Direct integration of *Hox* and segmentation gene inputs during *Drosophila* development

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During *Drosophila* embryogenesis, segments, each with an anterior and posterior compartment, are generated by the segmentation genes while the *Hox* genes provide each segment with a unique identity. These two processes have been thought to occur independently. Here we show that abdominal Hox proteins work directly with two different segmentation proteins, Sloppy paired and Engrailed, to repress the Hox target gene *Distalless* in anterior and posterior compartments, respectively. These results suggest that segmentation proteins can function as Hox cofactors and reveal a previously unanticipated use of compartments for gene regulation by Hox proteins. Our results suggest that these two classes of proteins may collaborate to directly control gene expression at many downstream target genes.

The segregation of groups of cells into compartments is fundamental to animal development[1–4]. Originally defined in *Drosophila melanogaster*, compartments are critical for providing cells with their unique positional address[5,6]. The first compartments to form during *Drosophila* development are the anterior and posterior compartments and the key step to defining them is the activation of the gene *engrailed* (*en*)[2]. Expression of *en*, which encodes a homeodomain transcription factor, results in a posterior compartment fate, and the absence of *en* expression results in an anterior compartment fate[7,8]. Once activated by gap and pair-rule genes, *en* expression and, consequently, the anterior–posterior compartment boundary later become dependent upon the protein Wingless (Wg), which is secreted from adjacent anterior compartment cells[9,10]. Concurrently with anterior–posterior compartmentalization and segmentation, the expression of the eight *Drosophila Hox* genes is also initially established by the gap and pair-rule genes. The *Hox* genes, however, which also encode homeodomain transcription factors, do not contribute to the formation or number of segments but instead specify their unique identities along the anterior–posterior axis[11–14].

This flow of genetic information during *Drosophila* embryogenesis has led to the idea that anterior–posterior compartmentalization and segment identity specification are independent processes[15–18]. In contrast to this view, we show here that these two pathways are interconnected in previously unrecognized ways. We provide evidence that Hox factors directly interact with segmentation proteins such as En to control gene expression. Moreover, Hox proteins collaborate with two different segmentation proteins in anterior and posterior cell types to regulate the same Hox target gene, revealing a previously unknown use of compartments to control gene expression by Hox proteins.

Distalless expression straddles the compartment boundary

Distalless (*Dll*) is a Hox target gene that is required for leg development in *Drosophila*[19]. In each thoracic hemisegment, *wg*, expressed by anterior cells adjacent to the anterior–posterior compartment boundary, activates *Dll* in a group of cells that straddle this boundary[20,21] (Fig. 1). A *cis*-regulatory element derived from *Dll*, called DMX, drives accurate *Dll*-like expression in the thorax (Fig. 1 and Supplementary Fig. 1a). The abdominal *Hox* genes *Ultrabithorax* (*Ubx*) and *abdominalA* (*abdA*) directly repress *Dll* and *DMX-lacZ* in both compartments, thereby blocking leg development in the abdomen[21–23] (Supplementary Fig. 1b, c). DMX is composed of a large activator element (DMXact) and a 57-base-pair (bp) repressor element referred to here as DMX-R (Fig. 1a–c). Previous work demonstrated that Ubx and AbdA cooperatively bind to DMX-R with two homeodomain cofactors, Extradenticle (Exd) and Homothorax (Hth)[23]. In contrast, the thoracic Hox protein Antennapedia (Antp) does not repress *Dll* and does not bind DMX-R with high affinity in the presence or absence of Exd and Hth[23]. Thus, repression of *Dll* in the abdomen depends in part on the ability of these cofactors to selectively enhance the binding of the abdominal Hox proteins to DMX-R[23].

Compartment-specific *Dll* repression

Exd and Hth, as well as their vertebrate counterparts, are used as Hox cofactors at many target genes[24]. Moreover, Hox/Exd/Hth complexes are used for both gene activation and repression, raising the question of how the decision to activate or repress is determined. One view posits that these complexes do not directly recruit co-activators or co-repressors, but instead are required for target gene selection[25]. Accordingly, other DNA sequences present at Hox/Exd/Hth-targeted elements would determine whether a target gene is activated or repressed. Consistent with this notion, DMX-R sequences isolated from six *Drosophila* species show extensive conservation outside the previously identified Hox (referred to here as Hox1) Exd and Hth binding sites (Supplementary Fig. 1d), suggesting that they also play a role in *Dll* regulation.

To test a role for these conserved sequences, we performed a thorough mutagenesis of DMX-R (Fig. 1; a complete summary of the mutagenesis is shown in Supplementary Fig. 1e). Each mutant DMX-R was cloned into an otherwise wild-type, full-length DMX and tested for activity in a standard reporter gene assay in transgenic embryos. Thoracic expression was normal in all cases. However, to our surprise, many of the DMX-R mutations, such as X5, resulted in abdominal de-repression only in En-positive posterior compartment cells, whereas other mutations, such as X2, resulted in abdominal de-repression only in En-negative anterior compartment cells (Fig. 1f, g). Single mutations in the Hox1, Exd, or Hth sites also resulted in de-repression predominantly in posterior cells (Supplementary Fig. 1e). In contrast, deletion of the entire DMX-R (*DMXact-lacZ*), or mutations in both the X2 and X5 sites

