Ras1 signaling and transcriptional competence in the R7 cell of *Drosophila*

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The receptor tyrosine kinase Sevenless determines R7 cell fate by activation of the Ras1 pathway in a subset of equivalent cells competent to respond in the *Drosophila* eye. We show that the *prospero* gene becomes transcriptionally activated at a low level in all Sevenless-competent cells prior to Sevenless signaling, and this requires the activities of Ras1 and two Ras1/MAP kinase-responsive ETS transcription factors. Restriction of high-level *prospero* expression to the R7 cell appears as a subsequent event, which requires Sevenless activation of the Ras1/MAP kinase pathway. We show that Phyllopod, a nuclear factor whose expression is induced by Sevenless, interacts with another nuclear factor, Sina, to form a complex, and that both factors are involved in upregulating transcription of the *prospero* gene in the eye. Ultimately, *prospero* expression is required for proper connectivity of R7 photoreceptor axons to their synaptic targets. Our results suggest that specific transcriptional responses are linked to the mode of activation of the Ras1/MAP kinase signal transduction pathway.

[Key Words: *Drosophila*; *prospero*; *sevenless*; Ras1; eye development]

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Receptor tyrosine kinases (RTKs) are involved in signaling between cells to regulate proliferation and differentiation. RTK signaling can be substituted by gain-of-function variants of Ras, MEK, and MAP kinase (MAPK), all of which are components of the same signal transduction pathway (for review, see Zipursky and Rubin 1994; Marshall 1995). This suggests that Ras-mediated activation of MAPK is a critical event in signal transduction from RTKs. Paradoxically, the Ras pathway is commonly required in many cell types during development, and yet activation of distinct RTKs generates different cellular responses. A central issue in understanding RTK signaling is how a cell-type specific response is generated by use of a common Ras pathway. In rat PC12 cells, the decision to differentiate into a neuron or to proliferate is guided by the duration of MAPK activation (Marshall 1995). In turn, this is regulated by the specific ligand-RTK interaction triggered in PC12 cells. In the *Drosophila* eye, specific responses are in part determined by cell-specific factors that function downstream of MAPK (Dickson 1995). Here we describe a gene, *prospero* (*pros*), that is necessary for cell-type specific differentiation in the *Drosophila* eye and is transcriptionally regulated by multiple signals that activate the same Ras1 pathway.

The *Drosophila* eye develops by the reiterative patterning of ~800 ommatidia initiated in the larval eye imaginal disc. Differentiation occurs progressively from posterior to anterior across the eye disc, with the leading edge of differentiation marked by an indentation called

the morphogenetic furrow. Assembly of each ommatidium behind the furrow occurs by the sequential recruitment of undifferentiated cells into distinct developmental programs (Zipursky and Rubin 1994). The R7 photoreceptor cell is the last photoreceptor to be recruited into the ommatidial cluster, and is followed by recruitment of four non-neuronal cone cells. Although only one R7 cell develops in each ommatidium, there is a potential for five cells to develop with an R7 cell identity. These cells include the R7 and cone cell precursors and are collectively known as the R7 equivalence group. Determination of R7 cell fate is dependent upon signaling by the Sevenless (*Sev*) RTK in response to an inductive interaction with the neighboring R8 cell. This is transduced within the presumptive R7 cell by a cascade of proteins including Ras1, Raf, and Rolled/MAPK. All of the cells within the R7 equivalence group can be transformed to an R7 cell fate by activation at any point along the *sev/Ras1/rolled* signaling pathway (Basler et al. 1991; Fortini et al. 1992; Brunner et al. 1994b). Conversely, genetic disruption of the pathway results in adoption of a cone cell fate (Tomlinson and Ready 1987; Simon et al. 1991; Biggs et al. 1994).

Biochemical studies of vertebrate MAPK have shown that activated MAPK translocates to the nucleus where it phosphorylates different transcription factors (Marshall 1995). Nuclear target molecules of activated *rolled*/MAPK in the R7 equivalence group include the ETS-domain transcription factors, Yan and Pointed (*Pnt*). A variety of studies support a model in which the balance between the activities of *Pnt* and Yan determine whether a cell adopts a cone or R7 cell fate (Lai and Rubin 1992;

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O'Neill et al. 1994; Brunner et al. 1994a; Rebay and Rubin 1995). When uninduced, Yan and Pnt are unphosphorylated, with Yan acting as a repressor of target gene expression. Upon induction, Yan and Pnt are phosphorylated, resulting in inhibition of Yan's repression and stimulation of target gene expression by Pnt.

The *phyllopod* (*phyl*) gene is a possible target gene of the Ras1 pathway (Chang et al. 1995; Dickson et al. 1995). It is expressed in the R1, R6, and R7 precursor cells where it functions to promote their determination as photoreceptor cells. In the absence of *phyl*, they are transformed into cone cells. Misexpression of *phyl* in cone cell precursors transforms their fate to an R7 cell provided the *sina* gene is present. This is consistent with the demonstrated requirements for *sina* in normal R7 determination (Carthew and Rubin 1990). Both *phyl* and *sina* encode nuclear factors of unknown biochemical function.

These studies have identified a number of nuclear factors that play important roles in R7 determination. However, target genes of the factors that are specifically expressed in R7 cells and are required for R7 differentiation have not been identified. With the aim of identifying genes that function downstream of *sina*, we performed a genetic screen for mutations that dominantly enhance a mutant *sina* phenotype. One of the genes we identified, *pros*, has been shown previously to encode a putative transcription factor required for differentiation of the embryonic nervous system (Doe et al. 1991; Vaessin et al. 1991). We show that *pros* is required in the eye for proper connectivity of R7 photoreceptor axons to their synaptic targets in the brain. We also show that *pros* transcription is initially activated in all cells of the R7 equivalence group by activation of the Ras1 pathway. Yan and Pnt proteins appear to mediate these effects. Later, R7 cells specifically transcribe *pros* at higher levels in response to Sev activation of Ras1. Yan and Pnt are also intermediates of the upregulation of transcription. In addition, *Phyl* is involved in transcriptional upregulation of *pros*, albeit in a functionally redundant capacity. We show that *sina* is required for *phyl*'s effect on *pros* transcription and that *phyl* protein forms a specific complex with *sina* protein both in vivo and in vitro. These results imply that *pros* transcription is regulated by *Ras1* through two different mechanisms, one that initiates transcription in the R7 equivalence group and one that stimulates increased levels of transcription in the R7 cell.

