eyelid antagonizes *wingless* signaling during *Drosophila* development and has homology to the Bright family of DNA-binding proteins

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In *Drosophila*, pattern formation at multiple stages of embryonic and imaginal development depends on the same intercellular signaling pathways. We have identified a novel gene, *eyelid* (*eld*), which is required for embryonic segmentation, development of the notum and wing margin, and photoreceptor differentiation. In these tissues, *eld* mutations have effects opposite to those caused by *wingless* (*wg*) mutations. *eld* encodes a widely expressed nuclear protein with a region homologous to a novel family of DNA-binding domains. Based on this homology and on the phenotypic analysis, we suggest that Eld could act as a transcription factor antagonistic to the Wg pathway.

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Development of a multicellular organism from a fertilized egg requires cells to sense their position relative to other cells and to use this information to differentiate appropriately. This process of pattern formation has been shown to involve the operation of a number of intercellular signaling molecules. In *Drosophila*, one such molecule is the secreted product of the *wingless* (*wg*) gene, a *Wnt* family member (Nusse and Varmus 1992). *wg* has multiple functions in development (for review, see Klingensmith and Nusse 1994; Siegfried and Perrimon 1994). In the embryo, the pattern of each segment is determined by the interaction between a stripe of cells expressing *wg* and an adjacent stripe expressing another secreted molecule encoded by *hedgehog* (*hh*) (for review, see DiNardo et al. 1994). In the absence of either *wg* or *hh*, expression of the other gene is not maintained; this leads to a segment polarity phenotype in which part of each segment is lost and the remaining region is duplicated with inverted polarity. *wg* subsequently acts locally on the row of cells posterior to it (Dougan and DiNardo 1992) and over a longer range on anterior cells (Baker 1988; Bejsovec and Martinez-Arias 1991), determining their cell fate. *wg* has additional functions later in embryogenesis, which include directing the development of a region of the gut (Immergluck et al. 1990).

In the imaginal discs, signaling pathways are used to establish compartment boundaries, defined as barriers to the movement of clonally-related cells (Garcia-Bellido et al. 1973). In the larval leg disc, *wg* is expressed along the ventral portion of the anterior–posterior compartment boundary (Cohen et al. 1993), where it determines ventral cell fates and distal outgrowth of the leg (Campbell et al. 1993; Struhl and Basler 1993; Wilder and Perrimon 1995). During wing development, *wg* functions at several stages, reflecting the sequential compartmentalization of the wing disc. *wg* is first required for the establishment of the wing as distinct from the body wall or notum (Couso et al. 1993; Ng et al. 1996), then for development of the ventral compartment (Williams et al. 1993), and finally, to determine the wing margin (Couso et al. 1994), which forms at the dorsal–ventral compartment boundary and has a characteristic pattern of bristles. *wg* also promotes cell proliferation in the wing hinge region (Neumann and Cohen 1996).

The eye disc, unlike the other imaginal discs, does not have distinct anterior and posterior compartments, but develops progressively, as a wave of differentiation moves from posterior to anterior; the transient boundary between differentiated and undifferentiated cells is known as the morphogenetic furrow (MF) (Ready et al.

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1976). Progression of the furrow depends on the expression of *hh* by differentiating photoreceptors located posterior to the MF (Heberlein et al. 1993; Ma et al. 1993; Heberlein et al. 1995). Hh protein induces more anteriorly-located cells to differentiate and themselves express *hh*, thereby creating a cycle of induction that moves the furrow across the eye disc. The initiation of differentiation requires *decapentaplegic* (*dpp*), a transforming growth-factor-β (TGF-β) homolog expressed at the margins of the disc (Wiersdorff et al. 1996; Chanut and Heberlein 1997; Pignoni and Zipursky 1997). *wg* is expressed at the dorsal and ventral margins of the eye disc, where it specifies regions of the head cuticle (Royet and Finkelstein 1996) and prevents *dpp* from inappropriately initiating photoreceptor differentiation, thereby restricting initiation to the posterior margin (Ma and Moses 1995; Treisman and Rubin 1995). Ectopic *wg* expression can block both initiation and progression of differentiation (Treisman and Rubin 1995).

Although many gaps remain in our understanding of the intracellular response to Wg signaling, some downstream elements have been identified. A frizzled-like molecule, Dfz2, has been proposed recently to act as the Wg receptor (Bhanot et al. 1996). Transmission of the Wg signal also requires a novel conserved protein encoded by *dishevelled* (*dsh*) (Klingensmith et al. 1994; Theisen et al. 1994) and the β-catenin homolog encoded by *armadillo* (*arm*) (Peifer and Wieschaus 1990). The protein kinase encoded by *shaggy/zeste-white3* (*sgg*) acts downstream of Dsh and upstream of Arm to antagonize Wg signaling (Bourouis et al. 1990; Siegfried et al. 1990, 1992, 1994; Noordemeer et al. 1994). In vertebrates, adenomatous polyposis coli (APC), a large protein with multiple sequence repeats present also in Arm, and the high mobility group (HMG) box proteins, Tcf-1 and LEF-1, have been proposed to act in Wnt signaling because of their molecular or functional interactions with the Arm homolog β-catenin or the *sgg* homolog GSK3 (Rubinfeld et al. 1993, 1996; Su et al. 1993; Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996). A *Drosophila* Tcf-1 homolog, pangolin (pan), has been shown recently to be an essential component of the Wg pathway and to bind to the Arm protein (Brunner et al. 1997; Riese et al. 1997; van de Wetering et al. 1997). However, the *Drosophila* APC homolog is not expressed at the time when *wg* acts on embryonic segmentation (Hayashi et al. 1997). The Arm–Pan complex may activate the transcription of Wg target genes (Brunner et al. 1997; van de Wetering et al. 1997) such as *engrailed* (*en*) in the embryonic epidermis (DiNardo et al. 1988), *Ultrabithorax* in the midgut (Riese et al. 1997), *achaete* and *cut* at the wing margin (Couso et al. 1994), and *optomotor-blind*, *nubbin*, *vestigial*, and *distalless* in the wing pouch (Diaz-Benjumea and Cohen 1995; Grimm and Pflugfelder 1996; Ng et al. 1996; Zecca et al. 1996). All of these genes encode transcription factors that mediate the subsequent effects of Wg.

wg appears to interact with other known pathways; in particular, it has many phenotypes in common with *Notch* (*N*) (Couso and Martinez-Arias 1994), which encodes a cell-surface receptor mediating cell fate choices triggered by local contact (for review, see Artavanis-Tsakonas et al. 1995). At the wing margin, an interaction between N and its ligands, Delta (Dl) in the ventral compartment and Serrate (Ser) in the dorsal compartment (Couso et al. 1995; Diaz-Benjumea and Cohen 1995; Kim et al. 1995; de Celis et al. 1996; Doherty et al. 1996), is required for *wg* expression along the margin (Diaz-Benjumea and Cohen 1995; Rulifson and Blair 1995; de Celis et al. 1996), explaining the loss of wing margin in both *N* and *wg* mutants. There also appears to be a negative interaction between *wg* and *N* signaling at the wing margin; Dsh, which interacts physically with the intracellular domain of N, inhibits N function, thereby limiting the domain of *wg* expression (Axelrod et al. 1996; Rulifson et al. 1996). In addition, the same gene, *sgg/zw3*, was isolated independently as an inhibitory element of the Wg pathway and a positive element of the N pathway (Bourouis et al. 1989; Perrimon and Smouse 1989).

