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A common set of DNA regulatory elements shapes *Drosophila* appendages

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SUMMARY

Animals have body parts made of similar cell types located at different axial positions (e.g. limbs). The identity and distinct morphology of each structure is often specified by the activity of different "master regulator" transcription factors. Although similarities in gene expression have been observed between body parts made of similar cell types, it is not known how regulatory information in the genome is differentially utilized to create morphologically diverse structures in development. Here, we use genome-wide open chromatin profiling to show that among the *Drosophila* appendages, the same DNA regulatory modules are accessible throughout the genome at a given stage of development, except at the loci encoding the master regulators themselves. In addition, while open chromatin profiles change over developmental time, these changes are coordinated between different appendages. We propose that master regulators create morphologically distinct structures by differentially influencing the function of the same set of DNA regulatory modules.

INTRODUCTION

Animals are comprised of a diversity of body parts, varied in form according to their function. Among species, changes in DNA sequence have been shown to underlie changes in morphology (Carroll, 2008; Wray, 2007). However, within a single animal, the same genome sequence gives rise to the full panoply of body parts through differential regulation of gene expression. During development, differences in body part identity are determined by the activity of master regulator transcription factors, often termed "selector" genes (Mann and Carroll, 2002). In *Drosophila*, the homeodomain transcription factor Distalless (Dll) (Gorfinkiel et al., 1997) and the zinc finger proteins Buttonhead and Sp1 (Estella and Mann, 2010) specify ventral appendage identities, including the legs. Dorsal appendage identities, such as the wing and haltere, are specified by Vestigial (Vg) and its TEA-domain DNA binding partner Scalloped (Sd) (Halder et al., 1998). Along the anterior-posterior axis, morphology of structures is diversified by other master regulator transcription factors such as the Hox proteins. For example, the Hox protein Ultrabithorax (Ubx) is responsible for

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specifying haltere identity over wing (Lewis, 1978). While many of the transcription factors that control growth and patterning during appendage development have been identified, little is known about how they access regulatory information in the genome to create different appendage morphologies. One possibility is that each master regulator, with its unique DNA binding specificity, accesses a unique set of *cis*-regulatory elements in the genome to differentially regulate gene expression between the appendages.

A major hurdle to understanding the mechanisms of developmental gene regulation is the identification of functional DNA regulatory elements in the genome. A variety of methods has been used to identify potential DNA regulatory elements with varying degrees of success, including prediction of transcription factor binding sites (Berman et al., 2002; Markstein et al., 2002; Rebeiz et al., 2002), DamID (van Steensel and Henikoff, 2000), chromatin immunoprecipitation (ChIP) (Fisher et al., 2012; Negre et al., 2011; Sandmann et al., 2007; Visel et al., 2013; Zinzen et al., 2009), STARR-seq (Arnold et al., 2013), and large-scale cloning efforts (Jory et al., 2012; Pfeiffer et al., 2008). Yet another approach to identify DNA regulatory elements is the identification of nucleosome-depleted or “open chromatin” sites. Methods such as DNase I hypersensitivity mapping (Dorschner et al., 2004) and FAIRE (Giresi et al., 2007; Nagy et al., 2003), provide a snapshot of genomic sites where nucleosomes have been depleted, often through competition with *trans*-acting factors. Nucleosome depletion identifies a variety of DNA regulatory elements, including those involved in DNA replication (MacAlpine et al., 2010), nuclear organization (Bartkuhn et al., 2009), and transcription (e.g. enhancers) (Song et al., 2011; Thomas et al., 2011). Thus, open chromatin profiling is an ideal method to compare how *trans*-acting factors read out the genome between different tissues, independently of the identity of those factors.

Here, we use development of the thoracic appendages in *Drosophila* to examine how a single genome sequence is utilized to give rise to morphologically diverse structures. We first demonstrate that open chromatin is an accurate and precise predictor of functional enhancer activity in developing embryos. Next, we ask how the genome is accessed in different appendages at two stages of their development. Although comprised of similar cell types, each appendage expresses a different combination of master regulator transcription factors that have different DNA binding domains, and therefore we hypothesized that in each appendage a significant subset of the enhancers used would be unique to that appendage. In contrast to our expectations, we find that the same set of enhancers is accessible in all three appendages, with the exception of enhancers that control expression of the master regulators themselves. We show that this shared set of appendage enhancers changes coordinately over developmental time. Finally, we provide functional evidence that the appendage master regulators differentially regulate the activity of the same enhancers to effect differences in gene expression between the appendages. Thus, morphologically distinct structures can be created using the same set of enhancers.

RESULTS

FAIRE identifies DNA bound by regulatory factors in developing animals

To identify genomic locations with gene regulatory activity, we performed Formaldehyde-Assisted Isolation of Regulatory Elements, which identifies nucleosome-depleted or “open” chromatin, followed by high-throughput sequencing (FAIRE-seq) (Giresi et al., 2007; Simon et al., 2012) and RNA-seq at three developmental timepoints in *Drosophila* embryos: 2–4 hr after egg laying (AEL) during initial establishment of the body axes and germ layers, 6–8 hr AEL during fine-scale cell fate specification through the action of local signaling pathways, and 16–18 hr AEL when many cells have terminally differentiated. Consistent with previous studies (Giresi et al., 2007; Song et al., 2011), we find FAIRE-enriched regions are bound by regulatory factors (Fig. 1, Fig. S1). FAIRE signal very closely resembles the aggregate

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transcription factor chromatin immunoprecipitation (ChIP) signal (Bradley et al., 2010) (Fig. 1A), supporting the well-established association between transcription factor binding and nucleosome depletion (Fig. 1B). Genomic locations with high FAIRE signal are evolutionarily conserved (Siepel et al., 2005) (Fig. S1) and are associated with high levels of "active" histone modifications (Fig. 1C, Fig. S1), including H3K4me1 and H3K27ac, marks associated with enhancer activity, and H3K4me3, a mark associated with active gene promoters. Correspondingly, high FAIRE signal is associated with low levels of "repressive" histone modifications such as H3K27me3 and H3K9me3 (Fig. 1C, Fig. S1). FAIRE data from embryos collected at 2–4 hr and 6–8 hr also closely match recent genome-wide DNase I hypersensitivity data from early *Drosophila* embryos (Thomas et al., 2011) (Fig. 1A, D, E). Thus, FAIRE identifies nucleosome-depleted regions during *Drosophila* development, which coincide with genomic sites bound by multiple regulatory factors. Both FAIRE-seq and RNA-seq experiments were highly reproducible (Fig. S1).

Open chromatin identifies enhancers and the timing of enhancer activity

A range of approaches has been used to identify functional DNA regulatory elements in the genome with varying degrees of success (Aerts et al., 2007). Since FAIRE identifies genomic regions that are bound by *trans*-acting proteins, it followed that FAIRE enrichment might be used as a predictor of enhancer activity at a given point in time. To test the sufficiency of individual FAIRE-enriched sites to control transcription, we cloned twenty-four different open chromatin regions for transgenic reporter assays (Table S1). To identify target regions for cloning, we used only FAIRE data, without consulting any other data sets (e.g. ChIP, evolutionary conservation). We chose previously uncharacterized regions that were differentially accessible across developmental stages or between tissues, and that are near developmentally important genes known to be expressed at these stages. We placed these selected regions upstream of a synthetic core promoter (Pfeiffer et al., 2010) to drive expression of the yeast transcription factor GAL4.

Despite extensive prior study of the loci selected for testing, we identified many previously undiscovered enhancers. Twenty-three of the twenty-four (96%) cloned regions recapitulated sharp, distinctive subsets of their gene's expression pattern in transgenic reporter assays (Data File S1). For example, several enhancers were identified at the *hunchback* (*hb*) locus. *Hb* was first identified due to its function in anterior-posterior patterning of the blastoderm embryo (Nusslein-Volhard and Wieschaus, 1980). Consistent with that role, all *hb* enhancers previously known to control blastoderm expression (Gallo et al., 2011; Perry et al., 2011) coincide precisely with regions of open chromatin specifically at the 2–4 hr FAIRE timepoint (Fig. 2A **black boxes**). However, little is known about control of *hb* expression later in development when *hb* is required for proper development of the central nervous system (Hirono et al., 2012) and the tracheal system (Merabet et al., 2005).

