Developmental Cell, Vol. 7, 687–696, November, 2004, Copyright ©2004 by Cell Press

EGF Receptor Signaling Triggers Recruitment of *Drosophila* Sense Organ Precursors by Stimulating Proneural Gene Autoregulation

Petra I. zur Lage,[1] Lynn M. Powell,[1] David R.A. Prentice,[1] Paul McLaughlin,[2] and Andrew P. Jarman[1,*]
[1]Division of Biomedical Sciences
Centre for Neuroscience Research
University of Edinburgh
Edinburgh EH8 9XD
United Kingdom
[2]Institute of Structural and Molecular Biology
School of Biological Sciences
University of Edinburgh
King's Buildings
Edinburgh EH9 3JR
United Kingdom

Summary

In *Drosophila*, commitment of a cell to a sense organ precursor (SOP) fate requires bHLH proneural transcription factor upregulation, a process that depends in most cases on the interplay of proneural gene autoregulation and inhibitory Notch signaling. A subset of SOPs are selected by a recruitment pathway involving EGFR signaling to ectodermal cells expressing the proneural gene *atonal*. We show that EGFR signaling drives recruitment by directly facilitating *atonal* autoregulation. Pointed, the transcription factor that mediates EGFR signaling, and Atonal protein itself bind cooperatively to adjacent conserved binding sites in an *atonal* enhancer. Recruitment is therefore contingent on the combined presence of Atonal protein (providing competence) and EGFR signaling (triggering recruitment). Thus, autoregulation is the nodal control point targeted by signaling. This exemplifies a simple and general mechanism for regulating the transition from competence to cell fate commitment whereby a cell signal directly targets the autoregulation of a selector gene.

Introduction

Paracrine signaling is a widespread trigger of cell fate determination during development. However, it is well known that the information that such signals impart depends on the context. Thus, signaling allows or prevents a target cell from committing to a fate for which it is already predisposed or competent (Freeman, 1997). Sense organ precursor (SOP) determination in the developing *Drosophila* PNS provides an important model system for understanding the mechanisms underlying competence and commitment, and particularly how the transition from competence to commitment is controlled. In this case, competence and commitment requires the function of the bHLH proneural genes *achaete* and *scute* (*ac/sc*), *atonal* (*ato*), and *amos*, which can be viewed as selector genes of SOP fate (Mann and Carroll, 2002). Much progress has been made in understanding this process during "classical" SOP selection. Proneural genes are initially expressed in groups of ectodermal cells known as proneural clusters (PNCs). This initial expression provides cells with neural competence but does not necessarily lead to commitment. The key event in SOP commitment is the upregulation of proneural protein expression in specific PNC cells (Cubas et al., 1991; Skeath and Carroll, 1991). For *ac/sc*, a complex network of cell interactions and signaling feedback loops determines whether a cell upregulates or downregulates *ac/sc* expression, thereby ensuring that a single SOP is selected (van Doren et al., 1992; Culí and Modolell, 1998). In this process, Notch signaling within proneural clusters inhibits *ac/sc* autoregulation by directly interacting with autoregulatory enhancers (Culí and Modolell, 1998; Jafar-Nejad et al., 2003).

In addition to this classical mode, there is a second mode of SOP formation that has been characterized for *ato* during chordotonal stretch receptor development: chordotonal SOPs can be recruited by EGFR signaling. In each embryonic abdominal segment, five chordotonal SOPs are selected from two *ato*-expressing PNCs in a conventional manner, involving the interplay of *ato* with Notch signaling (zur Lage et al., 1997; Okabe and Okano, 1997). These "primary" precursors express *rhomboid*, (*rho*) which activates secretion of the EGFR ligand Spitz, and the subsequent signaling to adjacent ectodermal cells leads to their recruitment as "secondary" chordotonal SOPs (Okabe and Okano, 1997; zur Lage et al., 1997). As a result, in each abdominal segment five primary precursors recruit three secondary precursors, which together differentiate as the eight chordotonal organs (Figure 1A). As in all cases of EGFR signaling, the recruited cell's immediate response is the activation of the ETS family transcription factor, Pointed (Pnt). The *pnt* gene encodes two isoforms, Pnt-P1 and P2 (we refer hereafter to the combined activity of the two isoforms as Pnt) (O'Neill et al., 1994). Pnt-P2 is activated by ERK MAPK phosphorylation upon EGFR stimulation, whereas Pnt-P1 is regulated by EGFR signaling at the transcriptional level (Gabay et al., 1996, 1997; Rawlins et al., 2003). The favored model is that ERK phosphorylates Pnt-P2, which then activates the transcription of Pnt-P1. Apart from their regulation, it is thought that Pnt-P1 and P2 function similarly as transcription factors by binding to the same sites via their common ETS domains (Albagli et al., 1996). Interestingly, signaling from the dorsal-most primary SOP triggers the recruitment of oenocyte precursors rather than chordotonal SOPs (Elstob et al., 2001; Rusten et al., 2001). Clearly, specificity of cellular response must depend on factors other than Pnt.

A similar process of recruitment occurs for the adult femoral chordotonal organ (FCO), but with key differences (Figure 1B). As in the embryo, chordotonal SOPs recruit further SOPs from the ectoderm, but in this case recruitment is reiterative: newly recruited SOPs themselves express *rho* and in turn recruit further SOPs from the *ato*-expressing PNC (zur Lage and Jarman, 1999).

*Correspondence: andrew.jarman@ed.ac.uk

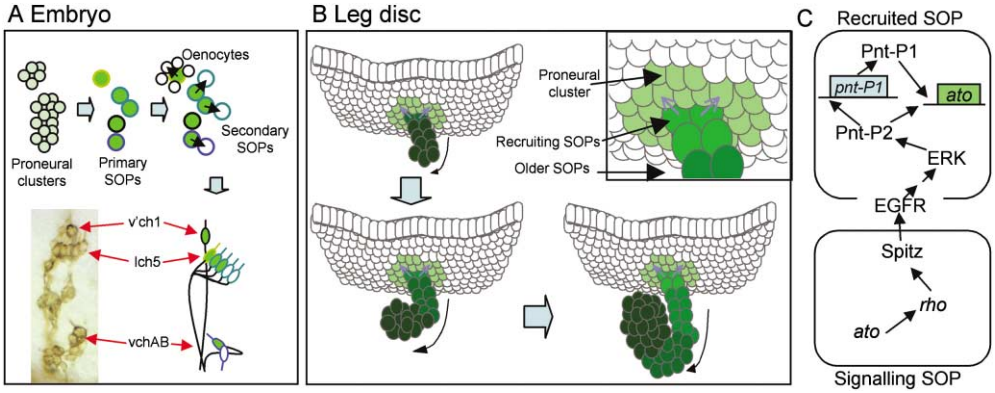

Figure 1. Chordotonal Precursor Recruitment

(A) Model of embryonic chordotonal precursor recruitment, based on zur Lage et al. (1997). The first, or primary, SOPs (filled green) are selected from *ato*-expressing PNCs by lateral inhibition. These then signal via the EGFR pathway (black arrows) to recruit adjacent cells (unfilled) as SOPs (secondary SOPs) or, in the case of the dorsal-most SOP, oenocytes. As a result, after differentiation the lch5 cluster consists of three primary and two secondary neurons while the vchAB pair consists of one primary and one secondary neuron. The v'ch1 neuron remains single. These neurons are color coded to indicate their inferred SOP origins and are also indicated on an immunohistochemical staining of an embryonic abdominal segment using the neuronal marker antibody, 22C10.

(B) Model of femoral chordotonal precursor recruitment in the leg imaginal disc, based on zur Lage and Jarman (1999). A small section of the leg epithelium is shown. SOPs arise from an *ato*-expressing PNC (light green) within this epithelium. EGFR signaling (blue arrows) from the latest born SOPs (darker green) recruits the next SOPs. This cycle of reiterative recruitment continues over an extended period to result in a large accumulation of SOPs. Three "snapshots" of this process are shown with younger SOPs indicated by increasingly lighter shades of green.

(C) Genetic model of *ato* and EGFR signaling function during in recruitment. Spitz is the ligand of EGFR.

