Cell, Vol. 83, 753–760, December 1, 1995, Copyright © 1995 by Cell Press

JUN Cooperates with the ETS Domain Protein Pointed to Induce Photoreceptor R7 Fate in the Drosophila Eye

Mathias Treier,* Dirk Bohmann, and Marek Mlodzik
Differentiation Programme
European Molecular Biology Laboratory
Meyerhofstrasse 1
D-69117 Heidelberg
Federal Republic of Germany

Summary

R7 photoreceptor fate in the Drosophila eye is induced by the activation of the Sevenless receptor tyrosine kinase and the RAS/MAP kinase signal transduction pathway. We show that expression of a constitutively activated JUN isoform in ommatidial precursor cells is sufficient to induce R7 fate independent of upstream signals normally required for photoreceptor determination. We present evidence that JUN interacts with the ETS domain protein Pointed to promote R7 formation. This interaction is cooperative when both proteins are targeted to the same promoter and is antagonized by another ETS domain protein, YAN, a negative regulator of R7 development. Furthermore, *phyllopod*, a putative transcriptional target of RAS pathway activation during R7 induction, behaves as a suppressor of activated JUN. Taken together, these data suggest that JUN and Pointed act on common target genes to promote neuronal differentiation in the Drosophila eye, and that *phyllopod* might be such a common target.

Introduction

Intercellular signaling mechanisms regulate many developmental processes, including cell proliferation, specification of cell fate, and differentiation. One commonly used signaling pathway is the ligand-induced activation of receptor tyrosine kinases (RTKs) and their downstream signaling cascade, including RAS and RAF, leading to the activation of a mitogen-activated protein kinase (MAPK-type enzymes) by phosphorylation and its subsequent translocation to the nucleus. Ultimately, the MAPK then activates (or inactivates) its nuclear targets by phosphorylation on serine or threonine residues (reviewed by Schlessinger, 1993; Marshall, 1994, 1995). It is thought that the combination of available nuclear target proteins determines the particular response that can be induced by RAS activation in a specific cell (reviewed by Hill and Treisman, 1995).

Induction of photoreceptor R7 during Drosophila eye development provides a powerful system to study the RTK/RAS pathway in vivo (Dickson and Hafen, 1993; Zipursky and Rubin, 1994; Dickson, 1995). Activation of the Sevenless (SEV) RTK and consequently the RAS/MAPK pathway triggers the differentiation of precursor cells as R7 photoreceptor neurons. Normally this event is restricted to a single precursor cell in each ommatidium (reviewed by Dickson and Hafen, 1993; Zipursky and Rubin, 1994). Nevertheless, several cells within an ommatidium have the potential to develop as R7 photoreceptors. These include the precursors for R7, the four cone cells, and the mystery cells, which together form the so-called R7 equivalence group (Dickson et al., 1992a). All these cells express the SEV receptor tyrosine kinase, and activation of the SEV/RAS pathway in these cells is sufficient to induce their development as R7 (Basler et al., 1991; Dickson et al., 1992a; Fortini et al., 1992). Moreover, the gain-of-function allele of the Drosophila MAPK encoded by the *rolled* gene (*rl*) $rl^{Sevenmaker}$, also causes a transformation of all cells of the equivalence group to the R7 fate (Brunner et al., 1994b).

Three nuclear targets of the SEV pathway have been implicated in R7 induction. The ETS (for E26-specific) domain protein isoforms encoded by *pointed* (*pnt*) and Drosophila JUN act as positive regulators of R7 cell fate (Bohmann et al., 1994; Brunner et al., 1994a; O'Neill et al., 1994), and YAN, also an ETS domain protein, acts as a negative regulator (Brunner et al., 1994a; O'Neill et al., 1994; Rebay and Rubin, 1995). Interestingly, JUN is expressed in all cells of the R7 equivalence group at the time when they are responsive to RAS activation (Bohmann et al., 1994). Similarly, PNTP2, one of the two protein isoforms encoded by *pnt* (Klämbt, 1993; Brunner et al., 1994a), is also present in most (if not all) cells of the equivalence group and is phosphorylated by Rolled/MAPK (Brunner et al., 1994a; O'Neill et al., 1994). In addition, *phyllopod* (*phyl*) mutants have been isolated as suppressors of constitutively activated RAS or RAF in the R7 equivalence group. *phyl* has been shown to be required for R7 development and to act as a transcriptional target of RAS pathway activation (Chang et al., 1995; Dickson et al., 1995). Overexpression of PHYL in the eye imaginal disc from the *sev* enhancer appears to be sufficient to induce the transformation of the nonneuronal cone cells to R7 photoreceptors, suggesting that *phyl* is an effector of RAS activation in this context. However, it is unclear whether one of the known nuclear targets of RAS/MAPK directly regulates *phyl* transcription.

Biochemical experiments and tissue culture expression studies of the proto-oncogene c-*jun* have shown that RAS/MAPK-mediated phosphorylation activates its transcriptional transactivation potential (Binetruy et al., 1991; Pulverer et al., 1991). Drosophila JUN and human c-JUN have very similar biochemical properties (Perkins et al., 1990; Zhang et al., 1990; data not shown). Moreover, a detailed mapping and characterization of phosphorylation sites implicated in the activation of c-JUN by MAP-type kinases has been performed (Pulverer et al., 1991; Smeal et al., 1991; Papavassiliou et al., 1995). Several point mutants

*Present address: Howard Hughes Medical Institute, University of California, San Diego, La Jolla, California 92093-0648.

were constructed, with the aim of generating a constitutively active form of c-JUN that could mimic the RAS-mediated phosphorylation and activation of JUN. Replacing six serine or threonine residues with phosphate-mimicking aspartic acid residues produced a protein, henceforth referred to as JUN^{Asp}, that behaved in several assays like the activated phosphoform of JUN (Papavassiliou et al., 1995; A. M. Musti and D. B., unpublished data). Conversely, mutating the same residues to alanine (JUN^{Ala}) created an inactive protein that proved impervious to activation by phosphorylation.

