Speed regulation of genetic cascades allows for evolvability in the body plan specification of insects

Xin Zhu[a,1], Heike Rudolf[b,1], Lucas Healey[b], Paul François[c], Susan J. Brown[a], Martin Klingler[b], and Ezzat El-Sherif[b,2]

[a]Division of Biology, Kansas State University, Manhattan, KS 66506; [b]Department of Biology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen 91058, Germany; and [c]Department of Physics, McGill University, Montréal, QC H3A 2T8, Canada

Edited by Naama Barkai, Weizmann Institute of Science, Rehovot, Israel, and accepted by Editorial Board Member Kathryn V. Anderson August 11, 2017 (received for review February 13, 2017)

During the anterior–posterior fate specification of insects, anterior fates arise in a nonelongating tissue (called the "blastoderm"), and posterior fates arise in an elongating tissue (called the "germband"). However, insects differ widely in the extent to which anterior–posterior fates are specified in the blastoderm versus the germband. Here we present a model in which patterning in both the blastoderm and germband of the beetle *Tribolium castaneum* is based on the same flexible mechanism: a gradient that modulates the speed of a genetic cascade of gap genes, resulting in the induction of sequential kinematic waves of gap gene expression. The mechanism is flexible and capable of patterning both elongating and nonelongating tissues, and hence converting blastodermal to germband fates and vice versa. Using RNAi perturbations, we found that blastodermal fates could be shifted to the germband, and germband fates could be generated in a blastoderm-like morphology. We also suggest a molecular mechanism underlying our model, in which gradient levels regulate the switch between two enhancers: One enhancer is responsible for sequential gene activation, and the other is responsible for freezing temporal rhythms into spatial patterns. This model is consistent with findings in *Drosophila melanogaster*, where gap genes were found to be regulated by two nonredundant "shadow" enhancers.

clock-and-wavefront | evolution | kinematic waves | cascade | enhancer switching

Rhythmic and sequential gene activity has been implicated in the spatial patterning of many embryonic structures. For example, a molecular clock mediates stripes of gene expression that delimit vertebrate somites (1–3), segments in short-germ arthropods (4–10), and lateral roots in plants (11, 12). Aperiodic sequential activation of genes regulates the spatial patterning of *Drosophila* neuroblasts (13, 14) and the vertebrate neural tube (15). However, different strategies are used in each case to translate a temporal process into a spatial one. Two main mechanisms have been described: (*i*) one based on the continuous retraction of a steep gradient or boundary (usually called a "wavefront") and (*ii*) the other based on a static or nonretracting gradient. The "clock-and-wavefront" model exemplifies the first type, and was originally proposed in the context of vertebrate somitogenesis (16). In this model, an arrest front sweeps the tissue and freezes oscillations of a molecular clock into stripes. The "spatial and temporal gradient" model exemplifies the latter, which was proposed in the context of vertebrate neural tube development (15, 17–19). In this model, the concentration of and exposure time to a more or less static (nonretracting) gradient regulates the sequential activation of genes.

Models that use a wavefront (henceforth called "wavefront-based" models) are best suited for patterning elongating tissues, since axial elongation offers a natural mechanism for continuous and sustained gradient retraction. On the other hand, models that use a static gradient (henceforth called "gradient-based" models) are best suited for patterning nonelongating tissues, since such tissues are stable enough to support the formation of gradients of the desired shape and level.

Often in evolution, the morphology of a certain embryonic structure changes, but still exhibits the same gene expression pattern. A notable example is the evolution of anterior–posterior (AP) patterning during early embryogenesis of insects. The AP fates of most insects are specified in two different phases (20): (*i*) the blastoderm, where the AP axis does not undergo any axial elongation, and (*ii*) the germband, where the AP axis undergoes gradual axis elongation. Insects differ in the number of fates specified in the blastoderm vs. germband. In short-germ insects [e.g., the grasshopper *Schistocerca americana* (21)], most fates form in the germband, while, in long-germ insects [e.g., the fruit fly *Drosophila melanogaster* (22) and the beetle *Callosobruchus maculatus* (23)], most fates form in the blastoderm. Intermediate-germ insects lie somewhere between those two extreme cases. For example, in the intermediate-germ insects *Tribolium castaneum* and *Oncopeltus fasciatus*, the gnathal and thoracic fates are specified in the blastoderm, while abdominal fates are specified in the germband (23, 24). In the intermediate-germ beetle, *Dermestes maculatus*, gnathal, thoracic, and some of the abdominal fates form in the blastoderm and the rest form in the germband (25), making it closer to the long-germ end of the short-germ/long-germ spectrum of insect embryogenesis. Throughout evolution, the specification of AP fates seems to shift easily from the germband to the blastoderm, resulting in a trend of short-germ to long-germ evolution [with some reports of the opposite evolutionary path (26)]. Given such dramatic flexibility of AP patterning in insects, we hypothesize that both blastoderm and germband are patterned using similar or related mechanisms.

Significance

How a homogeneous group of cells is partitioned into domains of different identities is a common problem in embryogenesis. Partitioning, in some cases, takes places within a static tissue field and, in other cases, in a progressively growing tissue. A curious case is the partitioning of insect bodies into a head, thorax, and abdomen, which may take place in an elongating or in a nonelongating embryo (short- vs. long-germ insects). Through evolution, the first type of segmentation can easily evolve into the second. In our studies of *Tribolium* segmentation, we elucidated a patterning mechanism based on speed regulation of genetic cascades. The mechanism functions in both elongating and nonelongating tissues, and could potentially have parallels in other tissues and organisms.

Author contributions: E.E.-S. designed research; X.Z., H.R., L.H., and E.E.-S. performed research; S.J.B. and M.K. contributed new reagents/analytic tools; X.Z., H.R., P.F., and E.E.-S. analyzed data; and E.E.-S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. N.B. is a guest editor invited by the Editorial Board.

[1]X.Z. and H.R. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: ezzat.el-sherif@fau.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1702478114/-/DCSupplemental.

In this paper, we propose the "speed regulation" model, in which the concentration of a molecular factor modulates the speed of sequential activation of genes. The model is flexible and can operate in a gradient-based or a wavefront-based fashion. We give theoretical and experimental evidence that the flexibility of the speed regulation model is behind the apparent ease with which many short-germ insects independently evolved into long-germ insects throughout evolution. Specifically, we show that, in the beetle, *Tribolium castaneum*, gap genes are activated sequentially in both the blastoderm and the germband. We also provide evidence that a gradient of the homeodomain transcription factor *caudal* (*cad*) (or a factor whose expression correlates with it) regulates the speed of gap genes sequential activation, patterning the blastoderm by the gradient-based mode and patterning the germband by the wavefront-based mode of the speed regulation model. Manipulating the levels and retraction dynamics of *cad* leads to the shifting of AP fates back and forth between the blastoderm and germband, mimicking short- to long-germ evolution and vice versa.

Based on insight from recent discoveries in *cis*-regulatory analysis, where most genes were found to be regulated by multiple "shadow" enhancers (27), we propose a computational model that realizes, in molecular terms, the concept of speed regulation. In this model, a gradient/wavefront regulates the switch between two enhancers: The first enhancer is responsible for sequential gene activation, and the second is responsible for freezing the temporal rhythms into spatial patterns. This model provides a molecular mechanism for short-germ to long-germ evolution in insects and functions equally well for gradient-based or wavefront-based patterning.

To place our experiments in context, we present the concept of "speed regulation" first. Based on this concept, we suggest a simple mechanism for short-germ to long-germ evolution. To test this idea, we studied gap gene dynamics in the beetle *Tribolium castaneum* in wild type (WT) and several RNAi knockdown backgrounds. We then present a computational model for a molecular realization of wavefront/gradient-mediated speed regulation. We show that a *Tribolium*-specific version of this model recapitulates the spatiotemporal patterns of gap genes in WT, gap gene RNAi knockdowns, and *cad* gradient manipulations. Finally, we present testable predictions for the suggested molecular model.

