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The role of Dpp signaling in maintaining the *Drosophila* anteroposterior compartment boundary

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Abstract

The subdivision of the developing *Drosophila* wing into anterior (A) and posterior (P) compartments is important for its development. The activities of the selector genes *engrailed* and *invected* in posterior cells and the transduction of the Hedgehog signal in anterior cells are required for maintaining the A/P boundary. Based on a previous study, it has been proposed that the signaling molecule Decapentaplegic (Dpp) is also important for this function by signaling from anterior to posterior cells. However, it was not known whether and in which cells Dpp signal transduction was required for maintaining the A/P boundary. Here, we have investigated the role of the Dpp signal transduction pathway and the epistatic relationship of Dpp and Hedgehog signaling in maintaining the A/P boundary by clonal analysis. We show that a transcriptional response to Dpp involving the T-box protein Optomotor-blind is required to maintain the A/P boundary. Further, we find that Dpp signal transduction is required in anterior cells, but not in posterior cells, indicating that anterior to posterior signaling by Dpp is not important for maintaining the A/P boundary. Finally, we provide evidence that Dpp signaling acts downstream of or in parallel with Hedgehog signaling to maintain the A/P boundary. We propose that Dpp signaling is required for anterior cells to interpret the Hedgehog signal in order to specify segregation properties important for maintaining the A/P boundary.

Keywords: *Drosophila*; Imaginal disc; Compartment boundary; Cell segregation; Decapentaplegic; Optomotor-blind; T-box

Introduction

Cell adhesion is fundamental for the development of multicellular organisms. However, cells do not simply adhere to one another randomly. For example, when disaggregated frog embryos were allowed to reaggregate, cells segregated out and reestablished the layers to which they initially belonged (Townes and Holtfreter, 1955). This property of cells to selectively aggregate with some cells and to segregate out from others was termed cell affinity (Garcia-Bellido, 1966, 1972; Holtfreter, 1939). The underlying cell biological mechanisms of this cell behavior and the molecular nature of cell affinity remain poorly understood.

One system for studying the mechanisms underlying the segregation of cells during development is the formation of lineage boundaries that subdivide a number of vertebrate and insect tissues into groups of non-intermingling cells termed compartments (Blair, 2003; Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; McNeill, 2000; Tepass et al., 2002; Vincent, 1998). Signaling across boundaries between adjacent compartments can lead to the local production of long-range signaling molecules that organize growth and patterning of the entire tissue (Lawrence and Struhl, 1996). The continuous segregation of cells at compartment boundaries is therefore important for the positioning and maintenance of such organizers and is crucial for the patterning of tissues.

The developing *Drosophila* wing is subdivided by two compartment boundaries. An early-arising compartment boundary separates anterior (A) and posterior (P) cells and a late-arising compartment boundary separates dorsal (D)

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and ventral (V) cells (Bryant, 1970; Garcia-Bellido and Merriam, 1971; Garcia-Bellido et al., 1973). It was originally proposed that the segregation of cells at these two compartment boundaries depends on compartment-wide cell affinities controlled by the activity of selector genes (Garcia-Bellido, 1975; Garcia-Bellido et al., 1973). However, more recently, it has become clear that signaling across compartment boundaries is at least equally important for segregating cells at these boundaries.

The homeobox transcription factors encoded by *engrailed* (*en*) and *invected* (*inv*) are expressed in P cells and act as selector genes for the P compartment (Brower, 1986; Coleman et al., 1987; Kornberg et al., 1985; Lawrence and Morata, 1976; Morata and Lawrence, 1975; Poole et al., 1985). Clonal analysis has shown that P cells lacking En and Inv activity often no longer segregate at the A/P boundary with P cells, but instead intermingle with A cells (Blair and Ralston, 1997; Hidalgo, 1994). Conversely, A cells ectopically expressing En, if in contact with P cells, segregate into the P territory (Dahmann and Basler, 2000). En regulates cell segregation mainly by controlling the signaling of the secreted molecule Hedgehog (Hh). In P cells, En both facilitates the expression of Hh and represses the transcription of the Zn-finger transcription factor Cubitus interruptus (Ci), an essential component of the Hh signal transduction pathway (Dominguez et al., 1996; Eaton and Kornberg, 1990; Tabata et al., 1992). Thus, P cells produce Hh but cannot respond to it. In contrast, A cells express Ci and can respond to Hh secreted from P cells. One response to this unidirectional signaling of Hh from P to A cells is the specification of an A cell affinity required to maintain the segregation of cells at the A/P boundary. Anterior cells lacking the function of the seven-pass transmembrane protein Smoothened (Smo), and hence the ability to transduce the Hh signal (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), no longer segregate with A cells but instead segregate into P territory (Blair and Ralston, 1997; Rodriguez and Basler, 1997). This control of cell segregation by Hh signaling requires the transcription factor Ci, indicating that Hh controls A/P cell segregation by regulating the transcription of target genes (Dahmann and Basler, 2000). Hh signaling is not only necessary, but also sufficient to control cell segregation. P cells ectopically expressing Ci, and thus activating the Hh pathway, segregate into the A territory (Dahmann and Basler, 2000). Recently, two subunits of the *Drosophila* mediator complex, Skuld (Skd) and Kohtalo (Kto), have been shown to be required for the normal segregation of cells at the A/P boundary (Janody et al., 2003). It has been proposed that Skd and Kto assist Ci to regulate some of its target genes, including those involved in cell segregation. Despite several efforts (e.g., Vegh and Basler, 2003), Hh target genes required for the segregation of cells at the A/P compartment boundary have not been identified.

Signaling across the A/P boundary is also bidirectional. In response to Hh, a narrow stripe of cells along the A side of the A/P boundary produces the long-range signaling molecule Decapentaplegic (Dpp), a member of the TGFβ superfamily (Masucci et al., 1990; Padgett et al., 1987). Dpp acts as a morphogen by specifying cell fates in both compartments along the A/P axis in a concentration-dependent manner (Lecuit et al., 1996; Nellen et al., 1996). To direct precise patterning, the shape of the source of the Dpp morphogen must be stably maintained and the continuous segregation of cells at the A/P boundary may contribute to this.

Dpp signals through a Ser/Thr kinase receptor complex including the type I and II receptors Thickveins (Tkv) and Punt, respectively (Brummel et al., 1994; Letsou et al., 1995; Nellen et al., 1994; Penton et al., 1994; Ruberte et al., 1995). The binding of Dpp to its receptors induces Punt to phosphorylate Tkv which in turn phosphorylates the transcription factor Mothers against dpp (Mad) (Raftery and Sutherland, 1999; Tanimoto et al., 2000). Phosphorylated Mad enters the nucleus and, in concert with the Zn-finger protein Schnurri (Arora et al., 1995; Grieder et al., 1995), represses the transcriptional repressor Brinker (Brk) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999; Muller et al., 2003). As a consequence, the extracellular Dpp gradient is converted into an inverse gradient of a transcriptional repressor. Brk, in a concentration-dependent manner, negatively controls the expression of Dpp target genes including *spalt-major* (*salm*), *spalt-related* (*salr*) (two neighboring and functionally related genes referred to in the following as *sal*), and *optomotor blind* (*omb*), which encodes a member of the T-box family of transcription factors (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999; Pflugfelder et al., 1992). As a consequence, *sal* and *omb* are expressed in nested regions centered around the Dpp expression domain with the *omb* expression domain being broader than the *sal* expression domain (Grimm and Pflugfelder, 1996; Sturtevant et al., 1997).