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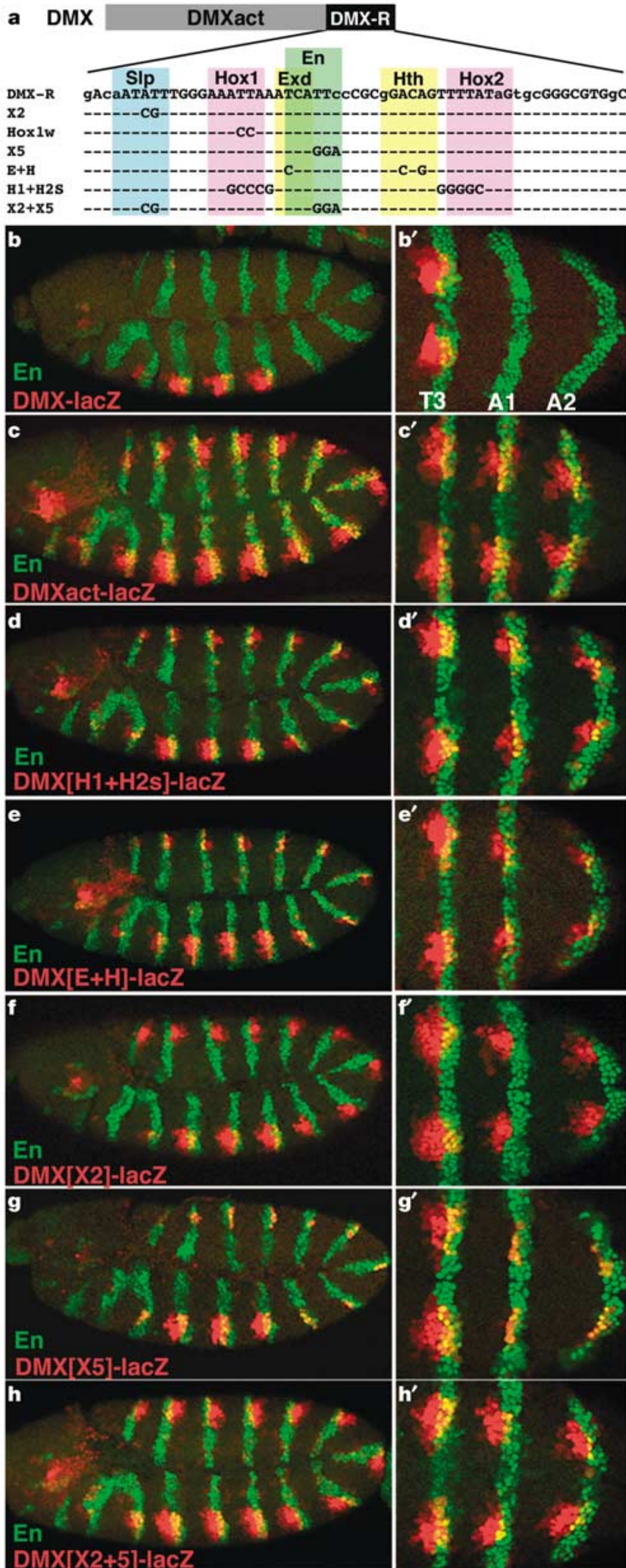
(*DMX[X2 + X5]-lacZ*), resulted in de-repression in both compartments (Fig. 1c, h). These results suggest that distinct repression complexes bind to the DMX-R in the anterior and posterior compartments and that segmentation genes play a role in *Dll* repression.



Hox input is mediated by a core Hox/Exd/Hth/Hox complex

One clue to the identity of the proteins in these repression complexes was that the sequence around the Hth site was nearly identical to a Hth/Hox binding site that had been identified previously by a systematic evolution of ligands by exponential enrichment (SELEX) approach using vertebrate Hox and Meis proteins[26] (Supplementary Fig. 1f). This similarity suggested the presence of a second, potentially redundant Hox binding site, Hox2. In agreement with this idea, mutations in both the Hox1 and Hox2 binding sites resulted in de-repression in both the anterior and posterior compartments of the abdominal segments (Fig. 1d). Similarly, although individual mutations in the Exd and Hth binding sites lead predominantly to de-repression in the posterior compartment, mutation of both sites resulted in de-repression in both compartments (Fig. 1e). These results suggest that a Hox/Exd/Hth/Hox complex may be used for repression in both compartments. Furthermore, they suggest that although single mutations in these binding sites are sufficient to disrupt the activity of this complex in the posterior compartment, double mutations are required to disrupt its activity in the anterior compartment.

To provide biochemical evidence for a Hox/Exd/Hth/Hox tetramer, we performed DNA binding experiments using DMX-R probes and proteins expressed and purified from *Escherichia coli* (Supplementary Fig. 2a). Previous experiments demonstrated that a Hox/Exd/Hth trimer cooperatively bound to the Hox1, Exd and Hth sites[23] (Supplementary Fig. 2b). We tested the function of the Hox2 site in two ways. First, we measured binding to a probe, DMX-R2, that includes the Exd, Hth and Hox2 sites, but not the Hox1 site (Supplementary Fig. 2c). We found that Exd/Hth/AbdA and Exd/Hth/Ubx trimers cooperatively bound to this probe and that mutations in the Hth, Exd or Hox2 binding sites reduced or eliminated complex formation (Supplementary Fig. 2c and data not shown).

Second, if both the Hox1 and Hox2 sites are functional, the full-length DMX-R may promote the assembly of Hox/Exd/Hth/Hox tetramers. Using a probe containing all four binding sites (DMX-R1+2), we observed the formation of such complexes (Fig. 2a, b). Mutation of any of the four binding sites reduced the amount of tetramer binding whereas mutation of both Hox sites or both the Exd and Hth sites eliminated tetramer binding (Fig. 2b and data not shown). Furthermore, Antp, which does not repress *Dll*, formed tetramers with Exd and Hth that were approximately tenfold weaker than with Ubx or AbdA (Fig. 2c), but bound well to a consensus Hox/Exd/Hth trimer binding site (Fig. 2d). Because mutation of both Hox sites or both the Exd and Hth sites resulted in de-repression in both compartments (Fig. 1), these experiments correlate the binding of a Hox/Exd/Hth/Hox complex on the

