Jun acts as a signal-regulated transcription factor in many cellular decisions, ranging from stress response to proliferation control and cell fate induction. Genetic interaction studies have suggested that Jun and JNK signaling are involved in Frizzled (Fz)-mediated planar polarity generation in the Drosophila eye. However, simple loss-of-function analysis of JNK signaling components did not show comparable planar polarity defects. To address the role of Jun and JNK in Fz signaling, we have used a combination of loss- and gain-of-function studies. Like Fz, Jun affects the bias between the R3/R4 photoreceptor pair that is critical for ommatidial polarity establishment. Detailed analysis of jun- clones reveals defects in R3 induction and planar polarity determination, whereas gain of Jun function induces the R3 fate and associated polarity phenotypes. We find also that affecting the levels of JNK signaling by either reduction or overexpression leads to planar polarity defects. Similarly, hypomorphic allelic combinations and overexpression of the negative JNK regulator Puckered causes planar polarity eye phenotypes, establishing that JNK acts in planar polarity signaling. The observation that Dl transcription in the early R3/R4 precursor cells is deregulated by Jun or Hep/JNKK activation, reminiscent of the effects seen with Fz overexpression, suggests that Jun is one of the transcription factors that mediates the effects of fz in planar polarity generation.

Key words: Jun, Frizzled, Puckered, JNK signaling, Planar polarity, Drosophila
Heberlein and Moses, 1995; Treisman and Heberlein, 1998). The DV alignment is generated in response to a polarizing signal that organizes the ommatidia around the dorsoventral midline, the equator (reviewed in Blair, 1999; Strutt and Strutt, 1999), and acts through frizzled. Ommatidia in the dorsal and ventral halves of the eye disc rotate 90° in opposite directions, thus creating a line of mirror-image symmetry running along the DV midline, the equator. Concomitant with their rotation, the ommatidia lose their symmetry and opposite chiral forms are established in each eye half as R3 takes a more polar position than R4 at the tip of the ommatidial trapezoids (reviewed in Tomlinson, 1988; Ready, 1989; Wolff and Ready, 1993; Blair, 1999; Mlodzik, 1999; Reifegerste and Moses, 1999; see also Fig. 1A,B).

Genetic analysis of fz mutants has shown that the R3/R4 photoreceptor pair is critical for the establishment of polarity (Zheng et al., 1995). Prior to rotation of the ommatidia the R3/R4 precursors are symmetrically arranged in the preclusters, with R3 being closer to the equator than R4. It is thought that the polarizing signal, emanating from the equator, is received first, or at higher levels, by Fz receptors in the R3/R4 precursor that is closer to the equator. Fz signaling specifies the R3 fate (Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999) and leads to stronger expression of Delta (Dl) in this cell. Subsequent Notch activation in the other cell specifies it as R4 (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). The correct cell fate specification of the R3/R4 cells with respect to one another determines the polarity of the ommatidium and thus the direction of rotation and chirality. Accordingly, indiscriminate activation or loss of Fz signaling in both R3 and R4 results in random stochastic Dl expression. The chirality decision thus becomes stochastic and the direction and degree of rotation become random.

In addition, some clusters can remain symmetrical, giving rise to either V- or U-shaped ommatidia with non-chiral R3/R3 or R4/R4 photoreceptor pairs (Gubb, 1993; Theisen et al., 1994; Zheng et al., 1995; Strutt et al., 1997; Zhang and Carthew, 1998; Fanto and Mlodzik, 1999; Paricio et al., 1999; Tomlinson and Struhl, 1999; see also Fig. 1B). Typically the R3/R3 type ommatidia have the rhabdomeres of R4-R6 also arranged in a line (like R1-R3 in wild type); in the R4/R4 type clusters, the rhabdomeres have a horseshoe-like arrangement. Although these results imply that a transcriptional event takes place downstream of Fz in R3, the respective transcription factors have not been identified.

Recently, a combination of genetic and biochemical studies has demonstrated that the planar polarity pathway downstream of fz and dsh consists of the small GTPase RhoA and the STE20-like kinase Misshapen (Msn) (Strutt et al., 1997; Paricio et al., 1999). Genetic interactions suggest that JNK-type MAPK modules might act downstream of Dsh and Msn (Boutros et al., 1998; Paricio et al., 1999). Consistently, Drosophila Dsh (and its human homologues) act as potent activators of JNK signaling (Boutros et al., 1998; Li et al., 1999). However, although mutations in the JNK cascade genetically suppress gain-of-function genotypes of fz, dsh and msn, they do not show polarity phenotypes in simple loss-of-function analyses, suggesting that there is redundancy at this level in the cascade (Boutros et al., 1998; Paricio et al., 1999). Several other polarity genes are known (e.g. strabismus (stbm) (Wolff and Rubin, 1998); prickly-spiny legs (pk-sple) (Gubb et al., 1999) and flamingo (fmi) (Usui et al., 1999), but their link to the Fz pathway is unclear and none of them encodes a transcription factor.

