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

Justin Crocker^{1*}, David L. Stern¹

Abstract

Transcriptional enhancers are regions of DNA that drive gene expression at precise times, levels, and locations. While 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 minimal *D. erecta eve* stripe 2 enhancer (*eveS2*) fails to drive appreciable expression in *D. melanogaster* [1]. 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 that regulatory information outside of the minimal *D. erecta eveS2* contains 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.

Keywords

Evolution – Transcription – Enhancer – *Drosophila* – *Even-skipped*

¹Janelia Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive, Ashburn, VA 20147 USA

*Corresponding author: crockerj@janelia.hhmi.org

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 [2, 3]. These studies have provided mechanistic insight into how transcriptional logic is encoded in individual enhancers [4, 5]. However, in even the earliest studies, it was clear that minimal enhancers are insufficient to define the normal gene expression pattern with complete fidelity [2]. 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 [6, 7, 8].

Phenotypic evolution results largely from sequence changes in enhancers [9, 10, 11, 12, 13], even between closely related species [14, 15, 16, 17, 18, 19]. 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 [20, 21]. Minimal enhancers have been identified that each drive expression in either one or two stripes that together drive expression in all seven stripes

[22, 23]. Of all these enhancers, the minimal element for stripe 2 has been studied in the greatest detail [2, 24, 25]. This enhancer contains multiple binding sites for transcriptional activators (Bicoid and Hunchback) and repressors (Giant, Krüppel, and Sloppy-paired). The collective activity of transcription factors binding to these sites drives *eve* expression specifically in stripe 2 [2, 21, 24, 25, 26].

Previously, reporter gene assays were used to investigate the functional evolution of *eve S2* from three divergent *Drosophila* species with transgenic assays in *D. melanogaster* [1, 27, 15, 28, 6, 29]. 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* [1]. First, the “minimal” functional *D. erecta eveS2* element may 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 may have evolved to accommodate differences in the *D. erecta* embryonic environment [30, 31]—for example, differences in transcription factor concentrations [17, 32]. Third, the true functional *eveS2* enhancer may 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 down-stream *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 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 [33, 34, 35, 36, 37, 38, 39, 40]. 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 critical transcription factor binding sites can shift during evolution between locations within and outside of “minimal” regions.

Results

D. melanogaster and *D. erecta* both express *eve* in seven embryonic stripes at similar levels and locations (Figure 1 a, 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 (Figure 1c). However, the orthologous *eve S2* fragment from *D. erecta* does not drive expression in transgenic *D. melanogaster* (Figure 1d) (see also Ludwig et al., [1]). A 20kb 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 [23]. 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 *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 the expression of the segmental polarity gene *engrailed* (Fig. 2a-c). Additionally, we found that the fosmid rescued all segmentation defects in the cuticles of

Figure 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 β -Gal RNA carrying the *D. melanogaster eveS2* (c) or *D. erecta eveS2*. (e) Schematic representation of the *eve* locus with enhancers indicated in dark 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 panel (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 indicates Arbitrary Units of fluorescence intensity.

larvae deficient for *eve* (Fig. 2d-f). Together, these results suggest that the 47kb *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 [41, 2, 25, 42]. Increasing the size of the DNA regions tested for these enhancers increases levels of transcription [41, 2,

Figure 2. Functional rescue of *eve* null flies by the *D. erecta* fosmid. (a, b, c) Stage 9 embryos stained for Engrailed (Eng) protein in either a wild-type background (a), *eve* null background (b), or an *eve* null background carrying the *D. erecta eve* fosmid (c). (d, e, f) First instar larval cuticle preps of either a wild-type (d), *eve* null background (e), or an *eve* null background carrying the *D. erecta eve* fosmid (f).

25, 42]. Additionally, while 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 [6]. Therefore, we suspected that sequences near the minimal *D. erecta eveS2* may be required for normal expression.

To test if regulatory information 5' of the minimal *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). While the 855 bp *D. erecta* minimal *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 two domain (Fig. 3d, e). Increasing the minimal construct size with an additional 1609 bp 5' of the minimal 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*, but also 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 the domain of *eve* stripe two, but increased 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 three transcription factors that regulate the spatial pat-

Figure 3. Sequences upstream of the minimal *D. erecta eveS2* enhancer drive expression in *D. melanogaster*. (a) Schematic representation 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 panel (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 indicates Arbitrary Units of fluorescence intensity.

tern of the *D. melanogaster eveS2* enhancer—Bicoid, Hunchback and Krüppel—across the regulatory region upstream of the minimal enhancer (Fig. S1). 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 [1].

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 [33, 34, 35, 36, 37, 38, 39, 40]. *Zelda* activity is correlated with chromatin accessibility [35, 39, 40] and *Zelda* appears to make enhancers accessible to transcription factors that drive specific patterns of gene expression [34, 35, 38, 39, 40, 43].

We searched for putative *Zelda* motifs in the *eveS2* mini-

mal element in *D. melanogaster*, *D. sechellia*, *D. yakuba*, and *D. erecta*. We found four sites that were perfectly conserved between these species, and six sites that were present in *D. melanogaster* but absent 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* and one of which is absent in *D. melanogaster* (Fig. 4a, b). 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 critical for stripe 2 expression in *D. erecta*.

To test if 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.

Figure 4. Zelda motifs are necessary and sufficient for the expression of the minimal *D. erecta* *eveS2*. (a, b) Schematic representation 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, d, e, f) Stage 5 *D. melanogaster* embryos stained for β -Gal RNA carrying the indicated *D. erecta* transgene

Discussion

Our results suggest that functional regulatory evolution has occurred outside the minimal *eveS2* enhancer between *D. melanogaster* and *D. erecta*. The minimal *D. erecta* *eveS2* region contains binding sites necessary for patterning of expression in stripe 2, but lacks sufficient Zelda binding sites to drive 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 [34, 35, 38, 39, 40, 43]. We have not explored the 777 bp upstream of these Zelda sites (the extra DNA in construct *D. ere eve 2+inter 3\7*) for the sequences that drive stronger expression in stripe 2. This is a poorly conserved genomic region and it is curious that it contains information both for bolstering stripe 2 expression and for patterning stripes 3 and 7. The function of this DNA region would repay further exploration.