We have shown previously that dominant negative forms of JUN are capable of suppressing RAS-induced R7 development (Bohmann et al., 1994). Here we took advantage of the existing constitutively activated form of c-JUN (JUN^{Asp}; Papavassiliou et al., 1995) to ask whether RAS-mediated activation of JUN is sufficient for R7 induction. We find that the JUN^{Asp} protein, expressed under the control of the *sev* enhancer, can transform the normally nonneuronal cone cells to functional R7 photoreceptor neurons. Furthermore, we provide evidence from genetic interaction experiments and tissue culture studies that JUN can interact with the ETS domain protein PNT in a cooperative manner and that this effect is antagonized by the negatively acting ETS domain protein YAN. In addition, we show that *phyl* acts as a dominant suppressor of JUN^{Asp}, which is consistent with the model that *phyl* is transcriptionally activated by JUN, possibly through its interaction with PNT.

Results

Mimicking RAS Activation of JUN in Cells of the R7 Equivalence Group Is Sufficient to Induce R7 Fate

Drosophila JUN is required for the development of R7 and probably also outer photoreceptors R1–R6. Since JUN appears necessary for photoreceptor induction and is normally activated by RAS-induced phosphorylation (F. Peverali, M. M., and D. B., unpublished data), we asked whether its activated form is sufficient to induce R7 cells in analogy to constitutively activated SEV and RAS proteins. To this end, the mutant form of c-JUN, JUN^{Asp}, was expressed under the control of the eye-specific *sev* enhancer (henceforth called *sE–jun^{Asp}*) during Drosophila eye development in cells of the R7 equivalence group. JUN^{Asp} mimics the RAS/MAPK-dependent phosphorylation of JUN and behaves like the active phosphoform of the protein (Papavassiliou et al., 1995; see also Experimental Procedures). Strikingly, in *sE–jun^{Asp}* flies the external eye surface is irregular, and ommatidia often contain additional R7-like photoreceptors, as judged by their morphology with small internally localized rhabdomeres (Figures 1B and 1E).

In control experiments, wild-type c-JUN and the JUN^{Ala} mutant, which is impervious to RAS-dependent phosphorylation, since all MAPK target serine and threonine residues have been mutated to alanine residues, were overexpressed in the same cells (*sE–jun^{wt}* and *sE–jun^{Ala}*). In contrast with *sE–jun^{Asp}*, *sE–jun^{wt}* causes a phenotype that is indistinguishable from the parental wild-type flies, with the regular ommatidial appearance and the correct number of six outer photoreceptors and one R7 cell (Figures 1A and 1D). On the other hand, *sE–jun^{Ala}*, like its *sE–jun^{Asp}* counterpart, causes rough, irregular eyes. Contrary to the phenotype elicited by activated JUN^{Asp}, however, R7 cells and some outer photoreceptors are often missing, consistent with a role for JUN^{Ala} as a dominant negative mutant (Figures 1C and 1F). We conclude that the development of the ectopic photoreceptors is specifically induced by the constitutively activated JUN^{Asp} protein.

JUN^{Asp}-Expressing Cone Cells Display Neuronal and R7-Specific Markers

To confirm the identity and to determine the origin of the ectopic R7-like photoreceptors, we analyzed ommatidial assembly in eye imaginal discs of *sE–jun^{Asp}* flies by using neuronal and R7-specific markers (Figure 2). ELAV (encoded by *embryonic lethal, abnormal vision*) is a nuclear antigen expressed in all cells that have initiated neural differentiation. In wild-type ommatidia, ELAV is only detected in differentiating photoreceptors. In the *sE–jun^{Asp}* genotype, however, several of the cone cell precursors (marked with the letter c in Figures 2A and 2B) have initiated neural differentiation in addition to the endogenous photoreceptor precursors. Thus, the ectopic R7-like cells originate from cone cell precursors, as judged from the expression of the neural marker ELAV (Figures 2A and 2B) and BP104 (data not shown).

To examine the identity of these ectopic R cells, we used the R7-specific marker H214, which in wild-type eye discs is only expressed at high levels in the endogenous R7 precursor (Figure 2C). In *sE–jun^{Asp}* eye imaginal discs, additional cells, the cone cell precursors, express H214, demonstrating that these cells have been induced to differentiate as ectopic R7 neurons (Figure 2D). Taken together, the cone cell precursors express neuronal and R7-specific markers in a temporal manner comparable to that of wild-type R7 cells (Figure 2), and therefore we conclude that they are transformed to additional R7 photoreceptors in *sE–jun^{Asp}* eyes. Moreover, since in *sE–jun^{Asp}* flies JUN^{Asp} is expressed in cone cell precursors (data not shown), this is consistent with these cells being cell-autonomously transformed to the neuronal fate by the activated JUN isoform.

JUN^{Asp} Induces Functional R7 Cells

The above experiments have established that JUN^{Asp} can transform the nonneuronal cone cell precursors to neuronal R7 cells, as judged by morphological criteria and the expression of R7 markers. Two remaining important questions are, first, whether the induction of R7 cells by JUN^{Asp} depends on upstream signals, and second, whether JUN^{Asp}-induced R7 cells are completely functional R7 photoreceptors. In a *sev^{-}* background, normally no R7 cells develop. Thus, in the *sev^{d2}* (a null allele of *sev*); *sE–jun^{Asp}* double mutant background, all R7 photoreceptors, if present, must have been induced, independent

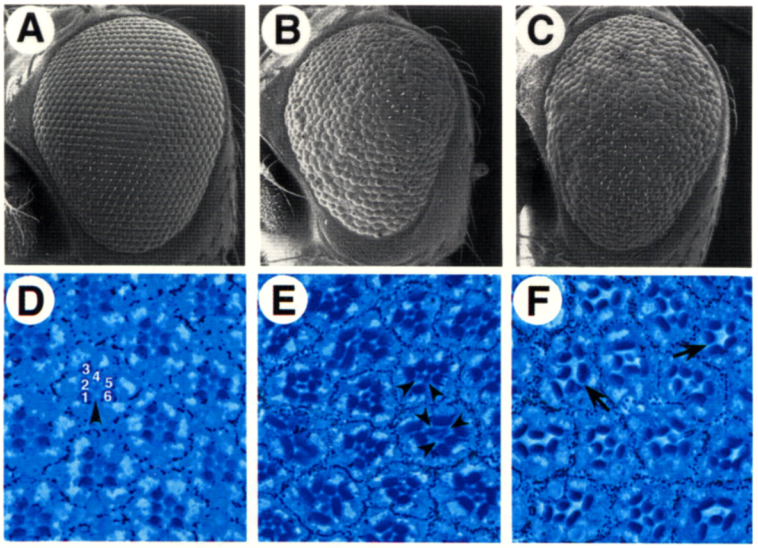

Figure 1. Eye Phenotypes Caused by Expression of Wild-Type, Gain-of-Function, and Dominant Negative Isoforms of JUN from the *sev* Enhancer in an Otherwise Wild-Type Background