Figure 1 Compartment-specific repression mediated by the DMX-R. **a**, Schematic of DMX and sequences of wild type and mutant DMX-Rs. See Supplementary Fig. 1e for the complete list of DMX-R mutants. Upper case letters in the top line indicate base pairs that are 100% conserved in six *Drosophila* species (Supplementary Fig. 1d). **b**–**h**, Stage-11 embryos containing various *DMX-lacZ* reporter genes stained for β-gal (red) and En (green). Panels on the left show lateral views of entire embryos; panels on the right show ventral views of the third thoracic segment (T3) to the second abdominal segment (A2). **b**, *DMX-lacZ*. Reporter expression is limited to the thorax and includes both En+ and En− cells. **c**, *DMX[act]-lacZ*. Reporter expression is throughout the embryo and includes both En+ and En− cells. **d**, *DMX[H1+H2s]-lacZ*. Reporter expression is throughout the embryo and includes both En+ and En− cells. **e**, *DMX[E+H]-lacZ*. Reporter expression is throughout the embryo and includes both En+ and En− cells. Expression in the abdomen is weaker, however, than *DMX[act]-lacZ*. **f**, *DMX[X2]-lacZ*. In the abdomen, reporter expression is only observed in En− anterior compartment cells. **g**, *DMX[X5]-lacZ*. In the abdomen, reporter expression is only observed in En+ posterior compartment cells. **h**, *DMX[X2+X5]-lacZ*. Reporter expression is throughout the embryo and includes both En+ and En− cells.

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DMX-R with the ability of this element to mediate repression in both compartments.

DMX expression overlaps Slp- or En-expressing cells

Although binding of a Hox/Exd/Hth/Hox tetramer is sufficient to account for the necessary abdominal Hox-input into *Dll* repression, it does not explain the compartment-specific de-repression exhibited by some DMX-R mutations (Fig. 1). The X2 and X5 mutations, for example, result in abdominal de-repression (Fig. 1) but do not prevent the formation of the Hox/Exd/Hth/Hox tetramer (Fig. 2b). Sequence inspection of the DMX-R revealed that the X2 mutation, which resulted in de-repression specifically in the anterior compartment, disrupts two partially overlapping matches to a consensus binding site for Forkhead (Fkh) domain proteins (Supplementary Fig. 1f). With this in mind, we examined the expression pattern of Sloppy paired 1 (Slp1), a Fkh domain factor encoded by one of two partially redundant segmentation genes, *slp1* and *slp2*[27–29]. The two

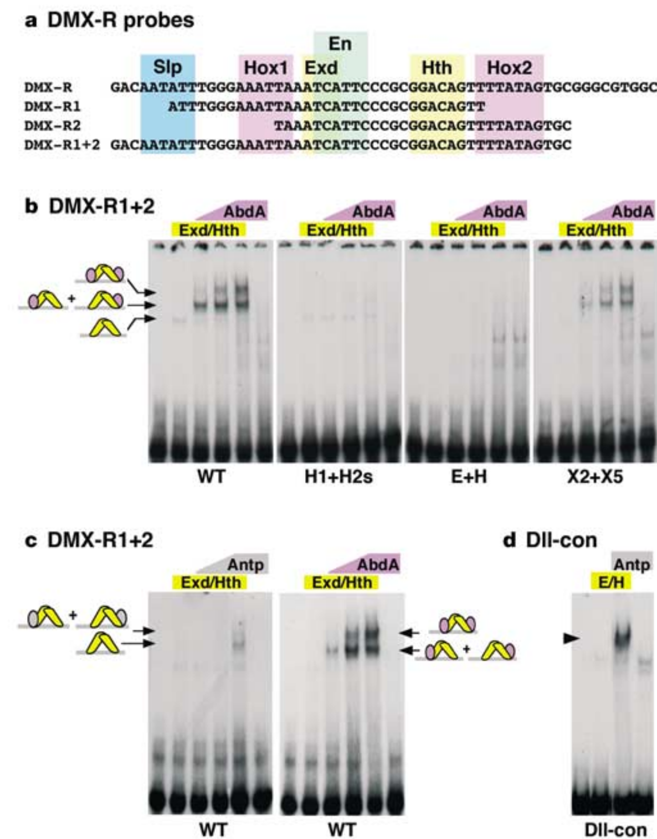

Figure 2 Assembly of a core Hox/Exd/Hth/Hox complex on the DMX-R. **a**, The sequences of full-length DMX-R and three probes used for EMSAs. **b**, **c**, EMSAs using wild type (WT) or mutant versions of the DMX-R1+2 probe. Although these experiments were performed with AbdA, Ubx has similar DNA binding properties (not shown). The complexes formed are indicated on the left using the symbols defined in Fig. 3i. The probes used (indicated below each EMSA) correspond to the mutations listed in Fig. 1a. **b**, On the wild type DMX-R1+2 probe, Exd/Hth, AbdA/Exd/Hth and Exd/Hth/AbdA trimers, and AbdA/Exd/Hth/AbdA tetramers form. Tetramers failed to form on probes with mutations in both the Exd and Hth sites (E+H) or in both Hox sites (H1+H2s). A probe with the X2 and X5 mutations still allowed tetramer binding. The Exdw and single Hox binding site mutations reduced the amount of tetramer formation approximately threefold, but still allowed trimer formation (data not shown). **c**, On the wild type DMX-R1+2 probe, Antp failed to form tetramers and formed trimers at least tenfold more weakly than AbdA. **d**, The same preparation of Antp used in **c** readily forms complexes with Exd/Hth on a consensus probe (Dll-con)[23].

