A dual role for DNA binding by Runt in activation and repression of sloppy paired transcription

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ABSTRACT This work investigates the role of DNA binding by Runt in regulating the sloppy paired 1 (slp1) gene and in particular two distinct cis-regulatory elements that mediate regulation by Runt and other pair-rule transcription factors during Drosophila segmentation. We find that a DNA-binding–defective form of Runt is ineffective at repressing both the distal (DESE) and proximal (PESE) early stripe elements of slp1 and is also compromised for DESE-dependent activation. The function of Runt-binding sites in DESE is further investigated using site-specific transgenesis and quantitative imaging techniques. When DESE is tested as an autonomous enhancer, mutagenesis of the Runt sites results in a clear loss of Runt-dependent repression but has little to no effect on Runt-dependent activation. Notably, mutagenesis of these same sites in the context of a reporter gene construct that also contains the PESE enhancer results in a significant reduction of DESE-dependent activation as well as the loss of repression observed for the autonomous mutant DESE enhancer. These results provide strong evidence that DNA binding by Runt directly contributes to the regulatory interplay of interactions between these two enhancers in the early embryo.

INTRODUCTION

The transcription factor encoded by the Drosophila runt gene provides a valuable model for investigating the regulation of gene expression by Runx proteins, a family of DNA-binding proteins that is conserved from basal metazoans, such as cnidarians and sponges, to humans (Sullivan et al., 2008). Runx proteins participate in multiple pathways, extending from pattern formation and sex determination in Drosophila to the specification of cell fates in all three germ layers during mammalian development (Duffy and Gergen, 1994; Lian et al., 2003; de Bruijn and Speck, 2004; Enomoto et al., 2004; Ito, 2004). Aberrations in Runx activity are associated with a number of pathological conditions in humans. Runx1, also known asAML1, was initially identified as a frequent target of chromosome rearrangements involved in leukemia (Miyoshi et al., 1993; Nucifora et al., 1993; Golub et al., 1995; Sammou et al., 1998) and is now implicated in a variety of hematologic disorders (Yamagata et al., 2005). Mutations in Runx2 are associated with cleidocranial dysplasia (Mundlos et al., 1997; Otto et al., 1997), and alterations in Runx2 activity are associated with osteosarcoma as well as forms of breast, pancreatic, and prostate cancer (Kayed et al., 2007; Pratap et al., 2008; Akech et al., 2010). Similarly, Runx3 has roles in melanoma, breast, and colon cancer (Goel et al., 2004; Lau et al., 2006; Kitago et al., 2009; Zhang et al., 2011; Shin et al., 2018) and has been identified as a susceptibility locus for ulcerative colitis (Weersma et al., 2008). The finding that polymorphisms in Runx-binding sites are associated with a range of autoimmune diseases in humans (Prokunina et al., 2002; Helms et al., 2003; de Bruijn and Speck, 2004) suggests that Runx proteins may be involved in the regulation of autoimmune processes. The hallmark of the Runx family is the Runt domain, a highly conserved 128-amino-acid domain that is responsible for DNA binding
and for interacting with the conserved CBFb/Bro partner proteins (Kagoshima et al., 1993; Tang et al., 2000; Bravo et al., 2001; Zhang et al., 2003). A mutant Runt protein that cannot bind to Bro partner proteins is defective in a number of different in vivo assays (Li and Gergen, 1999), strongly suggesting that all of Runt's regulatory activities involve this heteromeric transcription factor complex. In contrast, the DNA-binding activity of Runt does not appear to be required for the initial establishment of repression of the segment-polarity gene engrailed (Wheeler et al., 2002; Vander Zwan et al., 2003). This repression instead involves the Tramtrack transcription factor whose activity as a repressor is potentiated by Runt via an unknown mechanism. The DNA-binding–defective Runt[CK] protein retains certain activity, including the ability to disrupt anterior patterning in the embryo and to interfere with axonal targeting during eye development (Vander Zwan et al., 2003; Walrad et al., 2010). Runx proteins also regulate transcription via DNA-binding–independent pathways in mice (Wilday and Howe, 2009), indicating that this attribute is not unique to Drosophila. Indeed, the observation that factors such as the estrogen, glucocorticoid and PPARalpha nuclear receptors (Umayahara et al., 1994; Porter et al., 1997; Reichardt et al., 1998; Tuckermann et al., 1999; Delerieve et al., 2002; Cheung et al., 2005) and the bHLH proteins Scl/Tal1 and Hand2 (Ravet et al., 2004; Liu et al., 2009), as well as members of the STAT transcription factor family (Cao et al., 2011), all have regulatory functions that are DNA binding independent reinforces the need to validate the in vivo role of interactions between transcription factors and their specific cis-regulatory DNA targets.

Runt was initially characterized based on its vital role as a pair-rule gene in the Drosophila segmentation pathway (Nusslein-Volhard and Wieschaus, 1980; Gergen and Wieschaus, 1985, 1986). The extensive understanding of segmentation coupled with the tools available in the Drosophila system provides numerous advantages for investigating Runt function. The slp1 gene has been identified as a particularly useful model. Runt functions both as an activator and as a repressor of slp1 (Swantek and Gergen, 2004). The combinatorial rules that account for these dual regulatory properties were investigated using classical loss-of-function experiments in combination with an ectopic expression system that allows for quantitative manipulation of gene expression in the blastoderm embryo (Tracey et al., 2000). Important to this regulatory dissection was the sensitivity of slp1 to ectopic Runt and the finding that reproducible metameric changes in slp1 expression are obtained with ectopic Runt at levels that do not significantly alter the metameric expression of other pair-rule genes that are involved in slp1 regulation (Swantek and Gergen, 2004). Although this observation does not demonstrate that Runt directly regulates slp1, it strongly suggests that the initial response of slp1 to ectopic Runt is not mediated through other known pair-rule gene regulators.

The initial metameric slp1 pattern is composed of 14 two-cell-wide stripes in the posterior half of each parasegment (Figure 1A). This pattern of alternating stripes of slp1-expressing and nonexpressing cells can be divided into four different cellular contexts that repeat throughout the segmented region of the embryo, depending on the specific regulatory factors present in these cells (Figure 1A). Repression of slp1 in type I cells is due to the homeodomain transcription factor Even-skipped (Eve) (Swantek and Gergen, 2004). Expression in type II cells, comprising the odd-numbered slp1 stripes, is activated in response to Runt and Odd-paired (Opa), a Zn-finger transcription factor homologous to the mammalian Zic proteins (Mizugishi et al., 2001). Type III cells also express Runt and Opa, but the presence of the homeodomain transcription factor encoded by the fushi tarazu (ftz) gene in these cells converts Runt from an activator to a repressor of slp1 (Figure 1A). Finally, expression of slp1 in type IV cells, comprising the even-numbered slp1 stripes, is due to Opa plus a contribution from an unknown factor X.

