D-raf)*^{34B}, maps to 21.6 cM (Table I), a location similar to *D-mek* (Tsuda *et al.*, 1993). To test whether *Su(D-raf)*^{34B} represents a mutation in *D-mek*, *D-mek* genomic DNA derived from the *Su(D-raf)*^{34B} chromosome was sequenced and shown to contain a single base pair mutation which changes D83 → V. D83 is located eight amino acids N-terminal to the GXGXXG ATP binding motif. This position is usually occupied by an acidic residue in all serine/threonine kinases. To demonstrate that the D83V mutation in D-MEK is sufficient for the activity of *Su(D-raf)*^{34B}, we tested the ability of *P*-element transformants of *D-mek*, that contain the D83V mutation, to rescue the *D-raf*^{C110} mutation. Wild-type *D-mek* genomic DNA (*D-mek*^{wt}) was isolated and cloned into a *P*-element vector to generate a transgenic fly strain *D-mek*^{wt}*-20J*, which carries the *D-mek*^{wt} construct on the second chromosome (see Materials and methods). *D-mek*^{wt}*-20J* is able to rescue the zygotic lethality of a loss of function mutation in *D-mek*, *LH110* (see Materials and methods). However, it is unable to rescue the lethality associated with the *D-raf*^{C110} mutation. Next, D83 was mutated to V in *D-mek*^{wt} genomic DNA to generate a transgenic fly strain carrying *D-mek*^{D83V}. In contrast to *D-mek*^{wt}*-20J*, *D-mek*^{D83V} can rescue both *LH110* and *D-raf*^{C110} hemizygotes into fully viable and fertile males. These results demonstrate that the D83V mutation in *D-mek* is sufficient to generate the suppressing activity of *Su(D-raf)*^{34B}.

D-raf suppressors affect eye imaginal development

The seven extragenic suppressors were tested for their ability to rescue a temperature-sensitive *D-raf* allele, *D-raf*^{HM7}. At the non-permissive temperature of 29°C, *D-raf*^{HM7} causes a reduction of *D-raf* activity and the mutant animals die at late pupal stages. All seven extragenic suppressors rescued *D-raf*^{HM7}-associated lethality at the restrictive temperature (29°C). In addition, rescued *D-raf*^{C110} or *D-raf*^{HM7} males that carry a copy of the different suppressors have substantially more organized or near wild-type ommatidia in the eye (Figure 3C1 and C2; Table I). This allele non-specific nature of the suppressors suggests that they function by up-regulating *D-raf* signaling pathways. To further substantiate this observation, we decided to examine the

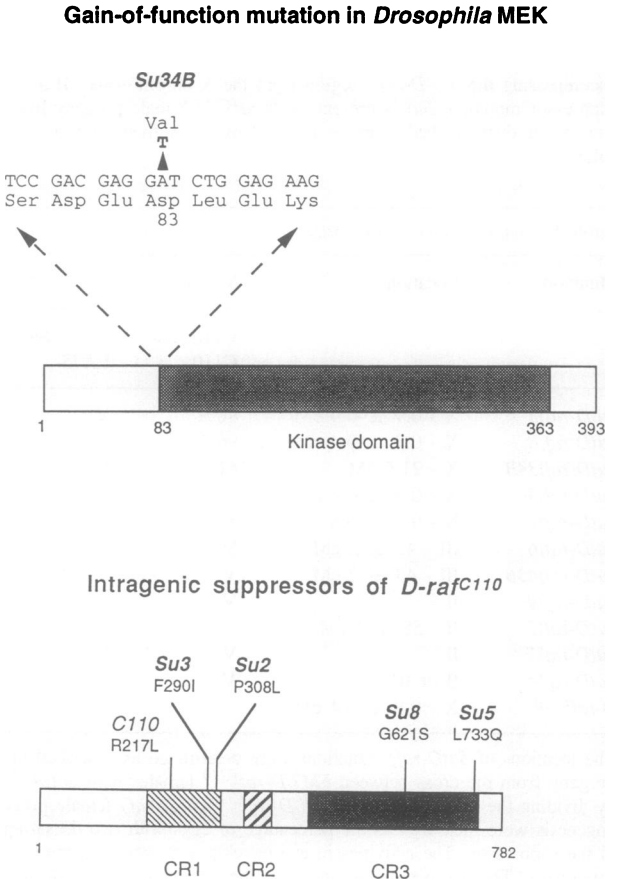

Fig. 2. Molecular lesions of intragenic *Su(D-raf)* and *Su(D-raf)*^{34B}. The horizontal bars represent polypeptide chains with amino acid positions indicated at the bottom. The amino acid changes associated with the different mutations are indicated.

