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Developmental Regulation of Chromatin Conformation by Hox Proteins in *Drosophila*

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SUMMARY

We present a strategy to examine the chromatin conformation of individual loci in specific cell types during *Drosophila* embryogenesis. Regulatory DNA is tagged with binding sites (*lacO*) for LacI, which is used to immunoprecipitate the tagged chromatin from specific cell types. We applied this approach to *Distalless* (*Dll*), a gene required for limb development in *Drosophila*. We show that the local chromatin conformation at *Dll* depends on the cell type: in cells that express *Dll*, the 5′ regulatory region is in close proximity to the *Dll* promoter. In *Dll*-nonexpressing cells this DNA is in a more extended configuration. In addition, transcriptional activators and repressors are bound to *Dll* regulatory DNA in a cell type-specific manner. The pattern of binding by GAGA factor and the variant histone H2Av suggest that they play a role in the regulation of *Dll* chromatin conformation in expressing and nonexpressing cell types, respectively.

INTRODUCTION

The regulation of transcription in higher eukaryotes depends on *cis*-regulatory modules (CRMs), DNA sequences that integrate temporal and spatial information by binding groups of transcription factors (Istrail and Davidson, 2005). CRMs can be very far—even tens or hundreds of kilobases—from a gene's promoter, where transcription initiates (Bartkuhn and Renkawitz, 2008). Moreover, in some cases, CRMs have been shown to regulate the transcription of genes located on other chromosomes (Apostolou and Thanos, 2008; Cavalli, 2007; Dekker, 2008; Ling et al., 2006; Lomvardas et al., 2006; Simonis et al., 2006). In many cases, communication between distant CRMs and promoters has been observed as a physical interaction between these elements, with intervening DNA looped out (Gothard et al., 1996; Heintzman and Ren, 2009; Liu and Garrard, 2005; Nolis et al., 2009; Petrascheck et al., 2005; Schneider and Grosschedl, 2007). Several transcription factors, such as GAGA factor (GAF) and CTCF, have been implicated in mediating such long-range interactions, which are thought to underlie much of gene regulation in eukaryotes (Ling et al., 2006; Mahmoudi et al., 2002; Ohtsuki and Levine, 1998).

Although chromatin structure can have a profound influence on gene expression, most approaches for analyzing chromatin during animal embryogenesis do not have cell type-specific resolution and thus cannot reveal biologically relevant differences if they exist. Capturing chromosome conformation (3C), for example, is capable of detecting interactions between DNA elements but, when applied to a whole embryo, cannot reveal in which cells these interactions occur (Dekker et al., 2002). Similarly, chromatin immunoprecipitation (ChIP) can also identify interactions between DNA elements, but unless some method is used to purify cell types (for example, by cell sorting), it also cannot determine if such interactions are cell type specific (Kadauke and Blobel, 2009). ChIP assays also suffer from the problem that it is difficult to determine if a DNA element is immunoprecipitated because of an interaction with another element or because both elements have a binding site for the immunoprecipitated transcription factor. In one study a solution to this problem was made possible by knocking in binding sites for the yeast transcription factor Gal4 into the imprinted *Igf-H19* locus (Murrell et al., 2004; Reik et al., 2004). Using antibodies against Gal4 to specifically ChIP this DNA, it was discovered that the pattern of long-range interactions differed depending on whether the locus was paternally or maternally inherited. Tissue-dependent differences in chromatin conformations have also been observed in *Drosophila* at the *Abd-B* locus (Cléard et al., 2006), as well as at *Sonic hedgehog* (*Shh*) (Amano et al., 2009), *β-globin* (Palstra et al., 2003), and vertebrate Hox gene complexes (Montavon et al., 2011; Noordermeer et al., 2011). However, these studies generally have limited resolution and compared tissues that have very distinct developmental origins. Moreover, most of the approaches used to identify long-range interactions in these studies cannot be used in a second step to identify the factors that mediate these interactions. Thus, it remains an open question whether changes in CRM-promoter interactions are used to regulate gene expression on a finer scale and, if so, which factors may be involved.

Distalless (*Dll*) is required for appendage development in *Drosophila* (Cohen et al., 1989; Cohen and Jürgens, 1989), and depends on multiple CRMs for its correct expression during embryogenesis and larval development (Estella et al., 2012; Galindo et al., 2011; McKay et al., 2009; Vachon et al., 1992). Two of these CRMs, *Dll304* and *LT*, are located next to each other and ~12 kb 5′ to the start of *Dll* transcription, suggesting that there is long-range communication between these CRMs and the *Dll* promoter (Estella et al., 2008) (Figure 1A). *Dll304* is

the first *Dll* CRM to be active at approximately stage 10 (~5 hr) of embryogenesis in a group of ~30 cells/thoracic hemisegment. *Dll304* is activated by Wingless (Wg) signaling but is repressed in abdominal segments by the abdominal Hox factors: Ultrabithorax (Ubx) and Abdominal-A (Abd-A) (Gebelein et al., 2002; Vachon et al., 1992) (Figure 1A). Ubx and Abd-A directly and cooperatively bind to *Dll304* with two Hox cofactors: Extradenticle (Exd) and Homothorax (Hth) (Gebelein et al., 2004). *LT*, which is activated later in embryogenesis (stage 13), requires direct input from both Wg and Decapentaplegic (Dpp) signaling, as well as input from the Zn finger transcription factors: Buttonhead (Btd) and Sp1 (Estella et al., 2003; McKay et al., 2009). In addition, *LT* requires *Dll* input, derived from the earlier acting *Dll304* CRM. As a consequence, direct Hox-mediated repression of *Dll304* is a key reason that *LT* is not activated and *Dll* is not expressed in the abdomen. Once *LT* is activated, *Dll* expression is maintained via a positive autoregulatory loop that requires direct binding of Dll to a maintenance (*M*) element, which encompasses the *Dll* promoter (Estella et al., 2003; McKay et al., 2009) (Figure 1A). In the experiments described here, we confirm that *Dll* CRMs interact with the *Dll* promoter. More interestingly, we show that this interaction depends on the cell type. Our results suggest that Hox proteins regulate *Dll* transcription in part by locally modifying chromatin structure at the *Dll* locus.