Thus, unlike in the embryo, the recruitment cycle is repeated many times as new SOPs become new signaling sources. As a result, some 80 SOPs are recruited over time. In the leg disc, SOP recruitment correlates with *ato* upregulation (Figure 1C; zur Lage and Jarman, 1999). To understand the basis of this, we have investigated *ato* regulation during recruitment. Analysis of key target gene enhancers has greatly increased our understanding of the logic of cell fate determination (Mann and Carroll, 2002). An enhancer upstream of *ato* is active specifically during recruitment in both the leg disc and the embryo. This enhancer is regulated directly by Pnt and Ato binding cooperatively to adjacent sites. The consequence of this is that the enhancer responds only to the combined input of both Pnt and Ato. Thus, Ato ensures the specificity of EGFR signaling in this context. Importantly, SOP recruitment depends on the direct manipulation of Ato autoregulation: such autoregulation is contingent on EGFR signaling. Thus, to promote the transition from competence to cell fate commitment, a cell signal directly targets the autoregulation of a selector gene.

Results

Identification of an *ato* SOP Recruitment Enhancer

Sun et al. (1998) described the approximate location of several regulatory elements up- and downstream of the *ato* ORF (Figure 2A). An enhancer supporting reporter gene expression in FCO precursors was inferred indirectly to exist between *Sma*I and *Bam*HI restriction sites 3.6–5.5 kb upstream of the *ato* ORF. When this 1.9 kb region was cloned into a Gal4 P element vector, it indeed supported Gal4 expression in the FCO precursors (data not shown). The fragment was subdivided and each subfragment was inserted into the GFP reporter vector, pHStinger (Figure 2A; Barolo et al., 2000). Using these transgenes, the FCO enhancer could be localized to a 367 bp fragment (Figures 2A and 2B). GFP expression driven by this fragment was observed in the chordotonal SOPs (marked by Ato and the SOP protein, Senseless [Sens] [Nolo et al., 2000]) but not in the overlying PNC (marked by Ato), suggesting that it is active during SOP commitment (Figure 2B).

The *ato* FCO enhancer is also active during embryonic chordotonal recruitment. The enhancer drives GFP expression in embryonic sensory cells that derive from a subset of Ato-dependent SOPs (Figure 2C). Owing to the delayed acquisition of GFP fluorescence, the onset of GFP expression appears shortly after Ato is downregulated in these SOPs. Nevertheless, examination of perduring GFP in older embryos relative to a sensory neuron differentiation marker, 22C10, revealed expression in two chordotonal sensilla of the five that make up the lateral chordotonal array (lch5) (Figures 2D–2F). This correlates with the two recruited SOPs that contribute to lch5 (Figure 1A; Okabe and Okano, 1997; zur Lage et al., 1997). The GFP-expressing sensilla are usually the most posterior of the lch5 cluster. Significantly, the anterior-most sensillum never expresses GFP, which is consistent with it deriving from a primary chordotonal precursor (zur Lage et al., 1997). Similarly, GFP expression is observed weakly in one of the two ventral chordotonal organs (vchB), which derives from a recruited SOP. In the head, there is notable expression in cells that give rise to the larval eye (Bolwig's organ). This is also an *ato*-specified sense organ that requires EGFR signaling (Dumstrei et al., 1998). Overall, these patterns suggest that this enhancer is responsible not for general *ato* regulation in SOPs, but specifically in situations where it depends on EGFR signaling. Moreover, the enhancer appears to mediate the EGFR signaling response in a

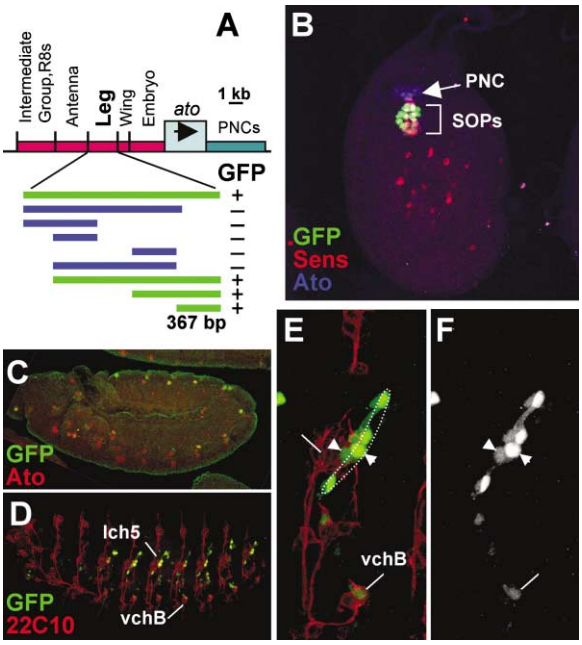

Figure 2. Identification of a Recruitment Enhancer for *ato*

(A) Schematic of constructs relative to the enhancer locations identified by Sun et al. (1998).

(B–F) GFP expression (green) driven by the 367 bp enhancer fragment.

(B) Leg disc, with GFP in the chordotonal SOPs (bracket). The SOP marker Sens (red) is present in these SOPs, while Ato (blue) has become switched off but is still present in the PNC (arrow). The scattered Sens cells are SOPs of external sense organs.

(C) Stage 11 embryo, Ato (red) is largely confined to chordotonal SOPs at this stage. GFP is beginning to appear in a subset of these.

(D–F) Late embryo, showing perduring GFP relative to sensory neurons (Mab22C10, red). (F) shows the GFP channel alone. Of the lateral chordotonal cluster (lch5), GFP perdures in two chordotonal sensilla (each consisting of four cells: one organ being ringed in [E], and the neuron of each organ is indicated by arrowheads). GFP expression is weakly observed in one of the ventral sensilla (vchB).

variety of developmental situations. We refer to this element as the *ato* recruitment enhancer (*ato*-RE). Significantly, *ato*-RE is not expressed in oenocytes, even though these are recruited by EGFR signaling from a primary chordotonal SOP (Elstob et al., 2001; Rusten et al., 2001).

The Recruitment Element Responds to Combined Pnt-P1 and Ato Misexpression

We analyzed how *ato*-RE-GFP expression responds to ectopic expression of *pnt-P1* using 109-68-*Gal4,* a Gal4 driver line that is expressed in many proneural clusters and SOPs in the imaginal discs (Jarman and Ahmed, 1998). However, misexpression of UAS-*pnt-P1* induced very little change in the *ato*-RE-GFP expression pattern in leg or wing discs (Figures 3A–3D). We reasoned that another tissue-restricted factor is required for the response of *ato*-RE to Pnt. Ato protein itself may be such a factor, since it is already expressed at a low level in the leg PNC cells that are recruited. UAS-*ato* misexpression (109-68-*Gal4* UAS-*ato*) resulted in a modest change in expression of *ato*-RE-GFP (Figures 3E and 3F), even though such misexpression induces significant (nonrecruitment) chordotonal SOP commitment (Jarman and Ahmed, 1998). In contrast, comisexpression of both Pnt-P1 and Ato resulted in a significant increase in ectopic *ato*-RE-GFP expression in the leg and wing (Figures 3G and 3H). This finding suggests two things. First, Pnt/EGFR activation of *ato*-RE is contingent on the presence of Ato. This restricts its function to Ato-expressing PNC cells. Second, *ato*-RE is an autoregulatory enhancer, but unlike other proneural autoregulatory enhancers (Van Doren, 1992; Culí and Modolell, 1998), autoregulation is contingent on the cell receiving an EGFR/Pnt signal.

Ato and Pnt-P1 Are Coexpressed during Recruitment

These results suggested that the *ato*-RE is activated by the simultaneous presence of Ato and Pnt in recruited SOPs. As expected from previous evidence (zur Lage and Jarman, 1999), Ato and Pnt-P1 are indeed both expressed in leg FCO SOPs (Figures 4A and 4B). In the embryo, too, in double labeling experiments, cells that coexpress Ato and Pnt-P1 are clearly present at the time and location expected of recruited SOPs (Figures 4C–4E). Consistent with this, a GFP reporter transgene that responds directly to Ato regulation (Powell et al., 2004) is expressed in both primary and recruited chordotonal SOPs and is coexpressed with Pnt-P1 (Figures 4F and 4G and data not shown).