Scanning electron micrographs (A–C) and tangential histological sections through eyes (D–F) of the following genotypes are shown.
(A and D) *sE–jun*^{wt}. Note wild-type appearance with the regular ommatidial array and correct number of six outer photoreceptors and one R7. Outer R cells are numbered, and R7 is indicated with an arrowhead in one ommatidium.
(B and E) *sE–jun*^{Asp}. Many ommatidia contain multiple R7 photoreceptors (indicated with arrowheads in two examples).
(C and F) *sE–jun*^{Ala}. The external eye surface is also irregular, but ommatidia often lack R7 cells and also some outer photoreceptors (two examples are indicated by arrows). In one out of seven independent transformants of *sE–jun*^{wt}, some ommatidia with seven instead of six outer photoreceptors in addition to R7 were found. This might reflect the weak photoreceptor-inducing activity of wild-type JUN when overexpressed by the *sev* enhancer in the mystery cells that can develop as outer R cells in some genetic backgrounds.

of normal signaling, by JUN^{Asp}, since there is no SEV protein, and thus also no activation of downstream effectors by SEV.

The most reliable assay for functional R7 cells is the assay for phototactic behavior. Wild-type adult flies are preferentially attracted by ultraviolet (UV) light when given a choice between UV and green light. As R7 photoreceptors are the only neurons that provide UV sensitivity, flies lacking (functional) R7 photoreceptors (e.g., *sev*$^{-}$ flies) are attracted preferentially by green light (Harris et al., 1976). The double mutant *sev*^{d2}; *sE–jun*^{Asp} flies are attracted to UV light, indicating that functional R7 cells form in this genetic background (Figure 3). Microscopic inspection of eyes of this genotype also shows the presence of R7 cells as judged by their morphology. About 50% of ommatidia contain one (or more) R7 photoreceptors (Figure 4B) that also express the R7-specific marker H214 (data not shown). Therefore, *sE–jun*^{Asp} is sufficient to induce functional R7 cells as determined by the correct phototactic UV-sensitive behavior and microscopic analysis, independently of upstream signals (see also Discussion).

PNT and YAN Affect the JUN^{Asp}-Mediated R7 Induction

The JUN^{Asp}-induced transformation of cone cell precursors to functional R7 photoreceptors is very similar to the transformation observed with constitutively activated components of the RAS/MAPK pathway (Basler et al., 1991; Dickson et al., 1992b; Fortini et al., 1992; Brunner et al., 1994b). Thus, our data demonstrate that the (artificial) activation of JUN is sufficient at least partially to mimic the effects of constitutively activated SEV or its downstream signaling cascade, and thus also recapitulates the nuclear events required for the induction of the R7 photoreceptor fate. This observation is in apparent contrast with the reported roles of the ETS domain proteins PNT and YAN as effectors of SEV signaling (Brunner et al., 1994a; O'Neill et al., 1994). In particular, inactivation of YAN appears to be a critical step in photoreceptor induction (Rebay and Rubin, 1995). To investigate their roles in the JUN^{Asp} context and to define the functional relationship between JUN, PNT, and YAN, we studied the genetic interactions between the constitutively activated *jun* and *pnt* or *yan*.

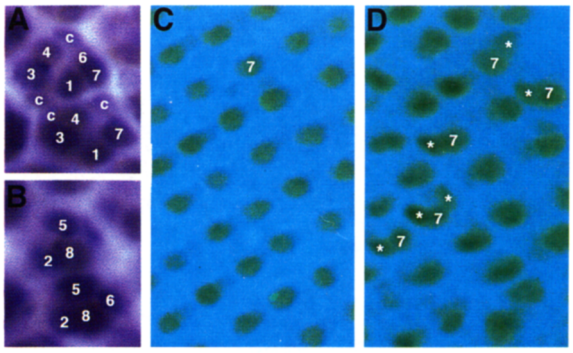

Figure 2. Expression of Neural and R7-Specific Markers in Eye Discs from *sE–jun*^{Asp} Flies

(A and B) ELAV expression in *sE–jun*^{Asp}/+ developing ommatidial clusters. Two representative clusters are shown at two different focal planes, R7 level in (A) and R8 level in (B). ELAV, a nuclear antigen, is normally expressed only in differentiating R cell precursors. Note that several of the cone cell precursors (marked with the letter c) have initiated neural differentiation in *sE–jun*^{Asp}. The endogenous R cell precursors are numbered, according to their identity, 1–8.
(C and D) Expression of the R7 marker H214 (Mlodzik et al., 1992). (C), wild-type eye imaginal disc; (D), *sE–jun*^{Asp}/+ disc. Note that H214 is only expressed at high levels in R7 precursors in wild type (an example is marked with 7) and that additional cells express H214 in *sE–jun*^{Asp} discs (a few examples are indicated with asterisks), demonstrating that they have been induced to differentiate as ectopic R7 cells.

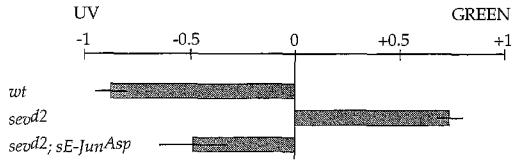

Figure 3. Color Choice Preference of Wild-Type, *sev*⁻, and *sev*⁻; *sE–jun*^{Asp} Flies

Flies were tested for light choice preference at 350 nm UV light and 550 nm green light. Wild-type flies (Oregon-R strain) with functional R7 photoreceptors are attracted by UV light, while *sev*⁻ flies do not see UV light and are thus attracted by green light. The *sev*⁻; *sE–jun*^{Asp} flies again prefer UV light over green light, proving that the *sE–jun*^{Asp}-induced R7 cells do not only have R7 morphology and express R7 markers (Figures 1 and 2; data not shown) but are fully functional R7 cells and make the correct connections in the medulla.

