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Regulation of the retinal determination gene *dachshund* in the embryonic head and developing eye of *Drosophila*

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Abstract

The retinal determination gene *dachshund* is distantly related to the family of Ski/Sno proto-oncogenes and influences the development of a wide range of tissues including the embryonic head, optic lobes, brain, central nervous system as well as the post-embryonic leg, wing, genital and eye-antennal discs. We were interested in the regulatory mechanisms that control the dynamic expression pattern of *dachshund* and in this report we set out to ascertain how the transcription of dachshund is modulated in the embryonic head and developing eye-antennal imaginal disc. We demonstrate that the TGFβ signaling cascade, the transcription factor *zerknullt* and several other patterning genes prevent *dachshund* from being expressed inappropriately within the embryonic head. Additionally, we show that several members of the eye specification cascade influence the transcription of *dachshund* during normal and ectopic eye development. Our results suggest that *dachshund* is regulated by a complex combinatorial code of transcription factors and signaling pathways. Unraveling this code may lead to an understanding of how *dachshund* regulates the development of many diverse tissue types including the eye.

Keywords

Dachshund; Zerknullt; TGFβ; Eye; Embryonic head

Introduction

The compound eye of *Drosophila melanogaster* is a simple nervous system whose stereotyped development and near crystalline cellular architecture make it an excellent experimental system for elucidating the mechanisms that govern tissue specification and patterning (Ready et al., 1976). The retina begins its development during embryogenesis when a small group of cells invaginates from the lateral surfaces of the embryonic head and begins to express several genes that initiate eye specification (Cohen, 1993). The determination of the eye is controlled, in part, by the concerted activity of eight nuclear proteins that comprise the retinal determination or eye specification cascade (Bonini and Choi, 1995; Heberlein and Treisman, 2000; Kumar and Moses, 2001b; Pappu and Mardon, 2004; Weasner et al., 2004). These include the Pax6 homologs *eyeless (ey)* and *twin of eyeless (toy)*, the Pax genes *eyegone (eyg)* and *twin of eyegone (toe)*, the Six family members *sine oculis (so)* and *optix* as well as *eyes absent (eya)* which encodes a protein tyrosine phosphatase *and dachshund (dac)* which is distantly related to the Ski/Sno family of proto-oncogenes (Bonini et al., 1993; Cheyette et al., 1994; Czerny et al., 1999; Hammond et al., 1998; Jun et al., 1998; Mardon et al., 1994; Quiring et al., 1994; Seimiya and Gehring, 2000; Serikaku and O'Tousa, 1994). Together, the eight genes that code for these proteins appear to control many of the earliest steps in the construction of compound eye.

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The importance of these genes in eye development is underscored by the presence of functional orthologs in other seeing animals including mammals and the demonstration that several human retinal disorders such as Aniridia, bilateral anophthalmia and congenital cataracts result from molecular lesions within the human homologs of these fly genes (Hanson, 2001; Kumar, 2001; Tomarev, 1999; Weasner et al., 2004). Furthermore, studies in *Drosophila* and mice have placed these eight genes at the top of the regulatory hierarchy that controls eye formation (Gehring, 1996; Gehring and Ikeo, 1999). Loss-of-function mutations within members of the network leads to a drastic if not total reduction in eye development while ectopic expression of any individual member, with the notable exception of *so*, is sufficient to redirect the development of non-retinal tissues into an eye fate. Several lines of evidence have suggested that the members of the retinal determination cascade function together to promote normal eye development.

Contrary to the perception that the expression and activity of members of the retinal determination network is limited to the developing eye, each member of the network is normally expressed within and regulates the development of several non-retinal tissues (Aldaz et al., 2003; Bonini et al., 1998; Cheyette et al., 1994; Czerny et al., 1999; Fabrizio et al., 2003; Jones et al., 1998; Kumar and Moses, 2001a; Leiserson et al., 1998; Martini et al., 2000a; Quiring et al., 1994; Seimiya and Gehring, 2000; Serikaku and O'Tousa, 1994). For example, the retinal determination gene *dachshund* regulates the development of the embryonic head, brain, optic lobes and central nervous system as well as the adult brain, mushroom bodies, antennae, legs and genitals (Keisman and Baker, 2001; Kurusu et al., 2000; Mardon et al., 1994; Martini et al., 2000b; Noveen et al., 2000b; Rauskolb, 2001). These roles are in addition to the parts played by *dac* during eye development, which includes tissue specification, morphogenetic furrow initiation and photoreceptor cell fate selection (Chen et al., 1997; Mardon et al., 1994; Shen and Mardon, 1997). In all of the aforementioned tissues, DAC protein is distributed in a dynamic temporal and spatial pattern that hints at a complex level of upstream regulation (Pappu et al., 2005).

As is the case in *Drosophila*, homologs of *dachshund* that have been identified in a wide range of organisms have interesting expression patterns, appear to be subject to intricate regulatory influences and are predicted to play major roles in the development of several tissues including the eye, ear, central nervous system and limbs. Dach genes have been isolated in the flour beetle, zebrafish, teleost fish, cricket and amphioxus, mouse and human (Candiani et al., 2003; Caubit et al., 1999; Davis et al., 1999; Davis et al., 2001b; Hammond et al., 2002; Hammond et al., 1999; Heanue et al., 2002; Inoue et al., 2004; Kozmik and Cvekl, 1999; Kozmik et al., 1999; Loosli et al., 2002; Prpic et al., 2001). While the effects of down-regulating *dachshund* is well documented in several instances, the role that mouse and human Dach genes play in development and disease remains elusive (Backman et al., 2003; Davis et al., 2001a). Understanding how DACH proteins function and how their distribution is regulated during development is still an open paradigm.

We set out to identify the upstream transcription factors and signaling pathways that regulate *dac* expression. Here we (1) describe the expression pattern of *dachshund* within the developing head at stage 9 of embryogenesis; (2) demonstrate that the transcription factor *zerknullt* and the TGFβ signaling cascade are essential to preventing the inappropriate expression of *dachshund* within the head; (3) present evidence that *zerknullt* can repress *dachshund* expression through both autonomous and non-autonomous mechanisms; and finally (4) describe the regulatory relationship between *dachshund* and each of the other eye specification cascade members. Two recent papers have demonstrated a crucial role for TGFβ signaling in the expression of genes within the neuroectoderm, a region in the embryonic head that encompasses the *dac* expression domain. The activity of the Dpp pathway occurs via its downstream target *zen* and is antagonized by the EGF Receptor signaling pathway (Chang et

al., 2001, 2003). The experimental evidence presented here supports the model proposed in these papers and extends them to a member of the retinal determination cascade that plays a pivotal role in the development of numerous tissue fates.

Experimental procedures

Fly stocks

The following stocks were obtained for the experiments performed in this report: UAS-ey, UAS-toy, ey-GAL4 (gift of Walter Gehring), UAS-so, UAS-optix (gift of Georg Halder), UAS-eyg, UAS-toe (gift of Claude Desplan), UAS-eya (gift of Nancy Bonini), UAS-dac (gift of Graeme Mardon), UAS-dpp, UAS-scw, UAS-Mad, UAS-Medea, UAS-shn (gift of Kavita Aurora), UAS-put, UAS-put^{DN}, UAS-tkv, UAS-tkv^{DN}, UAS-sax, UAS-tld, UAS-tsg (gift of Michael O'Connor), *brk*^{XA} (gift of Gerard Campbell), Bloomington Stock Center Deficiency Kit, Bloomington Stock Center GAL4 Collection v.05.06.29, actin5C-GAL4, UAS-GFP, *dl, dpp, Kr, lack, Mad, put, scw, sec5, sec13, srw, sim, sna, tkv, tsg, tok, tld, twi, zen* (gifts of the Bloomington Stock Center).

