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Opposing Transcriptional Outputs of Hedgehog Signaling and Engrailed Control Compartmental Cell Sorting at the *Drosophila* A/P Boundary

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Summary

The wing imaginal disc is subdivided into two nonintermingling sets of cells, the anterior (A) and posterior (P) compartments. Anterior cells require reception of the Hedgehog (Hh) signal to segregate from P cells. We provide evidence that Hh signaling controls A/P cell segregation not by directly modifying structural components but by a Cubitus interruptus (Ci)-mediated transcriptional response. A shift in the balance between repressor and activator forms of Ci toward the activator form is necessary and sufficient to define "A-type" cell sorting behavior. Moreover, we show that Engrailed (En), in the absence of Ci, is sufficient to specify "P-type" sorting. We propose that the opposing transcriptional activities of Ci and En control cell segregation at the A/P boundary by regulating a single cell adhesion molecule.

Introduction

During development of multicellular organisms, some cells preferentially intermingle with one another and sort out from other cells. This differential cell sorting is vital for the assembly of individual cells into distinct tissues. It is also the basis for the subdivision of a variety of insect and vertebrate tissues into adjacent but nonintermingling sets of cells termed compartments (reviewed by Blair, 1995; Lawrence and Struhl, 1996; Vincent, 1998; Dahmann and Basler, 1999). Signaling processes across boundaries between compartments can lead to the local production of secreted proteins that organize, at long range, patterning and growth of the entire tissue. The continuous segregation of cells into compartments is crucial for the positioning and maintenance of such organizers and thus for the precise implementation of body plans.

The *Drosophila melanogaster* wing imaginal disc is subdivided into an anterior (A) and a posterior (P) compartment (Garcia-Bellido et al., 1973). P cells heritably express the selector gene *engrailed* (*en*) (Kornberg et al., 1985), which directs these cells to secrete the short-range signaling molecule Hedgehog (Hh) and at the same time makes P cells refractory to the Hh signal (Tabata et al., 1992, 1995; Zecca et al., 1995; reviewed by Lawrence and Struhl, 1996). In contrast, A cells do not express En and, as a consequence, can receive and respond to Hh. The response to Hh requires Smoothened (Smo), a seven-pass transmembrane protein, and the transcription factor Cubitus interruptus (Ci), the *Drosophila* Gli homolog (reviewed by Alcedo and Noll, 1997; Ingham, 1998; Aza-Blanc and Kornberg, 1999). Ci is expressed exclusively in A cells, where it can exist in two forms. A repressor form of Ci (Ci[rep]) is generated in A cells that do not receive the Hh signal, and an activator form of Ci (Ci[act]) is generated in A cells that receive the Hh signal (Aza-Blanc et al., 1997; Ohlmeyer and Kalderon, 1998). Both forms of Ci control the transcription of the *decapentaplegic* (*dpp*) gene, which, as a consequence, is expressed only in a thin strip of A cells along the A/P boundary (Méthot and Basler, 1999). *dpp* encodes a member of the transforming growth factor β (TGFβ) superfamily (Padgett et al., 1987), which induces the expression of target genes in a concentration-dependent manner in both compartments (Lecuit et al., 1996; Nellen et al., 1996). The stable and precise positioning of the Dpp morphogen source is crucial for growth and patterning of the entire wing. It is critically dependent on the continuous segregation of En-expressing (Hh-secreting) and non-En-expressing (Hh-responsive) cells into distinct but apposing P and A compartments, respectively.

The segregation of A and P cells requires the activity of En. P cells lacking En sort out from neighboring cells and, if in contact with A cells, will mingle with them (Morata and Lawrence, 1975; Kornberg, 1981; Lawrence and Struhl, 1982; Blair and Ralston, 1997). How does En activity lead to the segregation of A and P cells? Recent experiments indicate that this is at least in part achieved by controlling Hh signaling. Anterior cells that have lost Smo function, and hence the ability to transduce the Hh signal, no longer segregate with A cells but instead take up positions normally only occupied by P cells (Blair and Ralston, 1997; Rodriguez and Basler, 1997). This observation was taken as evidence that En controls A/P cell segregation to a large extent indirectly, by creating an interface between Hh-receiving and non-Hh-receiving cells. However, such *smo* mutant A cells do not appear to intermingle well with P cells. This has raised the question of whether En might also play a role in cell segregation independently of Hh signaling (Lawrence, 1997). Moreover, while the experiments described above revealed a requirement for local Hh signaling in the establishment of the A/P boundary, they did not address the question of whether the Hh signal is sufficient to specify "A-type" cell sorting behavior.

The most attractive hypothesis to explain cell segregation at compartment boundaries is based on differential cell adhesion (or cell affinity [Garcia-Bellido, 1975]; reviewed by Dahmann and Basler, 1999). However, since the molecules involved in these processes have not been identified to date it remains uncertain whether such a mechanism would be sufficient to account for cell segregation phenomena in vivo. Regulation of cell adhesion can occur at several levels, including modulation of the adhesion complex itself or its interaction with the actin cytoskeleton. The organization of the actin

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cytoskeleton is, in part, under a direct influence of signaling pathways via the regulation of small GTPases (Van Aelst and D'Souza-Schorey, 1997). It is thus conceivable that Hh signaling affects cell sorting by directly modulating the activity of small GTPases. Alternatively, Hh signaling could regulate cell sorting at the A/P boundary via a transcriptional control of target genes that in turn modulate the segregation properties of responding cells.

Here, we show that Ci is required for all aspects of Hh-controlled cell segregation, thus demonstrating that Hh signaling governs cell sorting by transcriptionally regulating target genes. This transcriptional response is regulated by both Ci[act] and Ci[rep]. Hh signaling suffices to specify A-type cell sorting behavior irrespective of the activity of En. En, in addition to regulating Hh signaling, controls cell segregation also in a Hh-independent manner. In the absence of Ci, En is sufficient to specify "P-type" sorting. Cells lacking both Ci and En neither exhibit A- nor P-type sorting behavior, indicating that Ci and En control most, if not all, aspects of the distinct sorting properties of A and P cells, respectively. Finally, we show that cells expressing higher levels of a single cell adhesion molecule sort out in vivo from cells expressing it at lower levels, suggesting that differential cell adhesion is sufficient to account for cell segregation in vivo. We propose a model in which Ci and En control cell segregation at the A/P compartment boundary by causing an abrupt change in the activity of a single cell adhesion molecule.

Results

Experimental Design

To test the role of En and Hh-signaling components in controlling cell segregation, we applied two experimental assays. Both assays are based on the presumption that cells maximize contact (intermingle) with cells of the same adhesiveness and minimize contact with (sort out from) cells of different adhesiveness (Steinberg, 1963; reviewed by Dahmann and Basler, 1999; see also below [Figure 4]). In the "round-up assay", clones of mutant cells are assayed for their shape. Each clone is analyzed by how circular it is and how smoothly its border interfaces with surrounding tissue. The degree of roundness of the clone and smoothness of its border is taken as a measure for the difference in adhesiveness between cells inside and outside of the clone. In the wild-type wing imaginal disc, cell segregation is confined to the region of the compartment boundaries. Thus, in the more stringent "choice assay," clones generated in the vicinity of the A/P boundary are monitored for their sorting behavior. Clones have three choices: they can (1) remain within their compartment of origin, (2) sort completely into the territory of the adjacent compartment defining a straight border with cells of the compartment of origin at the normal position of the A/P boundary, or (3) sort out from cells of both compartments and take up positions overlapping the normal site of the A/P boundary. Depending on the genetic intervention, the compartment of origin of a clone was determined either by the state of the heritable and P-specific expression of an *en-lacZ* reporter gene or by the position of the "twin spot" clone, which is composed of sibling wild-type cells. The position of the A/P boundary was inferred from the expression of a *hh-lacZ* reporter gene expressed exclusively in P cells.

