© 2017. Published by The Company of Biologists Ltd | Development (2017) 144, 3095-3101 doi:10.1242/dev.149427

RESEARCH REPORT

Functional regulatory evolution outside of the minimal *even-skipped* stripe 2 enhancer

Justin Crocker*,‡ and David L. Stern

ABSTRACT

Transcriptional enhancers are regions of DNA that drive precise patterns of gene expression. Although many studies have elucidated how individual enhancers can evolve, most of this work has focused on what are called 'minimal' enhancers, the smallest DNA regions that drive expression that approximates an aspect of native gene expression. Here, we explore how the *Drosophila erecta even-skipped* (*eve*) locus has evolved by testing its activity in the divergent *D. melanogaster* genome. We found, as has been reported previously, that the *D. erecta eve* stripe 2 enhancer (*eveS2*) fails to drive appreciable expression in *D. melanogaster*. However, we found that a large transgene carrying the entire *D. erecta eve* locus drives normal *eve* expression, including in stripe 2. We performed a functional dissection of the region upstream of the *D. erecta eveS2* region and found multiple Zelda motifs that are required for normal expression. Our results illustrate how sequences outside of minimal enhancer regions can evolve functionally through mechanisms other than changes in transcription factor-binding sites that drive patterning.

KEY WORDS: Evolution, Transcription, Enhancer, *Drosophila*, *Even-skipped*

INTRODUCTION

Developmental enhancers contain multiple binding sites for transcription factors, together specifying the precise time, level and location of gene expression. Classically, minimal enhancers have been identified as the smallest DNA fragments that are sufficient to direct reporter-gene expression in a particular tissue or domain of normal gene expression (Crocker and Stern, 2013). These studies have provided mechanistic insight into how transcriptional logic is encoded in individual enhancers (Spitz and Furlong, 2012; Levo and Segal, 2014). However, in even the earliest studies, it was clear that minimal enhancers are insufficient to define the normal gene expression pattern with complete fidelity (Small et al., 1992). More recently, genomic studies have provided evidence that minimal enhancers are embedded within larger regions containing additional transcription factor-binding sites that may be required for normal enhancer function (Ludwig et al., 2011; Adkins et al., 2016).

Phenotypic evolution results largely from sequence changes in enhancers (Levine, 2010; Prescott et al., 2015), even between closely related species (MacArthur and Brookfield, 2004; Ludwig and Kreitman, 1995; Nord et al., 2013; Crocker et al., 2008, 2010, 2015b; Rebeiz et al., 2009). It is not clear, however, how often functional evolution includes changes within minimal enhancers versus outside of these regions. We have explored this problem through studies of the *even-skipped* (*eve*) gene.

The *Drosophila melanogaster eve* gene is expressed in seven transverse stripes along the anterior-posterior axis in the blastoderm embryo (Macdonald et al., 1986; Ilsley et al., 2013). Minimal enhancers have been identified that each direct expression in either one or two stripes and that together drive expression in all seven stripes (Fujioka et al., 1999). Of all these enhancers, the minimal element for stripe 2 has been studied in the greatest detail (Stanojevic et al., 1991; Arnosti et al., 1996; Small et al., 1992). This enhancer contains multiple binding sites for transcriptional activators (Bicoid and Hunchback) and repressors (Giant, Krüppel, and Sloppy-paired). The collective activity of transcription factor binding to these sites drives *eve* expression specifically in stripe 2 (Small et al., 1992; Ilsley et al., 2013; Arnosti et al., 1996).

Previously, reporter gene assays were used to investigate the functional evolution of *eveS2* from three divergent *Drosophila* species with transgenic assays in *D. melanogaster* (Ludwig et al., 2000, 2005; Ludwig et al., 1998). These studies revealed that the *eveS2* enhancers from *D. yakuba* and *D. pseudoobscura*, which diverged ∼10 and ∼40 million years ago, respectively, from *D. melanogaster*, drove apparently normal expression in stripe 2. However, *eveS2* from *D. erecta*, which is closely related to *D. yakuba*, failed to drive appreciable levels of expression.

There are several possible reasons for why the *D. erecta eveS2* element does not drive appreciable expression in *D. melanogaster* (Ludwig et al., 2005). First, the 'minimal' functional *D. erecta eveS2* element might have been replaced elsewhere in the *D. erecta eve* locus with a functionally equivalent enhancer. Second, the *D. erecta* enhancer may contain all the required information to drive native expression, but the enhancer might have evolved to accommodate differences in the *D. erecta* embryonic environment (Barrière et al., 2012; Fowlkes et al., 2011) – for example, differences in transcription factor concentrations (Crocker et al., 2008; Chahda et al., 2013). Third, the true functional *eveS2* enhancer might be larger than defined by the *D. melanogaster* minimal element and shifts in the locations of key transcription factor-binding sites may have rendered the *D. erecta* region corresponding to the *D. melanogaster* 'minimal' element unable to drive appropriate expression.

Here, we tested these hypotheses with a functional dissection of the *D. erecta eve* locus in transgenic *D. melanogaster*. We found that a large transgene carrying the entire *D. erecta eve* locus drove normal expression in all seven stripes. This *D. erecta* transgene rescued downstream *eve* targets and larval segmentation defects caused by an *eve* null mutation. We found that regulatory information required for *D. erecta* stripe 2 expression is located outside of the minimal stripe 2 enhancer region. Finally, we found

Janelia Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147, USA.
*Present address: European Molecular Biology Laboratory, D-69117 Heidelberg, Germany.

‡Author for correspondence (justin.crocker@embl.de)

J.C., 0000-0002-5113-0476

Received 19 January 2017; Accepted 19 July 2017

Development (2017) 144, 3095-3101 doi:10.1242/dev.149427

that the *D. erecta* minimal *eveS2* region lacks multiple Zelda transcription factor-binding sites that are found in *D. melanogaster* and we demonstrate that normal function of the *D. erecta eveS2* enhancer requires Zelda transcription factor-binding motifs located outside of the minimal enhancer element. Zelda is apparently not required for patterning, but instead for making enhancers accessible for regulation (Xu et al., 2014; Foo et al., 2014; Liang et al., 2008; Harrison et al. 2011; Nien et al., 2011; Li et al., 2014; Sun et al., 2015; Schulz et al., 2015). Many studies have suggested that the transcription factor-binding sites required for normal enhancer function are often distributed over regions larger than experimentally determined minimal enhancer elements and our results demonstrate that crucial transcription factor-binding sites can shift during evolution between locations within and outside of 'minimal' regions.