Jun is a phosphorylation-regulated transcription factor and a member of the basic region-leucine zipper family that can act as a homodimer or heterodimer with another leucine zipper family member (Wisdom, 1999). It is a phosphorylation target of several MAPkinase family members, including the ERK, JNK (Jun N-terminal kinase) and p38 kinases (Davis, 1999; Whitmarsh et al., 1997). Phosphorylation of Jun on its serine and threonine MAPK target sites renders the protein more stable and transcriptionally active (Wisdom, 1999). In Drosophila, Jun (dJun) is involved in dorsal closure downstream of JNK signaling (Glise and Noselli, 1997; Kocckel et al., 1997; Hou et al., 1997; Riesgo-Escovar and Hafen, 1997). It has also been implicated as an effector of Ras/JNK signaling during eye development (Treier et al., 1995; Kocckel et al., 1997), although it is largely redundant in this process (Kocckel et al., 1997). The potential involvement of JNK signaling downstream of Fz and Dsh in planar polarity generation makes Jun a candidate transcription factor for this process.

Here, we show that Jun acts downstream of Fz/Dsh signaling in R3 specification and planar polarity determination. Activated Jun displays very similar behavior to gain-of-function Fz, both at the phenotypic level and by its genetic requirement in R3. Like Fz, Jun affects the bias between cells of the R3/R4 pair that is critical for ommatidial polarity establishment. Analysis of Jun clones reveals deficits in R3 induction and associated polarity phenotypes. Affecting the levels of JNK activity with dominant negative and activated isoforms, or overexpression of the negative regulator Puckered (a dual specificity phosphatase (Martin-Blanco et al., 1998) also leads to typical planar polarity phenotypes. Moreover, we demonstrate that Dl transcription in the early R3/R4 precursor cells is deregulated by Jun (or Hep/JNK) activation, reminiscent of the effects seen with sev-Fz (Fanto and Mlodzik, 1999).

**MATERIALS AND METHODS**

**Flystrains and genetics**

Mutants used were JunR46 and DfE73 (Kocckel et al., 1997), junIA109 (Nüsslein-Volhard et al., 1984), Jun76-79 (Hou et al., 1997), pucK21(F2) (Bloomington), pucE69 (Ring and Martinez Arias, 1993), hep+ (Glise et al., 1995), hep1; pucE69 (kind gift of Julia Zeitlinger), and pnt19099 and pnt15166, which were isolated as domain suppressors of sev-Jun+ (U. Weber and David Jackson, unpublished results) in a collection of P-element-induced lethals (Guichet et al., 1997). Both alleles behave like very strong pnt alleles (O’Neill et al., 1994) in eye clones (U. Weber and David Jackson, unpublished results). Other fly strains and chromosomes are as described in Flybase.

**Sevenless enhancer driven constructs and Gal4 stocks used were:**

The genetics of the DfE73 (Kocckel et al., 1997), ser-Dsh rec2 (a recombinant of two copies of Pkb-Dsh, Boutros et al., 1998), sev-Jun+ (Treier et al., 1995), sevenless-Gal4 K24 and K25 for the second and third chromosome, respectively (gift from Konrad Basler). UAS constructs used were: UAS-puc (Martin-Blanco et al., 1998), UAS-Hep and UAS-BSk (Boutros et al., 1998).

Activated and dominant negative forms were generated by PCR mutagenesis. For Hep+ transgenic flies, the Ser and Thr residues at
positions 326 and 330 were replaced with Asp. In the Bsk\textsuperscript{DN} protein, Lys at position 53 was replaced by an Arg residue. The modified cDNAs were cloned into the pUAST vector (Brand and Perrimon, 1993) and transgenic flies were generated by standard P-element-mediated transformation (Spradling and Rubin, 1982).

Other fly strains used were: \textit{Ki}, \textit{p p}, \textit{delta2-3} to generate mosaic \textit{sev-Jun\textsuperscript{Asp}} eyes, \textit{w f 36a}; \textit{FRT42 w + f }\textit{+ M47/CyO} (gift from Fernando Diaz-Benjumea), \textit{eyFLpBD927T33,ry} (gift from Barry Dickson) and \textit{Dl-lacZ1282} (gift from Marc Haenlin).

For \textit{sev-Fz} interaction studies, the crosses were grown at 25°C and \textit{w 1118} was used as control. For \textit{sev>BskDN} interactions crosses a \textit{sevGal4}, \textit{UAS-BskDN} recombinant chromosome was used and grown at 29°C, \textit{OreR} was used as wild-type control. For imaginal disc stainings, the respective mutant chromosomes were established over the \textit{TM6B} or \textit{SM5a:TM6B} balancers.