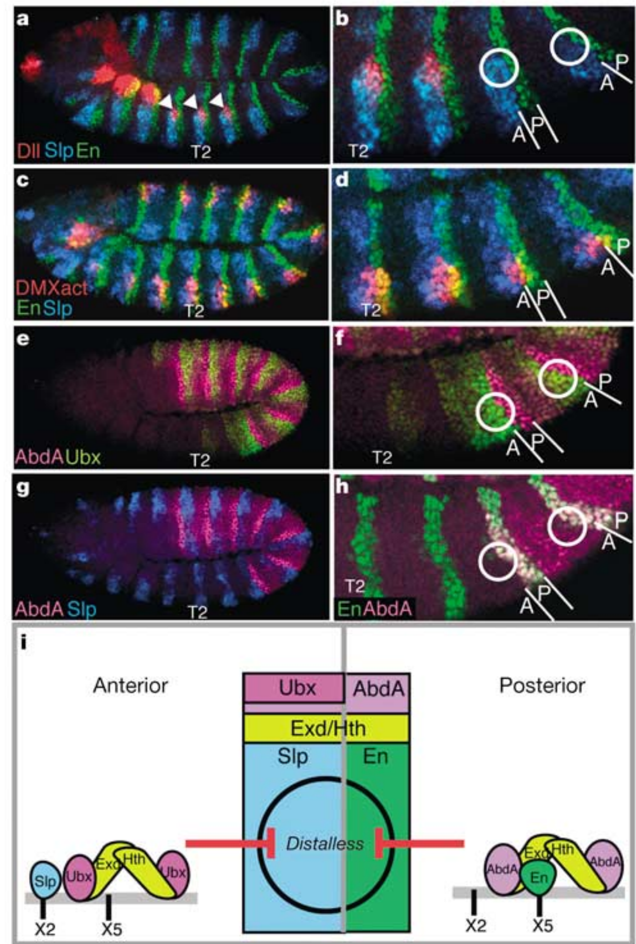

Figure 3 A model for DMX-R-mediated repression of *Dll*. **a**–**h**, All panels show lateral views of stage-11 embryos. Left-hand panels show entire embryos, right-hand panels show the second thoracic segment (T2) to the second abdomimal segment (A2). **a**, **b**, Wild type, stained for Dll (red), Slp (blue) and En (green). The white circles indicate the approximate location of the cells with the potential to express *Dll* in A1 and A2. 'A' and 'P' refer to the anterior and posterior compartments, respectively. In the thorax, Dll expression is included within the Slp and En stripes. In the abdomen, the cells where *Dll* is repressed (white circles) are also included within the Slp and En stripes. **c**, **d**, *DMXact-lacZ*, stained for β-gal (red), En (green) and Slp (blue). *DMXact-lacZ* expression in the abdomen marks the cells that have the potential to express *Dll*. These cells are included within the Slp and En stripes. **e**, **f**, Wild type, stained for Ubx (light green) and AbdA (pink). In the abdomen, Ubx levels are higher in the anterior compartment than in the posterior compartment, whereas AbdA levels are higher in the posterior compartment. **g**, Wild type, stained for AbdA (pink) and Slp (blue). In the abdomen, AbdA levels are highest in posterior compartment cells, adjacent to the Slp-expressing anterior cells. **h**, Wild type, stained for AbdA (purple) and En (green). The highest levels of AbdA overlap with En in the posterior compartment making these nuclei appear white. **i**, Model for DMX-R-mediated repression in the abdomen. In the centre is a summary of the expression patterns of Ubx, AbdA, Exd, Hth, Slp and En in the abdominal segments. In both anterior and posterior compartments we propose that a Hox/Exd/Hth/Hox tetramer binds to the Hox1/Exd/Hth/Hox2 binding sites. On the basis of their expression patterns, Ubx and AbdA are likely to be the predominant Hox proteins in these complexes in the anterior and posterior compartments, respectively. The model also posits that Slp is bound to the X2 site in the anterior compartment and En is bound to the X5 site in the posterior compartment. Our data cannot exclude, however, that in the posterior compartment, En is bound to the Hox1 site and AbdA is bound to the X5 site, or that an En/Exd/Hth/AbdA complex is used for repression in the posterior compartment. We favour the scheme shown here because it proposes a similar Hox/Exd/Hth/Hox tetramer in both compartments and it best accommodates the observed cooperative binding between En and AbdA to the DMX-R (Fig. 4a).

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slp genes are expressed in anterior compartment cells adjacent and anterior to En-expressing posterior compartment cells (Fig. 3a–d). In the thorax, cells expressing *Dll* and *DMX-lacZ* co-express either Slp or En at the time *Dll* is initially expressed (Fig. 3a, b and data not shown). In the abdomen, the homologous group of cells, which express *DMXact-lacZ* (a reporter lacking the DMX-R), co-express either Slp in the anterior compartment or En in the posterior compartment (Fig. 3c, d). We also compared the expression patterns of Slp and En with Ubx and AbdA. Ubx levels are highest in anterior, Slp-expressing cells whereas AbdA levels are elevated in posterior, En-expressing cells (Fig. 3e–h). In contrast, both Exd and Hth are present at similar levels in both compartments throughout the abdomen (data not shown).

A model for *Dll* repression in the abdomen

On the basis of these data, we present a model for Hox-mediated repression of *Dll* in both the anterior and posterior compartments of the abdominal segments (Fig. 3i). In the anterior compartment we propose that Slp binds to DMX-R directly with a Ubx/Exd/Hth/Ubx tetramer. In the posterior compartment we suggest that En binds to DMX-R directly with an AbdA/Exd/Hth/AbdA tetramer. One important feature of this model is that Antp/Exd/Hth/Antp complexes fail to form on this DNA, thereby accounting for the lack of repression in the thorax. Furthermore, the model proposes that Slp and En should, on their own, have only weak affinity for DMX-R sequences because repression does not occur in the thorax, despite the presence of these factors. The Hox/Exd/Hth/Hox complex, perhaps in conjunction with additional factors, is required to recruit or stabilize Slp and En binding to the DMX-R. Both Slp and En are known repressor proteins that directly bind the co-repressor Groucho[30–33]. Thus, the proposed complexes in both compartments provide a direct link to this co-repressor and, therefore, a mechanism for repression. Below we present DNA binding and genetic experiments that test and support this model.