Results

Speed Regulation Is a Flexible Mechanism That Can Pattern both Elongating and Nonelongating Tissues. Consider a group of cells, each of which has the capacity to transit through successive states. In Fig. 1*A*, each state is shown in a different color and represents the expression of one gene or the coexpression of several genes. The speed of state transitions is regulated by a molecular factor (that we call a "speed regulator," shown in gray in Fig. 1*A*). At low, intermediate, and high values of the speed regulator, cells transit through successive states at low, intermediate, and high speed, respectively (Fig. 1*A*, *Left*, *Middle*, and *Right*, respectively).

Next, consider a group of the aforementioned cells arranged along a spatial axis and subject to a static (i.e., not retracting) gradient of the speed regulator (which we will call a "speed gradient"; gray in Fig. 1*B*, *Left*). All cells transit through successive states, but they do so more slowly at lower values of the speed gradient, giving the appearance of waves of gene expression propagating from high to low levels of the speed gradient. With time, cells along the spatial axis are subdivided into domains of different states (Fig. 1*B*, *Left*, last row). We will call this mode of speed regulation gradient-based. Note that the "kinematic" or "pseudo" waves (described in refs. 1, 5, 7, and 28–33 and Fig. 1*B*, *Left*) do not require diffusion or cell–cell communication. However, the kinematic waves previously described in

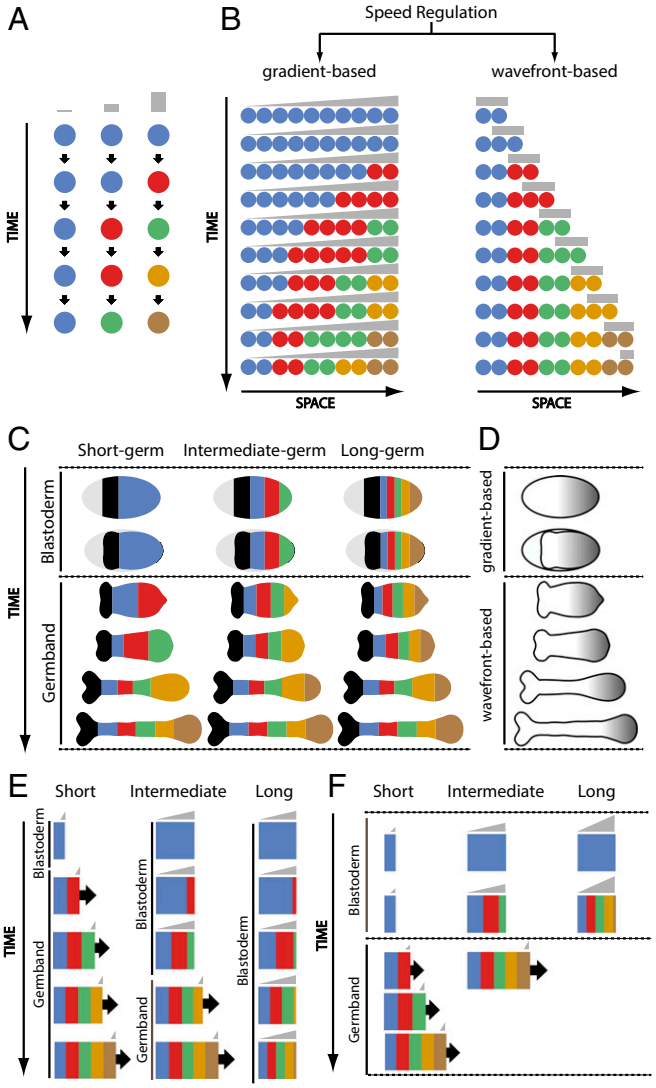

Fig. 1. The core mechanism of speed regulation model is flexible, can pattern elongating and nonelongating tissues, and can explain short-germ to long-germ evolution in insects. (*A*) Core mechanism of speed regulation model: The speed of sequential activation of states (or fates) is regulated by the concentration of a speed regulator. Different states are shown in different colors (in the order of sequential transitioning): blue, red, green, gold, and brown. The speed regulator is shown in gray. (*B*) Speed regulation model can operate in a gradient-based mode to pattern nonelongating tissues (*Left*) and in a wavefront-based mode to pattern elongating tissues (*Right*). (*C*) AP fates (shown in different colors) are specified during two different phases of insect early development: blastoderm and germband. Most AP fates are specified during the germband stage in short-germ insects (*Left*), and during the blastoderm stage in long-germ insects (*Right*). In intermediate-germ insects (*Middle*), anterior fates are specified in the blastoderm, whereas posterior fates are specified in the germband. (*D*) Presumed expression of speed regulator (gray) in insects. Blastoderm can be patterned with the gradient-based mode of speed regulation model, whereas germband can be patterned with the wavefront-based mode. (*E* and *F*) Computer simulation of two strategies for short- to intermediate- to long-germ evolution based on the speed regulation model. (*E*) A short-germ insect can evolve into an intermediate germ by delaying the blastoder-to-germband transition; similarly, an intermediate-germ insect can evolve into a long germ by introducing a further delay to blastoderm-to-germ transition (Movie S1). (*F*) A short-germ insect can evolve into an intermediate germ by boosting the speed regulator; similarly, an intermediate-germ insect can evolve into a long germ by further boosting the speed regulator (Movie S2).

these references are oscillatory waves generated by regulating an oscillator with a frequency gradient (Fig. S1*B*, *Left*), whereas the waves in Fig. 1*B*, *Left* are generated by regulating a sequential process by a speed gradient.

If the gradient is very steep (forming a step function or a boundary) and retracts toward high levels of the gradient (we will call this retracting boundary a wavefront), we end up with a model similar to the clock-and-wavefront model (with the clock replaced by an aperiodic sequential process; Fig. 1*B*, *Right*). In this mode, all cells start in the blue state, covered by the wavefront. As the wavefront retracts, some cells transit from the high to zero value of the wavefront. Cells still covered by the wavefront eventually transit to the next state (red in Fig. 1*B*, *Right*), whereas cells left behind by the wavefront stay in the blue state. This repeats until all cells along the spatial axis are subdivided into domains of different states (Fig. 1*B*, *Right*, last row of cells). We call this mode of speed regulation wavefront-based.

These considerations show that the same core mechanism (a temporal process whose speed of state transitioning is controlled by a speed regulator; Fig. 1*A*) can function in a gradient-based mode to pattern a nonelongating field of cells (Fig. 1*B*, *Left*), or in a wavefront-based mode to pattern an elongating field of cells (Fig. 1*B*, *Right*). Note that the same model can be used to generate a periodic pattern (i.e., segments), if the sequential process is replaced by an oscillator (Fig. S1).

Speed Regulation Model Offers a Mechanism for Short-Germ to Long-Germ Evolution in Insects. The anterior fates of insects arise in a blastoderm (a structure with a fixed AP length), whereas more-posterior fates are specified in a germband (whose AP axis lengthens by convergent extension and/or cell divisions) (Fig. 1*C*). Insects differ in the number of fates specified in blastoderm vs. germband. In short-germ insects, most fates form during the germband stage (Fig. 1*C*, *Left*), while, in long-germ insects, most fates form during the blastoderm stage (Fig. 1*C*, *Right*). Intermediate-germ insects lie somewhere between those two extreme cases (Fig. 1*C*, *Middle*). Short-germ embryogenesis is thought to be the ancestral mode of insect development, but it is not clear how it evolved into intermediate- and long-germ modes.