Hh-Transducing Cells Sort Out from Non-Hh-Transducing Cells

Cells on opposite sides of the A/P boundary differ in their response to Hh. To test whether a difference in Hh transduction between two cell populations is sufficient to cause their segregation, Hh transducing and non-transducing cells were juxtaposed far away from the A/P boundary. In two different experiments, the smoothness of the border between these cells was analyzed as a measure for their sorting behavior (round-up assay). In the first experiment, cells were generated that lost the ability to transduce the Hh signal (becoming mutant for *smo*) but at the same time gained the ability to produce and secrete the Hh ligand (using a *tubulin*α*1>CD2*, *smo*$^{+}$*> hh* transgene in a *smo*$^{-}$ background; see the Experimental Procedures). In the P compartment, where cells also express, but do not transduce, the Hh signal, these clones formed wiggly borders with surrounding cells (Figure 1A). In contrast, anterior *tubulin*α*1>hh smo*$^{-}$ clones formed significantly smoother borders with neighboring wild-type cells that received and responded to the Hh protein secreted from the cells in the clone (Figure 1A; $P << 0.001$, 35 A and 21 P clones in 18 discs were analyzed). This indicates that Hh-receiving cells sort out from Hh-producing cells that cannot respond to Hh.

In a complementary experiment, we created a Hh-receiving/non-Hh-receiving interface in the P compartment by inducing the expression of Ci in marked clones of cells (using an *act5c>CD2>Gal4 UAS-ci* transgene combination). The expression of Ci renders P cells competent to respond to the Hh signal (Dominguez et al., 1996). Compared to anterior clones that do not have access to the Hh signal and express Ci already from the endogenous gene, such clones had a significantly rounder shape in the P compartment (Figure 1B; $P << 0.001$, 31 A and 24 P clones in 10 discs were analyzed), providing further evidence that differences in the level of Hh transduction can lead to the separation of cell populations (see also Lawrence et al., 1999).

Evidence for the Involvement of Hh Target Genes in A/P Cell Segregation

The experiments described above indicate that one response of a cell to the Hh signal consists in the modification of its segregation behavior. Although the molecular basis is not known, a cell's segregation preference is presumably a function of its cytoskeletal or surface properties. The Hh signal is received on cell membranes by a receptor complex containing Smo (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). By a poorly understood mechanism, Smo activity impinges on a cytoplasmic high molecular weight complex that contains Ci and is bound to microtubules by the Kinesin superfamily protein Costal-2 (Robbins et al., 1997; Sisson et al., 1997). To test whether Hh signaling confers A segregation properties by directly altering structural components of responding cells or instead by regulating the transcription of one or several target genes, we assayed the role of Ci in the sorting behavior of A cells using the choice assay. Clones of P cells homozygous

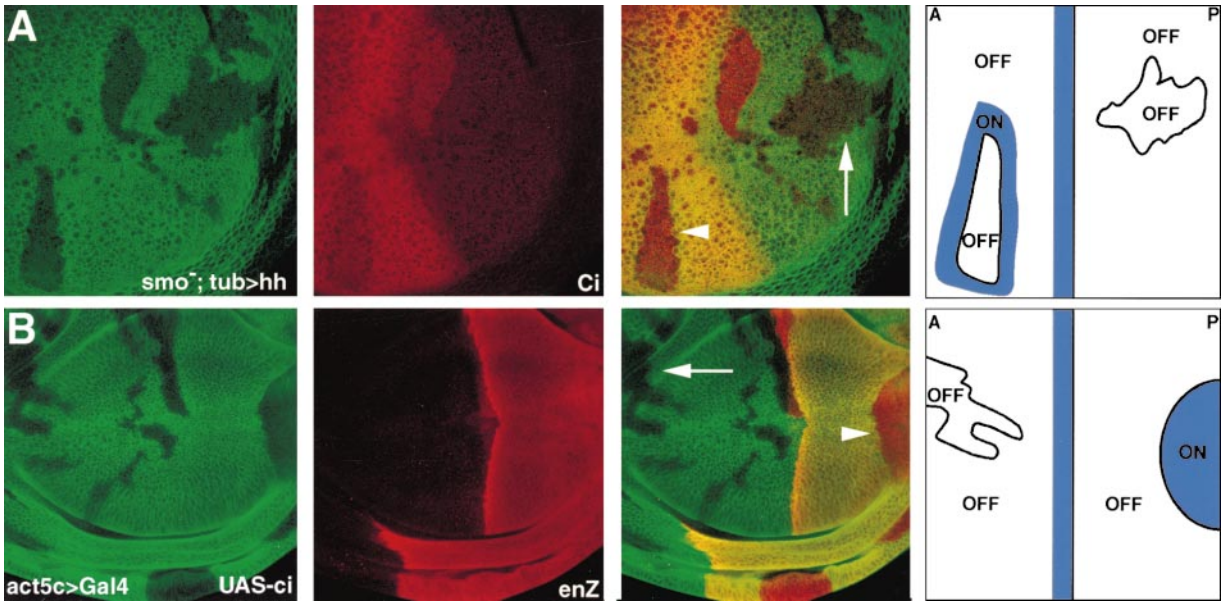

Figure 1. Hh-Transducing Cells Sort Out from Non-Hh-Transducing Cells

Clones of cells expressing Hh and being unable to respond to Hh (*smo*$^{-}$; *tub*>*hh* [A]) or expressing Ci from a transgene (*act5c*>*GAL4 UAS-ci* [B]) are marked by the absence of CD2 staining, in green (left column). Ci staining (A) or *en-lacZ* (enZ; [B]) expression is shown in red (second column). The superimposition of both stainings is shown in the third column. A schematic representation is shown in the right column where cells responding to the Hh signal are shown in blue. In this and subsequent figures, third instar wing imaginal discs are shown with the anterior to the left and dorsal up.

(A) Posterior *smo*$^{-}$, *tub*>*hh* clones (arrow) have wiggly borders to their neighbors. Neither cells within nor cells immediately outside of the clone transduce the Hh signal. In contrast, *smo*$^{-}$, *tub*>*hh* clones (arrowhead) have smooth borders when situated in the A compartment. Cells surrounding the clone respond to the Hh ligand secreted by *smo*$^{-}$; *tub*>*hh* cells. The large clone in the middle is of A origin (cells express Ci) and has taken up a position in the P territory due to the loss of Smo function. Since cells lack En, this clone has a smooth border to P cells (see below).