The phototactic value λ is calculated as follows: λ = N(green) − N(UV) / N(green) + N(UV). N(green) and N(UV) are the numbers of flies attracted by green light or UV light, respectively.

To this end, the *sE–jun*^{Asp} flies were combined with either the *yan*^{1}/+ or the *pnt* null allele, *pnt*$^{\Delta 88}$/+, genotype. Removal of one copy of the negative regulator *yan* (Lai and Rubin, 1992; Tei et al., 1992), which has to be inactivated by RAS-dependent phosphorylation during photoreceptor induction (Rebay and Rubin, 1995), enhances the *sE–jun*^{Asp} phenotype, leading to an increase in eye roughening and the number of ectopic R7 cells. This is apparent in both *sE–jun*^{Asp}, *yan*^{1}/+ (data not shown) and *sev*^{d2}; *sE–jun*^{Asp}, *yan*^{1}/+ flies (Figure 4C). The number of ommatidia with additional R7 cells and the average number of R7s per ommatidium are increased by the reduction of *yan* gene dosage. In contrast, a simple gene dosage reduction in *pnt* does not have a dominant effect on the *sE–jun*^{Asp} phenotype (data not shown). However, when *pnt* function is further reduced, the *sE–jun*^{Asp} phenotype is suppressed. Strikingly, even weak viable hypomorphic allele combinations of *pnt*, *pnt*^{1277}/*pnt*^{1230} and *pnt*^{1277}/*pnt*$^{\Delta 88}$, completely suppress the *sE–jun*^{Asp}-induced ectopic R7 cell development (Figures 4E and 4F; data not shown). Moreover, *sE–jun*^{Asp} is unable to rescue the eye phenotype of such *pnt* mutants, in which R7 and outer photoreceptors often fail to develop (Figures 4E and 4F). Therefore, the positive regulator PNT is still necessary in addition to activated JUN for R7 cell induction. However, in the presence of constitutively activated JUN^{Asp}, PNT does not appear to require SEV-mediated activation for R7 induction.

In addition to *pnt* and *yan*, we have tested the *sE–jun*^{Asp} genotype for dominant genetic interactions with many other mutations known to affect eye development and ommatidial assembly. Among these genes, we find only *phyl* as a modifier of the *sE–jun*^{Asp} phenotype. In *phyl*/+ heterozygous flies, the phenotype elicited by activated RAS and RAF is dominantly suppressed (Chang et al., 1995; Dickson et al., 1995). On the basis of its wild-type expression pattern and its transcriptional up-regulation in cone cell precursors in the activated RAS/RAF genetic backgrounds, *phyl* has been postulated to be a transcriptional target of RAS activation during photoreceptor induction (Chang et al., 1995; Dickson et al., 1995). In our assay, *phyl* is a strong suppressor of *sE–jun*^{Asp}; i.e., removal of one functional copy of *phyl* reverts the rough eye phenotype and the presence of ectopic R7 cells almost back to wild type (Figure 4D; data not shown). This result is consistent with the proposed idea that *phyl* is a transcriptional target of RAS activation in precursor cells and provides evidence that this activation is mediated (possibly directly) by JUN.

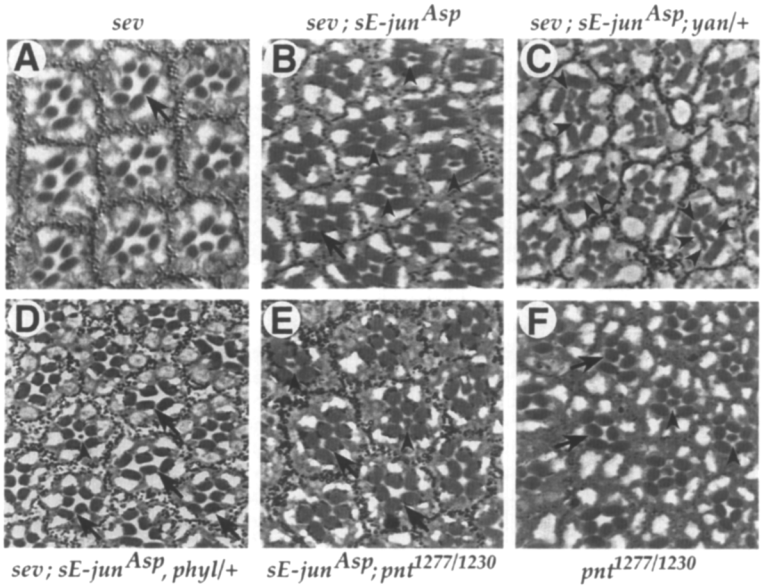

Figure 4. Eye Phenotypes of *sE–jun*^{Asp} in Different Genetic Backgrounds

Tangential eye sections at the level of R7 are shown. All flies carrying *sE–jun*^{Asp} contain one copy of the transgene.

(A) *sev*.

(B) *sev*⁻; *sE–jun*^{Asp}.

(C) *sev*⁻; *sE–jun*^{Asp}; *yan*^{1}/+.

(D) *sev*⁻; *sE–jun*^{Asp}, *phyl*^{3G6}/+.

(E) *sE–jun*^{Asp}; *pnt*^{1277}/*pnt*^{1230}.

(F) *pnt*^{1277}/*pnt*^{1230}. Analysis of another hypomorphic allelic combination of *pnt*, *pnt*^{1277}/*pnt*$^{\Delta 88}$, with *sE–jun*^{Asp} gives the same result: complete suppression of *sE–jun*^{Asp} induction of R7 cells. Examples of ommatidia with no R7 cells are indicated by arrows, and examples with one or multiple R7 cells are indicated by arrowheads. Note that *yan* and *phyl* mutants dominantly enhance or suppress the *sE–Jun*^{Asp} phenotype, respectively. This is evident in the *sev*⁻; *sE–jun*^{Asp} background (C, D) and also in *sev*⁺; *sE–jun*^{Asp} (data not shown), both externally and in tangential sections. All tested alleles of the respective genes show a very similar interaction with *sE–jun*^{Asp}. Note that even the weakest hypomorphic allelic *pnt* combination (E, F), previously found to have no mutant phenotype, completely suppresses *sE–jun*^{Asp}. Nevertheless, there are no dosage-sensitive interactions between *pnt* alleles and *sE–jun*^{Asp}.