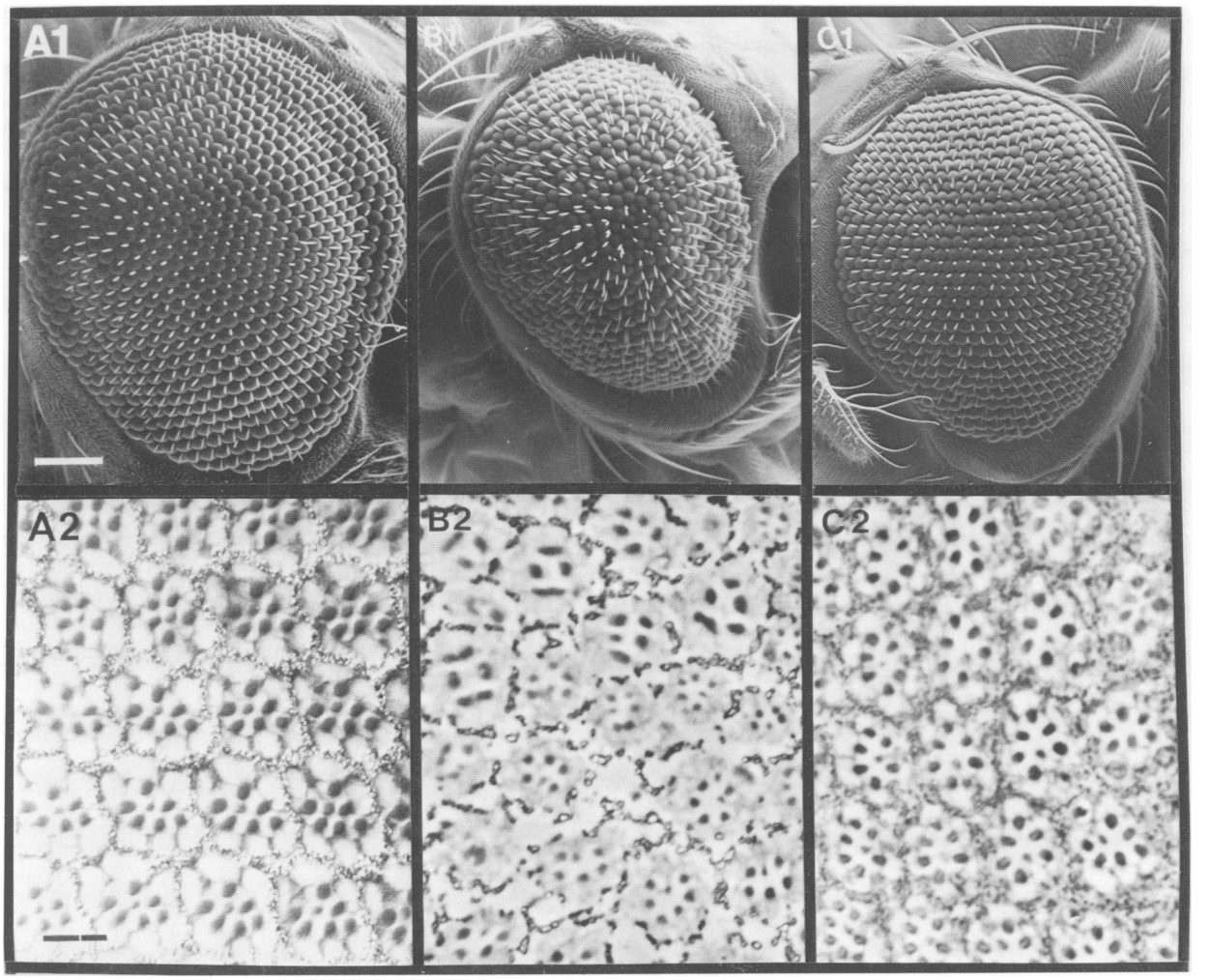

Fig. 3. *Su(D-raf)* rescues the eye defects associated with the *D-raf*^{C110} mutation. Scanning electron micrographs (top panel) and cross sections (bottom panels) of fly compound eyes. The eye derived from a wild-type male (**A1**) consists of regular arrays of ommatidia. The cross section of each ommatidium shows seven photoreceptor cells (**A2**). The eye derived from a *w D-raf*^{C110}*/Y* male (**B1**) is smaller and severely disorganized due to missing photoreceptor cells and fusion of ommatidium units (**B2**). The eye derived from a *w D-raf*^{C110} *Su(D-raf)*^{34B}*/Y* male (**C1** and **C2**) has rescued most of the cellular defects shown in (B1) and (B2).

