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Drosophila poised enhancers are generated during tissue patterning with the help of repression

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Abstract

Histone modifications are frequently used as markers for enhancer states, but how to interpret enhancer states in the context of embryonic development is not clear. The poised enhancer signature, involving H3K4me1 and low levels of H3K27ac, has been reported to mark inactive enhancers that are poised for future activation. However, future activation is not always observed and alternative reasons for the widespread occurrence of this enhancer signature have not been investigated. By analyzing enhancers during dorsal-ventral (DV) axis formation in the *Drosophila* embryo, we find that the poised enhancer signature is specifically generated during patterning in the tissue where the enhancers are not induced, including at enhancers that are known to be repressed by a transcriptional repressor. These results suggest that, rather than serving exclusively as an intermediate step before future activation, the poised enhancer state may be a mark for spatial regulation during tissue patterning. We discuss the possibility that the poised enhancer state is more generally the result of repression by transcriptional repressors.

Introduction

Understanding the mechanisms by which cis-regulatory elements, or enhancers, activate transcription has been intensively studied for the last three decades, yet our knowledge remains incomplete (Shlyueva et al. 2014). As shown by ChIP-seq experiments, transcription factors may bind to thousands of putative enhancer regions in the genome (Moorman et al. 2006; Li et al. 2008), yet a large fraction of these regions are likely inactive for a variety of reasons. For example, transcription factors may bind to so-called primed enhancers that have been made accessible by pioneer transcription factors but are not yet active (Zaret and Carroll 2011; Spitz and Furlong 2012) or they may bind to repressed enhancers, which are bound and actively prevented from activation by sequence-specific repressors (Sandmann et al. 2007; Zeitlinger et al. 2007). This raises the question of what types of enhancer states exist, how they relate to each other and how they help regulate the complex spatial and temporal expression patterns of genes during the development of multicellular organisms.

Good markers for enhancer states are the histone modifications found at the nucleosomes flanking enhancer regions. Most open enhancer regions are marked by histone mono-methylation on lysine 4 of histone H3 (H3K4me1), but only active enhancers carry lysine 27 acetylation on histone H3 (H3K27ac) (Creyghton et al. 2010; Ernst et al. 2011; Rada-Iglesias et al. 2011; Zentner et al. 2011; Bonn et al. 2012). Since some inactive enhancers show activation during later development, the combination of H3K4me1 along with low H3K27ac at inactive enhancers was termed the poised enhancer signature (Creyghton et al. 2010; Rada-Iglesias et al. 2011).

The mechanisms by which poised enhancers remain inactive and by which they become active under some conditions are poorly understood. For example, some studies have implicated the Polycomb-repressive mark H3K27me3 as a marker for poised enhancers (Rada-Iglesias et al. 2011), while others have not (Creyghton et al. 2010; Bonn et al. 2012). It is also possible that other mechanisms of repression might make enhancers susceptible to de-repression, thereby poising them for activation, but the relationship between poised enhancers and repressed enhancers has not yet been examined.

Poised enhancers are very common during the development of *Drosophila* and mammalian lineages, but their role in tissue patterning and lineage specification remains unclear. While originally described as being poised for future activation, this model is likely an oversimplification. The majority of enhancers become active without going through a poised state during prior developmental stages (Bonn et al. 2012; Choukrallah et al. 2015). Only a small fraction of poised enhancers are usually activated during lineage development (Rada-Iglesias et al. 2011; Bonn et al. 2012; Wamstad et al. 2012). Instead, many enhancers that are poised in a cell type are active in related cell types (Bonn et al. 2012; Junion et al. 2012; Wang et al. 2015). This not only questions the strict temporal model in which the poised enhancer state precedes enhancer activation, but also suggests a role for poised enhancers in tissue patterning.

A widespread role for poised enhancers in tissue patterning is consistent with large-scale DNase hypersensitivity (DHS) assays across a variety of cell types representing stages of human development (Stergachis et al. 2013). These data also show that enhancers are frequently accessible across broadly related cell types and only become active in specific lineages, raising the possibility that poised enhancers

in embryonic tissues are predisposed for activation spatially, and that enhancer activation is regulated by signals that control pattern formation.

During tissue patterning, developmental signals (or morphogens) are often generated at and propagated from precise locations within the embryo, typically leading to the graded activation of signal transduction pathways and transcription factors across fields of cells (Briscoe and Small 2015). Depending on the strength of signaling, different target genes are activated, giving rise to distinct cell fates across the gradient. Activation of already accessible enhancers is a logical mechanism by which signal transduction pathways could mediate precise cellular responses to morphogens. The broad distribution of poised enhancers may ensure that a sufficient number of cells can respond to specific developmental signals in the appropriate manner, thus facilitating pattern formation.

While a function of poised enhancers in pattern formation is plausible, in many systems the hypothesis is difficult to test due to the scarcity and heterogeneity of embryonic tissues. To analyze a possible role for poised enhancers during pattern formation in the embryo, we used the tractable *Drosophila* dorso-ventral (DV) patterning as model system. In the *Drosophila* embryo, DV patterning begins with localized activation of the Toll (Tl) receptor by maternal components, which leads to the formation of morphogen gradient of the transcription factor Dorsal (Dl) and gives rise to at least three cell fates with distinct gene expression programs along the DV axis: mesoderm on the ventral side, neurectoderm in the lateral regions and dorsal ectoderm on the dorsal side (Hong et al. 2008) (Fig. 1A). For simplicity, we focused on the cell fates at the ends of the gradient, mesoderm and dorsal ectoderm, which arise during cell cycle 14, around 2-4 h after egg deposition (AED).

The advantage of the *Drosophila* DV system is that large amounts of cells can be obtained from these two tissues without the need for cell sorting or tissue dissection. This is made possible by the availability of maternal mutants where all embryos in the progeny consist entirely of either mesodermal or dorsal ectodermal precursor cells. In *Tl* mutant embryos Tl^{10b}, Dl activity is uniformly high (but not above wild-type levels) leading to mesodermal precursor fate (Schneider et al. 1991). In *gastrulation defective (gd)* mutant embryos gd^{7}, Dl is not activated, resulting in uniformly high signaling activity of the fly BMP2/4

ortholog Decapentaplegic (Dpp) (but below wild-type maximum levels, see Ashe and Levine 1999) and the specification of dorsal ectodermal fate in the entire embryo. These mutants have frequently been used in the past because they allow the analysis of patterning across the Dl activity gradient (e.g. Stathopoulos et al. 2002; Zeitlinger et al. 2007; Holmqvist et al. 2012), and have helped DV patterning become one of the best-studied gene regulatory networks in development.

The DV patterning system also illustrates another important principle of pattern formation, the widespread use and requirement of sequence-specific transcriptional repressors. The extensive genetic screens in *Drosophila* have shown that transcriptional repressors are crucial for the correct interpretation of morphogen gradients, including DV patterning (Ip and Hemavathy 1997; Bier and De Robertis 2015; Briscoe and Small 2015). During DV patterning, Dl is able to specify three distinct cell fates because, in addition to its role as a transcriptional activator, it can also act as a repressor when certain additional repressive sequences in an enhancer are present next to a Dl motif (Pan and Courey 1992; Jiang et al. 1993). When Dl is converted into a repressor, it recruits co-repressors and histone deacetylases (Dubnicoff et al. 1997; Valentine et al. 1998; Chen et al. 1999; Flores-Saaib et al. 2001; Ratnaparkhi et al. 2006) and dominantly suppresses enhancer activation (Gray and Levine 1996; Dubnicoff et al. 1997). Three cis-regulatory sequences, those regulating *dpp*, *zerknüllt* (*zen*), and *tolloid* (*tld*), have been shown to be ventrally repressed by Dl, allowing spatially-restricted activation of these genes on the dorsal side of the embryo (Irish and Gelbart 1987; Rushlow et al. 1987; Ip et al. 1991; Huang et al. 1993; Kirov et al. 1994; Ratnaparkhi et al. 2006).

Using the DV system, we analyzed the state of uninduced enhancers during patterning, including those known to be repressed, and asked whether they correspond to a primed or poised enhancer state. We show that uninduced enhancers are primed and accessible to transcription factors but specifically acquire a poised enhancer signature during DV patterning, when enhancers are repressed. This supports a role for poised enhancers in tissue patterning and suggests that this enhancer signature can arise as a result of repression, a model with important mechanistic implications.

Results

Uninduced DV enhancers are accessible to transcription factors albeit at lower levels

To characterize the enhancer states during DV patterning, we first assembled a list of known DV enhancers that have been verified by transgenic *lacZ* reporter assays (Supplemental Table S1). We identified 37 mesoderm enhancers (MEs) that remain uninduced in the dorsal ectoderm, and 22 dorsal ectoderm enhancers (DEEs) that remain uninduced in the mesoderm (Fig. 1A, see Supplemental Material for a complete list and references). To validate our experimental system, we performed mRNA-seq experiments on Tl^{10b} and gd^{7} embryos at 2-4 h AED. As expected, most genes that were assigned to a known DV enhancer were more highly expressed in the tissue in which the enhancer is active (Supplemental Fig. S1).

