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Compartmental modulation of abdominal Hox expression by *engrailed* and *sloppy-paired* patterns the fly ectoderm

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Abstract

In *Drosophila*, segmentation genes partition the early embryo into reiterative segments along the anterior-posterior axis, while Hox genes assign segments their identities. Each segment is also subdivided into distinct anterior (A) and posterior (P) compartments based on the expression of the *engrailed* (*en*) segmentation gene. Differences in Hox expression often correlate with compartmental boundaries, but the genetic basis for these differences is not well understood. In this study, we extend previous results to describe a genetic circuit that controls the differential expression of two Hox genes, *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*), within the A and P compartments of the abdominal ectoderm. Consistent with earlier findings, we show that *en* is essential for high Abd-A levels and low Ubx levels in the P compartment, whereas *sloppy-paired* (*slp*) is required for high Ubx levels in the A compartment. Overall, these results demonstrate that the compartmental expression of *Ubx* and *abd-A* is established through a repressive regulatory network between *en*, *slp*, *Ubx* and *abd-A*. We also show that *abd-A* expression in the P compartment is important for the formation of abdominal-specific cell types, suggesting that *en* and *slp* modulation of Hox expression within the A and P compartments is essential for embryonic patterning.

Keywords

Segmentation; Hox; compartment

INTRODUCTION

Drosophila embryogenesis provides one of the best-characterized models for understanding how a fertilized egg develops into a complex organism. Early in development the fly embryo is subdivided into segments that give rise to specialized head, thoracic and abdominal structures in the larva and adult fly. Genetic studies have revealed that the processes of dividing the embryo into repetitive segments (segmentation) and the assignment of each segment its unique fate (segment identity) are controlled by two distinct groups of genes (Carroll et al., 2001; DiNardo et al., 1994; Hatini and DiNardo, 2001; Lawrence, 1992;

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Lawrence and Struhl, 1996; Manak et al., 1994; McGinnis and Krumlauf, 1992; Sanson, 2001; St Johnston and Nusslein-Volhard, 1992). The segmentation genes, which encode both transcription factors and cell signaling molecules, subdivide the embryo into segments, while the Hox genes encode a family of homeodomain transcription factors that specify segment identity.

The segmentation genes further subdivide each segment of the *Drosophila* embryo into distinct anterior (A) and posterior (P) compartments. A compartment consists of groups of cells that share a common ancestry and identity (Blair, 1995; Garcia-Bellido et al., 1973; Lawrence, 1992; Lawrence and Struhl, 1996; Martinez-Arias and Lawrence, 1985). Compartments are given their identities by the expression, or lack of expression, of selector genes. For example, the P compartments in *Drosophila* express *engrailed* (*en*) while the A compartments do not express this gene (DiNardo et al., 1985; Fjose et al., 1985; Kornberg, 1981; Kornberg et al., 1985; Lawrence and Morata, 1976; Morata and Lawrence, 1975; Vincent and O'Farrell, 1992). *en* encodes a homeodomain-containing transcription factor that regulates target genes that encode transcription factors, cell signaling molecules, and adhesion proteins in P cells, giving them a unique identity (Desplan et al., 1985; Gibert, 2002; Jaynes and O'Farrell, 1991; Solano et al., 2003). The lack of P cells in *en* mutant embryos results in the mis-regulation of other segment polarity genes such as the *wingless* and *hedgehog* signaling genes, and a subsequent failure in segmentation and patterning of the fly embryo (Lawrence, 1992; Lawrence et al., 1999; Lawrence et al., 1996; Lawrence and Struhl, 1996).

Compartments play important roles in regulating the expression of many key developmental genes, including the Hox genes. It has long been recognized, for example, that the expression domains of Hox genes respect compartment boundaries (Carroll et al., 1988; Karch et al., 1990; Lawrence, 1992; Macias et al., 1990). The anterior expression boundaries of many Hox genes, including *Ultrabithorax* (*Ubx*), *Antennapedia* (*Antp*), *abdominal-A* (*abd-A*), *Sex combs reduced* (*Scr*), and *Deformed* (*Dfd*) all coincide with an A/P compartment boundary. In addition, Hox expression patterns in *Drosophila* are typically modulated in a segmentally reiterated pattern suggesting that segmentation genes are likely to fine-tune Hox expression patterns. For example, *abd-A* is expressed highly in P compartments of the abdomen, and in a lower, more graded fashion in A compartments (Macias et al., 1994). This *abd-A* pattern is partially complementary to the expression of *Ubx*, which is low in P compartments and high in A compartments (Mann, 1994). Moreover, the *en* segmentation gene has been shown to modulate different expression levels of *abd-A* and *Ubx* within the P compartment (Macias et al., 1994; Mann, 1994). However, while we have some understanding of how Hox expression patterns are established, many aspects, including how the segmentally reiterated patterns of Ubx and Abd-A are established, are not well understood.

There are several indications in the literature suggesting that segmentally modulated Hox expression patterns are functionally important for development. One such example is how the two Hox genes, *Ubx* and *abd-A*, repress their target gene *Distalless* (*Dll*) in the abdomen (Cohen et al., 1989; Mann, 1994; Vachon et al., 1992). *Dll* is a leg selector gene that is also required for the formation of the Keilin's organ (KO), a thoracic-specific larval sensory structure (Cohen et al., 1991; Cohen et al., 1989). Both the leg and the KO are derived from cells that straddle the A/P compartment boundary (Struhl, 1984). In wild type embryos, repression of *Dll* by *Ubx* and *abd-A* blocks leg and KO formation in the abdomen (Vachon et al., 1992). In *Ubx* mutant larvae, *Dll* is de-repressed in the A compartment of the first abdominal segment resulting in the partial formation of a KO (Mann, 1994). Conversely, in *abd-A* mutant larvae, *Dll* is de-repressed in abdominal P compartments resulting in the partial formation of KO in abdominal segments A1 to A7 (Mann, 1994). Thus, Abd-A

represses *Dll* in the P compartments of A1 to A7 while Ubx represses *Dll* in the A compartment of A1. Recently, a dissection of a *cis*-regulatory element responsible for *Dll* repression in the abdomen has provided molecular insights underlying Hox-mediated repression (Gebelein et al., 2002; Gebelein et al., 2004). In the P compartment, Abd-A binds this element directly with En to repress *Dll*, while in the A compartment, Ubx binds this element with an anterior-specific segmentation gene, *sloppy paired* (*slp*), to repress *Dll*. Thus, abdominal repression of *Dll* and the suppression of KOs, is mediated by the collaboration of the compartment-specific factors En and Slp with the compartmentally-modulated Hox proteins Abd-A and Ubx, respectively. These findings suggest that interactions between segmentation and segment identity genes regulate gene expression and thereby cell fate in a compartment-specific manner.

In this study, we characterize the regulatory relationships between the *slp* and *en* segmentation genes and the *abd-A* and *Ubx* Hox genes. Previous results suggested that *en* regulates the expression of *abd-A* and *Ubx* in the P compartment (Macias et al., 1994; Mann, 1994). Here, we extend these observations to describe a repressive genetic circuit between *en, slp, abd-A* and *Ubx* by using mutations that also removed *engrailed's* sister gene (*invected*) as well as through a number of loss- and gain-of-function assays with *en* and *slp*. Our findings confirm that *en* regulation of abdominal Hox genes results in high levels of Abd-A and low levels of Ubx in the P compartment (Macias et al., 1994; Mann, 1994), and show that *slp* is required for the high levels of Ubx in the A compartment. Moreover, we found that *abd-A* expression in the P compartment correlates with the formation of abdominal-specific sensory organ structures (the lateral chordotonal organs, lch5) and secretory cells (oenocytes) (Brodu et al., 2002; Heuer and Kaufman, 1992; Wong and Merritt, 2002). Thus, the En and Slp transcription factors not only function as abdominal Hox co-factors in regulating key target genes like *Dll*, but *en* and *slp* modulation of Hox expression levels within the A and P compartments is critical for patterning the embryo.

