DECIPHERING SEGMENTATION EVOLUTION: IDENTIFYING *EVE* ENHANCERS IN *TRIBOLIUM CASTANEUM*

By

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Abstract

Arthropods are a phylum of invertebrates that are distinguished by their ability to generate segments in their body plan, utilizing at least two modes of development to do so. *Drosophila melanogaster* is a long-germband insect that can give rise to its segments in a simultaneous manner. The segmental development of *Tribolium castaneum* is characteristic of a short-germ band insect that develops its segments sequentially through the regulation of a molecular oscillator. These two arthropods have in common a segmentation gene termed *even-skipped* (*eve)* that oversees how the segments are created. Using this gene, we can examine how it is that these two different mechanisms of segmentation came to exist. In *Drosophila*, stripe-specific and modular enhancers regulate and drive expression of *eve; the enhancers of Tribolium eve* are unknown. To study the enhancers that regulate *eve* in *Tribolium*, we used a bioinformatics tool to aid us in predicting putative enhancers. *Tribolium* and *Drosophila* transgenics were then created that have one of five constructs integrated into their genome and enhancer-mCherry fusions were utilized to visualize the expression patterns. Immunohistochemistry revealed that the *Tc-eve* intron 2 enhancer drives expression in the posterior growth zone, hindgut, and nervous system in *Tribolium*, whereas in *Drosophila,* the *Tribolium* enhancer drove expression in the dorsal vessel (heart) and consistently the hindgut. To date, the transgenics and wild-type endogenous *eve* share expression only in the hindgut. Our current data suggests that the candidate *Tc-eve* intron 2 enhancer regulates eve during posterior growth, a function unique to sequentially segmenting insects and later-hindgut expression of *eve* - a function shared more broadly throughout metazoans.

Introduction

Segmentation is the morphological process that divides the developing embryo into segments that create the foundation of the adult body plan. Three phyla within the tree of life are known to carry out this process among which are arthropods, for which *Drosophila melanogaster* (fruit fly) has been the oldest and most well-studied model for segmentation. *Drosophila* segment patterning occurs in a rapid and nearly simultaneously manner during the first three hours of embryogenesis. A genetic cascade of sequentially expressed genes and their transcription factors governs this segmentation process (reviewed in (Clark, 2017). This cascade of transcription factors subdivides the embryo into successively more precise and smaller domains. Specifically, the pair-rule genes in the cascade are directly responsible for setting the location of segment boundaries and then activating the segment-polarity genes to maintain these boundaries ((DiNardo & O'Farrell, 1987); (Ingham et al., 1988); (Sackerson et al., 1999); (Clark, 2017); (Diaz-Cuadros et al., 2021)). Segment boundaries are set after the seven-striped pair-rule genes expression resolves into a 14-striped expression domain.

The nearly simultaneous manner of segmentation exhibited by *Drosophila* is a derived mechanism and rather most segmenting animals do so in a sequential manner, where segments are added one (or two) at a time (Diaz-Cuadros et al., 2021). *Tribolium castaneum* (red flour beetle) has emerged as a model for studying sequential segmentation. In *Tribolium*, the process is proposed to be controlled by a molecular oscillator that functions in the posterior of the embryo in a region known as the “growth zone,” from which the new segments originate ((El-Sherif et al., 2012); (El-Sherif et al., 2014); (Diaz-Cuadros et al., 2021)). The molecular oscillator, a segmentation clock composed of genes that dictate the periodicity of cellular and tissue events occurring within the embryo, propagates waves of gene expression to drive segment development ((El-Sherif et al., 2014); (Diaz-Cuadros et al., 2021)).

While the *Drosophila* segmentation hierarchy is very well studied, there is still much to be discovered about the genetic control of *Tribolium* segmentation. Over the years, various segmentation genes have been studied and one in particular has drawn our interest: *even-skipped* (*eve*).

Why *eve*? *Drosophila* and *Tribolium* segmentation both require the pair-rule gene *eve*. Pair-rule genes were named because their intermediate loss of function results in deletion of every other segment (Nüsslein-Volhard & Wieschaus, 1980); (O & Choe, 2020). *eve* is initially expressed in seven stripes in both arthropods, but the stripes come on all at once in *Drosophila*, whereas they come on one at a time, as an output of the oscillator, in *Tribolium*. When there is a complete loss of *Tribolium eve* function, the embryo does not develop any segments at all, as expected considering a component of the oscillator is knocked out, thus preventing it from oscillating at all. This led us to suspect that comparing the *eve* enhancers in *Drosophila* and *Tribolium* would provide insight about how the molecular oscillator functions and how segmentation evolved from one type to another.

In addition, the regulatory logic of how *Drosophila eve* is controlled is the best known on Earth and Mars. Elegant studies have shown that early striped *Drosophila eve* transcription is controlled by modular stripe-specific enhancers ((Small et al., 1991); (Small et al., 1996); (Fujioka et al., 1999); (Hare et al., 2008); (Peterson et al., 2009); (Berrocal et al., 2020)). These enhancers drive expression of one (*i.e.*, stripe 2) or a pair (*i.e.*, stripes 4+6) of *eve* stripes in the *Drosophila* embryo. Nothing is known at this time about what enhancers are responsible for the regulation of *eve* in *Tribolium*; maybe the enhancers are simpler and only need to respond to

the oscillator? Or maybe the stripe specific enhancers are conserved in *Tribolium* and used sequentially?

Lastly, pair-rule gene enhancers have also been studied in a few species outside of other *Drosophilids*. One study in sepsid insects, relatively close phylogenetically to *Drosophila*, showed that the primary DNA sequence of the stripe-specific enhancers is not conserved but their position relative to the open reading frame (ORF) is (Hare et al., 2008). Another study conducted on the *Tribolium* pair-rule gene and molecular oscillator member *runt,* provided evidence of the existence of a stripe-specific enhancer for *runt* stripes 1 and 2 in *Tribolium* (Christine et al., 2022). Another study demonstrated a small enhancer region located upstream of the *Tribolium hairy* gene that drove expression in both the growth zone and stripe 3 (Eckert et al., 2004). Based on the existing evidence, we predicted that *Tribolium eve* enhancers would be modular stripe-specific enhancers conserved in position relative to the ORF.

Our hypotheses were also influenced by models that have recently been proposed to account for how it is that evolution switches between simultaneous and sequential modes. One such model, the Speed Regulation Model, (Zhu et al., 2017); (Boos et al., 2018); (Rudolf et al., 2020); (Christine et al., 2022) proposes that segmentation is under the control of a speed regulator, or morphogen, that acts to sequentially activate gene expression domains in elongating and non-elongating tissues. *caudal* (*cad*) has been tendered as the "speed regulator," in *Tribolium* and *Drosophila*, but in other animals it may be another transcription factor that displays similar expression dynamics to that of *cad*. The speed regulator can be present in either a retracting anterior to posterior gradient to pattern elongating tissues, or a static gradient with its high point at the posterior to pattern non-elongating tissues. *Drosophila* is an example of non-elongating embryo that is patterned using a static gradient allowing for the near simultaneous gene expression domains. *Tribolium* is an example of an elongating embryo that is patterned by a retracting *cad* gradient ((El-Sherif et al., 2014); (Christine et al., 2022)). At the enhancer level, the Speed Regulation model posits that the speed regulator acts on two types of enhancers, dynamic and static. A dynamic enhancer is charged with establishing gene expression domains towards the posterior of the embryo and a static enhancer stabilizes these gene expression domains. Zhu proposes that static enhancers are mainly functioning in *Drosophila* to create the simultaneous expression patterning. If true, the dynamic enhancers are mainly at work in the *Tribolium* growth zone and static enhancers (modular stripe-specific enhancers) function transiently, as the pair-rule stripes resolve and the germband elongates.

The enhancers of *eve* in any species would also need to drive *eve*'s late, tissue-specific functional domains. *Drosophila eve* functions in four specific tissues later in embryogenesis, including the nervous system ((Doe, Smouse, et al., 1988); (Broadus et al., 1995); (Fujioka et al., 2003)), the anal pad of the hindgut ((Singer et al., 1996); (Gorfinkiel et al., 1999); (Moreno & Morata, 1999)), and the progenitors of the dorsal vessel (heart) of the *Drosophila* embryo ((Su et al., 1999); (Fujioka et al., 1999); (Tögel et al., 2008)). Those same progenitors also differentiate into dorsal acute muscle 1 (DA1 muscles), which are *eve* positive ((Doe, Smouse, et al., 1988); (Landgraf et al., 1999); (Fujioka et al., 2005)). Heart and muscle are regulated by a singular modular enhancer downstream of the ORF, but the neural enhancers are dispersed downstream of the ORF ((Sackerson et al., 1999); (Fujioka et al., 2005)). Anal pad enhancers are dispersed and located both upstream and downstream of the ORF ((Goto et al., 1989); (Jiang et al., 1991); (Sackerson et al., 1999); (Fujioka et al., 2005)).

Expression of *eve* late in *Tribolium* embryogenesis is yet to be thoroughly described, but some conserved tissue-specific roles for *eve* have been reported. *Tribolium eve* is expressed in the

same clusters of neurons and pericardial cells as seen for *Drosophila eve* ((Cande et al., 2009); (Biffar & Stollewerk, 2015)), suggesting it may be under the control of the same regulatory components. Whether they share similar enhancer distribution remains completely unknown.

To uncover the regulatory logic of the *Tribolium eve* locus, we have been studying the expression of putative enhancers of the *Tribolium eve* gene fused to a fluorescent reporter in both *Tribolium* and *Drosophila* transgenics. A team of previous students used MCAST (Bailey & Noble, 2003), a motif cluster alignment and search tool that searches for statistically significant clusters given a set of motifs, to find putative enhancer regions around the *Tribolium eve* locus, based on the binding site affinity of transcription factors known to regulate *Drosophila eve*. Using the clustered binding sites found with the tool, five fragments were chosen. These five putative *Tribolium eve* enhancers were cloned into constructs carrying the enhancer fragments fused to an mCherry reporter. Extensive research into previous studies was conducted to generate a comprehensive list of all the transcription factors known to associate and interact with the *eve* gene in both *Drosophila* and *Tribolium*. MCAST was then used again to predict where along the *Tribolium eve* locus the transcription factors would bind (Figure 1, red box denotes this works fragment of interest) to enable speculation of what expression patterns would be seen in the transgenics.

Interestingly, *Drosophila eve* only has one intron, whereas *Tribolium eve* contains two and the intron 2 fragment, not found in *Drosophila*, is what this thesis will focus on. Here I examine the role of *Tribolium eve* intron 2 (*Tc-eve* intron 2) enhancer throughout embryogenesis in transgenic *Tribolium*, using immunohistochemistry and live imaging techniques. I was particularly interested in its inability to drive stripe formation but instead provide evidence that it is a dynamic enhancer, expressed in the posterior growth zone. *Tc-eve* intron 2 enhancer additionally drives tissue-specific expression, overlapping with endogenous *Tribolium eve* in the hindgut. Though *Tc-eve* intron 2-mCherry expression is extensively observed in the *Tribolium* nervous system, it is not in the same cells that express *eve*. *Tc-eve* intron 2 also drives hindgut and heart expression when inserted into transgenic *Drosophila.* The capability of the *Tc-eve* intron 2 enhancer to drive hindgut expression in both species may indicate the relocation of the enhancer from within the intron in *Tribolium* to downstream of the ORF in *Drosophila*.

Results

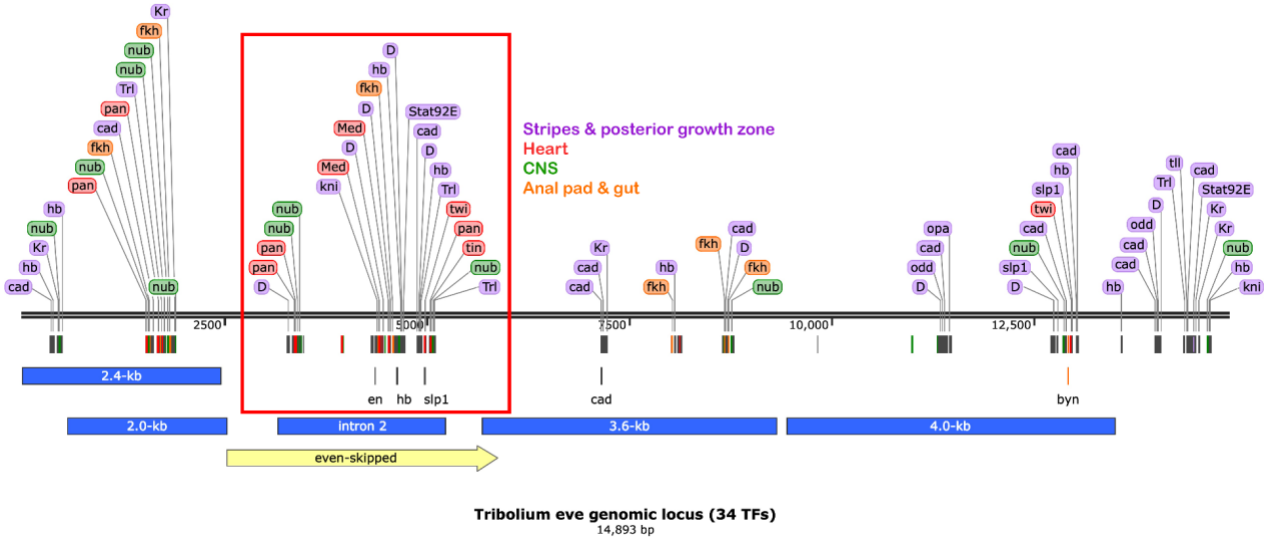


Figure 1: MCAST predicts expression patterns of putative *Tribolium eve* enhancers
Transcription factors that are known to interact with the *eve* locus in *Drosophila* and predicted to interact with the *eve* locus in *Tribolium* were run through MCAST against the *Tribolium* locus. Red box indicates this work's fragment of interest, intron 2 (intron 1 not shown), and all the TFs that have predicted binding sites on it. TFs are color-coded based on what expression patterns they are involved in; purple = early acting, red = heart, green = CNS, orange = gut/anal pad. *Tribolium eve* coding region and direction of transcription are depicted as yellow arrow and direction of arrow, respectively. Not all TF binding sites identified via MCAST are shown in Figure 1. All TFs ran are summarized in supplementary table 1.