We have isolated mutations in a novel gene, *eyelid* (*eld*), that have many similarities to *sgg/zw3* mutations, suggesting that *eld* may be involved in signaling through the Wg or N pathways. In the eye, wing, and embryo, *eld* appears to antagonize the function of *wg*. The *eld* gene has been cloned and encodes a highly proline-rich protein with a region homologous to the newly defined DNA-binding domains of the *Drosophila* dead ringer and mouse Bright proteins (Herrscher et al. 1995; Gregory et al. 1996). Eld protein is expressed ubiquitously in the early embryo and in imaginal discs and is localized to the nucleus, where we suggest that it counteracts the effects of *wg* on its target genes.

Results

eyelid *affects patterning of the eye imaginal disc*

A dominant mutation in the *rough* (*ro*) gene, ro^{DOM}, has been shown to arrest movement of the MF in the eye disc, resulting in a reduced adult eye that specifically lacks the anterior portion of the retina (Heberlein et al. 1993) (Fig. 1B). In wild-type eye discs, a smooth gradient of ommatidial maturation is observed behind the MF, reflecting the stepwise recruitment of photoreceptors (Tomlinson and Ready 1987) (Fig. 1E,I). Differentiating photoreceptors are recognized by the expression of the neuronal protein elav (Robinow and White 1991), and the MF is visualized by the expression of a *dpp* reporter (*dpp–lacZ*) (Blackman et al. 1991). Eye discs from ro^{DOM} third-instar larvae display a so-called ''furrow-stop'' phenotype, the landmarks of which are the presence of mature ommatidia that contain a full complement of photoreceptor cells in the anterior-most ommatidial row, and the loss of *dpp* expression in the furrow (Fig. 1F,J). To identify additional genes involved in furrow movement, a genetic screen for dominant enhancers and suppressors of the ro^{DOM} eye phenotype was carried out (A. Luk and U. Heberlein, unpubl.; see Materials and Methods). Several ethylmethane sulfonate (EMS)-induced suppressors (Fig. 1C; see Materials and Methods) failed to complement each other's recessive lethality and that of P-ele-

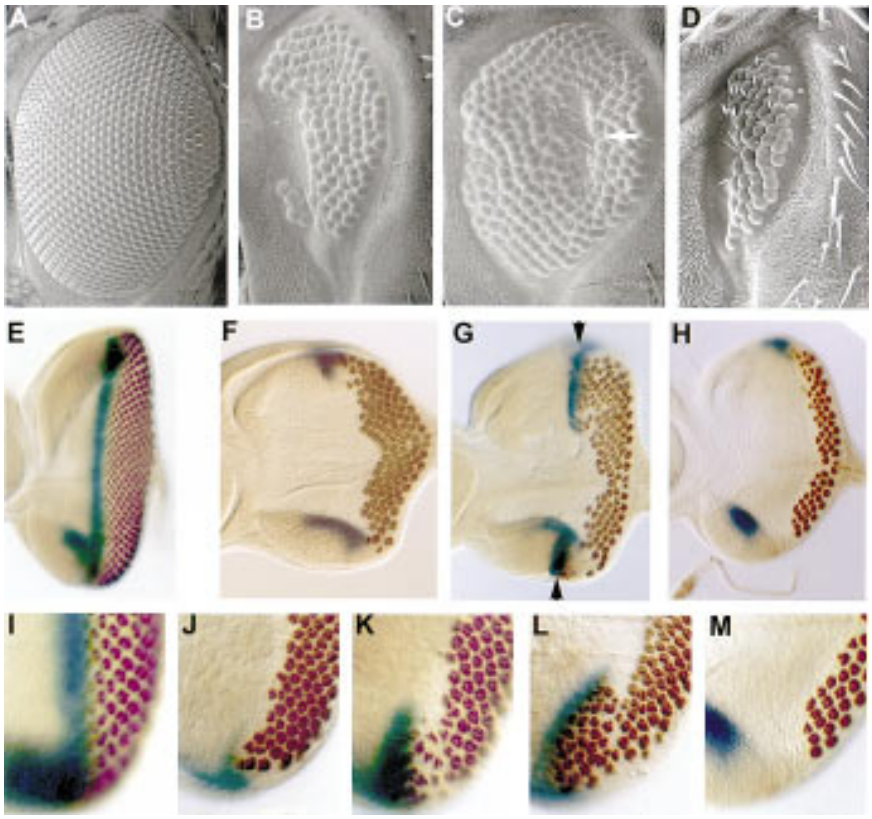

Figure 1. Effect of Eld and Wg on MF movement. (*A–D*) Scanning electron micrographs of adult eyes. (*A*) Wild type; (*B*) ro^{DOM}/+; (*C*) ro^{DOM}/eld^{308} (arrow indicates a scar in the eye); (*D*) wg^{CX4}/+; ro^{DOM}/+. *eld* suppresses the ro^{DOM} phenotype, whereas *wg* enhances it. *E–M*) Eye discs stained with antiElav antibody and X-gal to visualize a *dpp–lacZ* reporter. (*E,I*) Wild type; (*F,J*) ro^{DOM}/+; (*G,K,L*) ro^{DOM}/eld^{308}; (*H,M*) wg^{CX4}/+; ro^{DOM}/+. (*I–M*) Enlargements of the ventral posterior region of eye discs. *eld* restores furrow movement to the lateral edges of ro^{DOM} discs (arrowheads in *G*), whereas *wg* reduces the amount of furrow movement observed there.

ment insertions located at cytological position 90C1-2. Surprisingly, an analysis of eye discs from larvae heterozygous for both ro^{DOM} and any of the 90C1-2 mutants revealed that the observed increase in eye size was not attributable to relief of the ro^{DOM}-induced block to furrow progression in the central region of the disc. Rather, photoreceptor differentiation reinitiated at the dorsal and ventral edges of these discs (Fig. 1, arrowheads in G,K,L). This produced two ommatidial fields, each preceded by *dpp–lacZ*-expressing furrows, which moved anteriorly, presumably fusing later along the midline. The presence of a scar in the center of suppressed adult eyes (arrow in Fig. 1C) is consistent with this proposal.

The mechanism of ro^{DOM} suppression by the 90C1-2 mutants suggests that the dosage of this suppressor is most critical at the lateral edges of the disc, regions in which *wg* has been shown to inhibit precocious differentiation (Ma and Moses 1995; Treisman and Rubin 1995). A reduction of *wg* function had the opposite effect on ro^{DOM}, reducing the eye size (Fig. 1D) and the extent of photoreceptor differentiation along the disc margins (Fig. 1H,M). We do not understand why reducing the function of an inhibitor of differentiation, such as *wg*, enhances rather than suppresses the ro^{DOM} phenotype, as would be expected. Curiously, genetic interactions between ro^{DOM} and mutations in genes involved in furrow progression are also reversed. For example, mutations in *hh*, a gene necessary for furrow movement, suppress ro^{DOM}, whereas mutations in *patched*, an inhibitor of furrow movement, enhance ro^{DOM} (U. Heberlein, unpubl.). The 90C1-2 mutants also displayed a dominant genetic interaction with the *blink* allele of *dpp* ($dpp^{d\text{-}blk}$). They acted as enhancers of the small-eye phenotype displayed by $dpp^{d\text{-}blk}$, whereas *wg* is known to act as a suppressor (Treisman and Rubin 1995). The enhancement of *blink* led us to name the affected gene *eld*. In both of these situations *eld* mutations showed effects opposite to those of *wg* mutations, suggesting that *eld* may normally function as an antagonist of *wg* signaling.