effects of suppressors in eye imaginal development where *D-raf* mediates cell proliferation, specification and differentiation (Dickson *et al.*, 1992; Melnick *et al.*, 1993). As shown by an eye derived from a *D-raf*^{C110} male (Figure 3B1 and B2), reduction of *D-raf* activity by *D-raf*^{C110} causes three kinds of defects. First, cell proliferation is impaired resulting in smaller eyes with a decreased number of ommatidia. Second, outer photoreceptor cells (R1−6) in the ommatidia are frequently missing. Third, ~78% of the ommatidia lack the R7 photoreceptor cells due to subnormal levels of signaling in the *sevenless* pathway. These three aspects of cellular defects can be suppressed simultaneously by all the extragenic suppressors in varying degrees (Table I). For example, compared with eyes derived from *D-raf*^{C110} males, the eyes derived from *D-raf*^{C110} *Su(D-raf)*^{34B} males are larger; all the ommatidia contain the six outer photoreceptor cells and only 12% of ommatidia lack R7 cells (Figure 3C1 and C2). These results suggest that extragenic suppressors operate by up-regulating *D-raf* signaling in many aspects of eye imaginal development, including the *sevenless* pathway.

D-raf suppressors affect the DER pathway during oogenesis

The *Drosophila* egg has a distinct dorsal/ventral (D/V) polarity which is recognized easily by the presence of dorsal appendages on the anterior dorsal surface of the egg shell (Figure 4A). This D/V polarity arises during oogenesis in the egg chamber through an inductive mechanism involving *DER* (Schupbach, 1987; Price *et al.*, 1989). Reduction of *DER* activity by the *torpedo* mutation results in ventralized eggs with fused dorsal appendages (Price *et al.*, 1989). Similar ventralized eggs are produced by homozygous *D-raf*^{HM7} females (Figure 4B), suggesting that *D-raf* mediates *DER* signaling during oogenesis (Brand and Perrimon, 1994; J.Duffy and N.Perrimon, unpublished data). Because *D-raf*^{C110} has less activity than *D-raf*^{HM7} (Melnick *et al.*, 1993), it is conceivable that *D-raf*^{C110} homozygous females, if alive, would only produce ventralized eggs. To test if the suppressors may also affect the *DER* pathway during oogenesis, we generated *D-raf*^{C110} homozygous females that carry one copy of each of the seven extragenic suppressors. *D-raf*^{C110} homozygous females

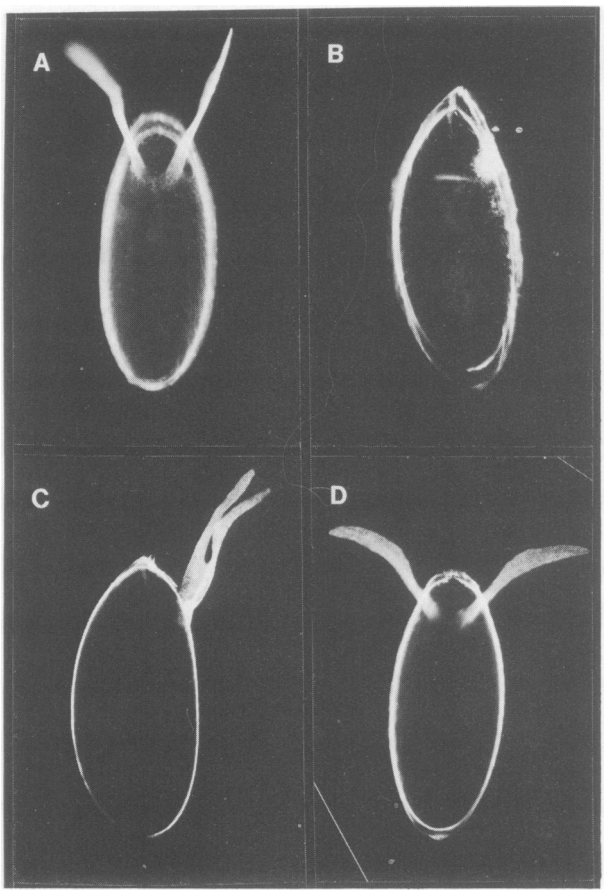

Fig. 4. Maternal activities of *Su(D-raf)* are involved in determining the dorsal/ventral polarity of the chorion. (**A**) An egg laid by a wild-type female. Note that the egg shell has a distinct dorsal/ventral polarity clearly marked by the location of the two dorsal chorionic appendages. (**B**) An egg laid by a *D-raf*^{HM7} homozygous female. Note that the egg shell is ventralized as the dorsal appendages are fused dorsally. This ventralized phenotype can be suppressed by extragenic *Su(D-raf)*. An example of suppressed chorion is shown in (**C**) and (**D**) which are derived from *D-raf*^{C110} homozygous females carrying one copy of *Su(D-raf)*^{43B} and *Su(D-raf)*^{1}, respectively.

carrying one copy of either *Su(D-raf)*^{1}, *Su(D-raf)*^{6}, *Su(D-raf)*^{7} and *Su(D-raf)*^{43B} produced a significant proportion of eggs with the normal two dorsal appendages (Figure 4D; Table I). In the case of *Su(D-raf)*^{1}, 62% of the eggs have acquired two dorsal appendages. The remaining eggs show variably fused dorsal appendages (Figure 4C). *D-raf*^{C110} homozygous females carrying one copy of either *Su(D-raf)*^{34B}, *Su(D-raf)*^{9} or the weak *Su(D-raf)*^{4}, produced few eggs with two appendages (Table I). These results suggest that mutations isolated as suppressors of *D-raf*^{C110} lethality affect the signaling pathway mediated by *DER*.