This study highlights the need to understand gene regulatory evolution at the level of the entire locus. While studies of minimal enhancers 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 [44, 45, 46, 47, 48, 49, 50]. Our results encourage great caution in comparative studies of enhancer evolution when functional studies have been 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.

Methods and Materials

0.1 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+ [51]. All enhancer constructs were cloned into the placZattB expression construct with a hsp70 promoter [18]. See supplemental materials for complete construct sequences.

0.2 Fly strains and crosses.

D. melanogaster strains were maintained under standard laboratory conditions. Transgenic enhancer constructs were created by Rainbow Transgenic Flies Inc. 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 Beta-galactosidase.

0.3 Embryo manipulations.

Embryos were raised at 25°C and fixed and stained according to standard protocols [52]. Briefly, primary antibodies obtained from the Developmental Studies Hybridoma Bank were used to detect Eve (3C10, used 1:20) and En (4D9, used 1:20) proteins, which was followed by detection of primary antibodies using secondary antibodies labeled with Alexa Fluor dyes (1:500, Invitrogen). Cuticle preps were performed using standard protocols [53].

0.4 Microscopy.

Each series of experiments to measure transcript levels was performed entirely in parallel. Embryo collections, fixations, and hybridizations, image acquisitions, and processing were performed side-by-side in 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.

Acknowledgments

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.

References

- [1] Michael Z Ludwig, Arnar Palsson, Elena Alekseeva, Casey M Bergman, Janaki Nathan, and Martin Kreitman. Functional evolution of a cis-regulatory module. *PLoS biology*, 3(4):e93, apr 2005.
- [2] S Small, A Blair, M Levine, S Small, and M Levine. Regulation of even-skipped stripe 2 in the *Drosophila* embryo. *The EMBO journal*, 11(11):4047–57, nov 1992.
- [3] Justin Crocker and David L Stern. TALE-mediated modulation of transcriptional enhancers in vivo. *Nature methods*, 10(8):762–7, aug 2013.
- [4] François Spitz and Eileen E M Furlong. Transcription factors: from enhancer binding to developmental control. *Nature reviews. Genetics*, 13(9):613–26, sep 2012.
- [5] Michal Levo and Eran Segal. In pursuit of design principles of regulatory sequences. *Nature reviews. Genetics*, 15(7):453–68, jul 2014.
- [6] Michael Z Ludwig, Manu, Ralf Kittler, Kevin P White, and Martin Kreitman. Consequences of eukaryotic enhancer architecture for gene expression dynamics, development, and fitness. *PLoS genetics*, 7(11):e1002364, nov 2011.
- [7] Cosmas D Arnold, Daniel Gerlach, Daniel Spies, Jessica A Matts, Yuliya A Sytnikova, Michaela Pagani, Nelson C Lau, and Alexander Stark. Quantitative genome-wide enhancer activity maps for five *Drosophila* species show functional enhancer conservation and turnover during cis-regulatory evolution. *Nature Genetics*, 46(7):685–692, jun 2014.
- [8] David S Lorberbaum, Andrea I Ramos, Kevin A Peterson, Brandon S Carpenter, David S Parker, Sandip De, Lauren E Hillers, Victoria M Blake, Yuichi Nishi, Matthew R McFarlane, Ason CY Chiang, Judith A Kassis, Benjamin L Allen, Andrew P McMahon, and Scott Barolo. An ancient yet flexible cis-regulatory architecture allows localized Hedgehog tuning by patched/Ptch1. *eLife*, 5, may 2016.
- [9] Mike Levine. Transcriptional enhancers in animal development and evolution. *Current biology : CB*, 20(17):R754–63, sep 2010.
- [10] Sara L. Prescott, Rajini Srinivasan, Maria Carolina Marchetto, Irina Grishina, Iñigo Narvaiza, Licia Sellari, Fred H. Gage, Tomek Swigut, and Joanna Wysocka. Enhancer Divergence and cis-Regulatory Evolution in the Human and Chimp Neural Crest. *Cell*, sep 2015.
- [11] William J. Glassford, Winslow C. Johnson, Natalie R. Dall, Sarah Jacquelyn Smith, Yang Liu, Werner Boll, Markus Noll, and Mark Rebeiz. Co-option of an Ancestral Hox-Regulated Network Underlies a Recently Evolved Morphological Novelty. *Developmental Cell*, 34(5):520–531, sep 2015.
- [12] Ella Preger-Ben Noon, Fred P. Davis, and David L. Stern. Evolved Repression Overcomes Enhancer Robustness. *Developmental Cell*, 39(5):572–584, dec 2016.
- [13] Evgeny Z. Kvon, Olga K. Kamneva, Uirá S. Melo, Iros Barozzi, Marco Osterwalder, Brandon J. Mannion, Virginie Tissières, Catherine S. Pickle, Ingrid Plajzer-Frick, Elizabeth A. Lee, Momoe Kato, Tyler H. Garvin, Jennifer A. Akiyama, Veena Afzal, Javier Lopez-Rios, Edward M. Rubin, Diane E. Dickel, Len A. Pennacchio, and Axel Visel. Progressive Loss of Function in a Limb Enhancer during Snake Evolution. *Cell*, 167(3):633–642.e11, oct 2016.
- [14] Stewart MacArthur and John F Y Brookfield. Expected rates and modes of evolution of enhancer sequences. *Molecular biology and evolution*, 21(6):1064–73, 2004.
- [15] M Z Ludwig and M Kreitman. Evolutionary dynamics of the enhancer region of even-skipped in *Drosophila*. *Molecular biology and evolution*, 12(6):1002–11, nov 1995.
- [16] Alex S Nord, Matthew J Blow, Catia Attanasio, Jennifer A Akiyama, Amy Holt, Roya Hosseini, Sengthavy

