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Yan Functions as a General Inhibitor of Differentiation and Is Negatively Regulated by Activation of the Ras1/MAPK Pathway

Ilaria Rebay and Gerald M. Rubin
Howard Hughes Medical Institute
Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720-3200

Summary

Drosophila yan has been postulated to act as an antagonist of the proneural signal mediated by the sevenless/Ras1/MAPK pathway. We have mutagenized the eight MAPK phosphorylation consensus sites of yan and examined the effects of overexpressing the mutant protein in transgenic flies and transfected S2 cultured cells. Our results suggest that phosphorylation by MAPK affects the stability and subcellular localization of yan, resulting in rapid down-regulation of yan activity. Furthermore, MAPK-mediated down-regulation of yan function appears to be critical for the proper differentiation of both neuronal and nonneuronal tissues throughout development, suggesting that yan is an essential component of a general timing mechanism controlling the competence of a cell to respond to inductive signals.

Introduction

Cell–cell interactions play an important role in controlling many developmental decisions in multicellular organisms, including cell fate specification, the process whereby different cell types are generated from a field of initially equipotent cells. Undetermined cells must integrate the positive and negative signals they receive in such a way that each cell assumes its proper identity in an appropriate spatial and temporal context. Many of these signaling events initiate at the cell surface via specific receptor–ligand interactions and are relayed through the cytoplasm to the nucleus, where transcription factors, thought to be among the ultimate targets of such signaling pathways, elicit an appropriate response by modulating gene expression. Thus, coordinated regulation of transcription factor activity represents a logical integration point for the various inductive and suppressive signals received by each cell as it selects a particular developmental program.

In Drosophila, the developing eye provides an excellent system for investigating the intricate molecular mechanisms underlying such cellular communication. The adult eye is composed of a regular array of ~800 ommatidial units, each one containing ~20 cells recruited in a stereotyped sequence of inductive interactions whereby differentiating cells signal their surrounding neighbors to follow specific developmental pathways (Tomlinson and Ready, 1987). One such inductive event, the recruitment of the R7 photoreceptor neuron, depends on proper signaling by the *sevenless* (*sev*) gene product, a member of the receptor tyrosine kinase (RTK) family (reviewed by Zipursky and Rubin, 1994). Genetic and biochemical investigations have revealed that sev activates an evolutionarily conserved signal transduction pathway used by numerous RTKs in many different organisms (reviewed by Zipursky and Rubin, 1994). Proteins implicated in sev-mediated signaling events include Ras1, along with numerous regulators of its activity (reviewed by Zipursky and Rubin, 1994), and the mitogen-activated protein kinase (MAPK) family of serine/threonine kinases that include Raf (MAPKKK), DSor (MAPKK), and rolled (MAPK) (reviewed by Marshall, 1994).

Efforts to identify downstream targets of MAPK have implicated several nuclear transcription factors. In vertebrate systems, activated MAPK translocates from the cytoplasm to the nucleus, where it phosphorylates several targets, including c-*fos*, NF-IL6, and Elk-1, a member of the Ets family of transcription factors (Chen et al., 1993; Nakajima et al., 1993; Marais et al., 1993; Jancknecht et al., 1993). In Drosophila, recent work has revealed that activated MAPK modulates the activities of two other Ets-related proteins, pointed and yan (O'Neill et al., 1994; Brunner et al., 1994). Genetically, *pointed* appears to be a positive regulator of photoreceptor development while *yan* appears to be a negative regulator, acting as an antagonist of the sev/Ras1 proneural signal (Lai and Rubin, 1992; O'Neill et al., 1994). Experiments assaying the activities of these proteins in transfected S2 cells have shown that pointed is a transcriptional activator whose activity is stimulated by the Ras1/MAPK pathway while yan functions as a transcriptional repressor negatively regulated by the Ras1/MAPK pathway (O'Neill et al., 1994). Yan contains eight putative MAPK phosphorylation consensus sequences (defined as P-X-S/T-P; P, proline; X, any amino acid; S/T, serine/threonine; Clark-Lewis et al., 1991) while pointed contains one such sequence (Lai and Rubin, 1992; Klämbt, 1993); both proteins can be directly phosphorylated in vitro by MAPK (Brunner et al., 1994).

As a negative regulator of a signal transduction pathway, yan offers a unique opportunity to investigate an intriguing aspect of development, namely, how cells are inhibited from responding inappropriately to the complex array of external signals to which they are exposed. Antibodies raised against the *yan* gene product show it is expressed prominently in basally located nuclei of undifferentiated cells in the larval imaginal eye disc (Lai and Rubin, 1992). However, as cells begin to differentiate and their nuclei migrate to a more apical position within the disc epithelium, yan expression is abruptly down-regulated (Lai and Rubin, 1992). In addition, examination of the yan sequence reveals the predicted protein to be rich in PEST sequences, a proline, serine/threonine, aspartic/glutamic acid–rich motif frequently found in proteins with short half-lives (Lai and Rubin, 1992; Rogers et al., 1986).

Based on these observations, it has been proposed that yan performs its role as a negative regulator of photoreceptor development by maintaining cells in an undifferentiated state (Lai and Rubin, 1992). According to the model, the

Figure 1. Structural Motifs of Yan

A schematic diagram depicting the structural motifs of the predicted *yan* gene product (Lai and Rubin, 1992). The Ets homologous putative DNA-binding domain is indicated by a box labeled ETS. The eight MAPK phosphorylation consensus sites are depicted as closed bars and are numbered 1–8. PEST sequences are delineated by the stippled boxes. The location of the molecular lesion associated with the yan^{S2382} allele is indicated with a vertical arrow. A 5 bp deletion (CGCCG; beginning with nucleotide 2649, according to Lai and Rubin, 1992) shifts the reading frame, thereby replacing the C-terminal 162 amino acids with a new 86 amino acid tail.

yan-mediated block to differentiation must be removed for a cell to respond to specific developmental cues. Perhaps either the same signaling cascade that eventually induces the cell to initiate a specific differentiation program or a different independent signaling event initially or simultaneously inactivates yan. Since yan can be negatively regulated by activated Ras1/MAPK in cell culture systems and is a direct target for phosphorylation by MAPK in vitro (O'Neill et al., 1994; Brunner et al., 1994), perhaps phosphorylation and subsequent PEST-mediated degradation or inactivation (or both) of yan in response to Ras1/MAPK activation is required to overcome the yan-mediated repression of differentiation in vivo.