JUN and PNT Show Cooperative Interactions When Targeted to the Same Promoter

The above genetic interactions suggest that the decision between the R7 photoreceptor and cone cell fate depends on the balance of JUN and PNT and possibly also YAN activities. Mechanistically, this would be most easily explained if PNT and JUN had common target genes, as is frequently found in the case of their vertebrate counterparts (e.g., Wasylyk et al., 1990; reviewed by Wasylyk et al., 1993). To test this idea, we have analyzed the interactions of JUN with the PNT isoforms in transient tissue culture transfection experiments. Since there are no Drosophila promoters available for these assays, we used reporter target promoters that carry single well-characterized composite AP-1/ETS promoter elements (e.g., the polyoma element) where the AP-1 and ETS binding sites are juxtaposed (Wasylyk et al., 1990; see also Experimental Procedures) upstream of a luciferase reporter gene. Undifferentiated F9 EC cells were used as recipients, because they reportedly have no endogenous JUN activity (Kryszke et al., 1987; Chiu et al., 1988).

When the reporter constructs are cotransfected with the different JUN protein isoforms alone, transcriptional activation is observed in the null background of the F9 cells. Activation by JUN^{Asp} is slightly higher and by JUN^{Ala} slightly lower than the effect of the wild-type JUN protein (Figure 5A; Discussion). Cotransfections of either PNTP1 or PNTP2 alone activate the same promoter element ~15-fold or 2- to 3-fold, respectively.

However, when both JUN and PNT proteins are simultaneously cotransfected, activation of the reporter plasmid is increased to 100- to 300-fold in a binding site–dependent manner, indicating a strong cooperative interaction between JUN and PNT (Figure 5A). In particular, JUN^{Asp} and PNTP1 cause an activation of over 300-fold, and JUN^{Asp} and PNTP2 lead to an over 100-fold activation of the common AP-1/ETS promoter element. Similar degrees of cooperativity are also observed when a different naturally occuring AP-1/ETS promoter element (e.g., as present in the *collagenase* gene) is used in these experiments (data not shown). Interestingly, JUN^{Asp} together with $PNTP2^{T151A}$ (referred to as $PNTP2^{Ala}$), the unphosphorylatable PNTP2 mutant (Brunner et al., 1994a; O'Neill et al., 1994), activates transcription also significantly stronger than JUN^{Asp} alone (Figure 5A). This result may explain why it is sufficient to activate JUN to induce R7 fate as long as PNT protein is present. Moreover, JUN^{Ala} with any PNT isoform also activates transcription of the common promoter element up to 100-fold (e.g., when combined with PNTP1), and thus significantly higher than either protein alone (Figure 5A). These observations might explain why in *yan*$^-$ mutants, ectopic R7 cells can form even when the RAS/MAPK pathway is not activated (Lai and Rubin, 1992; Tei et al., 1992) (see Discussion).

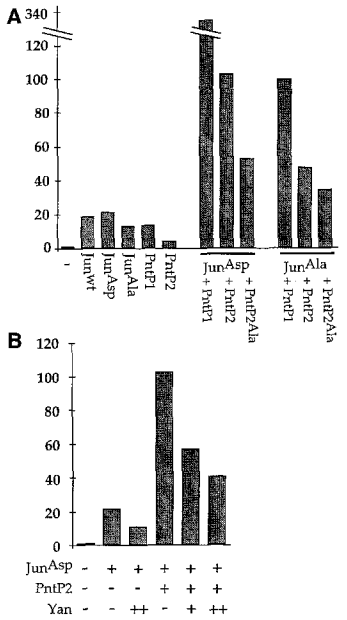

Figure 5. Interactions of PNT, YAN, and JUN on Common Promoter Elements

(A) Transcriptional activation of the Polyoma-Col-Luc reporter, containing a single AP-1/ETS promoter element, in response to the presence of the different JUN proteins with or without the addition of the different PNT isoforms. Note that all combinations of JUN and PNT isoforms cause a stronger transcriptional activation than either protein alone. The observation that even JUN^{Ala} activates transcription in this assay, although it behaves as a dominant negative mutant in vivo, is not surprising, since no endogenous JUN protein is present in F9 cells, and thus the JUN^{Ala} expression vector is the sole source of JUN protein in this assay. In wild-type eye imaginal discs, however, JUN^{Ala} is competing with activated endogenous JUN and causes a reduction in JUN activity and the observed phenotypic effect (Figures 1C and 1F).

(B) Activation of the same Polyoma-Col-Luc reporter when JUN or JUN and PNT are also cotransfected with the putative inhibitor YAN, as indicated by plus signs. All transfected plasmids were at 2 μg/plate, except YAN, where plus indicates 1 μg/plate and double plus indicates 2 μg/plate. Note increased inhibitory effect on reporter transcription by increased concentration of YAN-expressing plasmid.

In both (A) and (B), the relative luciferase activity (see Experimental Procedures) is shown in response to cotransfections of the respective proteins as indicated. F9 cells were transfected with 4 μg/plate of a Polyoma-Col-Luc reporter and RSV expression plasmids containing JUN, PNT, and YAN coding sequences or combinations thereof (2 μg/plate in [A] and [B], except for pRSV-yan [see legend to (B)]). Luciferase activity was determined 12–16 hr later. The results are the average of three independent experiments, each done in triplicate. Very similar results were also obtained with an AP-1/ETS promoter element from the *collagenase* gene (data not shown). The observed cooperativity between JUN and the PNT isoforms is binding site–dependent, as determined in experiments with mutant Polyoma-Col-LUC reporter (data not shown).

YAN Antagonizes the JUN-PNT-Mediated Transcriptional Activation

The *yan* gene acts as a negative regulator of R7 induction and encodes (like *pnt*) an ETS domain protein (Lai and Rubin, 1992; Tei et al., 1992). YAN has shown that it can act as a transcriptional repressor, and its down-regulation is critical for photoreceptor induction (O'Neill et al., 1994; Rebay and Rubin, 1995). Our results show that *yan* antagonizes the *sE–jun*^{Asp} effect in vivo (see Figure 4C). To test whether YAN can directly inhibit JUN-mediated transcriptional activation, we tested its effect in the above cotransfection assay. By use of the same AP-1/ETS promoter element as reporter construct (see Experimental Procedures), YAN was cotransfected either with JUN or together with both JUN and PNT, and transcriptional activation of

the luciferase reporter gene was assayed. In all combinations analyzed, YAN inhibited the activation of the reporter construct (Figure 5B). Significantly, increased concentration of the YAN expression plasmid caused a stronger inhibition of the JUN–PNT-mediated transcriptional activation. We conclude that YAN inhibits the transcriptional activation of JUN and PNT when targeted to the same promoter element.