Further insights on the regulation of slp1 by Runt and the other pair-rule transcription factors came from the characterization of two distinct cis-regulatory elements that mediate this regulation (Prazak et al., 2010). A proximal early stripe element (PESE) drives expression in type IV cells but fails to generate the Runt-dependent stripes in type II cells (Figure 1B). This enhancer mediates repression by Eve and by the combination of Runt and Ftz (Prazak et al., 2010; Hang and Gergen, 2017), which can account for the lack of expression in cell types I, II, and III. The distal early stripe element (DESE) drives strong expression in type II and IV cells and also gives lower levels of inappropriate expression in type I cells. Consistent with this observation, this enhancer mediates repression by the combination of Runt and Ftz but is insensitive to repression by Eve (Prazak et al., 2010). Important to this work, DESE has been demonstrated to mediate activation in response to Runt and Opa (Prazak et al., 2010; Hang and Gergen, 2017). Interestingly, a composite reporter containing both early stripe elements recapitulates pair-rule gene–dependent regulation in a manner beyond what is expected from the additive inputs of the two separate enhancer elements. Recent studies on the mechanisms by which the activity of these enhancers is regulated have led to a proposal that this nonadditive interaction can be accounted for by enhancer-specific differences in the mode of repression in different cell types (Hang and Gergen, 2017).

Although several of the key factors involved in the differential regulation of slp1 in these four different cell contexts are well defined, it remains to be established whether the Runt-dependent regulation of these enhancers is direct. Indeed, it has been suggested that Runt's role in this process is indirect (Clark and Akam, 2016). Previous work utilizing a temperature-sensitive allele of Runt provides evidence that there is a temporally acute role for Runt in slp1 regulation, consistent with a direct interaction, but does not rule out an indirect role possibly through protein–protein interactions with other factors that directly interact with the slp1 enhancers. Establishing whether the slp1 PESE and DESE enhancers are direct targets of Runt is critical for understanding the mechanisms that underlie the nonadditive interactions between these two cis-regulatory elements. Here we investigate the role of DNA binding in the Runt-dependent regulation of these two early stripe enhancers. The DNA-binding activity of Runt is required for both activation and repression of slp1 (Walrad et al., 2010). As observed for endogenous slp1, the DNA-binding–defective Runt[CK] protein is ineffective at repressing the separate DESE and PESE enhancers as well as a composite reporter gene construct that contains both enhancers. Similarly, Runt[CK] is also compromised for activation of the composite reporter and the DESE enhancer, indicating that DNA binding by Runt contributes to Opa-dependent transcriptional activation. We further explore the function of presumptive Runt-binding sites in the DESE enhancer using site-specific transgenesis and quantitative imaging techniques. The results indicate that these sites are critical in mediating Runt-dependent repression in cell type III but are less important for activation in cell type II when DESE activity is examined as an autonomous enhancer. However, mutagenesis of these same sites in the context of a composite reporter that also contains PESE results in reduced expression in cell type II in addition to affecting repression in cell type III. We consider the implications of these findings for understanding slp1 regulation in the context of models involving the competitive regulation of enhancer-promoter interactions by Runt and the pair-rule transcription factors and
Volume 32 | November 1, 2021

Runt directly regulates sloppy paired

FIGURE 1: Regulation of slp1 early stripe expression. (A) Schematic diagram of pair-rule regulatory inputs that generate the initial periodic expression of slp1. The phasing of the expression patterns of Runt, Eve, Ftz, and Opa are shown above a row of eight cells, representing two parasegmental repeats along the anterior–posterior axis. Shading indicates expression of slp1 in type II and type IV cells, with darker shading indicating the stronger early expression in type IV cells. The green arrow above type II cells indicates the requirement for both Runt and Opa in their activation (Swantek and Gergen, 2004). The contribution of Opa to expression in type IV cells is similarly indicated by the arrow above these cells. Repression of slp1 by Eve in type I cells is indicated by the orange horizontal bar and vertical line that connect to the domain of Eve expression indicated above these cells. The horizontal red bar above the slp1-repressed type III cells is connected to pink and blue vertical lines denoting the combined requirement for Runt and Ftz, respectively, in blocking expression (Swantek and Gergen, 2004). (B) The expression driven by slp1 and different slp-lacZ reporters within a region spanning two parasegments is diagrammed to the right as in A. A schematic of the slp1 locus indicating the relative position of the upstream DESE and PESE enhancers relative to the transcription unit (green arrow) is provided at the top. The black boxes identify the minimal regions for each early stripe enhancer, from 8.1 to 7.1 kb and from 3.1 to 2.5 kb upstream of the transcription start site for the DESE and PESE enhancers, respectively (Prazak et al., 2010). The white boxes identify the extent of DNA flanking the minimal enhancer segments contained in the composite reporter gene constructs used in this work to buffer potential short-range interactions between factors interacting with the different enhancer elements. These larger DNA segments extend from 8.7 to 6.5 kb and from 3.9 to 1.8 kb upstream of the transcription start site for DESE and PESE, respectively. The three lines below show schematics of the PESE-, DESE-, and [DESE + PESE]-containing lacZ (red arrow) reporter genes, with regions that are not contained in the reporters indicated by dotted lines. The pair-rule inputs mediated by the independent PESE and DESE enhancers are indicated above these elements (Prazak et al., 2010), with the resulting expression diagrammed to the right of each construct. The inappropriate expression in type I cells and stronger than normal expression in type II cells driven by the DESE enhancer are indicated by stippled and darkly shaded cells, respectively. DESE receives input from an unknown factor X that produces the residual expression in type IV cells in the absence of Opa. As shown at the bottom, the inappropriate activity of DESE is suppressed in a composite PESE-containing reporter that faithfully emulates the periodic expression of endogenous slp1.

RESULTS

Differential requirements for DNA binding in repressing the slp1 early stripe enhancers

The role of DNA binding in the regulatory activities of Runt has previously been investigated using a mutant derivative containing two point mutations in the Runt domain (C127S, K199A) that greatly reduce in vitro DNA-binding activity. This DNA-binding-defective Runt[CK] protein retains the ability to establish repression of en-grailed but is ineffective in activation of Sex-lethal as well as in both activation and repression of slp1 (Kramer et al., 1999; Vander Zwan et al., 2003; Walrad et al., 2010). To further clarify the requirement for DNA binding in slp1 regulation, we used in situ hybridization to compare the activities of the Runt and Runt[CK] proteins on reporter gene constructs containing different slp1 cis-regulatory elements.

