Jun in *Drosophila* development: redundant and nonredundant functions and regulation by two MAPK signal transduction pathways

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***Drosophila* Jun is shown to be involved in different signal transduction pathways and developmental decisions. Dorsal closure, a morphogenetic process occurring during *Drosophila* embryogenesis, is regulated by Hemipterous (Hep) and Basket (Bsk), homologs of JNKK and JNK, respectively. Embryos lacking Jun activity exhibit a dorsal closure phenotype, very similar to that of *bsk* and *hep* mutants, indicating that Jun is a target of Hep/Bsk signaling. In eye and wing development Jun participates in a separate signaling pathway that is comprised of Ras, Raf, and the ERK-type kinase Rolled. In contrast to the strict requirement for Jun in dorsal closure, its role in the eye is redundant but can be uncovered by mutations in other signaling components. The redundant function of Jun in eye development may contribute to the precision of photoreceptor differentiation and ommatidial assembly.**

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The paramount importance of cellular signal transduction pathways is particularly evident in multicellular organisms. These pathways not only mediate communication between cells but also processing of the exchanged information. In this manner they control complex functions during morphogenesis as well as in the adult organism. As the charting of signal transduction pathways proceeds toward an understanding in molecular detail, certain unexpected features emerge: At least some signaling components are used in more than one pathway. The small GTPase Ras, for example, is an element of growth factor response pathways but also of pathways that control differentiation of postmitotic cells (Zipursky and Rubin 1994; Marshall 1995). Likewise, many transcription factors such as Elk, cAMP response element binding (CREB) protein and NF- κ B are receptive to stimuli from a number of different pathways (Armstrong and Montminy 1993; Darnell et al. 1994; Siebenlist et al. 1994; Treier and Bohmann 1996).

Another recently discovered feature of cellular signaling is the existence of pathways that have quite different biological functions but consist of homologous components. The best-known examples are the different mitogen-activated protein kinase (MAPK) signaling path-

ways, first identified in yeast. The stimuli that lead to specific yeast responses, such as mating behavior after pheromone exposure or the reaction to osmotic stress, are relayed by distinct, yet related signal transduction pathways (Herskowitz 1995). The hallmark of these different pathways are cascades of at least three kinases which are now classified as MAPKKK, MAPKK, and MAPK (Marshall 1994).

Multiple functions of individual signaling components and interconnections of different signaling pathways indicate that the regulatory network that controls the behavior of cells in the context of multicellular organisms is very complex. Genetic studies in comparatively simple organisms have proved valuable in unraveling this complexity. Studies in *Drosophila melanogaster* and in *Caenorhabditis elegans* have contributed significantly to the deciphering of the Ras and other signal transduction pathways (Greenwald and Rubin 1992; Zipursky and Rubin 1994), and it may be assumed that genetics in combination with classic biochemical approaches will lead to a more complete understanding of regulatory networks.

Nuclear elements of cellular signal transduction include inducible transcription factors such as Jun. Jun activity responds to multiple cues such as growth factors, phorbol esters, activated oncogenes such as *ras*, and cellular stress (Angel and Karin 1991). Previously, we have studied the role of Jun as a nuclear target of the Ras/Raf/MAPK signal transduction pathway in *Drosophila* eye

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development (Zipursky and Rubin 1994). Transgene expression studies employing a panel of dominant-negative and constitutively activated mutants showed that Jun participates in the control of photoreceptor determination and differentiation during eye development (Bohmann et al. 1994, Treier et al. 1995; Peverali et al. 1996). Experiments in which the loss of R7 photoreceptors in mutants lacking the Sevenless (Sev) receptor, a tyrosine kinase, could be rescued by expression of activated *jun* alleles illustrated the potential of Jun to initiate neuronal differentiation in the developing eye (Treier et al. 1995). Biochemical data indicate that the neurogenic function of Jun depends on its phosphorylation by the extracellular signal-regulated kinase (ERK)-type MAPK Rolled (R1), a genetically defined downstream element of Ras signaling (Peverali et al. 1996).

Based primarily on mammalian tissue culture experiments, a related MAPK signal transduction pathway has been identified recently (Davis 1994). The MAPKs activated by this pathway are called JNKs (for Jun amino (N)-terminal kinase) or SAPKs (for stress-activated protein kinase) and are distantly related to ERK-type MAPK (Dérjard et al. 1994; Kyriakis et al. 1994). In vertebrate cells JNK signaling mediates cellular stress responses elicited, for example, by UV radiation, heat shock, and DNA damage (Minden et al. 1994). The relationship of ERK and JNK signaling and their relevance in a whole organism are understood only poorly.