In summary, the tissue culture data corroborate the genetic interactions among JUN, PNT, and YAN in vivo and support the hypothesis that they can act on common target gene promoters.

Discussion

We have shown that mimicking RAS-mediated activation of JUN by mutating the respective serine and threonine residues to aspartic acid (JUN^{Asp}) is sufficient to induce R7 photoreceptors during Drosophila eye development and to effect partial rescue of the *sev*⁻ eye phenotype, which normally lacks any R7 cells. In contrast, a JUN mutant where the same serine and threonine residues are mutated to alanine, which precludes RAS/MAPK-mediated phosphorylation, behaves as the product of a dominant negative mutant allele and blocks R7 formation. Even though JUN^{Ala} can activate transcription to a measurable base level, as detected in F9 cells where no endogenous JUN is present, in the context of R7 induction where it competes with endogenous activated JUN, it presumably causes a net reduction of JUN activity, which often results in the failure of R7 differentiation. The ability of the constitutively activated JUN^{Asp} isoform to trigger R7 differentiation independent of SEV is remarkable. At least one other gene, the ETS domain containing *pnt*, has been shown to act as a phosphorylation target and a positive nuclear effector of RAS/MAPK signaling in photoreceptor induction (Brunner et al., 1994a; O'Neill et al., 1994). In addition, it has been demonstrated that the negative regulator YAN, also an ETS domain protein, has to be inactivated by RAS/MAPK-mediated phosphorylation to allow R cell development (Rebay and Rubin, 1995). Nevertheless, our analyses of genetic interactions between *sE–jun*^{Asp} and these other nuclear components in vivo indicate that PNT is still required for photoreceptor induction, and YAN retains an inhibitory influence. On the basis of these data, and in correlation with the results from the cotransfection experiments, we propose a model for the nuclear events and regulation of target genes in response to RAS activation during photoreceptor induction as shown in Figure 6 and discussed below.

Do JUN and PNT Have Common Target Genes?

The presented model is based on the assumption that at least some genes, which are transcriptionally activated following RAS/MAPK induction, might be common target genes of both JUN and PNT. To date there has been only one putative photoreceptor-specific transcriptional target of the RAS/MAPK pathway described, which is *phyl*. It has been identified as a dominant suppressor of activated RAS and RAF in eye development (Chang et al., 1995; Dickson et al., 1995). Transcription of *phyl* is restricted to R7 and R1–R6, where it is also required. However, in *yan* mutants, in which cone cells develop as R7 photoreceptors, *phyl* expression is also detected in cone cell precursors, suggesting that YAN might repress *phyl* transcription in cells of the R7 equivalence group (Chang et al., 1995; Dickson et al., 1995). It is possible that this is a direct effect mediated by ETS binding site(s) in the so far uncharacterized *phyl* promoter region. Assuming that YAN and PNT can compete for the same binding sites, which is supported by genetic experiments (Brunner et al., 1994a) and tissue culture cotransfections (O'Neill et al., 1994; Figure 5B), *phyl* transcription might also be activated via *pnt*. Could *phyl* also be regulated by JUN? Our genetic experiments support this notion, since removing one gene dose of *phyl* strongly suppresses the *sE–jun*^{Asp}-induced cone cell transformation phenotype. Taken together, these data suggest that *phyl* might be a common target of both JUN and PNT. It is possible that other similarly regulated genes await identification.

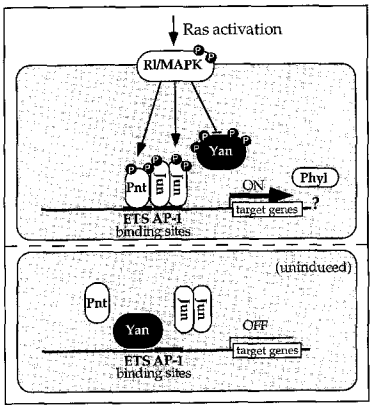

Figure 6. Model for the Action of JUN, PNT, and YAN in Ommatidial Precursor Cells Following RAS/MAPK Induction or Uninduced

Upon RAS activation (top), MAPK encoded by the *rolled* gene is phosphorylated via the RAF/DSOR (MEK or MAPKK) kinase cascade and translocates to the nucleus, where it phosphorylates JUN, PNTP2, and YAN. These phosphorylation events lead to the inactivation of the inhibitor YAN and simultaneously to the activation of the positive regulators JUN and PNTP2. This results in the transcriptional activation of target genes containing an AP-1/ETS promoter element.

In the uninduced state (bottom), the inhibitor YAN is active and causes repression of target gene expression (Rebay and Rubin, 1995; see also Discussion). Of the PNT isoforms, only PNTP2 has a MAPK phosphorylation site; PNTP1 is thought to be a constitutive transcriptional activator. Since the PNTP1 isoform contains its own promoter in vivo, it is possible that PNTP1 is transcriptionally activated in response to PNTP2 activation, as proposed by O'Neill et al. (1994).

The Sum of the Activities of JUN, PNT, and YAN Determines Whether a Precursor Develops as an R7 Photoreceptor

The complete suppression of the *sE–jun*^{Asp} phenotype by weak heteroallelic combinations of *pnt* combined with the tissue culture transcriptional activation data strongly suggests that JUN and PNT interact cooperatively to induce

photoreceptor fate. A strong cooperativity is observed between JUN and either PNTP1 or PNTP2 when targeted to the same promoter. The JUN–PNTP1 pair appears stronger than JUN–PNTP2, which is consistent with the observation that PNTP1 is a strong constitutive transcriptional activator, while PNTP2 activity depends on phosphorylation of its Thr-151 residue (O'Neill et al., 1994). Nevertheless, it is striking that all tested combinations of JUN and PNT isoforms, including unphosphorylatable mutants, display cooperativity. Interestingly, also the JUN^{Ala}–$PNTP2^{Ala}$ combination, in which neither protein can be phosphorylated upon RAS/MAPK activation, still activates the reporter construct significantly more strongly than any isoform of JUN, PNTP1, or PNTP2 alone (at least in the absence of wild-type JUN, e.g., as in F9 cells).