Discuss the potential importance of similar nonautonomous enhancer interactions in regulating gene expression in developmental systems.

Coexpression of Runt and Ftz using the nanos-GAL4-tubulin (NGT) maternal GAL4 expression system (Tracey et al., 2000) resulted in the nearly complete repression of slp1. In the context of the cell-type nomenclature used here, ectopic coexpression of Runt and Ftz represses slp1 in cell types II and IV and is driving all cells toward adopting the slp1 expression state characteristic of cell type III. This nearly complete repression was faithfully emulated by the slp[8765/3918]lacZ reporter, a composite P-element reporter gene construct containing both the DESE and PESE pair-rule response elements (Figure 2, A and A’ and B and B’). Indeed, the merged image demonstrates good concordance in the irregular expression of the slp1 and lacZ mRNAs that remains within the segmented region of the slp1-repressed embryos (Figure 2B’). In contrast, NGT-driven coexpression of Ftz and Runt[CK] had little effect on the expression of the even-numbered stripes in type IV cells of both slp1 and the composite slp[8765/3918]lacZ reporter (Figure 2, C and C’). The reduced expression of the odd-numbered stripes in type II cells observed in these embryos was expected and is due to the ectopic expression of Ftz in type II cells that normally express endogenous Runt but not endogenous Ftz.

Similar experiments were performed to investigate the importance of DNA binding for Runt-dependent repression of reporter genes that contain only the PESE or DESE enhancers and are integrated into the same chromosome site using F mis transgenesis. The PESE-containing slp[3918]lacZ reporter normally expresses only in type IV cells (Figure 2D). This expression was almost completely eliminated by NGT-driven coexpression of Runt and Ftz (Figure 2E). It is notable that the residual expression of this PESE-containing reporter does not overlap that of endogenous slp1. This discrepancy suggests that the integration of regulatory inputs by PESE does not contribute in a major way to the slp1 pattern in these embryos. More important to the issue of whether DNA binding is important for the Runt-dependent repression of PESE is the observation that the expression of this reporter in type IV cells appears to be insensitive to ectopic coexpression of Runt[CK] and Ftz (Figure 2F). Indeed, the lacZ (magenta) expression driven by this PESE reporter gene appears to be broadened anteriorly, suggesting that the Runt[CK] protein interferes with repression of the reporter by endogenous Runt and Ftz in type III cells. Taken altogether, these results provide strong evidence that the DNA-binding activity of Runt is important for repression of the slp1 PESE enhancer.

The response of the DESE-containing slp[8771]lacZ reporter is more complicated. This reporter faithfully emulates the response of the endogenous slp1 gene to ectopic coexpression of wild-type Runt and Ftz (Figure 2H). This concordance is in contrast to the nonoverlapping expression of slp1 and the PESE-containing slp[3918]
lacZ\textsuperscript{att} reporter in response to ectopic Runt and Ftz noted above (Figure 2E) and supports the proposal that the DESE enhancer is responsible for the regulation of slp\textsubscript{1} transcription in Runt-expressing cells (Hang and Gergen, 2017). However, there are interesting differences in the response of slp\textsubscript{1} and the slp\textsubscript{3918}lacZ\textsuperscript{att} reporter to ectopic coexpression of Runt[CK] and Ftz. It is most straightforward to interpret whether the effects of ectopic Runt[CK] expression are due to the inability to interact with DNA in cells that do not express endogenous Runt, that is, in cell type IV normally comprising the even-numbered slp\textsubscript{1} stripes and the type I cells posterior to these stripes. As observed for endogenous slp\textsubscript{1} and the PESE-containing reporter, ectopic expression of Runt[CK] in type IV cells does not result in repression of the slp\textsubscript{3918}lacZ\textsuperscript{att} reporter (Figure 2I). Somewhat unexpected is the strong expression of this reporter in type I cells, that is, in cells posterior to the even-numbered slp\textsubscript{1} stripes (Figure 2I). Although further work is needed to explain the DESE-dependent expression in type I cells in response to ectopic coexpression of Runt[CK] and Ftz, these results provide strong evidence that the DESE enhancer is responsible for the regulation of slp\textsubscript{1} transcription in Runt-expressing cells.
evidence that the DNA-binding activity of Runt is required for repression of both the PESE and DESE enhancers in type IV cells.