En and Slp bind DMX-R

To test the idea that En is playing a direct role in *Dll* repression, we examined the ability of En and Hox proteins to bind to DMX-R probes. On its own, En binds to DMX-R very poorly (Fig. 4a–c). Surprisingly, we found that En binds DMX-R with the abdominal Hox proteins Ubx or AbdA in a highly cooperative manner (Fig. 4a, b). The thoracic Hox protein Antp does not bind cooperatively with En to this probe (Fig. 4b). Mutations in the Hox1 or X5 binding sites block AbdA/En binding *in vitro*, consistent with these mutations showing posterior compartment de-repression *in vivo*. In contrast, the X6, X7 and Hth mutations do not affect AbdA/En complex formation (Fig. 4a; Supplementary Figs 1e and 3a).

On the basis of DMX-R's ability to assemble a Hox/Exd/Hth/Hox

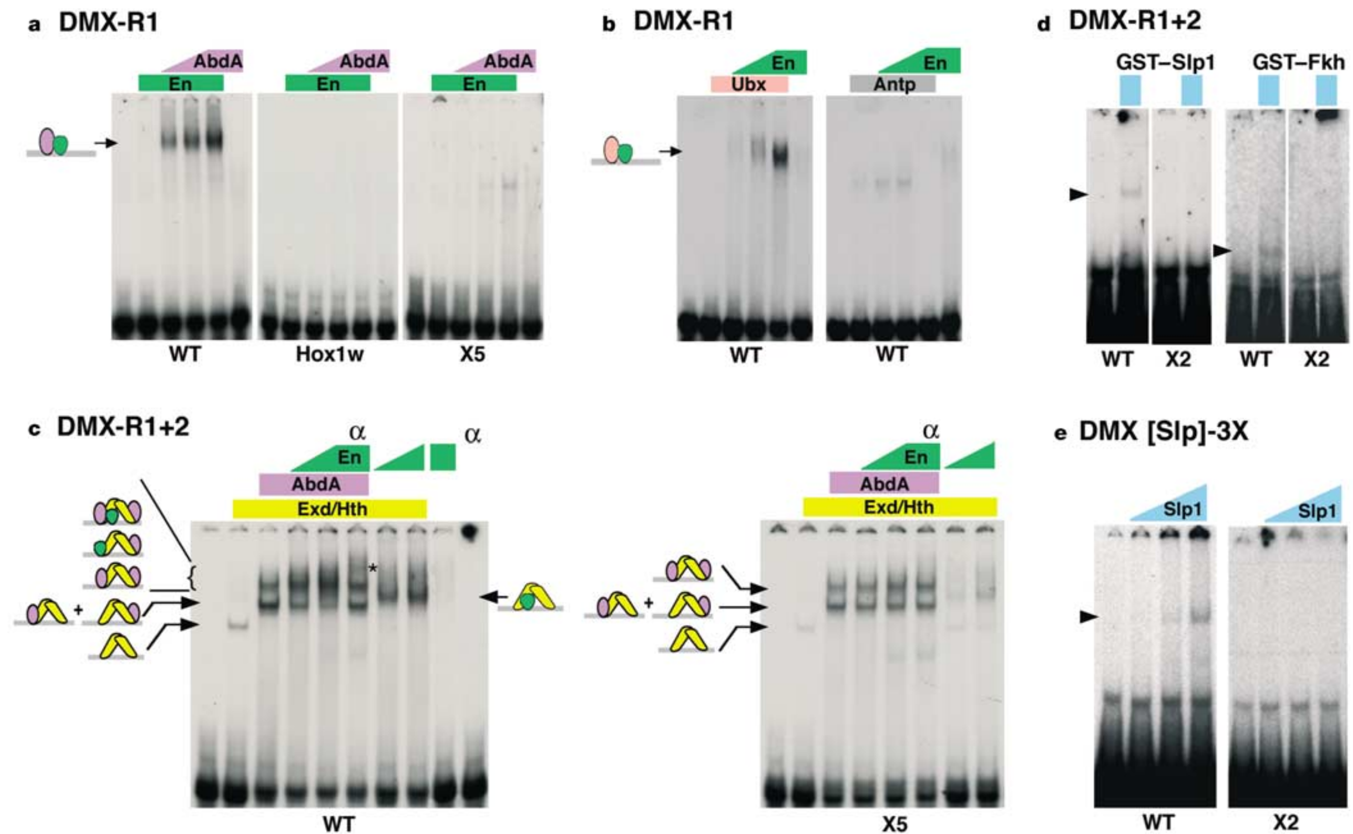

Figure 4 En and Slp bind to DMX-R. **a**, **b**, EMSAs showing that AbdA/En fails to bind probes with mutations in the Hox1 (Hox1w) or X5 sites, which also show posterior compartment de-repression. In contrast, the X6 mutation still allows AbdA/En complex formation (Supplementary Fig. 3). **b**, Ubx/En, like AbdA/En, binds cooperatively to the DMX-R1 probe but Antp does not bind with En in this assay. **c**, Combinations, as indicated, of En, AbdA, Exd and Hth were bound to wild type (WT) or X5 mutant DMX-R1+2 probes. The complexes proposed to form, including En/AbdA/Exd/Hth/AbdA and En/Exd/Hth/AbdA, are indicated. Formation of these higher order complexes is inhibited and partially supershifted (*) by the addition of anti-En antibody (α). On the X5 probe, addition of En does not generate higher order complexes (also see Supplementary Fig. 3b). On the WT probe, an En/Exd/Hth complex is also observed, but this complex is not seen upon addition of AbdA. **d**, **e**, EMSAs showing that Slp1 binds to DMX probes in an X2 dependent manner. **d**, Full-length Slp1 (GST–Slp1) and the forkhead domain of Slp1 (GST–Fkh) bind weakly to the wild type DMX-R1+2 probe (arrowhead) but not to a probe containing the X2 mutation. **e**, GST-Slp1 also binds to a probe containing three copies of the wild type X2 region of DMX-R, but not to a probe containing three copies of the X2 mutant (DMX[Slp]-3x; see Methods).