- Phouanenavong, Ingrid Plajzer-Frick, Malak Shoukry, Veena Afzal, John L R Rubenstein, Edward M Rubin, Len A Pennacchio, and Axel Visel. Rapid and pervasive changes in genome-wide enhancer usage during mammalian development. *Cell*, 155(7):1521–31, dec 2013.
- [17] Justin Crocker, Yoichiro Tamori, and Albert Erives. Evolution acts on enhancer organization to fine-tune gradient threshold readouts. *PLoS biology*, 6(11):e263, nov 2008.
- [18] Justin Crocker, Namiko Abe, Lucrezia Rinaldi, Alistair P McGregor, Nicolás Frankel, Shu Wang, Ahmad Alsawadi, Philippe Valenti, Serge Plaza, François Payre, Richard S Mann, and David L Stern. Low affinity binding site clusters confer hox specificity and regulatory robustness. *Cell*, 160(1-2):191–203, jan 2015.
- [19] Nicolás Frankel, Deniz F Erezylmaz, Alistair P McGregor, Shu Wang, François Payre, and David L Stern. Morphological evolution caused by many subtle-effect substitutions in regulatory DNA. *Nature*, 474(7353):598–603, jun 2011.
- [20] P M Macdonald, P Ingham, and G Struhl. Isolation, structure, and expression of even-skipped: a second pair-rule gene of *Drosophila* containing a homeo box. *Cell*, 47(5):721–34, dec 1986.
- [21] Garth R Ilsley, Jasmin Fisher, Rolf Apweiler, Angela H Depace, and Nicholas M Luscombe. Cellular resolution models for even skipped regulation in the entire *Drosophila* embryo. *eLife*, 2:e00522, jan 2013.
- [22] S Small, D N Arnosti, and M Levine. Spacing ensures autonomous expression of different stripe enhancers in the even-skipped promoter. *Development (Cambridge, England)*, 119(3):762–72, 1993.
- [23] M Fujioka, Y Emi-Sarker, G L Yusibova, T Goto, and J B Jaynes. 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 (Cambridge, England)*, 126(11):2527–38, jun 1999.
- [24] D Stanojevic, S Small, and M Levine. Regulation of a segmentation stripe by overlapping activators and repressors in the *Drosophila* embryo. *Science (New York, N.Y.)*, 254(5036):1385–7, nov 1991.
- [25] S Small, R Warrior, R Kraut, M Levine, T Hoey, R Warrior, and M Levine. Transcriptional regulation of a pair-rule stripe in *Drosophila*. *Genes & development*, 5(5):827–39, may 1991.
- [26] Justin Crocker, Garth R Ilsley, and David L Stern. Quantitatively predictable control of *Drosophila* transcriptional enhancers in vivo with engineered transcription factors. *Nature genetics*, 48(3):292–8, mar 2016.
- [27] M Z Ludwig, N H Patel, and M Kreitman. Functional analysis of eve stripe 2 enhancer evolution in *Drosophila*: rules governing conservation and change. *Development*, 125(5):949–958, mar 1998.
- [28] M Z Ludwig, C Bergman, N H Patel, and M Kreitman. Evidence for stabilizing selection in a eukaryotic enhancer element. *Nature*, 403(6769):564–7, feb 2000.
- [29] C. Martinez, J. S. Rest, A.-R. Kim, M. Ludwig, M. Kreitman, K. White, and J. Reinitz. Ancestral Resurrection of the *Drosophila* S2E Enhancer Reveals Accessible Evolutionary Paths through Compensatory Change. *Molecular Biology and Evolution*, 31(4):903–916, apr 2014.
- [30] Charles C Fowlkes, Kelly B. Eckenrode, Meghan D. Bragdon, Miriah Meyer, Zeba Wunderlich, Lisa Simirenko, Cris L. Luengo Hendriks, Soile V E Keränen, Clara Henriquez, David W Knowles, Mark D Biggin, Michael B Eisen, and Angela H. DePace. A Conserved Developmental Patterning Network Produces Quantitatively Different Output in Multiple Species of *Drosophila*. *PLoS Genetics*, 7(10):e1002346, oct 2011.
- [31] Antoine Barrière, Kacy L. Gordon, and Ilya Ruvinsky. Coevolution within and between Regulatory Loci Can Preserve Promoter Function Despite Evolutionary Rate Acceleration. *PLoS Genetics*, 8(9):e1002961, sep 2012.
- [32] Juan Sebastian Chahda, Rui Sousa-Neves, and Claudia Miekko Mizutani. Variation in the Dorsal Gradient Distribution Is a Source for Modified Scaling of Germ Layers in *Drosophila*. *Current Biology*, 23(8):710–716, apr 2013.
- [33] Hsiao-lan Liang, Chung-yi Nien, Hsiao-yun Liu, Mark M Metzstein, Nikolai Kirov, and Christine Rushlow. The zinc-finger protein Zelda is a key activator of the early zygotic genome in *Drosophila*. *Nature*, 456(November):400–404, 2008.
- [34] Zhe Xu, Hongtao Chen, Jia Ling, Danyang Yu, Paolo Struffi, and Stephen Small. Impacts of the ubiquitous factor Zelda on Bicoid-dependent DNA binding and transcription in *Drosophila*. *Genes & development*, 28(6):608–21, mar 2014.
- [35] Sun Melody Foo, Yujia Sun, Bomyi Lim, Ruta Ziukaite, Kevin O’Brien, Chung-Yi Nien, Nikolai Kirov, Stanislav Y Shvartsman, and Christine A Rushlow. Zelda potentiates morphogen activity by increasing chromatin accessibility. *Current biology : CB*, 24(12):1341–6, jun 2014.
- [36] Melissa M Harrison, Xiao-Yong Li, Tommy Kaplan, Michael R Botchan, and Michael B Eisen. Zelda binding in the early *Drosophila melanogaster* embryo marks regions subsequently activated at the maternal-to-zygotic transition. *PLoS genetics*, 7(10):e1002266, oct 2011.
- [37] Chung-Yi Nien, Hsiao-Lan Liang, Stephen Butcher, Yujia Sun, Shengbo Fu, Tenzin Gocha, Nikolai Kirov, J Robert Manak, and Christine Rushlow. Temporal coordination of gene networks by Zelda in the early *Drosophila* embryo. *PLoS genetics*, 7(10):e1002339, oct 2011.

