Caudal Regulates the Spatiotemporal Dynamics of Pair-Rule Waves in *Tribolium*

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Abstract

In the short-germ beetle *Tribolium castaneum*, waves of pair-rule gene expression propagate from the posterior end of the embryo towards the anterior and eventually freeze into stable stripes, partitioning the anterior-posterior axis into segments. Similar waves in vertebrates are assumed to arise due to the modulation of a molecular clock by a posterior-to-anterior frequency gradient. However, neither a molecular candidate nor a functional role has been identified to date for such a frequency gradient, neither in vertebrates or elsewhere. Here we provide evidence that the posterior gradient of *Tc-caudal* expression regulates the oscillation frequency of pair-rule gene expression in *Tribolium*. We show this by analyzing the spatiotemporal dynamics of *Tc-even-skipped* expression in strong and mild knockdown of *Tc-caudal*, and by correlating the extension, level and slope of the *Tc-caudal* expression gradient to the spatiotemporal dynamics of *Tc-even-skipped* expression in wild type as well as in different RNAi knockdowns of *Tc-caudal* regulators. Further, we show that besides its absolute importance for stripe generation in the static phase of the *Tribolium* blastoderm, a frequency gradient might serve as a buffer against noise during axis elongation phase in *Tribolium* as well as vertebrates. Our results highlight the role of frequency gradients in pattern formation.

Introduction

The anterior-posterior (AP) axis of arthropods, annelids, and vertebrates is partitioned into segments. The French flag model, in which threshold concentrations of morphogen gradients are interpreted by downstream genes to partition a developing tissue [1,2], provides the main theoretical framework explaining segmentation in *Drosophila*. Specifically, gradients of maternal factors span the AP axis of *Drosophila* providing positional information to downstream gap gene expression. In *Drosophila*, the shift in the posterior regions of the blastoderm forms the posterior gradient to the pair-rule gene expression [3].

In *Drosophila*, all segments form more or less simultaneously in a syncytial blastoderm of fixed AP axis length. In contrast, vertebrate segmentation (somitogenesis) takes place sequentially in an elongating and cellularized embryo. A different model, the ‘clock and wavefront’ explains segmentation in vertebrates [4,5]. Multiple genes (hairy/enhancer-of-split and genes of Notch, Wnt and FGF signaling pathways) show oscillatory expression in the presomitic mesoderm (PSM) of the vertebrate embryo and are thought to be constituents of a molecular clock [6,7]. In cells located anterior to a wavefront, oscillations are arrested into stable stripes. The wavefront is thought to be defined by a moving threshold that forms within the overlapping posterior gradients of Wnt and FGF [8,9] and an opposing retinoic acid gradient [10]. Oscillations seem to arrest gradually (i.e. they are modulated by a frequency gradient) as evidenced by kinematic expression waves that sweep the PSM from posterior to anterior [7].

In most short-germ arthropods, anterior segments form in a blastoderm, as in *Drosophila*, while posterior segments form subsequently during the germband stage out of a population of cells at the posterior end of the embryo termed the ‘growth zone’ [11], reminiscent of somitogenesis in vertebrates. Although it is conceivable that short-germ arthropods utilize a ‘French flag’-based segmentation mechanism in the blastoderm and a ‘clock and wavefront’ mechanism in the germband, it has recently been shown that a segmentation clock operates in both the germband [12] and blastoderm [13] of the short-germ insect *Tribolium castaneum*, where waves of pair-rule gene expression (specifically *Tc-even-skipped* (Tc-eve)) propagate from posterior to anterior [13].

The identification of factors that provide positional information for segmentation in the blastoderm of short-germ arthropods has been controversial [14–18]. Demonstration of the clock-based nature of short-germ segmentation fuels this debate as attention now turns to the search for factors functioning as a wavefront. The homeodomain transcription factor Caudal (Cad) has been implicated as playing a prominent role in AP patterning in...
The frequency of assuming a posterior-to-anterior gradient that positively regulates segmentation in Tribolium castaneum segments are demarcated by means of gene expression waves that propagate from posterior to anterior as the embryo elongates. These waves are assumed to arise due to the regulation of a molecular clock by a frequency gradient. However, to date, neither a candidate nor a functional role has been identified for such a frequency gradient. Here we provide evidence that a static expression gradient of caudal regulates pair-rule oscillations during blastoderm stage in Tribolium. In such a static setup, a frequency gradient is essential to convert clock oscillations into a striped pattern. We further show that a frequency gradient might be essential even in the presence of axis elongation as a buffer against noise. Our work also provides the best evidence to date that Caudal acts as a type of morphogen gradient in the blastoderm of short-germ arthropods; however, Caudal seems to convey positional information through frequency regulation of pair-rule oscillations, rather than through threshold concentration levels in the gradient.