**Mosaic analysis**

To generate predominantly jun\textsuperscript{-} eyes, \textit{w f 36a}; \textit{FRT42 w + f }\textit{+ M47/CyO} virgins were crossed to y \textit{w}; \textit{FRT42 jun\textsuperscript{-}}/\textit{SM5a:TM6B} males. Clones for analyzing the function of \textit{Jun\textsuperscript{Asp}} in single cells of an ommatidium were generated by crossing \textit{Ki}, \textit{p p}, \textit{A2-3} or \textit{w 1118} males to \textit{y w}; \textit{sev-Jun\textsuperscript{Asp}}/\textit{SM5a:TM6B} virgins. \textit{F1} progeny were X-irradiated as first larval instar larvae or grown normally (\textit{Ki}, \textit{p p}, \textit{A2-3}) and mosaic eyes were recovered in adults.

**Immunohistochemistry and histology**

Primary antibodies used were rat anti-Elav (a gift from Gerry Rubin), rat anti-Spalt (gift from Rosa Barrio) and rabbit anti-\textit{\beta}-gal (polyclonal from cappel). Secondary antibodies coupled to fluorochromes were from Jackson Laboratories. Imaginal disc stainings were performed as described previously (Paricio et al., 1999). Confocal images shown are projections of several single optical sections. Tangential eye sections were prepared as described (Tomlinson and Ready, 1987).

**RESULTS**

**Jun suppresses the gain-of-function \textit{Fz} eye phenotype**

Several lines of evidence have previously suggested an involvement of the JNK pathway in planar polarity generation. Mutations in the genes \textit{hep} (JNKK) and \textit{bsk} (JNK) cause a strong dominant suppression of the polarity-specific gain-of-function \textit{Fz}, \textit{Dsh} and \textit{Msn} eye phenotypes (Strutt et al., 1997; Boutros et al., 1998; Paricio et al., 1999). It was also shown that expression of Dsh and Msn in cell culture activates the JNK pathway, and ultimately induces phosphorylation of the

Fig. 1. \textit{dJun} loss-of-function mutants dominantly suppress the \textit{sev-Fz} eye polarity phenotype. Tangential sections of adult eyes are shown with a corresponding schematic diagram and arrows reflecting ommatidial polarity. Anterior is to the left and dorsal is up. In C and D only the dorsal part of the eye is shown. How arrows relate to actual photoreceptor arrangement is show in B. (A) Section of a wild-type eye (equator is indicated by a yellow or blue line). The correct ommatidial orientation is represented by black arrows. (B) Wild-type ommatidium with black arrow, the outer photoreceptors R1-6 are indicated. Green or blue straight arrows represent symmetric ommatidia with two photoreceptors of the R3 or the R4 type, respectively. In the R3/R3 type ommatidia, the rhabdomeres of R4-R6 are arranged in a line (like R1-R3 in WT); in R4/R4 type clusters, the rhabdomeres are arranged like a horseshoe. (C) \textit{sev>Fz/+} eye. Ommatidia are often misrotated (red arrows) and show chirality defects (green/blue arrows; green arrow with flag represents inverted chirality). Most of the symmetrical clusters in \textit{sev-Fz} eyes are R3/R3 symmetrical as also confirmed with molecular markers (H123 is expressed at high levels only in R4) in the eye disc (Fanto and Mlodzik, 1999). (D) \textit{jun\textsuperscript{IA109/+}; sev>Fz/+}. Note the suppression of the \textit{sev-Fz} phenotype. Whereas the control \textit{sev>Fz/+} had 42.6%\textpm{}9.1 (s.d.) of correctly polarized ommatidia, different \textit{jun} alleles showed a significant suppression: \textit{jun\textsuperscript{IA109/+}; sev>Fz/+} had 63.7%\textpm{}1.8%; \textit{jun\textsuperscript{76-19/+}; sev>Fz/+}, 72.0%\textpm{}7.1 and \textit{Df(2R)junE73; sev>Fz/+}, 74.3%\textpm{}4.5 of correctly polarized ommatidia, respectively (between 346 and 388 ommatidia were analyzed for each genotype).
target protein Jun (Boutros et al., 1998; Su et al., 1998; Paricio et al., 1999).

We have first tested whether mutations in D-jun, a target of JNK signaling in the Drosophila embryo (Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997), are also able to dominantly suppress the gain-of-function sev-Fz eye phenotype. Strikingly, all jun alleles tested, jun76–19, junA169 and a deficiency for the locus (Kockel et al., 1997), dominantly suppressed the sev-Fz phenotype (Fig. 1C,D) to the same extent as previously identified components of planar polarity signaling (Strutt et al., 1997). Jun alleles interacted in the same way with sev-Dsh and sev-Msn (Boutros et al., 1998; Paricio et al., 1999). These data support the idea that Jun acts downstream of Fz in planar polarity generation.