The identification of the product of the *hemipterous* (*hep*) gene as a *D. melanogaster* JNKK homolog (Glise et al. 1995) and of *basket* (*bsk*) as a JNK (Riesgo-Escovar et al. 1996; Sluss et al. 1996) has made a genetic approach to investigate the JNK pathway possible. Phenotypic analysis in the embryo, as well as biochemical data, indicate that Bsk and Hep are part of a *Drosophila* JNK signal transduction cascade (Knust 1996). Mutations in *bsk* and *hep* cause almost identical phenotypes. In both cases, mutant embryos fail to complete dorsal closure, a morphogenetic process occurring in late embryonic stages (Campos-Ortega and Hartenstein 1985). During dorsal closure the cells of the lateral epidermis undergo a concerted shape change and elongate along the dorsoventral axis. This process, which occurs in the absence of cell division, results in a dorsalward movement of the lateral epidermis enclosing gut and mesodermal derivatives, which until then are only covered by the amnioserosa. The cell shape changes of dorsal closure initiate in the cells of the leading edge of the lateral epidermis and may be controlled by a signal emanating from it (Knust 1996). Biochemical data indicate that Hep can phosphorylate Bsk, supporting the hypothesis that the two proteins are components of the same signal transduction pathway. It was also suggested by in vitro phosphorylation experiments that Bsk can phosphorylate *Drosophila* Jun (Riesgo-Escovar et al. 1996; Sluss et al. 1996). This might indicate that as in mammals, Jun is a target of the JNK signal transduction pathway in *D. melanogaster*. If true, this would provide a model in which the function of Jun as a target of both ERK and JNK signaling could be studied in vivo in the same organism.

Here we show that *Drosophila* Jun acts in multiple developmental control processes as a target of both ERK and JNK signaling. Our data indicate that Jun plays an essential role in dorsal closure, whereas in eye development, loss of Jun in an otherwise wild-type background can be tolerated with only minor phenotypic defects. Nevertheless, Jun becomes a limiting component if ERK signaling is reduced, demonstrating a redundant function of Jun in *Drosophila* similar to that proposed in mammals (Hilberg et al. 1993; Johnson et al. 1993).

Results

To investigate whether Jun might be involved in the Hep/Bsk signaling pathway in the control of dorsal closure, we followed three experimental strategies: (1) immunostaining of embryos for Jun protein in the relevant cells; (2) expression of dominant-negative forms of Jun in the relevant embryonic tissues; and (3) analysis of mutant embryos that lack a functional *jun* gene.

Jun expression in the early embryo

First, we asked whether the temporal and spatial expression pattern of Jun was compatible with a role in dorsal closure. Whole-mount immunostaining of wild-type *Drosophila* embryos with affinity-purified Jun antibodies (Bohmann et al. 1994; Peverali et al. 1996) reveals widespread nuclear Jun expression at varying levels throughout embryogenesis. One of the tissues showing elevated Jun expression is the amnioserosa (Fig. 1A). During and following germ-band retraction, cells of the dorsolateral epidermis also show increased Jun staining (Fig. 1B–D). Strikingly, highest Jun protein levels are detected in the cells at the leading dorsal edge of the epidermis. These cells initiate the process of dorsal closure and are also the first that undergo the associated cell shape changes (an elongation along the dorsoventral axis). The high expression of Jun in the cells of the leading edge of dorsal closure is maintained throughout this process until its completion in late embryogenesis (Fig. 1E,F). Cells along the cephalic furrow also express Jun at high levels (arrowheads in Fig. 1C,E).

The elevated staining in the leading edge of the lateral epidermal walls where dorsal closure initiates is consistent with a role for Jun in the process and lends credence to the hypothesis that Jun might be a target for the Hep/Bsk signaling pathway.

Dominant-negative forms of Jun interfere with dorsal closure

To obtain functional evidence for the involvement of Jun in dorsal closure, we employed a previously characterized dominant-negative mutant in which the transcriptional activation domains are deleted (Jun^{bZIP}) (Bohmann et al. 1994). This truncated derivative was expressed in the epidermis at relevant embryonic stages using the upstream activating sequence (UAS)–Gal4 expression system (Brand and Perrimon 1993). Embryonic expression of

Figure 1. Jun expression in embryos during dorsal closure. Wild-type embryos were stained with an affinity-purified polyclonal antiserum raised against bacterially expressed *Drosophila* Jun. All embryos are oriented anterior left; (A,B,D) Lateral views (dorsal up); (C,E,F) dorsal views. Stages are according to Campos-Ortega and Hartenstein (1985). (A) Stage 10: Note elevated nuclear expression in cells of the amnioserosa (arrows). (B) Stage 11: Transiently high levels of Jun are detected in the cells of the amnioserosa that are directly adjacent to the dorso-lateral epidermis (arrows). Lower levels of Jun staining remain present in all cells of the amnioserosa throughout their life time (D; data not shown). (C,D) Early stage 13: High Jun expression levels are detected in the cells of the leading edge of the lateral epidermis (arrows) and cells posteriorly adjacent to the head segments (arrowheads in C), which will undergo head involution. Note that lower levels of Jun are detectable in all cells of the lateral epidermis, and cells of the amnioserosa show also lower levels of Jun at this and later stages (arrowheads in D; data not shown). (E) Stage 15: Note that expression is maintained in cells of the leading edge of dorsal closure (arrows) and cells posteriorly adjacent to the head segments (arrowheads). (F) Stage 16: High Jun expression is maintained at the leading edge (arrows); dorsal closure is complete. Jun is also expressed in many other tissues and cell types throughout embryogenesis (not shown).