RESULTS AND DISCUSSION

D. melanogaster and *D. erecta* both express *eve* in seven embryonic stripes at similar levels and locations (Fig. 1A,B). A transgene of the *D. melanogaster eveS2* minimal enhancer, when re-introduced into *D. melanogaster*, drives robust expression in approximately the same region as the native expression of *eve* stripe 2 (Fig. 1C). However, the orthologous *eveS2* fragment from *D. erecta* does not drive expression in transgenic *D. melanogaster* (Fig. 1D) (see also Ludwig et al., 2005). A 20 kb region of the *D. melanogaster* genome surrounding the *eve* transcription unit is sufficient to drive apparently normal *eve* expression and rescues transcription of genes that are normally regulated by Eve (Fujioka et al., 1999). To maximize the likelihood that we would capture the entire *eve* locus from *D. erecta*, we tested the ability of a ~47 kb *D. erecta* fosmid to drive expression in *D. melanogaster* embryos deficient for native

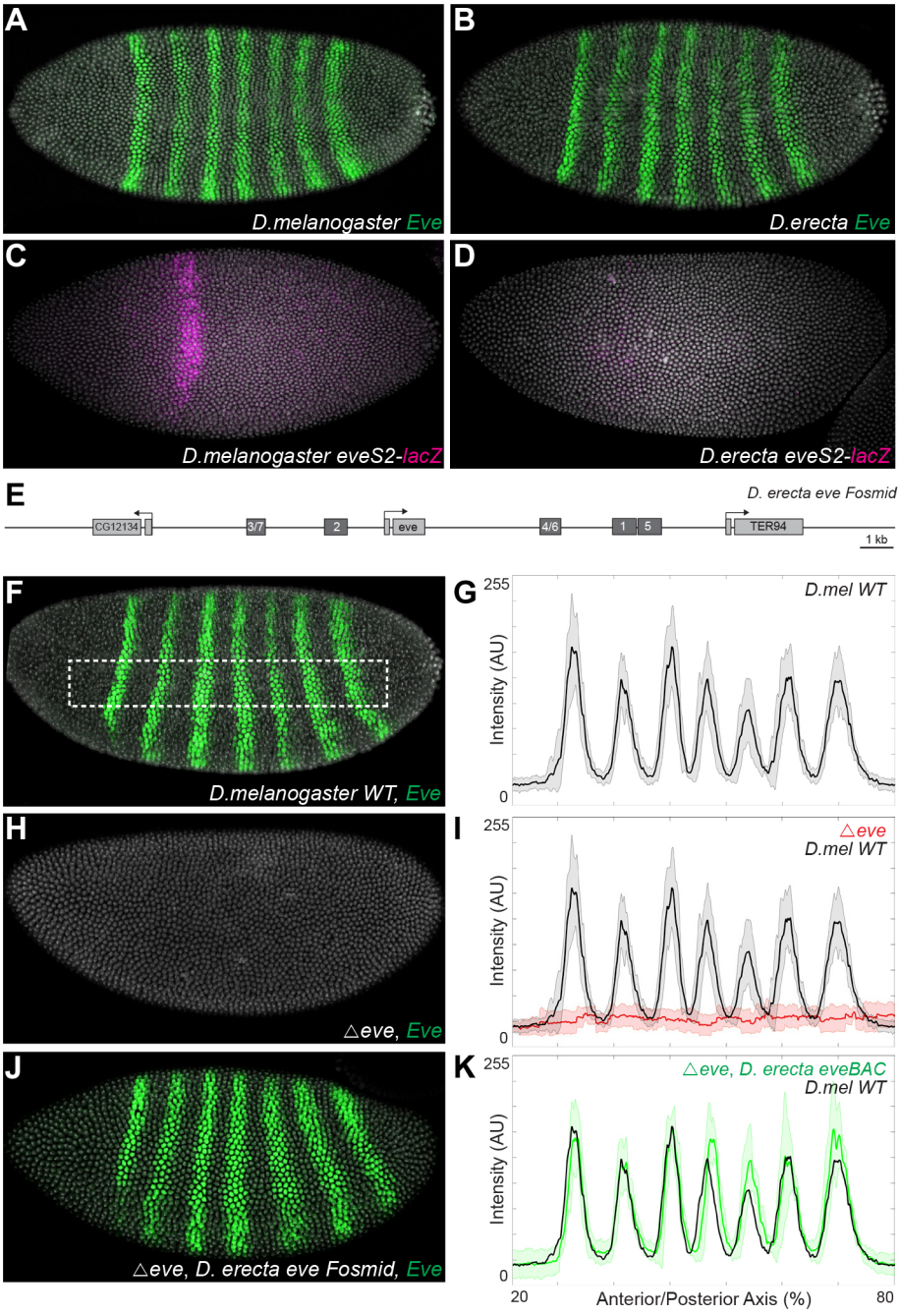

Fig. 1. The *D. erecta eve* locus drives normal *eve* expression in transgenic *D. melanogaster*. (A,B) Stage 5 embryos stained for Eve protein in either *D. melanogaster* (A) or *D. erecta* (B). (C,D) Stage 5 *D. melanogaster* embryos stained for stained for *lacZ* enhancer-driven RNA expression and carrying the *D. melanogaster eveS2* (C) or *D. erecta eveS2* (D). (E) Schematic of the *eve* locus with enhancers indicated by numbered boxes. (F,H,J) Stage 5 embryos stained for Eve protein in either a wild-type background (F), *eve* null background (h), or an *eve* null background carrying the *D. erecta eve* fosmid (J). (G,I,K) Profiles of average expression levels across the region indicated in F for the indicated genotype (n=10 for each genotype). In all plots, the solid black line denotes wild type, red denotes eve null (I), and green denotes an *eve* null background carrying the *D. erecta eve* fosmid (K). Bounding areas around experimental data indicate one standard deviation. AU, arbitrary units of fluorescence intensity.