In this paper we present the results of a series of experiments designed to test this model for yan function. In vitro mutagenesis was used to alter the eight MAPK phosphorylation consensus sequences in yan, and the effects of overexpressing these mutants both in vivo and in cell culture assays was examined. Our results suggest that phosphorylation of yan by MAPK affects the stability and subcellular localization of the protein, resulting in rapid down-regulation of yan activity. We further describe the phenotypes and molecular lesions associated with a unique dominant gain-of-function *yan* allele. Together, these results suggest yan not only functions as a negative regulator in the sev-mediated pathway of R7 photoreceptor development, but also acts as a much more general inhibitor of cell fate specification at multiple points during Drosophila development.

Results

Overexpression of MAPK Phosphorylation Site-Deficient Yan in the Developing Eye Blocks Photoreceptor Neuron Determination

To investigate the role of the MAPK phosphorylation consensus sequences in modulating yan function in vivo, we have used in vitro mutagenesis to replace the phosphoacceptor residues of all eight consensus sites with a non-phosphorylatable amino acid, alanine (Figure 1). If down-regulation of yan activity occurs via phosphorylation by MAPK, then removal of the phosphorylation sites should result in a constitutively active form of yan that fails to respond to the Ras1/MAPK signal and thereby inappropriately continues to inhibit differentiation. Two constructs, yan^{ACT}, in which all eight MAPK consensus sites were mutated, and yan^{WT}, the wild-type cDNA, were generated and placed under control of the eye-specific glass multimer reporter construct (pGMR; Hay et al., 1994; constructs designated GMR-yan^{ACT} and GMR-yan^{WT}) that drives expression in virtually all cells of the developing eye, beginning at the onset of differentiation in the morphogenetic furrow. Transgenic lines were established using P element–mediated transformation and were examined for dominant eye phenotypes. It should be emphasized that all phenotypes represent dominant effects of the specific transgene since the experiments were performed in a wild-type background.

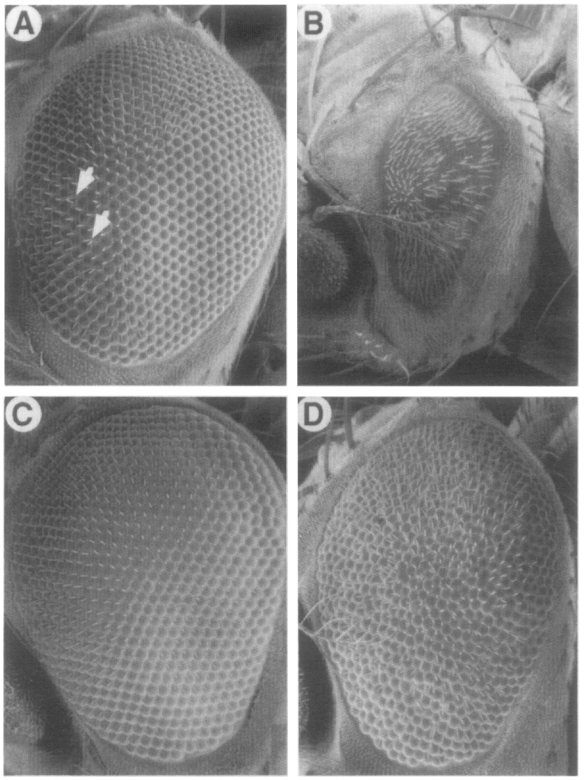

Figure 2. Adult Eye Phenotype of yan^{WT} and yan^{ACT} Overexpression

Scanning electron micrographs of adult eyes. All flies carry two copies of the specific transgene.

(A) GMR-yan^{WT}. The eye is virtually wild type, with the exception of occasional bristle duplications and irregularities, marked by white arrows, in the anterior portion. This phenotype is observed in only two of the lines; the remaining nine are indistinguishable from wild type.

(B) GMR-yan^{ACT}. Note the severe reduction in size as well as the complete abolition of the normal ommatidial lattice.

(C) Sev-yan^{WT}. This eye is indistinguishable from that of a wild-type fly.

(D) Sev-yan^{ACT}. Note the slight reduction in size and the mild rough phenotype.

Overexpression of GMR-yan^{WT} produces few discernible effects in the eye. In the majority of lines (nine out of 11), the external morphology of the adult eye, as judged by scanning electron microscopy, is virtually indistinguish-

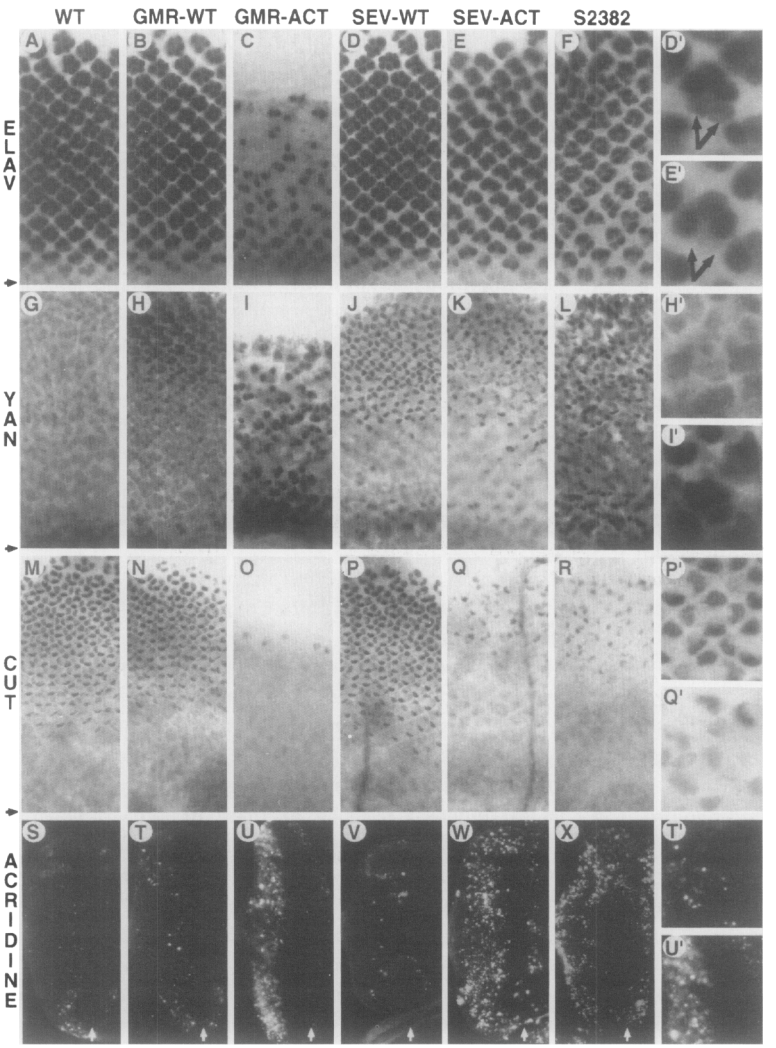

Figure 3. Third Instar Eye Disc Phenotype of yan^{WT} and yan^{ACT} Overexpression