MCAST

We extensively examined published studies that discussed TFs known to regulate *Drosophila eve* and added TFs predicted to be involved in regulation of the molecular oscillator that controls *Tribolium* segmentation or act early in *Tribolium* segmentation. From this, we generated a list of 34 TFs that we used to predict the locations of potential *Tribolium eve* enhancers (Figure 1; (Bailey & Noble, 2003). Intron 2 (2,063 bp) harbors the most predicted binding sites when compared to the other fragments: 19 out of the 34 TFs had predicted binding sites within the intron 2 fragment.

TFs were grouped based on the four domains of *eve* expression; 12 are regulators of *eve* stripes and/or PGZ, 3 are involved in anal pad and gut morphogenesis, 4 are known to drive heart development, and 7 are involved in CNS development. Not all TF binding sites identified via MCAST are shown in Figure 1 and most of these TFs are involved in development of more than one tissue.

The TFs colored in purple in Fig. 1 act early in embryonic development, which is when stripe formation and posterior growth zone (PGZ) expression of *eve* occur. TFs mainly involved in the PGZ are *D, hb, cad*, *pan, Trl, nub,* and *byn*. Stripe formation TFs are stat92e, *slp1, Trl, runt, kni, tll*, *en,* and *inv.* Tissue-specific TFs act later in embryogenesis, after stripe formation has concluded. The heart-specific TFs are *pan, Med, twi,* and *tin*. There were 7 TFs with binding sites that are involved in the CNS development, *nub*, *ftz*, *hb*, *D*, *runt*, *fkh*, and *en*. Again we only saw 3 anal pad and gut TFs that had predicted sites, *byn*, *cad*, and *fkh*. (Additional information on MCAST can be found in Supplementary Material - Sup. Doc. 1 & Sup. Table 1)

Based on the MCAST predicted TF binding sites, I hypothesized that the potential Tc-eve intron 2 enhancer will drive mCherry reporter expression in the PGZ & stripes, heart, CNS, and anal pad/gut.

***Tribolium* transgenics**

To assess the *Tc-eve* intron 2 expression forecasted by MCAST, a piggyGUM construct with the potential *Tc-eve* intron 2 enhancer driving expression of mCherry, along with the 3x Pax3 eye-GFP, enhancer was made by former postdoc Ben Goldman-Huertas. I injected this construct along with a piggyBac construct expressing a p-element transposase into 138 early embryos. 15 mating pairs were set up to collect and identify transgenic (green eyed) progeny. 1 green-eyed pupae was recovered from one mating pair on the first week of egg collections. One transgenic line was established. Immunohistochemical and HCR techniques were utilized to visualize *Tc-eve* intron 2 driven mCherry and endogenous Eve protein and mRNA expression patterns.

Tc-eve intron 2 contains a posterior growth zone enhancer

Endogenous Eve expression begins in the blastoderm and can be detected in the posterior half of the embryo (El-Sherif et al., 2012). This capped expression resolves into the first blastoderm stripe, with the second and third stripes coming on in the same manner prior to the onset of gastrulation and extension of the germband (El-Sherif et al., 2012). No mCherry was detected in these stages.

Eve expression next observed in stripes propagating from the PGZ, which split into two segmental primordia (white arrows Fig. 2A) as they mature, move anteriorly, and then ultimately disappear. mCherry expression is first detected in the PGZ starting at the early germband stage

in a broad posterior domain that overlaps with endogenous Eve expression (Fig. 2A). *Tc-eve* intron 2-mCherry expression in the PGZ never resolves into stripes. As germband elongation proceeds, the mCherry expression retracts posteriorly (Fig. 2B-D) and is maintained in the posterior region as this region becomes hindgut (Fig. 3). Note that both mCherry and Eve expression are more abundant in the midline of the posterior region, the presumptive mesoderm, during segment addition (Fig. 2A-C).

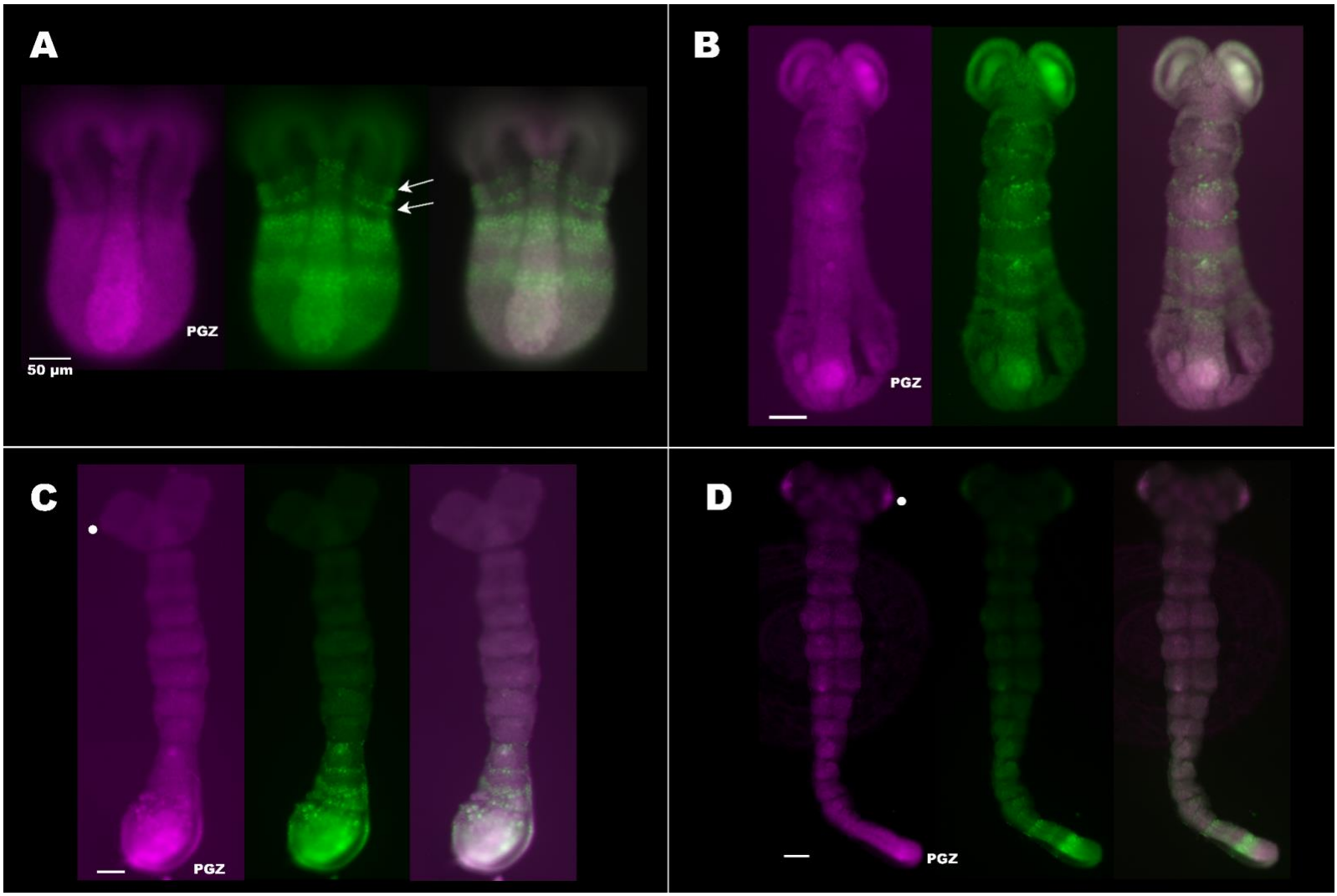


Figure 2: *Tc-eve* intron 2 harbors a posterior growth zone enhancer
Whole-mount embryos were stained with anti-mCherry (magenta) and anti-Eve (green,) antibodies, merged image (right image in each panel). Areas of overlap are seen in white. (A) Early germband embryo with *Tc-eve* directing broad PGZ mCherry expression, overlapping with Eve PGZ expression; arrows in Eve (green) image point to stripe that has split in two; n=2. (B, C, D) progressively older embryos as germband elongates and mCherry expression retracts towards the posterior. C and D have mCherry expression in the head nodules, discreetly in C but very pronounced in D and marked by white filled circles. PGZ = posterior growth zone.

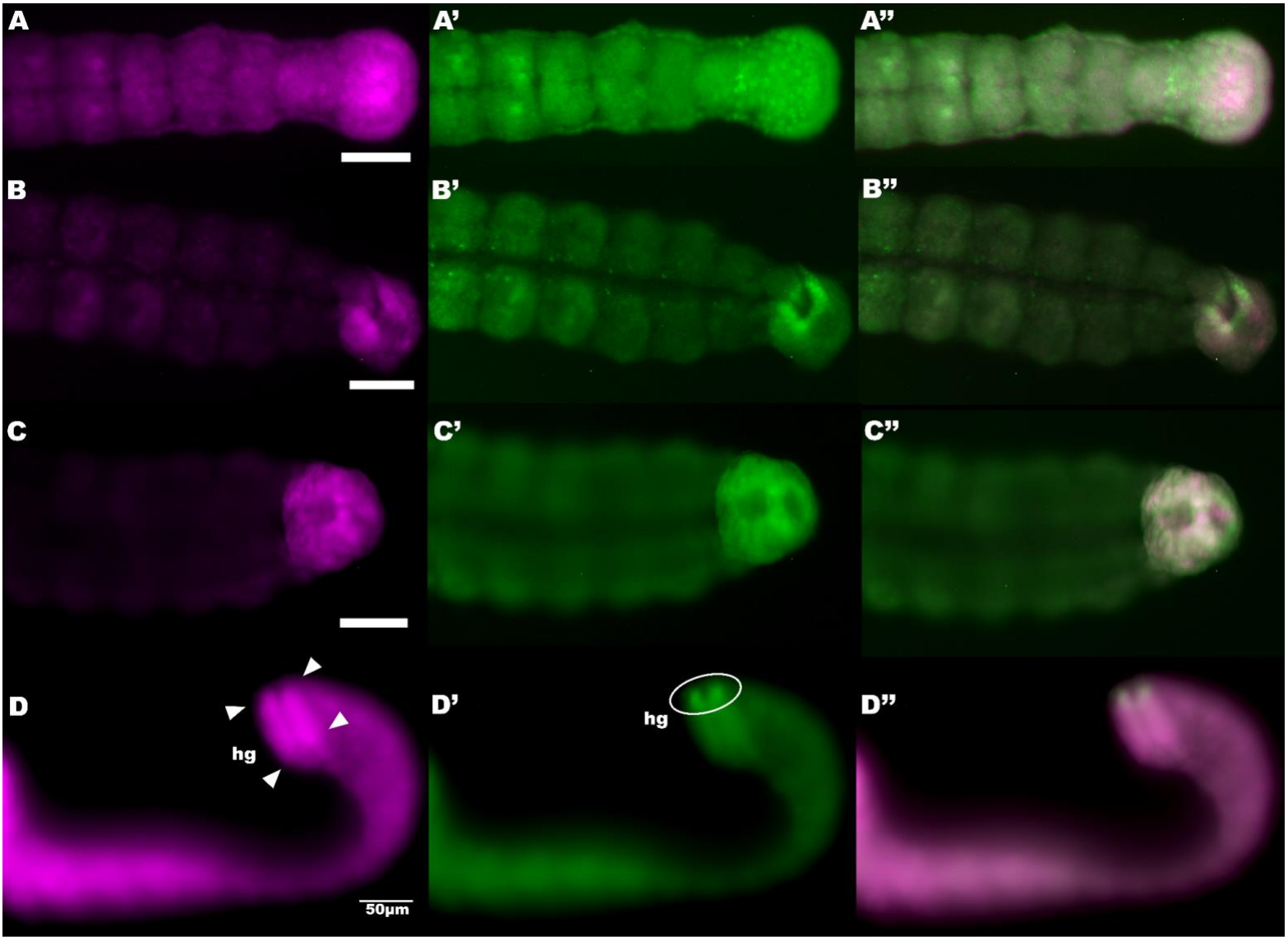


Figure 3: *Tc-eve* intron 2 enhancer activity in *Tribolium* hindgut morphogenesis
Whole-mount embryos were stained with anti-mCherry (magenta, left image in each panel) and anti-Eve (green, middle image in each panel) antibodies; merged images (right image in each panel) of anti-mCherry and anti-Eve, with areas of overlap seen in white. *Tc-eve* intron 2-mCherry PGZ expression is maintained in the posterior as it develops into a mature hindgut. (A-D, A'-D', A''-D'') Progressively older embryos. Arrows in A, A', and A'' point to expression in the hindgut anlage. Circles in B and B' highlight expression in inner proctodeum; overlap between mCherry and Eve can be seen in B''; early Eve-expressing CNS cells can be detected as well at the midline in B'. (D, D', D'') 20x insets of late retracting embryo with *Tc-eve* intron 2-mCherry active in the hindgut and enhancing Eve in the most posterior hindgut. pd = proctodeum; hg = hindgut.

Late tissue specific eve expression

eve in *Drosophila* and *Tribolium* has additional functions in three specific tissues: hindgut, nervous system and heart ((Fujioka et al., 1999); Figure 4)). It is plausible to speculate that the conservation of these late *eve*-expressing tissues might indicate the same TFs are regulating *eve* in the hindgut, nervous system, and heart formation of both species.