RESULTS AND DISCUSSION

To dissect the regulation of *Dll* beyond the characterization of CRMs, we initially carried out standard ChIP experiments with whole embryos using antibodies directed against several factors known to regulate *Dll*. In these ChIP experiments we typically surveyed the *LT/304* region, the *Dll* promoter (*M*) region, as well as three to four intermediate regions (*I1* to *I4*) in between *LT/304* and *M* (Figure 1A). We carried out ChIPs for both abdominal repressors (the Hox proteins Ubx and AbdA), known activators Mad (Mothers against Dpp, a transcriptional effector in the Dpp pathway), Armadillo (Arm [a coactivator in the Wg pathway]), and Dll, as well as two components of the basal transcription machinery (TATA-binding protein, TBP, and RNA Polymerase II [PolII]) (Figure 1B). Curiously, we found that all three activators, TBP, and PolII behaved differently in these ChIP experiments compared to the repressors. When immunoprecipitating for Ubx or AbdA, only the *LT/304* region, but not any of the intermediate or *M* regions, was robustly detected compared to control ChIPs (Figures 1D and 1E). In contrast all of these regions, even sequences far from the known CRMs and promoter, were detected in ChIPs for any of the activators (Mad, Arm, Dll), TBP, or PolII (Figures 1C, 1D, and 1F).

Two scenarios can account for the different abilities of activators and repressors to ChIP *Dll* DNA sequences. In one the activators and basal transcriptional machinery are bound, directly or indirectly, to binding sites scattered throughout the 12 kb 5′ *Dll* DNA, whereas the repressors are bound only to the *LT/304* region. Alternatively, the configuration of the chromatin may be different in cells where the activators are bound compared to cells in which the repressors are bound. According to this idea, in cells where the activators are bound, the chromatin may be configured such that multiple regions of the 12 kb 5′ DNA are close to each other, within a crosslinkable distance to the promoter. In contrast in cells where the repressors are bound, the *LT/304* region, which contains known binding sites for these factors, would not be in close proximity to the rest of the 5′ *Dll* DNA and promoter. These two configurations may correspond to cells that express or repress *Dll*, respectively.

Standard ChIP experiments with whole embryos, including 3C and its derivatives (Gavrilov et al., 2009), cannot discriminate between these two scenarios because they do not distinguish cells that express *Dll* from cells where *Dll* is repressed. Existing methods also have limited resolution and sensitivity, especially for genes such as *Dll* that are expressed transiently and in only a small subset of total embryonic cells. To overcome these obstacles, we established a method, called *c*ell and *g*ene-specific ChIP (cgChIP), in which one can monitor the chromatin structures of specific DNA sequences in specific cell types. We used this approach to characterize the 14 kb 5′ *Dll* region in both *Dll*-expressing and -nonexpressing cell types. cgChIP is a two-component system that relies on an interaction between the *E. coli* DNA binding protein LacI and its binding site, *lacO*. The first component of cgChIP consists of cell type-specific expression of a flag-tagged version of LacI. To study *Dll*, we generated two genotypes that differ only in the expression pattern of flag-LacI: (1) *thorax* > *lacI*, (*Dll304-Gal4; UAS-flag-lacI*), in which LacI is expressed in the *Dll*-expressing cells of the thoracic appendage primordia; and (2) *abdomen* > *lacI*, (*DME^{Act}-Gal4, Dll304-Gal80; UAS-flag-lacI*), in which LacI is expressed in the homologous cells of the abdomen (Figure 2A; see Experimental Procedures for details). Notably, although they do not express *Dll*, *abdomen* > *lacI*-expressing cells receive the same positive inputs (e.g., Wg and Dpp signaling) as *thorax* > *lacI*-expressing cells. In a second component of cgChIP, we generated *lacO*-tagged, *lacZ*-expressing transgenes under the control of ~14 kb of DNA 5′ to the start of *Dll* transcription, which includes *Dll304, LT*, and the native *Dll* promoter (Figures 2B and 2C). In one (*lacO:M*) eight copies of *lacO* were inserted adjacent to the *M* element, close to the *Dll* promoter. In a second (*lacO:LT/304*) eight copies of *lacO* were inserted into a non-conserved region at *LT/304*. Importantly, both *lacO:LT/304* and *lacO:M* drove expression of *lacZ* in a pattern that was indistinguishable from *Dll*, in the presence or absence of LacI, suggesting that the 14 kb region is sufficient to drive accurate *Dll*-like expression, and that binding of LacI to the *lacO* sequences did not interfere with the normal activities of the *Dll* CRMs or promoter (Figures 2A–2C). By combining these tools we expressed Flag-LacI in the *Dll*-expressing or nonexpressing cells in flies that also contained either the *lacO:LT/304* or *lacO:M* transgenes. Flag-lacI-bound chromatin was immunoprecipitated with anti-Flag antibody and analyzed by PCR (Figure 2D). The cell type-specific expression of Flag-lacI, coupled with the *lacO*-tagged *Dll* transgenes (*lacO:M* or *lacO:LT/304*), allowed us to ask questions about the state of *Dll* regulatory sequences in specific cell types that cannot be answered by conventional ChIP experiments.

The first set of results, shown in Figures 2E–2H by both ^{32}P-labeled and real-time qPCRs, demonstrates that the 14 kb of 5′ *Dll* DNA is in a distinct configuration in *Dll*-expressing cells in the thorax compared to *Dll*-nonexpressing cells in the abdomen. When Flag-lacI was expressed in the thorax in