We then asked whether uninduced enhancers are accessible to transcription factors and performed ChIP-seq experiments in Tl^{10b} and gd^{7} embryos at 2-4 h AED. Replicate experiments were highly correlated (Supplemental Material). We specifically analyzed DV transcription factors that are required for the cell fate specification of mesoderm and dorsal ectoderm (Fig. 1A). High Dl activity on the ventral side of the embryo induces expression of Twist (Twi), which together with Dl activates mesodermal target genes (Jiang et al. 1991; Ip et al. 1992). We therefore analyzed Dl and Twi occupancy in Tl^{10b} embryos and calculated their enrichments at active MEs and DEEs, which are actively repressed or remain uninduced (Fig. 1B). As a control, we used a set of 100 presumptive late enhancers that are inaccessible (“closed”) at 2-4 h AED but are accessible and marked by H3K27ac in the late embryo (see Methods). Active enhancers had the highest levels of Dl and Twi, the closed control regions had the lowest levels, and uninduced enhancers had statistically significant intermediate levels of occupancy (Fig. 1B).

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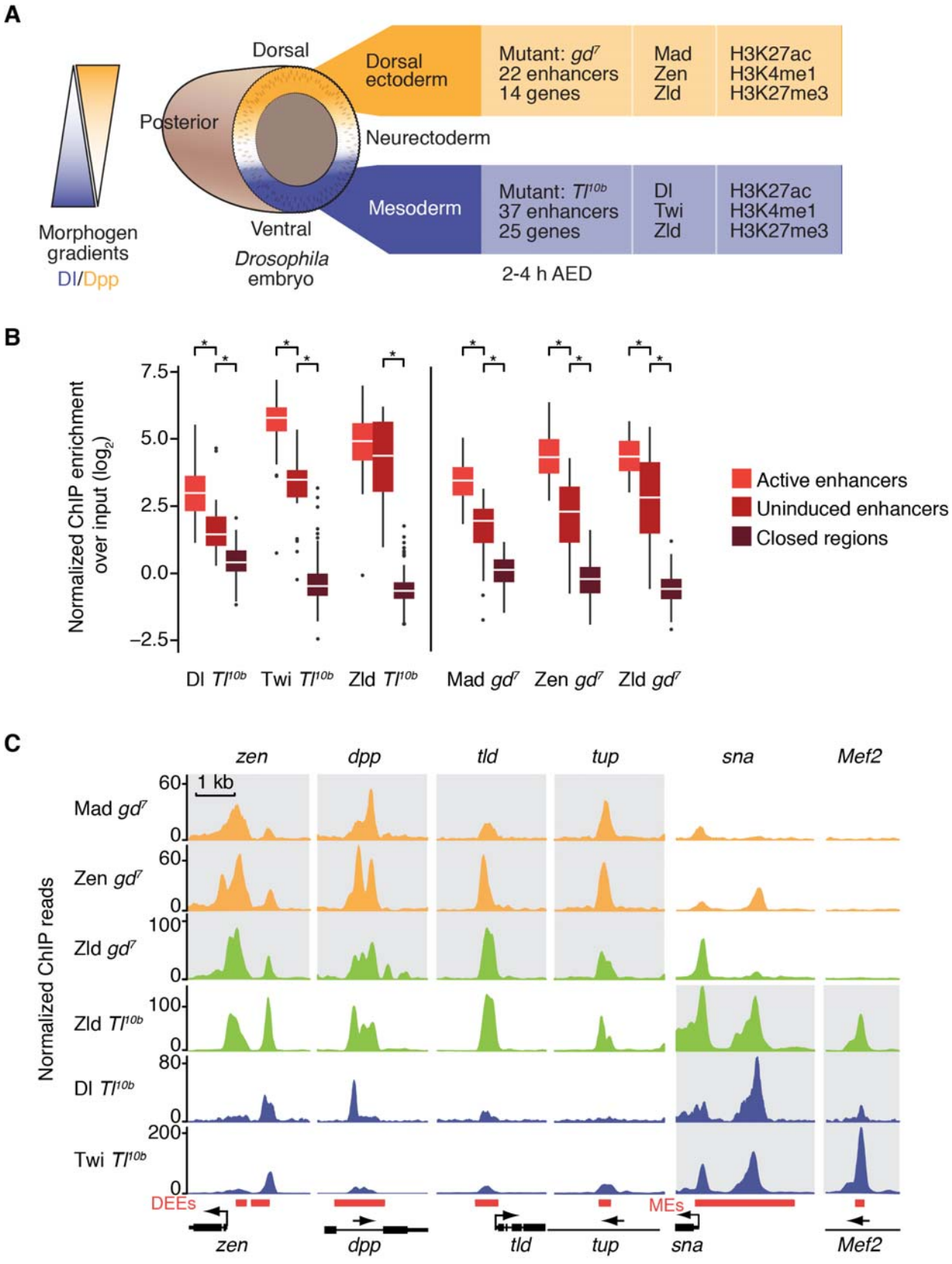

Figure 1. DV transcription factors occupy uninduced enhancers at intermediate levels

(A) Overview of the model system of dorsal-ventral (DV) patterning in the *Drosophila* embryo, in which homogeneous cell fates can be obtained through mutants such as Tl^{10b} and gd^{7} for which a large number of tissue-specific enhancers and their target genes are known. A summary of the analyzed ChIP-seq experiments of transcription factors and histone modifications is shown on the right. AED = after egg deposition (B) Boxplots of ChIP-seq enrichment over input for the DV transcription factors Dl, Twi, Mad, Zen and Zld at known DV enhancers. The ChIP-seq experiments were performed in either Tl^{10b} or gd^{7} or both, dependent on which tissue the transcription factor is expressed in. Note that DV transcription factors occupy uninduced enhancers less than active enhancers but significantly more than closed regions, indicating that uninduced enhancers are accessible. Closed regions are 100 presumptive late enhancers that are inaccessible by DHS (Thomas et al. 2011) at early stages and are enriched for H3K27ac at later stages (see Methods). Active enhancers are mesoderm enhancers (MEs) in Tl^{10b} embryos or dorsal ectoderm enhancers (DEEs) in gd^{7} embryos. Uninduced enhancers are MEs in gd^{7} embryos or DEEs in Tl^{10b} embryos. Whiskers show 1.5 times the interquartile range and outliers are shown as dots. Star indicates $p < 10^{-3}$ using the Wilcoxon rank sum test. (C) ChIP-seq binding profiles of the transcription factors at four DEEs and two MEs (red boxes with target genes shown in black) illustrate higher binding at active enhancers (grey shading) but some degree of binding at uninduced enhancers (white background). The DEEs of *zen, dpp* and *tld* are known be repressed by Dl, while the *snail (sna)* ME is activated by Dl (Ip et al. 1992). ChIP-seq reads are normalized to reads per million.

Dpp signaling, which activates the transcription factors Mothers against dpp (Mad), and Zen induce dorsal ectodermal fate (Raftery et al. 1995; Rusch and Levine 1997; Lin et al. 2006). We therefore analyzed the occupancy of Mad and Zen in gd^{7} embryos and found that their occupancy at both active DEEs and uninduced MEs was also significantly higher than at the closed control regions (Fig. 1B). Again, their occupancy at uninduced enhancers was significantly lower than at active enhancers (Fig. 1B),

further supporting the hypothesis that uninduced enhancers are bound by transcription factors, but to a lesser extent than active enhancers.

The observation that uninduced DV enhancers are bound by transcription factors suggests that these enhancers are accessible because they were primed by a pioneer transcription factor. A potential pioneer transcription factor is Zelda (Zld), which is present ubiquitously in the *Drosophila* early embryo and primes enhancers even before DV patterning begins (Liang et al. 2008; Harrison et al. 2011; Nien et al. 2011). While Zld is required to make some DV enhancers accessible (Yanez-Cuna et al. 2012; Foo et al. 2014; Schulz et al. 2015; Sun et al. 2015), it is not known whether Zld remains bound to uninduced enhancers at the same level as at active enhancers.

We therefore analyzed the occupancy of Zld at active enhancers, uninduced enhancers and closed regions. The closed regions that we used as controls were not bound by Zld or bound at very low levels. In contrast, uninduced enhancers remained highly bound by Zld in both tissues, albeit at slightly lower levels than at active enhancers (Fig. 1B). This suggests that Zld primes early enhancers in the entire embryo, whether or not the enhancers are induced. The sometimes higher levels of Zld at active enhancers compared to uninduced enhancers is consistent with our observation that active enhancers are more accessible to transcription factors than uninduced enhancers.

We next analyzed the three DEEs that are known to be repressed by Dl in Tl^{10b} mutants (*zen*, *dpp* and *tld* in Fig. 1C). We found that these enhancers follow similar trends as other DV enhancers. When these enhancers are active in gd^{7} embryos, they show high occupancy of Zld, Mad and Zen, as expected. When they are repressed in Tl^{10b} mutants, they are occupied by Zld, Dl and Twi. Since Twi is an activator and has no known role in repressing these enhancers, this result suggests that repressed enhancers are to some degree accessible to transcription factors, presumably due to the pioneering activity of Zld. Consistent with Zld being critical for enhancer access, in the rare case where Zld does not occupy an uninduced enhancer, other transcription factors are also not bound (e.g. *Mef2* in Fig. 1C).

Taken together, these results suggest that uninduced enhancers are frequently primed and bound by transcription factors, albeit to a lower degree than in the active state. This level of accessibility might allow these enhancers to be inactive but responsive to changes in signaling and transcription factor activity.

Uninduced enhancers are marked by H3K4me1 and low H3K27ac and thus carry a poised enhancer signature

Having identified three distinct enhancer states, we next investigated their histone modification status. We performed ChIP-seq experiments with antibodies against H3K27ac and H3K4me1 in both mutant embryos and calculated the enrichment (± 500 bp from enhancer center) at all active and uninduced enhancers from both mutants, as well as closed regions as a control.