Development (2017) 144, 3095-3101 doi:10.1242/dev.149427

eve function. This fosmid contains *D. erecta* sequences orthologous to all of the *D. melanogaster* stripe enhancers (Fig. 1E).

We found that this *D. erecta* fosmid drove apparently normal expression of all seven *eve* stripes (Fig. 1F-K). To determine whether the timing, levels and spatial distribution of Eve expression driven by the *D. erecta* fosmid were correct in *D. melanogaster*, we tested the ability of the *D. erecta* locus to functionally rescue gene regulatory networks that are normally regulated by Eve. We found that the *D. erecta eve* fosmid rescued expression of the segment polarity gene *engrailed* (Fig. 2A-C). Additionally, the fosmid rescued all segmentation defects in the cuticles of larvae deficient for *eve* (Fig. 2D-F). Finally, we found that the *D. erecta* fosmid rescued most flies to adult viability (86%, ±2.5 s.d., n=3), which is similar to rescue levels generated by the 20 kb *D. melanogaster eve* rescue construct (Ludwig et al., 2011). Together, these results suggest that the 47 kb *D. erecta* fosmid contains all of the regulatory information required for *eve* expression in stripe 2 and all other *eve* stripes.

The regulatory information for *D. melanogaster eve* stripe 2 and stripes 3/7 is located upstream of the *eve* promoter (Arnosti et al., 1996; Small et al., 1991, 1992, 1996). Increasing the size of the DNA regions tested for these enhancers increases levels of transcription (Arnosti et al., 1996; Small et al., 1991, 1992, 1996). Additionally, although the minimal *D. melanogaster* enhancer is sufficient to drive approximately normal expression of *eve* stripe 2, sequences surrounding the minimal *eveS2* element contribute to the robustness of the minimal enhancer (Ludwig et al., 2011). Therefore, we suspected that sequences near the *D. erecta eveS2* might be required for normal expression.

To test whether regulatory information 5′ of the *D. erecta eveS2* region is required for normal stripe 2 expression, we tested a series of constructs that included the minimal stripe 2 enhancer plus progressively more 5′ DNA (Fig. 3A). Whereas the 855 bp *D. erecta eveS2* construct drove very little expression in *D. melanogaster* (Fig. 3B,C), a fragment containing an additional 832 bp upstream of the minimal enhancer drove weak expression in the stripe 2 domain (Fig. 3D,E). We found that the expression levels driven by this larger *D. erecta* construct were similar to expression levels driven by the minimal *D. melanogaster eveS2* element (Fig. S1). Increasing the *D. erecta eveS2* construct size with an additional 1609 bp 5′ of the enhancer, up to the boundary with the minimal stripe 3/7 enhancer, further increased levels of stripe 2 expression and drove weak expression in stripes 3 and 7 (Fig. 3F,G). This indicates both that information critical for stripe 2 expression resides outside the minimal stripe 2 region in *D. erecta*, and that patterning information for stripes 3 and 7 resides outside of the minimal stripe 3/7 enhancer. Increasing the size of the element further, so that it encompassed the minimal *eve3/7* enhancer, did not further increase expression levels in stripe 2, but did increase expression in stripes 3 and 7 (Fig. 3H,I). Together, these results demonstrate that up to 1609 bp 5′ of the minimal *D. erecta eve* stripe-two enhancer contains regulatory information required for *eve* expression in transgenic *D. melanogaster*.

We next performed a computational search for binding sites of four transcription factors that regulate the spatial pattern of the *D. melanogaster eveS2* enhancer – Bicoid, Giant, Hunchback and Krüppel – across the regulatory region upstream of the minimal enhancer (Fig. S2). We observed no obvious turnover of these binding sites in *D. erecta* that would explain the loss of expression from the minimal *eveS2* enhancer, consistent with previous studies (Ludwig et al., 2005).

We next examined the spatial distribution of putative binding sites for the Zelda protein. *Zelda* is expressed ubiquitously in the blastoderm embryo and Zelda protein binds to many enhancers that drive transcription in the early blastoderm embryo (Xu et al., 2014; Foo et al., 2014; Liang et al., 2008; Harrison et al., 2011; Nien et al., 2011; Li et al., 2014). Zelda activity correlates with chromatin accessibility (Foo et al., 2014; Sun et al., 2015; Schulz et al., 2015) and Zelda appears to make enhancers accessible to transcription factors that drive specific patterns of gene expression (Xu et al., 2014; Foo et al., 2014; Li et al., 2014; Schulz et al., 2015; Crocker et al., 2017).

We searched for putative Zelda motifs in the *eveS2* minimal element in *D. melanogaster*, *D. sechellia*, *D. yakuba* and *D. erecta*. We found four sites that are perfectly conserved between these species, and six sites that are present in *D. melanogaster* but absent

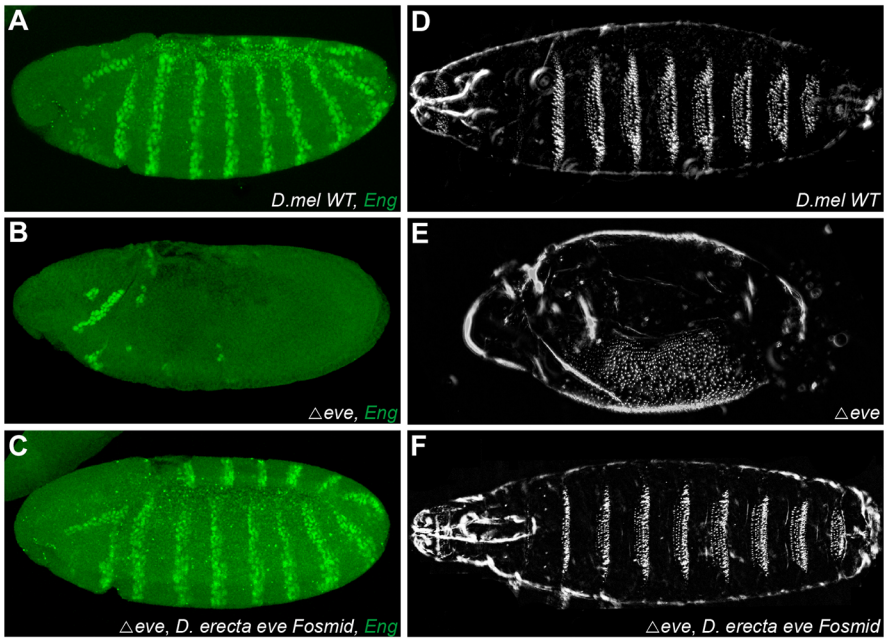

Fig. 2. Functional rescue of *eve* null flies by the *D. erecta* fosmid. (A-C) Stage 9 embryos stained for Engrailed (Eng; En) protein in either a wild-type background (A), an *eve* null background (B), or an *eve* null background carrying the *D. erecta eve* fosmid (C). (D-F) First instar larval cuticle preps of either wild type (D), *eve* null background (E), or *eve* null background carrying the *D. erecta eve* fosmid (F).

