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A temperature-sensitive MEK mutation demonstrates the conservation of the signaling pathways activated by receptor tyrosine kinases

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MEK, a dual specificity threonine/tyrosine kinase, has been postulated to be a convergent point for signaling from receptor protein tyrosine kinases (RTKs) and G-protein-coupled receptors. In contrast to yeast and mammalian cells where several MEKs have been isolated, only one *Drosophila* MEK (D-Mek) has been characterized to date. Previous studies have shown that D-Mek acts in the Torso RTK signaling pathway. To demonstrate that D-Mek also operates downstream of other RTKs, we generated a temperature-sensitive allele of *D-mek* (*D-mek*^{ts}) by site-directed mutagenesis based on the amino acid change of a yeast *cdc2*^{ts} mutation. Using *D-mek*^{ts}, we show that in addition to its role in Torso signaling, D-Mek operates in the Sevenless and in the *Drosophila* epidermal growth factor RTK pathways. Because loss-of-function mutations in *D-mek* and the upstream receptors give rise to similar phenotypes, it suggests that *D-mek* is the only MEK activated by *Drosophila* RTKs. In addition, we demonstrate that different RTK pathways respond differently to alteration in D-Mek activity.

[*Key Words*: *Drosophila*; signal transduction; pattern formation; Ras; Raf; MEK; MAPK]

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Mitogen-activated protein kinases (MAPKs), including p42^{mapk} and p44^{mapk}, are activated in response to a variety of extracellular signals that trigger cell proliferation or differentiation (Boulton et al. 1991; Her et al. 1991; Rossomando et al. 1991). These signals bind to receptors associated with tyrosine kinase activities such as the epidermal growth factor (EGF) receptor (DER) or receptors that are coupled to G proteins such as the thrombin receptor (L'Allemain et al. 1991). MAPKs are serine/threonine protein kinases that are uniformly distributed in the cytoplasm of quiescent cells. Within minutes following serum stimulation, MAPKs translocate to the nucleus (Chen et al. 1992). The substrates of MAPKs include ribosomal p90^{rsk} kinase (Sturgill et al. 1988), MAPK-activated protein kinase 2 (Stokoe et al. 1992), phospholipase A2 (Lin et al. 1993), and several transcriptional factors, such as c-Myc (Seth et al. 1992), p62^{TCF} (Gille et al. 1992) and Elk-1 (Marais et al. 1993).

Studies on p42 and p44 mammalian MAPKs have demonstrated that the activation of these kinases is dependent on the phosphorylation of two nearby tyrosine and threonine residues by a dual specificity MAPK/ERK kinase (MEK1) (Payne et al. 1991; Crews et al. 1992). In turn, activation of MEK1 requires phosphorylation of two conserved serine residues (Alessi et al. 1994; Zheng and Guan 1994). It appears that MEKs are a convergence point for various stimuli from the cell surface. MEKs can be activated by Raf (Dent et al. 1992; Howe et al. 1992; Kyriakis et al. 1992) following activation of receptor tyrosine kinases (RTKs). RTK pathways are mediated sequentially by adaptor proteins such as Grb-2, guanine nucleotide releasing factor, p21^{ras} GTPase, and Raf (for review, see Pawson and Schlessinger 1993; Vojtek et al. 1993). Alternatively, MEKs can be activated by MEKK following activation of G-protein-coupled receptors (Lange-Carter et al. 1993).

In *Saccharomyces cerevisiae*, related protein kinase modules are used in three different intracellular signal transduction pathways, including the mating response, protein kinase C-mediated cell wall construction, and osmolarity control, with specific MEKs and MAPKs in each pathway (for review, see Errede and Levin 1993). Genetic and biochemical analysis of the pheromone response signal pathway, mediated by a G-protein-coupled receptor, has demonstrated that Fus3 and Kss1 (Elion et al. 1990, 1991), a redundant pair of MAPK homologs, are phosphorylated and activated by STE7 (Gartner et al. 1992; Errede et al. 1993), which is structurally similar to vertebrate MEK. As in yeast, several mammalian MEKs have been isolated (Ashworth et al. 1992; Crews et al. 1992; Seger et al. 1992; Kosako et al. 1993; Wu et al. 1993a,b); however, it is not clear whether different re-

ceptors transmit signals through specific MEKs or whether MEKs are functionally redundant.

A *Drosophila* MEK, Dsor1, also referred to as D-Mek, has been characterized as a component of the Torso (Tor) RTK signaling pathway (Tsuda et al. 1993). Tor is required for the specification of terminal structures (for review, see Perrimon 1993). Activation of Tor initiates a signal transduction pathway that is mediated sequentially by the guanine nucleotide-releasing factor Son of sevenless (Sos), Ras1, and D-Raf. Screens for second site suppressors of a weak *D-raf* allele have led to the identification of gain-of-function (gof) *D-mek* mutations (Tsuda et al. 1993; Lu et al. 1994). Some *D-mek*^{gof} alleles can suppress the terminal defects associated with mutations that block Tor signaling upstream of *D-mek*. This indicates that these *D-mek*^{gof} mutations represent activated forms of D-Mek. In addition, analysis of loss-of-function (lof) *D-mek* mutations has established that D-Mek is an essential component of Tor signaling (Tsuda et al. 1993; Lu et al. 1994).

In *Drosophila* two additional RTK signaling pathways, the *Drosophila* DER and Sevenless (Sev) pathways, have been analyzed extensively. DER is involved in a number of developmental pathways that include the specification of dorsal–ventral polarity of the egg and wing vein formation (Schüpbach 1987; Clifford and Schüpbach 1989). Sev controls the specification of R7 photoreceptor cell fate in the developing eye (for review, see Dickson and Hafen 1993). Genetic studies of the DER and Sev signaling pathways have shown that components such as p21^{ras}, Sos, and D-Raf are common to these pathways (Simon et al. 1991; Dickson et al. 1992a; Melnick et al. 1993; Brand and Perrimon 1994; Diaz-Benjumea and Hafen 1994), contributing to the concept that all RTKs activate a conserved signaling "cassette" (for review, see Perrimon 1993). Because both yeast and mammalian cells contain various MEKs, we tested whether the specificity of RTK signaling lies at the level of MEKs. If D-Mek is required in the Tor, Sev, and DER RTK pathways, then the specificity of signaling may be at the level of a more downstream component.

Because flies carrying a *D-mek*^{lof} mutation die during larval or pupal stages (Tsuda et al. 1993; Lu et al. 1994), we generated a temperature-sensitive (ts) allele of *D-mek* (*D-mek*^{ts}) based on the sequence of a yeast *cdc2*^{ts} allele. By conducting a series of temperature-shift experiments, we were able to bypass the lethality associated with *D-mek*^{lof} mutations and analyze the function of *D-mek* in other RTK pathways. We demonstrate that *D-mek* is not only involved in Tor but also in Sev and DER signaling, suggesting that *D-mek* is likely to encode the only MEK activated by *Drosophila* RTKs. Our results indicate that the specificity of signaling is probably at the level of a more downstream component. However, our analysis also revealed that these pathways show different sensitivity to reduced levels of D-Mek. The possibility that specificity of various RTKs can be determined by the strength and/or duration of the activation of p21^{ras}/Raf/MEK is discussed.