Genetic screen for regulators of dachshund expression

We collected embryos that are homozygous for large chromosomal deletions from each of the approximately 235 stocks that constitute the Bloomington Stock Center Deficiency Kit, which provides nearly 95% coverage of the genome. These embryos were stained with an antibody that recognizes the DACSHUND protein and assayed for either the loss or gain of *dachshund* expression. We mapped the modifying activities by examining the expression pattern of *dachshund* in embryos that are homozygous for single gene disruptions that are uncovered by the larger deficiencies. The single gene mutations were assayed for their ability to mimic the phenotype of the overlying deletion. All genetic interactions were confirmed by testing multiple alleles of each complementation group.

Induction of ectopic eyes

A UAS-dac responder line (gift of Graeme Mardon) was crossed to the 219 GAL4 lines that comprise the Bloomington Stock Center GAL4 Collection v.05.06.29 and drive expression in unique patterns. The F1 progeny were scored for the presence of ectopic eye development using a light microscope.

Microscopy

The following reagents were used for the experiments described in this report: mouse anti-DACHSHUND (1:5, gift of Graeme Mardon), rat anti-ELAV (1:100, gift of Gerald Rubin) goat anti-mouse FITC (1:50, Jackson Laboratories), goat anti-mouse HRP (1:50, Jackson Laboratories), goat anti-mouse FITC minx (1:50, Jackson Laboratories), goat anti-rat TRITC minx (1:50, Jackson Laboratories), phalloidin-TRITC (1:100, Molecular Probes). Adult compound eyes and third instar eye-antennal imaginal discs were prepared for scanning electron microscopy and confocal microscopy respectively as described in (Kumar et al., 1998). Embryos were prepared for light microscopy as described in (Sullivan et al., 2000) with the exception that a HRP Conjugate Substrate Kit from Bio Rad was used to visualize the DACHSHUND protein profile.

Generation of UAS-zen, UAS-Kr and UAS-sna

Full-length transcripts of *zerknullt* (from genomic DNA), *Kruppel* and *snail* (gifts of Stephen Small) were isolated using PCR and cloned into the pUAST transformation vector using the Invitrogen Gateway Cloning System. The details of the cloning steps are available upon

request. Stable germline transformants were generated and the location of insertion lines was genetically mapped using standard techniques.

Results

Expression of dachshund within the embryonic head

The *dac* gene is expressed within and controls the development of several post-embryonic tissues including the developing eye, antenna, leg and genitals (Dong et al., 2000; Keisman and Baker, 2001; Mardon et al., 1994; Martini and Davis, 2005; Martini et al., 2000b; Noveen et al., 2000b; Pappu et al., 2005). In addition, *dac* influences the development of several embryonic tissues including the optic lobes, brain and central nervous system (Mardon et al., 1994). We used an antibody that recognizes the DAC protein to carefully map its distribution within the embryonic head. In late stage 9 embryos, DAC protein is seen in a cluster of roughly 25–30 cells within each hemisphere that make up a subset of the protocerebrum (pc). A single cell is selected to also express *dac* just anterior to each cluster (Fig. 1A). Additionally, *dac* expression expands to a subset of cells within the developing optic lobes (ol), maxillary and mandibular segments (Fig. 1B). It should be noted that cells within the mid dorsal head (mdh) do not express *dac* during normal development (Figs. 1A, B). Those cells, however, express so and *eya* (Cheyette et al., 1994; Kumar and Moses, 2001a; Serikaku and O'Tousa, 1994). During later stages of embryonic head development DAC protein is present within a significant proportion of cells within the developing brain (see below).

The genome harbors 39 upstream regulators of dachshund

The *dac* embryonic head expression pattern serves as a baseline of DAC protein distribution in normal development (Fig. 1). We then designed and conducted a genetic screen to find upstream genetic regulators of *dac* transcription (Fig. 2). In this screen, we were looking for mutants in which the distribution of DAC protein is significantly altered. The logic behind this screen is simple: situations in which *dac* expression is abolished are likely the result of mutations within positive regulators while instances in which *dac* is inappropriately expressed are most likely due to mutations within negative regulators. Using an antibody that recognizes the DAC protein, we screened the approximately 235 deletion stocks that constitute the Bloomington *Drosophila* Deficiency Kit for homozygous mutant embryos in which the profile of *dac* expression was altered. Since this kit provides coverage of nearly 95% of the genome we were able to rapidly determine which regions of the genome harbored putative upstream regulators. As a control, we examined the deficiency that removes the entire *dac* locus and determined that loss of dac does not significantly alter the overall morphology of stage 9 embryos (data not shown). In the course of the screen, we discarded mutant strains that either did not extend the germband or those in which the embryos had severe defects in head morphology. Of the remaining strains, we identified 39 deletion stocks with altered DAC protein distributions (Fig. 2). Homozygous mutant embryos of 20 of these deficiency strains showed a partial or complete loss of *dac* expression. These are briefly discussed (Figs. 1C–E, Table 1) but are not considered in detail within this report (see below). For the remaining 19 deletion strains that harbored putative negative regulators we refined the genetic maps and identified individual complementation groups by looking at DAC protein profiles in all single gene disruptions that were uncovered by the overlying deletion (Figs. 1F–K, Table 2). All complementation group identities were confirmed by testing multiple mutant alleles and they are discussed in detail here (see below).

Approximately 20 genes positively regulate dachshund in the embryonic head

Of the 39 deficiencies that scored positive in our assays 20 appeared to uncover putative positive regulators. In these cases, *dac* expression was either partially or completely eliminated. The locations of these deletions within the genome are listed in Table 1 and selected examples

of the phenotypes are presented in Figs. 1C–E. For instance, embryos homozygous for a deletion of the 7F1-8C6 cytological interval completely lack *dac* expression in all cells of the embryonic head (Fig. 1C). The gene that lies within this interval and regulates *dac* expression is predicted to be a global regulator of the *dac* locus. We also identified several deletions that we predict harbor region specific regulators and reside at lower positions within the regulatory hierarchy. In these cases, DAC protein was eliminated from most but not all cells within the embryonic head. For example, embryos that are homozygous for a deletion of the 49C1–50D2 cytological interval maintain *dac* expression only within 1–2 cells of the ventral maxillary segment (Fig. 1D). In another example, DAC protein is found only within a small group of cells within the protocerebrum and the mandibular segments in embryos that are homozygous for a deletion of the cytological interval 65A2–65E1 (Fig. 1E). Embryos that are homozygous for the remaining deletions show differing patterns of *dac* expression (Table 1; data not shown). Based on these examples presented here, it is predicted that the 20 putative genes that are located within the cytological regions listed in Table 1 are likely to represent a significant collection of upstream factors that regulate *dachshund* expression within the embryonic head (to be described in detail elsewhere). Since we focused on the regulation of *dac* within the embryonic head our list likely under represents the total number of positive regulators of the *dac* gene.

