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# Global repression by tailless during segmentation

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## Abstract

The orphan nuclear receptor Tailless (Tll) exhibits conserved roles in brain formation and maintenance that are shared, for example, with vertebrate orthologous forms (Tlx). However, the early expression of *tll* in two gap domains in the segmentation cascade of *Drosophila* is unusual even for most other insects. Here we investigate *tll* regulation on pair-rule stripes. With ectopic misexpression of *tll* we detected unexpected repression of almost all pair-rule stripes of *hairy (h), even-skipped (eve), runt (run), and fushi-tarazu (ftz)*. Examining Tll embryonic ChIP-chip data with regions mapped as Cis-Regulatory Modules (CRMs) of pair-rule stripes we verified Tll interactions to these regions. With the ChIP-chip data we also verified Tll interactions to the CRMs of gap domains and in the misexpression assay, Tll-mediated repression on *Kruppel* (*Kr*), *kni* (*kni*) and *giant* (*gt*) according to their differential sensitivity to Tll. These results with gap genes confirmed previous data from the literature and argue against indirect repression roles of Tll in the striped pattern. Moreover, the prediction of Tll binding sites in the CRMs of *eve* stripes and the mathematical modeling of their removal using an experimentally validated theoretical framework shows effects on *eve* stripes compatible with the absence of a repressor binding to the CRMs. In addition, modeling increased *tll* levels in the embryo results in the differential repression of *eve* stripes, agreeing well with the results of the misexpression assay. In genetic assays we investigated *eve* 5, that is strongly repressed by the ectopic domain and representative of more central stripes not previously implied to be under direct regulation of *tll*. While this stripe is little affected in *tll-*, its posterior border is expanded in *gt-* but detected with even greater expansion in *gt-;tll-*. We end up by discussing *tll* with key roles in combinatorial repression mechanisms to contain the expression of medial patterns of the segmentation cascade in the extremities of the embryo.

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Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2023.09.014.

**Keywords**

Segmentation; *Drosophila* ; *tailless* ; Transcription; Repression; Embryo; Patterning

## 1. Introduction

Nuclear receptors (NRs) comprise one of the largest family of transcription factors involved in diverse developmental and physiological processes in animals (Robinson-Rechavi et al., 2003). The NRs display a modular organization that includes two C4 zinc-finger motifs in the DNA binding domain (DBD) and a ligand binding domain (LBD) that interacts with lipophilic compounds such as small fatty acids and hormones (Finger et al., 2021; Germain et al., 2006; Mangelsdorf et al., 1995). NRs are considered ligand-inducible transcription factors mediating cell signaling and gene expression, however, the NR family also includes many members called orphan nuclear receptors (ONRs), whose physiological ligands have not yet been identified or because they function in a ligand-independent way (Bridgham et al., 2010; Markov and Laudet, 2011).

In *Drosophila*, there are 18 members representing each one of the subfamilies of the NR family, including the ONR *tailless* (*tll*) (Bodofsky et al., 2017; King-Jones and Thummel, 2005). *tll* was isolated from a genetic screen of blastoderm stages and was initially characterized as a gap gene with roles in the segmentation cascade (Jürgens et al., 1984). It turned out that Tll is the prototype of a class of ONRs that includes its vertebrate orthologue Tlx (Yu et al., 1994). Tll and Tlx share close structural resemblance and both the *Drosophila* and the vertebrate homologues display similar biological roles in the nervous system (Wang and Xiong, 2016; Yu et al., 1994). Thus, beyond segmentation, *tll* plays critical roles in the visual system and brain formation in gastrula and larval stages, as well as neural stem cell proliferation and self-renewal from embryonic to adult stages (Gui et al., 2011).

However, the expression of *tll* is detected comparatively earlier in the embryogenesis, displaying a dynamic pattern in the anterior and posterior regions of the embryo. (Gui et al., 2011; Pignoni et al., 1990, 1992; Strecker et al., 1986, 1988). The mRNA of *tll* is first detected forming two transient symmetrical expression patterns covering each end of the early syncytial blastoderm (the *cap* expression pattern). The expression at both poles then recedes giving rise to two asymmetrical expression gap domains with the posterior domain comparatively shorter at its anterior boundary. Immediately afterwards, the anterior domain further retreats from the pole and subsequently, also retreats from ventral regions of the embryo, which ends up forming a stripe traversing the dorsal midline (*horseshoe* shape) at the transition from the syncytial to the cellular blastoderm. The retraction of the anterior domain continues at the dorsal midline generating two dorsolateral patches anterior to the cephalic furrow (*triangle* shape) at the end of the cellular blastoderm stage. During cellular blastoderm, the posterior domain progressively weakens till it vanishes while at the anterior region, *tll* continues to be expressed in brain neuroblasts during gastrula stages (Rudolph et al., 1997; Younossi-Hartenstein et al., 1997).

The blastoderm expression of *tll* is a consequence of *tll* being an effector and integrator of responses coordinated by different maternal systems (Chen et al., 2009; Furriols and

Casanova, 2003; Gui et al., 2011; Liaw et al., 1995; Pignoni et al., 1992; Smits and Shvartsman, 2020), which also reflects its contribution in the formation of segmented and non-segmented regions of anterior and posterior extremities, as well as organs formed by different tissues. At the anterior end of the embryo, *tll*-affects the formation of the clypeolabrum and the procephalic and optic lobes, while in the posterior region, the 8th abdominal segment, telson and hindgut are missing as well as much of the posterior midgut (Mahoney and Lengyel, 1987; Pignoni et al., 1990; Rudolph et al., 1997; Strecker et al., 1988).

Interestingly, *tll* gap functions appear to act mainly by restricting the expression of more central segmentation genes at the ends of the embryo (Brönner and Jäckle, 1996; Pankratz et al., 1989; Steingrímsson et al., 1991; Strecker et al., 1988; Weigel et al., 1990). For example, the absence of *tll* expression in *tor*-is associated with the expansion of the central gap genes *Kruppel* (*Kr*) and *knirps* (*kni*) and the impairment of the formation of terminal parts of the embryo (Weigel et al., 1990). On the other hand, the gain of function of genetic alleles that expand the *tll* domains expand the formation of terminal structures into more central regions of the embryo through the repression of *Kr* and *kni* (Steingrímsson et al., 1991). Moreover, the homeotic genes *Deformed* (*Dfd*) and *Ultrabithorax* (*Ubx*) are under negative regulation of *tll* respectively at the anterior and posterior ends (Reinitz and Levine, 1990).

The gap activity of *tll* is not restricted to the gap genes, as it also acts for the correct positioning of the anterior pair-rule stripes *hairy* 1 (*h* 1) and *even-skipped* 1 (*eve* 1) (Andrioli et al., 2017). These stripes are detected with small expansions in *tll*-, which is consistent with the fact that *tll* is part of an additive repression mechanism formed by anterior gap domains that define the anterior border of these stripes (Baltruk et al., 2022). Furthermore, we checked *tll* repression with a misexpression system by creating an ectopic domain of *tll* along the ventral midline region of the embryo at the time of the pair-rule striped pattern formation (Andrioli et al., 2017). With this system, we verified *h* 1 and *eve* 1 strongly repressed by *tll* ectopic domain, and the set of aforementioned results prompt us to investigate other roles of *tll* in the segmentation.

Here we investigated the effects of ectopic *tll* on the other pair-rule stripes of *h*, *eve*, *runt* (*run*) and *fushi tarazu* (*ftz*), and on the expression patterns of the gap genes *Kr*, *kni*, *giant* (*gt*) and *hunchback* (*hb*). We also examined an embryonic ChIP-chip data for Tll (Li et al., 2008; MacArthur et al., 2009) and compared Tll-binding regions with regions previously mapped to Cis-Regulatory Modules (CRMs) for specific patterns of the segmentation genes. In the case of *eve*, we investigated Tll binding in more detail by examining predicted binding sites in that area of the *eve locus* containing open chromatin and detected differences in the binding affinity for Tll between CRMs. Moreover, with a previously validated chemical kinetic model of *eve* stripe formation (Barr and Reinitz, 2017) we simulated both the removal of Tll binding sites and its misexpression at uniform levels under conditions where other gap genes are unperturbed. Finally, in genetic assays we investigated *eve* 5 to obtain further information about Tll regulation, that could be representative of more central stripes not previously considered to be under direct regulation of *tll*. We end by discussing the mechanisms of Tll acting on CRMs and its physiological role in segmentation.

## 2. Material and methods

### 2.1. Ectopic misexpression

DNA construct preparation for the ventral ectopic expression of *tll* and the activation of the construct in transgenic lines was described before (Haecker et al., 2007). Briefly, to generate the ectopic ventral domain, *tll* cDNA was cloned under the regulation of the *snail* (*sna*) promoter into a CaSpeR vector construct. This construct is silenced by a cassette containing transcriptional stop signals flanked by FRT sites downstream of the *sna* promoter. Flies carrying the ectopic construct were initially crossed with flies containing the β2-tubulin-FLP transgene. Then, males containing both constructs were selected for crossing with *yw* flies. Flipase recombination and activation of the construct occurs during the spermatogenesis of these males and therefore, their progeny was collected for embryo inspection and larval cuticle preparation.