Materials and Methods

Fly stocks and antibody stainings

Immunocytochemistry was performed using the following antibodies: mouse anti-En (1:10, mAb4D9, the Developmental Studies Hybridoma Bank (DSHB) Univ of Iowa), mouse anti-Ubx (1:20, FP3.38) (White and Wilcox, 1985), rabbit anti-Ubx (1:500, a gift from Kevin White), mouse anti-Abd-A (1:400, a gift from Ian Duncan), rat anti-Abd-A (1:500) (Karch et al., 1990), guinea pig anti-Slp1 (1:500, a gift from John Reinitz) (Kosman et al., 1998), rabbit anti-β-gal (Cappell) and mouse anti-22C10 (1:20, DSHB). Mis-expression of *UAS-Ubx*, *UAS-Abd-A*, *UAS-En*, *UAS-VP16En*, and *UAS-Slp1* was driven by *prd-Gal4*. The *ato-lacZ* fly line (*ato7.2kb-lacZ*) was a gift from Yuh Nung Jan (Sun et al., 1998). The fly mutations used were as follows: *Df(2R)en-E* (removes *en* and *invected*, from Gary Struhl), ubx^{MX12}, $abd\text{-}A^{M1}$, $slp^{\Delta 34}$ (Cadigan et al., 1994b), and *slp/en* ($Df(2L)ed^{SZ1}$, *Df(2R)en-E)* double mutations (Cadigan et al., 1994b). Embryos were harvested, de-chorionated, fixed and immunostained using standard techniques. Images were taken using a Bio-Rad confocal microscope or a Zeiss Apotome fluorescent microscope.

RESULTS

Compartment-specific expression patterns of Ubx and Abd-A in the *Drosophila* abdomen

By stage 11 of embryogenesis, Ubx and Abd-A exhibit complex and segmentally reiterated expression patterns in the ectoderm (Fig. 1A). The primary anterior limits for Ubx and Abd-A are PS6 and PS7, respectively, although low levels of Ubx are observed in PS5 (Macias et al., 1990;White and Wilcox, 1985). At this stage, the highest levels of Ubx and Abd-A are in

adjacent anterior and posterior domains, respectively. Within each segment, cells within the ectoderm are assigned either an anterior (A) or posterior (P) compartment fate based on the expression of *en*. *en* encodes a transcription factor that specifies P cell identity and thus, is a useful marker for the P compartment (Kornberg, 1981;Kornberg et al., 1985;Lawrence and Morata, 1976;Morata and Lawrence, 1975). Analysis of Hox expression patterns with En revealed that, consistent with previous results (Carroll et al., 1988;Macias et al., 1990;Macias et al., 1994;Mann, 1994), Abd-A levels are high and Ubx levels are low within the P compartment (Fig. 1D and 1E). In the A compartment, high levels of *Ubx* expression are observed, but only in the half immediately anterior to En-positive P cells. As development progresses Abd-A levels increase in cells of the A compartment that lack significant *Ubx* expression (Fig. 1B and 1C), and *Ubx* expression in the P compartment weakly increases within PS6 but not within parasegments that express *abd-A* (Fig. 1E and F). Thus, throughout most of embryogenesis, the abdominal ectoderm from PS6 through PS12 express Ubx and Abd-A in an alternating pattern that largely correlates with compartmental identity.

Compartmental modulation of abdominal Hox expression is important for patterning the embryonic ectoderm

The expression patterns described above raise the question of why the levels of Ubx and Abd-A are modulated in the abdominal ectoderm. In *Drosophila*, the ectoderm differentiates to form the epidermis and the nervous system. The distinct expression of *Ubx* and *abd-A* in the A and P compartments suggest that these Hox factors may play different roles in patterning these tissues. In support of this idea, previous studies have shown that *abd-A*, but not *Ubx*, regulates the formation of specific abdominal cell types and sensory organs. For example, *abd-A* is required for the induction of secretory cells known as oenocytes (Brodu et al., 2002). To determine if oenocytes form within the P compartment, we immuno-stained *svp-lacZ* (an early marker of oenocytes (Elstob et al., 2001)) embryos for En, Abd-A and β-gal. As shown in Fig 2, *svp-lacZ* is expressed in En-positive cells that express high levels of Abd-A, demonstrating the origin of these cells is the P compartment. *abd-A* is also known to modulate the development of stretch receptors known as chordotonal (ch) organs (Heuer and Kaufman, 1992; Wong and Merritt, 2002). A ch organ consists of from one to 80 closely associated sensory structures called scolopodia, each of which develops from a single sensory organ precursor (SOP) cell (Lai and Orgogozo, 2004). In the fly embryo, a set of ch organs develops within the P compartment of each body segment (neurons visualized using mAb22C10 and the P compartment marked by *En-lacZ*, Fig 2b). However, the number and position of the scolopodia differ between the thorax and abdomen. The T2 and T3 thoracic ch organs contain three scolopodia with neurons located in a dorsal position (dch3), whereas the A1 through A7 ch organs contain five scolopodia with neurons located in a lateral position (lch5) (Fig. 2C). Previous studies have shown that the difference between thoracic and abdominal ch organs is dependent upon *abd-A*, as the ch organs within the A1 through A7 segments of *abd-A*$^{-}$ embryos are transformed into a dch3 fate (Heuer and Kaufman, 1992; Wong and Merritt, 2002). In contrast, the lch5 organs in the abdomen form normally in the P compartment of each abdominal segment in *Ubx*$^{-}$ embryos.

Each ch organ SOP cell is specified by the *atonal* proneural gene (Jarman et al., 1993). To determine if *ato* expression is modulated between the thoracic and abdominal segments, we co-stained *ato-lacZ* embryos for β-gal, Abd-A, and En (Sun et al., 1998). As expected most *ato-lacZ* expression is observed within the P compartment of each thoracic and abdominal segment (Fig. 2D). In early embryos (stage 11, Fig 2D), *ato-lacZ* levels are slightly higher in the abdominal than the thoracic segments, and this difference becomes more pronounced in older embryos (stage 14, Fig 2E). The high levels of *ato-lacZ* expression in the abdomen are lost in *abd-A*$^{-}$ embryos (Fig 2F). Moreover, ectopic *abd-A*, but not *Ubx*, expression in the

thorax is sufficient to induce higher levels of *ato-lacZ* in the thorax (Fig 2G, H). Overall, these results are consistent with previous studies demonstrating that *abd-A* induces oenocyte formation and modulates sensory organ development in the P compartment. In contrast, *Ubx* is expressed at low levels in this compartment and even when mis-expressed in P cells, it fails to perform these functions (Brodu et al., 2002). Thus, the compartmental modulation of abdominal Hox factor expression is important for the formation of distinct cell and organ types in the fly ectoderm. In the experiments described below, we address how this modulation of *Ubx* and *abd-A* expression arises.