The role of D-raf suppressors in torso signaling

To test the potential roles of various suppressors in the *torso* pathway, we determined whether one copy of the extragenic suppressor is able to suppress mutations that affect the receptor (*torso*) or its activation (*Nasrat*) [reviewed in Lu *et al.* (1993)]. In a previous study, *D-mek*^{Su1} has been shown to rescue *tailless* (*tll*) expression and the development of posterior terminal structures in *torso* and *D-raf* mutant embryos (Tsuda *et al.*, 1993). In contrast, the three strong suppressors, *Su(D-raf)*^{6}, *Su(D-raf)*^{34B} and *Su(D-raf)*^{43B}, when expressed maternally, do not appear to rescue the expression of *tll* in embryos derived from mothers devoid of either *torso* RPTK or *Nasrat* gene activities. However, it should be pointed out that the extragenic suppressors isolated in our screen are weak dominant activating mutations. This is apparent from data shown in Table I: our suppressors, unlike *D-mek*^{Su1}, do not rescue the viability of *D-raf*^{C110}/*D-raf*^{null} females. Thus, the observation that our suppressors do not rescue the *tll* expression in terminal mutants does not necessarily mean that these genes have no function in the *torso* pathway. In fact, *Su(D-raf)*^{34B} is allelic to *D-mek*^{Su1} which mediates *torso* signaling.

Discussion

We have isolated dominant mutations which rescue the late pupal lethality associated with a partial loss of function mutation *D-raf*^{C110}. Four intragenic and seven extragenic suppressors were recovered. The extragenic suppressors represent at least four genetic loci: one on the X [*Su(D-raf)*^{34B}] which identifies *D-mek*, one on the second [*Su(D-raf)*^{1}] and two on the third chromosome [*Su(D-raf)*^{43B} and *Su(D-raf)*^{6}]. The remaining three extragenic suppressors, 4, 7 and 9, have not been mapped to specific chromosomal regions (Table I).

Intragenic suppressors and their structural/functional relevance to the regulation of Raf kinase

The compensatory amino acid changes found in *D-raf*^{C110} intragenic suppressors are located either in the CR1 domain where the *D-raf*^{C110} mutation resides (Melnick *et al.*, 1993), or in the CR3 kinase domain. The CR1 domain has been shown recently to mediate the binding of Raf to the activated GTP-bound form of Ras (see Introduction). It is conceivable that the *D-raf*^{C110} mutation decreases the efficiency of this activating interaction, thus reducing D-raf activity (Vojtek *et al.*, 1993). Mutations in the CR1 domain that would increase the interaction with Ras could potentially suppress the *D-raf*^{C110} mutation. However, two compensatory mutations were found in the kinase domain suggesting that CR1 may also interact with the kinase domain. There are two potential roles for the putative CR1−kinase interaction. First, CR1 may bind and inhibit the kinase activity with a relief of inhibition once GTP-Ras binds CR1. In other words, GTP-Ras activates Raf by binding competitively to the CR1 domain. Second, GTP-Ras may serve as a positive allosteric factor to activate the kinase activity via contacts between CR1 and the kinase domain. Since truncation of the N-terminal half of the Raf molecule gives rise to a constitutively activated kinase, it is probable that the first explanation more accurately reflects how Raf responds to the interaction with GTP-Ras. We are in the process of testing the interaction between the CR1 and kinase domains.

D-raf extragenic suppressors are likely to encode novel signaling molecules

We have examined how *D-raf* extragenic suppressors affect the development of ommatidia in the eye and the *DER*-mediated dorsal/ventral patterning of egg chambers during oogenesis. These analyses have led us to demonstrate that the extragenic suppressors are strong candidates for new genes involved in signal transduction from RPTKs. First,

all the extragenic suppressors suppress *D-raf*^{HM7}, another hypomorphic mutation in *D-raf*. The allele non-specific nature of these suppressors suggests that they compensate for the reduction in *D-raf* activity by elevating the level of signaling at another step in the pathway. Second, *D-raf* is involved in cell proliferation, differentiation (Perrimon *et al.*, 1985; Nishida *et al.*, 1988) and *sevenless*-mediated specification of R7 photoreceptor cells in the eye imaginal disc (Dickson *et al.*, 1992). Reduction of *D-raf* activity by *D-raf*^{C110} mutation affects cell proliferation resulting in a decrease in the number of ommatidia in the eye. The ommatidia that do form are disorganized and most of them have lost R7 as well as other outer photoreceptor cells (Figure 3B2; Table I). All these cellular defects associated with *D-raf*^{C110} are rescued by the suppressors suggesting that the extragenic suppressors identify genes involved in the *sevenless* and other unidentified RPTKs involved in eye imaginal development. Consistent with this notion, the suppressors also show positive effects on the dorsal/ventral patterning of the egg shell, presumably by enhancing the level of *DER* signal transmission (Figure 4; Table I).