Since both gradient-based (Fig. 1*B*, *Left*) and wavefront-based (Fig. 1*B*, *Right*) patterning use the same core mechanism of speed regulation (Fig. 1*A*), it is easy to imagine one mode converting to the other, offering a mechanism for short-germ to long-germ evolution in insects. In this mechanism, a posteriorly localized gradient of a speed regulator (gray in Fig. 1*D*) is static in the blastoderm stage, and it retracts with AP axis elongation during the germband stage (Fig. 1*D*). In a short-germ insect, AP fates would be specified during the germband stage in a wavefront-based mode (Fig. 1*E*, *Left* and Movie S1*A*). To evolve into an intermediate-germ insect, the wavefront gets shallower (forming a speed gradient) and the blastoderm-to-germband transition is delayed, allowing the first few fates to propagate into the blastoderm in a gradient-based fashion. After the blastoderm-to-germband transition, fates continue to form in a wavefront-based fashion (Fig. 1*E*, *Middle* and Movie S1*B*). To further evolve into a long-germ insect, the blastoderm-to-germband transition is delayed until all fates are specified in the blastoderm (Fig. 1*E*, *Right* and Movie S1*C*). This strategy assumes that there exists a mechanism to control the timing of blastoderm-to-germband transition. Alternatively, the level of the speed regulator could be boosted such that fate transitions speed up, causing more fates to form in the blastoderm (Fig. 1*F* and Movie S2), or the sequential gene activation process itself could evolve to run faster at the same speed gradient level.

However, another possible mechanism for short- to long-germ evolution would be to start the patterning process at an advanced state (Fig. S2*B*). Such an advanced state (or initial pattern) could be reached using a threshold-based mechanism (Fig. S2*C*). For a more detailed discussion of the different possible mechanisms for short- to long-germ evolution, see *SI Comparison Between the Proposed Short-Germ to Long-Germ Evolutionary Mechanisms*.

Patterning the AP axis of insects has been previously proposed to depend on activation thresholds of a continuously increasing morphogen gradient (termed a "timer") (34). The speed regulation model stands in contrast to threshold-based models in that it does not require a morphogen of large dynamic range to pattern the whole AP axis or a tight control of the morphogen concentration over time.

Gap Genes Are Expressed in Sequential Waves in *Tribolium*. AP patterning in insects is carried out by two groups of genes: gap genes and pair-rule genes (35). Gap genes are primarily responsible for specifying AP fates by regulating Hox genes [in addition to a possible role in segment counting (36)], while pair-rule genes divide the AP axis into segments. Here we describe AP fate specification by gap genes in the beetle *Tribolium castaneum* and discuss them in the context of our patterning and evolutionary models.

First, we examined the expression patterns of four essential gap genes in *Tribolium*: *hunchback* (*hb*) (37, 38), *Krüppel* (*Kr*) (39), *milles-pattes* (*mlpt*) (40), and *giant* (*gt*) (41) in consecutive 3-h egg lays at 24 °C, starting at the onset of AP patterning (Fig. 2*A* and Fig. S3*A*; see *SI Detailed Description of Gap Gene Expression in WT and* axn *RNAi Embryos* for detailed description of WT expression patterns; see Fig. S4 for *gt* expression with higher temporal resolution). As shown in Fig. 2*A* and Fig. S3*A*, gap genes are expressed in sequential waves that emanate from the posterior, propagate toward the anterior, then stabilize for a while, before they slowly decay.

A temporal profile of gap gene expression patterns at the posterior end of the blastoderm was constructed based on analysis of gene activities at different time points (*Materials and Methods* and Fig. 2*C* and Fig. S5). As shown in Fig. 2*C*, gap genes are activated in sequential, yet overlapping, temporal order.

To determine whether the domains of gap gene expression propagate in waves, we analyzed the spatiotemporal dynamics of the anterior expression borders of *hb*, *Kr*, and *mlpt*, and the posterior border of *hb* during the blastoderm stage in timed egg collections (*Materials and Methods* and Fig. 2*D*). From Fig. 2*D*, it is clear that gap gene expression domains indeed propagate in waves from posterior to anterior in the *Tribolium* blastoderm. To examine if the shifting of *hb* and *Kr* expression domain borders is smooth, we analyzed their dynamics with higher temporal resolution between 17 h and 20 h after egg lay (AEL) (Fig. S6), and found them indeed to shift smoothly.

Gap Gene Waves Are Arrested into Stable Domains upon the Retraction of *cad*. The homeodomain transcription factor *caudal* (*cad*) is involved in posterior specification and patterning in many bilateria (42–44). In *Tribolium*, *cad* is expressed in a posterior-to-anterior gradient during the blastoderm stage (from 0 h to 23 h AEL; shown 14 h to 23 h AEL in Fig. 2*A*), then retracts to the posterior end during the germband stage (7, 42) (from 23 h to 38 h AEL, Fig. S3*A*) and remains restricted to the posterior end (usually called the "growth zone"), retracting posteriorly as the germband elongates (Fig. S3*A*).

We note that gap gene expression domains are only activated sequentially at the posterior end of the embryo, more specifically within the *cad* expression domain (Fig. 2*A* and Fig. S3*A*). Outside this region, gap gene expression domains seem to be stable for a while before eventually fading. To determine whether retraction of the *cad* gradient correlates with the arrest of gap gene expression waves, we compared gap gene expression within and outside of the *cad*-expressing domain in doubly stained embryos (Fig. S7). We found that, indeed, gap gene waves are dynamic in