Ubx and *abd-A* cross-regulation in the *Drosophila* abdomen

In general, Ubx and Abd-A are not expressed at high levels in the same cells of the ectoderm, suggesting they may repress each other. Consistent with this idea, *Ubx* is de-repressed in *abd-A* mutant embryos (Fig. 3A) (Struhl and White, 1985). However, Ubx de-repression is mainly observed in the A compartment and Ubx levels remain relatively low within En-positive cells of the P compartment (data not shown and (Mann, 1994)). In Ubx^{-} embryos, *abd-A* expression appears unaltered, indicating that *Ubx* is not required for *abd-A*'s wild type expression pattern (Fig. 3B). However, Castelli-Gair et al found that Ubx is able to transiently repress *abd-A* (Castelli-Gair et al., 1994). We addressed this question by mis-expressing Ubx using the Gal4-UAS system with the Paired Gal4 (PrdG4) driver. PrdG4 is ideal for this experiment as it is expressed in every other segment when *Ubx* and *abd-A* expression are being initiated, allowing for comparisons with wild type segments in the same embryo. Using this assay, we found that Abd-A represses *Ubx* in a cell autonomous manner (Fig. 3D), and ectopic Ubx was also able to partially repress *abd-A* (Fig. 3C) (Castelli-Gair et al., 1994). These results support two conclusions, first that Abd-A restricts *Ubx* expression, and second that Ubx has the potential to repress *abd-A* but is not required to perform this function.

en modulates *Ubx* and *abd-A* expression in the P compartment

Ubx and Abd-A cross-regulation may contribute to the stabilization of their expression patterns, but it does not reveal how these patterns are initiated. The *Ubx* and *abd-A* segmentally reiterated expression patterns suggest that segmentation genes may regulate abdominal Hox expression. The high levels of Abd-A and low levels of Ubx in the P compartment indicate that En regulates their expression (Macias et al., 1994; Mann, 1994). Although previous experiments suggested that *en* positively regulates *abd-A*, *en's* sister gene *invected* was not mutant in these experiments, raising the possibility that incomplete phenotypes were being measured (Macias et al., 1994). We therefore analyzed stage 11 *en inv* double mutants, hereafter referred to as en^{-}. At stage 11, high levels of Abd-A are not observed in en^{-} embryos except within the lateral regions of PS12 and 13 (Fig. 4B). As this expression is also observed in A cells of wild type embryos, it is not dependent on En (Fig. 4A and 4B). In older en^{-} embryos (stage 14), *Ubx* expression weakly expands posteriorly, which is most clearly seen in PS7 and PS8 (Fig. 4D). Compared to wild type, the *Ubx* and *abd-A* patterns are less organized in en^{-} embryos with most cells in PS7 and PS8 expressing *Ubx* and most cells in PS9 through PS12 expressing *abd-A* (Fig. 4D). However, even in these embryos most cells express either high levels of Ubx or Abd-A, but not both Hox factors.

If En activates *abd-A* then ectopic En should stimulate *abd-A*. Using *PrdG4* to mis-express En, we determined that En induces *abd-A* expression in a cell autonomous manner (Fig. 4E). Consistent with a repressive effect of En on *Ubx*, ectopic En repressed *Ubx* (Fig. 4G). En is most effective at repressing *Ubx* in parasegments that express *abd-A*, suggesting that at least some of its effects on Ubx levels may be mediated indirectly, through regulation of *abd-A*. Overall these data demonstrate that En modulates abdominal Hox expression within the P

compartment. Further, *abd-A* has at least two phases of expression in the embryo, first it is activated within P cells and second its expression increases in A cells that have low Ubx levels.

slp regulates *Ubx* and *abd-A* in the A compartment

Abdominal Hox expression within the A compartment is not uniform, suggesting that factors within this compartment regulate *Ubx* and *abd-A*. Previous studies have shown that the partially redundant *slp* genes (*slp1* and *slp2*, referred to here as *slp*) are expressed in approximately the posterior half of the A compartment (Fig. 5A) (Cadigan et al., 1994b;Grossniklaus et al., 1992). Much like *en*, *slp* is required for proper segmentation of the embryo, and the *slp* genes encode transcriptional regulatory proteins. Analysis of Slp1 and Ubx protein levels in stage 11 embryos reveals that where *slp1* expression is high so is *Ubx* (Fig. 5B). Consistently, *abd-A* expression is mutually exclusive with Slp1 (Fig. 5C). Later in embryogenesis (stage 12 and older) *slp1* expression is restricted ventrally (Fig. 5D). Interestingly, at the same time Abd-A levels increase in A compartment cells, but only within those that lack Slp1. This is most clearly seen in ventral views of a stage 12 embryo, where high levels of Abd-A surround Slp-positive cells (Fig. 5D). The correlation between low Abd-A levels and high Slp levels is maintained throughout embryogenesis. Thus, *slp* is a good candidate for an A compartment regulator of abdominal Hox expression.

To test this idea, we used both loss- and gain-of-function approaches to manipulate *slp* activity. As shown in Fig. 5E, *Ubx* and *abd-A* expression are altered in embryos lacking both *slp* genes (Δ34B is a deletion that removes *slp1* and *slp2*). As in wild type stage 11 embryos, *abd-A* is expressed in stripes within the abdomen of *slp* mutant embryos (Fig. 5E). However, these stripes tend to be wider and there are fewer of them. Previous studies have shown that in the absence of *slp* the odd-numbered stripes of *en* are lost and the even-numbered stripes are broadened (Cadigan et al., 1994b;Grossniklaus et al., 1992;Jaynes and Fujioka, 2004). Consistently, the slp^{-} embryo in Fig. 5F shows that the odd-numbered *en* stripes (PS7, 9, and 11 are shown) have mostly disappeared and that Abd-A is greatly reduced in these regions. In the even parasegments, *en* expression broadens and Abd-A levels are high. In addition, Abd-A levels increase in A cells immediately anterior to En-positive cells in slp^{-} embryos, which in wild type embryos is where *slp1* is expressed (Fig. 5G). The increase in *abd-A* expression is accompanied by a decrease in *Ubx* (Fig. 5E). Only in PS6, which lacks Abd-A, are Ubx levels normal. Strikingly, in older slp^{-} embryos (stage 14), *abd-A* is expressed uniformly from PS7 to PS12 at the expense of *Ubx* expression (Fig. 5H). Consistent with these findings, mis-expression of Slp1 using PrdG4 repressed *abd-A* and led to a moderate increase in *Ubx* levels (Fig. 5I and 5J). Taken together these results suggest that *slp* modulates Hox expression in the A compartment of the abdomen by repressing *abd-A*, which allows Ubx levels to increase.

Cross-regulation between En and Slp

The combined results of *en* and *slp* gain- and loss-of-function provide a possible explanation for how compartment-specific expression patterns of the abdominal Hox factors arise. In the P compartment, En stimulates *abd-A* and both En and Abd-A repress *Ubx*. In A compartment cells that express Slp, *abd-A* is repressed allowing *Ubx* expression. In A compartment cells that do not express Slp, Abd-A levels increase, which represses *Ubx*. Complicating our ability to determine how *en* and *slp* regulate the Hox factors is that Slp represses *en* and En represses *slp* (Suppl Fig. 1 and (Alexandre and Vincent, 2003; Cadigan et al., 1994a; Kobayashi et al., 2003)). Thus, it is possible that either En and/or Slp modulate Hox expression indirectly through mutual cross-repression. We address this question by assaying Hox expression in cells that 1) express an activator form of En (VP16En

(Alexandre and Vincent, 2003)), 2) express both En and Slp, and 3) express either En or Slp in *en*$^{-}$ *slp*$^{-}$ double mutant embryos.

En indirectly regulates *abd-A* and *Ubx*

To determine if En directly activates *abd-A* and/or directly represses *Ubx*, we used *UAS-VP16En* flies, in which the repression domain of En was replaced with the VP16 activation domain (Alexandre and Vincent, 2003). VP16En has previously been shown to activate other *en* targets, including *slp1* (Suppl Fig. 1C (Alexandre and Vincent, 2003)). If En directly activates *abd-A* then VP16En should also stimulate its expression. However, as shown in Fig. 4F, VP16En represses *abd-A*. This result is consistent with En stimulating *abd-A* indirectly, by repressing a repressor of *abd-A*. One obvious candidate that we test below is *slp*, as it is highly induced by VP16En and is capable of repressing *abd-A*. We also determined Vp16En's affect on *Ubx*. If En directly represses *Ubx*, then VP16En should activate *Ubx*. In stage 11 embryos, VP16En weakly stimulated *Ubx* in some cells (Fig. 4H). However, the dynamics of this increase are much slower for *Ubx* than for *slp1*, a known direct En-target gene, suggesting that En indirectly regulates *Ubx*. Because VP16En represses *abd-A*, the gradual increase in Ubx may be due to reduced Abd-A levels in these cells. In summary, the VP16En data suggests that En indirectly regulates the expression of both abdominal Hox proteins, perhaps through the repression of *slp1*.