Results

pros is a dominant enhancer of *sina*

Loss of *sina* activity in the R7 precursor cell leads to its transformation to a cone cell. In flies homozygous for a weak loss-of-function allele (*sina^d*), ~20% ($n=660$) of the ommatidia are missing the R7 photoreceptor cell (Fig. 1A). To identify genes that interact with *sina*, we screened a collection of lethal P-element enhancer trap insertions for loss-of-function mutations that dominantly enhance or suppress the *sina^d* phenotype. From

this screen, we identified an allele of *pros*, *l(3)10419*, which enhanced the *sina^d* phenotype, increasing the number of R7-less ommatidia to 50% ($n=316$; Fig. 1B). A transcript-null allele of *pros*, *pros¹⁰¹* (Vaessin et al. 1991), was tested for its ability to dominantly enhance the *sina^d* phenotype. Like the *pros* allele *l(3)10419* (*pros¹⁰⁴¹⁹*), *pros¹⁰¹* increased the number of R7-less ommatidia to 50% ($n=1420$; Fig. 1C). To demonstrate that a reduction in *pros* product was responsible for the enhanced phenotype, we increased the amount of Pros by generating transgenic flies in which a *pros* cDNA was put under the control of the *sev* enhancer (*sev-pros*). This enhancer is active in photoreceptor cells R3, R4, R7, the cone cell precursors, and (at low levels) in R1 and R6 (Zipursky and Rubin 1994). The enhancement of the *sina^d* phenotype was not observed in flies bearing one copy of the *sev-pros* transgene in a *sina^d/sina^d*; *pros¹⁰¹/+* background ($n=1859$; Fig. 1D). Therefore, reducing the dosage of the *pros* gene enhances the R7 cell defect caused by *sina^d*.

pros is required for R7 axon targeting

We wished to determine the homozygous mutant *pros* phenotype to further understand the function of *pros* in eye development. Since both *pros¹⁰⁴¹⁹* and *pros¹⁰¹* are homozygous lethal, we generated *pros¹⁰¹* mutant clones in a wild-type eye (Fig. 1E). Within the *pros⁻* clones, 23% ($n=215$) of R7 cells had an abnormal morphology, as evidenced by uncharacteristically small light-sensing organelles or rhabdomeres. We also examined the synaptic connections of *pros⁻* photoreceptors since *pros* plays a role in axon guidance within the embryonic nervous system (Doe et al. 1991; Vaessin et al. 1991). In a wild-type eye, the axons of the outer photoreceptor cells R1–R6 synapse onto a retinotopic unit in the first optic ganglion, the lamina (Fig. 1F). The axons of R7 and R8 bypass the lamina and retinotopically project to the second optic ganglion, the medulla. The R7 and R8 axons terminate at two distinct levels in the medulla. To visualize projections of wild-type R7 axons in the medulla, we filled retinal cells with horseradish peroxidase (HRP). The projections of R1–R6 were densely packed together in the lamina but the individual axons and terminals of R7 were clearly visible in whole mounts of the optic lobe (Fig. 1G). The axons formed optic columns and terminated at evenly spaced intervals to a unique layer of the medulla. HRP eye fills of *sev* mutant flies confirmed that these axon projections were from R7 cells (Fig. 1H). We filled retinal cells within *pros¹⁰¹* mutant clones and observed a disordered projection pattern (Fig. 1I). R7 axons were irregularly spaced, followed abnormal paths, and terminated at multiple layers in the medulla in a non-retinotopic fashion. The restriction of the phenotype to clones of mutant retinal cells indicates that *pros* is required in the retina for normal R7 axon connectivity.

pros expression is limited to the R7 equivalence group

The eye phenotype of *pros* mutants suggests that the gene is involved in determination and differentiation of

Figure 1. The phenotype of *prospero* in the eye. (A–D) Enhancement of *sina^d* phenotype by *pros*. Tangential sections from the dorsal-posterior region of compound eyes from (A) *sina^d*; (B) *sina^d; pros^{10419/+}*; (C) *sina^d; pros^{101/+}*; and (D) *sina^d; pros^{101/+}; P[sev-pros]* flies. Each ommatidium is composed of a cluster of photoreceptor cells, each with a light sensing rhabdomere which appears as a dark circular structure. The rhabdomeres of R1–R6 have a larger diameter than the central R7 rhabdomere. In a *sina^d* homozygote (A), the majority of ommatidia contain an R7 cell as indicated by the presence of R7 rhabdomeres. One ommatidium missing an R7 rhabdomere is marked by an arrowhead. Removing one functional copy of the *pros* gene prevents the formation of significantly more R7 cells (B,C). The expression of *pros* under the direction of the *sev* control elements suppresses the phenotype of the *pros¹⁰¹* allele and restores an R7 cell to most ommatidia (D). (E) Tangential section through a clone of homozygous *pros¹⁰¹* cells in the eye. A mixture of pigmented wild-type and unpigmented *pros⁻* cells are seen. In the center of the clone lie homozygous *pros⁻* ommatidia. Some of the mutant ommatidia have smaller sized R7 rhabdomeres than wild type. Arrowheads mark two clusters with small R7 rhabdomeres. The rhabdomeres of outer photoreceptors appear normal. The positioning of the R8 rhabdomere appears to be normal in these ommatidia, as indicated by exami-

nation of more-basal sections. Therefore, it is not likely that the loss of R7 and misplacement of R8 cells that accounts for the phenotype. (F–I) *pros* alters the R7 retinotopic map. (F) A transverse section through an adult head. The projections of R7 axons were visualized by application of HRP to particular locations in the retina, which resulted in localized filling of photoreceptors. Superficial and localized application ensures that only a small cluster of neighboring R7 cells (sometimes only one) and not R8 cells will be filled (Buckles et al. 1992). (G) Whole mount of a wild-type brain with the retina and lamina removed. A small group of ommatidia were filled with HRP, and the terminals and distal axonal segments are seen in this plane of focus. Evenly spaced terminals form a row which corresponds to the terminal zone of the R7 axons. (H) Whole mount of a *sevenless* brain, as in G. The optical plane of focus is at the level of the medulla. No HRP-filled axons were detected, indicating that the filled axons observed in G originate from R7 photoreceptors. (I) Whole mount of brain from a mosaic fly containing a clone of *pros⁻* eye cells in which photoreceptors within the clone were filled with HRP. Only flies containing large *white⁻ pros⁻* clones (at least 50 ommatidia) were used and only flies with fewer than ten filled R7 axons were examined. The mutant axons project together as a group to the appropriate region of the medulla. However, they terminate at several different levels of the medulla and are not retinotopically ordered in a single terminal zone. The axon paths are grossly normal but meander significantly more than wild-type axons. In some clones virtually all termini were located in different zonal positions. In all clones examined $\leq 50\%$ of termini were positioned in a single layer, suggesting that at least half of the R7 cells connect abnormally. This is significantly more penetrant than the abnormal rhabdomere phenotype of mutant R7 cells in *pros* clones.