The TGFβ signaling cascade represses dachshund expression

Our screen also identified two classes of negative regulators. The genes that fall into the first class (*dorsal* and *twist*) appear to inhibit *dac* transcription globally since loss-of-function mutations lead to a broad expansion of *dac* expression throughout the entire embryonic head (Fig. 1F, Table 2). Both genes are downstream members of the Toll pathway that regulates embryonic D/V patterning (Jiang et al., 1991;Levine, 1988;Rusch and Levine, 1994). The members of the TGFβ signaling cascade comprise a significant fraction of the second class of negative regulators and appear to inhibit the expression of *dac* in domain specific regions of the embryonic head (Table 2, plus sign). For instance, embryos homozygous for mutations in *twisted gastrulation (tsg)* lead to the expression of dac in the zone that lies between the two clusters of cells that normally express *dac* (“bridge”; Fig. 1G, arrow). Mutations within other TGFβ pathway elements lead to slightly different phenotypes. For example, embryos that are homozygous for mutations in *decapentalegic (dpp)* ectopically express *dac* in cells of the posterior–lateral head (“dumbbell”; Fig. 1H, arrow). Also de-repressed are two small clusters of cells that lie just posterior to the clusters of cells that normally express *dac.* Nearly all of the TGFβ pathway mutants that we identified, such as *brinker (brk), Mothers against Dpp (Mad), punt (put), screw (scw), shrew (srw)* and *thickveins (tkv),* show *dac* expression patterns that resemble that seen in *dpp* mutants. It should be noted that brinker, which is a repressor of TGFβ targets, gives a similar phenotype to other members of the pathway such as *dpp, tkv* and *scw* which positively influence the expression of downstream target genes. One possible explanation for this is that *brinker* represses the transcription of a gene or pathway that in turn promotes *dac* transcription in the embryonic dorsal head midline. In this case the loss of *brinker* expression would result in ectopic activation of *dac* transcription. In addition, a third pattern of DAC protein distribution can be seen in embryos that are mutant for the *tolloid (tld)* gene. In *tld* mutants DAC protein is ectopically distributed within both “dumbbell” and “bridge” zones (Fig. 1I, arrows). Taken together these results clearly implicate TGFβ signaling in the repression of *dachshund* within specific domains of the developing embryonic head.

zerknullt (zen) also negatively regulates dac expression

Several other genes were also identified as playing a role in the repression of dac transcription including *Kruppel (Kr), lethal with a checkpoint kinase (lack), sec5, sec13, single minded (sim), snail (sna), tolkin (tok)* and *zerknullt (zen).* Embryos that are homozygous for loss-of-function alleles of each of these genes display phenotypes that are similar to those of the TGFβ pathway

members (Table 2, Fig. 1J, K). For instance, in *zen* mutants DAC protein is ectopically distributed within the "dumbbell" zone in the posterior–lateral regions of the head (Fig. 1J, arrow) and within a single contiguous stretch of approximately 10 cells that spans the midline of the head (Fig. 1J, arrowhead). The TGFβ pathway is known to regulate *zen* in other developmental contexts within the embryo thus it is interesting that these two components appear to coordinately regulate *dac* transcription (Chang et al., 2001;Ray et al., 1991;Rushlow et al., 2001). Mutations within *Kr*, which is expressed at low levels within the embryonic head (Gaul et al., 1987;Sheng et al., 1997), also result in the expression of *dac* within the "dumbbell" zone but with two interesting exceptions. First *dac* transcription is still repressed along the midline and the zone of ectopic *dac* expression is located in a more anterior position (Fig. 1K). Based on the common patterns of *dac* derepression our results strongly suggest that these genes interact with the TGFβ signaling cascade to repress *dachshund* transcription.

It should be noted that Dpp signaling via *zen* also functions to regulate *so* transcription in the visual primordium (Chang et al., 2001). Differences in *so* and *dac* regulation are highlighted by stage 9 when *so* expression is activated in a significant proportion of cells that make up the visual primordium. Notable exceptions are the cells that lie along the dorsal midline (Chang et al., 2001). DAC protein, on the other hand, is completely excluded in the entire visual primordium at this stage (Fig. 1A; Kumar and Moses, 2001a,b). While loss of either *dpp* or *zen* leads to a de-repression of *dac* throughout the visual primordium, *so* expression is also de-repressed along the dorsal midline (Chang et al., 2001).

TGFβ signaling and zen is sufficient to represses dachshund expression

We next wanted to see if the genes listed in Table 2 had the ability to repress *dac* on their own. We used the UAS/GAL4 system to express a significant set of these genes (denoted by asterisks in Table 2) throughout the entire embryo and assayed the ability of each factor to repress *dac*. Expression of any TGFβ pathway member including the ligand *dpp* and the patterning genes of *Kr, zen, sim,* and *sna* leads to a complete abolition of *dac* expression throughout the developing head (Fig. 1L). These results support our suggestion that the genes and pathways listed in Table 2 are both necessary and sufficient to repress the expression of *dac*.

In order to rule out the possibility that the cells in the visual primordium were simply expressing *dac* precociously in response to reductions to Dpp signaling, we examined the distribution of DAC protein within the embryonic head of later stage embryos. We looked at *dac* expression in stage 10–14 embryos and in all cases we did not observe DAC protein in cells within the visual primordium. Instead *dac* expression appears limited to subsets of cells within the developing brain (Figs. 3A–E). These results suggest and confirm that the ectopic *dac* expression observed in *zen* and Dpp pathway mutants is due to a bona fide change in the *dac* regulation. It should be noted that dac is also expressed within subset of cells of the central nervous system (Mardon et al., 1994; data not shown).

Regulation of dachshund by zen is dependent on the developmental context

The regulation of *dac* by *zen* and *dpp* within the embryonic head is an interesting paradigm. One explanation for our results is that the TGFβ signaling cascade functions through *zen* to repress *dac* within particular subdomains of the embryonic head. This model would be consistent with prior reports, which indicate that TGFβ signaling lies upstream and regulates the expression of *zen* (Rusch and Levine, 1997; Chang et al., 2001). The distribution of pMAD, which demarcates TGFβ signaling activity, and *race*, which serves as a readout for ZEN protein activity, is observed along the dorsal head midline (Dorfman and Shilo, 2001; Tatei et al., 1995). It is easy to envision an autonomous mechanism by which TGFβ signaling activates the expression of *zen* and this in turn represses *dac* along the dorsal midline of the developing head (Figs. 4A, B, arrow). However, members of the TGFβ pathway and *zen* also appear to repress

dac in regions where pMAD and *race* are not expressed, most notably along the posterior–lateral regions of the head. One possible scenario is that TGFβ signaling activates the expression of *zen* along the midline and *zen*, in turn, utilizes a non-autonomous mechanism to repress *dac* in cells that are located away from the midline (Figs. 4A, B, arrowhead).

In order to examine the possibility that *zen* could regulate *dac* via different mechanisms we generated a UAS-zen responder and used a dpp-GAL4 driver to express *zen* within several developing tissues including the leg and antennal discs. We then assayed the effect that ectopic *zen* expression had on dac transcription (Fig. 5). During normal leg development *dac* is expressed in a series of concentric circles within mid-proximal regions of the leg while *dpp* is expressed along the A/P axis (Mardon et al., 1994;Masucci et al., 1990;Shen and Dahmann, 2005). When driven by the dpp-GAL4 driver, the expression pattern of *zen* intersects with that of *dac* at several locations (Figs. 5A, B). ZEN protein is capable of repressing *dac* expression in a cell autonomous fashion; repression occurs only in cells where the two expression patterns should intersect (Figs. 5E, F, arrow). This type of regulation is reminiscent of that seen within the midline of the embryonic head.