En regulates abdominal Hox expression independently of Slp

Cross-repression between *en* and *slp* complicates the analysis of how they regulate Hox expression. To determine if En activates *abd-A* and represses *Ubx* in the presence of Slp1, we co-expressed both factors using PrdG4 and analyzed *abd-A* and *Ubx* levels. As shown in Fig. 6, En failed to activate *abd-A* but repressed *Ubx* in the presence of Slp1 (Fig. 6A and 6B). This finding suggests that En stimulates *abd-A*, at least in part, by repressing *slp*. These data also demonstrate that Slp1 is unable to stimulate *Ubx* if En is present, indicating that Slp stimulates *Ubx*, at least in part, by repressing *en*.

To further test the role of *en* and *slp* in regulating abdominal Hox factors we analyzed *Ubx* and *abd-A* expression in *en*$^{-}$ *slp*$^{-}$ mutant embryos. Fig. 6C shows that *abd-A* and *Ubx* expression in early *en*$^{-}$ *slp*$^{-}$ embryos is relatively unpatterned with Ubx levels highest anterior to Abd-A expressing cells. In older *en*$^{-}$ *slp*$^{-}$ embryos, Abd-A increases throughout the abdominal ectoderm while Ubx levels decrease (Fig. 6D). This is in stark contrast to wild type embryos, which alternate high levels of Ubx and Abd-A throughout the abdomen (compare Fig. 6D to Fig. 4C). This finding further supports the idea that *en* and *slp* are essential for proper abdominal Hox gene expression.

The relatively uniform expression of *Ubx* and *abd-A* in *en*$^{-}$ *slp*$^{-}$ embryos provides an ideal genetic background to ectopically provide either En or Slp and analyze Hox expression. Mis-expression of En using PrdG4 in *en*$^{-}$ *slp*$^{-}$ mutants stimulated *abd-A* and repressed *Ubx* (Fig. 6E and 6F). These results suggest that En regulates these two Hox factors independently of its affect on *slp*. If En directly activates *abd-A* in *en*$^{-}$ *slp*$^{-}$ embryos, then VP16En should also stimulate *abd-A* in these embryos. However, Abd-A levels do not increase in response to VP16En, and in most, but not all, cells Abd-A levels decrease (Fig. 6G). This result indicates that En is repressing an additional repressor (R) of *abd-A*. According to this idea, VP16En activates R, which results in decreased Abd-A levels. We also examined *Ubx* expression in *en*$^{-}$ *slp*$^{-}$ embryos that express VP16En. In most stage 11 embryos, VP16En stimulated *Ubx* expression (Fig. 6H). In older embryos expressing VP16En, the increase in Ubx levels was correlated with a decrease in Abd-A (data not shown). Because the ability of VP16En to increase *Ubx* expression was slow, we propose the following model: En indirectly regulates abdominal Hox patterns by directly repressing

slp and another intermediary repressor (R) of *abd-A* (Figure 7). In P cells, En repression of *slp* and R allows Abd-A levels to increase. The combination of En and Abd-A in P cells represses Ubx.

Slp represses *abd-A* independently of En

The experiments described above demonstrate that Slp can repress *en* and *abd-A* (Fig. 5 and Suppl Fig. 1). The co-expression of Slp with En using PrdG4 indicates that Slp does so even in the presence of En (Fig. 6A). To determine if Slp1 represses *abd-A* in the absence of En, we ectopically expressed Slp1 in en^{-} slp^{-} embryos. Using this assay, we found that Slp1 represses *abd-A* in these embryos (Fig. 6I), and that Slp expressing cells have increased Ubx levels (Fig. 6J). This effect on abdominal Hox expression was also seen in older embryos (Fig. 6K) and is consistent with our previous results that suggest Slp1 represses *abd-A*, which thereby allows Ubx levels to increase. One possibility is that both Slp and Ubx are required to repress *abd-A*, as recent studies have shown that both are required for the repression of a common target gene (Gebelein et al., 2004). However, Slp does not require *Ubx* to repress *abd-A*, as Ubx^{-} embryos show wild type *slp1* and *abd-A* expression in the absence of *Ubx* function (Fig. 6L).

DISCUSSION

Regulation of Hox gene expression by segmentation genes

The Hox genes comprise a family of transcription factors that specify cell identities along the A-P axis in both vertebrates and invertebrates (Carroll et al., 2001). The precise regulation of Hox gene expression is therefore essential for the development of different cell types and morphological structures within the head, thorax, and abdomen of each organism. In *Drosophila*, the expression of the eight Hox genes during embryonic development is controlled by several types of transcriptional regulators. First, early in the fly embryo, the Gap genes demarcate the A-P limits of Hox gene expression. Hunchback (Hb), for example, represses the expression of the abdominal Hox genes to establish the anterior expression limits of both *Ubx* and *abd-A* (Shimell et al., 2000; White and Lehmann, 1986). Additional Gap genes expressed in distinct regions of the early embryo help establish the A-P limits for each Hox factor (Casares and Sanchez-Herrero, 1995; Qian et al., 1991; Reinitz and Levine, 1990). Once the Gap genes establish broad Hox expression domains, the Hox factors refine their own expression patterns. In general, the posterior Hox factors repress the expression of more anterior Hox factors and thereby establish distinct regions of Hox gene expression along the A-P axis (Capovilla and Botas, 1998; Struhl and White, 1985). Lastly, the *Polycomb* (*Pc*) and *trithorax* (*trx*) Group genes are required for the long-term repression (*Pc-G*) and activation (*trx-G*) of the Hox genes (Gould, 1997). Taken together, these transcriptional regulatory mechanisms provide a broad outline for how Hox gene expression patterns are established and maintained along the A-P axis.

In this study, we show that *en* and *slp*, which are expressed in cells of the P or A compartments, respectively, are also required to pattern abdominal Hox expression. Consistent with previous reports, we found that En is required for the high levels of Abd-A and low levels of Ubx observed in P compartment cells (Macias et al., 1994; Mann, 1994). We extend these observations by showing that En performs these functions not by directly activating *abd-A* but by repressing two repressors of *abd-A* (Fig. 7). First, En represses *slp* (Alexandre and Vincent, 2003; Cadigan et al., 1994a; Kobayashi et al., 2003), which we show is a potent repressor of *abd-A*. Second, we determined that even in the absence of *slp*, En induces *abd-A* expression whereas Vp16En represses *abd-A* expression. These results are consistent with En repressing an additional *abd-A* repressor, which we have called R (Fig. 7). Although the identity of R is currently unknown, we predict that R is repressed by En

and thus will not be expressed in the P compartment. Moreover, we predict R is expressed transiently in the A compartment and begins to fade by embryonic stage 12, allowing *abd-A* expression in these cells (Fig. 7). We have tested two likely candidates for R: Odd-skipped (Odd), which, like Slp, is expressed only in the A compartment (Mullen and DiNardo, 1995), and Cubitus-interruptis (Ci), which is repressed by En in the P compartment (Eaton and Kornberg, 1990). Both Odd and Ci are known to function as transcriptional repressors. However, mis-expression of either Odd or a constitutive repressor form of Ci in the P compartment using PrdG4 did not dramatically alter *abd-A* expression (data not shown). These results suggest that Odd and Ci do not repress *abd-A*, and that an as yet unidentified factor functions in this capacity.