**Author Summary**

One of the most popular problems in development is how the anterior-posterior axis of vertebrates, arthropods and annelids is partitioned into segments. In vertebrates, and recently shown in the beetle Tribolium castaneum, segments are demarcated by means of gene expression waves that propagate from posterior to anterior as the embryo elongates. These waves are assumed to arise due to the regulation of a molecular clock by a frequency gradient. However, to date, neither a candidate nor a functional role has been identified for such a frequency gradient. Here we provide evidence that a static expression gradient of caudal regulates pair-rule oscillations during blastoderm stage in Tribolium. In such a static setup, a frequency gradient is essential to convert clock oscillations into a striped pattern. We further show that a frequency gradient might be essential even in the presence of axis elongation as a buffer against noise. Our work also provides the best evidence to date that Caudal acts as a type of morphogen gradient in the blastoderm of short-germ arthropods; however, Caudal seems to convey positional information through frequency regulation of pair-rule oscillations, rather than through threshold concentration levels in the gradient.

arthropods since its expression overlaps with the newly forming stripes [19]. Cad is required for segmentation in the Drosophila abdomen [20], and for posterior patterning in other species [21,22]. It plays an even more prominent role in non-diptran insect segmentation; it is required for trunk segmentation in Nasonia vitripennis [23] and for both trunk and gnathal segmentation in Tribolium castaneum [24] and Gryllus bimaculatus [25]. However, the exact role of Cad in segmentation is still not known. Here we test the hypothesis that the posterior gradient of Tribolium cad (Tc-cad) expression regulates the oscillation frequency of pair-rule gene expression to produce kinematic waves in the Tribolium blastoderm. We found that the expression of Tc-eve was abolished in strong Tc-cad RNAi knock-down embryos, but in weak Tc-cad knock-down embryos, the Tc-eve expression domain was posteriorly shifted and its oscillation frequency reduced. Perturbing the Tc-cad gradient in different ways by knocking-down its regulators further demonstrated that the extent, intensity, and slope of the Tc-cad gradient correlated with the extension, frequency, and width of Tc-eve expression waves, respectively. As shown by computer simulations, these observations are consistent with the hypothesis that Tc-cad functions as a frequency gradient regulating the spatiotemporal dynamics of pair-rule gene oscillation in Tribolium. These observations, combined with the continued expression of Tc-cad in a posterior gradient suggest that Tc-cad also acts as a wavefront in the elongating germ band. Our study highlights the concept of a frequency gradient as a pattern formation mechanism. Using computer modeling, we also showed that a graded frequency profile might even be essential within the clock-and-wavefront model as a buffer against noise.

**Results**

**Characterizing Tc-cad expression in Tribolium**

The wave dynamics of Tc-eve in Tribolium can be explained by assuming a posterior-to-anterior gradient that positively regulates the frequency of Tc-eve oscillations [13]. Tc-cad is an obvious candidate to encode such a frequency gradient because its mRNA expression forms a posterior-to-anterior gradient that overlaps the Tc-eve expression waves arising at the posterior throughout Tribolium segmentation (Figure 1 A–D). Since studying segmentation in the germband phase of Tribolium development is hindered by the truncation phenotype generated by most segmentation gene knock-downs, we largely restricted our analysis to the stripes that form during the blastoderm stage. The expression of Tc-cad in the blastoderm (Figure 1 E) is approximated with reasonable accuracy by a posterior-to-anterior linear gradient that plateaus at the posterior end (Figure 1 F; Text S3). We used three descriptors to characterize this gradient: maximum posterior (plateau) value, position of anterior border, and slope (Figure 1 F). We analyzed the temporal dynamics of the Tc-cad gradient by calculating its three descriptors at 14–17 and 17–20 hours after egg lay (AEL) (Figure 1 G), spanning the formation of the first and second Tc-eve expression stripes in wild type (WT) [13] (analysis of later times was precluded by primitive pit formation, asterisk in Figure 1 C). As shown in Figure 1 G, the anterior border of Tc-cad expression gradient did not experience a significant shift during the formation of the first and second Tc-eve stripes, (which is also evident in Figure 1 A, B). However, both the maximum posterior value and the slope of the Tc-cad gradient increased over time. This indicates that the Tc-cad gradient was building up during the formation of the first and second Tc-eve stripes, but did not undergo a substantial shift along the AP axis (Figure 1 H). Characterizing Tc-cad gradient dynamics with higher temporal resolution (Figure S1) indicates that this buildup phase occurred between 14 to 16 hours AEL (i.e. before completion of the first Tc-eve stripe), after which the gradient was more or less static. This argues against a substantial influence of Tc-cad temporal dynamics on the wave dynamics of Tc-eve expression in the blastoderm. By the time the third stripe formed in the germ rudiment, the Tc-cad gradient had retreated toward posterior (Figure 1 C).

The spatial distribution of Tc-cad renders it a probable wavefront candidate in a clock-and-wavefront model. In the traditional model, a wavefront should retract posteriorly (like Tc-cad expression during the germband stage). However, a static but smooth gradient (like Tc-cad expression during the formation of first and second Tc-eve stripes in the blastoderm) that modulates the frequency of Tc-eve oscillation is, in principle, capable of forming a striped expression pattern (Movies S1, lower panel) [13,26]. Taking the initial buildup phase of the Tc-cad gradient into consideration (Movies S1, upper panel) yields similar results. However, this buildup phase is expected to slow down the formation of the first stripe (Figure S2). This agrees with experiment, since the first cycle of Tc-eve oscillations starts at 13.5 hours AEL and ends at 17 hours AEL (i.e. the first stripe takes 3.5 hours to form), while the second cycle starts at 17 hours AEL and ends at 20 hours (i.e. the second stripe takes 3 hours to form) [13]. However, this is not obvious in the timing results presented here (see below), since we chose to start our analysis at 14 hours AEL.

