Development 130, 4085-4096

doi:10.1242/dev.00624

EDL/MAE regulates EGF-mediated induction by antagonizing Ets transcription factor Pointed

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Accepted 22 May 2003

SUMMARY

Inductive patterning mechanisms often use negative regulators to coordinate the effects and efficiency of induction. During Spitz EGF-mediated neuronal induction in the *Drosophila* compound eye and chordotonal organs, Spitz causes activation of Ras signaling in the induced cells, resulting in the activation of Ets transcription factor Pointed P2. We describe developmental roles of a novel negative regulator of Ras signaling, EDL/MAE, a protein with an Ets-specific Pointed domain but not an ETS DNA-binding domain. The loss of EDL/MAE function results in reduced number of photoreceptor neurons and chordotonal organs, suggesting a positive role in the induction by Spitz EGF. However, EDL/MAE functions as an antagonist of Pointed P2, by binding to its Pointed domain and abolishing its transcriptional activation function. Furthermore, *edl*/*mae* appears to be specifically expressed in cells with inducing ability. This suggests that inducing cells, which can respond to Spitz they themselves produce, must somehow prevent activation of Pointed P2. Indeed hyperactivation of Pointed P2 in inducing cells interferes with their inducing ability, resulting in the reduction in inducing ability. We propose that EDL/MAE blocks autocrine activation of Pointed P2 so that inducing cells remain induction-competent. Inhibition of inducing ability by Pointed probably represents a novel negative feedback system that can prevent uncontrolled spread of induction of similar cell fates.

Key words: Eye, Chordotonal organ, Ras signaling

INTRODUCTION

In the development of a wide variety of animal tissues, a small number of pre-specified cells induce their neighbors to generate secondary cells with similar fates (Mangold and Spemann, 1927). This two-step induction strategy, termed homeogenetic induction, has the potential to produce an endless cascade of induction, leading to either malignant transformations or hyperproliferative diseases (reviewed by Tang et al., 1997; Zwick et al., 2002). In normal development, the induction must thus be regulated both in cells that release the inductive signal and in the cells that receive it, so that cells that receive the inductive signal do not continue to induce other cells. Although recent studies examining induction have identified many evolutionarily conserved signaling pathways acting in the induced cells (reviewed by Edlund and Jessell, 1999), much less is known about the distinct gene regulatory mechanisms that occur within the cells with inducing ability. We address two related questions: (1) 'how is inducing ability regulated?'; and (2) 'how do cells with inducing ability themselves respond to the inductive signals they release?'.

In *Drosophila*, two regions of the nervous system are known to use homeogenetic induction: (1) the specification of photoreceptor neurons during ommatidial assembly in the compound eye (Tomlinson and Ready, 1987), and (2) the formation of subepidermal stretch receptor precursors (chordotonal organ precursors: COPs) in the embryonic peripheral nervous system (Jarman and Jan, 1995). In both processes, cells already specified as photoreceptor neurons or COPs recruit surrounding cells to assume fates similar to their own. The inducing signal is a TGFα-like protein Spitz, that acts through EGF receptor to activate the Ras/MAPK signaling pathway in the induced cells (reviewed by Zipursky and Rubin, 1994). The outcome of the induction is remarkably constant: each ommatidium in the eye contains precisely eight photoreceptor neurons (R1-R8), and each hemisegment in the embryo generates exactly eight COPs (C1-C8). In order to achieve such constancy, the induction process must be exquisitely controlled.

Within the induced cells, the induction is regulated by phosphorylation of Ets transcription factors Pointed P2 (PNTP2) and YAN, which respectively acts as positive and negative regulators of neuronal differentiation (Brunner et al., 1994a; O'Neill et al., 1994; Rebay and Rubin, 1995). Both

proteins possess an Ets-specific domain called the Pointed domain (Klämbt, 1993) that are phosphorylated by MAPK upon stimulation by the induction signal. This modification leads to the degradation of YAN and activation of PNTP2 function. Activation of PNTP2 leads to the production of another isoform produced by the *pointed* (*pnt*) gene, PNTP1, a constitutive transcriptional activator that is necessary for neuronal development of the induced photoreceptor cells and COPs (O'Neill et al., 1994).

Induction is also regulated by restricting the cells with inducing ability. Inducing cells are selected from groups of cells, called proneural cluster, that express a bHLH transcription factor Atonal (reviewed by Jan and Jan, 1995). In the larval eye imaginal disc, proneural cluster consists of a moving front of differentiation called the morphogenetic furrow. From a stripe of cells in the morphogenetic furrow, evenly spaced R8 photoreceptor neurons are selected through lateral inhibition. In the embryo, lateral inhibition likewise selects COPs C1-C5 from Atonal$^+$ proneural clusters. In both tissues, Atonal expression is linked to inducing ability thorough expression of Rhomboid, a founding member of the Rhomboid family of intramembrane serine proteases, which is involved in the proteolytic activation of Spitz EGF (reviewed by Freeman and Gurdon, 2002). Rhomboid$^+$ R8 and C1-C5 secrete Spitz EGF and act as founder cells, inducing their neighbors to assume photoreceptor neuronal fates and COP fates, respectively (Jarman et al., 1993; Jarman et al., 1994; Lage et al., 1997; Okabe and Okano, 1997). R8 then induces the formation of R2 and R5, which in turn express *rhomboid* and serve as the secondary source of Spitz signal. Although in the eye Rhomboid paralog Roughoid plays a major role in induction, misexpression of Rhomboid causes recruitment of supernumerary photoreceptor neurons, implying that spatial regulation of Rhomboid is nevertheless essential for generating the correct ommatidium (Freeman et al., 1992; Wasserman, 2000). R8/R2/R5 and C1-C5 constitute EGF signaling centers, inducing R3/R4/R1/R6/R7 and C6-C8, respectively (Freeman et al., 1992; Tomlinson and Ready, 1987; Lage et al., 1997; Okabe and Okano, 1997). Thus, the transcriptional regulation of *rhomboid* constitutes a key element in specifying the cells with inducing ability.

Spitz EGF acts not only as a paracrine inducer, but also has an autocrine function; inducing cells secreting Spitz themselves receive the Spitz signal and must respond to it for cell survival (Tio and Moses, 1997). Although all cells within the ommatidium require EGF receptor function (Freeman, 1996), it is not known whether or not the Ras signaling pathway downstream of the receptor is used in the same way in all cells. We found that hyperactivation of *pnt* function abrogates the inducing ability of the inducing cells. Hence, inducing cells must possess a mechanism to escape the inhibitory effect of *pnt*. We have identified a novel Ets-related factor EDL (ETS-domain lacking), containing the Pointed domain but not the ETS domain (Shilo, 1998), that may mediate this mechanism. EDL has also been identified as MAE (modulator of the activity of ETS), a protein that binds YAN and promotes its phosphorylation by MAPK (Baker et al., 2001). Although such activity suggests a role of EDL/MAE in the induced cells, we find that EDL/MAE is specifically expressed in cells that act as the induction center by producing Spitz EGF. Furthermore, we show that EDL/MAE abolishes transcriptional activation function of PNTP2 through direct binding, rather than promoting it as suggested by Baker et al. (Baker et al., 2001). This antagonistic action on PNTP2 blocks an autocrine pathway downstream of Spitz EGF, thereby allowing inducing cells to express their inductive function.

MATERIALS AND METHODS

Drosophila strains

The P17 enhancer trap strain harbors an insertion of the P[*ry*$^+$; *lacZ*] enhancer trap P-element PZ (Mlodzik and Hiromi, 1992). This insertion reproduces the early embryonic expression pattern of *edl* in the ventral neuroectoderm, but does not express *lacZ* in the chordotonal precursor or in the eye imaginal disc. Local mobilization of the P-element was performed according to Tower et al. (Tower et al., 1993), and new insertions into the *edl* locus were screened by polymerase chain reaction (PCR). We identified two lines, edl^{JS} and edl^{JV}, harboring PZ element insertions at positions –38 and +4, respectively, from the putative transcription initiation site of *edl*. edl^{JV} insertion was again mobilized, and *rosy*$^-$ excision lines were screened for those that are lethal over the deficiency *Df(2R)P34* (55A; 55F). One such line, edl^{L19}, carried a deletion that includes the entire open reading frame of *edl*. Its 3′ breakpoint resides outside the region covered by our chromosomal walk. Excision lines were also generated from the P17 insertion. The U104 line, which extends farthest towards the *edl* ORF, fully complements edl^{L19}, and did not exhibit the *edl* mutant phenotype.

Somatic recombination clones were induced using the FRT technique (Xu and Rubin, 1993), over FRT43D, P[w^+]47A. Embryos homozygous for edl^{L19} were identified using the *CyO*, *wg-lacZ* balancer chromosome. FRT-mediated mitotic recombination clones of $pnt^{\Delta 88}$ (Klämbt, 1993) were made over FRT82B, *ub-GFP*, P[w^+]90D, $RpS3^2$ (also called $M(3)w^{124}$), and those for a null allele of *rhomboid*, $rhomboid^{P\Delta 5}$ (also called $ve^{rho\text{-}PDelta5}$) (Freeman et al., 1992) were made over P[w^+]70C, $RpS17^4$ (also called $M(3)i^{55}$), FRT80A.

Ectopic expression of *edl* was achieved by the GAL4/UAS system (Brand and Perrimon, 1993), using the following drivers: *elav-GAL4* C155 (Lin and Goodman, 1994), *sevE-GAL4* K25 (Brunner et al., 1994a), CY2 (gift of T. Schüpbach) and *en-GAL4* (gift of A. Brand and N. Perrimon). *UAS-pntP2* (Klaes et al., 1994), *UAS-phl.gof* (also called $D\text{-}raf^{F179}$) (Brand and Perrimon, 1994). $Ras85D^{v12.sev}$ (also called $sevE\text{-}Ras1^{Val12}$) (Fortini et al., 1992) has been described.