Development (2017) 144, 3095-3101 doi:10.1242/dev.149427

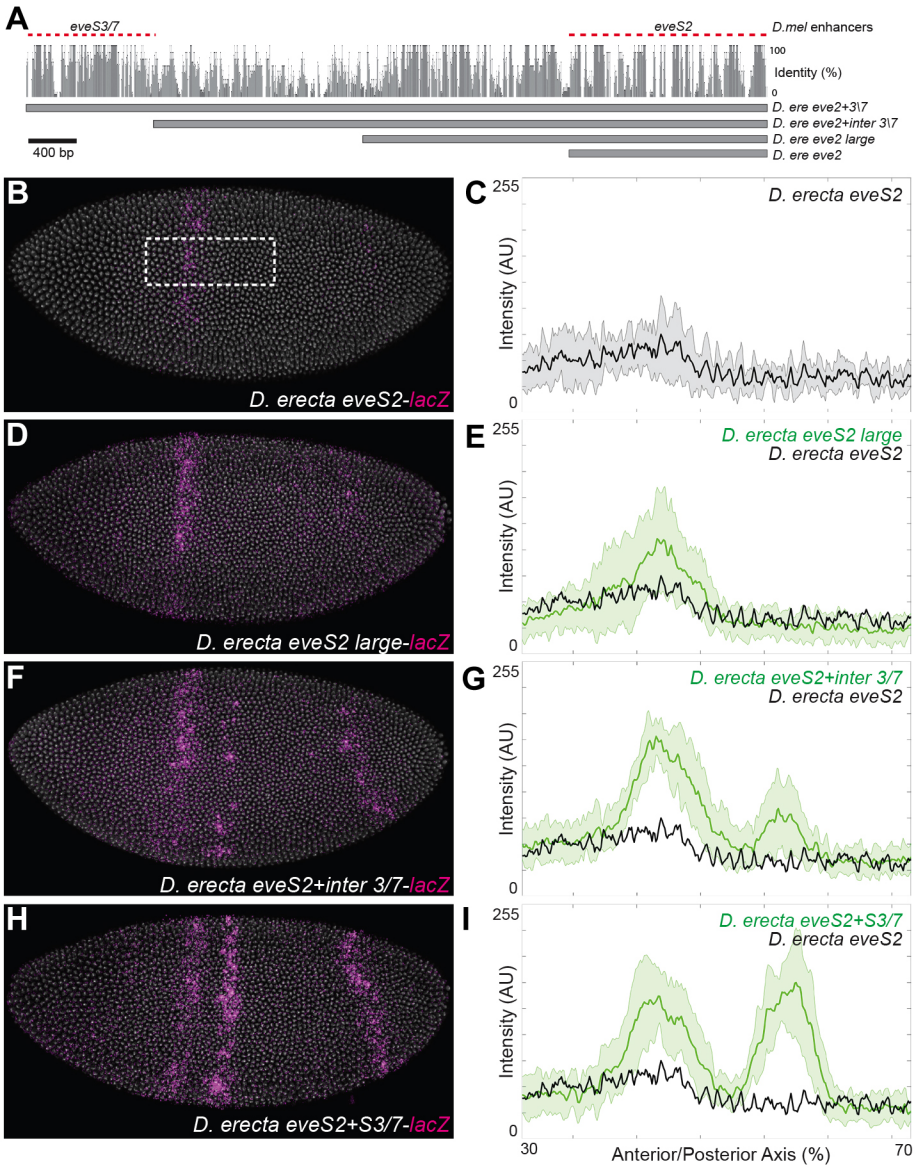

Fig. 3. Sequences upstream of the minimal *D. erecta eveS2* enhancer drive expression in *D. melanogaster*. (A) Schematic of the *eve* locus with enhancers tested indicated. The sequence conservation plot is a 5-way sequence alignment between *D. melanogaster*, *sechellia*, *yakuba*, *erecta* and *ananassae*. (B,D,F,H) Stage 5 *D. melanogaster* embryos stained for *β-Gal* RNA carrying the indicated transgene. (C,E,G,I) Profiles of average expression levels across the region indicated in B for the indicated enhancer (n=10 for each genotype). In all plots, the solid black line denotes wild type, and green denotes the *D. erecta eve* enhancer (K). Bounding areas around experimental data indicate one standard deviation. AU, arbitrary units of fluorescence intensity.

in *D. erecta*. It is possible that the loss of these motifs has led to the loss of activity of the *D. erecta eveS2* minimal element. Because the larger *D. erecta* reporter constructs we tested drove stripe 2 expression, we searched for additional Zelda motifs 5′ of the minimal element. We identified three Zelda motifs in this region in *D. erecta*, two of which are conserved in *D. melanogaster* (Fig. 4A,B; Fig. S3). We tested the activity of these motifs *in vivo* by deleting all three motifs from the large *D. erecta eveS2* construct (Fig. 4C,D). Removal of these upstream Zelda motifs abrogated reporter gene expression (Fig. 4D). These Zelda motifs are therefore essential for stripe 2 expression in *D. erecta*.