While En expression in the P compartment explains how Abd-A levels become high and Ubx levels become low, it does not reveal how Ubx expression is maintained in the A compartment of abdominal segments that express *abd-A*. In this study, we provide the following data demonstrating that Slp is required to establish alternating stripes of Ubx and Abd-A in the fly embryo. 1) Slp is highly co-expressed with Ubx, and Abd-A levels are low in Slp-positive cells. 2) In the absence of *slp* function, *abd-A* is de-repressed, resulting in a loss of *Ubx* expression within the abdominal ectoderm. 3) Ectopic Slp expression represses *abd-A* and allows for an expansion in *Ubx* expression. Based on these findings, we propose that Slp represses the expression of both *en* and *abd-A* to allow for the continued expression of Ubx in the A compartment (Fig. 7). In conclusion, these experiments reveal a symmetry between En and Slp in the establishment of abdominal Hox expression patterns in the A and P compartments: First, *en* and *slp* cross-repress each other to establish a sharp boundary between A and P cells, and second, *en* and *slp* repress either *Ubx* (En) or *abd-A* (Slp) to modulate abdominal Hox expression in a compartment-specific manner.

Compartment-specific Hox expression patterns and the development of the fly ectoderm

Our findings that *en* and *slp* modulate Ubx and Abd-A expression in the A and P compartments suggest that the abdominal Hox factors perform compartment-specific functions to pattern the ectoderm. The fly ectoderm gives rise to epidermal cells that secrete a patterned cuticle and neuronal cells that comprise the peripheral and central nervous system. Here we analyzed the development of two abdominal-specific cell and organ subtypes, the formation of secretory cells known as oenocytes and the formation of a specific sensory organ (the lateral chordotonal organ consisting of 5 scolopodia, lch5) in the PNS. Previous studies have shown that both oenocytes and the lch5 organs require *abd-A* but not *Ubx* function and that the lch5 organs form within the P compartment of the abdominal segments (Brodu et al., 2002; Heuer and Kaufman, 1992; Wong and Merritt, 2002). We determined that oenocytes also form in the P compartment of abdominal segments, and that the expression of a proneural reporter gene (*ato-lacZ*) that marks the formation of chordotonal organs is stimulated by Abd-A. Moreover, even the forced expression of Ubx within P compartment cells fails to induce oenocyte formation (Brodu et al., 2002), lch5 formation (Heuer and Kaufman, 1992; Wong and Merritt, 2002), or enhanced *ato-lacZ* expression (Fig. 2), revealing that these processes can only be regulated by Abd-A.

Overall, the *en* and *slp* genes are best known for their ability to regulate segmentation and the expression of signaling molecules that pattern the embryo (Cadigan et al., 1994b; Lawrence et al., 1999; Lawrence et al., 1996). In this study, we have demonstrated that the En and Slp factors also pattern the abdomen by differentially regulating the expression of Hox genes in the ectoderm. Moreover, our previous studies have revealed that En and Abd-A within the P compartment and Slp and Ubx within the A compartment work in concert to repress the expression of the leg selector gene, *Dll*, in the abdomen (Gebelein et al., 2004). Together with oenocyte and ch organ formation, these results suggest that the modulation of

Hox expression in the A and P compartments by En and Slp is essential for the compartment-specific control of gene expression during embryonic development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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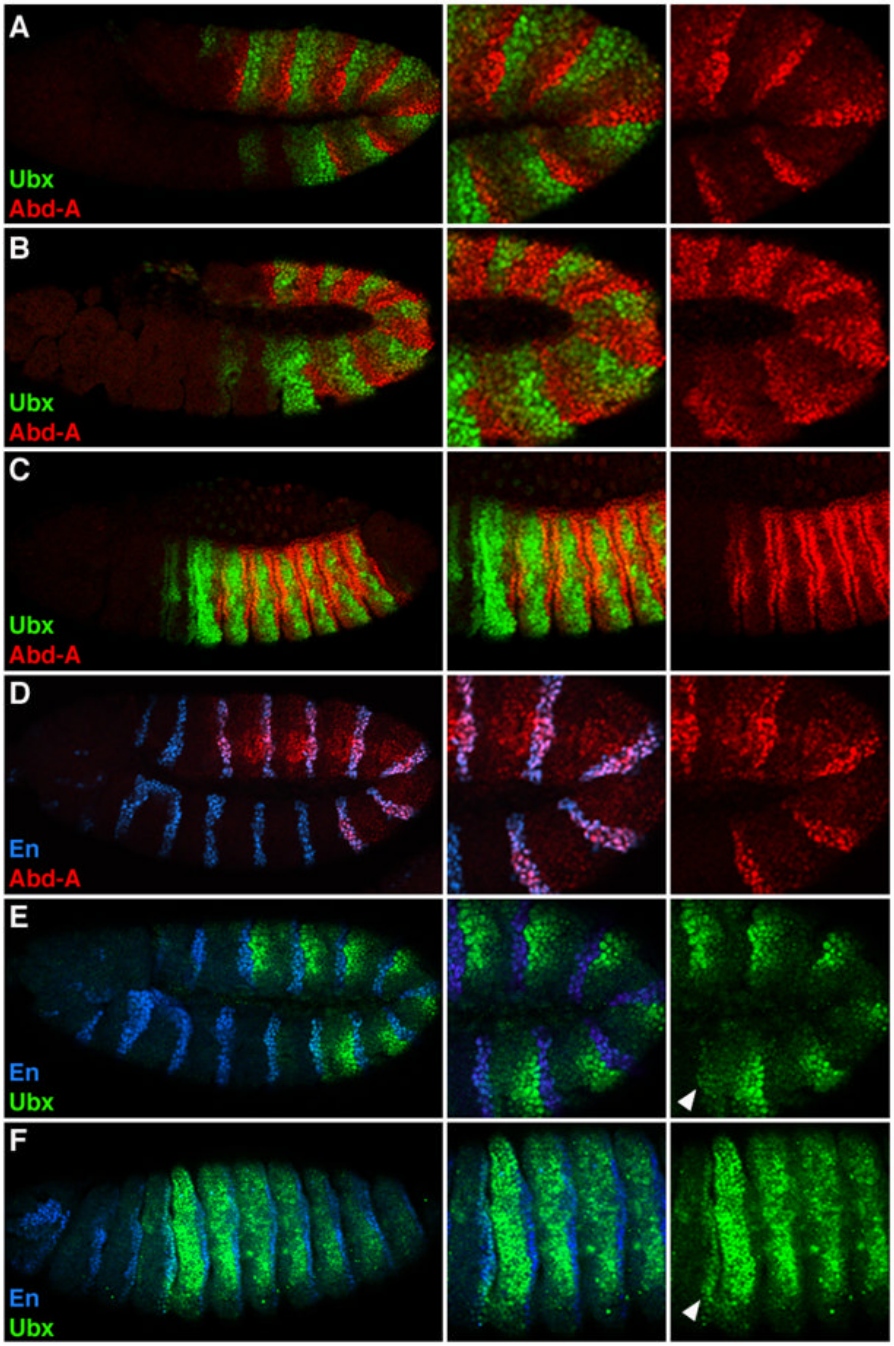

Figure 1. Compartmental expression pattern of *Ubx* and *abd-A*

Lateral views of *Drosophila* embryos immunostained for Ubx (green), Abd-A (red) and En (blue). **A, B and C.** Wild type embryos showing the expression patterns of Ubx and Abd-A at stage 11 (A), stage 12 (B), and stage 14 (C). **D and E.** Wild type stage 11 embryos have high levels of Abd-A and low levels of Ubx in En-positive cells. **F.** Stage 16 wild type embryo showing increased levels of Ubx in En-positive cells within ps7 (arrowhead).