Histology

In situ hybridization and antibody staining were performed as described (O'Neill and Bier, 1994; Tautz and Pfeifle, 1989; Tomlinson and Ready, 1987) with minor modifications. Confocal microscopy was done using BioRad MRC 1024 mounted on a Zeiss Axioplan2 microscope. For light microscopy, adult heads were fixed in 0.1 M cacodylate buffer or phosphate buffer (pH 7.4), 2.5% glutaraldehyde, 2% paraformaldehyde for 4 hours to overnight. Samples were postfixed in 1% OsO_4 in the same buffer for 3-4 hours, then dehydrated and embedded in Durcapan (Fluka). Sections (0.5 μm) were cut using a Reichert microtome, stained with Toluidine Blue and viewed in bright field microscopy. For scanning microscopy, flies were fixed in 0.1 M cacodylate buffer (pH 7.4), 2% OsO_4 overnight at room temperature. After dehydration, samples were dried using Peldry II (Ted Pella), coated with gold palladium in a Denton Desk II sputter coater and photographed in a JEOL 840 SEM.

Molecular analysis

DNA sequences flanking the P-element insertion point in the P17 line were recovered by plasmid rescue, and were used to initiate a chromosomal walk. Genomic restriction fragments were used to

screen a 4-6 hour embryonic cDNA library (Novagen). We isolated three classes of cDNAs, represented by N1, N4 and N9, that failed to hybridize with each other. Only N9 showed an expression pattern similar to the *lacZ* expression pattern of the P17 enhancer trap line in the ventral neuroectoderm. Screening of a 4-8 hour embryonic cDNA library (Brown and Kafatos, 1988) using N9 as a probe resulted in isolation of nine additional cDNAs, of which clone 115-3A was the longest. These cDNAs define the *edl* transcription unit that produces a 1.6 kb transcript. N9, whose length is 2 kb, possesses an upstream exon not found in other cDNAs. As the signal produced by a probe unique to N9 was weak, this cDNA appears to represent a minor transcript of *edl*. Nucleotide sequences of cDNAs N9 and 115-3A were determined by the chain termination method using Sequenase v.2 (US Biochemical Corporation), and was compared with the genomic sequence obtained by the *Drosophila* Genome Project (FlyBase, 1999). Within the 37 kb of DNA downstream of the *edl* gene, there was no potential exon capable of encoding an ETS domain. The genomic rescue transgene was made by subcloning a 18 kb *Xba*I genomic fragment into the pCaSpeR4 vector. To generate the UAS-*edl* effector construct, the *edl* open reading frame was PCR-amplified from cDNA clone 115-3A using primer 5′-TCAAGAACTCAAACGTTGCG-3′ and the T7 primer and subcloned into the *Eco*RI site of the pUAST vector (Brand and Perrimon, 1993). Sequences were verified by cycle sequencing according to the instructions of the manufacturer (ABI). GMR-GAL4 carries the GAL4 coding region in the pGMR vector (Hay et al., 1994).

Transfections and CAT assays

Transfections and CAT activity measurements were performed essentially as described (Pascal and Tjian, 1991; O'Neill et al., 1994) with minor modifications. In all transfections, 100 ng of each expression plasmid were cotransfected along with 2 μg of E_6BCAT and 3 μg of pBluescriptSK(–) (Stratagene) using the calcium phosphate method except various amount of EDL/MAE expression plasmid (12.5-200 ng) were used in Fig. 3E. For each plasmid, six (Fig. 3D) or two (Fig. 3E) transfections were performed in parallel and the resulting data were averaged. Expression plasmid pPacEdl was generated by amplifying the open reading frame of *edl* from the N9 cDNA clone using primers 5′-ACGGAAGCCATATGCAAG-TGGAATC-3′ and 5′-GAATCCTCGAGATATGTACAAC-3′, and subcloning into pPacUbx+Nde after digestion by *Nde*I and *Xho*I. Other expression plasmids are described in O'Neill et al. (O'Neill et al., 1994), and were generous gifts from I. Rebay. The pPacEdl clone used in Fig. 3D has a single base mutation causing a conservative amino acid change (K to R) at position 159. Identical results were obtained in small scale experiments using a pPacEdl clone without this mutation.

In vitro binding assay

PNT-derived GST fusion proteins were made by inserting appropriately digested fragments from pPacPntP2 or pPacPntP1 (O'Neill et al., 1994) into the pGEX-KG vector (Guan et al., 1991). Other GST-fusion constructs have been described (Brunner et al., 1994a; Lai and Rubin, 1992; Peverali et al., 1996). GST fusion proteins were purified from bacteria using glutathione-agarose beads (Sigma). Amounts of GST fusion proteins were estimated using CDNB assay as described in the manufacturer's protocol (Pharmacia Biotech). [^{35}S]methionine-labeled EDL/MAE was synthesized using an in vitro transcription/translation system (Promega). Agarose beads (10 μl) containing 0.5 μg GST fusion proteins were incubated with 5 μl of ^{35}S-labeled EDL/MAE for 2 hours at 4°C in 0.2 ml of binding buffer [12 mM HEPES (pH 7.0), 100 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 5% glycerol, 50 mg/ml BSA, 1 mM PMSF], and were washed with binding buffer. Bound proteins were released in SDS sample buffer, and analyzed by SDS-PAGE and autoradiography.

Electrophoretic mobility shift experiments

Proteins Myc-tagged at the N terminus were made by in vitro transcription/translation using TNT T7 Quick kit (Promega) and templates generated by PCR. Primers for the 5′ end contained a T7 promoter sequence for in vitro transcription. Electophoretic mobility shift experiments were carried out using DIG Gel Shift Kit (Roche) with minor modifications. Oligonucleotides containing EBS sequences with or without a mutation (EBS* or EBS) (Albagli et al., 1996) were self-annealed and 3′-end labeled with DIG-11-ddUTP using terminal transferase. Each 12 μl reaction contained 0.5 μl of protein solutions (or the sequential dilutions for myc-Edl), 0.6 ng labeled EBS probe and 0.5 μl anti-Myc monoclonal antibody (9E10). Maximum amount of unlabeled probes used for a competition assay was 12.5 ng per reaction. Solutions were mixed on ice and left for 20-25 minutes at room temperature. The order of mixing did not cause any significant changes in the results. Samples were then resolved on a 0.25× TBE, 2.5% glycerol, 5.25% polyacrylamid gel pre-electrophoresed at 4°C for 1 hour at 80 V. The gel was electroblotted onto GeneScreen Plus membrane (NEN), crosslinked by UV and subjected to a chemiluminescent detection using CDP-Star (Roche) as a substrate. Signals were recorded by Lumi-Imager (Boehringer-Mannheim).

RESULTS

EDL/MAE is specifically expressed in the EGF-signaling center

The *edl*/*mae* gene (hereafter referred to as *edl*) was identified through enhancer trap lines that harbor P-element insertions at 55E (Fig. 1A,B; see Materials and Methods). *edl* encodes a 177 amino acid polypeptide that contains a region similar to the Pointed domain found in many Ets proteins (Klämbt, 1993) (reviewed by Graves and Petersen, 1998) (Fig. 1C,D). In contrast to all other proteins that contain the Pointed domain, EDL/MAE lacks the conserved DNA-binding domain, the ETS domain. Because of the potential function of EDL/MAE in Ras/MAPK signaling, we examined the expression pattern of *edl* in two tissues where Ets proteins function as downstream targets of Ras/MAPK signaling. In the eye imaginal disc *edl* mRNA was expressed in clusters of cells in two rows in the morphogenetic furrow (Fig. 1E). Expression was seen in a small number of cells in each cluster, with a spacing roughly corresponding to that of the ommatidial clusters. To examine *edl* expression at the cellular level, we used an *edl* enhancer trap line edl^{JS} that expresses *lacZ* in the eye imaginal disc. Expression of this *edl-lacZ* reporter initiated in R8 cells within the morphogenetic furrow, corresponding to the stage in which R8 induces R2 and R5 (Fig. 1F). Subsequently, R2/R5, which act as the secondary source of induction, also initiated *edl-lacZ* expression at lower levels. During the development of the embryonic chordotonal organs, *edl* mRNA was present in COP C1-C5, but was undetectable in C6-C8 (Fig. 1G). As in the eye imaginal disc, *edl* expression was transient and disappeared from the COPs before they started dividing. Thus, in both the ommatidium and the chordotonal organ, *edl* expression is detectable only in cells with inducing ability.

edl affects neural inducing ability

To address the role of *edl* in inducing cells, we identified loss-of-function mutations in *edl*. The edl^{JV} line contains a P-element insertion in the vicinity of the presumptive transcription initiation site of *edl* (Fig. 1A) and has viability of