We identified five *hb* enhancers in this study. The HB01 enhancer, which is accessible at the 6–8 hr timepoint (and to a lesser extent at the 2–4 hr timepoint), is active in a subset of Hb-positive neuroblasts in the ventral nerve cord beginning at 4hr AEL (Fig. 2B), whereas the enhancers HB04 and HB05, which are also accessible at 6–8 hr, are active in the Hb-positive progeny of these cells beginning around 5 hr AEL (Fig. 2C and Data File S1). Enhancers HB03 and HB04 recapitulate *hb* expression patterns in cells required for tracheal system development, in the mesoderm, and in the nervous system (Data File S1). The expression patterns of these enhancers show (1) that regulation of *hb* expression is divided between different enhancers for different lineages of *hb*-expressing cells, and (2) that there is a temporal division in the regulation of *hb* expression between different enhancers within *hb*-expressing cells of the developing nervous system. Interestingly, none of the 3' *hb* enhancers

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we cloned are fully contained within the 10E1 *hb* construct (Fig. 2A **grey box**), which rescues *hb* function in blastoderm embryos, but is unable to provide appropriate hb function later in development, which leads to lethality (Margolis et al., 1995), This, along with our data from expression constructs, suggests that these newly-cloned enhancers are essential for regulating *hb* expression later in embryogenesis.

Finally, an important feature emerges from analysis of the newly-cloned enhancers: the timing of the appearance of open chromatin at enhancers coincides with the timing of their activity *in vivo* (Fig. 2, Table S1). Thus, FAIRE can identify not only the precise location in the genome of functional enhancers, but also the time at which these enhancers are active. Since FAIRE identifies any region of the genome that is depleted of nucleosomes, it is not expected that all FAIRE-enriched regions act as transcriptional enhancers. For example, many open chromatin regions identified by FAIRE correspond to Polycomb Response Elements (PREs) (Fig. 3C). Conversely, it is possible that regions of the genome that are not enriched by FAIRE act as transcriptional enhancers, or regulate gene expression through other mechanisms. Nevertheless, these reporter experiments demonstrate that FAIRE is an exceptionally accurate, sensitive, and precise predictor of gene regulatory activity.

Open chromatin profiles among leg, wing, and haltere imaginal discs are nearly identical at a given developmental stage

Similar to DNase I hypersensitivity patterns in embryos (Thomas et al., 2011), regulatory elements defined by FAIRE were highly dynamic from one embryonic stage to the next, with thousands of sites opening and closing between stages (Fig. S6). We next asked how information in the genome is utilized to generate morphologically diverse structures by mapping open chromatin during *Drosophila* appendage development. Insect appendages are thought to have evolutionary origins greater than 400 million years ago (Engel and Grimaldi, 2004; Garrouste et al., 2012), and they exhibit a stunning diversity of morphologies tailored to their functions (Grimaldi and Engel, 2005). The identity of each appendage is specified by a unique combination of master regulator transcription factors that differentially controls pattern formation, growth, and differentiation (Ashburner and Novitski, 1976; Estella and Mann, 2010; Gorfinkiel et al., 1997; Halder et al., 1998). Since the appendage master regulators possess different DNA-binding specificities, our hypothesis was that different transcriptional enhancers would be used to create each morphologically distinct appendage. To test this, we dissected the precursors of the thoracic appendages (called imaginal discs) from 3rd instar larvae (120 hAEL) and performed FAIRE. In sharp contrast to our findings from different stages of embryogenesis, and in refutation of our hypothesis, open chromatin profiles from the wing, haltere, and metathoracic (T3) leg imaginal discs were nearly identical to each other (Fig. 3A, Fig. S6). For example, the Spearman's rank correlation coefficients of FAIRE signals between the thoracic appendage imaginal discs ranged between 0.85–0.90, whereas the same measures between different stages of embryogenesis ranged between 0.20–0.64. We describe these findings in more detail below.

Nearly all the differences in open chromatin between wing and haltere imaginal discs occur at the *Ubx* locus

Comparison of wing and haltere imaginal disc open chromatin profiles revealed an especially striking result. Among the most pronounced FAIRE peaks in wing and haltere discs across the entire genome (the top 20%, 3,525 peaks), only five sites are specifically open in haltere imaginal discs relative to wing imaginal discs (Fig. S2, Table S2). Four of these five regions are located within the *Ubx* locus (Fig. 3B, C, Fig. S3A). The function of *Ubx* in transforming wing identity into haltere is one of the best-characterized examples of transcription-factor dependent morphogenesis in development (Crickmore and Mann, 2008).

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Mutations in *Ubx* can lead to transformation of haltere into wing, resulting in a four-winged fly (Lewis, 1978). Although *Ubx* has been shown to regulate hundreds of target genes at specific stages of haltere development (Hersh et al., 2007; Pavlopoulos and Akam, 2011) (Fig. S4), the molecular mechanisms by which *Ubx* controls growth and patterning are largely unknown. Recent ChIP-chip experiments have identified putative Ubx binding sites in the developing haltere and T3 leg imaginal discs (Choo et al., 2011; Slattery et al., 2011a), but the pattern of Ubx binding suggests that only a subset of these sites are functional (Slattery et al., 2011a). Moreover, since *Ubx* is expressed in the haltere but not in the wing, these ChIP experiments cannot be used to compare how regulatory information is accessed in the haltere relative to the wing. We asked whether our FAIRE data could help to define functional Ubx binding events. We found that open chromatin sites bound by Ubx tend to be more conserved, and occur at Ubx-responsive genes (Fig. S3B – D). These data, combined with the data showing that only five sites are open in the haltere disc but not the wing disc, with four of these residing at the *Ubx* locus itself, means that Ubx binds to regulatory DNA in the haltere (where it is expressed) that is also accessible for use in the wing (where *Ubx* is not expressed), rather than to a set of enhancers that are specific to the haltere. Thus, these data suggest that morphologically distinct structures with a shared evolutionary origin can be made by acquiring transcription factor binding sites in existing enhancers, rather than by introducing a new set of enhancers *de novo*.

Differences in appendage open chromatin profiles are at loci encoding key developmental regulators

Given their diverse morphologies and transcription factor expression profiles (Fig. S5), we were surprised to find that wing and leg imaginal discs also share very similar open chromatin profiles. Of the most pronounced open chromatin regions (the top 20%, 3,525 peaks), only 110 were differentially open (Fig. S2, Table S2). We speculated that these few differences in open chromatin between wing and leg imaginal discs were important in determining morphological differences, as was the case with wing and haltere imaginal discs. Indeed, genes with open chromatin specific to the leg imaginal discs include *Dll*, and *Sp1*, the master regulators of leg development (Estella and Mann, 2010; Gorfinkiel et al., 1997) (Fig. 4A, B). Similarly, genes with open regions specific to the dorsal imaginal discs (wing and haltere) include *vg* and *blistered*, transcription factors required for development of these appendages (Kim et al., 1996; Montagne et al., 1996) (Fig. 4, Fig. S5). We tested whether these disc-specific open chromatin regions identified by FAIRE function as appendage-specific enhancers, and found that 6 of 7 accurately recapitulate gene expression in imaginal discs of late 3rd instar larvae (Table S1, Data File S1). Similar to our observations from the embryonic time course, the presence of disc-specific open chromatin correlated with disc-specific enhancer activity - the cloned imaginal disc enhancers are active only in the imaginal discs in which they are accessible. For example, the VG01 enhancer identified by this study, which is open specifically in wing and haltere imaginal discs, recapitulates *vg* expression specifically in wing and haltere imaginal discs and is not active in leg imaginal discs (Fig. 4D). Together, these data demonstrate that genomic regions accessible for use in thoracic appendage imaginal discs are nearly identical, except for at appendage master regulator gene loci.

Leg, haltere, and wing open chromatin profiles change coordinately over developmental time

Although the fate of each disc is already determined by late third instar stages (Ashburner and Novitski, 1976), we thought that perhaps the similarity in thoracic imaginal disc open chromatin profiles might somehow be specific to this early stage of appendage formation. We therefore tested whether the terminally differentiated appendages that arose from these imaginal discs also share a similar open chromatin profile. We performed FAIRE on the

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fully-developed appendages of stage 13 and 14 pharate adults (~210hr). Like our observations in imaginal discs, the open chromatin profiles of the terminally differentiated appendages were strikingly similar to each other (Table S3). Spearman's rank correlation coefficients between the pharate appendages ranged between 0.67–0.80 (Fig. 3A). Despite their similarity to each other, the open chromatin profiles in pharate appendages were markedly different from the open chromatin profiles in imaginal discs (Fig. 5A, Fig. S6). These data lead to the unexpected conclusion that open chromatin profiles of different appendages at the same developmental stage are more similar to each other than they are to their own lineage in subsequent stages (Fig. 5B). Thus, an imaginal wing disc is more similar to an imaginal leg disc than it is to its cellular progeny, the adult wing. This conclusion holds true regardless of whether FAIRE-seq or RNA-seq data are used in the analysis (Fig. 5B), or whether the data are pooled or analyzed as individual replicates (Fig. S7A, B). Although larval discs also give rise to body wall regions that are not present in pharate adult appendages, the many new open chromatin regions in the adult appendages support a large-scale change in open chromatin profiles over time.