To test whether these Zelda motifs were sufficient to drive expression when appended to the minimal *D. erecta eveS2*, we placed the three Zelda motifs upstream of the minimal element. Strikingly, we found that this construct drove expression in stripe 2 (Fig. 4E,F). Addition of these Zelda sites is therefore sufficient to allow properly patterned expression of the minimal *D. erecta* stripe 2 element. Furthermore, the patterns of Zelda motif gain and loss suggest that loss of Zelda motifs within the minimal elements prevents the *D. erecta* minimal enhancer element from driving expression and that gain of a new Zelda motif upstream of the minimal element may be required for normal *D. erecta* stripe 2 expression.

Our results suggest that functional regulatory evolution has occurred outside the minimal *eveS2* enhancer between *D. melanogaster* and *D. erecta*. The *D. erecta eveS2* region contains binding sites necessary for patterning of expression in stripe 2, but lacks sufficient Zelda binding sites to initiate expression. These results support the hypothesis that Zelda contributes to determining the regulatory state of the *eveS2* enhancer in the blastoderm embryo – ON versus OFF – and can be decoupled, at least in part, from the patterns and levels of expression driven by the enhancer (Xu et al., 2014; Foo et al., 2014; Li et al., 2014; Schulz et al., 2015). The potential independence of enhancer state and patterning information has important evolutionary implications, as selection could act on each feature independently. Furthermore, Zelda binding sites are typically located near transcriptional activator-binding sites (Sun et al., 2015), suggesting there might be a constraint on enhancer size, which has widespread consequences for genome evolution.

We have not explored the 777 bp upstream of these Zelda sites, corresponding to the extra DNA in construct *D. ere cta eve 2+inter*

Development (2017) 144, 3095-3101 doi:10.1242/dev.149427

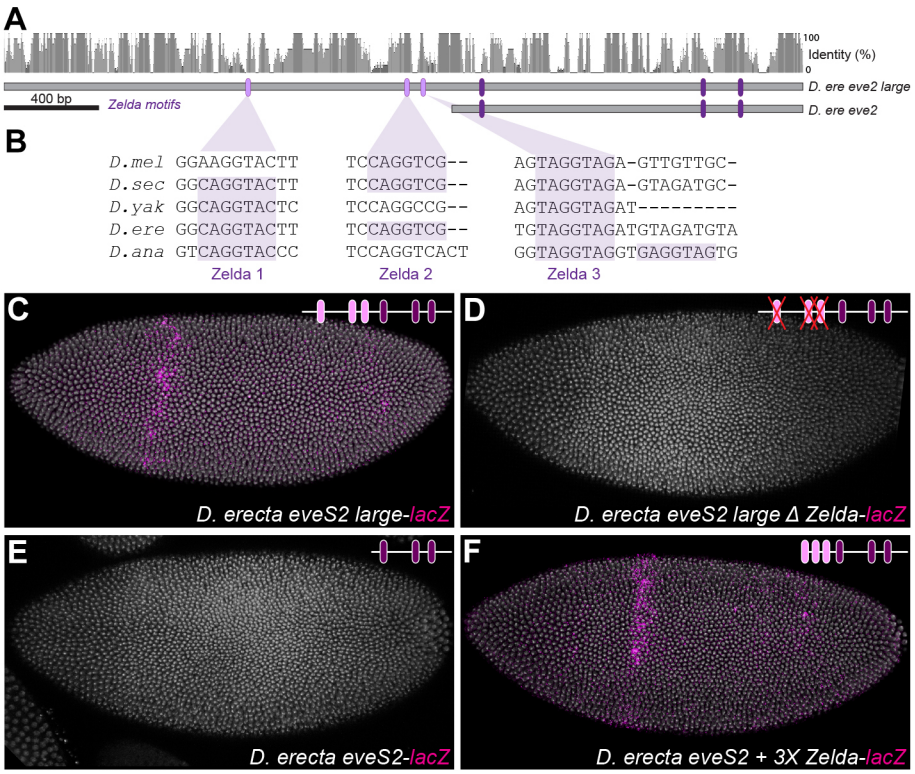

Fig. 4. Zelda motifs are necessary and sufficient for the expression of the *D. erecta eveS2*. (A,B) Schematic of the *eve* locus, with tested enhancers indicated, highlighting the upstream Zelda motifs in *D. erecta* (B). The sequence conservation plot is a 5-way sequence alignment between *D. melanogaster*, *sechellia*, *yakuba*, *erecta* and *ananassae*. (C-F) Stage 5 *D. melanogaster* embryos stained for *β-Gal* RNA carrying the indicated *D. erecta* transgene. (C) The 1609 bp *D. erecta eveS2* enhancer sequence encompassing the three Zelda motifs drives expression in *eve* stripe 2. (D) Mutation of the three Zelda motifs upstream of the 855 bp *D. erecta eveS2* sequence abrogates stripe 2 expression. (E) The 855 bp *D. erecta* enhancer alone does not drive appreciable expression in *D. melanogaster*. (F) The addition of three Zelda motifs, from the 1609 bp fragment (see B,C), to the 855 bp *D. erecta* enhancer is sufficient for transgenic enhancer expression.

3\7, for the sequences that drive stronger expression in stripe 2. This is a poorly conserved genomic region and it is therefore surprising that it contains information both for bolstering stripe 2 expression and for patterning stripes 3 and 7. Interestingly, there is an additional Bcd site in the extended region of *D. erecta*. Although we have not tested this Bcd site, it is possible that there are multiple ways to increase the activity of the *D. erecta eveS2*. This is consistent with a large body of data demonstrating the rapid evolution, and variable architectures, of transcriptional enhancers with conserved functions (Ilsley et al., 2013; Rastegar et al., 2008; Jin et al., 2013; Brown et al., 2007; Menoret et al., 2013; Lusk and Eisen, 2010; Hare et al., 2008; Junion et al., 2012; Crocker et al., 2016). The function of this upstream DNA region would benefit from further exploration.

There are several inherent caveats to cross-species enhancer analysis. For example, basal promoter interactions (Ohtsuki et al., 1998), changes in regulatory binding specificities (True and Haag, 2001; Gasch et al., 2004), and systems drift (True and Haag, 2001) can make it difficult to interpret results. There is also the possibility that additional regulatory sequences exist outside of the tested enhancer regions, including cryptic shadow enhancers (Frankel et al., 2010; Perry et al., 2010) and autoregulatory elements (Jiang et al., 1991). Interestingly, the minimal enhancers from *D. yakuba* and *D. ananassae* also contain only three Zelda sites, but they are both active in *D. melanogaster* (Ludwig et al., 2005). It is therefore possible that there are different elements in these enhancers that function to increase DNA accessibility. Alternatively, it is possible that the structure of Zelda sites, for example how tightly consolidated they are in *D. melanogaster* versus *D. erecta*, could contribute to nuanced differences in expression. In the future, it may be possible to take a synthetic biology approach to understand how the position of Zelda sites within enhancers influences regulatory function and evolution (Crocker et al., 2017).

