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Article

Coordinated Control of Cell Adhesion, Polarity, and Cytoskeleton Underlies Hox-Induced Organogenesis in *Drosophila*

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Summary

Background: Hox genes control animal body plans by directing the morphogenesis of segment-specific structures. As transcription factors, HOX proteins achieve this through the activation of downstream target genes. Much research has been devoted to the search for these targets and the characterization of their roles in organogenesis. This has shown that the direct targets of Hox activation are often transcription factors or signaling molecules, which form hierarchical genetic networks directing the morphogenesis of particular organs. Importantly, very few of the direct Hox targets known are "realizator" genes involved directly in the cellular processes of organogenesis.
Results: Here, we describe for the first time a complete network linking the Hox gene *Abdominal-B* to the realizator genes it controls during the organogenesis of the external respiratory organ of the larva. In this process, *Abdominal-B* induces the expression of four intermediate signaling molecules and transcription factors, and this expression results in the mosaic activation of several realizator genes. The ABD-B spiracle realizators include at least five cell-adhesion proteins, cell-polarity proteins, and GAP and GEF cytoskeleton regulators. Simultaneous ectopic expression of the *Abd-B* downstream targets can induce spiracle-like structure formation in the absence of ABD-B protein.
Conclusion: Hox realizators include cytoskeletal regulators and molecules required for the apico-basal cell organization. HOX-coordinated activation of these realizators in mosaic patterns confers to the organ primordium its assembling properties. We propose that during animal development, Hox-controlled genetic cascades coordinate the local cell-specific behaviors that result in organogenesis of segment-specific structures.

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Introduction

Hox genes control animal body plans by directing the morphogenesis of segment-specific structures [1]. As transcription factors, HOX proteins achieve this through the activation of downstream target genes [2]. In 1975, García-Bellido [3] proposed that homeotic genes (including Hox genes) were regulators of what he called realizator genes. Realizators would control local cell behaviors (cell adhesion, cell shape, etc.) that result in the development of a particular segment-specific organ. Since then, many HOX protein targets have been identified [2, 4–6], but most of them encode transcription factors and signaling molecules that activate the, largely unknown, realizator genes that "do the job." Thus, during organogenesis, most Hox realizator genes are generally controlled indirectly. Recently, however, the cell-death regulator gene *reaper* has been shown to be a direct realizator of the *Deformed* (*Dfd*) Hox gene of *Drosophila* [7]. In this case, localized cell death induced by *reaper* activation "sculpts" the segment's shape. Similarly, the Hox gene *lin-39* controls cell fusion in the *C. elegans* epidermis by activation of Eff-1, a protein that induces the mixing of membrane bilayers [8–10]. Despite these examples, we lack knowledge of how Hox realizators control the morphogenesis of segment-specific organs.

In *Drosophila*, two organs have been studied that address this issue: the salivary glands, which develop in the labial segment under the regulation of *Sex combs reduced* (*Scr*) [11] and the posterior spiracles, formed in the eighth abdominal (A8) segment under the control of *Abdominal-B (Abd-B)* [12]. Here, we show that ABD-B activates the JAK/STAT signaling cascade and transcription factors that activate regulatory molecules of the actin cytoskeleton, cell adhesion, and cell polarity. The interaction between these basic cell-regulatory molecules modifies the cellular behaviors that result in posterior spiracle organogenesis. Simultaneous ectopic expression of four early downstream targets of *Abd-B* is sufficient to activate the realizators required for spiracle formation in the absence of ABD-B. This work provides the most complete study to date describing how Hox genes control realizator genes during organogenesis.

Results

Primary Targets Controlled by ABD-B during Spiracle Organogenesis

The posterior spiracle forms the external connection of the respiratory system of the larva. It is composed of two morphologically different structures: the spiracular

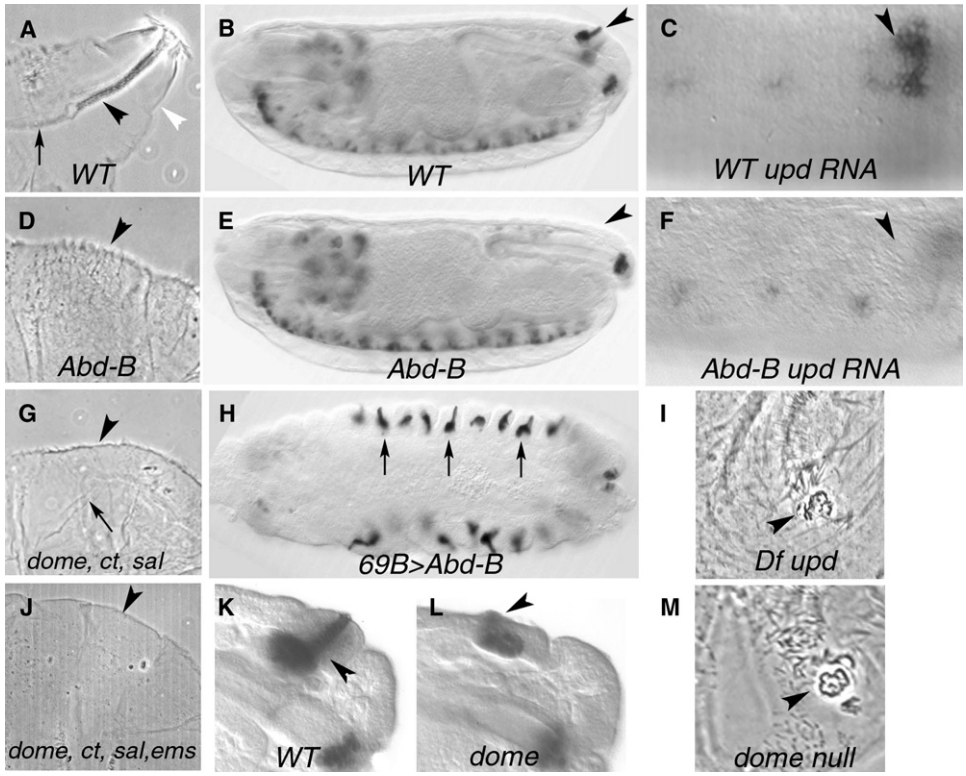

Figure 1. Spiracle Defects in *Abd-B* and Downstream Mutants

(A) The posterior spiracle is formed by two structures: the spiracular chamber (black arrowhead) where the refractile filzkörper forms and the stigmatophore (white arrowhead), a protruding structure where the spiracular chamber is lodged. The trachea (arrow) connects to the base of the spiracular chamber.
(B) A *grh-lacZ* marker labels the elongating spiracle cells forming in A8 (arrowhead).
(C) The JAK/STAT ligand *upd* is expressed in the A8 spiracle primordium prior to cell elongation.
(D) In *Abd-B* mutants, the posterior spiracle is completely missing (arrowhead).
(E) The *grh-lacZ* marker is not expressed in A8 in *Abd-B* mutants.
(F) In *Abd-B* mutants, *upd* is not expressed in A8.
(G) In *ct*, *dome*, *sal* triple mutants, no obvious spiracle structures remain, but the trachea (arrow) still joins to the outside.
(H) Ectopic ectodermal ABD-B expression induces ectopic spiracle structures as shown with *grh-lacZ*.
(I) Abnormal spiracles formed in the absence of *upd* ligands.
(J) In *ct*, *dome*, *sal*, *ems* quadruple mutants, no recognizable spiracle structures form.
(K) Close-up of the elongated spiracular chamber cells labeled with *grh-lacZ* in a st15 wild-type embryo.
(L) A *dome* zygotic mutant labeled as in (K) showing that the spiracular chamber cells do not elongate.
(M) Close up of the spiracles in *dome* null mutants.