**Runt’s DNA-binding activity potentiates Opa-dependent slp1 activation**

We used an additional assay to further investigate the role of DNA binding by Runt in slp1 activation. This assay takes advantage of the observation that coexpression of Runt and Opa results in slp1 activation in all somatic cells of the blastoderm embryo that do not express Ftz (Swantek and Gergen, 2004), including cells in the anterior head region that are not expressing other pair-rule transcription factors (Figure 3A). The resulting pattern is nearly uniform anterior expression of slp1 extending to cells at the anterior pole with five three- to four-cell-wide stripes of expression separated by four- to five-cell-wide bands of nonexpressing cells in the presegmental region of the embryo. The broader band of nonexpressing cells separating the fourth and fifth stripes is explained by the localized expression of Ftz throughout this region (Swantek and Gergen, 2004). The response of slp1 to ectopic coexpression of Runt and Opa was faithfully emulated by a composite reporter gene containing both the DESE and PESE enhancers (Figure 3, C′′ and D′′), suggesting that both activation and repression of these targets involves DNA binding by Runt. There is clear evidence for the ectopic expression of slp1 and both reporters in response to Runt[CK] and Opa, obtained in response to NGT-driven expression of Opa alone (E, E′, F, F′). The embryos in these experiments were obtained from crosses between females homozygous for the NGT40 driver and the appropriate reporter gene to males carrying the strong UAS-opaD10 transgene either alone or in combination with UAS-runt232 or UAS-runt[CK]77, two transgenes that are expressed at similar levels and have equivalent activities on DNA-binding–independent targets of Runt (Vander Zwan et al., 2003).
FIGURE 4: Identification of Runt-binding sites in the slp1 DESE enhancer. (A) Genome Surveyor screen shot depicting results of genome-wide chromatin immunoprecipitation experiments for an approximately 10 kb interval flanking the slp1 transcription unit performed with two different antisera directed against the Runt protein and one directed against the transcriptionally active phosphorylated form of RNA polymerase II on chromatin for stage 4–5 blastoderm embryos (MacArthur et al., 2009). The extents of DNA sequences included in the slp[DESE:8765] and slp[PESE:3918] enhancers are indicated by blue lines above this screen shot, with the thicker regions corresponding to the central minimal elements (Prazak et al., 2010). Data for all antibodies are shown with a threshold 1% false-discovery rate. (B) Results of electrophoretic mobility shift assays with recombinant Runt and Bro proteins and a labeled DNA probe containing the Runx site in the A core of the polyoma virus enhancer (Kamachi et al., 1990). The two leftmost lanes show the fast-migrating probe alone and the additional more slowly migrating complex detected with Runt and Bro near the top of the autoradiogram, respectively. The inset in the black box shows a longer exposure of the region with Runt:Bro:DNA complexes. The other lanes show competition experiments with 5, 12.5, and 100 ng of four different oligonucleotide probes as labeled across the top. These probes contain Runx sites of different affinities. Their effectiveness as competitors correlates with their rank order of affinity for Runx1, HA ($K_d = 2.6 \times 10^{-12} M$) > SAAB ($5.5 \times 10^{-12} M$) > Mo ($2.9 \times 10^{-11} M$) > Mx ($1.7 \times 10^{-10} M$) (Lewis et al., 1999). Comparable results were obtained in competition experiments done with oligonucleotide probes containing Runx sites from the SL3-3, TCRα, and SFFV (Rauscher) enhancers (unpublished data). (C) The sequence coordinates, orientation (Sense, Antisense), sequence, and Ri score based on a position weight matrix for a consensus Runx site are shown for candidate Runt sites in the region of the DESE enhancer. Nucleotides in a red font indicate base substitutions of one of the core cytosines in candidate sites with an Ri score greater than 5.1. The five sites with the highest Ri scores that also retain the core cytosines are numbered 1–5 on the right side of the table. The positions of these sites are indicated by red vertical lines below the schematic depiction of DESE in A. (D) Results of ChIP assays with control serum (light blue and light pink bars) and antibodies specific for Runt
Runt and Opa (Figure 3, A” and B”). A similar level of anterior activation was produced in response to NGT-driven expression of Opa alone (Figure 3, E” and F”). These observations indicate that ectopic expression of Opa alone can activate slp1 and the DESE-containing reporter gene constructs in the absence of Runt and suggests that this Opa-dependent transcription is potentiated by Runt in a DNA-binding–dependent manner.

**Runt-binding sites mediate repression by the slp1 DESE enhancer**

The results of the above experiments provide evidence that the DNA-binding activity of Runt contributes to both the activation and repression of the slp1 early stripe enhancers but do not demonstrate that this involves direct interactions between Runt and the slp1 enhancers. The sensitivity of slp1 expression to manipulations in Runt activity, as well as the temporal immediacy of the response to these manipulations (Swantek and Gergen, 2004), are consistent with slp1 being a direct target. Further, results from the Berkeley Drosophila Transcription Network Project (BDTNP) identify regions of the slp1 locus that are associated with Runt in the early Drosophila embryo (MacArthur et al., 2009). The two regions that show the highest levels of association with both Runt antisera used in these studies correspond remarkably well with the DESE and PESE enhancers identified by our functional studies on slp1 regulation (Figure 4A). Several studies have identified the sequence ACCRCA as a consensus Runx-binding site (Kamachi et al., 1990; Meinkova et al., 1993). The structure of Runx1 bound to DNA has been determined (Bravo et al., 2001; Tahir et al., 2001). The six amino acids that are the primary determinants of Runx1 DNA-binding specificity are all identical in Drosophila Runt, strongly suggesting that binding sites recognized by Runt will conform to the Runx consensus. The similar DNA-binding specificity of Runt was confirmed by electrophoretic mobility shift assays using a series of oligonucleotide competitors that have different affinities for mammalian Runx1 (Lewis et al., 1999). The results of these experiments revealed that Runt has the same rank order of affinities for these different sequences (Figure 4B). On the basis of these observations, we used a Runx position weight matrix to identify putative Runt-binding sites in DESE as a starting point for investigating whether direct interactions with the DESE enhancer are involved in the regulation of DESE-dependent transcription by Runt. This search identified a number of candidate sites of varying quality within the interval defined by the DESE-containing slp[8771]/lacZ<sup>att</sup> reporter gene (Figure 4C). Of these sites, five were chosen for mutagenesis based on having a high Ri value as well as containing the core cytosines in the consensus sequence that contribute most to DNA-binding specificity.

We used chromatin immunoprecipitation (ChIP) to investigate whether mutagenesis of these five putative binding sites affected the association of Runt with the DESE enhancer. ChIP assays were performed on chromatin from 3- to 4-h-old Drosophila embryos containing either the wild-type slp[8771]/lacZ<sup>att</sup> or the mutant slp[8771m1-5]/lacZ<sup>att</sup> reporter with mutations in all five of the candidate Runt sites. Results of quantitative PCR with the rDESE-7.8 primer pair that takes advantage of a sequence polymorphism to specifically amplify the region containing Runt sites 3 and 4 from the DESE-containing reporter genes revealed that the ChIP signal observed with the wild-type DESE-containing reporter is significantly reduced (p < 0.05) near to background levels for the reporter with the mutant Runt-binding sites (Figure 4D). The effect of these mutations on Runt association is further confirmed by direct sequencing of the PCR products obtained using primers that amplify a region containing Runt candidate binding site 4 for both endogenous slp1 and these reporter genes. The sequence chromatogram of the PCR product obtained with input chromatin from slp[8771m1-5]/lacZ<sup>att</sup> embryos shows both the wild-type GCCACA sequence from the endogenous slp1 locus and the GAAACA sequence for the mutant binding site in the reporter gene (Figure 4E). In contrast, the sequence of the PCR product from the ChIP assay with this mutant reporter gene shows a loss of adenine base calls from the mutant Runx-binding site (Figure 4E). These results confirm the in vivo association of Runt with the DESE-containing slp[8771]/lacZ<sup>att</sup> reporter gene and provide strong evidence that mutation of the Runt sites within this element interfere with this interaction.