The current model presented above assumes that signals controlling A/P cell segregation are exclusively unidirectional from P to A cells. Based on the findings that A cells signal back to P cells via Dpp (Lecuit et al., 1996; Nellen et al., 1996) and that wings from flies hypomorphic for *dpp* have a distorted A/P boundary (Hidalgo, 1994), it has been proposed that A and P cells are specified for their segregation behavior by P to A and A to P signaling, respectively, and that Dpp might be the A to P signal involved (Blair and Ralston, 1997; Vincent, 1998). However, it was not known whether and in which cells Dpp signal transduction is required for maintaining the segregation of cells at the A/P boundary. Here, we have addressed these questions by analyzing the segregation of marked clones of cells unable to transduce the Dpp signal at the A/P boundary. We find that an Omb-mediated transcriptional response to Dpp is required in A cells but not in P cells to maintain the A/P boundary. Thus, our results do not support the proposal that Dpp signaling from A to P cells is required

for maintaining the A/P boundary. Instead, our results suggest that an Omb-mediated transcriptional response to the Dpp signal is acting within the Dpp-producing A cells to maintain the A/P boundary.

Materials and methods

Drosophila stocks

The following deficiencies and mutant alleles were used: Df(2L)flp147E (a deficiency removing *bsk*), tkv^{a12}, mad^{B1} and mad^{12}, bks^{1} and bks^{2} (mutants in *mtv*), Df(2L)32FP5 (a deficiency removing *salm* and *salr*), omb^{3198}, and brk^{XH} (see http://flybase.bio.indiana.edu for description). Enhancer trap lines used in this study were hh^{P30} (Lee et al., 1992) and *hh-GAL4* (a gift from K. Basler). Transgenes used were *tubα1-mad* (Marty et al., 2000), *UAS-ci* (Dahmann and Basler, 2000), *UAS-tkv* (a gift from K. Basler), *UAS-GFP* (a gift from K. Basler), *tub>CD2>GAL4* (a gift from L. Zipursky, provided by K. Basler), and *tubP-GAL80* (Lee and Luo, 2001).

Clonal analysis

Marked clones of mutant cells were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993) subjecting first instar larvae to a 36–38°C heat-shock for 30 min. Transgenes were expressed using the GAL4-UAS system (Brand and Perrimon, 1993). Genotypes of the larvae were as follows:

bsk⁻: y w hsp70-Flp; Df(2L)flp147E FRT40/ubi-GFP FRT40
tkv⁻ *bsk*⁻: y w hsp70-Flp; tkv^{a12} Df(2L)flp147E FRT40/ubi-GFP FRT40
mad⁻ *bsk*⁻: y w hsp70-Flp; mad^{B1} Df(2L)flp147E FRT40/ubi-GFP FRT40
mad⁻ *brk*⁻: *tubα1-Mad* hsp70-GFP FRT18/brk^{XH} FRT18; mad^{B1}/mad^{12} hsp70-Flp
sal⁻: y w hsp70-Flp; Df(2L)32FP5 FRT40/πmyc FRT40; hh^{P30}/+
omb⁻: y w hsp-GFP hsp70-Flp FRT19/omb^{3198} sn FRT19; hh^{P30}/+
hh-GAL4 UAS-GFP: y w hsp70-Flp FRT19/hsp70-Flp tubP-GAL80 FRT19; hh-GAL4/UAS-GFP
hh-GAL4 UAS-ci UAS-GFP: y w hsp70-Flp FRT19/hsp70-Flp tubP-GAL80 FRT19; hh-GAL4/UAS-ci UAS-GFP
omb⁻ *hh-GAL4 UAS-ci UAS-GFP*: y w omb^{3198} sn FRT19/hsp70-Flp tubP-GAL80 FRT19; hh-GAL4/UAS-ci UAS-GFP
tub > GAL4 UAS-tkv: y w hsp70-Flp; tub>CD2>GAL4/UAS-tkv
mtv⁻: y w hsp70-Flp; bks^{2} FRT42/hsp-CD2 FRT42; hh^{P30}/+

Immunohistochemistry

Imaginal discs dissected from late third instar larvae were fixed and stained with appropriate antibodies to mark clones and monitor reporter gene expression. Primary antibodies used were rat monoclonal anti-Ci 2A1, 1:4 (gift from R. Holmgren), mouse monoclonal anti-Ptc, 1:50 (gift from I. Guerrero), rabbit polyclonal anti-GFP, 1:2000 (Clontech), mouse monoclonal anti-c-Myc 9E10, 1:1500 (Santa Cruz), mouse monoclonal anti-β-Gal, 1:2000 (Promega), rabbit polyclonal anti-β-Gal, 1:2000 (Cappel), mouse monoclonal anti-CD2 OX34, 1:2000 (Serotec), and rabbit polyclonal anti-pMad, 1:1500 (gift from P. ten Dijke). Secondary antibodies (diluted 1:200) used were anti-mouse Alexa 488, anti-mouse Alexa 594, anti-rabbit Alexa 488, anti-rabbit Alexa 594 (Molecular Probes), and anti-rat Texas Red (Jackson Immuno Research). Images were recorded on a LSM510 Zeiss confocal microscope.

Measurements and statistics

To quantify the segregation of clones at the A/P boundary, the position of the A/P boundary was determined by Ci or *hh-lacZ* staining and the outlines of clones were traced using the freehand selection tool of the NIH Image v. 1.61 program. The total area of a clone and the area of the clone outside the normal territory of the compartment of origin (misplaced area) were measured. The ratio of misplaced to total area of a clone expressed as a percent was calculated as a measure for the misplacement of clones. Only clones in direct contact with the A/P boundary were analyzed. To quantify the shape of clones, the area (A) and perimeter (L) of each clone were determined. To measure the shape of the clones, the formula $4\pi A/L^2$ 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 there was a significant difference between two sets of data.

Results

Experimental strategy

To test the role of Dpp signal transduction components in maintaining the segregation of cells at the A/P boundary, we generated genetically marked clones of cells using the Flp-FRT system (Xu and Rubin, 1993). Clones in the wing pouch region of the wing imaginal disc in contact with the A/P boundary were then assayed for their segregation behavior. Mutant clones may fully remain in the compartment in which they have been made, as wild-type clones would do. Alternatively, mutant clones may be partially or completely misplaced into the territory of the adjacent compartment, indicating that the mutation interfered with normal cell segregation. The compartment in which a clone

was generated was determined by the position of a sibling clone composed of wild-type cells. The position of the A/P boundary was inferred from the A-specific expression of Ci or the P-specific activity of the *hh-lacZ* enhancer trap line.