### 2.2. In situ hybridization, immunohistochemistry and cuticle preparations

Gene expression patterns were detected by whole mount *in situ* hybridization experiments with one or two antisense RNA probes in single or double stains (Kosman and Small, 1997). The collection of embryos and the *in situ* hybridization protocol using digoxigenin- or fluorescein-labeled antisense RNA probes were performed as described previously (Small, 2000). Genetic assays were performed with the null alleles $gt^{x11}$, $tll^{1}$ and $y^{1}w^{67c23}$ as "wild-type" flies, all originally obtained from the Bloomington Stock Center.

Tll protein was detected with a polyclonal rabbit anti-Tll antibody (Kosman et al., 1998) diluted 1:400 in PBT. For fluorescent detection, we used a donkey anti-rabbit conjugated with Alexa Fluor 488 nm dye. Embryos were mounted in Aqua Polymount (Polysciences) and stored at −20 °C. Images were collected on a Leica TCS SP2 confocal scanning microscope using the Leica confocal analysis software.

For cuticle preparations, larvae aged for 20–24 h after egg laying were fixed overnight at 65 °C in a 1:4 mixture of glycerol and acetic acid and then mounted in a 1:1 mixture of Hoyer's medium and lactic acid.

### 2.3. Bioinformatics and modeling

To investigate the participation of Tll in the stripe regulation of the pair-rule genes, we confronted embryonic ChIP-chip data for Tll (Li et al., 2008; MacArthur et al., 2009) with regions mapped as regulatory modules of these stripes. The ChIP-chip data were downloaded from the Berkeley Drosophila Transcription Network Project website (MacArthur et al., 2009), namely *D. melanogaster* version 6 genomic coordinates of Tll-bound regions as well as their peaks representing 1% and 25% FDR (false discovery rate) using the symmetric null test and raw data (.cel files). For the peaks they used two probability distributions: symmetrical null test (sym) and one based on the experimental IgG IP test (exp), resulting in four groups of peaks (exp-1%, exp-25%, sym-1%, sym-25%). For the bound regions they only applied symmetrical null test. Whereas the bound regions (BRs) represent the length-variable genomic regions where Tll bound the DNA, the peaks are regions of 500 bp where the maximal level of occupancy was observed inside the bound

regions. The 1% and 25% FDR bound regions were then united into a single bound region using a Python script based on their coordinates. We also searched for BRs and peaks of inter-CRM regions. The ChIP-chip score values, that define the vertical axis values of the binding curves in Figs. 7 and 8, were calculated as described in (Li et al., 2008; MacArthur et al., 2009) using Tll raw data (.cel files). These files are available as Supplementary Material (1–10).

The DNA sequences of the CRMs were obtained from REDfly (University at Buffalo Center for Computational Research (http://redfly.ccr.buffalo.edu/). In such instances, we noted the existence of two or more DNA sequences with different sizes assigned to the same CRM. In this case, we compared these sequences considering the expression pattern of these isolated elements and any other information from the original articles that could help us define CRMs more precisely (Figs. S1 and S2). Finally, the genomic coordinates of the CRM sequences were obtained using the tool Blast+ (Camacho, 2008) for *D. melanogaster* genome version 6 obtained from the UCSC Genome Browser database (Navarro Gonzalez et al., 2021). The overlapping of CRMs, peaks and unions of 1% and 25% FDR bound regions were calculated using a Python script based on their coordinates (Fig. S3). The graphical representations (Figs. 7 and 8) were generated using the tkinter library for Python.

Scans for individual Tll binding sites were performed using the curated set of *Positional Weight Matrices* (*PWMs*) described in Barr and Reinitz, 2017, which in the case of Tll a PCM (*Position Count Matrix*) was obtained from DNAseI footprinting data collected in Rajewsky et al., 2002. The Tll PCM $X$ was converted in a PWM $M$ using equation (1), where $\alpha$ indicates the PCM and PWM row corresponding to nucleotide $\alpha$ = A, C, G or T, $i$ indicates the PCM and PWM column position, $q_\alpha$ is the genomic frequency of nucleotide $\alpha$ (which in case of *D. melanogaster* is C = G = 0,21 e A = T = 0,29), $N$ is the number of binding sites used to create the PCM (that corresponds to the sum of each PCM column) and $c$ is a pseudocount used to smooth the distribution. In this work we used c = 1.

$$M_{[\alpha,i]} = log_2\left(\frac{X_{[\alpha,i]} + c \cdot q_\alpha}{(N+c)\cdot q_\alpha}\right) \quad (1)$$

Finally, the PWM score of a sequence was calculated according to equation (2):

$$PWM\ score = \sum_{j=1}^{l} M[\alpha_j, j] \quad (2)$$

where $l$ is the number of columns of the PWM, $j$ is each column of the PWM and also each position of a sequence of total length $l$, and $\alpha_j$ is the nucleotide occurring in the position j of the sequence.

The kinetic model for expression of *eve* is described in Barr and Reinitz, 2017. Transcriptional regulation is predicted based on *eve* locus sequence and amounts of

transcription factors described in a quantitative dataset of segmentation gene expression at cellular resolution (Pisarev et al., 2009; Poustelnikova et al., 2004; Surkova et al., 2008). Code and data for this model, including data for transcription factors, can be downloaded at https://github.com/kennethabarr/transcpp.

## 3. Results

### 3.1. Effects of the ventral ectopic domain of tll on pair-rule stripes

We developed an expression system that uses *snail* (*sna*) regulatory regions to form an ectopic domain at the ventral midline of the embryo to test the activities of gap genes on ventral parts of likely expression targets (Andrioli et al., 2002). Embryos carrying this construct for *tll* stained for Tll protein show the ventral ectopic domain in addition to the two endogenous gap domains (Fig. 1A). This system allows us to investigate the effects of gap genes on pair-rule stripes till mid-stages of nuclear cycle 14 of the syncytial blastoderm, when gap genes are surely responsible for the initial formation of the pair-rule striped pattern. With the ventral ectopic *tll* we previously showed ventral repression on *h* 1 and *eve* 1 (Andrioli et al., 2017). However, the ectopic domain of *tll* causes other effects on segmentation, as suggested by larvae with extensive modifications in the ventral cuticle pattern (Fig. 1C). The patterning problems in the cuticle are in line with the wide disturbance detected in the striped pattern of *h*, *eve*, *run* and *ftz*, and not restricted to *h* 1 and *eve* 1 (Fig. 2).

We focus on the ectopic effects detected in ventral regions of pair-rule stripes. Surprisingly, almost all stripes of *h*, *eve*, *run* and *ftz* are ventrally repressed by the ectopic expression of *tll* at mid-stage of nuclear cycle 14 (Fig. 2B, D, F, H). Only *run* 7 (Fig. 2F) and *ftz* 7 (Fig. 2H) appear to be unchanged from the wild-type pattern (respectively, Fig. 2E, G). In principle, *h* 7 (Fig. 2B) and *eve* 7 (Fig. 2D) are the only stripes showing effects that can be correlated to activation roles of *tll*, as they are ventrally expanded at their anterior borders compared to the wild-type pattern (respectively, Fig. 2A, C). The remaining stripes seem to be already repressed at different degrees by the misexpression assay (Fig. 2B, D, F, H). Interestingly, the repression effects are already detected at earlier stages at the beginning of the formation of the striated pattern, but not the expansion of *eve* 7 and *h* 7 (Fig. 3A; data not shown). The detection of ventral effects during the initial formation of the striped pattern favors the argument of direct repression roles of Tll.

We also noticed the tendency for corresponding stripes of different genes to exhibit comparable ventral effects due to the ectopic domain. Stripes 1 (*h* 1, *eve* 1, *run* 1 and *ftz* 1) are striking in this regard, being all severely repressed (Fig. 2B, D, F, H). Two other groups of stripes, formed by stripes 6 and by stripes 5 are also well-repressed. However, for stripes 6, h 6 (Fig. 2B) and eve 6 (Fig. 2D) are more strongly repressed than run 6 (Fig. 2F) and ftz 6 (Fig. 2H); while for stripes 5, run 5 (Fig. 2F) and ftz 5 (Fig. 2H) are more strongly repressed than h 5 (Fig. 2B) and eve 5 (Fig. 2D). Less pronounced repression effects are detected for stripes 2 and 4. These stripes also exhibit major differences when comparing corresponding stripes of different genes, most noticeable for stripes 4. For example, *ftz* 4 (Fig. 2H) is strongly repressed when compared to *h* 4 (Fig. 2D). Finally, stripes 3 are the least repressed ones at this embryonic stage. They are detected as thin stripes with weak

expression levels in relation to the wild-type pattern and even though they seem to be weakly ventrally repressed.

The interpretation of results sometimes is complicated by the dynamic changes of the striped patterns during nuclear cycle 14. As illustrated for *eve*, it is possible to detect a succession of events from a younger (Fig. 3A) to an older embryo (Fig. 3B), respectively from the beginning and from the refinement stage of the striped pattern, passing through the middle-aged embryo shown in Fig. 2D. For example, *eve* 3 shows progressive repression along nuclear cycle 14 (Fig. 3A and B). Other stripes show alterations in shape, for example, *eve* 2, that is initially partially repressed (Fig. 3A), and later, it is more intensively detected with an anterior bent (Fig. 3B). Moreover, there are effects triggered by the ectopic domain of *tll* that extend beyond the ventral parts of the stripes and that are not our focus here. For example, central stripes 3, 4 and 5 of *h*, *eve*, *run* and *ftz* seem to be less expressed along their longitudinal extensions, in addition to being thinner when compared to the respective wild-type stripes (Fig. 2).