We did a similar experiment within the developing antennal disc. During normal antennal development, *dac* is expressed in a crescent moon pattern within the dorso-lateral regions of the epithelium while *dpp* is expressed in a ventrally located pie shaped sector (Mardon et al., 1994; Masucci et al., 1990). The expression patterns of these two genes appear to be mutually exclusive (Figs. 5C, D). Expression of *zen* via the dpp-GAL4 driver completely abolishes *dac* expression within the antenna despite the lack of overlap in the expression patterns (Figs. 5G, H, arrow). The presence of DAC protein in the eye serves as an internal control for our staining procedures. Since the expression patterns of *dac* and the dpp-GAL4 driver are mutually exclusive, this result suggests that *zen* can repress dac within the antenna through a non-autonomous mechanism.

zerknullt, Kruppel and snail can repress dachshund in the eye

Several putative regulators of *dac* such as *zerknullt, Kruppel* and snail, appear to be expressed predominantly within the developing embryo (Doyle et al., 1986; Gaul et al., 1987; Leptin, 1991; Rushlow et al., 1987). We have already demonstrated that *zen* is sufficient, on its own, to repress *dac* transcription within the developing leg and antenna. We now wanted to extend these findings and determine if *zen, Kr* and sna are capable of repressing *dac* within the developing eye imaginal disc. We generated UAS-zen, UAS-Kr and UAS-sna responder lines and used an ey-GAL4 driver to express these genes ahead of the developing morphogenetic furrow. It should be noted that these three transcription factors are not normally expressed in the eye. However we were interested in determining if any or all of these factors were sufficient in a different developmental context to inhibit the transcription of dac. In each case we observed an inhibition in retinal development within the ventral half of the eye (Figs. 6A–D, arrows) while developing imaginal discs showed a repression of *dac* expression (data not shown). The effect on the ventral domain of the developing eye does not appear to be a consequence of the ey-GAL4 driver as eye discs of the ey-GAL4/UAS-GFP genotype appear to contain uniform levels of GFP within both dorsal and ventral halves of the tissue (data not shown). These results suggest that the embryonic patterning genes *zen, Kr* and *sna* can repress *dac* in very dissimilar developmental contexts.

The eye specification network positively regulate dac in the eye-antennal disc

We were interested in extending our findings into eye development and decided to focus on the role that several eye specification genes play in regulating *dac* expression in normal and ectopic eye development. We used a dpp-GAL4 driver to express each eye specification gene within the antenna and then assayed for the induction of *dac* expression. The individual

expression of *toy*, *ey*, *eya*, *optix*, *eyg* or *toe* is sufficient to induce the expression of dac within the developing antenna (Figs. 7B, C, E–H). The induction of dac by *toy*, *ey*, *eya* and *optix* is consistent with the ability of these genes to induce ectopic retinal development within the antenna (Bonini et al., 1997; Czerny et al., 1999; Halder et al., 1995; Seimiya and Gehring, 2000; Shen and Mardon, 1997). Interestingly, the while *eyg* and *toe* are able to induce *dac* transcription, they are incapable of inducing ectopic eyes within the antenna (Yao et al., 2005; C. Salzer, B.M. Weasner and J. Kumar unpublished data). Another interesting finding is that *so* is the only network member that is incapable of inducing dac expression (Fig. 7D), which is consistent with its inability to induce retinal development (Pignoni et al., 1997). These results suggest that other factors might cooperate with members of the eye specification network to regulate *dac* expression and ectopic eye development.

We also looked at the distribution of DAC protein in individual loss-of-function mutants. While mutations in *ey*, *so*, *eya* and *eyg* lead to a small eye disc and a complete loss of retinal development, DAC protein can still be detected within the eye field of these mutants (Figs. 7I–L, arrow). These results suggest that the transcriptional regulation of *dac* may be subject to multiple inputs from the other eye specification members. At a minimum, the reduced levels of DAC protein in the loss-of-function mutants suggest that *dac* expression is not dependent on a single member of the eye specification network. We did not analyze *toy*, *toe* and *optix* mutants since loss-of-function mutations in *toe* and *optix* do not yet exist and there are severe technical difficulties in analyzing *toy* mutants. However, as these mutants become available, it would be interesting to examine the role that these genes play in regulating *dac* transcription.

We wanted to further examine the regulatory relationship between *dac* and the other eye specification genes during the induction of eye formation. We expressed each eye specification gene along the A/P axis of eye-antenna, wing and leg imaginal discs via the dpp-GAL4 driver and assayed the ability of each gene to direct *dac* expression and photoreceptor development. Expression of *ey* along the A/P axis is sufficient to induce *dac* expression in only a subset of cells within the antennal and wing discs (Figs. 8A,D, compare to Fig. 5D). Additionally, only a further subset of *dac* expressing cells is capable of being transformed into photoreceptors (Figs. 8B, C, E, F). While *ey* had a greater effect on *dac* transcription in the leg disc (Fig. 8G) a similar level of induction of eye development was observed (Figs. 8H, I). We obtained similar results with each of the other eye specification genes (data not shown). These results have several implications. First, these results demonstrate the induction of *dac* transcription is likely regulated by an extensive regulatory network that cannot be recapitulated in all cell types by the sole expression of the retinal determination genes. Second, our results also imply that the decision to adopt a photoreceptor cell fate is dependent upon a regulatory network that includes but is not limited to *dac*. The regions of non-retinal tissues that can adopt a photoreceptor cell fate are likely to express the factors required to work with *dac* to induce retinal fates. Conversely, it is possible that there are inhibitory factors that are expressed in regions of the disc that are refractory to retinal specification.

dachshund induces ectopic eyes on the antenna, head and genitals

The above results suggest that the expression of *dac* alone is insufficient to induce ectopic eyes in all geographic locations and temporal patterns. *dac* had been previously shown to induce ectopic eyes on the ventral surface of the antenna when expressed via a dpp-GAL4 driver (Shen and Mardon, 1997). We have used an additional 219 GAL4 lines to express *dac* in unique patterns and have scored its ability to induce retinal development. Surprisingly, eye formation was seen in only 2 additional instances strongly suggesting that *dac* has only a limited capacity to induce retinal development (Table 3). In addition to the antenna (data not shown; Shen and Mardon, 1997), retinal development induced by *dac* expression was observed on the adult head and genitals (Figs. 9A, B). Interestingly, in most cases, ectopic *dac* expression had little effect

on the developing fly as 70% of all crosses had no visible effect (Table 3). The low frequency of ectopic eye development in other developing tissues is likely to be attributable to either the absence of critical eye promoting proteins or the presence of inhibitory factors or both. These results suggest that eye formation requires the activity of *dac* and several additional factors.

Discussion

In this report, we set out to further our understanding of how the eye specification gene *dac*, which is distantly related to the Ski/Sno family of proto-oncogenes (Hammond et al., 1998), is regulated in the developing eye and in non-retinal contexts, particularly during embryonic development. We were interested in *dac*, in part, because it is expressed and controls the development of a wide range of embryonic and post-embryonic tissues (Dong et al., 2000; Keisman and Baker, 2001; Mardon et al., 1994; Martini and Davis, 2005; Martini et al., 2000b; Noveen et al., 2000a). In contrast to eye development, the full set of remaining eye specification genes are not co-expressed with *dac* in these developmental contexts. We were interested in determining how, then, is *dac* regulated in other non-retinal tissues. We focused on the developing embryonic head in which DAC protein is present in a temporally and spatially dynamic pattern.

We have identified the TGFβ signaling cascade, the transcription factor *zen* and several other patterning genes as crucial players in preventing *dac* from being expressed within inappropriate regions of the head. The regulation of *dac* transcription by TGFβ signaling has been previously shown to exist within the developing leg and genital imaginal discs (Keisman and Baker, 2001; Lecuit and Cohen, 1997; Mardon et al., 1994). Our results further these studies by demonstrating that this regulatory relationship extends to the developing embryonic head. Our results also extend these reports by demonstrating that, in contrast to the leg and genital discs, the regulation of *dac* by Dpp signaling is unidirectional and independent of the Wingless signaling cascade. For example, within the leg, a mid-proximal ring of *dac* expression is initiated and maintained by intermediate levels of both Wg and Dpp signaling while high levels of these pathways cooperate to repress *dac* from distal regions (Lecuit and Cohen, 1997). In contrast, *dac* expression within the developing embryonic head is not dependent upon Wg signaling (data not shown) and unlike the situation within the leg, alterations of Dpp levels do not have differing effects on *dac* transcription. These features represent interesting and significant differences in how *dac* is regulated during development.