Uninduced enhancers had overall significantly higher levels of H3K27ac as compared to closed control regions (Fig. 2A, $p < 10^{-6}$, Wilcoxon rank sum test) but their levels were significantly lower than at active enhancers (Fig. 2A, $p < 10^{-6}$, Wilcoxon rank sum test). Indeed, when we plotted the relative difference for each enhancer between the two tissues, the difference in H3K27ac levels between active and uninduced enhancers became more significant ($p < 10^{-9}$, Wilcoxon rank sum test) (Fig. 2B). This suggests that uninduced enhancers have low levels of H3K27ac, and that the levels significantly increase when the enhancers are active.

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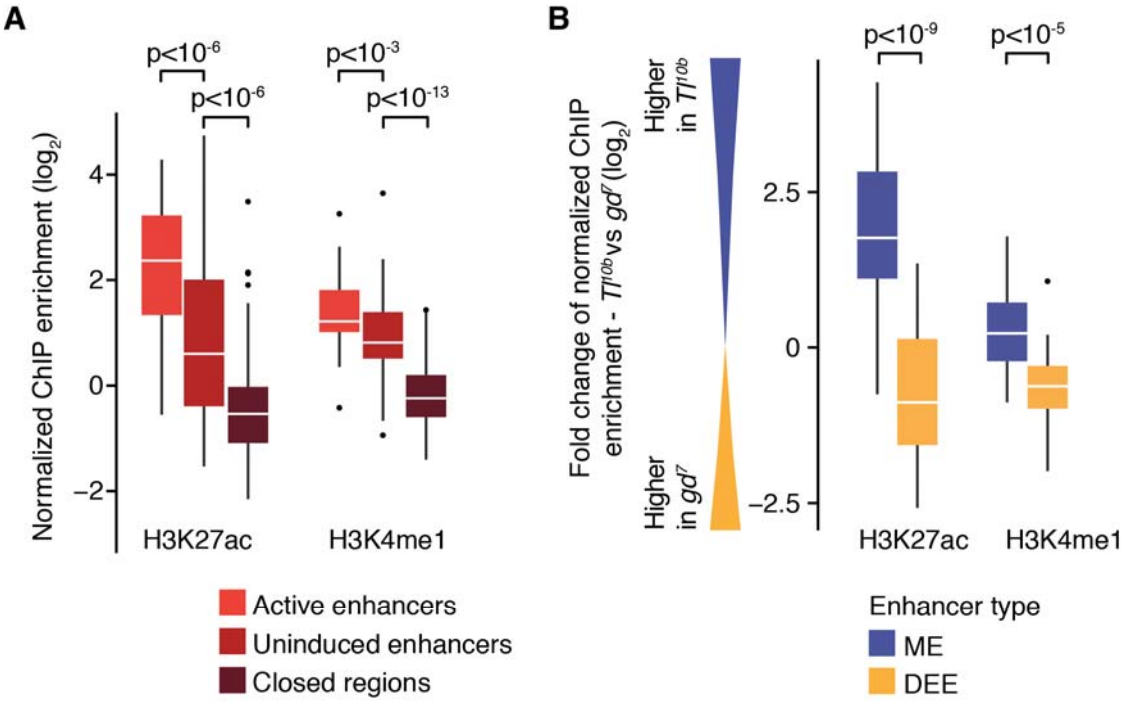

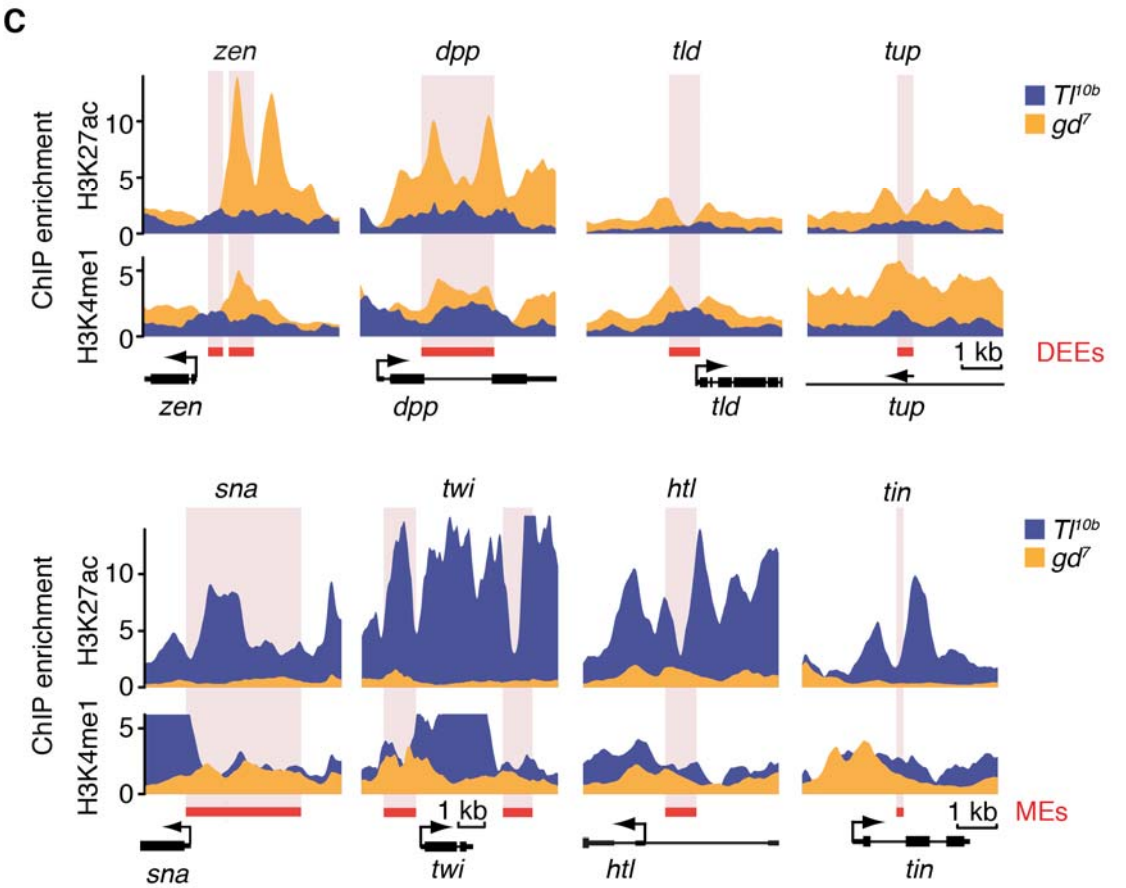

Figure 2. The histone modifications at uninduced enhancers resemble the poised enhancer signature

(A) Boxplots of normalized H3K27ac and H3K4me1 ChIP-seq enrichments show that all uninduced DV enhancers (n=59, from both mutants) have lower H3K27ac enrichment levels than the same enhancers in the active state, yet the levels of H3K4me1 are significantly above closed regions (n=100, same as in Fig. 1B), consistent with a poised enhancer signature. Whiskers show 1.5 times the interquartile range and outliers are shown as dots. Significance between enhancer groups was determined using the Wilcoxon rank sum test. (B) Boxplots of the fold-change of normalized histone modification ChIP-seq enrichments between mutant embryos show that H3K27ac and H3K4me1 levels are higher at active enhancers versus uninduced enhancers: the majority of mesoderm enhancers (MEs, blue) have higher H3K27ac enrichment in the Tl^{10b} mutant than in the gd^{7} mutant (thus $\log_2 Tl^{10b}$ - $\log_2 gd^{7}$ above 0), while the inverse is true for dorsal ectodermal enhancers (DEEs, yellow). Significance between MEs and DEEs was determined using the Wilcoxon rank sum test. (C) Binding profiles of histone modification ChIP-seq enrichments show higher enrichment of H3K27ac and H3K4me1 when the enhancer is active. At the four DEEs, the levels are higher in gd^{7} (yellow), while at four MEs, the levels are higher in Tl^{10b} (blue). The red box and the pink stripe show the position of the enhancers and the black arrow indicates the position and orientation of transcription start sites.

When we analyzed H3K4me1 levels, we found that uninduced enhancers are also enriched for H3K4me1 significantly above the levels of the control (Fig. 2A, $p < 10^{-13}$, Wilcoxon rank sum test), consistent with a poised enhancer signature. However, H3K4me1 enrichments were slightly lower in the uninduced state than in the active state (Fig. 2A, $p < 10^{-3}$, Wilcoxon rank sum test). This small but consistent difference became more significant when analyzing the relative difference in H3K4me1 at enhancers ($p < 10^{-5}$, Wilcoxon rank sum test) (Fig. 2B). The profiles of H3K4me1 at individual enhancers also confirm that H3K4me1 is more highly enriched at active enhancers (Fig. 2C). This finding is unexpected since H3K4me1 is used as a marker for both poised and active enhancers but closer examination reveals that it

is consistent with previous data (Rada-Iglesias et al. 2011; Bonn et al. 2012). The higher levels of H3K4me1 could potentially also be connected to the increased accessibility of active enhancers.

