Annual Progress Report for Moran Foundation Funded Work

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Summary of Progress

The overall goal of our original proposal was to look for protein-protein interactions that played a significant role during eye development in *Drosophila*. Specifically, we expected that the product of the *dachshund* gene would physically interact with other proteins required for normal retinal development. Our approach was two-fold: First, we looked for evidence of association of the Dachshund protein with other proteins using in vitro biochemistry. Second, we proposed to look for interactions with the Dachshund protein using the yeast two-hybrid assay. Both assays were extremely successful and a paper describing these results is now being reviewed for publication by Cell. A copy of this manuscript entitled "Dachshund and Eyes Absent Proteins Form a Complex and Function Synergistically to Induce Ectopic Eye Development in Drosophila" is attached. A summary of this work and its significance are presented below.

We initially proposed to look for interactions between the Dachshund protein and Eyeless, another protein that plays an important role in eye development in all animals, including *Drosophila* and humans. Although we found an interaction between Dachshund and Eyeless in both assays, we have not yet finished this analysis. We have genetic evidence that an important interaction occurs between Dachshund and Eyeless and will be submitting a manuscript on this work within the next few months, depending on the results of pending experiments. As a result, this project is still active.

We also looked for interaction between the Dachshund protein and Eyes absent, another conserved protein required for eye development in flies and expressed in the developing retina in mammals. These experiments were strongly positive: Dachshund and Eyes absent proteins form a complex and function together to control initiation of eye development (please see the attached manuscript). Moreover, the interaction between these proteins is mediated by domains that are highly conserved in the human homologs of these genes. We have gone further to show that the vertebrate Dachshund and Eyes absent proteins also physically interact, suggesting that these proteins function together during human development as well. The most plausible interpretation of these results is that a Dachshund-Eyes absent complex acts to regulate the expression of both themselves and other downstream target genes in the retinal development pathway. Thus, we have taken the level of our analysis of eye development from that of gene function to the biochemistry of the molecules involved.

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Dachshund and Eyes Absent Proteins Form a Complex and Function Synergistically to Induce Ectopic Eye Development in Drosophila

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Running Title: Dac and Eya Form a Complex and Act Synergistically

Summary

The *eyeless, dachshund* and *eyes absent* genes encode conserved, nuclear proteins that are essential for eye development in Drosophila. Misexpression of *eyeless* or *dachshund* is also sufficient to induce the formation of ectopic compound eyes. Here we show that the *dachshund* and *eyes absent* genes act synergistically to induce ectopic retinal development and positively regulate the expression of each other. Moreover, we show that the Dachshund and Eyes absent proteins can physically interact through conserved domains, suggesting a molecular basis for the genetic synergy observed and that a similar complex may function in mammals. We propose that a conserved regulatory network, rather than a linear hierarchy, controls retinal specification and involves multiple protein complexes that function during distinct steps of eye development.

Introduction

The molecular mechanisms controlling retinal cell-fate determination are rapidly being deciphered. One of the most striking aspects of recent findings is that many of the genes controlling eye development have been highly conserved between insects and vertebrates and perhaps throughout much of the metazoa (reviewed in Bonini and Choi, 1995; Callaerts et al., 1997; Freund et al., 1996; Heberlein and Moses, 1995). A group of four genes, all encoding conserved, nuclear proteins, play prominent roles during the early steps of retinal development in Drosophila. These are *eyeless (ey), dachshund (dac), eyes absent (eya)* and *sine oculis (so). ey* and *so* encode putative DNA-binding transcription factors while *dac* and *eya* both encode novel proteins. Loss-of-function mutations in each of these genes cause flies to develop with no eyes (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994). Moreover, targeted

expression of *ey* or *dac* is sufficient to induce ectopic retinal development in several tissues in Drosophila (Halder et al., 1995; Shen and Mardon, 1997). Strikingly, expression of a mouse homolog of *ey* is also sufficient to induce ectopic eye formation in Drosophila (Halder et al., 1995) and *ey* homologs are required for normal eye development in mammals (Glaser et al., 1992; Hill et al., 1991; Ton et al., 1991). In addition, homologs of *dac, eya* and *so* are expressed in the developing vertebrate retina (Oliver et al., 1995; Xu et al., 1997; G.M., manuscript in preparation). These results suggest that the function of these genes has been conserved for more than 500 million years since the divergence of insects and vertebrates and have lead to the proposal that visual systems throughout the metazoa may have a single common ancestor (Beverley and Wilson, 1984; Glardon et al., 1997; Halder et al., 1995; Tomarev et al., 1997). Nevertheless, the molecular mechanisms by which these genes act remain obscure.

The adult Drosophila compound eye is a precisely organized array of about 750 repeated units, called ommatidia. Each ommatidium contains eight photoreceptor cells and a set of non-neuronal accessory cells, including lens-secreting cone cells, pigment cells and interommatidial bristles (Tomlinson and Ready, 1987a; Tomlinson and Ready, 1987b). The adult eye is derived from a structure called the eye imaginal disc. During larval development, cells in the eye disc proliferate but remain largely undifferentiated until the beginning of the last or third instar larval stage (Wolff and Ready, 1993). Then, cells at the posterior margin of early third instar eye discs begin to organize into ommatidial precursors (Wolff and Ready, 1993). Differentiation of all cell types in the eye disc occurs progressively from posterior to anterior and is synchronized by a wave of changes termed the morphogenetic furrow (MF) (Ready et al., 1976). The MF is characterized by alterations in cell shape, cell cycle and patterns of gene expression (Ma et al., 1993). Neuronal differentiation requires MF movement and is apparent immediately posterior to the MF as it progresses across the eye disc. Movement of the MF requires the function of the secreted signaling molecules encoded by *decapentaplegic (dpp)* and *hedgehog (hh). dpp* is required for initiation and progression of the MF (Chanut and Heberlein, 1997) while *hh* is required only for

furrow progression (Ma et al., 1993). However, ectopic expression of *dpp* or *hh* during larval development does not change cell fates from one disc type to another but causes patterning defects specific to each disc type instead (Basler and Struhl, 1994; Chanut and Heberlein, 1997; Heberlein et al., 1995; Nellen et al., 1996; Pignoni and Zipursky, 1997). Thus, *dpp* and *hh* act as general patterning signals to control morphogenesis in all imaginal discs and are not sufficient to specify retinal cell fates. Other genes or combinations of genes more specific to eye development must control this process.

ey and *dac* are two of the key players that govern retinal specification during normal eye development: both genes are necessary and sufficient for eye development. Three types of evidence suggest that *dac* functions downstream of *ey* (Shen and Mardon, 1997). First, *dac* is not required for *ey* expression. Second, misexpression of *ey* can strongly induce *dac*. Third, *dac* is required for induction of ectopic retinal development by targeted *ey* expression. Although ectopic expression of each gene is sufficient to phenocopy initiation of the MF, *dac* is much less effective than *ey* in this regard (Halder et al., 1995; Mardon et al., 1994). While *ey* can induce large ectopic eyes with complete penetrance on all major appendages, *dac* induces retinal development in only a minority of animals and primarily on antennal disc-derived structures (Halder et al., 1995; Shen and Mardon, 1997). Thus, *ey* must be able to regulate other genes that control retinal cell fate specification in addition to *dac*.

The *eya* gene is a good candidate as another target of *ey* function. Like *dac, eya* is expressed in the eye disc prior to MF initiation and is essential for eye development but is not required for *ey* expression (Bonini et al., 1993; Halder et al., 1995). However, *dac* is necessary for only a subset of functions for which *eya* is essential during normal eye development. Specifically, *dac* is required for initiation of furrow movement but not for progression or photoreceptor differentiation (Mardon et al., 1994). In contrast, *eya* is required for both MF initiation and progression (L. Zipursky, personal communication). In addition, while *eya* null mutant clones result in cell

overproliferation and completely block photoreceptor differentiation throughout the eye disc, *dac* mutant clones present this phenotype only when they include the posterior margin of eye disc (Mardon et al., 1994; L. Zipursky, personal communication). *dpp* is also required for MF initiation and progression and *dac* and *eya* are both likely to act downstream of *dpp* during normal eye development (Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997). Interestingly, while *eya* is necessary to maintain *dpp* expression in the eye disc, *dac* is not (Mardon et al., 1994; L. Zipursky, personal communication). Thus, *eya* is required for *dpp* expression and the control of cell proliferation throughout the eye disc, initiation and progression of MF movement and neural differentiation, while *dac* is required for only a distinct subset of these steps.