Jun^{bZIP} is lethal and causes the appearance of large holes in the dorsal epidermis, which are indicative of a failure in dorsal closure (Fig. 2). Cuticle preparations of these embryos display very similar phenotypes compared to embryos that are homozygous for loss-of-function alleles of *bsk* or *hep*. Taken together with the previously reported biochemical data (Riesgo-Escovar et al. 1996; Sluss et al. 1996), the resemblance of the dorsal closure phenotypes caused by *hep*, *bsk*, and dominant-negative Jun suggests that the function of Jun in dorsal closure is that of a nuclear recipient of the *Drosophila* JNK signal transduction pathway. This hypothesis is further supported by the observation that Jun expression is not affected in *bsk* mutants. In zygotic *bsk*² homozygotes the Jun protein patterns and levels appear largely normal (not shown).

A *jun* null mutation disrupts dorsal closure

To obtain further proof for the role of Jun in dorsal closure, we wanted to examine the effect of endogenous

mutations in the *Drosophila jun* locus during embryogenesis. As a first step toward obtaining loss-of-function *jun* alleles, we generated a small deletion by imprecise excision of P-element 1(2)07103, which is inserted 2.8 kb downstream of the *jun* transcription unit (Fig. 3). This deletion causes a cytologically nonvisible deficiency termed E73 that, like 1(2)07103 (Kockel et al. 1997), is a lethal mutation. E73 completely removes the *jun* locus, as well as the first exon of the neighboring *14-3-3ζ* gene (Kockel et al. 1997). Based on the assumption that lack of *jun* would cause a lethal phenotype, we screened for ethylmethane sulfonate (EMS)-induced point mutations that failed to complement the lethality of E73 (see Materials and Methods). Among the isolated mutations, one *jun* allele, henceforth referred to as *jun*¹ was identified. As determined by PCR and sequence analysis, this allele carries a nonsense mutation at codon 177 of the *jun* open reading frame (Fig. 3). Because of the stop codon at this position, *jun*¹ encodes a truncated protein devoid of the

Figure 2. Expression of dominant negative Jun in the developing embryo can cause defects in head and dorsal cuticle development. The dominant-negative Jun^{bZIP} protein was expressed using the GAL4/UAS system with different embryonic epidermal GAL4 driver lines. Cuticle preparations of the following genotypes are shown: (A) Wild type; (B) C765/UAS-*jun*^{bZIP}; (C) 69B/UAS-*jun*^{bZIP}; (D) *hsGAL4/UAS-jun*^{bZIP}. Anterior is left and dorsal up except in D, which is a dorsal view. Note that all drivers in combination with UAS-*jun*^{bZIP} cause defects in head and dorsal cuticle and that they display graded severity: The C765 line induces predominantly an anterior open-like phenotype (marked with arrow), whereas 69B and *hsGAL4* (following heat shock) lead to phenotypes that progressively affect the entire dorsal cuticle. No defects in posterior structures, the Filzkörper, are observed.

Figure 3. Genomic organization and molecular lesions of the *jun* locus. The genomic region containing the *jun* and the 14-3-3 ξ genes [at band 46E on *Drosophila* chromosome II] is shown with landmark restriction sites. Jun and 14-3-3 transcripts are depicted with translated sequences (shaded). The area shown has been completely sequenced (EMBL database, accession no. Y12573). P-element insertion 07103 [obtained from the Berkeley *Drosophila* Genome Project as part of the Spradling collection] was mapped 2.8 kb to the 3' end of the *jun* transcription unit. Imprecise excision of this P element yielded deficiency *E73*, which removes the transcription start site and first exons of 14-3-3 ξ and the complete *jun* locus. *jun1* represents an EMS-induced mutant allele that was isolated by its inability to complement the lethality of *E73*. The *jun1* open reading frame is prematurely terminated (asterisks), and can only be translated into a truncated gene product of 176 amino acids. The *jun2* mutant was isolated originally, as *l(2)IA109*, in a screen for embryonic lethal mutants (Nüsslein-Volhard et al. 1984). *jun2* also originated from a nonsense mutation, this one in codon number 72. The amino acid sequence of wild-type Jun with the location of the point mutations, the basic region, and the leucine zipper is shown at the bottom.

basic region and leucine zipper. Consequently, the gene product, if stably expressed at all, would no longer be able to dimerize and bind to DNA, causing loss of the *jun* function. Thus, *jun1* can be regarded as a very strong and, possibly, a null allele. Immunostainings of *jun* mutant embryos with Jun antibodies revealed that *jun1* is likely to be a null allele, as no anti-Jun immunoreactivity was detected [not shown].

Homozygous *jun1* mutant embryos die in mid- to late embryogenesis with large anterior and dorsal holes, similar to the ones caused by *bsk* or *hep* (Fig. 4). Together with the experiments using dominant-negative forms of Jun, the *jun1* phenotype confirms the role of Jun as a regulator of dorsal closure and, to a variable degree, of head involution. These defects are consistent with the

elevated Jun expression in the leading edge of the lateral epidermis during dorsal closure and along the cephalic furrow prior to and during head involution (Fig. 1). In characterizing *jun1*, we noticed similarities to a mutant that was originally isolated in a screen for embryonic lethal mutations in *Drosophila* (Nüsslein-Volhard and Wieschaus 1980; Nüsslein-Volhard et al. 1984). This mutant *l(2)IA109*, like *jun1*, causes a dorsal closure defect and was genetically mapped to the same general region of chromosome IIR. Complementation analysis revealed that *jun1* and *l(2)IA109* are allelic, and sequence analysis showed that the *l(2)IA109* allele carries a stop codon at position 72 within the Jun open reading frame giving rise to a truncated translation product of only 71 amino acids (Fig. 3). Thus, *l(2)IA109* represents another *jun* allele, and we renamed it *jun2*. The phenotypes of *jun1* and *jun2* homozygous embryos, as well as those of embryos carrying various allelic combinations of *jun1*, *jun2*, and the *E73* deficiency are all indistinguishable (Fig. 4). This confirms that the dorsal closure defect is a specific consequence of a lesion in *jun* and is not caused by another unknown mutation. Furthermore, it supports the assumption that *jun1* and *jun2* are null alleles.