Results

Generation of a temperature-sensitive allele of D-mek

To assay the role of *D-mek* in various RTK signaling pathways, we analyzed the phenotypes associated with loss of *D-mek* activity in developmental processes controlled by Tor, Sev, and DER. Because *D-mek* is expressed throughout development (Tsuda et al. 1993) and available loss-of-function mutations are zygotic lethals, we decided to characterize the mutant phenotypes associated with a temperature-sensitive allele. Using a *D-mek*^{ts} mutation we can shift animals from permissive to restrictive temperatures at different stages and determine the function of *D-mek* in development.

One way to generate a *D-mek*^{ts} mutation is to alter, following site-directed mutagenesis, the *D-mek*-coding sequence such that an amino acid modification renders the activity of the protein temperature sensitive (see also Simon et al. 1991). MEK is a threonine/tyrosine kinase, and sequence comparison with conserved amino acids that distinguish serine/threonine kinases from tyrosine kinases shows that MEK resembles a serine/threonine kinase. Based on the sequence of two temperature-sensitive alleles of the serine/threonine *cdc2* kinase of *Schizosaccharomyces pombe*, we generated two *D-mek* mutations (Fig. 1). The first one, *D-mek*^{ts1}, is based on the *cdc2*^{ts} allele *M26/55* that changes proline^{137} to serine (Carr et al. 1989). This proline resides within the catalytic loop of the kinase subdomain VI (Hanks et al. 1988) but is only moderately conserved among serine/threonine kinases. The second one, *D-mek*^{ts2}, is based on the *cdc2*^{ts} allele *1w/2w*, which changes glycine^{146} to aspartic acid (Carr et al. 1989).

To assay the temperature-sensitive properties of *D-mek*^{ts1} and *D-mek*^{ts2}, we tested the ability of P-element transformants that carry the mutations to rescue the lethality associated with a null *D-mek* mutation, *LH110* (Fig. 2). In this assay *D-mek*^{ts1} was associated with a temperature-sensitive effect (Fig. 2) but not *D-mek*^{ts2} (data not shown). For example, *D-mek* mutant animals that carry one copy of the P-element insertion *D-mek*$^{ts1-6}$ are viable at both 18°C and 20°C but not above 25°C. (Note: We will refer to animals of genotype

cdc2$^{1w/2w}$	ts	D
cdc2$^{M26/55}$	ts	S
cdc2	WT	RDLKPQNLLI DKEGNLK
D-Mek	WT	RDVKPSNILVNS SGEIK
D-Mek^{ts1}	ts	S
D-Mek^{ts2}	mu	D

Figure 1. Generation of temperature-sensitive alleles of *D-mek*. Site-directed mutagenesis was used to generate two new alleles of *D-mek* (*D-mek*^{ts1} and *D-mek*^{ts2}). These changes were based on the sequences of two *cdc2*^{ts} alleles, *M26/55* and *1w/2w* (Carr et al. 1989). Sequence comparisons between portions of the wild-type cdc2, starting from amino acid 133, and D-Mek, starting from amino acid 205, are shown. Shaded boxes indicate the conserved amino acids. The phenotypes of flies carrying these *D-mek* mutations were determined following the viability test (see Fig. 2).

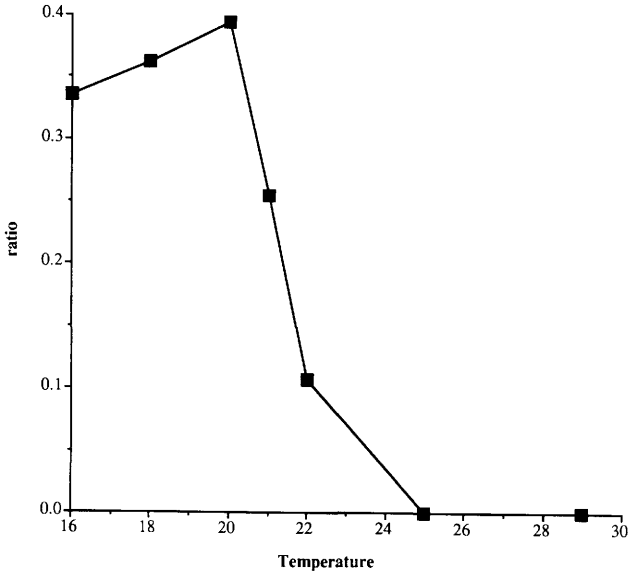

Figure 2. *D-mek*^{ts1} is a temperature-sensitive mutation. *FM7/ D-mek*^{LH110} females were crossed to *y w/Y; D-mek*$^{ts1\text{-}6}$/+ males, and the progeny scored at different temperatures. The temperature-sensitive properties of *D-mek*$^{ts1\text{-}6}$ were estimated by calculating the ratio of rescued *D-mek*^{LH110}/*Y; D-mek*$^{ts1\text{-}6}$/+ males vs. half the number of sibling females. The change associated with *D-mek*^{ts1} is able to confer temperature sensitivity because *D-mek*^{LH110}/*Y; D-mek*$^{ts1\text{-}6}$/+ animals are viable at both 18°C and 20°C. Their viability decreases dramatically at temperatures >20°C, and none are recovered at temperatures >25°C.

D-mek^{LH110}/*Y; D-mek*^{ts} or *D-mek*^{LH110}/*D-mek*^{LH110}; *D-mek*^{ts} as *D-mek*^{ts} animals in the following text.)

To further characterize the level of gene activity associated with *D-mek*$^{ts1\text{-}6}$, we tested the ability of this insertion line to rescue the *D-mek*^{lof} maternal-effect phenotype. Embryos derived from germ-line clones homozygous for *D-mek*^{LH110} fall into two phenotypic classes based on the paternal contribution: Class 1 rescued D-Mek embryos and class 2 null D-Mek embryos (of genotype *D-mek*^{LH110}/+) differentiate cuticle and show deletion of the telson that encompasses structures posterior to abdominal segment 7 (A7) and the acron that includes part of the head skeleton (Fig. 3B). This phenotype reflects the role of *D-mek* in Tor signaling (Tsuda et al. 1993; Lu et al. 1994). Class 2 null D-Mek embryos (of genotype *D-mek*^{LH110}/*Y*) fail to differentiate into structured embryos. To determine the level of gene activity provided by *D-mek*$^{ts1\text{-}6}$, the phenotypes of embryos derived from females that carry germ-line clones homozygous for *D-mek*^{LH110} and one copy of *D-mek*$^{ts1\text{-}6}$ were determined at various temperatures. At 18°C and 25°C, only embryos with wild-type terminal structures were observed. At 29°C, a fraction of the embryos were found that showed loss of A8 but retained part of the telson (Fig. 3C). Our results indicate that at 29°C, *D-mek*$^{ts1\text{-}6}$ does not behave as a complete null mutation but possesses residual activity to mediate Tor signaling.

D-mek *is involved in DER RTK signaling to define dorsal–ventral polarity during oogenesis*

During oogenesis, loss of *DER* activity in follicle cells, associated with a class of *DER* mutations called *torpedo* (*top*), causes ventralization of both the embryo and eggshell (Schüpbach 1987). Recently, studies on the gene *gurken* (*grk*), which encodes a TGF-α-like protein, have led to the model that secretion of Grk from the germ line activates the DER signaling pathway in dorsal follicle cells (Manseau and Schüpbach 1989; Neuman-Siderberg and Schüpbach 1993). Activation of DER in turn activates a pathway of which D-raf is a component (Brand and Perrimon 1994).