We used ΦC31-mediated site-specific transgenesis to investigate the effects of mutations in these five candidate Runt-binding sites, both singly and in a number of different combinations. None of the single site mutants that were tested showed obvious effects on reporter gene expression (Supplemental Figure 1). The region within DESE that showed the highest level of Runt association in the BDTNP results encompasses Runt sites 3 and 4 (Figure 4A). A DESE-lacZ reporter gene containing mutations in both of these sites, slp[8771m3,4]/lacZ<sup>att</sup>, produced an altered pattern. Expression of both the odd- and even-numbered stripes persisted, but with a clear loss of repression in the type III cells anterior to stripes 8, 10, and 12 (Figure 5, B’ and B”). The loss of repression is more apparent for a reporter containing mutations in all five of the candidate Runt transcription start sites as a negative control for Runt association. Primer pair rDESE −7.4 kb amplifies a region of DESE containing Runt site 5 for both endogenous slp1 and the DESE-containing reporter genes. Primer pair rDESE −7.8 kb takes advantage of a polymorphism to specifically amplify a region of the DESE-containing reporter genes containing Runt sites 3 and 4. The Eve primer pair amplifies a region of the eve minimal autoregulatory sequence (MAS) that has strong association with Runt (MacArthur et al., 2009) as a positive control. Primer pair PESE −2.8 kb amplifies a region of the endogenous slp1 PESE enhancer that provides a second positive control for the efficiency and sensitivity of these ChIP assays in detecting Runt binding with chromatin from embryos containing the wild-type DESE and DESEM1-5 reporter genes. The asterisk above the bar between the wild-type DESE and DESEM1-5 reporter genes for the PESE −7.8 kb primer pair indicates a statistically significant (p value < 0.05) reduction in the association of Runt with the DESEM1-5 reporter. Although the difference is not statistically significant, the reduced ChIP signal for the DESE −7.4 kb primer pair is expected based on the reduced association of Runt with the DESEM1-5 reporter but not with the endogenous slp1 DESE enhancer. (E) Chromatograms of sequence results from input DNA as well as three independent Runt ChIP assays for either the wild-type DESE or DESEM1-5 enhancer using primers that amplify a region of DESE containing Runt site 4 for both the endogenous and reporter DESE sequences. Shaded boxes indicate the two C > A mutations that were introduced to mutate Runt site 4 as well as the C > G sequence polymorphism that distinguishes the DESE-containing reporters from the endogenous slp1 locus.

Volume 32 November 1, 2021 Runt directly regulates sloppy paired | 7
FIGURE 5: Runt sites mediate repression of DESE-driven expression. Fluorescence in situ hybridization shows expression of slp1 (green) and lacZ (magenta) for reporter gene constructs containing the wild-type slp[8771]lacZ<sup>att</sup> reporter (A) compared with similar reporter gene constructs inserted at the identical genomic location that have mutations in Runt sites 3 and 4 (B) or in all five putative Runt-binding sites (C). The even-numbered stripes are labeled above the merged images for these three embryos shown in panels A′, B′, and C′, respectively. (D–F) Integrated expression patterns of these reporter gene constructs generated using an image processing method that allows averaging of results from multiple embryos (Janssens et al., 2005) that has been extended to include slp1 expression in stage 6 embryos as described in Materials and Methods. Relative mRNA accumulation levels are plotted as a function of position on the A–P axis, with slp1 and lacZ indicated by the green and magenta traces, respectively. As above, the even-numbered slp1 stripes are labeled to provide reference points for the descriptions provided in the text. It is notable that the trace of slp1 expression approaches background levels anterior to both the odd- and even-numbered
sites, slp[8771m1-5]lacZatt. The effect of these mutations was a loss of repression in the type III cells anterior to all of the even-numbered stripes (Figure 5, C′ and C″). This derepression occurs in cells where repression normally requires both Runt and Ftz. On the basis of these observations, we conclude that the Runt-binding sites in DESE are important for its repression by Runt and Ftz. Expression of the mutant reporter was not uniform, with somewhat higher levels of expression in type III cells anterior to even-numbered slp1 stripes than in the type I cells posterior to these stripes.

To validate the above observations and gain further insight into the possible effects of the Runt-binding site mutations on reporter gene expression, we used a process for quantifying confocal in situ hybridization data (see Materials and Methods). In this case data were collected and integrated for stage 6 Drosophila embryos, a brief (<10 min) stage with distinctive morphology during which the initial metameric slp1 pattern is evident. The differences in expression of endogenous slp1 and the DESE-containing slp[8771]lacZatt reporter were confirmed by this analysis (Figure 5D; Supplemental Figure 2). The difference in expression levels in even-versus-odd stripes observed for the slp1 mRNA (green trace) was much less apparent for lacZ (magenta trace). The inappropriate expression of this reporter gene in type I cells anterior to the odd stripes was also demonstrated by lacZ mRNA levels above background that are not observed for slp1, especially anterior to stripes 7 and 11 (i.e., posterior to stripes 6 and 10). A similar representation of the results for the reporter with mutations in Runt sites 3 and 4 confirmed the loss of repression in type III cells anterior to stripes 8, 10, and 12 and also provided an indication of partial loss of repression anterior to other even-numbered stripes (Figure 5E). Indeed, the lacZ mRNA levels due to loss of repression in type III cells anterior to the even stripes were consistently higher than in type I cells posterior to these stripes. The same pattern was observed for the quintuple mutant slp[8771m1-5]lacZatt reporter (Figure 5F), with consistently higher levels of expression occurring in type III cells anterior to the even slp1 stripes than in type I cells posterior to the even slp1 stripes.