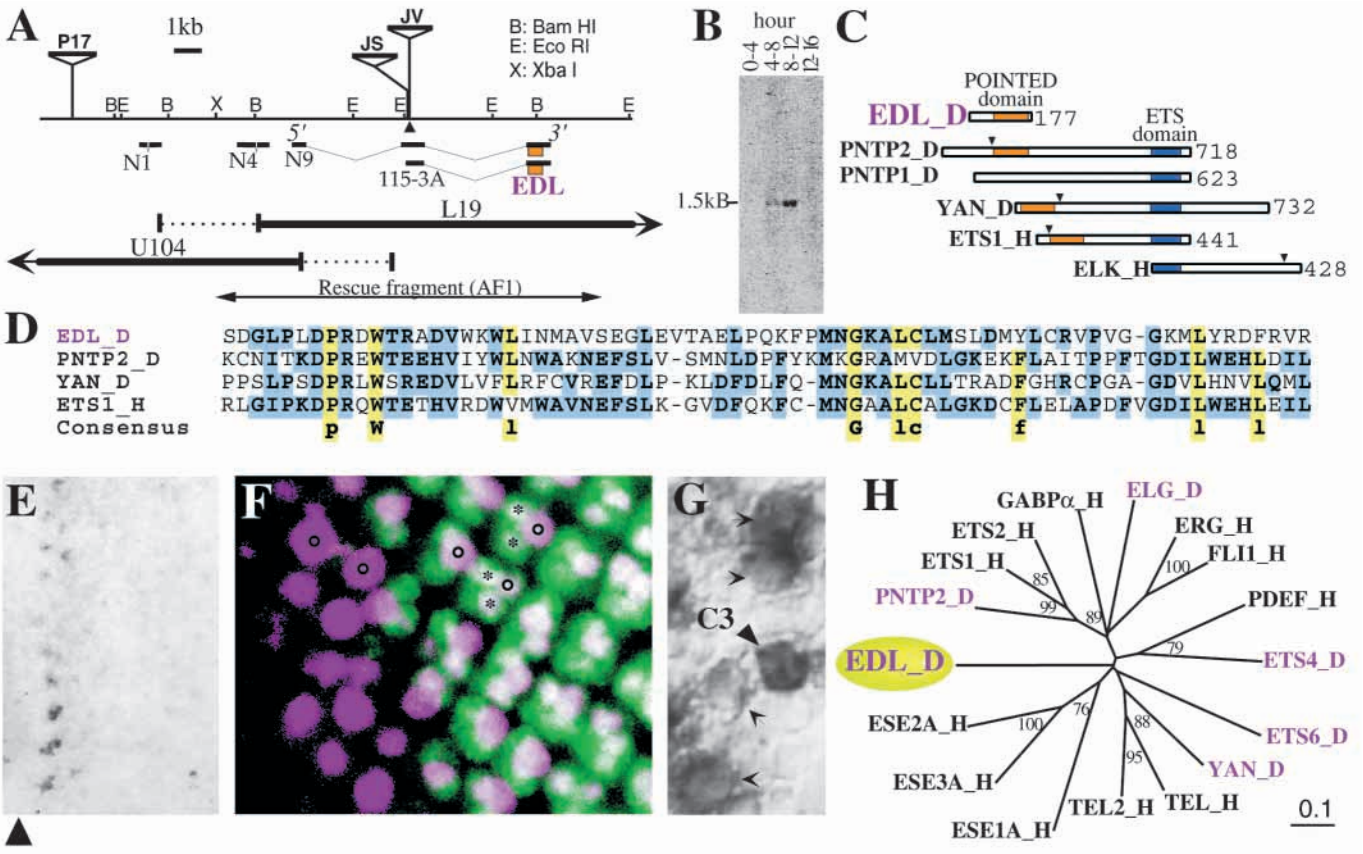

Fig. 1. The structure and expression pattern of *edl/mae*. (A) Genomic organization of the *edl* gene. Insertion points of enhancer trap lines P17, edl^{JS} and edl^{JV} are indicated. Structures of two *edl* cDNAs, N9 and 115-3A are shown below the map. Black triangle represents a possible major transcription initiation site. N1 and N4 are cDNAs unrelated to *edl*. L19 and U104 are chromosomal deletions that were generated by excising P-elements from P17 and edl^{JV} lines, respectively. Solid lines show DNA that are deleted, and broken lines represent segments where deletion end-points reside. The genomic region used to make the *edl* genomic rescue construct is shown by a double-headed arrow. Although N4 is contained within the rescue construct, it is unlikely to correspond to the *edl* gene because U104 that deletes N4 does not exhibit an *edl* mutant phenotype. (B) *edl* encodes a major transcript whose size is ~1.5 kb. N9 thus represents a minor product of *edl*. (C,D) *edl* represents a novel class of Ets proteins. (C) Line diagram showing the structures of EDL and examples of other Ets proteins (D, *Drosophila*; H, Human). The ETS domain is shown by a blue box, Pointed domain by an orange box. Essential MAPK phosphorylation sites are shown by triangles. Numbers on the right indicate the amino acid length. (D) An alignment of the Pointed domain. Amino acids that are conserved in all or most of the proteins (15-16 out of 17) analyzed in H are shaded yellow, and are shown with capital or lower case letters, respectively. Other amino acids that match those of EDL/MAE are shaded blue. (E,F) Expression of *edl* mRNA (E) and enhancer trap line edl^{JS} (F) in the eye imaginal disc. Anterior is towards the left, the position of the morphogenetic furrow is shown by an arrowhead. β-galactosidase expression (magenta) is seen in R8 (open circle) and R2/R5 (asterisk), whereas ELAV (green) is expressed in all neurons. Overlap of magenta and green is white. *edl-lacZ* reporter is also expressed in the subretinal glial cells, located below this focal plane. (G) In the developing chordotonal organ, *edl* mRNA is found in five COPs, C1 to C5 (arrowheads). (H) A phylogenic tree of the Pointed domain. Sequences of all Ets proteins containing Pointed domain from *Drosophila* (five sequences) and human (eleven sequences) are aligned with EDL/MAE using Clustal W. Bootstrap value more than forty (based on 100 replicates) are shown. Notice that all of the *Drosophila* members belong to different branches.

5% in *trans* to a deletion of the 55E/F region, *Df(2R)P34*. As this effect on viability was reverted upon excision of the P-element and was completely suppressed by a transgene containing the entire *edl* coding region, edl^{JV} represents a reduction of function allele of *edl*. In addition, we generated a lethal allele, edl^{L19}, that removes the entire *edl* gene. Both edl^{L19} homozygotes and edl^{L19}/*Df(2R)P34* animals die as late embryos or early larvae.

Analysis of *edl* mutants revealed that in both the eye and chordotonal organ, the loss of *edl* reduced the efficiency of Spitz-mediated induction. In retinal sections of edl^{JV}/*Df(2R)P34* and $edl^{JV/L19}$ animals, about 3% of ommatidia showed loss of photoreceptor cells, of the R1-R6 and R7 photoreceptor subtype (Fig. 2A,L). The R8 cell, which most strongly expresses *edl* expression within the ommatidium, was always present, even in ommatidia where other photoreceptor cells were missing (Fig. 2A, inset). A similar phenotype was seen in edl^{L19} mutant clones, which entirely lack *edl* function. This phenotype was almost completely rescued by an edl^{+} transgene (Fig. 2L). The requirement of *edl* was more pronounced when the level of the inducing signal was compromised. *Star* is a dosage-sensitive component of Spitz-mediated induction in the eye, and is required for the transport of Spitz EGF to the Golgi apparatus (Heberlein and Rubin, 1991; Kolodkin et al., 1994; Lee et al., 2001). In $Star^{-/+}$ animals, 30% of ommatidia show a reduction in the number of

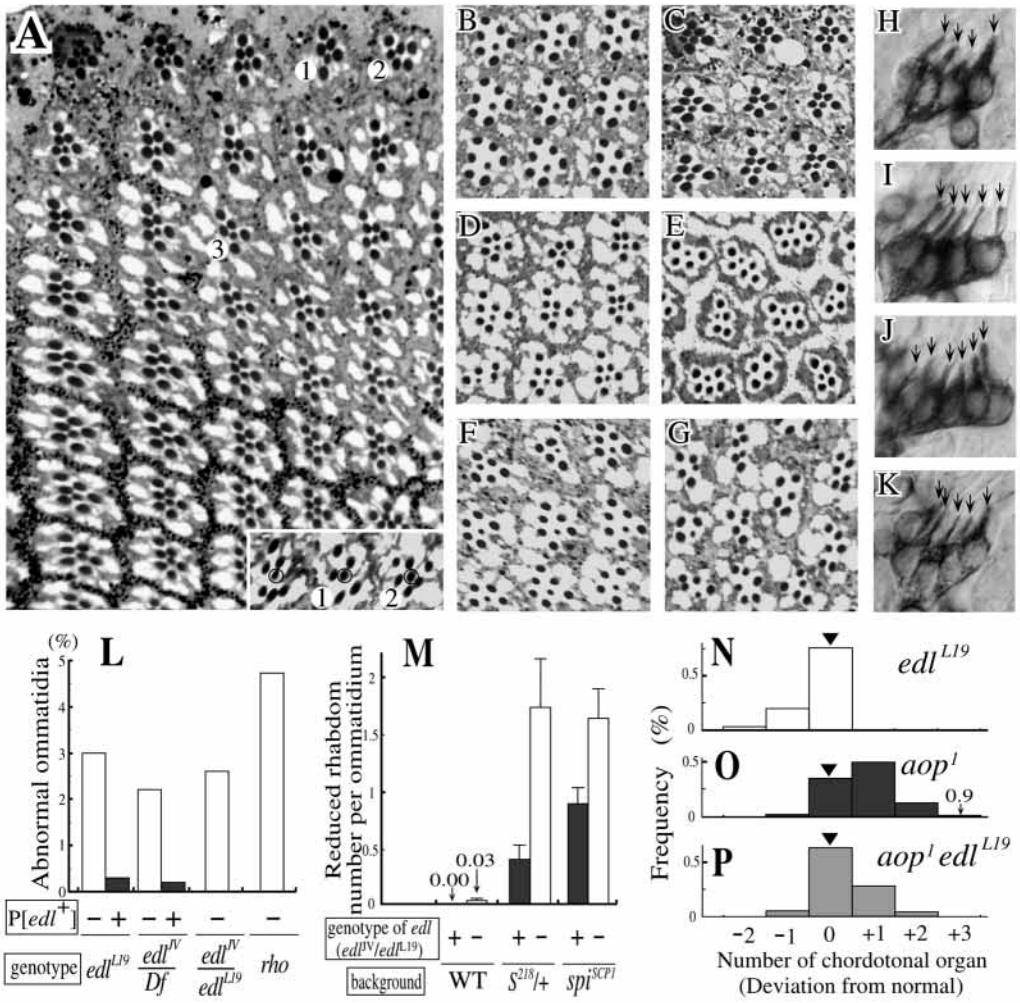

Fig. 2. Loss of *edl* affects recruitment of the induced cells. (A) An apical tangential section of a homozygous edl^{L19} mutant clone in the adult eye. The *edl* mutant region is marked by the absence of the $white^{+}$ marker gene, and can be recognized as a region that lacks pigments in the hexagonal lattice (upper right). Three ommatidia with missing R1-R6 photoreceptor cells (1, 3) or R7 cell (2) are numbered. Basal sections of such ommatidia reveal that in all such cases R8 is still present (ommatidia 1 and 2 are shown in inset). All three ommatidia are totally contained within the clone, and are composed solely of mutant cells. (L) The percentage of ommatidia with missing photoreceptor cells edl^{JV}, in trans to either *Df(2R)P34* (Df) or edl^{L19}, has a similar phenotype to the edl^{L19} homozygous clone. These phenotypes are rescued by one copy of the edl^{+} transgene. The percentage of ommatidia with missing photoreceptor cells in the $rhomboid^{P\Delta5}$ (*rho*) null mutant clone is included for comparison. (B-G,M) Loss of *edl* function dramatically enhances the phenotype of *Star* and *spitz*. Typical apical tangential sections of adult eyes of wild type (B), $Star^{218/+}$ (D), $spitz^{SCP1/SCP1}$ (F) and those in *edl* background, i.e. $edl^{L19/JV}$ (C), $Star^{218/+}$ $edl^{L19/JV}$ (E) and $spitz^{SCP1/SCP1}$ $edl^{L19/JV}$ (G). The average number of reduced R1-R7 photoreceptor neurons is summarized in (M). For each eye, about 100 ommatidia (or 70 for clonal analysis) were scored. (H-K,N-P) The phenotype of *edl* in the lateral chordotonal organ (Lch5) of the embryonic PNS. Neurons in scolopidia were visualized with a monoclonal antibody 22C10 (Fujita et al., 1982) (arrows). Number of scolopidia is reduced in the *edl* mutant (H,N), and is completely recovered by one copy of the edl^{+} transgene (I). In a null allele of *yan* (aop^{1}), which has an increase in the number of scolopidia (J,O), loss of *edl* still has an effect (K,P), indicating that EDL/MAE has target(s) other than YAN.