Because *eld* mutants die as embryos, we investigated the function of *eld* in eye development in mosaic retinae. Clones of cells homozygous mutant for *eld* were generated by the FLP–FRT method, consisting of the yeast FLP recombinase and its target FRT site (Golic 1991; Xu and Rubin 1993) (see Materials and Methods). Very few *eld* mutant cells were found when clones mutant for strong *eld* alleles were analyzed in adult eyes (Fig. 2A,B). However, such clones were associated with scars, suggesting that *eld* mutant cells were present at one stage and interfered with normal development. Clones analyzed in the eye disc did contain *eld* mutant cells, although in general the *eld* mutant clones were small in comparison to the wild-type twin spot clones (Fig. 2C), suggesting that *eld* is required for cell proliferation and/or survival. Exceptions were clones that included the posterior margin of the disc, which were frequently much larger than internal clones (Fig. 2D). The reason for this differential effect is unknown; however, it has been shown that initiation of development at the posterior margin is driven by *dpp* (Wiersdorff et al. 1996; Chanut and Heberlein

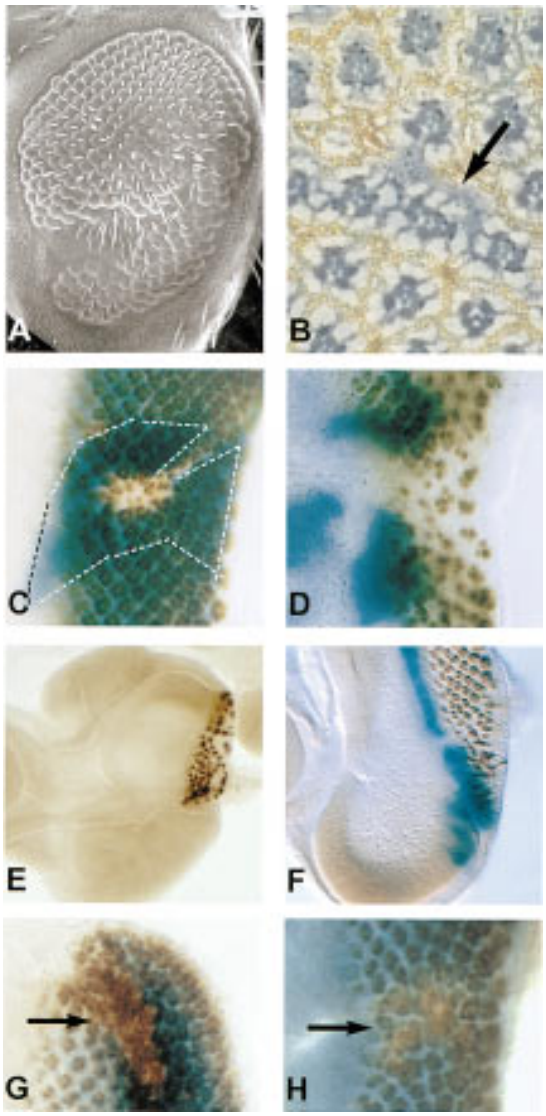

Figure 2. Phenotypes of Eld clones in the eye. (*A*) Scanning electron micrograph and (*B*) a tangential section of eld^{308} clones in the adult eye. The clones form scars with few remaining *eld* mutant cells, marked by the absence of pigment caused by the *white* mutation (arrow). (*C*,*D*) eld^{308} clones in eye discs stained with anti-Elav antibody (brown). The clones are marked by absence of X-gal staining (blue). (*C*) A small internal clone and (*D*) a large marginal clone. The wild-type twin spot is outlined with dotted lines in *C*. (*E*) An eld^{308}, M^{+} clone induced in a *M(3)be* background. Although this disc contains a *lacZ* marker, the clone encompasses all the cells posterior to the furrow that would normally express *lacZ;* it causes a failure to initiate over the ventral part of the posterior margin. (*F*) An eld^{308} clone visible by a loss of anti-Elav stained cells (arrow). The disc is double-labeled with X-gal to visualize a *dpp* reporter; *dpp* expression remains at the posterior margin within the *eld* clone. (*G*,*H*) Anti-Elav stained discs containing clones marked by absence of X-gal staining. (*G*) An $E(spl)^{r16}$ clone (arrow); (*H*) An $E(spl)^{r16}$, eld^{308} clone (arrow). Both show excess neuronal differentiation.

1997; Pignoni and Zipursky 1997), whereas its internal propagation depends on *hh* (Heberlein et al. 1993, 1995; Ma et al. 1993). Moreover, clones of cells mutant for the Dpp receptor *thick veins* proliferate significantly only when they include the posterior margin (Burke and Basler 1996).

In addition to its effect on cell proliferation, *eld* also affects neuronal differentiation. Most photoreceptor clusters that formed within *eld* clones contained fewer neuronal cells than normal (Fig. 2C,D); therefore, there seems to be a partial, though not absolute, requirement for *eld* for neuronal differentiation. Larger clones contained a reduced number of clusters as well as a reduced number of cells within each cluster (Fig. 2D). When we increased the size of the *eld* clones further by using a *Minute* mutation to slow the growth of the surrounding wild-type tissue (Morata and Ripoll 1975), a complete block of differentiation within the clone was observed frequently, although this usually did not encompass the entire clone (Fig. 2E,F). The lack of differentiation was not simply attributable to poor cell viability, as all the cells within *eld* clones could be induced to differentiate as neurons by removing the function of the *Enhancer of split* complex genes (*E(spl)*) (Fig. 2G,H), a group of helix–loop–helix (HLH) proteins that mediate lateral inhibition of neurogenesis by the *N* pathway (Delidakis et al. 1991). Therefore, lack of *eld* blocks neuronal differentiation at a point genetically upstream of *E(spl)* function and possibly upstream of the lateral inhibitory process.

The block to differentiation caused by loss of *eld* function in the eye resembles the effect of loss of *sgg* or ectopic expression of *wg* (Treisman and Rubin 1995), although it is less extreme unless the *eld* mutant cells are given a growth advantage. *eld* is required throughout the eye for differentiation, as is *sgg*, although *eld* is additionally required in internal regions of the eye disc for cell proliferation or survival. However, the effects of heterozygosity for *eld* appear most pronounced at the lateral margins, where *wg* is expressed, suggesting that *wg* signaling may be sensitive to the level of *eld* activity. Ectopic *wg* expression was not observed in *eld* mutant clones (data not shown), indicating that the effects of *eld* are not mediated primarily by activation of *wg* expression.

eld *affects wing patterning*

We next determined whether the *eld* mutant phenotype in other tissues was consistent with an effect on *wg* signaling. Clones of *eld* mutant cells induced in the wing disc also produced pattern alterations suggestive of antagonism to *wg*. One effect of clones produced early in development was the transformation of the posterior notum into a partial second wing (Fig. 3A). These wings had reversed anterior–posterior polarity; their most clearly differentiated structure was an alula produced consistently at their anterior margin. This transformation is the reverse of that produced by the wg^{1} mutation, which transforms the wing into a duplicated notum (Morata and Lawrence 1977), and is similar to that produced by