To determine whether D-Mek acts in DER signaling, we examined the phenotype of eggs laid by *D-mek*$^{ts1\text{-}6}$ females at different temperatures. Most of the eggs laid at 18°C show a weak ventralized eggshell phenotype with fused dorsal appendages of normal length (Fig. 4A2). The phenotype becomes more severe at higher temperatures. The dorsal appendages become shortened at 25°C (Fig. 4A3), and at 29°C only a knob of dorsal appendage material is found (Fig. 4A4). These phenotypes are reminiscent of the eggs derived from *top* females (Schüpbach 1987). To determine the temperature-sensitive period of D-Mek activity in dorsal appendage formation,

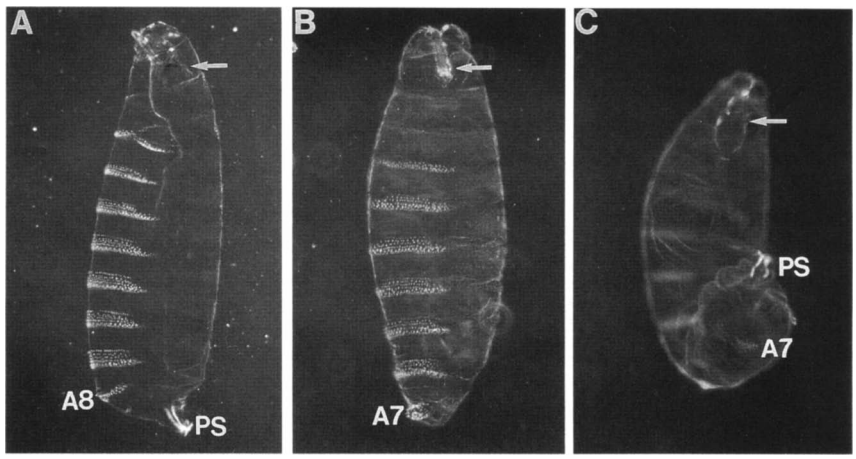

Figure 3. D-Mek is involved in the Torso pathway. (*A*) The cuticular pattern of a wild-type embryo. (*B*) A class 1 embryo derived from a female carrying a germ-line clone homozygous for *D-mek*^{LH110}. Note that in *B* the cephalopharyngeal head skeleton (arrow) is truncated and structures posterior to A7 (the telson) are deleted. At 29°C, the presence of one copy of *D-mek*$^{ts1\text{-}6}$ in females that carry *D-mek*^{lof} germ-line clones partially rescues the *D-mek*^{lof} maternal-effect phenotypes (*C*) Most embryos possess posterior spiracles (PS), anal pads, and A8 segments; however, 10–20% of them show partial or complete deletion of A8 segments and abnormal head skeleton (arrow in *C*).

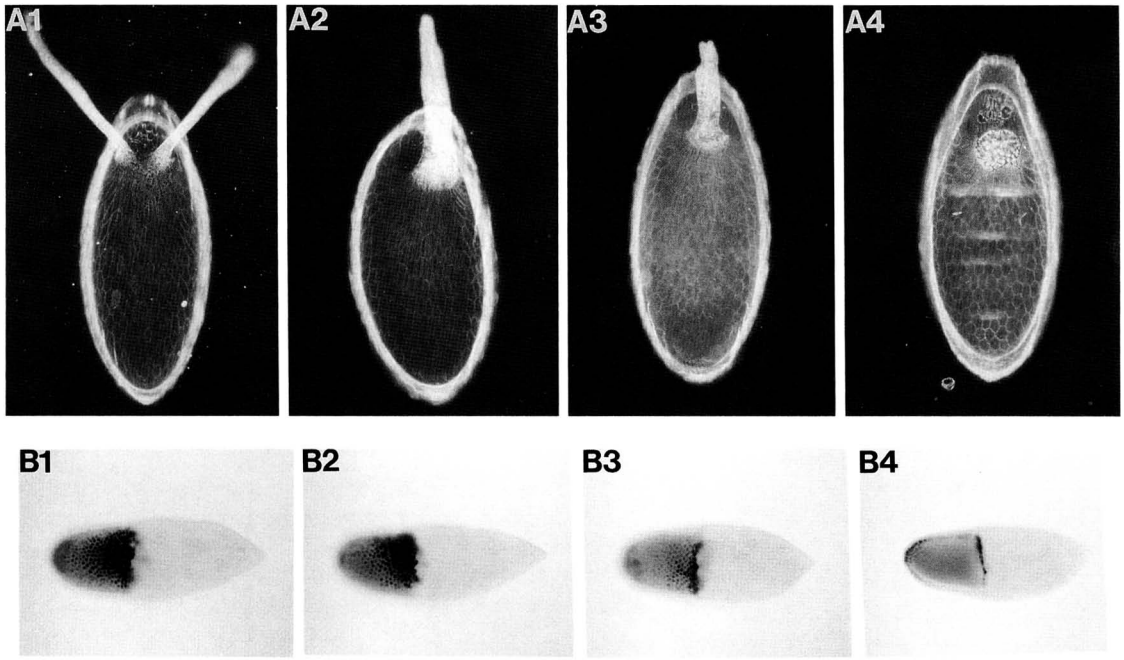

Figure 4. D-Mek is required for the establishment of D/V polarity. Chorion phenotypes of eggs were laid by wild-type (*A1*) and *D-mek*$^{ts1\text{-}6}$ females at 18°C (*A2*), 25°C (*A3*), and 29°C (*A4*). The dorsal appendages are fused and become progressively shortened as the temperature is increased. At 25°C, most eggs derived from those females are fertilized and give rise to normal embryos. At 29°C, eggs derived from these females are usually unfertilized; however, the eggs are not elongated and do not have the anterior chorion duplication as found in eggs derived from *grk* females (Schüpbach 1987). X-gal staining of stage 10 egg chambers of females containing one copy of the enhancer trap line *BB142* was performed. Wild-type (*B1*) and *D-mek*$^{ts1\text{-}6}$ egg chambers were at 18°C (*B2*), 25°C (*B3*), and 29°C (*B4*). The number of stained follicle cells located in the dorsal anterior region is reduced as the temperature is increased. Note that the pattern of centripetal staining does not change with temperature in *D-mek*$^{ts1\text{-}6}$; *B142* females.

D-mek$^{ts1\text{-}6}$ flies grown at 18°C were shifted to 29°C or vice versa (Fig. 5). The results of this temperature-shift experiment reveal that the temperature-sensitive period lasts ~7 hr, ranging between 20 and 27 hr before egg laying. This period corresponds approximately to the time of stages 7 and 8 (David and Merle 1968) or stages 8 and 9 (Lin and Spradling 1993) of oogenesis.