One of the extragenic suppressors, *Su(D-raf)*^{34B}, is an allele of *D-mek* which encodes the fly homolog of mammalian MEK. A single nucleotide mutation corresponding to the amino acid change D83V was identified in the *D-mek* coding region derived from the *Su(D-raf)*^{34B} chromosome. Mutational analysis demonstrated that this point mutation alone is sufficient to generate the suppressive activity of *Su(D-raf)*^{34B}. To date, MEK activity has been shown only to be activated following phosphorylation (Kyriakis *et al.*, 1992). The D83V mutation is the first dominant gain-of-function mutation so far identified in MEK. This mutation provides a convenient way to generate constitutively active MEK for biochemical studies.

RPTKs are known to activate a common set of signaling molecules. Molecular genetic analyses of the *torso*, *sevenless* and *DER* pathways have so far yielded few specific signal transducers unique to one of the pathways. Genes identified by various interactive screens designed specifically for one pathway have often turned out to affect other pathways as well [Simon *et al.*, 1991; Doyle and Bishop, 1993; reviewed by Perrimon (1993)]. Thus, it is not surprising that the suppressors we have isolated affect both the *DER* and the *sevenless* pathways. The fact that the extragenic mutations isolated in our screen are not specific to one RPTK pathway strengthens the conclusion that they encode crucial signaling molecules involved in *D-raf* signaling.

Differential sensitivities of signaling pathways

Genetic screens based on dosage interaction have been very successful in isolating genes involved in signaling (Gertler *et al.*, 1989; Simon *et al.*, 1991; Doyle and Bishop, 1993). This implies that the amount of a signal transducer present in a given cell can greatly affect its sensitivity to developmental cues. Expression of these molecules may normally be tightly regulated in various pathways to adapt to the needs of a particular developmental setting. It is apparent that different signaling pathways have different sensitivities to alteration in the activity of a signal transducer. For example, reduction of *D-raf* activity by the *D-raf*^{C110} mutation does not impair the *torso* pathway (Melnick *et al.*, 1993). However, the *D-raf*^{C110} mutation causes lethality and severe defects in the differentiation of photoreceptor cells. Similarly, the activity levels of *D-raf* suppressors in different assays (the viability test versus the oogenesis assay) do not always correlate well with each other (Table I and data not shown), indicating that there is differential utilization of signal transducers by different signaling pathways. In terms of assigning mutations to different pathways, additional confusion can arise from the fact that a given gene can be mutated to acquire different levels of constitutive activity. For example, even though *Su(D-raf)*^{34B} (*D-mek*^{Su34B}) and *D-mek*^{Su1} are allelic, the maternal activity of *Su(D-raf)*^{34B}, unlike *D-mek*^{Su1}, does not rescue *tll* expression and posterior terminal structures in *torso* or *D-raf* null mutant embryos. These data show that *D-mek*^{Su1} does not require an upstream signal for its activation, whereas full activation of *D-mek*^{Su34B} remains dependent on an upstream activating signal. The differences between *D-mek*^{Su34B} and *D-mek*^{Su1} suggest that *D-mek* can be mutated at different sites to become either a constitutive or a partially constitutive kinase. These observations stress the importance of characterizing null mutations for all genetic modifiers isolated to fully appreciate the function of a specific gene in signaling.