Based on the expression patterns of pMad protein and *race* mRNA (Sutherland et al., 2003; Wharton et al., 2004), it appears that both *dpp* and *zen* exert their influence on *dac* through cell-autonomous mechanisms along the midline while using non-autonomous mechanisms to repress *dac* in zones that lie at more distant locations. A non-autonomous role for *zen* in development would be novel. We set out to determine if *zen* can influence the expression of *dac* non-autonomously in other tissues. Using a dpp-GAL4 driver we expressed *zen* within the developing antennal and leg discs, two tissue in which *dac* is expressed. Within the developing leg *zen* appears to play a traditional role of being able to repress *dac* in only a cell autonomous manner. However, within the antennal disc, the expression of *zen* completely eliminated *dac* expression even within regions that were quite distant from *zen* positive cells. Thus it appears that within the embryonic head and the developing antennal disc there are factors that facilitate that non-autonomous repression of *dac* by *zen* and *dpp*. The identification of those factors will go a long way towards understanding how the regulation of dac has diverged in different tissues during evolution.

Interestingly, *dpp* has been shown to regulate both *so* and *eya* within the visual primordium during embryogenesis (Chang et al., 2001). In *dpp* loss-of-function mutants *so* and *eya* transcripts are lost from the visual primordium. These results are interesting in light of our

findings that in *dpp* mutants *dac* is now expressed in the visual primordium. A simple hypothesis is that *dpp* acts within the visual primordium to maintain both *so* and *eya* while simultaneously repressing *dac*. It should be noted that several other eye specification genes including *toy*, *ey optix*, *eyg* and *toe* are also excluded from this field at stage 9 of embryogenesis (Kumar and Moses, 2001a). Why would such an exclusion of these genes be required for the development of the visual primordium? One possible explanation is that the eye field requires a stepwise recruitment of "eye genes" with each additional gene making the field more competent to adopt a retinal fate. *so* and *eya* might serve as the catalysts for the development of the eye field with the other genes being added later as the eyes and optic lobes are specified.

Our results have shown that in loss-of-function mutants of the Dpp signaling pathway *dac* transcription is de-repressed in several distinct geographic locations within the embryonic head. Based on these phenotypes, a reasonable prediction would be that several different enhancer elements are negatively regulated by Dpp signaling. Each element would be further predicted to control expression of *dac* within a sub-domain of the head. Recently, two enhancer elements within the *dac* transcriptional unit have been identified and demonstrated to regulate expression within the developing eye imaginal disc. One of these elements is controlled by Dpp signaling and the eye specification genes *eya* and *so* (Pappu et al., 2005). Interestingly, both *eya* and *so* are expressed within regions of the embryonic visual primordium where *dac* transcription is absent. In *dpp* mutants, expression of both *eya* and *so* is lost while dac is ectopically expressed (Chang et al., 2001; this report Fig. 1H). A possible mechanism is that *eya* and *so* cooperate with the Dpp pathway at an embryonic enhancer element to shut off *dac* expression within the embryonic visual primordium. Later, in the developing eye imaginal disc, these same factors are then recruited again to turn on *dac* transcription through a separate eye imaginal disc enhancer.

The developing retina is the only place in the developing fly where all eight of the eye specification genes are co-expressed. We were interested in investigating the role that each member of the cascade plays in regulating *dac* expression during normal and ectopic eye development. Our results show that dac transcription is activated in response to the expression of each eye specification gene except for *so*. DAC protein was also present, albeit at lower levels, in the retinas of several loss-of-function mutants. These results imply that there might be several inputs by the members of the retinal determination network into the transcriptional regulation of *dac*. At a minimum, these results indicate that *dac* is not regulated within the retina by a single upstream factor.

We finally demonstrate that although most members of the eye specification network are capable of activating *dac* transcription, *dac* is unable to induce ectopic eye formation with the same vigor as these upstream factors. Previous reports by several groups had suggested that this is the case but these results were based on just a handful of GAL4 lines. We used a much larger collection (219 lines) to show that, in addition to the antenna, *dac* can only induce ectopic eyes in the anterior head and genitals. An obvious explanation is that the upstream factors regulate a large number of genes, of which *dac* is just one, and that it is this combination that leads to eye development. The identification of these additional factors by standard genetic and new genomic approaches will be crucial to furthering our understanding of how the eye is specified.

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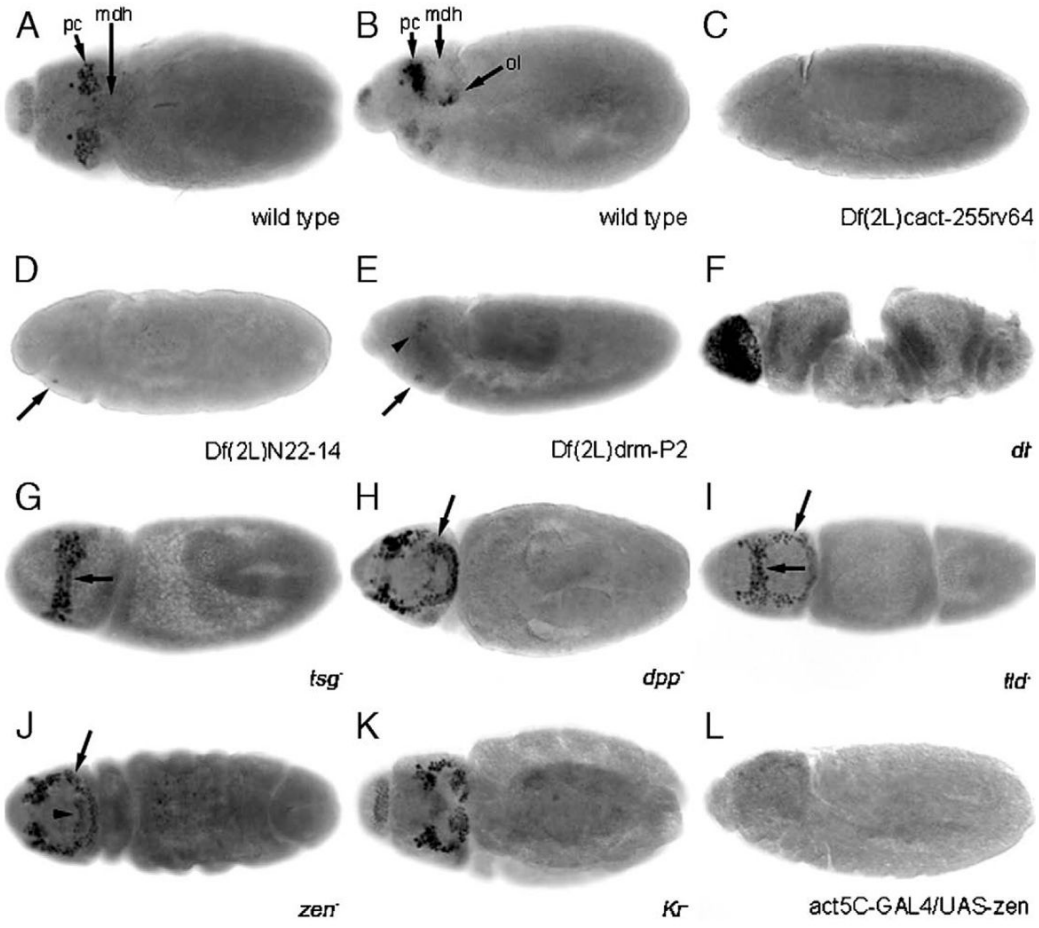

Fig. 1.