chamber and the stigmatophore. The spiracular chamber is the internal tube connecting the exterior with the trachea and forms a refractile filter, the filzkörper (Figure 1A, black arrowhead). The stigmatophore is the external protruding part of the spiracle in which the internal tube is located (Figure 1A, white arrowhead). The spiracular chamber develops by a two-stage process that begins with the localized invagination of 80–100 ectodermal cells and is followed by an elongation stage in which the cells increase their length 4-fold (Figures 1B and 1K). The stigmatophore uses a different morphogenetic mechanism, whereby the cells surrounding the future spiracular chamber change their relative position several times through movements similar to convergent extension and thereby create the proximo-distal axis of the stigmatophore [12].

Abd-B is the only Hox gene required for posterior spiracle formation. In *Abd-B* mutants, the posterior spiracles do not form (Figures 1D and 1E), whereas ectopic expression of *Abd-B* in the trunk segments results in the formation of ectopic spiracles (Figure 1H), indicating that this Hox gene is both necessary and sufficient to induce the specification and morphogenetic movements required for spiracle development [12–15].

The immediate effect of *Abd-B* expression in the posterior spiracle primordium is the activation of several spiracle-specific genes that will pattern the organ [12]. Among these genes are *empty spiracles* (*ems*) [16], *cut* (*ct*) [17] and *unpaired* (*upd*, the ligand of the *Drosophila* JAK/STAT pathway [18]), which are required for the formation of the spiracular chamber [12] (see Figure S1 in the Supplemental Data available with this article online). In the stigmatophore primordium, ABD-B activates *spalt* (*sal*) and, through *sal*, its downstream target *grain* (*grn*), which controls the cell rearrangements forming the stigmatophore [19]. *ems* is an ABD-B direct target [16] and *ct*, *upd*, and *sal* are also likely to be controlled directly by ABD-B because (1) their spiracle expression is independent of each other, (2) ectopic expression of ABD-B results in ectopic transcription of these genes in the

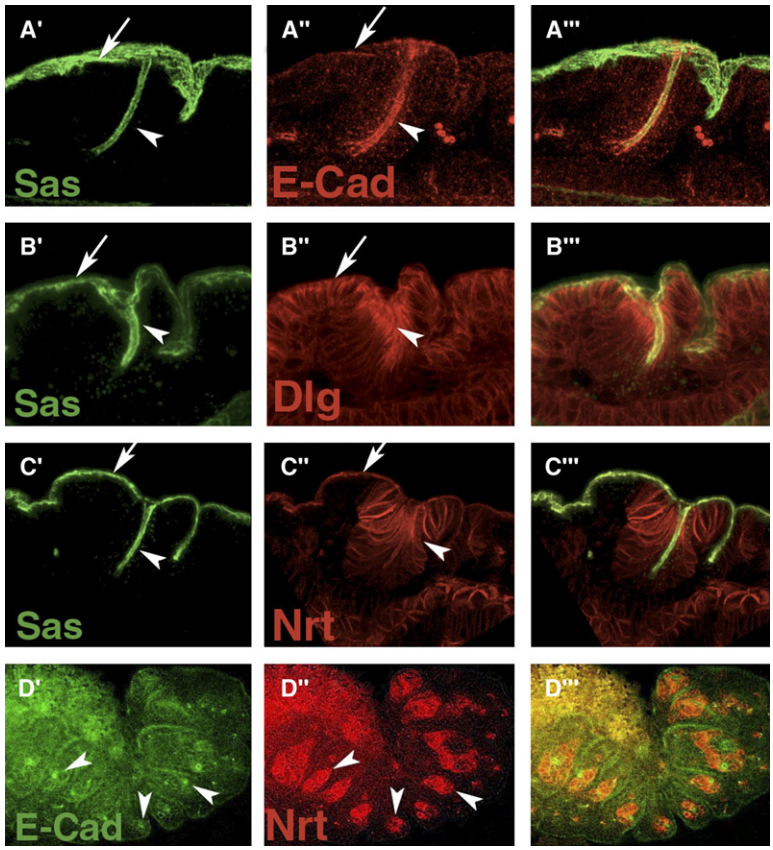

Figure 2. Localization of Apico-Basal Cell-Polarity Markers in the Posterior Spiracles

(A′), (B′), and (C′) show the expression of the apical marker Stranded at second (Sas) in three st14 embryos. The same embryos stained with E-Cad (A″), the lateral marker Dlg (B″) and the basolateral marker Nrt (C″). (A‴), (B‴), (C‴), and (D‴) show the merge of the two left panels. Note that only Sas is expressed homogeneously in the spiracle cells (arrowheads) and neighboring epithelial cells (arrows), whereas E-Cad, Dlg, and Nrt have higher levels of expression in the spiracular chamber. Higher levels of expression of E-Cad (D′) and Nrt (D″) can be induced in anterior segments by ectopic activation of ABD-B with the *69B-Gal4* ectodermal line. Arrowheads in (D) point at some of the ectopic spiracles.

areas where supernumerary spiracles are formed (Figure S1B), and (3) clustered putative binding sites for ABD-B [20, 21] are present in the adjoining genomic region (Table S1). Although *grn* and its upstream transcriptional regulator *sal* are required in the stigmatophore for cell rearrangements [19], neither *ems* nor *ct* abolish cell elongation in the spiracular chamber [12]. This led us to analyze the function of the JAK/STAT pathway in the posterior spiracles. Mutation of the transcription factor STAT, the pathway ligands (Upd) [22], or their receptor (Dome) [23] results in abnormal spiracles because of lack of cell elongation (Figures 1I and 1K–1M and Figure S1A).

To test whether these genes represent most of the primary targets activated by ABD-B during spiracle morphogenesis, we analyzed a quadruple mutant deleting *ct*, *sal, ems*, and *dome* (the receptor of the JAK/STAT pathway). In this combination, all spiracle structures are absent (Figure 1J), similar to the phenotype observed in *Abd-B* mutants (Figure 1D). The only clear difference is that *Abd-B* mutants develop a dorsal A7-like cuticle, probably because of the activation of ABD-A in A8 [24], whereas the quadruple mutants do not. These results show that *ct*, *ems*, *sal*, and *upd* represent a significant proportion of the primary targets downstream of ABD-B required for spiracle organogenesis (see below).