Different cell types have distinct chromatin profiles, but morphologically distinct tissues composed of similar cell types share open chromatin profiles at a given stage of development

Much like vertebrate limbs, the different *Drosophila* appendages are comprised of similar combinations of cell types (Klebes et al., 2002; Rodgers and Shearn, 1977; Taher et al., 2011). To test whether the similarities in thoracic imaginal disc open chromatin profiles also apply to body parts comprised of different combinations of cell types, we performed FAIRE on 3rd instar eye-antennal imaginal discs, which share developmental features of both dorsal and ventral appendages. The antenna is considered to be a ventral structure like the leg because mutations exist that transform antennal identity into leg (e.g. *homothorax*, *antennapedia*) (Casares and Mann, 1998). In contrast, the eye is considered to be a dorsal structure like the wing because mutations exist that transform eye tissue into wing (e.g. *ophthalmoptera*) (Morata and Lawrence, 1979). Therefore, since the wing and leg have very similar open chromatin profiles, one might expect the eye-antennal disc to have an open chromatin profile very similar to the wing and leg.

The open chromatin profile of the eye-antennal disc is indeed very similar to those of the thoracic imaginal discs (Fig. 3A, Fig. 6A,C). For example, many open chromatin regions are held in common between the eye-antennal disc and the thoracic imaginal discs at the *Delta* (*Dl*) locus (Fig. 6B). These similarities in open chromatin occur despite differences in *Dl* expression in these tissues. For example, *Dl* is transcribed in photoreceptors and cells within the morphogenetic furrow of the eye (Parks et al., 1995), whereas it is expressed in rings near the presumptive joints of leg imaginal discs (Bishop et al., 1999), and in stripes near the presumptive veins of wing imaginal discs (de Celis et al., 1997). While there are many similarities in the open chromatin profiles between these imaginal discs, the eye-antennal disc open chromatin profile also deviates from the thoracic disc open chromatin profiles at many locations in the genome (Fig. 6A, B). Many of these differences are found at genes that function in neural cells, particularly regions that are open in the eye-antennal disc but are closed in the thoracic discs (Fig. 6C). This is consistent with the known presence of neural cells in the eye half of the disc. To test this hypothesis, we compared the open chromatin profiles of the eye-antennal disc and the thoracic discs to those of the central nervous system of the same larval stage (late 3rd instar CNS). These data demonstrate that the open chromatin profile from the eye-antennal disc can be reconstructed nearly completely from the profiles of the thoracic discs plus the CNS (Fig. 6A, B). Thus, not all cells at a given developmental stage share the same open chromatin profiles. Instead, open

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chromatin profiles are likely shared by cells with similar identities. We have not yet explored the spatial heterogeneity of the open chromatin profiles within a given body part.

Appendage master regulator transcription factors differentially interpret the same enhancers

If the same set of enhancers is accessible between the developing appendages, how do master regulators such as *Ubx* produce differential gene expression? The *knot* (*kn*) gene is a known *Ubx* target that encodes a transcription factor required for cell fates between L3 and L4 wing veins (Vervoort et al., 1999). In wing imaginal discs, *kn* is expressed at high levels in a wide stripe of cells near the anterior-posterior boundary of the wing pouch, and at lower levels in the wing hinge (Vervoort et al., 1999) (Fig. 7A). In the haltere disc, *kn* is also expressed at low levels in the presumptive hinge region (Fig. 7B), but due to repression by Ubx, *kn* is not expressed in the pouch (Hersh and Carroll, 2005). Despite this difference in expression, the wing and haltere open chromatin profiles at the *kn* locus are identical (Fig. 7A). For example, a previously characterized enhancer that recapitulates *kn* expression specifically in the wing pouch (Hersh and Carroll, 2005) is open in both wing and haltere discs (Fig. 7A, **kn^{wing}**). We cloned a separate open chromatin region from the 4th *kn* intron that is highly accessible in both wing and haltere discs (KN01). Remarkably, the KN01 enhancer has strikingly different patterns of activity in the wing and haltere (Fig. 7B). In the wing, the KN01 enhancer is active in the pouch and hinge, whereas in the haltere, it is active only in the hinge.

A similarly noteworthy result was obtained with an enhancer we identified in this study from the *Dll* gene that is highly open in both wing and haltere discs (Fig. 7C, **DLL04**). Although *Dll* specifies leg identity, it is also required for development of cells near the margin of the wing, where *Dll* is expressed in late 3rd instar larvae (Gorfinkiel et al., 1997) (Fig. 7D). In the haltere, *Ubx* represses *Dll* expression in the center of the disc (Fig. S7C), such that *Dll* is expressed only at the extreme anterior aspect of the pouch (Fig. 7D); in contrast, *Ubx* does not repress *Dll* in the T3 leg disc despite *Ubx* expression because *Dll* is controlled by a different set of regulatory elements in leg discs (Estella et al., 2008; McKay et al., 2009) (Fig. S7C, D, E, Data File S1). Similar to our findings from the *kn* gene, the activity of the DLL04 enhancer in halteres is markedly different from its activity in wings, despite equivalent open chromatin profiles in both tissues (Fig. 7D). Importantly, ChIP data show that both KN01 and DLL04 are specifically bound by Ubx *in vivo* (Choo et al., 2011; Slattery et al., 2011a). These results provide functional evidence that *Ubx* controls haltere morphogenesis by modulating the activity of the set of enhancers utilized in the wing, rather than by creating a haltere-specific set of enhancers.

DISCUSSION

We address a longstanding question in developmental biology: How does a single genome give rise to a diversity of structures? Our results indicate that the unique combination of transcription factors expressed in each thoracic appendage acts upon a shared set of enhancers to create different morphological outputs, rather than operating on a set of enhancers that is specific to each tissue (Fig. 7E). This conclusion is based upon the surprising observation that the open chromatin profiles of the developing appendages are nearly identical at a given developmental stage. Therefore, rather than each master regulator operating on a set of enhancers that is specific to each tissue, the master regulators instead have access to the same set of enhancers in different tissues, which they differentially regulate. We also find that tissues composed of similar combinations of cell types have very similar open chromatin profiles, suggesting that a limited number of distinct open chromatin profiles may exist at a given stage of development, dependent on cell type identity.

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Considerations regarding the sensitivity of FAIRE and the spatial heterogeneity of open chromatin profiles within a given body part

We dissected different tissues from developing flies to compare their open chromatin profiles. These tissues are composed of different cell types, each with its own gene expression profile. Our FAIRE data thus represent the average signal across all cells present in a sample. However, data from embryos and imaginal discs indicate that FAIRE is a very sensitive detector of functional DNA regulatory elements. For example, the Dll01 enhancer is active in 2–4 neurons of the leg imaginal disc, yet the FAIRE signal at Dll01 is as strong as the Dll04 enhancer, which is active in hundreds of cells of the wing pouch (Figure 7B, D, Data File S1). Thus, FAIRE may detect nearly all of the DNA regulatory elements that are in use among the cells of an imaginal disc. Our study does not rule out the existence of DNA regulatory elements that are not marked by open chromatin or otherwise not detected by FAIRE.

Despite this sensitivity, our approach does not identify which cells within the tissue have a particular open chromatin profile. For a given locus, it is possible that all cells in the tissue share a single open chromatin profile, or that the FAIRE signal originates from only a subset of cells in which a given enhancer is active. Our comparisons between eye-antennal discs, larval CNS, and thoracic discs (Fig. 3A, Fig. 6) suggest that the latter scenario is most likely, with open chromatin profiles among cells within a tissue shared by cells with similar identities at a given developmental stage.