### 3.2. Effects of the ventral ectopic domain of tll on gap domains

The extent of the ventral effects caused by the ectopic expression domain suggests a widespread role of *tll* in the regulation of pair-rule stripes. Although the misexpression assay is not conclusive of Tll direct regulation, detection of effects at early and mid-stages during nuclear cycle 14, when gap genes are settling the seven-striped pattern, suggests direct regulation of *tll*. To further check that, we also investigated temporal and spatial patterns of gap genes in transgenic embryos misexpressing *tll*.

We inspected *Kr*, *kni*, *gt* and *hb* that are more central gap genes and known regulators of pair-rule stripes (for a review, Jaeger, 2011). Also, several studies investigated *tll* roles on these gap genes. According to genetic studies, *tll* represses *Kr* (Pankratz et al., 1989; Weigel et al., 1990), *kni* (Pankratz et al., 1992) and *gt* (Eldon and Pirrotta, 1991; Kraut and Levine, 1991b), and ubiquitous misexpression of *tll* represses *Kr* and *kni* endogenous domains (Steingrímsson et al., 1991). Consistent with these studies, we detected strong repression at ventral parts of *Kr* and *Kni* with the ectopic assay (Fig. 4F, H).

For *gt*, we detected different temporal effects on its expression pattern. In wild-type embryos, the blastoderm expression of *gt* starts as two broad gap domains (an anterior and a posterior domain) (Fig. 3C) and then, the large anterior domain is replaced by stripes 1, 2, 3, while the posterior domain (stripe 4) remains (Fig. 4C) (Eldon and Pirrotta, 1991; Kraut and Levine, 1991b). In the ectopic assay, the anterior and posterior gap domains of *gt* are detected with slight ventral repression at early stages of nuclear cycle 14 (Fig. 3D). The repression of the two *gt* gap domains at the early blastoderm suggests *tll* direct regulation. At later stages of the blastoderm, *gt* stripe 4 is slightly repressed and anteriorly expanded (Fig. 4D). In addition, *gt* stripe 3 seems to be repressed too (Fig. 4D). However, *gt* anterior stripes normally retract from the ventral region in wild-type embryos (Fig. 3C and 4C), a condition that makes it difficult to interpret effects for these stripes with the ventral ectopic domain.

Very interestingly though, we detected a strong correlation between the repression strength on *Kr*, *kni* and *gt* and positioning of these endogenous expression patterns in relation to

*tll* endogenous domains. *Kr* (Fig. 4H) is more severely affected compared to *kni* (Fig. 4F) and *gt* (Fig. 3D), and *Kr* has the farthest expression domain from anterior and posterior *tll* domains. Yet *kni* is intermediary regarding the position and repression extension. In this comparison, *gt* gap domains are the less repressed ones and closest to *tll* domains. Thus, repression caused by the ectopic domain indicates differential sensitivity to Tll increasing at a distance.

We also examined *hb* expression in the ectopic assay. In wild-type embryos, *hb* is initially expressed in the anterior half of the embryo, but this primary expression is replaced by a variable head domain, a stripe at the position of parasegment 4 (PS4-hb), and a posterior stripe (Fig. 4A) (Margolis et al., 1994). We detected *tll* effects only for the posterior domain at late stages during nuclear cycle 14 (Fig. 4B). The posterior domain of *hb* expands anteriorly in its ventral region (Fig. 4B), compatible to activation roles of *tll* like the situation detected for *gt* stripe 4 (Fig. 4D). Unlike *gt* (Fig. 4D), we did not detect weaker expressing levels at ventral regions of *hb* (Fig. 4B).

As well as for the striped pattern, we also detected effects not restricted to ventral regions of gap domains in embryos misexpressing *tll*. Most striking effects were detected for *Kr* (Fig. 4H). In addition to being ventrally repressed, *Kr* forms a narrow domain under the influence of *tll* ectopic domain. One possibility would be that *Kr* is differently expressed along its endogenous domain, being the expression less efficient in lateral nuclei compared to central nuclei. Thus, Tll emanating from the ventral region would be sufficient for lateral repression of the *Kr* domain. However, according to the protein gradient of Tll, one would expect to detect differences along the lateral domain of *Kr*, with repression progressively decreasing towards the dorsal region in consequence of the lower levels of Tll protein reaching the dorsal side of the embryo, a situation we did not detect.

### 3.3. Evidence of tll regulation on eve stripes by genetic assays

The repression of the main repressors of pair-rule stripes, *Kr*, *kni* and *gt*, by the ectopic *tll* in the early stages of nuclear cycle 14 argue against the early ventral repression of pair-rule stripes being caused by indirect regulation of *tll*.

We next investigated the possibility of *tll* repression for *eve* 5 in genetic assays. This stripe is ventrally well-repressed in the ectopic assay (Fig. 2D) and like the other more central pair-rule stripes, not previously suggested to be under direct regulation of Tll. Stripe *eve* 5 seems to be slightly shifted towards the posterior end of the embryo in *tll*- (Fig. 5C) when compared to the wild-type pattern (Fig. 5A). On the other hand, *eve* 1 is slightly expanded in the anterior border, stripe 6 is larger and shifted more posteriorly, and stripe 7 is missing, all results previously reported for these stripes in *tll*-background (Fig. 5C; Reinitz and Levine, 1990; Andrioli et al., 2017). It is known that Stat92 E (Dst) is the activator while Kr is the anterior repressor and Gt is the posterior repressor of *eve* 5 (Barr and Reinitz, 2017; Fujioka et al., 1999; Reinitz and Sharp, 1995; Ribeiro et al., 2010; Wu et al., 1998). As expected, this stripe is posteriorly expanded in *gt*- (Fig. 5B). However, the stain detected in the posterior region in *gt*-;*tll*-embryos probably corresponds to *eve* 5 (Fig. 5D), detected with an increased expansion compared to *gt*-only (Fig. 5B). That is because in double mutant embryos, *eve* 7 is not formed due the absence of *tll* and *eve* 6 is probably not formed either. In the case

of *eve* 6, the shadow of this stripe is detectable in *gt-* but not in *gt-*;*tll-* (compare Fig. 5B with 5D). The absence of *hb* and *tll*, both repressors for *kni* (Pankratz et al., 1989; Eldon and Pirrotta, 1991; Kraut and Levine, 1991b; Clyde et al., 2003), probably allows *kni* greater expansion in *gt-*; *tll-* and repression of *eve* 6, a target of *kni* (Fujioka et al., 1999; Clyde et al., 2003). Therefore, *eve* 5 is a common target for *gt* and *tll*, but *tll* repression is more evident only with the simultaneous absence of *gt*.

### 3.4. Evidence of tll regulation on eve stripes by computational modeling

We further analyzed the effects of ventral ectopic Tll, both at the level of binding sites and chemical kinetics of *eve* transcription, using an experimentally validated theoretical framework for studying the formation of *eve* stripes 2 through 7 under the control of the whole *locus* (Barr and Reinitz, 2017). The model leverages from availability of more data about regulation of all *eve* stripes and their CRMs when compared to stripes and CRMs of other pair-rule genes (Andrioli et al., 2002; Barr and Reinitz, 2017; Clyde et al., 2003; Janssens et al., 2013; Small et al., 1991, 1992). Expression of *eve* in the presence of ventrally expressed *Tll* (Fig. 6A) shows that stripes 1 and 6 are completely repressed ventrally, stripe 7 is unaffected and even expanded, while 2 is weakened but might be fused with 3, and stripe 5 is visible, but might be fused with 4 and weaker than the fused 2–3. Comparison of Tll-affinity to predicted binding sites (Fig. 6B) shows that the strongest Tll sites located in CRMs for stripes 2, 3, and 7 are weaker than the strongest Tll sites in the CRMs driving stripes 1, 4, 5, and 6. Thus, there is a strong correlation between the affinity of the strongest Tll sites in a CRM and its ventral repression by ectopic Tll.

With the aforementioned transcription model, we investigated the behavior of *eve* stripes after removal of Tll binding sites predicted in the CRMs of the *locus*. Stripes 4, 5, and 6 generated in this condition are wider when compared to stripes 2 and 3 (Fig. 6C), a result consistent with the absence of a repressor binding to the CRMs of these stripes. This result is also in accord with the strength of the binding site predictions for the CRMs (Fig. 6B), and with the differential sensitivity shown in 6 A. Together, binding sites prediction and removal based on chemical kinetic modeling results point to Tll direct regulation on *eve* stripes. It is important to note that this *in silico* experiment leaves the wild-type gap domains unchanged. This has the advantage of modeling only direct effects of Tll on *eve*, a condition corresponding to the ideal case of removal of binding sites for a specific transcription factor in genetic experiments without affecting potential superposing binding sites for additional transcription factors. Hence, one gets quantitative upper or lower bound effects of binding sites removal on transcription rates depending on whether we consider repressors or activators, respectively. As a disadvantage, we do not consider the changes in the gap system caused by ectopic ventral Tll (Fig. 4) that could explain the large derepression effect detected for *eve* 7 (Fig. 6C).

We also use the model to predict the effects of Tll protein added to native expression in a uniform manner along the A-P axis, representing the situation in the most ventral parts of embryos expressing *tll* under *sna* control (Fig. 1A and 6A). The results of the *in silico* experiment predict the repression of stripes 4, 5, and 6 but survival of 2, 3, and 7 at reduced levels (Fig. 6D), in accord with the ectopic results that shows stronger repression for stripes

4, 5 and 6 compared to stripes 2 and 3 (Fig. 6A). At sufficiently high levels of Tll, all *eve* stripes are well-repressed, but the differential sensitivity of stripes 4, 5, and versus 2, 3, and 7 are robust at all lower concentrations of ectopic Tll (Fig. S4).