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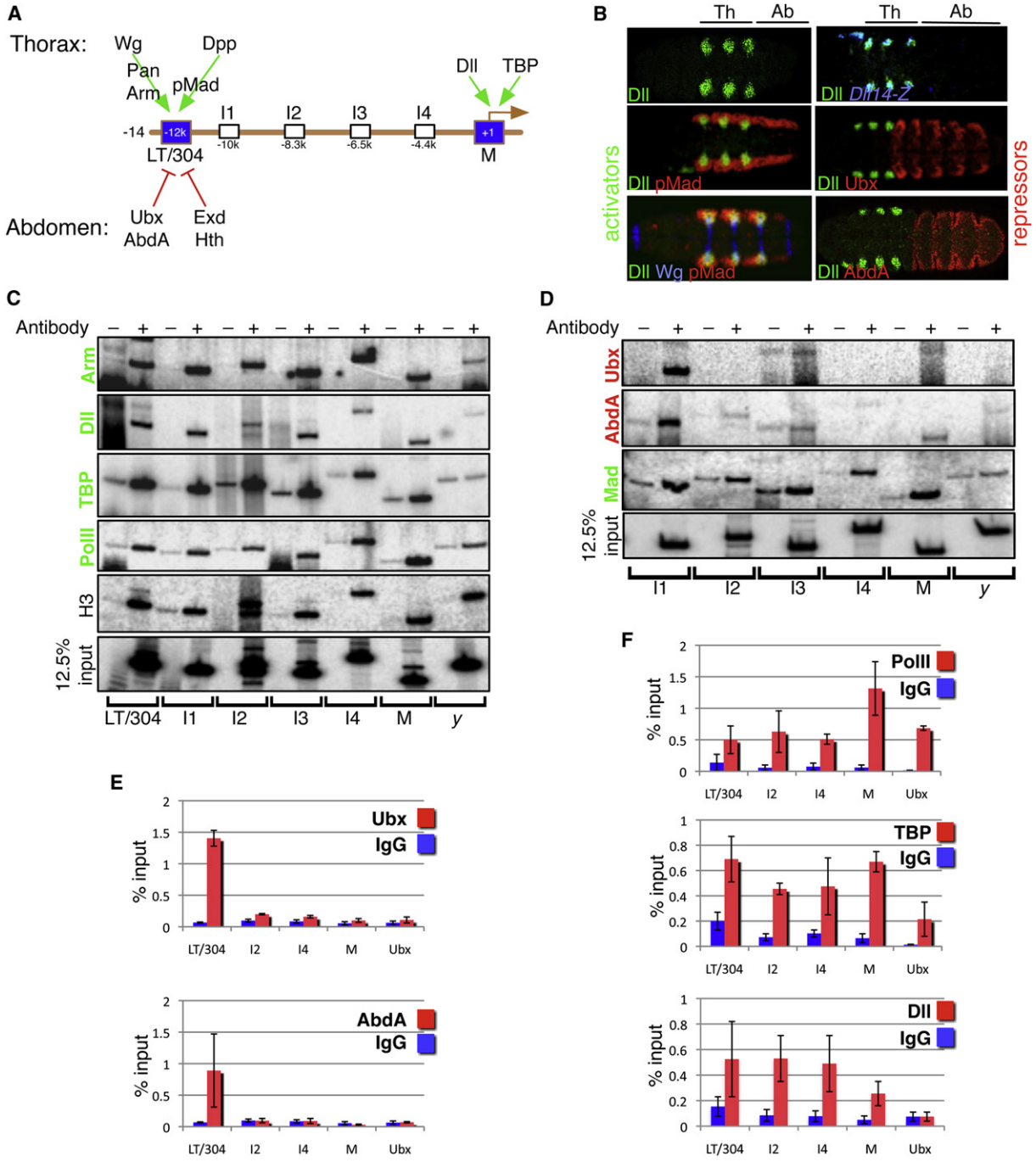

Figure 1. Whole-Embryo ChIPs Show Unique Distributions of Activators and Repressors Bound to *Dll* Regulatory Regions

(A) Schematic of the 14 kb of DNA 5′ to the start of *Dll* transcription, showing the positions of the *LT/304* CRMs and *M* element. Positive inputs in the thorax (above the DNA) and negative inputs in the abdomen (below the DNA) are indicated. *I1* to *I4* are intermediate regions that were monitored by PCR in ChIP experiments.

(B) Expression patterns of known *Dll* activators (Wg, blue; pMad, red) and repressors (Ubx and AbdA, red) relative to Dll expression in the thorax (green). Ab, abdomen; Th, thorax.

(C) Whole-embryo ChIPs using unprogrammed IgG (−) or antibodies (+) to known activators (Arm, Dll, TBP, PolII) and Histone3 (H3). Immunoprecipitated chromatin was used as a template for ^{32}P PCRs with the amplicons indicated below the gels. *y* refers to an amplicon in the *yellow* gene and serves as a negative control. The bottom row shows the amount of PCR product obtained when only water (−) or 12.5% of the input chromatin (+) was used for each amplicon.

(D) Whole-embryo ChIPs using unprogrammed IgG (−) or antibodies (+) to a known activator (Mad) or two known repressors (Ubx and AbdA). Immunoprecipitated chromatin was used as a template for ^{32}P PCRs with the amplicons shown below the gels. The bottom row shows the amount of PCR product obtained when only water (−) or 12.5% of the input chromatin (+) was used for each amplicon.

embryos containing *lacO:M*, the *M*, *LT/304*, *I2*, *I3*, and *I4* regions were all efficiently immunoprecipitated compared to control (IgG) ChIPs and negative control sequences in the *yellow* (*y*) gene and *Dll* exons (Figure 2E). In contrast when Flag-lacI was expressed in the abdomen in *lacO:M* embryos, only the *M* element was immunoprecipitated compared to the same negative controls (Figure 2E). Analogous results were obtained when Flag-lacI was expressed in the thorax or abdomen in embryos containing *lacO:LT/304*: *LT/304*, *M*, *I2*, *I3*, and *I4* were all immunoprecipitated from thoracic cells, whereas only the *LT/304* region was immunoprecipitated from abdominal cells (Figure 2F). These results were confirmed and quantified by carrying out real-time qPCR experiments (Figures 2G and 2H). We conclude that there is no detectable interaction between the *LT/304* region and the *Dll* promoter in abdominal cells, where *Dll* is repressed by Ubx and AbdA. In contrast such an interaction is readily observed in thoracic cells that express *Dll*. Interestingly, in *Dll*-expressing cells this interaction is not limited to the *LT/304* and promoter regions. Instead, the entire 12 kb region, including sequences in between *LT/304* and the promoter, is in close proximity to each other in *Dll*-expressing thoracic cells. The alternative scenario, that LacI "spreads" from its binding site into nearby DNA, is argued against because LacI is a highly specific DNA binding protein, and the version used here does not have its self-associating tetramerization domain (Robinett et al., 1996). Nevertheless, because our LacI cgChIPs show clear tissue-specific differences, both the spreading and interaction models argue that the local chromatin structure of the *Dll* 5′ region is different in *Dll*-expressing and nonexpressing cells. Together, these results suggest that abdominal Hox proteins repress *Dll* by modifying chromatin structure, in part by interfering with CRM-promoter communication.

We next used cgChIP to study the distribution of transcription factors in 5′ *Dll* sequences in thoracic and abdominal cell types. In these experiments two consecutive immunoprecipitations (IPs) were carried out: a primary IP using anti-Flag was used to pull down Flag-lacI bound to *lacO*-tagged chromatin, followed by a secondary ChIP using an antibody directed against a protein of interest (Figure 3A). In parallel to the secondary ChIP, we carried out two control IPs: a negative control with unprogrammed IgG, and a positive control with an antibody directed against LacI. Obtaining a strong signal (relative to IgG) with anti-LacI confirmed that both rounds of precipitation were successful. In addition we confirmed that primary anti-Flag cgChIPs using *thorax > lacI* embryos pulled down multiple *Dll* sequences (*M*, *LT/304*, and *I3*), whereas anti-Flag cgChIPs using *abdomen > lacI* embryos only detected sequences close to the *lacO* binding sites (Figure 3B). We again employed both ^{32}P-labeled and real-time qPCRs to quantify ChIP signals. Given the increased number of controls and the limiting quantities of material available for these sequential ChIP experiments, we limited this analysis to amplicons that detected the *LT/304*, *M*, and *I3* regions.