To demonstrate that lower levels of *D-mek* activity can induce follicle cells to adopt a more ventral cell fate, we analyzed the expression pattern of the enhancer trap line *BB142*. *BB142* is expressed asymmetrically in stage 10 wild-type egg chambers, with strongest expression in the dorsal anterior follicle cells (Fig. 4B1; T. Schüpbach, pers. comm.). At 18°C staining of *D-mek*$^{ts1\text{-}6}$; *BB142* female ovaries show an asymmetric expression of β-galactosidase as observed in wild-type egg chambers (Fig. 4B2). The number of stained follicle cells from the anterior–dorsal region of the egg chambers was greatly reduced, as flies were shifted to 25°C (Fig. 4B3), and almost no staining can be detected at 29°C (Fig. 4B4). The correlation between the amount of dorsal appendage and the number of stained follicle cells at different temperatures demonstrates that the level of D-Mek is instructive in the determination of dorsal follicle cells.

D-mek *is involved in embryonic dorsal–ventral patterning*

Studies on mutations affecting dorsal–ventral (D/V) patterning during oogenesis have shown that there is a good correlation between the D/V abnormalities of the eggshell and those of the corresponding embryo (Schüpbach 1987). Although reduced activity of *D-mek* leads to a ventralized eggshell phenotype, we have not been able to detect embryos displaying ventralized phenotypes even at 29°C (data not shown). One possibility is that *D-mek* does not function directly downstream of the DER signaling pathway but in a separate pathway required only for the establishment of eggshell polarity. To test this possibility, we examined the genetic interaction between *D-mek* and *fs(1)K10*, which affects early D/V patterning.

Eggs produced by homozygous *fs(1)K10* females have a dorsalized eggshell and develop into dorsalized embryos (Wieschaus et al. 1978). Females double mutant for *fs(1)K10* and either *grk* or *top* give rise to ventralized eggs and embryos that are identical to those produced by *grk* or *top* mutant females (Schüpbach 1987). The current model postulates that *fs(1)K10* is involved in determining the localization of Grk. We examined the eggs laid by females double mutant for *fs(1)K10* and *D-mek*$^{ts1\text{-}6}$. At 18°C, the eggs produced by these females have fused dorsal appendages (Fig. 6B) and the embryos are dorsalized, as in the case of embryos derived from females homozygous for *fs(1)K10*. When shifted to 29°C, the dorsalized eggshell phenotypes of all eggs are strongly suppressed (Fig. 6C) and a small fraction of the eggs are fertilized.

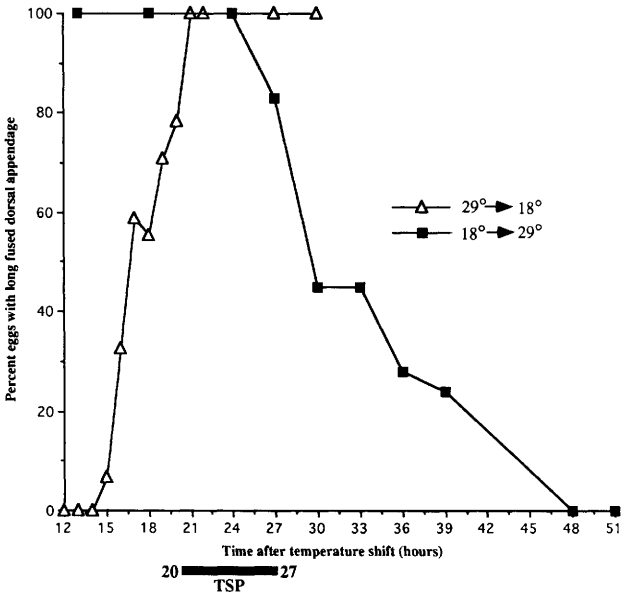

Figure 5. Temperature-sensitive period (TSP) of D-Mek in the formation of dorsal appendages. *D-mek*$^{ts1\text{-}6}$ females were reared at 18°C or 29°C and shifted to 29°C or 18°C, respectively. The morphology of the dorsal appendages was used to determine the period during oogenesis when D-Mek is required in dorsal appendage formation. Eggs laid at 18°C have a long fused dorsal appendage (Fig. 4A2), whereas eggs laid at 29°C only differentiate a dorsal knob (Fig. 4A4). To determine the period of *D-mek* activity, we have represented the percentage of eggs laid with the length of dorsal appendages similar to those at 18°C (*y*-axis). The time after which these eggs are scored following the temperature shift is indicated on the *x*-axis. The beginning of the TSP is defined as the earliest point at which the eggs laid by females shifted from the restrictive to the permissive temperature displayed a more severe phenotype than eggs laid by females raised at the permissive temperature (Suzuki 1970). Similarly, the end of the TSP is defined as the latest point at which the eggs laid by females shifted from the permissive to the restrictive temperature displayed a more severe phenotype than the eggs laid by females raised at the permisssive temperature. All developmental times were normalized to 25°C, by making the assumption that oogenesis takes place 2×as fast at 25°C as at 18°C and 1.5× as fast at 29°C as at 25°C. Shaded boxes below the graphs represent the temperature-sensitive period for D-Mek in the formation of dorsal appendages. This period corresponds to stages 7 and 8 (David and Merle 1968) or stages 8 and 9 (Lin and Sprading 1993) of oogenesis.

Among them, three classes of embryonic phenotypes are observed: Class 1 embryos are dorsalized as observed in eggs derived from *fs(1)K10* females; class 2 embryos have normal head skeleton and seven or eight ventral denticle bands, with filzkörper material often located inside the body (Fig. 6E); and class 3 embryos have a partial head skeleton and are missing the filzkörper (Fig. 6F). In class 3 embryos, the ventral denticle bands are usually incomplete or fused and their numbers are variable, possibly representing a phenotype intermediate between class 1 and class 2. We detect some class 2 embryos with wild-type cuticular structure, similar to those derived from *D-mek*$^{ts1\text{-}6}$ alone at 29°C, indicating that reducing the level of *D-mek* can suppress the *fs(1)K10* chorion phenotype but can only partially suppress the *fs(1)K10* embryonic phenotype. These results indicate that D-Mek is involved in both chorion and embryonic D/V patterning.

D-Mek acts downstream of D-Raf in DER signaling

D-Raf has been shown to act downstream of DER to define D/V polarity (Brand and Perrimon 1994). Females expressing a constitutively activated, truncated D-Raf protein under the heat shock promoter (*hsp70–D-raf*^{gof}) lay eggs with dorsalized chorions (Fig. 7B). We examined the epistatic relationship between D-Raf and D-Mek by expressing a constitutively activated form of D-Raf in a *D-mek* mutant background. If D-Mek acts downstream of D-Raf, reducing D-Mek activity will suppress the dorsalized phenotype associated with expression of the *D-raf*^{gof} mutation. We examined eggs laid by females homozygous for *D-mek*^{LH110} carrying *hsp70–D-raf*^{gof} and *D-mek*$^{7\text{-}B}$, which is associated with a weak D-Mek activity (Fig. 7A; see Materials and methods). Interestingly, reduced D-Mek activity markedly suppresses the *hsp70–D-raf*^{gof} dorsalized chorion phenotype (Fig. 7C), indicating that D-Mek functions downstream of D-Raf in the DER pathway.