Expression of Jun<sup>Asp</sup> mimics Fz pathway activation

To test whether expression of activated Jun in the eye can cause polarity phenotypes comparable to sev-Fz or sev-Dsh, due to ectopic activation of the pathway, we analyzed in detail the eye phenotype of flies expressing an activated Jun<sup>Asp</sup> isoform. Jun<sup>Asp</sup> mimics MAPK-mediated phosphorylation of Jun and behaves like the active form of the protein (Papavassiliou et al., 1995; Treier et al., 1995). As in the case of Fz and Dsh, Jun<sup>Asp</sup> was expressed under the control of the sevenless (sev) enhancer (sev-Jun<sup>Asp</sup>) transiently in the R3 and R4 precursors.

As previously reported, such flies have rough eyes with ommatidia that often contain additional R7 cells (Fig. 2A; see also Treier et al., 1995; Kockel et al., 1997), which is due to Jun<sup>Asp</sup> expression in the cone cells mimicking receptor tyrosine kinase (RTK)/Ras activation (Basler et al., 1991; Fortini et al., 1992; Freeman, 1996). However, in addition to this photoreceptor phenotype, such eyes also show an obvious planar polarity phenotype. Most ommatidia with the normal complement of photoreceptors are symmetrical R3/R3-type clusters and/or are misrotated (Fig. 2A), resembling the phenotypes of sev-Fz and sev-Dsh (Strutt et al., 1997; Boutros et al., 1998; Zhang and Carthew, 1998). This observation supports the notion that jun is a common target of two different pathways in the eye: RTK/Ras/ERK and Fz/Dsh signaling.

To demonstrate that the sev-Jun<sup>Asp</sup> phenotype is a consequence of mimicking the activation of both Ras/ERK and Fz signaling, we have tested the effect of removing one copy of the ERK target pnt. Pnt has been shown to act as a phosphorylation target and nuclear effector of Ras/ERK signaling in photoreceptor induction (Brunner et al., 1994; O’Neill et al., 1994), but has no effect on planar polarity (not shown). Reducing pnt function by hypomorphic allelic combinations suppresses the sev-Jun<sup>Asp</sup> induction of additional photoreceptors (Treier et al., 1995). Similarly, removing one copy of pnt by a strong (or null) allele dominantly suppresses the additional photoreceptor phenotype in sev-Jun<sup>Asp</sup> eyes (Fig. 2B). However, in such a genetic combination, the planar polarity defects of sev-Jun<sup>Asp</sup> persist (Fig. 2B), clearly revealing the additional role of jun in polarity generation.
**Jun<sup>Asp</sup> upregulates the JNK target puckered and induces polarity defects**

To test whether the *sev-Jun<sup>Asp</sup>*-mediated effects on polarity establishment are direct effects, we analyzed the respective eye imaginal discs.

Activation of the JNK pathway in the embryonic ectoderm can induce a strong upregulation of the JNK target gene *puckered* (*puc*) (Glise and Noselli, 1997; Martin-Blanco et al., 1998). *puc* is not only expressed in the epidermal cells but also in specific patterns in imaginal discs (Martin-Blanco et al., 1998). In the eye disc, *puc* is expressed at low levels in all photoreceptor cells posterior to the morphogenetic furrow (Fig. 2C; Martin-Blanco et al., 1998). To test whether expression of *Jun<sup>Asp</sup>* in the eye disc can mimic activation of the JNK pathway, we analyzed the expression of a *puc* enhancer trap line (*puc<sup>A251.1F3</sup>*) in a *sev-Jun<sup>Asp</sup>* background. In clusters close to the morphogenetic furrow, the *sev* enhancer drives expression in the R3 and R4 precursor cells and, accordingly, the analysis of β-gal expression in *sev-Jun<sup>Asp</sup>* shows that *puc* is specifically upregulated in most R3/R4 precursors (Fig. 2D).

In addition, this expression pattern reveals early polarity defects in the eye disc as visible by the irregular arrangement and rotation angles of the clusters. Thus *Jun<sup>Asp</sup>* has a primary effect on planar polarity establishment.

**Jun<sup>Asp</sup> induces the R3 cell fate**

It has been previously reported that *Fz* signaling is required in R3 for correct ommatidial chirality (Zheng et al., 1995). Another component of *Fz* signaling, *msn*, is also involved in the selection of the R3 fate (Paricio et al., 1999). Similarly, mosaic analysis of the gain-of-function *sev-Fz* genotype has shown that *Fz* induces the R3 fate (Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Since our genetic interaction experiments indicate that *Jun* acts downstream of *Fz* in polarity establishment (Fig. 1C,D) and *sev-Jun<sup>Asp</sup>* leads to symmetrical R3/R3 type ommatidia (Fig. 2B), we asked whether *Jun<sup>Asp</sup>* induces the R3 fate. Analyses of *sev-Jun<sup>Asp</sup>* clones in an otherwise wild-type background revealed that, in mosaic R3/R4 pairs, the *Jun<sup>Asp</sup>*-expressing cell tends to adopt the R3 fate (Fig. 3). For example, when the normal R3 precursor is *Jun<sup>Asp</sup>* positive and R4 is wild type, the ommatidium acquires the correct chirality and rotates normally. In contrast, when the R3 precursor is wild type and the cell in position of the normal R4 is *Jun<sup>Asp</sup>* positive, the ommatidia often become either R3/R3 symmetrical or adopt the opposite chirality (Fig. 3A,B). Thus *Jun<sup>Asp</sup>* induces the R3 fate, indicating that the genetic requirements of *jun* in single photoreceptors, in particular the R3/R4 pair, are similar to those of *fz*.

