The *Drosophila* Ste20-related kinase *misshapen* is required for embryonic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway

Yi-Chi Su,[1] Jessica E. Treisman,[2,3] and Edward Y. Skolnik[1,3]

[1]Department of Pharmacology and [2]Department of Cell Biology, New York University Medical Center, Skirball Institute of Biomolecular Medicine, New York, New York 10016 USA

Dorsal closure in the *Drosophila* embryo occurs during the later stages of embryogenesis and involves changes in cell shape leading to the juxtaposition and subsequent adherence of the lateral epidermal primordia over the amnioserosa. Dorsal closure requires the activation of a conserved c-*jun* amino-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) module, as it is blocked by null mutations in JNK kinase [hemipterous (*hep*)] and JNK [basket (*bsk*)]. *Drosophila* JNK (DJNK) functions by phosphorylating and activating DJun, which in turn induces the transcription of *decapentaplegic* (*dpp*). We provide biochemical and genetic evidence that a Ste20-related kinase, *misshapen* (*msn*), functions upstream of *hep* and *bsk* to stimulate dorsal closure in the *Drosophila* embryo. Mammalian (NCK-interacting kinase [NIK]) and *Caenorhabditis elegans* (*mig-15*) homologs of *msn* have been identified; *mig*-15 is necessary for several developmental processes in *C. elegans*. These data suggest that *msn*, *mig*-15, and NIK are components of a signaling pathway that is conserved among flies, worms, and mammals to control developmentally regulated pathways.

[*Key Words: Drosophila;* Ste20 kinase; NIK; misshapen; dorsal closure; JNK]

Received December 23, 1997; revised version accepted June 2, 1998.

Multiple mitogen-activated protein kinase (MAPK) modules have been identified as components of signal transduction pathways, including at least six in yeast and three in mammalian cells (Herskowitz 1995; Kyriakis and Avruch 1996). All MAPK modules are composed of three sequentially acting protein kinases: a MAP kinase, an enzyme that activates MAP kinase [known as MAP kinase kinase (MKK), MAP extracellular signal-related kinase (ERK) kinase (MEK), or Ste7-related kinase], and an enzyme that activates MKK [known as a MAP kinase kinase kinase (MKKK), MAP ERK kinase kinase (MEKK), or Ste11-related kinase] (Davis 1994; Cobb and Goldsmith 1995; Herskowitz 1995). The defining characteristic of these MAP kinase modules is the MAP kinase itself, and in mammalian cells the MAP kinases described thus far include the p42 and p44 MAP kinases (also known as ERK1 and ERK2), c-Jun amino-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and p38. In contrast to p42 and p44 MAP kinases, which are frequently downstream effectors of Ras and are central elements mediating cell proliferation by a variety of growth factors, JNK and p38 are activated most potently by cellular stresses and inflammatory cytokines (Kyriakis and Avruch 1996).

Genetic epistasis analysis in yeast as well as studies in mammalian cells have indicated that Ste20 related kinases function upstream of MKKKs to regulate the JNK MAPK module, and Ste20 kinases have been considered to be MAP kinase kinase kinase kinases (MKKKK) (Herskowitz 1995; Hu et al. 1996; Pombo et al. 1995; Su et al. 1997). Two families of protein kinases, which are closely related to Ste20 in their kinase domain, have been identified based on their structure and regulation. The first family includes the yeast Ste20 protein kinase and the mammalian and *Drosophila* p21-activated protein kinases (PAKs) (Manser et al. 1994; Martin et al. 1995; Harden et al. 1996). Kinases in this group contain a conserved p21Rac- and Cdc42-binding domain in their amino terminus (Burbelo et al. 1995; Martin et al. 1995) and are activated by binding GTP-bound Cdc42 and Rac (Manser et al. 1994; Bagrodia et al. 1995; Herskowitz 1995; Martin et al. 1995). The second family lacks

[3]**Corresponding authors.**
E-MAIL Skolnik@saturn.med.nyu.edu; FAX (212) 263-5711.
E-MAIL Treisman@saturn.med.nyu.edu; FAX (212) 263-7760.

p21Rac- and Cdc42-binding domains and is named for the yeast SPS1 protein kinase (Friesen et al. 1994). In addition, in contrast to PAKs that contain an amino-terminal regulatory and a carboxy-terminal kinase domain, members of this second family contain an amino-terminal kinase domain and a carboxy-terminal regulatory region. Several SPS1 family kinases have been identified in mammalian cells and include mammalian Ste20-like kinase 1 (MST1) and MST2, germinal center kinase (GCK), NCK-interacting kinase (NIK), Ste20/oxidant stress response kinase (SOK), hematopoietic progenitor kinase (HPK1), and GCK-like kinase (GLK) (Creasy and Chernoff 1995; Pombo et al. 1995; Hu et al. 1996; Diener et al. 1997; Su et al. 1997).

Although a clear pathway has been mapped out between cell surface receptors and the activation of p42 and p44 MAP kinase, the pathways that connect a specific extracellular stimulus to activation of the JNK pathway are not well established. A number of MKKKs or Ste11 related kinases have been found to activate JNK when overexpressed transiently in mammalian cells, but little is known about how these kinases are regulated or which of these kinases couple specific upstream signals to JNK activation. In addition, little is known about the regulation or function of Ste20 kinases; in fact, no mammalian Ste20 kinase has yet been shown to link a specific upstream signal to JNK activation. The ability of several mammalian SPS1 family members, such as GCK, NIK, GLK, and HPK1, to activate JNK when overexpressed transiently in mammalian cells is the strongest evidence that these kinases might activate JNK in response to upstream signals (Pombo et al. 1995; Hu et al. 1996; Diener et al. 1997; Su et al. 1997). However, these studies may not be conclusive because JNK activation has been measured under conditions in which the Ste20 kinases are expressed at very high levels and therefore, could have nonphysiological effects.

Genetic studies in lower organisms, such as *Drosophila* and *Caenorhabditis elegans,* have been particularly helpful in unraveling complex signaling pathways (Dickson and Hafen 1994). For example, studies of these organisms proved invaluable in furthering our understanding of the Ras–Raf–MAP kinase signaling pathway. Because of the difficulty in studying Ste20 kinases in mammalian cells, placement of a Ste20 kinase on a genetic pathway in *Drosophila* or *C. elegans* would greatly facilitate our understanding of the normal physiological functions of these kinases. Recently, we have identified a *Drosophila* homolog of NIK (a member of the mammalian SPS1 family of Ste20 kinases), called *misshapen* (*msn*). *msn* was identified in a screen for genes that are regulated by the photoreceptor transcription factor Glass. Its name derives from the finding that *msn* is required for the normal shape of *Drosophila* photoreceptor cells. However, *msn* is also required for normal embryogenesis, as null mutations are embryonic lethal. A *Drosophila* JNK, basket (*bsk*), and JNK kinase, hemipterous (*hep*), have also been shown to be essential for normal embryonic development (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). Loss of either kinase is embryonic lethal and causes a ''dorsal open'' phenotype. Dorsal closure of the *Drosophila* embryo involves changes in cell shape leading to elongation and migration of the lateral epithelial sheets (Campos-Ortega and Hartenstein 1985; Young et al. 1993). This coordinated movement of the lateral epithelia functions to internalize the amnioserosa and connect the two sides of the embryo. The finding that the mammalian homolog of *msn*, NIK, activates the JNK MAPK module, coupled with the finding that *msn* and JNK are required for normal embryonic development in *Drosophila,* led us to test whether *msn* is upstream of JNK activation in the *Drosophila* embryo.