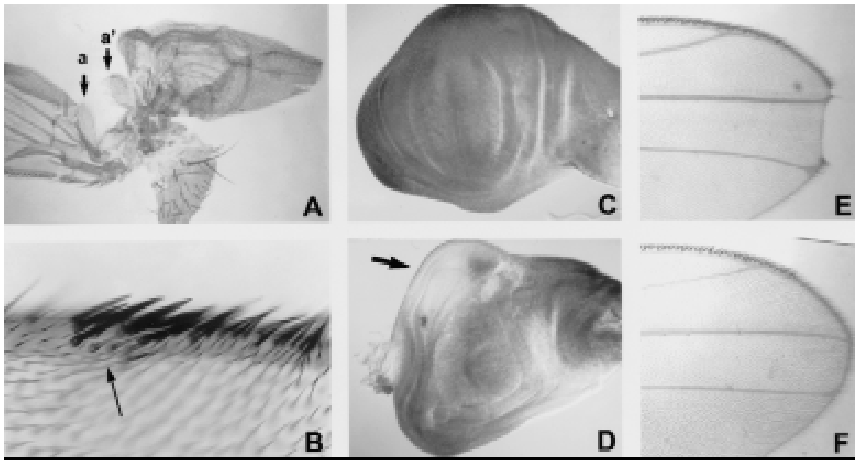

Figure 3. Phenotypes of *eld* in the wing. (*A*) A second wing formed posterior to the first wing. The alula at the posterior of the normal wing (a) is next to the alula at the anterior of the ectopic wing (a′), indicating reversed A-P polarity. (*B*) Extra wing margin bristles induced near the wing margin. eld^{616} mutant bristles are marked with yellow and appear lighter (arrow); they account for only a few of the ectopic bristles. The endogenous margin is out of the focal plane of this photograph. (*C*) A wing disc stained with an antibody to Eld, showing expression throughout the disc. (*D*) Distortion of the wing disc in the region of an unstained eld^{308} clone (arrow). (*C*) N^{5419}/+ wing; (*D*) N^{5419}/+; eld^{308}/+ wing. *eld* suppresses the notching of the distal wing margin caused by *N*.

overexpression of *wg, dpp,* or *optomotor-blind* (*omb*) in the notum (Grimm and Pflugfelder 1996; Ng et al. 1996). The marker we used to identify the *eld* mutant cells, *yellow,* marks only those cells that form wing margin bristles; no such mutant cells were detected in these ectopic wings. However, in wing discs containing *eld* clones and stained with an antibody to the Eld protein (see below), distortion of the disc was associated with regions failing to express Eld (Fig. 3D). These mutant cells must therefore either die at a later stage or fail to contribute to the wing margin, where they can be identified positively.

Clones induced later in wing development were associated with ectopic wing margin bristles (Fig. 3B). Many or all of these ectopic bristles were not mutant for *eld,* but they were sometimes seen to form adjacent to *eld* clones (Fig. 3B; data not shown). Ectopic bristle formation was restricted to the dorsal surface of the wing, within the anterior compartment, and was observed most commonly near the wing margin in tufts of the bristle type appropriate to their position along the anterior–posterior axis. Differentiation of the wing margin is controlled by the *wg* pathway, and ectopic wing margin bristles are produced in clones mutant for *sgg* (Simpson et al. 1988; Perrimon and Smouse 1989; Couso et al. 1994). However, *sgg* clones show neither the non-autonomy nor the positional restrictions observed for *eld* clones. Overexpression of *dsh* can also produce ectopic bristles, which are *wg*-dependent and therefore predominantly found close to the normal wing margin (Axelrod et al. 1996). The restriction of ectopic bristle formation attributable to loss of *eld* to the anterior dorsal region near the wing margin suggests either that *eld* represses bristle formation only in this area of the wing, or that *eld* is required for cell viability in other regions. The second explanation may be correct, as even within the observed clones few *eld* mutant cells are observed.

A reduction of *wg* expression at the wing margin caused by heterozygosity for *N* is associated with nicks in the distal wing margin (Fig. 3E) (Diaz-Benjumea and Cohen 1995; Rulifson and Blair 1995; de Celis et al. 1996). These nicks were suppressed completely by the removal of one copy of *eld* (Fig. 3F), indicating that reduction of *eld* expression can compensate for a reduction in either *N* function or *wg* expression. Loss of *N* activity is also associated with extra bristle-forming cells within the proneural region of the wing margin (Rulifson and Blair 1995), suggesting that *eld* and *N* might interact in bristle formation. *eld* clones do not seem to affect another *N*-dependent process, the formation of notal microchaetae, although the *wg*-dependent macrochaetae (Phillips and Whittle 1993) are often misplaced, duplicated, or triplicated (data not shown).

eld *is required in embryonic segmentation*

To further examine the interaction of *eld* with the *wg* pathway, we determined its effect on embryonic segmentation. Embryos zygotically mutant for *eld* appeared to have normal cuticle patterning and normal expression of the segment polarity gene *en* (data not shown). However, because Eld protein is present at high levels in the early embryo (see Fig. 6, below) and is presumably contributed maternally, these embryos would still contain Eld. To remove both the maternal and zygotic contributions of *eld,* the FLP–FRT–ovo^D system (Chou and Perrimon 1992) was used to produce *eld* mutant germ-line clones. Embryos derived from these clones showed severe defects in the cuticle pattern (Fig. 4B,C), with many denticle belts either missing, fused, or otherwise abnormal. Interestingly, the provision of a paternal wild-type copy of the *eld* gene failed to rescue the maternal mutants and made no perceptible difference to the phenotype (data not shown), suggesting either that early expression of *eld* is critical for its function or that the level of expression of the paternal copy is insufficient. Similarly, embryos maternally mutant for *sgg* are only partially rescued by a wild-type paternal copy of the gene (Perrimon and Smouse 1989; Siegfried et al. 1992), sug-