Control of Cell Polarity during Spiracle Organogenesis

The extreme cell elongation taking place in the spiracular chamber (Figure 1K) prompted us to investigate whether the apico-basal membrane domains in the spiracle cells were being remodeled. We used several cell-polarity markers that label different membrane domains. The apical Stranded at Second (Sas) protein [25, 26] highlights the lumen of the spiracle, indicating that it forms from the apical membranes of the invaginated spiracular chamber cells (Figures 2A′, 2B′, and 2C′). The subapical protein Crumbs (Crb) [27] is expressed at very high levels in the developing spiracle prior to invagination (Figure 3A) and later also occupies a lumenal position in the spiracular chamber (Figure 3B). The *Drosophila* E-cadherin protein Shotgun (E-Cad) [28] labels the adherens junctions immediately basal to the subapical region, and this organization is maintained in the spiracle cells (Figure 2A″). Staining of the septate junction protein Discs large (DLG) [29] shows that the spiracle cells have, compared to the cells in the adjacent epithelium, an expanded lateral domain (Figure 2B″). The expansion of the basolateral domain in the elongated spiracular chamber cells is most evident when staining for Neurotactin (Nrt) [30], an adhesion protein preferentially localized in this membrane compartment (Figure 2C″).

Interestingly, the levels of Crb, E-Cad, and Nrt proteins are higher in the developing spiracle than in the surrounding epithelium (Figures 2A″, 2C″, and 3B), suggesting that the ABD-B-induced spiracle-gene cascade modulates cell-polarity gene transcription. Accordingly, ectopic ABD-B activation in the ectoderm causes increased levels of these markers in the primordia of the ectopic spiracles (Figure 2D), and in *Abd-B* mutants, the levels of expression in A8 are identical to those in other abdominal segments (Figures S2B and S2F).

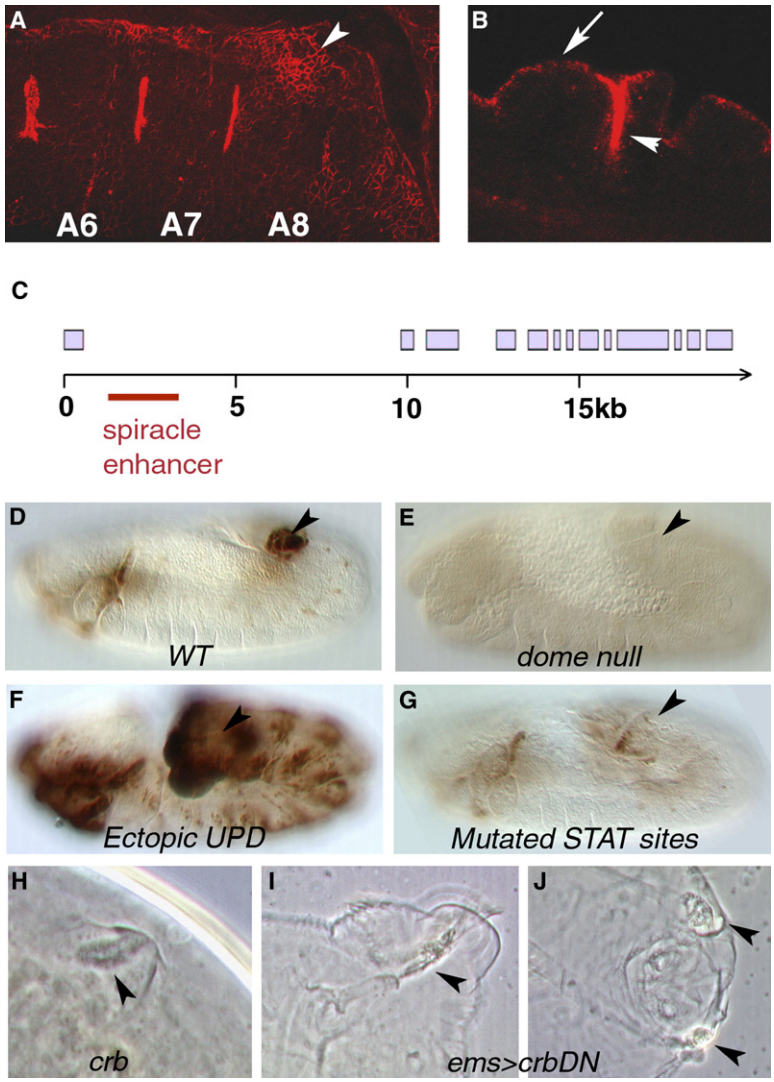

Figure 3. Posterior Spiracle Expression of the Apical Determinant *crb* Is Regulated by JAK/STAT

(A) shows that at st11, before spiracle invagination, there is an upregulation of Crb expression in the spiracular cells (arrowhead), which is maintained at later stages of development (B). (C) shows exon-intron representation of *crb* gene indicating the area where the 43.2 spiracle enhancer is located. (D) shows the expression of a 2 kb *crb43.2-lacZ* construct revealing the presence of a posterior spiracle enhancer (arrowhead). (E) shows that the enhancer is not expressed in null mutants for the JAK/STAT receptor. (F) shows that ectopic expression of the Upd ligand induces ectopic enhancer activation. (G) shows that the mutation of three putative STAT-binding sites reduces construct expression in the posterior spiracles. (H) shows a short spiracle formed in hypomorphic crb^{D88-3} embryos. (I) and (J) show that the expression of a *crbDN* construct in the spiracles by use of the *ems-Gal4* line results in various degrees of spiracle shortening that in the most extreme cases (J) resemble lack of JAK/STAT-function phenotypes. Arrowheads point to the posterior spiracles or their anlagen. Note in (A), the apical constriction of the spiracle cells is recognizable by the higher levels of Crb.

Because subcellular Crb redistribution has been shown to be important for the elongation of photoreceptor cells during eye organogenesis [31, 32], we decided to analyze the regulation of *crb* expression during spiracle-cell elongation. The elongation defects observed on JAK/STAT mutants (Figures 1I and 1M; see also Figure S1A) made us look for consensus STAT-DNA-binding sites in *crb*. Analysis of reporter genes revealed that a 2 kb intron fragment (Figure 3C) containing one perfect STAT site [33] and two partially conserved STAT-binding sites is capable of driving reporter-gene expression in the posterior spiracles (Figure 3D). This reporter gene is not expressed in embryos lacking the receptor or the ligands of the JAK/STAT pathway (Figure 3E). Conversely, ectopic expression of the spiracle *crb* reporter is observed when the JAK/STAT ligand Upd is ubiquitously expressed (Figure 3F), suggesting that the enhancer is directly activated by STAT92E. Directed mutagenesis of the three putative STAT-binding sites in the construct results in a decrease of spiracle expression, indicating that *crb* is a direct target of JAK/STAT regulation (Figure 3G).