(A–F) Discs labeled with anti-elav. (A) Wild type. Note the normal pattern of photoreceptor assembly. (B) GMR–yan^{WT}. Expression is indistinguishable from wild type. (C) GMR–yan^{ACT}. Note the loss of elav-positive photoreceptor nuclei, particularly in the posterior portion of the disc furthest from the morphogenetic furrow. The progression of the morphogenetic furrow is reduced relative to wild type (see also [I] and [O]), perhaps contributing to the small size of the adult eye (refer to Figure 2B). (D) Sev–yan^{WT}. Note the wild-type pattern. (D′) Higher magnification of a developing ommatidium. Arrows indicate the nuclei of the developing R3 and R4 photoreceptors. (E) Sev–yan^{ACT}. Photoreceptors R3, R4, and R7 are absent from most of the developing clusters. (E′) Higher magnification of a single ommatidium. Arrows indicate where the R3 and R4 nuclei would normally be found. (F) yan^{S2382}. Cluster formation appears irregular with occasional missing photoreceptor nuclei.

(G–L) Discs labeled with anti-yan MAb 8B12. (G) Wild type. Yan expression is not detected in the apically located nuclei of the developing photoreceptors and cone cells. Overdeveloped preparations reveal weak expression in the cone cells (data not shown). (H) GMR–yan^{WT}. Ectopic yan expression is detected only in photoreceptor nuclei in the most posterior portion of the disc farthest from the morphogenetic furrow. In weaker lines, this is not observed (data not shown). Near the morphogenetic furrow, where the clusters are first assembled, ectopically expressed yan is normally down-regulated in all lines; also shown in (H′) at higher magnification. (I) GMR–yan^{ACT}. Strong yan expression is found in apically located nuclei throughout the disc but primarily near the morphogenetic furrow; also shown at higher magnification in (I′). (J) Sev–yan^{WT}. Ectopic yan expression is detected only in the developing cone cell nuclei but is down-regulated in the R3, R4, and R7 photoreceptor precursors. (K) Sev–yan^{ACT}. Ectopic yan expression is observed in the irregularly patterned cone cell nuclei as well as in occasional photoreceptor nuclei. (L) yan^{S2382}. Abnormal stability of yan expression is detected in both photoreceptors and cone cell nuclei.

(M–R) Discs labeled with anti-cut. (M) Wild type. Note the regular pattern of the developing cone cell nuclei. (N) GMR–yan^{WT}. Cone cell assembly appears unaffected. (O) GMR–yan^{ACT}. Cut expression in the developing cone cell nuclei is virtually abolished. (P) Sev–yan^{WT}. Cone cell differentiation appears unaffected; also shown in higher magnification in (P′). (Q) Sev–yan^{ACT}. Cut expression is dramatically reduced; also shown in higher magnification in (Q′). (R) yan^{S2382}. Cut expression is reduced, and the pattern of the cone cell nuclei is irregular.

(S–X) Discs labeled with acridine orange. (S) Wild type. Only scattered cell death is observed. (T) GMR–yan^{WT}. Only scattered cell death is observed; also shown at higher magnification in (T′). (U) GMR–yan^{ACT}. A drastic increase in cell death occurs in the posterior half of the disc; also shown at higher magnification in (U′). (V) Sev–yan^{WT}. Only scattered cell death is observed. (W) Sev–yan^{ACT}. A moderate increase in cell death occurs. (X) yan^{S2382}. A mild increase in cell death is observed.

The black arrows on the left indicate the position of the morphogenetic furrow; thus, posterior is toward the top, except in (S)–(X), where posterior is to the left and the position of the morphogenetic furrow is indicated by vertical white arrows. Flies carry two copies of the specific transgene; all experiments were performed with at least two independent lines. WT, wild type; GMR–WT, GMR–yan^{WT}; GMR–ACT, GMR–yan^{ACT}; SEV–WT, sev–yan^{WT}; SEV–ACT, sev–yan^{ACT}; S2382, yan^{S2382}.

able from wild type, although in two lines, occasional bristle duplications are observed in the anterior portion of the eye (Figure 2A). Photoreceptor development in these flies was examined by labeling third larval instar eye imaginal discs with anti-elav antiserum, a nuclear marker for all Drosophila neurons. No obvious defects were discerned (Figures 3A and 3B).

In contrast, flies expressing GMR–yan^{ACT} have eyes that are severely reduced in size and devoid of most normal ommatidial morphology (see Figure 2B). In addition to the irregularly spaced bristles, small hairlike structures, similar to the hairs found on the surrounding head cuticle, cluster in the ventral–posterior region of the eye, suggesting a partial transformation of the remaining eye tissue into head cuticle. Of the ~50 lines obtained, ~75% show such a severe phenotype, while the remaining lines exhibit varying degrees of roughness (data not shown). Third instar eye imaginal discs stained with anti-elav antiserum

reveal a severe inhibition of photoreceptor development as only a few scattered elav-positive cells remain, primarily in the anterior portion of the disc (Figure 3C). A similar but less extreme inhibition of photoreceptor differentiation is observed in even the weakest lines (data not shown).

Mutation of the MAPK Phosphorylation Consensus Sites of Yan Results in Excessive Stability of the Protein

To assess the effects of mutating the MAPK phosphorylation consensus sequences on regulated yan expression, we stained third larval instar eye imaginal discs with anti-yan monoclonal antibodies (MAbs). In wild-type animals, yan is expressed strongly in the nuclei of most cells near the morphogenetic furrow as well as in the basally located nuclei of the undifferentiated cell population (Lai and Rubin, 1992). As the cells begin to differentiate, as marked by a concomitant migration of the nuclei to a more apical position within the epithelium, yan expression abruptly disappears from the nucleus (Lai and Rubin, 1992). Thus, nuclear yan expression is not detected in a more apical plane of focus, with the exception of very faint staining in the cone cells (Lai and Rubin, 1992; Figure 3G).