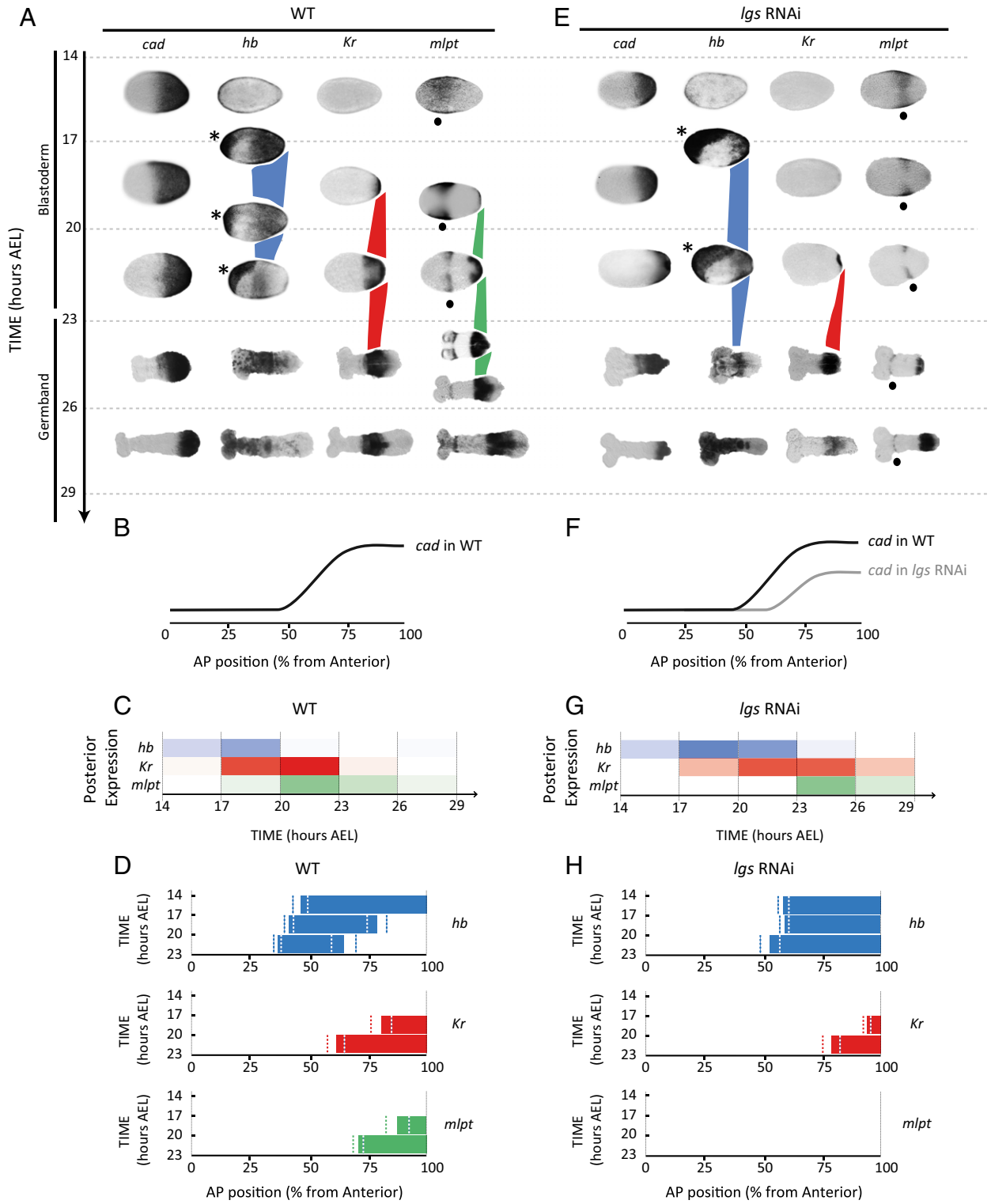

Fig. 2. Dynamics and regulation of gap genes in *Tribolium* blastoderm. (*A–D*) Gap genes are expressed as sequential waves in WT *Tribolium* embryos within *cad* expression domain; *cad* is expressed as a posterior-to-anterior gradient in the blastoderm (*cad* in *A*; quantification of the gradient is shown in *B*) and retracts to the posterior end (growth zone) of the embryo in the germband stage (23 h AEL onward). The *hb*, *Kr*, and *mlpt* waves are traced in blue, red, and green, respectively, in *A*. Extraembryonic expression of *hb* is marked with an asterisk. Head expression of *mlpt* (not considered in our analysis) is marked with a black dot. (*C*) Temporal profile of gap genes expression at the posterior end of WT embryo demonstrates their sequential (yet overlapping) expression. Color intensity of a bar within a time window reflects the percentage of embryos having a high level of gene expression of the corresponding gene in that time window (*Materials and Methods*). (*D*) Spatial distribution of gap genes along the AP axis of WT *Tribolium* blastoderm over time (*Materials and Methods*) demonstrates their posterior-to-anterior shifts over time. Dashed lines show expression domain borders ± SE. For detailed description of gap gene expression in WT, see *SI Detailed Description of Gap Gene Expression in WT and axn RNAi Embryos*. (*E–H*) The *cad* gradient is reduced and shifted toward posterior in *lgs* RNAi embryos. Correspondingly, gap gene waves are slower and shifted toward posterior. *B* and *F* are reproduced with permission from ref. 7. In all embryos shown, anterior is to the left.

the presence of *cad* and are stabilized upon retraction of the *cad* gradient.

Speed Regulation Model for Gap Genes in *Tribolium*. In the speed regulation model for AP patterning in insects (Fig. 1), a speed regulator can function in the form of a speed gradient to pattern the blastoderm and in the form of a wavefront to pattern the germband. In this model, the speed gradient induces waves of AP fate-determining genes to propagate from posterior to anterior. Upon speed gradient retraction (and transition into a wavefront), these gene expression patterns stabilize into specific expression domains. This describes the situation in *Tribolium*, where *cad* fills the role of a speed regulator, a hypothesis that will be examined in the subsequent sections.

In the basic speed regulation models shown in Fig. 1, we assumed that AP fate-determining genes are expressed in a nonoverlapping fashion. However, gap genes in *Tribolium* are expressed in overlapping and nested domains. Modifying the temporal process in our model to reflect that of gap genes in *Tribolium* embryos, and assuming *cad* (or a factor whose expression correlates with that of *cad*) to act as a speed regulator, simulates the experimentally observed results (Fig. 3, WT; Movie S3*A*; compare with gap genes expression in Fig. 2*A* and Fig. S3*A*).

The Intermediate-Germ Insect *Tribolium* Is Induced to Develop More as a Short Germ upon Reducing *cad*. If *cad* acts as the speed regulator of gap genes, then, according to the speed regulation model, changes affecting the *cad* gradient should also affect the dynamics of gap gene expression, including their sequential activation at the posterior end of the *Tribolium* embryo. To test this assumption, we analyzed the timing of gap gene regulation after knocking down *legless* (*lgs*, a positive regulator of *cad* through Wnt) (7, 45–47) by maternal RNAi. We have previously shown that, in *lgs* RNAi embryos, the *cad* gradient shifts posteriorly and its maximum value at the posterior end of the embryo is reduced (7) (compare *cad* in Fig. 2 *E* and *F* to Fig. 2 *A* and *B*; see ref. 7 for quantification of the *cad* gradient in WT and after knockdown of several Wnt regulators, some of which are summarized in Fig. 2 *B* and *F* and Fig. S8 *B*, *F*, and *J*). In *lgs* RNAi knockdown embryos, gap genes are still expressed in sequential waves emanating from the posterior end of the embryo (Fig. 2 *E*, *G*, and *H*). However, the timing of gap gene sequential activation in *lgs* RNAi embryos is slower than in WT (whereas the timing of morphological events is not affected, including the blastoderm-to-germband transition, which takes place around 20 h to 23 h AEL in both WT and *lgs* RNAi embryos). In addition, gap gene domains in the blastoderm are shifted posteriorly, in accordance with the posterior shift of the *cad* gradient (compare Fig. 2 *E*–*H* to Fig. 2 *A*–*D*). The temporal slowdown and the spatial shift of gap gene expression upon the reduction and posterior shift of *cad* are consistent with our speed regulation model for gap genes in *Tribolium* (compare *lgs* RNAi with WT in Fig. 3, and compare Movie S3 *B* and *A*).

In applying the speed regulation model to the evolution of insect patterning modes, one strategy for the evolution of long-germ to a more short-germ-like mode of embryogenesis (where more fates are specified in the germband) would be to reduce the level of the speed regulator gradient, while maintaining the timing of the blastoderm-to-germband transition (Fig. 1*F* and Movie S2). This was observed in the *lgs* RNAi knockdown embryos, where gap gene sequential activation was slower compared with WT, while the timing of the blastoderm-to-germband transition was not affected (compare Fig. 2 *E* and *G* to Fig. 2 *A* and *C*). We noticed that, while most of *hb*, *Kr*, and *mlpt* expression took place during the blastoderm stage in WT, most of *Kr* and all of the *mlpt* expression occurred during the germband