We have explored the functional and regulatory relationships among *ey, dac* and *eya.* We demonstrate that, like *dac, eya* is a target of *ey* activity and is required for *ey* function. Moreover, *dac* and *eya* show strong genetic synergy in their ability to induce ectopic retinal development. We provide evidence that a complex forms between the Dac and Eya proteins that is mediated by highly conserved domains, suggesting a molecular basis for the genetic synergy observed. We also show that while *eya* is genetically required upstream of *dac* during normal eye development, these genes are able to positively regulate each other at the level of transcription, indicating that a positive feedback loop is likely to exist between these genes. Finally, these results suggest a mechanism whereby complex formation between Dac and Eya may provide specificity to the function of Eya during MF initiation and that such interactions are likely to be conserved in vertebrates.

Results

While *dac* and *eya* play important roles in early retinal development the nature of the molecular and genetic association between these genes, if any, was not known. We examined the relationship

between dac and eya by misexpressing these genes employing the GAL4-UAS system and using ectopic eye induction as an assay.

***dac* and *eya* Act Synergistically to Induce Ectopic Eye Formation**

Like *ey* and *dac,* targeted expression of *eya* alone is sufficient to induce ectopic eye formation (Figures 1A and 1C). However, in contrast to *ey,* the penetrance of the ectopic eye phenotype induced by either *dac* or *eya* alone is incomplete and when induced, such eyes are small (Figures 1B and 1C). Even though *dac* expression is strongly induced in all imaginal discs, ectopic eye development is observed only on the anterior surface of the fly head ventral to the antenna and in just 56% (61/109) of animals examined (Figure 1B). Although no ectopic retinal structures are induced, the morphology of the legs and wings is severely disrupted (Figures 1F and 1J). Similarly, misexpression of *eya* causes ectopic eye development ventral to the antenna in only 34% (41/119) of animals inspected (Figure 1C). Although the gross morphology of the leg and wing is not significantly disrupted by ectopic *eya* expression, a tiny spot of red pigment is usually observed (>90% of cases examined) at the joint between the coxa and the femur of the leg and on the wing blade in 26% (30/115) of animals observed (data not shown). Thus, *dac* or *eya* alone are relatively weak inducers of ectopic retinal development in Drosophila.

In contrast, coexpression of *dac* and *eya* induces substantial ectopic eyes on the head, legs, wings and dorsal thorax of 100% of animals examined (n>100). On the head, the cuticle between the normal eye field and antennae is transformed into retinal cells such that the normal retinal field is expanded (Figure 1D). Large patches of pigment are induced on the dorsal surface of the femur and tibia of all legs, which are severely truncated (Figure 1G). Ommatidial structures are observed in each case (Figure 1H). Red pigment but no clear ommatidial morphology is also induced on the wing blade (Figure 1K). Ectopic eyes are also formed bilaterally on the dorsal thorax (notum) of the fly (Figures 1M and 1N), a place where no ectopic pigment or ommatidia are ever induced by

either *dac* or *eya* alone (data not shown). In all cases, these phenotypes are observed with 100% penetrance.

The phenotypes observed in imaginal discs are consistent with those observed in adults. Specifically, targeted *dac* or *eya* expression induces expression of the Glass protein, a visual system-specific marker (Moses et al., 1989; Moses and Rubin, 1991). Normally, *glass* is not expressed in the antennal, leg or wing imaginal discs (Figure 2A and data not shown). *dac* or *eya* alone induces ectopic Glass protein only in a small area of the ventral side of the antennal disc with about 50% penetrance (arrows in Figures 2B and 2C), but not in the leg disc or the part of the wing disc that gives rise to the dorsal thorax or notum (data not shown). hi addition, ectopic *eya* alone can induce small patches of *glass* expression in the pouch area of the wing disc with 25% penetrance (data not shown). In no case has ectopic Glass staining been observed in leg discs with either *dac* or *eya* alone. However, when *dac* and *eya* are coexpressed, ectopic Glass staining is induced with 100% penetrance along the ventral margin of the eye-antennal disc (Figure 2D), the dorsal half of the leg disc along the anterior-posterior compartment (A/P) boundary (Figure 2K) and along the A/P boundary of the dorsal wing disc (Figure 20). In each case, the sites of ectopic *glass* expression in discs correspond to the positions of ectopic retinal development observed in adults. Taken together, these data demonstrate that *dac* and *eya* show strong genetic synergy to induce ectopic retinal development in Drosophila.

Developmental Analysis of Ectopic Photoreceptor Differentiation

Synergistic induction of photoreceptor differentiation resulting from *dac* and *eya* coexpression can be seen in imaginal discs using a variety of neuronal markers. The nuclear protein Elav is expressed in all neurons of Drosophila (Robinow and White, 1991). Ectopic Elav-positive cells are induced in the antennal, leg and wing discs in response to *dac* and *eya* coexpression (Figures 2F, 2J and 2N). These ectopic neurons must be photoreceptor cells since the visual system-specific Glass protein is also induced in the same pattern (Figures 2D, 2K and 20). Moreover,

ectopic eyes observed in adults corresponding to these positions contain all of the normal cell types associated with the wild type eye, including pigment cells, lens-secreting cone cells and inter-ommatidial bristles (Figures 1D, 1H and 1N). In addition, the ectopic neurons induced by *dac* and *eya* misexpression send out axonal projections (Figures 2H, 2L and 2P). The axons of ectopic photoreceptors in the eye-antennal disc form a bundle that extends posteriorly into the eye imaginal disc. These axons appear to fuse with the axon tracts sent out by photoreceptors of the normal retinal field that exit through the optic stalk to synapse in the brain (arrowhead in Figure 2H). It is likely, therefore, that the fly can perceive light through ectopic photoreceptors formed in the eye-antennal disc as a result of *dac* and *eya* coexpression. In the leg and wing discs, ectopic photoreceptor axons are likely to fail to find any functional targets and retract during late larval and pupal development (Figures 2L and 2P). Ectopic neuronal marker induction is not observed in response to *dac* or *eya* alone in the eye, leg or part of the dorsal wing disc fated to give rise to the notum (data not shown). These data demonstrate that *dac* and *eya* act synergistically to induce cells to follow the normal retinal developmental pathway and elaborate all of the normal cell types found in the wild-type eye.

During normal retinal development, movement of the MF is required for photoreceptor differentiation (Heberlein and Moses, 1995; Heberlein et al., 1993; Ma et al., 1993). We looked for evidence of MF movement associated with ectopic eye formation using a *dpp-lacZ* reporter as an assay (Blackman et al., 1991). *dpp* expression marks the position of the MF as it crosses the eye imaginal disc and is not expressed along the dorsal or ventral margins of the eye disc anterior to the MF by the late third instar stage (Figure 2E). Specifically, *dpp* function is repressed in the anterior eye disc by *wg* (Treisman and Rubin, 1995). In addition, *dpp* is normally expressed in a wedge in the ventral half of the antennal disc (Figure 2E), along the A/P boundary in the dorsal half of the leg disc (Figure 21) and along the entire A/P boundary of the wing disc (Figure 2M). We found that coexpression of *dac* and *eya* induces ectopic *dpp* expression in the eye-antennal disc adjacent to the field of ectopic photoreceptors (arrow in Figure 2F). In the leg disc, *dpp*

expression is split by and forms a ring around the ectopic photoreceptors, again suggesting that an ectopic MF is initiated and propagates (Figure 2J). Although no obvious MF movement is observed in the wing disc, the level of *dpp* expression is significantly increased adjacent to the ectopic photoreceptor field (compare arrows in Figures 2M and 2N). These results indicate that ectopic expression of *dac* and *eya* may be sufficient to initiate MF movement along the ventral margin of the anterior eye-antennal disc, in the leg disc and perhaps the wing disc as well.