(B) Clones in the far A compartment (and thus not having access to Hh ligand) overexpressing Ci (arrow) have wiggly borders to their neighbors. Neither cells within nor cells immediately outside of the clone transduce the Hh signal. In contrast, P cells manipulated to transduce the Hh signal by ectopic expression of Ci (arrowhead) sort out from non-Hh-transducing wild-type P cells.

mutant for ci^{94} (and thus completely lacking *ci* product, referred to as *ci*$^{-}$; Méthot and Basler, 1999) exclusively occupied P territory and defined a straight border with A cells in the normal position of the A/P boundary (Figure 2B). In contrast, if derived from an A cell, *ci*$^{-}$ clones often occupied areas overlapping the normal position of the A/P boundary (i.e., straddled the boundary) forming straight borders both with neighboring A and P cells, indicating that A cells lacking Ci sort out from both A and P cells at the boundary (Figure 2A; see Table 1 for frequencies of clonal positions). We conclude that the Hh signal controlling cell segregation is at least in part transduced by the transcription factor Ci and hence involves a transcriptional response (see below). Because Ci is present in its activator form (Ci[act]) in Hh-receiving A cells from which *ci*$^{-}$ cells segregate, it can be inferred that Ci[act] plays an important role in conferring anterior segregation properties.

Opposing Roles of the Ci Activator and Repressor Forms in Cell Segregation

Anterior cells lacking Smo function sort entirely into the normal domain of the P compartment (Blair and Ralston, 1997; Rodriguez and Basler, 1997; data not shown). In contrast, as described above, *ci*$^{-}$ clones of A origin sort only partially into P territory (Figure 2A). One difference between *smo*$^{-}$ and *ci*$^{-}$ cells is that the former express Ci, which in the absence of Hh-signal transduction is processed to Ci[rep]. To test if the different sorting behaviors of *smo*$^{-}$ and *ci*$^{-}$ mutant clones are due to Ci[rep], we made use of an allele of *ci*, ci^{Cell}, which can only give rise to the repressor form of Ci (Méthot and Basler, 1999). Thirteen out of twenty-five anterior ci^{Cell}/ci^{-} clones that originated in the vicinity of the compartment boundary sorted entirely within the normal P territory, compared to none out of 20 *ci*$^{-}$/*ci*$^{-}$ clones originated at similar positions (compare Figures 2A and 2C; see Table 1). Furthermore, these ci^{Cell}/ci^{-} clones defined straight borders to A cells at the normal position of the A/P boundary. ci^{Cell}/ci^{-} clones of P origin remained exclusively in P territory (Figure 2D; see Table 1). Thus, the different sorting behavior of anterior *smo*$^{-}$/*smo*$^{-}$ and *ci*$^{-}$/*ci*$^{-}$ clones appears to be a consequence of the presence and absence, respectively, of Ci[rep] in these cells. From these results and those described above, we conclude that both the activator and repressor forms of Ci play important roles in controlling cell segregation at the A/P boundary. However, the two forms of Ci have opposing effects, Ci[act] conferring A properties and Ci[rep] conferring P properties. Thus, it must be the net balance of the two activities that determines whether a cell sorts with A or P cells.

To test whether a shift in the ratio between Ci[act] and Ci[rep] results in a corresponding change in segregation properties, we experimentally increased the levels of the repressor form of Ci in clones of A cells by expressing

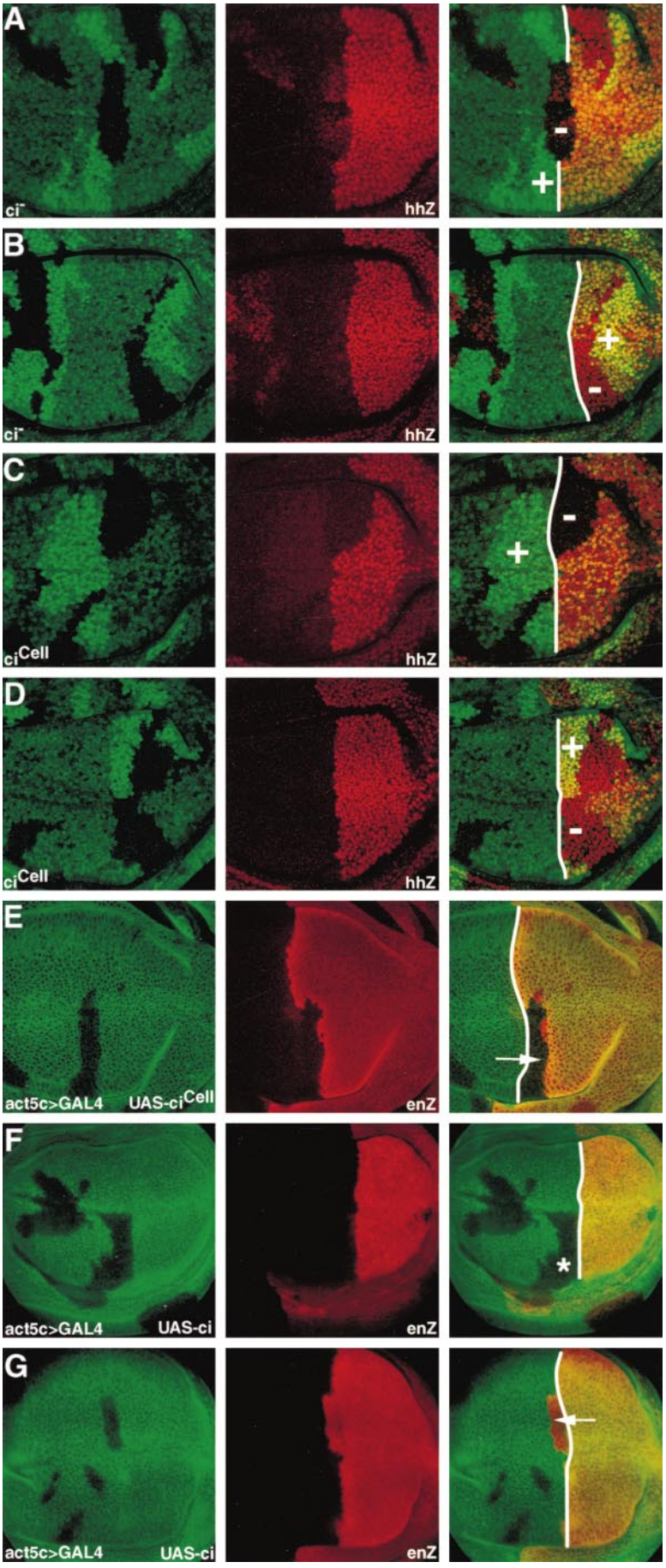

Figure 2. Transcriptional Response to Hh Signaling Is Necessary and Sufficient to Specify A-Type Cell Sorting Behavior

Clones of cells expressing no Ci (*ci*$^{-}$ homozygous clones [A and B]), only Ci^{Cell} (*ci*^{Cell}/*ci*$^{-}$ [C and D]), high levels of Ci^{Cell} (*act5c>GAL4 UAS-ci*^{Cell} [E]), or high levels of Ci (*act5c>GAL4 UAS-ci* [F and G]) are marked by the absence (−) of GFP staining (A–D) or CD2 staining (E–G) in green. The wild-type sister clones (in A-D) are marked by the elevated levels of GFP staining (+). *hh-lacZ* (*hhZ*) and *en-lacZ* (*enZ*) expression is shown in red. The white line in this and subsequent figures marks the normal position of the compartment boundary.

(A) *ci*$^{-}$ homozygous clones of A origin (as judged by the position of the *ci*$^{+}$/*ci*$^{+}$ sister clone and reduced expression of *hh-lacZ*) sort into territory overlapping the normal position of the compartment boundary.

