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The *Drosophila* STE20-like kinase Misshapen is required downstream of the Frizzled receptor in planar polarity signaling

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The *Drosophila misshapen* (*msn*) gene is a member of the STE20 kinase family. We show that *msn* acts in the Frizzled (Fz) mediated epithelial planar polarity (EPP) signaling pathway in eyes and wings. Both *msn* loss- and gain-of-function result in defective ommatidial polarity and wing hair formation. Genetic and biochemical analyses indicate that *msn* acts downstream of *fz* and *dishevelled* (*dsh*) in the planar polarity pathway, and thus implicates an STE20-like kinase in Fz/Dsh-mediated signaling. This demonstrates that seven-pass transmembrane receptors can signal via members of the STE20 kinase family in higher eukaryotes. We also show that Msn acts in EPP signaling through the JNK (Jun-N-terminal kinase) module as it does in dorsal closure. Although at the level of Fz/Dsh there is no apparent redundancy in this pathway, the downstream effector JNK/MAPK (mitogen-activated protein kinase) module is redundant in planar polarity generation. To address the nature of this redundancy, we provide evidence for an involvement of the related MAP kinases of the p38 subfamily in planar polarity signaling downstream of Msn.

Keywords: Dishevelled/ommatidia/p38 kinase/planar polarity/STE20 kinase

Introduction

The responses to extracellular stimuli and intercellular communication are important elements governing the development of higher eukaryotes. The resulting activation of signaling pathways leads to specific responses ranging from cell proliferation to cell shape changes. Although many signaling molecules have been identified through biochemical or genetic experiments, the picture of the respective signal transduction cascades is still incomplete. The genetic analysis of signaling pathways and mechanisms in multicellular model organisms like *Drosophila* or *Caenorhabditis elegans* has helped to identify some of the missing links and address the issues of signaling specificity *in vivo* (Greenwald and Rubin, 1992; Zipursky and Rubin, 1994; Artavanis-Tsakonas *et al*., 1995; Marshall, 1995; Sternberg *et al*., 1995).

Many distinct stimuli and signals lead to the activation of the so-called mitogen-activated protein kinase (MAPK) modules. Several related MAPK cascade modules have been identified in many organisms (including yeast, mammalian cells and *Drosophila*; Marshall, 1994; Herskowitz, 1995; Kyriakis and Avruch, 1996; Treisman, 1996) comprising the ERK (extracellular signal-related kinase)-type modules, the JNK (Jun-N-terminal kinase) or SAPK (stress-activated protein kinase) modules and the kinases of the p38 family. All the MAPK modules consist of a kinase cascade of at least three kinases acting sequentially (Marshall, 1994; Herskowitz, 1995). For any of these kinases there are multiple enzymes with the same (or very similar) specificity and thus it has been postulated that a high degree of redundancy is present within these cascades.

Despite this detailed knowledge about the MAPK modules, their upstream activating mechanisms are only partially known. Only the ERK-type MAPKs have a well defined mechanism of activation from the signal-responding cell surface receptors; they are effectors of Ras, mediating responses ranging from cell proliferation to cell fate induction (e.g. for *Drosophila*; Zipursky and Rubin, 1994; Freeman, 1997; Karim and Rubin, 1998). In contrast, the molecular mechanisms of activation of the JNK or p38 modules are poorly understood. Although they are potently activated by cellular stress and extracellular signals like LPS and IL-1 (for reviews see Kyriakis and Avruch, 1996; Minden and Karin, 1997; Gutkind, 1998) and factors involved in cell death signaling, such as DAXX and Fas (Coso *et al*., 1995; Minden *et al*., 1995; Yang *et al*., 1997), it is not well established how signals from the cell surface are linked to activation of these MAPKs (Gutkind, 1998).

Genetic experiments in yeast and more recently in *Drosophila*, as well as studies in mammalian cell cultures, have identified the STE20 kinase family as potential upstream activators of the JNK-type MAPK modules (Herskowitz, 1995; Pombo *et al*., 1995; Hu *et al*., 1996; Su *et al*., 1997, 1998), and thus the STE20-like kinases have also been referred to as MKKKKs (MAP kinase kinase kinase kinase). Despite the well documented yeast example where STE20, the founding member of the family, acts in the pheromone signaling pathway and activates the yeast MAPK module (Herskowitz, 1995), it is not known to what membrane receptors STE20-related kinases are linked in higher eukaryotes. Recently, a *Drosophila* STE20-related kinase Misshapen (Msn, the *Drosophila* homolog of mammalian NIK, an SPS1 subfamily STE20-like kinase) was demonstrated to act as an upstream activator of the JNK cascade required for dorsal closure (Noselli, 1998; Su *et al*., 1998). However, the upstream components in the dorsal closure pathway, as well as any link to membrane-associated receptors, are still elusive and thus it is not known how STE20-like kinases are activated in higher eukaryotic organisms.