In this report, we provide biochemical and genetic evidence that *msn* functions upstream of the JNK MAPK module to direct dorsal closure in *Drosophila.* To our knowledge, this is the first demonstration that an SPS1 family Ste20 kinase activates the JNK MAPK module under normal physiological circumstances. Understanding the upstream and downstream components of this signaling pathway should provide insights into the regulation of Ste20 kinases.

Results

msn *is the* Drosophila *homolog of NIK*

The *Drosophila msn* and *C. elegans* mig-15 proteins are highly homologous to mammalian NIK, an activator of the JNK module (Fig. 1A). These three proteins share the same overall structure, containing an amino-terminal kinase domain and a carboxy-terminal putative regulatory domain. Moreover, these three proteins are highly conserved within both the kinase domain and the carboxy-terminal regulatory domain. The high conservation within these two domains suggested that *msn* and *mig-15* would activate the JNK MAPK module, because previously we have shown that both of these domains are essential for NIK to fully activate JNK in mammalian cells (Su et al. 1997). To determine whether *msn* activates JNK, 293 cells were transfected with *msn* together with an epitope-tagged JNK, and kinase activity assays were performed on JNK precipitates. Overexpression of either *msn* or NIK led to about a four- to fivefold increase in JNK kinase activity as assessed by in vitro kinase reaction (Fig. 1B). In agreement with previous studies using NIK, a mutation abolishing *msn*'s kinase activity markedly reduced *msn*'s ability to activate JNK. The ability of *msn* to activate JNK was confirmed by examining its effect on an activated transcription factor 2 (ATF2)-stimulated luciferase reporter gene; JNK has been shown to phosphorylate and activate ATF2 (Gupta et al. 1995). Overexpression of *msn* in 293 cells led to about a 10-fold increase in the transcriptional activity of ATF2 (Fig. 1C). These findings indicate that *msn* and NIK are both structurally and functionally similar and suggests that *msn* may function to activate JNK in *Drosophila.*

Mutations in msn *impair dorsal closure in the* Drosophila *embryo*

Drosophila embryos mutant for JNK (*bsk*), JNK kinase

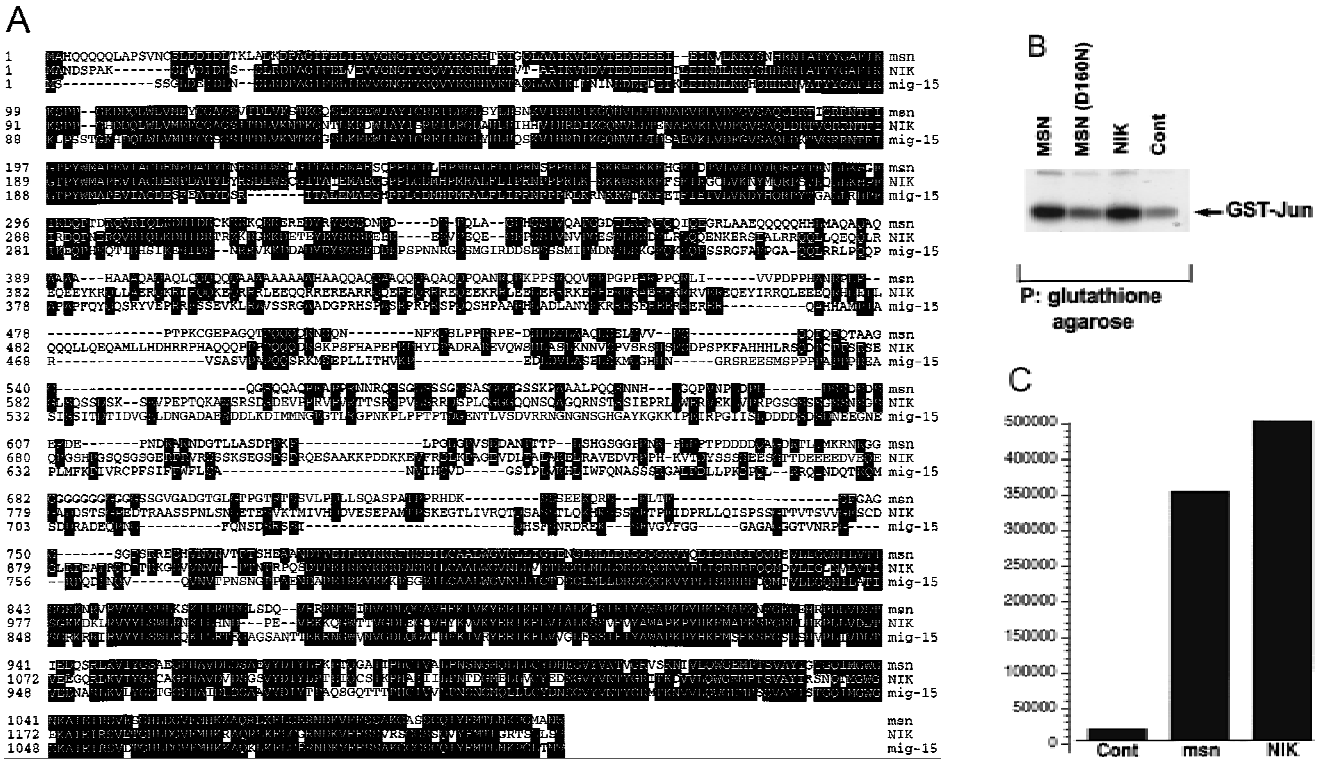

Figure 1. (*A*) Amino acid alignment of the deduced amino acid sequence of *msn,* NIK, and *mig-15.* Identical amino acids are shown in black. The amino-terminal kinase domain and the carboxy-terminal regulatory domain show a high degree of identity between the three proteins. (*B,C*) Activation of JNK by *msn* and NIK. (*B*) *msn,* NIK, and *msn* (D160N), which is a kinase inactive form of *msn* with asparagine substituted for aspartic acid at position 160, were transfected into 293 cells together with 1 μg of GST-tagged JNK. JNK activity in the transfected cell lysates was then determined as described in Materials and Methods. Equal amounts of GST–JNK were precipitated between samples (data not shown). (*C*) *msn* (0.2 μg) was cotransfected into 293 cells together with 10 ng of a plasmid expressing a fusion protein consisting of ATF2 and GAL4 DNA binding domain and 5 μg of a plasmid espressing a GAL4–luciferase reporter (Su et al. 1997). Transfection efficiency was assessed by cotransfecting 1 μg of a plasmid expressing β-galactosidase under the control of an SV40 promoter. Luciferase activity is expressed in arbitrary units after being standardized to β-galactosidase activity.