Regulation of dachshund in the embryonic head. (A–L) Light microscope images of wild type (A, B) and mutant (C–L) stage 9 embryos stained with an antibody against the DAC protein. (A) In this dorsal view of a wild type embryo, DAC protein can be seen within a subset of cells that comprise the protocerebrum. (B) In this lateral view of a wild type embryo, DAC protein can also be seen in the optic lobes. *dac* is not expressed within the mid-dorsal head. (C) In this example, *dac* expression is completely eliminated. (D) In this example, DAC protein is found within a few cells of the ventral maxillary (arrow) segment but is absent within the entire dorsal head region. (E) In this example DAC protein is present within a few cells of the protocerebrum (arrowhead) and mandibular segment (arrow). (F) Note that in *dorsal* mutants *dac* expression has expanded to fill in the entire embryonic head. (G) In *tsg* mutant embryos, repression of *dac* transcription is relieved within a small group of cells of the protocerebrum (arrow points to the "bridge"). (H) In *dpp* mutant embryos, the *dachshund* is ectopically expressed within several zones of the mid-dorsal head. The arrow points to a section of the expression pattern that looks like a bent dumbbell. (I) In *tld* mutants the repression of *dac* is observed in both the "bridge" and "dumbbell" regions (arrows). (J) In *zen* mutants, *dac* is ectopically expressed in the "dumbbell" zone (arrow) as well as a small strip of cells located just to the anterior (arrowhead). (K) In *Kr* mutants, cells that now ectopically express *dac* are located more towards the anterior than those in the other mutants shown in this figure. Also note that the cells that lie directly on the midline of the head do not express *dac*. (L) Expression of dac is nearly eliminated in embryos in which *dpp* has been expressed throughout the embryo via an act5C-GAL4 driver. Similar results were obtained when the remaining genes in Table 2 (asterisks)

were expressed throughout the embryo (data not shown). mdh = mid-dorsal head, pc = protocerebrum and ol = optic lobes. Anterior is towards the left.

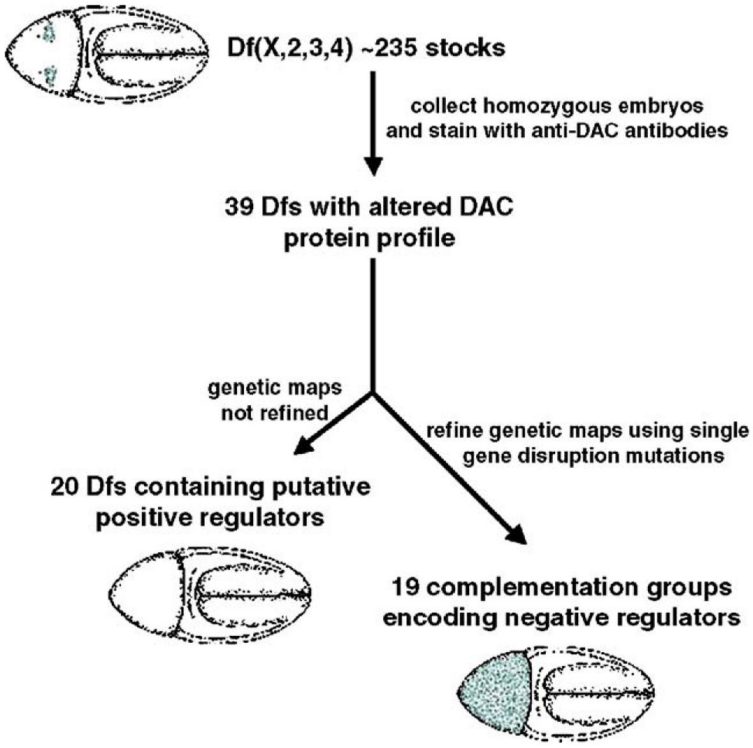

Fig. 2.

Schematic of the genetic screen for upstream regulators of *dachshund.* The Bloomington *Drosophila* Deficiency Kit was screened for alterations in the expression pattern of *dachshund* within the developing head of normal stage 9 embryos. Initially, 39 deletions stocks showed a modification of DAC protein distribution. 20 deficiency stocks are likely to harbor positive regulators and will be described elsewhere. 19 complementation groups that serve as negative regulators of *dachshund* were identified and are described in this report.

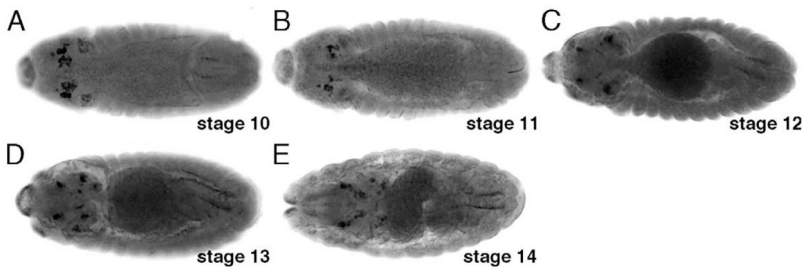

Fig. 3.

Expression of *dachshund* in late stages of embryogenesis. (A–D) Wild type embryos stained with an antibody to DAC. During stages 10–14, DAC protein distribution within the embryonic head is limited to subsets of cells within the brain. Note that DAC protein is absent from the visual primordium and the dorsal midline. Anterior is to the left.

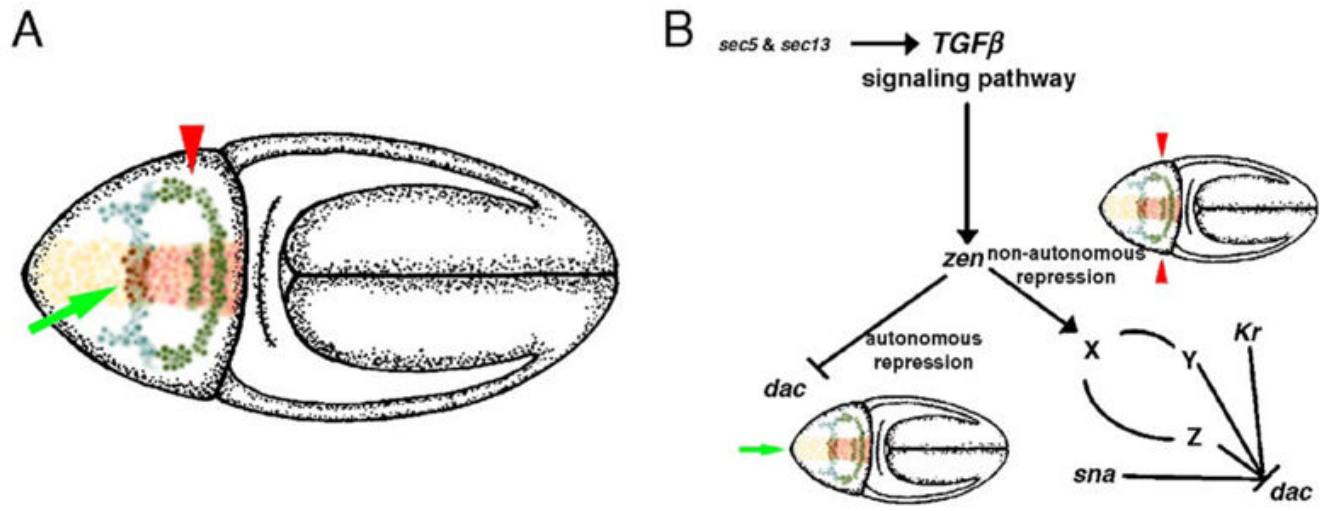

Fig. 4.