Tc-eve intron 2 enhances eve in the posterior hindgut

As segment addition comes to completion, the cells in the posterior begin to contribute to the development of the hindgut (Figure 3). It is highly likely that *Tc-eve* intron 2 enhances *eve* activity during the development of the mature hindgut, as overlap between the Eve and mCherry expressing cells can be detected. mCherry and Eve expression are first detected in the hindgut anlagen (arrows in Fig. 3A, 3A', 3A''). Eve is expressed in the last pair-rule stripe (Fig. 3A'), before becoming restricted to the inner proctodeum (Fig. 3B' & 3B''). As development continues, both signals are visible in the primordial tubing of the hindgut (Fig. 3C''). *Tc-eve* intron 2 is expressed in a slightly bigger domain than Eve: mCherry is expressed in the entire hindgut domain whereas Eve becomes confined to the posterior region of the developing hindgut (Fig. 3D, D', D'').

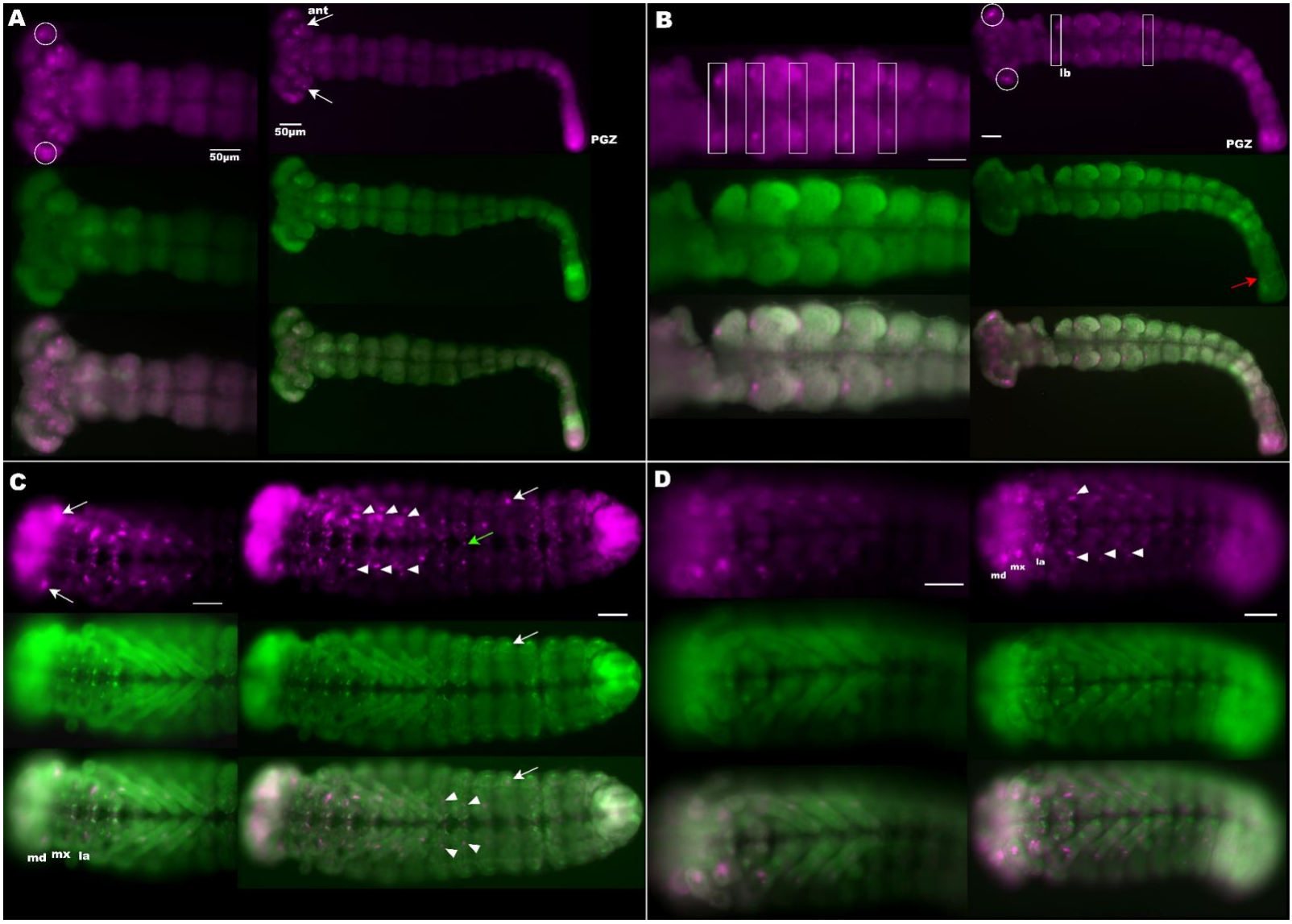


Figure 4: A dynamic neural expression pattern is modulated by *Tc-eve* intron 2
Whole-mount embryos were stained with anti-mCherry (magenta, top image in each panel) and anti-Eve (green, middle image in each panel) antibodies; merged images (bottom image in each panel) of anti-mCherry and anti-Eve. (A-D) Progressively older embryos; left panel is 20x inset of anterior germband. (A and B) Embryo shown in A is the same as in Fig. 2D. Eve and mCherry expression in head lobes (circles), developing brain, and bilaterally paired developing neurons in antennae (arrows), gnathal and limb buds (rectangles). mCherry expression persists in PGZ, Eve is expressed in the last of the pair-rule stripes (A & red arrow in B). (C) Dorsal view; mCherry and Eve expression expands in developing brain and head lobes; mCherry and Eve detected in non-overlapping bilaterally paired neurons in CNS; mCherry expressing neurons are posterior to the Eve-expressing neurons (arrowheads in merged image); Eve and mCherry overlap in hindgut; Eve is detected in pericardial cells on the dorsal margins (green arrow). (D) ventral view; mCherry expression persists in head and CNS; Eve is still expressed in CNS. (C & D) white arrowheads point to bilateral pairs of neuronal cells in legs. PGZ = posterior growth zone; ant = antennae; md = mandible; mx = maxilla; la = labrum. Anterior is left.

Tc-eve intron 2 directs neural activity during Tribolium embryogenesis

Tribolium and *Drosophila* have Eve expression in some ganglion mother cells (GMCs) and their progeny neurons, aCC, pCC, U/CQ, RP2, and along with that in EL neurons, but not their GMCs ((Doe, Smouse, et al., 1988); (Duman-Scheel & Patel, 1999); (Fujioka et al., 2003); (Biffar & Stollewerk, 2015)). Additionally, *Tribolium* Eve comes on late in neurogenesis in a subset of neurons named PEL (posterior Eve lateral cluster) solely due to their position (Biffar & Stollewerk, 2015).

Tc-eve intron 2 activates an intricate pattern of neural expression during germband elongation (Fig. 2C, D and Fig. 4). Neural activity is first detected on the lateral edges of the developing head lobes, (Fig. 2C, white circles), which later expands to cells in the presumptive brain as elongation continues (Fig. 2D & 4A). Next, two dots on either side of the developing thoracic appendages appear, (Fig. 4B, white rectangles), which we later observe as bilateral pairs of neurons as the appendages develop (Fig. 4C & 4D, white arrowheads in magenta images). As these mCherry patterns emerge, the Eve signal is only detected in stripes at the posterior (Fig. 4A & red arrow in 4B).

As expected, we observe neural expression of endogenous Eve expression in discrete, bilaterally paired neuroblasts: in aCC & pCC neurons, EL neurons, RP2 neurons, U/CQ neurons, and eventually PEL (posterior Eve lateral cluster) neurons (Fig. 3B and Fig. 4C & 4D not labeled). These Eve-expressing neuronal cells seem not to overlap with mCherry, instead we observe clusters of mCherry-expressing neuroblasts immediately posterior to the Eve expressing neuroblasts, in the maxillary to second thoracic segment. It is also feasible that the mCherry signal is located cytoplasmically and the Eve signal is nuclear, further experimentation would need to be done to determine this.

In addition, mCherry is detected in more laterally positioned neurons towards the middle and posterior of the embryo, (Fig. 4C, arrowheads in merge channel). At the center of each segment, in between the clusters of Eve-expressing neurons, a cell or cells are faintly expressing mCherry (Fig. 4C, white circles) and although these cells are not visible in every segment (Fig. 4D), that may be due to lack of a higher resolution image.

Despite the abundant levels of mCherry signal in the neural tissue, there is no direct overlap with endogenous Eve.

Tc-eve intron 2 does not drive expression in the cardioblasts or heart cells

A lineage of pericardial cells express *eve* in *Drosophila* and *Tribolium, Drosophila* expression begins earlier on during mesoderm differentiation ((Su et al., 1999); (Cande et al., 2009)) and *Tribolium* expression appears to start later in the course of germband retraction (Fig. 4C). Detection of Eve in the pericardial cells is discernible, (Fig. 4C, white arrow in green channel), yet no mCherry signal is present overlapping. Rather, an mCherry signal is detected directly next to the eve-expressing pericardial cell (Fig. 4C, white arrow in magenta whole mount) and has been observed in anterior segments as well. These mCherry-expressing cells appear to be more dorsal than the pericardial Eve cells. Better images or co-staining with other pericardial markers, such as tinman (Cande et al., 2009), would need to be acquired to make a more definitive conclusion about whether they are truly cardiac cells or not.

***Drosophila* transgenics**

The cluster of binding sites identified in the *Tribolium eve* intron enhancer are located within the second intron; the *Drosophila eve* gene only contains one intron. This leads to a hypothesis that features unique to *Tribolium eve* could be harbored in this intron. So, what expression patterns would be observable if you were to insert a putative *Tribolium eve* intron 2 enhancer into *Drosophila*? This question was answered using transgenic *Drosophila* lines.

Seven *Drosophila* lines were maintained, which differed in their chromosomal integration site of the intron 2-mCherry construct. Embryos from homozygous stocks of these lines were examined for mCherry fluorescence with live imaging throughout embryonic development. No expression was detected prior to germband retraction. One expression pattern was consistently seen across all transgenic lines: anterior and posterior hindgut expression. The intensity of the intron 2-mCherry expression patterns tended to vary between the balancer lines. Another expression pattern that stood out was all along the dorsal vessel (*Drosophila* heart), which only appeared in two transgenic lines.

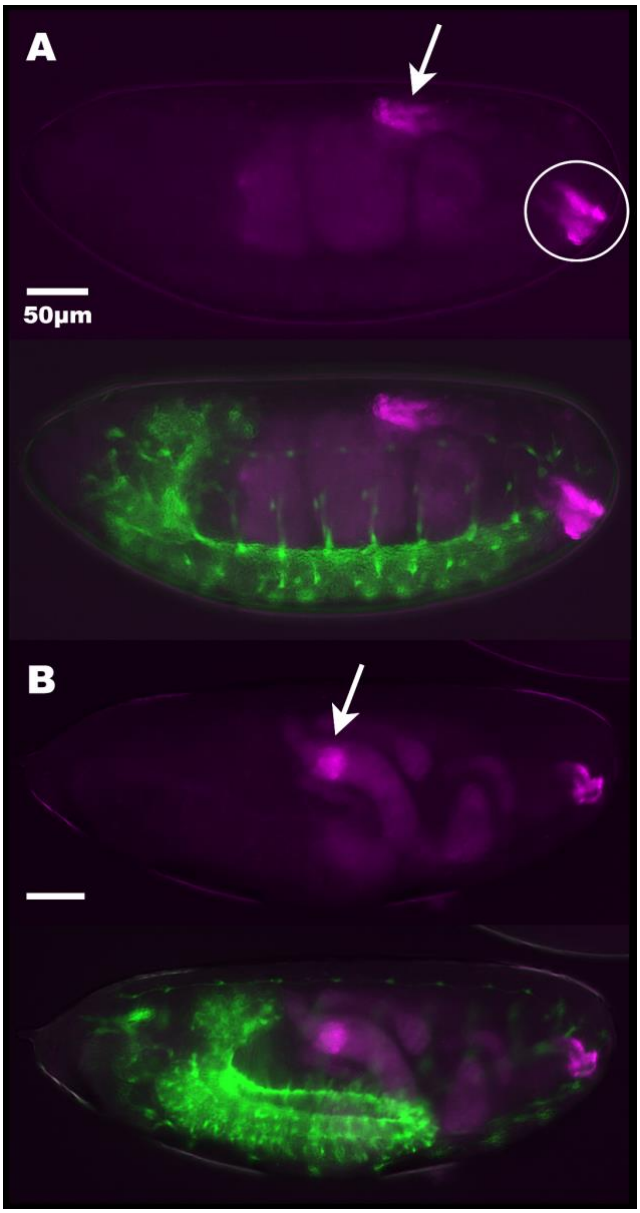


Figure 5: *Tc-eve* intron 2 transgene is active during late *Drosophila* hindgut development
Live imaging of transgenic *Drosophila* carrying *Tc-eve* intron 2-mCherry fusion construct. (A) Embryo right before ventral nerve cord shortening and (B) during ventral nerve cord shortening. The arrows follow the anterior hindgut expression as the hindgut becomes a tubular structure. Posterior hindgut expression is demarcated in A by the circle and still observed in B. Both embryos are shown in lateral views and were live-imaged for *Tc-eve* intron 2-mCherry fusion construct expression (magenta) and 3xP3 driven GFP (green). Anterior is left and dorsal is up.

Tc-eve intron 2 is active during late hindgut morphogenesis

Endogenous *eve* expression in *Drosophila* anal pad primordia, formed from part of the seventh *eve* stripe, begins after gastrulation ((Singer et al., 1996); (Sackerson et al., 1999); (Fujioka et al., 1999)) and persists in the anal pad as development continues. Whereas the posterior and most anterior hindgut expression, driven by the *Tc-eve* intron 2 enhancer, starts after germband retraction (Figure 5) and is confined to those regions for the rest of embryogenesis. Regionalized hindgut expression can be observed in late-staged embryos with completely retracted ventral nerve cords (Supplementary Fig. 1), but expression is still maintained and concentrated in the most posterior and anterior hindgut. HCR experiments revealed the endogenous *eve* expression is not enhanced by *Tc-eve* intron 2, in light of the fact that there is no overlap seen between the expression patterns (not shown). This *Tc-eve* intron 2 hindgut expression in transgenic *Drosophila* is similar to the hindgut expression that is driven by the same enhancer in transgenic *Tribolium* (Figure 3). All transgenic lines had this observed expression.