Dac and Eya Proteins Physically Interact

dac and *eya* both encode nuclear proteins that are expressed in similar temporal and spatial patterns in the eye imaginal disc, are required for MF initiation and, most importantly, show strong genetic synergy in our ectopic eye induction assay. These results lead us to hypothesize that the molecular basis for the genetic synergy observed may be a physical interaction between the Dac and Eya proteins. We used two independent methods to test and confirm this hypothesis: the yeast two-hybrid system and in vitro binding studies (Fields and Song, 1989; Harper et al., 1993). First, we fused full-length and truncated portions of the Dac protein to the DNA-binding domain of the yeast transcription factor GAL4 to make "bait" constructs and full-length and truncated portions of the Eya protein to the GAL4 transcriptional activation domain to make "prey" constructs (Figure 3). These constructs were transformed into yeast that contain a transgene with GAL4 binding sites upstream of the *lacZ* gene. We found that full-length Dac is sufficient to induce weak *lacZ* expression in the absence of a prey construct (Dac-F bait with no prey, lower panel, Figure 3). This result demonstrates that some portion of the Dac protein is able to act as a transcriptional activation domain in this assay. We have mapped the position of this activation domain to an amino-terminal portion of Dac by comparing the constructs Dac-N and Dac-NL. Specifically, the amino-terminal 165 amino acids (aa) of Dac, which contains a poly-glutamine rich region, does not activate transcription (Dac-N bait with no prey, Figure 3). In contrast, a construct that contains the first 392 aa of Dac is able to activate transcription, even in the absence of a prey construct (Dac-NL

bait and no prey, Figure 3). Thus, it is likely that an activation domain at least partially resides within amino acids 165-392 of the Drosophila Dac protein.

We also tested a fourth bait construct in this assay that contains a domain that is highly conserved in both mouse and human *dac* homologs (G.M., manuscript in preparation). When fused to the DNA binding portion of GAL4, this domain is incapable of activating transcription alone (Dac-C bait and no prey, Figure 3). However, when co-expressed with prey constructs containing either full-length or C-terminal portions of the Eya protein, strong activation of *lacZ* expression is observed (Dac-C bait and either Eya-C or Eya-F prey, Figure 3). Although the full-length Dac protein activates weakly on its own, a much stronger activation of *lacZ* is observed when co-expressed with the same Eya constructs (Dac-F bait and either Eya-C or Eya-F prey, Figure 3). The C-terminal portion of Eya (Eya-C) interacts with Dac while the amino-terminal portion does not (Eya-N), suggesting that the C-terminal conserved domain of the Eya protein (ED2) is contacting a portion of the Dac protein that is also conserved (Xu et al., 1997; Zimmerman et al., 1997).

We confirmed the physical interaction between Dac and Fyn using in vitro biochemistry. GST fusions of the conserved portions of Dac and Eya were used to bind in vitro translated, 35S-labeled Dac and Eya full-length proteins (Figure 4). GST::Eya was immobilized on glutathione-agarose beads and then incubated with in vitro translated, ^{35}S-labeled Dac protein. After extensive washing to remove nonspecifically adhered proteins, bound proteins were eluted, separated by SDS-PAGE, and visualized by autoradiography. While no ^{35}S-Dac bound to the control GST resin, it bound to the immobilized GST::Eya fusion protein (left panel, Figure 4). Similarly, ^{35}S-Eya can bind to immobilized GST::Dac but cannot be bound by GST alone. The same portions of Dac or Eya do not form homodimers in this assay (Figure 4).

Transcriptional Regulation of *dac* and *eya*

Since *dac* and *eya* are each able to induce ectopic eye development and act synergistically in this process, we investigated the regulatory relationships between these two genes and with *ey.* Determining the order of *dac* and *eya* function using traditional genetic epistasis analysis is not possible because loss-of-function mutations in each gene causes an eyeless phenotype. However, if *dac* and *eya* are acting in the same pathway, we expected that loss-of-function mutations in these genes would show dominant modification of the recessive eye phenotype of the other. Surprisingly, we have failed to observe any such interaction (data not shown). We were able to determine the regulatory relationship between *dac* and *eya* by analyzing the expression of each gene in wild-type and mutant backgrounds. We found that while *eya* expression in the eye disc does not depend on *dac* function, *dac* expression is greatly reduced in an eya^2 mutant background, demonstrating that *dac* expression requires *eya* activity (Figures 5A-5D). Similarly, *ey* induction of ectopic *dac* expression is greatly reduced in an eya^2 mutant background (Figures 5G and 5H). These results suggest that *dac* may function downstream of *eya.* Consistent with this interpretation, *eya* is unable to induce ectopic eye formation in a *dac* mutant background (data not shown).

Since *dac* expression is induced by both *ey* and *eya,* we explored the genetic and regulatory relationships among these genes. First, we found that *ey* misexpression is sufficient to induce *eya* (Figure 5E), suggesting that *eya* may be required for *ey* function. Indeed, ectopic retinal development driven by targeted *ey* expression fails to occur in an eya^2 mutant background (data not shown). We also found that induction of *eya* expression by *ey* does not depend on *dac* activity (Figure 5F), consistent with the idea that *eya* functions downstream of *ey* but upstream of *dac.* However, these genes do not act in a simple, linear pathway: targeted expression of *dac* and *eya* strongly induce the expression of each other (Figure 5I-5K) and *eya* is required for ectopic eye induction by *dac* (data not shown). In addition, misexpression of *dac* or *eya* is also sufficient to induce ectopic *ey* expression in the antennal disc (Shen and Mardon, 1997 and data not shown).

These results suggest that multiple positive feedback loops exist among these genes during normal eye development and raised the possibility that *ey* may be required for ectopic retinal induction by *eya* and *dac*. Indeed, ectopic eye formation driven by coexpression of *dac and eya* is completely blocked in an *ey⁴* mutant background, indicating that induction of *ey* is essential (data not shown). Finally, these regulatory events must occur at the level of transcription because *ey, dac* and *eya* all induce expression of *lacZ* reporter constructs specific for each gene (data not shown).

Discussion

eyeless, dachshund, eyes absent and *sine oculis* encode nuclear proteins that are required for early steps of Drosophila eye development (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994). Multiple vertebrate homologs of each gene have been identified and many of these are expressed in the developing vertebrate retina (Xu et al., 1997; Zimmerman et al., 1997; G.M., manuscript in preparation). The vertebrate homolog of *ey, Pax6,* is also required for eye development in humans and rodents (Glaser et al., 1992; Hill et al., 1991; Ton et al., 1991) and is sufficient to induce ectopic lens development in Xenopus (Altmann et al., 1997). Intriguingly, a mouse homolog of *so, Six3,* is also able to induce ectopic lens development in the teleost medaka (Oliver et al., 1996). Moreover, *Pax6* from a wide range of species is sufficient to induce compound eye formation in Drosophila, indicating that at least some of the crucial targets of *ey* function have been conserved throughout the metazoa (Glardon et al., 1997; Halder et al., 1995; Tomarev et al., 1997). In addition, some of the regulatory relationships among these genes are conserved between vertebrates and insects, suggesting that the regulatory hierarchy controlling visual system development has also been conserved. Specifically, expression of mouse *Eya1* and *Eya2* in the lens and nasal placodes during development requires *Pax6* function (Xu et al., 1997). Thus, studying the relationships among these four genes will not only help us to understand

mechanisms of cell-fate determination in general, but will also provide valuable insight regarding human retinal development.