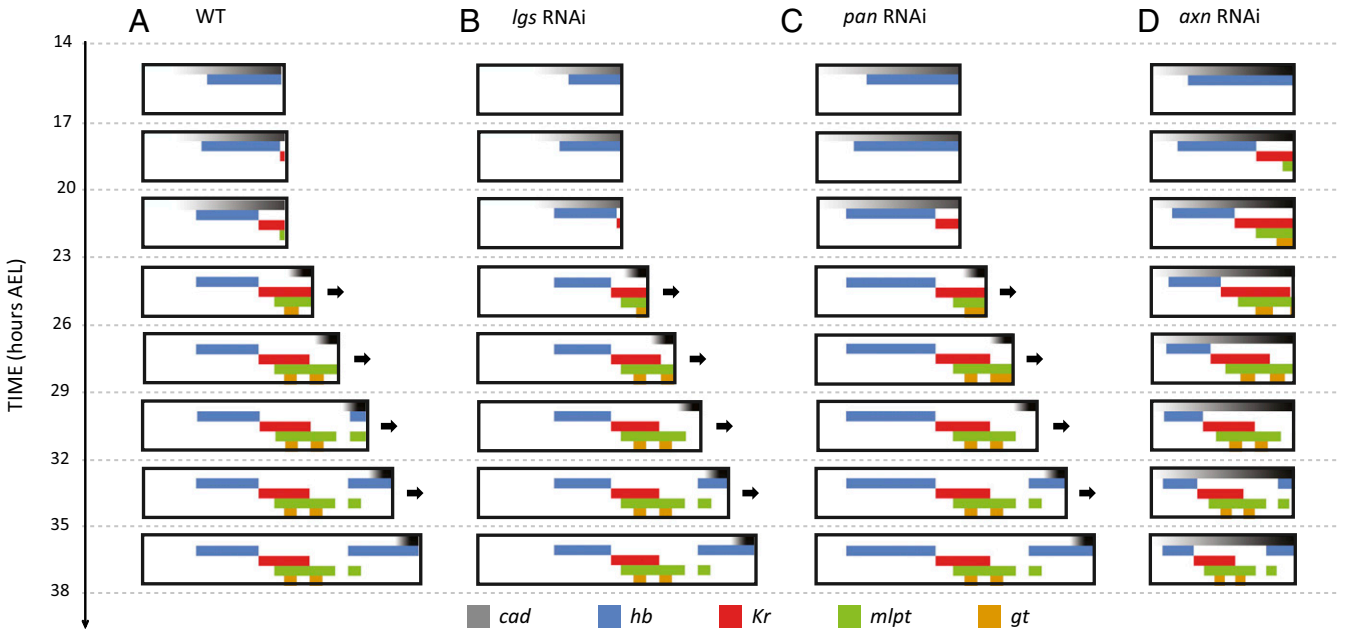

Fig. 3. Speed regulation model recapitulates gap gene expression in *Tribolium* WT, *lgs* RNAi, *pan* RNAi, and *axn* RNAi embryos. (*A*) In a computer simulation, where the speed of *Tribolium* gap gene sequence is regulated by *cad* gradient (black/gray; darker corresponds to higher concentration), gap gene (*hb*, blue; *Kr*, red; *mlpt*, green; *gt*, gold) spatiotemporal dynamics were recapitulated during blastoderm and germband stages of WT *Tribolium* embryos (compare with Fig. 2*A* and Fig. S3*A*). (*B*) To simulate *lgs* RNAi background, *cad* gradient was reduced and shifted toward posterior. Accordingly, gap gene waves were slower and shifted toward posterior (compare with Fig. 2*E*). (*C*) To simulate *pan* RNAi background, *cad* gradient was reduced, stretched, and shifted toward anterior. Accordingly, gap gene waves were slower, stretched, and shifted toward anterior (compare with Fig. S8*I*). (*D*) To simulate *axn* RNAi background, germband elongation and *cad* gradient retraction were halted. Accordingly, gap gene waves continued to propagate and shrink in the germband and never stabilized (compare with Fig. S3*B*). See Movie S3.

stage in *lgs* RNAi embryos (compare Fig. 2 *E* and *A*). Thus, in the *lgs* RNAi background, *Tribolium* embryos appear to develop in a more short-germ mode.

In our evolutionary model, reduction of the speed regulator concentration is essential for transitioning toward short-germ embryogenesis, but posterior shifting of the speed regulator gradient is not. We have previously shown that, in *pangolin* (*pan*, a Wnt effector) RNAi knockdown embryos, the *cad* gradient is reduced, stretched, and shifted anteriorly (in the opposite direction from the shift observed in *lgs* RNAi embryos; compare Fig. S8 *I* and *J* to Fig. S8 *A* and *B* and Fig. S8 *E* and *F*). Indeed, sequential gap gene activation is slower in *pan* RNAi embryos, in accord with reduced *cad*. The spatial extent of the leading gap gene (*hb*) is stretched and shifted anteriorly, in accord with the stretch and shift of the *cad* gradient (compare Fig. S8 *I–L* with Fig. S8 *A–D*). The anterior shift and reduced speed of gap gene expression upon the anterior shift and reduction of *cad* is consistent with our speed regulation model of gap genes in *Tribolium* (compare *pan* RNAi with WT in Fig. 3; compare Movie S3 *C* and *A*). Thus, *pan* RNAi embryos also developed more like a short-germ insect, similarly to the *lgs* RNAi embryos. In both *lgs* and *pan* RNAi embryos, most of *Kr* and *mlpt* expression occurred in the germband (compare Fig. S8 *A*, *E*, and *I*).

The Intermediate-Germ Insect *Tribolium* Is Induced to Develop More Like a Long Germ upon Halting *cad* Retraction. According to our speed regulation model of insect evolution, one strategy by which a short-germ mode of insect development might evolve into a long-germ mode is to arrest the blastoderm-to-germband transition (Fig. 1*E* and Movie S1). In this strategy, the speed gradient would not retract, and new fates would continue to propagate into the blastoderm. *Axin* (*axn*) is a negative regulator of Wnt signaling. In *axn* RNAi *Tribolium* embryos, the blastoderm develops into a germband that does not undergo appreciable axis elongation (compare Fig. 4*B* and *A*). Moreover, the *cad* gradient does not undergo any anterior-to-posterior retraction in these germbands (Fig. 4*B*, *cad*). Hence, in many aspects, *axn* RNAi germbands physically and molecularly resemble a blastoderm.

In a time series of *axn* RNAi embryos, the germband seemed to experience very limited axis elongation (Fig. 4*B*). However, since it is difficult to compare the sizes of different embryos due to embryo-to-embryo variation, we analyzed axis elongation in individual embryos using live imaging of a nuclear GFP line in *Tribolium* (4) for both WT and *axn* RNAi embryos (Movie S4). In Fig. S9, live imaging snapshots of single WT and *axn* RNAi embryos are shown. In the WT embryo, progressive elongation of the germband along the AP axis was evident. In comparison, *axn* RNAi embryos extended dorsoventrally but no appreciable AP axis elongation was observed. Although late *axn* RNAi germbands undergo some elongation in the AP direction, this was not accompanied by retraction of the *cad* gradient (Fig. 4*B*, *cad*; however, overall decay of *cad* expression in *axn* RNAi embryo was observed at 32 h to 38 h AEL).

Halting axis elongation and *cad* gradient retraction in *axn* RNAi embryos has dramatic consequences on gap gene expression dynamics. The gap gene expression domains never stabilize and continue to propagate anteriorly until they reach the edge of the *cad* gradient, abutting the tiny residual head lobes (see Fig. 4*B* for *mlpt* and *gt* expression, and see Fig. S3*B* for the full dataset; see *SI Detailed Description of Gap Gene Expression in WT and* axn *RNAi Embryos* for detailed description of the *axn* RNAi phenotype). This is consistent with our speed regulation model for gap genes in *Tribolium*, in which gap gene expression domains continue to shrink and propagate from posterior to anterior within the stabilized *cad* gradient (compare *axn* RNAi with WT in Fig. 3, Fig. S3, and Movie S3). In addition, these results suggest that germband elongation and *cad* retraction is not necessary for gap gene domain formation (however, they might be necessary for domain stabilization and for producing the required expression domain widths). This suggests a simple mechanism for short- to long-germ evolution: Halt axis elongation and *cad* retraction, such that more gap genes can be accommodated in the blastoderm. Retracting or reducing the *cad* gradient should occur at some point to stabilize the final pattern, however. This is consistent with the evolutionary model presented in Fig. 1*E*.