In GMR–yan^{WT} transgenic flies, the level of expression appears higher than usual in the basal nuclei (data not shown), while the pattern of protein expression in the apical nuclei is similar to wild type (Figure 3G). However, in the strongest lines, yan-positive apically located photoreceptor nuclei are detected in the most posterior region of the disc, although there is no detectable increase in yan expression in the nuclei of the differentiating photoreceptors immediately posterior to the morphogenetic furrow (Figures 3H and 3H′). Thus, in most cells, the mechanism responsible for down-regulating yan in the wild-type context is sufficiently robust to cope with abnormally high levels of protein.

In contrast, anti-yan antibody staining of eye imaginal discs from GMR–yan^{ACT} transgenic lines reveals in addition to exceptionally strong staining in the basally situated nuclei (data not shown), excessive stability of the protein in apically situated nuclei, particularly in the portion of the disc immediately posterior to the morphogenetic furrow (Figures 3I and 3I′). In our weakest lines, in which disruption of the eye structure is less severe, yan staining is clearly observed in the apical nuclei of developing photoreceptors throughout the disc (data not shown).

Overexpression of MAPK Phosphorylation Site-Deficient Yan Results in Increased Cell Death in the Developing Eye

Examination of both elav and yan expression in the developing eye discs of GMR–yan^{ACT} transgenic animals reveals most elav- or yan-positive cells are found in the anterior portion of the disc, while in the posterior half, the more highly differentiated section, only scattered elav- or yan-positive cells remain (Figures 3C and 3I). To determine the fate of the majority of cells in this posterior region, we labeled live discs with acridine orange to visualize the extent of cell death.

During normal eye development, cell death occurs at several stages to eliminate surplus cells as the ordered ommatidial lattice is constructed (Wolff and Ready, 1991). In a wild-type third instar imaginal eye disc labeled with acridine orange, dying cells are seen both anterior and posterior to the furrow (Figure 3S). GMR–yan^{WT} transgenic lines exhibit a similar cell death profile (Figures 3T and 3T′). However, the GMR–yan^{ACT} lines show a dramatic increase in cell death, particularly in the posterior half of the disc (Figures 3U and 3U′). The lack of a pronounced increase in cell death in the anterior portion of the disc suggests death is probably not a direct consequence of ectopic yan^{ACT} expression, but may be a secondary effect of prolonged yan-mediated inhibition of differentiation. Thus, cells that are continuously prevented from responding to inductive signals, owing to the excessive stability of yan^{ACT}, may eventually reach a point at which the only remaining option is death, thereby producing the small rough eye phenotype observed in GMR–yan^{ACT} lines.

Expression of MAPK Phosphorylation Site-Deficient Yan Inhibits Embryonic Neural Development

The effects of GMR–yan^{ACT} expression, in particular, the almost complete abolition of photoreceptor differentiation, suggest modulated yan activity is not only critical for R7 cell determination, but also for the development of all photoreceptor neurons. To determine whether yan functions as an inhibitor specific to photoreceptor neurons or whether it may act as an even more general repressor of neural development, we have overexpressed the yan^{ACT} mutant in early embryos by placing both yan^{ACT} and yan^{WT} sequences under control of the heat shock promoter *hsp70* (constructs designated HS–yan^{ACT} and HS–yan^{WT}). Yan is normally expressed in the embryonic ectoderm and mesoderm but is strikingly absent from the developing central nervous system (CNS) and underlying neurectoderm (Lai and Rubin, 1992; I. R. and G. M. R., unpublished data). To overexpress yan in cells of the developing CNS, we collected staged embryos from HS–yan^{WT} and HS–yan^{ACT} transgenic lines, aged them until cellularization, and then subjected them to two 30 min 37°C heat shock pulses separated by a 30 min recovery period at 25°C. After 4–8 hr of further development at 25°C, embryos were fixed and labeled with anti-elav antibodies to judge the extent of neuronal differentiation.

The results closely parallel the situation in the eye. Overexpression of HS–yan^{WT} in the developing CNS has no obvious effect on normal elav expression (Figure 4A), and the animals survive to adulthood in numbers similar to wild-type controls (data not shown). On the other hand, overexpression of HS–yan^{ACT} blocks expression of neural antigens as judged by the nearly complete abolition of elav staining (Figure 4B). The identical result was obtained using anti–horseradish peroxidase antibodies (data not shown), indicating that multiple neuronal antigens are repressed. In addition, induction of HS–yan^{ACT} expression results in completely penetrant embryonic lethality, with the embryos failing to complete germband retraction (data not shown).

We have repeated this experiment using the elav–GAL4

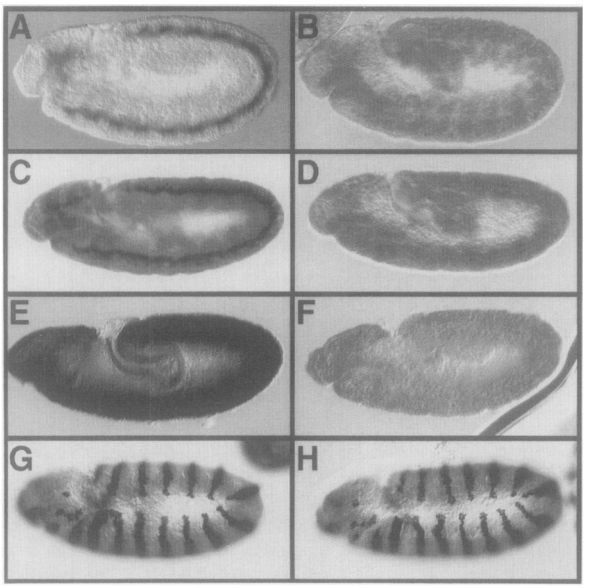

Figure 4. Effects of Embryonic Overexpression of yan^{WT} and yan^{ACT}

(A) HS–yan^{WT} embryo labeled with anti-elav. Elav staining in the developing CNS first becomes prominent at this stage.

(B) HS–yan^{ACT} embryo labeled with anti-elav. Elav staining is virtually abolished.

(C) UAS–yan^{WT} × GAL4 C155 embryo labeled with anti-elav. Normal elav staining in the developing CNS is detected.

(D) UAS–yan^{ACT} × GAL4 C155 embryo labeled with anti-elav. Elav staining is virtually abolished.

(E) HS–yan^{WT} embryo labeled with anti-twist. Strong twist expression is observed in the developing mesoderm.

(F) HS–yan^{ACT} embryo labeled with anti-twist. Twist expression is virtually absent.

(G) HS–yan^{WT} embryo labeled with anti-engrailed. The striped pattern of engrailed expression is shown.