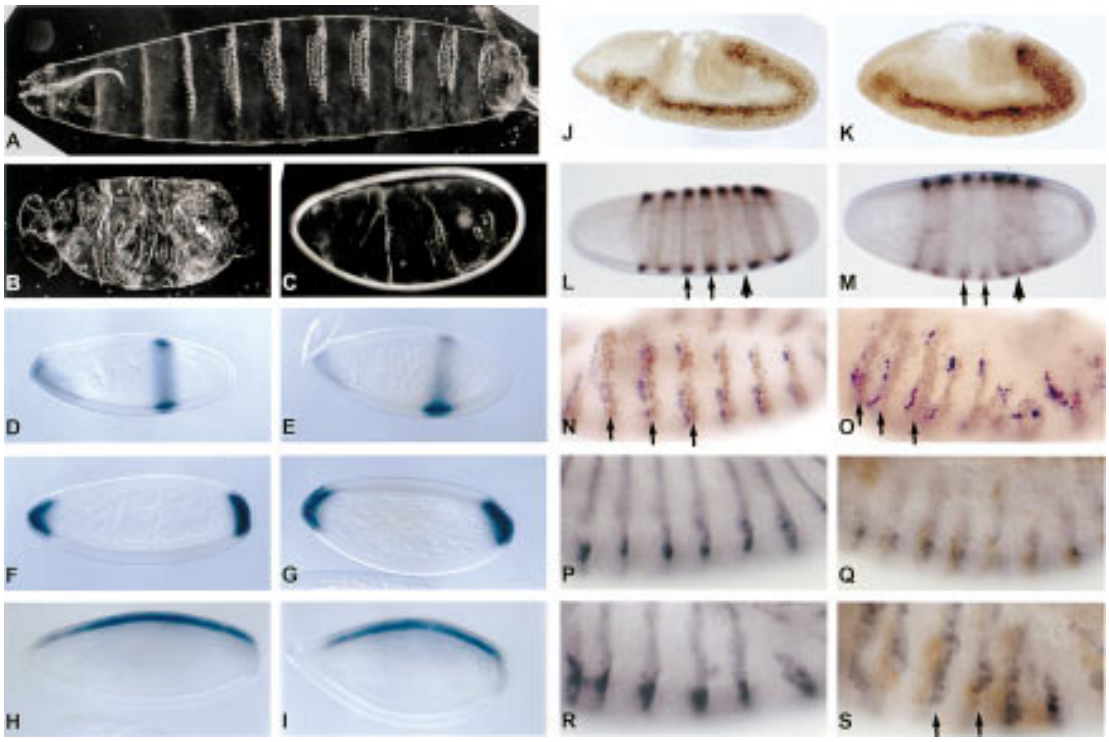

Figure 4. Embryonic phenotype of maternal and zygotic *eld* mutants. (*A,D,F,H,J,L,N*) Wild type. (*B,C,E,G,I,K,M,O*) Embryos derived from eld^{308} germ-line clones. (*A–C*) Cuticle preparations. *eld* embryos show missing and disorganized denticle belts. (*D,E*) In situ hybridization with a *knirps* probe. (*F,G*) In situ hybridization with a *huckebein* probe. Expression of gap genes is normal. (*H,I*) In situ hybridization with a *zerknullt* probe. (*J,K*) Anti-twist staining. Early patterning along the dorsoventral axis is normal. (*L,M*) Anti-even-skipped staining. The pattern is slightly altered in *eld* mutants. Stripes 3 and 4 are weaker (arrows) and the gap between stripes 5 and 6 is smaller (arrowhead). (*N,O*) Anti-En staining (brown) and in situ hybridization with a *wg* probe (blue). Wg RNA is present in stripes of approximately the normal width, but several En stripes are expanded (arrows) and others are disorganized. (*P–S*) Epistasis of *eld* over *wg*. (*P*) Wild type; (*Q*) wg^{P}; (*R*) eld^{616} maternal mutant; (*S*) wg^{P}; eld^{616} maternal mutant. The wg^{P} homozyotes, which contain a mutagenic enhancer trap insertion, are identified by staining with anti-β-galactosidase (brown). Embryos are also stained with anti-En (purple). En expression is lost from wg^{P} but is restored in the double mutant (arrows).

gesting that antagonism of the *wg* pathway may require high levels of gene expression.

Because the cuticle phenotype could be consistent with a number of possible defects in segmentation, we looked at the expression patterns of a variety of genes to determine the stage at which these defects arise. The gap genes *knirps* (Fig. 4D,E), *hunchback*, *Krüppel* (data not shown), *huckebein* (Fig. 4F,G), and *tailless* (data not shown) were expressed normally. Slight defects were seen in the expression pattern of the pair-rule gene *even-skipped* (*eve*) (Fig. 4L,M)—stripes 3 and 4 often appeared weaker than normal, stripe 2 wider, and stripes 5 and 6 closer together. This pattern is in fact quite similar to that of *eve* during its early expression in wild-type embryos, suggesting a failure of refinement. The *eld* mutant embryos also failed to extend their germ bands normally, arresting at a partially extended stage. The dorsal–ventral axis of the embryo is determined by a gradient of Dpp activity (Ferguson and Anderson 1992a), and a similar failure of germ-band extension is seen in ventralized embryos mutant for *dpp* or for genes in the *dpp* pathway (Ferguson and Anderson 1992b). However, both patterning along the dorsal–ventral axis, as judged by the expression of the dorsal gene *zen* (Fig. 4H,I) and the ventral gene *twist* (Fig. 4J,K), and formation of amnioserosa by the dorsal-most cells (data not shown) appeared normal in the absence of *eld*. Therefore, *eld* affects to some degree the expression of pair-rule genes and the morphogenetic movements that occur before the start of *wg* expression.

wg function is first required in the embryo to maintain the expression of *en* (DiNardo et al. 1988). Although *en* expression initiated relatively normally in maternally mutant *eld* embryos (data not shown), its later expression was abnormal—several stripes appeared broadened, others were partially missing, and their spacing was disrupted. Despite the significant increase in width of the *en* stripes, however, the *wg* stripes were not expanded, and ectopic *wg* was not generally induced posterior to the *en* stripes (Fig. 4, cf. N and O). These results are consistent with *eld* acting to counteract Wg signaling posterior to the *en* stripes, in addition to responding to earlier patterning signals that affect the positioning of pair-rule gene stripes.

We attempted to order *eld* relative to *wg* in the segmentation pathway using an epistasis test; we examined

embryos maternally mutant for *eld* and zygotically mutant for *wg*. *en* expression is reduced in *wg* single mutants (Fig. 4P,Q), but is present in both *eld* single mutants (Fig. 4R) and *eld, wg* double mutants (Fig. 4S); this would suggest that *eld* is epistatic to *wg*. However, we could not use null alleles in this experiment because the doubly heterozygous flies did not survive. Therefore, it is still possible that *eld* acts upstream of *wg* and that the absence of *eld* potentiates the activity of the small amount of remaining *wg*.

eld *encodes a nuclear proline-rich protein with a potential DNA-binding domain*

The three P-element alleles of *eld* allowed us to clone genomic DNA in the region by plasmid rescue. The resulting fragments were used to screen a cosmid genomic library. Probes extending at least 10 kb on each side of the P-element insertion sites were used to screen an eye disc cDNA library, and only a single class of cDNAs was isolated. All of the P elements are located in the first intron of this transcript, downstream of a noncoding exon (Fig. 5A). None of these cDNAs was full-length, and further screening of both eye disc and embryonic cDNA libraries was required to isolate overlapping cDNA clones covering the entire open reading frame (ORF). Eye disc and embryonic cDNAs were identical except for two small exons found only in the eye disc clones (Fig. 5A). The complete ORF deduced from these cDNAs would encode a protein of 2713 amino acids (Fig. 5B). The protein contains a region of homology to the DNA-binding domains of the *Drosophila* protein dead ringer (Gregory et al. 1996) and the mouse protein Bright (Herrscher et al. 1995; Fig. 5C). There is also a second region of homology to a protein predicted by the *Caenorhabditis elegans* genome project and to two human expressed sequence tags (ESTs) as well as to ESTs from mouse and rat (Fig. 5D; data not shown). Interestingly, the *C. elegans* ORF is adjacent to another ORF containing a region homologous to the potential DNA-binding domain of Eld (Fig. 5C), and it is possible that these actually belong to the same protein in *C. elegans* as well as in *Drosophila*. Another feature of the Eld sequence is that it is extremely proline-rich; proline comprises 17% of its amino acids. One of the EMS alleles of *eld*, eld^{616}, was found to contain a stop codon substituted for glutamine 338 (Fig. 5B), and therefore would produce only a small amino-terminal portion of the protein.

To examine the distribution and subcellular localization of the Eld protein, we raised a monoclonal antibody against a glutathionine *S*-transferase (GST) fusion protein containing amino acids 965–1049 of Eld. Staining of imaginal discs and embryos revealed that the protein was nuclear (Fig. 6A,B). Its distribution was quite ubiquitous in early embryos, showing no hint of a striped pattern (Fig. 6B), and was also ubiquitous in wing discs (Fig. 3C); although the protein was present everywhere in eye discs, its strongest expression occurred in a band just anterior to the MF, in the position where cells respond to Hh and Dpp signaling (Fig. 6A). To confirm that the antibody was specific for Eld, we stained embryos from *eld* germ-line clones; although strong staining was seen in those embryos carrying a paternal copy of the gene, the maternally and zygotically mutant embryos were completely unstained (Fig. 6C). This confirmed both that the antibody specifically recognizes the proline-rich protein, and that this protein is the product of the *eld* gene.