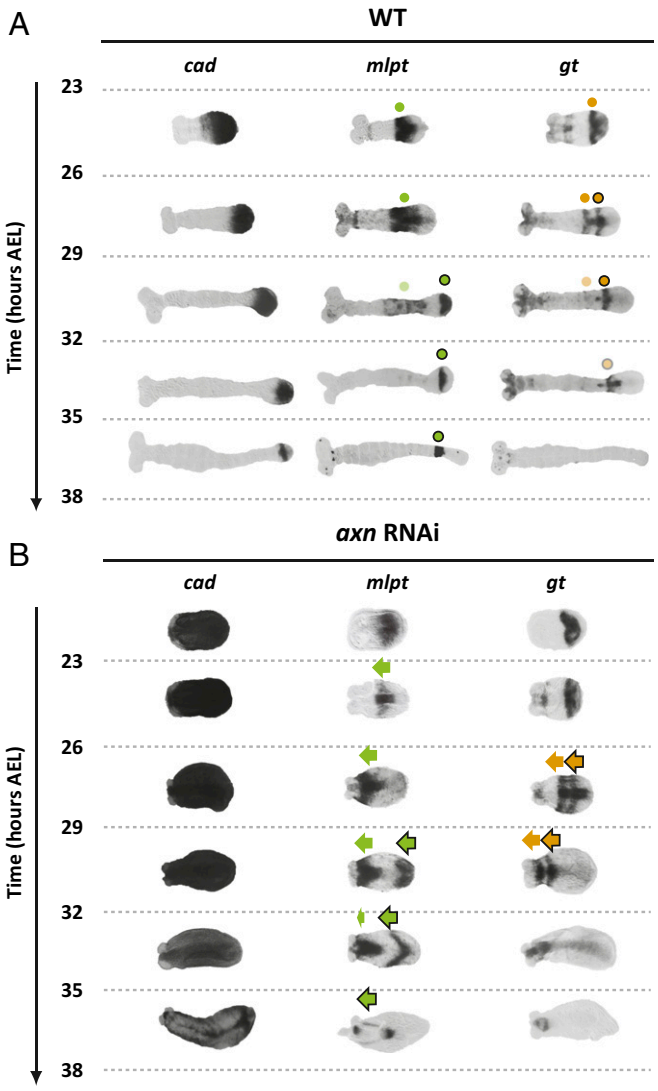

Fig. 4. Dynamics and regulation of gap genes in *Tribolium* germband. (*A*) Gap genes continue to be expressed sequentially in the posterior end of WT *Tribolium* germband (the so-called growth zone); *cad* is expressed in the growth zone and retracts as the germband elongates. More to the anterior, early expressed gap gene domains stabilize and eventually fade. Expression patterns are tracked by dots. Faint dots represent decaying expression. Dots outlined in black signify the second expression domains of *mlpt* and *gt*. (*B*) In *axn* RNAi embryos, germband experiences very limited axial elongation, and *cad* expression does not retract. Nevertheless, gap genes continue to emanate from the posterior and propagate toward anterior until they reach the (much reduced in size) head lobes. Expression patterns are tracked by arrows. Arrows outlined in black signify the second domain of *mlpt* and *gt*. For detailed description of *axn* RNAi phenotype, see *SI Detailed Description of Gap Gene Expression in WT and* axn *RNAi Embryos*. For the full dataset of all gap gene expression dynamics during germband stage, see Fig. S3. In all embryos shown, anterior is to the left.

A General Molecular Mechanism for the Speed Regulation Model: Gradual Enhancer Switching. We have described a phenomenological model (i.e., without specifying any molecular realization)

for speed regulation (Fig. 1). Now we attempt to find a general underlying molecular model (i.e., applicable but not specific to *Tribolium*). The phenomenological model of speed regulation has two components (Fig. 1*A*): (*i*) a mechanism for sequential gene activation and (*ii*) a mechanism for tuning the speed of sequential activation by the concentration of a molecular factor. One type of gene regulatory network (GRN) that generates sequential gene expression is the genetic cascade shown in ref. 48 and Fig. 5*A*, in which all of the genes mutually repress one another. However, the repression exerted by each gene on the gene immediately following it in the cascade is weak. For one cell, this genetic module works as follows. If the cell is initially expressing the first gene in the cascade (the blue gene in Fig. 5*A*), then all other genes are strongly repressed, except for the gene immediately following it (red). Hence, the red gene turns on. Since the red gene strongly represses the blue gene, the blue gene turns off. Now the blue gene is off, and since the red gene is only weakly repressing the green gene, the green gene turns on. Since the green gene strongly represses both the red and blue genes, the red gene turns off and the blue gene is kept off. In this way, we end up with a sequential activation of genes in time in the order: blue, red, and then green (Fig. 5*B*; Note that the simulation in this figure is done using a five-gene version of the cascade shown in Fig. 5*A*, with gene order blue, red, green, gold, then brown). We will call this network (and any network that induces sequential gene activation) a "dynamic module."

Next, we turn to a mechanism to regulate the speed of this dynamic module. To do so, we introduce a (tunable) brake or