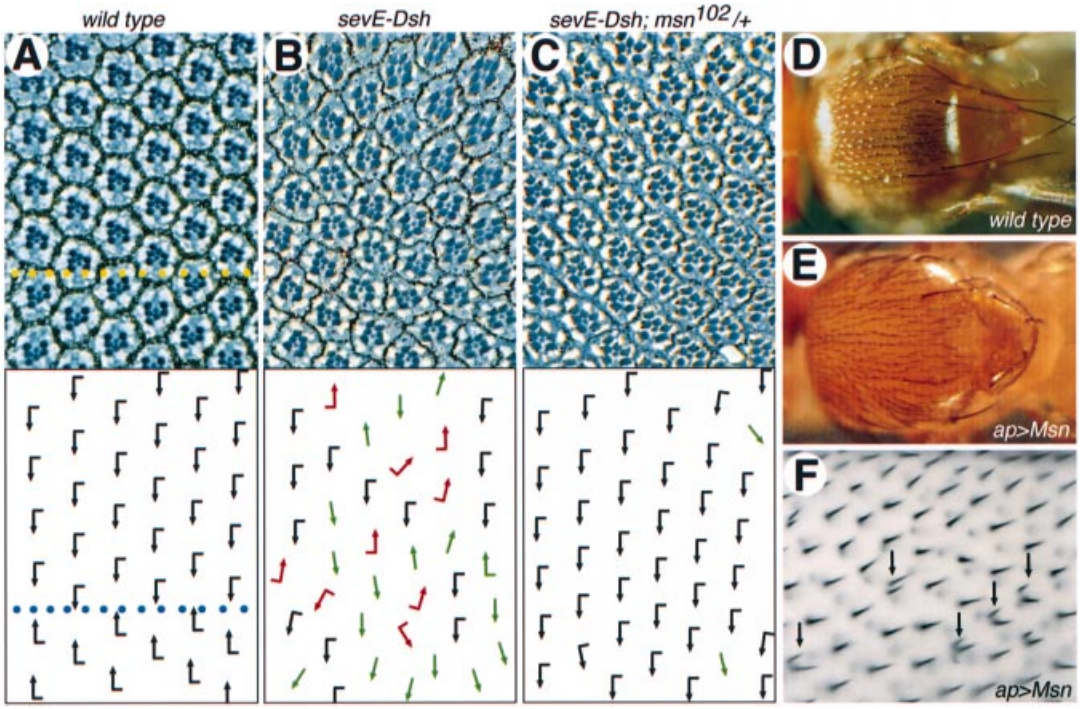

Fig. 1. Identification of Msn as a component of polarity signaling. (A), (B) and (C): tangential sections of adult eyes, and the corresponding schematic representations with arrows reflecting ommatidial polarity. Anterior is left, dorsal is up. Black arrows represent correct ommatidial orientation; red arrows, misrotated ommatidia; green arrows, opposite chirality for respective field; and green arrows without flags, symmetrical non-chiral ommatidia. (**A**) Wild-type eye. Note the regular arrangement of ommatidia relative to the equator (dotted line). (**B**) *sevE-Dsh* eye (dorsal field). Ommatidial arrangement is disrupted due to overactivation of planar polarity signaling. (**C**) *sev-Dsh*; msn^{102}/+ eye. The polarity defects [see (B)] are dominantly suppressed by mutations in *msn*. For quantitation of this suppression and other genotypes see Figure 2. (**D**) Wild-type notum. Microchaetae are regularly oriented and point distally. (**E**) *apGAL4*/+; *ep(3)*0549/+ notum. Misexpression of Msn in the notum results in disorganization of microchaetae. (**F**) *apGAL4*/+; *ep(3)0549*/+ wing. Misexpression of Msn in the wing causes duplication or triplication of hairs (arrows).

We analyze the role of the STE20-like Msn kinase in the epithelial planar polarity (EPP) pathway. Our phenotypic and genetic analyses indicate that Msn acts downstream of the Frizzled (Fz) receptor, being the first analysis that links an STE20-like kinase to a membrane receptor in higher eukaryotes.

The generation of planar polarity in *Drosophila* has emerged as a good model system to study signaling pathways mediated by the seven-pass transmembrane Fz receptor (Vinson *et al*., 1989; Strutt *et al*., 1997; Axelrod *et al*., 1998; Boutros *et al*., 1998). Planar polarity phenotypes are characterized by the misorientation of cells within epithelia, including the primordia of wings, legs and eyes (Adler, 1992; Gubb, 1993; Zheng *et al*., 1995; Strutt *et al*., 1997). Besides *fz*, several other genes have been implicated in planar polarity signaling based on their mutant phenotypes including *dishevelled* (*dsh*; Gubb, 1993; Adler *et al*., 1994; Theisen *et al*., 1994; Strutt *et al*., 1997; Wolff and Rubin, 1998). Biochemical and genetic studies have indicated that the planar polarity pathway downstream of Fz includes Dsh and small GTPases of the Rho subfamily (RhoA and Rac) and leads to the activation of a JNK-type MAPK module (Krasnow *et al*., 1995; Strutt *et al*., 1997; Boutros *et al*., 1998). However, it remains unclear how these components are linked, i.e. how Dsh is connected to the JNK cascade.

Here we provide evidence that the STE20-like Msn kinase acts in planar polarity signaling. We show genetically and in biochemical experiments that Msn is required downstream of the Fz receptor and its effector Dsh. This is not only the first analysis that implicates an STE20-like kinase in Fz-mediated signaling, but it is also the first report of higher eukaryotes linking STE20/Msn to a membrane-associated receptor. In addition, we show that in planar polarity, Msn acts through the JNK module as it does in dorsal closure, and also provide evidence for an involvement of the related MAP kinases of the p38 subfamily downstream of Msn.

Results

Identification of misshapen as a component of Dsh-mediated EPP signaling

In the *Drosophila* eye, EPP is reflected in the mirror-symmetric arrangement of ommatidial units relative to the dorso-ventral midline (the equator). This pattern is generated posterior to the morphogenetic furrow when ommatidial preclusters rotate 90° towards the equator, adopting opposite chirality depending on their dorsal or ventral positions (Gubb, 1993; Figure 1A). Polarity defects are manifested in the loss of mirror-image symmetry, with the ommatidia misrotating and adopting random chirality or remaining symmetrical (Gubb, 1993; Theisen *et al*., 1994; Zheng *et al*., 1995; Strutt *et al*., 1997; Boutros *et al*., 1998; Wolff and Rubin, 1998; see also Figure 1).

The gain-of-function *dsh* phenotype (*sev-Dsh*) has been successfully used in previous reports to identify new components of the Fz/Dsh planar polarity pathway (Strutt *et al*., 1997; Boutros *et al*., 1998). We have employed the same assay, dominant genetic modification of the *sev-Dsh*

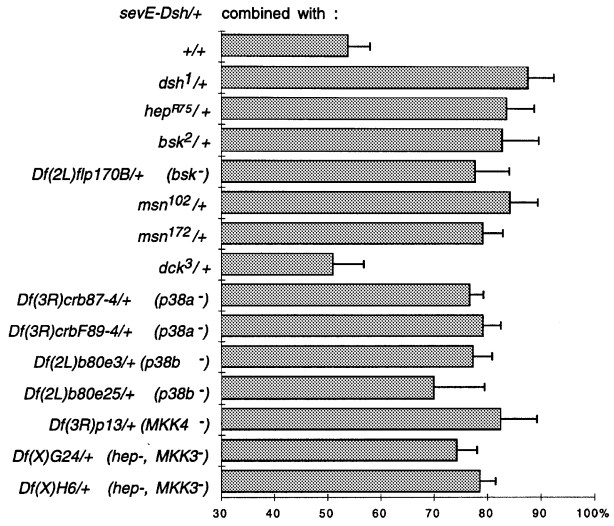

Fig. 2. Quantification of genetic interactions with the *sev-Dsh* phenotype. The percentage of correctly oriented ommatidia in the corresponding genetic backgrounds and allelic combinations with *sevE-Dsh* are shown. The data are based on scoring 3–12 independent eyes and between 400 and 1400 individual ommatidia per genotype. Standard deviations calculated across all eyes of a given phenotype are indicated by a line in each bar. In all cases flies analyzed were reared at 29°C.