**Regulation of the Tc-cad gradient**

In both vertebrates and arthropods, canonical Wnt is a positive regulator of cdx/cad [25,27–29]. Once bound by Wnt ligand, the receptor Frizzled recruits the β-catenin destruction complex (comprising Axin, APC, and other factors), rendering β-catenin free to enter the nucleus and bind Pangolin (TCF) with the help of Legless (Lgs), Pygopous (Pygo) and other coactivators [30] to activate Wnt targets. In Tribolium, wnt1 and wnt8 are expressed at the posterior pole of the blastoderm, and at the posterior end of the growth-zone in the germ band [31], which is expected to produce a posterior gradient of Wnt activity, the formation of
which is enhanced by the anterior localization of Wnt repressors in the blastoderm [28,32].

Manipulating Wnt activity affected Tc-cad expression in the Tribolium blastoderm. Knocking down Tc-lgs (a positive Wnt regulator) by means of maternal RNAi (Methods) shifted the Tc-cad expression gradient posteriorly (compare Figure 2 C–C’ to Figure 2 A–A’). In addition, the posterior maximum value of Tc-cad and slope of the gradient were reduced in Tc-lgs RNAi embryos compared to WT (Figure 2 D–D’).

Knocking down Tc-apc1 (a negative Wnt regulator) repositioned the Tc-cad gradient anteriorly (Figure 2 G–H’). Interestingly, the maximum posterior value of the Tc-cad expression gradient at 14–17 hours AEL was lower in Tc-apc1 RNAi embryos than in WT embryos (Figure 2 H’), but eventually reached WT levels by 17–20 hours AEL (Figure 2 H’). Thus, it appears that the Tc-cad expression gradient takes longer to mature in Tc-apc1 RNAi than in WT embryos, which might be indicative of early negative Wnt regulation of Tc-cad.

Knocking down another Wnt regulator, Tc-pan, also perturbed the Tc-cad expression gradient. Pan, a component of the activator complex, also acts as a repressor in the absence of nuclear β-catenin [33]. Hence, we expected Wnt activity to be reduced posteriorly but increased anteriorly in Tc-pan RNAi embryos compared to WT, resulting in a shallower Wnt gradient across the blastoderm, and consequently a shallower Tc-cad gradient. As expected, the border of the Tc-cad gradient in Tc-pan RNAi embryos shifted anteriorly, the gradient reached a lower maximum posterior value, and the slope was lower compared to WT (Figure 2 E–F’).

In Drosophila, two Hox3 type genes are involved in early patterning: bicoid (bed), which is expressed anteriorly and plays a major role in AP patterning, and zerknüllt (zen), which is expressed dorsally and specifies the amnioserosa [34]. Tribolium lacks bed [17] but one of its zen homologs, Tc-zen1, is expressed both anteriorly and dorsally [35]. Anterior expression precedes dorsal expression and is suspected to play a role in AP patterning [36]. As shown in Figure 2 I–J’, the Tc-cad gradient in Tc-zen1 RNAi embryos shifted anteriorly, but had the same slope and maximum posterior expression level as WT, indicating that Tc-zen1 represses Tc-cad anteriorly (see Figure 2 B for a summary of Tc-cad regulation).

Tc-cad gradient regulates Tc-eve waves in Tribolium

In Tribolium, Tc-eve is expressed in waves that shrink while propagating from posterior to anterior (Figure 3 A) [13]. Tc-eve and Tc-cad RNAi embryo display similar phenotypes lacking all post oral segments, and previous studies implicate cad in the regulation of eve in arthropods [24,25].

Tc-cad RNAi. To examine a possible role of Tc-cad in regulating Tc-eve, we characterized the dynamics of Tc-eve expression in WT and Tc-cad RNAi embryos. Strong Tc-cad RNAi completely abolished Tc-eve expression (Figure S3 A). We produced milder effects by injecting lower concentrations of Tc-cad dsRNA. In these embryos, waves of Tc-eve expression propagated from posterior to anterior (Figure 3 B); however, the final positions of the Tc-eve stripes were shifted posteriorly compared to WT (compare Figure 3 B with Figure 3 A; Figure 4 A).

In the mild Tc-cad RNAi embryos, the three expected stripes did not fully form prior to germ rudiment condensation (Figure 3 B). To determine if this is due to a reduction in Tc-eve oscillation frequency, we measured the maximum frequency of Tc-eve oscillations by tracing Tc-eve expression over time at the posterior
Figure 2. Characterization of *Tc-cad* gradient in WT and RNAi knockdowns. (A, A’) *Tc-cad* gradient in WT. (B) A model for *Tc-cad* regulation in the *Tribolium* blastoderm. (C–D’) *Tc-cad* gradient expression in a *Tc-lgs* RNAi embryo (C, C’), and the average of its three descriptors normalized to WT values (Text S3) in 14–17 AEL (D) and 17–20 AEL (D’). As inferred from (D, D’), a comparison between the spatial distribution of *Tc-cad* gradient in *Tc-lgs* RNAi embryos and that of WT is summarized in D” (not to scale). The same was performed for *Tc-pan* (E–F’), *Tc-opc1* (G–H’; in H’: dashed curve for 14–17 AEL and solid curve for 17–20 AEL), *Tc-zen1* (I–J’), and *Tc-lgs;Tc-zen1* (K–L’) RNAi embryos. (M–M’) the average of the three descriptors of the *Tc-cad* expression gradient in *Tc-pan* RNAi normalized to *Tc-lgs* RNAi values (Text S3). (N–N’) the average of the three descriptors of the *Tc-cad* expression gradient in *Tc-lgs;Tc-zen1* RNAi normalized to *Tc-lgs* RNAi values. Anterior to the left. Error bars represent 95% confidence intervals. Asterisk (*) represents p-value < 0.05.