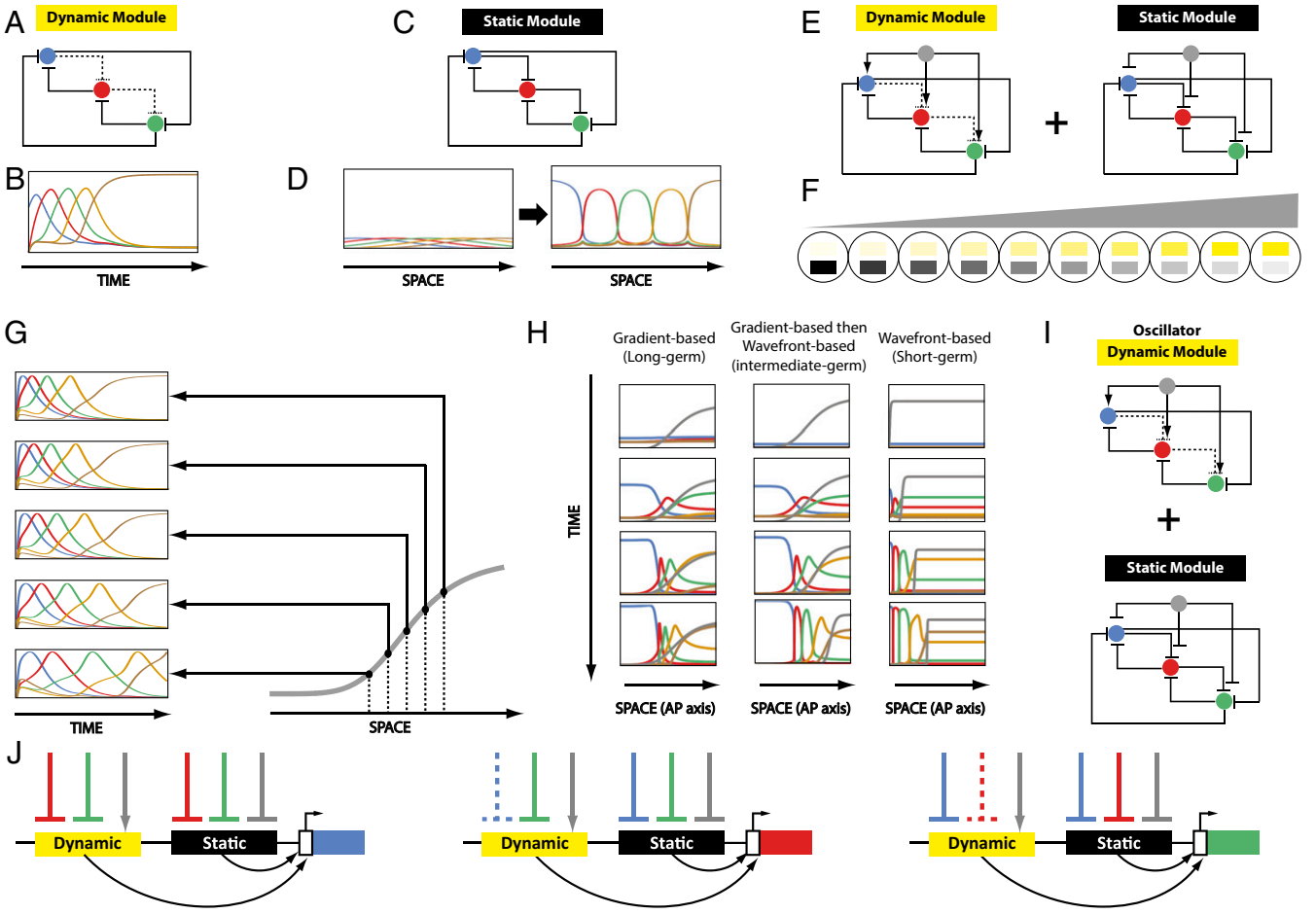

Fig. 5. Gradual enhancer switching model. (*A*) A three-gene cascade (dynamic module). Solid line, strong repression; dashed line, weak repression. (*B*) Computer simulation of a five-gene cascade (same structure as the three-gene cascade: Every gene is strongly repressing all other genes, except only weakly repressing its immediate successor in the cascade). Shown are expression profiles of the cascade constituent genes over time. (*C*) A three-gene mutual exclusion gene network (static module). (*D*) Shown is an array of cells along a spatial axis. Active in each cell is a five-gene mutual exclusion gene network (same structure as the three-gene mutual exclusion network: Every gene is strongly repressing all others). Cells along the spatial axis are initialized with a broad and diffuse domains of the expression of five genes (*Left*). Over time, the diffuse domains get stronger and sharper (*Right*). (*E*) The gradual enhancer switching gene network: A speed regulator (gray) is activating all of the genes in the dynamic module, while repressing all of the genes in the static module. (*F*) Shown is an array of cells in which the gradual enhancer switching gene network is active. Applied to the array is a gradient of the speed regulator (gray). This results in the gradual switching from the dynamic module (yellow) to the static module (black) as we go from high to low values of the gradient. (*G*) Computer simulations of the gradual enhancer switching model at different positions along the spatial axis (i.e., different values of the gradient, shown in gray): The higher the concentration of the gradient, the higher the speed of sequential activation of genes. (*H*) Computer simulations of the gradual enhancer switching model under different conditions: nonretracting speed regulator (gradient-based, long-germ insects; *Left*), retracting speed regulator (wavefront-based, short-germ insects; *Right*), and nonretracting then retracting speed regulator (gradient-based then wavefront-based, intermediate-germ insects; *Middle*). See Movie S5. (*I*) The gradual enhancer switching model with an oscillator as a dynamic module. The model induces oscillatory kinematic waves of gene expression, observed in vertebrates and insects (Movie S7). (*J*) Encoding dynamic and static modules by separate enhancers ensures modularity, since each module requires different regulatory logic. Shown is the regulatory wiring required for each gene in the combined dynamic+static gene network in *E*. Solid lines, strong interactions; dashed lines, weak interactions.

stabilizer to the sequential process, such as the mutually exclusive gene circuit shown in Fig. 5*C*. Here all genes are repressing each other equally strongly. In this circuit, an initial bias in the expression of one of the genes in one cell gets amplified and stabilized, while the expression of the other genes is attenuated. This circuit eventually sharpens initially overlapping and diffuse spatial patterns (Fig. 5*D*). This strategy is thought to be used in gap gene regulation in *Drosophila* (49). We call such a network (and any network that helps stabilize a pattern) a "static module."

Finally, we combine the static module with a gradient to modulate the speed of the dynamic module. We do so by regulating each gene by the additive activity of the two modules. If the dynamic module is positively regulated by the gradient, and the static module is negatively regulated by the same gradient (Fig. 5*E*, gradient is in gray), we see a gradual switching between the two modules as we go from high to low values of the gradient (Fig. 5*F*). In cells exposed to high doses of the gradient, the gene cascade will run full speed, whereas, in cells exposed to progressively lower doses of the gradient, the gene cascade will experience progressively higher resistance from the static module, and run progressively slower.

Indeed, simulations show that this scheme is able to modulate the speed of sequential gene activation: Progressively lower values of the gradient generate progressively more dilated (i.e., slower) temporal profiles of the gene cascade output (Fig. 5*G*). This is manifested in the induction of sequential waves of gene expression that propagate from high to low values of the gradient (Fig. 5*H*, *Left* and Movie S5*C*).

However, we noted that the generated gene expression domains in Movie S5*C* continue to propagate and shrink toward the lower end of the gradient to the limit of being diminishingly small. This is a characteristic of patterning by a nonretracing speed gradient (compare Movie S3 *D* and *A*), and can be remedied by retracting or decaying the gradient once a satisfactory expression pattern emerges. However, we also noted that the generated gene expression domains are compressed toward the lower end of the gradient compared with the higher end (Movie S5*C*). This is due to the fact that the effective speed gradient generated by our molecular model is more downwardly concave than the applied gradient (Fig. S10). This could be corrected by applying a more upwardly concave gradient, so that the effective speed gradient is linear and gene expression domains with equal widths are generated (Movie S6). For further discussion, see *SI Possible Limitations to the Proposed Patterning and Evolutionary Models and Their Applicability to Different Insects*.

All in all, our model was successful in molecularly implementing the gradient-based model of speed regulation (Fig. 1*B*, *Left*), suitable for patterning the AP axis of long-germ insects (Fig. 5*H*, *Left* and Movie S5*C*). The model also works if the gradient is continuously retracting (Fig. 5*H*, *Right* and Movie S5*A*), i.e., it is successful in implementing the wavefront-based mode of speed regulation (Fig. 1*B*, *Right*), which is suitable for patterning the AP axis of short-germ insects. Finally, the model tolerates the switching from gradient-based to wavefront-based patterning (Fig. 5*H*, *Middle* and Movie S5*B*); hence, it is suitable for patterning the AP axis of intermediate-germ insects as well.

Our model is also able to molecularly realize a "frequency gradient" by simply having an oscillator as a dynamic module instead of a genetic cascade (Fig. 5*I*). This scheme is able to generate oscillatory waves that can be stabilized into periodic stripes, suitable for segmenting the AP axis of vertebrates as well as short-, intermediate-, and long-germ insects (Movie S7).

The central idea behind the proposed molecular model is the gradual switching between two modules or GRNs. This scheme could possibly be realized by just one enhancer per gene, if such an enhancer would be able to switch its wiring scheme depending on the concentration of the graded molecular factor (gray in Fig. 5). However, we suggest a molecular strategy that uses a separate enhancer for each wiring scheme (Fig. 5*J*). We think that this strategy, while not the only conceivable, is a biologically plausible one, for two reasons. First, while it is possible to change the logic of an enhancer upon the binding of a certain factor, this might be too complex to implement, especially if the wiring of the two networks is very different. A modular (and possibly more feasible) approach is to use different enhancers for different wiring schemes. Second, if the wiring of one network needs to change with evolution, it is more feasible to evolve one enhancer independently from the other (although the feasibility of a particular molecular scheme is, in the end, highly dependent on specific evolutionary history). This is again a consequence of the better modularity of the two-enhancer solution, which is the main advantage of regulating genes with different independent enhancers. For these reasons, and mainly because it is a direct realization of our computational modeling (where we simply added the activities of two separate gene activation functions; see *SI Computational Modeling*), we call this model "gradual enhancer switching."

In summary, our gradual enhancer switching model (Fig. 5) is a molecular realization of the speed regulation principle (Fig. 1). It works under both gradient-based and wavefront-based conditions, and ensures convertibility between them.

A Molecular Realization for *Tribolium* Gap Gene Regulation. A gene network for *Tribolium* gap gene regulation has been previously constructed (35, 50). A summary of the genetic interactions in addition to a simplified schematic diagram for the relative positions of the final stable gap gene expression domains are shown in Fig. S11*A*. Although the gene network in Fig. S11*A* is indeed a genetic cascade and explains the sequential activation of gap genes at the very posterior end of *Tribolium* embryos, it is not obvious how the temporal output of the cascade is translated into a spatial pattern. However, a computational model in which *cad* mediates gradual switching between dynamic and static enhancers (Fig. S11 *B* and *C*; see *SI* Tribolium-*Specific Enhancer Switching Model* for detailed description of the model) successfully reproduced the spatiotemporal patterns of gap genes in WT (Fig. S11*D*, WT and Movie S8*A*). The model also recapitulates the spatiotemporal patterns of gap genes in *lgs* and *pan* RNAi embryos (Movie S8 *B* and *C*), which invoke evolution from a long- to a short-germ mode of development. The model recapitulates the spatiotemporal patterns of gap genes in *axn* RNAi embryos (Movie S8*D*), which appears equivalent to the evolution to a long-germ mode of development. The model also reproduces documented gap gene RNAi phenotypes in *Tribolium* (50) (Fig. S11*D* and Movie S9). We would like to note here that, while the dynamic module of our *Tribolium* model is data-driven, the wiring of the static module is mostly speculative (*SI* Tribolium-*Specific Enhancer Switching Model*), and, hence, we present the *Tribolium* enhancer switching model as a proof of principle and a working hypothesis.