(*hep*), or *Djun* display a dorsal open phenotype, indicating that activation of this pathway is essential for normal embryonic development (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996; Hou et al. 1997; Kockel et al. 1997). To test whether *msn* activates the JNK MAPK module in *Drosophila,* we determined whether embryos zygotically mutant for *msn* also display a dorsal open phenotype. Two inversion alleles of *msn*, msn^{102} and msn^{172}, were used in this analysis (Treisman et al. 1997). Embryos homozygous for either *msn* allele or transheterozygous for the two alleles display a defect in dorsal closure, resembling embryos zygotically mutant for *bsk* (Fig. 2). The observed defect in dorsal closure was observed at a frequency of ~15%, which is similar to that found for bsk^1 (data not shown). Expression of a *msn* cDNA in the epidermis rescues the dorsal closure defect in *msn* mutant embryos, allowing survival of all homozygotes to the pupal stage; this demonstrates that the phenotype is attributable to loss of *msn* function. GAL4 driven by the ectoderm-specific

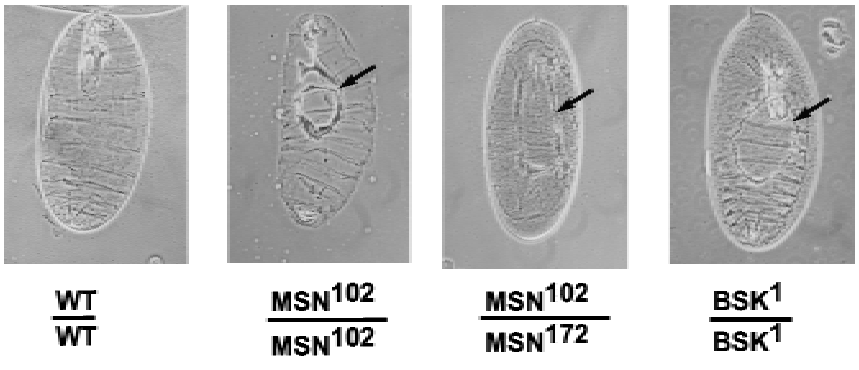

Figure 2. Effect of *msn* on dorsal closure. Cuticle preparations from embryos of the genotypes indicated. The hole in the dorsal cuticle is indicated with an arrow. Dorsal views are shown in which anterior is *up.*

promoter at 69B (Brand and Perrimon 1993) was used to direct the expression of UAS–*msn* in *msn* mutant embryos. *msn* mutant pupae that are rescued because of ectopic expression of *msn* are longer as the result of the lack of the dominant Tubby marker on the balancer. We found that 47 of 126 pupae obtained from a cross between UAS–*msn; msn*^{102}/SM6.TM6B and *69B*–GAL4, *msn*^{102}/TM6B were longer; the percentage of long pupae (36%) is very close to the 33% that is predicted if ectopic expression of *msn* rescued *msn* homozygous embryos.

msn *regulates* dpp *expression in the leading edge cells surrounding the amnioserosa*

The role of DJNK in dorsal closure is to phosphorylate and activate Djun, resulting in transcriptional activation of the *dpp* gene at the leading edge of the dorsal epidermis (Glise and Noselli 1997; Hou et al. 1997; Riesgo-Escovar and Hafen 1997a). Binding of *dpp* to its receptors *thick veins* (*tkv*) and *punt* (*put*) on the ventrally adjacent epithelial cells in turn induces reorganization of the cytoskeleton, leading to epithelial cell elongation and subsequent closure over the amnioserosa (Glise and Noselli 1997; Hou et al. 1997; Kockel et al. 1997; Riesgo-Escovar and Hafen 1997a). This is supported by the findings that *dpp* expression is decreased in the dorsal-most epithelial cells in embryos lacking *bsk* and *hep,* and that expression of activated forms of Djun or *tkv* rescues embryos zygotically mutant for *bsk* or *Djun* (Glise and Noselli 1997; Hou et al. 1997; Kockel et al. 1997; Riesgo-Escovar and Hafen 1997a). Therefore, if *msn* functions to initiate dorsal closure by activating DJNK, *dpp* expression in the leading edge epithelial cells should be decreased in *msn* mutant embryos. In agreement with the idea that *msn* functions upstream of *bsk,* we observed that *dpp* expression in leading edge cells surrounding the amnioserosa was decreased in embryos lacking *msn* to a comparable degree to *bsk*^{1} embryos (Fig. 3; data not shown). We found that ~20% of embryos derived from the *msn*^{102}/+; *msn*^{102}/+ cross displayed a decrease in *dpp* expression in the dorsal leading edge. The decrease in *dpp* staining in *msn* mutant embryos is limited to the dorsal rim cells; *dpp* expression in the visceral mesoderm and lateral ectoderm is normal in these embryos and serves as a control for *dpp* staining (Fig. 3).

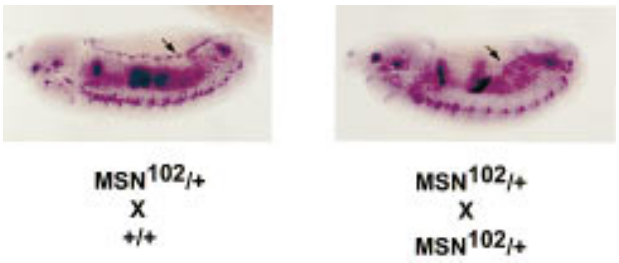

Figure 3. *dpp* expression in embryos lacking *msn*. Embryos (5 to 11 hr old) from a cross between *msn*^{102}/+ and *msn*^{102}/+ or between *msn*^{102}/+ and +/+ flies were hybridized with a *dpp* antisense probe. About 20% of embryos derived from the *msn*^{102}/+; *msn*^{102}/+ cross displayed a decrease in *dpp* expression in cells at the dorsal leading edge (arrow).

Genetic evidence that msn *functions upstream of the JNK MAP kinase pathway in* Drosophila

The above findings suggest that *msn* and *bsk* act in the same pathway and that *msn* functions upstream of DJNK activation. Further evidence that *msn* and *bsk* function in the same pathway comes from the observation that some embryos doubly heterozygous for *msn* and *bsk* displayed a dorsal open phenotype. We found that ~10% of embryos derived from a cross between *msn*/+ and *bsk*/+ flies exhibited a dorsal open phenotype (Fig. 4; Table 1). The defect in dorsal closure in embryos doubly heterozygous for *msn* and *bsk* is not a dominant effect of either gene; a defect in dorsal closure was very rarely observed when *msn*/+ or *bsk*/+ flies were crossed with wild-type flies (Table 1). The presence of such defects in doubly heterozygous flies strongly suggests that the genes function in the same pathway. Moreover, the severity of the phenotype correlated with the strength of the *bsk* allele with which *msn*/+ flies were crossed (Fig. 4; data not shown); *Df(2L)flp 147E* > *bsk*^{2} > *bsk*^{1} (Riesgo-Escovar et al. 1996; Sluss et al. 1996). To confirm genetically that *msn* also functions upstream of *hep,* we examined embryos doubly heterozygous for *msn* and *hep. hep* is on the X chromosome, and both maternal and zygotic *hep* contribute to dorsal closure. To obtain flies doubly heterozygous for *msn* and *hep, msn*/+ males were crossed with *hep*^{r75}/+ females. We found that ~35% of the flies obtained from this cross displayed a defect in dorsal closure. This finding is very close to the predicted frequency of 37% of embryos with a reduction in both the maternal and zygotic dosage of *hep* and the zygotic dosage of *msn* (Fig. 4; Table 1). A defect in dorsal closure is not observed when *hep*/+ females are crossed with +/Y males, indicating that embryos with the dorsal closure defect carry the *msn* mutation. Reduced dosage of both zygotic and maternal *hep* was required for the zygotic lethality of *msn*/+ embryos; +/+; *msn*/+ flies, which contain only a reduced dosage of maternal *hep,* emerged at the predicted frequency of 25% (26 of 117, 22%). In contrast, the viability of *msn*/+ flies lacking one copy of zygotic *hep* was reduced by >80%.