photoreceptor neurons, with the average number of R1-R7 cells reduced per ommatidium of 0.39 (Fig. 2B,D,M). When $edl^{JV/L19}$ mutation was placed in the $Star^{-/+}$ background, 65% of ommatidia lacked at least one neuron, with 1.71 photoreceptor cells missing per ommatidium on average (Fig. 2C,E,M). Similarly, the *edl* mutation enhanced the reduction in the number of photoreceptor neurons in a hypomorphic allele of *spitz* (Fig. 2F,G,M). These synergistic effects of *edl* and *Star*/*spitz* suggest that *edl* participates in the induction of R1-R7 by Spitz EGF.

The effect of the *edl* mutation in Spitz-mediated induction was also observed in the chordotonal organ. In each abdominal hemisegments of wild-type embryos, COP C1, C2, C3, C6 and C7 each generate a scolopidium that consists of a neuron and four support cells, and form a lateral chordotonal organ (Lch5) composed of five scolopidia. The loss of *edl* function caused a loss of one or two scolopidia from Lch5 in about 25% of the hemisegments (Fig. 2H,I,N).

Spitz EGF acts through the EGF receptor, resulting in the activation of the Ras/MAPK signaling leading to the phosphorylation of Ets proteins YAN and PNTP2. Baker et al. (Baker et al., 2001) have reported that EDL/MAE promotes the

MAPK-mediated phosphorylation of the repressor protein YAN, thus leading to its inactivation, and is also required for the transcriptional activation by PNTP2. Although the reduction in Spitz-mediated induction observed in *edl* mutants appears consistent with the role of EDL/MAE in promoting MAPK signaling, two lines of evidences argues against such model of EDL/MAE action. First, the expression of *edl* in chordotonal organs is detectable only in COPs C1-C5, which form using the proneural activity of *atonal* and do not require EGF receptor function for their specification (Okabe and Okano, 1997). It is also unlikely that *edl* acts solely by regulating YAN activity, because loss of *edl* function had an effect in the absence of YAN; while a null allele of *yan* (*aop*^{1})has increased number of scolopidia in Lch5, introduction

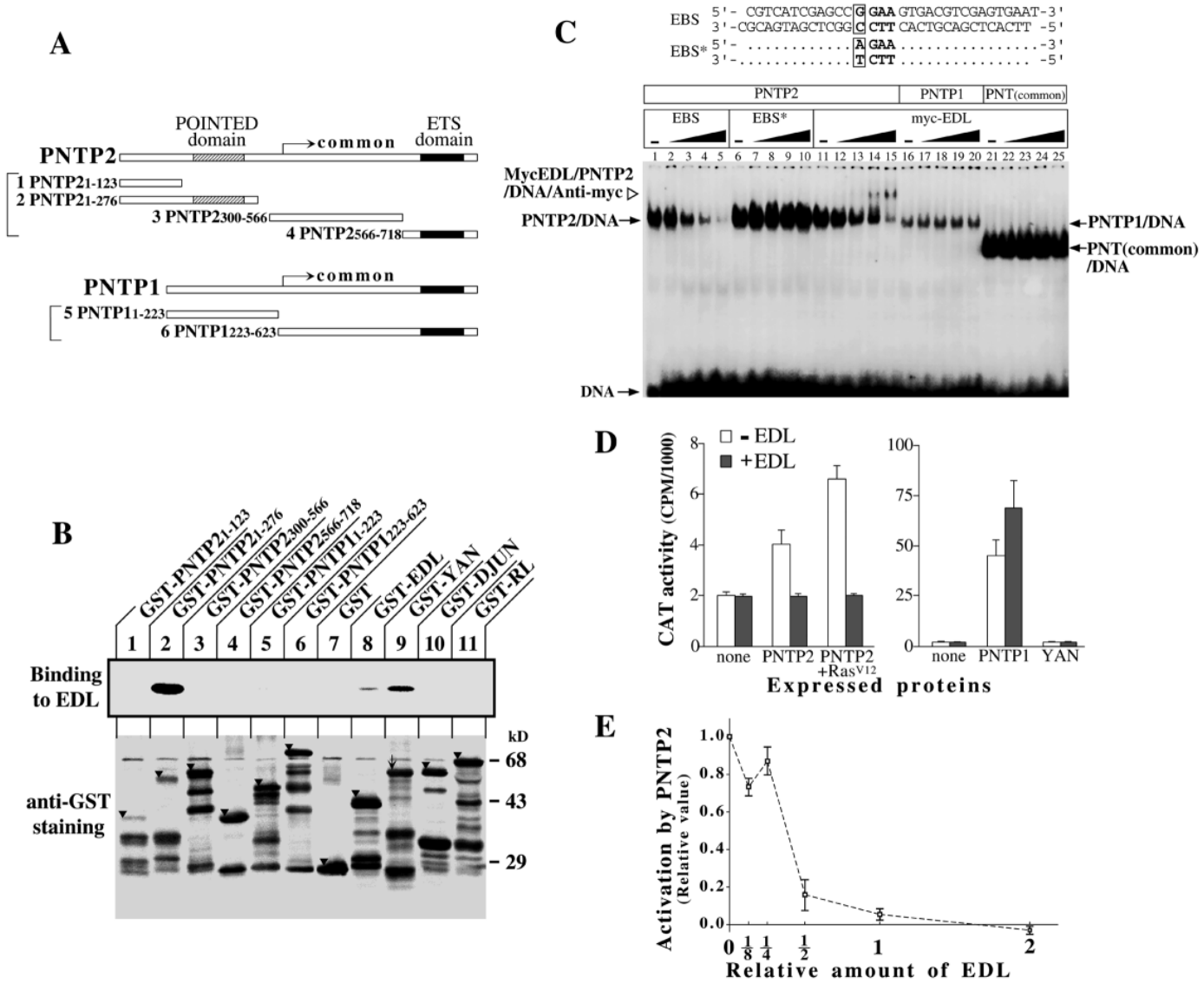

Fig. 3. EDL/MAE antagonizes PNTP2 function by direct binding. (A,B) GST pull-down assay using labeled EDL/MAE protein. (A) The region of PNT1 and PNTP2 that were used to make GST fusion proteins. (B) Upper gel is an autoradiogram showing the binding of ^{35}S-labeled EDL/MAE to the indicated GST fusion proteins. The lower part is a western blot using anti-GST antibody showing that most of the GST beads used contain a considerable amount of full length GST fusion proteins (triangles). YAN is the exception and its major degraded GST fusion product (which contains the Pointed domain) is shown by an arrow. (C) Electromobility shift assay (EMSA) of PNTP2 (lane 1-15), PNTP1 (lane 16-20) and a common sequence between PNTP1 and PNTP2 (lane 21-25) with the presence of labeled Ets-binding site probe (EBS) (Albagli et al., 1996) and anti-Myc antibody (9E10). Effects of increased amount of unlabeled EBS (lane 2-5), unlabeled mutated EBS (EBS*, lane 7-10) and EDL/MAE with Myc epitope at N-terminal (lane 12-15, 17-20 and 22-25) are shown. Arrows indicate labeled DNA or specific complexes while the white triangle shows the weaker supershifted band of complex containing EDL/MAE. For each reaction, quantities were adjusted with mock incubated reticulocyte lysates. (D) Tissue culture cell transfection assay for PNT-mediated transcription activation. Schneider cells were transfected with the effector construct alone (white bars) or the effector construct and the EDL/MAE expression construct (gray bars), and the expression of Ets-binding site-CAT reporter gene E_6BCAT was measured. (E) Dosage dependent effect of EDL/MAE (0-200 ng) on PNTP2 activation. Vertical axis shows the relative value of activation from zero (no effector) to 1.0 (PNTP2 alone), while horizontal axis shows the relative amount of EDL/MAE-expression construct compared with that of PNTP2, which was held constant (=100 ng).

of *edl* mutation caused a clear reduction in the number of scolopidia formed (Fig. 2J,K,O,P). EDL/MAE thus has targets other than repressor protein YAN. These results indicate that the mechanism by which EDL/MAE participates in Spitz-mediated induction is different from the one proposed by Baker et al. (Baker et al., 2001).

EDL/MAE inhibits transcriptional activation by PNTP2

As our genetic analysis indicated that EDL/MAE has targets other than YAN, we investigated the effect of EDL/MAE on PNTP2 activity. To test whether EDL/MAE directly binds PNTP2, we performed pull-down assays on bacterially produced GST-fusion proteins. EDL/MAE bound to the N-terminal region of the PNTP2 protein (PNTP2 1-276), which contains the Pointed domain and is specific to the PNTP2 isoform, consistent with the result obtained by Baker et al. (Baker et al., 2001) (Fig. 3A,B). Neither the region common to the PNTP1 and PNTP2 isoforms (PNTP1 223-623), nor the PNTP1-specific region (PNTP1 1-223) captured EDL/MAE (Fig. 3B). PNTP2 bound EDL/MAE much more efficiently than did YAN (Fig. 3B). MAPK, which is encoded by the *rolled* (*rl*) gene (Brunner et al., 1994b), and D-jun (Jra – FlyBase), which has been shown to act synergistically with PNT in transcription assays (Treier et al., 1995), showed only background levels of binding (Fig. 3B).