In summary, these results indicate that the *Jun<sup>Asp</sup>* polarity phenotype mimics overactivation of the *Fz* pathway in the R3/R4 pair early in development. Taken together with the genetic interactions between *sev-Fz* and *jun* mutants these data implicate *jun* in *Fz*-mediated R3/R4 specification and planar polarity signaling.

**Clones of *jun<sup>-</sup>* tissue show planar polarity defects in the eye**

Previous analysis of *jun<sup>-</sup>* clones has revealed defects in photoreceptor recruitment with very low penetrance (Kockel et al., 1997). To investigate in detail the role of *jun* in polarity signaling, we have analyzed the effect of *jun* mutant tissue on polarity establishment. In order to obtain clones encompassing large parts of the eye surface, we have used the *ey-FLP* construct (a kind gift of Barry Dickson) and the *Minute* technique (heterozygous *Minute* cells have a growth disadvantage and homozygous *Minute* cells die autonomously – Morata and Ripoll, 1975). Analyses of such clones of two *jun* alleles (*jun<sup>1A102</sup>* and *jun<sup>76-19</sup>* (Hou et al., 1997; Kockel et al., 1997) revealed that, although many ommatidia display correct polarity, *jun* mutant ommatidia can show chirality flips or symmetry of the R4/R4-type (Fig. 4), suggesting that, in the absence of *jun* function, R3 can develop as R4. In addition, we find occasionally misrotated clusters and also some ommatidia with missing photoreceptors (Fig. 4A and not shown; see Kockel et al., 1997). Consistent with the gain-of-function results, this phenotype indicates that *jun* function is required for chirality establishment and R3 specification. However, the low penetrance of the phenotype suggests that *jun* function in planar polarity establishment (as well as photoreceptor induction; Kockel et al., 1997) is largely redundant as compared to the complete loss of polarity in *fz* or *dsh* mutants (see Discussion).

**Inactivation of the JNK pathway causes polarity phenotypes**

The results described above indicate that Jun acts downstream...
of Fz in planar polarity establishment. How is Jun linked to Fz? Since Jun acts downstream of JNK and genetic interactions have suggested that JNK signaling might be involved in planar polarity generation, we looked for planar polarity defects caused by inactivation of the JNK kinase module.

Previous analyses of the hep null alleles or bsk hypomorphic alleles showed very weak or no phenotypes (our unpublished results and Riesgo-Escovar et al., 1996), suggesting redundancy at this level in the polarity signaling pathway. However, related kinases that could account for this redundancy might be also inhibited by a dominant negative JNK. Thus, to address this issue further, we have tested the effect of a kinase-defective version of bsk in planar polarity generation. Based on comparisons with other related protein kinases, we generated a Bsk^K53R mutation by altering an invariant lysine residue in the phosphate (ATP) binding pocket responsible for the catalytic activity of the kinase (Madhani et al., 1997). Expression of this dominant negative form of Bsk, BskDN, with the embryonic ectoderm-specific 69B-GAL4 driver (Brand and Perrimon, 1993) led to dorsally open embryos (not shown), as observed in bsk mutant embryos (Riesgo-Escovar et al., 1996; Sluss et al., 1996). This confirmed the notion that the K53R mutant is a dominant negative form of JNK/Bsk. Expression of BskDN with sev-GAL4 gave a typical eye planar polarity phenotype (Fig. 5A), with ommatidia being misrotated, displaying chirality flips or being symmetrical with variable frequencies. These results indicate that the presence of an inactive form of Bsk at the time of establishment of planar polarity interferes with the process, and that signaling through JNK or related kinases is required for polarity establishment.

It was suggested that other kinases belonging to the JNK/p38 class could possibly account for this redundancy (Paricio et al., 1999). To investigate how specific is the dominant negative Bsk^K53R isoform and to potentially identify related kinases that account for the redundancy, we tested whether a bsk loss-of-function allele and deficiencies removing p38 kinases had a dosage effect on a weak phenotype (one copy) of sev>^Bsk^K53R (Fig. 5D). Interestingly, the Bsk^K53R phenotype was not only enhanced by the bsk^2 allele, but also by deficiencies removing the related p38 type kinases (see quantification in Fig. 5D). This observation indicates that both related kinase types are inhibited by Bsk^K53R and thus involved in planar polarity signaling, suggesting that the redundancy is due to their combined function.