Genetic Synergy During Development

In this paper, we have begun to decipher the relationship between *dac* and *eya* using both genetic and molecular approaches. We have shown that coexpression of *dac* and *eya* induce ectopic eye development on the dorsal thorax of the fly with complete penetrance; this phenotype is never observed with either *dac* or *eya* alone. Similar results are also observed on the head, wing and leg, nearly every place where expression of these genes is driven by *dpp-GAL4.* Moreover, ectopic eyes induced by *dac* and *eya* misexpression contain all of the normal cell types comprising the wild-type compound eye. Consistent with these adult phenotypes, targeted coexpression of *dac* and *eya* induces imaginal disc expression of visual system-specific and neuronal markers with 100% penetrance in locations never observed with either gene alone. Thus, coexpression of *dac* and *eya* cause phenotypes that are greater than the predicted sum of the effects of each individual gene. These results indicate that *dac* and *eya* act synergistically to direct retinal cell-fate specification.

Genetic synergy often plays an important role in cell fate determination. For example, loss-of-function mutations in one copy of either the *achaete-scute* gene complex (AS-C) or the *daughterless (da)* gene have no phenotype in Drosophila. In contrast, animals doubly heterozygous for mutations in AS-C and *da* prevent bristle formation, suggesting that these genes act synergistically during external sense organ development (Dambly-Chaudiere et al., 1988). In addition, ectopic expression of *scute* or *lethal of scute,* members of the AS-C, is sufficient to induce ectopic bristle formation and this phenotype is modified by changes in the dosage of *da* (Brand et al., 1993; Hinz et al., 1994). Similarly, ectopic expression of *dac* and *eya* display dramatic synergy in their ability to induce ectopic retinal development. The observation that loss-of-function mutations in *dac* and *eya* do not exhibit dominant genetic interactions suggests that either the dose of these genes is less

critical for cell-fate specification, as compared to AS-C and *da,* or that there exist mechanisms to compensate for changes in *dac* and *eya* dosage.

Although substantial portions of each appendage can be induced to follow a retinal pathway, the overall patterning in adjacent tissues is not grossly disrupted by *dac* and *eya* misexpression. Specifically, clear signs of proper anterior-posterior compartment formation and proximal-distal axis formation are evident in both the legs and wings of such animals (Figure 1). The fact that an ectopic eye can form in the middle of an otherwise normally patterned appendage points to the remarkable plasticity of cells during development. Normally, the eye-antennal disc gives rise to a portion of the head cuticle, in addition to the retina and antenna. While the mechanisms controlling the choice between these fates are not understood, lineage relationships do not play a significant role (Baker, 1978). It is possible that synergistic interactions between proteins such as Dac and Eya help to define the sharp boundary between the cuticle and retinal fields. Consistent with such a model, both *dac* and *eya* are not expressed in the margin of the eye disc that is fated to give rise to head cuticle (Figure 5) and ectopic expression of these genes is sufficient to efficiently transform cuticle into retina. While these interpretations are based upon ectopic expression studies, it is likely that *dac* and *eya* also act together during normal retinal development: both genes are expressed in a largely overlapping set of cells at the same time, they are both required for initiation of retinal morphogenesis and together can induce ectopic retinal development in nearly all tissues in which they are expressed. Moreover, the Dac and Eya proteins can physically interact.

Protein-Protein Interactions Mediating Synergistic Function

The molecular basis of the genetic synergy observed between *dac* and *eya* may be a physical interaction between the protein products encoded by these two genes. We found that Dac interacts specifically with Eya in two independent assays and that this interaction occurs through domains that are highly conserved in humans and mice. Since *dac* and *eya* encode novel proteins, the biochemical consequence of this interaction is not known. However, Dac and Eya are nuclear

proteins that can activate the transcription of each other. In addition, these genes are sufficient to initiate the entire cascade of gene activity required to generate the compound eye and are required throughout eye development as well (Mardon et al., 1994; L. Zipursky, personal communication). Given that two to three thousand genes are involved in fly eye development (Halder et al., 1995; Thaker and Kankel, 1992), the most straightforward model to accommodate these data is that Dac and Eya are directly involved in gene regulation, either as DNA-binding proteins or as co-factors for such molecules. Intriguingly, So and Eya also physically interact through conserved domains and synergistically induce retinal development in Drosophila (L. Zipursky, personal communication). Since So is a homeodomain-containing transcription factor, it is likely that interaction between Eya and So alters the affinity or specificity of So binding to downstream targets. Taken together, these results suggest that Eya can form complexes with both Dac and So during normal eye development. Regulatory proteins that form multiple complexes during different developmental processes have been well-documented. For example, the Da protein forms heterodimers with members of the AS-C to control external sense organ formation but also regulates sex determination in complexes with the Deadpan and Sisterless-b proteins (Cabrera and Alonso, 1991; Liu and Belote, 1995).

Since *eya* and *so* are both required for the control of cell proliferation, MF movement and neural differentiation, it is likely that an Eya-So complex functions throughout retinal development (L. Zipursky, personal communication). In contrast, *dac* is essential for only a subset of these steps. Thus, interactions between Dac and Eya may provide specificity to an Eya-So complex during MF initiation. That is, furrow initiation-specific genes may be regulated by a trimer consisting of Dac, Eya and So. Alternatively, it is possible that Dac-Eya heterodimers regulate a subset of genes required for MF initiation and/or ommatidial patterning that are distinct from others that are regulated by an Eya-So complex. Genes that function during other steps of retinal development are likely to be regulated by Eya-So heterodimers, perhaps with the assistance of other factors yet to be identified.

A Network of Genes Controls Retinal Cell-Fate Specification

Although several lines of *evidence* suggest that *eya* acts upstream of *dac, these genes do not act* in a simple, linear pathway. *dac* and *eya* are able to induce the expression of each other, indicating that a positive feedback loop may exist between these two genes during normal eye development. In fact, a positive feedback loop could explain the absence of dominant genetic interactions between *dac* and *eya* mutants. That is, positive regulation between *dac* and *eya* may be sufficient to compensate for two-fold changes in the dose of either gene. In addition, if *dac* acts only as a downstream effector of *eya* function, then we would predict that ectopic eye induction by *dac* misexpression would not require *eya* activity. To the contrary, *dac* induction of ectopic retinal development is greatly reduced in an *eya* mutant background, suggesting that complex formation of Dac and Eya is important for their function. Thus, instead of acting in a linear pathway, *dac* and *eya* regulate each other and function synergistically during eye development.

We have characterized the regulatory and functional relationships among *ey, dac, eya* and *so* at three levels of analysis: transcriptional, genetic and protein interaction. These data are incorporated into the following model of retinal cell-fate specification (Figure 6). At the level of transcription, loss- and gain-of-function experiments suggest that *ey, dac, eya* and *so* can be placed in a primarily linear pathway. However, several lines of evidence suggest that these genes function in an interactive network. First, we have observed multiple regulatory feedback loops among these four genes. In addition, ectopic eye induction by *ey, dac* or *eya* requires the function of each member of this group. Specifically, *dac, so* and *eya* are all required for retinal induction driven by *ey* misexpression (Shen and Mardon, 1997; R.C. and G.M., unpublished results). Similarly, *ey* is required for ectopic eye formation driven by all combinations of *eya, dac* and *so (R.C.* and G.M., unpublished results; L. Zipursky, personal communication). Finally, the protein products encoded by these genes appear to function in one or more complexes. Thus, it seems likely that protein complexes formed by Dac, Eya, So and perhaps others act to regulate themselves and other downstream targets required for eye development. Some proteins, such as Dac, may function to

provide specificity during distinct stages of eye development to protein complexes that act throughout retinal development.

Finally, the interactions among Dac, Eya and So are mediated through domains in each of the proteins that are highly conserved in mammals (G.M., manuscript in preparation; L. Zipursky, personal communication). Given that members of each of these gene families are specifically expressed in the vertebrate retina during development, it is likely that the synergistic function and protein complex formation we have observed in Drosophila also plays an important role during human retinal development as well. Synergistic regulation mediated by protein-protein interactions is likely to be a common mechanism to specify cell fates throughout development.