In this context, it is worth noting that in *yan* mutant flies, ectopic R7 photoreceptors develop, even in the absence of the *sev*-mediated RAS/MAPK activation (Lai and Rubin, 1992). Several R7 cells develop in each ommatidium in this genetic background. The cooperative interaction between JUN and PNT, however, even in their unphosphorylated forms, as observed in the cotransfection experiments, might provide an explanation why R7 cells can be induced in the absence of RAS pathway activation when also the inhibiting YAN protein is absent. Similarly, in the *sE–jun*^{Asp} background, JUN is constitutively activated and thus can (at least partially) override the presence of the inhibitor YAN, which normally needs to be inactivated during photoreceptor induction (Rebay and Rubin, 1995). In accordance, reduction of *yan* gene dosage has an enhancing effect on the JUN^{Asp}-induced phenotype. Although the cone cell precursors contain a basal level of uninduced RAS activity (Gaul et al., 1992; Begemann et al., 1995) that is down-regulated by *Gap1*, the JUN^{Asp} phenotype is not sensitive to gene dosage reduction of RAS pathway components (data not shown).

In wild type, YAN activity is probably down-regulated by phosphorylation and not completely absent, and then the simultaneous activation of the positive regulators JUN and PNT would be required to ensure that a given cell enters the R cell fate program. Such a double-switch mechanism to inactivate a repressor and activate the positive regulators appears a safe way to ensure the proper developmental fate of the respective precursor. Assuming that JUN and PNT act on promoter elements of common target genes, all our data from the genetic interactions and the cotransfection experiments support the model first that JUN and PNT interact in a cooperative manner and second that a balance of activities of these positive regulators and their antagonist YAN determines whether a precursor cell becomes a neuronal photoreceptor or not.

Concluding Remarks

The strong cooperativity observed between JUN and PNT and the probable requirement for the sum of the activities of JUN, PNT, and YAN for R7 induction might also explain the phenotypic effect of dominant negative mutants for either protein (Bohmann et al., 1994; Brunner et al., 1994a). Similarly, although constitutive activation of JUN can be sufficient to induce R7 fate and (at least partially) to overcome the presence of the inhibitor YAN, gene dose reduction in *yan* still enhances this effect. Thus, we conclude that inactivation of YAN (Rebay and Rubin, 1995) and the cooperative interaction of JUN and PNT are the critical steps in R7 induction and differentiation.

Experimental Procedures

Generation of Point Mutations in JUN and Fly Strains

The point mutations in c-JUN were generated as described by Papavassiliou et al. (1995) and verified by sequencing. The JUN^{Asp} isoform contains aspartic acid residues in place of the serines or threonines at positions 58, 62, 63, 73, 91, and 93; JUN^{Ala} contains alanine residues in place of serines or threonines 58, 62, 63, 73, 89, 91, 93, and 95. The respective wild-type or mutant open reading frames were inserted into a germline transformation vector carrying the eye disc–specific *sev* enhancer expression module driving expression in the precursors for R3–R4, R7, and the cone cells, and somewhat weaker in the mystery cells and R1 and R6 during ommatidial assembly (Basler et al., 1991). Germline transformation was performed by standard procedures.

Several independent transformants of *sE–jun*^{Asp} were isolated, all displaying the same phenotypic effect as shown in Figure 1. However, only one insertion was viable and fertile enough to establish a stable stock. This is probably due to some leakiness of the expression system and the deleterious effects of JUN^{Asp} in other tissues.

Transfections and Luciferase Assays

F9 cells (100 mm dish) were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 supplemented with 10% FCS and 10^{-4} M β-mercaptoethanol. Cells were transfected by the $CaCl_2$ method (Graham and van der Eb, 1973). The −60/+63 collagenase LUC reporter plasmid was constructed by inserting the −60/+63 collagenase promoter sequence into the Asp-718–HindIII site of pG12 (Promega). Polyoma-Col-LUC is an extension of the −60/+63 Col-LUC reporter with the following sequence including the classical Polyoma site (Gutman and Wasylyk, 1990) 5′-ACAGGAAGTGACTAAGTACC-3′, which was cloned into the SmaI–Asp-718 site of −60/+63 Col-LUC. The expression vectors were RSV plasmids with the corresponding coding sequence cloned between the long terminal repeat and SV40 polyadenylation signal. The luciferase assays were performed as previously described (Smith and Bohmann, 1992). The relative luciferase activity as shown in Figure 5 was calculated by dividing actual activity obtained in the presence of the respective expression vectors by the activity of the reporter when cotransfected with an empty expression vector (pRSV-0).

Histological and Other Techniques

For scanning electron microscopy (SEM), heads were dehydrated and critical point dried and coated with 20 nm gold–palladium mix before they were viewed on a prototype SEM. Histological sections of adult eyes and antibody stainings on eye imaginal discs were performed as previously described (Tomlinson and Ready, 1987). The rat anti-ELAV monoclonal antibody was a gift from G. Rubin, and the mouse anti-β-galactosidase monoclonal used to detect expression of H214 was purchased from Promega (dilution, 1:500).

The UV/green light phototactic behavior assay was performed as previously described by Basler et al. (1991).

Acknowledgments

We thank Ernst Hafen, Barry Dickson, Christian Klämbt, Gerald Rubin, and Daisuke Yamamoto for fly strains and plasmid DNA. We are grateful to Lena Staszewski for technical assistance, and to Roger Wepf and Sandra Scianimanico for help with SEM analysis and embryo injections, respectively. Peter Becker, Steve Cohen, David Jackson, David Strutt, and Volker Wiersdorff made helpful comments on the manuscript.

Received July 4, 1995; revised October 4, 1995.

References

Basler, K., Christen, B., and Hafen, E. (1991). Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing Drosophila eye. Cell *64*, 1069–1081.

Begemann, G., Michon, A.M., van der Voorn, L., Wepf, R., and Mlodzik, M. (1995). The *Drosophila* orphan nuclear receptor Seven-up requires the Ras pathway for its function in photoreceptor determination. Development *121*, 225–235.