Overexpression of JNK pathway components produces EPP phenotypes

Overexpression of planar polarity genes such as fz, dsh and others in the R3/R4 precursors affects polarity generation (Strutt et al., 1997; Boutros et al., 1998; Zhang and Carthew, 1998; Paricio et al., 1999; see Fig. 1C). To test whether JNK pathway components can cause similar polarity defects, we generated flies containing UAS-Hep or UAS-Bsk, and crossed them to sev-GAL4 flies. Overexpression of the wild-type forms of either Hep or Bsk gives rise to flies with rough eyes showing polarity phenotypes, reminiscent of those observed with Fz, Dsh or Msn, where ommatidia are incorrectly rotated, and sometimes exhibit the wrong chiral form (Fig. 5B,C).

In addition, we have generated UAS strains carrying an activated form of Hep/JNKK (UAS-Hep^Act; where the relevant S and T residues were changed to aspartic acid to mimic an activated phosphorylated kinase; see Materials and methods). The sev-GAL4, UAS-Hep^Act flies displayed a phenotype that...
was more extreme with significantly reduced eyes and a very messy appearance in sections (possibly due to induction of cell death; data not shown). Interestingly, this phenotype is very similar to strong eye-specific overexpression of Dsh (not shown) suggesting that, although Dsh and the JNK cascade are involved in polarity generation, they can also share common functions outside planar polarity signaling. Nevertheless, when eye discs of such flies are analyzed for $puc\text{-}\text{lacZ}$ expression, which is specifically upregulated in many R3/R4 (Fig. 2E; very similar to activated Jun, Fig. 2D), the misalignment of the R3/R4 pair and thus polarity defects are also evident in early eye discs.

Taken together, these data are consistent with the JNK signaling module being involved in polarity establishment and the specification within the R3/R4 photoreceptor subtype.

The negative regulator of JNK, Puckered, causes polarity defects

The available results indicate that JNK/p38 signaling in planar polarity establishment is important, but that the removal of a single kinase does not significantly affect its level. Puckered (Puc) is a dual specificity MAPK phosphatase that negatively regulates the activity of JNK, and thus the whole pathway during dorsal closure (Ring and Martinez-Arias, 1993; Glise and Noselli, 1997; Martin-Blanco et al., 1998). It has been reported that overexpression of Puc results in inactivation of JNK and mimics $bsk$ mutant phenotypes in embryos (Martin-Blanco et al., 1998). Puc is also a target of JNK signaling and Jun in the eye (Fig. 2D,E). Thus, overexpression of Puc in the eye could also mimic the inactivation of JNK and related kinases, and inhibit signaling by the JNK module(s). To test this, we generated a $sev\text{-}\text{GAL4}$, $UAS\text{-}puc$ strain. The eyes of the resulting flies are generally mildly rough with sporadic very rough eyes. These show typical planar polarity phenotypes in tangential sections (Fig. 6A), consistent with the notion that overexpression of $puc$ is inhibiting JNK (and possibly p38) signaling.

Furthermore, we attempted to affect JNK signaling strength...
by removing \textit{puc} function. It was shown that during dorsal closure \textit{hep} and \textit{puc} have opposite effects on \textit{bsk} (Riesgo-Escovar et al., 1996; Sluss et al., 1996). JNK signaling is hyperactive in \textit{puc} mutants (Martin-Blanco et al., 1998), and mutations in \textit{puc} are able to suppress the adult phenotypes associated with \textit{hep} (Agnes et al., 1999). However, \textit{puc} is embryonic lethal (Martin-Blanco et al., 1998) and \textit{puc} cells do not survive in the eye disc (not shown; similar observations were made by E. Martin-Blanco, personal communication). This antagonistic effect is also evident in an allelic combination of \textit{hep} and \textit{puc} hypomorphic mutations, \textit{hep}^1 and \textit{puc}^{E69}. Flies of this genetic background give rise to homozygous escapers with mildly rough eyes that display typical planar polarity defects (Fig. 6B). These experiments demonstrate that affecting Puc/JNK activity causes defects in planar polarity generation, indicating that JNK signaling is required and that Puc/JNK levels have to be delicately balanced for polarity establishment in the eye.