In general these cgChIP experiments revealed that factors involved in *Dll* activation, including PolII, TBP, Mad, Tcf (a transcription factor in the Wg pathway), Arm, and Dll, bind to *Dll* in *Dll*-expressing thoracic cells, but not in *Dll*-nonexpressing abdominal cells (Figures 3C–3E; see Figure S1 available online). Moreover, *thorax > lacI* cgChIPs for these factors pulled down *LT/304*, the *Dll* promoter, and DNA sequences in between these two elements, regardless of where the *lacO* sequences were inserted. In contrast, cgChIPs for activators and RNA PolII failed to pull down any *Dll* sequences when *abdomen > lacI* was used to examine the *Dll*-nonexpressing cells in the abdomen (Figure 3E). These results suggest that these activators are bound to the structurally compact 5′ *Dll* sequences in thoracic *Dll*-expressing cells but are not bound to these sequences when they are in a more extended state in *Dll*-nonexpressing cells in the abdomen.

A different picture emerged when we examined factors known to be important for *Dll* repression, including the Hox proteins Ubx and AbdA and their cofactors Hth and Exd. In cgChIP experiments using *thorax > lacI* embryos, *Dll* sequences were not detected above background with anti-Ubx or anti-AbdA, consistent with the abdominal-specific expression of Ubx and AbdA (Figures 3C, 3D, and 3F). In contrast when *abdomen > lacI* was used to examine *Dll*-nonexpressing cells in *lacO:LT/304* embryos, cgChIPs for repressors pulled down the *LT/304* region, which contains essential binding sites for these factors (Figure 3E, left). Furthermore, consistent with the results shown in Figure 2, *M* sequences were not detected above background in *abdomen > lacI lacO:M* cgChIPs (Figure 3E, right). Thus, in the abdomen, factors used for *Dll* repression are bound only to the *LT/304* region, which is not in close proximity to other regions of the 5′ *Dll* regulatory DNA.

To gain insight into the factors contributing to the observed tissue-specific chromatin configurations, we examined the distributions of two proteins previously implicated in establishing distinct chromatin structures: GAF and the histone variant H2Av. GAF, encoded by the *Trithorax-like* (*Trl*) gene in *Drosophila*, has been shown to mediate long-range and even *trans*-interactions between DNA elements in vivo (Mahmoudi et al., 2002; Petrascheck et al., 2005), making it a good candidate for promoting CRM-promoter communication at *Dll*. Supporting this idea, whole-embryo ChIPs using an anti-GAF antibody were able to pull down multiple regions of the *Dll* 5′ regulatory DNA, including *LT/304*, *M*, and all four intermediate regions (*I1* to *I4*) (Figures 4A–4C). A robust signal of GAF binding was also detected at the Ubx promoter (Nègre et al., 2006). The distribution of GAF at *Dll* is identical to that observed for *Dll* activators (Figure 1), suggesting that GAF is also used to promote *Dll* expression. Due to its ability to self-interact via its BTB/POZ domain (Katsani et al., 1999),

(E) qPCRs of whole-embryo ChIPs comparing the signals obtained with IgG and either anti-Ubx or anti-AbdA for a subset of *Dll* amplicons. For these repressors a strong signal was only obtained for *LT/304*. An amplicon close to the *Ubx* promoter serves as a negative control. In these and all subsequent qPCRs, the error bars represent the SEM.

(F) qPCRs of whole-embryo ChIPs comparing the signals obtained with IgG and anti-PolII, anti-TBP, and anti-Dll. For these activators a strong signal was obtained for *LT/304*, *I2*, *I4*, and *M*. An amplicon close to the *Ubx* promoter served as a positive control for PolII and TBP binding but showed no binding to Dll, as expected. Error bars represent the SEM.

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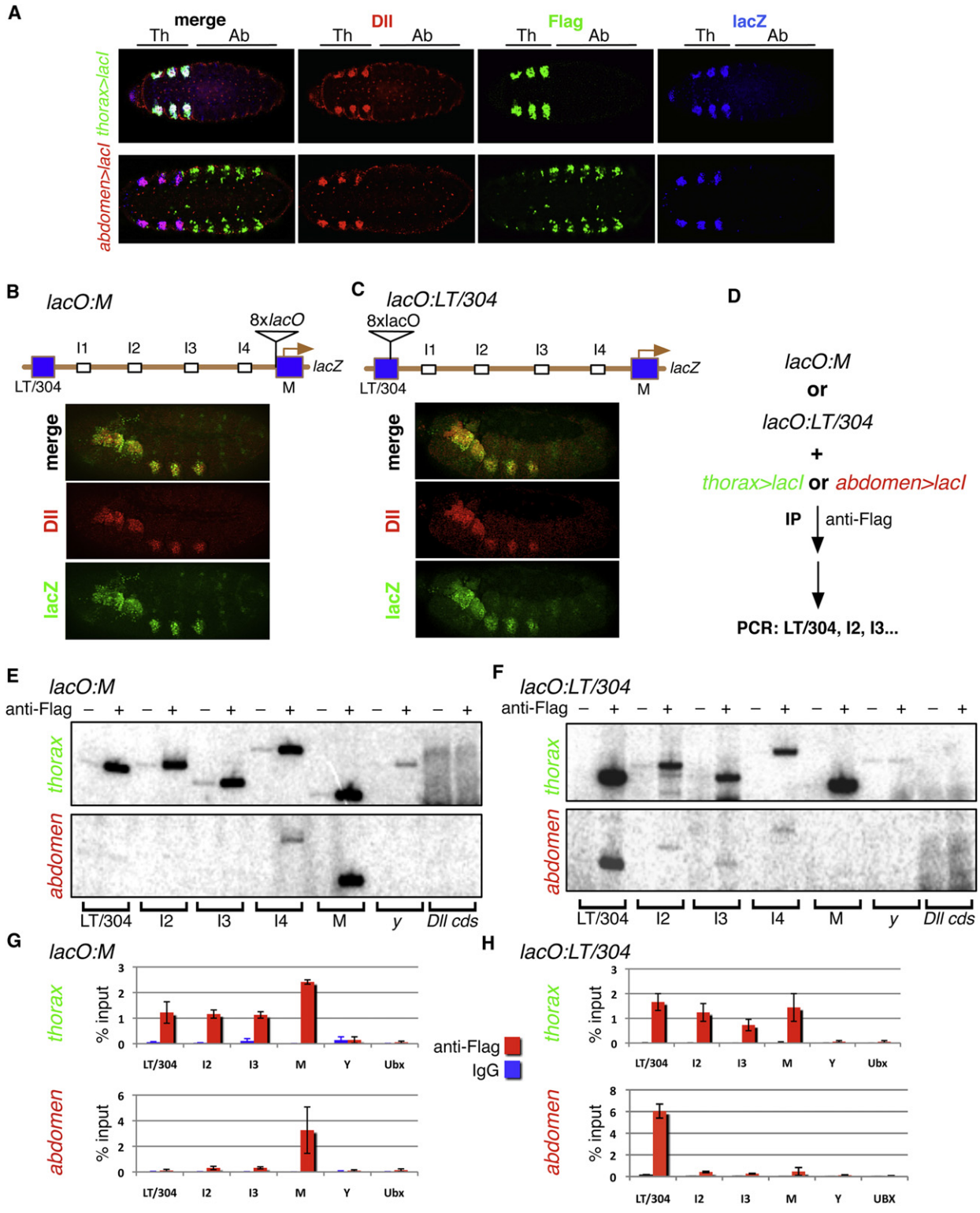