Binding Sites for Ato/Da and Pnt within the Recruitment Enhancer

ato-RE was tested for its ability to bind Pnt and Ato proteins in vitro (the latter as a heterodimer with Daughterless [Da] protein). In gel mobility shift assays using purified proteins and the entire *ato*-RE as a probe, Pnt and Ato/Da proteins both bind in a manner that is consistent with a single binding site each (Figure 5B). In general, Ato/Da binds to the bHLH A-class E-box core consensus CAGNTG (Jarman et al., 1993; Powell et al., 2004). On this criterion, there are two potential Ato/Da binding sites in *ato*-RE (E1 and E2) (Figure 5A). Ato/Da binding could be competed strongly by an E1-containing competitor oligonucleotide, but not strongly by an E2-containing competitor nor by a competitor with a mutated version of the E1 site (E1(M): CAGGTG→CCTAGG) (Figure 5B, lanes 3–5). This suggests that Ato/Da binds to *ato*-RE largely via E1. Mobility shifts with site-specific oligonucleotides as probes supported this (data not shown). To assess the in vivo function of these sites, we carried out site-directed mutagenesis on *ato*-RE and assessed the effect on reporter gene expression in transgenic flies. Mutation of E2 had no discernable effect on *ato*-RE-GFP expression (data not shown), but mutation of E1 completely abolished expression in the embryo, leg imaginal disc, and also the eye disc (Figures 5C and 5D). Thus, E1 is likely to be a binding site through which Ato regulates its own expression in recruited chordotonal precursors.

Pnt binds to a consensus sequence around a GGAA core that has been characterized for vertebrate ETS-1 (Albagli et al., 1996), and a number of functional Pnt binding sites have been characterized that conform to this consensus (Granderath et al., 2000; Xu et al., 2000).

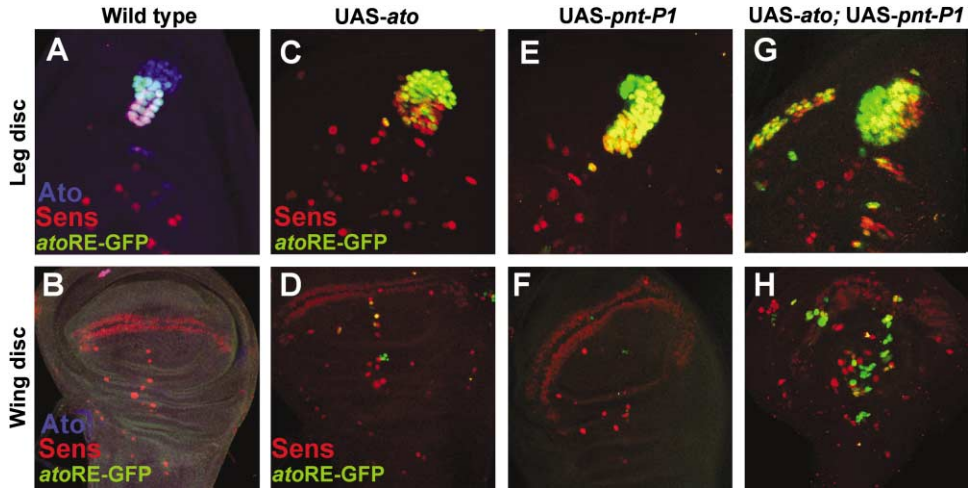

Figure 3. *ato*-RE Responds to Comisexpression of Pnt and Ato

GFP expression (green) driven by *ato*-RE in dorsal leg discs (A, C, E, G) and wing discs (B, D, F, H).
(A and B) Wild-type. There is no expression in the wing.
(C and D) 109-68*Gal4*; UAS-*ato*.
(E and F) 109-68*Gal4*, UAS-*Pnt-P1*.
(G and H) 109-68*Gal4* UAS-*ato*; UAS-*Pnt-P1*.
Whereas Ato or Pnt misexpression result in a larger cluster of femoral SOPs (and more GFP) (C–F), only comisexpression results in extensive ectopic GFP (G and H). Sens expression is in red, Ato expression (A and B) is in blue.

In *ato*-RE there are two potential Pnt binding sites (ETS-A and ETS-B). Purified Pnt-P1 protein binds to *ato*-RE (Figure 5, lane 6), and this binding is competed efficiently with a competitor oligonucleotide containing the ETS-A site but not one containing the ETS-B site (Figure 5B, lanes 7 and 8). This suggests that Pnt-P1 binds *ato*-RE via the ETS-A site. Mutating ETS-A (GGAAGC to GGCCTA) abolished GFP reporter gene expression in the embryo, leg FCO region, and eye (Figures 5C and 5E). A few GFP-expressing cells remaining in the leg may correspond to a tibial chordotonal organ. Mutation of ETS-B had no apparent effect (data not shown). Thus, the ETS-A site is likely to be a binding site through which Pnt regulates *ato* expression in the recruited chordotonal precursors.

In summary, virtually the entire activity of the *ato*-RE requires the E1 and ETS-A sites, most likely by binding Ato/Da and Pnt, respectively.

Cooperative Binding of Ato/Da and Pnt-P1

A remarkable feature of the E1 and ETS-A sites is their proximity, their core sequences being separated by 4 bp. This proximity is maintained precisely in the sequence upstream of *ato* in the genomes of *D. pseudoobscura* and *virilis*, where the sites are within an identical stretch of 58 bp (Figure 6A). The proximity and its conservation suggest that protein interactions between these transcription factors may contribute to the mechanism by which they specifically regulate *ato*. Molecular modeling suggests that the Pnt ETS domain and the E1 bHLH domains of Da/Ato heterodimer can bind simultaneously to this DNA sequence and may make direct contact with each other. Although no structures are known for any of these proteins, the conformations of the domains are likely to be highly similar to other proteins of similar sequence for which structures are available. The bHLH domains in the Ato:Da heterodimer were modeled using MyoD homodimer in complex with DNA (Ma et al., 1994), and Pnt was modeled using a structure of PU1's ETS domain in complex with DNA (Kodandapani et al., 1996). The DNA molecules in each complex were superposed such that the sites were the correct number of base pairs apart to resemble the E1-ETS-A sequence. The resultant model shows no serious steric clashes between Ato and Pnt domains and, indeed, the two proteins are close enough to form direct contacts (Figure 6B).

We addressed this possibility by investigating the binding of Ato/Da and Pnt in gel mobility shift assays. In the presence of all three proteins, a slower migrating protein-DNA complex was observed that represents all three proteins bound to the DNA (Figure 6C). In a supershift assay, this complex is lost if antibodies to Ato or Pnt-P1 are included (Figure 6D). Moreover, it appears that the triple binding is synergistic. In particular, although Pnt binds relatively poorly to ETS-A alone, the presence of all three proteins appears to drive strong binding of the ternary complex. Interestingly, the ternary complex also formed (albeit less efficiently) when the Pnt site was mutated such that it no longer bound Pnt when added alone (data not shown). Thus, although Pnt requires the ETS-A site in vivo, in vitro Ato/Da can pull Pnt into the DNA-protein complex even when Pnt cannot interact as efficiently with the DNA itself. Consistent with this, Pnt can also interact with Ato in a GST pull-down assay in the absence of DNA (Figure 6E). These data suggest that protein-protein interactions stabilize the DNA-protein complex and that cooperative binding may be important for this enhancer's function and specificity in vivo.