Binetruy, B., Smeal, T., and Karin, M. (1991). Ha-ras augments c-Jun activity and stimulates phosphorylation of its activation domain. Nature *351*, 122–127.

Bohmann, D., Ellis, M.C., Staszewski, L.M., and Mlodzik, M. (1994). Drosophila Jun mediates Ras-dependent photoreceptor determination. Cell *78*, 973–986.

Brunner, D., Dücker, K., Oellers, N., Hafen, E., Scholz, H., and Klämbt, C. (1994a). The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. Nature *370*, 386–389.

Brunner, D., Oellers, N., Szabad, J., Biggs, W., III, Zipursky, S.L., and Hafen, E. (1994b). A gain-of-function mutation in Drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell *76*, 875–888.

Chang, H.C., Solomon, N.M., Wassarman, D.A., Karim, F.D., Therrien, M., Rubin, G.M., and Wolff, T. (1995). *phyllopod* functions in the fate determination of a subset of photoreceptors in Drosophila. Cell *80*, 463–472.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell *54*, 541–552.

Dickson, B. (1995). Nuclear factors in sevenless signalling. Trends Genet. *11*, 106–111.

Dickson, B., and Hafen, E. (1993). Genetic dissection of eye development in *Drosophila*. In The Development of *Drosophila melanogaster*, M. Bates and A. Martinez Arias, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 1327–1362.

Dickson, B., Sprenger, F., and Hafen, E. (1992a). Prepattern in the developing *Drosophila* eye revealed by an activated torso–sevenless chimeric receptor. Genes Dev. *6*, 2327–2339.

Dickson, B., Sprenger, F., Morrison, D., and Hafen, E. (1992b). Raf functions downstream of Ras1 in the *sevenless* signal transduction pathway. Nature *360*, 600–603.

Dickson, B.J., Domínguez, M., van der Straaten, A., and Hafen, E. (1995). Control of Drosophila photoreceptor cell fates by phyllopod, a novel nuclear protein acting downstream of the Raf kinase. Cell *80*, 453–462.

Fortini, M.E., Simon, M.A., and Rubin, G.M. (1992). Signalling by the *sevenless* protein tyrosine kinase is mimicked by Ras1 activation. Nature *355*, 559–561.

Gaul, U., Mardon, G., and Rubin, G.M. (1992). A putative Ras GTPase-activating protein acts as a negative regulator of signaling by the sevenless receptor tyrosine kinase. Cell *68*, 1007–1019.

Graham, F.L., and van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52*, 456–457.

Gutman, A., and Wasylyk, B. (1990). The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. EMBO J. *9*, 2241–2246.

Harris, W.A., Stark, W.S., and Walker, J.A. (1976). Genetic dissection of the photoreceptor system in the compound eye of *Drosophila melanogaster*. J. Physiol. *256*, 415–439.

Hill, C.S., and Treisman, R. (1995). Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell *80*, 199–211.

Klämbt, C. (1993). The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. Development *117*, 163–176.

Kryszke, M.H., Piette, J., and Yaniv, M. (1987). Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. Nature *328*, 254–256.

Lai, Z.-C., and Rubin, G.M. (1992). Negative control of photoreceptor development in Drosophila by the product of the *yan* gene, an *ets* domain protein. Cell *70*, 609–620.

Marshall, C.J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr. Opin. Genet. Dev. *4*, 82–89.

Marshall, C.J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal–regulated kinase activation. Cell *80*, 179–185.

Mlodzik, M., Hiromi, Y., Goodman, C.S., and Rubin, G.M. (1992). The presumptive R7 cell of the developing *Drosophila* eye receives positional information independent of *sevenless*, *boss* and *sina*. Mech. Dev. *37*, 37–42.

O'Neill, E.M., Rebay, I., Tjian, R., and Rubin, G.M. (1994). The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell *78*, 137–147.

Papavassiliou, A.G., Treier, M., and Bohmann, D. (1995). Intramolecular signal transduction in c-Jun. EMBO J. *14*, 2014–2019.

Perkins, K.K., Admon, A., Patel, N., and Tjian, R. (1990). The *Drosophila* fos-related AP-1 protein is a developmentally regulated transcription factor. Genes Dev. *4*, 822–834.

Pulverer, B.J., Kyriakis, J.M., Aruch, J., Nikolakaki, E., and Woodgett, J.R. (1991). Phosphorylation of c-Jun mediated by MAP kinase. Nature *353*, 670–674.

Rebay, I., and Rubin, G.M. (1995). Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell *81*, 857–866.

Schlessinger, J. (1993). How receptor tyrosine kinases activate Ras. Trends Biochem. Sci. *18*, 273–275.

Smeal, T., Binetruy, B., Mercola, D.A., Birrer, M., and Karin, M. (1991). Oncogenic and transcriptional cooperation with Ha-ras requires phosphorylation of c-Jun on serines 63 and 73. Nature *354*, 494–498.

Smith, S.E., and Bohmann, D. (1992). Functional dissection of AP-1 transcription factors using the Gal4 adaptor assay. Cell Growth Differ. *3*, 523–529.

Tei, H., Nihonmatsu, I., Yokokura, T., Ueda, R., Sano, Y., Okuda, T., Sato, K., Hirata, K., Fujita, S.C., and Yamamoto, D. (1992). *pokkuri*, a *Drosophila* gene encoding an E-26-specific (Ets) domain protein, prevents overproduction of the R7 photoreceptor. Proc. Natl. Acad. Sci. USA *89*, 6856–6860.

Tomlinson, A., and Ready, D.F. (1987). Neuronal differentiation in the *Drosophila* ommatidium. Dev. Biol. *120*, 366–376.

Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., LePrince, D., and Stehelin, D. (1990). The *c-ets* proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature *346*, 191–193.

Wasylyk, B., Hahn, S.L., and Giovane, A. (1993). The Ets family of transcription factors. Eur. J. Biochem. *211*, 7–18.

Zhang, K., Chaillet, R., Perkins, L.A., Halazonetis, T.D., and Perrimon, N. (1990). *Drosophila* homolog of the mammalian *jun* oncogene is expressed during embryonic development and activates transcription in mammalian cells. Proc. Natl. Acad. Sci. USA *87*, 6281–6285.

Zipursky, S.L., and Rubin, G.M. (1994). Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila*. Annu. Rev. Neurosci. *17*, 373–397.