R7 photoreceptors. To examine the pattern of *pros* expression in eye imaginal discs, antibodies specific for the *pros* protein were generated. Antibody staining of eye discs revealed that *pros* protein is first detected in presumptive R7 cells of ommatidia ~7–8 columns posterior to the morphogenetic furrow (Fig. 2A,B). By columns 11–12 its expression is also detected in the four cone cells (Fig. 2C). Antibody staining is restricted to the nuclei of these five cells. Significantly, *Pros* is detected in R7 cells several hours prior to their overt differentiation into neurons, as marked by their expression of the neural antigen Elav (Fig. 2D). The timing of *pros* expression in R7 cells suggests that it is induced early in the R7 developmental program. The specificity of the antibody for *pros* protein was demonstrated by staining eye discs from *sev-pros* transgenic larvae. In these eye discs, *pros* should be detected in the cells which express *sev*: R3, R4, R7, and the four cone cells. The antibody specifically labeled the nu-

clei of these cells in *sev-pros* eye discs (Fig. 2E). Moreover, the antibody labeled cells in the developing nervous system of wild-type embryos in a pattern indistinguishable from the pattern reported previously for *pros* (Vaessin et al. 1991), whereas the antibody did not label any cells in homozygous mutant *pros¹⁰¹* embryos (data not shown).

The specific expression of *pros* in the R7 and cone cell precursors is primarily achieved through transcriptional regulation. We examined the expression pattern of *lacZ* in the enhancer-trap line *pros¹⁰⁴¹⁹*. The P element in *pros¹⁰⁴¹⁹* is inserted upstream of the transcription start site and expression of the *lacZ* reporter gene is restricted to the R7 and cone cells (Fig. 2F,G). Moreover, the onset of *lacZ* expression closely resembles the timing of *pros* expression as determined by antibody staining.

The initial level of *pros* protein in the presumptive R7 cell is equivalent to the level seen in the cone cells (cf.

Figure 2. Expression of *pros* in developing eyes. (A–E) Whole mounts of larval eye discs labeled with antibody against Pros. In A and E, discs are oriented with posterior up and are aligned to show similar regions of the disc. (A) Wild-type disc; (E) *P[sev-pros]* disc. No labeling is observed near the morphogenetic furrow (marked with an arrowhead) in A while staining of clusters of cells in E begins immediately adjacent to the furrow. These cells correspond to R3 and R4, as shown in the insert in E. (B–D) are higher magnifications of a wild-type disc labeled with antibody against Pros (shown in red). Anterior is to the right. (B) A basal focal plane showing weak labeling of nuclei from presumptive R7 cells as they are ascending in anterior ommatidia (marked with white arrowheads) and more intense labeling of R7 nuclei in ommatidia beginning three columns posterior. (C) An apical focal plane in a posterior region of the disc showing weak labeling of cone cell nuclei. (D) A disc labeled with antibody against Pros (shown in red) and antibody against Elav (shown in green). Strong staining in both channels appears yellow. The focal plane shows R7 nuclei (red or yellow) and other photoreceptor nuclei (green). (F,G) Larval eye disc from a *pros*^{10419/+} fly stained with X-Gal. High magnification view of staining of R7 cells (marked 7) at one focal plane (F) and cone cells (marked c) at a more apical focal plane (G). (H) A disc labeled with *pros* antibody coupled to FITC was optically sectioned (1 μ m sections) by confocal microscopy. Shown is an apical section of a single ommatidium showing cone cell labeling overlaid with a basal section showing R7 labeling. The signal contribution of the overlying equatorial cone cell was deleted from the composite to keep R7 signal strength constant. Fluorescence intensity was quantitated by NIH Image 6.0 and rendered on a color scale shown to the right. Numbers represent relative intensity values. (I–L) Pupal eye whole mounts (48 hr after pupation) of wild-type pupae labeled with anti-Pros antibody (I,J) and *pros*^{10419/+} pupae stained with X-Gal (K,L). Strong labeling of R7 nuclei with antibody and X-Gal observed at one focal plane (I,K) with labeling of underlying bristle neurons evident (arrowheads). A more-apical focal plane (J,L) shows weak labeling of cone cells. (M–P) Anterior is to the right. (M) Eye disc from a *P[sev-Notch^{act}]; pros*^{10419/+} fly stained with X-gal. Ommatidia viewed at a basal focal plane show two cells (marked with arrowheads) contacting each other in each cluster that stain at a level comparable to a wild-type R7. Apical focal planes not shown here exhibit four weakly staining cells typical of wild-type cone cell staining. No stained cell in the normal R7 position is seen. (N) Confocal image of a *P[sev-Notch^{act}]* disc labeled with anti-Pros antibody (green) and with the nuclear dye propidium iodide (red). Staining in both channels appears yellow. The R1, R3, R4, and R6 cells of one ommatidial cluster are labeled. (O,P) X-Gal strongly stains R1, R3, R4, and R6 cells in wild-type flies carrying the *H162-lacZ* enhancer trap insertion (O), but it stains two cells per ommatidium, R3 and R4, in a *P[sev-Notch^{act}]* background (P).

cell is equivalent to the level seen in the cone cells (cf. Fig. 2B,C). As the developing ommatidia mature, the level of *pros* protein in the R7 cell increases, whereas it remains constant in the cone cells. The increase of *pros* protein in R7 cells begins in column 11 which is coincident with the onset of *pros* expression in the cone cells. In the most mature ommatidia of the larval eye disc, there is a significant difference in levels of *pros* protein between R7 and cone cells (Fig. 2H). The level of *pros* protein in the R7 cell continues to increase as the ommatidia develop during pupal metamorphosis (Fig. 2I,J). *pros* expression is eventually turned off in all retinal cells between 56–64 hr after pupation (data not shown). The differential expression of *pros* protein in the eye was mimicked by a similar differential expression of *lacZ* in the *pros*¹⁰⁴¹⁹ enhancer trap line (Fig. 2K,L). This suggests that differential levels of *pros* protein in R7 and cone cells is achieved through differential transcriptional activity of *pros* in R7 and cone cells.

The restriction of *pros* expression to the R7 equivalence group is a consequence of the unique time at which these cells are recruited into the ommatidial program. Recruitment of other cells at the same time leads to their misexpression of *pros*. We overexpressed an activated form of the Notch receptor in defined cells, which transiently blocks their proper recruitment and causes them to adopt different fates (Fortini et al. 1993). In eye discs from flies carrying the *sev-N^{act}* gene, we observed two additional cells within each ommatidium that expressed *pros* at levels comparable to wild-type R7 cells (Fig. 2M). This result is consistent with the observation that two outer photoreceptor cells are developmentally delayed and adopt an R7 cell fate (Fortini et al. 1993). It suggests that any cell that is competent to receive a specific cue at this time in development can express *pros*. Interestingly, this period of competence appears to be fairly extended because developmental delay of the cone cell precursors by *sev-N^{act}* has no effect on their expression of *pros* (data not shown).