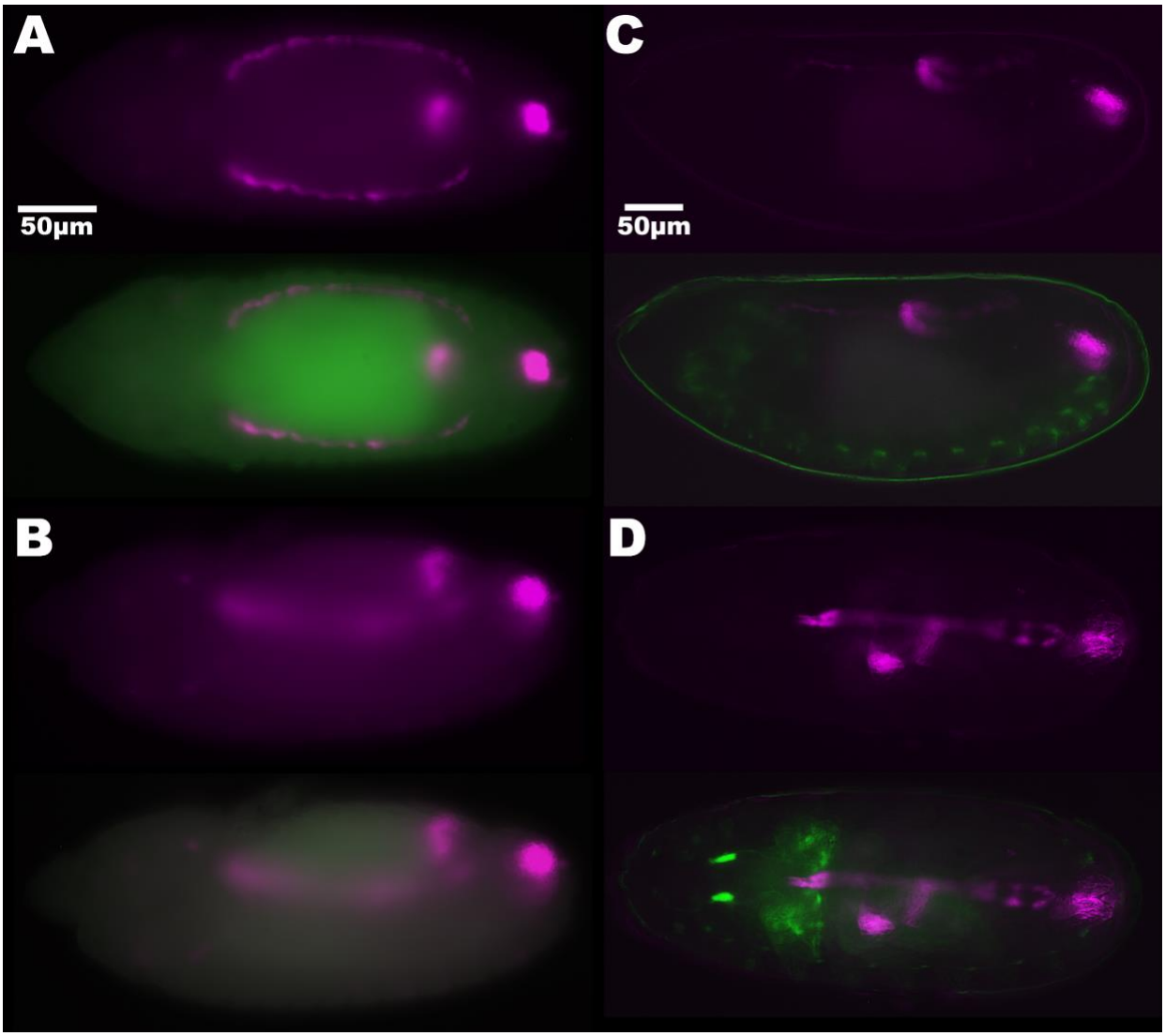


Figure 6: *Tc-eve* intron 2 activity in transgenic *Drosophila* heart
Live imaging of transgenic *Drosophila* carrying *Tc-eve* intron 2-mCherry fusion construct. (A,B) The same embryo in a dorsal view (A) and a lateral view (B) at the beginning of dorsal closure strongly expressing the *Tc-eve* intron 2 enhancer. (C) Later staged embryo than A and B, in a lateral view and faintly expressing enhancer construct. (D) Embryo at the end of ventral nerve cord shortening with expression in the dorsal vessel, but containing spaces of no expression; expression pattern that appears to be stemming off the dorsal vessel is part of the anterior hindgut expression. (A-D) Below each magenta image is a merge of mCherry and 3xp3-GFP; have anterior and posterior hindgut expression discussed in figure 5. Anterior is left.

Tc-eve intron 2 drives late heart expression

The putative *Tc-eve* intron 2 enhancer directs expression in the heart and aorta of the *Drosophila* dorsal vessel (Fig. 6). Expression is initially observed during dorsal closure (Fig. 6A), which begins about 11 hours into embryogenesis, and is sustained until hatching. *Drosophila eve* is expressed earlier in precursors for pericardial cells in segmentally repeated clusters that later differentiate into mature pericardial cells and muscle ((Frasch et al., 1987); (Sackerson et al., 1999); (Landgraf et al., 1999)). All the cardioblasts along the lateral edges are expressing the transgene (Fig. 6A) and as time goes on, the two lines of cells on either side migrate towards the dorsal midline eventually coming together to form the heart tube (Fig. 6C & 6D). As embryonic development nears the end, the pattern in the heart region changes and we now see areas of missing expression (Fig. 6D) that may correspond with ostial cells of the heart. There was no detectable cardiogenic mesodermal expression prior to dorsal closure detected by live imaging.

Discussion

Among the five predicted *Tc-eve* enhancers, intron 2 was particularly surprising and exciting. *Drosophila* only possess one small intron and none of the reported *Drosophila eve* enhancers fall within this region. In contrast, there is a 2.0-kb second intron in *Tribolium* that had the most MCAST predicted transcription factor binding sites, hence a strong likelihood of regulating patterns of *eve* unique to *Tribolium*. My findings demonstrate this to a degree- *Tc-eve* intron 2 is highly active and expressed in the PGZ, which retracts posteriorly as the germband grows. This PGZ expression overlaps with endogenous Tribolium eve expression and this domain is not found in *Drosophila*.

Wild type *Tribolium* expression

To understand which *Tc-eve* intron 2 driven expression domains represent regulation of endogenous *eve* we first had to complete a careful analysis of wildtype expression throughout development. Past studies have carefully characterized endogenous *eve* expression in early *Tribolium* embryos, but its expression past 24 hours of development has only briefly been reported ((Patel et al., 1994); (Brown et al., 1997); (El-Sherif et al., 2012); (O & Choe, 2020)). We found that as the last pair-rule stripe reconciles, CNS expression slowly becomes visible (Fig. 3B, green channel) as the subsets of eve-expressing neurons come on. Expression in the pericardial cells, along the dorsal margin, becomes noticeable (Fig. 4C) through germband retraction and hindgut expression is confined to the posterior (Fig. 3D, green channel). These previously unreported late expression domains were also verified with HCR (H. Garcia pers. comm).

Tc-eve intron 2 is not a stripe enhancer but is a PGZ enhancer

Tc-eve intron 2 had 19 out of the 34 of the assayed TFs bound and of those 19, 11 are known to be involved in *Drosophila* pair-rule stripe and/or *Tribolium* PGZ formation and regulation, therefore we would predict to see those patterns driven by the *Tc-eve* intron 2 enhancer. One of those predicted patterns held true. *Tc-eve* intron 2 drives strong mCherry expression in the PGZ of transgenic *Tribolium* samples (Figure 2), which coincides with the endogenous Eve PGZ expression providing compelling evidence that *Tc-eve* intron 2 is a PGZ *eve* enhancer. However, the posterior expression never resolves into pair-rule stripes, as the endogenous Eve does.

As described in the introduction, (Christine et al., 2022) propose that under their Speed Regulation model, enhancers downstream of a posterior graded morphogen would have both

'static' and 'dynamic' enhancers. 'Static' enhancers would stabilize stripes, dynamic would initialize gene expression. The "speed regulator," in the case of *Tribolium* is Wnt/*cad* because their gradients drive the posterior molecular oscillator that patterns segments. Following ATAC Seq analysis of Tribolium embryos, a 'static' stripe-specific enhancer for *Tribolium runt* (*Tc-runt*), was recently discovered, although the corresponding dynamic runt enhancer was not reported (Christine et al., 2022). All signs indicate that *Tc-eve* intron 2 is a dynamic PGZ enhancer of *eve* for the following reasons: (1) it had 11 TFs bound to it that are predicted to be expressed in the PGZ, of which *cad* and *pan* was one of them, (2) it is only active in the PGZ of the embryo as a dynamic enhancer would as described by Mau and colleagues.

Preliminary analysis of *Drosophila* transgenic for the *Tc-eve* intron 2 enhancer showed no presence of early *Tc-eve* intron 2 activity, not in a posterior domain nor stripes, even when *Dm-eve* stripes were observed. No mCherry stripes were detected in transgenic *Tribolium* either, considering mCherry expression never co-occurs with the endogenous Eve stripes (Fig. 2 & 3).

The absence of stripe specific enhancers could simply be explained that the spatial and temporal information for stripes is contained in one of the other predicted Tribolium eve enhancers or in the downstream uncloned region of the locus that has multiple predicted *cad* binding sites. This would be mitigated by creating and analyzing transgenic *Tribolium* containing each of the putative enhancers, a task others in my lab are currently partaking in. Another, less hopeful explanation, is that the maturation time and half-life of the mCherry reporter relative to the same parameters of the endogenous eve protein varies (Hebisch et al., 2013). Substantial differences could impact the dynamics of the expression pattern, potentially affecting whether the mCherry expression cycles in the posterior or resolves into stripes. An alternative explanation could be that the 'static', stripe stabilizing, enhancer may not have been captured in the intron 2 fragment.

Tc-eve **intron 2 in hindgut development**

As described earlier, *Tc-eve* intron 2 is active in the hindgut of transgenic *Tribolium* and *Drosophila*. Fascinating, albeit predicted by MCAST because of the *byn* and *fkh* binding sites on the intron 2 enhancer. *byn* and *fkh* are essential for the maintenance and specification of the hindgut in *Drosophila* ((Weigel et al., 1989); (Singer et al., 1996); (Wu & Lengyel, 1998); (Lengyel & Iwaki, 2002)) and *Tribolium* (Berns et al., 2008), the regulatory region of *Tc-byn* was able to even recapitulate the hindgut expression in transgenic *Drosophila*. Figure 3 depicts the activity of *Tc-eve* intron 2 throughout the whole process of *Tribolium* hindgut development and though *Tc-eve* intron 2 is only active late in transgenic *Drosophila* hindgut development (Figure 5), it is still just as strong and persistent as in *Tribolium*. This all supports the tentative notion that the *Tc-eve* intron 2 enhancer has Byn and Fkh TF activator sites that enhance *eve* expression in the hindgut. Also demonstrates MCAST ability to predict enhancer function based on conserved roles of TFs.

Tc-eve **intron 2 drives neural activity, outside of endogenous *eve***

Endogenous *Tribolium eve* CNS expression does not commence until the middle of germband retraction, NS11, and is expressed in the same *eve*-expressing cells as in *Drosophila* ((Doe, Smouse, et al., 1988); (Broadus et al., 1995); (Fujioka et al., 2003)) plus an additional *Tribolium* specific PEL cluster (Biffar & Stollewerk, 2015). We also detect endogenous Eve in the brain and in neurons in the developing appendages, expression domains not previously described. *Tc-eve* intron 2 enhancer activity is extensive in the nervous system and is active earlier than endogenous Eve, but none of it appears to coincide with Eve expression. How might we explain this? *hb*, *Dichaete*, nub, ftz and *en* all have predicted binding sites in the *Tc-eve* intron 2

enhancer. They are all *Tribolium* CNS TFs expressed in neuroblasts and in most cases in differentiated neurons ((Biffar & Stollewerk, 2014); (Janssen et al., 2018)). These CNS TFs may be abnormally activating the transgenic enhancer fragments, perhaps they typically are repressed from binding, but that repression is not accessible outside the normal chromatin context of endogenous eve. It is also possible the ectopic expression is driven by activity at the specific location the transgene hopped into.

Possible evolution in heart TFs

There are a multitude of previous studies that describe *eve* expression and function in the *Drosophila* dorsal vessel (heart & aorta; (Halfon et al., 2000); (Cande et al., 2009)). *Tc-eve* intron 2 drove very strong and precise dorsal vessel expression in two transgenic *Drosophila* lines (Fig. 6). *eve* is also endogenously expressed in the heart of *Tribolium* embryos, *Tc-eve* intron 2 did not drive expression in cardiac cells, *eve*-expressing or otherwise (Fig. 4). This suggests that the intron 2 contains binding sites for TFs that drive heart development in *Drosophila*, but either these TFs, or their binding site specificity has changed in *Tribolium*. Given that two independent Drosophila lines express the intron2 transgene in the heart argues against the expression resulting from the insertion site.

Predictions

This work has demonstrated that the *Tc-eve* intron 2 enhancer drives expression in transgenic *Tribolium* and *Drosophila* embryos. With that, we can speculate as to how the *eve* enhancers have evolved since the ancestral paradigm in *Tribolium*. Based on previous work, I had predicted that Tribolium eve enhancers would be modular and stripe and tissue specific, and that the positions of these enhancers would be conserved relative to the ORF. At my current level of understanding, this is not the case for the *Tc-eve* intron 2 enhancer, as this one fragment directs expression in the PGZ as well as two separate tissues, the hindgut and nervous system. However, it is possible that if we split the enhancer up into smaller fragments, the PGZ, hindgut, and nervous system patterns would be separable. To top it all off, the enhancer position is not conserved relative to the open reading frame (oRF) because the *Drosophila* muscle heart enhancer (MHE) is located downstream of the *eve* coding region, whereas the intron 2 fragment is located within the *Tribolium eve* coding region. In addition to this divergence in enhancer function/position, I identified a PGZ enhancer consistent with predictions from prior work that dynamic or timer enhancers would drive posterior expression. It will be fascinating to see if any other *Tc-eve* enhancers drive ‘static’ stripe expression.