doi:10.1371/journal.pgen.1004677.g002
end of the blastoderm (Figure 5 A; Text S3). In WT, a new Tc-eve cycle peaked in every 3-hour egg collection (Figure 5 A, blue bars), consistent with the 3 hour periodicity we previously reported for Tc-eve oscillations at 23–24°C [13]. For mild Tc-cad RNAi, while cycle I initiated at 14 to 17 hrs AEL similar to WT, it persisted through 17 to 20 hrs AEL (Figure 5 A, red bar). The duration of Tc-eve cycles I and II in Tc-cad RNAi embryos (Figure 5 A, Text S3) both lasted longer than in WT.

Tc-lgs RNAi. In Tc-lgs RNAi embryos, the anterior border of Tc-cad expression shifted posteriorly and the posterior maximum level decreased (Figure 2 C–D). The Tc-eve waves were also shifted posteriorly, in accordance with the posterior shift of the Tc-cad gradient (compare Figure 3 C with Figure 3 A; Figure 4 B). In addition, the Tc-eve oscillation frequency was reduced (Figure 5 B, B’), corresponding to the reduction in posterior Tc-cad levels. Both the posterior shift and the reduced frequency of Tc-eve oscillations at the posterior end of the blastoderm upon the reduction of the Tc-cad gradient (either in mild Tc-cad RNAi or Tc-lgs RNAi) is predicted by a model in which the Tc-cad gradient regulates the frequency of Tc-eve oscillations (Movie S2, compare Figure 3 C to Figure 3 A’).

Tc-pan RNAi. In contrast, the Tc-cad gradient shifted anteriorly in Tc-pan RNAi embryos (Figure 2 E–F). Correspondingly, Tc-eve waves were shifted anteriorly in Tc-pan RNAi compared to WT (compare Figure 3 D to Figure 3 A; Figure 4 C for 17–23 hours AEL). However, similar to Tc-lgs RNAi, Tc-cad mRNA levels were reduced at the posterior end of Tc-pan RNAi embryos (Figure 2 E–F’). The corresponding Tc-eve oscillation frequency was also reduced (Figure 5 C, C’). In addition to the anterior shift and frequency reduction of Tc-eve expression waves, the width of Tc-eve stripes in Tc-pan RNAi embryos was strikingly wider than those in WT (compare Figure 3 D to Figure 3 A; Figure 4 C’ and C”). This corresponds to the stretching effect of Tc-pan RNAi knock-down on the Tc-cad gradient, evident in the lower slope and anterior shift of this gradient in Tc-pan RNAi embryos compared to WT (Figure 2 F–F’).

Interestingly, in Tc-lgs RNAi embryos the first Tc-eve stripe, which formed at 17–20 hours AEL, was wider than that of WT.
(Figure 4 B') in accordance with the reduction of the slope of Tc-cad gradient there (Figure 2 D–D'), but by 20–23 hours AEL the width of Tc-eve stripes is similar to WT (Figure 4 B' and B''). In contrast to Tc-pan RNAi, Tc-cad slope reduction in Tc-lg5 RNAi embryos might not be severe enough to result in detectable differences in the final width of Tc-eve stripes. Comparison of the Tc-cad gradient in RNAi embryos that were fixed and stained in parallel confirmed that while the level of Tc-cad expression at the posterior end in both Tc-lg5 and Tc-pan was similar, the slope reduction in Tc-pan RNAI was more severe than in Tc-lg5 RNAI embryos (Figure 2 M–M').

The final anterior (but initial posterior) shift (Figure 4 C), the reduced frequency of Tc-eve oscillations at the posterior end of the blastoderm, and the wider Tc-eve stripe that were observed upon reducing and stretching the Tc-cad gradient in Tc-pan RNAI embryos is predicted by a model in which the Tc-cad gradient modulates the frequency of Tc-eve oscillations (Movie S3; Figure 3 D').

\textbf{Tc-apc1 RNAi.} In Tc-apc1 RNAI embryos, Tc-eve waves shifted towards the anterior (compare Figure 3 E to Figure 3 A; Figure 4 D) corresponding to the anterior shift in the Tc-cad gradient (Figure 2 G–H'). The first Tc-eve stripe took longer to form in Tc-apc1 RNAI embryos compared to WT (Figure 5 D'), corresponding to a lower maximum posterior value of Tc-cad in Tc-apc1 RNAI embryos during 14–17 AEL (Figure 2 H). The second stripe formed with near normal kinetics in Tc-apc1 RNAI embryos (Figure 5 D'), in accordance with the eventual increase of the maximum posterior value of Tc-cad in Tc-apc1 RNAI during 17–20 AEL (Figure 2 H').