**Run binding also contributes to DESE-dependent activation**

The DNA-binding activity of Runt contributes to slp1 activation (Walrad et al., 2010), an effect that is emulated by a composite reporter gene containing both the PESE and DESE enhancers (Figure 3). We previously showed that Runt-dependent activation is mediated by DESE and not by PESE (Prazak et al., 2010). However, as shown above, mutagenesis of five candidate Runtbinding sites in DESE greatly interferes with repression in type III cells but does not significantly reduce the expression of the reporter gene in type II cells. One explanation for these somewhat paradoxical results is that the relative importance of Runt DNA binding for DESE-dependent activation is increased by the presence of the PESE enhancer. This could be due to Runt interacting with the DESE and/or PESE enhancers. To further investigate whether activation involves Run binding to DESE we examined
FIGURE 6: Runt-binding sites in DESE contribute to both activation and repression of the composite DESE + PESE reporter gene. (A) Expression of slp1 (green), the wild-type composite *slp*[8765/3918]*lacZ* reporter (magenta, A'), and the merged image (A'') as visualized by fluorescence in situ hybridization. (B, B', B'') Similar representation of the expression of the mutant *slp*[8765m1-5/3918]*lacZ* reporter inserted in the same genetic location and identical in sequence to the wild-type reporter in A except for the mutations in the five Runt-binding sites in the DESE enhancer. In addition to having reduced expression of the odd-numbered stripes, the mutant reporter also shows evidence of...
the effect of mutating the Runt sites in DESE in the context of a composite reporter that also contains PESE. This experiment compares expression of wild-type and mutant composite reporter gene constructs integrated into the same genomic site using ΦC31 site-directed transgenesis. Similar to the composite slp[8765/3918]lacZΦ reporter described previously and used in Figures 2 and 3, the wild-type slp[8765/3918]lacZΦ reporter expressed both odd and even stripes, with weaker expression of the odd-numbered stripes as observed for endogenous slp1 (Figure 6, A and A′). The composite construct with mutations in the five Runt sites, slp[8765m1-5/3918]lacZΦ, also gave strong expression of the even stripes, but with reduced expression of the odd-numbered stripes (Figure 6, B, B′, and B″; Supplemental Figure 3). Quantification of the wild-type and mutant composite reporter gene expression patterns confirmed these qualitative observations. The mutant reporter showed greatly reduced expression in slp1-expressing type II cells comprising the odd stripes, with the residual expression most obvious for stripe 3 (Figure 6D). Further, the symmetric stripes of lacZ mRNA accumulation observed for the wild-type composite reporter (Figure 6C) are replaced by even-numbered stripes that show an anterior shoulder of lower-level expression in the slp[8765m1-5/3918]lacZΦ embryos (Figure 6D). This derepression in type III cells is analogous to that observed for slp[8771m1-5]lacZΦ. Statistical analysis of normalized expression data for the slp[8765/3918]lacZ WT and slp[8765m1-5/3918]lacZ MT reporters reveals reduced expression in type II cells, with a ratio of nMT to WT expression between 0.09 and 0.46 (Figure 6E). This reduction is statistically significant (p ≤ 0.05) for four of the five type II cells (shaded in yellow) included in this analysis. This analysis also indicates a statistically significant increase in expression in type III cells (p ≤ 0.01, shaded in green). Taken altogether, these observations indicate that mutating the Runt sites in the DESE enhancer affects both the repression and activation mediated by this enhancer. Notably the importance of the Runt-binding sites for activation is significant only in the composite reporter gene construct containing the PESE enhancer.

DISCUSSION

The experiments presented above used two different approaches to investigate the role of DNA binding by Runt in slp1 regulation. The results provide compelling evidence that direct interactions between the Runt protein and slp1 cis-regulatory DNA contribute to the transcriptional regulation of this gene during Drosophila segmentation. The DNA-binding–defective Runt(CK) protein is compromised for both repression and activation of slp1, and mutagenesis of Runt-binding sites within the DESE enhancer affects both repression and activation mediated by this element in response to the Runt protein. Perhaps most interesting are the different contributions of DNA binding by Runt to DESE-dependent regulation depending on the presence or absence of the PESE enhancer, a distinct cis-regulatory element normally separated from DESE by more than 2.5 kb of DNA. When DESE is tested separately as an autonomous element, the Runt-binding sites are important for repression in type III cells where Runt and Ftz cooperate to repress slp1 expression but have a small to no role in contributing to DESE-dependent activation in type II cells. However, when tested in a composite reporter gene construct that also includes the PESE enhancer, mutagenesis of the Runt-binding sites in DESE results in a significant reduction in the level of expression in type II cells.

One explanation for these observations is that a principal role of Runt’s binding to DESE is to increase the strength of interaction between DESE and the slp1 promoter, an activity that becomes most apparent when another enhancer, such as PESE, is competing for interaction with this promoter (Figure 7A). Given the context-dependent activities of the PESE and DESE enhancers, it is useful to consider this enhancer competition model in the context of the different slp1 expression states (Figure 7, B and C). The initial description of nonadditive interactions between the wild-type DESE and PESE enhancers was based on the inappropriate expression driven by the DESE enhancer in type I cells anterior to the odd-numbered slp1 stripes. This ectopic expression is suppressed in composite reporter gene constructs that also contain the PESE enhancer (Prazak et al., 2010). As expected, mutagenesis of the Runt-binding sites in the DESE enhancer does not affect the inappropriate expression in type
I cells (Figure 5G), as Runt is not normally expressed in these cells (Figure 7B). PESE also interferes with the ectopic expression driven by the mutant DESE enhancer in cell type I (Figures 6, A′, B′, and E, and 7C). Taken altogether these observations suggest that PESE, which normally mediates repression in cell type I, interferes with Runt-independent, DESE-driven expression in this cell type.

Further evidence in support of the repressive effect of PESE on DESE activity comes from considering the expression of these different reporter genes in cell type II. Expression of the odd-numbered slp1 stripes in cell type II requires both Runt and Opa and is mediated by the DESE enhancer (Prazak et al., 2010). When tested as an autonomous element, the wild-type DESE enhancer drives abnormally high levels of reporter gene expression in type II cells that are comparable to the levels observed in type IV cells (Figures 2G and 5, D and G). Inclusion of PESE in the composite reporter containing the DESE enhancer reduces expression in type II cells, giving a difference in the expression levels in these two cell types similar to that observed for slp1 (Figures 2A′ and 6A′). Mutagenesis of the Runt-binding sites in DESE in a composite reporter containing the PESE enhancer results in significantly reduced expression in type II cells (Figures 6 and 7C). In the context of the enhancer competition model, these observations suggest that Runt binding to DESE increases the ability to overcome the repressive effects of PESE in type II cells by augmenting interactions between DESE and the slp1 promoter.

The above discussion identifies the repressive effects of PESE on DESE-dependent expression in cell types I and II (Figure 7C). Prior work provides additional evidence that PESE is also capable of interfering with DESE-dependent expression in cell type IV. Like cell type I, Runt is not expressed in cell type IV, and this aspect of DESE-driven expression should be Runt independent. When tested as an autonomous enhancer, DESE drives expression in the type IV cells that normally comprise the even-numbered slp1 stripes, and this expression is insensitive to repression by Eve (Prazak et al., 2010). Ectopic Eve specifically represses the even-numbered stripes of both slp1 and composite reporter gene constructs containing both the PESE and DESE enhancers. Our interpretation is that ectopic Eve blocks DESE-dependent expression in these cells by repressing PESE in a manner that does not allow for DESE-driven expression. The Eve-dependent repression of PESE involves preventing release of promoter-proximal paused RNA polymerase. It has been proposed that repression at this step involves a stable association of PESE with the slp1 promoter that occludes access to the DESE enhancer (Hang and Gergen, 2017). The same explanation could also apply to the effects of PESE on DESE-dependent expression observed in cell types I and II.

