

# Insect oenocytes: a model system for studying cell-fate specification by *Hox* genes

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(Accepted 6 April 2001)

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## ABSTRACT

During insect development, morphological differences between segments are controlled by the *Hox* gene family of transcription factors. Recent evidence also suggests that variation in the regulatory elements of these genes and their downstream targets underlies the evolution of several segment-specific morphological traits. This review introduces a new model system, the larval oenocyte, for studying the evolution of fate specification by *Hox* genes at single-cell resolution. Oenocytes are found in a wide range of insects, including species using both the short and the long germ modes of development. Recent progress in our understanding of the genetics and cell biology of oenocyte development in the fruitfly *Drosophila melanogaster* is discussed. In the *D. melanogaster* embryo, the formation of this cell type is restricted to the first 7 abdominal segments and is under *Hox* gene control. Oenocytes delaminate from the dorsal ectoderm of A1–A7 in response to an induction that involves the epidermal growth factor receptor (EGFR) signalling pathway. Although the receptor itself is required in the presumptive oenocytes, its ligand Spitz (Spi) is secreted by a neighbouring chordotonal organ precursor (COP). Thus, in dorsal regions, local signalling from this component of the developing peripheral nervous system induces the formation of oenocytes. In contrast, in lateral regions of the ectoderm, Spi signal from a different COP induces the formation of secondary COPs in a homeogenetic manner. This dorsoventral difference in the fate induced by Spi ligand is controlled by a prepattern in the responding ectoderm that requires the Spalt (Sal) transcription factor. Sal protein is expressed in the dorsal but not lateral ectoderm and acts as a competence modifier to bias the response to Spi ligand in favour of the oenocyte fate. We discuss a recently proposed model that integrates the roles of Sal and the EGFR pathway in oenocyte/chordotonal organ induction. This model should provide a useful starting point for future comparative studies of these ectodermal derivatives in other insects.

*Key words:* *Hox*; homeotic; oenocytes; chordotonal organs; EGF receptor; *spalt*.

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## INTRODUCTION

The fruitfly *Drosophila melanogaster*, with its one hundred years of genetics and a completely sequenced genome, holds a central position in the study of insect development. However, several recent technical advances look extremely promising for applying the kind of sophisticated genetic tools, normally associated only with *D. melanogaster*, to other insects. These new approaches include forward genetic screens in other insects such as the red flour beetle (*Tribolium castaneum*), gene mapping using PCR based methods and cross-species transfer of genes via ‘universal’

transposable element vectors and retroviral infection. Furthermore, genome projects have already been started in several insect species other than *D. melanogaster* and it is expected that these will come on-line in the very near future. For all of these reasons, the comparative developmental biology of insects is rapidly becoming a very promising area for the detailed study of evolutionary mechanisms.

Genetic pathways that control differences between segments during development are likely to provide a rich substrate for segmental variation during evolution (Akam, 1998b). Perhaps the best characterised genes in these segment-specific pathways are the

*Hox*/homeotic family of transcription factors (McGinnis & Krumlauf, 1992; Akam, 1998a). Although the *Hox* protein sequences themselves are remarkably conserved across a wide range of animal species, their expression patterns and transcriptional targets appear to be highly variable (see for example, Palopoli & Patel, 1998; Weatherbee et al. 1999). Much current research is aimed at testing whether this source of variation is responsible for morphological innovation within Insecta.

The overall number of target genes modulated by the *Hox* genes during development is probably very large (Graba et al. 1997; Akam, 1998c; Pradel & White, 1998). Even in *D. melanogaster*, identifying all of the genetic networks under the control of any one *Hox* gene is a daunting prospect. Yet, if we are to understand how *Hox* genes modulate morphogenesis during development and evolution, the identification of these downstream targets is essential. This complex target problem can be reduced to something more manageable by focusing on the specification and differentiation of a single cell type that is under *Hox* control. Such a single-cell approach has been adopted recently in *D. melanogaster* for the larval oenocyte. The starting point for these studies is that larval oenocyte formation is completely blocked in embryos lacking all of the *Hox* genes of the bithorax complex: *Ultrabithorax*, *abdominal A* and *Abdominal B* (P. Elstob & A. Gould, unpublished observations). Thus the generation of the oenocyte fate is entirely dependent on *Hox* function. This dramatic *Hox*-dependence contrasts with many other cell types whose morphologies are only subtly modified by *Hox* inputs.