Dpp signal transduction is required in A cells to maintain the A/P boundary

Wings from flies hypomorphic for *dpp* have a partially distorted A/P boundary (Hidalgo, 1994), raising the possibility that Dpp signal transduction plays a role in maintaining the A/P boundary. To test whether and in which cells Dpp signal transduction is required for the segregation of A and P cells, we analyzed the segregation of clones of cells lacking the ability to transduce the Dpp signal at the A/P boundary. The type I Dpp receptor Tkv is essential for Dpp signal transduction (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994). However, within the wing pouch region of the wing disc, cells mutant for *tkv* undergo c-Jun amino-terminal kinase (JNK)-mediated apoptosis (Adachi-Yamada and O'Connor, 2002; Adachi-Yamada et al., 1999; Burke and Basler, 1996) and, thus, large tkv^- clones cannot be recovered. To obtain large clones, we generated clones double mutant for *tkv* and *Drosophila* JNK, encoded by *basket* (*bsk*) (Riesgo-Escovar et al., 1996; Sluss et al., 1996). As a control, we first analyzed the segregation of bsk^- single mutant clones at the A/P boundary. Both A ($n = 26$) and P ($n = 23$) bsk^- clones remained entirely in the compartment in which they had been generated (Figs. 1A–C), showing that *bsk* is not required to maintain the A/P boundary. In contrast, tkv^-bsk^- double mutant clones of A origin displaced the A/P boundary. In 1187 wing imaginal discs analyzed, 46 tkv^-bsk^- clones were found to be at the A/P boundary. Twenty-one clones were of P origin, the majority of which remained fully within the P compartment (Figs. 1E and F). In contrast, in 68% of the A origin clones ($n = 25$), at least 40% of the clonal area was misplaced into the P territory displacing the compartment boundary toward P (Figs. 1D and F). The propensity of A and P clones to displace the A/P boundary differed significantly ($P < 0.001$). These results suggest that A cells require Tkv activity for maintaining the A/P boundary.

Dpp target genes are involved in A/P cell segregation

The experiment described above indicated that a response of A cells to the Dpp signal is required for maintaining the A/P boundary. To test whether Dpp signal transduction controls cell segregation by regulating the transcription of one or several target genes, we tested the role of the transcription factor Mad (Raftery et al., 1995; Sekelsky et al., 1995), which is essential for Dpp signal transduction, in the segregation of cells at the A/P boundary.

Similar to tkv^- clones, clones of cells homozygous mutant for *mad* poorly survive in the pouch region of the wing imaginal disc. Thus, we used the same strategy as for obtaining large tkv^- clones and generated mad^-bsk^- double mutant clones. We analyzed 1121 wing imaginal discs and found 29 mad^-bsk^- clones at the A/P boundary. Fourteen clones were of P origin, the majority of which remained fully within the P compartment (Figs. 1H and I). In contrast, in 67% of A origin clones ($n = 15$), at least 40% of the clonal area was misplaced into the P territory, displacing the A/P boundary toward P (Figs. 1G and I). The propensity of clones from A and P origins to displace the A/P boundary differed significantly ($P < 0.001$). The segregation behavior of A tkv^-bsk^- clones and A mad^-bsk^- clones at the A/P boundary was not significantly different ($P > 0.05$). We conclude that the Dpp signal controlling cell segregation is for the most part, if not exclusively, transduced by the transcription factor Mad and thus involves a transcriptional response.

Loss of Dpp signal transduction in A boundary cells does not reduce Hh signal transduction

We have shown above that clones of A cells lacking either Tkv or Mad activity displace the A/P boundary. Likewise, A cells lacking the ability to respond to the Hh signal segregate out from other A cells and displace the A/P boundary (Blair and Ralston, 1997; Rodriguez and Basler, 1997). We therefore tested whether the displacement of the A/P boundary by tkv^-bsk^- clones was due to a reduced level of Hh signal transduction in these cells. A universal read-out for Hh signal transduction is the expression of the Hh-target gene *patched* (*ptc*) (Bijlsma et al., 2004). We therefore generated tkv^-bsk^- clones and analyzed the level of Ptc protein in A clones at the A/P boundary. As shown in Fig. 2B, A tkv^-bsk^- clones appeared to have normal levels of Ptc protein, indicating that the level of Hh signaling is not reduced in these cells. Likewise, the level of Ptc was not significantly reduced in A mad^-bsk^- clones (Fig. 2C). We conclude that Dpp signal transduction is not required to maintain Hh signal transduction and that the displacement of the A/P boundary by A tkv^-bsk^- and mad^-bsk^- clones is not due to a reduction of Hh signaling in these cells.

The repression of Brinker by Mad is required for maintaining the A/P boundary

Because the transcription factor Mad is required in A cells to maintain the normal position of the A/P boundary, we investigated the role of further downstream Dpp signal transduction components in maintaining the A/P boundary. In response to Dpp signaling, Mad represses the transcription of *brk* (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999), a gene encoding a transcriptional repressor. Brk, in turn, represses in a concentration-dependent manner the transcription of Dpp target genes and thereby shapes their expression domains (Muller et al., 2003). Cells mutant for either *tkv* or *mad* derepress *brk* (Jazwinska et al., 1999). To test whether the displacement of the A/P boundary