Is this evidence of divergence during *eve* enhancer evolution? One may be inclined to answer yes particularly because there is no intron 2 found in *Drosophila*. More extensive studies need to be conducted before conclusive statements can be made. Although, it will be interesting to see whether the hindgut enhancer is located in the expected position when the remaining *Tribolium eve* enhancers are studied.

Methods

Tribolium castaneum MCAST: 2.0-kb intron 2 enhancer

Prior literature was searched and a list of 34 TFs, known or suggested to directly modulate *eve* in *Drosophila* and/or *Tribolium*, was compiled and run through MCAST (Bailey & Noble, 2003) searching the 15-kb *Tribolium eve* locus to predict possible binding sites. Parameters used in all runs were hit p-value < 0.0005 and match E-value < 10. The motif files were retrieved from the JASPAR database and the *Tribolium eve* sequence was obtained from Ensembl. SnapGene was used to label the TF binding sites along the locus for presentation purposes and each was color-coded according to which of the four domains of *eve* regulation they are involved in. These four domains were, separately, run through MCAST as well.

Drosophila melanogaster transgenics

Using Gateway cloning, the *eve* intron 2 enhancer was cloned into a piggyGUM vector (Lai et al., 2018) by lab members prior to my arrival in the lab. With this expression construct, BestGene Inc. injected and generated transgenic *Drosophila* lines expressing the *eve* intron enhancer-mCherry fusion and the 3xP3 eye-GFP enhancer to indicate successful integration. We were sent eight balanced heterozygous lines, which we assigned CyO M1, CyO M6, TM3 M3, TM3 M4, TM3 M5, TM3 M7, TM3 M8, FM7i (died before experimentation could be done). The balancer chromosomes are denoted by the symbols CyO (2nd chromosome), TM3 (third chromosome), and FM7i (X Chromosome).
Drosophila were raised under standard conditions with yeast paste for food. To create homozygous stocks, movement was briefly stopped on large petri dishes filled with ice and flies with the homozygous or balanced phenotype (straight wings or long stubble) were selected under a stereoscopic microscope.

Tribolium castaneum transgenics

vermilion^{white} adults were put in 25 oz tupperware with unbleached All-Purpose flour and lentils spread on the top of the flour to conduct egg collections for injections. Eggs were collected 1-2 hrs AEL and incubated for another 1-2 hrs prior to injections. Embryos were dechorionated for 2 minutes using 5% Clorox hypochlorite.

Using P-element-mediated transgenesis, PiggyGUM (375µg/mL) and *piggyBac* (500µg/mL) plasmids (Handler & Harrell Ii, 1999) were injected into 3-4hr *vermilion*^{white} embryos (obtained from USDA *Tribolium* stock center) via a Leica MZFLIII dissection scope. *vermillion* mutants were used to permit easy visualization of eGFP in the eyes.

Embryos and adults were raised in a 30°C incubator and checked every week. Injected adults (G_0) were sexed as pupae and mated with WT *vermilion*^{white} beetles, giving rise to progeny possessing the transgenic green-eyed phenotype or not. Transgenic progeny (G_1), sexed as pupae, were then crossed with WT *vermilion*^{white} beetles to give rise to G_2 progeny, which constituted the intron 2 transgenic colony. Eggs from the colony were collected weekly and pupae were screened for green eyes 22 days later.

Egg collections

For *Drosophila* lines, egg pots were set up to carry out egg collections in. Flies are put in 250mL plastic Erlenmeyer flasks and trapped in with apple juice agar plates. Apple agar plates are a viscous mixture of 13g agar, 12.5g sucrose (dextrose or glucose can be used), 375mL dH_2O, 0.75g Nipagin, and 125mL apple juice that is poured into small petri dish tops and left to solidify before storage in a plastic bag at 4°C. A thin line of yeast paste, a mixture of yeast and water, is spread down the middle of the apple agar plate and small lines are cut into the agar. Egg pots are stored in a 25°C incubator, while egg collection is ongoing.

Transgenic *Tribolium* were reared in mason jars containing whole-wheat flour and 5% Brewer's yeast, with their surface covered in lentils to prevent beetle's getting stuck on their backs. Jars are kept in a 30°C incubator. Eggs are sifted out using a 300µm (0.0117in) fine mesh sieve and either put into a petri dish and put back in the incubator for further development or dechorionated.

AEL eggs are dechorionated, with 50% hypochlorite for *Drosophila* samples and 25% hypochlorite for *Tribolium* samples for 2 minutes, either for live imaging or fixed and stored at -20°C for HCR or immunohistochemistry.

Immunohistochemistry

Drosophila and *Tribolium* samples were fixed in the same manner as described by Bruce et al. 2021, with the exception that we used a 4.5% formaldehyde solution (derived from MI's buffer recipes for whole-mount fruit fly embryos) and fixed for 25 mins. Also, we added 4mL of MeOH for devitellinization.

Drosophila samples proceed to wash step with 0.1% PBTriton (PBT) twice right out of 100% MeOH, *Tribolium* samples need to be rehydrated in three 500µL MeOH/PTW solutions (75% MeOH, 50% MeOH, 25% MeOH) for 1 min before washing twice in 0.1% PBT for 2 mins. *Tribolium* samples that needed to be sonicated were put in a glass vial of 500µL 0.1% PBT, sonicated in 5 sec bursts using a vibrating water bath (Note: use figure 8 movements and be mindful of hot spots), and checked under the scope for successful membrane removal. Samples were blocked with 1% Bovine serum albumin (BSA) solution, in 0.1% PBT, for 1hr at RT while rocking gently. The samples were incubated overnight at 4°C in a primary antibody solution while rocking gently, washed with 0.1% PBT over the course of 1hr, incubated for 1hr rocking gently at RT in a secondary antibody solution, washed again with 0.1% PBT over 1hr, and some samples were incubated for 1hr at RT in a tertiary antibody solution while rocking gently. Then the samples were counterstained in a 1:1000 DAPI (10mg/mL) solution, for 10 minutes at RT, washed three times with 0.1% PBT, and incubated overnight in 80% glycerol & propyl gallate before imaging. Images taken to the confocal microscope were mounted in Aqua Poly/Mount (from Polysciences Inc.). Control embryos with anti-Biotin conjugated to anti-SA, no primary, had low levels of fluorescence in the thoracic and head region, primarily in germband retracted embryos, that was not coincident with mCherry nor Eve expression.

The following antibodies and dilutions were used: monoclonal primary mouse antibody 2B8 (Eve), 51µg/mL diluted to 1:50, from Developmental Studies Hybridoma Bank (DSHB) and conjugated to secondary antibody biotinylated goat anti-mouse IgG, 1.5mg/mL diluted to 1:300, from Vector Laboratories (VL), which is targeted by tertiary antibody Dylight-488 Streptavidin, 1 mg/mL diluted to 1:100, from VL; primary rabbit antibody mCherry, diluted to 1:100, from Cell Signaling Technology and conjugated to donkey anti-rabbit IgG-Alexa Fluor 568 (ordered from Thermo Fisher Scientific), 2 mg/mL diluted to 1:400.

Hybridization chain reaction (HCR)

Probe sets, hairpins, and reagents were as described by Molecular Instruments Inc. (MI). To help with embryo retention experiments, probe hybridization buffer was made with 10% dextran sulfate, instead of 5% (Tidswell et al., 2021). *Drosophila-eve* (labeled for 488 nm) had 20 probe pairs, *Tribolium-eve* (labeled for 488 nm) had 12 probe pairs, and mCherry reporter (labeled for 546 nm) had 12 probe pairs. We used the HCR protocol from (Bruce et al., 2021) (adapted from Molecular Instruments HCR version 3.0 protocol for whole-mount *Drosophila* embryos ((Choi et al., 2016); (Choi et al., 2018)).

To counterstain with DAPI, 1µL DAPI into 999µL 80% glycerol with propyl gallate for at least 30 minutes and then replace it with only 80% glycerol with propyl gallate. Samples were stored in 1.5mL eppendorf tubes, protected from light, at 4^0C.

Live Imaging

A Zeiss Axioplan fluorescence microscope equipped with a Plan-NEOFLUAR 10x/0.3 objective was utilized to image all embryos. Images were cropped and brightness and contrast were enhanced in Fiji/ImageJ. Scale bars were added to figures using Adobe Photoshop 2023.

Confocal Imaging

The Zeiss LSM 880 inverted confocal microscope was employed using laser lines 488 nm and 633 nm. All images were taken with the Plan-Apochromat 20x/0.8 objective lens as a montage, which was in turn made into a z-stack and the maximum intensity stacks were projected into one image. Images were cropped and edited in Fiji/ImageJ; red was replaced with magenta and fluorescence intensity was adjusted.

Supplementary Material

Early acting transcription factors

The molecular oscillator is proposed to be controlled by a posterior gradient of Wnt that directly activates the posterior gradient of *cad*, resulting in *eve* activating *runt*, *runt* activating *odd*, and *odd* repressing *eve* (Liao & Oates, 2017). To that end, we would have expected MCAST to predict pan (the downstream TF in Wnt signaling), *cad* and *odd* binding sites. *pangolin* (*pan*) has been described as part of the driving mechanism for the molecular oscillator because its activity in the posterior growth zone positively regulates the posterior *cad* gradient (El-Sherif et al., 2014). RNAi experimentation supports *pan*'s role as a repressor in the anterior of embryos seeing as knock down causes a disruption of the posterior *cad* gradient, shifting it anteriorly (El-Sherif et al., 2014). It is not evident whether *nubbin* (*nub*) has a direct role in the oscillator, but it is robustly expressed in the PGZ during germband elongation (Tidswell et al., 2021).

runt is proposed to be activated by *eve* in the oscillator, but the presence of *runt* in the *eve* MCAST results leads us to predict that it could directly regulate *eve* as well, as either an activator or repressor. Interestingly, no *odd* binding sites were predicted to be on intron 2, whereas it has a role in repressing *eve* according to the oscillator. The predicted *cad* binding sites all along the locus support its role in driving the oscillator. *eve*'s seven primary stripes resolve into 14 segmental stripes that are set and maintained at the anterior parasegment border by *engrailed* (*en*) (Lim & Choe, 2020). *invected* (*inv*) is a paralog of *en* and in *Drosophila* they have nearly identical functions and expression patterns (Gustavson et al., 1996), so it may be predicted that it would act in the same manner as *en* when interacting with *eve*.

hunchback (*hb*) had the largest abundance of hits on intron 2 and *Dichaete* (*D*) came in third, both are essential and expressed early in the growth zone although *D* is absent from the most posterior pit (Clark & Peel, 2018). The strong presence of these TFs predicted by MCAST provides support for the involvement of intron 2 regulating *eve* growth zone activity. Neither have ever been implicated in the molecular oscillator, but *D* has been suggested to interact with *cad* and *opa* (*odd-paired*) in an ancient gene regulatory network downstream of the oscillator in *Tribolium* (Clark & Peel, 2018). To go a step further, this ancient regulatory network, mainly *cad,* could activate *hb* expression; supported by the overlapping expression patterns of *hb* and *cad* (Wolff et al., 1998).

sloppy-paired 1 (*slp1*) being a secondary pair-rule gene, means we see its expression patterns in segmental stripes throughout segmentation. This is made possible by its indirect regulation by the molecular oscillator, specifically *slp1* is derepressed as *run* expression retracts posteriorly (Choe et al., 2006). At which time, it would be safe to assume *odd* is repressing *eve*, thus *run* is no longer being activated by *eve* so *slp1* could be predicted to have binding sites on the *eve* locus to allow for its derepression. In such a case, *slp1* would be acting as a repressor of *eve*.

In *Drosophila knirps* (*kni*) is well-studied as a gap gene, but this appears to not be the case in *Tribolium*, where RNAi does not result in normal gap gene phenotypes (Cerny et al., 2008). Blastodermal *Tribolium kni* expression is at the anterior border of *eve*'s posterior domain and stripe one and we see similarities in *kni*'s function in head patterning in both species (Cerny et al., 2008); (González-Gaitán et al., 1994). *tailless* (*tll*) is another identified gap gene in *Drosophila* and not identified as such in *Tribolium* but has a role in head development in both (Schröder et al., 2000); (Pignoni et al., 1990). *eve* has not been implicated in head patterning, but eve stripes do cover the posterior part of head parts, therefore it's possible the MCAST predicted binding sites are pointing towards *kni* and *tll* inhibiting eve in the head wherever they are active.

JAK/STAT signalling pathway regulates a diverse array of developmental processes, segmentation being one of them. *Drosophila* studies have uncovered t two stat92e (STAT) activator sites located on the enhancer for *eve* stripes 3+7 (Yan et al., 1996); (Small et al., 1996); (Binari & Perrimon, 1994), driving the possibility that the putative STAT binding site on intron 2 may be an activating site for *Tribolium eve*.

Trithorax-like (*Trl*) is important in maintaining gene expression patterns established early in embryogenesis in *Drosophila* (Bejarano & Busturia, 2004) and is found in *Tribolium* (*ensemble Gene: TC013586*) but has not yet been functionally analyzed. Its presence in the MCAST search implies retained function.

Heart transcription factors

pan has been well delineated in *Drosophila* and has quite a few functions, but important to this work is its involvement in cardiac development (Brunner et al., 1997); (Hare et al., 2008). As well as functioning as an activator, *pan* is also a repressor. *Tribolium* studies have not directly discussed its role in cardiogenesis, but it tied with *D* for third highest number of binding sites in MCAST, reinforcing its suggested role in regulating *eve*.

tinman (*tin*) and *twist* (*twi*) have direct roles that have been studied in *Tribolium* cardiogenesis and mesoderm formation, respectively (Sharma et al., 2015) (Handel et al., 2005). Granted these TFs only had one binding site each, they still may very well play a part in activating *eve* in the heart precursor and late cardiac cells.