A Synthetic Enhancer of E1 and ETS-A Sites Recreates Much of the *ato*-RE Expression Pattern

An interesting question is whether the synergistic interaction between Pnt and Ato/Da allows the E1/ETS-A

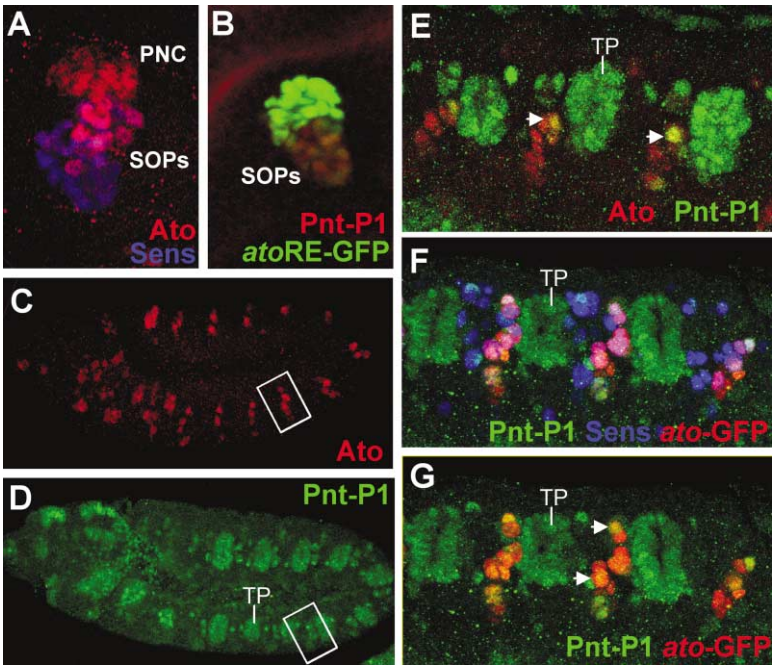

Figure 4. Coexpression of Ato and Pnt during Chordotonal Recruitment

(A) Expression of Ato (red) in leg disc femoral PNC and SOPs relative to Sens (blue).

(B) Expression of Pnt-P1 (red) in femoral SOPs compared with *ato*-RE-GFP (green).

(C) Ato in stage 11 embryo at a time when expression is resolved to SOPs. Box indicates chordotonal SOPs in one abdominal segment.

(D) Expression of Pnt-P1 in chordotonal SOPs (box). Most other abdominal expression at this stage is in the tracheal pits (TP).

(E) Stage 11 embryo, Ato (red) and Pnt-P1 (green) are coexpressed is some abdominal SOPs (arrows).

(F and G) Pnt-P1 (green) is coexpressed in some cells with an Ato-regulated GFP reporter construct (colored red to show better the overlap [arrows]).

sites to function in vivo outside the context of the *ato*-RE enhancer. A construct was made with GFP driven by two tandem repeats of a 35 bp fragment from the conserved *ato*-RE region, including the E1 and ETS-A sites ($[E1+ETS\text{-}A]_2$-GFP; Figure 7A). In the embryo, expression of $[E1+ETS\text{-}A]_2$-GFP was strikingly similar to *ato*-RE-GFP. It is strongly expressed in the precursors of vchAB, v'td2, and two sensilla of lch5 (Figures 7B–7E). In the head there is particularly strong expression in cells giving rise to Bolwig's organ, as well as other *ato*-dependent locations (Figure 7D). This construct, however, does not support any expression in the femoral precursors of the leg disc, suggesting that additional *ato*-RE sequences are required here for correct regulation (data not shown).

To ascertain the contribution of the ETS-A site, we examined a reporter transgene driven by six copies of the E1 site alone ($[E1]_6$-GFP; Powell et al., 2004). Unlike the $[E1+ETS\text{-}A]_2$-GFP construct, $[E1]_6$-GFP is expressed in all *ato*-dependent SOPs in the embryo (Figures 7F and 7G). Thus, the E1 site is capable of supporting Ato/Da-dependent regulation in all SOPs, but regulation is normally restricted to recruited SOPs by the need for Ato/Da to interact with Pnt. Presumably, this requirement is subverted when the E1 site is highly multimerized. A second possibility is that as well as binding Pnt, the ETS-A site can also bind a repressor. A likely candidate is the ETS repressor Yan. Yan acts in opposition to Pnt, and its repressor activity is relieved upon EGFR signaling by phosphorylation by ERK (Rebay and Rubin, 1995). Indeed, Yan protein is expressed during embryonic chordotonal recruitment (zur Lage et al., 1997), and recruitment is more extensive in *yan* mutant embryos (Okabe and Okano, 1997). Consistent with this, Yan protein is able to bind the ETS-A site in vitro (data not shown), and $[E1+ETS\text{-}A]_2$-GFP is expressed in more cells in *yan* mutant embryos (Figure 7H). This suggests that the ETS-A site is bound by Yan repressor. This repression of *ato*-RE is relieved by EGFR-dependent phosphorylation and by displacement by Pnt proteins. Interestingly, there is no evidence that Yan functions in leg disc SOP recruitment since its expression is undetectable during FCO development (data not shown). However, FCO recruitment is susceptible to Yan function, since expression of a UAS-yan^{Act} construct strongly inhibits chordotonal SOP recruitment (data not shown).

Discussion

The transition from competence to commitment during cell fate determination is a crucial decision point. In the case of SOP determination, proneural genes are required for both competence and commitment, and regulation of their expression is of central importance in deciding whether a cell proceeds from competence to commitment. We have shown that chordotonal SOP recruitment ultimately depends on the upregulation of the proneural gene *ato* via a specific recruitment enhancer. This enhancer requires the combined input of EGFR signaling (mediated by Pnt) and Ato/Da. It can be viewed as an EGFR-responsive enhancer that is contingent on the presence of Ato as a competence factor. This provides specificity of response to a widely utilized signaling pathway (Figure 7I). Regulation by a combination of versatile trigger (EGFR signaling mediated by Pnt) and a specific transcription factor (encoded by a selector gene) is a recurring theme in development (Mann and Carroll, 2002) and is known for several other EGFR functions, including the regulation of *even-skipped* (Halfon et al., 2000), *prospero* (Xu et al., 2000), *D-Pax2* (Flores et al., 2000), and *loco* (Granderath et al., 2000). However, in these cases, although genetic synergy was detected, a cooperative protein interaction was not described.

Chordotonal SOP recruitment also differs from these other cases of EGFR signaling in an important aspect:

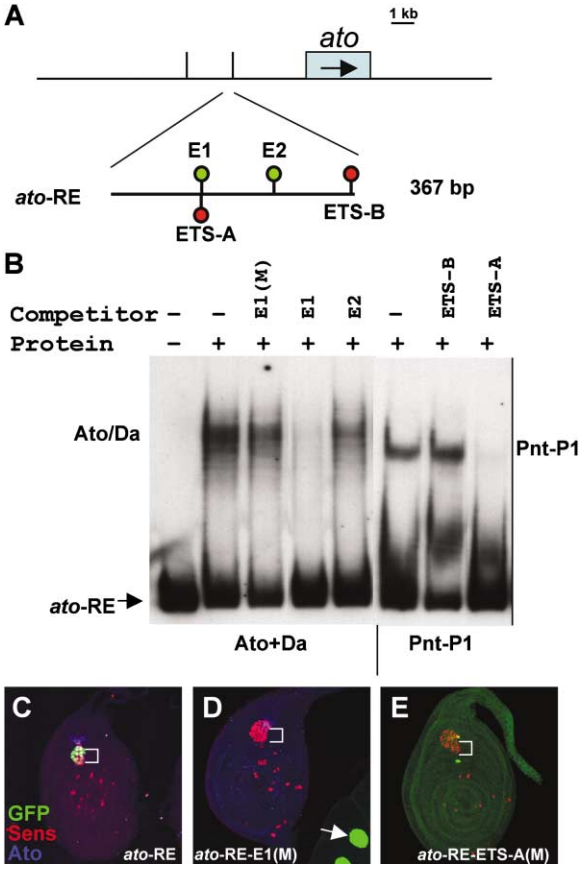

Figure 5. Ato/Da and Pnt-P1 Binding to the *ato*-RE

(A) Location of potential Ato/Da and Pnt binding sites in *ato*-RE.

(B) Gel mobility shift assay using the entire *ato*-RE fragment as DNA probe. Competitors are 50-fold excess of 20 bp double-stranded oligonucleotides containing the E1, E2, ETS-A, or ETS-B sites. E1(M) is the E1 competitor with a mutated E box.

(C and D) In vivo effect of mutation of E1 and ETS-A sites in *ato*-RE (green, GFP; blue, Ato; red, Sens).