Schematics describing the regulation of *dachshund* in the embryonic head. (A) A schematic drawing of a stage 9 embryo depicting the normal expression of *dachshund* (blue), the ectopic expression pattern of *dachshund* (brown and green), the normal expression pattern of *race* (red) and the normal distribution of pMAD (yellow). Note that autonomous regulation of *dachshund by zen* and the TGFβ pathway are predicted to occur along the midline of the embryonic head while a non-autonomous mechanism would be required to regulate *dachshund* in regions distant to the midline. The green arrow indicates regions that are predicted to use an autonomous regulatory mechanism while the red arrows indicate regions that would require the use of a non-autonomous mechanism. (B) A schematic depicting a potential regulatory cascade of *dachshund* within the embryonic head. The green arrow indicates regions that are predicted to use an autonomous regulatory mechanism while the red arrows indicate regions that would require the use of a non-autonomous mechanism. The X, Y and Z are meant to indicate that there are likely components to this cascade that are left to be identified. The placement of the genes and pathways within this diagram are based on known interactions and phenotypes observed in this report.

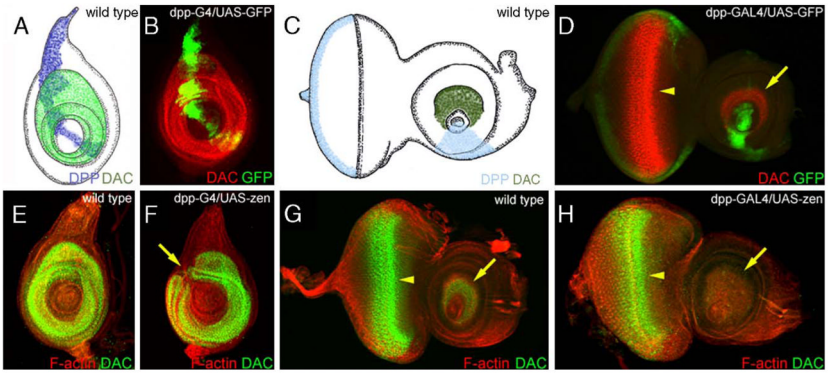

Fig. 5.

ZEN can repress dachshund transcription in the leg and antennal discs. (A, B, E, F) Schematic drawings and confocal images of leg discs. (C, D, G, H) Schematic drawings and confocal images of eye-antennal discs. (A) Schematic drawing of a wild type leg disc depicting the expression patterns of *dachshund* and *decapentaplegic*. (B) dpp-GAL4/UAS-GFP, note that dpp-GAL4 drives expression of GFP along the A/P axis of the leg disc and mimics endogenous *dpp* expression. Also note that the expression pattern of the *dpp* enhancer element (green) intersects that of *dac* (red). (E) A normal leg in which the distribution of DAC protein is depicted (green). (F) dpp-GAL4/UAS-zen, note that the expression of *zen* is sufficient to autonomously down-regulate *dac* expression in a regions where the dpp and dac expression patterns intersect (yellow arrow). (C) Schematic drawing of a wild type eye-antennal disc depicting the expression patterns of *dac* (green) and *dpp* (blue). (D) dpp-GAL4/UAS-GFP, note that within the eye disc dpp-GAL4 drives expression of GFP along the posterior–lateral margins and within a pie wedge region of the antennal disc (green). Also note that the patterns of the *dpp* enhancer element (green) and *dac* (red) do not overlap in either tissue. (G) A normal eye-antennal disc in which the distribution of DAC protein is depicted (green). (H) dpp-GAL4/UAS-zen, note that the expression of *zen* within the antennal disc is sufficient to non-autonomously repress the expression of *dac*. The arrows in panels D and G indicate areas within the antennal disc that normally contain DAC protein. The arrow in H indicates the region at which *dac* expression is repressed by the expression of zen. Arrowheads in panels D, G, H denote regions within the eye disc that normally express *dac*. Genotypes are listed within each panel. Visualized molecules are listed within each panel. Anterior is towards the right.

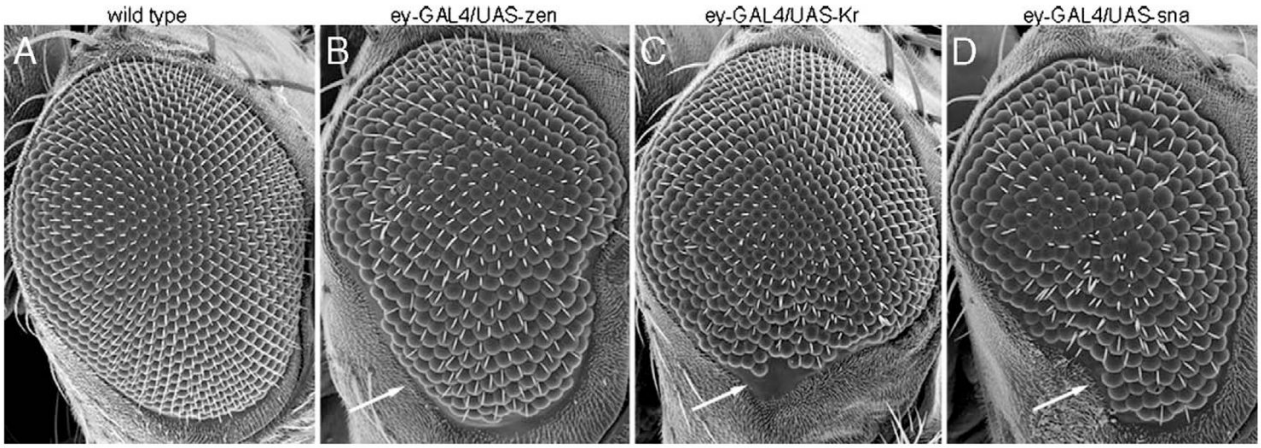

Fig. 6.

Expression of *zerknullt*, *Kruppel* and *snail* can repress eye development. (A–D) Scanning electron micrographs of adult wild type or mutant compound eyes. (A) Wild type. (B) ey-GAL4/UAS-zen. (C) ey-GAL4/UAS-Kr. (D) ey-GAL4/UAS-sna. Note that ey-GAL4 drives expression in all cells anterior to the morphogenetic furrow. As expected, the expression of *zen*, *Kr* and *sna* within the developing retina resulted in an inhibition of eye development. Note that the expression of *zen* and *Kr* appear more adversely affect the ventral domain of the eye while expression of *sna* results in a general roughening of the entire eye. Genotypes are listed above each panel. Arrows in panels B–D indicate areas in which ventral eye formation has been inhibited. Anterior is to the right.

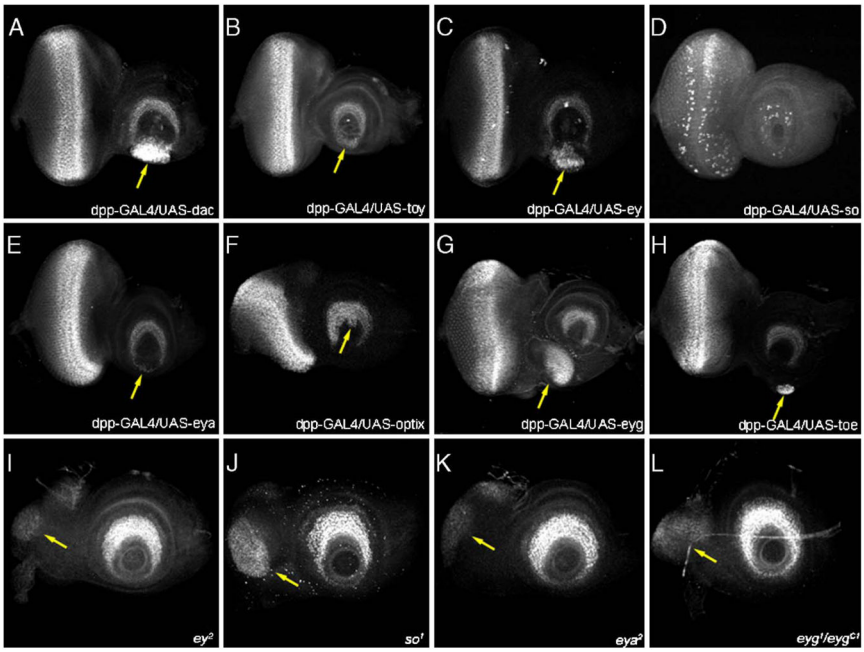

Fig. 7.