At present, it is not certain which of the 3 bithorax-complex genes are required for oenocyte formation. Neither is it known which *Hox* target genes are involved in making a larval oenocyte. Before we can hope to reconstruct the genetic cascade leading from *Hox* gene to oenocyte, it is important to understand the developmental origin of this rather mysterious cell type. The purpose of this article is to review the literature on larval oenocytes and to summarise recent progress in our understanding of their formation in *Drosophila*.

#### THE OENOCYTE AS A MODEL CELL FOR STUDYING *HOX* PATTERNING

The larval oenocyte is well suited to evolutionary-developmental studies of *Hox* patterning as it is both segment-specific and easily identifiable by morpho-

logical criteria across a range of insect species. Two separate populations of oenocytes exist in *D. melanogaster*, larval and imaginal (Koch, 1945). This review focuses only on the larval oenocytes (hereafter termed oenocytes) which are derived from the embryonic ectoderm of abdominal segments A1-A7 (Hartenstein et al. 1992; Elstob et al. 2001). In the mature embryo, the oenocytes occupy a characteristic lateral and subepidermal position and are clustered into a single group of cells within each hemisegment. Unlike many other ectodermal derivatives, such as the peripheral nervous system, the precise number of oenocytes in a cluster is not absolutely fixed and can vary anywhere between 4 and 9, with a mean of 6. After larval hatching, oenocytes undergo extensive cell growth without division so that by the late third instar they have attained a diameter of  $\sim 80 \mu\text{m}$  (Bodenstein, 1950). Due to their conspicuously large size and unusual ultrastructure, the oenocytes of *Drosophila* and other insects have long attracted the interest of invertebrate physiologists. The presence of densely packed smooth and rough endoplasmic reticulum, together with other morphological features characteristic of mammalian steroidogenic cells and hepatocytes, indicates that oenocytes are cells with a specialised secretory function (Koller, 1928; Wigglesworth, 1933; Rinterknecht & Matz, 1983). However, the repertoire of the substances that they secrete and their physiological function in the intact organism are far from clear. On the basis that oenocyte morphology varies with the moulting cycle and that maximal secretory potential is reached just prior to ecdysis, it was postulated that these cells secrete lipid and protein components of the insect cuticle (Wigglesworth, 1933, 1970; Baikova et al. 1993). An alternative, but not mutually exclusive, function in synthesising moulting hormones such as ecdysteroids has also been suggested (Locke, 1969; Dorn & Romer, 1976). Indeed, there is evidence that the oenocytes of 2 beetle species, *Tribolium castaneum* and *Tenebrio molitor*, are capable of secreting derivatives of ecdysone in vitro (Romer, 1971; Romer et al. 1974). Despite all of this historical interest in oenocytes, surprisingly little was known about the formation of these interesting cells until recently. Two new studies, however, have revealed that the embryonic origin of oenocytes in *D. melanogaster* is closely linked with that of another cell type: a proprioceptive component of the peripheral nervous system called a chordotonal organ (Elstob et al. 2001; Rusten et al. 2001). Therefore, in order to understand oenocyte development, it is necessary to be familiar with the mechanisms of chordotonal organ formation.

## PRIMARY AND SECONDARY CHORDOTONAL ORGAN FORMATION

In each abdominal hemisegment of the *Drosophila* embryo, there are 8 chordotonal organs that are partitioned into arrays consisting of 1 dorsolateral

(V'ch1), 5 lateral (Lch5) and 2 ventral (VchAB) organs (Brewster & Bodmer, 1995) (Fig. 1). The Lch5 array lies in the same lateral position, and just internal to, the mature oenocyte cluster of ~ 6 cells. As the remainder of this review will describe, this close association can be traced back in development to the