- [38] Xiao-Yong Li, Melissa M Harrison, Jacqueline E Villalta, Tommy Kaplan, and Michael B Eisen. Establishment of regions of genomic activity during the *Drosophila* maternal to zygotic transition. *eLife*, 3, jan 2014.
- [39] Yujia Sun, Chung-Yi Nien, Kai Chen, Hsiao-Yun Liu, Jeff Johnston, Julia Zeitlinger, and Christine Rushlow. Zelda overcomes the high intrinsic nucleosome barrier at enhancers during *Drosophila* zygotic genome activation. *Genome research*, sep 2015.
- [40] Katharine N Schulz, Eliana R Bondra, Arbel Moshe, Jacqueline E Villalta, Jason D Lieb, Tommy Kaplan, Daniel J McKay, and Melissa M Harrison. Zelda is differentially required for chromatin accessibility, transcription-factor binding and gene expression in the early *Drosophila* embryo. *Genome research*, sep 2015.
- [41] D N Arnosti, S Barolo, M Levine, and S Small. The eve stripe 2 enhancer employs multiple modes of transcriptional synergy. *Development (Cambridge, England)*, 122(1):205–14, 1996.
- [42] S Small, A Blair, and M Levine. Regulation of two pair-rule stripes by a single enhancer in the *Drosophila* embryo. *Developmental biology*, 175(2):314–24, 1996.
- [43] Justin Crocker, Albert Tsai, and David L. Stern. A Fully Synthetic Transcriptional Platform for a Multicellular Eukaryote. *Cell Reports*, 18(1):287–296, jan 2017.
- [44] Wouter de Laat and Denis Duboule. Topology of mammalian developmental enhancers and their regulatory landscapes. *Nature*, 502(7472):499–506, oct 2013.
- [45] Orsolya Symmons, Leslie Pan, Silvia Remeseiro, Tugce Aktas, Felix Klein, Wolfgang Huber, and François Spitz. The Shh Topological Domain Facilitates the Action of Remote Enhancers by Reducing the Effects of Genomic Distances. *Developmental Cell*, 39(5):529–543, dec 2016.
- [46] Hincio J Gierman, Mireille H G Indemans, Jan Koster, Sandra Goetze, Jurgen Seppen, Dirk Geerts, Roel van Driel, and Rogier Versteeg. Domain-wide regulation of gene expression in the human genome. *Genome research*, 17(9):1286–95, sep 2007.
- [47] Waseem Akhtar, Johann de Jong, Alexey V. V Pindyurin, Ludo Pagie, Wouter Meuleman, Jeroen de Ridder, Anton Berns, Lodewyk F.A. F A Wessels, Maarten van Lohuizen, Bas van Steensel, Johann de Jong, Alexey V. V Pindyurin, Ludo Pagie, Wouter Meuleman, Jeroen de Ridder, Anton Berns, Lodewyk F.A. F A Wessels, Maarten van Lohuizen, and Bas van Steensel. Chromatin position effects assayed by thousands of reporters integrated in parallel. *Cell*, 154(4):914–27, aug 2013.
- [48] Menzies Chen, Katherine Licon, Rei Otsuka, Lorraine Pilus, and Trey Ideker. Decoupling Epigenetic and Genetic Effects through Systematic Analysis of Gene Position. *Cell Reports*, 3(1):128–137, 2013.
- [49] Xiaoshu Chen and Jianzhi Zhang. The Genomic Landscape of Position Effects on Protein Expression Level and Noise in Yeast. 2016.
- [50] Leslie Dunipace, Anil Ozdemir, and Angelike Stathopoulos. Complex interactions between cis-regulatory modules in native conformation are critical for *Drosophila* snail expression. *Development*, 138(18), 2011.
- [51] Shu Kondo, Matthew Booker, and Norbert Perrimon. Cross-species RNAi rescue platform in *Drosophila melanogaster*. *Genetics*, 183(3):1165–73, nov 2009.
- [52] Justin Crocker and Albert Erives. A Schnurri/Mad/Medea complex attenuates the dorsal-twist gradient readout at vnd. *Developmental biology*, 378(1):64–72, jun 2013.
- [53] David L Stern and Elio Sucena. Preparation of cuticles from feeding *Drosophila* larvae. *Cold Spring Harbor protocols*, 2011(11):1394–8, nov 2011.
- [54] Gabriela G Loots, Ivan Ovcharenko, Lior Pachter, Inna Dubchak, and Edward M Rubin. rVista for comparative sequence-based discovery of functional transcription factor binding sites. *Genome research*, 12(5):832–9, may 2002.
- [55] Alexander P Lifanov, Vsevolod J Makeev, Anna G Nazina, and Dmitri A Papatsenko. Homotypic regulatory clusters in *Drosophila*. *Genome research*, 13(4):579–88, apr 2003.
- [56] Justin Crocker, Nathan Potter, and Albert Erives. Dynamic evolution of precise regulatory encodings creates the clustered site signature of enhancers. *Nature communications*, 1(7):99, jan 2010.

Appendices

Supplemental Figures

Figure S1. VISTA plot demonstrating extensive sequence conservation upstream of the *eve* start site [54]. Gray boxes denote regions homologous to the *D. melanogaster* minimal regulatory elements. Computationally predicted binding sites based on reported position-weighted matrices [55], demonstrate an extensive number of conserved binding sites for Bcd (Blue), Hb (Red), and Kr (Green)

Figure S2. Multiple species alignment of the *eveS2* minimal enhancer sequence with Zelda motifs and conservation noted. Highlighted Zelda motifs denote the minimal binding site sequence 5'-CAGGTA [40], allowing for one mismatch to identify possible low affinity Zelda binding sites [56, 18].