D-Mek is involved in the DER pathway to specify wing vein formation

DER loss-of-function mutations cause deletion of cross veins and longitudinal veins in the wing (Clifford and Schüpbach 1989). Flies with *D-mek*$^{ts1\text{-}6}$ cannot survive at 25°C; however, they all have wild-type wing veins when grown below 24°C. To demonstrate the involvement of *D-mek* in development, we used another transformant line, *D-mek*$^{ts1\text{-}11}$, which has slightly higher activity at 25°C (see Materials and methods). A few *D-mek*$^{ts1\text{-}11}$ survivors can be recovered at 25°C, and all show differing extents of deletions among the longitudinal veins. Vein L4 seems to be the most sensitive region to be affected as deletions from the distal end of L4 can be detected in each survivor. Some of the flies show additional distal deletions from veins L3 and L5 (Fig. 8B). Similarly, *D-mek*$^{ts1\text{-}6}$ emerging flies grown at 18°C but shifted to 29°C for 24 hr during the third-instar larval stage showed small gaps in vein L4 (data not shown).

In conclusion, the vein phenotypes observed following reduction of D-Mek during wing imaginal disc development are similar to those associated with DER mutations (Clifford and Schüpbach 1989), suggesting that *D-mek* is involved in DER signaling during vein formation.

D-Mek is involved in the Sevenless RTK signaling pathway

In the developing eye, specification of R7 cell fate requires activation of the Sev RTK. Sev, when activated by

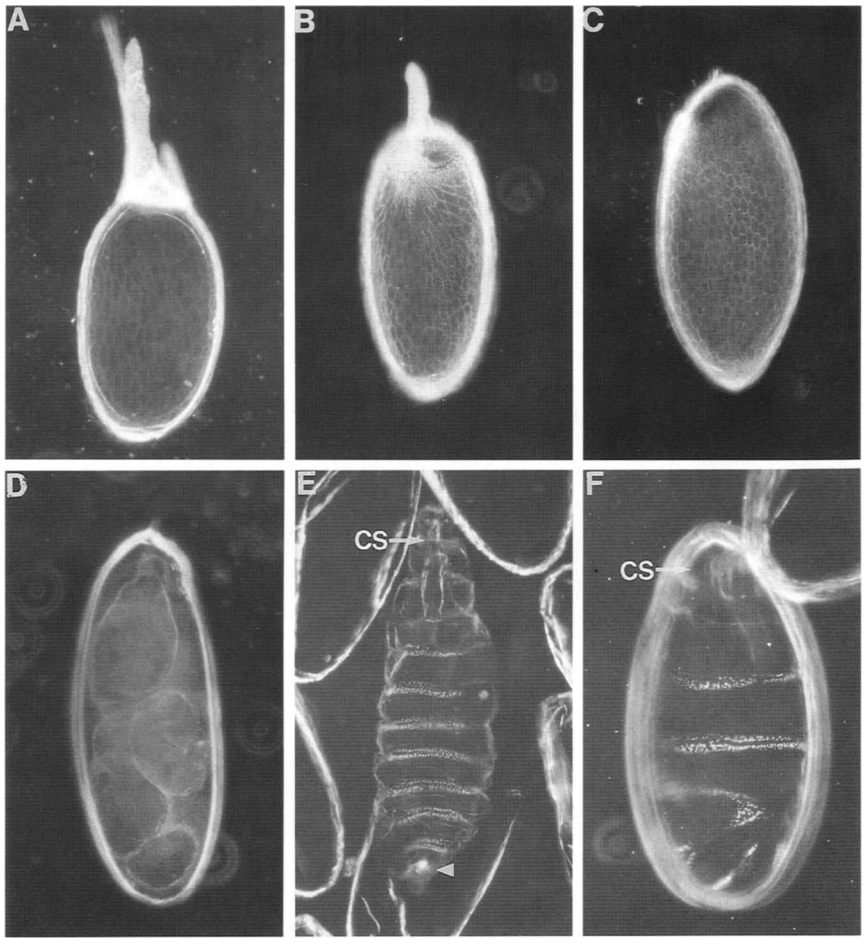

Figure 6. *fs(1)K10, D-mek* double-mutant phenotypes. Chorion phenotypes of eggs laid by females homozygous for *fs(1)K10* (*A*) or homozygous for both *fs(1)K10* and *D-mek*$^{ts1\text{-}6}$ at 18°C (*B*) and at 29°C (*C*). Cuticular pattern of embryos derived from females homozygous for *fs(1)K10* (*D*) or homozygous for *fs(1)K10, D-mek*$^{ts1\text{-}6}$ reared at 29°C: class 2 embryos (*E*) and class 3 embryos (*F*). At 18°C, reduced *D-mek* activity suppresses the *fs(1)K10* chorion phenotype but not the dorsalized embryonic phenotypes. At 29°C, residual *D-mek* activity completely suppresses the *fs(1)K10* chorion phenotype but only partially suppresses the dorsalized embryonic phenotype. Note that in *E* the cephalopharyngeal head skeleton (*CS*) and filzkörper (arrowhead) are present. However, the filzkörper are often found inside the body.

its ligand Bride of sevenless, activates a signaling pathway that includes Drk, Sos, Ras-1, and D-Raf (for review, see Dickson and Hafen 1993). To determine whether *D-mek* is a component of Sev signaling, we examined the eye phenotype of *D-mek*$^{ts1\text{-}6}$ animals. *D-mek*$^{ts1\text{-}6}$ animals have wild-type eyes at 18°C. However, when reared between 20°C and 25°C, they show progressively worse rough-eye phenotypes. At 25°C, the eye imaginal discs isolated from third-instar larvae show significant reduction in size, averaging one-quarter the size of discs from larvae raised at 18°C (data not shown). To overcome the growth defects associated with the imaginal discs, we examined the phenotype of *D-mek*$^{ts1\text{-}6}$ animals grown at 18°C with a 24-hr exposure to 29°C during the third-instar larval stage. *D-mek*$^{ts1\text{-}6}$ flies recovered under these conditions developed rough eyes that showed the absence of R7 in most ommatidia. In addition, many ommatidia have fewer than six outer photoreceptors (Fig. 9B), which is reminiscent of the phenotypes associated with weak *D-raf* alleles (Melnick et al. 1993).

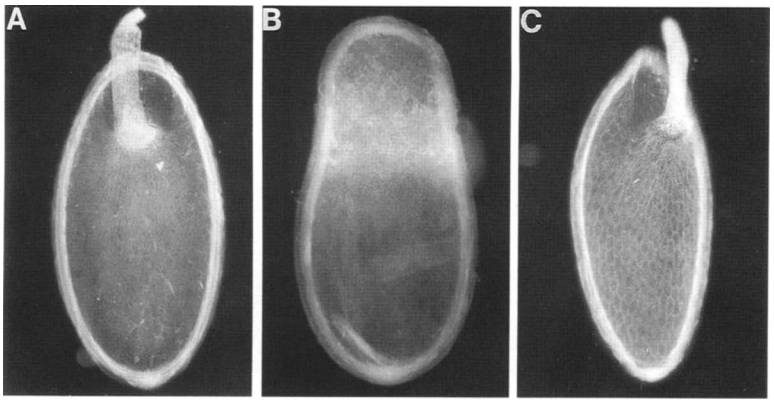

Figure 7. Chorion phenotypes of eggs laid by females of genotype *D-mek*$^{7\text{-}B}$ (*A*), *hsp70–D-raf*^{gof} (*B*), and *D-mek*$^{7\text{-}B}$/*hsp70–D-raf*^{gof} (*C*). Note that in *C* reduced *D-mek* activity suppresses the *hsp70–D-raf*^{gof} dorsalized eggshell phenotype.