We then tested whether a constitutively active form of Djun could rescue the dorsal open phenotype in *msn* mutant embryos. Previous studies have shown that activated *Djun* rescues the *bsk* phenotype, indicating that one of the main functions of JNK is to phosphorylate and activate Jun (Hou et al. 1997; Riesgo-Escovar and Hafen 1997a). A constitutively active form of Djun has been made by replacing the JNK phosphorylation sites with acidic residues (Treier et al. 1995). To test whether this activated Djun rescues the dorsal open phenotype in *msn* mutant embryos, we expressed it under the control of the *hsp70* heat shock promoter in the *msn* mutant background (Hou et al. 1997). Expression of activated Djun rescued the dorsal open phenotype in most of the *msn* mutant embryos (Fig. 5); heat shock decreased the num-

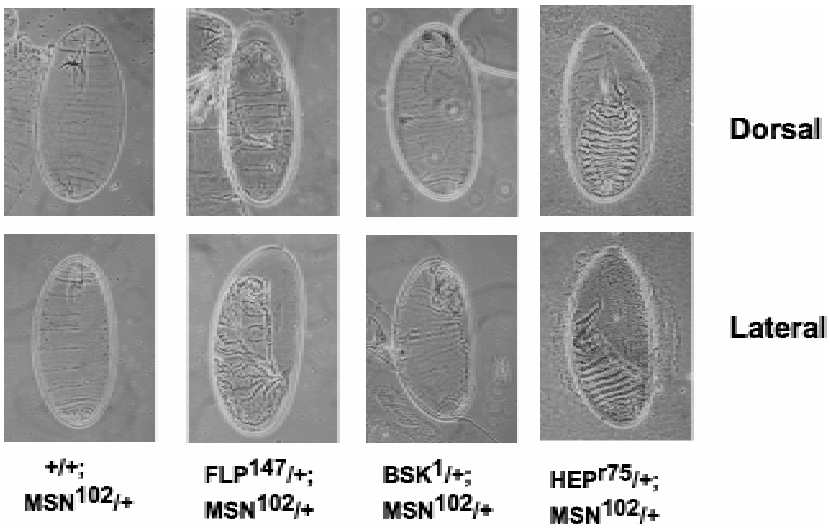

Figure 4. *msn* interacts genetically with *bsk* and *hep*. Cuticle preparations from embryos obtained from the crosses shown. Crossing *msn*/+ flies to wild-type flies (+/+) did not lead to a defect in dorsal closure (see Table 1). In contrast to the normal embryos obtained from crossing *msn*/+ flies with wild-type flies (+/+), crossing *msn*/+ flies with three different *bsk* alleles or with *hep* led to defects in dorsal closure. Note that embryos derived from a cross between *Df(2L)flp147*/+, which is a complete loss-of-function mutant of *bsk*, and *msn*/+ flies display the most severe defect in dorsal closure. (*Top*) Dorsal views with anterior *up;* (*bottom*) lateral views with anterior *up*.

ber of embryos with a dorsal open phenotype from ~50% (36 of 76) to <10% (6 of 65). In addition, we found that expression of an activated form of *tkv*, tkv^{Q253D} (Nellen et al. 1996), also rescued the dorsal open phenotype in *msn* mutant embryos. GAL4 driven by the ectoderm-specific promoter at 69B (Brand and Perrimon 1993) was used to direct the expression of UAS–tkv^{Q253D} in *msn* mutant embryos. This expression of activated *tkv* rescued partially the dorsal open phenotype caused by *msn;* it also had a dorsalizing effect on the ventral ectoderm of the embryos related to the earlier function of *dpp* in establishing the dorsoventral axis, which served to mark embryos expressing activated *tkv* (Fig. 5). Thus, these findings provide genetic evidence that *msn* functions upstream of the JNK MAP kinase module in leading edge cells.

GTP–Rac is required for msn *and NIK to fully activate JNK*

Rac activation is thought to be important for stimulating dorsal closure because expression of dominant negative forms of Rac (DN Rac) or Cdc42 inhibit dorsal closure in the *Drosophila* embryo (Harden et al. 1995; Riesgo-Escovar et al. 1996). The finding that activated Djun rescues the defect in dorsal closure induced by expression of DN Rac indicates that Rac probably functions upstream of JNK activation to stimulate dorsal closure (Hou et al. 1997). To begin to address the mechanism whereby Rac and *msn* cooperate to activate JNK, 293 cells were transfected with *msn* or NIK together with DN Rac and an epitope-tagged JNK, and kinase activity assays were performed on JNK precipitates. Although overexpression of NIK or *msn* led to a four- to fivefold increase in JNK activation, coexpression of DN Rac decreased markedly JNK activation (Fig. 6).

The PAK family of Ste20 kinases contain a conserved p21Rac and Cdc42-binding domain and are activated by

Table 1. *Embryos with defects in dorsal closure*

	No./total no. (%)
+/+ × msn^{102}/+	1/143 (0.7)
+/+ × bsk^{1}/+	1/108 (0.9)
bsk^{1}/+ × msn^{102}/+	15/137 (10.9)
bsk^{2}/+ × msn^{102}/+	20/198 (10.1)
flp147/+ × msn^{102}/+	39/388 (10.1)
hep^{1}/+ × msn^{102}/+	31/88 (35.2)
hep^{r75}/+ × msn^{103}/+	22/61 (36.0)

Embryos were collected and aged for 24 hr. Cuticles were prepared from unhatched embryos, and the number of embryos with an aberrant dorsal cuticle was determined. The total number of hatched and unhatched embryos was determined by resuspending all of the larvae and embryos in Hoyer's solution and counting under a microscope.