To address the functional significance of the binding of EDL/MAE to PNTP2, we tested the effect of EDL/MAE on the DNA binding activity of PNTP2. Mobility shift experiments revealed that PNTP2 can bind DNA in the presence of EDL/MAE, making a ternary complex with EDL/MAE and DNA (Fig. 3C). Such a ternary complex was not detected with PNTP1 protein, consistent with the finding that EDL/MAE does not bind PNTP1 (Fig. 3A,B). We then asked whether EDL/MAE affects transcriptional activation function of PNTP2 in a culture cell transfection assay (O'Neill et al., 1994). When PNTP2 was expressed in *Drosophila* Schneider cells, it activated transcription of a reporter gene harboring Ets-binding sites (Fig. 3D). Transcriptional activation by PNTP2 was enhanced by co-transfection with a plasmid encoding an activated form of Ras. However, in both cases, co-expression of EDL/MAE completely suppressed the transcriptional activation by PNTP2 (Fig. 3D). This suppression was dose dependent, and was specific to PNTP2, as EDL/MAE did not have such an inhibitory effect on PNTP1 (Fig. 3D,E). We conclude that EDL/MAE can bind PNTP2 and inactivate its function as a transcription activator. This activity is opposite to the one proposed by Baker et al. (Baker et al., 2001), who reported that EDL/MAE potentiates transcriptional activation by PNTP2 in monkey Cos-7 cells.

EDL/MAE antagonizes *pnt* function in vivo

To further study the role of EDL/MAE activity in vivo, we examined the effect of ectopic expression of *edl*. In the developing eye, *pnt* is required for the neuronal differentiation of photoreceptor cells, whereas *yan* is a negative regulator (O'Neill et al., 1994; Rebay and Rubin, 1995). If EDL/MAE inactivates PNTP2 activity, we would expect that misexpression of EDL/MAE in presumptive neurons would inhibit their neuronal differentiation. However, if EDL/MAE promoted MAPK-mediated phosphorylation of PNTP2 and YAN, misexpression of EDL/MAE should produce extra neurons. When *edl* was expressed in all neurons, the size of the compound eye was severely reduced (Fig. 4A,B). Hardly any photoreceptor neurons were present, and most of the retina was occupied by pigment cells (Fig. 4C). In the eye imaginal disc, a massive reduction of differentiating neurons was seen from the earliest stages of ommatidial assembly (Fig. 4D,E). The effect on neuronal specification was cell-type specific; we found little or no expression of markers for the recruited cells R1-R7, but R8 specification was largely unaffected (Fig. 4F,G, and data not shown). This phenotype was indistinguishable from the *pnt* mutant phenotype (Fig. 4H,I), consistent with the idea that ectopic EDL/MAE blocks *pnt* function. However, misexpression of *edl* in non-neuronal cone cells did not transform these cells towards a neuronal fate, as seen following the activation of Ras signaling (Fig. 4J-M). Furthermore, the suppressive effect of EDL/MAE on neuronal differentiation could be seen even in the presence of activated Ras; when *edl* was co-expressed with activated Ras, *edl* completely suppressed the ectopic neurons produced by Ras activation (Fig. 4J-Q). Thus, EDL/MAE can inhibit neuronal differentiation either downstream or parallel to Ras activation, consistent with EDL/MAE having an inhibitory effect on the transcriptional activation by PNTP2.

Additional genetic evidence supported the idea that EDL/MAE interferes with PNTP2 function in vivo. As expected from the model that EDL/MAE acted by titrating PNTP2, halving the gene dosage of *pnt* enhanced the rough eye phenotype caused by a weak misexpression of *edl* (Fig. 4R,S), and the effect of ectopic *edl* was suppressed by co-expression of PNTP2 (data not shown). Furthermore, ectopic expression of EDL/MAE in other tissues generated phenotypes that mimicked loss of *pnt* function (Fig. 4T-W) (Scholz et al., 1993; Morimoto et al., 1996). These results strongly suggest that EDL/MAE interferes with transcriptional activation by PNTP2 in vivo.

Overexpression of *pnt* interferes with induction

The inhibitory effect of EDL/MAE on transcriptional activation by PNTP2 suggests that EDL/MAE normally functions by suppressing PNTP2 function. As *edl* expression is most prominent at inducing cells in both chordotonal organ and the eye, EDL may exert its effect on PNTP2 function in inducing cells that produce Spiz. Inducing cells not only secrete Spitz EGF, but they also receive Spiz and thereby activate the downstream Ras/MAPK signaling pathway, which could result in PNT activation. Although the activation of PNTP2 is an obligatory step of neuronal specification of induced cells, the consequence of PNT activation in inducing cells has not been studied. EDL expression in inducing cells raises a possibility that these cells need to lower PNTP2 activity to ensure normal development. We thus examined the effects of hyperactivation of *pnt* on COP formation and photoreceptor recruitment.

When the PNTP2 isoform and an activated form of Raf (MAPKKK) was expressed in the posterior compartment of each segment in the embryo, the number of scolopidia in Lch5 was reduced from the normal number of five to three or four in 71% of hemisegments (n=60) (Fig. 5G,H). This phenotype is similar to the *edl* mutant phenotype, supporting the idea that the role of *edl* is to repress PNT activity in inducing cells. We

also hyperactivated *pnt* in all cells posterior to the morphogenetic furrow in the eye imaginal disc. Most ommatidial clusters in the early stages of ommatidial assembly contained fewer than the normal number of neurons, an effect that is opposite to the known role of *pnt* in promoting neuronal identity in induced cells (Fig. 5A,B). Cells that failed to initiate neural differentiation were of specific cell types; the specification of R3/R4/R1/R6 was severely disrupted, whereas the R8 cell was still present (Fig. 5C-F). Although a general disruptive effect on differentiation cannot be completely ruled