### 3.5. Tll binding to the CRMs of pair-rule stripes

We addressed Tll binding confronting the CRMs responsible for the regulation of pair-rule stripes with data from a genome-wide ChIP-chip assay of the blastoderm stage (MacArthur et al., 2009). We retrieved two data sets from these analyses, the bound regions (BRs), which are variable-length windows corresponding to Tll binding regions, and the peaks, which are regions of 500 bp indicating the maximal level of occupancy in certain BRs. The comparisons revealed several contiguous BRs that also contain peaks spanning regions coincident with CRMs (Fig. 7; Table 1; S3). Actually, the matches between the BRs covering almost all the extension of the CRMs of pair-rule stripes are really impressive, suggesting that the patterning problems detected for almost all stripes in the ectopic assay are caused by the direct regulation of Tll (Fig. 7; Table 1; S3). Moreover, BRs are low represented in *locus* regions not corresponding to CRMs (Fig. 7; Table 1; S3).

For peaks the situation is more difficult to interpret. On one hand, CRMs of stripes strongly affected in the misexpression system endorses the direct regulation of Tll. For example, Peak 6 (P6) retrieved from the ChIP-chip assay matches *eve* 1, which is severely repressed in the misexpression assays (not shown). Furthermore, the number of a peak represents the strength of Tll-binding to a peak region, meaning that P6 was one of the strongest genome-wide TLL-interaction regions detected in the ChIP-chip assay. On the other hand, there are stripes equally severely repressed which are not accompanied by any peak in its corresponding CRM, as it is the case for *eve* 5 (Fig. 7). One possibility is that *eve* 5 has few Tll binding sites for Tll. In this situation, it was not possible to generate a peak region matching the *eve* 5 CRM but still with relevant physiological roles for the regulation of *eve* 5. In agreement with this, we predicted a cluster of binding sites for *eve* 1 but an isolated strong site for *eve* 5 (Fig. 6B).

In addition, a closer inspection of peaks detected on CRMs and on regions between CRMs (Inter-CRMs) revealed that the occupation of peaks is significantly higher in regions corresponding to CRMs when compared to regions that are Inter-CRMs (Fig. 7; Table 1; S3). Therefore, the broad correlation between peaks and BRs to CRMs affected by the misexpression assay early in the formation of the striped patterns agrees well with expected direct regulatory roles for Tll gap activity.

We also investigated Tll binding to the CRMs of gap genes from the ChIP-chip data (MacArthur et al., 2009). Previous *in vitro* footprint assays revealed binding sites for Tll in the CRMs of *Kr* (Hoch et al., 1992), *kni* (Pankratz et al., 1992) and the P2 promoter of *hb* (Margolis et al., 1995). Accordingly, with that, we detected the presence of bound regions and peaks with the CRMs of *hb*, *gt*, *Kr* and *kni* (Fig. 8). These results confirmed previous Tll binding data and make us more confident of Tll interaction to the CRMs of the pair-rule genes retrieved from the ChIP-chip data. Together, the results point to a global regulation of *tll* in the segmentation, through the direct binding of its protein to the CRMs of pair-rule stripes and of gap domains.

# 4. Discussion

## 4.1. Direct regulation of Tll

The extensive ventral repression effects detected for virtually almost all pair-rule stripes verified with the ectopic domain of *tll* called our attention for an unsuspected global regulation of *tll* in the striped pattern of pair-rule genes. Although misexpression assays do not imply direct regulation *per se*, the effects detected with the ventral ectopic expression system suggests that the repression mediated by Tll on pair-rule stripes is direct: 1) the effects on pair-rule stripes are detected early during nuclear cycle 14 (Fig. 2) at the time the seven-striped patterns are being established by gap genes, probably without long enough time for gap genes affected by *tll* ectopic domain to have caused them; 2) the fact that *Kr*, *kni* and *gt* (Fig. 3D and 4) as well as the head gap domains of *empty-spiracles* (*ems*) and *buttonhead* (*btd*) (Andrioli et al., 2017) are repressed by the ectopic domain argues against the putative indirect repression of Tll through any of these gap genes. One alternative hypothesis to explain the ventral effects detected on the striped pattern would be the local failure of activation. Therefore, the non-staining at ventral regions of stripes could be caused by the local absence of gap activation. However, the most accepted model for the formation of the pair-rule striated pattern predicts maternal and long-range gap factors such as Bcd, Hb; Caudal (Cad) and Dst activating CRMs in broad areas of the embryo, while the local activity of repressors such as Kr, kni, and gt refine the expression of CRMs to specific stripes (Barr and Reinitz, 2017; Papatsenko et al., 2009; Reinitz and Sharp, 1995; Small et al., 1991, 1992).

More straight evidence of Tll direct regulation at physiological conditions come from two genome-wide studies. Here, we detected that the CRMs of pair-rule stripes affected in the misexpression assay interact with Tll (Fig. 7; Table 1) investigating the correlation between defined CRMs of targets of *tll* misexpression with genomic regions bound by Tll at blastoderm stages (Li et al., 2008; MacArthur et al., 2009). At least some of such findings are corroborated by the results shown in another study using a completely different approach, the bacterial one-hybrid (B1H) system (Noyes et al., 2008). Although Tll is one of the 35 transcription factors investigated in this study, the paper highlights the presence of binding sites for Tll predicted in the CRMs of *eve*, *h* and *ftz* stripes (Noyes et al., 2008). Consistent with this, these authors show that Tll in combination with Bcd, Hkb and Kr are with overrepresented binding sites predicted on *h* 1, *eve* 1 and *ftz* 1 + 5 that are in the top 5 matches in a genome-wide search for sequences for transcription factors that regulate anterior gene expression in both *D melanogaster* and *D pseudoobscura* (Noyes et al., 2008).

We also obtained support of Tll direct regulation with our theoretical analysis for *eve* stripes. First, we retrieved putative binding sites for Tll scanning the CRMs of *eve* stripes (Fig. 6B). Then, simulating the removal of these binding sites we were able to detect stripes that are differently affected (Fig. 6C), but with a behavior that matches very well the repression effects of the ectopic misexpression data (Fig. 6A). Very interestingly though, the *in vivo* and *in silico* experiments independent approaches converge to the same results. In conclusion, our results confirm previous data from the literature pointing to the direct regulation of *tll* and expand the view of *tll* roles in the segmentation.

### 4.2. Tll is a transcriptional repressor factor

The great majority of the ventral effects detected in the ectopic assay indicate Tll acting as a repressor (Figs. 2–4). In addition, the results for *eve* were nicely corroborated modeling *eve* stripes with additional levels of Tll that show repression for stripes *eve* 2 to *eve* 7 (Fig. 6D; S4). In fact, manipulations of Tll protein sequence converting Tll into an obligate repressor showed that this form behaves similarly to the endogenous protein in both gain and loss of function experiments (Morán and Jiménez, 2006). The authors concluded that Tll is a dedicated repressor and the activation roles attributed to Tll are probably indirect effects (Morán and Jiménez, 2006). Moreover, one important aspect of the NRs regulation, in particular the ones that act independently of ligands, is their necessity to recruit co-regulators (Germain et al., 2006). Tlx has been shown to function by forming corepressor complexes with histone deacetylases or Atrophin-1 at regulatory regions of target genes (Sun et al., 2017). Tll has also been shown to be dependent on the co-repressors Brakeless (Bks) and Atrophin-1 (ATN1) (Davis et al., 2014; Haecker et al., 2007; Wang et al., 2006).

Despite all this, Tll appears to act as an activator in four specific situations in the misexpression assay, in the ventral anterior expansion of *h* 7, *eve* 7 (Fig. 2B, D), and *hb* and *gt* posterior domains (Fig. 4B, D). One way to explain these results would be if the ventral ectopic domain Tll was repressing the repressors of these expression patterns. In fact, Kr is a strong repressor of *gt*, as indicated by the expansion of its gap domains in the absence of *Kr* (Kraut and Levine, 1991a), while *kni* expands posteriorly in *tll* mutant embryos and Kni is a strong repressor for *hb* (Clyde et al., 2003; Pankratz et al., 1989). Our results are consistent with this possibility: *kni* and *Kr* are severely repressed at ventral regions by ectopic *tll* (Fig. 4F, H), respectively enabling *hb* and *gt* anterior ventral expansions (Fig. 4B, D). According to that, the expansions of *hb* and *gt* are late effects in nuclear cycle 14, suggesting indirect roles of Tll. The expansions detected for *h* 7 and *eve* 7 (Fig. 2B, D) could be indirect late effects of Tll as well. Early in the striped pattern of *eve* and *h*, *eve* 7 and *h* 7 are not expanded while the other stripes are already repressed by the ectopic domain (Fig. 3A; data not shown). In fact, *h* 7 and *eve* 7 are also anteriorly positioned by *kni* in wild-type embryos (Clyde et al., 2003; La Rosée et al., 1997) and could be expanding with the repression of *kni* in transgenic embryos. Moreover, our modeling results suggest *tll* repression on *eve* 7 (Fig. 6C and D; S4). Taken together, most data points Tll as a dedicated repressor in the segmentation.