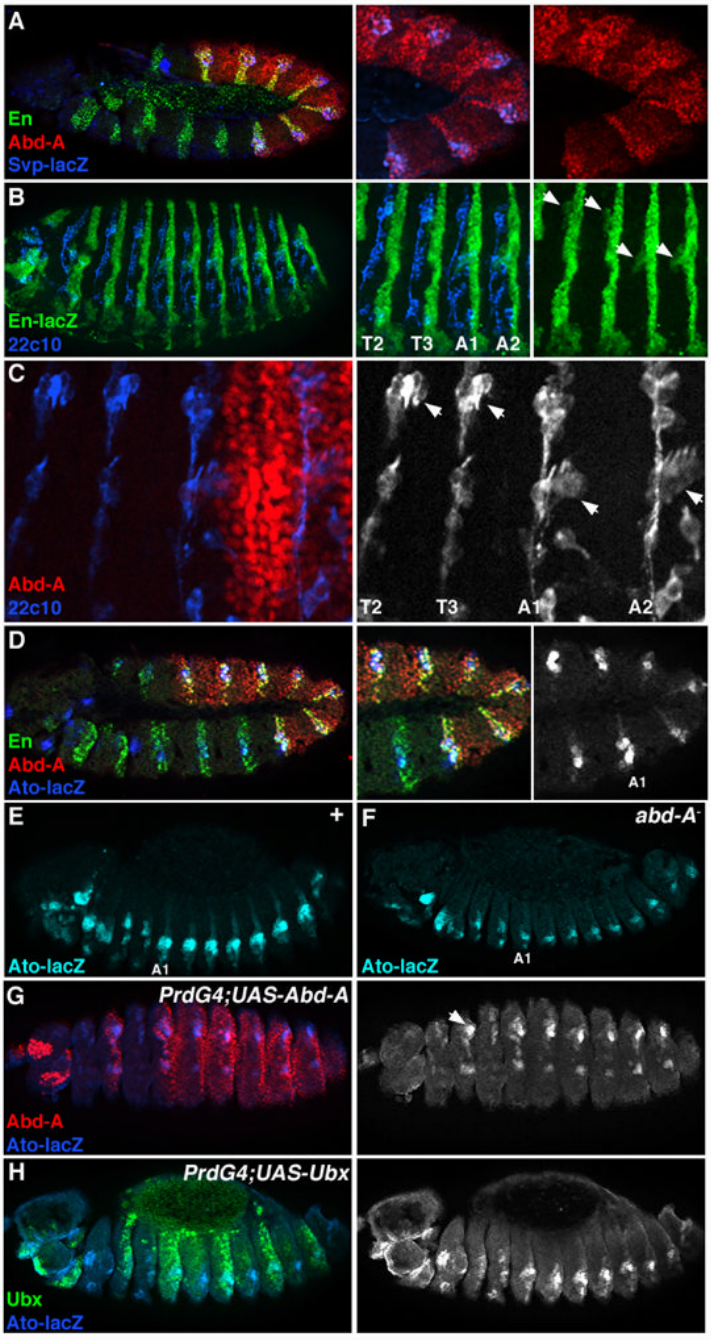

Figure 2. Developmental role of *abd-A* expression in the P compartment

A. Lateral view of a stage 11 *svp-lacZ Drosophila* embryo immunostained for Abd-A (red), En (green), and β-gal (blue). *svp-lacZ* expression serves as an early marker for oenocytes. **B.** Stage 16 *en-lacZ Drosophila* embryo immunostained for β-gal (green) and with a PNS-specific neuronal marker (mAb22C10, blue). Arrows (in right panel) point to where the dch3 (in T2/T3) and lch5 (A1/A2) sensory organs form within the P compartment. **C.** Close up view of the T2 and T3 thoracic and A1 and A2 abdominal segments immuostained with Abd-A (red) and mAb22C10 (blue, black and white at right). **D.** Lateral view of a stage 11 *ato-lacZ* embryo immunostained for Abd-A (red), En (green) and β-gal (blue, black and white at right). Note that the majority of *ato-lacZ* expression in the thorax and abdomen is

within the P compartment. **E.** Wild type *ato-lacZ* embryo (stage 14) immunostained for β-gal (blue). Note the higher levels of *ato-lacZ* within the abdomen than the thorax. **F.** *ato-lacZ* expression is the same in all body segments of *abd-A*$^{-}$ embryos. **G.** *PrdG4;UAS-Abd-A* embryos show that Abd-A (red) expression within the thorax stimulates *ato-lacZ* activity (blue, black and white at right). **H.** *PrdG4;UAS-Ubx* embryos show that ectopic Ubx (green) does not alter *ato-lacZ* levels within the thorax.

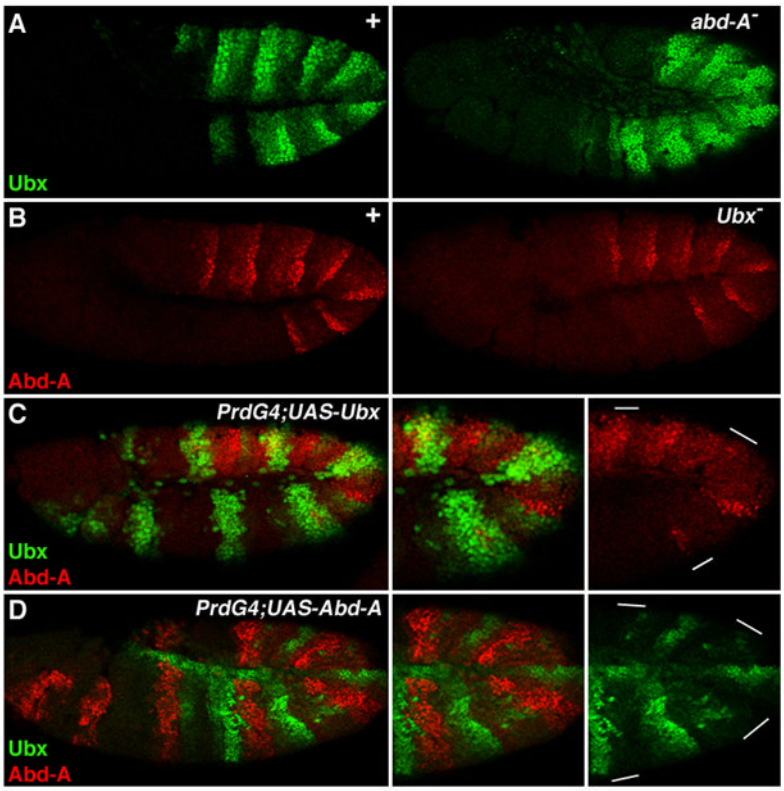

Figure 3. Cross-regulation between *Ubx* and *abd-A*

Lateral views of stage 11 *Drosophila* embryos immunostained for Ubx (green) and Abd-A (red). **A.** Pattern of *Ubx* expression in wild type (left) and *abd-A*$^{-}$ embryos. Note that *Ubx* is de-repressed in *abd-A* mutants. **B.** *abd-A* expression is the same in wild type (left) and *Ubx* mutant (right) embryos. **C.** *PrdG4;UAS-Ubx* embryo showing that ectopic Ubx represses *abd-A*. **D.** *PrdG4;UAS-Abd-A* embryo showing that ectopic Abd-A represses *Ubx*.