Differential regulation of a shared set of enhancers as a mechanism of generating morphological diversity

Our observation that halteres and wings share open chromatin profiles demonstrates that Hox proteins like Ubx can differentially interpret the DNA sequence within the same subset of enhancers to modify one structure into another. This is consistent with the idea that morphological differences are largely dependent on the precise location, duration, and magnitude of expression of similar genes (Crickmore and Mann, 2006; Weatherbee et al., 1998), and it is further supported by the similarity in gene expression profiles observed between *Drosophila* appendages (Klebes et al., 2002) (Fig. S4), and observed between vertebrate limbs (Taher et al., 2011). However, it was not known that such dramatic differences in morphology could be achieved by using the same subset of DNA regulatory modules in different tissues genome-wide. Our findings provide a molecular framework to support the hypothesis that Hox factors function as “versatile generalists”, rather than stable binary switches (Akam, 1998). The similarity in open chromatin profiles between wings and legs suggests that this framework also extends to other classes of master regulators beyond the Hox genes. We also note that, like the *Drosophila* appendages, vertebrate limbs are composed of similar combinations of cell types that differ in their pattern of organization. Moreover, the *Drosophila* appendage master regulators share a common evolutionary origin with the master regulators of vertebrate limb development (Mann and Carroll, 2002), suggesting that the concept of shared open chromatin profiles may also apply to human development.

Our data suggest that open chromatin profiles vary both over time for a given lineage and between cell types at a given stage of development. Given the dramatic differences in the FAIRE landscape observed during embryogenesis, and between the CNS and the appendage imaginal discs during larval stages, it appears as though the alteration of the chromatin landscape is especially important for specifying different cell types from a single genome. After cell-type specification, open chromatin profiles in the appendages continued to change as they proceeded toward terminal differentiation, suggesting that stage-specific functions require significant opening of new sites or the closing of existing sites. These findings

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contrast with those investigating hormone-induced changes in chromatin accessibility (John et al., 2011), in which the majority of open chromatin sites did not change after hormone treatment, including sites of *de novo* hormone-receptor binding. Thus, it may be that genome-wide remodeling of chromatin accessibility is reserved for the longer time-scales and eventual permanence of developmental processes rather than the shorter time-scales of environmental responses.

What determines the appendage open chromatin profiles?

Different combinations of "master regulator" transcription factors, often termed selector genes, are expressed in the developing appendages. Selectors are thought to specify the identity of distinct regions of developing animals by regulating the expression of transcription factors, signaling pathway components, and other genes that act as effectors of identity (Mann and Carroll, 2002). One property attributed to selectors to explain their unique power to specify identity during development is the ability to act as pioneer transcription factors (Budry et al., 2012; Fakhouri et al., 2010). In such models, selectors are the first factors to bind target genes; once bound, selectors then create a permissive chromatin environment for other transcription factors to bind. Our finding that the same set of enhancers are accessible for use in all three appendages, with the exception of the enhancers that control expression of the selector genes themselves and other primary determinants of appendage identity, suggests that the selectors expressed in each appendage do not absolutely control the chromatin accessibility profile; otherwise, the haltere chromatin profile (for example) would differ from that of the wing due to the expression of Ubx.

What then determines the appendage open chromatin profiles? Since open chromatin is likely a consequence of transcription factor binding, two non-exclusive models are possible. First, different combinations of transcription factors could specify the same open chromatin profiles. In this scenario, each appendage's selectors would bind to the same enhancers across the genome. For example, the wing selector Vg, with its DNA binding partner Sd, would bind the same enhancers in the wing as Dll and Sp1 bind in the leg. In the second model, transcription factors other than the selectors could specify the appendage open chromatin profiles. Selector genes are a small fraction of the total number of transcription factors expressed in the appendages (Fig. S5). Many of the non-selector transcription factors are expressed at similar levels in each appendage, and thermodynamic models would predict them to bind the same enhancers (Biggin, 2011). This model could also help to explain how the appendage open chromatin profiles coordinately change over developmental time despite the steady expression of the appendage selector genes during this same period. It is possible that stage-specific transcription factors determine which enhancers are accessible at a given stage of development. This would help to explain the temporal specificity of target genes observed for selectors such as Ubx (Pavlopoulos and Akam, 2011). Recent work supports the role of hormone-dependent transcription factors in specifying the temporal identity of target genes in the developing appendages (Mou et al., 2012). Further experiments, including ChIP of the selectors from each of the appendages, will be required to determine the extent to which either of these models is correct.

What determines the differential activity of enhancers in different appendages?

We show that binding of Ubx results in differential activity of enhancers in the haltere imaginal disc relative to the wing, despite equivalent accessibility of the enhancers in both discs, indicating that master regulators control morphogenesis by differentially regulating the activity of the same set of enhancers. It is likely that functional specificity of enhancers is achieved through multiple mechanisms. These include differential recruitment of co-activators and co-repressors, modulation of binding specificity through interactions with co-

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factors (Slattery et al., 2011b), differential utilization of binding sites within a single enhancer (Bradley et al., 2010), or regulation of binding dynamics through an altered chromatin context (Lickwar et al., 2012). This last mechanism would allow for epigenetic modifications early in development to impact subsequent gene regulatory events. For example, the activity of *Ubx* enhancers in the early embryo (Fig. 3C) may control recruitment of Trithorax or Polycomb complexes to the PREs within the *Ubx* locus, which then maintain *Ubx* in the ON or OFF state at subsequent stages of development (Papp and Muller, 2006; Pirrotta et al., 1995). Consistent with this model, *Ubx* enhancers active in the early embryo are only accessible in our 2–4 hr timepoint, whereas the accessibility of *Ubx* PREs varies little across developmental time or between tissues at a given developmental stage.

Evolutionary significance

Our results also have implications for the evolution of morphological diversity. Halteres and wings are considered to have a common evolutionary origin, but the relationship between insect wings and legs is unresolved (Averof and Cohen, 1997; Jockusch and Ober, 2004). Our observation that wings and legs share open chromatin profiles supports the hypothesis that wings and legs also share a common evolutionary origin in flies. Since legs appear in the fossil record before wings, the similarity in their open chromatin profiles suggests that the existing leg *cis*-regulatory network was co-opted for use in creation of dorsal appendages during insect evolution.

EXPERIMENTAL PROCEDURES

RNA and FAIRE sample collections

Drosophila strains were grown and collected as previously reported (Agelopoulos et al., 2012; Estella et al., 2008). RNA-seq and FAIRE-seq experiments were performed essentially as described (Simon et al., 2012). See Supplementary Experimental Procedures for details.

Sequence data analysis

FAIRE-seq data were processed essentially as previously described (Simon et al., 2012). FAIRE signal was converted to z-scores: genomic DNA signal (normalized to read depth) was subtracted from FAIRE signal (normalized to read depth) at each base, and z-scores were generated at each base by calculating the mean and standard deviation of the FAIRE base coverage signal for individual chromosome arms, subtracting the mean signal from the signal at each base on the given chromosome arm, and dividing by the standard deviation. FAIRE and DNaseI peaks were called with MACS2 (Zhang et al., 2008). Hierarchical clustering and principal component analysis was performed with Cluster 3.0 (de Hoon et al., 2004). RNA-seq data were aligned to the reference genome (dm3) using TopHat (version 1.1.4), and assembled into transcripts with Cufflinks (Trapnell et al., 2009) (version 0.9.3). Differential gene expression calls were made with Cuffdiff (version 0.9.3), as outlined in Fig. S4. The UCSC genome browser was used to visualize data (Kent et al., 2002) (http://genome.ucsc.edu). See Supplementary Experimental Procedures for details. Data has been deposited in the Gene Expression Omnibus under accession numbers GSE38727. Included in the dataset are raw sequencing reads, processed FAIRE signal tracks, FAIRE peaks calls, and RNA-seq FPKM values.

Defining regions of differential open chromatin in appendages

For the analysis shown in Figures S2, S5, and Tables S2 and S3, we focused on the most pronounced open chromatin regions because we hypothesized that these would be more

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likely to be associated with regulatory activity. We reasoned that DNA regulatory modules that are most likely to have mutually exclusive activity between appendages would exhibit large-scale differences in the degree to which they are open. Therefore, we defined a peak as differentially open if it was within the top 20% of FAIRE peaks (ranked by their MACS q-values) from the first sample, and did not intersect with a peak in the top 60% from the second sample. The number of FAIRE peaks in each of the two datasets being compared was kept equal for each comparison. See Fig. S2 for details.