Finally, we specifically examined the three enhancers known to be repressed by Dl (*zen*, *dpp* and *tld* in Fig. 2C) but found their histone signature of H3K4me1 and low H3K27ac to be indistinguishable from other uninduced enhancers. Thus, the poised enhancer signature is also characteristic for enhancers regulated by transcriptional repressors. Whether there is a histone modification that is specifically associated with transcriptional repressors is not known. H3K27me3 is a well-studied repressive mark but it is deposited by Polycomb group proteins, which are not known to associate with sequence-specific transcriptional repressors (Simon and Kingston 2013).

H3K27me3 is not a good marker for uninduced enhancers or sequence-specific repressors

The Polycomb-repressive mark H3K27me3 has been observed at poised enhancers (Rada-Iglesias et al. 2011) or repressed enhancers (Schwartz et al. 2006; Tolhuis et al. 2006; Oktaba et al. 2008; Bonn et al. 2012) but has not specifically been implicated in embryonic DV patterning because Polycomb group mutants are difficult to analyze in the early *Drosophila* embryo (Pelegri and Lehmann 1994).

When we analyzed H3K27me3 ChIP-seq data in Tl^{10b} and gd^{7} embryos, we found remarkably variable levels of H3K27me3 at DV enhancers. Some enhancers had very high levels of H3K27me3, while more than half of them had no enrichment above background (Fig. 3A). Despite the variance, however, there was a significant trend for enhancers to have higher H3K27me3 levels in the uninduced versus active state (Fig. 3B, $p < 10^{-2}$, Wilcoxon rank sum test), consistent with previous findings (Bonn et al. 2012).

Examination of individual DV enhancers confirms clear differences in H3K27me3 levels between the uninduced and active state in regions where the levels of H3K27me3 are high (Fig. 3C). However, H3K27me3 marks are distributed over broad regions, as expected (Schwartz et al. 2006; Tolhuis et al. 2006); the differences in H3K27me3 include the transcribed regions and thus are not specific to DV enhancers (Fig. 3C). This questions whether an enhancer's state directly regulates the surrounding levels

of H3K27me3 or may instead affect H3K27me3 levels more indirectly through its effect on gene activation. Indeed, an anti-correlation between H3K27me3 and transcriptional status has been observed previously (Klymenko and Muller 2004; Papp and Muller 2006; Tolhuis et al. 2006; Gaertner et al. 2012) and recent mammalian studies provide direct evidence that transcription status affects the levels of H3K27me3 (Riising et al. 2014; Beltran et al. 2016; Hosogane et al. 2016).

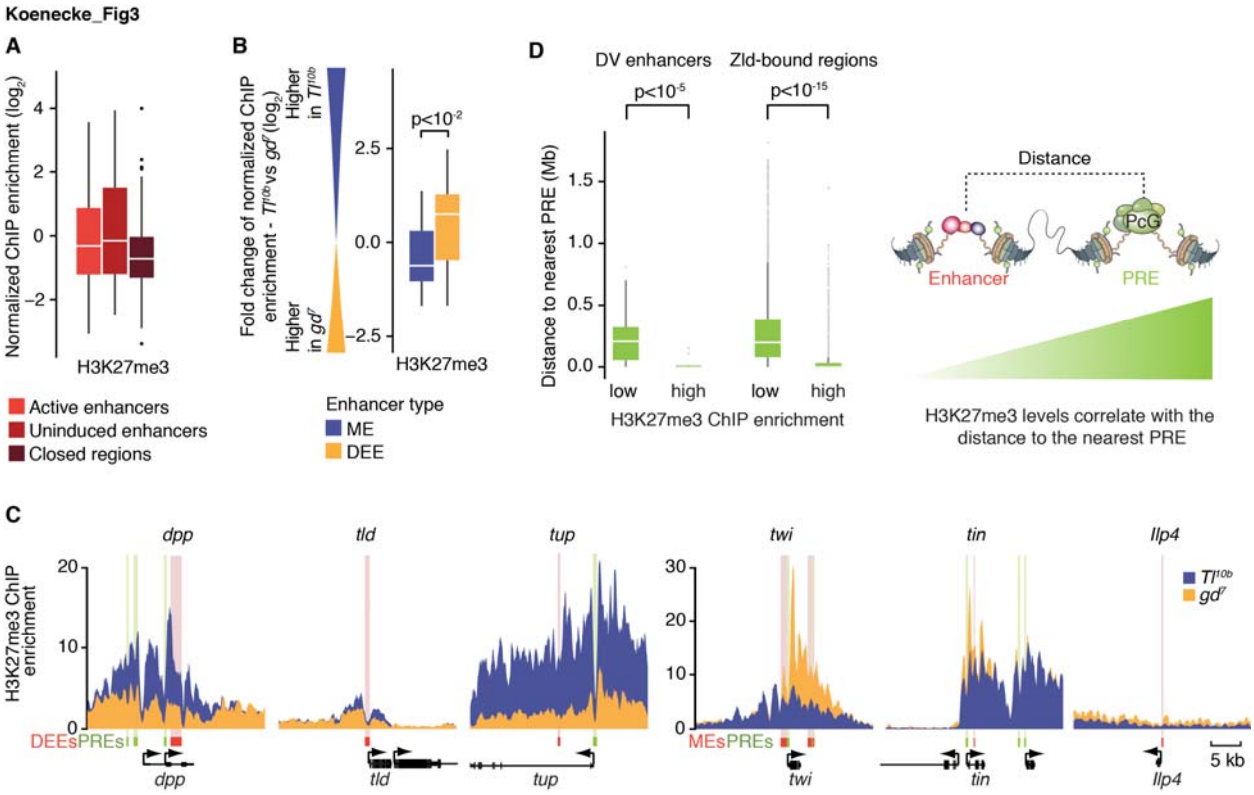

Figure 3. H3K27me3 levels are higher at uninduced enhancers but correlate more strongly with distance to the nearest PRE

(A) Boxplots show a wide range of different H3K27me3 levels at the different enhancer states, with no significant differences between them as determined by the Wilcoxon rank sum test. (B) The fold change difference in H3K27me3 enrichment between DV mutants shows that H3K27me3 at individual enhancers tends to be higher in the uninduced state versus active state. Significance was determined using the Wilcoxon rank sum test. (C) H3K27me3 ChIP-seq enrichment profiles for three dorsal ectoderm enhancers (DEEs) and three mesoderm enhancers (MEs) illustrate clear differences between mutants (yellow versus blue). H3K27me3 enrichment levels are highest near putative Polycomb response

elements (PREs, green). Enhancers are shown as red boxes with pink shading. (D) Boxplots showing the distance of enhancers to the nearest PRE, dependent on whether they have low or high H3K27me3 enrichment levels. For DV enhancers with low H3K27me3 enrichment (below two-fold enrichment, n=39), the distances between enhancers and putative PREs are much larger than for those with high H3K27me3 levels (above two-fold enrichment, n=20). This is also true for Zld-bound regions, which include a large number of putative early *Drosophila* enhancers (low H3K27me3 n=14,4425, high H3K27me3 n=2,720). Zld bound regions are the union of Zld ChIP-seq peaks in Tl^{10b} and gd^{7} with at least two-fold enrichment in either tissue. Putative PREs are defined as the overlap between Pc and GAF regions (n=602). Whiskers show 1.5 times the interquartile range and outliers are shown as dots.

If transcription reduces H3K27me3, what determines whether H3K27me3 is present in that region in the first place? Broad regions of H3K27me3 are catalyzed from specific nucleation sites in the DNA called Polycomb Responsive Elements (PREs) (Simon et al. 1993; Muller and Kassis 2006). In *Drosophila*, Polycomb group proteins are recruited to PREs by a combination of DNA-binding factors, including GAGA factor (GAF, also known as *Trithorax-like* or *Trl*) (Strutt et al. 1997). We therefore identified high-confidence PREs through the co-occupancy of GAF, which is not specific for PREs but gives high signal in ChIP experiments, and Polycomb (Pc) itself, which is indirectly bound to DNA but which is highly specific for PREs (Schuettengruber et al. 2009; Schuettengruber et al. 2014).

If the levels of H3K27me3 at enhancers depend on nearby PREs, we expect that DV enhancers with high H3K27me3 enrichment will be located closer to PREs than those without. Indeed, the median distance between DV enhancers with high H3K27me3 and the closest PRE is less than 10 kb, while for DV enhancers without H3K27me3 enrichment, the median distance to a PRE is ~200 kb (Fig. 3D, $p < 10^{-5}$, Wilcoxon rank sum test). The correlation between PREs and H3K27me3 can also be observed at individual DV enhancer regions, where the levels of H3K27me3 often peak close to PREs (Fig. 3C). Finally, the correlation between PREs and H3K27me3 is not specific for DV enhancers since the same

trend was observed for all Zld-bound regions (Fig. 3D). These results strongly support the traditional model that high levels of H3K27me3 depend on nearby PREs.

The anti-correlation between gene activation and H3K27me3 suggests that active enhancers can reduce the H3K27me3 mark deposited by nearby PREs. To consider alternative models, we also probed the possibility that repressors at enhancers might directly promote H3K27me3 deposition. However, the known Dl-repressed enhancers did not stand out in their H3K27me3 profile as compared to other uninduced enhancers (Fig. 3C). For example, the Dl-repressed *dpp* enhancer has very high levels of H3K27me3 in the repressed state, while another Dl-repressed enhancer, that of *tld*, has much lower levels. Furthermore, high levels are also observed at enhancers that are not repressed by Dl, including *tup*. Thus, while the levels of H3K27me3 correlate with the presence of PREs, they do not correlate with Dl-dependent repression. While we cannot rule out a subtle role for repressors in modulating H3K27me3 levels, our data suggest that the strongest determinants of H3K27me3 levels are nearby PREs and lack of gene activation. Therefore, H3K27me3 cannot be considered a specific marker for uninduced or repressed enhancers.