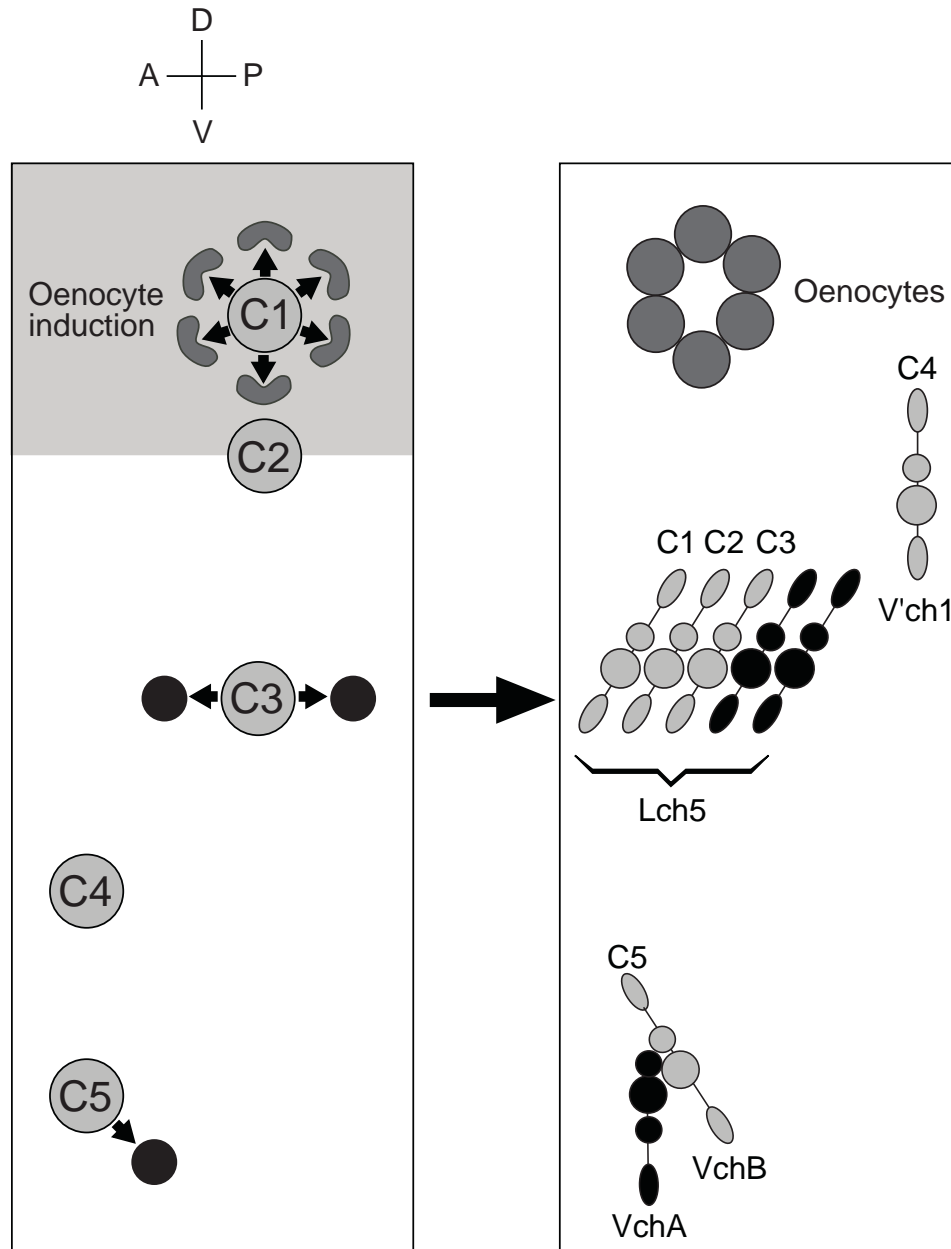


Fig. 1. Fate map for oenocytes and chordotonal organs. Each panel represents a single abdominal hemisegment at early (left) and late (right) stages of embryogenesis. Anterior (*A*) is to the left and dorsal (*D*) up, as indicated. The left panel shows the 5 primary COPs (C1-C5, light grey) that express *Atonal*. Arrowheads indicate the site of *Spitz/EGFR* induction events. Within the *Sal* dorsal domain (light grey shading), 6 oenocyte precursors (dark grey, sickle shape) are induced around C1. More ventrally, outside the *Sal* dorsal domain, 2 secondary COPs (black, circular) near C3 and one secondary COP near C5 are induced. The derivatives of all 14 precursor cells are shown in the right panel. The 6-cell oenocyte cluster (dark grey, circular) derived from the oenocyte precursors is depicted above the 5 primary chordotonal organs (C1-C5, light grey) and the 3 secondary chordotonal organs (black). The precursors for the 2 secondary chordotonal organs of the Lch5 are induced near C3, while the founder for the VchA is induced near C5. In the real embryo, there is spatial overlap between the oenocytes, V'ch1 and Lch5 but for clarity they have been drawn well separated. Note that there are 2 remaining uncertainties in the mapping. First, C2 is shown half-in and half-out of the *Sal* dorsal domain as its precise location is not clear. Secondly, within the Lch5 array, the anteroposterior order of the organs derived from C2 and C3 is not known. Data for this figure were compiled from zur Lage et al. (1997) and Elstob et al. (2001); see text for more details.