(H) HS–yan^{ACT} embryo labeled with anti-engrailed. The expression pattern is indistinguishable from (G).

Germband extended embryos, stages 8–9, are shown in all panels. All experiments were performed with at least two independent lines.

C155 line (Lin and Goodman, 1994) crossed to UAS–yan^{WT} and UAS–yan^{ACT} lines and obtained similar results (Figures 4C and 4D). Thus, expressing yan^{ACT} specifically in cells of the developing CNS blocks elav expression, while expressing yan^{WT} has no effect, indicating that loss of neuronal markers is a direct consequence of yan^{ACT} expression in the developing neural tissues. The UAS–yan^{WT} × elav–GAL4 C155 embryos develop and hatch normally, while the UAS–yan^{ACT} × elav–GAL4 C155 embryos exhibit a variable developmental arrest and a completely penetrant lethality (data not shown).

Yan-Mediated Inhibition of Differentiation Is Not Restricted to Neuronal Development

We have shown that yan appears to act as a general inhibitor of neuronal differentiation, as ectopic expression of the yan^{ACT} mutant blocks both photoreceptor and embryonic CNS development. We were therefore interested in determining whether yan activity is specific to neuronal precursors or whether it might similarly inhibit differentiation of nonneuronal cell types. To address this question, we examined the effects of ectopic yan^{ACT} expression in two different nonneuronal cell types, the embryonic mesoderm and the developing eye cone cell precursors.

In wild-type embryos, yan is expressed strongly in mesodermal cells during germband extension but is not detected in fully differentiated mesodermal derivatives such as muscle and gut, suggesting that down-regulation of yan expression, activity, or both in these tissues may be critical for their normal development (Lai and Rubin, 1992; I. R. and G. M. R., unpublished data). To determine the effect of overexpressing the yan^{ACT} mutant on embryonic mesodermal differentiation, we labeled heat shock–treated HS–yan^{ACT} embryos, as described in the previous section, with antiserum against twist, a mesodermal marker (Roth et al., 1989; Thisse et al., 1988). A drastic reduction in twist staining is observed in HS–yan^{ACT} embryos relative to HS–yan^{WT} embryos (Figures 4E and 4F), suggesting the function of yan as an inhibitor of differentiation is not restricted to neuronal cell types but may play a much more general role during development. We have confirmed the specificity of this result using the 24B GAL4 line that drives expression in the mesoderm (data not shown; Brand and Perrimon, 1993).

To assess the effect of ectopic yan^{ACT} expression in nonneuronal cone cell precursors of the eye, we placed both constructs under control of the sev regulatory sequences (designated sev–yan^{ACT} and sev–yan^{WT}) that drive expression in the developing R3, R4, and R7 photoreceptor neurons as well as in the four nonneuronal cone cell precursors (Tomlinson et al., 1987; Bowtell et al., 1988; Fortini et al., 1992). In accord with the results described for the corresponding GMR–*yan* constructs, flies expressing the sev–yan^{WT} transgene have completely wild-type eyes as judged by the adult external morphology as well as by elav expression in the imaginal discs (see Figures 2C, 3D, and 3D′). Interestingly, yan expression in these discs is normal with respect to the lack of staining in the developing R3, R4, and R7 photoreceptor precursors, yet is abnormally abundant in the cone cells (see Figure 3J). In wild-type flies, low levels of yan can be detected in the cone cells (I. R. and G. M. R., unpublished data), suggesting that down-regulation of yan may not be as critical in these cells as in the photoreceptors or that down-regulation may simply occur at a later stage. In contrast, sev–yan^{ACT} flies have moderately rough eyes (see Figure 2D), and elav staining of the imaginal discs reveals a failure of photoreceptors R3, R4, and R7 to develop (see Figures 3E and 3E′). As expected, abnormally high levels of yan expression are detected in a small subset of the apical photoreceptor nuclei and most prominently in many cone cell precursor nuclei (see Figure 3K). Finally, as revealed by acridine orange labeling, sev–yan^{ACT} discs show an increase in cell death, while sev–yan^{WT} discs exhibit a wild-type cell death profile (see Figures 3V and 3W).

To determine the effect of excessive yan^{ACT} expression in the nonneuronal cone cell precursors, we labeled discs with antiserum recognizing the *cut* gene product to mark the cone cell nuclei (Blochlinger et al., 1988). Sev–yan^{WT} discs labeled with anti-cut show the normal regular array of cone cell nuclei, while sev–yan^{ACT} discs show a dramatic reduction in cut expression, suggesting overexpression of

yan^{ACT} can inhibit the differentiation of these nonneuronal cells (see Figures 3P, 3P′, 3Q, and 3Q′). As expected, GMR–*yan*^{WT} and GMR–*yan*^{ACT} discs exhibited cut expression patterns similar to the corresponding sev–*yan* construct (see Figures 3N and 3O). Thus, regulated yan activity appears to be important for the differentiation of numerous cell types, both neuronal and nonneuronal, throughout development.

One important question arising from these results is whether yan functions as a universal inhibitor of differentiation in all tissues throughout development or whether it is somewhat restricted in its effects. Two experiments were designed to address this issue. First, we tested whether overexpression of HS–*yan*^{ACT} affects epidermal markers by labeling the same heat-treated HS–*yan*^{WT} and HS–*yan*^{ACT} embryo populations with anti-engrailed antiserum. In this instance, no obvious reduction or alteration in engrailed expression is detected (Figures 4G and 4H), indicating that overexpression of *yan*^{ACT} does not interfere with all aspects of embryonic development. Second, HS–*yan*^{WT} and HS–*yan*^{ACT} transgenic lines were subjected to extensive heat shock treatments during larval and pupal stages in order for us to examine the effects of ectopically expressing yan in developing tissues such as the wing and bristles where there is normally no detectable yan expression (Lai and Rubin, 1992; I. R. and G. M. R., unpublished data). Although uniformly high levels of ectopic yan were detected in all developing tissues examined, the only phenotype observed was a severely rough eye in the HS–*yan*^{ACT} lines; wings and bristles were completely wild type (data not shown). Thus, it appears the yan-mediated block to differentiation is specific to certain cell types at particular times in development.