(B) *ci*$^{-}$ homozygous clones of P origin (as judged by the position of the *ci*$^{+}$/*ci*$^{+}$ sister clone and expression of *hh-lacZ*) are strictly confined to the P compartment.

(C) *ci*^{Cell}/*ci*$^{-}$ clones of A origin (as judged by the position of the *ci*$^{+}$/*ci*$^{+}$ sister clone and lack of *hh-lacZ* expression) occupy positions normally taken up by P cells.

(D) *ci*^{Cell}/*ci*$^{-}$ clones of P origin (as judged by the position of the *ci*$^{+}$/*ci*$^{+}$ sister clone and expression of *hh-lacZ*) are strictly confined to the P compartment.

(E) Cells of A origin (as judged by the lack of *en-lacZ*) expressing Ci^{Cell} occupy positions normally only taken up by P cells (arrow).

(F) Anterior clones of cells expressing Ci sort exclusively within A territory and form straight borders to P cells at the normal position of the A/P boundary (asterisk).

(G) Cells of P origin (as judged by the expression of *en-lacZ*) occupy positions normally only taken up by A cells (arrow).

Table 1. Frequencies of Clonal Positions

	Discs Scored	A Origin			P Origin			Direction of Clonal Migration[a]
		A	A + P	P	A	A + P	P	
ci^-	190	2	18	0	0	0	13	A (>) P
ci^{Cell} [b]	221	1	11	13	0	0	8	A > P
en^-	219	26	0	0	35	1	6	P > A
smo^- [c]	>200	2	0	55	0	0	34	A > P
en^- ci^-	218	4	13	0	2	10	2	A (>) P A (<) P
en^- smo^-	176	0	1	18	0	0	10	A > P

Clones of cells generated either in the A or P compartment close to the A/P boundary were scored for their positions and subdivided into three classes: entirely within A compartment defining a straight border to P cells at the normal position of the compartment boundary (A), overlapping the normal position of the A/P boundary (A+P), and entirely within P compartment defining a straight border to A cells at the normal position of the compartment boundary (P). The number of clones for each class, genotype, and compartment of origin is indicated as well as the total number of discs scored for each genotype. Clones were only scored in the prospective wing blade region of the disc, where the boundary is particularly straight.

[a] A>P Clones of A origin sort entirely within P territory
P>A Clones of P origin sort entirely within A territory
A(>)P Clones of A origin take up positions overlapping the normal site of the A/P boundary
A(<)P Clones of P origin take up positions overlapping the normal site of the A/P boundary

[b] The observation that only about half of the A ci^{Cell}/ci^- clones appear to completely segregate into P territory, in contrast to >95% of smo^- clones, might be due to the presence of only one coding *ci* gene in the former case (*ciCell*) compared to two copies of ci^+ from which the smo^- cells can make Ci[rep].

[c] Rodriguez and Basler (1997).

Ci^{Cell} from a transgene (see the Experimental Procedures). Such clones took up positions normally occupied only by P cells and defined straight borders to A cells lacking the activity of the ci^{Cell} transgene (n = 20, Figure 2E). The anterior origin of these clones is inferred from the lack of *en-lacZ* reporter gene expression. P clones expressing Ci^{Cell} continue to express *en-lacZ* and remain in P territory (data not shown). We conclude that the relative amounts of activator and repressor forms of Ci determine an important aspect of the Hh-mediated control of cell segregation at the A/P boundary. High levels of Ci[act] or Ci[rep] confer A or P segregation properties, respectively, and absence of both forms causes A cells to segregate from both A cells near the compartment boundary and P cells.

Based on the observation that A cells expressing the repressor form of Ci (ci^{Cell}/ci^-) behave like cells unable to respond to the Hh signal (smo^- cells) in that they sort into P territory, we conclude that most if not all functions of Hh in controlling cell segregation are mediated by Ci and thus involve a transcriptional response.

Hh Signaling Is Sufficient for A-Type Cell Segregation

So far, we have shown that a difference in Hh transduction is sufficient to separate two cell populations and that Hh transduction is required for cell segregation at the A/P boundary. To test whether Hh signaling is also sufficient to direct cells to sort with A cells at the boundary, we activated Hh transduction in clones of P cells by ectopically expressing Ci and assayed their segregation at the A/P boundary. Clones of P origin, as inferred from the expression of the *en-lacZ* reporter gene, always took up positions normally only occupied by A cells and defined straight borders to wild-type P cells (n = 20, Figure 2G). Expression of Ci did not influence *en-lacZ* reporter gene expression. Anterior clones overexpressing Ci were strictly confined to A territory (Figure 2F). Thus, Hh signaling is sufficient to determine A cell segregation.

En Controls Cell Segregation Also in a Ci-Independent Manner

Posterior clones lacking *en* function sort, when in contact with A cells, into A territory (Morata and Lawrence, 1975; Kornberg, 1981; Lawrence and Struhl, 1982; Blair and Ralston, 1997; Table 1). However, posterior cells mutant for *en* express Ci (Tabata et al., 1995), which, as shown above, is sufficient for P cells to sort into A territory. To test whether the sorting of posterior en^- clones into A territory is solely due to expression of Ci, we monitored the behavior of en^- ci^- double mutant cells in the choice assay. The elimination of *en* function is complicated by the existence of a largely redundant homolog called *invected* (*inv*) (Coleman et al., 1987). However, the *enE* allele used throughout this study eliminates both genes (Gustavson et al., 1996), and we refer to this situation as en^-. Similar to ci^- single mutant clones, en^- ci^- double mutant clones of A origin straddled the A/P boundary (Figure 3A; Table 1). Unlike en^- single mutant P cells, however, en^- ci^- double mutant clones of P origin sorted only partially into A territory and straddled the A/P boundary (Figure 3B; Table 1), similar to en^- ci^- clones or ci^- clones originated in the A compartment. en^- ci^- clones of both A and P origin defined straight borders with neighboring wild-type A and P cells. en^- ci^- mutant clones also exhibit smooth borders with adjacent wild-type cells when situated entirely within the P compartment (Figure 3A). These results indicate that the complete sorting of posterior en^- clones into A territory depends indeed on the expression of Ci in these clones. However, because cells expressing neither En nor Ci segregate from P cells that also do not express Ci, it can be inferred that En controls cell segregation at the A/P boundary also in a Ci-independent manner.