## EGF Receptor Pathway

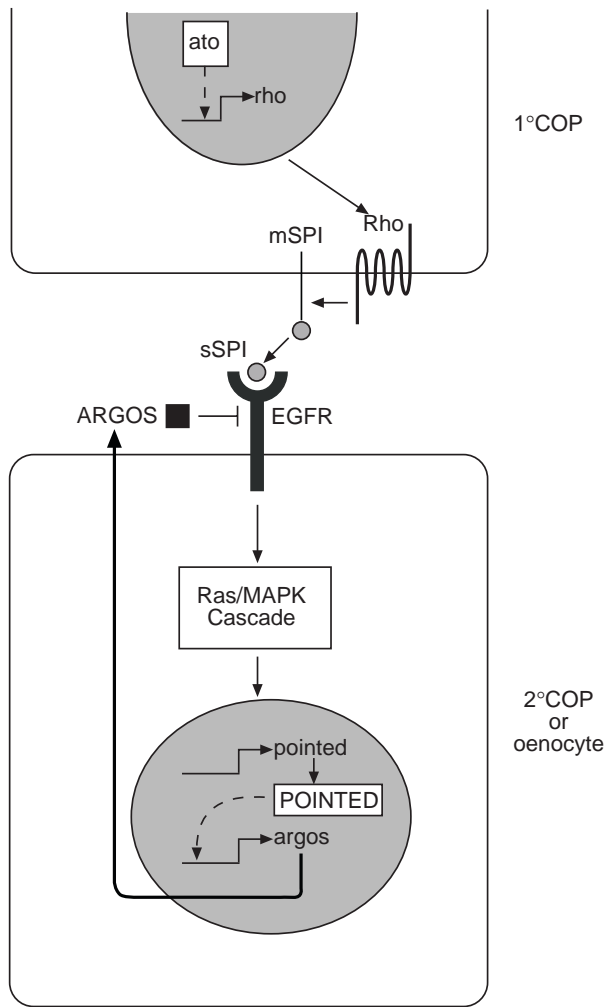


Fig. 2. The components of the EGFR Pathway in chordotonal organ/oenocyte induction. A primary COP (upper cell) signalling to a secondary COP or oenocyte (lower cell) is depicted. The expression of Atonal (Ato) protein in the nucleus of the primary COP results in the transcriptional activation of the *rhomboïd* (*rho*) gene. Rho, a 7-pass transmembrane protein, is required for the conversion of inactive, membrane-bound Spitz (mSpi) to its active, secreted form (sSpi). In turn, sSpi binds to and activates the EGFR, resulting in the stimulation of the RAS/MAPK signal transduction cascade. This results in transcriptional activation of *pointed* in the nucleus of the responding cell. One of the transcriptional targets for the Pointed protein is *argos*. Argos protein is secreted by the responding cell and inhibits further activation of the EGFR, thus completing a negative feedback loop that limits the extent of signalling.

time when the precursors for both cell types delaminate from the dorsolateral ectoderm of the extended-germ band embryo.

Each chordotonal organ is formed by a single chordotonal organ precursor (COP) that divides asymmetrically to produce a sensory neuron, scolopale, ligament and cap cell (Fig. 3A; Brewster &