Supplemental Construct Sequences

>D. erecta eveS2

CGGCGCAATATAACCCAATAATTTGAACCAACTCGCGGAGCAGCGAGGGCATCCTACCCGGTTACCCGGTACT
GCATAACAATGAAACGAAACTGGGACAGATCGGTGATGGTTTCTCGCTGTGTGTGCCGTGTTAATCCGTTTGC
CATCAGCCAGATTATTAGTCAATTGCAGTCGCAGTCGCAGTTGCAGTTGCAGGGTTTCGCTTTCCCTCGTCCTCG
TTTCACTTTTCGAGTTAGACTTTATTGCAGCATCTTGCAGCAACAATCGGCGCAGTTTGGTAACACGCTGTGCCC
TTCCACTTTCCACATTCCACGGCCCAATTCGGCGGATTTAGACGGAATCGAGGGACCCCTGACTATGTTTCGCATA
ATGAAAGCGAAACCAACCGGGTTGCAAAGTCAGGGCATTCCATCGCCGTCGCCATCGCCACCGCCATCTTCT
GCGGGCGTTTGTGTTGTTTGTGCTGGGATTAGCCAAGGGCTTGACTTGGAACCCAATCCCAATCCCAATCCCA
ATCCCAAAGCCAATCCCAGTGCCCATCCCGATCGCAGTCCCATGCCCTTTTCATTAGAAAGTCATAAAAACAC
ATAATAATGATGTGCAACGGATTAGCTGCGCGCAGTCCAGGCAGCGCAATTAACGGACTAGCAAACCTGGGTT
ATTTCTATTTTTTTATTTTCGCCGACTTAGCCCTGATCCGCGAGCTTAACCCGTTTGAGCCGCAGCAGGTAGCC
ATCCCCATCTGGCTTGCGCAAGTGGCAGGTTTCGCTGCCCCAGGAGAGCCGTGGAGGCACTCTGGCCACTGGC
CTGGTACAGTTGCCGCTGGGCATGATTATATCATCATAATAAATGTTT

>D. erecta eveS2 large

TCCTCGAGGATTTCCGGGCTGAATCACTTACTCAACCCGTCGATTCCGTCCGCGCAATCATCATAAATTCTCGGT
CTTTTGTCTGTAATTGTTTTATGGCAGAAATTACTCAATCATCAAGCATAATTCCCTCGTTTTTCGCCGTTTTATT
GCCAATTTTGCAGTGCCTCCCGCCTTTGCCACTCCCGGCCCTTCCCTCATCGTTTTGCGAATCTCCGACGGATT
CGCATTTCTATTGCGCGGACAATCCGGCCAGTGTGTTTGCCATTTACTTGCCATGATGACGGGCATAATCAGC
GAGATCGGCGCTTTGTGAGTGCAGAATGTGCAATAAAGCGGCAACAATCGGCTTGGGATTTCGCCTTCCCCTAT
TCCCAGTATTGCCCGAGTGCCCGGACGACTGCGAAAGTGTTGCGGATCGGGATCGGAATCGGAATAGGAAT
AGGAATACGAGACTGAGCAGAGGCAGGTACTTCCC GCCGGCCGACACTTTCGCCTAACCAAGCGGACTCAA
CCCAACCCAACCAACACAACCCAATCCAACCCACCCGATCGCCATAAAGGGTATTTACTGTGCTGCCGCAG
AGCCTCGCTTGACGACTTAACCCAAGCGGTCTGTTCCACGTCCATTCTCCGACGGAGTCAAAGACAAAGGCCG
GCGGAGCTGGACAATAGGCAAGGTTGTTGCTTGTGGGTAGAGGGTTTCAATCCCGAAATTGTACCTTTCCCGG
GCGAGAAGGGCTTGATGTGGGCCTTTTCCAGGTCGCAGATGTAGGTAGATGTAGATGTAGATGCCGATGGG
CCGGTCGAGTTAATGCCAATGCAAAATGCGGCGCAATATAACCCAATAATTTGAACCAACTCGCGGAGCAGC
GAGGGCATCCTACCCGGTTACCCGGTACTGCATAACAATGAAACGAAACTGGGACAGATCGGTGATGGTTTCT
CGCTGTGTGTGCCGTGTTAATCCGTTTGCCATCAGCCAGATTATTAGTCAATTGCAGTCGCAGTCGCAGTTGCA
GTTGCAGGGTTTTCGCTTTCCCTCGTCTCTGTTTCACTTTTCGAGTTAGACTTTATTGCAGCATCTTGCAGCAACAAT
CGGCGCAGTTTGGTAACACGCTGTGCCCTTCCACTTTCCACATTCCACGGCCCAATTCGGCGGATTTAGACGG
AATCGAGGGACCCCTGACTATGTTTCGCATAATGAAAGCGAAACCAACCGGGTTGCAAAGTCAGGGCATTCCA
TCGCCGTCGCCATCGCCACCGCCATCTTCTGCGGGCGTTTGTGTTGTTTGTGCTGGGATTAGCCAAGGGCTTG
ACTTGGAACCCAATCCCAATCCCAATCCCAATCCCAAGCCAATCCCAGTGCCCATCCCGATCGCAGTCCCAT
GCCCTTTTTCATTAGAAAGTCATAAAAACACATAATAATGATGTGCAACGGATTAGCTGCGCGCAGTCCAGGCA
GCGCAATTAACGGACTAGCAAACCTGGGTTATTTCTATTTTTTTATTTTCGCCGACTTAGCCCTGATCCGCGAGC
TTAACCCGTTTGAGCCGCAGCAGGTAGCCATCCCCATCCTGGCTTGCGCAAGTGGCAGGTTTCGCTGCCCCAGG
AGAGCCGTGGAGGCACTCTGGCCACTGGCCTGGTACAGTTGCCGCTGGGCATGATTATATCATCATAATAAAT
GTTT