To test the functional relevance of polarity-gene upregulation in spiracle cells, we investigated the spiracle defects caused by mutations in *crb*. These mutants form shorter spiracles (Figure 3H); however, given the general defects in the epithelial organization of these embryos, the spiracle defects could be indirect. To analyze the specific function of Crb after cellularization, we expressed in the spiracle a dominant negative form of Crb protein that cannot be phosphorylated [34]. In all cases, the spiracles are abnormal, with occasional embryos developing a spiracle phenotype reminiscent of the JAK/STAT loss of function phenotype (Figures 3I and 3J). However, expression of wild-type Crb in the spiracle is unable to rescue the JAK/STAT spiracle phenotypes (not shown), indicating that other STAT target genes besides *crb* are coregulated during spiracle-cell elongation by the JAK/STAT pathway.

Analysis of the regulation of the basolateral marker Nrt shows that its upregulation in the spiracle is controlled by the ABD-B cascade (Figures S2B, S2C, and S2F). The effects of Nrt mutants on spiracle development are not strong, however, suggesting that other basolateral

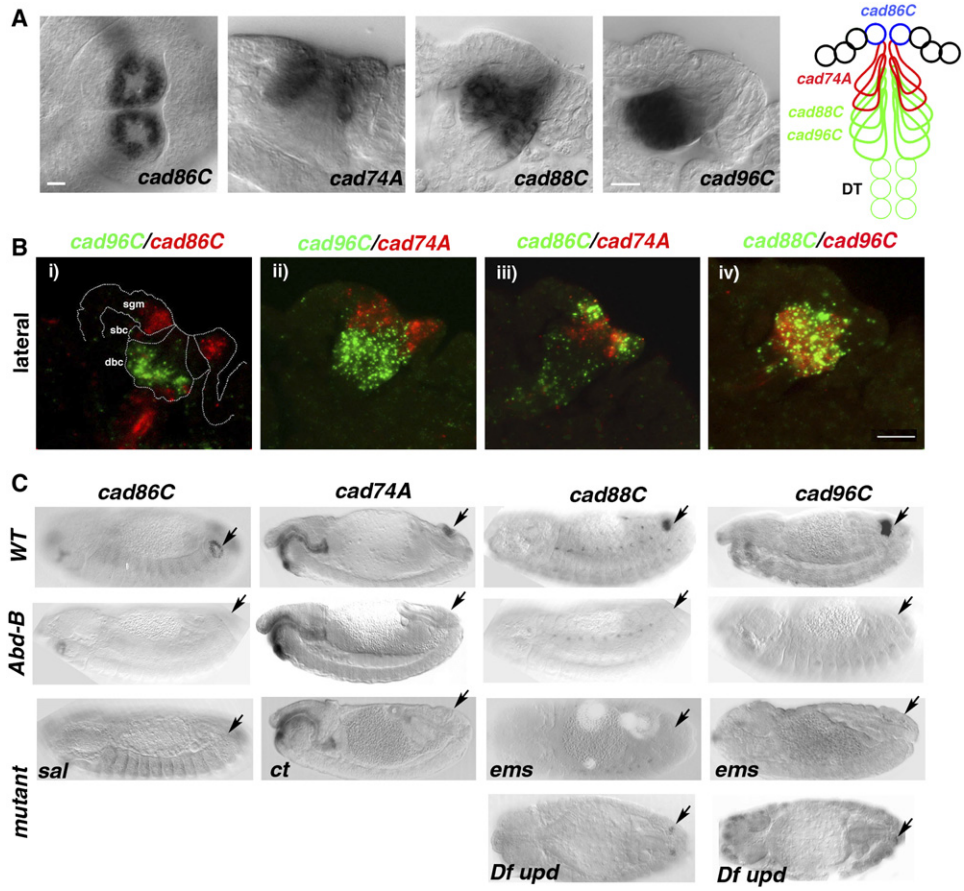

Figure 4. Mosaic of Expression of Nonclassical Cadherins in the Developing Posterior Spiracles

(A) In situ hybridization for *cad86C*, *cad74A*, *cad88C*, and *cad96C* and a scheme representing their expression in different subsets of spiracle cells (DT: dorsal trachea). The scale bar represents 10 μm.

(B) Lateral views of double in situ hybridized embryos showing complementary expression patterns among *cad96C*, *cad86C*, and *cad74A* and coexpression of *cad88C* and *cad96C* in the spiracle primordium. The scale bar represents 20 μm.

(C) Expression of nonclassical cadherins in *Abd-B* mutants and in mutants for ABD-B primary targets. Arrows in (C) point to spiracle primordia.

determinants are likely to be involved in the cell-elongation process.

Control of Cell Adhesion in Posterior Spiracle Cells

Given the observation that E-Cad is expressed at high levels in the cells of the spiracle primordium (Figure 2″), we investigated whether other cadherin molecules were also upregulated in the posterior spiracles. We found that four of the 14 *Drosophila* nonclassical cadherins [35] are activated in different subsets of spiracle cells (Figure 4A). A group of deeper spiracle cells coexpresses *cad88C* and *cad96C*, as assessed by double in situ hybridization (Figure 4B$_{iv}$). A second group of cells, less elongated and localized above the first group, expresses *cad74A* (Figure 4B$_{ii}$). Finally, a row of stigmatophore cells surrounding the spiracular chamber activates the expression of *cad86C* as cell intercalation proceeds (Figures 4B$_{i}$ and 4B$_{iii}$). We analyzed whether these cadherins are regulated by the ABD-B cascade. Our results show that expression of all spiracle cadherins requires *Abd-B* function (Figure 4C and Figure S2B). E-Cad upregulation in the posterior spiracles also requires JAK/STAT and *ct* function (Figures S2C and S2E); *cad86C* requires *sal* and *cad74A* requires *ct*; whereas *cad88C* and *cad96C* require *ems* and, to a lesser extent, activation of the JAK/STAT pathway by the *upd* ligands (Figure 4C). Thus, the expression of E-Cad and these four nonclassical cadherins in particular subsets of cells is regulated by different spiracle primary genes, which are themselves expressed in partially overlapping patterns, thus explaining their mosaic distribution (summarized in Figure 7M).

To show that nonclassical cadherins can mediate cell adhesion, we tested the subcellular localization and adhesive properties of Cad74A. Tagging GFP to Cad74A shows that it is enriched in the subapical and apical membrane of epithelial cells, apposing the localization of β-Catenin in the adherens junctions (Figure 5A). Cell aggregation assays with S2 cells demonstrate that Cad74A is able to promote homotypic cell-cell adhesion in a Ca^{2+}-dependent way, albeit less efficiently than E-Cad (Figure S3).

