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

Nina Koenecke,1 Jeff Johnston,1,3 Qiye He,1,4 Samuel Meier,1,5 and Julia Zeitlinger1,2

1Stowers Institute for Medical Research, Kansas City, Missouri 64110, USA; 2University of Kansas Medical Center, Department of Pathology, Kansas City, Kansas 66160, USA

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.

[Supplemental material is available for this article.]

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 monomethylation 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; Choukallah 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 (Stegachis 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 a 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 a 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, neuroectoderm in the lateral regions, and dorsal ectoderm on the dorsal side (Fig. 1A; Hong et al. 2008). 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 embryos in the progeny consist entirely of either mesodermal or dorsal ectodermal precursor cells. In Tl mutant embryos Tl10b, Dl activity is uniformly high (but not above wild-type levels), leading to a mesodermal precursor fate (Schneider et al. 2011). In gastrulation defective (gd) mutant embryos gd2, 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 DI is converted into a repressor, it recruits corepressors and histone deacetylases (Dubnicoff et al. 1997; Valentine et al. 1998; Chen et al. 1999; Flores-Saab 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, zerkniilt (zen), and tolloid (thd)—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; Kio et al. 1994; Ratnaparkhi et al. 2006).

By 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.

**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) for a complete list and references, see Supplemental Material. To validate our experimental system, we performed miRNA-seq experiments on T1106 and gd7 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 T1106 and gd7 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 the mesoderm and dorsal ectoderm (Fig. 1A). High DI activity on the ventral side of the embryo induces expression of Twist (Twi), which together with DI activates mesodermal target genes (Jiang et al. 1993; Ip et al. 1992). We therefore analyzed DI and Twi occupancy in T1106 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 DI and Twi, the closed control regions had the lowest levels, and uninduced enhancers had statistically significant intermediate levels of occupancy (Fig. 1B).

Dpp signaling, which activates the transcription factors Mothers against dpp (Mad), and Zen induce dorsal ectodermal fate (Raftery et al. 1995; Rushch and Levine 1997; Lin et al. 2006). We therefore analyzed the occupancy of Mad and Zen in gd7 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 were 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 with uninduced enhancers are 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 DI in T1106 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 gd7 embryos, they show high occupancy of Zld, Mad, and Zen, as expected. When they are repressed in T1106 mutants, they are occupied by Zld, DI, 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 compared with closed control regions (P < 10^-6, Wilcoxon rank-sum test) (Fig. 2A), but their levels were significantly lower than at active enhancers (P < 10^-6, Wilcoxon rank-sum test) (Fig. 2A). 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 lower levels of H3K27ac and that the levels significantly increase when the enhancers are active.

When we analyzed H3K4me1 levels, we found that uninduced enhancers are also enriched for H3K4me1 significantly above the levels of the control (P < 10^{-13}, Wilcoxon rank-sum test) (Fig. 2A), consistent with a poised enhancer signature. However, H3K4me1 enrichments were slightly lower in the uninduced state than in the active state (P < 10^{-3}, Wilcoxon rank-sum test) (Fig. 2A). 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 signatures 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; Tollu 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 *Tl10b* and *gf7* 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 (P < 10^{-3}, Wilcoxon rank-sum test) (Fig. 3B), 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; Tollu 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; Tollu et al. 2006; Gaertner et al. 2012), and recent mammalian studies provide direct evidence that transcription status affects the levels of H3K27me3 (Rülsing et al. 2014; Beltran et al. 2016; Hosogane et al. 2016).

**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 MEs (blue) have higher H3K27ac enrichment in the td^{10b} mutant than in the gd^{10b} mutant (thus log(Tl10b - log(gd^{10b}) 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^{10b} (yellow), while at four MEs, the levels are higher in Tl10b (blue). The red box and the pink shading show the position of the enhancers, and the black arrow indicates the position and orientation of transcription start sites. The 1 kb scale bar shown for the tin region also applies to the sna and htl region.

**Poised enhancers during tissue patterning**
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, 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 and putative PREs is much larger than for those with high H3K27me3 levels (above twofold enrichment, \(n = 20\)). This is also true for Zld-bound regions, which include a large number of putative early Drosophila enhancers (low H3K27me3 \(n = 14,442\), high H3K27me3 \(n = 2720\)). Zld-bound regions are the union of Zld ChIP-seq peaks in Tl10b and gd with at least twofold 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.