Bodmer, 1996). The proneural gene that specifies COPs and therefore mature chordotonal organs is *atonal* (*ato*) which encodes a basic helix-loop-helix transcription factor (Jarman et al. 1993). Previous studies have elegantly demonstrated that, in the embryo, COPs are produced in a 2-step delamination process (Okabe & Okano, 1997; zur Lage et al. 1997). Initially, several primary COPs that are Ato-positive delaminate from the ectoderm. A subset of these then induce the delamination of Ato-negative secondary COPs in a recruitment process that requires the EGFR signalling pathway (Fig. 2). In each abdominal hemisegment, 5 primary COPs (C1–5) are produced, with each occupying a characteristic dorsoventral position: C1 is the most dorsal and C5 the most ventral (Fig. 1). Ato expression in these primary COPs is thought to switch on the transcription of the *rhomboïd* (*rho*) gene, which encodes a transmembrane protein that is rate-limiting for the conversion of the EGFR ligand, Spitz (Spi), from an inactive to an active form (Freeman, 1994; Tio et al. 1994; Bang & Kintner, 2000). In turn, active Spi induces 3 secondary COPs via binding to and activation of the EGFR, giving the full complement of 8 COPs and thus 8 chordotonal organs per hemisegment (Fig. 1, 2). When EGFR signalling is abolished, such as in *rho* mutants, the 3 chordotonal organs that are descended from the secondary COPs are missing, producing a deficit of 2 units from the Lch5 array and a deletion of the VchA organ (Bier et al. 1990). Thus, 5 primary COPs induce just 3 secondary COPs, which raises the question of exactly which primary cells induce which secondary cells. Part of the answer to this anatomical puzzle came from observations suggesting that C5, the VchB precursor, induces the secondary COP that gives rise to VchA (zur Lage et al. 1997). However, as described below, the question of which primary COPs induce the 2 secondary COPs that contribute to the Lch5 was only resolved very recently (Elstob et al. 2001).

### OENOCYTES ARE INDUCED BY THE DEVELOPING PNS

By using several molecular markers to track the development of oenocytes and chordotonal organs, it was found that oenocyte precursors delaminate from the ectoderm overlying the most dorsally located primary COP (C1) and that the presence of this particular sensory mother cell is required for oenocyte formation (Elstob et al. 2001) (Fig. 1). Around the time of delamination, oenocyte precursors can be identified as a whorl of sickle-shaped cells that

surround the dividing C1 cell. Just before the onset of oenocyte precursor formation, C1 begins to express high levels of Rho and thus presumably acts as a source of active Spi. In *rho* and *spi* mutants, where production of Spi signal by C1 is blocked, oenocyte formation is completely abolished. Thus Spi signalling from C1 induces oenocyte precursor formation in the neighbouring ectoderm. Receipt of Spi ligand activates the EGFR and its target gene *pointed* (O'Neill et al. 1994) in the precursors themselves, triggering differentiation along the oenocyte pathway. Consistent with this scenario, reducing the level of EGFR activation using a dominant-negative form of the receptor or removing *pointed* function has the effect of abolishing induction. Oenocyte induction is a short-range signalling event, with only the cells immediately surrounding C1 switching on markers specific for this cell type. In *argos* mutants, however, the range of the response is increased from 1 to 2 concentric rings of cells. Argos is a secreted inhibitor of the EGFR that is produced in response to EGFR activation (Freeman, 1996; Okabe & Okano, 1997). Hence, Spi ligand is not intrinsically limited to immediate neighbours but the response is nevertheless kept short-range by *argos*-mediated negative feedback to the receptor.

By combining the recent oenocyte/chordotonal data together with that from earlier studies, most of the uncertainties in chordotonal organ fate mapping have now been resolved. The revised fate map for the complete set of all 14 oenocytes and chordotonal organs in an abdominal hemisegment is shown in Figure 1. With our current knowledge of the oenocyte induction event around C1, the previous suggestion (zur Lage et al. 1997), that C1 and C3 each induce one of the secondary COPs that contribute to the Lch5, now looks extremely unlikely. Therefore the revised map indicates that C1 induces approximately 6 oenocytes whilst C3 induces both of the secondary COPs for the Lch5.

#### CONTROL OF CELL NUMBER BY THE EXTENT OF EGFR PATHWAY SIGNALLING

The formation of both secondary COP and oenocyte fates requires the EGFR pathway. In *ato*, *rho*, *spi*, and *EGFR<sup>DN</sup>* backgrounds, where signalling is compromised, the induction of both cell types is blocked (Fig. 3A). Conversely, when there is more EGFR signalling both cell types become more numerous. This has been demonstrated in multiple ways, using mutants that lack *argos* function or when Rho or constitutively secreted Spi are overexpressed using the

GAL4/UAS system. Together, these results indicate that the number of recruited cells is controlled by the amount of EGFR pathway signal. Rather surprisingly, however, there is no parity between the numbers of additional oenocytes and lateral chordotonal organs that are produced by excess EGFR signalling. Thus, for a given degree of ligand overproduction, more oenocyte precursors than COPs are recruited (Fig. 3A). This implies the existence of an additional tier of control that restricts neural but not oenocyte induction. Such a selective inhibition process would ensure that the number of chordotonal organs is more tightly controlled than that of oenocytes, as is observed in wild-type embryos.