To determine the identities of the two ectopically stained cells, we used propidium iodide to label all of the nuclei in the eye disc and double-labeled with the Pros antibody. At the eight-cell cluster stage when the nuclei of R3, R4, R1, and R6 are at the apical surface, the Pros antibody labeled the two cells in the posterior position that by position correspond to R1 and R6 (Fig. 2N). The R1 and R6 precursor cells, developmentally delayed by *sev-N^{act}*, appear to be recruited into the R7 equivalence group and express *pros*. To verify that the R1 and R6 precursor cells were recruited into the R7 equivalence group, we examined the effect of *sev-N^{act}* on *seven-up* (*svp*) gene expression. *svp* is expressed in the outer photoreceptor cells R1, R3, R4, and R6 (Mlodzik et al. 1990; Fig. 2O). The addition of *sev-N^{act}* resulted in the loss of *svp* expression in the R1 and R6 cells (Fig. 2P).

Regulation of *pros* expression by the *Sev-Ras1* pathway

The R7 and cone cell precursors have an equivalent developmental potential that is restricted by Sev RTK ac-

tivity. This suggested the possibility that *sev* might induce *pros* expression to higher levels in developing R7 cells. We compared the *pros* expression pattern in wild-type and *sev* mutant eye discs (Fig. 3A,B). Cone cells in null-mutant *sev* discs expressed *pros* at a level comparable to wild type. However, no elevated level of *pros* expression was observed in the transformed R7 cells. Conversely, an average of three cells per ommatidium expressed elevated levels of *pros* in a constitutively activated *sev* mutant, *Sev^{S11}* (Fig. 3C). The positions of these cells suggest they are the endogenous R7 cell and cone cells transformed to an R7 fate. These results indicate that Sev signaling is not required for normal *pros* expression in cone cells, nor is it required for low level expression in the R7 cell. However, it is necessary and sufficient for upregulated expression of *pros*.

Sev signaling is mediated by Ras1. To determine the role of Ras1 in *pros* expression, a constitutively activated Ras1 mutant, *sev-Ras1^{V12}*, was examined (Fig. 3D). Like *Sev^{S11}*, this mutant exhibited elevated *pros* expression in the endogenous R7 cell and the cone cells that were transformed to an R7 fate. This was observed even in a

Figure 3. Regulation of *pros* expression by the Sev-Ras1 pathway. (A–D) X-Gal staining of *pros*^{10419/+} eye discs. All panels are oriented with anterior to the right. (A) Wild-type disc showing R7 cell staining; (B) *sev*^{d2} eye disc. Note that the transformed R7 cell staining (•) is equivalent to cone cell staining (marked c). (C) *Sev*^{S11} disc; (D) *P[sev-Ras1^{V12}]* disc. Arrowheads point to cells within one ommatidium that intensely stain with X-Gal. (E,F) Whole mounts of *P[sev-Ras1^{N17}]* eye discs labeled with anti-Pros antibody. The discs have been overstained to more readily visualize the weak labeling of cone cell nuclei. The region of the disc in E is ~7–10 rows behind the furrow, where Pros-labeled cells normally first appear. An R7 cell (arrowhead) is labeled at the same intensity as the cone cells. A reduced number of R7 precursor cells is labeled in this region of the disc. More posterior in E, a circle outlines a typical ommatidium containing two labeled cells that by position and morphology are indicative of cone cells. There is no evidence of intense R7 cell staining at this stage of development. The region of the disc in F is near the posterior edge where normally four cone cells are labeled. The circle in F highlights a typical ommatidium with two labeled cells that lie in the apical focal plane typical for cone cell nuclei.

sev null background (data not shown). Thus, *Ras1* is genetically downstream of *sev* in regulating *pros* expression. To examine the effects of removing *Ras1* function in retinal cells, a dominant negative form of *Ras1* (*sev-Ras1^{N17}*) was used. The eye phenotype of *sev-Ras1^{N17}* is the absence of R7 cells, presumably because of their transformation to a cone cell fate (Allard et al. 1996). We examined *pros* expression in *sev-Ras1^{N17}* mutant eye discs and found that ~50% of the cone and R7 cells did not express *pros*, nor did they express elevated levels of *pros* typical of an R7 cell (Fig. 3E,F). Therefore *Ras1*, like *sev*, appears to be both necessary and sufficient to activate high-level expression. However *Ras1*, unlike *sev*, is also required for low-level expression in both R7 and cone cell precursors.

Regulation of *pros* expression by MAPK-responsive transcription factors

To further define the role of the *Ras1* signaling pathway on *pros* expression, we examined the effects of mutations in two genes encoding ETS-transcription factors: *yan* and *pnt*. The activities of both factors are regulated by MAPK phosphorylation and both factors are critical for R7 fate determination (Brunner et al. 1994a; O'Neill et al. 1994; Rebay and Rubin 1995). We tested *pros* for dominant genetic interactions with partial loss-of-function alleles of *yan* and *pnt*. In flies carrying the viable allelic combination *pnt^{Δ88}/pnt^{T6}*, 25% ($n=820$) of the ommatidia are missing R7 cells (data not shown). The *pnt^{Δ88}/pnt^{T6}* phenotype was dominantly enhanced by *pros¹⁰¹*, leading to a roughening of the external eye (Fig. 4A,B). This was accompanied by a reduction in R7 cells from ~0.75 (± 0.03 S.D.) cells per ommatidium to ~0.55 (± 0.04 S.D.) cells per ommatidium ($n=1690$). Conversely, the *yan¹* rough eye phenotype was dominantly suppressed by *pros¹⁰¹* (Fig. 4C,D) and was accompanied by a reduction in supernumerary R7 cells from 1.8 (± 0.2) cells per ommatidium to 1.3 (± 0.1) cells per ommatidium ($n=1670$). The effect of *pros¹⁰¹* on these phenotypes was partial, suggesting that *pros* is not a major limiting component when *yan* and *pnt* activities are reduced.

To examine the effects of *yan* and *pnt* on *pros* expression, we stained mutant eye discs with anti-Pros antibodies. Partial loss-of-function *pnt^{Δ88}/pnt^{T6}* mutants contained a markedly reduced number of *pros*-expressing cells (Fig. 4E,F). Only two or three *pros*-positive cone cells per ommatidium were observed, and no elevated expression in R7 cells was observed. Thus, *pnt* is required for *pros* expression in both the cone cell and R7 cell precursors. In contrast, the partial loss-of-function *yan¹* mutant contained a number of cells of unknown identity that ectopically expressed *pros* in addition to the presumptive R7 and cone cells (Fig. 4G). The positions of the cells suggest they are recruited from the uncommitted cell population. This result is consistent with the ectopic transformation of uncommitted cells to an R7 fate observed in a *yan¹* mutant (Lai and Rubin 1992), and suggests that Yan normally represses *pros* expression.