This study highlights the importance of understanding gene regulatory evolution at the level of the entire locus. Although studies of minimal enhancer elements have provided enormous insight into the mechanisms of gene regulation, the broader DNA regions around minimal enhancers also contribute to enhancer function and evolution (de Laat and Duboule, 2013; Symmons et al., 2016; Gierman et al., 2007; Akhtar et al., 2013; Chen et al., 2013; Dey et al., 2015; Chen and Zhang, 2016; Dunipace et al., 2011). Our results encourage caution in comparative studies of enhancer evolution when functional studies are performed on enhancers from only one species. One goal for future research is to understand how gene regulation evolves at the level of the entire locus, including information such as binding-site composition, the arrangement of enhancers, the activities of intervening sequences, and the distance between functional sequences.

MATERIALS AND METHODS

Construction of enhancer constructs

The *D. erecta* locus was tested by cloning the BDERF01-4213 fosmid, obtained from BACPAC resources (https://bacpacresources.org/), corresponding to *D. melanogaster* chr2R:5,840,325-5,887,364, into vector TKBL-w+ (Kondo et al., 2009). All enhancer constructs were cloned into the *placZattB* expression construct with a *hsp70* promoter (Crocker et al., 2015b). See supplementary Materials and Methods for complete construct sequences.

Fly strains and crosses

D. melanogaster strains were maintained under standard laboratory conditions. Transgenic enhancer constructs were injected into flies by Rainbow Transgenic Flies (CA, USA) and were integrated at the attP2 landing site. The *eveR13* mutation was used to test for complementation using the *D. erecta* fosmid. The lethal mutations were balanced over marked balancer chromosome CyO P(hb-lacZ) to allow identification of mutant embryos by immunostaining for β-galactosidase (Promega, Z3781; 1:250). Relative viability of flies with the *D. erecta* fosmid, in the *eveR13* background, were determined based on the number of adult survivors, as previously described (Ludwig et al., 2011).

Development (2017) 144, 3095-3101 doi:10.1242/dev.149427

Embryo manipulations

Embryos were raised at 25°C and fixed and stained according to standard protocols (Crocker and Stern, 2013). Briefly, primary antibodies obtained from the Developmental Studies Hybridoma Bank were used to detect Eve (3C10; 1:20) and En (4D9; 1:20) proteins, which was followed by detection of primary antibodies using secondary antibodies labeled with Alexa Fluor dyes (1:500, Invitrogen, A31570). Cuticle preps were performed using standard protocols (Stern and Sucena, 2011). Details of the immunohistochemistry protocols can be found in the supplementary Materials and Methods.

Microscopy

Each series of experiments to measure transcript levels was performed entirely in parallel. Embryo collection, fixation and hybridization, and image acquisition and processing were performed side-by-side under identical conditions. Confocal exposures were identical for each series and were set to not exceed the 255 maximum level. Confocal images were obtained on a Leica DM5500 Q Microscope with an ACS APO 20×/0.60 IMM CORR lens and Leica Microsystems LAS AP software. Sum projections of confocal stacks were assembled, embryos were scaled to match sizes, background was subtracted using a 50-pixel rolling-ball radius and plot profiles of fluorescence intensity were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/). Data from the plot profiles were further analyzed in Matlab.

Acknowledgements

We thank the entire Stern lab for discussion and comments, Claire Standley for comments on the manuscript, and Jessica Cande for the Bourbon fruit cake that proved important for manuscript preparation.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.C.; Methodology: J.C.; Formal analysis: J.C.; Investigation: J.C.; Resources: J.C.; Data curation: J.C.; Writing - original draft: J.C.; Writing - review & editing: D.L.S.; Visualization: J.C.; Supervision: D.L.S.; Funding acquisition: D.L.S.

Funding

This work was funded by the Howard Hughes Medical Institute.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.149427.supplemental

References

Adkins, N. L., Hagerman, T. A. and Georgel, P. (2016). GAGA protein: a multi-faceted transcription factor. *Elife* **84**, 559-567.

Akhtar, W., de Jong, J., Pindyurin, A. V., Pagie, L., Meuleman, W., de Ridder, J., Berns, A., Wessels, L.F., van Lohuizen, M. and van Steensel, B. (2013). Chromatin position effects assayed by thousands of reporters integrated in parallel. *Cell* **154**, 914-927.

Arnosti, D. N., Barolo, S., Levine, M. and Small, S. (1996). The eve stripe 2 enhancer employs multiple modes of transcriptional synergy. *Development* **122**, 205-214.

Barrière, A., Gordon, K. L. and Ruvinsky, I. (2012). Coevolution within and between regulatory loci can preserve promoter function despite evolutionary rate acceleration. *PLoS Genet.* **8**, e1002961.

Brown, C. D., Johnson, D. S. and Sidow, A. (2007). Functional architecture and evolution of transcriptional elements that drive gene coexpression. *Science* **317**, 1557-1560.

Chahda, J. S., Sousa-Neves, R. and Mizutani, C. M. (2013). Variation in the dorsal gradient distribution is a source for modified scaling of germ layers in drosophila. *Curr. Biol.* **23**, 710-716.

Chen, X. and Zhang, J. (2016). The genomic landscape of position effects on protein expression level and noise in yeast. *Cell Syst.* **2**, 347-354.

Chen, M., Licon, K., Otsuka, R., Pillus, L. and Ideker, T. (2013). Decoupling epigenetic and genetic effects through systematic analysis of gene position. *Cell Rep.* **3**, 128-137.

Crocker, J. and Stern, D. L. (2013). TALE-mediated modulation of transcriptional enhancers in vivo. *Nat. Methods.* **10**, 762-767.