>D. erecta eveS2+inter 3/7

TTCGTTCCCAACTACGGCTAAGATATGCCAGTTTGTGTTTGTCTCCGGCAATTATTGGAAATTTTCATTGGGTTCGA
TTGGGTAGATGTGCGATTGGGTGCGATGTGCGATGCCTTCCCTCGGGAAAAGTGAATAGGTTGTGCCATAAAAATC
GCTGCTCTTGAGAGATGAAATGCTGTAGTAGTATGCCAAAGCATCATTCTGCTTTTTTATTTTCTCACTGCTAAA
TGCAGCTAATTTGTGCGATTGTCTGAAAAGTGTTCTCTAAGCCGAAGCACTTTTTTATGATGTTGCTAGAAAATG
AAATCACTTATGTTGCCATACATCCCCAGGCATTTTATGGCCATTTGAGTGCGGGGTGCGCAGTTCTGCTTAAG
TGGCGGATGGAAACCACCACATTTATTCGAGGGATGATGTGCTCTAATACCTCCTCATCAAATGGGATGGTCT
CTTCGCATGGAGAGTGGCAAACCTTTGAAAAGTGAGGCGGAGTTAAAAAGCCGTGCTATATAACAAGATTT
TTGATATTCAGTTATGTATATATGGACAAGAAATATCAAAGACCTTATCAAATATGTTGCCTTTTATCCTCGAA
ATGAAACAAATGCTCTACTAATTTGGCAAGTCAAAAGATCAGTTCAGCAATTATTCAAAGAACATAAAATAT
GCGTATATTTTTGGGAATGTACCAAGTGCTTTCCAAAATAGATTGCCAAACAAATCAACTAATAACTTTAATTTA
AAAAACTGGGCAATCCTGAGTTGGCAGTCTTCCCAAGAATGGCTCCTCGAGGATTTCCGGGCTGAATCACTTAC
TCAACCCGTCGATTCCGTCCGCGCAATCATCATAAATCTCGGTCTTTTTGCTGTAATTGTTTTATGGCAGAAA
TTACTCAATCATCAAGCATAATTCCTCGTTTTTCGCCGTTTTATTGCCAATTTTTGCAGTGCCTCCCGCCTTTGC
CACTCCCGGCCCTTCCCTCATCGTTTTGCGAATCTCCGACGGATTTCGCATTTCTATTGCGCGGACAATCCGGCC
AGTGTGTTTGCCATTTACTTGCCATGATGACGGGCATAATCAGCGAGATCGGCGCTTTGTGAGTGCAGAATGT

GCAATAAAGCGGCAACAATCGGCTTGGGATTGCGCTTCCCTATTCCCAGTATTGCCCGAGTGCCCGGACGAC
 TGCGAAAGTGTGTTGCGGATCGGGATCGGAATCGGAATAGGAATAGGAATACGAGACTGAGCAGAGGCAGGTA
 CTTCCCGCCGGCCGGACACTTTTCGCCTAACCAAGCGGACTCAACCCAACCAACCAACACAACCCAATCCAA
 CCCACCCGATCGCCATAAAGGGTATTTACTGTCGCTGCCGACAGAGCCTCGCTTGACGACTTAACCCAAGCGGT
 CGTTCCACGTCCATTCTCCGGACGGAGTCAAAGACAAAGGCCGGCGGAGCTGGACAATAGGCAAGGTTGTTG
 CTTGTGGGTAGAGGGTTTCAATCCCGAAATTTGACCTTTCCCGGGCGAGAAGGGCTTGCATGTGGGCCTTTTC
 CAGGTGCGCAGATGTAGGTAGATGTAGATGTAGATGCGGATGGGCCGGTTCGAGTTAATGCCAATGCAAATTGC
 GCGCAATATAACCCAATAATTTGAACCAACTCGCGGAGCAGCGAGGGCATCCTACCCGGTTACCCGGTACT
 GCATAACAATGAAACGAAACTGGGACAGATCGGTGATGGTTTCTCGCTGTGTGTGCGGTGTTAATCCGTTTGC
 CATCAGCCAGATTATTAGTCAATTGCAGTCGAGTCGAGTTGCAGTTGCAGGGTTTCGCTTTCTCGTCCTCG
 TTTCACTTTTCGAGTTAGACTTTATTGCAGCATCTTGACGCAACAATCGGCGCAGTTTGGTAACACGCTGTGCC
 TTCCACTTTCCACATTCCACGGCCCAATTCGGCGGATTTAGACGGAATCGAGGGACCCTGACTATGTTTCGCATA
 ATGAAAGCGAAACCAAAACCGGGTTGCAAAGTCAGGGCATTCCATCGCCGTCGCCATCGCCACCGCCATCTTCT
 GCGGGCGTTTGTGTTGTTTGTGCTGGGATTAGCCAAGGGCTTGACTTGGAACCCAATCCCAATCCCAATCCCA
 ATCCCAAGCCAATCCAGTGCCCATCCCGATCGCAGTCCCATGCCCTTTTCATTAGAAAGTCATAAAAACAC
 ATAATAATGATGTGAACGGATTAGCTGCGCGCAGTCCAGGCAGCGCAATTAACGGACTAGCAAACCTGGGTT
 ATTTCTATTTTTTTATTTTCGCCGACTTAGCCCTGATCCGCGAGCTTAACCCGTTTGAGCCGCGAGCAGGTAGCC
 ATCCCATCTGGCTTGCAGAGTGGCAGGTTGCTGCCCCAGGAGAGCCGTGGAGGCACTCTGGCCACTGGC
 CTGGTACAGTTGCCGCTGGGCATGATTATATCATCATAATAAATG