Regulation of *dachshund* by the eye specification network. (A–L) Confocal micrographs of developing eye-antennal discs stained with an antibody that recognizes the DAC protein. (A) dpp-GAL4/UAS-dac. (B) dpp-GAL4/UAS-toy. (C) dpp-GAL4/UAS-ey. (D) dpp-GAL4/UAS-so. (E) dpp-GAL4/UAS-eya. (F) dpp-GAL4/UAS-optix. (G) dpp-GAL4/UAS-eyg. (H) dpp-GAL4/UAS-toe. (I) ey^{2}. (J) so^{1}. (K) eya^{2}. (L) eyg^{1}/eyg^{C1}. Note that the expression of each construct in panels A–H, with the notable exception of *so*, is sufficient to induce the expression of *dachshund*. Also, note that in each mutant presented in panels I–L, DAC protein is still present albeit at lower levels than in wild type eye discs. Yellow arrows indicate regions of *dachshund* expression. Genotypes are listed within each panel. Anterior is towards the right.

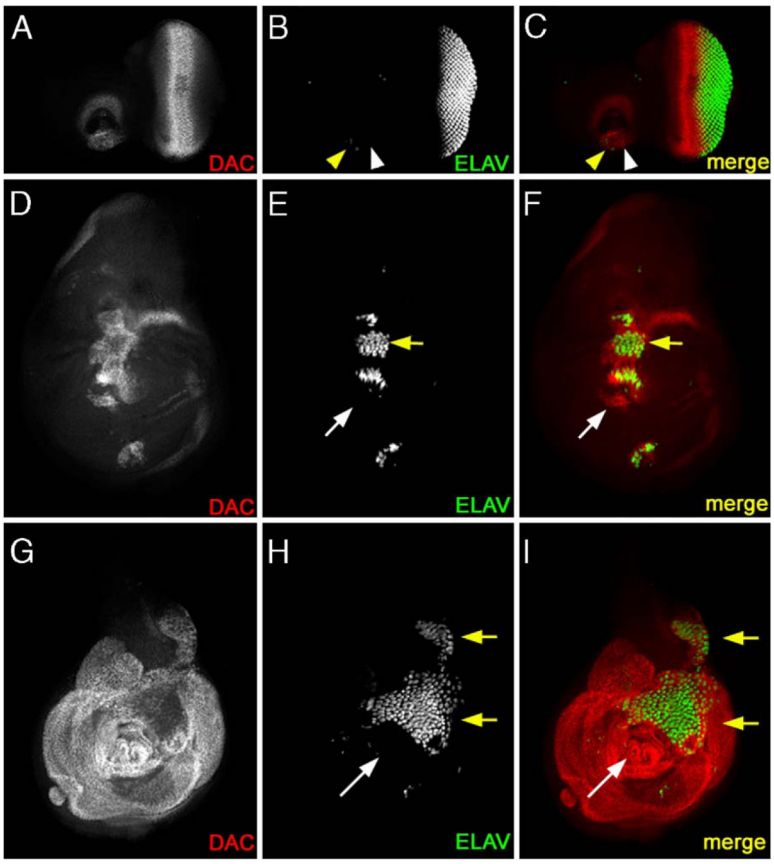

Fig. 8.

Only a subset of DAC positive cells are transformed into photoreceptors. (A–C) dpp-GAL4/UAS-ey eye-antennal imaginal discs. (A) Expression of ey within the dpp-GAL4 pattern is sufficient to induce dac expression within a subset of cells. (B) Of the cells that ectopically express dac only a subset of these cells are competent to express neuron specific markers. (C) Merge. (D–F) dpp-GAL4/UAS-ey wing imaginal discs. Similar to the antenna, expression of ey within the wing is sufficient to drive dac in subset of cells along the A/P axis and only a smaller domain of these cells are capable of becoming photoreceptors. (G–I) dpp-GAL4/UAS-ey leg discs. Note that a similar situation is seen in the leg discs. Red = DAC, green = ELAV. White arrowheads denote regions that ectopically express DAC but do not adopt a photoreceptor cell fate. Yellow areas denote cells that express both DAC and ELAV. Anterior is to the left.

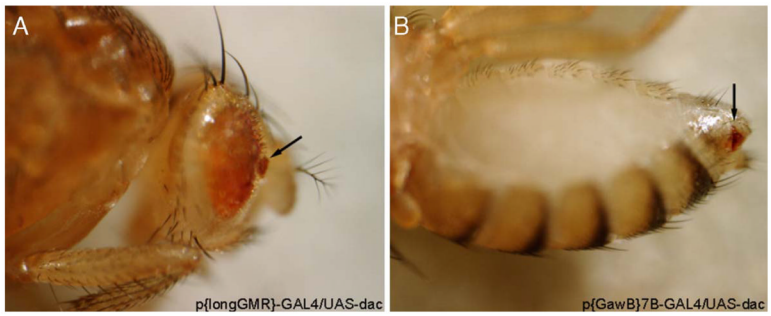

Fig. 9.

dachshund can direct ectopic eye development within the adult head and genitalia. (A) Expression of *dachshund* via the p(longGMR)-GAL4 driver results in the formation of a small ectopic eye along the head cuticle. The ectopic eye is always found along the anterior margin (arrow). (B) Expression of *dachshund* via the p(GawB)7B-GAL4 driver results in ectopic eye development within the female genitals (arrow). Male genitals do not show retinal tissue (data not shown).

Table 1

Deficiency stocks harboring complementation groups that encode for putative positive regulators of *dachshund* expression in the embryonic head

Df Stock	Interval
Df(1)BA1	1A1–2A1
Df(1)KA14	7F1–8C6
Df(2)BSC4	21B7–21C3
Df(2)drm-P2	23F3–24A2
Df(2)Dwee-1	27C2–27C5
Df(2)N22–14	29C1–30C9
Df(2)BSC17	30C3–30F1
Df(2)r10	35D1–36A7
Df(2)cact–255rv64	35F1–36D
Df(2)C′	40h35–40h38L
Df(2)nap9	42A1–42F1
Df(2)Np5	44F10–45E1
Df(2)CX1	49C1–50D2
Df(2)Jp1	51D3–52F9
Df(2)P34	55E2–56C11
Df(2)017	56F5–56F15
Df(3)R-G7	62B8–62F5
Df(3)XD198	65A2–65E1
Df(3)Ten-m	79C1–79E8

Table 2

Genes that negatively regulate *dachshund* expression in the embryonic head

brinker (brk)[a]
dorsal (dl) decapentaplegic (dpp)[a, b] *Kruppel (Kr)*[a]
lethal with a checkpoint kinase (lack) Mothers against Dpp (Mad)[a, b]
punt (put)[a, b] *screw (scw)*[a, b] *sec5*
sec13
shrew (srw)[b]
single minded (sim)
snail (sna)[a]
thick veins (tkv)[a, b]
twisted gastrulation (tsg)[a, b]
tolkin (tok)
tolloid (tld)[a, b]
twist (twi)
zerknullt (zen)[a]

[a] Dpp pathway mutant.

[b] UAS line tested.

Table 3

Dachshund has a limited ability to induce ectopic eyes in *Drosophila*

No. of GAL4 lines	% of total	Phenotype
155	70.77	normal
45	20.51	embryonic lethal
3	1.37	larval lethal
7	3.19	pupal lethal
3	1.37	eye defects
2	0.91	wing defects
3	**1.37**	**ectopic eyes**
1	0.46	leg defects
219 total		