Poised DV enhancers are specifically generated during tissue patterning and are not poised for future activation

Our results so far suggest that uninduced enhancers have a histone signature that is indistinguishable from the poised enhancer signature described in mammals, with or without H3K27me3. This raises the question whether the *Drosophila* DV enhancers are at some point poised for future activation.

We first considered the possibility that DV enhancers are poised prior to activation, when the enhancers are primed by Zld before Dl-dependent transcription begins. Based on a careful time-course analysis (Li et al. 2014), the primed DV enhancers do not show the poised signature until cell cycle 14, when DV patterning begins. While H3K27ac accumulates very early and gradually, H3K4me1 and H3K27me3 show a strong increase only during cell cycle 14 (Fig. 4A). This suggests that the DV enhancers do not have a poised enhancer signature when they are primed prior to activation.

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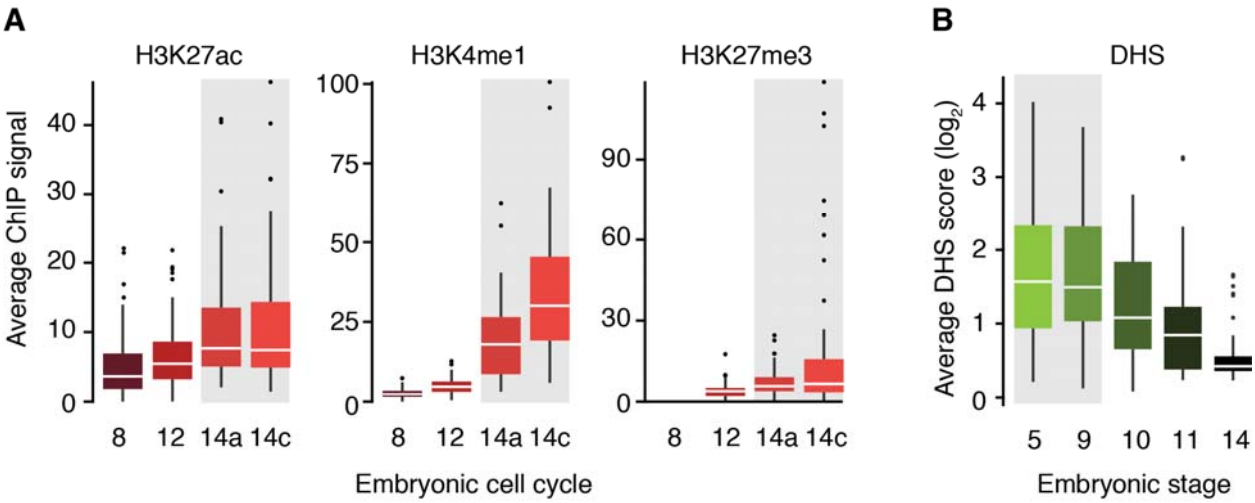

Figure 4. DV enhancers are not poised for future activation

(A) Histone modification levels at DV enhancers during the maternal-to-zygotic transition (Li et al. 2014) show that H3K27ac levels are accumulating early and gradually during development, and thus some H3K27ac is present during enhancer priming by Zld at cell cycle 8 and 12. In contrast, H3K4me1 and H3K27me3, which mark poised enhancers, are only detectable after Dl-dependent transcription begins at stage 5 or cell cycle 14 (shaded in grey). Data are shown as average ChIP-seq signal in a 1-kb window centered on each enhancer. (B) Boxplots of DNase I hypersensitivity (DHS) at DV enhancers during embryogenesis show that all DV enhancers are most accessible during stages 5 and 9 when DV patterning takes place (shaded in grey) and become less accessible at subsequent stages. The DHS score is the average signal per enhancer region derived from the data by Thomas et al. (2011). Whiskers show 1.5 times the interquartile range and outliers are shown as dots.

We next considered whether the DV enhancers are poised for activation beyond DV patterning during later stages of embryogenesis. This seems unlikely since enhancers are in the vast majority stage-specific. To nevertheless test the possibility, we analyzed DNase I hypersensitivity (DHS) data across embryogenesis (Thomas et al. 2011). We found that DV enhancers are most accessible during DV

patterning (stages 5 and 9), when they are active, and become less accessible at subsequent stages (Fig. 4B). This argues against additional roles for these enhancers past DV patterning.

Taken together, our analysis suggests that the poised enhancer signature of low H3K27ac and some H3K4me1 is specifically generated during DV patterning at uninduced enhancers. There is no evidence that it precedes enhancer activation, arguing that it marks spatial rather than temporal regulation in this system.

Discussion

The poised enhancer signature as a marker for spatial enhancer regulation

We found that DV enhancers acquire the poised enhancer signature (low H3K27ac, some H3K4me1) specifically during tissue patterning (model in Fig. 5). Before DV patterning, these enhancers are primed by the pioneer transcription factor Zld and have a very different enhancer signature (some H3K27ac but no H3K4me1). It is unclear whether this enhancer signature is typical for primed enhancers since the priming occurs during the maternal-to-zygotic transition. Nevertheless, it clearly shows that the poised enhancer signature does not precede enhancer activation in the DV system and thus is specifically generated in the tissue in which the enhancers are not activated. During subsequent stages, the DV enhancers close again, perhaps because key transcription factors such as Zld are no longer present (Kanodia et al. 2012). It is also possible that repressive chromatin modifying complexes help to decommission enhancers to reduce their activity in subsequent developmental programs (Whyte et al. 2012).

The *Drosophila* DV developmental system therefore demonstrates that poised enhancers are not always poised for activation in the future but may mark enhancers that did not receive the signal for activation during tissue patterning. These uninduced enhancers are accessible to transcription factors and thus likely remain responsive to the appropriate developmental signals for some time. This allows cells to adjust to changes in signals from surrounding cells during pattern formation. However, in the absence of

appropriate signals, a poised enhancer does not become active and instead proceeds directly to a closed state. Thus, poised enhancers in our system may be inducible during the time in which tissue patterning takes place, but their function is not to serve as a target for future patterning events.

In mammalian systems, a fraction of enhancers have been shown to be poised prior to activation (Creyghton et al. 2010; Rada-Iglesias et al. 2011; Wamstad et al. 2012; Wang et al. 2015). This may be because the primed state already resembles the poised state, or because enhancer activation occurs in a more sequential fashion by multiple patterning signals. However, many poised enhancers do not become active later, and many active enhancers are not poised before activation (Wamstad et al. 2012; Choukrallah et al. 2015). This suggests that many mammalian enhancers may also simply be poised as a side effect of tissue patterning, as we have observed in the *Drosophila* DV system. They are poised because they were not activated in a specific developmental context and they do not have another opportunity for activation in the future course of development.

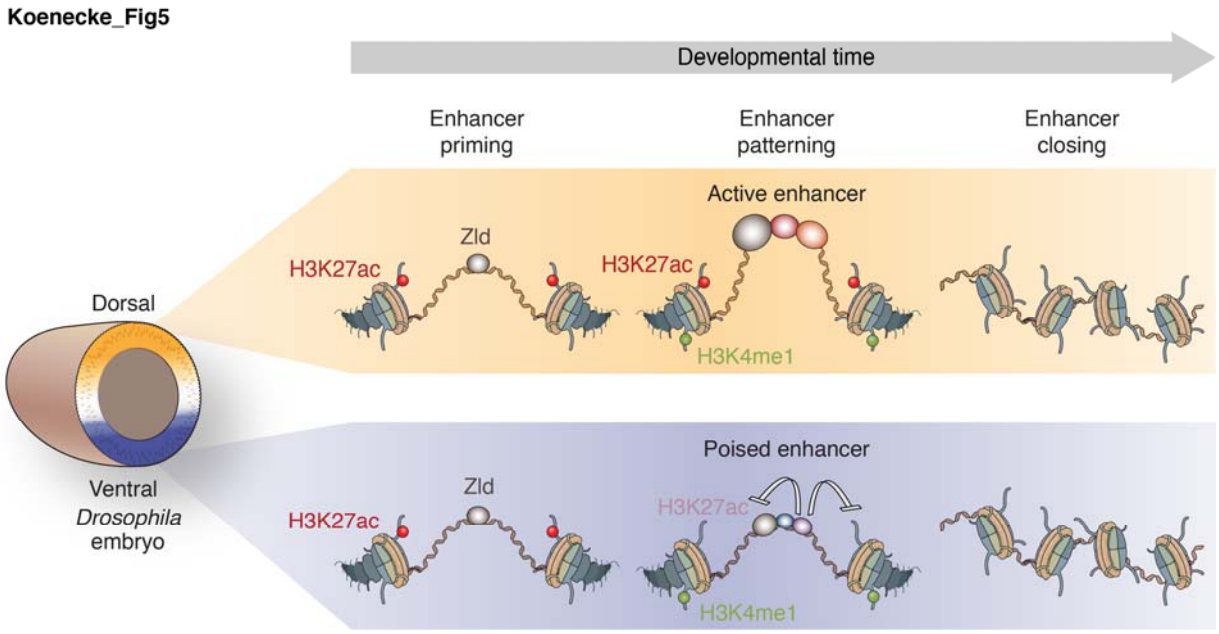

Figure 5. Summary model showing the poised enhancer signature arising specifically during tissue patterning in the Drosophila DV system

Before DV patterning begins in the *Drosophila* embryo, DV enhancers are primed by the pioneer transcription factor Zld and have low levels of H3K27ac. During DV patterning, DV enhancers may be active in one tissue but repressed by sequence-specific repressors in another tissue and thus remain uninduced. As has been studied extensively, these repressors recruit histone deacetylases, remove H3K27ac and thus produce the poised enhancer signature. After DV patterning is complete, DV enhancers gradually close, thus enhancers with the poised enhancer signature also close and are not poised for future activation.