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 compared with 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 tap. 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 DI-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.

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; Choukralah et al. 2015). This suggests that many mammalian enhancers may 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.

A role for repressors in keeping poised enhancers inactive

We found that the three DV enhancers that are actively repressed by DI 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 DI have been reported to reduce the occupancy of transcription factors and remove histone acetylation through the recruitment of corepressors and histone deacetylases (Chen et al. 1999; Kulkarni and Arnott 2005; Sekiya and Zaret 2007; Winkler et al. 2010; Li and Arnott 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 DI-mediated repression.
in parts of the embryo. The simplest explanation for this observa-
tion is 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 monocyte between opposing enzymes, thus acetylation by acetyl transferases and deacetylation by deacetylases. Analogous to phospho-
ylation–dephosphorylation dynamics found at some enzymes, such mono-
cycles 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 ultrasensi-
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Such zero-order ultrasensitivity predicts that a repressed en-
hancer 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 expres-
sion 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 mecha-
nism to explain the ultrasensitive response of enhancers to pat-
terning 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 ave-

cases. Embryos were collected from cages on apple juice plates for 2

Methods

Stock maintenance and embryo collection

The fly stock Tl108 was obtained from the Bloomington stock cen-
ter (no. 30914). The gd+ stock was kindly provided by Mike Levine. gd+/gd- females and gd+/Y males were collected from the gd+/winfo, hs-hid stock after heat shocking 1 day old larvae twice for 1 h at 37°C, 24 h apart. T(1;3)OR60/ Tl108, e1 females and Tl108/ TM3, e1, Sb1, Ser1 and T(1;3)OR60/ TM3, e1, Sb1, Ser1. Oregon-R embryos were used for wild-type ChIP-seq sam-
plees. Embryos were collected from cages on apple juice plates for 2

h at 25°C and then aged for another 2 h at 25°C (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 previously described (He et al. 2011, 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) or by Covance (Twi aa 340-490) or were commercially available: H3K27ac (Active motif, no. 39133), H3K4me1 (Active motif, no. 39635), H3K27me3 (Active motif, no. 39155), and Pc (Santa Cruz, no. sc-25762). Tl10b embryos were used for ChIP-seq for Dl, Twi, Zld, H3K27ac, H3K4me1, and H3K27me3; wild-type embryos for GAF and Pc; and gd7 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 the 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 Tl10b and gd7 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 1001-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 nondefault 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 Tl10b and gd7 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 Tl10b embryos in duplicates and gd7 embryos in triplicates using the Maxwell total mRNA purification kit (Promega, no. AS1225) according to the manufacturer’s instructions. PolyA-mRNA was isolated using Dynabeads oligo(dT) (Life Technologies, no. 61002). Libraries were prepared following the instructions of the TruSeq DNA sample preparation kit (Illumina, no. 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 nondefault parameters: tophat -G fly557_genomic.gtf -t 20 -i S500 --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 Tl10b and gd7 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, 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 Figure S1 (Supplemental Material).

Definition of active enhancers, uninduced enhancers, and closed regions

Active enhancers are MEs in the mutant Tl10b and DEEs in gd7 embryos. Uninduced enhancers are MEs in gd7 embryos and DEEs in Tl10b embryos. A total of 100 closed regions were randomly selected from published DHS regions (see more details below) (Thomas et al. 2011) 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 (obtained from modMine: modENCODE ID-4120) (Contrino et al. 2012); 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-hp 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 T450 and g4 ChIP-seq samples, and the union from the T450 and g4 peaks with at least twofold enrichment over background in one tissue was identified. Enrichment of H3K27me3 was calculated in a 1001-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 twofold 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.

DHS at known DV enhancers

DHS data sets (Thomas et al. 2011) were downloaded from the UCSC Genome Browser: http://hgdownload.cse.ucsc.edu/goldb/dm3/bdtnp/bdtnpDnaseS5R9481.bw; S9R127.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 1001-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 publicly accessible Linux virtual machine. For details, see http://research.stowers.org/zeitlingerlab/data.html.

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References


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