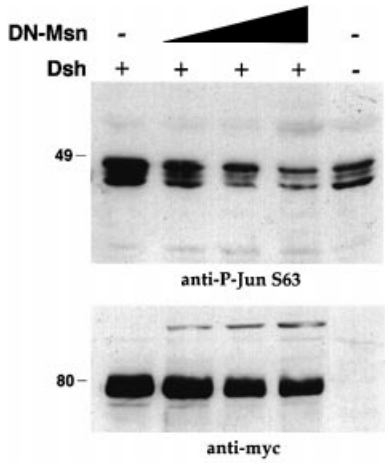

Fig. 3. Kinase-inactive Msn blocks Dsh-mediated Jun phosphorylation. Western blot analysis of cell extracts from NIH 3T3 cells that were transfected with wild-type Dsh (5 μg) and a kinase-inactive (DN) Msn derivative (concentration 5 μg each), with c-Jun as a reporter. Upper panel: phosphorylation of Jun was detected using phosphospecific antibodies to S63. Bottom panel: expression levels of the Dsh and DN-Msn proteins were monitored with anti-myc antibodies against a C-terminal myc tag. The phosphorylation pattern examined shows that Dsh caused activation of Jun-N-terminal kinases, and that increasing the concentration of DN-Msn blocks this activation and reduces Jun phosphorylation levels to endogenous background levels. Expression of either wild type or DN-Msn alone caused no significant effects.

phenotype (Figure 1B), to screen through a large number of known genes. Among the few mutants that showed a specific interaction, we found two alleles of the previously identified *misshapen* (*msn*) locus. msn^{102} and msn^{172} are X-ray-induced inversions with breakpoints in the *msn* gene (Treisman *et al*., 1997). This gene has recently been shown to encode the *Drosophila* homolog of NIK, a member of the mammalian SPS1 subfamily of the STE20 kinase family (Treisman *et al*., 1997; Su *et al*., 1998). Both loss-of-function alleles of *msn* act as dominant suppressors of *sev-Dsh*, comparable to other planar polarity-specific Dsh effectors (Figures 1C and 2; Boutros *et al*., 1998).

In addition, *msn* was isolated in a gain-of-function screen for genes involved in planar polarity generation (F.Feiguin and S.Eaton, unpublished data). Overexpression of genes required in planar polarity signaling at the relevant time often results in defects that are similar to the loss-of-function mutant phenotypes, e.g. with Fz and Dsh (Krasnow *et al*., 1995; Strutt *et al*., 1997; Boutros *et al*., 1998; see also Figure 1B). In such a screen, *ap-GAL4* flies (*ap-GAL4* induces overexpression of the corresponding gene in the notum and the dorsal part of the wing; Calleja *et al*., 1996) were crossed to the collection of 2200 E/P lines (Rørth *et al*., 1998) and the progeny were scored for disarranged microchaetae on the notum. One of the lines isolated in this screen, *ep(3)0549*, showed an abnormal orientation of the microchaetae (Figure 1D and E) similar to phenotypes obtained with *ap* driven Fz overexpression (F.Feiguin and S.Eaton, unpublished data). Similarly, *ap-GAL4*, *ep(3)0549* flies showed typical polarity phenotypes on the dorsal surface of the wing where these are manifest in the presence of multiple wing hairs (Figure 1F, see also below).

In situ hybridization experiments to polytene chromosomes (mapping to 62E6-7) and complementation analyses revealed that the EP-element insertion in line *ep(3)0549* is in the *msn* locus and represents a *msn* allele. Subsequent sequence analyses confirmed that the EP insertion is located 24 bp upstream of the 5′-end of a *msn* cDNA (data not shown). Taken together, these results suggest that *msn* is involved in EPP signaling and possibly acts downstream of Dsh.

A kinase-inactive form of Msn interferes with Dsh-mediated JNK activation

To gain further confirmation of the role of Msn in Fz/Dsh-mediated polarity signaling, we used an *in vitro* assay to determine whether Msn acts downstream of Dsh in JNK pathway activation. Previous experiments have shown that expression of Dsh in NIH 3T3 cells activates JNK and Jun phosphorylation, indicating that Dsh is a potent activator of a Jun-kinase pathway (Boutros *et al*., 1998). Using the same assay, we asked whether co-expression of a dominant-negative (kinase-inactive) Msn protein (DN-Msn) has an effect on Dsh-induced Jun phosphorylation. Significantly, co-expression of DN-Msn in this context caused a dramatic concentration-dependent inhibition of Jun phosphorylation (Figure 3).

Taken together with the genetic interactions, these experiments confirm that Msn is acting downstream of Fz/Dsh in planar polarity signaling.

misshapen mutants show planar polarity defects

Recently, it was shown that *msn* mutations affect both the morphology of the rhabdomeres in photoreceptors, causing