\textbf{Constitutive activation of Jun and JNK signaling causes deregulation of \textit{Dl} in R3/R4}

It has been recently shown genetically that Delta (\textit{Dl}) acts downstream of Fz signaling and is a transcriptional target of Fz in R3 (Cooper and Bray, 1999; Fanto and Mlodzik, 1999). In the eye disc, \textit{Dl} is expressed in a very dynamic pattern throughout eye development (Parks et al., 1995) and it has been implicated in several developmental aspects ranging from control of growth to lateral inhibition (Baker and Zitron, 1995; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). In the context of planar polarity signaling, \textit{Dl} is expressed transiently in the R3 precursor at higher levels in response to Fz activation (Fig. 7A; see also Parks et al., 1995; Fanto and Mlodzik, 1999; Cooper and Bray, 1999). This tightly regulated \textit{Dl} expression is critical for activating Notch in the neighboring R4 cell and specifying the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig6}
\caption{Puckered levels affect planar polarity. Tangential eye sections with the corresponding schematic drawings are shown; arrows as in Fig. 1. (A) \textit{sev-GAL4; UAS-puc}. Overexpression of the negative regulator of JNK produces strong polarity defects. (B) \textit{hep}^1, \textit{puc}^{E69} homozygous eye. The \textit{hep} loss-of-function mutation “rescues” the usually lethal \textit{puc}^{E69} mutation, giving rise to a viable double mutant with rough eyes. Note polarity defects and occasional loss of photoreceptors (circles in schematic).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7}
\caption{Differential Delta expression in the early R3/R4 pair is regulated by \textit{Jun} and JNK signaling. Confocal images of third instar eye imaginal discs showing \textit{Dl-lacZ} expression in green (expressed in a dynamic pattern in all photoreceptor precursors) and Spalt in red (present in the R3/R4 pair). Anterior is to the left, dorsal is up. The morphogenetic furrow is at the left margin of each panel. The bottom panels show the anti-Spalt staining highlighting the orientation of the preclusters (yellow arrows are examples of correctly oriented clusters; white arrows depict examples of misoriented clusters). (A) \textit{Dl-lacZ}+. In wild type, Delta expression is higher in the R3 photoreceptor compared to R4 at the time of polarity signaling and early ommatidial rotation. This is reflected by the yellow overlap with Spalt expression (examples of R3 and R4 precursors are numbered in black and white, respectively). (B) \textit{sev-Jun}^{hsP}+/+; \textit{Dl-lacZ}+. (C) \textit{sev-Gal4/UAS-Hept}/+; \textit{Dl-lacZ}+. (D) \textit{sev-Dsh} (rec2)/+ \textit{Dl-lacZ}. (B-D) Delta expression is generally deregulated, with the R3/R4 pair often showing equal expression levels (examples indicated with black asterisks), or with inverted expression levels within the R3/R4 pair (indicated with white/black asterisks; compare to wild type in A).}
\end{figure}
R4 fate (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). In the gain-of-function sev-Fz background, Dl expression is deregulated in the R3/R4 precursor pair, with both cells often expressing Dl at comparable levels or displaying random expression within the pair (Cooper and Bray, 1999; Fanto and Mlodzik, 1999).

The observation that Jun shows a specific requirement for R3 induction (as documented above) suggested that it might act as one of the transcription factors regulating Dl expression downstream of Fz. To test this hypothesis, we analyzed the expression of the Di-lacZ line, which mimics Dl expression, in the sev-Jun

\footnotesize{\textsuperscript{A}} and sev-Hep

\footnotesize{\textsuperscript{A}} backgrounds and compared it to those of Fz/Dsh signaling (sev-Dsh). Our results clearly show that, in all cases, Dl expression is not upregulated only in the R3 precursors as in wild type (Fig. 7A), but is often present in both cells of the R3/R4 pair, or expressed randomly within the pair (Fig. 7B-D). These data indicate that the Fz/Dsh-induced transcriptional regulation of Dl in R3 is mediated by Jun and the JNK module.

DISCUSSION

**Jun acts downstream of Fz in planar polarity generation**

Planar polarity generation in the *Drosophila* eye requires the specification of the R3/R4 cells by the Fz and N signaling cascades (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999). Here we have shown that the transcription factor Jun acts downstream of Fz signaling in R3 specification and planar polarity establishment in the eye. Both Fz and Jun specify the R3 fate and are involved in regulating *Delta* transcription, the only known transcriptional target of the planar polarity cascade to date. Although the gain-of-function analyses give very similar results, the loss-of-function phenotype of D-jun is weaker than that of fz, dsh or msn, indicating that the function of jun is redundant.

**The role of Jun in Ras/ERK and Fz/JNK signaling in the eye**

Jun, as a member of the AP-1 family, is activated by many distinct extracellular stimuli and acts downstream of several signaling pathways (Davis, 1999; Wisdom, 1999). Besides its involvement in stress response, Jun has been implicated in the control of proliferation, apoptosis, morphogenesis and cell fate induction (Wisdom, 1999). In *Drosophila*, Jun is critical for the process of dorsal closure in embryogenesis acting downstream of the JNK module (Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997). It has also been implicated in cell fate induction downstream of Ras/ERK signaling in the eye (Treier et al., 1995; Kockel et al., 1997). Our analysis shows that it also acts downstream of Fz in planar polarity signaling in the eye. It is the first transcription factor implicated in Fz/plantar polarity signaling. Fz signaling also requires a JNK (or related kinase) module (see below), and thus in the eye imaginal disc Jun acts downstream of both ERK and JNK.