To further characterize the dorsal closure defects caused by lack of Jun activity, mutant and wild-type embryos of stage 13 were stained with a marker to visualize cells at the leading edge of the lateral epidermis. Antibodies against the Coracle protein (Fehon et al. 1994)

Figure 4. Embryonic phenotype of *jun* mutants. Cuticle preparations of the following genotypes are shown: (A) *jun1/jun1*; (B) *jun2/jun2*; (C) *jun1/Df(2R)E73*; (D) *jun2/DfE73*; (E) *hep1/hep1*; (F) *bsk1/bsk1*. All cuticles are from animals raised at 18°C except *bsk1* which was raised at 25°C. For comparison a wild-type cuticle is shown in Fig. 2A. Note defects in dorsal closure that lead to a dorsally open cuticle. All allelic combinations of any *jun* alleles and the *E73* deficiency or homozygous deficiency (A–D; data not shown) have the same phenotype, which is very similar to the *hep* and *bsk* phenotypes (E and F, respectively; *bsk1* is a temperature-sensitive hypomorph and displays slightly weaker defects). None of the mutants shows defects affecting the posterior structures, the Filzkörper. All embryos are oriented anterior left and dorsal up.

Figure 5. Cell shape changes during dorsal closure in wild-type and *jun* mutants as visualized with Coracle staining. All panels shown are derived from late stage 13 embryos (the embryos were staged according to their state of gut development). Anterior is left; dorsal up. (A,B) Lateral epidermis of wild-type embryo (B is 4× magnification as compared to A); Note that cells have elongated along the dorsoventral axis, especially those of the leading edge (top cell layer). (C,D) *jun*¹ and *jun*² mutant embryos, respectively (shown at the same magnification as B); Note that the epidermal cells have not elongated.

outline individual epidermal cells and thus allow the detection of cell shape changes that take place during dorsal closure (Fig. 5). In wild type, dorsal closure initiates with a dorsoventral elongation of the cells at the leading edge of the lateral epidermis. The ventrally adjacent rows of epidermal cells subsequently undergo the same changes (Fig. 5A,B). In *jun* mutants the cells at the leading edge do not elongate to the same extent as in wild-type, and no cell shape changes are detectable more ventrally (Fig. C,D; data not shown). These data indicate that Jun is required for the initiation of dorsal closure, similar to *hep* and *bsk*.

Jun in eye development

The data described above provide evidence that the role of Jun in dorsal closure is that of a nuclear component in the *Drosophila* JNK signal transduction pathway. In the developing eye where we have studied the function of *Drosophila* Jun previously, the situation appears to be different. Here, Jun activity is regulated by a separate pathway that signals through Ras, Raf, and the ERK-type MAPK R1 (Biggs et al. 1994; Zipursky and Rubin 1994; Wassarman et al. 1995). Genetic and biochemical evidence illustrates that the phosphorylation of Jun by activated R1 can initiate neuronal differentiation in competent cells of the eye imaginal disc (Treier et al. 1995; Peverali et al. 1996).

To compare the role of Jun in photoreceptor differentiation versus dorsal closure, that is, as a target of ERK versus JNK signaling, we investigated the effect of *jun* mutant alleles on eye development. Clones of homozy-

gous mutant eye tissue were induced in *jun* heterozygous animals by somatic recombination using X rays or the FRT-FLP system (Xu and Rubin 1993). Surprisingly, *jun*¹, as well as *jun*² homozygous clones were obtained and grow to a size that is comparable to the corresponding wild-type twin spots, indicating that the loss of Jun does not interfere with imaginal cell proliferation. Moreover, mutant clones look externally indistinguishable from surrounding wild-type tissue and show, upon microscopic inspection of tangential sections, only minor defects (Fig. 6). Most ommatidia contain the wild-type complement of photoreceptors, and photoreceptor cells are missing only occasionally. This very mild phenotype contrasts with the effect of dominant-negative Jun mutants that cause a more pronounced, yet still weakly penetrant, loss of photoreceptor cells (Bohmann et al. 1994; Treier et al. 1995).