#### SPALT SWITCHES ECTODERMAL COMPETENCE FROM COP TO OENOCYTE

Both oenocytes and chordotonal organs are induced by the EGFR, but how can the same signalling pathway produce two such different outcomes? More specifically, this question centres on understanding how Spi signal from C1 induces oenocytes, while that from C3 induces secondary COPs. One possibility would be that the information for specifying the choice of cell fate is somehow contained in differences in the level or timing of active Spi production. This, however, seems unlikely as the fates induced in the vicinity of C1 and C3 are not qualitatively altered by over expressing Rho or active Spi, or by mimicking reduced Spi production using a dominant-negative EGFR. An alternative explanation for the C1/C3 difference is that the 2 populations of ectodermal cells responding to active Spi are differentially prepatterned prior to signalling. Strong evidence in favour of this scenario comes from a detailed analysis of the expression and function of the *spalt(sal)* gene which encodes a zinc-finger transcription factor that interacts with the EGFR signalling pathway (Kuhnlein et al. 1994; Chen et al. 1998; Elstob et al. 2001; Rusten et al. 2001).

Epistasis tests, using combinations of loss- or gain-of-function mutations in *sal* together with EGFR pathway genes were used to rule out a function for *sal* in the primary COP producing the Spi inductive signal (Elstob et al. 2001; Rusten et al. 2001). Instead, the results of these experiments are consistent with *sal* acting in the responding ectoderm. Here it appears to play a dual role: first acting prior to Spi signalling to modify ectodermal competence and secondly, functioning downstream of the EGFR, as part of the oenocyte-specific response (Elstob et al. 2001).