Discussion

eld *antagonizes wingless signaling*

We have shown for several different tissues in the fly that *eld* mutations have effects opposite to those caused by the absence of *wg* signaling. Loss of *eld* in the eye disc prevents normal photoreceptor differentiation, whereas differentiation is promoted by the loss of *wg* and inhibited completely by ectopic *wg* (Ma and Moses 1995; Treisman and Rubin 1995). The regions of the eye where *wg* is expressed are the most sensitive to a reduction in *eld* dosage. Loss of *eld* in the wing disc can cause a transformation of notum to wing, opposite to the wg^1 phenotype of wing-to-notum transformation and similar to the effect of ectopically expressed *wg* (Morata and Lawrence 1977; Ng et al. 1996). Another phenotype associated with loss of *eld* is ectopic formation of wing margin bristles, similar to the *sgg/zw3* mutant phenotype and opposite to the loss of margin seen in *wg* mutants (Simpson et al. 1988; Blair 1992; Couso et al. 1994). Finally, some of the stripes of *en* expression in the embryo are expanded in *eld* maternal mutants, as they all are in *sgg* maternal mutants (Siegfried et al. 1992); in contrast, *en* stripes are lost in *wg* mutants (DiNardo et al. 1988). *eld* also causes a mild overproduction of notal macrochaetae (data not shown), which are lost in *wg* mutants (Phillips and Whittle 1993).

We suggest that *eld* acts downstream rather than upstream of *wg* for the following reasons. First, *wg* expression is present in stripes of approximately normal width in embryos containing no Eld protein, showing that Eld is not required to repress *wg* transcription in the embryo. Second, ectopic *wg* expression is not found in clones of *eld* mutant cells in the eye disc, indicating again that *eld* does not repress *wg* expression. Third, En stripes are present in the *eld, wg* double mutant combination, suggesting that *eld* is not genetically upstream of *wg*. We have not been able to order *eld* relative to other known elements of the *wg* pathway, however, because of the technical difficulty in making clones on two different chromosomes simultaneously and the semi-lethality of heterozygous combinations of *eld* and *wg* pathway mutants. Another possibility that cannot be ruled out is that Eld could act by altering the activity of the Wg protein or the efficiency of *wg* signaling. Several possible models compatible with the data presented are shown in Figure 7. Further studies are necessary to determine whether Eld receives input from Sgg, Arm, or other factors, or belongs to a separate pathway.

eld *could be a nuclear effector of Wg*

Little is known about how the Wg signal is transmitted

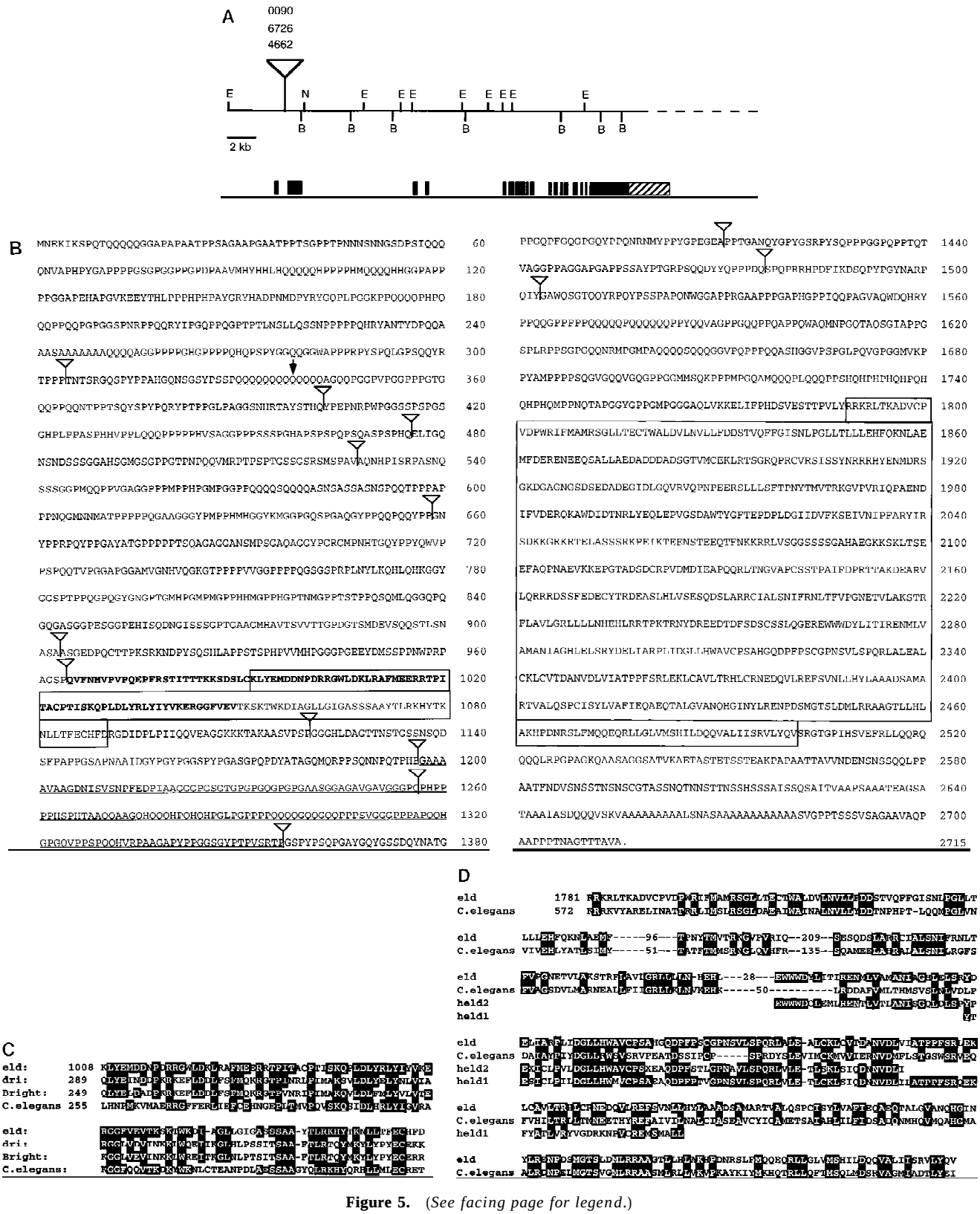

Figure 5. (*See facing page for legend.*)

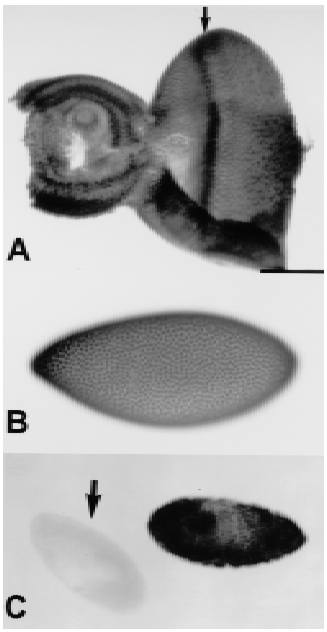

Figure 6. Expression of the Eld protein. All panels show staining with the Eld monoclonal antibody 15A8. (*A*) An eye disc with ubiquitous nuclear expression, which is enhanced just anterior to the MF (arrow). (*B*) An embryo at the cellular blastoderm stage, with staining in all nuclei. (*C*) Two embryos derived from an eld^{308} germ-line clone. One has a paternal copy of *eld* and stains with the antibody; the other is maternally and paternally mutant and shows no staining (arrow).