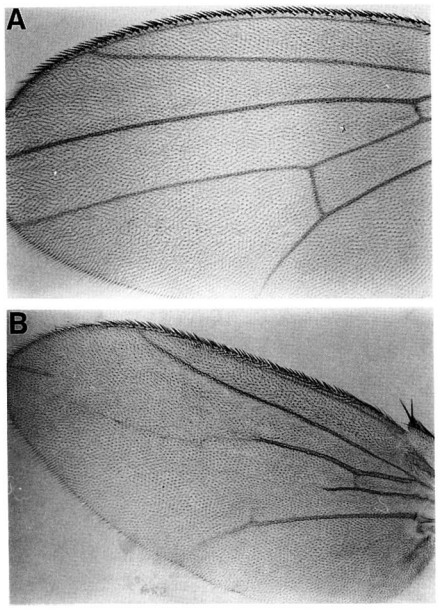

Figure 8. D-Mek is involved in wing vein formation. Wing of wild-type (*A*) and *D-mek*$^{ts1\text{-}11}$ (*B*) flies. *D-mek*$^{ts1\text{-}11}$ flies, grown at 25°C, have gaps in their third and fourth longitudinal veins.

Discussion

Generation of a temperature-sensitive allele of D-Mek

Although MEK is a threonine/tyrosine kinase, we were able to generate a D-Mek^{ts} allele based on the sequence similarity with a yeast cdc2^{ts} serine/threonine kinase mutation. The amino acid change from proline^{209} to serine that conferred temperature sensitivity to D-Mek lies within the catalytic loop that is positioned between amino acids 205 and 211. In this loop, 4 amino acids are highly conserved and participate in either executing catalysis or stabilizing the loop (Taylor et al. 1992). In contrast, proline^{209} is only moderately conserved among serine/threonine kinases. The replacement of a proline by a serine at this position may affect the local conformation of the catalytic loop in response to temperature and lead to different levels of catalytic activity. At 29°C, embryos derived from *D-mek*$^{ts1\text{-}6}$ females still possess posterior spiracles (Fig. 3C), and at 18°C, most of the eggs laid by *D-mek*$^{ts1\text{-}6}$ females have fused dorsal appendages (Fig. 4), indicating that *D-mek*$^{ts1\text{-}6}$ does not possess the full enzymatic activity of a wild-type copy at 18°C, and that at 29°C, *D-mek*$^{ts1\text{-}6}$ still has some residual activity.

Recently, a temperature-sensitive mutant of the avian sarcoma virus UR2 oncogene *ros*, which encodes a RTK, has been shown to have a mutation in a similar position in the catalytic loop (Chen et al. 1994). This mutation also confers temperature-sensitive properties when introduced in both the insulin and insulin-like growth factor I receptors (Chen et al. 1994), suggesting that an amino acid change at this position would probably confer temperature sensitivity on most kinases.

D-Mek is involved in multiple RTK signaling pathways

Using *D-mek*^{ts} we have shown that *D-mek* is required in a variety of temporally and spatially distinct developmental processes controlled by RTKs. These include the specification of D/V polarity during oogenesis (DER pathway), formation of terminal structures prior to the cellular blastoderm stage (Tor pathway), wing vein formation, and eye development during larval stages (DER and Sev pathways, respectively). In addition, clonal analysis of *D-mek* mutations in follicle cells revealed that homozygous *D-mek*^{lof} clones are recovered at a very low frequency and are of small size (J.-C. Hsu, unpubl.). This, together with the effect of loss of *D-mek* activity during imaginal disc development, may reflect the function of D-Mek in the DER pathway that is involved in the control of cellular proliferation (Xu and Rubin 1993). Because the phenotypes of loss-of-function mutations in both *D-mek* and the RTKs are similar, if not identical, this suggests that *D-mek* is the only MEK activated by these *Drosophila* RTKs.

Because of the temperature-sensitive properties associated with *D-mek*^{ts}, we have been able to visualize the effects of modulating the amount of active D-Mek in developmental processes controlled by RTKs. In the Tor signaling pathway, the extent of terminal structures that differentiate depends on the level of active D-Mek. In the presence of high levels of D-Mek activity, embryos develop posterior spiracles and A8; however, only the posterior spiracles are present if lower amounts of D-Mek activity are provided. The array of terminal structures that are determined in response to different amounts of active D-Mek reflects the instructive properties of Tor. This suggests that Tor may not simply act as an on/off switch in terminal cell fate determination (Casanova and Struhl 1989, 1993; Perkins et al. 1992; Sprenger and Nüs-

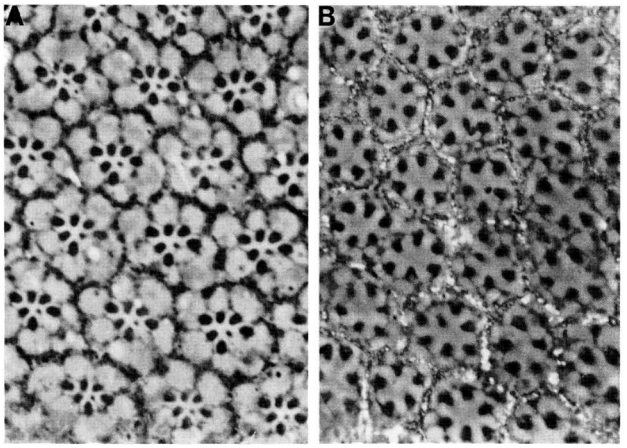

Figure 9. D-Mek is involved in the Sevenless pathway. Histological sections through the eye of wild-type (*A*) and *D-mek*$^{ts1\text{-}6}$ flies (*B*). *D-mek*$^{ts1\text{-}6}$ progeny were shifted to 29°C for 24 hr during the third-instar larval stage and grown to adulthood. Emerging flies were missing R7 photoreceptor cells in ~70% of the ommatidia. Some ommatidia have fewer than six outer photoreceptors.

slein-Volhard 1992; Melnick et al. 1993). Our results substantiate further that the strength of the signaling pathway generated by activated Tor is instructive in terminal cell fate determination. Similarly, in the DER signaling pathway, the strength of *D-mek* activity correlates well with the number of follicle cells adopting a dorsal fate. *D-mek*$^{ts1\text{-}6}$ females lay eggs with severe ventralized chorions at 29°C and weakly ventralized chorions at 18°C. As in the case of Tor, the level of activation of DER appears to be instructive in the establishment of dorsal follicle cell fate.

There is usually a good correlation between chorion defects and ventralization of the embryo in mutations that affect the establishment of D/V polarity during oogenesis (Schüpbach 1987). Interestingly, we found that although *D-mek*$^{ts1\text{-}6}$ females lay eggs with severe ventralized chorions at 29°C, the embryos have a normal pattern of denticle bands. This observation may indicate that *D-mek* activity is necessary and sufficient for the specification of D/V patterning of the chorion but not the embryo. Alternatively, in the follicle cells, the pathway that leads to the establishment of D/V chorionic cell fates may be more sensitive to a reduction of *D-mek* activity than the pathway that leads to the establishment of embryonic D/V polarity. Consistent with this hypothesis, we have shown that the dorsalized chorionic and embryonic phenotypes of *fs(1)K10* can be suppressed by *D-mek*^{ts} at 29°C, but with different sensitivity.