The removal of Jun function by mutation or by expression of dominant-negative mutants has essentially the same detrimental effect on dorsal closure. In the eye, however, dominant-negative mutants interfere with photoreceptor differentiation more strongly than the complete removal of Jun. One possible explanation for this discrepancy is that Jun function in the *Drosophila* eye is redundant and a complete removal of the protein under otherwise wild-type conditions is largely without phenotypic consequences. The slightly stronger effect of the dominant-negative mutants could be rationalized if occupancy of the Jun-binding sites in the relevant target genes by mutated proteins has a more disruptive effect on their expression and on photoreceptor differentiation than no binding at all. To test this, we examined whether a reduction in the level of wild-type Jun activity would enhance the effect of expressing dominant negative (*sE-jun*^{Ala}) and gain-of-function (*sE-jun*^{Asp}) versions of Jun (Treier et al. 1995). We established *Drosophila* strains that express the modified Jun proteins in a heterozygous *jun*[−] background. Phenotypic analysis shows that the loss of photoreceptor phenotype caused by *sE-*

Figure 6. Clonal analysis of *jun* mutant eye phenotypes. Clones homozygous for the *jun*¹ (A) and *jun*² (B) alleles in adult eyes are marked by the absence of pigment. Anterior is left and dorsal up. Note that most mutant ommatidia have wild-type appearance with the typical trapezoidal arrangement of the six outer photoreceptor rhabdomeres and the central R7 (R8 is below plane of section). Occasionally one or two photoreceptors are lost in mutant ommatidia (marked by arrows in A and B). The clone size is very similar to the wild-type twin clones (data not shown).

jun^{Ala} is enhanced when it is combined with a *jun¹/+* or a *jun²/+* genotype (Fig. 7, A,B, and E,F). Similarly, the removal of one copy of *jun* increases the general disorder and number of ectopic photoreceptors in lines carrying *sE-jun^{Asp}* (Fig. 7C,D and G,H). These results indicate that the gain- and loss-of-function forms of Jun interfere specifically with the endogenously expressed wild-type protein. If the levels of the latter are decreased by halving the gene dose, the mutants can bind to the corresponding promoter elements more efficiently and thus their effects are stronger.

Jun interacts genetically with the Sev/Ras/Raf/ERK signal transduction pathway

The above results are consistent with there being several redundant downstream targets of the Ras/Raf/ERK signal transduction pathway in the developing eye. These should individually be able to instruct cells to adopt photoreceptor fate, so that under normal circumstances loss of one of them, in this case, Jun, can be compensated. To search for such a redundant function of Jun, we examined the effect of a reduced *jun* gene dose, that is, het-

erozygosity for the newly defined mutant *jun* alleles, in the context of either too much or too little signaling through the neurogenic Ras/Raf/ERK pathway.

In the first approach, the neurogenic signaling pathway in the developing eye was artificially activated by expression of gain-of-function alleles, including *sev^{S11}*, *ras^{Val12}*, and *rl^{sem}* (Sevenmaker), which lead to the characteristic appearance of ectopic photoreceptor cells (Basler et al. 1991; Dickson et al. 1992; Fortini et al. 1992; Brunner et al. 1994a). Under these conditions downstream targets of the pathway are expected to be ectopically hyperactivated and might become limiting in this way. This creates a "sensitized background" in which decreased concentrations of such targets can suppress the appearance of ectopic photoreceptor cells. This principle has been applied successfully in several genetic screens for downstream targets of signal transduction components (Chang et al. 1995; Dickson et al. 1995; Karim et al. 1996). When one copy of *jun* is removed from transgenic lines expressing the gain-of-function alleles mentioned above, a clear suppression of the mutant extra photoreceptor phenotype can be observed. Although the *sev^{S11}* and *rl^{sem}* alleles cause the appearance of supernumerary photoreceptors in nearly all ommatidia in a wild-type background, the removal of one copy of *jun* suppresses this effect. In animals carrying the activated alleles in a *jun¹/+* or *jun²/+* background, approximately one quarter of the ommatidia revert to a wild-type appearance (Fig. 8A–F).

The *Ellipse^{B1}* (*Elp^{B1}*) gain-of-function allele of the *Drosophila* epidermal growth factor receptor (EGFR) represents another activated component of receptor tyrosine kinase (RTK)/Ras/ERK signaling (Baker and Rubin 1992). Both the eye and wing are affected in *Elp^{B1}*, and ectopic wing vein tissue develops in the wing (Fig. 8G). Strikingly, the removal of one copy of *jun* suppresses this phenotypic defect almost completely (Fig. 8H). Thus, reduction in *jun* gene dose can be rate limiting in several independent RTK/Ras/ERK signaling events, implying a general role for Jun function downstream of this signaling cascade. Nevertheless, analysis of *jun* mutant clones in wing tissue does not reveal any phenotypic features normally detected with mutants of components of the EGFR/Ras/ERK–Rl pathway (data not shown). Thus, as in eye development, Jun function in the wing appears to be redundant.

Next, we examined the effect of reducing *jun* gene dosage in the context of a weakened differentiation signal. Decreased ERK activity as a result of heterozygosity of the *rl* locus (genotype *rl^{10A}/+*) is phenotypically neutral with no detectable photoreceptor defects (Fig. 9A). Evidently, the system is flexible enough to accommodate moderate changes in signaling activity. However, if this genotype, *rl^{10A}/+*, is combined with one copy of a mutant *jun* allele or a *jun* deficiency (*jun⁻/+*), we observe nonallelic noncomplementation and a strong phenotype (Fig. 9A–C). Now in most of the ommatidia one or more photoreceptors are missing. We conclude that upon reduction of Rl activity, downstream components are activated less strongly, and loss of even one *jun* copy can