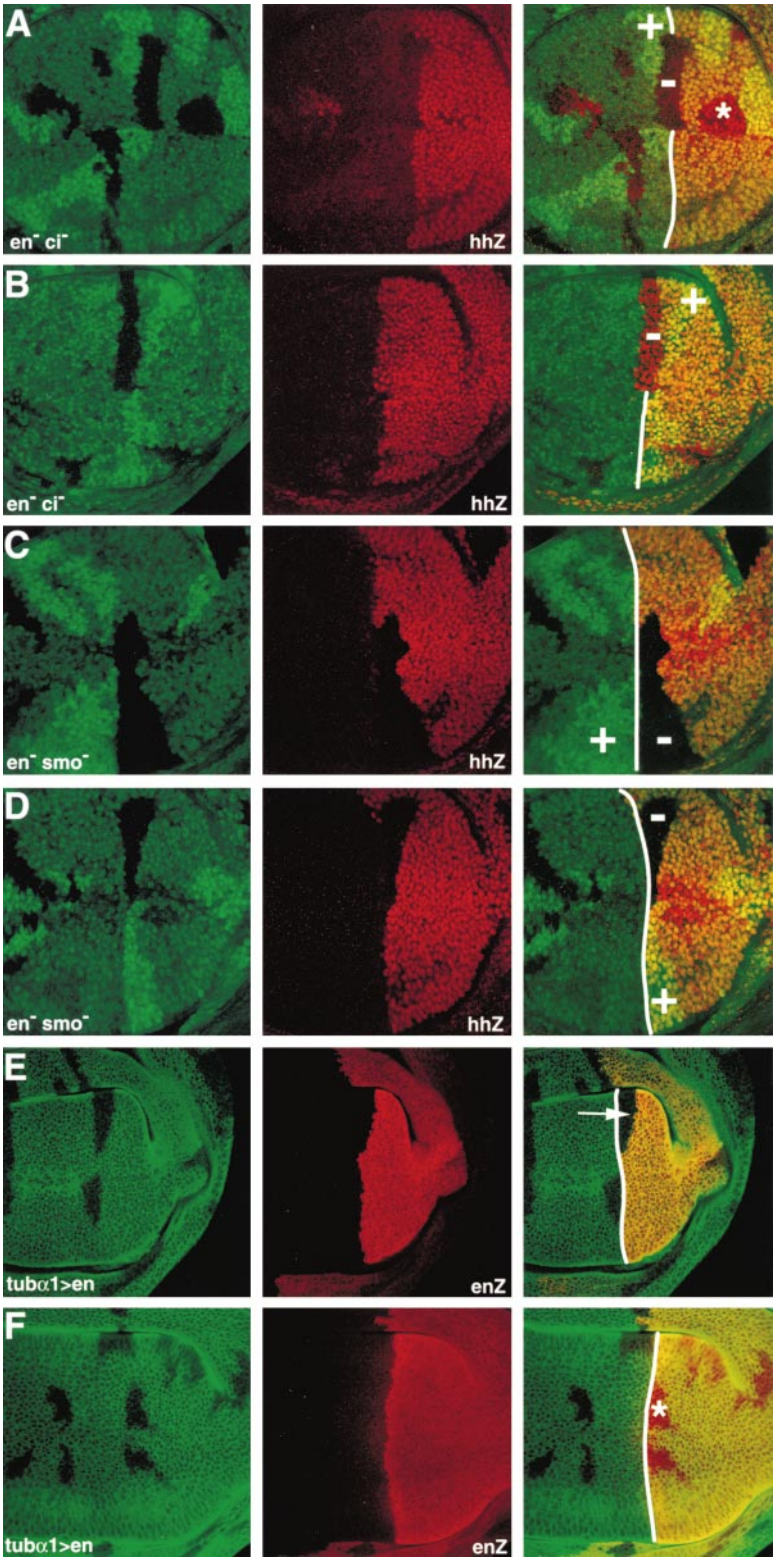

Figure 3. En Specifies P-Type Cell Segregation Independently of Ci

en⁻ *ci*⁻ homozygous clones (A and B) or *en*⁻ *smo*⁻ homozygous clones (C and D) are marked by the absence (−) of GFP staining, and the corresponding wild-type sister clones are marked by elevated levels (+) of GFP staining shown in green. Clones of cells expressing En at low levels (*tubα1>en*) are marked by the absence of CD2 staining shown in green (E and F). *hh-lacZ* and *en-lacZ* expressions are shown in red.

en⁻ *ci* clones of anterior (A) or posterior (B) origin take up positions overlapping the normal site of the A/P boundary. *en*⁻ *ci*⁻ clones have smooth borders to P cells ([A], asterisk). *en*⁻ *smo*⁻ clones of anterior (C) or posterior (D) origin occupy positions in the P territory and define straight borders with A cells at the normal site of the A/P boundary. Clones of cells of A origin expressing En at low level take up positions in the P territory ([E], arrow). P cells overexpressing En are confined to the P territory ([F], asterisk).

En Is Sufficient for P-Type Segregation in the Absence of Ci

The above experiments indicate that En is required for P cell segregation independent of Ci. To test whether En is also sufficient to confer P-type cell segregation to A cells, we expressed En in marked clones at low levels under control of the *tubulinα1* promoter and used *en-lacZ* as a marker for the compartmental origin of these clones. Clones derived from P cells were strictly confined to the territory of compartmental origin (Figure 3F). In contrast, among 16 clones derived from A cells at the boundary, 8 clones occupied positions normally taken

up only by P cells (Figure 3E). The levels of Ci were strongly reduced in these cells (data not shown), as expected from the repressive effects of En on *ci* transcription (Schwartz et al., 1995). We conclude that in the absence of Ci, En activity suffices to determine P-type segregation.

The Hh-Dependent Pathway Dominates Over the Hh-Independent Pathway

We have provided evidence that En acts both in a Hh-dependent and in a Hh-independent pathway to control cell segregation at the A/P boundary. To compare the relative contributions of these pathways, we analyzed the sorting behavior *of en*$^-$ cells that cannot transduce the Hh signal due to the lack of Smo activity (*en*$^-$ *smo*$^-$ cells). Unlike *en*$^-$ single mutant clones, *en*$^-$ *smo*$^-$ double mutant clones of P origin invariably occupied only P territory and defined straight borders to A cells at the normal position of the A/P boundary (Figure 3D; Table 1). Because *en*$^-$ cells remain in P territory in the absence of Hh transduction, we infer that the sorting of posterior *en*$^-$ clones into A territory critically depends on Hh signaling. From this and from experiments discussed above in which Ci was ectopically expressed in P cells, we conclude that the Hh-dependent pathway dominates over the Hh-independent pathway in its ability to influence the A/P sorting behavior of cells situated near the boundary.

Differences in Cell Adhesion Lead to Sorting Out In Vivo

Based on evidence presented above, we assume that both the Hh-dependent and -independent pathways control cell segregation at the transcriptional level. What are the target genes? It has been proposed that A and P cells segregate from each other due to differences in their adhesive strength (reviewed by Dahmann and Basler, 1999). To test whether such a mechanism could account for the sorting out of wing disc cells in vivo, we overexpressed the cell adhesion molecule DE-cadherin (Oda et al., 1994) from a transgene in clones of cells. As shown in Figure 4, control clones in which the levels of DE-cadherin are equal to those of surrounding cells form wiggly borders with neighboring tissue. Clones, however, with high DE-cadherin levels form smoother borders with their neighboring cells in both the A and P compartment ($P << 0.001$; 52 control and 44 DE-cadherin overexpressing clones, in 10 discs each, were analyzed), indicating that differences in the level of DE-cadherin lead to cell sorting. Furthermore, if in contact with each other, DE-cadherin overexpressing clones tended to fuse. As shown in Figures 4C and 4D, clones of different compartmental origins fused, overcoming even the normal segregation of A and P cells. It is thus conceivable that En and Ci could control cell segregation at the A/P boundary by regulating the transcription of a single cell adhesion gene.