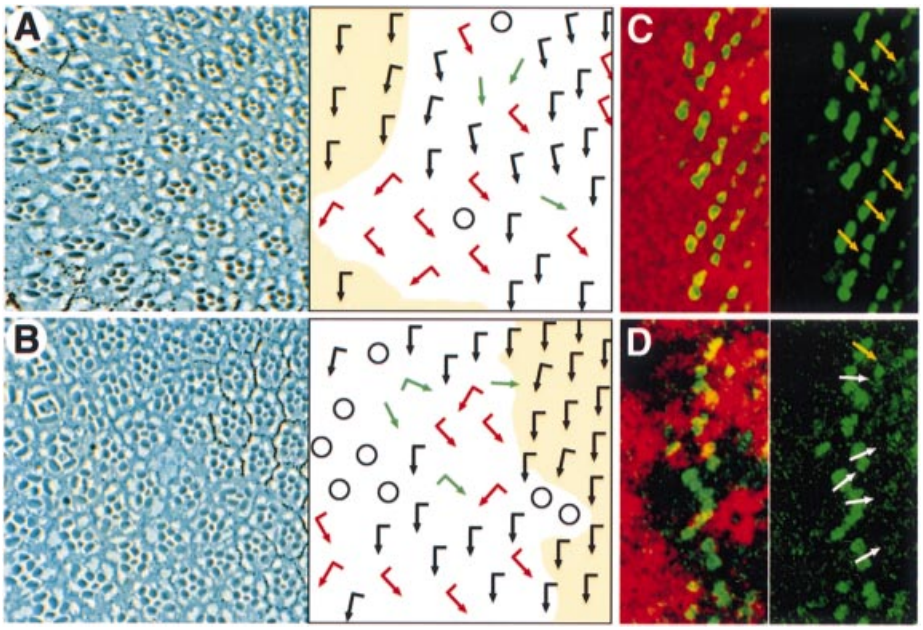

Fig. 4. *msn* mutants show planar polarity defects in the eye. (A) and (B) show tangential sections of homozygous mutant *msn* clones in adult eyes and the corresponding schematic representation. Clones are marked by the absence of pigment (unshaded regions in schematic). Arrows are drawn as in Figure 1: anterior is left, dorsal is up. Circles mark unscorable ommatidia due to missing or malformed photoreceptors. (**A**) msn^{102} allele. (**B**) msn^{172} allele. In both cases, ommatidia with polarity defects are present and also ommatidia with misshapen rhabdomeres, as previously reported (Treisman *et al*., 1997). (C) and (D) Confocal images of third instar eye imaginal discs with wild type (**C**) and an area with a homozygous msn^{102} clone (**D**). *msn* mutant cells are marked by the absence of cytoplasmic β-gal staining (red). Green nuclear staining (anti-Spalt) marks R3/R4 nuclei. Yellow arrows (in green channel) represent the normal 45° rotation of photoreceptors R3/R4 at this stage [also in (D)]. Note the misrotation of ommatidia in the mutant tissue (the orientation of some examples is highlighted with white arrows in the green channel).

malformed, 'misshapen' rhabdomeres (Treisman *et al*., 1997), and also, at lower frequency, the number of photoreceptors. In addition, *msn* is required for the process of dorsal closure, and embryos mutant for *msn* display a typical dorsal open phenotype (Su *et al*., 1998). To analyze its requirements in polarity generation, we looked in detail at *msn* mutant clones in the eye and the wing. Our phenotypic analysis of eye clones revealed that *msn* is required for the generation of planar polarity (Figure 4). *msn* mutant ommatidia containing the normal complement of photoreceptors are often misrotated and display the wrong chiral form or are symmetrical (non-chiral; Figure 4A and B). To confirm that the polarity defects of *msn* mutant ommatidia are primary defects, and thus implicate *msn* in polarity generation, we analyzed ommatidial polarity in *msn* mutant clones at the earliest possible stage in third instar larval imaginal discs (when tissue polarity genes are required). Spalt is expressed in the R3/R4 precursor pair for about two columns at this stage (Figure 4C and D), and in wild type this reflects the regular arrangement and direction of rotation of the preclusters (Figure 4C). In *msn* mutant tissue, ommatidial rotation, and thus polarity, is randomized (e.g. ommatidia rotate in the opposite direction to their wild-type neighbors) showing that these defects result from an early failure in polarity establishment (Figure 4D). Thus in the eye, the *msn* phenotype (defects in polarity, malformed, misshapen and missing photoreceptors) is very reminiscent of other genes involved in both polarity and terminal photoreceptor differentiation (e.g. *RhoA*; Strutt *et al*., 1997).

The *fz* gene has been implicated in the specification of the R3 cell within the R3/R4 pair in the process of chirality generation. The mosaic analysis of both loss-of-function (Zheng *et al*., 1995) and gain-of-function (Fanto and Mlodzik, 1999) *fz* alleles has shown that Fz signaling is required in R3 for correct ommatidial chirality generation and also induces R3 fate. The genetic interactions and cell culture experiments have shown that *msn* acts downstream of Fz/Dsh, and thus we asked whether *msn* is also involved in the selection of R3 in analogy to the *fz* requirement. We examined the genotypic composition of mosaic ommatidial clusters within the R3/R4 pair (Figure 5). This analysis revealed that, as is the case for *fz*, the msn^+ cell has a strong preference for adopting the R3 photoreceptor fate. This can often lead to chirality inversions, where the msn^+ R4 precursor adopts the R3 position and displaces the original msn^- R3 precursor (examples are shown in Figure 5A and B). In summary, the genetic requirements of *msn* in single photoreceptors, in particular the R3/R4 pair (Figure 5), are very similar to those of *fz*.

msn mutant clones in the wing affect the process of hair development and polarity. Phalloidin stainings of *msn* clones in pupal wings revealed that cells mutant for *msn* show defects in prehair initiation. These range from a complete failure of actin polymerization in the prehair to approximately wild-type levels of actin (Figure 6). Loss of Misshapen activity specifically affects wing hair actin organization, since adherens junction actin in *msn* clones appears normal. Although in some mutant cells an actin 'hair' is detected in the pupal wing, often at abnormal positions within the cell (Figure 6A), the adult hairs in msn^- tissue are either missing, branched or stunted (Figure 6C; arrows). Some cells that generate stunted hairs initiate them at multiple sites (a typical planar polarity phenotype; arrowhead in Figure 6C). These phenotypes are

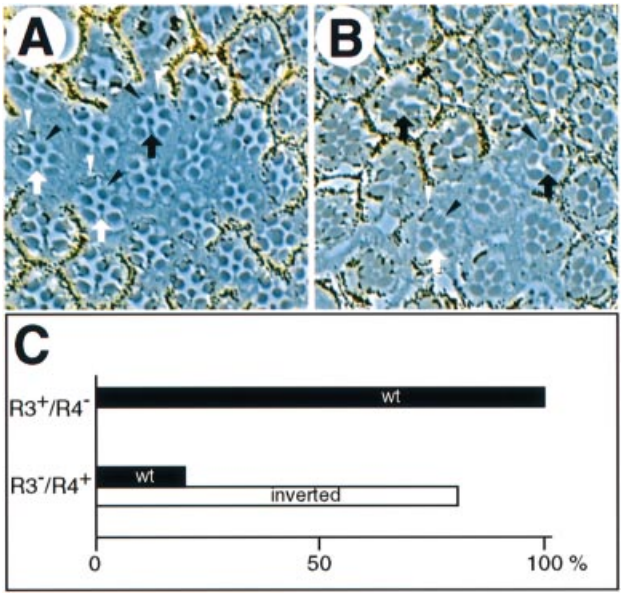

Fig. 5. The requirements of *msn* in the R3/R4 photoreceptor pair. Tangential sections of msn^{102} (**A**) and msn^{172} (**B**) clones. The high pigment level allows for an analysis with single cell resolution. Examples of ommatidia with mosaic R3/R4 pairs with either wild-type polarity/chirality or inverted chirality [including a symmetrical cluster in (B)] are highlighted with black and white arrows, respectively. The genotype of single photoreceptors can be determined due to the black pigment granules next to the rhabdomeres. Note that the genotype of the R3/R4 pair is critical for establishment of correct polarity. In addition to chirality inversions msn^- R3 cells can also lead to the formation of symmetrical clusters [an example is highlighted with a black arrow in (B)]. In such mosaic clusters the respective pigmented (wild-type) photoreceptors (white arrowheads) or mutant (unpigmented) photoreceptors (black arrowheads) are highlighted. Note that when the msn^+ precursor cell was originally positioned as R4, this cell can adopt the R3 fate and displace the msn^- cell leading to perfect chirality inversions. (**C**) Statitistical analysis of the R3/R4 requirements of *msn*. When the R3 precursor is msn^+ in 100% the ommatidium is of wild-type polarity; the opposite scenario, R4 being msn^+, leads to a high degree of chirality inversions or symmetrical clusters (81%). The number of R3/R4 mosaic clusters analyzed was 46 in 16 clones. The mosaic analysis also showed that for correct 90° rotation there was no bias for any particular photoreceptor (data not shown).