>D. erecta eveS2+S3/7

GGACACAAGGATCCTCGAAATCGAGAGCGACCTCGCTGCATTAGAAAACCTAGATCAGTTTTTTGTTTCGCGTC
 CGCTGATTTTTGTGCCCGTTTGCTCTCTTTACGGTTTATGGCCCCGTTTCCATTATTTTCGTCATTTTTCCACATTT
 CCCAGCTCCTTTGTGCCGCTCAAAGAAATCTGTACGGAATTATGGTATATGCAGATTTTTATGGGTCGCCGATC
 CGGTTTCGCGGAACGCGAGTGTCTGCCGCGAGGACCTCAGCGGCGATCCTTGTGCGGCCGTATTAAGAAAGTA
 GATCACGTTTTTTGTTCCCATTTGTGCGCTTTTTTCGCTGCGCCAGTTCTGTTCCCCGAACCCAGCGAACTGCTCT
 AATTTTTTAATTCTTCACGGACTTTTATTGGGCTCCTGGAAAAACACGCACAAGGCTAGCTCTAGGACTCTACT
 GGCATACCTGTAATGGTGTCCATAACGCGCACTGCTCTCGTTTTTAAGACCCGTTTGTGTTTCGTCCTCTGGAAG
 TAGATGGCATTACATTTTTATGAGTTTCGTTCCCAACTACGGCTAAGATATGCCAGTTTGTGTTGCTCTCGGCA
 ATTATTGGAAATTTTATTGGGTCGATTGGGTAGATGTGCGATTGGGTCGATGTGCGATGCCCTCCCTCGGGA
 GTGAATAGGTTGTGCCATAAAAAATCGCTGCTCTTGGAGATGAAATGCTGTAGTAGTATGCCAAAGCATCATTC
 TGCTTTTTTATTTTCTCACTGCTAAATGCAGCTAATTTGTGCGATTGTCTGAAAAGTGTTCTCTAAGCCGAAGCA
 CTTTTTATGATGTTGCTAGAAAATGAAATCACTTATGTTGCCATACATCCCCAGGCATTTTATGGCCATTTGA
 GTGCGGGGTGCGCAGTTCTGCTTAAGTGGCGGATGGAAACCACCACATTTATTCGAGGGATGATGTGCTCTAA
 TACCTCCTCATCAAATGGGATGGTCTCTTCGCATGGAGAGTGGCAAACCTCTTGGAAAAGTGAGGCGGAGTTAA
 AAAGCCGTGCTATATAACAAGATTTTTGATATTCACTTATGTATATATGGACAAGAAATATCAAAGACCTTAT
 CAAATATGTTGCCTTTTATCCTCGAAATGAAACAAATGCTCTACTAATTTGGCAAGTCAAAAGATCAGTTTCAG
 CAATTATTCAAAGAACATAAAATATGCGTATATTTTGGGAATGTACCAGTGCTTTCCAAAATAGATTGCCA
 AACAAATCAACTAATAACTTTAATTTAAAAAACTGGGCAATCCTGAGTTGGCAGTCTTCCCAAGAATGGCTCC
 TCGAGGATTTCCGGCTGAATCACTTACTCAACCCGTCGATTCCGTCGCGCAATCATCATAAATTCTCGGTCTT
 TTTGCTGTAATTGTTTTATGGCAGAAATTACTCAATCATCAAGCATAATTCCCTCGTTTTTCGCCGTTTTATTGCC
 AATTTTTGCACTGCCTCCCGCCTTTGCCACTCCCGGCCCTTCCCTCATCGTTTTTGCGAATCTCCGACGGATTGCG
 ATTTCTATTGCGCGGACAATCCGGCCAGTGTGTTTGCCATTTACTTGCCATGATGACGGGCATAATCAGCGAG
 ATCGGCGCTTTGTGAGTGCAGAATGTGCAATAAAGCGGCAACAATCGGCTTGGGATTGCGCTTCCCTATTCC
 CAGTATTGCCCGAGTGCCCGGACGACTGCGAAAGTGTGCGGATCGGGATCGGAATCGGAATAGGAATAGG
 AATACGAGACTGAGCAGAGGCAGGTACTTCCCGCCGGCCGGACACTTTTCGCTAACCAAGCGGACTCAACCC
 AACCCAACCAACACAACCCAATCCAACCCACCCGATCGCCATAAAGGGTATTTACTGTCGCTGCCGACAGAGC
 CTCGCTTGACGACTTAACCAAGCGGTCTTCCACGTCCATTCTCCGGACGGAGTCAAAGACAAAGGCCGGCG
 GAGCTGGACAATAGGCAAGGTTGTTGCTTGTGGGTAGAGGGTTTCAATCCCGAAATTTGACCTTTCCCGGGCG
 AGAAGGGCTTGCATGTGGGCCTTTTCCAGGTGCGCAGATGTAGGTAGATGTAGATGTAGATGCGGATGGGCCG
 GTCGAGTTAATGCCAATGCAAATTGCGGCGCAATATAACCCAATAATTTGAACCAACTCGCGGAGCAGCGAG
 GGCATCCTACCCGGTTACCCGGTACTGCATAACAATGAAACGAAACTGGGACAGATCGGTGATGGTTTCTCGC
 TGTGTGTGCCGTGTTAATCCGTTTGCCATCAGCCAGATTATTAGTCAATTGCAGTCGAGTCGAGTTGCAGTT
 GCAGGGTTTCGCTTTCTCGTCCTCGTTTCACTTTTCGAGTTAGACTTTATTGCAGCATCTTGACGCAACAATCG
 GCGCAGTTTGGTAACACGCTGTGCCCTTCCACTTTCCACATTCCACGGCCCAATTCGGCGGATTTAGACGGAAT
 CGAGGGACCCTGACTATGTTTCGCATAATGAAAGCGAAACCAAAACCGGGTTGCAAAGTCAGGGCATTCCATCG

CCGTCGCCATCGCCACCGCCATCTTCTGCGGGCGTTTGTGTTGTTGTTGTTGCTGGGATTAGCCAAGGGCTTGACT
TGGAACCCAATCCCAATCCCAATCCCAATCCCAAGCCAATCCCAGTGCCCATCCCGATCGCAGTCCCATGCC
CTTTTCATTAGAAAGTCATAAAAAACATAATAATGATGTGCAACGGATTAGCTGCGCGCAGTCCAGGCAGCG
CAATTAACGGACTAGCAAACCTGGGTTATTTCTATTTTTTTATTTTCGCCGACTTAGCCCTGATCCGCGAGCTTA
ACCCGTTTGAGCCGCGCAGCAGGTAGCCATCCCCATCCTGGCTTGCGCAAGTGGCAGGTTTCGCTGCCCCAGGAGA
GCCGTGGAGGCACTCTGGCCACTGGCCTGGTACAGTTGCCGCTGGGCATGATTATATCATCATAATAAATGTT
T