Materials and methods

Mutagenesis

The scheme used to isolate suppressors of the *D-raf*^{C110} mutation is shown in Figure 1. Males of genotype *D-raf*^{C110}*/Dp(1;Y) w*$^{+303}$ (screen A) or *D-raf*^{C110} *w/Dp(1;Y)w*$^{+303}$ (screen B) were fed with 1% sucrose solution containing 12 mM ethyl methane sulfonate (EMS; Sigma) for 18 h (Lewis and Bacher, 1968). Batches of mutagenized males were crossed at 25°C to virgin females obtained from the virginator stock *C(1)DX, y f/Y* crossed with *w v l*^{44ts}*/Y* males (Komitopoulou *et al.*, 1983). The parent flies were transferred daily for a period of 5 days and the F1 progeny were shifted to 29°C to kill any *w v l*^{44ts}*/Y* males that may be present in the F1 progeny produced by non-virgin females. Putative suppressors were identified among the progeny by the presence of viable *D-raf*^{C110} *w/Y* or *D-raf*^{C110}*/Y* males. Independent lines of suppressors were established by crossing a single *D-raf*^{C110} *w/Y* or *D-raf*^{C110}*/Y* viable male to *C(1)DX, y f /Y* virgin females. In screen A, ~100 000 mutagenized individuals were screened and two independent suppressors, *Su(D-raf)*^{34B} and *Su(D-raf)*^{43B}, were recovered. In screen B, ~300 000 chromosomes were screened and nine independent suppressors were recovered. The number of chromosomes screened was estimated from the number of *C(1)DX, y f/Dp(1;Y)w*$^{+303}$ females recovered. Descriptions of balancers and mutations that are not described in the text can be found in Lindsley and Zimm (1992).

We chose a dose of EMS that would generate ~30% of X-linked lethals. The amount of EMS used for these mutageneses was first determined and then controlled in each experiment. To control for the efficiency of the mutageneses, a set of wild-type males (*Ore R*) was mutagenized under the same conditions at the time of both screens and crossed to *C(1)DX, y f/Y* virgin females to determine the percentage of zygotic lethal mutations by calculating the ratio of males versus females following correction for the reduced viability of the attached-X females. The percentage of X-linked lethal mutations induced in our mutagenesis was ~30%, indicating that the low recovery of suppressors is not due to poor efficiency of the mutagenesis but instead the rarity of the event leading to their recovery.

Mapping of the suppressors

To assign mutations to specific chromosomes, *C(1)DX, y f/Y* virgin females from different suppressor stocks were crossed to *D-raf*^{C110} *w/Dp(1;Y) w*$^{+303}$ males. If no *D-raf*^{C110} *w* males are present in the F1 progeny, the suppressor is X-linked; otherwise, the suppressor is autosomal. The autosomal mutations were further localized to specific autosomes using two stocks, *w;Sco/CyO* for the second and *w;Cx*^{D}*/TM3,Sb* for the third chromosomes.

Suppressors were meiotically mapped by their suppressing activities. The X-linked suppressors were mapped with respect to *w*$^{+}$. Females of genotype *D-raf*^{C110} *w Su(D-raf)/D-raf*^{C110} + + were crossed to *FM7/Y* males. White eye males (genotype *D-raf*^{C110} *w Su(D-raf)/Y*) and red eye males [*D-raf*^{C110} *w*$^{+}$ *Su(D-raf)/Y*] were counted among F1 progeny to calculate the distance between *w* and the *Su(D-raf)*. Autosomal mutations

were mapped using dominant markers (*Ki*, *Sb*, *W* and *Sco*) in a manner analogous to the X-linked suppressors.

Identification of Su(D-raf)^{34B} null mutation

An independently isolated X-linked mutation, *LH110*, induced on the *y w FRT*^{101} chromosome (Chou and Perrimon, 1992) and exhibiting a terminal class phenotype, was sent to us by T.Schupbach (personal communication). We found that this mutation complemented all known terminal class genes on the X chromosome [*fs(1)Nasrat*, *fs(1)pole hole*, *l(1)corkscrew* and *l(1)pole hole*; data not shown]. Mapping of *LH110* using the polymarked X chromosome *sc ec cv ct g* indicated that the lethal mutation mapped roughly around the position of a previous mutation, *D-mek*^{Su1} (Tsuda *et al.*, 1993). Proof of the allelism between *D-mek*^{Su1} and *LH110* was obtained following a complementation test with a loss of function mutation in *D-mek*, *D-mek*^{r1} (Tsuda *et al.*, 1993), kindly performed by Y.Nishida.

Molecular characterization of Su(D-raf)^{34B}

Genomic DNA was isolated from *D-raf*^{C110} *Su(D-raf)*^{34B}*/Y* flies by the method of Junakovic and Angelucci (1986). DNA was PCR-amplified using five sets of primers designed according to the *D-mek* sequence (Tsuda *et al.*, 1993; Genbank accession number D13782). These primers generated five overlapping fragments from nucleotide 360 to 880, 820 to 1290, 1190 to 1690, 1690 to 2260 and 2150 to 2570 of the genomic sequence spanning *D-mek*. PCR primers contained *Bam*HI sites to facilitate cloning of the PCR products into pBSK (Stratagene). PCR conditions were as described in Melnick *et al.* (1993). DNA sequencing employed the dideoxy termination protocol described by Del Sal *et al.* (1989).