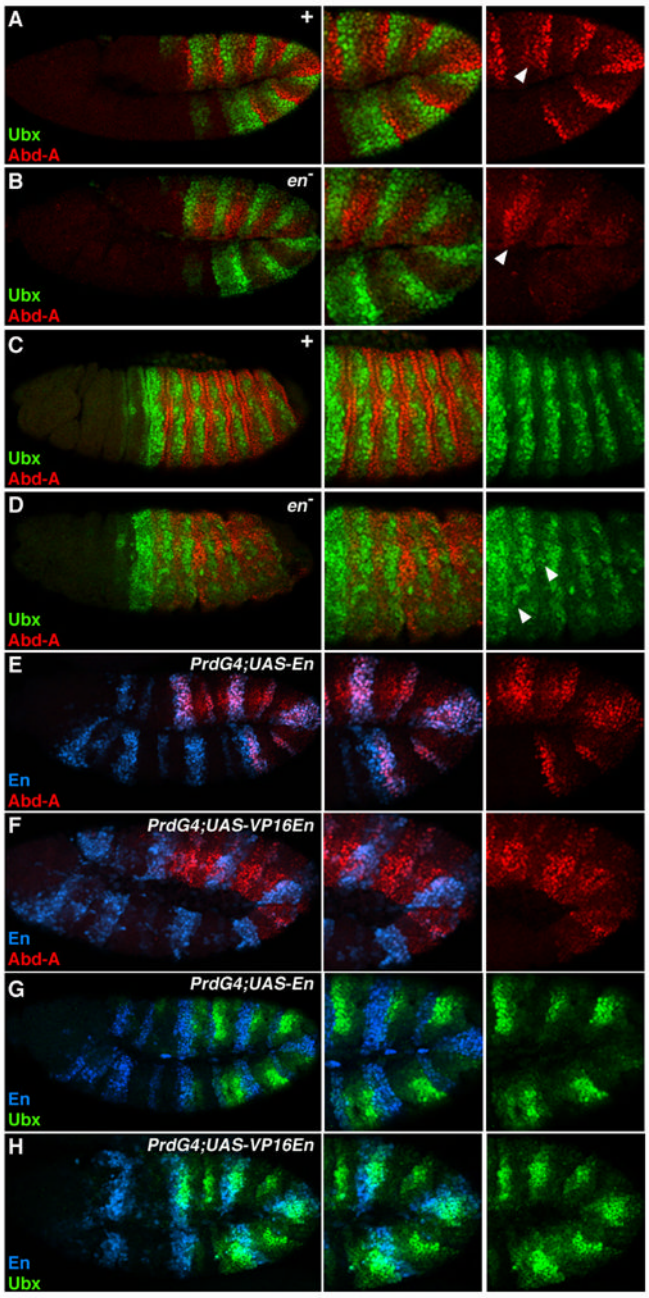

Figure 4. En regulates abdominal Hox expression in the P compartment

Lateral views of *Drosophila* embryos immunostained for Ubx (green), Abd-A (red), and En (blue). **A and B.** *Ubx* and *abd-A* expression in wild type (A) and *en* mutant (B) stage 11 embryos. Note that Ubx levels are relatively normal whereas Abd-A levels are decreased. Arrowhead points to lateral cells that express Abd-A independent of En. **C and D.** *Ubx* and *abd-A* expression in wild type (A) and *en* mutant (B) stage 14 embryos. Note that in older en^{-} embryos Ubx is de-repressed in the anterior abdominal parasegments (arrowhead), whereas posterior abdominal parasegments show increased *abd-A* expression. **E and G.** *PrdG4;UAS-En* embryos illustrating that ectopic En stimulates *abd-A* (E) and represses *Ubx* (G). **F and H.** *PrdG4;UAS-VP16En* embryos demonstrate that VP16En represses *abd-A* (F), and weakly activates *Ubx* (H).

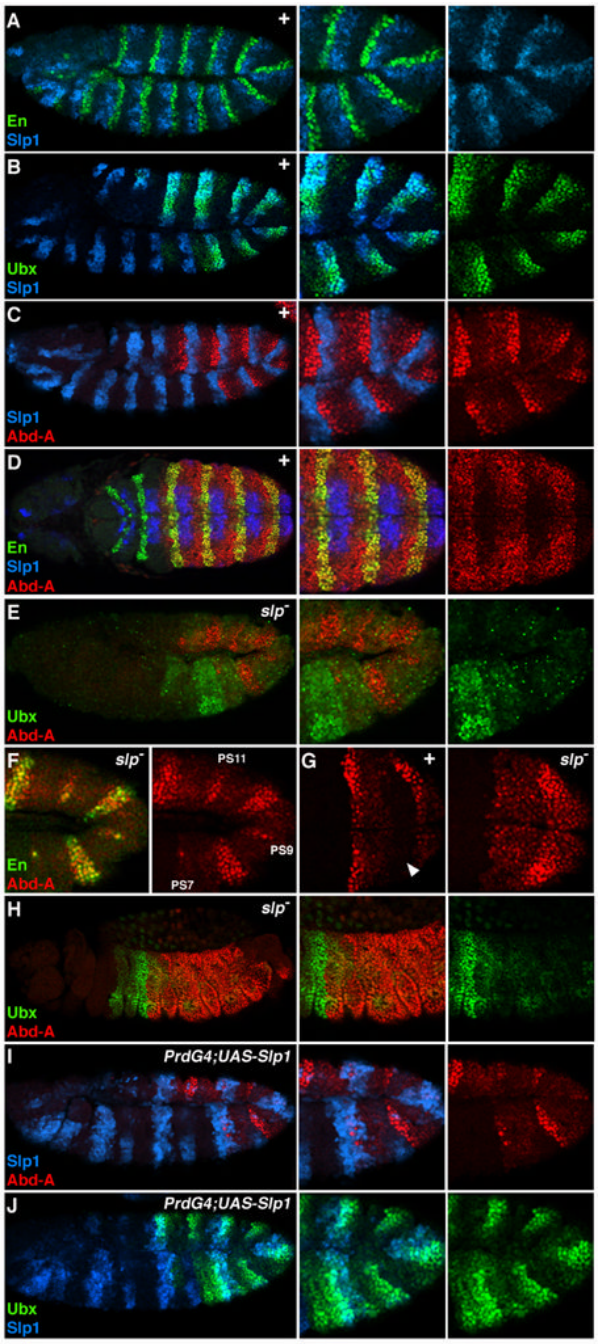

Figure 5. Slp regulates abdominal Hox expression in the A compartment

A, B, C, E, F, I, and J are lateral views of stage 11 *Drosophila* embryos. D and G are ventral views of the abdomen, and H is a lateral view of a stage 14 embryo. All embryos were immunostained for Slp1 (blue), Ubx or En (green), and Abd-A (red) as indicated. **A.** Slp1 is expressed in cells anterior to En-positive cells within each segment of the embryo. **B, C, and D.** Wild type embryos have high levels of Ubx (B) and low levels of Abd-A (C) in Slp1-positive cells. In stage 12 embryos Abd-A protein expression increases in Slp1-negative cells of the A compartment to similar levels as the P compartment (marked by En, green in D). **E-H.** *ubx* and *abd-A* expression in *slp*$^{-}$ embryos. **E.** Ubx levels are decreased in *slp*$^{-}$ embryos except in PS6 cells that lack *abd-A* expression. **F.** Close up view of Abd-A

and En expression in *slp* mutant embryos shows that the odd-numbered stripes (labeled) of En are lost and even-numbered stripes are broadened. As in wild type embryos, *abd-A* expression correlates with *en* expression. **G.** Close up ventral view of an slp^{-} embryo (right) showing that Abd-A levels increase in cells immediately preceding the posterior compartment compared to wild type embryos (left). In wild type embryos, these cells express Slp1 (arrowhead). **H.** *Ubx* expression remains restricted to PS6 in a stage 14 slp^{-} embryo and Abd-A levels are uniform in the ectoderm of PS7 through PS12. **I and J.** *PrdG4;UAS-Slp1* embryos show that ectopic Slp1 represses *abd-A* (I) and has little affect on *Ubx* (J).