Drosophila studies outline *Medea*'s (*Med*) function in patterning the dorsal-ventral axis during embryogenesis (Hudson et al., 1998); (Wisotzkey et al., 1998), moreover that role is necessary for proper cardiac formation. In which case, it is expected that *Med* is acting upstream of *tin* to establish the dorso-ventral axis to allow for *tin* to activate *eve* in cardiac cells, but the MCAST suggests *Med* could be activating *eve* itself in either the lateral ectoderm precursors to cardiac tissue or in cardiac mesoderm itself.

CNS transcription factors

Tribolium nub is first expressed in neuroectoderm, then progresses to neuroblasts (Biffar & Stollewerk, 2014). Neuroblasts also express *hb*, *runt*, *D*, *fkh*, and *en* (Schröder et al., 2000); (Clark & Peel, 2018); (Biffar & Stollewerk, 2014). Whether some or all the cells in the nervous system expressing the 6 TFs are the same cells expressing *eve* is unknown for now. Albeit the restricted expression of *eve* in the CNS may suggest these 6 TFs, broadly expressed in the CNS, are inhibiting it in all other neural cells. *nub* had the second highest number of binding site hits on intron 2, suggestive of a strong role possibly being a repressor.

Initially, *fushi tarazu* (*ftz*) is expressed in stripes that reconcile into weaker expression in odd-numbered parasegments before its neural expression comes on (Brown et al., 1994); (Heffer et al., 2013). Its stripes in parasegments overlap that of *eve* and *en*, indicative of its function in segmentation and *eve* regulation. More importantly, it is known that *ftz* is a direct regulator of *eve* in the *Drosophila* CNS (Doe, Hiromi, et al., 1988).

Gut/anal pad transcription factors

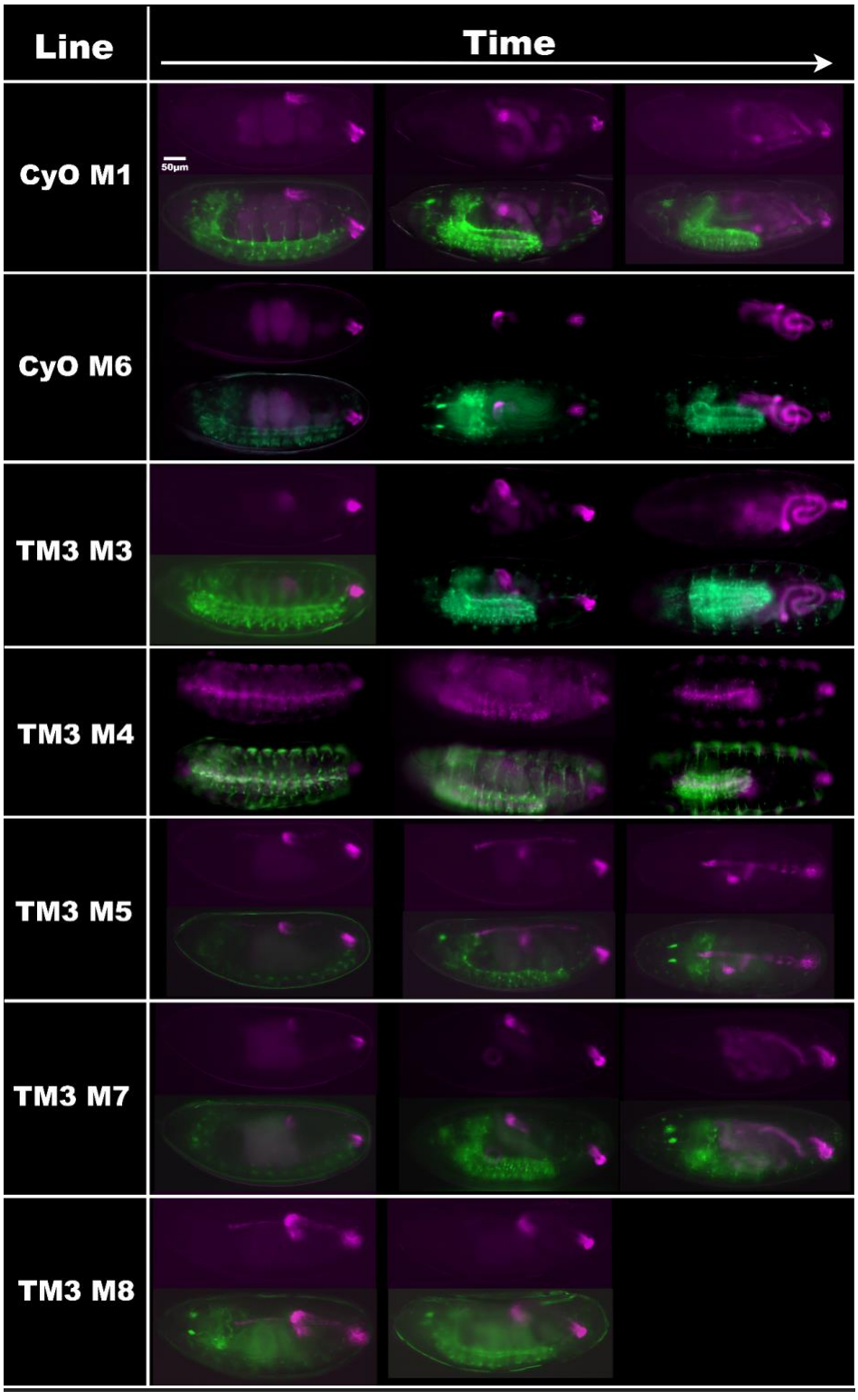
brachyenteron (*byn*) expression in *Tribolium* originates in the growth zone, prior to germband elongation (GBE), and persists throughout segmentation then later in development, it is seen in the hindgut (Moreno & Morata, 1999); (Berns et al., 2008). *cad* dynamics are similar, with expression visible in the anal pads (Schröder et al., 2000). *Tribolium forkhead* (*fkh*), as well as *Drosophila fkh*, are well characterized and expressed in the stomodaeum, proctodeum, midgut, and hindgut (Schröder et al., 2000); (Berns et al., 2008). Antibody experiments reveal endogenous *Tribolium eve* expression in the most posterior hindgut (Figure 3), which would overlap with *byn* and *fkh* supporting their possible roles as activators of *eve*.

Supplementary Document 1: Descriptions of MCAST predicted TFs.

Gene	Transcription factor binding or expression	Notes on Regulation	JASPAR ID or Fly Factor Survey URL	References
bicoid (bcd)	Not found in *Tribolium* genome	Not found in *Tribolium* genome	MA0212.1	(Small et al., 1992)
brachyenteron (byn)	GZ of extending germband; proctodeum and hindgut	Hindgut lost in RNAi; no effect on segmentation	mccb.umassmed.edu/ffs/TFdetails.php?FlybaseID=FBgn0011723	(Berns et al., 2008)
caudal (cad)	GZ gradient with high point in posterior; malpighian tubules; anal pad	Activated by Wnt gradient and works with it to control oscillator	MA0216.1. MA0216.2	(Schulz et al., 1998)
Dichaete (D)	GZ; neuroectoderm down midline and in head lobes	RNAi result in empty eggs	MA0445.1	(Oberhofer et al., 2014) (Clark & Peel, 2018)
engrailed (en)	Segmental stripes; neuroblasts	Activated by *eve*	MA0220.1	Choe & Brown 2009 (Biffar & Stollewerk, 2014)
even-skipped (eve)	Stripes; GZ; CNS (GMCs, RP2, aCC, pCC, EL, U/CQ, PEL); ap/posterior hindgut; cardioblasts	Component of molecular oscillator; repressed by *odd*	MA0221.1	(Choe et al., 2006) (El-Sherif et al., 2012) (Biffar & Stollewerk, 2015)
forkhead (fkh)	GZ; Stomodaeum; Primordia of hindgut and posterior midgut; midline CNS primordia; malpighian tubules	RNAi only leads to hindgut defects	MA0446.1	(Schröder et al., 2000) (Schoppmeier & Schröder, 2005) (Berns et al., 2008)
fushi tarazu (ftz)	Stripes; CNS	Stripes overlap with *eve* stripes	MA0225.1	(Brown et al., 1994)
giant (gt)	Ubiquitous blastoderm into a stripe; brief GZ into 2 stripes; brief brain cell clusters	Overlaps with *eve* stripes 1/3/4; RNAi results in missing or transformed segments	MA0447.1	(Bucher & Klingler, 2004)
huckebein (hkb)	Early segmental pattern; neuroblasts and clusters of neurons	Activates *eve* in neuroblasts	mccb.umassmed.edu/ffs/TFdetails.php?FlybaseID=FBgn0001204	(Kittelmann et al., 2013) (Biffar & Stollewerk, 2014) (Biffar & Stollewerk, 2015)
hunchback (hb)	Ubiquitous into anterior domain; neuroectoderm and all neuroblasts; late GZ	Possibly activated by *cad*	MA0049.1	(Wolff et al., 1998) (Tidswell et al., 2021)
invected (inv)	Segmental stripes	In *inv/en* KD *eve* expression increases	MA0229.1	Blunk Unpublished
knirps (kni)	Broad domain in blastoderm; GZ; tracheal pits and branches	RNAi causes fusions or deletions of partial segments	MA0049.1	(Cerny et al., 2008)
Kruppel (Kr)	Blastodermal posterior gap domain; head lobes; segments	RNAi transforms posterior segments to more anterior fates	MA0452.1 MA0452.2	(Cerny et al., 2005)
Mothers against Dpp (Mad)	Dorsal side of blastoderm; dorsal margins; stomodaeum	Dpp RNAi results in ventralized embryos	MA0535.1	(Zee et al., 2006)
Medea (Med)	Group of maternally provided TFs that kill progeny w/o it, using M poison	Inherited M or zygotically expressed H act as antidotes to M poison	mccb.umassmed.edu/ffs/TFdetails.php?FlybaseID=FBgn0259789	(Thomson, 2014)

nubbin (nub)	2 blastodermal patches; GZ; neuroectoderm & neuroblasts; limb buds	Possibly repressed by *hb*; RNAi leads to no cuticle or no hatching	MA0197.1 MA0197.2	(Tidswell et al., 2021)
odd-skipped (odd)	GZ; stripes; antennae	Activated by *runt* and represses *eve* in oscillator	MA0454.1	(Choe et al., 2006)
odd-paired (opa)	Ubiquitous in blastoderm into stripe; domain anterior to GZ; segmental stripes; posterior head lobes	Activated by *eve* and repressed by *odd*	MA0456.1	(Choe et al., 2017)
orthodenticle (otd)	Ubiquitous in blastoderm into anterior domain; brain; CNS midline	RNAi results in anterior and posterior segment deletions	mccb.umassmed.edu/ffs/TFdetails.php?FlybaseID=FBgn0004102	(Li et al., 1996)
shavenbaby (*ovo/svb*)	Posterior blastodermal domain; GZ; neuronal brain clusters; ventral midline; gnathal & thoracic appendages	Possibly *mlpt*; Mutants are shorter with leg defects and fused segments	MA0126.1	(Ray et al., 2019)
paired (prd)	Segmental stripes; mandible	Repressed by runt; RNAi yields typical pair-rule phenotype	MA0239.1	(Choe et al., 2006) (Choe & Brown, 2007)
pangolin (pan)	Maternally provided; anterior tip of blastoderm	Wnt signalling; abdominal segments fail to form with RNAi	MA0237.1 MA0237.2	(Bucher et al., 2005) (Bolognesi et al., 2009)
pleiohomeotic (pho)	Unknown in *Tribolium*	Unknown in *Tribolium*	MA1460.1	(Doe, Smouse, et al., 1988) (Fujioka et al., 2008)
pointed (pnt)	Embryonic leg development	Mutants arrest development at larval-pupal transition	mccb.umassmed.edu/ffs/TFdetails.php?FlybaseID=FBgn0259789	(Chafino et al., 2021)
runt (run)	Stripes; neuroblasts	Activated by *eve* in oscillator; activates *eve* in neuroblast	MA0242.1	(Choe et al., 2006) (Biffar & Stollewerk, 2015)
sloppy-paired 1 (slp1)	Segmental stripes; gnathal segments	Repressed by runt; RNAi mutants always missing gnathal segments	MA0458.1	(Choe et al., 2006) (Choe & Brown, 2007)
STAT	Maintain segment boundaries	RNAi severely reduces posterior segments	MA0532.1	(Bäumer et al., 2011)
tailless (tll)	Posterior pole; 2 patches in blastoderm; head lobes	Possibly torso-signaling	MA0459.1	(Schröder et al., 2000)
tinman (tin)	Cardiac mesoderm and developing heart; head spots	Possibly FGF signaling; possibly activates *eve*	MA0247.1 MA0247.2	(Janssen & Damen, 2008) (Sharma et al., 2015)
tramtrack (ttk)	Unknown in *Tribolium*	Unknown in *Tribolium*	MA0460.1	(Read et al., 1992)
Trithorax-like (Trl)	Unknown in *Tribolium*	Unknown in *Tribolium*	MA0205.1 MA0205.2	(Read et al., 1990)
twist (twi)	Blastodermal stripe along AP axis; posterior tip of GZ; mesoderm of segments; antennae	Possibly activated by Dorsal or *snail*; possibly repressed by *snail*; possibly regulated by torso-signaling	MA0249.1 MA0249.2	(Handel et al., 2005)
Zelda (Z)	Maternally provided; uniform in blastoderm into posterior domain; GZ; head lobes; CNS; maxilla	Possibly regulates *eve*; Mutants have continuous posterior domain of *eve* instead of stripes	mccb.umassmed.edu/ffs/TFdetails.php?FlybaseID=FBgn0259789	(Ribeiro et al., 2017)

Supplementary Table 1: Table of MCAST TFs.
Tribolium and *Drosophila* transcription factors used in MCAST and their respective roles in *eve* regulation. 34 TFs were compiled based on references, in the rightmost column. Matrices in MEME format were retrieved from JASPAR or Fly Factor Survey, denoted by the ID/URL in the fourth column. *bcd* does not exist in the *Tribolium* genome but was included in this list because of its well-characterized function in regulating *Drosophila eve*. *pho, ttk, & Trl* are only thus far predicted to exist in *Tribolium* and have direct interactions with *Drosophila eve*. Abbreviations: AP = anterior-posterior, CNS = central nervous system, GZ = growth zone, KD = knockdown, RNAi = RNA interference.