To address the role of cadherins during posterior spiracle organogenesis, we first studied strong zygotic *E-cad* mutants [28, 36]. In these embryos, maternally provided *E-cad* allows development to progress beyond gastrulation stages. However, when spiracle invagination occurs, many spiracular chamber cells remain on the surface, failing to form a lumen in either one (32%)

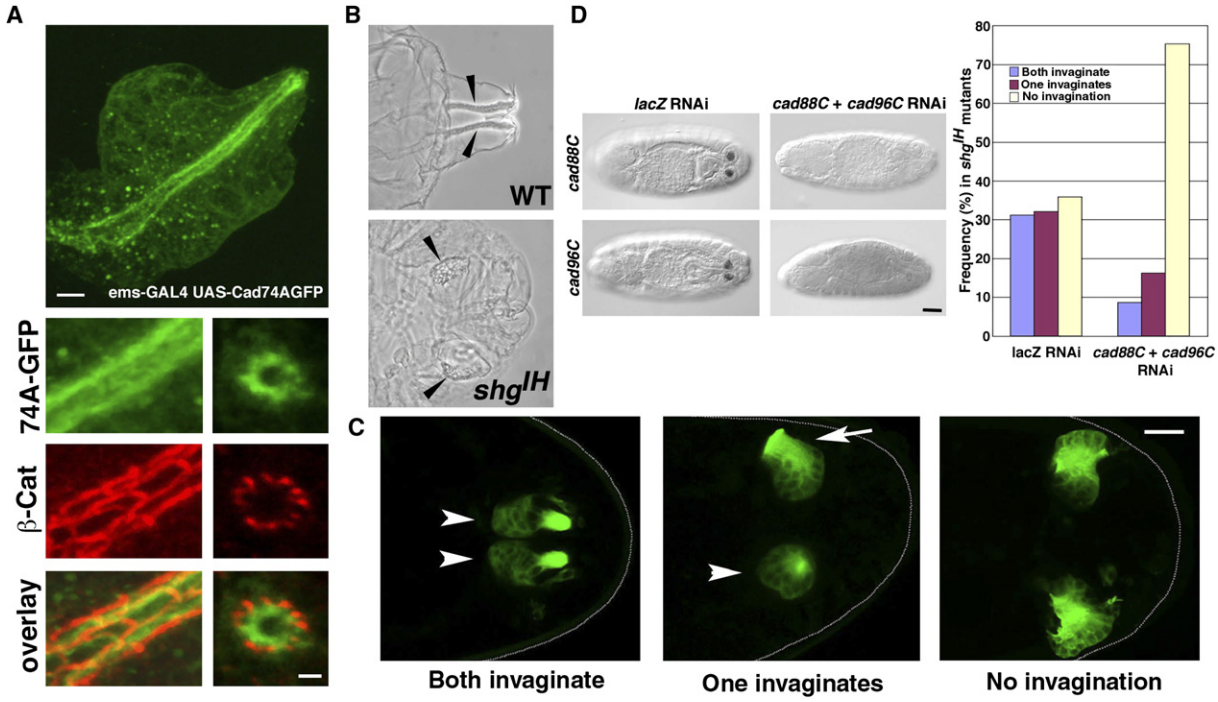

Figure 5. Nonclassical Cadherins Interact with *E-cad*

(A) Subcellular localization of Cad74A-GFP with the *ems-GAL4* driver (upper panel). Note apical localization lining the lumen of the spiracular chamber. Lower panels show the localization of Cad74A in the spiracular chamber cells, relative to β-Catenin, a marker of adherens junctions (left, lateral view; right, transversal view). Scale bars represent 5 μm (upper panel) and 10 μm (lower panel).

(B) Posterior spiracles in E-Cad shg^{IH} zygotic mutant embryos frequently fail to invaginate (arrowheads). (C) Three E-Cad shg^{IH} zygotic mutant embryos of the same genotype as in (B) with their spiracles stained with Actin-GFP to show the variable invagination phenotype. Note that in the middle panel, the uninvaginated spiracle cells (arrow) still elongate. Arrowheads point to normally invaginated spiracles. The scale bar represents 20 μm.

(D) Downregulation of *cad88C* and *cad96C* by RNAi, assessed by in situ hybridization, increases the frequency of spiracle defects in *E-cad* mutant embryos (no invagination, 75%, n = 106) compared to the control (*lacZ* dsRNA injected; no invagination, 36%, n = 105). The scale bar represents 50 μm.

or both spiracles (36%, n = 106) (Figures 5B and 5C). Labeling the cells with GPF-Actin shows that this invagination failure is not due to problems with the intrinsic process of cell elongation (Figure 5C, arrow).

We tested the function of nonclassical cadherins in posterior spiracle development by injection of dsRNA in syncytial embryos [37]. Knocking down the nonclassical cadherins, either singly or in double combinations, does not cause any noticeable spiracle defect. In order to test for a genetic interaction between *E-cad* and nonclassical cadherins during lumen formation, we coinjected dsRNA for *cad88C* and *cad96C* in strong zygotic *E-cad* mutants. This simultaneous reduction of cadherins duplicates the frequency of uninvaginated cells in both spiracles of mutant embryos (36% in *E-cad* mutants [n = 105]; 75% in *E-cad* mutants +*cad88C*/*cad96C* double RNAi [n = 106]; Figure 5D), without affecting cell elongation. Thus, we conclude that these adhesive molecules cooperate to control spiracle-cell invagination.

Control of Cytoskeleton during Spiracle Invagination

The extreme cell elongation and the apical-membrane constrictions observed during spiracle morphogenesis (Figures 1K and 3A) prompted us to study the function of the cytoskeletal regulators in these cells. Some of the most important regulators of the actin-cytoskeleton organization are the small Rho GTPases [38]. To explore their involvement on spiracle morphogenesis, we expressed constitutively active and dominant-negative forms of the *Rho* family of small GTPases. Expression of Rho1 dominant-negative or constitutively active forms interferes with spiracle-cell invagination (Figures 6A and 6B and [39]), whereas Rac1 dominant-negative and activated forms eliminate the spiracular chamber (Figures 6C and 6D).

Rho GTPases cycle from an active GTP-bound state to an inactive GDP-bound state. Given that Rho1 is ubiquitously expressed, we investigated whether any Rho GTPase regulatory protein is specifically controlled in spiracles by the ABD-B cascade. Mutations in the *crossveinless-c* (*cv-c*) gene, which encodes the GTPase-activating protein RhoGAP88C [40], present a very specific phenotype affecting spiracle invagination in about 30% of the embryos (Figure 6E). RNA in situ analysis reveals that at stage 11, *cv-c* is expressed in the tracheal pits, with higher levels of expression in the A8 pit that abuts the spiracle primordium (Figure 6F). This modulation is likely to depend on ABD-B because in *Abd-B* mutants, all pits have identical levels of expression (Figure 6G), and ectopic expression of ABD-B induces higher levels of *cv-c* (Figures 6H and 6I).