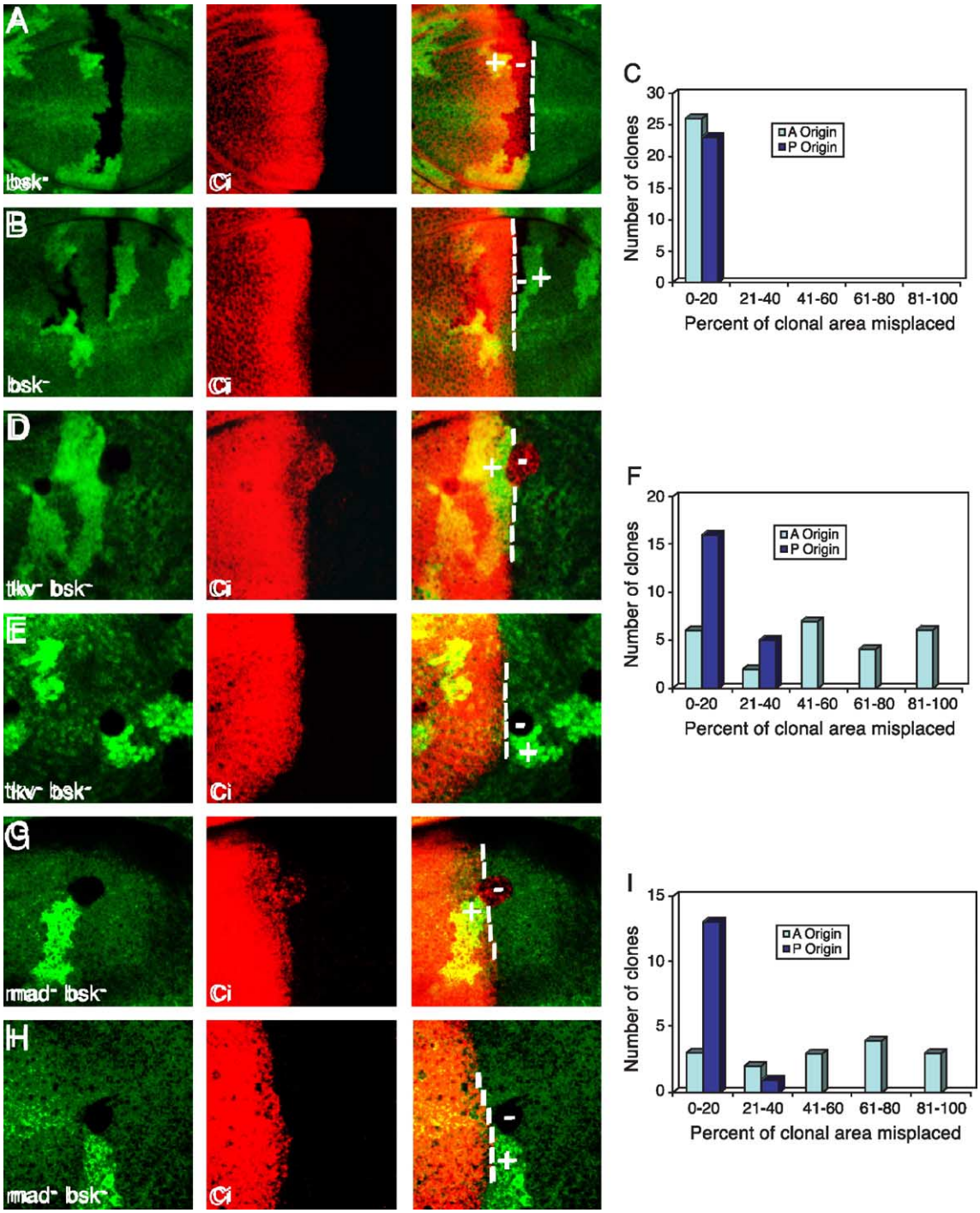


Fig. 1. *tkv* and *mad* are required to maintain the A/P boundary. Clones of cells homozygous for bsk^- (A and B), $tkv^- bsk^-$ (D and E), and $mad^- bsk^-$ (G and H) are marked by the absence (−) of GFP staining in green (left column). The wild-type sister clones are marked by the high levels of GFP staining (+). Ci staining is shown in red (middle column). The merge of both stainings is shown in the right column. (A) A bsk^- clone of A origin (as judged by the Ci staining and the position of sister clone) is strictly confined to the A compartment. (B) A bsk^- clone of P origin (as judged by the absence of Ci staining and the position of sister clone) is confined to the P territory. $tkv^- bsk^-$ (D) and $mad^- bsk^-$ (G) clones of A origin displace the A/P boundary toward P. $tkv^- bsk^-$ (E) and $mad^- bsk^-$ (H) clones of P origin are confined to the P compartment. In this and subsequent figures, third instar wing imaginal discs are shown with the anterior to the left and dorsal up. The white dashed line marks the normal position of the A/P boundary. Quantification of the area that bsk^- (C), $tkv^- bsk^-$ (F), and $mad^- bsk^-$ (I) clones located at the A/P boundary were misplaced into the adjacent compartment expressed as a percent.

by A $mad^- bsk^-$ clones was due to the derepression of *brk*, we analyzed the segregation of clones double mutant for *mad* and *brk* at the A/P boundary. $mad^- brk^-$ clones of A origin remained entirely in the A compartment (Figs. 3A and C). The segregation behavior of A $mad^- bsk^-$ clones and A $mad^- brk^-$ clones at the A/P boundary was significantly

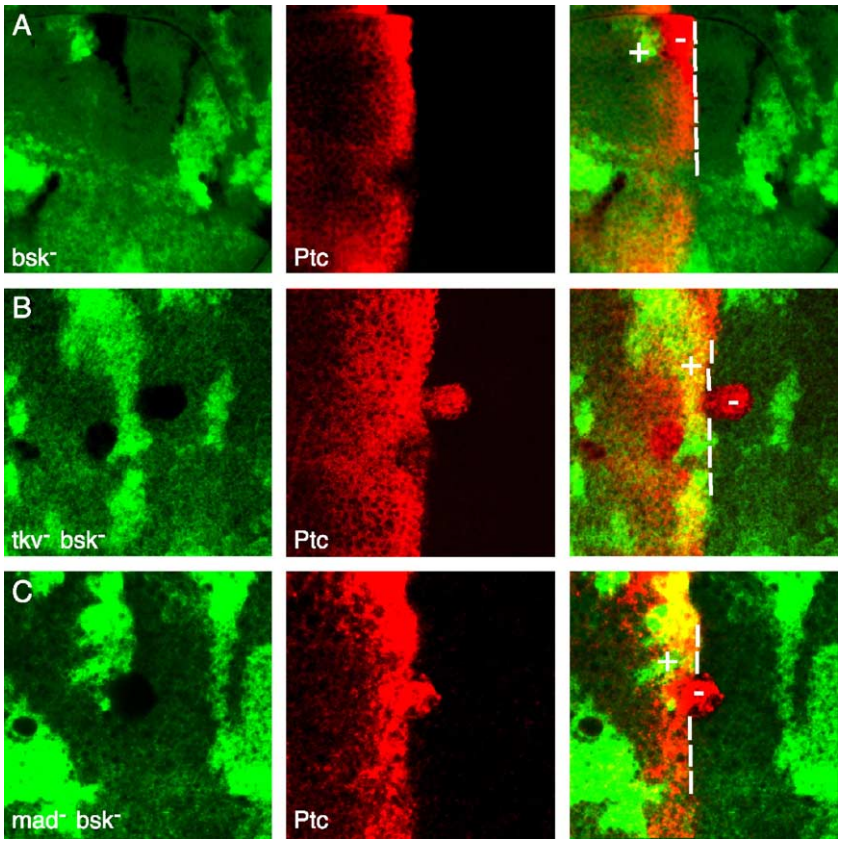


Fig. 2. Anterior *tkv*$^{-}$*bsk*$^{-}$ and *mad*$^{-}$*bsk*$^{-}$ cells at the A/P boundary transduce the Hh signal at a normal level. *bsk*$^{-}$ (A), *tkv*$^{-}$*bsk*$^{-}$ (B), and *mad*$^{-}$*bsk*$^{-}$ (C) clones of cells are marked as in Fig. 1. Antibody staining against Patched (Ptc), a marker for Hh signal transduction, is shown in red. (A–C) Clones of A origin and located in the vicinity of the A/P boundary show a normal Ptc staining.

different ($P < 0.001$). Thus, the derepression of *brk* is required for A *mad*$^{-}$*bsk*$^{-}$ clones to displace the A/P boundary. This suggests that ectopic expression of Brk in A cells at the A/P boundary interferes with their normal segregation at the A/P boundary.

The Dpp target gene omb is required to maintain the A/P boundary

The expression of Brk is normally confined to cells at the periphery of the wing disc where it acts to repress Dpp target genes (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Derepression of Brk in the central region of the wing disc in *mad* or *tkv* mutant clones leads to the repression of Dpp target genes like *sal* and *omb* (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Marty et al., 2000). This raises the possibility that the displacement of the A/P boundary by *mad*$^{-}$*bsk*$^{-}$ clones is due to the loss of expression of *sal* and/or *omb*. To test these possibilities, we generated clones of cells mutant for either *sal* or *omb* and analyzed their segregation at the A/P boundary. *sal*$^{-}$ clones of both A and P origin remained largely in the compartment of origin (Figs. 4A, B, and G). In contrast, *omb*$^{-}$ clones of A, but not P, origin were partly misplaced into the territory of the adjacent compartment displacing the A/P boundary (Figs. 4C–F and H). The displacement of the boundary was observed using markers for the A compartment (Ci, Fig. 4C) and P compartment (*hh-lacZ*, Fig. 4E). The frequency and extent of the displacement of the A/P boundary were not significantly different between A *omb*$^{-}$ and A *mad*$^{-}$*bsk*$^{-}$ clones ($P > 0.05$), indicating that Omb mediates most, if not all, aspects of Mad-controlled cell segregation at the A/P boundary.