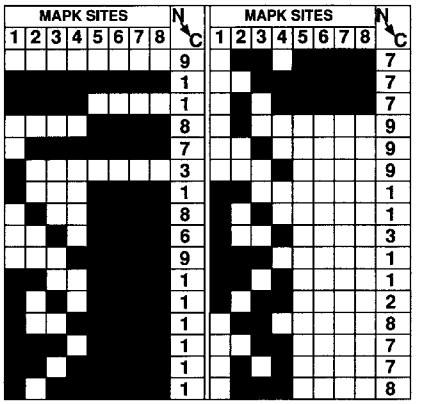

Figure 5. Effects of Mutational Analysis of the MAPK Phosphorylation Sites of Yan on Subcellular Localization

The MAPK phosphorylation sites of yan are designated 1–8. Open boxes indicate a wild-type phosphoacceptor residue (serine or threonine); closed boxes indicate the phosphoacceptor residue has been mutated to alanine. In the absence of activated Ras1, 90%–95% of cells transfected with all constructs exhibit nuclear yan expression (data not shown). The ability of a particular construct to translocate from the nucleus to the cytoplasm in the presence of activated Ras1 is given as a ratio of the percent of transfected cells with cytoplasmic staining in the presence of Ras1 divided by the percent of transfected cells with cytoplasmic staining in the absence of Ras1 and listed in the column designated N→C (nucleus to cytoplasm). Any cell with detectable cytoplasmic yan, such as the labeled cell in Figure 6A, is scored as cytoplasmic; the remaining cells in the field are scored as nuclear. For example, *yan*^{WT} is ~10% cytoplasmic (~90% nuclear) in the absence of activated Ras1 and ~90% cytoplasmic (~10% nuclear) in the presence of activated Ras1, giving a value of 9.

The Subcellular Distribution of Yan Is Altered by Activation of Ras1/MAPK in S2 Cultured Cells

In addition to examining the effects of ectopic yan expression in vivo, we have also examined yan expression in transfected S2 cells. In cells transfected with *yan*^{WT}, the protein is largely nuclear, although in a small percentage (5%–10%) of the transfected cell population, cytoplasmic localization is observed (Figures 5 and 6A). However, if the cells are cotransfected with *yan*^{WT} and activated Ras1, the subcellular distribution of yan is dramatically altered and the protein appears almost entirely cytoplasmic (Figures 5 and 6B). In contrast, the *yan*^{ACT} mutant is exclusively nuclear, even in the presence of activated Ras1/MAPK (Figures 5, 6C, and 6D).

We have used this nuclear to cytoplasmic shift in yan subcellular distribution to determine which subset of the eight MAPK phosphorylation consensus sites in yan are critical in the response to activated Ras1/MAPK. Many of the possible combinations of mutant MAPK sites were generated and tested in this assay, and the results are consistent with the first site (designated yan–MAPK1) being essential to the response (Figures 1 and 5). Thus, if the phosphoacceptor residue in yan–MAPK1 is mutated to alanine but the remaining seven sites are left wild type, the protein remains largely nuclear in the presence of activated Ras1 (Figures 5 and 6E). Conversely, if yan–MAPK1 is wild type and the other seven sites mutant, the protein is found predominantly in the cytoplasm in the presence of activated Ras1 (Figures 5 and 6F). However, while the alteration in subcellular localization of a particular construct in the presence of activated Ras1 is evident, the efficiency of the effect is frequently reduced relative to the *yan*^{WT} or *yan*^{ACT} constructs.

The first seven constructs listed in Figure 5 were further tested in the transcriptional assay developed by ONeill et al. (1994) in which yan was found to act as a transcriptional repressor whose repressing ability was itself inhibited by activated Ras1. In the absence of activated Ras1, all constructs repressed transcription at wild-type levels. However, in all constructs except the first (*yan*^{WT}) and the fourth (mutant MAPK sites 4–8), the repression was insensitive to the presence of activated Ras1, and even for the latter construct, abrogation of repression was only ~50% of wild type. The same set of seven constructs was also expressed in vivo using the GMR vector. Phenotypic analyses of the transgenic lines again confirm the essential role of yan–MAPK1. Thus, if MAPK sites 2–8 are mutant, the flies are wild type, and if only MAPK1 is mutant, the flies show a rough eye reminiscent of a mild GMR–*yan*^{ACT} phenotype (data not shown). Thus, while our results indicate yan–MAPK1 is absolutely required in the response to activation of the Ras1/MAPK pathway, it seems likely that phosphorylation at other sites will be important for modulation or amplification of the response.

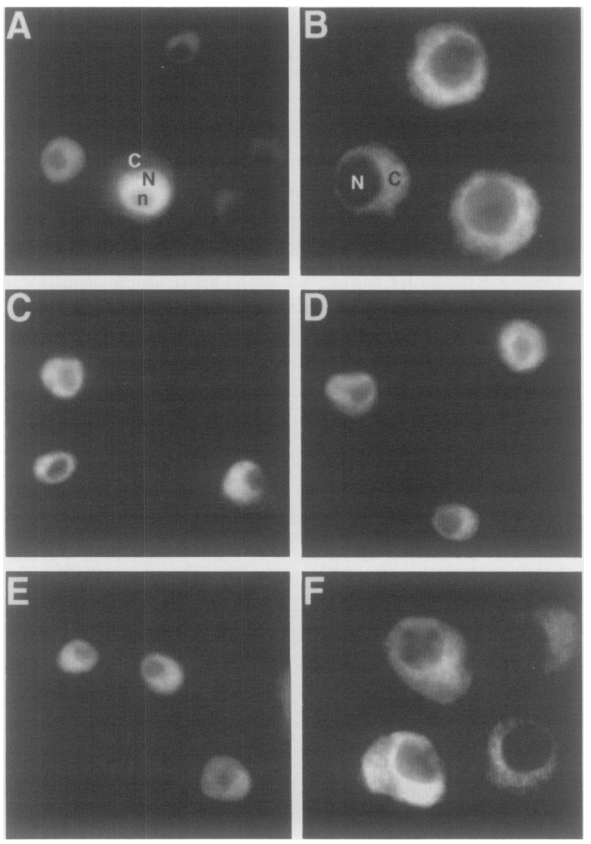

Figure 6. Activation of Ras1/MAPK Alters the Subcellular Distribution of Yan in Transfected S2 Cells

S2 cultured cells were transiently transfected with the indicated constructs and labeled with anti-yan MAb 8B12. Since transient transfections were used, only ~1%–5% of the cell population express yan; the remaining untransfected cells (~95% of the population) are not detected.

(A) yan^{WT}. Note the predominantly nuclear distribution of the protein. Yan staining is not observed in the nucleolus (n), is prominent in the nucleus (N), and in ~5%–10% of the cells is also detectable in the cytoplasm (C).

(B) yan^{WT} plus activated Ras1. Yan expression is apparent in the cytoplasm (C) and virtually absent from the nucleus (N).