For details on data processing, enhancer cloning, and immunofluorescence experiments, see Supplemental Experimental Procedures

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Open chromatin accurately predicts enhancer activity in developing animals
- *Drosophila* appendages use the same set of enhancers at a given developmental stage
- Appendage open chromatin profiles change coordinately over developmental time
- Master regulators differentially influence the same enhancers among appendages

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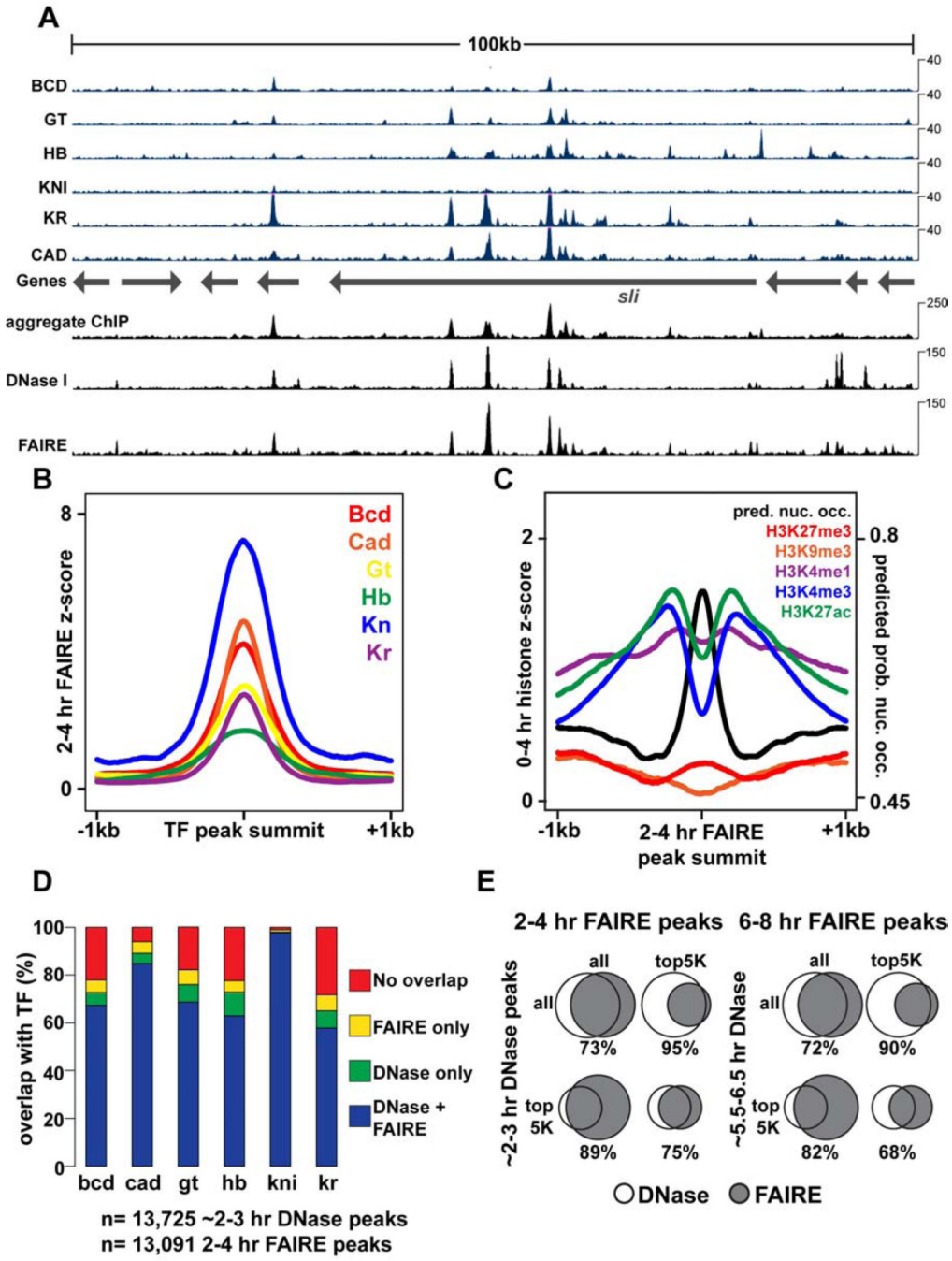

Figure 1. FAIRE identifies open chromatin bound by key developmental regulators

All times below refer to hours After Egg Laying (AEL), which are estimated for data from other studies. DNase I data are from (Thomas et al., 2011). 2–3 hr ChIP data are from (Bradley et al., 2010). Transcription Factors (TFs): Bcd, Bicoid; Cad, Caudal; Gt, Giant; Hb, Hunchback; Kn, Knirps; Kr, Kruppel. (**A**) Browser representation of the *slit* locus. Below the genes track is ChIP signal (blue, Counts Per Million (CPM)) from 2–3 hr embryos, plotted for individual TFs. Above the genes track, from bottom to top, is the aggregate ChIP signal generated by summing the normalized signal from each individual TF, followed by 2–3 hr DNase I signal (CPM), and 2–4 hr FAIRE data (CPM). (**B**) 2–4 hr FAIRE signal at TF

peaks from 2–3 hr embryos. (**C**) 0–4hr histone modification signals (Negre et al., 2011) and predicted probability of nucleosome occupancy based on DNA sequence (Kaplan et al., 2009) plotted for regions surrounding 2–4 hr AEL FAIRE peaks, centered on the maximum FAIRE signal for each peak. (**D**) Stacked bar charts showing overlap of ~2–3 hr DNase I and 2–4 hr FAIRE peaks with TF ChIP peaks from 2–3 hr embryos. (**E**) Venn diagrams depicting peak overlaps between ~2–3 hr DNase I peaks and 2–4 hr FAIRE peaks (left), and ~5.5–6.5 hr DNase I and 6–8 hr FAIRE peaks (right). See also Figure S1.

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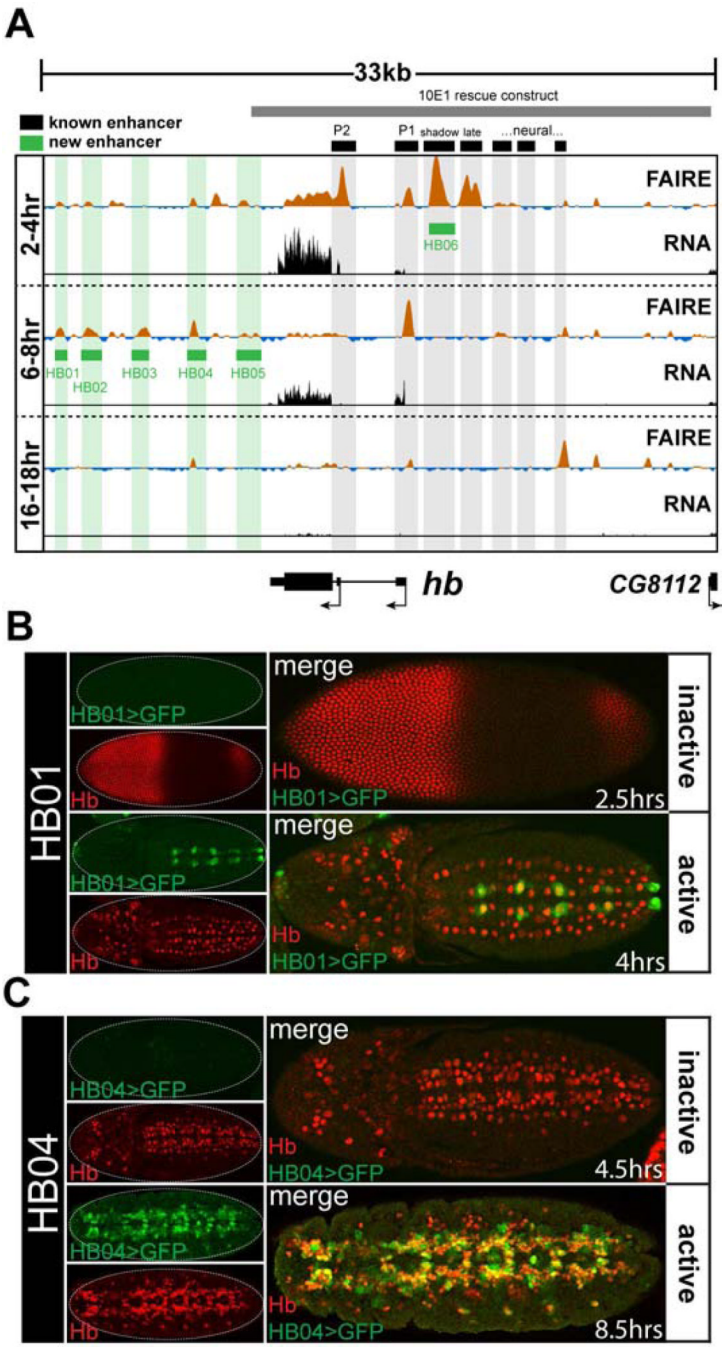

Figure 2. FAIRE signal accurately predicts enhancer activity

(**A**) FAIRE and RNA signals at the *hunchback* (*hb*) locus in embryos. Black boxes designate the locations of known enhancers: (left to right) P2 promoter, P1 promoter, blastoderm shadow enhancer, late blastoderm enhancer, and recently identified neural enhancers (Gallo et al., 2011; Hirono et al., 2012; Margolis et al., 1995; Perry et al., 2011). Green boxes designate enhancers that were identified and cloned in this study. The grey box indicates the boundaries of the 10E1 transgenic *hb* rescue construct (Margolis et al., 1995). (**B, C**) Confocal images of embryos from two transgenic lines (HB01, HB04) stained with antibodies for Hb (red) and GFP (green) protein. The estimated age of each embryo is

indicated. The timing of chromatin opening coincides with timing of reporter activity. See also Table S1 and Data File S1.