Crocker, J., Tamori, Y. and Erives, A. (2008). Evolution acts on enhancer organization to fine-tune gradient threshold readouts. *PLoS Biol.* **6**, e263.

Crocker, J., Potter, N. and Erives, A. (2010). Dynamic evolution of precise regulatory encodings creates the clustered site signature of enhancers. *Nat. Commun.* **1**, 99.

Crocker, J., Noon, E. P. B. and Stern, D. L. (2015a). The Soft touch: low-affinity transcription factor binding sites in development and evolution. *Curr. Top. Dev. Biol.* **117**, 455-469.

Crocker, J., Abe, N., Rinaldi, L., Mcgregor, A. P., Frankel, N. A., Wang, S., Alsawadi, A., Valenti, P., Plaza, S., Payre, F. et al. (2015b). Low affinity binding site clusters confer hox specificity and regulatory robustness. *Cell* **160**, 191-203.

Crocker, J., Ilsley, G. R. and Stern, D. L. (2016). Quantitatively predictable control of Drosophila transcriptional enhancers in vivo with engineered transcription factors. *Nat. Genet.* **48**, 292-298.

Crocker, J., Tsai, A. and Stern, D. L. (2017). A Fully Synthetic Transcriptional Platform for a Multicellular Eukaryote. *Cell Rep.* **18**, 287-296.

De Laat, W. and Duboule, D. (2013). Topology of mammalian developmental enhancers and their regulatory landscapes. *Nature* **502**, 499-506.

Dey, S. S., Foley, J. E., Limsirichai, P., Schaffer, D. V. and Arkin, A. P. (2015). Orthogonal control of expression mean and variance by epigenetic features at different genomic loci. *Mol. Syst. Biol.* **11**, 806.

Dunipace, L., Ozdemir, A. and Stathopoulos, A. (2011). Complex interactions between cis-regulatory modules in native conformation are critical for Drosophila snail expression. *Development* **138**, 4566.

Foo, S. M., Sun, Y., Lim, B., Ziukaite, R., O'Brien, K., Nien, C.-Y., Kirov, N., Shvartsman, S. Y. and Rushlow, C.A. (2014). Zelda potentiates morphogen activity by increasing chromatin accessibility. *Curr. Biol.* **24**, 1341-1346.

Fowlkes, C. C. Eckenrode, K. B., Bragdon, M. D., Meyer, M., Wunderlich, Z., Simirenko, L., Luengo Hendriks, C. L., Keränen, S. V. E., Henriquez, C., Knowles, D. W. et al. (2011). A conserved developmental patterning network produces quantitatively different output in multiple species of drosophila. *PLoS Genet.* **7**, e1002346.

Frankel, N., Davis, G. K., Vargas, D., Wang, S., Payre, F. and Stern, D. L. (2010). Phenotypic robustness conferred by apparently redundant transcriptional enhancers. *Nature* **466**, 490-493.

Fujioka, M., Emi-Sarker, Y., Yusibova, G. L., Goto, T. and Jaynes, J. B. (1999). Analysis of an even-skipped rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients. *Development.* **126**, 2527-2538.

Gasch, A. P., Moses, A. M., Chiang, D. Y., Fraser, H. B., Berardini, M. and Eisen, M. B. (2004). Conservation and evolution of cis-regulatory systems in ascomycete fungi. *PLoS Biol.* **2**, e398.

Gierman, H. J., Indemans, M. H. G., Koster, J., Goetze, S., Seppen, J., Geerts, D., Van Driel, R. and Versteeg, R. (2007). Domain-wide regulation of gene expression in the human genome. *Genome Res.* **17**, 1286-1295.

Hare, E. E., Peterson, B. K., Iyer, V. N., Meier, R. and Eisen, M. B. (2008). Sepsid even-skipped enhancers are functionally conserved in Drosophila despite lack of sequence conservation. *PLoS Genet.* **4**, e1000106.

Harrison, M. M., Li, X.-Y., Kaplan, T., Botchan, M. R. and Eisen, M. B. (2011). Zelda binding in the early Drosophila melanogaster embryo marks regions subsequently activated at the maternal-to-zygotic transition. *PLoS Genet.* **7**, e1002266.

Ilsley, G. R., Fisher, J., Apweiler, R., Depace, A. H. and Luscombe, N. M. (2013). Cellular resolution models for even skipped regulation in the entire Drosophila embryo. *Elife* **2**, e00522.

Jiang, J., Hoey, T. and Levine, M. (1991). Autoregulation of a segmentation gene in Drosophila: combinatorial interaction of the even-skipped homeo box protein with a distal enhancer element. *Genes Dev.* **5**, 265-277.

Jin, H., Stojnic, R., Adryan, B., Ozdemir, A., Stathopoulos, A. and Frasch, M. (2013). Genome-wide screens for in vivo Tinman binding sites identify cardiac enhancers with diverse functional architectures. *PLoS Genet.* **9**, e1003195.

Junion, G. Spivakov, M., Girardot, C., Braun, M., Gustafson, E. H., Birney, E. and Furlong, E. E. (2012). A transcription factor collective defines cardiac cell fate and reflects lineage history. *Cell* **148**, 473-486.

Kondo, S., Booker, M. and Perrimon, N. (2009). Cross-species RNAi rescue platform in Drosophila melanogaster. *Genetics* **183**, 1165-1173.

Levine, M. (2010). Transcriptional enhancers in animal development and evolution. *Curr. Biol.* **20**, R754-R763.

Levo, M. and Segal, E. (2014). In pursuit of design principles of regulatory sequences. *Nat. Rev. Genet.* **15**, 453-468.

Li, X.-Y., Harrison, M. M., Villalta, J. E., Kaplan, T. and Eisen, M. B. (2014). Establishment of regions of genomic activity during the Drosophila maternal to zygotic transition. *Elife*. **3**, e03737.

Liang, H., Nien, C.-Y., Liu, H.-Y., Metzstein, M. M., Kirov, N. and Rushlow, C. (2008). The zinc-finger protein Zelda is a key activator of the early zygotic genome in Drosophila. *Nature.* **456**, 400-403.

Ludwig, M. Z. and Kreitman, M. (1995). Evolutionary dynamics of the enhancer region of even-skipped in Drosophila. *Mol. Biol. Evol.* **12**, 1002-1011.