Experimental Procedures

Drosophila Genetics

All Drosophila crosses were carried out at 25°C on standard media. *dac* null mutant experiments were carried out using dac^3 and dac^4 mutant alleles (Mardon et al., 1994). The ey^4 and *eya2* mutations are eye-specific alleles that result in flies that have reduced or no eyes, respectively, but are otherwise viable and fertile (Bonini et al., 1993; Quiring et al., 1994). Previously published experiments with ectopic *dac* expression (Shen and Mardon, 1997) were carried out with a different transgene (UAS-dac^{21M5}) from that used in the present study (UAS-dac^{7c4}). Both transgenes were constructed and isolated as previously described but differ in their sites of insertion in the genome (Shen and Mardon, 1997). When induced by the same *dpp-GAL4* driver (Staehling-Hampton and Hoffmann, 1994), UAS-dac^{7c4} produces less detectable Dac protein and less severe phenotypes than UAS-dac^{21M5}. Specifically, in contrast to UAS-dac^{2115}, UAS-daC^{7c4} is unable to induce the formation of any ectopic pigment or ommatidia on the legs or thorax of flies when crossed to *dpp-GAL4.* The UA,S-dac^{7c4} line was used for all of the experiments reported in this paper. The UAS-eya line used in this study carries a full-length *eya* cDNA in pUAST and was a generous gift of Francesca Pignoni and Larry Zipursky. All experiments using the combination of UAS-dac^{7c4} and UAS-eya were conducted using a recombinant chromosome carrying both transgenes. Since these transgenes carry a white mini-gene, we were able to isolate recombinants using eye color as an assay. Putative recombinants were confirmed using single fly polymerase chain reaction (PCR) with primers specific for either *dac* or *eya.* Due to severe leg truncation resulting from UAS-dac misexpression, these animals fail to eclose from their pupal cases. Consequently, light microscope images of such animals were taken from dissected late pupae.

Immunohistochemistry

Imaginal discs were dissected and stained as previously described (Mardon et al., 1994). Anti-Eya (Bonini et al., 1993) stainings were performed using the same protocol except that imaginal discs were fixed in PLP for 20 min on ice. In addition, mouse anti-Eya antiserum was first preabsorbed to compete away non-specific staining as follows: 20 Ill of serum was incubated with 30 sets of *eya*^{2} mutant larval eye-brain complexes in one ml of PAXDG (Mardon et al., 1994) for one hour at room temperature (RT). Following preabsorption, this serum was used for staining with no further dilution. *dpp* expression was assayed using the BS3.0 *lacZ* reporter (Blackman et al., 1991). All discs were mounted in 80% glycerol in PBS.

Yeast Two-Hybrid Analysis

The yeast two-hybrid kit was a gift from Steve Elledge. An amino-terminal part of the *dac* coding region (corresponding to amino acids 1 to 366) was amplified by PCR so that an NcoI site was created at the AUG start codon. This PCR product was digested with NcoI to create a fragment representing amino acids 1-165 and was inserted into the unique NcoI site of the bait plasmid pAS2 (Harper et al., 1993) to create the Dac-N construct. This construct (Dac-N) was then digested with SacII *(dac* internal site) and Sall (pAS2 polylinker) and was used as a vector to clone a SacII, Sall fragment from the *dac* cDNA (Mardon et al., 1994), resulting in a full-length Dac construct (Dac-F). An NdeI fragment from Dac-F (representing amino acids 1 to 392) was inserted into the NdeI site of pAS2 to create the Dac-NL construct. A PCR fragment from the carboxy-terminal half of Dac (representing amino acids 653 to 850), flanked by artificial NcoI and BamHI sites, was cloned into pAS2 to yield the Dac-C construct. Similarly, an artificial NcoI site was introduced at the start codon of *eya* cDNA using PCR. The product was then digested with NcoI and BamHI (an internal site of the *eya* coding region) and cloned into the pACT2 prey plasmid (Durfee et al., 1993) to generate Eya-N (amino acids 1-223). A SmaI, Sall fragment (amino acids 209-760) from the *eya* type I cDNA (Bonini et al., 1993) was inserted into pACT2 to obtain Eya-C. This same fragment was also inserted into the Eya-N construct at the Smal and Sall

sites to make *the* Eya-F *construct. Yeast* transformations and X-gal *tests were* carried out as previously described (Harper et al., 1993).

In Vitro Biochemistry

To prepare the GST::Dac fusion protein, a fragment of the Drosophila *dac* cDNA (representing amino acids 711-869) was amplified by PCR such that artificial BamHI and Hindu sites flanked the product. Following digestion with BamHI and HindIII, this fragment was cloned into pGEX2 (Pharmacia) to generate pGST::Dac. Similarly, the GST::Eya fusion protein was prepared from the carboxy-terminus of *eya,* using PCR to amplify a fragment encoding amino acids 487-760 and creating an artificial EcoRI at the 5' end of the product. This fragment was then cloned into the EcoRI site of pGEX1 (Pharmacia) to generate pGST::Eya. pGST::Dac, pGST::Eya and pGEX1 (to generate GST alone), were then introduced into *E. coli* (strain BL21, Novagen). Recombinant proteins were purified from induced cultures (50 ptI■4 IPTG, 2 hrs, 30°C) as follows. One liter of bacterial culture was pelleted (4000xg, 10 min, 4°C) and resuspended in 20 ml of lysis buffer (PBS, 50 mM NaC1, 5 mM EDTA, 5 mM DTT, 1% Triton X-100, 1 mM PMSF, 5 µg/ml aprotinin, 5 tg/ml leupeptin) and sonicated for 1 min at 4°C. Lysates were pelleted again (10,000xg, 15 min, 4°C) and the supernatant was incubated (15 min, RT) with 2001.t1 of 50% glutathione resin (Pharmacia) per liter of original bacterial culture. Glutathione resin with bound GST proteins was pelleted (500xg, 3 mM, 4°C) and washed three times with 10 ml of lysis buffer and resuspended in 100 ill of binding buffer (20 mM Hepes-KOH (pH 7.7), 150 mM NaC1, 0.1% NP-40, 10% glycerol, 1 mM PMSF, 2 pg/ml aprotinin, 2 lig/mlleupeptin). ^{35}S-labeled Dac and Eya proteins were synthesized using a coupled in vitro transcription and translation kit (Promega) using *dac* or *eya* cDNAs as DNA templates (Bonini et al., 1993; Mardon et al., 1994). Translation products were separated from unincorporated label by passage over a 1 ml Sephadex G-25 column (Sigma). Labeled Dac and Eya proteins were incubated in 0.4 ml binding buffer with 101.t1 of glutathione resin containing 10 of bound GST, GST::Dac or GST::Eya for 2 hrs at 4°C. The resin was washed 3 times with 1 ml of binding buffer and labeled proteins were eluted by boiling

for 3 min in 25 41 of loading buffer, fractionated by SDS-PAGE and visualized by autoradiography (20-40 hr). About 4% of the labeled, full-length proteins were recovered.

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Figure Legends

Figure 1. *dac* and *eya* act synergistically to induce ectopic retinal development. (A) Ventral view of the head of an adult control fly carrying the *UAS-dac*^{7c4} and UAS-eya transgenes but without any GAL4 driver. (B to D) Ectopic eye induction (arrows) driven by *dpp-GAL4* (Staehling-Hampton and Hoffmann, 1994). UAS-dac ^{7c4} alone (B) or UAS-eya alone (C) cause very small ectopic eyes near the antennae. In contrast, *UAS-dac*^{7c4} plus UAS-eya (D) produces a large domain of ectopic retinal tissue that fuses with the normal eye field. (E) Wild-type leg. (F) Ectopic *dac* expression truncates the leg but does not induce eye development. Misexpression of *eya* alone has little effect on leg development (not shown). (G) Coexpression of *dac* and *eya* induces ectopic pigment on the dorsal side of all legs with complete penetrance. (H) A high magnification view of an ectopic eye on the leg where obvious ommatidial structures are visible. The inset shows the whole leg at the same magnification as in (E). (I) Wild-type wing. (J) Ectopic *dac* expression truncates the wing but does not induce retinal development. Targeted expression of *eya* alone has little effect on normal wing morphology but can induce very small patches of pigment on the wing blade in about 25% of animals examined (not shown). (K) Coexpression of *dac* and *eya* induces ectopic pigment on the wing blade in 100% of animals examined. (L) Lateral view of a wild-type notum (dorsal thorax). Expression of either *dac* or *eya* alone never induces retinal development in the notum (not shown). (M) Ectopic pigment is induced on the notum in 100% of animals expressing *dac* and *eya* together. (N) A higher magnification view of the ectopic pigment shown in (M). Scale bars in (E) and (F) are 100 pin and in (I) and (J) are 200 Rm.