Predictions. Our enhancer switching model (Fig. 5 and Fig. S11) predicts that each gap gene in *Tribolium* is regulated by two enhancers: a dynamic enhancer responsible for initialization and a static enhancer for stabilization. Movie S10 shows the predicted spatiotemporal expression of the dynamic and static enhancers for the gap gene *Kr* in our *Tribolium* enhancer switching model (Fig. S11 *B* and *C*). The model predicts both temporal and spatial differences in the expression driven by each of the two enhancers. The dynamic enhancer is activated early and posteriorly, and it drives progressively weaker expression in more-anterior cells. Meanwhile, the static enhancer turns on, and drives expression that progressively builds up and eventually stabilizes anteriorly (Fig. 6, *Left*).

Interestingly, the gap gene *Kr* in *Drosophila* is regulated by two enhancers: KrCD1 and KrCD2, whose expression dynamics were recently described in detail using live imaging techniques (51). KrCD1 is activated first. With time, KrCD1 loses expression, while the expression of KrCD2 builds up. The expression of

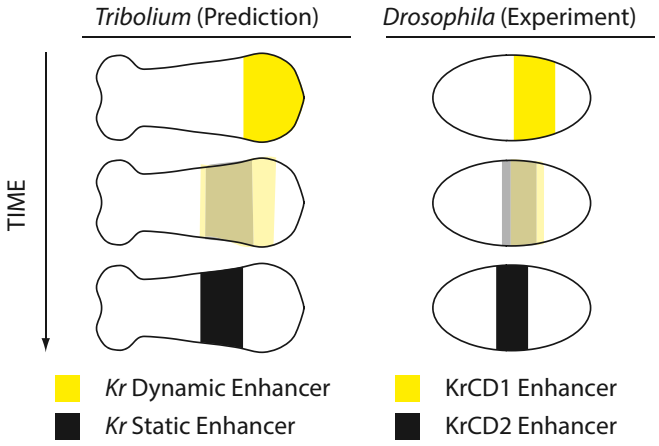

Fig. 6. Predictions of the enhancer switching model in *Tribolium* and recapitulation in *Drosophila*. (*Left*) The predicted expression of the dynamic (yellow) and static (black) enhancers of *Kr* in *Tribolium* according to the enhancer switching model (see also Movie S10). The dynamic enhancer turns on early and progressively decays with time while shifting toward anterior; meanwhile, the static enhancer is building up and forms a stable expression at a more anterior location. (*Right*) The expression dynamics of the two enhancers driving *Kr* expression in *Drosophila*: KrCD1 (yellow) and KrCD2 (black). KrCD1 is active first and progressively decays; meanwhile, KrCD2 turns on and stays active slightly more to the anterior. Faint colors signify weaker expression.

KrCD2 eventually stabilizes slightly more to the anterior (Fig. 6, *Right*). The gap gene *gt* is also regulated by two enhancers in *Drosophila*, one enhancer for initialization and the other for stabilization (52). Furthermore, two genetic programs have recently been implicated to be involved in regulating pair-rule genes in *Drosophila* (53, 54).

In summary, our gradual enhancer switching model predicts that each gap gene in *Tribolium* is regulated by two enhancers: one early and active posteriorly and the other late and active anteriorly (Fig. 6, *Left*).

Discussion

In this paper, we presented the speed regulation model, a patterning mechanism in which the speed of sequential activation of genes is regulated by a gradient. The model is capable of operating in two different modes, depending on the steepness of the gradient and its retraction dynamics. In the gradient-based mode (Fig. 1*B*, *Left*), the gradient is smooth and nonretracting. In the wavefront-based mode (Fig. 1*B*, *Right*), the gradient is steep (forming a boundary) and retracting. We used this observation to present a simple model for short-germ to long-germ evolution (Fig. 1 *E* and *F*).

The capacity of a nonretracting and smooth speed/frequency gradient (i.e., the gradient-based mode of speed regulation, Fig. 1*B*, *Left*) to generate spatial patterns by means of kinematic waves was first described in the context of oscillations in chemical reactions (30) and in the slime mold *Physarum* (29, 55), and later was shown to be involved in the patterning of *Tribolium* blastoderm (5). The gradient-based mode of the speed regulation model has also many similarities to the "temporal and spatial gradient" model proposed for neural tube patterning in vertebrates (15, 17, 19) and other models suggested for various developmental systems (56, 57). During neural tube patterning, for example, Shh forms a ventral to dorsal gradient. Neural fates emanate sequentially from the ventral end of the neural tube and expand toward more dorsal regions in the high-to-low directions of the Shh gradient. Moreover, both the concentration and duration of exposure of Shh regulate which fate a neural tube explant would reach in a given time window. This model is very similar to the gradient-based mode of speed regulation (Fig. 1*B*, *Left*), although usually described using different terminology. Curious similarities, indeed, between AP patterning in *Drosophila* and vertebrate neural tube patterning have been previously discussed (58).

The patterning capacity of the wavefront-based mode of speed regulation (Fig. 1*B*, *Right*) was first devised by Cooke and Zeeman (16) as a theoretical model for vertebrate somitogenesis and was termed the "clock-and-wavefront." In 1997, the clock-and-wavefront model received experimental support when oscillations in the expression of the gene *hairy* were detected in the presomitic mesoderm of chicken embryos during somitogenesis (1). However, it was also observed that *hairy* oscillations are expressed in kinematic waves that propagate from posterior to anterior. Julian Lewis proposed that these wave dynamics are due to the gradual arrest of *hairy* oscillations (appendix of ref. 1). In other words, the somitogenesis clock is regulated by a retracting smooth "frequency gradient" as opposed to the steep frequency gradient in the original formulation of the clock-and-wavefront. However, the frequency gradient in these models is used merely as a cosmetic means to reproduce the observed kinematic waves, with no significant effect on the pattern generated by the original clock-and-wavefront model. Indeed, the retraction of the wavefront is the cause of the resulting spatial pattern, whether the wavefront is realized by a steep or smooth frequency gradient (7, 32). However, it was argued that a smooth frequency (or speed) gradient might ensure scaling (59) and robustness of the clock-and-wavefront model against noise in the expression of the wavefront (7).

Two classes of genes are involved in the earliest stages of AP patterning of insects: gap genes and pair-rule genes. Gap genes have aperiodic expression and are responsible for dividing the AP axis into different fates, whereas pair-rule genes have periodic expression and are responsible for dividing the AP axis into segments. In this paper, we described a speed gradient model for gap gene regulation in insects, where *cad* (or a factor whose expression correlates with *cad*) acts as the speed regulator. In an earlier paper, we described a frequency gradient model for pair-rule regulation in insects, where *cad* acts as the frequency regulator (7). The two models are essentially similar, and could run independently and in parallel, with *cad* acting as a common regulator.

In Fig. 5, we suggested a molecular realization for our phenomenological model of speed regulation. Most gene network modeling studies in development assume a simple regulatory function (enhancer) to regulate the transcription of each gene in the network. However, complex spatial patterns (e.g., multiple *eve* stripes in *Drosophila*) were found to be regulated by multiple enhancers, each driving a subset of the complete pattern (60). Even some single bands of gene expression were found to be regulated by multiple enhancers; in some cases, these enhancers are redundant (61), but, in others, they drive different expression dynamics (51) and encode different regulatory logics (62). This inspired us to use a different formalism for modeling gene networks. In our molecular realization of speed regulation, a gradient regulates the gradual transition between two enhancers encoding two different regulatory logics. This model recapitulated the spatiotemporal dynamics involved in AP fate specification in insects and offered a molecular mechanism for their evolution. However, the main prediction of our molecular model, namely the regulation of *Tribolium* gap genes by sets of two enhancers and the gradient-mediated switching between them, awaits experimental verification.

Materials and Methods

Immunocytochemistry, in situ hybridization, RNAi, egg collections for developmental time windows, calculating class distribution graph, and performing spatial measurements are done using procedures similar to those described in ref. 7. For more details, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Michael Akam and James Briscoe for their comments on an early version of the manuscript.

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