A role for repressors in keeping poised enhancers inactive

We found that the three DV enhancers that are actively repressed by Dl have the poised enhancer signature. This raises the possibility that sequence-specific repressors actively help generate the poised enhancer signature and prevent these enhancers from becoming active.

In support of this hypothesis, the poised enhancer signature fits strikingly well with previous mechanistic studies on repression on individual loci in *Drosophila*. Transcriptional repressors such as Dl have been reported to reduce the occupancy of transcription factors and remove histone acetylation through the recruitment of co-repressors and histone deacetylases (Chen et al. 1999; Kulkarni and Arnosti 2005; Sekiya and Zaret 2007; Winkler et al. 2010; Li and Arnosti 2011). Thus, the low levels of H3K27ac and the reduced access to transcription factors that we observe for *zen*, *dpp* and *tld*, must be to some extent the result of Dl-mediated repression.

An even more intriguing hypothesis is that the poised enhancer signature is generally the product of enhancer-bound repressors. This would explain why the Dl-repressed enhancers did not stand out in their histone modification signature as compared to other uninduced enhancers. There are many sequence-specific repressors that modulate DV patterning, including Snail (Kosman et al. 1991; Leptin 1991), Capicua (Jimenez et al. 2000; Helman et al. 2012), Suppressor of Hairless (Morel and Schweisguth 2000; Ozdemir et al. 2014) and Schnurri (Crocker and Erives 2013). Thus, it is feasible that repressors play a

central role in preventing the inappropriate activation of accessible enhancers. Below, we discuss a number of reasons why this is not only plausible but also an attractive model.

Based on ChIP-seq data, regions of open chromatin are surprisingly susceptible to unspecific transcription factor binding (Moorman et al. 2006; MacArthur et al. 2009). Many transcription factors have strong activation domains, putting accessible enhancer regions at risk for unwarranted activation. For example, Zld has high transactivation potential and likely recruits the histone acetyl transferase CBP that mediates H3K27ac (Hamm et al. 2015; Stampfel et al. 2015), consistent with H3K27ac being present during enhancer priming by Zld (Li et al. 2014). Strikingly, we showed that Zld is still bound to DV enhancers during DV patterning, yet these enhancers have no or low H3K27ac and remain uninduced in parts of the embryo. The simplest explanation for this observation is that activation by Zld is repressed or "quenched" by repressors in these cells. Thus, repressors would serve to remove the histone acetylation that Zld induced during enhancer priming and prevent the accumulation of this activating mark throughout DV patterning.

Another reason is that the pattern by which poised enhancers occur during lineage development is consistent with the expected widespread use of repressors in signaling and tissue patterning. In addition to sequence-specific repressors employed during tissue patterning, most developmental signal transduction pathways have their own dedicated mechanism to repress target genes in the absence of signaling activity (Barolo and Posakony 2002; Affolter et al. 2008). The fact that these signal transduction pathways are highly conserved across evolution supports the notion that repression is an integral part of enhancer regulation.

Mechanistic implications for poised enhancers with repressors

Finally, the involvement of repressors in keeping poised enhancers inactive has important mechanistic implications and predictions that have not been discussed to our knowledge. An active battle between activators and repressors in controlling histone acetylation at the poised state implies a monocycle between opposing enzymes, thus acetylation by acetyl transferases and deacetylation by deacetylases. Analogous to phosphorylation-dephosphorylation dynamics found at some enzymes, such monocycles

can create switch-like behaviors and were therefore termed zero-order ultrasensitivity (Goldbeter and Koshland 1981; Ferrell and Ha 2014). In other words, repressors could make enhancers ultrasensitive in their response to activation signals.

Such zero-order ultrasensitivity predicts that a repressed enhancer can be very sensitive to activation, so that only a small amount of activation signal can lead to significant induction (Melen et al. 2005; Ferrell and Ha 2014). This is particularly important in the response to morphogen gradients, where a certain threshold concentration leads to enhancer activation and expression of downstream target genes. At the same time, zero-order ultrasensitivity also implies that a strongly repressed state is relatively stable against inappropriate activation. For example, the role of Polycomb repression, found at important developmental genes, could be to keep enhancers in the repressed regime until they are activated.

In summary, a model in which poised enhancers are actively balanced between activators and repressors could provide a mechanism to explain the ultrasensitive response of enhancers to patterning signals. This could explain the widespread occurrence of a distinct poised enhancer state during tissue patterning. Since the model makes clear mechanistic predictions, it opens new avenues for further exploration and tests in the future.

Methods

Stock maintenance and embryo collection

The fly stock *Tl*^{10b} was obtained from the Bloomington stock center (#30914). The *gd*^{7} stock was kindly provided by Mike Levine. *gd*^{7}/*gd*^{7} females *gd*^{7}/Y males and were collected from the *gd*^{7}*/winscy, hs-hid* stock after heat shocking 1 day-old larvae twice for 1 h at 37 °C, 24 h apart. *T(1;3)OR60/ Tl*^{10b}, *e*^{1} females and *Tl*^{10b}*/TM3, e*^{1}*, Sb*^{1}*, Ser*^{1} males were selected from the stock consisting of genotypes *Tl*^{10b}*/TM3, e*^{1}*, Sb*^{1}*, Ser*^{1} and *T(1;3)OR60/ TM3, e*^{1}*, Sb*^{1}*, Ser*^{1}*. Oregon-R* embryos were used for wild-type ChIP-seq samples. Embryos were collected from cages on apple juice plates for 2 h at 25 °C and then aged at 25 °C for another 2 h (to produce the time window of 2-4 h AED). Embryos were dechorionated with 100% bleach

and cross-linked for 15 min with 1.8% formaldehyde (final concentration in water phase) for ChIP-seq experiments.

ChIP-seq experiments

ChIP-seq experiments were performed as described (He et al. 2011; He et al. 2015) with ~100 mg embryos per ChIP and with more extensive washing with RIPA buffer after ChIP incubation to reduce background. The antibodies for ChIP-seq were custom-generated by Genscript (Dl aa 39-346, Mad aa 148-455, full-length Zen, Zld aa 1117-1327, GAF aa 1-382 of isoform PA), by Covance (Twi aa 340-490) or were commercially available: H3K27ac (Active motif, #39133), H3K4me1 (Active motif, #39635), H3K27me3 (Active motif, #39155), Pc (Santa Cruz, #sc-25762). Tl^{10b} embryos were used for ChIP-seq for Dl, Twi, Zld, H3K27ac, H3K4me1, and H3K27me3; wild-type embryos for GAF and Pc; and gd^{7} embryos for Mad, Zen, Zld, H3K27ac, H3K4me1, and H3K27me3.

Library preparation

Different combinations of library preparation kits and barcodes were used for ChIP-seq and mRNA-seq library preparations (Supplemental Table S3) and libraries were prepared according to manufacturer's instructions. ChIP-seq libraries were prepared from 5-15 ng ChIP DNA or 100 ng input DNA and sequenced on the GAIIX (Illumina) or the HiSeq 2500 (Illumina).

ChIP-seq data processing

Sequenced ChIP-seq reads were aligned to UCSC *Drosophila melanogaster* reference genome dm3 using Bowtie v1.1.1 (Langmead et al. 2009), allowing up to two mismatches and retaining only uniquely aligning reads. Aligned reads were extended to the sample's estimated fragment size using the *chipseq* Bioconductor library (Huber et al. 2015).

Replicates of genotype-specific whole cell extract (WCE) input samples for Tl^{10b} and gd^{7} were merged, and these merged inputs were used for enrichment calculations and peak calling.

Transcription factor ChIP-seq enrichments over input within each enhancer were calculated within a 201 bp window centered at the transcription factor's ChIP-seq signal summit. Enrichment calculations were

normalized for both differences in read count and estimated fragment size between ChIP and input samples. Histone modification enrichments were calculated similarly, but using a 1,001-bp window centered on the enhancer region. The replicates for each transcription factor and histone modification with the highest median enrichment were used for further analysis. To assess sample quality, MACS2 (Zhang et al. 2008) was run on all samples with their corresponding tissue's input control and these non-default parameters:

```
macs2 callpeak -t ip.bam -c wce.bam -g dm --keep-dup=all
```

Peak counts for each sample can be found in Supplemental Table S3.