Figure 2. Cell Type-Specific CRM-Promoter Interactions at *Dll*

(A) Ventral views of stage 14 embryos stained for Dll (red), β-gal (blue), and Flag-lacI (green). Top row shows *lacO:M-lacZ; thorax > lacI* (*thorax-Gal4; UAS-flag-lacI*); bottom row illustrates *lacO:M-lacZ; abdomen > lacI* (*abdomen-Gal4; UAS-flag-lacI*). The positions of the thoracic and abdominal segments are indicated above the images. Wild-type, *Dll*-like expression of *lacZ* is observed despite the presence of *lacO* binding sites and expression of LacI. Note that although *abdomen > lacI* is expressed in some nonabdominal cells, they do not express *Dll* (see Experimental Procedures for more details).

these observations suggest that GAF may play a role in promoting the compact chromatin structure present in *Dll*-expressing thoracic cells.

In contrast to the broad distribution of GAF, binding of H2Av, a histone variant implicated in both gene activation and repression (Clarkson et al., 1999; Hanai et al., 2008; Swaminathan et al., 2005), was only observed at *LT/304*, but not at any of the *I* regions or at the *Dll* promoter (Figures 4B and 4D). This polarized distribution of H2Av at *Dll* is similar to the binding pattern of Ubx and AbdA, implying that H2Av is present at *LT/304* in abdominal cells, where *Dll* is repressed. This conclusion was confirmed by carrying out cgChIP experiments for H2Av using *abdomen > lacI*; *lacO:LT/304* embryos (Figure 4E).

Together, these findings suggest that activation of *Dll* in thoracic cells may be mediated by GAF's ability to facilitate long-range interactions between distant regulatory elements and that abdominal Hox factors block these long-range interactions (Figure 4F). The association of H2Av with *LT/304* suggests that Hox-mediated recruitment of this histone variant may contribute to the lack of CRM-promoter interaction in abdominal cells. Indeed H2A.Z, the yeast homolog of H2Av, has been implicated in blocking fiber-fiber interactions in in vitro chromatin reconstitution experiments (Fan et al., 2004). Attempts to further test the proposed roles of GAF or H2Av at *Dll* using genetic approaches were unsuccessful, likely because of the pleiotropic requirement for these factors at many genes and in many cells during *Drosophila* development. Therefore, we cannot exclude that the presence of GAF or H2Av is a consequence, rather than a cause, of the distinct chromatin configurations present in abdominal and thoracic cells.

In summary the local chromatin conformation at *Dll* varies in a developmentally relevant manner: its 5′ regulatory DNA is present in different states depending on whether it is expressed or repressed by abdominal Hox proteins (Figure 4F). In contrast to previous studies where 3D chromatin organization was compared in very different tissues (e.g., forebrain versus limb; Noordermeer et al., 2011), our experiments compared a small group of *Dll*-expressing cells in the thorax that are fated to give rise to the appendages with the homologous groups of cells in the abdomen. The fates of these two populations of cells differ only due to the expression of Hox selector proteins. Because we observed long-distance interactions only in the thorax, our results suggest that abdominal Hox proteins suppress limb development at least in part by preventing distant enhancer elements from being brought into proximity with the *Dll* promoter. We further speculate that abdominal Hox proteins block these long-range interactions by interfering with the binding of GAF and other activators, perhaps by promoting the assembly of H2Av-containing nucleosomes.

It is also noteworthy that the interactions we observe in *Dll*-expressing cells are not limited to communication between individual enhancers and the promoter. Instead, the entire 5′ *Dll* regulatory region appears to be in a more compact state because many of these sequences are in close proximity to each other and to the *Dll* promoter. These observations suggest that the entire 5′ 12 kb region functions as a single unit, consistent with the presence of additional *Dll* CRMs within this region (Estella et al., 2008). Thus, whereas isolated CRMs and shadow enhancers (Hong et al., 2008) are often sufficient to drive accurate reporter gene expression, multiple CRMs may be integrated within larger functional regulons when in their native context.

Finally, our observations raise the question of whether other genes also have distinct chromatin conformations when activated. Consistent with this view, there are many examples of ChIP experiments that show broad transcription factor binding (>5 kb) that are reminiscent of what we observe for *Dll* activators (e.g., Li et al., 2011; MacArthur et al., 2009), and broad binding of the circadian rhythm factors Clock and Period was observed at some of their targets (Menet et al., 2010). As we suggest for *Dll*, these examples may represent the chromatin conformations of large regulons that contain multiple functionally related CRMs. In contrast to these examples, other transcription factor ChIPs typically pull down short (<1 kb) DNA fragments. However, because many of these experiments were carried out using heterogeneous populations of cells, such as whole embryos, cell type-specific chromatin conformations may be difficult to detect. In addition, chromatin interactions may occur between nonadjacent CRMs that function together to drive gene expression, leading to what appears to be independently immunoprecipitated DNA sequences. It follows that some fraction of the widespread binding observed in conventional ChIP experiments (Biggin, 2011; Li et al., 2011) may be an indirect consequence of interactions between regulatory elements. The recent identification of large chromatin interactomes, in which specific genomic regions interact with each other, is consistent with this view (Fullwood et al., 2009; Handoko et al., 2011; Schoenfelder et al., 2010). In addition to cell type-specific chromatin conformations, cell type-specific differences in transcription factor binding

(B and C) Lateral views of stage 14 embryos containing the *lacO:M* (B) and *lacO:LT/304* (C) transgenes, stained for Dll (red) and β-gal (green). Schematic diagrams of these two *lacZ*-expressing transgenes are shown above the images. The expression patterns of *Dll* and *lacZ* are indistinguishable.