\textbf{Tc-zen1 RNAi.} In Tc-zen1 RNAI embryos, Tc-eve waves shifted towards the anterior (compare Figure 3 F to Figure 3 A) corresponding to the anterior shift of the Tc-cad gradient (Figure 2 I–J'). The build-up of Tc-cad transcripts in the posterior in Tc-zen1 RNAI embryos was similar to those in WT (Figure 2 J,J'); correspondingly, the timing of Tc-eve waves in Tc-zen1 RNAI and WT embryos are very similar (Figure 5 E, E'). The anterior shift of Tc-eve waves upon anterior extension of the Tc-cad gradient (in Tc-apc1 and Tc-zen1 RNAI) is predicted by a model in which Tc-cad gradient modulates the frequency of Tc-eve oscillations (Movies S4 and S5; Figures 3 E' and 3 F').

The slope of Tc-cad gradient in both Tc-apc1 and Tc-zen1 RNAI embryos is largely similar to that of WT (Figure 2 H–H' and J–J'), and the corresponding width of Tc-eve stripes is also similar to WT (Figure 4 D', D', E', and E'), with the possibility of a slight initial reduction in the slope of the Tc-cad gradient in Tc-apc1 RNAI embryos (Figure 2 H) and the corresponding slight increase in Tc-eve stripe width (Figure 4 D'). The final stripe width reduction (at 20–23 hours AEL) in Tc-apc1 RNAI embryos (and possibly Tc-cad RNAI embryos; Figure 4 A' and D') could be due to a defect in the characteristic split of mature Tc-eve stripes into secondary, segmental stripes (compare Figure 3 E class III.1 embryo to Figure 3 A class III.1 embryo; while the splitting defect is variable in wild Tc-cad RNAI embryos, Figure S3 B).

\textbf{Tc-lg5;Tc-zen1 double RNAI.} Since Tc-lg5 and Tc-zen1 RNAI shifted the Tc-cad gradient (and Tc-eve stripes) in opposite directions, we sought to examine the effect of the double Tc-lg5;Tc-zen1 RNAI knock-down. Tc-zen1 RNAI rescued to some degree the posterior shift in Tc-cad gradient induced by Tc-lg5 RNAI (Figure 2 K–L'). The anterior border of the Tc-eve expression domain in Tc-lg5;Tc-zen1 double RNAI embryos is closer to that of WT than that of Tc-lg5 RNAI (Figure 3 G–H'). Surprisingly, although the Tc-cad posterior expression level is not altered in Tc-zen1 RNAI, the posterior maximum expression level of Tc-cad was partially rescued in Tc-lg5;Tc-zen1 double RNAI embryos at 17–20 hours AEL (Figure 2 N–N'). Corresponding to this, the first Tc-eve stripe forms more quickly in Tc-lg5;Tc-zen1 RNAI compared to Tc-lg5 RNAI (Figures 5 F, F'). However, this rescue effect eventually fades by the end of the blastoderm stage (20 to 23 hours AEL; Figure 5 F, F'), when Tc-zen1 is normally down-regulated (Figure S4).

The intermediate phenotype of Tc-lg5;Tc-zen1 RNAI between that of WT and Tc-lg5 RNAI is predicted by a model in which Tc-cad gradient modulates the frequency of Tc-eve oscillations (Movie S6; Figure 3 G').

\textbf{Graded frequency profile as a buffer against noise.} Axis elongation is an essential component of the clock-and-wavefront model. We have previously shown that blastoderm segmentation in Tribolium seems to be clock-based [13]. Despite the lack of axis elongation at the blastoderm stage, we did not exclude the possible existence of a retreating frequency gradient (wavefront). In the current study, we provide evidence that Tc-cad expression acts as a frequency gradient that modulates pair-rule gene oscillations in the blastoderm. Although a static step frequency gradient (i.e. suddenly dropping from non-zero to zero frequency) does not possess any patterning capacity, a static but gradually decreasing frequency gradient can generate a striped pattern [26]. Indeed, the first two stripes of Tc-eve form during a time period when the Tc-cad gradient is largely static. After the formation of the first two stripes, Tc-cad expression then abruptly retreats to the prospective growth zone (Figure 1 C). Later during axis elongation in the germ band stage, Tc-cad expression retreats posteriorly with every newly forming Tc-eve stripe (Figure 1 D).

However, in principle, a step frequency gradient is capable of generating a striped pattern during the germ band retraction phase. In vertebrates, a transition from high to low frequency (termed the 'arrest front') is thought to be determined by a threshold within a retreating posterior gradient. Such a mechanism might be very sensitive to the location of the threshold. Uncertainty in threshold location due to noise might lead to the generation of noisy patterns. On the other hand, gradually arresting oscillations would average out the noise and make the mechanism independent of precise threshold location. To investigate this, we developed two computer models for the clock-and-wavefront mechanism: one that utilizes a step frequency gradient by applying a threshold on a retreating smooth gradient (threshold-based model), and the other utilizes a smooth retreating frequency gradient without applying any thresholds (threshold-free model). Both generated similar striped patterns in the absence of noise (Figures 6 A–D; Movies S7 and S8). We then investigated the performance of both models after introducing random...
Fluctuations in the intensity of the posterior gradient at each cell. This is expected to result in independent random shifts in threshold locations across the lateral axis of the embryo, which ultimately leads to salt-and-pepper noise at the stripe borders; however, the threshold-free model is more robust to this type of noise than the threshold-based model (Figures 6 E–H; Movies S9 and S10).