Figure 2. Developmental analysis of ectopic photoreceptor induction. (A to D) Late larval eye-antennal discs were stained with an antibody to detect the visual system-specific Glass protein. No Glass staining is normally found in wild-type antennal discs (A). *UAS-dac*^{7c4} alone (B) or UAS-eya alone (C) can induce small amounts of *glass* expression in the antennal disc (arrows). Coexpression of *dac* and *eya* induces ectopic Glass expression anterior to the normal retinal field on the ventral side of the eye disc (arrow in D). (E and F) Late larval eye-antennal discs were stained for the neuron-specific Elav protein in brown and the MF marker *dpp-lacZ* in blue. Compared to wild-type (E), the combination of targeted *dac* and *eya* misexpression is sufficient to induce ectopic MF advancement from the ventral side of the eye and antennal discs and to cause substantial ectopic photoreceptor development (arrow in F). (G and H) The protein Neuroglian is present in all neurons and their axons and was detected using monoclonal antibody BP104 (Hortsch et al., 1990). In the wild-type, the only staining seen in the antennal disc is the larval Bolwig's nerve (G). *dac* and *eya* expressed together induce ectopic neurons in the eye and antennal disc which project axons that join those of the normal retinal field (arrow in H) and are likely to exit through the optic stalk (arrowhead in H) to synapse in the larval brain. (I and J) Late larval leg discs stained for Elav protein and *dpp-lacZ* expression. (I) Only a few neurons are normally observed in the wild-type leg disc and no extra neurons are observed with *dac* or *eya* alone (not shown). (J) Targeted expression of *dac* and *eya* together induces a large cluster of Elav-positive cells in the dorsal half of the leg disc along the A/P boundary. Glass (K) and Neuroglian (L) staining reveals that ectopic neurons induced in the leg disc by coexpression of *dac* and *eya* express Glass protein and extend axons as would be expected during normal photoreceptor development. (M) A wild-type wing disc stained for Elav protein and *dpp* expression. (N to P) Wing discs from late larvae expressing both *dac* and *eya* were stained for Elav and *dpp-lacZ (N),* Glass (0) and Neuroglian (P). Ectopic photoreceptor development is indicated (arrows in N to P). Posterior is to the left and dorsal is up for all discs. For panels (A) to (H), the eye disc is to the left and the antennal disc to the right.

Figure 3. Dac and Eya proteins interact in the yeast two-hybrid system. Portions of the Dac protein were fused to the GAL4 DNA-binding domain to create "bait" constructs (upper panel). Dac-F, full length Dac; Dac-N, N-terminal 165 amino acids (aa) of Dac, containing a glutamine-rich region (Q); Dac-NL, N-teiminal 392 aa of Dac; Dac-C, amino acids 653-850 from the carboxy-terminal half of the Dac protein. Portions of the Eya protein were fused to the GAL4 activation domain to create "prey" constructs (middle panel). Eya-F, full length Eya; Eya-N, amino-terminal 223 aa of Eya containing a glutamine-rich region (Q); Eya-C, carboxy-terminal portion of Eya beginning from amino acid 209. This portion of the Eya protein contains a highly conserved domain present in all three vertebrate *eya* homologs (ED2). All combinations of "bait" and "prey" constructs were transformed into yeast and then tested for activation of a *lacZ* reporter construct (lower panel). "-", no *lacZ* activity; "+" strong *lacZ* activity; "+/-" weak *lacZ* activity.

Figure 4. Dac and Eya proteins interact in vitro. GST alone (G) or GST fusions to Dac (D) or Eya (E) were used to bind in vitro transcribed and translated, ^{35}S-labeled Dac or Eya proteins (35D, ^{35}E, respectively). ^{35}S-labeled Dac binds to GST::Eya, but not GST alone or GST::Dac (lanes 1-3). ^{35}S-labeled Eya binds to GST::Dac, but not GST alone or GST::Eya (lanes 4-6). The in vitro translation products (lanes 7 and 8) and the GST fusions (lanes 9-11) before binding reactions are shown. MW indicates molecular weight standard in kilodaltons. Markers for lanes 1-8 are shown to the left and for lanes 9-11 to the right.

Figure 5. Regulatory relationships between *dac* and *eya* are complex. (A and B) *dac* is not required for *eya* expression. Wild-type (A) and *dac*^{3} null mutant (B) eye discs were stained with an antibody that specifically detects the Eya protein (Bonini et al., 1993). (C and D) *dac* expression is greatly reduced in *eya*^{2} mutant eye discs. Wild-type (C) and *eya*^{2} mutant (D) eye discs were stained with an antibody that specifically detects the Dac protein (Mardon et al., 1994). (E and F) *dac* is not required for *eyeless* to induce *eya* expression. Wing discs were dissected from late larvae carrying a *UAS-eyeless* transgene driven by *dpp-GAL4* in a wild-type (E) or in a *dac*^{3} null mutant background (F) and stained for *eya* expression. Eya protein is induced by *eyeless* misexpression even in the absence of *dac* function (arrows in E and F). (G and H) Ectopic *dac* expression induced by *UAS-eyeless* is greatly reduced in an *eya* mutant background. Wing discs were prepared from *UAS-eyeless, dpp-GAL4* larvae in either a wild-type (G) or *eya*^{2} mutant background (H). Dac protein induction by *eyeless* shows a strong requirement for *eya* function (arrows in G and H). (I to K) Misexpression of *dac* or *eya* can turn on the expression of each other. *eya* expression is induced in the ventral portion of the antennal disc in response to ectopic *dac* expression (arrow in I). Similarly, *dac* is induced by targeted *eya* expression (arrows) in the antennal disc (J) and the wing disc (K).

Figure 6. A model for retinal development in Drosophila. Arrows indicate positive transcriptional regulation. Whether *dac* induces *ey* directly, through induction of *eya* or by some other mechanism is not known ("?").