(D) Outline of cgChIP experiments for monitoring cell type-specific interactions between *LT/304* and *M* using the tools defined in (A)–(C).

(E) ^{32}P PCRs of cgChIPs from *lacO:M* embryos expressing either *thorax > lacI* (*thorax*) or *abdomen > lacI* (*abdomen*) as indicated. When Flag-lacI was expressed in the thorax, multiple *Dll* 5′ sequences, but not those from *y* or the *Dll*-coding sequence (*Dll cds*), were amplified. In contrast when Flag-lacI was expressed in the abdomen, only the *M* element (close to the *lacO* sites) was amplified. "−" and "+" above the gels indicate IPs with IgG or anti-Flag, respectively.

(F) ^{32}P PCRs of cgChIPs from *lacO:LT/304* embryos expressing either *thorax > lacI* (*thorax*) or *abdomen > lacI* (*abdomen*) as indicated. When Flag-lacI was expressed in the thorax, multiple *Dll* 5′ sequences, but not those from *y* or the *Dll* coding sequence (*Dll cds*), were amplified. In contrast when Flag-lacI was expressed in the abdomen, only the *LT/304* region (close to the *lacO* sites) was amplified. "−" and "+" above the gels indicate IPs with IgG or anti-Flag, respectively.

(G) qPCR results of cgChIP experiments with *lacO:M* and *thorax > lacI* or *abdomen > lacI* as indicated. The results confirm the ^{32}P PCR results shown in (E). Error bars represent the SEM.

(H) qPCR results for cgChIP experiments with *lacO:LT/304* and *thorax > lacI* or *abdomen > lacI* as indicated. The results confirm the ^{32}P PCR results shown in (F). Error bars represent the SEM.

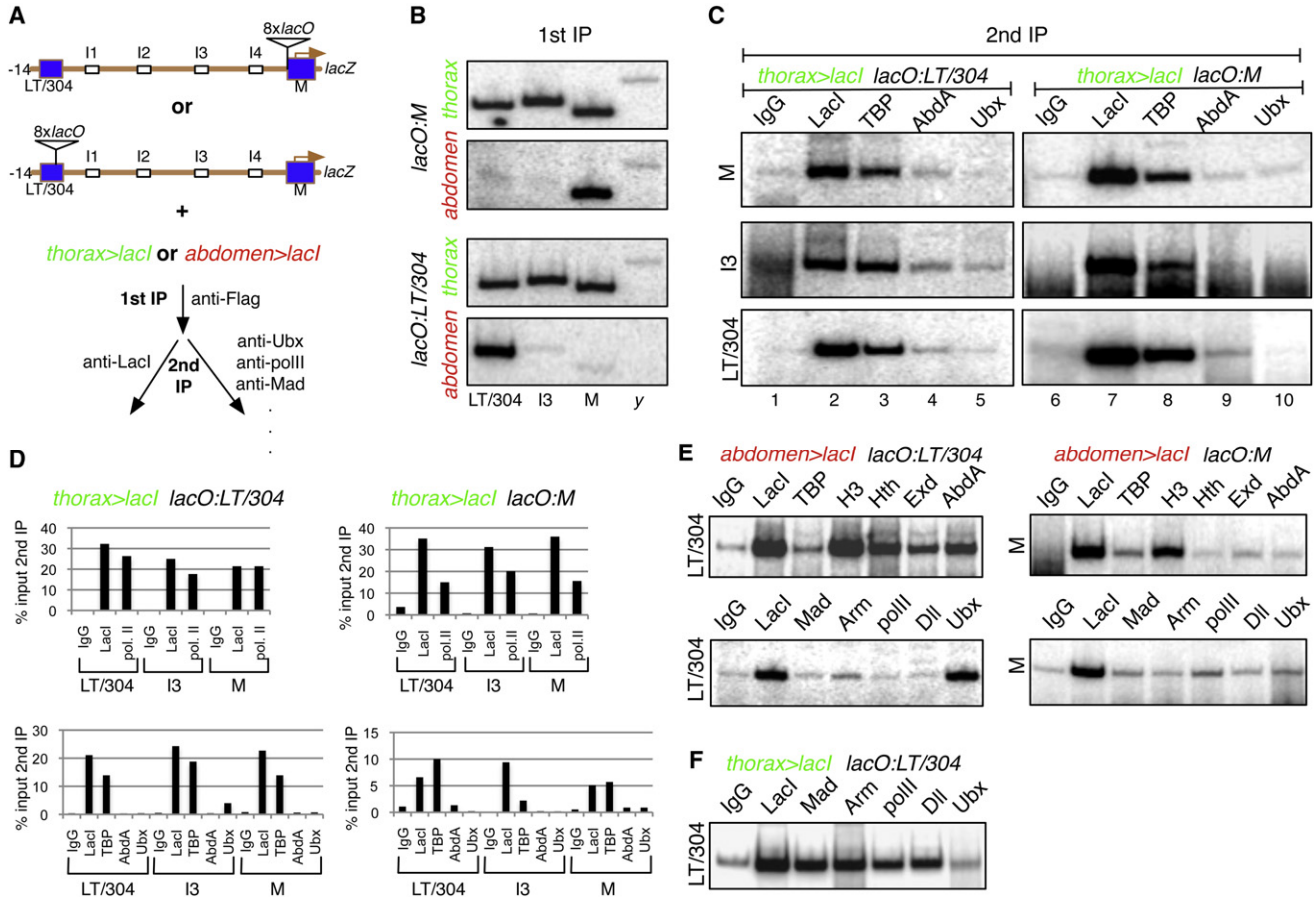

Figure 3. Cell Type-Specific Binding of Activators and Repressors at *Dll*

(A) Outline of cgChIP experiments for monitoring the presence of factors bound to *Dll* regulatory regions in thoracic and abdominal cells.

(B) ^{32}P PCRs confirming the thoracic-specific interaction between *Dll* regulatory elements after the primary anti-Flag IP. These data served as a quality control for the primary anti-Flag IP before carrying out any secondary ChIPs as in (C)–(F). Independent experiments are shown for both *lacO:M* and *lacO:LT/304*. *thorax* (green) and *abdomen* (red) refer to *thorax* > *lacI* and *abdomen* > *lacI*, respectively.

(C) ^{32}P PCRs of cgChIPs from *thorax* > *lacI; lacO:LT/304* (left) and *thorax* > *lacI; lacO:M* (right) embryos. These PCRs assess the presence of *M*, *I3*, and *LT/304* sequences following a secondary IP using the antibodies indicated above the gels (IgG, anti-LacI, anti-TBP, anti-AbdA, and anti-Ubx). The results confirm that IPs for LacI and TBP, but not abdominal Hox proteins, pull down multiple *Dll* 5′ regions in *Dll*-expressing cells in the thorax.