Normalization of histone modification ChIP-seq data

Fold-change in ChIP-seq enrichments of H3K27ac, H3K4me1 and H3K27me3 between Tl^{10b} and gd^{7} were normalized to account for differences in ChIP-seq efficiency. The normalization factor for each histone modification was determined by the median fold-change in ChIP-seq enrichment at MACS2 peaks that were detected in both mutant embryos.

mRNA-seq experiments

Embryos were dechorionated with 100% bleach and stored at -80 °C until used. Total mRNA was extracted from 50-100 mg 2-4 h AED Tl^{10b} embryos in duplicates and gd^{7} embryos in triplicates using the Maxwell Total mRNA purification kit (Promega, #AS1225) according to manufacturer's instructions. PolyA-mRNA was isolated using Dynabeads oligo(dT) (Life Technologies, #61002). Libraries were prepared following the instructions of the TruSeq DNA Sample Preparation Kit (Illumina, #FC-121-2001) and sequenced on the HiSeq 2500 (Illumina) or the NextSeq 500 (Illumina).

mRNA-seq data processing

mRNA-seq reads were aligned against the FlyBase r5.57 genome and gene annotations using Tophat2 v2.0.14 (Kim et al. 2013) with the following non-default parameters:

```
tophat -G fb557_genes.gtf -I 20 -I 5000 --no-coverage-search --segment-length 25
```

Cuffdiff, from Cufflinks v2.2.1 (Trapnell et al. 2010), was used to determine transcript abundance and differential expression between Tl^{10b} and gd^{7} mutants.

List of known DV enhancers

A list of known DV enhancers was assembled from the literature. Enhancers were only included if *lacZ* reporter assays confirmed a DV-biased expression pattern. The full list of known DV enhancer regions and the respective publication that shows the staining of the enhancer's *lacZ* reporter assay can be found in Supplemental Table S1. A target gene was assigned to DV enhancers only if the enhancer's expression domain overlapped and resembled the gene's expression domain (Supplemental Material). For this purpose, published mRNA in situ hybridization data from the Berkeley *Drosophila* Genome Project (BDGP) (Tomancak et al. 2002; Tomancak et al. 2007; Hammonds et al. 2013) database were used for enhancers identified by Kvon et al. (2012) and Ozdemir et al. (2011). For some of these enhancers, no target gene was identified with confidence and thus those enhancers were not included in mRNA-seq analysis shown in Supplemental Fig. S1 (Supplemental Material).

Definition of active enhancers, uninduced enhancers and closed regions

Active enhancers are MEs in the mutant Tl^{10b} and DEEs in gd^{7} embryos. Uninduced enhancers are MEs in gd^{7} embryos and DEEs in Tl^{10b} embryos. A total of 100 closed regions were randomly selected from published DHS regions (Thomas et al. 2011) (see more details below) based on the following criteria: they are only accessible at stage 14 and not in any of the earlier stages; they overlap with peaks from published H3K27ac ChIP-seq at 14-16 h AED in wild-type embryos (Contrino et al. 2012) (obtained from modMine: modENCODE ID:4120), and they do not overlap with a TSS (2 kb centered on a TSS).

ChIP-seq binding profile displays at single genes

Single gene profiles of histone modifications show ChIP-seq enrichment values over input calculated using a 501 bp sliding window. Transcription factor profiles are shown in reads per million.

Identification of putative PREs

Putative PREs were defined as regions that result from overlapping Pc and GAF peaks (minimum 50 bp overlap) from ChIP-seq in wild-type 2-4 h AED embryos. Overlapping regions were combined to one putative PRE region, resulting in 602 putative PREs. For Zld-bound regions, peaks were called by MACS2 (Zhang et al. 2008) on Tl^{10b} and gd^{7} ChIP-seq samples and the union from the Tl^{10b} and gd^{7} peaks with at least two-fold enrichment over background in one tissue was identified. Enrichment of H3K27me3 was calculated in a 1,001-bp region centered at each Zld peak. Both known enhancers and Zld regions were divided into H3K27me3 "low" and "high" groups based on an enrichment threshold of two-fold below or above input, respectively. Coordinates for putative PREs can be found in Supplemental Table S2 and distances of known DV enhancers to the closest putative PRE can be found in Supplemental Table S1.

DNase I hypersensitivity at known DV enhancers

DHS datasets (Thomas et al. 2011) were downloaded from the USCS genome browser: http://hgdownload.cse.ucsc.edu/gbdb/dm3/bdtnp/bdtnpDnaseS5R9481.bw; S9R9127.bw, S10R8816.bw, S11R9485.bw, and S14R9477.bw. Average DHS signal per base was calculated for all known DV enhancers at each of the five embryonic stages by summing the number of DHS reads that overlap each enhancer and dividing by the enhancer's width in base pairs.

Histone modification data during maternal-to-zygotic transition

Processed H3K27ac, H3K4me1 and H3K27me3 ChIP-seq data in the early embryo (Li et al. 2014) were obtained from GEO (GSE58935). ChIP-seq data are shown as average signal in a 1,001-bp region centered at each enhancer.

Data access

The whole-genome sequence data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE68983. In addition, all data analysis performed in this study, including raw data, processed data, software tools, and analysis

scripts, has been reproduced in a publically accessible Linux virtual machine. See http://research.stowers.org/zeitlingerlab/data.html for details.

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Disclosure declaration

The authors declare no conflict of interest.

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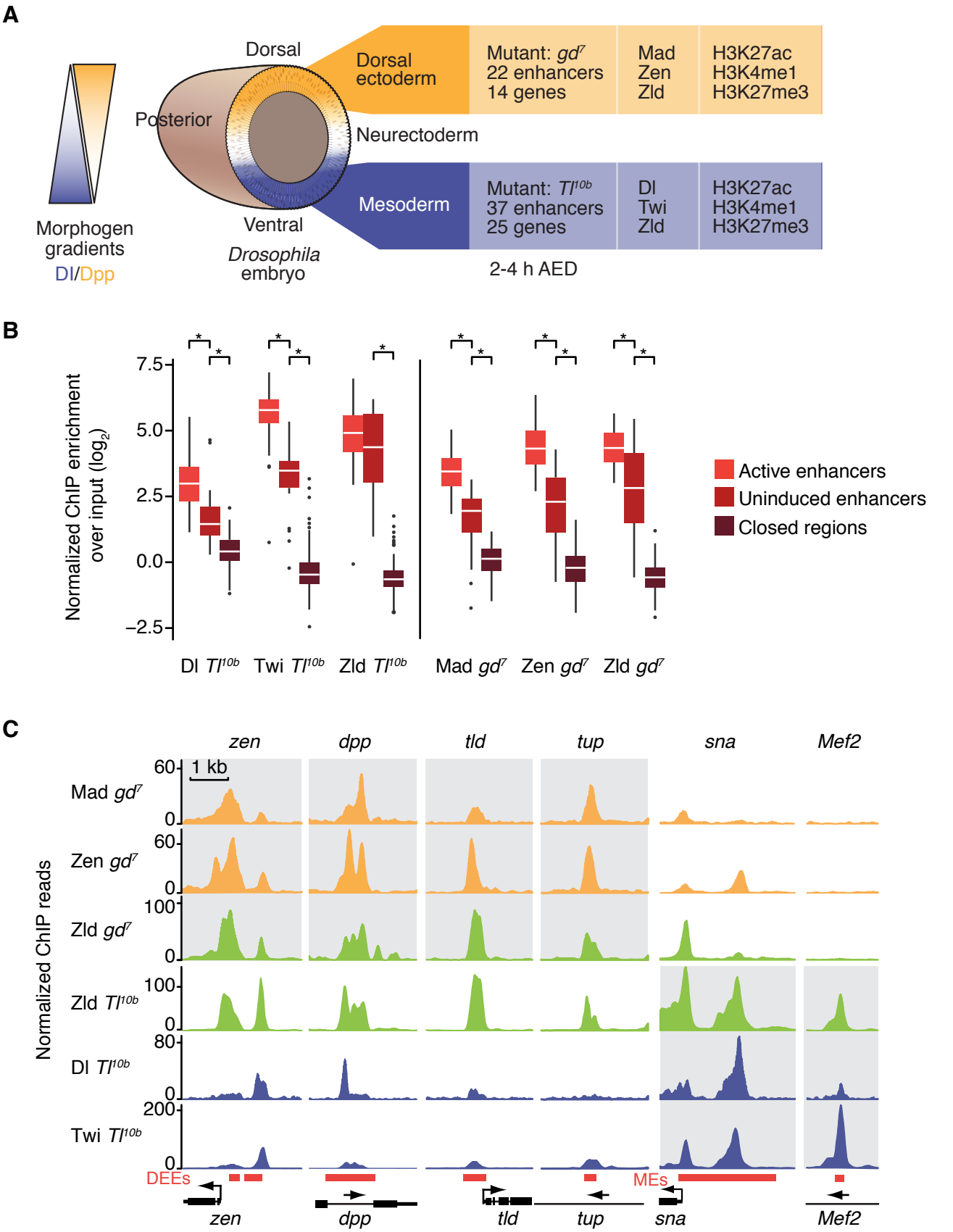
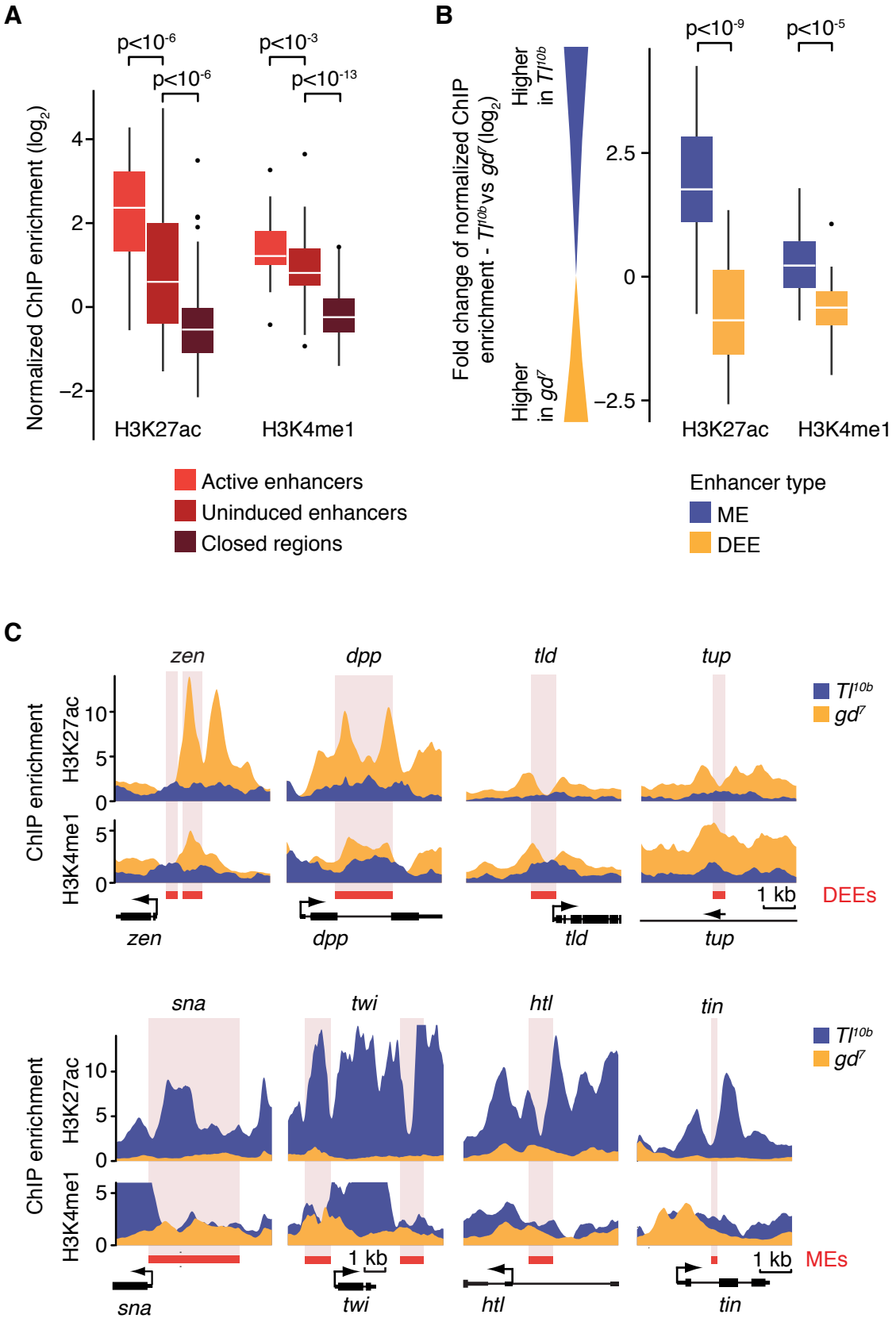
Koenecke_Fig1