Discussion

In this work we provide evidence that an anterior-to-posterior gradient of Tc-cad expression in Tribolium regulates waves of Tc-eve pair-rule gene expression. By examining the spatiotemporal dynamics of Tc-eve expression in WT and RNAi knockdowns of different Tc-cad regulators, three correlations were revealed: (1)
the spatial extent of Tc-cad correlates with that of Tc-eve waves, (2) the level of Tc-cad expression correlates with the frequency of Tc-eve oscillations at the posterior end of the blastoderm, and (3) the slope of the Tc-cad gradient correlates with the width of Tc-eve stripes. These three correlations are consistent with the hypothesis that the Tc-cad gradient modulates the frequency of pair-rule oscillations resulting in waves of gene expression [Figure 3 A, C’–G’; Movies S2, S3, S4, S5, S6]. A clock regulated by a frequency gradient is one way of transforming a temporally periodic process into a spatially periodic one; another would be the clock-and-wavefront model. One advantage of patterning with a frequency gradient, in contrast to the clock-and-wavefront model, is that it does not require axis elongation, which might explain how the Tribolium blastoderm is segmented. Another advantage, that we demonstrated using computer modelling, is that even within the framework of the clock-and-wavefront, utilizing a graded frequency profile renders the segmentation process more robust against noisy wavefront gene expression (Figure 6; Movies S7, S8, S9, S10).

The role of Caudal in segmentation

In Drosophila, maternal cad mRNA (Dm-cad) is ubiquitously expressed in the early blastoderm [37]. A posterior-to-anterior protein gradient of Dm-Cad forms due to translational repression by a reciprocal gradient of Dm-Bicoid [38]. Dm-Cad acts as an activator of posterior gap [39] and pair-rule genes [40] and binds to the enhancers of these genes [41,42]. However, the mild segmentation defects in embryos in which the shape of Dm-Cad gradient has been altered argues against its function as a morphogen gradient [20,43]. In the wasp Nasonia vitripennis, Nv-cad plays a more prominent role in activating gap and pair-rule genes, and a limited positioning role [23]. In the cricket Gryllus bimaculatus, Gb-cad was found to activate the pair-rule gene Gb-eve, and activate and position gap gene domains. This indicates that cad might act as a morphogen gradient in non-dipteran insects. In this study, we described similar results in Tribolium. We showed that in strong Tc-cad RNAi, expression of Tc-eve was abolished (Figure S2 A); while in weak Tc-cad RNAi, Tc-eve expression was posteriorly shifted (Figure 3 B). However, a morphogen gradient acting through concentration thresholds is less likely to act in positioning the highly dynamic pair-rule gene expression domains in Tribolium. Instead, we argue that Tc-cad regulates the frequency of a pair-rule clock to produce the observed wave dynamics.

Three cad homologs are found in mouse: Cdxl, Cd2x, and Cd1x. They are expressed in nested domains in the posterior end of the embryo. The Cd1x–Cd2x double mutant exhibits fused somites [44], suggesting a role in somitogenesis. However, the Cd1x–Cd2x double mutant also shows down-regulation of some caudalizing factors involved in somitogenesis (such as wnt3a) that are themselves Cd regulatory [45,46]. Cd1x genes also directly regulate Hox genes in a dose dependent manner [47,48], and even regulate their activation times [49].

In summary, cad-related genes are involved in posterior patterning in many species. While it is not clear whether they play a permissive or instructive role, there is evidence that they might act as a morphogen gradient for gap genes in basal insects (like in Gryllus) and for Hox genes in vertebrates. In this study, we showed that Tc-cad regulates the spatiotemporal dynamics of Tribolium pair-rule genes in a dose dependent manner, stressing the instructive role of cad in the development of a non-dipteran insect. However, we cannot exclude the possibility that Tc-cad regulates pair-rule genes indirectly. Indeed, Tc-cad and Wnt might cross-regulate in a positive feedback loop to form identical gradients. In this case, it is hard to decide which is the direct regulator (or whether both Wnt and Tc-Cad are direct regulators) of Tc-eve expression without performing detailed cis-regulatory analysis of the Tc-eve locus.

The patterning capacity of frequency gradients and the robustness of the clock-and-wavefront model

In the original formulation of the clock-and-wavefront model, the anterior-to-posterior movement of a step frequency profile (i.e. suddenly dropping from non-zero to zero frequency) over an oscillating field of cells sequentially generates a striped pattern in an anterior-to-posterior order [4]. Later, this mechanism was modified by assuming a graded frequency profile to accommodate the observation that oscillations organize into kinematic waves in the chick PSM [7]. Several efforts have been made to identify molecular gradient(s) that regulate the frequency of the vertebrate segmentation clock. A posterior-to-anterior Wnt activity gradient was found to define the PSM oscillation domain in the mouse [50,51]. Furthermore, down-regulation of Wnt activity reduced the clock frequency in both mouse and chick [32]. However, elevated and flattened constitutive stabilization of β-catenin in the mouse PSM only extended the oscillation domain, arguing against a role for the shape of Wnt activity gradient in segmentation [50]. A posterior-to-anterior FGF gradient in the PSM was found to define where oscillations arrest [9,53,34], but manipulating the level of FGF signaling does not alter the clock period [9,52]. A gradient of Her13.2 in zebrafish was suggested to modulate clock frequency through heterodimerization with other zebrafish clock constituents: Her1 and Her7 [55,56]. However, this idea was recently challenged and an alternative model of gradual switching between two oscillatory modules was suggested [57].