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tetramer (Fig. 2b), we also tested if En could bind together with an AbdA/Exd/Hth/AbdA complex. Addition of En to reactions containing AbdA, Exd and Hth resulted in the formation of a putative En/AbdA/Exd/Hth/AbdA complex (Fig. 4c and Supplementary Fig. 3b). This complex contains En because its formation is inhibited by an anti-En antibody. A weak antibody-induced supershift is also observed. Moreover, this complex fails to form on the X5 mutant, which causes posterior compartment-specific de-repression (Fig. 4c and Supplementary Fig. 3b). We note that En/Exd/Hth complexes also bind to the DMX-R (Fig. 4c) and that we cannot exclude that an En/Exd/Hth/AbdA complex may be important for *Dll* repression. The model (Fig. 3i) emphasizes a role for an En/AbdA/Exd/Hth/AbdA complex because it better accommodates the cooperative binding observed between En and AbdA on the DMX-R.

Repression in the anterior compartments of the abdominal segments requires the sequence defined by the X2 mutation, which is similar to a Fkh domain consensus binding site (Fig. 1a and Supplementary Fig. 1f). The model predicts that this sequence is bound by Slp (Fig. 3i). Consistent with this view, Slp1 binds weakly to wild type, but not to X2 mutant DMX-R probes (Fig. 4d, e). However, in contrast to En, we do not observe cooperative binding between Slp and Hox or Hox/Exd/Hth/Hox complexes (data not shown), suggesting that additional factors may be required to mediate interactions between Slp and the abdominal Hox factors.

Together, these results suggest that En and Slp play a direct role in *DMX-lacZ* and *Dll* repression. However, these experiments do not unambiguously determine the stoichiometry of binding by these factors. Furthermore, *in vivo*, additional factors may enhance the interaction between these segmentation proteins and Hox complexes, thereby increasing the stability and/or activity of the repression complexes.

Genetic tests of the model

The model for *Dll* repression is supported by previous genetic experiments that examined the effect of *Ubx* and *abdA* mutants on *Dll* expression in the abdomen. *Ubx abdA* double mutants de-repress *Dll* in both compartments of all abdominal segments. In contrast, *Ubx* mutants de-repress *Dll* in the anterior compartment of only the first abdominal segment, which lacks AbdA. *abdA* mutant embryos de-repress *Dll* in the posterior compartments of all abdominal segments, where Ubx levels are low[21,34] (Fig. 3).

We performed several genetic experiments to provide *in vivo* support for the idea that Slp and En work directly with Ubx and AbdA to repress *Dll*. The design of these experiments had to take into consideration that the activation of *Dll* in the thorax depends on *wg*, and that *wg* expression depends on both *slp* and *en*[29]. Consequently, *Dll* expression is mostly absent in *en* or *slp* mutants, making it impossible to characterize the role that these genes play in *Dll* repression from examining *en* or *slp* loss-of-function mutants (data not shown). However, some of the mutant DMX-Rs described here provide the opportunity to test the model in alternative ways.

According to the model, *DMX[X5]-lacZ* is de-repressed in the posterior compartments of the abdominal segments because it fails to assemble the posterior, En-containing complex (Fig. 3i). Repression of *DMX[X5]-lacZ* in the anterior compartments still occurs because it is able to assemble the anterior, Slp-containing complex. According to this model, *DMX[X5]-lacZ* should be fully repressed if Slp is provided in posterior cells. A negative control for this experiment is that ectopic Slp should be unable to repress *DMX[X2]-lacZ* because this reporter gene does not have a functional Slp binding site (Fig. 3i). To mis-express Slp, we used *paired-Gal4* (*prd-Gal4*), which overlaps both the Slp and En stripes in the odd-numbered abdominal segments (data not shown). As predicted, ectopic Slp repressed *DMX[X5]-lacZ* but not *DMX[X2]-lacZ* (Fig. 5b, i), providing strong *in vivo* support for Slp's direct role in *Dll* repression in the anterior compartments.

Conversely, the model posits that *DMX[X2]-lacZ* is de-repressed in the anterior compartment because it cannot bind Slp, but remains repressed in the posterior compartment because it is able to assemble the En-containing posterior complex (Fig. 3i). Thus, providing En in the anterior compartment should repress *DMX[X2]-lacZ*. A complication with this experiment is that En is a repressor of *Ubx*, which is the predominant abdominal Hox protein in the anterior compartment[21]. We confirmed that *prd-Gal4*-driven expression of En represses *Ubx* and that AbdA levels remain low at the time *Dll* is activated in the thorax (data not shown). Consequently, ectopic En expression is not sufficient to repress *DMX[X2]-lacZ*, consistent with the observation that low levels of abdominal Hox proteins are present (Fig. 5l). Therefore, to promote the assembly of the posterior complex in anterior cells, we co-expressed En with AbdA using *prd-Gal4*. As predicted, this combination of factors repressed *DMX[X2]-lacZ* but not *DMX[X5]-lacZ*, providing strong *in vivo* evidence for En playing an essential role in *Dll* repression in the posterior compartments (Fig. 5f, m).

Several observations provide additional support for the model. First, ectopic expression of AbdA or Ubx in the second thoracic segment (T2) represses *DMX[X5]-lacZ* in the anterior compartment, but not in the posterior compartment (Fig. 5d, g). Conversely, expression of AbdA or Ubx in T2 represses *DMX[X2]-lacZ* only in posterior compartment cells (Fig. 5k, n). Second, co-expression of Slp with Ubx completely represses *DMX[X5]-lacZ* in T2 but does not repress *DMX[X2]-lacZ* in T2 (Fig. 5c, j). Third, in those cases where repression is incomplete (for example, En+AbdA repression of *DMX[X2]-lacZ* in the abdomen), cells that escape repression have low levels of either an abdominal Hox protein or Slp/En (for example, Fig. 5m). Together, these data provide additional evidence that the abdominal Hox proteins work together with Slp and En to repress *Dll*.