On the other hand, footprinting assays revealed Tll binding to the CRMs of *hb* and *h* 7 (La Rośee et al., 1997; Margolis et al., 1995). Accordingly, data retrieved from the ChIP-chip assay (MacArthur et al., 2009) shows Tll genomic interactions on the CRMs of *h* 7, *eve* 7, *hb* and *gt* (Figs. 7 and 8). One possibility to reconcile all these contradictory results could be the counter-repression mechanism proposed for *eve* 2 (Vincent et al., 2018). According to this study, Hb is a repressor with sites on *eve* 2 but prevented from binding by the presence of Caudal (Cad) binding to nearby sites. It could be that Tll is also a repressor for *h* 7, *eve* 7, *hb* and *gt* because of a counter-repression mechanism also involving Cad. Differently from the anterior blastoderm where *eve* 2 is formed, Cad is expressed at high levels at the posterior of the embryo and locally activates segmentation genes (Rivera-Pomar and Jäckle, 1996). Moreover, Cad activator binding sites were detected at least for *kni* and

*h* 7 (La Rośee et al., 1997; Rivera-Pomar and Jäckle, 1996). Because the detection of Tll binding to *h* 7, *eve* 7, *hb* and *gt* at physiological levels (Figs. 7 and 8), it is possible that Tll is still able to bind to these CRMs but with unnoticed effects that are counteracted by Cad binding to the same CRMs even with the high levels of the ectopic domain of *tll*. Further experimental evidence is still necessary to check the interesting possibility of the counter-repression mechanism operating for these posterior targets.

### 4.3. Fine-tuned regulation of Tll

Our previous investigations revealed the need for at least the activities of Tll, Hkb and Slp1, acting together, to correctly position the anterior borders of stripes 1 of *h*, *eve*, *run* and *ftz* (Andrioli et al., 2012, 2017; Baltruk et al., 2022). The fact that Bcd is expressed in the anterior half of the embryo and, is probably the main activator of stripes 1 (Calhoun and Levine, 2003; Fujioka et al., 1999; Howard and Struhl, 1990; Klingler et al., 1996; Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991), is in agreement with the hypothesis that repressors are needed to bind the CRMs of these stripes, thus preventing Bcd activation in the anterior blastoderm and limiting the anterior borders of stripes 1 in wild-type embryos (Baltruk et al., 2022). This situation also matches the regulation proposed for *eve* 2 (Andrioli et al., 2002).

It is noteworthy though, that while investigating *tll* roles we did not detect changes in the anterior positioning of *run* 1 and *ftz* 1 in *tll*-(Andrioli et al., 2017), but these stripes are prompt and effectively repressed by the ectopic domain of *tll* (Fig. 2F, H). It is possible that Slp1, the common repressor for *run* 1 and *ftz* 1, and closer to these stripes than Tll, is more decisive for the combinatorial repression and masks the activity of Tll (Baltruk et al., 2022). Interestingly, besides the effective repression of Tll on *Kr* as reported in the literature and also shown here (Fig. 4H), the anterior border of *Kr* is not changed in *tll*-either (Weigel et al., 1990). *Kr* is also activated by Bcd (Hoch et al., 1990; Jacob et al., 1991) and it is anteriorly repressed by the anterior gap domains of Hb and Gt (Yu and Small, 2008). Therefore, it can be predicted that the anterior Hb and Gt gap domains, being closer to *Kr* in relation to Tll, are more relevant for *Kr* repression than Tll. Thus, evidence points out that Tll is part of combinatorial repression mechanisms necessary to position the anterior borders of stripes 1 and *Kr*, but the importance of *tll* in genetic assays can only be revealed when other repressors are absent.

The situations just described for stripes 1 and *Kr* illustrate the fine-tuned regulation involving Tll that could be operating for other pair-rule stripes as well. Genetic studies regarding the more central pair-rule stripes hint that maternal and central gap factors are sufficient to regulate their formation without the necessity of *tll* (Fujioka et al., 1999; Howard and Struhl, 1990; Klingler et al., 1996; Pankratz et al., 1990; Riddihough and Ish-Horowicz, 1991; Schroeder et al., 2011). Thus, the fine-tuned regulation of Tll for more central pair-rule seems to push this model to the limits.

Here, we investigated *eve* 5 in genetic assays as a case study of a stripe of more central regions of the striped pattern possibly under *tll* regulation. The detection of a posterior shift of *eve* 5 in *tll*-is not clear (Fig. 5C), but careful measurements and statistical analyzes confirmed this change (Janssens et al., 2013). However, *tll* regulation on *eve* 5 is most

noticeable when *gt* and *tll* activities are simultaneously absent in the double mutant embryo (Fig. 5D). Thus, the same points raised for Tll regulation of stripes 1 and *Kr* can be advocated for *eve* 5 as well: Gt domain is closer to *eve* 5 than Tll and a more effective repressor. Furthermore, these results also support the hypothesis that Tll is part of mechanisms composed of repressors that establish the borders of pair-rule stripes other than stripes 1. The presence of Tll might be of special importance to restrict the expression of CRMs towards the ends of the embryo in the absence of other repressors.

### 4.4. Regulation of Tll in the initial expression of pair-rule genes

One interesting point about the pair-rule genes *h*, *eve*, *run* and *ftz*, generally neglected, is that they are initially detected ubiquitously expressing all over the embryo before the establishment of the seven-striped pattern (Surkova et al., 2008; Andrioli et al., 2012). The ubiquitous expression of these genes is transient, and quickly retracts from the poles and then fade almost everywhere in the embryo, except for some regions that stain weakly, most notably at the position of stripes 1 (Andrioli et al., 2012). In the absence of any known CRM dedicated to this early expression pattern of the pair-rule genes, one possibility could be that the sum of different CRMs of stripes amenable to activation by maternal factors all over the embryo are responsible for these ubiquitous expression patterns. Then, the initial wave of expression of gap repressors in large domains shut down these CRMs almost all over the embryo. These early events are followed by an adjustment of the activity of activators and repressors with the refinement of gap domains, necessary to establish the composite pair-rule striped pattern.

In the case of *eve*, it could be that Bcd activates the CRMs of stripes 1 and 2 all over the anterior blastoderm, and then, Tll contribute to turn off most of the activation of these two CRMs at the anterior blastoderm even before the establishment of the striped pattern. The same rationale can apply for *eve* 5, as the ubiquitous maternal factor Dst is considered to be the activator of this and other *eve* stripes (Hou et al., 1996; Yan et al., 1996; Barr and Reinitz, 2017), and Tll could be repressing their CRMs at both ends. This possibility of regulation of pair-rule genes agrees well with the proposal that more than an instructive role, the main role of the gap domains of *tll* is to prevent gap genes expressed in more central regions from being expressed at the ends of the embryo (Hoch et al., 1992; Pankratz et al., 1992).

## 5. Conclusion

Here we show the broad extent of activity of *tll* in the segmentation cascade of *Drosophila*. With the ventral ectopic misexpression system previously used to test for *tll* repression on *h* 1, *eve* 1 and on head gap genes, we were able to detect the effects of *tll* on other gap domains and pair-rule stripes. We obtained expected results such as repression on *Kr*, *kni* and *gt* presenting convincingly differences to *tll* repression. Strikingly, the ectopic misexpression revealed drastic repression effects for almost all pair-rule stripes of *h*, *eve*, *run* and *ftz*, unpredicted targets for *tll* regulation. The coincidence of mapped CRMs to some Tll-genomic DNA interactions retrieved from a ChIP-chip data suggests that *tll* works through direct regulation on specific CRMS of the segmentation cascade. The direct binding

of Tll with repression roles was confirmed by modeling *eve* stripes in two situations: removing predicted binding sites from the CRMs of *eve* 2 to *eve* 7, and increasing Tll levels in the embryo. Investigating *eve* 5 in genetic assays, we detected *tll* regulation only when *gt*, the major repressor for the posterior border of this stripe was also absent. Our results reinforce previous evidence showing *tll* as a component of combinatorial repression mechanisms and extends the view of *tll* acting globally in the segmentation to represses the expression of medial segmentation patterns at the ends of the embryo.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability

Data will be made available on request.