Figure 7. Enhancement of the dominant-negative, *sE-jun^{Ala}*, and gain-of-function, *sE-jun^{Asp}*, Jun phenotypes by gene dose reduction in *jun*. (A–D) Scanning electron micrographs of adult eyes, and (E–H) sections through such eyes. Anterior is left and dorsal up. (A,E) *sE-jun^{Ala}/+*; (B,F) *sE-jun^{Ala}/jun²*; (C,G) *sE-jun^{Asp}/+*; (D,H) *sE-jun^{Asp}/jun²*. The dominant-negative *sE-jun^{Ala}* transgenic line used (no. 1) shows a weaker effect in wild-type than the line previously published (Treier et al. 1995): only a few ommatidia have lost one photoreceptor (an example is indicated by arrow) and some even contain an additional outer photoreceptor (arrowheads). Note that in the *sE-jun^{Ala}/jun²* genotype the number of ommatidia with missing photoreceptors is dramatically enhanced (examples indicated by arrows), and the presence of extra outer photoreceptors has disappeared as compared to *sE-jun^{Ala}/+*. The gain-of-function *sE-jun^{Asp}* phenotype (presence of additional R7 photoreceptors, example shown by arrow) and the general disorder of the eye are both enhanced by gene dose reduction in *jun* (*sE-jun^{Asp}/jun²*). Note that several ommatidia contain multiple extra R7 cells in H (examples shown by arrows). Both *jun¹* and *jun²* alleles have the same effect.

Figure 8. *jun* mutations dominantly suppress activated components of the RTK/Ras/Rl (ERK) pathway. (A–F) Eye phenotypes of activated components of Sev/Ras/ERK signaling in the presence and absence of *jun* mutants. (A,B,D,F) Sections through eyes of the respective genotypes; (C,E) the statistical analysis. (A–C) Gain-of-function Sev is suppressed by gene dose reduction in *jun*; (A) *sev^{S11}*; (B) *sev^{S11}; jun¹/+*; (C) corresponding statistical analysis, percentage of wild-type ommatidia are shown (for each genotype at least three independent eyes and >300 ommatidia were analyzed). (D–F) The gain-of-function allele of *rl/ERK*. *Sem* is suppressed by gene dose reduction in *jun*; (D) *rl^{Sem}*; (E) *rl^{Sem}; jun¹/+*; (F) corresponding statistical analysis (as in C). Note that both *jun* alleles have the same effect. Examples of wild-type ommatidia are marked with arrows. (G,H) The suppression effect of the wing phenotype of the gain-of-function allele of the *Drosophila* EGFR (*Elp*) by gene dose reduction in *jun*. (G) *Elp^{B1}/+* (note the presence of extra wing vein tissue, marked by arrow); (H) the wing has an almost wild-type appearance [note absence of extra vein tissue at arrow]. In 88% of the *Elp^{B1}/+* wings significant extra L2 vein tissue was visible. This number drops to 22% when one copy of *jun* is mutated (*Elp^{B1}/jun²*).

no longer be compensated by redundant factors anymore. The unusually strong phenotypic interaction of two heterozygous mutations unmasks the role of signal transduction from Rl to Jun in photoreceptor differentiation. The dominant interaction between Rl and Jun is not a peculiarity of this combination of alleles. Similar conclusions can be drawn from the interaction of mutant *jun* alleles with weak mutations elsewhere in the path-

way. For example, in the *sev^{E4}; Sos^{IC2}/+* genotype, Sev-mediated signaling is reduced because of a mutation in *sev* that is only partially rescued by a gain-of-function mutation in the GDP/GTP exchange factor Son of sevenless (*Sos*) (Rogge et al. 1991). Under these conditions, activation of Ras is impaired, causing loss of R7 photoreceptors in a fraction of the ommatidia. This weak defect is significantly enhanced when the *jun* locus is heterozygous mutant (Fig. 9D–F).

The enhancement of the *sev^{E4}; Sos^{IC2}/+* phenotype and, particularly, the striking synthetic mutant phenotype of *jun/rl^{10a}* unmasks the otherwise redundant role of Jun in the determination of photoreceptor cell fate and documents the relevance of the Rl–Jun interaction in the process.

Discussion

The identification of the mutant *Drosophila jun* alleles described in this report has made it possible to dissect the signaling and regulatory functions of Jun in a developing organism. Consistent with a wealth of data obtained in tissue culture experiments, Jun acts pleiotropically and influences morphogenesis and cell differentia-

Figure 9. Mutations in *jun* are limiting components of photoreceptor induction when Sev/Ras/Rolled(ERK) signaling is reduced. All panels show eye sections of the respective genotypes, except F, which shows a statistical analysis. (A–C) A combination of *rl/+* and *jun/+* mutations reveals nonallelic noncomplementation between both genes [(B) *rl^{10a}/+, jun²/+*; (C) *rl^{10a}/+, Df(jun)/+*], whereas both single heterozygotes, *rl^{10a}/+* (A) and the *jun* alleles over wild type (not shown) are completely wild type. The flies of the genotypes shown in B and C have also some missing bristles (not shown). (D–F) The phenotype of a combination of hypomorphic *sev* and *Sos* alleles is enhanced by gene dose reduction in *jun*. (D) *sev^{E4}; Sos^{IC2}/+*. (E) *sev^{E4}; Sos^{IC2}/+, jun¹/+*. (F) statistical analysis of the respective genotypes (percentage of mutant wild-type ommatidia are shown; analysis as in Fig. 7).

tion in various developmental situations. Previous results that had implicated Jun in the determination and differentiation of photoreceptor cells in the developing eye were confirmed and extended. Moreover, we could demonstrate the role of Jun in the regulation of dorsal closure in the embryo and of wing morphogenesis during pupal stages.