Yan is hypothesized to be a repressor of gene transcrip-

Figure 4. Regulation of Pros expression by two MAPK-responsive factors. (A–D) Scanning electron micrographs of the compound eyes of (A) *pnt^{T6}/pnt^{Δ88}*; (B) *pnt^{T6}/pnt^{Δ88}, pros¹⁰¹/+*; (C) *yan¹*; and (D) *yan¹, pros¹⁰¹/+*. (E–G) Larval eye discs from (E,F) *pnt^{T6}/pnt^{Δ88}* and (G) *yan¹* flies labeled with anti-Pros antibody. Anterior is to the right. (E) *pnt* mutant discs are often missing the strongly labeled nuclei that correspond to R7 nuclei in wild type. (F) *pnt* mutant ommatidia have an average of three labeled nuclei (an example is circled) which lie in an apical focal plane typical for cone cell nuclei. (G) Ommatidia in *yan* mutant discs typically contain six and occasionally seven labeled cells at different focal planes. This apical focal plane shows several ommatidia with five labeled cells. Two of these ommatidia are indicated with arrowheads. Note the variable labeling intensity between cells. Often three or more cells are strongly labeled with the antibody. (H) *P[sev-yan^{ACT}]; pros¹⁰⁴¹⁹/+* eye disc stained with X-gal shows an average of two labeled cells per ommatidium. A typical ommatidium is highlighted with a circle. The apical positions of the nuclei and their morphology indicate that they are cone cells. The intensity of staining is approximately equivalent between cells with no consistent single cell staining more strongly than the others.

tion that is down-regulated when phosphorylated by MAPK (Rebay and Rubin 1995). If *pros* transcription is normally activated by Yan phosphorylation, then unphosphorylated Yan should constitutively repress *pros* transcription. A dominant activated form of *yan* (*yan^{ACT}*), in which all potential MAPK phosphorylation sites are missing, blocks cell differentiation in the eye and constitutively represses transcription of reporter genes in cell culture (Rebay and Rubin 1995). We examined *pros* expression in flies carrying the *yan^{ACT}* gene under control of the *sev* enhancer (*sev-yan^{ACT}*). Most ommatidia contained substantially fewer cone and R7

cells that expressed *pros*, and elevated *pros* expression was not observed in R7 cells (Fig. 4H). This suggests that activation of *pros* transcription in all cells within the R7 equivalence group requires the down-regulation of Yan activity through phosphorylation by MAPK in the R7 and cone cell precursors. This is also consistent with the established role for Ras1 in activating *pros* expression.

The suppression of *pros* expression by *sev-Ras1^{N17}*, *pnt^{Δ88}/pnt^{T6}*, and *sev-yan^{ACT}* mutants was not complete, and indicates that these alleles may contain some residual activity. Although this is very likely for the *pnt^{Δ88}/pnt^{T6}* mutant, it is not as clear for the other two genes. It is possible that the signal needed to initiate the low level of *pros* expression in the equivalence group can occur before the *sev* enhancer becomes active, and, therefore, before *yan^{ACT}* and Ras1^{N17} are synthesized in these cells.

Activation of *pros* expression by a parallel pathway

The experiments described above suggest that Yan and Pnt may act on *pros* transcription in response to Ras1 activity. It was unclear whether their activities are sufficient for Ras1 signaling, because nuclear factors such as Sina and Phyl are also required for R7 determination. Therefore, we examined various *sina* and *phyl* mutants to see whether these genes are required for normal *pros* expression. A *sina* null mutant expressed levels of *pros* in the R7 equivalence group comparable to wild type, with elevated levels in transformed R7 cells equivalent to or sometimes greater than that observed in wild-type R7 cells (Fig. 5A,B). Because *sina* is absolutely required for R7 determination, this result suggests that elevated levels of *pros* expression are not strictly coupled to R7 fate determination. It also contrasts with the observed absence of elevated expression in the transformed cells of a *sev* null mutant. We examined *pros* expression in *sev sina* double mutant eye discs to determine the relationship between *sev* and *sina* in regulating *pros* transcription. Expression in the double mutant resembled expression in the *sev* mutant (Fig. 5C), indicating that *sev* is epistatic to *sina*. Consistent with this result, the elevated expression of *pros* in transformed cone cells in a *Sev^{S11}* mutant was not significantly affected by *sina* null mutations (data not shown). These epistasis results suggest that *sev* acts downstream or in parallel to *sina*.

We also examined the effect of removing *phyl* function during R7 development. The transformed R7 cells in a *phyl* mutant expressed *pros* at a level comparable to wild type (Fig. 5D). This result suggests that *phyl* is not absolutely required for elevated *pros* expression. However, the presence of *phyl* in cells is sufficient to induce them to express elevated levels of *pros*. We examined eye discs from flies bearing the *phyl* gene under control of the *sev* enhancer (*2sev-phyl*). Ectopic expression of *phyl* in the cone cell precursors resulted in supernumerary cells that express *pros* at levels comparable to the R7 cell (Fig. 5E). These cells appeared by position to correspond to transformed cone cells. We conclude that expression of *phyl* in cone cells is sufficient to induce elevated levels of *pros*

Figure 5. Sina and Phyl activate *pros* expression. X-gal staining of *pros¹⁰⁴¹⁹/+* eye discs (A–C, E,F) or labeling with antibody against Pros (D). All panels are oriented with anterior right. (A) wild-type disc showing R7 cell staining; (B) *sina²* eye disc. Staining of cone cells (marked c) is equivalent to wild type, and staining of transformed R7 cells (one is marked with •) is somewhat stronger than wild type. (C) *sev^{Δ2}; sina* eye disc. Intense staining of transformed R7 cell is abolished and weak cone cell staining appears unaffected. (D) *phyl²/phyl⁴* disc. An average of one cell per ommatidium is intensely stained (*), which by position corresponds to a transformed R7 cell. Posterior in the disc, the ommatidial cell clusters become disorganized and up to five cells display weak staining (data not shown). Because R1/R6 adopt a cone cell fate in *phyl* mutants, these ectopically expressing cells may be transformed R1/R6 cells. (E) *P[2sev-phyl]* eye disc. Multiple cells per ommatidium are staining intensely with X-Gal. The position of these cells correspond to transformed cone cells. They all lie on the same focal plane in a position characteristic of R7 cells in this region of the disc. Two strongly staining cells within one ommatidium are marked with arrowheads. The increased number of highly expressing cells was accompanied by a decrease in the number of cells expressing lower levels of *pros* normally observed in cone cells. The transformation of cone cell identity in *2sev-phyl* mutants results in fewer cone cells per ommatidium (Dickson et al. 1995) which likely accounts for the loss of lower-expressing cells. (F) *P[2sev-phyl] sina²* eye disc. One cell per ommatidium which occupies the position of the transformed R7 stains strongly with X-Gal (*). The cone cells stain comparably to wild type.

expression. Possibly, one reason that *phyl* is not absolutely required for *pros* upregulation in the R7 cell is that its function is redundant; other factors compensate for its absence.