Further evidence suggests that a competition between the PESE and DESE enhancers can account for the temporal dynamics of slp1 expression. Expression of the even-numbered slp1 stripes in cell type IV is strong in early cellular blastoderm stage embryos, whereas (DESE\textsuperscript{m1-5}) enhancer, either alone or in a composite reporter also containing the PESE enhancer. A high average expression at a level 80% or higher of that in type IV cells is indicated as ++. Intermediate expression levels, 25–50% of that in type IV cells, are indicated with a +. An average that is above background but less than 15% of that in type IV cells is indicated with a −. These numbers were generated by calculating the expression for each of the different types I, II, and III cells relative to the type IV cell used for normalizing expression values in Figures 5G and 6E and then averaging these values for the four different type I cells and five different type II and III cells included in these figures.

**FIGURE 7:** Runt-dependent regulation of enhancer-promoter interactions. (A) Schematic diagram of the slp1 locus. The DESE and PESE enhancers are depicted as blue boxes (labeled), and the blue arrow represents the slp1 transcription unit. The ability of DESE to mediate activation by Opa (O) is indicated by a green arrow pointing toward DESE. The effect of Runt (R) in potentiating this Opa-dependent activation is indicated by the curved green line connected to this arrow. The requirement for both Runt and Ftz (R + F) in repressing DESE is indicated by the red line with a bar at the end. The ability of PESE to mediate activation by Opa as well as repression by either Runt or Eve (E) are indicated similarly to the left of the PESE enhancer. The proposed competitions of these two enhancers for interacting with the slp1 promoter are represented by bidirectional black arrows between each enhancer and the promoter region. The expression of slp1 produced by these different regulatory inputs is diagramed below in B, which shows the relative phasing of Runt, Eve, Ftz, and Opa expression above a column of eight cells, corresponding to one odd and one even parasegment (PS). Cells that express slp1 are shaded green, with the darker shading representing the stronger early expression in the even-numbered parasegments. The PESE enhancer can mediate the Eve-dependent repression of slp1 in type I cells (Prazak et al., 2010). Runt prevents PESE-dependent expression in both type II and type III cells. The DESE-dependent expression of the odd-numbered slp1 stripes in type II cells requires both Runt and Opa, whereas this same enhancer mediates repression in response to the combination of both Runt and Ftz in type III cells. Both enhancers are capable of driving expression in type IV cells. (C) Table indicating the relative expression level in type I, II, and III cells relative to the high expression levels in type IV cells for DESE-containing reporter genes with the wild-type (DESE\textsuperscript{WT}) or Runt-binding site mutant (DESE\textsuperscript{m1-5}) enhancer, either alone or in a composite reporter also containing the PESE enhancer. A high average expression at a level 80% or higher of that in type IV cells is indicated as ++. Intermediate expression levels, 25–50% of that in type IV cells, are indicated with a +. An average that is above background but less than 15% of that in type IV cells is indicated with a −.
expression of the odd-numbered stripes in type II cells emerges later. Both PESE and DESE drive strong expression in type IV cells. The autonomous DESE enhancer can also drive strong expression in type II cells, but not when in competition with the PESE. The four-cell-wide pair-rule stripes of Eve shrink during the cellular blastoderm stage as expression is progressively lost from the more posterior cells in these stripes (Clark and Akam, 2016). We propose that the repression of PESE in cell type II is relaxed as Eve expression levels drop, thus allowing the DESE enhancer to access the slp1 promoter. The differences in expression of the wild-type and Runt-binding site mutant DESE enhancer in cell type II when these enhancers are confronted with competition from PESE indicate a role for DNA binding by Runt in promoting DESE activity during the dynamic regulatory interplay between these two enhancers.

Other examples of nonadditive interactions between distinct enhancers, in both Drosophila and mouse and human cells, have been interpreted to be due to competitive interactions between these enhancers and the promoter (Lin et al., 2007; Perry et al., 2010; Bhattacharyya et al., 2011; Dib et al., 2011; Dunipace et al., 2011; Bothma et al., 2015; El-Sherif and Levine, 2016; Waymack et al., 2020). Much of this work has focused on so-called shadow enhancers with largely overlapping expression patterns that are interpreted to buffer expression levels against changes in temperature or to suppress noise. The competitive repressive effect of PESE on DESE-dependent transcription that we describe is most similar to the dominant repressive effects that have been characterized for enhancer pairs from the Drosophila gap genes Kr and kni that contribute to dynamic shifts in the borders of expression (El-Sherif and Levine, 2016).

An important implication of these findings is that cis-regulatory elements that normally contribute to specific aspects of an expression pattern may have broader activities as autonomous elements, even when tested in a physiologically relevant cellular context. A survey of prospective enhancers from genes expressed in the Drosophila brain reveals that these elements frequently drive expression in cells that normally do not express the endogenous gene (Pfeiffer et al., 2008). This observation parallels our observations on the slp1 DESE enhancer and strongly suggests that nonautonomous regulation of enhancer activity is a phenomenon of widespread importance. Our knowledge on the set of transcription factors that are responsible for the nonadditive interactions between the two slp1 enhancers, coupled with the tools available for studies in the Drosophila embryo, provides an exceptional platform for further investigating the molecular basis of these regulatory phenomena.

**MATERIALS AND METHODS**

**Request a protocol** through Bio-protocol.

**Construction of slp1-lacZ reporters**

The composite P-element reporter gene construct slp1[8765:3918]lacZ obtained by standard P-element germline transformation and the slp1[3918]lacZ and slp1[8771]lacZ transgenes integrated into the same chromosomal using site-specific integration are as described (Prazak et al., 2010). The original slp1[3918]lacZ and slp1[8771]lacZ transgenes and the Runx-binding site mutant derivatives of this reporter gene contain slp1 basal promoter sequences from base pairs −72 to +57. The Runx binding site mutants were generated by first cloning a slp1 −8.7 kb to −6.5 kb XbaI/NotI fragment from pC:slp1[8765]lacZ (Prazak et al., 2010) into the corresponding sites of pBluescript to generate pB:slp1[8765]. This construct was then used for PCR using complementary primers (sequence available upon request) that change the two critical cytosines in the consensus sequence to adenine resulting in two fragments that extend to the flanking M13 forward and reverse primers. These fragments were mixed and amplified with M13 forward and reverse primers and cloned into the Xbal and NotI sites of pBluescript creating pB:slp1[8765m2], pB:slp1[8765m4], pB:slp1[8765m5], pB:slp1[8765m3,4], and pB:slp1[8765m1,2,3,4,5]. After confirming the sequence, primers that amplify from 8710 to 7136 base pairs upstream of slp1 were used to amplify DNA segments containing the binding site mutations such that these DNA segments could be cloned into the XhoI site of pC:slp1[8765m1-5]lacZ using the In-Fusion Dry-Down PCR Cloning Kit (Clontech) to generate pC:slp1[8771m2]lacZ, pC:slp1[8771m4]lacZ, pC:slp1[8771m5]lacZ, pC:slp1[8771m3,4]lacZ, and pC:slp1[8771-5]lacZ.