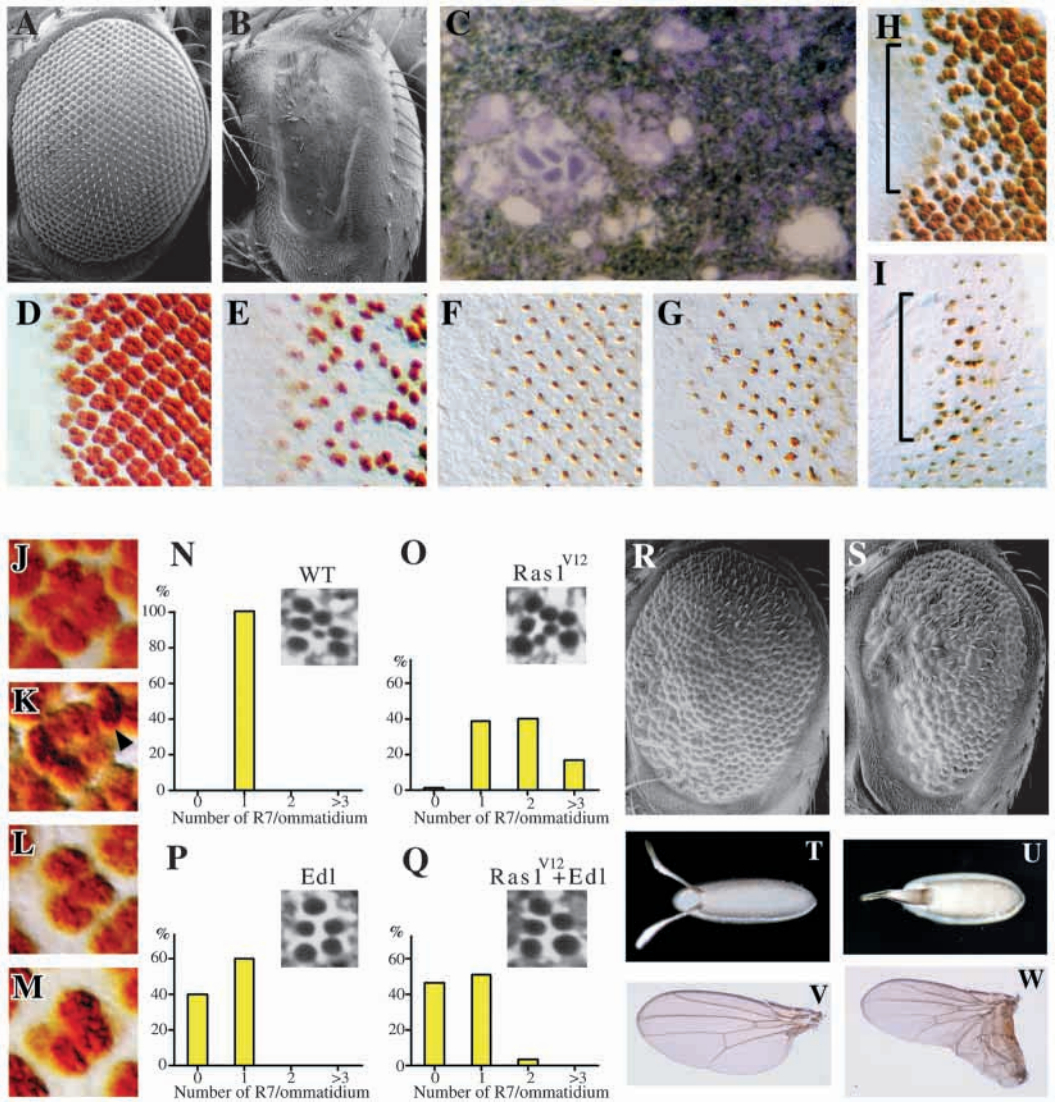

Fig. 4. Ectopic expression of EDL/MAE antagonizes *pnt* function in vivo. (A-I) The effect of misexpression of *edl* in all neurons. Scanning electron micrographs of eyes of wild type (A) and *elav-GAL4, UAS-edl* (B). Expression of *edl* in all neurons reduces the eye size. In the apical tangential section (C) photoreceptor neurons are hardly detected and most of the area is occupied by pigment cells. Neuronal marker ELAV expression (D,E,H) and R8 marker BOSS expression (F,G,I) in wild type (D,F), *elav-GAL4, UAS-edl* (E,G) discs, and discs with a clonal patch (bracketed) of $pnt^{\Delta 88}$ mutant cells (H,I). As misexpressing EDL/MAE by *elav*-GAL4 could down regulate the expression of *elav*-GAL4 driver itself, we examined GAL4 activity in *elav*-GAL4; UAS-*edl* animals using a UAS-NLS-*lacZ* reporter gene. Although in normal embryos expression of β-galactosidase coincided with ELAV expression, upon EDL/MAE misexpression many $ELAV^{-}$ $\beta\text{-gal}^{+}$ cells were present in a basal focal plane, where undifferentiated cells are present. Continued expression of GAL4 in these cells accounts for the strong effect of EDL/MAE misexpression on neuronal recruitment. (J-Q) EDL/MAE suppresses neuronal development even in the presence of activated Ras. The effect of strong (J-M) and weak (N-Q) ectopic EDL/MAE expression on both photoreceptor and cone cell development. Neuronal development was monitored by anti-ELAV staining of imaginal discs (J-M) and in sections of the adult eye (N-Q). (J,N) Wild type, (K,O) *sevE-*Ras^{V12}, (L,P) *sevE-GAL4, UAS-edl*, (M,Q) *sevE-*Ras^{V12}, *sevE-GAL4, UAS-edl*. Although expression of activated Ras transforms cone cells into R7 neurons (K,O), expression of EDL/MAE has no such effect and suppresses differentiation of endogenous neurons (L,P), even in the presence of activated Ras (M,Q). (R,S) The effect of ectopic EDL/MAE is enhanced by halving the dose of *pnt*. (R) *sevE-GAL4, UAS-edl*, (S) *sevE-GAL4, UAS-edl*, $pnt^{\Delta 88}$/+. (T-W) Ectopic expression of EDL/MAE phenocopies *pnt* loss of function phenotype. (U) EDL/MAE expression in ovarian follicle cells using *CY2-GAL4* line dorsalizes the chorion, resulting in fused dorsal appendages. (W) Expression of EDL/MAE in the posterior wing using *engrailed-GAL4* duplicates the wing. (T,V) Animals without transgenes.

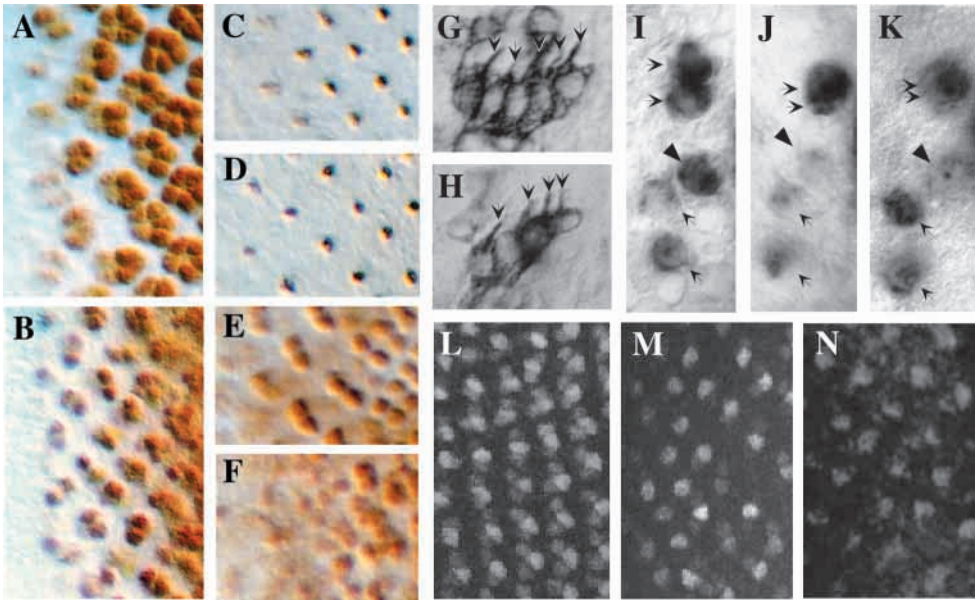

Fig. 5. Hyperactivation of *pnt* affects inducing ability. Eye imaginal discs of wild type (A,C,E) and discs after PNTP2 hyperactivation in all cells posterior to the morphogenetic furrow (*UAS-pntP2*, *UAS-phl.gof* / *GMR-GAL4*) (B,D,F). (A,B) Expression of ELAV, showing that hyperactivation of PNTP2 results in reduced number of ELAV^{+} photoreceptor neurons (B). In more posterior regions of the disc, massive neuronal differentiation occurred, consistent with the role of PNT in promoting neuronal differentiation (not shown). R8 marker BOSS (C,D) is expressed normally upon PNT hyperactivation (D), but R3/R4/R1/R6 marker *seven-up* fails to be induced, indicating a defect in induction (F). (G-K) Effect of *pnt* hyperactivation on chordotonal organ development. A wild-type cluster (G) contains five neurons in Lch5 (arrowheadss), but upon PNTP2 hyperactivation (*UAS-pntP2*, *UAS-phl.gof*/*en-GAL4*), many segments contain only four (H). Expression of *rhomboid* mRNA in wild type (I), and after *pnt* hyperactivation (J) and *edl* loss of function (K). Arrows indicate, from top to bottom, chordotonal organ precursors (COPs) C1, C2, C4, and C5. When *pnt* is hyperactivated, COP C3 (arrowhead) is present, but has undetectable levels of *rhomboid* mRNA. This phenotype is mimicked by the *edl* loss of function mutant edl^{L19}(K). (L-N) Effects of PNT hyperactivation and *edl* mutation on *rhomboid* expression. Expression level of a *rhomboid-lacZ* reporter is reduced upon PNTP2 hyperactivation (*UAS-pntP2*, *UAS-phl.gof* / *GMR-GAL4*) (M) and in $edl^{L19/JV}$ (N) imaginal discs, compared with wild type (L). The reduction is most pronounced in R2/R5. Anterior is towards the left.

out, these results suggest that hyperactivation of PNT may reduce the ability of inducing cells to recruit additional photoreceptors and COPs.

The production of the inducing signal requires the cleavage of Spitz by the Rhomboid family of intramembrane serine proteases (Freeman et al., 1992; Lee et al., 2001; Urban et al., 2001). In both the eye and the chordotonal organ, *rhomboid* expression marks cells with inducing ability. To test whether hyperactivation of PNT affects inducing ability by reducing *rhomboid* expression, we examined *rhomboid* mRNA during COP specification. Among the five COPs (C1-C5) that express *rhomboid* in wild-type embryos, C3 is particularly important for induction because one to two *rhomboid*-negative COP is recruited adjacent to COP C3 (Okabe and Okano, 1997). When PNT was hyperactivated, in many hemisegments *rhomboid* expression was reduced in COP C3 (Fig. 5I,J). It is thus likely that the reduction in the number of scolopidia of Lch5 is due to a non-autonomous effect through the loss of *rhomboid* expression in C3. If EDL/MAE acts by lowering PNTP2 activity, loss of *edl* function is expected to cause a similar effect on *rhomboid* expression as hyperactivation of PNT. Indeed, in edl^{L19} embryos *rhomboid* mRNA was undetectable in COP C3 in 25% of hemisegments (Fig. 5K), which is essentially identical to the penetrance of the segments with reduced number of scolopidia (Fig. 2). Similarly, in the eye both hyperactivation of PNT and reduction of EDL/MAE activity caused a reduction in the level of *rhomboid* expression (Fig. 5L-N). We propose that the role of EDL/MAE is to suppress PNTP2 activity in inducing cells, thereby allowing them to express *rhomboid* and produce the inducing factor Spitz EGF.

DISCUSSION

EDL/MAE antagonizes PNTP2 protein

We have analyzed the biological role of EDL/MAE, a protein that contains an Ets-type Pointed domain but no DNA-binding domain. Although the role of the Pointed domain as the target of the MAPK phosphorylation is well established (Wasylyk et al., 1998), the Pointed domain of EDL/MAE does not contain the consensus phosphorylation site and thus is unlikely to be regulated by the upstream signal. Emerging evidences indicate that this domain is also the site of protein-protein interaction, mediating homo- or hetero-oligomerization among Pointed domain-containing proteins (reviewed by Dittmer and Nordheim, 1998). The in vivo significance of such oligomerization, however, has not been demonstrated. Baker et al. (Baker et al., 2001) showed that the binding of EDL/MAE to YAN is required for MAPK-mediated phosphorylation of YAN, leading to inactivation of YAN function as a repressor of Ets target genes. As EDL/MAE has activities in the absence of YAN (Fig. 2N-P), EDL/MAE must have targets other than

YAN. Our results show that the binding of EDL/MAE to the Pointed domain of PNTP2 (Baker et al., 2001) (this work) causes a profound effect on the activity of PNT; expression of EDL/MAE abrogated the activity of PNTP2 as a transcription activator in culture cell transfection assays (Fig. 3D). This effect is supported by misexpression studies in vivo, which showed that EDL/MAE misexpression causes phenotypes that mimic the loss of PNTP2 function (Fig. 4). Phenotypes of *edl* loss of function were also similar to the consequences of PNT hyperactivation (Figs 2, 5). We propose that EDL/MAE acts by antagonizing PNTP2 protein in photoreceptor neuronal differentiation and chordotonal organ development.

These results contrasts with that of Baker et al. (Baker et al., 2001) who showed that EDL/MAE promotes transcriptional activation by PNTP2 protein in monkey Cos-7 cells. Our EDL/MAE misexpression experiments in vivo support the idea that EDL/MAE antagonizes, rather than promotes, PNTP2 activity. The effects of EDL/MAE misexpression cannot be explained by the promotion of phosphorylation of YAN, because phosphorylation causes the inactivation of YAN (O'Neill et al., 1994), and loss of *yan* produces effects that are opposite to what we have observed by EDL/MAE misexpression (Lai et al., 1992; Tei et al., 1992; Okabe and Okano, 1997). The opposite effects of EDL/MAE on PNTP2-mediated transcription may be due to the difference in the cell lines employed in the transfection assays. It is also possible that EDL/MAE activity is used differently in diverse tissues; for example, the effect seen on the ventral denticle belts in the embryonic cuticle (Baker et al., 2001) may be due to the promotion of YAN inactivation within the ventral neuroectoderm, allowing PNTP1 to function in the specification of medial fates (Kim and Crews, 1993).