In addition to providing new insight into the molecular basis of *Drosophila* development, these studies revealed interesting properties of Jun signaling in an intact organism: Jun can have both redundant and nonredundant functions during development, and it can act as a recipient of distinct signal transduction pathways.

Redundant and nonredundant functions of Jun

Jun is essential in embryonic development. Loss of Jun function in transgenic lines expressing dominant-negative mutants, or in animals that are homozygous for mutant *jun* alleles, causes lethality with defects in dorsal closure and in head involution. The demonstration that *jun* is essential for dorsal closure, even though not unexpected, based on suggestive biochemical evidence (Riesgo-Escovar et al. 1996; Sluss et al. 1996), is not a trivial result. In spite of its name, JNK has been shown in mammalian systems to phosphorylate a number of transcription factors besides Jun, and the JNK–Jun signaling connection has never before been implicated in the control of developmental processes or cell shape changes.

In contrast to the situation in the embryo, removal of Jun is much less disruptive in eye development, at least under otherwise wild-type conditions. In *jun*-deficient eye tissue, ommatidial assembly and photoreceptor differentiation are almost normal and only infrequent mild defects can be observed. However, when the Ras/ERK signaling pathway, which instructs the pluripotent imaginal disc cells to assume a neuronal fate, is disturbed, Jun function becomes critical for this process. Strikingly, a reduction of *jun* gene dose by 50% causes defects in most ommatidia in heterozygous ERK null mutants. Such nonallelic noncomplementation is observed only rarely in *Drosophila* and usually only between two genes acting in the same pathway, for example, some *dsh*^{+/+}; *wg*^{+/+} double heterozygotes display defects in the distal wing blade (Theisen et al. 1994). These results, together with the finding that mutant *jun* alleles give rise to significant loss of photoreceptor differentiation in sensitized genetic backgrounds, as well as biochemical evidence (Peverali et al. 1996), clearly document a function of Jun in the Ras/ERK signaling pathway that specifies photoreceptor cell fate (Fig. 10). This function appears however to be redundant in wild type flies.

Redundant functions of Jun are not unprecedented. In the mouse, null mutations of *c-jun*, the mammalian homolog of *Drosophila jun*, have an unexpectedly mild phenotype and cause death only late in embryogenesis (Hilberg et al. 1993; Johnson et al. 1993). It was concluded that some of the functions of Jun in the mouse are redundant, and lack of c-Jun can be compensated by

Figure 10. Model for Jun function in dorsal closure and eye development. During eye development activation of Jun by Rl-catalyzed phosphorylation can initiate photoreceptor differentiation, presumably through the regulation of appropriate target genes. In this process Jun cooperates with Pointed and this is antagonized by Yan. Jun function is redundant and can be substituted by another factor (marked X). The identity of this factor and its relationship to Pointed and Yan is not presently known. In dorsal closure Jun is regulated by the components of the *Drosophila* JNK pathway and activated by Basket-mediated phosphorylation. In this situation Jun is essential and no redundant function can be observed. Dominant-negative forms of Jun such as Jun^{bZIP} can interfere with target gene expression and thus disrupt photoreceptor differentiation and dorsal closure.

other gene products. Genetic redundancy is a phenomenon that is widespread in biology and has been found in eukaryotic organisms from yeast to mammals. The significance, the biological function, and the reason for the evolutionary stability of apparently redundant groups of genes are much debated topics. Our findings suggest that the redundant role of *jun* in photoreceptor differentiation serves to make eye development a precise and reliable process that is not easily disrupted. In contrast to many sensitized mutant phenotypes, the development of a wild-type eye is fairly insensitive to genetic or environmental disturbances, such as heterozygosity of important regulatory genes or temperature fluctuations. We

suggest that this robust fidelity of wild-type development is ensured by the action of several factors that have the same or very similar regulatory function and thus back each other up. Our results support this model as they show that Jun function becomes important when disturbances in the normal determination and differentiation program (such as a reduction in ERK activity because of a loss of one allele) need to be compensated (Fig. 10).

The question of which factors act redundantly with Jun cannot be answered based on the available results. It can be assumed that other ERK-responsive transcription factors could fulfill this function. Obvious candidates include Pointed P2 and Yan, which have been shown to be regulated by R1 phosphorylation and can cooperate with Jun in photoreceptor determination (Brunner et al. 1994b; O'Neill et al. 1994; Rebay and Rubin 1995; Treier et al. 1995) (Fig. 10). A more tentative possibility is *Drosophila* Fos, which can bind to DNA independently of Jun (Perkins et al. 1988) and is expressed in the developing eye (J. Zeitlinger, unpubl.).

Drosophila Jun is a target for both JNK and ERK signal transduction

There has been a certain controversy of whether Jun is regulated by ERK or by JNK (Minden et al. 1994). The results presented here, together with previously published biochemical data, indicate that Jun can be regulated by both types of MAPK signal transduction pathways. During eye development, and probably in the wing, Jun is phosphorylated by the ERK-type MAPK R1, whereas in dorsal closure Jun is a target for the JNK-type enzyme Bsk (Fig. 10).