The expression pattern of another Rho GTPase regulator, the GTPase exchange factor 64C (*Gef64C*) [41], was found to be activated in the spiracle primordia

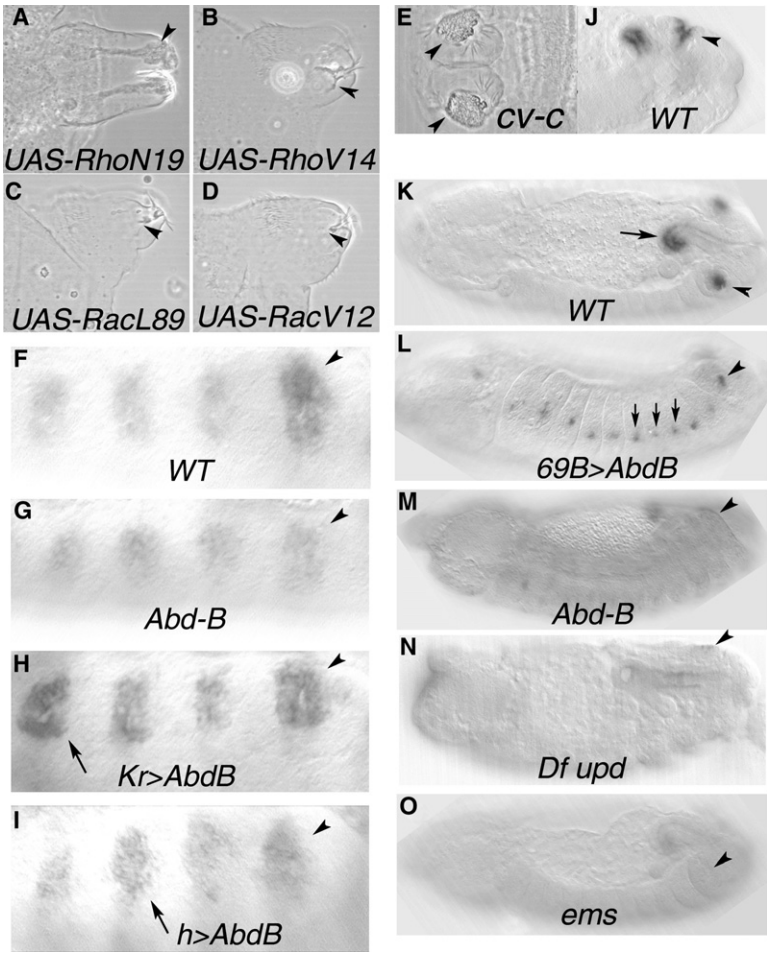

Figure 6. Activity of Rho GTPases in the Posterior Spiracle

(A–D) Expression of dominant-negative and constitutively activated GTPases in the spiracular chamber. (A) shows Rho1 dominant-negative and (B) shows constitutively activated perturb spiracular cell invagination. Expression of (C) Rac1 dominant-negative or (D) Rac1 constitutively activated results in the absence of spiracular chamber derivatives.

(E) Mutants for the RhoGAP *cv-c* gene have variable spiracle invagination defects.

(F–I) *cv-c* expression. In the wild-type (F), A8 shows increased levels of *cv-c* transcripts that are not present in (G) *Abd-B* mutants. (H–I) show that ectopic ABD-B induces higher *cv-c* levels in the central abdominal pits (arrows) when expressed with *Kr-Gal4* (H) or in alternate segments with *h-Gal4* (I).

(J–K) *Gef64C* posterior spiracle expression in the wild-type at the spiracle elongation stage. An arrow in (K) marks the hindgut

(L) Generalized expression of ABD-B in the ectoderm with the *69B-Gal4* line results in *Gef64C* activation on the ectopic spiracles. Arrowheads mark spiracles; small arrows mark ectopic *Gef64C* expression.

(M–O) *Gef64C* expression is downregulated in mutations abolishing JAK/STAT activation (N) and completely missing in *Abd-B* (M) or *ems* (O) mutants. Arrowheads mark spiracles.

under the control of ABD-B (Figures 6J–6M). The spiracle pattern of expression of *Gef64C* is activated by *ems* and JAK/STAT (Figures 6N and 6O), thereby linking the ABD-B spiracle-induced cascade and regulation of the cytoskeleton.

ABD-B Primary Targets Can Induce Spiracle Structures in the Absence of *Abd-B*

The above results suggest that ABD-B may be inducing spiracle morphogenesis indirectly by coordinately activating the realizator genes through the control of its primary targets.

To determine whether any of the ABD-B primary target is sufficient to induce the formation of spiracles by itself, we expressed Upd, Ems, Ct, and the *sal* downstream target Grn separately by using the *69B-Gal4* line and tested whether the spiracle realizators could be ectopically activated in the trunk (Figures 7I–7L and Figure S4). With the exception of Ct, none of the downstream primary targets, when expressed by itself, is capable of activating any of the spiracle realizators (Figure S4). Expression of Ct is unable to activate *Cad88C* (Figure 7I) but robustly activates *Cad96C* in dorsal patches of ectodermal cells (Figure 7J) and induces low levels of *Cad74A* and *Gef64C* in the trunk (Figures 7K and 7L). However, none of these cells invaginate to make spiracle-like structures.

We next analyzed the effects of simultaneous expression of Upd, Ems, Ct, and Grn. To make sure that any observed effects are not due to a feedback activation of the endogenous ABD-B protein, we performed the experiment in *Abd-B* mutants. Although the resulting embryos are very abnormal as a consequence of the unrestricted ectodermal expression of these four primary targets, they formed spiracle-like tubular structures that expressed spiracle-specific realizators (Figures 7A–7D). These spiracle-like structures are similar to the spiracles formed at that stage by ectopic ABD-B expression (Figures 7E–7H), supporting the conclusion that we have found most of the primary targets activated by ABD-B during spiracle organogenesis.