(C) Wild-type *ato*-RE showing GFP in leg chordotonal SOPs (bracket).

(D) *ato*-RE with E1 site mutated, showing loss of expression in SOPs (while nonspecific vector-driven expression is seen in salivary gland nuclei [arrow]).

(E) *ato*-RE with ETS-A site mutated, showing loss of expression in FCO SOPs, although expression remains in a few cells that are possibly other SOPs.

the target of the signaling (*ato*) is also one of the regulatory inputs. The *ato*-RE can thus be viewed as an autoregulatory enhancer whose function is only triggered by the concomitant input of EGFR signaling. On this basis, a model is proposed in which autoregulation of the key factor required for commitment is the nodal control point regulated by EGFR signaling. Such a self-contained mechanism is generally applicable to many other situations in which specific cell fates must be generated from among groups of competent cells. For instance, a similar situation potentially exists in *C. elegans*, where *lin-39* (a Hox gene) provides competence for vulval Ras signaling and is upregulated by signaling (Maloof and Kenyon, 1998).

A summary of the regulation of *ato* during recruitment is shown in Figure 7I. Initially, Ato is expressed at a low level in PNC cells as a result of regulation by an enhancer(s) that is distinct from *ato*-RE. Such PNC enhancers are known for both the leg and the embryo (Sun et al., 1998, and unpublished data). Coexpression of E(spl) in response to Notch signaling (zur Lage and Jarman, 1999) inhibits SOP commitment, but the low level of Ato provides the competence for these cells to respond to EGFR signaling from other SOPs. If an Ato-expressing cell receives this signal, the combined action of Pnt and Ato/Da acts via the *ato*-RE element to upregulate Ato expression, leading to SOP commitment. Although this model describes a mechanism for SOP recruitment, it is likely that the firstborn chordotonal SOPs (including the primary SOPs in the embryo) are selected from the PNC by mechanisms involving an interplay of *ato* regulation with *Notch/E(spl)* signaling, as described for *ac/sc*. This conventional SOP selection route would function via enhancers other than the *ato*-RE (Sun et al., 1998).

It is clear how this model applies to the leg disc, where SOPs are recruited cumulatively from a persistent pool of Ato-expressing competent cells (Figure 1B). Significantly, the same enhancer appears to mediate recruitment in multiple *ato*-dependent sense organs, including the embryonic Bolwig and chordotonal organs as well as the leg disc. This implies that control of autoregulation is a fundamentally common mechanism underlying recruitment that is adaptable to different circumstances. In the leg disc, recruitment is reiterative because Ato expression in the PNC is long lived relative to the recruitment process (zur Lage and Jarman, 1999). In the embryo, however, there is only one round of recruitment. A likely explanation is that Ato PNC expression is simply more transient in the embryo. After downregulation of Ato in the PNC, the *ato*-RE cannot respond to EGFR signaling. Since PNC expression depends on multiple *ato* enhancers (Figure 2A), the varying degree of recruitment in different locations would be determined by the differing abilities of these enhancers to drive PNC expression. In addition, the inhibitory ETS protein Yan modulates the process. Yan acts in opposition to Pnt, and its negative effect has to be relieved by phosphorylation by ERK (Gabay et al., 1996). There is good evidence that Yan inhibits recruitment in the embryo by antagonizing Pnt regulation of *ato*-RE (Figure 7I; Okabe and Okano, 1997; zur Lage and Jarman, 1999; this report). Conversely, Yan does not appear to function during leg disc chordotonal recruitment, although this recruitment can be inhibited by ectopic Yan expression.

An extreme case of modifying SOP recruitment is seen in the embryo, where EGFR signaling from one embryonic chordotonal SOP (the P1 cell) recruits oenocytes rather than secondary chordotonal SOPs (Figure 1; Elstob et al., 2001; Rusten et al., 2001). This difference can be explained by the competences of the receiving cells. The PNC that gives rise to the P1 SOP is very short lived, so that Ato expression is probably absent in cells adjacent to the P1 SOP at the time that it signals for recruitment. Instead of Ato, the presence of a different competence factor (Spalt) alters the response of the cells to an otherwise identical EGFR signal.

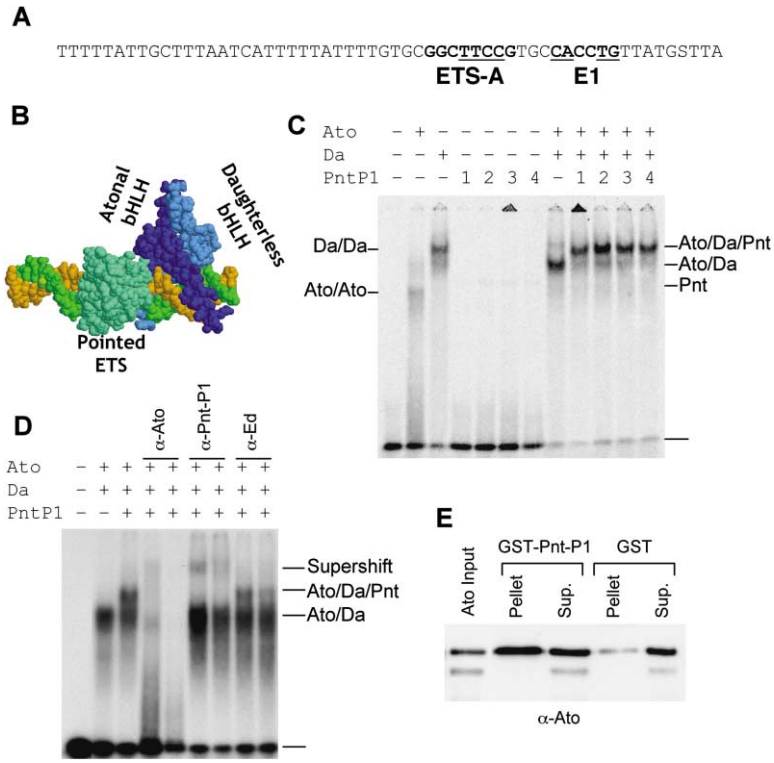

Figure 6. Cooperative Binding of Ato/Da and Pnt-P1 to Their Adjacent Sites

(A) 58 bp sequence around E1 and ETS-A that is conserved in *D. melanogaster*, *pseudoobscura*, and *virilis*.

(B) Space filling model of Pnt/Ato/Da ternary complex (green/dark blue/light blue) on the *ato*-RE DNA (lime green/orange). This model represents the bHLH and ETS domains only, which are based on known protein-DNA structures for MyoD and PU.1 proteins. The orientation of Ato/Da is based on Ato's binding to the 3′ half of the asymmetric E1 sequence (Powell et al., 2004).

(C) Gel mobility shift assay, showing the presence of a protein-DNA complex corresponding to the triple binding of Ato/Da and Pnt. Pnt-P1 notably binds poorly alone under the conditions of this assay, but is strongly enhanced by the presence of Ato/Da in the triple complex (cf. lanes 4–7 with lanes 9–12, which contain the same increasing amounts of Pnt protein).

(D) Supershift assay. The ternary complex is lost upon addition of antibodies to Ato or Pnt-P1, but not the unrelated protein, Echinoid. In this experiment, Ato/Da are present in excess over Pnt-P1. Anti-Pnt-P1 results in a supershift whereas Anti-Ato appears largely to abolish DNA binding.

(E) GST pull-down assay. GST-Pnt-P1 or GST were incubated with His_6-Ato and complexes collected with glutathione beads (pellet). Western blot with anti-Ato shows that Ato is pulled down preferentially by GST-Pnt-P1.