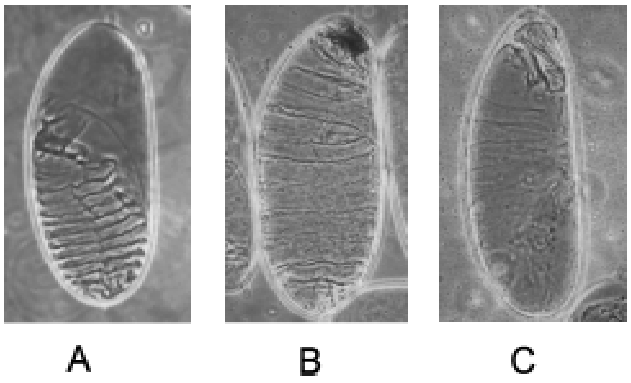

Figure 5. Expression of activated Djun or *tkv* rescues the dorsal open phenotype in *msn* mutant embryos. (*A*) *msn* mutant embryo containing hs-$Djun^{Asp}$ and not exposed to heat shock displays a severe defect in dorsal closure. (*B*) *msn* mutant embryos derived from the same cross and exposed to heat shock for 45 min (msn^{102}/msn^{172}; hs-$SEJun^{Asp}$). The dorsal cuticle is mostly closed in *msn* mutant embryos expressing activated Djun. (*C*) *msn* mutant embryo expressing activated tkv. The defect in dorsal closure is partially rescued by activated tkv (msn^{102}/msn^{172}; UAS–tkv^{Q252D}/*69B GAL4* embryo). Lateral views are shown, with anterior *up*.

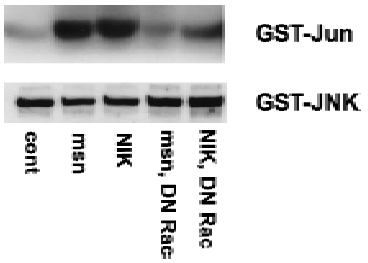

Figure 6. DN Rac inhibits JNK activation by *msn* and NIK. 293 cells were transfected with 2 μg of *msn* or NIK cDNA together with 1 μg of GST-tagged JNK with or without 2 μg of DN Rac. JNK activity in the transfected cell lysates was then determined as described in Fig. 1. To ensure that equal amounts of JNK were immunoprecipitated between samples, one-half of the precipitate was immunoblotted with antibodies to GST (JNK).

binding GTP-bound Cdc42 and Rac (Burbelo et al. 1995; Martin et al. 1995). To determine whether Rac may regulate directly *msn* or NIK in a similar manner to the way it activates PAK, we assessed whether *msn* or NIK bound activated Rac in vitro. Although *msn* and NIK do not contain a Rac-binding domain, other proteins without a Rac-binding motif have been found recently to bind Rac (Fanger et al. 1997). We were unable to detect binding of activated Rac to either *msn* or NIK using an overlay assay with GTP-bound Rac or by using the yeast two hybrid system, whereas binding to a positive control, mammalian PAK1, or *Drosophila* p21-activated protein kinase (DPAK), was easily detected (data not shown). These findings suggest that although Rac cooperates with both NIK and *msn* to activate JNK, Rac is unlikely to activate directly *msn* or NIK.

Discussion

At least two distinct MAPK modules have now been identified in *Drosophila*. Genetic evidence has established that these pathways function independently and are essential for the regulation of distinct developmentally regulated processes (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). The first MAPK module to be described is activated by several receptor tyrosine kinases including the sevenless, *Drosophila* epidermal growth factor (DEGF), and torso receptors (for review, see Dickson and Hafen 1994; Perrimon 1994). These receptors signal through DRas to activate DRaf (MKKK), which in turn phosphorylates and activates DSor1 (MKK), which phosphorylates and activates the *Drosophila* MAP kinase rolled (DERK). Activation of this MAPK module is essential for the sevenless receptor to specify the fate of R7 photoreceptors, the DEGF receptor to specify embryonic dorsoventral polarity or the development of other photoreceptors, and torso to specify embryonic termini. The second MAPK module to be described in *Drosophila* is the JNK pathway (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). Activation of this pathway stimulates changes in the cytoskeleton that enable lateral ectodermal cells to spread over the amnioserosa and join in the dorsal midline. Failure to activate the JNK MAP kinase module leads to a dorsal open phenotype and embryonic lethality. The JNK module is also activated during the *Drosophila* immune response to lipopolysaccharide (LPS) (Sluss et al. 1996). Unlike the Ras–Raf–MAPK module for which the upstream signals are well known, very little is known about the receptors or signaling molecules that mediate activation of the JNK MAPK module in either *Drosophila* or mammalian cells under normal biological conditions. Our findings indicate that the Ste20-related kinase *msn* functions upstream of the JNK MAPK module and is essential for initiating changes in cell shape that regulate dorsal closure of the *Drosophila* embryo. We found that *msn* mutant embryos exhibit a defect in dorsal closure that is similar to the defect in embryos lacking components of the JNK pathway. Furthermore, the finding that flies doubly heterozygous for *msn* and either *bsk* or *hep* display a defect in dorsal closure, coupled with genetic epistasis analysis showing that activated Djun and tkv at least partially rescue the defect in dorsal closure in *msn* mutant embryos, indicates that *msn* and *bsk* function in the same pathway and that *msn* functions upstream of *bsk* (JNK) activation.

Although several members of the SPS family of Ste20-related kinases have been shown to be specific and potent activators of the JNK pathway when overexpressed transiently in mammalian cells (Pombo et al. 1995; Hu et al. 1996; Su et al. 1997), little is known about the function or regulation of these kinases under normal physiological circumstances. Our finding that *msn* functions upstream of the JNK MAP kinase module in a genetically defined system indicates that kinases in this family are important activators of JNK under physiologically relevant conditions. The data put to rest the idea that activation of the JNK MAPK module observed in transient transfection assays by SPS1 Ste20 family members is an experimental artifact related to misexpressing these proteins at high levels.

As a result of several recent studies, a number of signaling molecules that are critical for stimulating dorsal closure can now be ordered on a signaling pathway. Genetic epistasis analysis in yeast, as well as studies in mammalian cells, lead us to predict that *msn* functions as a MKKKK in *Drosophila* and activates the JNK pathway by activating a yet to be defined *Drosophila* MKKK (Fig. 7). Therefore, *msn* becomes the most proximal molecule identified so far on this signaling pathway. Studies over the past several years have clarified which signaling molecules acting downstream of this putative *Drosophila* MKKK are important in stimulating dorsal closure. A *Drosophila* MKKK would be likely to phosphorylate and activate the JNK kinase *hep*, which in turn phosphorylates and activates JNK (*bsk*) (Glise et al. 1995; Riesgo-Escovar et al. 1996; Sluss et al. 1996). DJNK mediates its changes in the cytoskeleton primarily by regulating the expression of target genes; DJNK phosphorylates and activates DJun, which in turn cooperates with DFos to stimulate transcription of *dpp*, a member of the transforming growth factor-β (TGF-β) family (Glise and

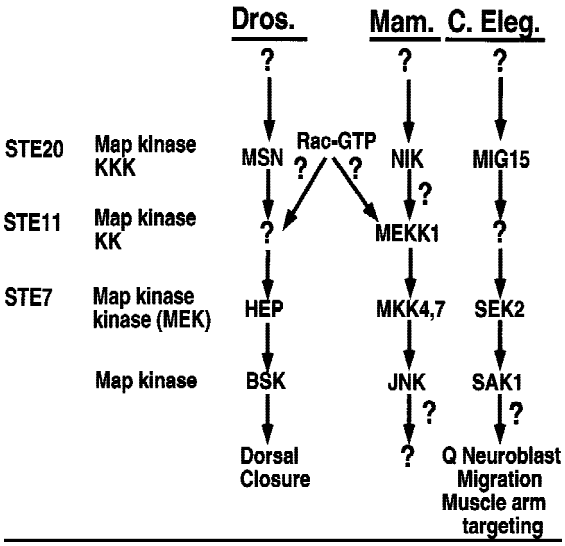

Figure 7. Schematic diagram showing the function of *msn*, NIK, and *mig-15* (see text for details). *mig-15* has not yet been shown to activate *C. elegans* JNK (SAK1) or JNK kinase (SEK). In addition, neither SAK nor SEK have yet been shown to function upstream of Q-neuroblast migration or muscle arm targeting in *C. elegans*.