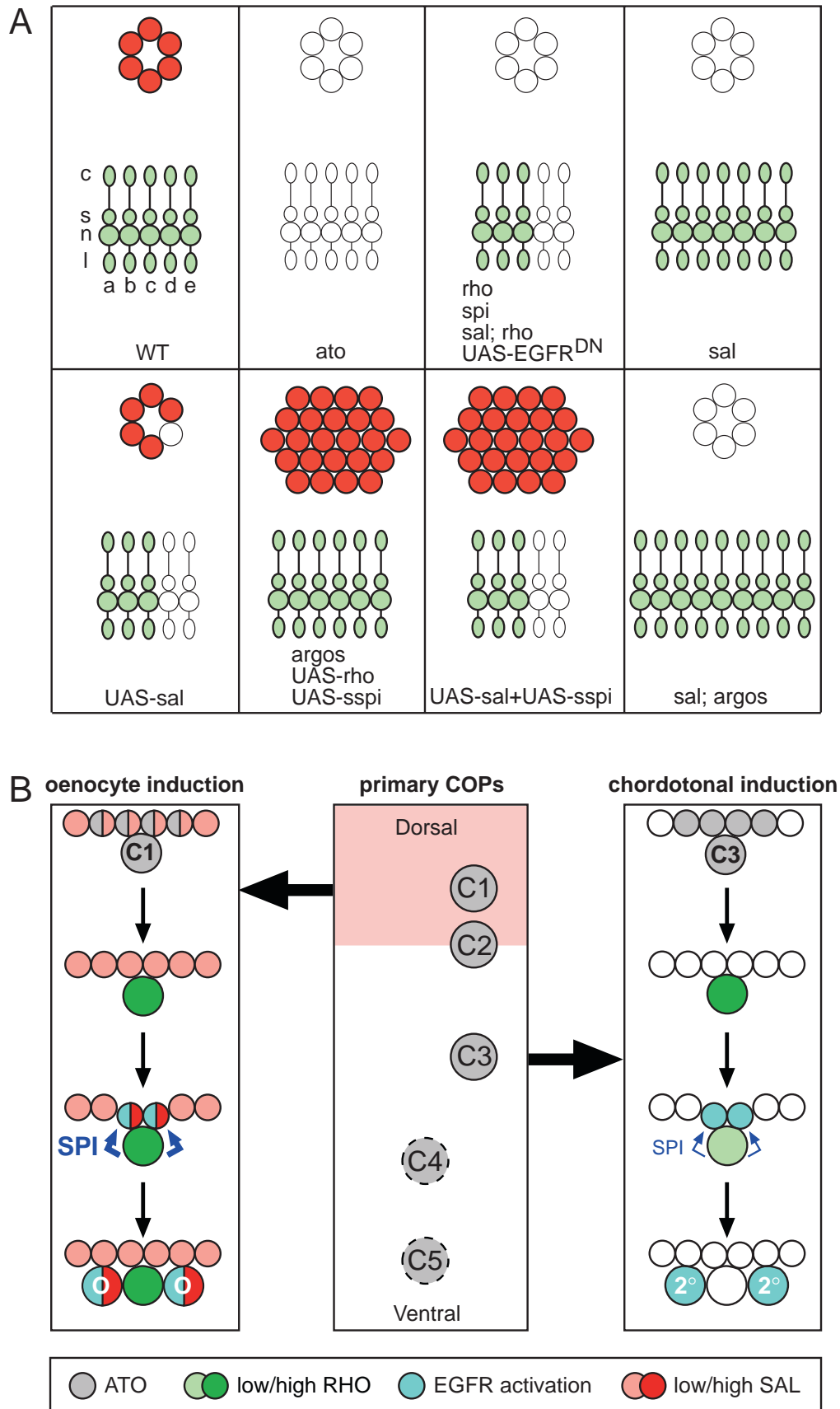


Fig. 3. Summary and prime-and-respond model for oenocyte and chordotonal induction. (A) Summary of the number of oenocytes (red) and lateral chordotonal organs (green) in wild-type (WT) and mutant backgrounds. Each panel represents a single abdominal hemisegment and V<sup>ch</sup>I and V<sup>ch</sup>AB are not shown. In the wild-type panel the relative positions of the cap (c), scolopale (s), neuron (n) and ligament (l) cells that constitute each of the 5 organs (a–e) of the Lch5 are shown. Missing lateral chordotonal organs and oenocytes are indicated by

Interestingly, these 2 roles for *sal* can be accounted for by 2 distinct phases of Sal protein expression. An early dorsal domain (the Sal dorsal domain), independent of *rho* and *spi* function, appears to define a zone of ectodermal competence to form oenocyte precursors. Later, at the time of induction, Sal levels are upregulated but only as a response to EGFR activation within the oenocyte precursors themselves.

The Sal dorsal domain is present in the extended germ-band embryo prior to oenocyte and secondary COP induction. It includes ectodermal territory in the vicinity of C1 but not C3, which lies too ventrally. Thus Sal protein is present in the right place and at the right time to have a role in modifying the competence of the ectoderm to respond to EGFR ligand from C1 but not C3. In support of this, oenocyte induction is blocked in *sal* null mutants (Elstob et al. 2001; Rusten et al. 2001). In contrast, in the same mutant background, 2 supernumerary lateral chordotonal organs are formed. As these ectopic organs can be suppressed by simultaneously removing *rho* function, they must be derived from supernumerary secondary and not primary COPs. This strongly suggests that, in the absence of Sal, C1 induces two secondary COPs at the expense of the entire oenocyte cluster. Hence, in the wild-type situation, the presence of Sal in the ectoderm overlying C1 plays 2 roles: the suppression of COP recruitment and the promotion of oenocyte induction. Sal is sufficient for the first of these roles, as when ectopically expressed in the ectoderm overlying C3 it can block the induction of the 2 secondary COPs that would normally form there (Elstob et al. 2001; Rusten et al. 2001). In this context, however, oenocytes fail to be induced near C3, arguing that Sal is not sufficient for oenocyte induction (Elstob et al. 2001). The most likely explanation for this lack-of-sufficiency is that Sal is only one of several essential components needed to define the ectodermal prepattern for oenocyte induction. Presumably these other competence factors are present within the wild-type dorsal Sal domain but, like Sal itself, are not expressed in more ventral regions.