Supplementary Figure 1: Live imaging of seven transgenic *Drosophila* lines carrying *Tc-eve* intron 2-mCherry fusion construct

Tc-eve intron 2-mCherry (magenta) and 3xP3 driven GFP (green). Each transgenic line also carries a balancer chromosome, CyO or TM3. Embryos get older from left to right. Anterior and posterior hindgut expression is observed in all lines, which becomes more regionalized in the gut towards hatching. mCherry expression in the dorsal vessel (heart) in only two lines, TM3 M5 & TM3 M8. Anterior is left.

Bibliography

Bailey, T. L., & Noble, W. S. (2003). Searching for statistically significant regulatory modules. *Bioinformatics*, *19*(suppl_2), ii16-ii25. https://doi.org/10.1093/bioinformatics/btg1054

Bejarano, F., & Busturia, A. (2004). Function of the Trithorax-like gene during Drosophila development. *Developmental Biology*, *268*(2), 327-341. https://doi.org/https://doi.org/10.1016/j.ydbio.2004.01.006

Berns, N., Kusch, T., Schröder, R., & Reuter, R. (2008). Expression, function and regulation of Brachyenteron in the short germband insect Tribolium castaneum. *Development Genes and Evolution*, *218*(3), 169-179. https://doi.org/10.1007/s00427-008-0210-7

Berrocal, A., Lammers, N. C., Garcia, H. G., & Eisen, M. B. (2020). Kinetic sculpting of the seven stripes of the Drosophila even-skipped gene. *eLife*, *9*, e61635. https://doi.org/10.7554/eLife.61635

Biffar, L., & Stollewerk, A. (2014). Conservation and evolutionary modifications of neuroblast expression patterns in insects. *Developmental Biology*, *388*(1), 103-116. https://doi.org/https://doi.org/10.1016/j.ydbio.2014.01.028

Biffar, L., & Stollewerk, A. (2015). Evolutionary variations in the expression of dorso-ventral patterning genes and the conservation of pioneer neurons in Tribolium castaneum. *Developmental Biology*, *400*(1), 159-167. https://doi.org/https://doi.org/10.1016/j.ydbio.2015.01.025

Binari, R., & Perrimon, N. (1994). Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. *Genes Dev*, *8*(3), 300-312. https://doi.org/10.1101/gad.8.3.300

Bolognesi, R., Fischer, T. D., & Brown, S. J. (2009). Loss of Tc-arrow and canonical Wnt signaling alters posterior morphology and pair-rule gene expression in the short-germ insect, Tribolium castaneum. *Development Genes and Evolution*, *219*(7), 369-375. https://doi.org/10.1007/s00427-009-0299-3

Boos, A., Distler, J., Rudolf, H., Klingler, M., & El-Sherif, E. (2018). A re-inducible gap gene cascade patterns the anterior–posterior axis of insects in a threshold-free fashion. *eLife*, *7*, e41208. https://doi.org/10.7554/eLife.41208

Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G., & Doe, C. Q. (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the Drosophila central nervous system. *Mechanisms of Development*, *53*(3), 393-402. https://doi.org/https://doi.org/10.1016/0925-4773(95)00454-8

Brown, S. J., Hilgenfeld, R. B., & Denell, R. E. (1994). The beetle Tribolium castaneum has a fushi tarazu homolog expressed in stripes during segmentation. *Proceedings of the National Academy of Sciences*, *91*(26), 12922-12926. https://doi.org/doi:10.1073/pnas.91.26.12922

Brown, S. J., Parrish, J. K., Beeman, R. W., & Denell, R. E. (1997). Molecular characterization and embryonic expression of the even-skipped ortholog of Tribolium castaneum. *Mechanisms of Development*, *61*(1), 165-173. https://doi.org/https://doi.org/10.1016/S0925-4773(96)00642-9

Bruce, H. S., Jerz, G., Kelly, S. R., McCarthy, J., Pomerantz, A., Senevirathne, G., . . . Patel, N. H. (2021). Hybridization Chain Reaction (HCR) In Situ Protocol. In. protocols.io.

Brunner, E., Peter, O., Schweizer, L., & Basler, K. (1997). pangolinencodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. *Nature*, *385*(6619), 829-833. https://doi.org/10.1038/385829a0

Bucher, G., Farzana, L., Brown, S. J., & Klingler, M. (2005). Anterior localization of maternal mRNAs in a short germ insect lacking bicoid. *Evolution & Development*, *7*(2), 142-149. https://doi.org/https://doi.org/10.1111/j.1525-142X.2005.05016.x

Bucher, G., & Klingler, M. (2004). Divergent segmentation mechanism in the short germ insect Tribolium revealed by giant expression and function. *Development*, *131*(8), 1729-1740. https://doi.org/10.1242/dev.01073

Bäumer, D., Trauner, J., Hollfelder, D., Cerny, A., & Schoppmeier, M. (2011). JAK-STAT signalling is required throughout telotrophic oogenesis and short-germ embryogenesis of the beetle Tribolium. *Developmental Biology*, *350*(1), 169-182. https://doi.org/https://doi.org/10.1016/j.ydbio.2010.10.020

Cande, J. D., Chopra, V. S., & Levine, M. (2009). Evolving enhancer-promoter interactions within the tinman complex of the flour beetle, Tribolium castaneum. *Development (Cambridge, England)*, *136*(18), 3153-3160. https://doi.org/10.1242/dev.038034

Cerny, A. C., Bucher, G., Schröder, R., & Klingler, M. (2005). Breakdown of abdominal patterning in the Tribolium Krüppel mutant jaws. *Development*, *132*(24), 5353-5363. https://doi.org/10.1242/dev.02154

Cerny, A. C., Grossmann, D., Bucher, G., & Klingler, M. (2008). The Tribolium ortholog of knirps and knirps-related is crucial for head segmentation but plays a minor role during abdominal patterning. *Developmental Biology*, *321*(1), 284-294. https://doi.org/https://doi.org/10.1016/j.ydbio.2008.05.527

Chafino, S., Martín, D., & Franch-Marro, X. (2021). Activation of EGFR signaling by Tc-Vein and Tc-Spitz regulates the metamorphic transition in the red flour beetle Tribolium castaneum. *Scientific Reports*, *11*(1), 18807. https://doi.org/10.1038/s41598-021-98334-9

Choe, C. P., & Brown, S. J. (2007). Evolutionary flexibility of pair-rule patterning revealed by functional analysis of secondary pair-rule genes, paired and sloppy-paired in the short-germ insect, Tribolium castaneum. *Developmental Biology*, *302*(1), 281-294. https://doi.org/https://doi.org/10.1016/j.ydbio.2006.09.037

Choe, C. P., Miller, S. C., & Brown, S. J. (2006). A pair-rule gene circuit defines segments sequentially in the short-germ insect Tribolium castaneum. *Proceedings of the National Academy of Sciences*, *103*(17), 6560-6564. https://doi.org/10.1073/pnas.0510440103

Choe, C. P., Stellabotte, F., & Brown, S. J. (2017). Regulation and function of odd-paired in Tribolium segmentation. *Development Genes and Evolution*, *227*(5), 309-317. https://doi.org/10.1007/s00427-017-0590-7

Choi, H. M. T., Calvert, C. R., Husain, N., Huss, D., Barsi, J. C., Deverman, B. E., . . . Pierce, N. A. (2016). Mapping a multiplexed zoo of mRNA expression. *Development*, *143*(19), 3632-3637. https://doi.org/10.1242/dev.140137

Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., . . . Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development*, *145*(12). https://doi.org/10.1242/dev.165753

Christine, M., Heike, R., Frederic, S., Benjamin, S., Timo, R., Ralf, P., . . . Ezzat, E.-S. (2022). How enhancers regulate wavelike gene expression patterns: Novel enhancer prediction and live reporter systems identify an enhancer associated with the arrest of pair-rule waves in the short-germ beetle Tribolium. *bioRxiv*, 2022.2009.2009.507237. https://doi.org/10.1101/2022.09.09.507237

Clark, E. (2017). Dynamic patterning by the Drosophila pair-rule network reconciles long-germ and short-germ segmentation. *PLOS Biology*, *15*(9), e2002439. https://doi.org/10.1371/journal.pbio.2002439

Clark, E., & Peel, A. D. (2018). Evidence for the temporal regulation of insect segmentation by a conserved sequence of transcription factors. *Development*, *145*(10). https://doi.org/10.1242/dev.155580

Diaz-Cuadros, M., Pourquié, O., & El-Sherif, E. (2021). Patterning with clocks and genetic cascades: Segmentation and regionalization of vertebrate versus insect body plans. *PLOS Genetics*, *17*(10), e1009812. https://doi.org/10.1371/journal.pgen.1009812

DiNardo, S., & O'Farrell, P. H. (1987). Establishment and refinement of segmental pattern in the Drosophila embryo: spatial control of engrailed expression by pair-rule genes. *Genes Dev*, *1*(10), 1212-1225. https://doi.org/10.1101/gad.1.10.1212

Doe, C. Q., Hiromi, Y., Gehring, W. J., & Goodman, C. S. (1988). Expression and Function of the Segmentation Gene fushi tarazu During Drosophila Neurogenesis. *Science*, *239*(4836), 170-175. https://doi.org/doi:10.1126/science.2892267

Doe, C. Q., Smouse, D., & Goodman, C. S. (1988). Control of neuronal fate by the Drosophila segmentation gene even-skipped. *Nature*, *333*(6171), 376-378. https://doi.org/10.1038/333376a0

Duman-Scheel, M., & Patel, N. H. (1999). Analysis of molecular marker expression reveals neuronal homology in distantly related arthropods. *Development*, *126*(11), 2327-2334. https://doi.org/10.1242/dev.126.11.2327

Eckert, C., Aranda, M., Wolff, C., & Tautz, D. (2004). Separable stripe enhancer elements for the pair-rule gene hairy in the beetle Tribolium. *EMBO Rep*, *5*(6), 638-642. https://doi.org/10.1038/sj.embor.7400148

El-Sherif, E., Averof, M., & Brown, S. J. (2012). A segmentation clock operating in blastoderm and germband stages of Tribolium development. *Development*, *139*(23), 4341-4346. https://doi.org/10.1242/dev.085126

El-Sherif, E., Zhu, X., Fu, J., & Brown, S. J. (2014). Caudal Regulates the Spatiotemporal Dynamics of Pair-Rule Waves in Tribolium. *PLOS Genetics*, *10*(10), e1004677. https://doi.org/10.1371/journal.pgen.1004677

Frasch, M., Hoey, T., Rushlow, C., Doyle, H., & Levine, M. (1987). Characterization and localization of the even-skipped protein of Drosophila. *Embo j*, *6*(3), 749-759. https://doi.org/10.1002/j.1460-2075.1987.tb04817.x

Fujioka, M., Emi-Sarker, Y., Yusibova, G. L., Goto, T., & 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*(11), 2527-2538. https://doi.org/10.1242/dev.126.11.2527

Fujioka, M., Lear, B. C., Landgraf, M., Yusibova, G. L., Zhou, J., Riley, K. M., . . . Jaynes, J. B. (2003). Even-skipped, acting as a repressor, regulates axonal projections in Drosophila. *Development*, *130*(22), 5385-5400. https://doi.org/10.1242/dev.00770

Fujioka, M., Wessells, R. J., Han, Z., Liu, J., Fitzgerald, K., Yusibova, G. L., . . . Jaynes, J. B. (2005). Embryonic even skipped-dependent muscle and heart cell fates are required for normal adult activity, heart function, and lifespan. *Circ Res*, *97*(11), 1108-1114. https://doi.org/10.1161/01.res.0000191546.08532.b2

Fujioka, M., Yusibova, G. L., Zhou, J., & Jaynes, J. B. (2008). The DNA-binding Polycomb-group protein Pleiohomeotic maintains both active and repressed transcriptional states through a single site. *Development*, *135*(24), 4131-4139. https://doi.org/10.1242/dev.024554

González-Gaitán, M., Rothe, M., Wimmer, E. A., Taubert, H., & Jäckle, H. (1994). Redundant functions of the genes knirps and knirps-related for the establishment of anterior Drosophila head structures. *Proceedings of the National Academy of Sciences*, *91*(18), 8567-8571. https://doi.org/doi:10.1073/pnas.91.18.8567

Gorfinkiel, N., Sánchez, L., & Guerrero, I. (1999). Drosophila terminalia as an appendage-like structure. *Mechanisms of Development*, *86*(1), 113-123. https://doi.org/https://doi.org/10.1016/S0925-4773(99)00122-7

Goto, T., Macdonald, P., & Maniatis, T. (1989). Early and late periodic patterns of even skipped expression are controlled by distinct regulatory elements that respond to different spatial cues. *Cell*, *57*(3), 413-422. https://doi.org/https://doi.org/10.1016/0092-8674(89)90916-1

Gustavson, E., Goldsborough, A. S., Ali, Z., & Kornberg, T. B. (1996). The Drosophila engrailed and invected Genes: Partners in Regulation, Expression and Function. *Genetics*, *142*(3), 893-906. https://doi.org/10.1093/genetics/142.3.893