It is not known whether the gradual arrest of oscillations and the resulting kinematic waves in vertebrates have any functional role or are a mere peculiarity, since, based on computer simulations of the clock-and-wavefront model, stripe widths depend only on the wavefront velocity and the maximum clock period, not on the shape of the frequency profile [5]. Although used for cosmetic means within the clock-and-wavefront model, a graded frequency profile (even a static one) by itself has a patterning capacity [26]; kinematic waves were observed in an oscillating Zhabotinskii chemical reaction, where a reactant controlling the frequency of oscillation is distributed in a gradient [58,59]. Since a static step frequency profile is unable to generate any stripes, the patterning capacity of a graded frequency profile might explain how blastodermal Tc-eve stripes in Tribolium form in the absence of axis elongation. Although the possibility of a yet unidentified frequency gradient that sweeps across the blastoderm still exists, we showed in this study that a strong candidate for the frequency gradient in Tribolium, Tc-cad, does not appreciably shift during the formation of the first two Tc-eve stripes (Figure 1 G, H).

In addition to its necessity in the absence of axis elongation, a graded frequency profile renders the clock-and-wavefront robust against noise in wavefront gene expression, as shown by computer simulations (Figure 6 and Movies S7, S8, S9, S10). This improvement in robustness might be due to the distributed nature by which oscillations are arrested in a graded frequency profile, in contrast to the total reliance on a single threshold in a step frequency profile. This and other recent works reinforce the importance of the concept of a frequency (or phase) gradient in sequential patterning [60,61].

In clock-based segmentation models that utilize a static frequency gradient, stripes continue to shrink and never stabilize (although stripe shrinkage rate decreases with time, Movie S1). Stripe stabilization can be achieved by the retraction of the
frequency gradient, kick-starting another ‘stabilizing’ genetic program that completely freezes the stripes. Such a stabilizing program might further refine the stripes and/or split them into secondary stripes. Interestingly, in the germ band stage (where Tc-cad retracts continuously along with germ band elongation), once a Tc-evi stripe forms, it splits into two secondary (segmental) stripes [13], whereas in the blastoderm stage, the first Tc-evi stripe does not split until Tc-evi expression completely retreats towards the posterior, at which time the second Tc-evi stripe is already formed and the third stripe is about to emerge (Figure 1 B-D). This suggests a link between Tc-cad retraction and Tc-evi splitting. Stabilizing and refinement/splitting strategies might rely on autoregulatory and cross-regulatory interactions between pair-rule genes or on a reaction diffusion mechanism [62] or both.

Materials and Methods

In situ hybridization, immunocytochemistry, and RNAi

In situ hybridization was performed using DIG-labeled RNA probes and anti-DIG::AP antibody (Roche). Signal was developed using NBT/BCIP (BM Purple, Roche), or Fast Red/HNPP (Roche). Immunocytochemistry was performed using anti-Eve mouse monoclonal antibody 2B8, hybridoma bank, University of Iowa) as primary, and anti-mouse::POD as secondary antibody (ABC kit, Vector). AlexaFluor 488 tyramide (Invitrogen) was used to give green fluorescent signal. All expression analyses were performed using embryos from uninjectected GA-1 strain (WT) or adult GA-1 females injected with double-stranded RNA (ds RNA) of the gene of interest. dsRNA was synthesized using the T7 megascript kit (Ambion) and mixed with injection buffer (5 mM KCl, 0.1 mM KPO4, pH 6.8) before injection. Used dsRNA concentrations: 200 ng/µl for severe Tc-cad, 7.5 µg/µl for mild Tc-cad, 200 ng/µl for Tc-lgs, 200 ng/µl for Tc-pan, 1 µg/µl for Tc-apc1, 1 µg/µl for Tc-evi, and 200 ng/µl; 1 µg/µl for Tc-lgs;Tc-zen double RNAi.

Egg collections for developmental time windows

One hour developmental windows were generated by incubating one hour egg collections at 23–24°C for the desired length of time. For 3-hour developmental windows, eggs were collected after three hours instead of one hour. The beetles were reared in whole-wheat flour supplemented with 5% dried yeast.

Supporting Information

Figure S1 Detailed temporal dynamics of Tc-cad expression gradient in the blastoderm. Shown is the three descriptors of Tc-cad expression gradient in one-hour timed egg collections (Text S3) spanning the time period 14 to 20 hours AEL. Tc-cad expression gradient builds up during 14–16 hours AEL (but without appreciable AP shift). During 16–19 hours AEL, the gradient is more or less static, but starts to drop after 19 hours AEL. Error bars represent 95% confidence intervals.

Figure S2 Stripes form slower during the build phase of the frequency gradient. Shown are the oscillation dynamics over time of a point at the posterior end (far right) in the computer simulations shown in (A) upper panel and (B) lower panel of Movie S1.