The original slp1[8765:3918]lacZ composite reporter construct contains slp1 basal promoter sequences spanning from −260 to +121. To generate similar composite reporter gene constructs for integration using ΦC31 transgenesis, this extended basal promoter was obtained by PCR amplification from genomic subclones with the addition of upstream XhoI and downstream KpnI sites and cloned into pBluescript to create pB:slp1[LVp]. This basal promoter fragment was moved into pC:slp1-link-lacZ+ w, where a linker was introduced to replace Bluescript polylinker between the EcoRI and XhoI sites upstream of the basal promoter with unique NotI, MfeI, Stul, and Spel sites as an EcoRI + KpnI fragment removing the smaller basal promoter and creating pC:slp1[8765m1-5]lacZ+. This vector was digested with EcoRI treated with Klenow, then digested with NotI, and then ligated to a gel-purified DESE-containing segment of either pB:slp1[8765] or pB:slp1[8765m1,2,3,4,5] that had been digested with Xbal treated with Klenow and digested with Notl to generate pC:slp1[8765lacZ]+ and pC:slp1[8765lacZ]+, respectively. The composite slp1[8765:3918]lacZ and slp1[8765m1-5:3918]lacZ constructs were obtained by inserting the PESE-containing NotI fragment from pB:slp1[1839] into the Notl site of the corresponding parental pC:slp1[8765lacZ]+ plasmid.

ΦC31-mediated transgenic lines were obtained using the attP integration site on the third chromosome (Groth et al., 2004). Constructs containing the attB sequence were coinfected with ΦC31 mRNA into y w; P[CaryllPlattP2 embryos, the surviving adult progeny backcrossed to the parental line, and the F1 generation screened for white transformants. ΦC31 mRNA was generated from BamHI linearized pET-phiC31-polA template with the mMessage mMACHINE high yield Capped RNA Transcription Kit (Ambion), and mRNA was recovered via LiCl precipitation without DNase treatment.

**Drosophila mutants and genetics**

Ectopic expression of pair-rule transcription factors was achieved using the NGT maternal expression system. The second chromosome linked P[GAL4-nos.NGT40 (NGT40) driver and the P[UAS-runt-T715, P[UAS-runt-T232], P[UAS-runt[CK]K43], P[UAS-runt[CK]777, P[UAS-opa.V2]D10, and P[UAS-opa.V2]14 transgenes have been described previously (Tracey et al., 2000; Vander Zwan et al., 2003; Swantek and Gegen, 2004). The P[UAS-fz]263 and P[UAS-eve]12 transgenes were provided to us by Leslie Pick (Lohr and Pick, 2005) and John Reinitz (Zallen and Wieschaus, 2004), respectively. Embryos were collected from crosses between females homozygous for NGT40 and for the different third chromosome–linked reporter genes and males carrying these different UAS transgenes.

**Whole-mount in situ hybridization**

Embryos were collected as described (Tsai and Gegen, 1994). Fluorescence in situ hybridization was carried out as described (Janssens et al., 2005) with the following modification: After fixation embryos
were cleared in xylenes:ethanol (9:1) and then postfixed in Phosphate buffered saline with 0.1% (v/v) Tween 20 (PBT) + 5% formaldehyde. Embryos were permeabilized for 10 min in 80% aceton in H2O at ~20°C. The fluorescein-labeled lacZ riboprobe was synthesized with fluorescein-12-UTP (Roche) in place of digoxigenin-conjugated UTP (Tsai and Gegen, 1994). After hybridization, lacZ mRNA was visualized by sequential incubation with rabbit anti-fluorescein (1 μg/ml final) and Alexa Fluor 647 donkey anti-rabbit (1 μg/ml) antibodies (Molecular Probes). The digoxigenin-labeled riboprobe for slp1 is as described in Wheeler et al. (2002). Digoxigenin-labeled probes were detected using mouse anti-digoxigenin antibody (Roche; 1.25 μg/ml final) followed by Alexa Fluor 555 goat anti-mouse (1 μg/ml) and Alexa Fluor 555 donkey anti-goat (1 μg/ml) antibodies (Molecular Probes). Blocking was done in 2× Western Blocking Reagent (Roche) diluted in PBT. All antibodies were preabsorbed at a 10× concentration in PBT with 1/10 volume of 0–12 h embryos and then diluted to 1×. PicoGreen (Molecular Probes) was used to stain nuclei at a 1:30,000 dilution. Before mounting, embryos were washed in phosphate-buffered saline:glycerol (1:1) for 20 min and then mounted in 45 μl mounting medium (2.5% Dabco [Sigma], 50 mM Tris [pH 8.0], and 90% glycerol) and covered with a Corning 22 × 40-mm cover glass (No. 1 ½). Images were obtained on a Leica TCS SP2 Spectral Confocal Microscope system as described (Janssens et al., 2005).

**Electrophoretic mobility shift assay (EMSA)**

EMSA experiments to detect DNA binding of Runt:Bro protein complexes were done as described by Kramer et al. (1999) with a 37 base pair radiolabeled DNA probe containing the Runx site in the A-element of the polycloma virus enhancer. Pure competitor oligonucleotides containing Runx sites of different affinities (Lewis et al., 1999) were obtained from Nancy Speck (University of Pennsylvania).

**ChiP analysis**

The ChiP assays were performed as described previously (Wang et al., 2007) using chromatin prepared from 3- to 4-h embryo collections with either a rabbit anti-Runt antibody or normal rabbit serum from Sigma. The primer sequences used in quantitative PCR (qPCR) are available upon request. (Error bars represent the mean SE from three independent immunoprecipitation experiments.)

**Sequencing results**

Input as well as Runt immunoprecipitated DNA were sequenced by the Stony Brook Sequencing Facility using primers specific to a region of the Stony Brook ChiP-seq data that average the patterns of individual embryos were generated to eliminate variability from individual embryos. To do this, the slp1 expression pattern was used to register the different embryos. Registration was performed by affine transformation of the nuclear coordinates along the A–P axis so that the ground control points (GCPs) in all patterns coincide as closely as possible (Myasnikova et al., 2001). The extrema of the 1D slp1 expression pattern were used as GCPs for registration. This set consisted of 23 points and did not include stripes 0 and 1. The GCPs were extracted using fast dyadic wavelet transform and the integrated data produced by averaging the registered individual patterns (Kozlov et al., 2009).

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