Regulation of homeogenetic induction by EDL/MAE

Within the developmental contexts examined in this study, *edl* expression appear to be confined to cells with the ability to induce other cells using Spitz EGF (Fig. 1D,E). This suggests that EDL may have a role in regulating induction by Spitz. Secreted Spitz acts not only on the induced cells, but is also received by the inducing cells themselves. Although the molecular events leading to the activation of PNT within the induced cells is well established, whether the same regulatory cascade operates within the inducing cells had not been studied. We found that hyperactivation of PNT in inducing cells has a deleterious effect on induction; in the embryo, COP C3 loses expression of *rhomboid*, a factor that is essential for the production of Spitz EGF. Although inducing cells are positioned so that they receive highest levels of Spitz EGF that they produce, they may possess a mechanism to prevent hyperactivation of PNT. The phenotypes of the *edl* loss-of-function mutants and the effect of PNT hyperactivation are similar in both ommatidial and chordotonal organ development (Figs 2, 5). EDL/MAE is thus likely to be a part of the machinery that antagonizes PNTP2 to prevent the negative effect of PNT on induction in the inducing cells (Fig. 6).

A major challenge to our proposal that EDL/MAE acts in inducing cells by antagonizing PNT is that the loss of EDL/MAE function produces a rather mild effect on induction; most ommatidia are constructed normally in the *edl* null clone, and the loss of scolopidia is observed in only 25% of hemisegments in *edl*$^{-}$ embryos. As this phenotype is weaker than that which can be achieved by an artificial activation of *pnt* using the GAL4/UAS-mediated overexpression (Figs 2, 5), it can be argued that the role that EDL/MAE plays in repressing PNT function might be minor. For example, inducing cells may possess multiple mechanisms to inhibit PNT activation, and deleting EDL alone may not lead to full activation. However, it is likely that our overexpression paradigm results in such a high level of PNT activation that cannot be achieved under physiological conditions. It is also possible that EDL does not completely block PNT activation in inducing cells, but just need to keep the level from reaching the state that results in interference of induction.

This raises the question when and where PNT uses the activity to curb induction. During both ommatidial assembly and the development of the chordotonal system, PNT promotes neuronal development in the induced cells. We suggest that PNT may also suppress inducing ability in such cells. This would create a negative feedback loop so that cell, once induced, does not itself acquire inducing ability. Although such mechanism would be effective in preventing uncontrolled spread of homeogenetic induction, the need for such regulatory system arises only if induced cells also have the opportunity to acquire inducing ability. This is indeed the case for R2/R5; these cells form via induction by R8, and then express *rhomboid* and become a secondary source of Spitz EGF. Other cells, such as R3/R4 could also potentially become inducers, because they have probably resided within the proneural

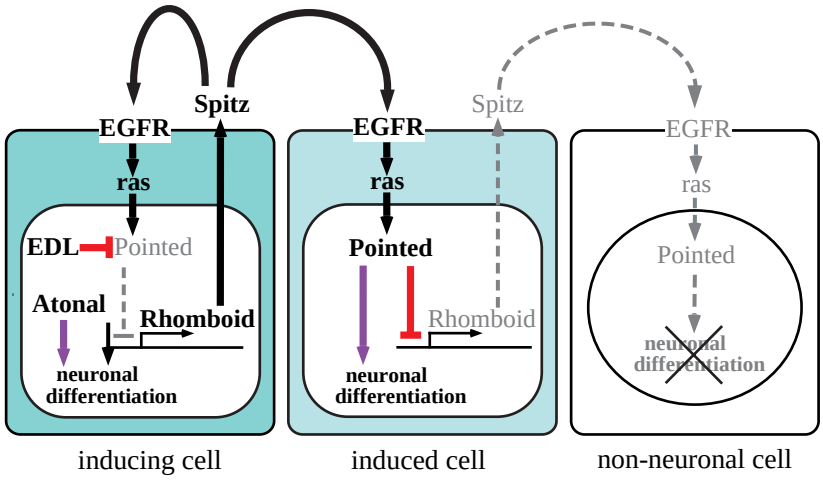

Fig. 6. A model for EDL/MAE function. In the induced cells Ets protein PNT has two functions: the promotion of neuronal development and inhibition of inducing ability through inhibiting *rhomboid* expression. The latter function ensures that induced cells do not participate in further induction. In the inducing cells, neuronal differentiation is triggered by the proneural gene *atonal*. Atonal also promotes expression of *rhomboid*. EDL/MAE expressed in the inducing cells antagonizes PNTP2 function, thus allowing *rhomboid* expression and Spitz-mediated induction. Broken lines indicate pathways that are inactive in the cells indicated.

cluster prior to the onset of induction and experienced Atonal expression, which promotes *rhomboid* expression (Lage et al., 1997) M.O., unpublished). Repressive effect of PNT on *rhomboid* would thus be a mechanism to safeguard against the potential activation of *rhomboid* by Atonal within the proneural cluster. PNT may cause this repression via activating expression of a repressor or by acting as a repressor itself.

The inhibition of *rhomboid* expression is not the only way that PNT negatively regulates induction. In the eye, a *rhomboid* paralog *roughoid* plays a critical role in generating mature Spitz EGF (Urban et al., 2001). It is possible that *roughoid* may also be regulated by PNT to control induction. Furthermore, upon activation of Ras signaling induced cells produce negative regulators of the Ras pathway, such as Sprouty, Argos and Kekkon, generating negative feedback loops (Casci et al., 1999; Ghiglione et al., 1999; Golembo et al., 1996; Kramer et al., 1999; Schweitzer et al., 1995a). Because Argos is a secreted antagonist of Spitz EGF, its production by inducing cells could be detrimental for induction. The inhibition of PNT function by EDL/MAE may also serve to reduce Argos production in the inducing cells, allowing efficient induction.

Although induction in *Drosophila* eye and the chordotonal organ discussed here is 'homeogenetic' in the sense that both the inducing cell and the induced cell are of the same cell type (photoreceptor neurons or COPs), they differ in genetic and molecular properties. Although neuronal specification of founder cells R8 and C1-C5 requires *atonal* function but not *pnt*, induced cells R1-R7 and C6-C8 depend on PNT activation and need *atonal* only indirectly. In addition, the induction itself generates a dichotomy between cells with inducing ability and those without, because induced cells acquire a different character (lack of inducing ability) from the inducing cell. Inducing cells, however, are prevented from expressing these characteristics through the repression of PNT function by EDL/MAE. Other instances of homeogenetic induction may also possess such properties, in order to generate cellular diversity, rather than equivalence. During the development of muscle progenitors in *Drosophila*, the size of the inductive field is defined by a group of cells similar to the proneural cluster; a small number of founder muscles are selected based on the activity of the bHLH transcription factor *lethal of scute* (Carmena et al., 1995) and EGF-mediated induction (Buff et al., 1998). Because *edl* is also expressed in a subset of muscle progenitors (data not shown), it may act in founder muscle selection in a similar way as it does in the eye and chordotonal organs.

Previous phylogenetic analysis revealed that the Ets protein family originated early during metazoan evolution and most of the functional diversity was already established prior to the separation of protostomes and deuterostomes (Degnan et al., 1993; Laudet et al., 1993; Laudet et al., 1999; Lautenberger et al., 1992; Price and Lai, 1999). Although it is likely that such an ancestral Ets protein already contained a Pointed domain, the Pointed domain of EDL/MAE could not be classified as similar to any of the previously known Ets protein subclasses (Fig. 1G). This suggests that EDL/MAE-like protein may have already existed before the divergence of Ets proteins. It is tempting to speculate that EDL/MAE or EDL/MAE-like proteins may regulate inductive processes in other developmental processes in *Drosophila* and vertebrates.

We thank A. Brand, M. Fortini, U. Heberlein, C. Klämbt, D. Lin, T. Schüpbach and Kathy Matthews at the Bloomington Stock Center for fly strains; and E. Bier, N. Brown, C. S. Goodman, E. Hafen, K. Klämbt, H. Krämer, K. Moses and I. Rebay for clones, antibodies and libraries. We acknowledge J. Goodhouse, S. Ray, E. Suzuki, T. Takano, K. Mita and D. Welsh for assistance. We are grateful to S. Butler, G. Deshpande, S. Hayashi, M. Jindra and S. Kramer for critical reading earlier versions of the manuscript, and K. Bhat, G. Deshpande, M. Go, D. Watson, E. Wieschaus and all members of the Hiromi laboratory for helpful discussions. T.Y. received postdoctoral grants from Human Frontier Science Project, Princeton University and JST (project leader Yoshiki Hotta). This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and JSPS (Research for the Future Program).

REFERENCES

Albagli, O., Klaes, A., Ferreira, E., Leprince, D. and Klambt, C. (1996). Function of ets genes is conserved between vertebrates and Drosophila. *Mech. Dev.* **59**, 29-40.

Baker, D. A., Mille-Baker, B., Wainwright, S. M., Ish-Horowicz, D. and Dibb, N. J. (2001). Mae mediates MAP kinase phosphorylation of Ets transcription factors in Drosophila. *Nature* **17**, 330-334.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brand, A. H. and Perrimon, N. (1994). Raf acts downstream of the EGF receptor to determine dorsoventral polarity during Drosophila oogenesis. *Genes Dev.* **8**, 629-639.

Brown, N. H. and Kafatos, F. C. (1988). Functional cDNA libraries from Drosophila embryos. *J. Mol. Biol.* **203**, 425-437.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H. and Klämbt, C. (1994a). The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. *Nature* **370**, 386-389.

Brunner, D., Oellers, N., Szabad, J., Biggs, W. H., 3rd, Zipursky, S. L. and Hafen, E. (1994b). A gain-of-function mutation in Drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. *Cell* **76**, 875-888.

Buff, E., Carmena, A., Gisselbrecht, S., Jimenez, F. and Michelson, A. M. (1998). Signalling by the Drosophila epidermal growth factor receptor is required for the specification and diversification of embryonic muscle progenitors. *Development* **125**, 2075-2086.

Carmena, A., Bate, M. and Jimenez, F. (1995). *Lethal of scute*, a proneural gene, participates in the specification of muscle progenitors during Drosophila embryogenesis. *Genes Dev.* **9**, 2373-2383.

Casci, T., Vinos, J. and Freeman, M. (1999). Sprouty, an intracellular inhibitor of Ras signaling. *Cell* **96**, 655-665.