Different RTK pathways show different sensitivities to reduced levels of D-mek

In addition to our observations that the level of activation of either the DER or Tor pathways is instructive in the establishment of cell fates, we find that different RTK pathways show different sensitivities to reduced levels of *D-mek* (Table 1). For example, at 25°C, *D-mek*$^{ts1\text{-}6}$ embryos derived from *D-mek*$^{ts1\text{-}6}$ females are wild type and develop into flies that exhibit both wing defects (DER pathway) and the absence of R7 photoreceptors (Sev pathway).

Table 1. *Differential sensitivity among different RTK pathways*

	DER pathway			
Temperature (°C)	D/V polarity	wing vein formation	Torso pathway	Sevenless pathway
18	m	wt	wt	wt
23	m	wt	wt	m
25	m	m	wt	m
29	m	m	wt/m	m

Wild-type (wt) or mutant (m) phenotypes associated with various amounts of *D-Mek*^{ts} are shown according to the temperature. The mutant phenotypes include fusion of dorsal appendages (D/V polarity, DER pathway), deletion of wing vein structures (wing vein formation, DER pathway), the absence of R7 photoreceptor cells (Sev pathway), and deletion of A8 (Tor pathway).

These observations suggest two models under our experimental conditions: (1) Different cells may possess different levels of functional D-Mek at the time of signaling; or (2) Tor requires a lower level of active D-Mek for activation of its target genes than either DER and Sev. In the first case, different RTKs may require the same level of D-Mek activity to activate their target genes; however, the difference in the synthesis rate or stability of D-Mek may allow the terminal regions of the embryo to accumulate more D-Mek than the imaginal and follicle cells. Therefore, the amount of functional D-Mek activity remaining following the temperature shift to 25°C may be sufficient to transmit Tor signal but not DER signaling. In the second model, the differential sensitivity of different RTK pathways in reducing levels of *D-mek* may reflect the fact that some RTKs generate signals of different strengths. This hypothesis is consistent with the observation that in PC12 cells, addition of nerve growth factor (NGF) or EGF results in neural differentiation or cell proliferation, respectively. NGF stimulation is associated with prolonged p21^{ras} and MAPK activation; however, EGF stimulation only leads to transient activation of both p21^{ras} and MAPK (Qui and Green 1992). Differences in the duration of the p21^{ras}/Raf/MEK activation may trigger different cellular responses depending on the amount of signal generated. If activation of different target genes by different RTKs depends on the strength of the signal, then a reduction in the amount of available MEK, or any of the other signal transducers, should reflect such dependency. Various RTKs may have evolved to tightly control the strength of the signal that they transmit to elicit different transcriptional responses.

Finally, different sensitivities to *D-mek* activity is also found with the same receptor. At 18°C or 23°C, *D-mek*$^{ts1\text{-}6}$ flies have wild-type wing, but the eggs laid by these females have a short fused dorsal appendage. Both of these developmental processes are under the control of the same RTK, DER. To account for such differences, it is possible that the binding of different ligands to the same receptors may induce different conformational changes in the cytoplasmic domain of the receptor that in turn may affect the efficiency of receptor auto- or transphosphorylation, receptor internalization, or degradation. These differences may allow receptors to generate different levels of signals.

A conserved signal transduction cassette generates different cellular responses

Because all RTKs activate a common set of molecules, one of the unresolved issues is how signals transmitted by the p21^{ras}, Raf, and MEK signaling cassette elicit specific transcriptional responses. Our results indicate that the specificity of RTK signaling is influenced by quantitative differences among different RTKs. However, the observations that expression of an activated form of D-Raf can dorsalize the chorion (Brand and Perrimon

1994), induce an embryonic phenotype reminiscent of ubiquitously activated Tor (A. Brand, X. Lu, and N. Perrimon, unpubl.), and induce extra R7 cell fates (Dickson et al. 1992) suggest that the level of a signal may not be the sole factor to trigger a specific pathway along a developmental process. In addition to the quantitative aspect of RTK signaling, specific components of each pathway downstream of MEK may exist.

MAPKs have been shown to act downstream of MEKs and control the activities of specific transcription factors. In *Drosophila* a MAPK known as *rolled* (*rl*) has been isolated (Biggs and Zipursky 1992; Biggs et al. 1994; Brunner et al. 1994). Loss-of-function *rl* mutations exhibit both R7 photoreceptor (Sev pathway) and wing defects (DER pathway), whereas a gain-of-function mutation, rl^{Sem}, can activate the Tor signaling pathway in the embryo, specify multiple R7 cells, and produce extra wing vein structures (Brunner et al. 1994). These results suggest that *rl* encodes a common MAPK for these three RTKs. However, because germ cells lacking *rl* activity do not develop (Brunner et al. 1994) and hyperactivation of the *rl* MAP kinase masks dorsal-mediated transcriptional repression less effectively than hyperactivation of the Tor receptor (Rusch and Levine 1994), it remains to be determined whether *rl* is directly involved in Tor signaling. In addition, females that carry the activated rl^{Sem} mutation lay wild-type eggs, which is in contrast to the expression of activated D-raf in follicle cells (Brand and Perrimon 1994; Brunner et al. 1994). Thus, *rl* may not act downstream of DER in the establishment of D/V polarity during oogenesis.

If MAPK is a common component of all RTKs, then the specificity may reside downstream of MAPK, that is, at the level of cell- or stage-specific transcription factors whose activity is modulated by phosphorylation. For example, the activation of the Tor pathway may be dependent on direct or indirect phosphorylation of an unidentified maternally derived transcription factor (referred to as gene *Y*; St Johnston and Nüsslein-Volhard 1992) by MAPK. Alternatively, the common signals generated by different RTKs may be interpreted by cell- or stage-specific transcription cofactors. For example, follicle cells may posses specific transcription coactivators that together with the common transcriptional factors activated by all receptors, may determine their binding specificity. Analysis of *Caenorhabditis elegans let-23*, a homolog of the EGF receptor, has indicated that the carboxyl terminus of *let-23* can be subdivided into different domains that each contribute to receptor function in different cell types (Aroian et al. 1994). Therefore, in addition to the common signaling cassette activated by RTKs, different cells may have factors that specifically interact with specific domains of receptors. Interestingly, it has been demonstrated recently that the SH2 containing transcription factor p91, a component of the JAK/STAT signaling system, also operates in EGF RTK signaling (for review, see Darnell et al. 1994). Possibly, some additional factors may be provided by components of the JAK/STAT signaling pathways.

To date, all components identified are shared in multiple RTK pathways. Genetic screens for novel components in each RTK pathway may allow us to identify branchpoints among different pathways and components specific to each pathway. Using the $D\text{-}mek^{ts}$ allele we can generate a sensitized background to carry out genetic screens for identification of genes that are involved in RTK signaling.