(D) qPCR measurements of cgChIP experiments for chromatin isolated from *thorax* > *lacI; lacO:LT/304* (left) and *thorax* > *lacI; lacO:M* (right). Measurements are for the three *Dll* sequences (*LT/304*, *I3*, and *M*) after secondary IPs with the antibodies indicated (top gels: IgG, anti-LacI, anti-PolII; bottom gels: IgG, anti-LacI, anti-TBP, anti-AbdA, anti-Ubx). Quantifications are presented as percentages (%) of the qPCR signals obtained from PCRs for the same amplicons after the primary, anti-Flag IP (i.e.; % input 2nd IP).

(E) ^{32}P PCRs of cgChIPs from *abdomen* > *lacI; lacO:LT/304* (left) and *abdomen* > *lacI; lacO:M* (right). These PCRs assess the presence of the *M* or *LT/304* sequences following a secondary IP using the antibodies indicated above each gel. IPs for repressors (e.g., Hth, Exd, AbdA, and Ubx) pull down *LT/304* sequences, but not *M* sequences; IPs for activators (TBP, Mad, Arm, PolII, and Dll) fail to pull down any *Dll* sequences from abdominal cells.

(F) ^{32}P PCRs of cgChIPs from *thorax* > *lacI; lacO:LT/304* embryos. IPs for activators (Mad, Arm, Dll, and PolII), but not repressors (Ubx), pull down these sequences from thoracic cells. See also Figure S1.

(e.g., Mad and Tcf binding to *Dll* in the thorax, but not in the abdomen) may also be missed when heterogeneous populations of cells are examined. Only by carrying out cell type-specific analyses, such as the cgChIP experiments described here, can such questions be fully resolved.

EXPERIMENTAL PROCEDURES

Antibodies

Immunostaining embryos was performed as in McKay et al. (2009) with minor modifications: (1) blocking was carried out overnight in PBST with 5% BSA at 4°C; and (2) both the primary and the secondary antibody incubations were 12 hr at 4°C. The antibodies used for immunostaining were anti-pMad (gift of G. Morata), anti-AbdA (gift of K. White), anti-Dll (Estella et al., 2008), anti-Wg (*Drosophila* Hybridoma Bank), anti-β-gal (MP Biomedicals), anti-Flag (Sigma-Aldrich; M2), and anti-Ubx (*Drosophila* Hybridoma Bank). The antibodies used for ChIPs were the following: anti-Ubx (modEncode; gift of K. White); anti-AbdA (Santa Cruz Biotechnology; SC-27063); anti-Mad (Santa Cruz Biotechnology; SC-25760); anti-Arm (Santa Cruz Biotechnology; SC-133180); anti-Dll (Santa Cruz Biotechnology; SC-15858); anti-Hth (Santa Cruz Biotechnology; SC-26187); anti-Exd (Santa Cruz Biotechnology; SC-26190); anti-GAF (Santa Cruz Biotechnology; SC-98263); anti-Flag (Sigma-Aldrich; M2); anti-LacI (Rockland; 600-401-B04); anti-PolII (Abcam;

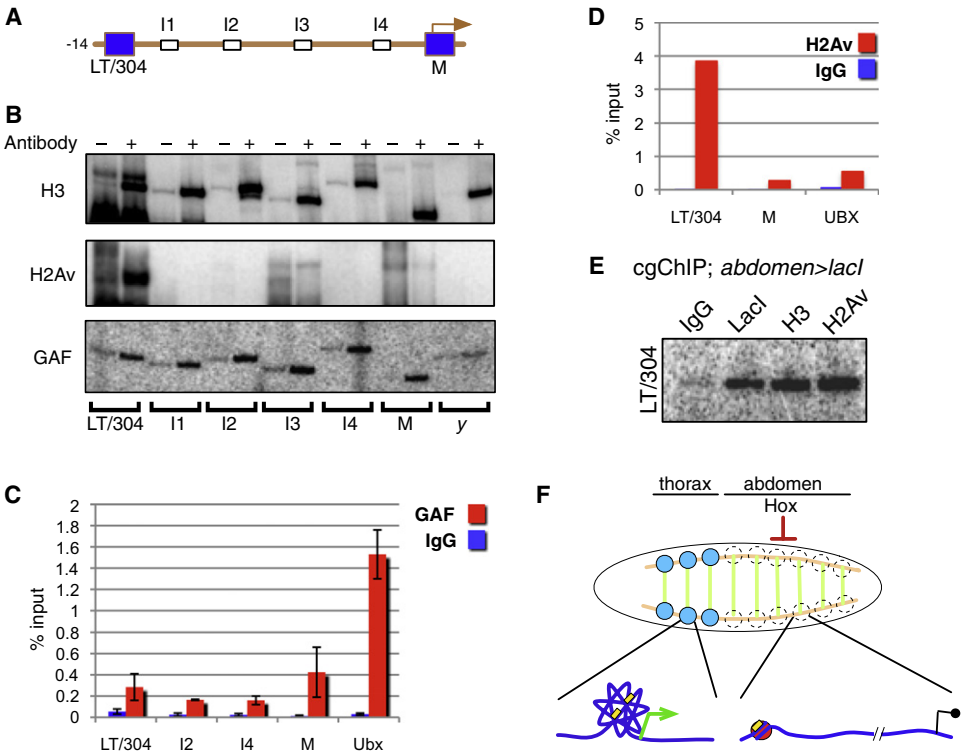

Figure 4. GAF and H2Av Have Distinct Patterns of Binding at *Dll*

(A) Schematic of the −14 kb 5′ *Dll* regulatory region.

(B) Whole-embryo ChIPs using anti-H3, anti-H2Av, and anti-GAF as indicated. H2Av, like other repressors, is bound to *LT/304*, but not other 5′ *Dll* regions. In contrast, binding of GAF appears to be widespread in the *Dll* 5′ region.

(C) qPCRs of whole-embryo ChIPs with anti-GAF, showing widespread binding to the *Dll* 5′ region, similar to the distribution of other activators (Figure 1). Error bars represent the SEM.

(D) qPCRs of whole-embryo ChIP with anti-H2Av, showing that it is bound to the *LT/304* region, but not to the *M* region.

(E) ^{32}P PCR of a cgChIP experiment from *abdomen > lacI; lacO:LT/304* embryos, showing that H2Av is bound to the *LT/304* region in abdominal cells.