reminiscent of the defects observed either when prehair actin organization is disrupted by dominant-negative Cdc42 (Eaton *et al*., 1996) or after cytochalasin D treatment of cultured pupal wing discs (Turner and Adler, 1998).

To test whether overexpression of Msn in the eye can cause polarity defects comparable to *sev-Fz* or *sev-Dsh* (Strutt *et al*., 1997; Boutros *et al*., 1998; see also above), we used the *msn* E/P-line *ep(3)0549* and a *UAS-msn* strain (Su *et al*., 1998), and crossed these to *sev-GAL4*. The eyes of the resulting flies (*sev>msn*) are externally rough and reveal typical polarity defects in tangential sections (Figure 7A). Taken together with the genetic requirements and the loss-of-function phenotypes, the suppression of the *sev-Dsh* genotype and the cell culture experiments, these data demonstrate that Msn acts in the Fz/Dsh-mediated polarity signaling downstream of Dsh.

The gain-of-function msn phenotype is modified by JNK pathway components

It has been shown recently that during *Drosophila* embryogenesis *msn* acts upstream of the JNK-type MAPK module

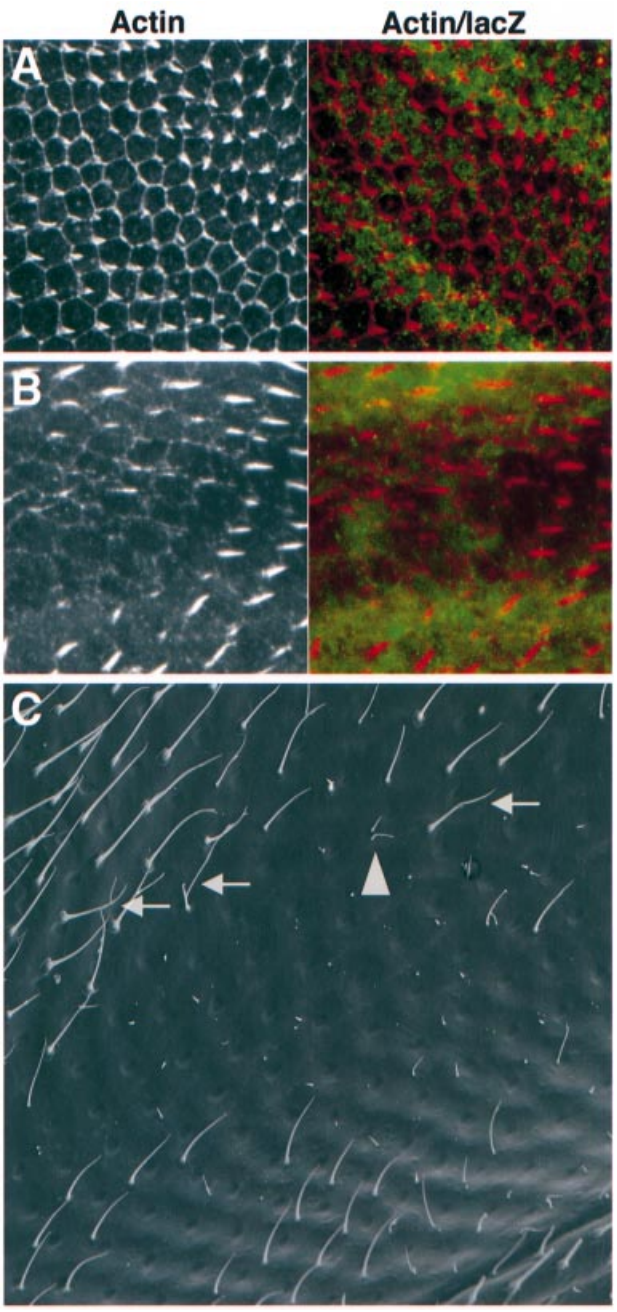

Fig. 6. *msn* mutant wing clones display missing or defective trichomes/hairs. [(**A**), (**B**)] *msn* clones in pupal wings stained with phalloidin to detect filamentous actin (left and right panels), and anti-β-gal (green channel in overlay in right panels). Cells not expressing β-gal are mutant for *msn*. The wing shown in (B) is slightly older (more developed) than the wing in (A). At the later stage, adherens junction actin becomes less abundant as the hairs extend. (**C**) SEM of adult wing containing a *msn* clone. Note that many cells have missing or stunted hairs that sometimes have the appearance of multiple wing hairs (vertical arrowhead). Other hairs (often at the edge of clones) appear forked or branched (arrows). Similar results were obtained in adult wing clones marked with *forked* (data not shown).

in dorsal closure signaling (Su *et al*., 1998). Several lines of evidence support a function of JNK cascade components in the generation of planar polarity (Strutt *et al*., 1997; Boutros *et al*., 1998).