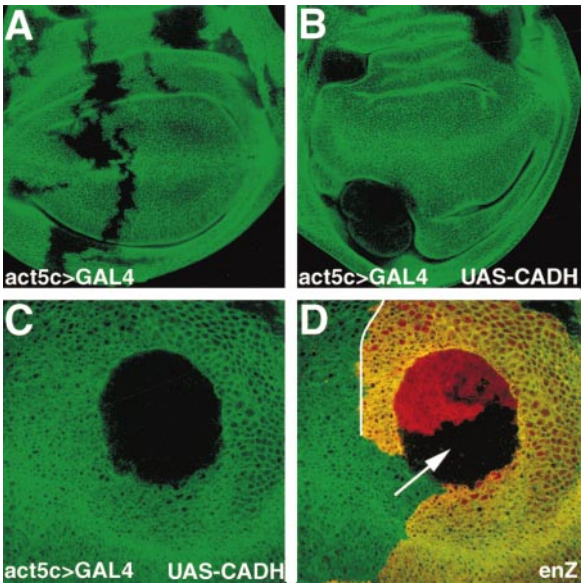

Figure 4. Differences in the Level of DE-Cadherin Lead to Sorting Out

Clones of cells expressing DE-cadherin at endogenous (A) or elevated levels ([B–D], *act5c>GAL4 UAS-CADH*) are marked by the loss of CD2 staining shown in green. *en-lacZ* expression is shown in red (D).

(A) Clones in which the levels of DE-cadherin are equal to those of surrounding cells form wiggly borders with neighboring tissue.

(B–D) Clones with high DE-cadherin levels form smooth borders with their neighboring cells both in the A and P compartment and tend to fuse with each other and interdigitate irrespective of the compartment boundary ([C and D], arrow).

Discussion

Cell segregation plays a fundamental role during animal development, yet how it is controlled is poorly understood. Here, we are concerned with the mechanisms regulating cell segregation at the A/P boundary of wing imaginal discs. Our results establish autonomous and direct roles for Ci[act] and En in specifying A and P cell segregation, respectively. We also provide evidence that Hh signaling is sufficient to specify A-type cell segregation and that it acts by shifting the balance between Ci[rep] and Ci[act] toward low levels of Ci[rep] and high levels of Ci[act]. We propose that the opposing transcriptional activities of Ci[act] and Ci[rep]/En lead to differences in the activity of a cell adhesion system at the boundary of A and P cells, thereby preventing these cell populations from intermingling.

Ci[Act] and En Autonomously Control Cell Segregation

The smooth and straight boundary between compartments has been ascribed to distinct adhesive properties of cells on opposite sides of the boundary causing these cell populations to minimize contact and sort out (Garcia-Bellido, 1975). In the case of the A/P boundary of the wing, one difference that could account for the distinct sorting behavior is the exclusive presence of two transcription factors, Ci[act] and En in adjacent A and P cells, respectively. For a long time, the view prevailed that En regulates cell segregation by autonomously and directly specifying P, as opposed to A, cell adhesiveness (Morata and Lawrence, 1975). This hypothesis has recently been challenged by two studies indicating that En acts, at least in part, by directing the expression of

Hh and that Hh secreted by P cells induces A cells to acquire a distinct cell adhesiveness (Blair and Ralston, 1997; Rodriguez and Basler, 1997). These studies, however, provided conflicting results as to whether or not En also had an autonomous, Hh-independent role in specifying cell segregation at the A/P boundary (see Lawrence, 1997). The same studies further raised, but did not address, the question of whether Hh signaling would specify cell segregation via its normal transduction pathway by leading to a transcriptional output depending on Ci. In various other systems, the activation of signaling receptors can lead to the posttranscriptional activation of small GTPases that can directly, without altering gene transcription, affect cytoskeletal components and thus conceivably cell adhesion (Van Aelst and D'Souza-Schorey, 1997). Key for us in addressing these questions was the choice assay. This assay allowed us to monitor whether altering the activity of a gene would change a cell's compartmental preference. Using this assay, we addressed the above questions by systematically considering three distinct situations (illustrated in Figure 5).

(1) Cells expressing neither Ci nor En

We first considered the "ground state" in which neither Ci nor En is present. Irrespective of their compartmental origin, clones of cells null mutant for both *ci* and *en* take up positions overlapping the normal site of the A/P boundary with smooth borders to wild-type A and P cells (Figures 3A and 3B). Because En is not required in A cells and because ci^- single mutant A cells behave like ci^- en^- double mutant A cells, we infer that Ci is required in A cells for their intermingling with other A cells at the compartment boundary. Since Ci acts in these cells as a transcriptional activator (Alexandre et al., 1996; Dominguez et al., 1996; Ohlmeyer and Kalderon, 1998; Méthot and Basler, 1999), we conclude that Hh signaling leads to a Ci-dependent transcriptional response in A cells and transcription of the immediate Hh target gene relevant for A segregation is induced, rather than repressed, in anterior boundary cells. The behavior of ci^- en^- double mutant clones also clarifies the role of En. Because clones of P cells lacking En and Ci form smooth borders with neighboring wild-type P cells that also lack Ci and, if in contact with A cells, sort partially into A territory, we infer that En has a function in specifying P segregation that is independent of Ci. Since Ci is required for all known responses to Hh signaling, we conclude that En has a Hh-independent role in determining P segregation. Our observation that clones of cells mutant for both *ci* and *en* occupy A and P territory to a similar extent leads us to conclude that Ci and En are required for most if not all aspects of the distinct segregation properties of A and P cells, and the difference between the ground state and the "A state" brought about by Ci[act] is similar to the difference between the ground state and the "P state" dependent on En.

(2) Cells expressing En but lacking Ci

A more direct argument for a Ci/Hh-independent role of En in the specification of cell sorting behavior can be derived from our experiment in which anterior clones were programmed to express low levels of En (*tub*α*1*>*en*). Such cells cease to express Ci and take up positions normally occupied only by P cells. The behavior of these cells is different from that of ground state cells that neither express Ci nor En (ci^- en^- double mutant cells, see above). In contrast to ci^- en^- cells, *tub*α*1*>*en* cells of A origin show a complete transgression to P territory, yet they do not intermingle well with P cells. We ascribe this latter observation to the unnaturally low levels of En produced in these cells (several-fold less than in wild-type P cells; data not shown). These levels may not repress *ci* completely and might not be sufficient to fully confer P cell adhesiveness.

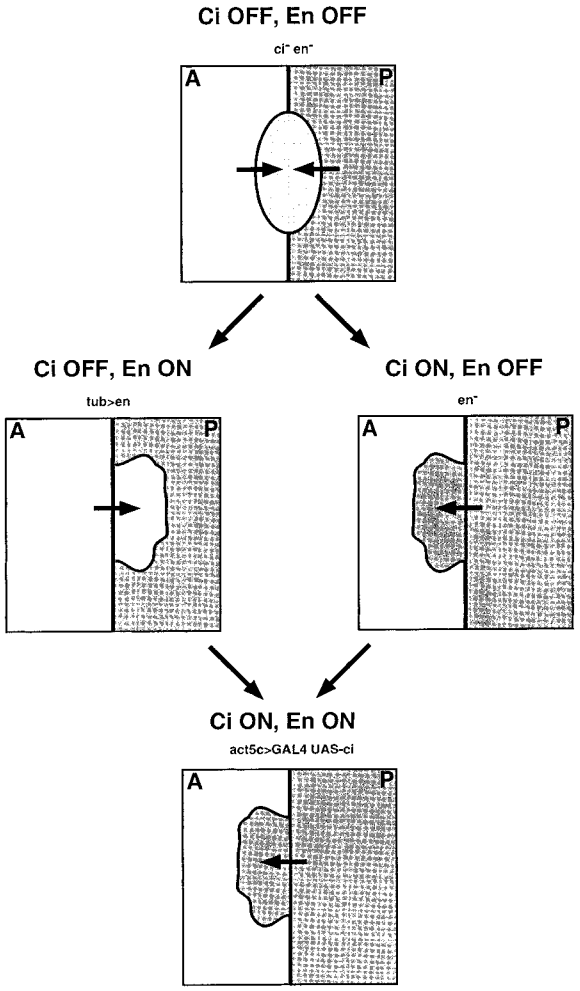

Figure 5. Scheme Illustrating the Opposing Roles of Ci and En in Separating A and P Cells