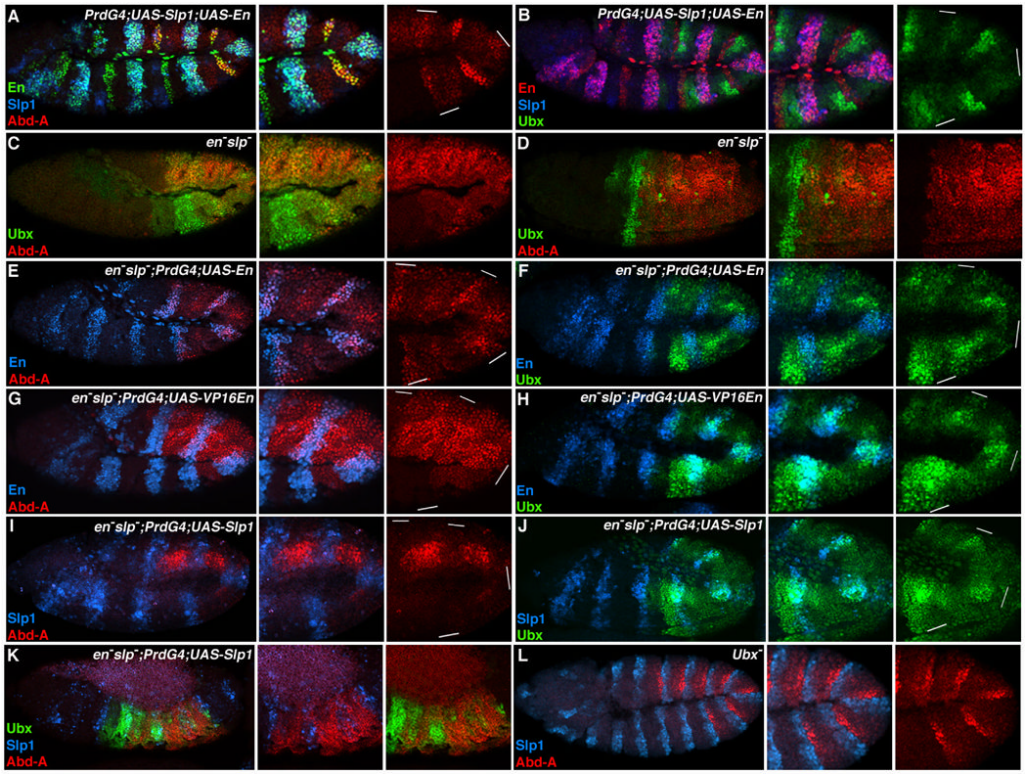

Figure 6. En and Slp regulate *Ubx* and *abd-A* independently

Lateral views of stage 11 (A, B, C, E, F, G, H, I, J, and L) and stage 14 *Drosophila* embryos (D and K). **A.** *PrdG4;UAS-Slp1;UAS-En* embryos show that ectopic Slp1 (blue) represses *abd-A* (red) in the presence of En (green). **B.** *PrdG4;UAS-Slp1;UAS-En* embryos. Ectopic En (red) represses *Ubx* (green) in the presence of Slp1(blue). **C and D.** Ubx (green) and Abd-A (red) expression in *en*$^{-}$ *slp*$^{-}$ embryos. Note the loss in patterned abdominal Hox expression within *en*$^{-}$ *slp*$^{-}$ embryos compared to wild type embryos (see Fig 1C). **E and F.** *en*$^{-}$ *slp*$^{-}$*;PrdG4;UAS-En* embryos demonstrates that ectopic En (blue) induces Abd-A (red in E) and represses Ubx (green in F). **G and H.** *en*$^{-}$ *slp*$^{-}$*;PrdG4;UAS-VP16En* embryos. VP16En (blue) represses Abd-A (red in G) and stimulates Ubx (green in H) levels. **I and J.** *en*$^{-}$ *slp*$^{-}$*;PrdG4;UAS-Slp1* embryos show that ectopic Slp1 (blue) represses *abd-A* (red in I) and increases *Ubx* (green in J). **K.** Stage 14 *en*$^{-}$ *slp*$^{-}$*;PrdG4;UAS-Slp1* embryo. Where Slp1 represses *abd-A*, *Ubx* expression increases. **L.** *Ubx*$^{-}$ embryos have normal *abd-A* and *slp1* expression, indicating that Slp1 does not require Ubx to repress *abd-A*.

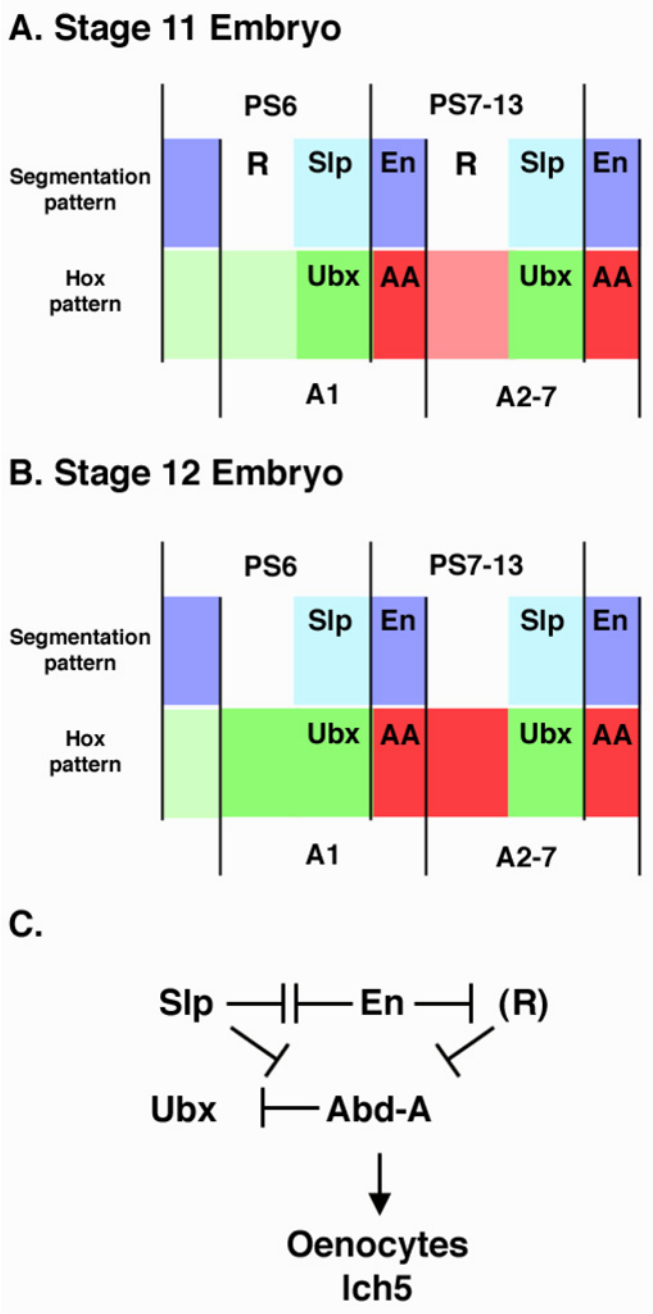

Figure 7. Model for the establishment of abdominal Hox expression patterns

A and B. Representation of En (purple), Slp (blue), Abd-A (red) and Ubx (green) expression levels in stage 11 (A) and stage 12 (B) within two parasegments/segments of an embryo. The segment and parasegment boundaries are denoted. The color intensities represent the relative expression levels of both Hox factors. Note that at stage 11 high levels of Abd-A are observed only within En-positive cells and that high levels of Ubx are observed within Slp-positive cells. We predict that an unknown repressor (R) keeps Abd-A levels low in cells anterior to *slp* expression. By stage 12, however, the expression of R decreases allowing Abd-A levels to increase in these A compartment cells. In the ventral ectoderm *slp* expression represses *abd-A* allowing *Ubx* levels to be maintained. **C.** A genetic diagram for

how the expression patterns of *Ubx* and *abd-A* are established by *en* and *slp*. R is shown in parentheses as we predict it is expressed transiently during stage 11 and fades by stage 12. The modulation of abdominal Hox gene expression in the P compartment correlates with the formation of abdominal specific cell fates, oenocytes and the lateral chordotanal organs (lch5).