at the molecular level. The Dfz2 molecule is able to act as a Wg receptor in cell culture (Bhanot et al. 1996), although its contribution to Wg signaling in vivo has not been ascertained. The cytoplasmic protein Dsh is phosphorylated by an unknown kinase in response to Wg signaling (Yanagawa et al. 1995). This event appears to lead to the inactivation of the Sgg serine/threonine kinase, which would otherwise phosphorylate the β-catenin homolog Arm. Active, unphosphorylated Arm accumulates in the cytoplasm and nucleus, whereas the phosphorylated form is restricted to adherens junctions (Peifer et al. 1994a,b; Orsulic and Peifer 1996). Pan, a *Drosophila* homolog of the HMG box protein Lef-1, is a good candidate to act in combination with Arm to activate target genes such as *en* or *Ubx* (Brunner et al. 1997; Riese et al. 1997; van de Wetering et al. 1997). These target genes are the only other known nuclear effectors of *wg*, and because their expression is induced by Wg, they cannot be directly involved in transducing the signal. Eld meets the criteria for a direct nuclear effector; its expression is ubiquitous in the early embryo and imaginal discs, and therefore cannot be dependent on localized Wg signaling. The distribution of Eld protein is not altered in embryos maternally and zygotically mutant for *sgg* or *dsh* (data not shown). However, the absence of *eld* has effects similar to those caused by *wg* overexpression, suggesting that its activity may normally be inhibited in the *wg*-expressing regions. Eld appears to function as a repressor of *en* expression, although this effect need not be direct. Intriguingly, its homolog dead ringer was identified by its ability to bind the consensus target sequence for the En protein (Gregory et al. 1996), which was derived from possible autoregulatory sites within the *en* genomic region (Desplan et al. 1988; Kassis 1990; Heemskerk et al. 1991). If Eld also binds to these sites, it might compete with En to prevent the establishment of autoregulation. This would imply that eld acts as a repressor; several active repression domains have been shown to have a similarly high proline content (Han and Manley 1993). Further study will be required to determine the DNA-binding specificity and biochemical function of Eld.

Interactions between signaling pathways

Our analysis indicates that the function of Eld is not restricted to the Wg signaling pathway. Although *eld* mutant cells behave similarly in many respects to cells that lack *sgg* or express *wg* ectopically, significant differences nevertheless exist. In particular, *eld* clearly functions in embryogenesis before *wg* is expressed. The effects of *eld* on individual Eve stripes are reminiscent of those of the JAK/STAT (*hopscotch*/*marelle;* Binari and Perrimon 1994; Hou et al. 1996; Yan et al. 1996) signaling pathway, although the particular stripes affected are different. In the eye, *eld* phenotypes are less extreme than those caused by ectopic *wg* (Treisman and Rubin 1995) although equally strong phenotypes can be seen if *Minute* mutations are used to increase the size of clones. However, *eld* appears to be required for proliferation, whereas ectopic *wg* stimulates proliferation. *eld* has been isolated independently as a dominant suppressor of activated *Ras1* expressed in the eye (Karim et al. 1996), and its requirement for *ras* signaling may explain its role in proliferation. The effects of *eld* on wing margin bristle

Figure 5. Molecular structure of the *eld* gene. (*A*) Genomic map of the *eld* region. Exons are shown as black boxes; the initiator methionine is in the second exon. The two gray exons are present in eye disc but not in embryonic cDNAs. The genomic structure corresponding to the last 2.3 kb of the *eld* transcript has not been characterized (hatched box and broken line). The three P-element *eld* alleles are inserted very close to each other in the first intron. (E) *Eco*RI; (B) *Bam*HI; (N) *Not*I. (*B*) Sequence of the Eld ORF. The positions of identified introns are shown by inverted triangles. The glutamine mutated to a stop codon in eld^{616} is indicated by an arrow. The region in bold type was used to generate the monoclonal antibody used in Figs. 3 and 6. The regions of homology shown in *C* and *D* are boxed. The GenBank accession number for this sequence is 1413215. (*C*) Homology of Eld to the DNA-binding domains of dead ringer (dri), Bright, and a related predicted protein from *C. elegans.* Amino acids identical to the Eld sequence appear white on a black background. (*D*) Homology of Eld to predicted *C. elegans* and human proteins (held1 and held2). Amino acids identical to the Eld sequence appear white on a black background.

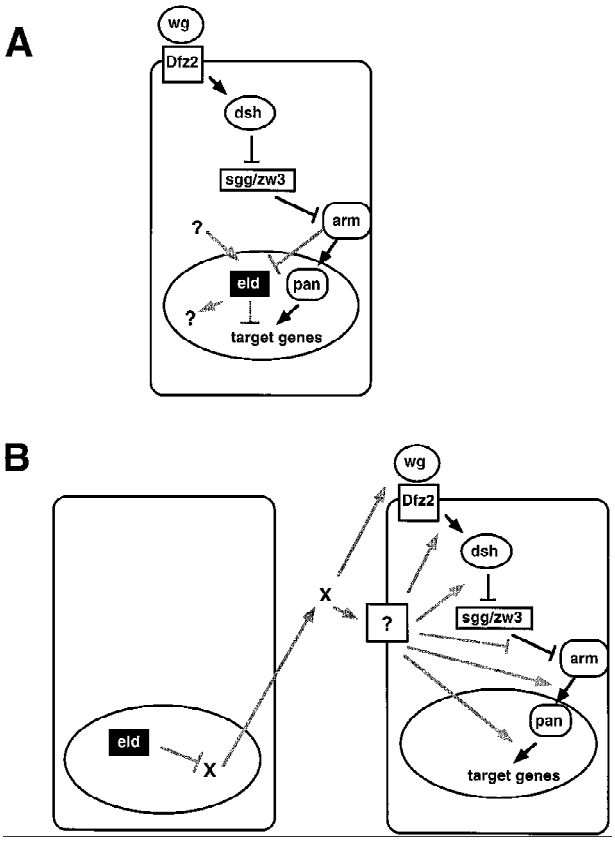

Figure 7. Possible roles of Eld in Wg signaling. (*A*) In this model, Eld acts as a nuclear repressor of Wg target genes (see Discussion). This model is supported by the finding that *eld* appears to be epistatic to *wg* in regulating the width of En stripes in the embryo and by the observation that *wg* expression is unaffected by loss of *eld* function. However, the non-autonomy of Eld function during wing development cannot be explained easily by this model. (*B*) In this model Eld acts as a repressor of a diffusible molecule X that could either augment Wg function directly or enhance the efficiency of Wg signaling at various steps in the signal transduction cascade. This model explains the phenotypes induced by loss of *eld* function in wing development. However, it fails to explain the epistatic relationship between *wg* and *eld* in the embryo. Shaded lines indicate postulated sites of genetic interaction. Arrows indicate positive genetic interactions, and perpendicular intersecting lines indicate negative genetic interactions.

formation differ from those of *sgg* in their nonautonomy and their positional restriction; again, the nonautonomy suggests that Eld does not function solely as a nuclear effector of Wg.

eld is not the only gene described with the potential to participate in multiple pathways. The *sgg* gene also has multiple functions in the embryo; in addition to regulating En maintenance, it is required for normal organization of the nervous system (Perrimon and Smouse 1989). Loss of *eld* leads to a similar disorganization that is considerably less extreme than the overproduction of neuroblasts seen in *N* mutants (data not shown). Additional precedents exist for effects on early stages of patterning caused by mutations that also affect neurogenesis; *strawberry notch* (*sno*) has defects at the blastoderm stage (Coyle-Thompson and Banerjee 1993), whereas *hairy* (*h*), which has neurogenic effects in the notum, wing and eye, acts as a pair-rule gene in the embryo (Rushlow et al. 1989; Brown et al. 1995). The *groucho* gene appears to function with *h* in segmentation, with the *E(spl)* genes in neurogenesis, and with *deadpan* in sex determination (Paroush et al. 1994), in addition to repressing *en* expression in the wing disc and embryo (de Celis and Ruiz-Gomez 1995). In the latter case, the phenotypes may be accounted for by a common mechanism, such as co-repression with basic HLH proteins (Paroush et al. 1994), rather than a common genetic pathway. Many questions remain about the mode of Eld action and its regulation by Wg and other signals, which can only be answered by biochemical analysis. Nevertheless, results to date suggest that Eld belongs to the growing class of signal integrators.