Noselli 1997; Hou et al. 1997; Riesgo-Escovar and Hafen 1997a,b; Noselli 1998). Signaling of *dpp* through its receptors *tkv* and *put* then stimulates changes in cell shape, thereby enabling the lateral epidermal cells to stretch over and subsequently close over the amnioserosa dorsally (Affolter et al. 1994; Brummel et al. 1994; Terracol and Lengyel 1994; Letsou et al. 1995; Ruperte et al. 1995).

Currently very little is known about the downstream MKKKs/MEKKs that are activated by Ste20 kinases or the mechanisms whereby these MKKKs are activated. The identification of a large number of kinases in mammalian cells that are capable of functioning as MKKKs, coupled with the finding that many of these kinases are constitutively active when overexpressed in cells, has hindered the identification of the specific downstream targets of Ste20 kinases or the mechanisms by which they activate MKKKs (Kyriakis and Avruch 1996). Recent evidence has suggested that direct interaction of Ste20 kinases with specific downstream MKKKs may be a critical component of their regulation (Hu et al. 1996; Su et al. 1997); we have found that the carboxy-terminal domain of NIK, which is highly conserved between NIK, *msn*, and *mig-15*, is critical for NIK to activate the JNK pathway and to associate with MEKK1 in cells (Su et al. 1997).

Our findings also indicate that Rac cooperates with SPS1 family Ste20 kinases to mediate activation of JNK. Previous studies have suggested that Rac functions upstream of the DJNK MAPK module in *Drosophila;* expression of DN Rac or Cdc42 results in a dorsal open phenotype (Harden et al. 1995; Riesgo-Escovar et al. 1996) and the defect in dorsal closure induced by expression of DN Rac can be reversed by expressing an activated form of Djun (Hou et al. 1997). Because PAK family Ste20 kinases are activated by GTP-bound Cdc42 and Rac, it had been assumed that this family of Ste20 kinases rather than an SPS1 Ste20 kinase family member would cooperate with Rac to activate JNK (Noselli 1998). Thus, these findings have led us to consider new paradigms for how Rac functions to activate JNK. We do not think that Rac activates *msn* directly. Unlike PAK family members, *msn* does not contain a consensus Rac-binding motif and we did not detect binding of *msn* to activated Rac in vitro (data not shown). Rather, we favor the hypothesis that Rac cooperates with *msn* to activate a downstream MKKK. MKKKs of the mixed lineage kinase family as well as MEKK1 and MEKK4 have been shown to bind GTP-bound Cdc42 or Rac (Teramoto et al. 1996; Fanger et al. 1997). Thus, Rac may cooperate with *msn* to regulate a downstream MKKK in a manner similar to the way Ras cooperates with a yet to be defined kinase to activate RAF. In this model, binding of a MKKK to activated Rac would facilitate interaction of this MKKK with *msn*, thereby enabling its activation by *msn* (Fig. 7). However, we cannot exclude the possibility that Rac and *msn* activate parallel pathways converging on JNK activation.

It is intriguing that the *C. elegans* homolog of *msn*, *mig-15*, is also an essential gene in development and, like *msn*, functions to regulate processes that undoubtedly require changes in the cytoskeleton and cell shape in developing worms (E. Hedgecock, pers. comm.). *mig-15* mutants have a variety of developmental defects including defects in Q-neuroblast migration and muscle arm targeting. Although it is not yet clear whether any or all of the phenotypes apparent in worms lacking *mig-15* are attributable to defective activation of the *C. elegans* JNK, these findings suggest a common theme in which JNK activation plays a central role in a variety of developmental processes by coordinating changes in cell shape and the cytoskeleton (Fig. 7). It is likely, however, that some of the phenotypes observed in embryos lacking these Ste20 kinases are independent of their effect on JNK activation. In addition to defects in dorsal closure, some embryos mutant for *msn* displayed a ventral defect (data not shown). Moreover, although *msn*, like *bsk* and *Djun*, is not required for specifying the fate of photoreceptor cells, clones of *msn* mutant photoreceptor cells display an abnormal shape (Riesgo-Escovar et al. 1996; Hou et al. 1997; Treisman et al. 1997). These defects are never observed in embryos mutant for *bsk* and therefore indicate that *msn* has other essential functions that are independent of JNK activation (Riesgo-Escovar et al. 1996). We were unable to evaluate the maternal contribution of *msn* to dorsal closure because of its requirement for oogenesis (Treisman et al. 1997).

Our findings also support the idea that the regulation of Ste20 kinases in mammalian cells is likely to be more complex than previously recognized. Several mammalian Ste20 kinases related to *msn* and NIK, which specifically activate the JNK pathway, such as GC kinase and HPK1, have been identified (Pombo et al. 1995; Hu et al. 1996; Su et al. 1997). It has not been clear whether the

function served by these kinases is redundant or whether each may function only under specific circumstances. Although the full repertoire of Ste20 kinases in *Drosophila* is not known, our results support the idea that members of this family are subject to different modes of regulation and, for at least some functions, are not redundant with other family members. Studying *msn* and *mig-15* in defined genetic systems will be a critical tool in the effort to unravel these complex pathways in mammalian cells.

The placement of *msn*, *mig-15*, and NIK on a MAP kinase pathway that is likely to be conserved between worms, flies, and mammals is reminiscent of the better studied Ras–Raf–MAPK module. Genetic analysis of this MAP kinase pathway in both *Drosophila* and *C. elegans* was critical in eventually elucidating key components of this pathway. Despite the large number of Ste20 kinases that have now been identified in mammalian cells, virtually nothing is known about either the upstream regulation of this family of kinases or their downstream targets. Thus, the identification of other components of this pathway in *Drosophila* and *C. elegans* will undoubtedly provide valuable insights into the signals that activate Ste20 kinases and their subsequent effects on cells.