Clones of cells lacking both Ci and En sort out from A and P cells taking up positions overlapping the normal site of the A/P boundary, suggesting that Ci and En are required for most if not all aspects of the distinct segregation properties of A and P cells. Cells of A origin manipulated to express En and thus lacking Ci (*tub*>*en*) sort into P territory, indicating that En, in the absence of Ci, suffices to specify P-type sorting properties. Conversely, cells of P origin lacking En activity and hence expressing Ci (en^-) sort into A territory, indicating that Ci suffices to specify A sorting properties. Cells expressing both Ci and En sort into A territory, demonstrating the prevalence of the Hh-dependent pathway over the Hh-independent pathway of En.

(3) Cells expressing Ci but lacking En

Posterior clones of cells expressing Ci at physiological levels, but lacking En (mutant for en^E), take up positions in the territory normally only occupied by A cells and

intermingle with A cells. This behavior is dependent on Ci, since *ci*$^{-}$ *en*$^{-}$ double mutant clones of P origin only partially occupy A territory and sort out from A cells. Furthermore, overexpression of Ci in P cells leads these cells to sort out from neighboring P cells, and, if in contact with A cells, sort into A territory. Together, by comparing situations (1) to (3), we conclude that Ci is necessary and sufficient to specify A segregation, and, in the absence of Ci, En is necessary and sufficient to specify P segregation.

The Hh-Dependent Pathway of En Predominates Over the Hh-Independent Pathway

We have shown that En has an autonomous, Hh-independent role in specifying cell segregation. In addition, we have demonstrated that Ci is necessary and sufficient to specify A segregation. Ci is activated in anterior boundary cells by Hh whose P-specific expression is in turn controlled by En. Thus, En controls cell segregation at the A/P boundary both by a Hh-dependent as well as a Hh-independent pathway. To determine the relative contributions of these two pathways, we generated and analyzed situations in which En activity was altered under conditions of constant Hh signaling, or conversely, situations in which the activity of Hh signal transduction was altered under constant En conditions. From these experiments, we conclude that for the segregation behavior of wing cells the state of the Hh pathway prevails over that of En activity. This conclusion is particularly well corroborated by our finding that cells in which both pathways are simultaneously "on" (P cells expressing Ci) sort with A cells. The behavior of such cells may also explain why the late expression of *en* in anterior boundary cells (Blair, 1992) has no deleterious effects on the integrity of the compartment boundary. Like our experimental cells, these cells are exposed to the Hh signal coexpress *ci* and *en*, yet associate with other A cells rather than with En-expressing P cells (Figure 5).

Hh Controls A Cell Segregation through Both Forms of Ci

Ci is required in A cells for proper cell segregation at the A/P boundary. Depending on the status of the Hh signaling pathway, Ci can exist in two forms with opposing transcriptional activities (Ci[rep] and Ci[act]). We have previously found that these two forms of Ci regulate the expression of different subsets of Hh target genes, some of which appear to be regulated exclusively by Ci[rep] or Ci[act] (Méthot and Basler, 1999). Here, we argue that the A/P sorting of wing cells is under control of both forms of Ci. This conclusion is based on our findings that both Ci[rep] and Ci[act] have a profound influence on the segregation behavior of A cells.

Two observations show that Ci[rep] determines a preference for sorting into P territory. First, A cells expressing Ci[rep] in the absence of Ci[act] (*ci*^{Cell}/*ci*$^{-}$ mutants) or A cells overexpressing Ci[rep] in the presence of Ci[act] both take up positions occupied normally only by P cells. This is in contrast to cells lacking Ci entirely, which take up positions overlapping the normal position of the A/P boundary. Second, P cells lacking En but expressing Ci[rep] (*en*$^{-}$ *smo*$^{-}$ mutants) are confined to the P compartment, unlike cells that lack En and Ci or cells that only lack En. We infer from this that one important function of Hh signaling in its role of specifying A-type segregation properties is to prevent the formation of Ci[rep] in cells close to the A/P boundary.

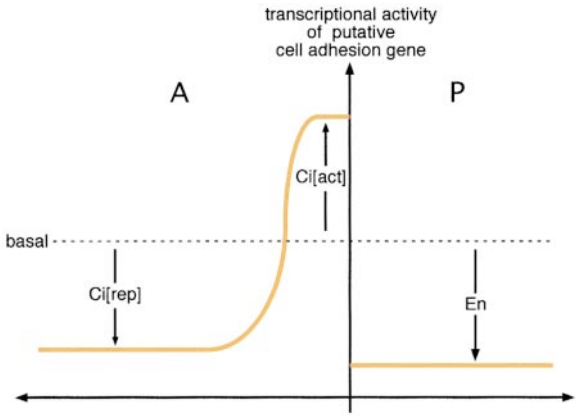

Figure 6. Model Illustrating How Ci[Rep], Ci[Act], and En Shape the Expression Profile of a Putative Cell Adhesion Gene

In the absence of Ci and En, cells segregate neither with A nor with P cells, indicating that they express a putative cell adhesion gene at an intermediate level (basal level) that is different from those in A or P cells. Since Ci[rep] can control cell segregation and is present in A cells far away from the boundary, the basal expression of this hypothetical gene may be downregulated by Ci[rep] in these cells. In A cells close to the boundary, Hh signaling prevents the formation of Ci[rep] and causes the formation of Ci[act], suggesting that in these cells the transcription of this target gene is upregulated. In P cells, En may repress this target gene, consistent with its role as a transcriptional repressor. We propose that the opposing transcriptional activities of Ci[act] and En lead to a large difference in the expression of this immediate target gene in cells on opposite sides of the A/P boundary.

The conclusion that not only prevention of Ci[rep] formation but also the induction of Ci[act] plays an important role in A/P sorting is deduced from the observation that cells lacking both forms of Ci do not mingle with wild-type A cells expressing Ci[act] due to their vicinity to the Hh source. Moreover, the addition of Ci to P cells, where Ci is readily converted to Ci[act], programs P cells to segregate with A cells.

Because Ci[rep] influences cell segregation, one might have expected that anterior *ci*$^{-}$ clones far away from the A/P boundary would sort out from neighboring Ci[rep]-expressing cells. However, *ci*$^{-}$ cells intermingle well with neighboring A cells. One likely explanation for this apparent discrepancy is the partial derepression of *hh* transcription in *ci*$^{-}$ mutant cells (Méthot and Basler, 1999). These low Hh levels induce in neighboring cells the formation of some Ci[act] that might neutralize remnant levels of Ci[rep]. In support of this assumption, we find that clones of cells double mutant for *ci* and *hh* do sort out at anterior positions (data not shown).