Omb is required for Ci to specify A-type cell segregation

Previous work has shown that a Ci-mediated response to the Hh signal is required in A cells to segregate from P cells (Dahmann and Basler, 2000). Here, we show that, in addition, an Omb-mediated response to Dpp signaling is required for normal A/P cell segregation. What is the epistatic relationship between Ci and Omb in this process? The failure of A *omb*$^{-}$ clones to properly maintain the position of the A/P boundary suggests that Omb might be required for Ci to specify A-type cell segregation. Ci expression is normally confined to A cells. When Ci is ectopically expressed in P clones at the A/P boundary, these clones segregate out from neighboring P cells and intermingle with A cells, indicating that Ci is sufficient to specify A-type cell segregation (Dahmann and Basler,

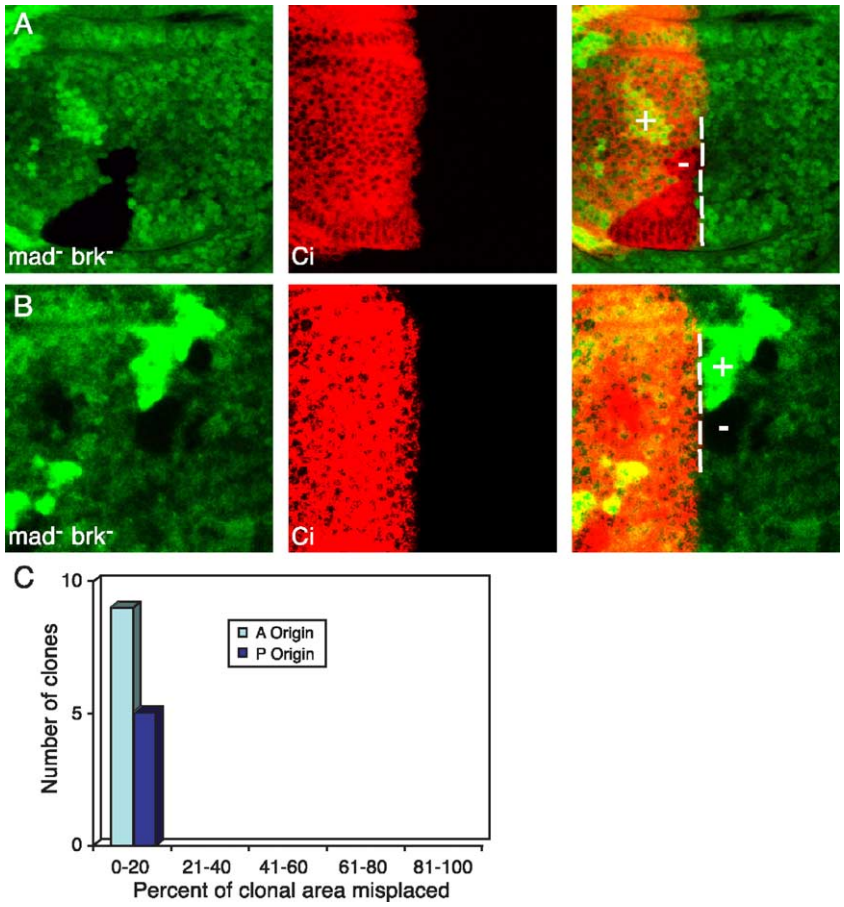
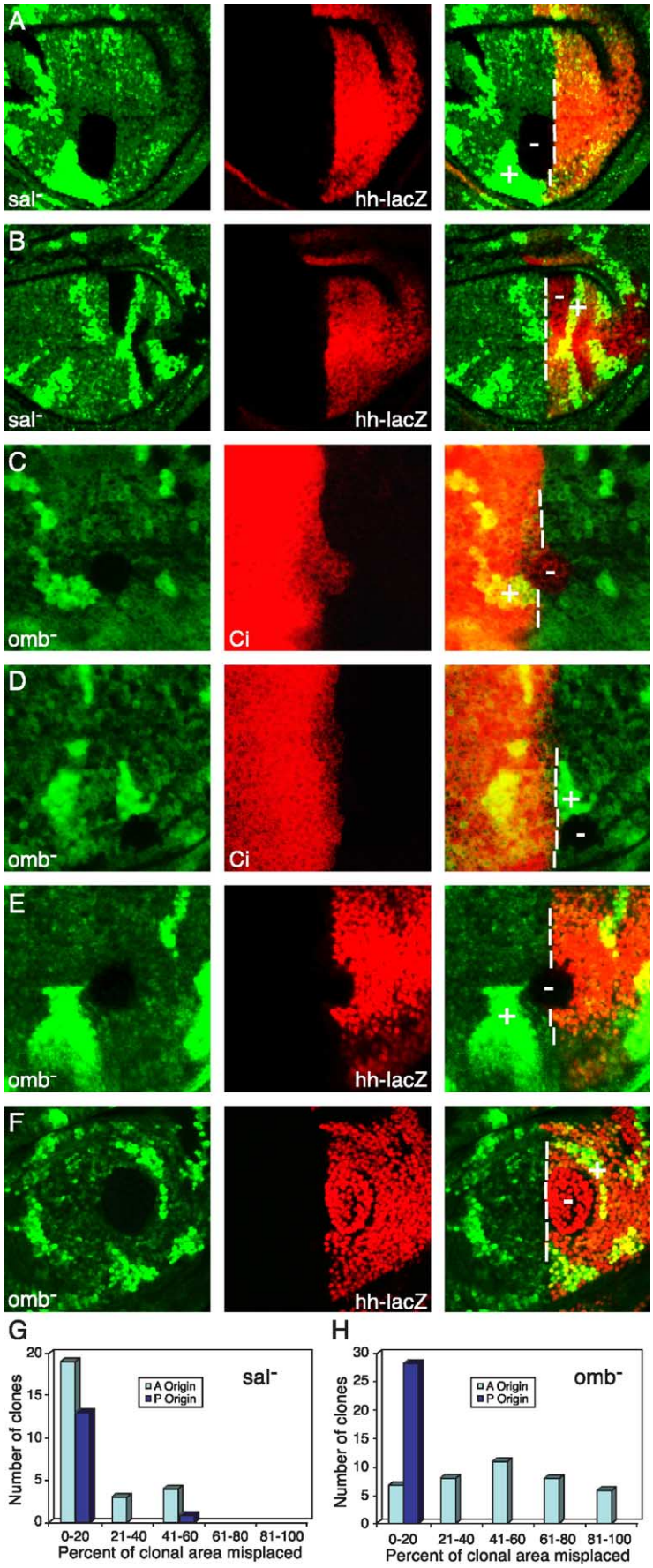


Fig. 3. The repression of Brinker by Mad is required for maintaining the normal position of the A/P boundary. Clones of cells homozygous mutant for *mad* and *brk* ($mad^{-}brk^{-}$) are marked by the absence (−) of GFP staining in green. The wild-type sister clones are marked by the elevated GFP staining (+). Ci staining is shown in red. (A) $mad^{-}brk^{-}$ clones of A origin remain entirely in the A territory and form borders with P cells at the normal position of the A/P boundary. (B) $mad^{-}brk^{-}$ clones of P origin remain in the P compartment. (C) Quantification of the area that $mad^{-}brk^{-}$ clones located at the A/P boundary were misplaced into the adjacent compartment expressed as a percent.