Materials and methods

Constructs, mutagenesis, and JNK assays

All constructs for tissue culture were *myc*-epitope tagged in their carboxyl terminus and subcloned into the vector pRK5 (Marcusohn et al. 1995; Su et al. 1997). To assess JNK activation, 2 μg of NIK, *msn*, or vector control were transfected into 293 cells together with 2 μg of GST-tagged JNK. GST–JNK was precipitated from 500 μg of cell lysates using glutathione agarose and subjected to an in vitro kinase assay using GST–Jun (Su et al. 1997). Reaction products were separated by SDS-PAGE and visualized by autoradiography. To make a kinase inactive form of *msn*, the *msn* cDNA was subcloned into the vector pALTER (Promega) and site directed mutagenesis was performed according to the manufacturer's specifications. The kinase inactive *msn* contained a substitution of N for D at position 160 (msn D160N). Transactivation of ATF2 was assessed by transfecting a fusion protein consisting of ATF2 (amino acids 1–505) and the GAL4–DNA-binding domain together with a vector control or *msn* and a reporter plasmid containing 5× GAL4–DNA-binding domains. All transfections were standardized by cotransfecting a control plasmid expressing β-galactosidase (Promega). Dominant negative N17 Rac was expressed using the vector RK5 as described (Marcusohn et al. 1995).

Fly strains and genetic analysis

msn^{102} and msn^{172} are x-ray-induced inversions with breakpoints within the *msn* gene (Treisman et al. 1997). Both inversions break within the coding region, although msn^{102} causes a larger disruption of the *msn* gene. The *Df(2L)flp 147E* line, a deletion of part of the *bsk*-coding region, was obtained from K. Beckingham (Rice University, Houston, TX). The bsk^1 and bsk^2 lines were obtained from the Nusslein-Volhard laboratory by way of T. Ip (University of Massachusetts Medical School, Worcester, MA). The hep^1 and hep^2 lines were obtained from S. Noselli [Centre de Biologie du Development, Centre National de la Recherche Scientifique (CNRS), Toulouse, France]. The activated Djun line has been described previously (Treier et al. 1995).

Genetic interactions

To test for genetic interaction between *msn* and *bsk*, or between *msn* and *hep*, embryos were collected from a cross between *msn*/+ flies with either +/+, *bsk*/+, or *hep*/+ flies. Embryos were collected for 6 hr and aged for an additional 16 hr and the total number of embryos with a dorsal open phenotype was determined.

To test whether activated Jun (hs-SEJun^{Asp}) rescues *msn* mutant embryos, activated Djun was introduced into the msn^{102} mutant background using the compound balancer SM6.TM6B to generate msn^{102}; hs-SEJun^{Asp}/SM6.TM6B. To induce expression of hs-SEJun^{Asp}, 5- to 7-hr-old embryos were heat-shocked for 45 min. Cuticle preparations were then performed 16 hr after heat shock.

To target expression of activated *tkv*, the activated type 1 *dpp* receptor, to the dorsal ectoderm we used the Gal4/UAS system (Brand and Perrimon 1993). A UAS–tkv^{Q253D} transgene on the third chromosome was recombined with msn^{102} to generate msn^{102}, UAS–tkv^{Q253D}/TM6B (Hoodless et al. 1996). To induce ectodermal expression of TKV^{Q252D}, msn^{102}, UAS–tkv^{Q253D}/TM6B flies were crossed to *69B–GAL4*, msn^{102}/TM6B.

Ecptopic expression of msn

The Gal4/UAS system was also used to test whether ectopic expression of *msn* rescues the dorsal defect in *msn* mutant embryos. The *msn* cDNA was cloned into the vector pUAST (Brand and Perrimon 1993). Germ-line transformations were then performed using standard methods following injection of pUAS–msn with a helper plasmid turbo δ2-3 (Spradling and Rubin 1982). Transgenic lines containing UAS–*msn* on the second chromosome were crossed to msn^{102} to generate UAS–*msn*; msn^{102}/SM6.TM6B. To induce ectodermal expression of *msn*, UAS–*msn*; msn^{102}/SM6.TM6B were crossed to *69B–GAL4*, msn^{102}/TM6B.

Cuticle preparations

Embryos were collected on yeasted agar plates, dechorionated in 100% bleach, rinsed with water, and then fixed for 10 min at 65°C in a solution containing acetic acid and glycerol at a 3:1 ratio. Embryos were then mounted in Hoyer's medium and incubated for 24 hr at 65°C.

In situ hybridization

In situ hybridization to embryos was performed using digoxigenin-labeled antisense *dpp* RNA probe according to the method of Tautz and Pfeifle (1989) as modified by Ronchi et al. (1993).

Acknowledgments

We thank Tony Ip of The University of Massachusetts Medical School for helpful discussions and for kindly providing the *dpp* cDNA and the bsk^1 and bsk^2 stocks. We thank S. Noselli for *hep* fly stocks and Gary Struhl for UAS–tkv^{Q2530}. We thank X. Steven Hou and Norbert Perrimon of Harvard Medical School for helpful discussions and hsSEJun^{Asp} fly stocks, and Kate Beckingham of Rice University for providing the *Df(2L)flp 147E*

stocks. We thank Hongzhi Liu for performing the yeast two-hybrid analysis of activated DRac and *msn*. The manuscript was improved by the critical comments of Corinne Zaffran. This work was supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and American Diabetes Association to E.Y.S.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked ''advertisement'' in accordance with 18 USC section 1734 solely to indicate this fact.

References

Affolter, M., D. Nellen, U. Nussbaumer, and K. Basler. 1994. Multiple requirements for the receptor serine/threonine kinase thick veins reveals a novel function of TGFβ homologs during *Drosophila* development. *Development* **120:** 3105–3117.

Bagrodia, S., B. Derijard, R.J. Davis, and R.A. Cerione. 1995. Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J. Biol. Chem*. **270:** 27995–27998.

Brand, A.H. and N. Perrimon. 1993. Targeting gene expression as a mean of altering cell fates and generating dominant phenotypes. *Development* **118:** 401–415.

Brummel, T.J., G. Twombly, G. Marques, J.L. Wrana, S.J. Newfeld, L. Attisano, and J. Massague. 1994. Characterization and relationship of Dpp receptors encoded by the *saxophone* and *thick veins* genes in Drosophila. *Cell* **78:** 251–261.

Burbelo, P.D., D. Drechsel, and A. Hall. 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J. Biol. Chem*. **270:** 29072–29074.

Campos-Ortega, J. and V. Hartenstein. 1985. *The embryonic development of* Drosophila melanogaster. Springer-Verlag, Berlin, Germany.

Cobb, M.H. and E.J. Goldsmith. 1995. How MAP kinases are regulated. *J. Biol. Chem*. **270:** 14843–14846.

Creasy, C.L. and J. Chernoff. 1995. Cloning and characterization of human protein kinase with homology to Ste20. *J. Biol. Chem*. **270:** 21695–21700.

Davis, R.J. 1994. MAPKs: New JNK expands the group. *Trends Biochem. Sci.* **19:** 470–473.

Dickson, B. and E. Hafen. 1994. Genetics of signal transduction in invertebrates. *Curr. Opin. Genet. Dev.* **4:** 64–70.

Diener, K., X.S. Wang, C. Chen, C.F. Meyer, G. Keesler, M. Zukowski, T.H. Tan, and Z. Yao. 1997. Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. *Proc. Natl. Acad. Sci.* **94:** 9687–9692.

Fanger, G.R., N.L. Johnson, and G.L. Johnson. 1997. MEK kinases are regulated by EGF and selectively interact with Rac/Cdc42. *EMBO J.* **16:** 4961–4972.