Increased expression of *pros* in transformed *2sev-phyl* cells required the *sina* gene. No supernumerary cells with elevated levels of *pros* were observed in eye discs from *2sev-phyl sina* flies (Fig. 5F). This result suggests that *phyl* requires *sina* activity to stimulate *pros* expression.

Sina protein forms a complex with *Phyl*

The close genetic interaction between *sina* and *phyl* suggested that the proteins may physically associate and

that this association may play an important role in regulating *pros* expression. To test this, we employed the yeast two-hybrid system that detects protein interactions [Gyuris et al. 1993]. Full-length Sina protein was fused to a transcriptional transactivation domain (Sina-TA) and Phyl protein was fused to a LexA DNA-binding domain (Phyl-LexA). Interaction was observed by both proteins together transactivating a *lacZ* reporter gene (Fig. 6A). In addition, both proteins together transactivated a Leucine reporter gene, resulting in transformant growth on Leu⁻ media (data not shown). Comparable transactivation was observed when the fusion domains were swapped, using Sina-LexA and Phyl-TA. Deletion of amino acids 108–400 of Phyl ($\Delta 108$) abolished the interaction, whereas deletion of amino acids 131–400 ($\Delta 131$) resulted in a strong interaction, as measured by *lacZ* transactivation. This suggests that amino acids between positions 108 and 130 are critical for interaction. The strength of the $\Delta 131$ interaction is 27-fold greater than the interaction with full-length Phyl. This difference may reflect a peculiarity of one of the constructs or may be of biological relevance. The interaction between Sina and Phyl is specific since bicoid fused to LexA only

Figure 6. Interaction of Sina and Phyl proteins. (A) Quantitative assays of β -galactosidase activity from the *lacZ* reporter gene in various yeast transformants. Exponential-phase yeast containing the indicated fusion proteins were assayed for β -galactosidase activity with ONPG substrate. LexA-Sina and LexA-Phyl are full-length fusion proteins; LexA-Bcd, a negative control fusion, expresses amino acids 2–160 of Bicoid [Gyuris et al. 1993]; TA-Sina and TA-Phyl are full length fusions to an acidic activation domain (TA); TA- $\Delta 131$ and TA- $\Delta 108$ are fusions of the TA domain to Phyl with amino acids 131–400 and 108–400 deleted, respectively. Activity was calculated as the difference in specific activity [Ausubel et al. 1993] between each transformant grown in galactose (induces TA-fusion protein synthesis) and each grown in glucose (TA-fusion synthesis uninduced). Values presented are the average of duplicate assays of 4–20 independent transformants. Error bars represent standard deviation. The error bar for Sina/Phyl $\Delta 131$ is ± 75 . (B) [35 S]methionine-labeled, in vitro translation of Phyl analyzed on a SDS-polyacrylamide gel. The protein was either electrophoresed directly following translation or following glutathione-sepharose adsorption in the presence of Sina-GST, GST, Yan119-GST, or Yan121-GST. Ten percent total *phyl* protein input was loaded into lane 1. The positions of molecular weight markers are given.

weakly transactivated *lacZ* in the presence of Sina-TA. To confirm the apparent in vivo association of Sina and Phyl, in vitro association assays were performed (Fig. 6B). Phyl protein was translated in vitro and incubated with a Sina-glutathione S-transferase (Sina-GST) fusion protein immobilized to glutathione beads. Phyl remained bound to immobilized Sina-GST following extensive washing of the beads, whereas it was not bound to immobilized Yan-GST or GST alone. Moreover, in control experiments Sina-GST did not associate with heterologous in vitro translated proteins such as TAF60, TAF30 α , and TFIIIE α (data not shown). These results establish that Phyl interacts specifically with Sina.

Discussion

The role of *pros* in photoreceptor development

We have found that *pros* is required for proper R7 differentiation and axon connectivity in the adult retina. It is not absolutely required for R7 fate determination because loss of *pros* function causes no significant transformation between different cell types. However, *pros* may participate in R7 determination because it genetically interacts with *sina*, *pnt*, and *yan* in forming R7 cells. *pros* appears to control neural connectivity indirectly by regulating gene expression because the protein is nuclear localized and contains a divergent homeo domain. Although *pros* is expressed in the cone cells at a low level, it is unclear if it is needed for their differentiation.

Regulation of *pros* expression

The expression of *pros* is initially activated in the five cells that comprise the R7 equivalence group of the eye disc. Later, R7 cells exhibit elevated levels of *pros* expression as a response to Sev-mediated activation while transcription remains constant in the cone cells. Several lines of evidence suggest that regulation occurs by a two-step process. First, *pros* expression is initially detected in the R7 precursor cells prior to the estimated point of time when *sev* is activated [Mullins and Rubin 1991]. Second, initial detection of *pros* expression in the R7 precursor cell of *sev* mutants occurs at the same time as in wild type. Finally, cone cell precursors transformed with activated *sev* exhibit an initial low level of expression followed by a high level of expression.

It is possible that there is a causal relationship between the initial phase of *pros* transcription and the subsequent ability to be induced by Sev. Mutations in *Ras1* and *yan* which block the first step also block the second step. It would imply that some genes such as *pros* are able to respond to induction only by having independently established transcriptional activity. This has been observed with MyoD activation during mesoderm induction in *Xenopus* [Rupp and Weintraub 1991]. Thus, competence of cells to respond to inductive signals could be regulated by the transcriptional responsiveness of genes. These genes might be required for determination or dif-

ferentiation. Some genes such as *phyl* may be regulated not by a two-step mechanism but by direct activation. Perhaps a single inductive interaction involves a combination of different modes of transcriptional activation. Our results suggest that after initiation of *pros* transcription, Sev-mediated induction leads to the accumulation of *pros* protein above a threshold level in the R7 cell. This level of *pros* protein might establish a positive feedback loop that stabilizes *pros* expression into pupal development, long after Sev signaling is complete. In cone cells, the low level of *pros* protein might lead to stable but low-level expression.