(C) yan^{ACT}. Yan expression is entirely nuclear.

(D) yan^{ACT} plus activated Ras1. Yan expression remains nuclear.

(E) $yan^{MAPK(1)S-A}$ plus activated Ras1. Yan expression remains largely nuclear.

(F) $yan^{MAPK(2-8)S-A}$ plus activated Ras1. Yan expression is predominantly cytoplasmic.

A Gain-of-Function *yan* Allele Blocks Photoreceptor Neuron Development and Shows Excessive Protein Stability

A genetic screen for modifiers of the extra photoreceptor phenotype caused by overexpressing activated Ras1 in the eye yielded a large complementation group of enhancers allelic to *yan* (F. Karim, M. Therrien, D. Wassarman, H. Chang, T. Laverty, and G. M. R., unpublished data). In addition, a single mutation, yan^{S2382}, isolated as a suppresser of activated Ras1, was genetically characterized as a neomorphic *yan* allele (F. Karim, M. Therrien, D. Wassarman, H. Chang, T. Laverty, and G. M. R., unpublished data). These flies are homozygously viable and have a dominant rough eye characterized by missing photoreceptors, predominantly R7 cells (F. Karim, M. Therrien, D. Wassarman, H. Chang, T. Laverty, and G. M. R., unpublished data). We have further characterized this neomorphic allele by examining the expression of specific markers in the developing eye imaginal disc. Eye discs stained with anti-elav antiserum reveal a disruption of the normal pattern of developing photoreceptors consistent with the adult phenotype (see Figure 3F; F. Karim, M. Therrien, D. Wassarman, H. Chang, T. Laverty, and G. M. R., unpublished data), while discs stained with anti-yan antiserum reveal an abnormal stability of the mutant protein in apically localized nuclei (see Figure 3L). Discs incubated with acridine orange show a moderate increase in cell death relative to wild-type controls (see Figure 3X). Cut expression in the developing cone cells is also reduced and disorganized (see Figure 3R). All of these phenotypes are highly reminiscent of those described for the engineered yan^{ACT} mutation, suggesting the yan^{S2382} mutation is a gain-of-function *yan* allele that produces an activated form of yan similar to, but weaker than, our yan^{ACT} construct.

To identify the molecular lesion associated with the yan^{S2382} allele, genomic DNA from homozygous adult flies was amplified and the entire coding region sequenced. A 5 bp deletion was identified that shifts the reading frame, thereby deleting the C-terminal 162 amino acids (see Figure 1). While the truncation removes only the last two MAPK phosphorylation consensus sites, which, based on our analyses, are not essential in regulating yan activity, the deleted region is rich in PEST sequences (Rogers et al., 1986; Lai and Rubin, 1992; see Figure 1). Thus, deletion of these sequences may alter the stability of yan, thereby producing a dominant phenotype very similar to, but milder than, that observed with the yan^{ACT} mutant. One might have expected the product of yan^{S2382} to be cytoplasmic since it retains the critical MAPK1 site; however, its retention in the nucleus suggests the deleted C-terminal sequences may be required to mediate translocation into the cytoplasm.

Discussion

We have demonstrated that in vivo overexpression of a MAPK phosphorylation site–deficient yan^{ACT} mutant effectively blocks neural development both in the photoreceptors of the eye and in cells of the embryonic CNS, as well as nonneural development in cone cells and embryonic mesodermal cells. Inhibition of cellular differentiation correlates with an increased stability of the mutant yan^{ACT} product. Together, these results indicate the MAPK phosphorylation sites of yan are critical for modulation of yan function and responsiveness to a variety of cell signaling events in vivo. Furthermore, based on a cell culture assay in conjunction with phenotypic analysis of a gain-of-function *yan* allele, we propose a possible mechanism for regulating yan activity during normal development, namely, a shift in the subcellular distribution of yan in response to activation of the Ras1/MAPK signaling cascade, followed by rapid degradation of the protein.

Yan Functions as an Inhibitor of Both Neural and Nonneural Differentiation

Initial genetic characterization of *yan* classified it as a negative regulator of R7 photoreceptor determination in the sev/Ras1 signaling pathway (Lai and Rubin, 1992). However, accumulating evidence suggests yan plays a much more complex and ubiquitous developmental role. Within the developing eye, yan appears to play a role in more than just R7 cell specification. Given that yan is expressed in seemingly all undifferentiated cells in the developing eye imaginal disc, including both neuronal and nonneuronal precursors, and is down-regulated in all cells as they differentiate (Lai and Rubin, 1992), there must be signaling mechanisms responsible for inactivating yan in all cells. These may include activation of MAPK through various RTKs such as sev, the epidermal growth factor receptor or other (as yet unidentified) receptors, activation of MAPK via a different non-RTK signaling cascade, or activation of other kinases that might similarly inactivate yan. It remains to be determined whether the same signaling cascade that induces a specific differentiation program initially or simultaneously inactivates yan or whether earlier or parallel independent signaling pathways are required.

Consistent with the idea of a more general role during development, yan is also expressed at high levels during embryonic development. During late stages, yan is particularly prominent in the developing trachea (Lai and Rubin, 1992), where breathless, the Drosophila fibroblast growth factor RTK homolog, is known to function (Glazer and Shilo, 1991). Yan is also abundantly expressed in the ectoderm, yet is down-regulated in the ventral neurectoderm and is not detectable in the developing embryonic CNS (Lai and Rubin, 1992; I. R. and G. M. R., unpublished data). This dynamic expression pattern, in conjunction with the inhibition of neural marker expression associated with ectopic expression of yan^{ACT} in these tissues, may indicate the existence of other RTK pathways that negatively regulate yan activity in cells of the developing embryonic CNS.

Yan activity does not appear to be restricted to neuronal tissues. For example, in the embryo, yan is expressed strongly in the developing mesoderm, yet is absent in more fully differentiated mesodermal derivatives such as the gut and musculature (Lai and Rubin, 1992; I. R. and G. M. R., unpublished data). Overexpression of the yan^{ACT} mutant inhibits expression of the twist mesodermal marker, suggesting a MAPK-mediated mechanism regulating yan activity may be critical for normal development of mesodermal derivatives. Similarly, overexpression of yan^{ACT} in the nonneuronal cone cell precursors of the developing eye inhibits expression of the cut cone cell marker. Thus, it is likely that yan functions as a very general inhibitor of differentiation and that inactivation of yan, presumably via phosphorylation by activated MAPK, must be a common effect of different signaling pathways.