Site-directed mutagenesis

To isolate *D-mek* genomic DNA, one of the PCR products mentioned above, namely nucleotides 360–880, was labeled by nick translation and used to probe a *Drosophila* genomic library in EMBL3 (Blackman *et al.*, 1987). A positive clone with a 15 kb insert was isolated and mapped by restriction enzymes. A 9 kb *Bam*HI–*Sal*I fragment which extends ~3 kb both 5′ and 3′ of the *D-mek* transcription unit was identified. This fragment was cloned into pCaSpeR4 (Thummel *et al.*, 1988) to create the *D-mek* rescuing construct *D-mek*^{wt}. A smaller 5 kb *Not*I–*Eco*RI fragment derived from the 9 kb genomic DNA was subcloned into pBSK for ease in performing site-directed mutagenesis. The unique change found in the *D-mek* open reading frame derived from a *Su(D-raf)*^{34B} chromosome was introduced into the 5 kb genomic clone using the method of Deng and Nickoloff (1992) with the following two modifications. First, the primer which incorporates the proposed change and the primer which changes a unique restriction site in the plasmid were chosen from opposite strands instead of the same strand. This pair of primers was used first to do PCR on wild-type DNA template. The resulting PCR product was then used to prime the synthesis of full-length mutated plasmid using T4 polymerase. We found that this approach is necessary to generate appreciable yields of plasmid that have incorporated both changes. Second, both mutagenesis primers were synthesized with phosphorylating amidite (Pharmacia) at the 5′ position instead of kinasing after synthesis. The sequence of the primer introducing the D83 → V amino acid change is 5′-PGCTTCTCCAGAACCTCGTCGGAC-3′; the sequence of the primer mutating the unique *Hin*dIII site in pBSK is 5′-PCC-TCGAGGTCGTCGGTATCGATATGCTTGATATC-3′.

For the PCR, 0.1 μg *Not*I–*Eco*RI 5 kb genomic DNA in pBSK were linearized with *Not*I and mixed with 1 μg of each primer, 10 μl 10 × PCR buffer (Perkin Elmer Cetus), 10 μl 2.5 mM dNTPs, 5 U Taq polymerase (Perkin Elmer Cetus) and dH_2O to the final volume of 100 μl. PCR conditions were 30 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C, followed by a final incubation of 10 min at 72°C. The PCR product was phenol extracted, ethanol precipitated and used instead of the selection and mutagenic primers in the Deng and Nickoloff (1992) protocol. After transformation, 25 colonies were picked and minipreps of plasmid DNA sequenced to check for the desired D83 → V mutation in *D-mek*. Even with these modifications only one-fifth of the colonies contained the mutation. We sequenced the coding portion of the mutagenized plasmid between the D83V mutation and the former *Hin*dIII site in pBSK to make sure we had not introduced PCR errors that might result in amino acid changes in *D-mek*. We then excised the *Not*I–*Eco*RI fragment and replaced it into the larger 9 kb *D-mek*/pCaSpeR4 genomic clone and checked this clone by sequencing for the presence of the D83V mutation before injecting the *D-mek*^{D83V} construct into fly embryos to make the transformant *D-mek*^{D83V} (Spradling, 1986).

Molecular characterization of the intragenic suppressors

Genomic DNA was isolated from male flies carrying *Su(D-raf)*^{2}, *Su(D-raf)*^{3}, *Su(D-raf)*^{5} and *Su(D-raf)*^{8} and used to isolate *D-raf* genomic DNA fragments by PCR amplification as described in Melnick *et al.* (1993). The consensus from three independent PCR clones was compared with the wild-type *D-raf* sequence (Genbank accession number L10626) to identify putative intragenic mutations.

Nomenclature

The nomenclature of *D-mek*, *D-mek*^{Su1} and *D-mek*^{r1} used above was suggested recently by Y.Nishida. *D-mek*^{Su1} is the same as *Dsor1*^{Su1} described by Tsuda *et al.* (1993).

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References

Aelst,L.V., Barr,M., Marcus,S., Polverino,A. and Wigler,M. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 6213–6217.

Ambrosio,L., Mahowald,A.P. and Perrimon,N. (1989) *Development*, **106**, 145–158.

Blackman,R.K., Grimalia,R., Koehler,M.M.D. amd Gelbart,W.M. (1987) *Cell*, **49**, 497–505.

Brand,A.H. and Perrimon,N. (1994) *Genes Dev.*, **8**, 629–639.

Bruder,J.T., Heidecker,G. and Rapp,U.R. (1992) *Genes Dev.*, **6**, 545–556.

Chou,T.B. and Perrimon,N. (1992) *Genetics*, **131**, 643–653.

Courchesne,W.E., Kunisawa,R. and Thorner,J. (1989) *Cell*, **58**, 1107–1119.

Del Sal,G., Manfioletti,G. and Schneider,C. (1989) *Biotechniques*, **7**, 514–519.

Deng,W.P. and Nickoloff,J.A. (1992) *Anal. Biochem.*, **200**, 81–88.