Implications of the model

The segregation of cells into anterior and posterior compartments during *Drosophila* embryogenesis is essential for many aspects of fly development[2,4–6,35]. The results presented here reveal an unanticipated intersection between anterior–posterior compartmentalization by segmentation genes and segment identity specification by Hox genes. Specifically, we suggest that the abdominal Hox proteins collaborate with two different segmentation proteins, Slp and En, to mediate repression of a Hox target gene in the anterior and posterior compartments of the abdomen, respectively. This mechanism of transcriptional repression suggests a previously unknown use of compartments in *Drosophila* development. The mechanism proposed here contrasts with the alternative and simpler hypothesis in which the abdominal Hox proteins would have used the same set of cofactors to repress *Dll* in all abdominal cells, regardless of their compartmental origin.

These results provide further support for the view that Hox/Exd/Hth complexes do not directly bind co-activators or co-repressors but instead indirectly recruit them to regulatory elements. Consistent with previous analyses[36–39], we suggest that Hox/Exd/Hth complexes are important for the Hox specificity of target gene selection. Additional factors, such as Slp or En in the case of *Dll* repression, are required to determine whether the target gene will be repressed or activated. In the future, it will be important to dissect in similar detail other Hox-regulated elements, to assess the generality of this mechanism.

These results also broaden the spectrum of cofactors used by Hox proteins to regulate gene expression. Although the analysis of Exd/Hth in *Drosophila* and Pbx/Meis in vertebrates has provided some insights into how Hox specificity is achieved, there are examples of tissues in which these proteins are not available to be Hox cofactors and of Hox targets in which Exd and Hth seem not to play a direct role[40–44]. We show here that En, a homeodomain segmentation protein, is used as a Hox cofactor to repress *Dll* in the

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abdomen. Although the complex defined at the DMX-R includes Exd and Hth, our DNA binding studies demonstrate that Hox and En proteins can bind cooperatively to DNA in the absence of Exd and Hth. These findings suggest that En may function with Ubx and/or AbdA to regulate target genes other than *Dll*, and perhaps independently of Exd and Hth. Consistent with this idea are genetic experiments showing that, in the absence of Exd, En can repress *slp* and this repression requires abdominal *Hox* activity[45]. Although these experiments were unable to distinguish whether the *Hox* input was direct or indirect, our results suggest that En may bind directly with Ubx and AbdA to repress *slp*, and perhaps other target genes.

Finally, these results raise the question of why a compartment-specific mechanism is used by Hox factors to repress *Dll*. The activation of *Dll* at the compartment boundary by *wg* may be important for accurately positioning the leg primordia within each thoracic hemisegment, but this mode of activation requires that *Dll* is repressed in both compartments in each abdominal segment. The utilization of segmentation proteins such as En and Slp may be the simplest solution to this problem. Compartment-specific mechanisms may also provide additional flexibility in the regulation of