Ludwig, M. Z., Patel, N. H. and Kreitman, M. (1998). Functional analysis of eve stripe 2 enhancer evolution in Drosophila: rules governing conservation and change. *Development* **125**, 949-958.

Ludwig, M. Z., Bergman, C., Patel, N. H. and Kreitman, M. (2000). Evidence for stabilizing selection in a eukaryotic enhancer element. *Nature* **403**, 564-567.
Ludwig, M. Z., Palsson, A., Alekseeva, E., Bergman, C. M., Nathan, J. and Kreitman, M. (2005). Functional evolution of a cis-regulatory module. *PLoS Biol.* **3**, e93.
Ludwig, M. Z., Manu, R. Kittler, K. P. White, and Kreitman, M. (2011). Consequences of eukaryotic enhancer architecture for gene expression dynamics, development, and fitness. *PLoS Genet*. **7**, e1002364.
Lusk, R. W. and Eisen, M. B. (2010). Evolutionary mirages: selection on binding site composition creates the illusion of conserved grammars in Drosophila enhancers. *PLoS Genet.* **6**, e1000829.
Macarthur, S. and Brookfield, J. F. Y. (2004). Expected rates and modes of evolution of enhancer sequences. *Mol. Biol. Evol.* **21**, 1064-1073.
Macdonald, P. M., Ingham, P. and Struhl, G. (1986). Isolation, structure, and expression of even-skipped: a second pair-rule gene of Drosophila containing a homeo box. *Cell* **47**, 721-734.
Menoret, D., Santolini, M., Fernandes, I., Spokony, R., Zanet, J., Gonzalez, I., Latapie, Y., Ferrer, P., Rouault, H., White, K. P. et al. (2013). Genome-wide analyses of Shavenbaby target genes reveals distinct features of enhancer organization. *Genome Biol.* **14**, R86.
Nien, C.-Y., Liang, H.-L., Butcher, S., Sun, Y., Fu, S., Gocha, T., Kirov, N., Manak, J. R. and Rushlow, C. (2011). Temporal coordination of gene networks by Zelda in the early Drosophila embryo. *PLoS Genet.* **7**, e1002339.
Nord, A. S. Blow, M. J., Attanasio, C., Akiyama, J. A., Holt, A., Hosseini, R., Phouanenavong, S., Plajzer-Frick, I., Shoukry, M., Afzal, V. et al. (2013). Rapid and pervasive changes in genome-wide enhancer usage during mammalian development. *Cell* **155**, 1521-1531.
Ohtsuki, S., Levine, M. and Cai, H. N. (1998). Different core promoters possess distinct regulatory activities in the Drosophila embryo. *Genes Dev.* **12**, 547-556.
Perry, M. W., Boettiger, A. N., Bothma, J. P. and Levine, M. (2010). Shadow enhancers foster robustness of Drosophila gastrulation. *Curr. Biol.* **20**, 1562-1567.
Prescott, S. L., Srinivasan, R., Marchetto, M. C., Grishina, I., Narvaiza, I., Selleri, L., Gage, F. H., Swigut, T. and Wysocka, J. (2015). Enhancer divergence and cis-regulatory evolution in the human and chimp neural crest. *Cell* **163**, 68-83.
Rastegar, S., Hess, I., Dickmeis, T., Nicod, J. C., Ertzer, R., Hadzhiev, Y., Thies, W.-G., Scherer, G. and Strähle, U. (2008). The words of the regulatory code are arranged in a variable manner in highly conserved enhancers. *Dev. Biol.* **318**, 366-377.
Rebeiz, M., Pool, J. E., Kassner, V. A., Aquadro, C. F. and Carroll, S. B. (2009). Stepwise modification of a modular enhancer underlies adaptation in a Drosophila population. *Science* **326**, 1663-1667.
Schulz, K. N., Bondra, E. R., Moshe, A., Villalta, J. E., Lieb, J. D., Kaplan, T., McKay, D. J. and Harrison, M. M. (2015). Zelda is differentially required for chromatin accessibility, transcription-factor binding and gene expression in the early Drosophila embryo. *Genome Res.* **25**, 1715-1726.
Small, S., Krau, R., Hoey, T., Warrior, R. and Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in Drosophila. *Genes Dev.* **5**, 827-839.
Small, S., Blair, A. and Levine, M. (1992). Regulation of even-skipped stripe 2 in the Drosophila embryo. *EMBO J.* **11**, 4047-4057.
Small, S., Blair, A. and Levine, M. (1996). Regulation of two pair-rule stripes by a single enhancer in the Drosophila embryo. *Dev. Biol.* **175**, 314-324.
Spitz, F. and Furlong, E. E. M. (2012). Transcription factors: from enhancer binding to developmental control. *Nat. Rev. Genet.* **13**, 613-626.
Stanojevic, D., Small, S. and Levine, M. (1991). Regulation of a segmentation stripe by overlapping activators and repressors in the Drosophila embryo. *Science* **254**, 1385-1387.
Stern, D. L. and Sucena, E. (2011). Preparation of cuticles from feeding Drosophila larvae. *Cold Spring Harb. Protoc.* **2011**, 1394-1398.
Sun, Y., Nien, C. Y., Chen, K., Liu, H. Y., Johnston, J., Zeitlinger, J. and Rushlow, C. (2015). Zelda overcomes the high intrinsic nucleosome barrier at enhancers during Drosophila zygotic genome activation. *Genome Res.* **25**, 1703-1714.
Symmons, O., Pan, L., Remeseiro, S., Aktas, T., Klein, F., Huber, W. and Spitz, F. (2016). The Shh topological domain facilitates the action of remote enhancers by reducing the effects of genomic distances. *Dev. Cell.* **39**, 529-543 .
True, J. R. and Haag, E. S. (2001). Developmental system drift and flexibility in evolutionary trajectories. *Evol. Dev.* **3**, 109-119.
Xu, Z., Chen, H., Ling, J., Yu, D., Struffi, P., Small, S. (2014). Impacts of the ubiquitous factor Zelda on Bicoid-dependent DNA binding and transcription in Drosophila. *Genes Dev.* **28**, 608-621.