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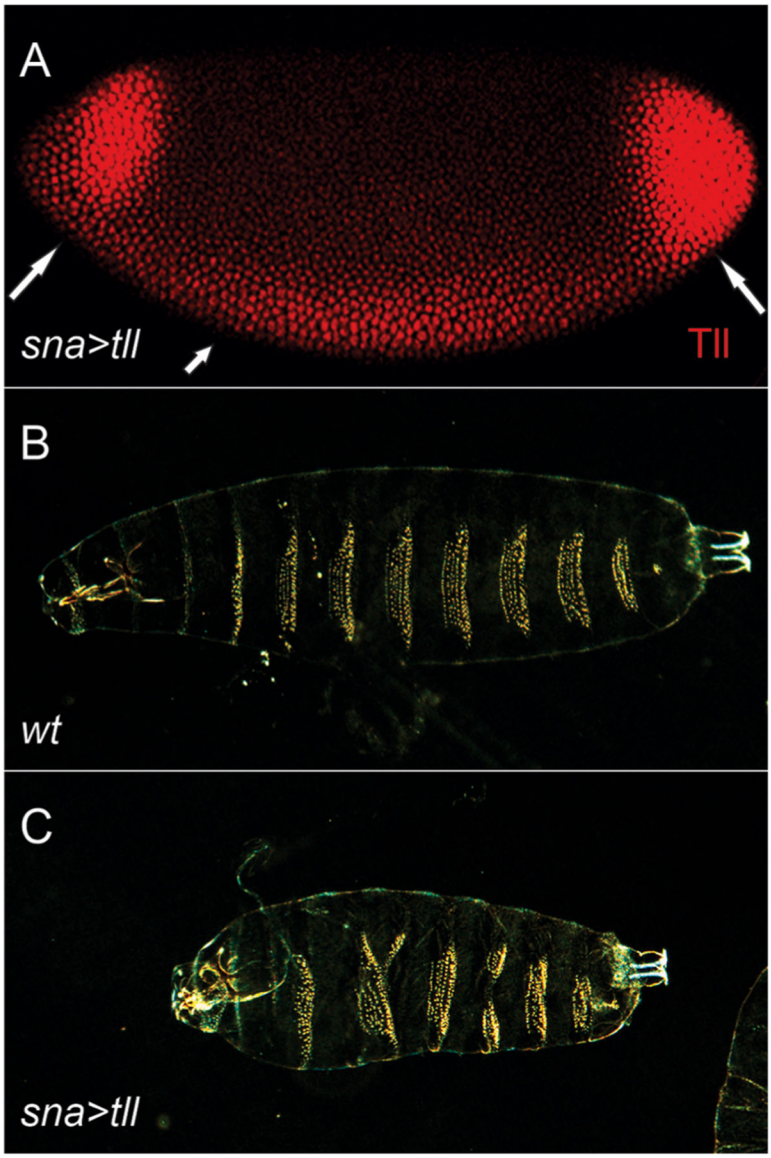


**Fig. 1.**

Ventral ectopic expression of *tll* changes the pattern of denticles of the larval cuticle. (A) Lateral view of a transgenic embryo (*sna* > *tll*) expressing the ectopic domain stained for Tll protein at an early stage of nuclear cycle 14. The longer arrows point to Tll gap endogenous domains, being the anterior (on the left) and posterior (on the right), and the short arrow points to Tll ventral ectopic domain. (B, C) Preparations of cuticles of first instar wild-type larva (B) and larva with the ectopic expression of *tll* (C). Note the disorganized pattern of the ventral denticles caused by the ectopic domain (C).

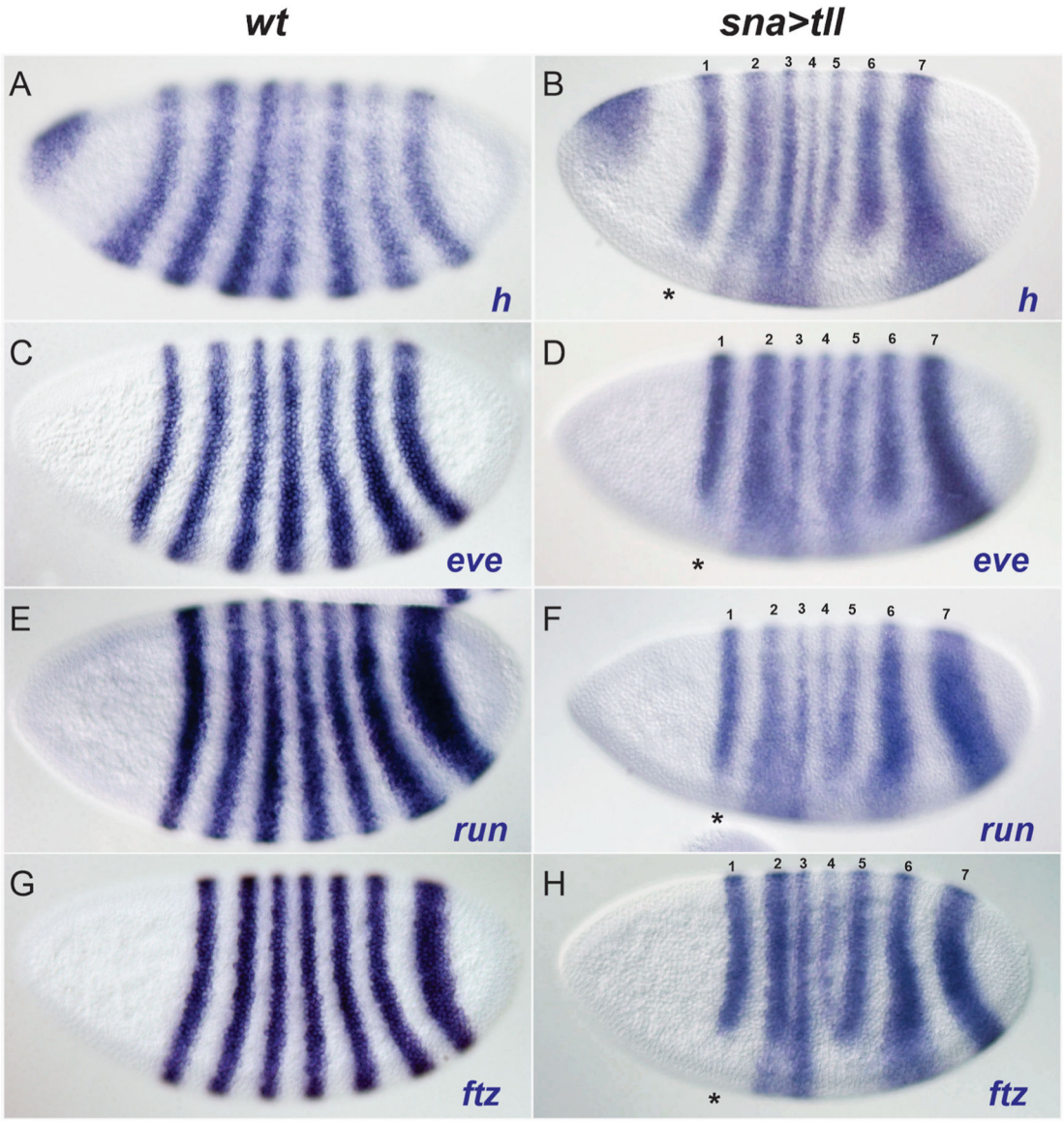


**Fig. 2.**

The ventral ectopic expression of *tll* dramatically disrupts the striped pattern of pair-rule genes. *In situ* hybridization in wild-type embryos (A, C, E, G) or embryos expressing the ventral ectopic domain of *tll* (B, D, F, H) detected for different probes as indicated in the figure. Note the broad range of effects in the striped patterns caused by the ectopic *tll* (B, D, F, H). For example, the strong repressions of *h* 1 (B), *eve* 1 (C), *run* 1 (D) and *ftz* 1 (E) that are marked with asterisks. The embryos are at mid-stage of nuclear cycle 14 of the syncytial blastoderm. All embryos in this figure (and in the remaining figures of the paper) are lateral views with anterior on the left and ventral is down.

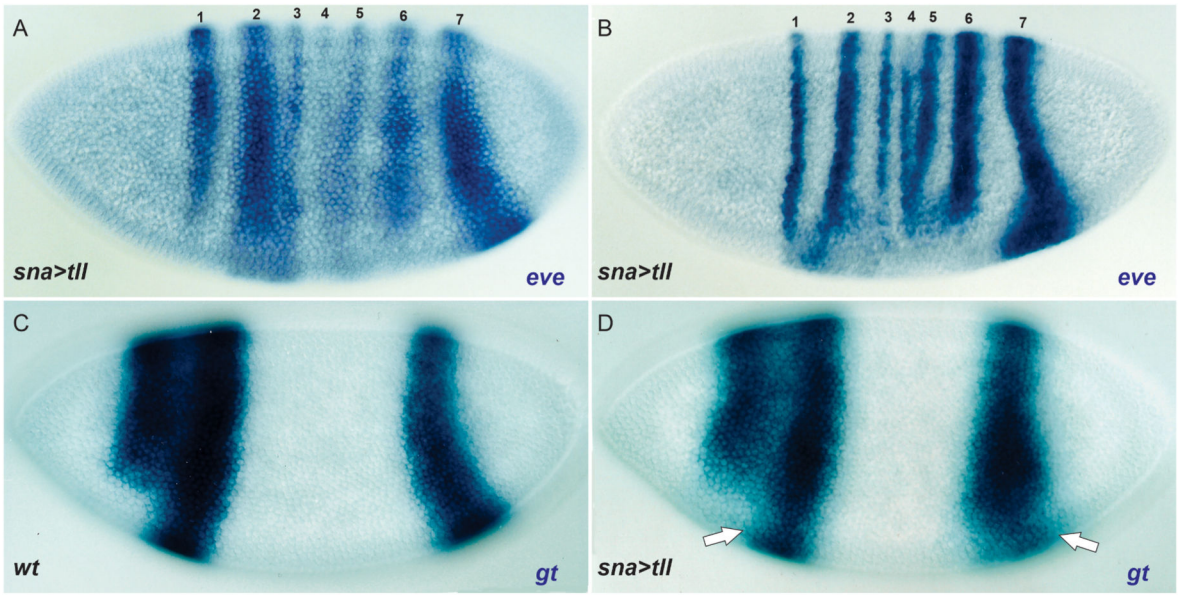


**Fig. 3.**

(A, B) Temporal effects of the ectopic expression of *tll* in the striped pattern of *eve* during nuclear cycle 14. Note the partial ventral recovery of *eve* 2 with an anterior bent in an older embryo (B) compared to a younger one (A). (C, D) *In situ* hybridization for *gt* in a wild-type embryo (C) and for an embryo with *tll* ectopic domain (D) at early-stage of nuclear cycle 14. The arrows point to the light repression of *gt* gap domains at the ventral midline of the embryo due to the ectopic domain of *tll* (D).