>D. erecta eveS2 large del. Zelda

TCCTCGAGGATTTCTGGGCTGAATCACTTACTCAACCCGTCGATTCCGTCCGCGCAATCATCATAAATTCTCGGT
CTTTTTGCTGTAATTGTTTTATGGCAGAAATTACTCAATCATCAAGCATAAATCCCTCGTTTTTCGCCGTTTTATT
GCCAATTTTTGCACTGCCTCCCGCCTTTGCCACTCCCGGCCCTTCCCTCATCGTTTTGCGAATCTCCGACGGATT
CGCATTTCTATTGCGCGGACAATCCGGCCAGTGTGTTTGCCATTTACTTGCCATGATGACGGGCATAATCAGC
GAGATCGGCGCTTTGTGAGTGCAGAATGTGCAATAAAGCGGCAACAATCGGCTTGGGATTTCGCTTCCCCTAT
TCCCAGTATTGCCCGAGTGCCCGGACGACTGCGAAAGTGTGCGGATCGGGATCGGAATCGGAATAGGAAT
AGGAATACGAGACTGAGCAGAGGAATTTACTTCCCGCCGGCCGACACTTTCGCCTAACCAAGCGGACTCAA
CCCAACCCAACCCAACACAACCCAATCCAACCCACCCGATCGCCATAAAGGGTATTTACTGTCGCTGCCGAG
AGCCTCGCTTGACGACTTAACCCAAGCGGTCTGTTCCACGTCCATTCTCCGACGAGGAGTCAAAGACAAAGGCCG
GCGGAGCTGGACAATAGGCAAGGTTGTTGCTTGTGGGTAGAGGGTTTCAATCCCGAAATTGTACCTTTCCCGG
GCGAGAAGGGCTTGCATGTGGGCCTTTTCAATTTGCGAGATGAATTTAGATGTAGATGTAGATGCGGATGGGC
CGGTGAGTTAATGCCAATGCAAATTGCGGCGCAATATAACCCAATAATTTGAACCAACTCGCGGAGCAGCG
AGGGCATCCTACCCGGTTACCCGGTACTGCATAACAATGAAACGAAACTGGGACAGATCGGTGATGGTTTCTC
GCTGTGTGTGCCGTGTTAATCCGTTTGCCATCAGCCAGATTATTAGTCAATTGCAGTTCGAGTTCGAGTTGCAG
TTGCAGGGTTTCGCTTTCCTCGTCCTCGTTTCACTTTCGAGTTAGACTTTATTGCAGCATCTTGAGCAACAATC
GGCGCAGTTTGGTAACACGCTGTGCCCTTCCACTTTCACATTCCACGGCCCAATTTCGGCGGATTTAGACGGA
ATCGAGGGACCCTGACTATGTTTCGCATAATGAAAGCGAAACCAAACCGGGTTGCAAAGTCAGGGCATTCCAT
CGCCGTCGCCATCGCCACCGCCATCTTCTGCGGGCGTTTGTGTTGTTGTTGCTGGGATTAGCCAAGGGCTTGA
CTTGGAACCCAATCCCAATCCCAATCCCAATCCCAAGCCAATCCCAGTGCCCATCCCGATCGCAGTCCCATG
CCTTTTTCATTAGAAAGTCATAAAAAACATAATAATGATGTGCAACGGATTAGCTGCGCGCAGTCCAGGCAG
CGCAATTAACGGACTAGCAAACCTGGGTTATTTCTATTTTTTTATTTTCGCCGACTTAGCCCTGATCCGCGAGCT
TAACCCGTTTGAGCCGCGCAGCAGGTAGCCATCCCCATCCTGGCTTGCGCAAGTGGCAGGTTTCGCTGCCCCAGGA
GAGCCGTGGAGGCACTCTGGCCACTGGCCTGGTACAGTTGCCGCTGGGCATGATTATATCATCATAATAAATG
TTT

>D. erecta eveS2 + 3X Zelda

GAGGCAGGTAATTCCTTTCCAGGTGCGCAGATGTAGGTAGATGTGCGGCGCAATATAACCCAATAATTTGAACCA
ACTCGCGGAGCAGCGAGGGCATCCTACCCGGTTACCCGGTACTGCATAACAATGAAACGAAACTGGGACAGA
TCGGTGATGGTTTCTCGCTGTGTGTGCCGTGTTAATCCGTTTGCCATCAGCCAGATTATTAGTCAATTGCAGTC
GCAGTCGAGTTGCAGTTGCAGGGTTTCGCTTTCCTCGTCCTCGTTTCACTTTCGAGTTAGACTTTATTGCAGC
ATCTTGAGCAACAATCGGCGCAGTTTGGTAACACGCTGTGCCCTTCCACTTTCACATTCCACGGCCCAATTC
GGCGGATTTAGACGGAATCGAGGGACCCTGACTATGTTTCGCATAATGAAAGCGAAACCAAACCGGGTTGCAA
AGTCAGGGCATTCCATCGCCGTCGCCATCGCCACCGCCATCTTCTGCGGGCGTTTGTGTTGTTGTTGCTGGGA
TTAGCCAAGGGCTTGACTTGGAACCCAATCCCAATCCCAATCCCAATCCCAAGCCAATCCCAGTGCCCATCC
CGATCGCAGTCCCATGCCCTTTTCATTAGAAAGTCATAAAAAACATAATAATGATGTGCAACGGATTAGCTG
CGCGCAGTCCAGGCAGCGCAATTAACGGACTAGCAAACCTGGGTTATTTCTATTTTTTTATTTTCGCCGACTTAG
CCCTGATCCGCGAGCTTAACCCGTTTGAGCCGCGCAGCAGGTAGCCATCCCCATCCTGGCTTGCGCAAGTGGCAG
GTTTCGCTGCCCCAGGAGAGCCGTGGAGGCACTCTGGCCACTGGCCTGGTACAGTTGCCGCTGGGCATGATTAT
ATCATCATAATAAATGTTT