#### TWO CELL FATES FROM ONE SIGNAL: A PRIME-AND-RESPOND MODEL FOR THE ROLE OF *SAL*

The dual roles for *sal* as a competence modifier, and also a part of the oenocyte-specific EGFR response, have been integrated in a prime-and-respond model (Elstob et al. 2001). This model illustrates how both the oenocyte and the chordotonal cell fates might be induced by one signal (Fig. 3B). First considering the early role of *sal*, where it functions prior to signalling as a competence switch: here, Sal prepatterns the dorsal ectoderm so that, on receipt of the Spi signal, oenocytes rather than COPs are induced. Experiments varying Sal concentration and levels of EGFR activity suggest that one consequence of the presence of Sal in the responding nucleus is to increase the apparent threshold for an inductive event (Elstob et al. 2001). This makes the prediction that a signalling cell inducing oenocytes may need to express more ligand than one that recruits secondary chordotonal organs. In fact, this does appear to be the case as in wild-type embryos C1 is known to express high levels of *rho* for longer than C3 (zur Lage et al. 1997). Hence, the EGFR pathway does contribute to the cell-type specificity of the induction event in the sense that more Spi ligand is required to overcome the higher induction threshold for oenocyte precursors than for secondary COPs.

Turning now to the later role of *sal* that is downstream of EGFR activation: here the up-regulation of Sal protein is an early oenocyte-specific response to Spi signalling. In turn, this high level of Sal appears to stimulate expression of *seven up* (*svp*), which encodes a member of the steroid receptor superfamily (Mlodzik et al. 1990; Elstob et al. 2001). Presumably a large set of genes is turned on in response to EGFR activation in oenocyte precursors but most of these have not yet been characterised. In the future, it will be interesting to see how much overlap there is between this gene set and the repertoire of genes that is switched on during COP induction.

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unfilled outlines and the UAS results shown are with the *en-GAL4* driver, which is active in all the precursors of the oenocytes and lateral chordotonal organs (Elstob et al. 2001). (B) The prime-and-respond model. The central panel indicates the position of the 5 Ato-expressing primary COPs (C1-C5), relative to the dorsal oenocyte prepattern of low Sal expression. It is not clear whether C2 lies within or just ventral to the Sal domain. Either way, it does not express *rho* strongly (zur Lage et al. 1997) and therefore is unlikely to induce oenocytes. C1-3 contribute to the Lch5 but C4 and C5 (dashed circles) do not. The left panel shows the induction of oenocytes (O) via strong and persistent Spi signalling (large blue arrows) from C1 to the EGFR in overlying ectodermal cells. The oenocyte prepattern of low Sal raises the apparent threshold for induction by Spi. Low Sal also serves to prime the responding cell so that *sal* can be subsequently upregulated as part of the response to EGFR activation. In turn, this stimulates the expression of the *sal* target gene, *svp* (not shown). The right panel shows the induction of secondary COPs (2°) by moderate Spi signalling (small blue arrows) from C3 to ectodermal cells that are Sal-negative. In this case, EGFR stimulation does not lead to the activation of *sal* or *svp*. Instead, sensory organ precursors that divide and differentiate into lateral chordotonal organs are produced. (From Elstob et al. 2001.)

A key feature of the prime-and-respond model is that moderate levels of *sal* expression serve to prime the responding cells to further upregulate Sal when they receive Spi ligand. In support of this priming mechanism, it was demonstrated that *sal* upregulation in response to constitutively secreted Spi is restricted to those cells that lie within the Sal dorsal domain (Elstob et al. 2001). Hence, Sal proteins appear to provide a molecular link between the prepattern and the EGFR response. As the levels of Sal are different in these 2 phases, it may be that there are at least 2 different concentration-dependent effects for this transcription factor. There is a precedent for this in another context, macrochaete formation, where low levels of Sal are known to promote sensory organ precursor formation but high levels have an inhibitory effect on the differentiation of this cell type (de Celis et al. 1999). Even more strikingly, during wing vein development, low and high levels of Sal are known to produce completely opposite transcriptional effects on the *knirps* target gene (de Celis & Barrio, 2000).

#### PERSPECTIVES

The oenocyte and secondary chordotonal organ fates are restricted to abdominal segments. At present, the mechanism by which Hox genes trigger the induction of these 2 cell types in such a segment-specific manner is unknown. The bithorax complex, *sal* and the EGFR pathway are all required for this process but exactly how these components interface is far from clear. It is hoped that by building on the present framework, future studies will successfully address this issue, first in *Drosophila* and then in other insects.

EGFR pathway components and *sal* orthologues are present in species as far apart as humans and worms. Furthermore, chordotonal organs and oenocytes have been described in several insects, including representative species employing the short and the long germ modes of embryogenesis. Due to their unique morphologies, it should be possible to identify both cell types in a wide range of insects and possibly even other arthropods. Such a comparative survey should help determine the extent to which the *Drosophila* prime-and-respond model described here is applicable to other species.

#### ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council.

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