2000). To test the epistatic relationship between Ci and Omb, we generated P clones that ectopically expressed Ci but lacked Omb function and analyzed their segregation at the A/P boundary. If Omb were required for Ci to specify A-type cell segregation, then P clones expressing Ci and lacking Omb should no longer segregate with A cells. P clones mutant for *omb* and expressing Ci from a transgene were generated using the MARCM system (Lee and Luo, 2001) in combination with the P-specific *hh*-GAL4 driver line. As a control, we drove expression of GFP alone in clones. All GFP-expressing clones ($n = 102$) were present in the P compartment, showing that clones expressing a UAS-transgene were indeed exclusively generated in the P compartment. Further, GFP-expressing control clones remained entirely within the P compartment (Fig. 5A). The majority of clones located at the A/P boundary expressing Ci and GFP were misplaced into the A territory of the wing disc (Fig. 5B), consistent with previous results (Dahmann and Basler, 2000). Few Ci and GFP coexpressing P clones lacking Omb function were misplaced into the A territory (Fig. 5C), suggesting that Ci may, in part, act independently of Omb. However, the majority of Ci and GFP coexpressing clones lacking Omb function remained in the P compartment (Fig. 5C). We conclude that Omb is required for Ci to specify A-type cell segregation, indicating that Omb acts either downstream of or in parallel with Ci.

Discussion

For many years, it was thought that En and Inv regulated the segregation of A and P cells by specifying a P-type cell segregation in a cell-autonomous fashion. Recent work has challenged this view by showing that a unidirectional Hh-mediated signal from P to A cells is required to specify the A-type segregation behavior of A cells and that the role of En and Inv is mainly to control Hh signaling (Blair and Ralston, 1997; Dahmann and Basler, 2000; Rodriguez and Basler, 1997). Based on the findings that A cells signal back to P cells via Dpp (Lecuit et al., 1996; Nellen et al., 1996) and that wings from flies hypomorphic for *dpp* have a distorted A/P boundary (Hidalgo, 1994), it has been proposed that A to P signaling by Dpp might also be important to maintain the A/P boundary (Blair and Ralston,


A
sal-
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D
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Ci
E
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hh-lacZ
F
omb-
hh-lacZ
G
sal-
A Origin
P Origin
Number of clones
0-20
21-40
41-60
61-80
81-100
Percent of clonal area misplaced
H
omb-
A Origin
P Origin
Number of clones
0-20
21-40
41-60
61-80
81-100
Percent of clonal area misplaced

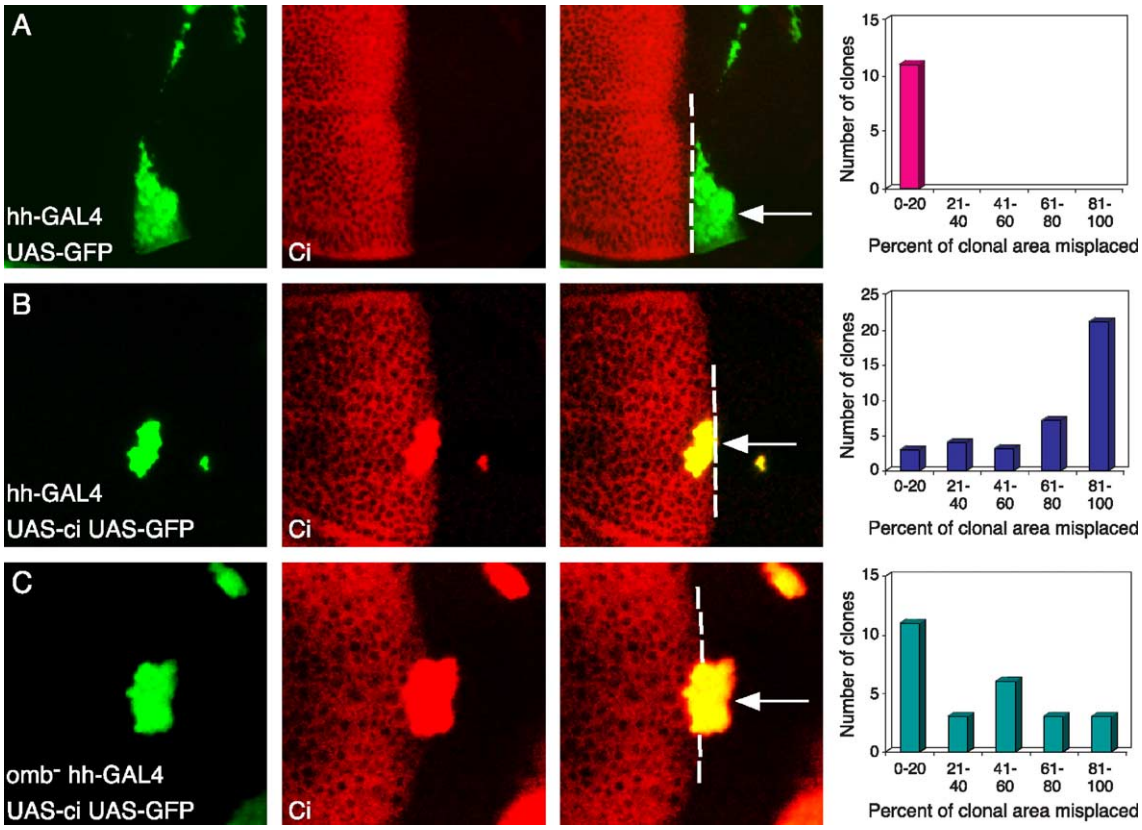

Fig. 5. Omb is required for Ci to specify A-type cell segregation. Clones of P origin expressing GFP (A), Ci and GFP (B), or Ci and GFP, and being mutant for *omb* (C) are marked by GFP staining in green. Ci staining is shown in red. The panel on the right shows quantification of the segregation of clones located at the A/P boundary. The percent of clonal area misplaced into the adjacent compartment is indicated. (A) GFP-expressing control clones remain in the P compartment (arrow). (B) Clones coexpressing Ci and GFP, when in contact with A cells, sort out from P cells and take up positions normally only occupied by A cells (arrow). (C) Clones coexpressing Ci and GFP but lacking Omb only partially take up positions normally occupied by A cells (arrow).

1997; Vincent, 1998). However, whether Dpp signal transduction is required for the maintenance of the A/P boundary and in which cells the Dpp signal is required was unknown. By analyzing clones mutant for *tkv*, *mad*, and *omb*, we now provide several independent lines of evidence that Dpp signal transduction is required to maintain the A/P boundary and that it is only required in A cells, but not in P cells. Thus, our results do not support the hypothesis that A to P signaling by Dpp is required to maintain the A/P boundary. Instead, our results suggest that Dpp signaling within Dpp-producing A cells is required to maintain the A/P boundary.