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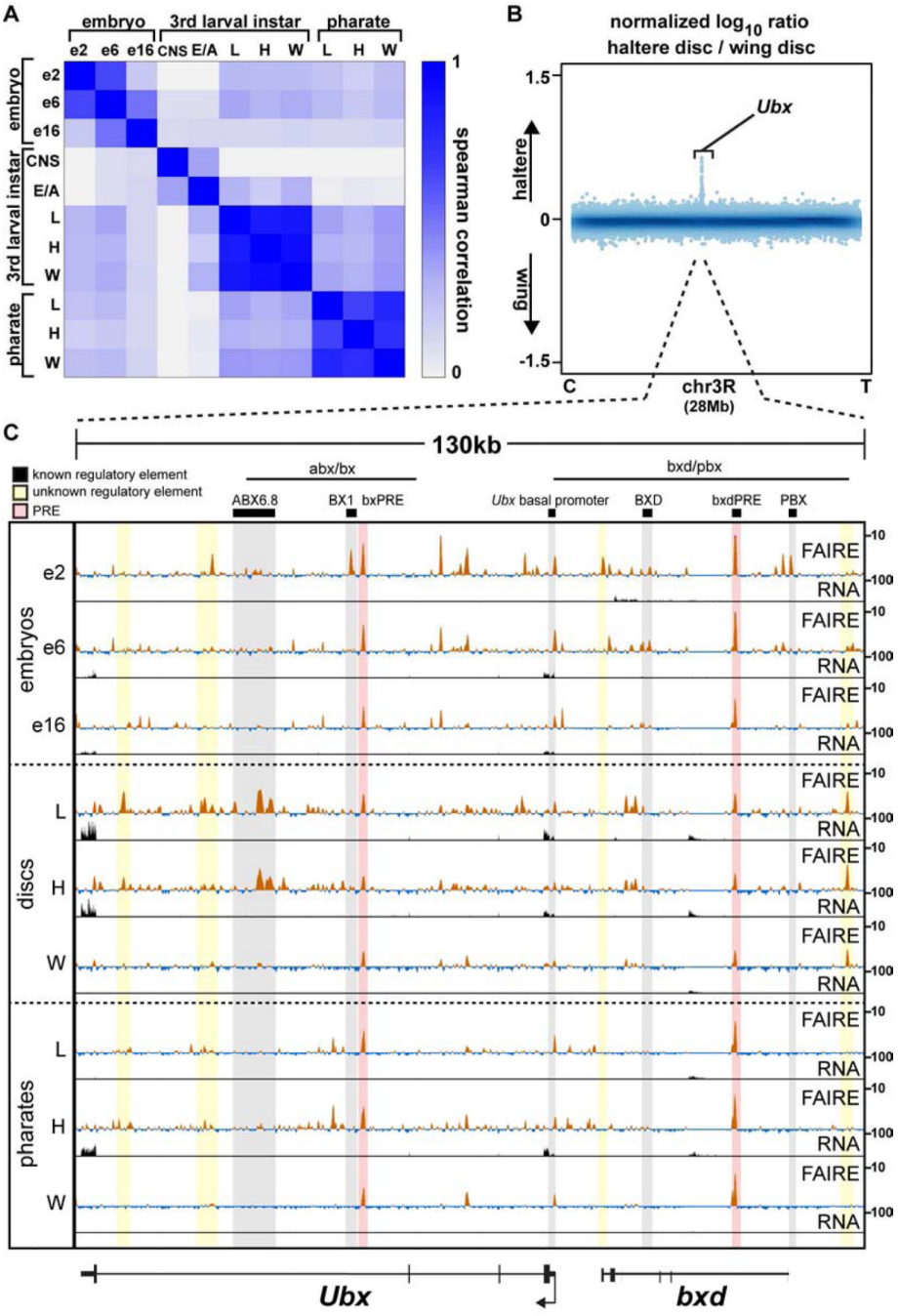

Figure 3. Appendage open chromatin profiles are very similar within a stage, except at master regulator loci

(**A**) Spearman correlation coefficients of FAIRE signal in 500-bp windows genome-wide for each pairwise comparison across all samples. (**B**) Log_{10} ratio (haltere/wing) of FAIRE signal from chromosome 3R (28Mb). Centromere (C), telomere (T), and the *Ultrabithorax* (*Ubx*) locus are indicated. (**C**) FAIRE (z score: −2 to 10) and RNA (FPKM: 0 to 100) signals at the *Ubx* and *bithoraxoid* (*bxd*) loci in embryos, imaginal discs, and pharate appendages. Horizontal black lines indicate the locations known *Ubx* regulatory regions (Simon et al., 1990). Black boxes designate the locations of known DNA regulatory elements: (left to right) ABX6.8 enhancer, BX1 enhancer, bxPRE, *Ubx* basal promoter,

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BXD enhancer, bxdPRE, PBX enhancer (Chan et al., 1994; Muller and Bienz, 1991; Pirrotta et al., 1995; Qian et al., 1991; Simon et al., 1990; Zhang et al., 1991). Shaded red rectangles indicate the locations of known PREs (Papp and Muller, 2006; Pirrotta et al., 1995). Shaded yellow rectangles indicate the locations of putative regulatory elements identified in this study. See also Figures S2, S3, S4.

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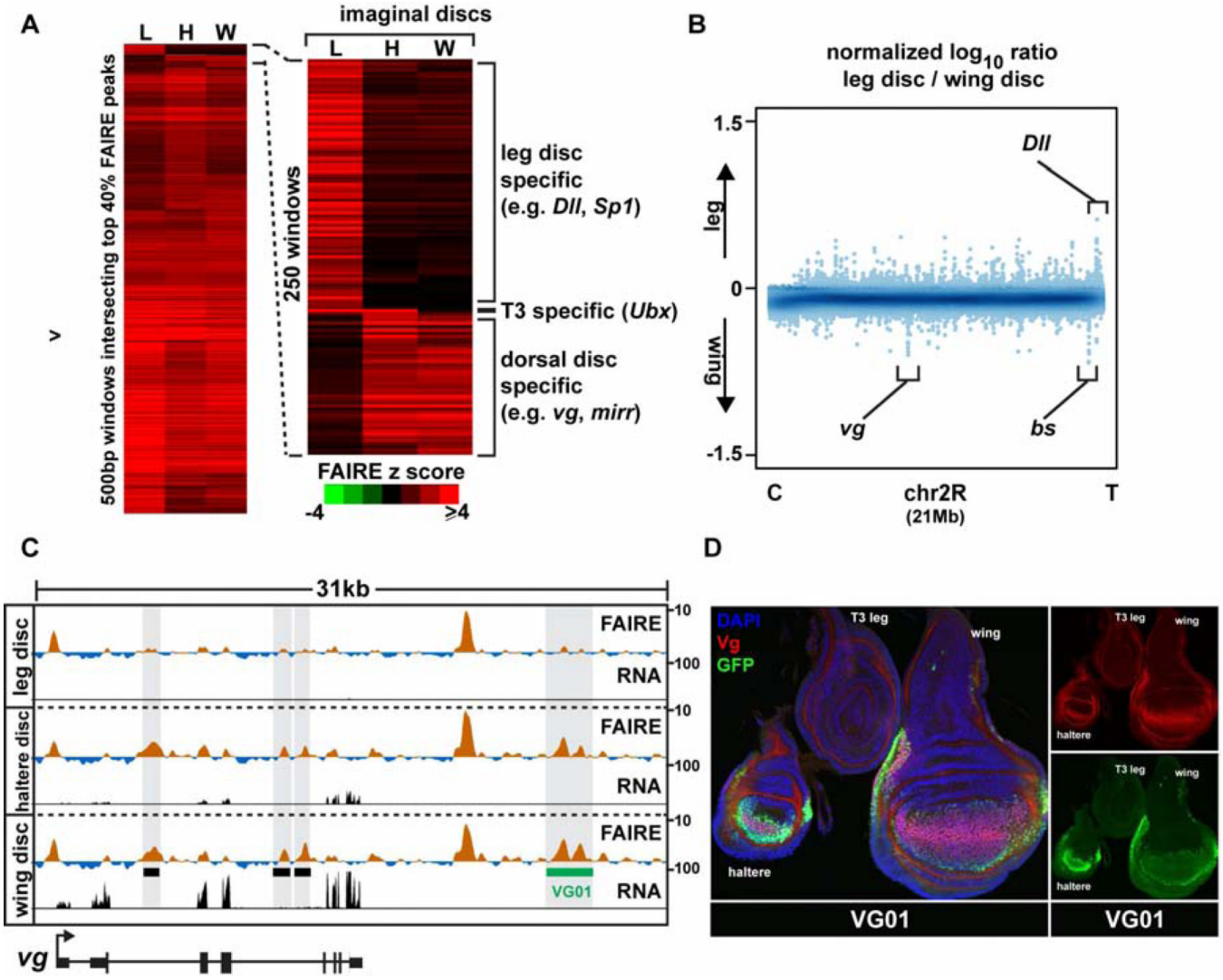