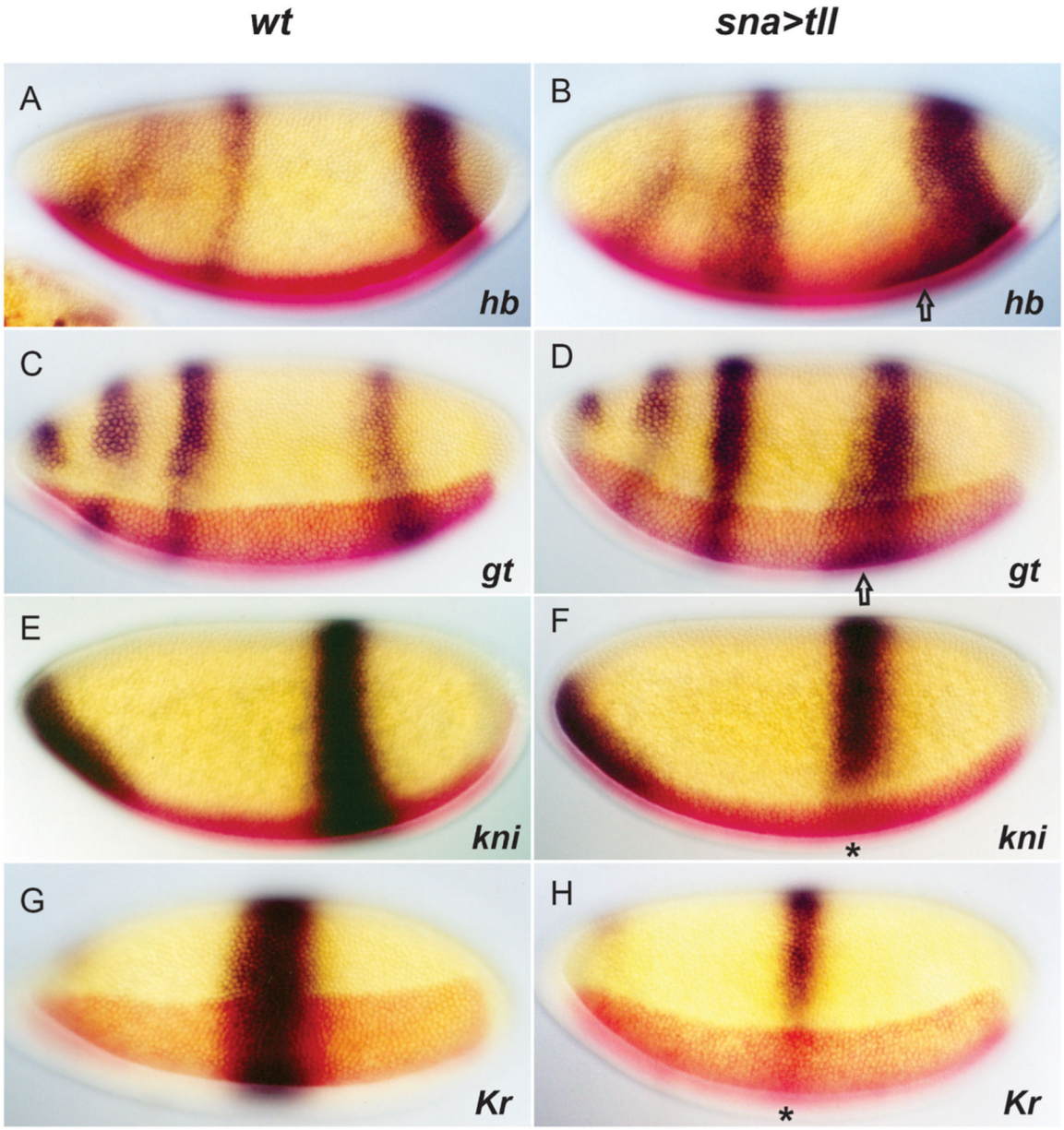


**Fig. 4.**

Gap genes response to the ventral ectopic domain of *tll* during the blastoderm. *In situ* hybridization in wild-type embryos (A, C, E, G) and embryos misexpressing *tll* (B, D, F, H) stained in black for different probes of gap genes as indicated. Embryos were also stained for *sna* which highlights their ventral midlines (and coincides with the ectopic expression domain). Arrows point to the anterior portions of *hb* posterior domain (B) and *gt* stripe 4 (D), both ventrally expanded. Asterisks indicate the strong repression of Tll on the gap domains of *kni* (F) and *Kr* (H). The embryos stained for *hb* (A, B) and *gt* (C, D) are older in nuclear cycle 14 compared to the embryos stained for *kni* (E, F) and *Kr* (G, H).

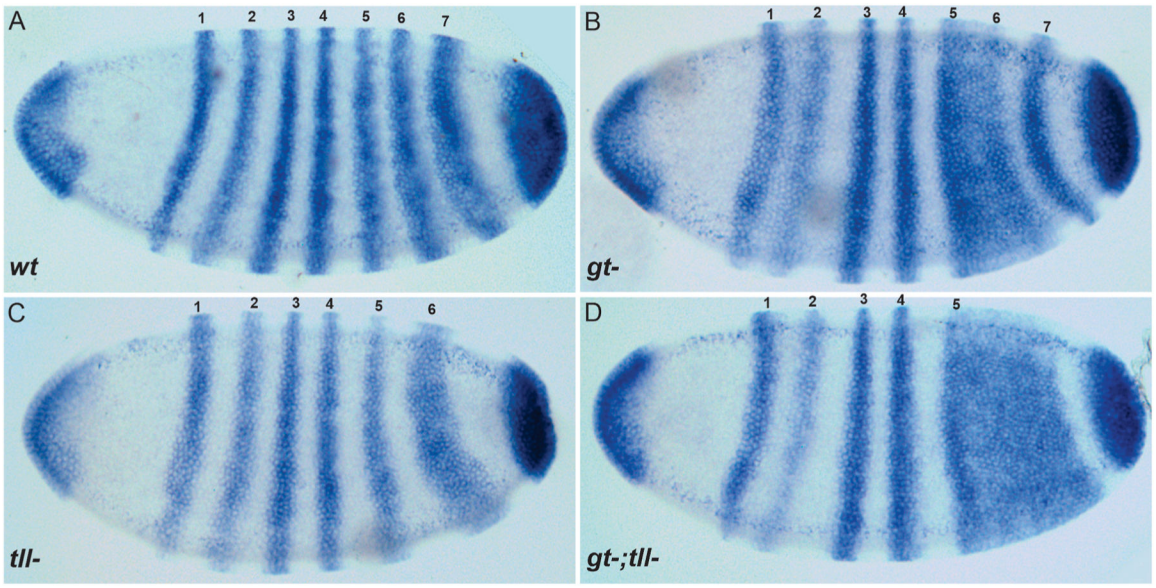


**Fig. 5.**

Expression pattern of *eve* in different genetic backgrounds. *In situ* hybridization in wild-type (A), *gt-* (B), *tll-* (C), *gt-;tll-* (D) embryos at mid-stage of nuclear cycle 14. Embryos were stained for *eve* and the gap gene *huckebein* (*hkb*) that is expressed at both ends of the embryo. *hkb* was used as a control as it is not affected at any genetic background. Note the expression between stripes 1 and 2 and between stripes 5 and 6 in *gt-* (B), while stripe 7 is missing in *tll-* (C). Note the larger posterior staining in *gt-;tll-* (D) compared to *gt-* (B). This staining might be due to *eve* 5 posterior expansion only, as *eve* 6 detectable in *gt-* (B) is not formed in *gt-; tll-* (D).