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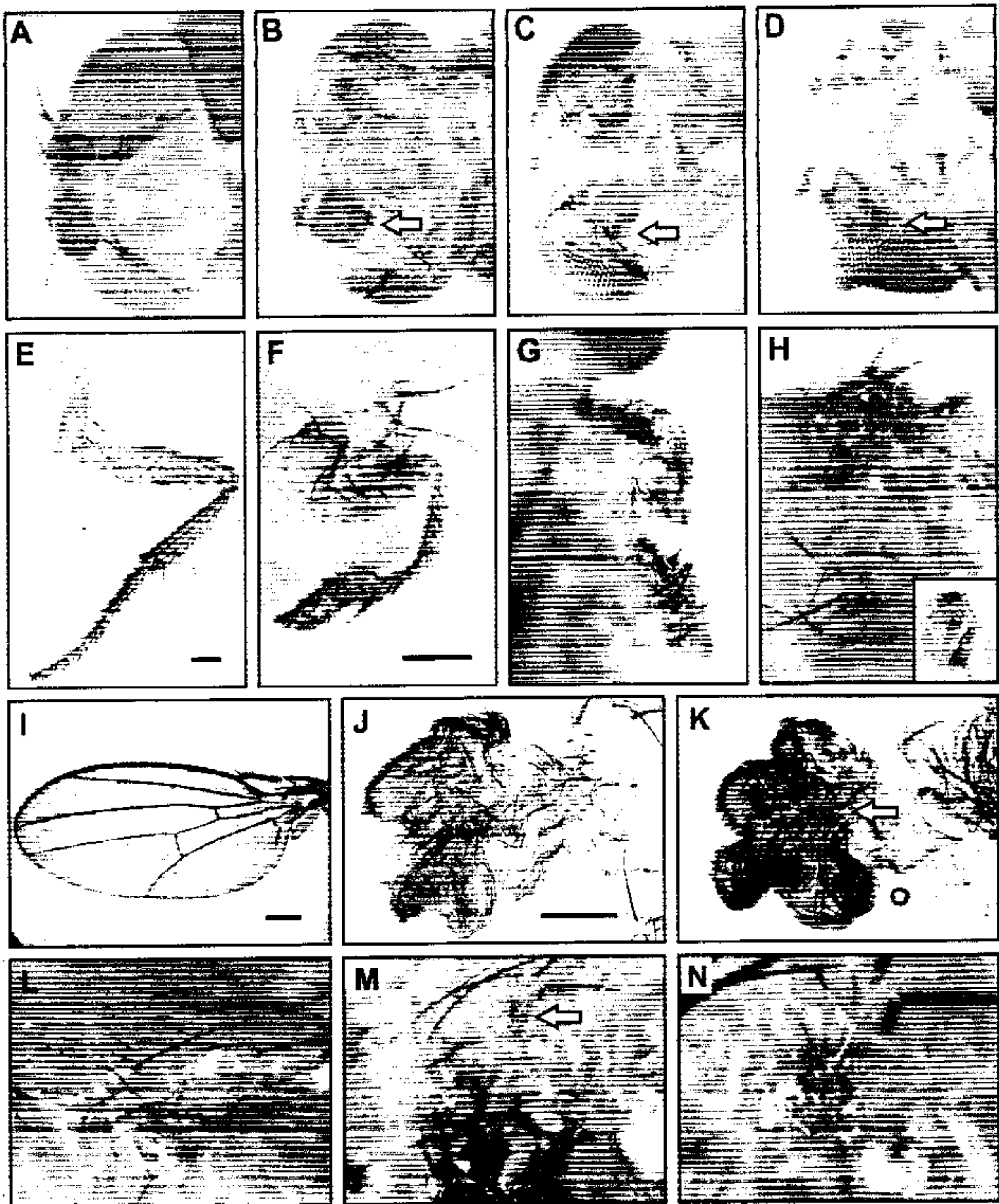
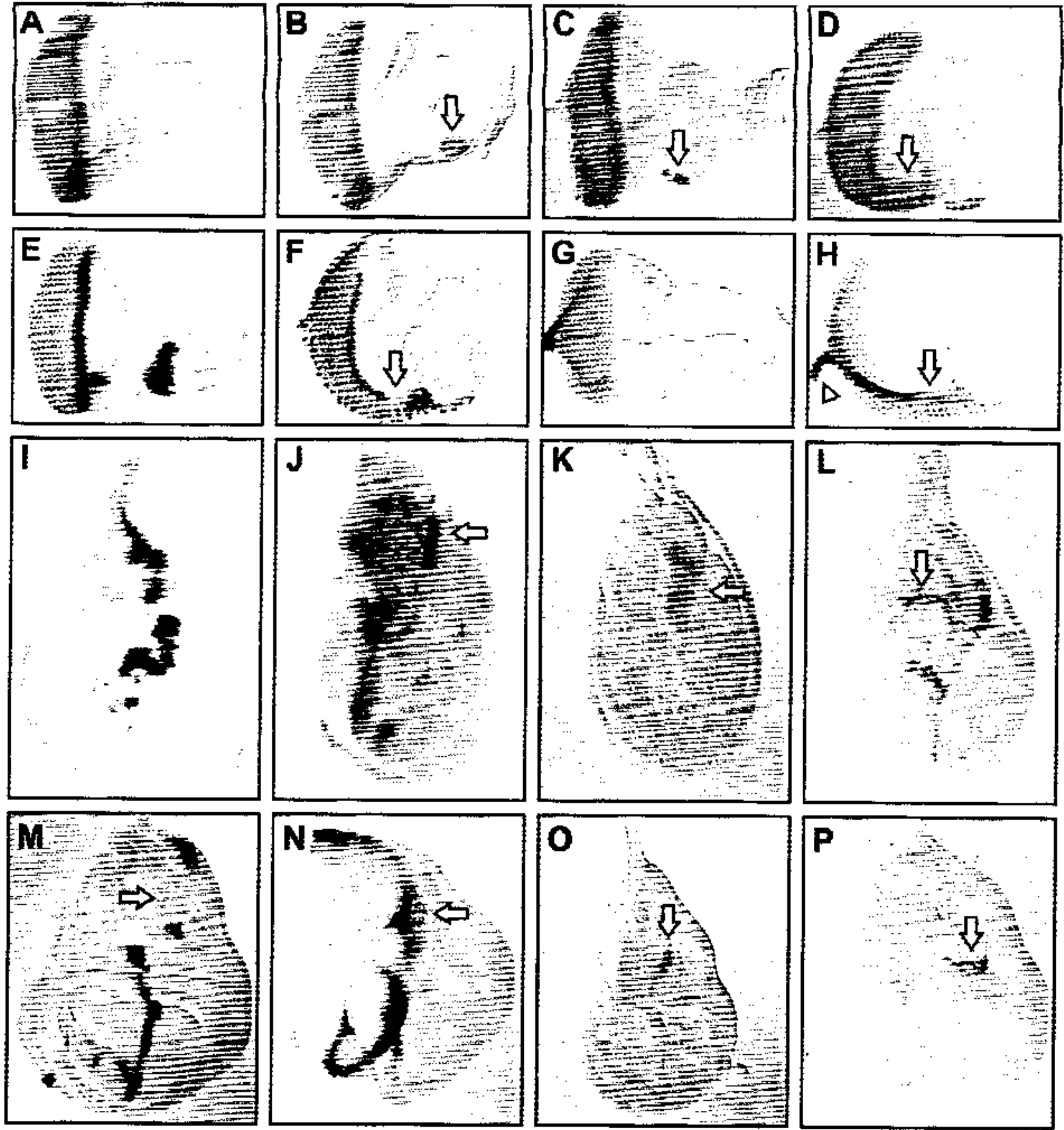
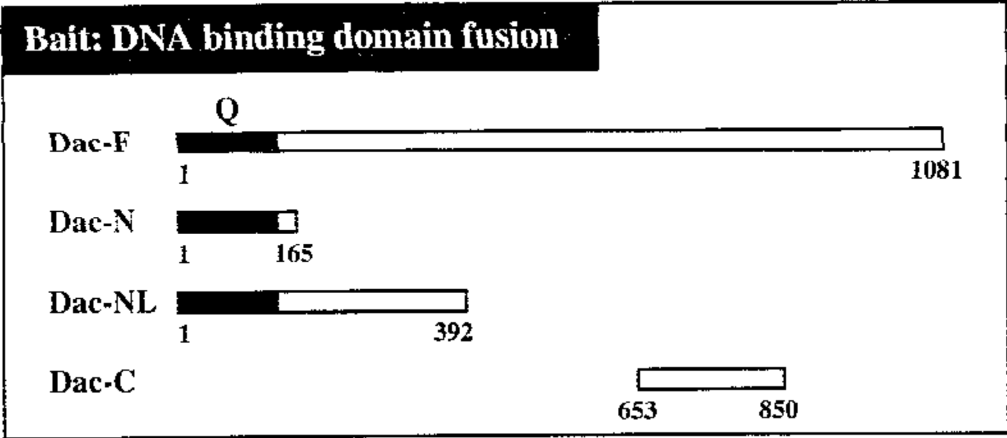
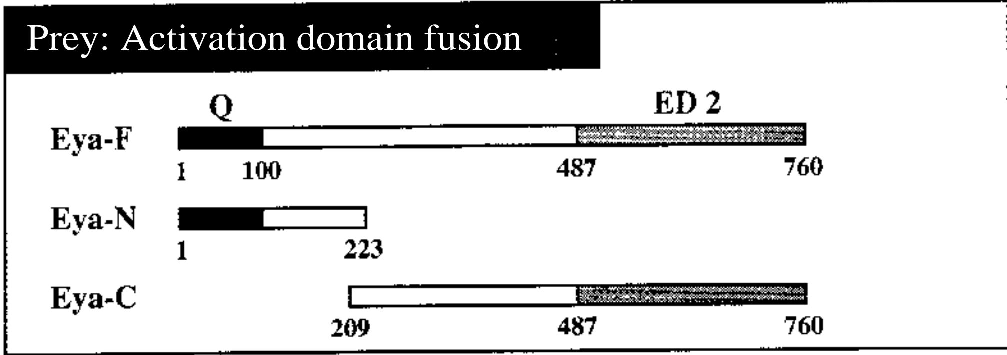