Figure S3 Tc-evi in severe and mild Tc-cad knockdowns. (A) Shown are two embryos with comparable stage (flattened posterior stage); Tc-evi is expressed in two stripes in WT while its expression is abolished in strong Tc-cad RNAi. (B) In mild Tc-cad RNAi, Tc-evi stripes split into secondary stripes (upper embryo; similar to WT; see class III.1 embryo in Figure 3 A) in some embryos, while in other embryos they do not (lower embryo). Anterior to left.

Figure S4 Early and late Tc-zen1 expression in Tribolium blastoderm. The dorsal anterior expression of Tc-zen1 (A) is down-regulated at the end of blastoderm stage (B) in WT Tribolium embryos.

Figure S5 Average AP axis lengths over time for WT and RNAi knockdowns. For 14–17, 17–20, 20–23 hours AEL egg collections, the average AP axis lengths were calculated and normalized to 14–17 hours AEL average value for WT, mild Tc-cad, Tc-lgs, Tc-pan, Tc-apc1, Tc-zen1, and Tc-lgs;Tc-zen1 RNAi.

Movie S1 Modeling Tc-evi waves in WT. Tc-evi expression (blue) in the blastoderm was modeled by an array of oscillators along the horizontal axis (representing the AP axis; posterior to the right). Each oscillator runs independently with a frequency determined by a spatial gradient (red). Simulations were run using a frequency gradient (red) corresponding to Tc-evi in WT. In lower panel, the frequency gradient is static. In upper panel, the frequency gradient builds up exponentially to steady state values equal to that in lower panel. Simulations were performed using Matlab (code is available in Text S1).

Movie S2 Modeling Tc-evi waves in Tc-lgs RNAi embryos versus WT. Simulation with a frequency gradient corresponding to Tc-cad in WT (upper panel, which is similar to the upper panel of Movie S1) was contrasted to a simulation run using a frequency gradient corresponding to Tc-cad in Tc-lgs RNAi (posteriorty shifted with reduced posterior value and small decrease in slope, compared to WT). Simulations were performed using Matlab (code is available in Text S1).

Movie S3 Modeling Tc-evi waves in Tc-pan RNAi embryos versus WT. Same as in Movie S2, but with a frequency gradient corresponding to Tc-cad in WT compared with simulations run using a frequency gradient corresponding to Tc-cad in Tc-pan RNAi (anteriorly shifted with reduced posterior value and large decrease in slope, compared to WT).

Movie S4 Modeling Tc-evi waves in Tc-apc1 RNAi embryos versus WT. Same as in Movie S2, but with a frequency gradient corresponding to Tc-cad in WT compared with simulations run using a frequency gradient corresponding to Tc-cad in Tc-apc1 RNAi (anteriorly shifted with the same posterior value and slope as WT, but with slower build up dynamics).

Movie S5 Modeling Tc-evi waves in Tc-zen1 RNAi embryos versus WT. Same as in Movie S2, but with a frequency gradient corresponding to Tc-cad in WT compared with simulations run using a frequency gradient corresponding to Tc-cad in Tc-zen1 RNAi (anteriorly shifted with the same posterior value, slope and build up dynamics as WT).

Movie S6 Modeling Tc-evi waves in Tc-lgs;Tc-zen1 double RNAi embryos versus WT. Same as in Movie S2, but with
a frequency gradient corresponding to *Te-ecd* in WT compared with simulations run using a frequency gradient corresponding to *Te-ecd* in *Tc-lgs;Te-zen1* RNAi (the anterior border is located between those of WT and *Te-lgs* RNAi; the posterior value is higher than that of *Te-lgs* RNAi but lower than that of WT; slope as *Te-lgs* RNAi and buildup dynamics as WT and *Te-lgs* RNAi).

(MVM)

**Movie S7** Performance of the threshold-free model in the absence of noise. Shown is a computer simulation of 2D lattice of oscillators (horizontal and vertical axes of the lattice represent the AP and lateral axes of the embryo, respectively, posterior to the right). Each oscillator (which high phase output is shown in white and low phase in black; lowermost panel) runs independently with a frequency determined by a smooth spatial gradient (shown in greyscale: the brighter the higher the gradient intensity, uppermost panel) that retracts posteriorly with time. Shown is a version of the model that utilizes the smooth gradient to regulate frequency directly (threshold-free model). Simulations were performed using Matlab (code is available in Text S2).

(MVM)

**Movie S8** Performance of the threshold-based model in the absence of noise. Same as in Movie S6, but with a version of the model that applies a threshold to the frequency gradient (threshold-based model; the thresholded gradient is shown in the middle panel).

(MVM)

**Movie S9** Performance of the threshold-free model subjected to gradient intensity noise. Same as in Movie S6, but with the threshold-free model subjected to gradient intensity noise.

(MVM)

**Movie S10** Performance of the threshold-based model subjected to gradient intensity noise. Same as in Movie S7, but with the threshold-based model subjected to gradient intensity noise.

(MVM)

**Text S1** Matlab code for Movies S1, S2, S3, S4, S5, S6.

(DOCX)

**Text S2** Matlab code for Movies S7, S8, S9, S10.

(DOCX)

**Text S3** Supplemental experimental procedures.

(DOCX)

**Author Contributions**

Conceived and designed the experiments: EES SJB. Performed the experiments: EES XZ JF. Analyzed the data: EES XZ SJB. Wrote the paper: EES SJB.

References