To confirm the cell culture experiments and to determine whether components of JNK signaling act downstream of *msn* in polarity signaling *in vivo*, we used the gain-

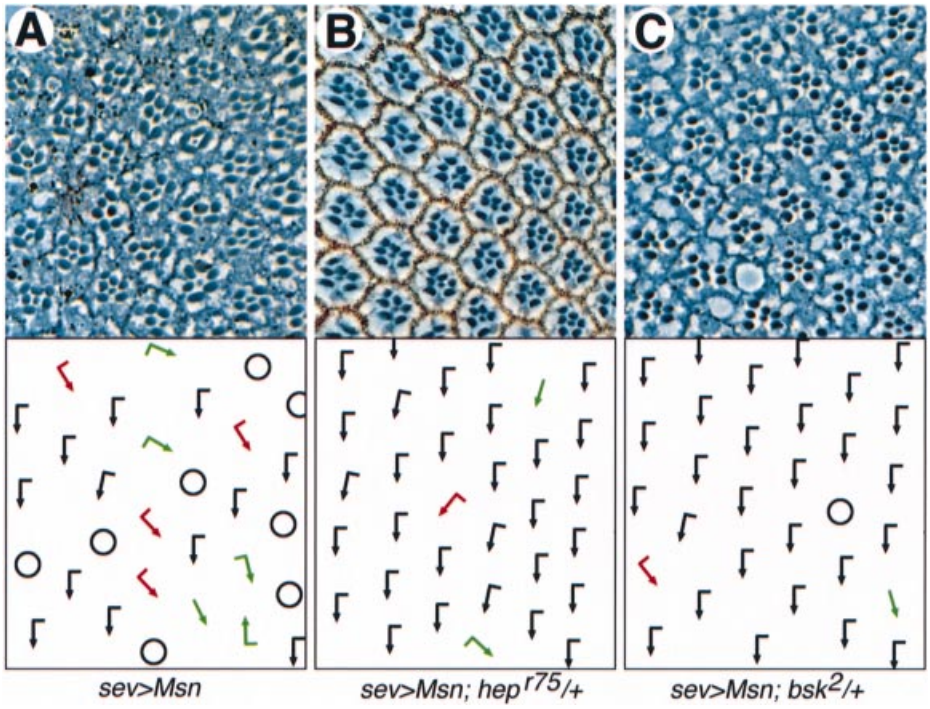

Fig. 7. Mutations in *hep/JNKK* and *bsk/JNK* dominantly suppress the *msn* gain-of-function phenotype. All panels show tangential sections of a dorsal field in adult eyes (anterior is left, dorsal is up) and corresponding schematic representations. Arrows are drawn as in Figure 1, circles mark unscorable ommatidia (due to missing or malformed photoreceptors). (**A**) *sevGAL4-ep(3)0549*. (**B**) *sevGAL4-ep(3)0549*/+; hep^{r75}/+. (**C**) *sevGAL4-ep(3)0549*/+; bsk^{2}/+. Overexpression of Msn in the eye causes polarity defects and also defects in photoreceptor differentiation (A). This phenotype is dominantly suppressed by mutations in the JNK components, *bsk* and *hep*. For quantitation of this suppression and other genotypes see Table I.

of-function eye polarity phenotype of *msn* (*sev>msn*; Figure 7A) to test for dominant interactions with mutations in JNK signaling components. In *sev>msn* eyes, only 62.6% of the ommatidia are correctly oriented (compared with 100% in wild-type eyes) with the remaining ommatidia showing polarity defects as well as defects in photoreceptor shape and differentiation. We find that reducing the dosage of known components of the JNK cascade causes a strong dominant suppression of *sev>msn* (Figure 7B and C and Table I). These results are consistent with *msn* acting upstream of the JNK module in polarity signaling and with the notion that Msn generally acts upstream of JNK-like cascades in higher eukaryotes.

Other components of planar polarity signaling that also participate in the process of dorsal closure upstream of the JNK module are the small GTPases RhoA and Rac (Strutt *et al*., 1997; Boutros *et al*., 1998). However, neither *RhoA* mutants nor deficiencies removing *Rac1* show a modification of *sev>msn* (Table I) suggesting that Msn acts downstream of these small GTPases. This is further supported by the observation that *sev-GAL4* driven co-expression of Rac^{N17} and Msn resembles the phenotype of Msn alone (Figure 7A and data not shown).

The *Drosophila dreadlocks* (*dock*) gene is highly related to the human proto-oncogene NCK (Garrity *et al*., 1996). Since Msn is a homolog of NIK which physically interacts with NCK, we tested whether *dock* alleles can modify *sev>msn*. None of the *dock* alleles showed an interaction in this assay (Table I). Moreover, the *dock* alleles do not interact with *sev-Dsh* (Figure 2) and do not display a polarity phenotype in homozygous mutant tissue (data not shown; Garrity *et al*., 1996). Thus, the potential Msn interactor Dock does not play a detectable role in polarity generation.

Table I. Quantification of genetic interactions with the gain-of-function *msn* phenotype

Genotype	Correctly rotated ommatidia (% ± SD)	Number of ommatidia scored
sE/ep(3)0549; +/+ (Control)	62.6 (± 5.1)	752
hep^{R75}/+	93.5 (± 6.7)	442
bsk^{2}/+	93.6 (± 2.4)	461
Df(2L)flp170B (bsk^{-})	92.4 (± 3.5)	445
jun^{2}/+	89.7 (± 6.3)	486
dck3/+	59.6 (± 2.6)	341
$Df(3R)crb^{87\text{-}4}$/+ ($p38a^{-}$)	85.7 (± 6.4)	706
$Df(3R)crb^{F89\text{-}4}$/+ ($p38a^{-}$)	84.8 (± 4.4)	564
Df(2L)b80e3/+ ($p38b^{-}$)	85.1 (± 4.2)	437
Df(2L)b87e25/+ ($p38b^{-}$)	86.1 (± 5.8)	475
Df(3R)p13/+ ($MKK4^{-}$)	88.4 (± 6.1)	542
Df(X)G24/+ ($MKK3^{-}$, hep^{-})	96.2 (± 2.5)	550
Df(X)H6/+ ($MKK3^{-}$, hep^{-})	87.4 (± 2.2)	446
$RhoA^{720}$/+	62.2 (± 9.0)	450
Df(3L)emc5/+ ($rac1^{-}$)	56.0 (± 8.4)	686

Quantification of genetic interaction of the overexpression phenotype of Msn with putative downstream components of EPP signaling. The quantifications of allelic combinations are based on scoring of four to seven independent eyes per genotype. The percentage shown in this table is the average number of correctly oriented ommatidia, with the standard deviation calculated across all eyes of a given genotype scored. In wild-type eyes, all ommatidia are correctly oriented.

The role of JNK-related kinase cascades in polarity signaling

Although there is accumulating evidence that JNK-type MAPK modules are involved in planar polarity signaling (Strutt *et al*., 1997; Boutros *et al*., 1998), the analysis of mutant clones of either *hep* or *bsk* alleles showed no or

weak phenotypes in imaginal tissues (data not shown). These observations suggest a high degree of redundancy at this level in the polarity signaling pathway.