Discussion

To initiate spiracle organogenesis, ABD-B, in combination with local signaling molecules [42], activates a set of targets within the dorsal area of A8 [12] (Figure 7M). Here, we show that there may be as few as four direct targets for the posterior spiracle. The expression of the primary targets, with their corresponding cofactors, subdivides the organ into specific regions. After this patterning stage, specific cell behaviors are controlled by another set of transcription factors that include the GATA transcription factor Grn to bring about cell rearrangements [19], and the JAK/STAT signaling pathway, which induces posterior spiracle-cell elongation. The partially overlapping expression of these transcription

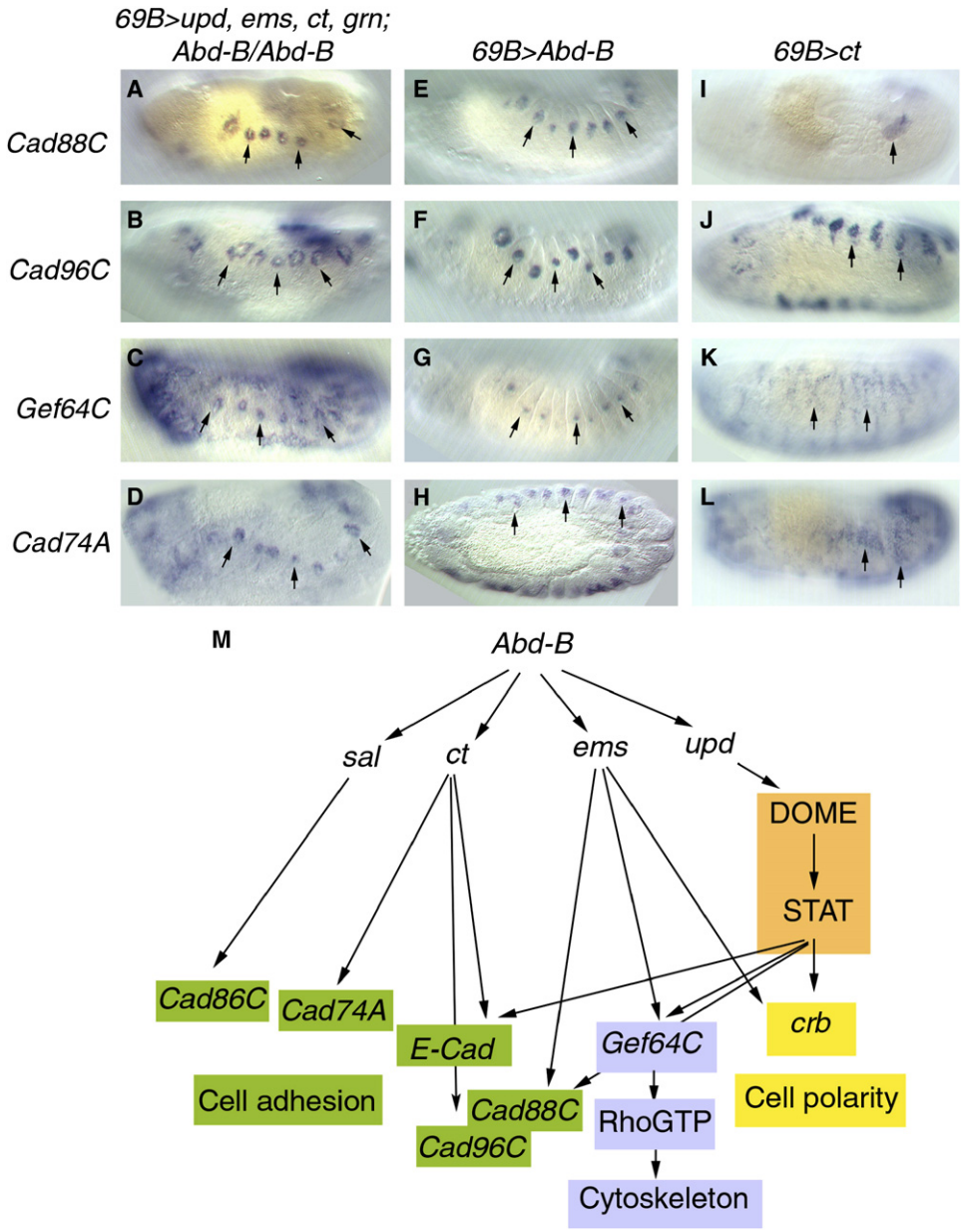

Figure 7. Simultaneous Expression of Four ABD-B Primary Targets Forms Spiracle-Like Tubular Structures and Activates Spiracle Realizators in the Absence of *Abd-B*

(A)–(D) show that in *Abd-B* mutant embryos, simultaneous ubiquitous ectodermal expression of the Ct, Ems, Upd, and the *sal* downstream target Grn can induce tubular spiracle-like structures that express spiracle-specific realizators. These structures are similar to those formed by ectopic expression of ABD-B in the ectoderm (E–H). (A), (E), and (I) show *cad88C*, (B), (F), and (J) show *cad96C*, (C), (G), and (K) show *Gef64C*, and (D), (H), and (L) show *cad74A*. (M) Summary of genetic interactions that are controlled downstream of ABD-B during posterior spiracle organogenesis. ABD-B, in combination with intrasegmental cues activates early-transcription factors and signaling molecules (*sal*, *ct*, *ems*, and *upd*) in the primordium of the posterior spiracle. These activate directly or indirectly the expression of cell adhesion (green), cell polarity (yellow) and cytoskeleton regulators (blue) that locally confer unique cell behaviors leading to spiracle morphogenesis. The orange box groups JAK/STAT pathway elements not directly regulated by ABD-B. To simplify (M), *grn* has been omitted. *grn* is activated in the stigmatophore downstream of *sal* and in the spiracular chamber downstream of *upd*. The genotype of embryos in (A)–(D) is *UAS-upd, UAS-grn, UAS-ct/+;69B-Gal4, Abd-B^{M1}/UAS-ems, Abd-B^{M1}*. The genotype of embryos in (E)–(H) is *69B-Gal4/UAS-AbdBm*. The genotype of embryos in (I)–(L) is *UAS-ct/+;69B-Gal4/+*.

factors has the potential to activate in particular subsets of spiracle cells different sets of realizator genes. In the spiracles, these realizators include cell-adhesion molecules, apico-basal polarity proteins, and cytoskeletal regulators. Thus, in this way, ABD-B activates a genetic cascade coordinating the local cell-specific behaviors that result in organogenesis.

Two main issues may explain why identification of the realizator genes has been so difficult. Primarily, by nature, many of these molecules are required for general functions in all cells. A screen for Hox realizators based on finding segment-specific defects would miss molecules like E-Cad or the Rho GTPases because of generalized embryonic malformations. Thus, their realizator

nature can only be uncovered when, through intermediate regulators, a link to the HOX protein is found. This is demonstrated in the case of *crb* where we found a specific spiracle enhancer, which directs its increased transcription. In the case of the cytoskeleton, the link is made through the use of specific regulatory GEF and GAP proteins that modulate the activity of the GTPases. A second problem has been that some of the realizator molecules function redundantly and therefore a mutational approach yields no result. This is the case with the nonclassic *cad88C* and *cad96C*, which only show a mutant phenotype if *E-cad* is also mutated. Although cell-adhesion molecules had been originally proposed to be realizators [3], it is surprising to find that there are four nonclassical cadherins with restricted expression in the spiracle.