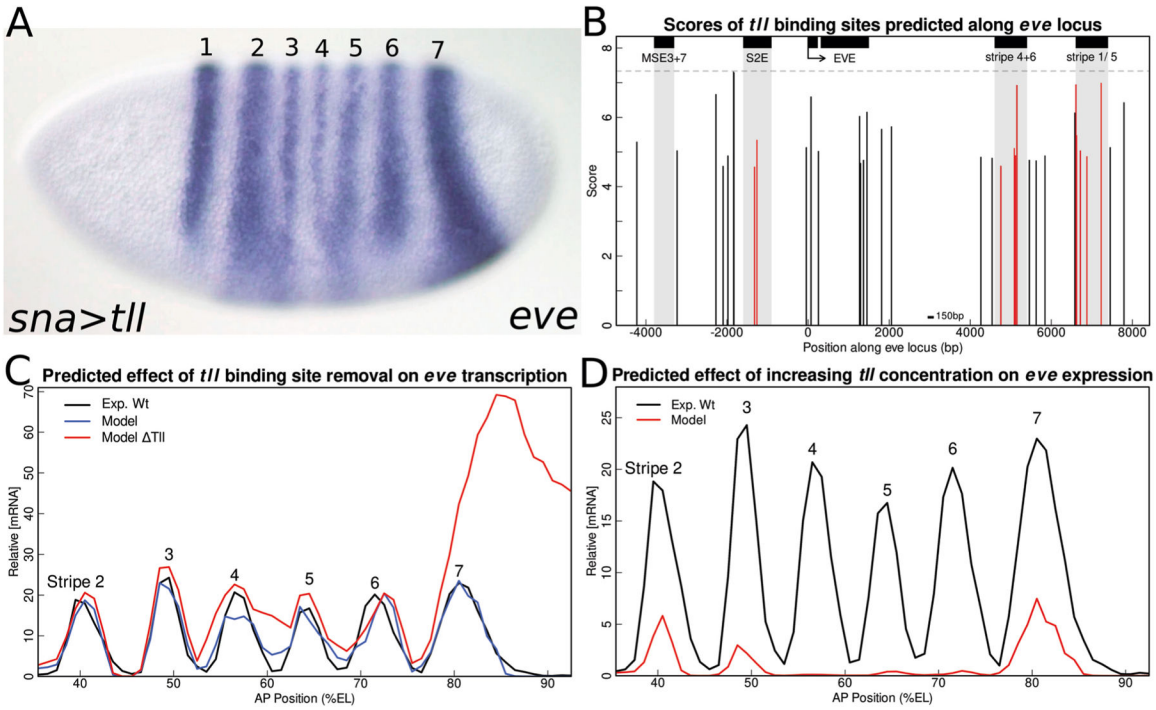


**Fig. 6.**

Computational analysis of the repression of *eve* stripes by *tll*. (A) *eve* RNA expression pattern in the presence of ectopic ventral expression of *tll*. (B) Predicted binding sites for Tll on the *eve locus* having PWM scores greater than 4.5 (black and red vertical bars). Bars denoted in red are Tll binding sites inside the CRMs (grey shadowed areas). Position is given relative to the transcription start site. The 150 bp scale bar gives the range of repression over which short range repressors act (Hewitt et al., 1999). Note that the high affinity sites near S2E are more than 150 bp away. (C) shows the *in silico* predicted *eve* patterns with Tll binding sites present (blue) or absent (red). (D) *In silico* predicted *eve* patterns after adding 10 concentration units of Tll distributed uniformly on the A-P axis. In (C) and (D), A-P position in percent egg length from the anterior pole is shown on the x-axis; stripes are numbered. The y-axis shows mRNA levels on a 0–255 fluorescence scale. The black curves indicate quantitative experimental data. In both panels, native gap domains are unaltered. In (B–D), the model, data, and all parameters therein are as described (Barr and Reinitz, 2017).

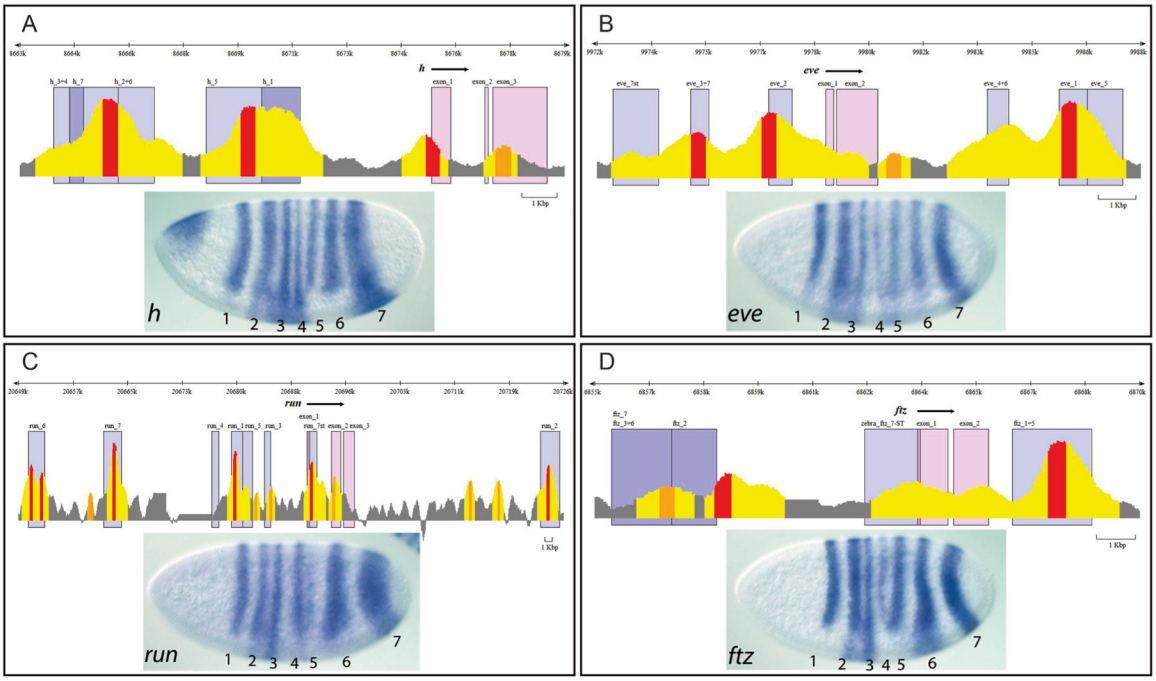


**Fig. 7.**

CRMs mapped for pair-rule stripes affected by the ventral ectopic expression domain of *tll* are Tll-bound regions in the embryo. Each scheme represents the superimposition of genomic coordinates of *h* (A), *eve* (B), *run* (C) or *ftz* (D) and respective CRMs, exons of the transcription unit and Tll binding intensities along these loci (represented as histograms), according to an embryonic ChIP-chip data (MacArthur et al., 2009). The coordinates of the CRMs are represented by blue stripes while exons are represented by pink stripes. Each histogram represents the binding intensity of Tll along the *locus*, where the vertical axis indicates the binding scores. In these histograms, regions corresponding to the union of 1% and 25% FDR bound regions are represented in yellow, 1% FDR peaks are represented in red, and 25% FDR peaks are represented in orange. Regions outside bound regions (and peaks) are represented in grey. The bottom part of the scheme shows the effects of the ventral ectopic domain of *tll* in the pair-rule stripes (embryos also shown in Fig. 2).

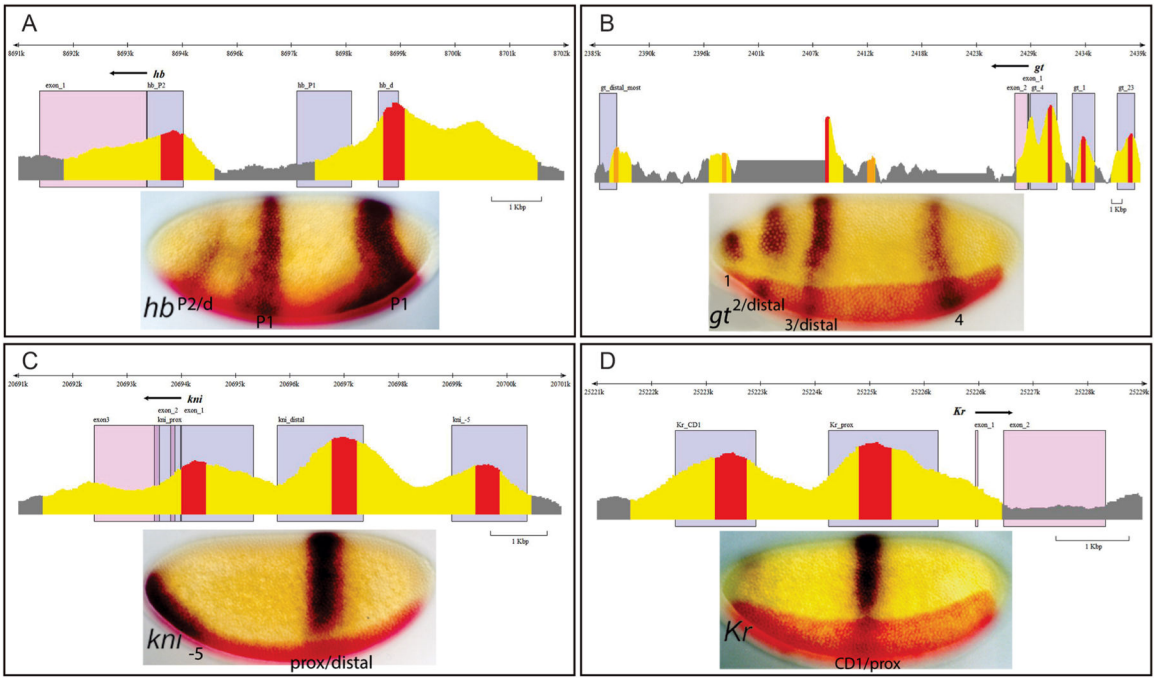


**Fig. 8.**

CRMs mapped for gap domains affected by the ventral ectopic expression domain of *tll* are Tll-bound regions in the embryo. Each scheme represents the superimposition of genomic coordinates of *hb*, *gt*, *kni* and *Kr* and respective CRMs, exons of the transcription unit and bound regions and peaks of Tll-interaction regions according to an embryonic ChIP-chip data (MacArthur et al., 2009). The coordinates of the CRM are represented by cyan rectangles while exons are represented by purple rectangles. The union of 1% and 25% FDR bound regions are represented by yellow rectangles. The 1% and 25% FDR peaks are respectively represented by red and pink rectangles. The bottom part of the scheme shows the effects of the ventral ectopic domain of *tll* in the gap domains (embryos also shown in Fig. 4).

**Table 1**

**Peak occupation in CRM or Inter-CRM regions.**

A region (CRM or Inter-CRMs) is the proportion of that region occupied by peaks, calculated as the total size of all peaks in that region divided by the total size of that region. Top graphic and bottom graphic respectively show the occupation of peaks of Tll detected at FDR 1% and FDR 25% for *h*, *eve*, *run* and *ftz*..