Thus, Jun acts as a convergence point for several signals and signal transduction pathways. Similar multiple responsiveness has also been reported in the case of other inducible transcription factors such as CREB or Elk. In tissue culture these factors are targets of more than one signal transduction pathway, and multiple kinases can phosphorylate them to modulate their activity (Dash et al. 1991; Lalli and Sassone-Corsi 1994; Gille et al. 1995; Whitmarsh et al. 1995; Treier and Bohmann 1996). A number of interesting questions arise from the concept of several signal transduction pathways converging on a single transcription factor: How is the specificity of different signal responses determined if they converge on the same "bottleneck", for example, Jun phosphorylation? Do Bsk and R1 have equivalent biochemical and regulatory effects? Can the two pathways act in the same cells, and if so, do they cooperate? In this regard it is worth pointing out that in light of current data it is not clear whether Bsk has a function in eye development. *bsk*⁻ clones, like *jun*⁻ clones, show essentially no mutant phenotype (Riesgo-Escovar et al. 1996), but it is conceivable that Bsk, which is expressed in the eye (Sluss et al. 1996), has a redundant function similar to that of Jun. These and related questions can now be addressed.

To better understand the role of Jun in *Drosophila* development, more has to be learned about the upstream

regulators and downstream targets of the transcription factor. Experiments with dominant-negative mutants point to Cdc42 and Rac-related proteins as potential upstream activators of the Hep/Bsk cascade in dorsal closure (Harden et al. 1995; Riesgo-Escovar et al. 1996). It will be important to determine how these proteins fit into the pathway, whether Jun activation by Rac or Cdc42 has a more general function in *Drosophila* development, and how these small G-proteins are themselves activated.

At this time we can only speculate about downstream targets of Jun. In dorsal closure the gene *puckered* is expressed with a pattern and a responsiveness that is consistent with it being a target gene of Jun (Ring and Martinez-Arias 1993; Glise et al. 1995). Another group of mutations affecting dorsal closure belong to the Decapentaplegic (Dpp) pathway. Certain alleles of *dpp*, as well as *punt* and *thick veins* (*tkv*) mutants cause a dorsal open phenotype (Letsou et al. 1995; Ruberte et al. 1995). It will be interesting to investigate whether there is a connection between these genes and the Bsk/Jun signaling pathway.

Materials and methods

Immunohistochemistry

Antibody staining was carried out according to standard methods using 0.1% Tween. The affinity-purified antibody against Jun (Bohmann et al. 1994; Peverali et al. 1996) was diluted 1:4000 and preincubated (Fig. 1) and 1:2000 for *jun* and *bsk* mutant embryos. The anti-Coracle antibody (Fehon et al. 1994) was used 1:5000. To identify homozygous mutant embryos, *jun* alleles were balanced over a *wg-lacZ* CyO marked balancer, and embryos were costained with a commercial anti- β -galactosidase antibody (Cappel). Coracle staining was detected using the European Molecular Biology Laboratory (EMBL) confocal microscope.

Genetics

The deficiency *Df(2R)E73* was obtained by mobilizing *P[ry⁺]/l(2)07103/CyO* by crossing with *Sp/CyO;Sb, Δ 2-3/TM6,Ubx* and subsequent F₂ screening for *ry* offspring which should include imprecise excisions of the P element. Established stocks were analyzed for genomic lesions by Southern blot and polytene chromosome in situ hybridization using genomic probes of the *jun* locus.

About 1000 isogenized *al*, *dp*, *bw*, *sp*-marked males were mutagenized by feeding with 1% sucrose 25 mM EMS solution for 8 hr. Chromosomes were extracted by mating to a CyO balancer stock. Male F₁ progeny (13183) were individually crossed to *Df(2R)E73/CyO* females. F₂ offspring were scored for non-complementation, and stocks of these fly lines were established. All obtained lethals fall into three complementation groups. One of them was complementary to *Drosophila* 14-3-3 ξ mutants. The *jun*¹ allele was cloned by PCR and sequenced by the EMBL sequencing facility.

Drosophila strains

The activated *jun* allele *sE-jun^{Asp}* is described in Treier et al. (1995). Dominant-negative *sE-jun^{Ala}* stocks were reestablished. The UAS-*jun^{bZIP}* fly strain was obtained by cloning the basic

region and leucine zipper (amino acids 183–289) of *Drosophila* Jun into the P-element transformation vector pUAST (Brand and Perrimon 1993), followed by germ-line transformation using standard methods (Spradling and Rubin 1982). The Gal4 drivers used in this study are described in Brand and Perrimon (1993). The following fly strains of sensitized or activated alleles of the receptor tyrosine kinase signaling pathway have been used: *sev*^{S11} (Basler et al. 1991); *rl*^{sem} (Brunner et al. 1994a); *se-ras*^{V12} (Fortini et al. 1992); *Elp*^{B1} (Baker and Rubin 1992); *rl*^{10a} and *sev*^{E4}; *sos*^{IC2} (Rogge et al. 1991; Biggs et al. 1994). The isogenized *al*, *dp*, *bw*, *sp* was a gift from Cristiana Molinari (EMBL) and Slava Bolshakov (EMBL).

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