(F) Summary of observed cell type-specific chromatin configurations in *Dll*-expressing (thorax) and *Dll*-nonexpressing (abdominal) cells. Thoracic *Dll*-expressing domains are indicated by the blue circles and occur close to the intersections of Wg expression (green) and Dpp expression (orange). Although Wg and Dpp are present in the same positions in abdominal segments, *Dll* is repressed in these segments by the abdominal Hox proteins. Our data suggest that in thoracic *Dll*-expressing cells the entire 5′ region of *Dll* (with its regulatory elements; yellow boxes) is in a compact state, whereas in abdominal segments the chromatin structure is more extended, and the *LT/304* region has H2Av-containing nucleosomes (red circle).

ab5408); anti-TBP (Abcam; ab61411); anti-Histone3 (Abcam; ab1791); and anti-Histone2Av (Abcam; ab18263).

Whole-Embryo ChIPs

Performed as in Orlando et al. (1997) with minor modifications: (1) ultracentrifugation was carried out for 30 hr; (2) 6 μg of primary antibody was used in an incubation step of 16 hr at 4°C; and (3) instead of agarose beads, magnetic beads (Invitrogen) were used and the coupling procedure we carried out for 1 hr at room temperature.

cgChip

The cgChIP experiments included several controls to assess any possible contamination. For one we routinely carried out anti-abdominal Hox ChIPs side by side with ChIPs for activators and basal factors from *thorax > lacI* embryos. Because abdominal Hox proteins are not expressed in the thorax, we did not continue with experiments in which these factors were detected in *thorax > lacI*-derived chromatin. Conversely, an anti-Dll ChIP was carried out in parallel with *abdominal > lacI* embryos. Because *Dll* is not expressed in the abdomen, we did not continue with experiments in which Dll binding was observed in *abdomen > lacI*-derived chromatin. In addition for both *thorax > lacI* and *abdomen > lacI* experiments, anti-LacI ChIPs were used as a positive control for both the primary and secondary IPs.

Genotypes

thorax > lacI flies were generated by combining *Dll304-Gal4* with *UAS-3Xflag-lacI* (simplified as *flag-lacI*). *abdomen > lacI* flies were generated by combining *Dll304-Gal80* and *DMX-Gal80* transgenes and a *DME^{Act}-Gal4* transgene with *UAS-3Xflag-lacI*. *DME^{Act}* is a mutant version of *Dll304* that is derepressed in the abdominal segments because the Hox, Exd, and Hth binding sites have been deleted (Gebelein et al., 2004), and the *Dll304-Gal80* and *DMX-Gal80* transgenes together block Gal4 activity in thoracic *Dll*-expressing cells. The result is predominant expression in cells of the abdominal segments that have the potential to express *Dll* (i.e., they receive the necessary positive inputs) in the absence of Hox repression. Because *DME^{Act}* is active in a slightly broader domain than *Dll304*, some non-*Dll*-expressing thoracic cells express *flag-lacI* in the *abdomen > lacI* embryos. *UAS-3Xflag-lacI* was generated from a *lacI* cDNA plasmid obtained from A. Belmont and expresses a form of LacI that has its tetramerization domain removed to avoid the formation of higher-order complexes and an NLS inserted at the N terminus (Robinett et al., 1996).

Collection and Fixing

Embryos ranging in age from 6 to 9 hr were grown at room temperature to ensure Gal80 (when present) was active. About 8 g of embryos was collected and dechorionated using standard procedures. Embryos were washed to remove any nonembryonic structures and fixed at room temperature for 30 min with 3:1 heptane:fix solutions. After washing, the embryos were transferred to Falcon tubes and placed at −80°C at least for 4 hr.

Chromatin Isolation

Embryos were pulverized and incubated twice in buffer A (0.25% Triton X-100, 10 mM Na-EDTA, 0.5 mM Na-EGTA,10 mM HEPES [pH 7.9]) for 10 min at room temperature and then twice with buffer B (0.2 M NaCl, 1 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM HEPES [pH 7.9]) for 15 min at 4°C. Sonication was on ice for at least seven times 40 s at maximum power. Upon centrifugation in 4,000 rpm for 10 min, the supernatant was separated to 1.5 ml vol followed by centrifugation for 20 min at 12,000 rpm at 4°C. Sheared isolated chromatin was stored at −80°C upon addition of glycerol (5% final).

Looping Experiments

Five hundred micrograms chromatin was precleared by incubation with 10 μl of magnetic beads for 1 hr at 4°C in 1x Ripa buffer. The reaction was divided into two tubes, and 2.5 μg of anti-Flag antibody or IgG was added, respectively. For the looping experiments the aforementioned reaction was at a final volume of 800 μl and incubated at 4°C for 4 hr with rocking. A total of 2 μl of beads was added for 1 hr at room temperature, followed by two rounds of incubation with 10 mM HEPES (pH 7.9), 0.5% Triton X-100, 140 mM Nacl, 0.14% DOC, 0.2% SDS. A final wash step was carried out before Proteinase K treatment and phenol/chloroform extraction and precipitations (Agelopoulos and Thanos, 2006). Formaldehyde crosslinking was reversed, and the extracted/precipitated DNA fragments were used as a template for the PCR amplification in which multiple domains of *Dll* 5′ DNA and control sequences were scanned. An equally divided sample was analyzed side by side with individual pairs of primers. Sequences of the primers are available upon request.

Double cgChIP Experiments

A total of 8 g of embryos was used in experiments with five antibodies in secondary IPs. Staged embryos were collected, harvested, and immunoprecipitated for Flag-lacI as described above. Precipitated *Dll* chromatin was eluted by the addition of 600 μl elution buffer and incubation at 4°C for 4 hr. The eluted material was precleared for a second time before further use. A small fraction of the eluted material was treated with Proteinase K, and after reversal of the crosslinks and extraction, the DNA was amplified with primers inside and outside of the transgene that contains the *lacO* binding sites. Thus, the purity of the first IP was tested before the second IP. PCR with primers that amplify lacZ sequences or sequences outside of the tagged transgene at irrelevant chromosomes was used to ensure the absence of any contamination of nonspecific chromatin.

The second round of IPs was carried out at 4°C. At this stage, two controls (IgG, a negative control, and anti-LacI, a positive control) were performed side by side to ensure that the first IP was successful. If confirmed the eluted chromatin was divided into equal samples and tested with 2 μg of a primary antibody in a total reaction of 300 μl. After 12 hr of incubation, chromatin/antibody complex was bound to magnetic beads as above. The reactions were washed twice with 1x Wash Ripa buffer and then treated with Proteinase K, and crosslinks were reversed. Finally, the extracted/precipitated DNA was analyzed with gene-specific primers in ^{32}P (Agelopoulos and Thanos, 2006) or SYBR Green based qPCR (Applied Biosystems).

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at doi:10.1016/j.celrep.2012.03.003.

LICENSING INFORMATION

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