Halfon, M. S., Carmena, A., Gisselbrecht, S., Sackerson, C. M., Jiménez, F., Baylies, M. K., & Michelson, A. M. (2000). Ras Pathway Specificity Is Determined by the Integration of Multiple Signal-Activated and Tissue-Restricted Transcription Factors. *Cell*, *103*(1), 63-74. https://doi.org/https://doi.org/10.1016/S0092-8674(00)00105-7

Handel, K., Basal, A., Fan, X., & Roth, S. (2005). Tribolium castaneum twist: gastrulation and mesoderm formation in a short-germ beetle. *Development Genes and Evolution*, *215*(1), 13-31. https://doi.org/10.1007/s00427-004-0446-9

Handler, A. M., & Harrell Ii, R. A. (1999). Germline transformation of Drosophila melanogaster with the piggyBac transposon vector. *Insect Molecular Biology*, *8*(4), 449-457. https://doi.org/https://doi.org/10.1046/j.1365-2583.1999.00139.x

Hare, E. E., Peterson, B. K., Iyer, V. N., Meier, R., & Eisen, M. B. (2008). Sepsid even-skipped Enhancers Are Functionally Conserved in Drosophila Despite Lack of Sequence Conservation. *PLOS Genetics*, *4*(6), e1000106. https://doi.org/10.1371/journal.pgen.1000106

Hebisch, E., Knebel, J., Landsberg, J., Frey, E., & Leisner, M. (2013). High variation of fluorescence protein maturation times in closely related Escherichia coli strains. *PLoS One*, *8*(10), e75991. https://doi.org/10.1371/journal.pone.0075991

Heffer, A., Xiang, J., & Pick, L. (2013). Variation and constraint in Hox gene evolution. *Proceedings of the National Academy of Sciences*, *110*(6), 2211-2216. https://doi.org/doi:10.1073/pnas.1210847110

Hudson, J. B., Podos, S. D., Keith, K., Simpson, S. L., & Ferguson, E. L. (1998). The Drosophila Medea gene is required downstream of dpp and encodes a functional homolog of human Smad4. *Development*, *125*(8), 1407-1420. https://doi.org/10.1242/dev.125.8.1407

Ingham, P. W., Baker, N. E., & Martinez-Arias, A. (1988). Regulation of segment polarity genes in the Drosophila blastoderm by fushi tarazu and even skipped. *Nature*, *331*(6151), 73-75. https://doi.org/10.1038/331073a0

Janssen, R., Andersson, E., Betnér, E., Bijl, S., Fowler, W., Höök, L., . . . Tiemann, S. (2018). Embryonic expression patterns and phylogenetic analysis of panarthropod sox genes: insight into nervous system development, segmentation and gonadogenesis. *BMC Evolutionary Biology*, *18*(1), 88. https://doi.org/10.1186/s12862-018-1196-z

Janssen, R., & Damen, W. G. M. (2008). Diverged and conserved aspects of heart formation in a spider. *Evolution & Development*, *10*(2), 155-165. https://doi.org/https://doi.org/10.1111/j.1525-142X.2008.00223.x

Jiang, J., Hoey, T., & 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*(2), 265-277. https://doi.org/10.1101/gad.5.2.265

Kittelmann, S., Ulrich, J., Posnien, N., & Bucher, G. (2013). Changes in anterior head patterning underlie the evolution of long germ embryogenesis. *Developmental Biology*, *374*(1), 174-184. https://doi.org/https://doi.org/10.1016/j.ydbio.2012.11.026

Lai, Y.-T., Deem, K. D., Borràs-Castells, F., Sambrani, N., Rudolf, H., Suryamohan, K., . . . Tomoyasu, Y. (2018). Enhancer identification and activity evaluation in the red flour beetle, Tribolium castaneum. *Development*, *145*(7). https://doi.org/10.1242/dev.160663

Landgraf, M., Roy, S., Prokop, A., VijayRaghavan, K., & Bate, M. (1999). even-skipped Determines the Dorsal Growth of Motor Axons in Drosophila. *Neuron*, *22*(1), 43-52. https://doi.org/https://doi.org/10.1016/S0896-6273(00)80677-7

Lengyel, J. A., & Iwaki, D. D. (2002). It Takes Guts: The Drosophila Hindgut as a Model System for Organogenesis. *Developmental Biology*, *243*(1), 1-19. https://doi.org/https://doi.org/10.1006/dbio.2002.0577

Li, Y., Brown, S. J., Hausdorf, B., Tautz, D., Denell, R. E., & Finkelstein, R. (1996). Two orthodenticle-related genes in the short-germ beetle Tribolium castaneum. *Development Genes and Evolution*, *206*(1), 35-45. https://doi.org/10.1007/s004270050028

Liao, B.-K., & Oates, A. C. (2017). Delta-Notch signalling in segmentation. *Arthropod Structure & Development*, *46*(3), 429-447. https://doi.org/https://doi.org/10.1016/j.asd.2016.11.007

Lim, J., & Choe, C. P. (2020). Functional analysis of engrailed in Tribolium segmentation. *Mechanisms of Development*, *161*, 103594. https://doi.org/https://doi.org/10.1016/j.mod.2019.103594

Moreno, E., & Morata, G. (1999). Caudal is the Hox gene that specifies the most posterior Drosophile segment. *Nature*, *400*(6747), 873-877. https://doi.org/10.1038/23709

Nüsslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature*, *287*(5785), 795-801. https://doi.org/10.1038/287795a0

O, J., & Choe, C. P. (2020). even-skipped acts as a pair-rule gene in germ band stages of Tribolium development. *Developmental Biology*, *462*(1), 1-6. https://doi.org/https://doi.org/10.1016/j.ydbio.2020.03.010

Oberhofer, G., Grossmann, D., Siemanowski, J. L., Beissbarth, T., & Bucher, G. (2014). Wnt/β-catenin signaling integrates patterning and metabolism of the insect growth zone. *Development*, *141*(24), 4740-4750. https://doi.org/10.1242/dev.112797

Patel, N. H., Condron, B. G., & Zinn, K. (1994). Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles. *Nature*, *367*(6462), 429-434. https://doi.org/10.1038/367429a0

Peterson, B. K., Hare, E. E., Iyer, V. N., Storage, S., Conner, L., Papaj, D. R., . . . Eisen, M. B. (2009). Big Genomes Facilitate the Comparative Identification of Regulatory Elements. *PLOS ONE*, *4*(3), e4688. https://doi.org/10.1371/journal.pone.0004688

Pignoni, F., Baldarelli, R. M., Steingrímsson, E., Diaz, R. J., Patapoutian, A., Merriam, J. R., & Lengyel, J. A. (1990). The Drosophila gene tailless is expressed at the embryonic termini and is a member of the steroid receptor superfamily. *Cell*, *62*(1), 151-163. https://doi.org/10.1016/0092-8674(90)90249-e

Ray, S., Rosenberg, M. I., Chanut-Delalande, H., Decaras, A., Schwertner, B., Toubiana, W., . . . Payre, F. (2019). The mlpt/Ubr3/Svb module comprises an ancient developmental switch for embryonic patterning. *eLife*, *8*, e39748. https://doi.org/10.7554/eLife.39748

Read, D., Levine, M., & Manley, J. L. (1992). Ectopic expression of the Drosophila tramtrack gene results in multiple embryonic defects, including repression of even-skipped and fushi tarazu. *Mechanisms of Development*, *38*(3), 183-195. https://doi.org/https://doi.org/10.1016/0925-4773(92)90052-L

Read, D., Nishigaki, T., & Manley, J. L. (1990). The Drosophila Even-Skipped Promoter Is Transcribed in a Stage-Specific Manner In Vitro and Contains Multiple, Overlapping Factor-Binding Sites. *Molecular and Cellular Biology*, *10*(8), 4334-4344. https://doi.org/10.1128/mcb.10.8.4334-4344.1990

Ribeiro, L., Tobias-Santos, V., Santos, D., Antunes, F., Feltran, G., de Souza Menezes, J., . . . Nunes da Fonseca, R. (2017). Evolution and multiple roles of the Pancrustacea specific transcription factor zelda in insects. *PLOS Genetics*, *13*(7), e1006868. https://doi.org/10.1371/journal.pgen.1006868

Rudolf, H., Zellner, C., & El-Sherif, E. (2020). Speeding up anterior-posterior patterning of insects by differential initialization of the gap gene cascade. *Developmental Biology*, *460*(1), 20-31. https://doi.org/https://doi.org/10.1016/j.ydbio.2019.04.015

Sackerson, C., Fujioka, M., & Goto, T. (1999). The even-skipped Locus Is Contained in a 16-kb Chromatin Domain. *Developmental Biology*, *211*(1), 39-52. https://doi.org/https://doi.org/10.1006/dbio.1999.9301

Schoppmeier, M., & Schröder, R. (2005). Maternal torso signaling controls body axis elongation in a short germ insect. *Curr Biol*, *15*(23), 2131-2136. https://doi.org/10.1016/j.cub.2005.10.036

Schröder, R., Eckert, C., Wolff, C., & Tautz, D. (2000). Conserved and divergent aspects of terminal patterning in the beetle Tribolium castaneum. *Proceedings of the National Academy of Sciences*, *97*(12), 6591-6596. https://doi.org/doi:10.1073/pnas.100005497

Schulz, C., Schröder, R., Hausdorf, B., Wolff, C., & Tautz, D. (1998). A caudal homologue in the short germ band beetle Tribolium shows similarities to both, the Drosophila and the vertebrate caudal expression patterns. *Development Genes and Evolution*, *208*(5), 283-289. https://doi.org/10.1007/s004270050183

Sharma, R., Beer, K., Iwanov, K., Schmöhl, F., Beckmann, P. I., & Schröder, R. (2015). The single fgf receptor gene in the beetle Tribolium castaneum codes for two isoforms that integrate FGF8- and Branchless-dependent signals. *Developmental Biology*, *402*(2), 264-275. https://doi.org/https://doi.org/10.1016/j.ydbio.2015.04.001

Singer, J. B., Harbecke, R., Kusch, T., Reuter, R., & Lengyel, J. A. (1996). Drosophila brachyenteron regulates gene activity and morphogenesis in the gut. *Development*, *122*(12), 3707-3718. https://doi.org/10.1242/dev.122.12.3707

Small, S., Blair, A., & Levine, M. (1992). Regulation of even-skipped stripe 2 in the Drosophila embryo. *The EMBO Journal*, *11*(11), 4047-4057. https://doi.org/https://doi.org/10.1002/j.1460-2075.1992.tb05498.x

Small, S., Blair, A., & Levine, M. (1996). Regulation of Two Pair-Rule Stripes by a Single Enhancer in theDrosophilaEmbryo. *Developmental Biology*, *175*(2), 314-324. https://doi.org/https://doi.org/10.1006/dbio.1996.0117

Small, S., Kraut, R., Hoey, T., Warrior, R., & Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in Drosophila. *Genes Dev*, *5*(5), 827-839. https://doi.org/10.1101/gad.5.5.827

Su, M. T., Fujioka, M., Goto, T., & Bodmer, R. (1999). The Drosophila homeobox genes zfh-1 and even-skipped are required for cardiac-specific differentiation of a numb-dependent lineage decision. *Development*, *126*(14), 3241-3251. https://doi.org/10.1242/dev.126.14.3241

Thomson, M. S. (2014). A selfish gene chastened: Tribolium castaneumMedeaM4is silenced by a complementary gene. *Genetica*, *142*(2), 161-167. https://doi.org/10.1007/s10709-014-9763-8

Tidswell, O. R. A., Benton, M. A., & Akam, M. (2021). The neuroblast timer gene nubbin exhibits functional redundancy with gap genes to regulate segment identity in Tribolium. *Development*, *148*(16). https://doi.org/10.1242/dev.199719

Tögel, M., Pass, G., & Paululat, A. (2008). The Drosophila wing hearts originate from pericardial cells and are essential for wing maturation. *Developmental Biology*, *318*(1), 29-37. https://doi.org/https://doi.org/10.1016/j.ydbio.2008.02.043

Weigel, D., Jürgens, G., Küttner, F., Seifert, E., & Jäckle, H. (1989). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell*, *57*(4), 645-658. https://doi.org/https://doi.org/10.1016/0092-8674(89)90133-5

Wisotzkey, R. G., Mehra, A., Sutherland, D. J., Dobens, L. L., Liu, X., Dohrmann, C., . . . Raftery, L. A. (1998). Medea is a Drosophila Smad4 homolog that is differentially required to potentiate DPP responses. *Development*, *125*(8), 1433-1445. https://doi.org/10.1242/dev.125.8.1433

Wolff, C., Schröder, R., Schulz, C., Tautz, D., & Klingler, M. (1998). Regulation of the Tribolium homologues of caudal and hunchback in Drosophila: evidence for maternal gradient systems in a short germ embryo. *Development*, *125*(18), 3645-3654. https://doi.org/10.1242/dev.125.18.3645

Wu, L. H., & Lengyel, J. A. (1998). Role of caudal in hindgut specification and gastrulation suggests homology between Drosophila amnioproctodeal invagination and vertebrate blastopore. *Development*, *125*(13), 2433-2442. https://doi.org/10.1242/dev.125.13.2433

Yan, R., Small, S., Desplan, C., Dearolf, C. R., & Darnell, J. E., Jr. (1996). Identification of a Stat gene that functions in Drosophila development. *Cell*, *84*(3), 421-430. https://doi.org/10.1016/s0092-8674(00)81287-8

Zee, M. v. d., Stockhammer, O., Levetzow, C. v., Fonseca, R. N. d., & Roth, S. (2006). Sog/Chordin is required for ventral-to-dorsal Dpp/BMP transport and head formation in a short germ insect. *Proceedings of the National Academy of Sciences*, *103*(44), 16307-16312. https://doi.org/doi:10.1073/pnas.0605154103

Zhu, X., Rudolf, H., Healey, L., François, P., Brown, S. J., Klingler, M., & El-Sherif, E. (2017). Speed regulation of genetic cascades allows for evolvability in the body plan specification of insects. *Proc Natl Acad Sci U S A*, *114*(41), E8646-e8655. https://doi.org/10.1073/pnas.1702478114