A Model for Transcriptional Regulation of Target Genes Controlling Cell Segregation

Ci and En are both DNA-binding proteins known to act as transcription factors, indicating that they control cell segregation by regulating the expression of target

genes. In analogy to *dpp*, a Hh target gene that is also controlled by En and both forms of Ci, we propose a model illustrating how Ci[rep], Ci[act], and En might shape the expression profile of a putative immediate target gene involved in cell segregation (Figure 6). Since in the absence of Ci and En, cells segregate neither with A nor with P cells, they are likely expressing an intermediate level of this gene (basal level) that is different from those in A or P cells. Since Ci[rep] can control cell segregation and is present in A cells far away from the boundary, we propose that the basal expression of this hypothetical gene is downregulated by Ci[rep] in these cells. In A cells close to the boundary, Hh signaling prevents the formation of Ci[rep] yet causes the formation of Ci[act], from which we infer that in these cells the transcription of this target gene is upregulated. In P cells, En may repress this target gene, consistent with its role as a transcriptional repressor (Jaynes and O'Farrell, 1991; Jimenez et al., 1997). We propose that the opposing transcriptional activities of Ci[act] and En lead to a large difference in the expression of this immediate target gene in cells on opposite sides of the A/P boundary.

In the above model, we assume that Ci and En control cell segregation by transcriptionally regulating one and the same gene, although it is also possible that they regulate different genes. While at present we cannot distinguish between these alternatives, we favor the simpler model that Ci and En control the same target gene for two reasons. First, as mentioned above, there is a precedent case for such a gene, *dpp*, which is known to be regulated by both Ci and En in a similar way as described above (Méthot and Basler, 1999). Second, a difference in the expression level of a single cell adhesion molecule (DE-cadherin) is sufficient for two cell populations to sort out (Steinberg, 1963; Friedlander et al., 1989; Steinberg and Takeichi, 1994; Figure 4). While it is conceivable that Ci and En directly regulate the expression of cell adhesion molecules like DE-cadherin, it is also possible that they act more indirectly by regulating genes whose products influence the activity of uniformly expressed cell adhesion molecules. Clones of cells lacking detectable amounts of DE-cadherin do sort out from neighboring wing disc cells; they are, however, exclusively confined to the compartment of origin (data not shown) indicating that DE-cadherin is not required for the separation of cells at the A/P boundary.

Why does cell segregation at the A/P boundary require two transcription factors with opposing activities? Based on our results, the differential activities of either Ci or En suffices for separating A and P cells. For Ci, this is best illustrated by the key finding that P cells forced to express Ci sort out from wild-type P cells and segregate into A territory. Conversely, we have shown that in the absence of Ci, expression of En suffices for A cells to sort into P territory. The use of two transcription factors with opposing activities may have the advantage of increasing the fidelity of the sorting process by further contrasting the expression levels of a common putative target gene in cells of opposite sides of the A/P boundary.

It seems to be a general mechanism that En controls cell segregation both in a Hh-dependent and -independent manner. In the *Drosophila* abdomen, En has also been implicated to control separation of A and P cells in Hh-dependent and -independent ways (Lawrence et al., 1999). The relative contributions of these two functions of En, however, appear to differ between the wing imaginal discs and the abdomen. While we find a prevalence of the Hh-dependent pathway in the wing disc, the two functions of En seem to contribute equally to the separation of abdominal A and P cells (Lawrence et al., 1999). This difference in dominance of the Hh-signal transduction pathway might be due to a more influential role of Ci[rep] in sorting of imaginal versus abdominal cells.

It is intriguing to notice that the same intricate network that defines the strip of cells expressing Dpp also appears to restrict the activity of a putative cell adhesion molecule to the very same cells. The use of Hh/En signaling for both setting up the Dpp organizer and segregating A and P cells may ensure that the position and shape of the morphogen source that organizes both compartments is stably maintained during development. Our prediction of a *dpp*-like expression pattern provides a novel criterion for the future identification of the elusive molecules conferring cell segregation.

Experimental Procedures

Transgenes

The *tubα1>CD2,smo⁺>hh* construct was generated by replacing the y^+ gene of *tubα1>CD2,y⁺>hh* (Zecca et al., 1995) with a 6.6 kb HindIII fragment of clone Y11–7 (Alcedo et al., 1996) containing the *smo* genomic sequence. The *UAS-ci* transgene was made by inserting a cDNA containing the full-length coding region of *ci* into *pUAST* (Brand and Perrimon, 1993). Reporter genes used in this study were hh^{P30} (Lee et al., 1992) and *enZ* (Hama et al., 1990). Additional transgenes used were *tubα1>CD2,y⁺>en* (Zecca et al., 1995), *act5c>CD2>GAL4* (Pignoni and Zipursky, 1997), P[smo^+, *hsp70-GFP*] (Méthot and Basler, 1999), *UAS-cadh* (Sanson et al., 1996), and *UAS-ciCell* (Méthot and Basler, 1999).

Marked Clones of Mutant Cells

Clones of mutant cells were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993), subjecting second instar larvae to a 33°C–35°C heat-shock for 30 min. Genotypes of the larvae were as follows:

- smo^-; tub>hh:
  y w hsp70-flp; smo^3 tubα1>CD2, smo^+> hh/smo^3
- ci^- clones, hhZ:
  y w hsp70-flp; FRT42 P[ci^+] hsp70-GFP/FRT42; hh-lacZ/+; ci^{94}/ci^{94}
- ci^{Cell}/ci^- clones, hhZ:
  y w hsp70-flp; FRT42 P[ci^+] hsp70-GFP/FRT42; hh-lacZ/+; ci^{Cell}/ci^{94}
- ci^- en^-, hhZ
  y w hsp70-flp; FRT42 P[ci^+] hsp70-GFP/FRT42 Df(2R)en^E; hh-Z/+ ci^{94}/ci^{94}
- smo^- en^- clones, hhZ:
  y w hsp70-flp; smo^3 FRT42 P[smo^+,hsp70-GFP]/smo^3 FRT42 Df(2R)en^E; hh-lacZ/+
- UAS-ci clones, enZ
  y w hsp70-flp; UAS-ci/en-lacZ; act5c>CD2>GAL4
- UAS-ci^{Cell} clones, enZ
  y w hsp70-flp; UAS-ciCell/en-lacZ; act5c>CD2>GAL4
- UAS-cadh clones, enZ
  y w hsp70-flp; UAS-cadh/en-lacZ; act5c>CD2>GAL4
- tubα1>en clones, enZ
  y w hsp70-flp; en-lacZ/+; tubα1>CD2,y^+>en/+

Immunohistochemistry

Imaginal discs dissected from late third instar larvae were fixed and stained with appropriate antibodies to mark clones and monitor reporter gene expression. Antibodies were rat monoclonal anti-Ci 2A1 (gift from R. Holmgren), mouse monoclonal anti-GFP (Clontech), mouse monoclonal anti-CD2 OX34 (Serotec), and rabbit polyclonal anti-βGAL (Cappel).

Measurements and Statistics

Wing imaginal discs were recorded on a Leica confocal microscope. The perimeters of the clones were traced with the help of the free-hand selection tool of the NIH Image v. 1.61 program. The area (A) and perimeter (L) of each clone were measured. A measure of the shape of the clones ($4\pi A/L2$) was used (Lawrence et al., 1999). The t-test of the difference between two means (Sokal and Rohlf, 1995) was carried out to determine if the shape of two different groups of clones differs.

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