Materials and methods

Drosophila *strains*

In this study we used the loss-of-function *D-mek* allele, $D\text{-}mek^{LH110}$, induced on the $y\ w\ FRT^{101}$ chromosome (Chou and Perrimon 1992). On the basis of the genetic map and complementation test with $D\text{-}mek^{r1}$ (Tsuda et al. 1993), $D\text{-}mek^{LH110}$ behaves as a null mutation (Lu et al. 1994). The lethal phase of $D\text{-}mek^{LH110}$ is very wide, ranging from early first-instar larval to pupal stages (data not shown). Previously, we have shown that a 9-kb genomic fragment is sufficient to rescue all aspects of the *D-mek* mutant phenotype (Lu et al. 1994), indicating that $D\text{-}mek^{LH110}$ is not associated with second-site mutations. The enhancer trap line *BB142*, carrying the P-transposable element on the second chromosome, was a gift from T. Schüpbach (Princeton University, NJ).

Description of other *Drosophila* mutations and chromosomes used in this study can be found in Lindsley and Zimm (1992).

Generation of temperature-sensitive D-mek *alleles*

To generate a $D\text{-}mek^{ts}$ mutation, we subcloned a 5-kb *Not*I–*Eco*RI fragment derived from the 9-kb genomic DNA into pBSK. This 5-kb fragment was used in the site-directed mutagenesis that was performed according to the method of Deng and Nickoloff (1992), with modifications (Lu et al. 1994). The sequence of the primer introducing the $proline^{209}$ to serine amino acid change (D-Mek^{ts1}) is 5′-PCGTGACGTGAAGTCGAGCAATATCCTC-3′. The mutated genomic DNA fragment was sequenced to confirm the presence of the mutation. Subsequently, the *Not*I–*Eco*RI fragment was excised and replaced into the 9-kb *D-mek–pCaSpeR4* genomic clone (Thummel et al. 1988) and injected into embryos of genotype *y w; delta 2-3, Sb/TM6, Ubx* (Spradling 1986; Robertson et al. 1988). Two independent transformant lines were recovered; one ($D\text{-}mek^{ts1\text{-}6}$) located on the *TM6, Ubx* chromosome, and the other one ($D\text{-}mek^{ts1\text{-}11}$), which contained two inserts, located on the second and third chromosomes. These insertions are followed by the presence of the *mini-white* gene that originates from *pCaSpeR4*. Either transformant line can rescue the lethality associated with $D\text{-}mek^{LH110}$ at 18°C but not at 29°C. Because we can recover a few survivors from $D\text{-}mek^{ts1\text{-}11}$ but not $D\text{-}mek^{ts1\text{-}6}$ at 25°C, we believe that $D\text{-}mek^{ts1\text{-}11}$ is associated with higher *D-mek* activity. Most of the experiments described in this paper were done using the $D\text{-}mek^{ts1\text{-}6}$ insertion line. Similar methods were used to change $glycine^{218}$ to aspartic acid (D-Mek^{ts2}) with the primer 5′-PTCAATAGCAGCGACGAGA-TCAAGAT-3′. Four independent transformant lines were recovered, but all failed to rescue the lethality associated with the $D\text{-}mek^{LH110}$ mutation at any temperature.

Other D-mek *insertion lines*

Previously, in an effort to rescue the lethality of the $D\text{-}mek^{LH110}$ mutation, we recovered four independent transformant lines carrying a 9-kb wild-type genomic fragment (Lu et al. 1994). One transformant line, $D\text{-}mek^{7\text{-}B}$, inserted on the *TM6, Ubx* chro-

mosome, can rescue *D-mek* mutations. However, $D\text{-}mek^{LH110}/D\text{-}mek^{LH110}$; $D\text{-}mek^{7\text{-}B}/+$ females (referred to as $D\text{-}mek^{7\text{-}B}$) lay eggs with fused dorsal appendages. The length of the dorsal appendages is shorter than those laid by $D\text{-}mek^{ts1\text{-}6}$ females at 18°C but longer than those at 25°C.

X-gal staining in ovary

Ovaries were dissected in PBS, 0.1% Triton X-100, fixed in 2.5% glutaraldehyde for 5 min, and stained at room temperature overnight as described previously (Brand and Perrimon 1994).

Histology

Fixation and sectioning (4 μm) of adult *Drosophila* eyes were performed as described previously (Tomlinson and Ready 1987).

Temperature shift experiments

As a stock, *y w* $D\text{-}mek^{LH110}$ FRT^{101}/*FM7*; *TM6, Ubx,* $D\text{-}mek^{ts1\text{-}6}/+$ flies were raised at 18°C. For the viability test, *y w* $D\text{-}mek^{LH110}$ FRT^{101}/*FM7* females were crossed to *y w*/*Y*; *TM6, Ubx,* $D\text{-}mek^{ts1\text{-}6}/+$ males. To study the temporal requirement for D-Mek during oogenesis, temperature-shift experiments were performed with the conditional allele $D\text{-}mek^{ts1\text{-}6}$. In the 18°C to 29°C shift experiment, $D\text{-}mek^{ts1\text{-}6}$ females and males were mated and allowed to lay eggs at 18°C for at least 3 days before being shifted to 29°C. In the 29°C to 18°C shift experiment, flies were mated and allowed to lay eggs at 29°C for at least 3 days before being shifted to 18°C.

Production of germ-line clones

Germ-line clones homozygous for *D-mek* with one copy of $D\text{-}mek^{ts}$ were generated using the FLP–DFS technique (Chou and Perrimon 1992). In brief, *y w* $D\text{-}mek^{LH110}$ FRT^{101}/*FM7*; *+/+*; *TM6, Ubx,* $D\text{-}mek^{ts1\text{-}6}/+$ females were crossed with *w* ovo^{D1} FRT^{101}/*Y*; FLP^{38}/FLP^{38}; *+/+* males at 25°C. Following eclosion, *y w* $D\text{-}mek^{LH110}$ FRT^{101}/*w* ovo^{D1} FRT^{101}; $FLP^{38}/+$; *TM6, Ubx,* $D\text{-}mek^{ts1\text{-}6}/+$ females that carried $D\text{-}mek^{LH110}$ germ-line clones were crossed to wild-type (*Oregon-R*) males and shifted to 29°C. The phenotypes of embryos derived from these females were examined 5 days following the matings.

Genetic epistasis experiments

To analyze the genetic interaction between *fs(1)K10* and *D-mek*, a recombinant between *fs(1)k10* and $D\text{-}mek^{LH110}$ was constructed. Females of genotype *fs(1)K10 w* $D\text{-}mek^{LH110}$ FRT^{101}/*fs(1)K10 w* $D\text{-}mek^{LH110}$ FRT^{101}; $D\text{-}mek^{ts1\text{-}6}/+$ were crossed to *Oregon-R* males and allowed to lay eggs at either 18°C or 29°C.

To determine the epistatic relationships between D-Raf and D-Mek, *y w* $D\text{-}mek^{LH110}$ FRT^{101}/*y w* $D\text{-}mek^{LH110}$ FRT^{101}; *hs–*$D\text{-}raf^{F22}/D\text{-}mek^{7\text{-}B}$ females were crossed to *Oregon-R* males and their eggs examined. *hs–*$D\text{-}raf^{F22}$ is a heat-inducible, gain-of-function *D-raf* gene (referred to as $hs\Delta Draf^{22}$ in Brand and Perrimon 1994). Heat shocks were performed as described previously (Brand and Perrimon 1994).

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