Further indications of the complexity of yan function comes from analysis of the null phenotype of the gene, the details of which will be reported elsewhere (I. R. and G. M. R., unpublished data). Based on genetic, molecular, and histochemical data, the original yan^{1} allele, which is homozygously viable and produces extra R7 cells (Lai and Rubin, 1992), appears to be a strongly hypomorphic mutation; true loss-of-function *yan* alleles are embryonic lethal and exhibit a complex clonal phenotype in the developing eye (F. Karim, M. Therrien, D. Wassarman, H. Chang, T. Laverty, and G. M. R., unpublished data). Thus, *yan* appears to be an essential gene required for numerous aspects of normal development.

However, yan does not appear to be a universal inhibitor of differentiation. For example, embryonic ectodermal markers such as engrailed were found to be unaffected by overexpression of yan^{ACT}. Furthermore, heat shock–induced overexpression of yan^{ACT} during postembryonic development failed to produce any bristle or wing phenotypes, indicating there are specific tissues, both neuronal and nonneuronal, whose differentiation is unaffected by unregulated yan activity. Given the importance of negative regulation during cell fate determination, it seems likely that other factors will be identified that will play a similar inhibitory role in regulating the differentiation of these cell types. One protein postulated to function in such a manner is Notch, a transmembrane receptor known to play an essential role in cell fate specification in numerous tissues throughout development. Based on the results of overexpressing a constitutively activated form of Notch in various developing tissues, including the eye, it has been proposed that Notch may act to delay or inhibit the differentiation of certain cell types, thereby preventing an inappropriate response to the numerous induction signals to which developing cells are exposed (Fortini et al., 1993). Although the details of the Notch-mediated signaling pathway remain to be elucidated, perhaps yan will offer a point of intersection between the Notch and Ras1/MAPK signal transduction cascades.

Ras1/MAPK-Mediated Regulation of Yan Activity May Involve Altering the Subcellular Localization and Stability of the Protein

In S2 cells transfected with the yan^{WT} expression construct, yan accumulates largely in the nucleus; however, if the cells are cotransfected with yan^{WT} and activated Ras1, yan becomes almost entirely localized to the cytoplasm. Furthermore, the yan^{ACT} mutant remains exclusively nuclear, even in the presence of activated Ras1.

These results suggest a model for yan function whereby unphosphorylated "active" yan resides in the nucleus, where it represses the transcription of specific genes required to promote a particular differentiation program. In response to specific intercellular signals, MAPK becomes activated and phosphorylates yan, resulting in a shift in subcellular localization, followed by rapid degradation of yan. This model predicts that, in vivo, yan should be transiently detected in the cytoplasm just as cells initiate their differentiation program, although to date anti-yan antisera reveal only that the protein is rapidly cleared from the nucleus. It is possible that the degradation machinery in S2 cells is not sufficiently robust to remove high levels of yan, whereas in the fly the system may manage to clear even abnormally high levels of protein so efficiently that yan is not detected in the cytoplasm.

Assuming the nucleus to cytoplasm translocation ob-

served in S2 cells accurately reflects the situation in vivo, it would identify an unusual mechanism for regulating transcription factor activity. Numerous examples of regulated alterations in subcellular localization of transcription factors have been reported, yet the predominant direction of transport is from the cytoplasm into the nucleus (reviewed by Whiteside and Goodbourn, 1993). Perhaps translocation from the nucleus to the cytoplasm will be a common mechanism for regulating negatively acting transcription factors such as yan, while transport in the opposite direction, from the cytoplasm to the nucleus, will be the common mechanism for regulating positively acting transcription factors.

It is also possible that the stability of yan is altered in response to phosphorylation. Thus, to account for the loss of nuclear expression observed in differentiating cells in vivo, phosphorylated yan should have a reduced half-life. In support of this idea, examination of the predicted yan sequence reveals it is rich in PEST sequences, with a particularly PEST-rich region covering the C-terminal ~50 amino acids (Lai and Rubin, 1992; Rogers et al., 1986). This is precisely the region that is deleted in the gain-of-function allele *yan*^{S2382}. The excessive stability of the truncated *yan*^{S2382} product is consistent with PEST-mediated degradation of yan acting as an essential component of Ras1/MAPK induced down-regulation of yan activity. Given that yan contains multiple stretches of putative PEST sequence scattered throughout the protein, only one of which is removed in the *yan*^{S2382} product, regulation of yan stability and degradation may be quite complex and tissue dependent.

Concluding Remarks

A critical aspect of normal development that is not yet fully understood is how cells integrate the complex array of positive and negative signals they receive to effect an appropriately timed developmental response. In this process, negative or inhibitory signals that prevent differentiation and maintain the competence of the cells to respond to the eventual correct signal are likely to be as important as the various inductive signals themselves. We have demonstrated a complex role for yan in inhibiting the differentiation of numerous cell types throughout development and have suggested a plausible mechanism whereby yan activity is down-regulated in response to Ras1/MAPK activation. Further identification and study of the activities and regulation of other proteins interacting with yan as well as the targets of yan transcriptional activity should further our understanding of this general timing mechanism that controls the competence of a cell to respond to inductive signals.

Experimental Procedures

Molecular Biology and P Element-Mediated Transformation

yan cDNA sequences (O'Neill et al., 1994) were subcloned into M13, and in vitro mutagenesis of the MAPK phosphorylation sites was performed using the Sculptor Mutagenesis kit (Amersham) and specifically designed primers. Incorporation of the mutation was confirmed by sequencing. Constructs were subcloned into the pRMHa-3 expression vector for S2 cell transformation and into the pGMR, pSEV, pUAS, and pCasPer-HS vectors for P element–mediated transformation (described by Rebay et al., 1993).

Transfections and CAT Assays

Transfections and CAT assays were performed as described by O'Neill et al. (1994).

Generation of Antibodies and Immunohistochemistry

MAb 8B12 was prepared from mice immunized with the yanA–pGEX fusion protein construct described by Lai and Rubin (1992). Fixation and staining of S2 cells, embryos, and imaginal discs were performed as described by Fehon et al. (1990, 1991). Anti-elav staining was performed as described by Lai and Rubin (1992). Anti-cut staining was performed using an affinity-purified rat polyclonal antibody provided by K. Blochlinger. Anti-twist staining was performed using a rabbit polyclonal antiserum provided by C. Nüsslein-Volhard via K. Anderson. Anti-engrailed staining was performed using a MAb provided by S. DiNardo. Appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were used at a 1:1000 dilution. Acridine orange labeling and scanning electron microscopy were performed as described by Hay et al. (1994).

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