Figure 4. Appendage open chromatin profiles differ primarily at loci of key developmental regulators

(**A**) Hierarchical clustering of FAIRE signal from windows intersecting the top 7,000 imaginal disc peaks. Right, zoom-in of the most variable windows. (**B**) Log_{10} ratio (leg/wing) of FAIRE signal from chromosome 2R (21 Mb). Loci encoding key transcription factors are indicated. (**C**) Browser representation of the *vg* locus showing FAIRE (z score: −2 to 10) and RNA (FPKM: 0 to 100) signals in imaginal discs. Black boxes designate the locations of known enhancers: (left to right) boundary, vgAME, and quadrant enhancers (Kim et al., 1996; Stergachis et al., 2013; Williams et al., 1994). The green box designates the newly-cloned VG01 enhancer, which is active in the wing and haltere but not the leg. (**D**) Confocal images of imaginal discs from the VG01 transgenic line, stained with DAPI (blue), and antibodies for GFP (green), and Vg (red). The VG01 enhancer recapitulates *vg* expression in haltere and wing imaginal discs, and lack of expression in the leg disc. See also Figure S5, and Table S2.

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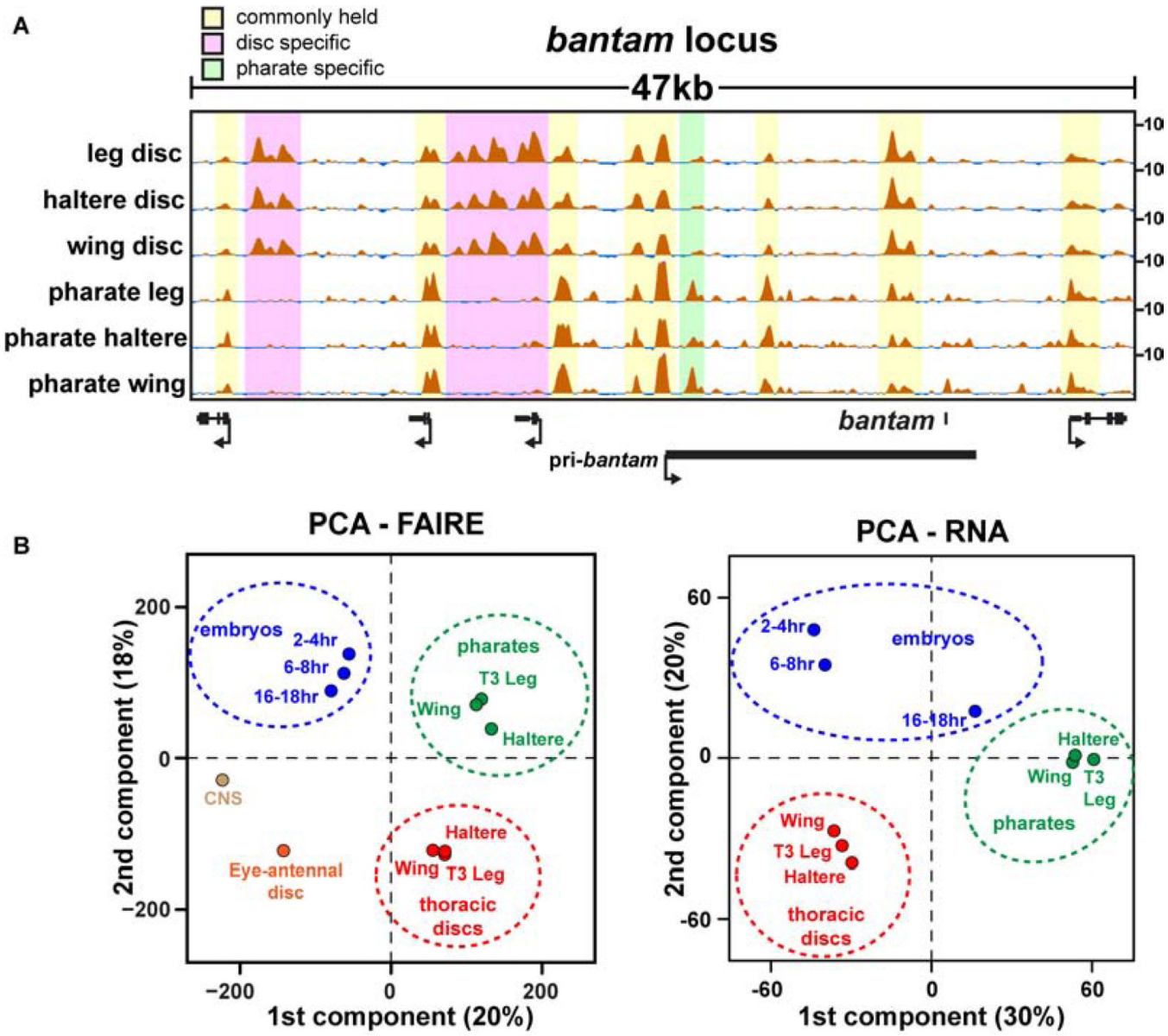

Figure 5. Different appendages are more similar to each other at a given timepoint than they are to their own cellular progeny at a later timepoint

(**A**) FAIRE signal surrounding the *bantam* locus from imaginal discs and pharate appendages. (**B**) Plots of PCA scores for the first two components from principal component analysis (PCA) of FAIRE and RNA signals. The percentage of the total variance represented by each component is shown in parentheses. See also Figure S6, and Table S2.