A
p<10-6
p<10-6
p<10-3
p<10-13
Normalized ChIP enrichment (log2)
4
2
0
−2
H3K27ac
H3K4me1
Active enhancers
Uninduced enhancers
Closed regions
B
Fold change of normalized ChIP enrichment - Tl10b vs gd7 (log2)
Higher in Tl10b
Higher in gd7
p<10-9
p<10-5
2.5
0
−2.5
H3K27ac
H3K4me1
Enhancer type
ME
DEE
C
zen
dpp
tld
tup
ChIP enrichment
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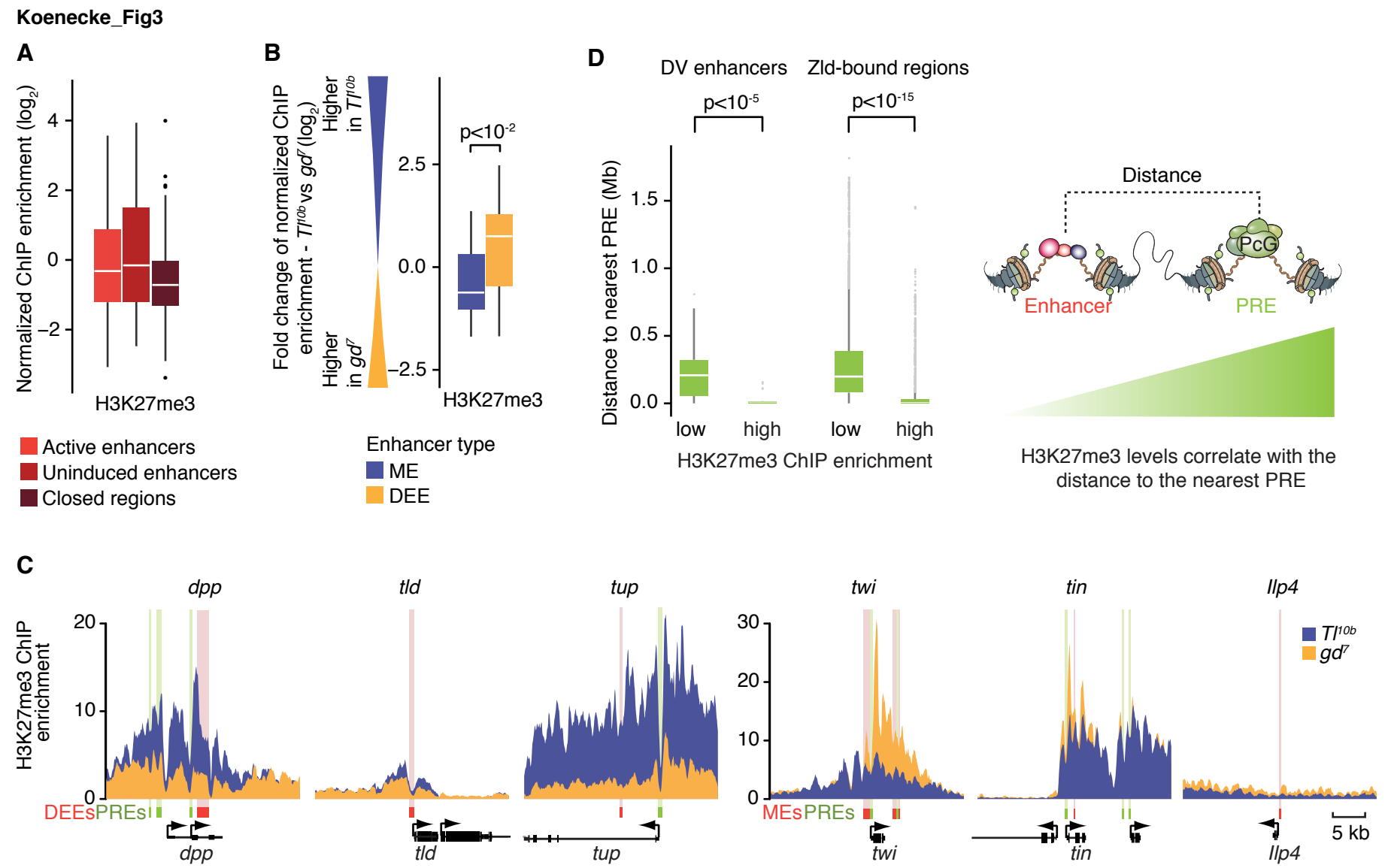
Koenecke_Fig3



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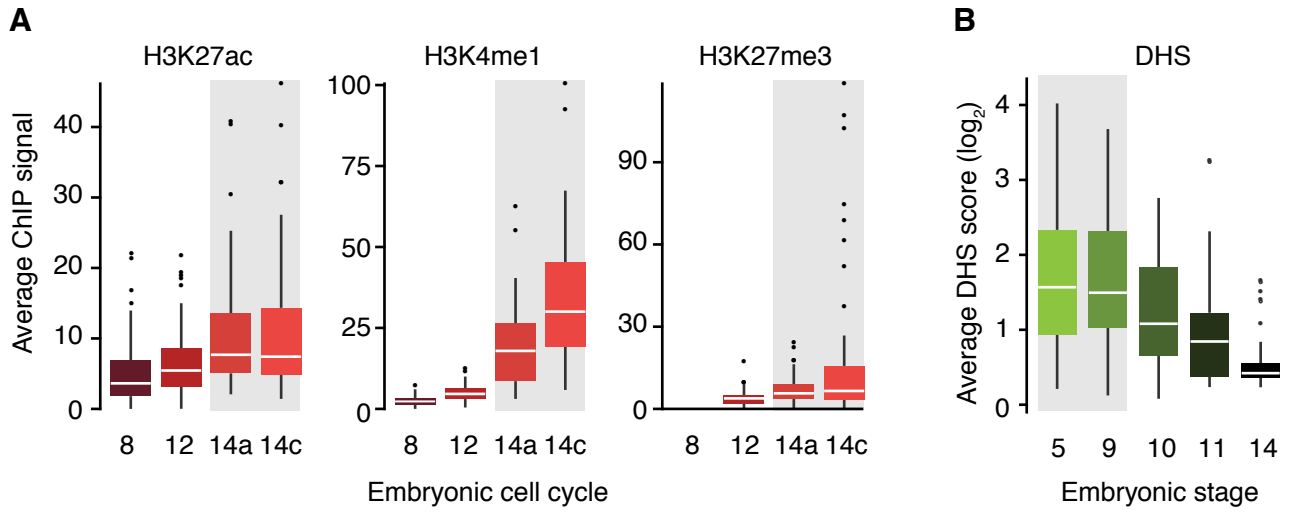

Koenecke_Fig5