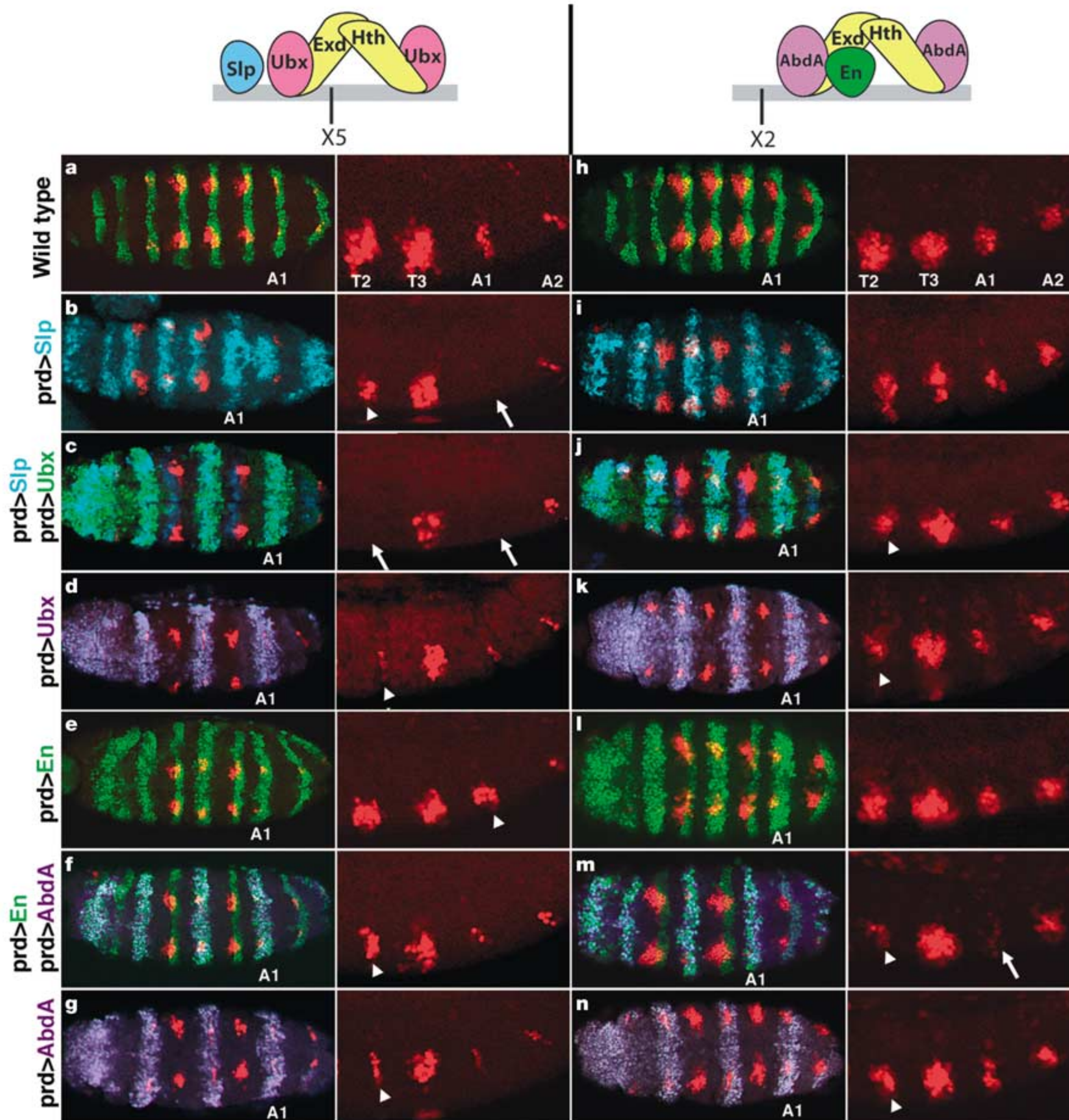

Figure 5 *Dll* is repressed by *Hox* and segmentation gene inputs. Shown on the top are the proposed complexes that can assemble onto the X5 (left) and X2 (right) mutant DMX-Rs. **a**–**g**, and **h**–**n**, show *DMX[X5]-lacZ* and *DMX[X2]-lacZ* embryos, respectively, ectopically expressing the indicated proteins via the *prd-Gal4* driver. *prd-Gal4* is expressed in T2 and the odd-numbered abdominal segments. Complete repression of the reporter gene is indicated with arrows, and partial effects are indicated with arrowheads. In all cases, low magnification ventral views are shown on the left and higher magnification lateral views are shown on the right. All embryos were stained for β-gal (red). The ectopically expressed proteins were monitored by antibody staining as indicated. In the embryos with no ectopically expressed proteins (**a** and **h**), the embryos were stained for En (green). In addition to the effects described in the text, two other results seen in these embryos are noteworthy. When Slp is expressed by itself, we observe partial repression in T2 of *DMX[X5]-lacZ* (arrowhead in **b**), but no effect on *DMX[X2]-lacZ* (**i**). We suggest that when Slp is present at very high levels (as with *prd-Gal4; UAS-Slp*) it can partially repress *DMX[X5]-lacZ* in a Hox-independent manner in the thorax. *DMX[X2]-lacZ* is not repressed, however, because it is unable to bind Slp. Expression of En by itself also resulted in some partial effects. For example, expression of En in A1 caused partial de-repression of *DMX[X5]-lacZ* (arrowhead in **e**) because, we suggest, En represses Slp, which is required for repression of this reporter gene in the anterior compartment.

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target genes by Hox proteins by allowing them to turn genes on or off specifically in anterior or posterior cell types. For these reasons, compartment-dependent mechanisms of gene regulation may turn out to be the general rule instead of the exception. □

Methods

Plasmids

The sequences of the DMX-R region from *Drosophila simulans*, *Drosophila teissieri*, *Drosophila erecta*, *Drosophila pseudoobscura* and *Drosophila hydei* were obtained using the polymerase chain reaction (PCR) (details available upon request). On the basis of this analysis the DMX-R was defined from bp 675 to 731 of Dll304[22], 18 bp longer than the previously described DME[23], which lacked the Hox2 site. Although shorter than the DMX-R, the DME mediated full repression due to compensating sequences present in adjacent vector sequences (data not shown). However, the truncated element was more sensitive to mutations in the remaining binding sites (for example, Hox1, Exd and Hth), which resulted in de-repression in both compartments. The DMX series of mutations and the DMXact (bps 1 to 680 of Dll304) constructs were generated by the PCR and cloned into the *hs43-nuc-lacZ* vector. All constructs were confirmed by DNA sequencing.

Fly stocks and antibody staining

Expression of *lacZ* (anti-β-gal, Cappell), En (4D9), Ubx (FP3.38), Abd-A[46], Dll[22] and Slp1[47] were detected by antibody staining and confocal microscopy. The degree of abdominal de-repression of lacZ between constructs was normalized to thoracic expression levels. En, Slp1, Ubx and Abd-A misexpression were driven by *prd-Gal4* in the presence of DMX-lacZ mutants as indicated.

Protein purification and EMSAs

Hox, Exd and Hth proteins used were purified from BL21 bacteria as His-tagged fusions using Ni-chromatography as described[23]. Full-length and the Fkh domain (residues 105–216) of Slp1 were cloned into pGEX5X-1. These proteins were purified from BL21 bacteria using the manufacturer's recommendations (Amersham–Pharmacia). Protein concentrations were measured by the Bradford assay and confirmed by SDS–polyacrylamide gel electrophoresis (SDSPAGE) and Coomassie blue analysis. Electrophoretic mobility shift assays (EMSAs) were performed as previously described[23]. In all cases, EMSAs within the same figure panel were performed at the same time and with the same amounts of proteins, so are directly comparable. The amount of protein used in each EMSA was: Fig. 2: Exd/Hth, 50 ng, AbdA and Antp, 15, 45 and 135 ng; Fig. 4b: Ubx and Antp, 40 ng, En, 25, 75 and 225 ng; Fig. 4a: En, 100 ng and AbdA, 15, 45 and 135 ng; Fig. 4c: Exd/Hth, 50 ng, AbdA, 135 ng and En, 75 or 225 ng; Fig. 4d: 500 ng glutathione *S*-transferase (GST)–Slp1 full-length and 500 ng GST–Fkh; Fig. 4e: 55, 167 or 500 ng GST–Slp1. The probes for Fig. 4e consist of three copies of the wild type (GACAATATTTGGGAA) or X2 mutant (GACAAT**CG**TTGGGAA) Slp region of DMX-R (DMX[Slp]-3X). 2 μl of anti-En antibody (mAb4F11) were used for supershifts.

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