To address this issue further, we looked for a potential involvement of related kinases that could account for the proposed redundancy. We tested the recently described *Drosophila* kinases, belonging to the JNK/p38 class within the MAPK modules (Han *et al*., 1998), for genetic interactions with the planar polarity phenotypes of *sev-Dsh* and *sev-msn*. These are obvious candidates to be cooperating with Hep and Bsk in polarity generation. At the level of Hep/JNKK, an MKK7 homolog, two other MKKs have been reported (DMKK3 and DMKK4). Similarly, at the level of Bsk/JNK, two p38-like kinases were isolated (Dp38a and Dp38b; Han *et al*., 1998). Since no mutants have yet been isolated for these genes, we asked whether deficiencies removing these kinases show an interaction with *sev-Dsh*. DMKK3 maps in the vicinity of *hep* and deficiencies removing DMKK3, *Df(X)G24* and *Df(X)H6,* also remove *hep* (S.Noselli; personal communication). These deficiencies show externally a very strong suppression of *sev-Dsh* (data not shown) with a marked decrease of misrotated ommatidia as observed in tangential sections (Figure 2). Deficiency *Df(3R)p13* removes the DMKK4 locus and also dominantly suppresses *sev-Dsh* (Figure 2). Similarly, deficiencies removing either Dp38a, $Df(3L)crb^{87\text{-}4}$ and $Df(3L)crb^{F89\text{-}4}$, or Dp38b, *Df(2L)b80e3* and *Df(2L)b87e25,* are suppressors of *sev-Dsh* (Figure 2). We also tested whether the respective deficiencies showed an interaction with *sev>msn*, and found that all of them act as dominant suppressors of this genotype as well (Table I). It is interesting to mention that the Msn-induced defects in rhabdomere morphology were also suppressed by those deficiencies. These interactions suggest that the p38 kinases are redundant with JNK in the context of planar polarity signaling.

Discussion

We have shown that the *Drosophila* STE20-related kinase, Msn, is a component of planar polarity signaling, linking Fz membrane receptors to JNK cascades. This is the first link of an STE20-like kinase to a membrane-associated receptor in metazoans. It was recently shown that Msn acts upstream of a JNK cascade in dorsal closure (Su *et al*., 1998). Based on genetic interactions with the Dsh and Msn gain-of-function polarity phenotypes, we show that not only JNK but also other related kinase cascades (p38) can act downstream of Fz/Dsh, cooperating with the Hep–Bsk module in planar polarity generation.

Role of Msn in the generation of polarity

Our phenotypic analysis of *msn* clones in the eye and wing shows typical planar polarity phenotypes. In the eye, *msn* loss-of-function mutants cause misrotated ommatidia and chirality defects. Similar to *fz* and *dsh*, these polarity phenotypes are already evident in third instar larval discs and display the same genetic requirements in the R3/R4 pair as *fz*. Other defects observed in *msn* mutant clones are malformed rhabdomeres and, at low frequency, defects in photoreceptor differentiation (Treisman *et al*., 1997; this study). As observed for other members of the polarity pathway (Boutros *et al*., 1998), overexpression of Msn (*sev>msn*) gives a similar phenotype to loss-of-function alleles. Although mutations in *fz* and *dsh* result in clean planar polarity defects (Theisen *et al*., 1994; Zheng *et al*., 1995; Strutt *et al*., 1997), the additional phenotypes observed in *msn* mutants are comparable to other members of polarity signaling, e.g. *RhoA* alleles also cause loss of photoreceptors or photoreceptors with abnormal morphology and malformed rhabdomeres (Strutt *et al*., 1997; and data not shown) very similar to *msn*.

The effects of Misshapen overexpression and loss-of-function in the wing suggest that it may represent a branchpoint in the eye and wing polarization pathways. Misshapen overexpression in the wing produces a multiple wing hair phenotype similar to that of the wing-specific tissue polarity genes *inturned* and *fuzzy.* In contrast, *msn* loss-of-function clones have defects similar to those of dominant-negative CDC42 expressing cells; hair actin polymerization is defective and adult hairs are missing or stunted.

One explanation of these data is that Misshapen acts through the JNK pathway, as it does in the eye, but that the targets of transcriptional activation are the components needed for hair formation. Excess production of these components may lead to multiple hairs and their loss to missing hairs. We do not favor this model because Dishevelled, which acts upstream of Misshapen in this pathway, affects only hair polarity and is not required for hair formation. Furthermore, expression in the wing of a kinase-inactive version of JNK, which acts as a dominant negative, has no effect on hair formation or polarity (F.Feiguin, N.Paricio and S.Eaton, unpublished data). Our data are more consistent with a model in which localized Misshapen activity directly promotes polarized cytoskeletal reorganization leading to hair formation; excess Misshapen might then expand the region of the cell competent for hair outgrowth. According to this model, activation of the Fz/Dsh signal transduction pathway in the wing would not necessarily increase the absolute level of Misshapen activity, but rather serve to localize Misshapen activity within the cell. It will be interesting to determine whether molecules such as CDC42, Inturned or Fuzzy represent wing-specific targets of Misshapen.

Taken together with the phenotypes in the eye and its role in dorsal closure (Su *et al*., 1998), these observations indicate that Msn has a function in both nuclear signaling and cytoskeletal rearrangements.

Msn links Dsh and JNKs in polarity signaling

Several studies in yeast and mammalian cells indicate that STE20 kinases function upstream of MKKKs regulating JNK (Herskowitz, 1995; Su *et al*., 1997). Msn is the *Drosophila* homolog of mammalian NIK, belonging to the SPS1 family of STE20-like kinases (Su *et al*., 1997, 1998; Treisman *et al*., 1997). Based on differences in structure and possible regulation, two subfamilies of STE20 kinases have been described: mammalian and *Drosophila* PAKs (p21 Activated Kinases), which are activated by binding GTP-bound Cdc42 and Rac (Manser *et al*., 1995; Martin *et al*., 1995; Harden *et al*., 1996) and contain N-terminal regulatory and a C-terminal kinase domain. Members of the second subfamily, containing the SPS1 kinase in yeast (Friesen *et al*., 1994), do not interact physically with Cdc42 or Rac and contain an N-terminal

kinase and a C-terminal regulatory domain. Several members of the SPS1 subfamily have been described in mammals, but only a subset of them, such as GCK (Pombo *et al*., 1995), HPK1 (Hu *et al*., 1996), GLK (Diener *et al*., 1997) and NIK (Su *et al*., 1997) have been shown to activate JNK. Genetic and biochemical studies have recently demonstrated that Msn can activate the JNK module and is required during dorsal closure (Su *et al*., 1998). Our observations indicate that Msn also acts upstream of the JNK module in polarity signaling in the eye and support this as a general mechanism.