Chen et al., Figure 1



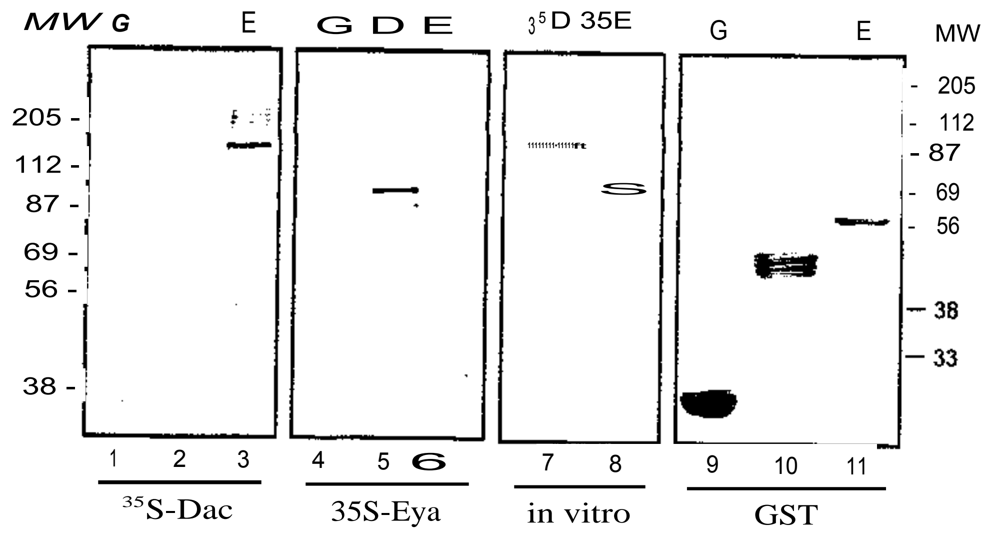
Chen et al., Figure 2



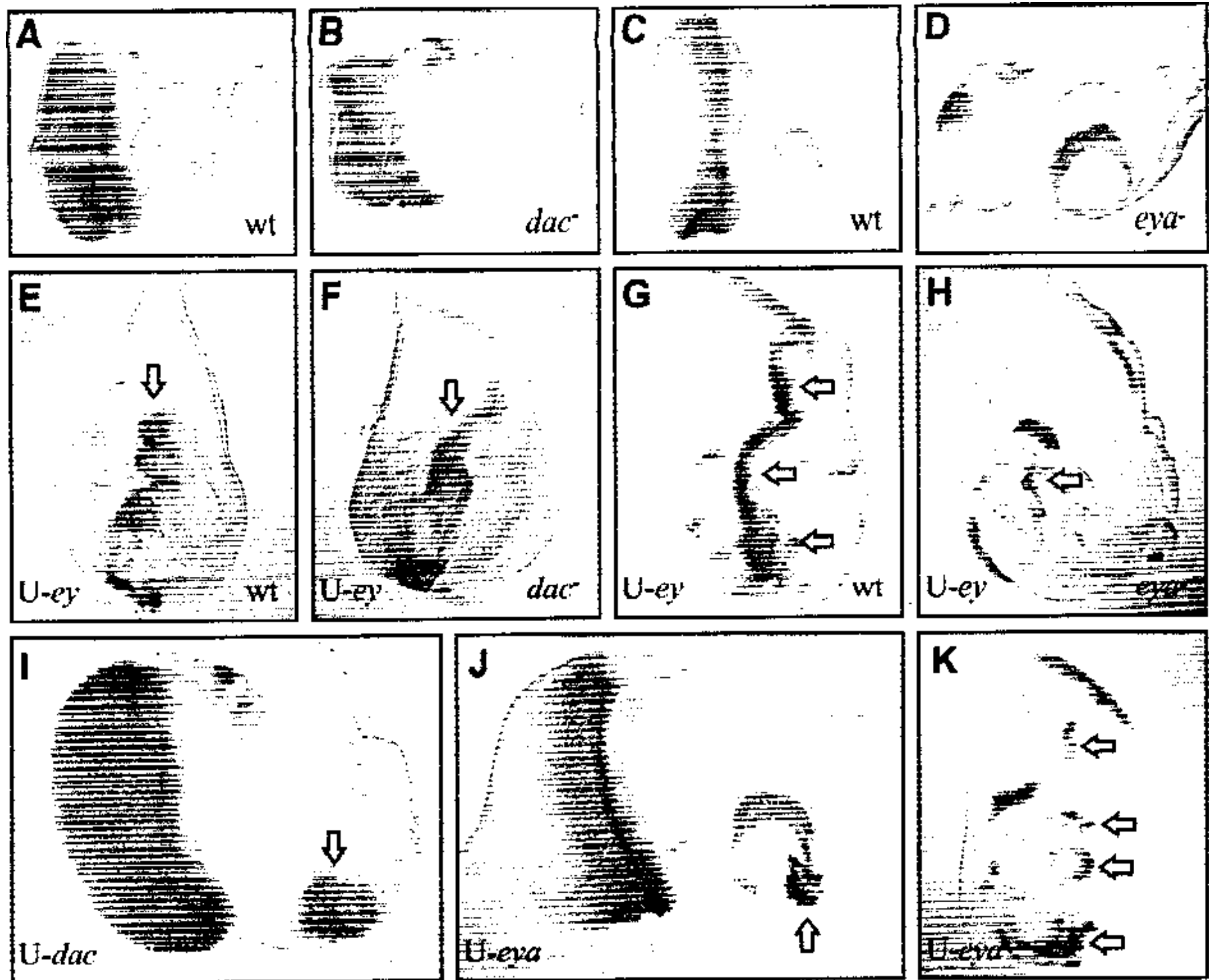


Prey\ Bait	Dac-N	Dac-NL	Dac-C	Dac-F
Eya-N	-	+	-	+1-
Eya-C	-	+	+	+
Eya-F	-	+	+	+
None	-	+	-	+/-

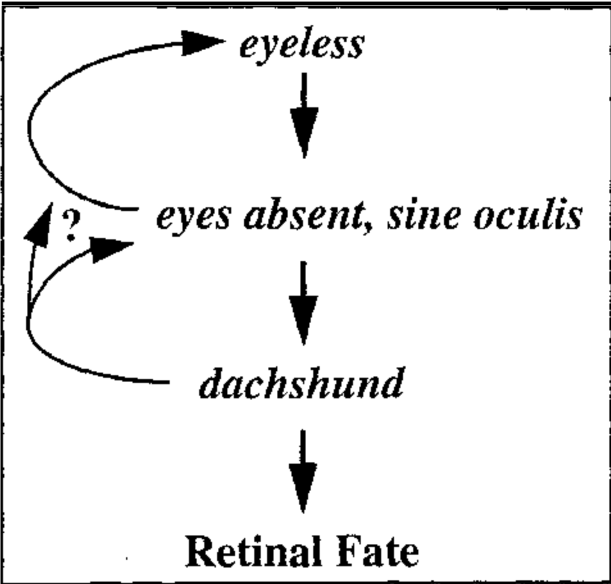
Chen et al., Figure 3



Chen et al., Figure 4



Chen et al., Figure 5



Chen et al., Figure 6