Molecular Basis of Enhancer Function

The combined response to Pnt and Ato/Da is mediated at the molecular level by cooperative binding of the transcription factors to adjacent sites that are evolutionarily conserved. The juxtaposition of sites allows high affinity of protein complex binding in vitro, and hence high specificity of enhancer activity in vivo even when the two sites form a synthetic enhancer in isolation from the rest of the enhancer. Binding by Pnt-P1 alone is rather poor but is much stronger in the presence of Ato/Da. Thus, cooperativity increases specificity. As in the case of Pnt, direct cooperative interaction with a selector gene product has been found to underlie the specificity of mammalian ETS-1 proteins, including interaction with Runx and Pax5 (Goetz et al., 2000; Fitzsimmons et al., 1996; reviewed in Verger and Duterque-Coquillaud, 2002). Significantly, cooperative binding has been characterized between ETS-1 and the bHLH protein, USF (Sieweke et al., 1998). Preliminary molecular modeling of bHLH and ETS domains on the *ato*-RE shows the feasibility of contact between the Ato HLH and Pnt ETS domains (Figure 6B). Among proneural proteins, this interaction may be very specific for Ato: bHLH residues available for interaction with Pnt are uniquely conserved in Ato and its vertebrate homologs compared with Sc and its homologs (Chien et al., 1996; Nakada et al., 2004; and unpublished observations). We suggest that Sc is unable to make appropriate interactions with Pnt. Consistent with this, when the Ato/Da E1 site is altered to conform to the binding consensus for Sc/Da, there is a dramatic loss of *ato*-RE enhancer activity (Powell et al., 2004). In this light, Pnt can be thought of as a specificity cofactor that ensures that Ato is the only proneural protein that can regulate the *ato*-RE.

In the embryo, the developmental logic of *ato*-RE function is wholly encapsulated in the interaction between the E1 and ETS-A sites. A synthetic enhancer consisting of two repeats of E1 and ETS-A reproduces the features of *ato*-RE expression in the embryo. Interestingly, a synthetic enhancer consisting of six repeats of the E1 site alone is active in all embryonic Ato-expressing SOPs (Figure 7; Powell et al., 2004). Thus, the E1 site is intrinsically capable of supporting Ato/Da-dependent regulation in all SOPs, but regulation is normally restricted to recruited SOPs by the need for Ato/Da to interact with Pnt. Presumably, this requirement is subverted when the E1 site is highly multimerized, perhaps because Ato/Da can then cooperatively bind to the tandem E1 sites. Unlike the embryo, the E1 and ETS-A sites are not sufficient for expression in the leg. These sites are part of an extensive region of sequence conservation. It is possible that other cofactors bind to sites in this region and interact with the Ato/Da/Pnt complex.

Ato Autoregulation as the Target of EGFR Signaling

Positive autoregulation is a common transcriptional control mechanism. We suggest that commonly, if not universally, positive autoregulation is contingent on other conditions being fulfilled in addition to the factor

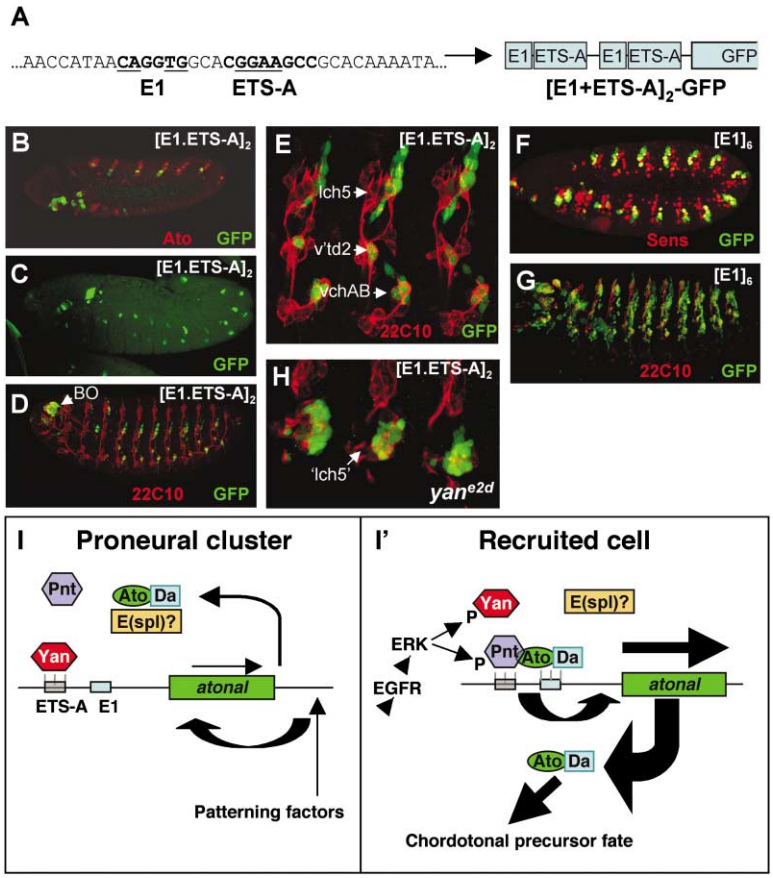

Figure 7. A Minimal Enhancer of Just the E1 and ETS-A Sites Can Support Recruitment-Pattern Expression

(A) Schematic of the 35 bp region around the E1 and ETS-A sites that was multimerized to generate the [E1+ETS-A]$_2$-GFP construct (note that this is the reverse complement of the sequence shown in Figure 6A).

(B–I) GFP expression (green) driven by [E1+ETS-A]$_2$-GFP.

(B) Stage 11 embryo (Ato in red).

(C) Stage 12 embryo.

(D and E) Stage 16 embryo (sensory neuron labeled by 22C10 in red), showing expression in vchAB and in two sensilla of lch5. The tracheal dendritic neurons (v'td2) also express GFP, which is consistent with them arising from the vchAB chordotonal lineages (Brewster and Bodmer, 1995). Expression is also observed in one sensillum of the thoracic dch3 cluster, which is not predicted to undergo recruitment, although *rho* is expressed transiently in dch3 SOPs (unpublished observation).

(F) Expression of an [E1]$_6$-GFP reporter in stage 11 embryo. Unlike the E1+ETS-A construct, expression is in all Ato-dependent SOPs (cf. B).

(G) [E1]$_6$-GFP reporter in stage 16 embryo, showing GFP expression in all cells derived from Ato-expressing SOPs (cf. D).

(H) Extra [E1+ETS-A]$_2$-GFP expression in *yan* mutant embryo. Only the lateral regions of three abdominal segments are shown.

(I and I′) Model of SOP recruitment by the interplay of signaling and competence.

(I) In PNC cells, low level of Ato expression does not lead to commitment but provides neural competence. The actions of Yan (in the embryo) and possibly E(spl) help to prevent Ato autoregulation via the *ato* recruitment enhancer.

(I′) Upon receiving signaling via EGFR, Yan and Pnt-P2 are phosphorylated and Pnt-P1 is expressed. Interaction between Pnt and Ato proteins allows their recruitment onto the *ato* enhancer, displacing Yan and E(spl), and resulting in Ato upregulation and SOP commitment.

itself being present. In consequence, promotion or inhibition of autoregulation provides a sensitive nodal point of regulation that can be modulated by extrinsic factors. In the case of *ato*, autoregulation provides the switch through which EGFR signaling can drive the transition from SOP competence to commitment. In a related way, autoregulation plays an important part in conventional SOP determination by Ac and Sc proneural proteins. In current models, Notch inhibits SOP selection by antagonizing the activity of Ac and Sc autoregulatory enhancers (van Doren et al., 1992; Culí and Modolell, 1998; Giagtzoglou et al., 2003). Genetically, chordotonal precursor recruitment is also inhibited by Notch signaling (zur Lage and Jarman, 1999). This does not appear to be by direct DNA binding, however, since there are no good candidate consensus sites for Su(H) or E(spl) in *ato*-RE (unpublished observations). One possibility is that E(spl) proteins interact directly with Ato/Da (Giagtzoglou et al., 2003) and that this inhibitory interaction is displaced by interaction with Pnt on the *ato*-RE (Figure 7I). Other possibilities include direct interaction between Notch and EGFR pathways farther upstream than Pnt and Ato (Berset et al., 2001) and the activation of *yan* expression by Notch signaling (Rohrbaugh et al., 2002).