Degnan, B. M., Degnan, S. M., Naganuma, T. and Morse, D. E. (1993). The ets multigene family is conserved throughout the Metazoa. *Nucleic Acids Res.* **21**, 3479-3484.

Dittmer, J. and Nordheim, A. (1998). Ets transcription factors and human disease. *Biochim. Biophys. Acta* **1377**, F1-11.

Edlund, T. and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. *Cell* **96**, 211-224.

FlyBase (1999). The FlyBase database of the Drosophila Genome Projects and community literature. The FlyBase Consortium. *Nucleic Acids Res.* **27**, 85-88.

Fortini, M. E., Simon, M. A. and Rubin, G. M. (1992). Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation. *Nature* **355**, 559-561.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. *Cell* **87**, 651-660.

Freeman, M. and Gurdon, J. B. (2002). Regulatory principles of developmental ignaling. *Annu. Rev. Cell Dev. Biol.* **18**, 515-539.

Freeman, M., Kimmel, B. E. and Rubin, G. M. (1992). Identifying targets of the *rough* homeobox gene of Drosophila: evidence that *rhomboid* functions in eye development. *Development* **116**, 335-346.

Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A. and Shotwell, S. L. (1982). Monoclonal antibodies against the Drosophila nervous system. *Proc. Natl. Acad. Sci. USA* **79**, 7929-7933.

Ghiglione, C., Carraway, K. L., 3rd, Amundadottir, L. T., Boswell, R. E., Perrimon, N. and Duffy, J. B. (1999). The transmembrane molecule kekkon 1 acts in a feedback loop to negatively regulate the activity of the *Drosophila* EGF receptor during oogenesis. *Cell* **96**, 847-856.

Golembo, M., Schweitzer, R., Freeman, M. and Shilo, B. Z. (1996). *Argos* transcription is induced by the Drosophila EGF receptor pathway to form an inhibitory feedback loop. *Development* **122**, 223-230.

Graves, B. J. and Petersen, J. M. (1998). Specificity within the ets family of transcription factors. *Adv. Cancer Res.* **75**, 1-55.

Guan, K. L. and Dixon, J. E. (1991). Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**, 262-267.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in Drosophila. *Development* **120**, 2121-2129.

Heberlein, U. and Rubin, G. M. (1991). *Star* is required in a subset of photoreceptor cells in the developing Drosophila retina and displays dosage sensitive interactions with rough. *Dev. Biol.* **144**, 353-361.

Jan, Y. N. and Jan, L. Y. (1995). Maggot's hair and bug's eye: role of cell interactions and intrinsic factors in cell fate specification. *Neuron* **14**, 1-5.

Jarman, A. P. and Jan, Y. N. (1995). Multiple roles for proneural genes in Drosophila neurogenesis. In *Neural Cell Specification: Molecular Mechanisms and Neurotherapeutic Implications* (ed. B. H. J. Juurlink, P. H. Krone, W. M. Kulyk, V. M. K. Verge and J. R. Doucette), pp. 97-104. New York: Plenum Press.

Jarman, A. P., Grau, Y., Jan, L. Y. and Jan, Y. N. (1993). *Atonal* is a proneural gene that directs chordotonal organ formation in the Drosophila peripheral nervous system. *Cell* **73**, 1307-1321.

Jarman, A. P., Grell, E. H., Ackerman, L., Jan, L. Y. and Jan, Y. N. (1994). *Atonal* is the proneural gene for Drosophila photoreceptors. *Nature* **369**, 398-400.

Kim, S. H. and Crews, S. T. (1993). Influence of Drosophila ventral epidermal development by the CNS midline cells and spitz class genes. *Development* **118**, 893-901.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H. and Klämbt, C. (1994). The Ets transcription factors encoded by the Drosophila gene *pointed* direct glial cell differentiation in the embryonic CNS. *Cell* **78**, 149-160.

Klämbt, C. (1993). The Drosophila gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* **117**, 163-176.

Kolodkin, A. L., Pickup, A. T., Lin, D. M., Goodman, C. S. and Banerjee, U. (1994). Characterization of *Star* and its interactions with *sevenless* and *EGF receptor* during photoreceptor cell development in Drosophila. *Development* **120**, 1731-1745.

Kramer, S., Okabe, M., Hacohen, N., Krasnow, M. A. and Hiromi, Y. (1999). Sprouty: a common antagonist of FGF and EGF signaling pathways in Drosophila. *Development* **126**, 2515-2525.

Lage, P., Jan, Y. N. and Jarman, A. P. (1997). Requirement for EGF receptor signalling in neural recruitment during formation of Drosophila chordotonal sense organ clusters. *Curr. Biol.* **7**, 166-175.

Lai, Z. C. and Rubin, G. M. (1992). Negative control of photoreceptor development in Drosophila by the product of the yan gene, an ETS domain protein. *Cell* **70**, 609-620.

Laudet, V., Niel, C., Duterque-Coquillaud, M., Leprince, D. and Stehelin, D. (1993). Evolution of the ets gene family. *Biochem. Biophys. Res. Commun.* **190**, 8-14.

Laudet, V., Hanni, C., Stehelin, D. and Duterque-Coquillaud, M. (1999). Molecular phylogeny of the ETS gene family. *Oncogene* **18**, 1351-1359.

Lautenberger, J. A., Burdett, L. A., Gunnell, M. A., Qi, S., Watson, D. K., O'Brien, S. J. and Papas, T. S. (1992). Genomic dispersal of the *ets* gene family during metazoan evolution. *Oncogene* **7**, 1713-1719.

Lee, J. R., Urban, S., Garvey, C. F. and Freeman, M. (2001). Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. *Cell* **107**, 161-171.

Lin, D. M. and Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* **13**, 507-523.

Mangold, O. and Spemann, H. (1927). Über Induktion von Medullarplatte durch Medullarplatte im jüngeren Keim, ein Beispiel homöogenetischer oder assimilatorischer Induktion. *W. Roux's Arch. Entw. Org.* **111**, 341-422.

Mlodzik, M. and Hiromi, Y. (1992). Enhancer trap method in Drosophila: its application to neurobiology. *Methods Neurosci.* **9**, 397-414.

Morimoto, A. M., Jordan, K. C., Tietze, K., Britton, J. S., O'Neill, E. M. and Ruohola-Baker, H. (1996) Pointed, an ETS domain transcription factor, negatively regulates the EGF receptor pathway in Drosophila oogenesis. *Development* **122**, 3745-3754.

Okabe, M. and Okano, H. (1997). Two-step induction of chordotonal organ precursors in Drosophila embryogenesis. *Development* **124**, 1045-1053.

O'Neill, E. M., Rebay, I., Tjian, R. and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. *Cell* **78**, 137-147.

O'Neill, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. *Biotechniques* **17**, 870, 874-875.

Pascal, E. and Tjian, R. (1991). Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. *Genes Dev.* **5**, 1646-1656.

Peverali, F. A., Isaksson, A., Papavassiliou, A. A., Plastina, P., Staszewski, L. M., Mlodzik, M. and Bohmann, D. (1996). Phosphorylation of Drosophila Jun by the MAP kinase rolled regulates photoreceptor differentiation. *EMBO J.* **15**, 3943-3950.

Price, M. D. and Lai, Z. C. (1999). The yan gene is highly conserved in Drosophila and its expression suggests a complex role throughout development. *Dev. Genes Evolution* **209**, 207-217.

Rebay, I. and Rubin, G. M. (1995). Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. *Cell* **81**, 857-866.

Scholz, H., Deatrick, J., Klaes, A. and Klämbt, C. (1993) Genetic dissection of pointed, a Drosophila gene encoding two ETS-related proteins. *Genetics* **135**, 455-468.

Schweitzer, R., Howes, R., Smith, R., Shilo, B. Z. and Freeman, M. (1995). Inhibition of Drosophila EGF receptor activation by the secreted protein Argos. *Nature* **376**, 699-702.

Shilo, B. Z. (1998). Flies over Crete: Drosophila molecular biology. Kolymbari, Crete, July 12-18, 1998. *EMBO J.* **17**, 6769-6771.

Tang, P., Steck, P. A. and Yung, W. K. (1997). The autocrine loop of TGF-alpha/EGFR and brain tumors. *J. Neurooncol.* **35**, 303-314.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-85.

Tei, H., Nihonmatsu, I., Yokokura, T., Ueda, R., Sano, Y., Okuda, T., Sato, K., Hirata, K., Fujita, S. C. and Yamamoto, D. (1992). pokkuri, a Drosophila gene encoding an E-26-specific (Ets) domain protein, prevents overproduction of the R7 photoreceptor. *Proc. Natl. Acad. Sci. USA* **89**, 6856-6860.

Tio, M. and Moses, K. (1997). The Drosophila TGF alpha homolog Spitz acts in photoreceptor recruitment in the developing retina. *Development* **124**, 343-351.

Tomlinson, A. and Ready, D. F. (1987). Neuronal differentiation in the Drosophila ommatidium. *Dev. Biol.* **120**, 366-376.

Tower, J., Karpen, G. H., Craig, N. and Spradling, A. C. (1993). Preferential transposition of Drosophila P elements to nearby chromosomal sites. *Genetics* **133**, 347-359.

Treier, M., Bohmann, D. and Mlodzik, M. (1995). JUN cooperates with the ETS domain protein pointed to induce photoreceptor R7 fate in the Drosophila eye. *Cell* **83**, 753-760.

Urban, S., Lee, J. R. and Freeman, M. (2001). Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell* **107**, 173-182.

Wasserman, J. D., Urban, S. and Freeman, M. (2000). A family of rhomboid-like genes: Drosophila *rhomboid-1* and *roughoid*/*rhomboid-3* cooperate to activate EGF receptor signaling. *Genes Dev.* **14**, 1651-1663.

Wasylyk, B., Hagman, J. and Gutierrez-Hartmann, A. (1998) Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem. Sci.* **23**, 213-216.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-1237.

Zipursky, S. L. and Rubin, G. M. (1994). Determination of neuronal cell fate: lessons from the R7 neuron of *Drosophila*. *Annu. Rev. Neurosci.* **17**, 373-397.

Zwick, E., Bange, J. and Ullrich, A. (2002). Receptor tyrosine kinases as targets for anticancer drugs. *Trends Mol. Med.* **8**, 17-23.