Transcription in the R7 equivalence group

Here, we report evidence implicating Ras1 signaling in the regulation of gene transcription in the R7 equivalence group. This is based on three findings that were made for *pros* transcription. First, a dominant negative form of *Ras1* can block *pros* expression in any cell of the equivalence group. Second, a constitutively repressive form of *yan* that is unable to be phosphorylated by MAPK can block *pros* expression in the equivalence group. Third, in the absence of *pnt* activity, cells in the equivalence group fail to express *pros*. The first result establishes that *Ras1* activity is required for *pros* transcription. The second result argues that *Ras1* activates *pros* transcription by downregulating *yan* activity through a modulation of MAPK activity. The third result argues that *pnt* activation is critical for *pros* transcription.

One simple hypothesis to account for the roles of these factors is that the basal level of *yan* activity is sufficient to repress *pros* transcription and the basal level of *pnt* activity is unable to activate *pros*. Activation of Ras1 in cells of the R7 equivalence group counteracts the repression of *pros* by *yan* (by stimulating MAPK to phosphorylate Yan) and activates *pnt* to regulate positively *pros* transcription (Fig. 7A). This model also explains our observation that the absence of *yan* causes *pros* expression in supernumerary cells. Several additional points about this model are worth noting. First, the nature of the signal that activates Ras1 in the R7 equivalence group is unknown. Possibly, the signal is mediated by contacts between uncommitted cells and differentiating photoreceptors in accordance with the sequential inductive mechanism operating at earlier stages of eye development (Zipursky and Rubin 1994). Formation of extra photoreceptor cells leads to formation of extra cone cells as a secondary event (Basler et al. 1991), possibly by increasing the number of cell contacts between differentiated and uncommitted cells. Second, activation of *pros* transcription depends on developmental timing. Ras1 is activated in all photoreceptor cells and yet only cells in the R7 equivalence group express *pros*. However, transient delay of R1 and R6 precursor cell fate determination leads to these cells expressing *pros*. We suggest that a signal timed to occur after R1 and R6 determination confers a specific transcriptional response within cells competent to receive the signal. This competence could

Figure 7. Model for the regulation of *pros* expression in the equivalence group. (A) Activation of Ras1 by an unknown mechanism results in the transcription of *pros*. Although our experiments do not distinguish whether Yan and Pnt act upstream or downstream from Ras1, we favor the view that they act downstream because of their demonstrated positions downstream of Ras1 in R7 determination. We propose that Ras1 acts through MAPK to alter the activities of the transcription factors Yan and Pnt. They, in turn, may bind to the ETS DNA binding sites upstream of *pros* to directly regulate *pros* transcription. (B) Within the presumptive R7 cell, Sev RTK is activated and signals through Ras1 to upregulate *pros* expression. Activation via Sev may result in a stronger or more sustained response of MAPK activity and modulate Yan and Pnt activity to a greater degree. The presumptive R7 cell also contains Phyl and Sina which interact, and together they also upregulate *pros* transcription. It is possible that synthesis of Phyl in the presumptive R7 cell is caused by activation of Sev and Ras1, with the participation of Yan and Pnt.

be manifested through use of alternative signaling pathways not normally utilized in the other photoreceptors or by inhibition of the signal response in cells already undergoing differentiation. Third, our experiments do not address the possibility that Yan and Pnt act antagonistically toward each other by direct competition for common binding sites in the *pros* gene. However, both purified Yan and Pnt proteins bind specifically to two 5'-flanking sequences upstream of the *pros* transcription start site (R.C. Kauffmann and J. Zhang, unpubl.). Possibly, Yan binds to regulatory sequences with higher affinity than Pnt, occupying these elements and occluding Pnt from the gene.

Transcription in the R7 cell

Expression of *pros* is upregulated in cells of the equivalence group by Sev induction. In Figure 7B, we outline a hypothetical pathway for the relationship between *sev* and *pros*. A critical intermediate is Ras1; constitutively activated Ras1 is sufficient to up-regulate *pros* transcription, while a dominant negative form of Ras1 blocks *sev* activation of *pros*. Likewise, *yan* and *pnt* are likely intermediates in *sev* signal transduction because absence

of *pnt* activity or constitutive *yan* activity blocks *sev*-mediated upregulation of *pros*.

The placement of *phyl* and *sina* into the pathway is more ambiguous. However, we propose that *sina* and *phyl* function in parallel to another pathway downstream of *Ras1*. There are two reasons for our proposal. First, only R7 cells that have activated *Sev* normally up-regulate *pros*. However, when *phyl* is ectopically synthesized in cone cells, *pros* is up-regulated in a *sev*-independent manner. This up-regulation depends on *sina* activity. Second, the absence of *phyl* or *sina* in an R7 cell has no effect on *sev*-dependent upregulation of *pros*. The first result argues that *phyl* activity is sufficient to up-regulate *pros* transcription provided *sina* is also present. The second result suggests that *sina* and *phyl* do not up-regulate *pros* transcription singlehandedly in an R7 cell. A second pathway makes their functions redundant. Possibly, the second pathway utilizes *yan* and *pnt*, which may be modulated to a greater degree in R7 than in other equivalence group members. In support of this hypothesis, it has been observed that *Yan* stability is lower in R7 than in cone cells (Rebay and Rubin 1995).

A simple hypothesis to account for placement of *sina* and *phyl* in a common pathway is that the two proteins must directly interact to be completely active. In this model, either protein by itself is inactive, but when complexed together they function synergistically to regulate transcription. Complex formation would normally be restricted to the R1/R6 and R7 cell subtypes, ensuring that synergistic activation is limited to these cells. Such a mechanism is reminiscent of muscle-specific gene expression that requires both ubiquitous and myogenic bHLH factors (Lassar et al. 1991). This model accounts for our finding that *Sina* and *Phyl* proteins interact in vivo and in vitro. An alternative model that also fits our data is that interaction between *Phyl* and *Sina* is transient, resulting in activation of *Sina*.

Concluding remarks

Ras1 signaling is required to regulate *pros* expression at low levels in the equivalence group and at high levels in R7. How does signaling through the *Ras1* pathway result in two different responses? In PC12 cells, treatment with NGF leads to neural differentiation, whereas treatment with EGF leads to cell proliferation (Marshall 1995). Both factors signal through *Ras* and MAPK activation. The addition of NGF results in an elevation of *RasGTP* levels and prolonged MAPK activation for several hours. In contrast, increased levels of *RasGTP* and activation of MAPK persist for only a short time following stimulation by EGF. Likewise, the signal that activates *Ras1* in the R7 equivalence group may lead only to transient activation of *Ras1* and, by inference, MAPK. Transient activation of the signaling pathway would result in modest activation of the *pros* gene. Conversely, activation of the *Sev* RTK may result in prolonged activation of *Ras1* and MAPK to generate higher levels of transcription of *pros* and initiate transcription of other target genes.