Autoregulation of *ac* and *sc* is relatively simple in the sense that a single autoregulatory enhancer functions in many or all of the locations in which these genes are expressed. In contrast, by requiring Pnt, *ato*-RE activity is limited to the subset of areas of *ato* expression in which recruitment signaling occurs. Such spatial restriction of autoregulatory enhancer activity appears to be an important part of *ato* regulation, since in addition to the *ato*-RE, the gene is proposed to have a number of distinct autoregulatory enhancers, with different ones required in different locations (Sun et al., 1998). Presumably, the autoregulatory action of each of these enhancers is contingent on spatially restricted factor(s) equivalent to Pnt. It will be important to find out what these factors are and whether they too interact directly with Ato/Da proteins at their respective binding sites.

Experimental Procedures

Fly Stocks

The *109-68Gal4* driver and the UAS line UAS-*ato* were described by Jarman and Ahmed (1998). *yan*^{e2d} is described by Rogge et al. (1995). UAS-*pntP1* and UAS-*GFP* lines were obtained from the Bloomington stock center. The misexpression crosses were carried out at 29°C, all others at 25°C. Stocks were maintained on standard fly medium.

Plasmid Constructs

The pBluescript plasmid containing the 1937 bp *Sma*I-*Bam*HI leg enhancer was a gift from Bassem Hassan. Eight subclones were

prepared by PCR using the standard T3 and T7 primers as well as five new primers designed from the sequence, which all contain an *Asp*718I site at their 5′ end: primer 1 (461–479) 5′-GTCGGTACCCGA ATTTCGCTGTTTCGCC-3′, primer 2 (860–841) 5′-GTCGGTACCGGC CACTCGAGCGCGAAAC-3′, primer 3 (1349–1468) 5′-GTCGGTACC GCAATCATGACAGTGAGTC-3′, primer 4 (1588–1567) 5′-GTCGGTA CCGCGATGCCTAGGAGAGATGC-3′, and primer 5 (1571–1590) 5′-GTC GGTACCGCATCTCTCCTAGGCATCGC-3′. After amplification, the fragments were cut with either *Asp*718I or *Asp*718I-*Bam*HI and after sequencing, cloned into pPTGAL (Sharma et al., 2002). Transformant flies were made by microinjection into syncytial blastoderm embryos. These were crossed to UAS-*GFP* lines and checked for GFP activity in the leg disc. For the binding site mutagenesis, the 367 bp fragment (PCR product of primer 5 and T7 amplified from 1937 bp pBluescript *Sma*I-*Bam*HI), *ato*-RE, was cloned into pHStinger (Barolo et al., 2000). Mutagenesis was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

The following primers were used: E-1, 5′-GCTTCCGTGCCCTAGG TTATGGTTAC-3′; E-2, 5′-GTGATTGCGTAGTTTTCCTAGGTTTTCTGT GTTCCAGC-3′; ETS-A, 5′-TTTTGTGCGTAGGCCGTGCCACCTG-3′; ETS-B, 5′-CTAGAACTAGTTAGGCCTGGTCCCACGAAAC-3′. Sequence changes are underlined.

Sequencing of *D. virilis* Enhancer

A phage clone containing the *ato* gene and its surrounding sequences had previously been isolated from a *D. virilis* genomic library (I. Ahmed and A.P.J., unpublished). The *ato*-RE was amplified from this phage by PCR and sequenced using primers based on the *D. melanogaster* sequence.

Protein Purification

Da and Pnt-P1 ORFs were cloned in-frame into pRSET (InVitrogen). Proteins from these and from pRSET-Ato were expressed in BL21 bacterial cells, purified by virtue of His_6 tags, and renatured as described by Jarman et al. (1993) using Ni-NTA agarose (Qiagen).

In Vitro GST Pull-Down Assay

GST-PntC fusion protein (Kauffmann et al., 1996) or GST alone were bound to glutathione-agarose beads according to manufacturer's instructions (Amersham Biosciences). The beads were washed in pull-down buffer (50 mM Tris-HCl [pH 7.5]; 100 mM NaCl; 0.1% Triton X-100; 10% glycerol; protease inhibitor cocktail [Roche]). Purified His_6-tagged Ato was incubated with the beads for 1 hr at 4°C. The beads were washed four times in pull-down buffer before resuspending in SDS-PAGE buffer and running on a 15% SDS-polyacrylamide gel.

Gel Mobility Shift Assay

In the initial experiments, the gel-purified 367 bp *ato*-RE was used as a probe using a DIG Gel Shift Kit (Roche). Labeling and binding reactions were carried out according to manufacturer's instructions. In other experiments, a 36 bp oligonucleotide (5′-TATTTTGTGCGGC TTCCGTGCCACCTGTTATGGTTA-3′) and its complement were used as a probe (ETS-A and E1 sites underlined). The oligonucleotide was labeled using T4 polynucleotide kinase and [γ-^{33}P]ATP prior to annealing to its complementary oligonucleotide. Before binding to the DNA, Ato and Da proteins were preincubated on ice for 20 min. When Pnt protein was added for the ternary binding, a further 20 min preincubation was carried out. For the supershift assay, 0.5–1.5 μl of rabbit polyclonal antibody were added to the protein mixture 15 min before the addition of the DNA probe. Antibody against Echinoid was used as a control (Rawlins et al., 2003). All samples were electrophoresed at RT on 4% nondenaturing polyacrylamide gels in 0.5× TBE. The following oligonucleotides and their complements were used for competition experiments (sites underlined): E1, GTGCGGCTTCCGTGCCACCTGTTATGGTTA; E1(M), GTGCGGCTTCCGTGCTGAAGTATATGGTTA; E2, TTGCGTAGTTTT CATCTGTTTTCTGTGTTC; ETS-A, CGGCTTCCGTGCCACCTGTTAT GGTTACTA; ETS-B, GTTTCGTGGGACCAGGATCCTCGCTTATAA.

Synthetic Enhancer Constructs

$[E1]_6$-GFP construct is based on a tandem repeat of a sequence containing the core E box (5′-ACCATAACAGGTGGCACGGC-3′; Powell et al., 2004). For $[E1+ETS\text{-}A]_2$-GFP, the following oligonucleotides were designed: 5′-GATCTAACCATAACAGGTGGCACGGAAG CCGCACAAAATAG-3′; 5′-GATCCTATTTTGTGCGGCTTCCGTGCC ACCTGTTATGGTTA-3′. These were annealed, ligated, and digested with *Bgl*II and *Bam*HI, and then multimers were gel purified. These were ligated into pBluescript (Stratagene) and sequenced. A dimer insert was subsequently transferred to pHStinger as a single *Bam*HI-*Xba*I fragment (Barolo et al., 2000). Finally, germline transformation was carried out, lines were established, and the sequence was confirmed from genomic DNA.

Immunohistochemistry

Antibody staining for embryos was as described by zur Lage et al. (1997) and for imaginal discs as described by zur Lage and Jarman (1999). Antibodies were: Ato (1:4000; Jarman et al., 1993), Sens (1:6250; Nolo et al., 2000), Pnt-P1 (1:1000; Alvarez et al., 2003), GFP (Molecular Probes), and 22C10 (1:200; Developmental Biology Hybridoma Bank, IA). Secondary antibodies (1:500) were from Molecular Probes. Microscopy analysis employed a Leica TCS-SP LSCM microscope.

Acknowledgments

We thank B. Hassan for the original *ato* plasmid; J. Skeath for Pnt-P1 antibodies; I. Ahmed for the *D. virilis* phage; and T. Carter for technical assistance. This work was supported by a Senior Fellowship to A.P.J. from the Wellcome Trust (042482).

Received: April 21, 2004
Revised: August 4, 2004
Accepted: September 14, 2004
Published: November 8, 2004

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.

Alvarez, A.D., Shi, W., Wilson, B.A., and Skeath, J.B. (2003). *pannier* and *pointedP2* act sequentially to regulate *Drosophila* heart development. Development *130*, 3015–3026.

Barolo, S., Carver, L.A., and Posakony, J.W. (2000). GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in *Drosophila*. Biotechniques *29*, 726–732.

Berset, T., Hoier, E.F., Battu, G., Canevascini, S., and Hajnal, A. (2001). Notch inhibition of RAS signalling through MAP kinase phosphatase LIP-1 during *C. elegans* vulval development. Science *291*, 1055–1058.