Friesen, H., R. Lunz, S. Doyle, and J. Segall. 1994. Mutation of Sps1-encoded protein kinase of *Sacchromyces cerevisiae* leads to defects in transcription and morphology during spore formation. *Genes* & *Dev*. **8:** 2162–2175.

Glise, B. and S. Noselli. 1997. Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in *Drosophila* morphogenesis. *Genes* & *Dev*. **11:** 1738–1747.

Glise, B., H. Bourbon, and S. Noselli. 1995. *Hemipterous* encodes a novel *Drosophila* MAP kinase kinase required for epithelial cell sheet movement. *Cell* **83:** 451–461.

Gupta, S., D. Campbell, B. Derijard, and R.J. Davis. 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267:** 389–393.

Harden, N., H.Y. Loh, W. Chia, and L. Lim. 1995. A dominant inhibitory version of the small GTP binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*. *Development* **121:** 930–914.

Harden, N.J., J. Lee, H.Y. Loh, Y.M. Ong, I. Tan, T. Leung, E. Manser, and L. Lim. 1996. A *Drosophila* homolog of the Rac and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. *Mol. Cell. Biol.* **16:** 1896–1908.

Herskowitz, I. 1995. MAP kinase pathways in yeast: For mating and more. *Cell* **80:** 187–197.

Hoodless, P.A., T. Haerry, S. Abdollah, M. Stapleton, M.B. O'Connor, L. Attisano, and J.L. Wrana. 1996. MADR1, a MAD-related protein that functions in BMP2 signaling pathways. *Cell* **85:** 489–500.

Hou, X.S., E.S. Goldstein, and N. Perrimon. 1997. *Drosophila* Jun relays the Jun amino-terminal kinase signal transduction pathway to the Decapentaplegic signal transduction pathway in regulating epithelial cell sheet movement. *Genes* & *Dev*. **11:** 1728–1737.

Hu, M.C., W.R. Qiu, X.R. Wang, C.F. Meyer, and T.-H. Tan. 1996. Human HPK1, a novel hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. *Genes* & *Dev*. **10:** 2251–2264.

Kockel, L., J. Zeitlinger, L.M. Staszewski, M. Moldzik, and D. Bohmann. 1997. Jun in *Drosophila* development: Redundant and nonredundant functions and regulation by two MAPK signal transduction pathways. *Genes* & *Dev*. **11:** 1748–1758.

Kyriakis, J.M. and J. Avruch. 1996. Sounding the alarm: Protein kinase cascades activated by stress and inflammation. *J. Biol. Chem*. **271:** 24313–24316.

Letsou, A.K., K. Arora, J.L. Wrana, K. Simin, V. Twombly, J. Jamal, M.B. Staehling-Hampton, W.M. Gelbert, J. Massague, and M.B. O'Connor. 1995. *Drosophila* dpp signaling is mediated by the *punt* gene product: A dual ligand binding type II receptor for the TGFβ receptor family. *Cell* **80:** 899–908.

Manser, E., T. Leung, H. Salihuddin, Z. Zhao, and L. Lim. 1994. A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature* **367:** 40–46.

Marcusohn, J., S.I. Isakoff, E. Rose, M. Symons, and E.Y. Skolnik. 1995. The GTP-binding protein Rac does not couple PI3-kinase to insulin-stimulated glucose transport. *Curr. Biol.* **5:** 1296–1302.

Martin, G.A., G. Bollag, F. McCormick, and A. Abo. 1995. A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20. *EMBO J.* **14:** 1970–1978.

Nellen, D., R. Burke, G. Struhl, and K. Basler. 1996. Direct and long-range action of a DPP morphogen gradient. *Cell* **85:** 357–368.

Noselli, S. 1998. JNK signaling and morphogenesis in *Drosophila*. *Trends Genet.* **14:** 33–38.

Perrimon, N. 1994. Signaling pathways initiated by receptor protein tyrosine kinases in *Drosophila*. *Curr. Opin. Cell Biol.* **6:** 260–266.

Pombo, C.M., J.H. Kehrl, I. Sanchez, P. Katz, J. Avruch, L.I. Zon, J.R. Woodgett, T. Force, and J.M. Kyriakis. 1995. Activation of the SAPK pathway by the human STE20 homologue germinal centre kinase. *Nature* **377:** 750–754.

Pombo, C.M., J.V. Bonventre, A. Molnar, J. Kyriakis, and T. Force. 1996. Activation of a human Ste20-like kinase by oxidant stress defines a novel stress response pathway. *EMBO J.* **15:** 4537–4536.

Riesgo-Escovar, J.R. and E. Hafen. 1997a. *Drosophila* Jun kinase

regulates expression of *decapentaplegic* via the ETS-domain protein Aop and the AP-1 transcription factor DJun during dorsal closure. *Genes* & *Dev.* **11:** 1717–1727.

———. 1997b. Common and distinct roles of DFos and DJun during *Drosophila* development. *Science* **278:** 669–671.

Riesgo-Escovar, J.R., M. Jenni, A. Fritz, and E. Hafen. 1996. The *Drosophila* Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. *Genes* & *Dev.* **10:** 2759–2768.

Ronchi, E., J.E. Treisman, N. Drostatni, G. Struhl, and C. Desplan. 1993. Down-regulation of the *Drosophila* morphogen Bicoid by the Torso receptor mediated signal transduction cascade. *Cell* **74:** 347–355.

Ruperte, E.T., M.D. Nellen, M. Affolter, and K. Basler. 1995. An absolute requirement for both type II and type I receptors, punt and thick veins, for dpp signaling in vivo. *Cell* **80:** 890–898.

Sluss, K.K., Z. Han, T. Barrett, R.J. Davis, and Y.T. Ip. 1996. A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila. Genes* & *Dev.* **10:** 2745–2758.

Spradling, A.C. and G.M. Rubin. 1982. Transposition of cloned P-elements into *Drosophila* germline chromosomes. *Science* **218:** 341–347.

Su, Y., J. Han, S. Xu, M. Cobb, and E.Y. Skolnik. 1997. NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. *EMBO J.* **16:** 1279–1290.

Tautz, D. and C. Pfeifle. 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98:** 81–95.

Teramoto, H., O.A. Coso, H. Miyata, T. Igishi, T. Miki, and J.S. Gutkin. 1996. Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. *J. Biol. Chem*. **271:** 27225–27228.

Terracol, R. and J.A. Lengyel. 1994. The *thick veins* gene of *Drosophila* is required for dorsoventral polarity of the embryo. *Genetics* **138:** 165–178.

Treier, M., D. Bohman, and M. Mlodzik. 1995. Jun cooperates with the ETS domain protein Pointed to induce photoreceptor R7 fate in the *Drosophila* eye. *Cell* **83:** 753–760.

Treisman, J.E., N. Ito, and G.M. Rubin. 1997. *misshapen* encodes a protein kinase involved in cell shape control in *Drosophila. Gene* **186:** 119–125.

Young, P.E., A.M. Richman, A.S. Ketchum, and D.P. Kiehart. 1993. Morphogenesis in *Drosophila* requires nonmuscle myosin heavy chain function. *Genes* & *Dev.* **7:** 29–41.