Materials and methods

Genetics

The following fly strains were used for genetic interactions: *sev-N^{Act}* (Fortini et al. 1993); *sev-Ras^{V12}* (Fortini et al. 1992); *sev-Ras^{N17}* (Allard et al. 1996); *yan¹* (Lai and Rubin 1992); *sev-Yan^{Act}* (Rebay and Rubin 1995); *2sev-phyl*, *phyl²*, and *phyl⁴* (Dickson et al. 1995); *Sev^{S11}* (Basler et al. 1991); *pnt^{Δ88}* and *pnt^{T6}* (Brunner et al. 1994a). The recessive lethal P-element insertion line *l(3)10419* or *pros¹⁰⁴¹⁹* was obtained from the Spradling enhancer trap stock collection. The cytological map position of *l(3)10419* was determined to be 86E (Berkeley Drosophila Genome Project). Complementation analysis of *l(3)10419* and *pros¹⁰¹* indicated they were allelic. The *l(3)10419* P-element inserted 5' of the gene in the promoter region as determined by Southern analysis. Clonal analysis in the eye was performed on *pros¹⁰¹* using the FLP/FRT system (Xu and Rubin 1993).

The *P[sev-pros]* transformation vector was made by cloning a 5.3-kb *pros* cDNA (Vaessin et al. 1991) into a *sev* expression plasmid (Chang et al. 1995). Transgenic lines were generated by P-element-mediated germ-line transformation.

Prospero antibodies

The start codon of *pros* cDNA was replaced with a DNA sequence encoding MetHis₆Gly₂ by PCR mediated site-directed mutagenesis. An *NdeI*–*BamHI* fragment from the engineered DNA corresponding to Met-1 to Ser-408 (Vaessin et al. 1991; GenBank accession number M81389) of the *Pros* protein was inserted into pET3c to generate pPros1. The *Pros1* protein was purified from bacterial lysate by Ni²⁺-NTA Agarose (Qiagen) affinity column and subsequently preparative SDS-PAGE for the immunization of mice (Harlow and Lane 1988). Affinity purified antibodies were prepared by passing serum over a *Pros1* affinity column and eluting with 0.1 M glycine-HCl (pH 2.5).

Histology and immunohistochemistry

Eye fills were done as previously described (Buckles et al. 1992). The adult heads were fixed and embedded, and sections were cut as described by Carthew and Rubin (1990). Samples were prepared for scanning electron microscopy as described by Kimmel et al. (1990) with the exception that hexamethyldisilazane (Ted Pella) was substituted for freon. The β-galactosidase activity of *pros¹⁰⁴¹⁹* discs was assayed with X-gal as described by Moses and Rubin (1991).

Immunolabeling of eye discs and pupal retinas by was done as described by Zheng et al. (1995). For immunofluorescence labeling, Cy3- and FITC-conjugated IgGs were used (Chemicon and Cappel, respectively). Staining of nuclei with propidium iodide was done as described in Neufeld and Rubin (1994).

Expression plasmids

Plasmids expressing Yan119–GST (amino-terminal region of Yan with amino acids 1–389) and Yan121–GST (carboxy-terminal region of Yan with amino acids 388–732) were provided by Z.C. Lai (Pennsylvania State University, State College). pPntC/GST was generated by ligating a 0.6-kb *SacI*–*SalI* fragment from pACpntP2 (O'Neill et al. 1994) into pGex-3X to generate pPntC/GST. The carboxy-terminal region of PntP2 from Ser-479 to Asp-718 is linked to GST through a single serine linker. pSina/GST was constructed by PCR amplification of *Sina* sequences from position 903 to 1851 (Carthew and Rubin 1990) and insertion into pGex-2T to create pSina/GST. *phyl* cDNA sequence was amplified by PCR from nucleotides 234–

1446 (Chang et al. 1995) and was inserted into pET3c to create pET/Phyl.

The Phyl two-hybrid expression plasmids were derived from the vectors pEG202 and pJG4-5 (Gyuris et al. 1993). Phyl PCR product (234–1446) was inserted into pJG4-5 and pEG202 to generate pJG4-5/Phyl and pEG202/Phyl, respectively. Deletion mutants Phyl Δ 130–400 and Phyl Δ 107–400 were constructed by a partial *Pst*I digestion of pJG4-5/Phyl and self-ligation to the *Xho*I site, resulting in removal of 3' coding sequences. The Sina two-hybrid expression plasmids were derived from pSina/GST by insertion of a fragment containing the entire *sina* coding sequence into pEG202 and pJG4-5 to make pEG202/Sina and pJG4-5/Sina, respectively. Met-1 of Sina is linked to the two-hybrid domains through a nine amino acid linker sequence (FELGTRGSP). All ligation junction sequences and complete sequences of PCR products were confirmed by automated DNA sequencing [ABI].

In vitro binding

GST fusion proteins were produced in strain *HMS174(DE3)-pLysS* induced with 1 mM IPTG and grown at 25°C for 3 hr in LB medium supplemented with 1 mM ZnSO₄. Proteins were purified from whole cell extracts as described (Ausubel et al. 1993) with the addition of a protease inhibitor cocktail (1 μ g/ml leupeptin, 1 mM benzamide, 1 μ g/ml pepstatin, 0.2 mM PMSF) to the lysis buffer. Phyl protein was produced by *in vitro* translation using pET/Phyl with the TNT Coupled Reticulocyte Lysate System (Promega). GST fusion proteins were prebound to glutathione–Sephadex (Pharmacia) such that the concentration of protein on beads was 0.2 mg/ml. The protein–bead complexes were equilibrated in Buffer B [50 mM HEPES (pH 7.4), 120 mM NaCl, 1 μ M ZnSO₄, 0.1% NP-40]. For *in vitro* binding, 12 μ l of equilibrated protein–bead complex was mixed with 150 μ l Buffer B, 7.5 μ l 10 mg/ml BSA, and 12 μ l of reticulocyte translate. The reaction was incubated at 4°C for 3 hr. Matrices were washed three times with 1 ml of Buffer B prior to electrophoresis and autoradiography.

Yeast two-hybrid association

Two-hybrid plasmids were transformed into the yeast strain EGY48 and selected as described (Gyuris et al. 1993). For one two-hybrid test, strains were tested for galactose-dependent growth on Leu[−] plates. For a second test, colonies were grown in liquid glucose/CM Ura[−] His[−] Trp[−] overnight and diluted into galactose/CM Ura[−] His[−] Trp[−] or glucose/CM Ura[−] His[−] Trp[−] to achieve log phase growth. β -Galactosidase activity was quantitatively assayed with ONPG as described (Ausubel et al. 1993).

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Kauffmann et al.

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