A transcriptional response to Dpp is required in A cells to maintain the A/P boundary

Here, through analysis of mutant clones located at the A/P boundary lacking the activity of the type I Dpp receptor Tkv, we provide evidence that the reception of the Dpp signal in A cells is required to maintain the A/P boundary. When generated in the P compartment, a few $tkv^{-}bsk^{-}$ clones displace the A/P boundary to a small extent, which we attribute to the unusual round shape of these clones (see below). However, the majority of P $tkv^{-}bsk^{-}$ clones do not displace the A/P boundary, suggesting that the reception of the Dpp signal is not required in P cells to maintain the A/P boundary. In contrast, mutant clones generated in the A compartment at the A/P boundary displace the position of the A/P boundary toward P, indicating that the reception of the Dpp signal is required in A cells to maintain the A/P boundary.

How does the reception of the Dpp signal control cell segregation at the A/P boundary? Although the molecular basis is unknown, a cell's segregation behavior presumably depends on its cytoskeletal or surface properties (cell

Fig. 4. The Dpp target gene *omb* is required for maintaining the normal position of the A/P boundary. Clones of cells homozygous mutant for *sal* (sal^{-}) (A and B) are marked by the absence (−) of πmyc staining in green. The wild-type sister clones are marked by the elevated πmyc staining (+). Clones of cells homozygous mutant for omb (omb^{-}) (C–F) are marked by the absence (−) of GFP staining in green. The wild-type sister clones are marked by the elevated GFP staining (+). *hh-lacZ* (A, B, E, and F) and Ci (C and D) staining are shown in red. (A) sal^{-} clones of A origin remain in the A territory. (B) sal^{-} clones of P origin remain in the P territory. (C and E) omb^{-} clones of A origin displace the A/P boundary toward P. (D and F) omb^{-} clones of P origin are confined to the P territory and form borders with A cells at the normal position of the A/P boundary. Quantification of the area that sal^{-} (G) and omb^{-} (H) clones located at the A/P boundary were misplaced into the adjacent compartment expressed as percent.

affinity). Members of the TGFβ superfamily have been observed in other systems to be able to activate regulators of the actin cytoskeleton independently of Mad/Smad transcription factors (Derynck and Zhang, 2003), raising the possibility that Dpp reception could control cell segregation by directly altering structural components of the responding cells. Alternatively, Dpp could control the segregation of cells by regulating the transcription of one or several target genes. To distinguish between these possibilities, we have analyzed the role of downstream components of the Dpp signal transduction pathway. We provide three independent lines of evidence that a transcriptional response to the Dpp signal is required to maintain the A/P boundary. First, the segregation behaviors of *mad*$^{-}$*bsk*$^{-}$ and *tkv*$^{-}$*bsk*$^{-}$ clones are indistinguishable. Like *tkv*$^{-}$*bsk*$^{-}$ clones, A *mad*$^{-}$*bsk*$^{-}$ clones displace the A/P boundary toward P (Figs. 1G and I), indicating a role for the transcription factor Mad in A cells to maintain the A/P boundary. Second, *mad*$^{-}$*brk*$^{-}$ clones respect the A/P boundary (Figs. 3A–C), indicating that repression of *brk* transcription by Mad is important for normal A/P cell segregation. Third, A *omb*$^{-}$ clones displace the A/P boundary toward P (Figs. 4C, E, and H). The frequency and extent of the boundary displacement of A *omb*$^{-}$, *tkv*$^{-}$*bsk*$^{-}$, and *mad*$^{-}$*bsk*$^{-}$ clones is comparable, suggesting that the Dpp target gene *omb* is the main mediator of this aspect of the Dpp signal. In contrast to *omb*$^{-}$ clones, most A clones mutant for the Dpp target gene *sal* do not displace the A/P boundary (Figs. 4A and G), indicating that *sal* does not play an important role in maintaining the A/P boundary. Together, these data suggest that the transduction of the Dpp signal controlling the maintenance of the A/P boundary bifurcates at the level of the Dpp target genes.

Cells of *tkv*$^{-}$*bsk*$^{-}$, *mad*$^{-}$*bsk*$^{-}$, and *omb*$^{-}$ clones displacing the A/P boundary do not appear to intermingle well with P cells. In fact, within the entire wing disc pouch, these mutant clones have a round shape and smooth borders ((Figs. 1D–H, 2B,C, and 4C–F), and Supplementary Fig. 1), suggesting that these mutant cells in general do not intermingle freely with wild-type cells. Similar clone shapes have been reported upon mutation or misexpression of several genes, including mutants in the Dpp target gene *sal* and misexpression of a constitutively active form of Tkv (Milan et al., 2002; Nellen et al., 1996). The round shapes and smooth borders of clones have been attributed to differences in the affinity of clone cells for their neighbors (Lawrence, 1997; Lawrence et al., 1999; Wright and Lawrence, 1981), suggesting that Tkv, Mad, and the Dpp target genes *omb* and *sal* may affect some aspects of wing pouch cell affinity. Therefore, we attribute the inability of A *tkv*$^{-}$*bsk*$^{-}$, *mad*$^{-}$*bsk*$^{-}$, and *omb*$^{-}$ clones displacing the A/P boundary to intermingle well with P cells to this particular role.

Taken together, our analysis indicates two roles for Dpp signal transduction. First, it provides some aspects of the cell affinity of both A and P wing pouch cells. Second, it is required in A cells to specify an A cell affinity important for maintaining the A/P boundary. These two roles of Dpp signal transduction could either be related or distinct. The finding that the Dpp target gene *sal* is required for the first (Milan et al., 2002), but not second, role provides a first indication that these two roles are implemented by partially distinct molecular mechanisms.

The role of Omb in maintaining the A/P boundary

How might Omb regulate the segregation behavior of cells at the A/P boundary? Recent work has shown that Omb has at least two roles during the patterning of the *Drosophila* wing. First, Omb is required for the expression of two Dpp target genes *sal* and *vestigial* (*vg*) (del Alamo Rodriguez et al., 2004). Since *sal* mutant clones do respect the A/P boundary (Figs. 4A and G), the role of Omb in maintaining the A/P boundary cannot depend on *sal* induction. Since Vg is required for wing cell proliferation (Kim et al., 1996), its role in maintaining the A/P boundary cannot be tested. Second, Omb is involved in shaping the expression pattern of *tkv* along the A/P axis of the wing disc (del Alamo Rodriguez et al., 2004). The expression of *tkv* is reduced in Dpp-producing A cells along the A/P boundary (Tanimoto et al., 2000). This reduction of *tkv* expression is mediated by the transcription factor Master of thickveins (Mtv, also known as Brakeless and Scribbler (Funakoshi et al., 2001; Senti et al., 2000; Yang et al., 2000)), which is expressed in these cells in response to the Hh signal. Since both *tkv* and *mtv* are upregulated in *omb* mutant clones, it has been proposed that Omb is required for Mtv to repress *tkv* (del Alamo Rodriguez et al., 2004). However, reduction of *tkv* transcription in A cells does not seem to be important for the segregation of cells at the A/P boundary, because A clones either mutant for *mtv*, in which *tkv* levels are increased, or overexpressing *tkv* respect the A/P boundary (Supplementary Fig. 2). Thus, neither the role of Omb in repressing *tkv* nor in activating *sal* transcription appears to be important for Omb's function in maintaining the A/P boundary. Therefore, other target genes of Omb must exist that mediate Omb's function in maintaining the A/P boundary.