Dent,P., Haser,W., Haystead,T.A.J., Vincent,L.A., Roberts,T.M. and Sturgill,T.W. (1992) *Science*, **257**, 1404–1406.

Dickson,B., Sprenger,F., Morrison,D. and Hafen,E. (1992) *Nature*, **360**, 600–602.

Doyle,H.J. and Bishop,M.J. (1993) *Genes Dev.*, **7**, 633–646.

Egan,S.E. and Weinberg,R.A. (1993) *Nature*, **365**, 781–783.

Elion,E.A., Grisafi,P.L. and Fink,G.R. (1990) *Cell*, **60**, 649–664.

Elion,E.A., Brill,J.A. and Fink,G.R. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9392–9396.

Gertler,F.B., Bennett,R.L., Clark,M.J. and Hoffmann,F.M. (1989) *Cell*, **58**, 103–113.

Han,M., Golden,A., Han,Y. and Sternberg,P.W. (1993) *Nature*, **363**, 133–140.

Heidecker,G., Huleihel,M., Cleveland,J.L., Kolch,W., Beck,T.W., Lioyd,P., Pawson,T. and Rapp,U.R. (1990) *Mol. Cell. Biol.*, **10**, 2503–2512.

Heidecker,G., Kolch,N., Morrison,D. and Rapp,U.R. (1992) *Adv. Cancer Res.*, **58**, 53–72.

Junakovic,N. and Angelucci,V. (1986) *J. Mol. Evol.*, **24**, 83–88.

Komitopoulou,K., Gans,M., Margaritis,L.H., Kafatos,F.C. and Masson,M. (1983) *Genetics*, **105**, 897–920.

Kyriakis,J.M., App,H., Zhang,X., Banerjee,P., Brautigan,D.L., Rapp,U.R. and Avruch,J. (1992) *Nature*, **358**, 417–421.

Lange-Carter,C.A., Pleiman,C.M., Gardner,A.M., Blumer,K.J. and Johnson,G.L. (1993) *Science*, **260**, 315–319.

Levin,D.E. and Errede,B. (1993) *NIH Res.*, **5**, 49–52.

Lewis,E.B. and Bacher,F. (1968) *Drosophila Inform. Serv.*, **43**, 193.

Lindsley,D. and Zimm,G. (1992) *The Genome of Drosophila melanogaster*. Academic Press, New York.

Lu,X., Perkins,L.A. and Perrimon,N. (1993) *Development*, Supplement in press.

Melnick,M.B., Perkins,L.A., Lee,M., Ambrosio,L. and Perrimon,N. (1993) *Development*, **118**, 127–138.

Nakeilny,S., Cohen,P., Wu,J. and Sturgill,T.W. (1992) *EMBO J.*, **11**, 2123–2129.

Nishida,Y., Hata,M., Ayaki,T., Ryo,H., Yamagata,M., Shimizu,K. and Nishizuka,Y. (1988) *EMBO J.*, **7**, 775–781.

Perrimon,N. (1993) *Cell*, **74**, 219–222.

Perrimon,N., Engstrom,L. and Mahowald,A.P. (1985) *Dev. Biol.*, **110**, 480–491.

Price,J.V., Clifford,R.J. and Schupbach,T. (1989) *Cell*, **56**, 1085–1092.
Rapp,U.R., Cleveland,J.L., Bonner,T.I. and Storm,S.M. (1988) In Reddy,P., Curran,T. and Skalra,A. (eds), *Handbook of Oncogenes*. Elsevier/North-Holland Publishing Co., Amsterdam, p. 213.
Rhodes,N., Connell,L. and Errede,B. (1990) *Genes Dev.*, **4**, 1862–1874.
Rossomando,A., Wu,J., Weber,M.J. and Sturgill,T.W. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 5221–5225.
Schupbach,T. (1987) *Cell*, **49**, 699–707.
Simon,M.A., Bowtell,D.D., Dodson,G.S., Larverty,T.R. and Rubin,G.M. (1991) *Cell*, **67**, 701–716.
Spradling,A. (1986) In Roberts,D.B. (ed.), *Drosophila: A Practical Approach*. IRL Press, Oxford, UK, pp. 175–198.
Stanton,V.P.,Jr, Nichols,D.W., Laudano,A.P. and Cooper,G.M. (1989) *Mol. Cell. Biol.*, **9**, 639–647.
Thummel,C.S., Boulet,A.M. and Lipshitz,H.D. (1988) *Gene*, **74**, 445–456.
Tsuda,L. *et al.* (1993) *Cell*, **72**, 407–414.
Vojtek,A.B., Hollenberg,S.M. and Cooper,J.A. (1993) *Cell*, **74**, 205–214.
Zhang,X. *et al.* (1993) *Nature*, **364**, 308–313.

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