Another unexpected finding has been the observation that the expression of apical- and basolateral-membrane proteins is modulated in the spiracle during the elongation stages. We have established a link between ABD-B and the apical determinant *crb* through the JAK/STAT pathway. During invagination, spiracle cells are going through major membrane reorganization, including apical constriction and basal elongation (Figures 2B, 2C, and 3A; [39]). Thus, Crb, which is required in many epithelia for maintenance of a proper zonula adherens, may be playing an important role for the polarized remodeling along the apico-basal cell axis. We have shown that Crb upregulation is functionally important for cell elongation but also that it is not the only function controlled by STAT. In this respect, it is important to note that spiracle-cell elongation occurs mainly through the increase of basolateral membranes. It is thus likely that the spiracle-gene network will also be controlling basal polarity determinants.

A role for ABD-B in regulation of the cytoskeleton in the posterior spiracles was expected because of the initial observations on cell elongation taking place in the spiracular chamber [12]. The observed effects of the dominant-negative and constitutively active forms of Rho GTPases on spiracle development support this hypothesis (Figure 6; [39]). The finding of *Gef64C* regulation by ABD-B in the spiracle cascade and the finding of spiracle invagination defects in RhoGAP *cv-c* mutants confirms that specific control of the Rho GTPases is an important feature of spiracle development.

Although all the realizators analyzed here are activated indirectly by ABD-B, we cannot disregard the possibility that ABD-B can also activate some others directly. Direct regulation of realizator genes by HOX may be important for differentiation of specific cell types [7, 43].

Conclusions

We have linked the activity of a HOX protein, through the regulation of a small number of intermediate regulators, to a battery of realizator genes. The local-specific modulation of these genes that in other contexts control cell adhesion, polarity, and organization of the cytoskeleton, would be sufficient to confer unique morphogenetic properties to the cells leading to the formation of a segment specific organ. Other examples in *Drosophila* include salivary-gland organogenesis, where SCR initially activates a cascade of downstream genes [44], and head formation where DFD activates *Dll* [45] but similar processes must be occurring in Hox-controlled organogenesis in vertebrates [4].

Experimental Procedures

Fly strains have been previously described [12, 22, 23, 27, 34, 40, 46]. The spiracle marker *grhD4-lacZ* and the *Abd-B^{M1}*, *ct^{db7}*, *ems^{9H}*, *Df(2L)5 sal salr^{-}*, *grn^{7J86}*, *cv-c^{7}*, *Df(1)os1A*, *stat92E^{mrl6346}*, *dome^{468}*, *crb^{D88-3}*, and *shg^{IH}* alleles were used. For rescuing of *Abd-B* mutants with its primary targets, *UAS-ct*, *UAS-upd* and *UAS-grn* were recombined on the second chromosome, *UAS-ems* (S. Brown, personal communication) was recombined to *Abd-B^{M1}*, and the ubiquitous ectodermal driver *69B-Gal4* was recombined to *Abd-B^{M1}*. Stocks carrying these mutations on the second and third chromosomes were established and crossed to generate all four UAS lines driven ectopically in an *Abd-B* mutant background. We used the *69B-Gal4*, *ems-Gal4*, *Klu-Gal4*, *Kr-Gal4*, and *h-Gal4* as driver lines.

The following antibodies were used: anti-Sas [26], anti-Crb, anti-E-Cad, anti-Dlg, anti-Nrt, anti-Ct (Developmental Studies Hybridoma Bank), anti-SAL (Schuh), and anti-ßGAL (Promega).

Whole-mount in situ hybridization was carried out according to standard methods with digoxigenin-labeled probes (Roche) generated by transcription of *cad74A* (CG6445; region 4887-5772), *cad86C* (CG4509; region 1388-2154), *cad88C* (CG3389, region 4961-5701), *cad96Cb* (CG10421, region 1100-1752), *sal*, *ems*, *Gef64C*, and *upd*. For double in situ, embryos were incubated with a mixture of digoxigenin- and fluorescein-labeled probes.

The construct pUAST-Cad74A-eGFP was generated from the EST RE10062. An ApaI site immediately before the stop codon of *Cad74A* was used to introduce eGFP, amplified by PCR from pEGFP N1 (Clontech). The fusion protein was then cloned into EcoRI/NotI sites of pUAST.

RNA Interference

dsRNA for *cad88C* and *cad96C* was synthesized as described [47], with the same DNA regions as for the antisense probes. A mixture of both dsRNAs (2 μg/μl each) was injected into 0- to 1-hr-old embryos derived from the cross between w; G13 *shg^{IH}* ems-GAL4/CyO females and w; *shg^{IH}* UAS-GFP-Actin/CyO males.

S2-Cell Aggregation Assays

Drosophila S2 cells were transfected according to [48] with pActin5.1-eGFP or with the following mixtures of plasmids: pActin5.1-GAL4 plus pUAST-E-Cad-GFP [49] and pActin5.1-GAL4 plus pUAST-Cad74A-eGFP. Cells were incubated for 24 hr at 25°C. The aggregation assay was done by distribution of 2.5 × 10^6 cells/well (24-well plate) in Schneider's medium followed by swirling in a horizontal shaker for 1 hr at 150 rpm. Schneider's medium was made Ca^{2+} free by addition of EGTA at 30 mM.

Analysis of ABD-B-Binding Sites

The *Drosophila melanogaster*, *virilis*, and *pseudobscura* genomic regions of *ct*, *upd*, and *sal* genes were compared with Vista alignments and putative ABD-B-binding sites were searched for in the conserved regions. We found clusters of ABD-B-binding sites in all three genes (Table S1). In the case of *upd*, one of them corresponded to an element capable of driving spiracle expression at stage 11 (S. Brown, personal communication).

Crb Spiracle Enhancer and Site-Directed Mutagenesis

Fragments of genomic *crb* were subcloned into pCaSpeRlacZ and injected into flies. The *crb43.2-lacZ* construct containing a 2 kb Ksp1 first-intron fragment (Figure 3) results predominantly in spiracle and head expression. The *43.2* fragment was directionally subcloned into pBluescript–SK+. Site-directed mutagenesis of the putative STAT-binding sites was then conducted on 43.2-pBluescript with the Stratagene Quikchange Kit according to manufacturers' instructions. Complementary primer pairs used for mutagenesis were as follows:

For the STAT92E-binding site at position 433 in the *43.2* fragment: 5′-ATTCATTCATTTCCATGTTCACATTTTCT-3′ (top strand) and 5′-AGAAAATGTGAACATGGAAATGAATGAAT-3′ (bottom strand). For the putative STAT92E site at position 711 in the *43.2* fragment: 5′-CTGTACGGCTTTCGTTTGAATTTCCGCCCT-3′ (top strand), 5′-AG GGCGGAAAAACAAACGAAAGCCGTACAG (bottom strand)-3′. For the putative STAT92E site at position 754 in the *43.2* fragment: 5′-ATCCATCATTTTCAGGGGTTAAATCGCGGC-3′ (top strand), 5′-GC CGCGATTTAACCCCTGAAAATGATGGAT-3′ (bottom strand). Mutant constructs were verified by sequencing.

Supplemental Data

Supplemental Data include four figures and one table and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/22/2206/DC1/.

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