The epistatic relationship of Dpp and Hh signaling in maintaining the A/P boundary

Anterior cells at the A/P boundary have been previously shown to require Hh signal transduction to segregate from P cells (Blair and Ralston, 1997; Rodriguez and Basler, 1997). We now provide evidence that A cells in addition need to transduce the Dpp signal for normal segregation. What is the epistatic relationship between Hh and Dpp signaling? The activity of the Hh transduction pathway is not affected in either *tkv*$^{-}$*bsk*$^{-}$ or *mad*$^{-}$*bsk*$^{-}$ clones as monitored by the expression of the Hh target gene *ptc* (Fig. 2), indicating that Hh signal transduction does not require Dpp signal transduction components for its activity. However, the Dpp target gene *omb* appears to be important for A cells to interpret the

Hh signal because the ability of Ci to specify A-type segregation properties depends, in part, on the activity of Omb (Fig. 5). Thus, Dpp signaling acts either downstream of or in parallel with Hh signaling in maintaining the A/P boundary.

A model for transcriptional regulation of genes mediating cell segregation at the A/P boundary

Previously, three transcription factors, a transcriptional activator form of Ci (hereafter referred to as Ci[act]) (Aza-Blanc et al., 1997), En, and Inv, have been shown to be required for the segregation of A and P cells (Blair and Ralston, 1997; Dahmann and Basler, 2000; Hidalgo, 1994; Morata and Lawrence, 1975). We now provide evidence for the involvement of a fourth transcription factor, the T-box protein Omb, and further show that Omb acts downstream of or in parallel with Ci. How could these four transcription factors regulate the segregation of A and P cells? In a simple model, Ci[act], En, Inv, and Omb could regulate the segregation of A and P cells by controlling the transcription of the same set of target genes that may encode cell affinity molecules or regulate the activity of cell affinity molecules (Fig. 6). Omb is activated in both A and P cells in a broad domain centered around the A/P boundary by Dpp signaling where it may upregulate the expression of this putative target gene(s). The activity of Ci[act] is restricted to Hh-responding A cells along the A/P boundary. In these A cells, the target gene(s) would be further induced. En and Inv expressions are mainly confined to P cells in which they are known to act as repressors of transcription. Thus, En and Inv would repress the putative target gene(s) in P cells. The abrupt difference in the expression of putative target gene(s) would contribute to the segregation of A and P cells. Anterior clones, but not P clones, of cells lacking Omb would displace the A/P boundary because normally the putative target gene would be highly expressed in A cells, but not in P cells, where it would be repressed by En and Inv. Omb may therefore provide a basal affinity to cells in the center of the wing disc that is modified by Ci[act], En, and Inv to create a sharp difference of this affinity in cells on both sides of the A/P boundary. In an alternative model, Omb, Ci[act], En, and Inv would regulate distinct sets of genes. To distinguish among these models, it will be necessary to identify the Ci[act], En, Inv, and Omb target genes mediating cell segregation.

The precise position and shape of the Dpp organizer along the A side of the A/P boundary are important for normal growth and patterning of the wing (e.g., Zecca et al., 1995). It has been proposed that the segregation of cells at the A/P boundary contributes to maintain this precise position and shape of the Dpp organizer in the growing wing disc epithelium (Dahmann and Basler, 1999). It is intriguing to notice that the Dpp-organizing activity itself plays a role in the segregation of A and P cells, suggesting that the Dpp-organizing activity contributes to maintain its own position. It will be interesting to investigate whether other organizers associated with compartment boundaries have similar functions.

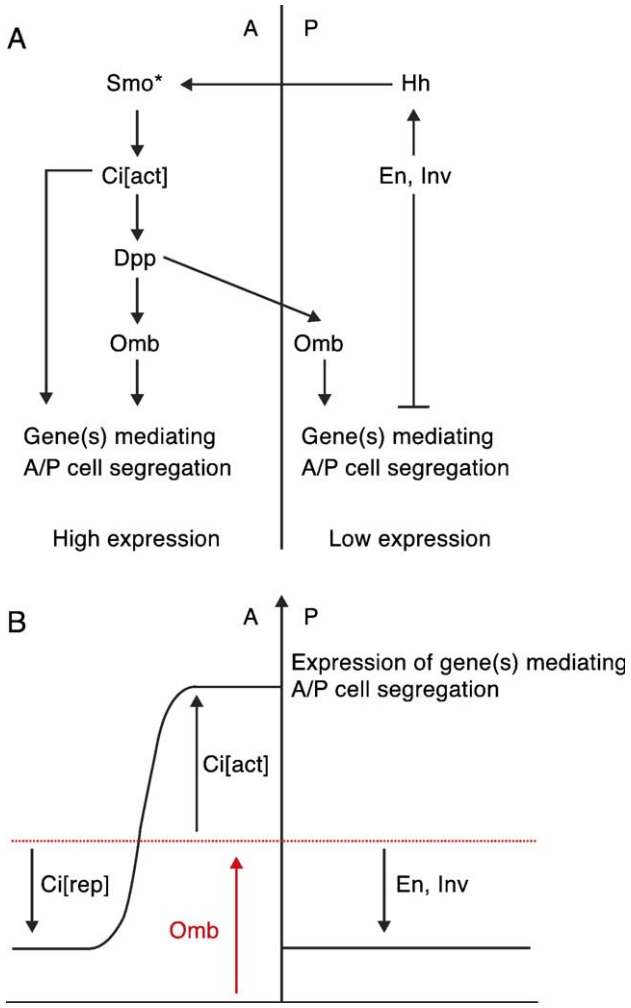

Fig. 6. A model of the control of cell segregation at the A/P boundary. (A) A scheme of the regulatory network controlling cell segregation. En and Inv promote the expression of Hh in P cells. Hh activates Ci in adjacent A cells, which induce the expression of Dpp. Dpp signaling leads to the expression of Omb in both A and P cells. In A cells along the A/P boundary, Ci[act] and Omb both activate the expression of a target gene(s) mediating A/P cell segregation that, as a consequence, will be expressed at high levels. In contrast, in P cells, the activation of this target gene(s) by Omb is counteracted by En and Inv. The target gene(s) is therefore expressed at low levels in these cells. The abrupt difference in the expression level of this target gene(s) leads to the segregation of A and P cells. (B) A scheme illustrating the predicted expression profile of target gene(s) mediating A/P cell segregation in the center of the wing disc. Omb provides a basal expression level that is increased in A cells at the A/P boundary by Ci[act] and decreased in P cells by En and Inv. Ci[rep] is a proteolytically cleaved form of Ci acting as a transcriptional repressor that is present in A cells far away from the A/P boundary receiving no or low levels of Hh (Aza-Blanc et al., 1997).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.11.033.

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