Brewster, R., and Bodmer, R. (1995). Origin and specification of type II sensory neurons in *Drosophila*. Development *121*, 2923–2936.

Chien, C.-T., Hsiao, C.-D., Jan, L.Y., and Jan, Y.N. (1996). Neuronal type information encoded in the basic-helix-loop-helix domain of proneural genes. Proc. Natl. Acad. Sci. USA *93*, 13239–13244.

Cubas, P., de Celis, J.-F., Campuzano, S., and Modolell, J. (1991). Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* wing disc. Genes Dev. *5*, 996–1008.

Culí, J., and Modolell, J. (1998). Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by *Notch* signalling. Genes Dev. *12*, 2036–2047.

Dumstrei, K., Nassif, C., Abboud, G., Aryai, A., and Hartenstein, V. (1998). EGFR signalling is required for the differentiation and maintenance of neural progenitors along the dorsal midline of the *Drosophila* embryonic head. Development *125*, 3417–3426.

Elstob, P.R., Brodu, V., and Gould, A.P. (2001). *spalt*-dependent switching between two cell fates that are induced by the *Drosophila* EGF receptor. Development *128*, 723–732.

Fitzsimmons, D., Hodson, W., Wheat, W., Maira, S.M., Wasylyk, B., and Hagman, J. (1996). Pax-5 (BSAP) recruits Ets proto-oncogene

family proteins to form functional ternary complexes on a B-cell-specific promoter. Genes Dev. *10*, 2198–2211.

Flores, G.V., Duan, H., Yan, H., Nagaraj, R., Fu, W., Zou, Y., Noll, M., and Banerjee, U. (2000). Combinatorial signaling in the specification of unique cell fates. Cell *103*, 75–85.

Freeman, M. (1997). Cell determination strategies in the *Drosophila* eye. Development *124*, 261–270.

Gabay, L., Scholz, H., Golembo, M., Klaes, A., Shilo, B.-Z., and Klaembt, C. (1996). EGF receptor signaling induces *pointed P1* transcription and inactivates Yan protein in the *Drosophila* embryonic ventral ectoderm. Development *122*, 3355–3362.

Gabay, L., Seger, R., and Shilo, B.-Z. (1997). In situ activation pattern of *Drosophila* EGF receptor pathway during development. Science *277*, 1103–1106.

Giagtzoglou, N., Alifragis, P., Koumbanakis, K.A., and Delidakis, C. (2003). Two modes of recruitment of E(spl) repressors onto target genes. Development *130*, 259–270.

Goetz, T.L., Gu, T.L., Speck, N.A., and Graves, B.J. (2000). Auto-inhibition of Ets-1 is counteracted by DNA binding cooperativity with core-binding factor alpha2. Mol. Cell. Biol. *20*, 81–90.

Granderath, S., Bunse, I., and Klambt, C. (2000). *gcm* and *pointed* synergistically control glial transcription of the *Drosophila* gene loco. Mech. Dev. *91*, 197–208.

Halfon, M.S., Carmena, A., Gisselbrecht, S., Sackerson, C.M., Jimenez, F., Baylies, M.K., and Michelson, A.M. (2000). Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. Cell *103*, 63–74.

Jafar-Nejad, H., Acar, M., Nolo, R., Lacin, H., Pan, H., Parkhurst, S.M., and Bellen, H.J. (2003). Senseless acts as a binary switch during sensory organ precursor selection. Genes Dev. *17*, 2966–2978.

Jarman, A.P., and Ahmed, I. (1998). The specificity of proneural genes in determining *Drosophila* sense organ identity. Mech. Dev. *76*, 117–125.

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.

Kauffmann, R.C., Li, S., Gallagher, P.A., Zhang, J., and Carthew, R.W. (1996). Ras1 signaling and transcriptional competence in the R7 cell of *Drosophila*. Genes Dev. *10*, 2167–2178.

Kodandapani, R., Pio, F., Ni, C.Z., Piccialli, G., Klemsz, M., McKercher, S., Maki, R.A., and Ely, K.R. (1996). A new pattern for helix-turn-helix recognition revealed by the PU.1 ETS-domain-DNA complex. Nature *380*, 456–460.

Ma, P.C., Rould, M.A., Weintraub, H., and Pabo, C.O. (1994). Crystal structure of MyoD bHLH-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. Cell *77*, 451–459.

Maloof, J.N., and Kenyon, C. (1998). The Hox gene *lin-39* is required during *C. elegans* vulval induction to select the outcome of Ras signalling. Development *125*, 181–190.

Mann, R.S., and Carroll, S.B. (2002). Molecular mechanisms of selector gene function and evolution. Curr. Opin. Genet. Dev. *12*, 592–600.

Nakada, Y., Hunsaker, T.L., Henke, M., and Johnson, J.E. (2004). Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus neuronal cell-type specification. Development *131*, 1319–1330.

Nolo, R., Abbott, L.A., and Bellen, H.J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. Cell *102*, 349–362.

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 2 Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/Mapk pathway. Cell *78*, 137–147.

Powell, L.M., zur Lage, P.I., Prentice, D.R.A., Senthinathan, B., and Jarman, A.P. (2004). The proneural proteins Atonal and Scute regulate neural target genes through different E-box binding sites. Mol. Cell. Biol., in press.

Rawlins, E.L., White, N.M., and Jarman, A.P. (2003). Echinoid limits R8 photoreceptor specification by inhibiting inappropriate EGF receptor signalling within R8 equivalence groups. Development *130*, 3715–3724.

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.

Rogge, R., Green, P.J., Urano, J., Horn-Saban, S., Mlodzik, M., Shilo, B.-Z., Hartenstein, V., and Banerjee, U. (1995). The role of *yan* in mediating choice between cell division and differentiation. Development *121*, 3947–3958.

Rohrbaugh, M., Ramos, E., Nguyen, D., Price, M., Wen, Y., and Lai, Z.C. (2002). Notch activation of *yan* expression is antagonized by RTK/Pointed signaling in the *Drosophila* eye. Curr. Biol. *12*, 576–581.

Rusten, T.E., Cantera, R., Urban, J., Technau, G., Kafatos, F.C., and Barrio, R. (2001). *spalt* modifies EGFR-mediated induction of chordotonal precursors in the embryonic PNS of *Drosophila* promoting the development of oenocytes. Development *128*, 711–722.

Sharma, Y., Cheung, U., Larsen, E.W., and Eberl, D.F. (2002). pPTGAL, a convenient Gal4 P-element vector for testing expression of enhancer fragments in *Drosophila*. Genesis *34*, 115–118.

Sieweke, M.H., Tekotte, H., Jarosch, U., and Graf, T. (1998). Cooperative interaction of Ets-1 with USF-1 required for HIV-1 enhancer activity in T cells. EMBO J. *17*, 1728–1739.

Skeath, J.B., and Carroll, S.B. (1991). Regulation of *achaete-scute* gene expression and sensory organ formation in the *Drosophila* wing. Genes Dev. *5*, 984–995.

Sun, Y., Jan, L.Y., and Jan, Y.N. (1998). Transcriptional regulation of *atonal* during development of the *Drosophila* peripheral nervous system. Development *125*, 3731–3740.

Van Doren, M., Powell, P.A., Pasternak, D., Singson, A., and Posakony, J.W. (1992). Spatial regulation of proneural gene activity: auto- and cross-activation of *achaete* is antagonized by *extramacrochaete*. Genes Dev. *6*, 2592–2605.

Verger, A., and Duterque-Coquillaud, M. (2002). When ETS transcription factors meet their partners. Bioessays *24*, 362–370.

Xu, C., Kauffmann, R.C., Zhang, J., Kladny, S., and Carthew, R.W. (2000). Overlapping activators and repressors delimit transcriptional response to receptor tyrosine kinase signals in the *Drosophila* eye. Cell *103*, 87–97.

zur Lage, P., and Jarman, A.P. (1999). Antagonism of EGFR and Notch signalling in the reiterative recruitment of *Drosophila* adult chordotonal sense organ precursors. Development *126*, 3149–3157.

zur 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.