Studies in *Saccharomyces cerevisiae* have shown that the STE20 kinase acts downstream of the pheromone receptor (Herskowitz, 1995). Our genetic and biochemical studies indicate that Msn acts downstream of Fz/Dsh in the transmission of the polarity signal. This is the first link of this family of kinases to membrane-associated receptors and upstream regulators in higher eukaryotes.

RhoA loss-of-function mutants and a deficiency removing the *rac1* gene do not interact with the *msn* gain-of-function genotype (*sev>Msn*), suggesting that these genes may act upstream of Msn. Msn does not contain Rac binding sites and does not physically interact with Rac or RhoA (Su *et al*., 1998). Dock, the *Drosophila* homolog of Nck (Garrity *et al*., 1996; Su *et al*., 1997), is an adapter protein that possibly interacts with Msn through its SH3 domains as has been described for the mammalian homologs NIK and Nck (Su *et al*., 1997). However, analyses of *dock* mutants revealed that it is required for photoreceptor axon guidance, but no polarity phenotypes are evident in eye clones (Garrity *et al*., 1996). Moreover, we have found no interactions between either *sev-Dsh* or *sev>Msn* and *dock* mutants, suggesting that *dock* is not required in polarity signaling or is redundant in this process. Thus, it remains unclear how Msn is linked to the Rho/Rac GTPases. Our genetic data argues for Msn acting downstream of RhoA/Rac. However, it has also been suggested that Rac and Msn act in parallel pathways (Su *et al*., 1998).

Redundancy among the JNK/p38 kinases in polarity signaling

Although genetic evidence suggests an involvement of *bsk* (JNK) and *hep* (JNKK) in polarity signaling (Strutt *et al*., 1997; Boutros *et al*., 1998), phenotypic analyses suggest that the JNK module components are highly redundant in this process. In the search for other kinases involved, we found that deficiencies uncovering the genes encoding the recently described p38 MAP kinases Dp38a and Dp38b, and the MAP kinase kinases DMKK3 and DMKK4 (Han *et al*., 1998) dominantly suppress the *sev>msn* and *sev-Dsh* phenotypes, suggesting that these proteins also function downstream of Dsh and Msn in polarity signaling. It is interesting to note that all phenotypic defects of *sev>Msn* were dominantly suppressed by mutations in both components of the JNK and the p38 kinase module.

In contrast to these interactions, tissue culture experiments in mammalian cells have shown that NIK overexpression leads to JNK phosphorylation, but no detectable p38 activation was observed (Su *et al*., 1997). This difference can be explained by cell- and tissue-specific requirements, e.g. in *Drosophila* during dorsal closure, JNK activation downstream of Msn is not redundant (Su *et al*., 1998), while redundancy and p38 interactions are observed in polarity signaling. Thus, it is tempting to speculate that both JNK and p38 kinases cooperate in polarity generation.

The reported promiscuity of the kinases at both the MKK and the MAPK levels (Madhani *et al*., 1997) could account for the redundancy. The *Drosophila* MKKs and JNK/p38 MAPKs also appear to act (at least partially) on overlapping downstream targets. Whereas DMKK3 appears rather specific for p38 activation (although it activates both p38s), DMKK4 and Hep (the MKK7 cognate) both activate Bsk/JNK (Han *et al*., 1998). Similarly, Bsk/JNK and both Dp38s can phosphorylate the downstream targets dJun and ATF2 (Han *et al*., 1998). Thus, a potential downstream target can still be phosphorylated when one of the upstream kinases is removed, and likewise for their upstream activators. An even more complicated picture may emerge when all relevant kinases are identified. Other examples of redundancy are described in yeast MAP kinases. Although KSS1 and FUS3 normally have specific roles in different pathways, it has been shown that they are redundant in the process of mating (Madhani *et al*., 1997) and in this case KSS1 replaces Fus3 when the latter is not present. The isolation and analysis of all the respective kinases and their mutants will be necessary to understand fully the contribution of each single kinase in planar polarity signaling.

In conclusion, the implication of Msn/STE20 in Fz/Dsh-mediated polarity signaling is an essential first step to unravel the detailed requirements of the relevant downstream kinase cascades.

Materials and methods

Fly strains and genetic interactions

Genetic interactions were perfomed using a *sev-Dsh* strain (Boutros *et al*., 1998); msn^{102} and msn^{172} (Treisman *et al*., 1997) were kindly provided by J.Treisman and are inversions with breakpoints in *msn*. The *ep(3)0549* line is from the pool of E/P lines previously described (Rørth *et al*., 1998). This line was also used to generate the *sev>Msn* gain-of-function allele (generated by recombination of *sev-GAL4* onto the *ep(3)0549* chromosome). *UAS-Msn* transgenic flies were a gift from J.Treisman. Deficiencies uncovering *p38a*, *p38b* and *MKK4* genes were obtained from the Bloomington stock center. Deficiencies *Df(X)G24* and *Df(X)H6*, which uncover *hep* and *MKK3*, were kindly provided by S.Noselli.

Genetic interactions with *sev-Dsh* (Boutros *et al*., 1998) and *sev>msn* were performed at 29°C. The flies analyzed were heterozygous for either *sev-Dsh* or *sev>msn* and the mutation of interest. w^{1118} was used as a negative control.

Mitotic clones

Clones of *msn* alleles were induced either by the FLP/FRT method (Xu and Rubin, 1993) or with X-rays. Larvae were heat shocked at 38°C for 120 min at 24–48 and 48–72 h after egg laying. Clones were marked by the absence of *arm-LacZ* staining in imaginal discs and by the loss of the pigment marker in adult eyes. To generate *msn* clones in pupal wings (marked by the absence of *arm-LacZ* staining), clones were induced at 48–72 h after egg laying. Such clones were also analyzed in adult wings where they were unmarked. X-ray-induced clones of both *msn* alleles in adult wings, marked with $forked^{-}$, were also generated giving rise to the same phenotype.

Histology and cell culture assays

Sections of adult eyes were performed as previously described (Tomlinson and Ready, 1987). Anti-Spalt and anti-β-gal stainings of eye discs were done in 0.1 M phosphate buffer, 0.2% saponin, 0.3% deoxycholate, 0.2% Triton X-100 and 10% normal goat serum. Spalt was detected with a rat antibody (a gift from R.Barrio) and β-gal with a rabbit

polyclonal antibody (Cappel). Secondary antibodies were purchased from the Jackson Laboratories. Pupal wing stainings and wing SEM analysis was performed as described (Eaton *et al*., 1996).

The NIH 3T3 cell culture assays with DN-Msn and Dsh were performed essentially as described (Boutros *et al*., 1998). The expression plasmids for the Msn protein were kindly provided by Ed Skolnik (Su *et al*., 1998).

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