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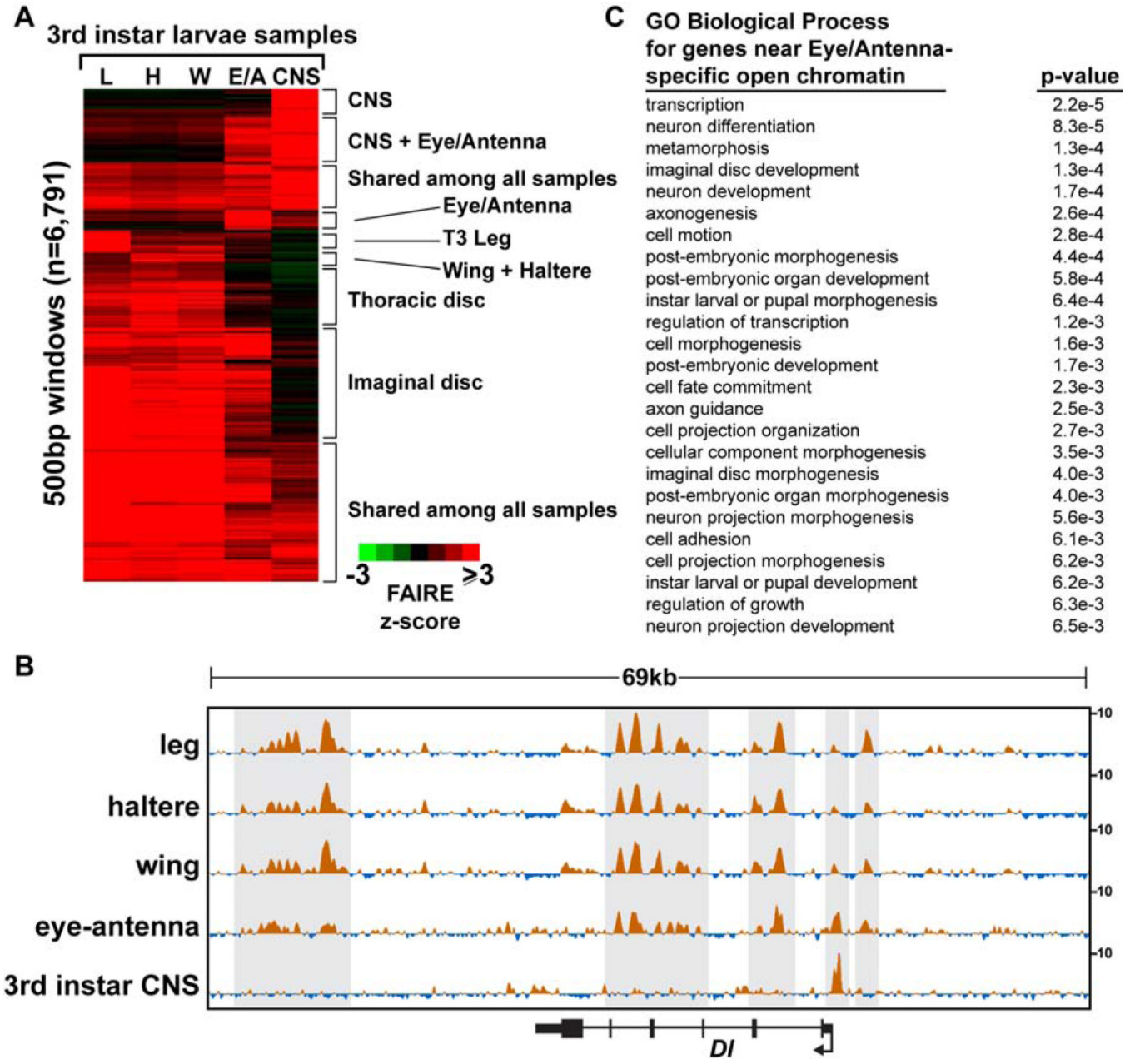

Figure 6. Eye-antennal open chromatin profiles share features with appendage and CNS open chromatin profiles

(**A**) Hierarchical clustering of FAIRE signal in windows intersecting the union set of top 5K FAIRE peaks from third instar larval samples. The eye-antennal signal can be reconstructed nearly completely from the profiles of the thoracic discs plus the CNS. (**B**) Browser representations of the *Delta* (*Dl*) locus, a gene with known roles in 3rd instar imaginal discs and CNS (see text). Note the eye-antennal signal shares features with both the thoracic discs and the CNS. (**C**) Gene ontology terms of the genes nearest to peaks that are present in eyeantennal discs but not present in the thoracic imaginal discs. Genes with neural cell functions are enriched. The Bonferroni corrected p-value is shown.

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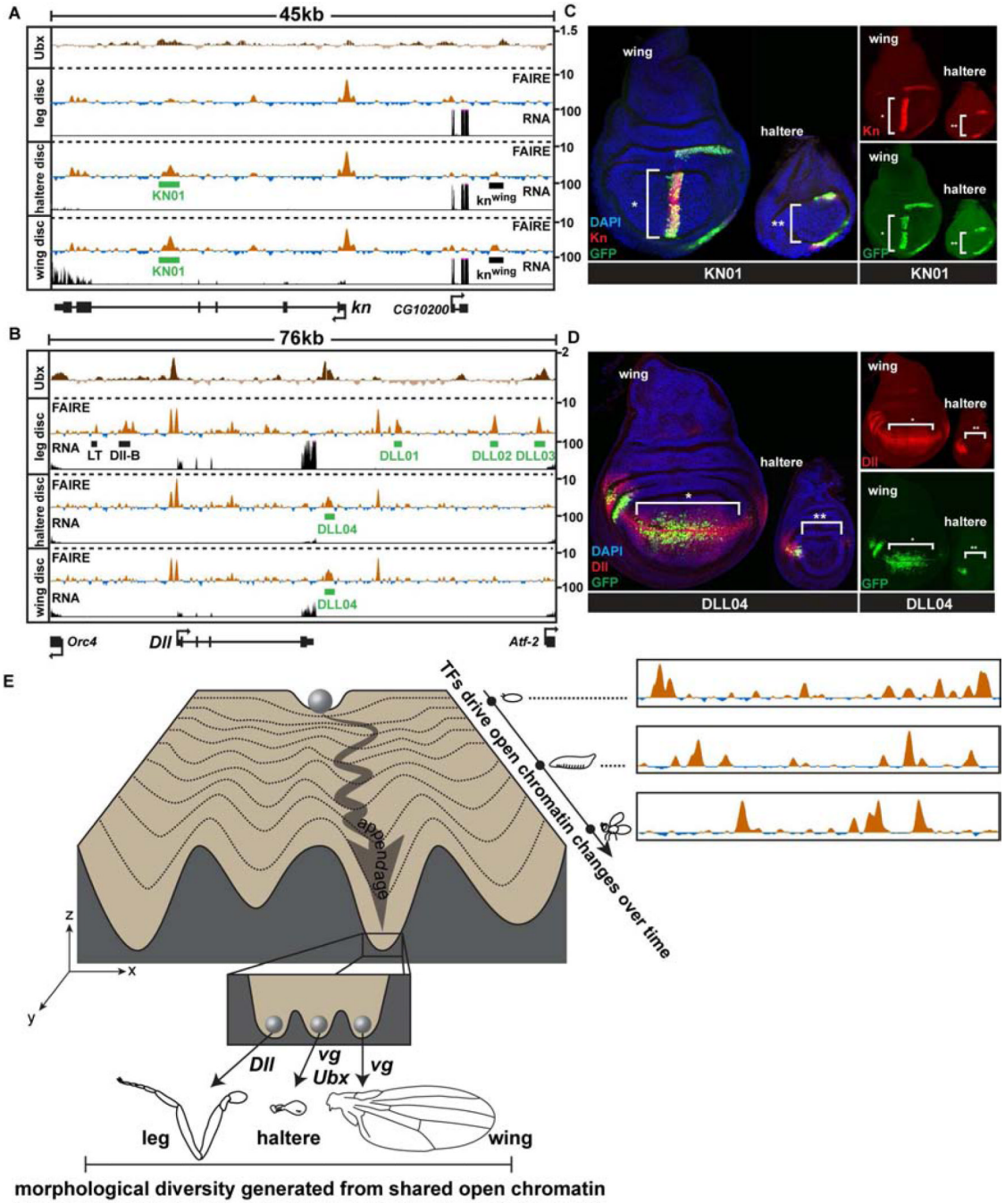

Figure 7. Transcription factors differentially regulate the activity of the same enhancers in different appendages

(**A**) FAIRE (z score), RNA (CPM), and Ubx ChIP (log_2 ratio) (Choo et al., 2011; Slattery et al., 2011a) signal at the *knot* (*kn*) and (**B**) *Distalless* (*Dll*) loci in imaginal discs, with locations of enhancers KN01 and DLL01-04 (green boxes) identified in this study, plotted as in Figure 3C. (**C**) Confocal images showing reporter activity of KN01 and (**D**) DLL04 in wing and haltere imaginal discs. Discs were stained for DAPI (blue), and antibodies to GFP (green), and Kn (**C**) or Dll (**D**) (red). (**E**) A conceptual model of the appendage shared open chromatin profiles, depicted within the framework of Waddington's epigenetic landscape (Waddington, 1957). A range of open chromatin profiles exists within the fly (x-axis) at any

single stage of development. These profiles are dynamic over time (y-axis), and differ by varying degrees between tissues (z-axis). Each valley (z-axis) represents the shared open chromatin profile of a developing anatomical structure or tissue (e.g. appendage). The inset depicts the specific group of selector genes expressed in each developing appendage acting upon the same set of open chromatin regions to create morphologically diverse tissues. See also Figure S7.

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