Materials and methods

Fly strains

The screen in which *eld* was identified will be described in detail elsewhere. Briefly, wild-type males were mutagenized with EMS or X-rays and mated to $ro^{DOM}/TM3, Sb$ virgin females. The F_1 progeny was screened for enhancers or suppressors of the furrow-stop phenotype of ro^{DOM}. Two EMS alleles of *eld* were used for all experiments: eld^{616}, which has a stop codon at position 338, and eld^{308}, which behaved as a stronger allele than eld^{616}, although we were unable to molecularly identify the mutation. Embryos maternally mutant for either allele do not express full-length protein as determined by immunohistochemistry with the monoclonal antibody directed against the predicted DNA-binding domain. The three lethal P-element alleles used were also identified as weak suppressors of ro^{DOM}; they failed to complement the lethality of the EMS alleles. l(3)00090, l(3)04662, and l(3)06726 have been described by Spradling et al. (1995). Other alleles used were ro^{DOM} (Heberlein et al. 1993), $dpp^{d\text{-}blk}$ (Masucci et al. 1990), *dpp–lacZ* BS3.0 (Blackman et al. 1991), wg^{P} (Kassis et al. 1992), wg^{IL114}, wg^{CX4}, $E(spl)^{r16}$, *M(3)be[36e]*, *M(3)w*, N^{317} (Karim et al. 1996), and N^{5419}. Two enhancer trap lines, a generous gift of Kevin Moses (University of Southern California, Los Angeles), were used to mark *eld* clones in the eye disc; one is at chromosomal position 94F, which expresses only posterior to the furrow (Fig. 2C,E,G), and the other at position 96C, which expresses at lower levels throughout the eye disc (Fig. 2D). An Arm–LacZ line on 3R was also used (Vincent et al. 1994; Fig. 2H).

Genetics

To make *eld* mutant clones in the adult eye, males of genotype $w; FRT82, eld^{308}/TM3$, or $w; FRT82, eld^{616}/TM3$ were crossed to females of genotype $w, hsFLP1; FRT82, P(w^{+})90C$. To make *eld* mutant clones in the eye disc, males of genotype $w; FRT82, eld^{308}/TM6B$, or $w; FRT82, eld^{616}/TM6B$ were crossed to females of genotype $w, hsFLP1; FRT82, P(lacZ, ry^{+})94F$ or $P(lacZ, ry^{+})96C$. Non-*Tb* larvae were selected for analysis. To make *eld, E(spl)* double mutant clones, the males used were $w; FRT82, eld^{308}, E(spl)^{r16}/TM6B$. To make large *eld* clones in the eye disc, the females used were $w, hsFLP1; FRT82, P(ry^{+})94F$,

M(3)be[36e]. To make *eld* clones in the wing disc, the females were *y, w, hsFLP1; FRT82, P(hs-πM)87E, Sb^{63b}, P(y^{+})96E*. To make *eld* mutant germ-line clones, males of genotype *w, hs-FLP1/Y; FRT82, ovo^{D1}/TM3* were crossed to females of genotype *w; FRT82, eld^{308}/TM3*, and non-*Sb* females resulting from this cross were crossed to *eld^{308}/TM3, eve–lacZ* males. To make maternal *eld*, zygotic *wg* double mutants, males of genotype *w, hsFLP1/Y; FRT82, ovo^{D1}/TM3* were crossed to females of genotype *w; FRT82, eld^{616}; wg^{P}/SM6.TM6B*, and the resulting *w, hsFLP1/w; FRT82, ovo^{D1}/FRT82, eld^{616}; wg^{P}/+* females were crossed to *w; FRT82, eld^{616}; wg^{P}/SM6.TM6B* males. All larvae were heat-shocked for 1 hr at 38.5°C in the first or second instar to induce *hsFLP* expression.

Histology and immunohistochemistry

Flies were prepared for scanning electron microscopy as described by Kimmel et al. (1990). Adult eyes were fixed, embedded, and sectioned as described by Tomlinson and Ready (1987). Eye imaginal discs were stained with antibodies as described by Xu and Rubin (1993), except that the detergent used was 0.2% Triton. Anti-Elav and anti-Eld were both diluted 1:2. Double labeling with antibody and X-gal was performed as described by Treisman et al. (1995). Embryos were stained with antibodies by blocking in PBS with 0.2% Triton and 5% donkey serum (PBSTS), incubating in primary antibody in PBSTS at 4°C overnight, washing in PBS with 0.2% Triton three times for 20 min at room temperature, incubating in secondary antibody (donkey anti-mouse or anti-rabbit, Jackson Immunoresearch) at a 1:200 dilution in PBSTS for 1 hr at room temperature, washing as above, and developing with a metal-stabilized DAB solution (Pierce). Rabbit anti-Twist was provided by Kathryn Anderson (University of California, Berkeley) and was used at a dilution of 1:5000. Rabbit anti-Eve was provided by Monica Boyle (Rockefeller University, New York, NY) and was used at a dilution of 1:5000. Mouse anti-En was provided by Peter Bokor (Rockefeller University, New York, NY) and was used undiluted. In situ hybridization and in situ/antibody double labeling were performed as described by Ronchi et al. (1993). To examine wings, flies were dehydrated through an ethanol series and the wings were mounted in methyl salicylate/Canada balsam (1:2).

Molecular biology

Standard procedures were used for DNA analysis (Sambrook et al. 1989). DNA surrounding the l(2)00090, l(2)04662, and l(2)06726 P elements was isolated by plasmid rescue (Mlodzik et al. 1990). All these probes were used to screen a cosmid library (Tamkun et al. 1992) and the genomic region was assembled by a cosmid walk. Fourteen cDNAs that cross-hybridized were isolated from a λgt10 third-instar eye-antennal disc cDNA library (constructed by Alan Cowman, Walter and Eliza Hall Research Institute, Victoria, Australia) using probes covering 24 kb of the walk. Further cDNA clones were isolated by a cDNA walk using both this library and a 9- to 12-hr embryonic λgt11 cDNA library (constructed by Kai Zinn, Caltech, Pasadena, CA). Six cDNAs were subcloned into p(Bluescript)SK+ and were sequenced completely on both strands using an Automated Laser Fluorescent DNA sequencer (Pharmacia), and several additional cDNAs were sequenced partially. The exon positions were mapped onto the genomic structure by hybridization and sequencing of junctional regions. Sequencing of the P-element rescue fragments was used to define their precise insertion positions. Sequences were analyzed using Staden and LaserGene software, and homology searches were done using the *blastp* program.

Antibody production

A fragment encoding amino acids 965–1049 of the Eld protein was generated by PCR, and was subcloned into the *Bam*HI and *Eco*RI sites of pGEX-1 (Pharmacia) using sites present in the PCR primers. The fusion protein was purified from bacteria on glutathione–agarose beads and injected into mice. Monoclonal antibodies were screened first by ELISA and then by staining embryos.

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