How does Jun achieve a specific response in this context? The S/T residues that are phosphorylated in Jun are the same for both ERK and JNK (Peverali et al., 1996; Sluss et al., 1996). Thus, although differences in phosphorylation level and/or preference for any of the serine/threonine target residues cannot be excluded in vivo, differential phosphorylation is unlikely to create specificity. A potential mechanism for specificity might be provided by other transcription factors that cooperate with Jun in the different processes. This is supported by the observation that the sev-Jun

\footnotescript{A} phenotype is a composite of two events, photoreceptor recruitment and ommatidial polarity generation. These two effects can, however, be separated by the reduction of specific interacting partners. In the process of Ras/ERK signaling in photoreceptor induction Jun interacts and synergizes with the ETS domain transcription factor Pointed (Pnt) (Treier et al., 1995). Pnt has been characterized as a target of the ERK/Rl kinase in *Drosophila* in all ERK-dependent processes analyzed (Freeman, 1997). However, it has not been linked to any JNK-mediated process. Removing one dose of pnt strongly suppresses the Ras/ERK-related extra photoreceptor phenotype of sev-Jun

\footnotescript{A}, whereas the polarity defects persist and thus are more prominent (Fig. 2, see also Treier et al., 1995). This observation indicates that, in the absence of normal Pnt levels, sev-Jun

\footnotescript{A} specifically affects polarity, suggesting that the interaction with Pnt is important for its role in the ERK-mediated induction. It is likely that for its planar polarity function other specific transcription factors provide the specificity cues.

**JNK signaling redundancy in the Frizzled pathway**

Although all components of the JNK module tested genetically interact with sev-Fz and sev-Dsh, analysis of existing loss-of-function mutants did not show defects in planar polarity establishment, suggesting a redundant role (Strutt et al., 1997; Boutros et al., 1998; Paricio et al., 1999). Even null alleles of the *Drosophila* homolog of JNKK hep (Df(X)H6 and Df(X)G24 deficiencies also affect licorne/MKK3, which is required during oogenesis – Suzanne et al., 1999) have no effects on planar polarity (data not shown).

Expression of a dominant negative (kinase dead) isoform of Bsk, however, interferes with planar polarity, giving rise to typical polarity phenotypes (Fig. 5), implying that Bsk and JNK signaling are important in this process. Consistently, homoygous mutant clones of the deficiency Df(2R)flp170B that removes bsk and other neighbouring loci (Sluss et al., 1996), and is considered a true null for bsk, showed a mild polarity phenotype in the eye, including the presence of symmetrical ommatidia (data not shown).

What are the redundant kinases in this process? Genetic interaction analysis with sev-Msn has shown that, besides hep and bsk, deficiencies affecting other MKKs and the *Drosophila* p38a and p38b loci suppress the sev-Msn phenotype (Paricio et al., 1999). This suggested that the p38 kinase module that is related to JNK and has been shown to have (at least partially) overlapping phosphorylation targets (Whitmarsh et al., 1997) might be responsible for the redundancy in this process. Our analysis with the dominant negative (DN) Bsk isoform and the respective deficiencies suggests that the p38 kinase(s) are contributing to this redundancy, as they enhance the DN-Bsk phenotype very similarly to the bsk deficiency. The identification of specific mutant alleles of p38a/b and double mutant analysis with bsk will be necessary to further clarify this issue.

The available results indicate that the level of JNK/p38 signaling in planar polarity establishment is important, but that the removal of a single kinase does not significantly affect this
level. In support, the observation that an allelic combination of hep and puc hypomorphic alleles can give rise to planar polarity eye phenotypes (Fig. 5) suggests that the balance between negative and positive regulators of JNK and related kinases is critical. Similarly, overexpression of the negative JNK regulator Puc, a dual specificity phosphatase (Martin-Blanco et al., 1998), causes typical polarity defects similar to those of fz or dsh mutants. It is likely that this phosphatase negatively regulates all JNK-related kinases and thus reduces the overall signaling more than the lack of a single kinase.

In summary, our data indicate that the transcriptional events downstream of Fz in R3 specification and chirality establishment (e.g. regulation of Dl) are mediated by Jun. The factors with which Jun is redundant in the imaginal disc are not yet identified. It is possible that other members of the AP-1 family are also involved in planar polarity signaling, as they are related to Jun and could dimerize with it via the leucine-zipper motif. A potential candidate is Fos as it is, like Jun, required downstream of JNK in the process of dorsal closure in the embryo. Similarly, the ETS domain protein Yan acts as negative regulator in dorsal closure and is inactivated by JNK in the process. Interestingly, in a gain-of-function planar polarity screen (Paricco et al., 1999), an EP-line in yan was identified. However, these factors do not show informative planar polarity phenotypes in clones and thus their involvement in this process remains unclear. Although AP-1 and ETS family members are attractive candidates, transcription factors belonging to other families cannot be excluded in this context.

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